Antagonistic Potential of Nutraceutical Microbial Bacteriocins against Seafood Pathogens

Tasleem Khan MD[1] & Azaz Ahmad Azad Z.R[2]
[1]Department of Microbiology, Faculty of Life Science, JJT University, Jhunjhunu, Rajasthan, India
[2]Department of Post-Harvest Engineering and Technology, Aligarh Muslim University, Aligarh, India

Received: September 03, 2018 Accepted: October 13, 2018

ABSTRACT *The interest on novel biopreservation has been increasing during recent years, supported by research indicating that antagonistic potential of nutraceautical microbial bacteriocins using probiotic cultures against seafood pathogens.Biopreservation using lactic acid bacteria (LAB) and their antimicrobial metabolites represents an alternative method for improving food safety. The important contribution of probiotic LAB in food preservation has been attracting much attention because of the nutritional qualities of the raw material through an extended shelf life of food and their ability to inhibit spoilage and foodborne pathogens, which is interesting for the food industry. In this study, we extracted and purified the bioactive metabolites (bacteriocins) from the culture filtrate of lactic acid bacteria. The MIC and MBC of bacteriocins were determined against pathogenic bacteria (Shigella sp, Vibrio sp and Salmonella sp). The inhibitory effects of bacteriocins on the viable cells of pathogens were evaluated using MTT assay. The use of competitive microbiota as a biotechnological tool for food preservation may lead to improve the optimization and quality assurance of food products while at the same time retaining the sensory qualities of the product such as color, flavor, texture and nutritional value.*

Keywords: *Lactic acid bacteria, Nutraceuticals, Biopreservation, Bacteriocins, Bioactive metabolite.*

In the current scenario, food industries are gaining lot of significant interest world-wide. It was noted that the food are packaged and transported under strict hygienic conditions to avoid contamination and also to improve the shelf life periods. Seafood is the perishable food contaminated due to certain types of spoilage organisms like *Vibrio, Salmonella, Shigella* spp. The problem mainly arises at the food packaging (post-processing) level, which is common to all foods including seafood products. Meticulous cooking of seafood products would almost eliminate all microbial and parasitic pathogens; but it will not destroy some microbial toxic metabolites (e.g., *Staphylococcus* toxins). Storage of seafood at −18°C (Hoa et al 2009) is considered as a common method. However, freezing forms ice crystals which can cause tissue damage. This process results in exudation, fluid loss, reduced nutritional value, and changes in the texture and appearance of feed after defrosting. The preservation methods of interest are drying, smoking, freezing, chilling, brining, fermentation and canning to extend the shelf-life of different types of seafood.

Biopreservation is the method of choice used recently in these industries. Even though several biopreservation methods and sources are available, probiotic organisms like lactic acid bacteria and their antimicrobial compounds are gaining more interest in the recent years. Lactic acid bacteria (LAB) are used as biopreservatives in different food industries (Akbar and Anal, 2014). LABs such as *Lactobacillus, Lactococcus, Pediococcus*and *Enterococcus* are reported as common genera falls under LABs (Khan et al 2010). Bacteriocins produced by probiotic lactobacillus are antagonistic to other (Cleveland et al 2001). Different types of bacteriocins like lactacin B, lactocin, plantaricin and helveticin are reported to be involved in inhibition of cell wall synthesis, increases the cell membrane permeability and inhibition of RNase or DNase activity in other pathogens (Galvez et al 2007).

Considering on these factors, the present work was designed to evaluate the effect of bacteriocin as biopreservative agents against the seafood pathogens in combination with other methods. The antagonistic potential of nutraceutical microbial bacteriocins was investigated by applying a novel hurdle factors with combined effect of probiotic cultures and its metabolites against seafood pathogens in the present research. This method is proposed to explain the significance of simple and combined use of different preservation factors as synergistic effects instead of using a large-intensity preservation factor to inhibit microbial growth.The principal hurdles employed in food safety are temperature (higher or lower), aw, pH, Eh, chemical preservatives, vacuum packaging, modified atmosphere, UV, and competitive flora (LAB producing antimicrobial compounds).

Method

The present research work was carried out in Department of Post-Harvest Engineering and Technology, Aligarh Muslim University, Aligarh, India. The research work was performed during the period of May 2018 to September 2018.

Procurement of food pathogens

Three different food spoilage causing organisms *Shigella* sp, *Vibrio* sp and *Salmonella* sp are collected from food processing laboratory and sub-cultured, stored under standard laboratory condition for the present research.

