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.

CENTRO INTERDIPARTIMENTALE DI RICERCA INDUSTRIALE
AGROALIMENTARE

Cesena, 22.02.2019

Dear Editor,

Enclosed you will find a revised copy of the manuscript **"Modelling of yeast thermal resistance and optimization of the pasteurization treatment applied to soft drinks"** (by Montanari, C., Tabanelli, G., Zamagna, I., Barbieri, F., Gardini, A., Ponzetto, M., Redaelli, E., Gardini, F.).

Following your precious suggestions, the work has been improved setting up the logistic regression with new data obtained with 200 ml bottles inoculated with the same initial concentrations used for the first design. The preparation of this large number of samples is the reason for which I asked you to delay the revision submission. We think that this extra work enhance the relevance of the study. As you can see, the parameters obviously changed but the trends described by the model remained quite similar.

We modified the text accordingly in green, leaving at the same time the previous revisions along the text in red and blue, to have a trace of the work done over time.

Thanking you in advance,

Sincerely,

Giulia Tabanelli

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Detailed Response to Reviewers

Comments:

Line 29: microbial stability can be overestimated: this is still an unclear statement.
If the stability is overestimated, it is less stable than you think so underprocessing. This is exactly the opposite of what I think you want to say. Or is the thermal treatment overestimated ? Then also the product is less stable than expected so underprocessed. REFORMULATE so that it is crystal clear what you mean and also correct.
You write in your response that you changed it in can be overprocessed. (What you did not do). That is getting in the right direction but still microbial stability can not be overprocessed and also thermal treatment can not be overprocessed.

The phrase has been changed: "The microbial stability is guaranteed by thermal treatments. However, excessive heat treatments can affect food sensorial quality".

line 78: carbonated beverages are also not fresh-like. You could make it "less heavily processed".

Changed

Lines 216-222: This is NOT results. You do not agree. But the editor does. You do not start your results with a method used and a discussion why. It should be results. You can start the results with 223:
The absence of ascospores and the limited presence of budding cells in the stationary phase were verified with a microscope (see Supplementary Figures 1 and 2). Table 1 reports the results of modelling of the Weibull equation, being able also to describe non-linear kinetics. In particular, the estimates of the parameters of the model (<delta> and p), the RMSE and the value of R as well as the time predicted to reach a 5 log CFU/ml inactivation are reported.

Changed

Line 219: You should also discuss the point about the curvature: you mention it is an interesting aspect. So then you should discuss that (somewhere at the end of the discussion, not in the start of results)

Moved at lines 252-259.

Line 332: You have to discuss also the relevance of the VOLUME on the probability. You tested the probability of a survivor in 10 ml. A consumer package is 1000 ml. This influences the probability. You now reason based on 10 ml only.
The effect of the thermal treatment indeed is on the concentration. But for the probability of a survivor in a package the volume DOES count. This is simple logic. If this is not mentioned/discussed, the paper will not be accepted.

The experimental plan of the previous version has been maintained. However, the test tubes containing 10 ml of beverage (8 for each combination) have been replaced with 200 ml bottles (20 for each combination), the same size used for commercial products. The bottles were pre-heated but the time for reaching room temperature (approx. 25°C) using a water/ice bath were longer (approx. 4 min). The samples were stored for 30 days but spoiled bottled were detected only in the first 10/12 days, after which the situation did not change. The repeatability of the results, if compared with the first (10 ml tubes) trial, was extremely satisfactory (indeed higher than our expectation). On the other hand, we used some time ago the same strain to test the stability of beverages thermally treated in the presence of essential oils in two different works (Belletti et al., Applied and Environmental Microbiology 2007, 73, pp. 5580 – 5586; Belletti et al., 2010, Int. J.

Food Microbiol. 136, 283-289) carried out two years away and, using the predictive model obtained for some conditions (regarding the presence of citral) we found differences of 10% or less. However, we agree with the reviewer that this new trial, which requires the preparation of more than 1000 bottles, makes the results obtained more robust and the model extremely more reliable. Corrected tables and figures have been included with the new results obtained.

Given the similarity of the trends described by the previous data and the new logistic model, we used only the second set of data excluding the first one (obtained with 10 ml), also in relation to the dimension of samples both in terms of numbers and in terms of volumes.

Table 1: 109.89 was indicated to be too much digits, you now even increased. I understand it is a handy windows setting in one column to have the same number of digits. But 109.891+-22.907 is nonsense accuracy. It should be 110+-23 (or maybe 109.9+-22.9). But then do not change 0.318+-0.057 since that level of digits is fine !
5*10^-4 has too few significant figures. Please read the instructions on this and correct meticulously.
6.25 log cfu/ml is overdone again. 253.97 min is overdone accuracy, while 0.27 min should remain like that (or even have one more digit).
[If you would determine your weight as 75.3 kg and report it in grams you would also not write it as 75315.8 g, this is overdone accuracy and more difficult to interpret]

The number of decimals in tables and text have been changed.

Lines 279-284: It remains too much M&M. Methods and choices are described in the M&M. In the results you can have an introductory sentence linked to the M&M but not 6 lines.
Like:
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 Figure 1.

Changed

Minor comments
1: Modelling (modeling is US English modelling is UK English) also in the rest of the paper you have modelling.

Corrected

Line 336: 14.23 and 16.84 are overdone accuracy. Use only maximally 3 significant digits, that is already a bit overdone. By presenting it as 14.23 you suggest too large accuracy and also you decrease the fast interpretability of your data. (see also instructions for authors).

Corrected

146 the aw=0.991 why is this not still not added to the paper ?

Added to beverage characteristics

change non carbonated non-carbonated throughout figure 3 axis legends are not still not well readable even not in the high resolution version. Make font size MUCH bigger.

Changed

Highlights

- High variability in yeast thermal resistance was found

- A logit model described *S. cerevisiae* inactivation in relation to process parameters

- Thermal treatment can be optimized to limit heat damage and energy cost

Modelling 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.

Keywords: Thermal treatment; yeast; fruit beverages; Weibull model; logistic regression.

