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Identification of Lantibiotic Producing Lactic Acid Bacteria and Its Use Combined With Irradiation For Food Preservation

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Abstract

The purpose of this study was to isolate lactic acid bacteria able to produce lantibiotics from dairy products containing them and evaluate their effects as food biopreservatives when combined with gamma irradiation. Twenty-five LAB isolates were isolated, among which fourteen proved to produce antibacterial substances at various levels. By using conventional methods, the two isolates that produced the highest yield were identified as *Streptococcus thermophilus*, and this identification was further verified by 16S ribosomal RNA (16S rRNA) sequence analysis. Lantibiotic was extracted from culture using chloroform. Liquid Chromatography Mass Spectrometry (LC/MS) and Gas chromatography–mass spectrometry (GC–MS) were used to characterize the extracted lantibiotic after it had been purified using Fast Protein Liquid Chromatography (FPLC). The resulting chromatograms indicated the presence of dehydrobutyrine amino acid, which is characteristic of lantibiotic compounds. Supernatant containing lantibiotics obtained from *Streptococcus thermophilus* was added to samples of minced meat and cut carrot at concentrations of 20 and 40 ml/kg, either separately or in combination with a dosage of 2 kGy gamma irradiation. Compared to the control samples, which had a storage life of only one week, these treatments effectively increased the samples' shelf life to two and three weeks at 4°C±1, respectively. To improve food preservation and microbiological safety, this study supports the use of natural biopreservatives as an alternative to chemical preservatives, either on their own or in combination with low doses of gamma irradiation.
Keywords: lantibiotics; lactic acid bacteria; irradiation; preservation of food

1. Introduction

Food safety and spoilage are right now the world's most serious issues. Traditionally, several techniques have been employed to raise food safety and prevent food from spoiling,one of these methods is the application of artificial chemical preservatives. However, some of these chemical preservatives are known to have adverse effects, while others are carcinogenic [1-3]. The solution to this issue is to apply "biopreservatives," which are described as the microbiota that naturally exists or their antibacterial chemicals, including bacteriocins, to prolong food's freshness and prevent spoiling. Lactic acid bacteria (LAB) and their antimicrobial substances are widely utilized in food biopreservation due to their high frequency in fermented foods and dairy products [4,5].

Most lactic acid bacteria species have been found to produce antimicrobial active peptides or peptide complexes such as bacteriocin during their growth [6,7]. The lactic acid bacterial bacteriocins were primarily divided into three classes [8,9]: Class (I) lantibiotics, which are identified by the presence of unusual amino acids (e.g., dehydrobutyrine, dehydroalanine, lanthionine, & 3-methyllanthionine); Class (II) bacteriocins, which are small heat-stable peptides; and Class (III) bacteriocins, which are large heat-stable proteins.

Lantibiotics are small, ribosomally synthesizedlanthipeptidescontaining translationally modified amino acids with antimicrobial activity [10]. They are produced by certain species of LAB and are active against Gram-positive bacteria, including important antibiotic-resistant pathogens [11]. Their specificity, stability, and ability to act even at nanomolar

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concentrations make them promising candidates for the process of food preservation [12,13], and recently, the application of natural antimicrobial compounds such as lantibiotics in food preservation has gained extensive attention [14].

Methods for irradiating food technology with ionizing radiation (X-rays, electron beams, gamma radiation, etc.) are currently applied in many countries for the preservation of food. It is a physical-cold process (non-thermal process) used to achieve numerous purposes, among which are shelf-life extension of food, reduction or elimination of foodborne pathogens, and disinfestation of insects, among others [15-17]. Several international authorities [18-20] have approved food irradiation technology. Recent advances in food irradiation technology have included the use of combination treatments to minimize the irradiation doses efficiently to prevent spoilage and eliminate pathogenic microorganisms contaminating food [21].

The current study's primary goals are to: 1) isolate and identify lactic acid bacteria that can produce compounds, "referred to as lantibiotics," that have antimicrobial activity; 2) extract, purify, and characterize the lantibiotics that are produced; 3) applylantibiotics that are contained in the supernatant in food preservation tests; and 4) combine lantibiotics with gamma radiation to extend the shelf life of certain food types.

2 Material and methods

2.1 Dairy Sampling

Samples of cream cheese, Romano cheese, feta cheese, pure goat cheese, and three types of yogurts were collected from retail dairy product supermarkets in the governorate of Cairo and sent in an ice box to the Food Microbiology Lab (NCRRT) of the National Center for Radiation Research and Technology. Samples were kept at 4oC and used for lactic acid bacterial species isolation as needed.

2.2 Isolation and identification

Twenty-five grams of each sample and 225 ml of saline solution (0.85% NaCl) were carefully combined in stomacher bags that had been sterilized by gamma radiation. A serial dilution (1:10) was made for each sample, and using the double-layer plate technique, one ml of the appropriate dilutions was plated on De Man–Rogosa–Sharpe (MRS) agar medium. Incubation for 24 to 48 hours at 35°C±1. Suspected colonies of lactic acid bacteria (pen point) were picked up and moved to another MRS plate to ensure their purity. These purified isolates were tested for catalase activity, Gram-stained, and examined under a light microscope.

2.3 *In vitro* evaluation of antimicrobial activity

Fourteen of the twenty-five LAB isolates were screened to produce antimicrobials (in general, referred to bacteriocins). Each of the pure isolates in MRS agar slants was enriched by culturing on pre-prepared MRS agar plates at 35°C±1 for 24 h. Pure colonies of each isolate were inoculated into MRS broth medium then incubated for 24 hours at 35°C±1. Each obtained culture was centrifuged at 15000 rpm for 10 min in a cooling centrifuge (HeraeusMegajuge 8 R centrifuge, Model No. 75007211, Thermofisher Scientific™) at 4°C to produce a cell-free supernatant CFS that was adjusted to pH 6.0 with 1 mol l-1 NaOH. The supernatant was filtered through a filter membrane having a pore size 0.22 µm, diam. 13 mm, PVDF membrane, hydrophilic (Millex®-GV Filter Unit, Millipore, Sigma-Aldrich®). The antimicrobial activity of each cell-free supernatant has been assessed using the agar well diffusion technique [22], against the standard *Staphylococcus aureus* strain as an indicator bacterium. Spreading 100µL (approx. 1x107 cfu/mL) was spread on the surface of MRS agar plates before incubation at 35°C±1 for 24 h. The cell-free supernatant of each isolate was tested for antibacterial activity by measuring the diameter of the inhibitory zone surrounding the well.

2.4 Antimicrobial activity against some food-borne pathogenic bacteria

Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Escherichia coli, and Salmonella spp. as testing bacteria were isolated from food samples at the Food Microbiology Lab, National Center for Radiation Research and Technology. The antimicrobial activity of the cell-free supernatant of each isolate towards them was measured here as for the cell-free extract (referred to as bacteriocin) was determined and expressed as arbitrary units per milliliter (Au/ml). The activity of bacteriocins was determined according to the formula [23].

Bacteriocin activity (mm2/mL) = Lz-Ls/V

Lz = area of clear zone (mm2); Ls = well area (mm2); V = volume of sample (mL).

2.5 Verification of lantibioticproduced by LAB isolates

The pH of the cell free supernatant of LAB isolates was adjusted to (6.0) with 1 M NaOH to eliminate the influence of organic acids. To overcome the influence of H2O2, the cell-free supernatant (pH 6.0) was incubated at 80 °C for 10 min.

