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Detection and Characterization of Bacteriocin-like Substances Produced by *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789

Ngozi Izuchukwu*

Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling, FK9 4LA, UK
Email: izuchukwu.ngozi@gmail.com

Abstract

The aims were to detect and characterize bacteriocin-like substances produced by *C. maltaromaticum* MMF-32 and *C. maltaromaticum* KOPRI 25789, isolated from smoked salmon, and to determine their inhibitory spectra against food-borne pathogens and food spoilage organisms. *Carnobacterium maltaromaticum* were isolated from smoked salmon, and identified phenotypically and by 16S rDNA-targeted PCR. *C. maltaromaticum* isolated from smoked salmon were capable of producing bacteriocin-like substances that inhibited Gram positive and Gram negative spoilage bacteria. The bacteriocin-like substances were stable at 56 ºC for 30 min and 100 ºC for 10 min and at acidic pH, and was proteinaceous in nature. This study shows that bacteriocin producing *C. maltaromaticum* MMF-32 and KOPRI 25789 could potentially be used as a biopreservative for minimally processed seafood.

Keywords: Bacteriocin-like substances; biopreservatives; *Carnobacterium maltaromaticum*; foodborne pathogens; food spoilage organisms; lactic acid bacteria.

1. Introduction

Lactic acid bacteria (LAB) are widely used for the production of fermented food products, their role being to improve the flavour, texture and shelf life [1]. The production of diverse antibacterial compounds, such as organic acids, diacetyl, hydrogen peroxide and proteinaceous molecules, known as bacteriocins leads to the inhibition of food spoilage and pathogenic bacteria [2].

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* Corresponding author.

Bacteriocins are antimicrobial peptides produced by ribosomes of bacteria, which have the characteristics of inhibiting other bacteria. Their spectrum of activity is narrow, notably of the same species or genus [3]. Many bacteriocins display strong activity against food spoilage and pathogenic bacteria, such as *Bacillus, Listeria, Clostridium*, methicillin-resistant *Staphylococcus aureus* and vancomycin resistant enterococci [4].

With the expansion of demand for seafood products, there have been considerable challenges related to preservation/spoilage [5]. LABs are generally regarded as safe (GRAS) for use in foods. Bacteriocins produced by LABs have been planned for future use in the biopreservation of food products by preventing spoilage microorganisms or pathogens [6]. In addition, nisin produced by *Lactococcus lactis* is the only bacteriocin with GRAS grade for use in some foods [7].

Various bacteriocins produced by *Carnobacterium* spp. have been isolated and characterized, including; divergicin M35, produced by *C. divergens* M35 which was isolated from frozen smoked mussels [8]; carnobacteriocin B2 produced by *C. piscicola* strain A9b was isolated from vacuum packed cold-smoked salmon [9]; divergicin A was produced by *C. divergens* NCIMB 702855 [10]. Divercin V41 was recovered from *C. divergens* 41 that was isolated from fish viscera [11]; piscicocin CS526 was produced by *C. piscicola* CS526, which was isolated from frozen surimi [12].

The lactic acid bacteria *C. maltaromaticum* MMF-32 and KOPRI 25789 are bacteriocin-like producing strain isolated from smoked salmon. The detection, characterisation of the bacteriocins and their inhibitory spectra against food-borne pathogens and food spoilage organisms are described in this study.

2. Materials and methods

2.1.1 Bacterial strains, culture media and growth conditions

Carnobacterium maltaromaticum isolates [from smoked salmon] were grown routinely on de Man Rogosa and Sharpe agar (MRS; Oxoid) at 30 °C for 48 h. Stock cultures were stored in tryptone soya broth (TSB; Oxoid) supplemented with 1% (w/v) sodium chloride [= TNB] and 20% (v/v) glycerol at -70 °C [13].

2.1.2 Extraction of LAB cell free supernatant

Carnobacterium maltaromaticum MMF-32 and KOPRI 25789 were grown in 500 ml volumes of MRS broth at 30 °C for 48 h. The optical density (OD) was 0.6. The bacterial cells were removed by centrifugation (10,000 x g for 10 min at 4 °C), and cell free supernatants were passed through 0.45 µm pore size filters (Sartorius; Stedim Biotech, GmbH Goettingen, Germany) and stored at -20 °C before use. To rule out the possibility that the inhibition might have been caused by acidification of the media induced by LAB metabolism, cell free supernatants were adjusted with 2 N NaOH to pH 6.5 ± 0.2 and filtered by passage through 0.45 µm of pore size filters. Dilutions of cell-free supernatants were made using TNB.

2.1.3 Preparation of bacterial indicator

Bacterial strains used as indicator were *Shewanella baltica* OS185, *Aeromonas* sp. HB-6, *Shewanella baltica*, *Shewanella baltica* OS678, *Serratia* sp. I-113-31, *Aeromonas salmonicida* subsp. *achromogene*s, *Aeromonas hydrophila* HX201006-3 and *Listeria monocytogenes* ATCC 19114. These bacterial indicators were incubated overnight at 30 °C in TNB. The overnight bacterial cultures of the indicator strains were centrifuged at 2000 g for 10 min at 4 °C, and the cell free supernatants were removed. The bacterial cells were washed twice in 0.85% (w/v) sterile saline, and serial dilutions of the washed bacterial cells were prepared in saline to realize10^5 CFU ml^{-1} as determined by [14].

2.1.4 Broth bioassays

Broth assays were performed as follows: 100 µl volumes of the serially diluted cell free supernatants from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 were pipetted into triplicate wells in 96-well microtitre plate together with 100 µl volumes of indicator bacteria containing 10^5 CFU ml^{-1}. Incubation of the microtitre plates was at 30 °C for 40 h and the presence of turbidity in the wells, was considered as indicative of the absence of inhibition. Hundred microlitres of positive and negative controls were also tested. Growth inhibition was measured spectrophotometrically at 600 nm and 610 nm every 8 h for 40 h with a microtitre plate reader (Fisher Scientific; BioTek, USA). Cell free supernatant extracts from the nisin-producer *Lactococcus lactis* subsp. *lactis* NCIMB 8586 were used against indicator bacteria as comparable controls.

