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Antifungal properties of phosphatidylcholine-oleic acid liposomes encapsulating garlic against environmental fungal in wheat bread

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ABSTRACT

Liposomes have gained great interest in the food and pharmaceutical industry as colloidal carriers of bioactive compounds. In this work, liposomes of phosphatidylcholine (PC) and oleic acid (OA) encapsulating garlic extract (GE) were developed to determine its aptitude as antifungal agent in wheat bread. The influence of GE on the properties of liposomes were followed by determination of size, Zeta potential, Fourier transform infrared patterns (FTIR), morphology, differential scanning calorimetry (DSC) and thermogravimetric (TGA) techniques. The produced PC-OA-GE liposomes showed spherical morphology with narrow size distribution, entrapment efficiency of 79.7% and zeta potential of −27.9 mV. *In vitro* antifungal test showed noticeable inhibitory activities for free and encapsulated GE against selected fungal strains. TGA analysis revealed that the presence of OA and GE in the formulation retards the liposomal thermal decomposition, as compared with the pure PC liposomes and the DSC enthalpy and main transition temperature variation in PC-OA-GE liposomes suggested a strong heat-induced rigidifying effect that could be attributed to the presence of garlic polysaccharides in the liposome surface, observed by FTIR. In the *in situ* test, the bread formulations with free or liposome-encapsulated GE (0.65 mL/100 g of dough) were microbiologically more stable as compared with the controls, showing mold inhibition for five days. Therefore, liposomes formulated with OA and GE showed potential as natural antifungal agent in bakery products.

1. Introduction

Bread is an important food constituent of the diet in many countries and is consumed daily almost over the world. Bread spoilage by microorganism is a serious concern for consumers and the resulting waste is also a problem that causes high economic losses in bakery industry and consumers (Melikoglu and Webb, 2013). Bacteria, yeast and molds can cause spoilage of bread. However, contamination originates mainly post baking by fungal spores being deposited from the bakery environment (Spicher, 1980). The principal factors to controlling the growth of spoilage fungi on food stuffs are oxygen, temperature, pH, and water activity (a_w); generally, breads have relatively high moisture content and a_w between 0.94 and 0.97 at a pH of about 6 on sliced, prepacked and wrapped breads being very susceptible bakery products for fungal spoilage (Hager et al., 2012). Apart from the unpleasant sight of visible mold growth, fungi can also produce of mycotoxins and off-flavors (Magan et al., 2003). Thus, spoiled breads represent a hazard and can cause health problems to the consumer.

The use of biopreservatives in food has become an increasingly important field of research. This trend is partially driven by the emergence of an increasing number of reports linking chemicals in foods with the development of chronic diseases (Tajkarimi et al., 2010; Seow et al., 2014). Additionally, this term can also be used in bio-active plant ingredients or plant extracts as alternative to the weak organic acids such as propionic and sorbic acids, which are commonly added as chemical preservatives to prevent the growth of unwanted microorganisms and improve the shelf life of bakery products (Marin et al., 2003; da Cruz Cabral et al., 2013). Nevertheless, the disadvantages associated with the use of chemical preservatives, has motivated the research of alternative natural agents to control spoilage fungi in food products.

Garlic (*Allium sativum*) has been known for centuries as important food flavoring agent with recognized medicinal properties (Martins et al., 2016). Several studies revealed that garlic has antimicrobial activity against bacteria and fungi (Borlinghaus et al., 2014; Fratianni et al., 2016; Pinilla et al., 2017). The principal constituents of garlic associated to its bio-active properties are the allicin, ajoene, thiosulfinates and others organosulphurate compounds (Ledezma and Apitz-

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Castro, 2006). It is related that allicin and thiosulfinates, the compounds responsible for the strong flavor of garlic, can decompose under different temperature and pH conditions to form additional sulfur constituents that include diallyl, methyl allyl, and diethyl polysulfides, the vinyldithiins and also the antimicrobial compounds (*E*)- and (*Z*)-ajoene (Rose et al., 2005). The sensitive character and especially the strong odor of garlic compounds, restricts its application in food products and only a few technological approaches have been reported to overcome this limitation (Wang et al., 2012).

In this context, the use of nanocarrier systems like liposomes may stabilize different bioactive compounds against environmental and chemical changes as well as increase their bioavailability and stability, improving its shelf-life (Mozafari et al., 2008). Liposomes are well known as spherical bilayer with an aqueous core inside. This vesicles are made by phospholipid membranes with amphiphilic characteristics and present multiple advantages such as biodegradability, biocompatibility and controlled release behavior of water-soluble, lipid-soluble, and amphiphilic compounds (Neethirajan and Jayas, 2011; Brandelli et al., 2017). The antimicrobial activity of liposomes containing garlic extract (GE) was related in our previous work (Pinilla et al., 2017) and it was possible to conclude that liposomes constitute a suitable system for encapsulation and controlled release of garlic active compounds with inhibitory effect against *Listeria* spp.

The present study aimed to produce phosphatidylcholine-oleic acid liposomes as carrier for GE and investigate its potential as inhibitor of spoilage fungi in wheat bread. The physicochemical and morphological characteristics of PC-OA-GE liposomes were investigated as well the antifungal properties through both *in-vitro* and *in-situ* assays.

