

# **Survival and growth of *Cronobacter* species (*Enterobacter sakazakii*) in wheat-based infant follow on formulas**

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## ABSTRACT

**Aim:** To determine the survival and growth characteristics of *Cronobacter* species (*Enterobacter sakazakii*) in infant wheat-based formulas reconstituted with water, milk, grape juice or apple juice during storage.

**Methods and Results:** Infant wheat-based formulas were reconstituted with water, UHT milk, pasteurized grape or apple juices. The reconstituted formulas were inoculated with *C. sakazakii* and *C. muytjensii* and stored at 4, 25 or 37°C for up to 24h. At 25 and 37°C, *Cronobacter* grew more ( $>5 \log_{10}$ ) in formulas reconstituted with water or milk than those prepared with grape or apple juices (*ca.* 2-3  $\log_{10}$ ). The organism persisted, but did not grow in any formulas stored at 4°C. Formulas reconstituted with water and milk decreased from pH 6.0 to 4.8-5.0 after 24h, whereas the pH of the formulas reconstituted with fruit juices remained at their initial pH values, *ca.* pH 4.8-5.0.

**Conclusion:** *C. sakazakii* and *C. muytjensii* can grow in reconstituted wheat-based formulas. If not immediately consumed, these formulas should be stored at refrigeration temperatures to reduce the risk of infant infection.

**Significance and Impact of the Study:** The results of this study will be of use to regulatory agencies and infant formula producers to recommend storage conditions that reduce the growth of *Cronobacter* in infant wheat-based formulas.

## INTRODUCTION

*Cronobacter* is a recently defined genus comprising of five species, and was previously known as *Enterobacter sakazakii* (Iversen *et al.* 2008). They are motile peritrichous Gram-negative rod-shaped non-spore forming bacteria, which belong to the *Enterobacteriaceae* family. *Cronobacter* species have been implicated in infant cases of meningitis, septicemia and necrotizing enterocolitis (Muytjens *et al.* 1983; van Acker *et al.* 2001; Bowen and Braden 2006; Caubilla-Barron *et al.* 2007).

*Cronobacter* species have been frequently isolated from the environment, plant material (wheat, rice, herbs and spices), and various other food products (Iversen and Forsythe 2004; Friedemann 2007; Shaker *et al.* 2007). Starches (from wheat and rice) are used in the manufacture of infant formula and can be source of *Cronobacter* (FAO/WHO 2004 and 2006). Iversen and Forsythe (2004) surveyed powdered infant formula and other food products for the presence of *Cronobacter*. They isolated the bacterium from 2 of 82 (2.4%) infant formulas, 5 of 49 (10.2%) infant follow on formulas, and 40 of 122 (37.8%) herbs and spices. Shaker *et al.* (2007) isolated *Cronobacter* from 2 of 8 (25.0%) infant formulas, 2 of 15 (13.3%) infant wheat-based follow on formulas, and from crushed wheat. Moreover, Restaino *et al.* (2006) isolated *Cronobacter* from 14 of 78 (17.9%) dried flour or meal including corn, soy, wheat, and rice, 2 of 8 (25%) dried adult cereals, 2 of 6 (33.3%) dried infant cereals, 1 of 5 (20.0%) dried vegetables and spices, and 4 of 24 (16.7%) dried sodium caseinate. Follow on formulas are non-sterile products, but are manufactured to comply with the same microbiological criteria as infant formula for pre-weaning infants (FAO/WHO 2006). The well publicized Tennessee outbreak of *C. sakazakii* was attributed to the accidental feeding of a non-infant formula to neonates; the

product was marketed for children and adults (Himmelright *et al.* 2002). A common risk factor in reported *Cronobacter* outbreaks in France was the temperature abuse of reconstituted formula (Coignard *et al.* 2006; Caubilla-Barron *et al.* 2007). This highlights the need for temperature control to reduce microbial growth in formula, especially as infants are not immune competent, and lack a competing gut flora (Townsend and Forsythe 2007).

The growth of *Cronobacter* in reconstituted pre-weaning infant formula has been studied. Iversen *et al.* (2004b) reported 6°C, 37-43°C, and 46°C as minimum, optimum and maximum growth temperature of *Cronobacter* in reconstituted infant milk formula, respectively. Osaili *et al.* (2008) confirmed that *Cronobacter* species did not grow in infant formula at 4°C, and that there was detectable increase in the viable count of *Cronobacter* during the first 2 h of storage at temperature 20-45°C. Currently, FAO/WHO (2006) recommends that the time between reconstitution and feeding is less than 4 h to reduce the opportunity for bacterial multiplication. The FAO/WHO (2006) also recommended that reconstitution should be with water at 70°C to reduce the bacterial load. However this has not been widely adopted and formulas may have instructions with reconstitution temperatures as low as 40°C. This is within the growth range of *Cronobacter*, and any other *Enterobacteriaceae* present in the formula.

