= Short Paper =

The Effect of Storage Temperature on the Growth of *Cronobacter sakazakii* in Prepared Powdered Infant Foods

Noriko FUKUDA^{*1}, Nozomi ARAI^{*1}, Aoi FUJIWARA^{*1},
Soichi FURUKAWA^{*1} and Hirokazu OGIHARA^{*1, †}

(^{*1} Department of Food Bioscience and Biotechnology, College of Bioresource Sciences, Nihon University,
1866 Kameino, Fujisawa-shi, Kanagawa 252-0880, Japan; [†] Corresponding author)

(Received: December 9, 2013)

(Accepted: October 16, 2014)

Effects of storage temperature (5, 10, and 25°C) on the survival and growth of *Cronobacter sakazakii* ATCC 29544 in prepared powdered infant foods (PIF); “vegetable and rice porridge” (VRP), “mixed vegetable and wheat paste” (MVWP), “liver, vegetable, and wheat paste” (LVWP) and “apple juice” (AJ) were investigated. *C. sakazakii* showed growth in VRP, MVWP, and LVWP at 25°C, but no growth at 5 or 10°C. In AJ, *C. sakazakii* was inactivated at 25°C for 24 hr, and there was no growth or slight decrease was observed at 5 and 10°C. These results indicate that, similarly to other PIFs, bacterial growth in the tested PIFs increase with storage under room temperature. However, it was suppressed by the tested AJ, and also a rapid decrease was found at 25°C.

Key words: *Cronobacter sakazakii*, powdered infant foods, growth

Introduction

Cronobacter spp. are Gram negative, non-spore-forming, facultative-anaerobic, motile bacteria belonging to the family Enterobacteriaceae. The genus *Cronobacter* was previously assigned to a single species, *Enterobacter sakazakii*. However, in 2008, it was renamed and reclassified as the following *Cronobacter* species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. dublinensis* subsp. *dublinensis*, *C. dublinensis* subsp. *lausannensis*, *C. dublinensis* subsp. *lactaridi*, and *C. genomospecies* 1 by Iversen et al.^{10, 11)}. *C. condimenti* and *C. universalis* were added as new species in 2012¹²⁾, and 7 species and 3 subspecies are currently included. These organisms are causative agents of meningitis, pneumonia, and sepsis in immunocompromised persons and the elderly^{1, 16)}. They are also known to cause necrotic enteritis and sepsis, particularly in newborns, infants, and premature babies through contaminated formula milk. Furthermore, they are known to cause food borne illness^{1, 4, 5, 7, 8)}. Ster-

ile preparation of infant powdered milk formula is challenging, typically requiring careful attention. *Cronobacter* is resistant to drying^{2, 17)}, and its presence in the kitchen and on surfaces in the home can contaminate infant milk upon preparation¹³⁾.

Commercially available PIFs, in Japan, are usually rice- or wheat paste- based, and vegetables and/or animal products are added. In this study, the effects of storage temperature (5, 10, and 25°C) on the survival and growth of *C. sakazakii* in three representative types of commercially available prepared powder infant food (PIFs), containing vegetables, and an apple juice was investigated.

Material and Methods

Foods samples: Commercially available PIFs; “vegetable and rice porridge” (VRP), “mixed vegetable and wheat paste” (MVWP), “liver, vegetable and wheat paste” (LVWP) and “apple juice” (AJ) were used in this study. PIFs were prepared according to the manufacturer’s instructions. Testing for the presence of *Cronobacter* was performed according to ISO/TS 22964⁹⁾; and the employed PIFs were *Cronobacter* negative.

Strain and culture conditions: *C. sakazakii*

[†] E-mail: Ogihara.hirokazu@nihon-u.ac.jp

^{*1} ☎252-0880 神奈川県藤沢市亀井野1866

ATCC 29544, a clinical isolate, was used in this study. Cells were sequentially cultured twice in tryptic soy broth (TSB; Difco, Detroit, USA) at 37°C for 24 hr, and the secondary cultures were used for experiments.

