

# Evaluation of the efficacy of nisin-loaded chitosan nanoparticles against foodborne pathogens in orange juice

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**Abstract** The current study aimed to fabricate nisin-loaded chitosan (N-CS) nanoparticles through ionic interactions between positive amino groups of chitosan and negatively charged tripolyphosphate ions in the presence of nisin and to evaluate their efficacy against foodborne pathogens in orange juice. The synthesized nanoparticles were sphere-shaped and homogenous with an average size of  $64.34 \pm 2.1$  and  $147.93 \pm 2.9$  for chitosan and N-CS nanoparticles, respectively. The encapsulation efficiency of nisin into nanoparticles was  $67.32 \pm 0.63\%$ . Both chitosan and N-CS nanoparticles showed greater stability, as indicated by a higher zeta potential value of  $+49.3$  and  $+33.4$  mV, respectively. The in vitro antibacterial activities of chitosan and N-CS nanoparticles were investigated against the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes* and the Gram-negative bacteria *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. N-CS nanoparticles showed higher activity compared with chitosan nanoparticles. The highest reduction of microorganisms was recorded for *S. aureus* of 3.82 log CFU/ml and *L. monocytogenes* of 3.61 log CFU/ml. The antimicrobial activity of N-CS nanoparticles in orange juice for 48 h revealed higher activity compared with the control against all the tested strains. The highest microbial reduction was recorded for N-CS nanoparticles against *S. aureus* with a 3.84 log CFU/ml reduction. *L. monocytogenes* and *E. coli* O157:H7 were reduced by 3.54 and 3.44

log CFU/ml, respectively. The results showed high potential for the N-CS nanoparticles to be used as potent antibacterial agents in food and other related areas.

**Keywords** Antimicrobial activity · Chitosan · Foodborne pathogens · Nanoparticles · Nisin

## Introduction

Each year, approximately 48 million people get sick, 128,000 are hospitalized, and 3000 die of foodborne diseases in the United States alone (CDC 2017). In the European Union in 2012, a total of 55,453 human illnesses, 5118 hospitalizations and 41 deaths were reported (Perez Pulido et al. 2016). In developing and underdeveloped countries major food-borne pathogens are widely predominant and pose a major threat to human health (Akhtar et al. 2014). Food products frequently associated with foodborne outbreaks include beef and poultry, eggs and egg products, mixed foods and fish and fish products (Khan et al. 2015; Perez Pulido et al. 2016; Tango et al. 2016). In addition, outbreaks of illness associated with the consumption of fruit juice have been a growing public health problem since the early 1990s. According to the CDC, 39 juice-associated outbreaks were reported from 1991 through 2010; 15 implicated apple juice or cider, 10 were linked to orange juice, 3 were linked to mixed fruit, and 11 involved other types of fruit juice (Danyluk et al. 2012).

A significant number of studies have been carried out on the application of pulsed electric fields, essential oils, ozone and bacteriocins from lactic acid bacteria in the preservation of fruit juices. Among these, the use of bacteriocins is a promising alternative (de Oliveira Junior et al.

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2015). Bacteriocins previously tested in fruit juices include nisin (Komitopoulou et al. 1999), enterocin AS48 (Grande et al. 2005), bovicin HC5 (De Carvalho et al. 2008), and bificin C6564 (Pei et al. 2014). Among these, only nisin has been approved as a food additive (de Oliveira Junior et al. 2015). However, the effectiveness of nisin in food products is influenced by many factors such as the interaction of nisin with the food matrix and its reactions with glutathione, sodium metabisulfite, proteases, and titanium oxide (Khan and Oh 2016; Quintavalla and Vicini 2002). The controlled release and delivery of nisin can be widely improved through the use of nanoparticulate systems (Khan and Oh 2016; Quintavalla and Vicini 2002). To overcome the issues linked with the use of nisin as a food preservative, the use of nanotechnology for the synthesis of nisin-loaded/coated nanoparticles has been introduced (da Silva Malheiros et al. 2010).

Chitosan is naturally obtained from chitin through N-deacetylation and is the second most abundant biopolymer after cellulose (Khan et al. 2015). Chitosan is considered to be the most widely distributed biopolymer having nontoxicity, biodegradability and biocompatibility properties. Chitosan has been extensively applied in the food industry as an antimicrobial and antioxidant agent (Khan et al. 2016). Moreover, chitosan has been extensively studied as a delivery agent in various fields, including medicine and the food industry (Alishahi 2014; Khan et al. 2015).

