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ORIGINAL PAPER

Mild Thermal Process Combined with Vanillin Plus Citral to Help Shorten the Inactivation Time for *Listeria innocua* in Orange Juice

Cielo D. Char · Sandra N. Guerrero · Stella M. Alzamora

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Abstract The response of *Listeria innocua* (surrogate for *Listeria monocytogenes*) to combined treatments involving moderate temperatures (57 to 61 °C) and the addition of different levels of citral (0 to 75 ppm) was assessed to obtain a minimally processed orange juice. The presence of citral notoriously increased the bactericidal effect of mild heating treatment. This effect did not depend on the amount of added citral at all assayed temperatures. In a second stage, combinations of two natural antimicrobials (vanillin and citral) were assessed in order to find the most effective inactivation treatment in orange juice. Vanillin (900–1,100 ppm) and citral (25 ppm) combined with mild heating treatment (52 or 57 °C) were tested against *L. innocua* in orange juice. The addition of 900 ppm vanillin and 25 ppm citral halved or more the time required to achieve five logarithmic cycles of reduction at both temperatures with respect to thermal treatment without antimicrobial addition. The increase in the maximum growth rate calculated from the modified Gompertz model properly correlated with the increasing vanillin level for a given citral concentration. Complementary information was obtained from successfully fitting a Weibullian model to the nonlinear semilogarithmic survival curves: The addition of vanillin and citral significantly increased the bactericidal effect of mild thermal treatment, changing the distribution of inactivation times and obtaining narrower frequency shapes with lower variance and mode values. The combination of vanillin and citral with mild heating treatment resulted in an innovative alternative to minimize detrimental effects caused by thermal processing of fruit juices. In addition, a consumer panel evaluated them with an acceptable overall pleasantness.

Keywords Orange juice · Vanillin · Citral · *Listeria innocua* · Gompertz · Weibull

Introduction

Fruit juices have been recognized to be targets of spoilage by yeasts, molds, and acid-tolerant bacteria (Fitzgerald et al. 2004). Visible deterioration of juice occurs through the production of lactic acid, ethanol, CO_2, diacetyl, and, eventually, mucilage, generating bubbles, opalescence, and/or buttery odor. These low pH high-acid products have traditionally been free of problems related to public health microorganisms. However, the number of documented outbreaks of human infections associated with consumption of raw fruits, vegetables, and unpasteurized fruit juices has recently increased (Beuchat and Ryu 1997). Unpasteurized fresh orange and apple juices and cider have been involved in major outbreaks caused by *Escherichia coli* O157:H7 and different serotypes of *Salmonella* (CDC 1999; Luedtke and Powel 2000; Parish 1998). It has been demonstrated that even though pathogens do not actively grow in fruit juices due to their low pH, they can survive and adapt to the acidic environment, increasing their tolerance to unfavorable growth conditions, thus extending their survivability (Mazzotta 2001).

Listeria monocytogenes, pathogen of ubiquitous nature, has been recovered from unpasteurized fruit juices (pH≅3.7;

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Sado et al. 1998; Suslick 1988). It is more heat-resistant than other non-spore-forming foodborne pathogens (i.e., *Salmonella* spp. or pathogenic *E. coli*). Therefore, processing recommendations based on data for other pathogens may not be severe enough to eliminate them (Baumann et al. 2005; Doyle et al. 2001). Besides, its potential damage increases because it is psychotropic, capable to grow at refrigeration temperatures on many raw or processed vegetables and fruits (Martinez et al. 2000).

Minimal preservation processes based on the combined factors technology have been successfully applied to preserve fruits like papaya, pineapple, mango, banana, chicozapote, apple, pear, and peach (Alzamora et al. 1995; Leúnda et al. 1999). This approach intelligently combines hurdles to improve the microbial stability and safety of foods as well as sensory and nutritive attributes (Leistner 2000).

Natural antimicrobials derived from plants are emerging as preserving factors which may be used in conjunction with conventional ones such as heating (Alzamora et al. 2003). These "green preservatives" accomplish to satisfy growing consumer demands for fresh products without chemical additives. Vanillin (4-hydroxy-3-methoxybenzaldehyde), the major constituent of vanilla beans, is a phenolic compound that not only has demonstrated to have antimicrobial and antioxidant properties but also impairs pleasant flavor notes to a wide variety of products such as confectionery, beverages, and pharmaceuticals (Perera and Owen 2007). It has been successfully used to inhibit bacteria, including *L. monocytogenes* (Ferrante et al. 2007), yeasts (Fitzgerald et al. 2004), and molds (López-Malo et al. 2000). Penney et al. (2004) found that the presence of 2,000 ppm vanillin resulted in suppression of initial microbial load (yeasts and bacterial cells) in fresh fruit yogurt containing wild blueberries over a 3-week period of storage at 4 °C. However, they determined that 1,000 ppm vanillin was effective in controlling growth, but it did not result in a significant spoilage reduction.

Citral (3,7-dimethyl-2,6-octadienal) is a terpenoid present in the oils of several plants, including lemongrass, verbena, lemon, and orange. This aroma compound is widely used in perfumery for its citrus notes and as a flavoring agent for foods and beverages. It has shown antifungal properties against *Penicillium digitatum*, *Penicillium italicum*, and *Aspergillus flavus* (Caccioni et al. 1995; López-Malo et al. 2000). Lanciotti et al. (2004) demonstrated that citrus essential oils from mandarin, lemon, and lime have increased the shelf life of minimally processed sliced fruit mixtures (apple, pear, grape, peach, and kiwifruit) packed under ordinary or modified atmosphere under temperature abuse conditions. They also verified that the addition of these substances increased the rate of death of *E. coli* inoculated at levels of 10^6 colony-forming unit (CFU) per gram.

The aim of this work was to investigate the response of *Listeria innocua* (as surrogate of *L. monocytogenes*) in orange juice as affected by certain combinations of vanillin, citral, and mild heating treatments. Inactivation kinetics was characterized by the application of the modified Gompertz model. Differences in microorganism resistance to the applied preservation factors were described through the use of a Weibullian model. Additionally, discriminative and affective sensory tests were performed.