2.2 Characterization of antimicrobial compounds

The tests were carried out with cell free supernatant extracts from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 strains grown at 30 °C for 48 h.

2.2.1 Effect of thermal treatment on bacteriocin activity

The effect of temperature on cell free supernatant extracts was determined as follows: the cell free supernatant extracts in sterile Bijoux bottles were placed in a water bath (Grant Instruments; Cambridge, Barrington, England). The cell free supernatant extracts were heated to 56 °C using a water bath for 30 min. For heating at 100 °C for 10 min, sterile 1.5 ml Eppendorf tubes containing the cell free supernatants were heated on a heating block (Techne; Cambridge, England).

2.2.2 Effect of pH range on bacteriocin activity

To check pH stability of the bacteriocins, the supernatant extracts from the two lactic acid bacteria were adjusted to pH 3.5, 4.9 and 6.5 by adding appropriate volumes of 2 N HCl or 2 N NaOH. Then, the samples were filtered through 0.45 µm filters (Sartorius), and antimicrobial activity was determined as described above. Negative controls aimed at making clear the possible role of acid pH values in the inhibition of *L. monocytogenes,* were prepared testing portions of non-inoculated MRS broth whose pH values were adjusted to 3.5, 4.9 and 6.5 [15].

2.2.3 Sensitivity of cell-free supernatants to proteases

The sensitivity of neutralized (i.e. cell-free supernatants adjusted to pH 6.5 using 2N NaOH) cell-free supernatants extracts to proteolytic digestion was investigated by the addition of proteinase K, trypsin, α-chymotrypsin (Sigma-Aldrich) and lysozyme (Fluka) at a final concentration of 1 mg ml^{-1} [16]. Samples with and without proteases were incubated aerobically for 3 h at 30 °C, and the residual activity was determined as described above. The presence of turbidity in the presence of proteases indicates absence of inhibition confirming the proteinaceous nature of the antimicrobial substances [17].

2.3 Statistical analysis

Statistical analysis involved use of IBM SPSS. A Kruskal-Wallis test was run to determine if there were differences in different bacteriocin samples and the treatments given to the groups. Pairwise comparisons were performed using [18] procedure with Bonferroni correction for multiple comparisons. Statistical significant level was fixed to $p < 0.05$.

3. Results

3.1 Identification and screening of bacteriocinogenic LABs

The strains were identified using 16S rDNA-targeted PCR, and demonstrated homology levels of 99%, [19], and by phenotypic characteristics using the API-50CH system based on [20,21]. The broth assay method using the 96-well microtitre plates were used to study the antimicrobial activity of the 2 LAB cultures. Cell-free supernatants were shown to exhibit inhibitory activity against *Shewanella baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Shewanella baltica*, *Shewanella baltica* OS678, *Serratia* I-113-31 and *A. salmonicida* subsp. *achromogenes*. Optical density measurements using microplate readers are used to determine the inhibitory effect of antimicrobials following the method of [22].

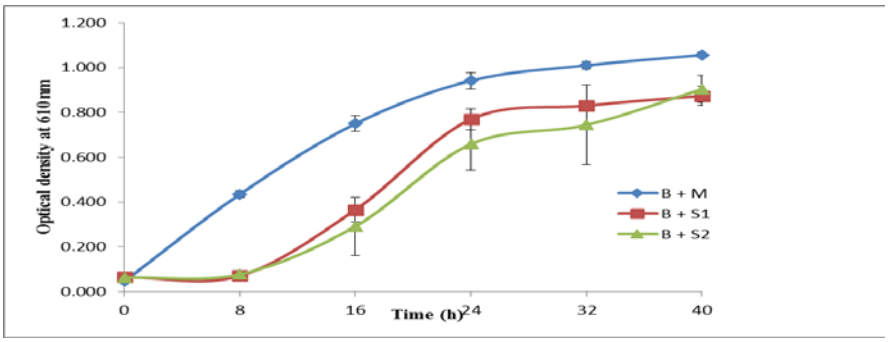
The inhibitory effect of cell-free supernatants from *C. maltaromaticum* KOPRI 25789 and MMF-32 against *Shewanella baltica* OS185 were shown in (Fig. 1 A.). The inhibitory assay revealed very weak inhibition of *Shewanella baltica* OS185 by the supernatants of KOPRI 25789 and MMF-32 during the 0-8 h incubation, respectively. The 0-8 h incubation period showed bacteriostasis.

C. maltaromaticum KOPRI 25789 and MMF-32 cell-free supernatants had no effect on the activity of *L. monocytogenes* ATCC 19114 after 8 h of incubation, respectively (Fig. 1 B.).

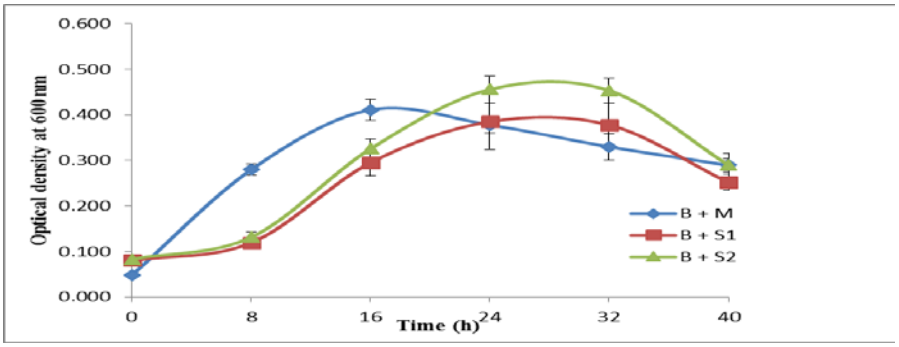
The addition of the cell-free supernatants of both LAB cultures to *Aeromonas* HB-6 (Fig 1 C.) revealed strong inhibition for 40 h. Fairly strong inhibition was observed with *A. hydrophila* HX201006-3 (Fig. 1 D.) from 0-40 h with the addition of the cell-free supernatants of both LAB cultures.

