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Effect of microbiologically active substances in powdered infant formula on the growth and detection of *Cronobacter* spp.

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List of Abbreviations

AFNOR	Association Française de Normalisation
BHA	Brain hearth infusion agar
BHI	Brain heart infusion
BPW	Buffered peptone water
CAC	Codex Alimentarius Commission
CFU	Colony forming unit
CLF	Central Laboratories Friedrichsdorf
CSB	*Cronobacter* screening broth
DFI agar	Druggan-Forsythe-Iversen agar
DIN	Deutsche Industrie Norm
DNA	Desoxyribonucleic acid
DSMZ	Deutsche Stammlung von Mikroorganismen und Zellkulturen
DT	Detection time
EC	European Commission
EE	Enterobacteriaceae Enrichment
ESE	*Enterobacter sakazakii* enrichment broth
ESIA	*Enterobacter sakazakii* isolation agar
ESPM	*Enterobacter sakazakii* chromogenic plating medium
ESSB	*Enterobacter sakazakii* selective broth
f-AFLP	fluorescent-labelled amplified fragment length polymorphism
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FIF	Fermented infant formula
FISH	Fluorescence in situ hybridization
FUF	Follow-up formula
h	hour(s)
HCl	Hydrogen chloride

HIV	Human immunodeficiency virus
ICMSF	International Commission on Microbiological Specification for Foods
ISO/TS	International Standards Organization/Technical Specification
LOD	Limit of detection
LPOS	Lactoperoxidase system
LST	Lauryl sulphate tryptose
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
min	minute(s)
MLSA	Multilocus sequence analysis
NEC	Necrotizing enterocolitis
PCR	Polymerase chain reaction
PIF	Powdered infant formula
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acid
TSA	Tryptic soy agar
VRBG	Violet red bile glucose
WHO	World Health Organization

Chapter 1

Introduction

The genus of *Cronobacter* spp. is an emerging opportunistic food borne pathogen, causing rare but serious infections of bacteraemia, meningitis and necrotizing enterocolitis in infants (Anonymous, 2006a; Anonymous, 2007b; Mullane et al., 2007). Contaminated powered infant formula (PIF) has been implicated as a most likely source of transmission of *Cronobacter* spp.. A microbiological association between consumption of PIF contaminated during manufacture and/or mishandling when reconstituted and a number of clinical outbreaks has been established (Van Acker et al., 2001). Understanding of biochemical characteristics and growth profiles of *Cronobacter* spp. is therefore essential for the investigation of inhibitive strategies against the growth of *Cronobacter* spp. as well as for the development of detection and identification methods of *Cronobacter* spp. in reconstituted PIF.

1.1 Taxonomy

Cronobacter spp. are a group of gram-negative, motile, non-spore forming, facultative anaerobic bacteria. These organisms were originally referred to as "yellow-pigmented *Enterobacter cloacae*" until they were classified as a new species, *Enterobacter sakazakii*, within the genus *Enterobacter* (Farmer et al., 1980). In year 2007 and 2008, a further classification of *E. sakazakii* with the creation of a new genus, *Cronobacter*, has been proposed. The genus *Cronobacter* consists of six species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C.* genomospecies 1 and *C. dublinensis* which contains three subspecies *dublinensis*, *lactaridi* and *lausannensis* (Iversen et al., 2007; Iversen et al., 2008). This genus proposal was based on data from a polyphasic taxonomic study of an extensive collection of target strains in which full-length 16S rRNA sequencing, fluorescent-labelled Amplified Fragment Length Polymorphism (f-AFLP) fingerprinting, ribotyping, DNA-DNA hybridization and characterization of phenotypic profiles were applied. Recently Joseph et al. (2012) used 16S rRNA sequencing and a Multilocus Sequence Analysis (MLSA) of seven housekeeping genes to re-evaluate the diversity of the genus. Based on his data, a new species *C. condimenti* was identified and the original *C.* genomospecies 1 was replaced by *C.*

universalis. *Cronobacter* genus comprises currently seven species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. condimenti* and *C. universalis*.

1.2 Biochemical Characteristics of *Cronobacter*

Farmer et al. (1980) found that *Cronobacter* spp. (previously *E. sakazakii*) possessed biochemical reactions very similar to those of *E. cloacae* but *Cronobacter* spp. were D-sorbitol negative and produced yellow-pigmented colonies on Tryptic Soy Agar (TSA) at 25°C after 48 h. An additional distinguishing property for *Cronobacter* spp. is the Tween 80 esterase activity, which was positive in 97.3% of isolates (Aldova et al., 1983). Muytjens et al. (1984) investigated the enzymatic profiles of 129 *Cronobacter* spp. strains and 97 *Enterobacter* strains including *E. cloacae*, *E. aerogenes* and *E. agglomerans* and determined the presence of α-glucosidase as a major difference between *Cronobacter* spp. and other *Enterobacter* species.

1.3 Epidemiology and Pathogenicity

Cronobacter spp. are considered as emerging opportunistic pathogens and have been identified as causes of bacteraemia, necrotizing enterocolitis and neonatal meningitis (Arseni et al., 1987; Bar-Oz et al., 2001; Giovannini et al., 2008; Mullane et al., 2007). Urmenyi and Franklin (1961) recorded the first isolation of *Cronobacter* spp. from a case of neonatal meningitis. Surveillance data of the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) (Anonymous, 2008b) showed that since 1961 and up to 2008, approximately 120 documented cases of *Cronobacter* infections in infants and young children less than three years of age have been reported worldwide with at least 27 associated deaths, showing an average case-fatality rate of 22%. This leads to the International Commission on Microbiological Specification for Foods (ICMSF) (Anonymous, 2002a) ranking *Cronobacter* spp. as “Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration.” An annual

incidence rate for *Cronobacter* invasive infections has been estimated to be 1 per 100,000 infants, whereas higher annual incidence rates among the low birth weight infants (< 2500 g) and very low birth weight infants (< 1500 g) of 8.7 and 9.4 per 100,000 infants respectively were reported (Anonymous, 2006a). The case-mortality rate caused by *Cronobacter* infection has been reported to be higher (50%) among premature or (very) low birth weight infants than full-term or infants with birth weight ≥ 2500 g (30%) (Lai, 2001). Although *Cronobacter* has caused diseases in all age groups, neonates under 28 days, particularly preterm-infants, low birth weight infants, immunocompromised or medically debilitated infants as well as infants with HIV-positive mothers are thought to be at the greatest risk for severe infection (Anonymous, 2007b). It has been concluded that infants during the first few weeks of life are particularly susceptible to meningitis rather than bacteraemia if exposed to *Cronobacter*. Data from 45 clinical cases have revealed that bacteraemia appears to occur at a later median age of 35 days of life in premature infants, whereas meningitis cases have been more frequently observed among full-term infants who generally develop symptoms before the age of one week. Infant with bacteraemia tend to fare better with a mortality of 10%. Conversely 44% of those with meningitis died and the majority of survivors experienced long-term neurological consequences (Anonymous, 2006a).

The mechanism of pathogenicity and potential virulence factors of *Cronobacter* remain elusive. Pagotte et al. (2003) were the first to investigate the virulence factor in *Cronobacter* spp. using a suckling mouse model and found four out of 18 *Cronobacter* strains producing enterotoxin. Townsend et al. (2007) reported that *Cronobacter* strains persisted or replicated in macrophages and showed attachment and invasion of human endothelial cells to different extents, indicating a diversity of virulence among *Cronobacter* isolates. However its relevance to human infant infections has yet to be established. To date only strains of *C. sakazakii*, *C. malonaticus* and *C. turicensis* have been found to be associated with reported neonatal infections. Particularly a variety of *C. sakazakii* with sequence type ST4 causes more infant meningitis (Joseph and Forsythe, 2011). Despite the inter- and intra-species variation in virulence, no data currently show that any of the *Cronobacter* species does not

pose a risk to neonatal and infant health. Therefore, all species in the genus *Cronobacter* are considered to be pathogenic (Anonymous, 2008b). In the investigation performed by Pagotte et al. (2003), a lethal dose of 10^8 CFU on infant mouse by intraperitoneal injection has been concluded for all the 18 *Cronobacter* strains, whereas only two isolates caused death when given orally, however, this value does not necessarily reflect the dose-response in human neonates. Till now no evident data for an infectious dose associated with *Cronobacter* spp. is available. An infectious dose of 1000 CFU similar to that of *Neisseria meningitidis* and *E. coli* O157 has been proposed by Iversen and Forsythe (2003). The FAO/WHO (Anonymous, 2007b) estimated a linear dose-response relationship at low dose below 10,000 CFU.

1.4 Production of Powdered Infant Formula, Contamination Routes and Prevalence of *Cronobacter* in Powdered Infant Formula

It has been internationally recognized that breast milk is the best source of nutrition to infants and young children. There are, however, occasions when breast milk is not available or where the mother is unable to breastfeed. In such cases, powdered formulae which are formulated to meet the special nutritional needs of infants represent one of the dietary options to replace the breast milk partially or totally (Anonymous, 2007c). Codex Alimentarius Commission (CAC) has divided powdered formulae into powdered infant formula (PIF), follow-up formula (FUF), human milk fortifiers and formula for special medical purposes. PIF is defined as a breast milk substitute specially manufactured to satisfy the nutritional requirements of infants as sole source of nutrition during the first months of life 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 the infant from the 6^{th} month on and for young children (Anonymous, 2008a).

PIF can be manufactured in a wet-mix process, a dry-blending process or a combined

process (Cordier, 2008). In the wet-mix process, all unprocessed raw materials as well as separately processed ingredients are handled in a liquid form, heat treated, dried and brought to the filling stage. The dry-mix procedure involves dry mixing of heat sensitive ingredients like vitamins, minerals, starch and carbohydrates into the powder after spray-drying. In the mix-process, the unprocessed raw materials and ingredients are processed in a liquid phase to obtain a base powder and other ingredients are added into the base powder and further blended. Due to the limitations of current technology, it is not possible to produce sterile PIF at a feasible price. PIF can be contaminated with low levels of pathogens e.g. *Cronobacter* spp. via intrinsic and extrinsic routes. Intrinsic contamination of PIF during the manufacturing process after pasteurization can occur either via addition of thermally sensitive plant-derived ingredients (e.g. vitamins, starch, protein and lecithin) or via transmission of materials in the processing environment such as aerosol, dust and water droplet (Iversen et al., 2009; Mullane et al., 2008). It is notable that although the standard pasteurization practices (71.6°C, 15 s or 74.4°C, 25 s) are sufficient to inactive *Cronobacter* spp, the organism may survive the spray drying (Arku et al., 2008). An extrinsic contamination of PIF can occur during the preparation and feeding when contaminated utensils such as spoons, blenders, bottles, teats are used (Bar-Oz et al., 2001; Simmons et al., 1989).

Being a ubiquitous organism, *Cronobacter* spp. have been isolated from a wide variety of food, environmental and clinical sources (Cottyn et al., 2001; Farber, 2004; Gallagher and Ball, 1991; Gassem, 1999; Kandhai et al., 2004; Leclercq et al., 2002). The presence of *Cronobacter* in PIF is of particular concern. A clear epidemiological correlation between *Cronobacter* infections in infants and the consumption of contaminated PIF has been described (Biering et al., 1989; Clark et al., 1990). Together with *Salmonella*, *Cronobacter* spp. have been categorized as "A" organisms. PIF contaminated with "A" organisms has been both epidemiologically and microbiologically shown to be the source and vehicle of infection in infants (Anonymous, 2007b). Several studies have investigated the prevalence of *Cronobacter* spp. in PIF and other infant foods. Muytjens et al. (1988) examined 141

different breast milk substitute powders from 35 countries and determined a contamination level of *Cronobacter* ranging from 0.36 to 66 CFU per 100 g. In a survey of 120 cans of Canadian infant formula samples, the prevalence of *Cronobacter* was 6.7%, with a contamination level of 0.36 CFU per 100 g (Nazarowec-White and Farber, 1997a). In a British survey, two samples (2.4%) were positive for *Cronobacter* among 82 PIF products purchased from retails (Iversen and Forsythe, 2004). Jongenburger et al. (2011) investigated 2291 samples of 1 g from a recalled batch and found a sporadic presence of *Cronobacter* cells in eight samples, with the two largest clusters containing 123 and 560 cells respectively.

1.5 Methodology

1.5.1 Conventional Microbiological Isolation of *Cronobacter*

1.5.1.1 Culture-based Isolation

The conventional culture–based procedure for detection of micro-organisms in foods comprises three basic steps: The first step is pre-enrichment in a non-selective medium which enables recovery of sub-lethally injured cells. The second step is selective enrichment containing compounds which promote the growth of the target micro-organisms to be isolated but are inhibitory to the majority of the background micro-organisms. The third step is streaking the selective broth onto selective solid media. In case of presumptive colonies on the agar, a confirmation step will be carried out.

The initial method for isolation and detection of *Cronobacter* from PIF applied by Muytjens et al. (1988) and Nazarowec-White and Farber (1997a) comprised 1) pre-enrichment in Buffered Peptone Water (BPW), 2) enrichment in Enterobacteriaceae Enrichment (EE) broth, 3) streaking onto Violet Red Bile Glucose (VRBG) agar, 4) picking five Enterobacteriaceae colonies on TSA agar and incubation at 25°C for 48-72h, 5) confirmation of yellow-pigmented colonies on TSA with API 20E (bioMérieux, France). This method was

adopted by the U.S. Food and Drug Administration (FDA) with modification of pre-enrichment in distilled water (Anonymous, 2002b). Being a time-consuming procedure, the FDA method requires a minimum of 5 days to finish. Also no selective pre-enrichment and enrichment broths were involved in this method. It is likely that *Cronobacter* spp. are outgrown by background Enterobacteriaceae, leading to few *Cronobacter* colonies isolated on VRGB and a subsequently reduced chance of picking the target organisms onto TSA. In contrast the European Community (EC) regulation refers to International Standards Organization (ISO) standard method ISO/TS 22964 which consists of pre-enrichment in BPW, followed by selective enrichment in modified lauryl sulphate tryptose (mLST) broth with vancomycin, plating on selective differential media and biochemical characterization of typical colonies (Anonymous, 2006b).

1.5.1.2 Chromogenic and Fluorogenic Media for *Cronobacter* Differentiation

Several media have been developed for a specific detection of *Cronobacter* from PIF. All of these selective media take advantage of the biochemical characteristic, the α-glucosidase activity of *Cronobacter* which was first described by Muytjens et al. (1988).

A differential selective medium, OK medium, described by Oh and Kang (2004) used a fluorogenic substrate, 4-methyl-umbelliferyl-α-D-glucopyranoside with the fluorogen serving as indicator to detect α-glucosidase activity. Druggan-Forsythe-Iversen (DFI) agar contains the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (Iversen et al., 2004a). Being hydrolysed by α-glucosidase positive organism, bromo-choloro-indigo will be liberated from the substrate and typical blue-green colonies will be produced. In addition, sodium thiosulphate and ammonium iron citrate were incorporated in the DFI agar as hydrogen sulphide indicator to differentiate *Cronobacter* from H_2S positive organisms like *Proteus* and *Salmonella* which appear black on the agar.

The *Enterobacter sakazakii* isolation agar (ESIA) recommended in the ISO method has been commercially available by AES Chemunex France since 2005 and it is based on the same chromogenic agent as DFI agar. Restaino et al. (2006) described the R&F *Enterobacter*

sakazakii chromogenic plating medium (ESPM) which contains three sugars (sorbitol, D-arabitol and adonitol) as well as two chromogens (X-α-D-glucopyranoside and X-α-D-cellobioside), causing *Cronobacter* colonies to create blue-black or blue-grey colonies on medium.

1.5.1.3 Other Culture Method: Impedance method

The impedance method is an alternative to the conventional microbiological methods. The term impedance refers to the electrical resistance that occurs in the alternating current circuit. Due to the microbial metabolism in the liquid culture medium, large molecules are broken down into smaller, electrically charged molecules. These changes in the molecular composition increase the conductivity of liquid nutrients and lower their electrical resistance. With the first application in clinical microbiology in 1975 (Wheeler and Goldschmidt, 1975), the technology has been widely used as a standardized rapid method in food microbiology in quality control laboratories in food industry, being recognized by numbers of official methods collections such as Association Française de Normalisation (AFNOR) (Anonymous, 2010a, 2010b) and Deutsche Industrie Norm (DIN NORM) (Anonymous, 1999, 2001, 2005). For detection and enumeration of several food relevant micro-organisms such as lactobacilli, *E. coli*, *Clostridia* and *Salmonella*, the impedance measurement has been assessed as a valid method (Colquhoun et al., 1995; Dromigny et al., 1997; Joosten et al., 1994; Lanzanova et al., 1993). To date the impedance technology has been optimized in its specificity for food relevant bacteria by the development of impedance specific media and the combination of impedance detection with immunological and molecular biological confirmation methods.

1.5.2 Identification of *Cronobacter*

1.5.2.1 Phenotypic Methods: Biochemical Identification Kits

Traditional phenotypic identification is based upon biochemical pathway and carbon source utilization. For confirmation of presumptive *Cronobacter* spp. isolated from agars, various commercially available biochemical systems, including API 20E, ID 32E, VITEK 2 (bioMérieux, France) and Biolog GN2 (Biolog, USA) have been used. Iversen et al. (2006)

investigated the original 15 biotypes described by Farmer et al. (1980) in correlation with four clusters formed in 16S rRNA sequences and established a new biogroup 16. As indicated by Iversen et al. (2007), five *Cronobacter* species *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinenesis* and *C. turicensis* can be distinguished using four key biochemical reactions indole, dulcitol, malonate and methyl-α-D-glucoside.

16 biogroups are distributed among *Cronobacter* species as following:

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

Two strains belonging to *Cronobacter universalis* (formerly *Cronobacter* genomospecies 1) have not been associated with any specific biogroup.

1.5.2.2 Genotypic Methods

1.5.2.2.1 Polymerase Chain Reaction (PCR)

To date several conventional and real time Polymerase Chain Reaction (PCR)-based systems that enable sensitive, specific and rapid end detection of *Cronobacter* from PIF, enrichment broths and media have been described. Targets for conventional PCR assays include e.g. the 16S rRNA gene (Hassan et al., 2007; Lehner et al., 2004), 1,6 α-gulcosidase gene (*glu*A) (Lehner et al., 2006b), outer membrane protein A (*omp*A) gene (Mohan Nair and Venkitanarayanan, 2006) and a gene encoding a zinc-containing metalloprotease (zpx) (Kothary et al., 2007). Seo and Brackett (2005) developed a quantitative real time PCR assay for *Cronobacter* detection targeting an internal segment of the *dna*G gene within the

macromolecular synthesis operon. Liu et al. (2006) evaluated a real time PCR method based on the amplification of an internal transcribed spacer sequence of the 16S–23S rDNA, using a Taqman probe and SYBR Green simultaneously.

1.5.2.2.2 Fluoreszenz *in situ* Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is an alternative to the DNA-targeted PCR-based approaches for rapid bacterial detection. FISH has been widely used for microbial identification in medical diagnoses (Kempf et al., 2005; Russmann et al., 2001) and has also allowed a rapid detection and identification of organisms including Enterobacteriaceae, and *Pseudomonas* in drink water and food (Baudart et al., 2005; Kitaguchi et al., 2005). Based on the binding of fluorescently labelled single-stranded DNA-probes to specific regions on the ribosomal RNA of the bacteria, the FISH system detects only living cells (Amann et al., 1995; DeLong et al., 1989). Using the commercially available test system based on FISH, the VIT® kit (Vermicon Identification Technology, Germany), presumptive *Cronobacter* isolates have been identified with 100% accuracy (Lehner et al., 2006a). The same test kit has been applied in the investigation of Sanjaq (2008), showing no false negative or false positive results in differentiating 101 *Cronobacter* isolates from 7 other Enterobacteriaceae species.

1.5.2.2.3 16S rRNA gene sequencing

Much effort has been put on the development of genotypic tools for *Cronobacter* identification with a high discrimination power to the species and subspecies level. The 16S rRNA gene is by far the most common housekeeping genetic marker because the ribosomal small subunit is present universally among bacteria and it includes both hypervariable regions with species-specific variability where sequences have diverged over evolution and strongly conserved regions, which makes the 16S rRNA gene sequencing a highly useful tool to study bacterial phylogeny, ecology and taxonomy (Janda and Abbott, 2007; Weisburg et al., 1991). Genomic DNA is extracted, amplified with universal 16S rRNA primer and sequenced. The cycle sequencing reaction is a modification of the traditional Sanger method using base-specific, dideoxynucleotide-terminated chain elongation method with modification for the use of reverse transcriptase and RNA template (Lane et al., 1985;

Sanger et al., 1977). By comparing sequence data in gene databases in public or private domains, it is possible to analyse relationships between various organisms and to identify unknown micro-organisms. This molecular approach has been extensively used for identification and classification of environmental and clinical bacterial isolates (Clarridge, 2004; Drancourt et al., 2000; Vandamme et al., 1996). Using 16S rRNA gene sequencing, the type strain of *Cronobacter* was found to be closer to *Citrobacter* (*C*.) *koseri* (97.8% similar) than to *E. cloacae* (97%) although the latter has been found to share most phenotypic features with *Cronobacter* (Iversen et al., 2004c). Four phylogenetic clusters have been defined among 189 *Cronobacter* strains analysed with partial 16S rRNA gene sequencing and this helped to form the basis of the taxonomic re-classification of these organisms (Iversen et al., 2006).

1.5.2.2.4 Matrix-assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Currently, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has been introduced as a new method for phylogenetic classification and identification of bacteria on the basis of protein profiling. The concept of bacterial differentiation/identification by detection of protein mass patterns is based on the principle that genomic sequences of organisms coding for production of proteins are determinant for phylogenetic differences between organisms. Since proteins reflect the genomic differences, the mass spectra should be able to serve as a differentiation/classification vector for bacteria (Holland et al., 1996; Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1996). Each mass spectrometer is composed of three units: an ion source to generate ion and transfer the analyte ions into the gas phase; a mass analyzer to separate ions by the mass-to charge ratio (m/z) and a detector for ion monitoring. In practice, bacterial samples are first subjected to a protein extraction method, embedded in small acidic molecules known as matrix such as α-cyano-4-hydroxycinnamic acid which desorbs laser energy and analyzed by mass spectrometry (Freiwald and Sauer, 2009). As a large majority of the bacterial proteins detected by this approach are of ribosomal origin, the generated masses of these ribosomal

proteins represent a specific fingerprint which can be used for phylogenetic classification and identification of bacteria. Based on comparison of protein biomarkers with high reproducibility and whole specific spectral fingerprints of protein peak patterns, MALDI-TOF MS identification system has been validated for foodborne pathogens such as *Campylobacter*, *Clostridia*, *Cronobacter*, *Salmonella* and *Listeria* (Alispahic et al., 2010; Barbuddhe et al., 2008; Dieckmann et al., 2008; Grosse-Herrenthey et al., 2008; Stephan et al., 2010).

1.6 Growth of *Cronobacter* in Reconstituted PIF

Previous work by Nazarowec-White and Farber (1997a) showed that the growth temperature of eleven *Cronobacter* strains in Brain Heart Infusion (BHI) ranged from 5.5°C to 45°C. Iversen et al. (2004b) reported that the organism grew in reconstituted PIF from 6°C to 47°C, optimally at 37°C-43°C. According to Nazarowec-White and Farber (1997a), average generation times of *Cronobacter* strains were 40 min at room temperature of 23°C and 4.98 h at 10°C in three different formulae. Iversen et al. (2004b) found the mean generation times of six *Cronobacter* strains in reconstituted PIF were 13.7 h, 1.7 h and 22 min at 6°C, 21°C and 37°C. Lag times of *Cronobacter* spp. reported by Nazarowec-White and Farber (1997a) ranged from 9 to 47 h at 10°C and from 2 to 3 h at 23°C respectively. Lag times have been estimated to be 83 h at 10°C and 1.7 h at 37°C in average (Kandhai et al., 2006). Based on a low contamination level of 0.36 *Cronobacter* cells/100 g PIF reported by Muytjens et al. (1988) and a single feeding of 18 g PIF, an infectious dose of 1000 *Cronobacter* cells would be achieved in 9 days at 8°C, 7.9 days at 10°C, 17.9 h at 21°C and 7 h at 37°C, without taking into account possible inactivation of *Cronobacter* cells during PIF preparation, bacterial proliferation in infant stomach or cumulative exposure due to 4-6 feeding of infants in a 24 h period (Iversen and Forsythe, 2003). From the data shown in this theoretical calculation model, it is evident that reconstituted PIF contaminated with low levels of *Cronobacter* is unlikely to cause an infection in a healthy baby unless inappropriate preparation, handling and storage of feeding bottle including e.g. gross temperature abuse or

extrinsic contamination through poor hygiene preparation exist. The FAO/WHO established a quantitative risk assessment model for *Cronobacter* in PIF and concluded reconstitution temperatures of 40°C and 50°C, extended holding time at room temperature and long feeding periods as determining factors regarding enhanced risk of *Cronobacter* infection (Anonymous, 2006a). Therefore any control strategy with bacteriostatic or bacteriocidal properties in reconstituted PIF are believed to be capable of minimizing the infection risk. Heat treatment of food just prior to consumption has long been used as a primary tool to reduce the risk of infection caused by food borne pathogen. Edelson-Mammel and Buchanan (2004) investigated the effect of reconstituting PIF with water of different temperatures on *Cronobacter* inactivation and concluded that preparing PIF with water of 70°C or greater lead to a more than 4-log reduction in *Cronobacter* levels. Based on their findings, FAO/WHO (Anonymous, 2007c) recommended use of water ≥ 70°C to reduce the infection risk in infants associated with *Cronobacter* contamination. Besides thermal inactivation, there has been an increasing interest in introducing external hurdles via incorporation of various natural antimicrobials to control *Cronobacter* in PIF. Lactoferrin and nisin have been shown to have detectable antimicrobial activity against *Cronobacter* depending on concentration and temperature (Al-Nabulsi et al., 2009). Gurtler and Beuchat (2007) reported that lactoperoxidase inhibited the growth of *Cronobacter* in reconstituted PIF. Plant-derived essential oils including caprylic acid and trans-cinnamaldehyde have been determined to be effective agents in the suppression of *Cronobacter* in reconstituted infant formula as well as in the inhibition and inactivation of *Cronobacter* biofilms (Amalaradjou et al., 2009; Amalaradjou and Venkitanarayanan, 2011). A study of Kim et al. (2009) concluded that muscadine seed extracts, as rich sources of phenolic compounds and organic acids, displayed a strong antimicrobial activity against *Cronobacter* strains which was mainly caused by organic acids like tartaric, malic and tannic acid. Back et al. (2009) screened the inhibitory effects of eight organic acids and found propionic and acetic acid as most effective against *Cronobacter* in liquid foods including baby food. Commercially available acidified infant formula by direct addition of lactic acid in 1.4 g/100 g of dry composition has been shown to be effective in limiting *Cronobacter* (Kreb, 2010). Besides

direct addition of organic acids in PIF as an alternative acidification intervention has been developed through the fermentation of the basic ingredients of infant formula with lactic acid bacteria. In a study of Joosten and Lardeau (2004), a commercially available biologically acidified (fermented) infant formula showed a clear bacteriostatic effect on pathogenic bacteria including *Cronobacter* spp..

