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POTENTIAL OF NISIN LOADED LIPID NANOPARTICLES ON INHIBITION OF *ENTEROBACTER CLOACAE* BIOFILM FORMATION

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ABSTRACT

The food borne pathogen *Enterobacter cloacae* contribute to food borne illness in humans. Biofilm formation in *Enterobacter cloacae* makes them more resistant to antibiotics. The main goal of the research is to prevent biofilm-forming *Enterobacter cloacae* by encapsulating nisin in liposomes using nanotechnology. The isolate was identified by 16S rRNA gene sequencing, and the biofilm-formed were characterized. Nisin was selected based on sensitivity testing. A microvesicle encapsulation method was used to encapsulate nisin in liposomes. Bacterial control was determined by colony forming units in an in vitro bioassay. Inhibition and eradication of *Enterobacter cloacae* was investigated using a microbial biofilm high-throughput antimicrobial susceptibility test using the Calgary biofilm apparatus. The food borne pathogen *Enterobacter cloaca was* isolated from the skin of grapes. After characterizing the biofilm formation on the isolate, the results showed that *Enterobacter cloacae* has the highest biofilm formation in tryptic broth (TSB) and brain heart infusion medium (BHI). In the antibiotic susceptibility test, the isolate is inhibited by antibiotics when presented in high concentrations. High-throughput analysis was performed using the Calgary biofilm apparatus, and the results showed that the nisin-loaded liposome exhibited good inhibition compared to antibiotics. One mM concentration of nisin (3.3 mg/10 mL) was used to encapsulate them in liposomes using a microvesicle encapsulation method. The results showed a tremendous inhibition of the food borne pathogen *Enterobacter cloacae* by the colony-forming units. This liposomal encapsulation of nisin promises high inhibition and can also be used for food safety.

1.Introduction

An *Enterobacter cloaca is* a food borne pathogen responsible for about 80% of all food borne illnesses. They tend to cause food borne illness by eating different foods (Shaker *et al.,* 2007). Despite the use of modern preservation techniques in food production, the number of food borne illnesses has increased in recent years. The use of chemicals such as preservatives to improve the shelf life and safety of food is a concern. However, the use of these chemical preservatives can harm human health (Anana *et al.,* 2020).

Bacterial biofilms fuse with a matrix of extracellular polymeric substances consisting of polysaccharides, proteins, lipids and extracellular DNA and adhere to the target surface (Zhong *et al.,* 2017). *Enterobacter cloacae* can form biofilms and is also more resistant to antibiotics. Because *Enterobacter cloacae* is able to form biofilms, they cannot be

prevented using low concentrations of antibiotics; therefore, inhibition is common with high doses of antibiotics (eg, 100 micrograms), which in turn can cause negative human health effects. Therefore, an alternative solution that can save us from the problem is nanotechnology.

Nanomaterials are specifically designed for incorporation into foods as nanoparticles for use as delivery systems or to modify optical, rheological or flow properties (Mc Clements & Xiao, 2017). Food-grade nanoparticles are used to encapsulate hydrophobic bioactive molecules such as phospholipids and lipids as an alternative to oral administration of vitamins, nutrients, and nutrients that can be used in the food industry.

Lactobacillus lactis produce an antimicrobial peptide Nisin, which consists of 34 amino acids and belongs to the lantibiotic family. They have been used as preservatives since ancient times and approved by the World Health Organization (Prombutara *et al.,* 2012). 'Nisin consists of unusual and distinct post-translationally modified amino acids: thioether-linked lanthionine and 3-methyllanthionine and unsaturated 2, 3-didehydroalanine and 2,3-didehydrobutyrine' (Breukink *et al.,* 1999). Nisin has several antimicrobial effects: it binds to peptidoglycan and lipid II precursors and inhibits cell wall biosynthesis. 'Nisin is widely used in foods such as cheeses, salads, canned soups, ice to preserve fish, baby food, shakes and baked goods' (Samelis *et al.,* 2005). Nisin can be used as a preservative because it is high in protein and non-toxic because the digestive system can convert protein into amino acids. Several dietary components such as 'glutathione, proteases, sodium metabisulfite and titanium dioxide' can damage nisin (Quintavalla & Vicini, 2002). Nisin, on the other hand, is typically incorporated directly into food systems as commercial products to avoid contamination with Listeria bacteria, an application in which activity declines over time as a result of enzyme degradation and interaction with food ingredients such as protein and lipids. (Jung *et al.,* 1992). Nanoparticulate systems can significantly enhance the nisin's controlled release and distribution. It seems that liposomes are appropriate vehicles for nisin's regulated movement and action within the cheese matrix. Nisin's distribution, stability, and availability within the cheese matrix are all enhanced by retention in liposomes. 'Both membrane-bound and encapsulated nisin, which can have both short-term (release of encapsulated nisin) and long-term (desorption of membrane-immobilized nisin) antibacterial activity, can be advantageously incorporated to this system' Benech *et al.* (2002). According to Benech *et al.* (2002), this technique can help reduce undesirable bacteria in foods that are kept for a long time, like cheese. Nisin can be encapsulated utilizing the microvesicle encapsulation method in liposomes, such as phosphatidyl and linolenic acids. The primary study's objective was to incorporate nisin into liposomes.

