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Modelling the effect of osmotic adaptation and temperature on the non–thermal inactivation of *Salmonella* spp. on brioche-type products

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ABSTRACT

Salmonella spp. is known to survive in intermediate– and low–moisture foods. Bakery products such as cream-filled brioche (a_w 0.82–0.84), depending mainly on the a_w of the fillings and the baking they receive for food preservation, may support survival of the pathogen. The study aimed to model the inactivation of osmotically adapted and non–adapted *Salmonella* in cream–fillings (praline and biscuit) and cream-filled brioche at different storage temperatures. All matrices were inoculated with *ca.* 6.0 log CFU/g of osmotically adapted and non–-adapted five–strain cocktail of *Salmonella* (Typhimurium, Agona, Reading, and Enteritidis) and stored aerobically in 120 mL screw-capped containers at 15, 20, and 30 °C. Adaptation of *Salmonella* was induced in cream–fillings (praline and biscuit) with a_w adjusted to 0.88, by adding sterile water to each of the original fillings (a_w 0.78–0.83) and incubating at 37 °C for 1 h. Survival of *Salmonella* was assessed at regular time intervals throughout storage using thin layer agar method to enhance the recovery of injured cells ($n = 4$). Inactivation curves were fitted best with the Weibull model using the freeware GInaFit tool and the estimated δ and β values were used to calculate the time for 4D reduction–t_{4D}. Results showed that inactivation of *Salmonella* increased with temperature, while osmotic adaptation enhanced its survival in a food matrix–related manner. Higher survival rates of adapted cells were observed in cream–fillings (t_{4D}: 79.9 ± 27.1 days on biscuit and 150.3 ± 19.6 days on praline) compared to brioche (t_{4D}: 61.3 ± 0.9 days on biscuit and 52.5 ± 4.6 days on praline) at 20 °C. Secondary (linear) modelling of t_{4D} showed that the survival of *Salmonella* was affected by temperature and osmotic adaptation. Model simulation of pathogen inactivation in independent trials on cream–fillings agreed well with observed data. In conclusion, the present data could be used as a means to identify areas for improving the performance of existing models quantifying the survival of *Salmonella* in bakery-confectionary products with intermediate a_w.

1. Introduction

Intermediate–(IMF) and low–moisture foods (LMF) are defined as foods with water activity (a_w) 0.65–0.90 and < 0.65, respectively (FAO, 2003; Smith et al., 2004). For many years, IMF and LMF have been considered as safe, hypothesizing that they constitute a retarding environment for the growth of foodborne pathogens (Pena-Meléndez et al., 2014). However, this assumption became disputable when several studies reported the increased ability of pathogens like *Salmonella* to survive in such food products after extended storage. In fact, a significant number of reported outbreaks has been associated with *Salmonella* contamination in IMF and LMF such as halva (Aavitsland et al., 2001; de Jong et al., 2001), tahini (CDC, 2012, 2013; Unicomb et al., 2005), chocolate (Bean and Post, 2014; Thompson et al., 2007), bread and bakery products filled with or without creams (Evans et al., 1996; Harvey et al., 1961; Kimura et al., 2005; Staff and Grover, 1936). The need to assess the risk of *Salmonella* survival in such products becomes more urgent, considering that the majority of IMF and LMF are ready-to-eat products of long shelf-life and that the infectious dose of *Salmonella* is low (*i.e.*, 2 to 3 CFU/g in chocolate) (Kapperud et al., 1990; Santillana Farakos et al., 2013).

Over the last few years, sweet bakery products such as croissant or brioche-type products filled with various kind of creams are highly popular to the consumers and especially the children. Except for their

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Table 1
The technological characteristics of the studied brioche and their respective cream–fillings (praline and biscuit).

Food matrix	Type	a_w	pH	Fat (%)	Sugars (%)	Preservatives	Shelf-life
Cream–filling	Biscuit	0.82–0.83	5.9–6.1	17.0	41.0	Potassium sorbate	6 months[a]
	Praline	0.78–0.81	6.1–6.3	16.1	41.4	2000 ppm	
Brioche	Biscuit	0.82–0.84	5.9–6.1	15.4	18.9	Calcium propionate	60 days[b]
	Praline	0.82–0.84	6.1–6.3	16.3	18.3	800 ppm	

[a] Under aerobic conditions at room temperature.
[b] Under MAP (10% O_2: 10% CO_2: 80% N_2) at room temperature.

moderate a_w (0.82–0.84), which classifies them into IMF, other intrinsic factors of these products, such as fat (*ca.* 15–16%) and/or sugars content (*ca.* 18–19%) (Table 1), intensify the need to study the survival of *Salmonella*, since these additional factors may protect the bacterium during its subsequent passage through the host gastrointestinal tract, thereby affecting the likelihood of illness, caused even by low numbers of the pathogen (FAO/WHO, 2016). In fact, *Salmonella* has been reported to survive in high-sugar and fat confectionery products for at least 182 days during storage at 25 °C (Beuchat and Mann, 2015) and over 12-months during storage at 20 °C (Kataoka et al., 2014). Moreover, as Li et al. (2014) highlighted, the presence of different components, such as in our case, flour, water, fat, sweet or savory cream–-fillings, chocolate chips, can impact the survival of *Salmonella* even after a thermal process (*i.e.*, baking). Specifically, components in multi-ingredient (complex) foods usually have dissimilar physicochemical properties, thus forming different local microenvironments (favorable or non-favorable) for pathogen survival in each food matrix (Li et al., 2014). In fact, based on estimates by the European Food Safety Authority, during the period 2004–2009, *Salmonella* caused about 85% of the 279 outbreaks associated with multi-ingredient foods that undergo a thermal process such as flour-based bakery foods, chocolate, and confectionery (EFSA, 2012).

Given that implementation of good sanitary practices may eliminate the in–factory presence of *Salmonella*, a potential source of contamination may be the raw materials (Finn et al., 2013). However, according to the technology of cream filled brioche–type products, all ingredients are subjected to thermal process with temperature reaching *ca.* > 90–100 °C in the center of the product for 2–3 min according to the manufacturer, since they are part of brioche dough, except for the filling, which constitutes a post-baking process. Considering the above, it is necessary to assess the risk of *Salmonella* to survive not only in the final product during its shelf-life, but also in the cream–filling. Beuchat and Mann (2015) showed that *Salmonella* can survive for at least 182 days in low-a_w fillings (peanut butter cream: at $a_w = 0.27$; chocolate cream: at $a_w = 0.31$) of cookie and cracker sandwiches during storage at 25 °C, highlighting the need to assure a *Salmonella*–free cream filling. However, there is limited information available related to the safety of intermediate–a_w multi-ingredient foods and their components.

Moreover, *Salmonella* might be exposed to various stresses in processing plants and environment before contaminating the final product (Osaili et al., 2008). In fact, when a potential exposure of *Salmonella* to osmotic stress takes place through IMF or LMF, cross-protection to similar or heterogeneous stresses may occur, enhancing their survival. Hence, in case of adopting the scenario of *Salmonella* cross-contamination from the cream–fillings to the final product and taking into consideration the stability of cream–fillings (*ca.* 6 months shelf-life), the pathogen osmotic or matrix adaptation, and how that may affect its survival in the final product, is a critical parameter for investigation. Therefore, the objective of the present study was to model the inactivation of osmotically adapted and non–adapted cells of *Salmonella* in two types (praline or biscuit) of: i) cream–fillings and ii) cream-filled brioche (final product) during storage at different temperatures.