1.7 Growth of *Cronobacter* in rehydrated PIF and Raw Materials as a Prerequisite for a Successful Detection

Except for one method developed by AES Laboratories which directly starts with an enrichment in a selective medium *Enterobacter sakazakii* selective broth (ESSB), all culture-based methods published to date including the FDA reference method and the ISO standard method involve an initial non-selective pre-enrichment step where PIF is diluted in a ratio of 1:10 in distilled water or in BPW and incubated at 37°C for 16-20 h (Fanning and Forsythe, 2008; Kandhai, 2010; Lampel and Chen, 2009). In addition, rapid methods such as PCR, real time PCR or impedance have to be combined with the pre-enrichment step as well due to the necessity for reconstitution of stressed *Cronobacter* cells in PIF and for acquisition of minimal cell numbers required for detection limit. The *Cronobacter* growth in reconstituted PIF during the pre-enrichment stage is hence an extremely important prerequisite for the whole detection, because the following isolation and confirmation steps would not make sense unless a sufficient growth of target micro-organism is guaranteed in the pre-enrichment broth. According to ISO/TS 22964:2006, 0.1 ml from the overnight pre-enrichment is applied in 10 ml mLST for further isolation. In order to make sure that at least 1 CFU *Cronobacter* would be included in this 0.1 ml aliquot, *Cronobacter* cells initially present at low levels in 100 g PIF must theoretically increase to a minimal density of 9000 CFU in 900 ml pre-enrichment broth after overnight incubation at 37°C. Conversely, no *Cronobacter* is detectable if this lowest detection limit in the pre-enrichment is not achieved. Using the same simplistic model of Iversen and Forsythe (2003) which assumed an average of 0.36 *Cronobacter* cells/100 g PIF, lag time of 2 h and doubling time of 0.5 h at

37°C, it can be calculated that 100 g PIF reconstituted in 900 ml water would need to be kept for approximately 7.5 h at 37°C before a cell number of *Cronobacter* of 9000 CFU is achieved. It implies that at ideal conditions a sufficient *Cronobacter* growth can be ensured during the pre-enrichment. In the reality, nevertheless, any non-target micro-organisms present in the sample that are able to grow in BPW or water might compete with *Cronobacter* during the pre-enrichment and further reduce the numbers of target colonies transferred into selective broth. In addition, special PIF products with antimicrobial agents have been shown to limit the *Cronobacter* growth. In commercially available biologically acidified infant formulae e.g. NAN PELARGON® as well as in acidified formula supplemented directly with organic acid, concentration of *Cronobacter* cells after 6 h incubation at 30°C were restricted to 10 CFU/ml comparative to the initial spiking level, while in the non-acidified formula *Cronobacter* grew to as high as 10^5 CFU/ml (Kreb, 2010). In the investigation of Joosten and Lardeau (2004), similarly, *Cronobacter* levels over a period of 6 h at 37°C in rehydrated acidified formula were 1000-fold lower than that detected in non-acidified formula. Oshima et al. (2012) screened thirty-three antimicrobial agents including peptides, organic acids, organic acid esters and lactoperoxidase system (LPOS) etc. for their anti-*Cronobacter* activity and determined final numbers of *C. sakazakii* grown in reconstituted milk powder at 37°C after 8 h at a very low level of < 1 log CFU/ml in the presence of LPOS combined with nisin or lacticin, showing the most potential in *Cronobacter* inhibition, whereas in the controlling infant formula group without these additives a high number of *Cronobacter* in 7 log CFU/ml was detected. Although the antimicrobial hurdles added to PIF have been shown to be beneficial in protecting against *Cronobacter* overgrowth due to inhibitory interactions, there is risk of target micro-organism not reaching the required cell limit in the reconstitution stage, which could lead to a false negative outcome by detection. It is strongly emphasized that samples from which no *Cronobacter* is detectable do not necessarily indicate their microbiological safety. Therefore, the limited growth of *Cronobacter* in PIF or PIF additives must be taken into consideration when a method for *Cronobacter* detection is developed, validated or applied.

1.8 Prevalence of non-*Cronobacter* Enterobacteriaceae in PIF and the related influence on *Cronobacter* Identification

Mutyjens et al. (1988) were the first to assess the microbiological quality of powdered substitutes for breast milk with regard to members of Enterobacteriaceae family. *Enterobacter* (*E.*) *agglomerans* has been found to be present in PIF most frequently with 27% of all isolates, followed by *Enterobacter* (*E.*) *cloacae* with 23% and *Klebsiella* (*K.*) *pneumoniae* with 10%. However the identity of *E. agglomerans* in this study is uncertain because since the revision of *Enterobacter-Pantoea-Citrobacter* group, the former *E. agglomerans* now encompasses both *Pantoea* spp. and *Escherichia vulneris* (Janda and Abbott, 1998). In another survey performed by Iversen and Forsythe (2004), a distribution of most common organisms for *E. cloacae* (25%) and *Pantoea (P.)* spp. (19%) was described. Estuningsih et al. (2006) found 24% *P. agglomerans*, 20% *Escherichia hermanni*, 16% *E. cloacae* and 6% *K. pneumoniae* in PIF products manufactured in Indonesia and Malaysia. *E. cloacae* and *E. agglomerans* were isolated from 15 infant food formula products in UK (Shaker et al., 2007). Popp et al. (2009) reported *E. cloacae, K. pneumoniae, K. oxytoca* and *Pantoea* spp. as most frequently isolated Enterobacteriaceae species from infant formula products. These available data allow the conclusion that besides *Cronobacter* spp., the most dominant Enterobacteriacea isolated from PIF included *Enterobacter* species, *Pantoea* species, and *Klebsiella* species. The available data do not indicate any relationship between Enterobacteriaceae and *Cronobacter* present in PIF samples. However neither is it possible to rule out a possible correlation with the available data. Cordier (2008) has shown that high levels of Enterobacteriaceae > 100 CFU/g are likely to represent an increased risk of product contamination with *Cronobacter*. Monitoring of Enterobacteriaceae is used as an indicator of hygienic status in production facilities (Anonymous, 2007b). As shown in the microbiological criteria for PIF applied by the European Commission, EC No 1441/2007 (Anonymous, 2007a), the detection of Enterobacteriaceae is a process hygienic criterion, while *Salmonella* and *Cronobacter* spp. serve as food safety criteria (Table 1).

Table 1. Current microbiological criteria for dried infant formula for infants up to 6 months of age and for formulae for special medical purposes of European Union

Micro-organisms	***n***	***c***	***m***	***M***	
Enterobacteriaceae (10 g)	10	0	0/10 g	NA	Process hygienic parameter
Cronobacter spp. (10 g)	30	0	0/30 g	NA	Food safety parameters
Salmonella (25 g)	30	0	0/30 g	NA	

n is the number of samples to be analyzed per lot;

c is the number of samples allowed between "m" and "M" value;

m is the microbiological limit separating good from marginally acceptable quality;

M is the microbiological limit separating marginally acceptable from unacceptable quality.

Based on findings of Guillaume-Gentil et al. (2005) and Nazarowec-White and Farber (1997b) that *Cronobacter* spp. showed a higher tolerance against osmotic stress and heat stress than most of other Enterbacteriaceae competitors except for four strains of *E. hermanni*, *E. cloacae*, *K. oxytoca* and *K. pneumoniae*, the new ISO specification for PIF ISO/TS 22964:2006 has been developed using mLST as the selective enrichment broth (Anonymous, 2006b). However, either using the selective enrichment media combined with elevated temperature in ISO method or using the non-differential FDA enrichment procedure, Enterobacteriaceae in different genera and species have been isolated from PIF, environmental samples or foods other than infant formula where competitive flora quantitatively occurred (Baumgartner et al., 2009; Iversen et al., 2009; Lehner et al., 2010; Shaker et al., 2007).

Objectives of the study

Consumption of reconstituted PIF contaminated with *Cronobacter* spp. has been shown to be associated with a high risk of infection diseases in infants. The present work aimed to get more insight into the effect of using hot water with a temperature of 70°C or higher for PIF rehydration on the growth and survival of *Cronobacter* present in fermented and non-fermented infant formula (Chapter 2). As an alternative to thermal inactivation, the influence of organic acids as natural antimicrobial barrier on the growth of *Cronobacter* spp. in laboratory medium as well as in the reconstituted PIF was investigated. Particularly the anti-*Cronobacter* effect of most inhibitive organic acids was studied under natural infant stomach acidity (Chapter 3). Due to the antimicrobial compounds or competing flora in PIF, the growth of *Cronobacter* spp. in the pre-enrichment stage can be inhibited, which could influence principally the overall effectiveness of the *Cronobacter* detection method. A newly developed impedance method combined with rRNA lateral flow assay for detection of *Cronobacter* spp. in PIF was evaluated, with possible effect of organism's history (healthy or stressed) and competing background flora assessed (Chapter 4). At last, various systems for the isolation, identification and differentiation of *Cronobacter* spp. from other micro-organisms with a common presence in PIF have been comparatively assessed in view of their sensitivity, selectivity, discrimination power, rapidity as well as convenience in performance (Chapter 5 and Addendum).

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Chapter 2

Thermal inactivation of *Cronobacter* spp. during rehydration of powdered infant formula with water of 70°C or higher

Abstract

The presence of *Cronobacter* spp. in powered infant formula (PIF) has been associated to outbreaks of rare, but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in newborns. This study was undertaken to explore the influence of reconstitution water of 70°C or higher on survival of *Cronobacter* spp. in PIF. A higher final temperature was obtained in plastic baby bottles than glass baby bottles after PIF was mixed with water of the same temperature. An average reduction in *Cronobacter* levels of 3.6 log was achieved if contaminated PIF was prepared with water at 80°C in plastic bottles. The inhibitory effect on *Cronobacter* in fermented infant formula (FIF) was reduced by reconstituting the formula with water of 80°C. Reconstitution temperature of 80°C did not lead to a greater reduction in vitamin C concentration in PIF compared to lower reconstitution temperatures of 20°C and 40°C.

Introduction

Cronobacter species, formerly known as *Enterobacter sakazakii*, are Gram-negative, rod-shaped pathogenic bacteria of the family Enterobactericeae. These organisms have been implicated as causes of life-threatening infections like meningitis, necrotizing enterocolitis and bacteremia predominantly in infants < 4 weeks of age with a mortality rate of 20%-50% (Anonymous, 2007a; Clark et al. 1990; Lehner and Stephan, 2004). Powdered infant formula (PIF) is not a sterile product. Contamination of PIF can occur intrinsically during the manufacturing process or from extrinsic sources through contaminated utensils (e.g. spoons, blenders, teats, bottles) (Anonymous, 2007b). PIF contaminated with *Cronobacter* spp. has been microbiologically and epidemiologically shown to be the vehicle and source of infection in infants. Heat treatment of food just prior to consumption has been used as a primary tool to reduce the risk associated with foodborne pathogens (Edelson-Mammel and Buchanan, 2004). FAO/WHO (Anonymous, 2007b) recommended reconstitution of PIF with water at 70°C or higher to reduce the potential risk associated with *Cronobacter* contamination. However, heating infant milk to ≥ 70°C raises other concerns such as loss of heat-sensitive nutrients and an increased risk of scalding. Besides, the high reconstitution

temperature may be inadequate for some PIF products containing probiotics because they could be killed by water of ≥ 70°C (Anonymous, 2006). This study was undertaken to evaluate the effect of preparing PIF and FIF with water ≥ 70°C on survival of *Cronobacter*.

Materials and Methods

Temperature of rehydrated PIF in baby bottles prepared with water at 70°C and 80°C

The tap water was first boiled in an electric kettle. The freshly boiled water was left at the room temperature to cool down to 80°C and 70°C respectively. According to the preparation instruction, 90 ml water were added to sterilized baby bottles made of glass and of plastic respectively. Three scoops (13.5 g) of PIF were added to the water. In the same manner, five scoops (22.5 g) PIF were dissolved in 150 ml water in glass and plastic baby bottles. The bottles were caped and gently agitated by hand at room temperature for 10 seconds. The temperature of rehydrated PIF in baby bottles was measured and three independent trials were performed.

Survival of *Cronobacter* strain in PIF prepared with water of 80°C

Commercially available PIF products were first screened for absence of *Cronobacter* spp. with ISO/TS 22964. 90 ml water of 80°C were poured in to a sterilized plastic baby bottle prior to adding 3 scoops of PIF. The rehydrated PIF was inoculated with 1 ml fresh overnight culture of *C. sakazakii* CLF2688 at levels of 10^8, 10^7, 10^6 and 10^5 and 10^4 CFU/90 ml respectively. The bottle was left at room temperature for 2 min and then cooled under the running tap water to 37°C. The *C. sakazakii* count in the bottle was determined by spread-plate technique using Brilliance™ *E. sakazakii* chromogenic agar Druggan-Forsythe-Iversen (DFI) formulation (Oxoid, UK). Three independent trials were performed.

Impact of reconstitution temperature of 80°C on the antibacterial effect of FIF

FIF in different fermentation levels (0%, 30%, 50% and 100%) were applied in this test. A non-fermented neutral PIF starter product served as reference. In the first group, 1-10 CFU

C. sakazakii (CLF2688) were added to 90 ml reconstituted FIF and PIF which were prepared with water of 40°C in a plastic baby bottle. To investigate the effect of a higher reconstitution temperature on the inhibitory activity of FIF against *Cronobacter*, 90 ml FIF and PIF were first prepared with water at 80°C and left at room temperature for 2 min. After being cooled down to 40°C under the running tap water, the infant formulae were artificially spiked with 1 ml *C. sakazakii* (CLF2688) in 1-10 CFU/90 ml of diets. Inoculated infant formulae in both groups were stored at room temperature for 24 h. The *Cronobacter* growth was followed by enumeration with DFI chromogenic agar after 5 and 24 h. Three independent trials were performed.

Vitamin C concentration in PIF prepared with water of 20°C, 40°C and 80°C

12.20 g commercially available PIF product were weighed in the flask and filled with 100 ml water of 20°C, 40°C and 80°C respectively. The flask was well shaken and stored at a household fridge temperature of 9°C for 8 h. The concentration of vitamin C in rehydrated PIF was determined with a potentiometric method on titrator (Mettler TOLEDO T50, Germany) using 2,6-dichchlorophenolindophenol at 0 h, 4 h and 8 h.

Statistic analysis

Data were analyzed with Student's t test using Microsoft® Excel 2003 software. Significant differences are presented at a 95% confidence level ($p < 0.05$).

Results

Compared with initial water temperatures of 70°C and 80°C added to bottles, the temperatures after PIF-mixing decreased in all bottle/serving volumes combinations (Table 1). However for both 90 ml and 150 ml serving volumes, the temperature in rehydrated PIF dropped to a less extend in bottles made of plastic than that of glass. Besides, a more rapid decrease in temperature after PIF-mixing was observed in smaller serving volume of 90 ml than in larger volume of 150 ml in glass bottles, while the final temperatures in plastic bottles did not differ between rehydration quantities.

Table 1. Temperature of rehydrated infant formula after hot water of 70°C and 80°C was added to glass and plastic baby bottles containing PIF

Water quantity	PIF reconstitution temperature			
	70°C		80°C	
	Glass bottle	Plastic bottle	Glass bottle	Plastic bottle
90 ml	54°C	62°C	63°C	70°C
150 ml	58°C	62°C	65°C	70°C

As higher final temperatures were reached in PIF rehydrated with water of 80°C than 70°C, the water of 80°C was used for preparing PIF in the following tests. With rehydration temperature of 80°C, the observed *Cronobacter* cell reduction in plastic bottles ranged from 3.5 log to 4.4 log when initial counts exceeded 10^6 CFU/90 ml, whereas in glass bottles a higher *Cronobacter* surviving number was obtained at initial inoculums of 10^8, 10^7 and 10^6 CFU. Especially at higher inoculums of 10^8 and 10^7 CFU *Cronobacter* cells, the thermal inactivation effect of preparing PIF with water at 80°C was very pronounced in plastic bottles: a 200 to 300 fold higher *C. sakazakii* counts surviving in glass bottles were obtained than in plastic ones. At lower inoculum of 10^6 CFU, the difference in *Cronobacter* survival between bottles of two materials was minimal, while at a contamination level of 10^5 CFU, *Cronobacter* counts dropped in both kinds of bottles to 10^2 CFU. For an inoculum of 10^4 CFU/90 ml PIF, the reduction was greater than 2 log and this is below the lowest detection limit of 1 CFU/ml corresponding to limit of detection (LOD) of 1.9 log CFU/90 ml (Table 2).

Table 2. Survival of *C. sakazakii* (CLF2688) during the rehydration of PIF with water of 80°C at different initial contamination levels

Initial inoculum of *Cronobacter* in infant formula (log CFU/90 ml)	Surviving *Cronobacter* (log CFU/90 ml) in Mean ± SD	
	Glass bottle	Plastic bottle
8	6.2 ± 0.3	3.7 ± 0.3
7	5.2 ± 0.2	2.8 ± 0.1
6	3.1 ± 0.1	2.5 ± 0.3
5	2.2 ± 0.5	2.5 ± 0.2
4	< 1.9 [a]	< 1.9 [a]

[a] Lower limit of detection: 1 CFU/ml = 90 CFU/90 ml (log 90 CFU/90 ml = 1.9)

After 5 h incubation at room temperature, the *C. sakazakii* populations were between 2 and 3 log CFU/ml in all products but in the 100% fermented FIF, regardless of reconstitution temperatures. In the FIF fermented to 100%, a slight but significant reduction in *Cronobacter* growth by 0.7 log was shown for reconstitution with 40°C when compared with the same product treated previously with water of 80°C (Fig. 1). On the other hand, among all products rehydrated with an ambient water temperature of 40°C as recommended by manufacturer, only limited inhibitory effect was shown in FIF fermented to 100% and no antibacterial effect was visible in formulae fermented to 30% and 50% compared with non-fermented FIF or reference PIF. In the group rehydrated with water of 80°C, the target micro-organism was able to grow to ≥ 7 log CFU/ml in all products within 24 h incubation, while a significant cell reduction ranging from 1.5 log to 2.4 log was shown in FIF fermented in 30%, 50% and 100% when prepared with water of 40°C (Fig. 2). The pH values at 0 h were 6.70, 6.25, 6.00, 5.60 and 6.80 for FIF fermented to 0%, 30%, 50%, 100% as well as in the control PIF respectively. After 5 h incubation slightly enhanced pH values were obtained for all products. After 24 h incubation decreased pH values of 4.16, 4.15 and 4.30 were obtained in FIF fermented to 30%, 50% and 100%. No obvious pH drop was observed in non-fermented FIF and reference product.

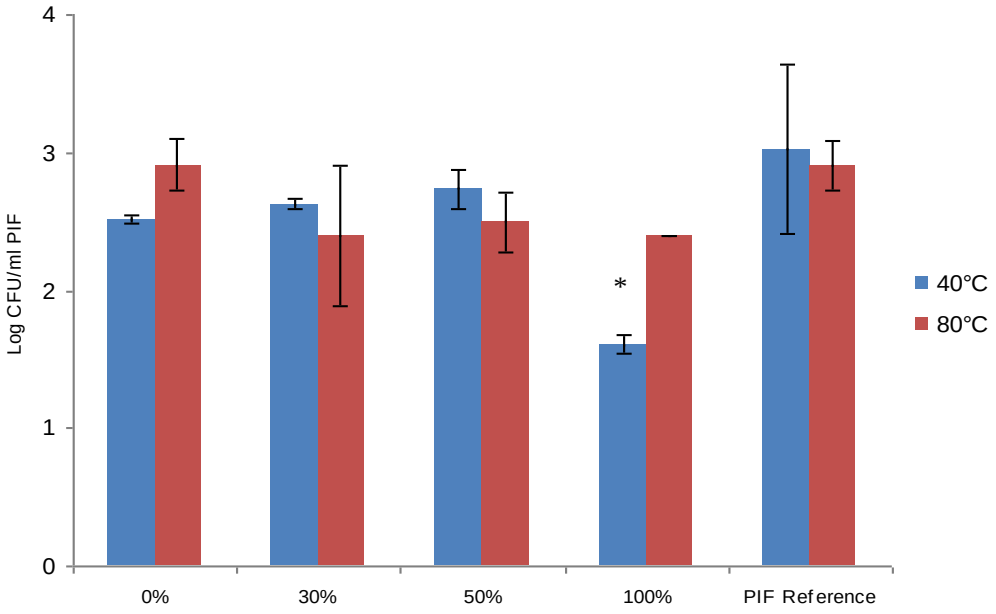

Figure 1. Growth of *C. sakazakii* (CLF2688) (log CFU/ml) in FIF/PIF hydrated with 40°C and 80°C after 5 h incubation at 22°C with an initial inoculum of 1-10 CFU *Cronobacter*/90 ml. Value with * is significantly different ($p < 0.05$) with remaining values without *.

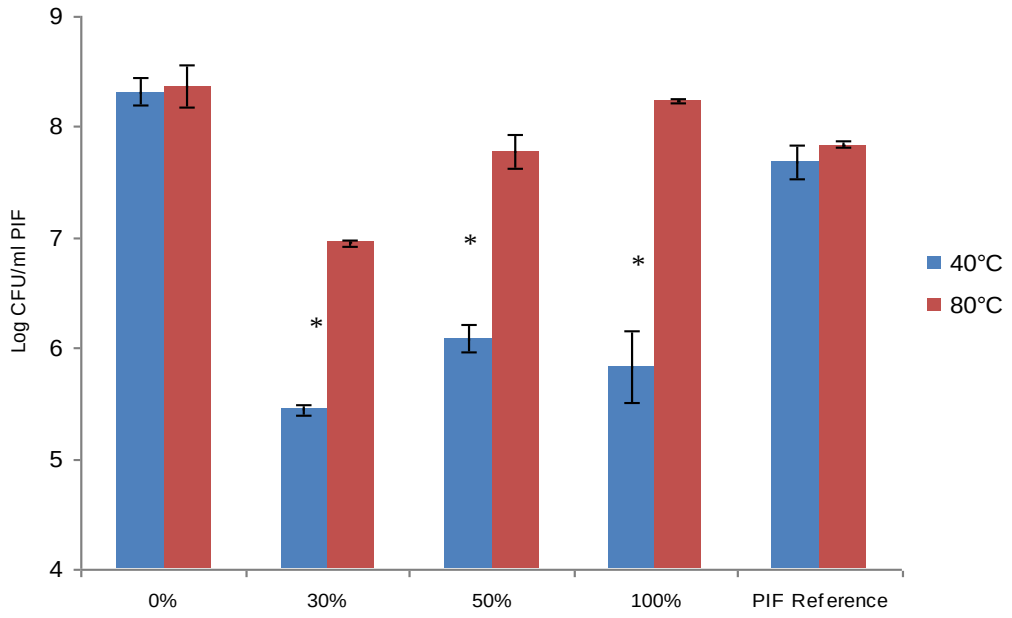

Figure 2. Growth of *C. sakazakii* (CLF2688) (log CFU/ml) in FIF/PIF hydrated with 40°C and 80°C after 24 h incubation at 22°C with an initial inoculum of 1-10 CFU *Cronobacter*/90 ml. Values with * are significantly different ($p < 0.05$) with remaining values without *. The three values with * revealed no significant differences with each other ($p > 0.05$).

As shown in Fig. 3, the vitamin C concentrations in rehydrated PIF were at the beginning 14.9, 14.5, 14.6 mg/100 ml and were reduced to 10.7, 10.2 and 10.9 mg/100 ml for preparation water temperature of 20°C, 40°C and 80°C respectively after 8 h storage at 9°C, which indicates that the vitamin C reduction caused by a high preparation temperature did not differ from that at lower temperatures.