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; ICSMF, 2005; Stratford, 2005). 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 (Azaredo et al., 2016; ICSMF, 2005; Stratford, 2005). 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 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 seconds 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 hours (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). Ten 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 0.1M), 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

155 The thermo-resistant *S. cerevisiae* SPA was tested in specific industrial pasteurization conditions in orange
156 beverages, taking into account different inoculum levels (1, 2, 3 and 4 log CFU/ml), temperatures (58 °C,
157 59.5°C, 61°C, 62.5°C and 64°C) and the lengths of thermal treatment (5, 10 and 20 minutes).
158 Glass bottles (200 ml) were washed with a 3% (v/v) solution of H_2O_2 and rinsed with water before usage.
159 Beverages were prepared as described above and distributed into the bottles. Pre-cultured yeast (72 h at
160 pH 4) was re-suspended at defined concentrations (1, 2, 3 and 4 log CFU/ml) in bottles pre-heated in water
161 baths at the different temperatures (58, 59.5, 61, 62.5 and 64°C) and immediately closed with aluminum
162 caps. The bottles were maintained at the programmed temperatures for 5, 10 and 20 min.
163 For the inoculum, after a presumptive quantification of *S. cerevisiae* SPA culture with a Bürker chamber
164 (Brand GMBH, Wertheim, Germany), serial dilutions were performed in order to obtain the yeast
165 concentration desired for every combination. At the same time, a precise counting of the initial yeast
166 concentration was carried out by plate count on SDA incubated at 28°C for 72 h. After the heat treatment,
167 samples were rapidly cooled to room temperature in a water/ice bath (cooling time approx. 4 min) and
168 stored at room temperature (21±2°C) and observed periodically over a 30-day period for the presence of
169 cloudiness, cell sediment on the bottom or CO_2 due to yeast growth. After this storage time, where no
170 growth was observed, a sample of 0.3 ml was spreaded onto a SDA plate in order to qualitatively confirm
171 the yeast death. All the sixty combinations of the three variables were considered and 20 repetitions were
172 tested for each combination.
173
174 **2.5. Models development**
175 The Weibull equation was used to fit the survivor data for each temperature in the death kinetics, both in
176 SDB and in beverages:

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

177 where N_t and N_0 are the number of cells before the treatment and after the treatment time t. δ and p are
178 temperature dependent parameters: δ is the time of first decimal reduction while p is the shape parameter
179 (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)

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

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 to investigate 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 $\alpha + \sum \beta_j x_j$. 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 Figures 1 and 2).

Table 1 reports the results of modelling 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 Figures 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 modelable resistance at 55°C. However, at this temperature, the initial cell concentration decreased of 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

232 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,
233 respectively). Intermediate results were observed for *S'codes ludwigii* G1, which did not survive the
234 treatment at 65°C and had a $t_{5\ log}$ of 5.6 min at 55°C.
235 A great variability in the thermal resistance was observed among the strains belonging to the *S. cerevisiae*
236 species. For one of the strains (MCKL15), no survivor was detected even after few seconds of treatment at
237 65 and 60°C. The other strains were characterized by different death kinetics. The strain *S. cerevisiae* SPA
238 showed the highest thermo-resistance. In fact, the $t_{5\ log}$ was not predictable (> 500 min) at 55°C and was
239 37.1 and 4.7 min at 60 and 65°C respectively. The high thermo-tolerance of this strain is not surprising and
240 it can be related to the source of isolation. In fact, it was isolated from orangeade bottles after an
241 important industrial case of spoilage (Ndagijimana et al., 2004). The harsh characteristics of an industrial
242 environment can exert a selective pressure on the microbial population, able to select strains more
243 resistant to the stress factors. The prolonged and continuous application of high temperature (for both
244 beverage stabilization and pasteurization) can determine the survival of high thermo-resistant strains. The
245 *S. cerevisiae* strains L118, I3_2Y, 67G3 and 34G2, having comparable behaviours, were less resistant. The t_{5}
246 $_{log}$ 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
247 intermediate resistance and a significantly higher thermo-tolerance at 55°C ($t_{5\ log}$ 254.0 min).
248 Commercial heat preservation processes are based on the assumption that thermal death kinetics follow
249 the classical first-order inactivation model (Stumbo, 1973). However, the linear model does not always
250 provide an adequate description of non-linear kinetics usually associated to the process conditions applied
251 in the food industry, which should be minimized in order to achieve an overall better food quality (Buzrul,
252 2007). The results obtained in this work applying the Weibull model confirmed that the linear model does
253 not always provide an adequate description of death kinetics. The goodness of fit indicated by the
254 diagnostics reported in Table 1 indicates the reliability and the flexibility of this model that allows also the
255 description of linear kinetics (with p=1).
256 Literature regarding the thermal resistance of yeasts is relatively scarce. In addition, the use of different
257 suspension media and different operative conditions make comparisons extremely difficult.

Heat shock responses in yeasts has 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 threalose, 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 5–10 min, while at 65°C it was lower than 1 min (Deák, 2008b). Studies carried out by Beuchat (1982) demonstrated that temperatures between 48–51°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 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 Figure 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 Figure 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

310 (sensitivity=0.976). Then, after the decision of *c*, a more accurate examination of the predictive credibility
311 of the model was effectuated through cross validation. If it is fixed *c*=0.25, the correct classification rate is
312 0.886; and this result is confirmed by average value obtained through the cross-validation method (0.883).
313 These results obtained with the cross-validation procedure are reported in Figure 2. The coherence of the
314 two values is fundamental to exclude that the high classification rate might be due to model overfitting.
315 According to the fitted model, Figure 3 shows the probability of spoilage for the four initial inoculum levels
316 with respect to time and temperature. As it is possible to observe, at the lowest yeast concentration tested,
317 (1 log CFU/ml) there is a relatively wide combination of factors able to stabilize the beverage (in particular
318 temperatures higher than 62°C or times longer than 10 min), while temperatures below 59°C are scarcely
319 effective, independently of treatment length. Obviously, increasing yeast concentration reduce the efficacy
320 of the thermal treatment and this "safe zone" is extremely reduced when the inoculum is 4 log CFU/ml
321 (63.5-64°C for 18-20 min). At 64°C, a 5 min treatment was not able to prevent the spoilage of more than
322 50% of samples, while, according to the results of Table 1, in SDB, 5 min of a 65°C treatment were sufficient
323 to inactivate more than 5 log units of the same *S. cerevisiae* strain.
324 This trend is better evidenced by Figure 4, which reports the combinations of time and temperature
325 allowing 99% of stability (*P*=0.01). In this case, the non-continuous effect of yeast inoculum can be
326 evidenced by the noteworthy shift of conditions passing from 2 to 3 log CFU/ml. It is possible to
327 hypothesise that this shift can be attributed to the results of the phenotypic heterogeneity, typical also of
328 clonal populations, known as "molecular noise", which can lead to different behaviour among genetically
329 identical cells in a homogeneous environment, as extensively described by Koutsoumanis and Aspridou
330 (2017). The same authors noted that this stochastic variation in gene responses decreases with initial
331 population size increase. They stated that, in bacterial populations higher than 100 cell per ml, this
332 variability was almost eliminated.
333 In any case, considering the lower initial contamination levels (1 or 2 log CFU/ml), which are considered
334 more representative of the expected contamination of industrial fruit juices or soft-drinks, few minutes at
335 64°C were enough to guarantee a satisfying microbial stability to the beverages. If we take into
336 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 modelling 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.