Growth inhibition of *Sh. baltica* and *Sh. baltica* OS678 by the addition of the cell-free supernatants of both LAB cultures showed strong inhibition (Figs. 1 E. and 1 F.). Addition of *C. maltaromaticum* KOPRI 25789 and MMF-32 to *Serratia* I-113-31 revealed weak inhibition during the 40 h of incubation (Fig.1 G.).

Very weak inhibitory effect of cell-free supernatants of *C. maltaromaticum* KOPRI 25789 and MMF-32 against *A. salmonicida* subsp. *achromogenes* was observed from 0-8 h of incubation (Fig. 1 H.). The inhibitory effects produced by organic acids were eliminated by adjusting the supernatants to pH 6.5 using 2 N NaOH. The effect of hydrogen peroxide was removed by using bovine liver catalase.

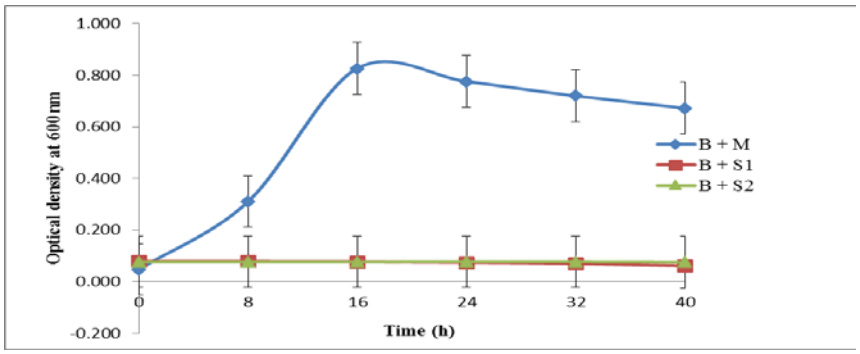


A



B

Figure 1: Growth inhibition test of *Sh. baltica* OS185 (B + M) (A) and *L. monocytogenes* ATCC19114 (B + M) (B) determined at 30 °C for 40 h by addition of cell-free supernatant of *C. maltaromaticum* KOPRI 25789 (B + S1) and *C. maltaromaticum* MMF-32 (B + S2). The values presented are the mean of six independent experiments. Points = Means ± SE.



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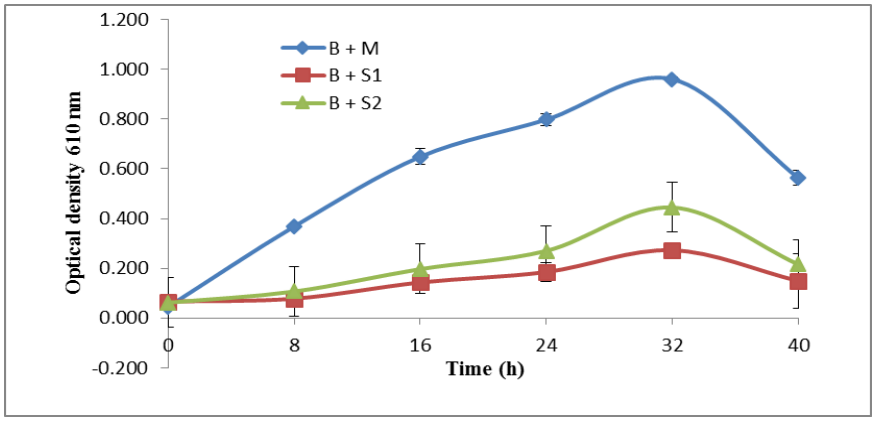

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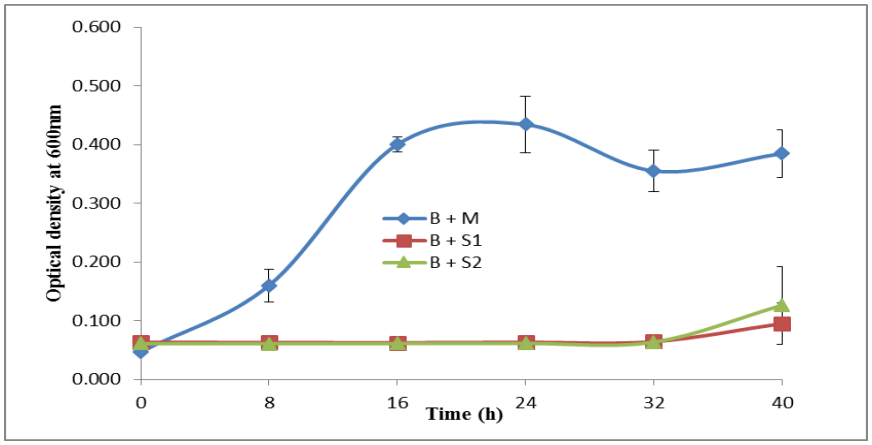
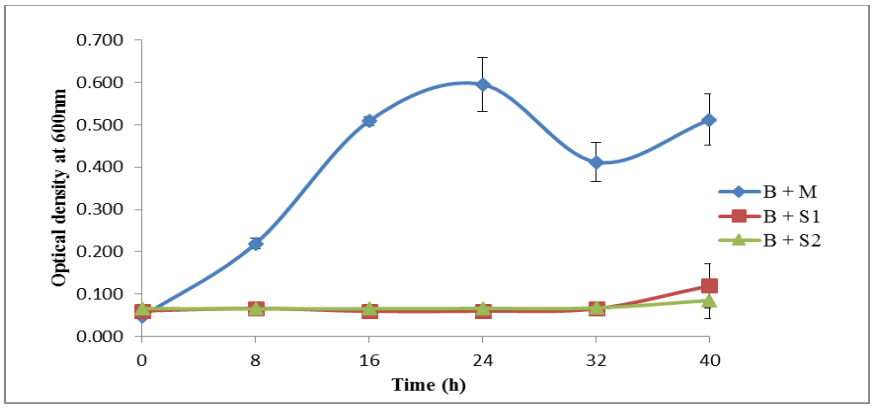