2.6 16S ribosomal RNA identification

According to Yeung et al. [24], 16S RNA sequence analysis was utilized to verify the isolate's identification at the species level. Using the gene JETTM genomic DNA purification kit (Fermantas, EU), bacterial DNA was isolated from bacteria grown in HM medium. Utilizing a UV lamb transilluminator on 1% agarose gel, the PCR product was visualized with ethidium bromide and captured on camera (Sony digital camera). Amplifying the DNA using a 50 µl PCR mixture. Using two primers, the following PCR reaction was carried out in a thermocycler (Thermo TM): in front: 5′AGT TCC TGG CTC AG 3′; in reverse: 5′-GGT TAC CTT GTT ACG ACT T-3 Initial denaturation at 94oC lasted five minutes for each cycle. cycles of

amplification were 35followed, with each cycle consisting of a 30-second denaturation phase at 94oC, a 1-minute annealing step at 65oC, and a 30-second extension step at 72oC. The reactions needed to be extended for ten minutes at 72°C to complete the reactions.We used the gene JETTM PCR purification kit to purify the PCR product. Solgent Co. Ltd. (Seoul, South Korea) used the automated sequence analyzer ABI 3730xl to sequence the amplified DNA. Using the pintail program, the chimera system examined the sequence for errors and compared it to standard alignments located in http://www.ncbi.nlm.nih.gov/BLAST, the NCBI database. Then, using the Mega-Blast software version 6, the phylogenic tree was created. GenBank provided access to the newly discovered nucleotide sequences through an accession number.

2.7 Chloroform extraction

From a 24 hr culture of the lantibiotic-producing *Streptococcus thermophilus*, 0.1% of the culture was inoculated into one liter of M17 broth medium and incubated for 24 hr at 37°C. Cells were centrifuged at 7100 xg (HeraeusMegafuge 8R centrifuge, Thermofisher Scientific) for 15 min in a cooling centrifuge at (12°C), and the lantibiotic-containing supernatant fluid was collected, and extracted with 500 mL of organic solvent (CHCl3) according to the method described by Burianek and Yousef [25].

2.8 Fast Protein Liquid Chromatography (FPLC)

The powder of lantibiotic containing extract was resuspended in 20 mM sodium phosphate buffer (pH 6.0) and then subjected to FPLC system (ÄKTA Avant 150-GE Healthcare Life Sciences) at the Central Laboratories Network, National Research Centre, Giza, Egypt) - cation exchange chromatography on a SP FF Sepharose Fast Flow column (5 mL) (GE Health Care, United States) using an ÄKTA Avant chromatography system (GE Healthcare, United States). Material that had been absorbed was eluted at a 1.5 mL/min flow rate with a linear gradient of 0–1 M NaCl. The eluted compounds were observed by a UV detector at a wavelength of 220 nm [26].

2.8.1 Antibacterial activity of (FPLC) protein fractions

Forty-eight fractions derived from (FPLC) were tested for their antibacterial activity using agar well diffusion technique against the standard *Staphylococcus aureus* strain as an indicator bacterium.

2.9 LC-MS/MS

The detection of lantibiotic was accomplished using a particular liquid chromatography-mass spectrometry (4000 QTRAP® LC-MS/MS System, SCIEX, United States) method located at Agriculture Research Center, Regional Center for Food & Feed – RCFF, LC-Ms/ Ms Lab, Giza, Egypt. The MS optimization was carried out by directly injecting an isocratic mobile phase that was a 50:50 v/v mix of water, methanol, and 0.1% formic acid and using a pump (Harvard Apparatus 11 Plus) and a flow rate of 0.2 ml/min. Scanning the obtained chromatograms in full-scan mode displayed the presence of lantibiotic [27].

2.10 GC/MS analysis

At The National Research Center, GC/MS Lab, Giza, Egypt, handled the GC/MS analysis. Utilizing a TG-5MS fused silica capillary column and a Thermo Scientific™ ISQ™ 7610 Single Quadrupole GC-MS (30m, 0.251mm, 0.1 mm film thickness). An electron ionization device with an ionization energy of 70 eV was employed for GC/MS analysis. Helium, the carrier gas, was applied at a constant 1 ml/min flow rate. The injector's temperatures and MS transfer line were adjusted to 280oC. The oven temperature was raised by 5°C every minute to reach the ultimate temperature of 280°C after starting at 150°C and holding it for 4 minutes (hold 4 min). A percent relative peak area was used to evaluate the quantification of all the components that were discovered. By comparing the compounds' respective retention times and mass spectra with those of the NIST and WILLY libraries of GC/MS system data, a preliminary identification of the compounds was carried out [28,29].

2.11 Study of shelf-life extension

Samples of fresh minced meat and cut carrots were used for applying lantibiotic-containing supernatant of Streptococcus thermophilus to study their shelf-life at refrigeration temperature (4°C±1). In this experiment, 20 and 40 mL/kg of freshly prepared lantibiotic-containing supernatant were sprayed over the surface of one kilogram of both minced meat and cut-carrot. Mixed samples were kept under aseptic conditions for 2 hr for full distribution of the crude lantibiotic extract in the samples. The sample from each product was divided into 50-gram portions and kept in 100-mL dishes covered with stretch biofilm. Treated samples were stored at 4°C±1 and periodically examined for their visual observation and microbial aspects.

2.11.1 Microbiological analysis

For determination of total bacterial counts (TBC), 10 g of each sample were homogenized in 90 ml sterile physiological saline solution (0.85% NaCl), then serial 10-fold dilutions were prepared. Appropriate serial dilutions were duplicate plated using standard pour plate method on plate count agar medium [30].

2.12 Irradiation process

An Indian Co-60 gamma Chamber 4000A, located at the NCRRT, EAEA, Nasr City, Cairo, Egypt, was used for all irradiation procedures. This source's dosage rate during the radiation process was 0.8144 kGy/h, and the procedures were carried out at room temperature. The radiation-absorbed dosage was measured using alanine dosimeters, which are traceable to the National Physical Laboratory in the United Kingdom. The Department of Radiation Protection and Dosimeter conducted detailed dosage mapping in accordance with Egyptian standards.

2.13 Combination treatment of lantibiotics and gamma irradiation

Three sets of samples were used to examine the impact of radiation on the preservation of food samples (minced meat and cut carrots). The first control set included samples without any treatments; the second set included samples that were exposed to a 2 kGy dose directly; and the third set included samples that were treated with 40 mL of lantibiotic-containing supernatants before being exposed to a 2 kGy dose directly.

Statistical analysis

Data are represented by mean ± SD (n= 3) using two-way ANOVA followed by tukey`s multiple comparisons test.

3 Results and discussion

3.1 Isolation and initial identification of LAB with Antibacterial Activity

Twenty-five presumptive LAB isolates pen point colony from different cheeses and yogurts were obtained on MRS agar plates, then Gram-stained and tested for catalase activity. Fourteen isolates were identified as non-spore formers, gram-positive, either cocci or rod-shaped, and catalase-negative (Table 1). These 14 isolates, when tested for antibacterial activity against pathogenic bacteria like *S. aureus, E. faecalis, B. cereus, Salmonella spp., and E. coli*, showed less activity against the Gram-negative bacteria used in this studye.g (*E. coli* and *Salmonella spp*.). However, all 14 isolates showed high antimicrobial activity against all Gram-positive indicator microorganisms. Specifically, isolates (Y.N.1) and (Y.N.2) scored the highest antibacterial activity, (Table 2) (Figure 1) and have been selected for further studies. This data is consistent with the findings of Castro et al. [31], who discovered that 27 LAB strains exhibited antimicrobial activity against Gram-positive indicator microorganisms, such as *S. aureus* and *Brochothrix spp.* However, antimicrobial activity against Gram-negative bacteria was absent from all tested isolates, including *E. coli* and *P. aeruginosa*. Ouzari et al. [32] screened a total of 81 Lactococcus lactis isolates obtained from traditional Tunisian dairy products for their capability to produce inhibitory substances in their culture supernatant. Twelve of them (15% of the collected isolates) were found to be able to produce bacteriocin-like substances with large inhibition zones detected. These isolates showed activity against many of the Gram-positive bacteria, including *Bacillus, Staphylococcus*, and *Listeria*, but not against any of the Gram-negative bacteria tested.

