HP JHWSR2025
Hummingbird Publications
Journal of Health, Wellness & Safety Research
www.hummingbirdjournals.com
© February, 2025
Vol. 7 No. 3

Evaluation of the Preservative Potentials of Lactic Acid Bacteria Isolated from Goat Milk and Finger Millet on Yoghurt

Ideh R.R., Agarry O.O., Anigo K.M. and Adeniran O.I

DOI: https://doi.org/10.70382/hujhwsr.v7i3.024

Key words: Lactic acid bacteria, antimicrobial, bacteriocin, preservative and Food-borne

The molecular identification protocol is neither caprured in the methodology nor stated the results

Abstract

Food fermentation using microorganisms is one the oldest technique for food production and preservation. Storage life can be extended with enhanced safety using the natural microflora and their antimicrobial products such as bacteriocins. The preservative potentials of bacteriocins extracted from lactic acid bacteria isolated from fresh goat milk and finger millet were studied. The isolated organisms were identified based on their morphological, biochemical and physiological characteristics. Isolates were screened for their ability to produce bacteriocin using chloroform extraction method. Isolates with good bacteriocin yield were subsequently identified molecularly. The stability of the bacteriocins extracted was evaluated at varying temperature (30°C - 100°C) and pH (2 - 8) conditions. Also, the antimicrobial activities of the isolated LAB were tested against selected food-borne pathogens using the agar well diffusion method. Yoghurt was produced using the organism with the most potent bacteriocin extracts after which for the effect of the bacteriocin extract on the yoghurt quality was assesed. Results indicated that LAB isolates with high

bacteriocin producing potential based on their antimicrobial activity were identified as *Lactobacillus plantarum, Enterococcus faecium,* and *Lactobacillus acidophilus*. The LAB isolates produced bacteriocin with a broad spectrum antibacterial activity against some food-borne pathogens namely *E.coli, S. aureus, S. typhi, Klebsiella* sp. and *P. aeruginosa* with inhibition zones ranging from 18 to 28 mm, *Lactobacillus plantarum* being most effective *had a zone of inbition* of …. against *Salmonella typhimurium.* The extracts were stable at temperatures of 30 - 80°C (>70%) and pH range of 2.0 to 6.0 (> 50%). All bacteriocin extracts maintained the proximate composition of yoghurt and extended the shelf life up to 35 days. The result of the sensory evaluation showed preference for yoghurt preserved with the extract acidocin, obtained from *Lactobacillus acidophilus* The findings from this study showed that finger millet and goat milk are rich sources of lactic acid bacteria with good bacteriocin producing potential. which were able to preserve the yoghurt for a period of 35 days at a temperature of 4°C.

Introduction

The beginning of the 21st century with its universal call to feed the hungry is an appropriate time to refocus attention on food security and especially the impact of biopatenting on poor communities who are the primary victims of hunger in our world. Awareness of human health when using chemical preservatives in food has increased, resulting in the use of alternative strategies for preserving food and enhancing its shelf-life (Dibyajit*et al.,* 2022) Antibacterial metabolites of Lactic Acid Bacteria (LAB) and *Bacillus* species have the potential as natural preservatives to control the growth of spoilage and pathogenic bacteria in food (Zhang *et al.,* 2024). Concentration in novel biological preservation has increased during recent years, supported by research indicating that antagonistic microorganisms or their antimicrobial metabolites may have some potential as natural preservatives to control the growth of pathogenic bacteria and mycotoxinogenic fungi in foods, (Amenu and Bacha 2024).

Due to the alarming rise in antibiotic resistance and adverse effects provoked by a number of antibiotics, bacteriocins have been applied in several fields which include: human health, food industry, animal health, and medicine, in particular as a substitute to the traditional growth promoters, antibiotics (Choi *et al*., 2023).

Bacteriocins are protein or protein complexes produced by bacteria and have antimicrobial activity against closely related species and various Gram-positive and some Gram-negative bacteria, including food spoilage bacteria and pathogens. (RenduelesMartínez *et al.,* 2022). The bacteriocins from LAB have arisen a great deal of attention as a novel approach to control pathogens in foodstuffs (Timothy *et al.,* 2021). Bacteriocins are used as preservative in food due to their heat stability, wider pH tolerance, and proteolytic activity. Due to their thermostability and pH tolerance, it can withstand heat and acidity/alkalinity of food during storage condition.

