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# Effect of acid, desiccation and heat stresses on the viability of *Cronobacter sakazakii* during rehydration of powdered infant formula and in simulated gastric fluid

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ABSTRACT

The aim of the present study was to evaluate survival of *Cronobacter sakazakii* adapted or shocked to heat, acid or desiccation during rehydration of powdered infant formula and in simulated gastric fluid (SGF). Four *C. sakazakii* strains isolated in South Korea were individually adapted or shocked under stress conditions, equal volumes of each strain were combined to form a cocktail. Heat and acid resistance of stressed cells were determined by subjecting the cells to rehydrated infant formula (RIF) reconstituted at 55, 58, 61, 64, 67, and 70 °C for 1 min, and in SGF (pH 2.5), respectively. The growth kinetic parameters of stressed cells at different storage temperatures were compared using the Baranyi model. Except for desiccation, all stress conditions significantly ($P < 0.05$) enhanced the heat resistance of *C. sakazakii* compared with unstressed cells. Long-term exposure of cells to heat and acid improved their acid resistance in SGF whereas acid resistance of heat shocked and desiccated cells were similar and significantly ($P < 0.05$) lower than that of unstressed cells, respectively. Heat, acid adapted and desiccated cells in RIF had longer lag time duration during storage at 12 °C than unstressed cells, while there was no significant ($P > 0.05$) difference in growth parameters between unstressed and stressed cells at 25 °C. No growth was detected at 5 °C. Thus, this study indicates that long-term exposure of *C. sakazakii* to mild heat and acid could enhance their survivability during rehydration of infant formula at high temperatures or in SGF. Furthermore, this study suggests that the prepared and leftover RIF must be stored at refrigeration temperature to inhibit the growth of *C. sakazakii*, minimizing the risk of possible outbreaks.


## 1. Introduction

*Cronobacter sakazakii* (formely called *Enterobacter sakazakii*) is a gram negative, facultative anaerobic, motile, and non-spore forming bacterium (Farmer, Asbury, Hickman, & Brenner, 1980). This microorganism has been implicated with neonatal illnesses, including bacteremia, septicemia, and meningitis (Bar-Oz, Preminger, Peleg, Block, & Arad, 2001; Van Acker et al., 2001) with high mortality rates (Nazarowec-White & Farber, 1997). Infants aged less than one year, especially <28 days old, can suffer from considerable chronic sequelae (ICMSF, 2002). Across the globe, about 160 infection cases have been reported in infants through a variety of origins and about 60 of these cases were in the United States (FAO/WHO, 2008). Despite the fact that *C. sakazakii* has been consistently isolated from infant foods in Korea, no outbreaks have been reported (KFDA, 2010). Powdered infant formula (PIF), of a wide range of sources, has been identified as one of the most common vehicles of *C. sakazakii* causing neonatal infections (Van Acker et al., 2001).

Rehydrated infant formula (RIF) may provide an optimal environment for growth of *C. sakazakii* due to its high nutritional value when it is not properly prepared and stored. It is recommended that PIF should be rehydrated at a temperature of at least 70 °C, which is a critical factor to drastically alleviate the risk of *C. sakazakii* (FAO/WHO, 2007). However, in household conditions it is difficult to control the desired temperature and thus the pathogen cannot be completely eliminated. In addition, the practices to reconstitute powdered infant formula may vary from person to

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person. Thus, there still remains the risk of *C. sakazakii* owing to the possibility of survival of the organism in the infant formula after rehydration. Furthermore, even though RIF ingested by infants is exposed to gastric acid while passing through their stomach, utterly eradicating the pathogen is arduous because gastric pH of infants is relatively higher than that of adults (Miclat, Hodgkinson, & Marx, 1978; Singh, Monterio, & Ghai, 1970).

It is known that bacterial pathogens have the ability to enhance their resistance to lethal stresses after their exposure to a sublethal one via genetic regulation or physiological adaptation (Beales, 2004). Besides, adaptation to a sublethal stress may provide protection in response to other stresses. This phenomenon so-called cross-protection has been a growing concern in the microbiological food safety area. It has been reported that the acid and heat resistance of *C. sakazakii* were greatly improved by heat or acid shock (Hsiao, Ho, & Chou, 2010; Kim, Bae, & Lee, 2012), while desiccation stress significantly decreased heat resistance of *C. sakazakii* in RIF (Shaker, Osaili, Abu Al-Hasan, Ayyash, & Forsythe, 2008). These studies indicated that cross-protection might be stress-dependent; therefore, it is necessary to take into account diverse stress types of *C. sakazakii* under multifarious environments to understand their resistance to lethal conditions.

Although the response of *C. sakazakii* exposed to various stresses such as heat, acid, and desiccation has been well documented (Hsiao et al., 2010; Kim et al., 2012; Shaker et al., 2008), these studies were only conducted in bacterial media or RIF at certain temperatures. Hence, the objective of this study was to investigate the effect of various stress conditions, including acid adaptation, heat adaptation, heat shock and desiccation on survival of *C. sakazakii* isolated in Korea during rehydration of PIF at different temperatures and in simulated gastric fluid. Growth of stressed cells in RIF was also compared during storage to predict the behavior of each stressed cell under temperature abuse conditions.

