

## **Opinion of the Scientific Panel on Biological Hazards on the request from the Commission related to the microbiological risks in infant formulae and follow-on formulae.<sup>1</sup>**

**(Question N° EFSA-Q-2003-111)**

**Adopted on 9<sup>th</sup> September 2004**

### **SUMMARY**

*Salmonella* and *Enterobacter sakazakii* are the microorganisms of greatest concern in infant formula. This Opinion concentrates on infant formula and *E. sakazakii*, rather than *Salmonella*, for which much information is available elsewhere (e.g. SCVPH Opinion on Salmonellae in Foodstuffs, adopted 14-15 April 2003).

Contamination of powdered infant formula with *E. sakazakii* and with salmonellae has been the cause of infection in infants, sometimes with serious sequelae or death. Although *E. sakazakii* has caused illness in all age groups of neonates (up to ca 4-6 weeks of age), pre-term or low birth weight infants and those immunocompromised are at greatest risk. There are no experimental or epidemiological studies on the specific dose/response relationships of *E. sakazakii* infections in humans. As is the case for other microorganisms, the dose/response relationship may vary according to the characteristics and physiological state of the organism, the state of the host and the food matrix. The widespread distribution of *E. sakazakii* suggests that consumption of low numbers in infant formula and follow-on formula by healthy infants and children does not lead to illness.

*Salmonella* and *E. sakazakii* do not survive the pasteurization processes used during manufacture but recontamination of the powdered infant formula during handling and filling processes may occur. *E. sakazakii*, due to its ubiquitous character, seems to be more difficult to control in the processing environment than *Salmonella*. Control measures at the manufacturing process for *E. sakazakii* include: the microbiological quality of ingredients; reducing the level of Enterobacteriaceae in the production environment; and avoiding recontamination of the final product. The presence of *E. sakazakii* in the processing environment can be minimized by strict hygiene measures including control of movement of personnel; the separation of wet and dry processes; and avoiding condensation and water ingress in dry areas.

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Environmental microbiological testing in the processing area is necessary to monitor the effectiveness of the hygiene measures. Testing the processing environment for Enterobacteriaceae is the most effective method of monitoring the efficacy of processing and hygiene since Enterobacteriaceae are more often present than *Salmonella* and *E. sakazakii*.

*Salmonella* and *E. sakazakii* can grow in the reconstituted product if stored above 5 °C for a sufficient time and multiply very rapidly at room temperatures. Good Hygienic Practices at reconstitution, storage and feeding are essential to avoid recontamination and/or multiplication of the pathogens in the reconstituted formula. The most effective control measure to minimise risks of *Salmonella* and *E. sakazakii* in high-risk infants (pre-term, underweight, immunocompromised), would be to use commercial sterile liquid formula.

It is recommended that a Performance Objective (PO) for powdered infant formula and follow-on formula, aiming at very low levels of *Salmonella* and *E. sakazakii* (e.g. absence in 1, 10 or 100 kg) is introduced and that verification of compliance with the PO is confirmed by testing for Enterobacteriaceae in the environment and in the product. In addition it is recommended that guidelines for preparation, handling, storage and use of infant formula in the home and in hospitals are developed.

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## BACKGROUND

Today there are on the market many foods specifically manufactured for infants less than 1 year of age and young children aged between 1-3 years. These foods form a diverse category of products covering different compositions and manufacturing processes and are intended to satisfy the specific nutritional requirements of these subgroups of the population.

Commission Directive 91/321/EEC<sup>2</sup> on infant formulae and follow-on formulae, as last amended by Commission Directive 2003/14/EC<sup>3</sup>, lays down detailed requirements for the composition and labelling of these products. This Directive also foresees that microbiological criteria for these products shall be established, as necessary.

Infants and young children are known to be particularly vulnerable to food borne infections. Therefore, the microbiological safety of these products is of utmost importance. For example, a number of sporadic cases and outbreaks caused by *Salmonella* spp., *Enterobacter sakazakii* and other species of Enterobacteriaceae family, have been reported among infants and young children. In many of these cases, the infant formulae and similar products have been either proved or suspected to be the origin of the infection.

The Community legislation on food hygiene is currently under revision. In this framework a revision of the microbiological criteria in Community legislation is taking place. During the discussions on the microbiological criteria, the wish to set criteria for infant foods and other baby foods has been expressed.

## TERMS of REFERENCE

The European Food Safety Authority is asked to:

- Identify the microbiological risks related to infant formulae and follow-on formulae.
- Evaluate the significance of these risks to public health
- Identify the best control options, with regard to effectiveness, to reduce these risks along the food chain. In doing so, special attention should be paid to assessing the possible use of microbiological testing, through guidelines or standards, as well as measures applicable at the time of preparation and storage of these foods until their consumption.

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<sup>2</sup> OJ L 175, 4.7.1991, p. 35.

<sup>3</sup> OJ L 41, 14.2.2003, p. 37.

## ASSESSMENT

### 1. INTRODUCTION

Contamination of powdered infant formula with *E. sakazakii* and with salmonellae has been the cause of infection in infants, sometimes with serious sequelae or death. Although *E. sakazakii* has caused illness in all age groups of neonates (up to ca 4 weeks of age), pre-term or low birth weight infants and those immunocompromised are at greatest risk.

The large scale of production of infant formula and follow-on formulae, both distributed worldwide, and the relatively low number of infections in infants indicates that the products are normally safe.

Revision\ of the Recommended International Code of Hygienic Practice for Foods for Infants and Children (CX/FH 04/11) led the 35<sup>th</sup> meeting of the Codex Committee on Food Hygiene to request that FAO and WHO convene an expert consultation on the genus *Enterobacter*, including *Enterobacter sakazakii*, and *Clostridium botulinum*. In response the FAO and WHO convened a meeting on *E. sakazakii* and other microorganisms in powdered infant formula in Geneva on 2-5 February 2004 and a report from the meeting is available on the internet (FAO/WHO, 2004) <http://www.who.int/foodsafety/micro/meetings/feb2004/en/>.

### 2. HAZARD IDENTIFICATION

#### 2.1. *Salmonella* and *E. sakazakii*

##### *Salmonella*:

Several outbreaks of salmonellosis have been traced to dried milk products. A nationwide outbreak occurred in the United States in 1964 - 1965 (Collins *et al.*, 1968) and was associated with non-fat dry milk produced in one plant and “instantized” in other factories. A total of 156 plants located in 23 states were investigated. Thirty-four samples of non-fat dry milk and 27 environmental samples contained salmonellae, resulting in improvements in processing and sanitation measures.

Non-fat dry milk contaminated with *S. Typhimurium* and *S. Agona* was identified in Oregon (USA) in 1979 (Anon., 1979). Recommended preventive measures from different bodies (IDF, 1994) and implementation of HACCP have improved product safety.

Outbreaks of salmonellosis traced to dried milk products have occurred in different countries (Becker and Terplan, 1986; Rowe *et al.*, 1987; Gelosa, 1994; Usera *et al.*, 1996; Threlfall *et al.*, 1998; Bornemann *et al.*, 2002). Failures in production, for example the presence of water in a zone that is normally dry, allowing multiplication of salmonellae, or the presence of salmonellae in zones

that are difficult to maintain and clean (e.g. a drying tower), were identified as the origins of contamination.

