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Modeling of yeast thermal resistance and optimization of the pasteurization treatment applied to soft drinks

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ABSTRACT

Yeast are usually responsible for spoilage of soft drinks and fruit beverages, because of the particular characteristics of these products (low pH, high C/N ratio). The microbial stability is guaranteed by thermal treatments. However, excessive heat treatments can affect food sensorial quality. In this work the thermal resistance of different yeasts strains (seven belonging to the species *Saccharomyces cerevisiae* and six belonging to the species *Kluyveromyces marxianus*, *Zygosaccharomyces bisporus*, *Z. mellis*, *Z. rouxii*, *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii*) was assessed in a model system. The results showed non-linear death curves and a high variability also within the same species. The most resistant strain, belonging to the species *S. cerevisiae*, was chosen for further experiments in orange juice based industrial beverages: first, death curves were performed; then, the probability of beverage spoilage in relation to process parameters (initial inoculum, temperature, treatment time) was evaluated using a logistic regression model. Finally, a cross-validation was performed to investigate the predictive capability of the fitted model. Pasteurization in the soft drink industry is commonly applied according to parameters defined several decades ago, which does not consider the successive findings concerning microbial physiology and stress response, the process improvement and the more recent tools provided by predictive microbiology. In this perspective, this study can fill a gap in the literature on this subject, going to be a basis for optimizing thermal processes. In fact, the data obtained indicated an interesting possibility for food industry to better modulated (and even reduce) thermal treatments, with the aim to guarantee microbial stability while reducing thermal damage and energy costs.

1. Introduction

Pasteurization in the soft drink industry is commonly applied according to parameters (time and temperature) defined several decades ago which often does not consider either the successive findings concerning microbial physiology and stress response or the hygienic level obtained in industrial plant which dramatically decreases the initial contamination of beverages (Azeredo et al., 2016; Deák, 2008a; Deák, 2008b; ICMSF, 2005; Stratford, 2006). In addition, the recent tools provided by predictive microbiology are often not considered in the definition of such parameters.

The characteristics of soft drinks and fruit juices usually make them a selective environment for microbial growth and spoilage. They are generally characterized by a low pH, ranging from 2.6 to 4.0, which is extremely selective and inhibits the growth (and often the survival) of many bacteria, including the pathogenic species. Other selective factors are a high C/N ratio and the limited presence of nutritional factors (Azeredo et al., 2016; Battey et al., 2002; ICMSF, 2005; Louriero and Querol, 1999; Stratford, 2006).

These conditions limit the possible growth to yeasts, moulds and some selected bacteria, such as *Alicyclobacillus* spp., lactic acid and acetic acid bacteria. The exclusion or the reduced availability of oxygen further reduces the growth potential of many of these microorganisms. Indeed, under these conditions, yeasts are accountable for the greatest part of acidified carbonated beverage spoilage (Azeredo et al., 2016; ICMSF, 2005; Stratford, 2006). Among the yeast species isolated from spoiled soft drinks, *Saccharomyces cerevisiae* is the most frequently involved in industrial spoilage cases (Lawlor et al., 2009; Ndagijimana

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et al., 2004; Stratford, 2006). Degradation caused by *S. cerevisiae* results in abundant CO_2 production (with consequent blowing/explosion of the packaging), off-odour and off-flavour production, cloudiness or sediment formation (ICMSF, 2005).

Several strategies are commonly used in the beverage industry in order to achieve microbial stability of acidified carbonated soft drinks, one of the most common being thermal treatment of finished and/or semi-finished products. Because of thermal treatment, however, the organoleptic characteristics of products may be affected. A second strategy is based upon the use of preservatives (*i.e.* weak acids such as benzoate and sorbate), especially for beverages in plastic bottle which cannot undergo high temperature thermal treatments (Lawlor et al., 2009). In any case, the trend toward less heavily processed foods induces the set up of milder treatment parameters safeguarding food safety and stability (Smelt and Brul, 2014).

It is generally reported that yeasts can be inactivated at temperatures higher than 55 °C in few minutes (*D*-value 5–10 min) and that an increase of 4–5 °C causes a tenfold increase (corresponding to *z*) in the death kinetics (Engel et al., 1994; Put and De Jong, 1982a; Put and De Jong, 1982b). However, only rough guidance values are available because several internal and external factors have to be taken into account. Generally, growth conditions before treatment (medium composition, age, sporulation, aerobic or anaerobic growth) affect yeast thermo-resistance (Deák, 2008a). Usually, cells are more resistant to thermal stress when in stationary, rather than exponential phase (Couto et al., 2005). Also low a_w values have a protective effect (Golden and Beuchat, 1992), while the effect of pH is more debated (Beuchat, 1983; Garza et al., 1994).

For orange juice based carbonated soft drinks, standard industrial thermal treatment consists of exposure to temperatures ranging from 65 to 70 °C for 10 to 20 min, not taking into account the extra time needed to reach the target temperature (ICMSF, 2005; Lawlor et al., 2009). In spite of the commercial importance of carbonated beverages, the literature concerning the effects of pasteurization temperature on yeast (and on *S. cerevisiae* in particular) is relatively scarce. In addition, the industrial thermal treatments are still based on the assumption of a linear correlation between logarithmic cell inactivation and time of isothermal treatment (Stumbo, 1973). The weakness of this assumption has been evidenced in many cases for microbial cells (including bacterial spores) and the behaviour of inactivation is often characterized by the presence of “shoulders” (*i.e.* an initial step of the treatment in which cells are less prone to inactivation) and “tails” (a cell thermal resistance increasing with the treatment time) (Buzrul, 2007; Mafart et al., 2002; Peleg, 2006).

The aim of this paper was to study the thermal death kinetics of seven yeast strains belonging to the species *S. cerevisiae* and of other six strains belonging to other species (*Kluyveromyces marxianus*, *Zygosaccharomyces bisporus*, *Z. mellis*, *Z. rouxii*, *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*), which can spoil soft drinks. Their death kinetics in culture medium at different temperature were modelled with the Weibull equation. The kinetics of the most thermo-resistant strain was also assessed in an orange juice based beverage to test the matrix effect. Finally, the probability of beverage spoilage was evaluated applying the logistic regression in relation to the inoculum variation (1 to 4 log cfu/ml), temperature (58 to 64 °C) and time of treatment (5 to 20 min). The model obtained was cross-validated. The attention was focused on yeast with the objective to establish pasteurization parameters for the carbonated soft drink industry, in order to reduce beverage organoleptic damages and energy costs, by maintaining the treatment efficiency required by an industrial process.