2. Materials and methods

2.1.Materials

2.1.1. Microbial Medium compositions

- Brain Heart Infusion Broth (g/L) **(**agar-15.0, brain extract -7.8, dextrose- 2.0, disodium phosphate- 2.5, heart extract- 9.7, peptone- 10.0, sodium chloride-5.0)
- Luria Bertani broth (g/L) (sodium chloride-0.5, tryptone-10, yeast extract- 5, agar-15)
- Tryptic Soy Broth (g/L) (Peptone from casein-17.0, Peptone from soymeal -3.0, D(+)-Glucose monohydrate- 2.5, Sodium chloride - 5.0 g/L, di-Potassium hydrogen phosphate - 2.5)
- Muller-Hinton agar (g/L) (agar- 17.0, beef infusion solids - 2.0, casein hydrolysate - 17.5, starch - 1.5)
- Congo red agar (g/L) (Brain heart infusion broth -37, sucrose -50, agar - 10, congo red stain -0.8)

2.2.Methods

2.2.1. Isolation and screening of Food borne Pathogen

Enterobacter cloacae, a food borne and hospital-acquired pathogen, was isolated from the skin of grapes. The isolate was identified as *Enterobacter cloacae* by 16s rRNA and the NCBI Genome Repository accession number is MK61597. Morphological and biochemical characterizations of the bacterial strain were

performed. Bacterial strains were grown at 37°C and maintained on nutrient agar plates at 4 °C.

2.2.2. Growth curve of food borne pathogen Enterobacter cloacae

The growth curve of the food borne pathogen *Enterobacter cloacae* was evaluated with a 1% stock culture of the foodborne pathogen. *Enterobacter cloacae* was inoculated into eight different growth media such as Brain Heart Infusion Broth, Brain Heart Infusion Modified Broth -1 and 25 g/L Protease Peptone, Brain Heart Infusion Modified Broth -2 and 4 g/L Protease Peptone, Feed Broth, Luria Bertani Broth, Tryptic Soy Broth, Tryptic Soy Broth Modified -1 with an additional 25 g/L tryptone, and Tryptic Soy Broth Modified - 2 with an additional 25 g/L Glucose and turbidity was analyzed every half hour with a spectrophotometer for six hours. The optical density values of the food pathogen *Enterobacter cloacae* cultured every 30 minutes were observed and graphed.

2.2.3 Antibiotic resistance test

The food borne pathogen *Enterobacter cloacae* was inoculated overnight in nutrient broth. Muller-Hinton agar plates were prepared and the food borne pathogen Enterobacter cloacae was swabbed with sterile cotton swabs. 20 different antibiotics were prepared at a high concentration (100 mcg/ml) and placed on swab plates. The plate was kept at 37°C overnight for incubation. The formation of groups indicated that they were sensitive, and the absence of groups indicated that they were resistant to that particular antibiotic.

2.2.4. Characterization of biofilm

2.2.4.1. Tube assay method

This method is mainly used to assess the biofilm formation in glass tubes. Cells were pre-grown in eight different growth mediums, and 1% of the culture was used to inoculate 10 ml of eight different growth mediums in a test tube. The tubes were incubated, and biofilm formation was monitored by crystal violet staining. This assay was carried out over 24- and 48-hour incubation periods.

2.2.4.2 Congo red agar (CRA) method

The food-borne pathogen *Enterobacter cloacae* was streaked on the CRA plate and incubated aerobically at 37°C for 24 h. Production of black or red colonies with a dry, crystalline consistency resulted in a positive result (Panda *et al.,* 2016).

2.2.4.3. Microtiter plate (MTP) method

In this method, 96-well plates were used to evaluate biofilm formation and optimize the formation of biofilm by the food-borne pathogen *Enterobacter cloacae* in eight different growth media. 10µl of the overnight culture was taken and diluted with 990µl of the fresh sterile eight different growth mediums and incubated for 24, 48, 72, and 96 h. Following a one-hour incubation period, a 15-minute staining period with 1% crystal violet, and a rinse with distilled water to eliminate any remaining stain, the planktonic cells were eliminated using phosphate buffered saline (PBS). Optical density (OD) was measured in the ELISA reader plate at 650 nm after the wells were filled with 33% glacial acetic acid.

2.2.4.4. Biofilm formation in different substrates

Different substrates were chosen, including glass, polyvinyl pyrrolidone, plastic, steel, and wood. They were then cleaned with soap, rinsed with distilled water, and allowed to air dry. Following an overnight culture inoculation, the dry substrates were incubated for 1 – 3 days at 37°C. Following the incubation period, sterile distilled water was used to wash the substrates after they were taken out of the broth culture. Following a 15-minute staining period with 1% crystal violet, the residual adhering bacteria were fixed using methanol. With the faucet running, the extra crystal violet was removed. Following resolubilization with 33% glacial acetic acid, the adhering bacteria were quantified at 570 nm versus optical density.

2.2.5. Screening for nisin that inhibit the growth of bacterial pathogen

The inhibition of the growth of the food-borne pathogen *Enterobacter cloacae* was carried out by the use of nisin. Muller-Hinton agar plates were prepared, and food-borne pathogens *Enterobacter cloacae* were swabbed with sterile cotton swabs. Nisin was introduced to the wells at varying amounts after the wells were perforated. The plates were stored at 37°C for an overnight incubation period. At various

concentrations, the zone of inhibition was seen.

2.2.6. Preparation of liposome encapsulated nisin

2.2.6.1 Nisin encapsulated linolenic acid

A stock solution was prepared by adding 12.5 mM alpha-linolenic acid to 27.5 mM NaOH. Nisin stock [3.3g of nisin (1 mM) in 0.02 N HCL]. Encapsulation of nisin was done by addition of 1.39 g of alpha-linolenic acid, which was dissolved in 0.25 mM chloroform in 50 ml of water. 0.5 ml of nisin was added to alpha-linolenic acid and stirred at 50 °C until the chloroform was completely evaporated. 5 ml of 27.5mM NaOH was added to the evaporated lipid, sonicated for 5 cycles (1 cycle = 10 sec), and kept in an ice bath for 3 min. The solution is filtered using a syringe filter and stored at room temperature.