*Enterobacter sakazakii*:

*E. sakazakii* has been implicated in sporadic severe forms of neonatal infections including meningitis and sepsis. Although the origin of the microorganism has not been established, several cases have been associated with the consumption of contaminated powdered infant formulae (Biering *et al.*, 1989; Simmons *et al.*, 1989; van Acker *et al.*, 2001; Himelright *et al.*, 2002).

In hospitals, environmental contamination and temperature abuse of the reconstituted formula have been contributory factors (Bar Oz *et al.* 2001). Outbreaks caused by *E. sakazakii* were reviewed by Nazarowec-White and Farber (1997a), Lai (2001) and Iversen and Forsythe (2003).

## 2.2. Other microorganisms

Illness in pre-term neonates has also been caused by other Enterobacteriaceae e.g. *Serratia marcescens* (Fleisch *et al.*, 2002) and enterotoxigenic *Escherichia coli* (Taneja *et al.*, 2003). Powdered infant formula was confirmed as the vehicle of infection by *Citrobacter freundii* (Thurm and Gericke, 1994; Ritter *et al.*, 1994), although the route of contamination was not identified.

*L. monocytogenes*:

There have been no documented outbreaks of listeriosis linked to dried dairy products.

*Staphylococcus aureus*:

A very large outbreak, not in infants, causing more than 13,000 cases in Japan (Asao *et al.*, 2003) was due to preformed staphylococcal enterotoxin in milk powder. This was traced back to poor hygienic and manufacturing practices during processing of liquid milk in particular the storage conditions. Illness has also been attributed to contamination and abuse of reconstituted non-fat dried milk (El Dairouty, 1989) and infant powdered milk (Umoh *et al.*, 1985).

*Bacillus cereus*:

The presence of *B. cereus* at low levels in dried milk has been reported (Becker *et al.*, 1989, 1994). Over 60% of milk powder samples supplied in the U.S. were positive for *B. cereus*. Although outbreaks of food poisoning due to *B. cereus* have not been directly attributed to dry dairy products, temperature abuse of reconstituted product is a concern.

### *B. licheniformis*

There is one report that *B. licheniformis* in infant formula may have contributed to a fatality (Salkinoja-Salonen *et al.*, 1999). A strain that produced a toxin with properties similar to the emetic toxin of *B. cereus* was isolated from a range of foods including “infant feed (formula)” and “infant feed formula (unused package)”. The link to a fatal food poisoning was not established or confirmed and there is no information whether the infant formula containing *B. licheniformis* also contained *E. sakazakii*.

### *Clostridium botulinum*

In the UK, infant botulism (type B) was diagnosed in a 5-month-old female (Anon., 2001a). Two foods from the household contained *C. botulinum*: dried rice pudding, which contained type A spores, and an infant formula milk powder, which contained type B spores (Anon., 2001b). Both products were already open when sampled for testing. Unopened samples of dried rice pudding from the same batch, and subsequent batches were tested but *C. botulinum* was not detected. Of five unopened samples of the same batch of infant formula, one was positive for *C. botulinum* type B. There was no conclusive link between the isolate from the baby and the isolate from the infant formula.

If honey is used in any infant formula or follow-on formula, caregivers should be aware of the occasional presence of *C. botulinum* and the possibility of infant botulism in infants less than one year of age. The Scientific Committee on Veterinary Measures related to Public Health of the European Commission issued an opinion on honey and microbiological hazards on 19-20 June 2002. In this opinion, the infant botulism is specifically addressed.

*C. botulinum* is not discussed here because there have been no cases of botulism with infant formula or follow-on formula confirmed as the source.

### *Mycotoxins*

Occasionally, dried milk has been found to contain aflatoxin M<sub>1</sub> (Galvano *et al.*, 1996). The amount of aflatoxin present in fluid milk is reduced somewhat by the drying process, but a significant percentage survives the process and will remain for extended periods in the dry product (Marth, 1987). The stability of other mycotoxins in dried dairy products has not been investigated.

## 2.3. Formulation and processing

Powdered infant formulae are manufactured by three different processes:

In the **wet-mix process** all constituents of the formula are handled in a liquid phase, heat-treated, ranging from pasteurization to sterilization, concentrated by evaporation, homogenized and then dried in a drying tower. The powder is then passed through a fluidized bed and cooled with filtered air.



In the **dry-mix process**, individual ingredients are dry-blended to obtain the desired formula, with no subsequent heat-treatment.

In the case of a **combined process**, some of the ingredients of the formula are wet-processed as described above to produce a base powder. The other ingredients are added either before the spray-drying, in the spray dryer itself, or blended into the base-powder in a mixer.

In all processes the powder is then usually stored either in big bags, tote bins or silos before final filling, for example into cans.

In these different processes, the only killing step (Critical Control Point, CCP) is in the wet-mix processes where a heat-treatment ranging between 75°C for 25 sec to ~105-125°C for at least 5 sec is applied. Processing conditions will vary depending on the products and the manufacturers, but are sufficient to destroy 6–10 log<sub>10</sub> of vegetative microorganisms. However, the product is not sterile because only a proportion of the spore-formers will be killed. In general, final products with total viable counts of the order of <100 cfu/g and up to a maximum of 500 cfu/g are obtained and are well within the usual legal requirements.

Ingredients used in the dry-mix process are processed by the suppliers so that they meet the same microbiological specifications as the finished product. Some ingredients have a high likelihood of carrying Enterobacteriaceae (e.g. sucrose, lactose) while others have a low likelihood (e.g. oils).

The main microbiological issues associated with ingredients are the occasional presence of *Salmonella* and Enterobacteriaceae, (including coliforms) and *E. sakazakii*.

Contamination can occur from ingredients not subjected to the heat-treatment during the process - this applies to dry mix and combined processes.

Contamination can also occur from the processing environment during the dry steps of the process, i.e. after the drying - this applies to dry, wet and combined processes.

Follow-on formulae and formulae for special medical processes are manufactured using similar processes.

#### **2.4. Isolation and identification**

Infant foods as dried milk products or milk-based powdered infant formulas are required to be sampled according to the ICMSF (ICMSF, 1986) or Codex Alimentarius methods (Codex, 1979) and prepared and diluted according to the European (EN) and international (ISO) standard EN/ISO 8261 for enterobacteriaceae (including coliforms). Official methods for detection and enumeration of *E. sakazakii* are being developed.

Detection or enumeration of microorganisms in infant foods has been standardized at European (EN standard) or International (ISO standard) levels (Table 1).

*Enterobacter sakazakii* is a motile, non-sporeforming, Gram-negative rod within the family Enterobacteriaceae, genus *Enterobacter*. It was referred to as 'yellow pigmented *Enterobacter cloacae*' until 1980, when it was designated as a new species (Farmer et al., 1980). It grows on media used to isolate enteric organisms such as MacConkey, eosin methylene blue and desoxycholate agars. On agar plates, it may form two colony types (glossy and matt) depending upon media and strain. There is no definitive information whether differences in virulence or any other phenotypic traits occur in the two colony types. Similarly, pathogenicity or associated virulence factors have not been characterised, except an enterotoxin-like compound (Pagotto et al., 2003).

