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In vitro control of food spoilage bacteria using bacteriocins from *Lactobacillus acidophilus*

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Abstract
Lactobacillus acidophilus is a lactic acid bacteria known for bacteriocin production. Bacteriocin are antimicrobials produced by lactic acid bacteria which retard the growth of other bacteria. *L acidophilus* NCDC 291 was used in the present work for bacteriocin production ability and efficacy to inhibit the growth of bacterial strains in the present work. Time course of bacteriocin production was determined initially by plotting the growth. Bacteriocin was harvested from different stages of growth and quantified by well diffusion assay. It produced 1000 AU/ml of the bacteriocin. Different concentrations of this bacteriocin was used to determine the inhibitory threshold against *Bacillis, Erwinia* and *Pseudomonas* strains. One log reduction of cells meaning 90% reduction in number of *Bacillus*, *Erwinia* and *Pseudomonas* was recorded with 5000 AU of bacteriocin produced by *L acidophilus* after 12h of co-incubation.

Keywords: *Bacteriocins*, Lactobacillus acidophilus, in vitro efficacy, *bacterial food spoilage*

Introduction
The principle of food preservation is to prevent or delay microbial activity and retard or eliminate enzyme activity so as to provide a reasonable shelf life to the product. Maintenance of the nutritional integrity and keeping the microbial load low are imperative. Various traditional methods of food preservation practiced include asepsis, removal of microorganisms, use of high temperature, use of low temperature, drying, use of chemical preservatives, irradiation and use of anaerobic environment. However, they can cause loss of nutrients. Chemical preservatives as propionates, benzoates, sulfides, etc. cause food allergies.
Production of minimally processed food with little use of chemical preservatives is a challenge in food processing. It can be done using bacteriocins. Bacteriocins are ribosomally produced antimicrobials of lactic acid bacteria which have immense application potential in food processing. Being biological it is referred to as biopreservation. Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and their antibacterial products [1]. Lactic acid bacteria have a major potential for use in biopreservation, because they are safe to consume and during storage they naturally dominate the microflora of many foods.
Lactobacillus acidophilus is a Gram positive, rod-shaped, non-spore forming, homo-fermentative (produces lactic acid via glycolyticpathway) bacterium that is normal inhabitant of our gastrointestinal and genitourinary tracts [2]. *Lactobacillus acidophilus* when added to food products as a preservative enhances their shelf-life and it acts as a probiotic as well [3].

Material and Methods
Lactobacillus acidophilus NCDC 291 was purchased from National Dairy Research Institute, Karnal. It was maintained on MRS medium. It was sub-cultured periodically to main viability. Time course for bacteriocin production was determined by inoculating metabolically active culture in MRS broth and recording optical density. Different stages of growth were identified from the graph. Bacteriocin was prepared by centrifuging the broth at 10,000 rpm/20 min to obtain supernatant. pH of CFS was neutralized by addition of 0.1N NaOH. It negates inhibition due to organic acids. It was then heated at 80ºC for 15 minutes to deactivate proteins. It was then filter sterilization through 0.22 µm pore size cellulose acetate filter (Millipore). Bacteriocin was prepared from broth cultures from late log and early stationary phase [4].
Antimicrobial assay was performed [5]. Plates containing solidified MRS agar (2% agar) were overlaid with soft MRS agar (0.8% agar). After solidification, the plates were inoculated with 0.1 ml of metabolically active culture of indicator strain *Lactobacillus delbrueckii* NCDC 290. Wells were made in soft agar and 100µl of bacteriocin preparation was transferred into each well. Plates were incubated at 37°C for 24h and observed for presence/absence of zone of inhibition. The bacteriocin was then quantified [6]. Serially ten-fold dilutions of the bacteriocin preparation was made and subjected to well diffusion assay. Arbitrary units (AU) is defined as the reciprocal of the highest dilution showing the minimum zone of inhibition of 2 mm [7]. *Bacillus*, *Erwinia carotovora* MTCC 1428 and *Pseudomonas marginalis* MTCC 2578 are commonly encountered bacterial food spoilage agents. Efficacy of bacteriocin was tested by growing the bacterial strains in presence of different concentrations of bacteriocin preparation. The growth was recorded at 12h and 24h of incubation by standard plate count method.

Results

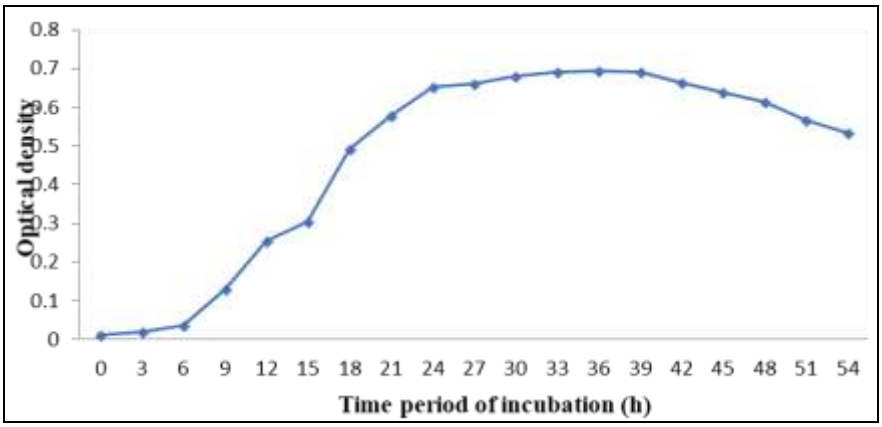

Fig 1: Growth kinetics of *Lactobacillus acidophilus* NCDC 291

L acidophilus NCDC 291 exhibited log phase upto 24 h, stationary phase upto 39h and decline phase upto 54h (Fig 1). *Lactobacillus acidophilus* NCDC 291 starts producing bacteriocins at early logarithmic growth phase and produces maximum bacteriocin at late logarithmic growth phase. It shows maximum zone of inhibition (i.e. 1.95 cm) at 24h of incubation. Subsequently, zone of inhibition decreases with increase in incubation period (Table 1).

