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Antibiofilm activity of carvacrol loaded chitosan nanoparticles against *Listeria monocytogenes*

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ABSTRACT

Aims: This study was designed to evaluate the effectiveness of the synthesised carvacrol loaded chitosan nanoparticles (CLCNPs) on the growing and pre-formed biofilms of *Listeria monocytogenes* isolated from slaughterhouses.
Methodology and results: The swab samples were collected from knives, hocks and cutting tables representing slaughterhouses meat contact surfaces (MCS), while those samples from walls and floors represent slaughterhouses meat non-contact surfaces (MNCS). The bacteriological analysis revealed the existence of *L. monocytogenes* with a prevalence rate of 3.3, 10 and 6.7% for knives, hocks and cutting tables, respectively and 2.2 and 6.6% for walls and floors, respectively. The isolates *L. monocytogenes* were assayed for biofilm production by the crystal violet binding assay method. Among the 10 *L. monocytogenes* isolates, 10%, 50% and 30% of the isolates were found to be strong, moderate and weak biofilm producers, respectively. The activities of carvacrol, chitosan nanoparticles (NPs) and CLCNPs against the only strong biofilm producer strain of *L. monocytogenes* were tested by microtiter plate assay. The minimum inhibitory concentrations (MIC) values were 3.75 mg/mL for CAR, 5 mg/mL for chitosan NPs and 0.62 mg/mL for CLCNPs. CLCNPs inhibit the produced biofilm by 35.79, 73.37 and 77.76%, when 0.5 MIC, 1 MIC and 2 MIC were used, respectively. Furthermore, the pre-formed *L. monocytogenes* biofilms were significantly reduced from 1.01 (control) OD_{570} to 0.40 and 0.29 OD_{570} by applying 2 MIC and 4 MIC doses, respectively.
Conclusion, significance and impact of study: The data generated is promising to develop bio-green disinfectants to inhibit biofilm formation by *L. monocytogenes* in the food processing environment and control its adverse effects for consumers.

Keywords: Biofilm, *L. monocytogenes*, carvacrol, chitosan nanoparticles, swabs

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium that has been associated with abattoir environment (Onyilokwu *et al.*, 2016), abattoir effluents and many raw foods related to these environments (Zhu et *al.*, 2017). Also, its existence in healthy food animals has been reported (Osman *et al.*, 2014). Furthermore, the organism was frequently detected in stool samples from hospitalized patients (El-Malek *et al.*, 2010). Meanwhile, it has been established that food-borne transmission constitutes the major acquisition route of listeriosis (Churchill *et al.*, 2006).

Listeria monocytogenes can persist for months or even years in a food processing environment (Ferreira *et al.*, 2014). Also, it can survive under food-related conditions that are stressful for other bacteria, such as low temperature and high salt content (Zoz *et al.*, 2017) and can form biofilms (Doijad *et al.*, 2015). The big problem of the produced biofilms is the dispersion and contamination of the food where the bacterial communities inside the biofilm are surrounded and protected by extracellular polymeric substances (Zhou *et al.*, 2012).

Foodborne *L. monocytogenes* strains were recorded to be less sensitive to cleansing, sanitization and disinfection processes (Gandhi and Chikindas, 2007; Mertins *et al.*, 2007; Dutta *et al.*, 2013; Møretrø *et al.*, 2017). Research trials to find effective alternatives to control bacterial biofilms have been reported the antibacterial activities of essential oils and their components. Carvacrol is a monoterpene phenol (2-methyl-5-(1-methylethyl) phenol) and exists in the volatile oils of *Thymus vulgaris* (Nabavi *et al.*, 2015). It has been

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approved by the U.S. Food and Drug Administration for use in foods and drinks (Marinelli *et al.*, 2018). However, the essential oil was less stable to use directly as antibacterial agents. So, the use of natural biopolymer such as chitosan, especially on a nanometer scale, was found to be a suitable carrier of bioactive materials. Thus, the use of essential oil conjugates with biopolymer could be an advantage to be safe, cost-effective and could be broadly used in food applications (Fan *et al.*, 2012).

Therefore, the aim of the present study is to isolate *L. monocytogenes* from slaughterhouses and determine its ability to produce biofilms as well as evaluate the efficiency of CLCNPs to inhibit the produced biofilm from developing a novel promising antibiofilm agent against *L. monocytogenes*.