Table 1. Reference methods in Europe for relevant bacteria (Anonymous, 2004b)

Parameters	Method(s) selected <sup>a</sup>	Comments
Enumeration of coliforms at 30°C	ISO 5541-1 <sup>b</sup>	For high levels ( $\geq 100$ cfu/g), total count on Petri dish
	ISO 5541-2 <sup>b</sup>	For low levels ( $\leq 100$ cfu/g), most probable number)
Enumeration of Enterobacteriaceae	ISO 7402 (pr ISO 21528)	
Enumeration of <i>Staph aureus</i>	EN ISO 6881 or 2	Equivalent status
Detection of staphylococcal enterotoxins	CRL screening method	Positive results to be confirmed by the CRL
Detection of <i>Salmonella</i>	EN ISO 6579 or ISO 6785	
Detection and enumeration of <i>L. monocytogenes</i>	EN ISO 11290-1 and 2	
<i>Clostridium botulinum</i>	EN/ISO project	ISO resolution No 242
		CEN resolution No 78
Enumeration of <i>Bacillus cereus</i>	EN ISO 7932	For high levels ( $\geq 100$ cfu/g), total count on Petri dish
Enumeration of <i>Bacillus cereus</i>	ISO 21871	For low levels ( $\leq 100$ cfu/g), most probable number)
Detection of <i>Enterobacter sakazakii</i>	ISO Project	ISO Resolution N°225
	Submitted as EN project	CEN Resolution N°78

(a) ISO: International Standardization Organization; EN: European Standardization Committee; CRL: EU Community reference laboratory for milk and milk products (b.), These standards will be replaced by the corresponding horizontal standards ISO 4831 and ISO 4832, once revised

Isolation and enumeration of *E. sakazakii* from dehydrated powdered infant formula was described in the FDA method (2002) derived from pioneering work of Muytjens *et al.* (1988) and modified by Nazarowec-White and Farber (1997b). This latter method takes 5 days and is based on the most probable number (MPN) approach, using a total of 333 g of product (3x100 g, 3x10 g, 3x1 g) (see Annex I).

Different microbiological methods are used by manufacturers of infant formula. No standardized method for *E. sakazakii* has been recognized at ISO (International Standardization Organisation) or at CEN (European Committee for Standardization) levels, except a EN/ISO standard project applicable first to milk powder and infant formula and, in the future, to food. During the Parma ISO/CEN meeting 2004, it was decided that this standard project will become a technical specification to provide urgently a European and an international reference method for this microorganism.

This standard project is based primarily on the method of Heuvelink *et al.* (2001) on isolation and detection (Detectable/Not Detectable microbiological criteria) in a 25 g sample pre-enriched in 225 ml of enrichment broth (BPW). The EN/ISO project, under validation is based on a 10 g sample directly enriched in 90 ml of lauryl sulphate tryptose broth modified with vancomycin (10 mg/l) and NaCl (0.5 M), and incubated 24 h at 45°C. This enrichment is streaked on trypticase soya agar modified with bile salts and incubated 24 h at 37°C with exposure to light. Yellow pigmented colonies were identified first with chromogenic agar based on  $\alpha$ -glucosidase activity detection (see Annex 1) significantly reducing the work-load, followed with an API20E microgallery (see Annex I). The rapid development of these new chromogenic media should simplify isolation and enumeration and improve our understanding of the distribution of *E. sakazakii* in the environment. Identification of *E. sakazakii* by classical methods presents some difficulties (see Annex I). That approach could soon be supplemented by a molecular method in a commercially available kit for automated real-time polymerase chain reaction using Bax® *E. sakazakii* kit (developed by Qualicon, a subsidiary of Dupont, USA and distributed by Oxoid, UK). Recent sequencing of *E. sakazakii* genome (4.8 Megabases) should permit further development of rapid methods based on molecular biology. Nevertheless, with this rapid/alternative method, positive results should be followed by isolation and molecular characterisation of the strain in order to perform epidemiological studies (see Annex I).

*E. sakazakii* enumeration and detection methods will be soon validated for the analysis of infant food formula, but not for production-line environmental analyses of air or surfaces or biofilm or industrial water control in the food industry (Kandhai *et al.*, 2004a). A new and simple method for screening environmental samples has recently been published (Kandhai *et al.*, 2004b).

There is an urgent need for a method for detection of this microorganism in infant formula (finished product), its raw materials and environmental samples. Absence of such a method could be a drawback for hazard control in the future. Nevertheless, it would seem reasonable to use a detection limit < 1 cell in 25 g of infant food formulas equivalent to *Salmonella* detection in milk powder (Mansfield and Forsythe, 2000). . Infant formula producers consider that a detection limit of 1 CFU/100g should be the target even for sublethally injured cells and also in the presence of large numbers of competitors.

Other species of Enterobacteriaceae family (*Citrobacter diversus*, *Klebsiella oxytoca*, *Serratia marcescens*, etc.) in infant formulas should also be regarded as opportunistic

pathogenic microorganisms. As no specific methods or standard exists, they can be investigated with ISO standard for Enterobacteriaceae (normally applied to food and environmental samples) but with a supplemental step of identification of distinct colonies (Leuschner and Bew, 2004; Leuschner *et al.*, 2004).

New methods have been published but need a thorough evaluation under manufacturing conditions (e.g. Iversen *et al.*, 2004b; Oh and Kang, 2004).

### 3. HAZARD CHARACTERIZATION

This Opinion concentrates on *E. sakazakii* rather than *Salmonella*, for which much information is already available (e.g. SCVPH Opinion on Salmonellae in Foodstuffs, adopted 14-15 April 2003).

Several members of the family Enterobacteriaceae have been cultured from infant formulas. The species most frequently isolated include *Enterobacter agglomerans*, *E. cloacae*, *E. sakazakii*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Escherichia coli*. Several Enterobacteriaceae have been responsible for systemic infections in neonates but only *E. sakazakii* has repeatedly been associated with illness where contaminated infant food has been shown to be the source of infection. One outbreak of *Citrobacter freundii* infections in a neonatal intensive care unit did however identify infant formula as the vehicle of infection. Thus, although other species within the family Enterobacteriaceae may potentially be transmitted through infant formulae and cause infections in neonates, this chapter deals only with the hazard characterisation of *E. sakazakii*.

#### 3.1. Epidemiology and pathogenicity

Sepsis, meningitis and necrotizing enterocolitis are the most common syndromes associated with *E. sakazakii*. Following an infection in the central nervous system, subsequent developmental delay occurs and hydrocephalus is a well-recognised and frequent sequela. Little is known about the specific virulence mechanism of *E. sakazakii*. Pagotto *et al.* (2003) examined clinical and foodborne isolates for enterotoxin production and found four of 18 strains tested positive. All strains of *E. sakazakii* were lethal to suckling mice at  $10^8$  CFU per mouse by intraperitoneal injection, while two strains were lethal by the oral route. Pagotto *et al.* (2003) was the first study describing putative virulence factors of *E. sakazakii*.