Table 1: Well Diffusion Assay of Bacteriocin Produced by *Lactobacillus acidophilus*

S. No	Time period of incubation (h)	Zone of inhibition (cm)
1.	15 (Early Log Phase)	1.5
2.	21 (Mid Log Phase)	1.62
3.	24 (Late Log Phase)	1.95
4.	27 (Stationary Phase)	1.55

Results of bacteriocin quantification are presented in Table 2. Serial ten-fold dilution done twice yielded a zone of inhibition of 7mm. No zone of inhibition was observed at higher dilution level. A threshold level of 2 mm zone of inhibition must be recorded to evidence the presence of antimicrobial activity. *L acidophilus* produced 1000 AU/ml of the bacteriocin preparation. Quantification of bacteriocin depends on many factors like growth stage and physiological state of the organism, method of detection, and also choice of indicator strain. *Lactobacillus delbrueckii* NCDC 291 have been widely used as an indicator strain as documented in literature.

Table 2: Quantification of Bacteriocin Produced by *Lactobacillus acidophilus*

S. No	Dilution factor	Zone of inhibition (mm)
1.	Bacteriocin preparation	16
2.	10^{-1}	12
3.	10^{-2}	7
4.	10^{-3}	ND
5.	10^{-4}	ND

In vitro antimicrobial activity of *L acidophilus* bacteriocin was observed by after co-incubation of the organism in presence of different concentrations of bacteriocins. Observations of standard plate count in terms of log cfu at 12h and 24 h is presented in Table 3.

Table 3: *In vitro* Antibacterial Activity of *Lactobacillus acidophilus* Bacteriocin.

Bacteriocin Concentration (AU)	*Bacillus*		*Erwinia carotovora*		*Pseudomonas marginalis*	
	12 h	24 h	12 h	24 h	12 h	24 h
1000	9.25	10.44	10.36	10.62	10.00	11.27
2000	9.17	10.25	10.34	9.61	9.93	9.96
3000	8.99	9.97	9.31	9.17	9.93	9.75
4000	8.52	9.25	9.27	9.85	9.89	9.83
5000	8.12	8.69	8.43	8.61	8.09	9.11
Control	9.26	10.03	10.17	10.36	9.97	10.04

One log reduction of cells meaning 90% reduction in number of *Bacillus*, *Erwinia* and *Pseudomonas* was recorded with 5000 AU of bacteriocin produced by *L acidophilus* after 12h of incubation. Similar reduction was recorded at 24h in *Erwinia.* Extent of inhibition of *Pseudomonas* however decreased at 24h of incubation.

Discussion

Lactic acid bacteria are among the most important groups of micro-organisms used in the food industry [8]. They have been used for flavor and texture development in fermented foods but also due to possession of property to prevent growth of pathogenic microbes [9]. An important role in antagonism of lactic acid bacteria is played by proteinaceous bacteriocins. Many bacteriocins produced by them inhibit closely related species i.e they exhibit narrow spectrum of activity [10]. They prevent the growth of food borne pathogens as *Listeria monocytogenes* and also food spoilage bacteria [11, 12].

In general, bacteriocins are reported to be active against Gram positive bacteria but some have also demonstrated efficacy against Gram negative species [13, 14, 15]. *L acidophilus* has been proven to kill pathogens as *E coli* and *Salmonella* [16]. Some bacteriocins produced by *L acidophilus* have been purified and characterized including acidocin 8912, acidocin A [17], acidocin B [18], lacticin B, lactocin LC-09 [19], Acidophilin 801, acidocin CH5 [20].

Erwinia and *Pseudomonas* are known bacterial food spoilage agents. They cause soft rot of fresh produce [21]. This problem is encountered specially during storage. *Erwinia* has been reported to be inhibited by bacteriocin produced by *Bacillus licheniformis* P40. The bacteriocin has bactericidal effect on

the cell. Electron micrography has shown wrinkled bacterial surfaces and shrinkage of whole cell indicating plasmolysis [22]. Growth of *Pseudomonas fragi* was reported to be inhibited by application of lactic acid bacteria in ground beef and also poultry [23]. The results of the present study also indicate that one log reduction of the viable cell count was achieved using 5000 AU of bacteriocin preparation within 12 h of incubation. Currently there are no bacteriocins from acidophilus which are being applied commercially as food bio preservatives. Many bacteriocins have been characterized for their properties but many aspects are yet unexplored. International research however strongly emphasize their safe use in food and pharmaceutical industry.

Conclusions

Bacteriocins are promising means of preservation of food. Their efficacy to retard growth of pathogens and food spoilage agents has been widely reported in literature. Combination of bacteriocins with other physical means of preservation offers good opportunity to be more effective. Antimicrobial spectrum spectrum of bacteriocins can be increased by use of concept of hurdle technology to improve shelf life and enhance food safety. Immobilization of bacteriocins into packaging films to control food spoilage and pathogenic organisms can be pursued. It may be possible to design bacteriocin molecules with improved stability and solubility by using DNA technology.

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