Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Food Engineering 67 (2005) 247–252
JOURNAL OF FOOD ENGINEERING
www.elsevier.com/locate/jfoodeng

Response surface analysis of the effects of *Capsicum* extract, temperature and pH on the growth and inactivation of *Listeria monocytogenes*

Claudia Acero-Ortega [a], Lidia Dorantes [a,*], Humberto Hernández-Sánchez [a], Maria Soledad Tapia [b], Gustavo Gutiérrez-López [a], Stella Alzamora [c], Aurelio López-Malo [d]

[a] *Graduados en Alimentos, Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala, Colonia Santo Tomás, México 11340 DF, Mexico*
[b] *Instituto de Ciencia y Tecnología de Alimentos, Facultad de Ciencias, Universidad Central de Venezuela, Apdo. 47097, Caracas 1049, Venezuela*
[c] *Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria 1428, Buenos Aires, Argentina*
[d] *Departamento de Ingeniería Química, Alimentos y Ambiental, Universidad de las Américas Puebla, Santa Catarina Mártir, Cholula, Puebla, México CP 72820, Mexico*

Received 10 October 2003; accepted 1 May 2004

Abstract

A surface response analysis was carried out using the Box-Behnken method in order to determine the effects and interactions of pH (4.5, 5.5, 6.5), temperature (2, 7, 12 °C) and *Capsicum* extract concentration (0%, 5%, 10%) on the growth kinetics of *Listeria monocytogenes* Scott A in trypticase soy broth. Survival ratio quadratic models (log N/N_0) were obtained for the combination of variables, valid only in the specified ranges. Temperature showed no effect on the bacterial inactivation; however both the extract concentration (5%) and pH value (4.5) had a relevant effect on the microbial counts. The models were validated on acidified milk inoculated with *L. monocytogenes*. According to their evaluation, it may be possible to use the models in order to obtain reasonable initial estimates of the impact of the *Capsicum* extract, as well as storage conditions, over the growth of *L. monocytogenes* after 4 and 8 days of storage.

Keywords: Modeling; *Listeria*; *Capsicum*; pH; Inactivation

1. Introduction

Listeria monocytogenes, an important pathogen associated to foodstuffs, is the cause for listeriosis. The appearance of this disease has been associated with the consumption of milk, cheese, vegetables and salads, as well as meat-based products. *Listeria* is particularly problematic for the food industry, due to its wide environmental distribution (Farber & Peterkin, 1991). The pathogen is able to grow in wide ranges of temperature (−1.5 to 45 °C), pH (4.39–9.4), and osmotic pressures (NaCl concentrations over 10%), besides from being an anaerobic facultative microorganism (ICMSF, 1996).

Recently, there has been an increasing interest on the development of mathematical models that describe the microbial growth as a function of various environmental

* Corresponding author. Tel.: +52 55 572963000x62458; fax: +52 55 53740959.
E-mail addresses: ldoran@ipn.mx, lidiadorantes@hotmail.com (D. Lidia).

0260-8774/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jfoodeng.2004.05.059

stress factors, such as water activity, pH, and temperature. Some of these models have been published for *L. monocytogenes*, particularly to describe the combined effect of temperature, pH, a_w, organic acids, $NaNO_2$, CO_2 concentration, and irradiation on the growth of the bacterium (Buchanan & Phillips, 1990; Grau & Vanderlinde, 1993; Patterson, Damoglou, & Buick, 1993; Duffy, Vanderlinde, & Grau, 1994; Farber, Cai, & Ross, 1996; George, Richardson, & Peck, 1996; Murphy, Rea, & Harrington, 1996; Fernández, George, Sills, & Peck, 1997; McClure, Beaumont, Sutherland, & Roberts, 1997). However, up to this date there are no reports on the inhibitory effect that *Capsicum* extracts, combined with other barrier factors such as temperature and pH, have on *L. monocytogenes*, even though there is a study that mentions the inhibitory effect of *Capsicum* extracts on *Listeria* (Dorantes et al., 2000). Since this extract is considered GRAS and its use in foodstuffs has been approved by the FDA, it is interesting to express quantitatively the effectiveness of *Capsicum* as a natural antimicrobial.

