Title Page

Comparative Analysis Of Non-Conventional Lactic Acid Bacteria Derived Bacteriocin Efficiency Determination Over Streptomycin In Order To Prevent Enteropathogenic *E. coli* Biofilm Formation

Prathibhaa.R[1], Ieshita Pan[2]

Prathibhaa.R[1]
Research Scholar,
Department of Biotechnology,
Saveetha School of Engineering,
Saveetha Institute of Medical and Technical Sciences,
Saveetha University, Chennai,Tamil Nadu, India, Pincode-602105.
192022023.sse@saveetha.com

Ieshita Pan[2]
Project Guide, Corresponding Author,
Department of Medical Biotechnology and Integrative Physiology,
Saveetha School of Engineering,
Saveetha Institute of Medical and Technical Sciences,
Saveetha University, Chennai, Tamil Nadu, India-602105.
ieshitapan.sse@saveetha.com

Keywords: *Lactobacillus*, Cabbage, Antimicrobial Activity, Streptomycin, Novel Bacteriocin, Biofilm, Food.

ABSTRACT

Aim: To compare the antimicrobial activity between novel bacteriocin isolated from *Lactobacillus* by fermenting cabbage and comparing its efficiency with streptomycin antibiotic by measuring the zone of clearance in order to prevent biofilm formation. **Materials and Methods:** Previously isolated *Lactobacillus* from fermented cabbage was taken for this study. A 150 µl of the freshly prepared culture was inoculated in nutrient broth media and incubated at 37°C for 72 hrs with a continuous shaking at 140 rpm. **Result:** Two groups with 20 (N=40) samples were used by keeping a threshold of 0.05, G power of 80%, coincidence interval (CI) 95%, and enrollment ratio as 1. *Lactobacillus* showed good results compared with streptomycin by measuring the zone of clearance of antimicrobial activity with a statistical significance of $p=0.000$ ($p<0.05$). **Discussion and Conclusion:** Novel bacteriocin isolated from *Lactobacillus* by non-conventional source cabbage fermentation has been clearly compared with the antibiotic streptomycin by measuring the zone of clearance and showed a promising result by killing >90% of pathogen compared to antibiotic streptomycin. The significance level of the investigation was determined to be $p=0.001$ ($p<0.05$). This suggests that there is a statistically significant difference among the test groups. Thus, to prevent biofilm formation, *Lactobacillus* isolated from fermented cabbage can be a potential source and has a wide range of applications in food industries.

Keywords: *Lactobacillus*, cabbage, Antimicrobial Activity, streptomycin, Novel Bacteriocin, Biofilm, Food.

INTRODUCTION:

Escherichia coli are a type of gram negative bacteria found in the environment, foods and intestines of people and animals (Mueller and Tainter 2023). *E.coli* are foodborne pathogens that have the capacity to form biofilms. A biofilm is an assembly of surface-associated bacterial cells that is enclosed in an extracellular polymeric substance matrix. These extracellular polymeric substance matrices are composed of polysaccharides, proteins, DNA and other macromolecules that help in providing structural support to improve the integrity of biofilm. Biofilm formation on food packaging can severely lead to recurrent cross-contamination of food products. The usage of chemical products on the biofilms is a serious health concern and they have also become resistant to the conventional sterilization and sanitation methods. Due to these problems, there's a need to develop natural alternatives to the conventional ones. Lactic acid bacteria along with other gram positive and gram negative bacterias produce an antimicrobial peptide called bacteriocin, that are traditionally used as a food preservative (Pang et al. 2022). They have a positive aptitude as a controlling agent against biofilms. Recent studies on biofilms have shown that bacteriocins can be effective against biofilms, but they cannot act on preformed biofilms and

also the use of these natural preservatives is restricted in some of the food industry due to their poor stability during food processing and storage.

