Effect of desiccation, starvation, heat and cold stresses on the thermal resistance of *Enterobacter sakazakii* in rehydrated infant milk formula

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Running Title: Thermal inactivation of stressed *E. sakazakii*

Abstract

Enterobacter sakazakii has been implicated in outbreaks of meningitis, septicemia, and necrotizing enterocolitis in immunocompromised and premature neonates. In this study, the effect of desiccation stress, starvation stress, heat shock and cold shock on thermal inactivation of *E. sakazakii* in rehydrated infant milk formula was evaluated. Stressed cells were mixed with rehydrated infant milk formula at 52, 54, 56, and 58°C for various time periods. The *D*- and *z*-values were determined by using linear regression analysis. *D*-values for unstressed *E. sakazakii* at 52, 54, 56 and 58°C were 15.33, 4.53, 2.00 and 0.53 min, respectively. Desiccation and heat stress, but not starvation or cold stress, caused significant reduction in *D*-values. For example, D_{52} was 15.33 min for unstressed cells compared with 8.72 and 7.36 after desiccation and heat stress. *Z-values* of desiccated, starved, heat shocked and cold shocked *E. sakazakii* were not significantly different from unstressed cells (4.22°C). The results of this study may be of use to regulatory agencies, infant milk producers and infant caregivers to design heating processes to eliminate *E. sakazakii* that may be present in infant milk formula.

1. Introduction

Enterobacter sakazakii is a Gram negative, facultatively anaerobic, motile, non-spore forming bacterium belonging to the *Enterobacter* genus and the *Enterobacteriaceae* family. It was known as "yellow-pigmented *Enterobacter cloacae*" but in 1980 was renamed *E. sakazakii* because of differences with *E. cloacae* in DNA-DNA hybridization, biochemical reactions, and pigment production (Farmer, Asbury, Hickmann, & Brenner, 1980).

E. sakazakii is considered an opportunistic pathogen, which can cause severe forms of infections including meningitis, bacteraemia, and necrotizing enterocolitis in neonates and infants (Farmer et al., 1980; Bar-Oz, Preminger, Peleg, Block, & Arad, 2001; Van Acker, De Smet, Muyldermans, Bougatef, Naessens, & Lauwers, 2001; Block, Peleg, Minster, Bar-Oz, Simhon, Arad, & Shapiro, 2002; Himelright, Harris, Lorch, Anderson, Jones, Craig, Kuehnert, Forster, Arduino, Jensen, & Jernigan, 2002; FAO/WHO, 2004). Although documented outbreaks caused by this pathogen are rare, *E. sakazakii* was grouped together with *Listeria monocytogenes*, *Clostridium perfringens* types A and B and *Cryptosporidium parvum*, into 'Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' by the International Commission for Microbiological Specification for Foods (2002).

This organism has been isolated from a variety of foods, food factories and environments (Muytjens, Zanen, Sonderkamp, Kolee, Wachsmuth, & Farmer, 1983; Iversen & Forsythe, 2004; Kandhai, Reij, Gorris, Guillaume-Gentil, & Van Schothorst, 2004; Guillaume-Gentil, Sonnard, Kandhai, Marugg, & Jousten, 2005; Nassereddin & Yamani, 2005; Restaino, Frampton, Lionberg, & Becker, 2006; Shaker, Osaili, Al-Omary, Jaradat, & Al-Zuby, 2007). However, *E. sakazakii* infections are associated with powder

infant formulas or preparation equipment (Bar-Oz et al., 2001; Van Acker et al., 2001; Block et al., 2002).

Because the potential risk of *E. sakazakii* in infant milk formula is a major concern to regulatory agencies and infant formula producers, different methods have been investigated to inactivate *E. sakazakii* in powdered and rehydrated infant milk formula such as gamma radiation (Osaili, Shaker, Abu Al-Hassan, Ayyash, & Martin, 2007a; Lee, Oh, Kim, Yook, & Byun, 2006), probiotics (Osaili, Shaker, Ayyash, & Holley, 2007b), bacteriophages (Kim, Klumpp, & Loessner, 2006), high pressure processing (Gonzalez, Flick, Arritt, Holliman, & Meadows, 2006), pulsed electrical field (Pérez, Aliaga, Bernat, Enguidanos, & López, 2007) and heat treatment (Edelson-Mammel & Buchanan, 2004). Heating rehydrated infant milk formula before feeding the infants has been recommended by FAO/WHO (2004) to eliminate the risk of *E. sakazakii* in infant milk formula.

