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Artículos Científicos

Estudio in vitro del efecto del aceite esencial de Cymbopogon citratus (AECC) en el control de Listeria monocytogenes NCTC 11994

Study in vitro of essential oil of Cymbopogon citratus (EOCC) on Listeria monocytogenes NCTC 11994 control

Estudo in vitro do efeito do óleo essencial de Cymbopogon citratus (AECC) no controle de Listeria monocytogenes NCTC 11994

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Resumen

Listeria monocytogenes tiene resistencia a diversos ambientes adversos. En condiciones de refrigeración puede sobrevivir periodos largos de tiempo. Además, el consumo de alimentos contaminados con *Listeria monocytogenes* puede causar listeriosis. Las bacterias del género *Listeria* spp., son bacilos anaerobios facultativos que no forman esporas, no contienen cápsula y son ubicuas, es decir, están ampliamente distribuidas en el medio ambiente. Algunos estudios indican que expresan mecanismos de resistencia fisiológica, *e.g.* al estrés térmico y ácido. Para el control de *Listeria monocytogenes* pueden usarse sustancias como el aceite esencial de *Cymbopogon citratus*, que tiene un contenido de citral > 70%. El citral es un monoterpeno compuesto de dos isómeros el neral y el geranial. Este compuesto tiene actividad antimicrobiana sobre *Enterobacter zakazakii* y *Echerichia coli*. El objetivo de este estudio fue evaluar el efecto de diferentes concentraciones (PICs, PBCs, MICs y MBCs) del aceite esencial de *Cymbopogon citratus* sobre el control de *Listeria monocytogenes* NCTC 11994 en un sistema modelo. Se realizó un análisis estadístico, con una comparación múltiple de medias de Duncan (SAS System, WindowsTM Versión 6.12, USA). Los

resultados del estudio a los tiempos de 1 min y 60 min mostraron en algunos tratamientos una inhibición significativa (P>0.05) de entre 45%-50% para las concentraciones de 10,000 mg/L, 2000 mg/L y 500 mg/L. Sin embargo, en algunos tratamientos se observó crecimiento significativo, específicamente en los tratamientos del AECC de 50 mg/L y 100 mg/L. Los resultados experimentales se ajustaron a un modelo lineal para ambos sistemas de estudio, el primero relacionado con la Inhibición (%) y el segundo con el Crecimiento (%) de la bacteria control (*Listeria monocytogenes* NCTC 11994). En este estudio se observó que a una concentración del AECC de 10000 mg/L se pudo realizar el control significativo del crecimiento de *Listeria monocytogenes*.

Palabras clave: *Listeria monocytogenes*, aceite esencial de citral, inhibición/crecimiento

Abstract

Listeria monocytogenes has great resistance to adverse environments. Under refrigeration conditions it can survive long periods of time. In addition, the consumption of foods contaminated with *Listeria monocytogenes* can cause listeriosis. The genus *Listeria* spp. are facultative anaerobic bacilli that do not form spores, do not contain a capsule and are ubiquitous, that is, they are widely distributed in the environment. Some studies mention mechanisms of physiological resistance, *e.g.* to thermal and acid stress. The control of this pathogenic microorganism can be carried out with natural preservatives such as the essential oil of *Cymbopogon citratus*; which has a citral content > 70%. Citral is a monoterpene composed of two isomers, neral and geranial. It has antimicrobial activity against *Enterobacter zakazakii* and *Echerichia coli*. In this study was evaluated the effect of the concentration (PICs, PBCs, MICs and MBCs) of the essential oil of *Cymbopogon citratus* on control of *Listeria monocytogenes* NCTC11994 in a model system. A statistical analysis was performed, with a multiple comparison test of Duncan means (SAS System, WindowsTM Versión 6.12, USA). Inhibition (%) and growth (%) was evaluated during study time: 1 and 60 min showed. In some treatments its were significant effect (P> 0.05) on variable respons. It was inhibition between 45-50% for concentrations of EOCC, 10,000 mg/L , 2000 mg/L and 500 mg/L. Significant growth was observed, specifically in the treatments of 50 mg/L and 100 mg/L. The experimental results were adjusted to a linear model for both study systems, the first related to inhibition (%) and the second to the growth (%) of *Listeria*

monocytogenes NCTC 11994. In this study it was observed at EOCC concentration of 10000 mg/L a significant control of the growth of *Listeria monocytogenes*,

Keywords: *Listeria monocytogenes*, essential oil of *Cymbopogon citratus*, inhibition/growth.

