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Use of free and encapsulated nerolidol to inhibit the survival of *Lactobacillus fermentum* in fresh orange juice

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ABSTRACT

Lactobacillus fermentum is commonly responsible for fruit juice fermentation and spoilage. The aim of this study was to investigate the potential use of nerolidol to control the spoilage of fresh orange juice by *L. fermentum*. Nerolidol was incorporated into hydroxypropyl-β-cyclodextrin inclusion complex, conventional liposome, and drug-in-cyclodextrin-in liposome systems. The systems were lyophilized and characterized with respect to their nerolidol content, size, and morphology. The effects of the acidity and cold storage of orange juice on the survival of *L. fermentum* were evaluated. Subsequently, the antibacterial activity of nerolidol in refrigerated orange juice was assessed at pH 3.3. Nerolidol showed a faster antibacterial activity at 4 000 μM (5 days) compared to 2 000 μM (8 days). Under the same conditions, the inclusion complex completely killed bacteria within 6 days of incubation at 4 000 μM, suggesting its potential application in fruit juices. Nerolidol-loaded liposomes did not exhibit an antibacterial activity and altered the appearance of juice.

1. Introduction

Orange juice is consumed worldwide and has a number of functional attributes, being rich in bioactive compounds such as polyphenols, carotenoids, and vitamin C. Protecting orange juice from chemical and microbiological spoilage has been a major focus of the juice industry for years. Orange juice is susceptible to spoilage by a wide range of acid-tolerant microorganisms, including fungi, lactic acid bacteria, and spore-forming *Alicyclobacillis* (Ephrem et al., 2018). Different strategies have been adopted to prevent this spoilage, including the use of food additives with or without the use of different pasteurization techniques. Various preservatives are currently added to orange juice, primarily nitrates, sorbates, and benzoates. However, because these preservatives can cause allergic responses and be converted to potential carcinogens (Anand and Sati, 2013), there is currently a need to identify alternative bioactive agents for orange juice preservation.

Nerolidol (Ner) is a sesquiterpene alcohol that is present in the essential oils of plants such as lemon grass (*Cymbopogon* spp.), lavender (*Lavandula* spp.), and tea tree (*Melaleuca alternifolia*). Ner has been approved for use as a flavouring agent in food products by the United States Food and Drug Administration (Chan et al., 2016) and has been shown to exhibit antimicrobial activity against different microorganisms, including *Streptococcus mutans*, *Staphylococcus aureus*, and *Salmonella enterica* (Chan et al., 2016). However, Ner has a low aqueous solubility and photostability (Azzi et al., 2018; McGinty et al., 2010), requiring its incorporation into appropriate encapsulation systems to promote its use in food preservation.

The use of encapsulation systems (e.g., liposomes, nanoemulsions, and cyclodextrin inclusion complexes) to protect natural antimicrobials and enhance their aqueous solubility in food systems has been described in a number of previous studies (Astray et al., 2009; Blanco-Padilla et al., 2014; Weiss et al., 2009). Antimicrobial-loaded encapsulation systems have also been demonstrated to be effective for fruit juice preservation in many studies (Ghosh et al., 2014; Shah et al., 2012; Sugumar et al., 2016; Truong et al., 2010). Azzi et al. (2018) previously prepared hydroxypropyl-β-cyclodextrin (HP-β-CD)/Ner inclusion complex, Ner-loaded conventional liposome (CL), and Ner-in-cyclodextrin-in-liposome (DCL) systems. The prepared aqueous formulations were optimized and characterized in terms of size, encapsulation efficiency, loading rate, release rate, photostability, and storage stability and yielded preparations with high encapsulation efficiencies and loading rates. Moreover, the DCL system exhibited the

Abbreviations: CFU, colony forming unit; CL, conventional liposome; DCL, drug-in-cyclodextrin-in-liposome; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; HP-β-CD, hydroxypropyl-β-cyclodextrin; HPLC, high performance liquid chromatography; Ner, nerolidol; MRS, de Man Rogosa and Sharpe; PdI, polydispersity index; SEM, scanning electron microscopy; TA, titratable acidity; TEM, transmission electron microscopy

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lowest release rate of Ner, followed by CLs and the inclusion complexes. Significant enhancements of the photostability and storage stability (4 °C) of Ner were also observed.

Ner has been previously shown to exhibit outstanding activity against *Lactobacillus fermentum* in de Man, Rogosa, and Sharpe (MRS) broth (pH 6.2, 37 °C) (Ephrem et al., 2019). In this study, *L. fermentum* ATCC 9338, an obligate heterofermentative, gram-positive, anaerobic, thermo-acidophilic lactic acid bacterium linked to fruit juice spoilage was used as a model strain. According to Lawlor et al. (2009), heterofermentative lactic acid bacteria are the major contributors to fruit juice spoilage. These bacteria can produce different products in fruit juices, including lactic acid, acetic acid, ethanol, and carbon dioxide, which are primarily responsible for the alteration of the organoleptic properties of juice (Ashurst, 2016). Aneja et al. (2014) reported 23.3% occurrence of lactic acid bacteria in orange juice. Garcia et al. (2016) identified up to 24 *L. fermentum* strains in different fruit pulp processing byproducts, including pineapple, mango, strawberry, soursop, and Barbados cherry. *L. fermentum* was also isolated from unpasteurized orange juice (Parish and Higgins, 1988) and from pasteurized mixed fruit juice (pineapple, orange, paw-paw, and guava) stored in the presence of sodium benzoate (0.08%) (Uzoukwu et al., 2015).