2.2.6.2. Nisin encapsulated phosphotidyl choline

A stock solution was prepared by adding 76 mg phosphotidyl choline to 10 mM phosphate buffered saline. Nisin stock was prepared by dissolving 3.3g of nisin in 0.02 N HCL. Encapsulation of nisin was done by adding 76 mg of phosphotidyl choline, in 100 ml of ethanol, and 0.5 ml of nisin stock to the phosphotidyl choline stirred at 50°C until the ethanol completely evaporated. 5 ml of 10 mM phosphate buffer saline was added to the evaporated lipid, sonicated for 5 cycles (1 cycle = 10 sec), and kept in an ice bath for 3 min. The solution is filtered using a syringe filter and stored at room temperature.

2.2.6.3. Quantification of nisin using spectrophotometer

Nisin present in the encapsulated liposomes were quantified by protein estimation by Lowry's method and the OD was taken at 660nm and the graph was plotted.

2.2.6.4. DL% (Drug loading) and EE % (Encapsulation efficiency) of nisin

$$\textbf{Active Loading (DL \%)} = \frac{\text{Entrapped drug}}{\text{Nanoparticle weight}} \text{ X } 100 \qquad (1)$$

Where entrapped drug is the weight of nisin-loaded nanoliposomes in Lowry's protein estimation and nanoparticle weight is the weight of nisin-loaded nanoliposomes.

$$\textbf{Encapsulation Efficiency(EE\%)} = \frac{C_{total} - C_{out}}{C_{total}} \textbf{ X 100} \qquad (2)$$

Where C_{out} - liposome suspension Diluted with water and filtered and C_{total} - hot ethanol (70°C) diluted liposome suspension to break up the liposomes and release the drugs contained in the solvent.

2.2.6.5 Calgary biofilm device method for testing of microbial biofilm

Step 1: Inoculation on modified MBEC Assay plate

Step 2: Setting up the antimicrobial challenge plate

Step 3: Neutralization and recovery

Step 4: Determination of MIC and MBEC

2.2.6.6 Susceptibility testing of nanoliposomes

The least dilution of the nanoliposomes that inhibit the organism was determined using the minimum inhibitory concentration. The *Enterobacter cloacae* from the overnight TSB modified-1 broth was added to each well in the 96-well plates at a concentration of 100µl. 50µl of the nisin-loaded nanoliposomes (linolenic acid and phosphatidylcholine) and fresh broth were added. The plate was incubated at 37 °C overnight. The OD recorded at 570 nm.

2.2.6.7 In vitro *liposome biological activity assay*

The bioavailability of the liposomes loaded with phosphotidyl choline and Linolenic acid was determined by CFU (Colony-Forming Units). The assays were performed after incubation with a 1% v/v suspension of food-borne pathogens (TSB modified 1) for 24 h (37 ^{o}C) with nisin loaded liposomes, at 3% concentration. The planktonic *Enterobacter cloacae* were also assayed.

2.2.7 Dynamic Light Scattering Zeta-Potential Analysis (DLS-Zeta Potential)

DLS determines the size of the liposome and Polydispersity Index (PDI). DLS was performed using a standard Brookhaven Instruments Goniometer, BL-200M (Biomolecular

Biomolecular Analyzer, BI- 9000AT Digital Correlation). DLS was used to determine the Zeta potential after dilution (1 mM NaCl) of nisin (nisin) and nisin (encapsulated) liposomes (Zetasizer®, nano ZS (ZEN 3600).

3. Result and discussion

3.1. Identification of *Enterobacter cloacae*

The food-borne pathogen *Enterobacter cloacae* was isolated from the grape peel of the grape fruit and observed by streaking on nutrient agar plates. The isolate was characterized as negative for Gram's staining and positive for catalase, oxidase, Voges Proskauer, citrate utilization, and nitrate reduction.

3.2 Cell growth analysis of *Enterobacter cloacae*

Cell growth was investigated in eight different growth media as shown in Figure 1 (NB, LB, BHI, BHI-1, BHI-2, TSB, TSB-1, and TSB-2). *Enterobacter cloacae* were able to grow for different periods of time, ranging from half an hour to six and a half hours. Among the eight different growth media, BHI media showed good growth in the logarithmic phase.

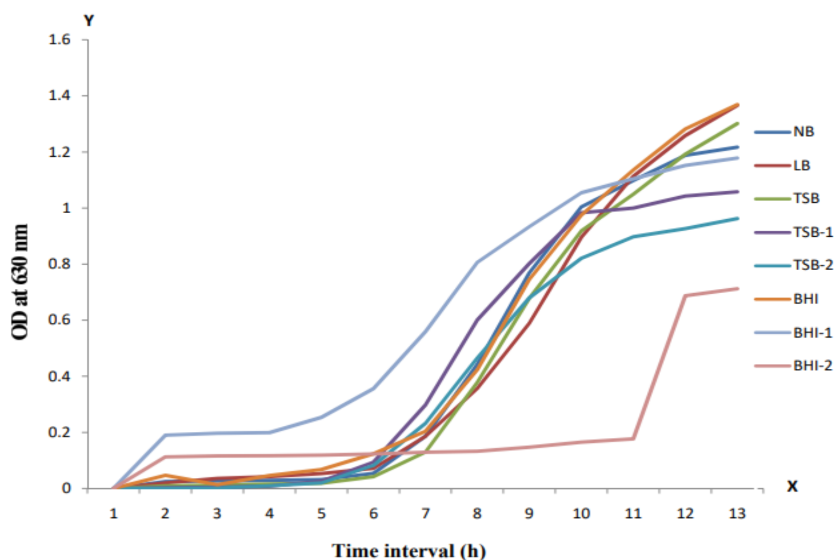


Figure 1. Analysis of growth curve of *Enterobacter cloacae* in different growth media (*BHI: Brain Heart Infusion broth, BHI-1: Brain Heart Infusion modified broth -1 BHI -2: Brain Heart Infusion modified broth- 2, NB: Nutrient broth, LB: Luria Bertani broth, TSB :Tryptic Soy broth, TSB-1: Tryptic Soy broth modified-1, TSB-2: Tryptic Soy Broth modified -2)* at a various period of time.

