ADASSA GAMA TAVARES

ENCAPSULAÇÃO DE CARVACROL EM LIPOSSOMAS PARA APLICAÇÃO EM EMBALAGEM ATIVA PARA ALIMENTOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae.*

Orientadora: Nilda de Fátima Ferreira Soares

Coorientadoras: Maria Cristina Dantas Vanetti
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RESUMO

TAVARES, Adassa Gama, D.Sc., Universidade Federal de Viçosa, outubro de 2020. **Encapsulação de carvacrol em lipossomas para aplicação em embalagem ativa para alimentos**. Orientadora: Nilda de Fátima Ferreira Soares. Coorientadoras: Maria Cristina Dantas Vanetti e Nathália Ramos de Melo.

O carvacrol é um antimicrobiano natural que tem sido estudado para aplicação como conservante em alimentos. No entanto, sua baixa solubilidade em água, alta volatilidade e sensibilidade à luz e calor prejudicam sua estabilidade e efeito quando incorporado a embalagens ou aplicado diretamente a produtos alimentícios. Uma das estratégias apontadas para superar estas limitações é a encapsulação do carvacrol em lipossomas. Os lipossomas são vesículas lipídicas esféricas que podem não apenas melhorar a solubilidade e estabilidade dos compostos antimicrobianos encapsulados, mas também interagir com as membranas das bactérias e, assim, aumentar sua eficiência. Neste trabalho, lipossomas à base de lecitina de soja e colesterol para encapsulação de carvacrol foram produzidos, caracterizados e incorporados ao filme de poli(vinil álcool). A atividade antibacteriana do carvacrol encapsulado em lipossomas e o seu modo de ação contra *Staphylococcus aureus* e *Escherichia coli* também foram investigados. A influência das concentrações de colesterol e carvacrol na formulação lipossomal foi explicada por um modelo de regressão e a formulação que resultou em maior eficiência de encapsulação foi obtida utilizando a função desejabilidade. As características físico-químicas dos lipossomas com carvacrol indicaram que este composto foi eficientemente encapsulado, reduziu a agregação e fusão das vesículas e aumentou a estabilidade térmica deste sistema. A incorporação em lipossomas melhorou o efeito antibacteriano do carvacrol. O extravasamento de material celular por *S. aureus* na presença de carvacrol não encapsulado sugere danos na membrana celular dessa bactéria. Entretanto, quando encapsulado em lipossomas, o carvacrol não causou a liberação de material intracelular. Para *E. coli,* os resultados obtidos não permitiram esclarecer o mecanismo de inativação do carvacrol livre e encapsulado em lipossomas. Quando incorporados ao filme de poli(vinil álcool), os lipossomas com carvacrol não foram eficientes na inibição de *S. aureus* e *E. coli*. Estes resultados sugerem que lipossomas podem ser um sistema promissor para utilização do carvacrol como conservante alimentar, entretanto, mais estudos são necessários para viabilizar sua aplicação em embalagens para alimentos.

Palavras-chave: Carvacrol. Conservante natural. Lipossomas. Embalagens de alimentos.

ABSTRACT

TAVARES, Adassa Gama, D.Sc., Universidade Federal de Viçosa, October, 2020. **Encapsulação de carvacrol em lipossomas para aplicação em embalagem ativa para alimentos**. Adviser: Nilda de Fátima Ferreira Soares. Co-advisers: Maria Cristina Dantas Vanetti and Nathália Ramos de Melo.

Carvacrol is a natural antimicrobial which has been studied for application as a food preservative. Nevertheless, it`s low water solubility, high volatility and sensitivity to light and heat harm it`s stability and effect when incorporated to packages or applied directly to foods. One strategy that has been pointed to overcome these limitations is the encapsulation of carvacrol to liposomes. The liposomes are spherical lipidic vesicles which can not only improve the solubility and stability of the encapsulated antimicrobial compounds, but also interact with the bacterial membranes and, therefore, improve it`s efficiency. On this work, lecithin soy and cholesterol based liposomes, for the encapsulation of carvacrol were produced, characterized and incorporated to poly(vinyl alcohol) film. The antibacterial activity of carvacrol encapsulated to liposomes and it`s mode of action against *Staphylococcus aureus* and *Escherichia coli* were also investigated. The influence of cholesterol and carvacrol concentrations on the liposomal formulation was explained using a regression model, and the formulation which resulted in the greatest encapsulation efficiency was obtained using a desirability function. The physicochemical attributes of liposomes with carvacrol showed that this compound was efficiently encapsulated, reduced the vesicles' aggregation and fusion, and increased the thermal stability of this system. The incorporation into liposomes improved the antimicrobial effect of the carvacrol. The leakage of cellular material by *S. aureus* on the presence of non-encapsulated carvacrol suggests there was damage to the cellular membrane of this bacteria, Nevertheless, when encapsulated to liposomes, the carvacrol did not cause intracellular material liberation. For *E. coli*, the obtained results did not clarify the mechanism of inactivation of free and encapsulated carvacrol. When incorporated to poly(vinyl alcohol) film, the liposomes with carvacrol were not efficient on the inhibition of *S. aureus* and *E. coli*. These results suggest that liposomes can be a promising system for the usage of carvacrol as a food preservative, nevertheless, more studies are required to make it's application in food packaging viable.

Keywords: Carvacrol. Natural preservatives. Liposomes. Food packaging.

SUMÁRIO

INTRODUÇÃO GERAL

O carvacrol é um monoterpenóide fenólico naturalmente presente nos óleos essenciais de orégano, tomilho e manjerona (LIOLIOS et al., 2009; VERGIS et al., 2015), que exibe a capacidade de inibir diferentes espécies de fungos e bactérias (CHAVAN; TUPE, 2014; HYLDGAARD; MYGIND; MEYER, 2012). O mecanismo de ação antimicrobiana deste composto envolve principalmente a ruptura das membranas citoplasmática e externa das bactérias Gram-negativas, levando ao vazamento do conteúdo intracelular e consequentemente à inativação das células (HYLDGAARD; MYGIND; MEYER, 2012; KACHUR; SUNTRES, 2019).

Além deste efeito, o carvacrol tem demonstrado habilidade de causar outros danos às células bacterianas. Chueca, Pagán e García-Gonzalo (2014) observaram o envolvimento de espécies reativas de oxigênio na inativação de *Escherichia coli* pelo carvacrol e detectaram que o DNA foi a molécula alvo destas substâncias. Churklam et al. (2020) sugeriram que este antimicrobiano inativou *Listeria monocytogenes* por meio de uma ação multi-alvo, que envolveu tanto o rompimento das membranas celulares, quanto a inibição da atividade respiratória e do metabolismo de nucleotídeos das células.

Devido ao seu potencial antimicrobiano, o carvacrol tem sido estudado com uma opção para a conservação de alimentos. Shrestha et al. (2019) observaram que a lavagem de cortes de pele de frango com carvacrol em suspensão resultou na redução das contagens de *Campylobacter jejuni* e bactérias aeróbicas previamente inoculadas neste produto e sugeriram que este tratamento pode ser uma boa alternativa aos produtos químicos convencionais para reduzir o número de células de *Campylobacter* e bactérias deteriorantes em carcaças de frango. O carvacrol também se mostrou eficaz na inibição do crescimento de *Salmonella* Typhimurium em pasta de tomate e, quando combinado ao

sorbato de potássio, estes compostos inibiram completamente o crescimento desta bactéria no terceiro dia de armazenamento do produto (PEREIRA BATISTA et al., 2019).

Filmes de quitosana e ciclodextrina incorporados com carvacrol inibiram o crescimento de microrganismos mesófilos, psicrófilos, *Pseudomonas* spp., Enterobactérias, bactérias lácticas, leveduras e fungos em filés de peito de frango armazenadas a 4 ° C por 9 dias e este efeito se mostrou dependente do tamanho do filme e do tempo de armazenamento (HIGUERAS et al., 2014). Quando incorporado a filme comestível, o carvacrol reduziu as contagens de *E. coli* em folhas verdes previamente contaminadas (ZHU et al., 2020).

Entretanto, a aplicação do carvacrol diretamente em alimentos ou embalagens tem sido limitada por seu sabor forte, baixa solubilidade em água e instabilidade, pois pode volatilizar e degradar durante o processamento de alimentos e a preparação de filmes ativos, já que estes são expostos diretamente ao calor, luz, umidade e oxigênio (KEAWCHAOON; YOKSAN, 2011; SUN; CAMERON; BAI, 2020). Pereira Batista et al. (2019) observaram que a adição de carvacrol a pasta de tomate reduziu a aceitação deste produto pelos consumidores, possivelmente devido a sua influência no sabor, que foi o atributo sensorial avaliado com a pontuação mais baixa.

Ao analisar a estrutura de filmes ativos produzidos com amido de milho, poli(vinil álcool) e nanoemulsão de carvacrol, foram observados gotas de óleo e poros na superfície dos filmes, o que indica a ocorrência de volatilização do carvacrol (KONG et al., 2020). Neira et al. (2019) reportaram que a perda de carvacrol incorporado à filmes de gelatina de peixe ao longo de 15 dias, diminuiu a eficiência antibacteriana *in vitro* do filme armazenado a 25 °C e umidade relativa de 65%. Além destas limitações, alguns componentes dos alimentos podem reduzir a atividade antimicrobiana do carvacrol. Quando adicionado à carne bovina moída, a atividade antimicrobiana deste composto foi

mais eficaz na carne magra comparada àquela obtida na carne com alto teor de gordura (WANG et al., 2020).

Uma das estratégias estudadas para superar estas limitações da aplicação do carvacrol como conservante em alimentos é o seu encapsulamento (HE et al., 2019). A encapsulação consiste no recobrimento de partículas sólidas, líquidas ou gasosas (núcleo) por um material polimérico natural ou sintético (parede) formando cápsulas, que isolam o material presente no núcleo interno (RIBEIRO-SANTOS; ANDRADE; SANCHES-SILVA, 2017; SILVA et al., 2014). Desta forma, as cápsulas podem aumentar a estabilidade das substâncias encapsuladas ao reduzirem o contato direto destas com as condições externas, como calor, luz e oxigênio (RAO et al., 2020).

Os sistemas de encapsulação também podem aumentar a estabilidade dos compostos encapsulados ao limitarem a ocorrência dos fenômenos de precipitação, cristalização, desnaturação, adsorção, difusão e interações químicas (FU et al., 2016). Sendo assim, a encapsulação pode tornar o composto aprisionado disponível por um tempo mais prolongado; aumentar a sua concentração nas áreas do alimento em que os microrganismos estão preferencialmente localizados, por exemplo, fases ricas em água ou interfaces líquido-sólidas; e impedir sua interação com ingredientes alimentares (DONSÌ et al., 2011). Além disto, a encapsulação pode reduzir o impacto sensorial do composto encapsulado sobre os alimentos (WEN et al., 2016).

Cenouras minimamente processadas higienizadas com uma solução contendo nanopartículas de tripolifosfato de quitosana carregadas com carvacrol não apresentaram sabor e aroma residuais deste antimicrobiano, mas as amostras tratadas com uma solução de carvacrol não encapsulado na mesma concentração apresentaram as maiores pontuações para estes atributos (MARTÍNEZ-HERNÁNDEZ; AMODIO; COLELLI, 2017). O estudo da estabilidade de nanopartículas lipídicas sólidas contendo carvacrol

sob condições ácidas, alcalinas e oxidativas demonstrou que a encapsulação protegeu este composto, mantendo até 98% das moléculas aprisionadas intactas nas nanopartículas (SHAKERI; RAZAVI; SHAKERI, 2019).

A beta-ciclodextrina também foi utilizada para encapsular o carvacrol, o que aumentou a solubilidade deste composto em água e resultou na inibição de *S.* Typhimurium e *E. coli* em uma concentração mais baixa do que a utilizada para o carvacrol não encapsulado, indicando que a encapsulação tornou a ação antimicrobiana mais eficiente e reduziu a concentração do composto necessária para a inibição destas bactérias (SANTOS et al., 2015). Resultado semelhante foi observado por Rao et al. (2020) ao avaliarem o efeito inibitório do carvacrol encapsulado em nanopartículas de ovalbumina sob o crescimento de *Bacillus cereus* e *Salmonella.* Estes pesquisadores sugeriram que a ovalbumina aumentou a solubilidade aquosa do carvacrol, o que provavelmente facilitou seu acesso à membrana e citoplasma das células bacterianas.

Além destes, outro sistema estudado para encapsulação de carvacrol é o lipossoma (ENGEL et al., 2017; HECKLER et al., 2020). Os lipossomas são vesículas esféricas, organizadas em uma ou várias bicamadas fosfolipídicas concêntricas em torno de um núcleo aquoso (SEBAALY et al., 2015). As membranas podem ser produzidas por fosfolipídios obtidos de fontes naturais, como ovo, soja ou leite, e também podem conter colesterol (SHARMA; SHARMA 1997, BLANCO-PADILLA et al. 2014). Sendo assim, estas vesículas são biocompatíveis, biodegradáveis, não imunogênicas e não tóxicas (ANWEKAR et al., 2011).

A composição das bicamadas lipossomais e sua interação com o composto encapsulado determinam sua espessura, fluidez, permeabilidade e polaridade (MAHERANI et al., 2013). Essas características, por sua vez, estão diretamente relacionadas à estabilidade dos lipossomas durante o armazenamento, à sua eficiência de

encapsulação e à liberação efetiva do composto encapsulado (MAHERANI et al., 2013; TAI et al. 2018).

Uma característica dos lipossomas que os tornam vantajosos na encapsulação de compostos antimicrobianos é a capacidade de interagir com membranas biológicas e, posteriormente, liberar sua carga no interior das células (HUANG et al., 2011; SACHETELLI et al., 2000). Essa capacidade facilita o acesso do antimicrobiano às bactérias, contornando seu mecanismo de resistência, e permite que o agente atue no interior da célula bacteriana (KHOSRAVI-DARANI; KHOOSFI; HOSSEINI, 2016; SACHETELLI et al., 2000; WANG et al., 2016). Por isto, supõe-se que os lipossomas podem melhorar a eficiência do antimicrobiano encapsulado.