A few studies have been conducted on the behavior of pathogenic organisms in reconstituted rice-based follow on formula. Deng *et al.* (1998) reported that *Escherichia coli* O157:H7 grew at 8 to 30°C in rice-based formula reconstituted with milk, but did not grow at 8°C in formula reconstituted with apple juice. Similarly, Jacquette and Beuchat (1998) found that *Bacillus cereus* grew in infant rice cereal reconstituted with milk during

storage at 8 to 30°C but not in rice cereal reconstituted with apple juice (pH 4.4). Richards *et al.* (2005) studied *Cronobacter* (unknown species) growth in rice-based follow on formula, which is significant as rice has previously been reported as a source of *Cronobacter* (Cottyn *et al.* 2001). However, to our knowledge, there have not been any equivalent studies of wheat-based products.

The objective of this study was to determine the survival and growth characteristics of *Cronobacter* species during storage at 4, 25 or 37°C in various reconstituted wheat-based infant formulas.

## **MATERIALS AND METHODS**

### **Preparation of *Cronobacter* cultures**

Five strains of *Cronobacter* were used in this study. *C. mytjensii* ATCC 51329, and four isolates of *C. sakazakii*; two from infant milk formula, one from infant cereal formula and one from semolina (Shaker *et al.* 2007).

All *Cronobacter* strains were stored at -40°C in brain heart infusion (BHI) (Oxoid Ltd, Basingstoke, UK) with 20% of glycerol. The organisms were grown in BHI at 37°C for 24 h. Immediately before inoculating the reconstituted formula, the cultures were combined to form a mixed culture which was then decimally diluted in peptone water (0.1%) (Becton Dickinson, Sparks, Md, USA) to 10<sup>5</sup> cfu ml<sup>-1</sup>.

### **Reconstitution and inoculation of wheat-based infant formulas**

Two different types of commercially available powdered infant follow on formulas were studied; wheat-based formula and wheat-based formula containing fruits. These were

purchased from a local market and were tested for intrinsic *Cronobacter* contamination and aerobic plate counts (Iversen *et al.* 2004a). Ultra High Temperature (UHT) milk (3% fat), pasteurized grape and apple juices were locally purchased to reconstitute the formulas. All liquids for reconstituting the formulas were kept at room temperature (20-22°C) for 1 h for temperature equilibration prior to use. Each dried formula (50 g) was weighed into Seward stomacher bags (BA6040, Seward Ltd., Norfolk, UK) before the addition of 180 ml of water, milk, grape juice or apple. The reconstituted formulas were inoculated with the mixed *Cronobacter* culture, to give an initial inoculum level of  $10^3$  cfu ml<sup>-1</sup>. The spiked formulas were then mixed thoroughly for two minutes before being stored at 4, 25 or 37°C for 24 h.

### ***Cronobacter* enumeration**

Samples of reconstituted formulas were taken at 0, 4, 8, and 24 h for *Cronobacter* enumeration, in duplicate, on violet red bile salt glucose agar (VRBGA) (Oxoid). The plates were incubated aerobically at 37°C for 24 to 48 h.

### **Measurement of pH**

The pH was measured by immersing the pH electrode (Cyberscan 500, Eutech instruments, Singapore) into well mixed samples.

### **Statistical analysis**

All experiments were repeated three times. Data were analyzed to determine significant differences ( $P \leq 0.05$ ) in the viable counts of *Cronobacter* using the General Linear Models of SAS version 8.1 (copyright 1999, 2000, SAS Institute Inc. Cary, NC, USA).

## RESULTS

The wheat-based formulas had aerobic plate counts of  $10^2$  cfu g<sup>-1</sup>, but were not intrinsically contaminated with *Cronobacter* species. The inoculated *C. sakazakii* and *C. muytjensii* cultures did not grow, in any reconstituted formula that was stored at 4°C for up to 24 h. In contrast, the *Cronobacter* grew at 25°C, and there were significant differences in the amount of growth according to the reconstitution liquid (Table 1). *Cronobacter* viable counts increased by 4.27 and 4.95 log<sub>10</sub> orders when the formulas were reconstituted with water and milk (Table 1). Whereas the viable counts only increased by 2.58 and 0.86 log<sub>10</sub> orders when the formulas were reconstituted with grape and apple juices. Similarly, *Cronobacter* numbers increased in wheat-based formulas containing fruits by 4.86 and 4.22 log<sub>10</sub> orders when reconstituted with water and milk, and by only 1.53 and 0.70 log<sub>10</sub> orders when the same product was reconstituted with grape and apple juices (Table 1).