Table 1: Gram-staining and catalase test of presumptive lactic acid bacterial isolates.

Isolates	Catalase	Gram-stain	Shape
Y.A.6	-ve	+ve	Rod
R.L.3	-ve	+ve	Oval
C.A.2	-ve	+ve	Cocci
Y.B.1	+ve	-ve	Cocci
Y.D.1	-ve	+ve	Cocci
Y.D.2	-ve	+ve	Rod
R.J.1	-ve	+ve	Cocci
C.R.3	-ve	+ve	Rod
Y.B.3	+ve	-ve	Oval
Y.B.4	+ve	-ve	Cocci
M.J.2	+ve	-ve	Oval
Y.J.5	-ve	+ve	Cocci
C.R.5	-ve	+ve	Rod
C.D.3	-ve	+ve	Rod
M.J.5	+ve	-ve	Cocci
C.A.1	+ve	-ve	Cocci
C.D.4	-ve	+ve	Rod
C.A.3	+ve	-ve	Rod
Y.N,1	-ve	+ve	Cocci
R.A.4	-ve	+ve	Cocci
S.S.1	+ve	-ve	Oval
S.S.2	+ve	-ve	Oval
Y.N.2	-ve	+ve	Cocci
R.L.1	+ve	-ve	Cocci
R.L.2	+ve	-ve	Rod

Table 2: Antibacterial activity of lactic acid bacteria against some foodborne-pathogenic bacteria.

	Antibacterial activity of different LAB isolates (AU/ml)				
LAB Isolates No.	***Staphylococcus aureus***	***Enterococcus faecalis***	***Bacillus cereus***	***Salmonella spp***	***Escherichia coli***
Y.A.6	220 ± 10	200±30	180±40	100±20	100±30
R.L.3	100 ± 20 ^{a}	80 ±10^{a}	60±10^{a}	0^{a}	0^{a}
C.A.2	160 ± 25	100±20^{a}	100±30^{a}	40±5^{a}	40±5^{a,b}
Y.D.1	150 ± 25	150±30	120±30	60±10^{b}	0^{a,c}
Y.2	160 ± 25	120±30	100±20^{a}	0^{a,d}	0^{a,c}
R.J.1	200 ± 30 ^{b}	160±20	160±30^{b}	100±20^{b,c,e}	80±20^{b,c,d,e}
C.R.3	140 ± 25	140±15	100±10^{a}	0^{a,d,f}	0^{a,c,f}
Y.J.5	200 ± 20	180±40^{b}	140±20^{b}	60±10^{b,e,g}	60±10^{b,d,e,g}
C.R.5	130 ± 20 ^{a}	100±10^{a}	40±10^{a,d,f,h}	0^{a,d,f,h}	0^{a,c,f,h}
C.D.3	160 ± 30	120±15	80±20^{a,f}	60±10^{b,e,g,i}	60±5^{a,b,d,e,g,i}
C.D.4	140 ± 20	120±15	100±20^{a}	60±5^{b,e,g,i}	40±4^{a,b,d,e,f,g,i}
Y.N.1	300±50 ^{b,c,d,e,f,g,h,I,j,k}	180±40^{b,e}	180±40^{b,c,e,g,I,j,k}	80±20^{b,e,g,i}	80±8^{b,c,d,e,g,I,k}
R.A.4	260 ± 30 ^{b,c,d,e,g,I,j,k}	150±30	140±20^{b,i}	80±23^{b,e,g,i}	60±6^{a,b,d,e,g,i}
Y.N.2	360±40^{a,b,c,d,e,f,g,h,I,j,k,l}	260±40^{b,c,d,f,g,I,j,k,l}	240±40^{b,c,d,e,f,g,h,I,j,k,l}	120±27^{b,c,d,e,g,h,I,j,k}	100±15^{b,c,d,e,g,h,I,j,k,l}

One-way ANOVA is used to report the data as mean ± SD (n = 3)at P ≤ 0.05, followed by Tukey's multiple comparisons test. a,b,c,d,e,f,g,h,I,j,k&l are significant difference from (Y.A.6; R.L.3; C.A.2; Y.D.1; Y.D.2; R.J.1; C.R.3; Y.J.5; C.R.5; C.D.3; C.D.4 & R.A.4).

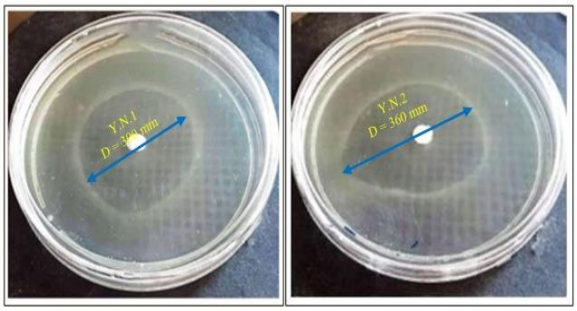

Figure 1: LAB isolates showed the highest antibacterial activity (Y.N.1 & Y.N.2).

3.2 Verification of lantibioticproduced by LAB

The PH value of MRS growing broth medium was adjusted to PH 6.0 using NaOH. Therefore, the antibacterial activity of cell-free supernatant was not affected by organic acids, and the antibacterial substances may be lantibiotic or hydrogen peroxide. Hydrogen peroxide produced by LAB can also impede the growth of bacteria, especially the growth of Gram-negative bacteria, so it is necessary to exclude the over-interference of hydrogen peroxide. The cell-free supernatant of isolates was incubated at 80°C for 10 min to eliminate the influence of H_2O_2. Therefore, it might be inferred that the antibacterial activity of the cell-free supernatant was also not caused by hydrogen peroxide. Organic acids, hydrogen peroxide, and bacteriocins are some of the well-known characterized antimicrobial agents synthesized by LAB [33]. Also, Ren et al [34] adjust the PH of strain A5 to (4.0) and found that the cell-free supernatant had an inhibitory activity to both indicators. Therefore, the antibacterial activity of cell-free supernatant was not caused by organic acids, and incubate the cell-free supernatant of strain A5 at 80°C for 10 min to eliminate the influence of H2O2, its antibacterial activity was almost unchanged.

3.3 16S ribosomal RNA identification.

The two isolated LAB strains with high antibacterial activity (Y.N.1 & Y.N.2) from yogurt samples had 16S rDNA sequencing results that were 99% identity to the 16S rRNA sequences of *Streptococcus thermophilus* OQ152625 &*Streptococcus thermophilus* OQ152628 by using 16S rRNA gene sequencing, according to the results of the BLAST similarity search. The isolate reported in this investigation and the 16S rRNA sequences of different *Streptococcus spp*. displayed in the database (Figures 2 and 3) appeared to have a highly similar phylogenetic relationship, according to the neighbor joining (NJ) technique and boot strap analysis. Therefore, *Streptococcus sp*. decent was suggested as the strain identification after 16S rDNA analysis revealed that the isolated bacterium for the biopreservation investigation was likely a unique strain Genbank accessions OQ152625 and OQ152628). Yang et al. [35] identified the various LAB strains isolated from cheese and yogurt as *Enterococcus faecium*, *Streptococcus thermophilus*, and *Lactobacillus casei*. El Kahlout et al. [36]

isolated five LAB isolates from homemade yogurt made in the Gaza Strip; using 16S rDNA sequencing, they identified three of these isolates as *Streptococcus thermophilus*and two as *Lactobacillus delbrueckii ssp.*