Determination of optimum growth temperature of *C. sakazakii*: Cultured *C. sakazakii* (10^3 CFU/ml) was inoculated into fresh TSB, and cultured under shaking for 24 hr at temperatures ranging from 5 to 60°C. Optical density at 660 nm was measured by a Biophotorecorder TSV126MA (Advantec Toyo Kaisha, Ltd., Japan). Relative growth rates were calculated by the culture time required to reach O.D. 660 nm=0.5 (100%).

Measurement of growth properties of *C. sakazakii* in TSB: Cultured *C. sakazakii* (10^{2-3} CFU/ml) was inoculated into fresh TSB (pH 6.8 and 3.6), and cultured at 5, 10, and 25°C for 6, 12, 24 and 48 hr. pH 3.6 was pH of AJ. Viable cell numbers were determined by culturing at 37°C for 48 hr on tryptic soy agar (TSA; Difco, Detroit, USA).

Measurement of growth properties of *C. sakazakii* in PIFs: Cultured *C. sakazakii* was inoculated (10^{2-3} CFU/ml) into prepared PIFs, and incubated at 5, 10, and 25°C for 48 hr. Thereafter, samples were homogenized in physiological saline with 0.1% peptone using a Stomacher. Samples from 6, 12, 24, and 48 hr-time points were cultured at $44\pm1^\circ\text{C}$ for 24 hr on XM-sakazakii (Nissui, Ltd., Japan), a *Cronobacter* selective media, and blue-green colonies were enumerated.

Results

Optimum growth temperature of *C. sakazakii*: The growth of *C. sakazakii* was assessed over a temperature range of 5 to 60°C; optimum growth was observed at approx. 37 to 44°C (Fig. 1). The optimum growth temperature of *C. sakazakii* was higher than typically observed for Enterobacteriaceae, but growth was not observed above 49°C. Growth properties of *C. sakazakii* in TSB: Growth properties of *C. sakazakii* at 5, 10 and 25°C are shown in Fig. 2. *C. sakazakii* reached greater than 10^8 CFU/ml in 24 hr at 25°C, and approx. 10^4 CFU/ml in 48 hr at 10°C; however, growth was not observed at 5°C. Growth properties of *C. sakazakii* in PIFs: Effects of storage temperature (5, 10, and 25°C) on the survival and growth of *C. sakazakii* in prepared PIFs (VRP, MVWP, LVWP) and AJ are shown in Fig 3. *C. sakazakii* grew after 6 hr at

Fig. 1. Growth curve of the *Cronobacter sakazakii* ATCC 29544.

Fig. 2. Effect of temperature on the growth of *Cronobacter sakazakii* ATCC 29544 in Tryptic Soy Broth for up to 48 hr.

25°C, and cell numbers reached 10^8 CFU/ml in VRP, MVWP and LVWP in 24 hr at 25°C. In MVWP and LVWP, *C. sakazakii* reached 10^9 CFU/ml in 48 hr at 25°C. On the other hand, *C. sakazakii* showed no growth at 5 and 10°C in all PIFs. In AJ, *C. sakazakii* was inactivated at 25°C, and no growth or slight decreases were observed at 5 and 10°C. These results suggest that the risk for bacterial growth in PIFs, but not in AJ, increases with room temperature storage of food after preparation.

Discussion

Nazaromec-White et al. studied the growth of *E. sakazakii* in infant formula; growth of *E. sakazakii* was observed after 6 days and the minimum growth temperature was 5.5°C²⁰. Gurtler et al. in-

Fig. 3. *Cronobacter sakazakii* ATCC 29544 growth and survival in reconstituted infant formula foods stored for up to 48 hr.