In this study, HP-β-CD/Ner inclusion complexes and Ner-loaded CLs and DCLs were prepared as previously reported by Azzi et al. (2018). The efficacy of free Ner, Ner solubilized in dimethyl sulfoxide (DMSO), HP-β-CD/Ner inclusion complexes, and Ner-loaded CLs and DCLs systems was investigated in orange juice against *L. fermentum*.

2. Materials and methods

2.1. Chemical and reagents

Nerolidol (98%, mixture of *cis* (40%) and *trans* (60%) isomers), thymol (> 99%), absolute ethanol, methanol (HPLC-grade), and DMSO were purchased from Sigma-Aldrich (Missouri, United States). HP-β-CD was purchased from Wacker-Chemie (Lyon, France). De Man, Rogosa, and Sharpe (MRS) broth and MRS agar were purchased from Laboratorios Conda (Madrid, Spain). Lipoid E80 (80–85% egg phosphatidylcholine, 7–9.5% phosphatidylethanolamine, 2% water, and 0.2% ethanol) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Citric acid was purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Bacterial strain and culture conditions

L. fermentum ATCC 9338 was purchased from the American Type Culture Collection (Virginia, USA). *L. fermentum* cultures were routinely maintained on MRS agar at 4 °C. Before each antimicrobial assay, fresh cultures were prepared in sterile MRS broth and incubated at 37 °C for 22 h under anaerobic conditions. A bacterial suspension was prepared by diluting the bacterial culture in MRS broth to a final concentration of 25×10^5 CFU(colony forming unit)/mL.

2.3. Preparation of HP-β-CD/Ner inclusion complexes, conventional liposomes and drug-in-cyclodextrin-in-liposomes loaded with Ner

HP-β-CD/Ner inclusion complexes as well as liposomes (CL and DCL systems) loaded with Ner were prepared as previously described by Azzi et al. (2018). Ner inclusion complexes were prepared using the freeze-drying method. First, an aqueous solution (300 mL) containing HP-β-CD (25 mM) and an excess of Ner (667 mg) was magnetically stirred at 300 rpm for 24 h at room temperature. Then, the suspension was filtered (0.45 µm, cellulose acetate membrane) to remove excess Ner, after which it was frozen at −80 °C and lyophilized. Blank HP-β-CD was prepared similarly without Ner. The obtained powders were stored at 4 °C until usage.

CLs and DCLs loaded with Ner were prepared using the ethanol injection method as previously described by Azzi et al. (2018). Briefly, to prepare CLs, E80 phospholipids were dissolved in ethanol at 50 mg/mL (0.066 M), after which Ner was added to the organic phase at an E80 phospholipid to Ner molar ratio of 100:10. The organic phase (10 mL) was then added to 20 mL of ultrapure water at an injection flow rate of 1 mL/min and under magnetic stirring (400 rpm) at room temperature. The obtained suspension was magnetically stirred for 15 min, after which ethanol and a small volume of water were evaporated under reduced pressure at 25 °C, and a final volume of 17 mL was obtained. Blank CLs were prepared similarly without Ner.

Ner-loaded DCLs were prepared following the same method as CL except that the organic phase (10 mL) was added to 20 mL of the HP-β-CD/Ner inclusion complex aqueous solution, which was prepared as described above. The obtained DCL system had an E80:HP-β-CD:Ner molar ratio of 100:75:30. For the preparation of blank DCLs, the organic phase was added to an aqueous solution of HP-β-CD (25 mM).

The CL and DCL suspensions were centrifuged at 15 000 rpm for 30 min at 4 °C to eliminate the excess aqueous media. HP-β-CD, used as cryoprotectant, was added to the suspensions at a concentration of 25 mM (Gharib et al., 2017; Sebaaly et al., 2016), after which the samples were frozen at −80 °C and lyophilized. The obtained powders were stored at 4 °C until usage.

2.4. Characterization of delivery systems

2.4.1. Determination of Ner incorporation in the delivery systems by HPLC analysis

The total concentration of Ner in the encapsulation systems was determined by HPLC as previously described (Azzi et al., 2018). The HPLC instrument used was a Hitachi LaChrom Elite® HPLC system (L-2130 pump, L-2200 autosampler) coupled to a L-2455 diode array detector. Samples were prepared at a concentration of 1 mg/mL in water. To generate a calibration curve, standard solutions of Ner in methanol were prepared at concentrations ranging from 2.5 to 250 µg/mL, with thymol used as an internal standard at a concentration of 100 µg/mL in methanol. For HPLC analyses, 100 µL of the Ner standard solutions, liposomal suspensions, or inclusion complex solutions were added to 200 µL of methanol and 100 µL of thymol solution (100 µg/mL). The samples were then sonicated for 10 min at 4 °C and centrifuged at 15 000 rpm for 30 min at 4 °C. The supernatant (20 µL) was then analysed using a reversed-phase C18 Agilent column (150 × 4.6 mm, 5 µm), using a mobile phase of methanol/ultrapure water (75/25 v/v) at a flow rate of 1 mL/min. The absorbance was measured at 212 nm and used to calculate the mass of Ner per mg of powder.