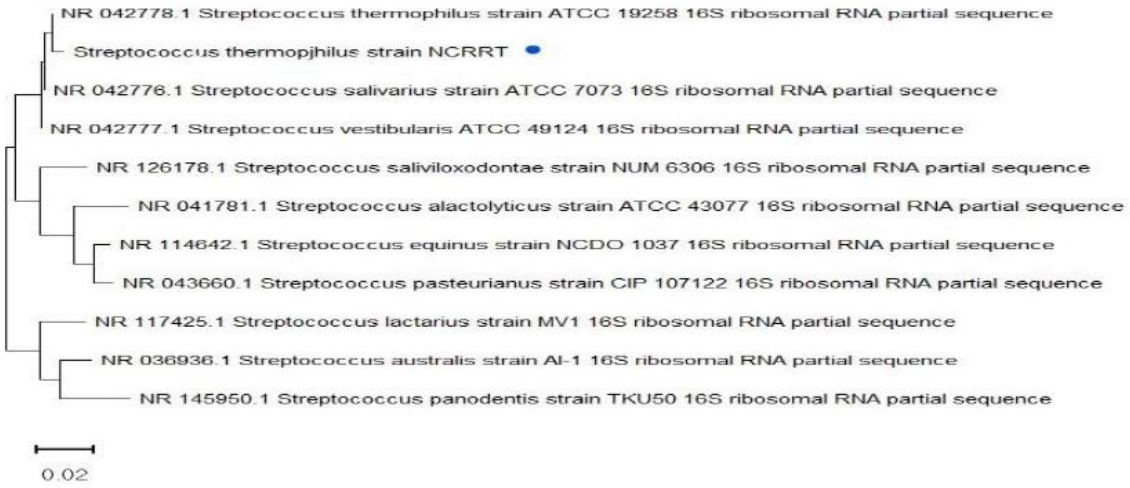

Figure 2: The phylogenetic tree obtained from the 16S rDNA genes demonstrated 99% sequence of isolate (Y.N.1) identity to *Streptococcus thermophilus*OQ152625.

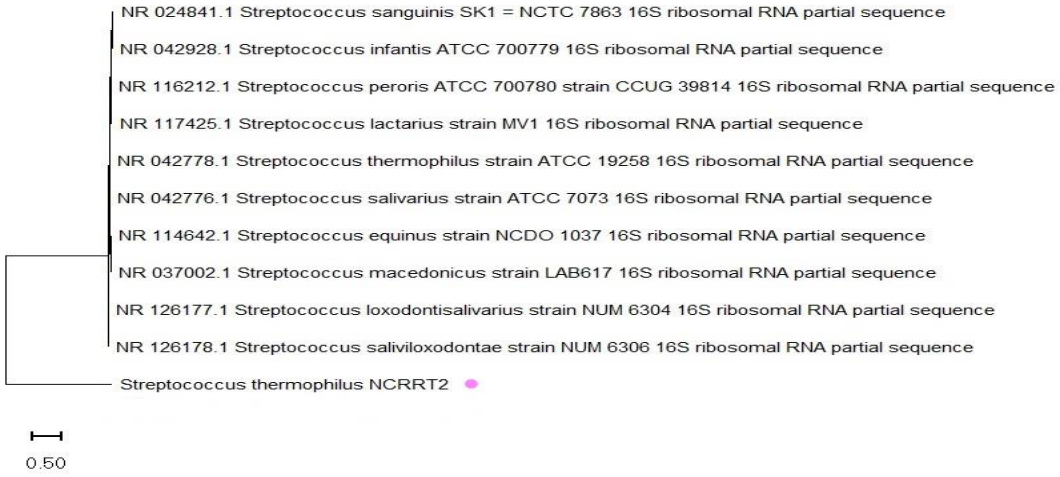

Figure 3: The phylogenetic tree obtained from the 16S rDNA genes demonstrated 99% sequence of isolate (Y.N.2) identity to *Streptococcus thermophilus*OQ152628

3.4 Activity of lantibiotics extracted by chloroform

Lantibiotics were extracted from the culture supernatant fluid using chloroform. Figure 4 represents the lantibiotics yield in 1 liter of inoculated M17 broth medium, which was 420 and 360 AU/mL, respectively. It is clear from the (figure 4) that *Streptococcus thermophilus* OQ152625 had higher lantibiotic activity in comparison to *Streptococcus thermophilus* OQ152628. The extraction process of lantibiotics using chloroform from *Streptococcus thermophilus* OQ152625, gave a final extract with high antibacterial activity that required less work than the other methods. This procedure was easy to perform and saved time, and chloroform solubility in water was only 0.815%. Therefore, chloroform is a solvent with intermediate polarity and is immiscible with water, which makes it most suitable for the extraction of lantibiotic from bacterial culture media. Burianek and Yousef [25] also used chloroform for the extraction of lacidin from the cultures of *Lactobacillus acidophilus* OSU133. Recovery of lacidin by chloroform extraction compared with precipitation with ammonium sulfate and cell acidification produced high yield lacidin and more clean preparations.Cheng et al[37]developed a simple peptide extraction procedure (chloroform extraction) using a semi-solid nutrient-rich agar medium. that leads to the rapid extraction of secreted bioactive bacteriocin peptides from the oral species *Streptococcus mutans*, a prolific bacteriocin-producing species, and its potential application for bacteriocin extraction from other LAB (*e.g., Streptococcus, Lactococcus, Enterococcus*).

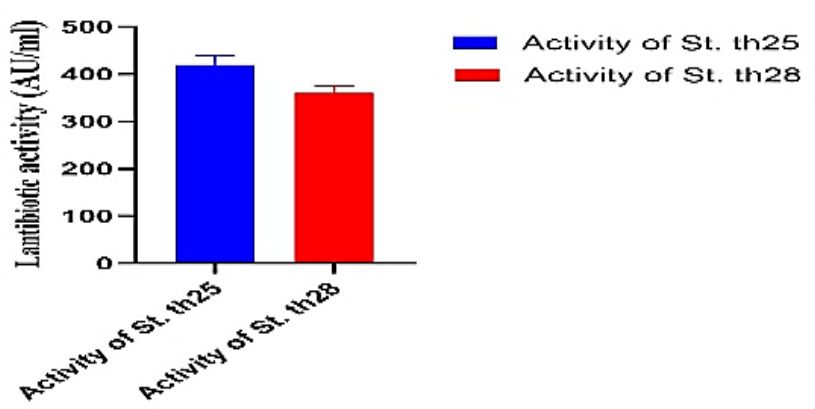

Figure 4: Antimicrobial activity (AU/ml) of lantibiotics extracted by chloroform from *Streptococcus thermophilus* OQ152625 &*Streptococcusthermophilus* OQ152628.

3.5 Purification of lantibiotic with Fast Protein Liquid Chromatography (FPLC)

Fast Protein Liquid Chromatography (FPLC) was used for purification and separation of protein fractions of the extract. Following the extraction of crude lantibiotics from *Streptococcus thermophilus* OQ152625, there was a separation of the protein fractions using Fast Protein Liquid Chromatography (FPLC). The resulting chromatogram (Figure 5) obtained at 220 UV shows a green peak, which indicates the concentration of buffer B (NaCl) used for the separation of protein fractions. Fractions derived from elution's were pure and tested for antibacterial activity. Tumbarski et al. [38] isolated crude bacteriocin from *Bacillus methylotrophicus* BM47 culture. An assay using the indicator microorganism *F. moniliforme* revealed that fractions 5 and 6 exhibited the most pronounced inhibitory effect of the crude bacteriocin substance. Using fast protein liquid chromatography (FPLC).

