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Use of Bacteriocin Producing *Lactococcus lactis* subsp. *lactis* LABW4 to Prevent *Listeria monocytogenes* Induced Spoilage of Meat

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Abstract

A bacteriocin producing strain of *Lactococcus lactis* subsp. *lactis* LABW4 was isolated from naturally fermented milk product which exhibited strong antibacterial activity against *Listeria monocytogenes* MTCC657, a food spoilage psychrophilic organism. Both cell free and heat killed supernatants of LABW4 were effective to produce zones of inhibition against *L. monocytogenes in vitro.* The antibacterial metabolite(s) of LABW4 showed strong cidal effect on the growth of *L. monocytogenes.* Meat samples, mixed with heat killed supernatant of LABW4 when inoculated with *Listeria,* remain fresh up to 25 days in refrigerated condition whereas spoilage started immediately after 24 hours of inoculation for control sets. Enhancement of *Lactate dehydrogenase* of *L. monocytogenes* upon treatment with LABW4 cell free supernatant suggested its lytic mode of action. Cell lysis or degradations were also supported by scanning electron micrograph of treated cells.

Keywords

Lactic Acid Bacteria, Bacteriocin, Spoilage of Meat, *Listeria monocytogenes*, *Lactate dehydrogenase*

1. Introduction

Lactic acid bacteria are Gram positive, usually non motile, non spore forming rods and cocci. The cellular energy of lactic acid bacteria is derived from the fermentation of carbohydrates to produce principally lactic acid. Along with their common fermentation properties these group of bacteria are the good source of different kinds

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of antimicrobial compounds like organic acids, diacetyl, hydrogen peroxide and proteinaceous bacteriocins [1]. From the very early periods till now lactic acid bacteria are used as biopreservatives because most of the bacteria in this group are reported as generally recognised as safe (GRAS). So now a day's lactic acid bacteria are used to prevent food spoilage replacing the commonly used chemical preservatives.

Bacteriocins produced by lactic acid bacteria are small, ribosomally synthesized, antimicrobial peptides or proteins. A large number of bacteriocin produced by different lactic acid bacteria have been characterized. Several studies have suggested that LAB starter cultures or co-cultures are able to produce their bacteriocins in food matrices, and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacteria [2].

The Gram positive bacteria *Listeria monocytogenes* is a common food borne pathogen and is responsible for several outbreak of food borne diseases. The organism occurs widely in different food products, with the highest incidences being found in poultry, meat, and seafood products [3]. Because of its widespread occurrence in nature and its ability to tolerate environmental stresses such as low pH, low temperature and salt concentration up to 10%, the potential for post processing contamination of foods with *L. monocytogenes* is high [4] [5].

Control of *Listeria monocytogens* using bacteriocin producing lactic acid bacterial strains has been reported by several workers. One such organism, *Pediococcus acidilactici*, produces a bacteriocin which was effective to inhibit *L. monocytogenes* in some dairy products [6]. Buyong *et al.* [7] studies on use of a genetically engineered *Lactococcus lactis* subsp. *lactis* MM217 to control *Listeria monocytogenes* in cheddar cheese.

In the present work we have tried to study the anti-listerial activity of a lactic acid bacterium LABW4 isolated from good quality fermented milk product. In addition to check the anti-listerial activity of LABW4 *in vivo*, we have also tried to control the spoilage of meat caused by *Listeria monocytogenes* by challenge study.

2. Materials and Methods

2.1. Isolation of Lactic acid Bacteria

Lactic acid bacterial strains were isolated from good quality fermented milk product on de Man Rogosa and Sharpe (MRS) [8] agar plates and their bacteriocin producing ability was initially screened using sensitive organism *Enterococcus faecalis* MB1(LAB8) by dual culture overlay method [9]. After the appearance of white colonies on MRS agar plates, the plates were overlaid with sensitive strain. The bacterial strains which can produce the inhibition zones against the sensitive organism were picked up and purified by streaking on MRS agar plates and the strain LABW4 was taken as potent one for further studies. Identification of the strain LABW4 was done based on morphological, biochemical and 16S rRNA gene sequence homology studies.