2. Materials and methods

2.1. Strains

The yeast strains used in this work belong to the strain collection of the Dipartimento di Scienze e Tecnologie Agroalimentari of the University of Bologna. In particular, seven *S. cerevisiae* strains were employed: SPA (isolated from spoiled soft drinks), Cw, L118, 24G2 and 67G3 (from wine) and I3_2y and MCKL15 (from sourdoughs). Moreover, strains belonging to other species were tested: *Sch. pombe* V1 and *S'codes ludwigii* G1 (from wine), *K. marxianus* YC (from fermented milk), *Z. bisporus* EN (from energy drinks), *Z. mellis* M1 (from honey) and *Z. rouxii* YF1 (from fruit concentrate).

The cultures were maintained until usage on Sabouraud Dextrose Agar (SDA) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) for all the strains with the exception of *Zygosaccharomyces* ones, for which SDA added with 20% (w/w) glucose was used. Before the experiments, strains were cultured twice for 72 h at 28 °C in Saboraud Dextrose Broth (SDB) (Oxoid). In the case of *Zygosaccharomyces* strains, SDB enriched with 20% (w/w) glucose was employed with an incubation time of 120 h. In order to assess the reaching of the stationary phase, a microscope was used to evaluate the presence of budding cells and ascospores.

Observations were performed under the bright field of a Nikon upright microscope (Eclipse Ti-U, Nikon Co, Tokyo, Japan) equipped with a Nikon digital video camera (digital sight DS-Qi1Mc, Nikon Co.) at a magnification of 1000× in phase contrast.

2.2. Thermal death kinetics in SDB

Strains precultured for 72 h (120 h for *Zygosaccharomyces* spp.) were inoculated (1:10 v/v) at a level of about 6.5 log CFU/ml in 30 ml SDB (pH 6.0) preheated to proper temperatures (55, 60 or 65 °C). The initial cell level in each experiment and for each strain was determined by plate counts on SDA or SDA added with 20% (w/w) glucose for *Zygosaccharomyces* strains. The decrease of the temperature after inoculation was negligible (a maximum of 0.5 °C for 2 to 3 s before returning to the treatment temperature); thus, the treatment was considered isothermal.

During the heat treatment, samples were periodically taken and were rapidly cooled to room temperature in a water/ice bath (cooling time approx. 1 min). Appropriate decimal dilutions in physiological solution (NaCl 0.9% w/w) were analysed by pour plate counting on SDA. The plates were incubated at 28 °C for 72 h (120 for the glucose-enriched medium). Each death kinetic experiment was performed twice and the data were combined to increase the number of raw data points and minimize the weight of outliers during the modeling process.

2.3. Thermal death kinetics in non-carbonated beverages

The strain *S. cerevisiae* SPA was chosen to test the thermal death kinetics in non-carbonated orange based beverages since it resulted the highest thermo-resistant among the tested strains. Beverages were prepared by aseptically diluting an industrial orange-based concentrate used for soft drink manufacturing with sterile water (dilution factor, 1:5.25; final 8.5°Bx, pH 3.1, a_w 0.991). 10 ml sterile tubes were filled with the beverage, pre-heated at the target temperature (55, 60 and 65 °C) in a LAUDA Ecoline (Lauda-Brinkmann, USA) water bath. They were inoculated, without agitation, with 72 h (120 for *Zygosaccharomyces* spp.) pre-cultured yeast cells at a level of about 6 log CFU/ml (volume of inoculum 1:10). Differently from the previous trials, the strain was pre-grown in SDB acidified at pH 4 (HCl 0.1 M), to adapt the cells to the acid environment of the beverages. During thermal treatment, samples were periodically collected, cooled at room temperature in a water/ice bath and analysed by plate counts on SDA.

2.4. Industrial beverage pasteurization

The thermo-resistant *S. cerevisiae* SPA was tested in specific industrial pasteurization conditions in orange beverages, taking into account different inoculum levels (1, 2, 3 and 4 log CFU/ml),

temperatures (58 °C, 59.5 °C, 61 °C, 62.5 °C and 64 °C) and the lengths of thermal treatment (5, 10 and 20 min).

Glass bottles (200 ml) were washed with a 3% (v/v) solution of H_2O_2 and rinsed with water before usage. Beverages were prepared as described above and distributed into the bottles. Pre-cultured yeast (72 h at pH 4) was re-suspended at defined concentrations (1, 2, 3 and 4 log CFU/ml) in bottles pre-heated in water baths at the different temperatures (58, 59.5, 61, 62.5 and 64 °C) and immediately closed with aluminum caps. The bottles were maintained at the programmed temperatures for 5, 10 and 20 min.

For the inoculum, after a presumptive quantification of *S. cerevisiae* SPA culture with a Bürker chamber (Brand GMBH, Wertheim, Germany), serial dilutions were performed in order to obtain the yeast concentration desired for every combination. At the same time, a precise counting of the initial yeast concentration was carried out by plate count on SDA incubated at 28 °C for 72 h. After the heat treatment, samples were rapidly cooled to room temperature in a water/ice bath (cooling time approx. 4 min) and stored at room temperature (21 ± 2 °C) and observed periodically over a 30-day period for the presence of cloudiness, cell sediment on the bottom or CO_2 due to yeast growth. After this storage time, where no growth was observed, a sample of 0.3 ml was spreaded onto a SDA plate in order to qualitatively confirm the yeast death. All the sixty combinations of the three variables were considered and 20 repetitions were tested for each combination.

2.5. Models development

The Weibull equation was used to fit the survivor data for each temperature in the death kinetics, both in SDB and in beverages:

$$LogN_t = LogN_0 - \left(\frac{t}{\delta}\right)^p$$

where N_t and N_0 are the number of cells before the treatment and after the treatment time t. δ and p are temperature dependent parameters: δ is the time of first decimal reduction while p is the shape parameter (Couvert et al., 2005; Mafart et al., 2002). $p < 1$ describes a concave upward curve (the curve presents a "tail"), $p > 1$ describes a concave downward curve (the curve presents a "shoulder"), while when $p = 1$ the curve describes a straight line (*i.e.* it coincides with the Bigelow model). $LogN_0$ for each strain considered was considered constant (the number of cell present in the samples before heating determined by plate counting). The parameters δ and p were estimated through non-linear regression. The goodness of fit of the regression has been evaluated using the coefficient of correlation between observed and fitted data (R) and the Root Mean Square Error (*RMSE*).

