Evaluation of the Listeria monocytogenes inactivation during post-process storage of fermented sausages: A basis for the development of a decision support tool

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Evaluation of the *Listeria monocytogenes* inactivation during post-process storage of fermented sausages: A basis for the development of a decision support tool

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Abstract

In situ quantitative data on *L. monocytogenes* survival during storage of vacuum-packaged fermented sausages at various temperatures were collected from the literature to develop a generic predictive model regarding its fate at a specific storage temperature. The development of the tool was based on the z-concept. The time needed for 4D reduction of the pathogen was estimated and its influence by the temperature was further described by linear regression. A secondary model was developed for describing the effect of sausage water activity on the z-concept parameters at the reference temperature. The decision support tool was successfully validated against the studies not used during the development of the model. Based on the model predictions, a decision can be made about the required time of product storage before its distribution to achieve an additional pathogen inactivation. Such tools can be incorporated in a HACCP plan of a food-producing company to assure food safety.

Keywords: Food safety, HACCP, non-thermal inactivation, predictive model, storage

1. Introduction

The role of the processes of fermentation and ripening of fermented meats is twofold, contributing to the organoleptic characteristics of the products, but also to its stability. Insufficient fermentation and/or maturation of the product may lead to an unsafe final product because pathogenic bacteria can survive in the process. There are instances, however, where fermentation and ripening are successful, but the conditions (pH and water activity in combination with fermentation temperature) prevailing during manufacturing facilitate the adaptation and better survival of pathogenic bacteria such as *Listeria monocytogenes* and *Salmonella enterica* (Mataragas et al., 2014a, Submitted for publication; Mataragas et al., 2014b). In those situations where the desired reduction of the pathogen is not induced by the current practices of fermentation and ripening, the existence of a mechanism which would allow further reduction of the pathogen in the finished product in order to enhance consumer safety would be highly beneficial for Food Business Operators (FBOs).

L. monocytogenes may contaminate food products at different steps of the manufacturing process since the organism is able to survive on equipment and in production facilities (Samelis & Metaxopoulos, 1999). The presence of the pathogen has been reported throughout the process of manufacturing fermented sausages (Buncic et al., 1991; Glass & Doyle, 1989; Hew et al., 2005). It has not unambiguously been shown to have caused listeriosis from the consumption of fermented sausages, and pathogens like salmonellae and STEC/VTEC are much more relevant to these products (Adams & Mitchell, 2002). Nevertheless, the detection of *L. monocytogenes* in fermented sausages may cause considerable problems to the manufacturer, and therefore, there is much interest in eliminating this bacterium, and the present study is topical.

Studies have shown that *L. monocytogenes* inactivation in fermented sausage is higher during its storage at ambient temperature (25^{o}C) compared to chilled temperatures (4^{o}C) (Gounadaki et al., 2007). The aim of this work was to gather from the literature the available quantitative data referred to *in situ* survival of *L. monocytogenes*, during storage of vacuum-packaged fermented sausage at various temperatures, and develop a generic model predicting the fate of the pathogen at a specific storage temperature. Based on the product characteristics, a suggestion is made about the storage conditions to be applied to achieve the additional reduction required. The tool will allow FBOs to introduce into their production process an additional step of *L. monocytogenes* inactivation before the final distribution of the product by keeping it at a specific temperature to ensure that the desired inactivation of *L. monocytogenes* is attained.

2. Materials and methods

2.1. Collection of data

Papers written in English and published until the execution of the current work were considered. Studies included were those reporting quantitative data relative to *in situ* survival of *L. monocytogenes* during storage of vacuum-packaged fermented sausages at various temperatures, and furthermore allowed the determination of the inactivation rate (per day). This meta-analysis involved fermented sausages inoculated with different *L. monocytogenes* strains being in different physiological state at the time of inoculation facilitating the determination of one or more inactivation rates in some cases. In addition, all fermented sausages were manufactured with the addition of starter cultures and nitrite. The search of the relevant studies was performed by consulting various literature databases, such as Sciencedirect, Scopus and PubMed.

Keywords used alone or in combination were "*Listeria monocytogenes*, non-thermal inactivation, survival, storage, fermented sausages, fermented meats and salami". Literature lists of the found relevant papers were also searched to uncover any additional publications.

As far as possible, the following information was extracted from the published works: number of *L. monocytogenes* strains inoculated in the sausages, type of fermented sausage, number of different inactivation rates determined, storage temperature conditions, storage duration, pH and water activity (a_w) range of each fermented sausage, and *L. monocytogenes* survival data.

2.2. Modeling L. monocytogenes inactivation during storage and parameters estimation

For each experimental case, representing a set of conditions (fermented sausage, storage temperature condition and physiological state of the inoculums), an inactivation rate of *L. monocytogenes* was determined by plotting pathogen's viability data (log CFU/g or cm^2) versus time. Two models describing the *L. monocytogenes* inactivation pattern observed in the studies were used, the log-linear (Bigelow & Esty, 1920) and biphasic (Cerf, 1977). The log-linear model is

$$N_t = N_0 - \frac{k_{max} \times t}{ln(10)} \qquad (1)$$

where N_t is the cell counts (log CFU/g or cm^2) at time t; N_0 is the initial population (log CFU/g or cm^2); t, the time (days); and k_{max}, the specific inactivation rate (per day) of the pathogen.

