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Growth and survival of *Cronobacter* species as measured by media performance

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Abstract: *Cronobacter sakazakii* is an emerging food borne pathogen which has been associated with outbreaks of a rare form of infant meningitis. Although the origin of the microorganism has not been established, several infection cases have been associated with the consumption of contaminated powdered infant formula (PIF). In the present study, growth characteristics of three *C. sakazakii* strains isolated from PIF samples and *C. muytjensii* strain ATCC 51329, which was formerly the ATCC Preceptrol™ strain for the quality control of '*Enterobacter sakazakii*' prior to the taxonomic revision, were investigated in Tryptone Soya broth (TSB) and reconstituted PIF at 4, 10, 25, 37, 45 and 50ºC. The viability of heat treated cells of *Cronobacter* strains was evaluated by plating on Violet Red Bile Glucose agar (VRBGA) and the Druggan-Forsythe-Iversen (DFI) chromogenic agar followed by incubation at 37ºC. These strains were also subjected to higher temperatures between 52 to 60ºC to measure their thermal tolerance. The mean generation time of all *Cronobacter* strains were slightly lower in PIF than in TSB. *C. muytjensii* ATCC 51329 showed lower generation time in all culture media and all temperatures compared to the *Cronobacter* food isolates, but the results were not significantly different ($P>0.05$). The results also indicated that combination of PIF: DFI culture media had higher recovery at all temperatures compared to other combinations. Survival study also indicated that *C. muytjensii* ATCC 51329 had higher D-value compared to food isolates at all incubation temperatures.

Keywords: *Cronobacter sakazakii*, powdered infant formula, chromogenic agar, generation time, thermotolerance

Introduction

Cronobacter sakazakii is a motile, non-sporeforming, Gram-negative foodborne pathogen belonging to the family Enterobacteriaceae (Iversen et al., 2008). This pathogenic organism has been implicated as a cause of infant meningitis, necrotizing entercolitis (NEC), bacteraemia and may cause death among neonates (Bar-Oz et al., 2001; Bowen and Braden, 2006; Caubilla-Barron et al., 2007; Lai, 2001). The groups at particular risk are infants (i.e., children < 1 year) and those who are immunocompromised. Neonates are considered to be at greatest risk, particularly neonates of low birth weight (FAO/WHO, 2004, 2006, 2008).

Cronobacter has been isolated from a wide range of foods including cereals, cheese, fruits, meat, milk, vegetables, grains, herbs and spices as well as their by products (Friedemann, 2007; Iversen and Forsythe, 2003). However, its presence in powdered infant formula (PIF) as the most common food has raised concern among the food microbiologists (Forsythe, 2005; Himelright et al., 2002; Van Acker et al., 2001). Unlike commercially ready to feed liquid formula, PIF are not sterile and must conform to national and international microbiological criteria (CAC, 2008a, b).

The use of high temperatures to preserve food is based on their destructive effects on microorganisms. Microbial thermotolerance varies very widely among different species and is influenced by a variety of factors. Thermal resistance of bacteria is influenced by the composition of culture medium in which the organisms are grown before heating, the composition of the recovery medium, the menstruum in which the organisms are heated, the density of the suspension, and the time and temperature of incubation before and after heating (Hansen and Riemann, 1963; Whiting and Buchanan, 1994). In this study, growth and survival characteristics of *Cronobacter* strains isolated from PIF available in Malaysia were measured using different media culture at different incubation temperatures.

Materials and Methods

Tryptone Soya broth (TSB, CM129), Maximum Recovery Diluent (MRD, CM733), Violet Red Bile Glucose agar (VRBGA, CM485), Druggan-Forsythe-Iversen (DFI formulation, CM 1055, Oxoid Ltd.,

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Basingstoke, UK), Tryptone Soya agar (TSA, CM131) were bought from Oxoid (UK). Sodium pyruvate (BDH 151TD) was bought from BDH Laboratory Supplies (UK) and PIF from local retailers.

Bacterial strains

Four strains were used in this study to determine the growth and survival characteristic of *Cronobacter* strains. Three strains of *Cronobacter* (MGG1, MGF1, and MGH1) have been isolated from PIF available in Malaysia in the previous study (Norrakiah et al., 2007) and one from American Type Culture Collection (ATCC), *C. muytjensii* strain ATCC 51329, which was formerly the ATCC Preceptrol™ (quality control) strain for the quality control of '*Enterobacter sakazakii*' prior to the taxonomic revision. All strains were activated by culturing on nutrient agar and incubating at 37ºC for 24 hr.

