# ANTIMICROBIAL ACTIVITY OF CARVACROL ON GROWTH KINETICS OF *LISTERIA INNOCUA*

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***Abstract.*** *Most perishable food products are stored at low temperature in order to extend their shelf-life. However, this step does not eliminate undesirable microorganisms from these products. Alternative preservation techniques such as natural antimicrobial agents are being used or investigated for their application to food products. The aim was to evaluate the antimicrobial capacity of carvacrol on growth kinetic of Listeria innocua (CECT 910T). The bacterial growth rates were obtained from viable cell counts on Tryptone Soya Agar supplemented with 0.6% yeast extract (TSA-YE).The tested concentrations of carvacrol ranged from 0.100 to 0.175 µL/mL. Control samples of bacterial growth were evaluated on the absence of carvacrol. Experimental data were fitted to the modified Gompertz equation. The kinetic parameters of bacterial growth were characterized based on the time needed to environment adaptation (λ) and on the maximum specific growth rate ($\mu_{max}$). Studies carried out using $10^6$ CFU/mL inoculum size of Listeria innocua shown that λ and $\mu_{max}$ were influenced by the concentration of carvacrol as antimicrobial agent ($p \leq 0.05$). As the concentration of car vacrol increased from 0.100 µL/mL to 0.175 µL/mL an increment of λ of 2.752 hours and a decrease of $\mu_{max}$ of 0.178 log/h were observed ($p \leq 0.05$). In conclusion, it was verified that the carvacrol showed an inhibitory effect on the kinetic parameters of Listeria innocua growth therefore it can be a natural agent to extend shelf life of foods products.*

**Keywords.** Carvacrol, natural antimicrobial, *Listeria innocua*, lag phase, maximum specific growth rate.

## Introduction

Most perishable food products are stored at low temperature in order to extend their shelf-life. However, this step does not eliminate undesirable microorganisms from these products. *Listeria* spp. is ubiquitous in the environment and it grows under refrigeration and survives in freezing environments (Gandhi & Chikindas, 2007).

Alternative preservation techniques such as natural antimicrobial agents are being used or investigated for their application to food products. A number of essential oils compounds have been registered by the European Commission for use as flavourings in foodstuffs. The flavourings registered are considered to present no risk to the health of the consumer and include amongst others carvacrol, carvone, cinnamaldehyde, citral, *p*-cymene, eugenol, limonene, menthol and thymol. Carvacrol ($C_{10}H_{14}O$) is a cyclic oxygenated monoterpene, phenolic, and the major component of the essential oils of oregano and thyme (Arrebola *et al.,* 1994). The aim was to evaluate the antimicrobial capacity of carvacrol on growth kinetic of *Listeria innocua* (CECT 910T) in reference media.

## Materials and methods

### *Chemicals*

Purified carvacrol ≥98% **(**5-Isopropyl-2-methylphenol**)** was purchased from Sigma Aldrich Company Ltd. Dimethyl sulfoxide (DMSO) was used as dilution agent for obtaining the levels of 0.100 and 0.175 µL of carvacrol/mL.

### *Bacterial strain and growth conditions*

The strain of *Listeria innocua* (CECT 910T) was obtained from a pure lyophilized culture supplied by the Spanish Type Culture Collection. During this investigation, stock culture at concentration of about 7.5 x $10^8$ colony forming units (cfu/mL) was maintained in cryovials at -80 °C. Bacterial broth subcultures from stock cultures were prepared by inoculating 200 µL of *Listeria innocua* in a test tube containing in 6 mL of Tryptone Soya Broth (TSB; Scharlab Chemie S.A., Barcelona, Spain) and incubated at 37 C for 12 h. The microbial suspension was mixed and diluted to obtain an inoculum size of about 1-2 x $10^6$ cfu/mL at time 0.