E

Figure 1: Growth inhibition test of *Aeromonas* HB-6 (B + M) (C), *A. hydrophila* HX201006-3 (B + M) (D) and *Sh. baltica* (B + M) (E) determined at 30 °C for 40 h by addition of cell-free supernatant of *C. maltaromaticum* KOPRI 25789 (B + S1) and *C. maltaromaticum* MMF-32 (B + S2). The values presented are the mean of six independent experiments. Points = Means ± SE.



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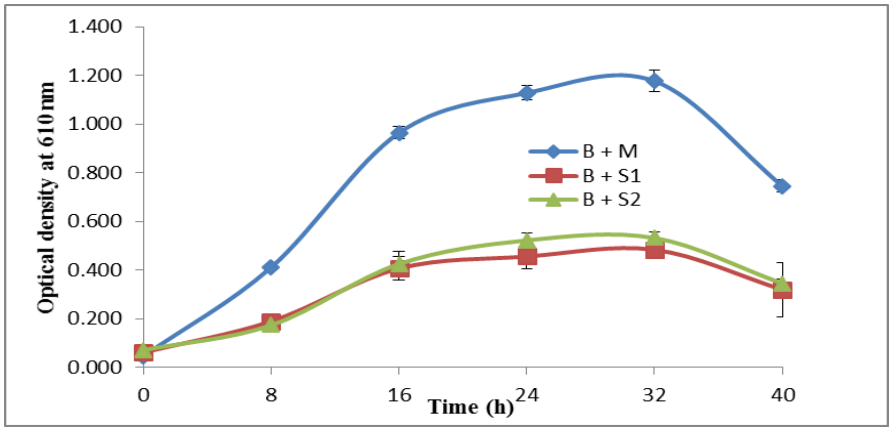
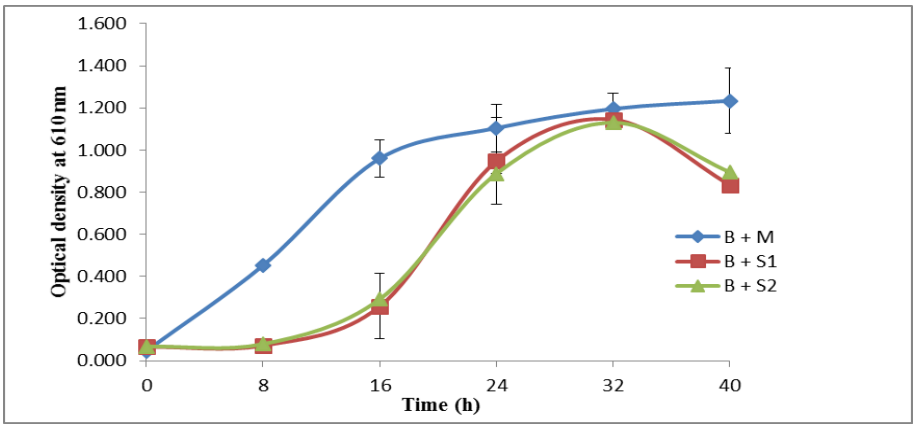


G



H

Figure 1: Growth inhibition test of *Sh. baltica* OS678 (B + M) (F), *Serratia* sp. I-113-31 (B + M) (G) and *A. salmonicida* subsp. *achromogenes* (B + M) (H) determined at 30 °C for 40 h by addition of cell-free supernatant of *C. maltaromaticum* KOPRI 25789 (B + S1) and *C. maltaromaticum* MMF-32 (B + S2). The values presented are the mean of six independent experiments. Points = Means ± SE.

3.2 Characterization of the antimicrobial compounds

3.2.1 Effect of temperature treatments

The thermal resistance of the bacteriocins produced by strains KOPRI 25789 and MMF-32 were determined. The treatment of the extracellular extracts of the two bacteriocin-producing LAB strains at 56 °C for 30 min and 100 °C for 10 min led to antimicrobial activity against *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* sp. I-113-31 and *A. salmonicida* subsp. *achromogenes* (Table 1.).

Inhibitory activities of KOPRI 25789 and MMF-32 were not completely destroyed by the heat treatment, i.e. 56 °C for 30 min (fairly weak inhibition) and 100 °C for 10 min (very weak inhibition), against *Sh. baltica* OS185.

Residual activity of 40% and 20% was retained respectively, at 56 °C for 30 min and 100 °C for 10 min by the supernatants of the 2 LAB after heat treatment.

The antibacterial activities of KOPRI 25789 and MMF-32 were not completely lost during heat incubation at 56 °C for 30 min (very weak inhibition) and 100 °C for 10 min (very weak inhibition) against *L. monocytogenes* ATCC 19114. Some 20% of the residual activity of the supernatants was retained from the 2 LABs after both heat treatments.

