

**Identification and further characterization of  
*Enterobacteriaceæ* and *Cronobacter* spp. in a milk  
powder and infant formula processing plant**

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*for Henri*

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## Abbreviations

CCP	Critical control point
CSB	<i>Cronobacter</i> screening broth
°C	Degree Celcius
DNA	Deoxyribonucleic acid
EC	European Community
EE	<i>Enterobacteriaceæ</i> enrichment broth
FAO	Food and Agriculture Organization
g	Gram
HACCP	Hazard analysis and critical control point concept
HEPA	High-efficiency particulate air filter
ISO	International Organization for Standardization
mLST	Modified lauryl sulphate tryptose broth
n	Number of samples
PIF	Powdered infant formula
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random-amplified polymorphic DNA
rt-PCR	Real-time polymerase chain reaction
spp.	Species pluralis
TS	Technical Standard
UHT	Ultra high temperature
WHO	World Health Organization

## 1 INTRODUCTION



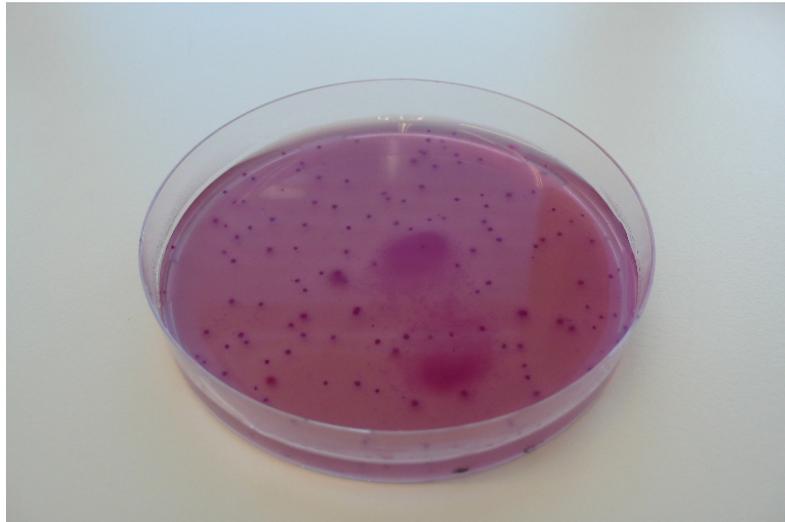
Members of the family *Enterobacteriaceae* are ubiquitous. Because of technically given non-sterility of milk powder and powdered infant formula (PIF) *Enterobacteriaceae* can be occasionally detected. For PIF a process hygiene criterion which requires the absence of *Enterobacteriaceae* in  $10 \times 10^6$  exists in the European Community (Anonymous, 2005). In case of positive results, improvements in process hygiene to minimize contamination are required.

Powdered infant formula is not a sterile product: due to technical reasons, a spray-drying tower always operates under conditions of slight under-pressure. The system is not hermetically sealed and therefore contaminations cannot entirely be excluded. This fact must be taken into consideration when it comes to trace back possible routes of contamination. Only few epidemiological data are available, industrial companies have to carry out their own investigations. Based on regulation (EC) 1441/2007 (Anonymous, 2007A) on microbiological criteria for foodstuffs PIF has to be analysed for *Enterobacteriaceae* and *Cronobacter* spp. in parallel unless a correlation of these micro-organisms has been established at an individual plant level. This criterion can hardly be fulfilled, which makes necessary additional analysis till the final release.

*Cronobacter* spp. is a newly described genus, formerly known as *Enterobacter sakazakii*. The World Health Organization (WHO) assessed the situation of *E. sakazakii* and other micro-organisms in PIF in 2004 (Anonymous, 2004), in 2006 there was made another risk assessment (Anonymous, 2006A) which states that industrial companies should effectively implement preventive measures (e.g. dry cleaning instead of wet cleaning) to avoid contaminations during manufacturing. WHO/FAO guidelines on safe preparation,

handling and storage of PIF were published in 2007 (Anonymous, 2007B), which show that not only production risks but also preparation risks have been taken into consideration. The first international scientific conference on *Cronobacter* in 2009 (<http://www.ucd.ie/crono09/>) presented a wide variety of information concerning taxonomy, epidemiology, methodology, risk assessment and field studies. Based on those data the following work should evaluate epidemiological data including analytical improvements.

## 2 DIVERSITY OF ENTEROBACTERIACEÆ IN POWDERED INFANT FORMULA (PIF) AND PIF PRODUCTION PLANTS



Until today, only few studies have focused on the diversity of *Enterobacteriaceæ* in PIF (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006). Since *Enterobacteriaceæ* are not likely to survive the implemented heating process during manufacturing, a recontamination of the powdered infant formula after this process step must be assumed. Thus also the presence and diversity of *Enterobacteriaceæ* in the production environment and in raw ingredients is of interest.

In Regulation (EC) 2073/2005 (Anonymous, 2005), the European Commission states that *Salmonella* and *Cronobacter* spp. are the micro-organisms of greatest concern in powdered infant formula. The presence of these pathogens constitutes a considerable risk when conditions allow growth of the bacteria. In particular, *Cronobacter* spp. are known to cause infections in neonates with severe outcomes such as sepsis, meningitis or necrotizing enterocolitis due to contaminated infant formula (Biering et al., 1989; Bar-Oz et al., 2001; van Acker et al., 2001; Himelright et al., 2002). Some reported cases have been linked to the ingestion of contaminated infant formula (Biering et al., 1989; Himelright et al., 2002; van Acker et al., 2001). However, the organisms appear to be ubiquitous having been isolated from a variety of food products, production environments and households (Friedemann, 2007; Gurtler and Beuchat, 2005; Kandhai et al., 2004A; Kandhai et al., 2004B).

The reported prevalence of *Cronobacter* in surveys of commercially available PIF appears to be gradually decreasing from estimates of 14% in 1988 (Muytjens et al., 1988) and 6.7% in 1997 (Nazarowec-White and Farber, 1997), to 2.5% in 2001 (Heuvelink et al., 2001). Recent estimates indicate that although small reductions may still occur, the prevalence appears to be stabilizing at 2.0-2.5% (Iversen and Forsythe, 2004; Iversen et al., 2008; Mullane et al., 2007).

This could be either due to particularly persistent phenotypes in production facilities which seem to be resistant even to the improved hygiene measures adopted by manufacturers, or to yet unidentified contamination routes. Fingerprint typing has become established as a useful method to support monitoring of micro-organisms in relation to public health, consumer protection and manufacturing hygiene practices. Over the years new typing methods have been developed and compared to existing methods to determine which have the most discriminatory power.

### 3 PRODUCTION OF POWDERED INFANT FORMULÆ



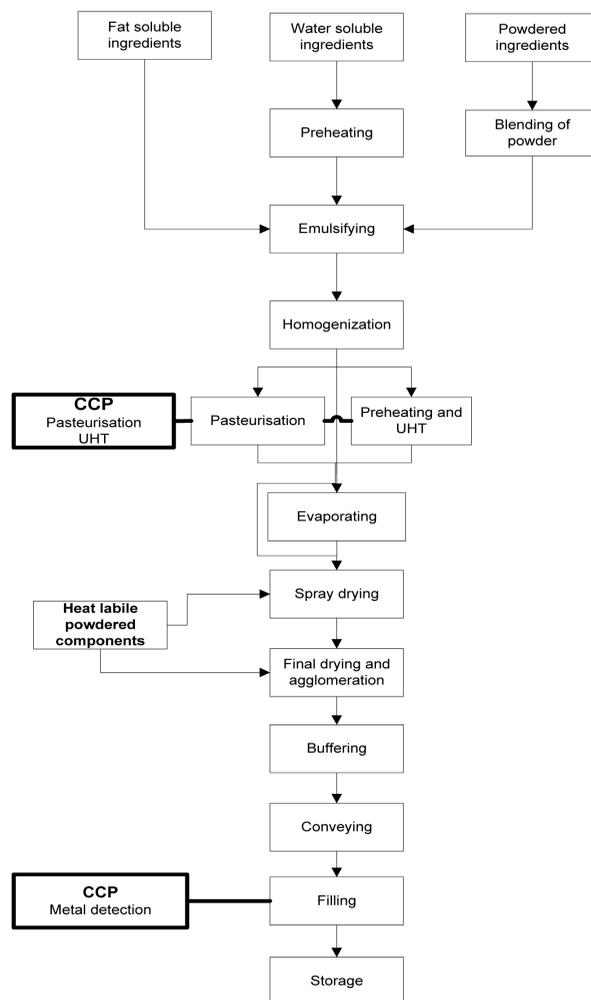
Powdered infant formulae (PIF) are foodstuff intended for particular nutritional use for infants under six months. PIF satisfy the baby's nutritional requirements until the introduction of appropriate complementary feeding. PIF are strictly regulated in Commission Directive 2006/141/EC (Anonymous, 2006B). These formulae are composed of the same main ingredients as human milk. For most of these ingredients there are upper and lower limits set in the above mentioned directive. In order to guarantee a constant level of product quality which well fulfils the strict parameters of PIF, the choice of raw material is of the utmost importance. For this reason a reliable quality agreement as well as certificates of analysis for each delivery are highly recommended. After a positive release of each incoming lot, milk and milk concentrate respectively, vegetable fats and powdered ingredients such as lactose, maltodextrin, whey protein, vitamins and minerals are blended, emulsified and homogenized in order to achieve a temporarily stable wet mix of similar droplet size. According to figure 1 the emulsion requires a heat treatment be it a pasteurisation (75°C) or a UHT treatment (>100°C). This step is necessary to eliminate bacteria which can originate from the raw material used. A critical control point (CCP) is therefore necessary. Commission Regulation (EC) No 1441/2007 (Anonymous, 2007A) sets the limits for food safety as well as for process hygiene criteria.

In order to achieve a maximization of dry matter in the liquid phase, which leads to a better efficiency of the spray-drying tower, the emulsion will in most cases require a concentration step to remove water from the mix. Afterwards, the emulsion is fed to the tower where the drying process takes place under conditions of slight under-pressure. Due to conditioned hot air (approximate 200°C) the water evaporates as the droplets fall. This step ensures the product's stability with a shelf life of 18-36 months. During the last

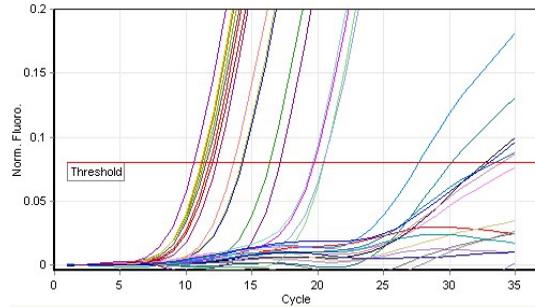
phase of the drying process, which takes place in an internal and external fluid bed, it is recommended that one should add heat labile ingredients such as probiotic bacteria, lactoferrine and heat sensitive trace elements. Throughout the last drying stage, an agglomeration of the powder occurs which leads to a better powder structure and solubility. A highly agglomerated powder also improves the product’s properties of reconstitution. After the buffering silo and the run off conveyor the powder is constantly checked for metal. This critical control point should take place during the last possible stage before the powder is filled into big-bags or directly in packaging units such as tins or pouches.

It is very important to ensure that the entire procedure from the wet mixing process until the packaging of the powder is designed in highly hygienic quality. This is important because after heat treatment a contamination could lead to non-conform products which could do harm to infants.

FIGURE 1: Flow chart of powdered infant formulæ (PIF)



## 4 AIMS OF THIS THESIS



This cumulative dissertation pursues the following objectives:

- Development and evaluation of *Cronobacter* spp. detection and identification methods.
- Determination of the occurrence of *Cronobacter* spp. in PIF manufacturing facilities in order to investigate persistence of specific strains and identify possible transmission routes.
- Genotyping of *Cronobacter* spp. isolates from infant formula processing facilities in Switzerland in order to characterize persistent strains and elucidate dissemination routes.
- Distribution of *Cronobacter* spp. pulso-types from powdered infant formula, ingredients and environment samples.
- Genetic diversity of multiple *Cronobacter* spp. isolates from cultural positive powdered samples – consequences for epidemiological studies in processing plants.
- Identification of *Enterobacteriaceae* isolates other than *Cronobacter* spp. and *Salmonella*, isolated from different sample types (raw material, environment, finished products) of an infant formula processing plant and genotyping isolates from frequently found species to elucidate and trace back transmission routes not only limited to *Cronobacter* spp.

## 5 PUBLICATIONS



### 5.1 Methodology

#### 5.1.1 Development of a novel screening method for the isolation of *Cronobacter* spp. (*Enterobacter sakazakii*)

Iversen, C.<sup>1,2</sup>, Druggan, P.<sup>3</sup>, Schumacher, S.<sup>1</sup>, Lehner, A.<sup>1</sup>, Feer, C.<sup>4</sup>, Gschwend, K.<sup>4</sup>, Joosten, H.<sup>2</sup>, Stephan, R.<sup>1,\*</sup> (2008).

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## Development of a Novel Screening Method for the Isolation of “*Cronobacter*” spp. (*Enterobacter sakazakii*)<sup>∇</sup>

Carol Iversen,<sup>1,2</sup> Patrick Druggan,<sup>3</sup> Sandra Schumacher,<sup>1</sup> Angelika Lehner,<sup>1</sup> Claudia Feer,<sup>4</sup>  
Karl Gschwend,<sup>4</sup> Han Joosten,<sup>2</sup> and Roger Stephan<sup>1\*</sup>

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**A differential medium, “*Cronobacter*” screening broth, has been designed to complement agars based on hydrolysis of chromogenic  $\alpha$ -glucopyranoside substrates. The broth was evaluated using 329 *Enterobacteriaceae* strains (229 target isolates), spiked/naturally contaminated samples, and a parallel comparison with current methods for raw materials, line/end products, and factory environment samples.**

*Enterobacter sakazakii* is an opportunistic neonatal pathogen that has been reported as an occasional contaminant of powdered infant formula (PIF) (2, 3). Recent polyphasic analysis led to the proposal of a reclassification of *E. sakazakii* as several novel genomospecies within a novel genus, “*Cronobacter*,” with the novel genus being synonymous with *E. sakazakii* (11, 13). Various chromogenic and fluorogenic agar media have been described for detection of *Cronobacter* (7, 17, 19, 20). These are based mainly on the enzyme  $\alpha$ -glucosidase, which is constitutively expressed in *Cronobacter* but in few other *Enterobacteriaceae* (18, 21, 22). Assessment of several of these media has shown that they provide comparable sensitivities and specificities (5, 12, 16). However, it has been established that some isolates of *Cronobacter* do not grow well in currently proposed enrichment broths, such as modified lauryl sulfate tryptose broth (mLST) and *Enterobacteriaceae* enrichment broth (6, 9, 16). Samples containing only such strains could give false-negative results; therefore, the enrichment procedure should be improved. Fermentation of sucrose and metabolism of 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-glucopyranoside have been shown to be useful distinguishing tests for *Cronobacter* spp. (12, 18). Therefore, a differential broth has been developed that enables samples to be screened for potential *Cronobacter* contamination without incorporating selective agents that may affect the recovery of sensitive strains (9).

**CSB.** *Cronobacter* screening broth (CSB) comprises 10.0 g liter<sup>-1</sup> peptone, 3.0 g liter<sup>-1</sup> meat extract, 5.0 g liter<sup>-1</sup> NaCl (7647-14-5), 0.04 g liter<sup>-1</sup> bromocresol purple (115-40-2), 10 g liter<sup>-1</sup> sucrose (57-50-1), and 10 mg liter<sup>-1</sup> vancomycin hydrochloride (1404-93-9). (All chemicals are described by their Chemical Abstract Service numbers to allow an exact identification of materials independent of supplier.) The final pH value is 7.4  $\pm$  0.1, and the optimum incubation temperature is 42°C.

**Microbiological strains.** Inclusivity and exclusivity of CSB were assessed using 329 strains, including 229 target *Cronobacter* isolates, covering a diversity of global distribution, species, and biogroups (11, 13). The 100 nontarget *Enterobacteriaceae* strains included 27 species from nine genera. After 24 h at 42°C, all 229 *Cronobacter* strains tested were able to grow in CSB and ferment the sucrose, thus lowering the pH and effecting a change in the color of the broth from purple to yellow. The minimum inoculum required to observe a color change in CSB within 24 h was 10<sup>2</sup> CFU ml<sup>-1</sup>, as determined with the slowest-growing strain (E770). This corresponds to a final concentration of 10<sup>4</sup> CFU ml<sup>-1</sup> after preenrichment of the sample in buffered peptone water (BPW) (CM0509; Oxoid AG, Pratteln, Switzerland). The level of competitive flora in PIF is generally low (8), the lag time for desiccated cells to recover after rehydration of PIF at 37°C has been estimated as 0.254 h (15), and the approximate doubling time at 37°C in infant formula for *Cronobacter* spp. is 20 min (10). Therefore, theoretically an initial contamination of only 1 CFU in a 300-g sample should be able to multiply to exceed a final concentration of 10<sup>4</sup> CFU ml<sup>-1</sup> well within an 18-h incubation time. The sensitivity and negative predictive value (NPV) of CSB were 100% when CSB was used in conjunction with a nonselective chromogenic medium (X-TSA) comprised of tryptone soya agar (TSA) (CM0131; Oxoid AG, Pratteln, Switzerland) containing 0.15 g liter<sup>-1</sup> 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-glucoside (108789-36-2). The specificity was 94%, and the positive predictive value (PPV) was 97.4%. The nontarget strains yielding presumptively positive reactions (6/100) were all isolates of the novel species *Enterobacter pulveris* (22), which ferment sucrose and metabolize 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-glucopyranoside. However, they can be distinguished from *Cronobacter* spp. by using phenotypic tests (11, 22). Caution needs to be used with interpretation of commercial biochemical galleries, as these species are not yet incorporated in the corresponding databases and can be designated a nearest match to *E. sakazakii*. Based on data from our culture collection, all *E. pulveris* strains are negative for arginine dihydrolase, ornithine decarboxylase, and Voges-Proskauer tests, whereas *Cronobacter* strains are, respectively, 97%, 92%, and 99.5% positive for these tests. Additionally, all *E. pulveris* strains are positive for fermentation of D-arabitol and the methyl red test,

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whereas *Cronobacter* strains are 100% negative for fermentation of D-arabitol and 99.5% negative for the methyl red test.

**Recovery from spiked samples.** To mimic the recovery of low numbers of cells from PIF, 100 g of PIF was diluted 1/10 with BPW. The rehydrated PIF was spiked with 20 target *Cronobacter* strains and 10 nontarget *Enterobacteriaceae* strains to achieve for each strain a final concentration of 1 to 10 CFU liter<sup>-1</sup>. The resulting spiked 1,000 ml of rehydrated formula was divided into 200 5-ml analytical samples; this gave an estimated average of 0.75 CFU 5 ml<sup>-1</sup>. The strains included representatives of the different *Cronobacter* species (13) as well as target strains that were previously found difficult to grow in *Cronobacter* isolation media (9). Nontarget organisms included the recently described species *Enterobacter helveticus*, *E. turicensis*, and *E. pulveris* (21, 22), which can be found in the same ecological niches as *Cronobacter*, such as dried food products and factory environments, and also yield presumptively positive colonies on chromogenic agar. The 200 spiked analytical samples were incubated for 24 h at 37°C. Each analytical sample was then diluted 1/100 into both CSB and mLST (4) and incubated for 24 h at 42°C and 44°C, respectively. All analytical samples were streaked onto X-TSA and the plates incubated at 37°C for 24 h to detect presumptively positive *Cronobacter* colonies. The presumptively positive colonies were distinguished using the methyl red and Voges-Proskauer tests. Bacteria were recovered from 147/200 spiked 5-ml analytical samples, with multiple isolates obtained from 17 samples. Fermentation of CSB occurred in 134 samples, and 105 gave presumptively positive colonies on chromogenic agar. *Cronobacter* was detected in a total of 91 of the spiked 5-ml samples, all of which were positive in CSB and produced presumptively positive colonies on the agar (Table 1). Of the 56 samples from which only nontarget strains were recovered, 43 were positive in CSB and 14 of these gave presumptively positive colonies on X-TSA. Fewer samples were found positive for *Cronobacter* when using mLST than when using CSB.

**Recovery from factory samples.** A total of 80 10-g samples (41 PIF, two dried infant cereals, 15 vitamin-mineral premixes, and 22 factory environmental samples) were preenriched for 24 h at 37°C in BPW at a 1/10 dilution. Each preenriched sample homogenate was then inoculated (100 µl) into 10 ml CSB, giving a 1/100 dilution of homogenate, and incubated at 42°C for 24 h, after which 10 µl was streaked onto X-TSA and incubated at 37°C for 24 h. The presumptively positive isolates were identified using ID 32E biochemical galleries (bioMérieux Industries, Marcy l'Etoile, France). No *Cronobacter* strains were detected in any of the PIF, infant food, or vitamin-mineral premixes, with only one nontarget presumptively positive (i.e., positive in CSB) out of 58 samples tested. Therefore, 98.3% (57/58) of these samples would correctly be identified as negative after 48 h and a decision to release could be made. For the environmental samples, 12/22 showed fermentation of CSB; nine of these produced presumptively positive colonies on chromogenic agar, of which eight were confirmed as *Cronobacter*.

**Comparison with current methods.** Samples were obtained from multiple factory sites and divided into 322 finished products (PIF), 146 ingredients, and 482 environmental samples (950 test samples in total, comprising a comprehensive survey of the infant formula manufacturing process). All samples were preenriched for 24 h at 37°C in BPW. Sample size varied

TABLE 1. Performance of mLST with chromogenic agar, CSB alone, and CSB with chromogenic agar for isolation of *Cronobacter* from spiked samples

Organism or result detected (total no. of strains)	No. of strains with results typical of <i>Cronobacter</i> spp.		
	mLST with X-TSA	CSB	CSB with X-TSA
<i>Cronobacter</i> (88)	84	88	88
<i>Cronobacter</i> plus nontarget strains (3)	2	3	3
Nontarget strains (56)	2	43	14
Uncontaminated aliquots (53)	0	0	0

depending on the nature of the material, and dilution in BPW was 1/10. For some samples, such as starches, chocolate powders, and vitamin-mineral premixes, a dilution of 1/100 was also used. Where feasible, raw materials were tested as 25-g aliquots, PIF were tested as 30 10-g, 10 30-g, or 2 150-g aliquots, and environmental swabs were preenriched in 10 ml BPW. Preenriched samples were analyzed using the FDA method (1) and the ISO/TS 22964 method (4) as well as the CSB method. For the last method, 0.1 ml of the preenriched sample was transferred to 10 ml CSB and incubated at 42°C for 24 h. In practice, only samples in which fermentation of the carbohydrate occurs, resulting in a color change from purple to yellow, need to be streaked onto chromogenic agar. However, in this study all broths were streaked onto a modified version of chromogenic *E. sakazakii* agar, DFI (Druggan, Forsythe, and Iverson) formulation (mDFI) (Oxoid Ltd., Basingstoke, United Kingdom). This medium comprised 7.0 g liter<sup>-1</sup> tryptone, 3.0 g liter<sup>-1</sup> yeast extract (8013-01-2), 5.0 g liter<sup>-1</sup> NaCl (7647-14-5), 0.15 g liter<sup>-1</sup> 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-glucopyranoside (108789-36-2), 0.25 g liter<sup>-1</sup> sodium desoxycholate (302-95-4), 1.0 g liter<sup>-1</sup> sodium thiosulfate (7772-98-7), 1.0 g liter<sup>-1</sup> ammonium iron(III) citrate (1185-57-5), and 13.0 g liter<sup>-1</sup> agar-agar (9002-18-0).

Although the FDA and ISO/TS 22964 methods cite the use of yellow pigmentation and biochemical tests as indicative of positive *Cronobacter* isolates (1, 4), these criteria have been shown to be unreliable (5, 9, 12). Therefore, in this study presumptive *Cronobacter* isolates obtained using the three methods were confirmed as positive by use of a *Cronobacter*-specific  $\alpha$ -glucosidase PCR assay (12). Biochemical tests and 16S rRNA gene sequencing (Fasteris S.A., Plan-les-Ouates, Switzerland) were used to indicate *Cronobacter* species identity (11, 13).

All three methods recovered *Cronobacter* from 2.17% (7/322) of end product samples. The CSB and ISO/TS 22964 methods both recovered target strains from 10.96% (16/146) of raw materials; however, the FDA method detected *Cronobacter* in only 3.42% (5/146) of raw materials. As these ingredients and products were from infant food production facilities, the sensitivity of the methods for detecting *Cronobacter* in these samples is of particular importance; both the ISO/TS 22964 and CSB methods were found to have 100% sensitivity and 100% NPV (Table 2). The advantage of using the CSB screening method is that a release decision can be made on negative samples within 48 h; also, the costs of materials and labor are considerably reduced, as only positive broths need to be streaked onto the chromogenic agars.