2. Materials and methods

2.1. Garlic extracts production

Preparation of garlic extract (GE) was carried out according to our previous work (Pinilla and Brandelli, 2016). Briefly, selected bulbs of garlic acquired from a local market at Porto Alegre (Brazil) without injuries or contamination were washed with purified water; then were cut in small pieces and mixed in a ratio 1:1 (w/v) with distillated water. The garlic juice (50%, w/v) was sonicated at 40 kHz for 15 min in a bath-type ultrasound (Unique USC 700, São Paulo, Brazil) at room temperature and centrifuged 10 min at 10,000*g* under refrigeration at 4 °C. Thereafter, the supernatant obtained was filter sterilized with 0.22 μm membrane (Sartorius, Göttingen, Germany) and stored at 4 °C for further use.

2.2. Preparation of liposomes

The GE liposomes were produced according to Pinilla and Brandelli (2016), by the thin-film hydration method with some modifications. Briefly, purified phosphatidylcholine (PC) Phospholipon 90G® (Lipoid, Ludwigshafen, Germany) and oleic acid (Labsinth, São Paulo, Brazil) at molar ratio (3:1) were dissolved in mixture chloroform/methanol (1:1 v/v) in a round-bottom flask, the solvent was removed by rotary evaporator at 40 °C, until obtaining a thin film on the flask wall and finally dried in desiccator for 24 h. The obtained lipid film was recovery by the addition of 4 mL of GE and 2 mL of 10 mmol/L phosphate buffer (pH 7), then vortexed at 50 °C in three times for 30 s, sonicated in an ultrasonic cell disrupter (Model DES500, Unique Group, São Paulo, Brazil) and finally sterilized by filtration through 0.22 μm membranes (Sartorius, Göttingen, Germany). In addition, empty liposomes with and without oleic acid (OA) were prepared with 10 mmol/L phosphate buffer pH 7.0 and used as control.

2.3. Size, polydispersity, zeta potential and morphological characterization

The size of and polydispersity index (PDI) of nanovesicles were determined by dynamic light scattering essentially as described elsewhere (Teixeira et al., 2008; Lopes et al., 2017). The measurements of size and PDI were made using a Brookhaven system (He−Ne laser of 35 mV) in a fixed scattering angle of 90°. The zeta potential analyses of liposomes were carried out after dilution (1:100) on purified water, using a Zetasizernano-ZS ZEN 3600 (Malvern Instruments, Worcestershire, UK), the mean value was determined by repeating the reading by 10 times. The vesicles morphology was observed by transmission electron microscopy (TEM). Each liposome sample was stained in 25 g/L uranyl acetate on a copper grid with formvar-coated of 300 mesh. The stained and dried samples were visualized on an electron microscope (JEM1200, Jeol Ltd., Tokyo, Japan).

2.4. Entrapment efficiency

The encapsulation efficiency (EE) of PC-OA-GE liposomes was determined considering the allicin as main component of the GE. The HPLC method for allicin described by Liang et al. (2013) was employed, using ethylparaben as secondary reference standard for allicin. Freshly prepared samples of PC-OA-GE liposomes were separated from the liquid media by membrane ultrafiltration (Ultracel YM-10; Millipore, MA, USA) and the filtrate without liposomes was used for determination of total allicin concentration by HPLC. The EE *v*alues were calculated by the equation:

$$\%EE = \frac{[\text{Allicin used in preparation}] - [\text{Allincin in the filtrate}]}{[\text{Allicin used in preparation}]} \times 100$$

The chromatographic analyses were performed in a HPLC (Shimadzu HPLC, Kyoto, Japan) equipped with quaternary pumps (LC-20 CE) and diode array detector (DAD) (SPD-M20A), using an XBridge® C18 (150 mm 4.6 mm, 5 mm) column, with an isocratic flow (0.7 mL/min) of methanol:water (65:35 v/v) acidified with formic acid (0.04%, v/v) for 15 min. The column temperature was maintained at 20 °C and the volume injected in the equipment was 10 μL for sample. Mobile phases were filtered using 0.22 μm nylon filters and degassed previous to use.

2.5. Fungal inhibition assays

For determination of *in-vitro* antifungal activity, the selected fungal strains *Penicillium expansum*, *Aspergillus niger*, *Penicillium herquei*, *Fusarium graminearum* and *Aspergillus flavus* were obtained from the culture collection of the Laboratório de Toxicologia de Alimentos (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) and cultured on PDA plates for 5 days at 30 °C. A sterile solution of Tween 80 at 0.05% (v/v) was transferred on each plate and the spores harvested with a Drigalski loop. This suspension was collected and the concentration of spores was determined and adjusted with sterile distilled water until reaches 1×10^6 spores/mL using a Neubauer chamber. For antifungal activity tests, a solution of sterile PDA with 1% of this spore suspension was homogenized at 45 °C and transferred to a sterile plate. After solidification of the media, sterile paper disks were placed on the agar plates and then 20 μL of each sample was transferred to the disks. The diameters of inhibition halos were measured after incubation for three days at 30 ± 2 °C.