364

365 **References**

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

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

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

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

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

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

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

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

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

388 • Couvert, O., Gaillard, S., Savy, N., Mafart, P., Leguerinel, I, 2005. Survival curves of heated bacteria
389 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 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.P., Brul, S., 2014. Thermal inactivation of microorganisms. Crit. Rev. Food Sci. Nutrit. 54, 1371-1385.

440 • Stratford, M., 2006. Food and beverage spoilage yeasts. In: Querol, A., Fleet, G. (Eds.), Yeasts in
441 food and beverages. Springer-Verlag, Berlin, pp. 335-379.
442 • Stumbo, C., 1973. Thermobacteriology in food processing, second ed. Academic Press, New York.
443

Figure caption:

Figure 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.

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

Figure 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.

Figure 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.

Table

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 (6.7 log CFU/ml)	**55°C**	109.9 (22.9)[a]	0.569 (0.110)	>500	0.876	0.098	*Sch. pombe* V1 (6.3 log CFU/ml)	**55°C**	2.50 (0.39)	0.515 (0.054)	56.9	0.974	0.290
	60°C	4.94 (0.50)	0.798 (0.050)	37.1	0.894	0.253		**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**	$5.3x10^{-4}$ ($6.4x10^{-5}$)	0.204 (0.014)	1.3	0.997	0.121
S. cerevisiae Cw (6.8 log CFU/ml)	**55°C**	12.0 (0.5)	0.527 (0.026)	254.0	0.993	0.216	*S'codes ludwigii* G1 (6.4 log CFU/ml)	**55°C**	3.75 (0.16)	2.285 (0.146)	5.6	0.997	0.116
	60°C	1.42 (0.12)	0.977 (0.058)	7.3	0.998	0.135		**60°C**	0.014 (0.004)	0.347 (0.028)	1.5	0.996	0.149
	65°C	$7.1x10^{-7}$ ($2.3x10^{-8}$)	0.119 (0.030)	0.27	0.993	0.318		**65°C**	-[b]	-	-		
S. cerevisiae L118 (6.5 log CFU/ml)	**55°C**	9.92 (0.86)	0.825 (0.065)	69.7	0.985	0.174	*K. marxianus* YC (6.3 log CFU/ml)	**55°C**	7.93 (0.53)	0.916 (0.058)	46.0	0.992	0.175
	60°C	0.600 (0.101)	0.751 (0.062)	5.1	0.982	0.431		**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**	$2.2x10^{-4}$ ($6.5x10^{-5}$)	0.203 (0.041)	0.42	0.991	0.271
S. cerevisiae I3_2Y (6.3 log CFU/ml)	**55°C**	5.25 (0.47)	0.774 (0.042)	42.0	0.990	0.224	*Z. bisporus* EN (6.7 log CFU/ml)	**55°C**	0.125 (0.038)	0.486 (0.017)	3.4	0.959	0.590
	60°C	0.222 (0.032)	0.558 (0.046)	3.9	0.982	0.315		**60°C**	-	-	-	-	
	65°C	$3.04x10^{-4}$ ($6.3x10^{-5}$)	0.221 (0.018)	0.44	0.986	0.352		**65°C**	-	-	-	-	
S. cerevisiae 67G3 (6.2 log CFU/ml)	**55°C**	4.15 (0.61)	0.568 (0.061)	70.6	0.973	0.282	*Z. mellis* M1 (6.5 log CFU/ml)	**55°C**	-	-	-	-	
	60°C	0.124 (0.024)	0.366 (0.040)	10.1	0.981	0.335		**60°C**	-	-	-	-	
	65°C	0.054 (0.009)	0.510 (0.098)	1.3	0.982	0.381		**65°C**	-	-	-	-	

S. cerevisiae 34G2 (6.2 log CFU/ml)	**55°C**	9.34 (0.97)	0.821 (0.088)	66.3	0.975	0.252	*Z. rouxii* YF1 (6.5 log CFU/ml)	**55°C**	-	-	-	-
	60°C	0.491 (0.090)	0.513 (0.032)	11.3	0.993	0.220		**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 (6.3 log CFU/ml)	**55°C**	1.03 (0.12)	0.679 (0.065)	12.0	0.988	0.304						
	60°C	-	-	-								
	65°C	-	-	-								