Growth characteristics of Cronobacter strains

To prepare inocula, one loopful of each strain was transferred into 10 mL TSB and incubated at 37°C without shaking for 18-24 hr. The inocula were serially diluted in sterile MRD to give a final concentration of 10^3 cfu/mL of infant formula and TSB and incubated at six temperatures namely 4, 10, 25, 37, 45 and 50°C. The first temperature (4°C) was selected as proper refrigeration temperature, 10°C was selected as slightly abusive temperature, 25°C was considered as room temperature in Malaysia, 37°C was used as an optimum temperature for pathogens, 45°C and 50°C was used as maximum growth temperatures.

Enumeration of viable Cronobacter strains

One mL sample of each culture at 25, 37, 45 and 50°C were withdrawn from inoculated TSB and reconstituted PIF separately every 2 hr over a 24-hr period and serially diluted in 9 mL sterile MRD, while at 10°C samples were taken every day for ten days and at 4°C, every other day for 20 days. After dilution in MRD, samples were plated onto duplicate plates of VRBG and DFI agars using spread surface method (Roberts and Greenwood, 2003), and incubated at 37°C for 24 hr in order to measure the viability of *Cronobacter* strains. *Cronobacter* strains formed entirely blue-green colonies on DFI agar.

Survival of Cronobacter strains

Each of four strains were sub-cultured in 5 mL TSB and incubated at 37°C for 16-17 hr and centrifuged at 2800 x g (2420 centrifuge, KUBOTA Corporation, Bunkyo-Ku, Tokyo) for 25 min and then the cell pellets were suspended in 10 mL reconstituted PIF. Prior to inoculation, the reconstituted PIF was pre-heated in a water bath (Fischer Scientifics, ISO TEMP 228) to the appropriate test temperatures of 52, 54, 56, 58 and 60°C. Water bath temperatures were monitored with a Digistrip 4C monitor/controller (Pyrometer Service, ECE Fast, Model AW 298). Each reconstituted PIF were inoculated with 1 mL of cell suspensions to give a final inoculum of 10^7 cfu/mL and heated at the required temperature. To measure viable *Cronobacter* strains, at various time intervals (52°C: 0, 10, 20, 30 & 35 min;, 54°C: 0, 20, 30 & 35 min; 56°C: 0, 5, 12, 15 & 20 min; 58°C: 0, 5, 8, 10 & 12 min; 60°C: 0, 2, 5, 7 & 10 min), 1 mL aliquots of each heating menstruum was serially diluted in MRD and plated on TSA plates containing 1% sodium pyruvate, and then incubated at 37°C for 24-48 hr using the surface drop method (Roberts and Greenwood, 2003).

Statistical analysis

For growth study the viable counts were expressed as Log_{10} cfu/mL and plotted against incubation time (hr) to obtain the growth curves for each sample of TSB and reconstituted PIF in different temperatures separately. Data were analyzed by the Gompertz equation to give fitted growth curves, using the ComBase statistical software package to obtain generation time. The generation time were then subjected to an analysis of variance (SPSS 14, SPSS Inc., 2005) in order to determine significant statistical differences between growth medium (PIF and TSB) and plating media (VRBGA and DFI) and among *Cronobacter* strains (ATCC 51329 and three isolated strains from PIF).

Themotolerance parameters (D- and z-values) were estimated using standard regression analysis based on log linear models. For each treatment, at each temperature, the viable counts (as $\log_{10}$ cfu/mL) were plotted as a function of time. A linear model for time versus $\log_{10}$ cfu/mL of the counts was used to estimate D-values. D-values were transformed into $\log_{10}$ values and plotted against temperature and the z-values were calculated as the negative reciprocal of the line. D-values were then subjected to an analysis of variance (SPSS 14, SPSS Inc., 2005) in order to determine significant statistical differences among strains or temperatures.

