Revista de la Sociedad Venezolana de Microbiología 2014; 34:10-14

Artículo original

Antibacterial activity of lime (*Citrus x aurantifolia*) essential oil against *Listeria monocytogenes* in tyndallised apple juice

Roberto Carrizo Flores[a,*], Noelia Audicio[a], Marcela Kurina Sanz[b], Marta Ponzi[a]

[a]*Instituto de Investigaciones en Tecnología Química (INTEQUI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ingeniería y Ciencias Agropecuarias (FICA), Universidad Nacional de San Luis. Argentina.*
[b]*INTEQUI, CONICET, Facultad de Química, Bioquímica y Farmacia (FQByF) Universidad Nacional de San Luis. Argentina.*

Recibido 6 de agosto de 2013; aceptado 3 de diciembre de 2013

***Abstract*:** The antibacterial activity of lime (*Citrus x aurantifolia*) essential oil (EO) against the foodborne pathogen *Listeria monocytogenes* in tyndallised apple juice was studied at two temperatures. The EO concentration required to produce a significant increase in the lag phase of bacterial growth was determined. The addition of 200 µL of lime EO per 100 mL of apple juice completely inhibited the growth of *L. monocytogenes* at 5 ºC and at 37 ºC. This concentration of EO extended the lag time at least 292.7% compared to juice without EO. This is especially important considering that *L. monocytogenes* was able to grow in the juice at low temperatures in the absence of EOs.

***Keywords*:** essential oil, lime, *Listeria monocytogenes*, antimicrobial, apple juice.

Actividad antimicrobiana del aceite de lima (*Citrus x aurantifolia*) contra *Listeria monocytogenes* en jugo de manzana tindalizado

***Resumen*:** En este trabajo se estudió la actividad antibacteriana del aceite esencial de lima (*Citrus x aurantifolia*) contra *Listeria monocytogenes*, patógeno alimentario, cultivado en jugo de manzana tindalizado a dos temperaturas. Se determinó la concentración necesaria del aceite esencial para producir una extensión significativa de la fase de retraso. La adición de 200 µL de aceite esencial de lima por 100 mL de jugo de manzana tindalizado, a 5 ºC produjo la inhibición total del crecimiento de *L. monocytogenes*, en tanto que con el mismo volumen a 37 ºC la fase de retraso se extendió a 24,7 h (292,7%). Esto es importante debido a que *L. monocytogenes* fue capaz de crecer en este sustrato a temperaturas bajas en ausencia de aceite esencial.

***Palabras clave*:** aceite esencial, lima, *Listeria monocytogenes*, antimicrobiano, jugo de manzana.

* Correspondencia:
E-mail: rcarrizo65@gmail.com

Introduction

Listeria monocytogenes is a grampositive psychotropic bacterium that is widely distributed in the environment and can be transmitted to humans through the consumption of contaminated foods. In recent decades, several outbreaks of listeriosis have been associated with the consumption of unpasteurized products. According to quantitative risk studies of *L. monocytogenes* in ready-to-eat foods conducted by the U.S. Food and Drug Administration (FDA), pasteurised or tyndallised fruit juices are moderate-risk products. Once foods have been tyndallised, controlling the temperature during their transport and storage of foods is critical to ensure that the foods remain safe; however, these conditions are often beyond the control of the manufacturer and there may be a break in the cold chain. This lack of temperature control could allow *L. monocytogenes* populations to reach levels that are harmful to consumer health ($>10^2$ CFU mL^{-1}) [1,2].

Current technologies to extend the preservation and shelf life of foods include chemical preservatives, heat processing and modified atmospheres in packaging or refrigeration [3]. However, these steps do not completely eliminate *L. monocytogenes* from products or delay microbial spoilage. Consequently, alternative preservation techniques for foods, such as non-thermal technologies and naturally derived antimicrobial products, are under investigation [4]. One of these alternative techniques is plant essential

oils (EOs), which have been used since antiquity to flavour drinks and food, and they are currently being used for their antimicrobial and antioxidant properties.

