Dept. of Food Hygiene,
Fac. of Vet. Med., Assiut Uni., Egypt.

INCIDENCE AND SURVIVAL OF *ENTEROBACTER SAKAZAKII* IN INFANTS POWDERED MILK-BASED FORMULAE USED BEFORE AND AFTER WEANING

(With 5 Tables and 5 Figures)

By

A.A-H. AHMED; ENAS EL-PRINCE; EMAN KORASHY* and MARWA M.N. AL-GENDI*

* Animal Health Research Institute, Assiut

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مدي تواجد ميكروب *Enterobacter sakazakii* في توليفات أغذية الأطفال الجافة المحتوية علي اللبن المستخدمة قبل وبعد الفطام

أحمد عبد الحميد ، إيناس البرنس ، إيمان قرشي ، مروة الجندي

تعد كل من الألبان الجافة والأغذية الجافة المحتوية علي اللبن من الأغذية واسعة التدوال لدي الكبـار والأطفال، وتلوثها بالميكروبات الضارة من الأمور التي تستوجب الأهتمام والدراسة. لذلك تضمنت هذه الدراسة فحص عدد 250 عينة عشوائية من أغذية الأطفال اللبنية الجافة بواقع 70 عينة من لبن البودرة للأطفال حديثي الولادة، 90 عينة من كل من أغذية الفطام الجافة المحتوية علي خلاصة الحبوب واللبن الجاف، وكانت صالحة للاستهلاك حيث تمتد فترة صلاحيتها لمدة لا تقل عن عام من تاريخ الإنتاج، وتم تجميع هذه العينات من العديد من المحال التجارية والصيدليات في مدينة وقري أسيوط لمعرفة مدي تلوثها بميكروب *E. sakazakii* لما له من خطورة كبيرة علي صحة الأطفال. وأظهرت النتائج تواجد *E. sakazakii* بالنسب الآتية : صفر، 6.7 و1.1% من عينات ألبان الأطفال، أغذية الأطفال الجافة االمحتوية علي اللبن واللبن الجاف علي التوالي. وهذا يدل علي أن أغذية الأطفال الجافة االمحتوية علي اللبن تعتبر الأسوأ من حيث تلوثها بميكروب *E. sakazakii*. وكذلك تم دراسة قدرة هذا الهيكروب علي النمو والبقاء في أغذية الأطفال الجافة االمحتوية علي اللبن متأثرا بالسائل الذي يستخدم في تحضيره (عصير التفاح والماء) ودرجة الحرارة التي يتم التخزين فيها (درجة حرارة الغرفة 16±2°م ودرجة حرارة الثلاجة 4 ±1°م) وذلك باستخدام ميكروب *E. sakazakii* الذي تم عزله من أغذية الأطفال المحتوية علي اللبن وتصنيفه معمليا باستخدام API ثم حقنه في أحد أنواع أغذية الأطفال المحضر ة معمليا . ولقد تبين من الفحص أن الميكروب لم ينمو باستخدام عصير التفاح عند درجتي الحرارة المستخدمة، وكذلك أيضا في حالة استخدام الماء عند درجة الحرارة (4 ±1 °م). أما في حالة حفظ الوجبة المحضرة باستخدام الماء في درجة حرارة الغرفة (16± 2 °م)، فقد وجد أن الميكروب بدأ بالزيادة بعد ساعة واحدة من حفظه وأخذ في الزيادة حتي وصل الي أعلي عدد بعد 4 ساعات وأنه كان هناك أيضا انخفاضا في العدد بعد 8 ساعات حتي وصل الي مستوي أقل من 100 ميكروب/جرام. ومن هذه النتائج

ينصح بعدم ترك الوجبة أكثر من ساعة في درجة حرارة الغرفة وحفظها في الثلاجة، كما يفضل استخدام عصير التفاح في تحضير هذه الوجبات. هذا وقد نوقشت الأهمية الصحية لميكروب *E. sakazakii* والشروط الواجب إتباعها لمنع تلوث ألبان وأغذية الأطفال المختلفة به.