Related to this study, the total number of articles published are as follows; Google Scholar contained 212 articles and in PubMed 13 articles were published. The most cited article is although some species of lactobacilli have been linked to food spoiling, the food and feed industries use them extensively in the fermentation of vegetables, silage, sourdough bread, and a variety of dairy and meat products (Stiles and Holzapfel 1997). Although a lactic acid bacteria strain derived from *Leymus chinensis* silage may be effective for encouraging good fermentation of cabbage (*Brassica oleracea)*, our prior investigation showed no evidence to support this study (Wang et al. 2019). An antibiotic is a chemical substance formed by microorganisms that has the ability to halt the development of bacteria and other microbes and even to kill them (Yang et al. 2020). *Streptomyces orientalis* produces the bactericidal glycopeptide antibiotic streptomycin, which was first used to treat newly developing penicillinase-producing staphylococci in 1956 (Ma et al. 2011). As opposed to other sources, lactic acid bacteria plays a more significant role in the creation of compounds with antimicrobial action due to their ease of availability, quick growth, and affordable manufacture (Kralik, Babak, and Dziedzinska 2018).

The difference between the former study and current paper is mostly *Lactobacillus* is isolated from dairy sources. In this research *Lactobacillus* is isolated from fermented cabbage which is easily available and cost effective. The aim of this study is to determine the comparative analysis of antimicrobial activity between *Lactobacillus* novel bacteriocin isolated from fermented cabbage and streptomycin antibiotic by measuring the zone of clearance to prevent biofilm formation.

MATERIALS AND METHODS

The experimental work was done in the Microbiology Laboratory, Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences. Sample is calculated in clinical.com and by keeping 0.05% threshold, 80% G power, coincidence interval 95% and 1 as enrollment ratio.

In this study bacteriocin production was analyzed by using one group (N=1) with 20 samples per group. To produce lactic acid bacteria from food sources such as cabbage (*Brassica oleracea var. capitata*), was fermented for 21 days under high salt concentrations. Cabbage was cut into equal size, length 8 cm and width 5 mm and kept in a sterile container with 70% moisture and aeration for faster decomposition (Zabat et al. 2018).

To prepare MRS medium [49190] (2.08g in 100ml water +/- 1.5 g agar) and Nutrient broth (1.3g in 100ml +/- 1.5g agar) medium ingredients were measured and mixed under stirring condition. After the pH checking volume make up was done and both the media were sterilized at 121℃ 15 lb pressure for 15 min in the autoclave. To check the bacterial load 100 µl samples from 10-4 dilution of each source were poured in 2 petri dishes. Each Petri plate was poured with MRS and Nutrient agar before solidification. The plates were rotated 10 times anticlockwise

and 10 times clockwise. Both the plates were incubated for 48 hrs at 37℃ (Wang et al. 2019). After 48 hrs of incubation the total number of colonies were counted and represented as CFU/ml.

Each colony was then grown in separate nutrient broth in test tubes of 5 ml each. It was left for 96 hours incubation in a shaker. After incubation 1ml of each of the growth culture was taken in autoclave eppendorf tubes and centrifuged at 15 000 r/min for 15 min, 4 °C (Elayaraja et al. 2014). The supernatant was collected in separate eppendorf tubes. Nutrient agar pour plates of serratia was prepared. Holes of 10µL capacity were punched using a sterile tip. Each hole was filled with 10µL of each of the centrifuged bacteriocin. Then it was left for 24 hrs incubation. After that, the zone of clearance was observed..

For the antibiotic test, antibiotics in different concentrations were prepared. The concentrations are water:antibiotic ratios 9:1, 19:1, 29:1, 39:1. A nutrient agar pour plate of serratia was prepared and holes were punched with a sterile tip. Each hole was filled with the controls and the tests of 10µL each and left for 24 hrs incubation. Then the zone of clearance was observed.

Statistical Analysis

The comparative analysis of antimicrobial activity between *Lactobacillus* novel bacteriocin and streptomycin checked with a paired t-test done with SPSS Version 29, where the statistical significance was evaluated by following standard assessment protocol (McCormick and Salcedo 2017). All the experiments were conducted in triplicate and values are taken as mean ± 1 SD. For this analysis medium composition, environmental parameters and concentration of novel bacteriocin and streptomycin are the dependable variables however sample sources are considered as independent variables.