Heat treatment remains the primary method of eliminating foodborne pathogens from foods. Although the thermotolerance of microorganisms is affected by their physiological states (Lou & Yousef, 1996; Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001; Wesche, Marks, & Ryser, 2005), all published thermal inactivation studies of *E. sakazakii* in rehydrated infant formula have employed cells that were prepared under optimal laboratory conditions (Nazarowec-White & Farber, 1997; Breeuwer, Lardeau, Peterz, & Joosten, 2003; Edelson-Mammel & Buchanan, 2004; Iversen, Lane, & Forsythe, 2004). In the environment, however, microorganisms are exposed to various stresses, e.g., chemical, physical or nutritional stresses. Therefore, it would be appropriate to study the thermotolerance properties of the stressed microbes that might contaminate the products from the food processing or preparation environment.

Desiccation stress can occur when microbes are exposed to dry conditions. Exposure of growing microbes to low nutrient conditions can lead to starvation stress (Dickson & Frank, 1993; Wesche et al., 2005). Heat shock can occur when bacteria are exposed for a short period to high temperature within or higher than their normal growth temperature (Bunning, Crawford, Tierney, & Peeler, 1990; Pagan, Condon, & Sala, 1997). Cold shock can occur when microbes are exposed to sudden drop in temperature of more than 15 degrees (Jones, Mitta, Kim, Jiang, & Inouyi, 1996).

The effect of stresses on the thermotolerance of pathogenic bacteria in the family of *Enterobacteriaceae* in food, water or broth system has been studied (Shenoy & Murano, 1996; Juneja, Klein, & Marmer, 1998; Leenanon & Drake, 2001; Wesche et al., 2005; Spinks, Dunstan, Harrison, Coombes, & Kuczera, 2006). However, there are no published studies investigating the effect of environmental stresses on thermal resistance of *E. sakazakii* in rehydrated infant milk formula. Thus, the present study was undertaken to quantify the effect of desiccation, starvation, heat and cold stresses on the thermal inactivation of *E. sakazakii* in rehydrated infant milk formula.

Such information may be useful to regulatory agencies, infant milk producers and infant care givers to design heating processes that are sufficient to kill *E. sakazakii* that may be present in infant milk formula.

2. Materials and Methods

2.1. E. sakazakii strains

Five *E. sakazakii* strains were used in this study; ATCC (51329) strain and four food isolates (Shaker et al., 2007). All cultures were stored in brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, UK) broth with 20% glycerol at -40°C. *E. sakazakii* cultures were subcultured in BHI three times before use.

2.2. Preparation of the unstressed E. sakazakii cultures

Equal volumes (1ml) of each *E. sakazakii* strain were combined to form a cocktail culture. The mixed culture was centrifuged (3008 *g*, 20 min). The supernatant was discarded and the pellet was resuspended in 1 ml of 0.1% peptone water (Becton Dickinson, Sparka, Md, USA) to a concentration of approximately 10^{10} CFU/ml.

2.3. Preparation of stressed E. sakazakii cultures

2.3.1. Desiccation treatment

The *E. sakazakii* cocktail was desiccated as described by Breeuwer et al. (2003) with minor modifications. One millilitre of freshly prepared *E. sakazakii* cocktail was divided into 50 µl portions in a sterile Petri dish. The plate was kept without lid in a 40°C incubator for drying. Dehydrated silica gel was placed in the incubator. After drying (< 2h) the plate was covered and kept at 21°C for 4 days. Preliminary study showed that the drying and storage times decreased the initial number of the cells 1 log and ≤ 1 log/ ml, respectively.

2.3.2. Starvation treatment

The starvation treatment method used in the present study was similar to that described by Leenanon and Drake (2001) for *E. coli* O157:H7. One millilitre of freshly prepared *E. sakazakii* cocktail was added to 9 ml of sterile saline solution (0.85% NaCl) in 15 ml screw cap test tube, mixed thoroughly for 1 min, and then incubated for 48h at 37°C.