2.6. FTIR analysis

In order to investigate the possible alterations occurred in phospholipid membranes, the FTIR spectra of the liposomes and its components were acquired, using a Bruker Alpha FTIR spectrometer (Bruker, Billerica, MA, USA) in the range of 4000 to 400 cm^{-1}. The spectral resolution of the spectrometer is 4 cm^{-1}. Samples of PC, PC-oleic acid and PC-OA-GE liposomes, were freeze dried before the analysis, mixed with KBr and then pressed into a tablet.

2.7. TGA and DSC analyses

The thermal stability evaluation was carried out in a thermogravimetric analyzer model TGA Pyris 1 (Perkin Elmer, Shelton, CT, USA). The lyophilized samples of liposomes were heated from 25 °C to 600 °C at the rate of 20 °C/min under nitrogen atmosphere (flow rate 40 mL/min). Differential scanning calorimetry (DSC) studies were developing in a DSC Q 8500 apparatus (Perkin Elmer, Shelton, CT, USA). Liquid samples were pre-concentrated in a factor of 1:3 using an Ultracel YM-50 Membrane (Millipore, Burlington, MA, USA), then approximately 10 mg were placed in aluminum pans and heated from −30 to 100 °C with a heating rate of 10 °C/min under nitrogen atmosphere. A clean and empty pan sealed with it cover was used as a reference sample.

2.8. Bread preparation and in situ antifungal activity

Bread was manufactured at Cereal Laboratory of Food Science and Technology Institute (Porto Alegre, Brazil) and all the bread ingredients were obtained from local suppliers of bakery products. The loaf bread recipe included 500 g wheat flour free of antifungal compounds (Orquidea, Caxias do Sul, Brazil), 15 g vegetal oil (Klemm & Cia, Santa Cruz do Sul, Brazil), 10 g baker yeast (Fleischmann, Petrópolis, Brazil), 10 g NaCl, 25 g sucrose, 0.045 g ascorbic acid (Granolab, Araucaria, Brazil), 300 mL tap water and 5 mL of free or liposome-encapsulated GE to achieve a concentration of 0.65 mL/100 g of dough. The ingredients were kneaded for 5 min, then the bread dough was maintain resting for 10 min and cut in pieces of 165 g before fermentation at 30 °C for 90 min. Baking was performed at 220 °C for 20 min in a deck oven (Tadesco ITT150E, Caxias do Sul, Brazil). The loaves were kept for 60 min on cooling racks at room temperature. Each bread was cut in 3 slices with approximately 10 cm high by 3 cm wide. The slices were exposed to the laboratory environment for 5 min and after that, packet in plastic bags, which were closed and stored at room temperature during 15 days. Every day until 15 day of analysis, the bread slices were examined to determine the visible fungal growth and to estimate if shelf life was improve or not. The breads were baked in triplicate, and the slides of the each assay were analyzed twice. The bread samples were monitored using the mold environmental challenge method, based on the analysis of the fungal outgrowth as a percentage of the total area of each bread slice (Axel et al., 2015).

2.9. Statistical analysis

Results are presented as average ± SD. Statistical analysis was carried out by ANOVA test followed by Tukey test, using SSPS software package 16.0 (IBM SPSS Statistics, Inc., Chicago, IL, USA), and differences between groups were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Liposome characterization

Different liposome formulations prepared by the thin film method were characterized. Garlic extract loaded PC-OA liposomes differ ($P < 0.05$) with the liposomes without GE, in the parameter of particle size, polydispersity index (PDI) and zeta potential (Table 1). The empty PC liposomes presented higher size and lower zeta potential than the PC-OA and PC-OA-GE liposomes that showed the narrow size distribution (110–140 nm), similar to previous reports on PC-OA liposomes (Srisuk et al., 2012). The polidispersivity index reflects the particle size distribution and is often considered satisfactory when it is below 0.3 that indicate a narrow distribution of the liposomes. In this work, PDI values oscillated between 0.32 for empty liposomes and 0.16 for PC-OA-GE liposomes. The size distribution histograms of PC-OA-GE liposomes and the controls PC and PC-OA liposomes are presented in the Fig. 1A. The formulations of control liposomes showed a broader monomodal size distribution profile, as result of variability in the size distribution profile in the samples. On the other hand, the PC-OA-GE liposomes showed a narrow monomodal curve, and 90% of the nanovesicles had diameters smaller than 150 nm (Fig. 1A). The PC-OA-GE liposome formulations formed stable translucent suspensions as compared with the controls without GE (Fig. 1B).

Table 1
Characterization of PC-OA liposomes containing garlic extract.

	PC	PC-OA	PC-OA-GE
Particle size (nm)	172.2 ± 5.5[a]	143.1 ± 21.7[b]	113.3 ± 3.3[c]
Polydispersity	0.33 ± 0.56[a]	0.32 ± 0.41[a]	0.16 ± 0.31[b]
Zeta potential (mV)	−13.9 ± 4.5[a]	−36.7 ± 7.8[b]	−27.9 ± 6.5[b]
EE (%)	–	–	79.7 ± 2.2

Different letters (a, b, c) indicate significant differences ($P < 0.05$).
Values are the means ± standard deviation of three independent experiments.

