Isolation, purification and characterisation of bacteriocin producing *Lactobacillus species* and its antimicrobial efficacy against food borne pathogens

R Anupama[1], Anusha Balasingh[2,*]

[1]PG Student, [2]Associate Professor, Dept. of Microbiology, Ethiraj College for Women, Chennai, Tamil Nadu 600008, India

***Corresponding Author:**
Email: anushamicro274@gmail.com

Abstract
Lactobacilli are a group of bacteria that are ubiquitous and contribute to the normal microbial flora of human beings. They are Generally Regarded as Safe (GRAS) and are not lethal to host. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by Lactic Acid Bacteria (LAB) that exhibit antagonistic activity against food contaminating microorganisms and inhibit similar or closely related bacterial strains. Hence, these bacteriocins are considered bio-preservative and are demonstrated as food preservatives, as therapeutics for veterinary and medicinal uses or as phytosanitary means for plant protection. The present study involved isolation of *Lactobacillus* species from dairy samples, purification of bacteriocin and the study of antagonistic activity against food borne pathogens. The study showed that purified bacteriocin exhibited inhibitory activity against *Escherichia coli* and *Shigella flexneri.*

Keywords: Bacteriocin, Lactic Acid bacteria, Pathogens, Cell free supernatants.

Introduction
The bacteriocins produced by bacteria are studied to greater extent in recent years in order to use them as bio-preservatives. These are antimicrobial peptides (AMPs) that are only toxic to bacteria that are closely related to the producing strain. These bacteriocins are ribosomally synthesized low molecular weight peptides. Presently, Nisin is a bacteriocin that is commercially used as food preservative (Delves-Broughton et al. 1996). Study of bacteriocin produced by lactic acid bacteria (LAB) has increased due to its potential as both a natural preservative and as therapeutic antibiotics. These peptides work by binding to specific surface receptors (Cascales et al.; Chavan & Riley, 2007) and killing only the cells that are recognized by them by altering membrane bound enzymes and disruption by pore formation.

These bacteriocins are produced by Gram-positive, Gram-negative and also by Archae. Bacteriocins produced by gram negative bacteria are of four main classes namely colicins, colicin like bacteriocins, phage-tail like bacteriocins and microcins (Chavan & Riley, 2007). Bacteriocins produced by gram positive bacteria are classified into four types based on size, morphology, physical and chemical properties (Lee & Kim, 2011) namely class I (<5kDa) (Field et al., 2007), class II (<10kDa) (Heng et al., 2007), class III (>10kDa) and class IV type bacteriocins.

These are "Live microorganisms which, when administered in adequate amounts, confers health benefit on host cells". They show various properties to prevent colonisation of pathogens such as synthesis of antimicrobial compounds, creating a competitive environment for pathogen adherence, competition for nutrients, and modulation of immune system. They also produce various compounds such as bacteriocins, long chain fatty acids, hydrogen peroxide for inhibiting intestinal pathogens.

These are used mainly in food and feed preservation to inhibit pathogenic infections. Lactic acid bacteria produce these bacteriocins naturally and hence, are used as starter cultures or co-cultures in fermented foods. These can be added in foods to prevent contamination and spoilage by pathogens and to extend shelf life of food product. Various chemicals are used as food preservatives which can have an impact on health benefits of humans. Instead, bacteriocins can be added as natural food preservative in place of various chemicals.

It was proven that pore-forming colicin A and E1 inhibited growth of human standard fibroblast line MRC5 and 11 human tumor cell-lines (Chumchalova and Smarda, 2003). Thus, bacteriocin could find a way to treat tumor cells that can prevent death of numerous lives that is increasing every year. It is preferable to use purified bacteriocin than using organisms which have ability to act upon pathogenic organisms. In addition a cocktail of more than one bacteriocin can be used as a tool to reduce and also treat various infections to save lives.

Materials and Methods
Isolation of *Lactobacillus* species from dairy foods
A total of 25 dairy samples were collected from regional dairy stores and homes were used for *Lactobacilli* isolation. They were transported at appropriate storage conditions and 1 ml of sample was serially diluted using 0.85% saline. Homogenisation of samples was done for some samples such as cheese, butter. The dilutions were plated on Lactobacillus MRS (Mann Rogosa Sharpe) agar by streaking and by spread plating of diluted samples. The culture was grown at

37ºC for 24- 48 hours. The colonies were sub cultured on a regular interval on Lactobacillus MRS agar (De Mann *et al*., 1960).