2.2. Antimicrobial Activity of LABW4 against *Listeria monocytogenes*

Antibacterial activity of LABW4 against *Listeria monocytogenes* MTCC657 was checked by agar well diffusion method [10] as well as by counting the colony forming units (CFUs). LABW4 was grown in MRS broth at 28˚C and the cell free supernatant was taken after centrifugation at 10,000 rpm for 10 minutes and pH was adjusted to 7. Cell free supernatant as well as heat killed cell free supernatants 50 µl each were added to the wells of nutrient agar plates containing *L. monocytogenes*. Uninoculated MRS broth (pH 7) was used as control. All the plates were incubated at 28˚C for 24 hours. Next day, the zones of inhibition were observed and diameters were measured. Antibacterial activity of the cell free supernatant of LABW4 was also checked after treatment with proteinase K (200 AU/ml).

The antilisterial activity of LABW4 was also checked by counting the colony forming units in the presence and absence of cell free supernatant (pH 7) of LABW4. *L. monocytogenes* was inoculated (1%) to 10 ml nutrient broth and treated with cell free supernatant of LABW4 (1% to 10%). In control set unionculated MRS broth was used in place of cell free supernatant. The tubes were incubated at 28˚C for 24 hours. Next day 100 µl of *Listeria* culture was spread on nutrient agar (NA) plates after proper dilution and the plates were incubated at 28˚C for 24 hours. The numbers of colony forming units were counted and compared.

2.3. Study of Mode of Action

To check the mode of action of bacteriocin produced by LABW4 on *L. monocytogenes*, growth pattern of the pathogen in the presence and absence of cell free supernatant of LABW4 were studied. 1% of *L. monocytogenes* culture was added in to two different sets of sterilised 10 ml nutrient broth and incubated at 28˚C. At the mid log phase of the growth (10.5 hours) cell free supernatant (10%) of LABW4 was added to one set and considered as

treated set [11]. The other set was considered as control where only uninoculated MRS broth was added and was incubated. Then the tubes were again incubated. Colony forming units were counted from the initial phase at every 1.5 hours intervals by spreading 100 µl of culture from both the sets after proper dilution. The growth pattern of *L. monocytogenes* in the presence of cell free supernatant was compared with the control set.

2.4. Control of Spoilage of Meat Caused by *Listeria monocytogenes* Using LABW4

Meat samples were cut into small pieces and were divided into four sets in petriplates in equal amounts (10 gm in each set). The petriplates with meat samples were wrapped with aluminium foil and autoclaved for proper sterilization. After sterilization the first and second sets were treated with 10 ml of *L. monocytogenes* (10^6 CFU/ml) suspended in 50 mM phosphate buffer. The second and third sets of meat samples were treated with cell free supernatant of LABW4 and the fourth set was considered as untreated one. So the second sets were treated with both *L. monocytogenes* as well as cell free supernatant of LABW4. All of the plates were incubated at 4˚C upto 25 days. The colony forming units of *L. monocytogenes* were counted for each set at five days of intervals. 1gm of meat sample was taken from each set, homogenized with sterilized tissue homogenizer and suspended in 10 ml of sterilized distilled water. 100 µl of suspension was taken and sprayed on nutrient agar plates after proper dilution to count the colony forming units.

2.5. *Lactate dehydrogenase* (LDH) Assay

The activity of the cytoplasmic enzyme *Lactate dehydrogenase* (LDH) from *L. monocytogenes* was measured following the method of Stockland and San Clemente [12] with little modification. The enzyme was prepared from overnight culture of *L. monocytogenes* resuspended in 50 mM Tris-chloride buffer (pH 7.5) (OD 2.0 at 620 nm). In one part, 1 ml of cell suspension was ruptured by sonication at 100% amplitude for 5 min with 0.9 cycles and in other part ten time concentrated cell free supernatant of LABW4 was introduced in cell suspension and incubated at 37˚C for 1 h. The cell free supernatant was collected by centrifugation at 8000 rpm for 5 min at 4˚C and used as enzyme source. The untreated CFS was used as the negative control. The rate of reduced NADH oxidation was measured in 3 ml cuvette at 37˚C and 340 nm after 5 min incubation. For each test 2 ml of Tris-chloride buffer (50 mM, pH 7.5), 150 µl of NADH (20 mM) [SIGMA-ALDRICH, USA], 750 µl of pyruvate (50 mM DC sodium form) [SIGMA-ALDRICH, USA] and 100 µl of enzyme preparation were added to the cuvette. One unit of enzyme activity is defined as a rate of NADH oxidation of 1 µmol per min per ml [13].