MATERIALS AND METHODS

Collection of samples

A total of 180 swab samples were collected from four slaughterhouses for large animals in Assiut Governorate, Egypt. Samples were collected separately from knives (n=30), hooks (n=30) and cutting tables (n=30) were considered as meat contact surfaces (MCS). In addition, 45 samples from walls and 45 samples were collected from floors were categorized as meat non-contact surfaces (MNCS). Sampling was performed with slight modification as reported by Bodur and Cagri-Mehmetoglu (2012). Briefly, sterile swabs were moistened with sterile peptone water then rubbed over the surface of the sample. Next, the swabs were cut aseptically, kept in 10 mL tubes of sterile peptone water and transported under the cooling condition to the laboratory.

Isolation of *L. monocytogenes*

Each 10 mL peptone water containing swab sample was mixed by vortex, followed by the addition of 90 mL sterile *Listeria* enrichment broth (Oxoid, CM0862) with supplements (10 mg/L acriflavine, 40 mg/L cycloheximide and 50 mg/L sodium nalidixic acid). Then, the mixture was incubated at 30 °C for 48 h. Next, loopful of enriched cultures were streaked onto plates of Oxford agar (Biolife, 401600) and incubated at 37 °C for 48 h. Five *Listeria* like colonies (about 1 mm diameter black colonies surrounded by black haloes) were picked, streaked onto tryptic soy agar with 0.6% yeast extract and differentiated according to Gram stain, hemolysis, fermentation of mannitol, xylose and rhamnose motility and CAMP tests (Bill and Doyle, 1991; FDA, 2001). Confirmation of the isolated *Listeria* was conducted by rapid latex agglutination test using Oxoid *Listeria* test kit (Oxoid, Basingstoke, Hampshire, England).

Assessment of biofilm formation ability of *L. monocytogenes* isolates

Bacterial strains

A total of 10 *L. monocytogenes* isolates identified in the present study were used in this assay. Each isolate was transferred from the stock culture into tryptic soy broth (TSB) and incubated overnight at 37 °C. The enriched isolates were further sub-cultured under the same conditions before investigating their ability of biofilm production**.**

Testing for the ability of biofilm production

The ability of biofilm production in polystyrene microtiter plates was based on the previously described methods (Stepanović *et al.*, 2004; 2007). Approximately 180 µL of TSB was aseptically transferred to the wells of sterile 96-well flat bottomed polystyrene microplate (Thermofisher Scientific, Spain). A quantity of 20 µL of overnight bacterial culture diluted to 0.5 McFarland scale was added into each well. The negative control wells contained broth only. The plates were incubated aerobically for 24 h at 37 °C. Each isolate was tested in triplicate. In each trial, after pouring off the content of the plate, the wells were washed three times using 300 µL of sterile distilled water. The attached cells were fixed using 200 µL of methanol per well and left to act for 15 min, then microplates were emptied and air-dried. The wells were then stained using 200 µL per well of 0.1% crystal violet for 15 min. The microplate was washed under running tap water to rinse excess stain. After the microplates were air-dried, the dye bound to the adherent cells was resolubilised with 200 µL of 96% ethanol per well. The optical density (OD_{570}) of each well was measured at 570 nm using a microplate reader (Epoch, 14041512, USA). The average OD values were calculated for all tested strains and negative controls since all tests were performed in triplicate and repeated three times. The cut-off value (ODc) was calculated as follow:

ODc = Average OD of negative control + (3 standard deviation (SD) of negative control). The final OD value of a tested strain is expressed as the average OD value of the strain reduced by ODc value where (OD = Average OD of a strain – ODc). ODc value was calculated for each microtiter plate separately.

Consequently, the results of strains can be divided into the following categories based upon the previously calculated OD values:

Non-biofilm producer (NBP) if (OD≤ODc), weak biofilm producer (WBP) if (ODc<OD≤2ODc), moderate biofilm producer (MBP) if (2ODc<OD≤4ODc) and strong biofilm producer (SBP) if (>4ODc).