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TABLE 2. Recovery of *Cronobacter* spp. from product, raw materials, and environment by use of CSB in combination with mDFI agar and comparison with the FDA and ISO/TS 22964 methods<sup>a</sup>

Method	No. of confirmed <i>Cronobacter</i> isolates			Result for product and RM samples				Result for ENV samples			
	Product	RM	ENV	SE (%)	SP (%)	PPV (%)	NPV (%)	SE (%)	SP (%)	PPV (%)	NPV (%)
FDA	7	5	13	52.2	73.5	9	96.8	26	35.6	4.6	80.2
ISO/TS 22964	7	16	46	100	94.2	46.9	100	92	99.3	93.9	99.1
CSB with mDFI agar	7	16	47	100	93.9	46	100	94	99.3	94	99.3

<sup>a</sup> RM, raw materials; ENV, environmental samples; SE, sensitivity; SP, specificity.

The testing of environmental samples in production facilities is performed to verify the effectiveness of hygiene control measures aimed at minimizing product recontamination. Due to the higher level of bacterial flora in these samples, the specificity of a detection method becomes of increased importance. In this study, *Cronobacter* was recovered from a total of 10.37% (50/482) of the environmental samples by one or more methods. The CSB method recovered *Cronobacter* from 9.75% (47/482), the ISO/TS 22964 from 9.54% (46/482), and the FDA method from only 2.7% (13/482) of environmental samples. The strains that were recovered using the CSB method but not the ISO/TS 22964 method and vice versa were tested for the ability to grow in the respective media. All seven of these strains grew to  $>10^9$  CFU ml<sup>-1</sup> in CSB after 24 h at 42°C and fermented the sucrose, yielding a distinct yellow coloration. Also, all of these strains were able to grow when incubated for 24 h on mDFI and *Enterobacter sakazakii* identification agar at 42°C and 44°C, respectively, producing distinct blue-green colonies. However, only three of these strains were able to grow to  $>10^9$  CFU ml<sup>-1</sup> in mLST when incubated for 24 h at 44°C. The other four strains did not produce turbid cultures, and plate counts on TSA incubated for 24 h at 37°C showed that the final concentrations reached in the mLST ranged from  $2.2 \times 10^7$  to  $7.3 \times 10^7$  CFU ml<sup>-1</sup>, an increase of approximately 2 log from the inoculum. Therefore, the inability of the methods to recover strains from some samples is probably due to a combination of competition from other bacterial species in the samples and reduced growth rate in mLST. McNemar's test for marginal homogeneity with continuity correction showed there was no significant difference in performance of the CSB and ISO/TS 22964 methods (two-tailed *P* value of 1.0 and odds ratio of 0.750, with a 95% confidence interval extending from 0.11 to 4.43). For the environmental samples, both methods showed a high specificity and high NPVs and PPVs (Table 2). The sensitivity of the CSB method (94%) was slightly higher than that of the ISO/TS 22964 method (92%), demonstrating that despite the differential rather than selective nature of CSB, this method is equally suited for testing environmental samples, ingredients, and end products. Moreover, examples of all proposed *Cronobacter* species were isolated during this study, indicating that this method is suitable for detection of all members of this genus.

**Conclusions.** A differential screening medium, CSB, has been developed to identify samples potentially contaminated with *Cronobacter* spp. The broth is designed to circumvent the problems encountered with selective enrichment media for these organisms and to be complementary to currently available chromogenic media in order to improve overall sensitivity

and selectivity of *Cronobacter* detection. The CSB screening method was able to detect *Cronobacter* in spiked and in naturally contaminated samples. Potentially, this screening method can enable the decision to release uncontaminated product after 48 h. As CSB is a differential rather than selective enrichment broth, all *Cronobacter* strains are able to grow in CSB. This differential screening broth is complementary to any agar medium that incorporates a test for metabolism of  $\alpha$ -glucopyranoside. However, while CSB has been shown to be 100% sensitive for *Cronobacter* species, the overall effectiveness of the method is dependent on the sensitivity and specificity of the chromogenic agar used. The numbers of positive samples found from ingredients and the environment are in line with previous findings that *Cronobacter* spp. are ubiquitous environmental organisms that can be isolated from various food products as well as from households (8, 14).

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### 5.1.2 Evaluation of three commercially available real-time PCR based systems for detection of *Cronobacter* species

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## Short Communication

Evaluation of three commercially available real-time PCR based systems for detection of *Cronobacter* speciesC. Fricker-Feer<sup>a</sup>, N. Cernela<sup>b</sup>, S. Bolzan<sup>a</sup>, A. Lehner<sup>b</sup>, R. Stephan<sup>b,\*</sup><sup>a</sup> QA and Food Safety Department Hochdorf Nutritec AG, CH-6280 Hochdorf, Switzerland<sup>b</sup> Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, CH-8057 Zurich, Switzerland

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## ABSTRACT

In the last few years, various PCR based methods have been developed that enable detection of *Cronobacter* spp. to the genus and species level. Moreover, several real-time PCR based systems for detection of *Cronobacter* spp. are available, however, comparative evaluation studies are not available.

The current study represents a comparative evaluation of three commercial diagnostic systems, namely the BAX® System PCR Assay *Enterobacter sakazakii* (DuPont, Qualicon, Wilmington, USA), the Assurance GDS™ *Enterobacter sakazakii* (BioControl, Bellvue, USA) and the foodproof® *Enterobacter sakazakii* Detection Kit (Biotecon Diagnostics, Potsdam, Germany) for the rapid identification of *Cronobacter* spp.

Twenty-one target and non-target strains were included in the study and results were compared for specificity and convenience in performance. A specificity of 100% was observed for two of the three real time PCR systems tested, namely the Assurance GDS™ *Enterobacter sakazakii* and the foodproof® *Enterobacter sakazakii* Detection Kit for pure cultures as well as artificially contaminated powdered infant formula (PIF) samples.

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## 1. Introduction

Updating the original taxonomy of *E. sakazakii* by using different approaches has resulted in the definition of a new genus *Cronobacter* sp. with six new species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter turicensis*, and *Cronobacter genomospecies 1* (Iversen et al., 2008a; Kuhnert et al., 2009; Stephan et al., 2010). *Cronobacter* spp. are Gram-negative opportunistic foodborne pathogens and known as rare but important causes of live-threatening neonatal infections, which can lead to severe disease manifestations such as brain abscesses, meningitis, necrotizing enterocolitis and systemic sepsis (Lehner and Stephan, 2004). Neonates and infants under 2 months of age that are borne prematurely are at greater risk for infections and illness (Hunter et al., 2008). For some clinical cases an epidemiological link to contaminated powdered infant formulas (PIF) was found (van Acker et al., 2001; Weir, 2002). Therefore, for this food product the absence of *Cronobacter* spp. in 300 g (n = 30; c = 0, m = M = absence in 10 g) is required (Anonymous, 2005).

The current ISO/TS 22964:2006 technical standard method for detection of *Cronobacter* spp. includes a pre-enrichment step in buffered peptone water (BPW), enrichment in modified lauryl sulfate

tryptose (mLST) broth, plating on ESIA agar and picking of presumptive positive colonies onto tryptone soy agar (TSA) plates, which are incubated at 25 °C for 48–72 h. Yellow-pigmented colonies on the TSA plates are confirmed as *Cronobacter* spp. using biochemical identification kits. This procedure requires up to six days to confirm a positive result.

Therefore, there is a strong need for more rapid methods for detection and identification of *Cronobacter* spp. During the last few years, several conventional PCR based methods have been developed that enable the identification of *Cronobacter* spp. to the genus (Lehner et al., 2006; Mohan Nair and Venkitanarayanan, 2006; Iversen et al., 2007) and species (Stoop et al., 2009) level.

More recently, several real-time PCR based systems for detection of *Cronobacter* spp. became commercially available. However, evaluation data of these systems are so far very limited. Therefore, the aim of the present study was to evaluate three commercially available real-time PCR based systems for the detection of *Cronobacter* by including *Cronobacter* target as well as non-target strains. Moreover, the tests were also compared in view of their convenience in performance.

## 2. Materials and methods

## 2.1. Bacterial strains

The following target strains, representing the six described *Cronobacter* species and the three *C. dublinensis* subspecies, were selected for the evaluation of the test specificity: *C. sakazakii* (E601 = ATCC 25944),

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*C. malonaticus* (E825 = LMG 23826), *C. turicensis* (3032 = LMG 23827), *C. myyjtjensii* (E603 = ATCC 51329), *C. dublinensis* subsp. *dublinensis* (CFS 237 = LMG 23823), *C. dublinensis* subsp. *lausannensis* (E 515 = LMG 23824), *C. dublinensis* subsp. *lactaridi* (E 464 = LMG 23825), and *Cronobacter* genomospecies 1 (E 680).

Moreover, thirteen non-target strains were included in the study (*Escherichia hermannii* (DSM 4560), *Escherichia vulneris* (DSM 4564), *Enterobacter (E.) cloacae* (DSM 30054), *E. ludwigii* (DSM 166889), *E. cancerogenus* (DSM 17580), *E. asburiae* (DSM 17506), *E. radicincitans* (DSM 16656), *Leclercia adecaboxylata* (DSM 5077), *E. turicensis* (508/05 = DSM 18397), *E. helveticus* (513/05 = DSM 18396), *E. pulveris* (516/05), *Pantoea (P.) conspicua* (LMG 24534), and *P. eucrina* (LMG 2781)) (Table 1).

The selected non-target strains included species closely related to *Cronobacter* spp. as well as members of species that are frequently isolated from infant formula products and the processing environment (Popp et al., 2009).

## 2.2. Detection systems evaluated

The following commercially available real-time systems were evaluated in this study: BAX® System PCR Assay *Enterobacter sakazakii* (DuPont, Qualicon, Wilmington, USA) applied on the BAX System Q7®, Assurance GDS™ *Enterobacter sakazakii* (BioControl, Bellvue, USA) applied on the GDS Rotor Gene®, and the foodproof® *Enterobacter sakazakii* Detection Kit (BOITECON Diagnostics, Potsdam, Germany) applied on the LightCycler 2.0 PCR instrument (Roche Diagnostics (Schweiz) AG, Rotkreuz, Switzerland). The PCR targets of these systems are confidential and not available.

## 2.3. Evaluation of the test specificity

### 2.3.1. Pure culture experiments

Pure culture experiments were performed in variants. As the foodproof® *Enterobacter sakazakii* Detection Kit requires the use of extracted DNA, pure culture experiments were performed with

DNA that had been extracted from 1 ml of BHI grown overnight cultures of the respective strains using the foodproof® StartPrep One Kit (Biotecon Diagnostics, Germany) according to the manufacturer's protocol as well as the Qiagen Blood and Tissue Kit (Qiagen, Hombrechtikon, Switzerland).

For the two other systems (BAX® System PCR Assay and the Assurance GDS™ *Enterobacter sakazakii*) a BHI grown overnight culture (5 µl for the BAX System PCR Assay, 10 µl for the GDS™ *Enterobacter sakazakii* system) was used as starting material and further processed (lysis procedure) according to the manufacturer's instructions.

### 2.3.2. Spiked PIF experiments

Powdered infant formula was obtained from Hochdorf Nutritec AG, Switzerland and tested for the absence of *Cronobacter* spp. prior to experimental use. For experiments using the foodproof® *Enterobacter sakazakii* Detection Kit and the BAX® System PCR Assay, 10 g PIF was diluted with a 90 ml BPW and spiked with 100 µl of BHI grown overnight culture of respective target and non-target strains. After incubation at 37 °C for 24 h samples were diluted 1/10 in mLST broth and *Cronobacter* screening broth (CSB) (Iversen et al., 2008b), respectively and incubated at 44 °C for 24 h. Following the GDS™ *Enterobacter sakazakii* system's protocol, 10 g of PIF was diluted in 90 ml of prewarmed (42 °C) *Cronobacter* broth and incubated for 24–28 h at 42 °C.

For the foodproof® *Enterobacter sakazakii* Detection Kit DNA was extracted according to the manufacturer's protocol from 1 ml PIF mLST and CSB grown material, respectively, using the StartPrep One Kit (Biotecon Diagnostics, Germany) as well as the Qiagen Blood and Tissue Kit (Qiagen, Hombrechtikon, Switzerland). Five microliters of sample DNA was used for the PCR reaction. For the two other systems (BAX® System PCR Assay and the Assurance GDS™ *Enterobacter sakazakii*) the grown cultures (5 µl for the BAX® System PCR Assay, 10 µl for the GDS™ *Enterobacter sakazakii* system), were used as starting material and further processed (lysis procedure) according to the manufacturer's instructions.

**Table 1**

Specificity results for the different real time PCR based systems tested on 8 target and 13 non-target strains.

Species	Strain	Assurance GDS™ <i>Enterobacter sakazakii</i> (BioControl)	foodproof® <i>Enterobacter sakazakii</i> Detection Kit (BIOTECON)	BAX® System PCR Assay <i>Enterobacter sakazakii</i> (DuPont, Qualicon)
		PC/spiked PIF	PC/spiked PIF	PC/spiked PIF
<i>C. sakazakii</i>	E601	+/+	+/+	+/+
<i>C. malonaticus</i>	E825	+/+	+/+	+/+
<i>C. turicensis</i>	3032	+/+	+/+	+/+
<i>C. myyjtjensii</i>	E603	+/+	+/+	+/+
<i>C. dublinensis</i> subsp. <i>dublinensis</i>	CFS 237	+/+	+/+	-/-
<i>C. dublinensis</i> subsp. <i>lausannensis</i>	E515	+/+	+/+	+/+
<i>C. dublinensis</i> subsp. <i>lactaridi</i>	E464	+/+	+/+	+/+
<i>C. genomospecies 1</i>	E680	+/+	+/+	+/+
<i>E. hermannii</i>	DSM 4560	-/-	-/-	-/-
<i>E. vulneris</i>	DSM 4564	-/-	-/-	-/-
<i>E. cloacae</i>	DSM 30054	-/-	-/-	+/+
<i>E. ludwigii</i>	DSM 166889	-/-	-/-	-/-
<i>E. cancerogenus</i>	DSM 17580	-/-	-/-	-/-
<i>E. asburiae</i>	DSM 17506	-/-	-/-	-/-
<i>E. radicincitans</i>	DSM 16656	-/-	-/-	-/-
<i>L. adecaboxylata</i>	DSM 5077	-/-	-/-	-/-
<i>E. turicensis</i>	DSM 18397	-/-	-/-	-/-
<i>E. helveticus</i>	DSM 18396	-/-	-/-	-/-
<i>E. pulveris</i>	516/05	-/-	-/-	-/-
<i>P. conspicua</i>	LMG 24534	-/-	-/-	-/-
<i>P. eucrina</i>	LMG 2781	-/-	-/-	-/-

+ positive.

- negative.

PC: Pure culture experiments.

## 2.4. Results and discussion

### 2.4.1. Specificity

Inclusivity and exclusivity tests for the systems were performed in two different setups, namely the pure culture and the spiked PIF format. A specificity of 100% was observed for two of the three real time PCR systems tested, namely the Assurance GDS™ *Enterobacter sakazakii* and the foodproof *Enterobacter sakazakii* Detection Kit. With both kits all target and non-target strains were identified correctly in either types of sample material (pure culture and spiked PIF samples). No false positives or false negatives were observed (Table 1).

For the foodproof® *Enterobacter sakazakii* Detection Kit comparable results were found using DNA extracted with the alternative extraction Kit.

Using the BAX® System PCR Assay *Enterobacter sakazakii* seven of the eight *Cronobacter* strains were identified correctly in the pure BHI culture as well as in the spiked PIF mLST type of experiments. However, *C. dublinensis* strain CFS 237 was not identified by the system using both sample types. Additionally, a false positive result was obtained with *E. cloacae* during experiments with BHI grown culture template.

Parallel to the enrichment of spiked PIF samples in mLST additional experiments using *Cronobacter* screening broth (CSB) as alternative enrichment broth were performed by the BAX® System PCR Assay *Enterobacter sakazakii* and the foodproof® *Enterobacter sakazakii* Detection Kit, respectively. The results were similar to the results obtained with mLST (data not shown).

For the three systems a detection limit between 1000 cfu/ml (foodproof®) and 10,000 cfu/ml (BAX® System; Assurance GDS™) enrichment broth was found.

### 2.4.2. Convenience in performance

Using the foodproof® *Enterobacter sakazakii* Detection Kit, a DNA extraction has to be performed prior to the PCR, whereas with the BAX® System PCR Assay *Enterobacter sakazakii* as well as with the Assurance GDS™ *Enterobacter sakazakii* system an easy to perform sample preparation (lysis) procedure with the enrichment broth is foreseen.

For the foodproof® *Enterobacter sakazakii* Detection Kit the format is designed such, that the assays can be run on standard real time PCR instruments, whereas the Assurance GDS™ *Enterobacter sakazakii* system and the BAX® System both represent platforms, where the respective instrument has to be purchased. However, both platforms can also be used for the analysis of several other organisms of interest.

One advantage of dedicated systems such as BAX® System PCR Assay *Enterobacter sakazakii* and Assurance GDS™ *Enterobacter sakazakii* system is that no separate working station or even another room for DNA extraction is needed. Besides, the hands-on time is shorter, even though there are substantial differences in view of the running time for PCR amplification and detection (75 min for Assurance GDS™ *Enterobacter sakazakii* system, 3.5 h for BAX® System PCR Assay *Enterobacter sakazakii*).

In conclusion, this study shows, that specific and well performing real-time based PCR Kits for the detection of *Cronobacter* spp. in PIF are commercially available which should help to further reduce the risk of bringing contaminated powdered infant formula on the market.

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### 5.1.3 Detection, identification and typing methods for *Cronobacter* spp. – a literature review

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## Detection, identification and typing methods for *Cronobacter* spp. – a review\*

*Detektion, Identifizierung und (Sub-)typisierung von Cronobacter spp. –  
eine Literaturübersicht\**

Angelika Lehner, Claudia Fricker-Feer, Roger Stephan

*\* Dedicated to Prof. Dr. Karsten Fehlhaber on the occasion of his 65<sup>th</sup> birthday  
Herrn Prof. Dr. Karsten Fehlhaber zum 65sten Geburtstag gewidmet*

### Summary

Rapid and reliable identification of strains of the genus *Cronobacter* and its differentiation from phenotypically similar, apathogenic *Enterobacter turicensis*, *Enterobacter helveticus* and *Enterobacter pulveris* is important for surveillance, prevention and control of this food-borne pathogen. Moreover, for *Cronobacter* a species differentiation is relevant for epidemiological studies. This review summarizes methods for detection, identification and typing of *Cronobacter* spp.

**Keywords:** *Cronobacter* spp., detection, identification, typing

### Zusammenfassung

Die zuverlässige Identifizierung von Stämmen der Gattung *Cronobacter*, welche eine Unterscheidung von den phänotypisch ähnlichen, jedoch apathogenen Arten *Enterobacter turicensis*, *Enterobacter helveticus* und *Enterobacter pulveris* ermöglicht, ist unerlässlich in Bezug auf die Überwachung, Prävention und Kontrolle dieses Lebensmittel-assoziierten pathogenen Organismus. Darüber hinaus stellt die Zuordnung von *Cronobacter* Isolaten auf Speziesebene die Voraussetzung zur Bearbeitung epidemiologischer Fragestellungen dar. Dieser Artikel gibt eine Übersicht über (etablierte) Methoden zur Detektion, Identifikation und (Sub-)typisierung von *Cronobacter* spp.

**Schlüsselwörter:** *Cronobacter* spp., Nachweis, Identifizierung, Typisierung

## Introduction

The genus *Cronobacter* (former *E. sakazakii*) comprises 6 species, *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter mytjensii*, *Cronobacter dublinensis*, *Cronobacter turicensis*, *Cronobacter* genomospecies 1, which have been recognized on the basis of a polyphasic approach using extensive geno- and phenotypic evaluations (Iversen et al., 2007a; Iversen et al., 2007b, Iversen et al., 2008b). The definition of six species within the genus *Cronobacter* is further supported by a recent study, where a multilocus sequence analysis (MLSA) approach was used (Kuhnert et al., 2009).

*Cronobacter* spp. are Gram-negative opportunistic foodborne pathogens and known as rare but important causes of live-threatening neonatal infections, which can lead to severe disease manifestations such as brain abscesses, meningitis, necrotizing enterocolitis and systemic sepsis (Lehner and Stephan, 2004). Neonates and infants under two months of age which were borne prematurely are at greater risk of *Cronobacter* infections from consuming *Cronobacter* contaminated powdered infant formulas (Hunter et al., 2008).

Rapid and reliable identification of strains of the genus *Cronobacter* and its differentiation from phenotypically similar, apathogenic *Enterobacter turicensis*, *Enterobacter helveticus* and *Enterobacter pulveris* is important for surveillance, prevention and control of food-borne diseases. Moreover, for *Cronobacter* spp. a species differentiation is relevant for epidemiological studies, and the different species show differences in sensitivity to chemical agents and antibiotics. The aim of this review is to summarize the knowledge on detection, identification and typing methods for *Cronobacter* spp.

## Detection procedures

In 2002, the United States Food and Drug Administration (FDA) published a method for detection of *E. sakazakii* which included a pre-enrichment step in buffered peptone water (BPW), enrichment in *Enterobacteriaceae* Enrichment (EE) broth, plating on Violet Red Bile Glucose agar (VRBG) and picking of five typical colonies onto tryptone soy agar (TSA) plates (Anonymous, 2002). After incubation at 25 °C for 48–72 hours, yellow pigmented colonies on TSA plates are confirmed using the API 20E system. The main weak points of this procedure are the inability of some target strains to grow in the selective EE broth; the lack of discrimination between *Enterobacteriaceae* strains on VRBG; the variation in intensity of the pigmentation, with occasional observation of non-pigmented strains; and the weak reliability of the API 20E system (Iversen et al., 2007b). Gentil et al. (2005) published an alternative procedure based on selective enrichment in a modified lauryl sulphate tryptose broth (mLST), incorporating 0.5 M NaCl and 10 mg l<sup>-1</sup> vancomycin hydrochloride. This method was further improved by the replacement of VRBG with a chromogenic agar and forms the basis of the ISO Technical Specification for detection of *E. sakazakii* in milk-based infant formula, ISO/TS 22964:2006 “Milk and milk products – detection of *Enterobacter sakazakii*”.

Various chromogenic and fluorogenic agar media have been described in recent years for detection of *Cronobacter* spp. (Iversen et al., 2004; Oh and Kang, 2004; Restaino et

al., 2006). These are based mainly on the enzyme  $\alpha$ -glucosidase, which is constitutively expressed in *Cronobacter* spp., other organisms also produce presumptive colonies on these agars, notably the recently described species *E. helveticus*, *E. turicensis* and *E. pulveris* (Stephan et al., 2007; Stephan et al., 2008). These species can be found in the same ecological niches as *Cronobacter*, such as dried food products and factory environments and present a challenge to both culture-based as well as molecular isolation and identification methods.

Meanwhile, it has been established that some isolates of *Cronobacter* spp. do not grow well in mLST currently proposed for isolation of these organisms (Iversen and Forsythe, 2007). Therefore, Iversen et al. (2008a) developed a new *Cronobacter* Screening Broth (CSB). The broth is designed to circumvent the problems encountered with selective enrichment media for these organisms and to be complementary to current available chromogenic media in order to improve overall sensitivity and selectivity of *Cronobacter* spp. detection.

Moreover, modifications to the composition of BPW with a view to inhibition of competing Gram-positive background flora may potentially improve recovery of Gram-negative organisms from samples and will be a challenge for the future. Such an improvement may have useful application as a pre-enrichment step in other microbiological culture methods for recovery of Gram-negative pathogens, such as *Salmonella* spp. and *Cronobacter* spp., as well as indicator organisms.

An alternative method to avoid the difficulties of selective broths is the MATRIX PSAK50 Method (Matrix MicroScience Ltd, UK), which uses cationic paramagnetic particle capture to concentrate contaminating microorganism in a pre-enriched sample before plating directly onto isolation agar.

Other proposed rapid methods for the detection of *E. sakazakii* include two enzyme-linked immunoassays (EIAs): the Assurance for *Enterobacter sakazakii* (Bio-Control Systems, USA) and the TECRA HELIX *E. sakazakii* Method (TECRA International, Australia).

## Phenotypic identification

To ensure the safety of infant formula and also to reduce unnecessary disposal of product, it is important to identify *Cronobacter* spp. as accurately as possible. The recently developed fluoro- and chromogenic media are useful tools in the phenotypic selection of presumptive *Cronobacter* spp. However *E. helveticus*, *E. pulveris*, *E. turicensis* and *E. cloacae*-complex species may also give characteristic colonies on some of these media and further confirmation is essential. The phenotypic identification of *Cronobacter* spp. with available commercial systems may be difficult (Iversen et al., 2007b). Molecular methods revealed that several strains identified as *E. sakazakii* by commercial biochemical kits belonged to *E. cloacae*-complex species (Iversen et al., 2007a).

## Molecular based identification

### Genus-specific detection and identification

A number of molecular approaches have been developed to specifically identify *Cronobacter* spp. strains. Conven-

tional and real-time PCR methods enable (quantitative), sensitive, specific and rapid detection from enrichment broths and agar media. Targets for conventional PCR systems include the 16S rRNA gene (Lehner et al., 2004), the *ompA* gene (Mohan Nair and Ventkitanarayanan, 2006), the gene coding for the 1,6  $\alpha$ -glucosidase (Lehner et al., 2006a) and a gene encoding a zinc-containing metalloprotease (Kothary et al., 2007). The 'real-time'-assays that have been described so far, target the 16S rRNA gene (Kang et al., 2007), a region located between the 16S rRNA and the 23 rRNA genes (Liu et al., 2006), a region between the tRNA-glu and 23S rRNA genes (Derzelle and Dilasser, 2006) and the *dnaG* gene (Seo and Brackett, 2005).