Lactic acid bacteria play essential role in food fermentation and are employed as starter culture in the manufacturing of dairy, meat, vegetable and bakery products. Bacteriocins produced by LAB are attracting considerable interest as food preservatives and safe alternatives to conventional antimicrobials in food industry as novel technology (Praveen *et al.,* 2023). LAB bacteriocins are mostly used due to their Generally Regarded As Safe (GRAS) status, especially in food industry as bio-preservatives (Abdulkareem, 2022). Lactic acid bacteria have a higher potential for biopreservation because they are safe to consume (Rathod, *et al.,* 2021).

MATERIALS AND METHODS

Study Area

This study was carried out in the laboratory of the Food and Industrial Biotechnology Department, National Biotechnology Development Agency, Lugbe Abuja.

Preparation and sterilization of Media

All microbiological media used in this study were prepared according to the manufacturer's instructions and sterilized in autoclave at 121°C for 15mins.

Bacteria Isolation from finger millet (*Eleusinecoracana*)

The fermented and dried finger millet samples were blended using a blender (Binatone BLG-412) for 1 minute. Lactic Acid Bacteria was isolated by

transferring 25 g of the flour into sterile sample bottles and 225 ml of buffered peptone water added and 1:10 dilution obtained for each sample. A serial dilution of 10^{-1} to 10^{-6} respectively was made. One mL of each dilution was plated. The culture samples were isolated by plating on MRS and M-17 agar; cultured plates were incubated at 37 ^{o}C for 48 -72 h following the method described by Vander Meulen *et al*., (2007).

Isolation of lactic acid bacteria from goat milk

The fresh goat milk was analyzed using the pour plate method of isolation described by Ali (2011). One ml of each sample was taken and homogenized in 9ml of peptone water. Serial dilutions of up to 10^{-6} were prepared and 1ml aliquots from 10^{-2}, 10^{-4} and 10^{-6} dilutions plated in duplicates into the MRS agar. All plates were incubated for 72 h at 37 ºC in microaerophilic conditions using an anaerobic gas jar pack system to reduce oxygen level as described by Frederick and Luc (2013).

Screening and Identification of Isolates

The isolates were screened using routine bacteriological methods which include observation of the cell colonial morphology: color, shape, elevation and size of the colonies were documented with preliminary tests carried out. After which the following tests were conducted after purification: Catalase, Gram stain, Oxidase, Motility test and other identification test. (Sneath and Holt, 2001).

Physiological Identification of Isolates

Each isolate was activated in 5 ml MRS broth for 24 h at 37ºC before use. Physiological identification of isolates was carried out in accordance with the methods described by Sneath and Holt (2001). The characteristics that were examined included growth at 10, 15, 25, 37 and 45^{o}C, salt tolerance at 2, 4 and 6.5% NaCl concentration and pH values of 2, 4 and 6.5 (Cheesbrough, 2003).

Sugar Fermentation Spectrum of Isolates

Isolates that yielded favourably to the tests above 3.5.4 were selected and examined for their ability to ferment different sugars such as, arabinose, fructose, mannose, raffinose, ribose, galactose, trehalose, maltose, lactose, xylose and glucose as described by the method of Bylund (1995). This involves inoculating a 0.1ml of overnight grown culture into tubes containing 5ml MRS

and M17 broth containing each sugar with bromocresol purple added as an indicator. Durham tubes were inserted into each tube and incubated for 48h at 37°C. Colour change from purple to yellow was taken as evidence for acid production and growth, while gas accumulation in the inverted tubes was taken for gas production.

Screening of Lactic acid bacteria for the potential to produce Bacteriocins
Bacteriocins production

Using a modified method of Cheng *et al.* (2020) a bacteriocin production semi solid agar medium (BAC) was prepared in a beaker and the sterile medium was kept overnight in a cold room before inoculation. A fresh overnight culture of selected LAB strains was prepared in a sterile culture tube containing 10ml of THYE broth and incubated for 18 h at 37 °C.

Using a sterile glass pipet, the semi solid BAC medium was stabbed severally, under aseptic condition. The overnight LAB preculture was poured over the stabbed surface and covered with foil paper then incubated at 37 °C for 72 h using an anaerobic jar (Cheng *et al.,* 2020).

At the end of 72 h, the mixture was mashed using a sterile spatula and transferred into a sterile 250 ml centrifuge bottles and subsequently stored in an ultra-low freezer for 18 h. It was then thawed by putting the bottles in a water bath at 65 °C for 1h, after which centrifugation at 15,000 x g for 40 min at 4 °C was done and the supernatant carefully collected.

