Isolation, identification and mode of action of partially purified bacteriocins from lactic acid bacteria in fermented cassava grits.

Abstract

The study on the isolation, identification and mode of action of partially purified bacteriocin from lactic acid bacteria found in fermented cassava grits was carried out. Fermented cassava grits were collected from different garri processing plants and transported with ice-cubed box to the laboratory for analysis. Using standard microbiological procedures, five different lactic acid bacteria were isolated using De man Rogossa sharpe agar and identified by morphological and biochemical tests. Standard laboratory procedures were used to extract and partially purify the bacteriocins from the different lactic acid bacteria isolates. The viable microbial count after the partially purified bacteriocin from the various lactic acid bacteria isolates were grown against the food borne bacteria (*S. aureus* and *B. subtilis*) ranged from 0.98 x 10^3 CFU/ml for partially purified bacteriocin from isolate 6 at 8 hrs to 9.2 x10^3 CFU/ml for isolate 3 at 24 hrs. Similar results were obtained against *B.subtilis* with microbial counts that ranged from 1.02 x10^2 CFU/ml for isolate 3 at 8 hrs to 9.2 x 10^2 CFU/ml at 24 hrs. Isolates 6, 7, 10 and 11 were bactericidal both to *S. aureus* and *B. subtilis* while isolate 3 was bacteriostatic. The viable microbial count after the partially purified bacteriocin was grown against the foodborne bacteria (*E. coli* and *S. typhi*) ranged from 1.0 x10^2 CFU/ml for PPB from isolate 3 at 8 hrs to 7.1 x 10^2 CFU/ml for PPB from isolate 6 at 24 hours. The microbial count against *S. typhi* ranged from 6.50 x 10^2 CFU/ml for isolate 6 at 8 hrs to 8.5 x 10^2 CFU/ml for isolate 7 at 24 hrs. Partially purified bacteriocins from isolates 3 and 7 were bacteriostatic while isolates 6, 10 and 11 are bactericidal to *E.coli* and *S.typhi*. This result showed the partially purified bacteriocins that were very efficacious in killing or inhibiting the growth of some foodborne pathogens which can be applied in biopreservation.

Keywords: Bactericidal, Biopreservation, Mode of action, Cassava grits, Microbiological.

Introduction

Lactic acid bacteria (LAB) are a large group of beneficial bacteria belonging to different taxonomic groups, but unified on the basis of their shared metabolic and physiological characteristics. Morphologically, they are gram positive, generally non-sporulating, non-respiring, either rod-shaped (bacilli) or spherical (cocci) bacteria which produce lactic acid as the major metabolic end product of carbohydrate fermentation. Their importance is associated mainly with their safe metabolic activity while growing in foods utilizing available sugar for the production of organic acid and other metabolites. Their common occurence in foods along with their long lived uses contributes to their natural acceptance as GRAS (Generally

Recognised as Safe for Human Consumption (Bourdichon *et al.*, 2012). Bacteriocins are ribosomally synthesized peptides that when secreted act selectively on other bacteria, permeabilizing its membrane and potentially leading to cell death (Jordan *et al.*, 2014). In recent times, bacteriocins eg nisin, a bacteriocin produced by *Lactococcus lactis*, is generally recognized as safe (GRAS) by the Food Drug Adminstration (FDA) and is currently being used as a preservative agent in the food industry to prevent the growth of *Listeria monocytogenes* and other food pathogens. The commercialization of nisin since the 1950s, triggered the research interest to isolate new bacteriocins from different sources so that by the 1990s, there was a variety of bacteriocins with different activity spectra, some of which are still in the process of seeking approval for use as food preservative. The aim of this research was to isolate, identify and determine the mode of action of partially purified bacteriocins from the lactic acid bacteria isolated from fermented cassava grits.

Materials and Methods

Sample Collection

Fifteen (15) samples of fermented cassava grits were randomly collected from different garri processing plants in Abakaliki metropolis. The samples were collected in ice-cubed box and transported to the Applied Microbiology Laboratory of Ebonyi State University, Abakaliki for analysis.