The U.S. Food and Drug Administration has also developed a revised method involving an enrichment step, centrifugation and a combination of two chromogenic agars and the *dnaG* gene PCR for confirmation (Chen et al., 2009).

In the last few years, several real-time PCR based systems for detection of *Cronobacter* spp. are also commercially available. In a recent study a comparative evaluation of three diagnostic systems, namely the BAX<sup>®</sup> System PCR Assay *Enterobacter sakazakii* (DuPont, Qualicon, Wilmington, USA), the Assurance GDS<sup>™</sup> *Enterobacter sakazakii* (BioControl, Bellvue, USA) and the foodproof<sup>®</sup> *Enterobacter sakazakii* Detection Kit (Biotecon Diagnostics, Potsdam, Germany) was done (Fricker-Feer et al., 2011). A specificity of 100 % was observed for two of the three real time PCR systems tested, namely the Assurance GDS<sup>™</sup> *Enterobacter sakazakii* and the foodproof<sup>®</sup> *Enterobacter sakazakii* Detection Kit for pure cultures as well as artificially contaminated powdered infant formula (PIF) samples.

The VIT<sup>®</sup> (vermicon identification technology, Munich, Germany) represents an alternative to the DNA-targeted PCR-based detection and identification systems for *Cronobacter* spp. It is based on fluorescently labelled gene probes targeting specified regions on the ribosomal RNA of the bacteria, therefore only live cells are detected by the system. The test is performed on 1 ml of overnight culture of enrichment broth or rich media broth (e.g. BHI, LB) after inoculation with presumptive colony material according to the instructions of the manufacturer. The method has successfully been used in a comparative study evaluating cultural and molecular identification systems for *Cronobacter* spp. (Lehner et al., 2006b) Another hybridization based method using a peptide nucleic acid probe for the specific detection of *Cronobacter* genospecies has been published (Almeida et al., 2008).

#### Species-specific identification

As identification of *Cronobacter* spp. isolates to the species level is required for epidemiological studies, a PCR system for the differentiation of the six proposed species was developed in a recent study (Stoop et al., 2009). The *rpoB* gene was chosen as target to develop different conventional PCR systems, which enable strains previously confirmed as belonging to the genus *Cronobacter* to be further discriminated to the species level. However, as the *rpoB* gene sequences for *C. sakazakii* and *C. malonaticus* are very closely related, a two-step procedure using the primers for the identification of *C. malonaticus* in a follow-up PCR on those strains positive for the *C. sakazakii* identification assay is necessary to reliably distinguish these species.

#### Identification by MALDI-TOF MS

The detection of protein mass patterns using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has become a convenient tool for the rapid analysis of bacteria. The method analyzes the profiles of proteins that are extracted from whole bacteria. In the study by Stephan et al. (2010) genus- and species-specific biomarker protein mass patterns were determined for the identification of the six *Cronobacter* species recognized so far. A reference MS database library including representative number of *Cronobacter* spp. target as well as non-target (e.g. phenotypically similar, non-pathogenic *Enterobacter helveticus*, *Enterobacter pulveris*) strains was established and included in the Spectral Archive and Microbial Identification System (SARAMIS) Super Spectrum database. Validation of the mass-spectrometry-based identification scheme yielded identical results as with PCR-based identification system demonstrating that MALDI-TOF MS is a reliable and powerful tool for the rapid identification of *Cronobacter* spp. strains to the genus and species level.

#### Subtyping methods

A number of methods have been developed that can be used to subtype strains of *Cronobacter* spp. including biotyping, serogrouping, plasmid profiling, ribotyping, random amplification of polymorphic DNA (RAPD), repetitive sequence primed PCR (REP-PCR), enterobacterial repetitive intergenic consensus (ERIC) PCR, amplified fragment length polymorphism (AFLP) and Pulsed-Field Gel Electrophoresis (PFGE). Techniques that generate a DNA fingerprint pattern are more discriminatory and reliable than classic techniques such as biotyping. PFGE is currently seen as the 'gold standard' for molecular subtyping of *Cronobacter* spp. and has been used to investigate outbreaks in neonatal intensive care units. It has also been successfully used to trace the dissemination of *Cronobacter* spp. strains within manufacturing facilities.

#### PFGE

Pulsed-Field Gel Electrophoresis (PFGE) is a non-amplified technique that separates long strands of DNA molecules (larger than 15–20 kb) previously digested with restriction enzyme(s). Periodical switching of the voltage in three directions results in the reorientation of DNA moving through the gel in a size dependent manner, which facilitates a finer resolution as it aligns and re-aligns to the applied electrical field.

In 1994 an outbreak occurred in France involving 17 neonates *Cronobacter* spp. isolates were obtained from various anatomical sites, prepared feeds and unused infant formula. PFGE analysis described four clusters, two of which contained isolates from neonates who ranged from asymptomatic colonised individuals to case fatalities. One other cluster contained isolates from the prepared feed, an asymptomatic neonate and a neonate with mild digestive problems. The fourth cluster comprised the isolates from the unused powdered formula. In this case no link could be made between the powdered formula and the outbreak and it is possible that the prepared feed became contaminated via an alternate source (Caubilla-Barron et al., 2007). A further outbreak occurred in France in 2004 involving 13

infants, nine were asymptotically colonised, two developed bacterial meningitis, one had conjunctivitis and one suffered haemorrhagic colitis. The outbreak was investigated using automated ribotyping (using restriction endonucleases *EcoRI*, *PstI* and *PvuII*) and by PFGE using the enzymes *XbaI* and *SpeI*. A total of nine isolates from eight neonates were found to have undistinguishable ribotypes and PFGE patterns from isolates obtained from four separate lots of infant formula, thus establishing a clear link between the outbreak and the product. Recently, factory surveillance studies have employed a PFGE method using *XbaI* for tracing *Cronobacter* spp. isolates within infant food manufacturing facilities (Mullane et al., 2008a; Iversen et al., 2009). Recently, in conjunction with the PulseNet Programme at the Centres for Disease Control and Prevention (CDC) in the US, a collaborative study was done in several international laboratories to develop a standard protocol for *Cronobacter* spp. PFGE typing. This protocol should be published soon.

#### REP-PCR

Although PFGE is considered the gold standard for the detection of clonality in disease outbreaks, PCR-based methods are cheaper, easier to perform, and provide faster results.

Recently the discriminative power of REP-PCR has been compared to PFGE for *Cronobacter* spp. isolates (Healy et al., 2008). Using Simpson's index of diversity, values of 0.974 and 0.998 were calculated for REP-PCR and PFGE respectively at a similarity cut-off of 95 % demonstrating good correlation with a high degree of genetic heterogeneity among the isolates.

#### RAPD

Random amplification of polymorphic DNA (RAPD) typing has been used to analyse clonal relationships between *Cronobacter* spp. strains in a number of independent studies (Drudy et al., 2006; Iversen et al., 2009). Nazarowec-White and Farber (1999) developed protocols for the molecular subtyping of *Cronobacter* spp. by ribotyping, RAPD and pulsed-field gel electrophoresis (PFGE). The authors showed that RAPD and PFGE were the most discriminatory sub-typing schemes for *Cronobacter* spp. followed by ribotyping and two microbiological typing methods – biotyping and antibiograms. However, the transferability of RAPD results to other labs can be a limitation of this technique. Moreover, in contrast to the PFGE method no databases of strain patterns exist for the RAPD technique.

#### Ribotyping

Ribotypes are generated by probing restriction fragments of genomic DNA for the highly conserved genes coding for the 16S and 23S rRNA. Small variations between strains occur in the less conserved, flanking genes and intergenic sections of the genome resulting in fragments of unequal size. These are separated using gel electrophoresis, transferred to a membrane and the individual strain fingerprints revealed by hybridization of chemiluminescent probes. Riboprint patterns are analyzed based on the number, size and signal intensity of the detected fragments. Comparison to existing entries in a riboprint database allows species- and subspecies-level identification.

The automated RiboPrinter<sup>™</sup> Microbial Characterization System (Dupont Qualicon, USA) was used as part of a polyphasic taxonomic characterisation of *Cronobacter*

spp. strains (Iversen et al., 2007a). Isolates were grown on TSA (18 h, 37 °C) and prepared according to standard procedures (Bruce, 1996) using the *EcoRI* restriction enzyme. The strains were divided into four distinct clusters, *C. dublinensis*, *C. turicensis* and *C. muytjensii* occupied individual clusters whereas *C. malonaticus* appeared as one subcluster among several subclusters of *C. sakazakii*. Nazarowec-White and Farber (1999) studying three *Cronobacter* spp. isolates obtained from one hospital over 11 years showed that they had indistinguishable ribotype patterns indicating possible persistence in the environment.

#### BOX-PCR

Proudy et al. (2008) examined the discriminative power of the 154 bp BOX element against the sequencing of the *fliC* gene and PFGE using 27 *Cronobacter* strains from clinical and environmental sources. The BOX-PCR results showed 92 % agreement with PFGE results indicating the potential of this typing method for epidemiological investigation, whereas *fliC* gene sequencing was poorly discriminative.

#### MLVA

Variable number tandem repeat (VNTR) motifs represent sources of genetic polymorphisms. These DNA sequence elements are often maintained within a bacterial species, with individual strains displaying different copy numbers. The length of a tandem repeat at a specific locus can vary as a consequence of DNA slippage during replication or unequal crossover elements. These differences can be analyzed by amplification of the region and sizing of the resulting amplicons. The high degree of polymorphism at these loci is particularly useful as a target for strain discrimination within bacterial species. Multi-locus VNTR analysis (MLVA) is a subtyping method that involves amplification and fragment size comparison of polymorphic VNTR regions. The availability of a complete *C. sakazakii* genome sequence ([http://genome.wustl.edu/pub/organism/Microbes/Enteric\\_Bacteria/Enterobacter\\_sakazakii/assembly/Enterobacter\\_sakazakii-4.0/](http://genome.wustl.edu/pub/organism/Microbes/Enteric_Bacteria/Enterobacter_sakazakii/assembly/Enterobacter_sakazakii-4.0/)) enabled the identification of VNTR motifs within *C. sakazakii*. Subsequently an MLVA subtyping scheme was developed and applied on a genotypically and phenotypically diverse collection of *Cronobacter* spp. isolates (Mullane et al., 2008c).

#### AFLP

The amplified fragment length polymorphisms (AFLP) technique has been employed in plant and microbiological research to describe the molecular ecology of various niches and can be used to determine inter- and intra-species relatedness. This technique was included in a study by Iversen et al. (2007a) to clarify the taxonomic relationship of over 200 strains previously identified as *Cronobacter* spp. and gave discriminatory results comparable to DNA-DNA hybridization.

#### Molecular based serotyping

Serotyping assays based on PCR specific O-antigen genes were developed for the identification of *C. sakazakii* serotypes O1 and O2 using PCR primers specific to the O1 *wehC* gene and the O2 *wehI* gene (Mullane et al., 2008b). In a more recent study, six *Cronobacter wzw* genes were sequenced and analyzed leading to the development of three O-antigen cluster-specific PCR primer pairs, which identify five new *Cronobacter* serotypes (Jarvis et al., 2011). Genomic DNA from 231 *Cronobacter* spp. isolates

were screened with these primer pairs with *C. sakazakii* having the most diverse serotype profile of all species included in this study. However, twenty-one percent of the isolates in their collection were negative by the *Cronobacter* serotype-specific PCR assays developed to date, including all of the *C. dublinensis* and *C. genomospecies 1* strains. Similarly, most of the *C. muytjensii* and *C. turicensis* strains tested in the study were negative by all of the serotype PCR assays, suggesting that there are additional serotypes within the *Cronobacter* genus.

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**5.1.4 Identification of the recently described *Cronobacter condimenti* by a *rpoB* based PCR system**

Lehner, A.<sup>1</sup>, Fricker-Feer, C.<sup>2</sup>, Stephan, R.<sup>1\*</sup> (2012).

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## Identification of the recently described *Cronobacter condimenti* by an *rpoB*-gene-based PCR system

*Enterobacter sakazakii* is an important foodborne pathogen, which can cause meningitis, necrotizing enterocolitis and bacteraemia in neonates. Updating the original taxonomy of *E. sakazakii* by using a polyphasic approach has resulted in the definition of six novel species (*C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis* and *Cronobacter* sp. genomospecies 1) and the transfer of these species to a novel genus, *Cronobacter* (Iversen *et al.*, 2007, 2008), in order to facilitate their continued inclusion in schemata for diagnosis, with the novel genus being contaxic with *E. sakazakii*. In a study by Stoop *et al.* (2009), an *rpoB*-gene-targeted PCR assay was developed, which enables the identification of members of the genus *Cronobacter* to the species level. In a recent study by Joseph *et al.* (2012) an additional species, *C. condimenti*, was described within the genus *Cronobacter*, and a novel species designation, *C. universalis*, was proposed for the formerly named *Cronobacter* sp. genomospecies 1. However, as of yet, no species-specific identification systems are available for *C. condimenti*. Here, we report an extension of the *rpoB*-gene-based PCR system (Stoop *et al.*, 2009) for identification of this species. The *rpoB* gene encodes the  $\beta$ -subunit of the bacterial ribosomal polymerase, which is a well-recognized polymorphic marker and is useful for the identification of bacteria. The *rpoB* gene sequence of *C. condimenti* 1330<sup>T</sup> (=CECT 7863<sup>T</sup> =LMG 26250<sup>T</sup>) was deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) under the accession number JQ316670.

A primer pair (Ccon-f: 5'-AACGCCAAGCCAATCTCG-3'; and Ccon-r: 5'-GTACCGCCACGTTTTGCT-3') was designed by multiple alignment analysis of the *rpoB* gene sequences of

The GenBank/EMBL/DDDBJ accession number for the *rpoB* gene sequence of *C. condimenti* 1330<sup>T</sup> obtained in this study is JQ316670.

members of all species of *Cronobacter* described thus far using CLUSTAL W (Thompson *et al.*, 1994). Specificity of the PCR assay was evaluated using *C. condimenti* 1330<sup>T</sup> as positive control and 27 non-target strains, comprising isolates from human, food and environmental origins, including: four *C. sakazakii* strains (E601, E602<sup>T</sup>=ATCC 29544<sup>T</sup>, E604 and E828), four *C. malonaticus* strains (E265, E621, E825 and E829<sup>T</sup>=LMG 23826<sup>T</sup>), five *C. turicensis* strains (z3032<sup>T</sup>=LMG 23827<sup>T</sup>, E626, E676, E681 and E688), two *C. universalis* (formerly *C. genomospecies* 1) strains (E797<sup>T</sup>=NCTC 9529<sup>T</sup> and E680), five *C. muytjensii* strains (E603<sup>T</sup>=ATCC 51329<sup>T</sup>, E456, E488, E616 and E769), and seven *C. dublinensis* strains (*C. dublinensis* subsp. *dublinensis* DES187<sup>T</sup>=LMG 23823<sup>T</sup>, *C. dublinensis* subsp. *lausannensis* E515<sup>T</sup>=LMG 23824<sup>T</sup> and *C. dublinensis* subsp. *lactaridi* strains E464<sup>T</sup>=LMG 23825<sup>T</sup>, E465, E791, E798 and E799). These strains were part of a taxonomic study performed by Iversen *et al.* (2007, 2008). The use of these previously characterized strains provided a valid basis for the assessment of the specificity data.

For PCR amplification, mixtures were prepared (total volume 50  $\mu$ l) containing GoTaq Green Master Mix (Promega) with 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs each and 10 pmol primers (final concentrations). Thermal cycling was carried out using an initial denaturation step of 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 30 s and elongation at 72 °C for 60 s. The amplification product was resolved on a 1% agarose gel followed by ethidium bromide staining and examination under UV light. The PCR assay was performed using both extracted DNA (10 ng per reaction) (DNeasy Blood & Tissue kit, Qiagen) as well as boiled colony material. The latter method included the resuspension of one colony (grown on tryptic soy agar) in 100  $\mu$ l distilled water, followed by lysis of cells by

heating the suspension at 100 °C for 10 min and final separation of the DNA from cellular debris by centrifugation for 2 min at 10 000 g. Five microlitre volumes of the resulting supernatants were used for the PCR.

With the primer pair Ccon-f/Ccon-r, the target strain yielded the expected amplification product size (689 bp), whereas no amplification product was obtained for all the non-target strains (100% specificity).

In conclusion, we propose that this extended *rpoB*-gene-based PCR assay, in combination with a *Cronobacter* genus specific PCR (Lehner *et al.*, 2006), is a reliable and time saving method to detect all members of the genus *Cronobacter* described so far. We would like to stress the fact that the PCR primers previously described for the identification of *C. genomospecies* 1 (Stoop *et al.*, 2009) may also be used for the identification of *C. universalis*.

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## 5.2 Epidemiology on *Enterobacteriaceæ*

### 5.2.1 Identification of *Enterobacteriaceæ* isolates from raw ingredients, environmental samples and products of an infant formula processing plant

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## Identification of *Enterobacteriaceae* isolates from raw ingredients, environmental samples and products of an infant formula processing plant

*Identifizierung von Enterobacteriaceae Isolaten aus verschiedenen Proben eines Produktionsbetriebes für Säuglingsanfangsnahrung*

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### Summary

Over the past few years the group of *Enterobacteriaceae* has considerably gained in importance for food industries, especially for infant formula processing plants. Up to now, only a few studies have focused on the diversity of this group in powdered infant formula and related products. Therefore, the aim of this study was to identify *Enterobacteriaceae* isolates from a previous study from different samples of an infant formula processing plant. A total of 470 isolates from raw ingredients (n = 117), environmental samples (n = 166) and finished products (n = 187) were further identified by biochemical tests as well as by *rpoB* sequencing. The most common species was *Enterobacter cloacae* (n = 161), followed by *Pantoea* spp. (n = 51) and *Klebsiella pneumoniae* (n = 39). In total, 65 isolates could not be identified. Using the partial *rpoB* gene sequence data as a similarity matrix, 22 different clusters could be formed within these 65 isolates. The fact that some isolates from the same *rpoB* cluster originate from different sample types might indicate that they are widely common within such a production environment and hence of practical relevance. These isolates which form possibly novel genus/species will be further investigated.

**Keywords:** powdered infant formula, infant food, *Enterobacteriaceae*

### Zusammenfassung

Im Verlauf der letzten Jahre hat die Bedeutung der Gruppe der *Enterobacteriaceae* in der Lebensmittelindustrie, speziell in Produktionsbetrieben für Säuglingsanfangsnahrung, deutlich zugenommen. Bis zum heutigen Zeitpunkt wurden nur wenige Studien durchgeführt, die schwerpunktmäßig die Diversität dieser Gruppe in Säuglingsanfangsnahrung in Pulverform und ähnlichen Produkten untersuchten. Demzufolge war das Ziel dieser Studie, *Enterobacteriaceae* Isolate, die in einer vorhergehenden Untersuchung aus verschiedenen Proben aus einem Produktionsbetrieb für Säuglingsanfangsnahrung isoliert wurden, näher zu identifizieren. Insgesamt wurden 470 Isolate aus Ausgangsmaterialien (n = 117), Umgebungsproben (n = 166) und Endprodukten (n = 187) mittels biochemischer Tests sowie *rpoB* Sequenzierung näher identifiziert. Die am häufigsten nachgewiesene Spezies stellte *Enterobacter cloacae* dar (n = 161), gefolgt von *Pantoea* spp. (n = 51) und *Klebsiella pneumoniae* (n = 39). Insgesamt konnten 65 Isolate nicht identifiziert werden. Unter Verwendung von *rpoB* Gensequenzdaten als Ähnlichkeitsmatrix konnten 22 verschiedene Cluster innerhalb dieser 65 Isolate gebildet werden. Die Tatsache, dass manche Isolate aus demselben *rpoB* Cluster aus verschiedenen Probentypen stammten, könnte darauf hinweisen, dass sie in derartigen Produktionsstätten weit verbreitet und somit von praktischer Bedeutung sind. Diese Isolate, welche möglicherweise neue Gattungen oder Arten darstellen, werden noch genaueren Untersuchungen unterzogen werden.

**Schlüsselwörter:** Säuglingsanfangsnahrung, *Enterobacteriaceae*

## Introduction

In Regulation (EC) 2073/2005 (Anonymous, 2005) the European Commission states that *Salmonella* and *Cronobacter* spp. are the microorganisms of greatest concern in powdered infant formula. The presence of these pathogens constitutes a considerable risk when conditions allow growth of the bacteria. In particular, *Cronobacter* spp. are known to cause infections in neonates with severe outcomes such as sepsis, meningitis or necrotizing enterocolitis due to contaminated infant formula (Biering et al., 1989; Bar-Oz et al., 2001; van Acker et al., 2001; Himelright et al., 2002).

*Enterobacteriaceae* can be used as process hygiene criteria for various products. Notably for powdered infant formula the tolerance levels are low (Anonymous, 2005). Up to now, only a few studies have focused on the diversity of this group in infant formula (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006). Since *Enterobacteriaceae* should not survive the heating processes used during manufacture, a recontamination of the powdered infant formula after this process step must be assumed. Thus, also the presence and diversity of *Enterobacteriaceae* in the production environment and

in raw ingredients is of interest. The aim of this study was therefore to identify *Enterobacteriaceae* isolates other than *Cronobacter* spp. and *Salmonella*, which were gained from different sample types (raw ingredients, environment, finished products) of an infant formula processing plant.

## Material and Methods

### Isolates

A total of 470 *Enterobacteriaceae* isolates (117 isolates from raw ingredients; 166 isolates from environmental samples; 187 isolates from finished products) were gained from an infant formula processing plant within a previous study (Iversen et al., 2009). The raw ingredients comprised milk, flours, starches, sugars, fruit and vegetable flakes and powders, vitamins and minerals, flavorings and emulsifiers. The environmental samples covered the manufacturing process including filling and storage equipment, walls, floors, handles, filters, cleaning equipment, dust, sinks, elevators and personnel. The finished products incorporated a range of food brands for infants of all ages and nutritional needs.

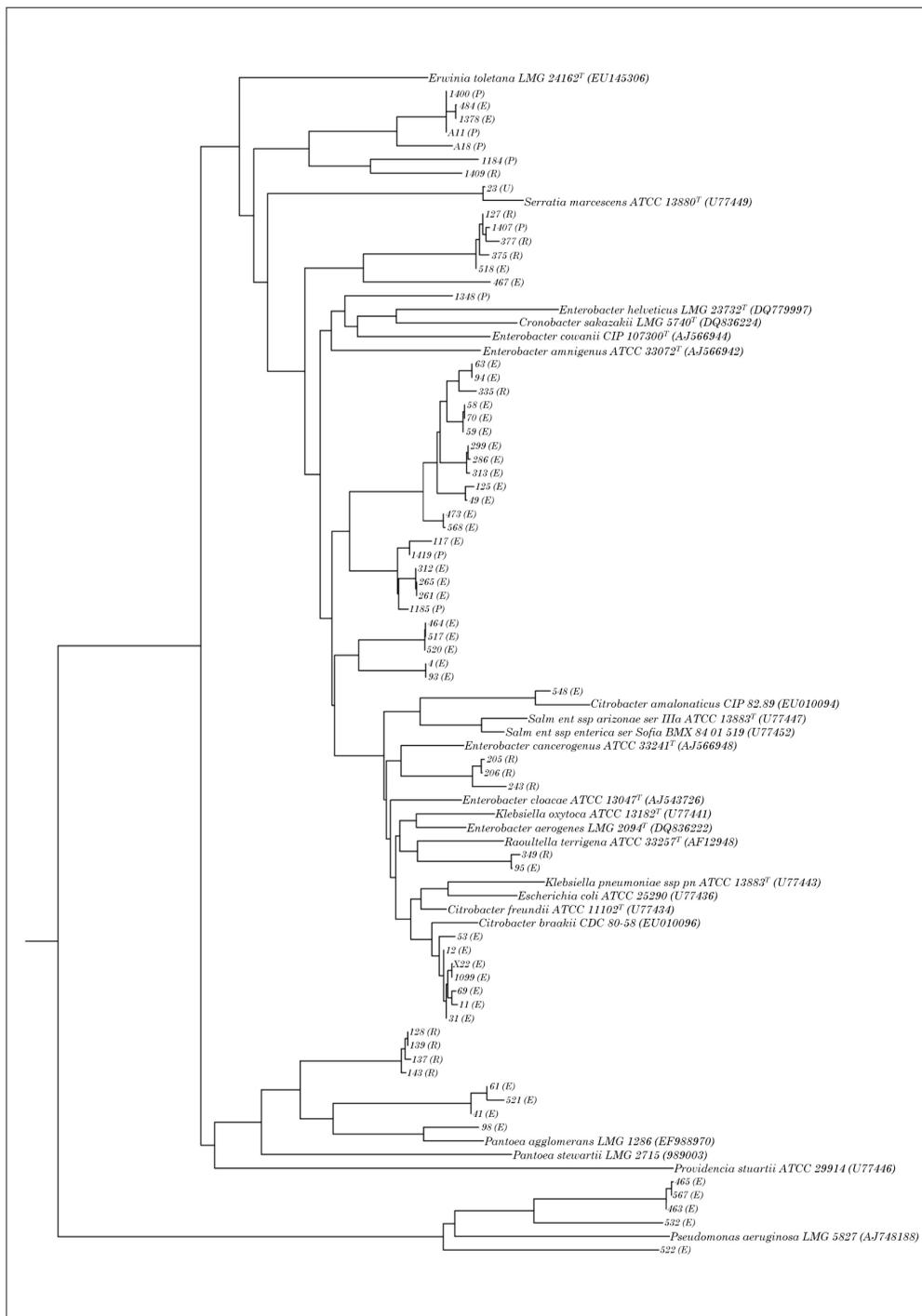
**TABLE 1:** Identification of 470 isolates from different samples of an infant formula processing plant by API ID32E and *rpoB* sequencing

Genus, species	Raw ingredient			Product			Environment		
	A <sup>1</sup>	S <sup>2</sup>	T <sup>3</sup>	A <sup>1</sup>	S <sup>2</sup>	T <sup>3</sup>	A <sup>1</sup>	S <sup>2</sup>	T <sup>3</sup>
<i>Burkholderia agrestis</i>	1	–	1	–	–	–	–	–	–
<i>Citrobacter amalonaticus/farmeri</i>	–	–	–	1	–	1	2	–	2
<i>Citrobacter braakii</i>	–	–	–	–	–	–	3	–	3
<i>Citrobacter freundii</i>	–	–	–	–	–	–	6	–	6
<i>Enterobacter cloacae</i>	23	7	30	72	23	95	22	14	36
<i>Enterobacter cancerogenus</i>	3	–	3	8	–	8	–	–	–
<i>Enterobacter pulveris</i>	–	4	4	–	2	2	–	–	–
<i>Enterobacter helveticus</i>	–	2	2	–	1	1	–	8	8
<i>Enterobacter amnigenus</i>	1	–	1	1	–	1	2	–	2
<i>Enterobacter asburiae</i>	–	1	1	–	–	–	–	–	–
<i>Enterobacter cowanii</i>	–	2	2	–	–	–	–	–	–
<i>Enterobacter turicensis</i>	–	3	3	–	–	–	–	–	–
<i>Escherichia coli</i>	3	–	3	8	–	8	8	1	9
<i>Escherichia hermannii</i>	3	–	3	6	–	6	7	–	7
<i>Escherichia vulneris</i>	1	–	1	–	–	–	4	–	4
<i>Hafnia alvei</i>	2	–	2	–	–	–	1	–	1
<i>Klebsiella pneumoniae</i>	4	–	4	27	1	28	6	1	7
<i>Klebsiella oxytoca</i>	2	2	4	10	2	12	7	4	11
<i>Klebsiella singaporensis</i>	–	–	–	–	–	–	–	1	1
<i>Klebsiella varicola</i>	–	1	1	–	–	–	–	–	–
<i>Leclercia adecarboxylata</i>	11	–	11	6	–	6	10	–	10
<i>Pantoea</i> spp.	26	–	26	11	–	11	13	1	14
<i>Raoultella terrigena</i>	1	1	2	–	–	–	–	–	–
<i>Serratia nematodiphila</i>	–	–	–	–	–	–	–	1	1
Unknown	–	13	13	–	8	8	–	44	44

<sup>1</sup> Isolates that could be identified with API ID32E.