High value of electronegative zeta potential is indicative of a good stability of the liposomal suspensions due to the increase of electrostatic repulsion among liposomes (Müller et al., 2001). The suspension of PC-OA-GE-liposomes had a zeta potential value estimated as −24.89 mV (Table 1) with no significant difference with the PC-OA. The addition of fatty acid resulted in a decrease in size and an increase in zeta potential of liposomes due to the changes in the membrane packing of vesicles (Lee et al., 2017), and this characteristics of size and charge make it possible that the particles with the same charge can repel each other providing stability and resistance to aggregation (Sou, 2011). The presence of oleic acid improved the size and PDI parameters and increased the surface charge, providing better stability as compared with the PC-GE liposomes developed in our previous work. Those liposomes encapsulating GE presented an average particle size of 174.6 nm with a PDI of 0.26, thus showing a larger mean diameter, broader size distribution, and zeta potential value of −16.2 mV (Pinilla et al., 2017).

Allicin concentration in the GE was 1.82 mg/mL. The developed liposomes containing GE and oleic acid showed an encapsulation efficiency (EE) of 79.7% for allicin. This value was higher as compared with those obtained in other studies that have been conducted for liposome encapsulation of allicin into matrices like lecithin-cholesterol at 3.77:1 ratio, reaching EE values about 75.2% (Lu et al., 2014) and using pure PC with maximum EE of 47.5% (Pinilla et al., 2017). Allicin was the unique component analyzed for EE determination, but the garlic extract has other water-soluble components that may be present in the liposomes. These include flavonoids, phenols and other radical scavenging compounds that potentially increase its bioactive properties and applications.

The prepared liposomes were analyzed by TEM. The structure of liposomes containing OA as wall material and loading GE can be observed in Fig. 1C. The electronic microscopy revealed spherical structures for both PC-OA-GE and PC liposomes, showing typical sphere-shaped morphologies found in PC liposomes containing peptides (Malheiros et al., 2011; Yan et al., 2014) (Fig. 1D). In our previous work (Pinilla et al., 2017), it was observed that the GE liposomes prepared only with PC presented amorphous structures with a tendency to form aggregates. Thus, the addition of OA to the preparation, improved its morphology and stability.

3.2. Antifungal activity

The formulated liposomes with GE were initially tested against environment and pathogenic fungi *P. expansum*, *A. niger*, *P. herquei*, *F. graminearum* and *A. flavus*. The control PC-OA liposomes caused no fungal growth inhibition, while GE and the liposomes containing GE showed inhibition against all tested fungi (Table 2). The inhibitory zones were significantly higher against *P. herquei*, *F. graminearum* and *A. flavus* as compared with those observed for *P. expansum* and *A. niger*. No significant difference ($P < 0.05$) was found between the inhibitory

