

Detection, occurrence, growth and inactivation of *Cronobacter* spp. M.C. Kandhai 2010

Detection, occurrence, growth and inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*)



Chantal Kandhai

Propositions

1. In the last decennium, detection methods for *Cronobacter* spp. have been improved, which has led to an increase in the isolation of *Cronobacter* spp. from a wide variety of food products and environments. This fact in itself is not a reason to consider *Cronobacter* spp. as an emerging pathogen.
2. The use of sterile liquid formulae is an effective control measure to reduce the probability of infections in low and very low birth weight infants due to *Cronobacter* spp..
3. As, generally, pathogens are relevant in foods only at such low numbers that multiplication is required to allow their detection, the enrichment step must be regarded as the most important step in the entire detection procedure. Yet, relatively little research effort is dedicated to optimizing this procedure.
4. Improvements in food safety management can only successfully be achieved in a food enterprise when there is a true food safety culture in the food enterprise.
5. Children are an extraordinary gift and enhance their mother's career by improving her multitasking skills.
6. Patience is the most important virtue in joint scientific writing.

Propositions belonging to the thesis, entitled:

“Detection, occurrence, growth, and inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*)”

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Wageningen, 23 April 2010.

**Detection, occurrence, growth
and inactivation
of *Cronobacter* spp.
(*Enterobacter sakazakii*)**

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(*Enterobacter sakazakii*)**

M.C. Kandhai

Thesis

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To Fons, Aditya,
Sarada & Ishaan

*“Life is constantly changing.
Whatever time you have is yours,
for tomorrow might never come”*

Abstract

The genus *Cronobacter* consists of Gram-negative, motile, non-spore forming, facultative anaerobic bacteria, and was originally defined as one species “*Enterobacter sakazakii*” within the genus *Enterobacter* in 1980. *Cronobacter* spp. have been documented as a rare cause of outbreaks and sporadic cases of neonatal meningitis, necrotizing enterocolitis, and sepsis in infants with a high mortality. Among these infants, those at greatest risk are infants less than 2 months of age, particularly pre-term infants, low birth weight (LBW) infants (< 2500 g), and immuno-compromised infants.

At the onset of the work for this thesis, *Cronobacter* spp. had been isolated from milk-based powdered formulae which have a direct link to the sub-population at greatest risk. However, there was a need to more closely investigate whether and where *Cronobacter* spp. occurs in environments in which these powdered are manufactured and packed but also to investigate other sources which could lead to exposure of vulnerable sub-populations. The main objective of this study was to develop isolation and detection methods that would allow quick and reliable investigation into the occurrence of the micro-organism in potential sources. Furthermore, more insight into the growth behavior of *Cronobacter* spp. in reconstituted infant formula was necessary in order to provide data to be used in Microbiological Risk Assessment (MRA) dedicated to this particular food product.

A selective enrichment method was developed for the rapid and reliable enrichment and detection of *Cronobacter* spp. in environmental samples. The detection method which was developed is based on two features of *Cronobacter* spp. combined: their yellow pigmented colonies when grown on tryptone soy agar and their constitutive α -glucosidase, which can be detected in a 4-h colorimetric assay. The initially developed method and refinements thereof were applied for routine screening for the presence of *Cronobacter* spp. in environmental samples and a variety of food products manufactured or marketed in The Netherlands. The detection method described in this thesis has been the basis for a series of media for *Cronobacter* spp. that have recently been commercialized.

Quantitative data on product contamination at manufacture, during preparation, and also growth after reconstitution are required in order to assess the risk associated with

Cronobacter spp. exposure. Next to that, tools are needed to assess the micro-organisms growth potential as well as its inactivation (thus, its survival) due to specific control measures applied. In this thesis, predictive growth models were developed that capture key growth parameters. Minimum- and maximum temperatures estimated with the Secondary Rosso equation were 3.6 °C and 47.6 °C, respectively. The estimated lag time of the micro-organisms was found to vary from 83.3 ± 18.7 h at 10 °C to 1.73 ± 0.43 h at 37 °C and could be described with the hyperbolic model and reciprocal square root relation. The models for growth rates and lag times as a function of temperatures obtained during this study allow estimating the potential growth of *Cronobacter* spp. in reconstituted infant formula stored at any temperature below 47 °C.

As growth rates of *Cronobacter* at refrigeration temperatures are relatively small, caregivers are advised to store reconstituted infant formula at low temperature as a control measure to prevent microbial growth. It is evident that storage of reconstituted formula in a refrigerator may require a significant amount of time before the formula reaches the targeted refrigeration temperature. Therefore, a mathematical model was built to predict the temperature profile and the resulting growth of *Cronobacter* spp. during cooling, i.e. under dynamic temperature conditions. Predictions showed that proliferation of *Cronobacter* spp. during cooling strongly depends on the size of the container used for storage and that it may be prevented by limiting the volume to be cooled to portion-size only or by reconstituting at temperatures of 25 °C or lower.

The survival of two *Cronobacter* strains in dry powdered infant formula (PIF) was tested and compared to the survival of six other bacterial strains after inoculation and storage at several temperatures between 7 and 42 °C. The effect of temperature on survival in PIF, was described using both the Weibull distribution model and the log-linear model. Differences were found in the rate of survival that can be due to difference in the resistance to inactivation in dry environments between *Cronobacter* species, which could be relevant to consider when establishing quantitative risk assessments on consumer risks related to PIF.

The research described in this thesis contributes to the existing knowledge on the natural habitat of *Cronobacter* spp. and its occurrence and behavior in PIF. The models developed

for quantifying the growth of *Cronobacter* spp. in reconstituted formulae under various conditions can be applied in risk assessments set-up to estimate the probability of vulnerable sub-populations becoming ill after consuming infant formulae. International and national governmental bodies may use these predictive models in risk assessments and to establish guidelines for health care professionals to provide effective hygiene training to parents and professional caregivers to ensure that PIF is prepared handled and stored appropriately.

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Chapter 1



Introduction

General

Cronobacter spp. (*Enterobacter sakazakii*) is an opportunistic food-borne pathogen, that has been linked with serious infections in infants, causing bacteraemia and meningitis and associated with necrotizing enterocolitis (NEC) (26, 27, 54). Most cases of *Cronobacter* disease have been detected among newborn and very young infants in hospital nurseries and neonatal intensive care units (28). The first reported cases attributed to this organism occurred in 1958 in England (See Figure 1) and resulted in the death of two infants (74). At the time the micro-organism was referred to as “the yellow pigmented *Enterobacter cloacae*”. In most outbreaks, in which a source could be established, the source of *Cronobacter* spp. was proven to be (dry) powdered infant formulae (PIF) (5, 6, 69, 75, 76). Interest has been increasingly raised in the public domain with notable activities of Codex Alimentarius, national governments, scientific communities, health care providers and the infant food industry, since an outbreak involving powdered infant formula (PIF) in the USA in 2002 (36).

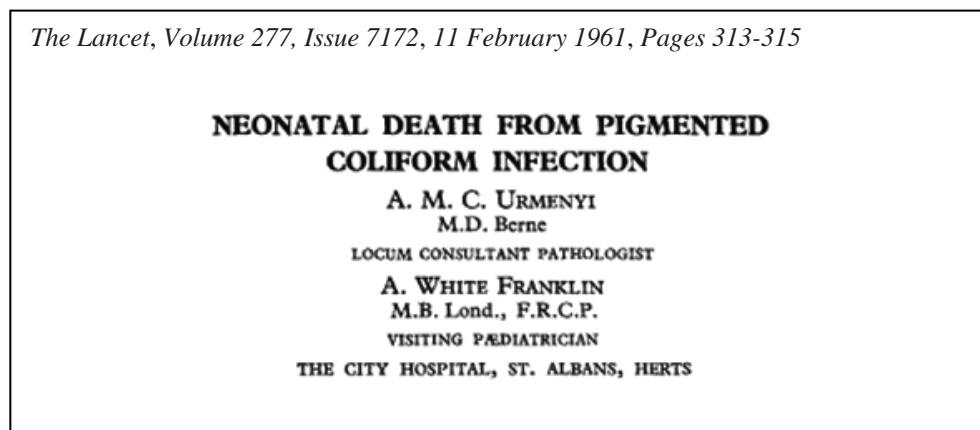


Figure 1. First report of an outbreak related to *Cronobacter* spp. in scientific literature.

In 2002, the International Commission on Microbiological Specifications for Food (ICMSF) categorised *Cronobacter* spp. as a severe hazard for restricted populations,

resulting in life-threatening diseases or substantial chronic sequelae or effects of illnesses of long duration (37). There is evidence that consumer groups vulnerable to *Cronobacter* infections are premature and low-birth-weight infants and those aged < 28 days (8, 22). More recently, the Codex Committee for Food Hygiene (CCFH) of Codex Alimentarius and the BIOHAZ scientific expert panel of the European Food Safety Authority (EFSA) established that all infants at or below 12 months are at particular risk for *Cronobacter* infections (21, 22). Among these infants, those at greatest risk are neonates (< 28 days), particularly pre-term infants, low birthweight (LBW) infants (< 2500 g), and immunocompromised infants, and those infants that are less than 2 months of age. Infants of HIV-positive mothers are also at risk for *Cronobacter* infection, as they may specifically require infant formula and may be more susceptible to infection (12, 21, 26-28).

Microbiological Risk Assessment approach

Microbiological risk assessment (MRA) is widely used to characterise health risks associated to the potential presence of microbial hazards in foods. An MRA consists of four stages: 1) hazard identification, 2) exposure assessment, 3) hazard characterization and 4) risk characterization (49). A hazard is defined as a biological, chemical or physiological agent in food (or condition of food) with the potential to cause an adverse health effect (11). The magnitude of the associated risk is obtained as well as insight in risk-contributing factors, and possible risk-mitigating control measures.

To determine the magnitude of the risk through MRA predictive models are often used as tools in such assessments. Suitable predictive models capture insights in the dynamics of the target micro-organisms, such as inactivation, survival and growth. Resulting parameters can be applied to estimate the consumer risk and to give directions to which preventive measures should be advised (51). Most value is derived from being able to quantify individual parameters. In this thesis the aspects that have been investigated are presented within the framework of the MRA. Information relevant for compiling a quantitative MRA relevant to *Cronobacter* spp. in PIF is presented. In Figure 2, the various steps of this MRA

approach are given with sub-topics indicated in the various boxes. Grey boxes indicate the topics that were investigated in more detail in this thesis.

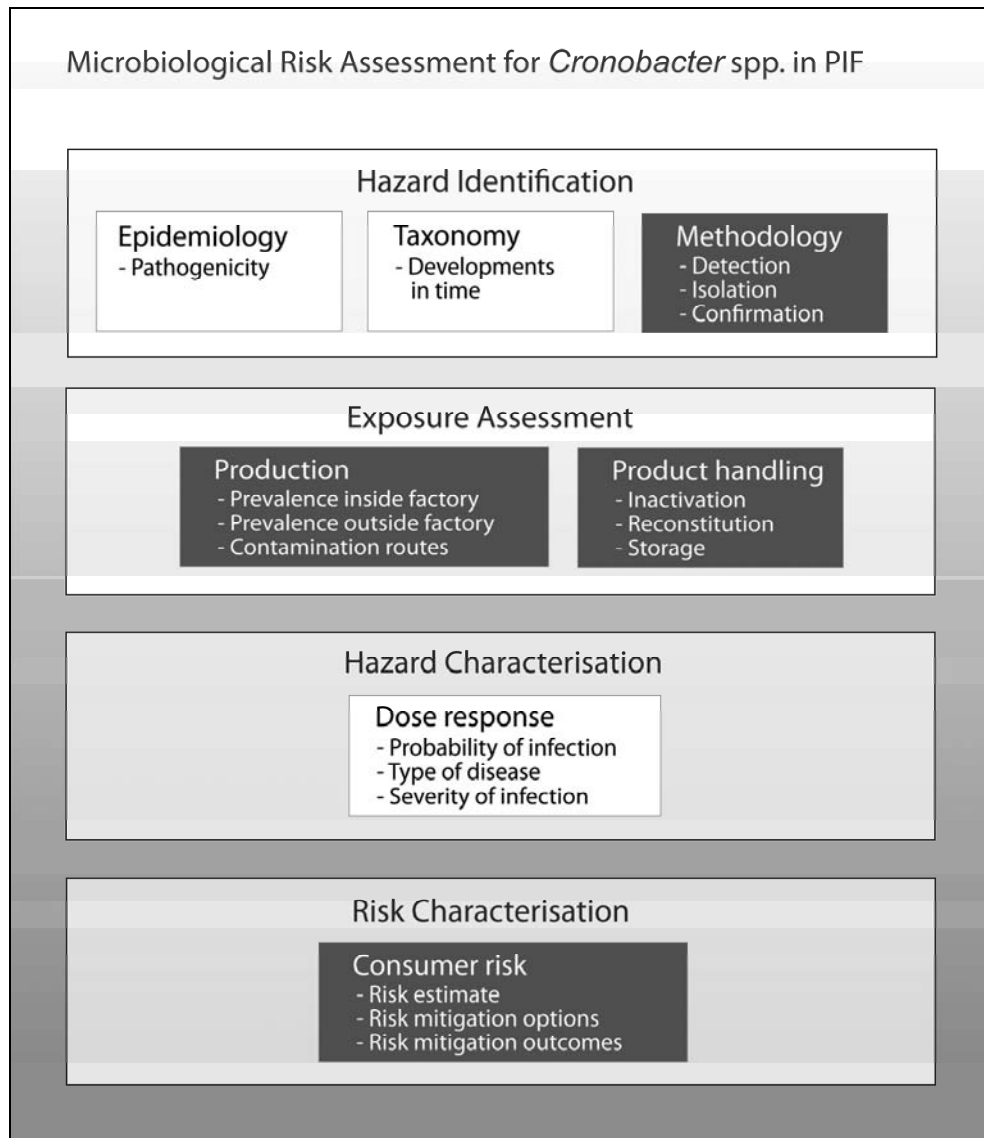


Figure 1. Schematic view of elements of microbiological risk assessment for *Cronobacter* spp. in powdered infant formula. All boxes are described in this introduction section. The grey boxes indicate the research topics focused on in this thesis.

Epidemiology

The epidemiology of *Cronobacter* spp. is poorly understood. *Cronobacter* infections are very rare and often underreported, especially in developing countries (23). Since 1961 and up to July 2008, 156 documented cases of *Cronobacter* spp. infections from all parts of the world have been reported in the published literature and in reports submitted by public health organizations and laboratories. Of these 156 cases, at least 29 cases (19%) resulted in death (28).

Specific international attention has been given to the safety of food for infants and young children as it relates to the possible presence of *Cronobacter* spp. in powdered formulae intended for this consumer group. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have established two international risk assessments on the topic (26, 27) that have provided a scientific basis for creating a new code for hygienic practices by CCFH (12), aimed at providing risk management guidance at the international level. In this code, products for infants up to 12 months of age are considered.

According to Codex Alimentarius, an infant is a person not more than 12 months of age, while young children are persons from 12 months up to the age of three years (36 months) (12). A neonate is a newborn infant, less than four weeks old, and a premature is an infant that was born prior to 37 weeks of gestation. An immunocompromised infant may have an immunodeficiency of any kind, and may therefore be particularly vulnerable to opportunistic infections in addition to normal infection that could affect anyone. A number of factors could contribute to an infant's immune status, i.e. nutritional status such as vitamin status, HIV status, clinical conditions, pharmaceutical treatment, low birth weight, and premature birth. Because the prevalence of these factors varies among countries, there is a wide variation in the prevalence of immunocompromised infants globally.

Diseases caused by *Cronobacter* spp. may affect the intestines (causing necrotizing enterocolitis), invade the blood stream (bacteraemia and/or sepsis) and/or invade the central nervous system, causing cerebritis and/or meningitis. Meningitis has a high mortality rate (42%) and many of the survivors (74%) suffer from neurological complications (66).

Premature infants, especially those with a low birth weight (< 2500 g) or a very low birth weight (< 1500 g), are thought to be at greater risk for severe infection than more mature infants, children or adults (26, 33, 48). The incidence of *Cronobacter* spp. invasive infections seems to be much higher among infants than older age groups such as young children. Cases of *Cronobacter* spp. meningitis are reported exclusively among infants, while *Cronobacter* bacteremia has occurred in all age groups (8). The group of infants at risk for necrotizing enterocolitis, however, seems to be similar to the group of neonates which is defined to be at risk for bacteraemia and meningitis (66).

Many infections in newborns are transmitted from mother to child. For several years passage of the organism through the mother's birth canal was therefore suspected to be the source of *Cronobacter* spp. infection. In most cases that infection has occurred, however both the route of exposure and the incubation period are generally unclear. Only in two outbreaks, occurring in neonatal intensive care units, a clear relationship was shown between *Cronobacter* spp. isolates from patients and isolates from unopened cans of powdered dry infant formulae of the same batch as consumed by the patient (6, 15). It has been reported that in outbreaks associated with powdered infant formulae, the illness started as soon as 3 to 4 days after the initial exposure to the implicated formula (2, 69, 75). Though *Cronobacter* spp. infections have been associated with contaminated powdered formulae, environmental sources of contamination should not be excluded (58). While relationships between sources of *Cronobacter* spp. other than from powdered infant formula and human illness are less well understood, documented *Cronobacter* spp. infections in immunocompromised adults (34, 48) may indicate potential other sources such as home environments, food and food manufacturing environments, as well as insect or animal reservoirs.

Table 1 shows an overview of documented outbreaks and cases of infections in infants and children per year per country caused by *Cronobacter* spp. as published by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (28). In various reports mortality rates between 40 and 80% are reported (48). A recent survey showed a mortality rate of invasive neonatal *Cronobacter* infections of 27%. The lethality was also calculated for *Cronobacter* meningitis (42%), *Cronobacter*

septicaemia (< 10%), *Cronobacter* necrotising enterocolitis (19%) (31). Considering the overview of invasive *Cronobacter* spp. outbreaks from 1961 to 2008 (28) as presented in Table 1, an average mortality rate of 19% can be calculated (i.e. 29 death in 156 cases). Analyzing the total number of cases did not yield consistent information about the cases per age group in the population, so currently it is not possible to quantify existing potential differences in the susceptibility of infants of different age groups. In the age group 6-11 months six cases are well documented, five of which were invasive. From the five invasive cases, three had other medical problems. In the age group 12–35 months only two cases have been well described. *Cronobacter* spp. infections have occurred in both hospital and home settings, but the incidence rate appears to be low. In the first expert meeting of the FAO/WHO on *Cronobacter* spp. in PIF, an annual incidence rate of 1 per 100,000 infants (i.e. children < 12 months of age) for *Cronobacter* spp. invasive infections was estimated for the United States of America (26), whereas the annual incidence rate among the low birth weight infants (< 2500 g) was found to be higher at 8.7 per 100,000 infants (27). Similarly, another study estimated an incidence rate of 9.4 per 100,000 very low birth weight infants (< 1500 g) (72).

Table 1. Overview of the number of invasive *Cronobacter* spp. outbreaks and cases in infants and children reported until July 2008 (adapted from reference (28)). In all cases the age at illness onset was < 1 year, except for *, where the child was 13 months of age

Publication Year	Country	No. cases	Deaths
1961	UK	2	2
1965	Denmark	1	
1979	USA	1	
1981	USA	2	
1983	Netherlands	8	6
1984	Greece	1	
1985	USA	1	
1987	Greece	11	4
1988	USA	2	
1989	Portugal	1	1
1989	Iceland	3	1

Table 1. Continued

Publication Year	Country	No. cases	Deaths
1989	USA	4	
1990	USA	1	
1991	USA	1	
1994	Germany	1	
1997	Scotland	1	
2000	USA	1	
2000	Brazil	5	
2001	USA	3	
2001	Belgium	12	2
2001	Israel	2	
2002	Israel	3	
2002	USA	20	2
2002	Belgium	1	1
2003	Brazil	1	
2003	Hungary	1	
2003	USA	5	1
2004	USA	2	
2004	Hungary	1	
2004	New Zealand	5	1
2005	USA	2	
2005	Hungary	1	
2006	USA	5	
2006	Hungary	1	
2006	France	9	2
2007	USA	9	1*
2007	Canada	1	
2007	India	2	1
2007	Spain	1	
2007	France	18	4
2008	USA	3	
2008	Japan	1	
Total		156	29

Pathogenicity

Enterobacter species are widely distributed in nature, occur in the intestinal tract of humans and animals, and are frequently a cause of nosocomial infections. The dose-response relationship for *Cronobacter* spp. causing illness in humans is not known and represents a challenging aspect requiring further investigation. To date, relatively little scientific attention has been given to unravelling the mechanism of pathogenicity or to potential virulence factors of *Cronobacter* spp. (63). Some strains of *C. sakazakii* have been shown to produce an enterotoxin (64) however, its relevance to pathogenesis has not been established yet. There is no data available that any one of the *Cronobacter* species is more pathogenic than another; each of the species have been linked retrospectively to clinical cases of infection in either infants or adults (28). It is known that *Cronobacter* spp. infection may cause a highly lethal syndrome of bacteraemia and meningitis with involvement of the central nervous system in neonates. In several cases and outbreaks, powdered infant formula has been identified as the source of infection, therefore the entry for the infection can be gastrointestinal (64). Pagotto *et al.*, (2003) used a suckling mice model to determine the pathogenicity of 18 *Cronobacter* isolates (9 clinical, 8 food and 1 Type strain), showing that a dose of 10^8 CFU/mouse was lethal to 58 out of 69 (84%) suckling mice within 3 days after intraperitoneal administration. A minimal number of 10^5 CFU of certain *Cronobacter* strains was lethal to 5 out 20 (25%) suckling mice, indicating that the pathogenicity between strains tested may differ. Orally dosed suckling mice, showed lethality at a dose of 10^7 CFU per mouse (2 out 8 suckling mice (20%) died) (64). These data have been extrapolated to estimate the minimum infectious dose for human infants to be 10^5 CFU in a number of studies.

Currently, very little information is available on the virulence factors of *Cronobacter* spp. and its pathogenic mechanisms.

Taxonomy

Cronobacter spp. are Gram-negative, motile, peritrichous non-spore forming, straight rods, within the family *Enterobacteriaceae* and originally belonging to the genus *Enterobacter*. Until 1980, *Cronobacter* spp. was referred to as the “yellow pigmented *Enterobacter cloacae*”. The reclassification to *Enterobacter sakazakii* was based on differences from *E. cloacae* in DNA-DNA hybridization, biochemical reactions, pigment production and antibiotic susceptibility. The micro-organism was named after the Japanese microbiologist Riichi Sakazaki in honor of his work on the enteric bacteriology. Based on DNA-DNA hybridization, 15 biogroups of 57 strains of *E. sakazakii* were described, with the wild type Biogroup 1 being the most common (29). In 2007, there was a proposal for a novel genus *Cronobacter*, comprising at least five genomospecies, including three subspecies. Also a new biogroup (biogroup 16) was identified (41). Figure 3 gives an overview of the place of the genus *Cronobacter* within the *Enterobacteriaceae* family. Throughout this thesis the name *Cronobacter* spp. is used, covering the various strains which were earlier named *Enterobacter sakazakii*.

The latest classification into *Cronobacter* gen. nov. is based on a polyphasic taxonomic approach, employing full-length 16S rRNA gene sequencing, ribotyping, fluorescent-amplified fragment length polymorphism (f-AFLP) and DNA-DNA hybridisation. Based on this approach and the different phenotypic profiles, the novel *Cronobacter* species are now divided in 16 biogroups.

The following five species are distinguished:

- *Cronobacter sakazakii* gen. nov., comb. nov. (Biogroup 1- 4, 7, 8, 11 and 13),
- *Cronobacter malonaticus* sp. nov. (Biogroup 5, 9, 14),
- *Cronobacter dublinensis* sp. nov. (Biogroup 6, 10, 12),
- *Cronobacter muytjensii* sp. nov. (Biogroup 15),
- *Cronobacter turicensis* sp. nov. (Biogroup 16).

Two *Cronobacter* strains appear to be a separate genomospecies and are indicated as *Cronobacter* genomospecies I. This genomospecies has not been associated with a specific biogroup.

A number of subspecies of *Cronobacter dublinensis* are: *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov. (Biogroup 12), *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. (Biogroup 10), and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. (Biogroup 6).

For the full taxonomic description on the genus *Cronobacter*, including (sub) species, the reader is referred to Iversen *et al.*, 2008 (42). Table 2 provides a list of the *Cronobacter* strains used throughout this research, including their new names.

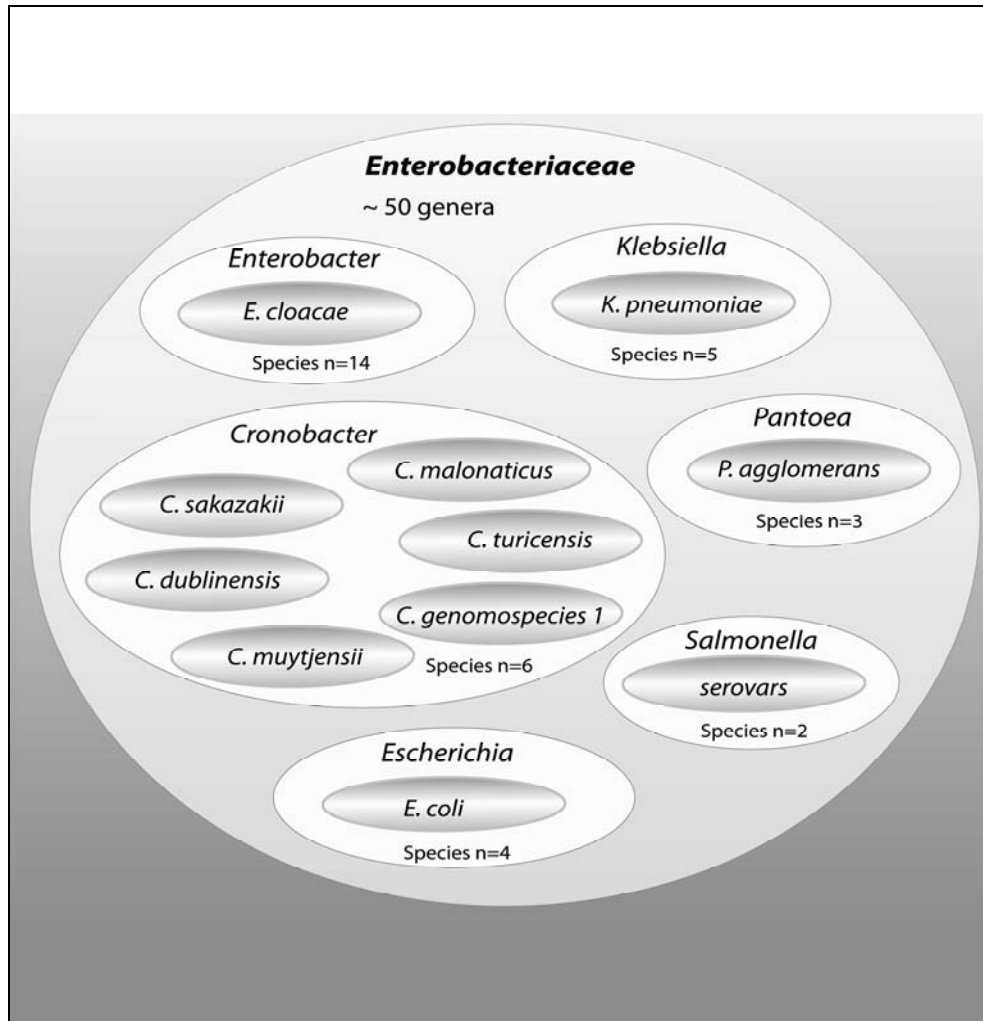


Figure 2. Schematic view of the place of *Cronobacter* spp. and *Salmonella* spp. within the *Enterobacteriaceae* family. Adapted from B. Healy (personal communication).

Table 2. Overview of the *Cronobacter* spp.^a strains investigated in this thesis

Old strain taxonomy and culture collection code(s)	New strain taxonomy
<i>Enterobacter sakazakii</i> NCTC 8155	<i>Cronobacter sakazakii</i>
<i>Enterobacter sakazakii</i> NCTC 9238	<i>Cronobacter sakazakii</i>
<i>Enterobacter sakazakii</i> NCTC 11467 = ATCC 29544 = DSM 4485	<i>Cronobacter sakazakii</i>
<i>Enterobacter sakazakii</i> NCTC 9844	<i>Cronobacter dublinensis</i>
<i>Enterobacter sakazakii</i> NCTC 9846	<i>Cronobacter dublinensis</i>
<i>Enterobacter sakazakii</i> NCTC 9529	<i>Cronobacter</i> genomospecies 1
<i>Enterobacter sakazakii</i> DSM 18705	<i>Cronobacter dublinensis</i> subsp. <i>dublinensis</i>
<i>Enterobacter sakazakii</i> ATCC 51329	<i>Cronobacter muytjensii</i>
<i>Enterobacter sakazakii</i> DSM 18703	<i>Cronobacter turicensis</i>
<i>Enterobacter sakazakii</i> DSM 18702T	<i>Cronobacter malonaticus</i>
<i>Enterobacter sakazakii</i> DSM 18706T	<i>Cronobacter dublinensis</i> subsp. <i>lausannensis</i>
<i>Enterobacter sakazakii</i> DSM 18707T	<i>Cronobacter dublinensis</i> subsp. <i>lactaridi</i>
<i>Enterobacter sakazakii</i> MC10	<i>Cronobacter</i> spp.
<i>Enterobacter sakazakii</i> MM9	<i>Cronobacter</i> spp.

^a According to Iversen *et al.*, 2008 (42).

Methodology

Detection and isolation

Cronobacter spp. can be present in low numbers in powdered infant formulae. Prevalences varying from 2 to 14% have been reported (35, 56, 60). Concentrations of the organism found in powdered infant formulae were between 0.2 and 92 CFU/100 g; levels greater than 1 CFU/g have not been reported (25).

Because of the low prevalence of *Cronobacter* spp. in PIF and powdered formula producing facilities, highly specific methods are required for enumeration/detection and identification that can be used for both product and environmental samples and that allow quick isolation of *Cronobacter* spp.. At the start of the studies documented in this thesis, there was a special need to improve the existing methods for isolation and detection of

Cronobacter spp. shown in Table 3 (56, 57, 73). Many methods and media have been described since then to isolate *Cronobacter* spp. (Chapter 9).

Table 3. Methods available in 2001 for detection of *Cronobacter* spp. in powdered infant formulae

Pre-enrichment	Enrichment (usually selective)	Isolation	Identification	Ref.
Buffered Peptone Water (BPW)	<i>Enterobacteriaceae</i> enrichment (EE) broth	Violet Red Bile Glucose Agar (VRBGA) plates	API 20 ^E and yellow colonies on Tryptone Soy Agar (TSA) plates	(56)
De-ionized Water	EE broth	VRBGA plates	API 20 ^E	(60)

Methods for detecting *Cronobacter* spp. are still under development to increasingly better meet the need to detect levels of approximately 1 cell per 100 g of PIF. There are three basic steps in the detection of micro-organism in foods. The first one is pre-enrichment in a non-selective medium allowing recovery of sub-lethally damaged cells. The second is selective enrichment containing one or more compounds that are inhibitory to the majority of micro-organisms, but significantly less to the species or group of species to be isolated. The third is streaking of (selective) enrichment broth onto selective solid media.

These procedures may be used in different combinations, depending on the number of cells expected in the sample. After the selective enrichment step, presumptive *Cronobacter* spp. isolated on agar need to be confirmed. The success rate of the above mentioned basic detection and isolation protocols depends on:

- 1) the number and the state of the micro-organisms in the sample,
- 2) the selectivity of the media (the balance between the inhibition of the competitors and inhibition of the target organism),
- 3) the conditions of the incubation (temperature, time, oxygen availability),
- 4) the visual distinction of the isolation medium (the distinction between the competitive flora and the target organism) (4).

Confirmation methods

To confirm presumptive *Cronobacter* spp. detected on selective plating media, biochemical tests, such as API 20^E can be used that distinguish *Cronobacter* spp. from other species by biochemical differentiation. A specific feature among *Enterobacter* species, namely the presence of α -glucosidase activity, described by Muytjens *et al.*, (57), was firstly employed in the research conducted in this thesis as a suitable confirmation method for *Cronobacter* spp. isolated from PIF – producing facilities (Chapter 2). The biochemical characteristic of α -glucosidase production by *Cronobacter* spp. has remained the basis for many detection media (22).

Much effort has been put on the development of molecular sub-typing techniques, like Pulsed Field Gel Electrophoresis (PFGE) (71), ribotyping and random amplification of polymorphic DNA (RAPD) (15, 62). Molecular subtyping of bacteria by profiling either proteins or nucleic acids could be a useful tool to investigate for example epidemiological relationships of isolates (25). Furthermore, DNA fingerprints obtained with standardized DNA-based protocols could be applied for direct comparison of isolates in outbreaks (70). Recently, also several genotypic methods including the Polymerase Chain Reaction (PCR) have been developed. These are based on amplifying specific fragments of DNA, allowing detection of a single copy of the target sequence (18, 52, 53, 65, 68, 77). The PCR confirmation method depends on the availability of two short oligonucleotide primer sequences that will hybridize to opposite strands of heat-denatured DNA at either end of the region which eventually be probed. A DNA polymerase then catalyses the extension of the primers to produce two double-stranded copies of the region of interest. Recently, a number of PCR probes for conserved genes such as *ompA* have been published (59). A potential disadvantage of molecular techniques can be their sensitivity that can be influenced by food components. Furthermore, molecular methods can not distinguish living from dead cells and organisms killed for example by a heat process can still be detected. Also these methods does not allow for isolation of the strains. Nevertheless, such methods can still be useful in rapid detection of the presence of very low numbers of target micro-organisms.

Production

Food production aims at delivering safe, nutritious and wholesome foods with an adequate shelf-life and at reasonable costs to the consumer. By taking adequate control measures and deploying sound assurance systems to manage food production, the food industry helps to minimize risks to consumers associated with the potential presence of foodborne hazards in foods. Food production processes often rely on control methods such as pasteurization or sterilization to reduce the number of vegetative pathogens and/or spore-formers in the final food product. Implementation of general food safety management systems such as Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP) are considered in many countries as pre-requisite conditions for facilities preparing or manufacturing foods. To better assure safe food production, specific food safety management systems such as the Hazard Analysis Critical Control Points (HACCP) system, have been deployed successfully. Using these food safety management systems, the effectiveness of various control measures is monitored to determine whether the process parameters for the control measures remain within predetermined limits designed to achieve food safety (50). To design adequate control measures and set-up effective food safety management systems it is important to understand the different possible contamination routes of relevant hazards as well as the efficacy of specific control measures to control them.

Production of Powdered Infant Formulae

Whereas it is generally accepted that breastfeeding is the best option for the nutrition and the health of infants and young children, there may be situations where a mother can not breastfeed her baby or chooses not to do so. In such cases, powdered formulae represent useful alternatives to replace breast feeding either partially or totally and these are therefore formulated to meet the nutritional needs of infants (78). Powdered formulae are divided in powdered infant formula (PIF), follow-up formula (FUF), and dietary foods for special medical purposes. PIF is defined as a breast milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding. FUF is defined as a food intended

for use as a liquid part of the weaning diet for infants from the 6th month onwards and for young children. Dietary foods for special medical purposes are defined as foods for particular nutritional uses, intended for dietary management of patients and to be used under medical supervision (12). The most significant difference between PIF and FUF is that FUF may contain a wider variety of dry-mix ingredients.

The composition, quality and labelling requirements of powdered infant formulae are clearly defined in national, regional as well as international standards or regulations (12). Production of powdered formulae can follow three procedures: the dry-mix-, the wet-mix- and the combined process. In the wet-mixing process, all unprocessed raw materials as well as separately processed ingredients are handled as a liquid product that is heat treated, dried and then further handled up to the filling stage. In the dry-mix process, all separate ingredients are dry blended to obtain the final product, which is further handled up to the filling process. The dry mixing is done when heat sensitive ingredients such as vitamins, minerals, starch, carbohydrates and others need to be added, according to the formulation. Ingredients as such have all been submitted to some thermal inactivation step during their manufacture and must fulfil the same microbiological requirements as the final product. The combined process may include and combine different mixing steps to obtain the final formulation. In the combined process the unprocessed raw material and part of the ingredients are processed according to the wet-mix process to obtain base powder, which is further used for the manufacture of different finished products. The type of process used depends mostly on the product manufactured and the processing facility. For a detailed description of the production process the reader is referred to Cordier (2008) (16). A schematic overview of PIF production is given in Figure 4.

Powdered formulae are produced with stringent control measures and strategies to prevent post-process contamination. Guidance and recommendations concerning control and elimination of *Salmonella* spp. and *Cronobacter* spp. in powdered infant formulae and reconstituted infant formula have been issued in joint reports by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (12, 26, 27). The microbiological criteria, applied by the European Commission, EC No 1441/2007, for PIF are shown in Table 4. Monitoring for *Enterobacteriaceae* is used as

process criterion that indicates the hygienic status of the production. For PIF for infants of 6 months and dietary products for special medical purposes parallel testing for *Enterobacteriaceae* and *Cronobacter* spp. is advised, unless a correlation between micro-organisms has been established at an individual plant level. If *Enterobacteriaceae* are detected in any of the products samples tested in such a plant, the batch must be tested for *Cronobacter* spp.. The detection of *Enterobacteriaceae* is a process hygiene parameter, while detection of *Salmonella* spp. and *Cronobacter* spp. include food safety parameters (24).

Table 4. Current European Union microbiological criteria for dried infant formulae for infants up to 6 months of age and for formulae for special medical purposes (24)

	Micro-organism(s)	Microbiological limit		
		<i>n</i>	<i>c</i>	<i>m</i>
Process hygiene criteria	<i>Enterobacteriaceae</i> (in 10-g samples)	10	0	Absence in 10 g
Food safety criteria	<i>Cronobacter</i> spp. (in 10-g samples)	30	0	Absence in 10 g
	<i>Salmonella</i> (in 25-g samples)	30	0	Absence in 25 g

n is the number of units comprising the sample.

c is number of acceptable sample units with values above *m*.

The recent review regarding the relationship between *Cronobacter* spp., *Salmonella* and other *Enterobacteriaceae* in PIF and FUF undertaken by the scientific Panel on Biological Hazards (BIOHAZ Panel) of the European Food Safety Authority (EFSA) concluded that there is no correlation between *Salmonella* spp. and other *Enterobacteriaceae*, and that there is also no apparent relationship between *Cronobacter* spp. and other *Enterobacteriaceae*. However, at individual plant level such a correlation between *Cronobacter* spp. and other *Enterobacteriaceae* may be established (22).

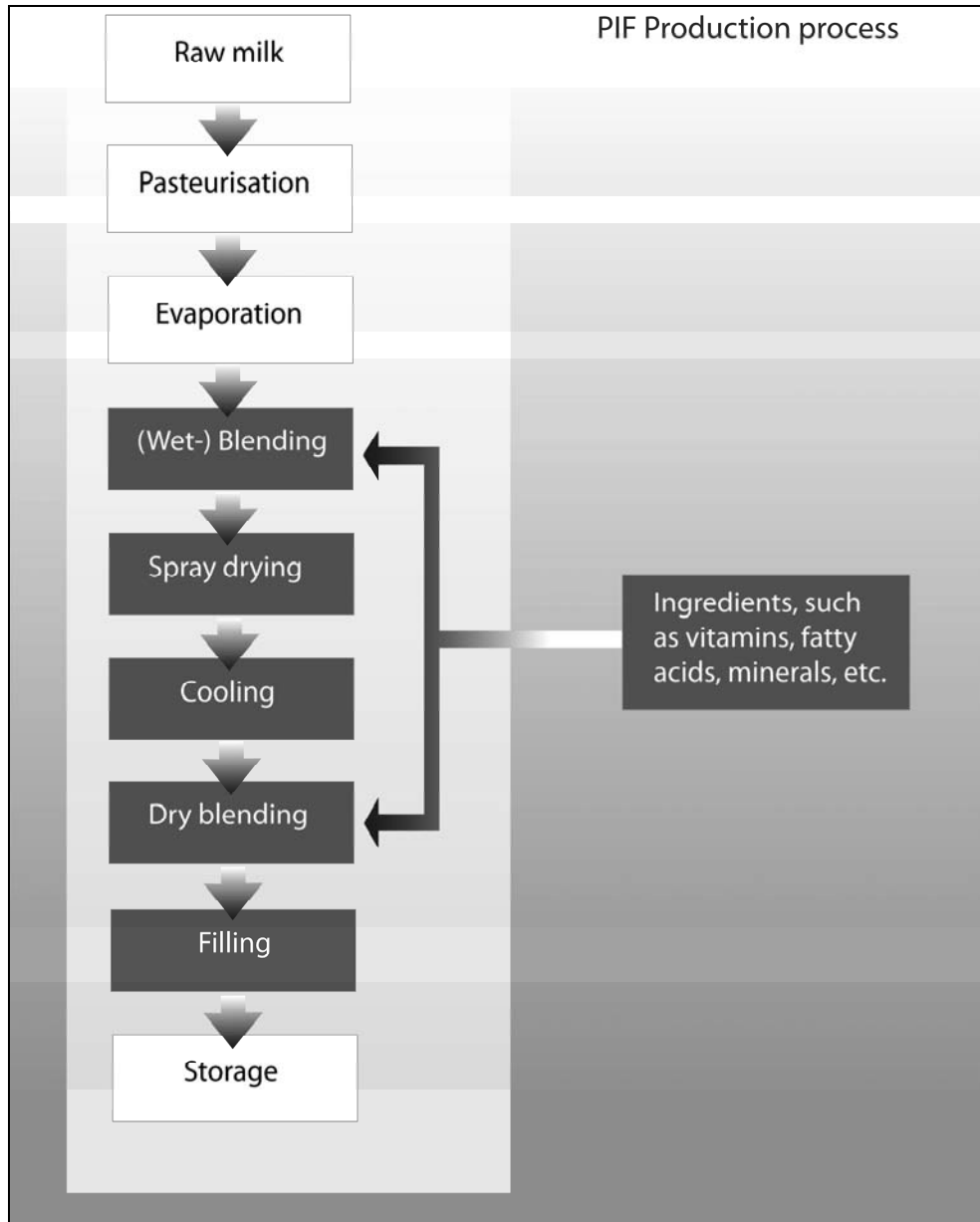


Figure 3. Schematic view of a production process for PIF, including both wet- and dry mixing. Contamination during manufacture could in particular occur in the steps highlighted in grey.

Contamination routes relevant for PIF production

Severe illness and sometimes death of infants has been attributed to PIF that has been contaminated with *Cronobacter* spp.. PIF has been found to be possibly contaminated during addition of ingredients after the pasteurization process, during the packing process or during reconstitution of the powder at homes or in the hospitals (14, 16, 54). Even though Good Practices (GMP and GHP) and correct HACCP plans are adhered to (26, 27), sporadically, PIF may be contaminated with this micro-organism. The relevant hazards in PIF requiring appropriate control measures, as pointed out by the FAO and WHO consultations, are *Cronobacter* spp. and *Salmonella* spp. (26, 27). Micro-organisms belonging to the family of *Enterobacteriaceae* (which includes *Salmonella* spp. and *Cronobacter* spp.) are known to be present in powdered infant formulae processing environments and in homes or other places where PIF is reconstituted, such as hospitals (14, 39, 45). The level of *Enterobacteriaceae* has been used for decades as an indicator for process hygiene. Literature data have shown that the levels of *Salmonella* spp. in the processing of PIF are much reduced in properly maintained high hygiene zones, and its occurrence generally is rare. Contrary to *Salmonella* spp., complete elimination of *Cronobacter* spp. during manufacture of PIF is, due to its ubiquitous nature, currently not considered to be feasible in the high-hygiene zone (27). Indeed, *Cronobacter* spp. has been detected in factory environments (46).

Following the production scheme depicted in Figure 4, *Cronobacter* spp. could enter powdered infant formulae via the processing environment or the processing line and through the addition of (contaminated) ingredients such as vitamins and starch after the heat-inactivation step (16, 27). In this Figure, the production steps where contamination could occur are depicted in grey. Next to contamination via ingredients, contamination from the environment could occur via aerosols, through air containing dust or skin particles of food handlers (17). The presence of *Cronobacter* spp. in the processing environment implies that there may always be a chance of product contamination. As the micro-organism occurs in homes and hospitals, powdered formulae can also be contaminated during preparation of the feed at homes and at hospitals (27).

Prevalence in factories

An adequate heating step is very important to significantly decrease the bacterial number in raw milk. Since *Cronobacter* spp. is a vegetative micro-organism, the pasteurization step should be sufficient to inactivate this micro-organism (9, 19, 40, 44, 61). This processing step is particularly important because *Cronobacter* spp. may survive the spray drying process (1). As air can be contaminated with bacteria, due to adherence to dust particles, water droplets or even skin particles, product–air contact could thus cause contamination after the pasteurization step. *Cronobacter* spp. may be introduced during dry cleaning of the spray dryer and also via air that is in contact with the product after the spray drying step. *Cronobacter* cells are able to survive in dry environments (45) such as powdered formulae producing facilities (55). In these factories the numbers of *Cronobacter* cells and the incidence of product contamination could increase after a wet cleaning process (16).

Prevalence outside factories

In order to identify potential vehicles for transmission, it is important to investigate potential environmental reservoirs of *Cronobacter* spp.. The micro-organism seems to be ubiquitously present as it has been isolated from a wide spectrum of environmental sources and food products (22). It has been detected in raw and fresh products of animal and vegetable origin as well as in processed and prepared foods such as dried, smoked, frozen, fermented, cooked or fried products, ready-to-eat and street foods. It was also isolated from fresh vegetables and spices (3). Primary *Cronobacter* spp. contamination may occur intrinsically, e.g. due to its endophytical presence in plants or through contact with water, soil and living vectors like insects or small vertebrates. Raw foods of animal origin may be contaminated additionally via the (faecal) micro-flora of the food source animal itself (30). In several investigations *Cronobacter* spp. was detected on kitchen equipment used for preparing powdered infant formulae and also on apparently clean equipment (58, 71). Furthermore, literature data showed that *Cronobacter* spp. was able to survive in a dish brush (58). In homes, *Cronobacter* spp. was isolated from household clothes (47), indicating that good hygiene practices are necessary to avoid contamination at homes and in

hospital kitchen facilities. Because of its ubiquitous nature, it is necessary to consider possible contamination during preparation and handling of prepared formulae.