KOPRI 25789 and MMF-32 retained their antagonistic activities when heat treatment was applied, i.e. 56 °C for 30 min (fairly strong and strong inhibition, respectively) and 100 °C for 10 min (fairly weak and strong inhibition, respectively) against *Aeromonas* HB-6. The supernatants from the two LABs retained 80% and100% residual activities respectively, after treatment at 56 °C for 30 min, while 40% and 100% activities were recorded respectively, at 100 °C for 10 min.

Antagonistic activity of KOPRI 25789 supernatant was not completely lost when heat treatment was applied at 56 °C for 30 min (very weak inhibition) against *A. hydrophila* HX201006-3, a residual activity of 20% was retained. Heat treatment at 56 °C for 30 min applied to the supernatant of strain MMF-32 against *A. hydrophila* HX201006-3 revealed that total activity was not completely lost (fairly weak inhibition). A residual activity of 40% was retained. Heating of KOPRI 25789 and MMF-32 supernatants at 100 °C for 10 min showed an activity loss after 8 h of incubation (very weak inhibition)., Residual activities of 20% were retained, respectively of 2 LAB after heat treatment at 56 °C for 30 min and 100 °C for 10 min.

The antagonistic activities of KOPRI 25789 and MMF-32 cell-free supernatants were retained after heat treatment at 56 °C for 30 min against *Sh. baltica* (fairly strong and strong inhibition, respectively). Residual activities of 80% and100% were retained by the 2 LABs respectively. Heat treatment of KOPRI 25789 and MMF-32 cell-free supernatants at 100 °C for 10 min against *Sh. baltica* revealed loss of activity at 16 and 32 h respectively by the 2 LABs (very weak and fairly strong inhibition). The residual activities retained by the 2 LABs were 20% and 80% respectively.

Heat treatment at 56 °C for 30 min for KOPRI 25789 and MMF-32 cell-free supernatants against *Sh. baltica* OS678 revealed that their antagonistic activities were retained. Complete residual activity was retained with KOPRI 25789 and MMF-32, respectively (strong inhibition). Antagonistic activity of KOPRI 25789 cell-free supernatant with heat treatment of 100 °C at 10 min against *Sh. baltica* OS678 revealed very weak inhibition at 40 h of incubation, with a residual activity of 20% retained. Antagonistic activity of MMF-32 cell-free supernatant was retained with heat treatment of 100 °C at 10 min against *Sh. baltica* OS678 (fairly strong inhibition), a residual activity of 80% was retained.

Antagonistic activities of KOPRI 25789 and MMF-32 cell-free supernatants with heat treatments at 56 °C for 30 min and 100 °C at 10 min against *Serratia* I-113-31 were retained (weak inhibition). A residual activity of 60% was retained.

Antagonistic activities of KOPRI 25789 and MMF-32 cell-free supernatants with treatments at 56 °C at 30 min

and 100 °C at 10 min against *A. salmonicida* subsp. *achromogenes* were not completely lost (very weak inhibition). Heat treatment at 56 °C for 30 min and 100 °C at 10 min revealed a loss of antagonistic activity of KOPRI 25789 and MMF-32 cell-free supernatants at 8 h of incubation. Residual activity retained was 20%, respectively.

Table 1: Effect of thermal treatments on cell-free supernatant activity of *C. maltaromaticum* KOPRI 25789 and MMF-32 against indicator strains using broth assay method.

Indicator strains	Time of thermal treatment	Temperature (°C)	KOPRI 25789	Thermal	MMF-32	Thermal
			Untreated[a]	Treated	Untreated[a]	Treated
Sh. baltica OS185	30 min	56	++	++	++	++
	10 min	100	+	+	+	+
L. monocytogenes ATCC19114	30 min	56	+	+	+	+
	10 min	100	+	+	+	+
Aeromonas sp. HB-6	30 min	56	++++	++++	++++	+++++
	10 min	100	++	++	+++	+++++
A. hydrophila HX201006-3	30 min	56	+	+	+	++
	10 min	100	+	+	+	+
Sh. baltica	30 min	56	+	++++	++++	+++++
	10 min	100	+	+	+	++++
Sh. baltica OS678	30 min	56	+++++	+++++	+++++	+++++
	10 min	100	+	+	++++	++++
Serratia I-113-31	30 min	56	+++	+++	+++	+++
	10 min	100	+++	+++	+++	+++
A. salmonicida subsp. *achromogenes*	30 min	56	+	+	+	+
	10 min	100	+	+	+	+

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of six independent experiments. Control samples consists of freshly prepared cell-free supernatants without thermal treatment.

3.2.2 Effect of pH treatment

Antagonistic activity of the cell-free supernatants of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum*

MMF-32 treated at pH 3.5 and 4.9 against indicator organisms was retained Residual activity retained was 100% (Table 2). The untreated cell-free supernatants used as positive control showed antagonistic activity based on indicator organisms used.

Table 2: Effect of pH on cell-free supernatant activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 against indicator strains using broth assay method.

Indicator strain	pH treatment	KOPRI 25789		MMF-32 Untreated	
		Untreated	Treated		Treated
Sh. baltica OS185	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++
L. monocytogenes ATCC19114	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++
Aeromonas HB-6	3.5	+++	+++++	++	+++++
	4.9	++	+++++	+	+++++
A. hydrophila HX201006-3	3.5	++	+++++	+	+++++
	4.9	++	+++++	+	+++++
Sh. baltica	3.5	+++++	+++++	+++	+++++
	4.9	++++	+++++	+++	+++++
Sh. baltica OS678	3.5	++	+++++	++	+++++
	4.9	++	+++++	++	+++++
Serratia I-113-31	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++
A. salmonicida subsp. *achromogenes*	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of six independent experiments. Control samples consist of freshly prepared cell-free supernatants without thermal treatment.