A: Vegetable and rice porridge; VIP, B: Mixed vegetable and wheat paste; MVWP, C: Liver, vegetable and wheat paste; LVWP, D: Apple juice; AJ.

investigated the growth of *C. sakazakii* in infant formula at 4, 12, 21 and 30°C, and no growth was observed at 4°C⁶⁾. We also studied the growth of *E. sakazakii* in powdered infant formula at 5, 10 and 25°C, and there was no growth at 5 and 10°C²¹⁾. Other related studies on the growth of *E. sakazakii* or *C. sakazakii* in PIFs revealed that the growth rates were repressed at 4°C^{18, 23, 24)}.

Fig. 4. Changes of pH in apple juice stored for up to 48 hr.

In this study, *C. sakazakii* grew in three representative PIFs containing vegetables, at room temperature after preparation. Vegetables has been considered to be a natural habitat of this bacterium^{25, 26)}, and in our previous investigation of *C. sakazakii* contamination of commercially available dried foods, including dried vegetables, we found that more than quarter of them were contaminated by *C. sakazakii*²²⁾. Therefore, as the possibility of vegetables to become a source of contamination of PIFs cannot be denied, it is important to control, decrease the number of viable cells, and eliminate this bacterium from vegetables used in PIFs, and decrease the number of viable cells in final PIF products.

In this study, *C. sakazakii* was not able to grow in AJ, and the low pH, below 3.5 at 25°C for 48 hr, of AJ repressed growth at room temperature (Fig. 4). In TSB (pH 3.6), decrease of the viable cell count of *C. sakazakii* was slower than that in AJ (data not shown), and it was hypothesized that some components in AJ would accelerate the inactivation of *C. sakazakii* or some components in TSB would repress the inactivation. Some previous reports showed that apple and its related juices had no remarkable effects on the growth of *C. sakazakii*^{3, 14, 15, 18)}. The differences of this and previous results should be investigated in the future.

This time it was showed that growth of *C. sakazakii* could be controlled by low temperature in commercially available PIFs in Japan. Nakamura *et al.* showed that temporal heating of PIFs during preparation could decrease *E. sakazakii* numbers; however, after a lag time, growth resumed at room temperature¹⁹⁾. The risk of bacterial growth in the tested PIFs increased at room

temperature storage after preparation, and that supported the importance in following the WHO guidelines for the safe preparation, storage, and handling of powdered infant formula²⁷⁾. In other hand, it demonstrated that there is some type of commercial manufactured apple juice that also inhibit the growth or even inactivate *C. sakazakii* in 24 hr at room temperature.

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調製乳幼児用食品における保存温度が *Cronobacter sakazakii* の増殖に及ぼす影響

福田典子^{*1}・新井のぞみ^{*1}・藤原 翠^{*1}
古川壮一^{*1}・萩原博和^{*1}

(^{*1} 日本大学生物資源科学部, 食品生命学科)

調製された乳幼児用食品に *Cronobacter sakazakii* を接種し, 25℃, 10℃, 5℃ の温度に 48 時間保存し, これらの保存期間中における *C. sakazakii* の挙動を検討した. その結果, 調製された乳幼児用食品の野菜粥 (VRP), 混合野菜と小麦ペースト (MVWP), レバー・野菜と小麦ペースト (LVWP) 中での *C. sakazakii* の挙動は, 25℃ ではいずれも急激な増殖が認められたのに対し, 5℃ と 10℃ では保存期間中菌数の増殖は認められなかった. 一方, リンゴ果汁 (AJ) では他の乳幼児用食品 3 種の結果と異なり, いずれの保存温度でも菌数の減少が観察され, 特に 25℃ では著しい減少が認められた. 以上のことより, 乳幼児用食品 4 種のうち, AJ を除き, VRP, MVWP, LVWP では, 室温 (25℃) において急激な増殖が確認されたことから, これらの乳幼児用食品を調製した後は室温に長時間放置せず, 速やかに消費することが感染リスクの低減になるものと考えられた.