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Enhanced antimicrobial, antibiofilm and ecotoxic activities of nanoencapsulated carvacrol and thymol as compared to their free counterparts

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ABSTRACT

Essential oils active components emerged as captivating antimicrobials to control biofilms developed on food contact surfaces. Free and nanoencapsulated carvacrol (CAR) and thymol (THY) were assessed as antimicrobials against *Salmonella* Enteritidis biofilms adhered to stainless steel. The developed spherical nanocapsules using the spray-drying technique showed an average size ranged between 159.25 and 234.76 nm and zeta potential values ranged between 23.60 and 24.66 mV. The minimal inhibitory concentrations (MIC) of free THY and CAR were both 1.25 mg L^{-1}. Nanoencapsulation reduced MIC values to 0.62 mg L^{-1} (THY) and 0.31 mg L^{-1} (CAR). Furthermore, the exposure to free and nanoencapsulated CAR and THY induced a destabilization of bacterial membranes with obvisous morphological deformations and a pronounced leakage of potassium ions and green fluorescent proteins. Eradication of *S.* Enteritidis biofilms developed on stainless steel was achieved following a 15 min treatment with nanoencapsulated CAR and THY at 2 MIC. Free antimicrobial exposures induced up to 4.27 log CFU cm^{-2} reductions. Additionally, the ecotoxicity tests against *Daphnia magna* crustaceans reported a non-toxicity of both free and nanoencapsulated CAR and THY after 48 h exposure. Thereby, both CAR and THY antimicrobials proved to be promising natural surface disinfectants that require further exploration and incorporation in food industries.

1. Introduction

Microbiological contaminations are one of the major concerns worldwide as they are involved in severe illnesses and outbreaks, along with costly economical losses in food industries. Lately in 2020, the European Union reported, 3086 foodborne outbreaks. *Salmonella* remained among the main causative pathogens and was responsible for up to 22.5% of the overall reported outbreaks with the majority (57.9%) being associated with *Salmonella* Enteritidis contaminations (European Food Safety Authority & European Centre for Disease Prevention and Control, 2021). Bacterial adhesion to food contact surfaces with subsequent formation of biofilms are a primary source of cross-contamination and recontamination in food industries. More than 80% of foodborne illnesses and outbreaks are related to biofilm formation with an estimated global annual impact of $324 billion in the agro-food sector (Byun et al., 2022; Cámara et al., 2022). Biofilms have shown a high resistance to different antimicrobials and are more resistant than their planktonic counterparts, increasing thus the microbiological risk in food environment. Cleaning followed by effective disinfection procedures should be commonly practiced in food industries to remove adhered bacterial cells and avoid biofilms formation. Disinfection should receive particular attention as cleaning is not designed to eradicate sufficient amounts of bacterial cells (Engel et al., 2017). Till present, the synthetic chemical disinfectants that are still being used in food processing environments, have shown a diminished efficacy due to the increased microbial resistance developed towards them with a reduced susceptibility

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to penetrate into the deep layers of biofilms, while providing toxic bi-products (Cai et al., 2022; Heckler et al., 2020). This urges the need to switch from the use of chemical disinfectants and find alternative strategies to control biofilms.

Consumers and producers' perceptions have been oriented lately towards the use of biosourced antimicrobial agents such as essential oils (EOs) and their active components that have glowed as potent antimicrobials. Carvacrol (CAR) and thymol (THY), two major active terpenes of several EOs, namely thyme and oregano, are Generally Recognized as Safe (GRAS) by the Food and Drug Administration and have emerged as potent antimicrobials with a broad spectrum of activity (Food and Drug Administration (FDA), 2016; Khan et al., 2020; Somrani et al., 2021). This offers EOs the advantage of substituting chemical disinfectants that are not safe and may produce toxic unhealthy or carcinogenic by-products (Cho et al., 2020; Falcó et al., 2019; Orhan-Yanıkan et al., 2019). Nevertheless, EOs compounds present several limitations such as their high volatility, low water solubility and stability (Bernal-Mercado et al., 2022). Therefore, encapsulation could be a prominent strategy to encounter these different potential limitations and boost the antimicrobial activity of the encapsulated agents. Capsules which are vesicular systems, can encapsulate active components within a carrier wall material shell that provides protection for the encapsulated agents and enhances their penetration into the biofilms matrix favoring their interaction with bacterial membranes and promoting thus their actions as surface disinfectants.

Although the use of free EOs as antimicrobials has been widely explored, fewer studies have shown the effect of encapsulation in the control of biofilms (Ashrafudoulla et al., 2021; Heckler et al., 2020; Mechmechani et al., 2022). Considering that no studies have yet reported the antibiofilm activity of free and nanoencapsulated CAR and THY against *S.* Enteritidis adhered to stainless steel (SS), this study aimed to nanoencapsulate CAR and THY, and to compare their antibiofilm activity with their free native forms against *S.* Enteritidis biofilms. The antimicrobial effect of both free and nanoencapsulated EOs components has been also investigated in addition to their ecotoxicological risk assessment toward aquatic organisms.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. Enteritidis (Collection Institut Pasteur CIP 8297 - ATCC 13076 - NCTC 12694) strains were maintained at - 80 °C in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) supplemented with 20% (v/v) glycerol. One hundred μL of the stock strain suspension were precultured in TSB for 24 h at 37 °C and then cultured by inoculating 100 μL in 50 mL of TSB and incubated under continuous shaking at 160 rpm, for 16 h at 37 °C. Overnight cultured cells were harvested by centrifugation (5000 rpm, 5 min, 20 °C) and washed twice with 20 mL of Potassium Phosphate Buffer (PPB; 100 mM, pH 7) before being resuspended in PPB.

2.2. Antimicrobial agents and chemicals

Carvacrol (98% purity) and thymol (≥99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (United Kingdom) and used at a final concentration <2% (v/v) to dissolve free CAR and THY. For the preparation of the nanocapsules, sodium caseinate and maltodextrins DE 21 were obtained from Fisher Scientific (United Kingdom) and Roquette-Frères SA (Lestrem, France), respectively. Maltodextrin was chosen as carrier material as it is a safe plant-based polysaccharide, biodegradable, non-toxic, widely abundant in nature with relatively good water solubility and low viscosity at high temperatures. While, sodium caseinate was selected as emulsifier as it is an abundant animal-based protein of low cost with relatively good emulsifying properties and good resistance to high temperatures (Aguiar et al., 2016; Fathi et al., 2019; Gharsallaoui et al., 2007).

2.3. Nanocapsules formation using spray-drying technique

2.3.1. Preparation of feed emulsions

The primary emulsions were first prepared by dissolving sodium caseinate in distilled water until complete hydration at room temperature. The pH of the solution was adjusted to 3 by adding HCl (0.1 or 1.0 M) or NaOH (0.1 or 1.0 M). Then, weighted amounts of CAR and THY were added to the sodium caseinate stock solution. Subsequently, emulsions were homogenized using an Ultra Turrax PT 4000 homegenizer (Polytron, Kinematica, Switzerland) for 5 min at 20 000 rpm. The obtained primary emulsions were further subject to high pressure microfluidization using an LM20 Microfluidizer (Microfluidics Co., MA, USA), at 500 bar and five recirculations. To the freshly prepared emulsions, stock maltodextrin DE 21 solutions (50% w/v) were added to obtain a final composition (w/w) of: 20.0% maltodextrins DE 21, 0.5% sodium caseinate and 1.0% CAR or 1.0% THY. The pH was readjusted to 3 before being used as feeds to the spray drying process.

