Predictive microbiology quantification of the antimicrobial effect of carvacrol

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**Abstract**

A study was carried out to evaluate quantitatively the effect of carvacrol on *Escherichia coli* K12 and *Listeria innocua* growth at different incubation temperatures (37, 30, 15 and 8 °C), from a kinetic point of view. Although the value of the minimum inhibitory concentration depended on microorganism and temperature, *Listeria innocua* was always more carvacrol-resistant than *Escherichia coli* K12. The lag time and the maximum specific growth rate achieved at different carvacrol concentrations and temperatures were calculated at non-inhibitory doses. The lower the temperature or the higher the carvacrol concentration, the greater the lag time and the smaller the growth rate. These results indicate that carvacrol can inhibit or slow *Escherichia coli* K12 and *Listeria innocua* growth, especially at low temperatures, because synergism was observed between the two factors. Consequently, carvacrol could be an effective hurdle when temperature or other factors compromise the microbial safety of minimally processed ready-to-eat foods.

**Keywords:** Food safety; carvacrol; *Escherichia coli*; *Listeria* *innocua*; growth kinetics; microbial hurdle

**1. Introduction**

Reducing and controlling the concentration and growth of microorganisms in foods is fundamental to prevent their spoilage and guarantee their safety. Foods are naturally contaminated and must undergo treatments making them microbiologically stable and safe. However, some treatments penalize the sensorial and nutritional qualities of foods. Consequently, the use of natural preservatives in combination with refrigerated storage is a suitable approach to avoid the proliferation of bacteria that resist the treatments applied. It is especially important to impede the multiplication of pathogens, such as *Escherichia coli* O157:H7 and *Listeria monocytogenes*, because illnesses transmitted by foods have a wide socioeconomic impact worldwide ([FAO and WHO, 2003](#_ENREF_11)).

In view of consumer demand for fresh, minimally processed products, without chemical additives but with a long shelf life, the application of mild thermal treatments and the use of non-thermal technologies are seen as promising because conventional processing at high temperatures modifies the flavour, odour, colour, texture and nutritional value of foods. Furthermore, for some years the use of natural substances with bacteriostatic and/or bactericidal properties has been promoted in order to obtain safe foods, maintaining or improving product characteristics and the effectiveness of the treatments applied ([Pina-Pérez et al., 2009a](#_ENREF_34); [Pina-Pérez et al., 2009b](#_ENREF_35)). Notable among these natural preservatives are the essential oils (EOs) derived from herbs and spices, traditionally used as flavourings, because some of their components can inhibit or control the growth of pathogenic and/or spoiling bacteria ([Debbarma et al., 2013](#_ENREF_10); [Ferrer et al., 2009](#_ENREF_12); [Nowak et al., 2013](#_ENREF_31)). EOs are complex aromatic mixtures of secondary metabolites, which have a broad commercial interest because they possess antimicrobial ([López et al., 2005](#_ENREF_27)), antiviral ([Saddi et al., 2007](#_ENREF_42)), antimycotic ([Chaieb et al., 2007](#_ENREF_7)), antiparasitic ([Pandey et al., 2000](#_ENREF_32)), antitoxigenic ([Juglal et al., 2002](#_ENREF_21)), insecticidal ([Rajkumar and Jebanesan, 2007](#_ENREF_37)), antioxidant ([Gachkar et al., 2007](#_ENREF_13)), analgesic ([Martínez et al., 2009](#_ENREF_28)), anti-inflammatory ([Sousa et al., 2008](#_ENREF_47)) and anti-cancer properties ([Ravizza et al., 2008](#_ENREF_39)). EOs antimicrobial activity has been attributed mainly to phenolic compounds, such as carvacrol. Carvacrol is the major component of the EOs obtained from oregano and thyme ([Arrebola et al., 1994](#_ENREF_2); [Burdock, 2002](#_ENREF_3)). It is a food additive generally recognized as safe (GRAS) and it is used in baked goods, frozen dairy foods, chewing gum, soft sweets, gelatines, puddings, sauces and beverages ([Burdock, 2002](#_ENREF_3)).

