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Cielo Char[a], Lorena Cisternas[a], Francisco Pérez[a] & Sandra Guerrero[b]

[a] Departamento de Agroindustria y Enología, Facultad de Ciencias Agronómicas, Universidad de Chile, P.O. Box 1004, Av. Santa Rosa 11315, La Pintana, Santiago, Chile

[b] Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina
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Effect of emulsification on the antimicrobial activity of carvacrol

Efecto de la emulsificación sobre la actividad antimicrobiana del carvacrol

Cielo Char[a]*, Lorena Cisternas[a], Francisco Pérez[a] and Sandra Guerrero[b]

[a]*Departamento de Agroindustria y Enología, Facultad de Ciencias Agronómicas, Universidad de Chile, P.O. Box 1004, Av. Santa Rosa 11315, La Pintana, Santiago, Chile;* [b]*Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina*

This work focuses on the emulsification of carvacrol for its incorporation into juices with the aim of retaining antimicrobial activity while enhancing the stability of the oil in aqueous systems. Carvacrol was emulsified (CA-E) using capsul® (1:2 emulsion) and its antimicrobial activity was determined on *Escherichia coli* and *Lactobacillus plantarum*. The combined effect of CA-E and pH reduction to 4.5 was assessed on different juices. The sensitivity of *L. plantarum* to carvacrol was not affected by emulsification, whereas *E. coli* presented higher minimal inhibitory concentrations. Combined treatments improved the effect: 0.5 µL/mL CA-E increased from 0.2 to 2.1 log reductions of *E. coli*. Carvacrol emulsion (1.0 µL/mL) successfully inactivated *E. coli* in apple and orange juices, attaining undetectable levels (<1 log CFU/mL). The efficacy of carvacrol emulsion was improved by acidification; therefore, its incorporation at low doses in acidic foods may be a useful alternative for multiple applications.

Keywords: natural antimicrobials; emulsion; carrot juice; *Escherichia coli*; *Lactobacillus plantarum*

Este trabajo se basa en la emulsificación del carvacrol para su incorporación a jugos con el objetivo de retener su actividad antimicrobiana y mejorar la estabilidad del aceite en sistemas acuosos. El carvacrol se emulsificó (CA-E) utilizando capsul® (emulsión1:2) y la actividad antimicrobiana se determinó sobre *Escherichia coli* y *Lactobacillus plantarum*. El efecto combinado de CA-E y la reducción del pH a 4,5 se evaluó en diferentes jugos. La emulsión no afectó la sensibilidad de *L. plantarum* al carvacrol; sin embargo, *E. coli* presentó mayor concentración mínima inhibitoria. Los tratamientos combinados incrementaron el efecto, 0,5 µL/mL CA-E aumentó desde 0,2 a 2,1 reducciones log de *E. coli*. La emulsión de carvacrol (1,0 µL/mL) inactivó exitosamente a *E. coli* en jugos de manzana y naranja, alcanzando niveles indetectables (<1 log UFC/mL). La eficacia de la emulsión de carvacrol aumentó por la acidificación, permitiendo su uso en bajas dosis en alimentos ácidos, constituyendo una alternativa para múltiples aplicaciones en alimentos.

Palabras clave: antimicrobianos naturales; emulsión; jugo de zanahoria; *Escherichia coli*; *Lactobacillus plantarum*

Introduction

The potential of essential oils as natural agents for food preservation is widely recognized and their antibacterial, antifungal and antioxidant activity, as well as their flavoring properties, have found applications in the pharmaceutical, food and cosmetic industries (Adorjan & Buchbauer, 2010; Char, Guerrero & Alzamora, 2010; Rivera, Bocanegra-García & Monge, 2010).

Carvacrol (CA), the major component of oregano (*Origanum* sp.) and thyme (*Thymus* sp.) essential oils, possesses a phenolic structure. One of the targets of this lipophilic compound in the microbial cell seems to be related to direct interaction with the hydrophobic regions of membrane proteins and protein complexes, increasing cell membrane permeability (Oussalah, Caillet & Lacroix, 2006; Ultee, Kets & Smid, 1999). Meaningful modifications of the cell surface structure have been demonstrated, along with a reduction in cell size that could reasonably be attributed to leakage of cytosolic fluids outside the cell (La Storia et al., 2011). Cell death due to oxidative damage to DNA structures in *Escherichia coli* has recently been suggested by Chueca, Pagán and García-Gonzalo (2014).

The addition of essential oils to aqueous foods is often limited by their poor solubility in water. Moreover, their strong flavor may change the sensory attributes of food when used in high doses. For these reasons, there is a need to assess new delivery systems to encapsulate and release essential oils in food products (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny & Martín-Belloso, 2015).

The effect of emulsification on the antimicrobial activity of natural antimicrobials depends on the formulation, the nature of the active compound, and the target microorganism (Donsì, Annunziata, Sessa & Ferrari, 2011; Weiss, Gaysinksy, Davidson & McClements, 2009). Decrease in droplet size (nanoemulsion) in many cases has increased antimicrobial activity; however, a significant reduction in droplet size does not necessary imply an increase in functionality, and antimicrobial activity may be provided by the whole delivery system (Buranasuksombat, Kwon, Turner & Bhandari, 2011; Salvia-Trujillo et al., 2015).

The aim of this study was to emulsify carvacrol for incorporation into natural juices with the aim of improving the physical stability of the oil in aqueous foods while retaining antimicrobial activity. The antimicrobial effect of CA and CA emulsion (CA-E) was determined on *E. coli* and *Lactobacillus plantarum*. Moreover, the combined effect of CA-E and pH reduction to 4.5 was assessed and subsequently verified during the storage of naturally acidic juices.

