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ANTIBACTERIAL ACTIVITY OF BACTERIOCIN PRODUCING *Lactobacillus spp.*, FROM VARIOUS MILK SAMPLES

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ABSTRACTBacteriocin are highly specific antibacterial protein (polypeptides) produced by gram positive bacteria ,in particular the lactic acid bacteria display fairly broad inhibitory spectra with food preservative and therapeutic potentials. This work aims at to isolate bacteriocin-producing *Lactobacillus* spp., and to determine the antibacterial activity against pathogenic bacteria. This work involves methodology as follows *Lactobacills* spp., was isolated from various milk samples by using MRS Agar (Hi-media, India) at 37°C for 48h. The isolates were identified by using conventional biochemical tests. Antibacterial characteristics of bacteriocin were studied by agar well diffusion method and it partially purified by Ammonium sulphate precipitation and dialyzed. The present study revealed that the *Lactobacillus* spp., strain isolated from various milk samples was capable of producing bacteriocin and inactivating the widest range of pathogenic bacteria. Totally 48 isolates of *Lactobacillus* spp., was identified in that only 8 (17%) *Lactobacillus* spp., have the antibacterial activity against pathogenic bacteria. The study revealed that the antibacterial property of bacteriocin act as a food preservative agent against pathogenic microorganisms.

Key words: *Lactobacillus* spp., Bacteriocin, Antibacterial activity, Pathogenic bacteria

Introduction

Lactic acid bacteria (LAB) are known to be Gram-positive, non-spore-forming rods, cocci and cocco-bacilli non-aerobic but aero tolerant, able to ferment carbohydrates into energy and lactic acid. Bacteriocin possess bactericidal activity against bacterial species, of which Gram positive bacteria of genus *Bacillus* and lactic acid bacteria (LAB) are main bacteriocin producing species. (Sharma et al., 2006). During the last few decades, investigation on food preservation has focused on meeting consumer demands for more natural and healthier food (Caminiti et al., 2011). This perception, joined with the increasing demand for minimally processed foods with

long shelf life and convenience, has stimulated research interest in finding natural and effective preservatives (Chen, 2003). The use of microorganisms and their natural products for the preservation of foods (biopreservation) has been a common practice in the history of mankind (Galvez, 2007). Biopreservation refers to the extension of the shelf-life and improvement of the safety of foods using microorganisms and/or their metabolites (Settanni and Corsetti, 2008).

There are two variants of this bacteriocin: nisin A and Z, which differ from each other onlyby the amino-acid 27. Histidine in nisin A is replaced by asparagin in nisin Z. This bacteriocin is used predominantly in canned foods and dairy products and is especially effective when utilized in the production of processed cheese and cheese spreads acting against heat-resistant spore forming organisms such as *Bacillus* and *Clostridium* (Deegan et al, 2006). Bacteriocin nisin was found to be safe for human consumption by the Food and Drug Administration and has thus gained popularity in probiotic research (Gillor, 2008).

The uses of bacteriocins in food preservation can offer several advantages: (a), an extended shelf life of foods, (b) offer extra protection during abnormal temperature conditions, (c) lessening the risk for spread of food borne pathogens through the food chain, (d) lessen the economic losses due to food spoilage, (e) decrease the application of chemical additives, (f) the process allow the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, (g), permit the marketing of “novel” foods (less acidic, with a lower salt content, and with a higher water content), and (h) they may serve to satisfy industrial and consumers demands (Ming, 1993).

Now a days, Bacteriocin can be applied on a purified or on a crude form or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (starter culture). Bacteriocins can also be used to improve food quality and sensory properties, for example increasing the rate of proteolysis or in the prevention of gas blowing defect in cheese. Another application of bacteriocins is bioactive packaging, a process that can protect the food from external contaminants (Paul Ross, (2002), Zacharof, (2012).

Although, lactic acid bacteria show a high impact on effective protection to human health, there is obvious evidence that lactic acid bacteria from different sources possess antimicrobial properties at different extent. Against these backdrops, this present study was undertaken to determine the antibacterial activity of partially purified bacteriocins of the *Lactobacillus* spp., from various milk samples.

Materials and Methods

Experimental design

The various raw milk samples were collected in around the Namakkal, Tamilnadu (India). The samples were processed on Man Rogosa Sharpe (MRS) medium and identified as *Lactobacillus* spp., then extract the bacteriocin in cell free supernatant by centrifugation method. The extracted product of bacteriocin from *Lactobacillus* spp., were subjected to study their antibacterial activity against pathogenic bacteria. After that the bacteriocin was partially purified by Ammonium sulfate method.