The biphasic model is

$$N_t = N_0 + log_{10}\{(f \times e^{-k_{max\ 1} \times t}) + [(1-f) \times e^{-k_{max\ 2} \times t}]\} \qquad (2)$$

where *f* is the fraction of initial population in the major population, 1 - *f* is the fraction of population in the subpopulation, and k_{max1} and k_{max2} are the inactivation rates of the major population and subpopulation, respectively (per day).

Because the inactivation curve of *L. monocytogenes* was not linear in all cases, the use of inactivation rates (k_{max}) and/or *D* values as a common metric between studies was difficult since they were not comparable, thus, the time (in days) needed for a 4-log reduction (t_{4D}) in the *L. monocytogenes* population was used (Buchanan et al., 1994). Model fitting by non linear regression and t_{4D} parameter calculation were carried out with the GInaFiT v1.6 Microsoft Excel 2007 (Microsoft, Redmond, WA, USA) add-in software (Geeraerd et al., 2005). The *L. monocytogenes* viability data (log CFU/g or cm^2) were extracted from the corresponding figures or tables of the published works. For the extraction of the data from the published figures the Ungraph 5 (Biosoft, Cambridge, UK) software was used.

2.3. Decision support tool development and validation

The development of the tool was based on the z-concept:

$$z_{4D} = \frac{\left(T - T_{ref}\right)}{log_{10}\left(t_{4D\ at\ T_{ref}}\right) - log_{10}(t_{4D\ at\ T})} \qquad (3)$$

Parameterization of the above equation, it gives:

$$t_{4D\ at\ T} = 10^{\left\{log_{10}\left(t_{4D\ at\ T_{ref}}\right) - [(T - T_{ref})/z_{4D}]\right\}} \qquad (4)$$

where z_{4D} is the temperature (°C) required for the non-thermal inactivation curve to move 1 log cycle; T_{ref} is the reference temperature (°C); $t_{4D\ at\ Tref}$ is the time (days)

needed for a 4-log reduction of the pathogen at the reference temperature; and T is the storage temperature at which the $t_{4D\ at\ T}$ parameter is estimated.

After calculating the t_{4D} parameter for each experimental case, a simple linear regression was used to model $\log(t_{4D})$ vs. temperature (T) [$\log(t_{4D}) = f(T)$] for determining the z_{4D} values for each study used to developed the model. The strength of the relationship was assessed by the coefficient of determination (R^2). A secondary model was developed for modeling the effect of the water activity (a_w) of fermented sausages on the z_{4D} and $(t_{4D})^{1/2}$ values at 25°C (reference temperature). Multiple regression was employed to identify significant predictors of the secondary model (predictor variables = mean values of pH and a_w of the final product, and response variable = t_{4D} or z_{4D} values). The reference temperature was chosen by comparing the observed and predicted values of the t_{4D} parameter at each temperature condition. Predicted values for the t_{4D} parameter were obtained from the Pathogen Modeling Program (PMP) 7.0 based on the storage conditions (temperature and vacuum packaging) and the mean value of the intrinsic properties of the final product (pH, a_w and nitrite). The temperature at which the t_{4D} parameter was within an acceptable prediction range based on the bias (B_f) and accuracy (A_f) factors (Ross, 1996) was selected as reference temperature.

A part of the studies found and temperatures were kept for validation purposes. The performance of the developed decision support tool was assessed by the root mean square error (RMSE), the bias (B_f) and accuracy (A_f) factors (Ross, 1996), and plotting observed against predicted values. Linear and multiple regression analysis were performed by using the GraphPad Prism 5.04 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS v15.0 (SPSS, Inc., Chicago, Ill., USA) computer-based programs.

3. Results and Discussion

3.1. Collection of data

Seven studies reporting on *in situ* non-thermal inactivation of *L. monocytogenes* during storage of fermented sausages at various temperatures were finally found (Table 1). Some of them contained more than one data set allowing the determination of 32 inactivation rates, in total, including 22 different *L. monocytogenes* strains, storage temperatures ranged from 4 to 30^{o}C, pH ranged from 4.5 to 5.0 and a_w ranged from 0.82 to 0.97. The limits, however, of the developed model were different regarding a_w (from 0.82 to 0.92).

