Quantitative assessment of citral antimicrobial potential at different temperatures

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A study was carried out to assess the effect of citral on the growth of *Escherichia coli* K12 and *Listeria innocua* at different temperatures [37–8 °C]. After defining the value of the lowest concentration able to inhibit their visible growth, the antimicrobial effect of non-inhibitory doses was determined and quantified based on the lag time duration (λ) and maximum growth rate (μ_{max}) parameters. In general terms, it was observed that the lower the temperature or the higher the citral concentration, the greater the lag time and the smaller the growth rate. Furthermore, synergy was detected between these two factors against *L. innocua,* since some of the results obtained in the presence of citral at low temperatures were better than the ones obtained at optimum growth temperature with the same citral concentration, or in the absence of citral under refrigeration conditions. Therefore, citral could be use as a natural preservative, even at non-inhibitory doses that enable a balance between the sensory acceptability of foods and the antimicrobial efficacy of the compound, especially if it is combined with storage at low temperatures.

Keywords Food safety; natural preservatives; citral; *Escherichia coli*; *Listeria* spp.; predictive microbiology

1. General remarks

The importance of a balanced diet, free of artificial chemicals, is well known. However, cooking is considered a chore and meal preparation is a very time-consuming activity [1]. In recent years, this has stimulated the intake of minimally processed, ready-to-eat foodstuffs that are healthy, tasty and safe, without synthetic additives.

For the industry, guaranteeing the innocuity of this kind of food is a real challenge. On the one hand, there is no strategy for achieving the complete elimination of hazardous microorganisms in fresh produce without affecting its quality [2], and on the other, post-processing contamination is always more than likely, despite the encouragement of good hygiene and manufacturing practices "from farm to fork".

To achieve this aim, taking market requirements into account, work is currently being done on the study and application of preservatives of animal, vegetable or microbial origin that enhance the effectiveness of treatments applied to products and avoid or control post-processing survival and growth of pathogenic microorganisms, such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* [3]. Notable among these natural preservatives are the essential oils of certain plants, because they contain *Generally Recognised As Safe* (GRAS) compounds, like citral, present in citrus such as orange, lemon and bergamot [4], which can inhibit or slow microbial growth.

Many authors have shown that citral is biostatic and/or biocidal against bacteria and fungi [5–8], but few data exist about its effects on microbial kinetics at non-inhibitory doses, more readily accepted by the consumer, given its strong, lemon-like odour and its bittersweet taste [9]. These studies are very interesting because they allow the prediction of bacterial behaviour *in vitro* and in foods, over time, as a function of various factors, as well as constructing a matrix of responses to a broad range of specific storage conditions [10, 11], which is the first step for a complete risk assessment and for the planning of an appropriate *Hazard Analysis and Critical Control Point* (HACCP) system in the food industry.

For this reason, a study was carried out in order to quantify the effect of citral on the growth of *E. coli* O157:H7 and *L. monocytogenes* non-pathogenic surrogates, at different temperatures (37, 30, 15 and 8 °C), based on lag time duration (λ) and maximum growth rate (μ_{max}) parameters, taking into account that both pathogens are commonly found in minimally processed foods.

2. Material and methods

2.1. Bacterial strains and culture preparation

Stock vials for *E. coli* K12 (CECT 433) and for *Listeria innocua* (CECT 910) were prepared and stored at –80 °C from samples provided by the Spanish Type Culture Collection, to a final concentration of 1×10^9 cfu/mL.

Vials containing *E. coli* K12 were obtained following the method described by Pina-Pérez, García-Fernández, Rodrigo and Martínez-López [12], while vials containing *L. innocua* were obtained following the method described by Saucedo-Reyes, Marco-Celdrán, Pina-Pérez, Rodrigo and Martínez-López [13].

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In both cases, the average cell density of the vials was established by viable plate count from several samples, using 1‰ buffered peptone water (Scharlau Chemie S. A., Barcelona, Spain) for their dilution.