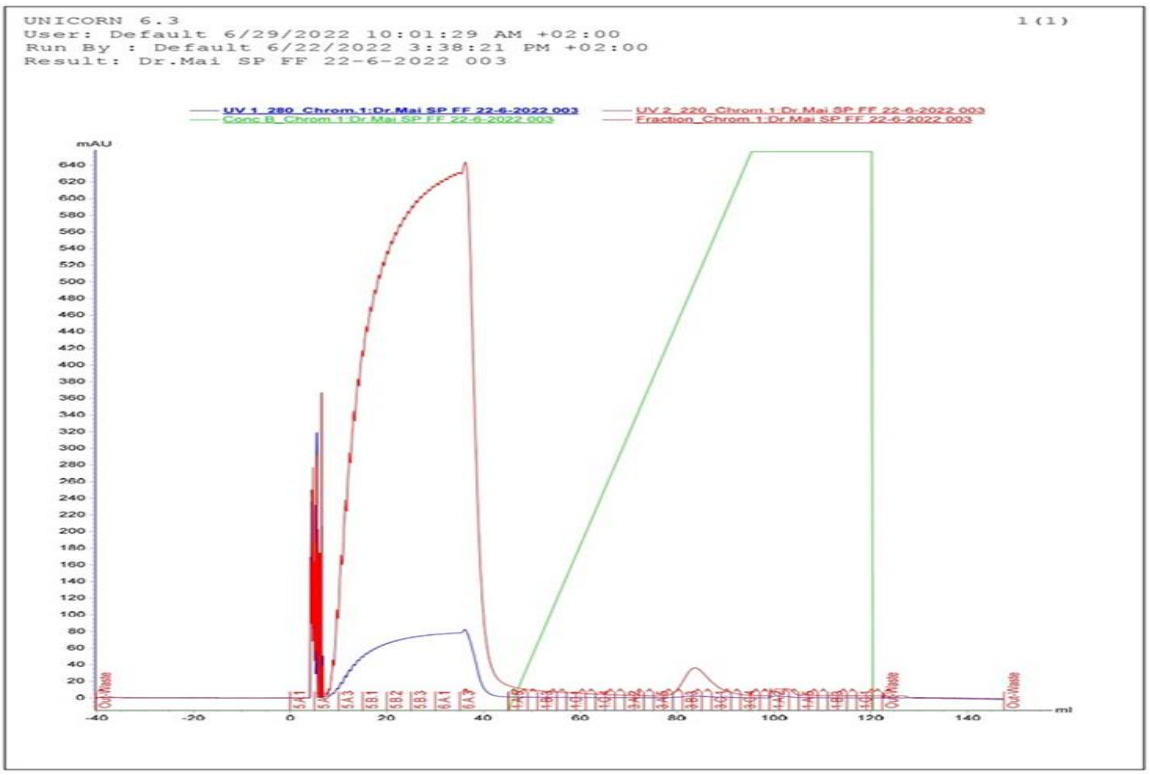

Figure 5: Fast Protein Liquid Chromatography (FPLC) of lantibiotic protein fractions.

3.5.1 Antibacterial activity of fractions purified with (FPLC)

Purified fractions derived from FPLC were tested for antibacterial activity. The results show that fractions 1C1, 1C4, 1C5, 3C3, 1A1, 3B1, and 1B4 had antibacterial activity against St. aureus bacteria. Two fractions, 1C1 and 1B4, had the greatest activity levels of these, measuring 380 and 360 AU/mL, respectively, which can be seen in Figure 6. Bashir et al. [39] recently purified bacteriocin from *Enterococcus faecium* and discovered that, out of all the purified enterocins, there was only one peak that exhibited antibacterial activity.

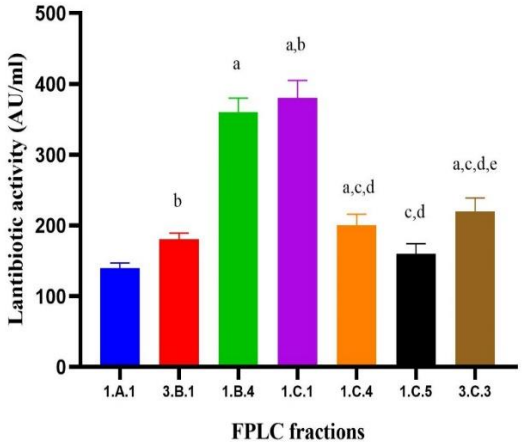

Figure 6: Antibacterial activity (AU/ml) of purified fractions derived from FPLC analysis

3.6 LC-MS/MS analysis of lantibiotic fractions obtained from FPLC

Lantibiotics are gene-encoded peptides that contain intramolecular ring structures, introduced through the thioether containing lanthionine and methyllanthionine residues. The overwhelming majority of the lantibiotics shows antibacterial activity

Pure fractions obtained from FPLC highlights the presence of lanthionine, an amino acid that is typical of lantibiotics (class I bacteriocins) made by lactic acid bacteria. Using ESI in negative ion (ESI-) mode, the lanthionine group has been dissected, and the molecular ion in ESI- mode has been detected at m/z 209.5 (Figure 7). Nielsen et al. [40] developed and used the LC-MS triple Q based MRM technique to determine the absolute quantity of lysinoalanine and lanthionine in various dairy products. Since they discovered that the lanthionine precursor ion mass for Q1 was 209.2 m/z and the fragment ion mass for Q3 was 119.9 m/z, their results took into consideration the results we obtained.

To determine the quantitative amount of lanthionine (119.9 m/z), the most prevalent fragment ions, also known as target ions, were chosen. According to Feldeková et al. [41], a positive APCI-MS spectrum revealed the distinctive molecular ion adduct (m/z 209.05904), and the 1H and 13C APT spectra showed intense CH and CH2 signals at α and β positions that were particularly characteristic of L-lanthionine.

After being post-translationally modified from a serine and a cysteine molecule, lanthionine is the thioether of two alanine residues. LC-MS/MS also emphasizes the presence of the amino acid cysteine. The molecular ion in ESI-mode was detected at m/z 122.2 (Figure 8). Fragments detected were: 104.4, 86.9, 74.1, 69.5, and 56.5, where m/z 122.2 is the dominant ion. It could be verified from Figure 8 that the amino acid cysteine showed up as a clear signal at 122.2 m/z. According to PubChem a public chemical database at the National Library of Medicine (NLM), the top five peaks in the LC-MS of the amino acid cysteine precursor are 122.0, 105.0, 87.0, 76.0, and 59.0, with a mz m/z of 122.06. Doğan et al. [42] applied liquid chromatography-mass spectrometry (LC-MS/MS) method for quantification of cysteine and cystine in wheat flour. Molecular ions of 121 and 240 m/z have been found to be the base peak in spectra for cysteine and cystine, respectively.

As demonstrated in Figure (9), LC-MS/MS technique allowed the characterization of the lactic acid that appeared produced a distinct signal at 89.3 m/z in negative ion mode, and the daughter ion following fragmentation was 43.1 m/z. Lactic acid has an ESI-mode molecular ion of m/z 89.1, according to Sobhy et al. [43], and its fragmentation of m/z 89.1 was (43.1).

As lantibiotics have application in food preservation it also being investigated for efficacy in a range of therapeutic applications. From this chart we investigate the presence of the amino acid (leucine) with parent ion (453.5) and the six fragments (435.5, 322.5, 228.3, 209.2, 114.2) figure (10). Leucine (symbol Leu or L) is an essential amino acid that is used in

the biosynthesis of proteins. Leucine is an α-amino acid, contains an α-amino group (which is in the protonated −NH3+ form under biological conditions), an α-carboxylic acid group (which is in the deprotonated −COO− form under biological conditions), and a side chain isobutyl group, making it a non-polar aliphatic amino acid. It is essential in humans, meaning the body cannot synthesize it: it must be obtained from the diet. Human dietary sources are foods that contain protein, such as meats, dairy products, soy products, and beans and other legumes.

leucine has been found to slow the degradation of muscle tissue by increasing the synthesis of muscle proteins in aged rats [44]. Similar results were found by Shi, et al. [45] Who showed a predominant precursor ion of leucine at (m/z 453.3431), (calculated for C24H44N4O4, 453.3441) in positive ion mode, MS/MS (453.3431, 435.3328, 322.2489, 227.1911, 209.1643, 114.0912).