While *E. sakazakii* has caused disease in all age groups, by far the majority of cases are seen in infants less than 2 months old. Approximately 50 cases have been reported world-wide in infants less than 60 days old (Iversen and Forsythe, 2003), although under-reporting is suspected. Data on these infants is incomplete but most of them were premature (<37 weeks gestation) and had a low birth weight below 2500 g (Lai, 2001; Anon. 2004a). Although premature infants and those with underlying medical conditions are at highest risk of developing an *E. sakazakii* infection, a healthy, full term, newborn infant in Iceland became ill prior to hospital discharge and suffered permanent neurological sequelae as a result of an *E. sakazakii* infection (Iversen and Forsythe, 2003).

Case fatality rates from *E. sakazakii* infection have been reported to be >50%, but have declined to <20% in recent years. The number of reported *E. sakazakii* cases in adults is low (below 10) and the majority of those adults had underlying diseases such as malignancies (Iversen and Forsythe, 2003; FAO/WHO, 2004).

The source of infection for *E. sakazakii* infections in many cases remains unknown, but an increasing number of reports have implicated dried-infant formula in both outbreaks and sporadic cases. In outbreaks among neonates in neonatal intensive care units, the strain of *E. sakazakii* recovered from ill neonates has been indistinguishable from strains recovered from unopened cans of infant formula used to feed the neonates. Thus van Acker *et al.* (2001) described 12 cases of necrotizing enterocolitis that occurred in neonates in 1998. *E. sakazakii* was isolated from 6 of the 12 patients. A review of feeding procedures revealed that 10 of the 12 patients were fed orally with the same brand of powdered formula for special medical purposes. *E. sakazakii* could be isolated from several unopened cans of a single batch of the implicated formula powder. Molecular typing confirmed strain similarity between all milk powder isolates and three patient isolates. When use of this formula was discontinued, the outbreak came to an end. Also, an outbreak in Tennessee in 2001 showed statistically significant association between *E. sakazakii* colonisation/infection and powdered milk formula ingestion (Himelright *et al.*, 2002). In that study, nine of nine infants infected/colonised with *E. sakazakii* had been fed a specific formula product compared to 21 of 40 infants who were not infected.

In addition to the above examples dealing with contaminated powdered infant formula, contamination at reconstitution has also been associated with disease in infants. In two cases contaminated blenders used to prepare formula from the dry powder were implicated in causing disease in infants (Noriega *et al.*, 1990; Bar-Oz *et al.*, 2001; Block *et al.*, 2002).

It should be emphasised that no incidents similar to those above have been associated with follow-on formulae.

### **3.2. Dose/response relationships**

There are no experimental or epidemiological studies on the specific dose/response relationships of *E. sakazakii* infections in humans. As is the case for other microorganisms, the dose/response relationship may vary according to the characteristics and physiological state of the organism, the state of the host and the food matrix. Because powdered infant formula is a source of nutrition for many infants at risk, a very large number of servings are consumed. Thus there is a small probability that even one or a few organisms in rehydrated powdered infant formula could cause illness. However, since there is no information on the actual number of organisms that ill patients were exposed to, it has not been possible to develop a dose-response curve for this pathogen. R values (number of cases/number of exposures to one organism) between  $8.9 \times 10^{-6}$  and  $2.5 \times 10^{-6}$  have

been theoretically estimated and the best guess is that the dose response relation at low doses is linear (FAO/WHO, 2004),

Relatively low levels of *E. sakazakii* were present in samples of powdered formula implicated as the cause of the outbreak described by van Acker *et al.* (2001). The manufacturer's microbiological control data for the batch of formula implicated in that outbreak in 1998 showed that of five samples analysed, one yielded 20 coliforms/g whereas in other four samples less than 1 coliform/g was found.

These results fulfilled the requirements of the Codex Alimentarius (a minimum of four of five samples with <3 coliforms/g and a maximum of one of five samples with >3 but <20 coliforms/g).

It is therefore apparent that application of these microbiological criteria cannot guarantee the safety of dried infant formula. Conditions and hygiene during reconstitution, and the temperature and time the reconstituted feed is held before consumption are both extremely important.

#### 4. EXPOSURE ASSESSMENT

##### 4.1. Inactivation and growth of pathogens during processing

*Enterobacter sakazakii* is not particularly thermotolerant. Nazarowec-White and Farber (1997c) calculated a  $D_{72^{\circ}\text{C}}$  of 1.3 seconds when heated in infant formula. According to Breeuwer *et al.* (2003), the  $D_{58^{\circ}\text{C}}$  for *E. sakazakii* ranged from 0.39 to 0.60 min, which is comparable with that of other Enterobacteriaceae, but much lower than reported previously by Nazarowec-White and Farber (1997c). Edelson-Mammel and Buchanan (2004) reported that the thermal resistance of *E. sakazakii* was such that exposure to temperatures at or above 70 °C should provide virtually instantaneous inactivation of the microorganisms. [BN&TR have deleted some sentences on z-values, as suggested at Plenary] Although the thermal resistance of *E. sakazakii* is greater than some enteric pathogens, it is less resistant than *L. monocytogenes*. Considering the thermal resistance of *E. sakazakii* (Nazarowec-White and Farber 1997c, Breeuwer *et al.*, 2003; Edelson-Mammel and Buchanan, 2004) minimum HTST pasteurization schedules given to the liquid mix prior to the drying step (15 s at 71.7 °C or 74.4 °C for 25 s) would achieve more than 11D reductions of *E. sakazakii* in infant formula, and therefore the organism would not survive the pasteurization process. Standard pasteurization practices have been reported to be effective for the destruction of *E. sakazakii* in bovine milk (Nazarowec-White *et al.*, 1999), 68°C for 16 sec giving a 5D reduction.

However, post-processing contamination of the dried product is still a problem. Although the microorganism cannot grow in such a dry substrate, it can survive for a long period of time and is a potential risk after the powder is rehydrated.

According to Breeuwer *et al.* (2003), stationary phase *E. sakazakii* is relatively resistant to osmotic and dry stress compared with other members of the family

Enterobacteriaceae. The survival of *E. sakazakii* at elevated temperatures (45°C) and its capacity for growth up to 47 °C, illustrate that in warm and dry environments, such as in the vicinity of drying equipment this bacterium has a competitive advantage compared with other members of the Enterobacteriaceae.

Environmental samples from eight of nine food factories, and from five of 16 households, contained *E. sakazakii*. The presence of *E. sakazakii* in factories producing milk powder, cereals, chocolate, potato flour, and pasta, as well as in domestic environment, indicates that it is widespread (Kandhai *et al.*, 2004a).

#### **4.2. Recontamination, preparation of infant formula and possibility of growth after reconstitution**

Airborne *E. sakazakii* can resist the stressing environment in the processing plant, survive adhered to equipment and can re-contaminate the powder during the handling and filling processes. Consequently, extreme care is taken by companies to prevent recontamination of the final formula. Liquid product and dry product areas in the factory are physically separated from one another. The drying and filling zone is managed to be dry, but condensation can lead to an increase in the normally very low numbers of *E. sakazakii* in that environment, putting the product at risk of contamination. Other sources of re-contamination are the ingredients added to the formulation. This could occur in the dry-blending process. To avoid such contamination companies have microbiological standards that must be met by suppliers of those ingredients. They require that dry ingredients meet the same microbiological standards as the final product because they received no final heat treatment. If recontamination of the powdered infant formula occurs, the microorganism can survive in the powder for up to 24 months (Iversen and Forsythe, 2003).