2.2. Citral

Citral ≥ 98% (3,7-dimethyl-2,6-octadienal) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.3. Antimicrobial assays

Flasks with 10 mL of sterile broth were prepared. Different amounts of citral freshly diluted in dimethyl sulfoxide (DMSO ACS reagent ≥ 99.9%; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) [14–17] were added to the culture media.

The mixtures were inoculated with *E. coli* K12 or *L. innocua*, the starting cell density being 1×10^7 cfu/mL. The concentrations tested in each case are shown in Table 1. The media selected were Nutrient Broth (NB) for *E. coli* K12 and Tryptic Soy Broth (TSB) for *L. innocua*. Both were supplied by Scharlau Chemie S. A. (Barcelona, Spain).

Table 1 Citral concentrations (µL/mL) tested, according to microorganism and incubation temperature.

Microorganism	Temperature (°C)	Concentrations tested (µL/mL)
Escherichia coli K12	37	0; 0.100; 0.200; 0.250; 0.275; 0.300; 0.325; 0.350
	30	0; 0.100; 0.200; 0.250; 0.275; 0.300; 0.325; 0.350
	15	0; 0.100; 0.200; 0.300; 0.500; 0.750; 1.000; 1.500; 3.000
	8	0; 0.100; 0.200; 0.300; 0.500; 0.750; 1.000; 1.500; 3.000
Listeria innocua	37	0; 0.100; 0.150; 0.175; 0.200; 0.250; 0.300
	30	0; 0.100; 0.150; 0.175; 0.200; 0.250; 0.300
	15	0; 0.100; 0.150; 0.200; 0.250; 0.300; 0.500; 1.000
	8	0; 0.100; 0.150; 0.200; 0.300; 0.500; 1.000; 1.500

Sterile polystyrene microplates (Deltalab S. L., Barcelona, Spain) were filled with the prepared samples, taking into account the citral concentrations to be tested for each temperature, for each of the bacteria studied, the volume added per well was 250 µL. In addition to the problem samples, non-inoculated samples with and without citral were included in all plates as negative controls for each assay. Peripheral wells were filled up with sterile distilled water.

Regardless of temperature, the plates were incubated with double orbital shaking (500 rpm) and culture absorbance was measured at 600 nm. Readings were taken at regular intervals, after 20 seconds of vigorous agitation and until the stationary phase was reached, for a maximum period of 300 hours (12.5 days). The time interval between readings was established on the basis of incubation temperature. At 30 and 37 °C the culture absorbance was measured every 30 minutes. At 15 and 8 °C, it was only recorded every 10 and 24 hours, respectively.

For this purpose, an automated microtiter plate reader (POLARstar Omega plate reader, BMG LABTECH GmbH, Offenburg, Germany) was used.

To ensure result reproducibility, at least three repetitions of each of the combinations studied were carried out, with a minimum of four replicates per repetition.

2.4. Modelling microbial growth and determination of kinetic parameters

Optical density data (OD data) were transformed to counts ($\log_{10}$ (cfu/mL)). The average absorbance of each of the negative controls was subtracted from the absorbance of the inoculated samples before being transformed [18, 19]. The transformation was carried out by means of calibration curves previously obtained, for each of the microorganisms studied and according to temperature [18–20]. The agreement between the observed values and the ones obtained from the curves was evaluated, based on the accuracy factor (*Af*) [21] associated with each of them.

$$A_f = 10^{\left(\frac{\sum|\log_{10}(predicted/observed)|}{number\ of\ observations}\right)} \quad (1)$$

Once transformed, the data obtained were fitted to a primary growth model [22], whose mathematical expression is as follows (Eq. 2):

$$\log_{10}(N_t) = A + C \times \mathrm{e}^{-\mathrm{e}^{-B\times(t-M)}} \quad (2)$$

In this equation, N_t represents the number of microorganisms at time t (cfu/mL); A the $\log_{10}$ of the initial count (N_0; $\log_{10}$ (cfu/mL)); C the difference between the curve asymptotes, i.e., between N_{max} and N_0 ($\log_{10}$ (cfu/mL)); B the relative growth rate when $t = M$ (($\log_{10}$ (cfu/mL))/h); M the elapsed time until the maximum growth rate is reached (h); and e is Euler's number, whose value is approximately equal to 2.718.