2.4.2. Size, PdI, and zeta potential of liposome formulations

The mean size and polydispersity index (PdI) values of the freeze-dried CLs and DCLs suspended in water were determined by dynamic light scattering (DLS) using a Malvern Zetasizer (3600, Orsay, France). The zeta potential was determined using a Malvern Zetasizer nanoSeries. Each sample was diluted to obtain a count rate between 150 and 300 kilo counts per second. The measurements were repeated in triplicates at 25 °C.

2.4.3. Transmission electron microscopy analysis of liposome formulations

The morphologies of the liposomal formulations were determined by transmission electron microscopy (TEM) using a Philips CM120 microscope (Eindhoven, Netherlands) at the "Centre Technologique des Microstructures" (CTµ) at the University of Lyon (Villeurbanne, France). The analyses were performed at an acceleration of 80 kV. One drop of each sample was placed on a copper grid coated with carbon. After 2 min, the excess sample was removed using a filter paper to obtain a thin film on the grid. Negative staining was performed using a 1% sodium silicotungstate solution. After 30 s, the excess sodium silicotungstate solution was removed with filter paper, and the sample was then left to dry at room temperature.

2.4.4. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) analyses were performed using an FEI Quanta 250 FEG microscope at the "Centre Technologique des Microstructures" (CTμ) at the University of Lyon (Villeurbanne, France). Freeze-dried samples of HP-β-CD, HP-β-CD/Ner complexes, blank liposomes (CLs or DCLs), or Ner-loaded liposomes (CLs or DCLs) were deposited on a flat steel holder. The samples were then coated under vacuum by cathodic sputtering with copper and observed by SEM under an accelerating voltage of 10 kV.

2.5. Preparation and quality evaluation of orange juice samples

Valencia oranges (*Citrus sinensis*) were purchased from a Lebanese local market. Unblemished fruits were selected, washed, and squeezed using a manual hand juicer. Physicochemical parameters (pH, titratable acidity, and Brix degree) were assessed to evaluate the quality of the orange juice during the experimental procedures. The pH of orange juice was determined using a pH meter (Orion Star A111, Thermo Scientific, USA). The pH measures the total concentration of free H^+ ions in a solution. The titratable acidity (TA) was determined by titration with a sodium hydroxide (0.1 N) solution. The TA represents the total concentration of organic and inorganic acids present in the juice and is expressed as grams of citric acid per litre of orange juice, as citric acid is the major organic acid in orange juice. The total soluble solids (Brix degree) was determined by refractometry using a hand refractometer (713528, Carl Zeiss Jena, Germany). The Brix degree is an indicator of the sugar content in juice, where 1 Brix degree is expressed as 1 g of sucrose in 100 g of juice.

2.6. Bacterial studies

2.6.1. Bacterial survival and proliferation in orange juice

The ability of *L. fermentum* to survive or proliferate in orange juice was investigated at two different storage temperatures (4 and 25 °C). In addition, the sensitivity of *L. fermentum* to the pH of orange juice was investigated at pH values between 2.5 and 3.9. When needed, the pH of the orange juice samples was lowered using a citric acid solution (3 M). A 200-μL volume of a diluted bacterial suspension (25×10^5 CFU/mL) was added to 4.8 mL of orange juice in glass culture tubes (20 × 100 mm) under sterile conditions to yield an initial bacterial count of 10^5 CFU/mL in juice. Control tubes contained orange juice without *L. fermentum*. The bacterial counts were then determined at 0, 24, 48, and 96 h using the plate count method. To this end, samples were serially diluted in peptone water, and 100 μL of each dilution was spread onto MRS agar. The bacterial counts were then determined after incubating the plates at 37 °C for 22 h. Each experiment was performed in triplicate.

2.6.2. Antibacterial activity of Ner solubilized in DMSO in refrigerated orange juice

Ner was solubilized in DMSO and tested against *L. fermentum* in orange juice (pH adjusted to 3.3) at different concentrations. Twenty five microliters of DMSO-solubilized Ner was added to 4.8 mL of orange juice (pH 3.3, TA of approximately 12 g/L) in glass culture tubes (20 × 100 mm) under sterile conditions to yield a range of Ner concentrations (between 50 and 4 000 μM). Subsequently, 200 μL of a diluted *L. fermentum* suspension (25×10^5 CFU/mL) was added, and the initial bacterial count in the culture medium was approximately 10^5 CFU/mL. Each test was performed in triplicate under sterile conditions. Orange juice supplemented with DMSO (25 μL), with or without *L. fermentum*, served as controls for bacterial growth. In addition, control tubes containing orange juice alone were used to exclude the possibility of contamination with lactic acid bacteria. The prepared tubes were then incubated at 4 °C, and changes in the bacterial counts were followed over 10 days of incubation at 4 °C by the plate count method as described above.

