Dissemination of *Cronobacter* spp. (*Enterobacter sakazakii*) in a Powdered Milk Protein Manufacturing Facility[∇]

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The microbial contamination of air filters and possible links to contaminated product in a powdered milk protein-processing facility were investigated. Over a 10-month period, seven air filters, the environment, and powdered product were analyzed for the presence of *Cronobacter* spp. The effects of air filter installation, maintenance, and subsequent dissemination of *Cronobacter* were investigated. A total of 30 isolates were characterized by pulsed-field gel electrophoresis (PFGE). PFGE revealed the presence of three clonal populations distributed throughout the manufacturing site. This study highlights the need for proper installation of air filters to limit the dissemination of microorganisms into processing sites.

Enterobacter sakazakii is an opportunistic pathogen that is occasionally associated with food-borne illness in neonates and infants (4, 6, 18). Recent taxonomic studies led to alternative classification of *E. sakazakii* into several new genomospecies in a novel genus, *Cronobacter* (12, 13).

The natural habitat of *Cronobacter* is currently unknown. The identity of a primary reservoir (if there is any) remains to be determined. Since 1958, numerous cases of *E. sakazakii* infant infection have been documented, and there have been at least 111 cases of infection and 26 associated deaths (18). Even though the incidence of reported infections is low, high mortality rates have been reported for infants who present with *Cronobacter* meningitis (4, 6, 17).

While *Cronobacter* has been isolated from a wide range of foods and environmental and clinical sources, powdered infant formula (PIF) has been identified as the dominant vehicle of transmission (10, 11, 18, 20). Unlike commercially available ready-to-feed liquid infant formula, which is sterile, PIF (including dried bovine milk and milk products) is not a sterile product. PIF has been known to be contaminated, on occasion, with bacterial pathogens, including *Bacillus* spp., *Clostridium* spp., *Staphylococcus* spp., and *Enterobacteriaceae*, notably *Cronobacter* (9). Therefore, hygienic measures and practices must be used during the manufacture of formula to minimize entry of contaminants into the process.

Cronobacter is often isolated from the environment in milk powder and PIF manufacturing facilities (7, 10, 14, 15, 19). It is generally assumed that *Cronobacter* contamination of a product occurs in the processing environment at stages after pasteurization, including drying or packing. The prevalence of *Cronobacter* following the drying stage may be due (in part) to the organism's ability to resist drying or osmotic stress (2, 5, 21). Limited information is available about the contamination entry points in facilities and the routes of dissemination into

powdered end products. Hazardous microorganisms found in milk powder products can be transferred by a number of routes, including air.

Air is a potential source of hazardous microorganisms. Air intake in low-risk areas and especially in high-risk areas must be controlled. Air-handling systems servicing high-risk areas must supply air that is a desirable quality and at an overpressure that prevents the ingress of external air. The types of airborne contamination found previously in a milk powder production facility include dust, water droplets, and airborne microorganisms (14, 15, 19). Non-spore-forming microorganisms can become aerosolized in water droplets or when they are attached to dust (1, 16). Dust is generated from a wide variety of processing events, while water droplets can be generated as a result of cleaning operations. Environmental air filters in the air-handling units provide clean air to a controlled area.

The aims of this study were to investigate *Cronobacter* contamination resulting from contaminated air filters in a powdered milk protein-processing facility and to identify subsequent routes of dissemination of *Cronobacter*.

MATERIALS AND METHODS

Facility. The study was conducted in a powdered milk protein production facility. Casein is the primary protein found in liquid milk, accounting for approximately 80% of total protein content. Rennet casein is produced when the enzyme chymosin causes casein to coagulate, and then indirect heat is applied to maximize casein precipitation. The casein curd is separated from the whey by centrifugation, washed, spray dried, milled, and bagged. The powdered protein is subsequently sold as an ingredient for food and nutritional products.

Air handling. A three-step air filtration system was used in this facility to reduce the microbial load associated with air intake prior to entry into the spray dryer, the mill, and other downstream processing areas. Externally drawn air coming into the facility was initially coarse filtered with fiberglass-type screens to trap large particulate matter entering the blower room (Fig. 1). These screens are low-efficiency dust filters used to limit the ingress of dust from external air entering the blower room. This coarse filtration was followed by primary and secondary filtration for the air intake of the dryer and the mill, using a first-stage G4 filter coupled with a second-stage F8 filter (as recommended by CEN EN 779:2002 European standard classification of air filters [8]). The pleated primary filters were low-efficiency filters, while the secondary filters were medium- to higher-efficiency filters and were designed to prevent the release of dust if the

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FIG. 1. Floor plan of the powdered milk protein production facility investigated in this study. Curved open arrows indicate the direction of the incoming airflow, and solid arrows indicate the process flow. The colored dots indicate the locations of shared clonal isolates (see Fig. 2).

primary filter failed. This filter set was employed to control the entry of hazardous material (biological and physical) into the process.