Materials and Methods

Inoculum

L. innocua ATCC 33090 and *L. monocytogenes* ATCC 19114 inocula were prepared by transferring a loopful of a stock culture maintained in trypteine soy agar with 0.6% (*w*/*w*) yeast extract (TSA-YE) slants to a 20-mL Erlenmeyer flask of trypteine soy broth with 0.6% (*w*/*w*) yeast extract (TSB-YE). Cultures were incubated 24 h with agitation at 37 °C, obtaining inocula of ≅5×10^7 CFU/mL. For the most severe treatment conditions, higher initial levels of inoculum were required. Thus, 100 mL of TSB-YE was incubated and centrifuged at 2,000×*g* for 20 min, and the pellet suspended in a proper volume of TSB-YE in order to obtain inocula of 9×10^8 CFU/mL. All microbiological media were purchased from Laboratorios Britania (Laboratorios Britania, Buenos Aires, Argentina).

Orange Juice Preparation

Orange juice (pH 3.5±0.1) was prepared from a concentrate (65 °Brix) without additives (ECA Agroindustrias S.A, Concordia, Argentina) by adding sterile water (water/juice=6:1). It was pasteurized at 72 °C for 14 s using a Microthermics UHT/HTST Lab-25DH unit (Microthermics, Raleigh, USA) and collected in a biological safety cabinet (Nuaire, Plymouth, USA). The pasteurized juice was dispensed into 100-mL flasks and stored at −18 °C for a maximum of 2 months until it was used for experimentation.

Verification of *L. innocua* as a Surrogate of *L. monocytogenes*

Suitable use of *L. innocua* as surrogate for *L. monocytogenes* was assayed by comparing its survivability in thermally treated orange juice (52 and 57 °C) without any additive (control) or added with 900 ppm vanillin plus 25 ppm citral, following exactly the same procedure as described above.

Combination of Citral and Mild Thermal Treatment

Thermal inactivation kinetics for *L. innocua* in orange juice supplemented or not with citral was carried out in a batch type reactor at constant temperature. It consisted of a 150-mL double-wall cylindrical vessel (diameter, 6.3 cm; height, 7.6 cm) connected to a thermostatically controlled water bath (HAAKE Mess-Technik, Karlsruhe, Germany) whose temperature was fixed to attain 57, 59, or 61±0.2 °C in the samples. Ninety-nine milliliters of juice was poured into the vessel and continuously stirred by using a magnetic shaker (Decalab SRL, Buenos Aires, Argentina). In the preliminary tests, citral (Firmenich SAICYF, Buenos Aires, Argentina) aqueous solution (5%, *w*/*w*) was added to each system in order to obtain 0, 25, 50, and 75 ppm final concentrations of citral. Once the desired temperature was reached and stabilized into the vessel, the orange juice sample was inoculated with 1 mL of *L. innocua* culture. Initial counts of $\cong 5\times10^6$ CFU/mL were obtained for regular inocula and $\cong 1\times10^8$ CFU/mL for concentrated inocula. During thermal treatment, 1-mL samples were withdrawn at preset intervals (every 20 or 30 s depending on the severity of the combined treatment), poured into 9-mL peptone water tubes, cooled in iced water, and immediately analyzed for survivors. Two plates were used for each dilution. When treatment resulted in low counts, up to 2-mL treated juice sample was directly poured and plated using four plates as replicates. Temperature was monitored throughout the experiment with a thermometer (±0.2 °C sensitivity). Juice was continuously stirred using a magnetic shaker. Agitation was vigorous enough to maintain homogeneous conditions of temperature and inoculum concentration within the whole vessel.

Combination of Vanillin, Citral, and Mild Thermal Treatment

Some combinations of both antimicrobials and thermal treatment were chosen (Table 1) and assessed for *L. innocua* inactivation following the same procedures as in previous tests. Vanillin levels were selected according to the results previously obtained by the same laboratory (Char et al. 2008).

Enumeration of Surviving Cells

Survival cells were enumerated by surface plate count and the most probable number techniques (for lower counts) using TSA-YE or TSB-YE plus 1% (*w*/*w*) sodium pyruvate (Laboratorios Britania, Buenos Aires, Argentina) in order to recover injured cells. Plates or tubes were incubated at 30 °C for 5 days. Each condition was assayed in triplicate and the average was reported.

Table 1 Experimental conditions for thermally treated orange juice systems with or without citral and/or vanillin addition

Temperature (°C)	Vanillin (ppm)	Citral (ppm)
52	0	0
	900	25
	1,000	25
57	0	0
	0	25
	0	50
	0	75
	900	25
	1,000	25
59	0	0
	0	25
	0	50
	0	75
61	0	0
	0	25
	0	50
	0	75

Modified Gompertz Model

Survival curves were obtained by plotting the logarithmic surviving fraction (log N_t/N_0, where N is the number of CFU/mL at a given time and N_0 the initial number of CFU/mL) versus time of treatment. Curves were modeled with the modified version of the general Gompertz equation for microbial survival described by Linton et al. (1995):

$$\mathrm{Log}\left(Nt/N_0\right) = Ce^{-e(A+Bt)} - Ce^{-e(A)} \quad (1)$$

where parameters A, B, and C represent the different regions in the survival curve [initial shoulder (A), maximum death rate (B), and the overall change in number of survivors (C)]. Parameter estimates associated with regression coefficients were derived from the nonlinear regression procedure.