The fits were carried out by nonlinear regression, using the Marquardt algorithm in order to determine the value of the model parameters by minimizing the residual sum of squares [23].

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A, *B*, *C* and *M* were used to calculate the lag phase duration (λ; h) and the maximum growth rate (μ_{max}; ($\log_{10}$ (cfu/mL))/h) reached by *E. coli* K12 and *L. innocua* in each of the scenarios studied (Eq. 3 and Eq. 4) [22–24].

$$\lambda = M - \left(\frac{1}{B}\right) + \frac{\log_{10}(N_0) - A}{\mu_{max}} \quad (3)$$

$$\mu_{max} = \frac{B \times C}{\mathrm{e}} \quad (4)$$

For this purpose, average values were calculated from the values obtained for each of the repetitions carried out, all being externally validated on the basis of the coefficient of variation (*CV*) associated with each average.

The goodness of fit was evaluated by calculating the corrected determination coefficient (*corrected* R^2) and the mean square error (*MSE*) associated with each of them. The formulas used were the following:

$$Corrected\ R^2 = \left[1 - \frac{n-1 \times \left(1 - \frac{SSQ_{regression}}{SSQ_{total}}\right)}{n-p}\right] \quad (5)$$

$$MSE = \frac{SSQ_{residual}}{n-p} \quad (6)$$

On them *n* represent the number of observations; *p* the number of model parameters; and *SSQ* the sum of squares [13].

3. Results and discussion

The antibacterial activity for different citral concentrations was evaluated in liquid media at optimum temperature (37 °C), at room temperature (30 °C), at abuse temperature (15 °C) and at refrigeration temperature (8 °C).

First the minimum inhibitory concentration (MIC) was established, i.e., the value of the lowest concentration able to inhibit visible growth of the microorganisms studied at the temperatures considered [25, 26]. The results obtained in each case are shown in Table 2.

The MIC values observed are comparable to those obtained by Kim, Marshall and Wei [8] for *E. coli* O157:H7 and *L. monocytogenes*, so *E. coli* K12 and *L. innocua* could be considered, respectively, as valid surrogates for these pathogens in order to carry out food pilot plant and in-factory bacterial challenge studies. These studies are often conducted with biosafety level 1 (surrogate) microorganisms [27], in order to validate potentially preservative compounds and processes, as well as to determine the shelf life of refrigerated and ambient-stored and to determine the ability of a food to support microbial growth, taking into account predetermined performance standards [28].

Table 2 Minimum inhibitory concentration values (μL/mL) obtained according to temperature.

Temperature (°C)	*Escherichia coli* K12	*Listeria innocua*
37	0.325	0.300
30	0.325	0.300
15	0.300	0.500
8	-[a]	1.000

[a] *No growth was detected even in the absence of citral. For more information, see section 3.*

At non-inhibitory doses, sigmoid growth curves were obtained (Figs. 1 and 2), except for *E. coli* K12 at 8 °C, where no growth was detected after 300 h of incubation. Valero, Rodríguez, Carrasco, Pérez-Rodríguez, García-Gimeno and Zurera [29] described 8 °C as an inhibition temperature for *E. coli* O157:H7. In view of this behaviour, the temperature of 8 °C was discarded in the subsequent kinetic studies on the antimicrobial effect of citral at different temperatures in *E. coli* K12.

Growth curves were fitted to the modified Gompertz equation, because it has been extensively used to describe microbial growth mathematically, both in culture media and in food matrices, under defined controlled environmental conditions [10, 30]. Also, among the existing growth models, the modified Gompertz equation was selected because all of its parameters have a biological meaning [30]. For this purpose, the absorbance values recorded were first transformed into counts by using previously obtained and validated calibration curves. In all cases, the *corrected* R^2 was higher than 0.97 and the maximum *MSE* obtained was 0.03.

Tables 3, 4, 5 and 6 show the λ and μ_{max} values reached in each of the scenarios studied.