Ao encapsularem o carvacrol em lipossomas à base de fosfatidilcolina, Heckler et al. (2020) observaram que as vesículas apresentaram uma distribuição homogênea de tamanho, alta eficiência de encapsulação e permanecerem estáveis por 28 dias sob refrigeração. No entanto, o carvacrol em lipossomas foi menos eficaz contra células de *Salmonella* planctônicas e aderidas ao vidro comparado ao composto não encapsulado. Em outro estudo, o contato de *pools* de *S. aureus* ou *S. enterica* aderidos ao aço inoxidável com lipossomas contendo uma mistura de timol e carvacrol por 10 minutos foi suficiente para inativar estas células bacterianas (ENGEL et al., 2017).

Os lipossomas também têm sido testados para produção de embalagens ativas. Filmes de polietileno foram revestidos com solução de quitosana contendo lipossomas com óleo essencial de louro e nanopartículas de prata e aplicados à carne suína (WU et al., 2019). Os pesquisadores reportaram que os lipossomas retardaram a liberação dos compostos encapsulados e que os filmes mostraram atividade antimicrobiana contra *E. coli* e *S. aureus*, além de manterem a qualidade da carne suína a 4 ° C por 15 dias, enquanto o filme de polietileno puro manteve por até 9 dias. Lipossomas contendo óleo

essencial de *Melaleuca alternifólia* incorporados a nanofibras de quitosana reduziram a viabilidade de células de *S.* Typhimurium e *S. enteritidis* em frango sem alterar a cor, sabor, suculência e aceitabilidade geral das amostras após 4 dias de armazenamento a 4 ° C e 12 ° C (CUI et al., 2018).

Neste trabalho, foram produzidos lipossomas à base de lecitina de soja e colesterol para encapsulação de carvacrol e foram apresentados os efeitos da concentração destes componentes sobre a eficiência de encapsulação, o tamanho, o índice de polidispersão e o potencial zeta dos lipossomas. A influência do carvacrol nas interações entre os componentes dos lipossomas, bem como sobre o comportamento térmico dos mesmos foi demonstrada. A atividade antibacteriana do carvacrol encapsulado em lipossomas e o seu modo de ação também foram investigados. Além disso, os lipossomas contendo carvacrol foram incorporados à filme de poli(vinil álcool) e o efeito antibacteriano deste filme foi avaliado.

Artigo 1*

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Carvacrol-loaded liposomes suspension: Optimization, characterization and incorporation into poly (vinyl alcohol) films

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Abstract

The purpose of this study was to encapsulate carvacrol in liposomes in order to promote its application in food preservation. Response surface methodology was used to evaluate the effect of the concentration of the liposomal components on its characteristics. The optimum formulation for the preparation of liposomes with the highest encapsulation efficiency (59.0 ± 1.99%) was found to be 3000 $\mu g \cdot mL^{-1}$ of cholesterol and 4000 $\mu g \cdot mL^{-1}$ of carvacrol. Carvacrol reduced the polydispersity index and increased the zeta potential and the thermal stability of liposomes. Fourier-transform infrared spectroscopy indicated that the interaction of carvacrol with liposomes occurred, probably through hydrogen-bonding. The incorporation into liposomes maintained the antibacterial effect of carvacrol, but when in the film, carvacrol liposomes were not effective in bacteria inhibition. It may be concluded that liposomes offer a viable option for stabilizing carvacrol, however, more studies are necessary to enable its application in food packaging.

Keywords: natural food preservatives; carvacrol; encapsulation; liposome

1 Introduction

Carvacrol (CAR) is a monoterpenoid phenol naturally occurring in oregano, thyme, marjoram, and pepperwort and can be obtained in their essential oil fraction (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009; Vergis, Gokulakrishnan, Agarwal, & Kumar, 2015). It is classified as 'generally recognized as safe' (GRAS) by the Food and Drug Administration (FDA) and is used in the food industry as a flavoring supplement in sweets, liquors, and baked goods without any restriction and in accordance with good manufacturing practices (Lahmar, Akcan, Chekir-Ghedira, & Estévez, 2018).

This phenolic compound exhibits antimicrobial effects on fungi and bacteria, and has therefore, been tested as a food preservative (Chavan & Tupe, 2014; Hyldgaard, Mygind, & Meyer, 2012). The antimicrobial mode of action of CAR primarily involves its ability to position itself in the cell membrane of the pathogen, thereby increasing its permeability (Hyldgaard et al., 2012). CAR displays a higher antimicrobial efficiency compared to other volatile compounds found in essential oils, due to its hydrophobicity and its free hydroxyl group (Ben Arfa, Combes, Preziosi-Belloy, Gontard, & Chalier, 2006).

However, the antimicrobial application of CAR directly in food or packaging has been limited by its strong flavor, poor water solubility, and instability, since it volatilizes and easily degrades during food processing and antimicrobial film preparation when exposed to direct heat, pressure, light or oxygen (Keawchaoon & Yoksan, 2011; Sun, Cameron, & Bai, 2020). Liposomal encapsulation can circumvent these limitations and also induce slower release of this compound thereby increase its bioactivity (Cui, Yuan, Li, & Lin, 2017; Engel, Heckler, Tondo, Daroit, & da Silva Malheiros, 2017; Tan et al., 2014).

Liposomes are closed spherical vesicles, organized in one or several concentric

phospholipid bilayers surrounding an aqueous core (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015). The main constituents of conventional liposomes are natural and safe such as lecithin (LEC) from egg yolk or soybeans, and cholesterol (CHO). Moreover, liposomes are biodegradable and biocompatible (Cui, Li, & Lin, 2017). The composition of liposomal bilayers and their interaction with the encapsulated compound determines their thickness, fluidity, permeability, and polarity. These characteristics, in turn, are directly related to encapsulation efficiency and effective release of the loaded compound (Maherani, Arab-Tehrany, Kheirolomoom, Geny, & Linder, 2013).

This study was aimed at bolstering the application of CAR in food preservation, through: 1) encapsulation of CAR in liposomes composed of soy LEC and CHO; 2) studying the effects of the concentration of the main components of liposomes and of the encapsulated compound in encapsulation efficiency (EE), particle size, polydispersity index (PDI) and zeta potential of the liposomes; 3) evaluating the influence of CAR on the particle size, PDI, zeta potential, component interactions and thermal behavior of liposomes; 4) determining the antibacterial properties of the resulting liposomes and 5) examining the antibacterial activity of the poly (vinyl alcohol) (PVA) films with carvacrol liposomes.

2 Materials and methods

2.1 Optimization of CAR liposome preparation

The Response Surface Method (RSM) was used to optimize the formulation of the liposomes and the encapsulated compound. A Rotational Central Composite Design (RCCD) was applied with three experimental factors – LEC, CHO, and CAR concentration, evaluated at five levels. The range of these three factors was based on preliminary experiments as follows: LEC (2000-6000 $\mu g \cdot mL^{-1}$), CHO (0-3000 $\mu g \cdot mL^{-1}$), and CAR (0-6000 $\mu g \cdot mL^{-1}$) (Table 1). The combinations of these levels and five replicates

of the central value generated a total of 19 treatments, which were evaluated based on four response variables: EE, particle size, PDI, and zeta potential (Table 1). The effects of unexplained variability in the observed response due to experimental errors were minimized by randomizing the order of the experiments.

The statistical models representing the influence of the three factors on the four response variables were obtained and validated using Analysis of Variance (ANOVA) with a 5% level of significance. Finally, the desirability function was used to optimize the significant response variables.

2.2 Preparation of CAR liposomes

The liposomes suspensions were prepared using the lipid film hydration technique (Bangham, Standish, & Watkins, 1965). Corresponding amounts of soy LEC (InLab, Brazil), CHO (Sigma–Aldrich, USA), sodium cholate (Sigma–Aldrich, USA) (200 $\mu g \cdot mL^{-1}$) and Tween 80 (Vetec, Brazil) (200 $\mu g \cdot mL^{-1}$) were dissolved in chloroform (Dinâmica, Brazil). The organic solvent of the solution was then removed under a nitrogen flow until a thin lipid film was formed. The lipid film was stored overnight in a desiccator to remove traces of chloroform. Thereafter, the film was hydrated with 30 mL of deionized water containing or not corresponding amounts of CAR (Sigma–Aldrich, USA) and the solution was sonicated in an ice bath (Sonics Vibra Cell VC 750, USA). Finally, the liposomes suspensions obtained were stored in dye flasks at 4 ± 0.5 °C.

2.3 Determination of the encapsulation efficiency of the liposomes

The loading efficiency of CAR into liposomes was determined by UV–vis spectrophotometry. Aliquots were taken from the CAR-loaded liposomes suspension (CLIP) to determine the total concentration of CAR ($[Car]_t$). To determine the concentration of the free CAR ($[Car]_f$), CLIP was ultracentrifuged at 149000 × g for 2 h at 4 °C and the supernatant was filtered through a 0.45 mm pore size filter. 0.15 mL of

CLIP and supernatant were diluted with ethyl alcohol (Vetec, Brazil) to 5 mL and their absorbances at 275 nm were measured using spectrophotometer UV-vis 1800 (Shimadzu, Japan). To calculate the CAR concentration, all of the absorbances were substituted into the regression equation obtained from the analytical curve of free CAR in ethyl alcohol ($R^2 = 0.996$). The EE (%) of CLIP was then calculated according to the following equation:

$$EE\ (\%) = \frac{[Car]_t - [Car]_f}{[Car]_t} \times 100 \quad (1)$$

2.4 Characterization of the optimized formulation of CLIP

The characterization of the CLIP – obtained from the optimized formulation – and of an empty liposomes suspension (LIP) were performed.

2.4.1 Particle size, PDI and zeta potential

The size distribution based on the hydrodynamic diameter and PDI of CLIP was measured using dynamic light scattering (DLS). The zeta potential was calculated from the electrophoretic mobility of the vesicles using the Henry equation. Measurements were carried out at 25 °C in Zetasizer Nano ZS (Malvern Zen 3500, United Kingdom), with the sample disposed in the DTS 1070 cuvette. Triplicate measurements from three samples were performed and expressed as the mean ± standard deviation. Statistically significant differences were determined by ANOVA following the Tukey's test ($p < 0.05$).

2.4.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of CAR and lyophilized CLIP and LIP were obtained on a Nicolet 6700 spectrometer (Thermo Scientific, USA), scanning in the wavenumber range of 4000 to 700 cm^{-1} with 32 scans and a resolution of 4 cm^{-1}.

2.4.3 Thermal analysis

The thermal decomposition of the liposomal components was analyzed using the thermogravimetric (TG) method (Shimadzu DTG-60H, Japan). Samples of approximately 5 mg of LEC, CHO, CAR, and lyophilized CLIP and LIP were heated from 30 to 600 °C, using a heating rate of 10 °C.min^{-1}. TG measurements were carried out under a nitrogen flow of 50 mL.min^{-1}.

The differential scanning calorimetry (DSC) measurements were made in a calorimeter associated with a thermal analyzer DSC-60 (Shimadzu, Japan). Samples of about 3 mg of LEC, CHO, CAR, and lyophilized CLIP and LIP were placed in aluminum crucibles and an empty crucible was placed in the reference cell. The temperature was set from 30 to 180 °C (90 °C.min^{-1}), maintained for 1 min at 180 °C, and from 180 to 600 °C (10 °C.min^{-1}) under a nitrogen gas flow of 30 mL.min^{-1}.

2.4.4 Antibacterial effects

Growth inhibition of two standard bacterial strains obtained from the American Type Culture Collection (ATCC), *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229, was evaluated. Prior to the testing, stock cultures of *S. aureus* and *E. coli* were cultivated in a Tryptic Soy broth (Difco, USA) and a Nutrient broth (Kasvi, Brazil), respectively, at 37 °C for 24 h. *S. aureus* and *E. coli* were then cultivated in Tryptic Soy agar (TSA) (Acumedia, USA) and Nutrient agar (Kasvi, Brazil), respectively, at 37 °C for 18 h and used to prepare bacterial inoculum in saline solution. The turbidity of the bacterial suspension was adjusted to an optical density (OD) of 0.09 to 0.11 at 600 nm using a spectrophotometer (GBC Scientific Equipment, Australia) to obtain a final concentration of 10^8 colony forming units (CFU).mL^{-1}.

Initial solutions of 0.250% v/v of CAR free (in 0.5% v/v Tween 80) and in liposomes were prepared in Mueller Hinton broth (MHB) (Difco, USA). Subsequently, two-fold serial dilutions were made in tubes containing MHB to obtain the final

concentrations (0.125-0.003% v/v) and the bacterial inoculum (10^8 CFU.mL^{-1}) was added to each tube. The concentration of each inoculum was confirmed using viable counts on TSA. The same amount of bacterial culture was added to the MHB without antimicrobial culture, as positive control and to the broth containing Tween 80 or LIP as negative control.

Tubes were incubated at 37 °C for 18 h, and cell viability was assessed using the drop plate method on TSA. After incubation at 37 °C for 8 to 12 h, the number of surviving organisms was determined. The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration that resulted in a significant decrease ($p < 0.05$) in inoculum viability and the Minimum Bactericidal Concentration (MBC) was the lowest concentration that could kill 99.9% of the treated cells. This analysis was run in five replicates from three independent experiments, and differences between the treatments were evaluated by Tukey's test ($p < 0.05$) using the Minitab 18 program, 2017.

2.5 Antibacterial activity of the films with CLIP

The films were prepared using the casting technique. PVA solution was prepared by dissolving PVA power (Sigma–Aldrich, USA) in heated Milli-Q water to a final concentration of 6% (w/v) under a constant stirring speed of 1000 rpm at 100 °C for 3 hours. Then, the film's solution was cooled to 30 °C and CAR in water or CLIP were incorporated into it to prepare the film at 5% (w/w) of CAR. Solutions of pure PVA and the mixture of PVA and LIP were also prepared as controls. Each mixture was stirred (300 rpm) at 30 °C for 30 min, and 10 mL of the obtained solutions were placed onto glass petri plates (diameter = 9 cm) and dried at room temperature (22 ± 2 °C) and room relative humidity (50 ± 2%) for 48 h.