2.6.3. Antibacterial activity of encapsulated Ner in refrigerated orange juice

HP-β-CD/Ner complex and Ner-loaded CLs and DCLs were tested against *L. fermentum* in orange juice. Each freeze-dried formulation was added to fresh orange juice (4.8 mL) under sterile conditions to yield the desired concentration of Ner (2 000 or 4 000 μM). The tubes were then inoculated with *L. fermentum* as previously described and incubated at 4 °C. The bacterial count was assessed over time (20 days) using the plate count method. The experiment was repeated in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using Student t-test, and *P* values equal to or less than 0.05 were considered to be significant.

3. Results and discussion

3.1. Characterization of the HP-β-CD/Ner complex, CL, and DCL systems

3.1.1. Determination of the mass of Ner/mass of freeze-dried encapsulation system

The HPLC analysis results showed that the retained mass of Ner in the HP-β-CD/Ner complexes, CLs, and DCLs was 40, 30, and 15 μg Ner/mg, respectively.

3.1.2. Size, homogeneity, and morphology of freeze-dried liposomes reconstituted with water

The DLS analysis of the four liposomal formulations (blank CLs, Ner-loaded CLs, blank DCLs, Ner-loaded DCLs) showed three populations, in contrast to the two populations obtained by laser granulometry analysis of non-freeze dried suspensions (Azzi et al., 2018). The PdI values of these samples were higher than 0.48 (Table 1), highlighting the low homogeneity of the suspensions. Moreover, the second population represents the major population and had a mean particle size of approximately 1 μm, which is higher than that previously reported by Azzi et al. (2018). Indeed, the lyophilization process may promote liposome aggregation and fusion. Gharib et al. (2017) previously reported on the increment of the size and the PdI of the unsaturated lipoid S100 liposomal suspensions during the freeze drying process, unlike liposomes prepared with saturated phospholipids (phospholipon 90 H). Similarly, in this study the liposomal formulations prepared with the unsaturated lipoid E80 demonstrated an increase of the size following the freeze-drying process.

Table 1
Mean size, PdI, and zeta potential values of blank CL and DCL, and Ner-loaded CL and DCL.

Delivery system	Population 1		Population 2		Population 3		PdI	Zeta potential (mV)
	(%)	Mean size (nm)	(%)	Mean size (nm)	(%)	Mean size (μm)		
Blank CL	24.6	176.3 ± 55.1	72.9	866.3 ± 392.4	2.6	5.0 ± 0.6	0.51	−32.7 ± 4.49
Ner-loaded CL	2.3	60.9 ± 15.2	97.7	1114 ± 917	–	–	0.52	−33.1 ± 3.79
Blank DCL	7.3	130.4 ± 33.1	76.2	925 ± 531.5	16.5	4.2 ± 0.9	0.50	−34.1 ± 5.39
Ner- loaded DCL	6.6	119.3 ± 21.6	74.0	925.7 ± 318.4	19.4	4.7 ± 0.7	0.49	−40.2 ± 5.42

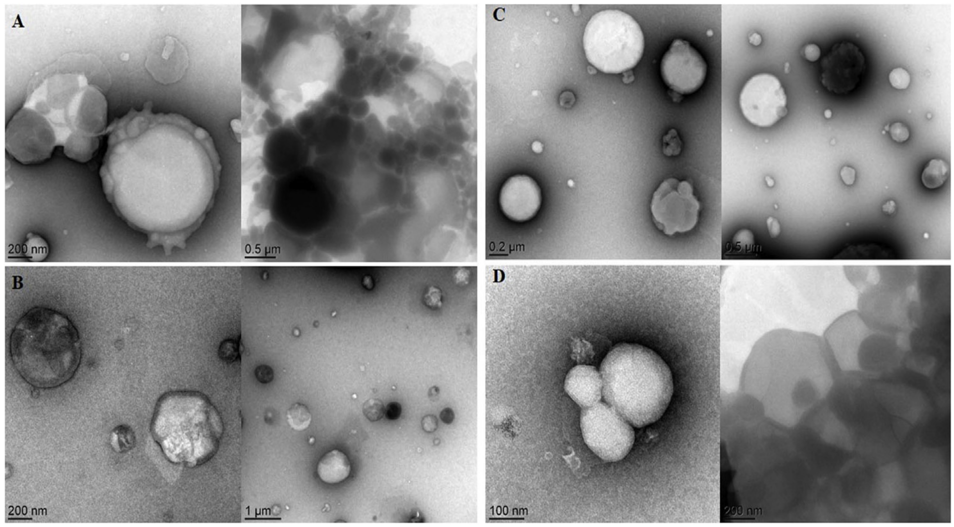

Fig. 1. TEM images of blank CL (A), Ner-loaded CL (B), blank DCL (C), and Ner-loaded DCL (D).

The incorporation of Ner in CL increased the size of particles, similarly to the results reported by Azzi et al. (2018). In contrast, Ner had no effect on the size of DCL particles. On the other hand, blank CL and DCL showed a negative zeta potential, which was not greatly affected by the presence of Ner. Also, the obtained zeta potential values were similar to those previously reported in literature for lipoid E80 liposomes (Karn et al., 2013).