Weibull Distribution of Resistances Model

An alternative description of *L. innocua* survival curves was performed by fitting the cumulative form of the Weibull-type distribution of resistances model (Peleg and Cole 1998):

$$S(t) = \mathrm{Log}\left(\frac{N_t}{N_0}\right) = -b \times t^n \quad (2)$$

where b and n are constants. They were derived using a nonlinear regression technique. The values of b and n were

then used to generate the resistance frequency curves using the following equation:

$$\frac{d\phi}{dt_c} = bnt_c^{n-1}\exp\left(-bt_c^n\right) \quad (3)$$

where t_c is a measure of the organism's resistance or sensitivity and $\frac{d\phi}{dt_c}$ is the Weibull distribution corresponding to t_c. Other statistical parameters which better explain the observed frequencies (distribution mode, t_{cm}; mean, $\bar{t}_c$; variance, $\sigma^2_{t_c}$; and coefficient of "skewness", υ_1) were calculated from the following equations (Peleg and Cole 1998):

$$t_{cm} = [(n-1)/nb]^{1/n} \quad (4)$$

$$\bar{t}_c = \{\Gamma[(n+1)/n]\}/b^{1/n} \quad (5)$$

$$\sigma^2_{t_c} = \left\{\Gamma[(n+2)/n] - (\Gamma[(n+1)/n])^2\right\}\Big/ b^{2/n} \quad (6)$$

$$\upsilon_1 = \frac{\left[\Gamma(n+3/n)/b^{3/n}\right]}{\left[\Gamma(n+2/n)/b^{2/n}\right]^{3/2}]} \quad (7)$$

where Γ is the gamma function. The distribution mode, t_{cm}, represents the treatment time at which the majority of population dies or inactivates. The mean, $\bar{t}_c$, corresponds to the inactivation time on average with its variance, $\sigma^2_{t_c}$. The coefficient of skewness, υ_1, represents the skew of the distribution.

Internal Validation

Internal validation was carried out to determine if the model could adequately describe the experimental data. Models were internally validated by means of analysis of variance test, the adjusted coefficient of determination $\left(R^2_{adj}\right)$, and Fisher's test (Alzamora et al. 2004). All regression analyses were applied using Statgraphics Plus for Windows 3.0® (StatPoint, Herndon, USA).

Sensory Evaluation

For sensory tests, the testing area followed the good practice guidelines recommended by Lawless and Heymann (1999). In order to determine whether a sensory difference existed between both proposed combined treatments emerging from the microbiological study, a triangle test was performed using 42 subjects. Three coded samples were presented to the panelists who were asked to select the odd sample. The number of correct replies was counted and referred to a table for critical number of correct answers extracted from Lawless and Heymann (1999).

Overall pleasantness of orange juice added with 900 ppm vanillin and 25 ppm citral and thermally treated at 52 °C for 4.7 min was assessed by 85 untrained volunteers, ranging from 22 to 45 years. Each judge evaluated the same sample presented at consume temperature (8–10 °C) in a white opaque cup but with a different code. Subjects were instructed to judge the pleasantness of the sample on a balanced nine-point hedonic scale (1: dislike extremely, 9: like extremely) followed by two open-ended questions for reasons of liking or disliking as recommended for consumer field tests (Lawless and Heymann 1999). Data obtained from panelists were analyzed by converting assigned positions to numbers. Results were reported as average of individual values.

Results and Discussion

Use of *L. innocua* as Surrogate of *L. monocytogenes*

L. innocua or *L. monocytogenes* survival curves in thermally treated (52 or 57 °C) orange juice without any additive (control) and with 900 ppm vanillin plus 25 ppm citral are presented in Fig. 1. Both combined methods were more effective in inactivating *L. monocytogenes* than *L. innocua*. Time to reach five log cycle reductions of *L. innocua* ranged from 1.5 to 0.5 times greater than the time to reach five log reductions required for *L. monocytogenes* at 52 and 57 °C, respectively. Time difference between strains was even larger in the control system. Therefore, *L. innocua* could be properly used as surrogate of *L. monocytogenes*.

Combination of Citral and Mild Thermal Treatment

A preliminary study to evaluate the survival of *L. innocua* in orange juice as affected by a combination of single citral addition (0, 25, 50, or 75 ppm) and mild heating treatment (57, 59, or 61 °C) was conducted. The range of citral concentrations was selected based on previous sensory results, which showed that the addition of 100 ppm citral or more to the orange juice was unacceptable to the senses (Ferrante et al. 2007; Char 2006). Addition of citral to the orange juice increased *L. innocua* inactivation at all heating temperatures, as shown by the difference between the control curve (without citral addition) and systems containing different concentrations of citral (Fig. 2). The addition of 25 ppm of citral reduced inactivation times needed to reach a five log cycle reduction of *L. innocua* from 6.2 to 3.8 min at 57 °C, from 3.8 to 2.8 min at 59 °C, and from 2.3 to 1.0 min at 61 °C. On the other hand, the increase of

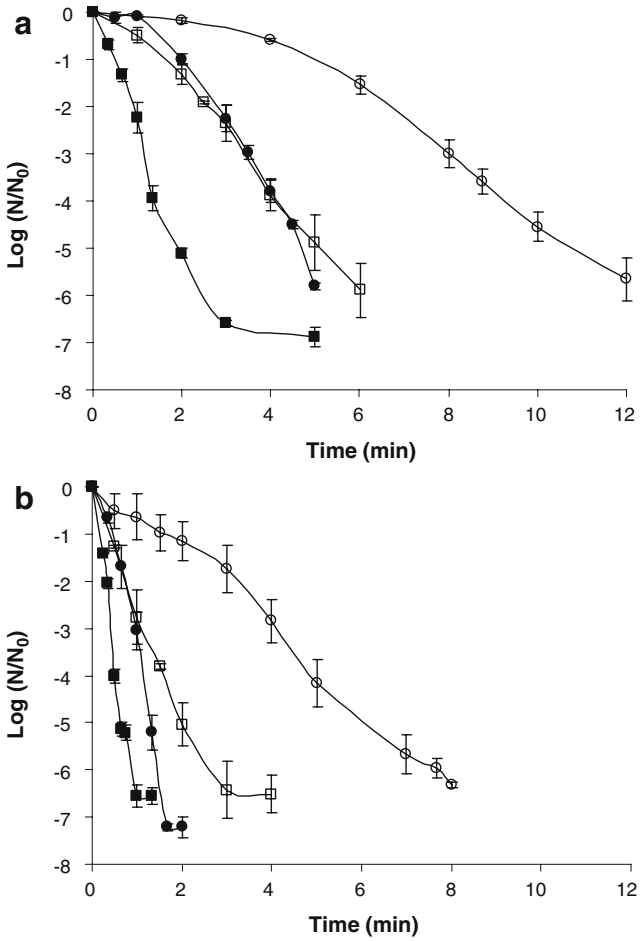

Fig. 1 Comparison of inactivation curves corresponding to *L. innocua* and *L. monocytogenes* in thermally treated orange juice supplemented or not with vanillin (*V*) and citral (*C*): (*open circle*) *L. innocua*, control; (*filled circle*) *L. innocua*, with 900 ppm V + 25 ppm C; (*open square*) *L. monocytogenes*, control; (*filled square*) *L. monocytogenes*, with 900 ppm V + 25 ppm C. **a** 52 °C; **b** 57 °C

citral concentration in the orange juice within the proposed range introduced a scarce difference on the response [$\log(N/N_o)$]. It was concluded that the addition of 25 ppm citral was useful in increasing mild thermosterilization. Higher citral concentrations within the evaluated range did not render an additional inactivation effect.