*Corresponding author. Email: cdchar@u.uchile.cl

Materials and methods

Inoculum preparation

Inocula of *E. coli* ATCC 35218 and *L. plantarum* ATCC 8014 were prepared by transferring a loopful of a stock culture to a 50 mL bottle of trypteine soy broth + 0.6% yeast extract (TSB-YE) or de Man, Rogosa and Sharpe Lactobacilli broth (MRSb), respectively. The cultures were incubated overnight with agitation at 37°C and enumerated by the spread plate count method. The appropriate dilution of *E. coli* culture was plated on trypti-case soy agar + 0.6% yeast extract (TSA-YE) and incubated at 37°C for 24 h; while *L. plantarum* was plated on de Man, Rogosa and Sharpe Lactobacilli agar (MRSa) and incubated at 37°C for 48 h in an anaerobic jar. The final cell density of the cultures was approximately 1×10^9 CFU/mL. All microbiological media used in this work were purchased from Merck (Darmstadt, Germany).

Juice preparation

Fresh carrot (pH 6.4; 12.2 °Brix) juice was aseptically obtained from carrots that were carefully washed and brushed with water in order to remove residual dirt, and decontaminated by immersion in a chlorine solution (sodium hypochlorite 100 mg/L) for 5 min. Following rinsing in distilled water and drying, the top and bottom (about 1 cm thickness) and any dirt spots were cut out. The juice was extracted with a semi-industrial juicer (ZJ 145 CTA, Panyu, China) then pasteurized at 75°C for 2 min using a batch-type reactor connected to a thermostatically controlled water bath (JeioTech, Seoul, South Korea) to eliminate the natural microbiota. The pasteurized juice was immediately cooled, dispensed into 100 mL amber flasks and stored at −18°C for a maximum of 1 month until required for experimentation.

Preparation of the emulsion

Carvacrol was purchased as a pure liquid substance (99%, molecular weight 150.2 g/mol) (Sigma Aldrich, Saint Louis, MO, USA). The emulsion was prepared using capsul® (Ingredion, Westchester, USA), a modified maize starch. The CA-capsul® (1:2 w/w) emulsion was prepared as follows: capsul® (15 g) was dissolved in water (77.5 g), heated to 70°C, then cooled to 30°C and mixed with the CA (7.5 g) with constant stirring. The resulting solution was homogenized using an Ultraturrax® (T18 IKA, Staufem, Germany) at increasing rates from 0 to 15,000 rpm within 5 min. The emulsion was stored at −20°C for subsequent studies.

The CA-E was characterized by measuring droplet size using a dynamic light scattering laser diffractometer (Mastersizer X, Malvern Instruments, Herrsching, Germany), giving a Sauter mean diameter ($D_{[3,2]}$) of 2.26 μm.

Determination of minimal inhibitory concentration and minimal bactericide concentration

The minimal inhibitory concentration (MIC) of carvacrol (CA) and carvacrol emulsion (CA-E) was determined on *E. coli* and *L. plantarum* using the tube dilution method (Rota, Herrera, Martínez, Sotomayor & Jordán, 2008). The tested concentrations were 25, 20, 15, 10, 5, 1 and 0.1 μL/mL of carvacrol (corresponding to the range 166–0.6 mM). In the case of the carvacrol emulsion, the appropriate amount of the emulsion containing 25–0.1 μL/mL of carvacrol was used. Tubes containing 10 mL of the appropriate broth and the corresponding concentration of CA or CA-E were inoculated with 100 μL of the microbial suspension, and incubated. *E. coli* was inoculated into TSB-YE tubes and incubated at 37°C for 24 h, while *L. plantarum* was inoculated into MRSb and incubated at 37°C for 48 h. MIC values were determined as the lowest concentration of the anti-microbial agent inhibiting visible microbial growth in the broth tube. The result was confirmed by measuring the absorbance at 615 nm of each tested system before and after incubation.

The minimal bactericide concentration (MBC) was determined by plating 1 mL of each incubated tube used for MIC determination in duplicate, using the appropriate solid culture medium and incubated under appropriate conditions according to the microorganism (*E. coli* was plated on TSA-YE and incubated at 37°C for 24 h, whereas *L. plantarum* was plated on MRSa and incubated at 37°C for 48 h in an anaerobic jar). MBC values were determined as the lowest concentration of the antimicrobial agent inhibiting microbial growth both in the broth and in subsequent transfer to the corresponding solid culture medium (Rota et al., 2008). Two independent experimental runs for each CA or CA-E concentrations were conducted.

Inactivation kinetics of two microbial strains as affected by CA-E in carrot juice

The effect of carvacrol emulsion was evaluated on a model bacterium (*E. coli*) and on an acid-tolerant bacterium (*L. plantarum*). The appropriate volume of emulsion was added to 99 mL of carrot juice (pH 6.4) under agitation with a magnetic stirrer to reach 0.5, 1.0 and 1.5 μL/mL (corresponding to 3.3, 6.6 and 9.9 mM, respectively). The inactivation kinetic tests were conducted by adding 1 mL of the inoculum and taking samples at regular intervals. After inoculation, the time count was immediately started and the first sample (1 mL) was simultaneously extracted ($t = 0$). Subsequent samples were taken every 15 or 30 min over 2 h depending on the severity of the treatment, poured into 9 mL peptone water (0.1% w/v) tubes and immediately analyzed for survivors. For systems where lower counts were expected, 5 mL samples were taken in empty tubes and immediately enumerated. Vigorous agitation (with the magnetic stirrer) before taking the samples was sufficient to maintain uniformity of the carvacrol emulsion and inoculum within the whole vessel.