Results and Discussions

Growth range of Cronobacter strains

Growth of microorganisms is influenced by the temperature. High temperatures refer to any temperature above ambient and may stop microbial

Table 1. Generation times of four *Cronobacter* strains in PIF and TSB at various temperatures

Cronobacter strain	Temperature (°C)	Generation time (hr)			
		PIF		TSB	
		DFI	VRBGA	DFI	VRBGA
ATCC 51329	10	2.49±0.048	3.19±0.006	3.23±0.089	3.27±1.484
	25	0.41±0.004	0.45±0.004	0.47±0.012	0.63±0.010
	37	0.27±0.034	0.28±0.051	0.28±0.058	0.30±0.029
	45	0.26±0.010	0.27±0.002	0.27±0.059	0.28±0.040
MGF1[a]	10	3.10±0.012	3.60±0.309	3.87±0.168	3.87±0.397
	25	0.51±0.001	0.52±0.024	0.62±0.134	0.64±0.033
	37	0.28±0.057	0.29±0.0512	0.29±0.047	0.30±0.002
	45	0.27±0.047	0.28±0.001	0.29±0.039	0.30±0.023
MGG1[a]	10	4.98±0.538	5.02±0.315	5.14±0.823	5.20±0.206
	25	0.49±0.0192	0.51±0.022	0.60±0.056	0.63±0.026
	37	0.28±0.019	0.29±0.001	0.31±0.032	0.32±0.002
	45	0.27±0.176	0.27±0.012	0.27±0.007	0.28±0.010
MGH1[a]	10	3.17±0.023	3.59±0.019	3.61±0.064	3.64±0.005
	25	0.52±0.005	0.57±0.055	0.63±0.059	0.65±0.105
	37	0.32±0.030	0.33±0.005	0.36±0.027	0.36±0.015
	45	0.26±0.117	0.27±0.007	0.28±0.098	0.29±0.070

± = Standard deviation based on two replicated experiments
a MGF1, MGG1 and MGH1 are *Cronobacter* strains isolated from PIF

Table 2. D and z-values of four *Cronobacter* strains in PIF samples

	D-value (min)					z-value (°C)
Temperature (°C)	52	54	56	58	60	
Cronobacter strains						
ATCC 51329	42.92±1.96	19.57±0.27	4.64±0.11	3.03±0.06	1.92±0.02	5.71±0.08
MGF1	34.6±0.34	18.79±0.18	4.56±0.05	2.98±0.02	1.88±0.01	6.01±0.01
MGG1	33.22±0.47	18.21±0.12	4.52±0.06	2.99±0.01	1.89±0.04	6.11±0.06
MGH1	38.31±1.24	19.01±0.18	4.53±0.01	2.98±0.01	1.86±0.01	5.83±0.03
Mean	39.01	18.89	4.56	2.99	1.89	5.91

± = Standard deviation based on two replication (n=2)

growth (Adams and Moss, 2000; Montville and Matthews, 2007). Growth and survival characteristics of *Cronobacter* strains in reconstituted PIF have been studied widely under different conditions of temperature and media cultures (Breeuwer et al., 2003; Edelson-Mammel and Buchanan, 2004; Iversen et al., 2004; Nazarowec-White and Farber, 1997). In the present study, the generation time of three *C. sakazakii* strains (MGH1, MGF1, MGG1) and *C. muytjensii* strain ATCC 51329 in PIF and TSB with four combinations of growth and plating media: TSB: VRBGA, TSB: DFI, PIF: VRBGA and PIF: DFI at 10, 25, 37 and 45°C were calculated using ComBase software (Table 1). The mean generation times for four *Cronobacter* strains were 3.64, 0.50, 0.29 and 0.27 hr in PIF and 3.98, 0.61, 0.31 and 0.28 hr in TSB at 10, 25, 37 and 45°C, respectively. There were no significant differences found in generation time among strains and growth media at 10, 25, 37 and 45°C ($P > 0.05$). Although strain *C. muytjensii* ATCC 51329 had a lower generation time compared to the other three *Cronobacter* food isolates in PIF and TSB at 10, 25 and 37°C, but the differences were insignificant ($P>0.05$).

At 4°C, in TSB and PIF and for all four strains, the concentration of *Cronobacter* remained at the initial inoculum levels (10^3 cfu/mL) and did not multiply or decline with time. These findings confirm the importance of proper refrigeration temperatures after reconstitution of infant formula powders to ensure that this organism does not grow. By increasing the temperature to 50°C, none of the food isolates and *C. muytjensii* ATCC 51329 grew either in TSB or PIF. The minimum growth temperature reported by

Nazarowec-White and Farber (1997) was 5.5°C, and none of the strains grew below 4ºC. The maximum temperature at which visible growth of *Cronobacter* was observed ranged from 41 to 45°C.