Combination of Vanillin, Citral, and Mild Thermal Treatment

Levels of vanillin concentration used in this study were chosen according to a previous work (Char et al. 2008) where *L. innocua* inactivation was assessed in orange juice added with 0, 500, 700, 900, or 1,100 ppm of vanillin combined with heat treatment at 57, 59, or 61 °C in a batch system. Char et al. (2008) observed that vanillin addition accelerated inactivation kinetics, diminishing the process time required to reach *L. innocua* inactivation at a given heating temperature. This effect considerably depended on the amount of vanillin when working at lower temperatures (57 and 59 °C) where higher vanillin concentrations increased observed inactivation. On the other hand, at the higher heating temperature (61 °C), vanillin addition was still

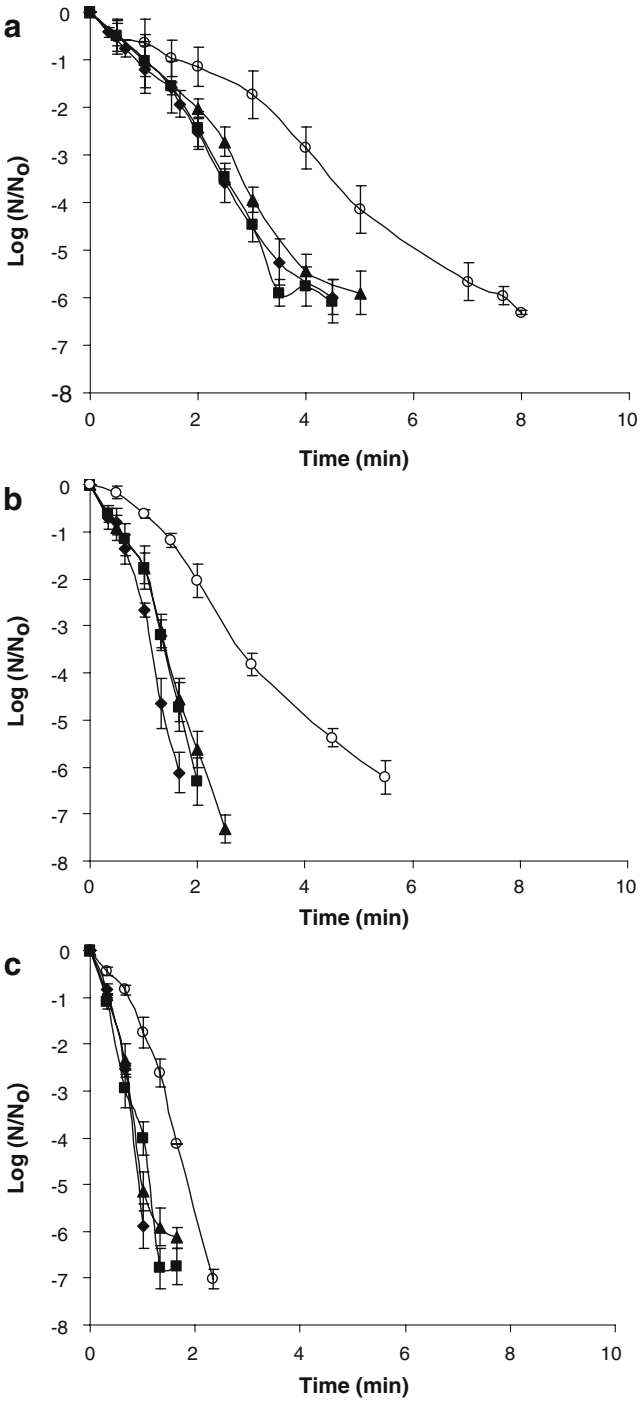

Fig. 2 Survival curves of *L. innocua* in heat-treated orange juice supplemented or not with citral: 0 ppm citral (*circle*); 25 ppm citral (*triangle*); 50 ppm citral (*square*); 75 ppm citral (*diamond*). **a** 57 °C; **b** 59 °C; **c** 61 °C

advantageous, but the microorganism inactivation was mostly due to thermal effect, as this temperature was closer to traditional pasteurization processes, rendering poorer sensory and nutritional quality. Delaquis et al. (2005) determined the minimal inhibitory concentration (MIC) of vanillin for several species of *Listeria* in laboratory media at various pH levels. Reported *L. monocytogenes* and *L. innocua* MICs at pH 5.0 were 20 and 23 mM (3,000 and 3,500 ppm), respectively, suggesting that the former was slightly more sensitive. This previous study demonstrated one way of increasing *L. innocua* sensitivity with a combined treatment using even lower vanillin concentrations.

In this work, alternative treatments combining vanillin and citral were assessed with the purpose of enhancing the efficiency of single antimicrobial mild heating treatment. One of the selected heating temperatures was the minimum temperature evaluated in the previous test (57 °C) in order to minimize modifications of sensory quality. A lower temperature (52 °C) was tested as well with the objective of proposing an equivalent combined treatment that would markedly diminish undesirable sensory changes derived from thermal treatments. Vanillin and citral combination levels (900 or 1,100 ppm vanillin + 25 ppm citral) were chosen according to the criteria of using lower concentrations, which demonstrated to be individually effective on the inactivation of *L. innocua* when combined with slight heating treatment, minimizing variations in flavor and taste of orange juice (Char et al. 2008).