The main antimicrobial components of spices and their EOs are eugenol in cloves, allicin in garlic, cinnamic aldehyde in cinnamon, carvacrol and thymol in oregano, and thyme and vanillin in vanilla beans [5,6]. In addition to these commonly cited components, there are other types of EOs that have the potential to control the growth of *L. monocytogenes* and other bacteria in food products [7]. Lime (*Citrus x aurantifolia*) and a variety of citrus EOs have shown efficacy in controlling bacterial growth in fruit juices, especially in processes associated with thermal treatments, such as tyndallisation [8]. Apple juice is one of the most common fruit juices consumed worldwide. It has a significant concentration of natural phenols, that may protect people from diseases associated with aging due to their antioxidant effects, which reduce the likelihood of developing cancer and Alzheimer's disease [9]. Furthermore, apple juice is a top product in a sector of high quality non-refrigerated and minimally processed products. We used a non-clarified juice that is rich in starch and pectins as a model substrate because it is an excellent medium for spoilage due to microorganism growth [10].

Thus, the main purposes of this work were as follows: i) to evaluate the growth of *L. monocytogenes* in tyndallised apple juice at 37 °C and 5 °C, and ii) to analyse the effect of increasing concentrations of lime EO on the growth of *L. monocytogenes* at 37 °C and 5 °C.

Materials and methods

Essential oil: The EO used in this study was lime (*Citrus x aurantifolia*). It was selected based on a previous analysis of 20 commercial EOs (Fritzsche SAICA, Argentina) that was conducted to determine the lowest minimum inhibitory concentration (MIC) against *L. monocytogenes*.

Bacteria: The bacterium used in this study was a strain of *L. monocytogenes* provided from the Pasteur Institute (Paris, France) and classified as CLIP 7125 ser/var 4b. It was stored at -20 °C in a 20% v/v glycerol aqueous solution and grown in Tryptic Soy Broth (TSB, pH 7.3, Britania, Argentina) for 24 h at 37 °C to obtain working cultures. These cultures were adjusted to a final concentration of 10^6 CFU mL^{-1} [11].

Preparation of apple juice: Apples of the Red Delicious variety were used. For each batch, 500 g of apples were washed, peeled, mashed and filtered through a piece of cotton fabric to obtain 250 mL of raw juice. Then, distilled water was added to obtain a final volume of 500 mL. The juice was dispensed in 100 mL aliquots into sterile vials and heated at 80 °C for 1 h for 3 consecutive days. The juice was stored at room temperature between each thermal treatment. This type of fractional treatment or tyndallisation provided a good level of decontamination (0-10 CFUmL^{-1} background micro flora remaining in the apple juice) for the inoculation studies without damaging the quality of the final product.

Inoculation of apple juice: Samples of 50 mL of tyndallised apple juice were diluted with 50 mL of sterile distilled water in 500 mL Erlenmeyer flasks. The final pH was 4.5. Each flask was inoculated with 1 mL of a *L. monocytogenes* suspension that was grown for 24 h and adjusted to a final concentration of 10^6 CFU mL^{-1}. The samples were incubated at 37 °C in a chamber and at 5 °C in a refrigerator.

Growth curves: The antibacterial activity of lime EO against *L. monocytogenes* CLIP 7125 ser/var 4b in apple juice was tested at 37 °C and 5 °C. Samples of 1 mL were collected from each Erlenmeyer flask containing apple juice every 2 h, which is the doubling time reported in the literature. The samples were diluted in Trypticase soy broth with (10^{-2}, 10^{-4} and 10^{-6}) and *L. monocytogenes* counts were performed on TSB agar plates. The colony forming units were counted after 24 h of incubation at 37 °C and two replicates were performed [12]. Each dilution was read on a visible Spectronic 20 spectrophotometer (Bausch and Lomb, USA) at 600 nm. Growth curves were obtained and fitted according to the Baranyi model [12]. The effects of 100, 200 and 500 µL of lime EO per 100 mL of apple juice (1%, 2% and 5% concentrations) on the growth of *L. monocytogenes* at the two temperatures were also investigated. Two replicate growth curves were performed for each condition. Finally, aliquots were collected and the pH of the samples were determined both prior to inoculation and after growth had finished.