Sampling plan. This study was performed in the powdered milk protein-processing facility from April 2007 to January 2008. Rennet casein powders and, in addition, once-off *Cronobacter* environmental monitoring were also included. Environmental monitoring for *Cronobacter* was sporadic, and the samples consisted of a production vacuum sample and samples obtained by swabbing the dryer air outlet, the inside of the housing of the dryer cyclone, and the air intake of the mill dehumidifier.

A total of seven air filters were analyzed as part of a routine factory overhaul of filter units (Table 1). The primary air filter servicing the spray dryer was subjected to further analysis by a *Cronobacter* most-probable-number (MPN) method and identification of non-*Cronobacter* *Enterobacteriaceae*. Twelve rennet casein powders were analyzed for the presence of *Cronobacter*.

Isolation and identification of *Cronobacter*. Five 10-cm² sections representing the middle and sides were aseptically cut from each filter unit. Swab samples were collected with sterile cotton swabs moistened in sterile peptone water and directed over a 5-cm² surface area. Swabs and air filter sections were each aseptically placed into 200 ml buffered peptone water (Difco Laboratories Le Pont de Claix, France) and homogenized gently. Powder and vacuum samples were diluted 1:10 (wt/vol) in

buffered peptone water. *Cronobacter* was isolated from all types of samples as recommended by ISO/TS 22964 (<http://webstore.ansi.org/ansidocstore/product.asp?sku=ISO%2FTS+22964%3A2006>) using DFI agar (CM1055; Oxoid, Hampshire, United Kingdom).

The identities of all presumptive *Cronobacter* isolates obtained during this study were further confirmed by real-time PCR using a primer set and probe that targeted the *dnaG* gene in the macromolecular synthesis operon (23). The species to which confirmed *Cronobacter* isolates belonged were determined using key biochemical tests as described by Iversen et al. (12).

Additional analysis of the primary filter of the spray dryer. With approximately 80% of the air filter remaining, 9.3 g of dust was aseptically removed from the surface of the primary filter of the dryer and collected in a sterile stomach bag. The number of *Cronobacter* CFU was determined by the MPN assay (22). Based on the growth observed on DFI agar at higher dilutions, the MPN of survivors was calculated using a "five-tube" technique. The dust was also analyzed using the Food and Drug Administration method for detection of *Cronobacter* with violet red bile glucose (Oxoid, Hampshire, United Kingdom) agar to detect *Enterobacteriaceae* (<http://www.cfsan.fda.gov/~comm/mmesakaz.html>). All isolates were identified using an ID32E biochemical gallery (bioMérieux, Marcy-l'Etoile, France).

TABLE 1. Overview of sampled air filters in the rennet casein facility

Location	No. tested	Filter		Efficiency (%) ^b	Arrestance (%) ^c	<i>Cronobacter</i> result
		Type	Classification ^a			
Wall (coarse dust filter)	2	Fiberglass	G1	<20	60	Positive
Dryer intake (primary filter)	2	Pleated panel filter	G4	25–30	90–95	Positive
Dryer intake (secondary filter)	1	Extended surface pocket filter	F8	90–95	90–95	Positive
Mill Intake (primary filter)	1	Pleated panel filter	G4	25–30	90–95	Positive
Mill Intake (secondary filter)	1	Extended surface rigid cell filter	F8	90–95	95	Positive

^a Classification was based on European standards EN 799 and EN 1882 (8).
^b Efficiency is the ability of a device to remove particulate matter from air, based on the ASHRAE 52.1-1992 test method (3).
^c Arrestance is the ability of a device to remove synthetic dust from air, based on the ASHRAE 52.1-1992 test method (3).

Determination of molecular subtypes by PFGE and band analysis. The subtypes of all *Cronobacter* isolates were determined by pulsed-field gel electrophoresis (PFGE) using previously described methods (19). DNA fingerprints were stored as tagged image file format (TIFF) files and imported into the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), and a dendrogram was created using the Dice coefficient and the unweighted-pair group method with arithmetic means. The genetic diversity of the *Cronobacter* isolates was compared at a level of similarity of 80%, with a position and optimization tolerance of 1.5%.