The number of *Cronobacter* only increased by 0.5 log<sub>10</sub> orders, which was not statistically significant, after 4 h of storage at 37°C in both types of formula irrespective of the reconstitution liquid (Table 1). However, after 8 h at 37°C the viable count in water and milk reconstituted formulas had increased by *ca.* 4.0 log orders. In comparison, the increases were only *ca.* 0.9 and *ca.* 0.4-0.7 log<sub>10</sub> orders when reconstituted with grape and apple juice, respectively. After 24 h at 37°C the

*Cronobacter* had multiplied to *ca.*  $10^7$ – $10^8$  cfu ml<sup>-1</sup> in formulas reconstituted with water and milk, compared with  $10^5$ – $10^6$  cfu ml<sup>-1</sup> for those reconstituted with grape and apple juices.

There was no significant difference between the populations of *Cronobacter* in wheat-based formula and wheat-based formula containing fruits regardless of storage temperature, storage time and hydration liquid (data not shown).

The initial pH of wheat-based formula and wheat-based formula with fruits were *ca.* pH 6.0 when reconstituted with water or milk, and *ca.* pH 5.0 when reconstituted with grape and apple juices. After incubation for 24h at 25°C, the pH had decreased to pH 5.3 and 5.8 in formulas reconstituted with water and milk, and to pH 4.8-5 when incubated at 37°C. The pH of reconstituted formulas did not change during storage at 4°C.

## **DISCUSSION**

Infant formulas can be intrinsically contaminated with *Cronobacter* species during production (Restaino *et al.* 2006; Shaker *et al.* 2007), or extrinsically through cross-contamination from the environment and equipment during preparation (Noriega *et al.* 1990). Despite the FAO/WHO (2006) recommendations, these formulas are not necessarily subjected to hot (70°C) water during reconstitution to reduce the bacterial load. Therefore, there is the opportunity for bacterial growth following reconstitution, which may increase the risk of *Cronobacter* infections.

Previous studies have considered *Cronobacter* growth in rice-based follow on formula, whereas this study was focused on wheat-based formulas. The *Cronobacter* grew in both wheat-based formulas at 25 and 37°C, but not 4°C (Table 1). Similarly,



Abushelaibi *et al.* (2003) reported that *Salmonella* did not grow in infant rice, oatmeal, and cereal mix formulas reconstituted with water, milk or apple juice during storage at 4°C. Therefore refrigeration at 4°C should be adequate to prevent the growth of *Cronobacter* species and *Salmonella* serovars in reconstituted infant formulas, regardless of the formula base or reconstitution liquid. Nevertheless, *Cronobacter* species persist at this temperature and hence could still be infectious after ingestion. The organism also persisted in formulas reconstituted with grape and apple juice, and stored at 4°C despite their low pH (*ca.* 4.8-5.0) (Table 1). Hence mild acidified feeds do not reduce the viability of *Cronobacter*.

The pH of the reconstituted feeds stored at ambient and above temperatures (25 and 37°C) did affect the growth of *Cronobacter*. Formulas reconstituted with water and milk, had initial pH values (*ca.* pH 6.0) that supported the growth of *Cronobacter* species more than those reconstituted with acidic fruit juices (initial pH 4.8-5.0). The affect of pH and organic acids on the growth of other pathogenic bacteria in formula preparations has been reported previously. Deng *et al.* (1998) reported that *E. coli* O157:H7 grew at 8°C in rice-based formula reconstituted with milk, but when reconstituted with apple juice. Abushelaibi *et al.* (2003) reported that *Salmonella* grew in rice, oatmeal, and cereal mix formulas reconstituted with apple juice during storage at 25°C. However the organism did not grow in formula reconstituted with apple juice stored at 15°C. They also did not find differences in the growth of *Salmonella* in rice, oatmeal and cereal mix when water or milk was used for rehydration. This is similar to our results for *Cronobacter*.

The pH of water and milk reconstituted formulas decreased during microbial growth to *ca.* pH 5.0. This was probably due to acetic and lactic acid production by the

*Cronobacter* (Skladal *et al.* 1993). The pH of wheat formulas reconstituted with apple and grape juices did not change substantially, and remained *ca.* pH 4.8-5.0. It is probable that the low pH of juices and organic acids retarded the growth of the microflora. *Cronobacter* is a moderately acid resistant member of the *Enterobacteriaceae*, being able to withstand exposure to pH 3.5 for > 5 h (Edelson-Mammel *et al.* 2006). Kim and Beuchat (2005) observed that *Cronobacter* spp. grew in tomato juice (pH 4.4), watermelon juice (pH 5.1), and cantaloupe juice (pH 6.8) but did not grow in apple juice (pH 4.0) and strawberry juice (pH 3.5) stored at 25°C.