Bacterial suspensions of *S. aureus* and *E. coli* (Section 2.4.4) were appropriately diluted to get a final inoculum of 10^5 CFU.mL^{-1}. Samples (8 cm^2) from the different types

of film formulation were placed in tubes with inoculated MHB – liquid medium test – and affixed on the lid of the plates with inoculated Mueller Hinton agar (MHA) (Becton Dickinson, Germany) – solid medium test. After 24 h incubation at 37 °C, the formation of the inhibition zones on plates with MHA was observed to evaluate the antibacterial activity of the films. In the liquid medium tests, serial dilutions were made and poured onto plates with TSA using the drop plate method. After incubation at 37 °C for 8 to 12 h, the number of $CFU.mL^{-1}$ was determined. These analyses were run in duplicates from three independent experiments. The results obtained for the liquid medium test were expressed as log $CFU.mL^{-1}$ and differences between the treatments were evaluated by Tukey's test ($p < 0.05$) using the Minitab 18 software, 2017.

3 Results and discussion

3.1 Optimization and validation of the CLIP preparation

RCCD permits the investigation of the individual and interactive effects of each factor among the response variables. This experimental design was aimed at estimating the contribution of synergistic factors, and to determine the optimum level of each independent variable through which it was possible to obtain the highest EE, smallest particle size, smallest PDI, and highest zeta potential. The results of the response variables studied for each combination of factor levels is shown in Table 1.

The observed values for each response variable were used for RCCD. ANOVA was used for the statistical validation of the polynomial equations, and the responses were fitted to linear, squared and interaction models. The statistically significant coefficients, non-significant lack of fit, and near 100% adjusted determination coefficient (adj R^2) indicate the adequacy and significance of the model.

The EE was influenced by two factors. The model presented an adjusted determination coefficient (adj $R^2 = 0.8649$) implying 86.49% of the variability in EE (%).

The ANOVA of the square regression model was significant ($p < 0.05$) and the lack of fit was not significant ($p > 0.05$) indicating that the model was adequate. The significance of each coefficient was determined and the *p*-values of CHO, CAR, and CAR^2 were lower than 0.05 suggesting that they had a significant influence on the EE. The regression equation obtained is expressed in Eq. (2):

$$EE(\%) = -4.74 + 0.00619CHO + 0.02173CAR - 0.000003CAR^2 \quad (2)$$

The regression coefficients of the response surface indicate that the EE was influenced by linear and positive effects of CHO, implying that higher amounts of CHO resulted in higher EE as shown in the response surface and contour plots (Figure 1). The CHO promotes the coupling of the lipid bilayer and reduces the electrostatic repulsion among head groups through hydrogen bonding with the phospholipids, increasing their ordered state and modulating lipid membrane fluidity, enhancing the stability of incorporated compounds (Sułkowski, Pentak, Nowak, & Sułkowska, 2005; Zhao, Temelli, Curtis, & Chen, 2015).

The linear and positive effects of CAR indicate that higher amounts of this component result in a higher EE of the CLIP, but the quadratic and negative effect, indicate that after reaching the optimum value of CAR (4000 $\mu g \cdot mL^{-1}$), the EE of the CLIP reduces quadratically with the increase in CAR as shown in Figure 1. Lu et al. (2014) observed that EE of CLIP was affected by the LEC-encapsulated compound ratio and concluded that the capacity of a certain amount of LEC to accommodate the encapsulated compound was limited. Therefore, at the maximum encapsulated amount, the EE decreased with increasing amounts of the added compound.

The concentration of phospholipid, sterol, and encapsulated compounds can affect the size, PDI, and zeta potential (Nahr et al., 2019; Rafiee, Barzegar, Sahari, & Maherani, 2017; Zhao et al., 2015). Nevertheless, in the ANOVA for particle size, PDI and zeta

potential, the *p*-value > 0.05 of linear, square and interaction models were not significant and the lack of fit was significant ($p < 0.05$), which mean the models' terms were not applicable, implying that the concentrations of LEC, CHO and CAR tested in the RCCD levels did not present a significant effect on these response variables.

The optimization of the concentrations of the main components of CLIP and the encapsulated compound using individual desirability was performed with the goal to maximize the EE, the only response variable that was shown to be significant in accordance with the RSM. The optimum formulation obtained was 3000 $\mu g \cdot mL^{-1}$ of CHO and 4000 $\mu g \cdot mL^{-1}$ of CAR, and the maximum response value for EE predicted by the model was 57.33% with a 95% confidence interval of 51.74% to 62.92%, obtained with the individual desirability value of 1.0. Based on our previous studies, 6000 $\mu g \cdot mL^{-1}$ of LEC was used in the optimized formulation.

In order to validate the reliability of the model equation, a verification experiment was performed with CLIP prepared based on the optimized formulation, and its EE determined. Experimental data were collected in duplicate from three samples and the mean experimental EE (59.0 $\pm$ 1.99%) was validated at the 95% confidence level.

3.2 Characterization of the CLIP of optimized formulation

3.2.1 Particle size, PDI and zeta potential

The particle sizes, PDI, and zeta potential of the CLIP, and the LIP were measured 24 h after the preparation. The size of the CLIP (192.0 $\pm$ 10.8 nm) was equal ($p > 0.05$) to the size of the LIP (203.6 $\pm$ 19.7 nm). The incorporation of bioactive material and sterols may increase or decrease the particle size of liposomes depending on whether compounds are able to induce a more optimal packing of phospholipids in the lipid bilayers (Sherry, Charcosset, Fessi, & Greige-Gerges, 2013; Nahr et al., 2019). However, in this case, it was not possible to attest any of these effects.

The mean PDI of the CLIP and the LIP were 0.204 ± 0.006 and 0.470 ± 0.046, respectively. PDI values lower than 0.3 are an indicator of homogeneous suspensions and PDI values above 0.3 demonstrate a broad particle size distribution of the sample (Yen, Wu, Lin, Cham, & Lin, 2008). Thus, values observed indicate narrow particle size distribution and high homogeneity of the resultant CAR system, and heterogeneous distribution of the particle size of the LIP (Pinilla, Thys, & Brandelli, 2019). It is possible that CAR promoted the cohesion of the lipid bilayer, which limited the fluidity of the membrane and slowed the destabilization. For liposomes co-encapsulating curcumin and resveratrol, Huang et al. (2019) observed that, with the addition of more resveratrol, the fluidity of the membrane was increased, resulting in the aggregation of particles. While curcumin-resveratrol 5:1 liposome presented a lower PDI value, probably due to the intense condensing effect on the lipid bilayer caused by curcumin.

The zeta potential value of the CLIP (-58.2 ± 1.7 mV) was more electronegative ($p < 0,05$) than that of LIP (-52.9 ± 0,7 mV). These systems are considered stable because particles with zeta potentials more positive than +30mV or more negative than -30mV prevent aggregation by incrementing the repulsive electrostatic force between charged particles (Rafiee et al., 2017). The increased absolute value of zeta potential caused by the addition of CAR may be due to the reorientation of head groups of lipids with favored exposure of the phosphate group in an outer plane (Jovanović et al., 2019).

3.2.2 FTIR

The FTIR spectrum of CAR (Figure 2A) showed bands and peaks that can be assigned to −OH stretching (3590 to 3000 cm^{-1}), the asymmetrical and symmetrical $-CH_3$ stretching (2960 cm^{-1} and 2869 cm^{-1}), C=C stretching in the aromatic ring (1625 to 1440 cm^{-1}), and the combination of the C−O stretching and O−H in-plane deformation (1260 to 1130 cm^{-1}). The FTIR spectra of the CLIP and LIP (Figure 2A) showed a

broadband corresponding to −OH stretching in associated CHO (3590 to 3000 cm^{-1}), peaks attributed to asymmetrical and symmetrical $=CH_2$ stretching of LEC and CHO (2923 cm^{-1} and 2853 cm^{-1}), a peak corresponding to C=O stretching of LEC (1739 cm^{-1}), and peaks that can be attributed the C−O stretching of CHO (1240 and 1130 cm^{-1}).

Entrapment of different compounds on the phospholipid bilayer can be identified by changes in the FTIR patterns, which may show intermolecular interaction between the loaded compounds and the components of the liposome wall (Pinilla et al., 2019). The higher peak intensities at 2923 cm^{-1}, 2853 cm^{-1}, and at 1240 to 1130 cm^{-1} in the spectrum of the CLIP (Figure 2B) can be attributed respectively to the presence of asymmetrical and symmetrical $-CH_3$ stretching, and the combination of the C−O stretching and O−H in-plane deformation in CAR. The large format band at 3590 to 3000 cm^{-1} suggests the presence of a hydrogen-type intermolecular bond indicating that the CAR was encapsulated in the liposomes.

3.2.3 Thermal analysis

The TG curves of liposomes and their compounds are displayed in Figure 3A. The degradation of liposomes occurred earlier than the degradation of pure LEC and CHO, suggesting an interaction between these compounds in the lipid bilayers. Curves for CAR showed mass loss (96.2%) in a unique step from around 144 °C. However, the higher weight loss in CLIP (41.1%) began at 271 °C, above the degradation temperature of CAR, indicating that CAR interacted with the liposomal material, granting it greater thermal stability. The earlier thermal decomposition of LIP (∼266 °C) in comparison to the CLIP (271 °C) might indicate that CHO causes a bigger disruption in the packing of lipids to accommodate it in the liposomes, in comparison to the CAR (Nkanga, Krause, Noundou, & Walker, 2017), as also suggested by DLS.

All DSC curves showed endothermic events (∼23-179 °C) (Figure 3B)

corresponding to water loss. DSC thermograms of LEC, CHO, and CAR showed a sharp endothermic peak at about 188 °C, 307 °C, and 257 °C, respectively, corresponding to their volatilization. The thermogram of CLIP exhibited a sharp peak at nearly 226 °C and the characteristic peaks of LEC, CHO, and CAR were not observed, which suggests that the CLIP was not a simple mixture of these components, but there exists interaction between CAR and the encapsulation material. The temperature of the main exothermic peak of the CLIP (430 °C) is higher than that of the LIP (387 °C). These peaks are related to the thermal degradation of the liposomes, which indicates that CAR increases the thermal stability of the liposomes, possibly contributing to a better organization of the liposome membrane, as showed in the discussion of these other techniques.

3.2.4 Antibacterial effects

The MIC and MBC for CAR and CLIP against *S. aureus* and *E. coli* strains in Table 2 indicate that both the free and the encapsulated CAR is an effective inhibitor and bactericidal against these bacteria. Previous studies reported the effectiveness of CAR in inhibiting the growth of the same bacteria (Ge & Ge, 2016; Engel et al., 2017). Evidence suggests that the cytoplasmic membrane is the site of action of the CAR and that its mode of action is to increase fluidity and permeability of the cellular membrane (Hyldgaard et al., 2012). In addition, CAR has been demonstrated to be able to disintegrate the outer membrane of Gram-negative bacteria like *E. coli*, causing the release of lipopolysaccharides (Burt, 2004).

The enhancement of antimicrobial properties of essential oils and their components after the encapsulation in liposomes was indicated by some reports (Liolios et al., 2009; Ge & Ge, 2016; Khatibi et al., 2017). But, according to the MIC and MBC results, the encapsulation of CAR into liposomes does not improve its antimicrobial activity, except for MBC in *S. aureus*. Likewise, Engel et al. (2017) detected that MIC

values of CAR, free and encapsulated into liposomes, were the same against *S. aureus* and *Salmonella* spp. It is supposed that the nanometric size of liposomes may improve its cellular absorption and the release of the active component inside the microbial cell increases its antimicrobial effect (Donsì, Annunziata, Sessa, & Ferrari, 2011; Liolios et al., 2009). However, the interaction between the liposomes and the cells depends on the cell wall/membrane composition, as well as the liposome membrane physicochemical characteristics (Liolios et al., 2009). Therefore, further research on this topic needs to elucidate this finding.

3.3 Antibacterial activity of the films with CLIP

The results of the solid medium test showed that films with CAR and CLIP did not affect the growth of *S. aureus* and *E. coli*, since inhibition zones for treatments and control films were not observed (data not shown). The bacteria counts (log $CFU.mL^{-1}$) exposed to films in the liquid medium for 24 hours are shown in Table 3. The films with CLIP led to a reduction in the growth of *S. aureus* compared to films of pure PVA (control) and with CAR ($p < 0.05$). However, the counts for the samples containing films with CLIP were not different than observed for LIP ($p > 0.05$). For *E. coli*, films with CAR or CLIP did not lead to a reduction in bacterial growth compared to those films of pure PVA (control) and with LIP ($p > 0.05$). These results indicate that free CAR and CAR encapsulated in liposomes were not effective in the inhibition of *S. aureus* and *E. coli* growth when incorporated in PVA film, though they were added in a concentration above the MIC for both bacteria. In order to explain this finding, the following hypotheses are presented.

It is possible that CAR and CLIP were not released from PVA matrix to the medium in sufficient concentration to affect the growth of the tested bacteria. According to Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez, & Fernández-López (2013), the

essential oils can interact with the polymer and form part of the chemical structure of the film, which reduces the release of these antimicrobial agents. When loaded by liposomes, the interaction between the active compound and the liposome increases its molecular weight, which reduces the speed of the compound migration from the film to the surface of the culture, and consequently the antimicrobial activity of the film (Aziz, Almasi, 2018). Thus, it is suggested that additional investigation is done with the aim to study the release kinetics of CAR-loaded in liposomes from the film.

