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INCIDENCE AND PUBLIC HEALTH HAZARD OF *Enterobacter sakazakii* IN MILK POWDER AND SOME DRIED MILK-BASED FOODS

(With 4 Tables and One Figure)

By

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مدى تواجد وخطورة ميكروب *Enterobacter sakazakii* في اللبن الجاف وبعض الأغذية الجافة المحتوية على اللبن

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تعد كل من الألبان الجافة والأغذية الجافة المحتوية على اللبن من الأغذية الواسعة التداول لدى الكبار والأطفال، وتلوثها بالميكروبات الضارة من الأمور التي تستوجب الاهتمام والدراسة، من هنا كان الاهتمام بتحديد مدى تواجد ميكروب *Enterobacter sakazakii* لما له من خطورة كبيرة على الصحة العامة للإنسان، لذلك تضمنت هذه الدراسة فحص عدد١٢٠ عينة عشوائية من اللبن الجاف وأغذية الأطفال الجافة المحتوية على اللبن والكابتشينو ومبيض الشاي (٣٠ عينة لكل منها) وكانت صالحة للاستهلاك حيث تمتد فترة صلاحيتها لمدة لا تقل عن عام من تاريخ الإنتاج. وتم جمع العينات من العديد من المحال التجارية والصيدليات في مدينة أسيوط لمعرفة مدى تلوثها بهذا الميكروب. وأظهرت النتائج تواجد *E. sakazakii* بالنسب الآتية: صفر و٣,٣٣ و٦,٦٧ وصفر% من العينات على التوالي باستخدام طريقة العزل بواسطة الوسط المنشط Enterobacteriaceae enrichment، أما طريقة العزل بواسطة الوسط المنشط peptone water فقد أظهرت تواجده بالنسب الآتية: صفر و١٠ وصفر وصفر% من العينات على التوالي، حيث تم استخدام ٣ طرق لعزل هذا الميكروب لتحديد الطريقة الأمثل لعزله، وكانت الطريقتان التي استخدم فيها وضع العينات في وسط منشط لنمو الميكروب أعلى فاعلية عن الطريقة الثالثة التي لم يستخدم فيها الوسط المنشط. وقد أثبتت النتائج أن أغذية الأطفال الجافة المحتوية على اللبن تعتبر الأسوأ من حيث تلوثها بميكروب *E. sakazakii*. بينما كانت عينات الكابتشينو أقل تلوثا ولم يتم عزل الميكروب من عينات اللبن الجاف ومبيض الشاي. ولقد تم عزل ميكروبات أخرى من genus Enterobacter كالتالي *E.aerogenes*، *E.cloacae*، *E.agglomerans*، *E.intermedius*. ذلك بالإضافة إلى عدد ٢٩عترة أخرى من family Enterobacteriaceae تم عزلها مع توضيح مدى تواجدها بعينات الدراسة بكل طريقة من

Cedecea species, *Escherichia coli, Ewingella americana, Hafnia alvei, Klebsiella pneumoniae, K.oxytoca, K.rhinoscleromatis, K.terrigena,* Pantoea species, *Salmonella paratyphi A, Serratia marcescens, S.liquefaciens, S.plymuthica,* Shigella species and Yersinia species. وقد تم مناقشة مدى خطورة ميكروب *E. sakazakii* على صحة وسلامة الإنسان والإجراءات الصحية والشروط الواجب اتباعها لتحسين جودة الألبان الجافة والأغذية الجافة المحتوية على اللبن. طرق العزل الثلاثة، وهم