This goal can be achieved through surface response analysis of experimental data, which would provide valuable information on the combined effect of environmental stress factors and the presence of a natural antimicrobial from the *Capsicum* genre.

Given the above, the aim of the present study is to (1) monitor the survival-inactivation of *L. monocytogenes* on trypticase soy broth and in a model foodstuff (partly skimmed, UHT, acidified milk), under different temperatures, pH, and concentrations of *Capsicum* extract; and (2) validate the culture media models by using it with data measured in the selected food (milk), and by interpolation, predicting the pathogen behavior in the food as a result of the combined conditions that were previously mentioned.

2. Materials and methods

2.1. Strain and culture conditions

L. monocytogenes Scott A was obtained from the Sanitary Microbiology Laboratory of the Escuela Nacional de Ciencias Biológicas (IPN, Mexico City) and was maintained on trypticase soy agar plates (TSA, Dibico, Mexico City) at 4 °C until use. The strain was subcultured monthly to ensure its viability.

2.2. Plant material and extract preparation

Capsicum annuum variety Guajillo San Luis was obtained from the National Institute of Agriculture and Forestry Researches (INIFAP), Tamaulipas and San Luis Experimental Center, Mexico. The peppers were washed and their stems cut out. The vegetables were weighed and placed in a blender (Osterizer, Mexico) with an equal amount of isopropanol (1:1 weight/volume). The mix was blended for 1 min, and then shaked for 15 min. Afterwards it was filtered through a large-pore filter paper, and 15% (w/w) of active charcoal was added to the filtrate. Then the mix was gently shaken for 5 min and filtered again through a Whatmann filter paper no. 1. The separated solids were discarded and the clear filtrate was evaporated under reduced pressure (Büchi B481) at a temperature of 71 °C, a pressure of 46 mbar, and a speed of 28 rpm in order to remove the alcohol. Finally, the extracts were stored at −20 °C until used (Dorantes et al., 2000).

2.3. Culture techniques

To obtain a standardization of the inoculum, the bacterium was cultured in trypticase soy broth (TSB, Dibico, Mexico City) for 18 h at 35–37 °C. Aliquots were taken and serial decimal dilutions were carried out using fresh broth until an absorbance of 0.05 at 590 nm was reached, to give a standard inoculum of about 10^7 CFU/ml. Bacterial counts were confirmed by culturing on TSA plates, incubating at 37 °C for 24 h.

The culture techniques employed were identical to those described by Pandit and Shelef (1994). Flasks containing 99 ml of TSB mixes and different concentrations of *Capsicum* extract were adjusted to the proper pH by means of the addition of a solution of 0.1 N HCl, sterilized at 121 °C for 15 min, and cooled at 35–37 °C. All the mixtures were inoculated with 1 ml of a bacterial culture that contained 10^7 CFU/ml, followed by incubation at 2, 7, and 12 °C. Periodically sampling was made and the populations of *L. monocytogenes* were determined by making serial dilutions of samples in a sterile saline solution (0.85%) and plating the appropriate dilutions on TSA, which was incubated at 35–37 °C for 24 h. Afterwards the typical colonies produced by *L. monocytogenes* were counted. All experiments were done in triplicate.

2.4. Experimental design

The study was developed in two stages. In the first, a Box-Behnken surface response design (Design Expert, ver. 5.0) was used to determine the effect of the combination of three temperature levels (2, 7, and 12 °C); three pH levels (4.5, 5.5, and 6.5), as well as three levels of extract concentration (0%, 5%, and 10%) on the survival of *L. monocytogenes* Scott A in TSB. Seventeen experimental runs, including five repetitions of the central point, were performed. The survival of *L. monocytogenes* was monitored in every treatment by counting the colonies developed on TSA plates after 1, 4, and 8 days. The experiments were done in triplicate and the results are shown as the average ± standard deviation.