2. Materials and methods

2.1. Salmonella strains

A five-strain cocktail of *Salmonella enterica* subp. *enterica* was used in the present study. In particular, *Salmonella* strains corresponded to serotype Typhimurium (4/74; Calf bowel, DT 193; human isolate), Agona (23; isolated from feeds), Reading (655; isolated from feeds), and Enteritidis (PT4; isolated from feeds). All strains were obtained from the microorganism collection of the Laboratory of Quality Control and Hygiene in Agricultural University of Athens and were maintained at −20 °C in Tryptic Soy Broth (TSB) (LAB M, Lancashire, UK) supplemented with 20% v/v glycerol.

2.2. Food matrices

The experiments were carried out in two types of freshly produced brioche filled with praline or biscuit cream as well as in their respective cream–fillings, which were kindly provided by a commercial Greek bakery company. Cream–fillings were obtained in bulk packages, while brioche were delivered in packages of 75 g and stored at room temperature as the bakery company recommended for *max.* 1 day. The technological characteristics of all food matrices used in the present study are shown in Table 1.

2.3. Preparation of non–osmotically adapted Salmonella cells

All strains were maintained on slants of Tryptic Soy Agar (Lab M, Lancashire, UK) at 4 °C and sub-cultured once a month. Single colony of each strain was grown separately in 10 mL TSB at 37 °C for 24 h and subsequently, 100 μL of each overnight culture were transferred to fresh TSB for 18 h incubation at 37 °C. Following activation stage, strains were harvested by centrifugation (3000*g* for 15 min at 4 °C) (Megafuge 1.0R, Heraeus, Buckinghamshire, England), washed twice, re–-suspended in 10 mL of ¼ strength Ringers' solution (LAB M, Lancashire, UK), and finally mixed in equal volumes, resulting in a *ca.* 9.0 log CFU/mL inoculum cocktail. The level of the inoculum was determined by plating 0.1 mL from appropriate decimal dilution of the cocktail on selective medium Xylose Lysine Deoxycholate (XLD) (LAB M, Lancashire, UK). The plates were incubated at 37 °C for 24 h.

2.4. Selection of adaptation medium–preparation of osmotically adapted Salmonella cells

With regard to osmotic adaptation, the selection of the adaptation medium was set as the first target of the study. Thus, a pre-experiment was carried out testing a common laboratory medium used for growth of *Salmonella* like TSB and two types of cream–fillings (biscuit and praline) (see Section 2.2). A_w of all matrices was adjusted to 0.88 and 0.95 using glycerol (as described below), corresponding to growth-inhibiting and growth-permitting conditions for *Salmonella*, respectively, thus resulting in six adaptation media. It is well-known that no growth of *Salmonella* may occur in $a_w < 0.85$ (Lund and Eklund, 2000), while values of a_w as low as 0.93 are sufficient to support growth of the pathogen (Beuchat, 2009). The steps followed for modifying a_w of TSB

Table 2
Steps followed for modifying a_w (0.95 and 0.88) in TSB and cream–fillings along with the adaptation procedure of initial *Salmonella* strain cocktail (*ca.* 9.0 log CFU/mL).

a_w level	a_w modifying and cells adaptation procedure
0.95	Re-suspension of cell biomass in 10 mL TSB a_w 0.95
	Addition of 1 mL inoculum[a] + 4 mL distilled sterile water in 5 g of biscuit cream–filling
	Addition of 1 mL inoculum[a] + 3.5 mL distilled sterile water in 5.5 g of praline cream–filling
0.88	Re-suspension of cells biomass in 10 mL TSB a_w 0.88
	Addition of 1 mL inoculum[a] in 9 g of biscuit cream–filling
	Addition of 1 mL inoculum[a] in 9 g of praline cream–filling

[a] In TSB and biscuit or praline cream–fillings followed by incubation at 37 °C for 1 h.

and biscuit or praline cream–fillings and the subsequent adaptation procedure of the pathogen in the latter substrates is summarized in Table 2. Specifically, with regard to TSB, glycerol was added at a final concentration of 25% and 15% v/v to reach a_w 0.88 and 0.95, respectively. On the other hand, the initial a_w of praline (0.80) and biscuit cream–fillings (0.83) was adjusted to 0.95 by adding 45% and 50% v/v sterile distilled water, respectively, while a_w 0.88 was achieved by adding 10% v/v sterile water on both cream–fillings. A_w adjustment took place in 50 mL sterile screw-capped plastic containers, followed by manual homogenization with a sterile spatula. The adjusted a_w levels of TSB (after sterilization) and cream–fillings were verified using a digital a_w meter (Hydrolab rotronic, Basserdorf, Switzerland). In all adaptation media, the *Salmonella* cocktail was added so as to obtain a final concentration of 8.0 log CFU/g, following incubation at 37 °C for 1 h. Approximately 50 g of each original cream–filling were weighted in 120 mL sterile screw-capped plastic containers. Aliquots of 0.5 mL non–adapted (controls; see Section 2.2) or osmotically adapted cells in TSB (a_w 0.88 and 0.95) or 0.5 g osmotically adapted cells in cream–-filling (a_w 0.88 and 0.95) were used to inoculate the original cream–-fillings followed by manual homogenization with a sterile spatula. The final population of *Salmonella* in cream–fillings was *ca.* 6.0 log CFU/g. All samples were stored at 25 °C (close to room temperature as recommended by the manufacturer) under aerobic conditions (containers were loosely capped) in high precision (± 0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan). Two independent storage experiments were performed and duplicate samples were used for each trial (n = 4).

The highest survival of *Salmonella* on the original biscuit cream–-filling during storage at 25 °C (Fig. 1(a)) was observed in cells adapted in a_w 0.88 biscuit cream, followed by, in decreasing order, non-adapted cells (controls), cells adapted in a_w 0.95 biscuit cream, cells adapted in a_w 0.88 TSB, and finally, cells adapted in a_w 0.95 TSB. Moreover, controls and cells adapted in praline cream–filling of a_w 0.88 or a_w 0.95 showed the highest survival at 25 °C of *Salmonella* when subsequently inoculated in the original praline–filling (non a_w–modified) (Fig. 1(b)). According to preliminary tests, and in order to use an adaptation medium of the same a_w, biscuit and praline cream–fillings with a_w adjusted to 0.88 were selected for the main experiment, hypothesizing that the potential contamination originated from the cream–filling, the only ingredient injected into brioche after the baking process.