Isolation and identification of lactic acid bacteria

Ten grams (10g) of the fermented cassava grits was added to 90ml of distilled water and homogenized in a stomacher (Seward Somacher Lab Blenders, UK) for 5 minutes. After ten fold serial dilution, 0.1ml of the sample homogenate was plated out on De Man Rogossa sharp agar (Uzoh *et al*., 2022) which was prepared and sterilized according to the manufacturer's instructions. The streaked plates were incubated anaerobically using an anaerobic jar (Gas pak) with CO_2 generating kit at 30^oC for 48 hours The pure colonies obtained were identified by morphological and biochemical characterization according to Cheesbrough, 2006. Only those isolates that conformed to the notable characteristics of lactic acid bacteria.

Isolation of bacteria pathogens from spoilt cucumber samples

The cucumber samples were processed by first removing the outer leaves and 20 g of each of the samples were weighed, washed with distilled water and placed on an electric blender. The blended samples were put on a clean beaker containing 20ml of sterile water and sieved. The resulting filtrate were plated on Cysteine Lactose Electrolyte Deficient (CLED) agar, Mannitol salt agar, *Salmonella-shigella* (SS) agar and Nutrient agar and incubated at 37^oC for 24hr. The discrete colonies of each of the bacterial isolates were identified by standard morphological and biochemical tests (Cheesbrough, 2006).

Production and Assay of Crude Bacteriocin

The preliminary isolation and characterization of lactic acid bacteria was carried out using standard microbiology technique after which the bacteriocin was extracted by growing the bacteriocin producing bacterium in 1000 ml of De Man Rogossa sharp (MRS) broth and incubated for 72 hours at 30^{o}C under anaerobic conditions. Extract was obtained by centrifuging the culture at 12, 000 rmp for 15 minutes to pellet down the cells (Hashim *et al*., 2017).

Partial Purification of Bacteriocins

The cell free supernatant from each of the bacterial culture (CFS) centrifuged at 10,000 x g, 4^{o}C, 10 minutes and the pH was adjusted to 6.5. The bacteriocin was precipitated by the addition of 40% ammonium sulphate in conical flask and mixtures were stirred overnight at 4^{o}C. Then the mixtures were centrifuged at 10, 000 x g, 4^{o}C, 10 min. The precipitated bacteriocins adhered on the wall of the tube were resuspended in 1ml of 0.2M phosphate – buffered saline (PBS). The precipitate was subjected to dialysis through a membrane (Dialysis bag). The partially purified bacteriocins obtained were stored at – 20^{o}C (Zhang *et al*., 2018).

Mode of action of the partially purified bacteriocin

The procedure followed was described by Faye *et al.,* (2000) and Nilsen *et al.,* (2003) in which 5 ml aliquots of the PPB from each of the LAB isolates was added to 20 ml of a suspension of 24 hour old 0.5 MacFarland standard of *Staphylococcus aureus, B. subtilis, E.coli and S. typhi* in Nutrient Broth in a McCartney bottle. *S. aureus, B. subtilis, E.coli and S. typhi* cells were allowed to grow for four hours before addition of the supernatant and their optical densities (OD at 640 nm) were measured and recorded at 8 hours intervals. The investigation was terminated by plating aliquots on nutrient agar plates and incubated at 37^{o}C for 24 hours to determine presence or absence of growth and the microbial count.

Results

Colony and Biochemical characteristics of Bacteriocinogenic Bacteria Isolated

Table 1 revealed that a total of five isolates were identified out of the 15 samples collected from locally fermented food. Out of the five isolated, all were Gram positive and catalase negative.

Table 1: Morphological and biochemical characteristics of lactic acid bacteria from fermented cassava grits.

Isolate code	Source	Cell shape	Oxidase	Gram reaction	Catalase activity	Spore formation	Anaerobic growth	Citrate
3	FCG	C	-	+	-	-	+	-
6	FCG	R	-	+	-	-	+	-
7	FCG	C	-	+	-	-	+	-
10	FCG	R	-	+	-	-	+	-
11	FCG	C	-	+	-	-	+	-

C = Cocci, R = Rod, FCG = Fermented Cassava Grits

Table 2: Carbohydrate utilization patterns of five isolates .

Carbohydrates	Isolates				
	3	6	7	10	11
Lactose	+	+	+	+	+
Xylose	+	+	+	+	+
D-fructose	+	+	+	+	+
Dextrose	+	+	+	+	+
Galactose	+	+	+	+	+
Maltose	+	+	+	+	+
Raffinose	+	+	+	+	+
Trehalose	+	+	+	+	+
Melibiose	+	+	+	+	+
Sucrose	+	+	+	-ve	+
L-Arabinose	+	+	+	-ve	-ve
Mannose	+	+	+	+	+
Sorbitol	+	+	+	+	+
Ribose	+	+	+	+	+
Adonitol	+	+	+	-	+
	Lactobacillus pentosus	*Lactobacillus spp*	*Lactobacillus spp*	*Lactobacillus plantarum*	*Lactobacillus fermentum*

The morphological and biochemical characteristics of bacteria isolated from cucumber samples are shown in table 3. It revealed that the probable bacteria isolated were *E. coli*, *S.aureus*, *Bacillus subtilis* and *Salmonella* species.