Recontamination can also take place during the preparation or reconstitution of the infant formula due to poorly cleaned baby bottles and poorly maintained equipment at home and hospitals.

At home the powder is mixed with warm or hot water in a clean/ “sterile” baby bottle. Manufacturers recommend boiling the water and then cooling it to 50 ° C before being mixed with the appropriate amount of powdered product. The use of water at 70 °C or more can produce 4-D, or more, reductions in the initial concentration of *E. sakazakii* (Edelson-Mammel and Buchanan, 2004), but may have nutritional consequences.

However, when a large amount of powder is rehydrated with hot water to produce large quantities of rehydrated formula, as in neo-natal units in hospitals, the temperature of the mixture can drop and, taking account the thermal resistance of *E. sakazakii*, the final temperature might not be sufficient to inactivate the microorganism should the powder be contaminated. Some pediatricians do not favour using hot water because of the risk of accidental scalding of babies. Although manufacturers recommend that formula be prepared before each feeding, sometimes several baby bottles are prepared in neo-natal



units and stored under refrigeration to satisfy the intake needs of the baby or babies during the whole day.

In hospitals, practices vary according to local arrangements, availability of trained personnel, facilities and number of babies to be attended. For example, there are hospitals where the centralized baby bottle preparation unit has two different areas, a dirty area and a clean area. In the dirty area, there are washing machines for bottles and the feeding rubbers (teats). After that, both pieces are sterilized, the bottles at 130°C and the teats at 120 °C. In some hospitals the heating processes are confirmed by inactivation of bacterial spores in both bottles and teats. The baby bottles are prepared in the clean area. The water used to prepare baby bottles is heated in an electric water heater and then passed through an ultraviolet unit. This water is sterile and it is tested for sterility by plate count. The powder is mixed with the water in a large container and, depending on the baby's nutritional needs, other components may be added. Those ingredients may present a risk of contamination of the product. Such added ingredients need to comply with the same requirements as the powdered infant formula. As is the case with home preparation, the temperature of the mixture could be insufficient to inactivate the microorganism. The reconstituted powder is manually dosed in the baby bottles and while some of them are used immediately for pre-term and newborn babies, others are stored at 4°C in the baby bottle preparation room until nurses from the baby care unit take and store them in the ward under refrigeration at 4 °C until used. Before feeding the baby, the bottle and contents are warmed. The contents of any remaining baby-bottles are discarded, so that the total storage time does not exceed 24 h. The central unit also prepares the infant formula for babies fed by tube, using special containers with a tube that can be connected to a baby. They arrive at the hospital, factory-sterilized and are not re-used. The entire preparation process is conducted by personnel wearing protective clothing, head covers and masks to avoid contaminating the liquid. Nevertheless, the clean room is not a “white” room or sterile room with special features. The quality of the room depends on the age of the hospital, etc. For example, in some cases the baby bottles are filled inside of a sterile air flow cabinet, while in others they are not, with a higher risk of recontamination.

Comprehensive guidelines for the preparation of formula in health care facilities from the Pediatric Nutrition Practice Group of the American Dietetic Association, covering the desirable physical facilities, equipment and utensils, personnel, formula preparation and handling, delivery and bed-side management of feeding, microbiology and infection control can be found at [http://www.eatright.org/Public/NutritionInformation/92\\_17242.cfm](http://www.eatright.org/Public/NutritionInformation/92_17242.cfm). Other groups of health professionals are responding similarly (e.g. Agostoni *et al.*, 2004; IFT, 2004)

*E. sakazakii* has a broad temperature range for growth, with a minimum between 5.5 to 8 and a maximum of 47 °C. Generation times for *E. sakazakii* at 10 °C can range from 4.18 to 5.52 h and at 22 °C from 37 to 44 min (Nazarowec-White and

Farber, 1997c). Iversen *et al.* (2004a) used clinical and food strains to establish that generation times for *E. sakazakii* in rehydrated infant formula were 13.7 h, 1.7 h and 21 min at 6, 21 and 37 °C respectively. Therefore, it is evident that storage of contaminated rehydrated powdered infant formula at too high temperatures could lead to rapid growth of *E. sakazakii*.

Temperature is the main factor responsible for the multiplication of the microorganism in the reconstituted formula. Rhodehamel (1992) found that temperature in many home refrigerators ranged from 7 to 10 °C. Harris (1989), reported that 20% of the home refrigerators surveyed were between 5 and 10 °C, while van Garde and Woodburn (1987) found that refrigerator temperatures in 21% of households surveyed were above 10 °C. Daniels (1991) reported that more than 25% of home refrigerators were above 7.2 °C and almost 10% above 10 °C. A study by Audits International (2000) on temperatures of food products at different stages of storage, including the home, showed that 18% of samples had a temperature between 5.5 and 6.6 °C and the 3% had a temperature between 8.8 and 10 °C. Those temperatures would allow growth of *E. sakazakii* if present in the reconstituted infant formula. The temperatures in domestic refrigerators from eight surveys were overviewed by James (2003). The average temperature in consumer refrigerators was calculated to be around 6-7 °C. However, from the minimum and maximum temperatures there was a large variation between individual refrigerators. Around 30 % of the refrigerators were at average temperatures above 7 °C.

No temperature surveys were found for refrigerators in hospitals.

In both the home and the hospital, preparation and storage of reconstituted infant formula is of the greatest concern with respect to avoiding recontamination and the possible growth of *E. sakazakii*. In hospitals, centralized preparation of ready-to-feed formula or on-ward preparation may occur. In both situations sterile water and aseptic conditions for reconstitution are essential. The transportation of ready-to-feed preparations to the wards under continuous refrigeration and refrigerated storage on the ward up to the time of feeding are also important factors to control

In the case of pre-term or newborn infants without coordinated sucking/swallowing, feeding by naso- or oro-gastric tube is practised. In such instances, flushing the tube after each feeding with sterile solutions may help to reduce the build-up of microbial contamination and the formation of biofilms within the feeding delivery system. Limiting the “hang time” to not more than 4 h is an important control measure.

Clinicians should be aware of the potential risk of infection from use of non-sterile enteral formula in the neonatal health-care setting.

#### 4.3. Contamination rate

Attempts have been made to determine the occurrence of *E. sakazakii* in production batches (lots) of powdered infant formula. According to Nazarowec and Farber (1977b) strains of the microorganism were isolated from dried infant formula available on the Canadian retail market and 0 to 12 % of samples from five different companies were contaminated. Muytjens *et al.* (1988) evaluated the quality of powdered substitutes for breast milk with respect to Enterobacteriaceae, isolating them from 52.2 % of the 141 different samples from 35 countries. *E. sakazakii* was one of the most frequent species isolated (14 %) (20 of 141 samples).

Heuvelink *et al.* (2001), using a presence / absence test for 25 g quantities, detected *E. sakazakii* in 1/40 infant formula powders and 7 of 170 milk powders.

