APPLICATION OF WEIBULL TYPE DISTRIBUTION OF RESISTANCES MODEL TO THERMAL RESISTANCE OF *LISTERIA INNOCUA* IN VANILLIN CONTAINING ORANGE JUICE.

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Abstract. Different methods are being explored for the preservation of orange juice for reducing the severity of conventional pasteurization. In order to decrease process temperature and/or time other stress factors must be included to assure food safety. Among natural antimicrobials, vanillin has been shown to be a natural inhibitor for molds and yeasts in fruit products. On the other hand, contamination of commercial products with *Listeria monocytogenes* has become a major concern for food processors. The purpose of the present work was to evaluate and model the influence of incorporated vanillin and process temperature on *Listeria innocua* (as surrogate microorganism for *L. monocytogenes*) survival in orange juice. Inactivation experiments were carried out in a 150 mL- double wall vessel containing orange juice supplemented with non inhibitory levels of vanillin (0, 500, 700, 900 or 1100 ppm). The temperature was controlled to attain 57 °C to 61°C. Samples were taken at regular intervals during ≅ 8 minutes and monitored by plate counting and NMP techniques using Triptone Soy Agar supplemented with 1% sodium piruvate in order to detect injured cells. *L. innocua* log reductions increased exponentially with temperature and vanillin concentration, reaching for some conditions 5 log CFU/ml as required by FDA to assure juice safety. Vanillin effect was more pronounced at higher temperatures. Weibull type distribution of resistances model was successfully fitted to survival curves. Statistic parameters which better explain the observed frequencies: *distribution mode*, *mean, variance,* and *coefficient of skewness,* were calculated. Some combinations of temperature-vanillin were highly effective exhibiting narrow frequency shapes, slightly skewed to the right, with low time media and variance, indicating that the majority of the population died at the beginning of treatment. The results suggest that the use of vanillin combined with mild heat treatment could inactivate *L. innocua* in minimally processed orange juice.

Keywords: *Listeria*, Orange juice, Weibull - type distribution

1. Introduction

Minimal preservation processes based on the combined factors technology have been successfully applied to preserve tropical and subtropical fruits like peach, papaya, pineapple, mango, banana and chicozapote (Sajur, 1985; Alzamora et al. 1989, 2003; Guerrero et al, 1994). This approach deliberately and intelligently combines hurdles to improve the microbial stability and safety of foods, as well as the sensory and nutritive qualities (Leistner, 2000).

Development of novel processing technologies involving the use of natural antimicrobials as a possible emergent preservation factor in conjunction with conventional ones, is been investigated (Guerrero et al., 2004; López Malo et al., 1995). This group of hurdles is at present of special interest for the food industry as "green preservatives" to satisfy growing consumer demands for fresh products without synthetic preservatives.

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Vanillin (4-hidroxi-3-methylbenzaldehide), the major constituent of vanilla beans, is a phenolic compound that not only has demonstrated to have antimicrobial and antioxidant properties, but also impairs good flavor to a wide variety of products such as bakeries, ice creams and drinks. It has been successfully used for inhibiting *Listeria monocytogenes* (Ferrante, 2004), yeasts (Cerruti and Alzamora, 1996) and moulds (López Malo, 2000).

Fruit and vegetables are frequently in contact with soil, insects and animals during growing and harvesting in the field. Even after the cleaning process, microorganisms may remain contaminating the fruit and be transferred to the juice during or after extraction. Pathogens do not grow in fruit juices due to their low pH, but can survive and become adapted to the acidic environment. The extended survival of these pathogens in acidic foods and the increased tolerance of acid-adapted cells to unfavourable growth conditions is well established (Mazzotta, 2001).

Many incidents of foodborne disease involving fruit juices prompted the U.S. Food and Drug Administration (FDA) to release a regulation which requires juice producers to implement a system that achieves a 5-$\log_{10}$ reduction of the most resistant organism of public health significance. It also recommends that in the absence of known specific pathogen-product associations, *E. coli* O157:H7 or *L. monocytogenes* should be used as target organisms, as appropriate.

Listeriae are more heat resistant than other non-spore-forming foodborne pathogens (i.e. *Salmonella spp.* or pathogenic *Eschericha coli*), and thus, processing recommendations based on data for other microorganisms may not be enough to eliminate them (Doyle et al., 2001). Reported data on *Listeria innocua* indicate that it is more heat resistant than some strains of *L. monocytogenes* when the two organisms are tested under identical conditions, therefore being a suitable model for estimating the thermal tolerance of *L. monocitogenes (*Kamat et al., 1996).