RESULTS

In Table 1, the analysis of paired samples statistics in paired tests was presented. To analyze the effect antimicrobial activity of *Lactobacillus* and streptomycin was checked for Pair (organism and antimicrobial agent, antimicrobial agent and concentration dose, concentration dose and avg zone clearance, antimicrobial agent and avg zone clearance, organism and avg zone clearance, organism and concentration dose). Maximum variation was found to be recorded with concentration dose. In Table 2, the analysis of Paired Samples Correlations was presented. For Pair (antimicrobial agent and concentration dose, concentration dose and avg zone clearance, antimicrobial agent and avg zone clearance, organism and avg zone clearance, organism and concentration dose) the difference was found to be recorded maximum. In Table 3, the analysis of paired differences in Paired samples test with a CI of 95% was presented. For Pair (antimicrobial agent and concentration dose, concentration dose and avg zone clearance, antimicrobial agent and avg zone clearance, organism and avg zone clearance, organism and concentration dose) the difference was found to be recorded maximum and the statistical significance was $p<0.05$.

Figure 1 shows the comparison of organism, antimicrobial agent, concentration dose with average zone clearance. The bar graph represents the comparison of organism, antimicrobial agent, concentration dose with average zone clearance. Maxima reached a concentration dose with a statistical significance of $p<0.05$.

DISCUSSION

Lactobacillus isolated from fermented cabbage was found promising towards health improvement. It produced novel bacteriocin which was analyzed for a zone of clearance and compared with the antibiotic streptomycin. The novel bacteriocin isolated from fermented cabbage produced a larger zone compared to the antibiotic streptomycin against *E.coli* with an efficiency >90% with a statistical significance of $p<0.05$. The significance level of the investigation was determined to be $p=0.000$. As a result, it's clear that the bacteriocin from *Lactobacillus* is better than streptomycin.

Fermentation conditions have a direct impact on the generation of novel bacteriocin (Mataragas et al. 2003). The synthesis of various antimicrobial peptides in isolates is controlled by fermentation and downstream processing (Benítez-Chao et al. 2021) Our earlier research revealed no evidence to support this study, despite the possibility that a lactic acid bacteria strain obtained from *Leymus chinensis* silage is useful for promoting excellent fermentation of cabbage ("Website," n.d.; Raveschot et al. 2018). Unfavorable fermentation conditions may have increased the amount of novel bacteriocin that lactic acid bacteria produce (De Vuyst and Vandamme 2012).

Considering the organism's genus-level identification, the measurement of bacterium size. The study's limitations involved 16s rDNA sequencing for molecular identification. The potential future scope still has to be completed, along with its characterization and use for complete growth optimization for bacterial fermentation.

CONCLUSION

Lactobacillus isolated from fermented cabbage has found potential for producing novel bacteriocin. Compared to the antibiotic Streptomycin, novel bacteriocin is effective against biofilm forming *E.coli*. The zone of clearance indicated that novel bacteriocin from *Lactobacillus* is efficient and can kill pathogens with >90% of efficiency compared to Streptomycin.

DECLARATION

Conflict of Interests

No conflict of interest in this manuscript

Authors Contribution

Author PR was involved in conceptualization, data collection, data analysis, and manuscript writing. Author IP was involved in conceptualization, data analysis, and critical review study of the manuscript.

Acknowledgment

The authors would like to express their gratitude towards Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, formerly known as Saveetha University for providing the necessary infrastructure to carry out this work successfully.

Funding: We thank our financial sponsor for the financial support that enabled us to complete this study.

1. LAB Chemicals, Chennai
2. Saveetha School of Engineering
3. Saveetha Institute of Medical and Technical Sciences
4. Saveetha University.

REFERENCES

Benítez-Chao, Diego Francisco, Angel León-Buitimea, Jordy Alexis Lerma-Escalera, and José Rubén Morones-Ramírez. 2021. "An Overview of Antimicrobial, Toxicity, and Biosafety Assessment by Models." *Frontiers in Microbiology* 12 (April): 630695.

De Vuyst, Luc, and Erick J. Vandamme. 2012. *Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications*. Springer.