However, the concentration is low, e.g. Nazarowec and Farber (1997b) indicated that Canadian samples contained less than 1 cfu/g. Muytjens *et al.* (1988) reported that the concentration ranged from 0.36 to 66 cfu/100g.

It is generally agreed that the concentration of *E. sakazakii* in powdered milk products needs to be re-determined using validated methods.

### 5. CONTROL MEASURES

#### 5.1. Control measures at the manufacturing level

The application of Good Manufacturing Practices, Good Hygienic Practices and HACCP, are designed to address the various possibilities of contamination at the manufacturing and processing levels. Wet processing and dry processing are located in different areas of the factory and are separated physically from each other.

On the wet part of the process, basic hygiene measures are applied such as for example the hygienic design of equipment, in particular for the sections after the heat-treatment, appropriate cleaning and sanitation procedures, etc

In terms of raw materials, suppliers are selected to minimise, as far as is possible, the occurrence of pathogenic organism and to avoid their ingress into the factory. In certain raw materials such as raw milk, however, the presence of Enterobacteriaceae including *Salmonella* and *E. sakazakii* is possible and the most effective control measure is appropriate heat-treatment. In the case of ingredients, which may be added dry after the heat-treatment, it is essential to ensure that those ingredients meet the same microbiological requirements as the finished infant formulae. This is achieved through the careful selection of suppliers, in particular those of critical ingredients such as lactose, including audits to assess their processes, control and monitoring procedures.

It is possible to control the introduction of *Salmonella* into the dry area of the processing line, considered as “high hygiene area”. This is performed through the application of strict hygienic zoning throughout the factory, i.e. from basic

hygiene zones through medium hygiene zones and finally to the high hygiene zone with the processing lines. This is achieved by ensuring the physical integrity of buildings and constructions, the hygienic design of internal premises and equipment, appropriate design of air intake and exhaust systems, hygienic design of drains (usually dry drains), etc.

Another critical element is the controlled access and flow of personnel, of ingredients added dry, of packaging material and of equipment. This is done from medium hygiene zones into the high hygiene zone through specifically designed access locks and following precise procedures such as shoe changes, use of protective clothing, the use of “strippable” bags, change of pallets, etc.

Due to their ubiquitous presence in this processing environment, Enterobacteriaceae are much more difficult to control than *Salmonella*. Enterobacteriaceae are found frequently, even in dry environments, but usually at low levels, from a few cells/g up to 100 cfu/g. Different species can be found, including *E. sakazakii*, and this seems to be related to the temperature and/or osmotic conditions prevailing in the high hygiene areas which has an impact on the microbial ecology of these processing zones.

To reduce and minimise the incidence of *E. sakazakii* in the processing environments, and thus in finished products, it is necessary to address and control Enterobacteriaceae. The same measures as described above for *Salmonella* apply to prevent or minimise the ingress of Enterobacteriaceae into the high hygiene zone. The application of the measures, however, needs to be tightened to take into account their much more frequent occurrence. A major difference from the *Salmonella* situation is the fact that Enterobacteriaceae, including *E. sakazakii*, are already present and established in high hygiene zones. Although levels of Enterobacteriaceae can be maintained at very low levels (a few cells per g) they are found ubiquitously throughout the areas (Kandhai *et al.*, 2004b). The mere presence of Enterobacteriaceae in the processing environment, even at such low levels, indicates the possibility of sporadic low levels of contamination in the finished product.

A further essential element of the preventive measures is the maintenance of dry conditions throughout the high hygiene area. While the presence of water does not necessarily have an immediate impact on the presence of salmonellae, it does have an immediate effect on the increase in numbers of Enterobacteriaceae. Dry conditions should therefore be ensured by elimination of any source of water (e.g. from accidental ingress, condensation in transport tubes, cleaning water etc.) and the application of dry cleaning procedures for the processing lines, the equipment, and the processing environment. This is achieved by appropriate training of personnel and using modified cleaning tools such as vacuum cleaners, brushes or scrapers. Wet cleaning must be avoided as far as possible. If absolutely necessary, then specific procedures need to be applied, e.g. limiting the wet cleaning to parts of equipment that can be taken out to dedicated rooms,

by applying specific protocols followed by immediate drying of the cleaned elements and areas.

The effectiveness of these control measures should be verified through the application of microbiological monitoring plans. These integrated plans include sampling of raw materials (for dry mixing), line samples in equipment (residues, food contact surfaces), and environmental samples (external surfaces of equipment and surroundings of the line). Enterobacteriaceae are particularly useful as indicators in environmental samples and increases in the levels are indicative of an increased risk of presence or even multiplication of *Salmonella* and *E. sakazakii*. These monitoring plans are dynamic and are adapted according to the results obtained.

All these additional measures contribute to minimise and reduce the possibilities and risk of recontamination. If applied correctly they will ensure minimal contamination of the products. Because the formulae are not sterile, control measures during preparation and reconstitution are also necessary.

New hygiene Directives to be introduced in the European Union on 01 January 2006 will make it mandatory for Codes of Hygienic Practice to be produced, and processing conditions and hygiene measures to be described:

1. Regulation on the hygiene of foodstuffs (Regulation (EC) No. 852/2004) [http://europa.eu.int/eurlex/pri/en/oj/dat/2004/l\\_139/l\\_13920040430en00010054.pdf](http://europa.eu.int/eurlex/pri/en/oj/dat/2004/l_139/l_13920040430en00010054.pdf)
2. Regulation laying down specific hygiene rules for food of animal origin (Regulation (EC) No. 853/2004) [http://europa.eu.int/eurlex/pri/en/oj/dat/2004/1139/l\\_13920040430en00550205.pdf](http://europa.eu.int/eurlex/pri/en/oj/dat/2004/1139/l_13920040430en00550205.pdf)
3. Regulation laying down detailed rules for the organisation of official controls on products of animal origin intended for human consumption (Regulation (EC) No.854/2004). [http://europa.eu.int/eurlex/pri/en/oj/dat/2004/l\\_139/l\\_13920040430en02060320.pdf](http://europa.eu.int/eurlex/pri/en/oj/dat/2004/l_139/l_13920040430en02060320.pdf)
4. Directive laying down the animal health rules governing the production, processing, distribution and introduction of products of animal origin for human consumption (Council Directive 2002/99/EC). [http://europa.eu.int/eurlex/pri/en/oj/dat/2003/l\\_018/l\\_01820030123en00110020.pdf](http://europa.eu.int/eurlex/pri/en/oj/dat/2003/l_018/l_01820030123en00110020.pdf)
5. Directive that repeals the 17 existing Directives on hygiene (Council Directive 2004/41/EC). [http://europa.eu.int/eurlex/pri/en/oj/dat/2004/l\\_157/l\\_15720040430en00330044.pdf](http://europa.eu.int/eurlex/pri/en/oj/dat/2004/l_157/l_15720040430en00330044.pdf)

## 5.2. Control measures during preparation and reconstitution

Some infant formula products are aseptically filled or retorted ready-to-feed formula, available in a range of containers (bottles, cans, pouches, Tetra packs, cups). Whenever possible, and especially in the case of infants at highest risk (pre-term, underweight, immunocompromised), caregivers should be encouraged to use commercially sterile liquid formulae. If that is not possible, formula should be reconstituted with water  $>70^{\circ}\text{C}$ , or heated after reconstitution, noting that there may be negative nutritional consequences. Caregivers should be repeatedly alerted that powdered infant formula is not a sterile product, and that infectious organisms (e.g. salmonellae, *E. sakazakii*) can survive in these products over prolonged periods of storage and multiply in the reconstituted feed if held at temperatures permitting their multiplication. Concern is sometimes expressed that using boiling water, or water close to boiling, may activate bacterial spores e.g. *B. cereus*, that are inevitably present in powdered infant formula. Those spores might then germinate and multiply, and pose a risk to the baby. However, there is no record of this happening.