<sup>2</sup> Isolates with insufficient API results where *rpoB* sequencing had to be done in addition.

<sup>3</sup> Total.



**FIGURE 1:** Neighbour-joining tree of *rpoB* gene sequences showing the estimated phylogenetic relationships between unidentified isolates and reference strains. Constructed with ClustalW (on <http://align.genome.jp/>) with the following multiple alignment parameters: gap open penalty: 15, gap extension penalty: 6.66, weight transition: no, Hydrophilic Gaps: yes, Selected Weight Matrix: IUB. Origin of the unidentified isolates in brackets. R = raw ingredients, P = finished products, E = environment.

## Identification

### Biochemical identification

For this study, only isolates which could not be identified in the previous study (Iversen et al., 2009) as *Cronobacter* spp. were further analyzed. All isolates were identified with API ID 32E (bioMérieux (Suisse) SA, Genève, Switzerland) according to the manufacturer's protocol. For each isolate with an identification result below „good“ a partial *rpoB* gene sequence analysis was performed in addition.

### *rpoB* sequencing

Bacterial DNA was extracted by boiling the colonies. PCR amplifications were performed in 50 µl reaction tubes containing the forward primer CM<sub>7</sub> (5'-aac cag ttc cgc gtt ggc ctg g-3') and reverse primer CM<sub>31b</sub> (5'-cct gaa caa cac gct cgg a-3') (Mollet et al., 1997) at 20 pmol each by using the GoTaq Green Master Mix (Promega, Madison, WI, USA). The PCR conditions were 90 s at 94°C followed by 40 cycles at 94°C for 10 s, 55°C for 20 s and 72°C for 50 s, completed by a final elongation step at 72°C for 5 min (Mollet et al., 1997). PCR products were resolved on a 1% agarose gel stained with ethidium bromide and examined under UV light. After purification with the PCR purification kit (Qiagen, Hombrechtikon, Switzerland), sequencing reactions were performed by Microsynth AG (Balgach, Switzerland). The obtained sequences were compared with the nucleotide collection of NCBI using the nucleotide BLAST optimized for highly similar sequences. With a similarity above 98% the isolate was assigned to the corresponding species. Isolates with a similarity less than 98% were considered as unidentified and hence as potential novel species.

### *rpoB* neighbour-joining tree

The *rpoB* sequences of these unidentified isolates were compared to those of 22 reference strains from the NCBI nucleotide database using ClustalW on <http://align.genome.jp/>. The *rpoB* neighbour-joining tree was calculated with the following multiple alignment parameters: gap open penalty: 15, gap extension penalty: 6.66, weight transition: no, Hydrophilic Gaps: yes, Selected Weight Matrix: IUB.

## Results and Discussion

The most common species out of all 470 isolates we identified were 161 *Enterobacter* (*Eb.*) *cloacae* (34%), followed by 51 *Pantoea* spp. (11%) and 39 *Klebsiella* (*K.*) *pneumoniae* (8%). If considering only the 187 isolates from finished

products, there were 95 *Eb. cloacae* (50%), 28 *K. pneumoniae* (15%) and 11 *Pantoea* spp. (6%) (Tab. 1).

Of the total of 470 isolates 322 (69%) could be identified with API ID32E to adequate quality, for 148 isolates (31%) *rpoB* sequencing was needed. We chose the *rpoB* gene which is known to produce more reliable outputs compared to sequencing the 16S rRNA gene (Mollet et al., 1997; Case et al., 2006; Brady et al., 2008; Paauw et al., 2008).

From the 148 isolates which were sequenced, 83 isolates (56%) showed the demanded *rpoB* gene similarity and could thus be assigned to an existing species, whereas 65 isolates (44%) could not be identified. From these 65 isolates 13 were found in raw ingredients, 44 in environmental samples and 8 in finished products. Using the partial *rpoB* gene sequence data as a similarity matrix 22 different clusters could be formed. These clusters contained one to 13 different isolates originating either from just one or up to all three sample types (Fig. 1). The fact that some isolates from the same *rpoB* cluster originate from different sample types (raw ingredients, environment and finished products) might indicate that they are widely common within this production environment and hence of practical relevance. These isolates which possibly form novel genus/species will be further investigated.

Muytjens et al. (1988) has been the first group that described the quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. They found *Eb. agglomerans* (now *Pantoea* [*P.*] *agglomerans*) with 27% of all isolates, to be the most common species, followed by *Eb. cloacae* with 23% and *K. pneumoniae* with 10%. However, in this study the identity of *Eb. agglomerans* is uncertain because since then the *Enterobacter-Pantoea-Citrobacter* group has been revised. Iversen and Forsythe (2004), looking at powdered infant formula milk and related products, described a distribution of 25% *Eb. cloacae*, 19% *P. agglomerans* and 2% *K. pneumoniae*. Recently, Estuningsih et al. (2006) found 24% *P. agglomerans*, 20% *Escherichia hermanii*, 16% *Eb. cloacae* and 6% *K. pneumoniae* in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. Compared to these results, we found higher numbers of *Eb. cloacae*.

*Eb. cloacae* is described as an emerging pathogen in neonatal care units causing mostly sepsis (Tresoldi et al., 2000; van Dijk et al., 2002; Talon et al., 2004; Dalben et al., 2008). Only rare cases of necrotizing enterocolitis were published (Powell et al., 1980; van Nierop et al., 1998). In a recently published review sixteen *Eb. cloacae* sepsis outbreaks were described (Dalben et al., 2008). In 50% of these outbreaks the source of infection could not be found. In other cases different sources were mentioned, like contaminated parenteral nutrition solution, ther-

mometers or hands of personnel (Shi et al., 1996; Tresoldi et al., 2000; van Dijk et al., 2002). In another study the gastrointestinal tracts of the patients and environmental surfaces have been assumed as principal sites of bacterial reservoir (Yu et al., 2000).

*Pantoea* spp. are actually known as plant pathogens (Coutinho et al., 2002; Cother et al., 2004; Goszczynska et al., 2007), which might explain the comparatively high number found in raw ingredients. In recent years, *Pantoea* strains have been consistently linked with human infections (Cruz et al., 2007). Van Rostenberghe et al. (2006) described eight cases of neonates with respiratory distress syndrome caused by *Pantoea* spp. where the source of infection was contaminated parenteral nutrition solution. Two outbreaks of sepsis have been reported, either caused by contaminated parenteral nutrition solution or by a contaminated tranference tube (Habsah et al., 2005; Bicudo et al., 2007).

*Klebsiella* is known as a health care associated pathogen (Gaillot et al., 1998; Cassettari et al., 2009). The urinary tract is the most common site of infection (Podschn and Ullmann, 1998). But also septicaemia, respiratory distress, meningitis or conjunctivitis have been described (Gaillot et al., 1998; Persson et al., 2002; Cassettari et al., 2009). Furthermore, outbreaks of extended-spectrum beta-lactamase producing *K. pneumoniae* in neonatal intensive care units have been reported (Gaillot et al., 1998; Macrae et al., 2001; Gupta et al., 2004; Cassettari et al., 2009). This creates considerable difficulty when empiric antibiotic therapy is needed for seriously ill neonates. Health care workers may act as a vehicle in the transmission between patients (Gupta et al., 2004; Cassettari et al., 2009), but situations in which there was a common source of infection have also been reported (Cassettari et al., 2009). The gastrointestinal tract of asymptomatic patients can serve as reservoir for this pathogen (Gupta et al., 2004), this is also confirmed by another study where 27 of 120 asymptomatic newborn infants were found to be colonized (Cassettari et al., 2009).

Although these frequently isolated species are known as pathogens for neonates, no correlation with contaminated infant formula has been described so far. However, these widely spread species might be used in an infant formula processing plant as indicator microorganisms to identify and trace back contamination routes.

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**5.2.2** PFGE-typing of *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Leclercia adecarboxylata* isolates from an infant formula processing plant

Popp, A.<sup>1</sup>, Fricker-Feer, C.<sup>2</sup>, Gschwend, K.<sup>2</sup>, Stephan, R.<sup>1,\*</sup> (2010).  
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## PFGE-typing of *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Leclercia* *adecarboxylata* isolates from an infant formula processing plant

*PFGE-Typisierung von Enterobacter cloacae, Klebsiella pneumoniae und Leclercia  
adecarboxylata Isolaten aus einem Verarbeitungsbetrieb für Säuglingsanfangsnahrung*

Alexandra Popp<sup>1</sup>, Claudia Fricker-Feer<sup>2</sup>, Karl Gschwend<sup>2</sup>, Roger Stephan<sup>1</sup>

### Summary

The family of *Enterobacteriaceae* is a useful indicator for hygiene conditions in food production facilities and food products. For powdered infant formula (PIF) the absence of *Enterobacteriaceae* in 100 g is required. Nevertheless, occasionally *Enterobacteriaceae* can be detected. A recent study concentrated on the occurrence of a specific foodborne pathogen out of the *Enterobacteriaceae* family, *Cronobacter* spp., in PIF manufacturing facilities in order to investigate clonal persistence and identify possible transmission routes. The aim of this study was to genotype isolates from frequently found other species within the family of *Enterobacteriaceae* in order to elucidate and trace back transmission routes not only limited on *Cronobacter* spp.

In total 216 isolates from three different species (*Enterobacter (E.) cloacae*, *Klebsiella (K.) pneumoniae* and *Leclercia (L.) adecarboxylata*) were genotyped. The isolates originated from raw ingredients, environment and products of an infant formula processing plant. Restriction digest with *Xba*I revealed discriminative PFGE patterns consisting of 10–20 bands for all three species. Heat sensitive additives could be traced back as contamination source for products. Furthermore, the production environment was found as a reservoir for persisting strains. Showing analogy to the situation described for *Cronobacter* spp., especially *E. cloacae* that can be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for hygiene monitoring along the processing chain. Certain genotypes of *E. cloacae*, that are able to persist within the factory environment, might possess special properties as e. g. enhanced desiccation tolerance enabling them to survive the harsh environmental conditions.

**Keywords:** PFGE, genotyping, *Enterobacteriaceae*, infant food

### Zusammenfassung

Für Säuglingsanfangsnahrung in Pulverform (PIF) ist der Parameter *Enterobacteriaceae* als Prozesshygienekriterium (nicht nachweisbar in 100 g) definiert. Dennoch können aber gelegentlich *Enterobacteriaceae* in solchen Produkten nachgewiesen werden.

Eine kürzlich publizierte Studie konzentrierte sich auf das Vorkommen von *Cronobacter* spp., einen pathogenen Vertreter aus der Familie der *Enterobacteriaceae*, um mögliche Kontaminationswege zu untersuchen.

Das Ziel der aktuellen Studie war es, andere häufig gefundene Spezies aus der Familie der *Enterobacteriaceae* aus Rohstoffen, dem Produktionsumfeld und von Endprodukten eines PIF Verarbeitungsbetriebes mittels PFGE zu genotypisieren, um mögliche Kontaminationswege aufzuzeigen und die Eignung dieser Spezies für betriebsepidemiologische Studien zu evaluieren. Insgesamt wurden 216 Isolate von drei verschiedenen Spezies (*Enterobacter (E.) cloacae*, *Klebsiella (K.) pneumoniae* und *Leclercia (L.) adecarboxylata*) in die Untersuchung miteinbezogen. Der Restriktionsverdau mittels *Xba*I ergab für alle drei Spezies aussagekräftige PFGE Muster. Endproduktkontaminationen konnten zum einen auf hitzeempfindliche Zusatzstoffe, aber vor allem auch auf das Produktionsumfeld zurückgeführt werden. Dabei hat sich vor allem die Spezies *E. cloacae*, die in den gleichen Nischen wie *Cronobacter* spp. gefunden werden kann, als ein guter Parameter für betriebsepidemiologische Untersuchungen erwiesen. Bestimmte Genotypen von *E. cloacae* scheinen fähig, im Produktionsumfeld zu persistieren, was möglicherweise auf besondere Eigenschaften, wie z. B. verstärkte Austrocknungstoleranz zurückzuführen ist.

**Schlüsselwörter:** PFGE, Genotypisierung, *Enterobacteriaceae*, Säuglingsnahrung

## Introduction

The family of *Enterobacteriaceae* is a useful indicator for a hygiene monitoring system in food production facilities and food products. For powdered infant formula the absence of *Enterobacteriaceae* in 100 g (n=10; M=absence in 10 g) is required (Anonymous, 2005). Nevertheless, occasionally *Enterobacteriaceae* can be detected in infant formula and production environment (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006; Popp et al., 2009). On one hand there are supplements that need to be added after the drying process since they are not heat stable, and on the other hand during the filling process contamination is possible while the system is not completely close.

Up to now, few studies have focused on the presence and the diversity of *Enterobacteriaceae* in infant formula and the related production environment (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006). Two recent surveys concentrated on the occurrence of a specific foodborne pathogen out of the *Enterobacteriaceae* family, *Cronobacter* spp., in powdered infant formula (PIF) manufacturing facilities in order to investigate clonal persistence and identify possible dissemination routes along the processing chain by the use of PFGE (Mullane et al., 2007; Iversen et al., 2009). Identical pulso-types of strains isolated from different locations indicated that the production environment is an important source for product contamination.

In a previous study (Popp et al., 2009) 470 *Enterobacteriaceae* isolates from different sample types of a Swiss infant formula processing plant were identified. Frequently isolated species were *Enterobacter cloacae* (34 %), *Klebsiella pneumoniae* (8 %) and *Leclercia adecarboxylata* (6 %). The aim of this study was to genotype these isolates in order to elucidate and trace back transmission routes not only limited on *Cronobacter* spp.

## Materials and Methods

### Manufacturing facility

The firm is one of the leading milk processing companies in Switzerland and has two milk-associated production sites (site A, site B) about 100 km apart. It handles about 350.000 tons of milk per year and fabricates various products like milk powder, powdered infant formula and follow on and growing up formula that are sold in over 60 countries. Customers include the food industry, the retail industry, bakeries and the catering trade.

### Isolates

Isolates were collected within a previous study (Popp et al., 2009) as different sample types from the two different production sites. Sample types were raw ingredients (for example milk, flours, starches, sugars, vitamins and flavorings), environmental samples (for example filling equipment, walls, floors, cleaning equipment, dust, sinks, elevators and personnel) and product samples. Product samples were taken from different steps of manufacturing and can be differentiated between semifinished products (before adding supplements) and finished products. Finished products were powdered infant formula and follow on

formula from different brands that can be bulk goods or shelf-ready products.

A total of 216 isolates from three different species were selected for genotyping. From the 155 *Enterobacter cloacae* isolates 25 were from raw ingredients, 16 from the environment and 94 from products. From the 37 *Klebsiella pneumoniae* isolates 4 were from raw ingredients, 6 from the environment and 27 from products. From the 24 *Leclercia adecarboxylata* isolates 11 were from raw ingredients, 7 from the environment and 6 from products.

### Pulsed-Field Gel Electrophoresis

Isolates were grown on blood agar overnight at 37 °C. Bacteria were harvested from the plate using a cotton swab and transferred into 2 ml cell suspension buffer (100 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0). The suspension was adjusted spectrophotometrically to an optical density of 1.0 at 600 nm. 400 µl of this suspension was mixed with 20 µl of Proteinase K solution (20 mg/ml) and 400 µl of 1.4 % (w/v) pulsed-field certified agarose (Bio-Rad Laboratories AG, Reinach BL, Switzerland). Agarose plugs were prepared in plug moulds (Bio-Rad) and allowed to solidify. Then plugs were placed into tubes containing 5 ml lysis buffer (50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1 % (w/v) N-Laurylsarcosine) with addition of 0.1 mg/ml Proteinase K immediately prior to use. After incubation at 55 °C overnight the plugs were washed with distilled water twice and with TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) four times with incubation time of 30 min and 60 min respectively at 50 °C. The washed plugs were stored in TE buffer at 4 °C. For restriction endonuclease digestion, agarose plugs were cut in 2 mm slices and equilibrated in 300 µl restriction buffer H (Roche) for 20 min. The restriction digest was performed in 300 µl fresh restriction buffer H containing 80 U of enzyme *Xba*I (Roche) for 12–14 h. Restriction fragments were separated in a 1 % (w:v) pulsed-field certified agarose gel (Bio-Rad) in 0.5 TBE running buffer with a CHEF-DR III system (Bio-Rad). The running buffer was supplemented with 50 mM Thiourea (Sigma); this overcomes the problem of non-typeable strains due to the nucleolytic activity of a peracid derivative of Tris, which can form at the anode during electrophoresis (Ray et al., 1995). *Salmonella* Braenderup strain H9812 digested with *Xba*I was used as a molecular size standard (Hunter et al., 2005). The following conditions were used for the separation of the digested fragments, pulse time: 5–50 sec, linear ramping for 20 h at 14 °C, 120 ° included angle. Following electrophoresis, gels were stained for 30 min with ethidium bromide (5 mg/l) and destained in distilled water for 1 minute. Fingerprint patterns were visualized and captured using a CCD photography system (Bio-Rad, Hercules, CA) from which tagged image file format (TIFF) files were imported into GelCompar II software version 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Clustering of the PFGE fingerprint patterns was performed using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA), with an optimization of 3 % and band position tolerance of 1.5 %. Cophenetic correlations ranged from 61–100 % and the relatedness of the fingerprint patterns was compared at 95 % similarity.

Isolates with identical band patterns were considered as a group.

**Results and Discussion**

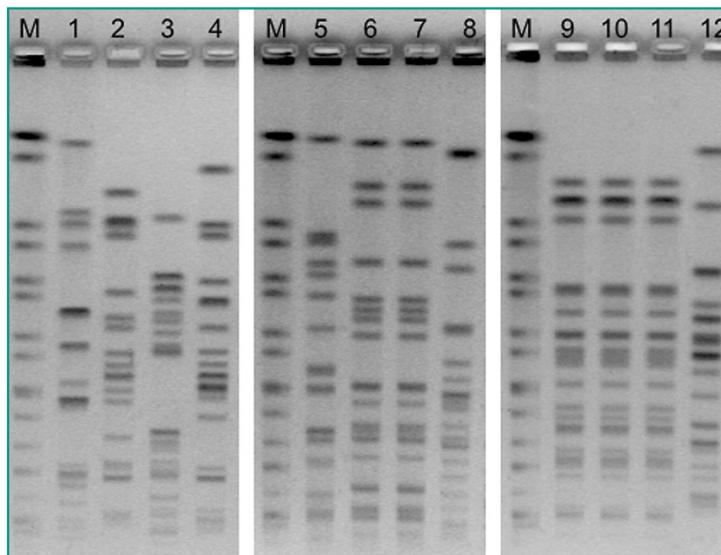
Restriction digest with *Xba*I revealed discriminative PFGE patterns for all three species. *E. cloacae* showed patterns consisting of 10–20 bands, *K. pneumoniae* had patterns with 11–20 bands and the patterns for *L. adedecarboxylata* contained 11–19 bands (fig. 1). In summary no difference in discriminative power could be observed between the three species, indicating that *Xba*I was appropriate for all of them.

The 155 *E. cloacae* isolates showed 92 different pulso-types, allocated in 24 groups with 2–11 isolates each. 17 groups contained only isolates from product samples, 3 groups only environmental samples. The other 4 groups contained isolates from different sample types. For *K. pneumoniae* there were 37 isolates resulting in 25 different pulso-types. 5 groups containing 2–6 isolates were formed, all containing product samples only. The 25 *L. adedecarboxylata* isolates resulted in 21 different pulso-types with 2 groups. One of them contained 2 isolates from raw ingredients, the other contained 3 isolates from product and environmental samples.

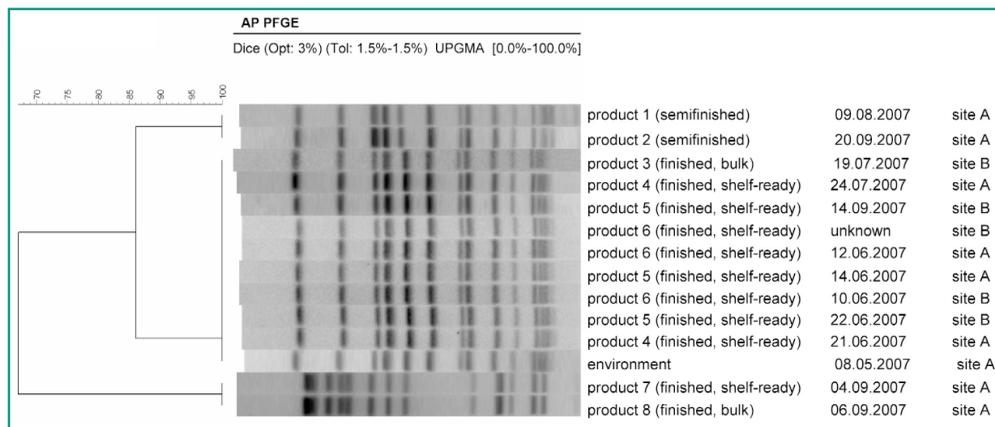
Various groups, especially for *E. cloacae*, were formed by isolates from different kind of finished products. A closer look at these products revealed that they were either produced on the same spray dryer, blended in the same mixer or packed in the same filling line. In other cases groups contained isolates from the same kind of finished product but manufactured on different dates. This possibly indicates a persistent contamination of the facilities. Since only dry cleaning is practicable in this kind of production environment, disinfectants are not efficient. Furthermore

dust formation can not totally be avoided and therefore air flow management is very important.

An other possible way of contamination is shown by the following example. One group (fig. 2) showed the same genotype of *E. cloacae* isolates from products that were manufactured in the two different production sites, 100 km apart, so the environment as possible source of contamination could be excluded. Checking the ingredients of those various products made evident that they all contained lactose, which is a carrier for heat sensitive additives, and therefore added after the drying process, from the same supplier. This indicates heat sensitive



**FIGURE 1:** PFGE patterns of chromosomal DNA restriction fragments digested with *Xba*I resolved in 1 % pulsed-field certified agarose gel (Bio-Rad) in 0.5 TBE running buffer. M, molecular size standard (*Salmonella Braenderup* strain H9812 digested with *Xba*I); lines 1–4 isolates of *E. cloacae*; lines 5–8, isolates of *L. adedecarboxylata*; lines 9–12 isolates of *K. pneumoniae*.