The chart in figure (10) indicates the presence of leucine precursor ion at m/z 453.5, 222.5, 228.3, 209.

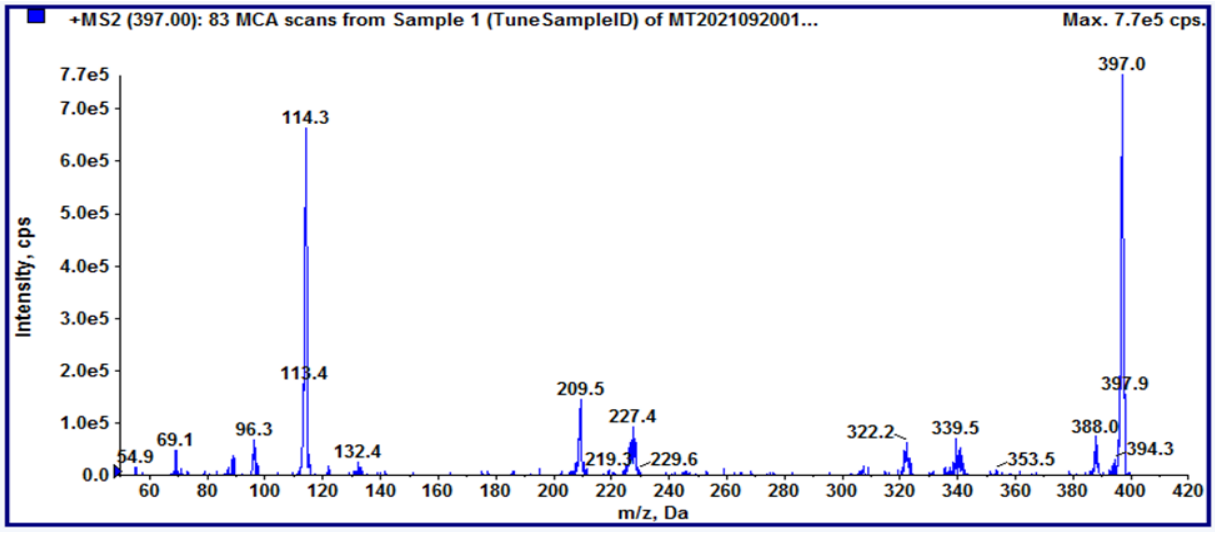

Figure 7: LC-MS/MS spectra of the lanthionine group obtained from FPLC fractions of *Streptococcus thermophilus* OQ152625.

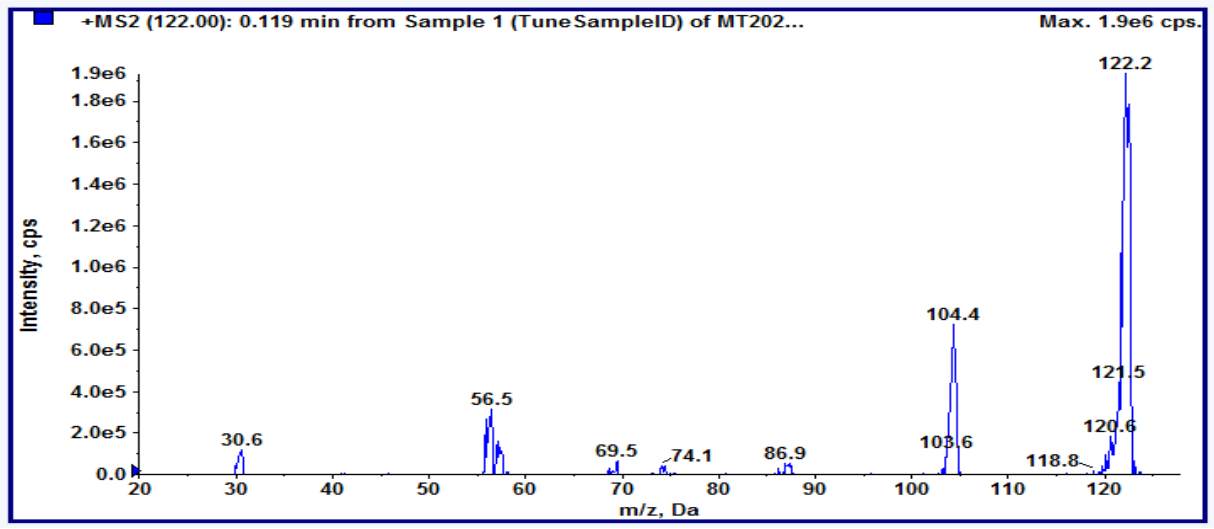

Figure 8: LC-MS/MS spectra of amino acid cysteine and its fragments.

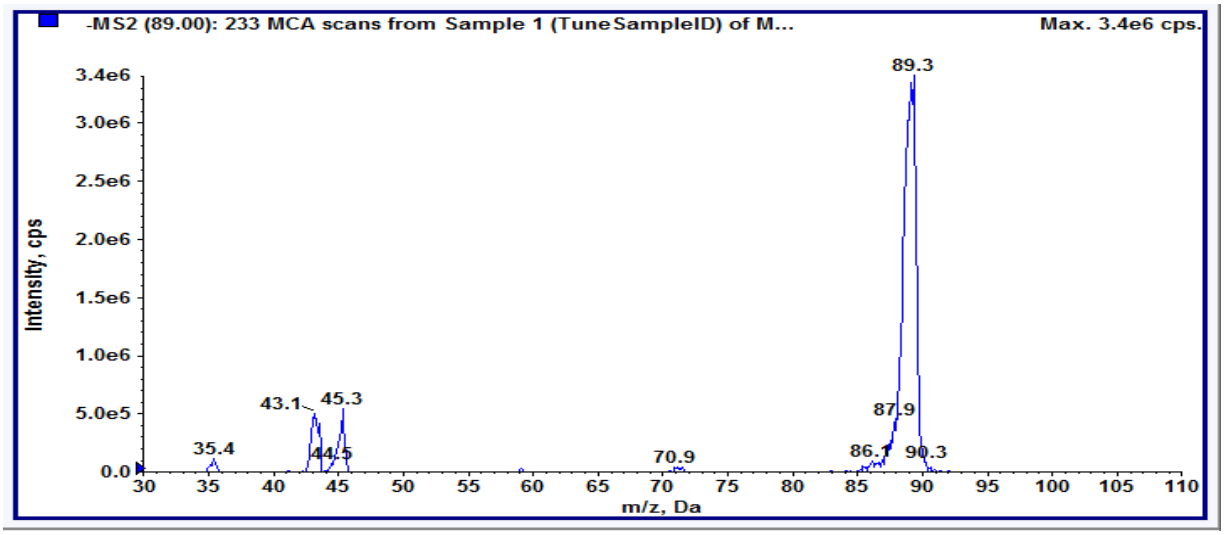

Figure 9: LC-MS/MS spectra of lactic acid transitions.