[a]: standard error; [b]: No survivor data available

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

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				

Figure

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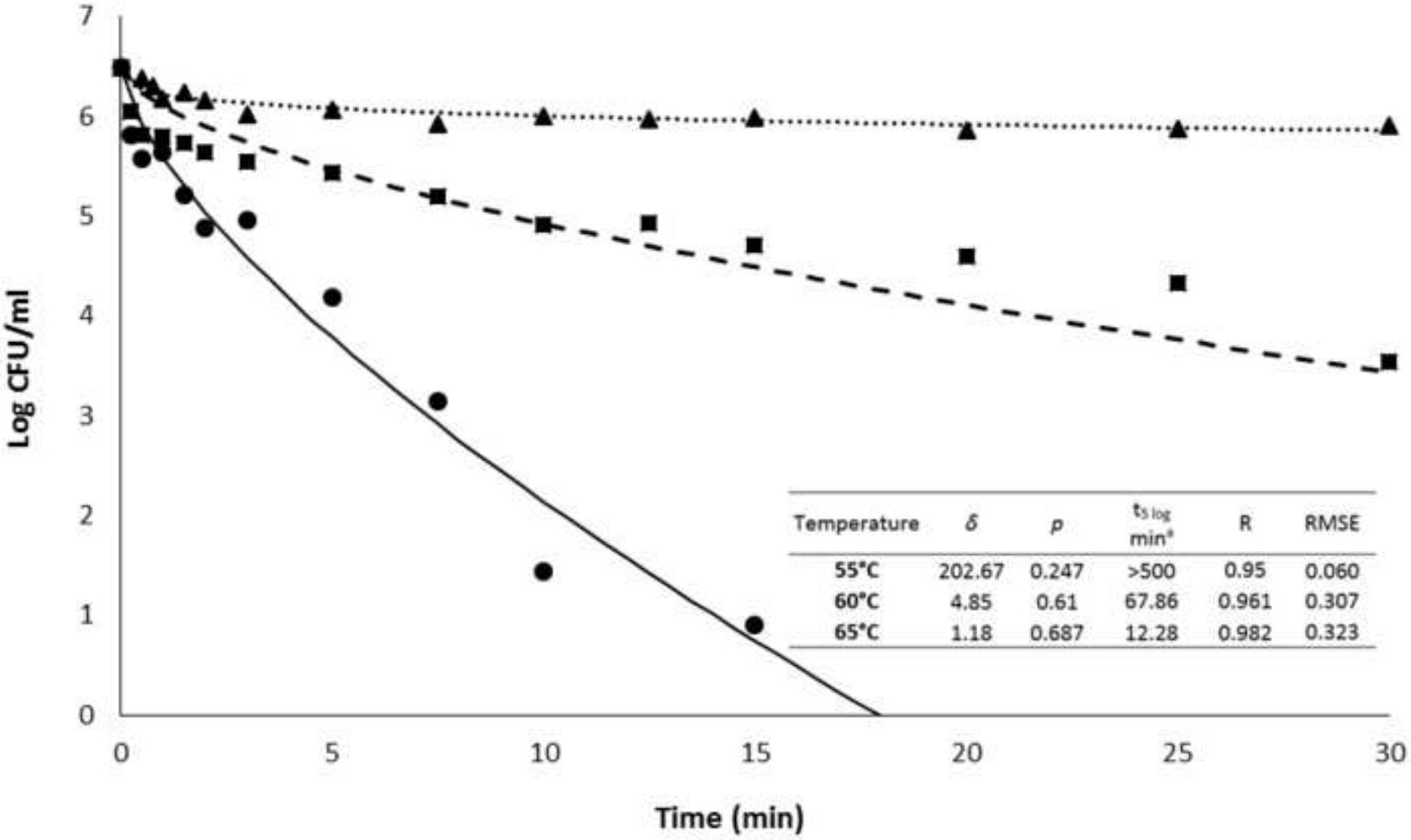

Temperature	δ	p	$t_{5\log}$ min^{a}	R	RMSE
55°C	202.67	0.247	>500	0.95	0.060
60°C	4.85	0.61	67.86	0.961	0.307
65°C	1.18	0.687	12.28	0.982	0.323

Figure
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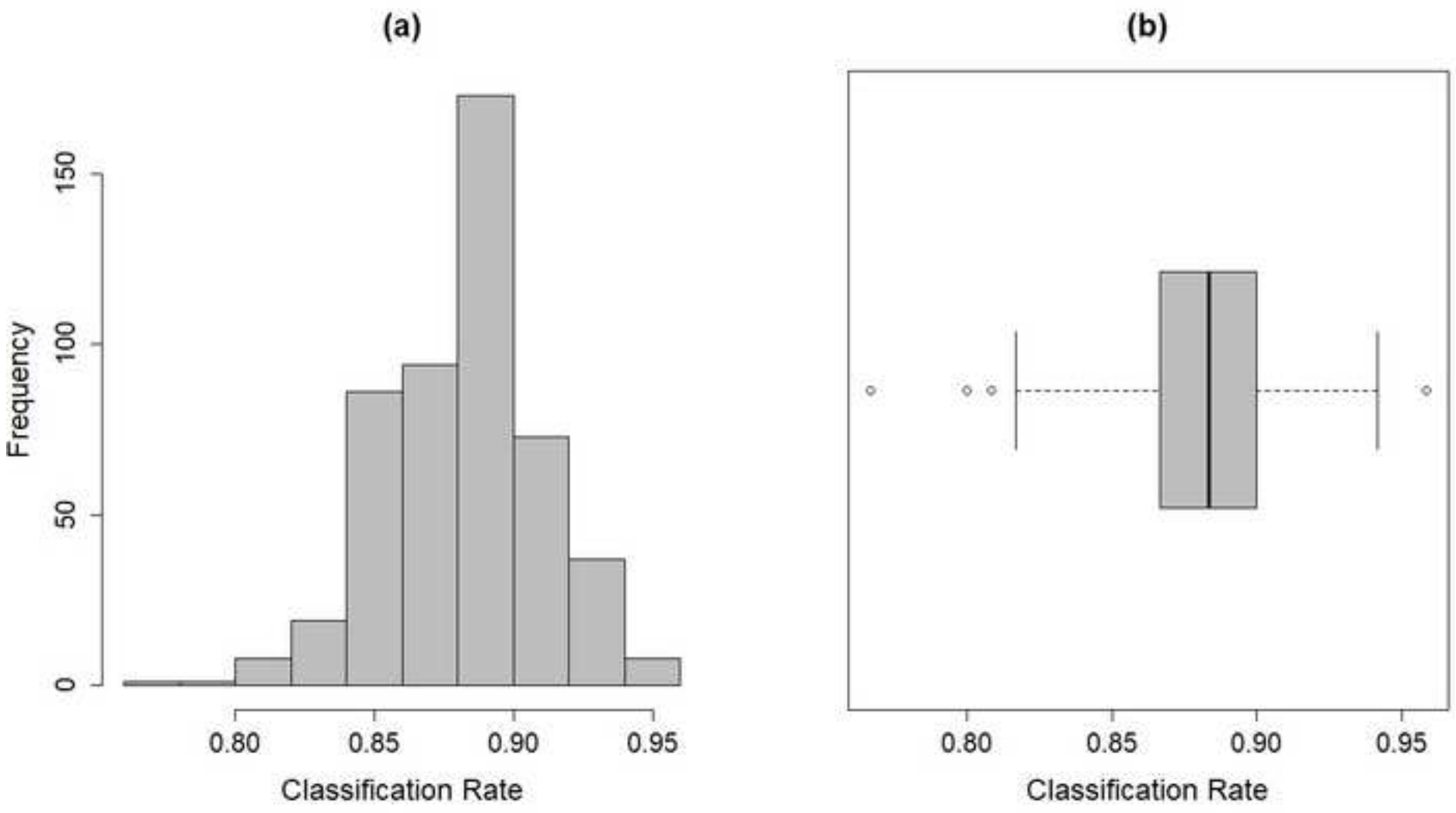