SUMMARY

A total of two hundred and fifty random samples of infant's milk powder for babies after birth (70 samples), milk-based cereal weaning food (90 samples) and dried milk powder (90 samples) were purchased from different shops and pharmacies in Assiut city and villages around the city. The samples were still valid for consumption as shelf life is at least to be more than one year from production time and they were transferred to the laboratory in their packages to be examined for prevalence of *Enterobacter sakazakii* which could be isolated from 6/90 milk-based cereal baby food samples and from 1/90 dried milk powder samples, however, failed to be detected in infant milk formulae which considered as non sterile products. The survival and growth of *E. sakazakii* in milk-based cereal weaning food using different reconstituted liquids (apple juice and water) stored at different temperatures (room temperature 16 ± 2°C and refrigerated temperature 4±1°C) were carried out. The results revealed that the growth did not occur in cereal reconstituted with apple juice, regardless of storage temperature, or in cereal reconstituted with water stored at 4±1°C. Upon reaching maximum populations of 4 log10 cfu/ g, in some instances populations decreased to nondetectable values during subsequent storage which was concurrent with decrease in pH values. *E. sakazakii* initially at very low populations can rapidly grow in infant cereal reconstituted with water. The public health hazards of *E.sakazakii* and the suggestive measures for improving the quality of infants' food were discussed.

Key words: *E.sakazakii, Incidence, Survival, Infants milk powder, Milk-based cereal weaning food, Dried milk powder.*

INTRODUCTION

Infants should be exclusively breast fed for the first 6 months of life and those, who are not, should be provided with a suitable breast milk substitute. The reconstitution of powdered infant formula (PIF) should be undertaken by caregivers using good hygienic measures and in accordance with the product manufacturer's food safety guidelines

(Drudy *et al.*, 2006). However, consumption of contaminated PIF has been epidemiologically linked with cases of infection. Contamination can occur during the manufacturing process or during post manufacture reconstitution of formula which is not a sterile product (Mullane *et al.*, 2007). *E. sakazakii* is an opportunistic pathogen and the etiological agent in rare but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in infants. Among infants, those at greatest risk are neonates (<28 days), particularly those born prematurely or of low birth weight (<2500 g). It was recorded that infant mortality for *E. sakazakii* meningitis is 40–80%, with death often occurring within hours of infection (Arseni *et al.*, 1987). Although there is no epidemiological evidence for the amount of an infectious dose for *E. sakazakii*, it would seem reasonable to use a detection limit <1 cell in 25 g of infant food formula, equivalent to that of detection of *Salmonellae* in milk powder (Mansfield and Forsythe, 2000).

Moreover, there have been few reports recording *E. sakazakii* infection in adults, and it is not usually life threatening as they had serious underlying diseases, such as malignancies. However, *E. sakazakii* is a rare cause of invasive disease when meningitis occurs, severe neurological complications, including cerebral abscess formation, are common, and death occurs in 33-80% of cases (Nazarowec-White and Farber, 1997a and Lai, 2001). The most frequently recorded method of entry of *Enterobacter* in patients with bacteremia was ingestion of contaminated foods or food ingredients like cereals, fruits and vegetables, legume products, herbs and spices as well as from animal food sources like milk, cheese, meat and fish and their products both raw and processed (Weischer and Kolmos, 1992 and Friedemann, 2007). It was hypothesized that the reservoir for *E. sakazakii*, in addition to other *coliforms* (*Klebsiella oxytoca*, *K. pneumoniae*, *E. cloacae*, and *Citrobacter spp.*) may be from primarily environmental and from plant materials (Mossel and Struijk, 1995).

Many authors could isolate *E. sakazakii* from infant milk formulae as Postupa and Aldová (1984); Simmons *et al.* (1989); Nazarowec-White and Farber (1997a); Nazarowec-White and Farber (1997c); Van Acker *et al.* (2001); MMWR (2002); Leuscher *et al.* (2004); Baiguini (2005); Jarvis (2005); Estuningsih *et al.* (2006); Iversen and Forsythe (2007); Shaker *et al.* (2007) and Torres-Chavolla *et al.* (2007). Moreover, Restaino *et al.* (2006), El-Prince *et al.* (2007) and Shaker *et al.* (2007) could isolate *E. sakazakii* from dried infant foods samples. Also, the survival and growth of *E. sakazakii* in infant cereals

as affected by composition, reconstitution liquid, and storage temperature were measured by many investigators (Richards *et al.*, 2005, Gurtler and Beuchat, 2007 and Lin and Beuchat, 2007).