Modelling of growth curves: Growth curves were fitted using the ComBase Modelling Toolbox, which was developed by the Food Standards Agency (FSA United Kingdom), U.S. Deparment of Agriculture (USDA USA) and Food Safety Centre Australia [13]. The major growth parameters such as specific growth rate (µ), lag time (λ) and maximum bacteria population (y_{max}) were estimated. The program also calculated the goodness of the fit (R^2) for each curve. For each combination of conditions, the bacterial numbers were plotted as a function of time. Only growth curves with at least 10 data points were used for modelling, as suggested by the program.

Statistical analysis: To analyse the significant differences between the samples of juice treated or not with EO we performed ANOVA tests. When significant differences were observed between samples treated at 37 ºC with or without EO, we used the Duncan´s test.

Results

Growth of L. monocytogenes in apple juice at 5 ºC and 37 °C: Experimental data were fitted using the Baranyi model, which gave a good fit in all cases. The lag times and specific growth rates derived from the modeled curves are shown

in table 1.

The estimated lag phase at 5 °C was 16.7 h, with a low growth rate (0.004 h^{-1}), whereas the lag time at 37 °C was 6.3 h and the maximum growth rate was 0.7 h^{-1}.

The pH values, prior to inoculation and at the end of the growth period were measured. No significant changes in pH were observed, with an initial pH of 4.5 and a final pH of 4.2.

Table 1. Growth parameters of *L. monocytogenes* incubated at 5 ºC and 37 °C in 100 mL of tyndallised apple juice (without essential oil).

Temp. (ºC)	Lag time (h)	y_o (log CFU/mL)	y_{max} (log CFU/mL)	Maximum specific growth rate (h^{-1})	R^2
5	16.7	4.9	5.2	0.004	0.995
37	6.3	5.3	9.7	0.7	0.9877

Curves were fitted with ComBase software. Data shown are the average of two replicate growth curves.

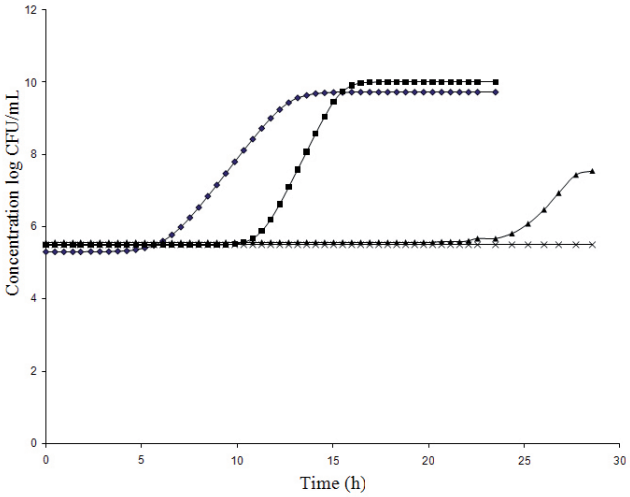

Figure 1. Effects of lime (♦:0 µL; ■: 100 µL; ▲: 200 µL; **x:** 500 µL) essential oil on the growth of *L. monocytogenes* CLIP 7125 strain in 100 mL of apple juice at 37 °C.

Influence of lime essential oil on the growth of L. monocytogenes in apple juice: To analyse the combined effect of added essential oil and refrigeration temperatures, growth curves were calculated for the samples grown at 5 °C. This temperature is recommended by the Risk Management Program (RMP) for food preservation [14,15]. At this temperature, no growth of *L. monocytogenes* was observed with the three concentrations of EO added to the apple juice. The main growth parameters were not calculated because the values could not be fitted with the ComBase Modelling Toolbox.