3.2. Modeling L. monocytogenes inactivation during storage and parameters estimation

The inactivation rates were derived from linear or biphasic inactivation curves between *L. monocytogenes* viable counts and storage time at isothermal temperature conditions (Fig. 1). This is in agreement with published works investigating the *in situ* non-thermal inactivation of the pathogen during manufacturing of fermented sausages (Degenhardt & Sant' Anna, 2007; Drosinos et al., 2006; Gareis et al., 2012; Mataragas et al., 2014a, Submitted for publication; Mataragas et al., 2014b). In those studies the inactivation curve varies between linear and various shapes of a non linear pattern indicating the potential presence of a more resistant subpopulation due to stress adaptation. Due to the different shape of the inactivation curves observed between studies, the t_{4D} parameter was calculated as common metric to describe the reduction rate of *L. monocytogenes* during storage of the fermented sausages.

3.3. Decision support tool development and validation

The observed values of the t_{4D} parameter were compared to the predicted t_{4D} as estimated by the PMP program. It was found that better matching between observed and predicted values were achieved at the temperature of 25°C based on the B_f and A_f values being in an acceptable prediction range (Table 2). Therefore, this temperature was selected as reference temperature (T_{ref}). At the temperature of 4°C the t_{4D} parameter was underestimated whereas at 12°C was overestimated. This would owing to strain and physiological state variation, use of different starter cultures, and presence of antimicrobial agents other than nitrite. For instance, the use of bacteriocinogenic lactic acid bacteria as starters significantly affected the *L. monocytogenes* survival during fermented sausages production (Drosinos et al., 2006). The z_{4D} values were determined by simple linear regression of $\log(t_{4D})$ versus temperature (T) as -1/slope. The modeling of $\log(t_{4D}) = f(T)$ showed good correlation (Fig. 2). The next step was to find significant predictors for the z_{4D} and the $t_{4D\ at\ 25^oC}$ parameters. Multiple regression was run in two stages. Firstly, it was run to identify which parameters influence the z_{4D} and the $t_{4D\ at\ 25^oC}$ (enter method) and then a stepwise method to identify which of the parameters is significant. The results showed that pH and a_w influence the z_{4D} (both explaining 99.3% of the variability in the data) and the $t_{4D\ at\ 25^oC}$ (both explaining 79.8% of the variability in the data). The stepwise method, however, revealed that z_{4D} and the $t_{4D\ at\ 25^oC}$ were dependent only on a_w (P = 0.041, explained variability = 92% and P = 0.007, explained variability = 79.3%, respectively) and not on pH (P = 0.266 and 0.769, respectively). Hansen et al. (2011) found that z values were dependent on salt and starter culture, but not on pH during challenge experiments with VTEC and storage of fermented sausages at various temperatures. Therefore, a_w was finally used as predictor variable to model z_{4D} and

$(t_{4D})^{1/2}$ at 25°C (secondary model) using the following equation (Mataragas et al., 2006):

$$z_{4D} or \sqrt{t_{4D\ at\ 25^{o}C}} = b \times \left(a_w - a_{w_{min}}\right) \quad (5)$$

where *b* is the slope of the regression line for z_{4D} or $(t_{4D})^{1/2}$ at 25°C; and a_{wmin} is the theoretical minimum a_w value where z_{4D} and $(t_{4D})^{1/2}$ at 25°C are equal to zero. The parameter estimates of the secondary model and its performance (R^2 and RMSE) are displayed in Table 3. Finally, Fig. 3 presents the comparison between [z_{4D} or $(t_{4D\ at\ 25^{o}C})^{1/2}$]$_{observed}$ and [z_{4D} or $(t_{4D\ at\ 25^{o}C})^{1/2}$]$_{predicted}$ values. The $(RMSE)_{z4D}$ was equal to 2.83 whereas the $(RMSE)_{(t4D)^{1/2}}$ was 0.251 for the reference temperature (25°C). Tool validation was performed with data kept aside during its development. The results showed good predictions by the model for the temperature range between 4 and 30°C resulting in B_f and A_f equal to 1.08 and 1.28, respectively (Fig. 4). B_f values above 1 indicate fail-safe predictions since an overestimation of the time needed for a 4-log reduction of *L. monocytogenes* is made. According to Ross (1999), models with B_f from 0.9 to 1.0 or 1.0 to 1.05 are considered as adequate whereas for B_f from 0.7 to 0.9 or 1.06 to 1.15 are acceptable. Furthermore, $A_f \leq 1.5$ is also acceptable (Hansen et al., 2011). Therefore based on the post-processing a_w of the fermented sausage, a prediction on the z_{4D} and $(t_{4D\ at\ 25^{o}C})^{1/2}$ parameters can be derived, which in combination with the desired storage temperature, an estimation for the time needed to reduce *L. monocytogenes* further by 4 logs can be given by the tool. Fig. 5 provides the time/temperature combinations leading to 4-log reduction of *L. monocytogenes* based on different post-processing a_w.