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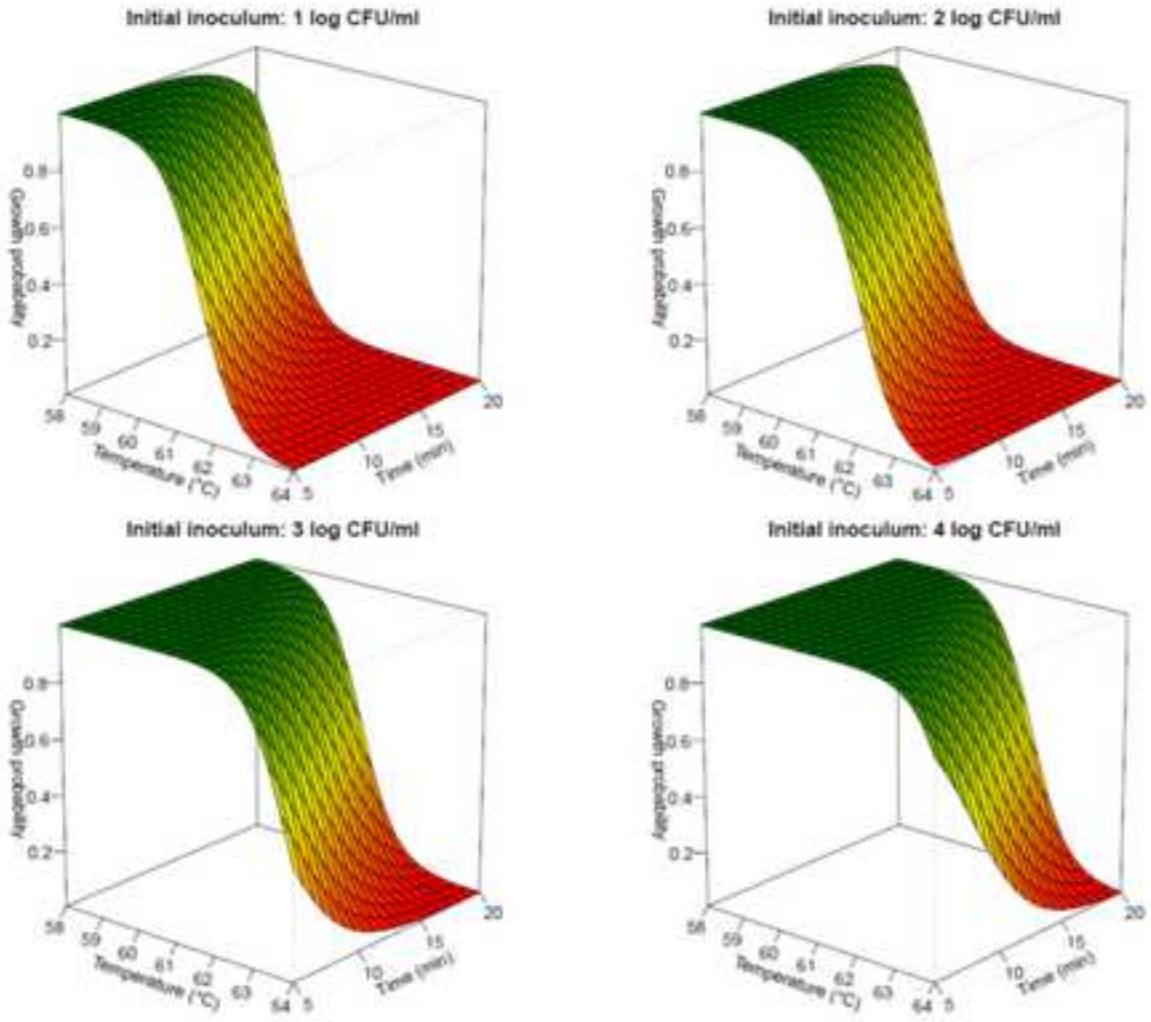