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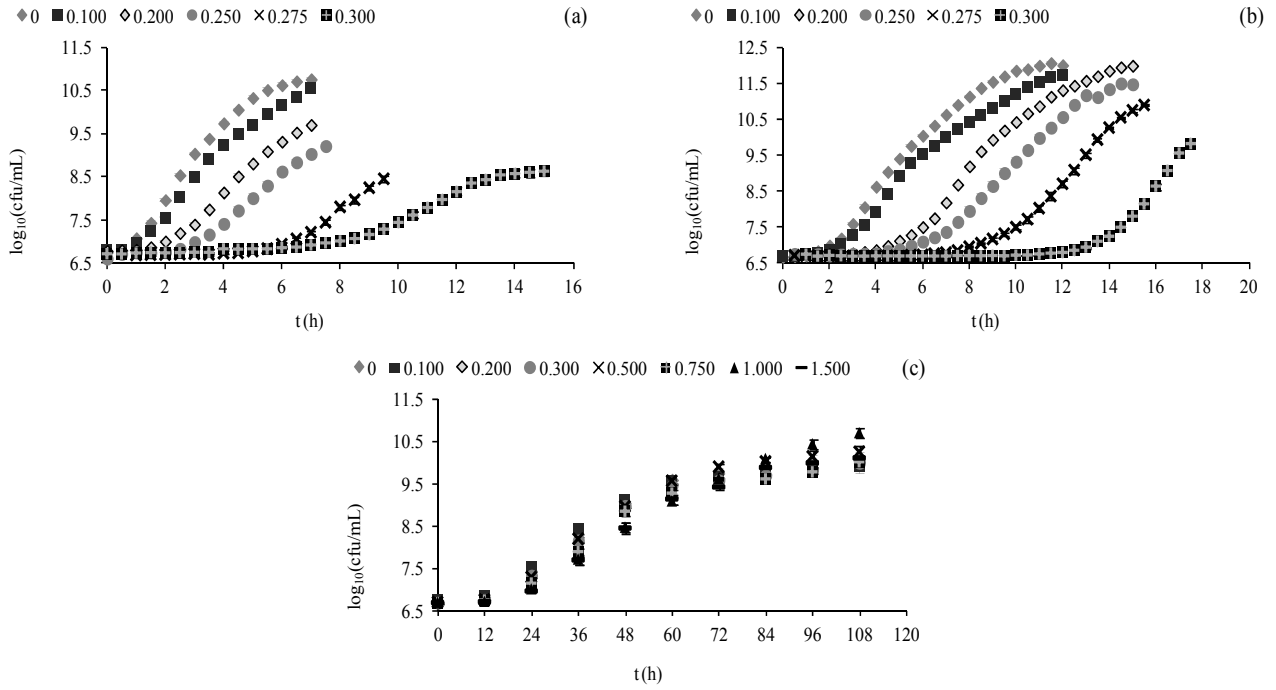

Fig. 1 *Escherichia coli* K12 growth at 37 °C (A), 30 °C (B) and 15 °C (C), depending on citral concentration (μL/mL). Error bars show the variation coefficient associated with each value.

