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Reassessment of *Cronobacter* spp. originally isolated as *Enterobacter sakazakii* from infant food

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ABSTRACT

Cronobacter spp. cause infant disease, several cases have been associated with powdered infant formulae (PIF). In the early 2000s, contamination of German PIF with these opportunistic pathogens was quite common. Before 2008, all isolates *Cronobacter* spp. had been classified as *Enterobacter sakazakii*, therefore little is known about species diversity within such isolates. Genetic, serologic, and biochemical traits of 80 *Cronobacter* isolates, originally obtained 2003–2006 within infant food surveys in Germany, were reassessed in this study. By sequencing of the *fusA* gene, all isolates were unambiguously assigned to two species, *C. sakazakii* (n = 73) and *C. malonaticus* (n = 7). PCR serotyping identified five *C. sakazakii* serotypes and two *C. malonaticus* serotypes, biochemical profiling yielded five biogroups. PFGE analysis also showed high heterogeneity in both species. Multilocus sequence typing of 26 selected isolates yielded 16 different sequence types (ST), including *C. sakazakii* ST 1 (n = 6) and the highly virulent ST 4 (n = 2). The results suggest that just two, but highly heterogeneous species were responsible for the *Cronobacter* contamination problem which challenged the German PIF industry in the beginning of this century. This fact may have influenced the success of efforts to identify and eliminate sources of contamination.

1. Introduction

Cronobacter spp. (formerly *Enterobacter (E.) sakazakii*) are opportunistic pathogens which have been associated worldwide with rare but life-threatening foodborne disease (meningitis, septicaemia, necrotizing enterocolitis) in newborn and premature infants. Powdered infant formula (PIF) have been identified as a source of infection (Block et al., 2002; Holý and Forsythe, 2014; Mullane et al., 2007; Van Acker et al., 2001).

Currently the Genus *Cronobacter* contains seven recognized species, but only strains of three species, *C. sakazakii*, *C. malonaticus*, and *C. turicensis*, have been associated with clinical infections. *C. sakazakii* and *C. malonaticus* isolates are responsible for the majority of infectious disease in infants (Joseph et al., 2012), and seem to be the most frequent *Cronobacter* species in PIF worldwide (Yang et al., 2016). For Germany, no data on species distribution within the genus *Cronobacter* from PIF are available.

In 2002, the International Commission on Microbiological Specification for Foods classified *E. sakazakii* as a severe hazard for restricted populations (ICMSF, 2002), and three risk assessments of *Cronobacter* spp. (*E. sakazakii*) have been published under the auspices of the FAO/WHO (FAO/WHO, 2004, 2006, 2008). European Union regulation (EC) No. 2073/2005 as amended by regulation (EU) No. 365/2010 requires the absence of *Cronobacter* spp. in 30 × 10 g test portions of PIF intended for infants below 6 months of age. No further identification of *Cronobacter* species is necessary (European Commission, 2010). As a "food safety criterion" in PIF, *Cronobacter*-positive lots need to be withdrawn from the market.

Before regulation (EC) No. 2073/2005 came into force, *Cronobacter* spp. had been found in PIF from the German market with considerable frequency, up to 30% in follow-on formulae (Kress et al., 2005). In recent years, however, alerts concerning *Cronobacter* in PIF and market withdrawals became a rare event in Germany, and none was published in the European Commission "rapid alert system for food and feed" (RASFF; https://webgate.ec.europa.eu/rasff-window/portal/) since 2012. Likewise, no report about infant disease caused by *Cronobacter* spp. from PIF has ever been published from Germany, although on a worldwide basis, sporadic

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but severe infections particularly in newborns continue to be reported (CDC, 2012). The problem of misidentification of *Cronobacter* spp., in particular in studies relying on phenotypic traits, has been discussed recently (Jackson et al., 2015).

Cronobacter spp. have been frequently isolated not only from PIF but also from environmental samples, water, plant material, and various food products (Akineden et al., 2015; Baumgartner et al., 2009; Chap et al., 2009; Friedemann, 2007; Vojkovska et al., 2016). *Cronobacter* spp. have also been isolated from various sites and equipment in PIF processing facilities (Craven et al., 2010; Fei et al., 2015; Iversen et al., 2009; Jacobs et al., 2011).

These aspects raise the question whether the contamination situation of PIF with *Cronobacter* spp. in Germany is, or was, different to other countries, and whether or not it has changed during the last decade. For this, it is necessary first to identify the situation concerning the dominant species in the years when the *Cronobacter* problem in PIF became a regulatory issue.

The aim of the present study was to retrospectively clarify the contamination situation of PIF from the German market with *Cronobacter* at species level for the years before and around the time when regulation (EC) No. 2073/2005 came into force. To this end, 80 *Cronobacter* isolates which had been isolated as *E. sakazakii* from different German infant foods in the years 2003–2006, were reassessed using current identification methods, including *fusA* sequencing, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and O-antigen serotyping. The phylogenetic relationships of the *rpo*B and *fus*A genes of these isolates were analysed.