As *E. sakazakii* was classified as a severe hazard for restricted populations, causing life-threatening or substantial chronic sequelae or illness of long duration (ICMSF, 2002), therefore, its incidence in infants powdered milk-based formulae used before and after weaning was studied. Also, its survival in cereals using different reconstitution liquids at various temperatures was investigated.

MATERIALS and METHODS

Collection of samples:

A total of two hundred and fifty random samples of infant's milk powder for babies after birth (70 samples), milk-based cereal weaning food and dried milk powder (90 samples each) were purchased from different shops and pharmacies in Assiut city and villages around the city. The samples were still valid for consumption as their shelf life is at least to be more than one year from the production time and they were transferred to the laboratory in their packages to be examined for the prevalence of *E. sakazakii.*

I- Isolation and identification of *E. sakazakii* (FDA., 2002):

Each sample was aseptically opened and 10 g were weighed and homogenized in 90 ml of sterile distilled water, shacked by hand until the powder was uniformly suspended, incubated overnight at 36°C. 10ml of the incubated sample were transferred to 90 ml of Enterobacteriaceae enrichment broth (EE) and incubated overnight at 36°C. Loopfuls were streaked onto violet red bile agar (VRBL) and incubated overnight at 36°C.

Isolation procedures:

From each VRBL plate, all colonies were purified on Tryptone Soy Agar (TSA). The TSA plates were incubated for 24 to 72 h in daylight at room temperature (about 25°C). The yellow pigmentation on TSA is a characteristic feature of *E. sakazakii*.

Identification of isolates:

Isolates were identified using biochemical tests including Triple Sugar Iron (TSI), Urease test, Sugar fermentation tests, IMViC tests, catalase test then oxidase test. Oxidase-negative isolates were further identified using API 20E biochemical identification test system (bioMerieux SA, France). API 20E systems have been used for

presumptive-positive confirmations of *E. sakazakii* via biochemical characteristics (Kandhai *et al.,* 2004 b).

II- Survival and growth of *E. sakazakii* in infant cereals using different reconstitution liquids at different storage temperatures.

Culture preparation:

E. sakazakii strain used in the present study was previously isolated and identified from the examined dried milk-based baby foods. The organism was propagated in EE broth at 37°C for 24 h. One ml of the culture was serially diluted in 0.1% peptone water to attain the desired inoculum level.

Preparation and inoculation of dried milk-based baby food samples:

Six portions of dried milk-based baby food obtained from Assiut markets, previously examined and found to be free from *E. sakazakii*, were reconstituted with sterile apple juice and sterile water as recommended by manufactures instructions (50 g of dried milk-based baby food reconstituted with 150 ml of reconstituted liquid). The prepared samples were inoculated with enough amount of broth culture to yield approximately 2 x 10, 2 x 10^2 and 2 x 10^3 cfu/ml of *E. sakazakii* and control samples were prepared for each treatment, without addition of the organism. Each sample was divided into 2 equal portions, one was stored at room temperature (16±2°C) and the other at refrigeration temperature (4±1°C) with their control.

Preparation of dried milk-based baby food samples for examination:

Samples were prepared following the procedures described by American Public Health Association (A.P.H.A., 1992).

Enumeration of *E. sakazakii* in the inoculated samples:

From the inoculated samples and their control, 10 fold serial dilutions were prepared using 0.1 % peptone water for determination of *E. sakazakii* count, using direct spreading method. Duplicate TSA plates were inoculated with each dilution by spreading 0.1 ml evenly onto the surface of each plate with a sterile glass spreading rod. Inoculated plates were incubated at 30°C for 48-72 h. The cfu/g was calculated and recorded. Samples and their control stored at room temperature (16±2°C) were examined for *E. sakazakii* at 0, 1, 4, 8 and 12 h, while those stored at refrigerating temperature (4±1°C) were tested every 2, 4, 8 12 and 48 h.