The results showed that the developed decision support tool was successfully validated. Caution, however, is needed when extrapolating predictions outside the

model limits (vacuum-packaged fermented sausages; post-ripening pH and a_w from 4.5 to 5.0 and 0.82 to 0.92, respectively; and storage at temperatures from 4 to 30^{o}C). For instance, the fermented sausage from the study of Lindqvist and Lindbland (2009), which used for validation purposes. The product was stored at 8 and 22^{o}C and its post-processing a_w was equal to 0.96-0.97. The tool was successfully validated at the elevated temperature of 22^{o}C, but the prediction error at the lower temperature of 8^{o}C was higher since the point was further away from the 45^o diagonal line (Fig. 4). The developed tool can be considered as a baseline model, which can be expanded by incorporating additional data from future studies relative to the *in situ L. monocytogenes* inactivation at conditions outside of the model domain and/or its prediction accuracy can be greatly enhanced by additional data within model domain. In this way the usefulness and workableness of the tool is increasing.

4. Conclusions

The developed $t_{4D\ at\ 25^oC}$ and z_{4D} models can be used to predict the desired time temperature combinations that lead to additional *L. monocytogenes* reduction during storage of fermented sausages. The decision support tool can predict the fate of *L. monocytogenes* at a specific storage temperature and based on that prediction a decision can be made (corrective action) about the time needed to store the product before its distribution in order to achieve an additional desired pathogen inactivation. Subsequently, such tools can be incorporated in HACCP plans of food-producing companies to assure the safety of their products. It should be noted, however, that the developed model is valid within the limits used for its development, i.e. various physiological states of the *L. monocytogenes* (non acid-adapted, acid-adapted, partially acid-adapted, non habituated and habituated in the environment of salami),

vacuum-packaged fermented sausages with post-ripening pH and a_w from 4.5 to 5.0 and 0.82 to 0.92, respectively, and storage at temperatures from 4 to 30^{o}C. For instance, if we use the tool for a fermented sausage with pH equal to 4.2, the obtained result could be not valid. This value is out of the model limits. Therefore, this factor should be checked again for its significance as predictor. Therefore, practitioners should understand the limitations of the model in order to use model predictions and interpretation of its results with caution.

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Figure legends

Fig. 1. Representative *in situ* inactivation curves of *L. monocytogenes* during storage of fermented sausage at a) 4^{o}C (Simpson et al., 2008) and b) 5^{o}C (Gounadaki et al., 2007) described by the biphasic and log-linear model, respectively. Closed circles are the observed data; solid line is the values estimated by the model; R^2 is the coefficient of determination; RMSE is the root mean square error; and t_{4D} is the time (days) needed for a 4-log reduction of the pathogen

Fig. 2. Modeling the log(t_{4D}) parameter as function of temperature by a simple linear regression. Closed circles are the observed data from a) Gounadaki et al. (2007), b) Simpson et al. (2008), c) Byelashov et al. (2009) and d) Porto-Fett et al. (2008); solid line is the regression line; R^2 is the coefficient of determination; and z_{4D} is the temperature (^{o}C) required for the non-thermal inactivation curve to move 1 log cycle and it is defined as -1/slope

Fig. 3. Comparison between the observed and estimated values of the a) z_{4D} and b) $(t_{4D\ at\ 25^{o}C})^{1/2}$ parameters

Fig. 4. External validation of the developed decision support tool for various storage temperatures from 4 to 30^{o}C by plotting the observed and predicted values of the t_{4D} parameter

Fig. 5. Time/temperatures combinations as estimated by the decision support tool leading to 4-log reduction of *L. monocytogenes* for various post-processing a_w values of the fermented sausages and storage temperature conditions used for validation

purposes. Solid line, the predicted values; dashed lines; the 95% confidence interval of the regression line

Table 1

Published studies included in the meta-analysis of the *in situ L. monocytogenes* inactivation during storage of fermented sausages[a] at various temperatures.

Reference	No. of strains	*L. monocytogenes* strain	Type of fermented sausage	No. of rates	Range			Comments
					Temperature (^{o}C)[b]	pH[c]	a_w[c]	
Model development								
Byelashov et al., 2009	10	N1-225, N1-227, R2-500, R2-501, R2-763, R2-764, R2-765 (all serotype 4b), 558 (serotype 1/2), NA-1 (serotype 3b), N-7150 (serotype 3a)	Pepperoni (American-style)	9	4, 12 and 25	4.5-4.8	0.83-0.85	Three different physiological states of the inoculated *L. monocyogenes*: acid-adapted, extract-habituated and non habituated.
Simpson et al., 2008	10	N1-225, N1-227, R2-500, R2-501, R2-763, R2-764, R2-765 (all serotype 4b), 558 (serotype 1/2), NA-1 (serotype 3b), N-7150 (serotype 3a)	Italian-style fermented sausage	12	4, 12 and 25	4.6-4.9	0.89-0.92	Four different physiological states of the inoculated *L. monocytogenes*: non acid-adapted, acid-adapted, partially acid-adapted and habituated.
Gounadaki et al., 2007	1	Scott A (serotype 4b)	Greek-style fermented sausage	3	5[d], 15[d] and 25	4.5-4.6	0.87-0.90	The data from the low inoculum and the aerobic storage of the inoculated fermented sausage were not considered.
Porto-Fett et al., 2008	5	MFS2, MFS102, MFS104, MFS105, MFS110	Soudjouk-style fermented sausage	4	4, 10[d], 21[d] and 30[d]	4.8-5.0	0.82-0.85	-
Model validation								
Barazi & Erkmen, 2008	2	ATCC 13932 and a strain of serotype 4a	Sucuk	1	4	5.0	0.84	The data from the fermented sausage stored under air or various modified atmospheres or made without starter cultures and stored under air were not considered.