**FIGURE 2:** Comparison of band patterns of three groups of *E. cloacae* isolates calculated with the GelCompar II software. Different product number means different kind of products, which means different brand names. Identical product numbers means the same product brand, which means an identical product.

additives as an important source of entry into the production facilities.

To summarize, the findings are comparable with the situation described for *Cronobacter* spp. (Mullane et al., 2007; Iversen et al., 2009). Especially *E. cloacae* that can be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for a hygiene monitoring program along the processing chain in order to trace back contamination routes. Certain genotypes of *E. cloacae*, that are able to persist within the factory environment, might possess special properties as e. g. enhanced desiccation tolerance and therefore demonstrate the need for further evaluation of stress response mechanisms in this species.

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## 5.3 Epidemiology on *Cronobacter* spp.

### 5.3.1 Genotyping of *Cronobacter* (*Enterobacter sakazakii*) strains isolated from an infant formula processing plant

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## Genotyping of *Cronobacter* (*Enterobacter sakazakii*) strains isolated from an infant formula processing plant

*Genotypisierung von Cronobacter (Enterobacter sakazakii) Stämmen aus einem Verarbeitungsbetrieb für Säuglingsanfangsnahrung*

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### Summary

*Cronobacter* spp. are occasional contaminants of infant food processing establishments and have been implicated in rare cases of neonatal infections. The control of these organisms during the processing of infant formula and identification of possible contaminating sources is of importance to manufacturers. In this study, infant formula processing sites were monitored for the presence of *Cronobacter* spp. (*Enterobacter* (*E.*) *sakazakii*) in 2007. *Cronobacter* were isolated using ISO/TS 22964 and simultaneously using a newly developed differential method, Cronobacter Screening Broth (CSB). Clonal types of *Cronobacter* strains in the environment, raw materials, and products were investigated using PFGE and RAPD in order to shed light on the possible dissemination routes within the facility. Over the course of the study, a total of 153 *Cronobacter* isolates were obtained from 46 end-products, 23 raw materials and 44 environmental samples. The 153 *Cronobacter* isolates represented 71 PFGE pulso-types, with 24 pulso-types containing multiple isolates. Clonal isolates present in products could be matched to indistinguishable pulso-types in raw materials and in the environment, however no strains were identified that were present in both the environment and raw materials. Although the majority of the isolates were obtained from products, the largest diversity of isolates was observed in raw materials. This may indicate raw materials as a significant source of *Cronobacter* entry into the production facility with subsequent selection for strains that are able to persist within the factory environment.

**Keywords:** *Cronobacter* spp., PFGE, RAPD, infant formula processing plant

### Zusammenfassung

*Cronobacter* spp. treten gelegentlich als Kontaminanten in Verarbeitungsbetrieben für Säuglingsanfangsnahrung auf und wurden in seltenen Fällen neonataler Infektionen nachgewiesen. Für die Lebensmittelunternehmer ist es entscheidend, diese Organismen während der Herstellung und Verarbeitung von Säuglingsnahrung zu kontrollieren und mögliche Kontaminationsquellen zu identifizieren. In der vorliegenden Studie wurden im Jahr 2007 Verarbeitungsbetriebe für Säuglingsanfangsnahrung im Hinblick auf das Vorkommen von *Cronobacter* spp. (*Enterobacter* (*E.*) *sakazakii*) untersucht. *Cronobacter* wurden mittels ISO/TS 22964 isoliert sowie parallel mit einer kürzlich entwickelten Methode unter Verwendung von Cronobacter Screening Broth (CSB). Klonale Typen von *Cronobacter* Stämmen wurden mittels PFGE und RAPD in Umgebungsproben, Ausgangsmaterialien und Endprodukten untersucht, um mögliche Ausbreitungswege innerhalb des Betriebs aufzudecken. Im Verlauf der Studie wurden aus 46 Proben von Endprodukten, 23 Proben von Ausgangsmaterialien und 44 Umgebungsproben insgesamt 153 *Cronobacter* Isolate gewonnen. Diese 153 Isolate entsprachen 71 PFGE-Typen, wobei 24 PFGE-Typen mehrere Isolate enthielten. Klonale Isolate aus Endprodukten konnten identischen PFGE-Typen aus Ausgangsmaterialien und Umgebungsproben zugeordnet werden, während keine Stämme zugleich in Umgebungsproben und Ausgangsmaterialien nachgewiesen werden konnten. Obwohl aus den Endprodukten die größte Zahl an Isolaten stammte, wurde in den Ausgangsmaterialien die größte Vielfalt an Isolaten festgestellt. Dies mag darauf hinweisen, dass die Ausgangsmaterialien eine bedeutende Quelle des Eintrags von *Cronobacter* in den Verarbeitungsbetrieb darstellen, wobei anschließend eine Selektion der Stämme stattfindet, die fähig sind, in dem Produktionsumfeld zu persistieren.

**Schlüsselwörter:** *Cronobacter* spp., PFGE, RAPD, Säuglingsnahrung

## Introduction

*Cronobacter* spp. (*Enterobacter* (*E.*) *sakazakii*) are environmental organisms that have been associated with rare opportunistic infections (Lai, 2001; Lehner and Stephan, 2004; Mullane et al., 2007a). Some reported cases have been linked to the ingestion of contaminated infant formula (Biering et al., 1989; Himelright et al., 2002; van Acker et al., 2001). However, the organisms appear to be ubiquitous having been isolated from a variety of food products, production environments and households (Friedemann, 2007; Gurtler et al., 2005; Kandhai et al., 2004a; Kandhai et al., 2004b).

The reported prevalence of *Cronobacter* in surveys of commercially available powdered infant formula appears to be gradually decreasing from estimates of 14% in 1988 (Muytjens et al., 1988) and 6.7% in 1997 (Nazarowec-White and Farber, 1997) to 2.5% in 2001 (Heuvelink, 2001). Recent estimates indicate that although small reductions may still be occurring the prevalence appears to be stabilizing at 2–2.5% (Iversen and Forsythe, 2004; Iversen et al., 2008a; Mullane et al., 2007b). This could be due to particularly persistent phenotypes in production facilities that are resistant to the improved hygiene measures adopted by manufacturers, or to the existence of as yet unidentified contamination routes. Fingerprint typing has become established as a useful method to support monitoring of micro-organisms in relation to public health, consumer protection and manufacturing hygiene practices. Over the years new typing methods have been developed and compared to existing methods to determine which have the most discriminatory power.

Nazarowec-White and Farber (1999) were the first to publish a detailed examination of typing methods for *E. sakazakii* strains, comparing antibiograms, biotypes, ribotypes, Random-Amplified Polymorphic DNA (RAPD) and Pulsed-Field Gel Electrophoresis (PFGE) patterns for isolates obtained from infant formula and hospital culture collections. PFGE and RAPD were found to be the most discriminatory methods. All 18 strains in the study gave distinguishable fingerprint patterns with *Xba*I (PFGE) and Primer UBC 245 (RAPD). In 1994, an outbreak of *Cronobacter* infections, involving 17 neonates occurred in France and isolates were obtained from patients, prepared feeds and unused infant formula. PFGE analysis using *Xba*I resulted in four clusters, two clusters contained isolates from neonates with conditions ranging from asymptomatic colonization to individuals with fatal disease. A third cluster contained isolates from prepared unfinished feed, an asymptomatic neonate and a neonate with mild digestive problems. The fourth cluster was comprised of isolates from unused powdered formula. In this case no link could be made between the powdered formula and the outbreak (Caubilla-Barron et al., 2007). Another outbreak involving 13 infants occurred in France in 2004. The outbreak was investigated using automated ribotyping with three restriction endonucleases (*Eco*RI, *Pst*I and *Pvu*II), and using PFGE with two enzymes (*Xba*I and *Spe*I). In this case a clear link was established between the infections and the infant formula, as nine isolates from eight neonates were found to have indistinguishable ribotypes and PFGE patterns to isolates obtained from four separate lots of the formula (Anonymous, 2004a).

Mullane et al. (2007b) undertook an extensive survey of the processing environment of an infant formula factory to characterize and trace the prevalence of *Cronobacter*. PFGE, using *Xba*I, resulted in 19 unique pulso-types. The majority of isolates were from the environment and large clonal groups were identified with apparently related isolates occurring throughout the production process and in the end-product. In this study no comparison with isolates from raw materials was undertaken and no clear route of entry into the factory was established. However, the manufacturing environment was identified as a source of sporadic product contamination.

This study examines *Cronobacter* isolates from infant formula processing facilities in Switzerland and employs both PFGE and a modified RAPD method to characterize persistent strains and elucidate dissemination routes. The starting premise for this current study was that isolates having  $\geq 95\%$  fingerprint pattern similarity would be considered indistinguishable and, given the limited sampling area, it would be reasonable to assume these were clonal being derived from a recent common ancestor.

## Material and Methods

### Samples

The manufacturing facilities were divided across two sites (Site 1 and Site 2), each site manufacturing various infant food products and intermediary ingredients. The sampling was performed from 1<sup>st</sup> May to 5<sup>th</sup> November, 2007. Samples were collected from finished products, raw materials and the production environment ( $n = 950$ ). The finished products incorporated a range of food brands for infants of all ages and nutritional needs. The raw ingredients included starches, flours and grains, sugars, fruit and vegetable flakes and powders, vegetable fats, milk proteins, egg powder, vitamins and minerals, yeast extract, flavorings and emulsifiers. The environmental sampling extensively covered the manufacturing process including production, filling and storage equipment, walls, floors, handles, vacuum cleaners, filters, air, cleaning equipment, dust, drains, sinks, refrigerators, office areas, canteen areas, washrooms, floor mats, metal detectors, light fittings, elevators, insect traps and personnel.

### Isolation of *Cronobacter* spp.

Sample size varied depending on the nature of the material. Powdered infant formulae were tested as 30 x 10 g, 10 x 30 g or 2 x 150 g aliquots and, when feasible, raw materials and environmental samples were tested as 25 g aliquots. All samples were diluted 1:10 in BPW and pre-enriched for 24 h at 37°C; environmental swabs were pre-enriched in 10 ml BPW. A dilution of 1:100 was simultaneously performed for samples such as starches, chocolate powders and vitamin-mineral premixes. *Cronobacter* were isolated using ISO/TS 22964 (Anonymous, 2006b) and simultaneously using a newly developed differential method, *Cronobacter* Screening Broth (CSB) (Iversen et al., 2008a). Pre-enriched sample (0.1 ml) was transferred to 10 ml modified Lauryl Sulphate Tryptone broth (mLST) and to 10 ml CSB; these were incubated at 44°C and 41.5°C, respectively, for 24 h. A 10  $\mu$ l aliquot from mLST was then streaked onto *E. sakazakii* isolation agar (ESIA, provided by Oxoid Ltd., Basingstoke, UK) and incubated at 44°C for 24 h. A 10  $\mu$ l aliquot from positive CSB broths was streaked onto modified Chromogenic *Enterobacter sakazakii* agar: DFI formulation (mDFI, provided by Oxoid Ltd., Basingstoke, UK) and incubated at 41.5°C for 24 h. Presumptive positive colonies from both agar plates were confirmed as *Cronobacter* using a specific  $\alpha$ -glucosidase PCR assay (Lehner et al., 2006; Iversen et al., 2007b). The identification of species was determined using biochemical tests as described previously (Iversen et al., 2007a; Iversen et al., 2008b) and 16S rRNA gene sequence comparison (Fasteris S.A., Plan-les-Ouates, Switzerland). All isolates were stored at -20°C.

### Random-Amplified Polymorphic DNA (RAPD)

Isolates were grown for 18–24 h in 10 ml Brain Heart Infusion (BHI) broth with shaking (200 rpm) until growth reached approximately  $10^9$  cfu/ml. Cells were harvested from 1 ml of culture by centrifugation (10 000 x g for 5 min). DNA was extracted using a DNeasy® Blood and Tissue kit (69505 QIAGEN AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol and quantified using a NanoDrop™ 1000 Spectropho-

**TABLE 1:** *Cronobacter* isolates from products, raw materials and the environment of infant food manufacturing facilities

<i>Cronobacter</i> isolates	Sample type			Total
	Product	Raw material	Environment	
<i>C. sakazakii</i>	63	22	44	129
<i>C. malonaticus</i>	4	7	10	21
<i>C. turicensis</i>	0	1	0	1
<i>C. dublinensis</i>	0	0	1	1
<i>C. muytjensii</i>	0	0	1	1
<b>Total</b>	<b>67</b>	<b>30</b>	<b>56</b>	<b>153</b>
<b>PFGE pulso-types</b>				
Total	31	20	27	(71)
Average no. strains/pulso-type	2.16	1.50	2.07	

tometer (Thermo Fisher Scientific GmbH, Schwerte, Germany). PCR amplification was performed in 50  $\mu$ l volumes comprising 22  $\mu$ l Nuclease-Free water, 25  $\mu$ l GoTaq<sup>®</sup> Green Master Mix (M7122, Promega, Madison, WI), 1  $\mu$ l 10  $\mu$ M Primer UBC 245 (5'-CGC GTG CCA G-3'), 1  $\mu$ M Primer UBC 282 (5'-GGG AAA GCA G-3'), 1  $\mu$ l DNA (30 ng/ $\mu$ l). The amplification conditions were 94°C for 120 s, followed by 34 cycles comprising 94°C for 60 s, 35°C for 60 s, and 72°C for 90 s, with a final extension of 72°C for 300 s. Duplicate DNA extractions and amplifications were performed separately by two personnel to examine the reproducibility of the method. Amplicons were separated using a 1.5% (w:v) agarose gel incorporating 0.5  $\mu$ g/ml ethidium bromide and run for 2 h at 50V in 0.5x TBE running buffer (45 mM Tris, 45 mM boric acid, 25 mM EDTA). The fingerprint patterns were visualized and captured using a CCD photography system (BioRad, Hercules, CA, USA) from which tagged image file format (TIFF) files were imported into GelCompar II software version 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Dendrograms were constructed using the Pearson correlation and the unweighted pair group method with arithmetic mean (UPGMA). Cophenetic correlations ranged from 61 to 100% and a rendered tree (rooted) with branch cut-off at 20% was constructed.

#### Pulsed-Field Gel Electrophoresis (PFGE)

For each isolate, 20 ml of Brain Heart Infusion (BHI) broth was inoculated with 200  $\mu$ l from an overnight culture grown in BHI. The inoculated broths were incubated for 3–4 h at 37°C with shaking (200 rpm) until growth reached an OD<sub>610 nm</sub> of approximately 0.8. Cells were harvested from 1 ml of broth by centrifugation (10 000  $\times$  g for 5 min) and washed once in 1 ml 0.85% (w:v) NaCl. Cells were resuspended in 0.5 ml 0.85% (w:v) NaCl and mixed with an equal volume of 1.2% (w:v) Pulsed-Field certified agarose (Bio-Rad Laboratories AG, Reinach BL, Switzerland) in distilled water. Aliquots (100  $\mu$ l) of the bacteria agarose mixture were dispensed into disposable plug moulds (Bio-Rad). The plugs were allowed to solidify at +4°C for 20 min, then placed into tubes containing 1 ml lysis buffer per plug and incubated at 55°C for 20–24 h. The lysis buffer comprised 50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1% (w:v) N-Laurylsarcosine; with addition of 2 mg/ml Proteinase K immediately prior to use. The plugs were then washed in TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) four times for 1 h at room temperature using 1 ml TE per plug. The washed plugs were stored in TE buffer at +4°C. For restriction endonuclease digestion, agarose plugs were cut in half and equilibrated twice in restriction buffer H (Roche) using 300  $\mu$ l per half-plug. The restriction digest was performed in 300  $\mu$ l fresh restriction buffer H containing 50 U of enzyme *Xba*I (Roche) for 4 h.

Restriction fragments were separated in a 1% (w:v) Pulsed-Field Certified agarose gel (Bio-Rad) in 0.5x TBE running buffer with a CHEF-DR III system (Bio-Rad Laboratories). The running buffer was supplemented with 50 mM thiourea (Sigma); this overcomes the problem of non-typeable strains due to the nucleolytic activity of a peracid derivative of Tris, which can form at the anode during electrophoresis (Ray et al., 1995). *Salmonella* serotype Braenderup strain H9812 digested with *Xba*I was used as a molecular size standard (Hunter et al., 2005). The following conditions were used for the separation of the digested fragments: pulse time 5–50 s, linear ramping for 20 h at 14°C, 120°C included angle. Following electrophoresis, gels were stained for 30 min with ethidium bromide (5 mg/l in distilled water) and de-stained in distilled water. Fingerprint patterns were visualized and captured using a CCD photography system (Bio-Rad, Hercules, CA) from which tagged image file format (TIFF) files were imported into GelCompar II software version 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Clustering of the PFGE fingerprint patterns was performed using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA), with an optimization of 1% and band position tolerance of 3%. Cophenetic correlations ranged from 61 to 100% and the relatedness of the fingerprint patterns was compared at 95% similarity.

## Results

### Prevalence of *Cronobacter* spp.

A total of 153 *Cronobacter* isolates were recovered from 46 products, 23 raw materials, and 44 environmental samples. When *Cronobacter* was recovered from a test aliquot this was considered an individual isolate having arisen from a discrete colony-forming unit in the sample. Examples of all currently named *Cronobacter* (*C.*) species were isolated, with *C. sakazakii* being the most prevalent (129 isolates) followed by *C. malonaticus* (21 isolates). One strain each of *C. muytjensii*, *C. turicensis* and *C. dublinensis* were isolated (Tab. 1). Details of the positive samples are given in Figures 1 and 2.

### RAPD Analysis

The RAPD fingerprints yielded patterns consisting of 4–13 amplified product bands. The RAPD rendered tree produced 44 distinguishable groups at a cut-off of 80% pattern similarity, with strains from individual species clustering together (Fig. 1). The cluster groups contained between 1 and 25 strains. Duplicate DNA extraction and analysis, performed on separate occasions by two personnel, proved the RAPD method used in this study to be reproducible (data not shown). The most distant groups in the RAPD rendered tree (Fig. 1) were formed by representatives of the different species; *C. muytjensii* (an isolate from an area of rubber flooring), *C. turicensis* (an isolate from gluten-free rice flour), *C. dublinensis* (an isolate from aspirated powder), and a group of *C. malonaticus* isolates from product, tapioca starch, vacuum and floor samples.

### PFGE Analysis

The PFGE fingerprint patterns consisted of 8–19 amplified product bands per strain and these clustered to form 71 distinguishable pulso-types at a cut-off value of 95% pattern similarity

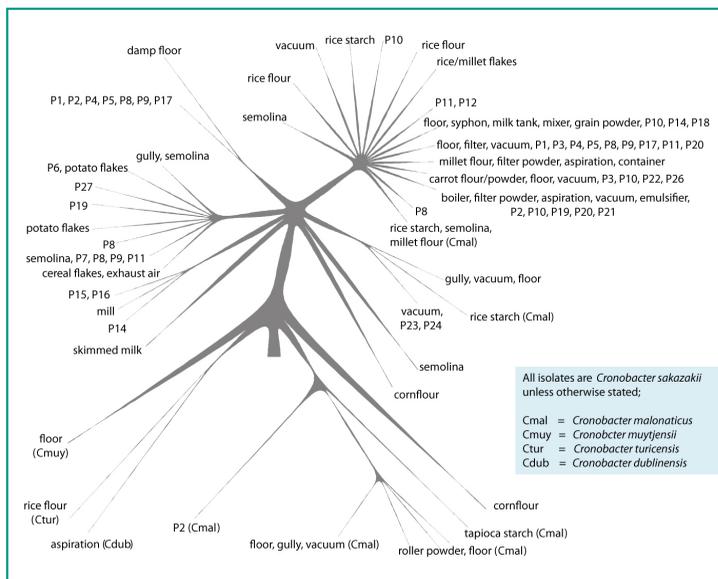
(Fig. 2). Pulso-types contained from 1 to 11 isolates, with some containing multiple clonal isolates from sample sites taken at different time points as well as clonal strains isolated from diverse samples. A representative dendrogram of 110 isolates from different sampling points is presented in Figure 2. A total of 24 pulso-types containing more than one isolate were identified and these are labelled A-X in Figure 2. The relationship between isolates in these groups was examined by an inherently post-hoc process where isolates were obtained and then the origin of related fingerprints traced back as far as possible to determine any linkages that may determine a common source. A Venn diagram to illustrate the presence of isolates from the three different sources (product, raw material and environmental) is presented in Figure 3. Clonal isolates present in products could be matched to indistinguishable pulso-types in raw materials and in the environment, however no strains were identified that were present in both the environment and raw materials.

**Discussion**

Investigation of the contamination routes and internal spread of *Cronobacter* isolates within production facilities is necessary to determine further effective strategies for reducing the presence of these organisms in the infant formula food chain. In this study we isolated *Cronobacter* spp. from a large number of end-product, raw material and environmental samples within infant food production facilities. These were characterised using phenotypic tests and the relatedness of strains from different samples was determined using RAPD and PFGE.

The RAPD analysis separated the isolates into 44 groups. Several *C. sakazakii* isolates (from cornflour, semolina and skimmed milk) appeared distinct from other strains of their species using this analysis method. The other *C. sakazakii* strains formed related groups. One *C. malonaticus* strain from rice starch and a group of three *C. malonaticus* strains from rice starch, semolina and millet flour grouped close to *C. sakazakii* strains. These two species appear to be closely related and are not distinguished from each other by 16S rRNA sequencing (Iversen et al., 2007a), which may indicate a more recent genetic divergence of these species than occurred with other *Cronobacter*.

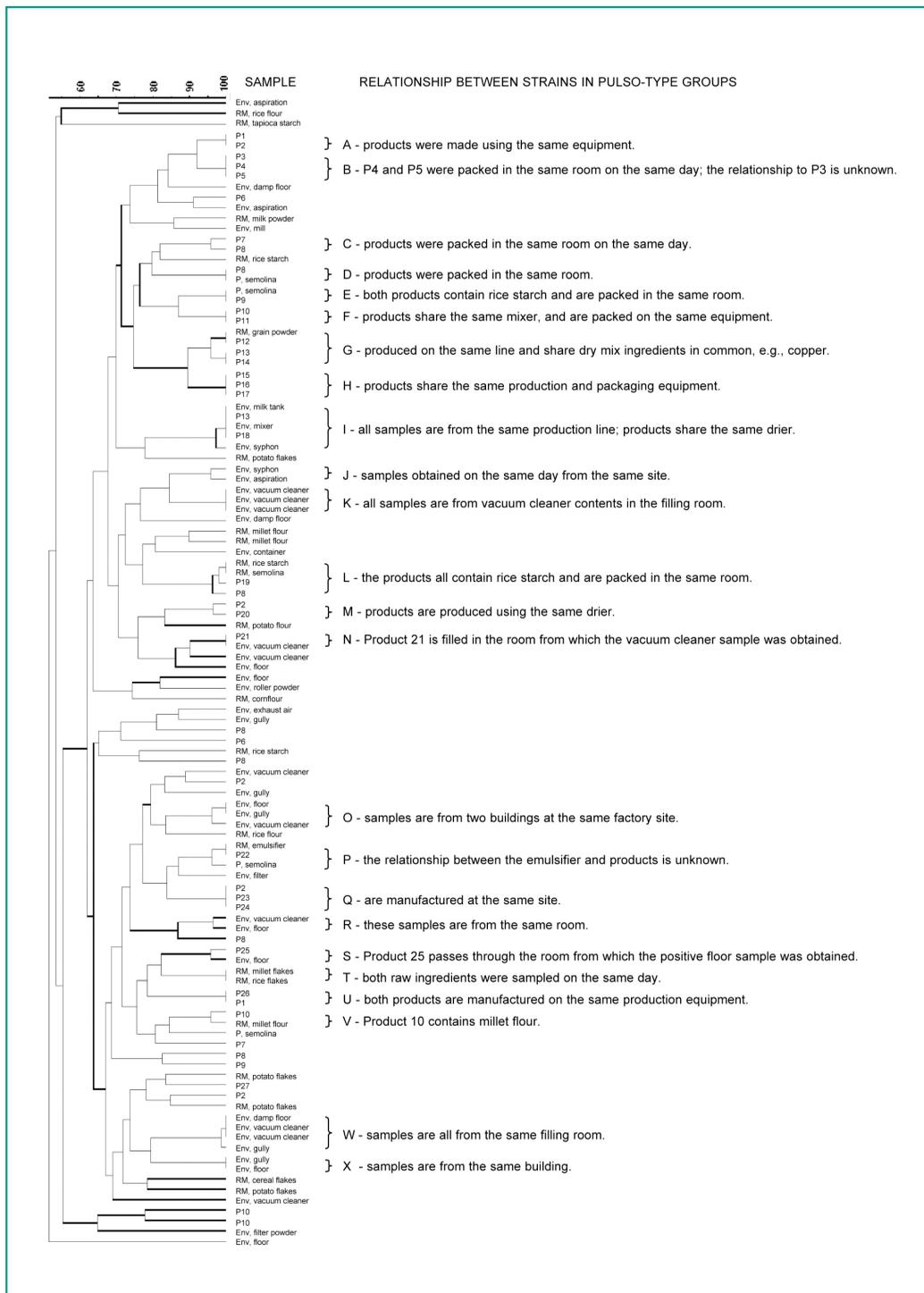
The PFGE analysis provided greater discrimination between the strains than the results obtained with RAPD. A total of 71 pulso-types were identified with 24 of these containing more than one isolate and 41 strains having distinct fingerprint patterns (Fig. 2). The pulso-types containing multiple isolates were labelled A-X and the relationship between the origins of the strains in these pulso-type groups is illustrated in Figure 3. Common raw ingredients could be determined for some end-products contaminated with the same pulso-types. For example pulso-type E includes products that all contain rice starch and



**FIGURE 1:** RAPD rendered tree (rooted) of *Cronobacter* isolates with branch cut-off at 20% similarity. Different infant food products are labelled P1–P27.

pulso-type G products share dry mix ingredients in common, such as zinc sulfate, iron, calcium, magnesium, potassium, copper and iodine (the latter two being dry-mixed into the powder post-pasteurization). However, another common factor between these product groups is that they are either produced on the same production line and/or packed in the same room, which leaves the possibility of a common environmental rather than raw ingredient contamination source. The link between raw material and product may be clearer in pulso-type L, which includes an isolate from rice starch as well as isolates from products that contain rice starch. Likewise, pulso-type V includes an isolate from millet flour, which is an ingredient in the product that was found to contain an indistinguishable isolate. Unfortunately, although pulso-type P contains an isolate from emulsifier, this raw material is not included in the end-products from which indistinguishable isolates were obtained. For other pulso-types that contain isolates from end-products, a possible linking factor is that they are produced and/or packed using common equipment. However, in most cases (A, B, D, E, F, H, M and Q) the isolates were obtained one to three months apart indicating persistent contamination with the same strains.