2.3.2. Spray-dyring process

Feed emulsions were stirred for 30 min and subsequently injected into a laboratory spray-dryer equipped with a 0.5 mm nozzle atomizer (Mini Spray-Dryer Büchi B-290, Switzerland). The operational conditions used for the spray-drying process were as follow: air pressure 3.2 bar, inlet air temperature 180 ± 2 °C, outlet air temperature 80 ± 5 °C and feed flow rate 0.5 L. h^{-1}. The formed nanocapsule powders were collected in sealed containers and stored at 4 °C until analysis. Then, to prepare reconstituted emulsions, the same weighted amounts of spray-dried powders (same amounts used prior to spray-drying) were dispersed in distilled water and stirred for 1 h.

2.4. Physicochemical characterization of nanoparticles

2.4.1. Determination of particles size and surface charge

The electrical charge known as zeta potential (ζ-Potential) and the size of the emulsions were determined using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The prepared particles were suspended in imidazole-acetate buffer (pH 3) and slightly agitated before analysis. All measurements were conducted in triplicates at 25 °C and the mean ζ-Potential values were expressed in millivolts (mV), while sizes were expressed in nanometers (nm).

2.4.2. Morphology observations

The external and internal morphologies of the spray-dried CAR and THY nanocapsules were carried out on the scanning electron microscopy (SEM) facility of the Advanced Characterization Platform of the Chevreul Institute (SEM; model JSM-7800FLV, JEOL, Japan) with a scanning voltage of 3 kV. Briefly, to prepare the samples for external observation, a thin layer of the dried nanocapsule powders was sprinkled and fixed on specimen stubs with double-sided adhesive tape (Agar scientific, Oxford). For the internal morphology observation, dried nanocapsules were smashed using a razor blade and mounted on a stub. Prior to observations, samples were coated with a thin carbon layer using a precision etching coating system (PECS; model Gatan 682) to achieve electrical conductivity.

2.5. Determination of the minimal inhibitory concentrations of free and nanoencapsulated carvacrol and thymol against S. Enteritidis

The minimal inhibitory concentrations (MIC) of free and nanoencapsulated CAR and THY were determined using the 96-wells microtiter plate assay. Initially, 100 μL of Müller-Hinton broth (MHB; Biokar Diagnostics, Pantin, France) were added to each well and then mixed with 100 μL of serial two-fold dilutions of free and nanoencapsulated antimicrobial agents to yield final concentrations ranging

from 10 to 0.156 mg mL^{-1}. One hundred μL of the bacterial suspensions (10^6 CFU mL^{-1}) were then added to the wells. Bacterial suspensions were not added to the negative controls, while in positive controls, DMSO (<2% v/v) without antimicrobials was added only to treatments with free antimicrobial agents. The microdilution plates were subsequently incubated in a Bioscreen C (Labsystems, Helsinki, Finland) at 37 °C with a continuous agitation. The optical density at 600 nm (OD_{600}) was measured every 2 h for 24 h. The MIC values were defined as the lowest concentration of each antimicrobial agent that inhibited the visible growth of bacterial cells. All tests were performed in triplicate.

2.6. Antimicrobial mechanism of action of free and nanoencapsulated carvacrol and thymol

2.6.1. Potassium ions efflux

The effect of free and nanoencapsulated CAR and THY on the leakage of potassium (K^+) ions from *S.* Enteritidis planktonic cells was assessed as follows. Briefly, tenfold dilutions of concentrated *S.* Enteritidis cells (10^{10} CFU mL^{-1}) were prepared in morpholinopropane sulfonic buffer (MOPS) (50 mM, pH 7.2; Fisher scientific, Belgium) containing free and encapsulated CAR and THY at MIC values. At 0, 5, and 10 min prior to the exposure to the antimicrobial solutions, K^+ concentration of the bacterial suspension filtrate (0.2 μm, Sartorius™ Minisart™ NML Syringe Filters, France) was measured. Then, after exposure of bacterial cells to MIC of the antimicrobial solutions, 4 mL were filter sterilized every 30 s for the first 5 min and then every 5 min until reaching 60 min. For the control, MOPS buffer was used. The concentration of leaked K^+ ions in the filtrate samples were measured by a Varian SpectrAA 55/B atomic absorption spectrometer in flame emission mode (slit 0.7 nm high; wavelength 766.5 nm; air-acetylene flame).

2.6.2. Extracellular green fluorescent proteins (GFP) monitoring

To monitor the extracellular intensity of GFP leaked out of the bacterial cells, an overnight culture of *Escherichia coli* GFP (ATCC 25922GFP) with 100 mg mL^{-1} of Ampicillin was prepared in TSB and incubated at 37 °C. Cells were then harvested by centrifugation (5000 *g*, 5 min, 20 °C), washed twice and resuspended in HEPES buffer (5 mM, pH 7.2) to obtain a final concentrated inoculum of 10^{10} CFU mL^{-1}. Prior to exposure to the antimicrobial solutions prepared in HEPES buffer at MICs, tenfold dilution of the concentrated inoculum was filter sterilized (Sartorius ™ Minisart™ NML 0.2 μm Syringe Filters, France) at 0, 5, and 10 min. Then, 100 μL of the bacterial inoculum were added to the antimicrobial solutions and samples were filter sterilized. HEPES buffer was used as negative control. Two hundred μL aliquots of the filtered samples were transferred into a 96-wells microplate and the GFP fluorescence was measured every 5 min until reaching 60 min, using a BioTek fluorescence spectrophotometer (BioTek Instruments SAS, France) with an emission at 510 nm and an excitation at 485 nm. Experiments were repeated in triplicate and the results were plotted as the fluorescence intensity ratio of the treated samples to HEPES buffer *versus* the contact time.

2.7. Formation of biofilms and treatment with free and nanoencapsulated carvacrol and thymol

2.7.1. Preparation of stainless steel coupons

Circular stainless steel (SS) (INOX 304L, Equinox, France) (Φ 41 mm × 1 mm thickness) were washed with 95% ethanol (Fluka, Sigma-Aldrich, France), properly rinsed with distilled water, and then immersed in 1% DDM ECO detergent (ANIOS, France) for 15 min. Subsequently, coupons were rinsed vigorously with distilled water, then air dried at 60 °C before being autoclaved at 121 °C for 20 min. The coupons were kept at room temperature in sterile vessels until testing.

