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ENHANCING THE ANTIBACTERIAL EFFECT OF BACTERIOCIN FROM *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* USING CHITOSAN NANOPARTICLES

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ABSTRACT

Chitosan, a cationic polymer derived from the hot alkali deacetylation of chitin, has numerous biological applications with non-toxicity, biocompatibility and biodegradability. Chitosan nanoparticles were prepared and encapsulated with bacteriocin extracted from *Lactococcus lactis* subsp *lactis* to produce chitosan nanoparticles conjugate bacteriocin using ionic gelation method. This formulation was examined for its antibacterial activity representing food bio-preservative against *Salmonella typhimurium, Escherichiae coli, Bacillus cereus* and *Staphylococcus aureus*, compared with chitosan nanoparticles, crud chitosan and free bacteriocin. Agar diffusion method was applied to evaluate the in-vitro drug release, effect of pH and temperature on its stability. The results revealed that the In-vitro release within 24 hours of chitosan nanoparticles conjugate bacteriocin was controlled by about (79%) with cumulative and sustained effect when compared with free bacteriocin (94%). Chitosan nanoparticles conjugate bacteriocin exhibit the highest antibacterial activity (with significant difference $p < 0.05$) followed by free bacteriocin and chitosan nanoparticles, while crud chitosan was the lowest representing thermal stability (≤ 70ºC) when subjected to low pH. Gram-positive bacteria were more susceptible than Gram-negative bacteria to all of the components. Chitosan nanoparticles contribute successful safe food preservative enhancement when incorporated with bacteriocin against the common food-borne pathogenic bacteria.

Keywords: Bacteriocin, Chitosan nanoparticles, Nanoparticles conjugate bacteriocin

INTRODUCTION

Chitosan is a non-toxic biodegradable copolymer consists of D-glucoseamine and N-acetyle-D-glucoseamine units from chitin deacetylation in the presence of hot alkali **(Zaghloul *et al*., 2015)**. It contains amino, primary and secondary hydroxyl as relative function groups in C2, C3 and C6 positions, respectively **(Ali and Toliba, 2018)**. Ionic gelation method with tripolyphosphate (TPP) ion was investigated by **Kahdestani *et al*. (2021)** to prepare chitosan nanoparticles (CSNps) from crude chitosan.

Chitosan reveal antimicrobial activity against many spoilage and pathogenic microorganisms representing Gram-positive and Gram-negative bacteria, molds and yeasts. Its antimicrobial effect is depending on the deacetylation degree, type of microorganism, molecular weight and pH value **(Eldaly *et al*., 2018)**. The antimicrobial properties of CSNps and its derivatives were proven in previous literature studies **(Acay *et al*., 2020)**. It encapsulates bioactive formulations in micro or nanoparticles form in addition to its antimicrobial properties **(Correa-Pacheco *et al*., 2018)**. The low toxic and ecological safe biocompatibility and admirable biodegradability with antimicrobial activity provided ample opportunities for further applications **(Chawla *et al*., 2014)**. CSNps are effective for sustained bioactive release because it mitigates the bioactive release **(Kahdestani *et al*., 2021)**.

Lactic acid bacteria (LAB), commonly used in food preservation, exhibit antagonistic activity and inhibiting pathogenic and spoilage microbiota in food and food products via bacteriocins and other metabolites which have vital antimicrobial capacities **(Akbar *et al*., 2019)**.

Bacteriocins, antimicrobial peptides synthesized by ribosomes, display bacteriostatic or bacteriocidal effect toward target specificity closely related and/or broad range bacterial strains **(Abdelsamei *et al*., 2015; Woraprayote *et al*., 2016)**. These bacteriocins have small cationic molecules containing 30 to 60 amino acids which form amphiphilic helices, have heat stability when subjected to temperature of 100ºC for 10 min and differ from each other in mode of action, spectrum of activity, biochemical properties, genetic origin and molecular weight **(Mokoena, 2017)**.

Among bacteriocinogenic LAB, *Lactococcus spp* produced bacteriocins **(Yusuf and Abdul Hamid, 2013)**. *Lactococcus lactis* generally isolated from fermented raw milk and known as Generally Recognized As Safe (GRAS) strain. This strain prevents pathogenic bacterial growth in the fermented products by converting lactose to lactic acid as a result of its proteolytic activity. The produced lactic acid considers an important role in the final taste and texture of fermented products **(Tenea and Suárez, 2020)**.

