To appear in:
International Journal of Nano Dimension (Int. J. Nano Dimens.)

Online ISSN: 2228-5059 Print ISSN: 2008-8868

Dates:
Received: 29 July 2025
Revised: 22 September 2025
Accepted: 08 October 2025

DOI: https://doi.org/10.57647/j.ijnd.2026.1702.02

Research Paper

Antibacterial Activity of Nisin Incorporated into Chitosan/Alginate Nanoparticles as a biopreservator agent in sausage

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ABSTRACT

This study investigates the antimicrobial effects of nisin extracted from *Lactococcus lactis* and the enhancement of its antimicrobial effect by incorporation into chitosan/alginate nanoparticles. Native strains of *Lactococcus lactis* were isolated from milk samples and the presence of the nisin gene was confirmed using polymerase chain reaction (PCR). Nisin concentration was determined using high-performance liquid chromatography (HPLC). The results showed that out of ten isolated strains, four strains (L1, L3, L8 and L9) showed genetic similarity to *Lactococcus lactis* with percentages of 82.45%, 79.87%, 93.33% and 79.87%, respectively. The optimal growth conditions for the most effective strain were determined at 30°C and pH 7 with an optical density of 5.51 at 600 nm. Nisin extract showed a significant inhibitory zone of 19 mm against *Bacillus cereus*, while nisin-containing nanoparticles showed an enhanced inhibitory effect of 20 mm. Furthermore, the results showed that the combination of nisin-containing nanoparticles significantly improved the antimicrobial activity compared to nisin alone. This study showed that nisin incorporated into nanoparticles effectively retained its antimicrobial properties, indicating its potential as a valuable model for biopreservation of food. These findings emphasize the importance of using natural antimicrobials in food preservation.

Keywords: *Antimicrobial properties; Lactococcus; Nanoparticles; Nisin; Preservation.*

INTRODUCTION

Today, lactic acid bacteria (LAB) are used as a biopreservation method to ensure food safety without the negative consequences of chemical preservatives and heat treatment [1]. These bacteria have long been of interest to humans in food preservation and have been used for many years to produce various types of processed foods. Their protective effect is attributed to the antimicrobial properties of bacteriocins and the production of lactic acid and hydrogen peroxide, which primarily inhibit the growth of Gram-positive bacteria, including spoilage and pathogenic microorganisms. *Lactococcus lactis* subspecies *lactis*, which can be isolated from dairy and vegetable products, is particularly notable among these bacteria. Bacteriocins produced by *Lactococcus lactis* subspecies *lactis* consist of small peptides with molecular weights between 3 and 6 kDa. Bacteriocins can be classified into groups based on their structure, molecular weight, and genetic sequence. These groups include class I (Lantibiotics), class II (Thermostable), and class III (Thermolabile). One of the most important antimicrobial bacteriocins produced by lactic acid bacteria is nisin [2-4], which is currently used in meat products to reduce the harmful effects of nitrite. However, due to its sensitivity to acid protease enzymes present in meat and reactivity with other substances present in the food matrix, nanomaterials are used in these products. Nanotechnology is expected to play a vital role in the future processing of various types of food, including internal (food additives) and external (packaging) applications [5]. Nanomaterials and nanocomposites, with their unique physicochemical properties including high surface area and enhanced bioactivity, have shown great potential in improving the performance and stability of antimicrobial agents like nisin. Metal nanoparticles such as silver, gold, and copper, especially when combined with natural polymers like chitosan and sodium alginate; demonstrate significant antibacterial effects [6]. Moreover, nanocomposite films and edible coatings based on biodegradable polymers such as polyvinyl alcohol (PVA), Nano cellulose, chitosan, and alginate not only enhance food preservation but also contribute to sustainable packaging solutions [7, 8]. Beyond food preservation, antibacterial nanomaterials have found diverse applications in environmental remediation, textile industries, and biomedical fields. For instance, metal-capped cobalt/zinc oxide nanoparticles and flame-retardant ZnO-coated silica nanofibers exhibit multifunctional properties including high antibacterial activity, durability, and reusability [9]. These advances highlight the versatility and broad applicability of nanotechnology in addressing microbial contamination and safety challenges [10, 11]. One commonly used nanoparticle is chitosan, which is a derivative of glucan with repeating units of chitin. It should be noted that commercial chitosan is derived from the exoskeleton of arthropods. This material is highly regarded for its compatibility with other materials, easy digestion, non-toxicity, high absorption rate, and availability, making it suitable for pharmaceutical, food, and industrial applications [12]. Sodium alginate, a salt of alginic acid and a polymer of L-glucuronic acid and D-mannuronic acid, is a polysaccharide derived from the primary structural carbohydrate of brown algae. It is a non-toxic, biodegradable, and edible material that can be directly used for food coatings. Films or coatings made from sodium alginate are effective in enhancing the quality of food

products and increasing their shelf life [13]. Polyionic chitosan/alginate complexes are formed through ionic bonding between the carboxyl groups of alginate and the amine groups of chitosan [14]. The aim of this study was to evaluate the antimicrobial activity of nisin-containing culture extract of *Lactococcus lactis* subspecies *lactis* and to investigate the effect of nanoparticles on it. For this purpose, the antimicrobial activity of nisin-containing culture extract related to isolated native bacteria and nisin-based nanoparticles in sausage was evaluated.

MATERIALS AND METHODS

Bacterial Isolation and Identification

10 samples of Milk from dairy farms in some areas of Kerman, Iran, were used to isolate lactic acid strains. The samples were transported to the laboratory in an ice bag within 2 hours. To isolate the strains, the first dilution was prepared by mixing 1 mL of sample with 9 mL of sterile normal saline and mixed thoroughly. Subsequently, serial dilutions were prepared and plated on Mann Rogosa Sharpe Agar. The plates were incubated anaerobically at 30°C for 48 hours. Bacterial colonies that dissolved calcium carbonate and formed a clear zone around them were carefully selected. To preserve these strains, they were stored in MRS medium containing 15% glycerol at a freezing temperature of -80°C [15, 16]. After morphological examination, the isolates were physiologically identified. These diagnostic tests included catalase test, determination of the inability to produce gas from glucose fermentation [15], growth assessment at temperatures of 10, 40 and 45°C, growth observation in the presence of 0.3% methylene blue in milk, growth assessment at pH 9.2, growth assessment at 4 and 6.5% NaCl, arginine hydrolysis, CO_2 production from citrate as well as diacetyl and acetoin production, maltose fermentation and starch hydrolysis [17, 18].