Nonetheless, it may also be the case that the CAR amount retained in the film was not enough to reduce the bacteria counts in the medium. During the film drying step, the water evaporation can result in the flocculation, coalescence, and creaming of the essential oils droplets in the forming film, which can lead to volatilization losses (Atarés & Chiralt, 2016; Dhumal, Ahmed, Bandara, & Sarkar, 2019). The disruption of the liposomes can also occur during this step due to evaporation of the solvent where the liposomes were originally dispersed, and the release of the active compound from liposomes made it more susceptible to volatilization (Andrade; González-Martínez; Chiralt, 2020; Sapper, Wilcaso, Santamarina, Roselló, & Chiralt, 2018). Despite this, liposomal encapsulation has been shown to improve the retention of the carvacrol in PVA film obtained by casting the polymer dispersions (Andrade; González-Martínez; Chiralt, 2020). Hence, strategies to avoid the destabilization of the liposomes during the film drying step also need to be investigated.

4 Conclusion

The experimental results show that the CLIP was successfully fabricated by the lipid film hydration method. The influence of the concentrations of CHO and CAR in the CLIP formulation was explained by a regression model and the formulation that resulted in the greatest EE was obtained using individual desirability. The physicochemical

characteristics of the CLIP indicate that the CAR was efficiently entrapped in liposomes, reduced the aggregation and fusion of vesicles and increased the thermal stability of this system. Moreover, CAR maintained its inhibitory effect against *S. aureus* and *E. coli* after its incorporation into liposomes. Therefore, this study suggests that liposomes of LEC and CHO can be considered natural delivery systems for CAR and provide good perspectives for the use of this antimicrobial as a food preservative, including heat-treated food. However, when incorporated in PVA film, CAR and CLIP were not shown to be effective in the inhibition of the growth of the tested bacteria, hence more research is necessary to improve the application of CAR-loaded liposomes in food packaging.

Abbreviations

CAR: carvacrol

CHO: cholesterol

CLIP: carvacrol-loaded liposomes suspension

EE: encapsulation efficiency

LEC: lecithin

LIP: empty liposomes suspension

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Table 1. Rotational Central Compound Design for independent variables lecithin (LEC) cholesterol (CHO) and carvacrol (CAR) concentrations and their response values for encapsulation efficiency (EE) particle size polydispersity index (PDI) and zeta potential.

Run	LEC (μg·mL^{-1})	CHO (μg·mL^{-1})	CAR (μg·mL^{-1})	EE (%)	Particle size (nm)	PDI	Zeta potential (mV)
1	2810 (−1)	608 (−1)	1216 (−1)	24.8	108.7	0.277	−61.5
2	5189 (+1)	608 (−1)	1216 (−1)	20.3	130.2	0.322	−54.4
3	2810 (−1)	2392 (+1)	1216 (−1)	39.7	238.6	0.407	−58.2
4	5189 (+1)	2392 (+1)	1216 (−1)	29.7	155.5	0.375	−57.1
5	2810 (−1)	608 (−1)	4784 (+1)	38.2	165.6	0.158	−57.0
6	5189 (+1)	608 (−1)	4784 (+1)	37.0	171.7	0.188	−59.3
7	2810 (−1)	2392 (+1)	4784 (+1)	48.0	182.6	0.200	−60.1
8	5189 (+1)	2392 (+1)	4784 (+1)	51.0	169.4	0.187	−57.9
9	2000 (−1.68)	1500 (0)	3000 (0)	53.0	193.5	0.203	−63.4
10	6000 (+1.68)	1500 (0)	3000 (0)	42.4	139.6	0.217	−58.0
11	4000 (0)	0 (−1.68)	3000 (0)	32.0	152.7	0.198	−57.1
12	4000 (0)	3000 (+1.68)	3000 (0)	48.2	171.3	0.224	−58.9
13	4000 (0)	1500 (0)	0 (−1.68)	0	129.4	0.265	−59.9
14	4000 (0)	1500 (0)	6000 (+1.68)	43.5	167.3	0.168	−57.6
15	4000 (0)	1500 (0)	3000 (0)	49.0	169.4	0.217	−71.1
16	4000 (0)	1500 (0)	3000 (0)	42.0	169.1	0.185	−57.1
17	4000 (0)	1500 (0)	3000 (0)	50.3	169.1	0.179	−54.0
18	4000 (0)	1500 (0)	3000 (0)	47.0	172.0	0.188	−60.7
19	4000 (0)	1500 (0)	3000 (0)	47.0	154.2	0.211	−60.9

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol (CAR) and carvacrol liposomes (CLIP) in % v/v against *Staphylococcus aureus* and *Escherichia coli*.

Bacteria	*S. aureus*		*E. coli*	
	MIC	MBC	MIC	MBC
CAR	0.015	0.250	0.015	0.031
CLIP	0.015	0.062	0.015	0.031

Table 3. Bacterial counts (log CFU.mL^{-1}) exposed to different films in liquid media stored at 37 °C for 24 hours.

Treatments	Bacteria	
	S. aureus	*E. coli*
PVA	7.65 ± 0.05^{a}	8.73 ± 0.29^{a}
PVA-CAR	7.51 ± 0.07^{a}	8.66 ± 0.24^{a}
PVA-CLIP	7.20 ± 0.01^{b}	8.49 ± 0.04^{a}
PVA-LIP	7.25 ± 0.10^{b}	8.55 ± 0.01^{a}

Results indicate mean values ± standard deviation (n = 3). Mean values followed by the same letter, within the same column, are not significantly different according to Tukey's test ($p > 0.05$).

PVA: Poly (vinyl alcohol) film; PVA-CAR: PVA film with carvacrol; PVA-CLIP: PVA film with carvacrol-loaded liposomes suspension; PVA-LIP: PVA film with empty liposomes suspension.

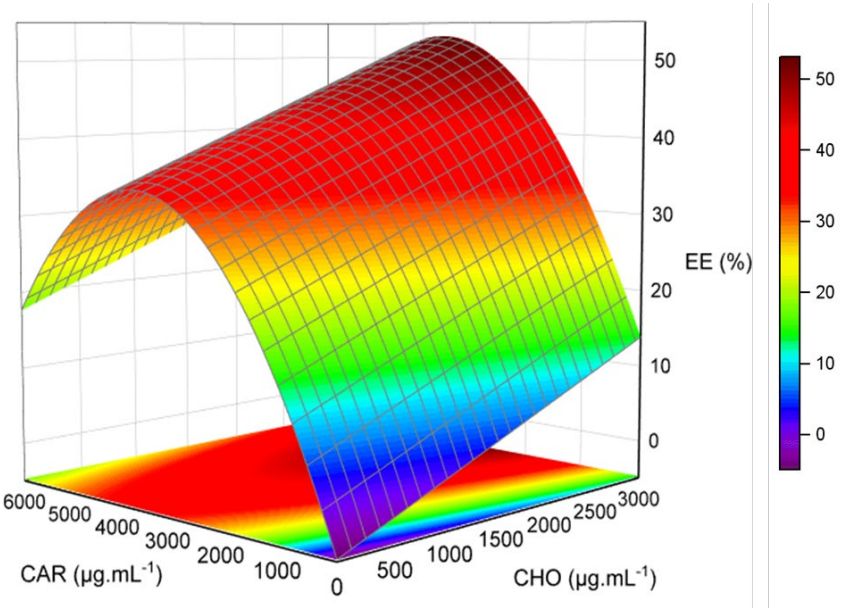

Figure 1. Response surface and contour plots for the analysis of the effect of cholesterol (CHO) and carvacrol (CAR) concentrations on the encapsulation efficiency (EE) of liposomes.

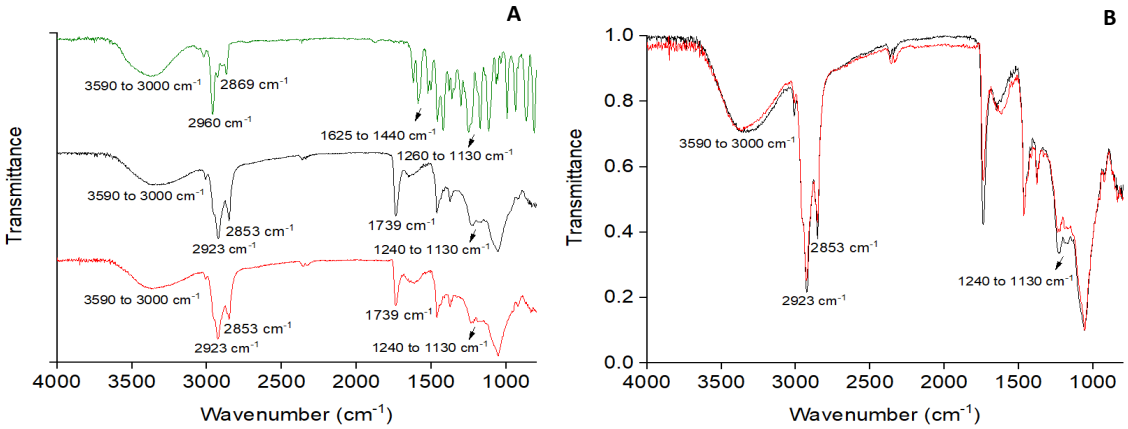

Figure 2. Fourier transform infrared (FTIR) spectrum of carvacrol (green curve), carvacrol-loaded liposomes suspension (black curve) and empty liposomes suspension (red curve) (A) and superposed FTIR spectrum of carvacrol-loaded liposomes suspension (black curve) and empty liposomes suspension (red curve) (B).

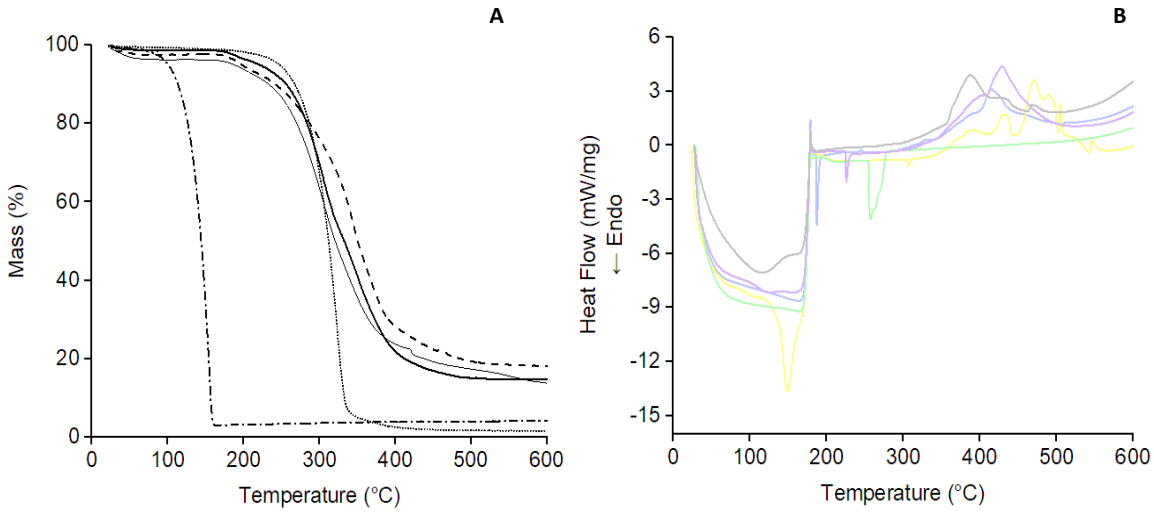

Figure 3. Thermogravimetric (TG) curves of lecithin (- - -) cholesterol (·····) carvacrol (- · - ·) carvacrol-loaded liposomes suspension (——) and empty liposomes suspension (——) (A). Differential scanning calorimetry (DSC) curves of lecithin (blue curve) cholesterol (yellow curve) carvacrol (green curve) carvacrol-loaded liposomes suspension (purple curve) and empty liposomes suspension (gray curve) (B).

Artigo 2*

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Stability and antibacterial activity of liposome-encapsulated carvacrol

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Abstract

Carvacrol is a natural compound whose antimicrobial activity is well known. However, its low solubility in water and high volatility impairs its stability and antibacterial activity in food products. To overcome these limitations, the encapsulation of carvacrol in liposomes was evaluated in this study. Encapsulation of carvacrol was confirmed by Fourier transform infrared spectroscopy, thermogravimetric analysis, and differential scanning calorimetry. Size, polydispersity index and zeta potential of the carvacrol-loaded liposomes remained stable for 30 days under refrigeration. The presence of carvacrol did not alter the size of the vesicles, improved the polydispersity index parameters, and increased the surface charge. We assume that carvacrol increased the rigidity of the liposomal membrane, which prevented aggregation of the vesicles. The encapsulation efficiency of the liposomes was 61.18 $\pm$ 1.50% and was also maintained for 30 days. Furthermore, encapsulated carvacrol was effective for inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* at 0.007% v/v and 0.15% v/v, respectively. These findings may help guide the design of liposomal encapsulation to efficiently utilize carvacrol in foods.

Keywords: liposomal stability, carvacrol encapsulation, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli*

1 Introduction

The use of essential oil components, such as carvacrol (CAR), is one of most widely studied techniques to prevent food spoilage and guarantee food safety (Ruiz-Rico et al., 2017). The antimicrobial activity of these components against foodborne pathogens is well known (García-Salinas et al., 2018; Klein et al., 2013), but their insolubility in aqueous media, high volatility, high susceptibility to degradation, and powerful aroma limit their application to food products (Kfoury et al., 2015). Therefore, encapsulation has been explored to increase the stability and utilization of these compounds (Cevallos et al., 2010).

Encapsulation is a process in which a particular component is incorporated within a matrix, which may be comprised of lipids, surfactants, and biopolymers (McClements, 2014). This technique can protect the antimicrobial compounds against chemical reactions, limit undesirable interactions with other components in food, improve solubility, prolong release, and preserve these compounds' stability during food processing and storage (Blanco-Padilla et al., 2014). Among the systems used to encapsulate essential oil components are liposomes, cyclodextrins, solid lipid nanoparticles, micelles, and polymer-based nanocarriers (Hammoud et al., 2019).