Product handling

As the manufacture of commercially sterile PIF is not feasible using current processing technologies, there is a low but potential risk of infection to infants through consumption of PIF. This risk is particularly relevant when prepared food is handled or stored incorrectly. Growth in PIF is not possible after the spray drying step because the water activity is too low to allow bacterial growth. Although the micro-organism may be able to survive in PIF, depending on the storage time and conditions, generally there is a gradual die-off. However, reconstituted PIF forms an excellent growth medium for *Cronobacter* spp. and other micro-organisms that may be present in such products. To avoid growth and further contamination, leading to infection and illness in infants, specific preparation and handling instructions are mentioned on the packaging label of powdered infant formulae. Nevertheless, several investigations showed that professional and home caregivers have their own way of preparing the formula. In hospitals, the preparation practices vary according to local arrangements and the availability of adequately trained personnel (23). Immediate consumption or rapid cooling and storage at low temperature are critical control measures to prevent microbial growth once PIF is reconstituted.

Inactivation of Cronobacter spp. in dry powder

Cronobacter spp. has an unusual surviving ability under dry conditions compared to other *Enterobacteriaceae* (9, 13, 20, 32), but there is a difference in the thermal tolerance between *Cronobacter* strains (19, 61). Previous studies showed the occurrence of *Cronobacter* spp. cells in the powdered infant formulae (39, 56, 60), and also that *Cronobacter* spp. cells were able to survive for at least twelve months in powdered infant formulae under favourable conditions (13, 20). Acidification could reduce the concentration of *Cronobacter* spp. cells in different types of powdered formulae and vegetable based

products (43, 67). Quantification of survival rates of *Cronobacter* spp. isolates in dry PIF may provide useful data for microbiological risk assessments and for product/process design studies, as it allows determining likely levels of the micro-organism in PIF dependent on for instance different scenarios of time and temperature during storage and distribution.

Growth of Cronobacter spp. in reconstituted infant formulae

As acknowledged by the Food and Agricultural Organization and the World Health Organization (26, 27), PIF is not a sterile product and several investigations have shown the occurrence of cells of *Cronobacter* spp. in powdered infant formulae (35, 39, 56) leading to it being associated to outbreaks after consumption of reconstituted infant formula (5, 8, 15, 36, 69, 75). Most probably, multiplication of the organism has taken place in these cases, as only low numbers of the micro-organism have been detected in powdered infant formulae (25). Literature data about the growth potential of *Cronobacter* spp. in reconstituted infant formulae at various temperatures is scarce. To determine its effect on risk it is therefore necessary to quantify the growth of *Cronobacter* spp. in reconstituted PIF. This information could lead to recommendations to health professionals and parents on proper preparation, handling, and storage of reconstituted PIF to ensure the product is safe when fed to the infants.

In order to predict the microbial behaviour in a food product and to determine the effect of growth on the product's safety and quality, it is a necessity to have good insights in the production, storage and handling conditions of PIF and to relate this understanding to quantitative data regarding the micro-organisms ability to survive, recontaminate and grow. Bacterial growth is defined as an orderly increase in the quantity of cellular constituents, depending on the ability of cells to form new protoplasm from available nutrients. Bacterial growth can be divided in four phases, being the lag phase, the exponential phase, the stationary phase and the death phase. In the lag phase, bacteria can adapt to their new environment and injured bacterial cells can recover. A wide variety of factors such as inoculum size, time necessary to recover from physical damage or shock after transfer from

other conditions, time needed for synthesis of essential (co-)enzymes or division factors and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium, can influence the length of the lag phase. In the exponential phase, cells divide at a constant rate depending on the composition of the growth medium and incubation conditions such as temperature. In the stationary phase, initially the cell number no longer increases but is kept in balance as a result of cells dying (due to exhaustion of available nutrients and/or accumulation of inhibitory metabolites or end products or exhaustion of space) and of cells multiplying. However, later in the stationary phase a gradual reduction in cell numbers may be seen as the balance is lost.

The duration of the lag phase is of particular interest, as an extended lag phase can be a means to prevent growth and multiplication of bacteria (10). Temperature is one of the major environmental factors influencing microbial growth, and fluctuations in temperature during storage and preparation may significantly affect the potential for outgrowth. Mathematical models are useful tools to describe the growth (7). Quantification of growth parameters, such as the specific growth rate as a function of temperature, help calculating possible outgrowth of *Cronobacter* spp. in reconstituted infant formulae at various time-temperature scenarios.

Hazard Characterization

In the hazard characterization step the possible impact of an identified hazard on consumer is determined, either qualitative or quantitative, using available dose-response relationships. The likelihood and severity of illness is directly related to the number of micro-organisms ingested, though in most cases this is a very variable relationship across consumer groups and individuals as well as possibly across different strains of micro-organisms. In the case of *Cronobacter* infections, the infectious dose relationship for humans has not been clearly determined yet. The minimum infectious dose (MID) of *Cronobacter* spp. for infants, is extrapolated from animal models (64): it is estimated that high levels of the organism ($> 10^5$ CFU/feeding) are necessary to cause illness. In other studies it is speculated that a reasonable estimate for infection might be close of that postulated for *Escherichia coli*

O157:H7 and *Listeria monocytogenes* 4b, i.e. circa 1000 CFU (38). Retrospective studies on epidemiological data and animal models could help to better estimate the dose-response relationship of *Cronobacter* spp..

Risk characterization

In the risk characterization step the results of the previous three phases are integrated to give an estimate of the probability of becoming ill after the food product, i.e reconstituted infant formula is consumed. Results from risk characterization and especially comparisons of the impact of different scenarios of exposure and possible mitigation could be used by risk managers to decide on appropriate controls, including regulatory measures. Furthermore, risk manager could decide whether to inform the consumer to implement actions to reduce the risk on infection.

Outline of this thesis and the research conducted

This research project aimed to get more insight into the habitat and growth characteristics of *Cronobacter* spp. and to study the factors that might ultimately affect consumer risk, in particular the survival of *Cronobacter* spp. in dry powder and growth after reconstitution of the powdered formula (Figure 2). Prior to this study, no specific enrichment method was available and therefore an enrichment method was developed as described in Chapter 2. To further improve enumeration and detection of *Cronobacter* spp., a more specific detection method than available at the onset of the study was designed based on the presence of α -glucosidase activity in the micro-organism (described in Chapter 3). The newly developed methods were then applied to dry environmental samples from households and food producing facilities (Chapter 4). To better explore the varied occurrence of the micro-organism in food products, an extensive survey on the occurrence of *Cronobacter* spp. in foods marketed in The Netherlands was conducted. In this survey, isolates were confirmed using a Polymerase Chain Reaction (PCR) method and typed with Pulse Field Gel Electrophoresis (PFGE) fingerprinting analysis (Chapter 5). As reported in Chapter 6, the effect of the pre-culturing conditions on key growth parameters of *Cronobacter* spp. was

quantified. Growth of *Cronobacter* spp. was then studied in reconstituted infant formulae cooled in refrigerators with stagnant air (normal household refrigerators) and in a refrigerator equipped with a fan (circulating air); growth parameters obtained earlier were used in different scenarios to predict the possible growth of *Cronobacter* spp. in reconstituted infant formula (Chapter 7). Finally, the survival and gradual inactivation of *Cronobacter* spp. in dry powdered infant formula under different storage conditions was investigated in detail and mathematically described (Chapter 8). In the general discussion, the scientific insights from the thesis work are put into the perspective of general progress in the field (Chapter 9).

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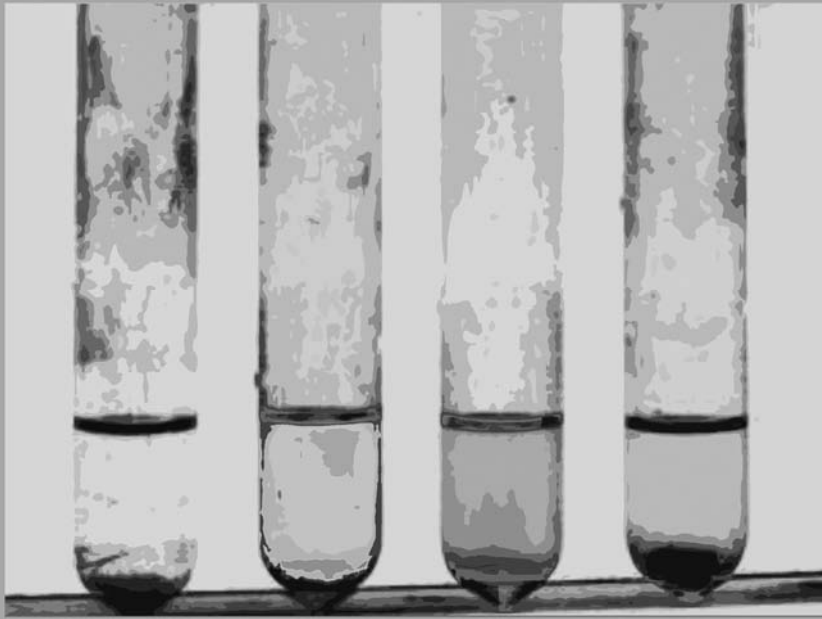
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Chapter 2



**A new protocol for the detection
of *Cronobacter* spp.
applied to environmental samples**

Abstract

Cronobacter spp. is a motile, peritrichous, Gram-negative rod that was previously known as a “yellow pigmented *Enterobacter cloacae*”. It is documented as a rare cause of outbreaks and sporadic cases of life-threatening neonatal meningitis, necrotizing enterocolitis, and sepsis. *Cronobacter spp.* have been isolated from milk powder-based formulas, and there is thus a need to investigate whether and where *Cronobacter spp.* occurs in these manufacturing environments. For this purpose, a simple detection method was developed based on two features of *Cronobacter spp.*: its yellow pigmented colonies when grown on tryptone soy agar and its constitutive α -glucosidase, which is detected in a 4-h colorimetric assay. Using this screening method, *Cronobacter spp.* strains were isolated from three individual factories from 18 of 152 environmental samples, such as scrapings from dust, vacuum cleaner bags, and spilled product near equipment. The method is useful for routine screening of environmental samples for the presence of *Cronobacter spp.*.

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“A new protocol for the detection of *Enterobacter sakazakii applied to environmental samples”**

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* Throughout this thesis the designation *Cronobacter spp.* has been used to indicate the taxonomic change.

Introduction

Cronobacter spp. is a member of the family *Enterobacteriaceae*. Until 1980, this organism was referred to as yellow pigmented *Enterobacter cloacae*. It was then reclassified as a unique species based on differences from *E. cloacae* in DNA relatedness, the specific yellow pigment production, biochemical reactions, and antibiotic susceptibility (4). Several outbreaks or sporadic cases of either severe neonatal meningitis in premature infants or necrotizing enterocolitis have been attributed to *Cronobacter* spp. (7). In some of these, contaminated dry infant formulas have been identified as the source of *Cronobacter* spp. (3, 13). Since heat treatments such as pasteurization readily kill the micro-organism (11), contamination must have occurred after processing. It is suspected that this micro-organism is present in the environment of the processing equipment (10, 11).

Most *Cronobacter* spp. strains described in the literature were isolated from cerebrospinal fluid of the patients (7, 13). Isolates were commonly obtained by streaking onto blood or chocolate agar, using the API 20^E system for identification. The isolation of *Cronobacter* spp. in milk powder is usually performed by enrichment in *Enterobacteriaceae* enrichment broth, followed by plating on violet red bile glucose agar and sub-culturing on sheep blood agar and eosin methylene blue agar (8) or on tryptone soy agar (TSA) (12). Often, isolates were screened for the presence of DNase activity on toluidine blue agar after 2 and 7 days of incubation at 36 °C and for the formation of yellow pigmented colonies on TSA at 25 °C for 48 h (4, 9). An additional characteristic feature is the production of Tween 80-esterase in 3 to 8 days (1). In all, this full confirmation protocol takes more than a week. A faster and elective protocol is preferred for the detection of *Cronobacter* spp. in environmental samples in food production, in which a variety of related coliforms may be present. This study describes a simple method for the detection of *Cronobacter* spp. and its application to environmental samples collected from three milk powder production plants.

Materials and methods

Detection

A total of 152 dry samples were obtained from the environments of three milk powder production plants. They were collected from floor sweepings, spilled dry products, scrapings, or vacuum cleaner bags. Approximately 65% of the samples were analyzed for the presence of *Cronobacter* spp. without enrichment, whereas for 35% an enrichment step was used. In case of enrichment, 10 g of dry sample was homogenized in 90 ml of buffered peptone water (Oxoid Ltd., Basingstoke, Hampshire, UK), incubated for 18 to 20 h at 37 °C, streaked onto violet red bile agar (VRBL; Oxoid), and incubated for 20 to 24 h at 37 °C, as shown in Figure 1. Other samples were streaked directly onto VRBL after homogenizing. From each VRBL plate, 10 colonies typical for coliforms were purified on TSA (Oxoid). On many plates, however, fewer than 10 colonies were present, and in that case all colonies were purified on plates. The TSA plates were incubated for 48 h in daylight at room temperature. The yellow pigmentation on TSA is a characteristic feature of *Cronobacter* spp..

Preliminary identification

Isolates were tested for oxidase, using Oxidase DrySlide OXIDASE (Becton Dickinson and Company, Sparks, Md.). Oxidase-negative isolates were further identified using the API 20^E test system (bioMérieux SA, Marcy l'Etoile, France) and the corresponding identification software (API Lab Plus version 3.3.3), using *Cronobacter sakazakii* DSM 4485, corresponding to ATCC 29544, as the type strain.

α -Glucosidase activity

For the assessment of α -glucosidase activity, paranitrophenyl- α -D-glucopyranoside (Fluka Chemie GmbH, Buchs, Switzerland) was dissolved in distilled water at 50 °C and added to 0.3 M (pH 7.0) phosphate buffer (Merck, KGaA, Darmstadt, Germany) in a final

concentration of 4 g/L of buffer. Individual colonies grown on TSA were suspended in 2 ml of physiological salt solution, 0.85% NaCl (Merck) in distilled water, whereupon 2 ml of the paranitrophenyl- α -D-glucopyranoside solution was added. Care was taken to transfer a whole colony to the test tube. The use of a standardized inoculum level of at least number 1 on the McFarland scale is recommended. The mixture was then incubated in a water bath at 37 °C. The formation of the yellow colored paranitrophenyl (PNP) hydrolysate was measured after 0, 4, and 24 h using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at 405 nm. A minimal absorption of 0.3 at 405 nm after 4 h, equivalent to 16 μ M PNP, was considered positive. Positive and negative controls were included as described below.

For some isolates, the α -glucosidase activity assay was also performed with paranitrophenyl- α -D-glucopyranoside tablets as follows. Five yellow, freshly grown colonies on TSA were individually tested for α -glucosidase activity. Each colony was suspended in glass tubes (10 by 100 mm; Emergo, Landsmeer, The Netherlands) containing 0.25 ml of a 0.85% NaCl solution and a ready-to-use diagnostic tablet (Diatab 50421, Rosco, Taastrup, Denmark). Negative controls were included each time consisting of a suspended tablet without bacteria and another one with an *E. cloacae* 218 strain that is α -glucosidase negative. A positive control was also included each time consisting of a suspended tablet with *Cronobacter sakazakii* DSM 4485. After vigorous vortexing for a few seconds, tubes were incubated in a water bath at 37 °C for 4 ± 0.1 h. A yellow color in the supernatant, caused by the release of PNP, indicated the presence of α -glucosidase.

Ribotyping

An automated ribotyping system, the RiboPrinter (DuPont Qualicon, Wilmington, Del.), was used to confirm the identity of presumptive *Cronobacter* spp. isolates (2). In this system DNA was extracted from a colony and then digested with *Eco*RI into discrete-sized fragments. The DNA was then transferred to a membrane and probed with a region of the rRNA operon to reveal the pattern of rRNA genes. The pattern was recorded, digitized, and stored in a database containing 6 DuPont *Cronobacter* spp. reference fingerprints combined

with the 2,000 in-house *Cronobacter* spp. fingerprints (5). Comparison of patterns in the database allows for the assessment of relatedness between *Cronobacter* spp. strains and provides further evidence of the identity of the isolates in addition to the biochemical identification.

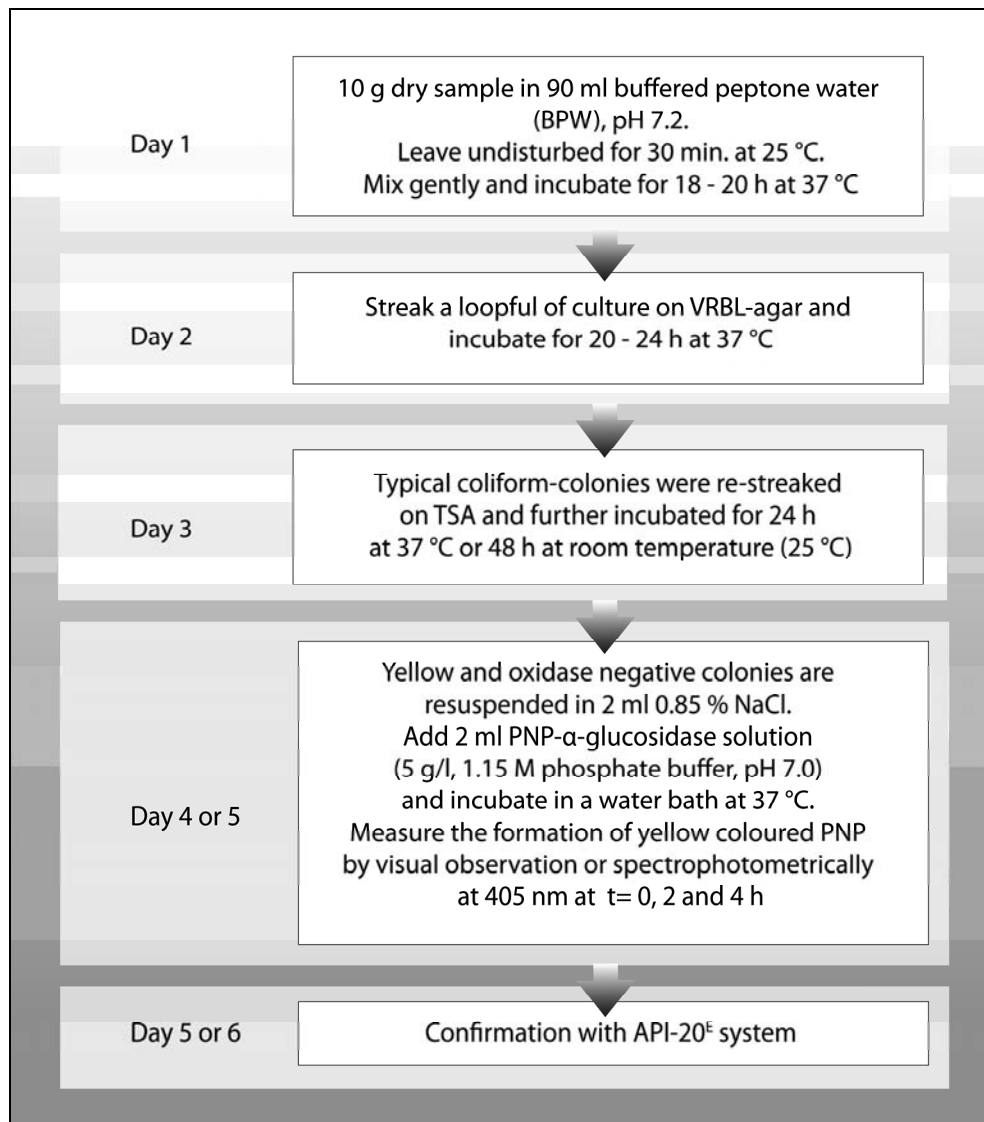


Figure 1. Schematic diagram for the enrichment and identification of *Cronobacter* spp..

Results and discussion

From the line environment of three milk powder plants, 152 dry samples were collected. Samples were analyzed with one of the two procedures, namely, a pre-enrichment step in buffered peptone water followed by streaking on VRBL agar as described in Figure 1 or a direct plating of diluted samples on VRBL agar. Coliforms were detected with both procedures. Some of these coliforms were subsequently identified as presumptive *Cronobacter* spp. on the basis of oxidase test and biochemical identification with API 20^E. A total of 100 samples were not pre-enriched and yielded in total 30 coliforms, 7 of which were presumptive *Cronobacter* spp. strains. Fifty-two samples were subjected to pre-enrichment, coliforms were isolated from 33 samples, and nine of the coliforms were presumptively *Cronobacter* spp.. From the data presented here, it appears that *Cronobacter* spp. can be isolated from environmental samples with and without pre-enrichment. In another study, 27 samples were analyzed using both methods. Presumptive *Cronobacter* spp. strains were isolated from 13 of 27 samples without enrichment and from 16 of the same 27 samples with enrichment (data not published).

From VRBL agar, all colonies were streaked on TSA plates and were identified with API 20^E. The API 20^E system was not able to identify isolates obtained from 38 samples. From samples positive for *Cronobacter* spp., numerous *Cronobacter* spp. isolates were obtained in several cases that could be differentiated by their API 20^E profiles and their ribotyping fingerprints. As shown in Table 1, many different species and strains of coliforms were found. Among the *Enterobacter* species, the presence of α -glucosidase is known to be specific for *Cronobacter* spp. (9). The combination of the yellow colonies on TSA and positive α -glucosidase activity was therefore considered further evidence for the identity of *Cronobacter* spp.. All 32 presumptive *Cronobacter* spp. isolates and 18 other coliforms, which had been isolated from environmental samples and had been identified using the API 20^E system, were tested for their α -glucosidase activity.

Table 1. Presumptive identification of coliforms isolated from environmental samples taken in three milk powder factories^a

Species	Factory A	Factory B	Factory C
No. of samples	56	49	47
<i>Chryseomonas luteola</i>	2		
<i>Citrobacter diversus</i>	1		3
<i>Enterobacter amnigenus</i>		1	
<i>Enterobacter cloacae</i>	5	8	9
<i>Cronobacter</i> spp. ^b	8 (16) ^c	4 (6) ^c	6 (10) ^c
<i>Erwinia nigrifluens</i>			2
<i>Escherichia coli</i>	1	2	
<i>Escherichia hermannii</i>		1	1
<i>Escherichia vulneris</i>		1	3
<i>Klebsiella ornithinolytica</i>		2	
<i>Klebsiella oxytoca</i>	1	1	
<i>Klebsiella pneumoniae</i>		2	1
<i>Kluyvera</i> spp.			1
<i>Leclercia adecarboxylata</i>	1	1	
<i>Pantoea</i> spp.	2	5	6
<i>Rahnella aquatilis</i>	1		1
<i>Serratia ficaria</i>		3	2
Unidentified	7	10	21

^a Identification was based on oxidase test and API 20^E. The Table indicate the number of samples from which a certain species was isolated.

^b Please note that 3 of the 32 presumptive isolates that were identified as *Cronobacter* spp. by the API 20^E system did not show α -glucosidase activity and were not confirmed to be *Cronobacter* spp. by their ribotype fingerprint.

^c Implies that 16, 6, and 10 different isolates were obtained from the samples based on ribotyping results.

As shown in Table 2, 29 *Cronobacter* spp. isolates hydrolyzed paranitrophenyl- α -D-glucopyranoside within 4 h. None of the other coliforms were positive in the 4-h assay. After 24 h, the difference between *Cronobacter* spp. and the other coliforms was less clear. One *Pantoea* isolate and one strain of *Erwinia* showed substantial α -glucosidase activity to be rated positive in the 24-h assay. This indicates that the specificity of the α -glucosidase

activity of *Cronobacter* spp. only applies for a short-term assay. No false-positive results were observed with the 4-h α -glucosidase assay.

Table 2. Yellow-pigment formation and the α -glucosidase assay after 4 and 24 h incubation by strains grown on TSA

Presumptive identification (no. of strains)	Yellow-pigment on TSA	Alpha-glucosidase ^a	
		4 h	24 h
<i>Citrobacter diversus</i> (3)	+	-	-
<i>Cronobacter</i> spp. (29)	+	+	+
<i>Cronobacter</i> spp. (3) ^b	+	-	-
<i>Enterobacter cloacae</i> (4)	-	-	-
<i>Erwinia nigrifluens</i> (1)	+	-	-
<i>Erwinia nigrifluens</i> (1)	+	-	+
<i>Escherichia coli</i> (1)	-	-	-
<i>Escherichia hermanii</i> (1)	+	-	-
<i>Escherichia vulneris</i> (1)	-	-	-
<i>Klebsiella ornithinolytica</i> (1)	-	-	-
<i>Klebsiella oxytoca</i> (1)	-	-	-
<i>Klebsiella pneumoniae</i> (1)	+	-	-
<i>Pantoea</i> spp. (1)	+	-	+
<i>Pantoea</i> spp. (1)	+	-	-
<i>Rahnella aquatilis</i> (1)	+	-	-

^a + : paranitrophenol concentration (PNP) greater than 16 μ M, as measured by OD at 405 nm.

^b Presumptively identified as *Cronobacter* spp. by the API 20E system but not confirmed by ribotyping or by α -glucosidase presence.

Three presumptive *Cronobacter* spp. isolates were α -glucosidase negative after 4 h. These three isolates were clearly α -glucosidase negative after retesting, although they were identified as *Cronobacter* spp. by the API 20^E system with a low percentage of match (80%) with the expected biochemical profile of *Cronobacter* spp..

The presumptive *Cronobacter* spp. strains were also characterized using the ribotyping technique. Ribotyping is a method that generates a highly reproducible and precise fingerprint of bacterial rRNA genes. Using a database of reference fingerprints, it can be

used to classify and identify bacteria. The technology can be used here as a further tool, next to yellow pigmentation, oxidase/API 20^E, and the α -glucosidase assay, to confirm the identity of *Cronobacter* spp. and also investigate relatedness among *Cronobacter* spp. isolates.

The results showed that 27 presumptive *Cronobacter* spp. isolates were confirmed to be *Cronobacter* spp. by ribotyping. Two other isolates gave unclear fingerprints due most likely to the lack of DNA digestion by the EcoRI enzyme. The remaining three strains were the three α -glucosidase-negative isolates and indeed had fingerprints that were significantly different from the six reference *Cronobacter* spp. strains of the Riboprinter database. Based on the absence of α -glucosidase and the ribotyping results, it can be concluded that the three isolates identified by the API 20^E system as *Cronobacter* spp. were not *Cronobacter* spp.. It should be noted that at least a 90% of biochemical reaction of the API 20^E is needed for a reliable identification. The results of the ribotyping also indicated that in none of the three factories was a specific strain predominant (results not shown).

In summary, it can be concluded that the combination of yellow pigmented colonies on TSA and the presence of α -glucosidase activity detected in a 4-h assay are a good method to differentiate *Cronobacter* spp. from other coliforms. This method is useful for routine screening of environmental samples taken from milk powder factories and most likely also for their dry milk powder-based formulas (6). To ensure the detection of low numbers of *Cronobacter* spp. in environmental samples, work is currently ongoing to optimize the enrichment step.

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Chapter 3



**A simple and rapid cultural method
for the detection of *Cronobacter* spp.
in environmental samples**

Abstract

A method was developed to detect and identify Cronobacter spp. in environmental samples. The method is based on selective enrichment at 45 ± 0.5 °C in lauryl sulfate tryptose broth supplemented with 0.5 M NaCl and 10 mg/L vancomycin (mLST) for 22 to 24 h followed by streaking on tryptone soy agar with bile salts. When exposed to light during incubation at 37 °C, Cronobacter spp. produces yellow colonies within 24 h; identification was confirmed by testing for α -glucosidase activity and by using API 20^E strips. All of the Cronobacter spp. strains tested (n=99) were able to grow in mLST at 45 ± 0.5 °C, whereas 35 of 39 strains of potential competitors, all belonging to the Enterobacteriaceae, were suppressed. A survey was carried out with 192 environmental samples from four different milk powder factories. Using this new protocol, Cronobacter spp. was isolated from almost 40% of the samples, whereas the reference procedure (enrichment in buffered peptone water, isolation on violet red bile glucose agar, and biochemical identification of randomly chosen colonies) only yielded 26% positive results. This selective method can be very useful for the rapid and reliable detection of Cronobacter spp. in environmental samples.

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*Throughout this thesis the designation *Cronobacter* spp. has been used to indicate the taxonomic change.

Introduction

Cronobacter spp., a yellow member of the family *Enterobacteriaceae*, has been associated with various cases of severe neonatal meningitis, bacteraemia, and necrotizing enterocolitis, particularly in premature babies (2–4, 8, 9, 15, 19, 20, 23, 25–27). In some of these cases, contaminated powdered milk infant formula was identified as the source of *Cronobacter* spp. (2, 4, 25, 26). In surveys of dry infant formulas obtained from various countries, *Cronobacter* spp. was indeed isolated (21, 24), albeit at very low concentrations of less than 1 CFU/g. The dose-response relationship for this opportunistic pathogen is not known. However, several cases have been associated with an exposure of prepared infant formula to improper holding temperatures, which suggests that multiplication of *Cronobacter* spp. is often a decisive factor in causing the outbreaks (4, 26). Nevertheless, it remains very important to reduce the prevalence of the micro-organism in infant formula. Management of factory environment hygiene is critical to the control of the organism, because the presence of *Cronobacter* spp. in infant formula is often attributed to contamination originating from dry factory environments, where it appears to survive better than other members of the *Enterobacteriaceae* (5).

To evaluate the effect of different cleaning strategies on the prevalence of *Cronobacter* spp. in the factory environment, sensitive and specific detection methods are required. The only methods described thus far are based on general methods for the enrichment and isolation of *Enterobacteriaceae* (e.g., pre-enrichment in buffered peptone water, sub-culturing in *Enterobacteriaceae* enrichment broth, and isolation on violet red bile glucose agar), followed by biochemical identification of typical colonies (1, 3, 21, 22). This approach is laborious and requires up to 7 days obtaining a confirmed positive result. Recently, an improved identification method was proposed (13), which is based on the detection of α -glucosidase and production of a yellow pigment. With this method, confirmed positive results can be obtained after 5 days. However, this procedure is not selective for *Cronobacter* spp. and may thus be prone to false-negative results in the presence of other *Enterobacteriaceae*.

Here, we describe the development of a selective enrichment procedure for *Cronobacter* spp. based on lauryl sulphate tryptose, a commonly used enrichment broth for coliforms. Selectivity was enhanced by the addition of 10 mg/L vancomycin and 0.5 M NaCl and by the application of a relatively high incubation temperature (45 ± 0.5 °C). We also introduce a new procedure that produces yellow colonies on the isolation agar within 24 h by exposing the plates to visible light during incubation at 37 °C.

Materials and methods

Bacterial strains

Ninety-nine strains of *Cronobacter* spp. were used in this study (Table 1), 70 of which had been isolated from the environment of milk powder factories in Europe and Asia using a non-selective enrichment and isolation method. These strains were selected to represent the major ribotypes of this species. The remaining strains were isolated from private households (14), or were kindly provided by Dr. S. Forsythe (Nottingham Trent University, Nottingham, UK) and by Dr. H. L. Muytjens (Radboud University, Nijmegen, The Netherlands). The identification of the *Cronobacter* spp. strains was based upon the combination of yellow pigmentation and a positive α -glucosidase reaction, as described by Kandhai *et al.*, (13). The identity of *Cronobacter* spp. was further confirmed with API 20^E strips (bioMérieux SA, Marcy l'Etoile, France) and by ribotyping with the RiboPrinter (DuPont Qualicon, Inc., Wilmington, Del.) (6). The ribopattern of each presumptive *Cronobacter* spp. strain was recorded, digitized, and stored in a database containing 11 DuPont reference *Cronobacter* spp. fingerprints combined with 2,000 in-house *Cronobacter* spp. fingerprints (unpublished results).

Thirty-nine non-*Cronobacter* spp. strains were selected to represent the major species of other *Enterobacteriaceae* found in factory environments. The strains either came from the Nestlé Food Safety Microbiology culture collection or had been isolated from milk powder or from the environment of milk powder factories. The selection focused on yellow non-*Cronobacter* spp. strains (*Citrobacter* spp., *Escherichia hermannii*, *Escherichia vulneris*, *Pantoea agglomerans*, *Pantoea* spp., and *Serratia ficaria*) that can easily be confused with yellow *Cronobacter* spp.. All strains were different from *Cronobacter* spp. by API 20^E and automated ribotyping (data not shown).

All strains were maintained at -30 °C in bacterial strain storage medium (Bio-Rad Laboratories, Inc., Hercules, Calif.). Working cultures were prepared by inoculation of brain heart infusion broth (BHI; Oxoid Ltd., Basingstoke, Hampshire, UK) and incubation for 20 h at 30 °C.

Table 1. *Cronobacter* spp. and other *Enterobacteriaceae* strains used in this study

Species	Strain code	Source ^a	Growth in ^b			Yellow pigment on TSBA
			LST 30 °C	LST 45 °C	mLST 45 °C	
<i>Cronobacter</i> spp.	48; 49; 145; 151; 155; 156; 158; 164; 166; 167; 262; 263; 265; 266; 270; 271; 272; 273; 274; 275; 276; 277; 278; 279; 280; 281; 282; 283; 284; 285; 286; 287; 288; 290; 291; 292; 293; 294; 295; 297; 298; 299; 300; 301; 302; 303; 304; 305; 306; 307; 308; 312; 314; 315; 316; 317; 318; 319; 320; 321; 323; 354; 356; 359; 366; 383; 384; 390; 391	E	++	++	++	+
<i>Cronobacter</i> spp.	365	E	++	++	+	+
<i>Cronobacter</i> spp.	309; 327; 368; 369	MP ^d	++	++	++	+
<i>Cronobacter</i> spp.	310; 328; 393	MP	++	++	++	+
<i>Cronobacter</i> spp.	313; 322; 376 ^e (=ATCC 29544); 468; 469; 470; 471; 472	C ^d	++	++	++	+
<i>Cronobacter</i> spp.	473	C ^d	++	++	+	+
<i>Cronobacter</i> spp.	375; 377; 378	H	++	++	++	+
<i>Cronobacter</i> spp.	454; 455; 457; 458; 459; 460; 461; 462; 463	Not known ^f	++	++	+	+
<i>Cronobacter</i> spp.	456	Not known ^f	++	++	++	+
<i>Citrobacter</i> spp.	31	NFSM ^g	++	+	-	-
<i>Citrobacter</i> spp.	189	NFSM	++	++	-	+
<i>Citrobacter</i> spp.	411	MP	++	+	-	+
<i>Enterobacter cloacae</i>	20; 62	NFSM	++	++	-	-
<i>Enterobacter cloacae</i>	84	NFSM	++	+	-	-
<i>Enterobacter cloacae</i>	86	MP	++	++	-	-
<i>Enterobacter cloacae</i>	418	MP	++	++	++	-
<i>E. aerogenes</i>	149	MP	++	++	-	-
<i>Escherchia coli</i>	186	NFSM	++	+	-	-
<i>Escherchia hermanii</i>	40	NFSM	++	++	++	-
<i>Escherchia hermanii</i>	73	NFSM	++	+	-	-
<i>Escherchia hermanii</i>	75	NFSM	++	++	-	+
<i>Escherchia vulneris</i>	417	MP	++	+	-	-
<i>Escherchia vulneris</i>	422; 428; 429; 435; 442	E	++	++	-	+
<i>Hafnia</i> spp.	42; 108	NFSM	++	++	-	-
<i>Hafnia</i> spp.	64	NFSM	++	+	-	-
<i>Klebsiella oxytoca</i>	104	NFSM	++	++	++	-
<i>Klebsiella pneumoniae</i>	254	NFSM	++	++	-	-
<i>Klebsiella pneumoniae</i>	255	NFSM	++	++	++	-
<i>Leclercia</i>	80	NFSM	-	-	-	-
<i>Pantoea agglomerans</i>	143	NFSM	++	+	-	-
<i>Pantoea agglomerans</i>	188	NFSM	-	-	-	+
<i>Pantoea agglomerans</i>	246	NFSM	++	++	-	-
<i>Pantoea</i> spp.	168	MP	++	++	-	+
<i>Pantoea</i> spp.	415	MP	+	+	-	+
<i>Pantoea</i> spp.	416	MP	++	++	-	+
<i>Pantoea</i> spp.	440; 441; 443; 450	E	++	++	-	+
<i>Providencia</i> spp.	65	NFSM	++	++	-	-
<i>Serratia ficaria</i>	142	NFSM	++	++	-	+
<i>Serratia ficaria</i>	427	E	++	++	-	+

^a E: Isolates from the environment of milk powder factories; MP: isolates from milk powder; C: clinical isolates; H: households. ^b Growth was assessed by measuring the optical density at 620 nm after incubation. “-”: OD₆₂₀ < 0.010; “+”: OD₆₂₀ = 0.010 – 0.100; “++” OD₆₂₀ < 0.010.

^c LST (Lauryl Sulphate Tryptose broth) supplemented with 0.5 M NaCL and 10 mg/L vancomycin (mLST). ^d Radboud University, Nijmegen (The Netherlands). ^e Type strain ^f Nottingham Trent University (UK). ^g NFSM: Nestlé Food Safety Microbiology Culture Collection.

Development of a selective enrichment broth for Cronobacter spp.

The conditions for selective enrichment culture of *Cronobacter* spp. were established in microtiter plate growth assays using 15 *Cronobacter* spp. strains (Table 2). Aliquots (5 μ l) of freshly grown BHI cultures were inoculated into wells containing 200 μ l lauryl sulfate tryptose broth (LST; Oxoid) supplemented with different concentrations (0, 0.25, 0.5, 0.75, and 1.0 M) of NaCl (Merck, Whitehouse Station, N.J.). The microtiter plates were incubated at 44, 45, 46, and 47 °C. Growth was assessed by determining the optical density at 620 nm after 0, 6, 24, and 48 h using a microtiter plate reader (Multiskan/MCC 340, Labsystems, Helsinki, Finland). Based on the results of these experiments, LST broth supplemented with 0.5 M NaCl and 10 mg/L vancomycin (Fluka Chemie, Buchs, Switzerland) (mLST) and incubated at 45 \pm 0.5 °C was selected for further evaluation with 99 *Cronobacter* spp. strains and 39 strains of other *Enterobacteriaceae*. For this part of the study, 0.1 ml aliquots of freshly grown BHI cultures diluted 1:100 in physiological saline were inoculated into 10 ml volumes of standard LST and mLST to give an initial count of ca. 10⁴ CFU/ml and then incubated at 30 or 45 °C for 24 h. The optical density was then measured at 620 nm.

Table 2. Growth of 15 *Cronobacter* strains in LST supplemented with various NaCl-concentrations

Growth ^a of 15 <i>Cronobacter</i> spp. strains ^b	Temperature				
	37 °C	44 °C	45 °C	46 °C	47 °C
Standard LST	++	++	++	++	+
LST supplemented with 0.25 M NaCL	++	++	++	++	+
LST supplemented with 0.50 M NaCL	++	++	++	+	-
LST supplemented with 0.75 M NaCL	++	++	+	-	-
LST supplemented with 1.00 M NaCL	+	-	-	-	-

^aGrowth was assessed by measuring the optical density at 620 nm after 24 h incubation.

“-”: OD₆₂₀ < 0.010; “+” : OD₆₂₀ 0.010 - 0.100; “++”:OD₆₂₀ > 0.010.

^bNestlé Food Safety Microbiology (NFSM) 262, 263, 266, 270, 280, 302, 310, 328, 359, 362, 369, 383, 384, 391, 393.

Samples

Dry environmental samples were obtained from different areas of four milk powder factories and included floor sweepings, scrapings of processing areas, spilled dry products, contents of vacuum cleaner bags, etc. From the same factories, moist environmental sponge samples (8 by 4 by 1 cm) were obtained.

Non-selective enrichment and isolation

For non-selective enrichment (BPW-VRBL) samples (10 g each) were suspended in 90 ml of buffered peptone water (BPW; Oxoid). When quantities smaller than 10 g were analyzed, the volume of BPW was adapted proportionally (1:10). Sponges were soaked with ca. 100 ml of BPW. The enrichment broth was incubated for 16 to 20 h at 37 °C. A loopful of pre-enrichment broth (ca. 10 µl) was streaked onto violet red bile lactose agar (VRBL; Oxoid) and incubated at 37 °C for 24 h. From each plate, five colonies demonstrating typical coliform characteristics were transferred to tryptone soy agar (TSA; Oxoid) and examined after 24 h of incubation at 37 °C for yellow pigment production, oxidase, and α -glucosidase activity as described by Kandhai *et al.*, (13) and identified with API 20^E strips.

Two-stage selective enrichment

For the two-stage selective enrichment (BPW-mLST-TSBA), samples were first pre-enriched in BPW as described above. A loopful of pre-enrichment broth (ca.10 µl) was transferred to 10 ml of mLST and incubated for 22 to 24 h at 45 ± 0.5 °C followed by streaking of a loopful onto a 14-cm petri dish containing TSA supplemented with 1.5 g/L bile salts 3 (TSBA; Oxoid). During incubation (24 h at 37 °C), plates were exposed to artificial white light (ca. 600 lx) to induce production of a yellow pigment by *Cronobacter* spp.. From each plate, five yellow colonies were selected for confirmation as described above.

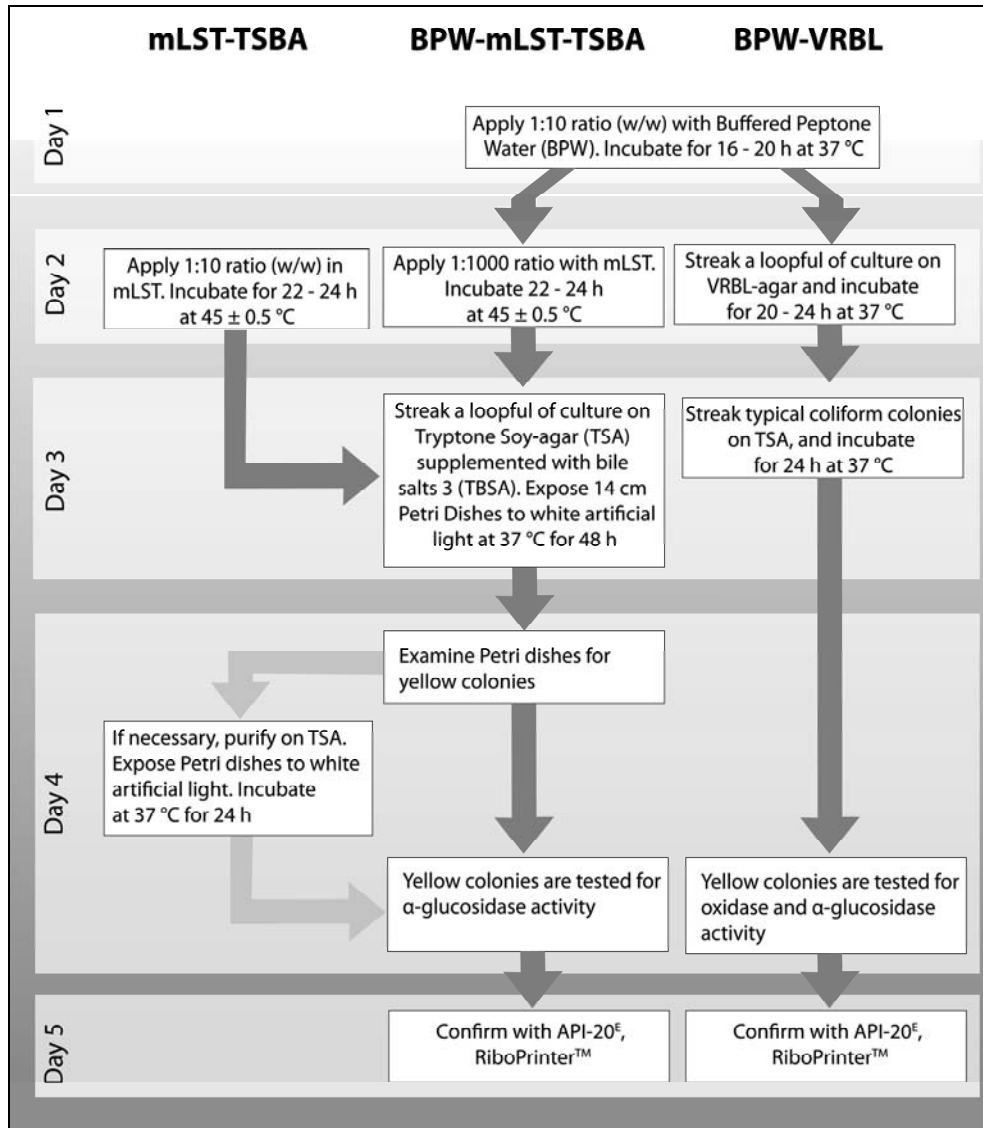


Figure 1. Schematic diagram for the enrichment and identification of *Cronobacter* spp. in environmental samples with mLST-TSBA, BPW-mLST-TSBA and BPW-VRBL.

Single-stage selective enrichment

For the single-stage selective enrichment (mLST-TSBA), 10 g of sample was suspended in 90 ml of mLST and incubated for 22 to 24 h at 45 ± 0.5 °C. When quantities smaller than

10 g were analyzed, the volume of mLST was adapted proportionally (1:10). Sponges were soaked with ca. 100 ml of mLST. After incubation, suspensions were transferred to TSBA for evaluation.