Guidelines are becoming available from a number of organisations for the preparation, handling, use and disposal of infant formula to minimise risk to the infants.

In hospitals, staff should be made aware of the numerous possibilities for recontamination of the product at filling into baby bottles or into specialised containers, and at reconstitution, and familiarised with storage conditions that would allow multiplication of the low numbers of the pathogens present.

Specific guidelines and good practices should be developed for hospitals and training seminars organized for the staff involved. It should be possible to use available estimates of rate of growth at different temperatures to calculate times the reconstitute product can safely be stored at different temperatures.

“Sterile” bottles (achieved by heating or chemical methods) should always be used.

In the home, formula may also be reconstituted with water that has been boiled and cooled.

Already some manufacturers recommend “Prepare the infant formula, bring to the right temperature as quickly as possible, feed the baby and discard any remaining feed”.

If reconstituted feed is stored, keep refrigerated (below  $4\text{-}5^{\circ}\text{C}$ ) and warm immediately before feeding.

If reconstituted formula is maintained at room temperature for continuous feeding by a tube, a “hang time” of 4 h should not be exceeded

The relatively short generation time and ability to grow at temperatures often found in domestic refrigerators (7-15°C) makes the presence of *E. sakazakii* in infant formula particularly hazardous. Kindle *et al.* (1996) reported that *E. sakazakii* and *Klebsiella pneumoniae* had higher growth rates in reconstituted infant formula than other organisms tested (*Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *Mycobacterium terrae* and *Candida albicans*).

### 5.3. Other possible control measures

Any process that would ensure elimination of *E. sakazakii* without affecting the nutritional status of the infant formula should be considered as a terminal treatment where powdered formula is fed to pre-term, underweight or immunocompromised infants.

### 5.4. Possible use of Food Safety Objectives and Performance Objectives

#### 5.4.1. Application of Food Safety Objectives (FSO's)

There is a low probability that one or a few organisms of *Salmonella* or *E. sakazakii* in a serving could cause illness. However, this risk increases rapidly if the level of *E. sakazakii* and/or *Salmonella* is allowed to increase. An FSO to be suggested for these microorganisms in reconstituted products is that they should not be present in servings for the "high risk" groups. If this FSO is reached then the risk of illness caused by these microorganisms in infant formula will be negligible.

This aim could be achieved either by using ready-to-feed commercially sterile formula or by using formula that has undergone an effective point-of-use decontamination procedure. A third possibility is to use powdered infant formula where *E. sakazakii* and *Salmonella* are not present and at the same time avoid recontamination during the reconstitution.

#### 5.4.2. Performance Objectives (PO's) for *E. sakazakii* and *Salmonella* in powdered infant formula

The main source of contamination of powdered infant formula by *E. sakazakii* and *Salmonella* appears to be the manufacturing environment. Reducing the numbers of Enterobacteriaceae in the production environment will also reduce the occurrence of Enterobacteriaceae including *E. sakazakii* and *Salmonella* in the finished product. It is suggested that a Performance Objective for both microorganisms in powdered infant formula is established. A performance objective could be, for example, absence of *E. sakazakii* and/or *Salmonella* in 1, 10, or 100 kg of infant formulae and/or follow-on formulae. No matter which of those PO's is selected, the assurance of compliance by microbiological testing will be impossible and ineffective.

Key aspects to ensure compliance with the PO would include an effective separation of wet and dry processing operations and an effective management programme of plant hygiene including an environmental monitoring programme

within a HACCP plan. Monitoring and testing of the concentration and prevalence of Enterobacteriaceae in both the environment and the finished product should be included.

#### 5.4.3. *Application of Microbiological Criteria*

In some situations, in order to ensure that a Performance Objective is reached, microbiological testing might be an option. In the case of *E. sakazakii* and *Salmonella* in infant formula the introduction of a microbiological criterion for these specific pathogen organisms is not recommended. Enterobacteriaceae, which are more often present than *E. sakazakii* and *Salmonella*, could be used as an indicator for risk and a criterion established for the presence of Enterobacteriaceae in powdered infant formula. It is suggested that the specific criteria be rather low (i.e. absence in 10 g) to comply with the Performance Objective for *E. sakazakii* and *Salmonella*.

### 5.5. Development of guidelines

It is recommended that guidelines be developed for reconstitution, storing and feeding.

#### 5.5.1. *Guidelines for reconstitution, handling, storage and use in the home:*

- Good hygienic measures are essential to avoid contamination.
- Prepare powdered infant formulae fresh for each meal
- Use “sanitized” containers to reconstitute the formula (in the home “sanitized” means a clean container, “sterilized” by immersion in hot water or chemically)
- Always reconstitute formulae in hot water (>70°C) or water that has been boiled and cooled, avoiding recontamination.
- Cool the reconstituted formula rapidly to use temperature.
- Use the reconstituted formula immediately.
- After feeding discard any remaining formula.

#### 5.5.2. *Guidelines for reconstitution, handling, storage and use at the hospital:*

- Caregivers should be trained to deal with dried formula in centralized units for reconstitution and in the neonatal health-care units.
- Good hygienic measures are essential to avoid contamination.
- Use sterile containers to reconstitute the formula under an air sterile cabinet avoiding the possibility of recontamination by the environment.



- Always reconstitute formulae in hot water (>70°C) avoiding recontamination.
- If continued feeding is necessary the maximum hang time should be not more than 4 hours.
- Cool the reconstituted formula rapidly to temperatures below the growth range of *E. sakazakii* (below 4-5°C).

## 6. CONCLUSIONS

- *Salmonella* and *E. sakazakii* are the pathogenic microorganisms of greatest concern in infant formulae and formulae for special medical purposes.
- *Salmonella* and *E. sakazakii* are also the microorganisms of greatest concern in follow-on formulae, although there is no history of their causing illness.
- The widespread distribution of *E. sakazakii* suggests that consumption of low numbers in infant formula and follow-on formula by healthy infants and children does not lead to illness
- The presence of *Salmonella* and *E. sakazakii* in infant formulae, follow-on formulae and formulae for special medical purposes constitute a considerable risk if conditions after reconstitution permit multiplication.
- *Salmonella* and *E. sakazakii* can grow in the reconstituted product if stored above 5 °C for a sufficient time and multiply very rapidly at room temperatures.
- Good Hygienic Practices at reconstitution, storage and feeding are essential to avoid recontamination and/or multiplication of the pathogens in the reconstituted formula.
- *E. sakazakii* has caused diseases in all ages group but by far the majority of cases are seen in infants less than 4-6 weeks of age, especially pre-term babies, underweight, immunocompromised or from immunocompromised mothers.
- *Salmonella* and *E. sakazakii* do not survive the pasteurization processes used during manufacture.
- *E. sakazakii*, due to its ubiquitous character, seems to be more difficult to control in the processing environment than *Salmonella*.