Regarding *S. cerevisiae* SPA resistance to industrial pasteurization conditions, value 1 was assigned to the samples in which growth was detected while 0 was assigned to samples in which no growth occurred. The growth/no growth data were modelled to understand the behaviour of the probability of growth (P) during the storage period using a logistic regression model (Kleinbaum and Klein, 2010)

$$P(y = growth \mid X) = \frac{1}{1 + e^{-(\alpha + \sum \beta_j x_j)}}$$

where α is an estimated constant and β_j is the fitted regression coefficient related to the covariate x_j. In the model, initial inoculum, temperature and time treatment were considered as explanatory variables.

The estimated coefficients allow investigating the effect that the explanatory variables have on the probability of the studied event (in our case the growth). Obviously, this kind of relation cannot be linear (the probability must be bounded between 0 and 1), and the popular logistic regression model is characterized by the linearity in the logit, *i.e.* the natural logarithm of the odds defined as P/(1-P). This particular assumption allows to deduce the previous expression of the probability P of the event as a function of the linear predictor $\alpha + \Sigma \beta_j x_j$. Moreover, the estimated coefficients β_j can be studied by exponentiating them and considering the obtained quantities as odds ratios, that must be carefully interpreted discriminating between continuous and categorical covariates (Hosmer and Lemeshow, 2004; Kleinbaum and Klein, 2010).

To assess the goodness of fit of the model, the Hosmer and Lemeshow test was performed. Furthermore, the capacity of the model in the discrimination between conditions that could lead to beverage spoilage (unstable) or not (stable) was investigated. Therefore, the performance of the model was also assessed by the usual techniques aimed to control the classification accuracy: ROC curve, classification rate and cross-validation (Hosmer and Lemeshow, 2004). In particular, the ROC curve shows the behavior of the sensitivity (*i.e.* the probability that the classifier is able to detect a true case) and the specificity (*i.e.* the probability to correctly classify a safe observation) with respect to a different value of the cutoff c (that is the value of probability that controls the classification: if the predicted probability is greater than c, then the observation is considered as a case) used. The area under the ROC curve and the classification rate (proportion of correctly classified observations) could be used as a first indicator of the predictive performance of the model.

Regarding the cross validation, the following steps were adopted: iteratively (500 times in our case) the dataset was randomly split into a training set (90% of the observations) and a test set (the remaining 10%); then, at each iteration, the model was fitted using the training set, and the correct classification rate was obtained predicting the test set. Finally, the mean of the correct classification rates was evaluated.

The whole statistical analysis was carried out using the R software (R Core Team, 2017).

3. Results and discussion

3.1. Thermal death kinetics in SDB

Before assessing the yeast thermal resistance, the absence of ascospores and the limited presence of budding cells were verified with a microscope (see Supplementary Figs. 1 and 2).

Table 1 reports the results of modeling and, in particular, the estimates of the parameters of the model (δ and p), the RMSE and the value of R as well as the time predicted to reach a 5 log CFU/ml inactivation. The experimental point for each temperature and strain together with the fitted models are reported in Supplementary Figs. 3 and 4 for *S. cerevisiae* strains and non-*Saccharomyces* strains, respectively. A high variability in thermal resistance was observed among the non-*Saccharomyces* strains. The strains belonging to the genus *Zygosaccharomyces* showed a weak thermal resistance: the two strains *Z. mellis* M1 and *Z. rouxii* YF1 were characterized by counts lower than the detection limit after few minutes of treatment at the lowest temperature (55 °C). Only *Z. bisporus* EN showed a appreciable resistance at 55 °C. However, at this temperature, the initial cell concentration decreased with 5 log CFU/ml ($t_{5\ log}$) in 3.4 min, confirming the high thermal sensitivity of this genus. Higher survival rates were observed in *Sch. pombe* V1 ($t_{5\ log}$ 56.9 and 1.3 min at 55 °C and 65 °C, respectively) and *K. marxianus* YC ($t_{5\ log}$ 46.0 and 0.4 min at 55 °C and 65 °C, respectively). Intermediate results were observed for *S'codes ludwigii* G1, which did not survive the treatment at 65 °C and had a $t_{5\ log}$ of 5.6 min at 55 °C.

A great variability in the thermal resistance was observed among the strains belonging to the *S. cerevisiae* species. For one of the strains (MCKL15), no survivor was detected even after few seconds of treatment at 65 and 60 °C. The other strains were characterized by different death kinetics. The strain *S. cerevisiae* SPA showed the highest thermoresistance. In fact, the $t_{5\ log}$ was not predictable (> 500 min) at 55 °C and was 37.1 and 4.7 min at 60 and 65 °C respectively. The high thermo-tolerance of this strain is not surprising and it can be related to the source of isolation. In fact, it was isolated from orangeade bottles after an important industrial case of spoilage (Ndagijimana et al., 2004). The harsh characteristics of an industrial environment can exert

Table 1
Estimates of the parameters (δ and p) obtained with the Weibull model for the isothermal inactivation curves of yeast strains at 55, 60 and 65 °C in Saboraud Dextrose Broth. In addition, some diagnostics (R and RSME) are reported, together with the time (minutes) needed, based on the model parameters, needed to have a 5 log reduction of the initial yeast population ($t_{5\ log}$). Initial concentration of each yeast strain is reported within brackets.