### *Determination of antimicrobial activity*

Briefly, it were added to a sterile tube, 20 mL of TSB, 150 µL of carvacrol at levels of 0.100 or 0.175 µL/mL and 5 mL of bacteria which were kept in a condition of incubation at 37 ºC under agitation. Control samples of bacterial growth were evaluated on the absence of carvacrol. Culture samples of the microorganism suspension were subsequently withdrawn every 60 minutes, up to population reached the stationary

phase. The bacterial growth rates were obtained from viable cell counts data. At least, four repetitions were conducted

***Counts of viable cells and kinect parameters of bacterial growth***

After treatments, the *L. innocua* growth curves were estimated from viable plate count on Tryptone Soya Agar (TSA; Scharlab Chemie S.A., Barcelona, Spain) supplemented with 0.6% yeast extract (TSA-YE). For the curves obtainment, samples of the culture were diluted in buffered peptone water (Scharlab Chemie S.A., Barcelona, Spain) and spread in plates containing TSA-YE. The plates were incubated at 37 ºC for 48 h after which the number of colony forming units was determined. After incubation, colonies were counted by image analyzer automatic counter. To determine the kinetic of microbial growth was used non-linear regression of GraphPadPrism v. 4.03, CA, USA. The experimental data were filtered to the modified Gompertz equation (Gibson *et al.,* 1987) to determine the maximum specific growth rate ($\mu_{max}$) and the lag phase duration ($\lambda$).

***Statistical analysis***

Analysis of variance (ANOVA) was conducted using Statistical Graphics System Centurion software (Statgraphics®) Centurion XV (StatPoint Technologies Inc., Virginia, USA). Fisher's LSD test was used to compare the mean values of data ($p \leq 0.05$).

## Results and discussion

Several food preservation systems such as heating, refrigeration and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of Listeria spp food poisoning. Studies carried out using $10^6$ CFU/mL inoculum size of *Listeria innocua*, used as a non-pathogenic surrogate of *L. monocytogenes*, showed that $\lambda$ and $\mu_{max}$ were influenced by the concentration of carvacrol as antimicrobial agent ($p \leq 0.05$). The control cells showed maximum specific growth rate and the lag phase duration of the 0.568 log/h and 1 x $10^{-7}$ hours, respectively. As the concentration of carvacrol increased from 0.100 µL/mL to 0.175 µL/mL an increment of $\lambda$ of 2.752 hours and a decrease of $\mu_{max}$ of 0.178 log/h were observed ($p \leq 0.05$). Probably, when the carvacrol concentration increases, more of the compound is expected to dissolve in the membrane and more damage of the membranes appears. Previous studies demonstrated bacteriostatic properties of phenols (thymol and carvacrol) on *L.*

*monocytogenes* strains with minimum inhibitory concentrations (MIC) ≤ 0.2 µl/mL (Ait-Ouazzou *et al.,* 2011) that support our findings because the tested concentrations of carvacrol at doses below the MIC value extended the lag phase and reduced the specific growth rate. Carvacrol is a lipophilic agent that is believed to preferentially insert into the cytoplasmic membrane (Sikkema *et al.,* 1994). The cytoplasmic membrane of bacteria has two principal functions: (i) barrier function and energy transduction, which allow the membrane to form ion gradients that can be used to drive various processes, and (ii) formation of a matrix for membrane-embedded proteins (such as the membrane-integrated $F_0$ complex of ATP-synthase) (Sikkema *et al.,* 1995). In the Gram positive bacterium, carvacrol causes increased permeability of the membrane for cations such as $H^+$ and $K^+$ (Ultee *et al.,* 1999). The dissipation of the ion gradients leads to impairment of essential processes in the cell and finally to cell death (Sikkema *et al.,* 1994; Ultee *et al.,* 1999).

## Conclusions

In conclusion, it was verified that the carvacrol showed an inhibitory effect on the kinetic parameters of *Listeria innocua* growth therefore it can be a natural agent to extend shelf life of foods products.

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## References

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., Pagán, R. 2011. The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innovative Food Science & Emerging Technologies*. **12** (3): 320–329.

Arrebola, M.L., Navarro, M.C., Jimenez, J. and Ocana, F.A. 1994. Yield and composition of the essential oil of *Thymus serpylloides*subsp. *Serpylloides Phytochemistry* **36**: 67–72.

Gandhi, M, Chikindas, M.L. 2007. *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology* **113**: 1–15.

Gibson, A.M., Bratchell, N. and Roberts, T.A. 1987. The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridiurn botulinum* type A in pasteurized pork slurry. *J. Appl. Bacteriol* **62**: 479-490.

Sikkema, J., de Bont, J.A.M. and Poolman, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *The Journal of Biological Chemistry* **11:** 8022–8028.

Sikkema, J, De Bont, J.A.M., Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Review* 59: 201–222.

Ultee, A., Kets, E.P.W., Smid, E.J. 1999. Mechanisms of action of carvacrol on the food borne pathogen *Bacillus cereus. Appl. Environ. Microbiol*. **65**: 4606–4610.