The purpose of this study was to investigate the response of *L._innocua* (as a surrogate microorganism for *L. monocytogenes*) in orange juice added with different levels of vanillin at four heating temperatures in an isothermal system, and to model survival curves in order to obtain the frequency distribution of resistances.

2. Materials and Methods

2.1. Inoculum

Cultures of *Listeria innocua* ATCC 33090 were grown in tryptone soya agar slants containing 0.6% yeast extract (TSA-YE), incubated at 37ºC for 24 hs and stored at 4ºC. Subcultures were made in 20 or 100 ml of triptone soya broth with 0.6% yeast extract (TSB-YE) using agitation. After 24 hour incubation, 100 ml cell cultures were sedimented at 2,000 x g for 20 minutes and the pellet was resuspended in the proper volume of TSB-YE in order to obtain initial counts of 10^7- 10^8 m.o./g.

2.2. Juice preparation

Concentrated orange juice (65 ºBrix) was specially prepared without additives by ECA Agroindustrias S.A. Diluted juice (pH 3.5) was prepared from concentrate by adding sterile water (water/juice = 6/1) and pasteurized at 72ºC for 14 seconds using a Microthermics UHT/HTST Lab- 25DH (Raleigh, USA) unit and collected in a biological safety cabinet (Nuaire Inc., USA).

2.3 Batch kinetic data

Thermal inactivation kinetics for *L. innocua* in orange juice supplemented or not with vanillin was determined in a reactor at constant temperature. Treatment was carried out in a 150 ml-double wall cylindrical vessel (diameter: 6.3 cm; height: 7.6 cm) connected to a thermostatically controlled water bath (whose temperature was fixed to attain 57, 59, 60 or 61 ± 0.2 ºC in the samples). Ninety-nine (99) ml of juice were poured into the vessel. Just before the heat treatment, vanillin solution (5% w/w) was added to each system in order to obtain 0, 500, 700, 900 or 1100 ppm. After inoculation, samples were taken at regular time intervals and survival cells were enumerated by superficial plate count and the most probable number techniques (for lower counts) using TSA-YE or TSB-YE plus 1% sodium piruvate in order to recover injured cells.

Plates were incubated at 30°C for 5 days. Each condition was assayed in triplicate and the average was reported. All microbiological media were purchased at Laboratorios Britania (Argentina).

2.4. Modeling routine

L. innocua survival curves were fitted using the cumulative form of the frequency Weibull type distribution of sensitivities (Peleg and Cole, 1998):

$$\log S(t) = \log(CFU_{(t)} / CFU_{(0)}) = -bt^{n} \qquad (1)$$

where *b* and *n* are constants. The values of *b* and *n* were then used to plot the resistances frequency curve using the following equation:

$$\frac{d\phi}{dt_c} = bnt_c^{n-1}\exp(-bt_c^{n}) \qquad (2)$$

Other statistic parameters which better explain the observed frequencies: distribution mode, t_{cm}; mean, $\bar{t}_c$; variance, σ_{tc}^2; and coefficient of skewness, υ_1, were calculated according to Peleg and Cole (1998). All model parameters were derived using nonlinear regression technique (Statgraphics Plus for Windows 3.0® package, Statistical Graphics Corp.).

3. Results and Discussion

L. innocua semi logarithmic survival curves during thermal treatment (57, 59, 60 or 61 ± 0.2°C) in orange juice (pH 3.5) with the addition of different levels of vanillin (0, 500, 700, 900 or 1100 ppm) were modeled applying the cumulative form of the Weibull type distribution of resistances. Results obtained at 57 and 61ºC are shown in Figure 1. Predicted curves were highly correlated to experimental data, leading to very significant determination coefficients (adj R^2), indicating that it was an appropriate mathematical model to represent survival data (Table 1).