The most common bacteriocin produced by *Lc. lactis* is nisin A and its variants. It represents Class I bacteriocin, Ripps, post-translationally modified peptides, heat stable, lanthionine and methyllanthionine containing peptides (<5 KDa) with members consisting lacticins **(Meade *et al*., 2020)**. Whereas, LAB produce one bacteriocin, *L. lactis* produce 2 synergistically Class II bacteriocins named LsbA and LsbB **(Duhan *et al*., 2013)**. Bacteriocins inhibit only Gram-positive bacteria including *Staphylococcus aureus* and *Listeria monocytogenes* because Gram negative bacteria exhibit high resistance to these compounds **(Moreno *et al*., 2000)**.

Where bacteriocins exploitation to be applied as preservatives showing slow pace moving is yet to be addressed for various limitations, bacteriocin-nanoconjugates utilization in food industry are basically focused to overcome the challenges of using bacteriocins alone. To combat the direct addition of bacteriocin and its susceptibility to storage conditions, changes in temperatures and production process, nanoparticles provides an efficient technology to protect and deliver their potential antibacterial effect **(Sidhu and Nehra, 2019)**.

Nanoparticles are known as particles with dimensions ranged from 1 to 100 nm with unique properties thanks to the reduction of its dimension to the atomic level increasing the atomic surface compared to the bulk equivalents **(Divya and Jisha, 2018)**. The suitable formulation technique using nanoparticles (nanoencapsulation) improve the antimicrobial activity of bacteriocins **(Namasivayam *et al*., 2015)**. These nanoparticles have the potential to diffuse and cross-biological cellular membrane barrier of different cell type. Several studies have modified chitosan as biocompatible nontoxic polymer with integrated ability to antimicrobial peptides to form chitosan-based nanoparticles vehicle for delivery applications **(Tamara *et al*., 2018; Kahdestani *et al*., 2021)**.

The aim of the present study is to evaluate the potential release and stability of bacteriocin encapsulated with CSNps under different values pH and temperatures and as well as its antibacterial activity against common food-borne pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains

Lyophilized strains of bacteriocin producing bacteria and food-borne pathogenic bacteria were obtained from different cultures collections as shown in (Table, 1).

Table 1 Source of bacterial strains.

Bacterial strains	Strain number/identification	Sources
Bacteriocin producing bacteria		
Lactococcus lactis subsp *lactis*	EMCC[a]11552	Dairy Department, Minia University
Food-borne pathogenic bacteria		
Salmonella typhimurium	ATCC[b]14028	Cairo MIRCEN[c]
Escherichiae coli	ATCC[b]10536	Cairo MIRCEN[c]
Bacillus cereus	ATCC[b]10876	Cairo MIRCEN[c]
Staphylococcus aureus	ATCC[b]6538	Cairo MIRCEN[c]

a) EMCC: Egyptian Microbial Culture Collection.
b) ATCC: American Type Culture Collection.
c) Cairo MIRCEN: Cairo Microbiological Resources Center, Faculty of Agriculture, Ain Shams University.

Bacterial activation

Different bacterial strains: bacteriocin producing bacteria and food-borne pathogenic bacteria, were activated at 37 ºC for 24h using MRS (De Man, Rogosa and Sharpe) broth CM0359 and Nutrient broth CM0001 media from Oxoid UK, respectively. In order to meet the active recommended levels, bacterial cultures were adjusted with sterile saline to McFarland standard ($1.5x10^8$ cfu/ml) **(Abdelsamei *et al*., 2015)**.

Extraction of free bacteriocin (free-B)

According to **Mostafa *et al*. (2019)**, free bacteriocin (free B) was extracted by incubation of active *Lc. lactis* subsp. *lactis* culture was inoculated into 250 ml of MRS broth (1% v/v), incubated at 37ºC for 18 h and then centrifuged (Centrifuge K2015, Centurion Scientific, UK) at 10,000 rpm for 10 min at 4ºC. The supernatant contained crude bacteriocin was subjected to salt saturation method to be partially purified by adding 70% saturation ammonium sulphate during a magnetic stirring (AccuPlate, Labnet, USA) at 4ºC then centrifuged at 10,000 rpm for 20 min at 4ºC to separate the precipitated proteins. The final protein pellet was dissolved in Phosphate-buffered saline (PBS) at pH 7.0 (Code: S3024, Agilent, USA) then sterilized through Seitz filter (Millipore, USA) with 0.22 µm pore size filter.