Results and discussion

To enhance the selectivity of *Cronobacter* spp., LST broth, widely used as semi-selective enrichment broth for coliforms (11, 12), was modified by adding extra NaCl and the antibiotic vancomycin. The NaCl was added because Breeuwer *et al.*, (5) demonstrated that *Cronobacter* spp. has a higher tolerance for osmotic stress than do other members of the *Enterobacteriaceae* such as *Salmonella* spp., *Escherichia coli*, *Klebsiella pneumoniae* and *Citrobacter freundii*. The vancomycin was added as an additional safeguard against the growth of competing gram-positive micro-organisms, in particular *Bacillus* spp. and lactic acid bacteria (10, 17). Preliminary tests with LST + 0.5 M NaCl had indicated that these gram-positive bacteria were often found in environmental samples and were apparently not sufficiently inhibited by the presence of lauryl sulfate (results not shown).

A relatively high incubation temperature was chosen on the basis of results of different studies (5, 7, 16, 24) indicating that *Cronobacter* spp. is able to grow at temperatures up to 46 or 47 °C. All 15 *Cronobacter* spp. strains evaluated grew well in standard LST up to 46 °C and were able to grow in LST at 37 °C at increasing osmolarities up to 1 M NaCl (Table 2). The combination of LST with 0.5 M NaCl and an incubation temperature of 45 ± 0.5 °C was selected for further evaluation.

The extended growth study with pure cultures (Table 1) revealed that many of the selected strains of *Enterobacteriaceae* were good competitors in LST at 45 ± 0.5 °C and that the addition of NaCl was essential for selective enrichment of *Cronobacter* spp. all of the tested *Cronobacter* spp. strains were able to grow, whereas 35 out of the 39 non-*Cronobacter* spp. *Enterobacteriaceae* strains were inhibited. The remaining competitors belonged to a variety of species: *Escherichia hermannii*, *Enterobacter cloacae*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*. However, none of these strains produces a yellow pigment and could be easily distinguished from *Cronobacter* spp. on the isolation medium TSBA.

The performance of mLST broth was assessed in a comparative study involving 192 environmental samples collected from four milk powder factories. Both the one and two-stage enrichment procedures (mLST-TSBA and BPW-mLST-TSBA, respectively) were used, and the non-selective enrichment method (BPW-VRBL) (14) was used as a reference. Similar numbers of *Cronobacter* spp. positive samples were obtained with BPW-mLST-TSBA and mLST-TSBA (73 and 75, respectively), whereas the reference method yielded only 51 positive results (Table 3). The difference in efficiency between the mLST-based procedures and the reference procedure is probably due to both the selectivity of mLST and the utilization of TSBA as isolation agar rather than randomly picking coliform colonies from VRBL plates.

Nevertheless, not all the yellow colonies growing on TSBA could be confirmed as *Cronobacter* spp.. Some strains were identified as *Pantoea* spp., *Pantoea agglomerans*, or *Escherichia vulneris*. Thus, additional tests such α -glucosidase activity and identification with API 20^E strips are necessary to eliminate false-positive results.

Table 3. Detection of *Cronobacter* spp. in environmental samples from four milk powder factories, using three different protocols

Source	Tested	Confirmed positive	No. (%) samples		
			Confirmed positive with:		
			mLST-TSBA	BPW-mLST-TSBA	BPW-VRBL
Factory A	48	20	18	18	9
Factory B	46	10	9	10	5
Factory C	44	24	23	19	19
Factory D	54	34	25	26	18
Total	192 (100)	88 (46)	75 (39)	73 (38)	51 (26.5)

The total number of positive samples found in this comparative study was still considerably higher (88) than that obtained with either of the two mLST-based methods (Table 3). This difference is likely due to an uneven distribution of *Cronobacter* spp. in the samples, which had been divided to allow comparison of the different procedures as outlined in Figure 1.

However, both methods may be prone to false-negative results, and further improvements of the sensitivity may be necessary.

Although the direct mLST-TSBA method is 1 day faster than the BPW-mLST-TSBA route, the latter method may be preferred for routine purposes because it can be combined with *Salmonella* testing; the first stage of this method also consists of a non-selective pre-enrichment. The direct mLST-TSBA method has been used in the development of a *Cronobacter* spp. specific PCR assay on the BAX system (Dupont Qualicon) that provides results within 30 to 32 h (18).

In this study, we also used an accelerated procedure to obtain yellow colonies on the isolation agar medium (TSBA) by exposing the plates to artificial white light during their incubation at 37 °C. Prior to implementing this method, we tested more than 100 different *Cronobacter* spp. strains from our collection, and in all cases the presence of yellow colonies could already be observed within 24 h (data not shown). This modification represents a clear advantage to the methods used previously, which are based on incubation at 25 °C for 48 to 72 h (3, 7, 21).

The procedures outlined in this study will enable efficient and rapid isolation of *Cronobacter* spp. from environmental samples. Effective environmental analysis can contribute to a better understanding of the route(s) by which *Cronobacter* spp. enters factories producing powdered infant formulas and will help managers identify in-factory ecological niches where this organism can persist. Such information is essential for developing control and prevention strategies for this emerging opportunistic pathogen.

Acknowledgments

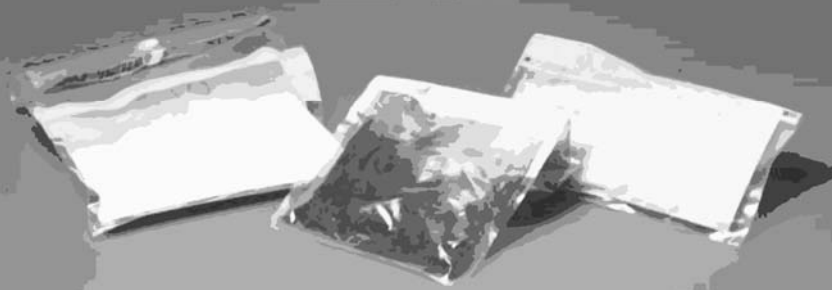
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Chapter 4



**Occurrence of *Cronobacter* spp.
in food production environments
and households**

Abstract

Cronobacter spp. occasionally causes illness in premature babies and neonates. Contamination of infant formulae during factory production or bottle preparation is implicated. Advice to health-care professionals focuses on bottle preparation, but the effectiveness of prevention depends on the degree of contamination and contamination sites, which are generally unknown. To keep contamination to a minimum in the finished product depends on knowledge of the occurrence of *Cronobacter* spp.. We used a refined isolation and detection method to investigate the presence of this micro-organism in various food factories and households. Environmental samples from eight of nine food factories and from five of 16 households contained *Cronobacter* spp.. The widespread nature of this micro-organism needs to be taken into account when designing preventive control measures.

This Chapter has been published as

“Occurrence of *Enterobacter sakazakii in food production environments and households”**

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* Throughout this thesis the designation *Cronobacter* spp. has been used to indicate the taxonomic change.

Introduction

Several species within the genus *Enterobacter* have been recognised as important causative agents of hospital acquired infections. *Cronobacter* spp. in particular has caused several outbreaks or sporadic cases of severe neonatal meningitis in premature babies, or necrotising enterocolitis. *Cronobacter* spp. has been isolated from various clinical materials, such as cerebrospinal fluid, blood, skin, wounds, respiratory tract (sputum, throat, and nose), digestive tract, and urine (1). *Cronobacter* spp. was isolated from powdered substitutes for breast milk (4). Infant formulae are pasteurised during manufacturing, and *Cronobacter* spp. does not survive such heat treatment (5). The micro-organism's presence in the finished product, therefore, probably originates from the factory environment, from heat-sensitive micronutrients added after pasteurisation or from bottle preparation.

In several investigations into outbreaks or cases of *Cronobacter* spp. infection in premature babies and neonates, the micro-organism was isolated from blenders, bottle cleaning brushes, etc. (3). Muytjens and Kollee investigated the occurrence of *Cronobacter* spp. more widely, but could not isolate the micro-organism from any environment they examined, which included surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic environments, cattle, and untreated cow's milk (3). *Cronobacter* spp. has, however, been isolated from cheese, minced beef, sausage meat, and vegetables (2), but we are unaware of any investigation into the natural habitat of *Cronobacter* spp.. Why *Cronobacter* spp. infection occurs only in neonates and premature babies, and why it is associated only with the consumption of infant formulae, is unclear.

The US Food and Drug Administration issued an alert in April, 2002, to health-care professionals about the risks associated with *Cronobacter* spp. infections among neonates fed with milk-based infant formulae, and recommended certain bottle-preparation practices. The alert stated that a major contribution to the prevention of *Cronobacter* spp. infections in premature babies and neonates is the prevention of contamination of infant formulae during production and bottle preparation. However, knowledge of the aetiological and ecological characteristics of *Cronobacter* spp. is sparse, and its occurrence in factories that

produce infant formulae, hospital kitchens, and households has hitherto not been studied in depth. We have started a systematic investigation of the habitat of *Cronobacter* spp..

Materials and methods

Environmental samples were obtained from nine factories and 16 households (Table1). In factories, samples were obtained by scraping or sweeping surfaces in the production-line environment or by sampling vacuum cleaner bags. Samples from households were taken from vacuum cleaner bags. From each sample, we analysed 10 g for the presence of *Cronobacter* spp. by selective enrichment in lauryl sulphate tryptose broth, supplemented with 0.5 mol/L salt, incubated at 45 °C for 22 to 24 h. A loopful was streaked on violet red bile lactose agar to select for coliforms during the subsequent incubation at 37 °C for 24 h. All coliform colonies on violet red bile lactose agar were streaked on to tryptone soy agar plates and incubated for 48 h in daylight at room temperature. For the identification of *Cronobacter* spp., we tested all yellow-coloured oxidase-negative colonies for the presence of α -glucosidase. Presumptive *Cronobacter* spp. isolates were identified with the API 20^E test system (bioMerieux, Marcy L'Etoile, France) and were also characterised as *Cronobacter* spp. with the ribotyping technique.

Results and discussion

Cronobacter spp. was isolated with varying frequency from nearly all environments examined. Although the proportion of positive samples differed between environments, the differences were not significantly different (Table 1). The presence of *Cronobacter* spp. in factories producing milk powder, cereals, chocolate, potato flour, and pasta, as well as in domestic environments, strongly indicates that it is a widespread micro-organism. Since measures to prevent neonatal cases of *Cronobacter* spp. infections are being discussed in various countries by industries, public-health workers, and consumers, our findings may contribute to the current knowledge. Most importantly, the widespread nature of *Cronobacter* spp. should be taken into account in the design of effective control measures.

Table 1. Environmental samples of various origins tested for the presence of *Cronobacter* spp.

Origin	Number of samples analysed	Number of samples positive for <i>Cronobacter</i> spp. (%)	95% confidential interval
Milk powder factory	23	2 (9)	0.01 - 0.27
Milk powder factory	26	9 (35)	0.19 - 0.53
Milk powder factory	11	1 (9)	0.002 - 0.404
Milk powder factory	8	2 (25)	0.03 - 0.64
Chocolate factory	8	2 (25)	0.03 - 0.64
Cereal factory	9	4 (44)	0.15 - 0.78
Potato flour factory	15	4 (27)	0.08 - 0.53
Pasta factory	26	6 (23)	0.10 - 0.42
Spice factory	5	0	0 - 0.52
Households	16	5 (31)	0.12 - 0.57

Acknowledgments

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Appendix: Correspondence

Cronobacter spp. in factories and households

The isolation of *Cronobacter* spp. from various factories, including infant formula factories (1), and the related contamination of infant formulae with *Cronobacter* spp. are causes for concern. I was surprised to see only one recommendation from the authors at the end of the article: “The widespread nature of this micro-organism needs to be taken into account when designing preventive control measures.” Two other recommendations quickly come to mind: (1) enhancement of the promotion of and support for breastfeeding, and (2) inclusion of a warning on infant formulae and other breast-milk substitutes that the product might be contaminated by *Cronobacter* spp. and other micro-organisms. I was wondering why the authors did not make such recommendations, when my eyes fell on the last paragraph of the article: “This research is part of a PhD research project . . . which is supported by the Nestlé Research Centre.” A few lines higher I read that no conflicts of interest were declared. Nestlé produces infant formula and other breast-milk substitutes. If this is not a conflict of interest, then what is?

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Authors' reply

Maaiké Arts asks why we did not include in our Research letter other recommendations about *Cronobacter* spp. in infant formulae. We believe that our conclusion “The widespread nature of this micro-organism needs to be taken into account when designing preventive control measures” is the only one justified on the basis of the results we reported in the paper. The purpose of our study was to gain a better understanding of the ecology of *Cronobacter* spp. as a starting point for designing effective control strategies. On the basis of the research we reported, we cannot substantiate the recommendations suggested by Arts. However, we would like to draw readers' attention to the joint FAO/WHO Expert Workshop on *E. sakazakii* and other micro-organisms in powdered infant formula (held in Geneva, Switzerland, Feb 2–5, 2004) (1) as a potential source of information.

With respect to the suggested conflict of interest, we are convinced that we clearly disclosed our affiliations and financial support as required by the journal. As stated in the original publication, “The sponsor had no role in the study design, data collection, data analysis, and data interpretation, or in writing the report.”

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Wageningen University, Laboratory of Food Microbiology, PO Box 8129, 6700 EV Wageningen, Netherlands (MCK, MWR, LGMG, MvS); and Nestlé Research Centre, Quality and Safety Assurance Department, Lausanne, Switzerland (OGG)

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Chapter 5



**A study into the occurrence of
Cronobacter spp. in The Netherlands
between 2001 and 2005**

Abstract

Cronobacter spp. is an opportunistic pathogen, possibly occurring in many different foods and environments causing illnesses predominantly through powdered infant formula. To add to the existing knowledge on the occurrence of *Cronobacter spp.* and to help identify habitats that could be potential sources of this organism, a broad survey of foods manufactured or marketed in The Netherlands, including relevant non-food environments, was conducted over a 5-years period (2001 – 2005). Using a specifically designed real-time polymerase chain reaction method for definite confirmation, *Cronobacter spp.* was isolated from milk powders (7/175), powdered formulae for consumers < 1 year (8 /395), follow-up formula for consumers > 1 year (1/5), other powdered instant products (1/182), dry cereals (6/123), fresh raw minced meats (7/222), vegetables (2/47), spices (1/28), samples of fresh human faeces (1/98), and human skin (1/116) samples. None of the samples taken from fresh raw bovine milk (0/51), fresh human milk (0/7), baby bottles (0/86) and bottle-nipples (0/95) were found to be positive for *Cronobacter spp.*. This study confirms that *Cronobacter spp.* can occur in a wide range of dry and wet foods, not limited to infant formulae or milk products, as well as on and in humans. This widespread occurrence is important to consider when manufacturing foods for consumers at potential risk for *Cronobacter spp.* infection.

Part of this Chapter has been accepted for publication as

“A study into the occurrence of *Cronobacter spp.* in The Netherlands between 2001 and 2005”

Kandhai, M.C., A.E. Heuvelink, M.W. Reij, R.R. Beumer, R. Dijk, J.J.H.C. van Tilburg, M. van Schothorst, and L.G.M. Gorris.

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Introduction

Cronobacter spp. (*Enterobacter sakazakii*) are Gram-negative, motile, non-spore forming, facultative anaerobic bacteria. The genus was originally defined as a *Enterobacter sakazakii* species in 1980 (21), but very recently it has been reclassified (39, 40) as six species in a new genus, *Cronobacter* gen. nov., within the family of *Enterobacteriaceae*. Five of the new species are *Cronobacter sakazakii*, *Cronobacter turicensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii* and *Cronobacter dublinensis*. The sixth species is preliminarily indicated as genomospecies I. To indicate the taxonomic change, the designation “*Cronobacter* spp.” is used consistently in the subsequent part of this paper, where the micro-organism is referred to in general terms or where historic information that is not specific to the newly recognized particular species is cited.

Although other *Enterobacter* species have been involved in nosocomial diseases, the importance of *Cronobacter* spp. is particularly due to the fact that it has been recognised as an opportunistic pathogen for infants, especially neonates (1, 11, 29). The micro-organism has been linked with serious infections in infants, causing bacteraemia and meningitis, and has also been isolated from infants in association with necrotizing enterocolitis (18, 19, 55). The International Commission on Microbiological Specifications for Foods (ICMSF) placed *Cronobacter* spp. in the category of severe hazards for restricted populations, being rare in the frequency of involvement in food-borne disease but with a lethality rate up to 70% among neonates (30). The Scientific Panel on Biological Hazards of the European Food Safety Authority (EFSA) noted that although *Cronobacter* spp. has caused illness in infants up to 1 year, neonates up to ca 4 to 6 weeks of age, pre-term or low birth-weight infants and those immuno-compromised are at greatest risk (16). Outbreaks of infant meningitis, necrotizing enterocolitis and or bacteraemia have been reported (2, 50, 61, 63, 72). All *Cronobacter* species have been linked retrospectively to clinical cases of infection in either infants or adults, and therefore all species should be considered pathogenic (20).

Cases of *Cronobacter* spp. disease have been detected most commonly among newborn and very young infants in hospital nurseries and neonatal intensive care units. The first cases attributed to this organism occurred in 1958 in England (73). Since then and up to July 2008, around 156 documented cases of *Cronobacter* spp. infection and at least 29 deaths

from all parts of the world appeared in the published literature and in reports submitted by public health organizations and laboratories (20). Considering the recent review of outbreaks caused by *Cronobacter*, an average lethality rate of 19% was calculated. More recently, the overall lethality of 67 invasive cases was estimated to be 26.7%. The lethality of *Cronobacter* meningitis, bacteraemia and necrotizing enterocolitis (NEC) was estimated to be 41.9%, < 10% and 19%, respectively (23).

Specific international attention has been given to the safety of food for infants and young children as it relates to the possible presence of *Cronobacter* spp. in powdered formulae intended for this consumer group. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have established two international risk assessments on the topic (18, 19) that have provided a scientific basis for the Codex Alimentarius Committee on Food Hygiene (CCFH), for drafting an new Code of Hygienic Practice (6) aimed at providing risk management guidance at the international level. In the Code of Hygienic Practice products for infants up to 12 months of age are considered, although several expert meetings have established that those younger than 2 months and infants of HIV positive mothers are at greatest risk for infection caused by *Cronobacter* spp. (18-20).

The occurrence of *Cronobacter* spp. at low concentrations in powdered infant formulae has been frequently reported (8, 28, 36, 59, 62). Notably, *Cronobacter* spp. has been isolated from a variety of environmental samples, including milk powder factories and other factories (43), as well as blenders and cleaning brushes used to prepare infant feeds in hospital kitchens (4, 61). The micro-organism has been isolated from several food products (i.e., beef, sausage meat, vegetables, cheese, powdered fruit flakes) and other environmental sources, including water (22), dust from households (43), insects (27, 49, 53) and rats (24). However, only powdered infant formulae have been linked to outbreaks of infections as an unequivocal source (1, 9, 23).

In order to design appropriate and effective control measures for powdered infant formula, the various possible sources of *Cronobacter* spp. need to be well understood. This would include identification of its natural habitats and/or specific niches, as well as its occurrence

in factory environments and as a contaminant of food ingredients and finished food (or feed) products. To increase our knowledge on sources and occurrence of the micro-organism, several investigations were conducted in The Netherlands over a five years period, namely between 2001 and 2005. These investigations considered food products marketed in the country in retail and wholesale, imported food and feed products, as well as food products, manufactured in The Netherlands. For part of the investigation period, in addition to analysis for *Cronobacter* spp., the level of *Enterobacteriaceae* was also assessed as an indicator of product hygiene, possibly related to the occurrence of the pathogen. A number of different methods were used in these investigations, reflecting the rapid evolution of detection and identification methodologies during the time of the investigation. Though methods differed, in all cases, a specifically designed real-time Polymerase Chain Reaction (PCR) protocol was used for definite confirmation of presumptive *Cronobacter* spp. isolates.

Material and methods

Samples

The occurrence of *Cronobacter* spp. was determined in milk powders (n=175), powdered formulae (PF) for young children below 1 year of age (n=395), follow-up formulae (FUF) for consumers > 1 year of age (n=15), other powdered instant foods (n=182), dry cereals (n=123), fresh raw minced meats (n=222), fresh raw cow's milk (n=51), dried vegetables (n=47), dry spices (n=28), fresh human breast milk (n=7), fresh human faeces (n=98), human skin (n=116), baby bottles (n=86), and bottle-nipples (n=95).

Of the 175 milk powders tested, 42 were produced in The Netherlands while the remainder (133) were taken from cargos and bulk goods as part of systematic examinations of food imports into The Netherlands. The milk powders originated from Middle- and Eastern-European countries: Ukraine (43), Poland (53), Estonia (18), the Czech Republic (8), Lithuania (5), Latvia (4) and Slovakia (2). All milk powders were intended for human consumption, with the exception of 27 samples imported from the Ukraine that were intended for animal feed.

A total of 295 powdered formulae obtained from retailers across the country were sampled, including 283 powdered formulae intended for use for infants up to the age of 12 months, and 12 follow-up formulae, intended for young children older than 12 months. An additional 115 powdered formulae were obtained directly from five manufacturers in The Netherlands, including 112 powdered formulae for consumers below 1 year of age and 3 follow-up formulae.

Several other food products were obtained from retail, comprising of dry powdered foods, including ice cream mixtures (n=62), instant pudding powders (n=25), flavoured instant drinks (n=33), dips (n=19), coffee creamers (n=4), and dry cereals (n=123), as well as raw meats, including beef (n=141), mixed beef and pork (n=50), pork (n=16), calf (n=10), and poultry (n=5), and vegetables (n=47) and spices (n=28). Additionally, 27 samples of ice cream mixtures were obtained from wholesale, whereas 12 instant pudding powders had been collected directly in factories in The Netherlands.

Fresh cow's milk (n=51) was collected from bulk tanks from 26 farms at two time points, two months apart. Human breast milk samples (n=7) were obtained from nurturing mothers. Samples of human faeces were collected from volunteers, enjoying a general healthy status and belonging to different age categories: 41 samples from children younger than 5 years of age, 9 from children between 9 and 14 years, and 48 from persons older than 14 years. Samples of human skin particles were obtained by using scrub gloves (Parfumerie Douglas Nederland B.V., Nijmegen, The Netherlands). Volunteers (n=116), belonging to the age categorised 0-20 years (n=8), 20-40 years (n=96) and 40-60 years (n=12), were asked to scrub off as much skin area as possible while taking a shower. Most volunteers scrubbed only their arms and legs. Used baby bottles and bottle-nipples were collected at day care facilities and households on the day of use.

Enrichment and isolation of Cronobacter spp.

During the period of investigation, isolation and identification methodology for this particular micro-organism experienced rapid improvement and innovation. For this reason,

different methods were employed in the course of the study. Table 1 gives an overview of the methods, which is further described in the following, with reference to the various abbreviated identification codes used to identify the specific methods. Irrespective of the method employed, all presumptive *Cronobacter* spp. colonies were further identified using the API 20^E system (bioMérieux, Marcy l'Etoile, France) and subsequently confirmed by a specific PCR assay (see below for PCR procedure details).

From the powdered food products collected in 2001-2003, 25 g or 50 g portions were carefully sprinkled onto a nine-fold volume of buffered peptone water (BPW) at a temperature of 45 °C. After soaking for 30 to 60 min on the work bench, the powders were dissolved by swirling slowly. Following overnight incubation at 37 °C, the pre-enrichments were streaked onto violet red bile lactose (VRBL; Oxoid Ltd., Basingstoke, Hampshire, England) agar (Method A1) and 10 ml portions were mixed with 90 ml of *Enterobacteriaceae* enrichment (EE; Oxoid Ltd.) broth. After overnight incubation at 37 °C, the EE cultures were streaked onto VRBL agar plates and incubated overnight at 37 °C (Method A2). Typical coliform-colonies were picked and tested for the production of yellow-pigmented colonies on Tryptone Soya agar (TSA; Oxoid Ltd.) and a positive α -glucosidase reaction within 4 h, as described previously (45). Positive isolates were further identified and confirmed as mentioned above.

From the BPW pre-enrichments of the dry powdered products and dry cereals collected in 2004 and 2005, 0.1 ml was added to 10 ml of lauryl sulphate tryptose broth (Oxoid Ltd.) supplemented with 0.5 M sodium chloride (NaCl) and 1 mg/ml vancomycin (Oxoid Ltd.), further referred to as mLST (25). After overnight incubation at 44 ± 0.5 °C, the selective enrichments were directly streaked onto TSA plates (Method B). Colonies that produced yellow pigment after overnight incubation at 37 °C were tested with the *E. sakazakii* specific 4-h α -glucosidase assay (45), whereupon positive isolates were further identified and confirmed.

The samples of raw meats were examined essentially according to procedure B, except that “*Enterobacter sakazakii* isolation agar” (ESIA; AES Chemunex, Bruz cedex, France) was used instead of TSA (Method C1). The ESIA plates were incubated overnight at 44 ± 0.5 °C

and turquoise-coloured colonies were picked off and streaked onto TSA for further identification and confirmation as described above.

The raw milk samples (10 ml) and the entire scrub gloves containing human skin particles were directly selectively enriched, in mLST (90 and 100 ml, respectively) without pre-enrichment in BPW. The mLST enrichments were streaked onto ESIA plates (Method C2). Turquoise coloured-colonies were streaked onto TSA for further identification and confirmation.

Samples of human faeces, used baby bottles and bottle-nipples, were enriched in “*Enterobacter sakazakii* selective broth” (ESSB; AES Chemunex). Ten grams of the human faeces samples were added to 90 ml ESSB. Samples of used baby bottles were obtained by rinsing the inside of the bottles with 10 ml sterile peptone physiological salt solution containing 0.1% Tween 80 (Van Merck-Schuchardt, Hohenbrunn, Germany) and adding the rinsing solution to 90 ml ESSB. Bottle-nipples were individually placed directly into 100 ml ESSB. Following 24 h incubation at 37 °C, the ESSB enrichments were streaked onto ESIA plates (Method D). Presumptive *Cronobacter* spp. colonies were further confirmed and identified as described above.

Table 1. Methods for the isolation of *Cronobacter* spp. used in this study

Method	Pre-enrichment	Selective enrichment	Isolation
A1	BPW ^a	n.d. ^b	VRBL ^c
A2	BPW	EE ^d	VRBL
B	BPW	mLST ^e	TSA ^f
C1	BPW	mLST	ESIA ^g
C2	n.d.	mLST	ESIA
D	n.d.	ESSB ^h	ESIA
E	n.d.	n.d.	ESIA+TSA+VRBL+DFI ⁱ

^a BPW, buffered peptone water.

^b n.d., not included in this method.

^c VRBL, violet red bile lactose agar.

^d EE, *Enterobacteriaceae* enrichment broth.

^e mLST, modified lauryl sulphate tryptose broth.

^f TSA, tryptone soy agar.

^g ESIA, “*Enterobacter sakazakii* isolation agar”.

^h ESSB, “*Enterobacter sakazakii* selective broth”.

ⁱ DFI, Druggan-Forsythe-Iversen agar.

The vegetables and spices were homogenized in peptone physiological salt solution (1:10 (w:v)) and dilutions were not enriched but directly streaked onto the following four media: ESIA, TSA, VRBL and Druggan-Forsythe-Iversen (DFI; Oxoid Ltd.) agar plates (Method E). Typical colonies were confirmed with the following biochemical identification methods: API 20^E (bioMérieux), Microbact (Oxoid Ltd.), BBL Crystal (Becton Dickinson and Company, Sparks Maryland, USA), Enterotube II (Becton Dickinson and Company) and the presence of α -glucosidase (45) and subsequently confirmed with the PCR assay.

Enumeration of Enterobacteriaceae

In order to assess the general hygienic status of all products, all samples collected in 2005, except for two samples of flavored instant drinks and one sample of coffee creamer, were examined for the number of *Enterobacteriaceae* (CFU/g) by using the pour plating technique, in violet red bile glucose (VRBG; Oxoid Ltd.) agar (32). Colonies of presumptive *Enterobacteriaceae* were, in accordance to the ISO 21528-1 method, confirmed by means of tests for fermentation of glucose and the presence of oxidase (31).

PCR confirmation of Cronobacter spp.

Presumptive *Cronobacter* spp. colonies were confirmed using a newly developed PCR assay, which is specific for all *Cronobacter* species tested. The new real-time PCR method targets a specific sequence within the gene encoding the major outer membrane protein (OmpA) of *Cronobacter* spp. (GenBank accession number DQ000206; National Centre for Biotechnology Information, Washington, D.C., USA). The set of oligonucleotides was designed using Primer Express software (Applied Biosystems, Foster City, USA) (Table 2). The specificity of the sequences was tested by a blast search in GenBank (BLASTn version 2.2.10, National Centre for Biotechnology Information). The oligonucleotides were purchased from Eurogentec (Liege, Belgium). The *Cronobacter* spp. probe was labelled at the 5' end with the reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with the black-hole quencher (BHQ).

Table 2. Primers and probe used for the real-time PCR assay specific for *Cronobacter* spp.

Designation	Sequence (5' → 3')	Position ^a
Esak ompA-F	ggt gaa gga ttt aac cgt gaa ctt	319-342
Esak ompA-R	gcg cct cgt tat cat cca aa	388-369
Esak ompA-probe	ccc gga aaa gcg cat ggc c	348-366

^a Positions correspond to GenBank accession no. DQ000206

For selectivity tests, 35 isolates of *Cronobacter* spp. and 44 isolates representing a range of other genera and species were grown overnight at 37 °C on TSA. One loop-full of bacterial culture was suspended in 200 µl sterile distilled water, whereupon 100 µl was used for DNA extraction. DNA was extracted using the automated MagNA Pure Compact® (Roche Diagnostics, Mannheim, Germany) system and the MagNA Pure Compact® Nucleic Acid Isolation kit I (Roche Diagnostics). Amplification and detection were carried out in a LightCycler Instrument (version 2.0, Roche Diagnostics) in a 20 µl PCR mixture containing 2.5 µl of the sample DNA, 500 nM each of the primers, 250 nM of the probe, and 4 µl LightCycler TaqMan Master mix (Roche Diagnostics). Samples were amplified with an initial denaturation step at 95 °C for 10 min to activate the FastStart Taq DNA polymerase and 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 15 s. The temperature transition rate was 20 °C/s. Samples positive for the target gene were identified by the instrument at the cycle number where the fluorescence attributable to the target sequences exceeded that measured for background. Those scored as positive by the instrument were confirmed by visual inspection of the graphical plot (cycle number versus fluorescence value) generated by the instrument.

Subtyping of Cronobacter spp.

The Pulsed Field Gel Electrophoresis (PFGE) technique of contour-clamped homogeneous electric fields (CHEF) was used for genomic typing of isolates confirmed as *Cronobacter* spp. by PCR. Genomic DNA was digested in agarose plugs with *Xba*I (10 U) (Roche Diagnostics). The resulting fragments were resolved by CHEF-PFGE with a CHEF DR-III apparatus (Bio-

Rad Laboratories, Richmond, California, USA) at a constant voltage of 200 V for 24 h at 13 °C and a linearly ramped pulse time of 5 to 60 s. The fingerprints generated were processed using BioNumerics software (Applied Maths, Kortrijk, Belgium). Isolates that had more than 95% fragments in common were designated “closely related”. Isolates were considered “indistinguishable” if 100% of fragments were identical.

Results

Selectivity of the real-time PCR

As shown in Table 3, all 35 *Cronobacter* spp. strains tested, which included 13 official reference strains and 22 isolates from various sources obtained in previous investigations at the Laboratory of Food Microbiology of the Wageningen University (43), gave a positive signal in the real-time PCR assay. All of the following 44 strains gave a fluorescence end-point signal below the threshold line (n=1 unless indicated otherwise): *Aeromonas hydrophila*, *Citrobacter* spp., *Citrobacter freundii* (n=2), *Enterobacter amnigenus*, *Enterobacter aerogenes*, *Enterobacter cloacae* (n=6), *Enterococcus faecium*, *Escherichia coli*, *Escherichia coli* O157, *Hafnia alveii*, *Klebsiella pneumoniae* (n=3), *Klebsiella* spp., *Klebsiella oxytoca* (n=2), *Listeria innocua*, *Listeria monocytogenes* (n=2), *Pseudomonas aeruginosa* (n=3), *Pseudomonas putida*, *Proteus mirabilis* (n=2), *Proteus vulgaris*, *Salmonella* Enteritidis, *Salmonella* Typhimurium (n=2), *Serratia marcescens*, *Shigella flexneri* (n=2), *Shigella sonnei* (n=3), and *Yersinia enterocolitica* (n=3).

Isolation of Cronobacter spp.

Table 4 summarizes the results of the isolation of *Cronobacter* spp. from the different samples investigated in this study. The micro-organism was isolated from the following dry powdered food sources tested: 8 out of a total of 395 powdered formulae, 1 out of 15 follow-up formulae, 7 out of 175 milk powders, 1 out of 182 of the other powdered instant products (including ice cream mixtures, instant pudding powders, flavoured instant drinks,

dips and coffee creamers). Other food products yielding *Cronobacter* spp. isolates were: dry cereals (6 out of 123), raw minced meats (7 out of 222), vegetables (2 out of 47) and spices (1 out of 28). In addition, the micro-organism was isolated twice from human sources, including faeces (1 out of 98 fresh human faeces) and skin scrubbings (1 out of 116 human skin scrub samples). *Cronobacter* spp. was not isolated from the fresh raw cow's milk samples nor from the samples of fresh human breast milk, used baby bottles or bottle-nipples. The presumptive *Cronobacter* spp. isolates obtained throughout the study period, with the exception of the human faecal isolate, were all confirmed using a newly developed real-time PCR assay, which proved to be highly specific for *Cronobacter* spp., as shown in Table 3 and shown below.

Table 3. Real-time PCR results of 79 test strains, using the probes as described in Table 2

Strain	Strain code/Origin	Results real-time PCR ^a
<i>Cronobacter sakazakii</i> ^b	<i>Enterobacter sakazakii</i> NCTC 8155	+
<i>Cronobacter sakazakii</i>	<i>Enterobacter sakazakii</i> NCTC 9238	+
<i>Cronobacter sakazakii</i>	<i>Enterobacter sakazakii</i> NCTC 11467	+
<i>Cronobacter sakazakii</i>	<i>Enterobacter sakazakii</i> DSM 4485	+
<i>Cronobacter dublinensis</i>	<i>Enterobacter sakazakii</i> NCTC 9844	
<i>Cronobacter dublinensis</i>	<i>Enterobacter sakazakii</i> NCTC 9846	+
<i>Cronobacter</i> genomospecies 1	<i>Enterobacter sakazakii</i> NCTC 9529	+
<i>Cronobacter dublinensis</i> subsp. <i>dublinensis</i>	<i>Enterobacter sakazakii</i> DSM 18705	+
<i>Cronobacter muytjensii</i>	<i>Enterobacter sakazakii</i> ATCC 51329	+
<i>Cronobacter turicensis</i>	<i>Enterobacter sakazakii</i> DSM 18703	+
<i>Cronobacter malonaticus</i>	<i>Enterobacter sakazakii</i> DSM 18702T	+
<i>Cronobacter dublinensis</i> subsp. <i>lausannensis</i>	<i>Enterobacter sakazakii</i> DSM 18706T	+
<i>Cronobacter dublinensis</i> subsp. <i>lactardi</i>	<i>Enterobacter sakazakii</i> DSM 18707T	+
<i>Cronobacter</i> spp. (n=22) ^c	<i>Enterobacter sakazakii</i> Isolates from Wageningen University culture collection	+
<i>Aeromonas hydrophila</i>	M800	-
<i>Citrobacter</i> spp.	PHLS 073-177	-
<i>Citrobacter freundii</i>	ATCC 8090	-
<i>Citrobacter freundii</i>	NCTC 6272	-

Table 3. Continued

Strain	Strain code/Origin	Results real-time PCR ^a
<i>Enterobacter amnigenus</i>	Isolate VWA	-
<i>Enterobacter aerogenes</i>	Isolate VWA	-
<i>Enterobacter cloacae</i>	ATCC 23355	-
<i>Enterobacter cloacae</i> (n=5)	Isolates VWA	-
<i>Enterococcus faecium</i>	ATCC 29212	-
<i>Escherichia coli</i>	ATCC 25922	-
<i>Escherichia coli</i> O157	NCTC 12900	-
<i>Hafnia alveii</i>	NCTC 8105	-
<i>Klebsiella</i> spp.	Isolate VWA	-
<i>Klebsiella oxytoca</i>	ATCC 49131	-
<i>Klebsiella oxytoca</i>	Isolate VWA	-
<i>Klebsiella pneumoniae</i>	ATCC 33495	-
<i>Klebsiella pneumoniae</i>	ATCC 13883	-
<i>Klebsiella pneumoniae</i>	Isolate VWA	-
<i>Listeria innocua</i>	NCTC 11288	-
<i>Listeria monocytogenes</i>	SLCC 2479	-
<i>Listeria monocytogenes</i>	NCTC 5348	-
<i>Pseudomonas aeruginosa</i>	ALM32	-
<i>Pseudomonas aeruginosa</i>	ATCC 27853	-
<i>Pseudomonas aeruginosa</i>	NCTC 10662	-
<i>Pseudomonas putida</i>	Isolate VWA	-
<i>Proteus mirabilis</i>	ALM29	-
<i>Proteus mirabilis</i>	NCTC 11938	-
<i>Proteus vulgaris</i>	ATCC 13315	-
<i>Salmonella</i> Enteritidis	ALM36	-
<i>Salmonella</i> Typhimurium	ALM40	-
<i>Salmonella</i> Typhimurium	ATCC 13311	-
<i>Serratia marcescens</i>	ATCC 13880	-
<i>Shigella flexneri</i>	ALM60	-
<i>Shigella flexneri</i>	Isolate VWA	-
<i>Shigella sonnei</i>	ATCC 11060	-
<i>Shigella sonnei</i>	Isolate GG&GD Haarlem	-
<i>Shigella sonnei</i>	Isolate VWA	-
<i>Yersinia enterocolitica</i>	ALM5	-
<i>Yersinia enterocolitica</i> O3	Isolate UMC St. Radboud, Nijmegen	-
<i>Yersinia enterocolitica</i> O9	CCUG8239A	-

^a +, positive; -, negative

^b The *Cronobacter* species identification is according to Iversen *et al.*, 2007 (39).

^c Presumptively identified by the α -glucosidase assay (45) and the API 20^E system (bioMérieux).

Table 4. Prevalence of *Cronobacter* spp. in samples of various origins, using methods as described in Table 1

Sample	Prevalence of <i>Cronobacter</i> spp. # positive/total (%)	95% Confidence interval (%) ^a	Year of sampling	Sample size	Method ^b
Powdered foods					
Milk powder	7 ^c / 171 ^d (4.1)	1.8 – 7.6	2001	25 g	A ^e
	0 / 4	0 – 60.2	2005	50 g	B
PF < 1 yr	1 / 40 (2.5)	0.1 – 12.8	2001	25 g	A
	2 / 101 (2.0)	0.3 – 6.6	2002	25 g	A
	0 / 101	0 – 3.6	2003	50 g	A
	5 / 139 (3.6)	1.3 – 7.6	2004	50 g	B
	0 / 14	0 – 23.1	2005	50 g	B
Follow-up formulae, >1 yr	1 / 11 (9.1)	0.2 – 40.4	2004	50 g	B
	0 / 4	0 – 60.2	2005	50 g	B
Ice cream mixtures	0 / 87	0 – 4.1	2003	50 g	A
	0 / 2	0 – 84.1	2005	50 g	B
Flavored instant drink	0 / 33	0 – 10.6	2005	50 g	B
Instant pudding powder	0 / 12	0 – 26.5	2003	50 g	A
	1 / 25 (4.0)	0.1 – 19.8	2005	50 g	B
Dip	0 / 19	0 – 17.7	2005	50 g	B
Coffee creamer	0 / 4	0 – 60.2	2005	50 g	B
Dry cereals	6 / 123 (4.9)	2.0 – 9.6	2005	50 g	B
Raw minced meats					
Beef	2 / 141 (1.4)	0.2 – 9.6	2005	50 g	C1
Mixed beef / pork	5 / 50 (10.0)	3.6 – 20.5	2005	50 g	C1
Pork	0 / 16	0 – 20.6	2005	50 g	C1
Calf	0 / 10	0 – 30.9	2005	50 g	C1
Poultry	0 / 5	0 – 52.2	2005	50 g	C1
Dry vegetables	2 / 47 (4.2)	0.5 – 13.9	2005	25 g	E
Spices	1 / 28 (3.6)	0.1 – 17.8	2005	10 g	E
Raw cow's milk	0 / 51	0 – 7.0	2005	10 ml	C2
Human milk	0 / 7	0 – 41.0	2005	10 ml	C2
Human faeces	1 / 98 (1.0)	0 – 5.4	2005	10 g	D
Human skin	1 / 116 (0.9)	0 – 4.6	2005	Scrub glove	C2

Table 4. Continued

Sample	Prevalence of <i>Cronobacter</i> spp. # positive / total (%)	95% Confidence interval (%) ^a	Year of sampling	Sample size	Method
Baby bottles	0 / 86	0 – 4.2	2005	10 ml rinse	D
Bottle-nipples	0 / 95	0 – 3.8	2005	each	D

^a The 95% confidence interval was calculated, assuming the drawing process is a series of independent draws from a binomial distribution with the probability p .

^b The methods used are summarized in Table 1.

^c 3 samples were produced in The Netherlands, 2 in Ukraine, and 2 in Poland.

^d Samples collected from cargos and bulk goods and included 27 products intended for animal feed.

^e A, includes use of either method A1 or method A2.

Subtyping of Cronobacter spp.

In total, 35 isolates were obtained of which 34 were positively confirmed by PCR, the remaining isolate (from human faeces) had not been kept for molecular confirmation and PFGE subtyping. Of the total of 35 *Cronobacter* spp. isolates, 34 were subjected to PFGE subtyping. Because of degradation of genomic DNA, four of the 34 isolates subjected to the subtyping procedure (one of the six isolates from dry cereals, the isolate from the pudding powder and one of the two beef isolates and the human skin isolate) did not yield a result. Of the remaining 30 isolates that had been successfully subtyped, 28 isolates generated a unique XbaI restriction pattern each, whereas two isolates generated patterns that could not be distinguished (Figure 1). These two isolates were cultured from two samples of minced mixed beef and pork, which had been purchased from two different outlets of the same supermarket chain, visited one month after each other.

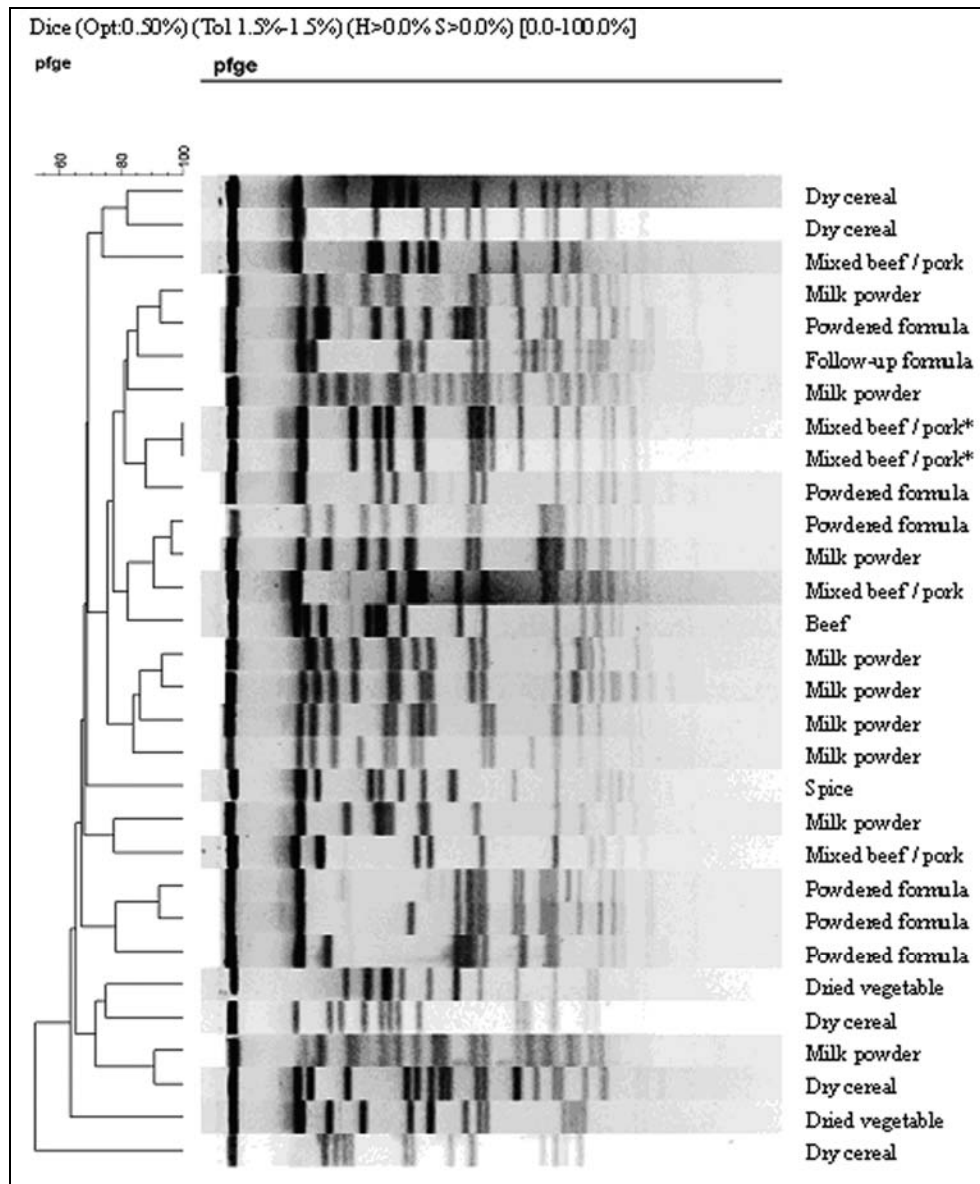


Figure 1. Cluster analysis of PFGE fingerprints of the 30 *Cronobacter* spp. isolates. Note that the PFGE fingerprints of the faeces isolate is not included in this Figure, as it was not analysed. * indicates the two generated patterns that could not be distinguished.