The aim of the current study was to enhance the antimicrobial activity of nisin in orange juice by encapsulation into chitosan nanoparticles. The synthesized nisin-loaded chitosan (N-CS) nanoparticles were characterized for morphology, zeta potential and encapsulation efficiency.

## Materials and methods

### Synthesis of nanoparticles

Chitosan (0.5%; LMW: 52 kDa; Sigma, USA) was dissolved in a 1% acetic acid (Sigma, USA) solution and stirred for 12 h to produce a homogeneous chitosan solution. Sodium tripolyphosphate (TPP; 0.5%; Sigma, USA) and nisin (990 IU/ml; Sigma, USA) were mixed in deionized water. A nisin/TPP solution at a 1:10 ratio of chitosan was added drop by drop to the chitosan solution to form ionically cross-linked nanoparticles. D-trehalose (3% w/v) was added to the reaction mixture to avoid nanoparticle aggregation and continuously stirred for 1 h. After centrifugation for 10 min at 20,000 rpm, the nanoparticles were collected and kept at  $-80\text{ }^{\circ}\text{C}$  for 24 h before freeze-drying. The nanoparticles were freeze-dried (Labtech

Freeze Dryer, Daihan, Korea) at  $-50\text{ }^{\circ}\text{C}$  and 0.15 mbar pressure. The powdered nanoparticles were stored at  $4\text{ }^{\circ}\text{C}$  until further analysis.

### Encapsulation efficiency

Nisin encapsulation efficiency was measured according to the method described by Zohri et al. (2010) with slight modifications. Briefly, the chitosan and N-CS nanoparticles dispersion was subjected to centrifugation at 13,800 rpm for 10 min at  $4\text{ }^{\circ}\text{C}$  by using 15 ml of a 100 kDa molecular weight cut off ultrafilter (Amicon Ultra-15 Ultracel-100 K). The amount of nisin in the upper tube was examined by Bradford protein assay. A calibration curve for nisin at various concentrations was produced before the experiment. The nisin encapsulation efficiency was determined using the following formula:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Total nisin} - \text{Unbound nisin}}{\text{Total nisin}} \times 100$$

### Zeta potential and size

The zeta potential and the size of the chitosan and N-CS nanoparticles were evaluated by a particle analyzer SZ-100 (Horibo, Japan). The diluted aqueous dispersion of nanoparticles was measured with the apparatus.

### Morphology

The morphology of the chitosan and N-CS nanoparticles was analyzed by scanning electron microscopy (SEM). Prior to observation, an aliquot (10  $\mu\text{l}$ ) of the nanoparticles was mounted on SEM plastic coverslips (Rinzl Plastic Coverslips, Electron Microscopy Science, USA). The operation condition of the SEM was at an accelerating voltage of 5 kV. SEM images were observed at 50,000 $\times$  magnification.

### In vitro antimicrobial activity

#### Preparation of bacterial strains

Four foodborne pathogens, two Gram-positive bacteria *Staphylococcus aureus* ATCC 12600 and *Listeria monocytogenes* ATCC 19115 and two Gram-negative bacteria *Escherichia coli* O157:H7 and *Salmonella* Typhimurium ATCC 14028 were used in the study. The stock culture of each strain was transferred to tryptic soy broth (TSB, 10 ml) and cultured at  $37\text{ }^{\circ}\text{C}$  for 18 h and then harvested. All the strains were washed two times with 0.1% buffer

peptone water (BPW) and centrifuged for 10 min at 4000 rpm. The final culture was resuspended in BPW to obtain a final concentration of approximately 8 log CFU/ml. The cell counts of each strain were recorded by plating an aliquot on TSA plates.

#### *Susceptibility testing*

The antimicrobial effect of chitosan and N-CS nanoparticles was determined by standard cell counting methods with minor modifications (Khan et al. 2016). Briefly, nanoparticles at concentrations of 1.25 and 2.5 mg/ml were added into a 96-well plate. Approximately 0.1 ml of each strain ( $\sim 8$  log CFU/ml) was poured into each well of the 96-well plate and the total volume was adjusted to 250 using BPW. The microtiter plates were incubated at 37 °C for 24 h. After incubation, 0.1 ml of a suitable dilution was spread on TSA and incubated at 37 °C for the next 24 h. The experiment was repeated twice and expressed as the mean  $\pm$  standard deviation.