Preparation of Chitosan nanoparticles (CSNps)

Ionic gelation method was performed by dissolving 0.2 g of chitosan (deacetylation degree of 93% from Sigma-Aldrich, USA.) was dissolved in 100 ml of 1% acetic acid, drop wise of sodium tripolyphosphate (TPP from Sigma-Aldrich, USA.) added during stirring for 3 h and subsequently centrifuged at 10,000 rpm for 10 min to obtain pellet contain CSNps **(Divya *et al*., 2017)**.

Preparation of Chitosan nanoparticles conjugate bacteriocin (CSNps-B)

To prepare chitosan incorporated bacteriocin, the above mentioned ionic gelation method was implemented with mixing 10 ml of free-B suspension after adding the 1% acetic acid. The centrifuged step (at 10,000 rpm/10 min) resulted in pellet contain CSNps-B **(Namasivayam *et al*., 2015)**.

In-vitro release of bacteriocin

To determine in-vitro bacteriocin release, dialysis bag method was used according to **Bohrey *et al*. (2016)** with modifications. An amount of 5 ml of release medium (0.1M PBS with pH 7.4) contained 50 mg of CSNps-B or free-B was pipette in dialysis bag at 37ºC, then placed in beaker contained 100 ml of PBS. The beaker was placed over magnetic stirrer at 100 rpm/37±1 ºC. An intermittent bacteriocin release was assessed by withdraw 2 ml samples at 1, 2, 3, …, up to 24 hrs while replacement with an equal amount with PBS. Samples were examined for bacteriocin release percent using US-Vis spectrophotometer (Jenway 6105, USA) at 291 nm.

Preparation of different formulations

Under aseptic conditions three-fold tubes (12*75 mm) representing 4 groups include crud chitosan, CSNps, free-B or CSNps-B were accurately quantified then added to 0.25% acetic acid to prepare 100 µg/ml concentration according to **Abdeltawab *et al*. (2019)**.

Assessments and characterizations

In-vitro release of bacteriocin

To determine in-vitro bacteriocin release, dialysis bag method was used according to **Bohrey *et al*. (2016)** with slight modifications. 5 ml of release medium (0.1M PBS, pH 7.4) containing 50 mg of CSNps-B or free-B was pipette in dialysis bag at 37ºC, then placed in beaker containing 100 ml of PBS. The beaker was stirred at 37±1°C using a magnetic stirrer at 100 rpm. An intermittent bacteriocin release was assessed by withdrawing 2 ml samples each hour for 24 hrs, with one hour as interval time, with replacing an equal amount of PBS. Samples were examined for the percentage of bacteriocin release using US-Vis spectrophotometer (Jenway 6105, USA) at 291 nm.

Antibacterial activity assay

Agar well diffusion method was applied to evaluate the food-borne pathogenic bacterial response against the formulation groups, by measuring the inhibition zone in millimeters (mm). Sterile cork borer well made wells by removing slug from inoculated Mueller Hinton Agar (Code: 64884, BIO-RAD, USA). The individual bacterial cultures were loaded with 100 µl of the separated treatments and then incubated at 37ºC for 24 h. The measurements were carried out in triplicates **(Balouiri *et al*., 2016)**.

Stability at different pH values

To assess the effect of pH on the prepared formulation groups, tubes containing 5 ml of the concentrates were pH adjusted at different values of pH which ranged from 4 to 11 with sterile lactic acid (1% w/v) or NaOH (1 M) at room temperature (22ºC) for 2 hours then readjusted to 7 as pH value. The antibacterial activity of these concentrates was determined using agar well diffusion method **(Mostafa *et al*., 2015)**.

Stability at different heating treatments

Tubes containing 5 ml of the above mentioned concentrates were over covered with paraffin oil to avoid evaporation, separately incubated in water bath (Memmert, WB14, Germany) for 30 min at different temperatures varied from 0 to 100C with interval 10°C and then cooled immediately at 4ºC. Concentrates were determined for antibacterial activity by agar well diffusion method **(Mostafa *et al*., 2019)**.

Statistical analysis

The obtained results representing the data of duplicated experiments were statistically analyzed. Analysis of variance among formulations and treatments were performed by entering data through one-way ANOVA and paired-samples T-test using (IBM-SPSS, 20; USA) with statistical significance declared at $p < 0.05$ **(Rabie *et al*., 2015)**.