2.7.2. Biofilm formation assay: NEC biofilm reactor

Biofilms were developed in the NEC biofilm reactor previsouly described in details (Abdallah et al., 2015). After autoclaving the different parts of the system, 3 mL of the bacterial suspension (10^7 CFU mL^{-1}) were added to the previously sterilized SS coupons deposited in each reactor and incubated for 60 min at 20 °C to allow bacterial adhesion onto surfaces. After incubation, coupons were gently washed twice with PPB to remove non-adherent cells. Subsequently, 5 mL of TSB were added to the coupons and the closed system was incubated at 37 °C for 24 h. Following the incubation, the culture medium was removed and biofilms were washed twice with PPB to remove planktonic bacterial cells. Coupons with attached biofilms were used for the quantification of biofilms biomass as well as for the antibiofilm treatment.

2.7.3. Antibiofilm activity of free and encapsulated carvacrol and thymol

In order to assess the activity of CAR and THY against 24 h *S.* Enteritidis biofilms, coupons with adhered biofilms were immersed in 3 mL of the antimicrobial solutions prepared in Tryptone Salt Broth (TS; Biokar Diagnostics, France) according to the MIC and 2 MIC values. The contact time between the antimicrobial solutions and biofilms was for 15 min. Following the treatment time, coupons were withdrawn from the antimicrobial solutions and immersed for 10 min into 5 mL of a neutralizing solution containing a mixture of Saponin (30 g L^{-1}), Tween 80 (30 g L^{-1}), L-Histidine (1 g L^{-1}), Sodium Thiosulphate (5 g L^{-1}), TS broth (9.5 g L^{-1}), and Lecithin (30 g L^{-1}). This neutralizing solution was used to stop the antimicrobial activity of the antimicrobial solutions. Then, for the quantification of biofilms biomass, each coupon was transferred to a sterile pot containing 20 mL of TS broth. To detach bacterial cells adhered to the coupon surface, the pots were vortexed for 30 s, sonicated for 5 min at 37 kHz (Elmasonic S60H, Elma, Germany), and then vortexed again for 30 s. Serial dilutions were perfomed in TS broth and 100 μL of each dilution were plated on Tryptic Soy Agar (TSA; Biokar Diagnostics, France) plates. The plates were incubated for 24 h at 37 °C. The number of culturable cells was counted and the results were expressed as log CFU cm^{-2}. Control assays were performed by immersing the coupons in TS broth instead of the antimicrobial solutions. All experiments were performed in triplicate and in each case, two slides were used.

2.8. Microscopic observations of biofilms before and after exposure to free and nanoencapsulated carvacrol and thymol

2.8.1. Epifluorescent microscopy

Fluorescent microscopy was used to observe the extent of membrane damage and viability of *S.* Enteritidis biofilms developed on SS coupons after exposure to the antimicrobial solutions. Live and dead staining experiment was carried out in the dark for 15 min according to the LIVE/DEAD® BacLight kit (Invitrogen Molecular Probes, USA) instructions. After exposure to the different antimicrobial solutions, the coupons were thouroughly rinsed with distilled water and kept to air dry in the dark. Then, observations of live bacterial cells stained in green by SYTO-9 and dead bacterial cells stained in red by propidium iodide (PI) were performed using the epifluorescent microscope (Olympus BX43, Germany).

2.8.2. Scanning electron microscopy

The effects of free and nanoencapsulated CAR and THY on *S.* Enteritidis biofilms morphologies were visualized through the SEM. In brief, after the formation and treatment of biofilms as described above, bacterial cells were recovered by scraping the surface, followed by aspirating and expelling with ultrapure water for at least 10 times. Recovered bacterial suspensions were vortexed (30 s) and sonicated (37 kHz, 5 min). The suspensions were tenfold diluted in TS broth and 1 mL was filter sterilized using a polycarbonate membrane filter of 0.2 μm pore size (Schleicher & Schuell, Dassel, Germany). The recovered filters were immersed into 2% glutaraldehyde in cacodylate buffer (sodium cacodylate trihydrate $(CH3)_2AsO_2Na.3H_2O$; 0.1 M, pH 7) at 4 °C for 4 h for fixation. The fixed samples were then carrefully dehydrated in a graded ethanol series (50, 70, 95, and twice with 100% v/v), and dried

overnight in a desiccator. Subsequently, the samples were critical point-dried in CO_2 and carbon coated by sputtering before observation on the SEM. For the control, cells were exposed to PPB instead of the antimicrobial solutions.

2.9. Assessment of the acute toxicity of free and nanoencapsulated carvacrol and thymol to Daphnia magna crustaceans

The acute toxicities of free and nanoencapsulated CAR and THY were tested using the Daphtoxkit F™ *magna* (MicroBioTests Inc., Belgium) according to the International Standardization Organization (ISO) standard procedure 6341 and OECD Guideline 202 based on the immobility or mortality of the crustacean cladoceran test species *Daphnia magna*. The toxicity test was performed in a multiwell test plate using neonates of *Daphnia magna* that were hatched from *ephippia* 72 h prior to the test at 20–22 °C under continuous illumination of 6000 LUX and then fed to provide live bioata. Briefly, test wells were filled with a series of dilutions from 4.8 to 78 mg L^{-1} for each antimicrobial agent previously prepared in standard freshwater solution supplied within the kits and prepared according to the ISO 6341 formula. Exactly, five selected moving *Daphnia* were added into each testing well. Multiwell test plates were sealed tightly with a strip of parafilm, covered with their lid, and further incubated at 20 ± 2 °C in the dark. After 24 h and 48 h, the number of *Daphnia* organisms that were unable to move within 15 s after gentle agitation were recorded and were considered as dead even if they can still move their antennae. The effective concentrations EC_{50} causing 50% immobilization of the exposed *Daphnia magna* were calculated using the Excel™ macro RegTox software and expressed in mg L^{-1} with 95% confidence limits. Control wells contained only standard freshwater. Each test was performed in four replicates for each tested concentration.

2.10. Statistical analysis

All experiments were performed in triplicates. The statistical significance of the results was performed by Analysis of variance (ANOVA) and Tukey's test using the Matplotlib software (Version 3.3.4., Python). Values of $p < 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. Physicochemical characterization of the nanoparticles

3.1.1. Size and zeta potential

The zetasizer is one of the most common techniques used to determine the colloidal stability of particles by measuring their average size and zeta potential. The results of the size and charges of the particles loaded with CAR and THY are represented in Table 1. Results show that the produced particles are nano-sized. ζ-Potential indicates the electrical charge difference between the ionic layer around the nanodroplets and the charges of the surrounding medium. These values may predict the stability of the suspensions as higher absolute values indicate greater repelling forces between dispersed droplets and thus lower aggregations and collision frequencies that increase the stability of particles (Cacciatore, 2020). Generally, absolute ζ-Potential values of 30 mV can be considered as high to get a good stability (Luna et al., 2022). Both ζ-Potential values obtained for THY and CAR nanocapsules were positive. This could be attributed to the presence of positively charged sodium caseinate as emulsifier. Nanocapsules were prepared at pH 3, and sodium caseinate is positively charged at a pH below its isoelectric point (pHi ~ 4.5). The nanodroplets positive charge will promote their electrostatic interactions with the negatively charged surfaces of bacterial cells. By this, the bioavailability and delivery of the antimicrobial compounds to the bacterial cell membrane would be faciliated and enhanced. Also, ζ-Potential values obtained in this study were high which could prevent possible aggregation between particles and subsequently result in a good stability.

