INTERNATIONAL SURVEY OF *CRONOBACTER SAKAZAKII* AND OTHER *CRONOBACTER* SPP. IN FOLLOW UP FORMULAS AND INFANT FOODS.

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Abstract

A coordinated survey for *Cronobacter* and related organisms in powdered infant formula, follow up formula and infant foods was undertaken by 8 laboratories in 7 countries in recognition of and in response to the data needs identified in an FAO/WHO call for data in order to develop global risk management guidance for these products. The products (domestic and imported) were purchased from the local market and were categorised according to their principle ingredients. A total of 290 products were analysed using a standardised procedure of pre-enrichment in 225 ml Buffered Peptone Water (BPW), followed by enrichment in Enterobacteriaceae Enrichment (EE) broth, plating on the chromogenic *Cronobacter* Druggan-Forsythe-Iversen (DFI) agar and presumptive identification with ID 32 E. Presumptive *Cronobacter* isolates were identified using 16S rRNA gene sequence analysis. Aerobic plate counts (APC) of the products were also determined on nutrient agar. Fourteen samples had APC $>10^5$ cfu/g, 3 of which contained probiotic cultures. *C. sakazakii* was isolated from 27 products; 3/91 (3%) follow up formulas (as defined by Codex Alimentarius Commission), and 24/199 (12%) infant foods and drinks. Hence *C. sakazakii* was less prevalent in follow up formula than other foods given to infants over the same age range. A range of other bacteria were also isolated from follow up formulas, including *Acinetobacter baumannii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Serratia ficaria*. There was significant variation in the reconstitution instructions for follow up formulas. These included using water at temperatures which would enable bacterial growth. Additionally, the definition of follow up formula varied between countries.

1. Introduction

Cronobacter is a recently defined genus comprising of at least five species, and was previously known as *Enterobacter sakazakii* (Iversen et al., 2008). They are motile peritrichous Gram-negative rod-shaped non-spore forming bacteria, which belong to the Enterobacteriaceae family. *Cronobacter* species have been implicated in neonatal intensive care unit outbreaks of meningitis, septicaemia and necrotizing enterocolitis (van Acker et al., 2001; Himelright et al., 2002; Caubilla-Barron et al., 2007). Bowen and Braden (2006) considered 46 cases of *Cronobacter* infections in neonates. They reported that the symptoms of very low birth weight neonates (age of onset *ca.* 1 month) tend to be bacteraemia, whereas those of birth weight *ca.* 2000g suffered from meningitis and an onset age of a few days. *Cronobacter* species are frequently isolated from the environment, plant material (wheat, rice, herbs and spices) and various other food products (Iversen and Forsythe, 2003 & 2004; Friedemann, 2007; Shaker et al., 2007; Osaili and Forsythe, 2009). The microbiological safety of powdered infant formula (PIF) is a major concern to regulatory agencies and formula producers as their intended use includes newborn infants who have undeveloped immune systems and lack a competing intestinal flora (Townsend and Forsythe 2008). Subsequently the control of the organism in these products has been studied intensively, and various detection methods have been developed (Cordlier, 2008; Fanning and Forsythe, 2008). It is known that starches from wheat and rice can be a source of *Cronobacter* and are PIF ingredients (FAO/WHO, 2004 & 2006). Muytjens et al. (1988) analysed 141 PIF samples from 35 countries and reported that 14% contained '*E. sakazaki*i'. It should be noted that recent re-identification of the strains using 16S rDNA sequence analysis, has

revealed that while most were *C. sakazakii*, some were *E. hormaechei* (Townsend et al., 2008). Iversen and Forsythe (2004) surveyed 486 PIF and other food products for the presence of *Cronobacter* (then *E. sakazakii*). They isolated the bacterium from 2/82 (2.4%) infant formulas, 5/49 (10.2%) infant weaning foods as well as 40/122 (37.8%) herbs and spices. More recently, Restaino et al. (2006) isolated *Cronobacter* from 2/6 dried infant cereals, and Shaker et al. (2007) isolated the organism from 2/8 infant formulas, and 2/15 infant wheat-based follow up formulas. Osaili et al. (2009) reported that *C. sakazakii* and *C. muytjensii* grew in infant wheat-based formulas whether they were reconstituted with water, UHT milk, pasteurized grape or apple juices. The *Cronobacter* grew more (>5 $\log_{10}$) in formulas reconstituted with water or milk than those prepared with grape or apple juices (*ca.* 2–3 $\log_{10}$).