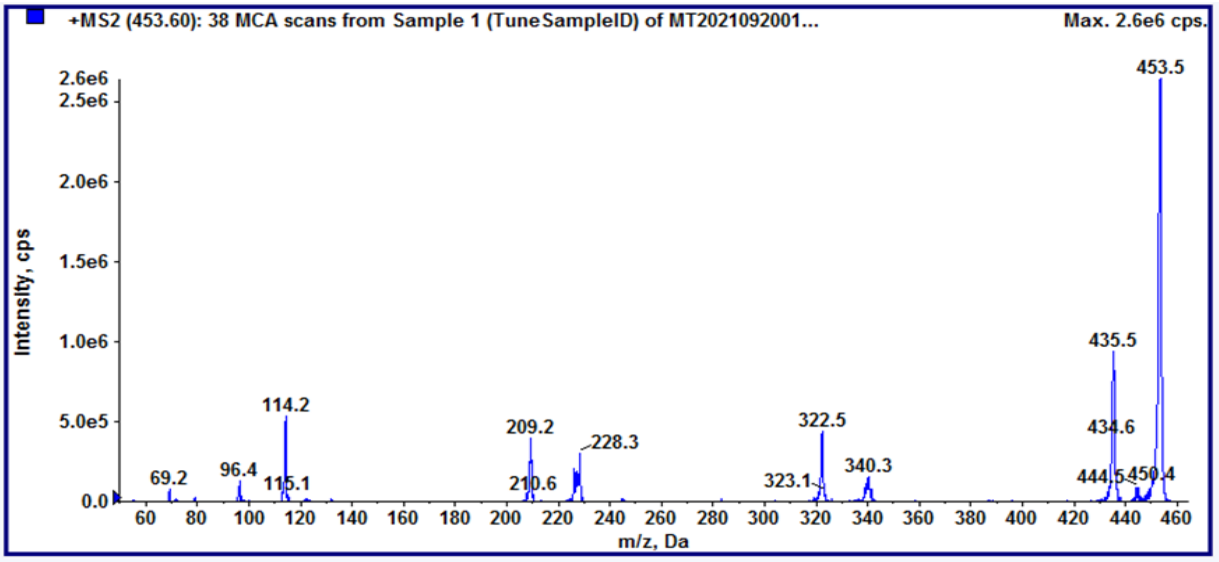

Figure 10: Parant and daughter peaks of amino acid leucine.

3.7 GC-Mass

The GC-mass shows the presence of the amino acid dehydrobutyrine (C4H7NO2), which has a molecular weight of 101 g/mol and is found in a range of peptides, including antimicrobial peptides that belong to the lantibiotic family (Figures 11 and 12). When analyzing the metabolites found in biological samples, the most popular analytical method has always been GC-MS. Due to the high sensitivity and specificity of mass spectrophotometry, this method exhibits a high chromatographic resolution [46]. A distinct class of antimicrobial peptides known as Lantibiotics is generated by ribosomes and includes modified amino acids such methyllanthionine, lanthionine, dehydroalanine, and dehydrobutyrine. The amino acid dehydrobutyrines with the chemical formula C4H7NO2 and molecular weight 101 g/mol are present in the current investigation, according to GC-Mass analysis [47].

Dehydrobutyrine is an amino acid found in a variety of peptide natural products. It is produced by reacting pentafluoropyridine with threonine and then using E1cb-type elimination. This process allows for the preparation of novel amino acids containing dehydrobutyrine [48]. According to PubChem [48], Z-dehydrobutyrine's chemical formula is (C4H7NO2), and its molecular weight is (101.10 g/mol).

Library Search Report

RT	Probability	Compound Name	SI	Area %	Area	Molecular Weight	Molecular Formula	Library
16.39	21.99	(D1)-1,3-DIOXOLEN-2-ONE	366	1.84	105103.63	86	C3HDO3	Wiley9
16.39	12.66	Tris(3,6-di-t-butyl-1-azulenyl)methane	350	1.84	105103.63	730	C55H70	Wiley9
16.39	4.24	2-Oxazolidinone, 3-methyl- (CAS)	326	1.84	105103.63	101	C4H7NO2	Wiley9

Mal-L #3351 RT: 16.39 AV: 1 RF: 6.00,3 NL: 1.58E3

RT	Probability	Compound Name	SI	Area %	Area	Molecular Weight	Molecular Formula	Library
16.39	4.24	2-Oxazolidinone, 3-methyl- (CAS)	0326	1.84	105103.63	101	C4H7NO2	Wiley9

Mal-L #3351 RT: 16.39 AV: 1 RF: 6.00,3 NL: 1.58E3

Figure 11: Library search report of GC-Mass analysis indicating the presence of dehydrobutyrine ($C_4H_7NO_2$) having molecular weight 101 g/ml.

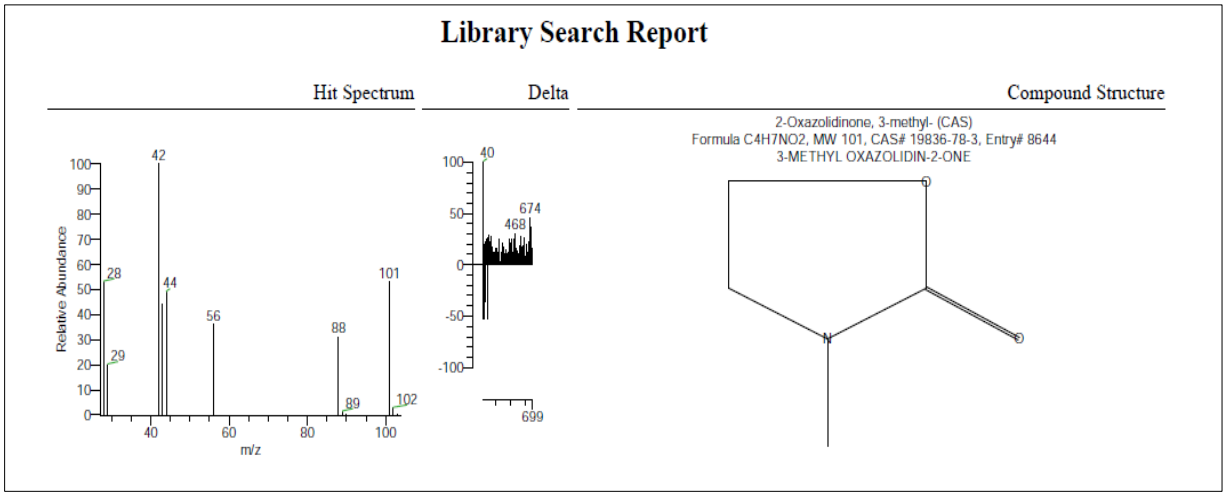

Figure 12: Library search report indicating the chemical structure of dehydrobutyrine

3.8 Food preservation experiments

3.8.1 Effect of lantibiotic-containing supernatants on preservation of minced meat

A sensory assessment of the quality of minced meat samples stored at 4°C ±1 revealed that after a week, the treated samples with Semi-purified fractions were elastic, odorless, and had surfaces that were clean, fresh, and dry, while the control sample began to change color and give off an odor. Samples of minced meat had an initial TBC count of 5.11 CFU/g. Semi-purified fractions were applied at 20 and 40 mL/kg, respectively, which resulted in a slight decrease in the count to 5.0 and 4.95 CFU/g. After a week, these counts raise in both the control and treated samples (containing Semi-purified fractions), although the treated samples showed a slower rate of increase. Table 3 shows that the treated samples (40 mL/kg of lantibiotic-containing supernatants) had a recorded count of 5.2 CFU/g, which was the set count for TBC, compared to 6.36 CFU/g for the control samples. Application of lantibiotic-containing supernatant enhanced preservation of minced meat, as the shelf-life extended to 2 weeks at 4°C against only one week for control samples. Skariyachan and Govindarajan [50] found that the cell-free supernatant from *Pediococcus spp*. at a concentration of 15 ml/kg extended the shelf-life of food samples (strawberries, tomatoes, fish, fresh meat, and corn samples) at room temperature, the fresh meat samples indicated a shelf life of 4 days in comparison with only 24 hours for the control samples.

Table 3: Effect of different concentrations of lantibiotic-containing supernatants on TBC of control and treated minced meats.