RESULTS

The obtained results are recorded in Tables 1-5 and Figures 1-5

Table 1: Incidence of *E. sakazakii* in the examined samples of powdered milk-based formulae.

Type of samples	No. of examined samples	Positive samples	
		No.	%
Infant milk formulae	70	-	-
Milk-based cereal weaning food	90	6	6.7%
Dried milk powder	90	1	1.1%

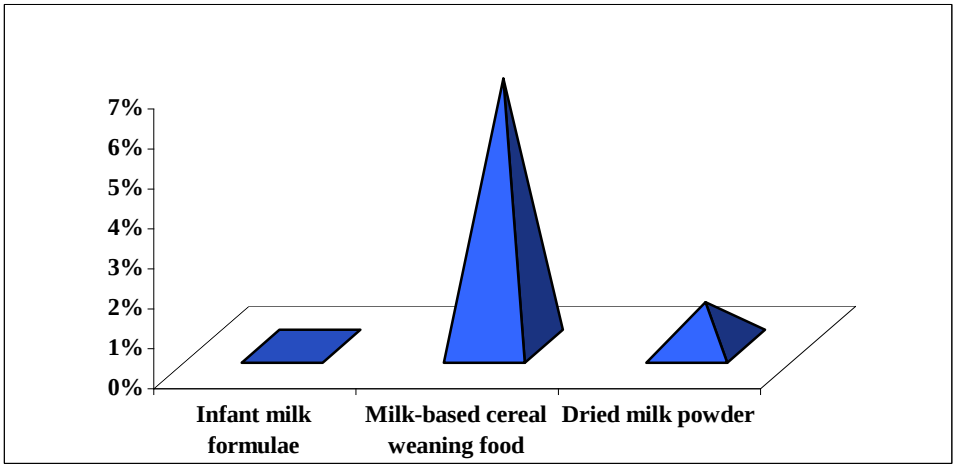

Fig. 1: Incidence of *E. sakazakii* in the examined samples of powdered milk-based formulae.

Table 2: Survival of *E. sakazakii* in infant cereal milk-based food using water as reconstitution liquid and stored at room temperature (16±2 °C).

Storage time	Inoculum 1/g	Inoculum 2/g	Inoculum 3/g
0	3 x 10	3×10^2	2×10^3
1 h	1×10^2	4×10^2	3×10^3
4 h	3×10^4	1×10^3	4×10^3
8 h	1×10^2	8×10^2	6×10^2
12 h	*< 100	*< 100	*< 100

* Not detected on the plate (<100), but the organisms could be isolated from the examined samples.

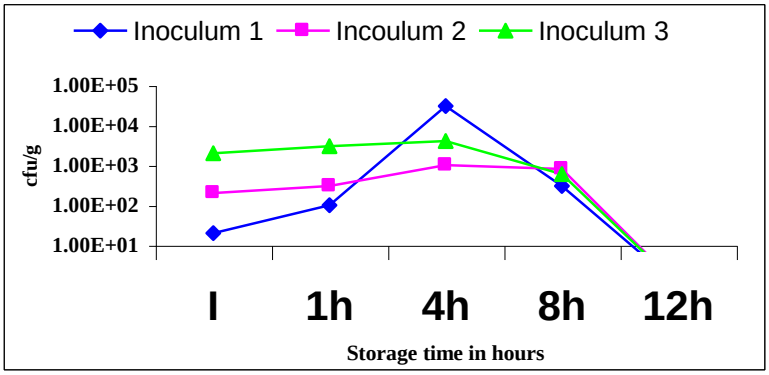

Fig. 2: Survival of *E. sakazakii* in infant cereal milk-based food using water as reconstitution liquid and stored at room temperature (16±2 °C).

Table 3: Survival of *E. sakazakii* in infant cereal milk-based food using apple juice as reconstitution liquid and stored at room temperature (16±2 °C).

Storage time	Inoculum 1/g	Inoculum 2/g	Inoculum 3/g
0	3 x 10	3 x 10^2	2 x 10^3
1 h	*<100	*<100	*<100
4 h	*<100	*<100	*<100
8 h	*<100	*<100	*<100
12 h	ND	ND	ND

* Not detected on the plate (<100) but could be isolated. ND = *E. sakazakii* could not be isolated in 1, 10 and 25 g of infant cereal milk-based food.