3.2.3 Enzymatic activity directed towards putative bacteriocins

Cell-free supernatants of KOPRI 25789 and MMF-32 exhibited no reduction, partially reduced, reduced, weakly reduced and very weakly reduced antimicrobial activity due to the action of proteases (Table 3.). The residual antagonistic activity of the LAB supernatants after protease treatment was determined by the role of the

microorganisms used as indicator and did not follow a particular pattern [15]. For example, the cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin showed very weak and fairly weak activity against *Sh. baltica* OS185, respectively. Treatment of cell-free supernatants with lysozyme, proteinase K and trypsin against *Sh. baltica* OS185 showed very weak reduced activity, respectively.

The antimicrobial activity exhibited by cell-free supernatants of KOPRI 25789 and MMF-32 treated with α-chymotrypsin against *L. monocytogenes* ATCC 19114 was very weakly reduced.

Treatment of the cell-free supernatants with lysozyme showed strong antimicrobial activity against *L. monocytogenes* ATCC 19114 for KOPRI 25789 and MMF-32 cell-free supernatants. Cell-free supernatants of KOPRI 25789 and MMF-32 showed a significant very weakly reduced antimicrobial activity against *L. monocytogenes* ATCC 19114, when treated with proteinase K and trypsin.

The antimicrobial activity of the cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin and proteinase K against *Aeromonas* HB-6 revealed strong activity. Cell-free supernatants of strain KOPRI 25789 treated with lysozyme led to a weak reduction. The cell-free supernatants treated with lysozyme and trypsin against *Aeromonas* HB-6 revealed weakly reduced activity.

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin, against *A. hydrophila* HX201006-3 revealed very weak and fairly weak activity, respectively. Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with lysozyme, proteinase K and trypsin showed very weak activity, respectively.

Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin and proteinase K against *Sh. baltica* showed strong activity. Trypsin and lysozyme treated cell-free supernatants of strains KOPRI 25789 and MMF-32, revealed fairly strong activity.

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin against *Sh. baltica* OS678 showed no reduction in antimicrobial activity of the cell-free supernatants. Treatment of cell-free supernatant of KOPRI 25789 and MMF-32 with lysozyme against *Sh. baltica* OS678 revealed very weak activity.

The antimicrobial activity of KOPRI 25789 and MMF-32 cell-free supernatants were treated with proteinase K against *Sh. baltica* OS678 revealed strong activity. Cell-free supernatants of strain KOPRI 25789 and MMF-32 treated with trypsin against *Sh. baltica* OS678 revealed weak activities.

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin, lysozyme, proteinase K and trypsin against *Serratia* I-113-31 showed revealed weak reduced antimicrobial activity.

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin, lysozyme, proteinase K and trypsin against *A. salmonicida* subsp. *achromogenes* revealed very weak activity.

Table 3: Effect of proteases on cell-free supernatant activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 against indicator strains using broth assay.

Indicator strain	Enzyme treatment	KOPRI 25789 Untreated	Treated	MMF-32 Untreated	Treated
Sh. baltica OS185	α-Chymotrypsin	+	+	+	++
	Lysozyme	+	+	+	+
	Protease K	+	++	+	++
	Trypsin	+	+	+	+
L. monocytogenes ATCC19114	α-Chymotrypsin	+	+	+	+
	Lysozyme	+	+++++	+	+++++
	Proteinase K	+	+	+	+
	Trypsin	+	+	+	+
Aeromonas HB-6	α-Chymotrypsin	+++++	+++++	+++++	++++
	Lysozyme	+++	+++	+++	+++
	Proteinase K	+++++	+++++	+++++	+++++
	Trypsin	+++	+++	+++	+++
A.hydrophila HX201006-3	α-Chymotrypsin	+	+	+	++
	Lysozyme	+	+	+	+
A.hydrophila HX201006-3	Proteinase K	+	+	+	+
	Trypsin	+	+	+	+
Sh. baltica	α-Chymotrypsin	+++++	+++++	+++++	+++++
	Lysozyme	++++	++++	++++	++++
	Proteinase K	+++++	+++++	+++++	+++++
	Trypsin	++++	++++	++++	++++
Sh. baltica OS678	α-Chymotrypsin	+++++	+++++	+++++	+++++
	Lysozyme	+	+	+	+
	Proteinase K	+++++	+++++	+++++	+++++
	Trypsin	+++	+++	+++	+++
Serratia I-113-31	α-Chymotrypsin	+++	+++	+++	++++
	Lysozyme	+++	+++	++	+++
	Proteinase K	++	+++	++	+++
	Trypsin	++	+++	++	+++
A. salmonicida subsp. *achromogenes*	α-Chymotrypsin	+	+	+	+
	Lysozyme	+	+	+	+
	Proteinase K	+	+	+	+
	Trypsin	+	+	+	+

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly

strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of six independent experiments. Control samples consist of freshly prepared cell-free supernatants without thermal treatment.

4. Discussion

Bacteriocin-producing *Carnobacterium* strains were isolated from smoked salmon and were identified using a 16S rDNA-targeted PCR method [19] and by morphological, cultural and biochemical characteristics using API-50CH, based on the methods of [20,21]. *C. maltaromaticum* often abounds as a member of the microflora of chilled vacuum or modified atmosphere-packed meat and seafood. Moreover, several studies have been dedicated to the application of carnobacteria for biopreservation of foods [23;24] including various sea foods [25;26;27].

The bacteriocin like substances (BLIS) from *C. maltaromaticum* KOPRI 25789 and MMF-32 are interesting antimicrobial compounds because they exhibit broad spectrum inhibitory activity against Gram-positive and Gram-negative bacteria (Figs. 1.A-H). Thus, the bacteriocins were tested for antibacterial activity against *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* I-113-31 and *A. salmonicida* subsp. *achromogenes*. The highest inhibitory activity was exhibited against *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678 and *Serratia* sp. I-113-31, whereas the least activity was exhibited against *A. salmonicida* subsp. *achromogenes*, *Sh. baltica* OS185 and *L. monocytogenes* ATCC 19114. The antimicrobial substances researched in this work are referred to as BLIS as they have not been isolated and their amino acid sequences have not been characterized.