2.5. Inactivation of osmotically adapted and non–adapted Salmonella cells in brioche and cream–fillings during storage at different temperatures

Brioche were first homogenized (under sterile conditions) and then inoculated, since brioche is considered to be a multi-ingredient food matrix (*i.e.*, dough, chocolate chips, and cream–fillings). The homogenization took place, in order to eliminate the potential impact of the variability in the physicochemical properties of the site, where

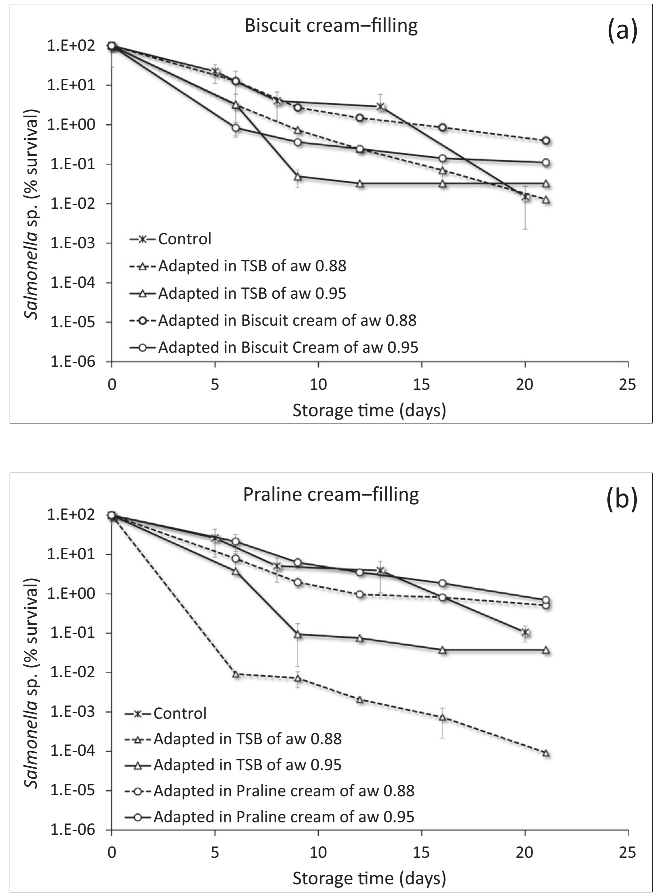

Fig. 1. Effect of TSB (-Δ-), biscuit and praline cream–filling (-○-) of a_w 0.95 (continuous line) and 0.88 (dotted line) as osmotic and matrix adaptation media of *Salmonella* spp. on the subsequent survival of the organism in cream–fillings (biscuit and praline) stored aerobically at 25 °C. Two independent storage experiments were performed and duplicate samples were used for each trial (n = 4).

inoculum could reside. Conversely, the inoculum was added directly in the cream–fillings, as they constitute an apparently homogenous matrix. Approximately 500 g of each cream–filling or homogenized brioche were weighted in sterile containers or in sterile stomacher bags, respectively. Five grams (5 g) of each cream–filling (as described in Section 2.4) inoculated with the osmotically adapted *Salmonella* cocktail, were added on the respective homogenized brioche and cream–-filling, while in case of non–osmotically adapted cells (as described in Section 2.3) aliquots of 5 mL were inoculated on the surface of all food matrices. Cream–fillings were manually homogenized with a sterile spatula, while smashed brioche were manually kneaded in order to obtain the best dispersion of the inoculum. The initial inoculum was 6.0 log CFU/g of food substrate. As Osaili et al. (2017) noticed, such high inoculation levels may not be reflective of actual contamination levels. However, they do help to more precisely and quantitatively delineate the decline of pathogens after exposure to environmental factors that are critical during processing. Ten grams (10 g) of all samples were weighted in 120 mL sterile screw-capped plastic containers and subsequently stored at 15, 20, and 30 °C, under aerobic conditions, as described in Section 2.4, in high precision (± 0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan). Two independent storage experiments (from different batches) were performed and duplicate samples were used for each trial (n = 4).

2.6. Microbiological analysis

On various days during storage at 15, 20, and 30 °C, 90 mL of sterile buffered peptone water were added aseptically to the plastic containers containing 10 g of food substrate and the samples were then homogenized by manual shaking for at least 60 s. Following homogenization, decimal dilutions in ¼ strength Ringer's solution were prepared. Aliquots of 0.1 mL and/or 1 mL of diluted sample were spread on TSA plates. Following incubation at 37 °C for 2 h, a second layer (8 mL) of XLD was applied to TSA plates, and the plates were further incubated at 37 °C for 24 h (thin agar layer method) in order to enhance recovery of sub-lethally injured cells (Kang and Fung, 1999). Colonies with typical *Salmonella* morphology were counted. Average numbers of colonies *per* plate were used to calculate the viable-cell concentrations, expressed as log CFU/g. Total viable counts (TVC) were estimated on TSA after incubation at 30 °C for 72 h. The enumeration limit was 1.0 log CFU/g. The pH values of samples were recorded at every sampling by using a digital pH meter (pH 526, Metrohm Ltd., Switzerland) *via* immersion of pH electrode in the homogenate.

2.7. Primary modelling

Survival curves of *Salmonella* were generated by plotting bacterial population (log CFU/g) against storage time. Survival data were fitted to Log-linear with tail (Geeraerd et al., 2000), as well as Weibull inactivation model (Mafart et al., 2002) using the excel-based freeware GInaFit tool Version 1.6 (Katholieke Universiteit Leuven, Leuven, Belgium) (Geeraerd et al., 2005). Root Mean Squared Error (RMSE) was used in order to determine the primary model that best described the data. As already shown in previous studies (Ma et al., 2009; Abd et al., 2012), survival curves of *Salmonella* in low–a_w foods often did not follow log–linear kinetics and show significant asymptotic tails. In fact, previous studies have shown Weibull model (Eq. (1)) as the most suitable model for describing *Salmonella* survival in LMF or IMF (Santillana Farakos et al., 2014). In the present study, experimental data fitted best to Weibull model, resulting in lower RMSE values than the log-linear model (data not shown). Thus, Weibull model was selected to determine the inactivation kinetic parameters (delta, *δ*; β values) of *Salmonella*.

$$\log(N_t) = \log(N_0) - \left(\frac{t}{\delta}\right)^{\beta} \quad (1)$$

where N_t is the bacterial population any time t, N_0 is the initial bacterial population, delta (*δ*) is the time of first decimal reduction, t is storage time, parameter β characterizes the shape of the curve ($\beta > 1$ produces curves of upward convexity, $\beta < 1$ describes curves of downward concavity and $\beta = 1$ corresponds to a straight line).

The estimated *δ* and β values were then used to calculate the time for four (4) decimal reductions (t_{4D}) *via* Eq. (2), which derived by setting the difference between Log (N_t) and log (N_0) equal to 4 in Eq. (1).

$$t_{4D} = \delta * 4^{1/\beta} \quad (2)$$

2.8. Secondary modelling

In order to model the effect of storage temperature on the inactivation of *Salmonella* in both cream–fillings and brioche products, a linear regression model was used. Natural logarithm transformations of *δ* and t_{4D} were selected among square root and no transformation for reducing the variance of the response variable and provided the best fit (Eqs. (3) and (4)). a_0, a_1, b_0 and b_1 are the constants to be estimated, and T is the temperature (°C) (independent or input variable). In particular, a_0 and b_0 parameters correspond to the slope of the linear regression, indicating the dependence of inactivation kinetics (ln (δ) and ln (t4D), respectively) on temperature, while a_1 and b_1 correspond to the ln (δ) and ln (t4D) values, respectively, when $T = 0$ °C. Even though, T = 0 °C is unlikely to occur regarding the particular products and therefore, is non-realistic, a_0 and b_0 indicate the beginning of the regression and are essential parameters to describe the linear model.

$$\ln(\delta) = a_0 * T + a_1 \quad (3)$$

$$\ln(t_{4D}) = b_0 * T + b_1 \quad (4)$$

To evaluate the performance of the models, the following statistical indicators were used: adjusted regression coefficient (R^2_{adj}) and F-test. The higher the R^2_{adj}, the better the fit of the model. Moreover, if the *p* value of the F-test (significance F) is < 0.05, then the model successfully describes the survival data.

