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, thermostolerance

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 enterocolitis (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 thermostolerance 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.,
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 37°C 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).

The motolerance 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
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Growth and survival of Cronobacter species as measured by media performance (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

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

<table>
<thead>
<tr>
<th>Cronobacter strain</th>
<th>Temperature (°C)</th>
<th>PIF Generation time (hr) VRBGA</th>
<th>TSB Generation time (hr) VRBGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 51329</td>
<td>10</td>
<td>2.49±0.048</td>
<td>3.19±0.006</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.41±0.004</td>
<td>0.45±0.004</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.27±0.034</td>
<td>0.28±0.051</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.26±0.010</td>
<td>0.27±0.002</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.10±0.012</td>
<td>3.60±0.309</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.51±0.001</td>
<td>0.51±0.024</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.28±0.057</td>
<td>0.29±0.0512</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.27±0.047</td>
<td>0.28±0.001</td>
</tr>
<tr>
<td>MGF1a</td>
<td>10</td>
<td>4.98±0.538</td>
<td>5.02±0.315</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.49±0.0192</td>
<td>0.51±0.022</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.28±0.0192</td>
<td>0.29±0.001</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.27±0.176</td>
<td>0.27±0.012</td>
</tr>
<tr>
<td>MGG1a</td>
<td>10</td>
<td>3.17±0.023</td>
<td>3.59±0.019</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.52±0.005</td>
<td>0.57±0.055</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.32±0.030</td>
<td>0.33±0.005</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.26±0.117</td>
<td>0.27±0.007</td>
</tr>
<tr>
<td>MGH1a</td>
<td>10</td>
<td>3.17±0.023</td>
<td>3.59±0.019</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.52±0.005</td>
<td>0.57±0.055</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.32±0.030</td>
<td>0.33±0.005</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.26±0.117</td>
<td>0.27±0.007</td>
</tr>
</tbody>
</table>

± = 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

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>52</th>
<th>54</th>
<th>56</th>
<th>58</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 51329</td>
<td>42.9±1.96</td>
<td>19.57±0.27</td>
<td>4.64±0.11</td>
<td>3.03±0.06</td>
<td>1.92±0.02</td>
</tr>
<tr>
<td>MGF1</td>
<td>34.6±0.34</td>
<td>18.79±0.18</td>
<td>4.56±0.05</td>
<td>2.98±0.02</td>
<td>1.88±0.01</td>
</tr>
<tr>
<td>MGG1</td>
<td>33.2±0.47</td>
<td>18.21±0.12</td>
<td>4.52±0.06</td>
<td>2.99±0.01</td>
<td>1.89±0.04</td>
</tr>
<tr>
<td>MGH1</td>
<td>38.31±1.24</td>
<td>19.01±0.18</td>
<td>4.53±0.01</td>
<td>2.98±0.01</td>
<td>1.86±0.01</td>
</tr>
<tr>
<td>Mean</td>
<td>39.01</td>
<td>18.89</td>
<td>4.56</td>
<td>2.99</td>
<td>1.89</td>
</tr>
</tbody>
</table>

± = Standard deviation based on two replication (n=2)
Nazarowec-White and Farber (1997) was 5.5°C, and none of the strains grew below 4°C. 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) indicated that 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.

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.

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 non-spore forming bacteria (Tomlins and Ordal, 1976).

The survival and growth of Cronobacter 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,
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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.

Acknowledgments

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References