Detection of nisin gene by PCR (polymerase chain reaction)

DNA was extracted from the isolated bacterial cells. For this purpose, the bacterial suspension was centrifuged for 10 min at 7000 rpm and then the cells were resuspended in 5 ml of lysis buffer. Subsequently, the cells were incubated for 2 h in a water bath at 37°C. Then, 500 µL of 10% SDS solution and 100 µL of 25 mg/mL proteinase K solution were added. The mixture was incubated for 2 hours in a water bath at 55°C. After that, 2 mL of 5 M sodium chloride solution and 6 mL of chloroform-isoamyle alcohol were added, and the resulting mixture was incubated for 30 minutes at room temperature. Then, 1 volume of 100% isopropanol was added and the DNA was washed with cold 70% ethanol. The DNA was air-dried and then suspended in 600 µL of TE buffer to prepare for polymerase chain reaction. For the PCR reaction, 2 µL of isolated DNA, 2.5 µL of 10X buffer, 3 µL of $MgCl_2$, 0.5 µL of dNTPs, 1 µL of Taq DNA polymerase, and 1 µL of primer (forward: ATAAGGAGGCACTCAAAATG, reverse: TACTATCCTTTGATTTGGTT) were used. The total reaction volume was adjusted to 25 µL by adding

distilled water. Finally, temperature cycles for the different steps of the PCR reaction were performed according to Table 1 [19].

Table 1. PCR temperature program; Repeat steps 2 to 4, 35 times.

To check the integrity of the DNA linkages, electrophoresis was performed using a 1.5% agarose gel immersed in EDTA-Tris solution. The samples were carefully placed in the agarose gel wells and subsequently subjected to an electric field of 85 V and 150 A. Finally, the DNA samples were analyzed for further examination within the gel [19].

Optimizing the growth conditions of Lactococcus lactis

A comprehensive study was conducted to improve the growth conditions of *Lactococcus lactis*, focusing on three critical parameters: pH (5, 7, and 9), salt content (0, 2, and 4%), and temperature (30, 35, and 40°C). The experiments involved inoculating 10% v/v of a concentration equivalent to a turbidity of 0.5 McFarland of the bacteria into MRS medium and incubating it for 24 hours at 180 rpm. The main goal was to identify the most favorable conditions for bacterial growth and measure them by determining the optical density at a wavelength of 600 nm. The optimal growth conditions were determined by selecting the highest optical absorption value [19].

Determination of nisin concentration by High-Performance Liquid Chromatography method

To identify and measure the presence of nisin, the sample extracted from *Lactococcus lactis* was analyzed using HPLC. Thus, a chromatographic column with dimensions of 4.6 × 250 mm and particle size of 5 µm, packed with C18 material, was used. In addition, a UV-visible detector with a wavelength of 200 nm was used. To prepare the nisin solution, 0.01 g of nisin powder was dissolved in water and the resulting solution was diluted in a flask to a final volume of 10 mL. Subsequently, each sample was filtered at ambient temperature with a 0.2 µm Millipore filter. Filtration was performed at a flow rate of 1 mL/min using a mobile phase consisting of a mixture of water and acetonitrile containing 0.1% trifluoroacetic acid in a ratio of 1:99. After filtration, the samples were injected into the HPLC apparatus for further analysis.

Investigating the antibacterial activity of the extract containing nisin on pathogenic bacteria

A 10% v/v bacterial suspension, equivalent to a turbidity of 0.5 McFarland, was inoculated into 250 ml flasks containing MRS broth. The flasks were then incubated for 48 h under optimal growth conditions. Subsequently, the cultures were centrifuged at 1000 × g for 10 min at 4 °C and the culture supernatant was used. The pH of the cell-free supernatant was adjusted to 6 using sterile 1 M sodium hydroxide solution to neutralize the inhibitory effect of lactic acid. In addition, the supernatant was placed in a water bath at 80°C for 10 min to inactivate extracellular proteases and hydrogen peroxide. Finally, the culture supernatant was passed through a 0.22 µm filter [20]. Then, the treated microorganisms from the Iranian Type Culture

Collection Center, namely *Staphylococcus aureus* (PTCC 1431), *Salmonella typhimurium* (PTCC 1709), *Bacillus cereus* (PTCC 1857), *Escherichia coli* (PTCC 1399), *Acinetobacter baumannii* (PTCC 1919), *Enterococcus faecalis* (PTCC 1858) and *Pseudomonas aeruginosa* (PTCC 1074), were cultured at a concentration of 0.5 McFarland on Mueller Hinton Agar. Subsequently, 10 µL of sterile supernatant was inoculated onto blank discs on the medium and incubated for 24 to 48 hours at 37°C, and inhibitory zones with a diameter greater than 5 mm were considered positive. Uncultured MRS broth was used as a negative control [20-22].

The effect of nanoparticles on nisin in increasing the shelf-life of sausage

In order to prepare the polymer solutions of alginate and chitosan, stock solutions of sodium alginate and chitosan were first created. This was done by dissolving 250 mg of each component in 50 mL of deionized water. To overcome the insolubility of chitosan in deionized water, 2 mL of 2% (w/w) acetic acid was added to the chitosan stock solution. The acidity of the chitosan/alginate solutions was adjusted to 4.5 and 2.5, respectively, and both solutions were filtered using a 0.22 µm filter before use. The preparation of nisin-loaded nanoparticles was carried out using a two-step method based on previous research [23]. To produce nanoparticles, 100 µL of nisin solution (10 mg/mL) was gradually added dropwise to 8 mL of aqueous sodium alginate solution (250 mg/mL) and the mixture was stirred for 30 min. Then, 4 mL of chitosan solution (250 mg/mL) was added to the resulting alginate solution and stirred for 1 h. Then, the nisin-based nanoparticles were centrifuged at 2500×g and 4°C for 10 minutes, which effectively separated free polymers from the nanoparticles [23]. Fourier transform infrared spectroscopy (FTIR) was used to analyze isolated nisin, chitosan/alginate nanoparticles, and nisin loaded with the respective nanoparticles. The analysis was performed at the central laboratory of Shahid Bahonar University. The sausage dough sample used in the experiment was obtained from Orial Meat Products Company in Kerman, Iran. Then, the samples were inoculated with nisin and nano/nisin solutions produced from the tested *Lactococcus lactis* at a concentration of 500 mg/L, and a control group was used for each. 200 grams of each sample was homogenized and divided into 1 gr steamed packets. Afterwards, bacterial strains including *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* were inoculated into the samples at a concentration of 10% w/v (1000 cfu/ml). The samples were stored in a refrigerator for different periods of time from 0 to 336 hours at 7°C. At each of the specified times, the contents of each package were suspended in 100 mL of sterile normal saline solution and microbial counts were performed using the standard plate counting method after 24 hours of incubation at 37°C [23, 24].