An experiment, similar to the one explained above, was carried out in the second stage, in order to validate the model. In this case, 99 ml of partly skimmed UHT milk were added with the corresponding concentrations of *Capsicum* extract, and the pH value was adjusted to 4.5, 5.5, and 6.5 using a 0.1 N HCl solution. The milk samples were then inoculated with 1 ml of the bacterial suspension, which contained 10^7 CFU/ml. Afterwards they were incubated at the temperatures given by the experimental design. The *L. monocytogenes* population was determined immediately after the inoculation and after 2, 4, 6, 8, 10, and 12 storage days. After an incubation period of 24–48 h at 35–37 °C, the surviving population was determined by culturing in Oxford agar (Oxoid, UK), and recounting the colonies.

2.5. Model development and validation

The counting data (initial count N_0 and final count N) for both the TSB and milk tests were transformed to $\log_{10}$ (N/N_0), and then plotted vs. time, as well as introduced to the surface response module of the Design Expert software. This was done with the aim of obtaining the model that better fitted the results.

On the other hand, and according to Ross (1996), a validation of the performance of the developed model was carried out by comparing the death (or survival) ratio of *L. monocytogenes* predicted by the model with the one observed in the actual foodstuff.

The indexes used for the performance evaluation of the predictive model were the bias and the accuracy factor, defined as follows (Ross, 1996):

$$\text{Bias factor} = 10^{\left(\sum \log(\mathrm{DR}_{\text{predicted}}/\mathrm{DR}_{\text{observed}})/n\right)}$$

$$\text{Accuracy factor} = 10^{\left(\sum \left|\log(\mathrm{DR}_{\text{predicted}}/\mathrm{DR}_{\text{observed}})\right|/n\right)}$$

where $\mathrm{DR}_{\text{predicted}}$ is the predicted death (or survival) ratio, in this case the interpolated death ratio value, $\mathrm{DR}_{\text{observed}}$ is the observed death ratio, and n is the number of observations used in the calculations.

3. Results and discussion

3.1. Effect of pH, temperature, and Capsicum extract concentration

The results obtained after carrying out the experimental tests are shown in Table 1. Examples of the plots of log (N/N_0) vs. time for the TSB and milk tests are shown in Figs. 1 and 2. The data from the fourth experimental day were analyzed with the Design Expert software version 5.0. Since no inhibition was observed until the 4th day, only the models for the 4th and 8th days were obtained. With the aim of obtaining adequate values for r^2, the variables temperature (T in °C) and pH were transformed to $1/T$ and 1/pH. In this way, an $r^2 = 0.7741$ was obtained, as seen in the following quadratic model valid only for an incubation period of 4 days and for interpolation within the data range:

$$\log\frac{N}{N_0} = 0.18 - \frac{5.95}{\mathrm{pH}} - 0.5[\text{extract}] + 0.041[\text{extract}]^2 \quad (1)$$

$$r^2 = 0.7741$$

It can be observed that, in this case, the temperature was not a relevant variable for the model (the temperature coefficient in the equation was not significant at a 0.05 significance level).

By repeating this process, a quadratic model valid only for the incubation period of 8 days and for interpolation within the data range was obtained, as follows:

$$\log\frac{N}{N_0} = 0.071 - \frac{6.80}{\mathrm{pH}} - 0.62[\text{extract}] + 0.053[\text{extract}]^2 \quad (2)$$

$$r^2 = 0.7416$$

As in the first case, temperature did not show any influence on the bacterial behavior. In this case, the model consisted of the two equations and time was taken as a parameter ($t = 4$ and $t = 8$) and not as a variable due to the complexity and the high number of variables involved in the experiments. Using both mathematical models, interpolated values for log N/N_0 were obtained, as shown in Table 1. As it can be seen, these values are very similar to each other.

The interpolated values were also compared to the experimental values obtained at 7 °C. This temperature was chosen because it is the intermediate point between the selected temperatures, bearing in mind that temperature was not a significant variable.