Enterobacteriaceae counts

Enterobacteriaceae counts were investigated in products sampled in 2005, including dry powders and cereals as well as raw minced meats, (Table 5). However, for three samples *Enterobacteriaceae* counts were not done.

A total of 223 dry powder samples had *Enterobacteriaceae* counts below the detection limit (≤ 10 CFU/g), and these included 5 of the 6 dry cereals samples that had been found positive for *Cronobacter* spp.. The sixth dry cereal sample (a type of muesli) that had been found positive for *Cronobacter* spp., contained *Enterobacteriaceae* at 15 CFU/g. The number of *Enterobacteriaceae* in the instant pudding powder that yielded *Cronobacter* spp. was below the detection limit. A wheat bran sample containing over 10,000 CFU/g *Enterobacteriaceae*, was negative for *Cronobacter* spp..

Enterobacteriaceae counts were found to be quite variable in the various minced meats. Out of 222 samples, 28 (13%) had counts below detection, 47 (21%) between 10 and 100 CFU/g, 80 (36%) between 100 and 1,000 CFU/g, 43 (19%) between 1,000 and 10,000 CFU/g, and 24 (11%) over 10,000 CFU/g. For the seven meat samples positive for *Cronobacter* spp., the *Enterobacteriaceae* counts were: ≤ 10 CFU/g, 55 CFU/g, 91 CFU/g, 110 CFU/g, 250 CFU/g, 680 CFU/g, and 2,000 CFU/g.

Table 5. Enumeration of *Enterobacteriaceae* in products collected in 2005

Enterobacteriaceae (CFU/g)	Dry products ^a	Raw minced meats
10 ^b	223	28
10 - 100	1	47
100 – 1,000	0	80
1,000 – 10,000	0	43
> 10,000	1 ^c	24

^a All dry samples collected in 2005, except for two samples of flavored instant drinks and one sample of coffee creamer.

^b Detection limit.

^c Wheat bran.

Discussion

Isolation and confirmation methods

An investigation of the occurrence of *Cronobacter* spp. and its prevalence in a variety of samples of different origin was carried out in The Netherlands between the years 2001 and 2005. During this study, various traditional and innovative isolation and (molecular) detection/confirmation methods were used, following in a timely way the ongoing developments and improvements in this field. Several isolation and (molecular) detection methods have been developed over the past years aiming to detect and type *Cronobacter* spp., in particular in powdered infant formulae (13, 25, 33, 34, 45, 46, 51, 52, 65, 70). Regarding detection, several chromogenic and fluorogenic agar media have been described for *Cronobacter* spp. (34, 52, 68), mainly based on its unique feature, being the presence of α -glucosidase, as compared to other *Enterobacter* species (60). The results obtained with the specific real-time PCR method, which we developed to further improve detection and definite confirmation, indicated that it represents a reliable confirmation test (Table 3) which is able to differentiate all *Cronobacter* spp. from many other *Enterobacteriaceae*. The PFGE subtyping method also showed to be a reliable method to subtype *Cronobacter* spp. isolates. It may prove useful for tracing infections to the source in sporadic cases or outbreak investigations, or for the identification of contamination sources in food manufacturing operations.

While most of the recent studies focused on the development of molecular detection/confirmation of *Cronobacter* spp., further research should focus on improving the enrichment step, ideally providing a specific and sensitive enrichment procedure, as this so far remains an important bottle-neck (13, 35, 37, 41, 64). This is a challenge considering the relative low levels of this micro-organism that are found in foods and the fact that it is present among other micro-organisms. The ISO procedure for milk and milk products published in 2006 (33) uses a selective enrichment procedure part of which was applied in the current study. In the selective enrichment step, mLST broth is used (25) in combination with a relatively high incubation temperature to suppress potential competitors, as in our Methods B, C1, and C2 (Table 2). However, it has been demonstrated that some

Cronobacter spp. isolates do not grow well in mLST (37); therefore recently, a new enrichment broth for *Cronobacter* spp., *Cronobacter* screening broth (CSB), has been developed (35).

Occurrence and prevalence of Cronobacter spp.

The results obtained (Table 4) show that also in The Netherlands, this opportunistic pathogen may be present in a variety of foods and feeds, either being produced within the country or reaching the national market via import from other countries, and substantiates humans as potential sources for contamination.

The prevalence of *Cronobacter* spp. on human skin and in fecal samples was rather low (~1%), with the micro-organism being isolated once from a faecal sample of a female adult, as well as once from skin particles collected from another female adult. Notably, the micro-organism was not detected in the seven human breast milk samples investigated. Farmer *et al.*, (1980) previously reported isolating the micro-organism from various clinical sources, including a child's stool (21). More recent studies also reported that *Cronobacter* spp. could be isolated from the stool of a child with diarrhea (66) and from stool samples of hospitalised infants (0.5%) and patients in the age group 61-70 years (6.5%) (48). While the occurrence of *Cronobacter* spp. in human faeces in the current investigation is not a new observation, its presence in skin scrapings is. These observations highlight that the micro-organism can be present on or in humans, making them a potential source of contamination both during product manufacture as well as food preparation.

Our study of *Cronobacter* spp. in a variety of food and feed products, with prevalences ranging from 1.4 to 10.0% of samples where positives were observed, confirms and further extend the results of previous studies on the occurrence of the micro-organism. Though prevalences have not been systematically reported, previously, *Cronobacter* spp. has been isolated from powdered infant formulae and milk powders (8, 10, 18-20, 36, 37, 57, 59) as well as from a variety of other foods (3, 22, 26), food factory environments (43, 58, 71), consumer homes (45), and environmental samples, including soil (15) and insects (27, 49,

53). In a recent review (22), the widespread occurrence of *Cronobacter* spp. in food and beverages other than infant formula was documented, noting that the presence of the micro-organism had been confirmed in several foods and food ingredients from plant origin, such as cereals, fruits and vegetables, legume products, herbs and spices, as well as in foods of animal origin such as milk, meat and fish and products made from these foods. The spectrum of food contaminated with *Cronobacter* spp. seems to cover both raw/fresh and processed food (being dried, frozen, ready-to-eat, fermented and cooked food products), as well as water suitable for food preparation.

Overseeing the variety of sources of *Cronobacter* spp. identified to-date, it is not apparent that there is one single, most important natural habitat for this micro-organism. In the course of previous research into the occurrence of *Cronobacter* spp. in dry environments, a range of other *Enterobacteriaceae* were isolated, such as *Klebsiella* spp., *Citrobacter* spp., *Erwinia* spp., *Enterobacter cloacae*, *Escherichia coli*, *Enterobacter agglomerans* (*Pantoea agglomerans*) and *Serratia* spp. (45). Based on the habitat profile of these possibly co-inhabiting micro-organisms and their physiological features, it is likely that these micro-organisms (and may be thus also *Cronobacter* spp.) are associated with the phytic flora (56, 69). Recent research indeed showed that *Cronobacter* spp. could be isolated from vegetable products (22, 42, 48, 69) and the vegetable drawer of domestic refrigerators (2.2% out of 137 refrigerators) (47). Our results extend these observations, as *Cronobacter* spp. was isolated from dried vegetables (4.2%) and spices (3.6%) (Table 4).

So far only powdered infant formulae have been definitely attributed to cases of *Cronobacter* spp. infection, and most of the investigative attention has been focused on these products. Nevertheless, actual data on prevalence and concentration are scarce. Concerning prevalence in powdered formulae, some reported prevalence figures in positive batches are about 1% and 3% (data quoted in FAO/WHO, 2008) (20), 6.7% (74) and less recently 14% (59). Relating to concentration, operations with good practices in place should be able to achieve levels of *Cronobacter* spp. of 10^{-4} CFU/g or better (6), while levels in contaminated products may be of the order of 10^{-3} CFU/g (12, 19). The current study did not determine the actual levels of *Cronobacter* spp. in various sources; it only investigated its prevalence. Following prevalence data were obtained (with prevalence

expressed per specific investigation, as indicated in Table 4): 4.1% for milk powders, 2.0-3.6% for powdered formulae intended for consumers < 1 year, 9.1% for follow up formula intended for children > 1 year, 4.0% for instant pudding powders, 4.9% for dry cereals, 1.4 to 10.0% for raw minced meats, 4.2% for dried vegetables and 3.6% for spices. Notably, in our specific survey some samples from milk powders, powdered formulae, instant pudding powders, and raw minced meats tested negative for *Cronobacter* spp. as did dry powder products such as ice-cream mixtures, instant drinks, dips and coffee creamers. It is apparent that prevalence rates of *Cronobacter* spp. in powdered formulae and other foods/feeds are in the similar low range as reported for PIF as mentioned above and concur with prevalence estimates of Jung and Park of 5 to 10% for agricultural food components such as brown rice and tomatoes (42). The relatively low frequency that *Cronobacter* spp. were detected in our study and others may be related to limitations of (selective) enrichment and isolation methods used, but may as well reflect that that the level at which *Cronobacter* spp. actually is present in various products/samples indeed is very low.

In powdered products and cereal samples taken in 2005 and raw minced meat samples, the *Enterobacteriaceae* levels were determined to possibly link product hygiene to the occurrence of *Cronobacter* spp.. Table 5 clearly shows that most of the dry products (including milk powders) contained low levels of *Enterobacteriaceae* (< 10 CFU/g). The apparently sound hygiene status of the products investigated is important because milk powders represent a basic ingredient in the majority of dry powdered foods, including dry-mixed powdered infant formulae and follow-up formulae. All these products (being powdered infant formula, follow-up formula, and special diet formula) thus are key to the health and well-being of infants and young children and our finding that the prevalence of *Cronobacter* spp. in these products is relatively infrequent or absent and that levels of *Enterobacteriaceae* in general were found to be below the detection limit, are positive indicators of the product safety and hygiene.

Of the two cereal samples showing levels of *Enterobacteriaceae* above detection (Table 5), the one with the lowest level of contamination (a muesli product) was positive for

Cronobacter spp., while the sample with the highest level of contamination had been found negative. Indeed, it has been reported before that although *Enterobacteriaceae* may be a good general hygiene indicator and are promoted as such by Codex Alimentarius for powdered infant formulae (6), they do not necessarily provide any insights in the occurrence of *Cronobacter* spp. (17).

Considering Cronobacter spp. as a hazard in food manufacture

This publication is one of a series of studies over the last several years by many investigators all dedicated to the elucidation of the occurrence and prevalence of *Cronobacter* spp.. Overall, a picture has emerged that the pathogen is widespread in nature, factory environments and people's homes, occurring in foods and food ingredients, soil, insects, in/on humans and utensils, etc. Thus, there may be many sources and vectors that can play a role in the spread and ultimate contamination of foods by the opportunistic pathogen. To consider all such sources and vectors may not be practical nor necessary in assuring food safety for the relevant consumers. There may be relatively fewer relevant sources and vectors to control, considering the actual consumer risk the micro-organism poses when present in a food. Consumer risk is determined by a combination of factors, including sensitivity of the consumer and the actual exposure of consumers to the pathogen through consumption.

Risk assessments or comparable studies developed under the auspices of the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) (18, 19) and of the European Food Safety Authority (EFSA) (16) have shown that *Cronobacter* spp. poses different levels of risk of infection for different consumer age groups. Among infants, those at greatest risk of infection are neonates (< 28 days), particularly pre-term, low-birth weight (< 2500 g), and immunocompromised infants and those less than two months of age and also infants of HIV-positive mothers (20). Cases occurring in this age-group are relatively well confirmed and documented. Also other infants below the age of 1 year are at particular risk, though solid case reports are fewer. Above that age limit, confirmed and documented cases are extremely sparse at the moment. To limit consumer risk, tightest control is needed for products intended for the consumers at

highest risk, i.e. for powdered formulae intended for very young infants. Recent guidance developed by Codex Alimentarius (6) therefore contains microbiological criteria for product safety related to *Cronobacter* spp. in powdered infant formula, formula for special medical purposes and human milk fortifiers.

Considering consumer exposure and risk, there are various routes by which *Cronobacter* spp. can end up in powdered formulae (6), two important ones relate to food manufacture:

- 1) through ingredients added in dry mixing operations during the manufacturing of powdered formula,
- 2) through contamination of the formula from the processing environment in the steps during or following the drying.

Whilst control of contamination of ingredients to some extent is possible by applying some form of thermal treatment during their production and assuring prevention of post treatment re-contamination, adherence to strict microbiological requirements and thorough selection and purchase procedures (12), current technology cannot completely eliminate this micro-organism from the manufacturing environment and prevent it gaining access to the processing line and final food product. Therefore, Codex provided guidance for the establishment of monitoring programs for *Cronobacter* spp. in high hygiene processing areas and in powdered formula preparation units (6).

A recent Microbiological Risk Assessment conducted under the auspices of the Food and Agricultural Organization of the United Nation and the World Health Organization (19) has shown that factory hygiene may be a factor contributing to the risk posed by *Cronobacter* spp. in powdered infant formulae as it impacts on the ultimate consumer exposure. The current study once more highlighted the possibility for humans as vectors for the micro-organism. These results suggest that also the hygiene awareness of personnel need to be taken into consideration to prevent or reduce the prevalence of *Cronobacter* spp. in the factory environments. Improvements in processing conditions such as very strict management of water in high hygiene zones, may help further reduce the level *Enterobacteriaceae*, including *Cronobacter* spp.. *Cronobacter* spp. are present in the milk powder processing environment (43, 54, 58), probably due to their resistance to desiccation

(5, 7, 14). Presence of water could lead to outgrowth, since strains of *Cronobacter* spp. have relatively short lag times and high specific growth rates at temperatures commonly found in those environments (38, 44). Due to its prevalence in dry environments, such as dust samples in factories and households (43), total elimination may be difficult, if not impossible, to achieve. Therefore and because contamination and proliferation of *Cronobacter* spp. in prepared formula prepared in hospitals and homes has been recognised as a key risk factor for sensitive consumers as well (19, 67), proper bottle preparation and handling practices are critical in order to sufficiently control the risk. It is noteworthy that used baby bottles and bottle-nipples were found to be negative for *Cronobacter* spp., which may indicate an appropriate level of hygiene at the day care facilities and homes sampled from. Nevertheless, caregivers should be mindful of these risk factors in preparing and handling infant formulae since *Cronobacter* spp. cells are able to survive over a long period in dry powdered infant formulae (7, 14) and can grow rapidly (44), once the powder has been reconstituted.

Conclusion

Cronobacter spp. is not a pathogen for the majority of the population, but rather a concern for specific sub-populations in the public. Measures for prevention should consider the actual target sub-population of a food as well as the consumer exposure due to the presence of the micro-organism in food. For relevant foods and sub-populations, strict control of contamination from food ingredients, during manufacture and processing as well as final preparation is paramount to limit the consumer risk. One should consider using sterile liquid formula, for those groups at greatest risk.

Our results do not point to a specific niche of *Cronobacter* spp., but rather extend already available evidence that *Cronobacter* spp. can be found in many different food products, food environments as well as on and in humans, making all these potential contamination sources of the micro-organism for foods during manufacture and preparation. The concentration and prevalence of *Cronobacter* spp. is not equal in all the potential sources and that not all sources are linked to the production and preparation of food for the

consumers that are at most risk. These foods are milk powder and infant formulae and the consumers at most risk are neonates (< 28 days), particular pre-term, low-birth weight (< 2500 g), and immuno-compromised infants, and those less than two months of age and also infants of HIV-positive mothers. Thus, it is a key that for these relevant foods and for these particular consumers, adequate control measures are being implemented that take into account the widespread occurrence of *Cronobacter* spp. as well as the contamination routes and growth opportunities that are still presented to the micro-organism in some of current practice. Over the past years several improvements have been made to the (pre)-enrichment step and microbiological culture methods for the recovery of *Cronobacter* spp. and this has helped to better investigate the prevalence in various locations.

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Chapter 6



Effect of pre-culturing conditions on lag time and specific growth rate of *Cronobacter* spp. in reconstituted powdered infant formula

Abstract

Cronobacter spp. can be present, although in low levels, in dry powdered infant formulae and it has been linked to cases of meningitis in neonates, especially those born prematurely. In order to prevent illness, product contamination at manufacture, during preparation, and also growth after reconstitution has to be minimised using appropriate control measures. In this publication several determinants of the growth of *Cronobacter spp.* in reconstituted infant formula are reported. The following key growth parameters were determined: lag time, specific growth rate and maximum population density. Cells were harvested at different phases of growth, and spiked into powdered infant formula. After reconstitution in sterile water, *Cronobacter spp.* was able to grow at temperatures between 8 and 47 °C. The estimated optimal growth temperature was 39.4 °C, whereas the optimal specific growth rate was 2.31 h⁻¹. The effect of temperature on the specific growth rate was described using two secondary growth models. The resulting minimum – and maximum temperature estimated with the Secondary Rosso equation were 3.6 °C and 47.6 °C, respectively. The estimated lag time varied from 83.3 ± 18.7 h at 10 °C to 1.73 ± 0.43 h at 37 °C and could be described with the hyperbolic model and reciprocal square root relation. Cells harvested at different phases of growth did not exhibit any significant difference in either specific growth rate or lag time. Strains did not have different lag times and lag times were short taking into account that the cells had spent several (3-10) days in dry powdered infant formula. The growth rates and lag times at various temperatures obtained in this study may help in calculation of the period for which reconstituted infant formula can be stored at a specific temperature without detrimental impact on health.

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* Throughout this thesis the designation *Cronobacter spp.* has been used to indicate the taxonomic change.

Introduction

Cronobacter spp. is a motile peritrichous, Gram-negative rod that occasionally causes neonatal meningitis and sepsis, with mortality rates of 40 to 80% (4). The recovery of *Cronobacter* spp. from samples of commercially available dry powdered infant formulae has been reported (5, 9, 10). *Cronobacter* spp. in infant formulae have been associated with outbreaks of meningitis, sepsis and necrotizing enterocolitis in preterm and full term infants, particularly those with predisposing medical conditions (3). Although the levels of *Cronobacter* spp. occurring in dry powdered infant formulae are generally very low, reconstituted infant formula is a good medium for growth. When present in dry formula *Cronobacter* spp. may grow during preparation, cooling, storage and holding of the bottles, increasing the probability of illness. Occasional contamination of the dried infant formula during manufacture is a source of the micro-organism occurring in reconstituted product. However, as *Cronobacter* spp. has been detected in various other dry environments (8), contamination may also occur during reconstitution of the dried infant formula in hospitals or at home.

In order to prevent illness, product contamination at manufacture and/or during preparation and growth after reconstitution has to be minimised using appropriate control measures. Mathematical models are useful tools to evaluate the effectiveness of control measures. Depending on the source and the history of contaminating bacterial cells, which influence their physiological state, and the suitability of the product to sustain their growth, i.e. the product's (intrinsic) conditions and the environmental (extrinsic) conditions, microbial cells will either start to grow immediately or show a distinct phase of no apparent growth (the lag phase). In the case of *Cronobacter* spp. cells, reconstituted infant formulae offer rich growth environments that allow immediate proliferation provided that the cells are in a sound physiological state, that the external conditions (mainly temperature) are favourable and that there is sufficient time for growth. Should lag times be apparent before growth, then this may be a result of an injured state of the *Cronobacter* spp. cells from which they may gradually recover, as is evidenced by the start of cell proliferation (17). Baranyi & Roberts (1) emphasized that the lag time is a period of adjustments to the new environment during which only intracellular conditions change. Growth models can simulate growth

after reconstitution and the effect of key intrinsic or extrinsic conditions can be determined. To develop growth models, insight into parameters describing growth of the micro-organism, such as the lag time and specific growth rate, is required.

This study describes the effects of a number of pre-culturing conditions on key growth parameters for *Cronobacter* spp. growing in reconstituted (with sterile water) powdered infant formula. Furthermore, the effects of temperature on specific growth rate and lag time were quantified and compared with literature values. Viable counts were used to construct growth curves that were used to derive the key growth parameters by curve fitting using the Modified Gompertz equation as primary growth model (20). During the secondary modelling step the Square root Ratkowsky model (13) and the Secondary Rosso model (14) were fitted to the estimates of the specific growth rates at various temperatures. Likewise, the lag time data were fitted, using the logarithm of the inverse of the Ratkowsky model and the hyperbolic equation (19). The resulting parameters permit useful predictions of the growth of *Cronobacter* spp. in reconstituted infant formula and aid in the design of effective control measures to reduce exposure of susceptible consumers in both hospital and household settings.

Materials and methods

Organisms

Four *Cronobacter* isolates were used in this study. Strain MM9 was isolated from milk powder and strain MC10 was isolated from a patient. Both isolates were obtained from Dr. H.L. Muytjens, University Medical Centre (UMC) St. Radboud, Nijmegen, The Netherlands. Strain 94 (S94) was isolated from a sample of a vacuum cleaner bag obtained from a domestic environment (8). *Cronobacter sakazakii* ATCC 29544 (4) was the reference strain. Stock cultures were maintained at –20 °C in cryo vials (Greiner Bio-one GmbH, Frickenhausen, Germany), containing 0.7 ml of a stationary-phase culture suspension in Brain Heart Infusion (BHI; Becton Dickinson and Co., 38800 Le Pont de Claix, France) broth with 0.3 ml 87% glycerol (Fluka-Chemica, GmbH CH9471 Buchs,

Switzerland). Characterization of these isolates together with 70 other *Cronobacter* isolates, using the pulsed field electrophoresis technique (15) revealed a large variability in molecular fingerprints. Specific groups could not be distinguished from the dendrograms (results not shown).

Preparation bacterial suspension

Strain ATCC 29544 was cultured by transferring 250 μ l of the stock culture into 250 ml BHI followed by incubation at 37 °C. Cells were incubated for 3, 6, 16, 24 and 72 hours to obtain respectively the exponential phase, early stationary phase, middle stationary phase, stationary phase and late stationary phase cells. Strains MC10, MM9 and S94 were incubated at 37 °C for 18 hours to obtain middle stationary cells. In order to obtain enough cells in the lag phase, 1 ml of a stock culture of ATCC 29544 from the -20 °C was diluted in 2 ml glycerol, whereupon the suspension was centrifuged (Hermle Z 231M, B. Hermle GmbH & Co., D-7209 Gosheim, Germany) for 10 minutes at 10,000 x g. Cells harvested were transferred into 30 ml BHI and incubated for 1.5 hour at 37 °C.

In order to obtain cells for spiking dry infant formula, these BHI grown cultures were centrifuged for 5 minutes at 20 °C at 2958 x g (MSE, Mistral 3000i, Leicester, United Kingdom). Cells were washed twice in 1% physiological salt solution and subsequently suspended in 30 ml 1% physiological salt solution for the lag phase cells or 250 ml 1% physiological salt solution, resulting in a cell suspension of about 10^4 CFU/ml for lag phase grown cells and between 10^5 and 10^7 CFU/ml for the other growth phases.

Spiking of the infant formula

Infant formula was bought in local shops; the numbers of the viable bacteria in the infant formula were sufficiently low to prevent them from influencing the growth of the spiked cells (data not shown). The obtained bacterial suspension was sprayed on commercial dry infant formula (1:50 (w/w)), using a perfume sprayer (Designed by Gérard Brinard, DA Drogisterij, Leusden, The Netherlands). The final bacterial concentration 3-4 days after

spiking was 10^2 - 10^4 CFU/g of dry powder and the infant formula maintained a water activity of $a_w < 0.22$. All growth experiments were performed within 10 days after spiking the infant formula.

Design

The initial estimates of the lowest temperature supporting growth (T_{\min}), highest temperature supporting growth (T_{\max}) and the specific growth rate (μ_{opt}) at optimal growth temperature (T_{opt}), shown in Table 1, were used to predict the specific growth rates at various temperatures. The lag time at each temperature was initially estimated by the k value, which is the product of the specific growth rate (μ_m) and lag time (λ) and which is known to have a large variability, but can be considered constant over a wide range of temperatures (18).

Table 1. Parameters for growth of *Cronobacter* spp. in reconstituted infant formula: initial estimates based on published data

Parameter	Initial estimate	Reference (s)
T_{\min}	5.5 °C	(10)
T_{\max}	47 °C	(4, 6)
T_{opt}	37 °C	(6)
μ_{opt}	2.5 h ⁻¹	(6)
k	3.75	(7)

In order to determine appropriate sampling times and dilutions for plate counting, the number of micro-organisms in each sample and at every sampling time was roughly predicted using the exponential growth function ($N_{(t)} = N_{(0)} e^{(\mu_m * t)}$) and the secondary Rosso equation (Equation 3), using the design values as shown in Table 1. Here N_t is the number of micro-organisms [CFU/ml] at time t [h], N_0 is the number of micro-organisms at time of inoculation, μ_m is the specific growth rate [h⁻¹], t is the time [h].

Growth experiments

To prepare samples for growth experiments, ten-gram portions of spiked infant formula were reconstituted in 100 ml sterilised tap water. In the experiments to determine the effect of various growth phases, bottles with strain ATCC 29544 were incubated as follows (the number of bottles incubated is given in parentheses: 10 °C (n=9); 21 °C (n=11); 29 °C (n=14); 37 °C (n=14). Middle stationary phase grown cells of strain ATCC 29544 were additionally used to assess (in duplicate) growth in reconstituted infant formula at the following temperatures (°C): 8, 14, 38, 39, 41, 43, 45, 46, 47, 48, 49, 50. Middle stationary phase grown cells of strains MM9, MC10 and S94 were incubated only at 29 and 37 °C.

Growth of *Cronobacter* spp. was measured at various time intervals, depending on the temperature of incubation, appropriate dilutions were made in Peptone Saline Solution (NaCl 8.5 g/L and neutralised Bacteriological Peptone 1g/L from Oxoid, Basingstoke, England). Samples were surface plated onto tryptone soy agar (TSA; Ltd., Basingstoke, Hampshire, England) with a spiral plater (Eddy Jet, IUL instruments, I.K.S. bv., Leerdam, The Netherlands). Inoculated plates were incubated for 20 to 24 h at 37 °C before manual counting.

Data analysis

For describing the evolution of the microbial count with time, a primary model was used. The three kinetic parameters, namely lag time, specific growth rate and maximum population density were estimated by fitting with the Modified Gompertz equation (Equation 1). This resulted in estimates for the lag time (λ ; [h]), specific growth rate (μ_m ; [h⁻¹]) and the asymptotic value (A ; [-]) (20) at the tested temperature for each growth curve.

$$\text{Ln}\left(\frac{N_t}{N_0}\right) = A \exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

In order to obtain reliable estimates for the growth parameters, experimental growth curves had to meet the following requirements:

- 1) at least 2 data points should fall within the lag-time, unless the lag time was shorter than 2 hours,
- 2) for data points within the exponential phase, there should be at least 3 data points over a range of 3-hours and 3-logs,
- 3) 3 data points should be in the stationary phase at least 1-hour apart.

Growth curves that did not meet these requirements were excluded.

A Bélehrádek type model (Equation 2), also known as the (expanded) square root model of Ratkowsky (13), was used to describe the relation between the specific growth rate and the temperature. This model contains four parameters of which two are easily interpretable, T_{\min} and T_{\max} .

If

$$T_{\min} < T < T_{\max} \quad (2)$$

then

$$\mu_m(T) = \left(b(T - T_{\min}) \left\{ 1 - \exp[-c(T - T_{\max})] \right\} \right)^2$$

$$\text{and if } \begin{cases} T \leq T_{\min} \\ T \geq T_{\max} \end{cases} \text{ then } \mu_m = 0$$

where T_{\min} is the extrapolated minimum temperature [°C] at which the specific growth rate (μ_m : [h⁻¹]) is zero, T_{\max} is the extrapolated maximum temperature at which $\mu_m = 0$, and b [°C⁻¹ h^{-0.5}], and c [°C⁻¹] are so-called Ratkowsky parameters (13).

The secondary growth model of Rosso *et al.*, (14) (Equation 3), was used as well to describe the effect of temperature on growth rate. This model contains all four interpretable parameters μ_{opt} , T_{\min} , T_{\max} and T_{opt} .

If

$$T_{\min} < T < T_{\max} \quad (3)$$

then

$$\mu_m(T) = \left(\frac{(T - T_{\max})(T - T_{\min})^2}{[(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T)](T_{\text{opt}} - T_{\min})} \right)^{\mu_{\text{opt}}}$$

$$\text{and if } \begin{cases} T \leq T_{\min} \\ T \geq T_{\max} \end{cases} \text{ then } \mu_m = 0$$

where T_{\min} and T_{\max} are defined in equation 2, T_{opt} [°C] is the temperature at which the specific growth rate μ_m [h⁻¹] is optimal, and μ_{opt} is the μ_m at optimal temperature.

The logarithm of the inverse of the secondary Ratkowsky model (Equation 4) and the hyperbolic equation (Equation 5) were used (19) to describe the lag time temperature relation.

$$\ln(\lambda(T)) = \ln \left[\left(b(T - T_{\min}) \left\{ 1 - \exp \left[c(T - T_{\max}) \right] \right\} \right)^{-2} \right] \quad (4)$$

where b [°C⁻¹ h^{-0.5}], and c [°C⁻¹] are the so-called Ratkowsky parameters. The T_{\min} and T_{\max} values were assumed to be equal to the T_{\min} and T_{\max} of equation 3 (Secondary Rosso growth model), describing the specific growth rate.

$$\ln(\lambda) = \frac{p}{T - q} \quad (5)$$

where p is a measure for the decrease of the lag time when the temperature is increased and q is the temperature at which the lag time is infinite (no growth). The q value is comparable to T_{\min} .

Statistical analysis

In order to determine whether pre-culturing conditions have a significant effect on lag times and/ or specific growth rates, the data obtained with strain ATCC 29544 at 10, 21, 29 and 37 °C were subjected to univariate analysis of variance. Lag time data were log transformed and specific growth rate data were square root transformed in order to obtain homogeneity of variance. A significance level of 5% was used. All data analyses were performed using SPSS (SPSS release 11.5 for Microsoft Windows 95/98/NT/2000; SPSS Inc., Chicago, U.S.A). Fitting was done by minimizing the residual sum of squares (RSS) with both the solver function in Excel and Table curve 2D, windows 2.03, for verification. Standard deviations were calculated with Excel and are reported as plus or minus the mean.

Results

Determination of growth parameters, using pre-cultured middle stationary ATCC 29544 cells

In order to design the experiments optimally and to obtain growth curves meeting the specified requirements, the course of each individual growth curve of *Cronobacter* spp. at 10, 21, 29, and 37 °C was predicted based on published growth parameters (see Table 1, for initial estimates). Examples of the design growth curve at 21 °C and 37 °C are shown in Figures 1A and 1B, respectively.

These figures show, furthermore, the resulting experimental count data of growth in reconstituted, contaminated infant formula. The modified Gompertz equation (Equation 1) was fitted to the observed number of micro-organisms in time, resulting in estimates for the lag time, specific growth rate, asymptote and the initial number of organisms (19). For the growth curves at 10 °C and 29 °C comparable results were obtained (data not shown). Variations in the initial number of micro-organisms at time zero may have been due to a gradual decline of the cell number in the dry infant formula.

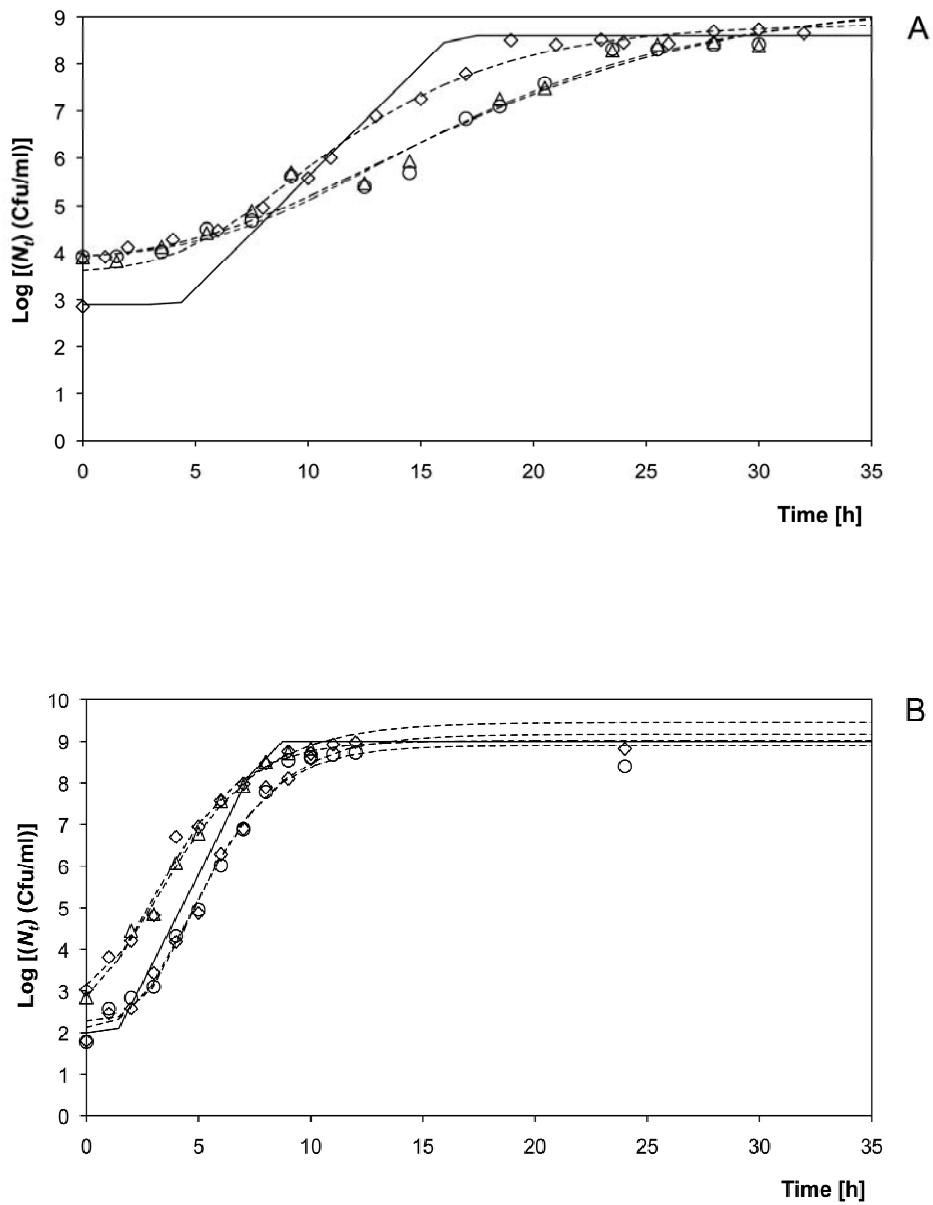


Figure 1. Predicted and measured growth curves at 21 °C (A) and 37 °C (B) of *Cronobacter sakazakii* ATCC 29544, pre-cultured to middle stationary state. The different symbols indicate different replicate experiments. The straight line represents the design growth curve at 21 °C, using the cardinal values as shown in Table 1 under “initial estimate”; Dotted lines represent fits of the Modified Gompertz equation to each single experiment.

Effects of the physiological growth phase on μ_m and λ

Six different pre-cultures of the reference strain, ATCC 29544 were prepared to yield a variety of physiological growth phases at the moment of spiking of the powdered infant formula. After reconstitution with sterile tap water, all six types were incubated with various replications at 10, 21, 29 and 37 °C to represent a temperature range relevant for reconstituted infant formulae. Growth was observed in all cases and at every temperature. Temperature had a marked effect on both the specific growth rate (μ_m) and the lag phase (λ) (Figures 2A and 2B). With increasing temperature, μ_m strongly increased. Values for specific growth rates varied from $0.12 \pm 0.04 \text{ h}^{-1}$ at 10 °C to $2.29 \pm 0.45 \text{ h}^{-1}$ at 37 °C, and no apparent effect of the physiological state was found. In Figure 2B it is shown that the lag time decreased with increasing temperature and that there was also no apparent effect of the various physiological growth phases on the lag time.

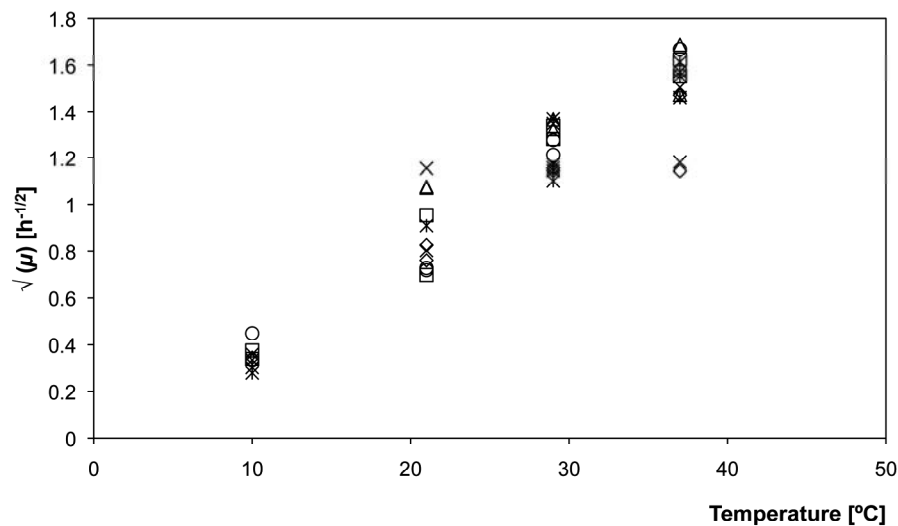


Figure 2A. Square root of specific growth rate data for various physiological growth states of strain ATCC 29544 as function of temperature; × (◇) SKDMHJURZ Q FH0V exponential phase cells; ◇: early stationary phase cells; *: middle stationary phase cells; o: stationary phase cells; □: late stationary phase cells.

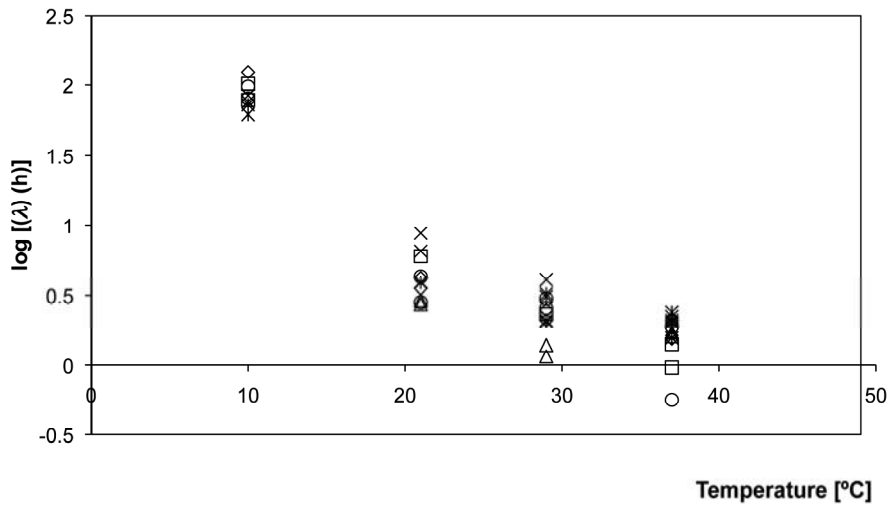


Figure 2B. Log lag time for various physiological growth states of ATCC 29544 as a function of the temperature; ×: lag phase cells; △: exponential phase cells; ◇: early stationary phase cells; *: middle stationary phase cells; o: stationary phase cells; □: late stationary phase cells.

Estimates of the specific growth rates and lag times for the various pre-culturing conditions (lag phase, exponential phase, early stationary, middle stationary phase and late stationary phase) of strain ATCC 29544 as shown in Figures 2A and 2B were analysed with the univariate analysis of variance test. As depicted in Table 2, this resulted in p -values > 0.05 , except for the lag time at 21 °C ($p=0.048$). However, this value can be considered as borderline significance. This test corroborates the visual observation (Figures 2A and 2B) that the cell history had no significant effect on either the specific growth rate or on the lag time during subsequent cultivation in reconstituted infant formula. Furthermore, statistical analysis showed that the k -value (product of λ and μ_m) was also not significantly influenced by the cell history (Table 2).

Effects of the strain variability on μ_m and λ

The effect of strain variability on the growth parameters was studied by growing three other strains MC10, MM9 and S94, pre-cultured to middle stationary phase, at 29 and 37 °C. It is shown in Figures 3 and 4 that neither the lag time nor the maximum specific growth rate was significantly different for strains of different origin.

Table 2. Statistical evaluation (*p*-values) of the univariate analysis of variance for the effects of the physiological growth phase of *Cronobacter sakazakii* ATCC 29544 cells on lag time, specific growth rate and the product of both (*k*) at 10, 21, 29 and 37 °C. Bold value indicates *p*-value < 0.05 meaning a significant effect of the physiological state on the variable

Parameter	Value at:			
	10 °C	21 °C	29 °C	37 °C
Lag time (λ) [h]	0.092	0.048	0.629	0.254
Specific growth rate (μ_m) [h ⁻¹]	0.461	0.295	0.314	0.166
<i>k</i> -value ($\lambda \times \mu_m$) [-]	0.407	0.408	0.539	0.512

Estimations of growth parameters

Since statistical analysis showed that the physiological growth phases of strain ATCC 29544 at 10, 21, 29 and 37 °C did not significantly influence the specific growth rates and lag times, further experiments were performed over a wide range of temperatures, from 8 up to 50 °C with middle stationary pre-cultured cells of strain ATCC 29544 only. Although growth was observed up to 47 °C, no reliable estimates of the specific growth rate and lag time could be derived from the models at that temperature as the growth curves did not meet the requirements as formulated in the materials and method section. All of the estimated specific growth rates were combined and modelled with equation 2 and equation 3 as shown in Figure 3, where the square root of the specific growth rate ($\sqrt{\mu_m}$) is plotted as function of the temperature. The secondary growth parameters derived from optimal fits are shown in Table 3 and 4.

Table 3. Parameter values for the effect of the temperature on the specific growth rate (μ_m) for *Cronobacter* spp. in reconstituted infant formula resulting from fits by the Ratkowsky Secondary growth model (Equation 2)

Parameter	Estimate	95% Confidence interval	Dimensions
T_{\min}	2.19	-0.26 - 4.64	°C
T_{\max}	48.9	47.9 - 49.9	°C
b	0.047	0.0407 - 0.0521	°C ⁻¹ h ^{-0.5}
c	0.239	0.144 - 0.335	°C ⁻¹
RSS	1.28		

Table 4. Parameter values for the effect of the temperature on the specific growth rate (μ_m) for *Cronobacter* spp. in reconstituted infant formula resulting from fits by the Rosso secondary growth (Equation 3)

Parameter	Estimate	95% Confidence interval	Dimensions
T_{\min}	3.60	1.42 - 5.79	°C
T_{\max}	47.6	47.1 - 48.2	°C
T_{opt}	39.4	38.1- 40.7	°C
μ_{opt}	2.31	2.13 - 2.48	h ⁻¹
RSS	1.31		

From Figure 3 it is apparent that the differences between the fits of these models are smaller than the experimental variability. The residual sum of squares (RSS) for the secondary Rosso model (Equation 3) was 1.31 and for the square root Ratkowsky model (Equation 2) was 1.28. Although the RSS was slightly higher, the secondary Rosso model was used for further evaluation since it consists of four parameters that all have a biological meaning and can be interpreted as such. Transforming the square root μ_m data from Figure 3 back to μ_m , it can be seen that the specific growth rate of *Cronobacter* spp. varied from 0.115 h⁻¹ ($\sqrt{\mu_m} = 0.339 \text{ h}^{-0.5}$) at 10 °C to 1.113 h⁻¹ ($\sqrt{\mu_m} = 1.063 \text{ h}^{-0.5}$) at 46 °C, and 2.242 h⁻¹ ($\sqrt{\mu_m} = 1.498 \text{ h}^{-0.5}$) at 37 °C. For comparison the experimental specific growth data

measured by Iversen *et al.*, (6) and Nazarowec-White *et al.*, (10) have been transformed and included in Figure 3.

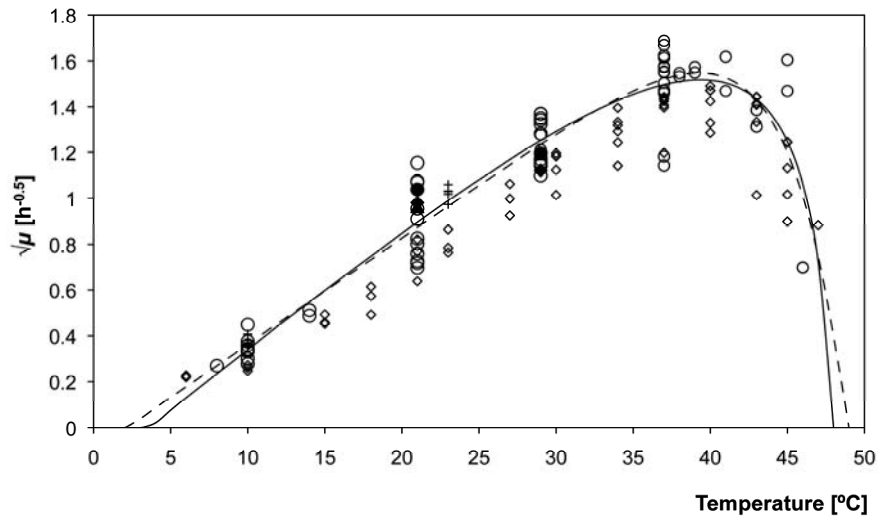


Figure 3. Square root of measured and fitted specific growth rates as function of the temperature. ○: growth rates of ATCC 29544 pre-cultures to various growth states as estimated by the fit of the Modified Gompertz model to each individual growth curve. Also for middle stationary phase grown cells of ● = MM9, ◆ = S94 and ▲ = MC10. +: growth rates published by Nazarowec-White *et al.*, (9); ◇: growth rates published by Iversen *et al.*, (7); fits by secondary growth model of - - - Ratkowsky and —: Rosso (fitted to square root transformed data of the current study only).