RESULTS AND DISCUSSION

In-vitro release of bacteriocin

The efficiency of loaded CSNps with bacteriocin produced by *Lc. lactis* subsp *lactis* as safe food preservative was in vitro evaluated by comparing formulations, i.e. CSNps-B and its components (chitosan, CSNps and free-B), against Gram positive and Gram negative food-borne pathogenic bacteria.
The release behavior of bacteriocin from CSNps-B and free-B during 24 hours was carried out using dialysis bag method and shown in Figure (1). Gradual increase of bacteriocin release was found to be 79 and 95% from CSNps-B and free-B tubes, respectively. During the initial 4 hours, burst release about 83% of bacteriocin from free-B tubes then slightly increase to release about 94% by the end of 24 hours. On the other hand, cumulative sustained release from tubes containing CSNps-B was observed reaching about 28% within 4 hours and 79% by the end of 24 hours. A significant difference at $p < 0.05$ was observed when compared with the paired-samples T-test.

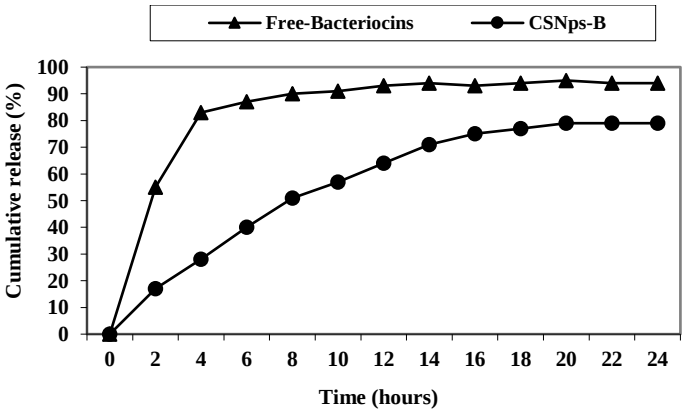

Figure 1 In vitro profile of bacteriocin release. *Significant difference ($p < 0.05$) was observed.

The obtained results are in agreement with those reported by **Namasivayam *et al*. (2015)** and **Bohrey *et al*. (2016)**. The bacteriocins are contained within or covalently linked to CSNps, attained a high free-B concentrate, resulting in lower release over the time with a better efficacy of comparable doses which exploited for preventing bacterial growth **(Teixeira *et al*., 2020)**. Chitosan encapsulated active compound increases their control release and protect its components against environmental factors **(Alishahi, 2014)**. So, CSNps-B could be used to control bacteriocin release to improve its bioavailability **(Singh *et al*., 2018)**.

Antibacterial activity

In order to investigate the efficiency of impregnate CSNps with bacteriocin produced from *Lc. lactis* subsp. *lactis*, 4 formulations were prepared including CSNps-B, CSNps, chitosan and free-B were examined for their antibacterial activity against food-borne pathogenic bacteria. The bio-preservation effect of these formulations against Gram-positive (G^+) (*B. cereus* and *Staph. aureus*) and Gram-negative (G^-) bacteria (*E. coli* and *S. typhimurium*) by agar well diffusion method was illustrated in Figure (2). The results revealed that Gram-negative bacteria were more resistant than Gram-positive ones toward the 4 formulations.
According to **Prudêncio *et al*. (2015)**, the outer membrane of Gram-negative bacteria may act as an effective barrier protecting it against Gram-positive bacterial bacteriocins. The meticulous nature of cellular envelope of Gram-negative bacteria could resist the bacteriocins throughout the adsorption phenomena **(Goraya *et al*., 2013)**.
The measured inhibition zone (mm) indicates that all of the bacteria showed high sensitivity against CSNps-B with inhibition zones ranged from 20 to 23 mm for gram-positive bacteria and from 14 to 16 mm for Gram-negative bacteria. A significant difference ($p < 0.05$) was observed when the inhibition zone obtained by CSNps-B was compared with those obtained by the other 3 formulations: chitosan (6 to 8), CSNps (8 to 10) and Free-B (6 to 14).

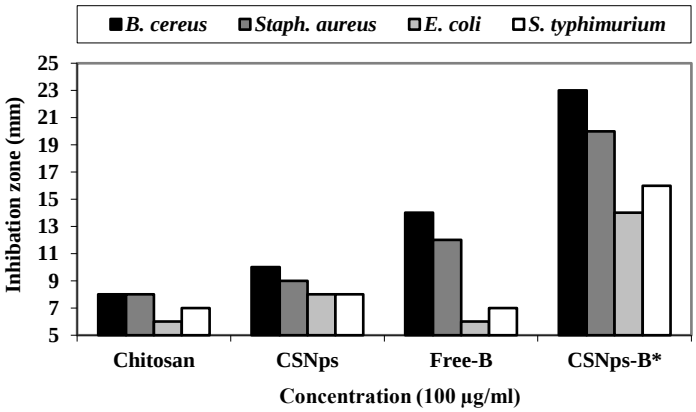

Figure 2 Inhibition zone (mm) of chitosan, CSNps, free-B and CSNps-B against food-borne pathogenic bacteria. * Significant difference ($p < 0.05$) between CSNps-B and the other formulations was observed.