Morphology of the Nanoparticles

The morphology of nanoparticles, such as shape and occurrence of aggregation, was studied by scanning electron microscopy. Before observation, samples of nanoparticle suspensions (5–10 µl) were mounted on metal supports, vacuum coated, and then examined by scanning electron microscopy (SEM, TESCAN MIRA3, CZE) [14].

Statistical analysis

Statistical analysis of data was performed using SPSS 16 software using a completely randomized block design with three replications. One-way analysis of variance and Duncan's multiple range tests were used to evaluate differences between subjects, and their significance was obtained at $p < 0.05$.

RESULTS AND DISCUSSION

Isolation and identification tests

A total of ten different types of microorganisms were isolated from the culture medium, four of which were identified as *Lactococcus lactis* based on colony morphology, Gram stain, and diagnostic tests. The results of these diagnostic tests are shown in Table 2. The strains were designated with the hypothetical letter "L". Figures 1A and 1B show the macroscopic and microscopic views of one of the isolated strains, respectively.

Table 2. The results of biochemical diagnostic tests for the relevant strains.

Fig. 1. L1: A) on MRS agar B) Microscopic image.

The effect of temperature, salt concentration and pH levels on the optimal growth of the L1 strain

To investigate the effect of temperature, salt, and pH (Figs 2A, 2B and 2C) on the optimal growth conditions of strain L1, bacterial cultivation was performed in triplicate and the optical density was evaluated at a wavelength of 600 nm, and its values under optimal conditions were 5.51, 5.51, and 2.1, respectively, for a temperature of 30°C, lack of salt, and an acidity of 7. To investigate the significance of the effects of temperature, salt concentration, and acidity, a one-way analysis of variance test was performed, which was significant in all cases at a $p<0.05$.

Fig. 2. The effect of temperature (A), salt concentration (B) & pH (C) on the growth of strain L1 (error bar 95 %)

Presence of nisin gene in the screened strain

Genomic DNA extraction was performed using a mixture of chloroform and isoamyle alcohol. The resulting product was evaluated by observing it on an agarose gel (see figure 3A). After that, PCR was performed

using primers specific for the nisin gene and the target gene was successfully amplified. The image taken from the agarose gel analysis shows the 208 bp band corresponding to the nisin gene of strain L1, located adjacent to the positive and negative controls and the ladder (see figure 3B).

Fig. 3. A, Extracted bacterial genomic DNA product on agarose gel, B, Amplification of nisin gene fragment obtained by PCR (ladder size 1000 bp).

After sequencing the PCR product, the obtained data were analyzed using the BLAST algorithm in the NCBI database. Notably, the results showed that strain L1 had 82.45% similarity to *Lactococcus lactis* strain 19.3 nisin A gene, complete cds (Accession no: MG913137.1). The phylogenetic tree of the isolated strain was drawn based on the following sequence, which can be seen in figure 4. For this purpose, the 16S rRNA gene sequence of the corresponding strain was determined, and its similarity with other strains was examined.

ATAAGGTAGGCACTCAAAATGAGTTGAGCGCGAAAGATTTATGTAACTTGGATTTGTTATCTGT
TTCAAAGAAAACGGATTCAGTCGCATCACCCCGTGACGCATTACAGGTATTTCGCTATGTATAC
CCAGTTGCAAAACAGGAGCTCTCATGGTTGTTCCAACATGAAAATAGCAACTTGTCATTGCAAT
TACCCT

Fig. 4. The phylogenetic tree of the isolated strain (L1).

Nisin concentration in the sample corresponding to L1

High-performance liquid chromatography was performed to evaluate the concentration of nisin in the L1 sample, which showed that the concentration of nisin in the sample was 41.5 mg/L. The chromatogram of the L1 strain showed a distinct peak at 4.946 min. The area under this peak in the L1 strain was measured to be 725.579 units (Figure 5).

Fig. 5. Chromatogram of L1 strain nisin.

Microscopic Image Assessment

SEM confirmed the presence of nanoparticles and provided morphological information about the chitosan/alginate nanoparticles containing nisin. Through SEM images, spherical, regular, smooth and aggregate shape was observed for the nanoparticles (Figure 6). The particle size range of the nanoparticles was 50–120 nm.

Fig. 6. SEM image of chitosan/alginate nanoparticles containing nisin.

Results of FTIR test on samples containing nisin and nisin loaded on chitosan/alginate nanoparticles

Figure 7A shows a sample containing nisin-free chitosan/alginate nanoparticles. This figure clearly shows the OH, NH2, and C=O groups in the infrared (IR) spectrum. On the other hand, figure 7B shows both the nisin sample and the chitosan/alginate nanoparticles. The IR spectrum of this figure confirms the presence of functional groups and shows that the binding of chitosan/alginate nanoparticles has caused changes in the peak size and position. Notably, a distinct and elongated peak is observed at 3421 and the IR spectrum also shows the appearance of C-H stretching frequencies at 2921 and 2855.

Fig. 7. FTIR analysis of nisin-free chitosan/alginate nanoparticle sample (A), and nisin combined with chitosan/alginate nanoparticles presented by strain L1 (B).

Antibacterial activity of nisin and L1 extracted nisin with chitosan/alginate nanoparticles on pathogenic bacteria

The results obtained regarding the antimicrobial properties of nisin showed that the studied strain was not effective against *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, but it had a significant effect on *Bacillus cereus*, as shown in figure 8. Figures 8A, 8B and, 8C show the results of the disk diffusion test with nisin L1, chitosan/alginate nanoparticles with nisin L1 and negative control on the indicator bacteria, respectively. *Bacillus cereus* showed the highest sensitivity, with an average zone of inhibition of 20 mm. To investigate the importance of the type of bacteria treated with chitosan/alginate nanoparticles in relation to different types of bacterial nisin, a one-way analysis of variance test was performed and the results showed that the growth of treated bacteria was significantly affected by the antimicrobial agent at a significance level of $p<0.05$. To determine the effect of the antimicrobial substance on the bacteria under treatment, Duncan's test was performed and showed that *Bacillus cereus* showed the highest sensitivity to the substance.