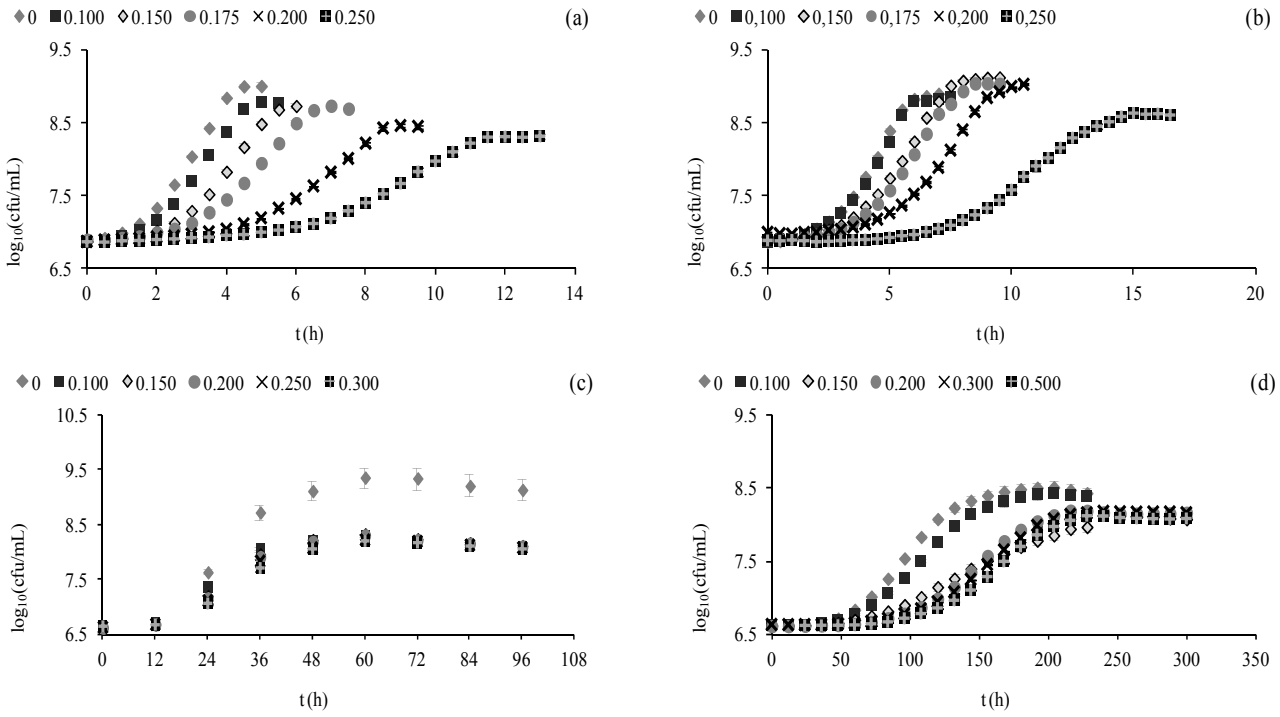

Fig. 2 *Listeria innocua* growth at 37 °C (A), 30 °C (B), 15 °C (C) and 8 °C (D), depending on citral concentration (μL/mL). Error bars show the variation coefficient associated with each value.

3.1. Effect of temperature and citral on *Escherichia coli* K12 growth

In general terms, irrespective of citral concentration, as the temperature decreases, λ increases and μ_{max} decreases (Tables 3 and 4).

At 37 °C, without citral, after a period of adaptation to the environment that lasted almost 1 hour (0.881 h), *E. coli* K12 grew at a rate of 1.070 ($\log_{10}$ (cfu/mL))/h. At 15 °C, μ_{max} was 10 times smaller (0.105 ($\log_{10}$ (cfu/mL))/h), and λ was approximately 21 times longer (18.570 h).

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Regardless of temperature, as citral concentration increased, λ increased, multiplying by as much as 7 at 37 °C. Under these conditions, i.e., when temperature remained constant and citral was added to the broth culture, it was observed that μ_{max} decreased whenever incubation temperature was above 15 °C. At this temperature, typical when a cold chain failure occurs or when foods are stored incorrectly, citral could increase λ up to a maximum value of 49.518 h when the concentration of the compound is 1.500 µL/mL. This means that the growth of *E. coli* K12 and *E. coli* O157:H7 would begin after 2 days instead of after 18 h of incubation. This extra time without growth could mean that the microbial population size avoids exceeding defined levels considered safe.

3.2. Effect of temperature and citral on *Listeria innocua* growth

In general terms, regardless of the citral concentration, it was observed that the lower the temperature, the higher the value of λ and the lower the value of μ_{max} (Table 5 and Table 6).

The value of λ at 37 °C, in the absence of citral, was 1.543 h. This means that, under optimal conditions, *L. innocua* needed about one and a half hours to adapt to the environment. A reduction of only 7 °C multiplied this value by almost 2 (1.543 vs. 2.464 h). Incubation at lower temperatures made the lag phase even longer. The λ values obtained at 15 and 8 °C are 9.5 and 30.5 times higher than the value obtained at 37 °C (14.708 and 46.989 vs. 1.543 h), respectively. Once adapted to incubation conditions, the μ_{max} reached at 30, 15 and 8 °C was approximately 1.3, 10.5 and 34 times smaller, respectively, than the μ_{max} reached at 37 °C.