When analyzing the effect of pH on the inhibition of *L. monocytogenes* in the models developed for the 4th and 8th days of incubation, it was observed that a higher inhibitory effect was obtained when pH values were lower, being the highest effect at pH 4.5. In regard to the *Capsicum* extract, the best inhibitory effect was observed at a concentration of 5%. This is noteworthy for both the 4th and 8th days (Table 1). Such results can probably be due to the inhibitory action of the various hydroxycinnamic acids that are known to be present in the *Capsicum annuum* var. Guajillo, as identified in a previous report (Acero et al., in revision). These acids include the *t*-cinnamic, *o*-cumaric, *m*-cumaric, ferulic, and caffeic acids, which can be classified as weak lipophilic acids. It is also known that the antimicrobial activity of these compounds is due to their non-dissociated molecules (Davidson & Banden, 1981). On this regard, it is also known that at pH 4.5, the proportion of

Table 1
Results of the response surface design for the survival of *L. monocytogenes* at different temperatures, pH values and concentrations of *Capsicum* extract after 4 and 8 days of incubation

Days of incubation	Temperature (°C)	pH	Extract concentration (%, v/v)	log N/N_0 (interpolated from the model)	N/N_0 (experimental)
4	2	4.5	5.0	−2.61	−2.85 ± 0.014
		5.5	0.0	−0.90	−0.81 ± 0.003
			10	−1.80	−1.62 ± 0.015
		6.5	5	−2.20	−1.98 ± 0.005
	7	4.5	0	−1.14	−0.99 ± 0.052
			10	−1.63	−1.53 ± 0.032
		5.5	5	−2.38	−2.69 ± 0.037
				−2.38	−1.72 ± 0.028
				−2.38	−2.49 ± 0.009
				−2.38	−2.43 ± 0.015
				−2.38	−2.69 ± 0.018
		6.5	0	−0.73	−1.19 ± 0.017
			10	−1.63	−1.53 ± 0.110
	12	4.5	5	−2.61	−2.22 ± 0.014
		5.5	0	−0.90	−0.70 ± 0.012
			10	−1.80	−1.35 ± 0.007
		6.5	5	−2.20	−2.32 ± 0.046
8	2	4.5	5.0	−3.21	−3.64 ± 0.003
		5.5	0.0	−1.16	−0.80 ± 0.107
			10	−2.06	−1.67 ± 0.047
		6.5	5	−2.75	−2.33 ± 0.008
	7	4.5	0	−1.43	−1.94 ± 0.032
			10	−1.86	−2.00 ± 0.019
		5.5	5	−2.94	−3.18 ± 0.013
				−2.94	−3.15 ± 0.058
				−2.94	−2.94 ± 0.038
				−2.94	−2.75 ± 0.020
				−2.94	−3.16 ± 0.024
		6.5	0	−0.97	−1.83 ± 0.004
			10	−1.86	−2.00 ± 0.033
	12	4.5	5	−3.21	−2.66 ± 0.059
		5.5	0	−1.16	−0.18 ± 0.009
			10	−2.06	−2.04 ± 0.011
		6.5	5	−2.75	−2.81 ± 0.022

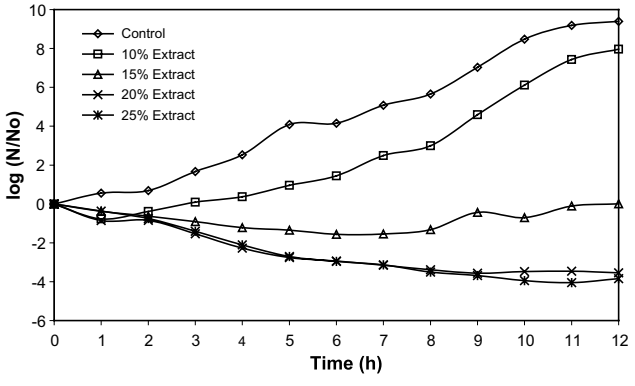

Fig. 1. Growth and survival curves for *L. monocytogenes* Scott A in the presence of 0%, 10%, 15%, 20% and 25% *Capsicum* extract in TSB at pH 7.2 and 37 °C.