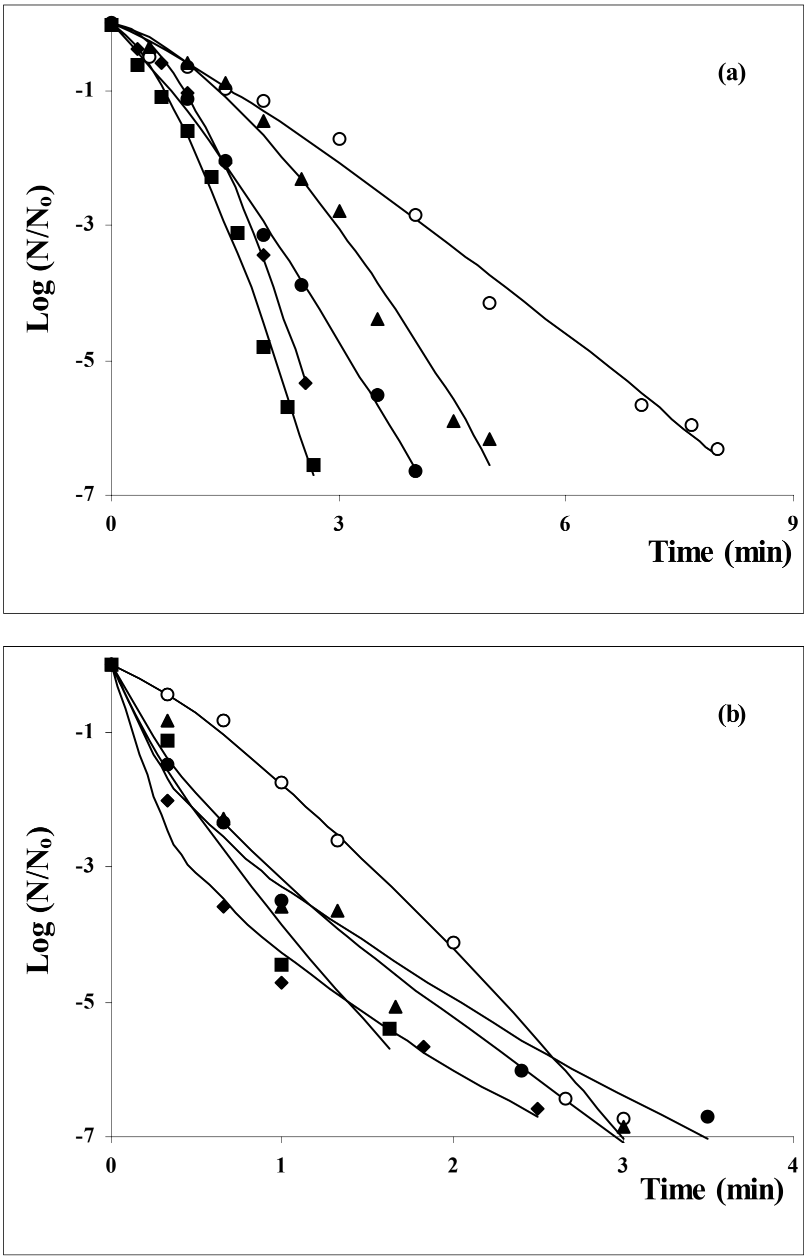

Figure 1. Semilogarithmic survival curves of *L. innocua* in orange juice (pH 3.5) fitted according Eq.1: predicted values (—); experimental data: control (○), 500 ppm vanillin (▲), 700 ppm vanillin (●), 900 ppm vanillin (◆), 1100 ppm vanillin (■). a) 57°C; b) 61°C.

Survival curves exhibited different shapes according to the severity of the heat treatment, which was reflected in the "n" value. When n>1 (lower temperatures), survival curves showed a pronounced downward concavity; systems with n values close to 1 were linear indicating first-order kinetics; and for systems with n<1 (higher temperatures), the semilogarithmic survival curve had a noticeable upward concavity. In all cases the concavity was more evident as vanillin concentration increased.

Table 1. *L. innocua* predicted survival curves constants and statistics obtained by the application of the cumulative form of the Weibull type distribution of resistances.

Temperature (°C)	*Vanillin (ppm)*	*b*	*n*	*adj R^2*
57	***0***	0.59	1.15	99.1
	500	0.59	1.49	98.3
	700	1.31	1.17	99.6
	900	1.06	1.72	99.7
	1100	1.66	1.43	98.9
59	***0***	0.93	1.15	98.1
	500	2.06	1.10	98.5
	700	2.02	1.04	97.9
	900	2.61	1.05	98.4
	1100	3.23	0.81	99.6
60	***0***	2.90	1.04	99.6
	500	3.46	0.96	98.9
	700	3.95	0.74	96.3
	900	3.65	0.85	99.2
	1100	3.91	0.66	98.7
61	***0***	1.77	1.26	99.3
	500	3.17	0.73	97.0
	700	3.29	0.6	98.5
	900	4.26	0.5	98.1
	1100	3.85	0.8	95.3

The significant deviations from log-linear kinetics, encountered under mild heating regimes, mean that the method incorporating *D* and *z* values cannot be relied upon to predict product safety. Similar results have been addressed by other authors (Stephens et al., 1994; Peleg and Cole, 1998).