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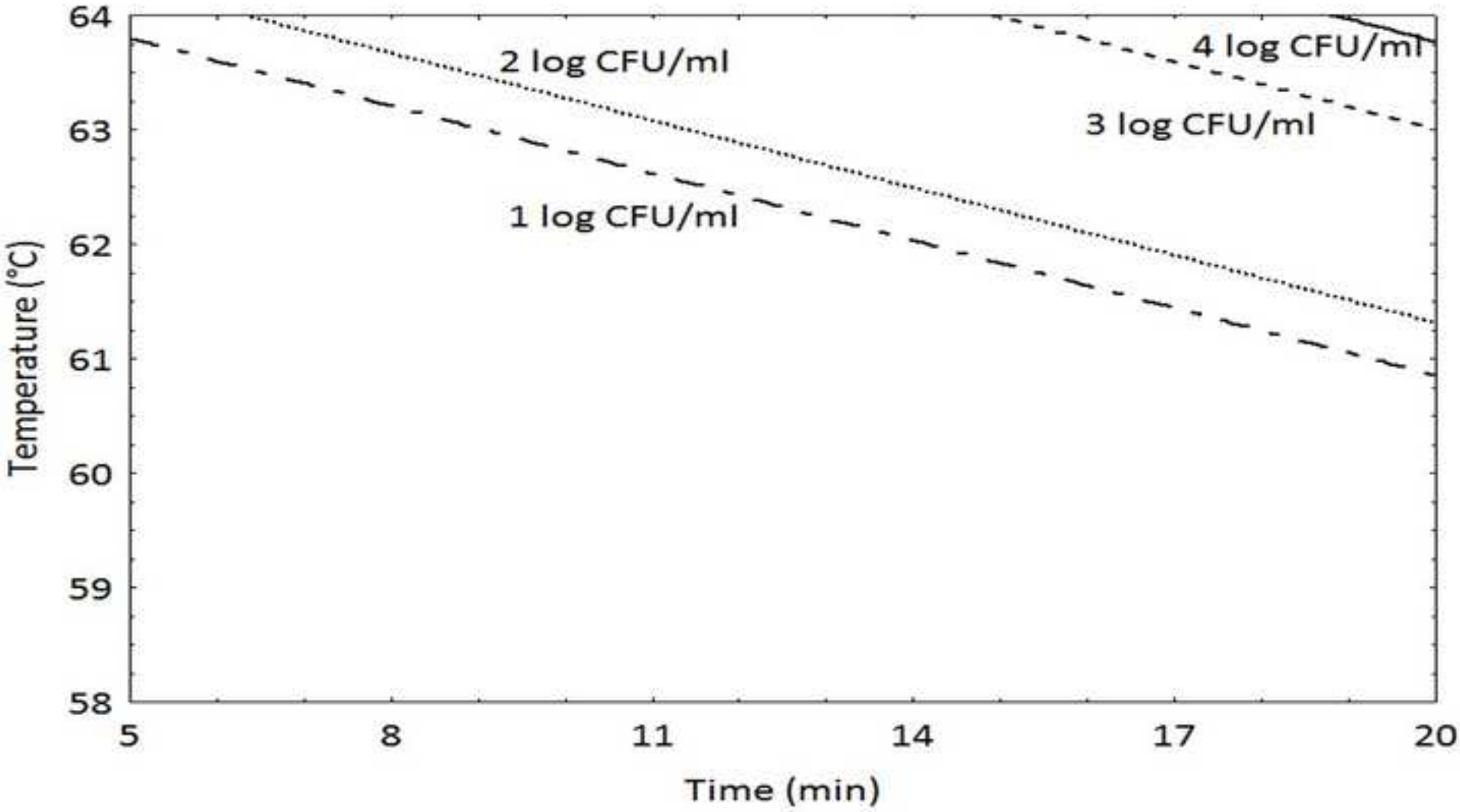
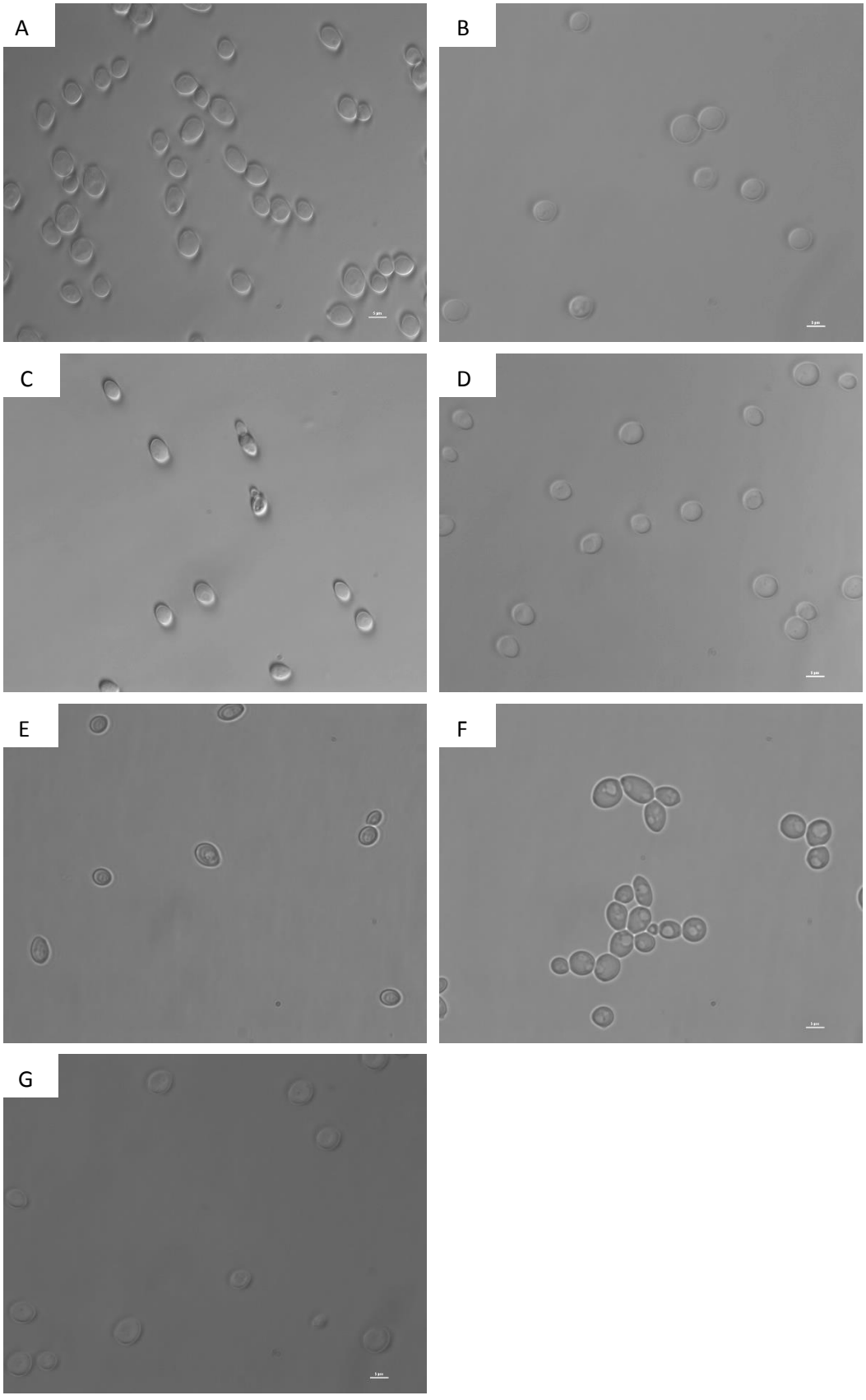