Lahti et al., 2001	1	A strain of serotype 4b	Dry fermented sausage	1	15	4.7-4.8	0.92	-
Lindqvist & Lindblad, 2009	3	L8, L58, L67	Dry fermented sausage	2	8 and 22	4.5-4.7	0.96-0.97	The data from the inactivation in broths were not considered.

[a] Only vacuum-packaged fermented sausages and manufactured with the addition of starter cultures and nitrite were considered

[b] Storage temperature

[c] Values of the final commercial product

[d] Temperatures used for validation of the decision support tool

Table 2

Comparison between $(t_{4D})_{observed}$ and $(t_{4D})_{predicted}$ by the PMP program for finding suitable reference temperature (T_{ref}) based on the B_f and A_f values.

Storage temperature (°C)[a]	n[b]	B_f	A_f
4	7	0.84	1.54
12	6	1.39	2.45
25	8	1.01	1.40

[a] These three temperatures were used to find the T_{ref} since the remaining temperatures contained only one repetition (see Table 1) and the comparison was not valid

[b] Number of experimental cases at each temperature condition of all studies considered during model development, and from which the observed t_{4D} value was estimated

Table 3

Estimation of the secondary model parameters for the variables z_{4D} and $(t_{4D\ at\ 25^oC})^{1/2}$, and its performance.

Variables	Parameters estimates		Performance	
	b[a]	a_{wmin}[a,b]	R^2	RMSE
z_{4D}	238.5 ± 49.8	0.728 ± 0.029	0.92	2.954
$(t_{4D\ at\ 25^oC})^{1/2}$	16.46 ± 3.76	0.670 ± 0.049	0.79	0.282

[a] mean value ± standard error

[b] The comparison between the two a_{wmin} values showed no significant difference as indicated also by the overlapping 95% confidence interval (CI) of the two values, i.e. the mean value is included in the 95% CI of the other, 0.602-0.855 (z_{4D}) and 0.543-0.796 [$(t_{4D\ at\ 25^oC})^{1/2}$]