#### **Evaluation of antimicrobial activity in orange juice**

##### *Preparation of orange juice*

Fresh navel oranges were purchased from a local market in Chuncheon, South Korea and transferred immediately into the laboratory. The oranges were washed with tap water two times and halved, then squeezed using an ordinary orange squeezer. Milder action was taken as the maximum point of penetration was reached so that the oil and juice from the orange rind were not released into the juice. The juice was then filtered using a stainless filter with a net square hole of 1 mm<sup>2</sup>. The juice was kept in a sterile reagent bottle at 4 °C until further use.

##### *Microorganisms and growth conditions*

*S. aureus*, *L. monocytogenes*, *E. coli* 0157:H7 and *S. Typhimurium* were grown in TSB according to the methods described by Tango et al. (2016).

##### *Antimicrobial activity*

In vivo antimicrobial activity of the test samples was evaluated against that of *S. aureus*, *L. monocytogenes*, *E. coli* 0157:H7 and *S. Typhimurium*. Test samples, i.e., chitosan (2.5 mg/ml), nisin (1 mg/ml), chitosan nanoparticles (2.5 mg/ml) and N-CS nanoparticles (2.5 mg/ml) were mixed with 25 ml of orange juice. Approximately 6 log CFU/mL (18 h culture) of the microorganisms was inoculated into the fruit juices that were supplemented with different test samples. The fruit juices were incubated at

the optimal microorganism growth temperature, i.e., 25 °C for up to 48 h. After 0, 8, 24 and 48 h of incubation, samples were withdrawn and diluted (in tenfold increments), and viable cell numbers were determined by plate counting. Pure orange juice without any additive was used as a negative control.

#### **Statistical analysis**

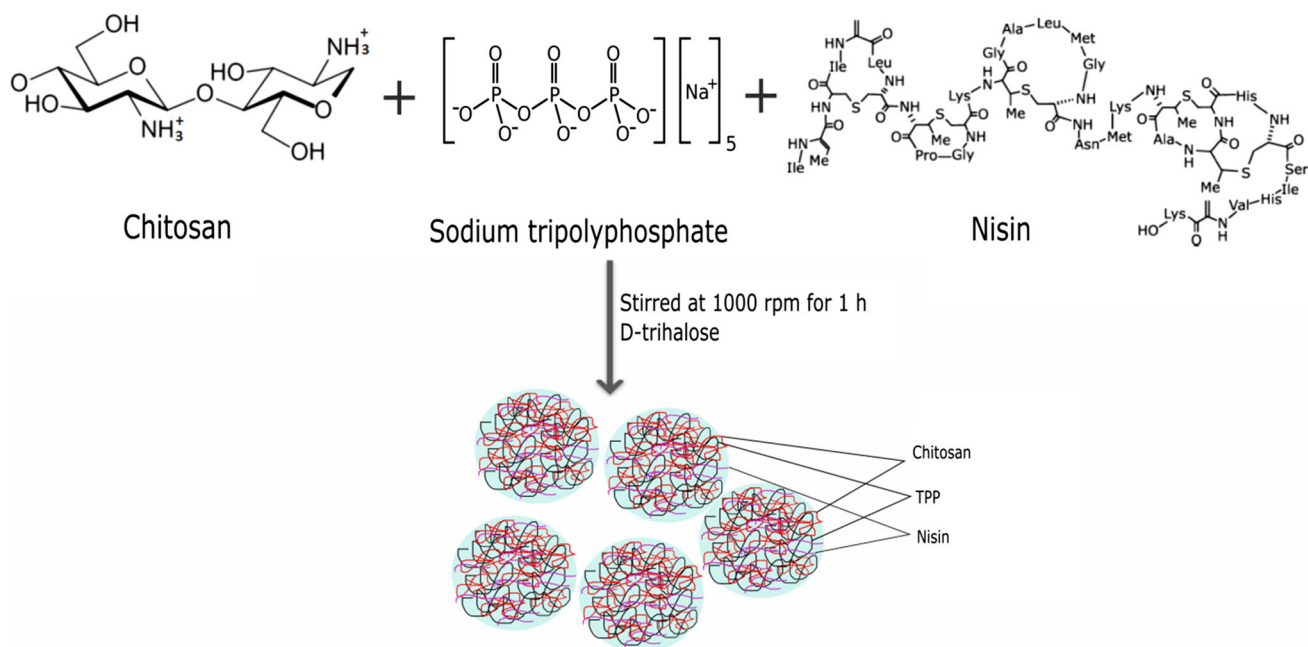
All data are expressed as the mean  $\pm$  standard deviation. The data were analyzed by one-way ANOVA using SPSS software (IBM SPSS version 21.0; IBM Corp, Chicago, USA). Tukey's multiple range tests were used to determine the significant differences at  $P < 0.05$  between treatments.