2.9. Evaluation of models performance

The developed inactivation models were validated by obtaining survival data of adapted *Salmonella* (in duplicate) on biscuit and praline cream–fillings at 25 °C and storage time (from 0 to 30 days) within the range of the modeled data. For validation purposes, though, the average β was used among temperatures within each product, assuming that it sufficiently accounts for the small variations among β values (σ ≤ 0.2) at different storage temperatures and thus, accepting that inactivation curves of *Salmonella* spp. were of similar shape. Nevertheless, in order to describe the potential variation of the predicted *δ* values caused by the variation of β values, three predicted *δ* values were estimated using a) the average beta value for a given food matrix as well as b) β + stdev and c) β-stdev. The performance of the developed model was assessed by using the accuracy (A*f*) (Eq. (5)) and the bias (B*f*) factors (Eq. (6)) (Ross, 1996; Baranyi and Pin, 1999).

$$A_f = 10^{\wedge}\left[\frac{\sum_1^n \left|\log\left(\log N_{model}/\log N_{data}\right)\right|}{n}\right] \quad (5)$$

$$B_f = 10^{\wedge}\left[\frac{\sum_1^n \log\left(\log N_{model}/\log N_{data}\right)}{n}\right] \quad (6)$$

2.10. Statistical analysis

Data analysis was performed by using SPSS statistical software program (SPSS for Windows version 16, SPSS Inc., USA). One-way analysis of variance (ANOVA) with Duncan post-hoc multiple comparisons test was used in order to assess the effect of storage temperature as well as the previous osmotic adaptation of *Salmonella* on the inactivation kinetic parameters (*δ* and t_{4D}). Differences were considered significant at $p < 0.05$.

3. Results and discussion

Kinetics of inactivation revealed that the survival of *Salmonella* was temperature dependent, regardless of food matrix and cells status (osmotically adapted or non–adapted). Specifically, inactivation rates of *Salmonella* were decreased as the storage temperature was increasing from 15 °C to 30 °C (Fig. 2(a)–(d)), suggesting that temperature was the controlling factor of the observed non-thermal inactivation as documented by Ross et al. (2008). This principle was also reflected in the *δ* and $t_{4D,}$ which significantly decreased ($p < 0.05$) as temperature increased, indicating that less time is required for the first and four decimal reductions respectively, at all studied assays (Table 3). With regard to storage of biscuit and praline brioche at 20 °C, the population of *Salmonella* decreased to the enumeration limit (1.0 log CFU/g) after 48 days (non–adapted) and 61 days (adapted), indicating that a few cells of osmotically adapted *Salmonella* may be capable of surviving during the 60-days shelf-life of brioche at room temperature (25 °C)

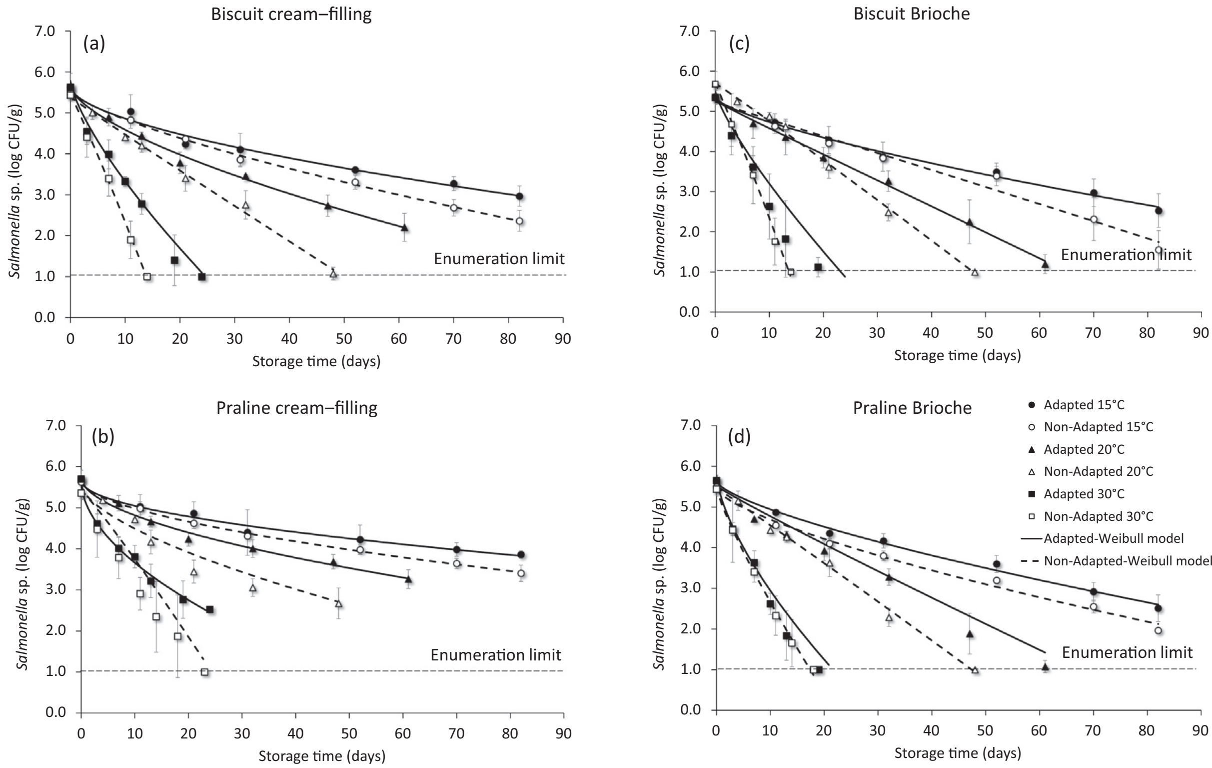

Fig. 2. Survival curves of osmotically non–adapted (dotted lines) and adapted *Salmonella* spp. (in biscuit and praline cream–fillings of a_w 0.88) (continuous lines) after subsequent inoculation in homogenized brioche and their respective, original cream–fillings (non a_w–modified) during storage at 15, 20, and 30 °C for up to 82 days (mean of samplings ± standard deviation; lines represent the statistical fit of the Weibull model). Two independent storage experiments were performed and duplicate samples were used for each trial (n = 4).
*Enumeration limit was 1.0 log CFU/g.

(Fig. 2(c) and (d); Table 1). However, it must be also taken into account that the shelf-life was estimated under modified atmosphere packaging (10% O_2: 10% CO_2: 80% N_2). Previous studies have reported that *Salmonella* may better survive under limited oxygen levels (Christian and Stewart, 1973; Flowers, 2004). During storage at 30 °C, it took only 2 weeks for the pathogen to reach the enumeration limit

Table 3
Estimated parameters (δ, t_{4D}, and β) (mean ± stdev) derived from fitting the Weibull model to the recorded data describing the inactivation of osmotically adapted and non–adapted cells of *Salmonella* spp. inoculated in cream–filling and brioche (biscuit and praline), stored at 15, 20, and 30 °C.