Isolation and identification of *Lactobacillus*spp

Nutraceutical bacteriocin producing *Lactobacillus*spp(Figure 1) was isolated and identified as per the method described bySaharKarami et al (2017). The isolated species was used for the production of nutraceutical bacteriocin compounds using the lab scale process(Figure 2). The compounds were extracted and purified using standard bioprocess technology. The production and purification methods are described below.

Production and extraction of bioactive metabolites (bacteriocins) by *Lactobacillus*spp in the selective media (Muhammad Zahid*et al.*, 2015)

The bioactive metabolite, bacteriocin as extracellular substance from *Lactobacillus* sppwas produced in the selective MRS broth. The procedure was explained briefly below about the extraction of bacteriocin from *Lactobacillus* spp used in this present research. About 200 ml of MRS broth was prepared and the selected strains (*Lactobacillus* spp) were inoculated and kept in the incubator shaker for 72 hours with 170rpm and 37°C. The entire 100ml culture was inoculated onto 200ml media and incubated at similar condition. As a scale up process, the 200ml cultured cells were transferred to 500ml production media and incubated for the production of nutraceutical bacteriocins. After incubation the broth was centrifuged and the supernatant containing the bacteriocin was collected in a clean separate flask. The concentrated bacteriocin extracts were re-dissolved in 2ml of sterile distilled water; filter sterilized and stored in sterile eppendorf tubes until further use.

Purification of the bioactive metabolites (bacteriocins) from the culture filtrates of lactic acid bacteria (Goraya et al 2013)

Cell free culture filtrate from three *Lactobacillus*spp extracted as above was further subjected for partial purification step. The protocol was described in brief below. About 200 ml of extracted cell free supernatant was presented to ammonium sulphate precipitation (4°C) at 80% saturation level. The resultant precipitate obtained was re-suspended in 20 mM Phosphate buffer (pH 7 ± 0.5). The precipitate was further processed for dialysis process. Activation of dialysis membrane was done by immersing the membrane into 20mM Phosphate buffer (40°C) for 10 minutes. The entire content was filled into the dialysis bag and tied at both ends without any leakage. The bag was dialyzed for 12 h against 20mM Phosphate buffer (pH 7 ± 0.5) at 4°C. After dialysis the proteinous suspension containing bacteriocin was carefully collected, centrifuged and the pellet obtained was re-suspended in 2 ml of Phosphate buffer and stored at 4°C for further use.

Determining the MIC and MBC of bacteriocins against sea food pathogenic bacteria (Savadogo*et al.*, 2004)

Minimum inhibitory concentration of bacteriocin extracts was tested against three selected sea food pathogen (*Shigella* sp, *Vibrio* sp and *Salmonella* sp) using agar well diffusion method using four different known concentrates of purified bacteriocins (1X, 2X, 3X and 4X). Antibacterial drug gentamicin (100µg/ml) was used as standard antibiotic.

Evaluating the inhibitory effect of bacteriocins on the viable cells of sea food pathogens using MTT assay methods (Weijia Li et al 2015)

The inhibitory effects of the bacteriocin extracts from the *Lactobacillus*spp are determined on the viable bacterial cells (*Shigella* sp, *Vibrio* sp and *Salmonella* sp) using a standard MTT assay method. In brief, about 1ml of actively growing culture was taken and its absorbance indicating the growth of the organisms was measured at 600nm using a UV- Vis Spectrophotometer. To this growing culture, 1 ml of MTT reagent mixed with bacteriocin was added and incubated for 10 min. The above mixture was centrifuged at 8000 rpm for 5 min. Then, the pellet was collected and re-suspended in 1 ml of sterile water. The CFU was enumerated for both bacteriocin-MTT exposed and Control cells. The difference in CFU for test sample and control was calculated to express in percentage of cell inhibition (viable cells).

Cell inhibition (%) = A – B/A X 100

Where A - Control cells, and B - MTT exposed cells

Results and Discussion

Purification of the nutraceutical bacteriocins

Bacteriocins of lactic acid bacteria have been widely studied in recent years.There arefew studies that describe their chemical structure. This may be due to the many challenges associated with the purification of theseantimicrobial peptides (Mackay *et al*., 1997).Different strategies for the partial purification of bacteriocins from complex cultivation broths have performed their cationic and hydrophobic characteristics (Cheigh*et al*., 2004). According to Burlanek and Yousef (2000) the presence of hydrophobic regions in bacteriocin molecules isessential for their activity against sensitivebacteria, since inactivation of microorganisms by bacteriocins depends on the hydrophobic interaction between the bacterial cells and bacteriocin molecules (Cintas *et al*., 2001).