## **Results and discussion**

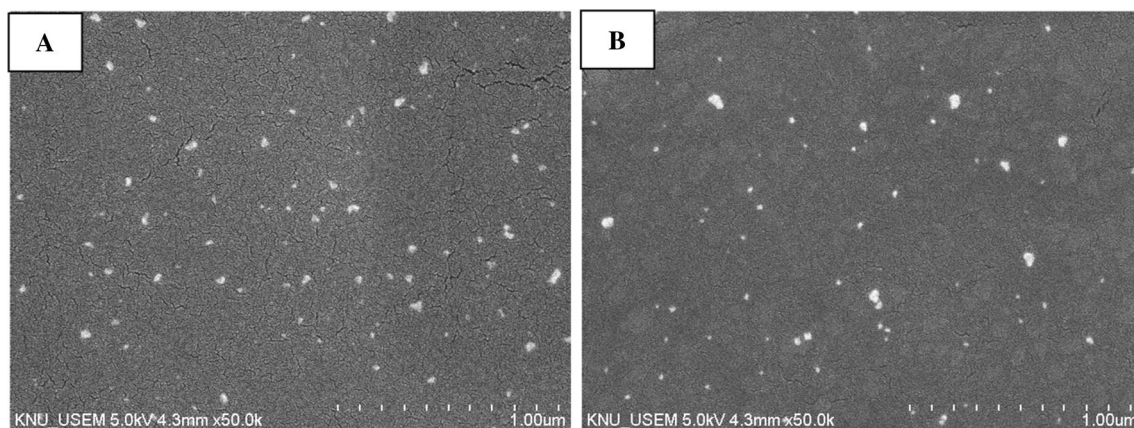
### **Nanoparticle synthesis and characterization**

Chitosan and N-CS nanoparticles were successfully synthesized according to the scheme shown in Fig. 1. Chitosan/TPP interacted with nisin through hydrogen and ionic bonding (Alishahi 2014). Figure 2 shows the morphology of the chitosan and N-CS nanoparticles observed by SEM. The nanoparticles have spherical shapes and smooth surfaces (Mohammadpour Dounighi et al. 2012). Chitosan and N-CS nanoparticles have similar morphology. The mean size of the chitosan and N-CS nanoparticles was  $64.34 \pm 2.12$  and  $147.93 \pm 2.9$  nm, respectively. The size of chitosan nanoparticles is highly influenced by the solution pH, TPP and chitosan concentration (Zhao et al. 2011). Various studies have reported different sizes and shapes of chitosan nanoparticles that ranged from 42 to 350 nm (Khan et al. 2015; Liu and Gao 2009; Muhammed et al. 2010; Zhao et al. 2011; Zohri et al. 2010). In the present study, the N-CS nanoparticles were larger than the chitosan nanoparticles, possibly due to the molecular weight and size of the nisin peptide.

The zeta potential of N-CS nanoparticles can greatly influence their stability in suspension by means of electrostatic repulsion between the particles (Mohammadpour Dounighi et al. 2012). Our results demonstrated the zeta potential of chitosan and N-CS nanoparticles was + 49.3 and + 33.4 mV, respectively. Similar results were obtained by Khan et al. (2017), who reported a zeta potential of + 48.34 and + 39.4 mV for chitosan and N-CS nanoparticles, respectively. The zeta potential of the nanoparticle was positive, indicating the presence of amino groups of chitosan on the surface (Alishahi 2014; Dorkoosh et al. 2002). However, these results showed that the nisin loading into the nanoparticles led to reduction of the particle's zeta potential. It was supposed that the nisin