SUMMARY

A total of 120 random samples of milk powder, dried milk-based baby foods, cappuccino and tea creamer (30 samples each) were obtained from different shops and pharmacies in Assiut city. The samples were still valid for consumption as their shelf life was at least one year from the production date. These samples were examined for prevalence of *Enterobacter sakazakii* which could be isolated in percentage rates of 0, 3.33, 6.67 and 0%, respectively using the isolation procedure with Enterobacteriaceae enrichment broth, while the isolation procedure with peptone water showed its percentage rates as 0, 10, 0 and 0%, respectively. Thus 3 different procedures were used for isolation of such organism, the enrichment broth was used in 2 procedures and one procedure was carried out without enrichment broth. The 2 procedures using enrichment broth were valuable for isolation of *E. sakazakii* than that used without enrichment. The results obtained in this study showed that dried milk-based baby foods were the worst in its contamination by *E. sakazakii*. However, cappuccino samples were of less contamination while milk powder and tea creamer samples failed to recover the organism. Other organisms related to genus Enterobacter could be isolated as *E. aerogenes, E. cloacae, E. agglomerans* and *E. intermedius*. Also, 29 isolates related to family Enterobacteriaceae could be isolated from the examined samples using the 3 different methods of isolation. The isolates were found to be Cedecea species, *Escherichia coli, Ewingella americana, Hafnia alvei, Klebsiella pneumoniae, K. oxytoca, K. rhinoscleromatis, K. terrigena,* Pantoea species, *Salmonella paratyphi A, Serratia marcescens, S. liquefaciens, S. plymuthica,* Shigella species and Yersinia species. Suggestive hygienic measures for improving the quality of milk powder and some dried milk-based foods and the public health hazard of *E. sakazakii* were recommended.

Key words: *Enterobacter sakazakii,* Milk powder, Dried milk-based foods, Dried milk-based baby foods, Cappuccino, Tea creamer.

INTRODUCTION

E. sakazakii is a motile, peritrichous, Gram-negative, non-sporulating straight rod within the family Enterobacteriaceae, genus Enterobacter. The organism was called "yellow-pigmented *Enterobacter cloacae*" until 1980 when it was reclassified as a unique species based on differences from *E. cloacae* in DNA relatedness, the specific yellow pigment production, biochemical reactions and antibiotic susceptibility, then was renamed as *E. sakazakii* (Farmer *et al.*, 1980). Genus Enterobacter has 14 species or biogroups (Farmer, 1995) and can create community infections that are responsible for approximately half of all nosocomially acquired infections and are often implicated in co-infections (Hervas *et al.*, 2001).

E. sakazakii is recognized nowadays as a potential emerging foodborne pathogen linked to several outbreaks causing sepsis, meningitis or enteritis especially in neonates and infants. Contributing factors in neonates that might increase the risk of infection include immunosuppression, premature birth and low birth weight (Himelright *et al.*, 2002). At least 76 cases of neonatal *E. sakazakii* infections were documented to occur worldwide between 1958 and 2003 (Iversen and Forsythe, 2003). The first 2 known cases of meningitis were reported in 1961 (Urmenyi and White-Franklin, 1961). Subsequently, cases of meningitis, septicemia and necrotizing enterocolitis due to *E. sakazakii* have been reported worldwide. Although most documented cases involved infants, reports describe infections in adults as well (Lai, 2001). Overall, case-fatality rates have varied considerably with rates as high as 80% in some instants. A mortality rate of 40 to 80% has been recorded, and in many cases, neonates die within days of birth. Moreover, in surviving patients with meningitis, severe neurological sequlae such as ventriculitis, brain abscess, hydrocephalus and retarded neural development can occur (Willis and Robinson, 1988). Neonatal necrotizing enterocolities (NEC) characterized by intestinal necrosis and pneumatosis intestinalis with a mortality rate of 10 to 55% were documented (Peter *et al.*, 1999).

Fecal carriage of *E. sakazakii* has been seen in infants up to 18 weeks, emphasizing potential not only for attachment and possible long-term survival in human and animal gut, and environment but also for cross-infection (Block *et al.*, 2002). There are only few reports of infections among adults and most adult patients with *E. sakazakii* infections have serious underlying diseases such as malignancies

(Burdette and Santos, 2000). Isolates of *E. sakazakii* could be obtained from patients suffering from bactermia and osteomyelitis (Lai, 2001).