Fig. 8. Disk diffusion tests of L1 strain on indicator bacteria, A: Antimicrobial activity of nisin L1, B: Chitosan/alginate nanoparticles with nisin L1, C: Negative control (Error Bar 95%).

Determining the fractional inhibitory concentration index of each case of Nano/Nisin to nisin strain L1

Considering the minimum inhibitory concentration (MIC) of the nisin-containing nanoparticle extract and the nisin-containing culture medium extract, partial inhibitory concentration (FIC) analysis revealed a synergistic effect between the two agents. This positive interaction was observed for the products associated with the L1 strain, as shown in Table 3.

Table 3. F.I.C. of L1 strain on indicator bacteria.

Effectiveness of L1 nisin and nono-nisin in increasing sausage shelf life in the various time and process

Figure 9 shows the changes in the overall microbial population of sausage samples at seven time points, showing the effect of nisin L1, nano-nisin L1, and the negative control sample. To assess the importance of different factors, including bacterial type, time, and sample type, on the number of bacteria found in the sample, a one-way ANOVA test showed that all three factors significantly affected the number of bacterial colonies present (p<0.05). To further investigate the effect of the type of bacteria on different processes and time points, Duncan's test showed that *Bacillus cereus* had the greatest effect in the relevant experiments, especially in terms of its abundance. To determine the optimal duration for disinfection of meat samples, Duncan's test showed that it takes approximately 24 hours for the antimicrobial agent to effectively exert its effect after treatment. However, extending the treatment time beyond the seventh day had a negative impact on the antimicrobial properties of the process. Finally, to evaluate the effect of treatment type on the number of indicator bacteria at different disinfection times, Duncan's test showed that commercial nano/nisin had the greatest effect on the number of treated bacteria at different time points, and L1 nano/nisin was followed by a small difference.

Fig. 9. Changes in total count of indicator bacteria in various kinds of samples and times (h).

The present study demonstrates the antimicrobial potential of nisin, especially when combined with chitosan/alginate nanoparticles, which can be used as an effective preservative in meat products. Molecular analysis confirmed the presence of nisin biosynthesis gene in isolated strains of *Lactococcus lactis*, which have significant inhibitory activity against pathogenic bacteria. Recent research has highlighted the importance of lactic acid bacteria as effective producers of bacteriocins and their role in the biological preservation of foods [15, 25]. These compounds have the potential to be used as natural preservatives in the food industry. The use of lactic acid bacteria (LAB) and their metabolic derivatives is widely documented and classified as generally recognized as safe (GRAS) and has a qualified presumed safe status (QPS), supporting their suitability for incorporation into food matrices. Previous microbiological studies have shown that antimicrobial metabolites produced by LAB, such as organic acids, bacteriocins, and other bioactive compounds, act as effective natural barriers against pathogenic bacteria and inhibit food spoilage microorganisms, thereby enhancing microbial safety and extending product shelf life [1, 26]. The antimicrobial mechanism of bacteriocins, such as nisin, involves electrostatic interactions between their cationic domains and the anionic phospholipid components of the bacterial cell membrane. These primary electrostatic attractions facilitate the binding of bacteriocins to the target membrane surface. It is

hypothesized that the hydrophobic domains of bacteriocins play a role in membrane entry and pore formation. Two mechanistic models have been proposed to explain bacteriocin-induced cell death: The first model suggests that nisin molecules enter the membrane vertically to form membrane ion channels. When enough molecules accumulate, they collectively form a functional pore. Furthermore, nisin exerts its antimicrobial effect by inhibiting cell wall biosynthesis through binding to lipid II, thereby disrupting the transport of peptidoglycan precursors. This interaction suggests that nisin may use lipid II as a receptor or scaffold to facilitate membrane attachment and pore formation, contributing to its bactericidal activity [27]. Recently, due to their antimicrobial properties, there has been a growing interest in using bacteriocins as natural preservatives to replace synthetic chemical preservatives. However, the application of bacteriocins as effective antimicrobial agents has been limited by several challenges such as susceptibility to proteolytic degradation and interaction with food matrix components. To overcome these limitations and increase the shelf life of food products, nisin-based nanostructures such as chitosan/alginate nanoparticles have been developed as a promising drug delivery system. Chitosan is a biopolymer widely used in biomedical applications and has attracted attention due to its biocompatibility, biodegradability, non-toxicity, high film-forming ability, and capacity as a drug carrier, making it suitable for incorporation into nanoparticle systems for food preservation purposes [28, 29]. Alginate is a natural polysaccharide classified in the glycosaminoglycan family and shares structural similarities with chitosan. Both alginate and chitosan have emulsifying and stabilizing properties, which are related to the high density of hydroxyl and carboxylate functional groups on their polymer chains, which facilitates interaction with various biomolecules. The abundance of hydroxyl groups, combined with carboxylate features, endows them with unique physicochemical properties such as gelation, biocompatibility, and biodegradability, thus enabling their application in encapsulation systems for bioactive compounds. Notably, alginate exhibits exceptional biocompatibility, low immunogenicity, and effective substrate and carrier functions, which underpin its widespread use in biomedical applications including drug delivery, tissue engineering, and regenerative medicine [30]. In a previous study, a comparative microbiological evaluation was conducted to evaluate the antibacterial efficacy of free nisin versus nisin encapsulated in chitosan/alginate nanoparticles against *Staphylococcus aureus* in raw and pasteurized milk matrices. The results showed that the intrinsic antimicrobial activity of free nisin decreases after repeated incubation periods, while nisin encapsulated in chitosan/alginate nanoparticles maintains its biological activity over time. Notably, the antimicrobial potency of nisin encapsulated in nanoparticles increases over incubation periods compared to free nisin. The cationic nature of chitosan facilitates electrostatic interactions with negatively charged bacterial cell surfaces, leading to disruption of the cell membrane. At the same time, alginate acts as a hydrogel matrix that, by absorbing water, reduces water activity, thereby creating unfavorable conditions for bacterial proliferation. These biopolymers also play a protective role, protecting nisin from physicochemical instability caused by temperature changes and pH fluctuations. In addition, the nanoparticle matrix protects nisin from enzymatic

degradation by hiding its active sites, thereby maintaining its antimicrobial function in complex food environments such as meat. In further research, chitosan/alginate nanoparticles containing nisin at a concentration of 450 IU/mL were formulated to enhance physicochemical stability. The antimicrobial activity of these encapsulated formulations was evaluated against *Listeria monocytogenes* ATCC 25923 and *Staphylococcus aureus* ATCC 19117 in feta cheese.