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Preparation of antimicrobials nanoparticles

Synthesis of chitosan nanoparticles

Chitosan nanoparticles (NPs) were prepared according to procedures described by Zimet *et al.* (2018). Briefly, 0.5 g of chitosan (75-85% degree of deacetylation and Mw= 50,000-190,000 Da, Sigma-Aldrich, USA) was dissolved in (1% v/v) acetic acid solution (Sigma-Aldrich, USA). Next, the solution was continuously stirred at 500 rpm for 12 h at room temperature. After that, the pH of chitosan solution was adjusted by the addition of NaOH solution (1 M) dropwise until the formation of chitosan NPs with a turbid solution. The solution was kept under vigorous stirring for 1 h. The NPs was centrifuged at 6000 rpm for 20 min (Beckman, Germany). Later, the NPs were washed using double distilled water and kept at –20 °C overnight. Finally, the chitosan NPs was freeze-dried using lyophilisation (Virtis freeze dryer, Model 6KBTES-55, SP scientific, USA).

Preparation of carvacrol loaded chitosan nanoparticles

Carvacrol loaded chitosan nanoparticles were prepared according to Keawchaoon and Yoksan (2011). Forty mL of chitosan solution (1.2% w/v) was prepared by dissolving chitosan flakes in an aqueous solution of acetic acid (1% v/v) at an ambient temperature overnight. Tween 80 was added to the solution, and the mixture was stirred for 2 h at 60 °C. Carvacrol was gradually dropped into the stirring mixture and agitation was carried out for 20 min. A weight of 0.48 g of carvacrol were used to obtain a weight ratio of chitosan to carvacrol of 1:1 (w:w). Subsequently, 40 mL of sodium tripolyphosphate (Sigma-Aldrich) solution (0.5% w/v) was slowly dropped into an oil/water (o/w) emulsion while stirring, with continuous agitation for 30 min. The final pH of the resulted solution was 5.0. The particles were collected by centrifugation at 6,000 rpm for 20 min at 25 °C and washed with an aqueous solution of Tween 80 (1% v/v) four times. The collected wet particles were dispersed in 25 mL of distilled water. CLCNPs was freeze-dried by lyophilisation.

Characterization of chitosan NPs and CLCNPs

Characterization of nanoparticles was done following the procedures of Keawchaoon and Yoksan (2011) using:

Fourier transform infrared (FTIR) spectroscopy

The functional groups of the nanoparticles were identified using a Thermo Nicolet Nexus 470 ESP FTIR spectrometer (Thermo Nicolet, Madison, WI, USA). Thirty-two scans at a resolution of 4 cm^{-1} were evaluated and referenced against air. The infrared spectra between 400 and 4000 cm⁻1 were obtained with a tablet containing KBr. Typically, Briefly, 5 mg of each chitosan NPs and CLCNPs were thoroughly mixed with dry potassium bromide.

High resolution transmission electron microscopy

High resolution transmission electron microscopy (HRTEM) coupled with Energy-dispersive spectroscopy (EDS) (JEOL JEM 2100F) used to characterize the morphology and sizes of the NPs. Briefly, 5 mg of each chitosan NPs and CLCNPs were dispersed in ethanol using ultrasonication for 10 min, then 10 µL of each NPs solution was deposited on copper grid.

Zeta potential and particle size analysis

Zeta potential and particle diameter were measured at 20 °C using a Zetasizer (Malvern model 3600, UK). Samples were prepared by dispersing a fixed 5 mg of the nanomaterial in a 5 mL of deionised water by ultrasonication treatments.

Determination of anti-listerial activity of carvacrol, chitosan NPs and CLCNPs

The minimum inhibitory concentrations against the bacterial isolate were determined according to the procedures described by Barry (2007). Ninety-six-well plates were prepared by dispensing 50 µL of different concentrations of CAR, chitosan NPs and CLCNPs dissolved in Mueller-Hinton broth (MHB). Then, 50 µL of the calculated bacterial suspension (6 log CFU/mL) from the strong biofilm producer isolate were added to each well, providing final concentration of 120 to 0.47 mg/mL (w/v) for carvacrol and 20 to 0.16 mg/mL (w/v) for chitosan NPs and CLCNPs. A positive control containing MHB and bacterial culture without compounds, and a negative control containing no bacteria were included in each experiment. The only microplate of carvacrol was loosely wrapped with parafilm because it is a volatile essential oil while, the carvacrol loaded into chitosan NPs was stabilized in the chitosan NPs. Plates including controls were incubated at 37 °C for 24 h. Then, 30 µL aliquot of an aqueous solution of resazurin 0.015% was added to each well. After 2 h of incubation at 37 °C, colour changes were assessed. Colour change in well from purple to pink (or colourless) indicates bacterial growth. The MIC value is the lowest concentration that inhibits the growth of the tested strains (no change in colour).