Food matrix	Cells treatment	Temperature (°C)	δ ± stdev (days)	t_{4D} ± stdev (days)	β ± stdev
Biscuit filling	Non – adapted	15	16.1 ± 0.7^{a}	115.2 ± 22.9^{a}	0.7 ± 0.1
		20	11.2 ± 4.0^{ab}	45.1 ± 4.5^{b}	1.0 ± 0.2
		30	3.5 ± 1.1^{b}	12.5 ± 0.4^{c}	1.1 ± 0.2
	Adapted	15	15.0 ± 0.3^{a}	208.7 ± 130.5^{a}	0.6 ± 0.2
		20	9.5 ± 3.1^{ab}	79.9 ± 27.1^{b,*}	0.7 ± 0.0
		30	4.1 ± 1.6^{a}	15.1 ± 4.0^{c}	1.1 ± 0.1
Praline filling	Non – adapted	15	21.0 ± 1.5^{a}	228.2 ± 13.3^{a}	0.6 ± 0.0
		20	9.2 ± 0.1^{b}	74.9 ± 10.8^{b}	0.7 ± 0.1
		30	4.7 ± 2.2^{b}	19.4 ± 4.00^{c}	1.0 ± 0.2
	Adapted	15	23.6 ± 8.3^{a}	495.6 ± 358.9^{a,*}	0.5 ± 0.2
		20	12.1 ± 1.6^{ab}	150.3 ± 19.6^{a,*}	0.6 ± 0.0
		30	2.8 ± 1.9^{b}	35.1 ± 0.1^{b,*}	0.5 ± 0.2
Biscuit brioche	Non – adapted	15	23.2 ± 4.1^{a}	96.7 ± 16.0^{a}	1.0 ± 0.2
		20	10.8 ± 2.0^{b}	40.8 ± 2.6^{b}	1.0 ± 0.1
		30	3.2 ± 1.4^{c}	11.7 ± 0.2^{c}	1.1 ± 0.4
	Adapted	15	20.3 ± 3.8^{a}	141.3 ± 14.5^{a,*}	0.7 ± 0.0
		20	14.9 ± 4.9^{ab}	61.3 ± 0.9^{b,*}	1.0 ± 0.2
		30	3.6 ± 0.2^{b}	16.3 ± 5.3^{c}	1.0 ± 0.2
Praline brioche	Non – adapted	15	14.9 ± 1.4^{a}	106.6 ± 7.9^{a}	0.7 ± 0.0
		20	11.0 ± 3.8^{ab}	42.2 ± 3.2^{b}	1.0 ± 0.2
		30	2.9 ± 0.8^{b}	15.2 ± 0.7^{c}	0.8 ± 0.2
	Adapted	15	17.5 ± 0.7^{a}	123.8 ± 14.3^{a}	0.71 ± 0.0
		20	12.1 ± 0.9^{b}	52.5 ± 4.6^{b,*}	1.0 ± 0.0
		30	2.1 ± 0.1^{c}	15.3 ± 1.9^{c}	0.7 ± 0.0

δ and t_{4D} values within the same food matrix and cells treatment having different letter are significantly different from each other ($p < 0.05$).
Star (*) indicates statistical significance ($p < 0.05$) between adapted and non – adapted samples at the same food matrix and temperature.

(t_{4D} = 12–16 days), regardless of cells pre-treatment and food matrix (Fig. 2(c) and (d); Table 3). In cream–fillings, *Salmonella* showed a significantly lower inactivation rate ($p < 0.05$) in praline compared to biscuit (Fig. 2(a) and (b)). In fact, t_{4D} on praline cream–filling reached values *ca.* 2–fold higher than on biscuit ($p < 0.05$), especially as storage temperature decreased (Table 3). Specifically, at 20 °C, the estimated values of t_{4D} were 150.3 ± 19.6 days (*ca.* 5 months) and 74.9 ± 10.8 days (*ca.* 2.5 months) for the adapted and non–adapted cells, respectively (Table 3), suggesting that a potential exposure of *Salmonella* to osmotic stress may result in prolonged survival of the pathogen, even at the end of the shelf-life of praline cream–filling (Table 1). Experimental-wise, we used a high initial inoculation level (*ca.* 10^6 CFU/g), in order to enable the assessment of the increase in time to kill of adapted as compared to non-adapted cells. The results clearly suggest the development of adaptive osmotic tolerance of *Salmonella* as a result of residence in osmotically stressful habitats. Subsequently, these results, also indicate that a few *Salmonella* cells in a processing environment, may develop adaptive stress tolerance upon exposure to food-related stresses, such as acid-, osmotic- and heat-stress, as well as preservatives, thus representing an important source of risk by persistent cells. Such persistent cells that could also proliferate under favorable conditions in their adaptation niches, may constitute a reservoir of *Salmonella*, with inherent resistance to osmotic stress. Apart from stressing the potential of emergence of persistent strains derived from osmotically stressful habitats, this information could be also used to set (or revise) a performance criterion for *Salmonella*, expressed as log reductions in the final product. Concomitantly, the industry could update the product formulations (*e.g.*, a_w of cream fillings), the processing parameters (*e.g.*, of dough baking), or the shelf-life of final product to achieve such a criterion. The direct comparison of our results with previous data was difficult due to the lack of studies for *Salmonella* inactivation on IMF, as compared to abundant information in the behavior of the bacterium under low moisture foods. However, previous studies have also reported that the pathogen can persist for long storage periods in low–a_w substrates such as sucrose (a_w = 0.24–0.54), tahini (a_w = 0.17–0.23), peanut butter (a_w = 0.22–0.33), almonds (a_w = 0.36–0.45), and pistachios (a_w = 0.43–0.49), especially when storage takes place under refrigerated temperatures compared to room temperature (Burnett et al., 2000; Beuchat et al., 2017; Kimber et al., 2012; Osaili et al., 2017). Overall, the results indicated that the osmotic adaptation of *Salmonella* in cream–filling of a_w 0.88 may enhance its subsequent survival in temperature–related manner.

With regard to the effect of adaptation on *Salmonella* survival in brioche and cream fillings, inactivation rates were lower for osmotically adapted cells compared to non–adapted, regardless of food matrix and storage temperature (Fig. 2(a)–(d)). In terms of δ and t_{4D}, the influence of osmotic adaptation was more pronounced as the storage temperature decreased, especially in cream–fillings compared to brioche (Table 3). In fact, during storage at 15 and 20 °C, the t_{4D} values for adapted cells was 1.5 to 2–fold higher ($p < 0.05$) than the respective values for non–adapted cells, at all food matrices, while at 30 °C the inactivation rates were similar ($p \geq 0.05$), except for praline filling ($p < 0.05$) (Table 3). On the contrary, the recorded differences of δ between adapted and non–adapted cells at the same storage temperature and product were of lower magnitude and, in fact, not even significant ($p \geq 0.05$) (Table 3). The latter indicates that osmotic adaptation of *Salmonella* may result in the development of resistant sub-population(s) that manage to survive subsequent exposure to lethal osmotic stress in the food environment. Even though the effect of osmotic adaptation may not be evident in the beginning of storage (no significant differences between δ values), it may result in prolonged survival of *Salmonella* during storage as manifested by the increased t_{4D} values of adapted cultures. Our results are in agreement with Beuchat and Mann (2015), who also reported that inactivation of *Salmonella* was more rapid if cells had not been exposed to a low–a_w environment before their exposure to subsequent low–a_w fillings (chocolate cream–a_w 0.30 and peanut butter-based filling–a_w 0.27). Osaili et al. (2017) showed that the populations of *Salmonella* spp. in halva (a_w 0.18) after exposure to desiccation stress, were similar compared to controls during a 12– and 9– month storage at 10 and 25 °C, respectively. Such differences could be potentially attributed to the conditions of osmotic adaptation protocol (*i.e.*, time, level of a_w, type of humectant) (Pena-Meléndez et al., 2014), as well as to the food composition.