Values b and n where used to plot the underlying frequency distribution of sensitivities, or resistances (Fig. 2) and to calculate the distribution associated statistical parameters (Table 2).

Frequency distributions clearly show the reduction in spread and the increase in frequency value as the concentration of vanillin and heating temperature increased. Indicate that most part of the population had the same resistance at a specific time under more severe conditions. It is also noticeable that all resistance distributions where strongly skewed to the right. This may be explained arguing that a comparatively small number of survivors were still viable after a considerable long time. As stressful conditions increased, the time

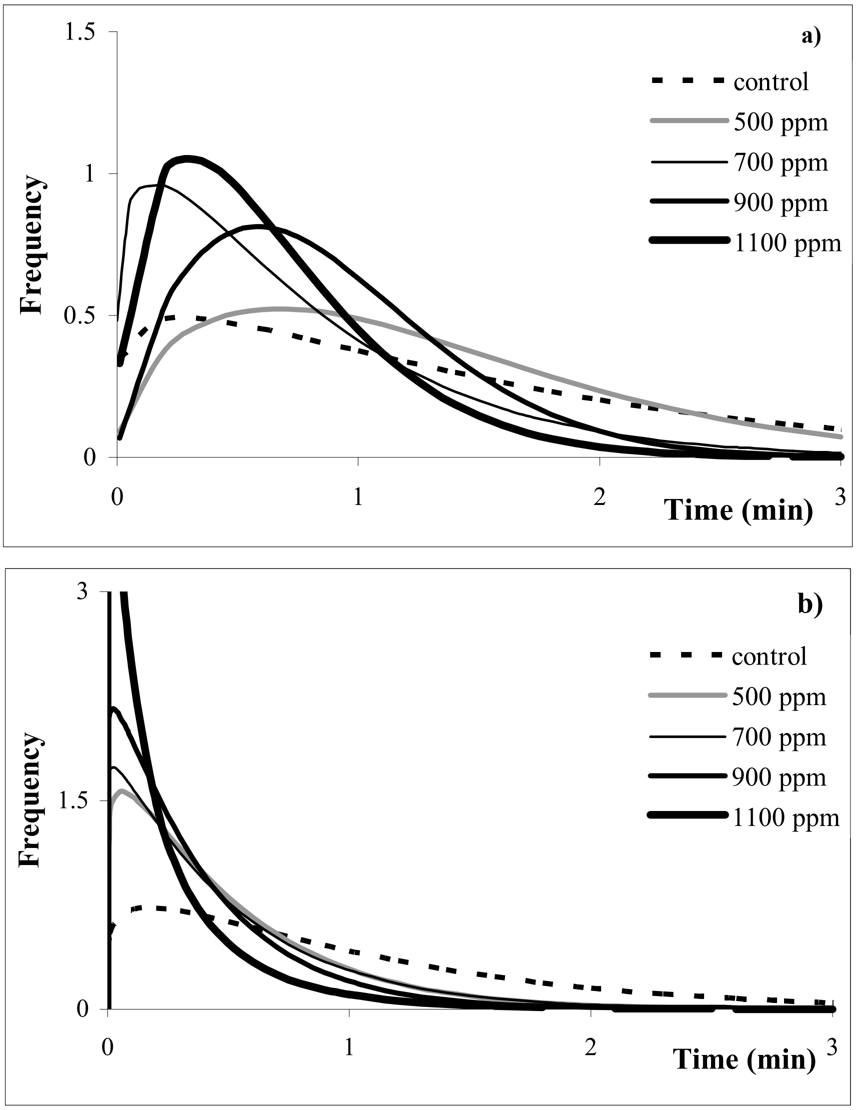

Figure 2. Frequency distribution of resistances of *L. innocua* in orange juice (pH 3.5) with different vanillin concentrations, fitted with the cumulative Weibull distribution function: a) 57°C; b) 59°C.

needed to destroy the cells diminished as indicated by the mode, excepting for the case of 700 ppm of vanillin at 57°C, further tests will be done in order to clarify this last behavior.