Resumo

Listeria monocytogenes tem resistência a vários ambientes adversos. Em condições de refrigeração, pode sobreviver a longos períodos de tempo. Além disso, a ingestão de alimentos contaminados com *Listeria monocytogenes* pode causar listeriose. As bactérias do gênero Listeria spp. são bacilos anaeróbicos facultativos que não formam esporos, não contêm cápsulas e são onipresentes, ou seja, estão amplamente distribuídos no ambiente. Alguns estudos indicam que eles expressam mecanismos de resistência fisiológica, p. ao estresse térmico e ácido. Para o controle de *Listeria monocytogenes*, podem ser utilizadas substâncias como o óleo essencial de *Cymbopogon citratus*, com conteúdo citral> 70%. Citral é um monoterpeno composto por dois isômeros, o neral e o geranial. Este composto possui atividade antimicrobiana em *Enterobacter zakazakii* e *Echerichia coli*. O objetivo deste estudo foi avaliar o efeito de diferentes concentrações (PICs, PBCs, MICs e MBCs) do óleo essencial de *Cymbopogon citratus* no controle de *Listeria monocytogenes* NCTC 11994 em um sistema modelo. Foi realizada análise estatística, com uma comparação múltipla das médias de Duncan (SAS System, WindowsTM Versão 6.12, EUA). Os resultados do estudo em tempos de 1 e 60 minutos mostraram em alguns tratamentos uma inibição significativa (P> 0,05) entre 45% -50% para as concentrações de 10.000 mg / L, 2000 mg / L e 500 mg / L . Em alguns tratamentos, foi observado um crescimento significativo, especificamente nos tratamentos AECC de 50 mg / L e 100 mg / L. Os resultados experimentais foram ajustados para um modelo linear para ambos os sistemas de estudo, o primeiro relacionado à Inibição (%) e o segundo relacionado ao Crescimento (%) das bactérias controle (*Listeria monocytogenes* NCTC 11994). Neste estudo, observou-se que a uma concentração de 10.000 mg / L no AECC, um controle significativo do crescimento de *Listeria monocytogenes* poderia ser realizado,

Palavras-chave: Listeria monocytogenes, óleo essencial citral, inibição / crescimento.

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Introduction

Currently, the food industry has as one of its main objectives to develop products with better sensory characteristics, which have a higher proportion of natural components and with a high nutritional quality. An important aspect in the design and development of food are conservation strategies to obtain a longer shelf life and that the food is safe. This aspect has been little explored in products that require refrigeration and those considered ready to be consumed (LPC) [ICMSF, 2011; Jay, 2005].

In recent years food contamination has been reported by a wide variety of microorganisms that can harm the health of humans, e.g. According to the report on the tendency of outbreaks of food origin, the common microorganisms are: Campylobacter, Salmonella, Listeria, Escherichia coli, Bacillus, Clostridium, Staphylococcus and Yersinia. The main foods involved are eggs, mixed foods, fish and frozen products [EFSA, 2007; Mortimore].

The above bacteria are of importance in terms of health quality, however, Listeria monocytogenes, has become more relevant because it causes listeriosis, with a mortality rate between 20% and 30% of hospitalized patients (United Nations Organization for Agriculture and Food, 2000). It manifests in adults, especially in immunosuppressed people, including effects such as septicemia, meningitis (or meningoencephalitis), encephalitis and intrauterine infections in pregnant women, which could result in spontaneous abortions [Marzocca, Marucci, Siga, and Alvarez, 2004; Torres-Vitela and Castillo-Ayala, 2010].