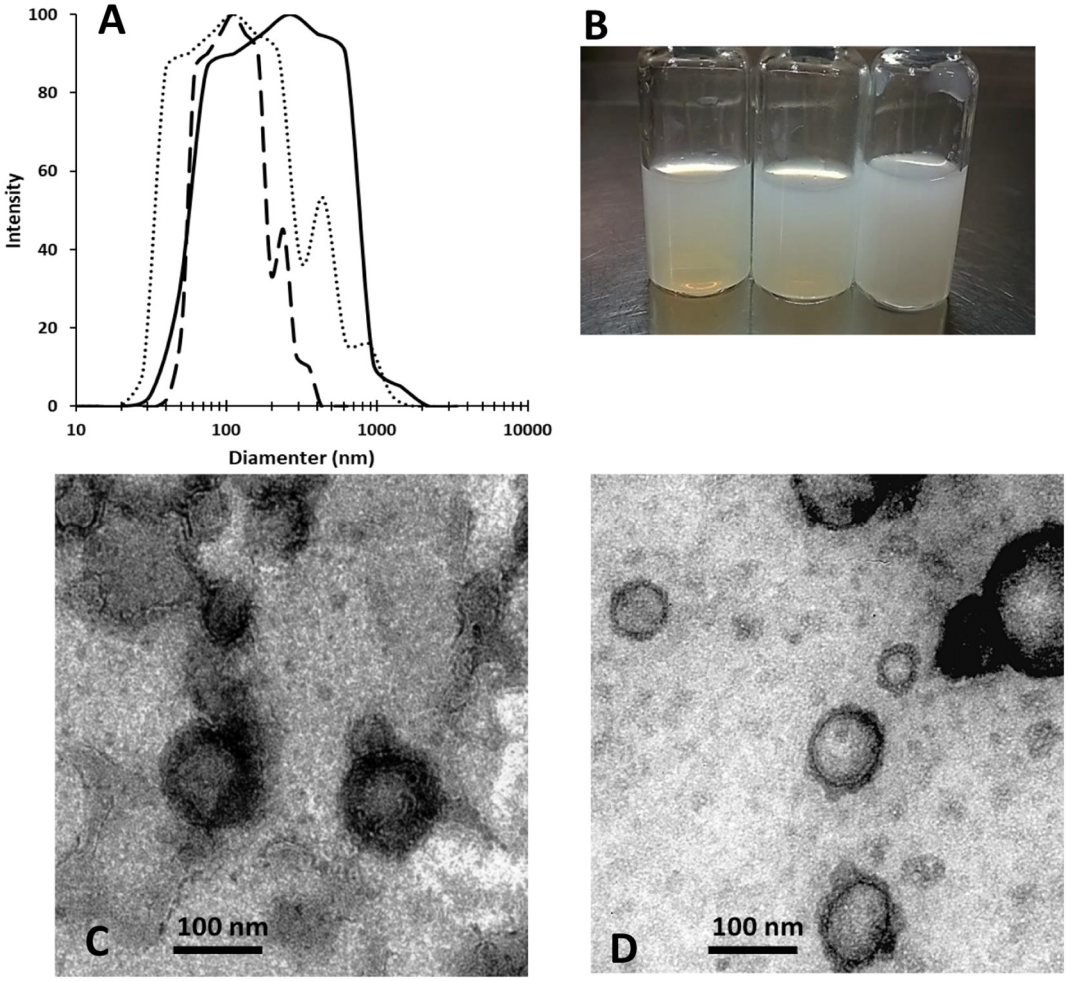

Fig. 1. (A) Histograms of size distribution obtained by laser dynamic light scattering of PC liposomes (full line), PC-OA liposomes (dotted line) and GE-PC-OA liposomes (dash line). (B) Image of the liposome suspensions, from left to right: GE-PC-OA, PC-OA and PC liposomes. (C,D) Transmission electron microscopy images of PC-OA-GE and control PC liposomes containing phosphate buffer, respectively. Bar = 100 nm.

Table 2
Inhibition of fungal growth by PC-OA liposomes containing garlic extract.

Fungi	GE	PC-OA-GE	PC-OA
	Inhibition halo (mm)		
Penicillium expansum	6.2 ± 0.8	6.5 ± 0.7	0.0
Penicillium herquei	34.4 ± 0.2	35.3 ± 0.5	0.0
Fusarium graminearum	33.6 ± 0.7	34.1 ± 0.9	0.0
Aspergillus flavus	34.1 ± 0.6	35.3 ± 0.5	0.0
Aspergillus niger	6.3 ± 0.4	6.1 ± 0.6	0.0

Results are the means ± standard deviations of three independent experiments. Garlic extract and PC-OA were used as controls.

zones caused by GE and PC-OA-GE liposomes. Using the same antifungal assay, a 16.3 mm inhibition halo was observed for 20 µg pure ketoconazole against the aflatoxin producer fungus *A. flavus* (Veras et al., 2016). In addition, inhibition halos ranging from 5 to 11.33 mm were reported in the antifungal activity of some GE prepared with two different varieties of garlic from the Campania Region, Southern Italy, against *P. expansum*, a mycotoxin-producer strain (Fratianni et al., 2016). According with the results obtained in this work (Table 2), it can be concluded that nanoencapsulation did not affect the antifungal activity of the GE.

3.3. Fourier transform infrared spectroscopy

The intermolecular interaction between the load compounds and the wall materials of liposomes commonly leads to changes in the FTIR patterns. Infrared spectroscopy can be used to evaluate structural and conformational changes produced by entrapment of different compounds at different parts of the phospholipid bilayer (Toyran and Severcan, 2003). The FTIR spectrum of the PC-OA-GE liposomes (Fig. 2D) showed characteristic peaks of phosphatidylcholine and oleic acid (Fig. 2A and B) corresponding to the asymmetrical and symmetrical $-CH_2$ stretching at 2924 and 2853 cm^{-1} and the C=O stretching at 1738 cm^{-1}. For the GE (Fig. 2C), it was observed the C=C stretching frequency of diallyl disulfide at 1630 cm^{-1}, vinyl group that appeared at 1026 cm^{-1} and the −OH broad stretching in 3358 cm^{-1} (Pinilla et al., 2017). The presence of these characteristic peaks in the FTIR spectrum of PC-OA-GE liposomes (Fig. 2F), indicated the presence and effective incorporation of GE into the nanovesicles. The infrared spectrum of PC-OA-GE liposomes showed no other specific peaks and no variations were found in wavenumbers at 2924 cm^{-1} and 2853 cm^{-1}, revealing no changes in the bilayer acyl chains. Recently, Ezer et al. (2017) reported that alliin increases membrane dynamics of DMPC liposomes and induces compositional and structural changes with significant increase due to the increased hydration of these polar groups. However, in the present work, no differences were observed in the absorption band at 1738 cm^{-1} (C=O stretching), evidencing the absence of structural changes associated with the hydration state of