Juice samples were tenfold diluted in 0.1% w/v peptone water for the enumeration of surviving cells. *E. coli* was plated on TSA-YE and incubated at 37°C for 24 h, while *L. plantarum* was plated on MRSa and incubated at 37°C for 48 h in an anaerobic jar. Two serial dilutions of each sample were plated in duplicate and the average was reported. Each condition was assayed in three independent runs ($n = 3$).

Combined effect of CA-E and acidification on carrot juice

The effect of CA-E (0.5, 1.0 and 1.5 μL/mL) was assessed on *E. coli* in carrot (pH 6.4, 12.2 °Brix) and acidified carrot juice (pH 4.5, 12.2 °Brix), which was obtained and pasteurized as described before and subsequently acidified using 30% p/v citric acid, depending on the treatment. The inactivation kinetic tests were conducted as described before, by adding 1 mL of the inoculum to 99 mL of each juice added with the appropriate CA-E concentration. The samples were taken at regular intervals

and the surviving cells were immediately enumerated as described before. Single CA-E and acidification (pH 4.5) treatments were also performed to be compared to the combined treatments.

Effect of CA-E on E. coli *inoculated in different juices stored under refrigeration*

The effect of CA-E (1.0 and 1.5 µL/mL) was evaluated on *E. coli* inoculated in carrot (pH 6.4; 12.2 °Brix), apple (pH 4.3; 13.8 °Brix) and orange (pH 3.6; 9.8 °Brix) juices during refrigerated storage (5°C). For this experiment, carrot and apple juices were sanitized and extracted as described before. The orange juice was extracted with a citrus juicer and filtered with a mesh to reduce the pulp content. The juices were inoculated with $1.3 - 5.0 \times 10^5$ CFU/mL of *E. coli* and stored at 5°C for 14 days. Samples (1 mL) were taken every 3 or 4 days and *E. coli* was enumerated as described before. Additionally, inoculated juices of each type without CA-E were used as controls. Two independent runs ($n = 2$) of each treatment were conducted.

Modeling of survival curves

Survival curves were fitted to the cumulative form of a Weibull-type distribution of resistance (Peleg & Cole, 1998):

$$S_{(t)} = \log\left(\frac{N}{N_0}\right) = -b \cdot t^n \tag{1}$$

where S is the survival fraction of the microorganism, t is the reaction time, and b and n are the scale and the shape parameters, respectively. The b parameter in the Weibull function represents the rate of inactivation of the cells, whereas, the n parameter indicates the concavity of the survival curve ($n > 1$, downward concavity; $n < 1$, upward concavity (Ferrario, Alzamora, & Guerrero, 2013)).

The values of b and n were then used to generate the resistance frequency curves using the following equation:

$$\frac{d\phi}{dt_c} = bnt_c^{n-1}\exp(-bt_c^n) \tag{2}$$

where t_c is a measure of the resistance or sensitivity of the organism and $\frac{d\phi}{dt_c}$ is the Weibull distribution corresponding to t_c. Other statistical parameters which better explain the observed frequencies (distribution mode, t_{cm}; mean, $\bar{t}_c$; variance, σ_{tc}^2; and coefficient of "skewness", υ_1) were calculated from the equations reported by Peleg and Cole (1998). The distribution mode, t_{cm}, represents the treatment time at which the majority of the population either dies or is inactivated. The mean, $\bar{t}_c$, corresponds to the inactivation time on average with its variance, σ_{tc}^2. The coefficient of skewness, υ_1, represents the skew of the distribution.

Experimental design and statistical analysis

The tests were carried out following a completely randomized design with three replicates ($n = 3$), unless otherwise indicated. Parameter estimates for the Weibull model were derived using a nonlinear regression technique. Internal validation of the model was carried out to determine whether it could adequately describe the experimental data by means of the analysis of variance (ANOVA) with a 95% confidence level and adjusted coefficient of determination (R^2_{adj}). All regression analyses

were applied using Statgraphics Centurion XV.II® (StatPoint Technologies Inc., Warrenton, VA, USA).

Results and discussion

Determination of MIC and MBC

The minimum concentration of CA and CA-E necessary to inhibit the growth of bacteria was determined in culture media in order to compare the antimicrobial effectiveness and to select the lowest doses that were microbiologically active.

Lactobacillus plantarum was the more resistant strain to carvacrol, presenting MIC and MBC values of 5 µL/mL for both CA-E and CA. The *E. coli* MIC and MBC values for CA were 0.1 µL/mL which are within the published range (Ait-Ouazzou et al., 2011; Burt, 2004; Guarda, Rubilar, Miltz, & Galotto, 2011). This bacterium was more sensitive to CA than to CA-E, with 1 µL/mL required to inactivate *E. coli* when carvacrol was emulsified. Therefore, the effect of the emulsion on the antimicrobial activity of carvacrol depended on the microbial strain.

Inactivation kinetics of two microbial strains as affected by CA-E in carrot juice

The inactivation kinetics of *E. coli* and *L. plantarum* as affected by CA-E (0.5; 1.0 and 1.5 µL/mL) in carrot juice is presented in Figure 1. The Weibull-type model (lines) was fitted to the experimental data (points). The survival curves demonstrated that *E. coli* (Figure 1a) was more sensitive than *L. plantarum* (Figure 1b) to the antimicrobial effect of emulsified carvacrol in carrot juice, resulting in higher inactivation (3.8 and 2.7 log cycles for *E. coli* and *L. plantarum*, respectively). These results are in agreement with the MIC and MBC data obtained in culture media as shown by previous analysis.