The bacterium is widespread in the environment and is particularly resistant to the effects of freezing, drying, heating, among others. It is resistant to low pH, high concentration of NaCl and is considered a psychotrophic bacterium [Lou and Yousef. nineteen ninety six].

With regard to the origins of listeriosis outbreaks, some reports indicate that some specific foods are more dangerous than others, considering the so-called ready-to-eat foods, preserved for a prolonged period of time at refrigeration, freezing temperatures and those that they have a high population of Listeria monocytogenes, greater than 100 UFC/g or mL [ICMS, 2011].

The control of pathogenic microorganisms in food can be carried out with various strategies, such as the use of substances of natural origin, e.g. The essential oils of some vegetables. These are oily liquids obtained from different parts of plants such as: flowers, buds, seeds,

leaves, branches, bark, herbs, wood, fruits and roots. Some are known for their antimicrobial activity against a wide range of bacteria and fungi [ICMFS, 2011].

Each essential oil has characteristic properties, generally they are complex mixtures of up to more than 100 components. The chemical structures are of the low molecular weight aliphatic type (alkanes, alcohols, aldehydes, ketones, esters and acids), terpenoids (monoterpenes, sesquiterpenes) and phenylpropanes. One of the essential oils that has demonstrated antimicrobial activity on Listeria monocytogenes is the essential oil of Cymbopogon citratus (AECC). This has as its main component a mixture of two aldehydes with the same molecular formula but different structures, citral a or geranial and citral b or neral [Plazas-Tovar, Wolf-Maciel, Ferreira-Pinto, Maciel-Filho and Ramalho-Gomes, 2010]. This compound has antimicrobial activity on Enterobacter zakazakii and Echerichia coli [Arroyo, Somolinos, Cebrián, Condón and Pagán, 2010; Somolinos, García, Mackey and Pagán, 2010a]. These compounds provide the property of damaging the cell membrane of fungi and gram positive or negative bacteria [Paranagama, 1991; Doyle-Beuchat, 2001].

In this study the effect of concentrations (PICs, PBCs, MICs and MBCs) of the essential oil of Cymbopogon citratus on the control of Listeria monocytogenes NCTC11994 in a model system was evaluated. It is intended to generate information on the response of the control bacteria to different adverse environmental conditions, to subsequently develop efficient control strategies for Listeria monocytogenes, such as the combined processes using the essential oil of Cymbopogon citratus and low temperatures in both systems *in vitro* como *in situ*.

Methodology

Preparation of the essential oil of Cymbopogon citratus

The AECC was acquired in the company "Herbalise and essence oils, S.A. of C.V. " located in Ecatepec de Morelos, State of Mexico. This was filtered with cellulose acetate membranes (0.22 µm, Durapore® Membrane Filters Millipore) to remove contaminating microbial flora. Subsequently stock solutions of 106 mg / L, 105 mg / L, 104 mg / L and 103 mg / L in sterile distilled water (added 1% Tween 80) were prepared. These solutions were stored in amber bottles at a temperature of -20 ° C. The density of each solution was determined to calculate the volumes to be used in the different concentrations of the AECC.

Cepa microbiana and growing conditions

Listeria monocytogenes NCTC 11994 It belongs to the microbial collection of the University of Murcia, Spain. During this study the strain was conserved in cryovials at -80 ° C. This was sown by cross streak in TSAYE medium (Tryptic Soy Broth, Bioxon, Mexico) supplemented with 0.6% yeast extract (w / v Bioxon, Mexico) and 1.5% bacteriological agar (w / v Bioxon, Mexico). The sample was incubated at 37 ° C for 24 h. A colony of the control bacteria was used to inoculate in TSBYE broth (Tryptic Soy Broth, Bioxon, Mexico) with 0.6% yeast extract (w / v Bioxon, Mexico) and incubated at 37 ° C for 12 h. 100 µL of this pre-culture was taken to be inoculated in 5 mL of TSBYE and the sample was incubated for 24 h at 37 ° C, to obtain the study culture.