2.3.3. Heat shock treatment

Heat shocked cultures were prepared as described by Gurtler and Beuchat (2005). One milliltre of freshly prepared *E. sakazakii* cocktail was added to 9 ml of sterile potassium

phosphate buffer (0.1 M: pH 6.8) in 15 ml screw cap test tube that was placed in a water bath (Memmert, Germany) at temperature of 55°C. After 5 min of holding, the test tube was removed and cooled immediately under running tap water. Preliminary studies showed that heat stress decreased the number of *E. sakazakii* ≤ 1 log/ml

2.3.4. Cold shock treatment

The cold shock culture was prepared as described by Wesche et al. (2005) for *Salmonella*. One millilitre of freshly prepared *E. sakazakii* cocktail was added to 9 ml of sterile potassium phosphate buffer (0.1 M, pH 6.8) in 15 ml screw cap test tube and mixed thoroughly for 1 min then stored for 24 h at 4°C. Preliminary study showed that cold stress decreased the number of *E. sakazakii* ≤ 1 log/ml

2.4. Infant formula

Commercial dehydrated infant milk formula (56.6% carbohydrate, 11.4% protein, and 25.4% fat) was rehydrated according to the manufacture's instruction at the ratio of 1/6.7 (w/v). The infant milk formula was screened before use and no *E. sakazakii* were detected.

2.5. Thermal inactivation

Prior to heat treatments, unstressed, starved, heat shocked or cold shocked cultures were centrifuged, as described before, and resuspended in 1 ml peptone water (0.1%) to be used in the thermal inactivation studies. Desiccated cells were rehydrated by adding 1 ml of peptone water.

Fifty millilitre of rehydrated infant milk formula were prepared in sterile 100-ml capacity Duran bottles. The formula was heated prior of inoculation to 52, 54, 56, or 58°C in a

temperature-controlled shaking water bath. A calibrated thermocouple was placed in a replicate diluent bottle to monitor the temperature profile over the experimental periods. One millilitre of the unstressed, desiccated, starved, heat shocked or cold shocked cocktails was mixed individually with heated rehydrated infant formula at each temperature. At timed intervals, depending on temperature, samples (1 ml) were transferred to sterile tubes and cooled in an ice-water bath. Aliquots (0.1ml) of appropriate dilutions of the samples were plated in duplicate on tryptone soy agar (TSA) (Oxoid Ltd., Basingstoke, UK) supplemented with 0.1% sodium pyrovate, and incubated at 37°C. After 48h of incubation survivor cells were enumerated. Triplicate thermal inactivation trials were performed at each studied temperature.

2.6. D- and z-value determinations

The logarithms of the number of *E. sakazakii* survivors in rehydrated infant milk formula after each heat treatment were plotted against the heating time. The *D*-value for the microorganism at each temperature was calculated from the linear regression model for the $\log_{10}$ of surviving bacterial cells and heating time. The *D*-value is the negative inverse slope of the plot:

$$\log(N) = \log(N_0) - \frac{t}{D}$$

where N is the number of survivors (CFU/ml) at time t and N_0 is the number of survivors at time 0.

The *z*-values for *E. sakazakii* were calculated by determining the linear regression of the $\log_{10}$ of *D*-values and temperatures (T). The *z*-value is the negative inverse slope of the plot:

$$\log(D) = \log(D_0) - \frac{T}{z}$$

where D is the decimal reduction time (min) at temperature T (°C). D_0 is the decimal reduction time at temperature 52°C and z is thermal resistant constant (°C).

2.7. Process lethality calculation

Sixty millilitre of rehydrated infant milk formula in 125 ml-capacity sterile Duran bottle was heated in water bath to 63°C (minimum temperature used for pasteurization process) then immediately cooled under running tap water to 40°C. The temperature during heating and cooling the rehydrated formula was monitored using a calibrated thermometer at 5 second timed intervals. Process lethality was calculated from the integration of the time-temperature relationship during heating and cooling of the milk formula:

$$F = \int_0^t 10^{\frac{[T(t)-T(\mathrm{Ref})]}{z}} dt$$

Where T(t) is the temperature of rehydrated infant milk formula at time t, and T(Ref) is the reference temperature. The T(Ref) was 58°C and the D_{58} and z values obtained for *E. sakazakii* in rehydrated infant milk formula were used in predicting pathogen lethality for the heating processes.