Elayaraja, Sivaramasamy, Neelamegam Annamalai, Packiyam Mayavu, and Thangavel Balasubramanian. 2014. "Production, Purification and Characterization of Bacteriocin from Lactobacillus Murinus AU06 and Its Broad Antibacterial Spectrum." *Asian Pacific Journal of Tropical Biomedicine* 4 (Suppl 1): S305.

Kralik, Petr, Vladimir Babak, and Radka Dziedzinska. 2018. "The Impact of the Antimicrobial Compounds Produced by Lactic Acid Bacteria on the Growth Performance of Mycobacterium Avium Subsp. Paratuberculosis." *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2018.00638.

Mataragas, M., J. Metaxopoulos, M. Galiotou, and E. H. Drosinos. 2003. "Influence of pH and Temperature on Growth and Bacteriocin Production by Leuconostoc Mesenteroides L124 and Lactobacillus Curvatus L442." *Meat Science* 64 (3): 265–71.

Ma, Xiao Xue, En Hua Wang, Yong Liu, and En Jie Luo. 2011. "Antibiotic Susceptibility of Coagulase-Negative Staphylococci (CoNS): Emergence of Teicoplanin-Non-Susceptible CoNS

Strains with Inducible Resistance to Vancomycin." *Journal of Medical Microbiology*. https://doi.org/10.1099/jmm.0.034066-0.
McCormick, Keith, and Jesus Salcedo. 2017. "SPSS[reg] Statistics for Data Analysis and Visualization." https://doi.org/10.1002/9781119183426.
Mueller, Matthew, and Christopher R. Tainter. 2023. "Escherichia Coli Infection." In *StatPearls [Internet]*. StatPearls Publishing.
Pang, Xinyi, Xiaoye Song, Minjie Chen, Shuhua Tian, Zhaoxin Lu, Jing Sun, Xiangfei Li, Yingjian Lu, and Hyun-Gyun Yuk. 2022. "Combating Biofilms of Foodborne Pathogens with Bacteriocins by Lactic Acid Bacteria in the Food Industry." *Comprehensive Reviews in Food Science and Food Safety* 21 (2): 1657–76.
Raveschot, Cyril, Benoit Cudennec, François Coutte, Christophe Flahaut, Marc Fremont, Djamel Drider, and Pascal Dhulster. 2018. "Production of Bioactive Peptides by Species: From Gene to Application." *Frontiers in Microbiology* 9 (October): 2354.
Stiles, M. E., and W. H. Holzapfel. 1997. "Lactic Acid Bacteria of Foods and Their Current Taxonomy." *International Journal of Food Microbiology* 36 (1): 1–29.
Wang, Cheng, Liwen He, Yaqi Xing, Wei Zhou, Fuyu Yang, Xiaoyang Chen, and Qing Zhang. 2019. "Fermentation Quality and Microbial Community of Alfalfa and Stylo Silage Mixed with Moringa Oleifera Leaves." *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2019.03.129.
"Website." n.d. https://www.researchgate.net/publication/355231570_Fermentative_Production_of_Alternative_Antimicrobial_Peptides_and_Enzymes.
Yang, Yong, Olga Babich, Stanislav Sukhikh, Mariya Zimina, and Irina Milentyeva. 2020. "Antibiotic Activity and Resistance of Lactic Acid Bacteria and Other Antagonistic Bacteriocin-Producing Microorganisms." *Foods and Raw Materials*. https://doi.org/10.21603/2308-4057-2020-2-377-384.
Zabat, Michelle A., William H. Sano, Jenna I. Wurster, Damien J. Cabral, and Peter Belenky. 2018. "Microbial Community Analysis of Sauerkraut Fermentation Reveals a Stable and Rapidly Established Community." *Foods* 7 (5). https://doi.org/10.3390/foods7050077.