Microbiological study

A 100 µL volume of the previously obtained culture was emptied into Eppendorf tubes with different volumes of a sterile water solution + Tween 80 (1%). The essential oil was added to obtain the different study concentrations (0 mg / L, 50 mg / L, 100 mg / L, 250 mg / L, 500 mg / L, 750 mg / L, 1000 mg / L, 2000 mg / L, 5000 mg / L and 10000 mg / L). The samples were shaken in a Vortex-Mixer (Cole-Parmer, USA) for 1 min. All treatments were maintained at 37 ° C in a temperature controlled bath (Polystat Temperature Controller, Cole-Parmer, USA). At the time of 1 min and 60 min, a plate count was made using the technique of gout (Miles and Misra, 1938). All treatments were analyzed in duplicate. In previous studies (not shown) the different inhibitory concentrations were determined: MIC (Minimum Inhibitory Concentration) was defined as the concentration of AECC that maintained the initial population of Listeria monocytogenes without any change throughout the incubation time. The PICs (Partial Inhibitory Concentration) was defined as the concentration of AECC lower than the MIC that inhibited Listeria monocytogenes populations compared to the control sample (without AECC). The PBCs (Partial Bactericidal Concentration) was considered as the highest concentration of AECC than the MIC where there was a significant reduction of Listeria monocytogenes during the study time. The MBCs (Minimum Bactericidal Concentration) was defined as the concentration of AECC in which 99.9% of

Listeria monocytogenes was inactivated under the experimental conditions evaluated (Cava y col. 2007).

Statistic analysis

The treatments were statistically analyzed with an ANOVA and a multiple comparison of means with the Duncan test. (SAS System, WindowsTM Versión 6.12, USA).

Results and Discussion

Listeria monocytogenes It is a pathogenic bacterium of importance in food. Its control is difficult due to its biological characteristics that allow it to adapt to different adverse environmental conditions (ICMFS, 2011). Figures 1,2 and 3 show the populations of Listeria monocytogenes at the time of 1 min and 60 min. It is observed that in most of the treatments evaluated there was inhibition. Figures 2 and 3 show the results of inhibition and growth at the times of 1 min and 60 min. In the first time in all treatments, significant inhibition was observed (P> 0.05, Table 5) greater than between 45-50% for concentrations of 10,000 mg / L, 2000 mg / L and 500 mg / L. However, in some treatments significant growth was observed (P> 0.05, Table 5), specifically in the 50 mg / L and 100 mg / L treatments. This condition can be explained because at low concentrations of AECC the control bacteria can use it, as a substrate. There were treatments, possibly showing a specific physiological response of Listeria monocytogenes to the presence of AECC, for example at a concentration of 5000 mg / L the highest growth was observed (only less than in the treatments of 50 mg / L and 100 mg / L). This behavior suggests that Listeria monocytogenes has a physiological defense response to this concentration of AECC, so growth is favored. Some studies have shown the formation of specific protective proteins (e.g. thermal stress proteins) when the strain is subjected to heat treatments (Lou and Yousef, 1996).

Statistical analysis (Tables 1-3) shows that the experimental results were adjusted to a linear model for both study systems, the first related to Inhibition (%) and the second related to the Growth (%) of the control bacteria (Listeria monocytogenes NCTC 11994). The models describe the influence of the factors investigated independently: time (A) and concentration (B) of the essential oil of Cymbopogon citratus (AECC, mg / mL), as well as the effect of the interaction (A * B). These parameters had a significant effect (Table 1-5) on the response variable (Log CFU / mL). The coefficients of determination for the linear models in the study