Few researchers reported the mechanisms of pathogenicity of potential virulence factors of *E. sakazakii*. As Gram-negative organisms, Enterobacter species possess endotoxin and therefore they are considered opportunistic pathogens. They have been implicated in a broad range of clinical syndromes as infections of skin, soft tissue, respiratory, urinary and gastrointestinal tract (Sanders and Sanders, 1997). However, the reservoir for *E. sakazakii* is unknown, a growing number of reports suggest a role for dried milk-based infant formulas as a vehicle and a potential source of *E. sakazakii* (Van Acker *et al.*, 2001). Farmer *et al.* (1980) reported that one the original isolates in the National Collection of Type Cultures was from an unopened can of milk powder. Also, *E. sakazakii* was isolated from dried milk-based infant formulas (Muytjens *et al.*, 1988), environmental samples collected from milk powder factories and from 5 of 16 households (Kandhai *et al.*, 2004a and Kandhai *et al.*, 2004b). Sources of *E. sakazakii* with infant infections have implicated rehydrated powdered infant formula (a non sterile product) as well as equipments and utensils used to prepare rehydrated formula in hospital settings (Bar-Oz *et al.*, 2001).

The widespread nature of this organism and its severity of infections led to this study. Therefore, the objective was to determine the incidence of *E. sakazakii* in high-risk products including milk powder, dried milk-based baby foods, cappuccino and tea creamer.

MATERIALS and METHODS

Collection of samples:

A total of 120 random samples of milk powder, dried milk-based baby foods, cappuccino and tea creamer (30 samples each) were obtained from different shops and pharmacies in Assiut city. The samples were still valid for consumption as their shelf life was at least one year from the production date. All samples were dispatched directly to the laboratory under strict hygienic measures with a minimum of delay. The surface of samples' packages and the spoons used for withdrawal of the samples were disinfected by flaming with 70% (wt/vol) ethanol.

Analyses procedures:

3 different procedures for analyses were applied for each sample: 1st procedure using Enterobacteriaceae Enrichment broth (EE broth) according to U.S.FDA (2002a):

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. 10 ml of the incubated sample were transferred to 90 ml of EE broth (buffered glucose, brilliant green, bile broth) and incubated overnight at 36°C. Loopfuls were streaked onto violet red bile agar (VRBL) and incubated overnight at 36°C.

2nd procedure using peptone water according to Kandhai *et al.* (2004b):

10 g of each sample were weighed and homogenized in 90 ml of buffered peptone water, incubated for 18 to 20 hours at 37°C. Loopfuls were streaked onto VRBL and incubated for 20 to 24 hours at 37°C.

3rd procedure without enrichment according to Kandhai *et al.* (2004b):

As described in the instruction label of each package, a part of the sample was homogenized with distilled water and directly streaked onto VRBL.

Isolation procedures:

From each VRBL plate, all colonies were purified on Tryptone Soy Agar (TSA). The TSA plates were incubated for 24 to 72 hours 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), IMViC tests, carbohydrates fermentation tests, catalase test and then oxidase test. Oxidase-negative isolates were further identified using API 20E biochemical identification test system (bioMerieux SA, Marcy l'Etoile, France). API 20E systems have been used for presumptive-positive confirmations of *E. sakazakii* via biochemical characteristics (Kandhai *et al.*, 2004b).

RESULTS

Table 1: Incidence of *E. sakazakii* in the examined samples using 3 different procedures.

Examined samples	EE[a]		Peptone[b]		Without[c]	
	No./30	%	No./30	%	No./30	%
Milk powder	0	0	0	0	0	0
Baby foods*	1	3.33	3	10	0	0
Cappuccino	2	6.67	0	0	0	0
Tea creamer	0	0	0	0	0	0

*: Dried milk-based baby foods.
EE[a]: 1st procedure using Enterobacteriaceae Enrichment broth.
Peptone[b]: 2nd procedure using peptone water enrichment.
Without[c]: 3rd procedure without enrichment.

Table 2: Frequency distribution of *E. sakazakii* in relation to Enterobacteriaceae isolates.

Procedure	Total number of isolates	Baby foods*		Cappuccino	
		No.	%	No.	%
EE[a]	20	1	5	2	10
Peptone[b]	24	3	12.5	0	0
Without[c]	3	0	0	0	0

*: Dried milk-based baby foods.
EE[a]: 1st procedure using Enterobacteriaceae Enrichment broth.
Peptone[b]: 2nd procedure using peptone water enrichment.
Without[c]: 3rd procedure without enrichment.