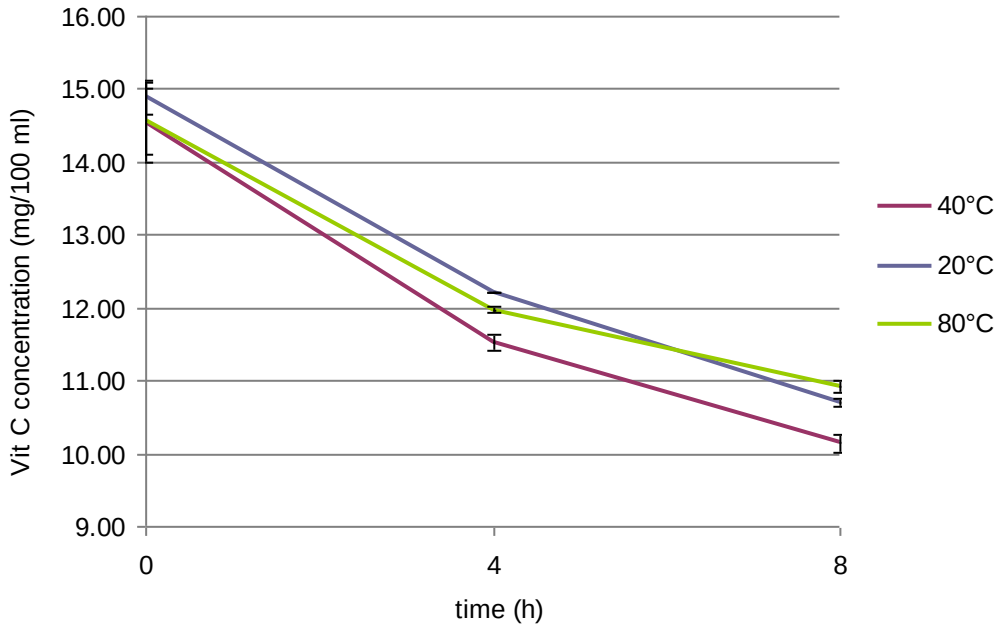

Figure 3. Vitamin C concentration in PIF rehydrated with water of 20°C, 40°C and 80°C over 8 h at a household fridge temperature.

Discussion

In Germany, use of water at 40°C or 50°C for reconstituting PIF is recommended by the manufacturers for products such as Milumil, Aptamil, Hipp and Alete. However, FAO/WHO (Anonymous, 2006) performed a risk assessment model for *Cronobacter* in PIF and concluded that PIF reconstitution with water at 40°C and 50°C is highly associated with an increased risk of *Cronobacter* infection. Edelson-Mammel and Buchanan (2004) reported that using water at 70°C or greater for PIF preparation was able to cause a more than 4-log reduction in *Cronobacter* levels and could therefore significantly reduce the risk of infection. In addition, Chen et al. (2009) showed less survival of *Cronobacter* cells in reconstitution water ≥ 70°C in larger volumes than in smaller volumes because a higher temperature was maintained for a longer time in larger volumes, exhibiting a more pronounced lethality effect. In the present work, temperature differences between PIF prepared in smaller and larger volumes were only observed in feeding bottles made of glass. It is known that lower final temperatures achieved in PIF after reconstitution water is added allow a better survival of *Cronobacter* cells (Chen et al., 2009; Kim and Park, 2007). Due to the fact that younger

infants often receive liquid formula prepared in a smaller quantity per feeding than older infants and the younger babies are more vulnerable to *Cronobacter* infections than older babies (Anonymous, 2006), the consumption of contaminated PIF in a smaller serving volume could therefore pose a high risk of *Cronobacter* infection particularly to infants at younger age. As shown in Table 1, the highest final temperature of 70°C was achieved in PIF prepared with water of 80°C in plastic bottle regardless of serving volumes, while the lowest final temperature of 54°C after PIF-mixing was obtained in 90 ml PIF rehydrated with water of 70°C in glass bottle. This might indicate that the plastic bottle can hold heat for longer period of time than glass one probably due to the high capacity of glass material for heat absorption. The temperature drop in PIF after it was mixed with hot water has been documented in various previous studies (Chen et al., 2009; Edelson-Mammel and Buchanan, 2004; Kim and Park, 2007). However, no information about the material of baby bottles used was given in details: while only a rough description as “feeding bottles” or “baby bottles” was given in publications of Edelson-Mammel and Buchanan (2004) and Kim and Park (2007), Chen et al. (2009) did not mention at all in what kind of container the PIF was reconstituted and the growth of *Cronobacter* was determined. From the data presented in Table 1, it is evident that the material of baby bottles represents an important determinant for temperature drop in rehydrated PIF impacting the *Cronobacter* survival. In the present work, rehydration of 90 ml PIF in 80°C hot water resulted in greater than 3 log and 2 log reduction in the *Cronobacter* strain tested in plastic and glass bottle respectively (Table 2). By contrast, in the pilot study of Edelson-Mammel and Buchanan (2004), a stronger effect of reconstitution water of 80°C on *Cronobacter* inactivation in PIF of $\geq$ 4 log has been reported. However it must be noted that in their investigation a higher volume of formula (180 ml) was prepared using 25.5 g PIF contributing to decrease of temperature in a less extent and further a lower *Cronobacter* survival. In the study of Chen et al. (2009), *Cronobacter* cell inactivation of greater than 4 log by using water of 80°C was observed on two pre-selected heat-sensitive target strains inoculated in larger serving volumes. Last but not least, different incubation time could be responsible for the observed differences in *Cronobacter* survival rate as well. In the present study the heat in PIF prepared with hot

water of 80°C was kept for 2 min before *Cronobacter* counts were determined, whereas the bottles were hold for 10 min and analyzed for *Cronobacter* by Edelson-Mammel and Buchanan (2004). A relative low level of *Cronobacter* cells of 0.36 to 66 CFU *Cronobacter*/100 g PIF has been determined by Muytjens et al. (1988). This implies that preparing reconstituted formula using the best option presented here (80°C in plastic bottle) is likely to result in a high probability that a serving would not contain this micro-organism. Concerns have been raised in using water of ≥ 70°C to prepare PIF due to possible loss of nutrient in product or inactivation of probiotic added in specialized infant formula (Anonymous, 2006). Fermented infant formula is infant formula fermented with lactic acid-producing bacteria e.g. *Bifidobacterium* and *Streptococcus* during the production process. The FIF in the present work is based on a mixture of fully fermented PIF and non-fermented PIF product. The fermentation levels of FIF refer to the percentage of fully fermented product in the whole mixture. FIF usually contains low numbers of viable bacteria (< 10^3 CFU/g dry weight) in the final product due to the inactivation of the fermenting bacteria by physical treatment. Clinical trials have shown that some FIF could reduce the occurrence or severity of infectious diarrhea in infants which is likely to be associated with its bacteriostatic effect (Brunser et al., 1989; Joosten and Lardeau, 2004; Thibault et al., 2004). It has been concluded that the additional benefits of FIF result from the remaining bacterial components such as cell membrane or bacterial DNA, and/or bacterial metabolites such as organic acids or protein with enzyme activity rather than surviving intact living bacteria (Agostoni, et al., 2007; Ludwig, et al., 2012). As shown in Fig. 2, the FIF fermented to 30%, 50% and 100% showed an inhibition effect on *Cronobacter* growth in 24 h if rehydrated with water at 40°C. The cell reduction might be caused by the relatively lower pH values in these products after 24 h incubation. No inhibitive effect was demonstrated in FIF when it was prepared with 80°C water, indicating the thermal sensitivity of the compositions produced by fermentation. On the other hand, the inhibition effect of FIF is very limited. In FIF with lower fermentation levels of 30% and 50%, *Cronobacter* grew to higher than 100 CFU/ml equivalent to 9000 CFU/90 ml diet within 5 h at room temperature of 22°C if the PIF was reconstituted with water of 40°C

according to recommendation. 5 h is regarded as a critical timeframe in consumer practices where an extended feeding or holding can really occur. Most importantly, the cell concentration of 9000 CFU/90 ml exceeds the infectious dose of 1000 CFU estimated by Iversen and Forsythe (2003) and is very close to the infectious level of 10,000 CFU proposed by FAO/WHO (Anonymous, 2007a). In 24 h *Cronobacter* grew to a cell density of between 10^5 and 10^6 CFU/ml in FIF treated with water of 40°C, posing a high risk of infection. By comparison, the alternative *Cronobacter* inactivation strategy by using water of ≥ 70°C for reconstitution which should provide virtually instantaneous inactivation of this micro-organism is presumed to kill all *Cronobacter* cells present initially at low level in infant formula and is therefore likely to control the risk more effectively. Reduction of vitamin C ranging from 5.6% to 65.6% in four particular formulae due to preparation with boiling water has been noted in the report of the FAO/WHO expert meeting (Anonymous, 2006; Anonymous, 2007b). To solve this problem, dry formulae actually contain higher levels of vitamin C than labelled. After reconstituting with boiling water, the vitamin C levels in the four formulae mentioned above still exceeded the can label or were higher than the minimum recommendation (8 mg vitamin C/100 calories) required by the CAC Codex Standard for Infant Formula (Anonymous, 1981). Based on data in Fig. 4, the vitamin C concentration in reconstituted PIF decreased over the incubation duration at refrigerator temperature. However the reduction of vitamin C is not directly related to the enhanced water temperature. Rehydration of PIF with 80°C water resulted in a slighter vitamin C reduction of 18% and 25% at 4 h and 8 h respectively than reductions achieved with 20°C (21% and 30%) and 40°C (18% and 28%).

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Chapter 3

Growth inhibition of *Cronobacter* spp. strains in reconstituted powdered infant formula acidified with organic acids supported by natural stomach acidity

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Growth inhibition of *Cronobacter* spp. strains in reconstituted powdered infant formula acidified with organic acids supported by natural stomach acidity

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ABSTRACT

Cronobacter is associated with outbreaks of rare, but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in newborns. This study was conducted to determine the effect of organic acids on growth of *Cronobacter* in laboratory medium and reconstituted powdered infant formula (PIF) as well as the bacteriostatic effect of slightly acidified infant formula when combined with neonatal gastric acidity. Inhibitory effect of seven organic acids on four acid sensitive *Cronobacter* strains was determined in laboratory medium with broth dilution method at pH 5.0, 5.5 and 6.0. Acetic, butyric and propionic acids were most inhibitive against *Cronobacter* in the laboratory medium. The killing effect of these three acids was partially buffered in reconstituted PIF. Under neonatal gastric acid condition of pH 5.0, the slightly acidified formula which did not exert inhibition effect solely reduced significantly the *Cronobacter* populations. A synergistic effect of formula moderately acidified with organic acid combined with the physiological infant gastric acid was visible in preventing the rapid growth of *Cronobacter* in neonatal stomach. The study contributed to a better understanding of the inhibitory effect of organic acids on *Cronobacter* growth in different matrixes and provided new ideas in terms of controlling bacteria colonization and translocation by acidified formula.

1. Introduction

Cronobacter has recently been described as a novel genus in 2008 and it comprises seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter turicensis*, *Cronobacter universalis* and *Cronobacter condimenti* (Iversen et al., 2007; Joseph et al., 2012). Known as emerging food borne pathogens, *Cronobacter* spp. have been implicated in neonatal infections such as meningitis, septicemia or necrotizing enterocolitis (NEC) with a mortality of 20–50% (Clark et al., 1990; Lehner and Stephan, 2004). Due to their ubiquitous occurrence, *Cronobacter* spp. have been isolated from a variety of environmental and clinical sources including hospitals, households and factories, as well as from a wide range of food and food ingredients (Baumgartner et al., 2009; Kandhai et al., 2004; Mullane et al., 2008). Especially, contaminated powdered infant formula (PIF) has been epidemiologically identified as one of the most likely vehicles of *Cronobacter* transmission (Clark et al., 1990; Van Acker et al., 2001). There have been approximately 120 cases of *Cronobacter* infections in neonates and young children reported and a number of outbreaks have been tracked back to the presence of these pathogens in PIF or in food preparation equipment (Anonymous, 2008).

Reconstituted PIF provides optimal growth conditions for *Cronobacter*, especially when stored inappropriately i.e. at temperatures higher than 20 °C over extended periods of several hours (Anonymous, 2006). FAO/WHO recommends the preparation of PIF at a temperature of at least 70 °C, which dramatically reduces the risk of a *Cronobacter* infection (Anonymous, 2007b). However this procedure cannot eradicate the problem completely. Due to the unpredictable consumer behaviours the 70 °C treatment is not reliable and in a number of cases not applied at all. There has been hence a growing interest in development of ingredients which can inhibit the microbiological growth in reconstituted formula. One option would be the utilization of antimicrobial fortifiers in PIF to limit the multiplication of *Cronobacter* and thereby reduce the risk of infections in infants by consumption of contaminated PIF (Back et al., 2009; Nair et al., 2004). Given that synthetic compounds or antibiotics may pose some level of safety risk to infants and young children, the development of substances intrinsically of food origin which can inhibit bacterial growth has become the desired approach in controlling the number of pathogens in infant foods.

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Organic acids have a long history of being used as food additives and preservatives for preventing microbial contamination and dissemination in food production, processing and storage. In animal nutrition, formic acid and propionic acid have been added to chicken feeds due to their antifungal and bactericidal activity particularly against *Salmonella* (Hinton and Linton, 1988; Kwon et al., 1998; Paster, 1979). In human food and beverage product, benzoic, acetic, sorbic and propionic acid constitute the most extensively used acid preservatives for reasons of solubility, taste and low toxicity (Cherrington et al., 1991a; Hazan et al., 2004). Acidification of infant formulas with propionic, acetic and lactic acid has been reported as an effective way in preventing the rapid proliferation of pathogenic bacteria like *Cronobacter* spp., *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* etc (Back et al., 2009; Joosten and Lardeau, 2004). In the present study, the effect of organic acids in inhibiting the growth of *Cronobacter* strains in laboratory medium and reconstituted PIF was evaluated. Additionally, a simplified static gastric model was applied to investigate the growth kinetics of *Cronobacter* strains in organic acid-treated reconstituted PIF under physiological infant gastric conditions.

2. Materials and methods

2.1. Bacterial strains

30 *Cronobacter* strains of food, clinical and environmental sources were used in this study: CLF39, CLF70, CLF90, CLF128, CLF155, CLF162, CLF187, CLF209, CLF247, CLF248, CLF259, CLF282, CLF323, CLF352, CLF353, CLF462, CLF468, CLF527, CLF548, CLF587, CLF588, CLF772, CLF2682, CLF2683, CLF2684, CLF2687, CLF2688, CLF2689 and CLF2691. Strains frozen at −80 °C on biobeads (Transia, Germany) were individually overnight grown in 90 ml buffered peptone water (BPW) (E. Merck, Germany) for 24 h at 37 °C.

2.2. Screening of Cronobacter strains for growth properties over a wide pH spectrum in laboratory medium

BiMedia 001B (Sylab, Austria) was prepared according to the manufacturer's directions. The pH of the broth was adjusted to pH 4.5, 5.0, 5.5, 6.0 and 7.0 with 1 mol/l hydrochloric acid (HCl) (E. Merck, Germany) and then sterilized by 0.22 μm vacuum filtration (Millipore, Germany). The pH values were monitored with pH-Meter CG 840 (Schott, Germany). The pH-adjusted BiMedia 001B was then aseptically dispensed into 10-ml proportions into sterile culture tubes. *Cronobacter* overnight cultures were inoculated in pH-adjusted BiMedia 001B at a level of 1–10 CFU/10 ml. Each strain/pH combination was then incubated at 37 °C. After 24 h incubation, all samples and dilutions in peptone saline solution (PSS) (maximum recovery diluents, E. Merck, Germany) were spread-plated on Tryptic Soy Agar (TSA) (E. Merck, Germany) by using 1 ml of suspension for *Cronobacter* enumeration.

2.3. Chemical preparation for organic acids in laboratory medium

Several kinds of organic acids including tartaric acid (E. Merck, Germany), citric acid (E. Merck, Germany), malic acid (E. Merck, Germany), lactic acid (E. Merck, Germany), acetic acid (Prolabo, Germany), butyric acid (E. Merck, Germany) and propionic acid (E. Merck, Germany) were used in the study. Each acid was made up into stock solutions (1 mol/l) in sterile distilled water and then added to 200 ml BiMedia 001B to give final pH values of pH 5.0, pH 5.5 and pH 6.0, respectively. The acidified mediums were sterilely filtered with 0.22 μm vacuum-driven filtration systems.

2.4. Effect of organic acids on Cronobacter growth in laboratory medium

Four acid sensitive strains, *C. sakazakii* CLF70, *C. universalis* CLF2684, *C. malonaticus* CLF248 and *C. turicensis* CLF587 that showed a limited growth at pH 5.0 in the initial screening trial were grown in BPW at 37 °C for 24 h. After dilution with PSS, strain suspensions were added to 10 ml acids-treated BiMedia 001B at pH 5.0, 5.5 and 6.0, respectively, yielding an initial concentration of approximately 1–10 CFU/10 ml and incubated at 37 °C for 24 h. After incubation, all samples and dilutions in PSS were spread-plated on TSA by using 1 ml of suspension for *Cronobacter* enumeration.

2.5. Effect of selected organic acids on Cronobacter growth in reconstituted PIF

10 g commercial PIF sample were reconstituted in 90 ml sterile water. To achieve specific pH values, acetic, butyric and propionic acid were added into freshly prepared reconstituted infant formula at high concentrations (19.50, 22.00, and 20.00 mmol/l) and low concentrations (7.25, 6.50 and 7.00 mmol/l), respectively. Each of acidified PIF samples was inoculated with *Cronobacter* strains at a level of 1–10 CFU/90 ml respectively and incubated at 37 °C for 24 h. After incubation, all samples and dilutions in PSS were spread-plated on Brilliance™ *Enterobacter sakazakii* chromogenic agar Druggan–Forsythe–Iversen (DFI) formulation (Oxoid, UK) by using 1 ml of suspension for *Cronobacter* enumeration.

2.6. Cronobacter growth in acidified PIF under influence of physiological gastric acid

90 ml reconstituted PIF were acidified with acetic acid at 7.25 mmol/l to achieve a pH value of 6.0. 90 ml reconstitution of the same diet without organic acid supplementation was used as control (pH 7.0). 1.2 ml HCl (1 mol/l) was added into the acidified/non-acidified infant formulas to give an environmental pH value of 5 to simulate the infant physiological gastric acidity. A bacterial challenge was performed by adding *C. sakazakii* CLF2688 and *C. universalis* CLF2684 respectively, at a level of 1–10 CFU/90 ml of diets. Inoculated formulas were incubated at 37 °C for 24 h and the *Cronobacter* growth was followed by enumeration with DFI chromogenic agar after 5 and 24 h.

2.7. Cronobacter growth in acidified PIF in a simulated gastric model

90 ml acetic acid acidified and non-acidified reconstituted PIF samples were prepared as described above. 1.2 ml hydrochloric acid (1 mol/l) was added into the acidified/non-acidified infant formulas to give a pH value of 5. Based on data documented by Groiss (2011) and by Hamosh and Hamosh (2000), simulation of gastric fluid constitutions of newborns was attempted in a static model: Gastric secretions including 100 mmol/l NaCl, 5 mmol/l KCl, 0.9 mmol/l $CaCl_2$, 3 mmol/l $MgCl_2$ as well as 3338 U/mg pepsin (Sigma, Germany) and 16.5 U/mg lipase (AppliChem, Germany) were added into infant formulas additionally. A bacterial challenge was performed by adding *C. sakazakii* CLF2688 and *C. universalis* CLF2684 respectively, at a level of 1–10 CFU/90 ml. Inoculated formulas were incubated at 37 °C for 24 h and growth of micro-organisms was followed by enumeration DFI chromogenic agar after 5 and 24 h.

2.8. Statistic analysis

All experiments were performed in three independent trials. Data were analysed with Student's *t* test using Microsoft® Excel

Table 1
Populations of *Cronobacter* strains in HCl-acidified laboratory medium BiMedia 001B ranging from pH 4.5 to pH 11.0 for 24 h.

Strain	Population of *Cronobacter* (log CFU/ml)				
	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 7.0
C. sakazakii CLF2688	A ND a	A 7.41 ± 0.26 b	A 7.45 ± 0.51 b	A 8.13 ± 0.37 b	A 7.99 ± 0.27 b
C. sakazakii CLF70	A ND a	B 3.89 ± 0.89 b	A 7.22 ± 0.27 b	A 7.46 ± 0.52 b	A 8.25 ± 0.63 b
C. universalis CLF2684	A ND a	B 3.31 ± 0.88 b	A 7.77 ± 0.09 b	A 7.64 ± 0.30 b	A 7.85 ± 0.39 b
C. malonaticus CLF248	A ND a	B 3.60 ± 0.24 b	A 7.82 ± 0.08 b	A 7.64 ± 0.43 b	A 7.52 ± 0.16 b
C. turicensis CLF587	A ND a	B 4.11 ± 1.04 b	A 7.58 ± 0.13 b	A 7.34 ± 0.11 b	A 7.32 ± 0.22 b

Within the same row, mean values for populations that are not followed by the same small letter are significantly different ($P \leq 0.05$).
Within the same column, mean values for populations that are not preceded by the same capital letter are significantly different ($P \leq 0.05$).
ND, no detection. Log CFU/ml of *Cronobacter* was <0.

2003 software. Significant differences are presented at a 95% confidence level ($P \leq 0.05$).

3. Results

3.1. Growth characteristics of Cronobacter strains at various pH conditions in laboratory medium

The growth properties of *Cronobacter* strains in the laboratory medium varied with the pH conditions in the environment. Among 30 *Cronobacter* isolates, 26 strains showed a similar manner of growth response over the complete pH spectrum. The isolate *C. sakazakii* CLF2688 was thus selected as representative for this panel of strain collection and listed comparatively to the remaining strains. As shown in Table 1, all of the *Cronobacter* strains were able to grow to $\geq 10^7$ CFU/ml at pH values ranging from 5.5 to 7.0, whilst at pH 4.5 no growth was detected at 37 °C. The growth was substantially different at pH 5.0. 26 strains including *C. sakazakii* CLF2688 were clearly resistant to pH 5.0 for 24 h and displayed a full growth to 10^7–10^8 CFU/ml. In contrast, exposure to pH 5.0 caused an extensive and significant growth suppression on four isolates, *C. sakazakii* CLF70, *C. universalis* CLF2684, *C. malonaticus* CLF248 and *C. turicensis* CLF587, with a maximum viable count from 3.89 to 4.11 log CFU/ml after 24 h incubation. These four strains were therefore defined as acid sensitive strains in the present study. They were used to explore the inhibitive effect of organic acids and to make the effect visible. The acid resistant strain *C. sakazakii* CLF2688 was used additionally to study the inhibition effects of organic acid-treated PIF in a simulated infant gastric model.

3.2. Growth of acid sensitive Cronobacter strains in laboratory medium and reconstituted PIF supplemented with organic acids

In the laboratory medium acidified with different acidic substances, the growth characteristics of the four acid sensitive strains differed dramatically depending on the acidulant identity used to decrease the pH. Table 2 shows the growth behaviour of the *Cronobacter* strains in BiMedia 001B containing seven organic acids as well as hydrochloric acid at various pH levels. Acetic and propionic acid showed the strongest inhibition effect against *Cronobacter* spp. Certain sensitive strains were not able to grow in the acetic and propionic acid-treated medium even at pH 6.0, while at the same pH value a full bacterial multiplication was shown for the remaining strain/acid combinations. At pH 5.5 all investigated strains were able to grow to 10^7 CFU/ml except when exposed to acetic, butyric or propionic acid. At pH 5.0 the bacterial growth was always incomplete when treated with hydrochloric, tartaric, citric and malic acid, resulting in a delayed growth with viable counts of 3–5 log after 24 h incubation. An absolute growth inhibition at pH 5.0 was observed under the treatments with lactic, acetic, butyric and propionic acid.

The reconstituted PIF could partially buffer the acidic inhibition effects observed in the laboratory medium when used as the incubation matrix for *Cronobacter*. As shown in Table 3, acetic, butyric and propionic acid were applied to acidify the reconstituted PIF at low concentrations of 7.25 mmol/l, 6.50 mmol/l and 7.60 mmol/l, respectively, by which an initial pH value of 6.0 was achieved in BiMedia 001B. In reconstituted PIF supplemented with organic acids with the same concentrations, a maintained or an increased

Table 2
Populations of *Cronobacter* strains in acidified BiMedia 001B at pH 5.0, 5.5 and 6.0 for 24 h.

Strain	Population of *Cronobacter* (log CFU/ml) in various acidic substances							
	Hydrochloric acid	Tartaric acid	Citric acid	Malic acid	Lactic acid	Acetic acid	Butyric acid	Propionic acid
pH 5.0								
C. sakazakii CLF70	A 3.89 ± 0.89 a	A 3.92 ± 1.06 a	A 5.09 ± 0.10 a	A 3.53 ± 0.54 a	A ND b	A ND b	A ND b	A ND b
C. universalis CLF2684	A 3.31 ± 0.88 a	A 4.70 ± 0.24 a	A 3.80 ± 0.43 a	A 4.87 ± 0.73 a	A ND b	A ND b	A ND b	A ND b
C. malonaticus CLF248	A 3.60 ± 0.24 a	A 4.85 ± 0.47 a	A 4.09 ± 0.61 a	A 4.13 ± 0.16 a	A 0.60 ± 1.04 b	A ND b	A ND b	A ND b
C. turicensis CLF587	A 4.11 ± 1.04 a	A 5.28 ± 0.29 a	A 5.27 ± 0.40 a	A 4.69 ± 0.27 a	A ND b	A ND b	A ND b	A ND b
pH 5.5								
C. sakazakii CLF70	B 7.22 ± 0.27 a	B 7.21 ± 0.28 a	B 7.86 ± 0.04 a	B 7.29 ± 0.43 a	B 7.74 ± 0.11 a	A ND b	A ND b	A ND b
C. universalis CLF2684	B 7.77 ± 0.09 a	B 7.47 ± 0.42 a	B 7.69 ± 0.14 a	B 7.70 ± 0.05 a	B 7.77 ± 0.10 a	A ND b	A ND b	A ND b
C. malonaticus CLF248	B 7.82 ± 0.08 a	B 7.53 ± 0.15 a	B 7.70 ± 0.02 a	B 7.53 ± 0.22 a	B 7.33 ± 0.04 a	A ND b	A ND b	A ND b
C. turicensis CLF587	B 7.58 ± 0.13 a	B 7.82 ± 0.12 a	B 7.72 ± 0.11 a	B 7.86 ± 0.07 a	B 7.83 ± 0.08 a	A ND b	A 0.94 ± 0.27 b	A ND b
pH 6.0								
C. sakazakii CLF70	B 7.46 ± 0.52 a	B 7.55 ± 0.30 a	B 7.80 ± 0.09 a	B 7.52 ± 0.38 a	B 7.70 ± 0.08 a	A 0.49 ± 0.85 b	B 7.40 ± 0.18 b	B 7.48 ± 0.19 a
C. universalis CLF2684	B 7.64 ± 0.30 a	B 7.57 ± 0.25 a	B 7.67 ± 0.23 a	B 7.75 ± 0.09 a	B 7.67 ± 0.23 a	B 7.59 ± 0.36 a	B 7.85 ± 0.10 a	B 7.80 ± 0.07 a
C. malonaticus CLF248	B 7.63 ± 0.43 a	B 7.46 ± 0.04 a	B 7.48 ± 0.03 a	B 7.31 ± 0.03 a	B 7.33 ± 0.06 a	B 7.34 ± 0.14 a	B 7.29 ± 0.19 a	A ND b
C. turicensis CLF587	B 7.34 ± 0.11 a	B 7.67 ± 0.34 a	B 7.79 ± 0.08 a	B 7.70 ± 0.03 a	B 7.87 ± 0.03 a	B 7.78 ± 0.07 a	B 7.85 ± 0.07 a	B 7.48 ± 0.16 a

Within the same row, mean values for populations that are not followed by the same small letter are significantly different ($P \leq 0.05$).
Within the same column for each species and acid, mean values for populations that are not preceded by the same capital letter are significantly different ($P \leq 0.05$).
ND, no detection. Log CFU/ml of *Cronobacter* was <0.