TEM analysis supported the results obtained by DLS, as two populations were observed for blank and Ner-loaded CL and DCL systems (Fig. 1). However, particles with a mean diameter close to or higher than 2 µm were not observed by TEM for all formulations (Fig. 1).

SEM analysis of the freeze-dried CL and DCL showed irregular particles structures (Fig. 2). On the other hand, untreated HP-β-CD sample appeared as amorphous spherical particles, and the HP-β-CD/Ner inclusion complexes were observed as irregular particles (Fig. 3). Similar results were obtained by Figueiras et al. (2007) and Li et al. (2016) for untreated CD powder, CD/omeprazole, and CD/catechin inclusion complexes prepared by freeze-drying. The SEM images of HP-β-CD prepared in the same manner as the HP-β-CD/Ner complexes (dissolution, filtration, and lyophilization) were similar to those of the inclusion complexes. The SEM analysis results could not demonstrate the formation of the inclusion complexes, as the newly observed structures are a direct consequence of the preparation method.

3.2. Bacterial studies

3.2.1. Evaluation of orange juice quality parameters

Physicochemical quality parameters were assessed for all of the freshly-squeezed orange juice samples, which exhibited pH values between 3.5 and 3.9, titratable acidity (TA) values between 5 and 6 g/L of citric acid, and Brix degree values of approximately 11–12 °Brix. When orange juice was stored at 4 °C, the values of these parameters were maintained and were unaffected by the presence of *L. fermentum* due to the inhibition of bacterial proliferation and metabolic activity at this temperature. At 25 °C and after 96 h of incubation, the TA increased to 9.4 ± 0.45 g/L of citric acid, equivalent to that of orange juice spiked with *L. fermentum*, and the Brix degree was decreased by approximately 1 °Brix, whereas no significant changes were noted in the pH value (data not shown). In addition, similar variations in TA and Brix degree values were observed in the tubes without *L. fermentum* at 96 h due to the proliferation of naturally occurring spoilage bacteria or fungi in orange juice.

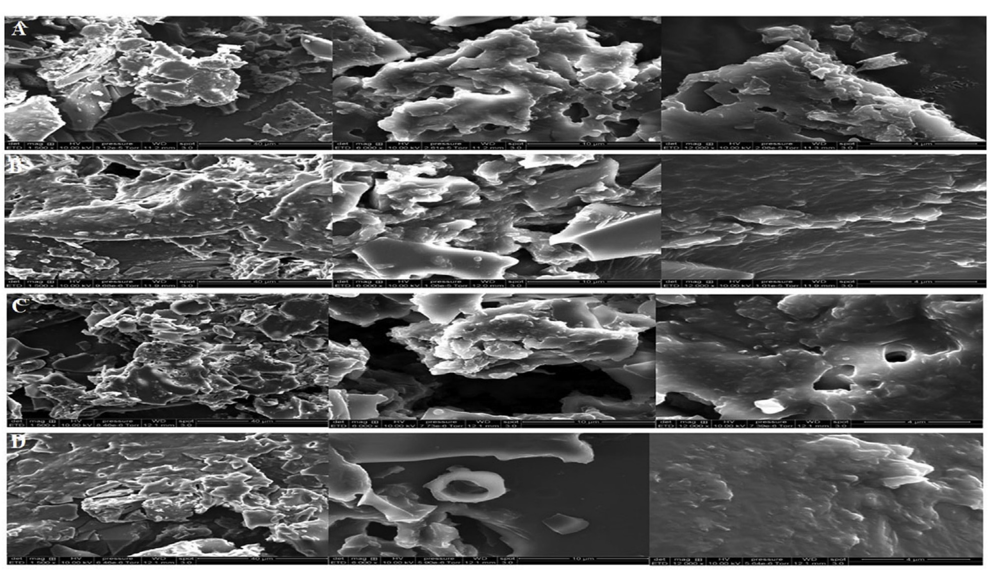

Fig. 2. SEM images of blank CL (A), Ner-loaded CL (B), blank DCL (C), and Ner-loaded DCL (D).