Many studies based on the minimal inhibitory concentration (MIC) or the minimal bactericidal concentration (MBC) have shown that carvacrol is biostatic and/or biocidal against bacteria and fungi ([Ait-Ouazzou et al., 2011](#_ENREF_1); [Chami et al., 2004](#_ENREF_8); [Kim et al., 1995](#_ENREF_22); [Ultee et al., 1998](#_ENREF_48)), but few data exist about its effects on growth kinetics at non-inhibitory doses, more readily accepted by the consumer, given its marked flavour and its pungent, warm aroma ([Burdock, 2002](#_ENREF_3)). From a practical point of view, however, these kind of studies are very interesting because mathematical models allow the prediction of bacterial behaviour 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 ([Ross and McMeekin, 1991](#_ENREF_41); [Scott et al., 2005](#_ENREF_45); [Whiting, 1995](#_ENREF_54)), 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 industry. Kinetic studies allow food processors to know whether a product is safe or not, regardless of production, distribution and storage conditions, from farm to fork. Furthermore, they allow taking advantage of economically interesting minimal effective doses. The MIC and the MBC depend on factors such as temperature, so, its establishment to a practical application, avoiding over-processing, is more than complex due to foods are never kept under well-controlled conditions. For this purpose, sensorially unacceptable high doses would be needed, especially because EOs efficacy in foods is reduced. Consequently, the main goal of this work was to evaluate and model the effect of carvacrol and storage temperature on the growth kinetics of *E. coli* K12 and *Listeria innocua*, as non-pathogenic surrogates of *E. coli* O157:H7 and *L. monocytogenes* ([Jadhav et al., 2013](#_ENREF_20); [Kim et al., 2013](#_ENREF_23))*,* taking into account that these last are pathogens commonly found in minimally processed ready-to-eat foods. For this purpose, the modified Gompertz model was selected because all of its parameters have a biological meaning ([Corbo et al., 2009](#_ENREF_9)).

**2. Material and methods**

*2.1 Microorganisms*

*E. coli* K12 (CECT 433) and *L. innocua* (CECT 910) in stationary phase were prepared from freeze-dried pure cultures provided by the Spanish Type Culture Collection, following methods previously described ([Pina-Pérez et al., 2010](#_ENREF_33); [Saucedo-Reyes et al., 2009](#_ENREF_44)).

The average cell concentration of stocks obtained was established by viable plate count from 4 samples. The media used and the incubation time at 37 °C were the followings: Nutrient Agar (NA; Scharlau Chemie, SA, Barcelona, Spain), 24 h, for *E. coli* K12, and Tryptic Soy Agar (TSA; Scharlau Chemie, SA, Barcelona, Spain), 48 h, for *L. innocua*.

*2.2 Antimicrobial assays*

Flasks with 10 mL of sterile Nutrient Broth (NB; Scharlau Chemie, SA, Barcelona, Spain) for *E. coli* K12 and Tryptic Soy Broth (TSB; Scharlau Chemie, SA, Barcelona, Spain) for *L. innocua* were prepared. Carvacrol (≥ 98%; Sigma-Aldrich® Chemie GmbH, Steinheim, Germany) freshly diluted in dimethyl sulfoxide (DMSO ACS reagent ≥ 99.9%; Sigma-Aldrich® Chemie GmbH, Steinheim, Germany) was added to the culture media to obtain flasks containing different concentrations of carvacrol (ranging from 0.02 to 2.00 µL/mL; Table 1). All were determined from preliminary experiments establishing the MIC as the lowest concentration inhibiting visible growth of the microorganisms studied at the temperatures considered (37, 30, 15 and 8 °C), in presence of which no absorbance increase was observed after 300 h of incubation (12.5 d). The mixtures were inoculated with 1 × 107 cfu/mL *E. coli* K12 or *L. innocua* by diluting stocks in buffered peptone water (Scharlau Chemie, SA, Barcelona, Spain), if necessary. Sterile polystyrene microtiter plates (Deltalab, SL, Barcelona, Spain) were filled with inoculated samples (250 µL) with up to 9 carvacrol concentrations above the MIC (minimum number: 5). In addition, un-inoculated samples with and without carvacrol and DMSO were included in all plates as negative controls for each assay.