Table 3: Morphological, Microscopic and Biochemical Characteristics of Bacteria Isolated from Cucumber Sample

Morphological Characteristics		Biochemical Tests						Sugar Fermentation Test							
Shape	**Colour**	**Gram reaction**	**Motility Test**	**Citrate Test**	**Oxidase Test**	**Coagulase Test**	**Indole Test**	**Lactose**	**Glucose**	**Sucrose**	**Catalase Test**	**Urease Test**	**Voges Proskauer**	**Methyl Red**	**Probable isolates**
Cocci	Yellow	+	-	-	-	+	-	+	+	-	+	+	+	+	*E. coli*
Rods	opaque yellow colonies with slightly yellow center on CLED	-	+	-	-	-	+	+	+	+	+	-	-	+	*S. aureus*
															Bacillus subtilis
Gray-white round, opaque, flat on NA		+	+	+	+	-	-	-	+	+	+	-	+	-	*Salmonella specie*
Rods and	black	-	+	-	-	+	-	-	+	-	+	-	-	+	

Key: + = Positive, - Negative, NA- Nutrient Agar

Production and Assay of Crude bacteriocin

Figure 1 below showed the crude bacteriocin produced by the various LAB isolates. This showed that isolate 10 produced the highest crude bacteriocin.

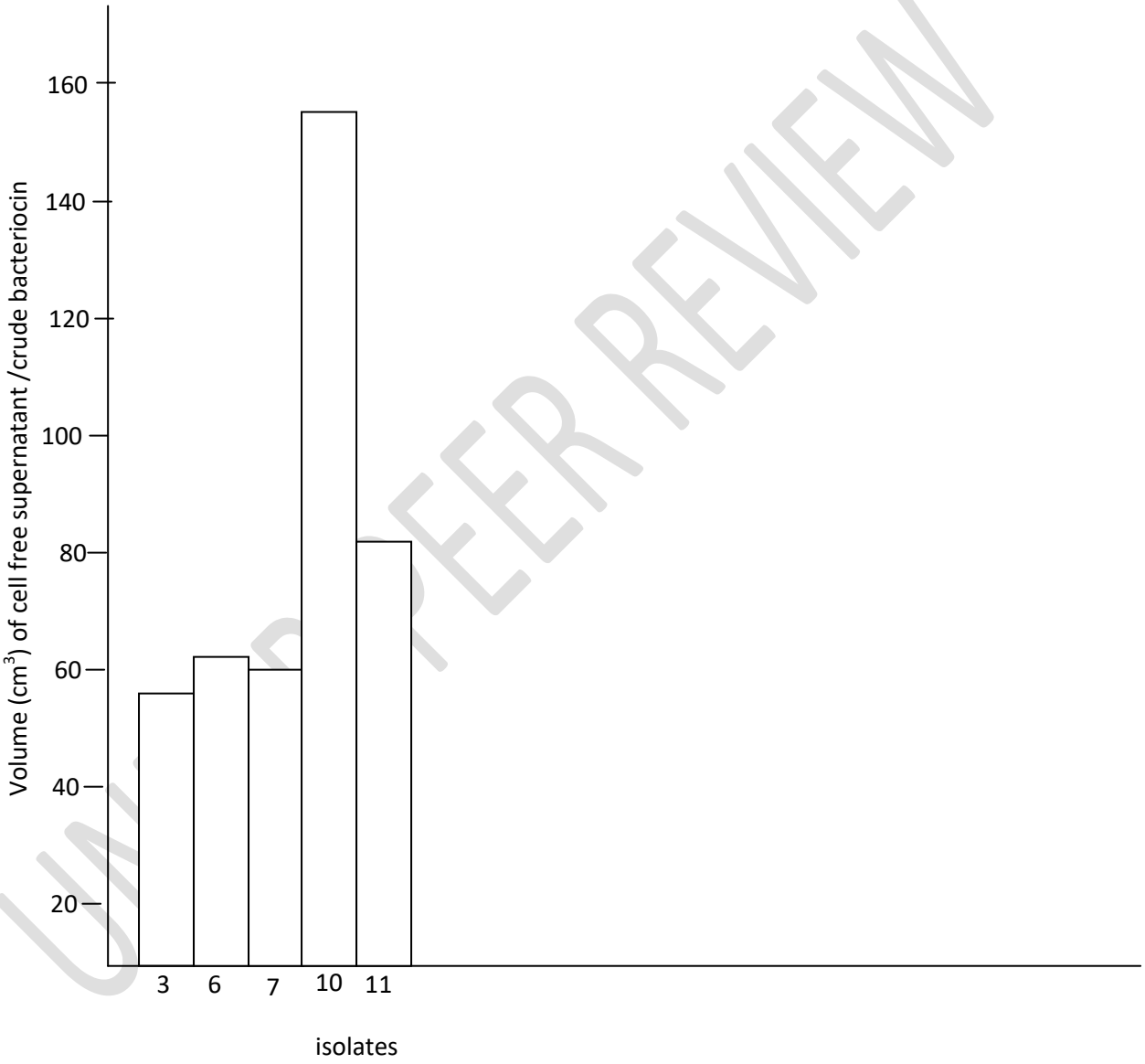

Figure 1: A table showing the volume of crude bacteriocin produced by each lactic acid bacteria isolates

Table 4 below showed the viable microbial count of *S. aureus* and *B. subtilis* after incubation with PPB from the various isolates at 8 hours and 24 hours interval. The result revealed that PPB from isolates 6,7,10 and 11 were bactericidal while isolate 3 was bacteriostatic.

Table 4: Microbial counts against Gram positive foodborne bacteria showing mode of action of PPB.

	Microbial counts (*S.aureus*)		**Microbial counts (*B.subtilis*)**CFU/ml	
Isolates	**8hours**	**24 hours**	**8hours**	**24 hours**
3	1.22×10^3	9.2×10^3	1.02×10^2	8.8×10^2
6	0.98×10^3	8.5×10^2	7.1×10^3	6.2×10^2
7	1.12×10^3	8.2×10^2	7.0×10^3	9.2×10^2
10	1.42×10^3	1.3×10^2	4.2×10^3	3.0×10^2
11	1.65×10^3	1.2×10^2	1.42×10^3	1.05×10^2

Table 5 below showed the viable microbial count of *E.coli* and *S.typhi* after incubation with PPB from the various isolates at 8 hours and 24 hours interval. The result revealed that PPB from isolates 6, 10 and 11 were bacteriocidal while isolates 3 and 7 were bacteriostatic.

Table 5: Microbial counts against Gram negative foodborne bacteria showing mode of action of PPB.