An equal volume of chloroform was added to the collected supernatant, the mixture was shaken thoroughly by hand for about 20 min and stored overnight at 4 °C. The upper layer (chloroform waste) was carefully removed and white layer containing the secreted peptides was transferred into a 50ml tube and the emulsion was allowed to settle on its own.

The floating white interfacial layer was transferred into a glass beaker and the material kept in a fume hood until the residual chloroform was completely evaporated. After about 7h days?? , a bacteriocin-containing powder was obtained.

The bacteriocin-containing powder was dissolved using 25ml of ACN-TFA solvent and the re-suspended extract was transferred into a 50ml tube and centrifuged at 3200 x g for 40 min using a benchtop centrifuge. The clear supernatant was transferred into a clean 50ml tube with the extract (bacteriocin) stored in the freezer for further use.

Bacteriocins Assay

Screening for Antibacterial Activity

The antimicrobial activities of the bacteriocins extracted from the LAB were determined using the agar well diffusion method as described by Swapnil *et al*. (2020). Indicator organisms used for antimicrobial test were *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* were obtained from Fleming's Laboratory, Department of Microbiology, Veterinary Teaching Hospital, Usman Dan Fodio University, Sokoto.

Agar plates seeded with the indicator bacteria were used (one plate for each indicator bacteria). Wells of 6-mm diameter were made in each of the plate using a cork borer under controlled conditions. Fifty microlitre of the bacteriocin was placed in the 6-mm diameter wells that had been cut in agar plates previously seeded with the indicator bacteria. The plates were kept inside refrigerator for complete diffusion of the added bacteriocins in wells for about 10 minutes. After 10 min of diffusion process plaes were allowed to incubate at 37 °C for 24 h. Observation of zones of inhibition were noted for each pathogen after 24 h incubation period (Daeschel *et al*., 1990).

Evaluation of the Preservative Effect of Extracted Bacteriocin in Yoghurt

To determine the preservative effect of bacteriocin in yoghurt, yoghurt was prepared in the laboratory, the effect of bacteriocin on the coagulation time, microbial load, curd syneresis, pH and titratable acidity as well as the proximate composition of the yoghurt were analyzed at 4°c for 35 days (AOAC, 2013).

Preparation of yoghurt

Freeze-dried yoghurt starter culture containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (*Yogourmet* original) was obtained from where?. Yoghurt was produced in the laboratory using the methods of Tamime (2006). Eight glass beakers were sterilized and to each of the beakers containing 500ml of distilled water, 200g of commercially purchased skimmed milk (Dano milk) was added. The mixture was blended using a sterile spatula till a homogenous solution was formed. The glass beakers were covered with aluminum foil and pasteurized at 85°C for 10 minutes, then allowed to cool to 42°C and labelled R1, R4, R9, L1, L4, L9, C1 and C2. Each beaker was inoculated with a standard yoghurt starter culture and different treatment as follows:

The inoculated milk were dispensed into sterile 50ml tubes and incubated at 42°C for 8h for further analysis.

Microbiological analysis of yoghurt

The microbiological analysis carried out on the treated yoghurt include total bacterial count to assess the overall bacterial population, lactic acid bacteria count, yeast and mold count following the methods of Hussein *et al*. (2022).

Coagulation time

Coagulation time for each treatment yoghurt group was calculated using the initial incubation time till when the curd was formed as described by Hassan and Amjad (2010).

Proximate Composition of Treated Yoghurt

The proximate composition of each yoghurt treatment was analyzed using the method described by AOAC (2023).

RESULTS

Biochemical Characteristics of Isolates

Isolates	Cell shape	Gram stain	Catalase test	Oxidase test	Motility test	Spore formation	Arginine hydrolysis
Gm001	Rod	+	-	-	-	-	+
Gm003	Cocci	+	-	-	-	-	-
Gm006	Cocci	+	-	-	-	-	-
Gm008	Rod	+	-	-	-	-	+
Gm011	Cocci	+	-	-	-	-	-
Gm019	Cocci	+	-	-	-	-	+
Gm027	Cocci	+	-	-	-	-	+
Gm035	Rod	+	-	-	-	-	+
Gm046	Cocci	+	-	-	-	-	+
Gm052	Rod	+	-	-	-	-	-
Gm071	Rod	+	-	-	-	-	+
Fm009	Rod	+	-	-	-	-	+
Fm011	Rod	+	-	-	-	-	+
Fm014	Cocci	+	-	-	-	-	+
Fm016	Rod	+	-	-	-	-	-
Fm022	Rod	+	-	-	-	-	+
Fm047	Cocci	+	-	-	-	-	+
Fm065	Cocci	+	-	-	-	-	+