In summary, it is evident that reconstituted wheat-based formulas, if not immediately consumed, should be stored at refrigeration temperatures to prevent the growth of *Cronobacter* species. Reconstitution with milk and water supported the growth of *Cronobacter* spp. between 25 and 37°C. In contrast, reconstitution with fruit juices retarded bacterial growth, and this was probably due to the lower pH (pH 4.8-5.0 compared with pH 6.0). In addition, the practice of good personal hygiene during the reconstitution of infant formulas is necessary to reduce the risk of contamination by *Cronobacter* spp. and other infectious organisms.

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1 Table 1: Growth of a cocktail culture of *Cronobacter* species (cfu ml<sup>-1</sup>) in infant wheat-  
 2 based formulas reconstituted with water, milk, grape juice or apple juice and stored for up  
 3 to 24h.  
 4

Temperature	Storage time (h)	Reconstitution liquid*			
		Water	Milk	Grape juice	Apple juice
Wheat-based formula					
4 (°C)	0	† A 2.67±0.42 a‡	A 2.90±0.27 a	A 2.90±0.18 a	A 2.84±0.15 a
	4	A 2.98±0.13 a	A 2.94±0.52 a	A 3.10±0.34 a	A 3.11±0.41 a
	8	A 2.72±0.17 a	A 2.71±0.22 a	A 3.12±0.19 a	A 2.81±0.36 a
	24	A 2.81±0.39 a	A 2.73±0.38 a	A 2.74±0.39 a	A 2.97±0.15 a
25 (°C)	0	C 2.72±0.14 a	C 2.73±0.28 a	B 2.89±0.19 a	A 2.95±0.35 a
	4	C 2.71±0.24 a	C 2.63±0.40 a	B 3.16±0.11 a	A 2.85±0.33 a
	8	B 3.54±0.47 a	B 3.68±0.16 a	B 3.03±0.18 a	A 2.95±0.60 a
	24	A 6.99±0.62 a	A 7.68±0.81 a	A 5.47±0.24 b	A 3.81±0.76 c
37 (°C)	0	C 2.78±0.45 a	C 2.89±0.16 a	C 2.96±0.08 a	C 2.88±0.22 a
	4	B 3.89±0.15 a	C 3.92±0.15 a	C 3.18±0.36 b	CB 3.38±0.16 b
	8	A 7.18±0.23 a	B 7.51±0.96 a	B 4.07±0.37 b	B 3.70±0.08 b
	24	A 7.70±0.20 a	A 8.43±0.80 a	A 5.79±0.27 b	A 5.34±0.42 b
Wheat-based formula with fruits					
4 (°C)	0	A 2.83±0.38 a	A 3.03±0.18 a	A 2.81±0.25 a	A 3.03±0.30 a
	4	A 3.18±0.32 a	A 2.84±0.26 a	A 2.88±0.15 a	A 2.94±0.47 a
	8	A 2.84±0.23 a	A 2.82±0.23 a	A 2.81±0.20 a	A 2.86±0.21 a
	24	A 2.64±0.22 a	A 2.89±0.30 a	A 2.94±0.25 a	A 2.77±0.25 a
25 (°C)	0	B 2.92±0.10 a	B 3.04±0.23 a	B 2.92±0.15 a	B 2.89±0.20 a
	4	B 2.98±0.27 a	B 3.06±0.29 a	B 2.74±0.24 a	B 2.90±0.25 a
	8	B 3.03±0.23 b	B 3.64±0.08 a	B 3.13±0.30 b	B 3.09±0.28 b
	24	A 7.78±0.69 a	A 7.26±0.73 a	A 4.45±0.25 b	A 3.59±0.25 b
37 (°C)	0	C 2.86±0.27 a	C 3.19±0.51 a	C 3.03±0.28 a	B 2.90±0.28 a
	4	C 3.33±0.25 ab	C 3.63±0.34 a	C 3.32±0.35 ab	B 3.02±0.25 b
	8	B 6.91±0.41 a	B 6.30±0.37 a	B 3.97±0.25 b	B 3.34±0.27 b
	24	A 7.95±0.65 a	A 7.48±0.34 a	A 5.99±0.47 b	A 5.58±0.37 b

5 \* Tabulated values represent means of 3 replications (n=6) ± SD (log<sub>10</sub> cfu ml<sup>-1</sup>)

6 † Within the same formula type and storage temperature, means having the same  
 7 uppercase letters within the same column are not significantly different ( $P > 0.05$ ).

8 ‡ Within the same row, means having the same lowercase letter are not significantly  
 9 different ( $P > 0.05$ )