2.6. Scanning Electron Microscopic Study

To study the effect of bacteriocin produced by LABW4 on pathogenic bacterium, scanning electron microscopy was done for both treated and untreated *L. monocytogenes*. *Listeria* cells resuspended in 50 mM Tris-chloride buffer (pH 7.5) was treated with 10% ten time concentrated cell free supernatant of LABW4 for 4 hours. Ten times concentrated MRS broth was used for control set. Cells were harvested by centrifugation at 6000 rpm for 10 minutes and prepared for SEM studies. The cells were prefixed with 2% glutaraldehyde in 20 mM Na-P buffer (pH 6.5) plus 5% dimethyl sulphoxide (DMSO) [Merck, Germany] for 30 minute. After pre-fixation the cells were gently washed with 20 mM Na-P buffer (pH 6.5) and post fixed with osmium tetraoxide [SIGMA-ALDRICH, USA] dissolved in 50 mM Na-P buffer (pH 6.5). Cells were then dehydrated in series of alcohol grades starting from 30% to absolute alcohol, 10 minutes in every dilution. The dehydrated cells were coated with gold using an ion sputter (Coater IB-2, Gike Engineering, Japan) and observed under scanning electron microscope (HITACHI S-530, Japan) [14].

2.7. Data Analysis

The results presented are average of at least three observations. The means and standard deviations were calculated using Microsoft Excel 2007 program.

3. Results

3.1. Isolation of Lactic Acid Bacteria

The lactic acid bacterial strain LABW4 was isolated from the good quality fermented milk product. Initially numbers of bacterial colonies were isolated on MRS agar plates and finally the strain LABW4 was selected as a

potent bacteriocin producer. LABW4 was able to produce prominent zone of inhibition (**Figure 1**) against bacteriocin sensitive strain *Enterococcus faecalis* MB1 (LAB8). Based on morphological, biochemical and 16S rRNA gene sequence homology studies the strain LABW4 was identified as *Lactococcus lactis* subsp. *lactis.* The cells were Gram positive non-motile, non-endospore forming cocci with lactose utilizing and catalase non-utilizing properties.

3.2. Anti-Listerial Activity of Isolated Strain LABW4

The antibacterial activity of LABW4 was checked against *Listeria monocytogenes* MTCC657 which is a Gram positive psychrophilic bacterium responsible for spoilage of meat and other food products. The isolated strain LABW4 produced distinct zone of inhibition against *L.monocytogenes* when checked by agar well diffusion method. Both the normal as well as heat killed cell free supernatant was effective to produce 12.5 ± 0.5 mm zones of inhibition (**Figure 2**).

The anti listerial activity of LABW4 was also checked by counting the colony forming units of *L. monocytogenes* after treatment with cell free supernatant of LABW4. During this study it was found that the number of colony forming units decreased significantly with increased percentage of cell free supernatant of LABW4 (**Table 1**). Similar results were also obtained when heat killed supernatant was used (**Table 1**).

3.3. Effect of Cell Free Supernatant of LABW4 on Growth Pattern of *L. monocytogenes*

To check the mode of action of the cell free supernatant of LABW4 on *L. monocytogenes*, growth curve patterns of *Listeria* were studied in the presence and absence of cell free supernatant of LABW4. From the growth curve pattern it was found that the number of CFUs decreased rapidly after the addition of LABW4 supernatant and in next 10 hours, all the cells died (**Figure 3**).