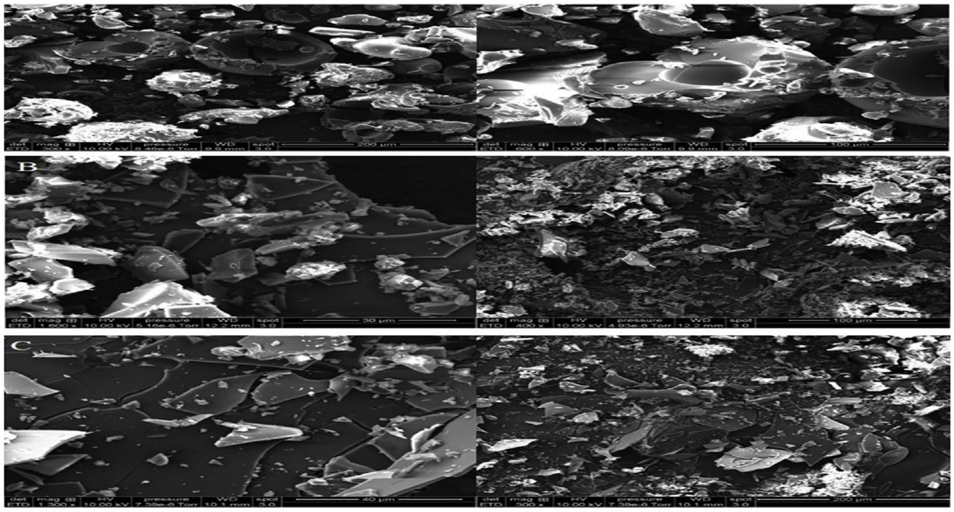

Fig. 3. SEM images of untreated HP-β-CD (A), HP-β-CD treated as HP-β-CD/Ner complex (B), and HP-β-CD/Ner inclusion complex (C).

3.2.2. *L. fermentum* survival and proliferation in orange juice

L. fermentum was able to survive in freshly-squeezed orange juice (pH 3.5–3.9; TA 5–6 g/L citric acid; 11–12 °Brix) at 4 °C until the end of the experiment (96 h) (Fig. 4) and exhibited exponential growth at 25 °C, with an approximately 5.5 log increase of the bacterial count after 96 h of incubation (Fig. 4). To further investigate the tolerance of *L. fermentum* to acidity in orange juice, the survival and proliferation of *L. fermentum* was tested at 4 and 25 °C in orange juice samples in which the pH was adjusted with citric acid (3 M) to values ranging from 2.5 to 3.3. At a pH value of 3.3 (TA of approximately 12 g/L citric acid), the bacteria were able to survive at 4 °C, and only a slight proliferation was observed at 25 °C (Fig. 4). At pH values lower than or equal to 3.0 (TA > 22 g/L citric acid), *L. fermentum* was unable to survive in orange juice at 4 and 25 °C (data not shown). These results indicate that a low pH and/or a high organic acid content in juice may inhibit bacterial growth and even lead to cell death. Furthermore, the effectiveness of organic acids as preservatives is enhanced at lower pH values, as they are active when present in their undissociated form. In the latter state, organic acids can easily penetrate the cytoplasmic membrane of bacteria and alter their internal pH. The pKa values of citric and malic acids, which are the primary organic acids of orange juice, are 3.13 and 3.40, respectively (Kim and Rhee, 2013). Thus, at pH values below 3.5, the two organic acids are primarily in their antibacterial, undissociated forms.

To avoid possible interference by other microorganisms and to perform the experiments under the storage conditions used for fresh orange juice, the antibacterial activity of Ner was assessed at 4 °C.

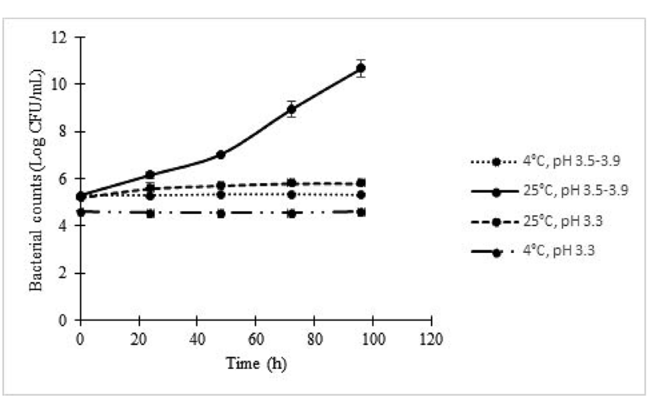

Fig. 4. *L. fermentum* survival and proliferation in orange juice at 4 and 25 °C, at different initial pH values. TA 5–6 g/L corresponds to a pH of 3.5–3.9; TA of 12 g/L corresponds to pH value of 3.3.

3.2.3. Antibacterial activity of Ner in refrigerated orange juice

Based on the ability of *L. fermentum* to survive in orange juice at a pH values higher than 3.0, the antibacterial activity of Ner was assessed in orange juice with a pH value of 3.3 (TA approximately 12 g/L citric acid) at 4 °C. Ner solubilized in DMSO was tested at concentrations between 50 and 4 000 µM. At concentrations lower than 2 000 µM (50-1 000 µM), Ner showed no antibacterial activity for up to 10 days of incubation (data not shown). At 2 000 and 4 000 µM, total bacterial death was obtained within 8 and 5 days of incubation, respectively (Fig. 5). Surprisingly, the effective concentration of Ner and the time needed to reach total bactericidal activity in orange juice (pH 3.3, 4 °C) increased by 40- and 12-fold, respectively, compared to that observed in MRS broth (pH 6.2, 37 °C) (Ephrem et al., 2019). The less potent and delayed effect of Ner in orange juice may be due to the influence of the incubation temperature and the rich matrix of the juice. Interestingly, Friedman et al. (2004) reported that the antibacterial activity of essential oil components against *Escherichia coli* and *Salmonella enterica* in apple juice was enhanced as the incubation temperature increased from 4 to 37 °C. Some orange juice components may also be major suppressors of Ner activity. For instance, the interaction between essential oils and food components may decrease the bioactivity of essential oil components in food products (Perricone et al., 2015).