Table 1
Average size and zeta potential values for the developed carvacrol and thymol nanocapsules.

Encapsulated component	Mean Size ± S.D. (nm)	ζ-Potential ± S.D. (mV)
Carvacrol	234.76 ± 7.88	24.66 ± 0.90
Thymol	159.25 ± 7.23	23.60 ± 1.35

S.D.: Standard deviation.

Several studies about the encapsulation of EOs have reported slightly similar values for the size and ζ-Potential. Cacciatore (2020) reported an average size of 159.3 nm and a ζ-Potential of +44.8 mV for carvacrol nanodroplets. Also, the size and ζ-Potential of chitosan nanoparticles loading carvacrol were in the range of 140–166 nm and 10.5–14.4 mV, respectively (Bernal-Mercado et al., 2022). Compared to our results, Mahdi et al. (2021) reported slighly bigger size of nanoemulsions of *Citrus reticulata* EO with 411.15 nm and a ζ-Potential of +30.66 mV.

3.1.2. Morphology

The external as well as the internal morphologies of CAR and THY nanoparticles obtained by spray-drying were determined using the SEM (Fig. 1). The results confirmed the spherical external structure of nanoparticles with a smooth and continuous surface. Some of the observed nanoparticles showed a rough collapsed structure that might be attributed to the thermal expansion of air and subsequently to the evaporation of moisture from the dry particles (Ma et al., 2022). Upon spray-drying, the morphology of the particles is mainly related to the formulation composition as well as the droplet evaporation rate (Fernandes et al., 2008). The internal morphologies of the nanocapsules showed a core-shell structure with a hollow void in the center. The observed air holes in the shell wall of nanoparticles demonstrates the encapsulated CAR and THY droplets that are stuck onto the surface.

3.2. Minimal inhibitory concentrations of free and nanoencapsulated carvacrol and thymol

MIC of free and nanoencapsulated CAR and THY were determined against *S.* Enteritidis planktonic cells. MIC values for both free CAR (F-CAR) and free THY (F-THY) were 1.25 mg mL^{-1}, while for the nanoencapsulated CAR (NE-CAR) and nanoencapsulated THY (NE-THY), MIC values were 0.31 and 0.62 mg mL^{-1}, respectively. MIC values determined after nanoencapsulation were respectively four- and two-fold lower than values reported with F-CAR and F-THY. Nanocapsules possessed lower MIC values against *S.* Enteritidis and thus a higher inhibitory activity compared to free terpenes. This could be attributed to the reduced size of particles to a nano-scale that increases the surface area and favors the diffusion of CAR and THY across *S.* Enteritidis cell membranes. Additionally, the larger surface area of nanocapsules promotes the interaction of terpenes with water, with modifications in oil-water partition coefficents that enhance the water solubility of terpenes and their subsequent antimicrobial activity (Barradas & de Holanda e Silva, 2021; McClements, 2011; Siva et al., 2020). Moreover, encapsulation enhances the stability of encapsulated agents by decreasing their volatilization and degradation rates as compared to their free counterparts which favors their antimicrobial effect (da Silva Gündel et al., 2018; Gong et al., 2021; Wadhwa et al., 2019). It should be also noted that DMSO used at a concentration <2% to solubilize free CAR and THY was not toxic to bacterial cells, as we observed regular bacterial growth (data not shown).

These results were in agreement with other studies that showed that the nanoencapsulation of several EOs greatly improved their antibacterial activity. As an example *Thymus daenensis* EO with thymol being its main active component against *Escherichia coli* (Moghimi et al., 2016), *Cymbopogon flexuosus* EO against mycobacteria (Rossi et al., 2017), and carvacrol against *Esherichia coli* K12 and *S.* Enteritidis (Kamimura et al.,

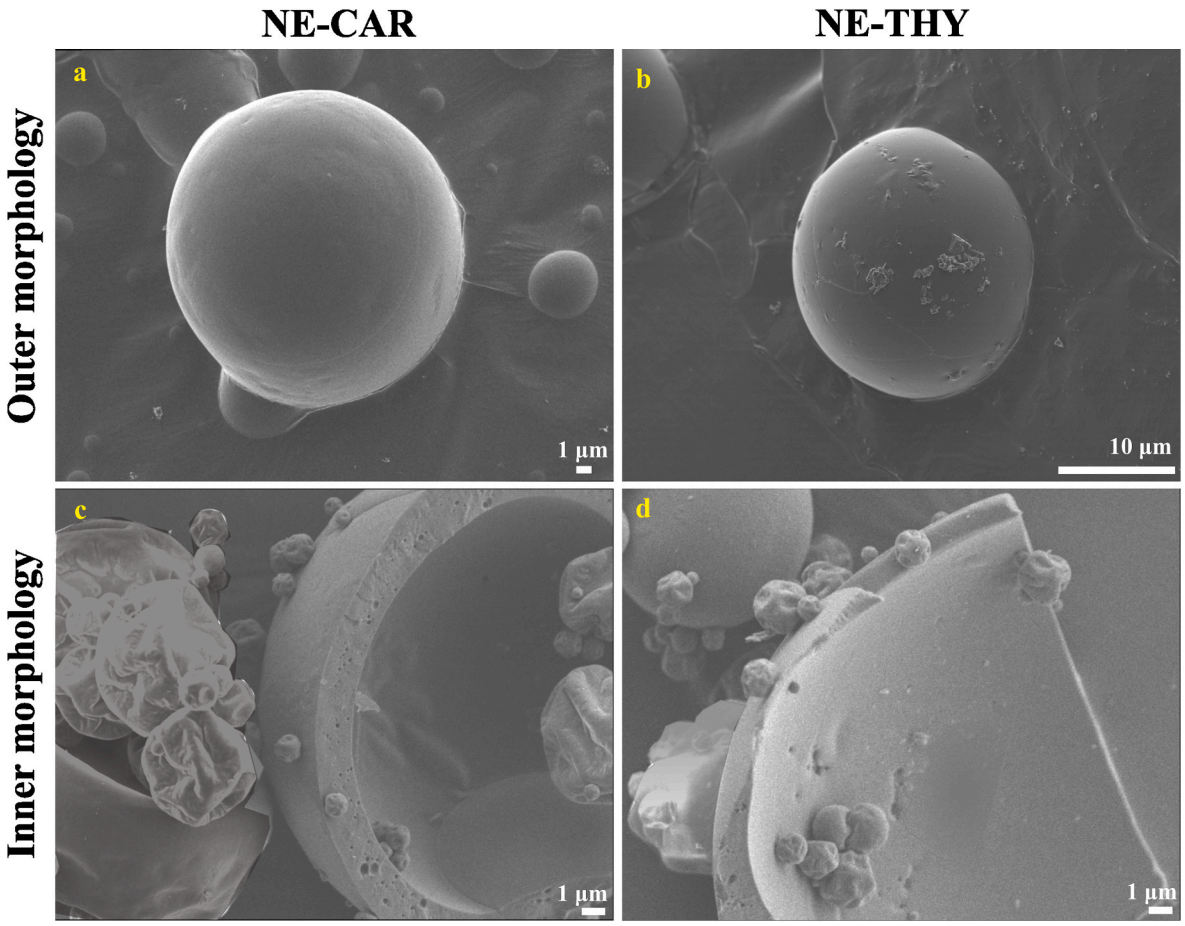

Fig. 1. Scanning electron micrographs of the external nanocapsules structures of a) carvacrol (3000 x), b) thymol (2500 x), and the internal nanocapsules structures of c) carvacrol (5000 x), and d) thymol (5000 x).