The estimated lag times as resulting from the fit by the modified Gompertz model were plotted against the temperature and presented in Figure 4. The longest lag time observed was 83.3 ± 18.7 h at temperatures around 10 °C. The temperature at which the lag time was minimal was between 37 and 39 °C, with an estimated minimal lag time of 1.73 ± 0.44 h.

The effect of the temperature on the lag time was described by fitting the reciprocal square root relation (Equation 4) and the hyperbolic model (Equation 5) to the logarithmic transformation of the data. It is assumed that the T_{\min} and T_{\max} value are equal to the T_{\min} and T_{\max} value of Equation 3 describing the specific growth rate. Parameters estimated with both models are given in Table 5.

Table 5. Parameter values for the effects of temperature on the lag time (λ) for *Cronobacter* spp. in reconstituted infant formula

Model	Parameter	Estimate	95% Confidence interval
Inverse Ratkowsky (Equation 4)	T_{\min}	3.60	- ^a
	T_{\max}	47.6	- ^a
	b	0.023	0.021 - 0.024
	c	0.645	0.275 - 1.176
	RSS	2.29	
Hyperbolic (Equation 5)	p	25.8	22.3 - 29.3
	q	3.75	2.88 - 4.62
	RSS	2.11	

^a – fixed (from Table 4) by using the values estimated with the secondary Rosso model for growth (Equation 3).

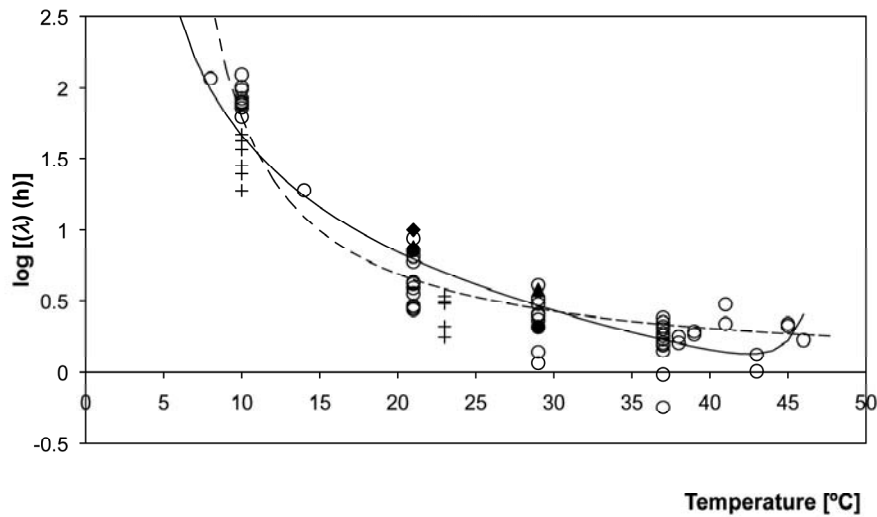


Figure 4. Log lag time as function of temperature. ○ = ATCC 29544 pre-cultures to various growth states. Also for middle stationary phase grown cells of ● = MM9, ◆ = S94 and ▲ = MC10. + indicates the lag times at 10 and 23 °C as published by Nazarowec-White *et al.*, (10). Logarithmic transformed lag time data of current study, modelled with the hyperbola model (-----) and reciprocal Ratkowsky model (—).

The logarithmic transformed lag times as fitted by the reciprocal square root relation and the hyperbolic model are represented in Figure 4. From this graph it can be concluded that both models fits the data reasonably well. The residual sum of squares (RSS) for the hyperbolic model was 2.11 and for the reciprocal square root relation 2.29. In Figure 5, the parameter k , the product of the lag time and the specific growth rate ($\mu_m \times \lambda$), as function of temperature is shown. The k -value between 8 and 47 °C was 5.08 ± 3.37 [-]. At temperatures ranging from 20 to 46 °C values for k were between 0.82 and 11.6 [-], with an average of 4.05 ± 1.92 [-]. Below 20 °C it increased to an average value of 10.06 ± 4.49 [-]. Using the k -value of 5.08 to predict the lag time at any temperature and equation 3 to determine the specific growth rate resulted in a RSS value of 16.7.

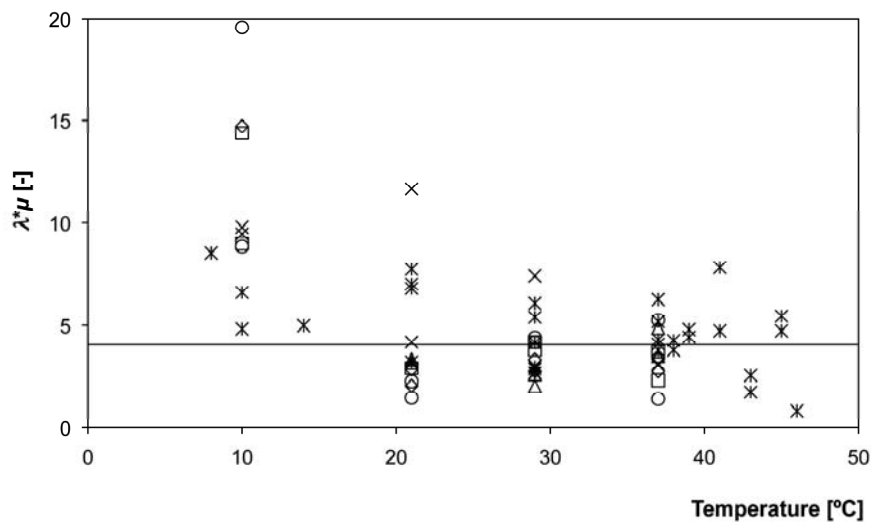


Figure 5. Parameter k (product of λ and μ_m) as function of the temperature for each growth experiment of strain ATCC 29544 pre-cultured to various growth phases and grown at temperatures from 8 up to 47 °C; × □ SKDMHJRZQFHØ Exponential phase grown cells; ◇: early stationary phase grown cells; *: middle stationary phase grown cells; o: stationary phase grown cells; □: late stationary phase grown cells. The dotted line represents the average value for the data from 20 up to 46 °C.

Based on the RSS values for the models describing the lag time it can be concluded that the hyperbolic model and reciprocal square root model best convey the experimental lag times.

The reciprocal square root model is recommended, since it has the ability of increasing the lag time at higher temperatures and it contains more interpretable parameters.

The maximum number of cells of strain ATCC 29544 reached at the various incubation temperatures between 8 and 46 °C varied between approximately 10^7 and 10^9 CFU/ml, with an average of $10^{8.2}$ CFU/ml (Figure 6). At temperatures tested above 45 °C, the maximum number of cells reached was $\pm 10^6$ CFU/ml. The initial inoculum level, which varied between $\pm 10^2$ and 10^5 CFU/ml did not seem to affect the maximum population density.

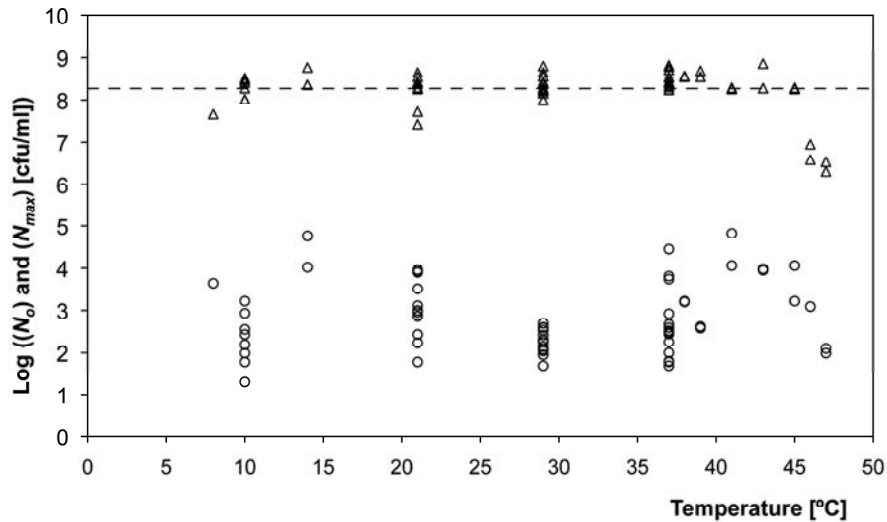


Figure 6. The initial inoculum of strain ATCC 29544, pre-cultured to various physiological states and its maximum population density at various temperatures. Δ : maximum population density; o: initial number of cells. The dotted line represents the average value for the maximum population density data from 8 up to 46 °C.

Discussion

Cronobacter spp. may contaminate infant formulae either during production or during bottle preparation. In factory environments *Cronobacter* spp. may grow in wet spots and survive in dust containing residues of infant formulae and contaminate the product after the drying process. In hospital and household kitchens infant formulae may be contaminated likewise with dry or wet residues by utensils and via environmental vectors. In all situations

the physiological state of the contaminating cells is not known. But it is known that the duration of the lag time may depend not only on the growth environment but also on the previous history of cells (12, 16). It was envisaged that the time of pre-culturing, i.e. the physiological growth phase of the inoculum, could have an effect on the lag time but probably not on the specific growth rate (16). In our study, based on visual observation of growth curves and on statistical analysis of growth data, no effect on either parameter was found. A possible explanation might be that the cells were spiked into dry powdered infant formula prior to the growth experiments, and their metabolic activity had possibly changed in all cases to a comparable level. The period (3 to 10 days) the cells were in the powdered infant formulae before carrying out a growth experiment was found not to influence the growth parameters. Cells that had been in the powdered infant formula for up to 4 weeks did not show a longer lag phase either (data not shown). Another explanation for not measuring differences in resuscitation times might be the rich growth medium (reconstituted powdered infant formula) used. In this medium *Cronobacter* spp. cells had a rather short lag time of 1.71 ± 0.50 h at 37 °C and small differences in lag phase may not become apparent.

Our specific growth rate data were compared to specific growth rates published by Nazarowec-White *et al.*, (1997) and Iversen *et al.*, (2004) (6, 10) as shown in Figure 3. In our study the specific growth rates of *Cronobacter* spp. spiked into dry infant formula is comparable to Nazarowec-White *et al.*, (1997) who reported similar specific growth rates. Specific growth rates reported by Iversen *et al.*, (2004) were consistently over 10% lower. One cause for the difference with the latter study may be due to differences in growth conditions: in our experiments spiked dry powdered infant formula samples reconstituted with sterile tap water were used as growth medium, whereas Iversen *et al.*, measured growth in other media by inoculating with an overnight TSB culture (6). Another difference is the use of the rapid automated bacterial impedance technique, which measures growth only at much higher levels ($> 10^6$ CFU/ml) than the plate count method used in our study (16). In the current study, the plate count technique was used and growth was measured in a more representative range and over multiple logs of bacterial counts, thus the estimates for the lag time and specific growth rate can be considered more accurate (2).

The experimental design, based on initial estimates of specific growth rate and lag time, allowed prediction of the growth with enough accuracy to determine the appropriate sampling times and dilutions to measure growth curves, such that our quality requirements were met for 95% of the experimental growth curves. The modified Gompertz model (Equation 1), the secondary Rosso model (Equation 3) and the reciprocal square root model (Equation 4) successfully estimated the lag time and specific growth rate for the whole growth temperature range (Table 4 and 5). The theoretical minimal-, and maximum growth temperatures as fitted with both the secondary growth models are 2.19 °C and 48.9 °C (Equation 2) and 3.60 °C and 47.6 °C (Equation 3), respectively. In practice, however, growth of *Cronobacter* spp. strains has been observed between 5.5 °C and 47 °C (4).

The fact that low numbers of *Cronobacter* spp. cells have occurred in dry infant formulae (5, 9, 10) in combination with the relatively short lag time and high specific growth rates found in this study, underscores the need for careful preparation and use. A study by Pagotto *et al.*, (2003), however, showed that a number of 10⁵ CFU/ml of certain *Cronobacter* spp. strains could be lethal to suckling mice after ingestion, but not all strains appeared to be pathogenic (11). As there is a lack of information about virulence factors, not all the *Cronobacter* spp. strains necessarily need to be regarded as potential pathogens. Nevertheless the results described in this paper (Table 4 and 5) allow to predict growth of *Cronobacter* spp. in reconstituted infant formulae under conditions that closely mimic the conditions in the actual food product in both hospital and household settings and will be useful in designing effective control measures.

Acknowledgements

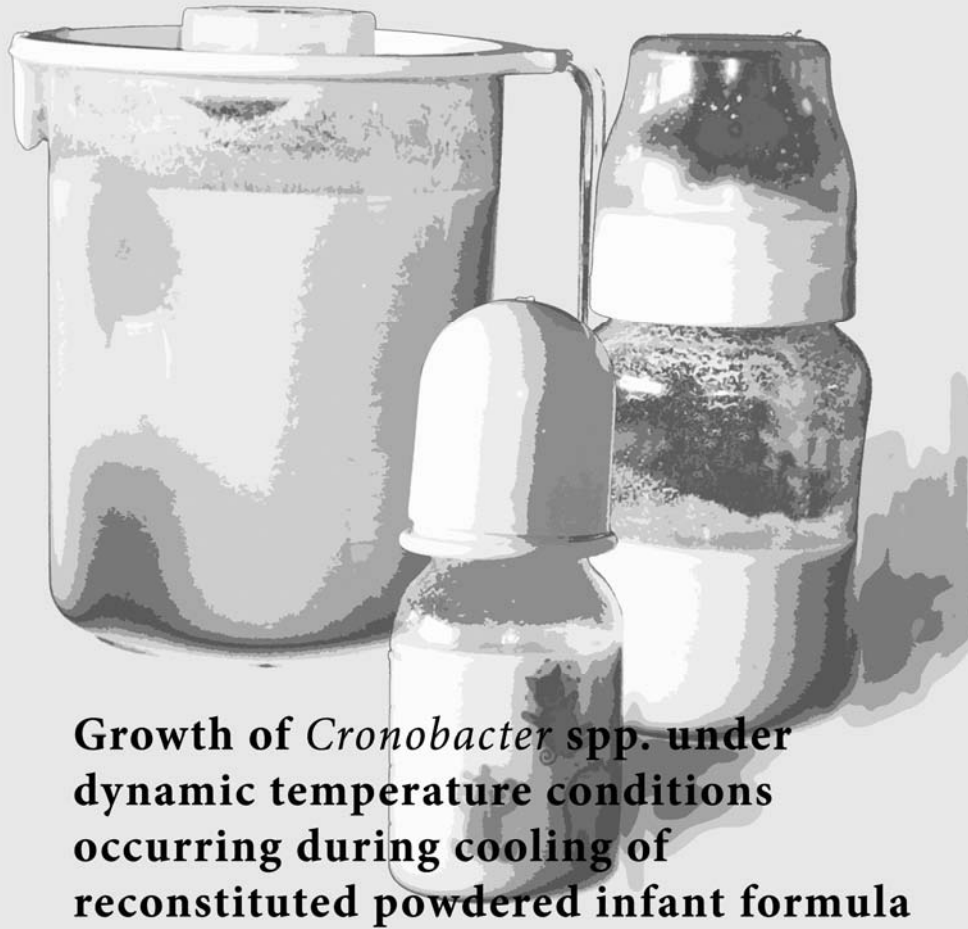
The authors wish to thank Ingrid Maas for skilful technical assistance. The authors thank Ingrid Hombergen and Etienne Vidal for carrying out part of the experiments. Dr. H.L. Muytjens is thanked for providing the *Cronobacter* spp. strains MM9 and MC10. The financial support by Nestlé Research Center, Lausanne, Switzerland, is gratefully acknowledged.

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Chapter 7



Abstract

Reconstituted infant formulae are excellent growth media for Cronobacter spp. (formerly Enterobacter sakazakii) and other micro-organisms that may be present in such products. Immediate consumption or rapid cooling and storage at low temperature are therefore recommended as control measures to prevent microbial growth. Placing a container filled with reconstituted liquid formula in the refrigerator, however, does not mean that the temperature of the liquid is directly the same as the set-point of the refrigerator. This study describes the temperature profiles and methods to predict lag time and possible growth of Cronobacter spp. during the cooling process in three types of containers. The overall heat transfer coefficients (α) were determined and were shown to have a very large variability in both household refrigerators and an air-ventilated refrigerator equipped with a fan. A mathematical model was built to predict the growth of Cronobacter spp. under dynamic temperature conditions using three models for the lag time. The various estimations for the lag time had a remarkably strong impact on the predicted growth. The assumption of a constant k -value ($k = \text{lag time} \times \text{specific growth rate} = \lambda \times \mu = 2.88$) fitted the experimental data best. Predictions taking into account the large variability in heat transfer showed that proliferation of Cronobacter spp. during cooling may be prevented by limiting the volume to be cooled to portion-size only, or by reconstituting at temperatures of 25 °C or lower. The model may also be used to predict growth in other situations where dynamic temperature conditions exist.

This Chapter has been published as

“Growth of *Cronobacter* spp. under dynamic temperature conditions occurring during cooling of reconstituted powdered infant formula”

Kandhai, M.C., P. Breeuwer, L.G.M. Gorris, M.H. Zwietering, and M.W. Reij
Journal of Food Protection, 2009; 72: 2489-2498.

Introduction

Cronobacter spp., until recently known as the species *Enterobacter sakazakii*, is a group of opportunistic pathogenic micro-organisms belonging to the family of *Enterobacteriaceae* (9, 13). Although only recently brought to wider attention of infant formulae manufacturers and the public through several outbreaks and public recalls, *Cronobacter* spp. have been associated with neonatal deaths as early as 1958 (32). Cases of neonatal meningitis or necrotizing enterocolitis due to *Cronobacter* infection have in certain cases been associated with powdered infant formulae (4, 6, 29). Several investigations showed that the micro-organism can be found in a variety of environments and that contamination may occur during manufacturing and during preparation of the bottles (12, 16, 19, 20, 22). Reported concentrations in dry powdered formulae ranged from 0.002 to 0.92 CFU/g (7).

In dry powdered infant formula *Cronobacter* spp. are not able to grow, but after the addition of water, reconstituted infant formula is a good medium for growth with only the barriers of short storage and low temperature to prevent bacterial growth. After reconstitution *Cronobacter* spp. can multiply at temperatures between 3.6 and 47.6 °C (12, 17, 23). At room temperature (25 °C) the generation time was determined to be 0.59 h (17). A study by Pagotto *et al.*, (2003), suggests that a number of 10⁵ CFU/ml of certain *Cronobacter* spp. strains could cause illness in suckling mice after ingestion (24).

To prevent multiplication of micro-organisms most manufacturers prescribe consumption of infant formula directly after preparation. For practical reasons, however, caregivers sometimes prefer to prepare all bottles needed for one day in advance (26). Specifically in hospitals it is not uncommon that infant formulae are prepared once per day and placed in a refrigerator to be used within 24 h (27) or within 30 h with the refrigerator set at 4 °C (1).

However, even if the temperature of the refrigerator is controlled, the temperature of the reconstituted formula itself is not controlled yet. Many manufacturers instruct that their powdered infant formulae should be reconstituted with moderately warm (lukewarm) water to assure that the powder dissolves well. The liquid formula then needs time to cool down in the refrigerator. The cooling rate of the liquid depends on the temperature, the filling

rate, and the air velocities in the refrigerator, the geometry of the food container, the thermal properties of food and bottle, and the volume of the container to be cooled.

In household refrigerators, also referred to as static refrigerators, heat transfer at the container surface is principally due to natural convection by a very limited airflow caused by variations in air density. These variations are mainly related to differences in temperature, filling of the fridge, and humidity gradients. In air-ventilated refrigerators mechanic ventilation forces air convection which improves heat transfer (18). Air-ventilated refrigerators are often, but not always, used in hospitals and nurseries.

To our knowledge, investigations into the cooling process of baby bottles are scarce. Rosset *et al.*, (27) studied temperature profiles in 25 neonatal care units and concluded that cold storage was the operation with the largest impact on potential growth of *Cronobacter* spp.. In the report of the technical meeting on *Enterobacter sakazakii* and *Salmonella* in powdered infant formula (8) a risk assessment model has been discussed that estimates the fate of *Cronobacter* spp. during various scenarios of preparation and storage. The aim of our study was to make a detailed investigation, both experimentally and by modeling, of the growth opportunities of *Cronobacter* spp. during cooling of reconstituted infant formula in refrigerators and to use the findings to propose control measures that may reduce and prevent the growth of *Cronobacter* spp. during the cooling process.

Materials and methods

Preparation of reconstituted infant formula for cooling experiments

Powdered infant formula suitable for infants less than 6 months of age, was bought locally and had a bacterial count of less than 10^2 CFU/g, which did not influence the experiments (data not shown). In accordance with the instructions on the label, tap water was brought to a full boil in a covered water boiler, cooled down on the bench to approximately 45 °C and mixed with the powdered infant formula to obtain reconstituted formula with a temperature of 40 ± 3 °C.

Cooling experiments

A total of 25 cooling experiments were performed using an experimental set up whereby three different container types with reconstituted infant formulae were placed in the refrigerator and the temperature during cooling was recorded. The following three polycarbonate containers were used: a measuring beaker equipped with a lid with a volume of 1,000 ml and a diameter of 0.10 m, (baby) bottle Type 1 (maximum 260 ml) with a diameter of 0.05 m, and (baby) bottle Type 2 (maximum 120 ml) with a diameter of 0.04 m (Table 1).

Table 1. Parameters used in Equations 2 and 3

Parameter	Value	Dimension	Reference
Specific heat capacity of infant formula (c_p)	3.93	$\text{kJ kg}^{-1} \text{ }^\circ\text{C}^{-1}$	(3)
Density of infant formula (ρ)	1,032	kg m^{-3}	(30)
Radius of 1-liter measuring beaker (r)	0.05	m	-
Radius of bottle Type 1 (r)	0.025	m	-
Radius of bottle Type 2 (r)	0.02	m	-
Height of 1-liter measuring beaker (ht)	0.14	m	-
Height of bottle Type 1 (ht)	0.115	m	-
Height of bottle Type 2 (ht)	0.95	m	-
Thermal conductivity of infant formula (k_{liquid})	0.52	$\text{W m}^{-1} \text{ }^\circ\text{C}^{-1}$	(30)
Thermal conductivity of bottle wall, polycarbonate ($k_{containerwall}$)	0.21	$\text{W m}^{-1} \text{ }^\circ\text{C}^{-1}$	(2)

Reconstituted infant formula was divided among the measuring beaker and the two types of baby bottles. While the bottles had different geometries, both were filled with 120 ml of formula. The measuring beaker held 1,000 ml. The three containers were placed, in the center of, either an incubator ventilated with air or in one of the 9 different static air refrigerators (further referred to as household refrigerators). Eight out of nine household refrigerators were located in households, and contained different amounts of other food materials. The ninth household type refrigerator was located in the laboratory, and was

empty except for the three containers. The temperature of the household refrigerators varied, which was recorded as a given parameter and not set as a controlled experimental parameter. During cooling the temperature was recorded in the center and near the wall of each container every 5 minutes for 24 h using thermistor metal oxide sensors (CM-UU) and a squirrel data logger (both: Eltek Ltd., Cambridge, UK). The temperature measured in the center of the bottles was slightly higher than the temperature measured near the wall and the difference was 0.5 °C at most. The temperature measured in the center of the bottle was chosen for use in the model predictions, for a more fail safe approach. The internal air temperature in the refrigerator was measured with a thermistor as well.

Cooling model for reconstituted infant formula

Equation 1 describes the change in temperature of a bottle or beaker (T_C) assuming that the temperature in the liquid is homogeneous and that heat is transferred from the liquid via the wall of the bottle to the air.

$$V \cdot \rho \cdot c_p \frac{dT_C}{dt} = -\alpha \cdot A (T_C - T_R) \quad (1)$$

where V [m³] is the volume of the reconstituted infant formula in the measuring beaker/bottle, ρ [kg m⁻³] is the density of the reconstituted infant formula; c_p [J kg⁻¹ °C⁻¹] is the specific heat capacity of the reconstituted infant formula; t [s] is the time; α [J m⁻² °C⁻¹ s⁻¹ = W m⁻² °C⁻¹] is the overall heat transfer coefficient; A [m²] is the surface area of the object; T_C [°C] is the temperature of the prepared liquid formula and T_R [°C] is the air temperature in the refrigerator.

Values of the parameters are shown in Table 1. After integration and rewriting this equation transforms into:

$$T_{C,t} = T_R + (T_{C,0} - T_R) e^{\left(\frac{-2\alpha t}{r\rho c_p}\right)} \quad (2)$$

where $T_{C,0}$ [°C] is the temperature in the center of formula in the measuring beaker or bottle at the beginning of the cooling process and r [m] is the radius of the measuring beaker or bottle.

Here, we assume that both the bottles and the measuring beaker can be described as a cylinder and that only the side surface area of the liquid ($A = 2 \cdot \pi \cdot r \cdot h$) contributes to heat transfer, not the top or the bottom of the container. The ratio of surface area and volume (A/V) can then be described as $2 r^{-1}$.

As shown in equation 3, the overall heat transfer coefficient α consists of the external heat transfer coefficient in air, $\alpha_{external}$, the coefficient of the wall, $\alpha_{containerwall}$ [the ratio of thermal conductivity of the container material ($k_{containerwall}$) and the thickness of the wall], and the internal heat transfer coefficient, $\alpha_{product}$.

$$\frac{1}{\alpha} = \frac{1}{\alpha_{external}} + \frac{1}{\alpha_{containerwall}} + \frac{1}{\alpha_{product}} \quad (3)$$

The external heat transfer coefficient largely depends on the movement of air in the refrigerator. When a fan is present, this coefficient can be expected to be higher than in household refrigerators that are not equipped with a fan. The internal heat transfer coefficient depends on the movement of liquid in the bottle. During cooling of the bottles the liquid may circulate slowly due to free convection. If free convection does not occur, the internal heat transfer coefficient can be estimated by the ratio of the thermal conductivity of the reconstituted infant formula (k_{liquid}) and the radius of the container (r).

Test organism

Stock cultures of *Cronobacter sakazakii* (formerly named *Enterobacter sakazakii*) ATCC 29544 were maintained at -80 °C in cryo vials (Greiner Bio-one GmbH, Frickenhausen, Germany), containing a stationary-phase culture suspension in Brain Heart Infusion (BHI; Difco, Becton Dickson, Maryland, U.S.A) broth with 30% (vol/vol) glycerol (Fluka-chemica, Buchs, Switzerland).

Growth medium, inoculation and sampling

The inoculum of strain ATCC 29544 was prepared by transferring 100 µl of the stock culture into 100 ml BHI broth, followed by incubation for 18 to 20 h at 37 °C. Cells were harvested, washed, sprayed onto dry infant formula to obtain a final concentration of 10^4 to 10^6 CFU/g and stored for 3 to 10 days as described elsewhere (17). The powder containing *Cronobacter* spp. was then mixed with water, similar to the procedure described at “Preparation of reconstituted infant formula for cooling experiments”, to obtain reconstituted formula with 10^3 to 10^5 CFU/ml at a temperature of 37 ± 3 °C. This formula was distributed between the Type 1 bottle and the measuring beaker and placed in the laboratory refrigerators immediately. No other products were present in the refrigerators. Samples were taken every 30 min during the first 4 h and subsequently every 60 min, by inserting a 1-ml disposable pipette with balloon, leaving the bottles in the refrigerator. If appropriate, samples were first diluted in Peptone Saline Solution (PPS; NaCl 8.5 g/L supplemented with 1 g/L neutralized Bacteriological Peptone from Oxoid, Basingstoke, England) and then plated in duplicate onto Tryptone Soy Agar (TSA; Oxoid LTD., Basingstoke, Hampshire, England) using a spiral plater device (Eddy Jet, Leerdam, The Netherlands). Plates were incubated for 20 to 24 h at 37 °C and counted manually. The inoculation levels chosen are higher than the very low numbers found in infant formulae (7, 11, 21, 23). A minimum level required to accurately count the organisms plated by the spiral plater, however, is 6×10^2 CFU/ml, and inoculum levels were chosen to be slightly higher than this minimum.

Predicting growth of Cronobacter spp. under dynamic temperature conditions

A growth model was built that uses the lag time and the specific growth rate of *Cronobacter* spp. during the constantly changing temperature conditions that occur during cooling. The specific growth rate (μ_m) for *Cronobacter* spp. at each temperature (T_i) was

described with the secondary growth model (Equation 4) of Rosso *et al.*, (28) using cardinal parameters previously established (Table 2) (17).

If

$$T_{\min} < T < T_{\max} \quad (4)$$

then

$$\mu_m(T_i) = \left(\frac{(T - T_{\max})(T_i - T_{\min})^2}{[(T_{\text{opt}} - T_{\min})(T_i - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T_i)](T_{\text{opt}} - T_{\min})} \right) \mu_{\text{opt}}$$

$$\text{and if } \begin{cases} T_i \leq T_{\min} \\ T_i \geq T_{\max} \end{cases} \text{ then } \mu_m(T_i) = 0$$

where T_i is the temperature [°C] at each moment in the center of the volume of the infant formula in the measuring beaker or the bottle; T_{\min} [C] is the extrapolated minimum temperature, T_{\max} [C] is the extrapolated maximum temperature, T_{opt} [C] is the temperature at which the specific growth rate is maximal, and μ_{opt} [h⁻¹] is the μ_m [h⁻¹] at this optimal temperature (28).

Three models were used to estimate the lag time. First, lag times were estimated using the inverse of the secondary Ratkowsky model (Equation 5) with parameters previously determined as shown in Table 2 (17).

$$\ln(\lambda(T_i)) = \ln \left[\left(b(T_i - T_{\min}) \cdot \{1 - \exp[c(T_i - T_{\max})]\} \right)^{-2} \right] \quad (5)$$

where b [°C⁻¹ h^{-0.5}], and c [°C⁻¹] are the so-called Ratkowsky parameters (34). T_i [°C] used is the average temperature of the simulation interval. The T_{\min} and T_{\max} parameters were determined from previous growth experiments at temperatures ranging from 8 up to 47 °C (17).

Second, the lag times were estimated using a constant k -value, which is the product of the specific growth rate and the lag time (Equation 6). It is known that the k -value has a large variability but it can be considered constant over a wide range of temperatures (34).

$$k = \lambda(T_i) \cdot \mu(T_i) \quad (6)$$

Third, the logarithmic model was used to estimate lag times (Equation 7) (25).

$$\log_{10}(\lambda(T_i)) = c_l \ln(T_i) + b_l \quad (7)$$

where, λ [h] is the lag time, b_l and c_l are parameters as used in the microbial risk assessment model (25) (available at <http://www.mramodels.org/>).

Table 2. Earlier determined cardinal growth parameters taken from literature, to calculate the specific growth rate (μ [h⁻¹]) and the duration of the lag phase (λ [h]) as a function of temperature

Model	Parameter	Value	Dimension	Equation	Reference
Secondary Rosso growth model	T_{\min}	3.6	°C	4	(17)
	T_{\max}	47.6	°C	4	(17)
	T_{opt}	39.4	°C	4	(17)
	μ_{opt}	2.31	h ⁻¹	4	(17)
Inverse of secondary Ratkowsky model	T_{\min}	2.19		5	(17)
	T_{\max}	48.9		5	(17)
	b	0.023	°C ⁻¹ h ^{-0.5}	5	(17)
	c	0.645	°C ⁻¹	5	(17)
Logarithmic model	b_l	4.309	-	7	(25)
	c_l	-1.141	-	7	(25)

It should be noted that all specific growth rate and the lag time models (Equation 4, 5, 7) are used with earlier estimated parameters (17) and are therefore predictions. To specifically estimate lag time effects in this study, the k -value (Equation 6) was determined from experiments taking into account the effect of a dynamically changing temperature on a lag time value.

To determine the length of the lag time at constantly changing temperature, the fraction of the lag time elapsed (λf_i) in every time step was calculated numerically according to Equation 8.

$$\lambda f_i = \left(\frac{\frac{\Delta t}{60}}{\overline{\lambda(T_{i-1,i})}} \right) + \lambda f_{i-1} \quad (8)$$

where Δt is the time step of the simulation [5 min] and λf_i [-] is the fraction of the lag time elapsed at step i and $\overline{\lambda(T_{i-1,i})}$ [h] is the average of the lag time of the current and the previous time step.

Each fraction λf_i was added to the value of the previous time step. Once $\lambda f_i \geq 1$, the lag time was assumed to be completed and growth was assumed to be starting with a specific growth rate corresponding to the temperature prevailing in the bottle at that moment.

To include changing temperature also during the growth stage, the dynamic first order model for bacterial growth was used and was solved numerically. In each time step the average temperature (and consequently the specific growth rate) was taken as constant, providing the exponential function as outcome (Equations 9A to C). As a conservative estimate, growth was predicted assuming $\lambda=0$, using Equation 9A only, thus assuming that *Cronobacter* spp. started growing instantaneously after addition of water. So, any further steps can be described by Equation 9A, assuming that during the full time step (Δt) there is exponential growth. When there was a lag time, the fraction of the lag time elapsed was

calculated using Equation 8. When the fraction of the lag time elapsed was less than 1, the number of micro-organisms was stable (Equation 9B). To exactly determine the commencement of growth after the lag phase, in the step that λf_i has become greater than 1, Equation 9C describes growth in the remaining fraction ($\lambda f_i - 1$) of this time step. Equation 9C is valid only in the first iteration after the lag phase has been completed.

After the first $\lambda f_i > 1$:

$$N_i = N_{i-1} e^{\mu_m(T_i) \Delta t} \quad (9A)$$

For $\lambda f_i < 1$:

$$N_i = N_0 \quad (9B)$$

For the first value of $\lambda f_i \geq 1$:

$$N_i = N_0 e^{\mu_m(T_i) (\lambda f_i - 1) \Delta t} \quad (9C)$$

where N_i [CFU/ml] is the number of micro-organisms present at time step i ; $\mu_m(T_i)$ [h^{-1}] is the specific growth rate at the temperature prevailing in the current time step i and λf_i [-] is the fraction of the lag time elapsed at step i .

Data analysis

Temperature profiles during cooling were fitted into Equation 2 using the solver function in Microsoft Office Excel 2003 by minimizing the residual sum of squares (RSS) to estimate the overall heat transfer coefficient (α) and the air temperature (T_R). Experimental growth curves were fitted to the dynamic growth model to estimate the k -value using the same software. Standard deviations were calculated with Excel and are reported as plus or minus the mean. Confidence intervals were calculated in Excel. The increase in numbers of *Cronobacter* spp. cells assessed in the duplicate growth experiments and the α estimated for the different type of refrigerators were analyzed by analysis of variance (ANOVA),

univariate analyses using SPSS (SPSS release 12.01 for Microsoft Windows; SPSS Inc., Chicago, U.S.A.). Significant differences are presented at a 95%-confidence level ($P \leq 0.05$).

Results and discussion

Temperature variability within refrigerator

In our experiments large variations in air temperatures were observed both in time and between different locations in the same empty household refrigerator (Figure 1A). Starting one hour after closing the door with the temperature set at 6.3 °C, the temperature near the cooling element in the back of the upper department varied between -8.4 °C and +10.4 °C. This variation is due to switching on and off the refrigerator's engine. The lower, middle and upper compartments of the refrigerator showed more constant temperatures and were on average of 5.5 ± 0.8 , 6.5 ± 1.3 and 9.9 ± 0.7 °C respectively. These results are in line with data reported elsewhere. James and Evans (14) surveyed 252 household refrigerators without a fan and found that the coldest spot was on average 2.9 °C colder than the warmest spot within the same refrigerator, whereas the greatest range of average temperatures observed in a refrigerator was 12 °C. Individual (not averaged) temperature differences ranged from 4.5 up to 30.5 °C in the same refrigerator. Door openings resulted in an increase in average temperature and in an even larger spatial variability (15). A variation in local air temperature does not immediately change the temperature of a bottle placed in that refrigerator, but varying temperature profiles may change the air flow and thus affect the heat transfer coefficient (α). Additionally, the speed of cooling as described in Equation 1, will be affected by variations in air temperature.

In air-ventilated refrigerators equipped with a fan, the air is continuously in motion and the variation in air temperature is limited (Figure 1B). Near the air inlet the temperature varied from 5.4 to 7.3 °C (average 6.3 ± 0.5 °C) and near the door the temperature was 7.7 ± 0.1 °C, but all other locations had temperatures of 6.7 ± 0.1 °C.

As a result of the variations in air temperature it was not possible to establish one value for the air temperature required to fit the data to equation 2. Therefore, both the heat transfer coefficient (α) and the air temperature (T_R) were fitted simultaneously to the experimental data according to Equation 2. The differences between the measured T_R and the fitted T_R were on average less than 1 °C (data not shown).

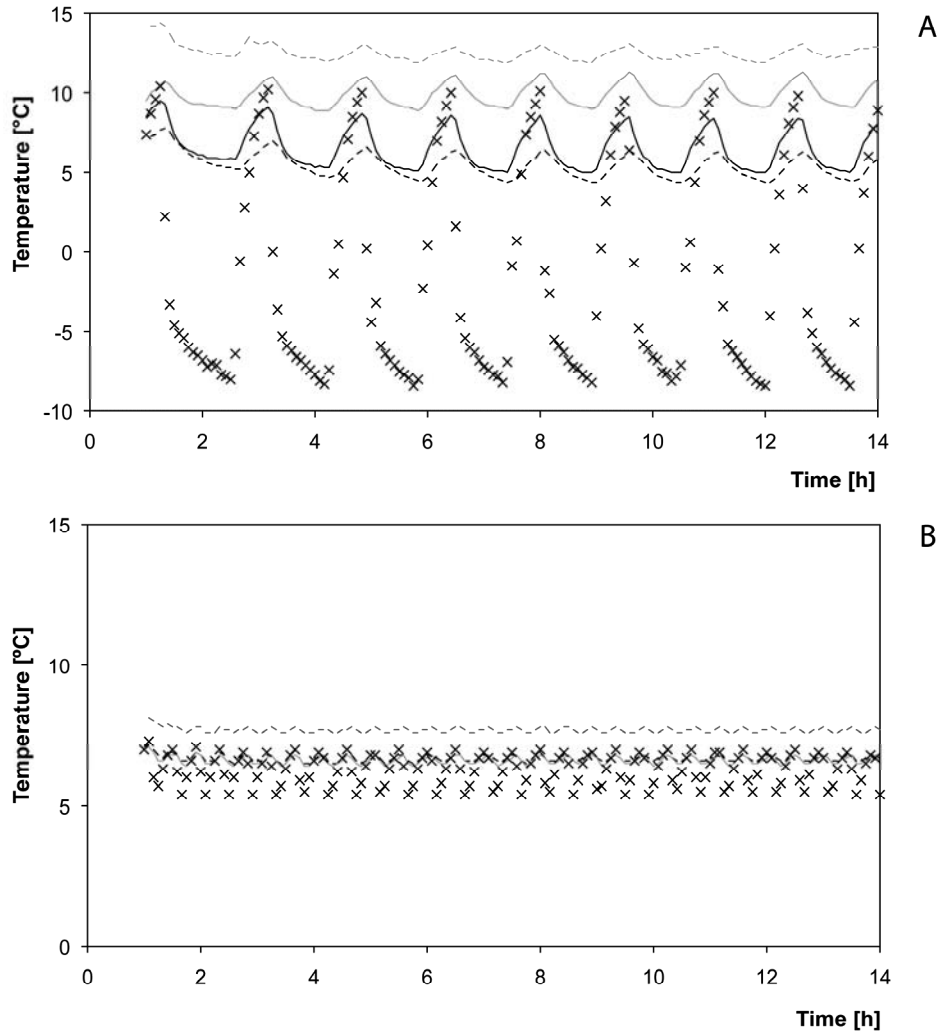


Figure 1. Air temperatures in an empty household set at 6.3 °C (A) and an air-ventilated refrigerator set at 6.3 °C (B). Measurements started one hour after closing the door. Locations were (grey dashed line), door of upper shelf; (grey solid line), center of the upper shelf; (x), back wall of upper shelf, close to the cooling element (A) or the air inlet (B); (black dashed line), center of the lower shelf.

Overall heat transfer coefficient (α)

Figure 2 shows typical cooling profiles of the 1-liter beaker and both bottles placed in either a household refrigerator (2A) and an air-ventilated refrigerator (2B). During the cooling process the temperature fluctuated slightly due to variations in the air temperature. In a household refrigerator set at 6.3 °C the time required for cooling the prepared formula to 10 °C was typically 7.5 hours for 1,000 ml formula in the measuring beaker and 3.5 hours for the 120 ml volume in the two types of bottles. In an air-ventilated refrigerator, cooling times typically were 2 hours less.

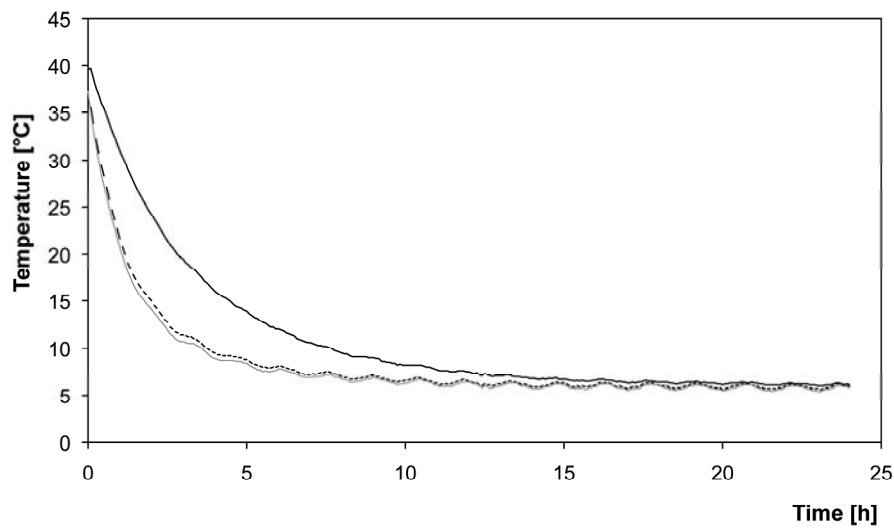


Figure 2A. Example of temperature profiles of reconstituted powdered infant formula in (black solid line), 1-liter measuring beaker; (grey solid line), Type 1; and (black dashed line), Type 2 bottles, cooled down in a household type refrigerator. The refrigerator had an average air temperature of 6.3 °C.

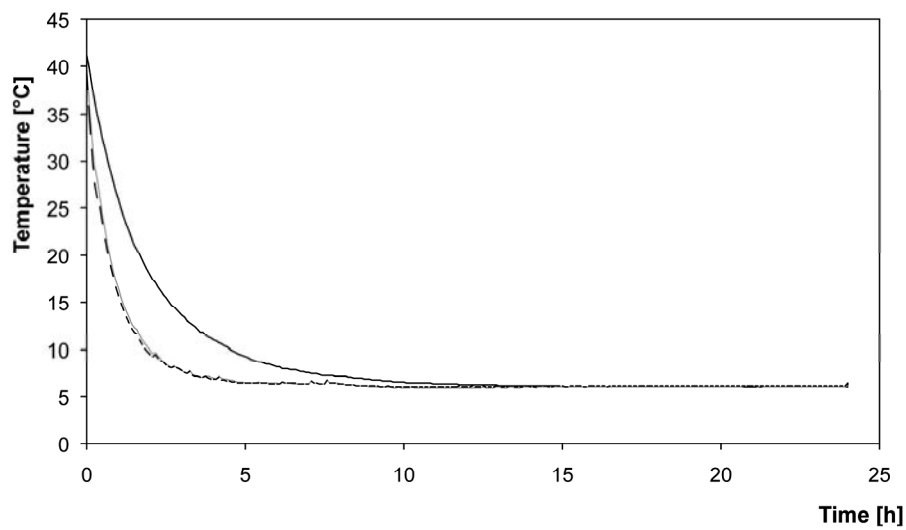


Figure 2B. Example of temperature profiles of reconstituted powdered infant formula in (black solid line), 1-liter measuring beaker; (grey solid line), Type 1; and (black dashed line), Type 2 bottles, cooled down in an air-ventilated refrigerator. The refrigerator had an average air temperature of 6.3 °C.

With the three containers, 25 cooling experiments were performed in nine different household refrigerators and 9 experiments were performed in an air-ventilated refrigerator. All cooling profiles ($3 \times 25 + 3 \times 9$) were fitted to Equation 1 to obtain the overall heat transfer coefficient (α [$\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$]), a parameter independent of the reconstitution temperature, the air temperature, and within a limited range independent of the size of the object. No consistent differences were found between the α -values determined for the refrigerators that were empty and those that contained also other materials, therefore the data were pooled and used as a single data set. Figure 3A shows the α -values with their standard deviation as a function of air temperature in household refrigerators. An effect of air temperature on the heat transfer coefficient (α) was not observed indeed. The α -values ranged from 5.4 to 12.9 [$\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$] in this series of experiments in which temperature settings and filling rates of the 9 household refrigerators were variable.

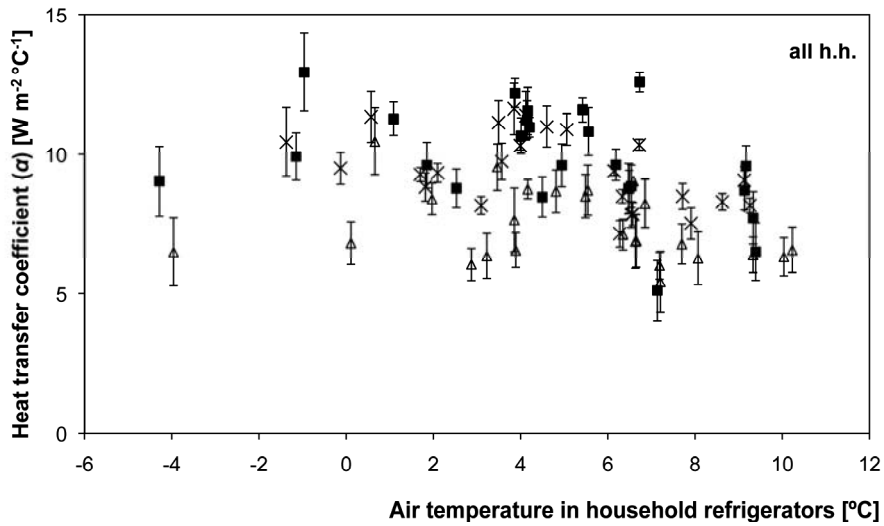


Figure 3A. Overall heat transfer coefficient (α) ($\text{Wm}^{-2} \text{ } ^\circ\text{C}^{-1}$) with their standard deviations, as determined in 9 different household refrigerators at various temperatures. Markers indicate (\times , $+$), 1-liter measuring beaker; (\blacksquare , \square), Type 1 bottle; (\blacktriangle , \triangle), Type 2 bottle.