Table 3
Populations of *Cronobacter* strains in acidified reconstituted PIF at low acid concentrations and high acid concentrations for 24 h.

Strain	Population of *Cronobacter* (log CFU/ml)					
	Acetic acid		Butyric acid		Propionic acid	
	7.25 mmol/l	19.50 mmol/l	6.50 mmol/l	22.00 mmol/l	7.60 mmol/l	20.00 mmol/l
	pH 6.0	pH 4.7	pH 6.5	pH 4.8	pH 6.5	pH 4.7
C. sakazakii CLF70	A 7.05 ± 0.13 a	A ND b	A 7.29 ± 0.19 a	A ND b	A 7.08 ± 0.34 a	A ND b
C. universalis CLF2684	A 7.47 ± 0.23 a	A ND b	A 7.64 ± 0.21 a	A ND b	A 7.47 ± 0.43 a	A ND b
C. malonaticus CLF248	A 7.05 ± 0.21 a	A ND b	A 7.35 ± 0.13 a	A ND b	A 7.20 ± 0.64 a	A ND b
C. turicensis CLF587	A 7.19 ± 0.10 a	A ND b	A 7.90 ± 0.09 a	A ND b	A 7.51 ± 0.48 a	A ND b

Within the same row, mean values for populations that are not followed by the same small letter are significantly different ($P \leq 0.05$).
Within the same column, mean values for populations that are not preceded by the same capital letter are significantly different ($P \leq 0.05$).
ND, no detection. Log CFU/ml of *Cronobacter* was <0.

pH of 6.0, 6.5, 6.5 for acetic, butyric and propionic acid was recorded respectively. An inhibition effect described in BiMedia 001B was not shown in a single case in reconstituted PIF, including isolate CLF70 in acetic acid and CLF248 in propionic acid. With high acid concentrations (19.50 mmol/l, 22.00 mmol/l and 20.00 mmol/l) which produced pH 5.0 in BiMedia 001B, slightly decreased pH values of 4.7, 4.8, and 4.7 were obtained in the reconstituted PIF for acetic, butyric and propionic acid. At these high acid concentrations no *Cronobacter* strains were detectable after 24 h at 37 °C.

3.3. *Cronobacter* growth in acidified PIF under infant gastric condition

Although no apparent inhibition of *Cronobacter* growth was obtained in acetic acid-treated reconstituted PIF with pH 6.0 (Table 3), it is visible that the multiplication of bacteria was severely prolonged when the same formula was exposed to an infant gastric environment (pH 5 caused by HCl). As shown in Figs. 1 and 2, under the gastric pH of 5.0, the numbers of *Cronobacter* were significantly lower ($P \leq 0.05$) in acetic acid-acidified samples compared to non-acidified samples at 5 h and 24 h. After 5 h incubation, the bacterial populations in 90 ml formula supplemented with acetic acid were 3.26 log and 3.25 log for acid resistant *C. sakazakii* CLF2688 and acid sensitive *C. universalis* CLF2684 respectively, showing a decrease in growth of 1.36 log and 0.81 log when compared to the incubation in unchanged formula. At 24 h, both *Cronobacter* strains grew to 10^9 CFU/90 ml in unacidified samples, whereas an inhibited growth with a cell number reduction by 3.90 log for *C. sakazakii* CLF2688 and 3.32 log for *C. universalis* CLF2684 occurred in acidified infant formulas. In the simplified gastric compartment where electrolytes and digestive enzymes were additionally introduced, a considerable difference ($P \leq 0.05$) in viable counts was obtained between acidified treatment and non-acidified treatment for both *Cronobacter* strains at 5 h and 24 h (Figs. 3 and 4).

4. Discussion

Lambert and Bidlas (2006) used a predictive modelling to investigate the effect of multiple antimicrobial factors on the growth and inhibition of *Cronobacter* and concluded pH as an independent inhibiting determinant. In a previous study of bacterial survival under acid stress conducted by Edelson-Mammel et al. (2006), ten of 12 *Cronobacter* strains could withstand exposure to pH 3.5 with HCl for >5 h. At pH 3.0, the decline of *Cronbacter* populations over 5 h incubation period was approximately >6 log cycles. Substantial diversity between the strains at pH 3.0 was observed for less than 2 h. In contrast to the data reported by Edelson-Mammel et al. (2006), the *Cronobacter* strains examined in the current study grew at a minimum pH between 4.5 and 5.0 by acidification with HCl.

Different concentrations of organic acids in the laboratory medium were tested against the growth of four acid sensitive *Cronobacter* strains which were determined in the initial screening test. The effect was best visible by using these acid sensitive strains. The acid resistant strain CLF2688 has been introduced for the growth inhibition studies in the infant gastric model. As shown in Table 2, at the same pH value the inhibitory effects were different between acids: acetic acid, butyric acid, propionic acid were the most potent inhibitors of *Cronobacter* growth, followed by lactic acid, whereas tartaric, citric, malic, and hydrochloric acid showed the least antibacterial activity. Similarly, Back et al. (2009) reported that *C. sakazakii* was most susceptible to propionic acid, followed by acetic,

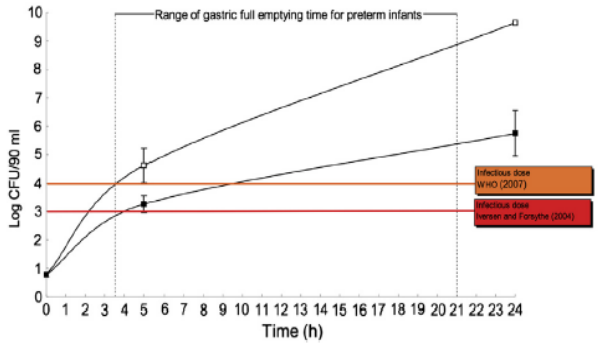

Fig. 1. Growth of *C. sakazakii* (CLF2688) in acetic acid-treated infant formula of pH 6.0 (■) and in control infant formula of pH 7.0 (□) under gastric pH of 5.0. The gastric full emptying time ranges from 3.5 h to 21 h for preterm infants (Bodé et al., 2004). Values are presented in log CFU/90 ml.

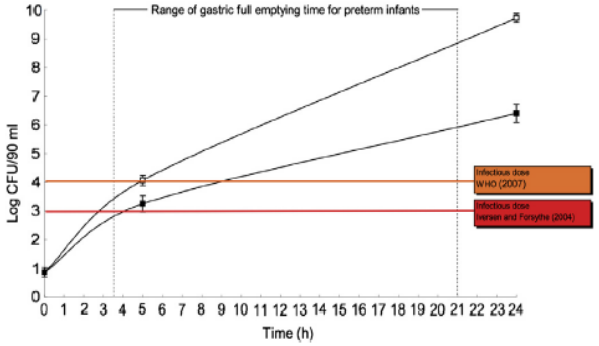

Fig. 2. Growth of *C. universalis* (CLF2684) in acetic acid-treated infant formula of pH 6.0 (■) and in control infant formula (□) under gastric pH of 5.0. The gastric full emptying time ranges from 3.5 h to 21 h for preterm infants (Bodé et al., 2004). Values are presented in log CFU/90 ml.

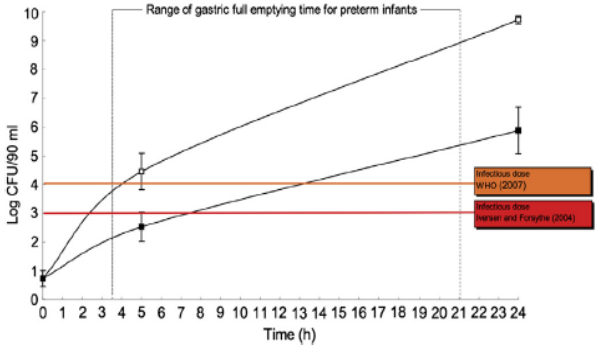

Fig. 3. Growth of *C. sakazakii* (CLF2688) in acetic acid-treated infant formula of pH 6.0 (■) and in control infant formula of pH 7.0 (□) in the simulated static gastric compartment. The gastric full emptying time ranges from 3.5 h to 21 h for preterm infants (Bodé et al., 2004). Values are presented in log CFU/90 ml.

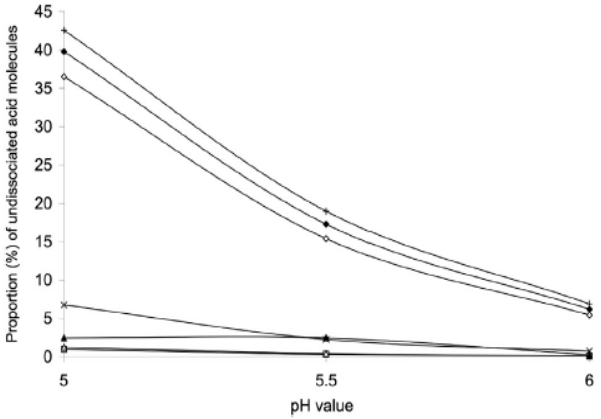

Fig. 5. Proportion of undissociated organic acids: propionic acid (+), butyric acid (◆), acetic acid (◇), lactic acid (×), malic acid (▲), citric acid (△) and tartaric acid (□) at pH 5.0, 5.5 and 6.0 in the laboratory medium.

malic, citric and formic acid in descending order. Oshima et al. (2012) concluded propionate and acetate as the most inhibitive organic acid salts against *C. sakazakii* in reconstituted whole milk powder. Hsiao et al. (2010) identified lactic acid as the most active agent against *C. sakazakii* at pH 4.0, followed by acetic, propionic, citric and tartaric acids. These findings indicate that the inhibition potency of acids was not only caused by the low pH value. Previous studies have revealed that the potential bacterial inhibitory activity of acids correlates strongly with the undissociated forms (Cherrington et al., 1991a; Salmond et al., 1984). It has been assumed that only undissociated organic acids can easily penetrate the bacterial cell membrane, dissociate within the cytoplasm and cause the intracellular pH to drop. Export of excess protons could result in the depletion of proton motive force and a consequent cell death due to irreversible denaturation of acid-labile protein and DNA (Cherrington et al., 1991a; Cherrington et al., 1991b; Davidson, 2001; Salmond et al., 1984). Fig. 5 shows the proportion of undissociated molecules for each organic acid at pH 5.0, 5.5 and 6.0 in BiMedia 001B calculated according to the Henderson–Hasselbalch equation (Hasselbalch, 1917; Henderson, 1908). At all pH levels, acetic, butyric and propionic acid showed an obviously greater proportion of acid molecules in undissociated form than other organic acids. The results support strongly the concept that the undissociated molecule is the primary factor determining the inhibitory potential of organic acids. In general the inhibition on *Cronobacter* growth did not differ apparently between the three most effective organic acids. However it was evident that certain

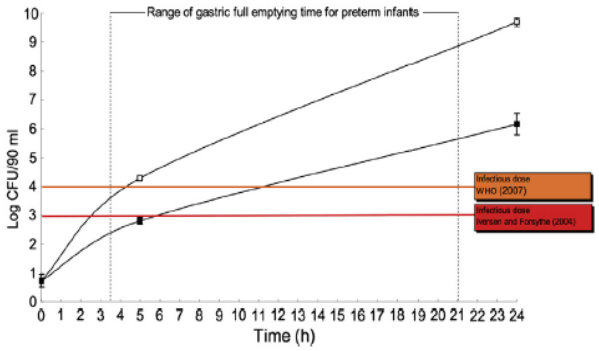

Fig. 4. Growth of *C. universalis* (CLF2684) in acetic acid-treated infant formula of pH 6.0 (■) and in control infant formula of pH 7.0 (□) in the simulated static gastric compartment. The gastric full emptying time ranges from 3.5 h to 21 h for preterm infants (Bodé et al., 2004). Values are presented in log CFU/90 ml.

Cronobacter strains showed a higher sensitivity against certain organic acids. *C. sakazakii* CLF70 was found to be sensitive against acetic acid at pH 6.0 but grew to 10^7 CFU/ml under treatment of propionic acid and butyric acid at the same pH value. A similar observation was obtained for *C. malonaticus* CLF248. In contrast to the fact that two *Cronobacter* strains were suppressed even under mild acidic condition of pH 6.0 in the laboratory medium, no growth inhibition was observed in the reconstituted PIF as matrix with same acid concentration. Medium composition could influence the antibacterial activity of organic acids (Back et al., 2009; Cherrington et al., 1991a). Tabib et al. (1984) reported that protein ingredients in feed could alter the effective concentration of propionic acid by buffering and conversion to its less active form, and thus result in an inconsistent antifungal effect.

The benefits of acidification of milk fed to infants have been shown by Carrion and Egan (1990). Hypochlorhydric neonates were fed on breast milk or commercial premature formula supplemented with 1 mol/l HCl/ml milk which decreased the pH from 7 to lower than 5.5. Compared to the control group, the HCl supplemented-group had a lower gastric pH, less gastric colonization and a lower incidence of NEC. However, HCl-supplemented diets are not always well tolerated, mainly because of systematic acidemia caused by the absorption of acid in the diet (Mehall et al., 2002). The poor tolerance of strong acid acidification has thus pushed the interest in the use of organic acid compounds in infant formula for controlling and inhibiting the growth of natural contaminants, either as direct additives or accumulate as a consequence of the fermentation activity of indigenous or starter cultures added to diet (Ricke, 2003). NAN Pelargon® Nestle, a commercially available biologically acidified formula with pH 4.55, has been reported as effective in reducing pulmonary and gastric colonization in animal models due to lactic acid production through probiotic fermentation (Boneti et al., 2009). Joosten and Lardeau (2004) found that acidified diets either through lactic acid bacteria fermentation or by direct lactic acid fortification were equally effective in preventing rapid bacterial proliferation in infant formulas, when a pH value lower than 5.0 was ensured. Combinations of lactic acid and its salt forms have been reported to be bacteriostatic and bactericidal on major pathogens when supplemented at appropriated concentrations in liquid infant formulas, giving a pH ranging from 4.8 to 5.1 (Kreb, 2010). Back et al. (2009) found in a recent study that propionic and acetic acid in 100 mM were effective in eliminating the *Cronobacter* contamination in infant food, accompanied with a pH drop from 6.6 to 4.5. It is not surprising that organic acids are

bactericidal when fortified in an adequate amount because the acidity is the mechanism of bacterial killing. However, the sensorial acceptance could be strongly affected if the pH value is too low. In the opinion of the UK Committee on Medical Aspects of Food and Nutrition Policy (Anonymous, 1980), the pH value of a new infant formula should not be too dissimilar from that of well established infant formula commonly with a neutral pH. Brunser et al. (1989) reported a high population of children refusing the commercially acidified milk. The question thus raised is whether there could be an acidification intervention in infant formula which prevents the growth of pathogen without deteriorates its taste significantly.

The fact that *Cronobacter* is implicated as a causal agent of neonatal NEC which is characterized by ulceration and necrosis primarily in distal small intestine and colon suggests that the pathogen can survive the gastric acidity and reach the intestinal lumen. In the present study, the minimum pH value of *Cronobacter* growth under the HCl-induced acid stress was between 4.5 and 5.0. This pH limit is within the infant gastric pH ranging from pH 4-5 reported by Agunod et al. (1969). The survival and perhaps even the growth of *Cronobacter* in the gastric compartment are hence predictive for subsequent bacterial colonization and translocation in the gut. On the other hand, it seems reasonable to postulate that the relatively low pH of the stomach plays a role in enhancing the inhibitory effect of organic acids by shifting the equilibrium to their undissociated forms. This postulation is supported by results of our in vitro experiments in which the native gastric acidity and gastric secretions were simulated. A significant decrease in *Cronobacter* populations in the 24 h incubation period was shown in mild acidified formula with pH 6.0, compared to neutral pH diet (pH 7.0) when exposed to gastric condition of pH 5.0 (Figs. 2–5). Although the physiological infant gastric acidity condition around pH 4–5 has been reported in the majority of cases by Agunod et al. (1969), this suppressive effect could be limited if the gastric pH exceeds 5.5. Sondheimer et al. (1985) reported that the gastric pH of infants six days old varies from a fasting pH of 2.9 to a value of 5.2 directly after feeding. The gastric pH in infants 7–15 days old is relatively higher ranging between 4.6 and 5.8. On the other hand, the epidemiological data of invasive *Cronobacter* infection showed that newborns within 7 days of life tend to develop meningitis with a high mortality of 44%, while infants older than one week of life develop bacteraemia more often with a better prognosis indicating that the vulnerability to lethal *Cronobacter* infections starts to decrease after the 7th day of life (Anonymous, 2006).

The growth inhibition effect on *Cronobacter* achieved by combining the acidification of strong acid in the stomach and the slow dissociated organic acid in the formula exceed the effects of HCl or organic acid alone. *Cronobacter* has been determined to be present in PIF at a level of ca. 0.36–66 CFU/100 g by Muytjens et al. (1988) and Nazarowec-White and Farber (1997). Given that 13.5 g PIF are reconstituted in a single feeding, a prepared feeding of 90 ml at the highest contamination level could contain between 1 and 10 cells. Until now no infectious dose has been epidemiologically established for *Cronobacter*. The FAO/WHO (Anonymous, 2007a) proposed an infectious dose of 10,000 CFU in a single feeding. According to the data presented here, after 5 h incubation the bacterial counts of both *Cronobacter* strains would be below this level in acidified formula and would have exceeded it in unchanged formula. It can be estimated that the critical number would be achieved in acidified formula with a delay of several hours. The gastric emptying of infants follows an exponential model where the flux decreases with the residue chyme. The emptying rate is influenced by the composition and volume of the meal as well as the age and the gender of neonates (Heyman, 1998). Bodé et al. (2004) reported the half emptying time ($T_{1/2}$) in preterm infants ranging from 30 min to 180 min. Extrapolation of these data indicates a total gastric emptying time between 3.5 (for $T_{1/2} = 30$ min) and 21 h (for $T_{1/2} = 180$ min), until less than 1 ml milk remains in the stomach, when started with an initial feeding volume of 90 ml. Given the minimum full emptying time, the growth of *Cronobacter* in unacidified diets could approach a critical value of 10,000 CFU before the gastric digestion phase is nearly completed, with a low initial contamination of 1–10 cells per 90 ml feeding. This could proceed a further bacterial translocation in upper intestinal tract and cause an infection. In acidified formula, on the other hand, the infectious dose would not be reached during the stomach passage. The bacteria numbers in the chyme by entering the small intestine would be below the hazardous level, which could contribute to a reduced risk of *Cronobacter* infection in following intestinal sections. Even in the extreme case with a long gastric emptying time of 21 h, a clearly retarded *Cronobacter* growth in acidified PIF in infant stomach could be of interest in minimising the potential infection risk. This is especially important as the longest emptying time is often seen in preterm infants who belong to the most vulnerable group to *Cronobacter* infections (Cavell, 1981; Gurtler et al., 2005). Iversen and Forsythe (2003) estimated that the infectious dose may be as low as 1000 CFU. Although this critical level was exceeded by both *Cronobacter* strains in acidified PIF under the gastric acidic environment at 5 h (Figs. 1 and 2), a retarded growth through the acidification of formula is visible. In the simulated gastric model, numbers of both *Cronbacter* strains were below 1000 CFU in 90 ml acidified formula and over 1000 CFU in unacidified formula at 5 h (Figs. 3 and 4). Moreover it should be noted that if such as low contamination level is really dangerous, the hazard could hardly be controlled by the growth suppression.

The proposed pathogenicity of bacteria can be attributed to a series of determinants including a certain reduction of the initially incorporated number of cells during the stomach passage, the cell counts required to enable a contact to the target structure in the gut wall as well as the number of cells in the final stage of entering the bloodstream to develop infections. The pathomechanisms involves not only a minimum quantity of bacterial cells but also the intact cellular structural integrity to withstand the immune response and to invade the organism actively. The growth inhibition caused by organic acids under the gastric fluid in the present study would presumably consists of two components: Reduction of the number of bacterial cells surviving the stomach passage and the severe damage on some of the surviving cells which leads to a decreased infectious ability (Pagotto et al., 2008). As discussed above, different *Cronobacter* strains are sensitive to low pH value in varying intensities. Therefore the inhibition by organic acids could probably be much more effective on some strains than on others. Moreover it is assumed that the inhibition effect might not be limited to *Cronobacter* spp. Other closely related pathogens and opportunistic pathogens of the *Enterobacteriaceae* group would most likely show a similar range of sensitivities. The pH range for the growth of *Salmonella* has been reported between 4 and 9, whereby the minimum pH value is affected by acidulant used. Acetic acid, for example, has been reported to limit the pH minimum of *Salmonella* growth to about pH 5 (D'Aoust and Maurer, 2007).

In the present study, the physiological condition of infants has been taken into consideration for the first time by designing the acid-based intervention. The gastric acidity of pH 5.0 in neonates resulting from the native gastric acid production is not low enough to suppress the *Cronobacter* growth when a normal infant formula is mixed with the gastric fluid. On the other hand, reconstituted PIF acidified to pH 6.0 solely might not have been sufficiently bactericidal or bacteriostatic. However, owing to the synergistic counteractions, H^+ ions fully dissociated from gastric hydrochloric acid can reinforce the accumulation in the undissociated organic acids and thus alter its effective concentration. Compared to other acidified

feedings where the protective function is achieved by direct acid supplementation with a pH drop to 5 or lower, the acidification concept for formula presented in this study is likely to be better accepted and tolerated by children due to the mild acidic pH value. Combined with the gastric acid, it is equally effective in *Cronobacter* inhibition as other acid interventions. Kim et al. (2012) reported that *C. sakazakii* strains pre-exposed to acidic conditions with pH varying from 3.0 to 5.0 showed a higher tolerance against the subsequent environmental acidic stress. The *Cronobacter* cells in formulas directly acidified to lower pH values hence probably develop this adaptation mechanism and could survive better in the infant gastric acidic milieu, while in the current described intervention with pH 6 pathogens do not undergo a pre-acid shock before entering the stomach. It has to be noted that the inhibition effect presented here becomes active only when the gastric acidity is incorporated, while the risk management of abused bacterial growth caused by mishandling the formula e.g. prolonged storage at elevated temperatures etc. which is principally evaluable by other acidification models reported, is not addressed by this approach. Short-chain fatty acids are produced quantitatively in intestinal regions of neonates where strictly anaerobic microbiota is predominant (Siigur et al., 1993), indicating a possible reinforcement in prevention of bacterial colonization in conjunction with acidified infant formula. Therefore, further in-depth studies on the survival of *Cronobacter* cells in acid-treated PIF under physiological conditions in proximal and distal intestine need to be pursued.

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Chapter 4

Rapid detection of *Cronobacter* spp. with a method combining impedance technology and rRNA based lateral flow assay

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Short communication

Rapid detection of *Cronobacter* spp. with a method combining impedance technology and rRNA based lateral flow assay

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ABSTRACT

Cronobacter spp. is an important release test parameter for powered infant formula (PIF). An impedance method is proposed for the rapid detection of this pathogen in PIF. An impedance based method (BacTrac 4300 Microbiological Analyzer) combined with a RNA hybridisation assay (RiboFlow™) was evaluated using 23 strains in PIF samples and compared to a culture based reference method (ISO/TS 22964). The influences of competitive flora, heat and dry stress on the reliability of the impedance method were investigated. Seven different *Cronobacter* species were included in the evaluation, among them are strains with high susceptibility to low pH and high temperatures. Compared to the reference method, a higher sensitivity (85%) and specificity (100%) was observed using the impedance method, combined with the commercial rRNA based lateral flow test kit as a confirmation tool. The detection time was substantially shortened by using the impedance technique and RiboFlow™. *Cronobacter* could be detected within maximally 29 h, while the reference method takes up to five days when including confirmation of positive results.

1. Introduction

The genus *Cronobacter* comprises seven species: *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, *C. turicensis*, *C. uiversalis* and *C. condimenti* (Iversen et al., 2007a; Joseph et al., 2011). *Cronobacter* spp. is regarded as a causative agent of meningitis, septicemia or necrotizing enterocolitis in infants with a mortality of 20 to 50% (Lehner and Stephan, 2004). Contaminated powdered infant formulas (PIF) have been identified as the most likely vehicle of transmission of *Cronobacter* associated with hospital outbreaks (van Acker et al., 2001). A rapid and accurate detection of *Cronobacter* spp. is of particular importance for the risk management of *Cronobacter* related food borne diseases.