2014). Other studies reported that encaspsulation of EOs did not reduce the MIC values compared to free EOs. Sun et al., 2019 and Wattanasatcha et al., 2012, reported equal MIC values for both free and encapsulated carvacrol and thymol against *E. coli* and *Staphylococcus aureus*. This was explained by the slower diffusion of terpenes out of the nanoparticles whether due to the non-degradability of capsules' shells

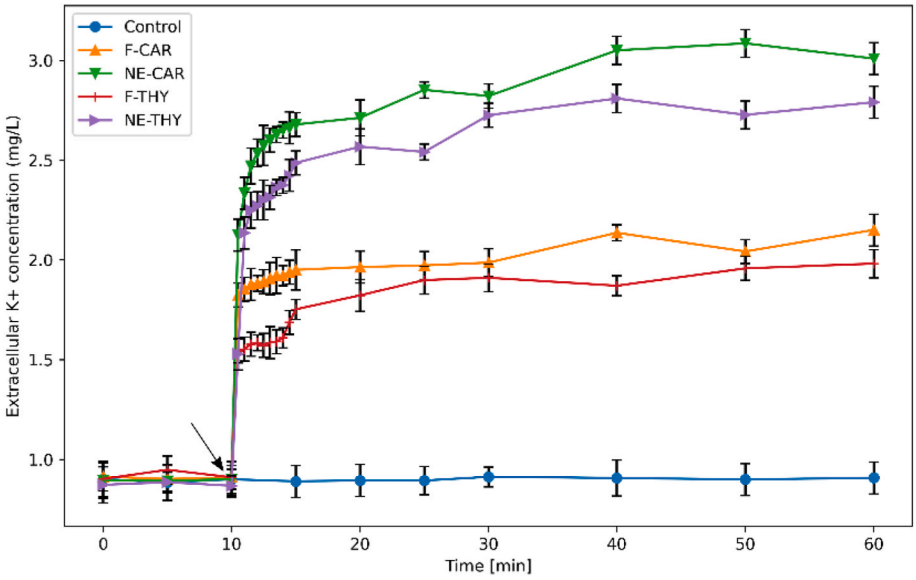

Fig. 2. Kinetics of potassium ions leakage from the intracellular medium of S. Enteritidis planktonic cells after exposure to free and nanoencapsulated CAR and THY at MIC values. Control represents S. Enteritidis cells exposed to MOPS buffer alone. The black arrow represents the time of the addition of the different treatments. Experiments were performed in triplicates and data are represented as mean (±SD).

under the performed experimental conditions or due to the relatively high encapsulation efficiency that decreases the release rate of terpenes.

3.3. Antimicrobial effect of free and nanoencapsulated carvacrol and thymol against S. Enteritidis planktonic cells

3.3.1. Leakage of K+ across cell membranes

To investigate one of the possible antimicrobial mechanisms of action induced by CAR and THY on the integrity of *S.* Enteritidis cell membranes, the extracellular concentrations of K^+ ions leaked out of bacterial cells were assessed after exposure to free and nanoencapsulated CAR and THY (Fig. 2). In fact, intracellular constituents as ions could be leaked out when bacterial membranes permeability changes after exposure to antimicrobial agents. During the first 10 min prior to the exposure of bacterial cells to terpenes, the K^+ extracelullar concentrationremained stable below 1 mg L^{-1}. Whereas, after exposure of *S.* Enteritidis cells to free and nanoencapsulated CAR and THY, the K^+ concentrations in the extracellular medium increased immediately within the first 30 s of exposure. K^+ concentration increased with increased exposure time to the antimicrobial agents. One hour after the exposure to F-CAR and F-THY at MIC = 1.25 mg L^{-1}, K^+ concentrations reached 2.15 and 1.98 mg L^{-1}, respectively. However, the concentrations recorded after 1 h exposure to NE-CAR (at MIC = 0.31 mg L^{-1}) and NE-THY (at MIC = 0.62 mg L^{-1}) were 3.00 and 2.79 mg L^{-1}, respectively. CAR and THY showed the ability to disrupt the outer membrane of the Gram-negative bacterial cells of *S.* Enteritidis, leading subsequently to functional and structural damages of the cytoplasmic membrane that increased its permeability and promoted an excessive leakage of intracellular material as K^+ ions with a subsequent bacterial death. Nanoencapsulation induced more pronounced leakages of K^+ even at lower concentrations. This could be related to the reduced size of nanoparticles that allow a greater interaction with bacterial cell membranes leading thus to more destabilization of the membranes with an increased permeability and a subsequent leakage of micromolecules to the outer medium.

3.3.2. Leakage of low molecular weight proteins across cell membranes

To further investigate the effect of CAR and THY on the permeability of bacterial cell membranes, the leakage of macromolecular proteins from bacterial cells was assessed after exposure to different treatments. The choice of *E coli* GFP was done due to the absence of *Salmonella* GFP. Moreover, what is demonstrated on *E. coli* cells could be applied on *Salmonella* as they are both Gram-negative microorganisms. The reported fluorescence intensity of the leaked proteins to the outer medium of bacterial cells are presented in Fig. 3. The negative control showed no effect on the extracellular fluorescence intensity of GFP *E. coli* cells. However, directly in the first 5 min after exposure of *E. coli* cells to F-CAR and F-THY, the GFP fluorescent intensity increased by 3.19 and 2.85 folds, respectively, and by 5.09 and 4.75 folds after exposure to NE-CAR and NE-THY, respectively. The GFP fluorescence intensity increase is time dependent as after 1 h, the intensities were further increased. Compared to the treatments with free terpenes, NE-CAR and NE-THY induced 2.14 and 2.47 higher folds, respectively even at lower concentrations. This further proves that nanocapsules reinforce and enhance the antimicrobial activity of CAR and THY on bacterial membranes. According to the obtained results, exposure to CAR and THY caused damage to bacterial membranes and provoked an increased permeability that led to the leakage of both micro- and macromolecular substances. Our findings add new results and give evidence that the antimicrobial mechanism of action of several EOs can be attributed to the loss of bacterial membranes integrity and the increased permeability with a subsequent excessive leakage of vital micro and macromolecular materials as reported by Bouyahya et al. (2019), Cui et al. (2018), Prasad et al. (2022) and Tang et al. (2020).

3.4. Assessment of the antibiofilm activity of free and nanoencapsulated carvacrol and thymol against S. Enteritidis biofilms

The antibiofilm activity of free and nanoencaspulated CAR and THY was assessed against *S.* Enteritidis biofilms grown for 24 h on SS coupons. SS is a widely used material in food industries due to its remarkable physicochemical properties, high resistance to corrosion and mechanical strength (Engel et al., 2017). It is considered as an abiotic surface to which bacterial cells including *S.* Enteritidis have the ability to adhere in relatively short period times (2 h or less) (Engel et al., 2017). The NEC biofilm system was used as a bioreactor for biofilms development. This system is a closed reactor that ensures a high repeatability with easier handling conditions for biofilms deposition (Abdallah et al., 2015).