S. cerevisiae strains	Temperature	δ	p	$t_{5\ log}$ min	R	*RMSE*	Non-*Saccharomyces* strains	Temperature	δ	p	$t_{5\ log}$ min	R	*RMSE*
S. cerevisiae SPA	**55 °C**	109.9 (22.9)[a]	0.569 (0.110)	> 500	0.876	0.098	*Sch. pombe* V1	**55 °C**	2.50 (0.39)	0.515 (0.054)	56.9	0.974	0.290
(6.7 log CFU/ml)	**60 °C**	4.94 (0.50)	0.798 (0.050)	37.1	0.894	0.253	(6.3 log CFU/ml)	**60 °C**	0.073 (0.009)	0.406 (0.050)	8.9	0.967	0.512
	65 °C	0.318 (0.057)	0.597 (0.040)	4.7	0.988	0.267		**65 °C**	5.3×10^{-4} (6.4×10^{-5})	0.204 (0.014)	1.3	0.997	0.121
S. cerevisiae Cw	**55 °C**	12.0 (0.5)	0.527 (0.026)	254.0	0.993	0.216	*S'codes ludwigii* G1	**55 °C**	3.75 (0.16)	2.285 (0.146)	5.6	0.997	0.116
(6.8 log CFU/ml)	**60 °C**	1.42 (0.12)	0.977 (0.058)	7.3	0.998	0.135	(6.4 log CFU/ml)	**60 °C**	0.014 (0.004)	0.347 (0.028)	1.5	0.996	0.149
	65 °C	7.1×10^{-7} (2.3×10^{-8})	0.119 (0.030)	0.27	0.993	0.318		**65 °C**	–[b]	–	–		
S. cerevisiae L118	**55 °C**	9.92 (0.86)	0.825 (0.065)	69.7	0.985	0.174	*K. marxianus* YC	**55 °C**	7.93 (0.53)	0.916 (0.058)	46.0	0.992	0.175
(6.5 log CFU/ml)	**60 °C**	0.600 (0.101)	0.751 (0.062)	5.1	0.982	0.431	(6.3 log CFU/ml)	**60 °C**	0.134 (0.030)	0.494 (0.056)	3.3	0.993	0.175
	65 °C	0.048 (0.007)	0.510 (0.061)	1.1	0.975	0.508		**65 °C**	2.2×10^{-4} (6.5×10^{-5})	0.203 (0.041)	0.42	0.991	0.271
S. cerevisiae I3_2Y	**55 °C**	5.25 (0.47)	0.774 (0.042)	42.0	0.990	0.224	*Z. bisporus* EN	**55 °C**	0.125 (0.038)	0.486 (0.017)	3.4	0.959	0.590
(6.3 log CFU/ml)	**60 °C**	0.222 (0.032)	0.558 (0.046)	3.9	0.982	0.315	(6.7 log CFU/ml)	**60 °C**	–	–	–	–	
	65 °C	3.04×10^{-4} (6.3×10^{-5})	0.221 (0.018)	0.44	0.986	0.352		**65 °C**	–	–	–	–	
S. cerevisiae 67G3	**55 °C**	4.15 (0.61)	0.568 (0.061)	70.6	0.973	0.282	*Z. mellis* M1	**55 °C**	–	–	–	–	
(6.2 log CFU/ml)	**60 °C**	0.124 (0.024)	0.366 (0.040)	10.1	0.981	0.335	(6.5 log CFU/ml)	**60 °C**	–	–	–	–	
	65 °C	0.054 (0.009)	0.510 (0.098)	1.3	0.982	0.381		**65 °C**	–	–	–	–	
S. cerevisiae 34G2	**55 °C**	9.34 (0.97)	0.821 (0.088)	66.3	0.975	0.252	*Z. rouxii* YF1	**55 °C**	–	–	–	–	
(6.2 log CFU/ml)	**60 °C**	0.491 (0.090)	0.513 (0.032)	11.3	0.993	0.220	(6.5 log CFU/ml)	**60 °C**	–	–	–	–	
	65 °C	1.2×10^{-3} (3.4×10^{-4})	0.257 (0.033)	0.73	0.983	0.407		**65 °C**	–	–	–	–	
S. cerevisiae MCKL15	**55 °C**	1.03 (0.12)	0.679 (0.065)	12.0	0.988	0.304							
(6.3 log CFU/ml)	**60 °C**	–	–	–									
	65 °C	–	–	–									

[a] Standard error.
[b] No survivor data available.

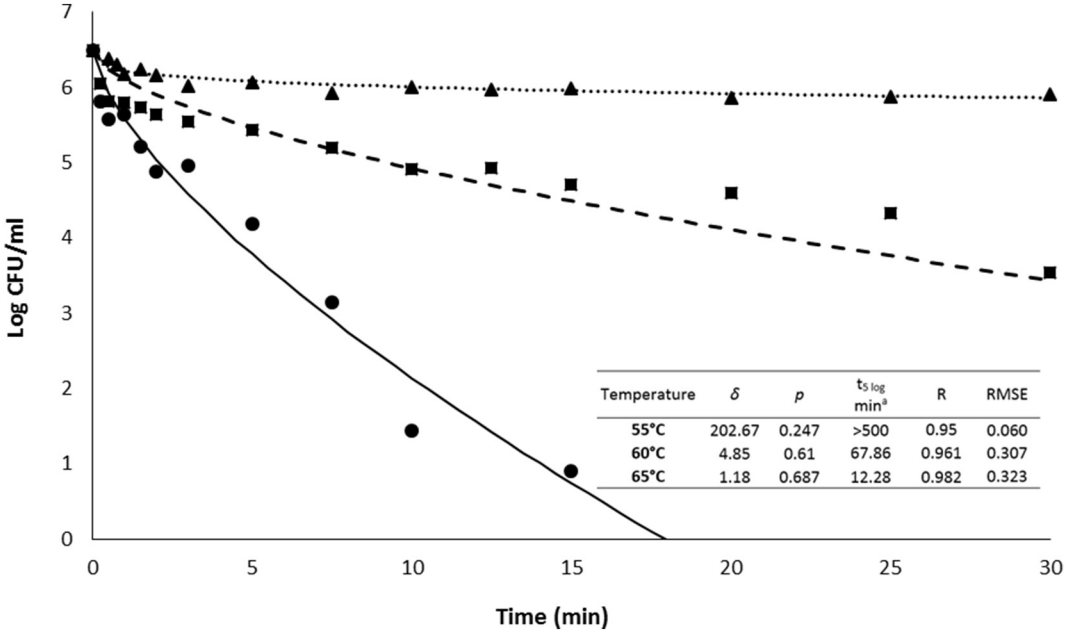

Fig. 1. Thermal death curves of *S. cerevisiae* SPA at 55 (triangle), 60 (square) and 65 °C (circle) in non-carbonated orange beverages. Points represent experimental data and lines the fitted Weibull models. Model parameters (δ and p), diagnostics and the time (minutes) needed to inactivate 5 log CFU/ml are also reported.

Table 2
Frequency of *S. cerevisiae* SPA growth observed in orange based beverages in relation to initial inoculum, different treatment temperature and time. The frequencies reported are the results of twenty repetitions for each condition tested. Growth/no growth was assessed after a 30 days storage at room temperature. Samples were considered spoiled when visible growth occurred or when survived cells were detected in the absence of any evidence of yeast growth.