2.8. Statistical analysis

The means of the D-and z-values of desiccated, starved, heat shocked or cold shocked *E. sakazakii* in rehydrated infant milk formula were compared with those values of unstressed *E. sakazakii* in rehydrated infant milk formula by using the student's t-test at 0.05 significant level.

3. Results

The *E. sakazakii* death kinetics were modeled using linear regression analysis. The regression curves were fitted with r^2 values (coefficient of determination) of > 0.90 for all four temperatures. Figures 1 - 4 show the survivor curves of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* at temperature range of 52 to 58°C (Table 1). Desiccation and heat stress, but not starvation or cold stress, caused significant reductions in D-values. For example, D_{52} was 15.33 min for unstressed cells compared with 8.72 and 7.36 after desiccation and heat stress. Desiccation and heat stresses significantly ($P < 0.05$) decreased thermal resistance of *E. sakazakii* in rehydrated infant milk formula. Desiccation stress decreased the D-values by 43.1, 54.8, 57.8 and 43.4% and heat stress decreased the D-values by 52.0, 54.3, 62.0, and 49.1% at temperatures 52, 54, 56 and 58°C, respectively. Starvation and cold stresses did not affect significantly ($P > 0.05$) the thermal resistance of *E. sakazakii* in rehydrated infant milk formula.

The z-values of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* were 4.22 (± 0.16, SD), 4.20 (± 0.09), 4.23 (± 0.40), 4.22 (± 0.09), and 4.12 (±0.13), respectively, with correlation coefficients >0.97 (Figure 5). There were no statistical differences ($P > 0.05$) between the z-values of the unstressed and stressed cells.

The time-temperature profile during heating and cooling the rehydrated infant milk formula was monitored (Figure 6). The maximum temperature (63°C) was reached after 4 minutes, and reduced to 40°C within 7 minutes.

4. Discussion

Previously the thermal resistance of *E. sakazakii* in rehydrated infant milk formula has been studied using unstressed cultures that were grown under optimal laboratory conditions. Breeuwer et al. (2003) reported *D*-values for two strains of *E. sakazakii* within

the same range as those reported in the present study at 54, 56, and 58°C. Iversen et al. (2004) and Nazarowec-White and Farber (1997) reported higher *D*-values at 52, 54, 56, and 58°C for *E. sakazakii* in infant milk formula than our values for stressed and unstressed cultures.

The effect of desiccation on the thermotolerance of *E. sakazakii* has not previously been reported. Breeuwer et al. (2003) studied the survival of *E. sakazakii* in dry conditions and found that *E. sakazakii* is more resistant to osmotic and dry stresses than other *Enterobacteriaceae* members and that resistance is most likely linked to the accumulation of trehalose in the cells. In our study, desiccation stress failed to provide cross-protection against heat treatment. This is possibly because simultaneous exposure of the microbe to different stresses, as in this study exposure to desiccation stress is combined with starvation stress, required energy-consuming production of a number of protective stress shock proteins, which may cause the microorganisms to be metabolically exhausted (Beales, 2004) and thus less heat resistant.

The changes in *D*-values obtained for starved *E. sakazakii* were consistent with those reported for other starved pathogens. Lou and Yousef (1996) observed that exposing *L. monocytogenes* Scott A to starvation stress for 48h increased the *D*-value at 56°C 5.5-fold compared with the control sample. Bang and Drake (2002) reported that starvation increased the *D*-values at 47°C for three strains of *Vibrio vulnificus* by 6 to 26% compared with control cultures. Leenanon and Drake (2001) found that starvation enhanced the thermotolerance in two *E. coli* strains. They reported that the *D*-values at 56°C increased from 7.1 and 5.4 min to 9.7and 7.2 min, respectively.