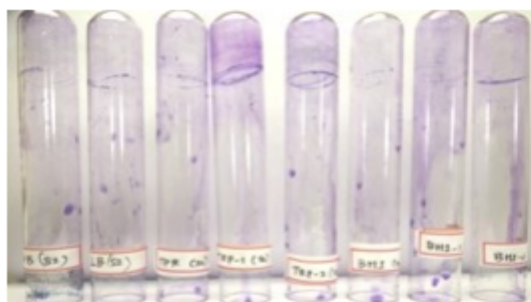


Figure 2 (A): 24 h

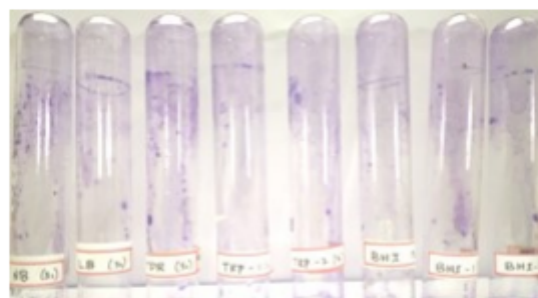


Figure 2(B): 48 h

Figure 2 (A): The violet color ring shows that the biofilm formation of food borne pathogen *Enterobacter cloacae* at a time period of 24 h.

Figure 2 (B): The violet color ring shows that the biofilm formation of food borne pathogen *Enterobacter cloacae* at a time period of 48 h.

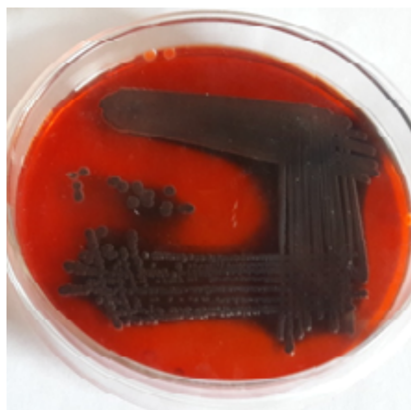


Figure 2(C): The Black Crystalline Colonies produced by *Enterobacter cloacae* showing strong biofilm formation streaked in Congo Red Agar Plate.

3.3. Antibiotic resistance of *Enterobacter cloacae*

The antibiotic resistance pattern of the foodborne pathogen *Enterobacter cloacae* was observed using the filter paper method. The absence of the zone shows that the food-borne pathogen *Enterobacter cloacae* was resistant to the antibiotics used, and the presence of the zone indicates that the same were sensitive to the antibiotics**.** When lower concentration antibiotics are provided, the isolate shows resistance to most of the antibiotics and is sensitive to Roxithyromycin and Azithromycin, whereas it is multidrug sensitive to all the antibiotics in the tests except for Erythromycin, Nystatin, and Bacteriocin when given at higher concentrations. *Enterobacter cloacae* are a multiresistant organism that shows resistance against most of the antibiotics when lower concentrations are provided. The spread of zone in antibiotics with the food-borne pathogen *Enterobacter cloacae* was earlier explained in a study (Mezzatesta *et al.,* 2012).

3.4 Characterization of biofilm formation of *Enterobacter cloacae*

3.4.1 Biofilm formation by tube assay method

The biofilm formation in the tube assay method was carried out for different time periods of 24 h and 48 h. 1% of the overnight culture was inoculated in the eight different growth media and incubated statically. After staining with the crystal violet stain and a half-hour incubation, they result in a violet color ring (Figure 2(A,B) around the tube after staining with crystal violet, which shows the attachment of biofilm formation on the foodborne pathogen *Enterobacter cloacae* at a time period of 24 h and 48 h. The more visible the ring around the walls of the glass, the more biofilm formation occurs in different media at different periods of time.

3.4.2 Biofilm formation by Congo red agar (CRA) method

The Congo Red Method (CRA) was carried out after an incubation period of 24 h. The overnight culture from the broth was streaked on BHI medium containing Congo red stain. After the incubation time, the organism showed black colonies on the streaked line on the Congo red agar plate (Figure 2(C)). Mirriam *et al.,* (2013) in their work demonstrated that *Enterobacter cloacae* readily forms biofilms on microtiter plates and the study also indicated the suitability fo BHI and TSB medium for the cultivation of the *Enterobacter cloacae* biofilm. In the study done by Kim *et al.* (2012), twenty two clinical isolates of *Enterobacteriaceae* were investigated for the biofilm forming ability by Congo Red staining and calcofluor staining methods and the highest biofilm forming ability was seen among the isolates of *Enterobacter cloacae* when compared to the isolates of *Enterobacter aerogenes.*

3.4.3 Biofilm formation by Microtiter plate method

The microtiter plate was carried out to evaluate the relationship between the biofilm

formation of *Enterobacter cloacae* in different media and at different time intervals, as shown in Table 1.