Regardless of temperature, the plates were incubated with double orbital shaking (500 rpm) and culture absorbance was measured at 600 nm. The readings were taken at regular intervals, after 20 s of vigorous agitation and until the stationary phase was reached, for a maximum period of 300 h (12.5 d). At 30 and 37 °C the culture absorbance was measured every 30 min. At 15 and 8 °C it was only recorded every 10 and 24 h, 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 3 repetitions of each of the combinations studied were carried out, with a minimum of 4 replicates per repetition.

*2.4 Modelling microbial growth and determination of kinetic parameters*

When growth was observed, optical density (OD) data were transformed to counts (log10cfu/mL). The average absorbance of each of the negative controls was subtracted from the absorbance of the inoculated samples before being transformed ([Sampath et al., 2011](#_ENREF_43); [Valero et al., 2006](#_ENREF_50)). The transformation was carried out by means of calibration curves previously obtained in reference media for each of the microorganisms and test temperatures ([Gupta et al., 2012](#_ENREF_16); [Valero et al., 2006](#_ENREF_50)), taking into account that a significant linear relation (*r* > 0.90) should exist between the OD data and the counts obtained. The agreement between the observed values and the ones obtained from the curves was evaluated by means of the accuracy factor (*Af*) ([Ross, 1996](#_ENREF_40)). Once transformed, the data obtained were fitted to the modified Gompertz model ([Gibson et al., 1988](#_ENREF_15)), whose mathematical expression is as follows (Eq. (1)):

$log\_{10}N\_{t}=A+C×e^{-e^{-B×(t-M)}}$ (1)

In this equation, *Nt* represents the number of microorganisms at time *t* (cfu/mL); *A* the log10 of the initial count (*N0*; log10cfu/mL); *C* the difference between the final count (*Nmax*; log10cfu/mL) and *N0* (log10cfu/mL); *B* the relative growth rate when *t* = *M* ((log10cfu/mL)/h); and, *M* the elapsed time until the maximum growth rate is reached (h).

The modified Gompertz model has been used extensively to describe microbial growth mathematically, both in culture media and in foods, under defined and controlled environmental conditions ([Corbo et al., 2009](#_ENREF_9); [Whiting, 1995](#_ENREF_54)). Fits were performed by nonlinear regression, using the Marquardt algorithm to determine the value of the model parameters by minimizing the residual sum of squares ([Zwietering et al., 1990](#_ENREF_55)). *A*, *B*, *C* and *M* were then used to calculate the lag phase duration (*λ*; h) and the maximum growth rate (*μmax*; (log10cfu/mL)/h) reached by *E. coli* K12 and *L. innocua* in each of the scenarios studied (Eqs. (2) and (3)) ([Gibson et al., 1988](#_ENREF_15); [McMeekin et al., 1993](#_ENREF_29)).

$λ=M-\left(\frac{1}{B}\right)+\frac{log\_{10}N\_{0}-A}{μ\_{max}}$ (2)

$μ\_{max}=\frac{B×C}{e}$ (3)

For this purpose, average values were calculated from the values obtained for each of the repetitions carried out. To validate these averages, the coefficient of variation (*CV*) associated with each one was calculated, not accepting values obtained from replicates statistically different (*CV* > 20%). The goodness of fits was evaluated by calculating the corrected determination coefficient (*corrected R2*) and the mean square error (*MSE*) associated with each of them ([Saucedo-Reyes et al., 2009](#_ENREF_44)). The data analysis previously described was implemented using Statgraphics® Centurion XV software (Statpoint Technologies, Inc., Virginia, USA).