The grown colonies were observed for their colour, size, texture, margination. They were also observed for their Gram's staining, sugar fermentation and catalase test and morphological properties.

Screening for the production of bacteriocin

1. **Primary streak method:** The isolated colonies were sub cultured and checked for their inhibitory activity by primary streak method. The pathogens were lawn cultured on Mueller-Hinton agar and the isolated *Lactobacillus* was streaked against the pathogens and incubated at 37°C for 24 hours and observed for zone of inhibition.
2. **Well diffusion assay:** Well diffusion assay was performed using cell free supernatants of culture. The organisms were grown on Lactobacillus MRS both and after an incubation for 48 hours, the broth was centrifuged at 6000rpm for 10 minutes at 4ºC. The efficacy of supernatants was tested by well diffusion assay against pathogens. The plates were incubated at 37ºC for 24 hours and observed for zone of inhibition.

Purification of bacteriocin

1. **Ammonium sulphate precipitation:** The isolated cultures were grown in MRS broth for 48 hours until turbidity was observed. Cell free supernatant (CFS) was obtained by centrifuging the broth in a cooling centrifuge for 10 minutes at 6000rpm. The CFS was purified using ammonium sulphate precipitation. The cell free supernatants were saturated with 70% and 40% ammonium sulphate solution and the suspended protein was obtained as pellet. Finally, the pellet was dissolved in phosphate buffered saline (pH 7). And it was dialysed in a magnetic stirrer in Phosphate buffered saline (PBS) for 12 hours changing the buffer for every 3 hours.
2. **Study of antagonistic activity against pathogens:** Well diffusion assay was performed using purified supernatants against pathogens. Nutrient agar plates were prepared and lawn of pathogenic organisms was done and kept for 15 minutes at room temperature. Wells were done on nutrient agar using a sterile core borer. The purified supernatants were added to the wells after marking appropriate isolate details. The plates were incubated in an incubator at 37ºC for 24 hours in an inverted position and observed for zone of inhibition.
3. **SDS Page:** The purified supernatants were run under SDS PAGE to detect the presence of protein bands.

Results and Discussion

Isolation and identification of *Lactobacillus* species

The present study that included 26 dairy samples such as curd, butter, cheese, lassi, buttermilk, yogurt, raw milk etc., a total of 35 isolates were obtained. On Lactobacillus MRS Agar, after incubation for 24 hours at 37ºC, colonies were observed. They were mostly creamy, white/off-white, round, regular/irregular, entire colonies showing a characteristic smell.

Table 1: Morphology of isolated *Lactobacillus* species

S. No.	Sample	Colony morphology
1	Butter	Creamy white , raised, round, elevated
2	Lassie	Off-white, large, irregular, entire
3	Cheese	Raised, white, small
4	Yoghurt 1	White/off-white colonies, circular, small
5	Yoghurt 2	White colonies, slightly yellowish later
6	Curd	Small, creamy white colonies

Gram's staining of the isolates revealed gram-positive long rods mainly, and sometimes with slightly short. Catalase producing ability was identified by placing an isolated colony on Hydrogen peroxide solution. No effervescence was observed indicating a negative catalase test which is a characteristic of *Lactobacillus* species. Fermenting ability of various sugars was also determined by phenol red broth base containing sugars in a tube containing inverted durham's tube.

Table 2: Staining, catalase and sugar fermentation

Isolate	Gram's staining	Catalase	Fructose	Dextrose	Maltose	Mannitol
1	Positive rods	-	+	+	-	+
2	Positive rods	-	+	+	+	-
3	Positive rods	-	+	-	+	+
4	Positive rods	-	-	+	+	+
5	Positive rods	-	+	+	-	-

6	Positive rods	-	-	+	-	+

The same kind of morphology was observed by Ashok V. Gomashe *et al.,* in 2014. They studied the antibacterial activity of bacteriocin producing bacteria from milk and curd against *Staphylococcus aureus*. Other pathogens such as *Escherichia coli, Klebsiella pneumonia* were inhibited by probiotics.