2. Materials and methods

2.1. Bacterial isolates

A total number of 80 *Cronobacter* isolates, previously identified as *E. sakazakii*, most of which had been obtained from PIF within several surveys performed in 2003–2006 (Kress et al., 2005; Kurz, 2009), were included in this study. A few isolates were from apple sauce and fruit pulp specifically labelled as suitable for infant nutrition. The isolates were the yield of a total sample size of approximately 400 packages (16 different producers) of PIF, all had been purchased as offered to the consumer from German retail shops. All isolates had been identified biochemically using the api 20E, api ID 32, by growth characteristics on different media (TSA agar, DFI agar, ESIA agar), and by a *E. sakazakii*-specific PCR (Hassan et al., 2007). For all isolates, a full documentation with regard to sample origin was available (Table 1). The vast majority of isolates was obtained from follow-on formulae (>4 months), but some isolates were from three different infant formulae recommended from birth on. All isolates had been stored at -80 °C using the Cryobank bacteria conservation system. The type strains *C. sakazakii* (DSM 4485^T), *C. malonaticus* (DSM 18702 ^{T}), *C. turicensis* (DSM 18703^T), *C. universalis* (NCTC 9529 ^{T}), *C. muytjensii* (DSM 21870^T), *C. dublinensis* ssp. *dublinensis* (DSM 18705^T), *C. dublinensis* ssp. *lactaridi* (DSM 18707^T), *C. dublinensis* ssp. *lausannensis* (DSM 18706^T), *C. condimenti* (LMG 26250^T) were used as controls.

Table 1
Information on original sources and designation of *Cronobacter* spp. (*E. sakazakii*) isolates (n = 80) from PIF and other infant food used in this study.

Product category	Recommended age from	Ingredients, specific information	Number of different samples	Manufacturer	No. of isolates	Isolate designation[a]
infant milk formula	birth	–	3	B, O, R	3	DB-24b, DB-258-1-05, DB-376/2e-06
	4 months	–	4	B, C, E, F	5	DB-271-3-05, DB-71b/3-05, DB-71b/4-05, DB-74c/2-05, DB-89c/1-05
		hypoallergenic	3	A, B, D	4	DB-212b-3-05, DB-211a-3-05, DB-255-2-05, DB-255-5-05
		spelt	3	J	3	DB-338-3j-06, DB-359/1c-06, DB-372/2k-06
		semolina	2	B, D	2	(DB)37c, DB-321-2c-06
		semolina, fruits	1	A	1	DB-299-6y-06
		grain flour, fruits	10	B, C, M, O, I	10	DB-32c, (DB)38a, (DB)41c, (DB)54b, DB-262-1-05, DB-193a-2-05, DB-275-1-05, DB-223/c2-05, DB-264-3-05, DB-370/1j-06
		fruits	1	C	1	DB-129a/4-05
infant formula without milk	4 months	buckwheat	2	E	3	DB-367-1c-06, DB-382-2N, DB-382-3D3-06
		grain cereals flour	3	C, G, N	6	DB-80c/5-05, DB-80c/7-05, DB-144a-2a-05, DB-144a-5-05, DB-200a-1-05, DB-200c-4-05
		porridge	2	A, J	3	DB-163a-3-05, DB-168b-5-05, DB-168c-2-05
		semolina, pumpkin	1	J	1	DB-141a-1-05
		millet gruel	1	J	2	DB-164a/2-05, DB-164b/1-05
follow-up milk formula	6 months	whole-grains flour	2	A	3	DB-M2, DB-M8, DB-1b-04
		semolina	3	C, B	3	DB-31a, (DB)36a/04, DB-361-2b-06
		biscuits	7	C, D, M	8	DB-33a, (DB)42a/04, (DB)53c, (DB)55a/04, (DB)55b/04, DB191a-4-05, DB-232-2-05, DB-277-1-05
		fruits	2	B	3	DB-122b/4-05, DB-169a-5-05, DB-169b-2-05
		grain flour, fruits	2	C, P	2	DB-238-2, DB-246-3-05
	8 months	–	1	I	1	DB-84a/1-05
		grain flour, fruits	1	A	2	DB-65c/15-2-05, DB-65c/18-05
		fruits, yogurt	1	C	1	DB-172a-1-05
	10 months	grain flour	1	A	1	DB-280-2-05
follow-up formula without milk	6 months	durum wheat semolina	1	H	1	DB-81b/1-05
	10 months	grain flour	1	A	1	DB-152b-2-05
milk powder	not specified	soya	1	K	1	DB-160a-6-05
milk powder	not specified	soya	1	L	2	DB-186a-3-05, DB-186b-3-05
whey powder	not specified	fruits, yogurt	1	C	2	DB-73a/1-05, DB-73a/3-05
apple sauce	4 months	–	1	C	3	DB-143a-8-05, DB-143b-6-05, DB-143c-7-05
fruit pulp	6 months	fruits	1	A	2	DB-185a-1-05, DB-185c-3-05

[a] First numbers in isolate designation code indicate sample number, for example DB-24b refers to an isolate from sample number 24. Identical numbers indicate that isolates are from the same sample.