Sugar Fermentation Spectra of Isolates and Presumed LAB

Isolates Sugar	Gm006	Gm027	Gm052	Fm011	Fm016
Arabinose	-	-	-	-	-
Fructose	+	+	+	+	+
Mannose	+	-	+	+	+
Raffinose	+	-	-	+	+
Ribose	-	+	+	+	+
Galactose	+	+	+	+	+
Trehalose	-	-	+	-	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Xylose	-	-	+	+	+
Glucose	+	+	+	+	+
Gas production from glucose	+	+	+	+	+
Presumed LAB	***Lactococcus* species**	***Enterococcus* species**	***Lactobacillus plantarum***	***Lactobacillus acidophilus***	***Lactobacillus fermentum***

Key - + growth -no growth

Antimicrobial assay of crude bacteriocin

Test organisms	Isolates				
	Gm006	Gm027	Gm052	Fm011	Fm016
E. coli	+	++	++	++	+
S. aureus	+	+++	+++	++	++
*Shigella*sp	+	+	-	++	++
S. typhi	++	+	+	+	+

Key –

- No clear zone

\+ Clear zone of inhibition > 5mm

++ Clear zone of inhibition > 10mm

+++ Clear zone of inhibition > 20mm

Preservative effect of bacteriocin and shelf life of yoghurt

Microbiological analysis

The effect of bacteriocin on the microbial composition of yoghurt produced was evaluated within 35 days storage period. The lactic acid bacteria counts were

higher in the untreated yoghurt (C2) than all the treated yoghurt including nisin treated yoghurt and freshly produced yoghurt. Nisin treated yoghurt had the highest effect on total bacterial growth. Nisin and bacteriocin-treated yoghurt retarded the rate of bacterial growth throughout the storage period followed by yoghurt treated with R4. The untreated yoghurt was spoilt at the end of day 14. The yoghurt treated with bacteriocin-producing LAB were stable for 28 days and began to deteriorate after day 35.

Effect of bacteriocin on coagulation time of treated yoghurt

The result of the effect of bacteriocin on the coagulation time of yoghurt indicated that bacteriocin extracts and bacteriocin producing LAB increased the coagulation time of the yoghurt. However, the coagulation time of yoghurt treated with acidocin (R4) increased by 12.83% compared to untreated yoghurt. Nisin extended the coagulation time by 13.30%, R1 increased coagulation time by 12.10% and R9 11.84% while yoghurt treated with bacteriocin producing culture L1, L4 and L9 increased coagulation time of 11.78%, 10.47% and 10.37% respectively.

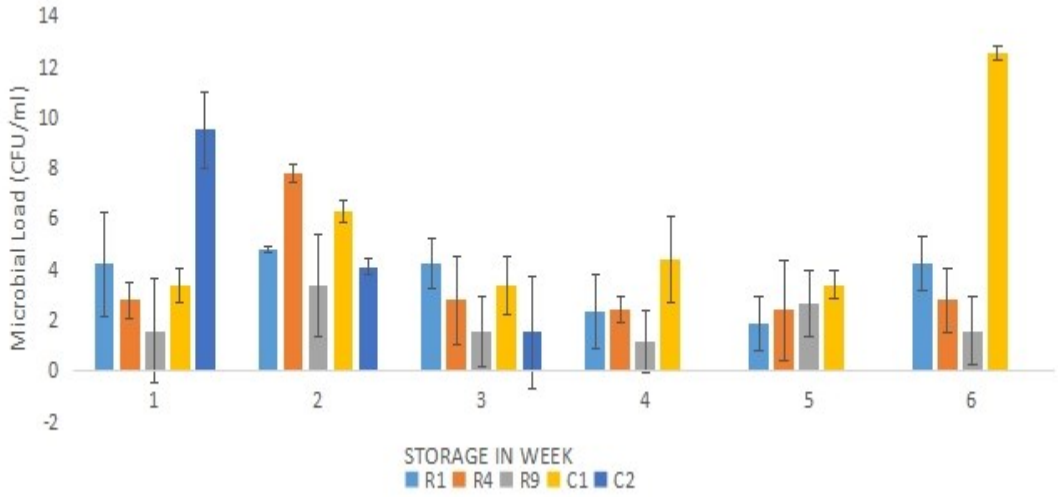

Fig. 4.1: Lactic acid bacteria population in stored yoghurt preserved with bacteriocin.