Follow up formula (also known as 'follow on formulas') are defined as 'a food intended for use as a liquid part of the weaning diet for the infant from the 6th month on and for young children' (CAC 1987, CCFH 2009). Similar to PIF, follow up formulas are non-sterile products. The microbiological criteria for PIF used to be based on the 1979 Codex Alimentarius Commission (CAC) guidelines which covered infant formulas for intended use up to 12 months. These criteria have recently been amended (CAC, 2008 a & b). However the issue of whether criteria for *Cronobacter* spp. were necessary for the follow up formulas, which by definition only form part of an infant's diet, had not been decided. This issue was the focus of the FAO/WHO (2008) risk assessment which also reviewed the known cases *Cronobacter* infections which have been reported in infants >6 months in age. It is of particular interest, that the well publicized Tennessee outbreak of *C. sakazakii* on a neonatal intensive unit

in which one neonate died was attributed to the accidental feeding of a non-infant formula. This product was marketed for children and adults and was not intended for use with neonates or infants (Himelright et al., 2002).

The objective of this study was to survey and compare a wide range of infant follow up formulas and weaning foods for *Cronobacter* spp. using a specific chromogenic agar (Iversen et al., 2004) and general microbial load (aerobic plate count) using laboratories in number of countries. The products were chosen according the general description of 'follow up formulas' in each country with an intended use by infants aged between 6-12 months. However, it was subsequently found that many products did not meet the CAC definition of 'follow up formula' especially in that they were not consumed as a liquid (CAC, 1987). In this paper, the CAC definition of 'follow up formulas' has been applied and other products are referred to as 'infant foods'. The latter may also be called 'weaning foods' in some countries and are commonly cereal-based products.

2. Materials and Methods

2.1. Participants and sample collection

Eight laboratories participated in seven countries; Brazil, Indonesia, Jordan (2 independent laboratories), Korea, Malaysia, Portugal, and UK. One hundred and thirty-six follow up formulas and 179 other infant foods were purchased from local markets. These for primarily products with intended use of infants aged 6-12 months, and were both imported and domestically produced. Three herbal tea samples were

included in the survey as these are given to infants as drinks. The list of ingredients and preparation instructions were recorded for each sample.

2.2. Microbiological analysis

The microbiological analysis was limited to the aerobic plate count (APC), and *Cronobacter* detection using standardised methods for all participants. For APC enumeration, 25 gram samples were aseptically taken from each product and mixed with 225 ml Buffered Peptone Water (BPW; Oxoid Thermo Fisher, UK). After 10 minutes, 1 ml aliquots were aseptically removed, decimally diluted in sterile saline, and plated on Nutrient Agar (Oxoid Thermo Fisher) using the spread plate technique (0.1ml volume). The plates were incubated overnight at 37°C. Discrete colonies were enumerated to determine the aerobic plate count. The remaining BPW-sample mixture was incubated at 37°C, overnight as a pre-enrichment step. After incubation, a 10 ml aliquot was transferred to 90ml Enterobacteriaceae Enrichment (EE) broth (Oxoid Thermo Fisher, UK), and after a further overnight incubation at 37°C, the broth was streaked on the Brilliance *Enterobacter sakazakii* chromogenic DFI agar (Oxoid Thermo Fisher, UK). The plates were incubated at 37°C, for 18 hours.

2.3. Identification of isolates

Presumptive *Cronobacter* colonies (blue-green colouration) were picked from DFI agar plates for phenotypic identification using ID 32 E (bioMerieux), and were speciated using 16S rDNA sequence analysis (Accugenix, Delaware, USA) and

phenotyping. Other non-*Cronobacter* colonies were also picked for phenotypic identification using ID 32 E.

3. Results

In total, 318 products were sampled in seven countries by eight laboratories, comprising 136 follow up formulas (according to CAC 2008a definition), and 182 other products. *C. sakazakii* was isolated from 1/136 (1%) follow up formulas, and 22/179 (12%) of infant foods, and none of the three herbal teas. Table 1 summarises the results of the survey for aerobic plates counts, and incidence of *Cronobacter* spp. for each country.