Pulso-type T contains only isolates from raw materials (millet flakes and rice flakes), which were sampled on the same day. It is not known if these products originated from a common supplier where they could have acquired the same contaminating *Cronobacter* strain, or whether they could have been simultaneously contaminated after arrival at the production facility. The pulso-types containing multiple environmental isolates (J, K, O, R, W and X) could be linked by the fact that the samples within these groups were usually obtained from the same room/building, in most cases on the same day. Pulso-types I, N and S contain isolates from products and environmental samples. The products in pulso-type I pass through the equipment from which the environmental samples were obtained. The environmental samples were taken in May 2007, and one product sample was taken two months later in July 2007, indicating persistence of this pulso-type and the most likely scenario that the product was contaminated from the environment. Similarly, the product in pulso-type S



**FIGURE 2:** PFGE dendrogram constructed using the DICE coefficient optimization of 1% and band position tolerance of 3%. Cophenetic correlations ranged from 61–100% and the relatedness of the fingerprint patterns was compared at 95% similarity (dashed line). Different infant food products are labelled P1–P27.

passes through the room from which the indistinguishable floor sample was obtained. The product sample was taken 16 weeks later than the environmental sample again indicating persistence of this pulso-type and contamination of the product from the environment. The product in pulso-type N is packed in the room from which the indistinguishable vacuum cleaner isolate was obtained, however in this case the product isolate was obtained two weeks before the environmental isolate. Therefore, it is not possible to ascertain whether the environmental isolate originated from spillage of contaminated product or whether the product was contaminated from an existing problem in the environment.

Not all of the strains contaminating the product could be linked to raw materials or environment samples, indicating that although the sampling in this study was extensive it did not cover all the possible reservoirs or contamination routes. Also, four test aliquots contained more than one *Cronobacter* species, indicating multiple contamination events may have occurred in these samples. A filter, millet flour and grain powder were found to contain *C. malonaticus* as well as *C. sakazakii*, while aspiration powder was found to contain both *C. sakazakii* and *C. dublinensis*. The greatest diversity of *Cronobacter* species was seen in the environment, where four species were recovered compared to only two species from products. The difference in species diversity in the sample types may indicate that selection is occurring for isolates that are better adapted to survival in dried products and/or production environment. Interestingly a group of *C. malonaticus* strains with an unusual, distinct phenotype was isolated. These strains were non-pigmented and were all located in the same locality on one production site. The largest pulso-type diversity was seen in raw materials with an average of 1.5 isolates per pulso-type compared to an average of 2.16 and 2.07 for the product and environmental isolates respectively. This indicates that raw materials could be a continual source of new isolates, but that certain strains may become established within the production environment where they are able to persist (and possibly proliferate) leading to more frequent isolation/contamination. Further characterization of these persistent strains may lead to more effective prevention and elimination strategies.

Unlike in a previous factory study where the majority of strains fell into clonal groups (Mullane et al., 2007b) and comparisons of clinical and food *Cronobacter* isolates which involved limited numbers of strains (Anonymous, 2004a; Caubilla-Barron et al., 2007), in this study a great diversity of pulso-types was generated which made interpretation of the information more complex. Although the descriptions of some possible linkages are limited, from a manufacturing stance this is useful information to help focus resources on potential problem areas for further consideration.

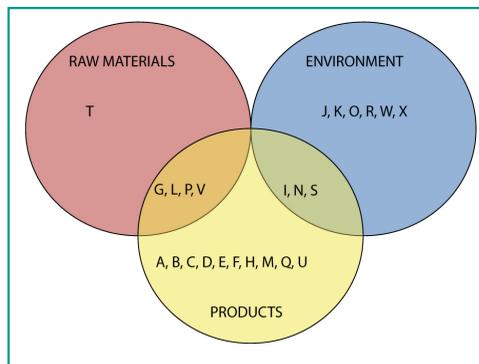


FIGURE 3: Venn diagram showing the relationship between isolates from different sources within pulso-type groups.

### Conclusion

The results from this study indicate that raw materials may be a significant source for *Cronobacter* contamination of powdered infant formula production facilities and also that selection is probably occurring for certain persistent strains of *Cronobacter* within production environments and powdered infant food products. A complex interaction between raw materials, products and the environment appears to be in process. However, the lack of common strains between environment and raw materials may suggest that the net flow of contamination events is from raw materials to the product and then to the environment, from which contamination of later products can occur. It was found that the most common raw ingredients contaminated with *Cronobacter* were plant-derived materials, such as flours, powders and flakes of rice, millet, wheat, corn, tapioca and other cereal grains, as well as of vegetables such as potatoes and carrots. This is in contrast to isolation of *Cronobacter* from only one sample of a milk-derived raw material (milk powder), with no isolates obtained from either egg powder, yeast extract, minerals or vitamin premixes.

However, there were a significant number of isolates from end-products that had fingerprint patterns which could not be linked to raw material or environment samples. This could be due to undetermined environmental reservoirs or to a low level of contamination in raw materials that was below the limit of detection for the sampling and isolation methods employed. Ef-

fective cleaning of the production environment to control the post-process contamination events is needed, with spilled powder in vacuum cleaners appearing to be a rich source of *Cronobacter* isolates. Constant monitoring of the production process is necessary to ascertain the areas of most risk and identify further contamination reservoirs. In this study RAPD proved to be a quick and reliable tool for initial genotyping of isolates. PFGE was used as a means of obtaining more detailed information to link isolates from samples with different origins together to track contamination flow. In the absence of complete epidemiological data and/or a more stringent typing method this kind of study using PFGE and retrospective analysis of data provides the first steps to understanding dissemination of contamination in factory sites and can help improve the safety of infant food. Furthermore, this approach contributes to the strengthening of existing HACCP plans to improve the microbiological quality of the manufacturing environment and end products.

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**5.3.2 Identification of *Enterobacteriaceæ* and *Cronobacter* spp. in raw milk, milk concentrate and milk powder: prevalence and genotyping**

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## Identification of *Enterobacteriaceae* and *Cronobacter* spp. in raw milk, milk concentrate and milk powder: prevalence and genotyping

*Identifizierung von Enterobacteriaceae und Cronobacter spp. aus Rohmilch, Milchkonzentrat und Milchpulver: Prävalenz und Genotypisierung*

Angelika Lehner<sup>1</sup>, Claudia Fricker-Feer<sup>2</sup>, Karl Gschwend<sup>2</sup>, Roger Stephan<sup>1</sup>

### Summary

*Cronobacter* spp. (former *Enterobacter sakazakii*) are occasional contaminants of powdered infant formula (PIF) and have been implicated in rare cases of neonatal infections. Surveys on the prevalence of these organisms and/or contamination routes during the processing of the infant formula are of importance to the manufacturers in order to reduce the level of contamination of these products. Increasing customer awareness on possible contamination of other milk powder based products intended for consumption by (older) infants posed the question about the presence of *Enterobacteriaceae* and especially *Cronobacter* spp. in products other than PIF e. g. milk concentrate (intermediate) and milk powder, both added to a variety of infant foods. It was the aim of this study to create data on the prevalence of *Enterobacteriaceae* and possible epidemiologic correlation of *Cronobacter* spp. in raw milk, milk concentrate and milk powder obtained from a Swiss milk powder production facility (2 production sites). A total of 100 raw milk samples, 91 milk concentrate samples and 172 milk powder samples were collected and tested for the presence of *Enterobacteriaceae* including *Cronobacter* spp. by cultural means. Subsets of isolates from each sample category were selected for further molecular identification and subtyping analysis. A variety of members of the *Enterobacteriaceae* family were observed in all types of samples, whereas *Cronobacter* spp. was isolated from milk powder only. Subtyping revealed a relatively high degree of heterogeneity among *Cronobacter* spp. isolates from both production sites suggesting continuous entry and dissemination of organisms from the production environment into the products.

**Keywords:** *Cronobacter* spp. prevalence raw milk, milk concentrate, milk powder, PFGE

### Zusammenfassung

*Cronobacter* spp. (*Enterobacter sakazakii*) treten gelegentlich als Kontaminanten von Säuglingsanfangsnahrung auf und wurden in seltenen Fällen als ursächlich für neonatale Infektionen nachgewiesen. Studien die sich mit dem Vorkommen und möglichen Kontaminationsrouten von *Cronobacter* spp. während der Herstellung dieser Produkte befassen haben die Verminderung des Kontaminationsdruckes dieser Produkte durch diesen opportunistisch pathogenen Organismus zum Ziel. Die zunehmende Sensibilisierung von Abnehmern bezüglich *Cronobacter* spp. in Säuglingsanfangsnahrung führte auch zu der Frage nach der Präsenz von *Enterobacteriaceae* im allgemeinen und *Cronobacter* spp. im speziellen in anderen Milchprodukten, wie Milchkonzentrat und Milchpulver, welche in der Folge einer Reihe von Kindernahrungsmitteln zugesetzt werden. Ziel dieser Studie war es Daten zur Prävalenz von *Enterobacteriaceae* und *Cronobacter* spp. sowie eventuellen epidemiologischen Zusammenhängen von *Cronobacter* spp. Isolat aus Rohmilch-, Milchkonzentrat- und Milchpulverproben zu ermitteln, welche aus einem Schweizer Milchpulverproduktionsbetrieb mit zwei Betriebsstandorten stammten. Einhundert Proben aus Rohmilch, 91 Milchkonzentrat- sowie 172 Milchpulverproben wurden mit Hilfe von kulturellen Methoden auf die Präsenz von *Enterobacteriaceae* und *Cronobacter* spp. untersucht. Eine ausgewählte Teilmenge an Isolat aus jeder Kategorie wurde in der Folge mittels molekularer Methoden weiterführend identifiziert und genotypisiert. Verschiedene Mitglieder der Familie der *Enterobacteriaceae* konnten aus allen Probenkategorien isoliert werden, wohingegen *Cronobacter* spp. nur aus Milchpulverproben isoliert wurde. Die Genotypisierung der *Cronobacter* spp. Isolate brachte eine relativ hohe Varianz der Stämme von beiden Betriebsstandorten zu Tage, was auf einen ständigen Eintrag bzw. eine Verteilung der Organismen aus dem Betriebsumfeld in die Endprodukte schliessen lässt.

**Schlüsselwörter:** *Cronobacter* spp. Prävalenz, Rohmilch, Milchkonzentrat, Milchpulver, PFGE

## Introduction

Unlike commercially available ready-to-feed liquid infant formula, which is sterile, powdered infant formula (PIF), including dried bovine milk and milk products, are a non-sterile products. PIF has been known to be contaminated, on occasion, with *Enterobacteriaceae* including bacterial pathogens, notably *Cronobacter* spp. (Forsythe, 2005). Therefore, hygienic measures and practices must be applied during manufacture of the formula to minimize entry and dissemination of contaminants into the process.

*Cronobacter* spp. (former *Enterobacter sakazakii*) are environmental organisms that have been associated with food-borne illness in neonates and immunocompromised infants (Lai, 2001; Lehner and Stephan, 2004). While *Cronobacter* spp. has been isolated from a wide range of foods, environmental and clinical sources, PIF has been identified as the dominant vehicle of transmission (Nazarowec-White and Farber, 1997, Iversen and Forsythe 2004, Gurtler and Beuchat., 2005, Mullane et al., 2007).

*Cronobacter* spp. can be isolated from the environment in milk powder and PIF manufacturing facilities (Kandhai et al., 2004, Gurtler and Beuchat, 2005, Drudy et al., 2006, Mullane et al., 2008, Hein et al., 2009). It is generally assumed that *Cronobacter* contamination of the products occurs in the processing environment at stages after pasteurization or drying e. g. storage or packaging. The occurrence of *Cronobacter* spp. in the dry environment may be due (in part) to the organism's ability to resist drying and osmotic stress (Breeuwer et al., 2003, Riedel and Lehner, 2007).

Only limited information is available about the contamination entry points in facilities and the routes of dissemination into powdered end products. A recent study investigated the possibility of microbial contamination of air filters and possible links to contaminated products in a powdered milk protein-processing facility (Mullane et al., 2008). The authors explained, that microorganisms can become aerosolized in water droplets or when they are attached to dust. Dust is generated from a variety of processing events, while water droplets can be generated as a result of cleaning operations.

In another study by Iversen et al. (2009), it was shown, that raw materials such as starches, fruit powders, milk proteins, vitamins or emulsifiers are potentially contaminated with *Cronobacter* and thus may be a significant source of entry of these organisms into the milk powder

production facility (or the final products when added as supplements).

Increasing customer awareness on possible contamination of powdered formula intended for consumption by (older) infants and children posed the question about the presence of *Enterobacteriaceae* in general and *Cronobacter* spp. in particular in products other than PIF e. g. the raw material milk, milk concentrate and milk powder, the latter two which are subsequently added to a number of milk based (infant) foods such as ice cream.

It was the aim of this study to create data on the prevalence of *Enterobacteriaceae* as well as of *Cronobacter* spp. in samples gained from raw milk, milk concentrate and milk powder and thus perform a process step analysis in a milk powder production facility in Switzerland.

## Material and Methods

### Sampling

A total of 363 samples were collected from a Swiss infant formula processing facility, divided across two sites (site 1 and site 2) each site manufacturing various infant food products and intermediates. Sampling was performed from May to September 2009. Samples were retrieved from raw milk (site 1: n = 50, site 2: n = 50), milk concentrate (site 1: n = 50, site 2: n = 41) and milk powder (site 1: n = 72, site 2: n = 100). The 100 raw milk samples represented pooled tank milk samples (from approx. 1240 sampling points in Switzerland), the 91 milk concentrate samples were obtained directly from evaporators using syringes and the 172 milk powder samples represented pooled samples from batches from both spray dried or roller dried powders.

### Isolation of *Enterobacteriaceae* and *Cronobacter* spp.

All samples were tested as 10 and 100 g aliquots. Samples were diluted 1:10 in buffered peptone water (BPW) and pre-enriched for > 16 h at 37 °C. For isolation of *Enterobacteriaceae*, enriched samples were streaked onto violet red glucose bile agar (VRBG) agar and incubated for 24 h at 37 °C.

*Cronobacter* spp. were isolated using a recently developed differential method (Iversen et al., 2008). Pre-enriched samples (0.1 ml) were transferred to 10 ml *Cronobacter* screening broth (CSB) and incubated for 24 h at 42 °C. Aliquots from presumptive (yellow) positive CSB broth tubes were streaked onto *Enterobacter sakazakii*

isolation agar (ESIA, Oxoid Ltd, Basinstoke, UK) and incubated for 24 h at 44 °C.

Considering the expectation that most if not all of the raw milk samples would be positive for growth on VRBG a subset (n= 14) of VRBG isolates as well as 4 presumptive *Cronobacter* isolates from ESIA were selected for cryopreservation and further identification of *Enterobacteriaceae* and *Cronobacter* spp. from raw milk. For the other matrices from the study (concentrates and milk powder samples) selected VRBG and all ESIA grown isolates were cryopreserved and stored until further use.

#### Identification

Presumptive *Enterobacteriaceae* isolates from cryocultures were re-streaked onto blood agar and subjected to API ID32E (bioMérieux (Suisse), SA, Geneve, Switzerland) identification according to the manufacturer's protocol. For each isolate with an identification result below "good" a partial *rpoB* gene sequence analysis was performed as described (Popp et al., 2009). Sequencing of the *rpoB* amplification products was outsourced (Microsynth AG, Balgach, Switzerland). Sequences were subjected to the nucleotide collection of NCBI using the nucleotide BLAST function optimized for highly similar sequences. With a similarity > 98 % the isolate was assigned to the corresponding species.

Presumptive *Cronobacter* spp. isolates from ESIA agar plates were confirmed to the genus level using the  $\alpha$ -glucosidase PCR assay (Lehner et al., 2006) and identification to the *Cronobacter* species level was completed employing the recently developed *rpoB* targeted PCR assay (Stoop et al., 2009).

#### Subtyping of isolates by PFGE

PFGE analysis on confirmed *Cronobacter sakazakii* isolates was performed according to the protocol published by Iversen et al., (2009).

## Results and Discussion

#### Selection of isolates

Of originally 100 raw milk samples, 99 showed growth on VRBG agar plates after an enrichment step and 14 isolates were selected exemplarily and preserved for further analysis. Additionally, 4 (3 from site 1 and 1 from site 2)

presumptive positive *Cronobacter* spp. colonies, were stored for identification experiments. Of the 91 milk concentrate samples, 35 (31 from site 1 and 4 from site 2) showed growth on VRBG after an enrichment step of which 20 were selected for further analysis. No presumptive positive *Cronobacter* spp. colonies were observed for these samples on ESIA plates. Of the 172 milk powder samples 64 (40 from site 1 and 24 from site 2) showed growth on VRBG and 30 (20 from site 1 and 10 from site 2) were selected for further analysis. For 12 milk powder samples (7 from site 1 and 5 from site 2) presumptive positive *Cronobacter* spp. colonies were observed on ESIA plates and isolates were stored for identification purpose.

#### Prevalence and identification of *Enterobacteriaceae* and *Cronobacter* spp.

Of the 18 raw milk isolates (14 from VRBG and 4 from ESIA), 13 could be identified with API ID32E adequately. The 4 presumptive positive *Cronobacter* spp. isolates (ESIA plates) from raw milk were negative in the *Cronobacter* genus identification PCR assay. Two of these isolates were identified by API ID32E as *E. cloacae* (ID 99.9 %, T 0.42) and *Proteus mirabilis* (ID 99.9 %, T 0.71) respectively.

All of the 20 selected milk concentrate isolates were typable by API ID32E.

Of the 30 milk powder isolates (from VRBG plates) only 4 isolates were untypable by API ID32E and were thus subjected to *rpoB* sequencing. Sequence analysis of the 4 isolates identified three of the isolates as *E. cloacae* (98 %, 98 % and 99 %) and one isolate as *E. coli* (99 %).

Of the 12 presumptive positive *Cronobacter* spp. isolates (from ESIA) from milk powder samples, 10 (5 from site one and 5 from site 2) isolates could be confirmed as *Cronobacter* spp. by genus specific PCR. The remaining 2 non-*Cronobacter* isolates (from ESIA plates) were identified by API ID32E as *Klebsiella pneumoniae* (ID 99.9 %; T 0.98), and *E. cloacae* (99.9 %, T 0.54). Results of the biochemical identification and *rpoB* sequencing are shown in Table 1.

The most dominant species out of the raw milk samples were *E. coli* (9) followed by *Hafnia alvei* (2), *E. cloacae* (1) and *Proteus mirabilis* (1). These as well as other organisms have previously been isolated from bovine raw milk on occasion (Ercolini et al., 2008, Kagkli et al., 2006).

Concerning the milk concentrate samples, 70 % (35/70) from site one and 9.7 % (4/41) of the samples from site 2 showed growth on VRBG plates. *E. coli* (8) and *E. cloacae* (4) were the most frequent organisms identified in the 20 strains isolated from milk concentrate. Additionally, *Enterobacter* spp. (1), *Klebsiella pneumoniae* (5) and *Acinetobacter baumannii* (2) were found. The latter organisms (*A. baumannii*) do not belong to the *Enterobacteriaceae* family, but were nevertheless identified from VRBG plates.

The milk concentrates are either further processed to condensed milk or used in stuffings for chocolates and candies or represent an inter-

**TABLE 1:** Identification of 63 isolates from different samples of an infant formula processing plant by API ID32E and *rpoB* sequencing.

Genus, species	Raw milk			Concentrate			Milk powder		
	A <sup>1D</sup>	S <sup>2</sup>	T <sup>3</sup>	A <sup>1</sup>	S <sup>2</sup>	T <sup>3</sup>	A <sup>1</sup>	S <sup>2</sup>	T <sup>3</sup>
<i>Acinetobacter baumannii</i>	-	-	-	2	-	2	8	-	8
<i>Citrobacter sedlakii</i>	-	-	-	-	-	-	1	-	1
<i>Enterobacter</i> spp.	-	-	-	1	-	1	4	-	4
<i>Enterobacter cloacae</i>	1	-	1	4	-	4	4	3	7
<i>Escherichia coli</i>	9	-	9	8	-	8	2	1	3
<i>Escherichia hermannii</i>	-	-	-	-	-	-	2	-	2
<i>Hafnia alvei</i>	2	-	2	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	-	-	-	5	-	5	3	-	3
<i>Leclercia adecarboxylata</i>	-	-	-	-	-	-	1	-	1
<i>Pantoea</i> spp.	-	-	-	-	-	-	1	-	1
<i>Proteus mirabilis</i>	1	-	1	-	-	-	-	-	-

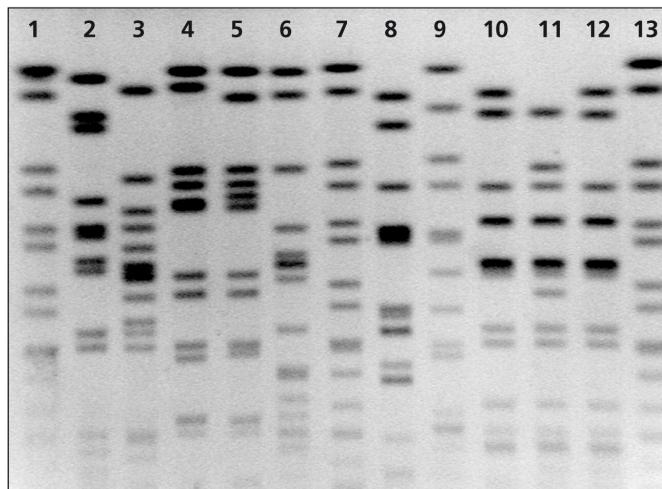
<sup>1</sup>Identification by API ID32E; <sup>2</sup>Identification by *rpoB* sequencing; <sup>3</sup>Total

mediate within the milk powder processing chain. Concentration in evaporators leads to a reduction of the water content to a dry weight of 40–52 %. From the moment of the application of the raw milk to the evaporator this process represents a closed loop circuit, which can be sanitized frequently and effectively (cleaning in place, CIP). The discrepancy between the numbers of positives from the two sites may be (in parts) explained by the fact that in site two the raw milk is subjected to a pasteurization process before the milk is passed on to the concentration process. Additionally, two different concentration technologies are applied at the two sites, which also might have an influence.

Identification of the isolates from 30 milk powder products revealed the highest degree of diversity in organisms and represents in general a combination of the organisms already identified in the other material. However, four more opportunistic pathogenic members of the *Enterobacteriaceae* appeared in the milk powder material namely *Citrobacter sedlakii* (1), *Escherichia hermannii* (2), *Pantoea* spp. (1) and *Leclercia adecarboxylata* (1). Most of these species have frequently been isolated from PIF, related products and production environments (Muytjens et al., 1988, Iversen and Forsythe, 2004, Estuningsih et al., 2006, Mullane et al., 2008, Popp et al., 2009).

Two different drying strategies are conducted in this factory in order to obtain milk powder: spray-drying for milk concentrate, originating from low-fat milk or roller drying for concentrate using full-fat milk as raw material (both types of powder are produced at both sites). Depending on which temperatures are applied during dehydration and drying the resulting milk powder is classified as low heat powder or high heat powder. Low heat powder has a high whey protein nitrogen index and is therefore used mainly in animal feeding (calf mast), whereas high heat powder consists of highly denatured milk proteins and is used as supplement in food industry and especially for the production of milk based infant food, yoghurt, pastries and ice cream.