The counts of adhered *S.* Enteritidis cells to SS coupons after 24 h

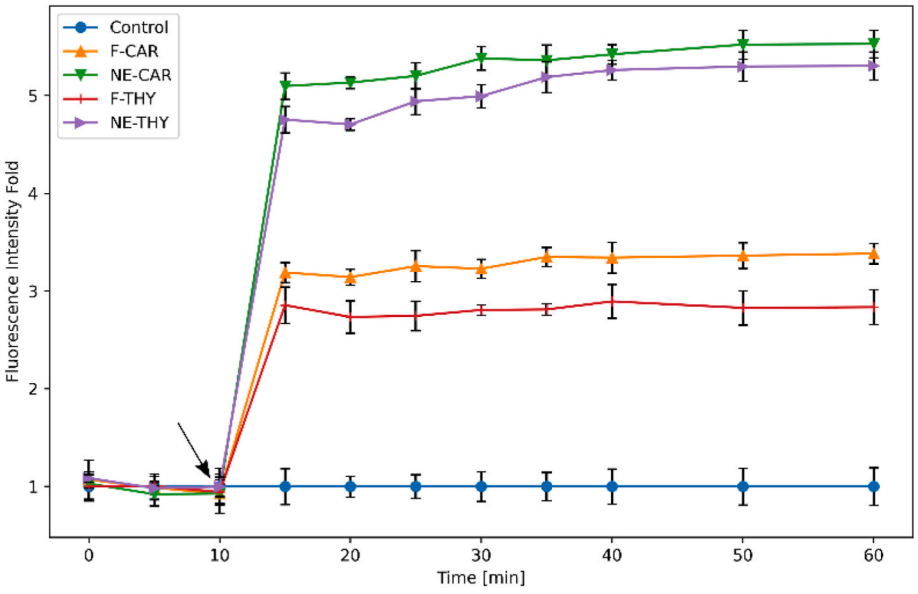

Fig. 3. Kinetics of green fluorescent proteins leakage from the intracellular medium of *E. coli* planktonic cells after treatment with free and nanoencapsulated CAR and THY at MIC. Control represents *E. coli* cells exposed to HEPES buffer alone. The black arrow represents the time of the addition of the different treatments. Experiments were performed in triplicates and data are represented as mean (±SD).

were 7.31 log CFU cm^2. The results of the exposure of biofilms to 1 MIC and 2 MIC of free and nanoencaspulated CAR and THY solutions for 15 min, are presented in Fig. 4. Exposure to MIC of F-CAR and F-THY reduced the number of culturable cells in the preformed biofilms and induced 1.73 and 1.48 log reductions, respectively. After an exposure to MIC of NE-CAR and NE-THY, further significant ($p < 0.05$) reductions of biofilms biomass were induced with 4.23 and 3.72 log reductions, respectively. As shown, the antibiofilm activity of free and nanoencapsulated CAR and THY was dose dependent ($p < 0.05$), as more pronounced reductions were reported with increased concentrations at 2 MIC values. No significant differences ($p > 0.05$) were reported between F-CAR and F-THY at 2 MIC values compared to their nanoencapsulated forms at MIC values suggesting that nanoencaspulation promoted the antibiofilm activity of both CAR and THY against *S.* Enteritidis biofilms even when using lower concentrations. Exposure to 2 MIC of NE-CAR and NE-THY for 15 min represented the most effective treatments with no significant difference ($p > 0.05$) as they both achieved a 99.99% eradication of biofilms. The nano-sized capsules ensured probably a good diffusion of CAR and THY across the biofilm matrix. In addition, it is known that once the particles' size decreases, the surface area increases allowing thus a greater contact with microorganisms (Hasheminejad et al., 2020; Kavaz et al., 2019). Nanoencapsulation provided also a higher stability and water solubility of the encapsulated terpenic compounds, promoting their antimicrobial efficiency. Similarly, Cai et al. (2022) demonstrated a 78.33% and 80.81% inhibition of *E. coli* and *S. aureus* planktonic cells treated with MIC of chitosan nanoparticles encapsulating *Ocimum basilicum* EO. Heckler et al. (2020) reported a total inactivation of *Salmonella* biofilms adhered to glass after 1 min treatment with free carvacrol and thymol, while nanoliposomes showed lower reductions between 3.79 and 4.03 log CFU cm^{-2} for the same exposure time and concentration. Trevisan et al. (2018) demonstrated that an exposure to 2 MIC and 4 MIC carvacrol for 1 h, induced 46.3% and 60.6% reduction of 48 h old *S. typhimurium* biofilms developed on stainless steel. It has been established that to induce biofilms reduction or eradication, the antimicrobial compounds should penetrate into the deep layers of biofilms, damage the extrapolymeric matrix by disrupting the cytoplasmic membranes or through other mechanisms of action (Bernal-Mercado et al., 2022).

3.5. Direct assessemnt of the antibiofilm activity of free and nanoencapsulated carvacrol and thymol by epifluorescent microscopy

S. Enteritidis biofilms exposed to MIC of free and nanoencapsulated THY and CAR were visualized using the epifluorescent microscopy after being stained with SYTO-9 and PI (Fig. 5). Bacteria that had intact bacterial membranes were stained with SYTO-9 and appeared as green, while bacteria with lost membrane integrity were stained with PI and resulted in red coloration. For control cells treated with TS broth, dense viable green stained biofilms were mainly observed. Exposure to free and nanoencapsulated CAR and THY resulted in a disruptive effect on the biofilm structure with a domination of dead red cells. Increased damage to bacterial membranes and death were recorded for biofilms treated with NE-CAR and NE-THY as red was the dominant coloration. The obtained epifluorescent microscopy images were in agreement with the previous results obtained after the biofilm treatment which confirm an efficient antibiofilm activity of both free and nanoencapsulated CAR and THY with a primary action on the cell membranes integrity. Nanoencaspulated antimicrobials showed more pronounced damages to bacterial cells membranes which might be attributed to the enhanced facilited diffusion of nanocapsules to the deep layers of biofilms.

3.6. Bacterial morphology changes after exposure to free and nanoencapsulated carvacrol and thymol

The effect of free and nanoencapsulated CAR and THY, against *S.* Enteritidis cells recovered from biofilms was investigated using SEM. Morphological alterations induced to the bacterial membranes were recorded in micrographs of Fig. 6. Control biofilm cells showed intact structures with normal morphologies of *S.* Enteritidis membranes. However, after exposure to free and nanoencapsulated CAR and THY, bacterial cells showed obvious morphological alterations. Bacterial cells were pitted, deformed, shrinked and shriveled as CAR and THY led to an irreversible destruction of membranes and to the leakage of intracellular vital constituents with subsequent death of bacterial cells. This confirms the effect of the penetration of free and nanoencapsulated CAR and THY across biofilms structures.