Farmer et al. (1980) examined 57 strains of *Cronobacter* and reported growth of the organism at 25, 36 and 45°C. Fifty of the tested strains grew at 47°C, but not at 4 or 50°C. In another study Nazarowec-White and Farber (1997) reported the growth of 10 strains of *Cronobacter* (5 clinical, 5 food isolates) at 4, 10 and 23°C. The minimum growth temperature of *Cronobacter* was reported at 5.5-8.0°C and the maximum temperature at 41-45°C using a temperature-gradient incubator. The temperatures of many home refrigerators range from 7 to 10°C (Rhodehamel, 1992). Harris and Oriel (1989) reported 20% of the home refrigerators surveyed were found to be between 5 and 10°C; however, none of home refrigerators were found above 10°C.

Figure 1 and Figure 2 show the comparison of the growth curves of four *Cronobacter* strains with the two combinations of growth and plating media, PIF: DFI and TSB: VRBGA, at 10, 25, 37 and 45°C respectively. Comparison of growth kinetics of *Cronobacter* strains indicated that *C. muytjensii* ATCC 51329 has a higher growth rate in all temperatures and its generation time in PIF and TSB is slightly shorter than the other strains. However these comparisons also suggest that other three food isolates in PIF and TSB grow approximately at the same rate of ATCC 51329 at various temperatures (Figure 1 and Figure 2).

The lag time and generation time of 10 *Cronobacter* strains in reconstituted dried-infant formula, *Salmonella* and *E. coli* in Brain Heart Infusion broth was evaluated by Nazarowec-White and Farber (1997) at 10 and 23°C. At 23°C, both *E. coli* and *Salmonella* spp. had a predicted generation time of 44.4 min, as compared to a mean generation time of 40 min for *Cronobacter* and at 10°C the average generation time for *Cronobacter* was 4.64 hr which was shorter than generation time of *Salmonella* and *E. coli* (Nazarowec-White and Farber, 1997). In comparison with this study, the mean generation time of *Cronobacter* strains in TSB and PIF at 10 and 25°C were 3.98 hr and 60.88 min and 3.64 hr and 49.75 min respectively. Iversen et al. (2004) investigated the specific growth rates of 6 *Cronobacter* strains in different microbiological media and PIF. All *Cronobacter* strains grew between 6 to 45°C with the optimum of 37-43°C. The mean generation time for *Cronobacter* in PIF at 6, 21 and 37°C was 13.7 hr, 1.7 hr and 20 min, respectively. In comparison with this study, the mean generation times of *Cronobacter* strains in TSB and PIF at 37°C were 31.50 and 29.25 min respectively.

Thermal resistance of Cronobacter strains

Table 2 shows the D-values at each temperature for all four strains of *Cronobacter*. The D-values ranged from 42.92 min at 52°C for type strain ATCC 51329 to 1.86 min at 60°C for *Cronobacter* strain MGH1. D-values for type strain ATCC 51329 were higher at each temperature in comparison to the D-values for the food strains. As Table 2 shows, the z-values for *Cronobacter* isolated strains were higher than the type strain ATCC 51329 (5.71°C) which were in the same range (4-6°C) reported for most none-spore forming bacteria (Tomlins and Ordal, 1976).

The survival and growth of *Cronobacte*r in other products has been evaluated. Richards et al. (2005) reported the growth of *Cronobacter* strains in infant rice cereal reconstituted with water, apple juice, milk and infant formula at 4, 12, 21 and 30°C. No growth was reported when reconstituted with apple juice, regardless of the storage temperature, nor with water,

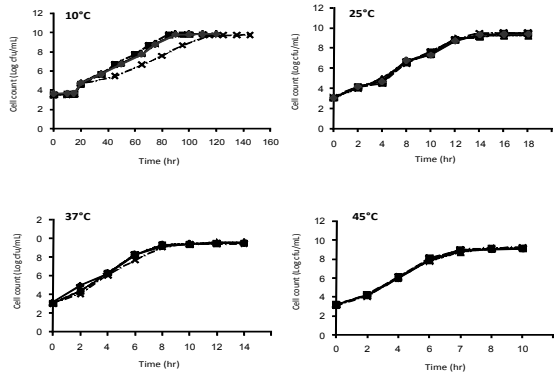

Figure 1. Growth curves of four *Cronobacter* strains *C. muytjensii* strain ATCC 51329 (■), MGF1 (▲), MGG1 (×), MGH1 (●) recovered in TSB: VRBGA at 10, 25, 37 and 45°C