Microbial counts showed that nisin with nanoparticles, compared to free nisin, resulted in a five-log and seven-log reduction in the population of *S. aureus* and *L. monocytogenes*, respectively. Furthermore, encapsulated nisin showed better bactericidal effect with minimal adverse effects on cheese quality parameters. These findings indicate that chitosan/alginate nanocarriers significantly enhance the antimicrobial spectrum and stability of nisin, confirming their application as effective biopreservatives in dairy products. The increase in antimicrobial activity and stability is attributed to the protective and controlled release properties provided by the nanoparticle matrix [14, 23]. In one study, nisin produced by *Lactococcus lactis* was isolated from fermented dairy products, including milk and cheese. The isolates were cultured under optimal conditions on de Man Rogosa and Sharpe (MRS) agar to evaluate bacteriocin activity. Antimicrobial activity was assessed using the agar well diffusion method against standard indicator strains: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Bacillus cereus* ATCC 14579. The results showed that *E. coli* showed the least susceptibility to bacteriocin compared to other indicator strains [15]. In our current study, bacteriocins produced by the tested *Lactococcus lactis* strains showed no inhibitory activity against *E. coli*, while significant inhibition was observed against *S. aureus*. The highest antimicrobial effect was observed against *Bacillus cereus*, which confirms the findings of Mitra *et al.* (2005). In one study, a total of 23 lactic acid bacteria isolates derived from dairy and non-dairy sources were subjected to microbiological investigations, which resulted in the isolation of eight strains of *Lactococcus lactis*. These strains showed the capacity to synthesize nisin, a class I bacteriocin with proven activity against Gram-positive bacteria, including *Bacillus cereus*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Nisin biosynthesis by these strains was confirmed by polymerase chain reaction (PCR) targeting the nisA gene locus [31]. In the present study, the presence of the nisin gene in four isolates of Lactococcus lactis obtained from goat milk samples was detected by species-specific PCR assays. The PCR assay failed to amplify the nisA gene in the control Escherichia coli, indicating its specificity, while successful amplification was observed for *S. aureus* and *E. faecalis*, confirming the antimicrobial activity of the nisin-producing strains. The most pronounced inhibitory effect was observed against *Bacillus cereus*, confirming previous findings. The antimicrobial effect of nisin mainly targets Gram-positive bacteria, which is related to the difference in cell wall structure. The thick peptidoglycan layer in Gram-positive bacteria provides a binding site for nisin, which facilitates the formation of pores in the cytoplasmic membrane, thereby causing ion leakage and cell death. In contrast, Gram-negative bacteria have an outer membrane composed of lipopolysaccharides that acts as a permeability barrier, preventing the penetration of

bacteriocins such as nisin, thereby conferring intrinsic resistance. These structural and biological distinctions are consistent with us in vitro antimicrobial assays that have shown significant activity against Gram-positive pathogens. It is worth noting that although several bacteriocins have been identified, nisin is the only bacteriocin approved for use as a food additive by regulatory agencies such as the European Food Safety Authority (EFSA) and the United States Food and Drug Administration (FDA). Nisin is designated as E234 as documented by Hassan *et al.* (2021) [32]. Despite the significant advantages of nano/nisin systems in enhancing stability and antimicrobial efficacy, their industrial implementation faces several challenges. These include limited stability in certain food matrices, particularly in the presence of fats, enzymes, or unfavorable pH; scalability and high production costs of nanoformulations; regulatory and safety concerns related to the use of nanotechnology in foods; and potential consumer acceptance issues. Nevertheless, these systems hold great potential for applications in active packaging, ready-to-eat foods, and products requiring extended shelf life, provided that formulation design and application conditions are carefully optimized according to the specific food product [33, 34].

CONCLUSION

In conclusion, the antimicrobial effect of nisin derived from *Lactococcus lactis* and its incorporation of chitosan/alginate nanoparticles show significant potential for controlling bacterial pathogens in the sausage matrix, thereby enhancing food safety and reducing spoilage. This study emphasizes the importance of utilizing natural antimicrobial compounds and novel nanocarrier drug delivery systems in the food industry. Isolation of indigenous bacterial strains capable of producing nisin, combined with nanoparticle technology, is a promising biological preservative alternative to synthetic chemical preservatives such as sodium nitrite. For future research, it is recommended to evaluate the synergistic effects of plant essential oils in combination with nisin and nanoparticles to further increase product shelf life. Furthermore, increasing the sample size to assess the prevalence and concentration of target bacterial populations, along with testing with different nisin concentrations and nanoparticle formulations, will facilitate optimization and a deeper understanding of their antimicrobial interactions.

Acknowledgments

We would like to thank the Islamic Azad University, Kerman Branch, for administrative support and the Orial Meat Products Company for providing the Frankfurter sausage dough.

CONFLICTS OF INTEREST

The authors of this work declare that they have no conflicts of interest.

RESEARCH LIMITATIONS

Experiments on release kinetics and stability of nisin were not conducted in this phase. Moreover, sensory assessments, and toxicity evaluations were not included, as the study was designed as a preliminary in vitro investigation.

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Table 1. PCR temperature program; Repeat steps 2 to 4, 35 times.

Step No	PCR Stages	Temperature(°C)	Time(minute)
1	Primary Denaturation	94	3
2	Secondary Denaturation	94	1
3	Primer annealing	45	0.5
4	Primer extension	72	2

Table 2. the results of biochemical diagnostic tests for the relevant strains.

Diagnostic tests	Isolated strains
	L1, L3, L8, L9
Gram-positive cocci, double-chain	+
Catalase test	-
Oxidase test	-
Growth at 10 and 40°C	+
Growth at 45°C	-
Growth in 4% NaCl	+
Growth in 6.5% NaCl	-
Growth in pH 9.2	+
Growth in 0.3% methylene blue	+
Homolactic fermentation	+
Arginine hydrolysis	+

Starch hydrolysis	-
CO_2 production from citrate	-

Table 3. F.I.C. of L1 strain on indicator bacteria.