Survival curves of *L. innocua* (Fig. 3) showed that there were certain vanillin and citral combinations that halved the treatment time to achieve five logarithmic cycles reduction with respect to thermal treatment without antimicrobial addition (control systems), accomplishing the requirements of the US Food and Drug Administration (FDA 2004). The combination of 900 ppm vanillin and 25 ppm citral reduced time from 10.8 to 5.7 min at 52 °C and from 6 to 1.5 min at 57 °C to achieve that requirement. It was also observed that an increase in vanillin concentration (1,000 ppm) in orange juice added with 25 ppm citral reduced this time by 1 min at 52 °C and 0.5 min at 57 °C (Fig. 3).

Since most inactivation curves of *L. innocua* in orange juice were notoriously deviated from linearity, kinetic data were quantified by means of a nonlinear model like the modified Gompertz equation. In thermal studies, this model is likely to provide a more accurate estimation of a microorganism's resistance than the first-order model (Bhaduri et al. 1991). Statistical validation performed using the F values (Table 2) indicated that observed values were not significantly different ($\alpha<0.001$) from those predicted by the modified Gompertz model. The values of adjusted determination coefficients R^2_{adj} which represent the fraction (%) of variation that is explained by the model were also high (between 98.0% and 99.9%).

By analyzing Gompertz parameters (Table 2), it can be concluded that maximum growth rate (B) increased with vanillin concentration for a given citral level. This effect was more notorious as heating temperature increased (57 °C) and reflects a decrease in microorganism resistance to the combined treatment. Unclear trend was obtained for the A parameter (shoulder region of survival curve). Parameter C values (associated with the overall change in survivors at the end of treatment) exceeded six log cycle reductions for tested conditions. This fact was predictable because most curves did not show a noticeable tailing region. The modified Gompertz model was more appropriate to describe survival curves in such situations where less severe combinations of stress factors were used (absence of vanillin and citral or lower heating temperatures) with approximately sigmoid curves. When these conditions became more stressing, survival curves became closer to linearity with slight or no tailing region, diminishing the accuracy of the model.

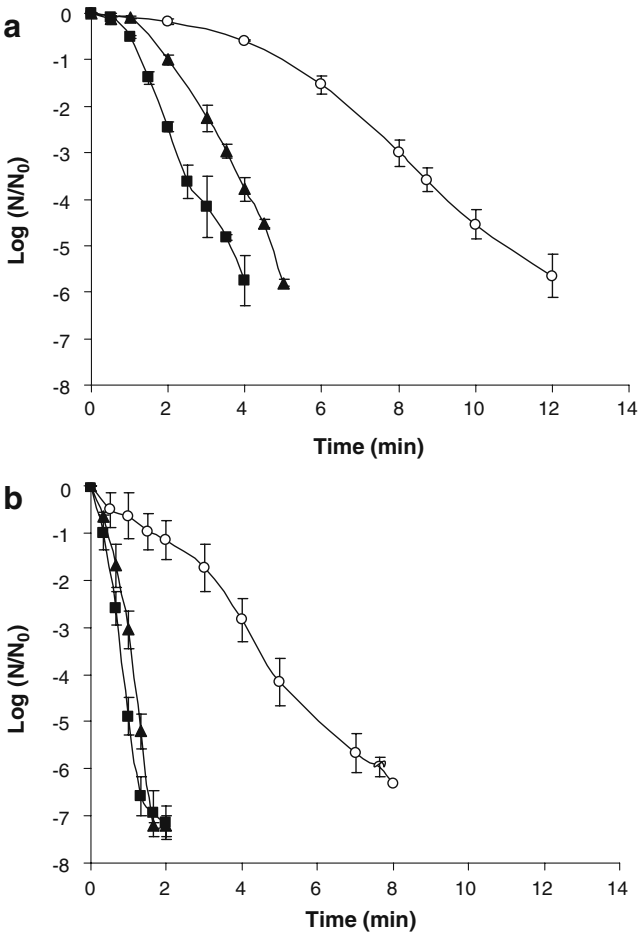

Fig. 3 Survival curves of *L. innocua* in orange juice supplemented or not with combinations of vanillin (V), citral (C), and mild heating treatment. Control (*circle*); 900 ppm V + 25 ppm C (*triangle*); 1100 ppm V + 25 ppm C (*square*). **a** 52 °C; **b** 57 °C

Table 2 Estimated Gompertz parameters (initial shoulder, *A*; maximum death rate, *B*; and global change in survivors number, *C*) and their standard errors (SE) corresponding to *L. innocua* survival in thermally treated orange juice supplemented with vanillin and citral

Temperature (°C)	Vanillin (ppm)	Citral (ppm)	Gompertz parameter						Variability explained % $\left(R^2_{adj}\right)$	Fisher
			A (min)	SE	*B* (min^{-1})	SE	*C*	SE		
52	0	0	1.97	0.07	−0.24	0.02	−8.4	0.42	99.9	7,044[a]
	900	25	1.65	0.10	−0.32	0.09	−16.6	6.65	99.6	1,553[a]
	1,100	25	1.72	0.17	−0.85	0.12	−6.8	0.54	99.4	1,174[a]
57	0	0	1.06	0.19	−0.25	0.07	−10.1	2.28	99.3	1,163[a]
	900	25	1.69	0.37	−1.74	0.50	−8.9	1.40	98.0	262[a]
	1,100	25	1.65	0.21	−2.52	0.32	−7.6	0.31	99.4	1,095[a]

[a] Significant at 0.01% level

The effect of combined treatments was also analyzed from a different approach by means of the Weibull distribution of resistances model. This model considers that the entire microbial population is not equally resistant to the proposed treatment and so each cell is not destroyed at the same time during processing. As a result, the survival curve is the cumulative form of a temporal distribution of lethal events where each individual organism is inactivated at a specific time, thus generating a spectrum of heat resistances (Peleg and Cole 2000).