The International Standards Organization (ISO) (Anonymous, 2006) and the US Food and Drug Administration (FDA) (Anonymous, 2002) have published standardised methods for detection of *Cronobacter* in PIF. For rapid detection and identification of the micro-organism, a number of conventional PCR as well as real-time PCR systems based on different target regions have been developed (Lehner et al., 2006; Nair and Ventkitanarayanan, 2006; Seo and Brackett, 2005).

Another alternative to conventional microbiological method is the impedance method. The concept of using impedance for bacterial growth detection by measuring the change of electrical conductivity caused by bacterial metabolism has been described as early as 1899 (Steward, 1899). For the detection and enumeration of foodborne pathogens such as *Clostridia* and *Salmonella*, the impedance measurement has been assessed as a valid method (Dromigny et al., 1997; Joosten et al., 1994). In the recent years, the impedance technology has been optimised in its specificity by the development of the specific media and the combination with immunological or molecular biological confirmation tools.

The aim of the present study is to evaluate the performance of an impedance method combined with a commercially available RNA hybridisation assay for *Cronobacter* detection in PIF.

2. Materials and methods

2.1. Bacterial strains

The following strains were used in this study: *C. sakazakii* (2688), *C. malonaticus* (2689), *C. malonaticus* (248), *C. muytjensii* (2682), *C. dublinensis* (2687), *C. turicensis* (2683), *C. universalis* (2684), *Enterobacter pulveris* (DSM 19144), *Enterobacter helveticus* (DSM 18306), *Enterobacter turicensis* (DSM 18307), *Enterobacter cloacae* (ATCC 13047), *Escherichia hermanni* (330), *Citrobacter freundii* (ATCC 8454), *Klebsiella pneumoniae* (1947), *Serratia ficaria* (1955), *Pseudomonas aeruginosa* (ATCC 9027), *Leclercia adecarboxylata* (770), *Acenitobacter baumanni* (ATCC 15308), *Bacillus cereus* (ATCC 11778), *Pantoea agglomerans* (ATCC 27155), *Staphylococcus aureus* (ATCC 27733), *Enterococcus casseliflavus* (ATCC 27284) and *Enterococcus faecalis* (DSM 20380).

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2.2. Specificity test

Specificity was determined by analysing seven *Cronobacter* strains and 16 non-*Cronobacter* strains. Strains frozen at −80 °C on biobeads (Transia, Germany) were first grown overnight in Buffered Peptone Water (BPW) (Merck, Germany) at 37 °C and diluted in bacteriological peptone (Oxoid, UK). For specificity analysis, PIF was first sterilised with 25 kGray gamma irradiation. 100 g sterilised PIF was spiked with respective target and non-target strains at 1–10 CFU/100 g. For each sample the impedance analyses were performed in six replicates.

2.3. Cronobacter detection with competitive flora

Sterilised PIF was inoculated with fresh overnight cultures of *C. sakazakii* or *C. universalis* in 1–10 CFU/100 g respectively and with competing micro-organisms at levels of 1, 100, 1000 CFU/100 g. The impedance analyses were performed in six replicates for *C. sakazakii* and duplicates for *C. universalis*. In an extended experiment focusing on acid- and heat-sensitive *Cronobacter* strains, PIF was inoculated with *C. malonaticus* 248 and *C. universalis* at 10 and 100 CFU/100 g respectively and simultaneously with *C. feundii* at 1 CFU/100 g. There were five replicates per target strain at each inoculation level.

2.4. Detection of heat and dry stressed Cronobacter strains

100 g sterilised PIF was spiked with *C. sakazakii* in 8×10^4 CFU/g and desiccated at 50 °C for 4 h and kept dry at 20° for 96 h. This resulted in a reduction of the *Cronobacter* load by 2 to 3 log units.

2.5. Cronobacter detection in commercial PIF samples

Ten commercially manufactured PIF samples and 50 skim milk powder samples of various manufacturers were randomly collected. Each sample was tested in 100 g with the impedance–RiboFlow™ method and the reference method (Section 2.6.2) in parallel.

2.6. Method performance comparison

2.6.1. Impedance measurement combined with RiboFlow™ assay

100 g spiked or commercial PIF samples were diluted in 900 ml BPW. After incubation at 37 °C for 16 h 0.1 ml of the pre-enriched samples was transferred into the measuring cells filled with 10 ml BiMedia 144A (Sylab, Austria) supplemented with 10 mg/l vancomycin. The impedance measurement was performed at 42 °C for 24 h in the BacTrac 4300 Microbiological Analyzer (Sylab, Austria). The medium impedance (M-value) detects the change of the impedance caused by the changed conductivity of the growth medium. A detection time (DT) is defined as incubation time required from analysis start to reach a certain change percentage in the impedance which is selected as threshold value. For the current evaluation a 5% threshold for the M-value was defined. As soon as the threshold value was achieved, samples were subjected to further confirmation using the RiboFlow™ *Cronobacter* kit (Sylab, Austria). 0.5 ml of the cultured BiMedia 144A was used and processed according to the manufacturer's instructions (briefly: spin at 13,000 g and reconstitution with specific kit buffer, application of 0.1 ml to the lateral flow assay, incubation for 0.25 h at 37 °C).

2.6.2. Conventional reference procedure

After pre-enrichment of 100 g samples in 900 ml BPW for 16 h at 37 °C, 0.1 ml of pre-enriched culture medium was transferred into 10 ml modified lauryl sulphate tryptose (mLST) broth (Merck, Germany) with 0.5 M NaCl and 10 mg/l vancomycin for 24 h at 44 °C and streaked on Brilliance™ *E. sakazakii* chromogenic agar Druggan–Forsythe–Iversen (DFI) formulation (Oxoid, UK) and incubated for 24 h at 37 °C. For each sample in this study, the reference method was performed in parallel to the impedance method.

3. Results and discussion

3.1. Determination of the cut-off time and specificity test for cronobacter screening with impedance

The slowest-growing *Cronobacter* species *C. universalis* was used to establish the cut off time (incubation time in BacTrac required to exceed the preset threshold of M-value) for the impedance-based screening step on *Cronobacter* in PIF. The average DT of 4.24 h with a standard deviation of 1.09 was used to calculate a tolerance interval of m±2 s (2.06 h to 6.42 h) and the upper limit was rounded to 7 h. Samples with DTs longer than 7 h would presumptively be free of *Cronobacter* with a confidence of at least 95%. Therefore the cut-off time to discriminate presumptively positive samples from negative ones was set at 7 h. Totally 138 impedance analyses were performed on 23 PIF samples spiked with *Cronobacter* or non-*Cronobacter* species as pure inoculum respectively (Table 1). All seven samples spiked with *Cronobacter* were detected within 7 h, with the maximum DT of 4.24 h observed in *C. universalis*. 56 of the 96 analyses spiked with non-*Cronobacter* strains were detected within 7 h in the impedance assay. These 56 positive results included eight samples containing non-*Cronobacter Enterobacteriaceae* strains, with the longest impedance response time of 5.60 h obtained in *S. ficaria*. Of the samples with non-*Enterobacteriaceae* strains, only one inoculated with *P. aeruginosa* was detected within 7 h. The RiboFlow™ assay detected all samples containing *Cronobacter* correctly and confirmed all non-target strains as negative. The reference method provided presumptive positive results on DFI for samples spiked with *E. pulveris*, *E. helveticus* and *E. turicensis*.

3.2. Cronobacter analyses with competitive flora in spiked and plain commercial PIF samples

In an earlier investigation, isolates of *C. universalis* 2684 and *C. malonaticus* 248 were found to be sensitive to low pH (inhibited growth at pH 5.0) and sensitive to a higher temperature (no growth at 45 °C), while the strain *C. sakazakii* 2688 grew well up to 46 °C and at pH 5.0 (data not shown). The recovery of the acid- and heat-resistant strain of *C. sakazakii* and the sensitive strain of *C. universalis* with competitive flora in spiked PIF is shown in Table 2. All mixed culture samples spiked with *C. sakazakii* were detected within 7 h and were positively identified in all cases using RiboFlow™, fully comparable with the results obtained from the reference method. *C. universalis* provided delayed DT responses except when co-contaminated with *E. cloacae* or *C. freundii* in amounts of 100 and 1000 CFU/100 g respectively and in the latter cases DTs were around 3 h. While *C. sakazakii* in co-cultures was always detectable with both methods, *C. universalis* was out-competed by *E. cloacae* or *C. freundii* and therefore not recovered in the RiboFlow™ assay or with the reference method. DTs exceeding 7 h were found in six samples with *S. aureus*, *E. faecalis* and *P. aeruginosa* although the target strains were detected in all cases with both methods. This has been investigated in more detailed experiments involving two sensitive *Cronobacter* strains and *C. freundi*. These results revealed for samples inoculated with critical target strains at levels of 10 CFU/100 g a recovery rate of 60% (6/10) and 40% (4/10) for the impedance–RiboFlow™ and the reference method respectively. Samples spiked with *Cronobacter* at higher levels of 100 CFU/100 g were correctly identified in 9 of 10 (90%) cases with the impedance–RiboFlow™ and in 5 of 10 (50%) cases with the reference method.

The detection of bacteria in the impedance devices requires that the micro-organisms grow to a cell density of 10^6–10^7 CFU/ml (Firstenberg-Eden, 1984). After the pre-enrichment in BPW for 16 h, a bacterial count of 10^8 CFU/ml was obtained for *C. sakazakii* 2688 (data not shown), indicating that the bacterial count is still in the detectable level of approximately 10^6 CFU/ml or would reach this level within a short incubation period when 0.1 ml aliquot is transferred from pre-enrichment into the impedance measuring

Table 1
Specificity test on target and nontarget strains.

Strain	Species	Impedance DT (h) (mean n=6)	Impedance–RiboFlow™	Reference method
2682	*C. mutyjensii*	2.47	+	+
2683	*C. turicensis*	1.22	+	+
2684	*C. universalis*	4.24	+	+
2687	*C. dublinensis*	1.80	+	+
2688	*C. sakazakii*	0.77	+	+
2689	*C. malonaticus*	1.31	+	+
248	*C. malonaticus*	3.96	+	+
DSM 19144	*E. pulveris*	1.44	−	+
DSM 18306	*E. helveticus*	2.28	−	+
DSM 18307	*E. turicensis*	3.02	−	+
ATCC 13047	*E. cloacae*	1.86	−	−
330	*E. hermanni*	1.15	−	−
ATCC 8454	*C. freundii*	1.41	−	−
1947	*K. pneumoniae*	0.95	−	−
1955	*S. ficaria*	5.60	−	−
ATCC 27155	*P. agglomerans*	10.50	−	−
ATCC 9027	*P. aeruginosa*	6.78	−	−
770	*L. adecarboxylata*	8.96	−	−
ATCC 15308	*A. baumanni*	10.11	−	−
ATCC 11778	*B. cereus*	–	−	−
ATCC 27733	*S. aureus*	–	−	−
ATCC 27284	*E. casseliflavus*	–	−	−
DSM 20380	*E. faecalis*	11.39	−	−

vials. A short DT of the target micro-organism<1–2 h is therefore likely. However the DT can be prolonged if the bacterial count achieved after the pre-enrichment stage is lower than 10^6–10^7 CFU/ml due to cell injury or interaction with competitive flora. A longer incubation time in impedance medium is therefore necessary to ensure a reliable negative result. The interaction between the background flora and the target micro-organism could lead to a growth suppression of *Cronobacter* in the pre-enrichment stage or in the selective incubation in the impedance medium and thus result in a retarded DT response. Miled et al. (2010) investigated the influence of background flora on the detection of *Cronobacter* and found that the pooling of PIF samples could decrease the sensitivity of the standardised method due to the interactions with increasing competitive flora, which could be limited by splitting the sample into smaller portions. In the current study, the competitive non-coliform bacteria prolonged clearly the DT for *C. sakazakii* in a direct correlation to the contamination load. On the other hand, the DT was decreased with an increasing amount of competitive flora belonging to the coliform group like *Cronobacter* spp. (Table 2).

The influence of prolonged lag times on the detection time was investigated in a heat and dry stress simulation. The surviving bacteria showed only a slight difference in the DT (1.3 h) in BacTrac compared to not stressed strains (0.77 h). This is probably due to the fact that prolonged lag times are compensated during the pre-enrichment step.

Table 3 shows the recovery of *Cronobacter* from spiked PIF samples with both methods. A higher sensitivity was concluded for the impedance–RiboFlow™ assay (85%) compared to the reference method (76%). Competing *Enterobacteriaceae*, such as *E. cloacae* and *C. freundii*,

Table 2
Detection of acid- and heat-resistant *C. sakazakii* and acid- and heat-sensitive *C. universalis* (10^0–10^1 CFU/100 g PIF) with competing flora.

Competitive flora (CFU/100 g)		Impedance DT (h)		Impedance–RiboFlow™		Reference method	
		C. sakazakii (mean n=6)	*C. universalis* (mean n=2)	*C. sakazakii*	*C. universalis*	*C. sakazakii*	*C. universalis*
S. aureus	10^0	0.73	8.08	+	+	+	+
	10^2	1.26	8.46	+	+	+	+
	10^3	1.09	6.57	+	+	+	+
B. cereus	10^0	0.62	6.17	+	+	+	+
	10^2	1.84	6.37	+	+	+	+
	10^3	1.19	6.61	+	+	+	+
E. faecalis	10^0	1.57	9.53	+	+	+	+
	10^2	2.56	10.59	+	+	+	+
	10^3	4.71	10.37	+	+	+	+
P. aeruginosa	10^0	0.72	7.29	+	+	+	+
	10^2	0.93	4.71	+	+	+	+
	10^3	1.18	4.85	+	+	+	+
P. agglomerans	10^0	1.29	6.38	+	+	+	+
	10^2	0.79	6.48	+	+	+	+
	10^3	0.75	4.56	+	+	+	+
E. cloacae	10^0	1.09	1.19	+	−	+	−
	10^2	0.95	0.95	+	−	+	−
	10^3	0.61	1.43	+	−	+	−
C. freundii	10^0	2.87	4.05[a]	+	+	+	+/−[b]
	10^2	0.77	3.19	+	−	+	−
	10^3	1.58	3.02	+	−	+	−

[a] Mean value of five independent trails for mixed samples of *C. universalis* and *C. freundii* both in 10^0–10^1 CFU/100 g PIF.
[b] Reference method results differed in independent trails (s. 3.2).

Table 3
Comparison of impedance–RiboFlow™ with the reference method for *Cronobacter* detection from PIF spiked with target strains (n = 68) and non-target strains (n = 16).

	Positive samples	Negative samples	False-positive samples	False-negative samples	Sensitivity (%)	Specificity (%)
Impedance–RiboFlow™	58	26	0	10	85%	100%
Reference method	55	29	3	16	76%	81%

were interfering in the detection of sensitive *Cronobacter*. The inability of both systems to recover the target strains in some samples is probably due to the outgrowth of certain competitive *Enterobacteriaceae* over *Cronobacter* in the pre-enrichment stage. Both selective enrichment media, BiMedia 144A and mLST, are not specific enough to provide *Cronobacter* cells the crucial advantage over the competitive flora. As an important parameter for process hygiene criteria for foodstuffs, only very low levels of *Enterobacteriaceae* are tolerated in PIF (Joosten et al., 2008). Dried infant formula samples positive for *Enterobacteriaceae* in 10×10 g indicate insufficient hygiene and improvements in food production process are required (Anonymous, 2005). The fact that the presence of *Cronobacter* spp. could be masked by certain *Enterobacteriaceae* strains present in the sample has to be taken into consideration by making the release decision. A positive result in the usually available *Enterobacteriaceae* test makes the *Cronobacter* result less reliable.

Compared to the impedance–RiboFlow™ method with a specificity of 100%, the reference method produced three false positive results on novel *Enterobacter* strains. Indeed the false positive rate could be reduced when molecular based confirmation like PCR or fluorescence in situ hybridisation assays are performed (Iversen et al., 2007b; Lehner et al., 2006). With the RiboFlow™ assay, however, a differentiation of the novel *Enterobacter* strains from *Cronobacter* is possible following directly the impedance enrichment, thus reducing the time required for the final confirmation process to 15 min.

In total 228 impedance analyses were performed on PIF samples spiked with target strains either as pure inoculum or with competitive flora, with DTs ranging from 0.57 to 12.70 h. In 94.3% (215/228) of the cases the DT was <7 h and in 5.7% (13/228) of the cases the DT was between 7 h and 12.70 h. In order to exclude false negative results, a three way screening approach based on two cut-off times at 7 h and 13 h was established. Any sample with a DT of less than 7 h has been classified as highly presumptive positive for *Cronobacter* contamination and has to be confirmed with the RiboFlow™. Samples with DT between 7 and 13 h have been classified as questionable regarding the risk of *Cronobacter* contamination. In this study, all target strains-containing samples detected between 7 and 13 h referred to special cases with sensitive *Cronobacter* strains and competing flora. Practically, however, the prevalence of such strain combinations in PIF is extremely low. Composed of only two strains to date, the group of *C. universalis* is of low incidence (Iversen et al., 2007a). Schlegelová et al. (2002) reported that Enterococci isolated from milk are usually susceptible to vancomycin, while the vancomycin-resistant *E. faecalis* strain DSM 20380 used in this study is originally of a clinical source (Anonymous, 2011). Furthermore, the competitive micro-organism *C. freundii*, as a potential source in the nosocomial infection, is accepted in PIF in very low levels only (Anonymous, 2007). Nevertheless to ensure there are no omissions of target micro-organisms even in such rare cases, it is recommended that questionable samples are confirmed by the RiboFlow™ assay. All samples detected longer than 13 h would be classified as pass samples negative for *Cronobacter*.

From ten commercial PIF products of various manufacturers, one sample gave DT shorter than 7 h and two samples were detected between 7 h and 13 h using the impedance method, indicating that a confirmation was necessary in 30% of cases. *Cronobacter* was not detected either with the impedance–RiboFlow™ or the reference method. Thirty-five out of 50 skim milk samples were detected within 7 h and were confirmed by RiboFlow™. Eight of 35 samples gave a positive result while only five samples were detected positive with the reference method. *E. coli* and *E. cloacae* were isolated from the three samples which produced conflicting results, indicating an out-compete of *Enterobacteriaceae* during the enrichment step of the reference method.

The discriminatory power of the impedance enrichment is limited due to the fact that the medium used is targeted on coliforms and the added vancomycin as well as the elevated incubation temperature provides only limited selectivity. The final decision is left to the RNA hybridisation assay, which makes the method more expensive as approximately 30% of the samples have to be confirmed by RiboFlow™. This disadvantage is compensated by the advantages such as higher sensitivity and specificity compared to the reference method and a considerable shortening of the analysis time.

In the current study, a positive detection result was obtained in the majority of the *Cronobacter*-containing samples within 20 h using impedance measurement in combination with RiboFlow™ and thus enabled a final decision 4 days earlier than based on the conventional method.

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Chapter 5

Matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry, 16S rRNA gene sequencing, and API 32E for identification of *Cronobacter* spp.: A comparative study

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Research Note

Matrix-Assisted Laser Desorption and Ionization–Time-of-Flight Mass Spectrometry, 16S rRNA Gene Sequencing, and API 32E for Identification of *Cronobacter* spp.: A Comparative Study

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ABSTRACT

Twenty-two isolates of the family *Enterobacteriaceae,* with focus on *Cronobacter* isolated from infant formula and the environment of milk powder plants, were comparatively identified using API 32E (bioMérieux, Marcy l'Etoile, France), 16S rRNA gene sequencing (Accugenix, Newark, USA), and matrix-assisted laser desorption and ionization–time-of-flight mass spectrometry (MALDI-TOF MS; Mabritec, Riehen, Switzerland and AnagnosTec, Potsdam, Germany). With API 32E, 22% of the isolates were assigned to species, 64% were assigned to a genus, and 14% could not be discriminated at any taxonomic level. Both 16S rRNA gene sequencing and MALDI-TOF MS assigned 100% of the isolates to species, but the identifications based on MALDI-TOF MS results were more discriminating and unequivocal. Our data indicate that MALDI-TOF MS provides the most rapid and unambiguous identification of *Cronobacter* and closely related *Enterobacteriaceae* isolates.

The genus *Cronobacter* was described in 2008 and comprises six species: *C. sakazakii, C. malonaticus, C. muytjensii, C. dublinensis, C. turicensis,* and *C.* genomospecies 1 *(14, 16)*. Known as emerging foodborne pathogens, *Cronobacter* species are regarded as causative agents of meningitis, septicemia, and necrotizing enterocolitis in infants, with a mortality rate of 20 to 50% *(5, 22)*. Because of their ubiquitous nature, *Cronobacter* species have been isolated from various environmental and clinical sources, including hospitals, households, and factories *(5, 18, 25)*. Contaminated powdered infant formula (PIF) is believed to be the likely vehicle of transmission for *Cronobacter* species associated with hospital outbreaks *(5, 19)*. In previous prevalence studies, *C. sakazakii* and *C. malonaticus* were the most common *Cronobacter* species in PIF and environmental samples *(23, 25)*. Osaili and Forsythe *(26)* reported differences among *Cronobacter* species in PIF concerning their thermotolerance and resistance to gamma radiation. Thus, species identification within the genus *Cronobacter* is relevant for epidemiological studies, for clinical diagnostics and the selection of chemical agents and antibiotics, and for risk assessment during PIF manufacturing. Rapid and accurate identification of *Cronobacter* species and other *Enterobacteriaceae* strains, particularly differentiation among closely related and phenotypically similar apathogenic *Enterobacter turicensis, Enterobacter pulveris,* and *Enterobacter helveticus,* is of great importance for risk assessment and management, for reducing the risk of contamination in final products, and for reducing the incidence of relevant foodborne diseases.

Various commercially available phenotypic identification systems have been used in food microbiology laboratories. Traditional phenotypic identification is based on biochemical pathways and carbon source utilization. As one of the most commonly used commercial kits, the API test system (bioMérieux, Marcy l'Etoile, France) employs a series of cupules that test for the metabolizing ability of bacteria in different substrates and provides a metabolic fingerprint of the test organism *(30)*. Genotypic identification methods also are emerging as an alternative to phenotypic identification. The 16S rRNA gene sequence has become an important characteristic for phylogenetic identification of microorganisms. As sequence libraries grow, 16S rRNA gene sequencing is being widely applied to bacterial identification and classification *(3, 7, 17)*. Matrix-assisted laser desorption and ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced as a new method for phylogenetic classification of bacteria on the basis of protein profiling. Protein extracted from the whole bacterial cell can be used for bacterial identification at the genus, species, and in some cases subspecies level through comparing protein mass spectral fingerprints of unknown isolates with reference mass spectra in databases. For several foodborne pathogens in the genera *Arcobacter, Helicobacter, Campylobacter, Clostridia,* and *Salmonella* and the species *Listeria monocytogenes, Staphylococcus aureus,* and *Vibrio para-*

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haemolyticus, protein mass spectrum detection methods with MALDI-TOF MS has been validated *(1, 2, 4, 6, 10, 11)*. Recently, a well-validated highly sensitive and specific protein mass pattern identification system for *Cronobacter* has been developed *(31)*.

In the present study, three bacterial identification systems based on biochemical reactions (API 32E), genotypic analysis (partial 16S rRNA gene sequencing), and protein mass patterns (MALDI-TOF MS) were compared. These systems were applied in parallel for identification of various *Cronobacter* species and closely related *Enterobacteriaceae* species.

MATERIALS AND METHODS

Bacterial isolates. Twelve wild isolates from PIF and environment samples and 10 well-identified reference strains from the Centre of Food Safety (Veterinary Sciences Centre, University College, Dublin, Ireland) and the Institute for Food Safety and Hygiene (Vetsuisse Faculty, University of Zurich, Zurich, Switzerland) were used in this study. The reference strains were seven described species and subspecies of the genus *Cronobacter* and three newly described *Enterobacter* species.

Identification with the API 32E system. The API 32E system included 32 biochemical reactions representing 106 taxa of *Enterobacteriaceae*. The test was performed according to the manufacturer's instructions. Substrate utilization was determined after 24 $\pm$ 2 h at 37°C. Identification was performed using the API 32E database (version 3.0) with the analytical profile index and with api*web* identification software (version 3.0). Identification was classified into four groups: (i) excellent identification, identification % $\geq$ 99.9%, and *T* index $\geq$ 0.75; (ii) very good identification, identification % $\geq$ 99.0%, and *T* index $\geq$ 0.5; (iii) good identification, identification % $\geq$ 90.0%, and *T* index $\geq$ 0.25; and (iv) acceptable identification, identification % $\geq$ 80.0%, and *T* index $\geq$ 0.0. These four categories were assigned to correct identifications. In this study, isolates with low discrimination levels, such as doubtful profiles, were categorized as unidentified.

Sequencing of the 16S rRNA gene. Genomic DNA was extracted using a proprietary process (Accugenix, Newark, NJ) incorporating FTA Elute Micro Cards. Extracted DNA was amplified with universal 16S rDNA primers 5F *(27)* and 536R *(33)* and the GeneAmp enzyme (Applied Biosystems, Foster City, CA). An amplicon of 500 bp of the bacterial 16S ribosomal gene was purified with exonuclease and shrimp alkaline phosphatase. The cycle sequencing was performed with a BigDye Terminator v. 1.1 cycling sequencing kit (Applied Biosystems) incorporating a universal primer and was followed by nucleotide purification with paramagnetic beads. The fragment analysis was run on ABI 3130xl genetic analyzer (Applied Biosystems). Sequences obtained were analyzed with proprietary software (Accugenix) and compared with those in the Accugenix database and the open source databases from the National Center for Biotechnology Information and the Ribosomal Database Project.