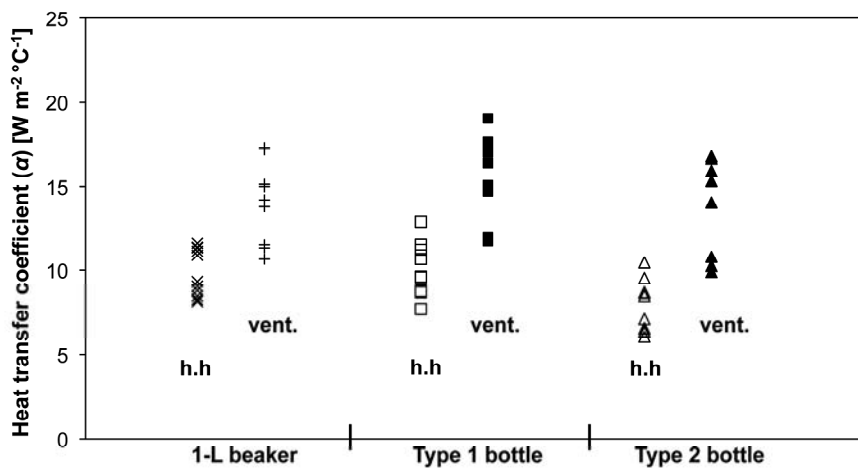


Figure 3B. Overall heat transfer coefficient (α) ($\text{Wm}^{-2} \text{ } ^\circ\text{C}^{-1}$) with their standard deviations, as determined in a selection of 11 experiments performed in a single, empty household refrigerator (h.h.) and 9 experiments in a single air-ventilated (vent.) refrigerator.

Figure 3B shows a selection of the α -values, which were measured in one single household refrigerator and one air-ventilated refrigerator, both empty except for the experimental setup consisting of the 3 containers. In this well-defined series of experiments the range of α -values (6.1 to 12.9 [$\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$]) in the single household refrigerator was almost as wide as in the 9 different household refrigerators shown in Figure 3A. The fitted heat transfer coefficients as shown in Figure 3B are significantly higher ($P = 0.01$) than in the household type refrigerators. The coefficients in this well-controlled refrigerator varied over the range of 9.9-19.0 $\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$. Average values and confidence intervals are shown in Table 3.

Table 3. Estimated overall heat transfer coefficient (α) [$\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$] of bottles Type 1 and Type 2, each containing 120 ml, and a measuring beaker containing 1000 ml of reconstituted infant formula as measured in one household-type refrigerator and one air-ventilated refrigerator

	Type of refrigerator	Average overall α [$\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$]	Number of experiments	Standard deviation of average	95%-confidence interval [$\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$]
Measuring beaker	household	9.9	11	1.4	6.8 – 13.0
	air-ventilated	14.0	9	2.4	8.4 – 19.7
Bottle Type 1	household	10.0	11	1.5	6.7 – 13.3
	air-ventilated	15.7	9	2.5	9.8 – 21.5
Bottle Type 2	household	7.7	11	1.5	4.4 – 11.1
	air-ventilated	13.9	9	2.8	7.4 – 20.4

The average values for the beaker and the Type 1 bottle corresponded to cooling rates of 1.0×10^{-4} and $2.0 \times 10^{-4} \text{ s}^{-1}$, identical to the cooling rates assumed in the FAO/WHO microbial risk assessment model (25). For bottle Type 2, α was significantly lower in household refrigerators (ANOVA univariate analysis, $P=0.001$) than the corresponding values of the measuring beaker and bottle Type 1. The Type 2 bottle, which is the smallest baby bottle available on the Dutch market, was filled to its maximum volume. It might be that the area available for heat transfer was slightly less than assumed in Equation 2, since the bottle is rounded at the bottom and not a perfect cylinder. Another explanation is that the lower α might be due to the fact that in this narrow bottle, with a radius of 0.02 m, internal heat transfer can not be overlooked. When a circular flow does develop in a

container during cooling, the internal heat transfer coefficient (α) can be estimated to be in the range of 150 to 250 $\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$ using the theory of free convection from a vertical plate (5) assuming a temperature difference of 0.5 $^\circ\text{C}$ between core and the wall. If in a narrow bottle such a circular flow does not develop, the internal heat transfer coefficient depends solely on the ratio of the thermal conductivity of the reconstituted infant formula (k_{liquid} , 0.52 $\text{W m}^{-1} \text{ }^\circ\text{C}^{-1}$) and radius of the container (0.02 m). The resulting internal heat transfer coefficient is 26 $\text{W m}^{-2} \text{ }^\circ\text{C}^{-1}$, and thus is in the same order of magnitude as the resulting overall coefficient (see Figure 3). It may therefore have contributed significantly to heat transfer resistance. To rule out the potential effect of insufficient liquid mixing, the experiments testing the growth of *Cronobacter* spp. during cooling were continued using bottle Type 1. Furthermore this bottle size and type is used by the majority of the caregivers in The Netherlands .

Growth during cooling

On the basis of the estimated overall heat transfer coefficient, the temperature in a bottle or measuring beaker could be predicted during the entire cooling process. Figure 4A shows the temperature of a 1-liter beaker placed in a household refrigerator at 7.1 $^\circ\text{C}$. With the temperature at each time point known, both the specific growth rate and the lag time of *Cronobacter* spp. were calculated using Equations 4 through 7.

Figure 4B shows that the specific growth rate dropped sharply during the first 5 hours of the cooling process. In the same 5-h period, the lag time increased to values of 20 h and more. The construction of the lag time under dynamic temperature conditions is shown in Figure 4C. According to the inverse of the Ratkowsky model (Equation 5) the lag time was 3.2 h. An initial estimate for the k -value of 4.05 [-] taken from previous research (17) led to a predicted lag time of 3.3 h (data not shown), while the logarithmic model (Equation 7) predicted a lag time of 4.8 h. The resulting predicted and experimental numbers of *Cronobacter* spp. under dynamic temperature conditions are shown in Figure 4D. Growth of the organism was apparent in the 1-liter measuring beaker that was cooling down from 38 $^\circ\text{C}$ in the household-type refrigerator set at 7.1 $^\circ\text{C}$. The increase in numbers during the

entire 24 hours was 0.7 log CFU/ml. Figure 5 shows graphs similar to Figure 4D for both the 1-liter measuring beaker and the Type 1 bottle at 5, 7, 10 and 16 °C. This range of temperatures was chosen to mimic the range of temperatures that can be found in household refrigerators, including the 30% of household refrigerators that exceed standard temperatures (10, 31).

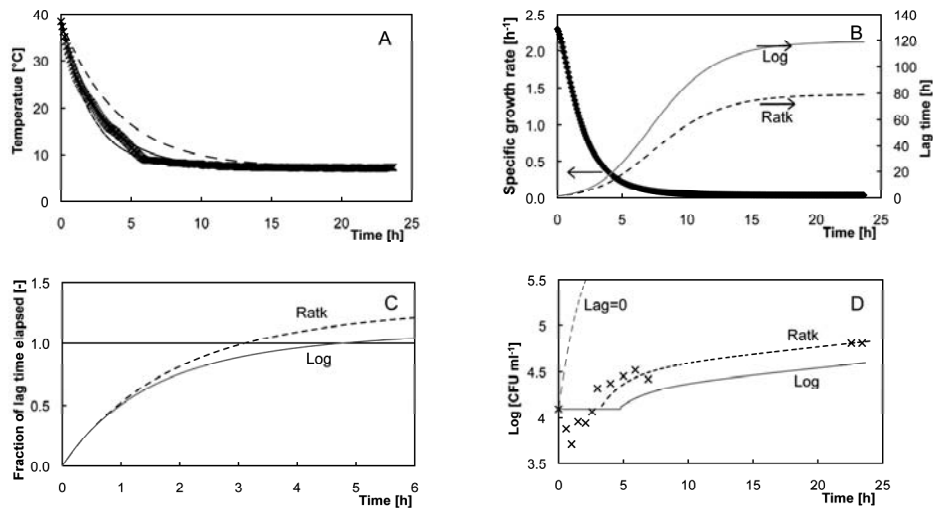


Figure 4. Prediction and experimental values of growth during cooling of infant formula in a 1-liter measuring beaker in a household refrigerator set at 7.1 °C. The reconstituted formula was prepared from artificially contaminated powder and had a temperature of 38 °C at the start of the experiment. A: (x), measured and (solid line), predicted temperature with 95%-confidence interval (dashed lines). B: Predictions of (♦), specific growth rate and (lines), lag times at the prevailing temperature. C: Fraction of lag time elapsed and D: number of *C. sakazakii* (x), measured and (lines), predicted. Various lines represent: (grey dashed line), lag = 0; (black dashed line - - - -), lag time according to Ratkowsky; and (grey line), lag time predicted with the logarithmic model.

Impact of lag time

In the predictions a small difference in lag time resulted in considerable differences in the estimated growth. With a longer lag time (Figure 4C), the temperature of the formula (Figure 4A) will be lower at the moment that growth commences, whereas also the specific

growth rate (Figure 4B) will be lower, together resulting in a considerably smaller increase in cell count.

When the lag time was assumed to be zero (grey dashed lines), the exponential growth function overestimated the experimental values considerably in all cases shown in Figure 5. Although *Cronobacter* spp. cells are known to commence their growth quickly in infant formula, they thus seem to require some time to adjust to their new environment and start multiplying. The logarithmic model overestimated the lag time and thus underestimated the growth in all cases (25). The results of the use of a k -value were often comparable to the Ratkowsky model. The Ratkowsky model (black dashed lines) predicted the growth during cooling at 7 and 10 °C quite well, but at other temperatures this approach underestimated growth. Optimal fits to the experimental data were obtained with $k = 1.75$ at 4 °C, $k = 3.49$ at 7 °C, $k = 4.07$ at 10 °C, $k = 2.06$ [-] at 16 °C (grey solid lines). The best fit over all 8 experiments was obtained with $k = 2.88$ [-] (black solid line). These values are all within the range of lag values previously reported for individual experiments at a constant temperature (17, 34).

It should be noted that the experiments shown in Figures 4 and 5 were performed with *Cronobacter* spp. cells that were present in the dry powder, which can be expected to be injured and therefore show relatively long lag phases. It could be that, even longer lag times might have been observed when lower levels of inocula were used than in the current study and when naturally contaminated powdered infant formula had been used. On the other hand, in an additional series of experiments using an inoculum of cells grown overnight in BHI, lag times were shorter and could be predicted best by assuming k -values ranging from 1.2 to 1.9 [-] (results not shown).

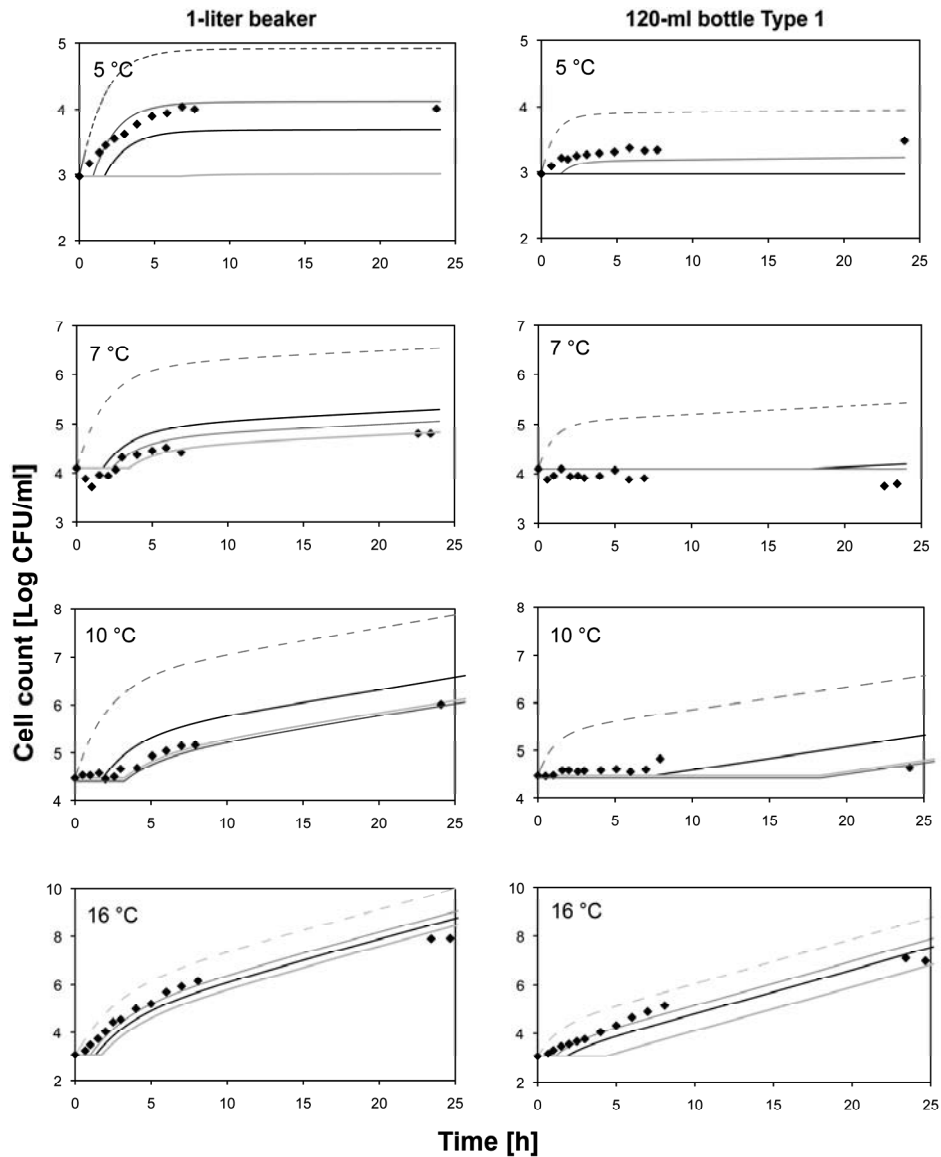


Figure 5. Growth during cooling of infant formula in (left) 1-liter measuring beaker and (right) the Type 1 baby bottle at air temperatures of 5 °C, 7 °C, 10 °C, and 16 °C in an empty household-type refrigerator. The markers represent experimental data, while the lines are predictions, assuming (grey dashed line ----), lag = 0; (light grey solid line) lag time according to Ratkowsky; (black solid line), lag time as predicted with $k = 2.88$; (grey solid line), lag as predicted with an optimal fit of the k -value to the two experiments at each specific temperature: $k = 1.75$ at 5 °C; $k = 3.49$ at 7 °C; $k = 4.07$ at 10 °C; $k = 2.06$ at 16 °C.

Table 4. Prediction of the time required to cool down from 40 to 10 °C and the increase in cell counts after 24 h in bottles Type 1 and Type 2, each containing 120 ml, and a measuring beaker containing 1,000 ml of infant formula. Infant formula was reconstituted at 40 °C and placed in refrigerators with an air temperature of 7 °C. Overall heat transfer coefficients with their confidence intervals were taken from Table 3 and for lag time the optimal value $k = 2.88$ [-] was assumed. In parentheses are 95% confidence intervals

	Type of refrigerator	Time to reach 10 °C [h]	Predicted increase in cell counts in 24 h [log CFU/ml]
Measuring beaker	household	6.8 (5.3 – 10.0)	1.3 (0.8 – 2.3)
	air-ventilated	4.8 (3.5 – 8.1)	0.7 (0.2 – 1.7)
Bottle Type 1	household	3.4 (2.6 – 5.1)	0.2 (0.0 – 0.7)
	air-ventilated	2.2 (1.6 – 3.5)	0.0 (0.0 – 0.2)
Bottle Type 2	household	3.6 (2.5 – 6.2)	0.3 (0.0 – 1.1)
	air-ventilated	2.0 (1.3 – 3.7)	0.0 (0.0 – 0.3)

Prediction of growth during cooling

Using the parameters for heat transfer and lag time derived from the experiments, a series of simulations were performed, to evaluate the effect of reconstitution temperature and refrigerator temperature. Regarding the lag time, the value $k = 2.88$ [-] as found in this study was assumed in all predictions.

Cooling time and growth during 24 hours

Table 4 presents an overview of the estimated time required to cool down infant formula from 40 °C to 10 °C in refrigerators set at 7 °C and the increase in *Cronobacter* spp. cells in bottles placed in such refrigerator for 24 h. In a household refrigerator, a 1-liter portion of formula reconstituted at 40 °C needed at least 5.3 and up to 10 h to reach a temperature of 10 °C and in an air-ventilated refrigerator at least 3.5 h. Bottles needed on average 3.5 h in the household type and 2.1 h in the air-ventilated type to cool down to 10 °C.

In 1-liter portions, growth of *Cronobacter* spp. was predicted to vary in both types of refrigerators from 0.2 to 2.3 log CFU/ml. In bottles placed in household refrigerators, the

increase in numbers was on average limited to 0.2 or 0.3 log CFU/ml. The maximum increase in bottles placed in household refrigerators was 1.1 log CFU/ml for refrigerators with the poorest heat transfer properties. In most air-ventilated refrigerators set at 7 °C no increase (0.0 log on average) was predicted in bottles during 24 h, while the upper limit of the confidence interval was 0.3 log CFU/ml. The variability of both the time to reach 10 °C and the increase in cells over 24 h mainly originated from the considerable variability of the overall heat transfer coefficient (Table 3).

Varying refrigerator temperature

Figure 6 shows the effect of air temperature in the refrigerator on the predicted growth of *Cronobacter* spp. during cooling down from 40 °C in the 1-liter-measuring beaker and in the Type 1 bottle filled with 120 ml. Assuming an average heat transfer coefficient of 10 W m⁻² °C⁻¹, no growth was predicted to occur in the bottle if the air temperature was 6 °C or lower. Over 6 °C, the numbers were predicted to increase slightly within 4 h, whereas up to 24 h more significant multiplication was predicted, depending on the temperature.

In contrast to the bottle, the 1-liter container was predicted to support growth at all refrigerator temperatures. This can be explained by the fact that the resulting temperature of the formula (T_c) during cooling in this container is higher than in the bottles, due to the higher V/A ratio (Equation 1) in the 1-liter container.

At the common range of refrigerator temperatures (below 7 °C), growth was predominantly in the first 4 h of cooling, while growth was limited in the period between 4 and 24 h. At higher temperatures, however, *Cronobacter* spp. was able to continue multiplying after the first 4 h. With the refrigerator set at 16 °C a 4.2-log increase in the bottle and an almost 5.5-log increase in the beaker were predicted.

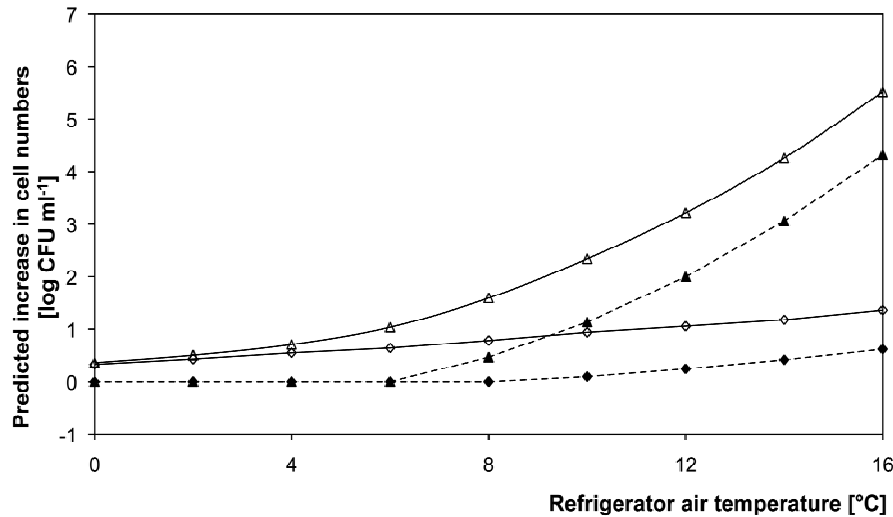


Figure 6. Increase in cell counts as a function of refrigerator temperature at (diamonds), 4 h and (triangles), 24 hours after reconstitution at 40 °C, assuming an average ($10 \text{ W m}^{-2} \text{ °C}^{-1}$) heat transfer coefficient. Open symbols and solid lines represent the 1-liter measuring beaker and closed symbols, dotted lines the Type 1 baby bottle.

Effect of reconstitution temperature

Figure 7A shows the impact of reconstitution temperature on the predicted growth of *Cronobacter* spp. in formula placed for 24 h in a household refrigerator at 4 °C with a heat transfer coefficient that is either average, or at the upper or lower limits of its 95%-confidence interval. In the 1-liter beaker growth was predicted to occur at reconstitution temperatures over 25, 32 or 37 °C, depending on the assumption for the heat transfer coefficient. Below a reconstitution temperature of 25 °C no growth was predicted under any circumstances. For the 120-ml bottle our model did not predict growth, unless the reconstitution was over 35 °C and the heat transfer coefficient was at its lower confidence limit. Reconstitution at more than 50 °C was not included, since our experiments and simulations were not suitable to measure and to predict cell numbers after thermal inactivation which likely plays a role at these temperatures. For comparison with our study, results obtained using the microbial risk assessment model (25) are shown in Figure 7B as an increase in relative risk compared to a base-line scenario, which is reconstitution at

25 °C. Simulations were limited to 24 h cooling. Although the end-points were not identical, the microbial risk assessment model and our study concluded that in bottles growth will either not occur (both studies), or will be limited to 0.45 log units in case of poor heat transfer (this study). When cooling 1-liter containers, however, the reconstitution temperature was found to affect the proliferation of *Cronobacter* spp. and it may well affect growth of other micro-organisms, possible present in the reconstituted infant formula. Our study predicted growth of *Cronobacter* spp. already in 1 liter of formula after reconstituting at 26 °C when placed in a household refrigerator with the poorest heat transfer properties.

It should be noted that the latter situation is not a worst case scenario. The heat transfer coefficient assumed in our study was measured in an empty refrigerator. Refrigerators in a home situations may have even poorer heat transfer properties, because the refrigerator may be packed with other food products, and the door may be opened to frequently, or bottles may be placed in the refrigerator door, where heat transfer is at its minimum (15). Moreover, the *Cronobacter* spp. cells used in this study were present in the powder and can be expected to have a longer lag phase than actively growing cells. When, formula is contaminated during reconstitution with cells that are actively growing, a shorter lag phase and quicker multiplication may be envisioned.

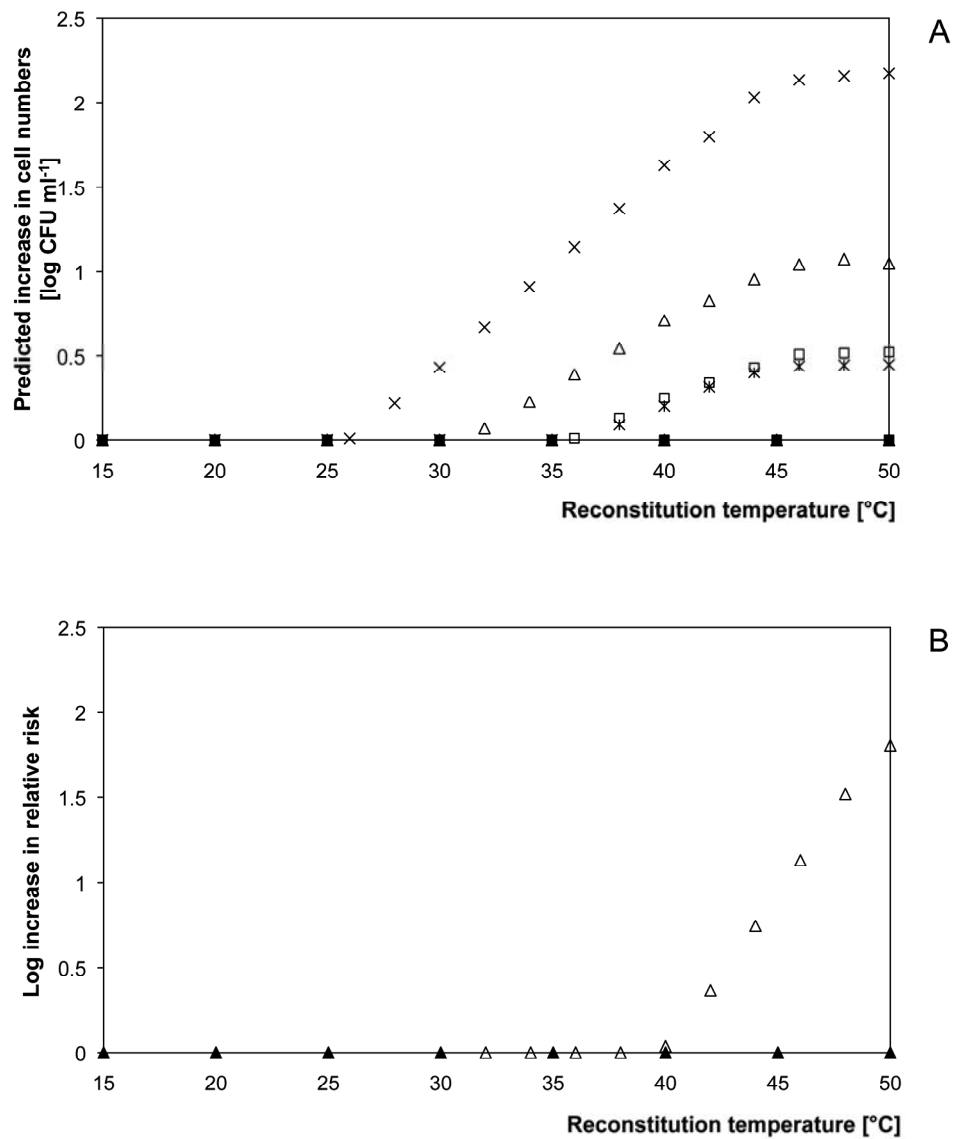


Figure 7. The effect of reconstitution temperature on (A) increase in log count (this study) and (B) increase in relative risk (25) during cooling for 24 hours in a refrigerator with an air temperature of 4°C. Heat transfer coefficients are assumed to be (triangles), average ($10 \text{ W m}^{-2} \text{ °C}^{-1}$); (crosses) at lower ($6.7 \text{ W m}^{-2} \text{ °C}^{-1}$); and (squares) at upper ($13 \text{ W m}^{-2} \text{ °C}^{-1}$) limit of confidence interval. Open symbols represent a 1-liter beaker and closed symbols the Type 1 bottle filled with 120 ml of infant formula.

Possible control measures

Focusing on the cooling phase only, there are several control measures that may help to prevent and minimize growth of *Cronobacter* spp. in situations where bottles and/or syringes of infant formula have to be prepared in advance from powdered formula for use within 24 h. For example:

- Cooling reconstituted infant formula in portion size containers only, such as bottles, may prevent multiplication quite drastically as compared to cooling of 1-liter portions.
- Replacing household-type refrigerators with air-ventilated refrigerators with better heat transfer properties is effective in reducing growth during cooling. However, air-ventilated refrigerators can not totally prevent growth in 1-liter portions of prepared formula with reconstitution temperatures around 40 °C.
- Our study predicts no growth during cooling after formula reconstitution at or below 25 °C. This may suggest that limiting the reconstitution temperature to 25 °C might be an effective control measure, provided that reconstitution is followed by immediate cooling of bottle-sized portions in a refrigerator. The formulae used in this study dissolved well at lower temperatures; for those powders that may not there may be not dissolve at room temperature and need to be reconstituted at 40 °C or more, quick cooling of bottle-sized portions (for instance using running water or ice) may be an alternative to prevent growth during cooling. Reformulation to allow powders to dissolve at lower temperature would, however, be preferred.
- Although lowering the set points of refrigerators in neonatal care units and/or households to 2 °C or less might seem a likely control measure, our results suggests that this may have only a very limited effect on multiplication during cooling. Only when the current set point is above 7 °C such measure might have a significant effect.

This study provides measurements of both heat transfer characteristics and microbiological growth of *Cronobacter* spp. in containers of reconstituted infant formula, along with a model that is able to estimate the variability of these processes taking place simultaneously

under dynamic temperature conditions. Powdered infant formula manufacturers, neonatal health care professionals and (inter)governmental organizations may take benefit from the specific findings to further improve guidelines and best handling practices (33).

The lag time, which is known to be highly variable, has a remarkable impact on the overall growth opportunities of *Cronobacter* spp., as it affects both the moment in time and the temperature at which the organism starts to grow. The predictive model proposed can be used to estimate the proliferation of *Cronobacter* spp. and potentially of other micro-organisms that might be present in powdered infant formula. Using the predictive model, exposure of consumers can be simulated for different scenarios of preparation and consumption of infant formulae, thereby aiding governments and industries to identify effective control measures to protect the vulnerable consumers of this product.

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Chapter 8

**Inactivation rates of *Cronobacter* spp.
and selected other bacterial strains
in powdered infant formulae stored
at different temperatures**

Abstract

The aim of the study was to determine the survival of two strains of Cronobacter (Enterobacter sakazakii) and six other bacterial strains inoculated into dry powdered infant formula (PIF) stored for 22 weeks at several temperatures between 7 and 42 °C. The experimental setup involved a relatively high initial concentration of bacteria, around 10⁴ CFU/g of powder, and enumeration of survivors with a minimum detection level of 100 CFU/g. For all strains tested, it was found that the number of bacterial cells decreased faster with increasing temperature. Cronobacter spp. cells generally survived better at high temperatures (37 and 42 °C) than the other bacteria, while such a difference in survival was not apparent at other temperatures. To describe the effect of temperature on survival, both the Weibull distribution model and the log-linear model were tested. At 22 °C, decline rates of 0.011 and 0.008 log units/day were found for Cronobacter sakazakii ATCC 29544 and Cronobacter strain MC 10, respectively. Assuming a linear relationship between log transformed D-values and temperature, z-values estimated for C. sakazakii ATCC 29544 and Cronobacter MC10, were 13.3 and 23.5 °C, respectively. Such differences found in resistance among Cronobacter spp. would be relevant to consider when establishing quantitative risk assessments on consumer risks related to PIF.

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“Inactivation rates of *Cronobacter* spp. and selected other bacterial strains in powdered infant formulae stored at different temperatures”

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Introduction

The International Commission on Microbiological Specifications for Foods (ICMSF) has placed *Enterobacter sakazakii* in the category of “severe hazards for restricted population”, being rarely involved in food-borne disease among neonates (14). Over the past fifty years, an overall mortality rate of 19% was estimated, based on data presented in the Food and Agricultural Organization and the World Health Organization report (11). Only recently, this Gram-negative micro-organism has been reclassified (16, 17) as 6 species in a new genus, *Cronobacter* gen. nov., within the family of *Enterobacteriaceae*. Five of the new species are *Cronobacter sakazakii*, *C. turicensis*, *C. malonaticus*, *C. muytjensii* and *C. dublinensis*. The sixth species is referred to as genomospecies I and currently includes two representative strains. The collective of species originally known as *E. sakazakii*, can be referred to as *Cronobacter* spp. The designation *Cronobacter* spp. is used consistently in the subsequent part of this paper when the micro-organisms are referred to in general terms or when historic information that is not specific to the various newly recognized species is cited.

Cronobacter spp. are opportunistic pathogens that have caused illness particularly in premature and neonatal infants. In several cases, the source of infection has been identified as powdered infant formula (PIF) (4, 13, 26). Levels of *Cronobacter* spp. in PIF are thought to be low, when present at all, with the mean level in contaminated product possibly being in the order of 10^{-3} CFU/g (8, 10). Such levels, however, reflect operations that lack stringent hygiene measures as reductions ranging from about 10^{-4} to 10^{-6} CFU/g may be possible in adequately hygienic operations (5, 11). The prevalence of these and other micro-organisms in PIF may depend on many factors, including factors related to manufacturing process, choice of ingredients and postprocess handling. Prevalence figures for *Cronobacter* spp. in PIF are scarce, but may differ widely as they have been reported to be about 1% and 3% (data quoted by Food and Agricultural Organization/World Health Organization in 2008) (11), 6.7% (29) and less recently 14% (22). *Cronobacter* spp. has not been consistently isolated from PIF. As an example in one study it could not be isolated in milk- or soy based infant formulae or the infant herb drinks (24).

Cronobacter spp. are vegetative micro-organisms that are not particularly heat resistant (23). Reconstitution with water of 70 °C before final use of the dry PIF will decrease the number of micro-organisms by at least 100,000 fold, rendering the final product safe (10). It has been reported that the micro-organism will not survive pasteurization treatments normally given during manufacture of wet-processed infant formulae (15, 23). However, recent research had shown that several isolates of *Cronobacter* spp. were able to survive spray drying, a process that is not intended to be a pasteurization treatment (1). Though PIF manufacturing is advocated to be undertaken respecting high hygiene conditions and keeping the environment essentially dry, it is important to note that PIF is not manufactured to be a sterile product. Contamination after pasteurization can occur through at least two routes. One such route is via heat-sensitive ingredients, which may have to be added after pasteurization and may introduce *Cronobacter* spp. into the final product when they are present in the ingredients. A second route is environmental contamination. In the case where micro-organisms are present in the factory environment, recontamination after pasteurization may occur from such environmental sources (5). The presence of the micro-organisms in the factory environment may pose a special problem where factory environments become wet, for instance during wet cleaning, allowing *Cronobacter* spp. to multiply rapidly (18). Because the temperature in the factory environments may allow the micro-organisms to proliferate in residues to relatively high numbers in a short time, the rate of survival in dried-up material is a key factor determining the extent of recontamination possible during PIF manufacturing. Quite long survival of *Cronobacter* spp. in dry residues and powder has been reported by several authors (3, 6, 12), but the dynamics have not yet been described mathematically. Conceivably, low water activity (a_w) is an important determinant of the survival of *Cronobacter* spp. in dry factory environments and the same may hold true for the final product during storage and retail. Unfortunately, the impact of a_w on the growth potential of these micro-organisms under realistic conditions has not been reported in great detail.

In this study, the survival rates of two strains of *Cronobacter* spp. was determined in PIF stored at a number of different temperatures and compared to those of six other micro-organisms. The specific aim was to quantify key parameters that express the inactivation

rate of *Cronobacter* spp. due to desiccation in PIF at low a_w at several different temperatures. Such parameters are considered important for microbiological risk assessments and for product/process design studies. Formula manufacturers may be able to use the data in establishing adequate Good Hygienic Practices (GHP) and Hazard Analysis Critical Control Point (HACCP) plans.

Materials and Methods

Preparation of the inoculum

The following strains, *Cronobacter sakazakii* ATCC 29544 (*Enterobacter sakazakii*), *Cronobacter strain* MC10 (*Enterobacter sakazakii* MC10), *Escherichia coli* O2K- ec0001, *Enterobacter cloacae* eb0001, *Klebsiella pneumoniae* kl0001, *Salmonella* serovar Enteritidis (phage type 4), *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 14458 SEB were used. The stock cultures were stored at -20 °C in cryo vials (Greiner Bio-one GmbH, Frickenhausen, Germany), containing 0.3 ml 87% glycerol (Fluka-Chemica GmbH, Buchs, Switzerland) and 0.7 ml of the bacterial culture. *Cronobacter* MC10 is a clinical isolate, kindly provided by Dr. Harry Muytjens (University Medical Center St. Radboud, Nijmegen, The Netherlands). *Bacillus cereus* ATCC 14579 is an air isolate, *St. aureus* ATCC 14458 SEB is a human feces isolate, while the other strains were food isolates. These strains were obtained from the culture collection of the Laboratory of Food Microbiology (Wageningen University, Wageningen, The Netherlands). All the strains were pre-cultured by adding 100 µl of the stock to 100 ml of Brain Heart Infusion Broth (BHI; Becton Dickinson and Co., Le Pont de Claix, France) and incubating at 37 °C for 22 hours to obtain a mature culture. Grown cells were centrifuged for 10 min at 20 °C at 2958 x g (MSE, Mistral 3000i, Leicester, United Kingdom) and washed twice in 40 ml of 0.85% physiological salt solution. The obtained bacterial suspension with a final concentration of around 10^7 CFU/ml was transferred into tubes of a perfume sprayer (Designed by Gérard Brinard, DA Drogisterij, Leusden, The Netherlands) to artificially contaminate PIF (18).

Inoculation of the powdered infant formula

Powdered infant formula for infants from 0 to 6 months was bought locally and aseptically divided in sterilized square boxes (Greiner bio-one GmbH, Alphen aan de Rijn, The Netherlands), each containing 20 grams. Powdered infant formula was sprayed six times, using a perfume sprayer with an individual bacterial suspension and rigorously shaken, resulting in a final concentration of bacterial cells in the dry powder between 10^4 and 10^5 CFU/g. The a_w of the contaminated powder was determined using a water activity (a_w) meter (Novasina Aw-box, Pedak, Heythuysen, The Netherlands). The contaminated powder was divided over small plastic containers and placed in desiccators containing saturated lithium chloride (LiCl; Normapur, VWR International Prolab, Fontenay sous Bois France), which has a constant a_w of 0.11. The a_w of the powdered infant formula measured immediately after spraying with the bacterial suspension remained below 0.3. The desiccators with the inoculated PIF were incubated at the following different temperatures: 7, 15, 22, 30, 37, and 42 °C for 22 weeks.

The survival of the micro-organisms during storage at 7, 15, 22 and 30 °C was determined by sampling the contaminated PIF once every two weeks, while contaminated powder stored at 37 and 42 °C was sampled once every week. As the decline in the numbers of bacteria was found to progress very rapidly at 37 and 42 °C and very few data-points reflecting this decline were obtained, an additional experiment was conducted with five of the strains of bacteria (i.e. *Cronobacter sakazakii* ATCC 29544, *Cronobacter* MC10, *E. coli*, *E. cloacae*, and *St. aureus*) with storage at 37 °C. In this additional experiment, samples were taken every day, except on week-end days, for 16 days.

Samples of 1 g of PIF were reconstituted in 9 ml peptone saline solution (NaCl, 8.5 g/L, and neutralized bacteriological peptone, 1 g/L; Oxoid Ltd, Basingstoke, England), and appropriate dilutions were surface plated in duplicate onto tryptone soya agar (TSA; Oxoid Ltd., Basingstoke, England) by using a spiral plater device (Eddy Jet, IUL instruments, IKS BV, Leerdam, The Netherlands). Inoculated plates were incubated for 20 to 24 h at 37 °C and typical colonies were counted by manual enumeration, the detection limit was 100 CFU/g.

Determination of the D-value

To describe the rate of survival of the two strains of *Cronobacter* spp. and the other bacterial strains evaluated over time, both the Weibull distribution model (Equation 1) and the log-linear function (Equation 2) were applied to the bacterial counts and used to determine the decimal reduction time D_w and D_l (20, 28).

$$\log(N_t) = \log(N_0) - \left(\frac{t}{D_w} \right)^p \quad (1)$$

$$\log(N_t) = \log(N_0) - \left(\frac{t}{D_l} \right) \quad (2)$$

where t [days] is the time, $N(t)$ [CFU/g] is the bacterial concentration at time t , N_0 is the initial bacterial concentration at time $t = 0$, the start of the incubation period. D [days] is the time of the first decimal reduction (time required to reduce the population by 1 log unit from the initial level at $t = 0$) as obtained by the log-linear model (D_l) and the Weibull distribution model (D_w).

Parameter p [-] ($p > 0$) in equation 1 has no direct biological relevance; it is a parameter that describes the shape of the inactivation curve. If $p = 1$ then equation 1 is identical to equation 2 and the decline of the Log count is linear (Equation 2).

In order to obtain reliable estimates for D_w and D_l parameters, the experimental data had to meet the following requirements:

- 1) a minimum of three data points were required,
- 2) at least two data points had to be above the detection limit of 100 CFU/g.

Survival data that did not meet these requirements were excluded from fitting using the Weibull model.

All parameters in the Weibull distribution model and the log-linear model were obtained via minimizing the residual sum of squares (RSS) using the solver option in Microsoft Office Excel 2003 and were verified using Table curve 2D, for Windows 2.03. (Jandel Scientific, Erkrath, Germany). The goodness of fit of both the Weibull model and the log-

linear model was assessed by calculating the mean square error (*MSE*). This is done by dividing the *RSS* value by the degrees of freedom (*DF*). In addition, for each *p*-value the 95% confidence interval was calculated; when $p = 1$ falls within the 95% confidence interval, then the log-linear model was also suitable to describe the data.

Calculation of the z-value

Traditionally, it has been assumed that the temperature dependence of the *D*-value versus the temperature is log linear and can be expressed with the *z*-value. The *z*-value is the increase in temperature (°C) that is needed to reduce or extend the *D*-value by a factor of ten. The estimated *D*-values were therefore log transformed in order to obtain a linear relationship with temperature (27). Equation 3 was used to estimate the *z*-values.

$$\log(D_{ref}) = \log(D_{refmodel}) - \left(\frac{T - T_{ref}}{z} \right) \quad (3)$$

where $\log(D_T)$ [log days], is the logarithm of the *D*-value [days], $\log(D_{refmodel})$ is the log *D_{ref}* value at T_{ref} , and T_{ref} is the reference temperature [°C].

Results

This study assessed the survival of eight strains of bacteria, i.e. *Cronobacter sakazakii* ATCC 29544, *Cronobacter* MC10, *E. coli*, *E. cloacae*, *Kl. pneumoniae*, *Salmonella*, *B. cereus* and *S. aureus*, in powdered infant formula during storage for 22 weeks at 7, 15, 22, 30, 37 and 42 °C. A key parameter determining the potential survival in PIF is water activity, and this parameter was monitored during storage. After 5 days of storage at 30 °C, 37 °C and 42 °C, the average water activity had dropped to 0.129 (-). The water activity decreased more slowly at 7, 15 and 22 °C, reaching values of 0.286, 0.180, and 0.152 [-] after 5 days storage. Since the water activity varies with temperature, and products were stored at different temperatures some level of inaccuracy may be associated to the results reported here.

Figures 1 and 2 show the enumeration results for the various bacterial strains at the various temperatures over time. Symbols on the detection limit, 100 CFU/g, indicate that the results were either on or below the detection limit. At room temperature (Figure 1; results at 22 °C) the trend for all strains was gradual inactivation, although the data for individual strains showed a somewhat erratic profile. Notably, the first strain that declined below the detection limit was *B. cereus* at 7 weeks of incubation. The other strains were detectable significantly longer, with *S. aureus* detectable up to 17 weeks, *Kl. pneumoniae* and *Salmonella* up to 18 weeks and *E. coli* and *E. cloacae* up to 20 weeks. After 22 weeks, at the end of storage, *C. sakazakii* and *Cronobacter* MC10 were still detectable, with the latter at the higher level of the two.

Compared to room temperature storage, the apparent decline at 37 and at 42 °C progressed very rapidly for all bacteria investigated, while a somewhat faster decline could be seen at 30 °C and a somewhat slower decline was noticeable at 15 and 7 °C. At the two highest temperatures evaluated (37 and 42 °C), *Cronobacter* spp. strains, *E. cloacae*, *E. coli* and *S. aureus* could be recovered incidentally beyond the first week of storage. All the other strains tested were below the detection limit of 100 CFU/g already after one week. Because of the limited number of data points obtained in this experiment, decline rates at 42 °C could only be estimated for *C. sakazakii* and *Cronobacter* MC10 and these were calculated to be 0.07 and 0.08 log units/days, respectively.

In order to gather more data points at 37 °C, which would allow to more accurately quantify the decline in cell numbers during the initial incubation period, a second experiment was performed with five of the strains, namely *C. sakazakii*, *Cronobacter* MC10, *E. coli*, *E. cloacae*, and *S. aureus*. In this experiment, incubation was for a period of 16 days, with more frequent monitoring of the levels of bacteria than in the first experiment. The results obtained are depicted in Figure 2. For all the strains tested in the second experiment, at least a 2-log CFU/g decrease was found within the 16 days of the incubation period. Solid and broken lines indicate fitting of the data-points with a nonlinear model (the Weibull model shown in Equation 1) and a linear model (the log-linear model shown in Equation 2). Data fitting did include data points on or below the detection limit. Figure 2 indicates that for several bacteria, inactivation in PIF at 37 °C shows a rather nonlinear curve.

For the various data obtained in the first and second storage experiment the times for the first decimal reduction, where possible, were calculated as a reflection of the inactivation rate (Table 1). To quantify the progressive decline in cell numbers during storage, two different modeling approaches were used to establish times for the first decimal reduction (D). Decimal reduction times were estimated by fitting the log-transformed data with either the Weibull distribution model (Equation 1), giving D values referred to as D_w , or the log-linear model (Equation 2), giving D values designated D_l . In both cases, D expresses the rate of the first decimal reduction, which for the log-linear model is equal to the rate of subsequent decimal reductions, whereas for the Weibull model this D_w value can change depending on the value for parameter p . With $0 < p < 1$, a fitted curve shows a decline in rate over time and thus a slow down of the speed of inactivation. From Table 1 it can be deduced for all bacteria investigated that, while there were sometimes notable differences between the absolute values of D_w and D_l , they were often in the same order of magnitude for individual bacteria at specific temperatures but more importantly consistently showed higher initial inactivation rates with an increase in storage temperature.