Liposomes are spherical vesicles formed by amphipathic phospholipid bilayer membranes that isolate an aqueous internal compartment from the external environment (Lu et al., 2014). The membranes can be produced by phospholipids obtained from natural sources, such as egg, soy, or milk, and may also contain cholesterol (Sharma and Sharma, 1997; Blanco-Padilla et al., 2014). Thus, they are biocompatible, biodegradable, non-immunogenic, and non-toxic (Anwekar et al., 2011). It has been reported that the incorporation of essential oil components in liposomes improves their solubility, chemical stability, and bioactivity (Baranauskaite, 2018; Liolios et al., 2009).

Liposome particles can be unstable during storage, which can result in aggregation, fusion, flocculation, and precipitation (Xia and Xu, 2005). The addition of cholesterol during liposome preparation can modulate membrane fluidity and promote stability of phospholipid bilayers since it fills the gaps within bilayers and reduces leakage created by imperfect packing (Zhao et al., 2015). The organization of the encapsulated compound in lipid bilayer also may affect the natural properties of the membrane (Huang et al., 2019). The rigidity of the liposomal membrane, in turn, may affect the delivery efficiency, storage stability, and release profile of the bioactive compounds (Tai et al., 2018).

This study focused on the encapsulation of CAR in liposomes composed of soy lecithin (LEC) and cholesterol (CHO). Fourier transform infrared spectroscopy, thermogravimetric analysis, and differential scanning calorimetry were used to verify the success of the encapsulation. The effects of CAR on stability, mean particle size, polydispersity index (PDI), zeta potential, morphology, and antibacterial activity of liposomes were investigated, and the encapsulation efficiency was determined.

2 Materials and methods

2.1 Materials

Soy LEC was purchased from InLab (Brazil). CAR, CHO, and sodium cholate hydrate were obtained from Sigma–Aldrich (USA). Absolute ethanol and Tween 80 were obtained from Vetec (Brazil). Chloroform was purchased from Dinâmica (Brazil). Mueller Hinton broth (MHB) and Tryptic soy agar (TSA) were obtained from Difco (USA) and Acumedia (USA), respectively.

2.2 Preparation of liposome suspension

CAR-loaded liposome suspension (CLIP) was prepared using a modified version of the method of lipid film hydration demonstrated by Bangham et al. (1965). Applied

amounts of LEC (6 mg·mL^{-1}), CHO (3 mg·mL^{-1}), sodium cholate (0.2 mg·mL^{-1}), and Tween 80 (0.2 mg·mL^{-1}) were dissolved in chloroform (2 mL) by stirring. Next, the mixture was subjected to a nitrogen flow until the organic solvent was completely removed, resulting in a thin lipid layer. The corresponding amount of CAR (4.42 mg·mL^{-1}) and 30 mL of deionized water were added into the thin lipid layer followed by sonication in an ice bath (Sonics Vibra Cell VC 750, USA). Empty liposome suspension (LIP) was prepared using the same procedure but without CAR. Finally, the liposomal suspensions were stored in amber flasks at 4 ± 0.5 °C for further analysis.

2.3 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of LEC, CHO, CAR, and lyophilized CLIP and LIP were obtained on a Nicolet 6700 spectrometer (Thermo Scientific, USA), scanning in the wavenumber range of 4000 to 700 cm^{-1} with 32 scans and a resolution of 4 cm^{-1} (Barbosa 2013). The data were normalized and baseline corrected.

2.4 Thermogravimetric analysis (TGA)

The thermal decomposition of CAR and the liposomes was examined using a thermal analyzer model DTG-60H (Shimadzu, Japan). Samples of approximately 5 mg of CAR and lyophilized CLIP and LIP were heated from 30 to 600 °C using a heating rate of 10 °C.min^{-1} (Nkanga et al., 2017). Thermogravimetric measurements were carried out under a nitrogen flow of 50 mL.min^{-1}. The derivative thermogravimetric (DTG) curves were calculated using the software Thermal Analyzer 60 version 2.21.

2.5 Differential scanning calorimetry (DSC)

The thermal behavior of the liposomes and their components was investigated by DSC, and the measurements were made in a calorimeter associated with the thermal analyzer model DSC-60 (Shimadzu). About 3 mg of LEC, CHO, CAR, and lyophilized CLIP and LIP were placed in an aluminum pan. The temperature was set from 30 to 180

°C (90 °C.min^{-1}), maintained for 1 min at 180 °C, and again set from 180 to 600 °C (10 °C.min^{-1}) under a nitrogen gas flow of 30 mL.min^{-1} (Detoni et al., 2012). An empty pan sealed with its cover was used as a reference. The enthalpy values were calculated using the software Thermal Analyzer 60 version 2.21.

2.6 Analysis of liposomal stability

The stability of the size, PDI, and zeta potential of the CLIP and LIP were analyzed individually for 30 days. The size distribution, based on the hydrodynamic diameter and PDI, was measured through dynamic light scattering. The zeta potential was calculated by the Henry equation from the electrophoretic mobility of the vesicles. Sample was placed in a DTS 1070 cuvette and the measurements were carried out at 25 °C in the Zetasizer Nano ZS (Malvern Zen 3500, United Kingdom). The average size, PDI, and zeta potential were calculated from three runs for each measure in three independent experiments. Statistically significant differences were determined by Analysis of Variance (ANOVA) following the Tukey test (p<0.05) using the Minitab 18 program, 2017.

2.7 Analysis of encapsulation efficiency (EE) of CLIP

The amount of CAR loaded in the liposomes was determined by UV–vis spectrophotometry (Lu et al., 2014). A volume of 3.5 mL of CLIP was ultracentrifuged at 149000 × g for 2 h at 4 °C to separate the unloaded CAR (Zhang et al., 2019). The supernatant was removed immediately and filtered through a 0.45 mm pore size filter. We then diluted a 0.15 mL sample with ethanol to a final volume of 5 mL, and its absorbance was measured using the spectrophotometer UV-vis 1800 (Shimadzu) at 275 nm to quantify the free CAR (Keawchaoon and Yoksan, 2011). To quantify the total CAR into CLIP, 0.15 mL of the CLIP sample was diluted in 5 mL of ethanol so that the liposomes would rupture and release entrapped CAR. Then, the absorbance of this

solution was measured at 275 nm. The amount of CAR was calculated using an appropriate analytical curve constructed using free CAR in ethanol ($R^2 = 0.996$). A blank sample was made from LIP and treated similarly to CLIP. The EE (%) of CLIP was determined as follows:

$$EE\ (\%) = \frac{[Car]_t - [Car]_f}{[Car]_t} \times 100 \quad (1)$$

where $[Car]_t$ is the total concentration of CAR and $[Car]_f$ is the concentration of free CAR in the liposomal suspension.

The measurements were duplicated in three independent experiments during the 1st, 15th and 30th days. Statistically significant differences were determined by ANOVA following the Tukey test (p<0.05) using the Minitab 18 program, 2017.

2.8 Atomic force microscopy (AFM)

The morphology and sizes of the liposomes were analyzed by AFM. Diluted liposome suspension (10% v/v) was deposited on freshly cleaved mica substrates and oven-dried at 60 °C. The AFM measurements were made using the scanning probe microscope NT-MDT (Integra Prima, Russia). Scans were performed on 10 x 10 μm and 2 x 2 μm areas in the intermittent contact mode using a probe with a 10 nm radius of curvature, a force constant of 10 N/m, and a resonance frequency of approximately 280 kHz. The scanning speed was between 1 and 3 lines per second with a resolution of 512 x 512 points. Both plane and three-dimensional images were obtained for more detailed information on the surface morphology.

2.9 Analysis of antibacterial activity

To quantify the antibacterial activity of CAR and CLIP against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229, Minimal Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) were

determined. Initial solutions of 0.250% v/v of CAR (in 0.5% v/v Tween 80) free and encapsulated in liposomes were prepared in MHB. Subsequently, two-fold serial dilutions were made in tubes containing MHB to obtain the final concentrations (0.125-0.003% v/v). A standardized bacteria suspension of 10^8 colony forming units (CFU).mL^{-1} was transferred to each tube. The concentration of each inoculum was confirmed using viable counts on TSA. Positive and negative growth controls were included in every test. Tubes were incubated at 37 °C for 18 h, and viability was assessed using the drop plate method on TSA. After incubation at 37 °C for 8 to 12 h, the number of surviving organisms was determined.

The MIC was defined as the lowest concentration that resulted in a significant decrease (p<0.05) in inoculum viability and the MBC was the lowest concentration that could kill 99.9% of the treated cells (Carson et al., 1995). This experiment was repeated three times, and differences between the treatments were evaluated by Tukey's test (p<0.05) using the Minitab 18 program, 2017.

3 Results and discussion

3.1 FTIR

The principal absorption frequencies observed in the FTIR spectra of LEC, CHO and CAR are listed in Table 1 with their respectively assignments (Barbosa, 2013). The FTIR spectra of CLIP and LIP (Figure 1) showed a broadband at 3590 to 3000 cm^{-1}, peaks at 2923 cm^{-1} and 2853 cm^{-1}, a peak at 1739 cm^{-1}, and peaks between 1240 and 1130 cm^{-1}. This bands e peaks are attributed to characteristic functional groups of LEC and CHO (Table 1). The interaction of CAR with LEC in the liposomes is highlighted by the broader band at 3590 to 3000 cm^{-1} in the CLIP spectrum (Figure 1), which is attributed, according to Barbosa (2013), to −OH stretching in associated phenols by the hydrogen-type intermolecular bond. The entrapment of CAR in CLIP may also be suggested by the

highest peaks' intensities between 1240 and 1130 cm^{-1} in the spectrum of the CLIP (Figure 1), which can be attributed to the combination of C−O stretching and the O−H in-plane angular deformation in CAR (Barbosa, 2013).

3.2 TGA

The TG and DTG curves are presented in Figure 2. The DTG curve shows that CAR mass is lost in a unique step between 144 and 160 °C. Three steps can be observed in the DTG curve of CLIP, the first of which is located around 186 °C, which suggests that CAR was incorporated into the liposomal structure, thereby increasing its thermal stability. The interaction of CAR with liposome compounds also can be indicated by the lower mass loss of CLIP (∼87%) in comparison with LIP mass loss (∼100%) up to 480 °C as noted in the TG curves. This difference suggests that CAR also improved the thermal stability of the liposomes, possibly reducing phospholipid mobility in the liposomal membrane. A similar effect was also observed by Pinilla et al. (2019), who detected better thermal stability of liposomes containing intercalated oleic acid with the molecules in the bilayer compared to pure phosphatidylcholine liposomes.

3.3 DSC

The DSC results are presented in Table 2. DSC is a reliable method to detect possible interaction of the bioactive material with liposomes through the change in their thermal behavior (Nahr et al., 2019). The endothermic events at about 257 °C (ΔH = -24.73 $J.g^{-1}$) and 272 °C (ΔH = -5.61 $J.g^{-1}$) observed for CLIP suggest volatilization of the LEC and CAR that did not form liposomes. Furthermore, LIP showed an exothermic event early (∼388 °C) and with a higher enthalpy value (363.60 $J.g^{-1}$) compared to CLIP (ΔH = 312.82 $J.g^{-1}$ at about 443 °C), which is also in accordance with the higher mass loss for LIP observed in TG analyses and resulted in greater liberation of energy. This finding also indicates structural changes in the bilayer membrane containing CAR, which

increased the thermal stability of the liposomes.

3.4 Liposomal stability

The variations in the mean size, PDI, and zeta potential of CLIP and LIP during the 30-day period (Figure 3) were not significant (p>0.05). These results indicate that both systems remained stable throughout the experiment duration under refrigerated conditions, possibly because zeta potentials more negative than the -30 mV of the particles prevented aggregation by the increment of the repulsive electrostatic force between them (Rafiee et al., 2017).

In addition, it was observed that CAR encapsulation had no significant effect on the size of the liposomes (p>0.05). Similarly, Hasan et al. (2019) detected that the size of the liposomes encapsulating curcumin was equal to the size of empty liposomes. However, the mean PDIs of CLIP and LIP during the 30-day period remained lower than 0.3 and above 0.3, respectively. A PDI value greater than 0.3 implies a high degree of heterogeneity in the size distribution (Maitani et al., 2001).

The broad size distribution of LIP may have occurred because the liposomes are a dynamic system in aqueous suspension and the acyl-chains of its membrane are moving, which can cause the fusion of vesicles. From this finding, it is hypothesized that CAR inserted itself among the fatty acyl chains of phospholipid molecules, compacting the liposomal membrane and reducing its fluidity, which prevented aggregation of particles and resulted in a monodisperse distribution size profile for CLIP.

The CLIP and LIP displayed a negatively charged surface associated with the head group (PO_4^{-3}) of the LEC's phospholipids and a hydroxyl group (OH^-) of the CHO. The CAR increased (p<0.05) the negatively charged surface of the vesicles, possibly due to the hydroxyl group (OH^-) present in its structure. Similarly, Homayouni et al. (2017) observed that the interaction of CAR and starch, which has a slightly negative charge,

increased the negative charges on the surfaces of the starch-carvacrol particles. Further, the higher zeta potential value for CLIP in comparison with the value for LIP suggests that part of the CAR molecule is in the liposomal surface, facing the water, and that CAR contributes to greater suspension stability.

3.5 EE of the CLIP

To evaluate the stability of CAR loaded in liposomes, the EE was measured on the 1st, 15th, and 30th days after suspension preparation. After storage for 15 days, the EE of CLIP decreased (p<0.05) from 61.18 ± 1.50% to 55.17 ± 1.99% but increased on the 30th day (60.78 ± 1.22%), attaining a value similar to the one obtained on the first day (p>0.05). This result indicates the strong CAR-retaining ability of the liposomal membrane during storage at 4 °C. It also suggests that CAR affected membrane structure as previously mentioned, leading to low membrane permeability, but without stopping the movement of its membrane acyl-chains completely, which may explain the variation of the EE value. In addition, these data were consistent with stability of size < 200 nm, PDI < 0.3, and zeta potential more negative than the -30 mV observed for CLIP. Huang et al. (2017) observed that lower particle size and PDI < 0.3 were related to higher retention capacity of the encapsulated compound by liposomes.