The Weibullian model accurately fitted experimental data as shown by adjusted R^2 values (between 97.0% and 99.8%) and Fisher values which were highly significant ($\alpha<0.0001$; Table 3). Most treatments presented $n>1$ values related with an observed downward concavity of the survival curve. *L. innocua* inactivation response to the combined treatment was better described by the frequency distribution of resistance plots (Fig. 4) generated from *b* and *n* values. These parameters were also used to calculate the associated statistics: mode, mean, variance, and coefficient of skewness according to Eqs. 4, 5, 6, and 7. Frequency distributions of resistances showed that *L. innocua* inactivation in heat-treated orange juice without antimicrobial addition rendered a wide dispersion of frequencies with a broad range of inactivation times and had high mode, mean, and variance values, especially at the lowest temperature (52 °C). This behavior would indicate that the members of the population were inactivated at very different treating times, leaving a large proportion of resistant microorganisms, which was evidenced by a wide tail on the distribution (Schenk et al. 2008). Increasing the heating temperature to 57 °C produced a reduction in frequency spread with lower associated statistics. The distribution was more skewed to the right, but still kept it very wide (Fig. 4 and Table 3). Even if the highest temperature rapidly inactivated most part of the population, it left behind a fraction of more resistant survivors. Therefore, applied thermal treatment (52 or 57 °C) used as a single stress factor was not enough to reach the desired inactivation effect at short times.

The use of vanillin and citral in combination was very useful, since narrower distributions were obtained with lesser variance, mode, and mean values than those corresponding to only heat-treated orange juice. This

Table 3 Weibull-type distribution parameters *b* and *n*, standard errors (SE) and related statistics corresponding to *L. innocua* survival in thermally treated orange juice supplemented with vanillin and citral

Temperature (°C)	Vanillin (ppm)	Citral (ppm)	*b* (min^{-n})	SE	*n*	SE	Variability explained % $\left(R^2_{adj}\right)$	Fisher	Mode (min)	Mean (min)	Skewness	Variance (min^2)
52	0	0	0.08	0.02	1.76	0.12	98.9	772[a]	2.70	3.89	1.41	5.22
	900	25	0.27	0.03	1.89	0.07	99.7	3,569[a]	1.34	1.77	1.36	0.94
	1,100	25	0.91	0.12	1.35	0.11	98.2	574[a]	0.39	0.98	1.65	0.54
57	0	0	0.59	0.06	1.15	0.06	99.1	1,412[a]	0.26	1.51	1.87	1.74
	900	25	3.22	0.08	1.59	0.06	99.8	2,977[a]	0.26	0.43	1.49	0.08
	1,100	25	4.45	0.28	1.03	0.15	97.0	213[a]	–	0.23	2.06	0.05

[a] Significant at 0.01% level

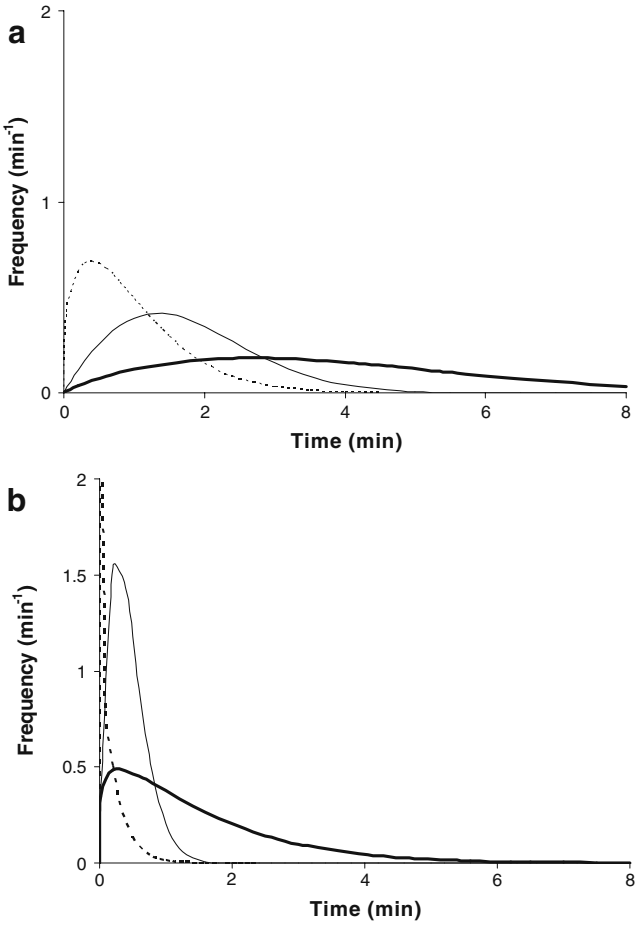

Fig. 4 Weibullian frequency distribution of resistances corresponding to survival curves of *L. innocua* in heat-treated orange juice supplemented or not with vanillin (*V*) and citral (*C*): (*thick line*) control; (*thin line*) 900 ppm V + 25 ppm C; (*dashed line*) 1,100 ppm V + 25 ppm C. **a** 52 °C; **b** 57 °C

effect was enhanced by an increase in vanillin concentration and/or heating temperature. Such is the case of orange juice with 1,100 ppm vanillin and 25 ppm citral heat-treated at 57 °C which presented a distribution without peak, indicating that the biggest proportion of the population was destroyed in a very short time, while a very little proportion with higher resistance remained viable.

It is worthy to note that thermally treated orange juice with only vanillin or citral addition at the same concentrations as used in this study generated frequency distributions with wider data spread and higher mean, mode, and variance values than those obtained in this study (data not shown). This fact remarks the benefits of the use of ternary combined treatments (Char et al. 2008).

Moon et al. (2006) determined the effectiveness of higher levels of vanillin (40 or 80 mM) used as a single stress factor on *L. monocytogenes* and *E. coli* O157:H7 inoculated in apple juice at two pH values (3.4 or 4.0). They observed that no viable cells of *L. monocytogenes* were recovered from juice supplemented with 40 mM vanillin after 3 days of incubation at 4 or 15 °C, while it persisted up to 13 days in the same juice without vanillin addition. *E. coli* O157:H7 resulted slightly more sensitive to vanillin effect, declining in 2 days. This shows that vanillin not only diminishes the severity required to the thermal process but also produces a long-term effect preventing microbial growth of any cell that might have survived while stored by refrigeration.

