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Korrespondenzadresse:
lehnera@fsafety.uzh.ch

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; [2]Hochdorf Nutritec AG, Hochdorf, Switzerland

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[1], Claudia Fricker-Feer[2], Karl Gschwend[2], Roger Stephan[1]

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. Isolaten aus Rohmlich-, 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 Isolaten 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 routs 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*

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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 form 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 (bioMerieux (Suisse), SA, Geneve, Switzerland) identification according to the manufacturer's protocol. For each isolate with an identification result below "good" a partial *rpo*B gene sequence analysis was performed as described (Popp et al., 2009). Sequencing of the *rpo*B 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 α-glucosidase PCR assay (Lehner et al., 2006) and identification to the *Cronobacter* species level was completed employing the recently developed *rpo*B 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 *Enterobacetriaceae* 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 *rpo*B 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 *rpo*B 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[1D]	S[2]	T[3]	A[1]	S[2]	T[3]	A[1]	S[2]	T[3]
Acinetobacter baumannii	–	–	–	2	–	2	8	–	8
Citrobacter sedlakii	–	–	–	–	–	–	1	–	1
Enterobacter spp.	–	–	–	1	–	1	4	–	4
Enterobacter cloacae	1	–	1	4	–	4	4	3	7
Escherichia coli	9	–	9	8	–	8	2	1	3
Escherichia hermannii	–	–	–	–	–	–	2	–	2
Hafnia alvei	2	–	2	–	–	–	–	–	–
Klebsiella pneumoniae	–	–	–	5	–	5	3	–	3
Leclercia adecarboxylata	–	–	–	–	–	–	1	–	1
Pantoea spp.	–	–	–	–	–	–	1	–	1
Proteus mirabilis	1	–	1	–	–	–	–	–	–

[1]Identification by API ID32E; [2]Identification by *rpo*B sequencing; [3]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).

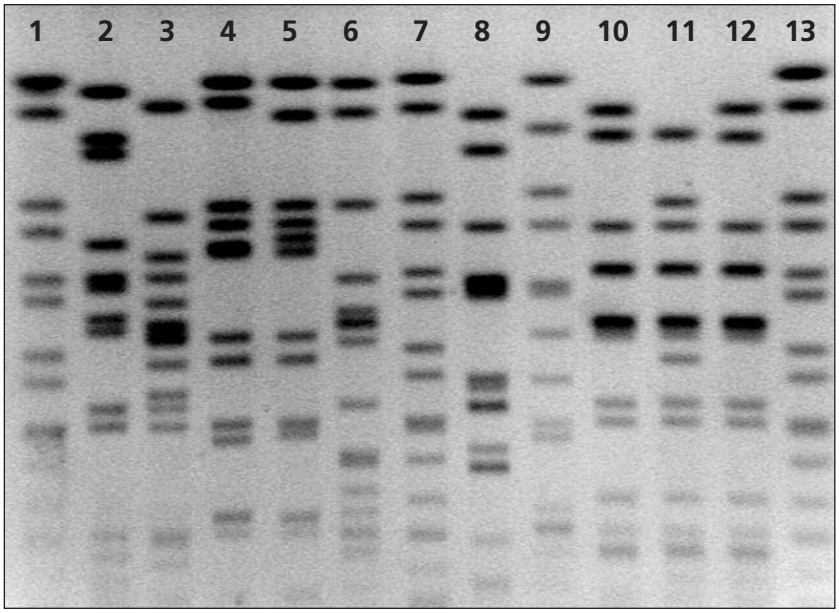

FIGURE 1: *PFGE analysis of 10 Cronobacter spp. isolates from milk powder. Lane 1, 7 and 13: S. Braenderup size standard. Lane 2–6 Cronobacter sakazakii isolates from site 1; lane 8: Cronobacter malonaticus, lane 9–12: Cronobacter sakazakii isolates from site 2.*

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 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 suggested 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.

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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 *Enterobacteriacae* 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 *Enterobacetriaceae* 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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Address for correspondence:
Dr. Angelika Lehner
Institute for Food Safety and Hygiene,
Vetsuisse Faculty, University of Zürich,
Winterthurerstraße 272
8057 Zürich, Switzerland,
lehnera@fsafety.uzh.ch