	Microbial counts (*E.coli*)		**Microbial counts (*S.typhi*)** CFU/ml	
Isolates	**8hours**	**24 hours**	**8hours**	**24 hours**
3	1.0×10^2	3.6×10^2	8.50×10^2	7.2×10^2
6	8.5×10^3	7.1×10^2	6.50×10^2	5.8×10^1
7	8.2×10^2	7.0×10^2	9.10×10^2	8.5×10^2
10	1.28×10^3	1.1×10^2	1.10×10^3	2.3×10^2
11	1.16×10^3	1.7×10^2	1.25×10^3	2.50×10^2

Discussion

A total of 15 fermented cassava grits were collected from different garri processing plants. The colony morphology, Gram's reaction, catalase test and other biochemical tests were used to identify the five (5) lactic acid bacteria in these fermented foods. This result was similar to the work of Ohenhen *et al*., 2015 who isolated 5 different species of *Lactobacillus* species from fermented ogi samples. The result of the morphological and biochemical characteristics of bacteria isolated from spoilt cucumber showed that *E. coli, Staphylococcus aureus, Bacillus subtilis* and *Salmonella* species were present. These bacteria are mostly enteric microbes which suggests possible fecal contamination from faecal materials. This is in agreement with the work of Sujeet and Vipin, 2017 who reported the presence of the same bacterial species in cabbage and other salad vegetables. The viable microbial count after the partially purified bacteriocin from the various lactic acid bacteria isolates were grown against the food borne bacteria (*S. aureus* and *B. subtilis*) ranged from 0.98 $x10^3$ CFU/ml for PPB from isolate 6 at 8 hrs to 9.2 $x10^3$ CFU/ml for PPB from isolate 3 and 1.02 x 10^2CFU/ml for PPB from isolate 3 at 8 hours to 9.2 x 10^2CFU/ml for PPB from isolate 7 at 24 hrs. The microbial count for PPB from isolate 3 at 8 hours and 24 hours intervals were 1.22 x 10^3CFU/ml and 9.2x10^3CFU/ml for *S. aureus*. There was no remarkable reduction in the microbial count over a long period of time, hence they are bacteriostatic. For PPB from isolate 6, isolate 7, isolate 10 and isolate 11, microbial counts at 8 hrs and 24 hrs intervals were 0.98 $x10^3$ CFU/ml to 8.5 $x10^2$ CFU/ml. This showed that the partially purified bacteriocin (PPB) was bactericidal in its mode of action. This result corroborates the work of