Table 3: Prevalence of other Enterobacter species recovered from the examined samples.

Enterobacter species	Baby foods*				Cappuccino			
	EE[a]		Peptone[b]		EE[a]		Peptone[b]	
	No./30	%	No./30	%	No./30	%	No./30	%
E. aerogenes	-	-	1	3.33	-	-	-	-
E. cloacae	-	-	1	3.33	-	-	-	-
E. agglomerans	4	13.33	4	13.33	-	-	1	3.33
E. intermedius	1	3.33	-	-	-	-	-	-

*: Dried milk-based baby foods.
EE[a]: 1st procedure using Enterobacteriaceae Enrichment broth.
Peptone[b]: 2nd procedure using peptone water enrichment.

Table 4: Frequency distribution of Enterobacteriaceae isolates other than Enterobacter species.

Enterobacteriaceae species	Total isolates		Baby foods*						Cappuccino	
			EE[a]		Peptone[b]		Without[c]		Peptone[b]	
	No./29	%	No./29	%	No./29	%	No./29	%	No./29	%
Cedecea species	2	6.89	1	3.45	1	3.45	-	-	-	-
Escherichia coli	2	6.89	-	-	1	3.45	-	-	1	3.45
Ewingella americana	1	3.45	1	3.45	-	-	-	-	-	-
Hafnia alvei	6	20.69	3	10.34	3	10.34	-	-	-	-
Klebsiella pneumoniae	1	3.45	1	3.45	-	-	-	-	-	-
Klebsiella oxytoca	1	3.45	1	3.45	-	-	-	-	-	-
Klebsiella rhinoscleromatis	1	3.45	1	3.45	-	-	-	-	-	-
Klebsiella terrigena	2	6.89	-	-	2	6.89	-	-	-	-
Pantoea species	6	20.69	3	10.34	2	6.89	-	-	1	3.45
Salmonella paratyphi A	1	3.45	-	-	-	-	1	3.45	-	-
Serratia marcescens	2	6.89	1	3.45	1	3.45	-	-	-	-
Serratia liquefaciens	1	3.45	-	-	-	-	1	3.45	-	-
Serratia plymuthica	1	3.45	-	-	-	-	1	3.45	-	-
Shigella species	1	3.45	-	-	1	3.45	-	-	-	-
Yersinia species	1	3.45	-	-	-	-	-	-	1	3.45
Total	29	100	12	41.38	11	37.93	3	10.34	3	10.34

*: Dried milk-based baby foods.
EE[a]: 1st procedure using Enterobacteriaceae Enrichment broth.
Peptone[b]: 2nd procedure using peptone water enrichment.
Without[c]: 3rd procedure without enrichment.

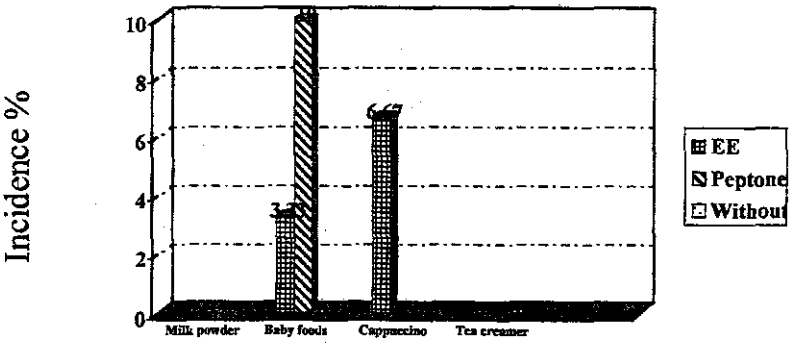

Fig. 1: Incidence of *E. sakazakii* in the examined samples using 3 different procedures.

DISCUSSION

E. sakazakii that designated as a unique species in 1980 (Farmer *et al.*, 1980), has implicated as the causal organism in a rare but severe form of neonatal meningitis (Gallagher and Ball, 1991). A mortality rate of 50 to 75% has been reported by Willis and Robinson (1988). Muytjens and Kollee (1990) investigated the occurrence of *E. sakazakii* more widely, but could not isolate the organism from any environment they examined, which included surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic environments, cattle and untreated cow's milk.