Table 1. Relationship between biofilm formations of *Enterobacter cloacae* in static microtiter plates in different media at different time intervals

Different Media	Time in Hours			
	24 hours	48 hours	72 hours	96 hours
NB	0.0050 ± 0.003 [b,c]	0.0095 ± 0.0035 [d,e]	0.0295 ± 0.0035 [b,d,f]	0.799 ± 0.0707 [c,e,f]
LB	0.0285 ± 0.0085	0.011 ± 0.0042 [d,e]	0.031 ± 0.0085 [d]	0.06 ± 0.0212 [e]
TSB	1.771 ±0.101 [a,b,c]	0.026 ± 0.0085 [a,d,e]	0.0715 ± 0.0078 [b,d,f]	1.151 ± 0.0707 [c,e,f]
TSB-1	0.188 ± 0.025 [a,b,c]	0.0265 ± 0.0078 [a,d,e]	0.0615 ± 0.0106 [b,d]	2.2075 ± 0.0912 [c,e]
TSB-2	0.199 ± 0.033 [a,b,c]	0.010 ± 0.0042 [a,d,e]	0.058 ± 0.0226 [b,d,f]	0.6545 ± 0.0742 [c,e,f]
BHI	0.108 ± 0.001 [a,b,c]	0.0065 ± 0.0078 [a,d,e]	0.048 ± 0.0156 [b,d,f]	0.169 ± 0.0071 [c,e,f]
BHI-1	0.0915 ± 0.0055 [a,b,c]	0.032 ± 0.0141 [a,e]	0.0305 ± 0.0092 [b,f]	0.5175 ± 0.0078 [c,e,f]
BHI-2	0.077 ± 0.011 [b,c]	0.0485 ± 0.012 [d,e]	0.387 ± 0.0071 [b,d,f]	0.829 ± 0.0566 [c,e,f]

The values are obtained by measuring the OD_{650} at four different time intervals and expressed as mean ± SD at significance $p<0.05$.

[a] the significant relationship between the formation of biofilm at 24 h and 48 h in different mediums used
[b] the significant relationship between the formation of biofilm at 24 h and 72 h in the different mediums used.
[c] the significant relationship between the formation of biofilm at 24 h and 96 h in the different mediums used.
[d] the significant relationship between the formation of biofilm at 48 h and 72 h in different mediums used
[e] the significant relationship between the formation of biofilm at 48 h and 96 h in different mediums used.
[F] the significant relationship between the formation of biofilm at 72 h and 96 h in the different mediums used.

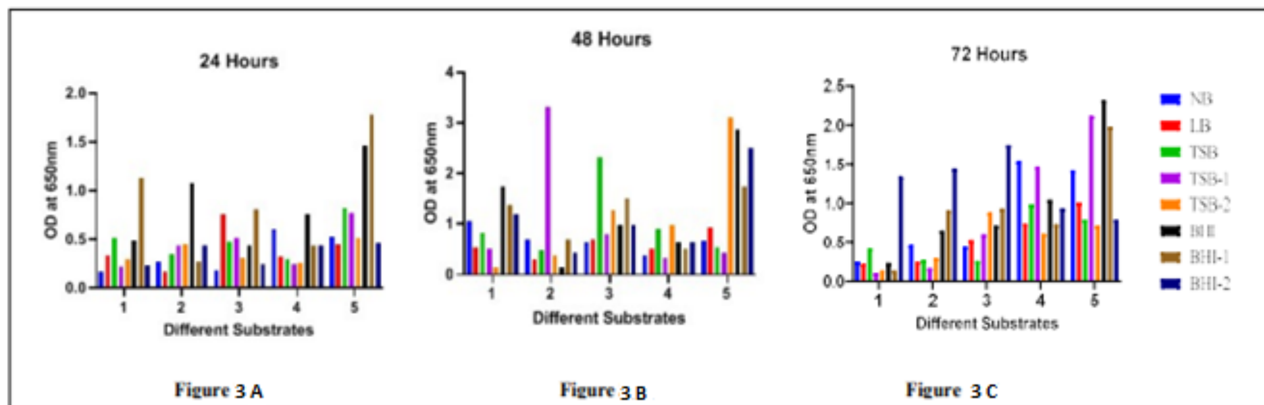


Figure 3(A): Biofilm formation of *Enterobacter cloacae* on different substrates in eight different media over a 24-hour period of time.
Figure 3(B): Biofilm formation of *Enterobacter cloacae* on different substrates in eight different media over a 48-hour period of time.
Figure 3(C): Biofilm formation of *Enterobacter cloacae* on different substrates in eight different media over a 72-hour period of time.

The cultures were loaded into a 96-well plate and incubated for1 to 4 days. Followed by the addition of crystal violet and glacial acetic acid. OD_{650} recorded to determine how the biofilm is adhered to the surface of the 96-well plates. *Enterobacter cloacae* form good biofilm formation and show a significant relationship between the time intervals in modified TSB-1 broth when compared to the other different growth media. A study conducted in 2013

demonstrated that *Enterobacter cloacae* readily forms biofilms on microtiter plates, in BHI and TSB medium. However, temperature and incubation time significantly affected biofilm formation by these bacteria (Nyenje *et al.,* 2013).

3.4.4. Biofilm formation and attachment on different substrates

After overnight incubation of the media along with the culture and substrates (glass, PVC, plastic, steel, and wood), are subjected to crystal violet staining. The biofilm attachment in the substrate is measured by taking an optical density value at 650nm. The substrate attachment was higher in wood at 24 h and 72 h and produced good attachment in plastic at 48 h (Figure 3 (A, B, C)**.** Mohana Priya and her colleagues reported that different substrates, such as glass, wood, teflon, steel, and plastic, showed biofilm attachment in *Myroides odorarimimus.* Among the five substrates, teflon and plastic were found to support high biofilm formation in food flavobacterium media (Mohana Priya *et al.,* 2018). Crystal violet staining at 650nm (A_{650}) on a 96-well plate reader at three different time intervals (1 to 4 days) determines the biofilm formation. The cell concentration of 6.57 x 10 cfu/ml was used. The mediums used were NB, LB, TSB, TSB-1, TSB-1, BHI, BHI-1, and BHI-2, and the substrates used were glass (1), PVC (2), plastic (3), steel (4), and wood (5).