The lowest concentration of CA-E (0.5 µL/mL) was not enough to inactivate either of the assayed microorganisms; however, 1.0 and 1.5 µL/mL were effective in reducing between 3.4 and 3.8 log cycles of *E. coli* and between 1.6 and 2.7 log cycles of *L. plantarum* in carrot juice after 2 h of contact time. Further incubation time did not significantly increase inactivation according to 24 h studies (data not shown).

The effect was also analyzed using a different approach by means of the Weibull distribution of resistances model (Figure 2). This model considers that the entire microbial population is not equally resistant to the proposed treatment so each individual organism is inactivated at a specific time, thus generating a spectrum of resistance (Peleg & Cole, 1998). The Weibullian model accurately fitted experimental data of the survival curves, as shown by the adjusted R^2 values (94.4–99.8%) (Table 1), except for the 0.5 µL/mL CA-E system inoculated with *L. plantarum* in which the estimated parameters were not significant due to the low level of inactivation obtained.

The most effective treatments for *E. coli* inactivation presented $n < 1$ values, which are related to the observed upward concavity of the survival curves (Table 1; Figure 1a) and b values between 2.36 and 3.09 h^{-n} corresponding to the highest observed inactivation rates among all systems evaluated. For both strains, the scale parameter b increased as the concentration of CA-E increased (Table 1). The same trend for the parameters of the Weibull model was observed for *Listeria innocua* in orange juice as affected by a combination of vanillin and thermal treatment, where the combinations of the highest vanillin
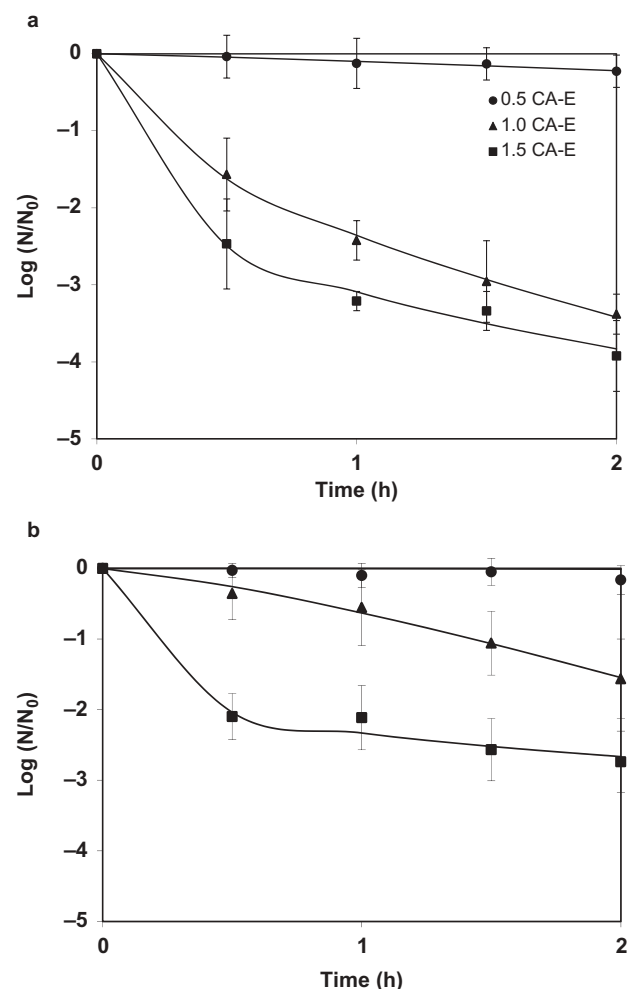
Figure 1.   Fit of Weibull model to survival data sets of *E. coli* (a) and *L. plantarum* (b) as affected by different concentrations of carvacrol emulsion (CA-E) in carrot juice. Experimental (points) and predicted (lines) values. (●) 0.5 μL/mL CA-E; (▲) 1.0 μL/mL CA-E; (■) 1.5 μL/mL CA-E. The vertical bars represent the standard deviation of the means ($n = 3$).

Figura 1.   Ajuste del modelo de Weibull a los datos de supervivencia de *E. coli* (a) y *L. plantarum* (b) según el efecto de diferentes concentraciones de carvacrol emulsionado (CA-E) en jugo de zanahoria. Valores experimentales (puntos) y predichos (lineas). (●) 0,5 μL/mL CA-E; (▲) 1,0 μL/mL CA-E; (■) 1,5 μL/mL CA-E. Las barras verticales representan la desviación estándar de las medias ($n = 3$).

concentration and treatment temperatures presented the highest *b* and the lowest *n* values (Char, Guerrero & Alzamora, 2009).