Wayah and Philip, 2018 who reported that a bacteriocin, Pentocin MQ1 was bactericidal to *L. monocytogenes* and *B. cereus*. Similarly, the results for PPB against *B.subtilis* followed the same trend as PPB from isolates 6, 7, 10 and 11 were bacteriocidal to the foodborne bacteria while isolate 3 was bacteriostatic. The viable microbial count after the PPB was grown against the foodborne bacteria (*E. coli* and *S. typhi*) ranged from 1.0 $x10^2$ CFU/ml for PPB from isolate 3 at 8hrs to 8.2 $x10^2$CFU/ml for PPB from isolate 7 at 24 hours. The microbial count for partially purified bacteriocin from isolate 3 at 8 hours and 24 hours intervals were 8.5 $x10^2$ CFU/ml and 7.2 $x10^2$ for *S. typhi*. There was no appreciable reduction in the microbial count over a long period of time, hence they are bacteriostatic. It was observed that they exhibited bacteriostatic mode of action. PPB from isolates 6, 10 and isolate 11 had microbial counts of 8.5 $x10^3$ to 7.1 $x10^2$CFU/ml, 1.28 $x10^3$ to 1.1 $x10^2$CFU/ml, 1.16 $x10^3$ to 1.7 $x10^2$CFU/ml at 8 hrs and 24 hrs respectively. It was observed that the PPB were bactericidal. This was consistent with the work of Zhao *et al*., 2022 who reported the bactericidal mode of action of plantaricin 827 on *S. aureus* thereby extending the shelf life of skin milk. Similarly, Yi *et al*., 2016 equally stated that a novel bacteriocin produced by *L. crustorum* MNO47 from China, had bactericidal action on indicator organisms. Similarly, the microbial count for PPB from isolate 3 against *S. typhi* were 8.50 $x10^2$CFU/ml at 8 hrs while 7.2 $x10^2$CFU/ml after 24 hours. Same trend of results were recorded for PPB from isolate 7 was 9.10 $x10^2$ to 8.5 $x10^2$CFU/ml. This result showed that the partially purified bacteriocins had bacteriostatic activity on *S. typhi*. There was observed decrease in microbial counts for PPB from isolate 6 against *S.typhi* from 8 hrs to 24 hrs with a value of 6.50 $x10^2$ to 5.1 $x10^1$CFU/ml and isolate 10 with a value of 1.10 $x10^3$ to 2.3 $x10^2$CFU/ml. These indicated bactericidal mode of action of the PPB on *S.typhi*. This corroborates the report of Jiang *et al*., 2017 who reported the bactericidal mode of action of Pentocil JL-1 from *L. pentosus* against *S.aureus*. The result in this research corroborates the work of Xinran *et al*., 2018 who reported that plantaricin JY22, a novel bacteriocin isolated from *L. plantarum* JY22 had bactericidal activity on *B.cereus*.

Conclusion

The different partially purified bacteriocins from the various isolates exhibited different modes of action against the different foodborne bacteria. This showed that some partially purified bacteriocins or bacteriocins can be used for biopreservation and this study will help researchers and industries to choose the most appropriate bacteriocins to be used specifically to prevent and preserve certain foods from fermented foodborne pathogens.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement

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REFERENCES

Bourdichon F., Casaregola S. , Farrokh C., Frisvad J. C., Gerds M. L, Hammes W. P., Harnett J,Huys G., Laulund S., Ouwehand A., Powell I. B., Prajapati J. B. , Seto Y, Schure E.T , Boven A.V, Vankerckhoven V., Zgoda A, Tuijtelaars S., Hansen E. B (2012).Food fermentations : Microorganisms with technological beneficial use. *International J Food Microbiol.*154 (3): 87- 97.