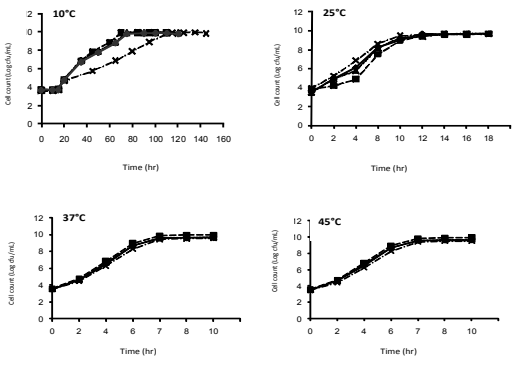

Figure 2. Growth curves of four *Cronobacter* strains, *C. muytjensii* strain ATCC 51329(■), MGF1 (▲), MGG1 (×), MGH1 (●) recovered in PIF: DFI at 10, 25, 37 and 45°C

milk and infant formula at 4°C. Kim and Beuchat (2005) also investigated growth of *Cronobacter* on fresh-cut fruits and vegetables and their juices such as fresh-cut apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, and tomato at 4, 12, or 25°C. There was no growth of *Cronobacter* strains when stored at 4°C but grew at 12°C on fresh-cut apple, cantaloupe, watermelon, cucumber, and tomato and in all juices except apple, strawberry, cabbage, and tomato juices. All fresh-cut fruits and vegetables except strawberry supported growth of *Cronobacter* strains at 25°C. The results of this study indicate the importance of proper preparation and storage of reconstitute dried-infant formula with respect to the survival and growth of *Cronobacter* strains.

Although *Cronobacter* strains do not grow at temperature of 4°C, it can grow at slightly abusive temperature of 10°C (3.64 hr). At room temperature of 25°C, the organism has a generation time of 49.75 min in reconstituted PIF. The ability of *Cronobacter* strains to multiply very quickly during holding time at room temperature (25°C) increases the risk of *Cronobacter* infection. Due to the exponential nature of bacterial growth, the risk will also increase exponentially once the organism comes out of the lag period. For example, after 6 hr at 25°C, the relative risk increases thirty fold and after 10 hr at 25°C, the relative risk increases 30 000- fold compared to the baseline (FAO/WHO, 2004). To reduce this risk, reconstituted infant formula must be immediately used and if not must be kept below 5°C. *Cronobacter* strains do not survive the pasteurization processes used during manufacturing but recontamination of the PIF during handling and reconstitution processes may occur.

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References

Adams, M.R. and Moss, M.O. 2000. Factors affecting the growth and survival of microorganism in foods, In: Adams, M.R. and Moss, M.O. (Eds.), Food Microbiology 2nd Ed. Cambridge, UK, pp: 48–50.

Bar-Oz, B., Preminger, A., Peleg, O., Block, C. and Arad, I. 2001. *Enterobacter sakazakii* in the newborn. Acta-Paediatrica 90: 356-358.

Bowen, A.B. and Branden, C.R. 2006. Invasive *Enterobacter sakazakii* disease in infants. Emerging Infectious Diseases 12: 1185–1189.

Breeuwer, P., Lardeau, A., Peterz, M. and Joosten, H.M. 2003. Desiccation and heat tolerance of *Enterobacter sakazakii*. Journal of Applied Microbiology 95: 967–973.

Caubilla-Barron, J. and Forsythe, S. 2007. Dry stress and survival time of *Enterobacter sakazakii* and other Enterobacteriaceae. Journal of Food Protection 70: 2111-7.

Codex Alimentarius Commission (CAC) 2008a. Report of the thirty-first session of the Codex Alimentarius Commission. Geneva, Switzerland, 30 June–4 July. Alinorm 08/31/rep. available at: ftp://ftp.fao.org/codex/alinorm08/al31rep_adv.pdf.

Codex Alimentarius Commission (CAC) 2008b. Code of hygienic practice for powdered formulae for infants and young children. CAC/RCP 66-2008. http://www.codexalimentarius.net/download/standards/11026/cxp-066e.pdf.

Edelson-Mammel, S.G. and Buchanan, R.L. 2004. Thermal inactivation of *Enterobacter sakazakii* in rehydrated infant formula. Journal of Food Protection 67: 60–63.

Food and Agriculture Organization /World Health Organization (FAO/WHO) 2004. *Enterobacter sakazakii* and other microorganisms in powdered infant formula. Microbiological risk assessment. Series, no. 6. ISBN: 924156265.) Geneva. *www.who.inf/publications/miro/en/es.pdf*.