In the present study, the cell free culture filtrate from *Lactobacillus* sp production media was extracted and purified. The crude bacteriocin samples were partially purified by treating with the ammonium sulphate at 60% saturation and then centrifuged at 10,000 rpm for 10 min. The black colored pellets obtained were subjected to dialysis using a Molecular weight cut off (1200 KDa) dialysis membrane and purified. The dialysis method was implied in the study based on the concept of Cintas *et al*., (2001). Once the bacteriocins are recovered from the cell-free supernatants, they can be concentrated by techniques permitting separation of the fractions according to their size and their physicochemical properties.

The steps handled in the current research for production and purification of nutraceutical compounds was in accordance to the method described by Jack et al., (1995). The researchers handled the method for extraction of non-lantibiotic bacteriocins. For the non-lantibiotic bacteriocins, they used the method involving growth in a suitable nutrient broth under optimal conditions for bacteriocin production, removal of the cells followed by fractionated precipitation of the proteins from the culture supernatant by addition of ammonium sulfate. The precipitated proteins are subsequently dissolved in in a weak buffer, and bacteriocin molecules are separated by use of different procedures including hydrophobic, ion-exchange, and size exclusion chromatography. These techniques have facilitated production of highly purified bacteriocin preparations; the final yield has generally been below 20% and involves several days of processing.

In comparison with the method handled in the present study, Joerger and Klaenhammer (1986) and Cheigh*et al* (2004) have developed a simple onestep purification method for obtaining antimicrobial peptides like Helveticin and nisin Z respectively. , The first authors produced and purified a peptide Helveticin from *Lactobacillus helveticus.* The compound is a class III bacteriocin, sensitiveto proteolytic enzymes and heat. It was purified by usingammonium sulfate precipitation; the pellet was ressuspended in sodium acetate buffer anddialysed against the same buffer. The sample was applied to a Sephadex column for gel chromatography. Cheigh et al (2004) used an expanded bed ion-exchange chromatography method for the fractionation of nisin Z produced by *Lactococcuslactis*subsp. *lactis*A164.

Determining the MIC and MBC of nutraceutical bacteriocins

The results of antibacterial activity of bacteriocin extractsshowed good activity against the food pathogens (*Shigella* sp, *Vibrio* sp and *Salmonella* sp) (Figure 3). In Table 1, the inhibitory action for different concentrates (1X, 2X, 3X and 4X) was recorded. Among the selected, 3X concentrate were identified as the minimal inhibitory concentration (MIC) for *Shigella* sp and *Vibrio* sp; whereas for *Salmonella* sp 2X was identified as MIC. In addition to MIC, the significant MBC (minimal bactericidal concentration) was also identified. The MBC was determined by sub-culturing the test dilution (3X) on fresh solid medium and further incubated at 37ºC for 24h. The lowest concentration of MIC tubes with no visible bacterial growth on solid medium was regarded as MBC (Table 2). In the present study, the same concentrate (3X) when tested showed no significant growth of pathogens on the solid nutrient medium.

In this study *Shigella* sp showed good inhibitory of 12mm and 17mm for 3X, and 4X concentrate respectively. Similarly *Vibrio* sp also exhibited the inhibitory zonesof 12mm and 19mm respectivelyfor 3X, and 4X concentrate. Inhibitory zones of 10mm, 17mm and 21mm for *Salmonella* sp were found against their respective concentrates (2X, 3X and 4X). In Fig.3 the obtained inhibitory zones were presented in comparison with the standard antibiotic gentamicin. All the obtained inhibitory zones were found almost similar to the antibiotic gentamicin during the study. The extended antibacterial potential of the extracted nutraceutical compounds was mainly due to their significant mode of actions as reported earlier. Montville *et al*., (1998) described that bacteriocins would dissipate trans-membrane potential and increase the membrane permeability of organisms to ions. The permeability thus leads to collapse of proton motive force in the organism. The collapse will subsequently damage the plasma membrane or cell wall of the organism leading to release of all cytoplasmic constituents from the cell and cell death.

Inhibitory effect of bacteriocins on the viable cells

The inhibitory effect of bacteriocins on the viable cells of food pathogens was evaluated using MTT assay methods. In this method, the CFU of the control cells was compared with the CFU of the bacteriocin exposed cells. During the analysis, all the three pathogens exposed to MTT showed maximum cell inhibition (Table 3). The bacteriocin exposed *Shigella* cells reduced upto 86% after enumerating the CFU in a Plate Count Agar media. *Salmonella* sp and *Vibrio* sp exhibited 90% and 85% respectively. The obtained results were found to be well in accordance to the results of MIC and MBC values; where the bacteriocin in the well diffusion method showed good inhibitory zones against all the three test organisms.