The *b* and *n* parameters were then used to generate the frequency distribution of resistance (Figure 2) according to Equation (2), and to calculate the associated statistics mean, mode, variance and skewness coefficient (Table 1). *E. coli* distribution of resistance corresponding to 1.0 and 1.5 μL/mL CA-E lacked mode and were strongly skewed to the right, showing that the majority of the cells in the population were sensitive at very low contact times. *L. plantarum* exhibited the same behavior for the 1.5 μL/mL CA-E system; however, the 1.0 μL/mL CA-E system showed a distribution of resistance with mode and was also skewed to the right (Figure 2b). Notwithstanding the effectiveness of the 1.5 μL/mL CA-E system inactivating *L. plantarum* (Figure 2b), it is worth noting that this distribution exhibited high variance
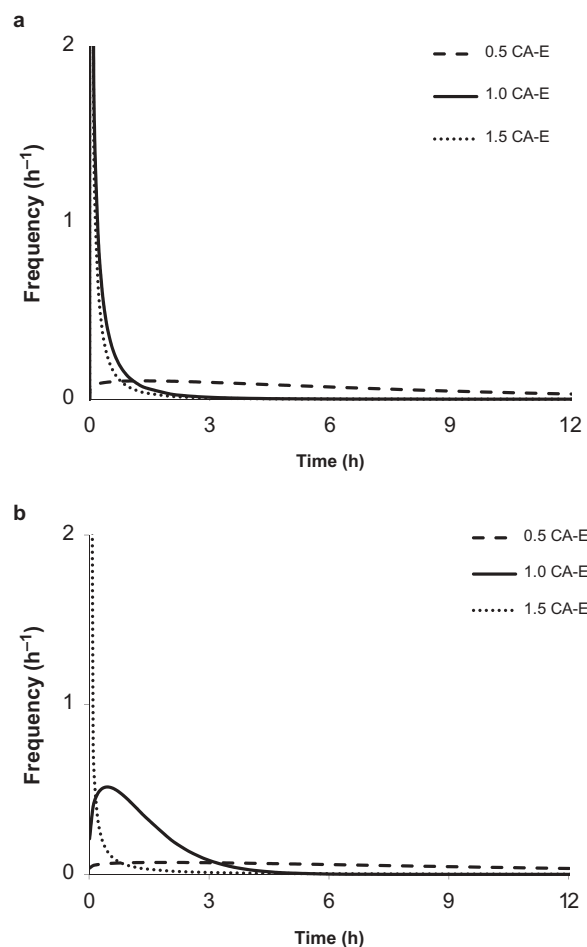

Figure 2.   Frequency distribution of resistance corresponding to *E. coli* (a) and *L. plantarum* (b) survival curves as affected by different concentrations of carvacrol emulsion (CA-E) in carrot juice.

Figura 2.   Distribuciones de frecuencias de resistencias correspondientes a las curvas de supervivencia de *E. coli* (a) y *L. plantarum* (b) según el efecto de diferentes concentraciones de carvacrol emulsionado (CA-E) en jugo de zanahoria.

values (Table 1) suggesting that the heterogeneity of the response was important as it was reflected by the tail of the distribution. This behavior was also observed by Ferrario et al. (2013) for *Saccharomyces cerevisiae* in melon juice treated with pulsed light (71.6 J/cm² for 60 s).

Frequency distributions corresponding to the lowest CA-E concentration (0.5 μL/mL) were notoriously flat, with high variance values (Figure 2a and b) indicating significant spread of inactivation time among members in the population; therefore, a poor efficacy for this treatment was evident.

These results are in agreement with those reported by Salvia-Trujillo et al. (2015) for the inactivation kinetics of *E. coli* as affected by thyme essential oil (which contains carvacrol and thymol terpenes) either emulsified or nanoemulsified in alginate. They reported that thyme essential oil (1.0 μL/mL) exhibited 2.1 and 2.6 log reductions of *E. coli* after 30 min contact time with the emulsion or nanoemulsion, respectively. Moreover, the survival curves presented $n < 1$ values describing a similar upward concavity to the 1.0 and 1.5 μL/mL CA-E systems when fitted to the Weibull model.

Table 1. Estimated parameters of the Weibull model proposed to describe the inactivation of two microorganisms by different concentrations of carvacrol emulsion (CA-E) in carrot juice.

Tabla 1. Parámetros estimados del modelo de Weibull propuesto para describir la inactivación de dos microorganismos por diferentes concentraciones de carvacrol emulsionado (CA-E) en jugo de zanahoria.

Microorganism	CA-E (mL/L)	b (h^{-n})	SE	n	SE	Variability explained % ($R^2_{adj.}$)	Mode (h)	Mean (h)	Variance (h^2)	Skewness
E. coli	0.5	0.10	0.01	1.16	0.25	94.4	1.33	7.0	37	2
E. coli	1.0	2.36	0.03	0.54	0.03	99.8	(–)	0.4	1	5
E. coli	1.5	3.09	0.07	0.31	0.04	99.3	(–)	0.2	1	23
L. plantarum	0.5	0.06	0.03	1.15	0.70	63.8	1.83	10.5	83	2
L. plantarum	1.0	0.63	0.05	1.29	0.13	98.6	0.45	1.3	1	2
L. plantarum	1.5	2.31	0.07	0.21	0.06	98.7	(–)	1.4	300	127

Notes: (–) Frequency distribution lack of mode.
SE: Standard error.

Notas: (–) La distribución de frecuencias carece de moda.
SE: Error estándar.

Combined effect of CA-E and acidification in carrot juice

Carrot juice is a low-acid juice (pH 6.0–6.5) and its acidification is current commercial practice due to microbiological safety issues. The effect of the combination of CA-E (0.5, 1.0 and 1.5 µL/mL) and acidification (pH 4.5) on the inactivation of *E. coli* in carrot juice was assessed and compared to the regular juice (Figure 3). The experimental survival curves (data points) were also modeled using the Weibull model (lines). Inoculated carrot juice with no CA-E addition and/or acidification served as controls and exhibited no changes in population during the trial (data not shown).