3.5. Encapsulation efficiency of nisin-loaded liposomes

Screening of nisin, the zone of inhibition was observed at a high concentration (3.3 mg/10 ml). Nisin was encapsulated in the liposomes by the microencapsulation vesicle method. The encapsulation of nisin was evaluated by protein estimation. Nisin showed higher encapsulation efficiency and loading capacity at pH 7 in linolenic acid and pH 8.5 in phosphotidyl choline (Figures 4 (A) and 4 (B)). In a study conducted by Tomoko and Fumiyoshi in 2005, 'the encapsulation efficiency of various drugs with three kinds of egg yolk lecithin namely purified egg yolk lecithin (PEL), partially hydrogenated purified egg yolk lecithin (R-20) and completely hydrogenated purified egg yolk lecithin (R-5) with different iodine values were done. Among the three kinds of lecithin PEL tendered to show a higher encapsulation efficiency than R-20 and R-5'.Microencapsulation of nisin Z in lipid vesicle made of high melting point phospholipids was successfully achieved using liposome prepared with a high EE of 47% (Lardi *et al.,* 2003). This study indicated that 'the nisin loaded vesicles or nisin immobilized on the unlamellar membranes may provide a powerful tool for controlling spoilage and pathogenic organisms in food and can improve nisin stability, efficacy and distribution in food matrices'.

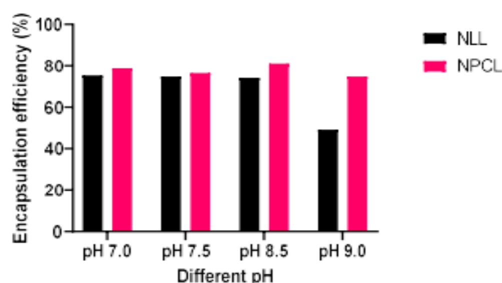


Figure 4 (A)

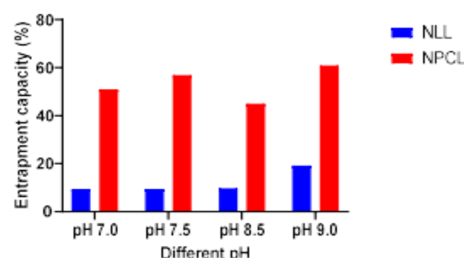


Figure 4 (B)

Figure 4(A): Encapsulation efficiency of the nisin loaded phosphatidyl choline and nisin loaded linolenic acid were analysed and the results showed higher encapsulation efficiency pH 7 in linolenic acid and pH 8.5 in phosphatidyl choline.

Figure 4(B): Loading capacity of the nisin loaded phosphatidyl choline and nisin loaded linolenic acid were analysed and the results showed higher loading capacity in pH 9 in linolenic acid and phosphatidyl choline.

3.6. Susceptibility testing of *Enterobacter cloacae* in nisin and nisin loaded liposomes

Minimal Inhibitory Concentration of *Enterobacter cloacae* for nisin and nisin loaded liposomes were done in 96 well plates as in table 2. Overnight culture was added to the well plate along with media and nisin, nisin loaded liposomes. The minimum inhibitory concentration was evaluated at an optical density of 650nm. The concentration ranging from 10µg/ ml to 0.017 µg/ ml are used. From this, 0.156 µg/ ml is enough for inhibition of *Enterobacter cloacae* nisin linolenic acid, 2.5 µg/ ml showed Minimal Inhibitory Concentration for nisin phosphotidyl choline liposome and 5 µg/ ml showed Minimal Inhibitory Concentration for nisin.

Table 2. Susceptibility test of *Enterobacter cloacae* for Nisin and Nisin liposomes.

Concentration (µg/ml)	Nisin	Nisin Phosphatidyl choline Liposome	Nisin linolenic acid Liposome
10	-	-	-
5	0.0545 ± 0.060	-	-
2.5	0.0695 ± 0.040	0.2315 ± 0.302	-
1.25	0.0885 ± 0.039	0.2660 ± 0.342	-
0.625	0.0955 ± 0.060	0.2990 ± 0.356	-
0.312	0.1200 ± 0.063	0.3205 ± 0.181	-
0.156	0.1425 ± 0.055	0.3475 ± 0.181	0.0795 ± 0.078
0.007	0.1695 ± 0.052	0.3745 ± 0.183	0.1010 ± 0.033
0.035	0.1985 ± 0.048	0.4745 ± 0.179	0.1410± 0.062
0.017	0.2815 ± 0.101	0.6520 ± 0.203	0.2240 ± 0.061

The values given are obtained by measuring the turbidity at 650nm $_{(A650)}$ on a 96-well plate reader and expressed as mean ± SD.

3.6.1. *In vitro* biological activity assay

The *in vitro* nisin and nisin liposome activity was tested by colony-forming units. The fresh broth was inoculated with inoculums, nisin, and nisin-loaded lipid nanoparticles.