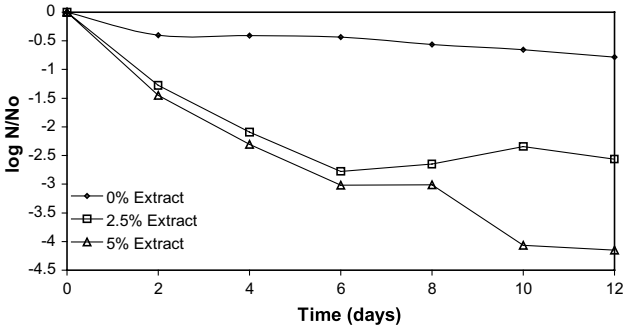

Fig. 2. Survival curves of *L. monocytogenes* Scott A in the presence of 0%, 2.5% and 5% *Capsicum* extract in acidified milk at pH 4.5 and 7 °C.

non-dissociated molecules in these acids increases (Rico-Muñoz, Bargiota, & Davidson, 1987; Kouassi & Shelef, 1998) and therefore when the pH of the media approaches 4.5, the activity of the hydroxycinnamic acids

Table 2
Values predicted by the response surface model for the survival of *L. monocytogenes* in acidified milk at different pH values and *Capsicum* extract concentrations

Days of incubation	pH	Extract concentration (%, v/v)	log N/N_0 (interpolated from the model)	log N/N_0 (experimental)
4	4.5	2.5	−2.13	−2.07 ± 0.160
		5.0	−2.61	−2.30 ± 0.071
8		2.5	−2.78	−2.65 ± 0.014
		5.0	−3.21	−3.01 ± 0.016
4	5.5	5.0	−2.38	−2.37 ± 0.168
8			−2.93	−2.95 ± 0.103
4	6.5	5.0	−2.20	−2.34 ± 0.031
		10.0	−1.63	−1.92 ± 1.111
8		5.0	−2.62	−2.68 ± 0.015
		10.0	−1.74	−1.62 ± 0.950

increases (ICMSF, 1994). This agrees with the results of the present study.

Juven, Kanner, Schved, and Weisslowicz (1994) reported that the increase of antibacterial activity in essential oils and plant extracts at low pH values may be due to the increased hydrophobicity of extract constituents at acid pH levels, and thus are better dissolved in the lipidic phase of the bacterial membrane. Helander et al. (1998) and Sikkema, de Bont, and Poolman (1994) mention that phenolic compounds form plant extracts accumulate in the lipidic bilayer according to the partition coefficient, which is specific for these compounds. This causes a disruption in both the structure and functionality of the cell membrane, probably due to the expansion that the membrane surface undergoes as a result of the accumulation of lipophilic compounds.

3.2. Development and validation of the model

Table 2 shows the results obtained experimentally, as well as the results interpolated from the mathematical models for the test in acidified milk. As it can be observed, the differences between experimental and interpolated values are not significant. However, with the aim of corroborate this, the validation method of Ross (1996) was applied. A bias factor of 1.11 and an accuracy factor of 1.19 were obtained. According to Ross (1996) the bias factor answers the question whether, on average, the observed values lie above or below the line of equivalence and, if so, by how much. Thus it assesses whether the model is "fail–safe". The accuracy factor averages the minimum "distance" between each point and the line of equivalence as a measure of how close, on average, predictions are to observations. The accuracy factor is, thus, a measure of average deviation and may be used as a simple measure of the level of confidence one may have in the model's predictions. Based on the accuracy factor, the interpolations are, on average, within 19% of the observations. Nonetheless, that the bias factor remains close to unit for these data sets is a reassuring feature of the predictive equation generated, and supports the validity of the interpolation modeling approach in this case.

From the above, it may be concluded that it is possible to use the model to provide valuable information on the combined effect of pH, and *Capsicum* extract concentration on the growth and inactivation of *L. monocytogenes*.

Acknowledgments

The authors wish to thank the Government of Mexico, through the Secretaría de Relaciones Exteriores, the Instituto Politécnico Nacional, and the CYTED XI.15 Project "Tecnologías emergentes para la conservación de alimentos de interés para Iberoamerica", for the support given during the completion of this study. The authors also thank Ing. Octavio Pozos for providing the plant samples.

References

Buchanan, R. L., & Phillips, J. G. (1990). Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. *Journal of Food Protection, 53*, 370–376.

Davidson, P. M., & Banden, A. L. (1981). Antimicrobial activity of non-halogenated phenolic compounds. *Journal of Food Protection, 44*, 623–632.