Pathogenic bacteria

Escherichia coli (MTCC 1687), *Bacillus cereus* (MTCC 1272), *Staphylococcus aureus* (MTCC 96) *Klebsiella pneumoniae* (MTCC 530), *Pseudomonas aeruginosa* (MTCC 1688) were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Sample collection

The various raw milk samples such as Cow milk, Buffalo milk, Goat milk, Donkey milk, Mother milk were collected during the period of March 2015 to August 2015.The milk samples was collected in a sterile container and immediately transported to laboratory at 4°C for bacterial isolation.

Isolation and Identification of *Lactobacillus* spp.,

The collected milk samples were serially diluted in MRS broth (10^{-1} to 10^{-9}). Then *Lactobacillus* spp. was isolated by spread plate (0.1ml) technique on MRS medium from the dilution of 10^{-4} to 10^{-6} for bacterial isolation. The plates were incubated at 37°C for 24-48 hours in an anaerobic container. The suspected colony was maintained on the MRS agar slants for further use. Then to identify the *Lactobacillus* spp., based on the gram staining, colony morphology and carbohydrate fermentation of the sugars.

Production of bacteriocin

The strain was grown in MRS broth at 37°C for 48 h. After incubation, the broth was centrifuged at 5000 X g for 10 minutes and the cells were separated out. The cell free supernatant was used as crude bacteriocin (Narayanapillai Udhayashree et al., 2012).

Extraction of cell-free supernatant (CFS)

Following identification of *Lactobacillus*, the pure isolate was propagated in 1000 ml flask containing MRS broth (pH-6.0) and was incubated at 37°C for 72 hours anaerobically. A supernatant that may contain crude bacteriocin, a cell-free solution was obtained by centrifuging the culture at 10,000 rpm for 20 minutes at 4°C. After centrifugation the supernatant was collected in a fresh sterile tube and pellets were discarded. The CFS was adjusted to pH-6.0 using 1N NaOH (Karthikeyan and Santosh, 2009).

Antibacterial activity of bacteriocin producing *Lactobacillus* spp.,

The cultures of the indicator strains (*Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, E. coli* and *Bacillus cereus*) were prepared and swabbed on MHA plates to completely cover the surface of the agar. Six mm diameter wells were punched into the agar using sterilized well cutter, which were cut to obtain a 6mm diameter bore. 150 µl of each probiotic inoculum (bacteriocin) (150 µl) was carefully pipetted into each well. Then observed the diameter of the inhibition zones around the wells were recorded after incubating the plates for 48 h *at* 37°C (Rajesh singh et al., 2013).

Partial purification of CFS

The CFS extracted from *Lactobacillus spp.*, was subjected to following partial purification methods.

a. Ammonium sulphate precipitation: A concentration of 60% of ammonium sulphate was added to 10 ml of CFS in sterile test tubes. It was allowed to precipitate for 24 hours at 4°C. The mixture was then centrifuged at 10,000 rpm for 20 minutes and the precipitate was re-suspended in 25 ml of 0.05 M potassium phosphate buffer. The mixture was stirred at 4°C for 24 hours.

b. Dialysis: Following ammonium sulphate precipitation, the suspension was dialysed in a tubular cellulose membrane against 2000 ml distilled water for 48 hours with 3 changes. After dialysis, the purified sample was collected in sterile tubes (Karthikeyan and Santosh, 2009).

Results and Discussion

A total of 48 *Lactobacillus* spp., were isolated from 5 various raw milk samples such as milk, Buffalo milk, Goat milk, Donkey milk, Mother milk. 48 isolates were obtained from MRS agar.

All of these were gram positive rod and catalase negative. *The Lactobacillus* spp., produced Creamy, round, convex and smooth colonies (Figure 1).Several types of bacteriocins from food associated lactic acid bacteria have been identified and characterized, of which nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, lactolin and plantaricins are the important bacteriocins. Of these, bacteriocin and nisin produced by *Lactococcus lactis* sp. lactis, has been the most extensively characterized. Nisin is the only bacteriocin commercially available. It has been reported that nisin is more active against Gram-positive bacteria, particularly the spore-formers. Other bacteriocins of *Lactobacilli* have been reported to be effective against closely related species of mesophilic *Lactobacillus* and therefore considered as potential natural food preservatives (Ray et al., 2001).

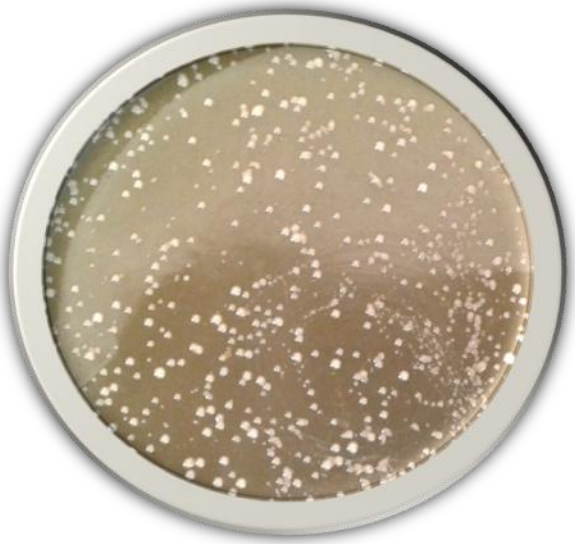

Figure 1. Colony morphology of *Lactobacillus* spp., on MRS Agar

Our results suggested that bacteriocins producing *Lactobacillus* spp., naturally occurs and survive in various raw milk samples.