3.4. Control of Spoilage of Meat Caused by *L. monocytogenes*

The anti-listerial activity of LABW4 was also checked by *in vivo* challenge study where we have tried to control

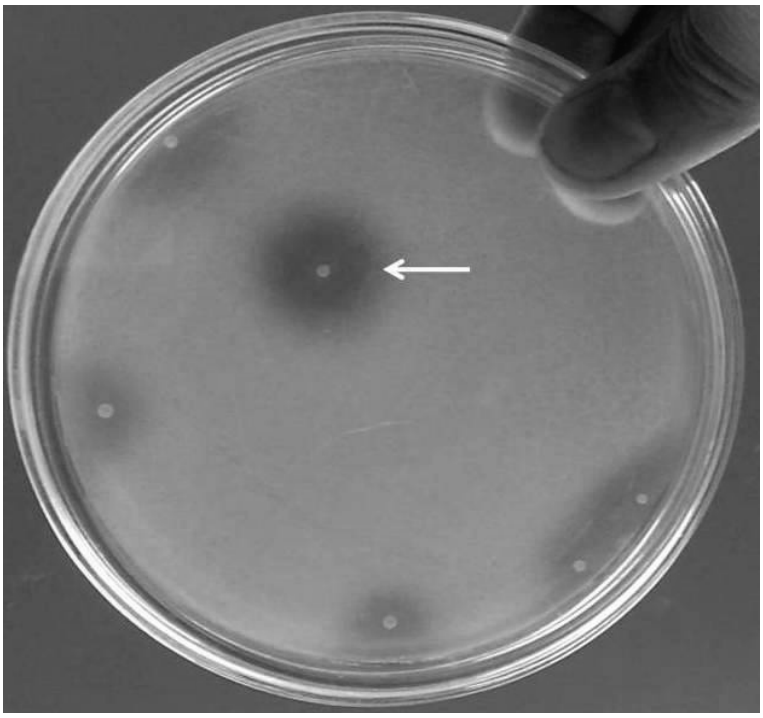


Figure 1. Zone of inhibition produced by Lactic acid bacterial strain LABW4 against bacteriocin sensitive *Enterococcus faecalis* MB1 (LAB8) on dual culture overlay plate.

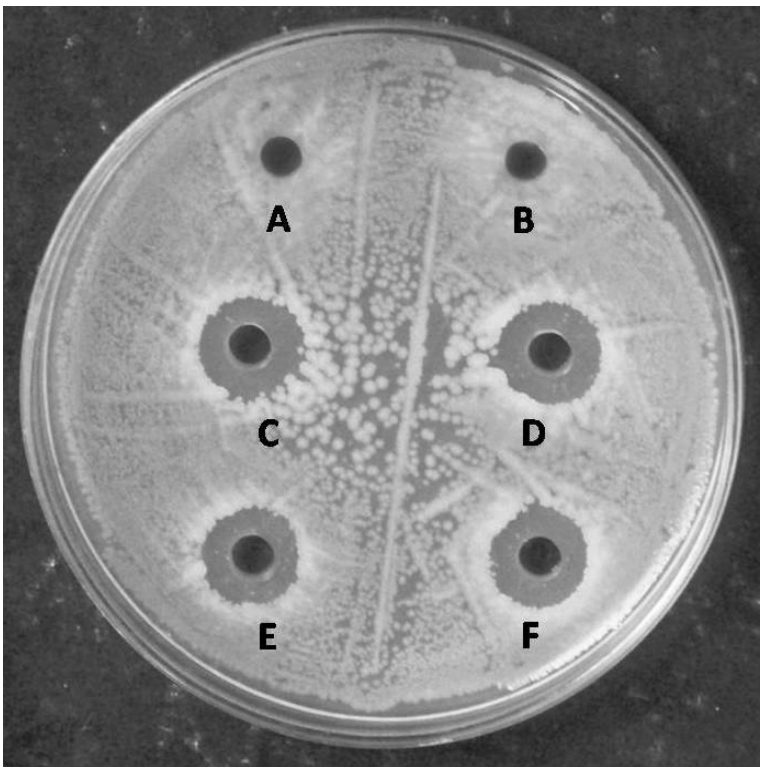


Figure 2. Zones of inhibition produced by cell free supernatant (CFS) of LABW4 against *Listeria monocytogenes* MTCC657: (A) & (B) control; (C) & (D) normal cell free *supernatant*; (E) & (F) Heat killed cell free supernatant.