In contradistinction to *E. coli* K12, *L. innocua* was able to grow at refrigeration temperature because, like *L. monocytogenes*, it is a psychrotrophic microorganism. In these cases, the use of citral as a measure for avoiding and controlling bacterial multiplication could be useful even if no failures occur in the refrigeration chain. At 8 °C, it was observed that λ increases as citral concentration increases, reaching a maximum value in the presence of 0.500 µL/mL of citral. This value is 2.4 times bigger than the value observed in the absence of citral (46.989 vs. 111.867 h).

At higher temperatures, the greater the concentration of citral, the higher the λ and the lower the μ_{max}. These results show that the use of non-inhibitory citral doses could also be an effective way to control *L. monocytogenes* growth if a cold chain failure occurs or if foods are not stored correctly.

In this case, the combination of the two factors improved the effects achieved with each of them separately. In the presence of 0.200 µL/mL of citral, at 8 °C the λ value was equal to 92.210 h; this value is almost 2 times greater than the value obtained at 8 °C in the absence of citral (92.210 vs. 46.989 h), approximately 21 times greater than the maximum value obtained by adding citral at 37 °C (92.210 vs. 4.328 h), and almost 60 times greater than the value obtained in non-stressful environments (92.210 vs. 1.543 h).

The same was true for the μ_{max} parameter: the combined application of factors was more effective than the application of one alone. In the presence of 0.200 µL/mL of citral, at 8 °C μ_{max} was equal to 0.019 ($\log_{10}$ (cfu/mL))/h; this value is approximately 1.3 times smaller than the value obtained at 8 °C in the absence of citral (0.019 vs. 0.024 ($\log_{10}$ (cfu/mL))/h), 19 times smaller than the maximum value obtained by adding citral at 37 °C (0.019 vs. 0.359 ($\log_{10}$ (cfu/mL))/h), and 43 times greater than the value obtained in non-stressful environments (0.019 vs. 0.821 ($\log_{10}$ (cfu/mL))/h).

These results show that each factor could enhance the effect of the other by synergy. The existence of synergies, on which the hurdle technology proposed by Leistner and Gorris [31] for food preservation is based, is of outstanding importance because it allows a reduction in the concentration of citral used to achieve a specific objective since it maximizes the effectiveness of a given dose.

Table 3 Lag time (λ; h) shown by *Escherichia coli* K12 according to citral concentration and incubation temperature.

Citral (µL/mL)	37 °C	30 °C	15 °C
0	0.881 ± 0.183	2.152 ± 0.359	18.570 ± 0.523
0.100	1.206 ± 0.171	2.512 ± 0.285	18.861 ± 2.101
0.200	2.225 ± 0.334	5.206 ± 0.334	20.441 ± 2.561
0.250	3.074 ± 0.614	6.745 ± 1.436	*nt*
0.275	6.221 ± 1.318	9.400 ± 1.281	*nt*
0.300	*nt*	13.760 ± 0.598	21.695 ± 3.543
0.325	MIC	MIC	*nt*
0.350			*nt*
0.500			24.026 ± 0.074
0.750			31.157 ± 2.821
1.000			40.083 ± 1.102
1.500			49.518 ± 3.313
3.000			MIC

nt = not tested; MIC = Minimum Inhibitory Concentration.

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Table 4 Maximum growth rate (μ_{max}; ($\log_{10}$ (cfu/mL))/h) reached by *Escherichia coli* K12 according to citral concentration and incubation temperature.