Accession numbers for 16S rRNA sequences. The 22 strains included in this study (and their 16S rRNA sequence accession no.) were designated cc135 (GenBank JN255111), cc197 (GenBank JN255112), cc204 (GenBank JN255113), cc208 (GenBank JN255114), cc320 (GenBank JN255115), cc553 (GenBank JN255116), cc770 (GenBank JN255117), cc1549 (GenBank JN255118), cc1552 (GenBank JN255119), cc2136 (GenBank JN255120), cc2326 (GenBank JN255121), cc2498 (GenBank JN255122), cc2682 (GenBank JN255123), cc2683 (GenBank JN255124), cc2684 (GenBank JN255125), cc2685 (GenBank JN255126), cc2686 (GenBank JN255127), cc2687 (GenBank JN255128), cc2688 (GenBank JN255129), cc2689 (GenBank JN255130), cc2690 (GenBank JN255131), cc2691 (GenBank JN255132).

Identification with MALDI-TOF MS. All strains were streaked on tryptic soya agar (Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. A disposable polymeric FlexiMass-DS target (Shimadzu-Biotech Corp., Kyoto, Japan) with 48 sample positions was used for sample preparation. Single colonies were directly smeared onto the target plate, which was overlaid with 1 μl of CHCA (α-cyano-4-hydroxy-cinnamic acid) matrix (49508, Sigma-Aldrich, Buchs, Switzerland), and air dried within minutes at room temperature. To generate the protein mass fingerprints, the FlexiMass target was loaded into a MALDI-TOF mass spectrometry Axima Confidence machine (Shimadzu-Biotech). All raw spectra were processed with the Launchpad v. 2.8 software (Shimadzu-Biotech). Obtained protein mass fingerprints were analyzed using SAMARIS software (AnagnosTec, Potsdam, Germany). Peak lists of isolates were analyzed by comparison with spectra in the SARAMIS reference MS database (Marbritec, Riehen, Switzerland).

Confirmation of *Cronobacter* species with fluorescence in situ hybridization (FISH). The VIT *Cronobacter* assay (Vermicon, Munich, Germany), in which a fluorescently labeled probe targets regions of 16S rRNA, was performed with wild isolates according to the manufacturer's instructions.

Differentiation of *Cronobacter* to species based on biochemical parameters. For wild strains confirmed as *Cronobacter* by FISH, differentiation to species was performed as described by Iversen et al. *(14)* based on four biochemical parameters.

RESULTS

API 32E identified 5 (22%) of 22 isolates to species, yielding excellent, good, and acceptable identifications for 2, 2, and 1 isolate, respectively. Fourteen isolates (64%) were identified to genus, yielding excellent and very good for 10 and 4 isolates, respectively. Three isolates (14%) were classified as unidentified because of doubtful profiles. Both 16S rRNA gene sequencing and MALDI-TOF MS assigned 100% of the isolates to genus.

Table 1 shows the comparative identification results for reference strains. All the *Cronobacter* isolates were correctly identified to genus by API 32E. *E. helveticus* and *E. turicensis* were identified as *Enterobacter vulneris* with good and acceptable identifications. For *E. pulveris*, the API system assigned *Enterobacter sakazakii* with doubtful profiles. By using partial 16S rRNA gene sequencing (<500 bp), the results of four *Cronobacter* and three *Enterobacter* isolates were in full agreement with reference designations. *C.* genomospecies 1 was misidentified as *C. turicensis*. Differentiation between *C. sakazakii* and *C. malonaticus* was not possible. With MALDI-TOF MS, all reference isolates were assigned unambiguously to the correct species.

TABLE 1. *Reference strains identified with API 32E, 16S rRNA gene sequencing, and MALDI-TOF MS*

Strain	Reference designation	API 32E				16S rRNA gene sequencing	MALDI-TOF MS
		Identification results	Identification %	*T* index	Identification level		
cc2682	*C. muytjensii*	*E. sakazakii*	99.9	0.58	Very good	*C. muytjensii*	*C. muytjensii*
cc2683	*C. turicensis*	*E. sakazakii*	99.9	0.68	Very good	*C. turicensis*	*C. turicensis*
cc2684	*C.* genomospecies 1	*E. sakazakii*	99.9	0.53	Very good	*C. turicensis*	*C.* genomospecies 1
cc2685	*E. pulveris*	*E. sakazakii*	99.9	0.12	Doubtful profiles	*E. pulveris*	*E. pulveris*
cc2686	*E. helveticus*	*E. vulneris*	98.7	0.56	Good	*E. helveticus*	*E. helveticus*
cc2687	*C. dublinensis* subsp. *lausannensis*	*E. sakazakii*	99.9	0.75	Excellent	*C. dublinensis*	*C. dublinensis*
cc2688	*C. sakazakii*	*E. sakazakii*	99.9	1.00	Excellent	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*
cc2689	*C. malonaticus*	*E. sakazakii*	99.9	0.68	Very good	*C. malonaticus* or *C. sakazakii*	*C. malonaticus*
cc2690	*E. turicensis*	*E. vulneris*	83.2	0.41	Acceptable	*E. turicensis*	*E. turicensis*
cc2691	*C. dublinensis* subsp. *lactaridi*	*E. sakazakii*	99.9	0.83	Excellent	*C. dublinensis*	*C. dublinensis*

Comparative identifications for wild isolates are shown in Table 2. For six isolates identified as *E. sakazakii* with excellent results by API 32E, the other two methods gave concordant but more discriminative identifications; they were identified as belonging to either of the two species in the *C. sakazakii–C. malonaticus* complex by partial 16S rRNA gene sequencing and as *C. sakazakii* by MALDI-TOF MS. The higher discriminative power of the molecular identification methods also was observed for strain cc197. Discrepant results were obtained for two isolates. For strains with doubtful profiles as *E. sakazakii* by API 32E, unambiguous assignments as *E. pulveris* and *E. helveticus* were obtained with 16S rRNA sequence analysis and MALDI-TOF MS. Six wild isolates were confirmed as *Cronobacter* species by FISH. Further phenotypic differentiation of these isolates based on key parameters yielded the set of profiles typical for *C. sakazakii*: they were indole negative, methyl-α-D-glucopyranoside positive, malonate negative, and dulcitol negative.

DISCUSSION

In this study, we compared API 32E, 16S rRNA gene sequencing, and MALDI-TOF MS for the identification of *Cronobacter* and *Enterobacteriaceae* species relevant to evaluation of PIF in the food microbiology laboratory. Other researchers have concluded that API 32E version 3.0 is well suited for *Cronobacter* identification without giving false-negative results *(8, 15)*. In this study, all *Cronobacter* strains were positively identified to genus by API 32E, but false-positive results as *E. sakazakii* were obtained for isolates cc1549, cc2136, and cc 2685, although the profiles were doubtful. Differentiation of the genus *Cronobacter* into species was limited, possibly because of database limitations or limited numbers of reactions included in API 32E strips. Based on the results of tests for four biochemical parameters, six wild isolates were identified as *C. sakazakii,* in concordance with identifications by MALDI-TOF MS and 16S rRNA gene sequencing at the species or complex level. The same approach was used by Miled-Bennour et al. *(23)* and Mullane et al. *(25)* to differentiate previously confirmed *Cronobacter* spp. into species. The feasibility of these discriminative biochemical tests not yet included in current API 32E test strips indicates areas of improvement for the commercial biochemical identification systems for *Cronobacter* characterization. Five isolates belonging to *E. helveticus, E. pulveris,* and *E. turicensis,* which are not part of the API 32E database, were identified as *E. sakazakii* and *E. vulneris,* with doubtful profiles, good results, and acceptable results. Lehner et al. *(21)* reported API 32E identifications as *E. vulneris*, *Pantoea* spp., and *Buttiauxella agrestis* for these newly classified species, with low levels of confidence for all. This findings suggest that a result obtained with API 32E is reliable only when the result is classified as excellent or very good. In our study, the API 32E results for all isolates with excellent or very good identification levels were consistent with final designations to species or genus. For the two strains with an identification level of good, one was correctly identified. None of the

TABLE 2. *Wild isolates identified with API 32E, 16S rRNA gene sequencing, and MALDI-TOF MS*

Strain	API 32E				VIT-FISH	Key biochemical parameters[a]				16S rRNA gene sequencing	MALDI-TOF MS	Conclusive species identification
	Identification results	Identification %	*T* index	Identification level		Ind	Dul	Malo	AMG			
cc208	*E. sakazakii*	99.9	1.00	Excellent	+	−	−	−	+	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*	*C. sakazakii*
cc320	*E. sakazakii*	99.9	1.00	Excellent	+	−	−	−	+	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*	*C. sakazakii*
cc553	*E. sakazakii*	99.9	1.00	Excellent	+	−	−	−	+	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*	*C. sakazakii*
cc1552	*E. sakazakii*	99.9	0.85	Excellent	+	−	−	−	+	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*	*C. sakazakii*
cc2326	*E. sakazakii*	99.9	1.00	Excellent	+	−	−	−	+	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*	*C. sakazakii*
cc2498	*E. sakazakii*	99.9	0.92	Excellent	+	−	−	−	+	*C. malonaticus* or *C. sakazakii*	*C. sakazakii*	*C. sakazakii*
cc197	*Pantoea* sp. I	99.9	0.93	Excellent	−	ND[b]	ND	ND	ND	*P. gavinia* or *P. calida*	*P. agglomerans*	Most likely *P. agglomerans* or *P. gavinias* or *P. calida*
cc770	*Leclercia adecarboxylata*	99.9	1.00	Excellent	−	ND	ND	ND	ND	*E. cloacae* or *E. ludwigii* or *L. adecarboxylata* or *Pantoea* sp.	*L. adecarboxylata*	Most likely *L. adecarboxylata*
cc135	*Escherichia vulneris*	97.9	0.87	Good	−	ND	ND	ND	ND	*E. hormaechei* or *E. vulneris*	*E. vulneris*	Most likely *E. vulneris*
cc204	*E. vulneris*	99.9	0.76	Excellent	−	ND	ND	ND	ND	*E. hormaechei* or *E. vulneris*	*E. vulneris*	Most likely *E. vulneris*
cc1549	*E. sakazakii*	97.4	0.18	Doubtful profiles	−	ND	ND	ND	ND	*E. pulveris*	*E. pulveris*	Most likely *E. pulveris*
cc2136	*E. sakazakii*	99.9	0.38	Doubtful profiles	−	ND	ND	ND	ND	*E. helveticus*	*E. helveticus*	Most likely *E. helveticus*

[a] Ind, production of indole; Dul, production of acid from dulcitol; Malo, malonate utilization; AMG, production of acid from methyl-α-D-glucoside. Profiles of −, −, −, and + are characteristic of *C. sakazakii* species.
[b] ND, not determined.

results for strains with doubtful or acceptable identification levels were consistent with final outcomes. The reliability of results interpreted with a high identification level in phenotypic systems also has been demonstrated in the studies with gram-negative bacteria using API 20NE and VITEK 2 *(3, 34)*.

In many laboratories, 16S rRNA gene sequencing has been extensively applied to bacterial identification, especially for those bacteria that are rarely encountered or cannot be unambiguously identified with a commercial biochemical system *(3, 17)*. Drancourt et al. *(7)* reported a resolution rate of 90% with 16S rRNA gene sequencing for the identification of biochemically unidentifiable isolates covering a wide range of phenotypes. In the present study, strains cc1549, cc2136, cc2685, cc2686, and cc2690, which were incorrectly identified by phenotypic typing method, were assigned to *E. helveticus*, *E. pulveris*, and *E. turicensis* by partial 16S rRNA gene sequencing. Another advantage of 16S rRNA–based identification methods is the robust read-out from the bacterial sample. Although phenotypic typing parameters can be influenced considerably by environmental factors such as culture conditions, subculture, and incubation status, results of 16S rRNA gene sequencing are not usually affected by external factors because of the stability of the genome over time and its resistance to environmental influences *(29)*.

The restricted phylogenetic power of 16S rRNA gene sequencing for *Enterobacteriaceae* species has been discussed previously *(7, 22)*. In the present study, 12 of 22 isolates could not be assigned to a single species because of the high similarity of the 16S rRNA gene in closely related bacteria. This problem was evident particularly for members of the *C. malonaticus*–*C. sakazakii* complex. *C.* genomospecies 1 was given a single species assignment as *C. turicensis*. The inability of 16S rRNA gene sequencing to identify *C.* genomospecies 1 is due to two facts: high sequence similarity between the two species and the noninclusion of *C.* genomospecies 1 in 16S rRNA databases. Thus, for an absolute phylogenetic resolution of *Cronobacter*, supplementary tests including multilocus sequence analysis, DNA-DNA hybridization, amplified fragment length polymorphisms analysis, ribotyping, and phenotypic descriptions are recommended *(14, 16, 20)*.

As a newly developed rapid method for bacterial classification, MALDI-TOF MS has been widely applied for food quality control, clinical screening, and environmental and taxonomical research. Hotta et al. *(13)* and Teramoto et al. *(32)* proposed the use of selected ribosomal subunit proteins encoded by the *S10-spc-alpha* operon as biomarkers for *Pseudomonas* classification and found a strong correlation between phylogenetic topologies derived from MALDI-TOF MS and *gyrB* genetic sequencing. Stephan et al. *(31)* analyzed proteins of 2,000 to 30,000 Da from whole bacteria for establishment of a reference MS database library for *Cronobacter* identification. The obtained phylogenetic affiliations corresponded with the current taxonomic classification. In our study, 10 references strains were assigned to species with MALDI-TOF MS with 100% accuracy. For wild isolates, no reference designations were available to evaluate the accuracy of the MALDI-TOF MS results. Thus, an additional test with FISH *(21)* and biochemical parameters was performed to determine the species identification for these *Cronobacter* isolates. The final outcomes were fully compatible with those from MALDI-TOF MS. For non-*Cronobacter* wild isolates, alternative tools such as sequence analysis of the protein coding genes *hsp60*, *rpoB*, and *hemB* might be useful for assessing the accuracy of MALDI-TOF MS *(12, 24)*. In the present study, MALDI-TOF MS allowed a distinct differentiation of *Cronobacter* species from relevant *Enterobacteriaceae* strains. Such differentiation was sufficient for this study because risk assessment of *Cronobacter* species was our main concern. Compared with 16S rRNA gene sequencing and API 32E, which require usually 1 or 2 days for final outcomes, MALDI-TOF MS is less time-consuming and less laborious because of minimal sample preparation, high detection speed, and high throughput. Beginning with a single colony, results can be obtained within a few minutes *(29, 31)*.

MALDI-TOF MS has some limitations. The high equipment cost must be taken into consideration, and cell culture conditions and sample preparation processes can affect the derived protein patterns *(9, 28, 29)*. During the establishment of an MS library for *Cronobacter*, reproducibility and discrimination of species in a cluster analysis were better for samples grown on tryptic soya agar than for samples grown on blood agar *(31)*. Therefore, to guarantee the reproducibility of mass spectra, standardized sample preparation and incubation procedures are important.

According to our data, MALDI-TOF MS is most suitable of the three methods evaluated for identification of *Cronobacter* and relevant *Enterobacteriaceae* strains. However, this study did not include a large number of isolates. The suitability of this system for a larger culture collection still needs to be established.

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Chapter 6

Discussion

6.1 Inactivation of *Cronobacter* in Reconstituted PIF

PIF is not a sterile product. Being a nutrient-rich medium, reconstituted PIF provides a good substrate for growth of *Cronobacter* spp. (Nazarowec-White and Farber, 1997b). Once rehydrated, storage time and temperature have been considered as only limiting factors for bacterial growth and related infection (Amalaradjou et al., 2009). Rapid generation times of *Cronobacter* of 37-44 min and 19-21 min at 22°C and 37°C, respectively, have been reported by Iversen et al. (2004b) and Nazarowec-White and Farber (1997a). The FAO/WHO Expert Group developed a quantitative risk assessment model in which the risk of *Cronobacter* infection posed to infants from intrinsically contaminated PIF was estimated and concluded that improper reconstitution water temperature, holding bottles at room temperature and prolonged feeding periods and storage practices could lead to an increased risk of *Cronobacter* infection (Anonymous, 2006a).

Thermal treatment of food right prior to consumption has long been used as a primary tool of reducing the contamination risk of pathogens. Use of heat-treated water for PIF reconstitution to eliminate *Cronobacter* may represent a final opportunity to prevent infection. However, the recommendations on the product labelling given by manufacturer for PIF reconstitution temperature are inconsistent between different countries: water of 20°C, 40°C, 50°C and at least 70°C is recommended for PIF reconstitution in France, Germany, Japan and UK. As predicted by FAO/WHO, PIF reconstitution with water 40°C or 50°C (unless consumed immediately) is associated with the greatest risk of *Cronobacter* infection (Anonymous, 2006a). In a preliminary experiment in the present work, when 90 ml PIF spiked with *C. sakazakii* CLF2688 at a low level of 1-10 CFU was reconstituted with water of 40°C, temperature in rehydrated PIF decreased to 37°C and an appreciable bacteria growth up to 100-1000 CFU/90 ml occurred after incubation at 37°C for 2 h. Iversen et al. (2004) reported 35-37°C as the optimal growth temperature for *Cronobacter*. Mixing water of 30-50°C did not cause a substantial inactivation of *Cronobacter* in PIF but provided conditions favourable for bacteria growth (Chen et al., 2009; Kim and Park, 2007). It must be noted that in the risk assessment model of FAO/WHO (Anonymous, 2006a) the results

are presented as relative risk based on definitive preparation scenarios. Bacterial growth in infant gastrointestinal tract after PIF consumption was not taken into consideration and the highest environmental temperature simulated in the model was 30°C instead of a physiological infant body temperature of 37°C. Using the calculation of gastric emptying reported in Chapter 3, the infectious dose of 1000 CFU for *Cronobacter* proposed by Iversen and Forsythe (2003) would be easily exceeded during stomach emptying by the consumption of contaminated PIF reconstituted with water of 40°C. Even the higher infectious dose of 10,000 CFU estimated by FAO/WHO (Anonymous, 2007a) could be achieved in a preterm infant with an extremely prolonged gastric emptying time of 21 h. The rapid growth of *Cronobacter* at ambient temperatures indicates that even if the PIF reconstituted with water of 40°C or 50°C is consumed immediately, it apparently still poses an enhanced risk of infection under infant physiological conditions. FIF has proven to be effective in inhibiting pathogen due to competition for nutrients and production of antimicrobial substances (Agostoni et al., 2007). In the present work, only limited bacteria-killing effect of FIF was demonstrated. In the FIF product fermented to 100% where the most pronounced inhibitory effect was demonstrated, the *Cronobacter* grew from a low initial level to a relatively high level close to the infectious dose 5 h after FIF was mixed with water of 40°C as recommended. It suggests that the risk associated with *Cronobacter* is still likely to exist if FIF is reconstituted with water of 40°C and the reconstituted FIF is kept for an increased hang-time.

Edelson-Mammel and Buchanan (2004) were the first to report a greater than 4 log reduction of *Cronobacter* by rehydrating PIF with water ≥ 70°C. Consequently, the FAO/WHO (Anonymous, 2007b) has recommended rehydration of PIF with water at 70°C or higher to reduce the potential infection risk associated with *Cronobacter* contamination. In the present work, water of 70°C and 80°C was used to mix PIF and lower temperatures were obtained in rehydrated PIF. We are the first to indicate that not only the serving volumes, but the material of baby bottles as well can influence the decline of temperature in rehydrated PIF. Chen et al. (2009) reported higher temperature of reconstituted PIF when a

larger amount of water was mixed with PIF, which could be explained by the slower cooling pattern of the container for larger servings. Correspondingly, in the same study a lower number of survivors as well as a retarded growth of *Cronobacter* spp. has been recorded in two serving (120 ml) than in single serving (60 ml) preparation(s) of PIF reconstituted with water of same temperature. Notable that compared with older babies, the more vulnerable neonates ≤ 28 days are usually fed on infant formula in smaller volumes and could thus be exposed to a higher risk of *Cronobacter* infection.

As shown in Table 2 Chapter 2, 90 ml PIF rehydrated with water of 80°C lead to a greater *Cronobacter* cell reduction in average in plastic bottle (3.6 log) than in glass one (2.3 log), which could be due to the higher temperature of rehydrated PIF in the plastic container with a better heat preservation capability (Table 1 Chapter 2). Moreover, the *Cronobacter* reduction in plastic bottle was < 4 log for lower inoculums and > 4 log for higher inoculums. This is probably explained by the uneven heating of rehydrated PIF caused by insufficient shaking, which could lead to lower than anticipated microbial inactivation in cold points where bacteria are clustered.

Thermal inactivation effect of hot water on *Cronobacter* in reconstituted PIF has been reported by other investigators: Chen et al. (2009) reported no inactivation and 4-6 log reduction in *Cronobacter* numbers using water of 50°C and 90°C respectively. For two heat resistant strains in single serving a decrease in cell counts by 1-2 log at 60°C and 70°C was achieved. Kim and Park (2007) studied the inactivation of one *Cronobacter* isolate during PIF rehydration and reported reduction by 1-2 log at 60°C and 4-6 log at 65°C and 70°C. Kindle et al. (1996) reported over 4 log reduction at 82°C-93°C for 85-93 sec by microwaves. Despite serving volume and container materials, other factors like initial reconstitution water temperature, cooling duration and environmental temperature could influence the “final real temperature” in rehydrated PIF and consequently the surviving rate of *Cronobacter*. On the other hand, thermal inactivation is strongly strain dependent (Chen et al., 2009). Thermal stability of *Cronobacter* isolates varied as much as twentyfold

(Edelson-Mammel and Buchanan, 2004). All biochemical differences between strains (e.g. heat sensitive or not; type strain or capsulated strain; heat injured or not) are responsible for diversity of heat resistance among *Cronobacter* strains (Chen et al., 2009; Kim and Park, 2007). Furthermore, nutritional aspects of media as well as components in different infant formula formulations (sugar and fat concentration, total solid content) may influence the thermal resistance of bacteria and further the bacterial survival during heat treatment (Chen et al., 2009; Nazarowec-White and Farber 1997c).

To date low levels of *Cronobacter* have been found in PIF in most cases ranging from 0.36 to 66 CFU/100 g (Muytjens et al., 1988). The most highly contaminated PIF cluster contained 560 *Cronobacter* cells/g which corresponds to a cell density of approximately 5600 CFU/90 ml infant formula (Jongenburger et al., 2011). Even at this worst case scenario the heat treatment with 80°C water is likely to result in a high probability that no target organism would be present in a single serving anymore, thereby reducing the infection risk associated with consumption of contaminated PIF. Although the antimicrobial effect of FIF was slightly affected by heat treatment with water of 80°C, this effect which is linked to bacterial competition or lowered pH is not strong enough to replace the effectiveness of thermal inactivation of pathogens using hot water ≥ 70°C for reconstitution. Loss of vitamin C by preparing PIF with boiling water varied between different infant formula formulations. Data shown in the present study (Fig 3, Chapter 2) suggest no effect of water of 80°C on substantial vitamin C reduction.

According to FAO/WHO recommendation (Anonymous, 2007b), the PIF is prepared with water ≥ 70°C, cooled to feeding temperature under a running tap and fed immediately. Any feed that has not been consumed within two hours has to be discarded. Although it is recommended to make PIF fresh for each feed, for practical reasons, feeds need to be prepared in advance. Prepared feeds should be stored in the refrigerator of no higher than 5°C of maximally up to 24 h.

Besides loss of heat-sensitive nutrients, other concerns about using water ≥ 70°C for PIF reconstitution such as a scalding risk and clumping of the powder have been raised (Anonymous, 2006a). Although these potential problems can be avoided by careful handling or technological improvement, unfortunately, the FAO/WHO recommendation is not always followed. Fein and Falci (1999) and Herbold and Scott (2008) reported a high percentage of mothers who failed to comply with PIF preparation and feeding instructions. It poses a high risk of recontamination of prepared PIF from unwashed hands, utensils, insects, etc. before it is consumed. Therefore, there is increasing interest in fortifying PIF with antimicrobial barrier to allow for reconstitution at lower temperatures. The use of various natural antimicrobials to limit *Cronobacter* growth in rehydrated PIF or milk products e.g. lactoperoxidase (Gurtler and Beuchat, 2007), probiotics (Osaili et al., 2008; Shaker et al., 2008), prebiotics (Quintero et al., 2011) and essential oils derived from plants (Amalaradjou et al., 2009; Amalaradjou and Venkitanarayanan, 2011) have been reported. Acidification of PIF or weaning food has been a popular strategy since the first half of 20th century as a treatment for infant intestinal disease and chronic diarrhoea (Carrion and Egan, 1990). Both chemically and biologically acidified formula have been reported to be effective in limiting bacterial proliferation and/or the consequent infection disease in *in vitro* and *in vivo* studies (Back et al., 2009; Boneti et al., 2009; Brunser et al., 1989; Carrion and Egan, 1990; Joosten and Lardeau; 2004; Mehall et al., 2001; Mehall et al., 2002; Nout et al., 1989). On the other hand, the downsides of these approaches like systemic infection caused by some probiotic strains and acidosis caused by HCl have been documented in infant receiving acidified formulae (Boneti et al., 2009; Carrion and Egan, 1990; Mehall, et al., 2002). Additionally, a high proportion of children who rejected the commercial biologically acidified infant formula (pH 4.8) due to the taste have been reported by Brunser et al. (1989). In the present work the physiological gastric acidity was taken into consideration for the first time. In the experimental model simulating the infant gastric condition, infant formula mildly acidified with acetic acid to pH 6.0 showed a significant antibacterial effect against *Cronobacter* combined with natural physiological acidity of pH 5.0. Compared to acidification interventions previously reported with pH values ranging from < 4.0 to 5.0, the current

intervention with a higher pH has a lesser impact of taste. A better sensorial acceptance and a higher tolerance of the diet are to be expected, whilst the antimicrobial activity is preserved (Chapter 3).