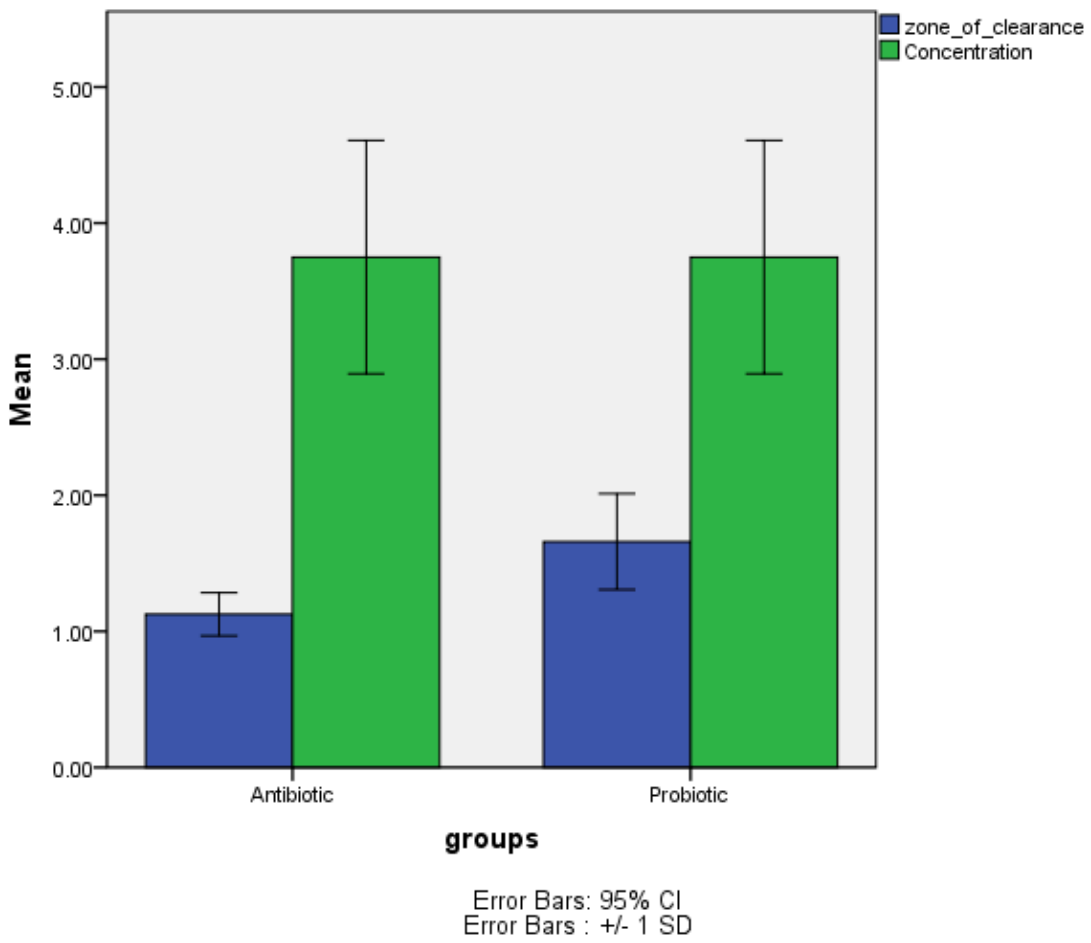

Fig 1: represents the mean of zone of clearance and concentration of the 2 groups

Group 1-antibiotic

Group 2-probiotic

Table 1

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Groups	1.5000	40	.50637	.08006
	zone_of_clearance	1.3920	40	.63738	.10078
Pair 2	Groups	1.5000	40	.50637	.08006
	Concentration	3.7500	40	1.80810	.28589
Pair 3	Zone_of_clearance	1.3920	40	.63738	.10078
	Concentration	3.7500	40	1.80810	.28589

Represents paired samples statistics for the 3 pairs of zone of clearance

Table 2:

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Groups & zone_of_clearance	40	.424	.006
Pair 2	Groups & Concentration	40	.000	1.000
Pair 3	Zone_of_clearance & Concentration	40	-.579	.000

Represents the correlations of the aired samples of groups and zone of clearance

Table 3:

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Groups - zone_of_ clearance	.10800	.62355	.09859	-.09142	.30742	1.095	39	.280
Pair 2	Groups Concentration	-2.25000	1.87767	.29689	-2.85051	-1.64949	-7.579	39	.000
Pair 3	Zone_of_ clearance - Concentration	-2.35800	2.23850	.35394	-3.07391	-1.64209	-6.662	39	.000

Represents the paired samples test of groups of zone of clearance, concentrations.