Bacteriocins produced by *Lc. lactis* subsp. *lactis* may contains lantibiotic nisin-A which in turn binds lipid II, inhibiting the synthesis of peptidoglycan and forming a complex of membrane pore **(Kim *et al*., 2020)**. This exhibits different action mechanisms including the interaction with cell wall, cell membrane and intracellular organelles in addition to binding the genetic material. With prokaryotic selectivity, it adheres to bacterial cells and penetrates phospholipids membranes as a result of cationic small size and alteration of hydrophobic, hydrophilic and charge properties **(Meade *et al*., 2020)**.
Furthermore, bacteriocins bind to charged phospholipids headgroups and proteinaceous receptors in the bacterial cell membrane resulting in pores formation within the cytoplasmic membrane and depletion the proton motive force leading to interfere with cell biosynthesis and further cell death **(Abamecha, 2017)**.
Chitosan had an antibacterial activity thanks of the positive charges which interact with plasma membrane phospholipids (negative charges) causing leakage of components then cell death by altering the cell permeability, chelating property of metal ions, and inhibiting the mRNA synthesis by penetrating the cell wall and binding to the DNA **(Divya and Jisha, 2018)**.
The obtained results are in concurrence with that reported by **Divya *et al*. (2017)** who found that CSNps exhibited large inhibition zone for all microorganisms, i.e. *Klebsiella pneumonia, E. coli, Staph. aureus* and *Pseudomonas aeruginosa*, with superior antimicrobial activity when compared with chitosan. These results could be explained by the fact that CSNps could diffuse better than chitosan and cross the cell membrane as biological barrier **(Kahdestani *et al*., 2021)**. The increase in the antibacterial activity using these nanoparticles may be due to the compactable arrangement and strong interaction of smaller particle size increase its surface to volume ratio **(Divya *et al*., 2017; Acay *et al*., 2020)**.
The combination of nanoparticles and loaded active compounds may exert a synergistic effect as nanoparticles themselves have antibacterial activity. The modified nanoparticles with target active molecules could attack the specific biological sites **(Teixeira *et al*., 2020)**. CSNps-B could provide nanoencapsulation which in turn protects bacteriocins from the degradation by proteolytic enzymes degradation rendering bioactive component stability and increase the shelf life of food **(Sidhu and Nehra, 2019)**. In food, nisin as a free bacteriocin is exhausted due to its interaction with lipids, proteins, enzymatic degradation and uneven distribution within food matrix. Encapsulation of bacteriocin with CSNps has a synergistic demonstrated antibacterial effect against bacterial growth rather than its individual components **(Alishahi, 2014)**. CSNps may cause morphological changes such as formation of pores in Gram-positive and Gram-negative bacterial cell membrane which in turn facilitate the bacteriocin cell penetration **(Pan *et al*. 2011)**.Several studies reported that bacteriocin conjugated with nanoparticles enhanced and increased the antibacterial activity against food borne pathogenic bacteria by about two to four times more higher than the use of bacteriocin alone **(Zohri *et al*., 2010; Zohri *et al*., 2013)**.

Effect of pH on the antibacterial activity

Environmental parameters such as pH and temperature were investigated to define the optimized conditions in order to maximize the antibacterial activity. Figure (3) shows the effect of different pH values on the antibacterial activity of the 4 formulations (chitosan, CSNps, free-B and CSNps-B), on the growth of food borne pathogenic bacteria; *B. cereus, Staph. aureus, E. coli*, and *S. typhimurium*. An increase in inhibition zone was observed at lower pH values using the different formulations for all studied strains wherein the highest inhibition zone was recorded at pH = 4. Whereas, a gradual decrease trend in inhibition zone was found with increasing the pH value wherein the lowest inhibition zone was observed at pH = 11. The release of bacteriocin (nisin) is pH depending; **Wu *et al*. (2016)** found higher significant release of bacteriocin (nisin) at pH value of 3 (with 46% cumulative bacteriocin during 72 hrs) than those at 6 as pH value.
It was found that CSNps-B exhibited the highest antibacterial activity for all bacterial strains followed by free-B, CSNps, and chitosan at different pH values. Furthermore, CSNps and chitosan did not exhibit any antibacterial activity at pH values of 10 and 11 against both *B. cereus* and *Staph. aureus.*
It is worth to mention here that at pH value of 10; only the CSNps-B exhibited an antibacterial activity on the growth of *E. coli* and *S. typhimurium* while, no effect was observed at pH value of 11. Moreover, the Gram-negative bacteria; *E. coli* and *S. typhimurium*, were more resistant to all formulations compared to Gram-positive bacteria; *B. cereus* and *Staph. aureus.*
The obtained results are in agreement with those reported by **Adesina and Enerijiofi (2016)** and **Kumari *et al*. (2018)** who reported that the most active bacteriocin against bacterial indicators was in pH values ranged from 6 to 7 with slight sensitivity at pH = 7, while a decrease in antibacterial activity was observed at pH values varied from 2 to 5.