The actual pH in infant intestinal lumen is substantially influenced by food intake. McClendon et al. (1917) reported pH 3.1 and 6 in infant duodenal and ileum contents respectively. In a clinical study performed by Senger et al. (1987), the pH values in duodenal juice of infants ranged approximately between 5.5 and 6.5. It thus raises the question whether the beneficial effects of acid-treated PIF reported in the present work still exist in the intestinal lumen, once the acidified product is neutralised in the small intestines. Short-chain fatty acids (SCFAs) including acetic, propionic and butyric acid have been reported to be produced quantitatively in intestinal regions of both breast-fed and bottle-fed neonates due to gut microbial fermentation of prebiotic substances such as human milk oligosaccharides or GOS/FOS supplemented in infant formulation (Roberfroid et al., 2010). The presence of SCFA lowers the pH and might exert synergic antimicrobial effect in intestinal lumen after acidified PIF is ingested. Siigur et al. (1993) showed differences on SCFA pattern between formula feeding and breast feeding. At age of two months, the breast-fed infants had a significantly higher proportion of acetic acid in the SCFA spectra than the bottle-fed infants. Whereas acetic acid is the dominant SCFA produced by *Bifidobacterium*, *Bacterioides* and *Clostridium* produce a variety of other SCFAs e.g. butyric, propionic, formic and lactic acids in addition to acetic acid (Madigan et al., 2010). As shown by Back et al. (2009) as well as in Chapter 3, the bactericidal effect against *Cronobacter* spp. varies between organic acids. Therefore further investigation on additional contribution of SFCA to growth inhibition of *Cronobacter* in acidified or non-acidified PIF in intestinal lumen as well as *in vivo* tests of feeding tolerance and sensory acceptance would be beneficial.

6.2 Improvement of the Screening Procedure for the *Cronobacter* Detection from PIF

Most published methods included a pre-enrichment step, a selective enrichment broth and plating on chromogenic agars. A pre-enrichment step is essential in enabling detection of target micro-organisms in low levels, as it can promote the bacterial multiplication from a single cell to levels of > 10^3 CFU/ml in the enrichment broth (Kandhai, 2010). A pre-enrichment step is necessary for the recovery of injured or stressed cells which were exposed to chemical or physical treatments during the food processing (Van Schothorst, 1976). Selective media have been shown not suitable to be applied in the initial enrichment step due to their poor ability to recover stressed cells (Chen et al., 2010). Chen et al. (2010) evaluated three enrichment broths BPW, BHI and EE for their recovery of healthy as well as heat- and desiccation-stressed *Cronobacter* in PIF and concluded BPW as most sufficient for recovering target micro-organisms.

The selective enrichment step aims at providing the specific growth condition of the target micro-organisms via promoting their growth while inhibiting other background micro-flora to the greatest extent. The mLST broth recommended in the standard ISO method ISO/TS 22964:2006 modifies the protocol of Guillaume-Gentil et al. (2005) by adding 0.5 M NaCl against competing non-*Cronobacter* Enterobacteriaceae and the vancomycin against gram-positive micro-organisms. To enhance the selectivity, a relatively high incubation temperature (44°C ± 0.5°C) was applied. The U.S. FDA-recommended method (Anonymous, 2002) proposed EE broth as the screening enrichment, a medium which was designed for isolation of the total Enterobacteriaceae strains. It has been reported that the EE broth was not selective enough as it could allow other Enterobacteriaceae to outgrow *Cronobacter* and give false negative results (Iversen et al., 2004a). In a comparative study of Iversen and Forsythe (2007), mLST has been evaluated as more selective than other three selective media including EE, *Enterobacter sakazakii* selective broth (ESSB) and *Enterobacter sakazakii* enrichment broth (ESE) which replaced dextrose and/or lactose with

sucrose. On the other hand, however, it has been established that there are *Cronobacter* isolates unable to grow in mLST or brilliant green bile broth or do not grow at an elevated temperature of 44°C (Iversen et al., 2007b; Iversen and Forsythe, 2007; Joosten et al., 2008). In this respect, improvements in detection methods for *Cronobacter* have been focused on the optimization of selective enrichment media. A new protocol using a *Cronobacter* screening broth (CSB) which enables the growth of *Cronobacter* strains sensitive to EE and mLST broths has been developed (Iversen et al., 2008a). Both this new protocol and the ISO standard method showed equally high sensitivity and specificity for testing *Cronobacter* spp. from PIF end products, raw materials and environmental samples. One of the greatest advantages of using the CSB screening method is that a release decision on *Cronobacter* negative sample can be made within 48 h. To confirm a positive result, all protocols reported to date require still up to 5 to 6 days (Table 2).

The practicability and feasibility of a rapid impedance method with complementary molecular-based confirmation system for *Cronobacter* detection in PIF samples were explored in Chapter 4. This new protocol involves a pre-enrichment step in BPW for 16 h, which allowed the full growth of a healthy *C. sakazakii* strain in 100 g PIF reconstituted in 900 ml BPW from an initial level of 1 CFU to approximately 10^8 CFU. Injured cells might have a prolonged lag time and this may add several hours to the overall detection time. With this new protocol, heat and desiccation-injured *C. sakazakii* strains were recovered successfully within a short DT as well. Kandhai et al. (2006) reported that different pre-culturing conditions exhibited no effect on either lag time or growth rate of *Cronobacter* spp. and concluded that the rich growth medium, the reconstituted PIF, may be responsible for not measuring differences. The lag time for desiccated cells to recover after PIF rehydration at 37°C has been estimated to be 1.73 ± 0.44 h (Kandhai et al., 2006). The doubling time of 20 min at 37°C for *Cronobacter* has been reported by Iversen et al. (2004b). Theoretically, stressed *Cronobacter* cells present initially at 10^2 CFU/100 g sterile PIF sample would well multiply to exceed a final concentration of 10^8 CFU/ml within a 16 h incubation time in BPW. In the impedance devices, the signal for a successful bacterial

detection is given only when the target micro-organisms have increased to at least 10^6-10^7 CFU/ml (Firstenberg-Eden, 1984). A bacterial density of approximately 10^6 CFU/ml would be reached for both the healthy and stressed cells if 0.1 ml aliquot is transferred from pre-enrichment into the impedance measuring cells giving an explanation of short DTs < 1-2 h obtained for most of the target micro-organisms handled in the present work.

In the current new protocol, BiMedia 144A is a coliform selective media that has been adapted to enable the growth of laurylsulfate– and/or crystal violet sensitive *Cronobacter* strains. Despite the addition of vancomycin and the relatively enhanced incubation temperature of 42°C, the selective nature of the BiMedia 144A is limited, as it allows well the growth of non-*Cronobacter* Enterobacteriaceae competitors. As a result, BiMedia 144A remains a screening tool in the current protocol and has to be used complementary with confirmation systems like RiboFlow™ or any agar medium. The overall effectiveness of the method is therefore dependent on the sensitivity and specificity of the confirmation assay used. In the present study, the 16S rRNA-based RiboFlow™ identification system has been shown to be 100% sensitive and specific to *Cronobacter* species. For this assay a minimum population for *Cronobacter* spp. of 10^5 CFU/ml is required to obtain a positive signal (personal communication). The new protocol described in Chapter 4 was not capable of covering sensitive *Cronobacter* strains CLF2684 and CLF248 in the presence of rapidly growing Enterobacteriaceae strains *E. cloacae* and *C. freundii* in 15 of 32 (47%) cases. These false negative results are probably due to the reduced growth rate of *Cronobacter* spp. in the pre-enrichment broth and/or selective enrichment broth BiMedia 144A caused by the interference of competing micro-flora which might prevent the target micro-organism from multiplying to a minimum cell concentration needed for a positive confirmation.

Based on data of artificially spiked PIF experiments, the impedance-RiboFlow™ protocol exhibited an overall higher sensitivity (85%) and specificity (100%) for detection of *Cronobacter* compared with the reference procedure comprising pre-enrichment in BPW, selective enrichment in mLST and isolation on DFI agar. With the employment of the

impedance method, the maximally incubation time in impedance media was shortened to 13 h. Samples by which the M-value of 5% is surpassed with DT > 13 h are negative for *Cronobacter* and the release decision can thus be made 29 h after start of sample pre-enrichment; also, the work and cost outlays are substantially reduced, as only samples with DT < 13 h need to be further confirmed.

As mentioned in Chapter 1, high numbers of complex competing flora contained in raw ingredients as well as antimicrobial substances in special infant formulae may reduce the growth rate of *Cronobacter* spp. in the pre-enrichment and hence lead to a false negative outcome by detection. Table 1 showed the results of follow-up tests of detection of *Cronobacter* from artificially spiked raw ingredients and fermented infant formulae (FIF) using the impedance-RiboFlow™ method and the reference method identical to that described in Chapter 4. Fewer samples were found positive for *Cronobacter* when using reference method than when using impedance-RiboFlow™: While *Cronobacter* was recoverable by both means in maltodextrin, RiboFlow™ recovered the target strains from 100% (3/3) and reference method from 66% (2/3) of the thickening agent samples. For three maize meal samples from which endogenous *K. pneumoniae* was isolated, the target strain was not detected by both methods, yet the sensitivity of the impedance-RiboFlow™ being slightly higher than that of reference method. In two spiked FIF products, *Cronobacter* spp. were successfully recovered by both impedance-RiboFlow™ and the reference procedure. Significantly prolonged DTs exceeding 7 h were obtained for FIF product Gallia Lactofidus which achieved a pH value of 5.0 after fermentation, indicating that the growth of *C. sakazakii* was slowed down in the pre-enrichment in reconstituted product probably due to the relatively lower pH value and the inhibition of bioactive substances emerging after product fermentation.

Table 1. Detection of *C. sakazakii* CLF2688 (10^0–10^1 CFU/100 g) from raw ingredients and fermented infant formulae

	Impedance DT (h)[a]	Impedance-RiboFlow™	mLST-DFI
Maltodextrin	1.64	+	+
	1.51	+	+
	1.06	+	+
Xanthan gum	1.67	+	-
	1.59	+	+
	1.54	+	+
Maize meal	1.27	+	-
	1.14	-	-
	1.38	-	-
Gallia Calisma	1.07	+	+
	1.44	+	+
	1.88	+	+
Gallia Lactofidus	7.81	+	+
	8.70	+	+
	7.93	+	+

[a] The results of triplicate tests are shown for each of three raw materials and two fermented infant formulae.

More recently, the FDA method has been revised to incorporate a slightly modified form of the real time PCR assay developed by Seo and Brackett (2005) and two chromogenic agars for *Cronobacter* detection from PIF. The revised FDA method has been proved to shorten the analysis time substantially and to increase the sensitivity and specificity as compared to the original FDA protocol (Chen et al., 2010). As reported by Fanning and Forsythe (2008), a pre-enrichment procedure is necessary to increase the population to 10^2 CFU/ml to meet the detection limit of PCR. When serving as a screening tool in the revised FDA protocol, the real time PCR assay showed a higher detection limit: a population of target micro-organism of 10^3 CFU/ml in the pre-enrichment step in BPW is required to enable successful detection of *Cronobacter* in PIF contaminated with natural background flora in low amounts, while for recovering target micro-organism with a strong interference of background flora such *Samonella* and *E. cloacae* in PIF, a population of 10^5 CFU/ml is necessary (Chen et al., 2010). One limitation of PCR analysis is that DNA from dead cells can be amplified, resulting in false-positive outcomes. Another disadvantage using molecular based detection technologies is that the micro-organism itself cannot be isolated

and studied further. Furthermore, matrix interference on PCR assay has been reported (Wilson, 1997). Therefore, in the revised FDA method, the PCR analysis utilized as screening tool must be followed by a subsequent culture-based confirmation step.

Table 2. Overview of detection methods for *Cronobacter* in PIF

	Pre-enrichment	Selective enrichment	Isolation	Confirmation/ Identification	Detection duration
ISO/TS 22964	BPW (37°C)	mLST + vancomycin (44°C)	ESIA (44°C)	TSA followed by biochemical characterization	6 d
Reference FDA	Distilled water (37°C)	EE broth (37°C)	VRBG (37°C)	TSA followed by API 20E	6-7 d
Revised FDA	BPW (37°C)		6 h culture of pre-enrichment for real time PCR analysis; DFI and R&F agar (37°C)	Real time PCR; RAPID ID 32E	1-2 d
CSB	BPW (37°C)	CSB (42°C)	X-TSA[a] (37°C)	Biochemical characterization	5 d
Impedance	BPW (37°C)	BiMedia 144A (42°C)		RiboFlow™	1-2 d

[a] Chromogenic media based on TSA formulation containing 0.15 g/L 5-romo-4-chloro-3-indolyl-α-D-glucoside

6.3 Chromogenic Isolation Media and Identification Systems for the Detection of *Cronobacter*

As discussed above, none of the selective enrichment broths developed so far is able to support the growth of all currently known *Cronobacter* phenotypes and ensures at the same time a high selectivity. Therefore an isolation step of streaking the selective enrichment broth onto selective solid media is incorporated in the conventional procedure after the selective enrichment stage. Being used as the primary isolation agar in the FDA method (Anonymous, 2002), the VRBGA is selective only for Enterobacteriaceae and coliforms and is not specific for *Cronobacter* due to its limited screening property for only one differential characteristic (acid production from glucose). On VRBGA, *Cronobacter* spp. and all other enteric bacteria can produce red-pink colonies (Iversen and Forsythe, 2007; Restaino et al., 2006). The chance of isolating of *Cronobacter* spp. colony from VRBGA is thus highly dependent on the numbers of competing enteric bacteria grown on it. Based on the unique feature of α-glucosidase activity of *Cronobacter* spp., several chromogenic media have been developed to differentiate *Cronobacter* spp. from other members of Enterobacteriaceae. DFI agar which makes use of two differential characteristics, X-α-D-glucopyranoside and hydrogen sulphide production, showed a 100% sensitivity for *Cronobacter* spp. yielding typical blue-green colonies and a specificity of 87.2% at 37°C, producing false positive results on certain *Pantoea* spp., *C. koseri* and *Escherichia vulneris* strains (Iversen et al., 2004a). The ISO/TS 22964 (Anonymous, 2006b) proposed the use of ESIA agar for *Cronobacter* isolation with an incubation temperature of 44°C. Though an excellent specificity of ESIA was demonstrated in several studies, reduction in its sensitivity has been determined as some *Cronobacter* strains were inhibited by crystal violet as the medium component and by the high incubation temperature of 44°C (Iversen and Forsythe, 2007; Iversen et al., 2007b). The inclusion of two chromogenic substrates X-α-D-glucopyranoside and X-β-D-cellobioside, three sugars, a pH indicator and inhibitors like bile salts, cefsulodin and vancomycin in the ESPM agar was therefore intended to improve the sensitivity and selectivity of this new medium for *Cronobacter* spp. (Restaino et al., 2006). Compared with

the FDA procedure which involves VRBG and TSA, a greater number of *Cronobacter* spp. were recovered from a variety of food and environmental sources using ESPM with a higher sensitivity and specificity (Restaino et al., 2006). Iversen et al. (2007b) compared the efficacy of ESIA, ESPM and DFI on 210 target and 102 non-target strains at recommended temperatures and found 3.8% of *Cronobacter* strains grew but did not produce blue-green-pigmented colonies on DFI due to the weak α-glucosidase activity. To enable the production of typical blue-green colonies, the concentration of X-α-D-glucopyranoside was increased by 50%. The modified DFI showed the highest sensitivity of 100%, followed by ESPM (99.5%). In the comparative test in the present work (s. Addendum), ChromoCult™ showed the highest specificity with only one non-*Cronobacter* strain CLF2685 *Enterobacter pulveris* producing typical colonies on it. On the other hand, the sensitivity of ChromoCult™ agar (92%) was lower than other media tested due to the misidentification of *C. malonaticus* CLF2689. All the other five chromogenic media showed a sensitivity of 100%, while the specificity varied from 44% for DFI to 67% for Compass and ESPM.

In a study of Lehner et al. (2006), twelve strains isolated from fruit powder produced presumptive colonies on DFI and ESIA. However, data of 16S rRNA gene sequencing and ribotyping analysis clearly revealed that they did not belong to the group *Cronobacter* spp. To distinguish these questionable, unclassified members of Enterobacteriaceae from *Cronobacter* spp., Stephan et al. (2007, 2008) performed a taxonomic polyphasic study and proposed a new taxonomic assignment for these strains of the genus *Enterobacter* as *E. helveticus*, *E. turicensis* and *E. pulveris*. Isolates of the three novel *Enterobacter* species have been found in the same ecological inhabitants as *Cronobacter* spp. such as infant formula, dried food products and infant formula processing environments (Stephan et al., 2007; Stephan et al., 2008). Sharing typical characteristics with *Cronobacter* spp. e.g. production of yellow pigment and hydrolysis of XαGlc and 4NPαGlc due to α-glucosidase activity, *E. helveticus*, *E. turicensis* and *E. pulveris* have been determined to present as the main false positive presumptive organisms on chromogenic media containing XαGlc like ESIA, DFI or ESPM and to pose a challenge for isolation methods of *Cronobacter*

(Addendum, Iversen et al., 2007b; Lehner et al., 2006).

The presence of typical colony morphologies on isolation agar is only a presumptive identification. A confirmation step is needed. Among a variety of identification systems, commercially available biochemical galleries based on phenotypic characteristics such as API 20E, ID 32E, Biolog etc. are still preferably used in the laboratories for *Cronobacter* identification due to the simple performance and low costs. Iversen et al. (2004a) assessed two commercial tests kits, FDA recommended-API 20E and ISO/TS 26994 recommended-ID 32E, and reported apparent disagreement between them in the efficacy of identification of *Cronobacter* spp.: of 12 strains identified as *Pantoea* spp. strains using API 20E, ten had higher levels of confidence as *Cronobacter* spp. (*E. sakazakii*) using the ID 32E panel. The limited number of tests included in the API 20E test kit e.g. the lack of α-glucosidase reaction might be responsible for its inadequacy in identifying *Cronobacter*. Further studies of Iversen et al. (2007b) showed that ID 32E was more reliable than API 20E for *Cronobacter* identification, with 99.5% of the *Cronobacter* strains correctly identified, while the API 20E gallery was only 70% successful for identifying the target strains. On the other hand, Fanning and Forsythe (2008) reported a higher sensitivity and specificity of Biolog GN2 than ID 32E and API 20E. For the three novel *Enterobacter* species, the ID 32E test performed by Lehner et al. (2006) suggested ambiguous identification results as *E. vulneris*, *Pantoea* spp. and *Buttiauxiella agrestis*. In the study of Iversen et al. (2007b), no false positive identifications as *Cronobacter* spp. were obtained on these three species by using ID 32E gallery. As shown in Table 1 Chapter 5, an isolate of *E. pulveris* was identified as *Cronobacter* spp., however, with a low level of confidence using ID 32E. Other kits including Rapid ID 32E have been reported to give equally variable results e.g. with misidentification of *E. pulveris* as *Cronobacter* spp. (Druggan and Iversen, 2009). Due to the inter- and intra-species biochemical heterogeneity and the instable expression of phenotypic features of *Cronobacter* and related organisms, none of the commercially available biochemical kits showed an accuracy of 100% for *Cronobacter* identification. In this respect, Restaino et al. (2006) suggested that the *Cronobacter* identification should be

based on more than one identification system. Notably, none of the databases of these commercial biochemical kits have been updated with the 2007 taxonomic revisions and the discrimination is limited at the *Cronobacter* genus level.

It is known that α-glucosidase is a characteristic feature for *Cronobacter* species. Depending on the α-glucosidase substrate, different profiles can be generated (Iversen et al., 2007b). The fermentation of XαGlc is e.g. typical for the α-glucosidase activity of all *Cronobacter* species. Hydrolysis of α-methyl-D-glucoside (αMGlc) is a further test of α-glucosidase. All *Cronobacter* spp. except for one species *C. muytjensii* can ferment this substrate. Interestingly, *E. helveticus*, *E. turicensis* and *E. pulveris* are positive for hydrolysis of XαGlc and are therefore the main false positive organisms on chromogenic media containing XαGlc, but they can not ferment αMGlc. It would appear that using a combination of α-glucosidase substrates might facilitate a more accurate identification of presumptive *Cronobacter* spp. (Druggan and Iversen, 2009).

Compared to phenotypic methods, molecular-based techniques have been increasingly used because they usually enable a more sensitive, more specific and more rapid identification of *Cronobacter*. To date two molecular-based methods, the α-glucosidase (*gluA*) PCR and the real time PCR assay targeting the *dnaG* gene encoding the DNA-primase have been broadly evaluated on an extensive collection of target and non-target strains. In most cases, both systems have proven to be 100% sensitive and specific for *Cronobacter* spp. (Drudy et al., 2006; Iversen et al., 2007b; Lehner et al., 2006). The only exception was shown on a type strain of *E. turicensis* (LMG 23730) where a false positive result was obtained using the real time PCR protocol targeting *dnaG* gene (Lampel and Chen, 2009). In a comparative evaluation of several commercially available real time PCR-based systems for *Cronobacter* identification, the Assurance GDS™ *Enterobacter sakazakii* and the foodproof® *Enterobacter sakazakii* Detection Kit were 100% successful for differentiating *Cronobacter* spp. from other Enterobacteriaceae strains including *E. helveticus*, *E. pulveris* and *E. turicensis* (Fricker-Feer et al., 2011). Similarly, a high accuracy of 100% of the foodproof®

Enterobacter sakazakii Detection Kit for *Cronobacter* identification has been shown in the present work (s. Addendum). In the study of Lehner et al. (2006), twelve non-*Cronobacter* strains isolated from fruit powder reliably identified using 16S rRNA gene analysis were correctly identified as negative for *Cronobacter* with α-glucosidase PCR, although they produced typical colonies on ESIA and DFI. In the subsequent investigations performed by Stephan et al. (2007; 2008), it has been proposed that these isolates represent novel *Enterobacter* species *E. pulveris*, *E. turicensis* and *E. helveticus*. The results suggest strongly that the α-glucosidase PCR system exclusively target the gene encoding the α-glucosidase in *Cronobacter* spp. Furthermore, in the same study of Lehner et al. (2006), a 100% sensitivity and specificity for *Cronobacter* spp. was shown on the 16S r RNA-based FISH assay VIT® as well.

The PCR-based technique requires a step of DNA extraction prior to amplification set up and is thus restricted to especially equipped laboratory. Results are obtained within 4 h when using PCR for *Cronobacter* identification. The commercial FISH kit VIT® and RiboFlow™ assay are based on fluorescently labelled gene probes targeting special regions of 16S rRNA of the bacteria, therefore only vital *Cronobacter* cells containing a high ribosomal content are detected by the systems. Both kits are easy and convenient to handle. Especially the detection time required by RiboFlow™ is substantially decreased to 15 min. As shown in the study of Lehner et al. (2006) as well as in Chapter 5 and in Addendum, they performed equally well in identifying *Cronobacter* spp. with a high sensitivity and specificity.

All the above-mentioned methods facilitate an identification of *Cronobacter* spp. only at genus level. Using polyphasic methods including f-AFLP fingerprints, ribopatterns and full-length 16S rRNA gene sequences, a set of 210 *E. sakazakii* (*Cronobacter*) strains have been divided into separate groups and a reclassification of these organisms as *Cronobacter* on the genus level containing eight (sub-) species has been proposed (Iversen et al., 2007a; Iversen et al., 2008b). In the present work in which totally 22 target and non-target strains were tested, *Cronobacter* strains were 100% assigned to species level and were successfully

separated from closely related Enterobacteriaceae strains using 16S rRNA gene sequencing (Chapter 5). A distinguishment between *C. sakazakii* and *C. malonaticus* was not possible due to the high similarity of their 16S rRNA gene sequences. The restricted discriminatory power for Enterobacteriaceae reported by Drancourt et al. (2000) and Lehner and Stephan (2004) has been demonstrated on three of non-target strains, for which ambiguous identifications were suggested (Table 2 Chapter 5). Overall the 16S rRNA gene sequencing is regarded as a superior identification tool compared to phenotypic methods due to the stability of the genome over time, high reproducibility and resistance to external influences rapidity and robust read-out (Clarridge, 2004; Sauer and Kliem, 2010). Providing phylogenetically meaningful information, it has been used in several studies as adjunct to phenotypic methods for the identification of difficult-to-identify bacteria or as reference standard to evaluate the accuracy of phenotypic identification systems (Bosshard, et al., 2006; Fanning and Forsythe, 2008; Iversen et al., 2007c, Lehner et al., 2006). On the other hand, some well-known limitations of 16S rRNA gene sequencing have been listed. Firstly, the quality of the sequence is critical and undetermined positions > 1% can interfere with the interpretation (Clarridge, 2004; Drancourt et al., 2000). Secondly, although the sequence itself is objective, the interpretation of data is subjective and no universally acceptable guideline of 16S rRNA sequence similarity for bacterial designation is available so far. A 97% similarity was previously proposed for species delineation (Stackebrandt and Goebel, 1994); nevertheless, a cut-off value ranging between 98.7-99.5% has been recently amended (Palys et al., 1997). Thirdly the interpretation of sequences highly depends on the quality of the database. The quality of public database has been questioned because of lack of peer review (Boudewijns et al., 2006; Mignard and Flandrois, 2006). On the other hand, the commercial database of a better quality with more correct sequence entries might have the disadvantage of limited number of sequences and the enhanced cost of analysis (Clarridge, 2004).