RESULTS

A sequential series of air filters was examined microbiologically following replacement during a routine overhaul in a powdered milk protein production facility. Five sections were sampled from each filter. In addition, environmental and rennet casein powder samples were included in the study. All seven air filters were positive for *Cronobacter*, yielding 24 isolates. The results were as follows. The sections analyzed from two coarse dust wall filters, labeled a and b (Fig. 2), were 40 and 20% positive, respectively; the sections analyzed from two primary filters, also labeled a and b (Fig. 2), of the spray

dryer were both 100% positive; and the sections analyzed from the secondary filter were 60% positive. The sections from the primary and secondary filters of the mill were both 80% positive. A total of three *Cronobacter* isolates were obtained from environmental samples, including the production vacuum sample and samples from swabs of the dryer outlet and dehumidifier air intake. Additionally, three isolates were recovered from rennet casein powder at a frequency of 25%.
XbaI subtyping by PFGE was conducted for all 30 strains. Each strain produced between 8 and 13 DNA fragments ranging from 48.5 to 1,000 kbp long. A dendrogram showing the fingerprint pattern similarities among the isolates was constructed (Fig. 2). A low degree of genetic diversity was observed among the isolates belonging to three DNA pulsotypes (designated pulsotypes P-1 through P-3), as shown in the dendrogram. Each pulsotype contained between 3 and 14 isolates. Pulsotype P-1 (Fig. 2) contained the largest number of isolates ($n = 14$; 47%), including isolates from filter samples from the coarse wall filter and both the primary and secondary filters for the drying and milling operations. In addition, this pulsotype

FIG. 2. Representative XbaI macrorestriction DNA profiles of *Cronobacter* isolates from a milk-processing facility. A dendrogram showing the relationships between isolates was created using the BioNumerics software. The colored dots indicate three disseminated strain types.

included isolates from rennet casein powder and from a swab of the dryer air outlet. Pulsotype P-2 (Fig. 2) was the second largest cluster and contained 13 (43%) isolates recovered from both primary filters for the dryer and both secondary filters for the dryer and the mill. This pulsotype also contained isolates from a swab of the air intake dehumidifier of the mill and from a production vacuum sample. The third pulsotype (pulsotype P-3) (Fig. 2) contained three isolates (10%), was 73% similar to pulsotype P-2, and included isolates from the coarse wall screen and from the primary filter for the dryer. No single sample or location contained all three pulsotypes. The three pulsotypes were identified as *Cronobacter sakazakii* (pulsotype P-1) and *Cronobacter malonaticus* (pulsotypes P-2 and P3).

All locations for the three pulsotypes were mapped to locations in the facility (Fig. 1). Pulsotype P-1 was the most ubiquitous pulsotype and was recovered from six of seven filters, the dryer outlet, and the final product. Pulsotype P-2 was isolated from four of seven filters tested, the production vacuum, and the air intake dehumidifier for the mill. Pulsotype P-3 was found on the coarse wall filter and the primary filter of the dryer.

Analysis of the dust from the primary filter for the spray dryer revealed 111 MPN *Cronobacter* per g of dust. Members of the *Enterobacteriaceae* that were not members of *Cronobacter* recovered from this dust included *Enterobacter cloacae*, *Citrobacter braakii*, *Citrobacter sedlakii*, and *Klebsiella pneumoniae*. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were also detected on the filters. These organisms were not investigated further.

DISCUSSION

The production site studied supplied rennet casein powder that was subsequently included as an ingredient in various food products but not in PIF. A total of 30 *Cronobacter* isolates were recovered from different processing sites, including air filters ($n = 24$), environmental samples ($n = 3$), and the final powdered product ($n = 3$). This study used PFGE to assess the fingerprint pattern similarities between these isolates in order to establish possible routes of dissemination of contamination and to identify failures at critical control points to limit contamination of the final product.

General handling of ingredients, spray drying, and milling operations can create aerosols. Aerosols can also be generated from cleaning operations, including wet cleaning (in the form of water droplets) and dry cleaning (where aerosols are generated by sweeping actions or directly from the exhaust of vacuum cleaners, which represents a hazard unless appropriate filters are used). Dust particles are a significant hazard as even in slowly moving air, small particles (0.1 to 5.0 μm) can remain airborne for hours and potentially travel long distances from their source (16). Therefore, it is recommended that high-pressure hoses not be used in high-hygiene areas and that cleaning equipment be sanitized regularly to prevent buildup of microorganisms. Correct selection, fitting, and maintenance of air filters are essential to control the ingress of airborne contaminants into processes and ultimately to exclude hazards from the final powdered product.