Initial inoculum (log CFU/ml)	Treatment time (min)	Temperature (°C)				
		58	59.5	61	62.5	64
1	5	1	1	0.70	0	0
1	10	1	1	0.35	0.05	0
1	20	0.75	0.15	0	0	0
2	5	1	1	0.95	0.30	0.05
2	10	1	1	0.30	0.10	0
2	20	1	0.20	0	0	0
3	5	1	1	1	0.95	0.40
3	10	1	1	0.85	0.75	0.10
3	20	1	0.85	0.35	0	0
4	5	1	1	1	1	0.50
4	10	1	1	1	0.95	0.25
4	20	1	1	0.85	0.15	0

a selective pressure on the microbial population, able to select strains more resistant to the stress factors. The prolonged and continuous application of high temperature (for both beverage stabilization and pasteurization) can determine the survival of high thermo-resistant strains. The *S. cerevisiae* strains L118, I3_2Y, 67G3 and 34G2, having comparable behaviours, were less resistant. The $t_{5\ log}$ varied from 3.9 and 11.3 min at 60 °C and 0.4 and 1.1 min at 65 °C. The strain *S. cerevisiae* CW showed an intermediate resistance and a significantly higher thermo-tolerance at 55 °C ($t_{5\ log}$ 254.0 min).

Commercial heat preservation processes are based on the assumption that thermal death kinetics follow the classical first-order inactivation model (Stumbo, 1973). However, the linear model does not always provide an adequate description of non-linear kinetics usually associated to the process conditions applied in the food industry, which should be minimized in order to achieve an overall better food quality (Buzrul, 2007). The results obtained in this work applying the Weibull model confirmed that the linear model does not always provide an adequate description of death kinetics. The goodness of fit indicated by the diagnostics reported in Table 1 indicates the reliability and the flexibility of this model that allows also the description of linear kinetics (with $p = 1$).

Literature regarding the thermal resistance of yeasts is relatively scarce. In addition, the use of different suspension media and different operative conditions make comparisons extremely difficult.

Heat shock responses in yeasts have been mainly studied in relation to high fermentation temperature, which characterize many biotechnological industrial processes. In addition, heat shock can accelerate the production of reactive oxygen species. Several mechanisms are responsible for this response. They include the production of heat shock proteins, protecting proteins from thermal damage, the accumulation of trehalose, which stabilizes biological membrane and nucleic acids, ATPase proton pumping activity, and antioxidant defenses (superoxide dismutase, catalase and peroxiredoxin) (Gao et al., 2016).

Generally, yeasts are killed within a few minutes at temperatures between 55 °C and 65 °C (Put and De Jong, 1982a, 1982b; Engel et al., 1994) and the *D*-value at 55 °C was reported to range between 5 and 10 min, while at 65 °C it was lower than 1 min (Deák, 2008b). Studies carried out by Beuchat (1982) demonstrated that temperatures between 48 and 51 °C were sufficient to inactivate all vegetative yeasts in sweet fruit juices, as their *D*-value varied from 10 min at 51 °C to 30 min at 48 °C. Raso et al. (1998) reported D_{50} values for *Z. bailii* in different acidic juices ranging from 1.97 and 4.48 min, which increased up to 10.4 and 37.0 min for ascospores. Beuchat (1981) also described the unusual sensitivity to heat of *Z. rouxii* and *Debaryomyces hansenii*. A D_{50} value of 14.6 min was observed for *S. cerevisiae* DSMZ1848, but this value increased up to 62 min for the ascospores in 4% alcoholic beer. In the same matrix, the D_{65} values for the ascospores of different *S. cerevisiae* strains ranged from 2.2 and 3.6 min (Milani et al., 2015). Chueca et al. (2015) tested several *S. cerevisiae* strains at 54 °C for 10 min and the counts of more resistant ones decreased of 1–2 log units. López-Malo et al. (1999) described *D*-values for *S. cerevisiae* of 739 min at 45 °C, 18.3 min at 50 °C and 2.7 min at 55 °C. These values were drastically reduced if the same treatment was carried out combined with an ultrasound treatment at 20 kHz.

In beer, the presence of ethanol and hops (which contains natural

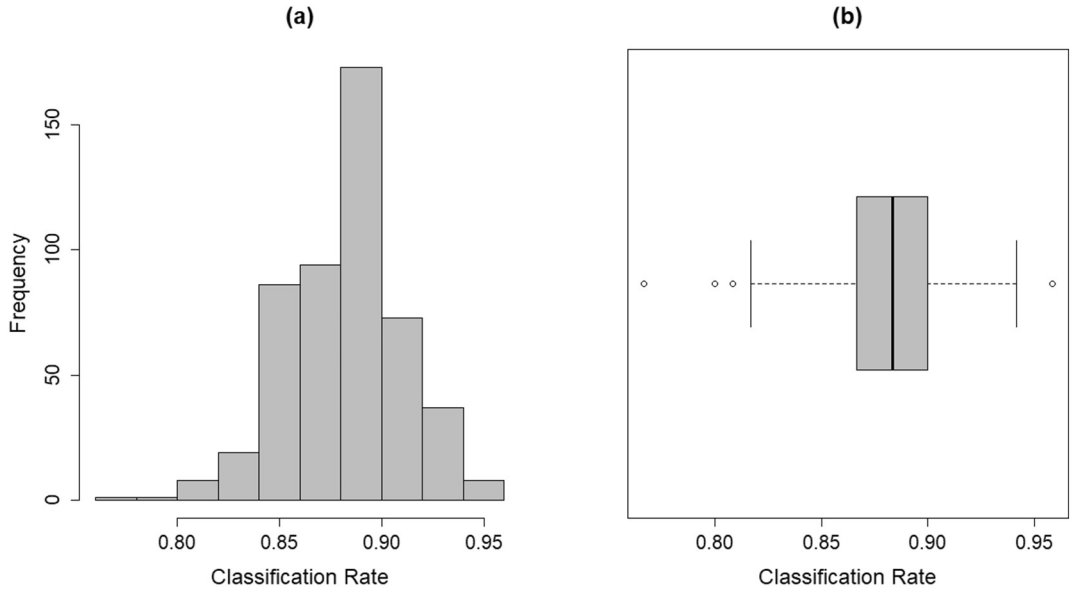

Fig. 2. Histogram (a) and boxplot (b) showing the distribution of the classification rate in the cross validation procedure.

Table 3
Parameter estimates and diagnostics for the logistic regression analysis.

Variables	Estimates	Standard error	*Z*-value	*p*-Value
Intercept (1 log CFU/ml)	134.03	9.26	14.47	< 0.001
2 log CFU/ml	0.99	0.34	2.93	0.003
3 log CFU/ml	4.60	0.45	10.14	< 0.001
4 log CFU/ml	6.24	0.53	11.82	< 0.001
Temperature (°C)	−2.14	0.15	−14.46	< 0.001
Treatment time (min)	−0.42	0.03	−12.50	< 0.001

Hosmer and Lemeshow goodness of fit test: *X*-squared 1.402 (df = 7) *p*-value 0.986. AIC (Akaike Information Criterion) 476.06. Residual deviance 464.06.

antimicrobials) confers more effectiveness to pasteurization (Milani et al., 2015). However, the amount of yeast cells to be inactivated is considerably higher than in soft drinks (several log units against few cells per ml).