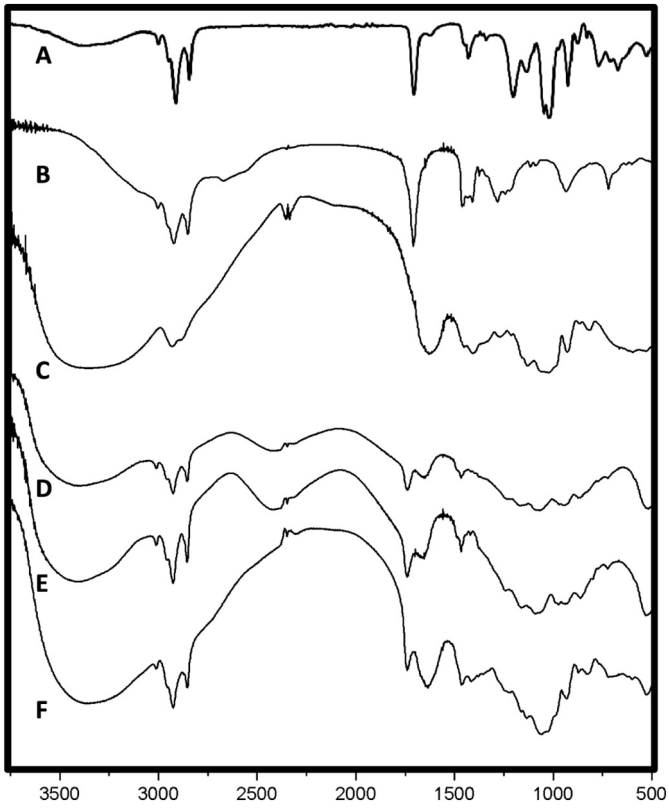

Fig. 2. FTIR spectra of (A) pure PC, (B) pure OA, (C) GE, (D) PC-liposomes, (E) PC-OA liposomes, (F) PC-OA-GE liposomes.

carbonyl groups on the membrane interfacial parts (Toyran and Severcan, 2003). For PC-OA-GE liposomes (Fig. 2F) in the spectral range of 1200–900 cm^{-1}, was possible observe a prevalence of bands attributed to C—C, C—O stretching and C—O—H, C—O—C bending, characteristic of polysaccharides as inulin, which could be present in the GE. The band at 1646 cm^{-1} observed in the PC and PC-OA empty liposomes (Fig. 2 D and E) used as controls, could be attributed to the bending vibration of water molecules in a weak phospholipid interlayer space (Marcos and Rodriguez, 2016). The FTIR pattern in the PC-OA-GE liposomes showed that no new chemical bonds were formed, indicating that the compounds of GE, PC and OA were combined through physical interaction.

3.4. Thermal properties

Thermogravimetric analysis (TGA) was used to determinate the physical and chemical changes of the freeze dried nanoliposomes when subjected to high temperatures. From TGA analysis, PC liposomes moisture content was 10.1%, for PC-OA liposomes 7.9% and for PC-OA-GE liposomes 6.9%. The TGA analysis (Fig. 3A) showed the degradation curves of the liposome samples. The first decay is related to the loss of moisture and loss of volatile compounds from de samples. Control PC liposomes showed an important weight loss (about 25 wt%) at 120 °C, corresponding to the evaporation of adsorbed water. In contrast, PC-OA and PC-OA-GE liposomes presented a slight weight loss (about 9 wt%) at 190 °C. From the curve of liposomes containing GE, the highest rate of degradation started at 190 °C and when reached 600 °C the residue was 28%, while the liposomes without GE presented a final residue of 48%. The better thermal stability below 200 °C of liposomes containing OA compared to pure PC liposomes could be due to the intercalated OA molecules in the bilayer. This interruption of tight packing of lipids may promote chain disorder and reducing the lipid mobility by the formation of a liquid/crystalline solution at room temperature (Ricker et al., 2003; Cacela and Hincha, 2006).

DSC results of fresh liposomal suspensions are presented in Fig. 3B. Fully hydrated PC-OA liposomes incorporating GE showed thermograms consisting of broad melting transitions, high enthalpy, abolition of the pre-transition and without visible liquid–crystalline phase transition in the range of temperatures tested (−30 to 90 °C). In DSC analysis, small molecules presented a very sharp peak, while for larger molecules, such as polymers or lipid bilayers, the melting transition is broad (Demetzos, 2008). Liposome suspensions were characterized by a highly cooperative endothermic and broad transition at temperatures between 3 °C to 9 °C. The PC-OA empty liposomes used as control, showed a minor endothermic event at 3.5 °C (ΔH = 128.36 J/g). The empty PC liposomes present the higher melting temperature (9.1 °C) and an enthalpy value of 263.20 J/g. In contrast, the PC-OA-GE liposomes showed a temperature transition of 5.41 °C and the higher endothermic event of 293.79 J/g. These findings indicated structural changes in the bilayer membrane of the liposomes containing OA and GE, probably owing to interactions with the polar head groups and their intercalation in the bilayer membrane must be lowered. Thus, the addition of GE into liposomes probably result in domains with heterogeneous distribution of PC and OA, increasing the ΔH values by induction of interdigitated gel phase and gives rise to more rigid bilayers. In agreement with our results, Marín et al. (2018) reported a subzero melting transition of phosphatidylcholine liposomes prepared with different food waste compounds. The ΔH was lower for empty PC liposomes (120.6 J/g) and higher (166.8 J/g) for PC liposomes containing shrimp lipid extract, concluding that liposomes loaded with lipophilic compounds may suffer a strong heat-induced rigidifying effect.