Effective concentrations (2 000 and 4 000 µM) of Ner were directly added without DMSO to orange juice, and the antibacterial activities of solubilized and unsolubilized Ner in orange juice were compared. The results showed that similar antibacterial activities were obtained.

Nevertheless, at 2 000 and 4 000 µM, undissolved Ner particles were observed in the juice stored at 4 °C, demonstrating the need to solubilize Ner for use in juice. Thus, the antibacterial activities of the HP-β-CD/Ner inclusion complexes, Ner-loaded CL, and Ner-loaded DCL systems were investigated in fresh orange juice stored at 4 °C.

3.2.4. Antibacterial activity of encapsulated Ner in refrigerated orange juice

The HP-β-CD inclusion complex, CL, and DCL systems were tested for their ability to maintain or improve the antibacterial activity of Ner in orange juice (pH 3.3; TA: approximately 12 g/L citric acid; 11–12 °Brix). When Ner-loaded CLs and DCLs were added at a final Ner concentration of 2 000 µM, the antibacterial activity was considerably delayed, and no antibacterial activity was observed for up to 20 days of storage (data not shown). Compared to the CD inclusion complexes, the CL and DCL systems extended the release of Ner in water at 25 °C (Azzi et al., 2018). This result could be due to the strong retention of the

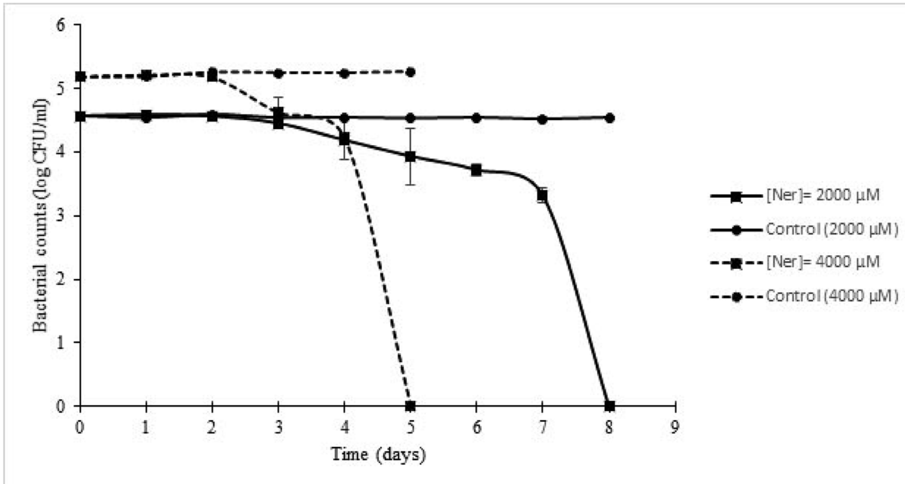

Fig. 5. Antibacterial activity of Ner at 2 000 and 4 000 µM in orange juice (pH 3.3; TA: approximately 12 g/L citric acid; 11–12 °Brix) at 4 °C against *L. fermentum*.

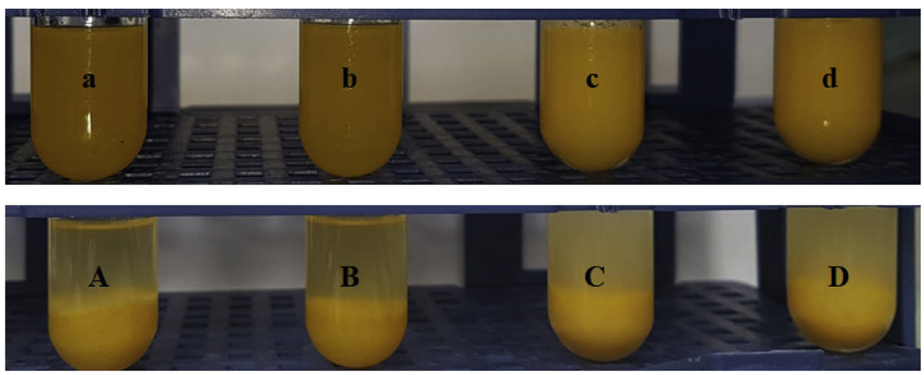

Fig. 6. Appearance of orange juice stored at 4 °C in the absence (a and A) and presence of HP-β- CD/Ner complex (b and B), Ner-loaded CL (c and C), and Ner-loaded DCL (d and D), at t = 0 and after 24 h of incubation, respectively. All delivery systems were added to give a final Ner concentration of 2 000 µM.