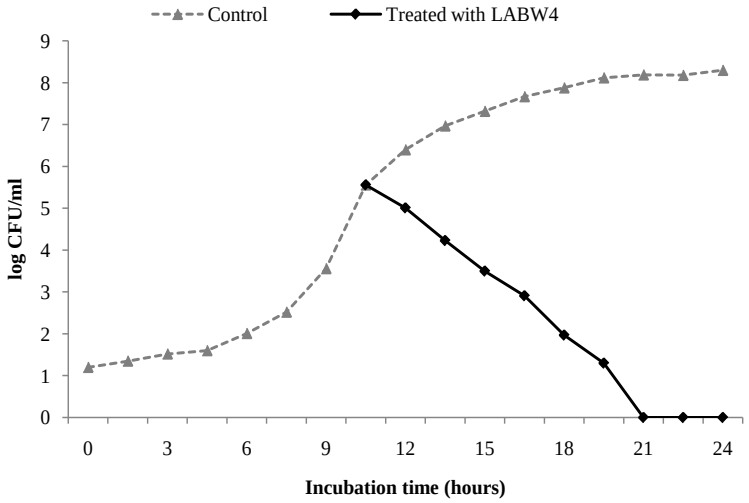


Figure 3. Effect of cell free supernatant of LABW4 on the growth of *Listeria moocytogenes* MTCC657. The results were the mean of triplicate trials.

the spoilage of meat samples using LABW4. It was found that the cell free supernatant of LABW4 was also able to inhibit the growth of *L. monocytogenes* in meat samples and therefore able to prevent the spoilage of meat up to 25 days or more at 4˚C. When the characteristics of meat samples were checked it was also found that spoilage of meat started within 24 hours in case of control set. When the colony forming units of *Listeria* were counted from meat samples, only negligible numbers of 1.08×10^1 CFUs in 10 days were found in case of LABW4 treated samples in comparison to its untreated control (**Figure 4**).

Table 1. Number of colony forming units of *L. monocytogenes* after treatment with cell free supernatant of LABW4.

Control (CFU/ml)	**Treated with cell free supernatant of LABW4**		
	Percentage (%) of cell free supernatant	CFU/ml	
		Unboiled	Boiled
9.1×10^8	1	7.5×10^8	8.9×10^8
	2	6.9×10^8	8.6×10^8
	3	5.6×10^7	6.9×10^7
	4	2.1×10^7	2.7×10^7
	5	7.6×10^6	8.1×10^6
	6	3.2×10^6	3.8×10^6
	7	6.3×10^4	7.4×10^4
	8	9.2×10^2	9.6×10^2
	9	0	0
	10	0	0

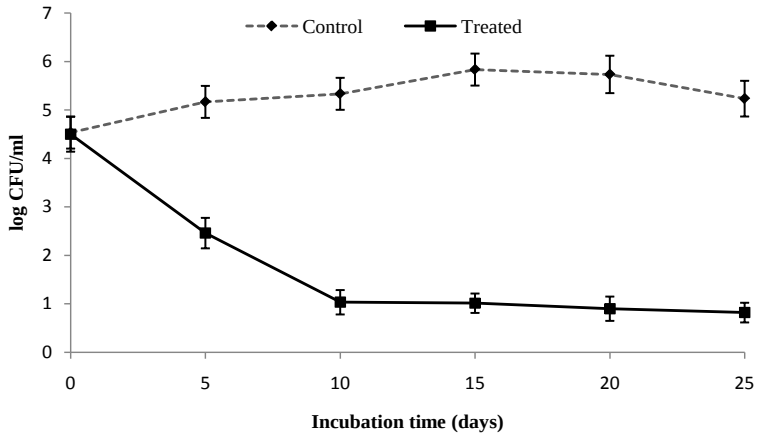


Figure 4. Numbers of *Listeria monocytogenes* in meat samples by cell free supernatant of LABW4 after storage at 4˚C. The results were the mean of triplicate trials.