Testing the anti-biofilm activity of carvacrol loaded chitosan nanoparticles (CLCNPs)

Effect on inhibition of biofilm formation

The ability of nanomaterials to inhibit the formation of biofilm was carried out by the crystal violet spectrophotometric method (Plyuta *et al.*, 2013). Approximately 180 µL of different concentrations of CLCNPs (2 MIC, MIC and 0.5 MIC) prepared separately in TSB were distributed in 96-well microtiter plates. Wells were inoculated with 20 µL of the overnight-grown culture of the tested bacterial strains and incubated at 37 °C for

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Table 1: Incidence of *Listeria* spp. on meat contact surfaces and meat non-contact surfaces in slaughterhouses.

Samples	No. of samples	Number of positive isolates (percentage)					
		Listeria spp. No. (%)	*L. monocytogenes* No. (%)	*L. innocua* No. (%)	*L. ivanovii* No. (%)	*L. welshimeri* No. (%)	*L. grayi* No. (%)
Meat contact surfaces (MCS)							
Knives	30	5 (16.7)	1 (3.3)	1 (3.3)	1 (3.3)	0 (0)	2 (6.6)
Hooks	30	7 (23.3)	3 (10)	2 (6.7)	0 (0)	1 (3.3)	1 (3.3)
Cutting boards	30	4 (13.3)	2 (6.7)	1 (3.3)	1 (3.3)	0 (0)	0 (0)
Total	90	16 (17.8)	6 (6.7)	4 (4.4)	2 (2.2)	0 (0)	3 (3.3)
Meat non-contact surfaces (MNCS)							
Walls	45	5 (11.1)	1 (2.2)	2 (4.4)	1(2.2)	0 (0)	1 (2.2)
Floors	45	8 (17.8)	3 (6.7)	3 (6.7)	0 (0)	1(2.2)	1 (2.2)
Total	90	13 (14.4)	4 (4.4)	5 (5.6)	1(1.1)	1(1.1)	2 (2.2)

24 h. The suspensions were then removed, and the wells were washed with 200 µL of phosphate buffer saline (PBS) to remove free-floating bacteria. Biofilms formed by adherent cells in the plate were fixed by 200 µL methanol for 20 min, then stained with 200 µL of 0.1% crystal violet and incubated at room temperature for 15 min. Excess stain was rinsed off by thorough washing with PBS. After air-drying of wells, dye of biofilms lining the walls was resolubilised by 200 µL of ethanol (96%), then incubated for 15 min. The resulting reaction was evaluated spectrophotometrically at 570 nm. Triplicate samples were included for each treatment and the experiment was repeated three times. In each case, a set of wells inoculated with bacteria without any treatment were used as the controls.

The percentage of biofilm inhibition = $[(OD_{control} - OD_{treatment})/OD_{control}] \times 100$

Effect on pre-formed biofilms

The effect of different concentrations (2 MIC and 4 MIC) of CLCNPs on the reduction of pre-formed biofilms was tested on polystyrene flat-bottomed microtiter plates as described by Soni *et al.* (2013). A volume of 180 µL of TSB was pipetted to each well and further inoculated with 20 µL of overnight-grown bacterial cultures. Plates were incubated at 37 °C for 24 h in a static condition. After incubation, the contents were poured off and each well was washed three times. Subsequently, the attached cells were treated separately by 200 µL per well of TSB containing different concentrations of CLCNPs (2 MIC and 4 MIC). A set of wells containing TSB only without any treatment was also used as a control. Plates were incubated at 37 °C for 24 h. The biofilm was evaluated according to the procedures described above using the crystal violet staining method.

Statistical analysis

The one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (GraphPad Prism, version 8.4.2, LLC) was used in the statistical analyses. The differences between groups were considered significant when (P<0.05). The results for MIC determination assay are expressed as modal values because the values were the same in all repetitions.