Dorantes, L., Colmenero, R., Hernández, H., Mota, L., Jaramillo, M. E., Fernández, E., & Solano, C. (2000). Inhibition of growth of some food borne pathogenic bacteria by *Capsicum annum* extracts. *International Journal of Food Microbiology, 57*, 125–128.

Duffy, L. L., Vanderlinde, P. B., & Grau, F. H. (1994). Growth of *Listeria monocytogenes* on vacuum-packed cooked meats: effects of pH, a_w, nitrite and ascorbate. *International Journal of Food Microbiology, 23*, 377–390.

Farber, J. M., & Peterkin, P. I. (1991). *Listeria monocytogenes*, a foodborne pathogen. *Microbiological Review, 55*, 476–511.

Farber, J. M., Cai, Y., & Ross, W. H. (1996). Predictive modeling of the growth of *Listeria monocytogenes* in CO_2 environments. *International Journal of Food Microbiology, 32*, 133–144.

Fernández, P. S., George, S. M., Sills, C. C., & Peck, M. W. (1997). Predictive model of the effect of CO_2, pH, temperature and NaCl on the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology, 37*, 37–45.

George, S. M., Richardson, L. C. C., & Peck, M. W. (1996). Predictive models of the effect of temperature, pH and acetic and lactic acids on the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology, 32*, 73–90.

Grau, F. H., & Vanderlinde, P. B. (1993). Aerobic growth of *Listeria monocytogenes* on beef lean and fatty tissue: equations describing the effects of temperature and pH. *Journal Food Protection, 56*, 96–101.

Helander, I. M., Alakomi, H. L., Latva-Kala, K., Matilla-Sandholm, T., Pol, I., Smid, E. J., Gorris, L. G., & von-Wright, A. (1998). Characterization of the action of selected essential oil components on Gram negative bacteria. *Journal of Agricultural and Food Chemistry, 46*, 3590–3595.

ICMSF (1994). *Ecología microbiana de los alimentos. Factores que afectan la vida y muerte de microorganismos* (Vol. 1). New York: Academic Press (pp. 1–38, 97–117, 132–142).

ICMSF (1996). *Listeria*. In *Microorganisms in foods. Microbiological specifications of food pathogens* (Vol. 5, pp. 141–182). London: Blackie Academic & Professional.

Juven, B. J., Kanner, J., Schved, F., & Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *Journal of Applied Bacteriology, 76*, 626–631.

Kouassi, Y., & Shelef, L. A. (1998). Inhibition of *Listeria monocytogenes* by cinnamic acid: possible interaction of the acid with cysteynil residues. *Journal of Food Safety, 18*, 231–242.

McClure, P. J., Beaumont, A. L., Sutherland, J. P., & Roberts, T. A. (1997). Predictive modeling of growth of *Listeria monocytogenes*. The effects on growth of NaCl, pH, storage temperature and $NaNO_2$. *International Journal of Food Microbiology, 34*, 221–232.

Murphy, P. M., Rea, M. C., & Harrington, D. (1996). Development of a predictive model for growth of *Listeria monocytogenes* in a skim milk medium and validation studies in a range of dairy products. *Journal of Applied Bacteriology, 80*, 557–564.

Pandit, V. A., & Shelef, L. A. (1994). Sensitivity of *Listeria monocytogenes* to rosemary (*Rosmarinus officinialis* L.). *Food Microbiology, 11*, 57–63.

Patterson, M. F., Damoglou, A. P., & Buick, R. K. (1993). Effects of irradiation dose and storage temperature on the growth of *Listeria monocytogenes* on poultry meat. *Food Microbiology, 10*, 197–206.

Rico-Muñoz, E., Bargiota, E. E., & Davidson, P. M. (1987). Effect of selected phenolic compounds on the membrane-bound adenosine triphosphatase of *Staphylococcus aureus*. *Food Microbiology, 4*, 239–249.

Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Bacteriology, 81*, 501–508.

Sikkema, J., de Bont, J. A. M., & Poolman, B. (1994). Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry, 269*, 8022–8028.