KEY: C1= Control with Nisin

C2 = Control (untreated)

R1 = Yoghurt with extract from *Enterococcus faecium*

R4 = Yoghurt with extract from *Lactobacillus acidophilus*

R9 = Yoghurt with extract from *Lactobacillus plantarum*

MRS =Man Rogosa agar, NG= No growth

Discussion

Isolation, Identification and Screening of LAB from goat milk and fermented finger millets with Antimicrobial Activity

Some Lactic acid bacteria from goat milk and finger millet were identified and screened for their ability to produce bacteriocin as secondary bio-active metabolites using Gram-staining (positive), oxidase reaction (negative), and sugar fermentation spectra. The major LAB identified in this work were *Lactobacillus plantarum, Enterococcus faecium,* and *Lactobacillus acidophilus*. This observation agrees with several reports that dairy products are the common resources for the isolation of Lactic acid bacteria for local and commercial fermentation (Choi *et al.*, 2021). Common among these LAB are lactoccoci, lactobacilli, enteroocii, and other strains which are reported to be prevalent as active producers of bio-active and acidifying components of the yoghurts and other products produced by them through fermentation (Bangar *et al*., 2022).Subsequently, the three isolates (*Lactobacillus plantarum, Enterococcus faecium,* and *Lactobacillus acidophilus*) were genotyped by 16S rDNA sequencing and confirmed using BLAST analysis with 99% sequence identity.

In total 258 LAB were isolated in this study and among all isolates screened, three candidates exhibited antimicrobial activity against the test bacteria (*E. coli, S. aureus, S. typhi, Klebsiella*sp. and *P. aeruginosa*) using agar well diffusion method. Bacteriocin extract from these LAB isolates exhibited broad-spectrum antimicrobial activity against selected food borne pathogens with inhibition zones ranging from 18 to 28 mm with extract from *Lactobacillus plantarum* being most effective against *S.typhi.* This also agreed with the work of Collins *et al*., (2017) that screening for bacteriocins from dairy-derived LAB is the common strategy, and many bacteriocins have been reported using the strategy with few modifications in some cases (Alvarez-Sieiro*et al*., 2016). Among common bacteriocins, lactococci bacteriocins and garvicin M were reported to have relatively narrow inhibition spectra while nisin, garvicin KS are broad-spectrum in activity (Arbulu*et al*., 2019). In the present study however, the three LAB strains showed antimicrobial activity against three different genera of indicator strains, indicating that the three LAB strains which have broad inhibition spectra. Other researchers have shown that bacteriocins derived from enterococci and lactococci strains have been extensively researched in several instances. Reports also indicates that Lactoccocins, such as lactococcin A, lactococcin G, and others, have been observed to have narrow inhibition spectra mainly against related bacteria genera and species which they typically used to prevail in their given habitats (Antonio*et al*., 2019). This concept is used or taken

advantage of in the preservation of fermented dairy products where they inhibit a wide range of spoilage bacteria and foodborne pathogens (Kasimin*et al.,* 2022). Preservation of yoghurt and other dairy products utilizing the antagonistic activities of LABs is hinged on the ability of the respective isolate bacteria involved in the fermentation to produce copious number of biological substances that are potent enough to directly lyse the cell membrane of other invading bacteria or indirectly interfere with the protein /amino acid production ability thereby killing them (Kumariya*et al.,* 2019). There have also been some studies on the antagonistic activities of enterocins against Gram-negative bacteria, fungi, yeasts, and viruses, although the present study did not test the activity against fungi strains. The antagonistic activity of the isolated strains in this present work against wide range of bacteria (Gram positive and Gram negative), either pathogenic or spoilage bacteria strains was observed, indicating that they may likely be able to produce different bacteriocins compounds especially at different time of fermentation and conditions. This observation might be as a result of the natural abilities of dairy-derived LABs to produce metabolites with reported activities against other bacteria strains for preservation purposes (Lasik-Kurdyś*et al*., 2019). Since this beneficial discovery, many bacteriocins derived from LAB have been further researched and packaged for longer shelf life and application convenience.