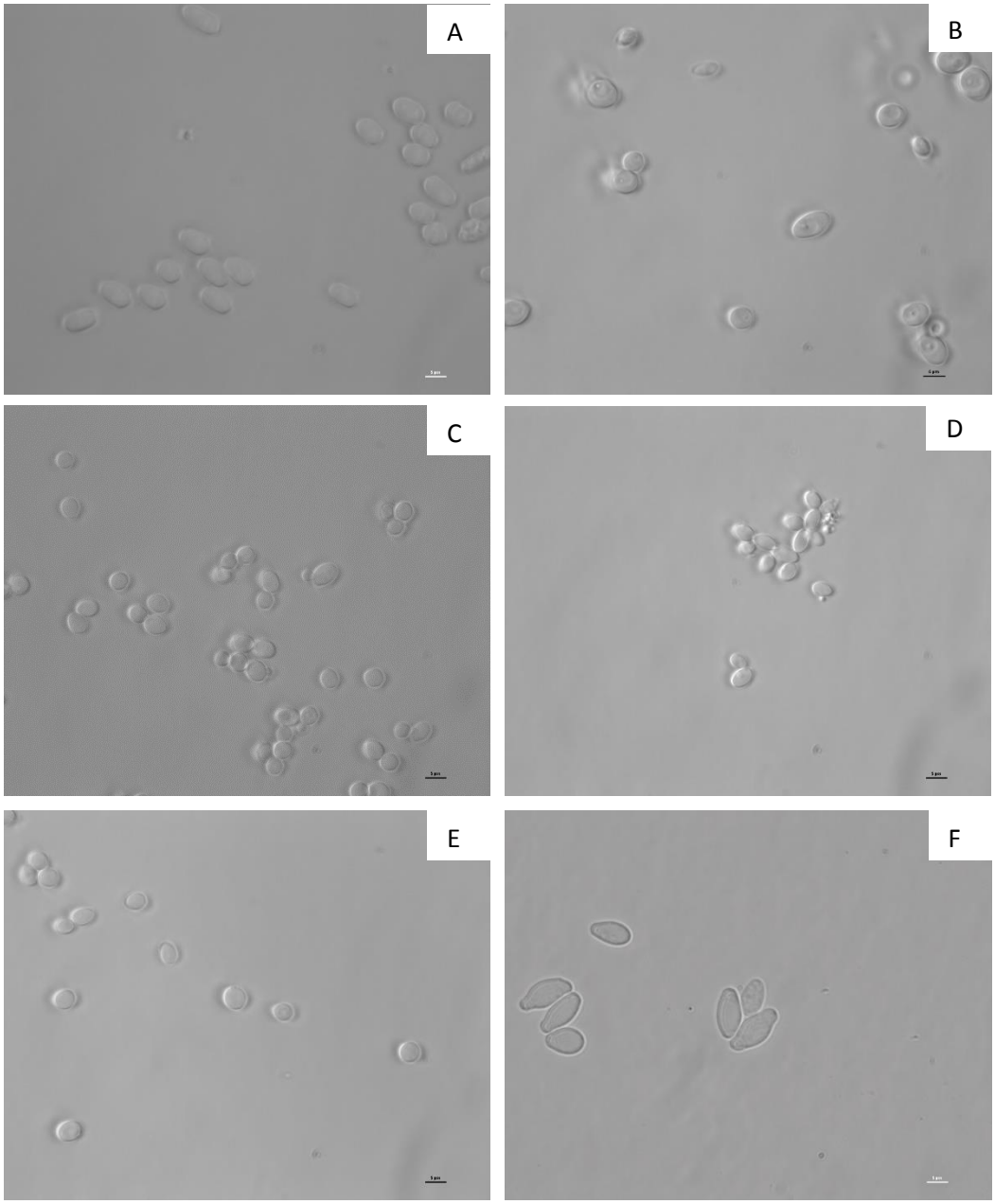
Figure

Supplementary figure 1: Photographs of the *S. cerevisiae* strains after 72 h of incubation in SDB (magnification of 1000× in phase contrast). A) *S. cerevisiae* SPA; B) *S. cerevisiae* Cw; C) *S. cerevisiae* I3_2Y; D) *S. cerevisiae* L118; E) *S. cerevisiae* 67G3; F) *S. cerevisiae* 34G2; G) *S. cerevisiae* MCKL15.