Fig. 1

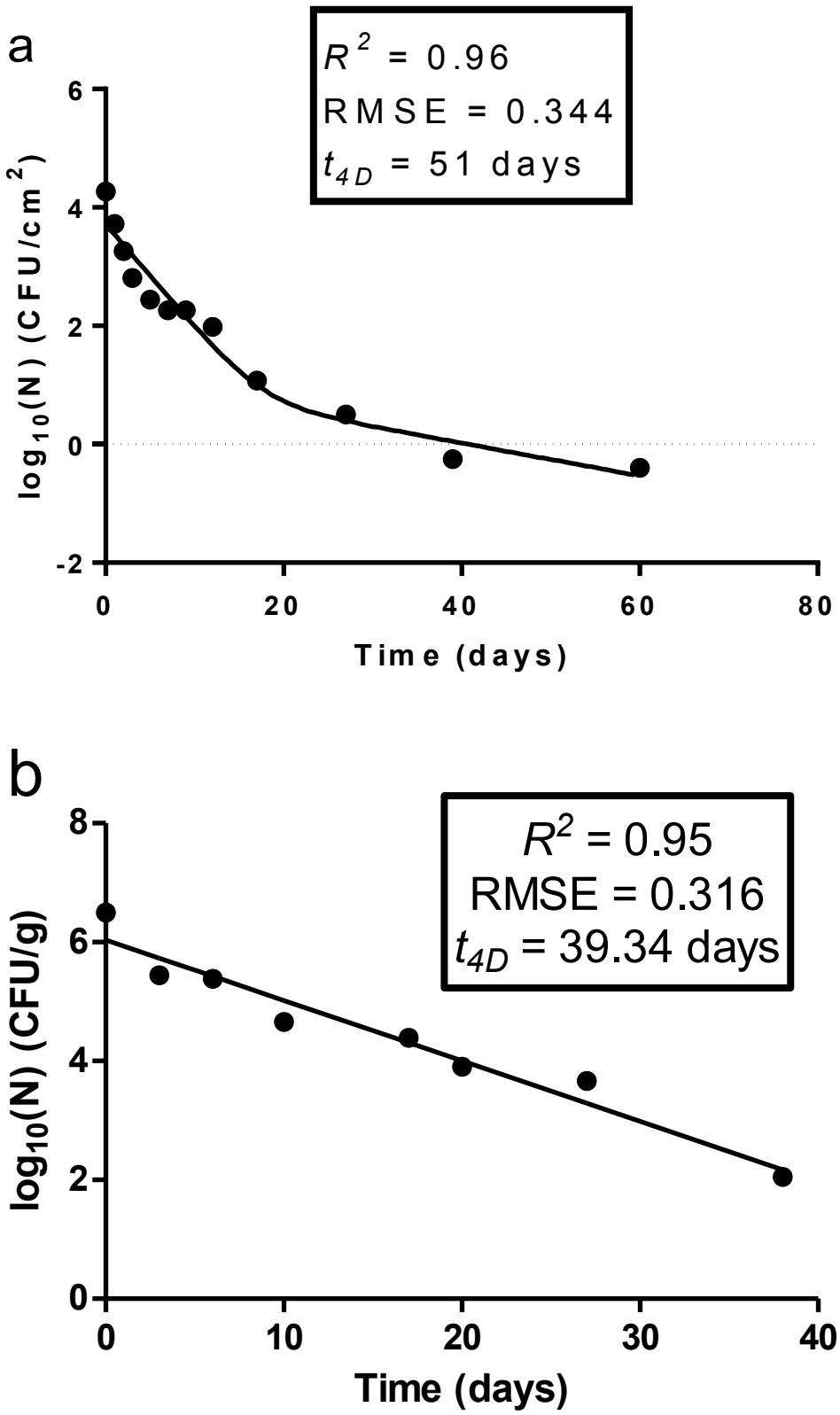

a
R^2 = 0.96
RMSE = 0.344
t_{4D} = 51 days
$\log_{10}$(N) (CFU/cm^2)
6
4
2
0
-2
0
20
40
60
80
Time (days)
b
R^2 = 0.95
RMSE = 0.316
t_{4D} = 39.34 days
$\log_{10}$(N) (CFU/g)
8
6
4
2
0
0
10
20
30
40
Time (days)