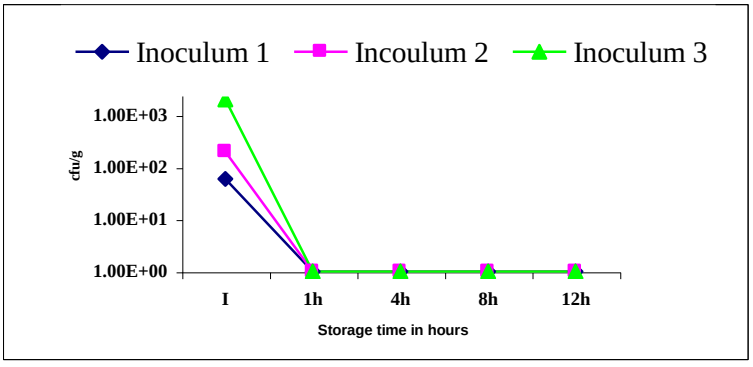

Fig. 3: Survival of *E. sakazakii* in infant cereal milk-based food using apple juice as reconstitution liquid and stored at room temperature (16±2 °C).

Table 4: Survival of *E. sakazakii* in infant cereal milk-based food water as reconstitution liquid and stored at refrigerated temperature (4±1°C).

Storage time	Inoculum 1/g	Inoculum 2/g	Inoculum 3/g
0	3 x 10	3 x 10^2	2 x 10^3
1 h	*<100	*<100	*<100
4 h	*<100	*<100	*<100
8 h	*<100	*<100	*<100
12 h	ND	ND	ND

* Not detected on the plate (count < 100) but the organisms could be isolated.

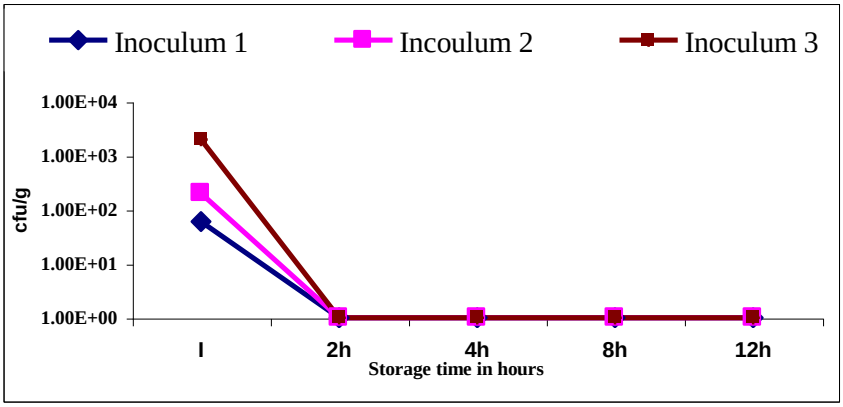

Fig. 4: Survival of *E. sakazakii* in infant cereal milk-based food using water as reconstitution liquid and stored at refrigerated temperature (4 ±1°C).

Table 5: Survival of *E. sakazakii* in infant cereal milk-based food apple juice as reconstitution liquid and stored at refrigerated temperature (4±1°C).

Storage time	Inoculum 1/g	Inoculum 2/g	Inoculum 3/g
0	3 x 10	3 x 10^2	2 x 10^3
1 h	*<100	*<100	*<100
4 h	*<100	*<100	*<100
8 h	*<100	*<100	*<100
12 h	*<100	*<100	*<100

* Could not be detected on the plate (count < 100) but the organisms could be isolated.
ND = *E. sakazakii* could not be isolated in 1, 10 and 25 g of infant cereal milk-based food.