of inhibition (%) and growth (%) of Listeria monocytogenes indicate that only 0.05% and 0.19% respectively of the variation in the response variable cannot be explained by the models developed . The F values for the fixed variables and the study interactions were useful to explain the degree of significance. For the study of inhibition of Listeria monocytogenes, the fixed variable that had the greatest significance according to F and Pr> F, was the time subsequently the concentration of AECC and finally the interaction. In the study of the growth of Listeria monocytogenes a similar behavior is observed. Under these experimental conditions, for the growth of the control strain, it is observed that the value of F (for growth) for time, is higher, compared to all other parameters (both inhibition and growth) which indicates that Changes in the fixed variable, time (A) have the greatest effect on the response variable. Some studies mention that Listeria monocytogenes can develop mechanisms for resistance to essential oils and even use them as a substrate source under specific conditions such as some concentrations of the AECC (PICs or GDPs). In this study it was observed that at a concentration of the AECC of 10,000 mg / mL, growth control of Listeria monocytogenes could be carried out. Significant inhibition was observed (Figures 1 and 2, Table 5). In vitro experimental results and statistical analysis provide information on the significant control of Listeria monocytogenes with the AECC. However, its application in food systems may have some limitations, because a food has other components that affect the antimicrobial activity of the AECC. In situ studies with model foods are necessary to determine the MICs concentrations that should be used for the significant control of Listeria monocytogenes; Likewise, government regulations regarding food additives should be considered.

Figura 1. (a) Poblaciones de *Listeria monocytogenes* NCTC 11994 a diferentes concentraciones de aceite esencial de *Cymbopogon citratus* (mg/L) a los tiempos de 1 in y 60 min y temperatura de 37°C

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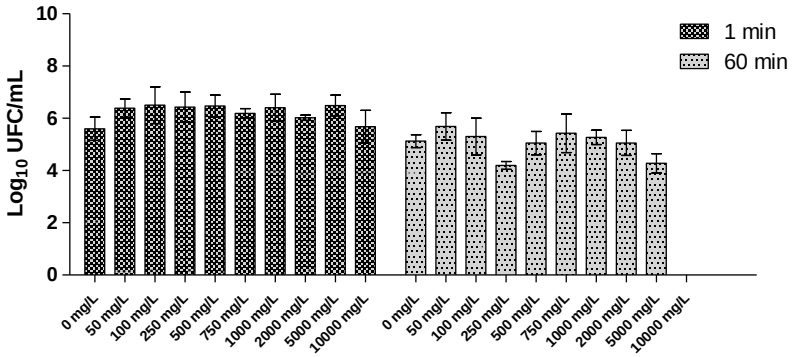
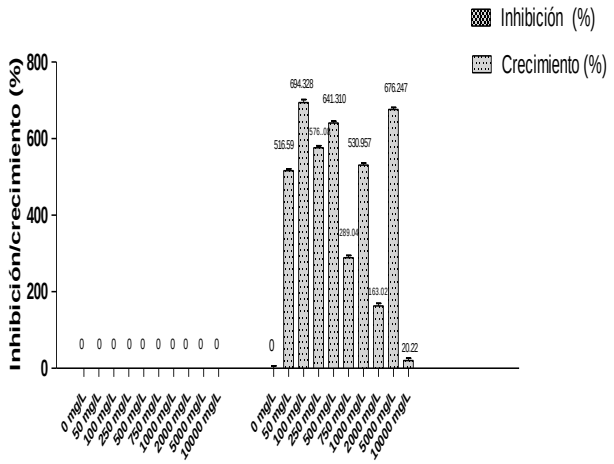


Fuente: Elaboración propia

Figura 2. Inhibición/crecimiento (%) de *Listeria monocytogenes* NCTC 11994 con diferentes concentraciones de aceite esencial de *Cymbopogon citratus* (mg/L) al tiempo de 1 min



Fuente: Elaboración propia.

Figura 3. Inhibición/crecimiento de *Listeria monocytogenes* NCTC 11994 con diferentes concentraciones (mg/L) de aceite esencial de *Cymbopogon citratus* al tiempo de 60 min.

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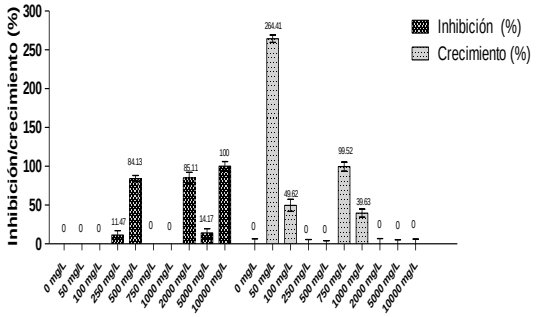

Fuente: Elaboración propia.