3.6 AFM

The AFM images (Figure 4) illustrate that the liposomes presented almost spherical shapes and smooth surfaces without any perceptible orifices. CLIP exhibited vesicles were uniformly dispersed and had a homogeneous size distribution while LIP presented particle aggregation with a heterogeneous size distribution, as was observed in PDI analysis. These findings provide more evidence that CAR affects the lipid membrane structure.

3.7 Antibacterial activity

The antibacterial activity of CAR against *S. aureus* and *E. coli* was determined before and after encapsulation in liposomes (Table 3). CAR and CLIP appeared able to inhibit and kill the two bacterial strains effectively even though no inhibition was found in the negative control. The exposure to LIP increased (p<0.05) or maintained (p>0.05) the number of viable cells of the bacteria compared to the initial inoculum.

According to the MBC results, *E. coli* was more sensitive to CAR than *S. aureus* and this result could be attributed to the ability of CAR in disintegrate the outer membrane present only in Gram-negative bacteria (Helander et al., 1998). Ultee et al. (2002) suggest that CAR also distorts the structure and destabilization of the cytoplasmic membrane, resulting in the collapse of the proton motive force, which harms the essential processes in the cell and leads to its death.

As argued in the previous sections, CAR interacts with liposome membranes, leading to low membrane permeability. However, given that the MIC and MBC values of CAR were the same as the values of CLIP against *E. coli,* this interaction does not limit the antibacterial effect of the compound. On the other hand, the results show that the encapsulation of CAR into liposomes has improved its antibacterial activity against *S. aureus*. This improvement may have occurred because liposomes can enhance the solubility and reduce the volatility of the encapsulated compound (Ge and Ge, 2016). Furthermore, the liposomes can transport the antimicrobial compound across the bacterial membrane, where it is released and can act on the inside of the cell (Khosravi-Darani et al., 2016).

4 Conclusion

Based on these findings, we conclude that the liposomes of LEC and CHO are suitable carriers for the encapsulation of CAR. The results of FTIR, TG, and DSC indicate that CAR was successfully introduced into liposomes. In addition, according to the PDI,

zeta potential, and EE of the CLIP, it is suggested that CAR can be located among the fatty acyl chains of phospholipid molecules with its hydroxyl group facing the water, therefore rigidifying the lipid bilayers that directly contribute to the good storage stability of the CAR liposomes. The prepared CLIP also demonstrated the ability to inhibit and kill *S. aureus* and *E. coli*. These findings suggest that liposomes should be further analyzed for future applications as CAR carriers in food preservation.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Table 1. Assignments of the principal absorption frequencies observed in Fourier transform infrared spectra of lecithin, cholesterol and carvacrol

	Wavenumber (cm^{-1})	Assignment
Lecithin	3625 - 3029	−OH stretching
	2923	Asymmetrical $=CH_2$ stretching
	2854	Symmetrical $=CH_2$ stretching
	1739	C=O stretching
	1646	N−H deformation
	1465	$=CH_2$ and $-CH_3$ deformation
	1224	P=O stretching
		Asymmetrical C−O−C stretching
	1062	Symmetrical C−O−C stretching
Cholesterol	3531 - 3095	−OH stretching
	2933, 2900	Asymmetrical $=CH_2$ and $-CH_3$ stretching
	2867, 2848	Symmetrical $=CH_2$ and $-CH_3$ stretching
	1465, 1365	$=CH_2$ and $-CH_3$ deformation
	1234 - 1010	C−O stretching
Carvacrol	3602 - 3093	−OH stretching
	2960	Asymmetrical $-CH_3$ stretching
	2869	Symmetrical $-CH_3$ stretching
	1625 - 1440	C=C stretching in the aromatic ring
	1260 - 1130	Combination of the C−O stretching and O−H in-plane deformation

Table 2. Differential scanning calorimetry results of lecithin (LEC), cholesterol (CHO), carvacrol (CAR), CAR-loaded liposome suspension (CLIP), and empty liposome suspension (LIP).

	T_{peak} (°C)	T_{onset} (°C)	T_{endset} (°C)	ΔH(J.g^{-1})	Event
LEC	187.45	186.32	190.41	-60.32	Volatilization
CHO	307.05	305.50	314.21	-5.15	Volatilization
CAR	257.21	254.76	276.59	-288.61	Volatilization
CLIP	187.47	185.36	190.89	-24.73	Volatilization
	271.83	270.45	275.18	-5.61	Volatilization
	443.01	422.72	466.14	312.82	Degradation
LIP	387.53	356.77	408.38	363.60	Degradation

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of carvacrol (CAR) and CAR-loaded liposome suspension (CLIP) in % v/v against *Staphylococcus aureus* and *Escherichia coli*.

Bacteria	*S. aureus*		*E. coli*	
	MIC	MBC	MIC	MBC
CAR	0.015	0.250	0.015	0.031
CLIP	0.007	0.125	0.015	0.031

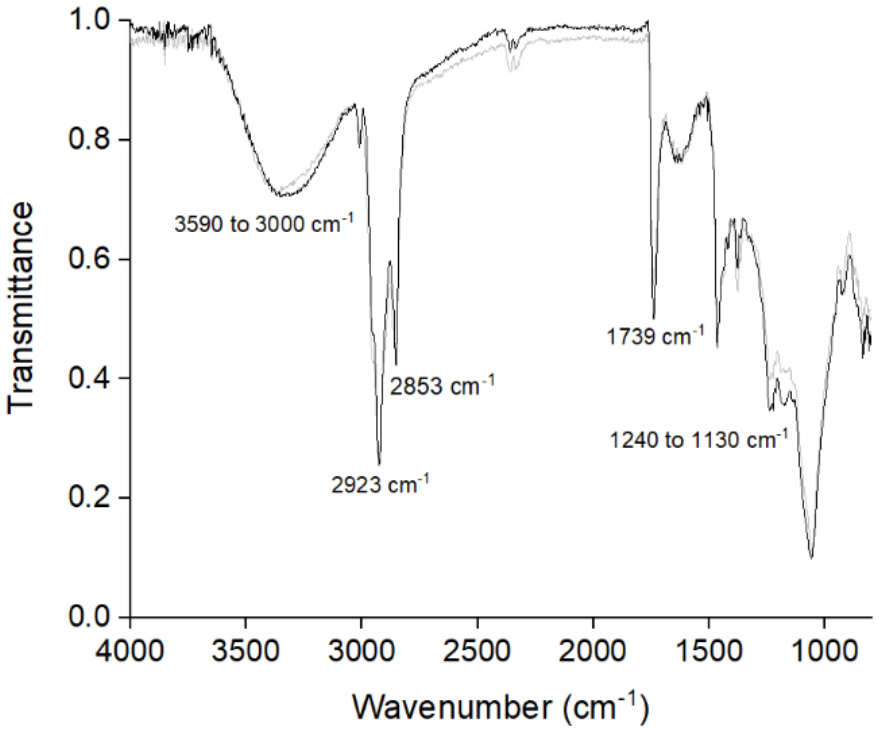

Figure 1. Fourier transform infrared spectrum of carvacrol liposomes (black curve) and empty liposomes (gray curve).

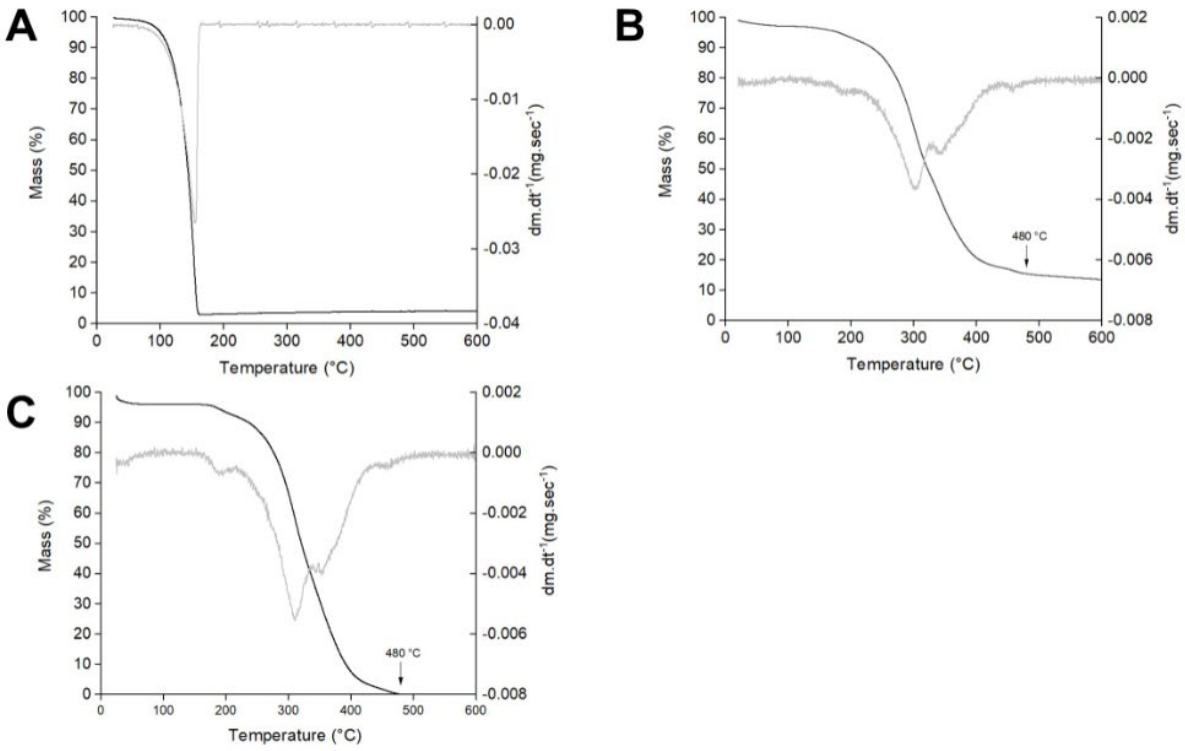

Figure 2. Thermogravimetric (black) and derivative (gray) curves of carvacrol (A), carvacrol liposomes (B), and empty liposomes (C).

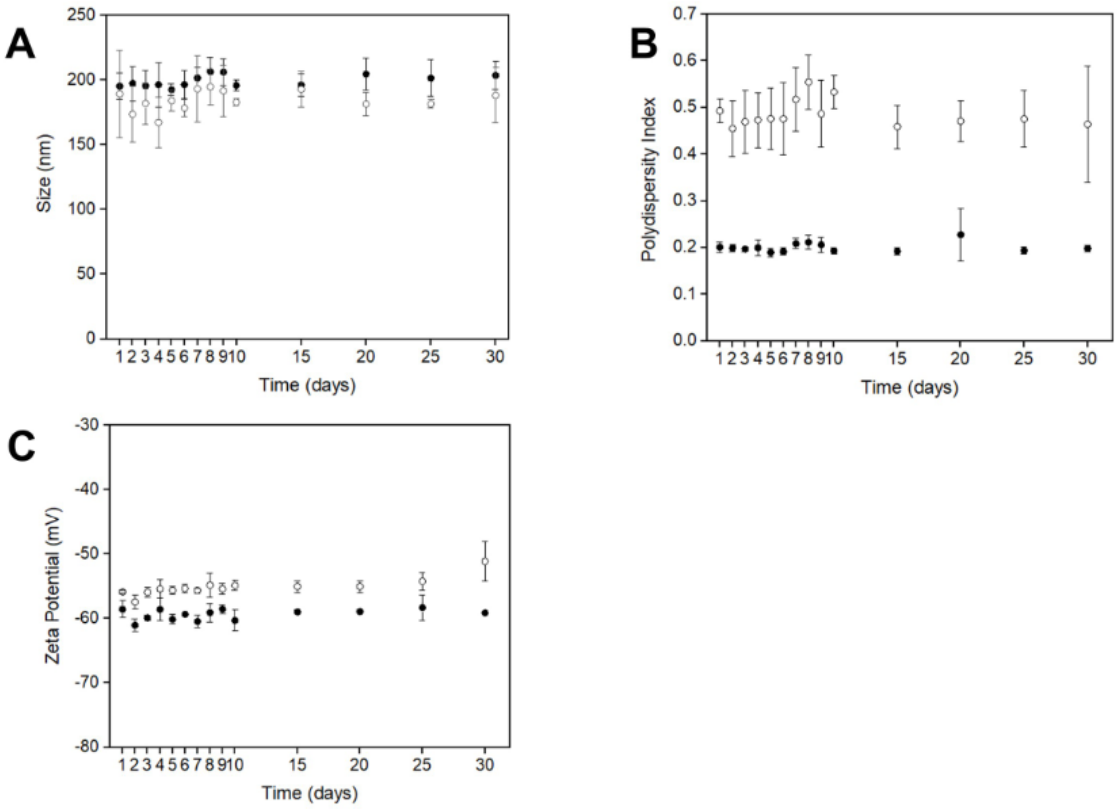

Figure 3. Variations in the mean size (A), polydispersity index (B), and zeta potential (C) of carvacrol liposomes (●) and empty liposomes (○) during storage at 4 °C in the dark for 30 days. The data are expressed as the mean ± standard deviation (n = 3).