3.2. Thermal death kinetics in non-carbonated beverages

The experimental points of the highest thermo-resistance strain *S. cerevisiae* SPA, pre-grown in acidified SDB, were fitted with the Weibull equation and the results, together with estimates of the parameters and the diagnostics of the models, are shown in Fig. 1.

The times required for a unit inactivation were higher than those observed in SDB in this work, especially at the maximum temperature applied (12.2 *vs.* 4.7 min).

The composition of the growth medium and the cell physiological state affect the thermal resistance of yeasts. Variable survival rates have been described in the presence of different sugar concentrations (or different a_w), depending on the age (stationary or exponential phase) or starvation under aerobic or anaerobic conditions. Generally, increasing carbohydrate amounts favour a higher resistance while few data are available for the effect of pH (Deák, 2008b). However, de Melo et al. (2010) demonstrated that the stress response mechanisms in *S. cerevisiae* induced by pH determined also an increase in its thermo-resistance.

3.3. Growth/no growth at specific industrial pasteurization conditions

After these trials, the heat resistance of yeast was further assessed in beverages at 55, 60 and 65 °C. The most thermo-resistant among the tested strains (*S. cerevisiae* SPA) was inoculated in non-carbonated orange beverages according to the conditions showed in Table 2, where the frequency of growth observed within 30 days for each condition is reported. The observations were used to fit a logistic regression model (Belletti et al., 2010) to predict the probability of growth in relation to the variables considered.

The yeast concentration variable was considered as a categorical one: some evidence of non-linear effect was pointed out. On the other hand, length and temperature of the thermal treatment were considered as continuous variables. The parameter estimates of the model together with the basic goodness of fit diagnostics are reported in Table 3. According to the Hosmer and Lemeshow (as shown in Table 3) test no evidence of poor fit was detected. To confirm this positive result also from a predictive point of view, ROC curve was analysed (Supplementary Fig. 5). The area under the ROC curve is 0.976, which suggests an excellent behaviour of the model. The value of *c* was chosen in order to minimize the probability to wrongly classify an unsafe case as a safe observation (false negative): $c = 0.25$ was considered as appropriate (sensitivity = 0.976). Then, after the decision of *c*, a more accurate examination of the predictive credibility of the model was effectuated through cross validation. If it is fixed $c = 0.25$, the correct classification rate is 0.886; and this result is confirmed by average value obtained through the cross-validation method (0.883). These results obtained with the cross-validation procedure are reported in Fig. 2. The coherence of the two values is fundamental to exclude that the high classification rate might be due to model overfitting.

According to the fitted model, Fig. 3 shows the probability of spoilage for the four initial inoculum levels with respect to time and temperature. As it is possible to observe, at the lowest yeast concentration tested, (1 log CFU/ml) there is a relatively wide combination of factors able to stabilize the beverage (in particular temperatures higher than 62 °C or times longer than 10 min), while temperatures below 59 °C are scarcely effective, independently of treatment length. Obviously, increasing yeast concentration reduces the efficacy of the thermal treatment and this “safe zone” is extremely reduced when the inoculum is 4 log CFU/ml (63.5–64 °C for 18–20 min). At 64 °C, a 5 min treatment was not able to prevent the spoilage of > 50% of samples, while, according to the results of Table 1, in SDB, 5 min of a 65 °C treatment were sufficient to inactivate > 5 log units of the same *S. cerevisiae* strain.

This trend is better evidenced by Fig. 4, which reports the combinations of time and temperature allowing 99% of stability ($p = 0.01$). In this case, the non-continuous effect of yeast inoculum can be