Indistinguishable *Cronobacter* isolates were observed at various processing sites (Fig. 1 and 2). A total of three pulsotypes

were identified, indicating that the genetic diversity was low. Pulsotype P-1 was recovered from all seven air filters, including both coarse wall filters. We could not definitively conclude that the isolates originated from external air as aerosolization of dust within the blower room could also have accounted for the bacteria on the filter. Both the P-1 and P-2 pulsotypes were also found on the primary and secondary air filters from the spray dryer and the mill. Dust recovered from a primary pleated air filter from the dryer yielded 111 *Cronobacter* MPN per g. The elevated number of *Cronobacter* bacteria present on this filter indicates that there was recurring contamination from the processing environment and/or proliferation due to the presence of moisture on the primary filter. The presence of pulsotypes P-1 and P-2 on both secondary filters suggests that both primary filter units failed to control microbial contamination. The presence of pulsotype P-1 in the final rennet powder and the presence of pulsotype P-2 in the dehumidifier could be explained by failure of the secondary filtration units.

Furthermore, pulsotypes P-1 and P-3 were indistinguishable from isolates recovered from an adjoining acid casein facility (data not shown). Walls physically separated the two processes, but open doorways between the two areas allowed exchange of air and unrestricted access for personnel. Positive samples in the acid casein plant included samples from the production vacuum, the bag-off filter (used to decrease product loss and avoid release of powder dust into the atmosphere from exhaust air), and the final powdered product, suggesting that there was wider dissemination between these sites.

A primary G4 air filter, with medium efficiency (25 to 30%), removes large dust particles from air, while the F8 air filter specifications state that this filter type is 90 to 95% efficient at trapping 0.4- μm -diameter particles. A common misconception is that filters should be selected based on particle size. However, a microorganism is often larger when it is suspended in a water droplet or attached to a dust particle. This filter combination has long been used by the milk-processing industry (and PIF industry) to supply air to "high-care" areas and is a well-established means of controlling microbial contamination, but it works only when it is installed and maintained at a satisfactory level (16; J.-L. Cordier, personal communication). Inspection of the primary air filters of the spray dryer and mill revealed significant accumulations of dust on the intake sides of the filters. Examination of the filter housings showed that the filters were inadequately fixed in position and that the gasket between the filter and the housing was often damaged. We hypothesize that, as the pressure progressively increased due to accumulation of dust, an increased amount of air bypassed the filter via a break in the gasket, thereby rendering the primary filtration step ineffective. It was not possible to assess the operation of the secondary filters, as these filters were inaccessible at the time of inspection.

It is acknowledged that hazard analysis and critical control point plans play an important role in the control of microbiological hazards. All milk powder and PIF manufacturers incorporate the principles of such plans into their control programs, and air filters are an important critical control point that must be considered. Air intake filters are used to remove particulate matter and other physical hazards, but correct installation and maintenance are critical. Our findings highlighted the need to monitor air-handling units to ensure that the desired perfor-

mance is being achieved. Control of moisture and humidity in air and on filters must also be considered in the control of microbial contamination. Humidity is controlled by passing hot air through the cooling coil of a dehumidifier, where condensation and drainage of the resulting water occur. Our study identified *Cronobacter* in the air intake of the mill dehumidifier. This finding indicates that there is increased risk to the downstream process due to the likelihood of the presence of water and subsequent proliferation of bacteria at this site. The cooling coils must be considered a critical control point and disinfected as part of a manufacturing control program.

The ducts of air-handling systems should have inspection and cleaning access panels located at regular intervals. This allows cleaning of the ducts, especially critical areas such as filters and coils, when necessary. The cleanliness of internal duct surfaces should be monitored visually in conjunction with microbiological sampling either by swabbing or by using agar contact plates.

It is important to ensure that filters are maintained according to manufacturers' specifications and that performance of filters is controlled by using planned visual inspection schedules. Criteria for replacement are based on (i) when the filter manufacturer's maximum differential pressure of a filter has been reached, (ii) when the integrity of a filter has been compromised, and (iii) when company practice indicates that a planned filter change is required.

Satisfactory performance of air filters requires regular checking, and documentation of all maintenance and filter change is essential.

In conclusion, the installation of differential pressure gauges to monitor filter resistance and pressure drops, along with regular replacement of filters, especially those with low load capacities, should be considered. Appropriate air filter maintenance along with surveillance for *Cronobacter* spp. would contribute to reducing the dissemination of these pathogens in the food chain.

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