The results of the present study show the presence of bacteriocin in the LAB. Studies have shown bacteriocins to be inhibitory against several other bacteria [2;28;3]. Some carnobacterial bacteriocin producing strains have been reported to be active against *Lactobacillus farciminis*, *Brochothrix thermosphacta*, *Shewanella putrefaciens*, *L. monocyogenes*, *Staphylococcus xylosus*, *Pseudomonas* spp., *Serratia liquefaciens* and *Staphylococcus aureus* [29]. These compounds may serve as natural substitutes for chemical food preservatives to enhance shelf-life of food, [6].

Furthermore, the results revealed a high level of antibacterial activity of *C. maltaromaticum* KOPRI 25789 and MMF-32 against Gram-negative bacteria (Figs 1.A-H), which is unusual, and has been reported for only a few LAB bacteriocins [30;31]. For example, [32] demonstrated the activity of sakacin C2 produced by *Lactobacillus sakei* C2 against *E. coli* ATCC 25922, *Salmonella enterica* serovar Typhimuriu*m* CMCC 47729 and *Shigella flexineri* CMCC 51606. Furthermore, sakacin LSJ618 inhibited *E. coli* ECX4 and *Proteus* sp. [33]. In contrast, [34] demonstrated that the antagonistic effect of cell-free supernatant from *C. piscicola* 526 did not inhibit *E. coli* IFO15034, *Salmonella enterica* serovar Enteritidis RIMD1933001, *Ps. fluorescens* JCM5963m and *A. hydrophila* IFO3820.

In addition, the results from this study showed a reduced level of activity against *L. monocytogenes* ATCC 19114 by the cell-free supernatants of *C. maltaromaticum* KOPRI 25789 and MMF-32 (Fig. 1.B). The work is consistent with the results of [29], who demonstrated that their LAB strains revealed only weak inhibition of *L. monocytogenes*. However, it should be mentioned that several studies have confirmed the ability of LAB strains, isolated from various food products, to inhibit *L. monocytogenes* [35;36;37;38]. *Listeria* spp. comprises part of the inhibition range of carnobacterial class IIa bacteriocins, and the inhibitory activity is the result of pore formation, dispersion of membrane potential, and leakage of internal low molecular weight substances [39]; [40]. Resistance of *L. monocytogenes* to divergicin M35 a bacteriocin produced by *C. divergens* M35 was likely due to change in structure of the cell wall fatty acid composition [41]. According to [42], resistance of *L. monocytogenes* strains to class IIa divercin V41 also revealed considerable differences in protein expression as compared with the wild type. This means that for the resistant strain of *L. monocytogenes* the molecular mass of the protein ranges from 25 – 65 kDa, while the sensitive type had a protein molecular mass ranging from <20 – 35 kDa. Thus changes in cell envelope fatty acid composition [43;41], D-alanine content of teichoic acids i.e. α-amino acid that occurs in fortified (teichoic acids) bacterial cell wall [43], alterations in cell surface charge [43] or changes in the major cell membrane components [44;45] were reported to be involved in the mechanisms of resistance to class IIa bacteriocins.

Reference [46] reported that bacteriocin production was strongly dependent on temperature, pH and nutrient source. Different physicochemical factors seemed to affect bacteriocin production as well as its activity [2]. Nevertheless, it is important to note that in the present study the thermal effect of these BLIS on indicator strains revealed strong activity, fairly strong activity, weak activity, fairly weak and very weak activity (Table 1). The BLIS under the thermal regimes of (56 °C for 30 min and 100 °C for 10 min) against *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *A. hydrophila* HX201006-3 and *A. salmonicida* subsp. *achromogenes* showed fairly weak and very weak activity. The thermal regimes of the BLIS against *Aeromonas* sp. HB-6, *Sh. baltica* and *Sh. baltica* OS678, revealed strong activity, fairly strong activity and weak activity, respectively, whereas for *Serratia* sp. I-113-31 it showed fairly strong activity. Thus the inhibitory activity of both cell-free supernatant under both heat regimes treated against *Aeromonas* sp. HB-6, *Sh. baltica*, *Sh. baltica* OS678 and *Serratia* sp. I-113-31 were stable.

Reference [47] reported the stability of partially purified bacteriocin by *L. lactis* subsp. *lactis* H-559 at 100 °C for 10 min. According to [48], the thermal stability may be caused by the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkages, and high glycine content. Piscicolin 126 produced by *C. piscicola* JG was stable at 100 °C over a wide range of pH [49]. As reported in the present study (Table 1), [50] demonstrated that both treated (at 100 °C for 30 min) and nontreated supernatants of B26 and B33 of carnobacteria isolates revealed stability against various bacteria. The inhibitory activity of cell-free supernatant of *C. piscicola* CS526 was completely stable after 30 min at 100°C [34]. Thermal treated and untreated supernatants exhibited reduced or stable activity against indicator microorganisms, showing that it is proteinaceous in nature and that it denatures at certain temperature [51].