Storage time	Control	20 (ml/kg)	40 (ml/Kg)
0 time	5.11±0.2	5.0± 0.3	$4.95\pm0.4^{a,}$
7 days	$6.36\pm 0.3^{b,c}$	$5.53\pm0.5^{b,c}$	5.2 ± 0.5^{c}
14 days	$8\pm1^{a,b,c,d,e,f}$	$6.18\pm 0.4^{b,c,g}$	$5.91\pm0.5^{b,c,g}$

Two-way ANOVA is used to report the data as mean ± SD (n = 3),at $P \leq 0.05$ followed by Tukey's multiple comparisons test. a,b,c,d,e,f& g are significant difference from 0 time (control), 0 time (20 ml/kg), 0 time (40 ml/kg), 7 days (control), 7 days (20 ml/kg), 7 days (40 ml/kg), 14 days (control).

3.8.2 Effect of lantibiotic-containing supernatants of *Streptococcusthermophilus* on TBC of cut-carrot.

The effectiveness of preservation for the cut-carrot samples following treatment is shown in Table (4). The initial log count of the control samples was 2.7 CFU/g. When treated with Semi-purified fractions, the treated samples' log counts decreased to 2.47 and 2.3 CFU/g at doses of 20 and 40 mL/kg, respectively. The treated samples containing Semi-purified fractions demonstrated a significant decrease in log counts to 4.1 and 3.34 CFU/g, respectively, when compared to the control samples (Table 4). After a three-week period, the log counts of the control samples increased to over 6.0. Application of lantibiotic-containing supernatant enhanced preservation of cut-carrot samples, as the shelf-life was extended to 3 weeks at 4°C against 2 weeks for control samples. Delcarlo et al. [51] showed that the application of cell-free supernatant (CFS) of *E. mundtii* STw38 could reduce the growth of native flora in fish for 6 days on air-packed hake paste compared to the control.

Table 4: Effect of different concentrations of lantibiotic-containing supernatant on TBC of control and treated cut-carrot samples.

Storage time	Control	20 (ml/Kg)	40 (ml/Kg)
0 time	2.78±0.2	2.47± 0.3	2.3±0.5
7 days	3.7± 0.3	2.9±0.4	2.78±0.5
14 days	4.18 ± 0.5^{c}	3.08± 0.7	3±0.5
21 days	$6\pm1^{a,b,c,d,e,f,g,h,i}$	3.6 ± 0.6^{j}	3.34 ± 0.3^{j}

Two-way ANOVA is used to report the data as mean ± SD (n = 3, at $P \leq 0.05$ followed by Tukey's multiple comparisons test. a,b,c,d,e,f,g,h,i& j are significant difference from 0 time (control), 0 time (20 mL/kg), 0 time (40 mL/kg), 7 days (control), 7 days (20 ml/kg), 7 days (40 ml/kg), 14 days (control), 14 days (20 ml/kg), 14 days (40 ml/Kg), & 21 days (control).

3.8.3 Combination treatment of (minced meat and cut-carrot) with lantibiotic-containing supernatants and gamma irradiation.

The technology of irradiation was effective on food preservation. The application of Semi-purified fractions in conjunction with gamma irradiation to food samples resulted in a noticeable impact on the longest possible food preservation. Our results confirm these findings, as all food samples (cut carrot and minced meat) treated with 40 ml/kg of Semi-purified fractions and exposed to 2 kGy of radiation showed undetectable bacterial levels after two weeks for the minced meat samples and three weeks for the cut-carrot samples (tables 5 and 6).

Food irradiation technology is a convenient technology that can be successfully used for food preservation [52]. It is currently applied in many countries for extending the shelf-life of foods and for reducing or eliminating pathogenic bacteria andmycotoxins producing molds, as well as for many other purposes [53]. Zahran [54] studied the effects of combination treatments of nisin (100 or 200 µg/g) and γ irradiation (1 or 2 kGy) on minced chicken samples stored at refrigeration temperature (4± 1oC). It was found that such a combination treatment reduced the TBC and total count of both mold and yeasts, in addition, extended the shelf-life of the samples to 14 days.

The immediate reduction after γ irradiation may be mainly due to the direct effect of gamma rays on the microbial cell by causing damage to the genetic material of the cell, preventing it from carrying out the biological processes necessary for its existence.

Table 5: Combination treatment of meat samples with lantibiotic-containing supernatants and gamma irradiation.

Storage time	Control	40 (ml/Kg)	2 kGy	40 (ml/Kg) &2 kGy
0 d	3.6±0.4	2.36±0.2	2.2±0.2	<1
7 d	$4.43\pm0.6^{b,c,d}$	3.54 ± 0.5^{d}	3.6 ± 0.6^{d}	<1
14 d	$>7\pm1^{a,b,c,d,e,f,g,h}$	$3.9\pm0.6^{b,c,d,h,i}$	$3.74\pm0.7^{c,d,h,i}$	<1

Two-way ANOVA is used to report the data as mean ± SD (n = 3),at $P \leq 0.05$ followed by Tukey's multiple comparisons test. a,b,c,d,e,h&i are significant difference from 0 time (control), 0 time (40 ml/kg), 0 time (2 kGy), 0 time (40 & 2 kGy), 7 days (control), 7 days (40 ml/kg), 7 days (2 kGy), 7 days (40 & 2 kGy), 14 days (control), 14 days (40 ml/kg), 14 days (2 kGy).

Table 6: Combination treatment of cut-carrot samples with lantibiotic-containing supernatants and gamma irradiation.

Storage time	Control	40 (ml/Kg)	2 kGy	40 (ml/Kg) & 2kGy
0 d	2.5±0.5	1.4 ± 0.2^{a}	2.1±0.3	<1
7 d	$2.8\pm0.4^{b,d}$	2.4 ± 0.4^{d}	2.38 ± 0.3^{d}	<1
14 d	$3.2\pm0.3^{b,c,d,h}$	$2.74\pm0.3^{b,d,h}$	$2.43\pm0.4^{b,d,h}$	<1
21 d	$3.4\pm0.4^{b,c,d,h,l}$	$2.9\pm0.5^{b,d,h,l}$	$3\pm0.5^{b,d,h,l}$	<1

Two-way ANOVA is used to report the data as mean ± SD (n = 3),at $P \leq 0.05$ followed by Tukey's multiple comparisons test. a,b,c,d,e,h&i are significant difference from 0 time (control), 0 time (40 ml/Kg), 0 time (2 kGy), 0 time (40 & 2 kGy), 7 days (control), 7 days (40 ml/kg), 7 days (2 kGy), 7 days (40 & 2 kGy), 14 days (control), 14 days (40 ml/kg), 14 days (2 kGy), 14 days (40 & 2 kGy), 21 days (control), 21 days (40 ml/kg) & 21 days (2 kGy).

4 Conclusion

In conclusion, this research identified lantibiotic lactic acid producing bacteria, *Streptococcus thermophilus*, from dairy products. Extraction of lantibiotic with chloroform is the most suitable method. Characterization of the lantibiotic revealed the presence of dehydrobutyrine amino acid. Applying lantibiotic rich supernatant to minced beef and carrot samples, alone or with 2 kGy gamma irradiation, substantially increased shelf life to 2 and 3 weeks at 4°C±1, compared to control samples' one-week storage life. Natural biopreservatives like lantibiotics can enhance food preservation and microbiological safety, especially when combined with low doses of gamma irradiation.

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Author Contributions:

Conceiving the proposed idea, study conception and design and participation in the development of the research plan: A.H., M.AZ. Material preparation, data collection M.Z. and S.M. Analysis and interpretation of results were performed by M.Z., S.AE. The first draft main manuscript text was written by M.AZ. and A.H.; S.M. prepared figures; S.AE. prepared tables; and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials: All data generated or analyzed during this study is included in this published paper.

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