Phenolic compounds damage the structure and functionality of cell membrane proteins, altering cell permeability (Ultee et al. 1999). It also has been suggested that terpenes cause cell membrane disruption in molds and bacteria (Cox et al. 2000). This would explain the enhanced simultaneous effect of these active agents against membrane. The combined vanillin and citral effect was even more accentuated by thermal treatment. Lanciotti et al. (2004) proposed that the biological activity of most essential oils depends in first instance on the partition in cell membrane attributing the main role on toxicity to vapor pressure, which can be considered an indirect measure of hydrophobicity. Therefore, any factor capable of increasing vapor pressure of these substances may enhance antimicrobial activity, increasing solubility in cell membranes. Such is the case of an increment in heating temperature that favors the tendency of molecules to change to gaseous phase, consequently enhancing antimicrobial effect. Once these antimicrobials trespass cell membrane, they are able to interact with membrane enzymes altering their structure with functionality loss, which may cause reverse proton flux altering cell activity. Due to their hydrophobicity, these antimicrobials could accumulate in cell lipidic bilayer, altering membrane function, provoking disruption, and finally conducting to macromolecular loss from the inside, facilitating the entry of more antimicrobial from the cell outside (Ramos-Nino et al. 1996).

Selection of Proposed Treatments

For the selection of the suitable vanillin and citral combination, besides the antimicrobial efficiency, the modification of orange juice sensory properties was considered. For that reason, a preliminary sensory test was conducted in the laboratory where all acceptable antimicrobial combinations were tested (data not shown). Combinations that produced strong orange juice flavor modification were dismissed, such as the use of 1,100 ppm vanillin + 25 ppm citral combination.

Treatment times were calculated from the respective *L. innocua* survival curves in orange juice for an observed reduction of five logarithmic cycles in the population.

According to these results, a combination of stress factors at two different temperatures and times were chosen as proposed treatments:

Treatment A: orange juice with 900 ppm vanillin + 25 ppm citral, thermally treated at 52 °C for 4.7 min;
Treatment B: orange juice with 900 ppm vanillin + 25 ppm citral, thermally treated at 57 °C for 1.3 min.

Sensory Evaluation

The proposed combined treatments A and B were sensory-evaluated in order to establish overall differences. Results obtained for the discriminative test established that 14 subjects correctly identified the odd sample. According to the triangle test table, the minimal number of correct answers for 42 panelists is 20 (Lawless and Heymann 1999); thus, it was concluded that the two combined treatments A and B did not overall differ at the 5% level of significance, and any of them could be selected.

Treatment A was selected to assess consumer acceptance. The affective test showed that these concentrations of vanillin and citral introduced small sensory changes to orange juice. Juice added with citral and vanillin and thermally treated at 52 °C obtained an acceptable overall pleasantness with a mean rating around 6 (corresponding to "like slightly" category). Comments made by panelists through the open-ended questions remarked that the addition of vanillin and citral imparted pleasant flavor notes to the orange juice, but were also unfamiliar to consumers.

Conclusions

The use of plant-derived antimicrobials to inhibit microorganisms is limited due to high MICs in current foods altering flavor and/or aroma. Combination of sub-MIC levels of vanillin and citral with mild heating treatment showed to be a good alternative in minimizing undesirable thermal effects as well as increasing safety of minimally processed fruits. The Weibull-type distribution model evidenced differences between treatments that did not surge by applying a kinetic approach like the modified Gompertz model. This analysis allowed a better understanding of inactivation kinetics and was very helpful in choosing a more appropriate treatment to assess product safety. Orange juice added with 25 ppm citral and 900 ppm vanillin and thermally treated (52 °C) was acceptable to the taste panel. Extensive further work on applications of these combined treatments taking into account other strains would be required before making these results more generally extended.

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References

Alzamora, S. M., Cerrutti, P., Guerrero, S., & López Malo, A. (1995). Minimally processed fruits by combined methods. In G. Barbosa-Cánovas & J. Welti (Eds.), *Food preservation by moisture control. Fundamentals and applications* (pp. 463–491). Lancaster, PA: Technomic Publishing.

Alzamora, S. M., Guerrero, S., Viollaz, P., & Welti, J. (2004). Experimental protocols for modeling the response of microbial populations exposed to emerging technologies: Some points of concern. In G. V. Barbosa-Cánovas, M. Tapia, & P. Cano (Eds.), *Novel food processing technologies* (pp. 591–608). New York: CRC.

Alzamora, S. M., López-Malo, A., Guerrero, S., & Palou, E. (2003). Plant antimicrobials combined with conventional preservatives for fruit products. In S. Roller (Ed.), *Natural antimicrobials for the minimal processing of foods* (pp. 235–249). London: Woodhead Publishing.

Baumann, A. R., Martin, S. E., & Feng, H. (2005). Power ultrasound treatment of *Listeria monocytogenes* in apple cider. *Journal of Food Protection*, *68*, 2333–2340.

Beuchat, L. R., & Ryu, J. H. (1997). Produce handling and processing practices. *Emerging Infectious Diseases*, *3*, 459–465.

Bhaduri, S., Smith, P. W., Palumbo, S. A., Turner-Jones, C. O., Smith, J. L., Marner, B. S., et al. (1991). Thermal destruction of *Listeria monocytogenes* in liver sausage slurry. *Food Microbiology*, *8*, 75–78. doi:10.1016/0740-0020(91)90019-X.

Caccioni, D. R. L., Deans, S. G., & Ruberto, G. (1995). Inhibitory effect of citrus fruits essential oil components on *Penicillium italicum* and *Penicillium digitatum*. *Petria*, *5*, 177–182.

Centers for Disease Control and Prevention (CDC) (1999). Outbreak of *Salmonella* serotype Muenchen infections associated with unpasteurized orange juice. United States and Canada, June 1999. *Morbidity and Mortality Weekly Report*, *48*, 582–585. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4827a2.htm.

Char, C. (2006). Procesamiento mínimo de jugo de naranja mediante tratamiento térmico combinado con otros factores de estrés. PhD thesis, University of Buenos Aires.

Char, C., Guerrero, S., & Alzamora, S. M. (2008). Survival of *Listeria innocua* in thermally processed orange juice as affected by vanillin addition. *Food Control*, *20*, 67–74. doi:10.1016/j.foodcont.2008.02.004.

Cox, S. D., Mann, C. M., & Markham, J. L. (2000). The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology*, *88*, 170–175. doi:10.1046/j.1365-2672.2000.00943.x.