Citral (µL/mL)	37 °C	30 °C	15 °C
0	1.070 ± 0.028	1.018 ± 0.049	0.105 ± 0.004
0.100	0.916 ± 0.062	0.873 ± 0.018	0.099 ± 0.010
0.200	0.736 ± 0.088	0.851 ± 0.024	0.099 ± 0.013
0.250	0.649 ± 0.134	0.822 ± 0.043	*nt*
0.275	0.727 ± 0.095	0.804 ± 0.026	*nt*
0.300	*nt*	0.787 ± 0.059	0.091 ± 0.016
0.325	MIC	MIC	*nt*
0.350			*nt*
0.500			0.095 ± 0.002
0.750			0.103 ± 0.006
1.000			0.099 ± 0.009
1.500			0.108 ± 0.001
3.000			MIC

nt = not tested; MIC = Minimum Inhibitory Concentration.

Table 5 Lag time (λ; h) shown by *Listeria innocua* according to citral concentration and incubation temperature.

Citral (µL/mL)	37 °C	30 °C	15 °C	8 °C
0	1.543 ± 0.081	2.464 ± 0.163	14.708 ± 2.099	46.989 ± 7.465
0.100	1.769 ± 0.116	2.541 ± 0.100	15.885 ± 1.956	67.235 ± 11.605
0.150	2.433 ± 0.140	3.385 ± 0.362	17.054 ± 0.531	80.444 ± 3.542
0.175	3.030 ± 0.201	3.718 ± 0.529	*nt*	*nt*
0.200	4.328 ± 0.138	4.807 ± 0.576	18.167 ± 0.571	92.210 ± 15.458
0.250	6.534 ± 1.334	8.706 ± 1.315	18.917 ± 2.806	*nt*
0.300	MIC	MIC	18.939 ± 1.807	99.465 ± 8.476
0.500			MIC	111.867 ± 4.934
1.000				MIC
1.500				

nt = not tested; MIC = Minimum Inhibitory Concentration.

Table 6 Maximum growth rate (μ_{max}; ($\log_{10}$ (cfu/mL))/h) reached by *Listeria innocua* according to citral concentration and incubation temperature.

Citral (µL/mL)	37 °C	30 °C	15 °C	8 °C
0	0.821 ± 0.093	0.611 ± 0.015	0.078 ± 0.015	0.024 ± 0.004
0.100	0.692 ± 0.097	0.607 ± 0.045	0.078 ± 0.015	0.022 ± 0.004
0.150	0.645 ± 0.138	0.555 ± 0.042	0.079 ± 0.015	0.020 ± 0.004
0.175	0.527 ± 0.070	0.531 ± 0.047	*nt*	*nt*
0.200	0.359 ± 0.049	0.443 ± 0.015	0.058 ± 0.006	0.019 ± 0.002
0.250	0.302 ± 0.034	0.416 ± 0.052	0.052 ± 0.003	*nt*
0.300	MIC	MIC	0.046 ± 0.005	0.020 ± 0.000
0.500			MIC	0.018 ± 0.001
1.000				MIC
1.500				

nt = not tested; MIC = Minimum Inhibitory Concentration.

4. Conclusions

Worldwide, alimentary toxic infections[32] have an enormous socioeconomic impact. Many recent foodborne outbreaks resulted from consumption of undercooked or processed ready-to-eat meats, dairy products, or minimally processed fruits and vegetables [3].

In order to prolong shelf life and enhance quality of these goods, the study of the antimicrobial activity of plant origin substances [33], such as citral, is being promoted.

The results obtained demonstrate that the addition of citral in concentrations higher than 3 and 0.50 µL/mL could inhibit the growth of *E. coli* K12 and *L. innocua*, respectively, in foods kept at 15 °C, i.e., if a cold chain failure occurs or if the storage temperature is higher than recommended. Under these conditions, lower doses could modify the bacterial growth pattern, increasing the time that both microorganisms need to start growing, and decreasing the

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maximum growth rate reached in log phase, in the case of *L. innocua*, especially at low temperatures, given that synergy was detected between these two factors against this Gram-positive bacterium.

Although further studies are needed to evaluate the effect of citral on bacterial growth in food matrices, this preliminary study quantitatively shows that citral could be used as a natural preservative at suitable concentrations to obtain acceptable and safe ready-to-eat foodstuffs, in view of its ability to modify the growth of valid surrogates for *E. coli* O157:H7 and *L. monocytogenes*, whose incidence has recently increased.

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