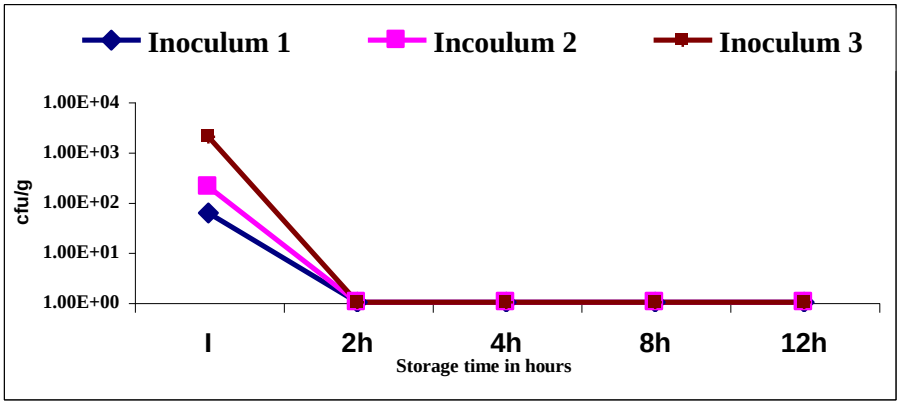

Fig. 5: Survival of *E. sakazakii* in infant cereal milk-based food using apple juice as reconstitution liquid and stored at refrigerated temperature (4±1 °C).

DISCUSSION

The results achieved in Table 1 & Fig. 1, indicated that IMF samples were found to be free from *E. sakazakii*. However, many investigators could isolate *E. sakazakii* from IMF as Postupa and Aldová (1984); Simmons *et al.* (1989); Nazarowec-White and Farber (1997a); Nazarowec-White and Farber (1997c); Van Acker *et al.* (2001); MMWR (2002); Leuscher *et al.* (2004); Baiguini (2005); Jarvis (2005); Estuningsih *et al.* (2006); Iversen and Forsythe (2007) and Shaker *et al.* (2007). Moreover, higher incidence of *E. sakazakii* could be detected by Torres-Chavolla *et al.* (2007). Also, *E. sakazakii* could be isolated from 6 (6.7%) of the examined milk-based cereal weaning food samples. Somewhat similar percentage was obtained by El-Prince *et al.* (2007) who could isolate *E. sakazakii* from 1 out of 30 (3.33%), of examined dried infant foods samples. While, higher percentages were detected by Restaino *et al.* (2006) and Shaker *et al.* (2007). Moreover, 1 strain (1.1%) of *E. sakazakii* was recovered from the examined samples of dried milk powder, however it failed to be detected as postulated by El-Prince *et al.* (2007) and Shaker *et al.* (2007). While, slightly higher percentage was estimated by Postupa and Aldová (1984) who could isolate 4 strains of *E. sakazakii* from milk powder in Czechoslovakia. Nazarowec-White and Farber (1997b) stated that microbial pathogens could gain access to the milk powder from the environment or from the addition of ingredients at the powder stage.

According to Commission Regulation (EC) (2005) on the microbiological criteria for foodstuffs, *E. sakazakii* is considered a microorganism of greatest concern in infant formulae and follow-on formulae. *E. sakazakii* is included between "safety criteria" and it must be absent in 10 g of dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age (Conte and Passantino, 2007). The infectious dose can vary according to the organism's history (stress response factors), host state (healthy or immunocompromised) and food matrix. Several physiological characteristics of *E. sakazakii* appear to play an important role in this transmission route: (i) it can grow in a broad range of temperatures of 6–45 °C (Iversen *et al.,* 2004 a); (ii) it can survive longer than other *Enterobacteriaceae* under conditions of dry or osmotic stress, which might provide a selection pressure in dried milk products (Breeuwer *et al.,* 2003); (iii) it can efficiently form biofilms on various materials, including the frequently used polycarbonate material from infant-feeding bottles (Iversen *et al.,* 2004 b and Lehner and Stephan, 2004); (iv) in reconstituted infant formula, *E. sakazakii* seems to be more heat-resistant than other species of *Enterobacteriaceae* (Nazarowec-White and Farber, 1997 b).

Hygiene mismanagement due to incorrect temperature and time factors as well as due to the contact transmission of microorganisms via hands, insects, small vertebrates and equipment should be avoided during production, preparation and storage of food and drink. *E. sakazakii* may be associated with food spoilage, but, the detection of the ubiquitous *E. sakazakii* in food is not always an indicator for hygiene mismanagement.