- *E. sakazakii* can resist the stressful environment in the processing plant and can re-contaminate the powdered infant formula during handling and filling processes.
- Enterobacteriaceae can be used as an indicator of the presence of *E. sakazakii*.
- Control measures at the manufacturing process for *E. sakazakii* include: the microbiological quality of ingredients; reducing the level of Enterobacteriaceae in the production environment; and avoiding recontamination in the final product.
- The presence of *E. sakazakii* in the processing environment can be minimized by strict hygiene measures including control of movement of personnel; the separation of wet and dry processes; and avoiding condensation and water ingress in dry areas.
- Environmental microbiological testing in the processing area is necessary to monitor the effectiveness of the hygiene measures
- Testing the processing environment for Enterobacteriaceae is the most effective method of monitoring the efficacy of processing and hygiene since Enterobacteriaceae are more often present than *Salmonella* and *E. sakazakii*.
- Verification of the effectiveness of the above control measures should be by application of monitoring plans for Enterobacteriaceae.
- New methods of detection have been developed promising to improve the detection of *E. sakazakii*.
- The prevalence and concentration of the organism in powdered milk products and in the processing environment needs to be re-determined using validated detection methods.
- If honey is used in any infant formula or follow-on formula, caregivers should be aware of the occasional presence of *C. botulinum* and the possibility of infant botulism in infants less than one year of age. The Scientific Committee on Veterinary Measures related to Public Health of the European Commission issued an opinion on honey and microbiological hazards on 19-20 June 2002. In this opinion, the infant botulism is specifically addressed.

## 7. RECOMMENDATIONS

- The most effective control measure to minimise risks of *Salmonella* and *E. sakazakii* in high-risk infants (pre-term, underweight,

immunocompromised), would be to use commercial sterile liquid formula.

- A Performance Objective (PO) should be introduced for powdered infant formula and follow-on formula, aiming at very low levels of *Salmonella* and *E. sakazakii* (e.g. absence in 1, 10 or 100 kg).
- Compliance with the PO should be achieved through Good Manufacturing Practices (GMP), Good Hygienic Practices (GHP), hygienic measures and processing conditions.
- Verification of compliance with the PO should be confirmed by testing for Enterobacteriaceae in the environment and in the product.
- Guidelines should be developed for preparation, handling, storage and use of infant formula in the home and in hospitals.
- Storage temperatures and times of reconstituted formula in hospitals should be recorded, preferably continuously.

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## ANNEX

### Identification of *Enterobacter sakazakii*

In the FDA (2002) method, the first step is a pre-enrichment (overnight, 36°C) where initial suspension (1/10) was made in sterile distilled water. An enrichment (overnight, 36°C) of this subculture in Enterobacteriaceae Enrichment broth was performed and followed by isolation using Violet Red Bile Glucose (VRBG) selective media incubated overnight at 36°C which is only selective for Enterobacteriaceae. Inoculation of VRBG plates can be by direct spreading method (0.1 ml) or direct streaking method with an inoculating loop (10 µl). Five presumptive *E. sakazakii* colonies on VRBG are subcultured on Trypticase soya agar (TSA) at 25°C for 48-72 h for yellow pigment production, followed by confirmation using the API 20E (bioMérieux, Marcy l'Etoile, France) biochemical identification system and the oxidase test. In contrast, Muytjens *et al.* (1988) and Nazarowec-White and Farber (1997b) resuspend milk-based powdered infant formulas in buffered peptone water for the pre-enrichment step which was advantageous for laboratories of food industries in order to perform with the same pre-enrichment the detection of *Salmonella*, and direct pour plate 1 ml of the enrichment EE broth in VRBG medium for enumeration.

Identification of *Enterobacter sakazakii* presents some difficulties. The main phenotypic characters, such as D-Sorbitol negative, delayed positive DNase test on toluidine blue agar (36°C, 7 days), Tween™ 80 esterase and production of non diffusible yellow pigment at 25°C (Farmer *et al.*, 1980; Aldova *et al.*, 1983) are not reliable (Heuvelink *et al.*, 2001). Stable and species-specific characters seem to be α-glucosidase activity (Muytjens *et al.*, 1984) and phosphoamidase. α-glucosidase activity detected using 4-nitrophenyl-α-D-glucopyranoside (36°C, 4h) or 5-bromo-4-chloro-3-indolyl-α, D—glucopyranoside, which have been used to develop chromogenic isolation media. *Enterobacter sakazakii* can be reliably identified by the API 20E identification system. However, it is recommended to perform D-sorbitol in a classical tube test in parallel (incubation: 48-72 h, 25°C), because this test is problematic in the API 20E system for accurate identification. Other equivalent identification systems are Biolog GN2 (AES laboratories, Combourg, France) or Microbial ID systems or Microbact™ 24E (Oxoid, Basingstoke, USA).

Recently, two commercially available chromogenic isolation media for *E. sakazakii* have been developed. The ESIA media from AES laboratories (Combourg, France) requires incubation for 18-24 h at 44°C after inoculation with a culture (24 h, 37°C) of the samples in a specific enrichment broth (ESSB) from AES laboratories. The typical colonies are blue. The second medium was developed by Iverson et al., (2004b) for Oxoid (Basingstoke, USA) and named “Chromogenic *Enterobacter sakazakii* Agar (DFI formulation)”. This medium can be inoculated with a specific enrichment broth (incubated 20-22 h at 45°C) comprising Lauryl Sulphate Tryptose broth supplemented with sodium chloride and vancomycin (3 mg/L) and incubated 24 h at 37°C. Typical colonies are blue-green against the yellow background of the medium. This medium reduces the time taken to identify *E. sakazakii* by at least two days compared to the FDA recommended method. These two commercial media should be used for enumeration and differentiation of *E. sakazakii*. They should be second media to VRBG, under the choice of users, in the standardized ISO method and other detection/enumeration ISO standards for pathogens. A new medium was recently developed by Leuschner *et al.* (2004) , based on  $\alpha$ -glucosidase activity, but it is not commercially available.

*Enterobacter sakazakii* should be typed by biotyping according to the scheme of Farmer *et al.* (1980) or by molecular typing as plasmid analysis, ribotyping with *Hind*III or the automatic system of ribotyping Riboprinter® (Dupont Qualicon, USA), chromosomal restriction endonuclease analysis, multilocus enzyme electrophoresis, tDNA-PCR, Random amplification polymorphism (RAPD), pulsed field gel electrophoresis with *Xba*I and *Spe*I enzymes (Goulet and Picard, 1986; Clark *et al.*, 1990; Nazarowec-White and Farber, 1999; Clementino and Martins, 2001) The two last molecular typing seems to be the more discriminating between strains in epidemiological investigations (Nazarowec-White and Farber, 1999).



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