**3. Results and discussion**

The antibacterial activity for different carvacrol concentrations was evaluated in liquid media at 37 °C (optimum growth temperature), 30 °C, 15 °C (abuse temperature in case of cold chain failure) and 8 °C (upper end refrigeration temperature).

The MIC at the test temperatures is shown in Table 2 (maximum incubation time: 300 h). All are in accordance with those published by other authors for *E. coli* O157:H7 and *L. monocytogenes* ([Burt et al., 2005](#_ENREF_5); [Gutiérrez-Larraínzar et al., 2012](#_ENREF_18); [Pol and Smid, 1999](#_ENREF_36); [Veldhuizen et al., 2007](#_ENREF_52)). The difference observed between the previously reported values and the ones obtained is less than 0.05 µL/mL, when the incubation temperature is equal or higher than 30 °C, and less than 0.11 µL/mL, when the incubation temperature is equal or lower than 20 °C. [Gutiérrez-Larraínzar et al. (2012](#_ENREF_18)) established that at 35 °C visible growth of *E. coli* O157:H7 in Mueller-Hinton broth is inhibited in the presence of 0.15 µL/mL of carvacrol. The results obtained shown that visible growth of *E. coli* K12 is inhibited at temperatures above 30 °C in presence of 0.14 µL/mL of carvacrol (Table 2). Furthermore, [Veldhuizen et al. (2007](#_ENREF_52)) determined that *L. monocytogenes* visible growth in TSB, at 37 °C, is inhibited in the presence of approximately 0.25 µL/mL of carvacrol. This value is close to the value observed for *L. innocua* under the same incubation conditions (Table 2). This agreement between results shown in Table 2 and the ones obtained by [Veldhuizen et al. (2007](#_ENREF_52)) as well as by [Gutiérrez-Larraínzar et al. (2012](#_ENREF_18)), among others, validates the use of *E. coli* K12 and *L. innocua* to assess the antimicrobial activity of carvacrol against *E. coli* O157:H7 and *L. monocytogenes* since their response to exposure is similar. This is important because food pilot plant and in-factory bacterial challenge studies are often conducted with biosafety level 1 (surrogate) microorganisms ([Gurtler et al., 2010](#_ENREF_17)), as recommended by the [NACMCF (2010](#_ENREF_30)).

In general, MIC increases for lower temperatures (e.g., 15 and 8 °C). Perhaps the activation of stress response mechanisms only happens in hostile environments where only more resistant bacteria survive. Moreover, microorganisms which survive or adapt to a given stress often gain resistance to other stresses ([Wesche et al., 2009](#_ENREF_53)). When bacteria are grown at low temperatures, they modify their membrane composition to increase their cold tolerance; these membrane changes could also increase carvacrol resistance ([Rattanachaikunsopon and Phumkhachorn, 2010](#_ENREF_38); [Veldhuizen et al., 2007](#_ENREF_52)). Although its mechanism of action is not fully clear, it appears that this compound is capable of destabilizing cell membranes and producing damage to the membrane which leads to an increase in its permeability, depletion of the intracellular ATP pool and disruption of the proton-motive force ([Helander et al., 1998](#_ENREF_19); [Ultee et al., 1999](#_ENREF_49)). To do this, carvacrol must interact with cells, and this may depend on the physiological state of the membrane. On the other hand, this phenomenon could be due to the effect that temperature has on the vapour pressure. Previous studies have shown that the antimicrobial potential of aroma compounds could be reduced at lower temperatures because the lower the temperature, the lower the vapour pressure and, therefore, the lower the solubility of these volatiles ([Lanciotti et al., 2004](#_ENREF_26)). In any case, this trend has also been observed by other researchers for other bacteria and food matrices ([Rattanachaikunsopon and Phumkhachorn, 2010](#_ENREF_38); [Veldhuizen et al., 2007](#_ENREF_52)).