Several investigators have reported that heat shock increases the thermotolerance of bacteria through the induction of a specific set of proteins known as heat shock proteins (Juneja et al., 1998; Wesche et al., 2005). However, this study found that heat shock prior

to heat treatment made *E. sakazakii* more sensitive to heat. Yousef and Courtney (2003) mentioned that there are three levels of microbial stresses; mild stress that does not cause viability loss but arrests growth rate, moderate stress that causes some viability loss and arrests growth, and severe stress that causes microbial death. It seems that the heat shock (55°C for 5 min) used in this study resulted in injured cells which when were exposed to heat treatment died quickly.

Cold stress can decrease the heat tolerance of bacteria. Leenanon and Drake (2001) reported that the *D*-values of three *E. coli* strains at 56°C in broth system decreased after exposure to cold stress. Generally, change in thermotolerance of microorganisms after environmental stresses may be explained by stress induced physiological changes (Lou and Yousef, 1996). In our study, the decrease in heat tolerance following cold stress may be due to the induction of cold shock proteins and the repression of heat shock proteins or to the incorporation of more unsaturated fatty acids into cell membranes to maintain membrane fluidity (Beales, 2004).

The *z*-values for *E. sakazakii* (4.22, 4.20, 4.23, 4.22, and 4.12 °C) were similar to those previously published optimally grown, unstressed cells. Nazarowec-White and Farber (1997) reported *z*-value of 5.8°C for a cocktail of *E. sakazakii*. Breeuwer et al. (2003) reported *z*-values of 3.1 and 3.6°C for *E. sakazakii* 1787-2 and 16, respectively. Edelson-Mammel and Buchanan (2004) and Iversen et al. (2004) have reported *z*-value of 5.6°C for *E. sakazakii* in rehydrated infant milk formula. The similarity between the *z*-values of unstressed and shocked *E. sakazakii* indicating that stresses had no effect on the sensitivity of the microbe to temperature changes.

The *z*-value of *E. sakazakii* in rehydrated infant milk formula is required to calculate process lethality (*F*). For instance, heating the infant milk formula to temperature of 63°C then cooling to 40°C will achieve average process lethality at reference temperature 58°C

of 18 min. This process lethality will result in *ca* 60, 27, 67, and 38 log reduction (F/ $D_{58°C}$) of desiccated, starved, heat shocked, and cold shocked *E. sakazakii* and 40 log reduction of unstressed *E. sakazakii* in rehydrated infant milk formula. Therefore the presence of *E. sakazakii* in reconstituted powdered infant milk formula will probably be due to contamination after pasteurization during the manufacturing process. This study would be useful to infant milk formula processors, regulatory agencies and infant care givers to design heating processes that are sufficient to destroy *E. sakazakii* that may be present in dehydrated infant milk formula.

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Table1. *D*-values of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* in rehydrated infant milk formula.

	D-values (min)[a]				
	Treatment				
Temperature (°C)	Unstressed	Desiccation	Starvation	Heat	Cold
52	15.33± 2.19	8.72±0.92*	17.47 ± 3.11	7.36 ± 0.52*	14.43 ± 1.36
54	4.53 ± 0.55	2.05±0.11*	6.67 ± 1.27	2.07 ± 0.35*	3.93 ± 0.15
56	2.00 ± 0.35	0.84±0.07*	2.07 ± 0.21	0.76 ± 0.20*	1.47 ± 0.04
58	0.53 ± 0.03	0.30±0.04*	0.67 ± 0.06*	0.27 ± 0.01*	0.48 ± 0.03

[a] Arithmetic mean of three replications ± standard deviation.

* The value is significantly different ($P < 0.05$) compared with that of unstressed cells at the same temperature.