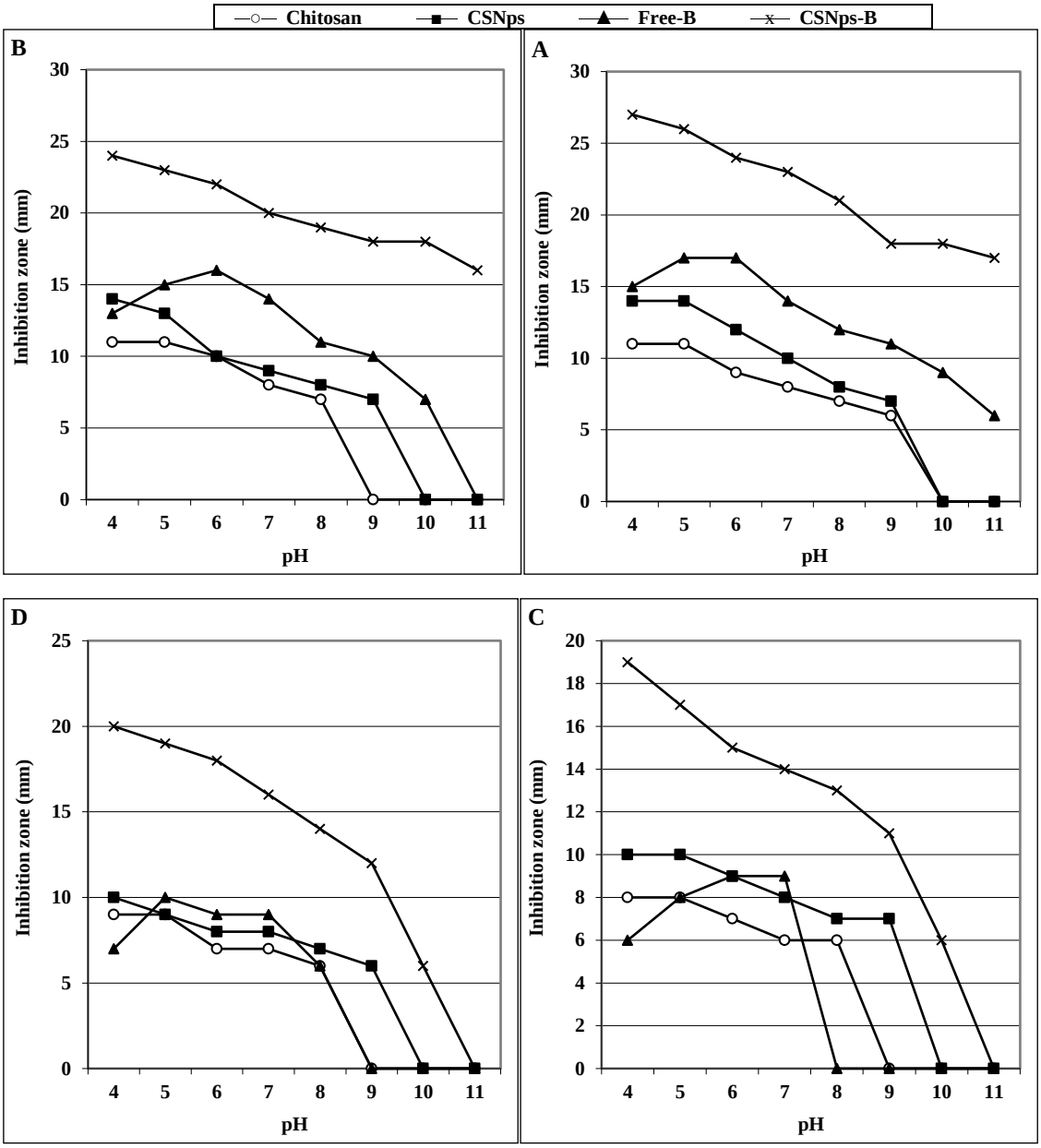

Figure 3 Stability of chitosan, CSNps, Free-B and CSNps-B at different pH against food-borne pathogenic bacteria: A) *B. cereus*, B) *Staph. aureus*, C) *E. coli* and D) *S. typhimurium*. [* Significant difference ($p < 0.05$) between CSNps-B and the other formulations was observed.]