Cronobacter spp. were not isolated from any products in Brazil, Korea or Malaysia (Table 1). Brazil analysed 31 follow up formulas. One sample contained aerobic plate count 10^2-10^3 range, all the others were $<10^2$ cfu/g. Other organisms isolated included *Acinetobacter baumannii*, *E. amnigenus*, *E. cloacae*, and *Pantoea* spp. All products had labels informing the user that the product should be reconstituted with water that had been boiled, but not the specific temperature of rehydration. Korea did not detect any *Cronobacter* in the 30 products sampled; 24 follow up formulas, and 6 infant foods. High aerobic plate counts ($>10^5$ cfu/g) were obtained with cereal-based infant foods. All products had labels advising the use of water >70°C for reconstituting. Malaysia found high APC values for 7/12 follow up formulas; four were 10^4-10^5 cfu/g and three were $>10^5$ cfu/g. One sample with APC $>10^5$ cfu/g contained a probiotic culture. The packaging instructions for 7 follow up

formulas advised reconstituting with water that was 40-45°C, one stated 'lukewarm' water and the remainder advised 50-55°C. The infant foods also had a wide range of APC, with 4 having values >10^5 cfu/g.

C. sakazakii was isolated from infant foods, but not follow up formulas in the UK, Indonesia, and Portugal (Table 1). The UK isolated *C. sakazakii* from 6/64 of infant foods, and these products had instructions advising the use of warm or cold milk for reconstitution. Other organisms isolated included *Aeromonas sobria*, *K. pneumoniae*, *Pantoea* spp., *E. cloacae*, *Stenotrophomonas maltophila*, *A. baumannii*, *Ps. oryzihabitans, Citrobacter amalonaticus*, and *Esch. vulneris*. The APC ranged from <10^2 to >10^5 cfu/g for 55/64 and 2/64 infant foods, respectively. Indonesia recovered *C. sakazakii* from 6/15 infant foods. A range of other organisms were also isolated; *K. pneumoniae*, *K. terrigena*, *Pantoea* spp., *Esch. vulneris*, *S. ficaria*, *S. plymuthycia*, *S. rubidaea*, *E. cloacae*, and *Cirobacter freundii*. For Portugal, the packaging instructions for two *Cronobacter* positive samples advised the use of warm milk (50°C) for reconstitution. *Leclercia adecarboxylata* and *E. helveticus* were also isolated from infant foods. The APC were primarily <10^2 cfu/g, with 8/30 in the range 10^2-10^3 cfu/g. *C. sakazakii* was isolated from a transition infant milk formula. This is a product for special medical purposes, and is subject to the same microbiological criteria as PIF by CAC (2008b). The instructions advised reconstituting with water that had been boiled for 5 minutes, and had cooled to 40°C. The APC of this product was 23 cfu/g.

Jordan analysed 11 follow up formulas, 46 infant foods, and 3 herbal teas which are given to infants >4 months. It was the only country to isolate *Cronobacter* spp. from a follow up formula sample (Table 1). No other Enterobacteriaceae were

isolated from this sample, and the APC was <10^2 cfu/g. The instructions on the packaging advised that reconstitution should use water which had been boiled for 5 minutes, and allowed to cool to an unspecified temperature. *C. sakazakii* was also isolated from 7/46 infant foods, and the highest APC was >10^5 cfu/g. Other Enterobacteriaceae isolated were *E. hormaechei* subsp. steigerwaltii and *E. helveticus*. The labelling on infant food products advised using boiled water, but gave no specific temperature for reconstitution. No *Cronobacter* were isolated from the herbal teas. These had APC <10^2 cfu/g.

4. Discussion

This study was focused on follow up formula and other foods given to infants >6 months in response to the FAO/WHO (2008) call for appropriate microbiological data. A major advantage of our study was the use of laboratories in seven countries which sampled imported and domestically-produced formulas in local retail, and therefore a wide range of products were analysed. However on compilation of the data, the study revealed that the term 'follow up formula' varied between countries. For consistency, the description of follow up formulas from CAC (1987, 2009) was followed, that is products with the intended age >6 months which are part of the infant's diet and consumed as a liquid. Consequently some products which had been were purchased as follow up formulas were re-categorised as 'infant foods' in Table 1.

C. sakazakii was the only *Cronobacter* species isolated from any products. It was isolated from 1/136 (0.7%) follow up formulas, and 22/182 (12%) other products. This is the first large study of follow up formulas and shows the organism to be present at a lower frequency than infant foods which are given to the same age range. Previously it was shown that *Cronobacter* spp. were present in 5/49 (10%) infant foods (Iversen and Forsythe, 2004).

Since the FAO/WHO (2006) and WHO (2007) recommended the use of water >70°C for reconstitution of powdered infant formula, it was of interest to record the guidance instructions on the follow up formula packaging. The only country which clearly stated the use of water >70°C for reconstitution was Korea. The advice given in other countries varied considerably including non-specified temperature (lukewarm) and as low as 40°C which is within the growth range of *Cronobacter* spp. and other Enterobacteriaceae.