The inactivation response was markedly increased by the acidification for all CA-E concentrations; in particular, enhancement was most marked for the 0.5 µL/mL CA-E system which changed from 0.2 to 2.1 log reductions of *E. coli* after 2 h of contact time.

Most of the *E. coli* survival curves presented an initial period of rapid decrease in population followed by a second period of slower decline (Figure 3). This shape correlates with the $n < 1$ values and with the increase in the b parameter of the Weibull model as the severity of the combined treatment increased (Table 2). The corresponding frequency distribution of resistance was similar in shape to that obtained in Figure 2a (data not shown) for each CA-E concentration.

The acidification of these systems generated distribution of resistance lacking mode and exhibited the lowest mean values, indicating that the majority of the population died at the very beginning of the combined treatment (Table 2).
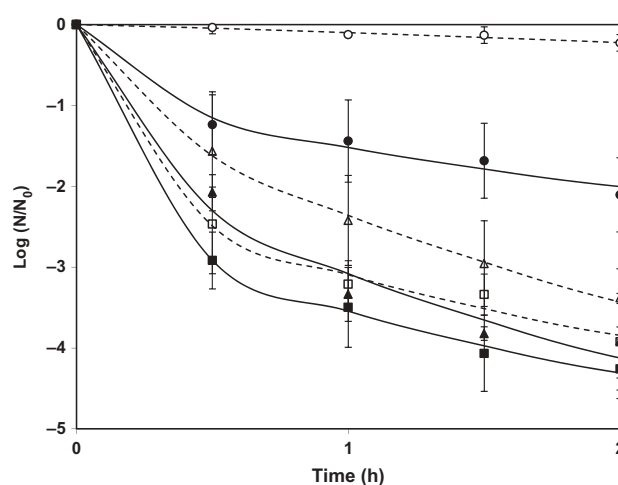

Figure 3. Fit of Weibull model to survival data sets of *E. coli* as affected by different concentrations of carvacrol emulsion (CA-E) in carrot juice ((---) pH 6.4) and acidified carrot juice ((—) pH 4.5). Experimental (points) and predicted (lines) values. (●) 0.5 µL/mL CA-E; (▲) 1.0 µL/mL CA-E; (■) 1.5 µL/mL CA-E. The vertical bars represent the standard deviation of the means ($n = 3$).

Figura 3. Ajuste del modelo de Weibull a los datos de supervivencia de *E. coli* según el efecto de diferentes concentraciones de carvacrol emulsionado (CA-E) en jugo de zanahoria ((---) pH 6,4) y jugo de zanahoria acidificado ((—) pH 4,5). Valores experimentales (puntos) y predichos (líneas). (●) 0,5 µL/mL CA-E; (▲) 1,0 µL/mL CA-E; (■) 1,5 µL/mL CA-E. Las barras verticales representan la desviación estándar de las medias ($n = 3$).

Table 2. Estimated parameters of the Weibull model proposed to describe the inactivation of *E. coli* in carrot juice by different concentrations of carvacrol emulsion (CA-E) and the combination of CA-E with acidification (pH 4.5).

Tabla 2. Parámetros estimados del modelo de Weibull propuesto para describir la inactivación de *E. coli* en jugo de zanahoria por diferentes concentraciones de carvacrol emulsionado (CA-E) y la combinación de CA-E con acidificación (pH 4,5).

CA-E (mL/L)	Carrot juice pH	b (h^{-n})	SE	n	SE	Variability explained % ($R^2_{adj.}$)	Mode (h)	Mean (h)	Variance (h^2)	Skewness
0.5	6.4	0.10	0.01	1.16	0.26	94.4	1.33	7.0	37	2
1.0	6.4	2.36	0.13	0.54	0.04	99.8	(–)	0.4	1	5
1.5	6.4	3.09	0.04	0.31	0.01	99.3	(–)	0.2	1	23
0.5	4.5	1.52	0.14	0.40	0.05	98.2	(–)	1.2	14	12
1.0	4.5	3.08	0.11	0.42	0.03	97.6	(–)	0.2	0	9
1.5	4.5	3.54	0.23	0.28	0.05	99.8	(–)	0.1	1	35

Notes: (–) Frequency distribution lack of mode.
SE: Standard error.

Notas: (–) La distribución de frecuencia carece de moda.
SE: Error estándar.

This synergistic effect of carvacrol combined with acidification was also described by Ait-Ouazzou et al. (2011), who evaluated the inactivation of *E. coli* by different constituents of essential oils, such as carvacrol, at pH 7.0 and 4.0 in citrate-phosphate buffer. They observed that 0.2 µL/L of carvacrol at pH 7.0 resulted in approximately 1.8 and 2.8 log cycles reduction of *E. coli* after 6 and 24 h of storage, respectively. Moreover, at pH 4.0 the same concentration of carvacrol inactivated 5 log cycles of *E. coli* after 6 h of contact time. They also determined that the whole population suffered sublethal injuries on both the cytoplasmatic and outer membranes. The higher degree of inactivation reported by these authors may be related with the intrinsic factors in the carrot juice, which exert a protective effect unlike the buffer. It is well known that pH level can modify not only microbial resistance to physical and chemical agents but also the mechanism of action of synthetic antimicrobials (Espina et al., 2012). However, there is still little information available about the effect of pH on natural antimicrobial activity and the results are variable among studies (Ait-Ouazzou et al., 2011; Somolinos, García, Mackey & Pagán, 2010).