The susceptibility of various pathogenic organisms to growth inhibition by CFS of *Lactobacillus* spp., *is* shown in Table 1.

Table 1. Inhibition of pathogenic bacteria by bacteriocin of *Lactobacillus* spp.,

Clinical Isolates	Degree of Inhibition
Escherichia coli	++++
Klebsiella pneumonia	++
Pseudomonas aeruginosa	++
Staphylococcus aureus	+++
Bacillus cereus	++

++ Optimal inhibition, ++++Maximum inhibition

Maximum inhibition denoted by ++++ inferred that growth of the test pathogenic bacteria was not observed on Muller Hinton Agar (MHA agar). While, optimal inhibition denoted by ++ inferred bactericidal inhibition being observed. The highest inhibitory activity was demonstrated against *Escherichia coli*, while, the lowest inhibitory activity of the cell-free supernatant was demonstrated against *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Figure 2). In this study, out of 48 isolates, only 8 (17%) isolates of Lactobacillus spp., showed antibacterial activity against pathogenic bacteria.

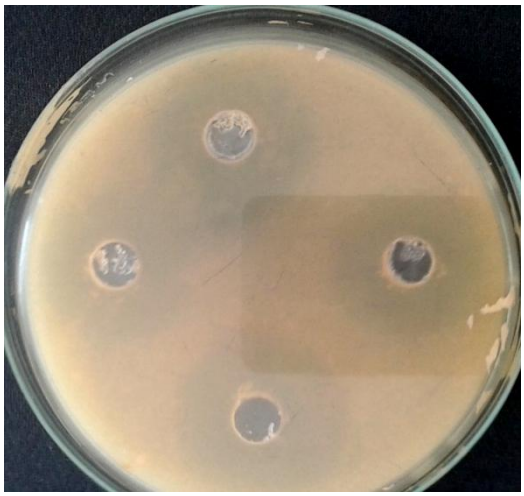

Figure 2. Antibacterial activity by Cell Free Supernatant (Bacteriocin)

The isolated *Lactobacillus paracasei* was used for separation of CFS containing extracellular crude bacteriocin. This CFS was tested for antimicrobial susceptibility to a spectrum of

pathogenic Gram-positive and Gram-negative bacteria commonly known to be associated with various clinical manifestations by Spot assay method. The highest inhibitory activity was demonstrated against *Salmonella typhi, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* while, the lowest activity seen against *Staphylococcus aureus*. The inhibitory activity demonstrated by crude bacteriocin against these organisms is a firm indicator of the presence of active bacteriocin in the test supernatant. The inhibitory activity of bacteriocins has also been reported against a number of other bacteria (Ogunbanwo et al., 2003). Similar results have been observed in experiments related to inhibitory effect of bacteriocin produced by other *Lactobacillus* species (Tatsadjieu et al., 2009).

The CFS extracted in the present study showed maximum antimicrobial activity against *Escherichia coli* which was also well supported by the results obtained in the well diffusion assay with an index of 150 µl. This assay conducted resulted in an index of 150 µl against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus*. In an earlier study conducted by Tzu-Hsing Lin, cell-free supernatants of *L. paracasei* sub sp. paracasei NTU101 showed maximum inhibitory effects on pathogenic strains especially against *Vibrio paraheamolyticus* followed by a optimum inhibition against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, the latter as observed in the present study where 150 µl of crude bacteriocin was effective against these organisms (Klaenhammer, 1983; Flythe et al., 2004). The present study also included purification of bacteriocin from the extracellular CFS. The partially purified dialysate obtained through ammonium sulphate precipitation.

Conclusion

Bacteriocin is a biologically active antimicrobial substance produced by living microorganisms, especially Lactic acid bacteria. This protein has a specific role in food and pharmaceutical industry. It will be more useful to protect the food items from unwanted microbes. It reduce the use of antibiotics food substances because over use of antibiotics lead to the drug resistance in bacteria and make the unwanted illness in the humans beings mostly Lactic acid bacteria have an ability to produce the protein to protect the food items. When compared to other microorganisms. In this study, the screening milk isolates of *Lactobacillus* spp., showed the highest antibacterial activity against human pathogens based on the presented results, it may be concluded that the

antimicrobial substance from the culture supernatant of *Lactobacillus* spp., have a diverse spectrum of antimicrobial activity.

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