3.7. Assessment of the acute toxicity test with Daphnia magna

The ecotoxicities of free and nanoencapsulated CAR and THY against

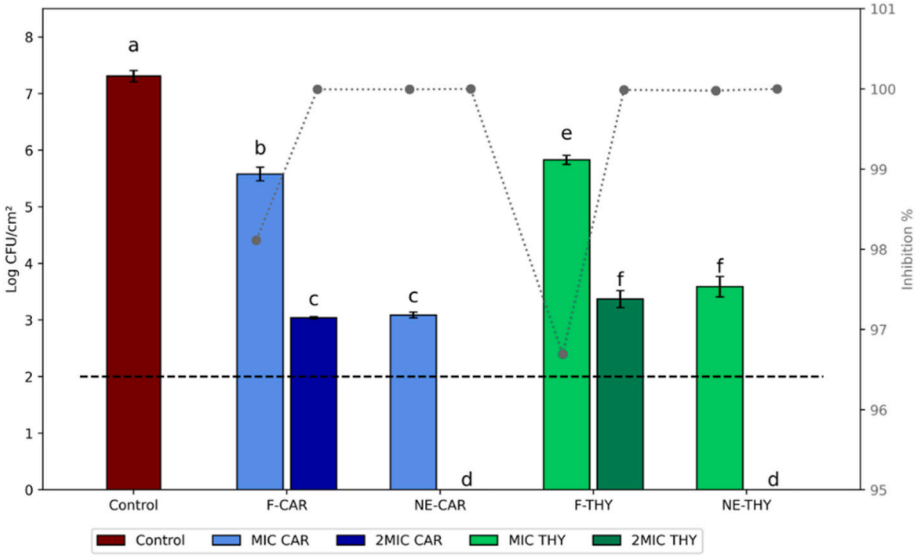

Fig. 4. *Antibiofilm activity of free and nanoencapsulated CAR and THY (at MIC and 2 MIC), after* 15 min *contact with S.* Enteritidis *biofilms adhered to stainless steel. Control represents biofilms treated with TS broth. Results are expressed as bars representing the mean (± SD) of three independent experiments. The different letters indicate significant differences ($p < 0.05$). The detection limit of this method is 2* log *CFU* cm^{-2} *which is indicated by the dashed line.*

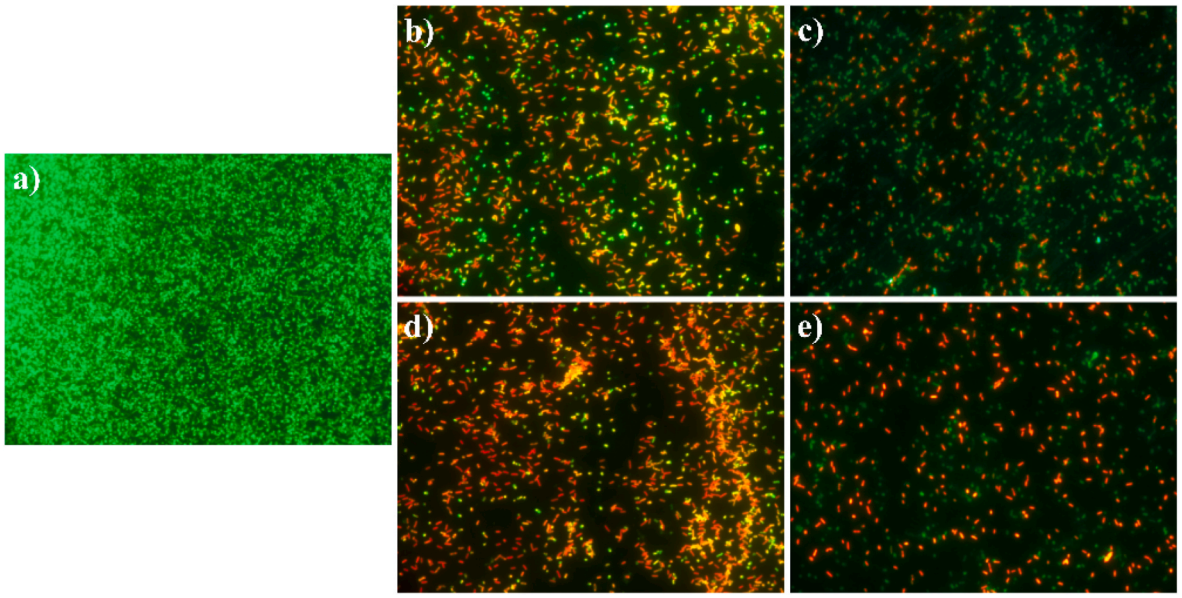

Fig. 5. *Fluorescent microscopy micrographs of S.* Enteritidis *biofilms control (a), exposed to F-CAR (b), F-THY (c), NE-CAR (d) and NE-THY (e). Green cells represent viable bacteria stained with SYTO-9 and red cells represent dead bacteria stained with propidium iodide. Control represents biofilms treated with TS broth.*

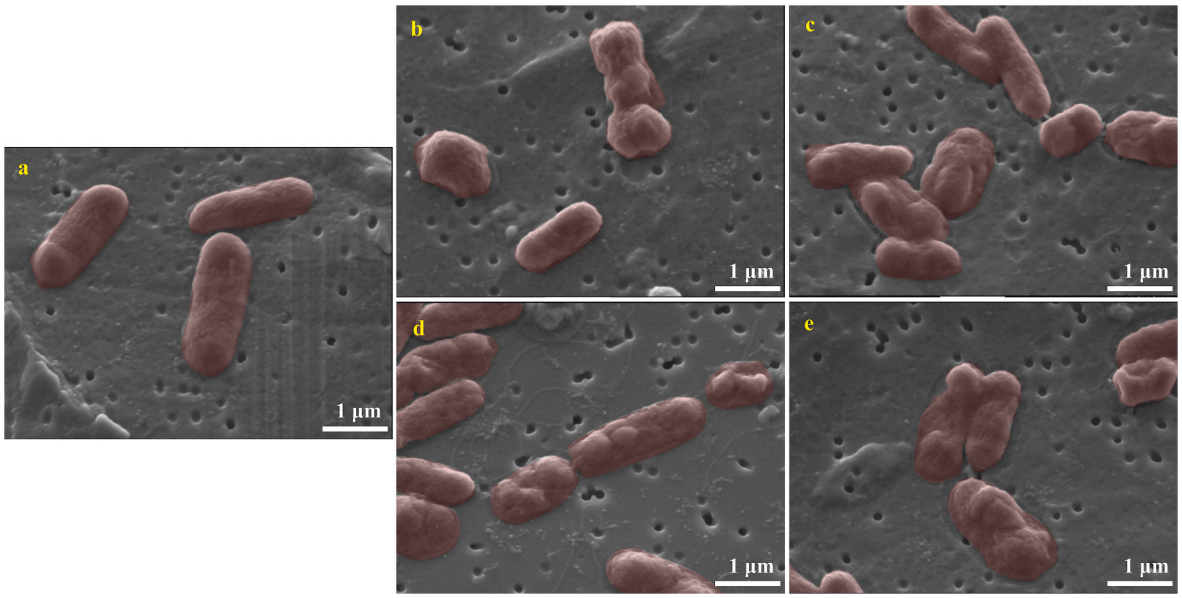

Fig. 6. *SEM micrographs of S.* Enteritidis *biofilms a) control, and after being exposed to b) F-CAR, c) F-THY, d) NE-CAR, and e) NE-THY at MIC values (20 000 x). Control represents biofilms treated with TS broth.*

Daphnia magna crustaceans, were evaluated using the Daphtoxkit F™ *magna* kits. *Daphnia magna* organisms are recommended by international organizations as the Organization for Economic Co-operation and Development (OECD) and the American Society for Testing and Materials (ASTM), as well as the European Union (EC Regulation n°1907/2006) to perform toxicity tests and provide information regarding the ecotoxicity of substances towards aquatic organisms (Ferraz et al., 2022). These evaluations remain of high importance to predict the toxic effect of using terpenes on the freshwater aquatic ecosystems.