Effect of CA-E on E. coli *inoculated in different juices stored under refrigeration*

The effect of CA-E (0, 1.0 and 1.5 µL/mL) on *E. coli* inoculated in carrot, apple and orange juices was evaluated during 15 days of storage at 5°C (Figure 4). Most of the curves showed that the addition of CA-E demonstrated its maximum antimicrobial activity within the first 24 h; moreover, this effect was maintained during the 15 days of refrigerated storage.

The addition of CA-E 1.0 and even 1.5 µL/mL to carrot juice (pH 6.4; 12.2 °Brix) did not completely inactivate the inoculated *E. coli* (5.0×10^5 CFU/mL), leaving a remaining population throughout the whole storage period (approximately 5.0×10^1 CFU/mL for 1.5 µL/mL CA-E addition) (Figure 4a). Conversely, CA-E at both concentrations was effective in the inactivation of *E. coli* inoculated in apple (pH 4.3; 13.8 °Brix) and orange (pH 3.6; 9.8 °Brix) juices, reaching undetectable levels ($<1 \times 10^1$ CFU/mL) (Figure 4b and c, respectively). The effect of the acidic condition of these two juices did not seem to be dependent on the nature of the main organic acid in the juice, since similar results were obtained for apple (malic acid) and orange juice (citric acid).

Nevertheless, no effect of acidification was observed for the untreated controls in apple and orange juices, which maintained an *E. coli* population through the storage period. Therefore, the acidic condition itself had little impact on microbial inactivation and it was the combination with CA-E which resulted in a synergistic effect. This fact may be of considerable industrial relevance since the increase in bactericidal activity of carvacrol emulsion by acidification allows the use of very low doses of natural antimicrobials to enhance the microbial safety of foods and beverages while avoiding adverse sensory effects.

Conclusions

The effect of emulsification on the antimicrobial activity of carvacrol was dependent on both microbial strain and CA-E concentration. In that sense, *L. plantarum* sensitivity to carvacrol was not affected by emulsification whereas that of *E. coli* decreased, giving higher MIC and MBC values for the emulsified carvacrol.

The additive and/or synergistic effects observed for the combination of CA-E with acidification encourage its use at very low
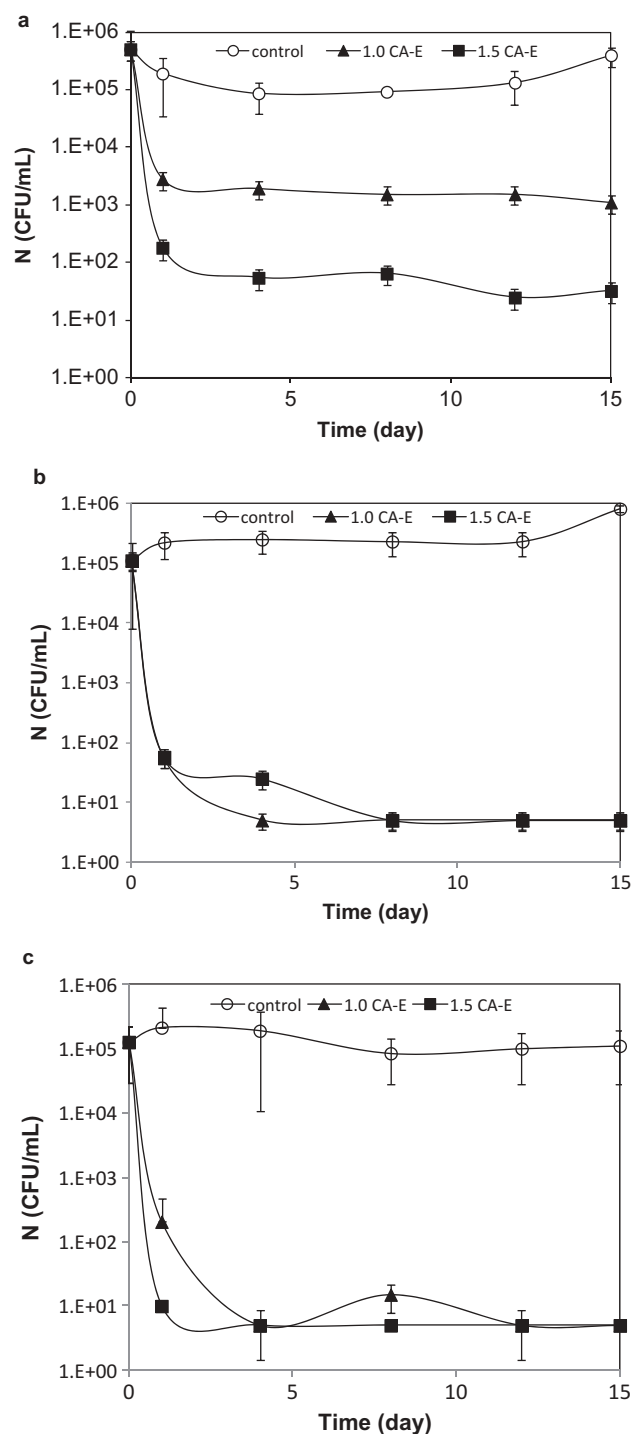

Figure 4.   Inactivation curves of *E. coli* inoculated in carrot (a), apple (b) and orange (c) juices as affected by carvacrol emulsion (CA-E 0, 1.0 and 1.5 µL/mL) evaluated during 15 days of storage at 4°C. The vertical bars represent the standard deviation of the means (*n* = 2).