MALDI-TOF MS has become a convenient tool for bacterial identification due to its promising advantages e. g. rapidity, high-throughput, simple sample preparation, ability to

detect minor differences between strains and direct comparison between strains-characteristic patterns (Holland et al., 1996; Rupf et al., 2005; Stephan et al., 2010; Wang et al., 1998). Stephan et al. (2010) were the first to describe the identification of *Cronobacter* with MALDI-TOF MS technique with development of a reference mass spectrometry library based on 54 *Cronobacter* strains and 17 *Enterobacter* species. With the same database, all *Cronobacter* species and non-*Cronobacter* species including phenotypically similar *E. helveticus*, *E. turicensis* and *E. pulveris* were unambiguously identified using MALDI-TOF MS with a high accuracy of 100% in the present work (Chapter 5). Moreover, instead of using pure cultures, Javůrková et al. (2012) spiked PIF products artificially with *Cronobacter* species and identified them correctly at genus level using MALDI BioTyper™ system (Bruker Daltonics, Germany). More recently, the potential of rapid taxonomic resolution for *Cronobacter* using MALDI-TOF MS has been expanded at biogroup level. Karamonová et al. (2012) created a protein peak pattern database for eight biogroups of *C. sakazakii* proposed by Iversen et al. (2008b) by analyzing 300 spectra of 19 *C. sakazakii* reference strains and 5 non-*Cronobacter Enterobacter* strains as negative controls. The validity of this protocol based on biogroup-specific patterns was successfully confirmed by using another ten non-identified strains and the results obtained using MALDI-TOF MS were in accordance with that using a series of reference biochemical tests. On the other hand, Cetinkaya et al. (2013) reported discrepancy on two *Cronobacter* isolates at species level between MALDI-TOF MS and 16S rRNA gene sequencing. It has been reported that peak patterns generated by mass spectrometry measurement may vary strongly with various culture and sample preparation conditions like growth time, cultivation media, solvent condition and extraction method, thereby affecting the identification result of MALDI-TOF MS (Wang et al., 1998). The results were more reproducible and the peaks were generated in a higher intensity and higher number by incubation on TSA for 48 h than on Brain Hearth Infusion Agar (BHA), Blood Agar and ESIA for 24 h (Karamonová et al., 2012; Stephan et al., 2010). Therefore to guarantee the accuracy and stability, it is paramount to standardize the sample preparation and incubation procedures. Importantly, the protein mass peaks represent complex and variable fingerprints

of peptides and small proteins extracted from the whole cell and the m/z value alone is not sufficient to allow an assignment of individual peaks to special molecules (Rupf et al., 2005). Identification of bacteria based on specific biomarkers should rely on known peptides, which indicates the importance of identifying unique conserved biomarkers instead of only observing spectral pattern difference for bacterial identification and differentiation (Wang et al., 1998). Limited database entries for specific proteins and peptides can be a crucial factor for incorrect bacterial identification with MALDI-TOF MS.

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Summary

Contaminated powered infant formula (PIF) has been identified to be linked with a number of clinical outbreaks of rare but life-threatening infections in infants associated with *Cronobacter*. The overall objective of this thesis is to evaluate the impact of microbiologically active substances in PIF on the growth and detection of *Cronobacter* spp. Depending on preparation, storage and feeding practices, a potential rapid growth of *Cronobacter* spp. in reconstituted PIF is possible. The first aim was to investigate the effectiveness of using hot water of 70°C or higher on inactivation of *Cronobacter* spp. during PIF rehydration. It was showed that PIF reconstituted with water of 80°C in plastic bottle caused a cell reduction of *Cronobacter* spp. of greater than 3 log and this might thus reduce substantially the risk of infection in infants. Although the effect of fermented infant formula (FIF) on suppression of *Cronobacter* spp. growth was shown to be partially offset by water of 80°C, this inhibitive effect based on the bioactive substances emerging after infant formula fermentation was not strong enough. It was assumed that a direct reconstitution of FIF with water of 80°C may lead to a greater inactivation of *Cronobacter* spp. and result in a reduction of potential infection risk in higher magnitude.

Incorporation of organic acids in PIF contributes to an alternative in controlling *Cronobacter* growth in rehydrated PIF. The present study first reported the synergistic effect of combination of infant formula moderately acidified with organic acid to pH 6.0 and physiological infant gastric acidity of pH 5.0: Under the simulated neonatal gastric acid condition, the slightly acidified infant formula which did not show inhibitive effect solely reduced significantly the *Cronobacter* population. Compared with other acidification interventions reported to date where a low pH of ≤ 5.0 was achieved, the acidified formula presented in this work is expected to improve the taste due to the natural pH value and yet preserve the bacteriostatic activity.

Microbiologically active substances present in infant formula product such competitive flora and/or antimicrobial components can inhibit the growth of *Cronobacter* and further affect the effectiveness of the detection method for *Cronobacter*. A new protocol for rapid detection of *Cronobacter* spp. from PIF and raw materials using impedance method combined with rRNA-based lateral flow assay has been evaluated, particularly on products/matrices with high amounts of competing background flora and on inhibitive specialised infant formulae. Compared with the reference method based on ISO/TS 22964, a higher sensitivity and specificity were observed, and the detection time was substantially reduced to 29 hours to obtain a final confirmation for *Cronobacter* in this new protocol whereas the standard protocol needs 6 days.

The present work has also represented a comparative assessment of various systems including chromogenic agars, FISH, real time PCR, 16S rRNA gene sequencing, API and MALDI-TOF MS for the detection and identification of *Cronobacter* spp.. While the phenotypic systems based on biochemical reactions gave false positive or false negative outcomes for *Cronobacter* spp., the molecular-based identification systems have shown an accuracy of 100% in differentiating *Cronobacter* from non-*Cronobacter* strains. Both 16S rRNA gene sequencing and MALDI-TOF MS assigned isolates to species level, but the identification of MALDI-TOF MS was more discriminating and unambiguous, with results provided more rapidly within a few minutes.

Zusammenfassung

Cronobacter verursacht bakterielle Infektionen mit nekrotisierenden Enterokolitiden, Meningitiden und Septikämien bei Neu- und Frühgeborenen, deren Ursprung in Verbindung mit kontaminierter Säuglingsnahrung gebracht wird. Ziel dieser Arbeit war es Nachweismethoden für *Cronobacter* zu vergleichen, um ein möglichst schnelles und sicheres Nachweissystem zu entwickeln. Darüber hinaus sollten Substanzen identifiziert werden, die das Wachstum von *Cronobacter* in der Säuglingsmilchnahrung reduzieren. Der Einfluß der Zubreitung, Lagerung und Handhabung der gefertigten Milchnahrung auf das Wachstum von *Cronobacter* wurde untersucht. Zunächst wurde Wasser mit einer Temperatur von 70°C oder höher zur Rekonstituierung der Trockenmilch verwendet und die Überlebensrate von *Cronobacter* geprüft. Die Ergebnisse zeigten, dass sich die Keimzahl von *Cronobacter* in Säuglingsnahrung, welche mit 80°C heißem Wasser in Babyflaschen aus Kunststoff zubereitet wurde, um mindestens drei Zehnerpotenzen verringerte. Somit könnte das Infektionsrisiko von *Cronobacter* durch die Verwendung von 80°C heißem Wasser und Kunststoffflaschen erheblich reduziert werden. Auch die mit 40°C warmen Wasser zubereitete fermentierte Säuglingsnahrung zeigte leicht reduziertes Wachstum von *Cronobacter*. Allerdings ist dieser Hemmungseffekt, der auf die nach der Fermentierung aufgetauchten bioaktiven Substanzen zurückzuführen ist, nicht stark genug. Es wird vermutet, dass eine Rekonstituierung der fermentierten Säuglingsnahrung mit 80°C heißem Wasser direkt zu einer stärkeren Verringerung der Keimzahl von *Cronobacter* führen und demzufolge das Infektionsrisiko im höheren Maße reduzieren könnte.

Die Supplementierung von organischen Säuren in Säuglingsnahrung stellt somit eine Option zur Limitierung des Wachstums von *Cronobacter* in rekonstitutierter Säuglingsnahrung dar. Die vorliegende Arbeit ist die erste Studie, die einen synergistischen pH-Effekt zur Wachstumshemmung von *Cronobacter* beschreibt, der durch die Zugabe von organische Säure zur Säuglingsnahrung auf einem pH-Wert von 6,0 und der physiologischen Azidität

von pH 5,0 im Säuglingsmagen berichtete. In dem simulierten Model der Säuglingsmagensäure wurde eine eindeutige Hemmwirkung gegen *Cronobacter* durch diese leicht angesäuerte Säuglingsnahrung gezeigt, die aber keine vollständige Inhibierung gegen das Wachstum von *Cronobacter* ermöglicht. Im Vergleich zu anderen Ansäuerungsinterventionen, bei denen die pH-Werte unter 5,0 erreicht wurden, ist bei der angesäuerten Säuglingsnahrung in der vorliegenden Arbeit eine Optimierung des sensorischen Geschmacks gleichzeitig ohne Verlust der Wachstumshemmung zu erwarten.

Mikrobiologisch aktive Substanzen in Säuglingsnahrung, zu denen auch die kompetitive Begleitflora und antimikrobielle Komponente gehören, können das Wachstum von *Cronobacter* inhibieren aber zudem die Effektivität der Nachweismethoden für *Cronobacter* beeinträchtigen. In diesem Zusammenhang wurde eine neue Methode zum Schnellnachweis von *Cronobacter* mittels Impedanz-Verfahren gekoppelt mit rRNA Lateral Flow Assay etabliert, insbesondere mit Produkten und Rohmaterialen mit einer hohen Anzahl von Begleitflora sowie mit speziellen Säuglingsnahrungen mit Hemmeffekt. Gegenüber der standardisierten konventionellen Methode ISO/TS 22964 zeigte die neue Methode in dieser Arbeit eine höhere Sensitivität und Selektivität. Bei dem neuen Testverfahren ließ sich die gesamte Dauer wesentlich auf 29 Stunden verkürzen, während die klassische Methode sechs Tage brauchte, bis ein Bestätigungsergebnis für diejenige Probe, die verdächtigt positiv für *Cronobacter* war, vorlag.

In dieser Arbeit wurden vielfältige Isolate, die zum Genus *Cronobacter* sowie zur nicht-*Cronobacter* Enterobacteriaceae-Familie gehören, vergleichend mit verschiedenen Testsystemen einschließlich chromogener Media, Fluorescenz in situ Hydridisierung (FISH), real time PCR, 16S rRNA Gensequenzierung, API und MALDI-TOF MS analysiert. Während die phänotypischen Methoden, deren Prinzipien auf biochemische Reaktionen basieren, falsch positive oder falsch negative Resultate für die Identifizierung der *Cronobacter* lieferten, zeigten die molekularbiologischen Identifizierungssysteme eine Genauigkeit von 100% in der Differenzierung zwischen *Cronobacter* und nicht-*Cronobacter*

Isolaten. Eine Identifizierung auf Artebene war sowohl mit der 16S rRNA Gensequenzierung als auch mit der MALDI-TOF MS-Methode möglich. Im Vergleich zu 16S rRNA Gensequenzierung waren die Ergebnisse, die von der MALDI-TOF MS-Methode geliefert wurden, diskriminierender und eindeutiger. Außerdem zeichnet sich diese neue Technologie durch einen hohen Durchsatz und eine Schnelligkeit für die Bakterienidentifizierung in wenigen Minuten aus.

Addendum

Poster: Detection of *Cronobacter* spp. with different standard methods

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An International Meeting on *Cronobacter*, University College Dublin, Dublin, Ireland, 2009

Detection of *Cronobacter spp.* with different standard methods

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CLF Analytical Services for uncompromising Food Safety

Introduction

Cronobacter (C.) spp. is an important opportunistic pathogen which has been isolated from a variety of foods and is thought to be common in the environment. Occasionally found as contaminant of powdered infant formula (PIF), this organism is associated in neonatal and infant cases of meningitis, septicemia and necrotizing enterocolitis. This has brought the organism into the focus of food microbiology over the recent years and a number of standard methods based on chromogenic agars and molecular biological methods have been developed.

The topic of the current investigations was to compare the different approaches in terms of sensitivity and specificity. This has been done especially against the background of the species *Enterobacter sakazakii* recently being renamed to *Cronobacter spp.* and showing a broader diversity now. We have included several *Cronobacter* species in the study to investigate differences in the detection ability of standard methods.

Materials and Methods

Bacterial strains: *Enterobacter sakazakii* (CC 208, 320, 553, 1549, 1552, 2136, 2326, 2498), *Enterobacter asburiae* (CC 135), *Escherichia vulneris* (CC 204), *Leclercia adecarboxylata* (CC 770), *Cronobacter muytjensii* (CC 2682)*, *Cronobacter turicensis* (CC 2683)*, *Cronobacter genomospecies* (CC 2684)*, *Cronobacter dublinensis subsp. lausannensis* (CC 2687)*, *Cronobacter sakazakii* (CC 2688)*, *Cronobacter malonaticus* (CC 2689)*, *Cronobacter dublinensis subsp. lactaridi* (CC 2691), *Enterobacter pulveris* (CC 2685)*, *Enterobacter helveticus* (CC 2686)* and *Enterobacter turicensis* (CC 2690)*.

Chromogenic media: ESIA (AES CHEMUNEX), Compass (Doenitz Prolab), ChromoCult (MERCK), DFI (OXOID), ESPM/ChromID E.sakazakii (Biomerieux) and HarlequinT (Lab M)

FISH (Fluorescence in situ hybridisation): VIT-*Enterobacter sakazakii* (Vermicon)

Real time PCR: StarPrep One Kit, foodproof® *Enterobacteriaceae* plus *E.sakazakii* Detection Kit – TqM Probes, Reagent D (BIOTECON Diagnostics)

Biochemical identification: API 32E (Biomérieux)

Results

NAME [a]	ID 32E	API Profile	real time PCR	FISH
Enterobacter sakazakii (CC 208)	Enterobacter sakazakii 99.9%. T=0.92	3427675?050	E.sakazakii	positive
Enterobacter sakazakii (CC 320)	Enterobacter sakazakii 99.9%. T=1.00	3427675?250	E.sakazakii	positive
Enterobacter sakazakii (CC 553)	Enterobacter sakazakii 99.9%. T=1.00	3427675?250	E.sakazakii	positive
Enterobacter sakazakii (CC 1549)[b]	Enterobacter sakazakii 97.4%. T=0.18	3427677?050	EB	negative
Enterobacter sakazakii (CC 1552)	Enterobacter sakazakii 99.9%. T=0.85	7527675?050	E.sakazakii	positive
Enterobacter sakazakii (CC 2136)[c]	Enterobacter sakazakii 99.9%. T=0.71	3427656?050	EB	negative
Enterobacter sakazakii (CC 2326)	Enterobacter sakazakii 99.9%. T=1.00	3427675?250	E.sakazakii	positive
Enterobacter sakazakii (CC 2498)	Enterobacter sakazakii 99.9%. T=0.92	3427675?050	E.sakazakii	positive
Cronobacter muytjensii (CC 2682)	Enterobacter sakazakii 99.9%. T=0.58	3427776?051	E.sakazakii	positive
Cronobacter turicensis (CC 2683)	Enterobacter sakazakii 99.9%. T=0.45	7427675?251	E.sakazakii	positive
Cronobacter genomospecies (CC2684)	Enterobacter sakazaki 99.9%. T=0.53	3407776?251	E.sakazakii	positive
Enterobacter pulveris (CC 2685)	Enterobacter sakazakii 99.9%. T=0.28	3447673?050	EB	negative
Cronobacter dublinensis subsp. lausannensis (CC2687)	Enterobacter sakazakii 99.9%. T=0.75	3427576?250	E.sakazakii	positive
Cronobacter sakazakii (CC 2688)	Enterobacter sakazakii 99.9%. T=1.00	3423675?250	E.sakazakii	positive
Cronobacter malonaticus (CC 2689)	Enterobacter sakazakii 99.9%. T=0.68	3407676?251	E.sakazakii	positive
Cronobacter dublinensis subsp. lactaridi (CC 2691)	Enterobacter sakazakii 99.9%. T=0.83.	3427776?250	E.sakazakii	positive
Enterobacter helveticus (CC 2686)	Escherichia vulneris 96.1%. T=0.25	3447456?051	EB	negative
Enterobacter turicensis (CC2690)	Escherichia vulneris 93.2%. T=0.41	4474563?11	EB	negative
Enterobacter asburiae (CC 135)	Escherichia vulneris 98.1%. T=0.87	4474543?51	EB	negative
Leclercia adecarboxylata (CC 770)	Leclercia adecarboxylata 99.9%. T=1.0	4475753111	EB	negative
Pantoea (CC 197)	Pantoea spp 1 99.9%. T=0.97	74703001	EB	negative

Table 1: Comparison of confirmation methods for *Cronobacter spp.*
a: *E. sakazakii* designation according to ID 32E. *b,c*: cc1549 and cc2136 were identified as *E. pulveris* and *E. helveticus* by 16S rRNA sequencing

In this study biochemical (API) and molecular biological methods such as Real time PCR and FISH were applied to identify *Cronobacter spp.* strains (tab.1).
Six chromogenic agars were compared for their ability to recover *Cronobacter spp.* The media ESIA, DFI, Compass and HarlequinT have shown identical results for Cronobacter species. ChromoCult and ESPM have displayed deviating results (fig.1, tab.2).

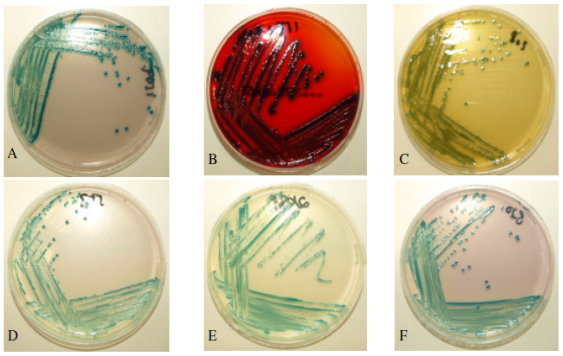

Figure 1: Appearance of *Cronobacter sakazakii* on chromogenic media (A: Compass; B: ESPM; C: DFI; D: HarlequinT; E: ChromoCult; F: ESIA)

Species	ESIA	Compass	HarlequinT	ChromoCult	DFI	ESPM	Reference methods *Cronobacter*
Enterobacter sakazakii (CC 208)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 320)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 553)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 1552)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 2326)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 3498)	+	+	+	+	+	+	positive
Cronobacter muytjensii (CC 2682)	+	+	+	+	+	+	positive
Cronobacter turicensis (CC 2683)	+	+	+	+	+	+	positive
Cronobacter genomospecies (CC 2684)	+	+	+	+	+	+	positive
Cronobacter dublinensis subsp. lausannensis (CC 2687)	+	+	+	+	+	+	positive
Cronobacter sakazakii (CC 2688)	+	+	+	+	+	+	positive
Cronobacter malonaticus (CC 2689)	+	+	+	-	+	+	positive
Cronobacter dublinensis subsp. lactaridi (CC 2691)	+	+	+	+	+	+	positive
Enterobacter pulveris (CC 2685)	+	+	+	+	+	-	negative
Enterobacter helveticus (CC 2686)	+	-	+	-	+	+	negative
Enterobacter turicensis (CC 2690)	-	-	-	-	+	+	negative
Enterobacter sakazakii (CC 1549)	+	+	+	-	+	-	negative
Enterobacter sakazakii (CC 2136)	+	+	+	-	+	+	negative
Enterobacter asburiae (CC 135)	-	-	-	-	-	-	negative
Escherichia vulneris (CC 204)	-	-	-	-	-	-	negative
Leclercia adecarboxylata (CC 770)	-	-	-	-	-	-	negative
Pantoea (CC 197)	-	-	-	-	-	-	negative
Specificity against PCR/FISH as reference method	56%	67%	56%	89%	44%	67%	
Sensitivity against PCR/FISH as reference method	100%	100%	100%	92%	100%	100%	

Table 2: Sensitivity and specificity of different chromogenic *Cronobacter* media

Discussion

The comparison of detection methods has demonstrated that in line with the new taxonomy the molecular biological techniques are able to distinguish between the members of the new genus *Cronobacter* and those *Enterobacter* spp. which belonged before to the *Enterobacter sakazakii* group and was dropped out now.
However the new established genus *Cronobacter* is rather inhomogenous, therefore neither the molecular techniques nor the classical microbiological methods are able to detect 100 % of the included species. The ChromoCult agar shows the lowest sensitivity, which means that 92 % of the confirmed *Cronobacter* species could be detected.

Literature:

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Restaino, L. et al (2006) J Food Prot. 69 (2), 315-322
Iversen, C. et al. (2006) BMC Microbiology. 6:94
*The *C. spp* strains have been gratefully provided by C. Iversen UCD

An International Meeting on Cronobacter (*Enterobacter sakazakii*) 2009, University College Dublin, Ireland

Table 1. Comparison of confirmation methods for *Cronobacter* spp.

NAME [a]	ID 32E	API Profile	real time PCR	FISH
Enterobacter sakazakii (CC 208)	Enterobacter sakazakii 99.9%. T=0.92	34276767050	E.sakazakii	positive
Enterobacter sakazakii (CC 320)	Enterobacter sakazakii 99.9%. T=1.00	34276763250	E.sakazakii	positive
Enterobacter sakazakii (CC 553)	Enterobacter sakazakii 99.9%. T=1.00	34276767250	E.sakazakii	positive
Enterobacter sakazakii (CC 1549) [b]	Enterobacter sakazakii 97.4%. T=0.18	34276773050	EB	negative
Enterobacter sakazakii (CC 1552)	Enterobacter sakazakii 99.9%. T=0.85	75276767050	E.sakazakii	positive
Enterobacter sakazakii (CC 2136) [c]	Enterobacter sakazakii 99.9%. T=0.71	34276563050	EB	negative
Enterobacter sakazakii (CC 2326)	Enterobacter sakazakii 99.9%. T=1.00	34276767250	E.sakazakii	positive
Enterobacter sakazakii (CC 2498)	Enterobacter sakazakii 99.9%. T=0.92	34276767050	E.sakazakii	positive
Cronobacter muytjensii (CC 2682)	Enterobacter sakazakii 99.9%. T=0.58	34277767051	E.sakazakii	positive
Cronobacter turicensis (CC 2683)	Enterobacter sakazakii 99.9%. T=0.45	74276767251	E.sakazakii	positive
Cronobacter genomospecies (CC2684)	Enterobacter sakazakii 99.9%. T=0.53	34077767251	E.sakazakii	positive
Enterobacter pulveris (CC 2685)	Enterobacter sakazakii 99.9%. T=0.28	34476773050	EB	negative
Cronobacter dublinensis subsp. lausannensis (CC2687)	Enterobacter sakazakii 99.9%. T=0.75	34275763250	E.sakazakii	positive
Cronobacter sakazakii (CC 2688)	Enterobacter sakazakii 99.9%. T=1.00	34236767250	E.sakazakii	positive
Cronobacter malonaticus (CC 2689)	Enterobacter sakazakii 99.9%. T=0.68	34076763251	E.sakazakii	positive
Cronobacter dublinensis subsp. lactaridi (CC 2691)	Enterobacter sakazakii 99.9%. T=0.83.	34277767250	E.sakazakii	positive
Enterobacter helveticus (CC 2686)	Escherichia vulneris 96.1%. T=0.25	34474561051	EB	negative
Enterobacter turicensis (CC2690)	Escherichia vulneris 83.2%. T=0.41	4474563011	EB	negative
Enterobacter asburiae (CC 135)	Escherichia vulneris 98.1%. T=0.87	4474543051	EB	negative
Leclercia adecarboxylata (CC 770)	Leclercia adecarboxylata 99.9%. T=1.0	4475753111	EB	negative
Pantoea (CC 197)	Pantoea spp 1 99.9%. T=0.97	74703001	EB	negative

[a]: *E. sakazakii* designation according to ID 32E. [b] [c]: cc1549 and cc2136 were identified as *E. pulveris* and *E. helveticus* by 16S rRNA gene sequencing.

Table 2. Sensitivity and specificity of different chromogenic *Cronobacter* media

Species	ESIA	Compass	HarlequinT	ChromoCult	DFI	ESPM	Reference methods *Cronobacter*
Enterobacter sakazakii (CC 208)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 320)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 553)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 1552)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 2326)	+	+	+	+	+	+	positive
Enterobacter sakazakii (CC 2498)	+	+	+	+	+	+	positive
Cronobacter muytjensii (CC 2682)	+	+	+	+	+	+	positive
Cronobacter turicensis (CC 2683)	+	+	+	+	+	+	positive
Cronobacter genomospecies (CC 2684)	+	+	+	+	+	+	positive
Cronobacter dublinensis subsp. lausannensis (CC 2687)	+	+	+	+	+	+	positive
Cronobacter sakazakii (CC 2688)	+	+	+	+	+	+	positive
Cronobacter malonaticus (CC 2689)	+	+	+	-	+	+	positive
Cronobacter dublinensis subsp. lactaridi (CC 2691)	+	+	+	+	+	+	positive
Enterobacter pulveris (CC 2685)	+	+	+	+	+	-	negative
Enterobacter helveticus (CC 2686)	+	-	+	-	+	+	negative
Enterobacter turicensis (CC 2690)	-	-	-	-	+	+	negative
Enteroacter sakazakii (CC 1549)	+	+	+	-	+	-	negative
Enteroacter sakazakii (CC 2136)	+	+	+	-	+	+	negative
Enterobacter asburiae (CC 135)	-	-	-	-	-	-	negative
Escherichia vulneris (CC 204)	-	-	-	-	-	-	negative
Leclercia adecarboxylata (CC 770)	-	-	-	-	-	-	negative
Pantoea (CC 197)	-	-	-	-	-	-	negative
Specificity against PCR/FISH as reference method	56%	67%	56%	89%	44%	67%	
Sensitivity against PCR/FISH as reference method	100%	100%	100%	92%	100%	100%	

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Es ist wahr. Ohne Euch hätte ich die Arbeit nicht schaffen können.

Vielen Dank!

谢谢!

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

Eidesstattliche Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die ich wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Frankfurt am Main, den 01. August 2013

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