Plots of frequency distribution at 57ºC (Fig. 2a) show a wider overall spread of bacterial sensitivities (as indicated by the correspondent higher variance values) than plots at 59ºC (Fig 2b). The increase of heating temperature to 59ºC dramatically narrowed the frequency distribution and increased the skewness to the right. This effect was greater as vanillin concentration increased. For juice containing 1100 ppm of vanillin, the distribution did not appear to have a peak. Probably, most of *Listeria* population was destroyed within a very short time after being exposed to heat and a very small number of survivors were still viable after a considerably long time.

Table 2. Weibull type distribution statistical parameters corresponding to *L. innocua* as affected by thermical treatment in combination with different concentrations of vanillin.

Temperature (°C)	*Vanillin (ppm)*	*Mode (min)*	*Mean (min)*	*Coeff. of skewness*	*Variance (min^2)*
57	***0***	0.26	1.51	1.87	1.74
	500	0.68	1.28	1.54	0.77
	700	0.15	0.75	1.84	0.42
	900	0.58	0.86	1.43	0.27
	1100	0.30	0.64	1.59	0.21
59	***0***	0.18	1.02	1.87	0.80
	500	0.06	0.50	1.94	0.21
	700	0.02	0.50	2.04	0.23
	900	0.02	0.39	2.02	0.14
	1100	0.04	0.26	2.70	0.11
60	***0***	0.02	0.35	2.04	0.12
	500	0.01	0.28	2.22	0.08
	700	0.04	0.19	3.05	0.07
	900	0.03	0.24	2.53	0.08
	1100	0.05	0.17	3.65	0.07
61	***0***	0.18	0.59	1.74	0.22
	500	0.05	0.25	3.09	0.12
	700	0.07	0.21	4.20	0.13
	900	0.06	0.11	6.25	0.06
	1100	0.03	0.21	2.73	0.07

The same pattern could be more clearly observed by plotting the effect of different heating temperatures at a selected concentration of vanillin (Fig. 3). It can be seen that heating of juice with 900 ppm of vanillin at lower temperatures (57-59ºC) promptly affected the more sensitive cells but leaved a long tail of resistant ones. On the other hand, the variance dramatically diminished as temperature increased to 60 or 61ºC, reducing the exposure time needed to inactivate *L. innocua*.

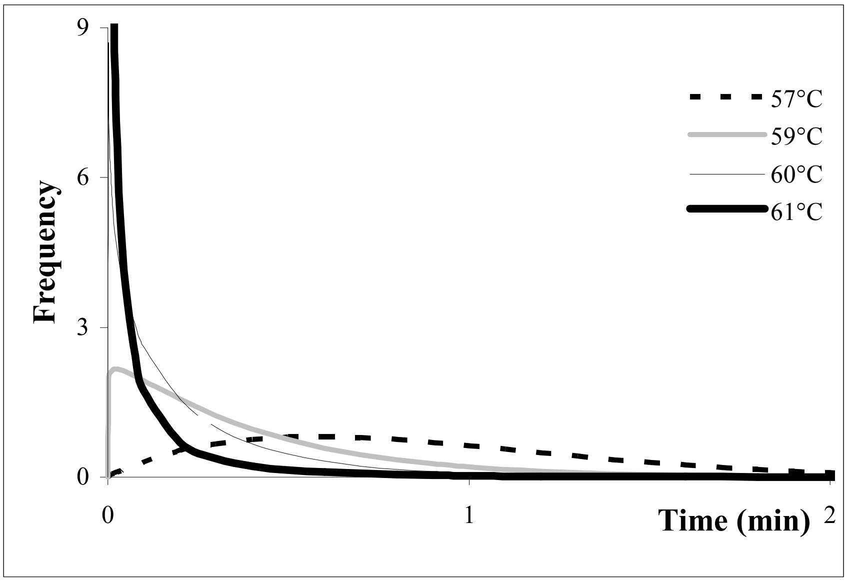

Figure 3. Frequency distribution of resistances of *L. innocua* in orange juice (pH 3.5) with 900 ppm vanillin at different heating temperatures fitted with the cumulative Weibull distribution function.

4. Conclusions

Vanillin at relatively low concentrations was highly effective enhancing thermal lethal effect on *L. innocua* in orange juice. The Weibull distribution of resistances model accurately fitted the inactivation curves resulting in a good description of the underlying phenomena. This model is a useful tool to select vanillin concentration-treatment temperature combination necessary to achieve the inactivation of most part of bacterial population, assuming the least reminding viable pathogens. Heating at 61°C for forty seconds where sufficient for *Listeria innocua* inactivation in orange juice with the addition of 900 ppm of vanillin.

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