3.5. *Lactate dehydrogenase* Assay

It was found that treatment of *Listeria* cells with ten time concentrated cell free supernatant of LABW4 significantly enhanced the LDH activity in comparison to its untreated control (**Figure 5**). The increase of LDH activity after treatment indicated leakiness or lysis of bacterial cell membrane to release of LDH into extracellular environment.

3.6. Effect of Cell Free Supernatant of LABW4 on Morphology of *L. monocytogenes*

Scanning electron microscopic study was performed to observe the morphological changes of *L. moncytogenes* after treatment with ten time concentrated cell free supernatant of LABW4. During this study it was observed that cell free supernatant significantly affected the cellular morphologies of *L. monocytogenes*. Degradations of wall structures were noticed in case of treated cells which supported the lytic mode of action of bacteriocin (**Figure 6(b)** & **Figure 6(c)**). In some cases shrinkages of treated cells were also observed which may be due to

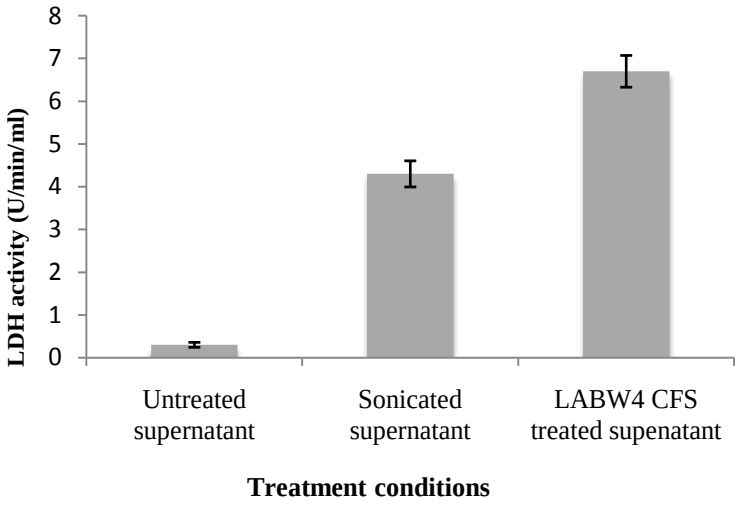
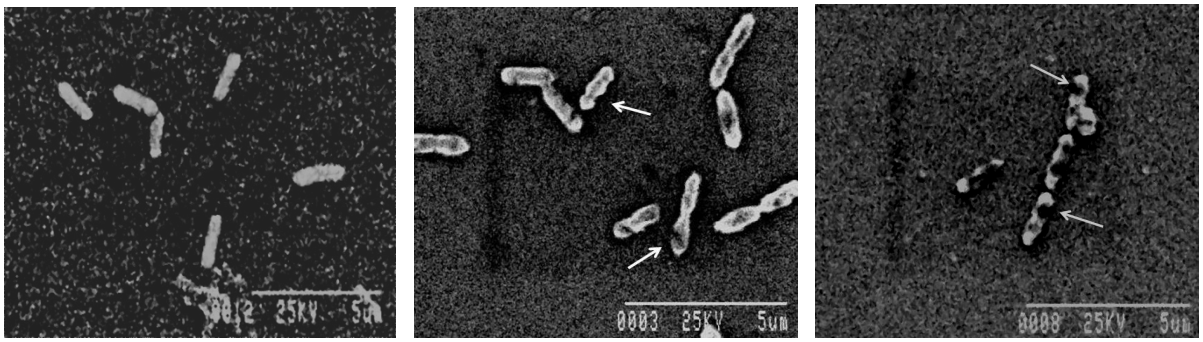


Figure 5. *Lactate dehydrogenase* assay of *Listeria monocytogenes* MTCC657. Values were the averages of three independent trials.