In contrast, **Zacharof and Lovitt (2012)** showed that bacteriocins produced from LAB had high antibacterial activity at pH values below 5.

Similar results concerning the antibacterial activity of chitosan were obtained by **Qi *et al*. (2004)** who reported a high antibacterial activity of chitosan only in acidic medium, as it loses its solubility at pH value higher than 6.5. A complete loss of bacteriocin activity especially nisin was also observed by **Benkerroum *et al*. (2002)** at neutral pH as a result of decreasing its solubility Furthermore, the free amino groups in the d-glucosamine units may result in its protonation **(Kahdestani *et al*., 2021)**. The obtained results are in disagreement with those found by **Alishahi, (2014)** who reported that the release of bacteriocin was faster at higher pH than that at the lower pH wherein the diffusion process controlled the release of bacteriocin at acidic environment.

The high antibacterial effect of CSNps compared to chitosan may be due to the interfacial interaction between CSNps small particle size and the bacterial cell membrane throughout the endocytosis **(Divya and Jisha, 2018; Lee *et al*., 2018)**. The high bacteriocin release at lower pH values may be due to the dissolution and swelling of CSNps **(Khan *et al*., 2020)**. Bacteriocins exhibited high activity at pH 2-5 however, a loss by about 5.9-10% of its activity was observed at alkaline levels **(Abanoz and Kunduhoglu, 2018; Kaktcham *et al*., 2019)**. While, the antibacterial activity of chitosan may be due to the interactions between the positive charged amino groups and the negative charged bacterial membrane **(Kravanja *et al*., 2019)**.

The obtained results could be explained by 1) changes in the molecular interactions which take place between biopolymers as a result of the variation in pH values **(Wu *et al*., 2016)**; 2) increasing the positive charges of chitosan as a result of lowering pH value which in turn increases its affinity towards the bacterial cell wall; increased protonated amino groups such as –NH3 groups with positive charges can bind to bacterial membrane components with negative charges. Moreover, the antibacterial activity at pH values below 6 may be due to the consequent of positively charged protonation which interacts with teichoic acid in Gram-positive bacteria and anionic lipopolysaccharides in Gram-negative bacteria **(Malinowska-Pañczyk *et al*., 2015)**, and 3) alteration of mRNA functions and limiting the interactions of DNA through their binding with the low molecular weight which can pass through the cell **(Rizeq *et al*., 2019)**.

Effect of temperature on the antibacterial activity

In order to investigate the heating effect on the antibacterial activity of the 4 formulations expressed by the inhibition zone using different temperatures which varied from 0 to 100°C for 30 min. Figure (4) shows that the 4 formulations exhibited different heat stability representing different antimicrobial activity against Gram-positive and Gram-negative bacteria. The highest stability was recorded for temperatures ranged from 0 to 70°C. Beyond these temperatures, a gradual decrease trend was observed until the absence of inhibition zone except CSNps-B at 100°C. The highest inhibition zone was observed for CSNps-B ($p < 0.05$) followed by free-B, CSNps, and chitosan. CSNps-B showed higher antibacterial activity against Gram-positive more than Gram-negative bacteria. The obtained results are in agreement with those reported by **Azhar *et al*. (2017), Le *et al*. (2019)** and **Mostafa *et al*. (2019)**. The combined use of CSNps-B with high temperature may provide synergistic effect which includes the effect of high

temperature and the antibacterial activity of CSNps-B increasing the inhibition zone which reflects the growth inhibition of food-borne bacteria **(Prudêncio *et al*., 2015)**. Therefore, the heat stability of CSNps-B, Free-bacteriocin, CSNps and chitosan allows their applications as food preservative under the mentioned conditions of temperatures.