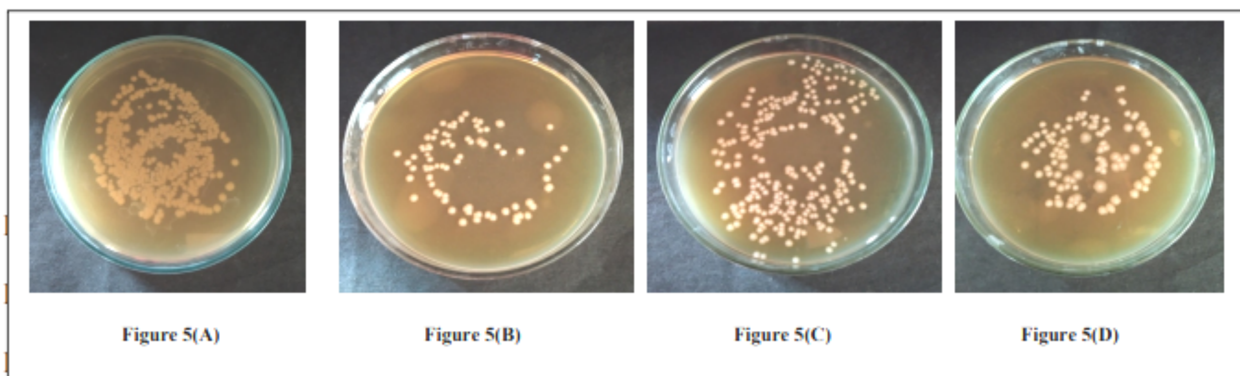


Figure 5(A): *Enterobacter cloacae* 6.57 x 10^8 CFU/ml
Figure 5(B): Nisin loaded linolenic acid 7.4 x 10^7 CFU/ml
Figure 5 (C): Nisin 1.54 x 10^8 CFU/ml
Figure 5(D): Nisin loaded phosphatidyl choline 1.02 x 10^8 CFU/ml

Enterobacter cloacae was kept as a control without adding any inhibitors for colony-forming units, which produced 6.54 x 10^8 CFU/ml (Figure 5(A)), and for liposomes added

at the same concentration of nisin, a nisin-loaded linolenic acid liposome, and a nisin-loaded phosphatidylcholine liposome. We observed 7.4 x 10^7 CFU/ml (Figure 5(B)) in nisin-loaded linolenic acid liposomes, 1.54 x 10^8 CFU/ml (Figure 5(C)) in nisin, and 1.02 x 10^8 CFU/ml (Figure 5(D)) in nisin-loaded phosphatidylcholine liposomes. It is observed that the numbers of colony-forming units were lower in liposome-loaded nisin compared to nisin and control. This shows the growth inhibition of liposomes. In a previous study, the biological activity and nisin demonstrated that nisin was entrapped in the nanoparticles in an active form, and hot homogenization did not substantially deteriorate the nisin activity (Prombutara *et al.*, 2012).

3.7. High-throughput assay of antibiotics, nisin, and nisin-loaded lipid nanoparticles

High-throughput assays were performed to evaluate the minimum inhibitory concentration and the minimum eradication biofilm concentration, as shown in Table 3.

Table 3. MIC and MBEC of antibiotics, Nisin, and Nisin liposomes when treated on a preformed biofilm of *Enterobacter cloacae*

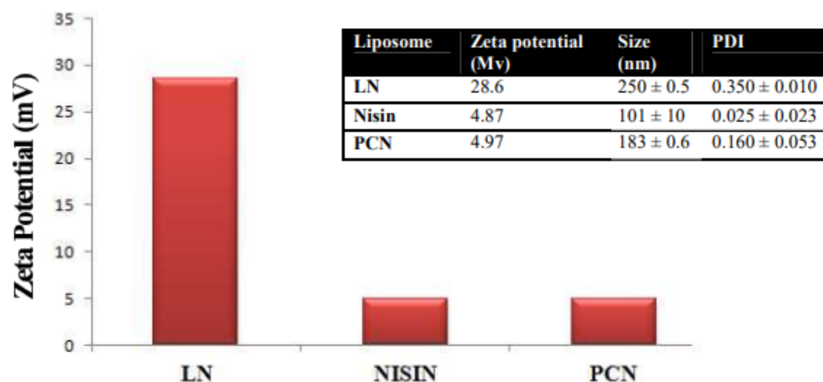
S.No	Antibiotics / Nisin / Liposomes	MIC in µg/ml	MBEC in µg/ml
1.	CM	>25	>100
2.	NM	>0.78	>12.5
3.	TET	>0.39	>100
4.	STR	No inhibition	>25
5.	AMP	>0.39	>100
6.	KAN	>100	>25
7.	ERY	>0.39	>6.25
8.	CAZ	No inhibition	>100
9.	NYS	>0.39	>100
10.	CHL	>0.39	>100
11.	CRO	>0.39	>100
12.	VAN	>3.125	>0.195
13.	BAC	>6.25	>100
14.	AMX	>0.195	>25
15.	CIP	No inhibition	>0.195
16.	CAR	>6.25	>50
17.	P	>0.39	>6.25
18.	CEF	No inhibition	>1.56
19.	CPH	>0.39	>3.125
20.	HYG	No inhibition	>100
21.	NISIN	>5	>0.625
22.	Nisin linolenic acid Liposome	>0.312	>0.035
23.	Nisin Phosphatidylcholine Liposome	>2.5	>0.017

The minimum inhibitory concentration of different antibiotics to inhibit the foodborne pathogen *Enterobacter cloacae* at a concentration of 25 g/ml is enough. But for the eradication of the foodborne pathogen *Enterobacter cloacae,* a higher concentration of 100 µg/ ml is needed. For nisin encapsulated linolenic liposomes, the Minimal Inhibitory Concentration is greater than 0.312 µg/ ml and the eradication, the minimal concentration is greater than 0.035 µg/ ml. For nisin encapsulated phosphotidyl choline liposomes, the Minimal Inhibitory Concentration is greater than 2.5 µg/ ml and the eradication, the minimal concentration is greater than 0.017 µg/ ml. Similar descriptive studies in other Gram negative shows indulgences of proton motive force in membranes of *E. coli* when AgNPs are exposed in various concentrations (Lok *et al.,*2007).