Comparing the two food matrices, lower inactivation rates were observed in cream–fillings compared to brioche, in all cases (Fig. 2(a)–(d)). Pre-exposure of the pathogen to cream fillings of adjusted a_w at 0.88 may have induced a combination of osmotic- and matrix-specific adaptation, considering that both cream fillings and brioche are complex foods. In fact, they are characterized by different intrinsic factors that could affect survival of *Salmonella*, such as composition, microstructure, as well as the preservatives used in each product. Cream fillings and brioche dough include potassium sorbate and calcium propionate respectively. Considering the above, the osmotic and matrix adaptation of *Salmonella* taken place in cream fillings, could enhance subsequent survival of the pathogen to a similar matrix (cream fillings) but not in the homogenized brioche products. In fact, during storage at 20 °C *i.e.*, close to the recommended storage temperature, biscuit and praline cream–fillings showed the highest survival rates of adapted *Salmonella* (t_{4D}: 79.9 ± 27.1 days and 150.3 ± 19.6 days, respectively) compared to biscuit and praline brioche (61.3 ± 0.9 days and 52.5 ± 4.6 days, respectively). In particular, *Salmonella* in praline cream–filling survived in significantly higher populations ($p < 0.05$) compared to the other three studied food matrices, at all storage temperatures (Fig. 2(a)–(d)). This could be partially attributed to the lower initial a_w of praline (0.78–0.81) compared to the other studied food matrices (Beuchat and Mann, 2015) (Table 1), as well as the fact that praline acquires a higher pH (6.1–6.3) compared to biscuit (5.9–6.1) that may also favor the survival of *Salmonella* (Table 1). Other intrinsic factors of the studied food matrices like fat (15–17%) and/or sugars content (*ca.* 18–19% in brioche; *ca.* 41% in cream–filling) may protect *Salmonella* against the acidic conditions of the stomach, potentially increasing the likelihood of illness in case of consuming low numbers of the pathogen (FAO/WHO, 2016). Previous studies have reported that *Salmonella* is capable of surviving in high–sugar (Beuchat and Mann, 2015; Nummer et al., 2012) and high–fat confectionery products (Kataoka et al., 2014). *S.* Typhimurium was found to survive in peanut butter fondant candy for a minimum period of 12 months (Nummer et al., 2012), while *S.* Enteritidis survived in halva for at least 8 months (Kotzekidou, 1998). Given the absence of the competitive effect of indigenous flora (data not shown), the observed inactivation of *Salmonella* was collectively attributable to the other intrinsic factors such as a_w, pH, fat, and sugars content (Wu et al., 2017).

In an effort to compare the data of the present study with existing predictive models, the recorded inactivation curves of *Salmonella* (osmotically adapted and non–adapted cells) *per* food matrix were compared to simulation curves retrieved by existing polynomial models of ComBase (www.combase.cc) and the non-thermal inactivation model of Pin et al. (2011) in GroPIN modelling software (http://www.aua.gr/psomas/gropin/), under the closest possible experimental conditions to those applied here. Specifically, simulation curves were generated by taking into account the parameters of: i) pH (5.9–6.3) and a_w (0.78–0.84) *per* food matrix (Table 1), ii) storage temperature (20 °C), and iii) initial population of *Salmonella* (N_0 = 6.0 log CFU/g). Models from both databases underestimated the survival of *Salmonella* in both food matrices (Fig. 3(a)–(d)); however, the predicted inactivation curve of *Salmonella* retrieved by the model of Pin et al. (2011) was closer to the experimental data of the present study, especially in the case of brioche. With regards to ComBase, the recorded underestimation may potentially be attributed to the fact that inactivation curves are derived from *in vitro* models that, inevitably, do not take into account matrix-related parameters of the particular products, which apparently

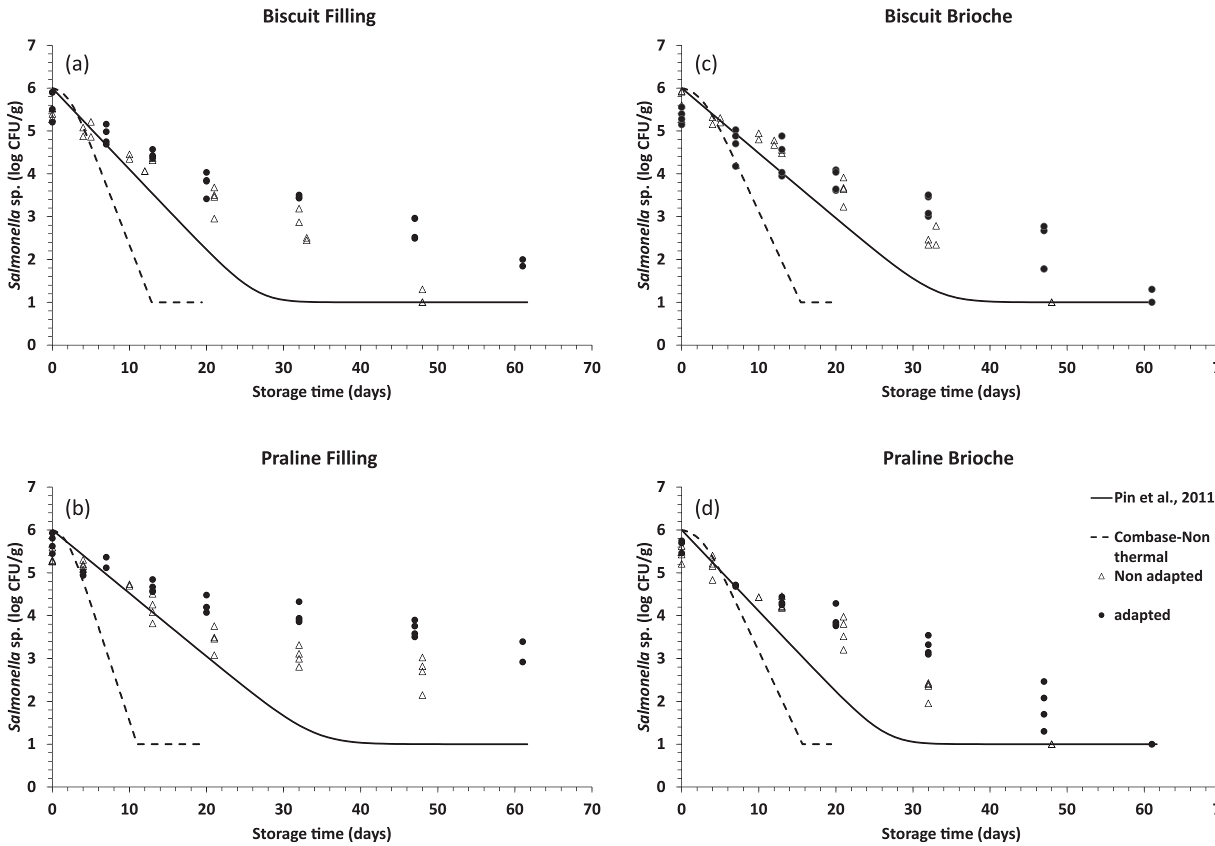

Fig. 3. Observed inactivation of *Salmonella* spp. of (a) biscuit cream–filling, (b) praline cream–filling, (c) biscuit brioche, and (d) praline brioche stored under aerobic conditions at 20 °C *co*-plotted with the simulated survival of the pathogen by previously existed polynomial models of ComBase and GroPIN (Pin et al., 2011) under the same experimental conditions of initial inoculum (6.0 log CFU/g), pH (5.9–6.3), a_w (0.78–0.84), and storage temperature (20 °C).