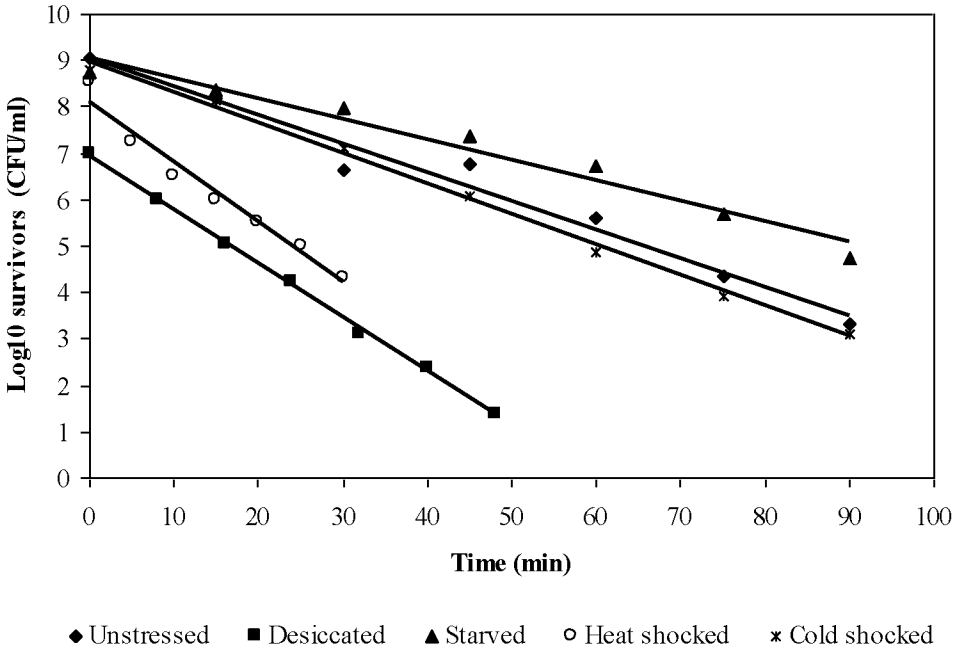

Figure 1- Survivor curves of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* at 52°C in rehydrated infant milk formula.

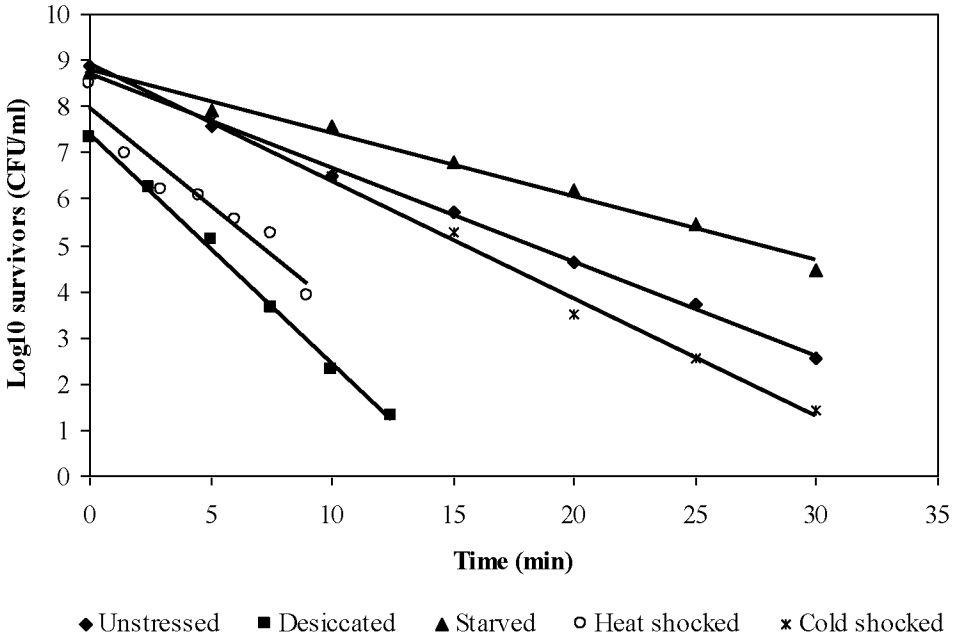

Figure 2- Survivor curves of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* at 54°C in rehydrated infant milk formula.