## 2. Materials and methods

### 2.1. Bacterial culture

Four strains of *C. sakazakii* isolated from different sources -EB 1, stool; EB 5, throat of human; EB 7, Sunsik (ready-to-eat dried grain food); EB 41, soil-were used for this study (Gyeonggi Institute of Health Environment, Korea). The frozen cultures were stored at −78 °C in a vial containing 20% (v/v) glycerol. To activate the frozen cultures, the vials were thawed at room temperature and 20 μl of the thawed cultures were transferred into test tubes containing 9.9 ml of sterile Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA), followed by incubation at 37 °C for 24 h. Two successive transfers were performed before the cultures were used for the experiments.

### 2.2. Preparation of stressed C. sakazakii

To prepare acid-adapted cells, 0.1 ml of each 24-h culture was transferred to 9.9 ml TSB supplemented with 1% (w/v) D-glucose (Sigma–Aldrich, St Louis, MO, USA) and incubated at 37 °C for 24 h (Deng, Ryu, & Beuchat, 1999). For heat adaptation, 0.1 ml of culture was transferred to 9.9 ml TSB and incubated at 42 °C for 24 h. To prepare heat-shocked cells, 10 ml of each culture was centrifuged at 3124 × *g* for 10 min at 4 °C and the pellets were resuspended in 1 ml of TSB. The bacterial suspension was transferred to 9 ml of TSB pre-warmed at 47 °C in a circulating water bath (NTT-2200; EYELA, Tokyo, Japan). The culture was heat shocked for 15 min, cooled in the water bath at 25 °C for 1 min (Chang, Chiang, & Chou, 2009) and immediately inoculated into rehydrated infant formula and simulated gastric fluid as described below. Desiccated cells were prepared as described by Breeuwer, Lardeau, Peterz, and Joosten (2003) with a slight modification. Briefly, 10 spots (0.5 ml/spot) of each culture were prepared in a sterile Petri dish. The plate without a lid was placed in a 40 °C incubator with dehydrated silica gel for 4 h. After desiccation, the plate was covered with a lid and kept at 25 °C for 24 h. The desiccated cells were rehydrated with 10 ml of sterile phosphate buffered saline (PBS; pH 7.4). Desiccation stress led to about 2-log reduction (CFU/ml) of the cell population, while other stress conditions did now cause log reductions. *C. sakazakii* grown in TSB at 37 °C for 24 h served as unstressed control cells in this study.

### 2.3. Preparation of inoculum

Unstressed or stressed bacterial cells (a four-strain cocktail used for each condition) were aseptically combined with equal volumes to produce a cocktail. The cocktail was centrifuged at 3124 × *g* for 10 min at 4 °C and the pellet was resuspended in PBS. The washing and centrifugation steps were repeated twice. The harvested pellets were resuspended in PBS to obtain a final cell density of $10^9$ CFU/ml.

### 2.4. Infant formula

Powdered infant formula (PIF) aged from 0 to 100 days was purchased from a local supermarket (Changwon, Korea). To confirm natural contamination, PIF was reconstituted according to manufacturer's instruction and stored at 37 °C for 24 h. One-ml aliquot of the rehydrated infant formula (RIF) was inoculated onto Tryptic Soy Agar (TSA; Difco) and incubated at 37 °C for 24 h and no background microflora was observed.

### 2.5. Determination of heat resistance

Ten gram of PIF and 1 ml of unstressed or stressed cocktail cell suspension (ca. $10^9$ CFU/ml) were transferred into a 100 ml bottle containing 80 ml of sterile deionized water pre-warmed at 55, 58, 61, 64, 67, and 70 °C in a circulating water bath. The bottles were removed from the water bath, agitated manually for 1 min and immediately cooled in a water bath at 25 °C until the temperature of bottles decreased to proper temperature (37 °C) that was measured using a thermometer. Once the temperature reached 37 °C, 1 ml of each inoculated rehydrated infant formula (RIF) was serially diluted in PBS to determine the number of viable cells. One-ml aliquot of RIF was incubated in 9 ml of TSB at 37 °C for 24 h when no viable cells were detected after rehydration of PIF.

### 2.6. Determination of acid resistance

Acid resistance of unstressed or stressed cocktail cells was determined by subjecting to simulated gastric fluid (SGF; pH 2.5). The SGF consisted of 8.3 g/l proteose-peptone, 3.5 g/l D-glucose, 2.05 g/l NaCl, 0.6 g/l $KH_2PO_4$, 0.11 g/l $CaCl_2$, 0.37 g/l KCl, 0.05 g/l oxgall, 1 g/l lysozyme and 13.3 mg/l of pepsin. All compounds were dissolved in deionized water, autoclaved together except for oxgall, lysozyme, and pepsin, which were filter sterilized (0.25 μm). The final pH was adjusted with a sterile 5.0 N HCl solution. Ten gram of PIF was rehydrated into a 100 ml bottle including 80 ml of sterile deionized water pre-warmed at 37 °C, followed by the addition of 1 ml of unstressed or stressed cocktail cell suspension (ca. $10^9$ CFU/ml). After agitation, a 0.2 ml aliquot of the RIF was added to 19.8 ml of SGF solution pre-warmed at 37 °C in a circulating water bath. Survival was monitored with appropriate time intervals and survival curves were constructed by plotting the $\log_{10}$ number of viable cells against time. The best fit line for survival curves was determined by linear regression with Microsoft Excel (Microsoft