Treated bacteria	F.I.C. index of strain L1
Escherichia coli	0.5
Salmonella typhimurium	0.25
Staphylococcus aureus	0.249
Bacillus cereus	0.124
Acinetobacter baumannii	0.25
Enterococcus faecalis	0.249
Pseudomonas aeruginosa	0.25

F.I.C. fractional inhibitory concentration

A B

Fig.1. L1: A) on MRS agar B) Microscopic image.

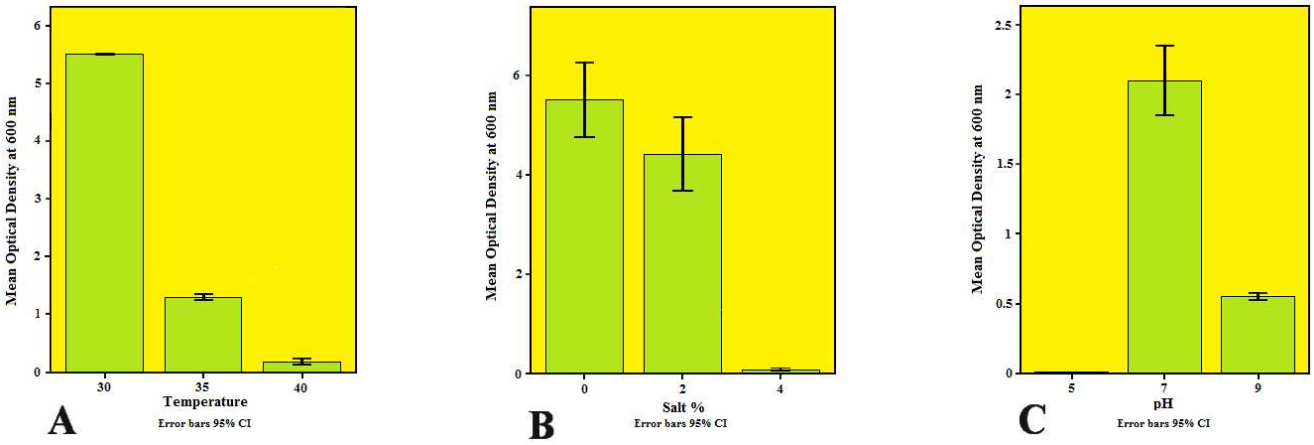

Fig. 2. The effect of temperature (A), salt concentration (B) and pH (C) on the growth of strain L1 (error bar 95 %).

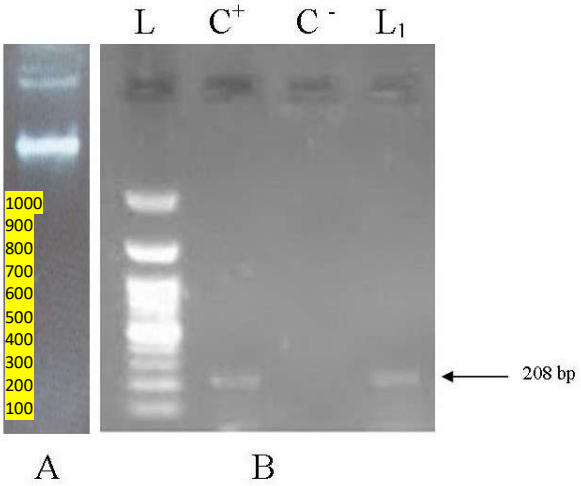

Fig. 3. A, Extracted bacterial genomic DNA product on agarose gel, B, Amplification of nisin gene fragment obtained by PCR (ladder size 1000 bp).

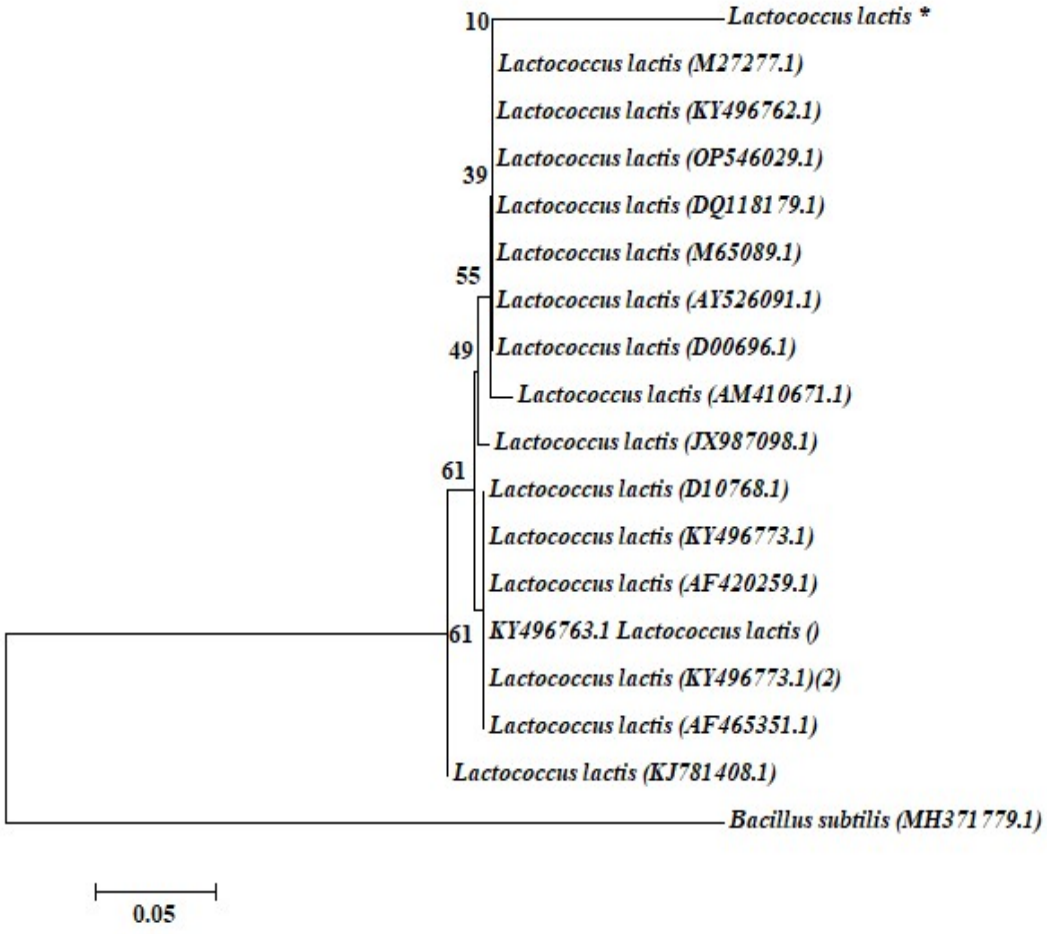

Fig. 4. The phylogenetic tree of the isolated strain (L1).