Delaquis, P., Stanich, K., & Toivonen, P. (2005). Effect of pH on the inhibition of *Listeria* spp. by vanillin and vanillic acid. *Journal of Food Protection*, *68*, 1472–1476.

Doyle, M. E., Mazzotta, A. S., Wang, T., Wiseman, D. W., & Scott, V. N. (2001). Heat resistance of *Listeria monocytogenes*. *Journal of Food Protection*, *64*, 410–429.

Ferrante, S., Guerrero, S., & Alzamora, S. M. (2007). Combined use of ultrasound and natural antimicrobials to inactivate *Listeria monocytogenes* in orange juice. *Journal of Food Protection*, *70*, 1850–1857.

Fitzgerald, D. J., Stratford, M., Gasson, M. J., & Narbad, A. (2004). The potential application of vanillin in preventing yeast spoilage

of soft drinks and fruit juices. *Journal of Food Protection*, *67*, 391–395.

Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M. E., & Gardini, F. (2004). Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. *Trends in Food Science & Technology*, *15*, 201–208. doi:10.1016/j.tifs.2003.10.004.

Lawless, H. T., & Heymann, H. (1999). Sensory evaluation of food. In H. T. Lawless, & H. Heymann (Eds.), *Principles and practices*. Maryland: Aspen Publishers.

Leistner, L. (2000). Hurdle technology in the design of minimally processed foods. In S. M. Alzamora, M. S. Tapia, & A. López-Malo (Eds.), *Minimally processed fruits and vegetables: Fundamental aspects and applications* (pp. 13–27). Maryland: Aspen Publishers.

Leúnda, A., Guerrero, S., & Alzamora, S. M. (1999). Efecto de la impregnación con vainillina en frutas mínimamente procesadas. In *Anales del VIII Congreso Argentino de Ciencia y Tecnología de Alimentos*, Santa Fe, Argentina, Asociación Argentina de Tecnólogos Alimentarios.

Linton, R. H., Carter, W. H., Pierson, M. D., & Hackney, C. R. (1995). Use of a modified Gompertz equation to model nonlinear survival curves for *Listeria monocytogenes* Scott A. *Journal of Food Protection*, *58*, 946–954.

López-Malo, A., Alzamora, S. M., & Guerrero, S. N. (2000). Natural antimicrobials from plants. In S. M. Alzamora, M. S. Tapia, & A. López-Malo (Eds.), *Minimally processed fruits and vegetables: Fundamental aspects and applications* (pp. 237–263). Maryland: Aspen.

Luedtke, A., & Powel, D. (2000). Fact sheet: A timeline of fresh juice outbreaks. Food safety risk management and communications project, International Food Safety Network. Available at: <http://www.foodsafetynetwork.ca/en/article-details.php?a=2&c=6&sc=37&id=427>.

Martinez, A., Díaz, R. V., & Tapia, M. S. (2000). Microbial ecology of spoilage and pathogenic flora associated to fruits and vegetables. In S. M. Alzamora, M. S. Tapia, & A. López-Malo (Eds.), *Minimally processed fruits and vegetables: Fundamental aspects and applications* (pp. 43–62). Maryland: Aspen.

Mazzotta, A. S. (2001). Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. *Journal of Food Protection*, *64*, 315–320.

Moon, K. D., Delaquis, P., Toivonen, P., & Stanich, K. (2006). Effect of vanillin on the fate of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in a model apple juice medium and in apple juice. *Food Microbiology*, *23*, 169–174. doi:10.1016/j.fm.2005.02.005.

Parish, M. E. (1998). Coliforms, *Escherichia coli* and *Salmonella* serovars associated with a citrus-processing facility implicated in a salmonellosis outbreak. *Journal of Food Protection*, *61*, 280–284.

Peleg, M., & Cole, M. B. (1998). Reinterpretation of microbial survival curves. *Critical Reviews in Food Science and Nutrition*, *38*, 353–380. doi:10.1080/10408699891274246.

Peleg, M., & Cole, M. B. (2000). Estimating the survival of *Clostridium botulinum* spores during heat treatments. *Journal of Food Protection*, *63*, 190–195.

Penney, V., Henderson, H., Blum, C., & Johnson-Green, P. (2004). The potential of phytopreservatives and nisin to control microbial spoilage of minimally processed fruit yogurts. *Innovative Food Science & Emerging Technologies*, *5*, 369–375. doi:10.1016/j.ifset.2003.10.006.

Perera, C. O., & Owen, E. (2008). Effect of tissue disruption by different methods followed by incubation with hydrolyzing enzymes on the production of vanillin from tongan vanilla beans. *Food Bioprocess Technology*. doi:10.1007/s11947-007-0048-4.

Ramos-Nino, M. E., Ramírez-Rodríguez, C. A., Clifford, M. N., & Adams, M. R. (1996). A comparison of quantitative structure-activity relationships for the effect of benzoic and cinnamic acids on *Listeria monocytogenes* using multiple linear regression, artificial neural network and fuzzy systems. *Journal of Applied Microbiology*, *82*, 68–76.

Sado, P. N., Jinneman, K. C., Husby, G. J., Sorg, S. M., & Omiecinsky, C. J. (1998). Identification of *Listeria monocytogenes* from pasteurized apple juice using rapid test kits. *Journal of Food Protection*, *61*, 1199–1202.

Schenk, M., Guerrero, S., & Alzamora, S. M. (2008). Response of some microorganisms to ultraviolet treatment on fresh-cut pear. *Food Bioprocess Technology*, *1*, 384–392. doi:10.1007/s11947-007-0029-7.

Suslick, K. S. (1988). Homogeneous sonochemistry. In K. S. Suslick (Ed.), *Ultrasound, its chemical, physical and biological effects*. New York: VCH.

Ultee, A., Kets, E. P. W., & Smid, E. J. (1999). Mechanism of action of carvacrol on the foodborne poathogen *Bacillus cereus*. *Applied and Environmental Microbiology*, *65*, 4606–4610.

United States Food and Drug Administration. Center for Food Safety and Applied Nutrition (US FDA/CFSAN) (2004). *Juice HACCP hazards and controls guidance. Guidance for industry, 1st ed*. Available at <http://www.cfsan.fda.gov/~dms/juicgu10.html>.