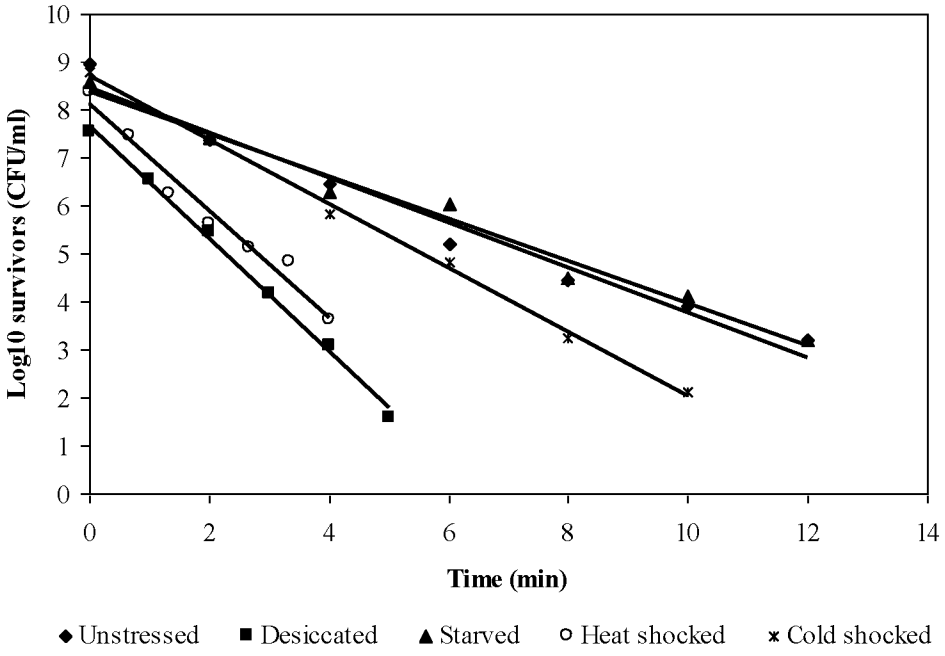

Figure 3- Survivor curves of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* at 56°C in rehydrated infant milk formula.