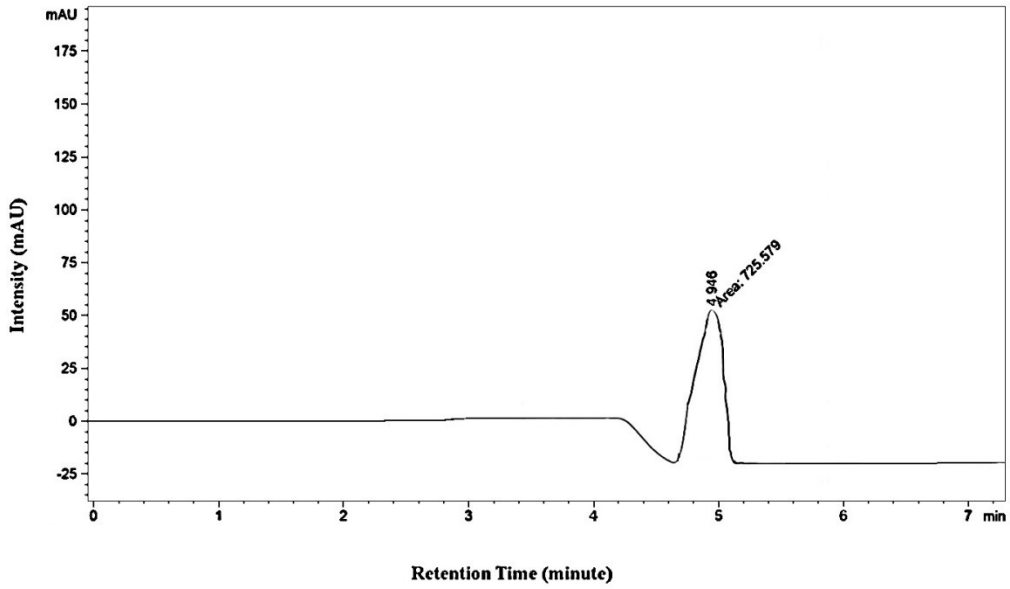

Fig. 5. Chromatogram of L1 strain nisin.

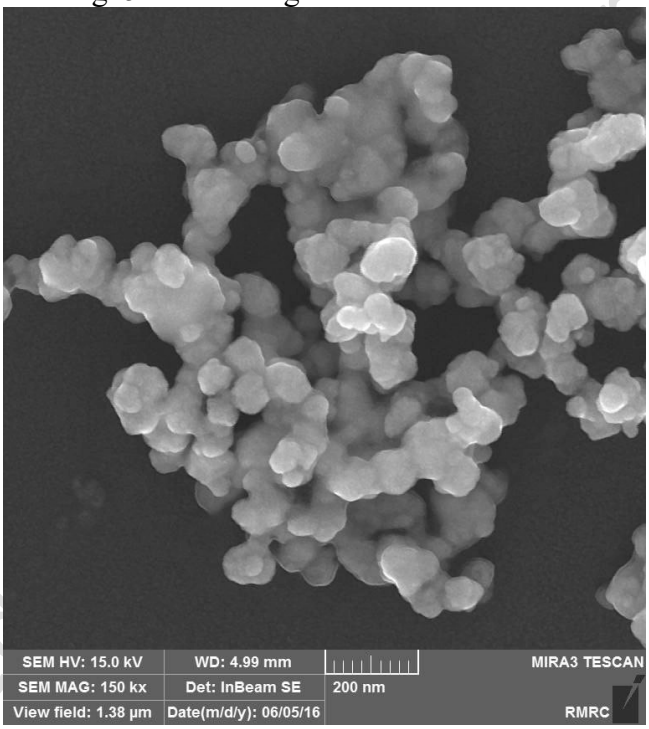

Fig. 6. SEM image of chitosan/alginate nanoparticles containing nisin.

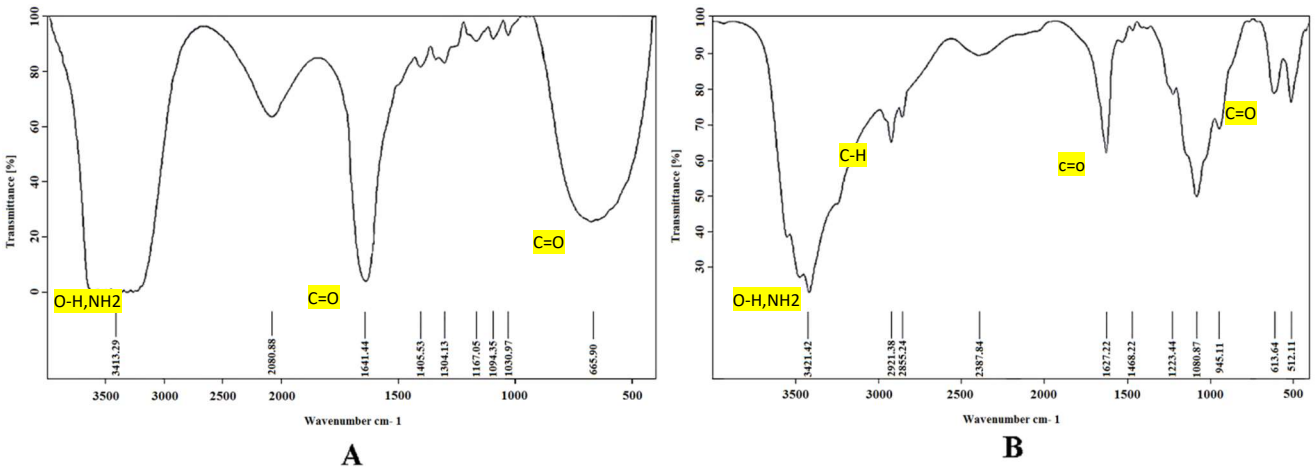

Fig. 7. FTIR analysis of nisin-free chitosan/alginate nanoparticle sample (A), and nisin combined with chitosan/alginate nanoparticles presented by strain L1 (B).