In addition, other bacteria which the FAO/WHO (2004) listed as Category B (causality plausible-but not yet demonstrated) were also isolated from follow up formulas and infant foods; *E. cloacae*, *K. pneumoniae*, *C. freundii*, *Esch. vulneris*, *Pantoea* spp., *S. ficaria* and *A. baumannii*. These opportunistic pathogens would not have been killed by the mild temperatures used on reconstitution, and could multiply if the feed was left at ambient temperature.

The APC of the follow up formulas ranged from $<10^2$ for 102/136 (75%) samples to $>10^5$ cfu/g for 3/136 (2%). These can be compared with 103/179 (58 %) and 11/179 (6%) for infant foods. A similar APC range was reported by Iversen and Forsythe (2004) for infant foods; 35/49 (71%) $<10^2$ cfu/g, and 3/49 (6%) 10^4-10^5 cfu/g. The latter data is included in Table 1 for comparative purposes of samples

taken 4 years previously in the UK. In the current study, 7 products contained probiotics, three of which gave very high APC (>10^5 cfu/g). Whether the presence of these organisms affects the detection of *Cronobacter* is unknown and is a matter warranting further investigation. It is important to note that Joosten et al (2006) reported the need to modify the *Salmonella* detection method due the presence of probiotic cultures. The three herbal tea samples were included in the survey, as these are also given to infants (>4 months) and *Cronobacter* have been isolated from similar products (Tamura et al., 1995; Friedemann 2007; Osaili and Forsythe, 2009). These products are prepared with boiled water which is cooled to about 50°C.

Infants >6 months are exposed to a wider range of sources of micro-organisms (environmental and foodstuffs) than neonates, who are more susceptible to infection due to the lack of a developed immune system and intestinal flora (Townsend and Forsythe, 2008). Nevertheless infants are still prone to infections and general good hygienic practices in food preparation are necessary to reduce this risk. This survey has shown the presence of *C. sakazakii* in follow up formulas and infants foods. In addition, it has shown that the packaging instructions are inconsistent with regard to the temperature of reconstitution, which is a known step in reducing bacterial load. *Cronobacter* spp. infections have been reported for all age groups. Worldwide there have been *ca.* 120 reported cases in infants and children <3 years in age, of which 8 were cases aged between 6-35 months (FAO/WHO, 2008). In the UK between 1999-2007, 15/570 laboratory reported infections were from infants (<12 months in age), and 16/570 were from children (1-4 years in age) (FAO/WHO, 2008). Previous surveys of PIF have shown the incidence of *Cronobacter* spp. to be between 2-22 % (FAO/WHO 2006). In contrast, this

international study has shown that the incidence of *Cronobacter* spp. is lower (0.7%) in follow up formula. Since follow up formula is only part of the infant's diet, this data supports the recent CAC (2009) decision for microbiological criteria for follow up formula to include *Salmonella* and not *Cronobacter* spp. Nevertheless good hygienic practices are always required in the preparation of such foods.

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Table 1. Microbiological analysis of follow up formulas and infant foods.

Product	Country	Number of samples	Aerobic plate counts (cfu/g)					*Cronobacter* positive samples (%)
			$<10^2$	10^2-10^3	10^3-10^4	10^4-10^5	$>10^5$	
Follow up formula[a]	Brazil	31	30	1				
	Korea	24	21	3				
	Malaysia	12	5(1)[c]			4	3 (1)	
	UK	38	22	12	4(1)			
	Indonesia	0	ND[d]					
	Portugal	20	18	2				
	Jordan 1	6	6					1
	Jordan 2	5	ND					
	Total	136	102	18	4	4	3	1 (0.7)
Infant foods	Brazil	0						
	Korea	6	2				4	
	Malaysia	18	11	1	1	1(1)	4(2)	
	UK	64	55	5	2		2	6
	Indonesia	15	ND					6
	Portugal	30	23	8				3
	Jordan 1	21	12(1)	7	1		1	4
	Jordan 2	25	ND					3
	Total	179	103	21	4	1	11	22(12)
Herbal tea	Jordan 1	3	3					
Previous infant food published data[b]	UK	49	35	8	3	3		5 (10)

a. Description matches that of CAC (1987 & 2009) definition of 'follow up formulas'.

b. Iversen and Forsythe, 2004.

c. Number in parenthesis indicates the number of samples which contained probiotic cultures.

d. Not done.