In view of the MIC values obtained, carvacrol seems more effective against *E. coli* K12 than against *L. innocua*. There is some controversy in the scientific literature about the effectiveness of natural antimicrobials; some studies argue that they or their active components are more effective against Gram-positive bacteria, while others have shown that EOs obtained from oregano, as well as other EOs, are more effective against Gram-negative bacteria because they are able to damage their outer membrane ([Burt, 2004](#_ENREF_4)).

At non-inhibitory doses, sigmoid growth curves were obtained (Figs. 1 and 2) except for *E. coli* K12 at 8 °C, after 300 h of incubation. [Valero et al. (2010](#_ENREF_51)) described 8 °C as an inhibition temperature for *E. coli* O157:H7. In view of this behaviour, 8 °C was discarded in subsequent kinetic data analysis on the antimicrobial effect of carvacrol on *E. coli* K12.

Growth data transformed into counts by calibration curves previously obtained and validated taking into account that a significant linear relation (*r* > 0.90) was detected between the OD data and the counts, when the bacterial density ranged from ca. 6 to ca. 10 log10cfu/mL, were fitted to the modified Gompertz equation. In all cases, the *corrected R2* was higher than 0.99 and the maximum *MSE* obtained was 0.01. Tables 3 and 4 show the *λ* and *μmax* values reached in each of the scenarios studied.

*3.1 Effect of temperature and carvacrol on* Escherichia coli *K12 growth*

In general, irrespective of the carvacrol concentration in the temperature range studied, as temperature decreases, *μmax* decreases as well, while *λ* increases (Table 3). At 37 °C, without carvacrol, after a period of adaptation to the environment lasting about 30 min (0.47 ± 0.09 h), *E. coli* K12 grew at a rate of 0.954 ± 0.065 (log10cfu/mL)/h. At 15 °C, *μmax* was 7 times smaller (0.138 ± 0.024 (log10cfu/mL)/h), and *λ* was almost 40 times longer (17.63 ± 4.49 h). The increase in *λ* associated with temperature reduction was notable for the temperature interval studied. However, an appreciable decrease in *μmax* was only achieved at 15 °C, while the values obtained at 30 and 37 °C were similar indicating that *μmax* is less temperature dependent than *λ*. At the same carvacrol concentration, decreasing temperature increase the time needed by *E. coli* K12 to adapt to its environment, but once adapted it grows at the same rate as at 37 °C, except at 15 °C or lower temperatures.

Regardless of temperature, as carvacrol concentration increases *λ* increases as well, while *μmax* decreases (Table 3). At 15 °C, with carvacrol (0.15 µL/mL), *λ* reached a maximum value of 35.82 h. Furthermore, at this temperature it was observed that the compound reduced *μmax* to values very close to 0 (0.086 ± 0.004 (log10cfu/mL)/h). These results show that carvacrol could control the growth of *E. coli* O157:H7 in refrigerated products if a cold chain failure occurs, or if the refrigeration is deficient. Controlling and maintaining the temperature from production to consumption is difficult because in the supply chain there are many points where a failure could take place ([Laguerre et al., 2013](#_ENREF_25)). Several surveys on refrigeration temperatures have shown that a large percentage of retail and home refrigerators operate at temperatures above the recommended level of 4 °C ([Garrido et al., 2010](#_ENREF_14)). In many cases, the storage temperature was equal or higher than 9 °C ([Carrasco et al., 2007](#_ENREF_6); [Laguerre et al., 2002](#_ENREF_24); [Sergelidis et al., 1997](#_ENREF_46)). These abuses could easily permit the growth of pathogenic and/or spoilage microorganisms in foodstuffs stored under refrigeration. Moreover, it has been demonstrated that consumers (i) are unaware of the real temperature of their refrigerator, and (ii) place items, depending on how full it is, exceeding sometimes the refrigerating capacity of the system by overstocking ([Sergelidis et al., 1997](#_ENREF_46)). Hence the importance of the use of natural antimicrobials capable of inhibiting or modifying bacterial growth, while not forgetting the encouragement of good hygiene and storage practices ([Garrido et al., 2010](#_ENREF_14)).