Fang ZHAO *et al.,* has also isolated and identified *Lactobacillus* using Lactobacillus MRS (de Mann, Rogosa, Sharpe, Aoboxing, Beijing, China) agar from Chinese pickle using Gram's stain and catalase test. En Yang *et al.,* (2014) have also identified *Lactobacillus* in a similar way by Gram's staining catalase negative property and observation under light microscope.

Screening of bacteriocin activity

Primary streak method performed against pathogens such as *Escherichia coli, Salmonella typhi, Salmonella paratyphi A, Salmonella paratyphi B* and *Shigella flexneri.* One isolate of *Lactobacillus* showed a zone of inhibition of around 4mm against *Shigella flexneri* during the Primary screening.

Ranganath *et al.,* has also reported the same results showing zone of inhibition against test pathogens and also quoted the antimicrobial activity of bacteriocinic substances. Amit Bharal *et al.,* has also demonstrated the antimicrobial activity of bacteriocin from *L. acidophilus* and showed their activity against human and food borne pathogens.

Antagonistic activity of Purified supernatants

The protein purified by ammonium sulphate precipitation was tested for antimicrobial activity by well diffusion assay. During this, purified proteins from isolate 1 and 5 showed antimicrobial activity against *Escherichia coli*. The antagonistic activity was found to increase after purification.

Timothy *et al.,* (2014) has also done the antagonistic activity of supernatants by centrifuging the colonies in broth at 3000 rpm for 15 minutes and R3 strain has shown inhibition of around 0.5cm against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Rajaram *et al.,* 2010 has also demonstrated the antimicrobial activity of cell free supernatant using crude and purified protein derivative and has compare its activity with antibiotics.

The zone of inhibition of test organisms indicates that the growth of test organisms has been halted by the supernatant containing antimicrobial compounds. When the purified supernatant was studied under SDS-PAGE a protein band was observed showing the presence of protein. The purified proteins were also studied using SDS-PAGE and band was observed after staining and destaining of gel (Gupta *et al*., 2010).

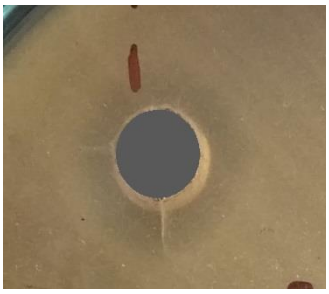
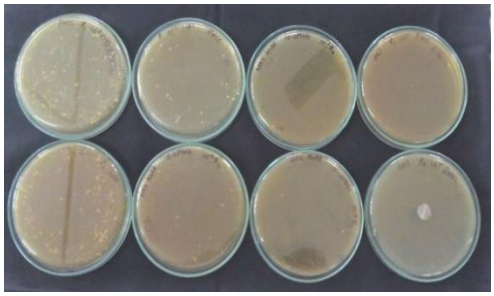


Fig. 1: Zone of inhibition produced by the purified isolate



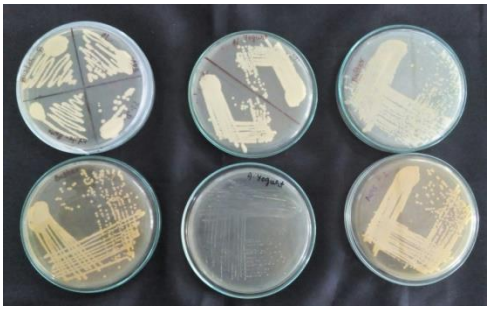


Fig. 2: Isolation of Lactobacillus species from dairy products

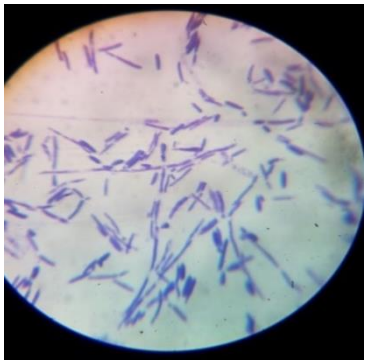


Fig. 3: Gram positive bacilli under microscopic observation

Fig. 4: Visible band under SDS PAGE gel

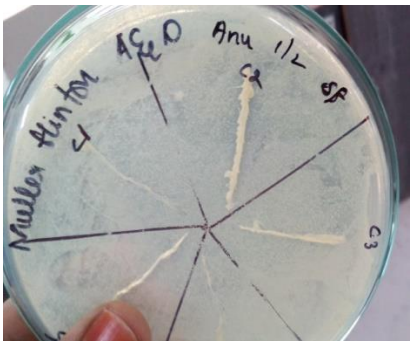


Fig. 5: Zone of inhibition produced by the isolated culture

Conclusion

Living a healthy disease free life remains a challenge throughout life. Microorganism's role in producing antimicrobial compounds appears to be significant. In such aspect bacteriocin produced by *Lactobacilli* appears to be a preventive measure for various invasions by pathogens. Purification of bacteriocin increases its application in many fields.