Cheesbrough M.(2006).District Laboratory Practice in Tropical Countries. 2nd Edition Cambridge University Press, Cambridge Edition pp 72-75.

Faye T, Langsrud T, Nes IF, Holo H (2000). Biochemical and genetic characterization of propionicin T1, a new bacteriocin from *Propionibacterium thoenii*. *Appl Environ Microbiol* **66**: 4230 - 4236.

Hashim Gasal M., Saad B.Almasaudi , Esam Azhar, Soad K. Al Jaouni Steve Harakeh(2017). Biological activity of *Cymbopogon schoenanthus* essential oil *Saudi J Biol Sci.* **24(**7): 1458-1464.

Jiang H, Zou J, Cheng H, Fang J and Huang G (2017). Purification, Characterization, and Mode of Action of Pentocin JL-1, a Novel Bacteriocin isolated from *Lactobacillus pentosus,* against Drug-Resistant *Staphylococcus aureus BioMed Res International* 20 : 1-12.

Jordan, K., Dalmasso, M., Zentek, J., Mader, A., Bruggeman, G., Wallace, J. (2014). Microbes versus microbes: control of pathogens in the food chain. *J. Sci. Food Agric.* 94, 3079–3089.

Nilsen, T.,Nes, I.F. and Holo, H. (2003). Enterolysin A, a Cell Wall-Degrading Bacteriocin from *Enterococcus faecalis* LMG 2333. *Appl. Environ. Microbiol.* **69**: 2975–298.

Ohenhen R.E, Isibor J.O, Emonfonmwan G., Enabulele S.A (2015).Effect of pH and Storage Temperature on Antimicrobial Activity of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Ogi. *British Microbiol. Research J.* **9**(3):1-9

Sujeet K.M, Vipin K (2017). A Study on Prevalence of Microbial Contamination on the Surface of Raw Salad Vegetable. International *J Food Microbiol*. 6:411 – 418.

Uzoh C.V, Orji J.O, Okeh C.O, Nworie C.O, Igwe P. C(2022). Preliminary probiotic properties of lactic acid bacteria isolated from locally fermented food condiment-Ogiri. *International J Advanced Res Biol Sci*.9(4): 21-30.

Wayah S.B and Philip K(2018). Pentocin MQ1: A novel, broad spectrum, pore forming bacteriocin from Lactobacillus pentosus CS2 with Quorum sensing regulatory mechanism and biopreservative potential. *Front Microbiol*. 9 : 564-569.

Xinran L, Luhuan M., Huanhuan M, Fengling B., Yang L., Mengtong S. and Jianrong L. (2018). Purification, characterization and action mechanism of plantaricin JY22, a novel bacteriocin against Bacillus cereus produced by *Lactobacillus plantarum* JY22 from golden carp intestine *Food Sci Biotechnol* **27**(3):695–703.

Yi L., Dang Y, Wu J, Zhang L, Liu X, Liu B, Zhou Y and Lu X (2016). Purification and characterization of a novel bacteriocin produced by *Lactobacillus crustorum* MN047 isolated from koumiss from Xinjiang, China J. Dairy Sci. 99:7002–7015.

Zhang J, Yang Y, Yang H, Bu Y, Yi H, Zhang L, Han X and Ai L (2018) Purification and Partial Characterization of Bacteriocin Lac-B23, a Novel Bacteriocin Production by *Lactobacillus plantarum* J23, Isolated From Chinese Traditional Fermented Milk. *Front. Microbiol*. **9**:2165-2172.

Zhao D , Wang Q , Lu F , Bie X , Zhao H , Lu Z and Lu Y (2022). A novel plantaricin 827 effectively inhibits *Staphylococcus aureus* and extends shelf life of skim milk *LWT - Food Science and Technol* 154 : 1-11.

Bourdichon F., Casaregola S. , Farrokh C., Frisvad J. C., Gerds M. L, Hammes W. P., Harnett J. ,Huys G., Laulund S., Ouwehand A., Powell I. B., Prajapati J. B. , Seto Y, Schure E.T , Boven A.V, Vankerckhoven V., A. , Zgoda , Tuijtelaars S., Hansen E. B (2012). Food fermentations : Microorganisms with technological beneficial use. *International J Food Microbiol.*154 (3): 87-97.