Antimicrobial (Bacteriocin) Stability assay

Several Lactic acid bacteria (LAB) have been reported as alluded to in this present study, to be able to produce bacteriocins, organic acids, and other bacteriostatic compounds (Li*et al*, 2021). However, reports have also indicated that the stabilities of the compound are influenced by several factors such as heat, pH and proteases among others (Negash andTsehai (2020). In this present investigation, the extracts were stable (>70%) at temperatures of 30 - 80°C and > 50% for pH range of 2.0 to 6.0. as well as extended ….days. All bacteriocin extracts maintained the proximate composition of yoghurt and extended the shelf life up to ….days. This preservative ability might be because bacteriocins have high thermal stability and are not easily decomposed by proteases. The stability of bacteriocins produced by the three LAB strains after heat and enzyme treatments is presented. This result confirmed that the inhibitory substances produced by the three LAB strains had the basic properties of bacteriocins which might be a good factor for their consideration for both further studies and adoption for industrial applications.

Conclusion and Recommendations

In this study, the LAB strains isolated from fermented goat milk and finger millet were confirmed by Gram staining (positive), peroxidase reaction (negative), ability to metabolize wide range of carbohydrates. However, three

out of the numerous LAB strains inhibited the test bacteria strains (*E. coli, S. aureus, S. typhi, Klebsiella*sp. and *P. aeruginosa*). The application of molecular 16S rDNA identification confirmed these three LAB strains. The bacteriocins produced by these three strains prevented the growth of all the test bacteria *in vitro* using agar well diffusion method. The respective bacteriocins produced by the strains completely inhibited the bacteria test strains owing to the high potency of the compound in the supernatant. In addition, the three strains of bacteriocin-producing LAB as well as their products were safe and stable at the tested conditions including high temperature and enzyme. The overall findings provided a theoretical basis for further development of probiotic resources and subsequent *in vivo* experiments with the isolates for use in the preservation of yoghurt and other products on commercial basis. Further studies are however recommended to understand the specific mechanisms of the action of the bacteriocin compounds derived from the studied isolates. Pilot plant should be designed to upscale the optimal conditions for mass production of the bacteriocin in the study.

References

Choi GH, Fugaban JII, Dioso CM, Bucheliet JEV, Holzapfelal WH, Todorov SD (2021): Selection of bacteriocinogenic *Bacillus spp*. from traditional fermented Korean food products with additional beneficial properties. Fermentation 7(4): 271.

Bangar SP, Chaudhary VP, Singh TP, Ozogul F (2022): Retrospecting the concept and industrial significance of LAB bacteriocins. Food Bioscience 46.

Alvarez-SieiroP, Montalban-Lopez M, Mu DD, Kuipers OP (2016):Bacteriocins of lactic acid bacteria: extending the family. ApplMicrobiolBiotechnol 100(7):2939–2951.

Arbulu S, Jimenez JJ, Gutiez L, Feito J, Cintas LM, Herranz C, Hernandez PE (2019) Cloning and expression of synthetic genes encoding native, hybrid- and bacteriocin-derived chimeras from mature class IIabacteriocins, by *Pichia pastoris* (syn. *Komagataella* spp.). Food Res Int 121:888–899.

Collins FWJ, O'Connor PM, O'Sullivan O, Gomez-Sala B, Rea MC, Hill C, Ross RP (2017): Bacteriocin Gene-Trait matching across the complete *Lactobacillus Pan-genome*. Sci Rep 7.

Antonio LA, Alfonso PA, Tolentino EJC, Tabios CP, Sebastian PDS, Salamat GV, Gracila DC, Tabug MIN, Hagosojos BM (2021): A Review of Lactic Acid Bacteria and their Bacteriocins: Classification, Biosynthesis, and Mechanism against Oral Pathogens. Asian Journal of Biological and Life Sciences 10(2):291.

Kasimin ME, Shamsuddin S, Molujin AM, Sabullah MK, Gansau JA, Jawan R (2022):Enterocin: Promising Biopreservative Produced by *Enterococcus sp*. Microorganisms 10(4):684.

Kumariya R, Garsa AK, Rajput YS, Sood SK, Akhtar N, Patel S (2019) Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. MicrobPathog 128:171–177.

Lasik-Kurdyś M, Sip A (2019): Evaluation of the antimicrobial activity of bacteriocin-like inhibitory substances of enological importance produced by *Oenococcusoeni* isolated from wine. Eur Food Res Technol 245:375–382.

Li P, Long CH, Zhao X (2021): Biosynthesis of class II bacteriocins and their applications in the food industry. Chinese Journal of Food 21(10):269–286.

Negash AW andTsehai BA (2020): Current applications of bacteriocin. International Journal of Microbiology 2020:4374891.