The applications of bacteriocin can be abundant in fields like food and feed preservative. Further organisms that can produce high quantity of bacteriocin can be screened and extraction procedure can be optimized. By doing this, the application of bacteriocin can be done in various fields. And usage of these organisms as probiotics can also be encouraged to maintain a healthy immune system and to prevent colonization of pathogens.

References

1. Amit Bharnal and Vipan Kumar Sohpal, "Evaluation of Antimicrobial Activity of Bacteriocin (L. Acidophilus) against human pathogenic and food borne microorganisms," International journal of Innovative Research in Sciences, vol. 2, issue 8, pp. 4221-5, 2013.
2. Ashok V. Gomashe, Anjali A. Sharma, and Mamta A. Wankhede, "Screening and Evaluation of Antibacterial Activity of Bacteriocin Producing Lab against some Selected Bacteria Causing Food Spoilage ," International Journal of Current and Microbiology and Applied Sciences, vol. 3, no. 8, pp. 658-65, 2014.
3. Bamgbose Timothy and Sumit Sharma, "Isolation and characterization of microbes producing bacteriocin from curd, raw milk and soil and its preservative effects," International Journal of Pharmaceutical Sciences and Research, vol. 5, issue 5, pp. 1942-8, 2014.
4. Chavan, M.A., & Riley, M.A., "Molecular Evolution of Bacteriocins in Gram-Negative Bacteria", Bacteriocins: Ecology and Evolution, pp. 19-43, 2007.
5. Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J (1996) Applications of Bacteriocin, nisin. Anton Leewenhoek 69;193-202,1996.
6. Chumchalova, J., and Smarda, J, "Human tumor cells are selectively inhibited by colicins", Folia Microbiology, pp. 111-5, 2003.
7. De man J.C., RogosaM. And Sharpe M.E. 1960."A medium for the cultivation of Lactobacilli", Journal of Applied Bacteriology, 23:130-5.
8. En Yang, Lihua Fan, Yueming Jiang, Craig Doucette and Sherry Fillmore, "Antimicrobial activity of bacteriocin - producing lactic acid bacteria isolated from cheeses and yogurts," AMB Express, vol. 2, issue 28, 2012.
9. Fang ZHOU, Hongfei ZHAO, Fengling BAI, Piotr DZIUGAN, Yuen LIU and Bolin ZHANG, "Purification and Characterisation of the Bacteriocin Produced by Lactobacillus plantarum, Isolated from Chinese Pickle," Czech Journal of Food Sciences, vol. 32, no. 5, pp. 430-6, 2014.
10. H. Gupta, R. K. Malik, A. Bhardwaj, G. Kaur, S. De and J. K. Kaushik, "Purification and characterization of enterocin FH 99 produced by a faecal isolate Enterococcus faecium FH 99," Indian Journal of Microbiology, vol. 50, pp. 145-55, 2010.
11. Heng, N.C.K., Wescombe, P.A., Burton,J.P., Jack, R.W& Tagg, J.R, "The Diversity of Bacteriocins in Gram Positive Bacteria" , Bacteriocins: Ecology and Evolution,pp. 45-92, 2007
12. G. Rajaram, P. Manivasagan, B. Thilagavathi and A. Saravanakumar, "Purification and Characterization of a Bacteriocin Produced by Lactobacillus lactis Isolated from Marine Environment," Advance Journal of Food Science and Technology, vol. 2, issue 2, pp. 138-44, 2010.
13. S.B. Ranganath and T. Sharmila, "Isolation and Identification of a Bacteriocinic Substance Producing Bacteria from Various Sources," International Journal of Current Microbiology and Applied Sciences, vol. 5, no. 6, pp. 655-64, 2016.