Figura 4. Curvas de inactivación de *E. coli* inoculada en jugos de zanahoria (a), manzana (b) y naranja (c) según el efecto de carvacrol emulsionado (CA-E 0; 1,0 y 1,5 µL/mL) evaluado durante 15 días de almacenamiento a 4°C. Las barras verticales representan la desviación estándar de las medias (*n* = 2).

doses, enhancing physical stability and reducing the impact on sensory attributes as an alternative for many applications in foods and beverages. Moreover, the combination with low doses of carvacrol emulsion may improve other processing technologies

traditionally applied to juices, such as thermal treatments, by reducing the required severity and consequently reducing the adverse effects on food quality. Furthermore, low concentrations of CA-E combined with juice acidification proved to be an efficient alternative and could be used to enhance the effects of emerging nonthermal technologies while preserving the bioactive compounds and sensory attributes of minimally processed products.

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References

Adorjan, B., & Buchbauer, G. (2010). Biological properties of essential oils: An updated review. *Flavour and Fragrance Journal*, *25*, 407–426. doi:10.1002/ffj.2024

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., & Pagán, R. (2011). The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innovative Food Science & Emerging Technologies*, *12*, 320–329. doi:10.1016/j.ifset.2011.04.004

Buranasuksombat, U., Kwon, Y. J., Turner, M., & Bhandari, B. (2011). Influence of emulsion droplet size on antimicrobial properties. *Food Science and Biotechnology*, *20*, 793–800. doi:10.1007/s10068-011-0110-x

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods – A review. *International Journal of Food Microbiology*, *94*, 223–253. doi:10.1016/j.ijfoodmicro.2004.03.022

Char, C., Guerrero, S., & Alzamora, S. M. (2009). Survival of *Listeria innocua* in thermally processed orange juice as affected by vanillin addition. *Food Control*, *20*, 67–74. doi:10.1016/j.foodcont.2008.02.004

Char, C., Guerrero, S., & Alzamora, S. M. (2010). Mild thermal process combined with vanillin plus citral to help shorten the inactivation time for *Listeria innocua* in orange juice. *Food and Bioprocess Technology*, *3*, 752–761. doi:10.1007/s11947-008-0155-x

Chueca, B., Pagán, R., & García-Gonzalo, D. (2014). Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction. *International Journal of Food Microbiology*, *189*, 126–131. doi:10.1016/j.ijfoodmicro.2014.08.008

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT - Food Science and Technology*, *44*, 1908–1914. doi:10.1016/j.lwt.2011.03.003

Espina, L., Somolinos, M., Ait Ouazzou, A., Condón, S., García-Gonzalo, D., & Pagán, R. (2012). Inactivation of *Escherichia coli* O157: H7 in fruit juices by combined treatments of citrus fruit essential oils and heat. *International Journal of Food Microbiology*, *159*, 9–16. doi:10.1016/j.ijfoodmicro.2012.07.020

Ferrario, M., Alzamora, S. M., & Guerrero, S. (2013). Inactivation kinetics of some microorganisms in apple, melon, orange and strawberry juices by high intensity light pulses. *Journal of Food Engineering*, *118*, 302–311. doi:10.1016/j.jfoodeng.2013.04.007

Guarda, A., Rubilar, J. F., Miltz, J., & Galotto, M. J. (2011). The antimicrobial activity of microencapsulated thymol and carvacrol. *International Journal of Food Microbiology*, *146*, 144–150. doi:10.1016/j.ijfoodmicro.2011.02.011

La Storia, A., Ercolini, D., Marinello, F., Di Pasqua, R., Villani, F., & Mauriello, G. (2011). Atomic force microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells. *Research in Microbiology*, *162*, 164–172. doi:10.1016/j.resmic.2010.11.006

Oussalah, M., Caillet, S., & Lacroix, M. (2006). Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157: H7 and *Listeria monocytogenes*. *Journal of Food Protection*, *69*, 1046–1055.

Peleg, M., & Cole, M. B. (1998). Reinterpretation of microbial survival curves. *Critical Reviews in Food Science and Nutrition*, *38*, 353–380. doi:10.1080/10408699891274246

Rivera, G., Bocanegra-García, V., & Monge, A. (2010). Traditional plants as source of functional foods: A review. Plantas tradicionales como fuente de alimentos funcionales: Una revisión. *CyTA – Journal of Food*, *8*, 159–167. doi:10.1080/19476330903322978

Rota, M. C., Herrera, A., Martínez, R. M., Sotomayor, J. A., & Jordán, M. J. (2008). Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control*, *19*, 681–687. doi:10.1016/j.foodcont.2007.07.007

Salvia-Trujillo, L., Rojas-Graü, A., Soliva-Fortuny, R., & Martín-Belloso, O. (2015). Physicochemical characterization and antimicrobial activity of foodgrade emulsions and nanoemulsions incorporating essential oils. *Food Hydrocolloids*, *43*, 547–556. doi:10.1016/j.foodhyd.2014.07.012

Somolinos, M., García, D., Mackey, B., & Pagán, R. (2010). Inactivation of *Escherichia coli* by citral. *Journal of Applied Microbiology*, *108*, 1928–1939.

Ultee, A., Kets, E. P. W., & Smid, E. J. (1999). Mechanisms of action of carvacrol on the foodborne pathogen *Bacillus cereus*. *Journal of Applied and Environmental Microbiology*, *65*, 4606–4610.

Weiss, J., Gaysinksy, S., Davidson, M., & McClements, J. (2009). Nanostructured encapsulation systems: Food antimicrobials. In G. V. Barbosa-Cánovas, A. Mortimer, D. Lineback, W. Spiess, & K. Buckle (Eds.), *IUFoST world congress book: Global issues in food science and technology* (pp. 425–479). Amsterdam: Elsevier.