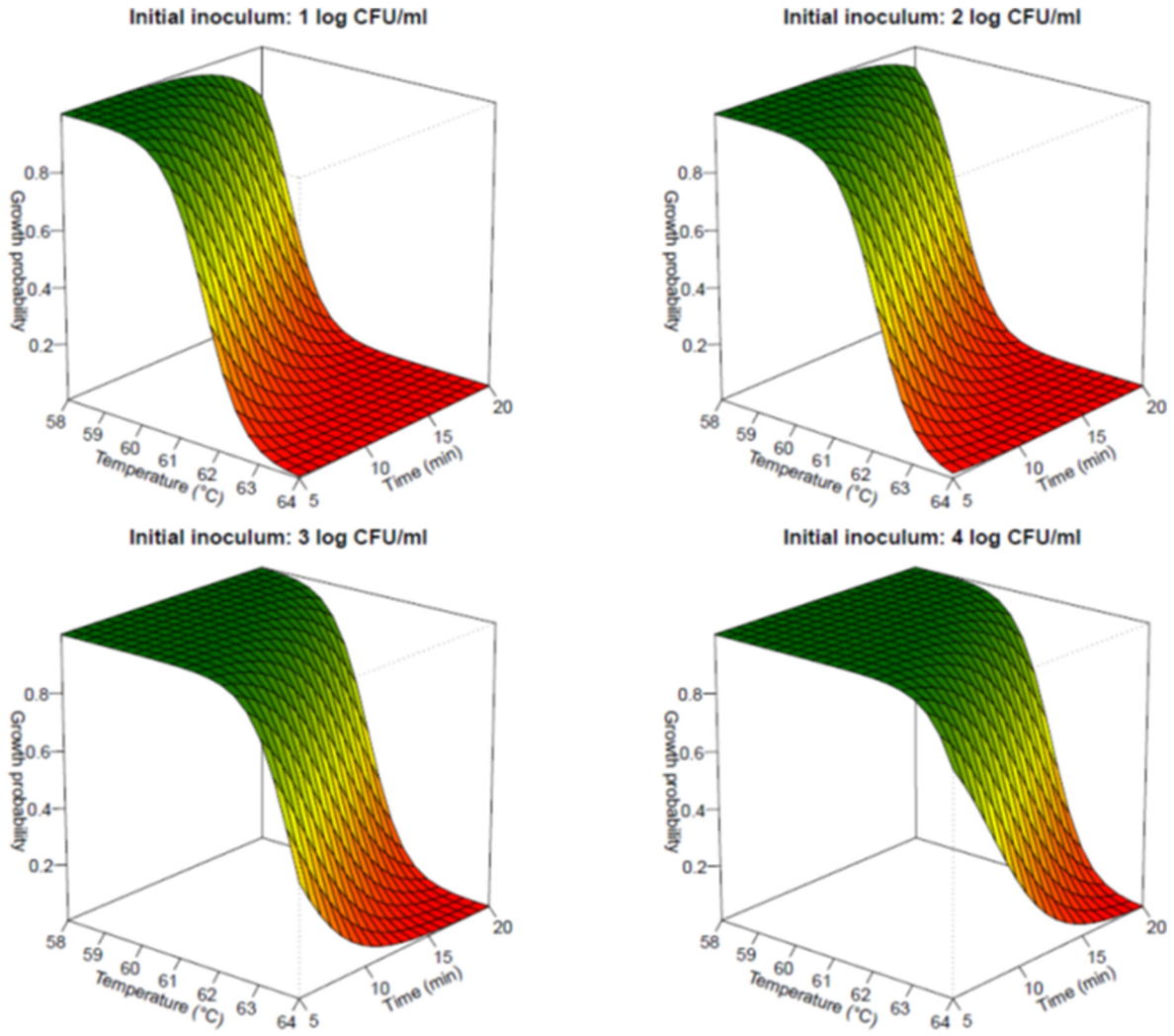

Fig. 3. Probability of spoilage in relation to the temperature and time adopted for the beverage thermal treatment for the four initial inoculum levels of *S. cerevisiae* SPA.

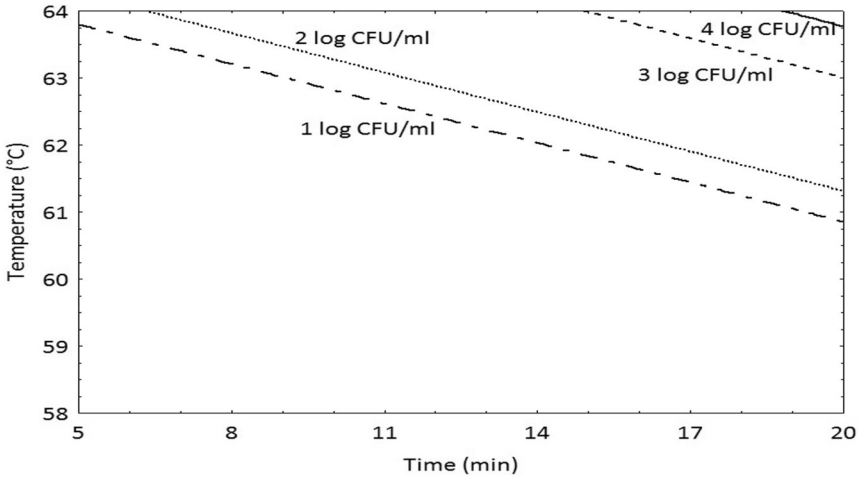

Fig. 4. Combination of time and temperature treatment able to assure a predicted probability of having 99% of stable bottles ($p = 0.01$) according to the fitted logistic regression model for beverages inoculated with different concentrations of *S. cerevisiae* SPA.

evidenced by the noteworthy shift of conditions passing from 2 to 3 log CFU/ml. It is possible to hypothesise that this shift can be attributed to the results of the phenotypic heterogeneity, typical also of clonal populations, known as “molecular noise”, which can lead to different behaviour among genetically identical cells in a homogeneous environment, as extensively described by Koutsoumanis and Aspridou (2017). The same authors noted that this stochastic variation in gene responses decreases with initial population size increase. They stated

that, in bacterial populations higher than 100 cell per ml, this variability was almost eliminated.

In any case, considering the lower initial contamination levels (1 or 2 log CFU/ml), which are considered more representative of the expected contamination of industrial fruit juices or soft-drinks, few minutes at 64 °C were enough to guarantee a satisfying microbial stability to the beverages. If we take into consideration lower P, *i.e.* 0.0001, the length of the treatment predicted at a treatment temperature of 64 °C was 15.0 and 17.3 min at cell concentration of 1, 2 log cfu/ml, respectively. *Vice versa*, if a constant treatment time (20 min) is kept, the temperatures to be reached to have the same P were 63.0 and 63.5 °C at 1 and 2 log cfu/ml, respectively.

According to the data obtained through these experiments, there is an interesting possibility for the beverage industry to better modulate the thermal treatment against yeasts. Treatments at 68 °C and more, prolonged for 15–20 min (without considering the time needed to reach the target temperature) are commonly applied for the pasteurization of soft drinks in cans or glass bottles (ICMSF, 2005). These treatments might be excessive in relation to the normal yeast concentration, especially in carbonated soft drinks, in which the high level of CO_2 added reinforces the inactivation effect of the thermal treatment (Deák, 2008b).

4. Conclusions

The definition of the parameters (time and temperature) of industrial thermal treatments for the stabilization of beverages is often based on standard protocols established several years ago which might be revaluated. In addition to the conditions under which the thermal resistance of microorganisms (in this case yeasts) is assessed, the recent studies about death kinetics demonstrated that basic assumptions of the linear model of thermal inactivation are disputable. The recent predictive tools for modeling the cell death are important instruments to optimize the pasteurization process of beverages.

A more appropriate thermal treatment would likely prevent organoleptic damages, which can strongly affect the flavour and aroma of the beverages. This could reduce also the energy costs, leading to environmental and economic benefits for the producers. However, further and more accurate studies on yeast thermo-resistance are needed to set up safe pasteurization protocols. These studies should take into consideration the high variability of yeasts in response to thermal treatment, related to the selective pressure exerted by the isolation source, and their physiological and growth conditions as well as the characteristics of the beverage. In this perspective, the choice of the target microorganisms is crucial for the correct application of the process parameters aimed to obtain a compromise between the stabilization of the product and the organoleptic quality of the beverages.

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References

Azeredo, D.R.P., Alvarenga, V., Sant'Ana, A.S., Sabaa Srur, A.U.O., 2016. An overview of microorganisms and factors contributing for the microbial stability of carbonated soft drinks. Food Res. Int. 82, 136–144.

Battey, A.S., Duffy, S., Schaffner, D.W., 2002. Modeling yeast spoilage in cold-filled ready-to-drink beverages with *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Candida lipolytica*. Appl. Environ. Microbiol. 68, 1901–1906.

Belletti, N., Sado Kamdem, S., Tabanelli, G., Lanciotti, R., Gardini, F., 2010. Modeling of combined effects of citral, linalool and β-pinene used against *Saccharomyces cerevisiae* in citrus-based beverages subjected to a mild heat treatment. Int. J. Food Microbiol. 136, 283–289.