**Fig. 1** Schematic representation of N-CS nanoparticles



**Fig. 2** Scanning electron microscopy of chitosan nanoparticles (a) and N-CS nanoparticles (b)

encapsulation in long chain chitosan molecules was not uniform. This can be explained by the fact that chitosan can possibly adopt a diffuse conformation in an aqueous solution due to the electrostatic repulsion force present between amine groups through the long chitosan chain. Furthermore, the carboxyl groups of nisin may form hydrogen bonds with amine groups at certain locations along the chitosan chain but still maintain a compact structure without diffusing in the relatively acidic solution to keep an inner hydrophobic core. Therefore, nisin attachment did not sufficiently neutralize the positive surface charge of chitosan (Mohammadpour Dounighi et al. 2012).

The encapsulation efficiency of nisin into nanoparticles was  $67.32 \pm 0.63\%$ . The encapsulation efficiency of nisin

is consistent with previously reported work (Alishahi 2014; Gan et al. 2005). It is well established that increasing the chitosan to nisin concentration results in an increase of encapsulation efficiency. When the concentration of chitosan was 2.0%, the encapsulation efficiency of nisin was 78.8% (Alishahi 2014).

### In vitro antimicrobial activity

The in vitro antimicrobial activity of chitosan and chitosan nanoparticles against four foodborne pathogens is depicted in Table 1. The highest reduction was observed for *S. aureus* with a 3.82 log CFU/ml reduction followed by *L. monocytogenes* with a  $3.61 \pm 0.05$  log CFU/ml reduction for N-CS nanoparticles compared to chitosan

**Table 1** Antimicrobial activity of chitosan nanoparticles and N-CS nanoparticles against some foodborne pathogens

Foodborne pathogens	Chitosan (2.5 mg/ml) Log reduction (CFU/ml)	Nisin (1 mg/ml)	Chitosan nanoparticles (2.5 mg/ml)	N-CS nanoparticles (1.5 mg/ml)
<i>S. aureus</i>	1.75 ± 0.02d	2.73 ± 0.01d	2.21 ± 0.02d	3.82 ± 0.03d
<i>L. monocytogenes</i>	1.58 ± 0.01c	2.34 ± 0.05c	2.15 ± 0.04c	3.61 ± 0.05c
<i>E. coli</i> O157:H7	1.45 ± 0.06b	1.72 ± 0.03b	2.03 ± 0.03b	3.49 ± 0.01b
<i>S. Typhimurium</i>	1.32 ± 0.03a	1.56 ± 0.03a	1.96 ± 0.01a	2.88 ± 0.03a

Initial count of the bacterial strains *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *S. Typhimurium* was 8.35, 8.12, 7.98, and 8.02 log CFU/ml, respectively. Letters (a–d) are assigned according to increasing mean values. Values sharing the same letters (column wise) are not significantly different at  $P < 0.05$

nanoparticles. However, Gram-negative bacteria have shown slight resistance to both chitosan and N-CS nanoparticles. Although various mechanisms of interaction between chitosan and bacteria have been reported, the most likely mechanism of interaction is facilitated by the electrostatic forces between the protonated-NH<sub>3</sub><sup>+</sup> groups of chitosan and the electronegative charges on the cell surface of microbes, causing the leakage of intracellular materials (Hosseini et al. 2016). Nisin has been shown to be effective against a wide range of Gram-positive bacteria. However, it shows little or no activity against Gram-negative bacteria (Deegan et al. 2006; Karam et al. 2013). This can be explained by the fact that Gram-positive bacteria have comparatively higher amounts of anionic lipid in the cytoplasmic membrane, facilitating nisin insertion, compared to Gram-negative bacteria (Breukink and de Kruijff 1999). Moreover, nisin disrupts the integrity of the microbial cell membrane by forming pores that lead to the efflux of small metabolites such as amino acids, nucleotides, ions and other cytoplasmic solutes, resulting in cell death as depicted in Fig. 3 (Reiners et al. 2017).