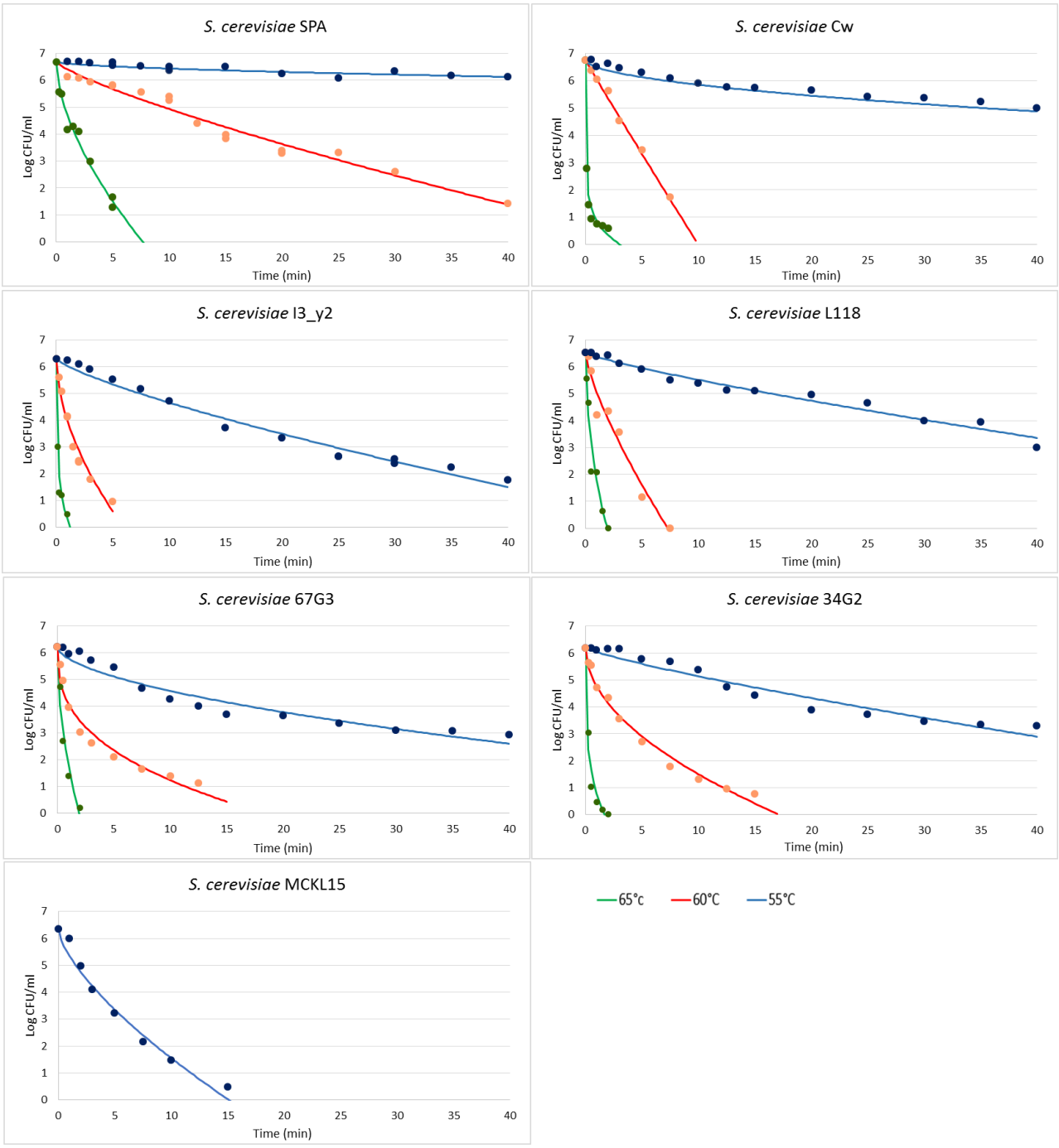
Figure

Supplementary figure 2: Photographs of the Non-*Saccharomyces* strains after 72 h of incubation in SDB or 120 h for *Zygosaccharomyces* in SDB enriched with 20% (w/w) glucose (magnification of 1000× in phase contrast). A) *Sch. pombe* V1; B) *K. marxianus* YC; C) *Z. bisporus* EN; D) *Z. mellis* M1; E) *Z. rouxii* YF1; F) *S'codes ludwigii* G1



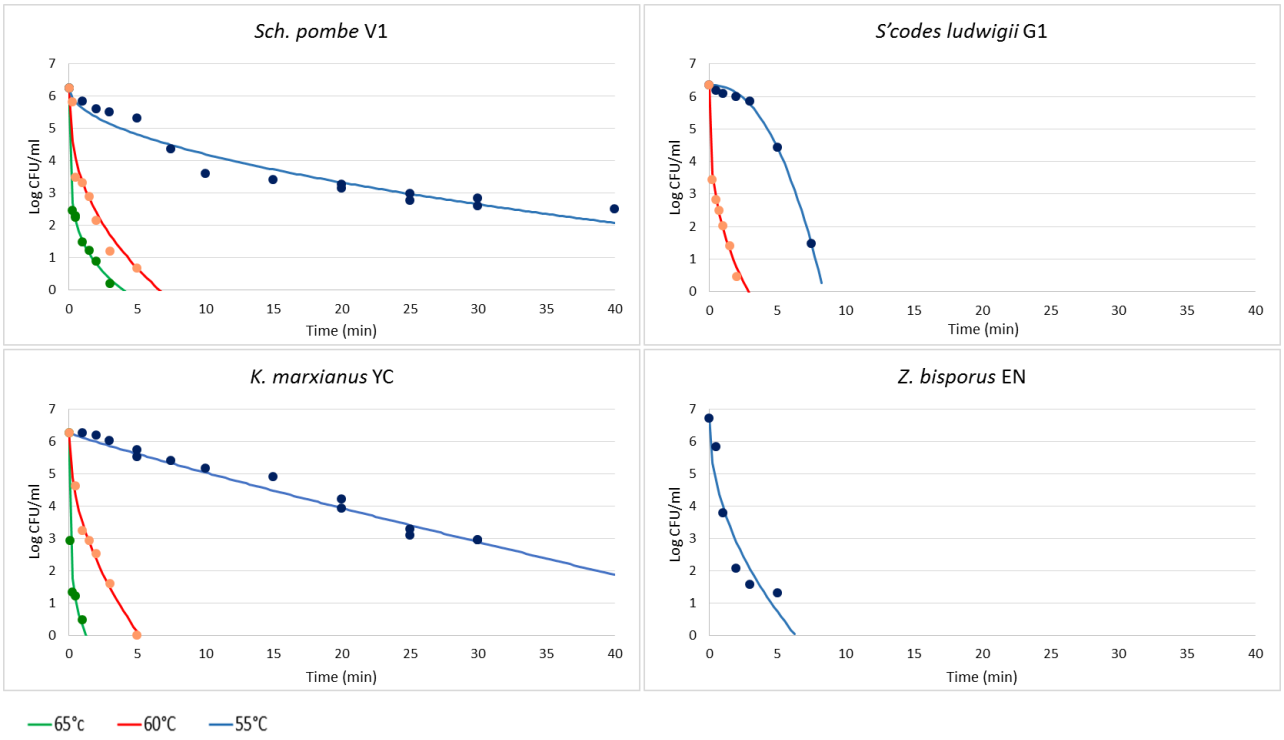
Figure

Supplementary figure 3: Modeling with the Weibull equation of the inactivation curves at 55, 60, 65°C for *Saccharomyces* strains in SDB. In each figure the experimental data and the fitted model are reported.



Figure

Supplementary figure 4: Modeling with the Weibull equation of the inactivation curves at 55, 60, 65°C for non-*Saccharomyces* strains in SDB. In each figure, the experimental data and the fitted model are reported.



Figure