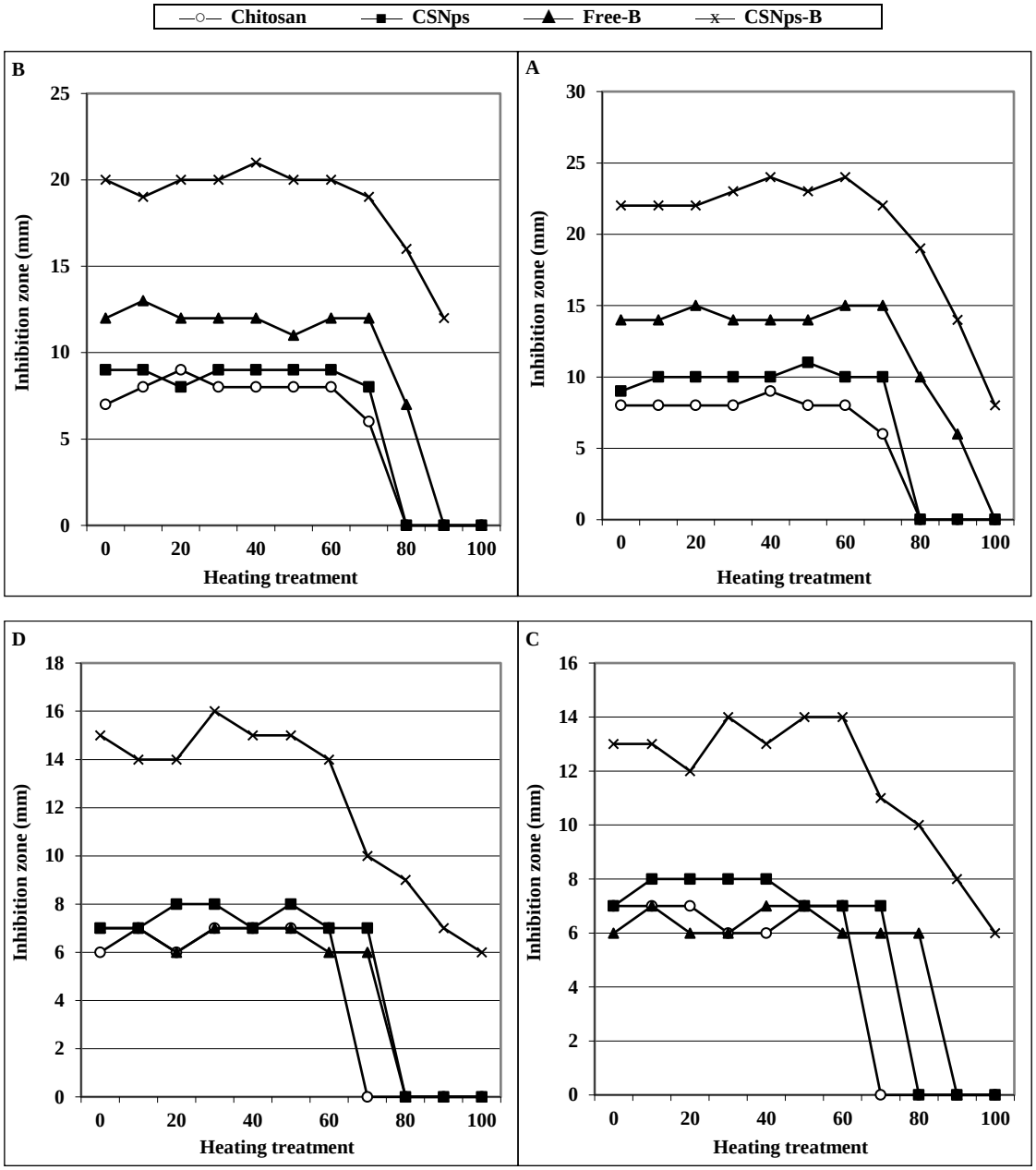

Figure 4 Stability of chitosan, CSNps, Free-B and CSNps-B at different heating treatment against food-borne pathogenic bacteria: A) *B. cereus*, B) *Staph. aureus*, C) *E. coli* and D) *S. typhimurium.* [* Significant difference ($p < 0.05$) between CSNps-B and the other formulations was observed.]

CONCLUSION

The combination of bacteriocins with CSNps (CSNps-B) increased the antibacterial activity of bacteriocin as food preservative extending the shelf life of food without altering its quality attributes. CSNps-B showed high antibacterial activity at wide range of temperature and pH. It exhibited higher antibacterial activity against Gram-positive pathogenic bacteria which was observed at lower values of pH than Gram-negative pathogenic bacteria. So, CSNps-B could be used as food bio-preservative inhibiting the growth of food-borne bacteria.

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