(a) (b) (c)

Figure 6. Scanning electron micrographs of *Listeria monocytogenes*: (a) untreated; (b) & (c) Treated with cell free supernatant of LABW4 for 4 hours.

loss of internal cellular fluids after treatments (**Figure 6(b)** & **Figure 6(c)**). No such degradations or shrinkages were noticed in untreated cells (**Figure 6(a)**).

4. Discussion

The isolated food grade lactic acid bacterial strain *Lactococcus lactis* subsp. *lactis* was effective to produce significant zone of inhibition against *L. monocytogenes*. Inhibition zone produced by cell supernatant indicated the secretion of antibacterial principle to the extracellular environment. The antibacterial potential of heat killed supernatant also supported the thermostable nature of the antibacterial compound(s). Loss of antibacterial activity after treatment with proteinase K demonstrated the proteinaceous nature of the antibacterial metabolite(s). It has already been reported by several workers that the bacteriocin produced by lactic acid bacteria are highly thermostable proteinaceous compounds which showed anti bacterial activity even after autoclaving (121˚C) [15]. Significant decreases in the numbers of colony forming units of *L. monocytogenes* were also noticed after treatment with cell free supernatant of LABW4. Pinto *et al.* [16] have reported the antilisterial activities of bacteriocin produced by *Enterococcus faecium* and *Pediococcus pentosaceus.* Cosentino *et al.* [17] have checked the antimicrobial properties of 117 *Lactococcus lactis* subsp. *lactis* isolated from artisanal Sardinian dairy products of which six strains were found to produce bacteriocin-like substances having anti-listerial activity. To check the mode of action of the antibacterial metabolites cell free supernatant of LABW4 was added to mid log phase of *L. monocytogenes.* A rapid downward trend of growth curve in treated set indicated the strong cidal nature of the antibacterial molecule(s) on *L. monocytogenes*.

During *in vivo* challenge studies using meat samples it was found that cell free supernatant of LABW4 was highly effective to prevent the spoilage of meat samples upto 25 days or more at 4˚C even after the treatment with high concentration of spoilage pathogen *L. monocytogenes*. As LABW4 is a bacteriocin producing strain therefore it is assumed that it produces sufficient bacteriocin to prevent the growth of *L. monocytogenes*. Presence of lactic acid in the medium also decreased the medium pH and creates an acidic environment which may also inhibit the growth of *L. monocytogenes*. *L. monocytogenes* has been shown to be able to grow under a variety of conditions. It is able to survive and grow on meat and meat products at refrigeration temperatures. The organism is able to survive in acid conditions with pH as low as 4.8 in foods and as low as 4.4 in laboratory media. Nielsen *et al.* [18] reported an inhibitory and bactericidal effect of a bacteriocin produced by *Pediococcus acidilactici* on *L. monocytogenes* associated with spoilage of fresh meat.

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme and its activity is routinely used to measure the cellular integrity [13]. The significant increase in LDH activity of *L. monocytogenes* after treatment indicated the lytic mode of action of the bacteriocin produced by LABW4. This type of observation was also reported by previous workers [13]. The lysis of treated cells was also evidentially proved when prominent degradations of wall structures were observed by scanning electron microscopic studies. Such type of cell lysis was also reported in case of *Pediococus acidilactici* B1153 treated with bacteriocin from *Pediococcus pentosaceus* CFR SIII [19]. On the other hand Mandal *et al.* [13] reported pore formation in case *L. monocytogenes* by bacteriocin of *Pediococcus acidilactici* LAB 5.

5. Conclusion

Lactococcus lactis subsp. *lactis* LABW4 was able to inhibit the growth and activity of *Listeria monocytogenes* and was effective for long-term preservation of meat samples under refrigeration. It showed strong cidal effect and lytic mode of action on *Listeria monocytogenes* which cause severe meat-spoilage. Traditional methods of preservation such as refrigeration, pasteurization, and low pH are not completely effective in controlling or eliminating *L. monocytogenes* from food. The use of lactic acid bacteria or its metabolite(s) in combination with traditional methods of preservation could be effective in controlling *L. monocytogenes* to prevent spoilage of meat products. The lactic acid bacteria also have GRAS status and several probiotic activities, therefore using of such organism to preserve the food products may also confer various health benefits.

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