The results obtained by interaction between factors (temperature and carvacrol concentration) are better than the ones obtained by just reducing temperature or just increasing the carvacrol concentration. This means that each factor could enhance the effects of the other by synergy. The *λ* value under optimal growth conditions, i.e., without carvacrol, at 37 °C, was 0.47 ± 0.09 h. The value at 15 °C, without carvacrol, was 17.63 ± 4.49 h. At 37 °C but with 0.10 µL/mL of carvacrol, it was 1.34 ± 0.02 h. At 15 °C and with 0.10 µL/mL, i.e., combining both factors, *λ* was equal to 24.00 ± 0.13 h; approximately 51 times greater than the value obtained under non-stressful conditions (24.00 vs. 0.47 h), almost 1.4 times greater than the maximum value obtained by temperature reduction (24.00 vs. 17.63 h), and approximately 18 times greater than the maximum value obtained by addition of carvacrol to the culture medium (24.00 vs. 1.34 h). The same was true of the *μmax* parameter: the combined application of factors was more effective than the application of one alone.

*3.2 Effect of temperature and carvacrol on* Listeria innocua *growth*

Regardless of the carvacrol concentration, it was observed that the lower the temperature, the higher the value of *λ* and the lower the value of *μmax* (Table 4). The *λ* value at 37 °C without carvacrol was 1.22 ± 0.21 h. This means that, under optimal conditions, *L. innocua* needed approximately an hour and a quarter for adaptation to the environment. A reduction of only 7 °C multiplied this value by approximately 2 (1.22 vs. 2.05 h). Incubation at lower temperatures made this lag phase even longer. The *λ* values obtained at 15 and 8 °C are 15 and 44 times higher than the values obtained at 37 °C (15.31 and 44.26 vs. 1.22 h), respectively. Once adapted to storage conditions, the *μmax* reached at 30, 15 and 8 °C was approximately 1.4, 11.5 and 44 times smaller, respectively, than the *μmax* reached at 37 °C.

Unlike *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 carvacrol as an additional measure for controlling bacterial proliferation could be interesting even when there are no cold chain failures. At 8 °C, it was observed that *μmax* decreases as carvacrol concentration increases, reaching a minimum value with 0.125 µL/mL of carvacrol. Under these conditions, the parameter value is 3 times smaller than the value reached by the bacterium without carvacrol (0.006 vs. 0.020 (log10cfu/mL)/h). At 15 °C, the higher the carvacrol concentration, the higher the value of *λ* and the lower the value of *μmax*, and therefore the use of non-inhibitory carvacrol doses could be an effective way to control *L. monocytogenes* growth if a cold chain failure occurs or if foods are not stored correctly.

Assays at 15 °C showed that carvacrol could double *λ* and multiply *μmax* by 10 the values obtained without carvacrol when compared with those observed at the highest non-inhibitory dose tested (0.250 µL/mL). At 30 and 37 °C, the same phenomenon was observed: the higher the carvacrol concentration, the higher the value of *λ* and the lower the value of *μmax*. In both cases, 0.250 µL/mL of carvacrol could increase the *λ* value twofold and reduce *μmax* about 5.5 times. In this case, the combination of the two factors also improved the effects achieved with each of them separately. Between 37 and 15 °C, the lower the temperature and the higher the carvacrol concentration, the lower the value of *μmax* and the higher the value of *λ*.

**4. Conclusion**

The preservative potential of naturally occurring compounds was studied as an alternative to reduce the use of artificial chemicals. Non-inhibitory doses are recommended to reduce their impact on the organoleptic characteristics of foods. Mathematical models are appropriate tools to evaluate the effectiveness of natural antimicrobials at those non-inhibitory doses and under changing environmental conditions. The results obtained demonstrate that the addition of carvacrol at concentrations higher than 0.20 and 0.50 µL/mL could inhibit the visible growth of *E. coli* K12 and *L.* *innocua*, respectively, in foods kept at 15 °C. Under these conditions, lower carvacrol doses (0.15 µL/mL in the case of *E. coli* K12 and 0.25 µL/mL in the case of *L.* *innocua*) doubled the time that both microorganisms needed to start growing and decreased the maximum growth rate reached in the log phase. Further studies are needed to evaluate its effect on the growth of these bacteria in food matrices. Carvacrol could be used as a natural preservative at concentrations that allow the production of safe foodstuffs when mild preservation technologies are used to meet consumer preferences.