It is obvious from the achieved results in Table 1 and Figure 1 that dried milk-based baby foods samples were the highest in their contamination with *E. sakazakii*. The organism could be isolated in a percentage of 3.33 and 10% by using EE broth and peptone water, respectively. Somewhat similar percentage was obtained by Iversen and Forsythe (2004) who could isolate *E. sakazakii* from 5 out of 49 (10.20%) examined dried infant foods samples, however higher incidence in dried food ingredients samples (22.73%) was recorded.

Moreover, *E. sakazakii* was isolated from 20 out of 141 samples of powdered substitute for human breast milk (Muytjens *et al.*, 1988), and from an average of 6.7% of examined samples collected from 5 different companies of dried infant formula available on the Canadian retail market (Nazarowec-White and Farber, 1997a). Also, Kandhai *et al.* (2004b) stated that 22% of this pathogen could be isolated from 68 environmental samples.

In addition, an incidence of *E. sakazakii* (6.67%) was recovered from the examined cappuccino samples. While, *E. sakazakii* failed to be detected in milk powder and tea creamer samples (Table 1 and Figure 1). On contrast, Postupa and Aldova (1984) isolated 4 strains of *E. sakazakii* from milk powder in Czechoslovakia, while Iversen and Forsythe (2004) could isolate *E. sakazakii* from 3 out of 72 examined milk powder samples (4.17%).

The presence of *E. sakazakii* 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 organism is widespread (Kandhai *et al.*, 2004a). Although, many of dried food ingredients would be heat-treated prior to ingestion, the high prevalence of the organism and the potential for cross contamination means that these ingredients could be a source of *E. sakazakii* in a food manufacturing process (Iversen and Forsythe, 2004). Also, the high tolerance of the organism to desiccation provides a competitive advantage for *E. sakazakii* in dry environments, as found in milk powder factories, and thereby increases the risk of postpasteurization contamination of the finished product (Breeuwer *et al.*, 2003 and Kandhai *et al.*, 2004a). Moreover, the stationary phase of *E. sakazakii* cells are remarkably resistant to osmotic and drying stresses compared with other species of the Enterobacteriaceae (Lehner and Stephan, 2004). Therefore, Nazarowec-White and Farber (1997b) reported that this organism was among the most thermotolerant members of Enterobacteriaceae encountered in dairy products.

From the aforementioned results (Tables 1 and 2), it is evident that the ability of 1st and 2nd procedures using enrichment step to yield *E. sakazakii* is better than 3rd procedure (without enrichment). This could be attributed to the low numbers of *E. sakazakii* in examined samples and their need to be enriched to grow and multiply to enable detection. This result goes parallel with that recorded by Iversen and Forsythe (2004) who could not isolate any of Enterobacteriaceae without using enrichment procedure. Moreover, Kandhai *et al.* (2004b) optimized the enrichment step, as 7% of samples yielded *E. sakazakii* on using a procedure without enrichment while 17% of samples yielded *E. sakazakii* on using peptone water as enrichment broth. In another study, presumptive *E. sakazakii* strains were isolated from 13 out of 27 (48.15%) samples examined without enrichment and from 16 out of the 27 (59.26%) samples examined after the enrichment step.

E. sakazakii percentages were found to be 5 & 10% and 12.5 & 0% out of Enterobacteriaceae isolates recovered from dried milk-based baby foods and cappuccino after this enrichment in EE broth and peptone water, respectively as presented in Table 2.

Concerning other species of Enterobacter (Table 3), it is evident that the incidences of these microorganisms in dried milk-based baby foods samples were 1 (3.33%) for each *E. aerogenes* and *E. cloacae* (using peptone water enrichment) and *E. intermedius* (using EE broth). *E. agglomerans* was the only species which could be isolated by using both of EE broth and peptone water enrichment procedures from 4 samples each of dried milk-based baby foods in a percentage of 13.33%, while its prevalence in cappuccino was 3.33% by using peptone water enrichment. Additionally, it was also noticed that, *E. agglomerans* constituted the highest prevalence (9 isolates) among all Enterobacter species even *E. sakazakii* (6 isolates). It was found that several strains of *E. cloacae* are genetically related to *E. sakazakii* and can produce exotoxins, aerobactin and haemagglutinin (Sanders and Sanders, 1997). Moreover, Enterobacter species can create community infections that are responsible for approximately half of all nosocomially acquired infections and have been recognized as important agents of other hospital-acquired infections (Hervas *et al.*, 2001).