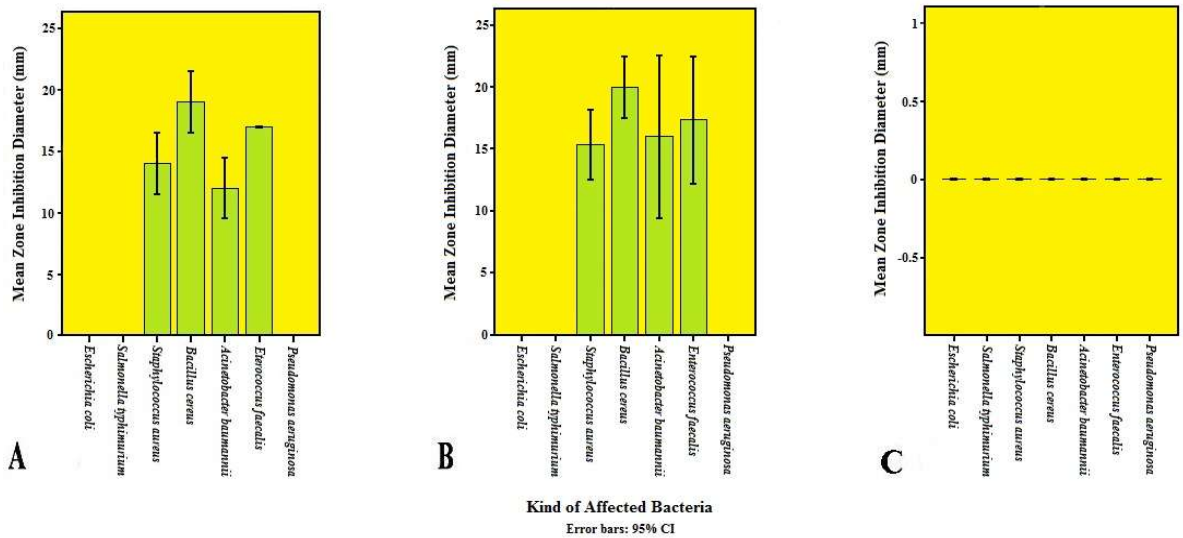

Fig. 8. Disk diffusion tests of L1 strain on indicator bacteria, A: Antimicrobial activity of nisin L1, B: Chitosan/alginate nanoparticles with nisin L1, C: Negative control (Error Bar 95%).

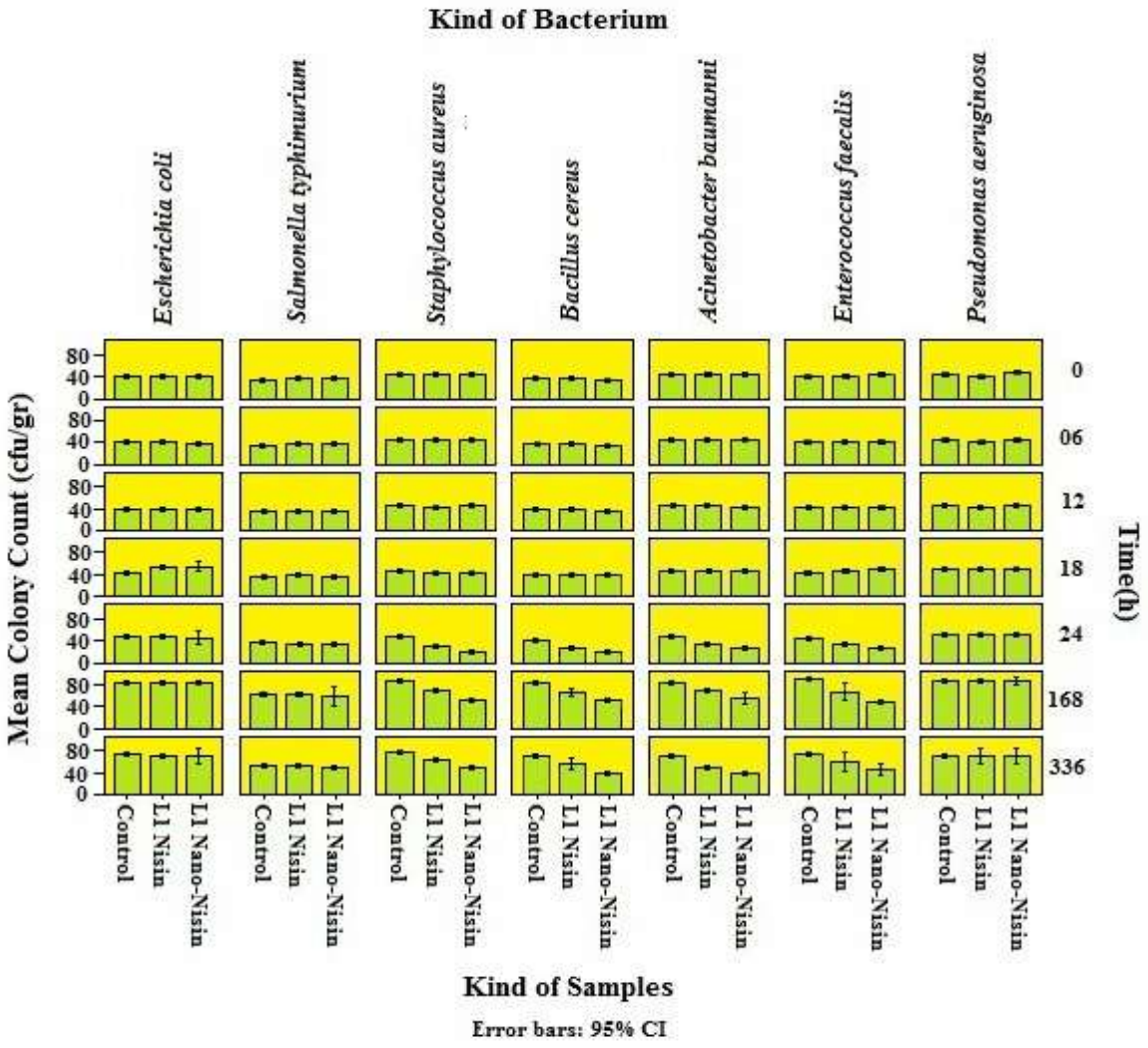

Fig. 9. Changes in total count of indicator bacteria in various kinds of samples and times (h).