Interestingly, the effect of N-CS nanoparticles on *E. coli* O157:H7 was higher with 3.49 ± 0.04 log CFU/ml compared to chitosan nanoparticles with 2.03 ± 0.03 log CFU/ml reduction. The lowest log CFU/ml reduction was

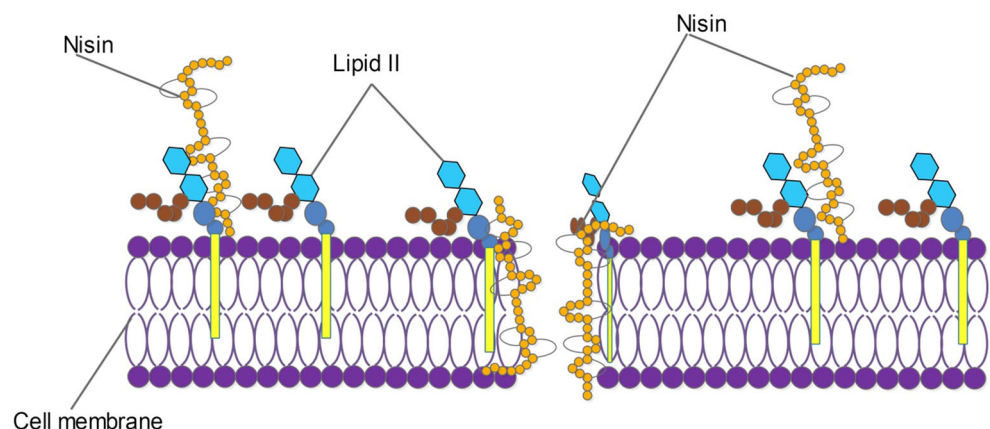
observed for *S. Typhimurium* with 2.88 ± 0.03 and 1.96 ± 0.01 for chitosan and N-CS nanoparticles, respectively. *S. Typhimurium* showed resistance against all the tested samples compared with the other tested foodborne pathogens.

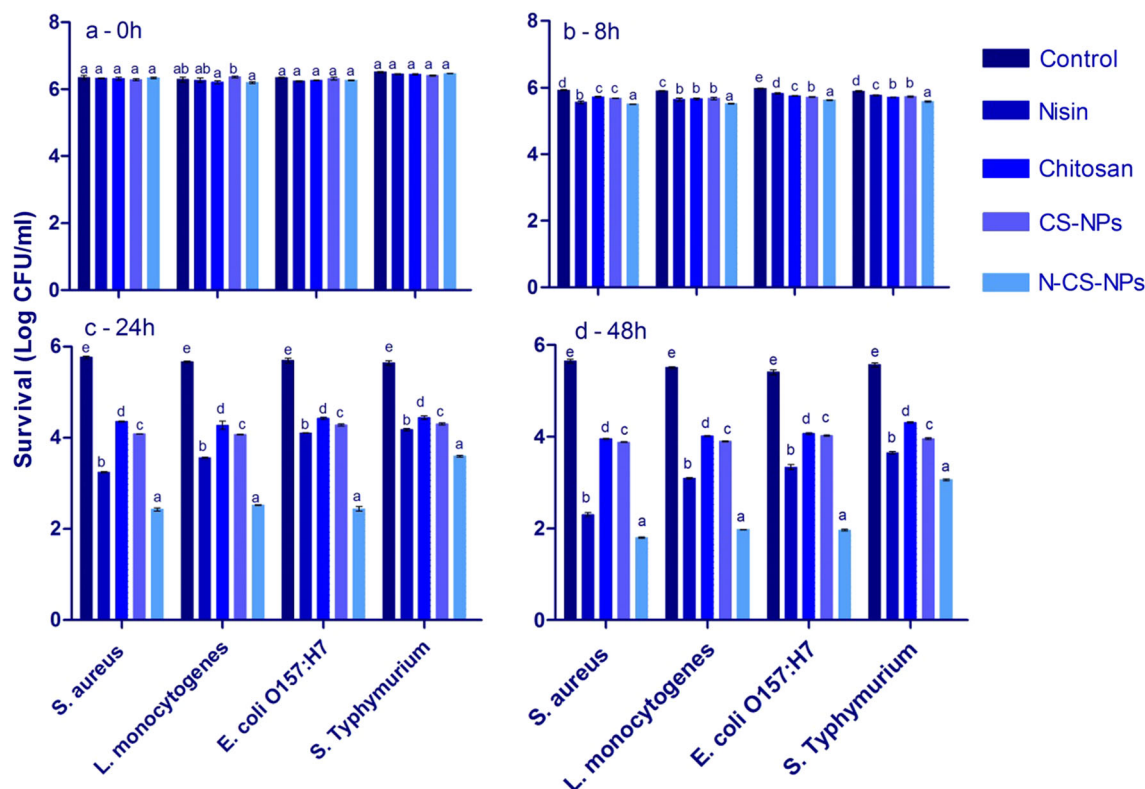
The results have revealed that a synergistic antibacterial effect between chitosan nanoparticles and nisin has been observed. Our results coincide with those obtained by Alishahi (2014), where N-CS nanoparticles showed a synergistic effect compared with the control.