During the roller drying process, the full-fat milk concentrate is exposed to temperatures > 100 °C. As roller drying is not a process which can be completed in an enclosed system, the hygienic monitoring, including the air within this room is crucial and specialized filters (HEPA) are applied to meet microbiological criteria. The powder has a final water content between 1.5–4.5 %. As these processes including the filling of the final products into “big-bags” are executed in an area, where wet cleaning is not possible, the presence of powder dust/particles especially throughout the packaging area is inevitable and entry of organisms may occur via personnel/material flow from/between other areas of the facility. Thin layers of powder/dust are present in the dry areas of the production facilities which may enable bacterial organisms to persist and to get disseminated among the different areas in the facility. However, these organisms must have undergone some selection processes since survival and persistence in dry (product) environments represents a challenge for most bacteria. In this respect it is interesting to note, that most of the organisms identified in this study may have their original habitat in the environment and thus have developed strategies to quickly adapt to adverse



**FIGURE 1:** PFGE analysis of 10 *Cronobacter* spp. isolates from milk powder. Lane 1, 7 and 13: *S. Braenderup* size standard. Lane 2–6 *Cronobacter sakazakii* isolates from site 1; lane 8: *Cronobacter malonaticus*, lane 9–12: *Cronobacter sakazakii* isolates from site 2.

conditions. Concerning *Cronobacter* spp., such capabilities have already been reported (Schmid et al., 2009).

#### Further characterization of *Cronobacter* spp. isolates and subtyping

*Cronobacter* spp. was exclusively isolated from milk powder from both facility sites. Of originally twelve presumptive positive isolates, ten were confirmed as *Cronobacter* spp. whereas nine of them were further identified as *Cronobacter sakazakii* (5 from site 1 and 4 from site 2) and one as *Cronobacter malonaticus* (site 2).

All five *C. sakazakii* isolates from site 1 exhibited different PFGE profiles. Among the 4 *C. sakazakii* isolates from site 2, three different profiles were observed. Additionally, the *C. malonaticus* isolate displayed a unique fingerprint profile. No identical profiles were observed across the two facility sites. The PFGE patterns of the ten *Cronobacter* spp. isolates from both sites are shown in Figure 1.

This relatively high degree of diversity among *Cronobacter* spp. isolates from both sites suggests continuous entry/dissemination of organisms into/among different buildings/areas. The results support findings from a recent study performed in the infant formula production area from the same processing plant investigated in this study. In that study, the presence of *Cronobacter* spp. originating from products, environment and raw materials was investigated and subtyping analysis performed on 153 isolates revealed 71 pulsotypes (Iversen et al., 2009). The results from this study indicated that both raw and environmental material may be a significant source for *Cronobacter* spp. contamination of powdered infant formula production facilities and also that a selection is probably occurring for certain persistent strains of *Cronobacter* spp. within production environments and powdered infant food products. Moreover, the lack of common strains between environment and plant raw materials may suggest that the net flow of the contamination events is from raw materials to the product and then to the environment, from which contamination of the later products can occur.

The final product milk powders from the current study are not supplemented with any additives, thus contamination of the final products seems constricted to environmental material.

However, the areas of PIF and milk powder production in this facility are not strictly separated, and there is a constant flow of personnel and/or material which may count responsible for the entry/dissemination of *Cronobacter* spp. and other *Enterobacteriaceae* among production areas.

## Conclusions

There are two major conclusions that can be made from the results from this study: (1) the presence of potentially pathogenic members of the *Enterobacteriaceae* family in milk concentrate may be (although not completely eliminated) “reduced” by implementing a pasteurization step before continuing with the concentration process and (2) the contamination of the milk powders during the drying/packaging process is most likely originating from the environment.

In order to reduce the risk of post process contaminations of milk powder products, effective cleaning of the production environments seems crucial, with spilled powder in vacuum cleaners appearing a rich source of organisms including *Cronobacter* spp. (Iversen et al., 2009). Constant monitoring of the production process is necessary to ascertain the areas of most risk and identify further contamination reservoirs.

*Enterobacteriaceae* are often used as process hygiene criteria and indicator organisms for the presence of *Cronobacter* spp. However, it has been shown, that some isolates of *Cronobacter* spp. do not grow well in currently proposed enrichment broths notably the *Enterobacteriaceae* enrichment broth (ISO 21526-1:2004), as well as the modified Lauryl Sulphate Tryptone broth included in the ISO procedure for the identification of *Cronobacter* spp. (ISO/TS 22964). Therefore it is recommended for monitoring purposes to test for both *Enterobacteriaceae* using the standard procedure (21526-1:2004) as well as for *Cronobacter* spp. using a modified differential media developed by Iversen et al., 2008.

Moreover, the installation of personal locks and the strict separation of areas, where different products are processed seem necessary, to disrupt the flow of (contaminated) powders and/or supplements.

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**5.3.3 Genetic diversity of multiple *Cronobacter* spp. isolates from cultural positive powdered infant formulæ, ingredients and environment samples – consequences for epidemiological studies in processing plants**

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## Genetic diversity of multiple *Cronobacter* spp. isolates from cultural positive powdered infant formulae, ingredients and environment samples – consequences for epidemiological studies in processing plants

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*Cronobacter* spp. is an opportunistic pathogen and an occasional contaminant of powdered infant formulae and the production environment. During plant epidemiological surveys usually one isolate of sample (originating from one colony forming unit on selective plate) is being used for genotype analysis. The aim of this study was to investigate if different colony forming units from one original sample show the same PFGE pattern. Therefore, 5 colony forming units from 25 positive samples including powdered infant formulae (8), product environment samples (16) and ingredients (1) have been analysed. Twenty-one of 25 (84%) showed the same PFGE patterns whereas 2 finished products (25%) and 2 environmental samples (12.5%) showed different PFGE fingerprints. The observation of the presence of multiple isolates within individual samples suggests that analysis of one isolate per sample may not be sufficient for trace back studies. The analysis of at least five colony forming units per sample is therefore suggested.

### Genetische Unterschiede von multiplen *Cronobacter* spp.-Isolaten aus positiver pulverförmiger Säuglingsnahrung, Rohstoff- und Umgebungsproben – Folgen für epidemiologische Untersuchungen in Produktionsstätten

Bei *Cronobacter* spp. handelt es sich um opportunistisch pathogene Mikroorganismen, welche in pulverförmigen Säuglingsnährmitteln und in deren Produktionsumgebung gelegentlich nachgewiesen werden können. Für epidemiologische Untersuchungen auf Ebene des Produktionsbetriebes wird häufig ein einziges Probenisolat, welches von einer Kolonie-bildenden Einheit auf einem Selektivmedium stammt, für die Genotypisierung verwendet. Im Rahmen dieser Studie wurde untersucht, ob verschiedene Kolonie-bildenden Einheiten derselben Ursprungsprobe dasselbe PFGE Muster zeigten. Zu diesem Zweck wurden je 5 Kolonie-bildende Einheiten von 25 positiven Proben analysiert (8 pulverförmige Säuglingsnahrungen, 16 Umgebungsproben, 1 Rohstoff). 21/25 Proben (84%) zeigten dasselbe PFGE Muster, bei 2 Fertigprodukten (25%) und 2 Umgebungsproben (12.5%) unterschieden sich die PFGE Fingerprints. Die Tatsache, dass mehrere Isolate mit unterschiedlichen PFGE-Mustern in derselben Probe vorhanden waren, lässt darauf schließen, dass die Genotypisierung von lediglich einem Isolat pro Probe für epidemiologische Studien nicht genügend aussagekräftig ist. Deshalb ist es ratsam, mindestens 5 Kolonie-bildende Einheiten pro Probe zu genotypisieren.

**26 *Cronobacter* spp.** (PFGE, epidemiology, powdered infant formula, manufacturing plant)

**26 *Cronobacter* spp.** (PFGE, Epidemiologie, pulverförmige Säuglingsnahrung, Produktionsstätte)

## 1. Introduction

*Cronobacter* spp. (former *Enterobacter sakazakii*) are Gram-negative opportunistic foodborne pathogens that have been associated with sepsis, meningitis and necrotizing enterocolitis in neonates and pre-term babies due to ingestion of contaminated infant formulae (1, 2, 3, 4, 5). The organisms appear to be ubiquitous and have been isolated from a variety of food products, production environments and households (6, 7, 8, 9). In addition, *Cronobacter* spp. is highly tolerant to osmotic and dry stresses and has the ability to persist for extended periods in the environment as well as in powdered products (10, 11, 2, 13).

The reported prevalence of *Cronobacter* spp. in surveys of commercially available powdered infant formulae appears to be gradually decreasing from estimates of 14% in 1988 (14) and 6.7% in 1997 (15), to 2.5% in 2001 (16). Recent estimates indicate that although small reductions may still occur, the prevalence appears to be stabilizing at 2-2.5% (17, 18, 19). This could be either due to particularly persistent phenotypes in production facilities which seem to be resistant even to the improved hygiene measures adopted by manufacturers, or to yet unidentified con-

tamination routes. Fingerprint typing has become established as a useful method to support monitoring of microorganisms in relation to public health, consumer protection and manufacturing hygiene practices.

Generally, during plant epidemiological surveys one isolate of the sample (originating from one colony forming unit on a selective plate) is being analysed by a fingerprint analysis. Within this study we wanted to gain information on the genetic diversity of different isolates originating from one sample. The samples were collected from two different infant formula processing plants and comprised finished powdered infant formula products, ingredients and environmental samples. The generated information on the presence of different PFGE pulso-types of individual isolates from the same sample should help improving epidemiological discussion and implementing hygiene measures.

## 2. Material and methods

### 2.1 Sampling

The selected cultural positive samples (n = 25) originated from two manufacturing plants producing different kinds of milk powder and powdered infant

formulae in Switzerland. Samples comprised finished products (n=8), ingredients (n=1) and processing environment (n=16). Environmental samples comprised the content of vacuum cleaner bags and waste water drainages as well as air filter powder. Finished products included starter formulae which were not intended for selling. The names and origin of the samples used in this study are given in Table 1.

Table 1: Descriptions and codes of used samples (n=25)		
Sample code	Description of sample	Location
Prod 1	Finished product in tins	site 1, building 3
Prod 2	finished product in big bags	site 1, building 3
Prod 3	finished product in tins	site 1, building 3
Prod 4	finished product in pouches	site 2, building 2
Prod 5	finished product in pouches	site 2, building 2
Prod 6	finished product in big bags	site 1, building 3
Prod 7	finished product in big bags	site 1, building 3
Prod 8	finished product in big bags	site 2, building 1
Ing 1	whey protein	site 2, building 1
Env 1	waste water drainage	site 1, building 1
Env 2	vacuum cleaner bag	site 2, building 1
Env 3	vacuum cleaner bag	site 2, building 2
Env 4	waste water drainage	site 1, building 1
Env 5	waste water drainage	site 1, building 2
Env 6	waste water drainage	site 1, building 1
Env 7	waste water drainage	site 1, building 3
Env 8	waste water drainage	site 1, building 2
Env 9	waste water drainage	site 1, building 3
Env 10	waste water drainage	site 1, building 3
Env 11	air filter powder	site 1, building 1
Env 12	waste water drainage	site 1, building 3
Env 13	vacuum cleaner bag	site 2, building 2
Env 14	vacuum cleaner bag	site 2, building 1
Env 15	vacuum cleaner bag	site 2, building 3
Env 16	air filter powder	site 1, building 1

2.2 Isolation, identification and fingerprinting method

The tested sample volume depended on the type of material: product samples were analysed in 1 x 300g, environmental samples and raw material in 1 x 10g and swabs covering a surface of 10x10cm<sup>2</sup>, respectively. Samples were pre-enriched 1:10 (w/v) in buffered peptone water (37°C, 18-24h), subsequently enriched 1:100 (w/v) in *Cronobacter* screening broth (CSB, 42°C, 22-24h) and a loopful of CSB was streaked directly onto chromogenic agar, namely DFI (Druggan, Forsythe and Iversen agar, CM1055, Oxoid Ltd., Basingstoke, UK) and/or ESIA (*Cronobacter* Isolation agar, CM1134, Oxoid Ltd., Basingstoke, UK) and incubated as recommended by the supplier.

Five colony forming units per sample were picked from the selective plates and isolates were genus and species identified by PCR according to the methods by LEHNER *et al.* (20) and STOOP *et al.* (21), respectively. Macrorestriction digest using the restriction enzyme XbaI and PFGE was performed according to the published method by IVERSEN *et al.* (19) with the modification that the digestion step was prolonged for 12 h.

The PFGE patterns were analysed using the Gel-Compar II software version 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Cluster analysis and construction of dendrograms were performed using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The parameters for optimization and position tolerance were both set to 3%.

3. Results

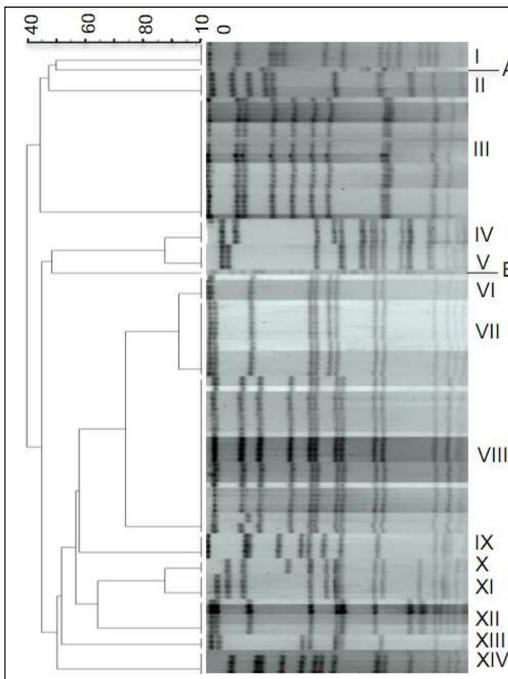


Fig. 1: Dendrogram of 125 isolates from 25 sample sets. 14 clusters (I-XIV) consisting of several identical isolates each and 2 unique pulso-types (A, B) found in only one isolate each have been identified. Identification codes of samples in downward order (according to appearance within dendrogram; top to bottom): Cluster I (Ing 1\_1, Ing 1\_2, Ing 1\_3, Ing 1\_4, Ing 1\_5), A (Prod 1\_2) Cluster II (Env 15\_1, Env 15\_2, Env 15\_3, Env 15\_4, Env 15\_5), Cluster III (Prod 1\_1, Prod 2\_2, Prod 2\_3, Prod 2\_4, Prod 2\_5, Prod 1\_3, Prod 1\_4, Prod 1\_5, Prod 3\_3, Prod 3\_4, Prod 3\_5, Prod 3\_1, Prod 3\_2, Prod 4\_1, Prod 4\_2, Prod 4\_3, Prod 4\_4, Prod 4\_5, Prod 5\_1, Prod 5\_2, Prod 5\_3, Prod 5\_4, Prod 5\_5, Prod 2\_1), Cluster IV (Prod 8\_5, Prod 8\_1, Prod 8\_2, Prod 8\_3, Prod 8\_4), Cluster V (Env 13\_1, Env 13\_2, Env 13\_3, Env 13\_4, Env 13\_5) B (Env 8\_5), Cluster VI ( Env 7\_1, Env 7\_2, Env 7\_3, Env 7\_4, Env 7\_5), Cluster VII (Env 4\_1, Env 4\_2, Env 4\_3, Env 4\_4, Env 4\_5, Env 5\_1, Env 5\_2, Env 5\_3, Env 5\_4, Env 5\_5, Env 6\_1, Env 6\_2, Env 6\_3, Env 6\_4, Env 6\_5), Cluster VIII (Env 8\_1, Env 8\_2, Env 9\_1, Env 9\_2, Env 9\_3, Env 9\_4, Env 9\_5, Env 10\_1, Env 10\_2, Env 10\_3, Env 10\_4, Env 10\_5, Env 11\_1, Env 11\_2, Env 11\_3, Env 11\_4, Env 11\_5, Env 16\_1, Env 16\_2, Env 16\_3, Env 16\_4, Env 16\_5, Prod 7\_1, Prod 7\_2, Prod 7\_3, Prod 7\_4, Prod 7\_5, Prod 6\_2, Prod 6\_5, Env 8\_3, Env 8\_4), Cluster IX (Env 14\_1, Env 14\_2, Env 14\_3, Env 14\_4, Env 14\_5), Cluster X (Prod 6\_1, Prod 6\_3, Prod 6\_4), Cluster XI (Env 1\_1, Env 1\_2, Env 1\_3, Env 1\_4, Env 1\_5), Cluster XII (Env 2\_1, Env 2\_2, Env 2\_3, Env 2\_4, Env 2\_5, Env 3\_4, Env 3\_5), Cluster XIII (Env 3\_1, Env 3\_2, Env 3\_3), Cluster XIV (Env 12\_1, Env 12\_2, Env 12\_3, Env 12\_4, Env 12\_5)

Twenty-five sample sets each consisting of five isolates were obtained by picking five presumptively positive colony forming units from the respective selective plate. The isolates were genus and species identified by PCR. All of the isolates were confirmed

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Fricker-Feer, Genetic diversity of multiple *Cronobacter* spp

as *Cronobacter* spp. and all but one isolate were identified as *Cronobacter sakazakii*. This isolate was part of the finished product sample set "Prod 1" and was PCR identified as *Cronobacter malonicus*.

Fingerprint analysis using macrorestriction digest and PFGE revealed identical pulso-types among the five selected isolates from one sample in the majority (21) of the cases. However, in four of the samples (two environmental and two finished product samples) two different pulso-types were observed among the set of five isolates per sample tested. In Fig. 1 the overall dendrogram of the pulso-types of the 125 (5 x 25) individual isolates is given. Fourteen different pulso-types comprising 3 – 31 isolates and two separate patterns were identified.

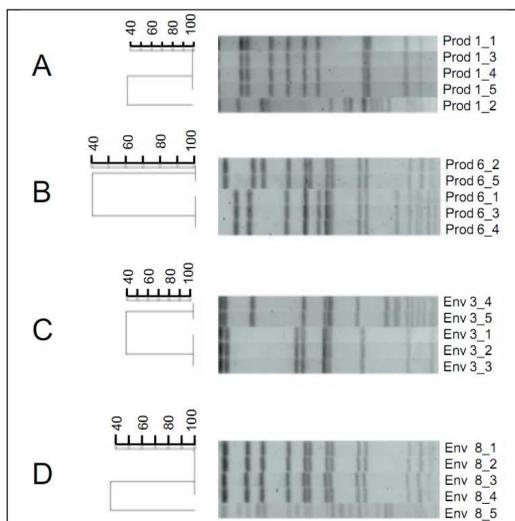


Fig. 2: Dendrograms of 4 sample sets showing divergent pulso-types among isolates

In Fig. 2 the four sub-trees of the sample sets exhibiting different pulso-types among isolates is depicted. Two out of eight product sample sets (Prod 1 and Prod 6) as well as two out of sixteen environmental sample sets (Env 3, Env 8) showed diverging PFGE patterns among isolates of the same sample set. These environmental samples originated from a waste water drainage and a vacuum cleaner bag respectively. The ratio of varying patterns ranged from 1:4 (Prod 1, Env 8) to 2:3 (Prod 6, Env 3). The similarity of diverging patterns among sample sets was below 60%.

Analysis of the finished product sample set 6 (Prod 6) revealed that three out of five isolates exhibited separate patterns with no direct connection to pulso-types of other samples, whereas the two other isolates had pulso-types that were identical to another finished product sample set (Prod 7) manufactured on the same spray drying tower. Moreover, the very same pattern was observed in isolates from five different environmental sample sets (Env 8, Env 9, Env 10, Env 11, Env 16) which originated from waste water drainage material collected in two different buildings and from air filter powder.

From a long-term study which is still ongoing, we have evidence that this is a strain, which has been persisting in the processing environment for an extensive period of time (data not shown).

The two environmental sample sets, that exhibited diverging patterns originated from a vacuum cleaner belonging to a filling line and from a waste water drainage, respectively. Two of the isolates from the vacuum cleaner bag sample showed identical pattern to the one from an isolate, sampled at the filling station of a spray drying tower which is likely to be a possible dissemination route within the production area. The patterns of the remaining three isolates did not show any correlation with fingerprints from other sample isolates.

Four PFGE fingerprints of the waste water drainage sample isolates (Env 8) showed the same patterns as 20 other environmental strains (namely from other buildings but the same production site) and seven finished product sample isolates.

It is known that contaminations with *Cronobacter* spp. often occur after the spray drying process either through an insufficient hygienic environment or through dry blended raw materials (11, 18, 22). Considering different pulso-types observed in some sample sets implies that elucidation of dissemination routes is even more complex and must be discussed under different aspects.

#### 4. Conclusion

Fingerprint analyses have become valuable tools in processing epidemiology surveys. Thereby, in most of the cases, one isolate per sample is analysed by using a fingerprinting method of choice. However, the results from the present study show that multiple fingerprint patterns of *Cronobacter* spp. can be obtained when analysing several isolates that originated from the same sample. This clearly indicates that samples can be contaminated with multiple strains of the same species and/or even members of different species. Interestingly, this was not just true for the environmental sample material, where one could expect a higher variation in species and/or strains but it was also observed in finished product samples.

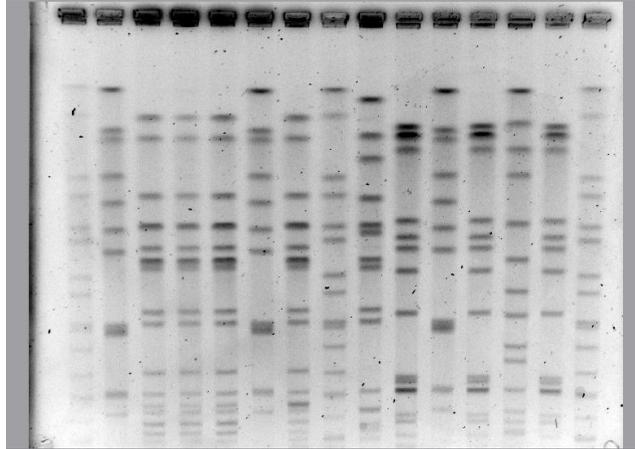
The distribution of *Cronobacter* spp. in industrial batches of powdered infant formula is heterogeneous and the average concentration of  $-4.41 \log \text{CFU/g}$  is determined very low (23). Against this background the implementation of suitable sampling strategies as well as the use of specific and sensitive detection methods is of central importance.

However, for manufacturers it is important to generate epidemiological data in order to detect possible dissemination routes and in consequence to avoid possible (cross)-contaminations especially in finished products. Unfortunately, PCR-based fingerprinting methods such as RAPD have been proven to be of poor discriminative power (11). Therefore, the application of a macrorestriction-based method in combination with PFGE seems indispensable. In addition, the observation of the presence of multiple isolates within individual samples suggests that analysis of one isolate per sample may not be sufficient for trace back studies. The analysis of at least five colony forming units per sample is therefore suggested.

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## 6 DISCUSSION



### 6.1 Methodology

The novel proposed screening broth for the isolation of *Cronobacter* spp. is based on less selective components than the currently used modified lauryl sulphate tryptose broth (mLST according to ISO/TS 22964, Anonymous, 2006c) and *Enterobacteriaceae* enrichment broth (EE, according to FDA-method, Anonymous, 2002) where some strains of *Cronobacter* spp. do not grow well (Guillame-Gentil et al., 2005; Iversen and Forsythe, 2007; Lehner et al., 2006). Among other experiments, 950 samples (322 PIF, 146 ingredients, 482 environmental samples) have been analysed with CSB, mLST and EE. The comparison showed >90% sensitivity, >95% specificity and >99% negative predictive value for CSB and mLST, whereas the FDA-method with EE was less accurate (33% sensitivity, 83% specificity, 94% negative predictive value). The advantage of CSB in contrast to the current ISO/TS 22964 is a product release within 48 hours based on negative samples. The costs and working time are considerably reduced, as only positive broths need to be streaked onto chromogenic agars. However, the overall effectiveness of the method depends on the sensitivity and specificity of the chromogenic agar (**chapter 5.1.1**).

In the last few years, various PCR based methods have been developed, namely an *rpoB* based PCR system to differentiate the six proposed *Cronobacter* species (Stoop et al., 2009). A recent study described an additional species *C. condimenti* within the genus *Cronobacter* and a novel species designation – *C. universalis* – was proposed for the former *C. genomospecies 1* (Joseph et al., 2011). In this context we extended the existing *rpoB* based PCR system for the new species *C. condimenti* (**chapter 5.1.4**).

More recently, several real-time PCR based systems for detection of *Cronobacter* spp. have become commercially available. This improvement provides industrial laboratories with the possibility to release product in a

short time and based on reliable results. Two out of three tested systems generated neither false positive nor false negative results (22 pure culture experiments and spiked samples).

In addition, one system needs only 24h for pre-enrichment which means another saving of time and faster release of product. The main difference of the tested platforms is the DNA extraction procedure: for one system a previous DNA extraction was needed. This separate step requires professional lab personnel and more time. The two dedicated systems though, require pre-enriched broth as starting material. This is more convenient and can prevent handling faults. In conclusion, there are specific and well performing real-time PCR kits available (**chapter 5.1.2**).

An actual literature review about different systems of detection, identification and typing methods for *Cronobacter* spp. summarises the latest methods which are commonly available and often used for detection, identification and typing of *Cronobacter* spp. (**chapter 5.1.3**).

In the future, extended experiments with different pre-enrichment broths should be done. Weber et al. (2009) showed that stressed *Enterobacteriaceae* grow poorly when background competitors (e.g. gram-positive bacteria) are present. Desiccated cells, which are often present in milk powder and infant nutrition plants, are able to recover if specific supplements are added to enrichment broth under adequate temperature-time conditions. Premium powdered infant formulae which often contain probiotics such as *Lactobacilli* and *Bifidobacteriae* need to be further investigated in order to exclude false negative results.