RESULTS AND DISCUSSION

The prevalence of *Listeria* on surfaces

The role of abattoirs in disseminating *Listeria* still constituting a problem elsewhere. Under uncontrolled hygiene, abattoirs are considered feasible transmission sources of Listeria to humans (EFSA, 2007). Also, Mahmoud *et al.* (2019) and Gebremedhin *et al.* (2020) proved a link contribution of contamination with *Listeria* in three spots of beef chain (abattoir, butcher and restaurant). In the present study, both meat contact surfaces (MCS) and meat non-contact surfaces (MNCS) showed *Listeria*. As illustrated in Table 1, swab samples from MCS showed a high level (17.8%) of *Listeria* species compared to MNCS (14.4%). *Listeria monocytogenes* predominated within MCS (6.7%) followed by *L. innocua* (4.4%) while in MNCS *L. innocua* was predominant (5.6%) compared to *L. monocytogenes* (4.4%). The level of *L. monocytogenes* in the present study nearly coordinates with those of Babiker *et al.* (2020), who attributed that the level of contamination (5.5%) was due to an unclean working environment and improper handling of meat in abattoirs. Meanwhile, Gebremedhin *et al.* (2020) explored that the widespread of *L. monocytogenes* (4.4%) was due to inadequate hygiene practice and absence of law for hazard analysis critical control point implementation in an abattoir.

The current study shared the observation with Onyilokwu *et al.* (2016) that butcher tables are significant sources of *Listeria*. Also, the general poor hygiene practices by the butchers and workers was reflected in the high prevalence of *Listeria* in hocks, cutting tables and floor in the present study.

Biofilm forming ability of *L. monocytogenes* isolates

Listeriosis, caused by *L. monocytogenes*, is a serious food-borne illness with high rates of morbidity and mortality. Ten *L. monocytogenes* isolates were

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categorized based on the optical density (OD_{570}) produced by biofilms. The data presented in Table 2 revealed that out of 10 tested isolates of *L. monocytogenes* for biofilm production, 9 isolates were capable of forming biofilm and classified as SBP (N=1; 10%), MBP (N=5; 50%) and WBP (N=3; 30%). Based on the results, most of the tested *L. monocytogenes* were MBP. This finding was parallel with that of Henriques and Fraqueza (2017), where 47% of *L. monocytogenes* isolates were MBP. Meanwhile, Doijad *et al.* (2015) found that the majority of *L. monocytogenes* isolated were WBP. Also, previous studies by Djordjevic *et al.* (2002) and Borucki *et al.* (2003) indicated that *Listeria* isolates were generally weak to moderate biofilm producers.

The strong biofilm producer (SBP) in the present study represented 10% of *L. monocytogenes* isolates. It is in accordance with the findings of Barbosa *et al.* (2013). In addition, the food-borne strains of *L. monocytogenes* were found to be SBP even in limited nutrient conditions (Sharar *et al.*, 2018). Besides, studies investigating *L. monocytogenes* biofilm transfer showed that the SBP strains could transfer a significantly higher number of bacteria to beef products (Midelet and Carpentier, 2002).

The exact mechanism of biofilm formation by *L. monocytogenes* has not been fully established yet. Studies showed that microbial adherence is largely dependent upon the surface charge and hydrophobicity (Guo *et al*., 2013). Other studies revealed that bacterial biofilm is influenced by temperature (Barbosa *et al.*, 2013), availability of nutrients (Kadam *et al.*, 2013) and biofilm maturation (de Oliveira *et al.*, 2010).

The present study also cleared that *L. monocytogenes* isolates had the ability to form biofilm on polystyrene microtiter plates. Polystyrene represents a widely used matrixes, in commercial food containers. This coordinates with the findings of Grudlewska-Buda *et al.* (2020) where environmental strains were strong biofilm producers on the polypropylene surfaces. In this respect, Giaouris and Simões (2018) declared that *L. monocytogenes* had the capability to adhere to and create biofilms on various surfaces in food processing industries. Related studies have reported that hydrophilic surfaces such as steel are hydrophobic ones e.g. polystyrene (Bonsaglia *et al.*, 2014).

Pathogens capable of forming biofilms constitutes microbiological hazards. In biofilms, *L. monocytogenes* has been found to acquire resistance to adverse environmental conditions and killing substances (Fagerlund *et al.*, 2017). That resulted in enhanced persistence in the food processing environment (Gandhi and Chikindas, 2007) and processed foods (Carpentier and Cerf, 2011).