### Inactivation of foodborne pathogens in orange juice

The inactivation effect of nisin, chitosan, CS nanoparticles and N-CS nanoparticles against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, and *S. Typhimurium* in orange juice (Fig. 4). The bacterial count in the control treatment was slightly reduced after 48 h of incubation (Fig. 4). The slight decline in the microbial count of the control samples can be attributed to the antimicrobial effect of orange juice itself (Oikeh et al. 2016). Tomotake et al. (2006) reported the antimicrobial effects of citrus juices against seven *Vibrio* spp. The results indicated that all juices were effective in inhibiting the growth of the *Vibrio* strains.

**Fig. 3** Mechanism of action of nisin by inhibiting cell wall synthesis and pore formation. In both cases, nisin interacts with lipid II molecules, a main transporter for peptidoglycan subunits, leading to the death of targeted cells





**Fig. 4** The effect of test samples on the inactivation of four foodborne pathogens in orange juice at different time intervals. The data are given as log CFU/ml survival. The letters a–e represent statistically significant differences among the same microorganism

using different test samples. Bars showing the same letter are not significantly different at  $P < 0.05$ . CS-NPs: chitosan nanoparticles, N-CS-NPs: nisin loaded chitosan nanoparticles

The effect of N-CS nanoparticles was prominent among all the treatments against the microorganisms tested. At 8 h of incubation with test samples, the N-CS nanoparticles showed the highest ( $P < 0.05$ ) antimicrobial activity against all the strains compared with other test samples (Fig. 4b). The antimicrobial effect of the test samples was significantly ( $P < 0.05$ ) enhanced after 24 h of incubation against all the microorganisms with approximately 1.47–3.33 log CFU/ml reduction (Fig. 4c). The highest ( $P < 0.05$ ) microbial reduction was observed for N-CS nanoparticles against *S. aureus* (3.33 log CFU/ml) followed by *E. coli* O157:H7 (3.25 log CFU/ml). At 48 h of incubation with test samples, the highest ( $P < 0.05$ ) microbial reduction was recorded for N-CS nanoparticles against *S. aureus* with 3.84 log CFU/ml, followed by *L. monocytogenes* with 3.54 log CFU/ml reduction in total count. The *E. coli* O157:H7 count was reduced by 3.44 after 48 h of incubation with N-CS nanoparticles. However, *S. Typhimurium* was the most resistant strain with 2.51 log CFU/ml reduction after 48 h of incubation (Fig. 4d).

The antimicrobial effect of chitosan and nisin was lower ( $P < 0.05$ ) compared with that of N-CS nanoparticles. The antimicrobial activity of nisin and chitosan was dose-dependent, and therefore, the activity varies from study to

study (Bhatia and Bharti 2015; Goy et al. 2016). In addition, the antimicrobial effect of chitosan and nisin varies both from strain to strain and species to species (Bergholz et al. 2013; Goy et al. 2009). The enhanced antimicrobial effect of N-CS nanoparticles could be attributed to the stable structure of nanoparticles and controlled release of nisin into the medium (Alishahi 2014). The enhanced antimicrobial effect of N-CS nanoparticles against *E. coli* O157:H7 and *S. aureus* could be explained by the fact that treatment with the nanoparticle causes a release of intracellular components from the cell membrane, which is increased through destroying the integrity of the bacterial cell membranes (Alishahi 2014).

## Conclusion

Chitosan has the ability to form stable and homogenous nanoparticles for the successful encapsulation of nisin. N-CS nanoparticles were successfully prepared through ionic interactions. N-CS nanoparticles showed higher antimicrobial activity compared to free nisin and chitosan alone. Higher antimicrobial activity was observed against *S. aureus* and *L. monocytogenes*. N-CS nanoparticles

showed a higher reduction of pathogenic microbes in orange juice compared with control samples. Higher encapsulation efficiency and stability make them a good candidate for the preservation of foods against pathogenic bacteria.

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