Fig. 2

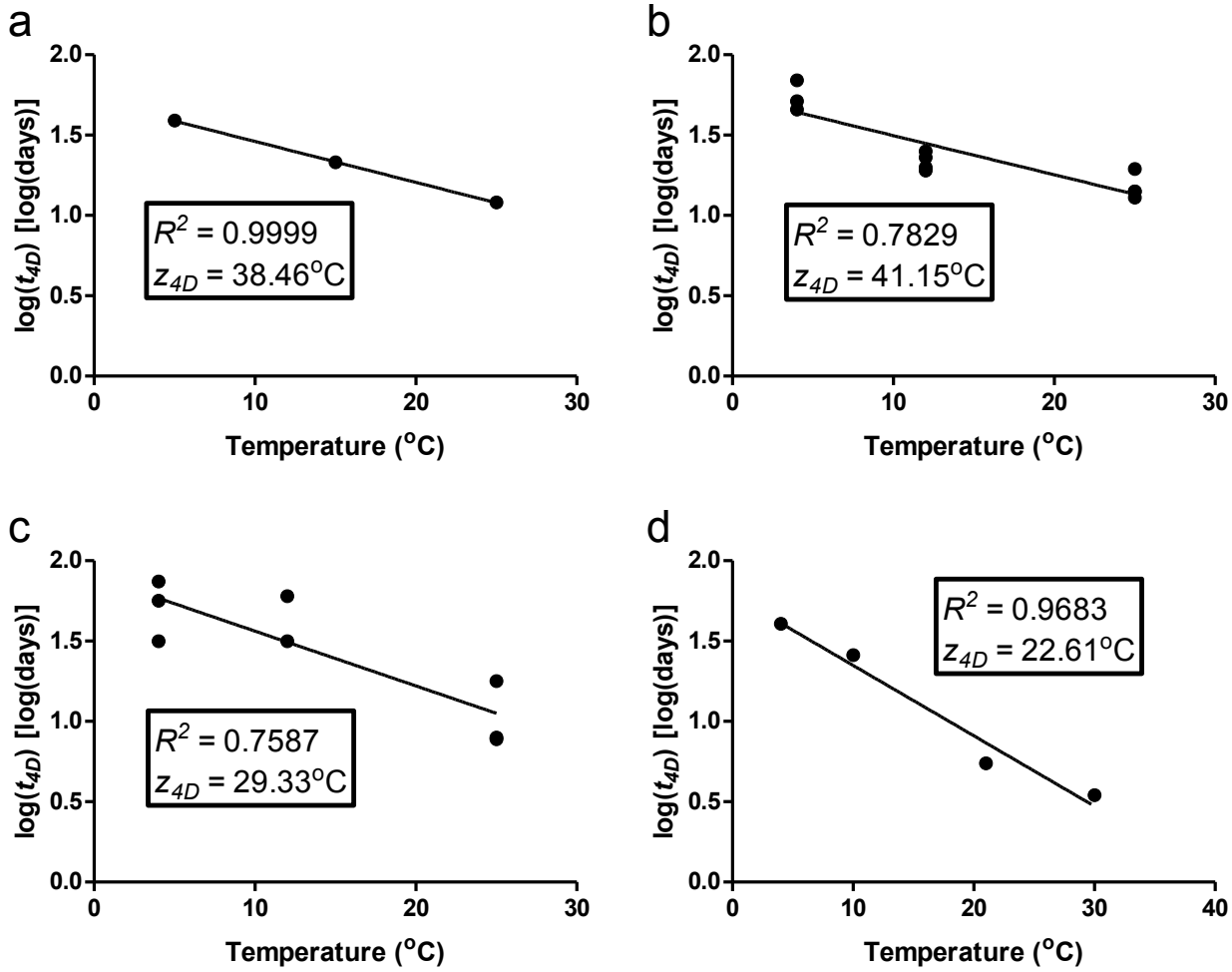

a
b
c
d
log(t4D) [log(days)]
Temperature (°C)
R2 = 0.9999
z4D = 38.46°C
R2 = 0.7829
z4D = 41.15°C
R2 = 0.7587
z4D = 29.33°C
R2 = 0.9683
z4D = 22.61°C

Fig. 3

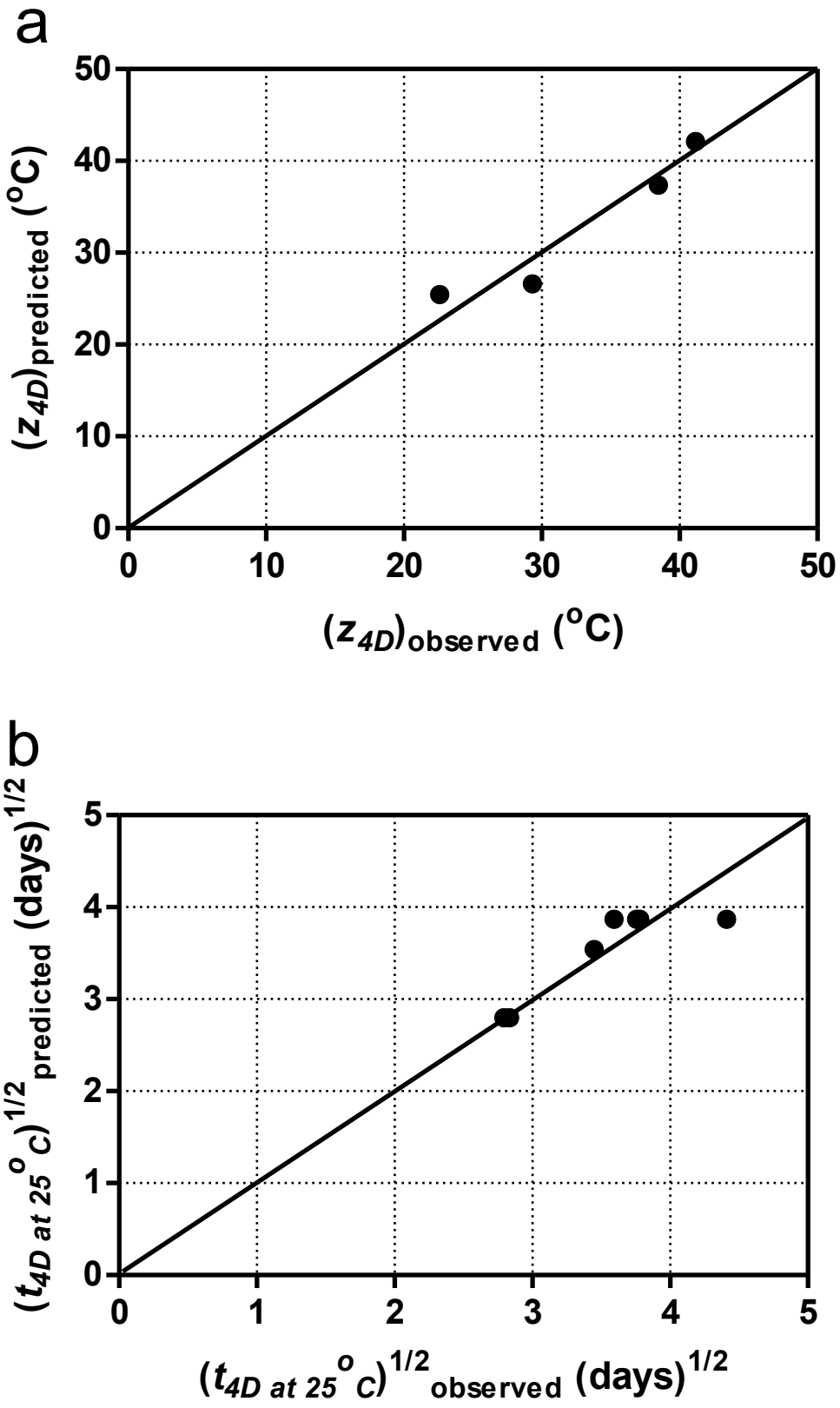

a
50
40
30
20
10
0
0
10
20
30
40
50
$(z_{4D})_{predicted}$ (°C)
$(z_{4D})_{observed}$ (°C)
b
5
4
3
2
1
0
0
1
2
3
4
5
$(t_{4D\ at\ 25^oC})^{1/2}_{predicted}$ $(days)^{1/2}$
$(t_{4D\ at\ 25^oC})^{1/2}_{observed}$ $(days)^{1/2}$