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**Figure captions**

**Fig. 1.** *Escherichia coli* K12 growth curves at 37 °C (a), 30 °C (b), and 15 °C (c) as a function of carvacrol concentration (μL/mL). Error bars show the variation coefficient associated with each value.

**Fig. 2.** *Listeria innocua* growth curves at 37 °C (a), 30 °C (b), 15 °C (c) and 8 °C (d) as a function of carvacrol concentration (μL/mL). Error bars show the variation coefficient associated with each value.

****

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

|  |  |  |
| --- | --- | --- |
| **Microorganism** | **Temperature (°C)** | **Concentrations tested (µL/mL)** |
| *Escherichia coli* K12 | 37 | 0; 0.04; 0.06; 0.08; 0.10; 0.12; 0.14; 0.15; 0.16 |
| 30 | 0; 0.04; 0.06; 0.08; 0.10; 0.12; 0.14; 0.15; 0.16 |
| 15 | 0; 0.02; 0.04; 0.06; 0.08; 0.10; 0.15; 0.20; 0.40; 0.80; 1.60; 2.00 |
| 8 | 0; 0.02; 0.04; 0.06; 0.08; 0.10; 0.15; 0.20; 0.40; 0.80; 1.60; 2.00 |
| *Listeria innocua* | 37 | 0; 0.050; 0.100; 0.125; 0.150; 0.175; 0.200; 0.250 |
| 30 | 0; 0.050; 0.100; 0.125; 0.150; 0.175; 0.200; 0.250 |
| 15 | 0; 0.050; 0.075; 0.100; 0.125; 0.150; 0.175; 0.200; 0.250; 0.500; 1.000; 1.250; 1.500; 1.750; 2.000 |
| 8 | 0; 0.050; 0.075; 0.100; 0.125; 0.150; 0.175; 0.200; 0.250; 0.500; 1.000; 1.250; 1.500; 1.750; 2.000 |

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

|  |  |  |
| --- | --- | --- |
| **Temperature (°C)**  | ***Escherichia coli* K12** | ***Listeria innocua*** |
| 37 | 0.14 | 0.20 |
| 30 | 0.14 | 0.20 |
| 15 | 0.20 | 0.50 |
| 8 | -a | 0.50 |

a No growth was detected even in the absence of carvacrol. For more information, see section 3

**Table 3** Lag time (*λ*; h) and maximum growth rate (*μmax*; (log10cfu/mL)/h) reached by *Escherichia coli* K12 as a function of carvacrol concentration and incubation temperature.

|  |  |  |  |
| --- | --- | --- | --- |
| **Carvacrol****(µL/mL)** | ***λ*** |  | ***μmax*** |
| **37 °C** | **30 °C** | **15 °C** |  | **37 °C** | **30 °C** | **15 °C** |
| 0 | 0.47 ± 0.09 | 1.31 ± 0.11 | 17.63 ± 4.49 |  | 0.954 ± 0.065 | 1.058 ± 0.091 | 0.138 ± 0.024 |
| 0.02 | *nt* | *nt* | 17.70 ± 1.39 |  | *nt* | *nt* | 0.131 ± 0.018 |
| 0.04 | 0.67 ± 0.07 | 1.33 ± 0.16 | 18.73 ± 0.40 |  | 0.861 ± 0.103 | 0.911 ± 0.078 | 0.124 ± 0.024 |
| 0.06 | 0.81 ± 0.15 | 1.49 ± 0.26 | 19.24 ± 0.97 |  | 0.762 ± 0.115 | 0.844 ± 0.079 | 0.120 ± 0.024 |
| 0.08 | 0.82 ± 0.11 | 1.79 ± 0.32 | 20.68 ± 3.01 |  | 0.739 ± 0.125 | 0.760 ± 0.086 | 0.113 ± 0.016 |
| 0.10 | 1.34 ± 0.02 | 5.93 ± 0.46 | 24.00 ± 0.13 |  | 0.416 ± 0.021 | 0.619 ± 0.100 | 0.101 ± 0.003 |
| 0.12 | 7.95 ± 1.62 | 14.74 ± 2.39 | *Nt* |  | 0.467 ± 0.021 | 0.522 ± 0.120 | *nt* |
| 0.14 | MIC | MIC | *Nt* |  | MIC | MIC | *nt* |
| 0.15 |  |  | 35.82 ± 7.12- |  |  |  | 0.086 ± 0.004 |
| 0.20 |  |  | MIC |  |  |  | MIC |