Characterization of NPs

Morphology of CLCNPs by high resolution transmission electron microscope (TEM)

The morphology of the synthesized nanoparticles such as chitosan and carvacrol loaded chitosan NPs were observed using TEM. The individual chitosan NPs exhibited spherical shape with diameters ranging from 52-88 nm as shown in Figure 1A and CLCNPs was demonstrated by covering the chitosan NPs with carvacrol as illustrated in Figure 1B. CLCNPs were prepared in two steps; droplet formation and solidification. The droplet of carvacrol in chitosan solution was formed by the oil-in- water emulsion technique. Then, the droplet was solidified by ion cross-linked of the amino group corresponding to chitosan and polyphosphate group from tripolyphosphate. The obtained results are in agreement with Keawchaoon and Yoksan (2011), who reported that the individual CLCNPs exhibited a spherical shape with a diameter size of 40-80 nm.

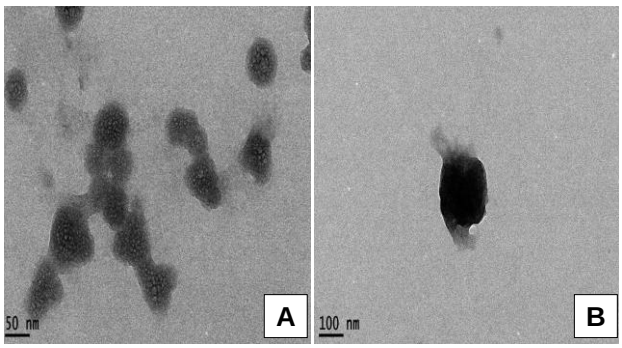

Figure 1: TEM images of synthesized (A) chitosan NPs and (B) carvacrol loaded chitosan NPs.

Table 2: Classification of *L. monocytogenes* slaughterhouses isolates based on biofilm-forming ability.

Biofilm forming abilities	Total (N=10)	
	No.	%
Non-biofilm producers (NBP)	1	10
Weak biofilm producers (WBP)	3	30
Moderate biofilm producers (MBP)	5	50
Strong biofilm producers (SBP)	1	10

Size and surface charge of CLCNPs

Zeta potential (ZP) is usually used as a key parameter for evaluating the stability of dispersions, emulsions and suspensions (Dickinson, 2009). The average particle size of CLCNPs was about 60 nm, as shown in Figure 2. In addition, the ZP of chitosan NPs was found to be +37.44 ± 0.94 mV as showed in Table 3, implying a positively charged surface of the particles. Moreover, the ZP value for CLCNPs being to be +31.50 ± 0.30 mV. This showed that the loading of carvacrol reduced the positive surface charge. These findings agreed with Keawchaoon and Yoksan (2011) who revealed that a positively charged surface of CLCNPs with a ZP value of 25-29 mV. The decrease of the positive charge of ZP in CLCNPs could be attributed to the coverage of carvacrol onto the surface of chitosan NPs, which means the decrease of the free amino groups of chitosan.

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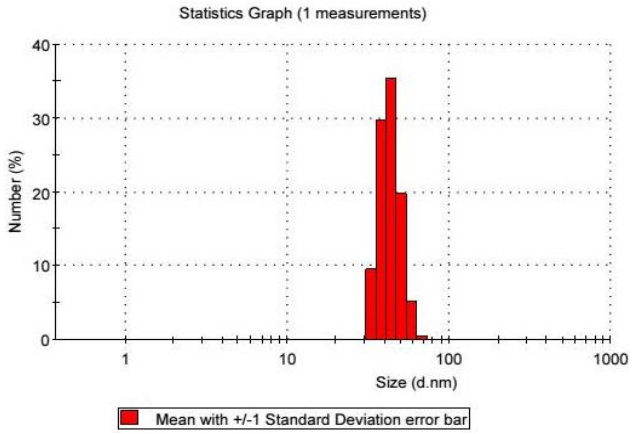

Figure 2: Particle size distribution of carvacrol loaded chitosan NPs using Zetasizer.

Table 3: Zeta potential of chitosan NPs and carvacrol loaded chitosan NPs.