Furthermore, the 47 Enterobacteriaceae isolates, which could be recovered from all of the examined samples, 18 of them were belonging to Enterobacter species (including 6 *E. sakazakii*) and the rest of them (29 isolates) were identified in Table 4, with their frequency distribution according the procedures of isolation. It should be noted that at least 90% of the biochemical reactions of the API 20E are needed for reliable identification (Kandhai *et al.*, 2004b).

Also, as shown in Table 4 different species of Enterobacteriaceae were isolated in low incidences varying from 1 to 2%. The most prevalent isolates of Enterobacteriaceae were *Hafnia alvei* and Pantoea species (each 20.69%). In heat-processed foods such as the samples of the present study (all are sensitive to pasteurization) and in ready-to-eat foods, the presence of species of family Enterobacteriaceae should have public health significance. Furthermore, it follows from the foregoing that contamination of the examined food samples in this study with Enterobacteriaceae could be taken as an index of fecal pollution and could be attributed to the unsanitary practices, poor hygienic quality of ingredients used and/or absence of efficient pasteurization during manufacturing processes. It has been hypothesized that the reservoir for

E. sakazakii, in addition to other coliforms as *Klebsiella oxytoca, K. pneumoniae, E. cloacae* and Citrobacter species, may be primarily environmental and from plant materials (Mossel and Struijk, 1995). 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. At least one strain of *E. sakazakii* originated from dried milk (Iversen and Forsythe, 2003).

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 formula at ambient temperatures. Moreover, trained personnel should prepare dried milk-based products under aseptic techniques and conditions in a designated area following the manufacturer's instructions. The product should be used or refrigerated immediately and discarded if not used within 24 hours (Weir, 2002). ICMSF (2002) has ranked *E. sakazakii* as severe hazard for restricted populations, life threatening or substantial chronic sequelae or illness of long duration. Due to the raised awareness of the organism and its implied role as an emerging foodborne pathogen are of concern to clinicians, the food industry and consumers. As the infection with *E. sakazakii* has been reported in a number of developed countries, it is likely that there is a significant underreporting of infections in all countries (INFOSAN, 2005).

This study stresses the importance of using aseptic methods and proper temperature control in preparation, use and storage of dried milk-based foods to limit contamination and multiplication of such organism and to prevent infections among infants, public health workers and consumers. Understanding of the physiology and survival strategies of *E. sakazakii* is an important step in the efforts to eliminate this bacterium from the critical food production environments (Breeuwer *et al.*, 2003). U.S.FDA (2002b) highlighted the dangers of bacterial contamination of enteral formula products, most of which contain powdered milk as the major ingredient. Consequently, powdered infant formula is not commercially sterile and may harbor *E. sakazakii*. However, studies have not established the minimum number of cells needed to cause clinical symptoms, barring poor preparation, temperature abuse, refrigeration and hygienic practices that have been frequently implicated as contributing factors to infections (Iversen and Forsythe, 2003). The widespread nature of *E. sakazakii* needs to be taken into consideration when designing preventive control measures (Kandhai *et al.*, 2004a).

Arts (2004) made 2 additional recommendations: enhancement of the promotion of and support for breast feeding and inclusion of a warning on infant formula and other breast-milk substitutes that the product might be contaminated with *E. sakazakii* and other microorganisms.

In conclusion, good manufacturing practices and the implementation of Hazard Analysis Critical Control Point (HACCP) program in food manufacturing and food preparation should be done to improve quality and control pathogenic microorganisms contaminating milk powder and dried milk-based foods. In addition, public health authorities and researchers are exploring ways to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *E. sakazakii* and on ways to eliminate and control its growth in milk powder, dried milk-based foods, processing environments and formula preparations areas in hospitals (Gurtler *et al.*, 2005).

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