The activity of BLIS in this study was dependent on the producer strain and indicator microorganisms. The results showed that antibacterial activities of KOPRI 25789 and MMF-32 were lost at 8 h of incubation against

L. monocytogenes ATCC 19114 when the heat regimes of 56 °C for 30 min and 100 °C for 10 min were applied. Similarly, the antibacterial activity of BLIS substances were lost at 8 h of incubation against *A. salmonicida* subsp. *achromogenes* when heat treatment of 56 °C for 30 min was applied. Conclusively, there was no antibacterial effect on *L. monocytogenes* ATCC 19114 and *A. salmonicida* subsp. *achromogenes* by the 2 LAB with the application of the heat regimes. The thermal stability at 56 °C for 30 min and 100 °C for 10 min (Table 1) of BLIS produced by the bacteriocin-producing LAB isolates described here may suggest possible use as biopreservatives in combination with heat processing in order to preserve food products, in procedures like pasteurization, drying, refrigeration and freezing.

pH can enhance the antimicrobial activity of LAB, it must be an increase, decrease or within certain range. pH of media affected the antimicrobial activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 against for *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* I-113-31 and *A. salmonicida* subsp. *achromogenes* (Table 2). The maximum antimicrobial activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 was increased significantly at pH 3.5 and 4.9. A reduced activity was observed at pH 6.5 used in analysing the inhibitory effect of the cell-free supernatants against the indicator microorganisms. Thus, the antimicrobial activity increased significantly with decrease in pH. Residual activity of 100% was observed for all samples treated to pH 3.5 and 4.9. [52] hypothesized that organic acids act on the cytoplasmic membrane by neutralizing its electrochemical potential and increasing its permeability, thus leading to bacteriostasis and eventual death of the susceptible bacteria. Further to the effect of bacteriocin produced by these strains, the potent activity could be attributed to the production of lactic acid at lower pH value [53] or plausibly because of the better penetration of organic acids produced by LAB into the microbial cell wall at pH around 4.9 [54]. Reduced activity at higher pH is a mark of alkali lysis of bacteriocin at high pH [55]. The activity of cell-free supernatant of *C. piscicola* CS526 was completely stable at pH 2 to 8 but reduced at values pH of 9 to 11, antimicrobial activity of 67, 44 and 30% was observed respectively, of the cell-free supernatant [34].

Bacteriocins produced by lactic acid bacteria are endowed with general stability at acidic or neutral pH, showing that these substances are well modified to the environmental conditions produced by the bacteria [56]. The maximum antimicrobial activity of *L. lactis* subsp. *lactis* TI-4 was observed at pH 5 [57]. Reference [3] demonstrated the stability at broader pH value between 4.0 and 9.0 of bacteriocin from *L. acidophilus* NCIM5426. *L. brevis* OG1 exhibited the highest antibacterial activity in an acidic pH range of 2 to 6, while inactivation occurred at pH 8 to 12 [58]. Our result showed that *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 inhibited the growth of *L. monocytogenes* ATCC 19114 and *A. salmonicida* subsp. *achromogenes* at the pH range of 3.5 and 4.9 in contrast to the result at pH 6.5. These results indicate that the BLIS described in this study might be possibly used for application in both low and medium–acid fermented food product having final pH with in such ranges and may well be used in fermented foods.

Bacteriocins may be degraded by some proteolytic enzymes resulting in a loss in their antimicrobial activity [28]. In this study, *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* sp. I-113-31 and *A. salmonicida* subsp. *achromogenes* were used as indicators and the BLIS produced by *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum*

MMF-32 had various inhibitory effects as a result of treatment with α-chymotrypsin, lysozyme, proteinase K and trypsin (Table 3).

Antimicrobial activity was reduced against *L. monocytogenes* ATCC 19114 with cell-free supernatant treated with α-chymotrypsin, protease K and trypsin residual activity of 20% was retained respectively. In contrast, complete activity was lost against *L. monocytogenes* when cell-free supernatant from *C. piscicola* CS526 treated with α-chymotrypsin and protease K, whereas trypsin partially inhibited with a residual activity of 67% [34]. Antimicrobial activity was not lost against *L. monocytogenes* ATCC 19114 with cell-free supernatants treated with lysozyme, residual activity of 100% was recorded.

Reference [28] demonstrated the use of *Listeria innocua* and *Lactobacillus sakei* as indicators, and the BLIS produced by different LAB strains had various inhibitory effects following use the treatment with proteolytic enzymes.

With regard to the antibacterial activity exhibited by proteolytic enzyme treatments and the fact that the antimicrobial activities of the BLIS were not affected by catalase, I presume that antibacterial activities could be mainly due to proteinaceous compounds. The failure of the proteolytic enzymes to modify the antimicrobial activity of the bacteriocin is not unusual. Reference [59] demonstrated the use of pepsin, papain, trypsin, chymotrypsin, protease K, lysozyme, catalase, DNase and RNase to treat thuricin 7, a bacteriocin produced *Bacillus thuringiensis* BNG 1.7 and they observed that the inhibitory activity was only susceptible to protease K. Thus, the observed resistance to proteolytic enzymes might be due to the presence of unusual amino acids in the bacteriocin structure, or cyclic N- and C-terminal blocked peptides [60]. The resistance of cyclic peptide to hydrolysis by proteases may be mainly due to their cyclic structure rendering them relatively inflexible, which may make cleavage sites inaccessible due to steric hindrance [61].

5. Conclusion

In conclusion, the antimicrobial activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 were similar. The strains might be useful in preventing the development of spoilage microorganisms in foodstuffs since the bacteriocins produced by the lactic acid bacteria are stable at 100°C for 10 min and 56°C for 30 min and pH ranges of (3.5, 4.9 and 6.5), and susceptible to enzyme treatments which confirms their potential application as biopreservatives for food products subjected to pasteurization, cook-chilling, sterilization, fermentation and other heat processing treatments.

6. Constraints/Limitations

The cell free supernatants of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 were not heated at 100°C for 10 min before storage at -20°C. This resulted to the partial degradation of the cell free supernatant by the proteolytic enzymes present, thereby reducing its bioactivity against *Listeria monocytogenes*. A more effective recovery of bacteriocin activity may have been realised if the method described by [62] was used for the treatment of the cell free supernatants before use.

7. Recommendation

Cell free supernatant from bacteriocin cultures should be heated to 100°C for 10 min before storage at -20°C.

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