Tabla 1. ANOVA del efecto de las variables fijas: concentración (A) del aceite esencial de *Cymbopogon citratus* (AECC) y tiempo (B) sobre la variable de respuesta: inhibición (%) de *Listeria monocytogenes* NCTC 11994

Fuente de variación	Grados de libertad	Suma de cuadrados	Cuadrados medios	Valor de F	Pr > f
Modelo	19	40837.671	2149.351	211.18	0.0001
Error	20	203.567	10.177		
Total	39	41041.228			
R-Square= 0.9950					

*Los valores de "Prob > F" menores que 0.050 indican que el modelo estadístico evaluado es significativo

Fuente: Elaboración propia.

Tabla 2. ANOVA del efecto de las variables fijas: concentración (A) del aceite esencial de *Cymbopogon citratus* (AECC) y tiempo (B) la variable respuesta: crecimiento (%) de *Listeria monocytogenes* NCTC 11994

Fuente de variación	Grados de libertad	Suma de cuadrados	Cuadrados medios	Valor de F	Pr > f
Modelo	35	2806510.384	147711.072	581.73	0.0001
Error	36	5078.332	253.916		
Total	71	2811588.717			
R-Square= 0.9981					

*Los valores de "Prob > F" menores que 0.050 indican que los parámetros evaluados son significativos

Fuente: Elaboración propia.

Tabla 3. ANOVA para el efecto de los factores: concentración del AECC (mg/L) y tiempo (min) sobre la inhibición/crecimiento (%) de *Listeria monocytogenes* NCTC 11994

Fuente de variación	Valor de F	Pr > F
Inhibición (%)		
A-Tiempo	876.42	0.0001
B- Concentración de AECC (mg/L)	176.16	0.0001
A*B – Tiempo*AECC	172.29	0.0001
Crecimiento (%)		
A – Tiempo	5195.35	0.0001
B – Concentración de AECC (mg/L)	350.75	0.0001
A*B – Tiempo*AECC	300.09	0.0001

Fuente: Elaboración propia

Tabla 4. Prueba de Duncan para la comparación del efecto del tiempo sobre la inhibición/crecimiento (%) de *Listeria monocytogenes* NCTC 11994

Inhibición (%)			**Crecimiento (%)**		
Tiempo (min)	Promedio	Prueba de Duncan*	Tiempo (min)	Promedio	Prueba de Duncan*
60	31.492	A	1	411.390	A
1	1.625	B	60	48.184	B

*Medias con la misma letra no son significativamente diferentes con una α = 0 0.05

Fuente: Elaboración propia.

Tabla 5. Prueba de Duncan para la comparación del efecto de la concentración del aceite esencial de *Cymbopogon citratus* (AECC) y el tiempo sobre la inhibición/crecimiento (%) de *Listeria monocytogenes* NCTC 11994

Inhibición (%)	**Crecimiento (%)**

Concentración de AECC (mg/L)	Promedio (%)	Prueba de Duncan*	Concentración de AECC (mg/L)	Promedio (%)	Prueba de Duncan*
10000	50	A	50	390.25	A
2000	45.528	A	100	375.99	A
500	45.408	A	5000	345.31	B
5000	8.905	B	500	330.33	B
250	6.618	B C	1000	292.65	C
0	2.5	C D	250	282.65	C
750	2.250	C D	750	192.14	D
1000	7.875	C D	2000	87.25	E
50	1.5	C D	10000	7.56	F
100	1	D	0	3.75	F

*Medias con la misma letra no son significativamente diferentes con una α = 0 0.05

Fuente: Elaboración propia.

Conclusions

Under the experimental conditions evaluated, a significant effect on the inhibition and growth of Listeria monocytogenes was found in some treatments of essential oil concentrations of Cymbopogon citratus. This can be explained due to the effect of the different study variables: concentration of essential oil and time. In addition to the possible development of microbial resistance mechanisms by the study strain.

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