Table 4
Estimated parameters of secondary linear model for ln (δ) and goodness-of-fit indices (R^2_{adj}; F-significance) for inactivation of osmotically adapted and non–adapted cells of *Salmonella* spp. inoculated in cream–filling and brioche (biscuit and praline), stored at 15, 20, and 30 °C.

ln (δ)								
Parameter	Biscuit cream–filling		Praline cream–filling		Biscuit brioche		Praline brioche	
	Non–adapted	Adapted	Non–adapted	Adapted	Non–adapted	Adapted	Non–adapted	Adapted
a_1	4.40 ± 0.37	4.03 ± 0.38	4.41 ± 0.44	5.44 ± 0.63	5.10 ± 0.39	4.86 ± 0.34	4.52 ± 0.38	5.20 ± 0.29
a_0 (°C^{-1})	−0.10 ± 0.01	−0.08 ± 0.01	−0.09 ± 0.01	−0.15 ± 0.02	−0.13 ± 0.01	−0.11 ± 0.01	−0.11 ± 0.01	−0.14 ± 0.01
R^2_{adj}	0.889	0.842	0.832	0.847	0.920	0.919	0.898	0.962
F-significance	0.0031	0.0063	0.0071	0.0058	0.0016	0.0016	0.0026	0.0003

Table 5
Estimated parameters of secondary linear model for ln (t_{4D}) and goodness-of-fit indices (R^2_{adj}; F significance) for inactivation of osmotically adapted and non–adapted cells of *Salmonella* spp. inoculated in cream–filling and brioche (biscuit and praline), stored at 15, 20, and 30 °C.

ln (t_{4D})								
Parameter	Biscuit cream–filling		Praline cream–filling		Biscuit brioche		Praline brioche	
	Non–adapted	Adapted	Non–adapted	Adapted	Non–adapted	Adapted	Non–adapted	Adapted
b_1	6.82 ± 0.23	7.74 ± 0.59	7.71 ± 0.31	8.41 ± 0.62	6.57 ± 0.18	7.06 ± 0.27	6.43 ± 0.24	6.80 ± 0.19
b_0 (°C^{-1})	−0.14 ± 0.01	−0.16 ± 0.02	−0.16 ± 0.01	−0.16 ± 0.02	−0.13 ± 0.00	−0.14 ± 0.01	−0.12 ± 0.01	−0.13 ± 0.00
R^2_{adj}	0.975	0.890	0.964	0.871	0.983	0.966	0.963	0.981
F-significance	0.0002	0.0030	0.0003	0.0041	0.0001	0.0003	0.0003	0.0001

improved survival of *Salmonella* compared to laboratory broths. In addition, it is conceivable that the bacterial cultures used for generating the data of the *in vitro* models, were not osmotically adapted as in the present study. Thus, the latter observations collectively underline the need for updating the current non–thermal inactivation models, which are commonly based on laboratory media and non–adapted overnight cultures.

From the aspect of modelling, and considering the aforementioned

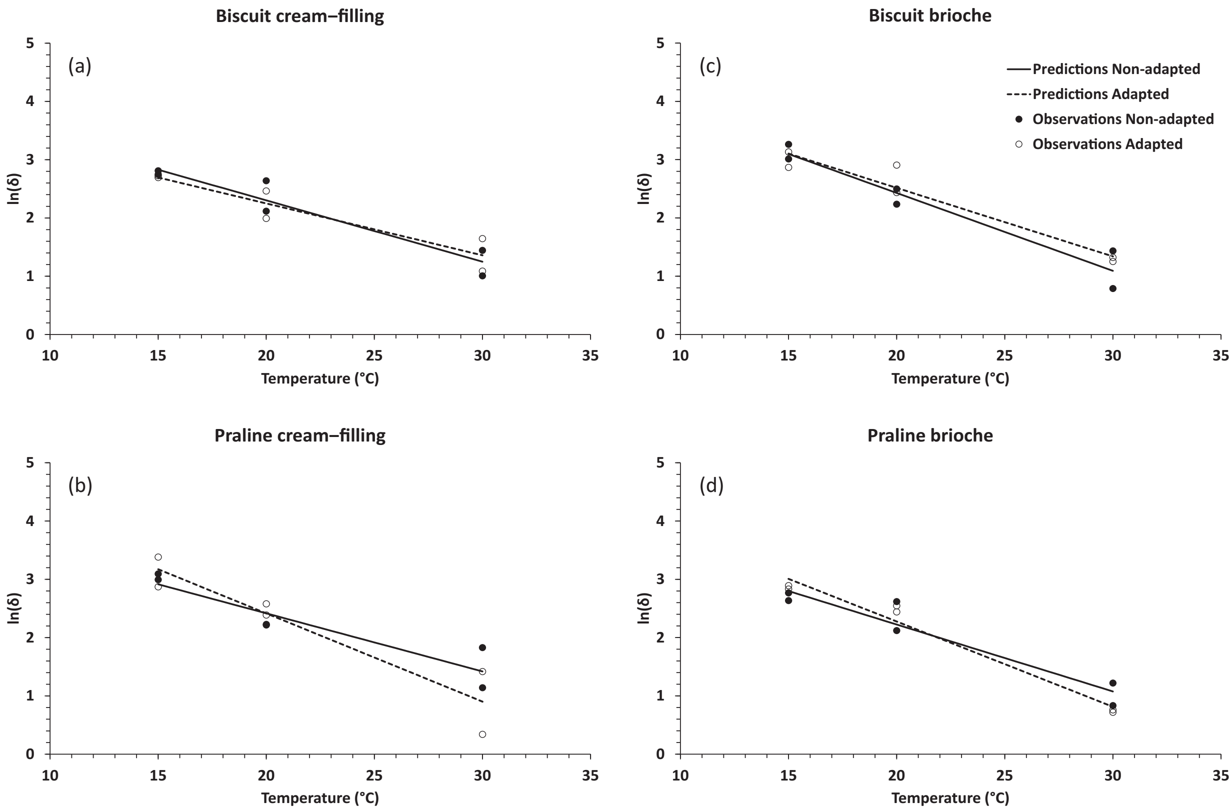

Fig. 4. Graphical illustration of fitted curves of the linear regression model of ln(δ) of *Salmonella* spp. on (a) biscuit cream–filling, (b) praline cream–filling, (c) biscuit brioche, and (d) praline brioche stored under aerobic conditions at 15, 20, and 30 °C in comparison with experimental data used for the fitting.