Nanoparticles	Zeta potential (mV)
Chitosan NPs	+37.44 ± 0.94
Carvacrol loaded chitosan NPs	+31.5 ± 0.30

FTIR analysis of CLCNPs

The chemical structure of carvacrol, chitosan NPs and CLCNPs were characterized by FTIR technique. As demonstrated in Figure 3, carvacrol showed characteristic peaks at 3423, 2960, (1459, 1383 and 1362) and (866 and 812) $cm^{-1,}$ which correspond to OH, CH stretching, CH deformation and aromatic ring, respectively. In addition, the peaks appeared of chitosan NPs at 3441 (OH), 2922 (CH stretching), 1639 (amide I), 1560 (amide II), 1149 (P=O), 1076 (C-O-C) and 879 cm^{-1} (pyranose ring) indicating electrostatic associations between polyphosphate group of tripolyphosphate (TPP) and amine group of chitosan which agrees with Hosseini *et al.* (2013). It can be seen from the FTIR spectra that the addition of carvacrol to chitosan NPs resulted in a significant increase in the intensity of CH stretching peak at 2871-2958 cm^{-1}, reflecting the existence of carvacrol in the chitosan matrix. The CH stretching peak was thus used as a probe band for an indirect determination of loaded carvacrol content.

Anti-listerial activity of carvacrol, chitosan NPs and CLCNPs

Carvacrol is a major component of essential oils from oregano and thyme responsible for their antimicrobial activity, according to Božik *et al.* (2018). As a phenolic, it fights bacteria through destabilization of the cell membrane (Nostro *et al.*, 2007; Ait-Ouazzou *et al.*, 2012). Carvacrol is approved as a safe food additive in the USA and Europe, Center for Food Safety and Applied Nutrition (2006). The results in Table 4 showed that carvacrol

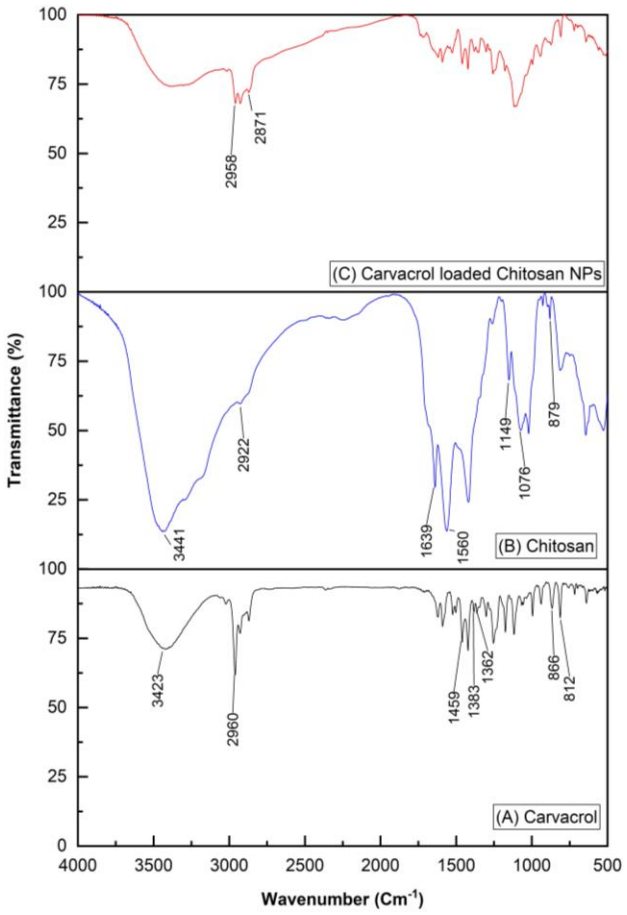

Figure 3: FTIR spectra for (A) carvacrol, (B) chitosan NPs and (C) carvacrol loaded chitosan NPs.

could inhibit *L. monocytogenes* at a concentration of 3.75 mg/mL. According to Božik *et al.* (2018), Cosentino *et al.* (2002) and Li *et al.* (2014), the MIC of carvacrol were found to be 500, 10.45 and 0.25 mg/mL, respectively. While, listericidal concentrations of 750 mg/mL and 0.5 mg/mL were reported by Gill and Holley (2006) and Li *et al.* (2014), respectively. Strain susceptibility, as well as methodological differences, may account for the variation in results.

Chitosan NPs were used in the current study to serve as a carrier to carvacrol. It has advantageous characteristics as cationic nature, biodegradability and good adsorption capacity (Fan *et al.*, 2012). The 5 mg/mL could inhibit *Listeria*, as depicted in Table 4. The antimicrobial function of chitosan is known against a wide variety of Gram-positive, Gram-negative bacteria, mould and yeast. The broad activity was attributed to its polycationic property (Goy *et al.*, 2009).