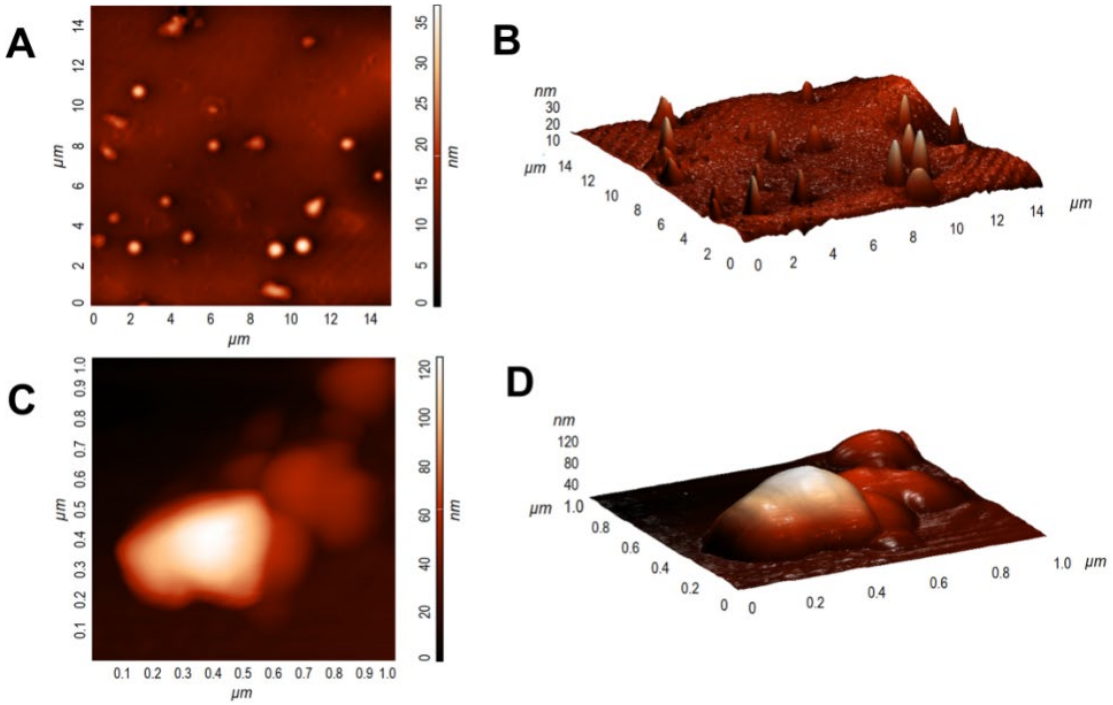

Figure 4. Atomic force microscopy (AFM) 2D and 3D images of carvacrol liposomes (A and B) and empty liposomes (C and D).

Artigo 3

Antibacterial effect of free and liposome-encapsulated carvacrol against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

The liposomal encapsulation of antimicrobial agents has been explored in order to enhance its activity in foods. The liposomes are spherical lipid vesicles that can not only improve the solubility of entrapped compounds but also interact with biological membranes and release their load into the bacteria. In this study, carvacrol, a natural antimicrobial, was incorporated into liposomes, and growth assays were used to evaluate its antimicrobial activity in comparison to its free form. Time-kill curves demonstrated that carvacrol in liposomes had a superior effect on the inhibition of *Staphylococcus aureus* and *Escherichia coli* growth when compared to the free carvacrol. The mechanism involved in the inactivation of these bacteria by free and encapsulated carvacrol was investigated by measuring the leakage of intracellular contents. Based on results obtained with *S. aureus*, carvacrol probably disrupted the cells membranes whereas the liposomes interacted with the membranes and released carvacrol inside the cells. Submitting *E. coli* both to free carvacrol and to carvacrol into liposomes, the only cell constituent release was ATP. These data suggest that liposome might be a promising system to carry carvacrol in foods, but more studies are needed to fully understand their mechanisms of inactivation against *E. coli*.

Keywords: carvacrol liposomes, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli*, cell permeability

1 Introduction

Bacterial foodborne pathogens are an important cause of morbidity and mortality worldwide (Zhang et al., 2019). *Staphylococcus aureus* is a Gram-positive, spherical-shaped bacterium, of about 1 μm, that is associated with food poisoning, as some of its pathogenic strains have the ability to produce enterotoxins in food (Huong et al., 2010; Rubab, Shahbaz, Olaimat, & Oh, 2018). *Escherichia coli* is a Gram-negative and rod-shaped bacterium, of which most strains are commensal organisms found in the gastrointestinal tract of humans, but a few harbor different virulence factors that can cause intestinal or extraintestinal infections (Croxen et al., 2013; Dell'Orco et al., 2019). The prevalence of *E. coli* infections is generally related to the ingestion of contaminated food (Ali et al., 2020).

The use of carvacrol (CAR), the major constituent of oregano essential oil, has been studied as a way of ensuring food safety (Churklam, Chaturongakul, Ngamwongsatit, & Aunpad, 2020; Santos et al., 2016). This compound has been shown to exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria, including *S. aureus* and *E. coli* (Guarda, Rubilar, Miltz, & Galotto, 2011; Stratakos et al., 2018). The mechanism of antibacterial action of CAR that is most often reported involves the disruption of cell envelopes, cytoplasmic membranes, and outer membranes of Gram-negative bacteria, leading to leakage of intracellular contents and consequently to death (Hyldgaard, Mygind, & Meyer, 2012; Kachur & Suntres, 2019). It is proposed that this compound could also interact with intracellular targets (Hyldgaard, Mygind, & Meyer, 2012; Wang et al., 2016). However, CAR has low water solubility, high volatility, and low chemical stability, all of which hamper its antibacterial activity in foods (Engel, Heckler, Tondo, Daroit, & da Silva Malheiros, 2017).

Therefore, CAR encapsulation in liposomes has been evaluated in order to preserve

it and distribute it evenly when applied directly in food or packaging (Tavares et al., 2020). Liposomes are spherical vesicles consisting of phospholipid bilayers that can improve the effectiveness of the entrapped antimicrobial since it can interact with biological membranes, and subsequently release their load into the bacteria (Huang et al., 2011; Sachetelli et al., 2000). This ability facilitates the access of the antimicrobial to the bacteria, circumventing its resistance mechanism and allowing the agent to act inside the bacterial cell (Khosravi-Darani, Khoosfi, & Hosseini, 2016; Sachetelli et al., 2000; Wang et al., 2016). Despite the antibacterial activity of CAR entrapped in liposomes (CLIP) having already been reported (Liolios et al., 2009; Heckler et al., 2020; Tavares et al., 2020), its mode of action has not yet been clarified, this study aimed to add information to elucidate that mechanism.

2 Materials and methods

2.1 Preparation of the liposomes

Liposomes composed of soy lecithin (InLab, Brazil) and cholesterol (Sigma–Aldrich, USA) were prepared as described by Tavares et al. (2020).

2.2 Bacterial strains and growth conditions

Two standard bacterial strains obtained from the American Type Culture Collection (ATCC), *S. aureus* ATCC 6538 and *E. coli* ATCC 11229, were evaluated in this study. The stock cultures were stored at – 80 °C and, before the experiments, *S. aureus* and *E. coli* were cultivated in a Tryptic Soy Broth (TSB) (Difco, USA) and Nutrient Broth (NB) (Kasvi, Brazil), respectively, at 37 °C for 24 h followed by streaking on Tryptic Soy Agar (TSA) (Acumedia, USA) and Nutrient Agar (NA) (Kasvi, Brazil), respectively, and incubation at 37 °C for 18 h. This culture was used to prepare bacterial suspension in a saline solution, that had its absorbance adjusted of 0.09 to 0.11 at 600 nm using a spectrophotometer (GBC Scientific Equipment, Australia) to obtain a final concentration

of 10^8 Colony Forming Units (CFU).mL^{-1}.

2.3 Effect of the CAR and CLIP on bacteria growth

The drop plate method was used to assess the viability of the bacteria exposed to free and encapsulated CAR. Tubes (50 mL) with CAR free (in 0.5% v/v Tween 80) and in liposomes were diluted in TSB at final concentration of 0.03, 0.06 and 0.12% v/v and were inoculated with bacteria (10^8 CFU.mL^{-1}) at final volume of 20 mL. *S. aureus* was exposed to the solutions of 0.03, 0.06 and 0.12% v/v and *E. coli* to the solutions of 0.03, 0.06% v/v. These concentrations tested were based on preliminary experiments. The tubes were incubated at 37 °C for periods of 0, 18, 48, 72, and 96 h. After each period, 1 mL of liquid were removed from the test tube and centrifuged at 2000 *g* for 15 min (VWR International, USA). The pellets were washed, resuspended, and diluted in saline solution to dropped on the surface of TSA. After incubation of the plates at 37 °C for 8-12 h, viable cells were counted and results were converted to CFU.mL^{-1}. Each assay included a growth control with no antimicrobial. The time-kill curves were constructed by plotting $\log_{10}$ CFU.mL^{-1} against time (h).

2.4 Measurement of cellular leakage

The antimicrobial mode of action of CAR, free and in liposomes, against *S. aureus* and *E. coli*, was investigated based on the release of short-chain DNAs and RNAs, amino acids and short peptides, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP).

The individual cell suspension (10^8 CFU.mL^{-1}) was placed into tubes (15 mL) containing PBS (pH 7.2) supplemented with 0.03% v/v of CAR, free (in 0.06% v/v Tween 80) and encapsulated, at final volume of 6 mL. In this concentration, the CAR presented an inhibitory effect against *S. aureus* and *E. coli* and was used to make a comparison between the effects of the free and encapsulated CAR. The samples were maintained at a

temperature of 37 °C for 1 h and were then, centrifuged at 2500 *g* for 10 min, and the absorbance of the supernatant was measured at 260 nm and 280 nm. The samples were maintained at a temperature of 37 °C for 1 h and were then, centrifuged at 2500 g for 10 min. The absorbance of the supernatant was measured at 260 nm, to detected the leakage of short-chain DNAs and RNAs, and at 280 nm, to identify the loss of amino acids and short peptides (Thermo Fisher Scientific, USA). The bacterial suspension in sterile PBS without CAR was tested as a control.

The individual cell suspension (10^8 CFU.mL^{-1}) was placed into tubes containing PBS (pH 7.2) supplemented with 0.18% v/v of the CAR, free (in 0.36% v/v Tween 80) and encapsulated, against *S. aureus* and 0.06% v/v of the CAR, free (in 0.12% v/v Tween 80) and encapsulated, against *E. coli*. Free CAR presented a bactericidal effect against *S. aureus* and *E. coli* in these concentrations, which were used to make a comparison between the effects of the free and the encapsulated CAR. The samples were kept at 37 °C for 1 h and then were centrifuged at 2500 *g* for 10 min. The supernatant was filtered through a membrane with pores of 0.2 µm. Concentrations of NADPH in the supernatant were quantitated by measuring the absorbance at 340 nm (SpectraMax M5, USA). The ATP release from the cells was measured by the Bac Titer-Glo™ Microbial Cell Viability Assay Reagent Kit (Promega, USA). Fifty microliters of the supernatant were mixed with 50 µL of this reagent reagent in a black 96-well plate. Light transmission was measured using a luminometer (SpectraMax M5, USA) at 560 nm. Analytical curves, to calculate the concentrations of the NADPH (mM) and ATP (µM) from the absorbance and luminescence level, were produced using an NADPH and ATP standard (Promega, USA), respectively. Untreated samples were used as controls.

2.5 Statistical analysis

The experiments were performed three times and differences between the treatments were evaluated by Analysis of Variance (ANOVA) following Tukey's test ($p < 0.05$), using the software Minitab 18, 2017.

3 Results and discussion

3.1 Effect of the CAR and CLIP on bacteria growth

A preliminary experiment demonstrated that unloaded liposomes were ineffective against the tested bacteria (data not shown). Figure 1A shows the growth of *S. aureus* exposed to CAR and CLIP. The number of cells in the control experiment differed ($p < 0.05$) from all treatments after 18 h. CAR and CLIP at 0.03% v/v after 18 h showed an inhibitory effect, decreasing the bacteria's growth about 4.5 log cycles ($p < 0.05$) in comparison with the control. As of 48 h, CLIP at 0.03% v/v presented bactericidal effect while the number of viable cells of the bacteria exposed to CAR in this same concentration remained constant ($p > 0.05$). CLIP was also more efficient at reducing the viability of the cell than CAR at 0.06 and 0.12% v/v ($p < 0.05$) at 0 h and after 18 h. CAR and CLIP at 0.06 and 0.12% v/v decreased *S. aureus* number below the detection level after 48 h of incubation, indicating bactericidal effects.

The growth of *E. coli* exposed to CAR and CLIP is presented in Figure 1B. At 18 h, the number of viable cells of *E. coli* in the control treatment was higher ($p < 0.05$) than the number observed for the other treatments. The CAR at 0.03% v/v reduced the cell viability after 18 h and decreased bacteria counts about 4 log cycles ($p < 0.05$) in comparison to the control. The inhibitory effect of the CAR at 0.03% v/v was sustained until 96 h. At 0.06% v/v, the CAR caused a bactericidal effect as of 18 h. However, CLIP was bactericidal in the two concentrations tested, since bacterial counts remained below detection level at 0.03% as of 18 h and at 0.06% as immediately after inoculation.

The results indicate that CAR and CLIP had a significant inhibitory and bactericidal effects concentration dependent against *S. aureus* and *E. coli*. Furthermore, they show that *E. coli* was more sensitive than *S. aureus* for both free and encapsulated CAR, even though the Gram-negative bacteria are generally considered more resistant to the action of CAR since they have lipopolysaccharides in the outer membrane, that hamper the access of hydrophobic compounds to the cytoplasmic membrane (Arrieta et al., 2014; Guarda et al., 2011; Kurek, Moundanga, Favier, Galić, & Debeaufort, 2013). It was suggested that a greater concentration of this antimicrobial against Gram-negative bacteria is needed to cause the same effect obtained in the Gram-positive bacteria, because CAR first causes the disintegration of the outer membrane and later damage to the cytoplasmic membrane, leading to cell inactivation (Guarda et al., 2011). However, interspecific and intraspecific differences in the resistance of Gram-positive and Gram-negative bacteria can contribute to variation in its susceptibility to CAR (Kurek et al., 2013).