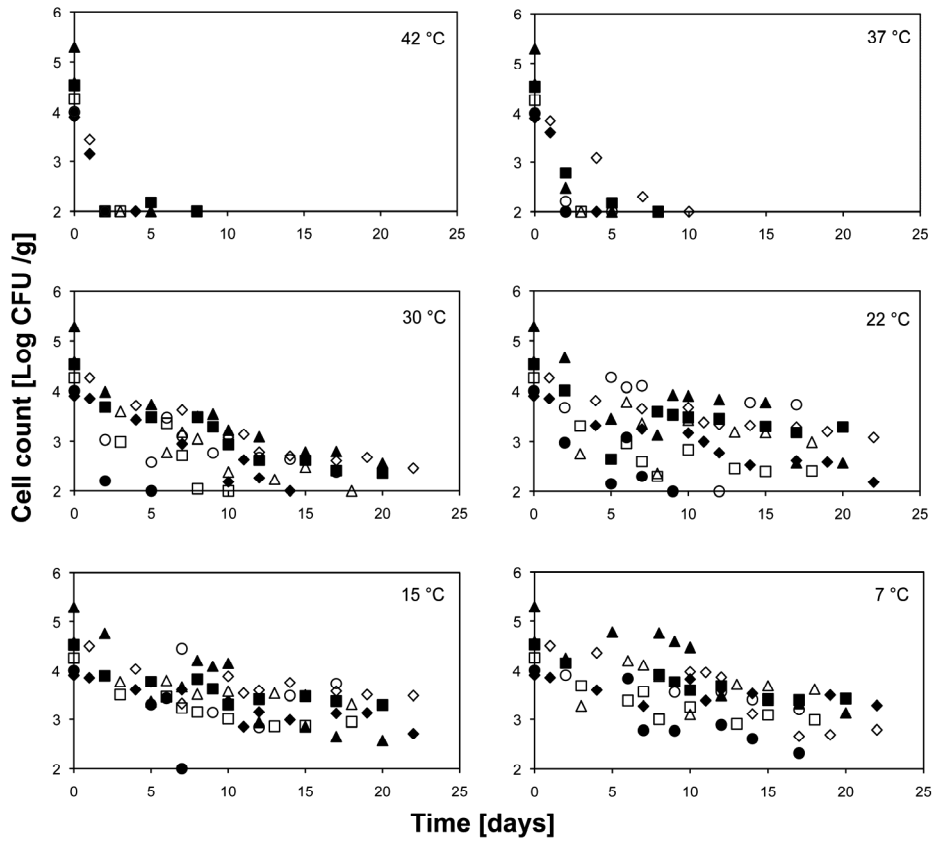


Figure 1. Enumeration of various bacterial strains in powdered infant formula at 42 °C, 37 °C, 30 °C, 22 °C, 15 °C and 7 °C for a period of 22 weeks. ◆ = *Cronobacter sakazakii* ATCC 29544; ◇ = *Cronobacter* MC10 ; ▲ = *E. cloacae* eb0001; ■ = *E. coli* ec0001; ○ = *S. aureus* 14458 SEB; ● = *B. cereus* ATCC 14579; □ = *Kl. pneumoniae* k10001 ; △ = *Salmonella*.

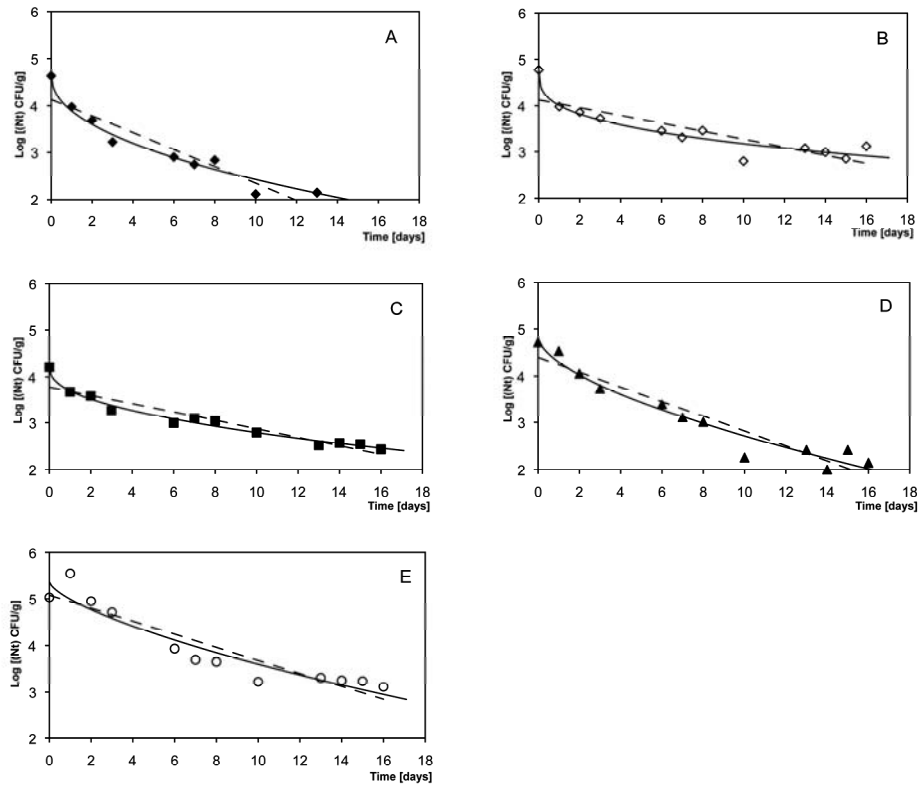


Figure 2. Survival of various bacterial strains in powdered infant formula at 37 °C for a period of 16 days, the fit of the Weibull model (—) and the log linear regression (-----). ♦ = *Cronobacter sakazakii* ATCC 29544 (A); ◇ = *Cronobacter* spp. (B); ■ = *E. coli* ec0001 (C); ▲ = *E. cloacae* eb0001 (D); ○ = *S. aureus* 14458 SEB (E).

Table 1. Overview of the estimated D_I -en D_w -values, using both the Weibull distribution model and the log-linear model, of *Cronobacter sakazakii* ATCC 29544 ; *Cronobacter* MC10 ; *E. cloacae* eb0001; *E. coli* ec0001; *S. aureus* 14458 SEB; *B. cereus* ATCC 14579; *Kl. pneumoniae* kl0001; *Salmonella* at various temperatures

Strain	Weibull model					Log-linear model				
	Temp. [°C]	D_w [days]	95% Confidence interval	p	MSE_w	D_I [days]	95% Confidence interval	MSE_I	$MSE_w \leq MSE_I$	$p = 1$ in 95% confidence interval
<i>C. sakazakii</i> ATCC 29544	7	713	-1,521 to 2,948	0.40	0.031	332	55 - 608	0.031	Yes	Yes
	15	128	47 - 209	0.67	0.035	142	88 - 196	0.035	Yes	Yes
	22	78	44 - 113	0.75	0.019	95	77 - 114	0.023	Yes	Yes
	30	49	14 - 84	1.00	0.047	49	35 - 64	0.037	No	Yes
	37	- ^a	-	-	-	14	-3 to 32	0.019	No	No
	37 ^b	2	1 - 3	0.46	0.021	7	5 - 9	0.097	Yes	Yes
	42	-	-	-	-	15	-18 to 49	0.049	No	No
	<i>Cronobacter</i> MC10	7	88	48 - 127	1.32	0.075	73	52 - 95	0.075	Yes
	15	102	50 - 253	0.38	0.053	162	59 - 265	0.077	Yes	Yes
	22	67	36 - 97	0.46	0.007	119	84 - 154	0.030	Yes	No
	30	38	14 - 63	0.57	0.020	76	57 - 94	0.050	Yes	No

Table 1. continued

Strain	Temp. [°C]	Weibull model				Log-linear model				
		D_w [days]	95% Confidence interval	p	MSE_w	D_l [days]	95% Confidence interval	MSE_l	$MSE_w \leq$ MSE_l	$p = 1$ in 95 % confidence interval
	37	15	-26 to 56	0.66	0.010	23	12 - 35	0.039	Yes	Yes
	37 ^b	2	-1 to 5	0.32	0.025	12	7 - 16	0.089	Yes	No
	42	-	-	-	-	12	-19 to 42	0.123		
<i>Salmonella</i> spp.	7	121	69 - 174	0.70	0.008	128	86 - 170	0.011	Yes	Yes
	15	64	13 - 116	0.26	0.006	133	53 - 214	0.048	Yes	No
	22	47	3 - 91	0.46	0.017	86	45 - 128	0.511	Yes	No
	30	17	-12 to 46	0.47	0.069	50	25 - 75	0.135	Yes	No
	37	-	-	-	-	-	-	-		
<i>B. cereus</i>	7	63	-26 to 151	0.82	0.135	71	26 - 116	0.112	No	Yes
ATCC 14579	15	45	-10 to 100	6.96	0.204	33	-42 to 108	0.407	Yes	Yes
	22	14	-50 to 79	0.41	0.199	37	4 t-69	0.209	Yes	Yes
	30	-	-	-	-	-	-	-	No	No
	37	-	-	-	-	-	-	-	No	No

Table 1. continued

Strain	Temp. [°C]	Weibull model				Log-linear model				$p=1$ in 95% confidence interval
		D_w [days]	95% Confidence interval	p	MSE_w	D_l [days]	95% Confidence interval	MSE_l	$MSE_w \leq MSE_l$	
<i>Kl. pneumoniae</i> KI0001	7	59	-9 to 127	0.41	0.030	109	49 - 170	0.059	Yes	No
	15	46	9 - 82	0.37	0.011	104	52 - 156	0.054	Yes	No
	22	13	-18 to 44	0.30	0.054	81	26 - 135	0.161	Yes	Yes
	30	23	-91 to 137	0.63	0.320	32	-2 - 66	0.225	No	Yes
	37	-	-	-	-	-	-	-	No	No
<i>S. aureus</i> 14458 SEB	7	147	113 - 183	1.17	0.004	151	116 - 186	0.003	No	Yes
	15	253	-1345 to 1852	0.55	0.293	200	-287 to 687	0.251	No	Yes
	22	169	-35 to 373	2.34	0.018	281	-60 to 623	0.023	Yes	Yes
	30	35	-66 to 136	0.29	0.095	101	24 - 178	0.124	Yes	No
	37 ^b	4	-1 to 9	0.67	0.094	7	5 to 9	0.113	Yes	Yes

Table 1. continued

Strain	Temp. [°C]	Weibull model				Log-linear model				
		D_w [days]	95% Confidence interval	p	MSE_w	D_l [days]	95% Confidence interval	MSE_l	$MSE_w \leq$ MSE_l	$p = 1$ in 95 % confidence interval
<i>E. colovacae</i> eb0001	7	67	16 - 118	1.17	0.009	59	38 - 80	0.082	Yes	Yes
	15	35	-24 to 93	0.72	0.230	54	31 - 76	0.215	No	Yes
	22	22	-39 to 83	0.47	0.257	65	27 - 103	0.275	Yes	Yes
	30	11	1 - 20	0.38	0.016	63	40 - 85	0.114	Yes	No
	37 [†]	3	0 - 6	0.61	0.051	6	5 - 8	0.086	Yes	No
<i>E. coli ec0001</i>	7	101	55 - 147	0.52	0.008	127	87 - 168	0.019	Yes	No
	15	73	-2 to 149	0.32	0.017	140	68 - 212	0.046	Yes	No
	22	64	37 - 91	0.38	0.004	119	71 - 168	0.035	Yes	No
	30	35	5 - 65	0.56	0.034	70	50 - 90	0.057	Yes	No
	37 ^b	5	3 - 7	0.45	0.007	11	8 - 14	0.034	Yes	No

^a experimental data did not meet the criteria for determination of D -values (see "Materials and Methods").

^b estimates obtained from data from the second experiment at 37 °C

Comparing the D_w -values in Table 1 for the various bacteria, *St. aureus* did not show a consistent decrease in numbers at 22 °C, resulting in a D_w -value of 169 days, whereas *E. cloacae*, *B. cereus* and *Kl. pneumoniae* showed very fast decimal reduction (D_w -value of 22, 14 and 13 days, respectively). The D_w -values at 22 °C calculated for the two *Cronobacter* strains (78 and 67 days) were comparable with the D_w -value estimated for the *E. coli* strain (D_w -value of 64 days) and somewhat higher than that of *Salmonella* (D_w -value 47 days). For individual micro-organisms, D_w -values at 22 °C were generally higher at 7 or 15 °C, while these values were lower at 30 °C or above. For the two strains of *Cronobacter* spp., D_w - and D_l values at 22 °C were quite different, being 78 days and 95 days, respectively 67 days and 119 days for *C. sakazakii* and *Cronobacter* spp. MC10, respectively.

The fit of the two models with the data points, expressed in mean square error (MSE) values, showed that the Weibull model generally provided the better fit as compared to the log-linear model (Table 1). The estimated values for MSE_w and MSE_l , respectively for the Weibull model and the log-linear regression model, were similar in many instances, with better fits often at the lower storage temperatures. In 31 (84%) of the 37 cases (as indicated in the former last column in Table 1), the Weibull model showed equal or lower MSE values than the log-linear model meaning that, overall, this model provided for the better fit of the data, reflecting a rapid first decline followed by successively slower inactivation.

Table 1 shows the values computed with the Weibull model for parameter p (Equation 1). In the last column an indication is given whether the value for parameter p is equal to 1 within the confidence interval of the data obtained and thus Equation 1 equals Equation 2, the log-linear model. From the total of 37 cases, the log-linear model is valid 19 times (51%) on the basis of parameter p . However, generally, differences in MSE values between models were relatively small and also on the basis of the p parameter in the Weibull model it appeared that either model might be applicable in certain cases.

In an effort to characterize the effect of temperature on the decline of bacteria in PIF in quantitative terms, the log transformed first decimal reduction times calculated with the Weibull model (D_w -values), were plotted against the incubation temperature (Figure 3).

D_w values rather than D_l values were chosen as the Weibull model fitted the data generally better than the log-linear model. Assuming a linear relation for the temperature impact on survival, z -values were calculated with Equation 3 using 22 °C as the reference temperature.

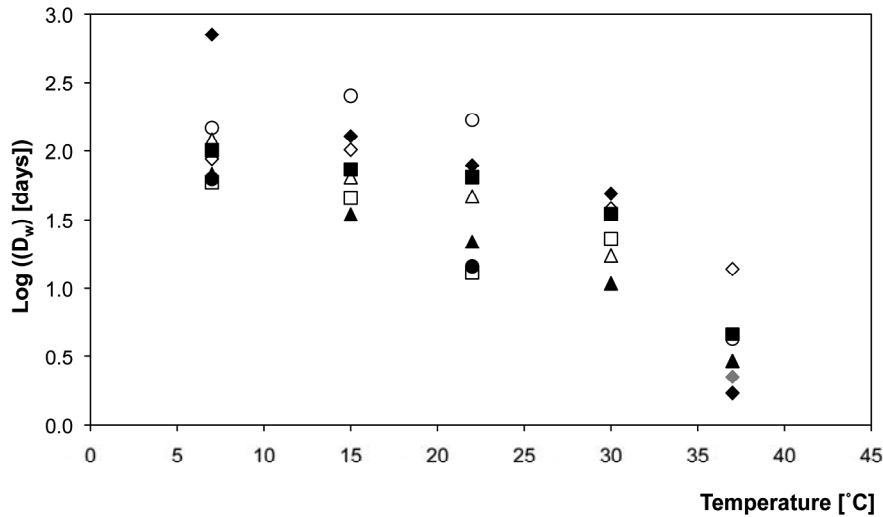


Figure 3. The log transformed estimated first decimal reduction time (D_w -values) for several microorganisms in powdered infant formula at various temperatures. ◆ = *Cronobacter sakazakii* ATCC 29544; ◇ = *Cronobacter* MC10; ▲ = *E. cloacae* eb0001; ■ = *E. coli* ec0001; ○ = *S. aureus* 14458 SEB; ● = *B. cereus* ATCC 14579; □ = *Kl. pneumoniae* kl0001; △ = *Salmonella*.

Table 2 presents the calculated z -values and the log $D_{ref\ model}$ values for the various bacterial strains investigated. From these data the inactivation rate of strain *C. sakazakii* ATCC 29544 ($z=13.3$ °C) seemed to be the most temperature depended while the inactivation rate of *Kl. pneumoniae* was the least temperature dependent ($z = 43.3$ °C) during incubation in powdered infant formula. Estimated z -values for *Cronobacter* MC10, *B. cereus*, *E. cloacae* and *E. coli* were all very close together (range: 23.3 to 25.2 °C) while the z -value for *St. aureus* was relatively low (19.2 °C) and that of *Salmonella* relatively high (28.2 °C).

Table 2. Estimates of the z -values for *Cronobacter sakazakii* ATCC 29544; *Cronobacter* MC10; *E. cloacae* eb0001; *E. coli* ec0001; *S. aureus* 14458 SEB; *B. cereus* ATCC 14579; *Kl. pneumoniae* kl0001; *Salmonella* at 22 °C

Strain	z -value [°C]	Log ($D_{ref\ model}$ [days]) ^a
<i>C. sakazakii</i> ATCC 29544	13.3	1.77
<i>Cronobacter</i> MC10	23.5	1.59
<i>Salmonella</i>	28.2	1.58
<i>B. cereus</i> ATCC 14579	23.5	1.23
<i>Kl. pneumoniae</i> kl0001	43.3	1.40
<i>S. aureus</i> 14458 SEB	19.2	1.81
<i>E. cloacae</i> eb0001	23.3	1.25
<i>E. coli</i> ec0001	25.2	1.59

^a D_{ref} with T_{ref} 22 °C.

Discussion

In this study we investigated the dynamics of several different bacteria possibly associated with powdered infant formula including two strains of *Cronobacter* spp., the genus that has been identified as an opportunistic pathogen potentially present in PIF and a risk factor for specific vulnerable consumers. PIF is not designed and processed to be a sterile product and as a consequence storage, handling and preparation before consumption have to take account of the presence of potentially harmful bacteria. Though much has been done in manufacturing operations in terms of process optimization and control of hygiene, at present it is not possible to totally eliminate *Enterobacteriaceae*, including *Cronobacter* spp. from the processing environment in practice (10, 21). In effect, bacteria may be present in PIF after manufacture and packaging. However, adherence to proper hygiene and manufacturing practices tailored to PIF production should ensure levels of, for instance, *Cronobacter* spp. to be below 10^{-4} CFU/g (5). Levels of *Cronobacter* spp. in contaminated PIF have been reported, in three surveys, in the range of 0.002 to 0.92 CFU/g (8). Whether and how the level of such bacteria changes from the manufacturing phase, during storage

up to consumer use, may depend on factors such as the length of storage and storage temperature.

In this study it was found for all bacteria, that levels in PIF declined over time and that the rate of decline was influenced by storage temperature (Figure 1). At temperatures of 37 and 42 °C, reductions were generally very rapid, but then these temperatures are usually not temperatures at which PIF is stored in practice. However, such temperatures (and higher) are not uncommon in environments surrounding spray-dryers. At 30 °C, the rate of decline was found to be somewhat more rapid than at 22 °C. The latter is a realistic storage temperature generally, while the former could be a valid storage temperature in countries with a tropical climate. Decline at 7 and 15 °C progressed more slowly than at 22 °C. In terms of achieving a reduction in the level of potentially hazardous micro-organisms remaining in PIF when present from the manufacturing phase, storage at room temperature or above may thus have an advantageous effect over cold storage. However, there may be a detrimental impact on product quality and/or nutritional properties of high temperature storage.

For the various bacteria tested, the estimated values for D -values were found to decrease with increasing temperature (Table 1) putting the observations made regarding Figures 1 and 2 on a quantitative basis. Consistently, higher storage temperatures seem to cause or contribute to a progressively rapid die-off of micro-organisms. At 37 and 42 °C, loss of viability is particularly rapid compared to storage at 22 °C. The underlying mechanism for this may relate to metabolic exhaustion at elevated temperatures as reported before (19). Vegetative cells tend to initiate every possible repair mechanism to overcome the impact of temperature stress on metabolism and cell integrity, exhausting their energy reserves, as well as genetic and structural building blocks. Notably, rather the low water activity of the product would render the micro-organisms metabolically inactive within a short space of time. Comparing the five strains of which the decline rate could be derived at 37 °C (*St. aureus*, *E. cloacae*, *E. coli* and both *Cronobacter* spp. strains), it seems that the D_w -value of the two *Cronobacter* spp. strains were slightly lower (2 days at 37 °C) than those of the others (3 to 5 days). For *C. sakazakii* and *Cronobacter* MC10, the estimated D -values at 37 °C were 2 days (for D_w) and 7 and 12 days (for D_l), respectively. D -values between 30 °C

and 7 °C ranged between 49 and 713 days and between 49 and 332 days for *C. sakazakii* and *Cronobacter* MC10, respectively. As indicated by the 95% confidence interval data for the D_w - and D_l -values in Table 1, these ranges occasionally varied considerably. Also the D_w - and D_l -values estimated for the other six bacteria at the six different incubation temperatures evaluated showed significant individual variation. Nevertheless, the data indicated that at 22 and 30 °C, temperatures that can be room temperature in practice, progressive inactivation of most bacteria may take place in the course of storage. At 7 and 15 °C, overall, inactivation was limited to about 2 log cycles for the various bacteria. Notably, at these lower temperatures, the two strains of *Cronobacter* spp. remained viable and well over the detection limit of the experimental set-up for the whole 22 weeks storage period. The fact that these and other bacteria tested here survived under the dry conditions prevailing in PIF, indicates that the micro-organisms possess a certain level of resistance or tolerance to dryness. Many other studies reported resistance to low a_w (2, 3, 6, 12, 15), and together these findings underscore that the survival of *Cronobacter* spp. in PIF is a matter to be carefully considered in assessing the safe preparation of infant formula for final consumption.

Previous studies on the inactivation of *Cronobacter* spp. in dry powdered infant formulae only considered storage at room temperature and expressed the decline as a rate in log units per day. To compare the current findings with those of previous studies, the decimal reduction times found at 22 °C were converted to decline rates (log units/day) by taking the reciprocal of the D -values. Thus, decline rates observed in this study at room temperature were found to be 0.011 and 0.013 log units per day for *C. sakazakii* and 0.008 and 0.015 log units per day for *Cronobacter* MC10, as calculated from the D_l - and D_w -values, respectively. These values compare well with the decline rate of 0.014 log units per day reported in literature (7, 10). However, these literature values were computed considering the inactivation curves to be biphasic. While the first rapid phase of inactivation was characterized by a decline rate of 0.014 log units per day, the second phase showed a much lower decline rate for *Cronobacter* spp. of 0.001 log units per day (10). However, as seen frequently in the current study for this bacterium and several others investigated, decline rates may gradually slow down in the course of storage, a (so-called) tailing effect that was

most pronounced at low storage temperatures (7 and 15 °C). Rather than choosing one decline rate, e.g. the most rapid as the best case or the slowest as the most conservative case, it would be more realistic to consider the actual dynamic change in decline rate over storage in predictions, for instance as part of a microbiological risk assessment study. The data obtained in the current investigation show that a dynamic change in decline rate can be reflected well by using the Weibull model. In Table 3, our findings considering the impact of storage temperature on the decline of *Cronobacter* spp. in PIF are projected as times required for a certain decimal reduction using both the Weibull and the log-linear model. The projected times are in months and are best interpreted considering that the maximum shelf-life of PIF is about 30 months, though it is likely that in most cases PIF is consumed within a shorter time frame (10). Comparing times predicted using the Weibull model, as this generally fitted the dynamic behavior of most bacteria evaluated here best, it can be seen that at 7 °C, an 1-log inactivation in the level of *Cronobacter* spp. may occur within 3 to 24 months of storage. At 22 °C, a 1-log reduction may occur within 2 to 3 months while a 3-log reduction may take place in 11 to 25 months. At elevated temperatures, reduction is faster than at room temperature, while under refrigeration, survival will exceed the maximum shelf life of the product. Interpretation of these calculations should take into account the significant variation in the estimated *D*-values determined in this study and that samples were taken during a 22-week period. Extrapolations beyond that time frame have been indicated in parentheses in Table 3 and should be interpreted as orders of magnitude only.

Table 3. Calculation of a 1-D, 2-D, 3-D and 5-D log reduction, with the Weibull and log-linear model for *Cronobacter sakazakii* ATCC 29544 and *Cronobacter* MC10 at 7, 15, 22, 30, 37, and 42 °C^a

Strain	Temp. [°C]	Time (months) calculated by indicated model to achieve reduction of:									
		1-D log		2-D log		3-D log		5-D log			
		Weibull model	Log-linear model	Weibull model	Log-linear model	Weibull model	Log-linear model	Weibull model	Log-linear model		
<i>C. sakazakii</i> ATCC 29544	7	(24)	(11)	(> 30)	(22)	(> 30)	(> 30)	(> 30)	(> 30)		
	15	4	5	(12)	(9)	(22)	(14)	(> 30)	(24)		
	22	3	3	(7)	6	(11)	(10)	(22)	(16)		
	30	2	2	3	3	5	5	(8)	(8)		
	37 ^b	0.1	0.2	0.3	0.5	1	1	2	1		
<i>Cronobacter</i> MC10	7	3	2	5	5	(7)	(7)	(10)	(12)		
	15	3	5	(21)	(11)	(> 30)	(16)	(> 30)	(27)		
	22	2	4	(10)	(8)	(25)	(12)	(> 30)	(20)		
	30	1	3	4	5	(9)	(8)	(22)	(13)		
	37 ^b	0.1	0.4	1	1	2	1	(10)	2		

^a Values in parentheses exceeded the duration of the experimental and must be interpreted as orders of magnitude only.

^b Calculations of the log reduction according to the $D_{n,r}$ and D_p -values obtained from the second experiment.

Since inactivation kinetics data have been accumulated for several different temperatures in the present investigation, it has been possible to estimate z -values which can be used to relate inactivation kinetics between temperatures. Considering the better fit of the Weibull distribution model with experimental data, z -values for the various bacteria were established on the basis of the D_w -values at different storage temperatures. As shown in Table 2, z -value of 13.3 °C and 23.5 °C were estimated for *C. sakazakii* and *Cronobacter* spp. MC10, respectively. It should be noted that z -values reported in this paper are based on D_w -values as estimated in dry PIF and do not apply to earlier reported thermal inactivation in liquid (25). Thus the given D/z -concept is specific for inactivation in dry PIF at moderate temperatures.

In conclusion, quantification of survival rates of *Cronobacter* spp. in dry PIF may provide useful data for microbiological risk assessments and for product/process design studies, as it allows determining likely levels of the micro-organism in PIF subjected to different scenarios of time and temperature during storage and distribution. Our findings suggest that there may be wide differences in the inactivation rates of *Cronobacter* spp. in desiccated PIF, *Cronobacter* cells are rapidly inactivated in dry powder at temperatures of 37 °C and 42 °C, but that inactivation rates at ambient temperatures or lower tend to progressively slow-down. In most cases, however, inactivation rates may be higher than the rather slow decline rate of 0.001 log units per day assumed in the Food and Agricultural Organization/World Health Organization risk assessment study (11). The Weibull model in conjunction with the inactivation data generated in this study, may be useful for reflecting the dynamic reduction of *Cronobacter* spp. in PIF, for instance, in microbiological risk assessments. Formula manufacturers may be able to use the model and other results in establishing adequate Good Hygienic Practices (GHP) and Hazard Analysis Critical Control Point (HACCP) plans. Several observations may need to be considered in the design of Good Manufacturing Practices (GMP) and HACCP systems for PIF manufacture, including the impact of storage at temperatures over ambient on *Cronobacter* spp. inactivation, D - and z -values and the quantitative substantiation of the very long survival time of *Cronobacter* spp. in PIF. Further work may need to address interstrain differences of *Cronobacter* spp. in

their ability to survive dry environments. Our data, collected from two clinical isolates in this study, should not be assumed to represent a worst-case scenario of the most desiccation-resistant strains that may be present in the environment.

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Chapter 9

Discussion and concluding remarks

Introduction

Cronobacter spp., previously referred to as *Enterobacter sakazakii*, is more and more widely recognized as an opportunistic foodborne pathogen that can potentially cause illness in vulnerable sub-populations of consumers. *Cronobacter* infection is a rare illness and no active surveillance systems exist, though notification of the illness is mandatory in a few countries. All known species of *Cronobacter* have been linked retrospectively in either infants or adults to clinical cases of infection (24). Sub-populations at risk for *Cronobacter* infection can be found among infants and elderly consumers (53). As detailed in the Chapter 1, the number of cases of confirmed *Cronobacter* infections in children worldwide is limited (n=156), as is the number of confirmed death (n=29). In this regard, *Cronobacter* spp. may not appear to be as important a pathogen as many other foodborne pathogens that challenge the safety of our food to date, but because it particularly affects very young infants it is causing a high level of public concern and outrage.

Although the natural source or habitat of *Cronobacter* spp. is not clearly understood, it is increasingly apparent that this group of micro-organisms is present in many different environments and can be found in a wide range of foods. Epidemiological studies have implicated powdered infant formulae (PIF) as a prime vehicle for possible exposure of infants to *Cronobacter* species (1, 77, 82). Since an outbreak of *Cronobacter* infections occurred in the United States (Figure 1), there have been recalls of *Cronobacter* spp. contaminated infant formulae in several countries around the world (28).

Like many other governmental agencies, the United States Food and Drug Administration (FDA) issued an alert to health care professionals regarding the risk associated with *Cronobacter* infections among neonates fed with milk-based powdered infant formulae (26). For the last decade there has been a strong and focused effort amongst all relevant stakeholders (i.e. governments, health care professionals, industry, academia, consumers) to establish harmonized safety criteria, good manufacturing practices and sound guidelines for PIF preparation and use in order to protect the vulnerable consumers from the threat of infection with *Cronobacter* spp..

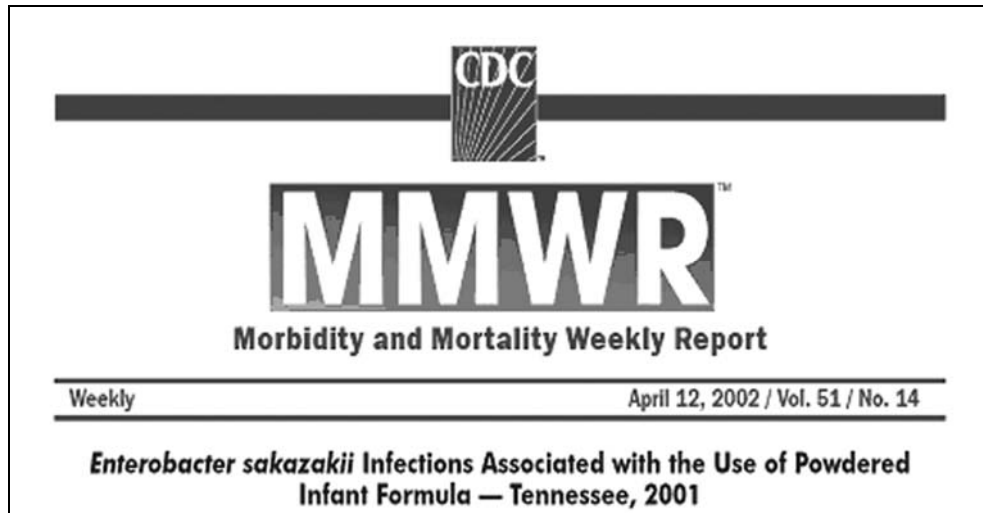


Figure 1. Reported outbreak related to *Cronobacter* spp. in 2002 in the United States (14).

Because the ability to prevent and control *Cronobacter* spp. is intimately associated to the ability to detect and identify the micro-organism in foods and food environments, the progress in methodologies is first discussed. Following that, new insights from the research reported here as well as from other sources as related to our ability to establish sound assessments of consumer exposure to the micro-organism, are described. Finally, potential exposure is simulated for different scenarios of preparation and consumption of infant formulae, using the results of this research and relevant external data to compile particular simulations and to draw conclusions relating to prevention and control of the risk to infants of *Cronobacter* spp. associated with powdered infant formulae.

Methodology

Before the new systematic categorization and nomenclature as described in Chapter 1, *Cronobacter* spp. belonged to the genus *Enterobacter* that contains species such as *E. cloacae*, *Pantoea* spp. (formerly *E. agglomerans*), *E. aerogenes* and *E. gergoviae*. The differentiation of *Cronobacter* spp. among these species is based on biochemical reactions,

and serological and molecular techniques (25). A key feature for *Cronobacter* spp., as compared to other *Enterobacter* species, is the presence of α -glucosidase (60). Based on this feature and the fact that *Cronobacter* spp. typically produce yellow pigmented colonies when grown on tryptone soy agar, early in this research a new practical method for screening *Cronobacter* spp. in foods and food environments was developed. This method is elaborated on in Chapter 2 and it has been used with a range of isolates obtained from environmental samples taken in several different (food) factories and food products (Chapter 3 and 4). The biochemical characteristic of α -glucosidase production has remained the basis for many subsequently developed detection media. Selective media developed using this feature include “*Enterobacter sakazakii* Isolation Agar” – ESIA (AES, France), R&F[®] (*E. sakazakii* chromogenic plating medium) and Druggan-Forsythe-Iversen-medium (DFI) (37). Notably, other *Enterobacteriaceae* species may possess α -glucosidase after 24 hours, but only after induction of the enzyme by a suitable substrate (Chapter 2).

Over the years, methods for the isolation, detection and enumeration of *Enterobacteriaceae*, *Cronobacter* spp. and *Salmonella* spp. have been developed to various degrees of specificity, sensitivity and robustness. Many new methods have been introduced and further refined over the last decade by academics and media manufacturers (Table 1), in particular methods aiming to detect and trace *Cronobacter* spp. in powdered infant formulae.

All methods have a number of steps such as pre-enrichment and (selective) enrichment. Sometimes a resuscitation step is used for the recovery of stressed cells or cells injured by exposure to chemical or physical treatments used in food production or processing (79). In the (selective) enrichment step the specific growth conditions of the target organism(s) are met while growth of other micro-organisms is inhibited to the greatest extent possible. An enrichment procedure may be an essential step in enabling detection of low contamination levels as it can promote the amplification of a single cell to levels of $> 10^3$ CFU/ml of enrichment broth. In an enrichment procedure, typically, a 1:10 dilution of the food matrix is made, thus reducing interferences from the food in the detection assay (9). Reducing the detection level has been an important aim for method development and currently *Cronobacter* spp. can be detected at 1 CFU/100g of PIF, when 100 g is pre-enriched.

The initial detection methods established by the US FDA (2002) (78) and ISO (TS 22964) (36) are currently being re-evaluated, as knowledge regarding *Cronobacter* further improves. For example, the reliance on yellow-pigmentation (25) in ISO TS 22964 is no longer accepted as valid because a high proportion of strains (up to 25%) are non-pigmented on tryptone soy agar (6). It was also shown that some *Cronobacter* strains (2%) (43) are unable to grow in standard *Enterobacteriaceae* enrichment (EE) broth and, therefore, would be missed using only the general *Enterobacteriaceae* test procedures.

Improvements in detection methods for *Cronobacter* have been focussing much on optimising the isolation of the micro-organism from powdered infant formulae. Most published methods to date include a pre-enrichment step, which is followed by a selective enrichment broth prior to chromogenic agar plating. Currently, the international standard method for detection of *Cronobacter* from milk products issued by the International Organization for Standardization (ISO) and designed by the International Dairy Federation (IDF) involves a two-step enrichment (36). The selective enrichment method used in this international standard method has been adapted from the one which has been developed in the research described in this thesis as described in Chapter 3.

Conventional methods are generally more time consuming than the molecular methods described more recently (2, 55, 62, 76, 85). However, the conventional methods showed to be efficient even when low levels of *Cronobacter* cells were found in a sample (0.36 to 66 CFU/100 g) (59). Iversen *et al.*, (40) showed that current cultural media do not support the growth of all *Cronobacter* strains and, therefore, efforts have to be made to improve the cultural methods such that all strains are captured. Although real-time PCR methods for specific detection of *Cronobacter* spp. that target 16S rRNA of the micro-organism are highly sensitive and useful for rapid and direct detection of *Cronobacter* spp. in foods, they are rather qualitative and still rely on a time consuming (pre-)enrichment step. Moreover, for this method a population of 10^2 CFU/ml is required to obtain a 100% detection probability (21). Recently, a fluorescence *in situ* hybridisation (FISH) technique has been described for the detection of *Cronobacter* spp. This method is based on binding of specific probes to target nucleic acid regions and is able to detect *Cronobacter* spp. at a population

level of 10^{-2} CFU/ml after an enrichment of 8 h (2). Note that an enrichment step is necessary to detect such low levels of the micro-organism.

Further research on methodology should best focus on improving the enrichment step, ideally providing a more specific and sensitive enrichment procedure, as this so far remains an important bottle-neck. A key physiological parameter of importance to consider in further methodology development is the lag time (i.e. the time required for adjustment of the microbial cells to a new environment after transfer from another, including recovery from an injured state) of the target micro-organism, as it may add several hours to the overall detection time. Further optimising the sensitivity and speed of detection remains a challenging task, considering the relatively low level of the micro-organism amidst many other micro-organisms and given the heterogeneous distribution within a bulk production of PIF and the complexity and diversity of the food matrix itself. Molecular based detection technologies offer opportunities for increased sensitivity but they may be sensitive to product matrix interference. A major drawback of applying molecular based detection technologies as compared to traditional culture based methods is that they can not clearly differentiate living from dead bacteria. Another disadvantage is that the micro-organism itself is not isolated and can not be studied further. Therefore, while PCR methods for *Cronobacter* spp. detection may offer advantages in identification of the micro-organism, they need to be used in conjunction with culture based confirmation methods.

Table 1. Methods for detection of *Cronobacter* spp. in powdered infant formulae available until 2009

Pre-enrichment (usually selective)	Enrichment (usually selective)	Isolation medium	Identification/Confirmation	Isolation / Identification time (days)	Reference
Sterile water (37 °C)	EE broth (37 °C)	VRBGA plates (36 °C)	Presumptive colonies are streaked onto TSA, followed by API 20 ^E	5-7	(15, 78)
BPW (37 °C)	EE broth (37 °C)	DFI Agar (37 °C)	API 20 ^F	3	(37)
BPW (37 °C)	mLST + vancomycin (45 °C)	TSA (37 °C)	Presence of α -glucosidase	5	(29)
BPW (37 °C)	mLST + vancomycin (45 °C)	ESIA (44 °C)	API 20 ^F	5	(36)
Sterile water (37 °C)	EE broth (37 °C)	ESPM (35-37 °C), ESSM (35-37 °C), and TSA (23-25 °C)	API 20 ^E	3-6	(72)
BPW (37 °C)	CSB (\pm 42 °C)	XTSA (37 °C)	ID 32 ^E , and α -glucosidase based PCR assay	2	(38)
	BPW (37 \pm 1 °C)	DNA extraction after 6 h of enrichment DFI agar (37 °C) and R&F agar (37 °C)	PCR assay	1-2	(15)
	CEB (37 and 41 °C)	Chrom ID Sakazakii agar (37 °C)	PCR assay	2	(66)

API 20^E = biochemical identification,
 BPW = Buffered Peptone Water,
 CSB = *Cronobacter* Screening Broth,
 CEB = *Cronobacter* Enrichment Broth,
 DFI agar = Druggan-Forsythe-Iversen-Agar = Brilliance *Enterobacter sakazakii* Agar,
 EE broth = "*Enterobacteriaceae* Enrichment broth",
 ESIA = "*Enterobacter Sakazakii* Isolation Agar",
 ESSM = R&F *Enterobacter Sakazakii* Screening Medium,
 ESPM = *Enterobacter sakazakii* Chromogenic plating medium = R&F® Agar = ChromID *Sakazakii* = Chrom ID™ *sakazakii* Agar,
 ID 32^E = biochemical identification,
 mLST = modified Lauryl Sulphate Tryptose broth,
 PCR = real time polymerase chain reaction,
 VRBGA = Violet Red Bile Glucose Agar,
 TSA = Tryptone Soy Agar,
 XTSA = modified TSA,
 containing 0.15 g/L 5-bromo-4-chloro-3-indolyl- α -D-glucoside.

Occurrence of *Cronobacter* spp.

Chapter 4 and 5 showed that *Cronobacter* spp. can be found in the production environment of various (dry) food products, including the production facilities for milk powder, chocolate, and cereals and also in various foods present on the Dutch market. Notably, *Cronobacter* spp. were also isolated from vacuum cleaner bags collected in households. Several other studies have since investigated the prevalence of *Cronobacter* spp. in a wide variety of products (4, 13, 27, 39, 45, 54). Overall, these studies have established the ubiquitous or widespread occurrence of *Cronobacter* spp. in food and beverages other than infant formulae, with the presence of the micro-organism being confirmed in food ingredients from plant origin, like cereals, fruits and vegetables, legume products, herbs and spices, as well as in foods of animal origin like milk, meat and fish and products made from these foods (27). *Cronobacter* spp. were isolated from scraping or sweeping surfaces in the production-line environment of factories producing milk powder, cereals, chocolate, potato flour, and pasta, as well as in vacuum cleaner bags from domestic environments (Chapter 4). *Cronobacter* spp. have also been isolated from household cleaning cloths (52) and kitchen brushes (61). Chapter 5 showed that the prevalence of *Cronobacter* spp. on human skin and in human fecal samples may be rather low, at ~1%. The isolation of *Cronobacter* spp. from human samples is not new, as it was earlier isolated from various clinical sources, including a child's stool (25). More recently, *Cronobacter* spp. could be isolated from a diarrhoeal child's stool (69) and from stool samples of hospitalised infants (0.5% prevalence) and patients in the age group 61-70 year (51). These findings may suggest that humans may be carrier of the micro-organism without any symptoms. Consequently, it should be recognized that PIF can be contaminated with *Cronobacter* spp. through human vectors both during manufacture and preparation.

Although *Cronobacter* spp. have been isolated from various environmental and clinical sources and a wide range of food products, so far only powdered infant formulae have been epidemiologically linked with *Cronobacter* infections (1, 7, 16, 61, 65, 77). PIF manufacturing industries around the world have tightened control of *Cronobacter* spp. contamination in PIF and the PIF batches that are marketed generally comply with stringent safety standards established for the presence on *Cronobacter* spp.. However, the bacterium

has been isolated from powdered infant formulae by several investigators (13, 22-24, 32, 39, 57, 59).

It needs to be stressed that PIFs are not designed to be sterile products and that they can occasionally be contaminated with micro-organism, including pathogens. Micro-organisms that can occasionally be present, depending on many factors relating to choice of ingredients, processing, and other manufacturing activities, include *Bacillus* spp., *Clostridium* spp., *Staphylococcus* spp., and *Enterobacteriaceae*, especially *Cronobacter* spp. and *Salmonella* spp. (23).

Regulatory agencies worldwide (22, 23, 83) have issued warnings to professional and domestic carers that the safety of powdered infant formulae needs to include careful handling and preparation of PIF for consumption. While generally healthy infants should be at no elevated risk consuming product prepared according to instructions provided by manufacturers, certain more sensitive infant groups may require that additional care is taken in the preparation and handling of infant formulae. Most notably, this is the case for consumption of PIF by infants less than two months of age, especially those that are of low birth weight, premature and or otherwise immunocompromised.

It is generally accepted that preventive measures implemented to control *Salmonella* spp. occurring in PIF, comprise the basis of possible control measures and are a prerequisite to address and control *Cronobacter* spp. in infant formulae production facilities (17). Until now, contrary to what can be achieved for *Salmonella* spp., *Cronobacter* spp. can not be totally eliminated from milk powder production facilities (30, 48, 58) possibly due to its ubiquitous nature. Surveys in powdered milk facilities have identified the presence of *Cronobacter* spp. throughout the manufacturing operation. Results obtained by sub-typing of *Cronobacter* isolates revealed a genetic diversity of isolates, with most isolates being detected in air filtering samples (42, 56). Prevalence figures of 39% have been recorded for *Cronobacter* spp. in certain environmental samples from processing lines. Notably, such high prevalence figures may be due to ongoing improvements in enrichment and biochemical methods. Some isolates recovered from the PIF final product reported were identical to those from the environmental samples on the basis of similarity of PFGE

profiles, and could be found over a year period in product samples, which may suggest that *Cronobacter* spp. can persist in the production environment (30).

Future research on occurrence might focus on determining the spatial and statistical distribution of contamination, especially in PIF.

Contamination routes of *Cronobacter* spp.

There are three main routes by which micro-organisms may enter PIF during its production and at reconstitution:

- 1) through exposing the product to contaminated equipment, the environment and people handling the product in the course of the manufacture,
- 2) through adding dry ingredients after the pasteurization step,
- 3) through contamination during reconstitution prior to feeding, from the environment or due to poorly cleaned feeding bottles or poorly maintained equipment in hospitals and homes.

Because of the importance to control infections caused by *Cronobacter* spp. potentially present in PIF, it is important that the occurrence of *Cronobacter* spp. in the product ready for consumption is prevented or that its presence in the prepared product is controlled to a level that is considered acceptable for consumer safety. Preventive and control measures need to be taken 1) during production of powdered infant formula (i.e. regarding contamination of product ingredients and recontamination from environmental sources), 2) during reconstitution of PIF (e.g. regarding recontamination from environmental sources and product preparation conditions) and 3) before the reconstituted formula is consumed (e.g. regarding product handling and storage and consumption conditions). Together, prevention and control measures need to ensure that exposure of consumers to *Cronobacter* spp. is minimized.

Regarding the possible occurrence of micro-organisms in PIF, it is worthwhile to discuss a number of characteristics/issues such as their survival in dry powdered infant formulae,

their inactivation by pasteurisation and their growth in reconstituted infant formulae. Such considerations can lead risk managers to specific advice to caregivers of at risk consumers, such as health care professionals in neonatal care units, and to further improve the existing guidelines and handling practices (83).