3.5. Antifungal activity in wheat bread

The presence of visible molds on the surface of bread slices is considered a critical point of spoilage evaluation for the consumers. The Fig. 4 presents the results of the assay and the characteristic behavior of mold growth in breads with the addition of PC-OA-GE liposomes and also for the control samples, during the 15 days of testing. A significant difference was observed between the results of mold growth in the breads treated with GE and the control ones without antifungal agent. With the addition of GE and PC-OA-GE liposomes the absence of molds was prolonged for at least 5 days compared to the non-treated control and the PC-OA control liposome, where the molds were visible and cover large portions of the slices after 2 days. This early presence of molds can be explained because wrapping can prevent moisture loss from the bread slices allowing suitable growth conditions for fungi in the humid atmosphere. At the end of the 11th day of storage, the control bread slices were completely covered by molds. In contrast, the treatments with free GE and PC-OA-GE liposomes showed only 4 and 2 slides totally moldy at the day 15, respectively.

Breads were baked at 220 °C and at this condition the crumb temperature is around 98 °C (Bosmans et al., 2013). Therefore, bread preservatives could resist to losses due to evaporation or decomposition during the baking process. Heat stability of liposomes content GE was showed in the Fig. 3. The results indicated good liposomal stability in temperatures around 100 °C and a rapid decomposition over 225 °C. The GE encapsulation into liposomes could prevent the loss of antifungal compounds by the high temperatures used in the bakery process, improving its retention in the matrix. In addition, the antifungal compound ajoene, formed when garlic juice is exposing at high temperatures could be formed during baking. Yoo et al. (2014) studied the optimal conditions for ajoene production from garlic juice, reporting that the temperature of reaction, possibly will be the key factor in the ajoene formation, due to the increases of temperature resulted in higher ajoene content in oil-macerated garlic and the optimal conditions for *E*- and *Z*-ajoene formation was 98.80 °C in 6.87 h and 42.24 °C in 9.71 h. Thus, the presence of native antifungal compounds of garlic and the possible formation of other preservatives such as ajoene could be

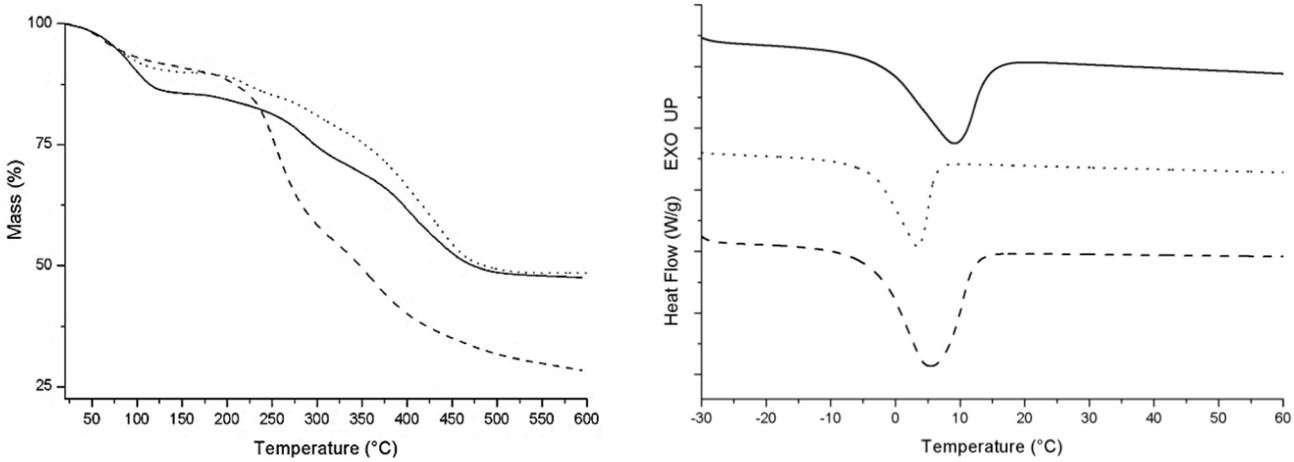

Fig. 3. Thermal analysis of liposomes. Samples of control PC liposomes (full lines), PC-OA liposomes (dotted lines) and GE-PC-OA liposomes (dash lines) were subjected to TGA (A) and DSC (B) analysis.

responsible for the positive results of free and encapsulated GE in mold control. However, additional studies must be conducted to determine the changes in the volatile compounds profile of garlic extract into liposomes at high temperatures.