It is obvious from the aforementioned results that milk-based cereal weaning food samples were the highest in contamination with *E. sakazakii*. Its presence in the examined samples probably originated from factories producing milk powder, cereals, chocolate, potato flour and pasta as well as in households strongly indicates that the organisms are widespread. Also, the high tolerance of *E. sakazakii* to desiccation provides a competitive advantage in dry environments thereby increases the risk of post-pasteurization contamination of the finished product (Breeuwer *et al.,* 2003 and Kandhai *et al.,* 2004 a).

The survival of *E. sakazakii* in infant cereal milk-based food using water as reconstitution liquid and stored at room temperature (16±2 °C) was recorded in Table 2 & Fig.2. It is evident by using 3 different inoculums (3 x 10, 3 x 10^2 and 2 x 10^3) that, the numbers of

E. sakazakii increased to 1 x 10^2, 4 x 10^2 and 3 x 10^3 cfu/g after 1 h of inoculation and reach the peak after 4 h to be 3 x 10^4, 1 x 10^3 and 4 x 10^3 cfu/ g, respectively. The organisms began to decline rapidly after 8 h to reach 1 x 10^2, 8 x 10^2, 6 x 10^2 cfu / g, respectively, then reached to undetectable numbers after 12 h of storage (<100/g). However, it was detected by enrichment of one g of each treatment. From the previous results, it is clear that *E. sakazakii* initially at very low populations can rapidly grow in infant cereal reconstituted with water. These results are harmony with those obtained by Richards *et al.* (2005), Gurtler and Beuchat (2007) and Lin and Beuchat (2007).

From the data recorded in Table 3 & Fig. 3, it is apparent that *E. sakazakii* failed to be counted (<100) in the 3 different inoculated samples after one hour of inoculation when apple juice was used as a reconstituted liquid stored at room temperature (16±2 °C). After 12 h, *E. sakazakii* could not be isolated from 1, 10, 25 g of examined samples using E E broth. Absence of *E. sakazakii* in examined samples reconstituted with apple juice coincided with decreases in pH and an increase in the population of lactic acid bacteria. These results go parallel with the findings obtained by Weir (2002), Richards *et al.* (2005) and Lin and Beuchat (2007).

On the other hand, *E.sakazakii* did not grow in infant cereal milk-based food reconstituted either with water or apple juice and stored at 4±1 °C. *E.sakazakii* failed to be detectable in both water and apple juice reconstituted samples after 2 h of storage and thereafter. However, the organisms could be isolated by enrichment procedures from water reconstituted samples after 4, 8 and 12 h and failed to be isolated from 1, 10 and 25 g after 12 h of storage in apple juice reconstituted samples (Tables 4 & 5 and Fig. 4 & 5). Our results were in accordance with those obtained by Farmer *et al.* (1980), Iversen *et al.* (2004 b), Richards *et al.* (2005), Gurtler and Beuchat (2007) and Lin and Beuchat (2007).

E.sakazakii is a pathogen of great concern to food industry, especially in foods normally stored under refrigeration conditions, unlike most food borne pathogens; it is able to multiply at refrigerator temperature (6°C). These findings confirm the importance of proper refrigeration, consequently, refrigeration should not be relied upon as the sole method for control of *E. sakazakii*, but should be incorporated with other means of preservation. Simmons *et al.* (1989) recommended the using of refrigeration and limiting the hang time to prevent or retard the growth of *E. sakazakii*. Nazarowec-White and Farber (1997a) found that *E. sakazakii* did not grow at 4°C and began to die off during storage at

this temperature and warned improper preparation and storage of reconstituted dried infant formulae at ambient temperature. Moreover, trained personnel should prepare dried milk-based products under aseptic techniques and conditions in a designated area following the manufacturer's instructions. The composition of infant cereals did not markedly affect the survival or growth of *E. sakazakii* in reconstituted cereals (Lin and Beuchat, 2007).

It is clear from the present study that reconstituted infant cereal can support luxuriant growth of *E. sakazakii* and when it is not immediately consumed should be discarded or stored at a temperature at which it cannot grow for only 24 h. Also, the reconstituted infant cereals stored at 30 ºC should be discarded within 1 h after preparation.

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