Cronobacter spp. have the ability to survive under dry conditions (8) as has been documented in the research described in Chapter 8 and it can do so for a long period. Previous studies conclusively showed that *Cronobacter* spp. can be present in various dry environments (46) and that *Cronobacter* spp. cells are able to survive for at least twelve months in powdered infant formulae (12, 18). The micro-organism is clearly able to survive in very dry environments such as those with a water activity < 0.2 [-]. In factories, the incidence of *Cronobacter* spp. could increase after a wet cleaning process or due to uncontrolled presence of water, for instance following an emergency shower (17). Conceivably, therefore, the incidence of *Cronobacter* spp. in infant formula production factories should be reduced by effectively eliminating water throughout the manufacturing operation, by applying appropriate control measures such as the application of adequate dry cleaning procedures within the processing environment and on the processing equipment.

Pasteurization, a heat process applied typically in the range of 60-80 °C for a few minutes, can lead to elimination of specific micro-organisms. Pasteurization is indeed effective in destroying *Cronobacter* spp. cells during dry powdered infant formulae manufacture even if the thermal tolerance of individual *Cronobacter* spp. isolates may differ (41, 64, 67). Recent studies showed that *Cronobacter* spp. strains could survive the spray drying process (3). Acidification could reduce the concentration of *Cronobacter* cells in different types of infant formulae even further (44).

Notably, occasional contamination of PIF may occur after the pasteurization step, either via addition of contaminated ingredients, such as vitamins, or via the factory environment, though in general the incidence of *Cronobacter* in PIF remains low with levels, reportedly between 0.002 to 0.92 CFU/g (21, 63). In 2007, there has been an increase of Rapid Alert System for Food and Feed (RASFF) notifications concerning potential microbiological

contaminations mainly due to some batches of vitamins contaminated with *Cronobacter* spp. (20).

A factor possibly contributing to exposure of infants to *Cronobacter* spp. that has received rather little scientific attention is biofilm formation. Some *Cronobacter* strains can produce exopolysaccharides and thus it is possible that biofilm formation may occur on surfaces commonly associated with PIF feeding equipment (41, 49). Biofilms may be present in a variety of environments, such as process equipment, and kitchens sinks (33) and such biofilms may be a source of contamination. In fact, formation of biofilms may protect bacteria from aggressive conditions, such as resulting from detergents and disinfectants. Disinfectants are used routinely in hospitals, day care facilities and food service kitchens, however they have been shown to be quite inadequate in eliminating biofilms comprised of *Cronobacter* spp. (50). Biofilm may also be formed in enteral feeding, as described further on.

PIF reconstitution and growth of *Cronobacter* spp.

Reconstituted infant formula can either be used immediately or, if indicated on the package, can be covered, refrigerated and used within 24 h (83). Previous results (Chapter 4 an 5) showed the ubiquitous occurrence of *Cronobacter* spp., and the possible outgrowth of the *Cronobacter* spp. in reconstituted infant formula (Chapter 6) (23). It is likely that storage at ambient temperatures may lead to substantial growth. Cells of *Cronobacter* spp. may recover quickly after dry stress (23), in Chapter 6 the quick recovery of *Cronobacter* cells at 37 °C is highlighted. The bacteria were furthermore found to be able to grow at 7 °C, with an optimum being between 37 and 43 °C (39, 47, 63). In Chapter 6 the effect of pre-culturing conditions on the growth was investigated at several temperatures between 8 and 47 °C, and the growth data obtained were used to develop predictive growth models for *Cronobacter* spp. The key growth parameters were quantified applying the secondary growth model of Rosso, resulting in $T_{\min}= 3.6$ °C, $T_{\max}= 47.6$ °C, $\mu_{\text{opt}}= 2.31$ h⁻¹, $T_{\text{opt}}= 39.4$ °C. These parameters could be used to calculate the proliferation of *Cronobacter* spp. in reconstituted infant formula at any temperature below the maximum growth temperature of

47.6 °C. Studying the microbial growth of *Cronobacter* spp. in reconstituted infant formulae, using cells with a different pre-culturing background (being pre-cultured to lag phase, exponential phase and stationary phase, respectively), it was found that the various physiological phases of the cells did not significantly influence its growth in reconstituted powdered infant formulae.

Future research might focus on determining the lag phase of the organism in naturally contaminated powder, although this will not be easy due to initially very low levels.

Chapter 7 focused on the cooling time required for reconstituted infant formula to reach the set refrigerator temperature. A model was established to predict the cooling profiles of reconstituted infant formulae given different containers or refrigerators used. The growth parameters obtained from the research in Chapter 6, were combined with this model into one that allows predicting growth of *Cronobacter* spp. during the cooling process of prepared formulae irrespective of container volume. The model enabled to show quantitatively to what extent practical control measures such as proper cooling or limiting the time between preparation and consumption could help reduce growth of *Cronobacter* spp., thereby likely leading to a reduction in risk as was also reported elsewhere (23, 67).

Cronobacter spp. infections have occurred in both hospitals and outpatient settings. Many professionals and home cares for infants lack information on proper handling, storage, preparation and use practices should be performed (31). In practice, various practices are followed in hospitals and in the home, including mixing PIF with cold or hot water, immediate feeding or storage for short or extended times with or without refrigeration, re-warming or feeding without re-warming, or several feedings from a single container or a single feeding taking an extended amount of time (11, 23, 31, 70). Based on the results in this thesis, the relative risk associated with the various preparation practices can be assessed.

Risk mitigation options

Several risk mitigation options have been proposed for various stakeholders (10, 23, 67, 83) in order to prevent *Cronobacter* infections due to consumption of PIF. These include the following:

- For manufacturers
 - Utilizing good manufacturing and good hygiene practices to further minimize the entry of *Cronobacter* spp. into in manufacturing environment and avoid their persistence/growth (10, 23),
 - Improving product labeling, e.g. including harmonized guidance to create awareness among caregivers that PIF is not a sterile product and could cause illness in infants, and providing clear information to caregivers for them to adequately control microbiological hazards in reconstituted formula (10).
- For governments and intergovernmental bodies:
 - Setting a microbiological criteria for *Cronobacter* spp. and/or suitable indicator micro-organisms in order to reduce the risk of *Cronobacter* infection (23),
 - Educating health care professionals to provide effective hygiene training to parents and professional caregivers to ensure that PIF is prepared, handled and stored properly (10).
- For hospitals:
 - Prevention of prolonged storage time of prepared formulae,
 - Restricting feeding times, especially regarding tube feeding,
 - Exclusively using sterilized liquid infant formula for high risk infants,
 - Quick cooling of reconstituted formula using ice or running water and storing only small volumes of prepared formulae in refrigerators (83),
 - Reconstituting PIF with water of at least 70 °C, in order to take benefit of the heat to inactivate viable *Cronobacter* spp. possibly present (23, 67).

In the next paragraphs four mitigation options are discussed in some more detail, where relevant, employing the estimated parameters and predictive models developed in this thesis (Chapter 6 and 7) to determine the effectiveness of the mitigation options in reducing absolute or relative risks.

Risk mitigation option 1: Preparation of PIF with water of at least 70 °C

The Food and Agricultural Organization and the World Health Organization (FAO/WHO) guidelines for safe preparation, storage and handling of powdered infant formula (83) advise several steps to reconstitute PIF, namely 1) heat-treat the bottle at each feeding; 2) wash hands with warm water and soap before preparation; 3) use boiled water of a temperature no less than 70 °C; 4) add the PIF; 5) quickly cool prepared formula to feeding temperature by holding the bottle under running tap water, or by placing in a container of cold or iced water; 6) check the temperature before feeding in order to avoid scalding the infant's mouth; 7) discard any feed that is not consumed within 2 hours after feeding. If prepared formula is not to be consumed within two hours after preparation it should be quickly cooled immediately after preparation and stored in a refrigerator (at a maximum temperature of 5 °C) and used within 24 h (83). These guidelines have been implemented or advised in some countries, e.g. Italy (11) and Ireland (75), while in other countries, such as in the Netherlands, caregivers are still advised to prepare one or two bottles in advance following the instructions on the label, and place the bottles immediately in a refrigerator with a temperature of no more than 4 °C with a maximum storage time of 8 h (81).

Following the advice provided in FAO/WHO guidelines to reduce the risk of *Cronobacter* infection from PIF, reconstituting PIF with water > 70 °C should result in at least a 4-log reduction of *Cronobacter* spp.(67).

A study in Italy on home preparation of PIF showed that only 22% (n=29) of caregivers use water of at least 70 °C when preparing infant formula. Furthermore, after preparing a bottle, 10% of the parents stored the bottle at room temperature and 3% in a bottle warmer; 16% of the parents used the stored formula within 4 h after preparation (11). These observations

indicate that the guidelines are implemented only by a certain percentage of the caregivers. Evidently, it may take quite some time and effort for training caregivers to properly implement best practices in reliable way.

A key beneficial effect of using water of at least 70 °C is the on-site pasteurization of the bottle. Baby bottles may be heavily contaminated if small amounts of infant formulae are not immediately discarded after feeding, left at room temperature (70) and not properly cleaned and heat inactivated before re-use. This may allow *Cronobacter* spp. to outgrow to hazardous numbers (Chapter 6). In the Netherlands, it is advised to clean bottles using a dishwasher with a washing temperature of at least 55 °C or using warm soapy water in combination with a dish brush (80). Note that *Cronobacter* spp. may be introduced into a bottle due to its presence in for example a contaminated dish cloth or dish brush used for cleaning.

A potential disadvantage of reconstitution with hot water is its effect on heat sensitive vitamins. Furthermore, the caregiver or the infant may be wounded by scalding. Notably, the effect of the high temperature on dissolving the powder should also be considered. It would be relevant to dedicate further research on suitable control measures to minimizing an impact on vitamins and to minimizing the risk of scalding.

Another important factor that needs to be taken into account is the potential activation of *Bacillus* spores. Infant formulae are frequently contaminated with low numbers of *Bacillus* spores (5). It has been reported that reconstitution at temperatures ranging from 65 to 95 °C may lead to germination of endospores of *B. cereus*, which may cause foodborne illnesses in infants such as diarrhea (73, 74). Thus, the high temperatures as recommended may encourage the proliferation of other bacterial cells, germination of spores and possible toxin production. For this reason, more research is encouraged to investigate the effect of using hot water for the preparation of PIF on sporulation of spores, such as *B. cereus*.

Risk mitigation option 2: The relative risk reduction achieved when reconstitution is not with water of 70 °C but with water of 65 or 60 °C

The expert consultation of the FAO/WHO (2006) pointed out that reconstitution with liquid of at least 70 °C is an effective risk mitigation strategy (23). A relevant risk management question then is: how much less risk reduction is achieved at lower reconstitution temperature?

In order to investigate the effect of different water temperatures, the following scenarios were calculated: use of water of 40, 50, 60, 65 and 70 °C to reconstitute the powder in either a can or a bottle and to store it in both a refrigerator with still air or convection. Furthermore, the effect of rapid cooling on the relative risk was estimated. The baseline scenario for relative risk calculations was preparation of the formula with water of 40 °C in a bottle and storing the bottle in a refrigerator with still air at 6 °C for 8 hours, as shown in Table 2. The web-based tool of the risk assessment model of the FAO/WHO (68) was used to assess the relative risk associated with the various preparation and handling scenarios. The initial *Cronobacter* spp. concentration was assumed to be 1 CFU/g.

Table 2. Preparation and storing conditions for scenario's

	(Room) temperature [°C]	Time [h]
Preparation	25 ^a	0.25
Refrigeration ^b	6	8
Re-warming	37	0.5
Feeding	30	0.5

^a Please note that the temperature of the water used for reconstitution is between 40 and 70 °C whereas preparation was at room temperature is 25 °C.

^b Refrigeration conditions used in the various scenarios included immediate refrigeration after preparation in still air or convection refrigerator. Where rapid cooling was part of the option it was part of the refrigeration step.

For the given preparation and storage scenarios, the temperature of the formula was calculated by the web-tool at successive time points between preparation and consumption.

From these, the duration of the lag time, growth or inactivation rates were estimated and the resulting cell concentration calculated. The probability of infection (P) resulting from consumption of an estimated average dose of *Cronobacter* spp. can be described by the exponential dose-response model (Equation 1) (71).

$$P = 1 - \exp(-rD) \quad (1)$$

where r [1/CFU] is the exponential dose-response parameter and D [CFU] the estimated dose consumed.

The value of the dose-response parameter r has not been established with any certainty. Values between 10^{-10} and 10^{-5} have been suggested (23). For the low values of the dose (D), it is assumed that the risk (P) and the dose (D) are linearly correlated. Therefore, even without knowing the exact value of the r value a relative risk can be determined.

Figure 2 shows the estimated relative risk for the various scenarios. At a reconstitution temperature of 40 °C, there is a slight reduction in estimated risk for reconstituting PIF in the bottles for rapid cooling and convection refrigeration compared with the baseline scenario. Reconstitution of PIF in a can shows an increase (100%) in the relative risk. When water of 50 °C is used for reconstitution in a bottle, the relative risk increases (32%) for the scenario with still air and the bottle while it does not change for convection refrigeration but it is greatly reduced (38%) in the case of rapid cooling (for example using ice or running water). Note that these comparisons are made in relation to the baseline scenario. The relative risk increases a lot (247%) when a can volume is used rather than a bottle. Rapid cooling of PIF reconstituted at 50 °C in a bottle, could lead to a decrease in relative risk of 68% compared to cooling in a refrigerator with still air.

Reconstituting PIF with water of 60 °C gives a variable effect in risk, with a reduction for all storage scenarios involving bottles but with increased risk for formula preparation in a can followed by storage in a still air refrigerator.

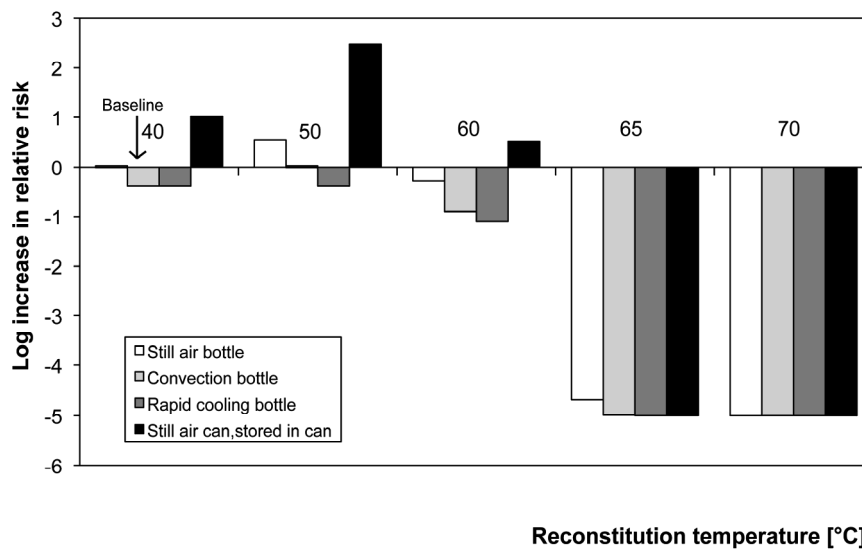


Figure 2. Increase in relative risk of different preparation, storage and handling practices for prepared formula as simulated using the web based tool (68). Note that the chosen setting (at 40 °C), as shown in Table 2, was used as baseline scenario for comparing relative risk levels.

At the higher temperatures, 65 and 70 °C, there was no apparent effect of convection and rapid cooling for both the bottle and can on the relative risk. In all cases a similar risk reduction of at least 4 log units or more was observed. Reconstituting at a temperature somewhat colder than 70 °C, still can be expected to achieve a reduction in *Cronobacter*, subsequently in relative risk reduction. However, when the temperature of the water is 60 °C, instead of 70 °C, a slight inactivation of *Cronobacter* spp. is achieved and the relative risk may even be increased compared with the baseline scenario in case PIF is prepared and stored in a can. Results from these simulations can be compared with reported reductions achieved when water of 60 °C and 70 °C was used to reconstitute PIF, where a 1.2- and 5.26-log reduction of *Cronobacter* populations was obtained (67).

Therefore, reconstituting PIF with water of more than 70 °C may have a safety margin, as this action would yield at least a 4 to 6-log reduction of *Cronobacter* spp., depending on the type of formula used and the type of *Cronobacter* strain present.

Risk mitigation option 3: Impact of hang-time in case of continuous tube-feeding on consumer risk

The WHO guidelines for safe preparation, storage and handling of PIF do not consider the use of enteral feeding tubes, which can be in place for various time periods, ranging from < 6 h to > 48 h (35), at a relatively high temperature (warm rooms may be 35 °C) and therefore may be a cause of large outgrowth of bacteria, or may accommodate micro-organisms as these may possibly attach and form biofilms. Enteral feeding tubes may be used to especially feed low birth weight infants in neonatal care units, which are the typical weak and the group at greatest risk (84).

Depending on the regime in the hospital, tube-fed formula is refreshed every 2 to 3 hours (34). Research has shown that *Cronobacter* spp. can form biofilms on plastic surfaces (41) and attach to enteral feeding tubes at ambient temperatures (49). It is therefore reasonable to assume that *Cronobacter* spp. will multiply in the tube, which is at about body temperature and also in the feeding container (syringe) which is at ambient temperature.

Considering the advice from the FAO/WHO expert meetings (22, 23) and the EFSA panel (19) to limit the hang time of tube-fed prepared infant formulae to 4h, the impact of the following scenarios on the level of *Cronobacter* spp. was estimated: the feeding container was assumed to be a baby bottle containing 120 ml of reconstituted infant formula was assumed to contain an initial concentration of *Cronobacter* spp. of 1 CFU/ml. Therefore, the estimated parameters for the baby bottle from Chapter 7 were presumed to be similar for the feeding container. The tube feeding started immediately after preparation and the formula was prepared with lukewarm water (40 °C) in a baby bottle. The feeding time was simulated up to 6 hours in a warm room (35 °C) and in a cold room (20 °C). The growth of *Cronobacter* spp. was predicted, using the specific growth rate calculated with the secondary growth model of Rosso with parameters as determined in Chapter 6. The increase in cell count was predicted with the exponential growth function, both neglecting the lag time and including the lag time according to the optimal fit of the k -value: $k = \mu \times \lambda = 2.88$ as described in Chapter 7.

Figure 3 gives the temperature profile, together with the predicted increase in cell count during tube feeding in a cold room (20 °C), while the temperature profile and predicted increase in cell count during tube feeding in a warm room are depicted in Figure 4. As can be seen, the decrease in temperature from 40 to 20 °C is slow and takes more than 6 hours, while cell numbers increase in time. The exponential growth function, which neglects lag time, overestimates growth as it predicts the increase to start immediately. When considering a lag time, growth will start after 2 hours. The predicted growth shows a 1-log unit increase in 4 h.

Tube feeding in a warm room (35 °C), may result in a considerably larger increase in cell numbers as can be seen in Figure 4. After 4 hours, an increase of at least 4-log units is predicted with the exponential growth function. This prediction must be assumed to be an overestimation, as lag time is neglected (Chapter 7). Including the lag time, as estimated with a k -value of 2.88, an increase of 2.5 log units is predicted in 4 h. These predictions show that the room temperature (Figures 3 A and 4A) and thus, the temperature of the formula being fed may significantly affect growth of *Cronobacter* spp., and needs to be considered for tube feeding infant formula to sensitive infants.

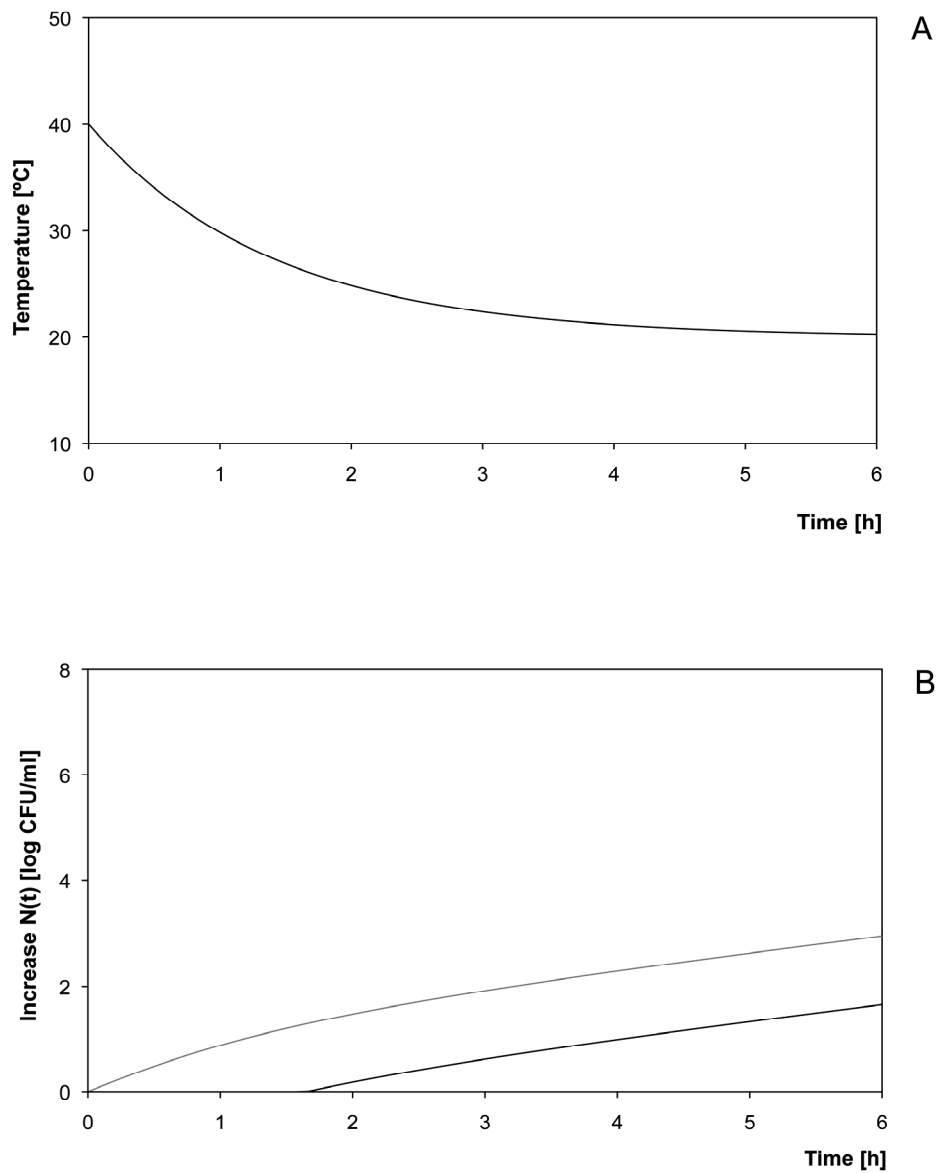


Figure 3. Temperature profile (A) and the number of *Cronobacter* spp. (B) in a bottle used for tube feeding in a room which is at 20 °C. Grey line = prediction with exponential growth function; black line = prediction with optimal fit $k=2.88$.

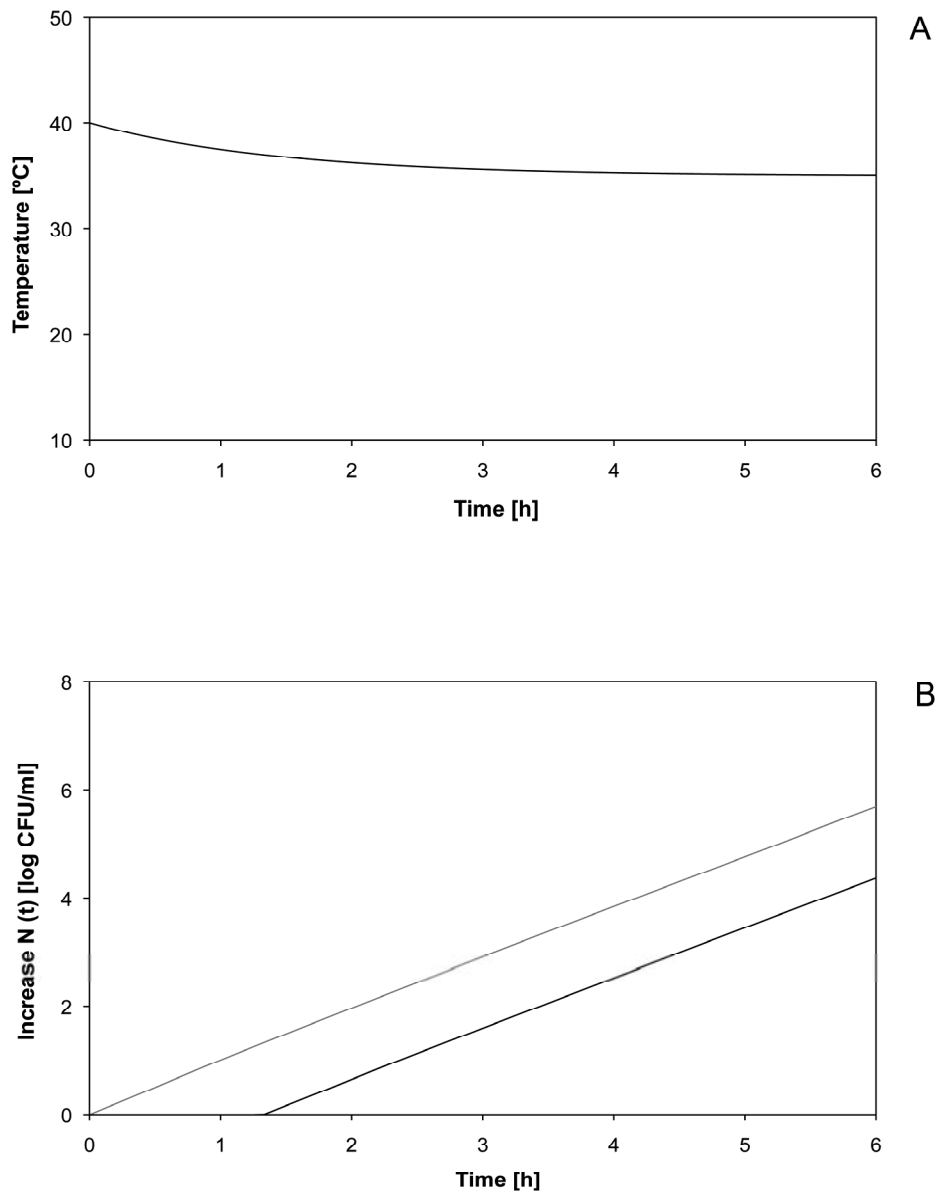


Figure 4. Temperature profile (A) and the number of *Cronobacter* spp. (B) in a bottle used for tube feeding in a room which is at 35 °C. Grey line = prediction with exponential growth function; black line = prediction with optimal fit $k=2.88$.

Risk mitigation option 4: Use sterile formula for the population at greatest risk.

The two FAO/WHO expert meetings have established that all infants at or below 12 months are at particular risk for *Cronobacter* infections (22, 23). Among these infants, those at greatest risk are neonates (< 28 days), particularly pre-term infants, low birthweight (LBW) infants (< 2500 g), and immuno-compromised infants, and those infants that are less than 2 months of age. Infants of HIV-positive mothers are also at risk, because they may specifically require infant formula and be more susceptible to infection (10, 24).

Table 3 gives an overview of data on reported cases adapted from the FAO/WHO expert meetings. Cases are divided in subgroups, being all infants, the group at greatest risk, low birthweight infants, and neonates. It is assumed that all infants have been formula fed only and that all cases were due to the presence of *Cronobacter* spp. in PIF; both assumptions are conservative and may cause overestimation of risks. It was estimated what the risk reduction could be of providing sterile formula to the whole or to a subset of the group at greatest risk. A 100% risk reduction was assumed when using sterile formula for each individual infant.

Using liquid sterile formula for all infants of the group at greatest risk, would possibly lead to a 100% reduction of *Cronobacter* infection cases for this group and a 72% (111/155), reduction in the total group of infants. Subsequently, a mortality reduction of 90% (26/29) would be achieved. Feeding only the LBW group with sterile formula is expected to have reduced the morbidity of *Cronobacter* infection with 37% (57/155), and the motility by 52% (15/29). If all neonates, in both homes and household settings, are fed with sterile formula only a 48% (75/155) reduction of *Cronobacter* infections and a 24% (7/29) reduction in deaths would be reached.

Table 3. Estimation of the risk reduction, feeding the group particularly at risk and the at greatest risk group with sterile formula. Data adapted from WHO/FAO 2008 report (24)

	No. cases	Recovered	Outcome unknown	Number of deaths
All infants (≤ 12 months of age and including age not known) ^a	155	85	41	29
Group indicated to be at greatest risk (≤ 2 months) ^a	111	71	14	26
LBW group (< 2500 g)	57	38	4	15
Neonates (< 28 days) ^a	75	49	9	7
Age at moment of illness not known	10	2	6	2

^a in all groups is age indicated to be age at onset of the *Cronobacter* infection.

These estimates show that it would be worthwhile to consider using sterile formula for the group at greatest risk, especially for the LBW group a larger reduction in the mortality rate was estimated compared to the neonate group. Although prematurity and low birth-weight are the most important risk factors for diseases, like necrotising enterocolitis in newborn infants, also other strategies, next to using sterile formula, should be considered to address effect in preventing illness.

Concluding remarks

In conclusion; results in this thesis showed that *Cronobacter* spp. is an ubiquitous pathogen, because of its widespread occurrence. It was isolated from a wide variety of food production environments and food products, including PIF. Therefore, it should be considered that PIF is not a sterile product. Most of reported *Cronobacter* infections are related to PIF, although infections were also reported in adults, showing that young infants are at greatest risk. For this reason, care should be taken while preparing, storing and handling PIF, as results in this thesis showed that potential rapid growth *Cronobacter* spp. in reconstituted infant formula is possible, depending on storage and feeding practices. Overall, the results described in this thesis can be used to estimate the exposure and thus the

probability of illness due to *Cronobacter* spp. after the consumption of reconstituted infant formulae.

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Samenvatting

Het genus *Cronobacter* bestaat uit 6 verschillende species (= soorten = spp.) en is onderdeel van de familie van de *Enterobacteriaceae*. Vóór 2007 werd *Cronobacter* aangeduid als *Enterobacter sakazakii*, en tot 1980 werd het genus aangegeven met de naam “geelgekleurde *Enterobacter cloacae*”. Het zijn Gram-negatieve, staafvormige bacteriën die bij specifieke groepen gevoelige personen ziekte kunnen veroorzaken. De meeste *Cronobacter* stammen maken een kenmerkend geel pigment aan als ze op Trypton Soya Agar gekweekt worden.

Cronobacter heeft ziektes veroorzaakt in alle leeftijdsgroepen, maar het merendeel van de infecties komt voor bij zuigelingen jonger dan één jaar. In het bijzonder baby's jonger dan twee maanden, die te vroeg zijn geboren, ondergewicht of een verzwakt afweersysteem hebben, lopen een verhoogd risico. *Cronobacter* infecties zijn zeldzaam, maar kunnen resulteren in vormen van neonatale meningitis (hersenvliesontsteking bij pasgeborenen), sepsis (bloedvergiftiging) en necrotiserende enterocolitis (ernstige ontsteking en afsterving van het maagdarmkanaal). Van de gerapporteerde patiënten is 19% overleden tussen 1961 en 2008 en van degenen die een infectie overleven, houdt een groot gedeelte blijvende neurologische complicaties. In nationaal en internationaal verband wordt sinds enige jaren aandacht besteed aan de mogelijkheid dat de bacterie via poedervormige zuigelingenvoeding ziekte kan veroorzaken.

Cronobacter is uit verschillende voedingsmiddelen en omgevingen geïsoleerd. Zuigelingenvoeding is echter het enige levensmiddel dat als mogelijke infectiebron geïdentificeerd is. In dit proefschrift worden in hoofdstuk 2 en 3 methoden beschreven om snel en nauwkeurig vast te stellen of er *Cronobacter* voorkomt in een product of in een omgeving (detectie) en of de gevonden bacteriesoort inderdaad een *Cronobacter* is (identificatie). De ontwikkelde selectieve methoden zijn toegepast in diverse producten en omgevingen, waaronder huishoudens. Het blijkt dat *Cronobacter* spp. voorkomt in onder andere fabrieken waar graanproducten, aardappelzetmeel of melkpoeder verwerkt worden. Eveneens werd *Cronobacter* spp. geïsoleerd uit vuil uit stofzuigerzakken, verzameld in verschillende huishoudens.

Hoofdstuk 4 en 5 van dit proefschrift geven meer inzicht in het voorkomen van *Cronobacter* spp., eventuele besmettingsroutes en bieden tevens mogelijkheden voor maatregelen om *Cronobacter* spp. infecties te voorkomen.

Eerder onderzoek heeft aangetoond dat *Cronobacter* spp. in vergelijking tot andere bacteriën uit de *Enterobacteriaceae* familie, goed kan overleven in droge omgevingen. De bacterie kan dan ook via herbesmetting vanuit productieruimten, met name in de afvalruimten, in zuigelingenvoeding terechtkomen. Een andere besmettingsroute is via het toevoegen van besmette, droge ingrediënten aan de zuigelingenvoeding, waarna tijdens het verdere productie proces geen hittebehandeling meer plaatsvindt.

Aangezien *Cronobacter* spp. in de fabrieksomgeving aanwezig kan zijn, is het zaak te voorkómen dat de bacterie in zuigelingenvoeding aanwezig is, door toepassing van kwaliteitssystemen zoals Good Manufacturing Practices (GMP) en Good Hygiene Practices (GHP). De in dit proefschrift beschreven resultaten kunnen onder ander door zuigelingenvoeding fabrikanten worden geïmplementeerd in de bestaande Hazard Analysis Critical Control Point (HACCP) plan om zo de veiligheid beter te beheersen. Ondanks alle voorzorgsmaatregelen, kan het toch voorkomen dat *Cronobacter* spp. in de zuigelingenvoeding aanwezig is. Het is belangrijk te realiseren dat zuigelingenvoeding niet een steriel product is en dat onjuiste bereiding en bewaring van de voeding de kans op besmetting kunnen vergroten. Over de groei van *Cronobacter* spp. in aangemaakte zuigelingenvoeding was tot voor kort niet veel bekend. Resultaten uit een experimentele dierstudie doen suggereren dat hoge aantallen levende cellen van *Cronobacter* spp. (meer dan 100.000 cellen per voeding ofwel $> 10^5$) nodig zouden zijn om ziekte of dood te veroorzaken. Deze hoge aantallen zijn nooit in droge poedervormige zuigelingenvoeding aangetroffen, dankzij een goede beheersing van de productie en de hygiëne bij het vullen van de verpakkingen. Besmetting kan echter ook optreden tijdens het bereiden van de zuigelingenvoeding zowel in het ziekenhuis als thuis. Wanneer lage aantallen *Cronobacter* spp. voorkomen in de poedervormige zuigelingenvoeding kunnen deze redelijk goed overleven (hoofdstuk 8) en eventueel verder uitgroeien nadat de poedervormige zuigelingenvoeding aangemaakt is met water. De snelheid en mate van uitgroeï is sterk afhankelijk van de temperatuur van de aangemaakte voeding. In hoofdstuk 6 worden

voorspellende modellen gebruikt om de groeiparameters, waaronder de groeisnelheid van *Cronobacter* spp. in aangemaakte zuigelingenvoeding, te kunnen bepalen bij diverse temperaturen. *Cronobacter* spp. hebben optimale groei tussen 37 en 39 °C.

Aangemaakte zuigelingenvoeding kan worden bewaard in de koelkast. De temperatuur van huishoudkoelkasten is echter niet altijd beneden de 7 °C, wat in Nederland de voorgeschreven maximum temperatuur is. Een nog belangrijker probleem is echter dat de voeding niet onmiddellijk dezelfde temperatuur heeft als de lucht in de koelkast. Na het plaatsen in de koelkast duurt het geruime tijd voordat de koelkasttemperatuur bereikt is. De verkregen groeiparameters uit hoofdstuk 6 zijn in hoofdstuk 7 gebruikt om een model te ontwikkelen om de proliferatie van *Cronobacter* spp. in aangemaakte zuigelingenvoeding te voorspellen gedurende het afkoelproces. Voorspellingen laten zien dat de groei van *Cronobacter* spp. kan worden voorkomen door de aangemaakte zuigelingenvoeding in kleine porties ter grootte van één voeding te bewaren in de koelkast, en door de poedervormige zuigelingenvoeding aan te maken met water dat een temperatuur van 25 °C of minder heeft.

De in dit proefschrift beschreven resultaten zijn gebruikt in de expert meetings van de FAO (Food and Agricultural Organization of the United Nations) en de WHO (World Health Organization) waarbij het risico van *Cronobacter* spp. in zuigelingenvoeding is onderzocht. De ontwikkelde modellen kunnen worden toegepast voor het kwantificeren van de groei van *Cronobacter* spp. in de zuigelingenvoeding onder verschillende aanmaak- en bewaaromstandigheden om zo de effecten van opslag en bereiding van zuigelingenvoeding door te rekenen, bijvoorbeeld in het kader van microbiologische bepalingen (Microbiologisch Risk Assessments) die ontwikkeld worden om de kans op ziekte na consumptie te kunnen schatten.

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Finally, I reached the last pages of this thesis. I would therefore like to take the opportunity to thank the various people who helped to finalize this dissertation.

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Esther, I learned a lot from you, sharing the same office with you for some years was a very pleasant time. I hope our friendship will last forever. Thank you for being one of my paranymphs.

The cover is beautiful. Thank you, Dorothée, for designing it and for helping out with the layout of this thesis.

Dear family thank you for all your support, now I can answer your question that I finally have finished my “study”. Late aunt Rinia and Ine, you were always there for me during the past years. Thank you! Geeta thank you for your support and the discussions we had especially on the last part of this thesis.

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A handwritten signature in cursive script that reads "Chantal". The signature is written in black ink on a light-colored background. The word "Chantal" is written in a fluid, connected style, with a long, sweeping underline that curves under the letters.

List of Publications

Peer-reviewed papers

Kandhai, M. C., A. E. Heuvelink, M. W. Reij, R. R. Beumer, R. Dijk, M. Van Schothorst, and L. G. M. Gorris. 2010. A study into the occurrence of *Cronobacter* spp. in The Netherlands between 2001 and 2005. *Food Control*, doi:10.1016/j.foodcont.2010.01.007.

Kandhai, M. C., M. W. Reij, M. Van Schothorst, L. G. M. Gorris and M. H. Zwietering. 2010. Inactivation rates of *Cronobacter* spp. and other bacteria in dry powdered infant formula at various temperatures. *Accepted for publication in Journal of Food Protection*

Kandhai, M. C., C. J. H. Booij, and H. J. Van der Fels-Klerx. 2010. A Delphi based expert study to identify indicators for emerging *Fusarium* related mycotoxins in wheat based supply chains. *Accepted for publication in Risk Analysis*.

Van der Fels-Klerx, H. J., S. Dekkers, **M. C. Kandhai**, S. M. F. Jeurissen, C. J. H. Booij, and C. De Heer. 2010. Expert study to select indicators for identification of re-emerging mycotoxins. *NJAS - Wageningen Journal of Life Sciences*, doi:10.1016/j.njas.2010.02.003.

Kandhai, M. C., P. Breeuwer, M. H. Zwietering, L. G. M. Gorris, and M. W. Reij. 2009. Growth of *Cronobacter sakazakii* under dynamic temperature conditions during bottle cooling. *Journal of Food Protection*, 72:2489-2498.

Van der Fels-Klerx, H. J., **M. C. Kandhai**, S. Brynestad, M. Dreyer, T. Borjesson, M. Martins, E. Morrison, and C. J. H. Booij. 2009. Development of an European system for identification of emerging mycotoxins in wheat based supply chains. *World Mycotoxin Journal*, 2:2; 119-127.

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Guillaume-Gentil, O., V. Sonnard, **M. C. Kandhai**, J. D. Marugg, and H. Joosten. 2005. A simple and rapid cultural method for detection of *Enterobacter sakazakii* in environmental samples. *Journal of Food Protection*, 68: 64-69.

Kandhai, M. C., M. W. Reij, L. G. M. Gorris, O. Guillaume-Gentil, and M. Van Schothorst. 2004. Occurrence of *Enterobacter sakazakii* in the food production environments and households. *The Lancet*, 363: 39-40.

Kandhai, M. C., M. W. Reij, K. Van Puyvelde, O. Guillaume-Gentil, R. R. Beumer, and M. Van Schothorst. 2004. A new protocol for the detection of *Enterobacter sakazakii* applied to environmental samples. *Journal of Food Protection*, 67: 1267-1270.

Kandhai, M. C., M. W. Reij, L. G. M. Gorris, O. Guillaume-Gentil, and M. Van Schothorst. 2004. *Enterobacter sakazakii* in factories and households. *The Lancet*. 364: 414-415.

Non peer-reviewed scientific publications

Kandhai, M. C., P. Breeuwer, E. G. Biesta-Peters, L. G. M. Gorris, M. H. Zwietering, and M. W. Reij. 2009. Growth of *Cronobacter* spp. under dynamic temperature conditions occurring during cooling of reconstituted powdered infant formula. In: V.K. Juneja & D.W. Schaffner (Eds.), 6th International Conference Predictive Modeling in Foods Meeting Abstracts and Information Booklet, Washington DC, USA. p. 46.

Kandhai, M. C., H. J. Van der Fels-Klerx, and C. J. H. Booij. 2008. Early Identification of Emerging Mycotoxins in Wheat. In: The 21st international ICFMH Symposium, Food Micro 2008, Evolving microbial food quality and safety, Aberdeen, Scotland, 1-4 September 2008. p. Z4.

Van Asselt, E. D.; H. Van der Voet, H. J. Van der Fels-Klerx, and **M. C. Kandhai**. 2008. *Salmonella* serotypes in the broiler supply chain. In: The 21st international ICFMH Symposium, Food Micro 2008, Evolving microbial food quality and safety, Aberdeen, Scotland. p. AA3.

Dekkers, S., H. J. Van der Fels-Klerx, S. M. F. Jeurissen, **M. C. Kandhai**, C. J. H. Booij, C. de Heer, and P. M. J. Bos. 2008. Development of a model to assess the occurrence of mycotoxins in wheat, maize and nuts using a holistic approach-draft report. RIVM report 320111002/RIKILT report 2008.004/PRI report 186. Available at:
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Van der Fels-Klerx, H. J., C. J. H. Booij, and **M. C. Kandhai**. Indicators for emerging mycotoxins. 2008. In: Van der Fels-Klerx, H. J. and C. J. H. Booij (Eds.). European network of information sources for an identification system for emerging mycotoxins in wheat based supply chains (MYCONET). RIKILT – Institute of Food Safety, Wageningen UR, Wageningen, The Netherlands. Report R2008.008. Available at:
<http://www.rikilt.wur.nl/NR/rdonlyres/BDEEDD31-F58C-47EB-A0AA-23CB9956CE18/83031/R2008008.pdf>.

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Kandhai M. C. 2003. *Enterobacter sakazakii* en zuigelingen voeding Symposium 'Voedselvergiftigen en -infecties' : EFFI symposium , 2003-05-15 / Stichting EFFI, - Wageningen : Stichting EFFI , 2003. p. 1–4.

Curriculum Vitae

Morea Chantal Kandhai was born on April 3rd 1974 in Paramaribo, Suriname, as the first daughter of Johan Kit Rameshchandra Narain and late Jien-Tjauw Melie Narain-Tjon Sien Foek. She completed her secondary education (Lyceum II in Paramaribo) in 1993. From 1994 until 2000 she studied Food Science at the Wageningen University (then the Agricultural University). As part of her MSc study thesis research projects were carried out within the Food chemistry and Food Microbiology specialization. During her internship at Numico Research, Wageningen, The Netherlands, she worked on the effect of antioxidant supplementation on the antioxidant status of healthy volunteers. In April 2001 she started with her PhD project entitled: “Detection, occurrence, growth and inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*)” at the European chair in Food Safety Microbiology and the Laboratory of Food Microbiology of the Wageningen University. The results obtained during this research are described in this thesis. In 2006 she worked part time as education coordinator at the Graduate school VLAG. From 2007 till 2009 she worked as a researcher at RIKILT - Institute of Food Safety, on microbiological safety of various food supply chains. Currently, Chantal is working at the Board for the Authorisation of Plant Protection Products and Biocides (Ctgb).

Completed Training Activities

Courses

VLAG Management on food safety and microbiological risks (2002)
VLAG Physiology of food associated micro-organisms (2004)
VLAG Food Fermentation (2004)
Basic and advanced statistic course (2003/2004)
Bionumerics software course (2002)

Meetings

EFFI symposium: Overleving en na-besmetting (2001)
NVVM symposium (2001)
HACCP en de garantie van voedselveiligheid (2002)
Safe: new emerging pathogens, Brussel, Belgium (2003)
EFFI symposium: Voedsel vergiftigingen en – infecties (2003)
Fourth International Conference on Predictive Modeling in foods, Quimper, France (2003)
NVvM symposium: Microbiologische voedselveiligheid: van gen tot wet (2003)
Nationale Hygiënedag (2003)
EFFI symposium: Microbiologisch onderzoek van Levensmiddelen (2003)
EFFI symposium: Microbiële gevaren: voorkomen en prevention (2004)
EUROFORUM: 5^e Nationaal Congres Voedselveiligheid (2004)
NVVM symposium: Microbiologisch onderzoek van levensmiddelen: eigen werk (2004)
Food Micro: New tools for improving microbial food safety and quality, Slovenia (2004)
NIZO Dairy Conference: Prospects for health, well-being and safety, Papendal (2005)
EFFI symposium: Micro-organismen op weg (2006)

General courses

English for PhD students (2001)
Endnote course (2001)
Presenting in English (2003)
PE&RC Time and project management (2004)
Career perspectives (2004)
OWU Gespreksvaardigheden (2005)
OWU Basis cursus didactiek (2004/2005)
OWU Afstudeervak organiseren en begeleiden (2003)

Other activities

Preparation research proposal (2001)
Training at Nestlé Research Centre in Lausanne, Switzerland (2001)
Foreign students trip Slovakia, June 2004
General meetings Laboratory of Food Microbiology (2001, 2002, 2003, 2004, 2005)

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