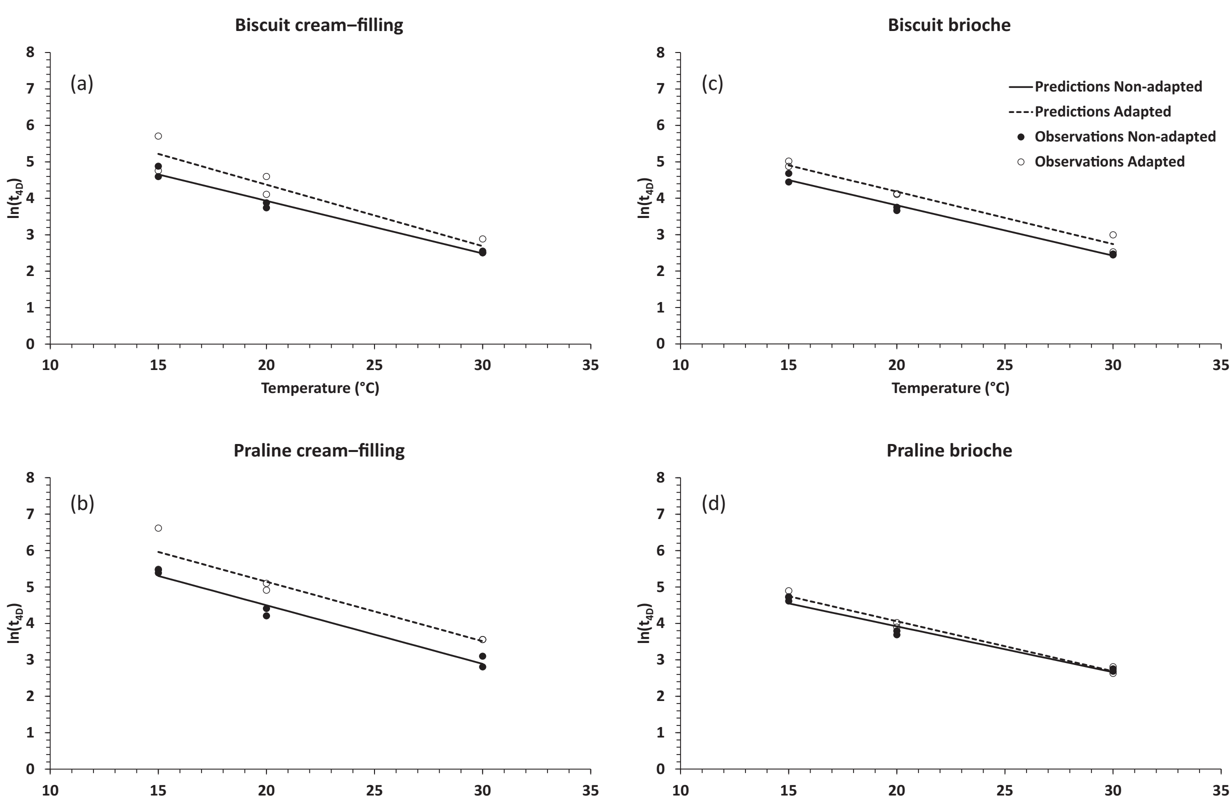

Fig. 5. Graphical illustration of fitted curves of the linear regression model of ln(t_{4D}) of *Salmonella* spp. on (a) biscuit cream–filling, (b) praline cream–filling, (c) biscuit brioche, and (d) praline brioche stored under aerobic conditions at 15, 20, and 30 °C in comparison with experimental data used for the fitting.

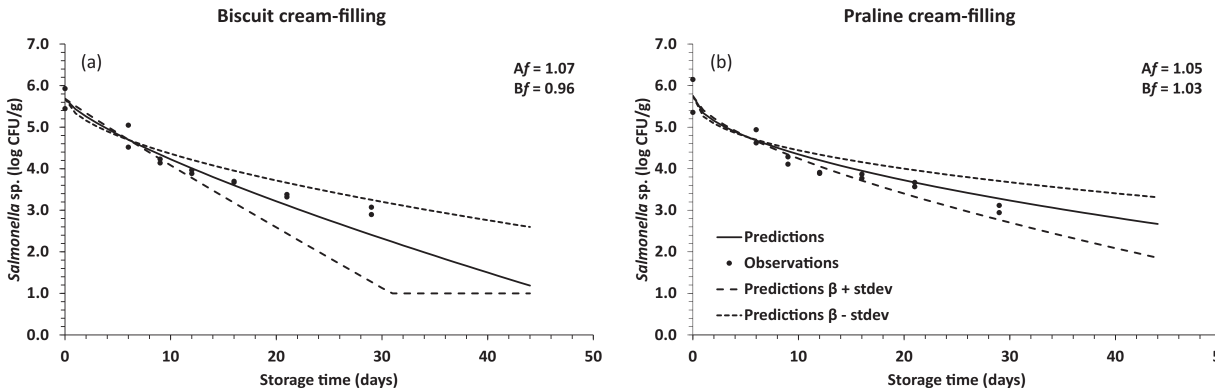

Fig. 6. Model simulation of adapted *Salmonella* spp. inactivation in (a) biscuit and (b) praline cream–fillings during storage at 25 °C for 30 days and comparison with observed data.

literature limitations, linear regression model was used in order to describe the effect of storage temperature on inactivation of osmotically adapted and non–adapted cells of *Salmonella* in the present food matrices (*i.e.*, cream–fillings and brioche). As already mentioned in material and methods, the goodness of fit was determined by the estimated parameters of R^2_{adj} and F-test. The secondary models for ln (δ) and ln (t_{4D}) showed R^2_{adj} of 0.83–0.96 and 0.87–0.98, respectively, depending on the food matrix (Tables 4 and 5), while the significance of F-test was < 0.05 for all fitted models. Predictive modelling revealed that survival of *Salmonella* is dependent on storage temperature and cells status (osmotically non– or adapted) (Figs. 4 and 5). As mentioned above, the organism was inactivated faster at elevated storage temperatures (Figs. 4 and 5). The developed secondary models for *Salmonella* inactivation showed that osmotically adapted cells had higher ln (t_{4D}), compared to non–adapted cells (Fig. 5). The maximum differences for the predictions curves of ln (t_{4D}) between osmotically adapted and non–adapted pathogen cells were observed on praline cream–filling, followed by biscuit cream–filling, biscuit brioche, and finally, praline brioche (Fig. 5(a)–(d)).

Both cream–fillings showed lower inactivation rates (expressed as increased δ and t_{4D}) compared to brioche, thus it was decided to be tested for the validation experiments as the worst case scenario of contamination. The observed inactivation data of adapted *Salmonella* cells showed good agreement with model simulations on both cream–fillings (Fig. 6), with the simulated inactivation curve being slightly lower than the recorded data on biscuit cream–filling (Fig. 6(a)). On the contrary, model predictions were higher than the observations on praline cream–filling (Fig. 6(b)). Bias factors (0.96 on biscuit cream–filling and 1.03 on praline cream–filling) were both close to 1, indicating no substantial bias of the model and fail-safe predictions, respectively (Mataragas et al., 2008). Ross (1999) has reported that models with B*f* values from 1.00 to 1.05 or 1.06 to 1.15 are considered adequate or acceptable, respectively. Moreover, accuracy factors (A*f*) showed good agreement between predictions and observations (1.07 on biscuit cream–filling and 1.05 on praline cream–filling) at both studied cream–fillings. In general, a good agreement between predictions and observations was obtained with the *Salmonella* inactivation model for both cream–fillings, suggesting that it may be a useful tool for food safety.

4. Conclusions

Overall, *Salmonella* response in cream–fillings and brioche may be affected by food matrix, storage temperature, as well as potential prior adaptation of pathogens' cells. The take away message of the present study is summarized in that: i) the insufficient control of a_w may increase the risk of *Salmonella* survival in food products, and ii) the existing non–thermal inactivation models in laboratory media do not take into account potential adaptation of cells, neither matrix-assisted survival or adaptation, thereby, underestimating the actual pathogen response in IMF food matrices. Hence, the data from the present study could be used as a means to identify areas of improving the performance of existing models quantifying the behavior of the pathogen in bakery-confectionary products.

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