In addition, further experiments concerning the sampling size should be performed. In Regulation (EC) 2073/2005 (Anonymous, 2005) only the sampling number is mentioned ( $n$  = number of using comprising the sample). This number has a statistical relevance for establishing the sampling plan but has no significance for the pooling size of analytical samples. In routine laboratories it is common to analyse *Enterobacteriaceae* in composite samples of 100g instead of 10 samples of 10g and *Cronobacter* spp. in samples of 300g instead of 30 samples of 10g. This causes less labour, is more convenient and needs less space for preparation and incubation. Recently, Miled et al. (2010) described the effect of sample pooling on *Cronobacter* spp. growth. Bacterial interactions, as they occur in case of pooled samples, can cover the presence of low quantity of *Cronobacter* spp. and lead to false negative results. In case of very low contamination levels, the variability of individual cells can have an important impact on pathogen bacteria growth (Guillier and Augustin, 2006). Only few published data about the effect of different sampling size concerning *Enterobacteriaceae* and *Cronobacter* spp. is available. Industrial company laboratories need simple rules how to pool and analyse their samples. The procedure must be 100% sensitive and 100% specific in order to avoid product release based on false negative results. Further studies on a robust and reliable procedure for enrichment, isolation and detection of *Cronobacter* spp. are ongoing.

## 6.2 Epidemiology

Till the end of 2005 *Enterobacteriaceae* used to be detected by quantitative method and proof of *Cronobacter* spp. was not required and therefore seldom analysed. With the introduction of Regulation (EC) 2073/2005 (Anonymous, 2005), a completely new situation for manufacturers arose: First of all, collected data was needed to identify the prevalence of those bacteria and to gain a general overview of the whole situation. According to literature data, the estimated prevalence has decreased during the last 20 years and seems to stabilise at 2.0-2.5% (Iversen and Forsythe, 2004; Iversen et al., 2008; Mullane et al., 2007). These figures are based on commercially available PIF and do not necessarily have to correlate with the actual situation in industrial plants. Results from raw materials, intermediates, finished products and environmental samples had to be analysed accurately in order to find correlations between them. Based on a reliable analytical method, which was a challenge in itself (**chapter 5.1.1**), we analysed more than 1000 samples to obtain evidence where *Enterobacteriaceae* and especially *Cronobacter* spp. come from (**chapter 5.2.1 and 5.3.1**). It is important not only to identify these *Enterobacteriaceae* but also to subtype the strains in order to reveal possible routes of contamination.

*Enterobacteriaceae* are ubiquitous micro-organisms which are common in powder factories. The study in **chapter 5.2.1** shows a high diversity of 24 different species and 65 samples which could not be identified. Out of 470 isolates > 30% were *E. cloacae*, a species which can be found in the same niches as *Cronobacter* spp. Compared with previous studies, we detected higher amounts of *E. cloacae* (25% Iversen and Forsythe, 2004; 16% Estuningsih et al., 2006). One possible reason could be that both production sites fabricate not only PIF but also other products such as milk powder and different blendings in which *E. cloacae* can occasionally be detected. In addition, *Pantoea* spp. (11%) and *Klebsiella pneumoniae* (8%) were identified. Prevalence data of these bacteria were not available for PIF, therefore more studies are required. For this reason we performed a PFGE-typing in order to achieve a better understanding in relation to possible cross-contaminations and dissemination (**chapter 5.2.2**). A closer look at the wide variety of different clusters revealed that different finished products containing the same pulso-type were manufactured on the same line (e.g. spray dryer, blender, packaging line). In these cases a carryover seems likely. In other cases, possible routes of dissemination via raw material or environment could be identified. These results demonstrate that the air flow management, different types of cleaning and heat instable raw material are potential sources of cross-contamination. Raw milk itself contains *Enterobacteriaceae* but no *Cronobacter* spp. were found (**chapter 5.3.2**). These results have been confirmed by Baumgartner and Niederhauser (2010) who tested 875 samples of raw milk. Their conclusion supports our theory that milk is not a relevant source of *Cronobacter* spp. whereas milk powder as an ingredient in PIF might be contaminated by environmental sources and should be monitored. The discrepancy of the positive *Enterobacteriaceae* results for milk concentrate between site 1 (70%) and site 2 (10%)

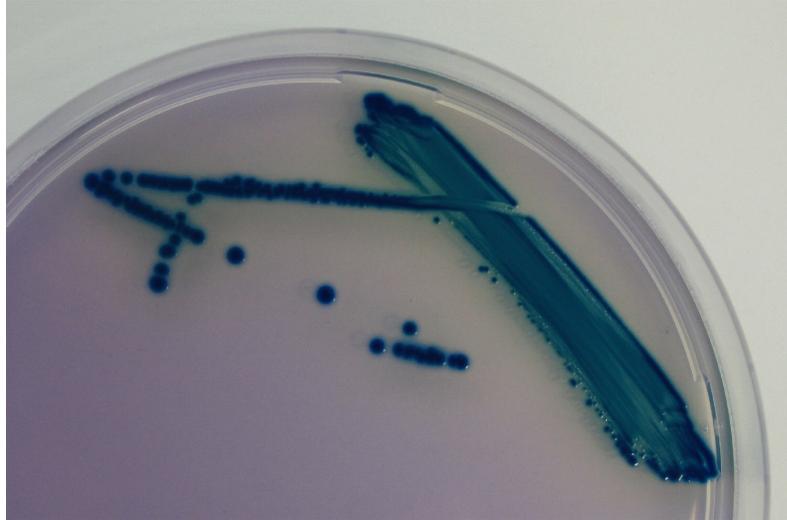
indicates that different pasteurization procedures can have a major influence on the microbiological status of intermediates such as milk concentrate. It seems likely that *Enterobacteriaceæ* form biofilm in evaporators if the running times are too long. Lehner et al. (2005) proved the ability of *Cronobacter* spp. to produce biofilm. PIF-suspensions contain protein, carbohydrates, fat and some minor components like vitamins and minerals. These nutrient-rich media should be further investigated in order to get a better understanding of biofilm formation during PIF production. Temperature-time relations of emulsion preparation, heating steps (e.g. evaporation and pasteurization) and cleaning procedures need to be considered for better process management.

In order to study different dissemination routes of *Cronobacter* spp., 950 samples have been analysed where of 153 isolates could be identified as *Cronobacter* spp. (**chapter 5.3.1**). The largest diversity was found in raw material, but no correlation between raw material and environment could be made. The sample analysis was performed according to ISO/TS 22964 (Anonymous, 2006c) and parallel to CSB-method (**chapter 5.1.1**). Clonal types were further examined using RAPD (Random-Amplified Polymorphic DNA) and PFGE (Pulsed-Field Gel Electrophoresis). Whereas the RAPD tree produced 44 distinguishable groups in a quick and reliable manner, the PFGE fingerprint patterns clustered 71 different pulso-types. The PFGE analysis provides greater discrimination between the strains than the results obtained with RAPD. But nevertheless, even with RAPD several typical clusters were built (e.g. plant-derived raw materials). The PFGE dendrogram shows various correlations between both raw material and finished product and between environmental samples and finished product. These facts lead to the hypothesis that *Cronobacter* spp. is brought into the plant via raw material. The bacteria disseminate to finished products and later on to the environment. It seems likely that some strains are persistent. Environmental niches that are suspected to be a source of contamination should be examined more carefully. A steady monitoring of several production steps (e.g. emulsion preparation, heat treatment, storage tank, internal fluid bed, external fluid bed, sieves, pipes, big bag filling station) should be implemented. In addition, storage and handling of raw materials should be improved. The secondary wrapping of palletised raw material should be removed before entering the hygienic zone. Bulk products should be conveyed into tanks using a continuously sterile air system (HEPA filtered). The air flow management for the spray drying tower must be taken into consideration, especially during intermittent standstills, e.g. weekends.

Epidemiological data is essential for each manufacturer. Based on scientific information it is possible to establish an adequate HACCP system which can guarantee food safety.

Usually, for plant epidemiological surveys one isolate of the sample (originating from one colony forming unit on selective plate) is being used for pulso-type analysis. The analysis of five different colony forming units from 25 samples (finished products, raw material, environmental samples) showed in 16% different PFGE fingerprints. This observation suggests that analysis of one isolate per sample may not be sufficient for trace back studies. The analysis of at least five colony forming units per sample is suggested (**chapter 5.3.3**).

## 7 CONCLUSION



The presented data reveal that *Enterobacteriaceae* in general and *Cronobacter* spp. in particular are not likely to disappear from PIF production plants in the near future. Manufacturers have to put up with the situation and consequently have to take adequate measures such as:

- Reduce re-contamination after the last heating step (e.g. UHT treatment):
  - determine possible niches where the bacteria can survive and take measures to eliminate them, e.g. dead end pipes, thermal bridges.
- Establish step controls over the whole process, e.g. rinsing water, dust, conditioned air.
- Carry out as less wet cleaning as possible in high hygienic areas. Dry cleaning should be performed with vacuum cleaners equipped with HEPA-filters.
- Establish defined personal and material flow by implementing efficient gates.
- Avoid visitors and external personal.
- Install hygienic designed equipment.
- Implement validated procedures, e.g. for start and end of production and cleaning processes.
- Evaluate appropriate temperature-time conditions during preparation of emulsion in order to achieve a sufficient pasteurization effect and to avoid biofilm.
- Establish appropriate air flow management.

It is important to mention that only the sum of implemented measurements (equipments, personal and material flows, environment, process media, process procedures, analytics) lead to successful finished products. This work has to be done in a multi disciplinary team.

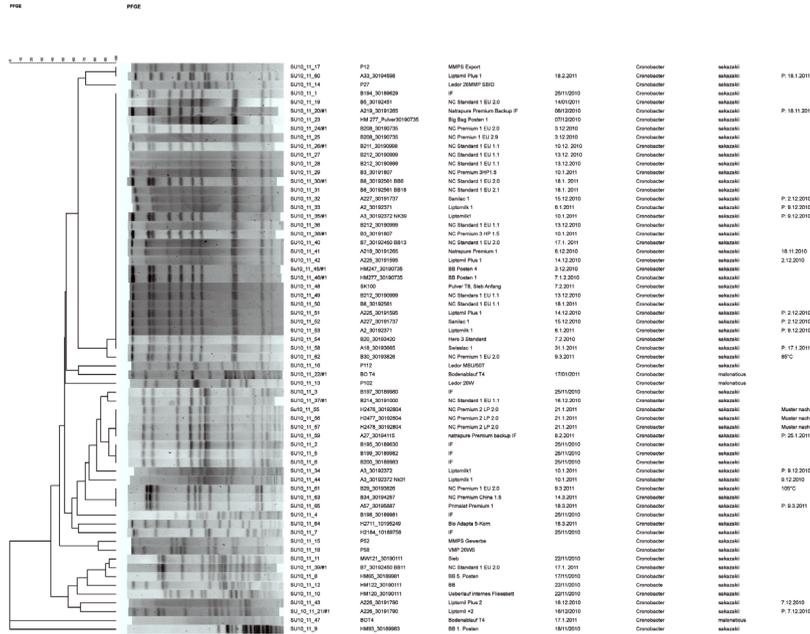
Scientific support is needed to answer the following questions:

How does the sampling size influence the growth of bacteria during pre-enrichment and enrichment procedure? The maximum pool size has to be defined for each germ in order to generate no false negative results.

What is the ideal media to resuscitate stressed (e.g. desiccated) cells? The possibility of a pre-enrichment broth for several gram-negative bacteria should be evaluated.

My work containing the mentioned publications represents only a puzzle-piece in the field of research into *Enterobacteriaceae* and *Cronobacter* spp. Further studies focussing on more epidemiological aspects are of great scientific importance. Epidemiologic data combined with adequate analytical techniques is needed for manufacturers to produce safe PIF. A close collaboration between industry and science can prevent infections in newborns.

# 8 SUMMARY



Due to technical reasons, milk powder and powdered infant formulae (PIF) are not sterile products. In order to achieve the requirements set by the Swiss and European regulations for microbiologic criteria extensive epidemiological studies are needed on each individual plant level. In this way contamination routes can be identified and appropriate measurements taken. A legally reglemented pasteurization process eliminates *Enterobacteriaceae*. Therefore recontamination must now be focussed on. PIF contaminated with *Cronobacter* spp. can lead to severe infections in neonates such a sepsis, meningitis or necrotizing enterocolitis. The reported prevalence of commercially available PIF appears to be gradually decreasing from estimates of 14% in 1988 to 2.0-2.5%, where it now seems to have become stabilized.

In order to make a reasonable estimate concerning the prevalence of *Cronobacter* spp. on an individual plant level, 950 samples (raw material, finished products, environmental samples) were analysed. The high prevalence of 16% comes from the intentional sampling of critical raw material and environmental samples. The PFGE analysis, however, did not reveal any correlation between raw material and environmental samples which would indicate a possible contamination via finished products. 470 PIF *Enterobacteriaceae* isolates were identified through biochemical tests as well as by *rpoB* sequencing. *E. cloacae* (35%), *Pantoea* spp. (11%) and *K. pneumoniae* (8%) were the most prevalent genus and species. In order to reveal possible contamination routes, a subtyping was conducted. The species *E. cloacae*, which can be found in the same niches as *Cronobacter* spp., could be used as a significant hygienic indicator organism. To complete the epidemiological picture, 363 milk based

samples were analysed (raw milk, milk concentrate, milk powder). Raw milk contains *Enterobacteriaceae* but no *Cronobacter* spp. were detected. However, 12/172 samples of milk powder contained *Cronobacter* spp. due to re-contamination (during the packaging process and/or further processing steps).

In order to increase the sensitivity and specificity of today's available analysis for the detection of *Cronobacter* spp. methodological improvements had to be undertaken. The currently used enrichment media (mLST, EE) contain components of too selective nature which can therefore lead to false negative results. The new "*Cronobacter* Screening Broth" (CSB) contains sucrose, bromocresol purple and vancomycin hydrochloride which now leads to a sensitivity of 100% and a negative predicting value of 100% as well. The change in colour of the broth indicates a presumptively positive result whereby only these samples need to be streaked onto chromogenic agar. The visual intermediate result leads to a reduction in costs and working time.

In order to increase specificity as well as the commercial pressure of fast product release, a PCR-based system where positive and negative results are clearly available in short time is recommended. Several real-time PCR based systems for detection of *Cronobacter* spp. have become commercially available. Two systems (one open platform (Biotecon Diagnostics, Potsdam, Germany) and one dedicated system (BioControl, Bellvue, USA)) generated neither false positive nor false negative results. Both systems were able to detect 9 target and 13 non-target strains. The dedicated system has the advantage of shorter hands-on and analysis time. In addition, contaminations due to handling faults are reduced.

The existing *rpoB* based *Cronobacter* species PCR was upgraded for the recently described species *C. condimenti* which can now be detected with high reliability.

Additional epidemiological data is needed in order to monitor the microbiological situation in industrial plants constantly as well as consequently. Based on information on individual plant level it is possible to implement adequate measurements such as HEPA filters, exact time for adding heat labile ingredients, personal and material flow, air management and cleaning (type, time). Scientific support is needed concerning adequate analytical methods, formation of biofilm, desiccation data, types of enrichment media, sample size as well as additional epidemiological data.

Our recent study concerning genetic diversity showed that different *Cronobacter* isolates from one sample can contain different PFGE fingerprints. This observation suggests that analysis of one isolate per sample may not be sufficient for trace back studies. The analysis of at least five colony forming units per sample is suggested. This example shows that through a close collaboration between industrial companies and scientific institutes, knowledge can be actively turned into practice. – This helps prevent pre-term babies and newborns from falling ill to *Cronobacter* spp.

KEY WORDS: *Enterobacteriaceae*, *Cronobacter* spp., epidemiology, prevalence, PCR, PFGE, powdered infant formulæ, industrial production plant

## 9 ZUSAMMENFASSUNG



Bedingt durch den Produktionsprozess, handelt es sich sowohl bei Milchpulver wie auch bei pulverförmiger Säuglingsnahrung nicht um sterile Produkte. Um die Anforderungen der Hygieneverordnungen der Schweiz und der EU zu erfüllen, sind umfassende betriebsepidemiologische Studien notwendig. Dadurch können Kontaminationswege aufgezeigt und die notwendigen Massnahmen ergriffen werden. Da *Enterobacteriaceae* durch die gesetzlich reglementierten Pasteurisationsbedingungen abgetötet werden, liegt der Fokus vor allem bei der Rekontamination. Gelangen *Cronobacter* spp. in pulverförmige Säuglingsnahrung, können sie bei Neugeborenen Sepsis, Meningitis oder necrotisierende Enterocolitis auslösen. Die Prävalenz in pulverförmiger Säuglingsanfangsnahrung lag 1988 bei geschätzten 14% und sank kontinuierlich auf 2.0-2.5%, wo sie sich nun zu stabilisieren scheint.

Um auf Ebene des Produktionsbetriebes Aussagen über die aktuelle Prävalenz machen zu können, wurden insgesamt 950 Proben (Rohstoffe, Endprodukte, Umfeld) auf *Cronobacter* spp. analysiert. Die Prävalenz lag mit 16% sehr hoch, dies ist durch den bewussten Musterzug von kritischen Rohstoffen und Umfeldproben zu erklären. Die PFGE-Analyse zeigte keine Korrelation zwischen Rohstoff- und Umfeldproben, was auf eine Kontamination via Endprodukt hindeutet. 470 pulverförmige *Enterobacteriaceae*-Isolate wurden mittels biochemischer Tests und *rpoB* Sequenzierung näher identifiziert.

*E. cloacæ* (35%), *Pantoea* spp. (11%) und *K. pneumoniae* (8%) waren die am häufigsten nachgewiesenen Genus und Spezies. Die identifizierten Stämme wurden genotypisiert, um mögliche Kontaminationswege in den Betriebsstätten aufzuzeigen. Die Spezies *E. cloacæ*, die in denselben Nischen wie *Cronobacter* spp. nachgewiesen wurde, könnte künftig als aussagekräftiger Indikatororganismus dienen. Um das epidemiologische Bild zu vervollständigen, wurden neben pulverförmigen Ingredienzien für Säuglingsnahrung auch 363 milchbasierte Proben analysiert, es handelte sich dabei um Rohmilch, Milch-konzentrat und Milchpulver. Die Untersuchung zeigte, dass Rohmilch *Enterobacteriaceæ*, allerdings keine *Cronobacter* spp. enthält, wohingegen 12/172 Milchpulverisolate auf Grund von Rekontaminationen während des Verpackungs- und Weiterverarbeitungsprozesses mit *Cronobacter* spp. belastet waren.

Um sowohl die Sensitivität wie auch die Spezifität der heute für *Cronobacter* spp. angewendeten Analytik zu erhöhen, bedurfte es methodischer Anpassungen. Die bisherigen Anreicherungsmedien für *Cronobacter* spp. (mLST, EE) können auf Grund ihrer selektiven Ingredienzien zu falsch negativen Resultaten führen. Durch die Supplementierung von Saccharose, Vancomycin und Bromocresol Purple wurde mit der neu entwickelten „*Cronobacter* Screening Broth“ (CSB) eine Sensitivität von 100% und ein negativer Voraussagewert (NPV) von 100% erreicht. Die Bouillon zeigt einen Farbumschlag bei Verdacht auf *Cronobacter* spp., so dass nur umgeschlagene Proben weiter analysiert werden müssen. Das optisch sichtbare Zwischenresultat bedeutet eine Zeit- und Kostenersparnis.

Um die Spezifität zu erhöhen, ist die Anwendung von PCR-basierten Methoden zu empfehlen. Der Vorteil der Verfügbarkeit von eindeutigen Resultaten ist vor allem in Industriebetrieben mit kurzen Quarantänezeiten massgebend. Zur Zeit sind auf dem Markt mehrere kommerziell erhältliche real-time PCR-Systeme verfügbar, wovon je ein offenes System (Biotecon Diagnostics, Potsdam, Deutschland) und ein geschlossenes System (BioControl, Bellvue, USA) empfehlenswert sind. Mit beiden Systemen liessen sich 9 target und 13 non-target-Stämme detektieren. Das geschlossene System bietet zusätzlich den Vorteil von wesentlich kürzeren Vorbereitungs- und Analysenzeiten und geringeren Kontaminationsgefahren.

Für die neulich beschriebene Spezies *C. condimenti* wurde die existierende *rpoB* basierenden Spezies-PCR erweitert, so dass *C. condimenti* neu ebenfalls zuverlässig nachgewiesen werden kann.

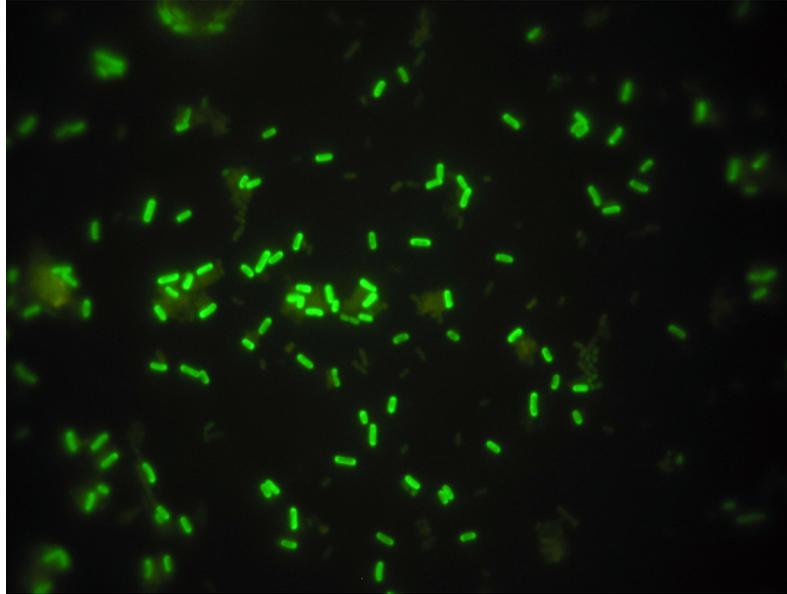
Um die mikrobiologische Situation in Industriebetrieben gezielt und konsequent zu überwachen, sind weitere betriebsepidemiologische Daten notwendig. Auf Grund der Informationen auf Betriebsebene können effiziente Massnahmen, wie der Einsatz von HEPA-Filtern, rezepturielle Überlegungen betreffend Trockenzudosierung, Personal- und Warenflusskonzept, Luftmanagement und Reinigungen (Art, Zeit) definiert und umgesetzt werden.

Die wissenschaftliche Unterstützung betreffend adäquater analytischer Methoden, Biofilmbildung, Trockenresistenz, Medienwahl, Mustergrösse und weiterer betriebsepidemiologischer Informationen ist sinnvoll und notwendig.

Eine Studie über die genetische Diversität verschiedener *Cronobacter* Isolate hat gezeigt, dass in einer Probe verschiedene PFGE-Fingerprints vorkommen können. Um gesicherte epidemiologische Aussagen auf Ebene des Betriebes machen zu können, bedingt dies die Typisierung von mindestens fünf koloniebildenden Einheiten. Dieses Beispiel zeigt, dass durch eine enge Zusammenarbeit zwischen Industrie und Forschung das Wissen aktiv in die Praxis umgesetzt werden kann – dies hilft zu verhindern, dass Früh- und Neugeborene an *Cronobacter* spp. erkranken.

SCHLÜSSELWÖRTER: *Enterobacteriaceae*, *Cronobacter* spp., Betriebs-epidemiologie, Prävalenz, PCR, PFGE, Säuglingsnahrung, Industriebetrieb

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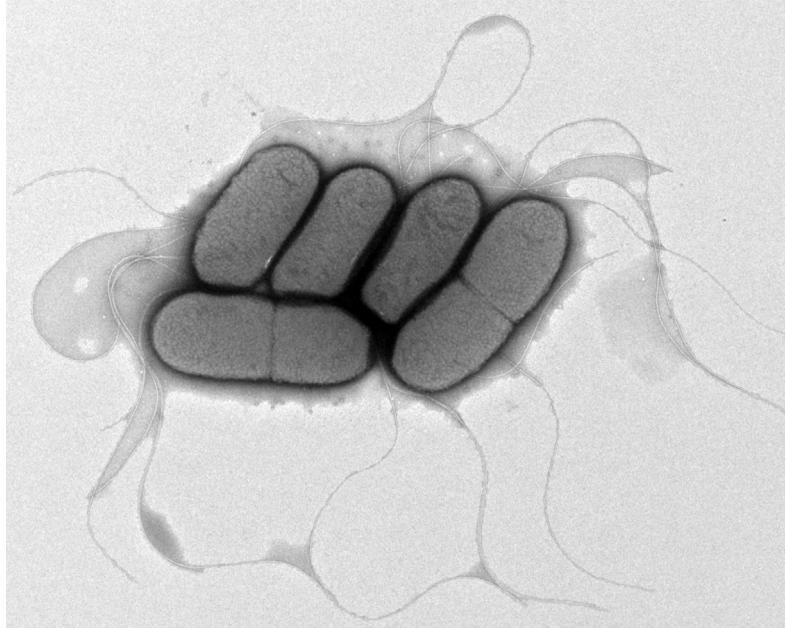


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