Fig. 4

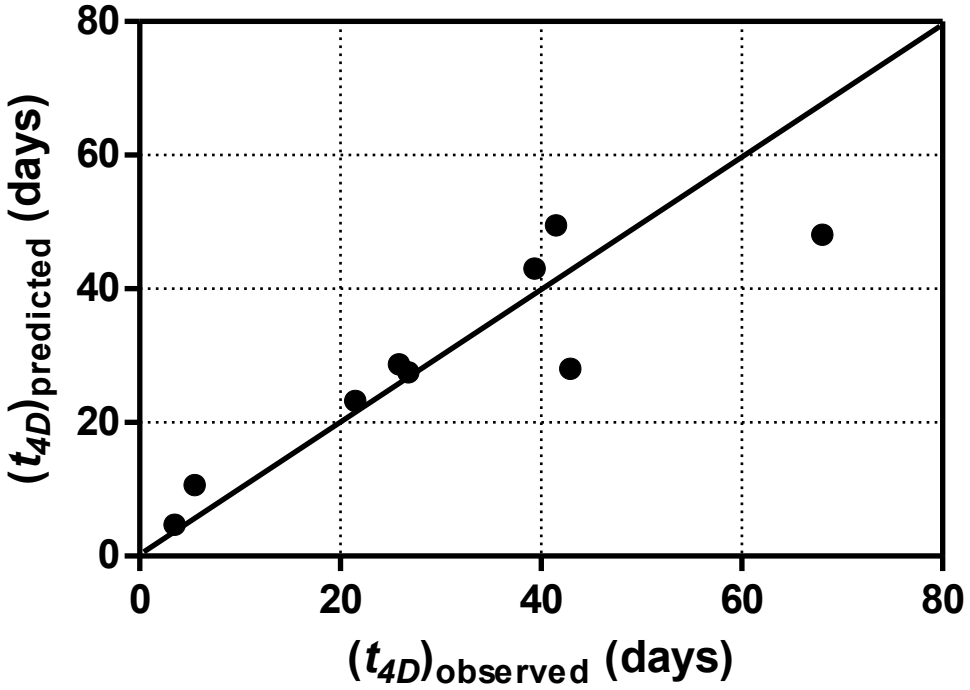
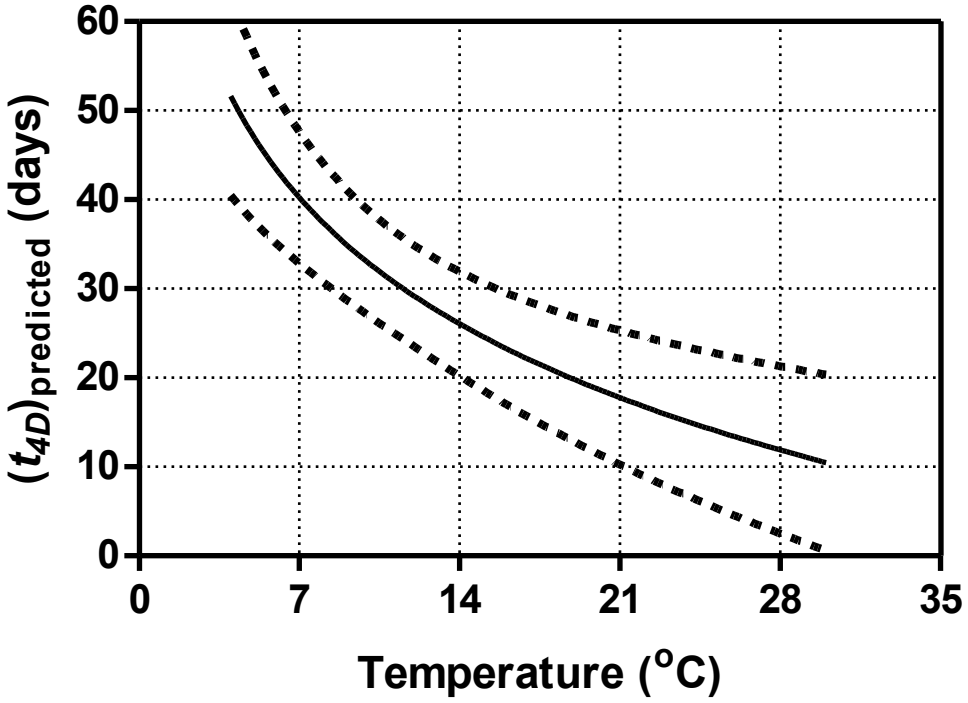


Fig. 5


60
50
40
30
20
10
0
$(t_{4D})_{predicted}$ (days)
0
7
14
21
28
35
Temperature (^{o}C)