Beuchat, L.R., 1981. Synergistic effects of potassium sorbate and sodium benzoate on thermal inactivation of yeasts. J. Food Sci. 49, 771–777.

Beuchat, L.R., 1982. Thermal inactivation of yeasts in fruit juices supplemented with food preservatives and sucrose. J. Food Sci. 47, 1679–1682.

Beuchat, L.R., 1983. Combined effects of food preservatives and organic acids on thermal inactivation of yeasts in fruit juices. Lebensm. Wiss. Technol. 16, 51–54.

Buzrul, S., 2007. A suitable model of microbial survival curves for beer pasteurization. LWT Food Sci. Technol. 40, 1330–1336.

Chueca, B., Ramírez, N., Arvizu-Medrano, S.M., García-Gonzalo, D., Pagán, R., 2015. Inactivation of spoiling microorganisms in apple juice by a combination of essential oils' constituents and physical treatments. Food Sci. Technol. Int. 22, 389–398.

Couto, J.A., Neves, F., Campos, F., Hogg, T., 2005. Thermal inactivation of the wine spoilage yeasts *Dekkera/Brettanomyces*. Int. J. Food Microbiol. 104, 337–344.

Couvert, O., Gaillard, S., Savy, N., Mafart, P., Leguerinel, I., 2005. Survival curves of heated bacteria spores: effects of environmental factors on Weibull parameters. Int. J. Food Microbiol. 101, 73–81.

de Melo, H.F., Bonini, B.M., Thevelein, J., Simões, D.A., Morais Jr., M.A., 2010. Physiological and molecular analysis of the stress response of *Saccharomyces cerevisiae* imposed by strong inorganic acid with implication to industrial fermentations. J. Appl. Microbiol. 109, 116–127.

Deák, T., 2008a. Growth, life cycle, death. In: Deák, T. (Ed.), Handbook of Food Spoilage Yeasts, second ed. CRC Press, Boca Raton, pp. 71–86.

Deák, T., 2008b. Preservation: inhibition and inactivation of yeasts. In: Deák, T. (Ed.), Handbook of Food Spoilage Yeasts, second ed. CRC Press, Boca Raton, pp. 87–116.

Engel, G., Rosch, N., Heller, K.J., 1994. Heat tolerance of yeasts. Kieler Milchw. Forsch. 46, 81–90.

Gao, L., Liu, Y., Sun, H., Li, C., Zhao, Z., Liu, G., 2016. Advances in mechanisms and modifications for rendering yeast thermotolerance. J. Biosci. Bioeng. 121, 599–606.

Garza, S., Teixido, J.A., Sanchis, V., Vinas, I., Condon, S., 1994. Heat resistance of *Saccharomyces cerevisiae* strains isolated from spoiled peach puree. Int. J. Food Microbiol. 23, 209–213.

Golden, D.A., Beuchat, L.R., 1992. Interactive effects of solutes, potassium sorbate and incubation temperature on growth, heat resistance and tolerance to freezing of *Zygosaccharomyces rouxii*. J. Appl. Bacteriol. 73, 524–530.

Hosmer Jr., D.W., Lemeshow, S., 2004. Applied Logistic Regression. John Wiley & Sons, New York.

International Commission on Microbiological Specifications for Foods (ICMSF), 2005. Soft drinks, fruit juices, concentrates and fruit preserves. In: ICMSF (Ed.), Microorganisms in Foods vol. 6: Microbial Ecology of Food Commodities. Kluwer Academic/Plenum Publishers, New York, pp. 544–573.

Kleinbaum, D.G., Klein, M., 2010. Logistic regression, third ed. Springer, New York.

Koutsoumanis, K.P., Aspridou, Z., 2017. Individual cell heterogeneity in predictive food microbiology: challenges in predicting a “noisy” world. Int. J. Food Microbiol. 240, 3–10.

Lawlor, K.A., Schuman, J.D., Simpson, P.G., Taormina, P.J., 2009. Microbiological spoilage of beverages. In: Sperber, H.S., Doyle, M.P. (Eds.), Compendium of the Microbiological Spoilage of Foods and Beverages. Springer-Verlag, Berlin, pp. 245–284.

López-Malo, A., Guerrero, S., Alzamora, S.M., 1999. *Saccharomyces cerevisiae* thermal inactivation kinetics combined with ultrasound. J. Food Prot. (10), 1215–1217.

Louriero, V., Querol, A., 1999. The prevalence and control of spoilage yeasts in foods and beverages. Trends Food Sci. Technol. 10, 356–365.

Mafart, P., Couvert, O., Gaillard, S., Leguerinel, I., 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. Int. J. Food Microbiol. 72, 107–113.

Milani, E.A., Gardner, R.C., Silva, F.V.M., 2015. Thermal resistance of *Saccharomyces* yeast ascospores in beers. Int. J. Food Microbiol. 206, 75–80.

Ndagijimana, M., Belletti, N., Lanciotti, R., Guerzoni, M.E., Gardini, F., 2004. Effect of aroma compounds on the microbial stabilization of orange based soft drinks. J. Food Sci. 69, 20–24.

Peleg, M., 2006. Advanced Quantitative Microbiology for Food and Biosystems: Models for Predictive Growth and Inactivation. CRC Press, Boca Raton.

Put, H.M.C., De Jong, J., 1982a. The heat resistance of ascospores of four *Saccharomyces* spp. isolated from spoiled heat processed soft drinks and fruit products. J. Appl. Bacteriol. 52, 235–244.

Put, H.M.C., De Jong, J., 1982b. Heat resistance studies on yeasts—vegetative cells versus ascospores—erythromycin inhibition of sporulation in *Kluyveromyces* and *Saccharomyces* species. J. Appl. Bacteriol. 53, 73–79.

R Core Team, 2017. R: A language and environment for statistical computing. https://www.R-project.org/. Accessed 31 January 2018.

Raso, J., Calderon, M.L., Gongora, M., Barbosa-Canovas, G.V., Swanson, B.G., 1998. Inactivation of *Zygosaccharomyces bailii* in fruit juices by heat, high hydrostatic pressure and pulsed electric fields. J. Food Sci. 63, 1042–1044.

Smelt, J.P., Brul, S., 2014. Thermal inactivation of microorganisms. Crit. Rev. Food Sci. Nutr. 54, 1371–1385.

Stratford, M., 2006. Food and beverage spoilage yeasts. In: Querol, A., Fleet, G. (Eds.), Yeasts in Food and Beverages. Springer-Verlag, Berlin, pp. 335–379.

Stumbo, C., 1973. Thermobacteriology in Food Processing, second ed. Academic Press, New York.