*nt* = not tested; MIC = Minimum inhibitory concentration

**Table 4** Lag time (*λ*; h) and maximum growth rate (*μmax*; (log10cfu/mL)/h) reached by *Listeria innocua* as a function of carvacrol concentration and incubation temperature.

|  |  |  |  |
| --- | --- | --- | --- |
| **Carvacrol****(µL/mL)** | ***λ*** |  | ***μmax*** |
| **37 °C** | **30 °C** | **15 °C** | **8 °C** |  | **37 °C** | **30 °C** | **15 °C** | **8 °C** |
| 0 | 1.22 ± 0.21 | 2.05 ± 0.21 | 15.31 ± 1.46 | 44.26 ± 1.86 |  | 0.879 ± 0.056 | 0.635 ± 0.012 | 0.077 ± 0.005 | 0.020 ± 0.001 |
| 0.050 | 1.33 ± 0.20 | 2.11 ± 0.23 | 15.85 ± 1.42 | 44.26 ± 5.67 |  | 0.781 ± 0.069 | 0.635 ± 0.056 | 0.068 ± 0.004 | 0.017 ± 0.002 |
| 0.075 | *nt* | *nt* | 16.33 ± 1.94 | 44.03 ± 1.17 |  | *nt* | *nt* | 0.068 ± 0.004 | 0.016 ± 0.002 |
| 0.100 | 1.86 ± 0.24 | 3.18 ± 0.32 | 17.09 ± 2.17 | 46.39 ± 1.80 |  | 0.727 ± 0.021 | 0.621 ± 0.038 | 0.064 ± 0.002 | 0.013 ± 0.002 |
| 0.125 | 2.22 ± 0.38 | 3.48 ± 0.51 | 17.31 ± 1.62 | 43.63 ± 1.12 |  | 0.541 ± 0.087 | 0.448 ± 0.079 | 0.054 ± 0.009 | 0.007 ± 0.001 |
| 0.150 | 2.95 ± 0.30 | 4.23 ± 0.29 | 19.42 ± 2.96 | 43.69 ± 2.23 |  | 0.241 ± 0.041 | 0.242 ± 0.044 | 0.044 ± 0.003 | 0.006 ± 0.000 |
| 0.175 | 2.98 ± 0.15 | 5.39 ± 0.53 | 20.94 ± 3.16 | 45.98 ± 1.24 |  | 0.161 ± 0.071 | 0.111 ± 0.018 | 0.042 ± 0.008 | 0.006 ± 0.001 |
| 0.200 | MIC | MIC | 30.09 ± 1.30 | 43.92 ± 2.23 |  | MIC | MIC | 0.025 ± 0.001 | 0.006 ± 0.000 |
| 0.250 |  |  | 33.78 ± 2.81 | 42.95 ± 4.56 |  |  |  | 0.006 ± 0.001 | 0.006 ± 0.000 |
| 0.500 |  |  | MIC | MIC |  |  |  | MIC | MIC |

*nt* = not tested; MIC = Minimum inhibitory concentration