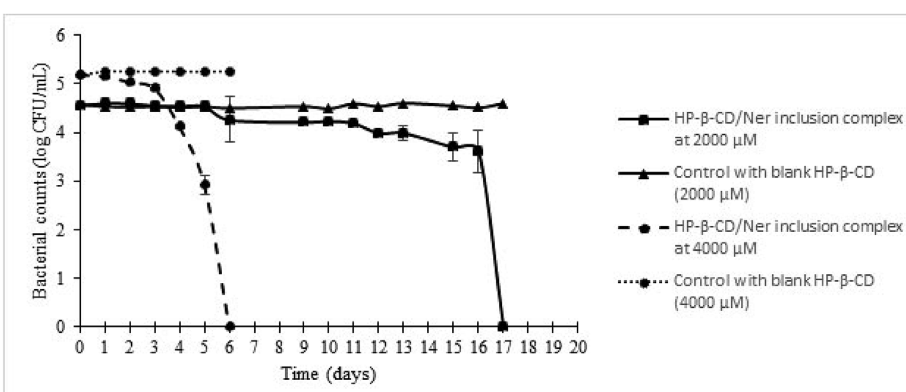

Fig. 7. Antibacterial activity of HP-β-CD/Ner complex in orange juice (pH 3.3; TA: approximately 12 g/L citric acid; 11–12 °Brix) at 4 °C against *L. fermentum*. HP-β-CD/Ner complex is added to orange juice to give a final concentration of 2 000 µM and 4 000 µM of Ner.

loaded agent in liposomes (CLs and DCLs) compared to the inclusion complex system. Furthermore, to be released, Ner needs to overcome the bilayered membrane of CLs and the inclusion complex in addition to the membrane in DCL. Even though this approach increases the photostability of Ner and enhances its stability during storage (Azzi et al., 2018), the delayed release abrogates the antibacterial activity of Ner in orange juice. Thus, the maintained Ner concentration in contact with *L. fermentum* is not effective against the bacterial cells. Moreover, the CL and DCL systems may have specific limitations in fruit juice applications at the tested concentration due to their milky appearance (Fig. 6). However, the HP-β-CD/Ner inclusion complexes did not alter the appearance of the juice (Fig. 6) and showed a total bactericidal activity in orange juice against *L. fermentum* within 17 days of incubation at 2 000 µM and 4 °C (Fig. 7). Therefore, only the inclusion complex was assessed in subsequent investigations.

When the HP-β-CD/Ner inclusion complexes were added at a final Ner concentration of 4 000 µM, the appearance, pH (3.3), and TA (approximately 12 g/L citric acid) of the juice were not altered. However, a slight change was observed in the Brix degree value, which increased from 11 to 12 to 13 °Brix. Moreover, HP-β-CD/Ner inclusion complex killed all bacteria within 6 days of incubation at 4 °C (Fig. 7). Thus, the inclusion complex showed a bactericidal activity against *L. fermentum* approximately that was 3-fold faster at 4 000 μM than at 2 000 μM (Fig. 7). This result may highlight the higher aqueous solubility of Ner incorporated in inclusion complexes compared to free Ner. Furthermore, the incorporation of Ner into inclusion complexes was observed to increase its aqueous solubility by more than 100-fold at 25 °C (Azzi et al., 2018). However, HP-β-CD/Ner inclusion complexes showed delayed antibacterial activity compared to the free form (8 versus 17 days at 2 000 μM and 5 versus 6 days at 4 000 μM). This delay was even more pronounced at 2 000 μM (nine-days delay) compared to 4 000 μM (one-day delay). This delay may be due to the release kinetics of Ner from the inclusion complexes. Azzi et al. (2018) reported the release of approximately 45% of Ner from inclusion complexes within 8 h, followed by a slow release over 7 days at 25 °C in water. Therefore, at 4 000 μM, the available concentration of Ner for interacting with *L. fermentum* in juice is approximately 2 000 μM after 8–10 h of incubation, with complete bacterial killing observed within 6 days compared to 5 days for free Ner. Shah et al. (2012) also reported a better antibacterial activity of free thymol compared to thymol nanodispersion. However, different factors limit the application of free hydrophobic molecules in food rich in water, such as fruit juice, including the appearance of undissolved particles (Shah et al., 2012) and the low physicochemical stability of the added agent. In this study, agglomerates of free Ner were observed in orange juice at the assayed concentrations. On the other hand, Ner has a poor photostability, which was overcome by its incorporation into HP-β-CD inclusion complexes (Azzi et al., 2018).

4. Conclusions

In this study, the use of HP-β-CD/Ner inclusion complexes was observed to be effective against *L. fermentum* in orange juice (pH 3.3; TA: approximately 12 g/L citric acid; 11–12 °Brix) at 2 000 and 4 000 μM, although faster bacterial killing was obtained at 4 000 μM. The addition of the inclusion complexes to the juice at 4 000 μM did not alter the appearance, pH, and TA of juice, and only a slight increase in the Brix degree value was observed. In contrast, the Ner-loaded CL and DCL systems (2 000 μM) strongly altered the physical appearance of the juice and did not exhibit an antibacterial effect within 20 days. According to the results obtained in this study, we can suggest that a combination of cold storage and HP-β-CD/Ner inclusion complexes limits the bacterial spoilage of fresh orange juice. In addition, assessing the use of a combination of the three systems may be interesting, as the inclusion complexes exhibits rapid activity against the bacterium, whereas the liposomal formulations extended the presence of Ner in the juice.

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.

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