Minimal Inhibitory Concentration and Minimal Biofilm Eradication Concentration were evaluated for 21 different antibiotics and nisin-loaded liposomes using the Calgary device, and the minimum inhibition concentration and minimal biofilm eradication concentration were noted. Yamakami *et al.,* (2014) stated that 'acyl chain length of phosphatidyl choline mediates the sustained bactericidal activity of encapsulated nisin. Liposomal nisin composed of distearoyl phoshatidylcholine (DSPC) exerted the highest bactericidal activity as compared to those containing dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoyl phoshatidylcholine (DSPC)'. The results might suggest that DSPC-based liposomes are capable of preserving nisin's bioactivity.

3.8. Zeta Potential of Nisin and Nisin-Loaded Lipid Nanoparticles

The zeta potential of nisin-loaded linolenic acid liposomes (LN), nisin, and nisin-loaded phosphatidyl choline liposomes (PCN) is represented in the Zeta potential analysis (Figure 6).



Liposome	Zeta potential (Mv)	Size (nm)	PDI
LN	28.6	250 ± 0.5	0.350 ± 0.010
Nisin	4.87	101 ± 10	0.025 ± 0.023
PCN	4.97	183 ± 0.6	0.160 ± 0.053

Figure 6. Graph representing the zeta potential of nisin loaded linolenic acid liposome (LN), nisin and nisin loaded phosphatidyl choline liposomes (PCN). Nisin loaded phosphatidyl choline showed an effective zeta potential value when compared to nisin and nisin loaded linolenic acid liposomes. Polydispersity index (PDI).

Nisin-loaded phosphatidyl choline showed `effective zeta potential value when compared to nisin and nisin-loaded linolenic acid liposomes. The zeta potential value of nisin is 4.87 Mv, nisin-loaded linolenic lipid nanoparticles are 28.6 Mv, and nisin-loaded phosphatidylcholine lipid nanoparticles are 4.97 Mv. When compared to nisin-loaded linolenic lipid nanoparticles, nisin-loaded phosphatidyl choline lipid nanoparticles show good and efficient zeta potential values. Previous studies reported that 'the incorporation of freshly prepared nisin into solid lipid nanoparticles (SLNs) resulted in a slight increase in the average particle size with a slight decrease in zeta potential. Whereas after 28 days of storage at 30°C, nisin-loaded solid lipid nanoparticles had significantly increased their average sizes with an overall decrease in

zeta potential' (Prombutara *et al.,* 2012).

Finally, to overcome the hindrances associated with nisin as a food preservative, the use of nanotechnology for the synthesis of nisin-loaded or coated nanoparticles has been introduced (Silva, 2010). Nisin inhibits food-borne pathogens and the prevention of spore germination. For nisin's antimicrobial activity adsorption of nisin molecules onto the bacteria's surface and disruption of the membrane is very important. Nisin efficacy in food could be improved through incorporation or encapsulation in natural or synthetic polymers (Silva, 2010).

Adsorption of bacteriocins to various surfaces with retention of activity may be successfully achieved (Bower *et al.,* 1995a; Bower *et al.,* 1995b; Daeschel *et al.,* 1992; Ming *et al.,* 1997). Membrane-immobilised nisin or surfaces could provide several advantages as a nisin delivery system, such as reducing the amount of nisin that would be used and improving its stability (Cutter & Siragusa, 1997; Siragusa & Cutter, 1999). Nisin adsorbed to polyethylene used for meat packaging has been shown to be more stable and active against gram-positive pathogens and the food spoilage organisms *L. monocytogenes* and *Brochothrix thermosphacta* than nisin applied directly in a free form (Bower *et al.,* 1995a; Bower *et al.,* 1995b; Cutter & Siragusa, 1996; Cutter & Siragusa, 1997). Previous studies have shown that nisin adsorbed to lipid membranes can retain its antimicrobial activity and may have potential for use as a food-grade antimicrobial agent (Bower *et al.,* 1995a; Bower *et al.,* 1995b; Cutter & Siragusa, 1996; Scannel *et al.,* 2000). The desorption of nisin from lipid membranes occurs on contact with bacterial cells (Daeschel *et al.,* 1992).

It appears that liposomes are the appropriate carriers for the controlled delivery and action of nisin in the chevre matrix. The binding of nisin to liposomes enhances the stability, availability and distribution of nisin within the chevre. The presence of encapsulated as well as membrane-related nisin may provide an additive effect to this system, providing short-term antibacterial action (release of encapsulate nisin) as well as long-term antibacterial activity (desorption of nisin membrane-imobilised). This technique could improve the management of undesirable bacteria in goods (like cheese) that are kept in storage for long periods of time.

According to Daghastanli *et al.,* (2004) with regard to 'the efficiency of liposomal nisin, the selection of the acyl chain length of phospholipids is quite evident from our findings'. It has been postulated that liposome stabilisation is increased with long acyl groups, which explains why DSPC-nisin releases nisin more slowly than DPPC-nisin or DMPC-nisin (Daghastanli *et al.,* 2004). The current review shows that by varying the lipid arrangement, nisin-discharge profiles from the liposomes can be made depending upon the length of the acyl chain of phospholipids. The revelations appear that the hydrophobic collaborations between determinedly charged nisin and the acyl chain of phospholipids activate supported bactericidal development.

4. Conclusions

The food-borne pathogen *Enterobacter cloacae* Biofilm activity was observed for the samples nisin, nisin-loaded linolenic acid liposomes, and nisin-loaded phosphatidylcholine liposomes against control *Enterobacter cloacae*. The results showed that the sample in nisin-loaded liposomes had more inhibition against the control Enterobacter cloacae. So therefore, the study concluded that nisin-loaded nanoliposomes had better biofilm inhibition activity in a high-throughput assay.

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