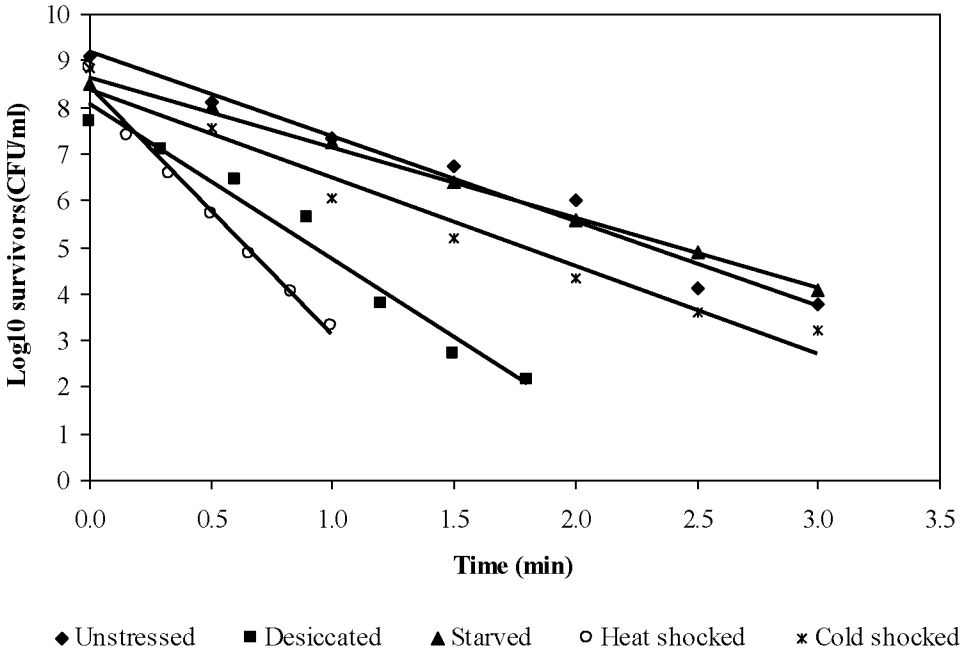

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Figure 4- Survivor curves of unstressed, desiccated, starved, heat shocked and cold shocked *E*675
sakazakii at 58°C in rehydrated infant milk formula. 676
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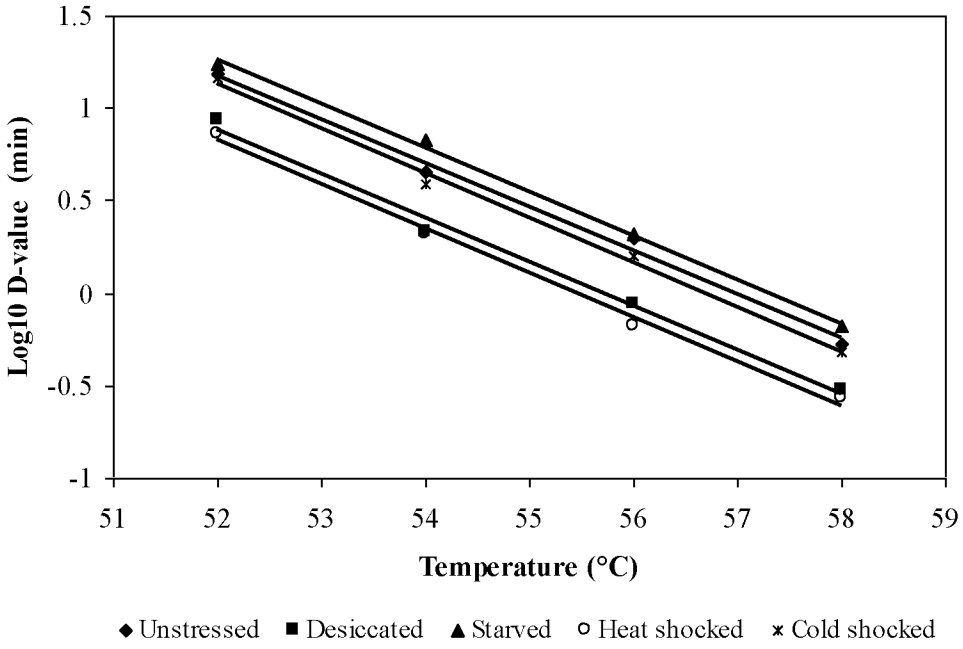

Figure 5- Thermal resistance curves of unstressed, desiccated, starved, heat shocked and cold shocked *E. sakazakii* in rehydrated infant milk formula.

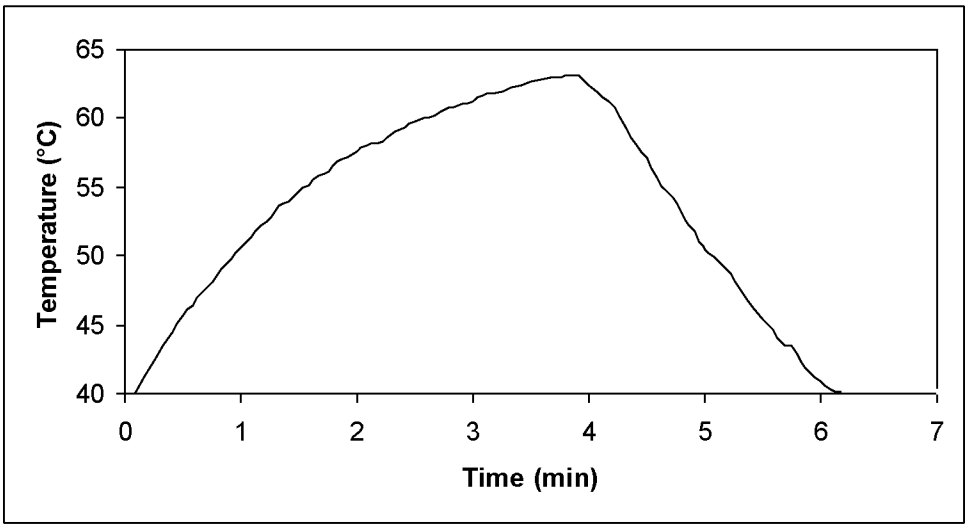

Figure 6- Time-temperature profile during heating and cooling of rehydrated infant milk formula.