MTT assay is a tetrazolium salt, which in the presence of metabolically active cells is reduced into a product that can be measured colorimetrically, serving as a respiratory indicator of live cells (Krom et al. 2007). The results of the MTT assay confirmed that the bacteriocin inhibited the metabolic activity of test organisms. The decrease in cell metabolism occurs because most antimicrobial peptides work by interacting with the bacterial cell surface, followed by disruption of cellular integrity (Nawrocki et al. 2014). The mode of action of bacteriocin was found to be well correlated with study conducted by Motta et al. (2008). In their study, the bacteriocin-like substance (BLS P34 – an antimicrobial peptide) showed good inhibitory and lethal effect on the bacterial wall of *Listeria monocytogenes* (ATCC 7644).

Conclusion

Bacteriocin from LAB is having bright prospects to be used as aneffective food bio-preservative and as therapeutics, as ithas strong antagonism against a broad range ofchallenging and commonest sea food spoilage pathogens. The study paves the way ofexploring the possibilities of large scale production ofbacteriocin from LAB to be used as anantibiotic and as bio-preservative in various seafood stuffs. Abudding expediency of bacteriocins justifying a more inprofound research for their identification and applicationas food bio preservatives is thusrevealed. Bactericidal mode of action described in the present research would eradicate the major population of undesirable microorganisms from food items. The antibacterial action of the extracted nutraceutical compounds shall be increased by handling further purification techniques like chromatography and other methods.

References

1. Akbar A, Ali and Anal AK (2014), Occurrence of Staphylococcus aureus and evaluation of antistaphylococcal activity of Lactococcuslactissubsp. lactisin ready-to-eat poultry meat, Ann. Microbiol., 64(1),131-138
2. Amanda S,Florencia O and Adriano B (2004).Screening for antimicrobial activity among bacteria isolated from Amazon basin. Brazilian Journal of Microbiology;35(1)42-48.
3. Bizani D and BrandelliA.Characterization of a Lisboa and bacteriocin produced by a newly isolated Bacillus sp(2002).strain 8. Advanced Journal of Applied Microbiology; 93:521-519.
4. Burlanek, L. L. and Yousef, A. E (2000), Solvent extraction of bacteriocins from liquid cultures.L.Appl. Microbiol., 31, 193-197.
5. Cheigh C, Kook M, Kim S. Hong, Y. and Pyun, Y (2004), Simple one-step purification of nisin Z from unclarified culture broth of Lactococcuslactissubsp. lactisA164 using expanded bed ion exchange chromatography.Biotechnol.Lett.,26, 1341-1345.
6. Cintas LM, Herranz C, Hernández PE, Casaus MP and Nes LF (2001), Review: Bacteriocins of lactic acid bacteria. Food Sci. Tech. Int., 7, 281-305.
7. Cleveland J, Montville TJ, Nes IF. andChikindas ML (2001), Bacteriocins: safe, natural antimicrobials for food preservation, Int. J. Food Microbiol., 71, 1-20
8. Galvez A, Abriouel H, Lopez RL and Omar NB (2007), Bacteriocin-based strategies for food biopreservation, Int. J. Food Microbiol. 120, 51-70
9. Goraya MU, Ashraf M., Rahman S. U. and Habib A. (2013). Determination of antibacterial activity of bacteriocins of lactic acid producing bacteria, J. Inf. Mol. Biol., 1(1), 8-10.
10. Hoa ND, Wouters R, Wille M, Thanh V, Dong TK, Hao NV and Sorgeloos P (2009). A fresh food maturation diet with an adequate HUFA composition for brood-stock nutrition studies in black tiger shrimp Penaeusmonodon,Aquacul., 297, 116-121
11. JackRW, Tagg JR and Ray B (1995), Bacteriocins of Gram-positive bacteria.Microbiol.Rev.,59, 171-200.
12. Joerger MC and Klaenhammer T.R (1986), Characterization and purification of helveticin J and evidence for a choromosomally determined bacteriocin produced by Lactobacillus helveticus481.J. Bacteriol., 167, 439-446.
13. Khan H, Flint S and Yu PL (2010), Enterocins in food preservation, Int. J. Food Microbiol.,141, 1- 10.
14. Krom BP, Cohen JB, McelhaneyFeser GE and Cihlar RL (2007). Optimized candidal biofilm microtiter assay. J Microbiol Meth 68: 421-423
15. Mackay, V. C.; Arenose, G. and Hastings, J. W. (1997), Purification of bacteriocins of lactic acid bacteria: problems and pointers. J. Food Microbiol.,34, 1-16.