It was also observed that the incorporation of the CAR into liposomes improved the antibacterial activity, especially for the *E. coli*. This can be attributed to the greater stability and interaction with bacterial cells of the CAR when encapsulated in liposome. Liposomes can limit the coalescence of the active ingredient encapsulated and its loss by volatilization, oxidation, degradation by light and heat, thus, making it available for more time (Cui, Wu, Lin, 2016). The CLIP presented a zeta potential of -58.2 ± 1.7 mV, which increases the repulsive electrostatic force between charged vesicles and prevents their aggregation (Tavares et al., 2020). Juveriya Fathima, Fathima, Abhishek, & Khanum (2016) observed that liposomes containing thiamine maintained their size (< 200 nm), polydispersity index (< 0.3) and zeta potential (< -30 mV) in the stability range with no significant variation for up to 30 days at 37 °C. Liposomes loading curcumin and

resveratrol retained 90% of the polyphenols initially encapsulated even after being submitted to a temperature of 37 °C and pH 7.4 for 2 hours (Huang et al., 2019).

Liolios et al. (2009) proposed that liposome improves the transportation of the active component to the bacteria and makes it possible for it to be released inside the cell by fusion with the cell membrane, contact release, inter-membrane transfer, and absorption. The CLIP presented a size of 192.0 ± 10.8 nm (Tavares et al., 2020) and the subcellular size of the liposomes reduces mass transfer resistance and may enable its cellular absorption in a passive form (Donsì, Annunziata, Sessa, & Ferrari, 2011). Also, the interaction of the liposome with cells could increase the permeability of the bacterial membrane to loaded antimicrobial and cause its liberation more quickly and in high concentration (Ge; Ge, 2016; Huang et al., 2011). Other authors also reported an increase in the antimicrobial efficiency of essential oils and their constituents after its encapsulation in liposomes, namely *Zataria multiflora* Boiss essential oil (Khosravi-Darani, Khoosfi, & Hosseini, 2016), tea tree oil (Ge; Ge, 2016), and cinnamaldehyde (Makwana, Choudhary, Dogra, Kohli, & Haddock, 2014).

The enhanced penetration into bacterial cells for antimicrobials encapsulated in liposomes was observed by some researchers (Mugabe, Halwani, Azghani, Lafrenie, & Omri, 2006; Sachetelli et al., 2000). Wang et al. (2016) proposed that components of bacterial membranes can induce the fusion of the liposome and bacteria and that the mechanisms involved can be different in Gram-negative and Gram-positive bacteria. The mode by which the liposomes cross the membrane is not completely clear, but it is likely that a destabilization of the vesicle bilayers occurs during its fusion with the cell membrane, facilitating the release of the liposome's content into the cytoplasm (Nicolosi, Scalia, Nicolosi, & Pignatello, 2010). It was also reported that liposomes were able to bypass the outer membrane and release the entrapped substance inside the periplasmic

space of the Gram-negative bacterial cells, where its antibacterial activity can be exerted (Mugabe et al., 2006; Nicolosi, Scalia, Nicolosi, & Pignatello, 2010). It is possible that this effect was what caused the improved activity of the CLIP against *E. coli* in comparison with free CAR.

3.2 Measurement of cellular leakage

Nucleic acid has strong UV absorption at 260 nm and amino acids and short peptides at 280 nm (Lee, Kim, Lim, & Ahn, 2014; Prudêncio, Vanetti, & Prieto, 2015). Therefore, the loss of absorbing material at these wavelengths from bacterial suspensions treated with CAR and CLIP was monitored. The measure of the absorbance at 260 nm of the supernatant indicated that DNA and RNA loss by *S. aureus* was higher for the cells exposed to CAR than for both the control ($p < 0.05$) and the cells exposed to CLIP ($p < 0.05$) (Figure 2A). The cells exposed to CAR also demonstrated higher leakage of intracellular content of amino acids and short peptides in comparison to the control ($p < 0.05$) and cells treated with CLIP ($p < 0.05$) (Figure 2B).

The NADPH absorbs UV light at a wavelength of 340 nm (Ruyck et al., 2007). The *S. aureus* cells treated with CAR leak more of their intracellular content of NADPH into the supernatant than the untreated cells ($p < 0.05$) and the ones treated with CLIP ($p < 0.05$) (Figure 3A). Results of the ATP luminescence assay showed that the release of the intracellular ATP contents by *S. aureus* was higher in the cells treated with CAR compared to the control ($p < 0.05$) and to cells submitted to CLIP ($p < 0.05$) (Figure 3B).

The potential disruption of the cell membranes by CAR may lead to an increase in the cell membrane permeability and, consequently, to leakage of internal cell contents (Hyldgaard, Mygind, & Meyer, 2012). The detection of intracellular substances in the cell's supernatant suggests that CAR caused damage to the cellular membranes of *S. aureus*. However, CLIP did not cause considerable leakage of cell constituents by *S.*

aureus, even though it inhibited the growth of this bacterium. This indicates that the mechanism by which CAR inhibits *S. aureus* when encapsulated in liposomes is different than when free. As discussed before, it is suggested that the liposomes fused with the membrane or were absorbed by the bacteria, releasing the CAR contents into the cytoplasm, where they acted. *In vitro* studies using the fluorescent method demonstrated that oleic acid-loaded liposomes can rapidly fuse into the bacterial membranes, thereby significantly improving the ability of oleic acid to kill methicillin-resistant *S. aureus* compared with the use of free oleic acid (Huang et al., 2011).

The release of cell constituents by *E. coli* submitted to CAR and CLIP was not observed, except for ATP (Figures 2 and 3). *E. coli* cells exposed to CAR demonstrated leakage of ATP in a concentration similar to cells treated with CLIP ($p > 0.05$). According to other authors, the antimicrobial effect of carvacrol against *E. coli* was induced by changes in cell membrane permeability, the release of proteins and nucleic acid and reduction in intracellular ATP levels (Ait-Ouazzou et al., 2012; Khan et al., 2020; Stratakos et al., 2018). Jung, Thamphiwatana, Zhang, & Obonyo (2015) observed that linolenic acid-loaded liposomes increased the outer membrane permeability of *Helicobacter pylori* and caused the release of the intracellular ATP. The authors suggested that the liposomes fused with the bacteria and released the encapsulated compound, which resulted in the disruption of the cytoplasmic membrane. Considering that CAR and CLIP have inhibited and killed *E. coli* cells in the concentrations tested, it is suggested that more research be performed to better understand their mode of action against this bacterium.

4 Conclusion

The encapsulation of CAR into liposomes greatly enhanced its inhibitory and bactericidal effects against *S. aureus* and *E. coli*, important foodborne bacteria. Therefore,

liposome might be a promising system to carry the CAR in foods as a strategy to increase its safety. Based on the measurement of cellular leakage, CAR is likely to exert its effect against *S. aureus* by the disruption of the cell membranes, which caused the release of the intracellular contents. The CLIP probably released CAR inside of the *S. aureus* cell, where it acted. The release of the cell constituents by *E. coli* submitted to CAR and CLIP, except for ATP, was not observed. Thus, more studies are needed to fully understand their mechanisms of inactivation against this bacterium.

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Declaration of Interests

None

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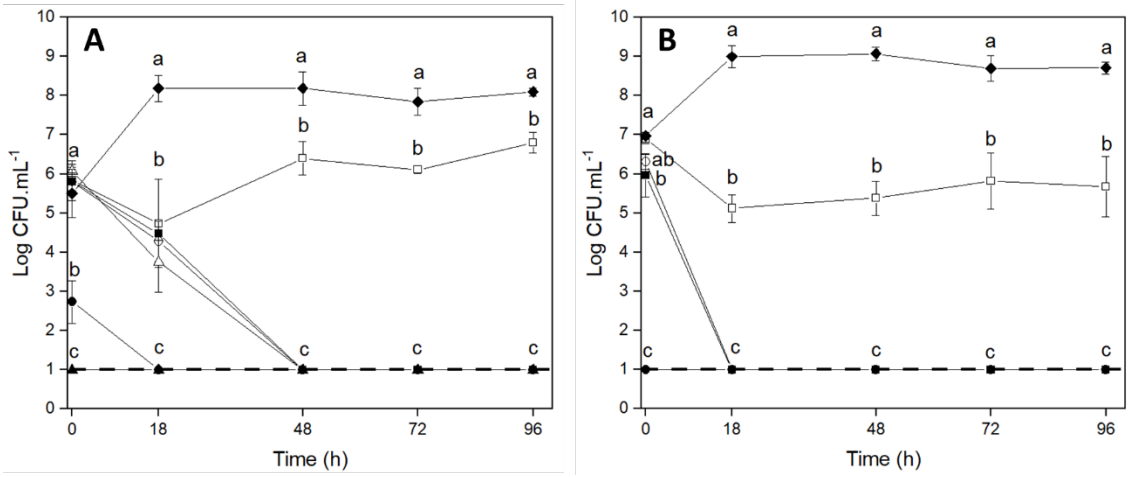

Figure 1. Effects of free carvacrol (CAR) and carvacrol in liposomes (CLIP) on the growth of *Staphylococcus aureus* (A) and *Escherichia coli* (B). CAR at 0.03% v/v (□), CAR at 0.06% v/v (○), CAR at 0.12% v/v (△), CLIP at 0.03% v/v (■), CLIP at 0.06% v/v (●), CLIP at 0.12% v/v (▲), Control (♦). Each point represents the mean ± standard deviation of three independent experiments. Counts with the same letter in each time interval do not differ from each other by Tukey's test ($p > 0.05$). The detection limit of the technique was indicated with a dotted horizontal line.

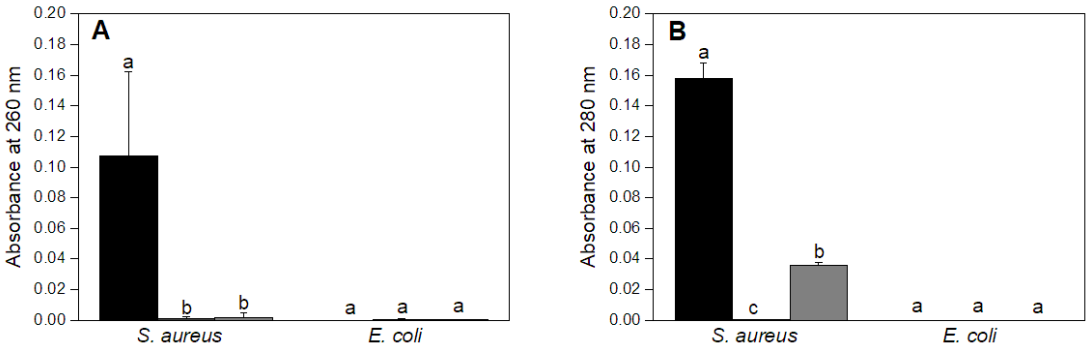

Figure 2. Leakage of material absorbing at 260 nm (A) and 280 nm (B) from *Staphylococcus aureus* and *Escherichia coli* cells treated with 0.03% v/v of carvacrol (■), 0.03% v/v of carvacrol into liposomes (■) and without antimicrobial (■). Each point represents the mean ± standard deviation of three independent experiments. Means with the same letter for each bacteria do not differ from each other by Tukey's test ($p > 0.05$).

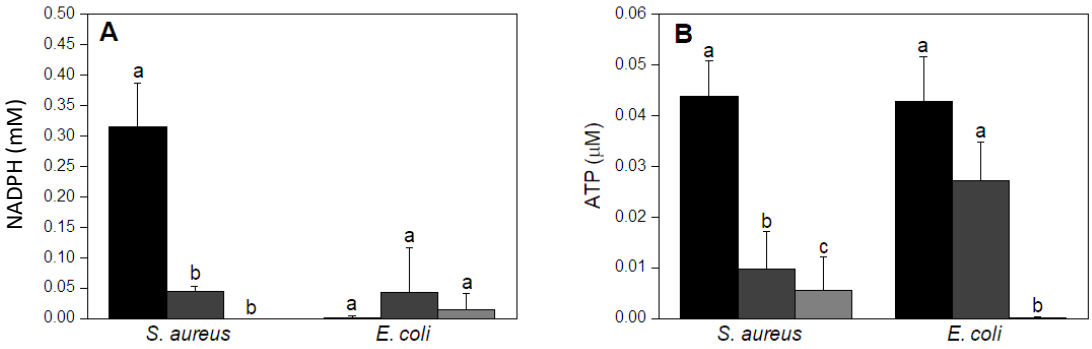

Figure 3. Leakage of NADPH (A) and ATP (B) from *Staphylococcus aureus* and *Escherichia coli* cells treated with carvacrol (■), carvacrol into liposomes (■), and without antimicrobial (■). For *S. aureus*, the concentration tested was 0.18 % v/v and for *E. coli*, 0.06 % v/v. Each point represents the mean ± standard deviation of three independent experiments. Means with the same letter for each bacteria do not differ from each other by Tukey's test ($p > 0.05$).

CONCLUSÃO GERAL

Lipossomas compostos por lecitina de soja e colesterol para encapsulação de carvacrol foram produzidos pela técnica de hidratação de filme lipídico. Um modelo de regressão explicou a influência das concentrações de colesterol e carvacrol na eficiência de encapsulação dos lipossomas produzidos. A formulação com maior eficiência de encapsulação foi obtida por meio da função desejabilidade e a mesma foi posteriormente validada. As análises físico-químicas indicaram que o carvacrol, além de eficientemente encapsulado nos lipossomas, reduziu a agregação e fusão das vesículas e aumentou a estabilidade térmica das mesmas.

A encapsulação do carvacrol em lipossomas manteve seus efeitos inibitórios e bactericidas contra *S. aureus* e *E. coli* e aumentou o seu tempo de ação antibacteriana. Ao avaliar o extravasamento de material celular por *S. aureus*, foi sugerido que o carvacrol causou o rompimento da membrana celular dessa bactéria, mas quando encapsulado, agiu no interior da célula, já que os lipossomas podem se fundir às membranas celulares. O mecanismo de inativação do carvacrol livre e encapsulado em lipossomas contra *E. coli* não foi esclarecido. Quando incorporados à filmes de poli(vinil álcool), os lipossomas com carvacrol não foram eficientes na inibição de *S. aureus* e *E. coli*.

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