Food and Agriculture Organization /World Health Organization (FAO/WHO) 2006. Meeting on *Enterobacter sakazakii* and *Salmonella* in powdered infant formula, Rome. Food And Agriculture Organization/World Health Organization *http://www.who.int/foodsafety/publications/ micro/mra6/en/*.

Food and Agriculture Organization /World Health Organization (FAO/WHO) 2008. *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae, Washington. Microbiological risk assessment Series 15. http://www.who.int/foodsafety/publications/micro/mra_followup/en/index.html.

Farmer, J.J. Iii., Asbury, M.A., Hickman, F.W. and Brenner, D.J. 1980. Enterobacteriaceae study group *Enterobacter sakazakii*; a new species of Enterobacteriaceae isolated from clinical specimen. International Journal of Systematic Bacteriology 30: 569-584.

Forsythe, S., 2005. *Enterobacter sakazakii* and other bacteria in powdered infant milk formula. Mother and Child Nutrition 1: 44-50.

Friedemann, M. 2007. *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). International Journal of Food Microbiology 116: 1–10.

Hansen, N.H. and Rieman, H. 1963. Factors affecting the heat resistance of non-sporing organisms. Journal of Applied Bacteriology 26:314-333.

Harris, L.S. and Oriel, P.J. 1989. Heteropolysaccharide produced by *Enterobacter sakazakii*. US Patent 4806636.

Himelright, I., Harris, E., Lorch, V. and Anderson, M. 2002. *Enterobacter sakazakii* infections associated with the use of powdered infant formula-tennessee-2001. Journal of the American Medical Association 287: 2204-2205.

Iversen, C. and Forsythe, S.J. 2003. Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. Trends in Food Science and Technology 14: 443-454.

Iversen, C., Lane, M. and Forsythe, S.J. 2004. The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Letters in Applied Microbiology 38: 378-382.

Iversen, C., Druggan, P., Schumacher, S., Lehner, A., Feer, C., Joosten, H. and Stephan, R. 2008. Development of a novel screening method for the isolation of *Cronobacter* spp. (*Enterobacter sakazakii*). Applied Environmental Microbiology 74: 2550–2553.

Kim, H. and Beuchat, L.R. 2005. Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurized juices as affected by storage temperature. Journal of Food Protection 68: 2541-2552.

Lai, K.K. 2001. *Enterobacter sakazakii* infections among neonates, infants, children and adults: Case reports and a review of the literate. Medicine (Baltimore). 80(2): 113-122.

Montville, T.J. and Matthews, K.R. 2007.Growth, survival, and death of microbes in foods, In: Doyle, M.P. and Beuchat, L.R. (Eds.), Food microbiology: fundamentals and frontiers, 3rd Ed. ASM press, NW, Washington, pp: 5–6.

Nazarowec-White, M. and Farber, J.M. 1997. Incidence, survival and growth of *Enterobacter sakazakii* in infant formula. Journal of Food Protection 60: 226-230.

Norrakiah, A. S., Ghassem, M. & Babji, A. S. 2007. *Enterobacter sakazakii* and growth characterization in infant formula milk. Proceedings 10th Asean Food Conference 07, pp. 1-3.

Rhodehamel, E.J. 1992. FDA's concerns with sous vide processing. Food Technology 46: 72-76.

Richards, G.M., Gurtler, J.B. and Beuchat, L.R. 2005. Survival and growth of *Enterobacter sakazakii* in infant rice cereal reconstituted with water, milk, liquid infant formula, or apple juice. Journal of Applied Microbiology 99: 844-850.

Roberts, D. and Greenwood, M. 2003. Practical food microbiology. Massachusetts: Blackwell Publishing Inc.

Tomlins, R.I. and Ordal, Z.J. 1976. Thermal injury and inactivation in vegetative bacteria. In Sinner, F.A. and Hugo, W.B. (Eds). Inhibition and inactivation of vegetative microbes. The Society for Applied Bacteriology symposium series, no. 5, London : Academic Press.

Van Acker, J. De Smet, F. Muyldermans, G. Bougatef, A. Naessens, A. and Lauwers, S. 2001. Outbreak of necrotizing entercolitis associated with *Enterobacter sakazakii* in powdered milk formula. Journal of Clinical Microbiology 39: 293-297.

Whiting, R.C. and Buchanan, R.L. 1994. Microbial modelling. Journal of Food. Technology 48: 113-120.