16. Montville TJ.and Chen Y (1998).Mechanistic Action of Pediocin and Nisin: Recent Progress and Unresolved Questions.Applied Microbiology and Biotechnology;50:267-274.
17. Motta AS and Brandelli A (2008). Evaluation of environmental conditions for production of bacteriocinlike substance by Bacillus sp. strain P34. World J MicrobiolBiotechnol 24: 641-646
18. Muhammad Zahid, Muhammad Ashraf, Muhammad Arshad, Ghulam Muhammad, AqeelaYasmin, and Hafiz Muhammad Adnan Hameed. (2015), Antimicrobial Activity of Bacteriocins isolated from Lactic Acid Bacteria against Resistant Pathogenic Strains, Int. J. Nut. Food Sci., 4(3), 326-331.
19. Nawrocki KL, Crispell EK and McBride SM.(2014). Antimicrobial peptide resistance mechanisms of Gram-positive bacteria.Antibiotics 3: 461-492.
20. SaharKarami, Mohammad Roayaei, HosnaHamzavi, Mahmoud Bahmani, Hassan Hassanzad-Azar,Mahmoodnia Leila, and Mahmoud Rafieian-Kopaei (2017). Isolation and identification of probiotic Lactobacillus from local dairy and evaluating their antagonistic effect on pathogens.Int J Pharm Investig.7(3): 137–141.
21. Savadogo A, Cheik AO, Imael HB and Traore AS (2004), Antimicrobial activity of lactic acid bacteria strains isolated from Burkina Faso Fermented Milk, Pakistan J. Nut., 3(3), 172-179.
22. Vieira GA, Soares M., Ramirez NB, Schleder DD, Correa da Silva B. and Mourino JP (2016), Lactic acid bacteria used as preservative in fresh feed for marine shrimp maturation, Pesq. agropec. bras., 51(11), 1799-1805.
23. Weijia Li, Zhou and YuyinXu. (2015), Study of the in vitro cytotoxicity testing of medical devices, Biomed Rep., 3(5), 617–620.

Table 1
MIC of nutraceutical bacteriocins

S.No	Test organisms	Zone of inhibition (in mm)				
		STD*	1x	2x	3x	4x
1	*Shigella* sp	19	0	0	12	17
2	*Salmonella* sp	23	0	10	17	21
3	*Vibrio* sp	21	0	0	12	19

*STD: gentamycin (antibiotic)

Table 2
MBC of nutraceutical bacteriocins

S.No	Test organisms	MBC for 3X concentrate
1	*Shigella* sp	No growth visible on the solid media
2	*Salmonella* sp	No growth visible on the solid media
3	*Vibrio* sp	No growth visible on the solid media

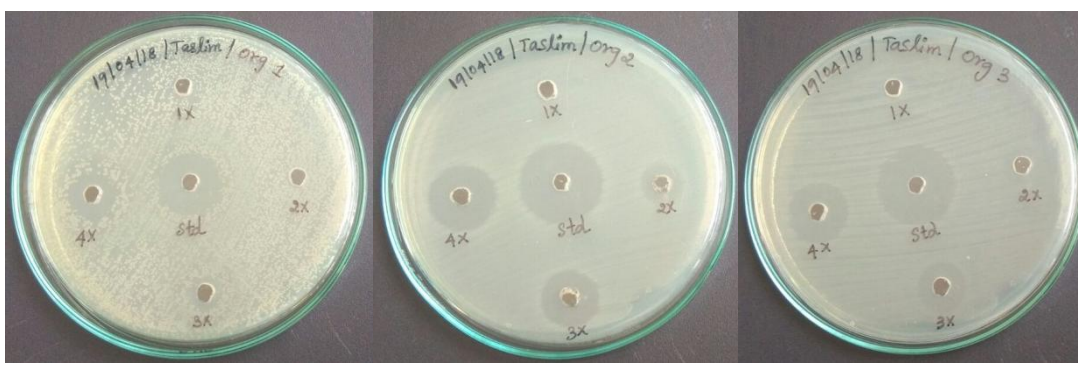
Table3
Inhibitory effect of bacteriocins on the viable cells

S.No	Test organisms	Percentage of cell inhibiton
1	*Shigella* sp	86
2	*Salmonella* sp	90
3	*Vibrio* sp	85

Figure 1. Isolated Nutraceutical bacteriocin producing Lactobacillus spp

Figure 2. Nutraceutical bacteriocin Production - Laboratory scale up process

Figure 3.Antibacterial activity of Nutraceutical bacteriocins



Shigella sp *Salmonella* sp *Vibrio* sp