Loading carvacrol on chitosan NPs potentiated its anti-listerial activity. A concentration of 0.62 mg/mL of CLCNPs was enough to inhibit *L. monocytogenes*. This parallel with the findings of Guarda *et al.* (2011), where microencapsulation of carvacrol potentiate its effect

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Table 4: Minimum inhibitory concentrations (MICs) of carvacrol, chitosan NPs and carvacrol loaded chitosan NPs against *L. monocytogenes*.

Bacterial strains	MICs		
	Carvacrol (mg/mL)	Chitosan NPs (mg/mL)	Carvacrol loaded chitosan NPs (mg/mL)
L. monocytogenes	3.75	5	0.62

Table 5: The effect of carvacrol loaded chitosan NPs on the inhibition of biofilm formation by *L. monocytogenes*.

Strain	% of biofilm inhibition		
	0.5 MIC	MIC	2 MIC
L. monocytogenes	35.79 ± 9.32^{a}	73.37 ± 3.62^{b}	77.76 ± 4.06^{b}

Means ± SD with different superscript letters in the same row for each concentration are significantly different (P<0.05).

Table 6: Effect of carvacrol loaded chitosan NPs on the pre-formed biofilm by *L. monocytogenes*.

Strain	OD_{570} Mean ± SD		
	2 MIC	4 MIC	Control
L. monocytogenes	0.40 ± 0.05^{a}	0.29 ± 0.03^{b}	1.01 ± 0.12^{c}

Means ± SD with different superscript letters are significantly different (P<0.05).

against *L. monocytogenes*. Also, Soto-Chilaca *et al.* (2016) explained that polymeric nanoparticles binding the core with phenolic compounds function for the protection of bioactive compounds. In addition, the small size in combination with the chemical composition and surface structure gives nanoparticles their unique features and huge potential for applications (Ravichandran *et al.*, 2011; de Faria *et al.*, 2014).

Anti-biofilm activity of CLCNPs

Interference with biofilm formation

The ability of CLCNPs to interfere with the formation of biofilm by *L. monocytogenes*, as strong biofilm producer, was illustrated in Table 5. The degree of interference was proportional to CLCNPs where inhibitions of 35.79, 73.37 and 77.76% corresponded to 0.5 MIC, MIC and 2 MIC doses, respectively. In a related study, Knowles and Roller (2001) cleared that both chitosan and carvacrol in the bulk size have biocidal properties against biofilms of *L. monocytogenes*. The mechanism by which carvacrol inhibits biofilm formation is not yet fully cleared. Carvacrol could inhibit the biofilm formation due to its amphipathic properties (Nostro *et al.*, 2009), interfere with the quorum sensing system (Burt *et al.*, 2014) or it may decrease the bacterial motility and pathogenicity (Inamuco *et al.*, 2012; van Alphen *et al.*, 2012).

Effect on pre-formed biofilm

From Table 6, biofilm biomass was significantly (p<0.05) reduced to 0.4 and 0.29 OD_{570} after treatment with CLCNPs at 2 MIC, 4 MIC doses, respectively as compared with control (1.01 OD_{570}). In a related study by Desai *et al.* (2012), they found that 0.025% carvacrol significantly reduced pre-formed biofilms of *L. monocytogenes*. The effect of carvacrol against pre-formed bacterial biofilm of *Pseudomonas* and *Staphylococcus aureus* was also recorded (Nostro *et al.*, 2007; El abed *et al.*, 2011), respectively. Carvacrol has been reported to possess relative hydrophilicity, having a water solubility of 830 ± 10 ppm (Griffin *et al.*, 1999); thus, in a liquid state was found effective against pre-formed bacterial biofilms (Nostro *et al.*, 2007). Besides, carvacrol is a volatile molecule that evaporates easily, and its vapour phase has shown antimicrobial activity (Jesus *et al.*, 2015). In addition, the advantage of the volatility of carvacrol permits its diffusion into inaccessible areas (Chami *et al.*, 2005; Kristinsson *et al.*, 2005). These properties made carvacrol promising anti *L. monocytogenes* phenolic (Pérez-Conesa *et al.*, 2006; Shemesh *et al.*, 2015).

CONCLUSION

From the achieved results, it can be concluded that carvacrol and nano chitosan appeared as efficient anti-listerial biofilm agents. These properties, together with the small size, are successfully collected in active biopolymer CLCNPs with promising antibiofilm activity against *L. monocytogenes*. The obtained biopolymer is important in the food industry to overcome the problem of chronic contamination. These data have the potential for the development of novel disinfectant agents for food-processing surfaces.

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