This is the first report on free and nanoencapsulated GE used to prevent of mold spoilage for wheat bread. A complete review about the recent strategies for bread shelf life extension was published by Axel et al. (2016), where they highlighted the use of specific compounds produced by lactic acid bacteria that extend the shelf life of bread as the main area of study, followed by the use of antifungal peptides, ethanol and plant extracts as antifungal preservatives. These authors found only a few studies dealing with the direct addition of plant extracts in bakery products. In a more recent work, a challenge and shelf life tests were performed to investigate the mold inhibition potential of thyme oil in par-baked wheat and sourdough bread, finding initially *in-vitro* antifungal activity, but when performed an *in situ* test, using different concentrations of thyme oil (0.08, 0.15 and 0.20 mL/100 g dough), no clear shelf-life extension was observed in the samples of par-baked bread. According to the authors, the activity was affected by food matrix and for a not homogeneous distribution on the thyme oil in the bread (Debonne et al., 2018). In another study, Rizzello et al. (2017) used legume flour hydrolysates as ingredient for making bread under

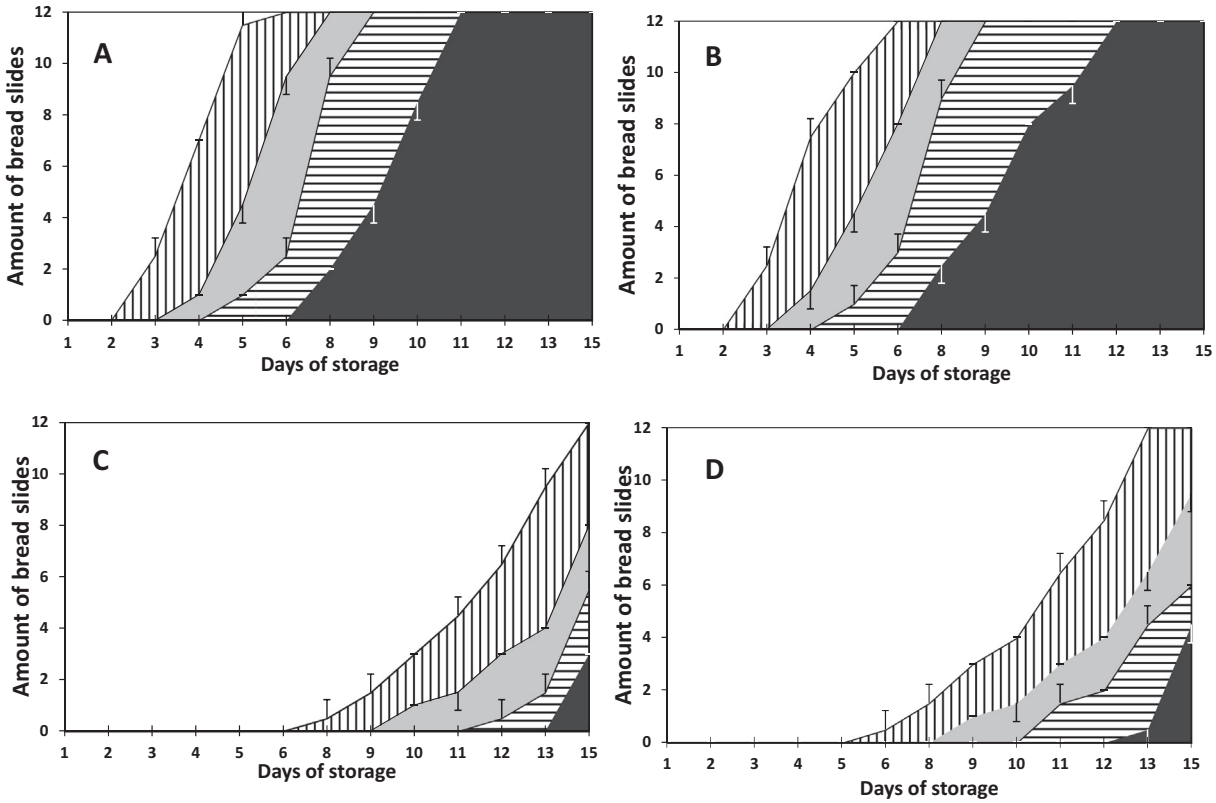

Fig. 4. Shelf life wheat bread slices with liposomes after challenging against environmental molds during a 15-day storage period. Bread spoilage is designated as percentage of the total surface area of each slice where fungal outgrowth was observed: Mold free slices (white area), 10% moldy (black vertical striped area), 10–24% (grey area), 25–49% (black horizontally striped area) and 50% moldy (black area). (A) Control without treatment, (B) control PC-OA liposomes, (C) free GE, and (D) PC-OA-GE liposomes. Values are means of duplicate samples originated for three independent fermentations each; error bars indicate standard deviations.

pilot plant conditions and observed that the hydrolysates extended the bread shelf-life, without affecting the rheological and sensory properties. According to the authors, the antifungal activity was attributed to specific peptides produced during the legume flour hydrolysis.

4. Conclusions

The addition of OA improved multiple properties of liposomes, such as size distribution, PDI and the entrapment efficiency of GE. The maintenance of antifungal properties of encapsulated GE compounds resulted in the absence of molds for prolonged time in baked wheat bread. PC-OA-liposomes entrapping GE could serve potential ingredients that improve the microbiological stability of baked food products, due to its thermal properties that make it possible their use at baking temperatures.

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Conflicts of interest

Authors declare no conflicts of interest regarding this study.

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