However, we were able to determine growth parameters for the samples incubated at 37 °C. These parameters were derived from the fitted growth curves and indicated substantial delays in bacterial growth with increased lag times (Figure 1). When we added EO at a concentration of 1% the lag time was extended to 11.2 h (72.8% greater than that in untreated juice) and the maximum growth rate was 1.1 h^{-1}. With a concentration of 2% of EO in the juice, the lag time was 24.7 h (292.7% greater than untreated juice) and the maximum growth rate was 0.6197 h^{-1}. These observations imply that once the bacteria began multiplying, the presence of the EO had no effect on the growth rate. Finally, with a concentration of 5% of EO there was no growth detected after 48 h of incubation. Table 2 shows the summarised data for both temperatures and all concentrations of EO. The differences in growth rates between samples with and without EO were analysed with an ANOVA test and found to be significant (p=0.0344). For the experiments conducted at 37 ºC, we found that there were significant differences (95% of confidence) between the samples containing 2% or 5% EO compared with the samples containing 1% EO. In addition, the sample with 1% EO did not exhibit significantly different bacterial growth compared with the samples without EO added.

Discussion

There are several studies that have examined methods of inhibiting the growth of *L. monocytogenes*. However,

Table 2. Growth parameters of *L. monocytogenes* incubated at 5 ºC and 37 °C in 100 mL of tyndallised apple juice containing 1%, 2% or 5% lime essential oil.

Concentration (%)	Temperature (°C)	Lag time (h)	y_o (log CFU/mL)	y_{max} (log CFU/mL)	Maximum specific growth rate (h^{-1})	R^2
1	5	nd	4.9	4.9	nd	nd
2	5	nd	4.9	4.9	nd	nd
5	5	nd	4.9	4.9	nd	nd
1	37	11.2	5.5	10.0	1.0803	0.9838
2	37	24.7	5.6	9.8	0.6197	0.9949
5	37	nd	nd	nd	nd	nd

nd: not data. Curves were fitted with ComBase software. Data shown are the average of two replicate growth curves.

while synthetic products are efficient at inhibiting bacterial growth, consumers increasingly prefer natural products and naturally derived compounds from plants, which also have the ability to control pathogen growth in food products [16,17]. The challenge is to isolate, purify and incorporate natural antimicrobial substances into foods without adversely affecting their taste, nutritional and safety characteristics. Furthermore, this process must be achieved without significantly increasing production, processing and marketing costs.

In this paper, we describe the effects of adding three amounts of lime EO to apple juice (which was used as a growth substrate) on the growth of the food borne pathogen *L. monocytogenes*. From the range of EO concentrations that should be effective at controlling growth, we tried to use the lowest amount to avoid any possible negative impacts on sensory properties [18]. The use of EO as food preservative is well documented. For instance, Desai *et al.* [19] report the control of *L. monocyotgenes* growth in fish treated with EO and confirmed the activity of certain active compounds such as carvacrol. However, it is difficult to perform a detailed comparison with results from other studies for a variety of reasons. For example, there is variability in vegetable material used in terms of its nature (spice, extract or EO), origin (country, altitude and harvest season), extraction methods, purity and preservation; all of which these factors together affect the concentrations of antimicrobials agents in the final product. Furthermore, some tests have been performed on synthetic growth media or other types of foods as growth models [20, 21]. However, in many food substrates, there have not been many studies on the antimicrobial activity of EOs and extracts or their components.

Conclusions

The data presented in this paper indicate that lime EO significantly increased the lag times of *L. monocytogenes* growth ($p=0.0344$), which leads to the conclusion that lime EO is a potential antimicrobial agent against *L. monocytogenes* in tyndallised apple juice. The effectiveness of this method is based on the high activity of lime EO at moderate doses and the fact that these amounts do not affect the safety of flavoured tyndallised juices. The combined use of refrigeration and addition of EOs represents an exciting potential for future research in the field of food conservation with non chemical compounds.

Acknowledgements

The authors are grateful to MSc Jorge Leporati. This study was conducted with financial support from the Universidad Nacional de San Luis (PROICO 50207) and (PIIC 0209).

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