In this study, the mortality (immobilization) percentages and the EC_{50} values were determined after 24 h and 48 h exposure to CAR and THY. EC_{50} values are considered as the effective concentrations of the antimicrobial agents that have resulted in a 50% immobilization of *Daphnia magna* organisms. International organizations recommend using the 48 h- EC_{50} values to determine the toxicity of a substance. In this study, no significant difference ($p > 0.05$) was reported between the mortality percentages as well as the EC_{50} values reported at 24 h and 48 h for both free and nanoencapsulated CAR and THY treatments (data not shown). Thus, the calculated percentages of mortality and the EC_{50} values determined by the Excel™ macro RegTox software only after 48 h exposure to antimicrobial agents are presented in Fig. 7. To relate the EC_{50} values of a substance with its toxicity, the United Nations Globally Harmonized System of Classification and Labelling of Chemicals Categorization (GHS) has proposed different acute toxicity categories: category 1 very toxic to aquatic life if $EC_{50} \leq 1$ mg L^{-1}, category 2 toxic to aquatic life if $1 < EC_{50} \leq 10$ mg L^{-1}, category 3 harmful to aquatic life if $10 < EC_{50} \leq 100$ mg L^{-1}, and category 4 not classified as harmful to aquatic life if $EC_{50} > 100$ mg L^{-1} (UN, 2019). In this study, significantly ($p < 0.05$) higher EC_{50} values were recorded for both nanoencapsulated CAR and THY compared to their free forms. Free CAR and THY showed

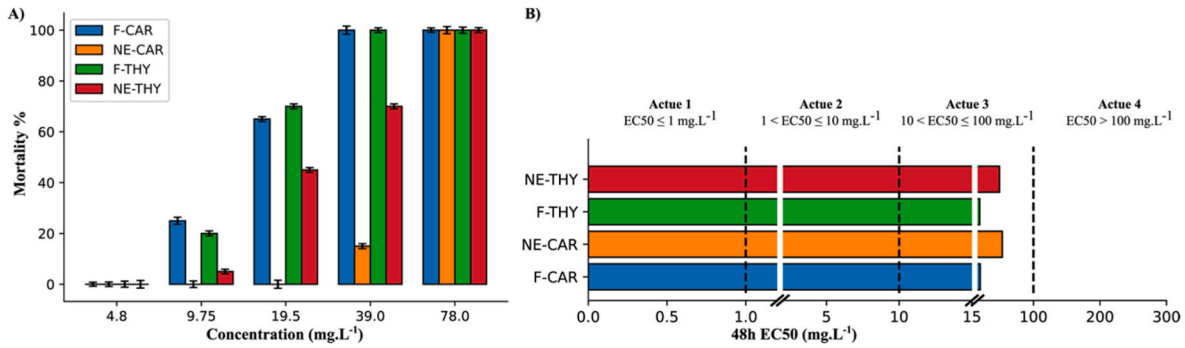

Fig. 7. (A) Mortality percentages of Daphnia magna in function of different concentrations of free and nanoencapsulated CAR and THY after 48 h exposure, and (B) the 48 h - EC_{50} values of free and nanoencapsulated CAR and THY with the acute toxicity indications of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals.

the highest harmfullness to *Daphnia magna* with the lowest EC_{50} values recorded (19.81 and 19.18 mg L^{-1}, respectively), which were significantly ($p < 0.05$) lower than EC_{50} values recorded for nanocapsules (48.89–53.05 mg L^{-1}). Nanocapsules provoked lower acute toxicity than free terpenes on *Daphnia magna* organisms which might be attributed to the lower concentrations of CAR and THY that are being released into the aquatic environment due to the progressive release of encapsulated compounds. Nevertheless, both free and nanoencapsulated CAR and THY belonged to the third acute toxicity category which indicates that they are harmful but not toxic to the aquatic life as their 48 h – EC_{50} values recorded were >10 but ≤100 mg L^{-1}. For the control, *Daphnia magna* remained mobile with no mortality or other signs of toxicity. It should be also noted that only a direct massive input of terpenes in the freshwater environments might represent a high ecotoxicological risk. However, under natural conditions, the possible concentrations of terpenes that might reach the aquatic ecosystem are very diluted which tend to reduce their toxicity.

4. Conclusions and perspectives

This study provides more evidence on the potent applications of EOs active components CAR and THY as natural tools to control *S.* Enteritidis biofilms developed on SS surfaces. The nanoencapsulated terpenes exhibited higher efficiencies in inhibiting bacterial cells than free terpenes, indicating better prospect applications. Both NE-CAR and NE-THY were effective to eradicate totally *S.* Enteritidis biofilms after 15 min contact at 2 MIC. To explain viable cells reductions, microscopic observations as well as measurements of the leakage of intracellular micro and macromolecular substances was assessed. Both free and nanoencapsulated CAR and THY distrupted the normal cellular integrity of *S.* Enteritidis membranes, increasing their permeability and leakage of vital components that was responsible for their death.

Although, several EOs and their active components have been Generally Recognized as Safe (GRAS) by the FDA and are being used in food packaging and as food additives in the food industries, their ecotoxicological risk assessments are still being neglected. The 48 h - EC_{50} values determined for CAR and THY in this study, have shown their non-toxicity to *Daphnia magna* organisms. However, these results are still preliminary and further studies should focus on the longterm ecotoxicological risk assessments of more EO components in the aquatic ecosystems.

Overall, the maltodextrin and sodium caseinate nano-based delivery systems loaded with CAR and THY demonstrated a high efficiency as disinfectant agents with potent applications in the food industries while representing no ecotoxicity. However, before being applied, they have to be registered and approved by national and international regulatory bodies.

CRediT authorship contribution statement

Jina Yammine: Design, Conceptualization, Experiments, Data curation, Writing. **Adem Gharsallaoui:** Nanocapsules development, Writing – review & editing. **Alexandre Fadel:** Scanning electron microscopy observations. **Samah Mechmechani:** Experiments, Data curation. **Layal Karam:** Writing – review & editing. **Ali Ismail:** Writing – review & editing. **Nour-Eddine Chihib:** Conceptualization, Supervision, Writing – review & editing, All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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