NOTTINGHAM TRENT UNIVERSITY

GENOTYPIC AND PHENOTYPIC DIVERSITY OF *CRONOBACTER* SPECIES FROM FOOD AND ENVIRONMENTAL SOURCES

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DECLARATION

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PUBLICATIONS

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Abstract.

The genus *Cronobacter* includes food-borne pathogens causing neonatal infections such as meningitis, and necrotizing enterocolitis as well as bacteraemia in immunocompromised adults. Understanding the molecular characterisation, clonality and phenotypic diversity of *Cronobacter* species is essential to reduce the source of microbial contamination of powdered infant formula (PIF) and other food. Therefore, an improved understanding of the diversity of the genus is warranted.

In the first part of the study, the 7-loci Multilocus sequence typing (MLST) scheme was applied to investigate the diversity of *Cronobacter* spp. isolated from food and environmental sources. Twenty-six strains that had not previously been profiled were divided into 21 sequence type (STs), and 9 new STs were identified, which had not been previously reported. *Cronobacter* strains isolated from food and environmental sources were highly diverse with respect to their ST, particularly those from different sources of food.

This study is the first to describe the development and application of variable number tandem repeat analysis (VNTRA) typing method for *C. sakazakii* ST4 strains. Nineteen *C. sakazakii* ST4 strains, which were widely distributed geographically, temporally and origin of source were profiled. These strains were divided into 15 distinct groups based on the number of tandem repeats of 6 VNTR loci. It was concluded that VNTRA profiling could contribute to further understanding of *C. sakazakii* ST4 diversity and tracking of infection sources. Of particular interest in this research was the finding that the analysis of the lipopolysaccharide (LPS) profiling using BioNumerics software, (version 7.1) showed great ability to discriminate between strains within the same serotype. Furthermore, the present study developed a multiplex PCR assay targeting capsular polysaccharide genes such as *kpsS* (K1 and K2) and *galE* (CA1 and CA2) for the specific detection and rapid identification of K-capsule type and colanic acid type respectively.

Another important finding was that a strong correlation between the amount of mucoid production, type and ratio of monosaccharides production and type of O-antigen serotype. This study indicated also that rhamnose is the main sugar in *C. sakazakii* serotype O:2 strain. The most interesting observation was that *C. sakazakii* strains with serotype O:1 and O:4 had high numbers of sublethally injured cells after desiccation, while strains with serotype O:2 and O:3 showed low numbers of sublethally injured cells. *C. sakazakii* strains showed a higher survival rate after the exposure to drying (90 days) than other *Cronobacter* species. Strains (6 of 54) containing the thermotolerance genomic island tended to survive better at 58°C than other strains. In general, *C. sakazakii* strains were much more resistant than other *Cronobacter* species to environmental stresses such as desiccation, long-term drying and heat. This might explain why *C. sakazakii* strains are associated with PIF, milk powder and dry powdered foods, and more frequently isolated than other *Cronobacter* species.

This study analysed multiple methods for typing *Cronobacter* at the species and strain level, and showed different discriminatory powers. The present study recommended that using a combination of genomic cluster, chromogenic agar (DFI), and sialic acid, malonate, indole and inositol utilisation tests can be useful tools to distinguish the seven species of *Cronobacter*, distinguish pathogenic *C.* ST7 from other *C. malonaticus* STs, as well as to characterize and distinguish *C. dublinensis* strains to the subspecies level. The present study is an important contribution to the understanding of the diversity and characteristics of the *Cronobacter* genus

using different typing methods, which is essential to reduce the risk of contaminations for the food products, in particular baby food such as PIF, milk powder and weaning food.

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List of abbreviation.

ABC ATP binding cassette

ATP Adenosine triphosphate

APS Ammonium persulphate

ANI Average nucleotide identity

BLAST Basic local alignment search tool

BPW Buffered peptone water

CA Colanic acid

CAC Codex Alimentarius Commission

CC Clonal complex

CDC Centres for disease control and prevention

CFU Colony forming units

CGH Comparative genomic hybridization

COG-MLST Clusters of orthologous genes- Multilocus sequence analysis

COSHH Control of substances hazardous to health

CPS Polysaccharide capsules

CRISPR Clustered regularly interspaced short palindromic repeats

CSF Cerebrospinal fluid

DDH DNA-DNA hybridization

DFI Druggan-Forsythe-Iversen

DNA Deoxyribonucleic acid

EDTA Ethylenediamine tetra-acetic acid

EE Enterobacteriaceae enrichment

FAO Food and Agriculture Organization of the UN

FDA Food and Drug Administration

LPS Lipopolysaccharide

LBA Luria-Bertani agar

ICMSF International Commission for Microbiological Specification for Foods

ISO International Organization for Standardization

ITS Internal transcribed spacer

KDa Kilodalton

MEGA Molecular Evolutionary Genetics Analysis

MLSA Multilocus sequence analysis

MLST Multilocus sequence typing

MLVA Multi-locus variable-number tandem-repeat analysis

NCBI National Centre for Biotechnology Information

NEC Necrotising enterocolitis

NGS Next generation sequencing

NMEC Neonatal meningitis *Escherichia coli*

NICU Neonatal intensive care unit

NMR Nuclear magnetic resonance

NTU Nottingham Trent University

OD Optical density

O-LPS Oligo-lipopolysaccharide

OM Outer membrane

OMP Outer membrane proteins

ORF Open reading frame

PBS Phosphate buffered saline

PCA Plate count agar

PCR Polymerase chain reaction

PFGE Pulsed field gel electrophoresis

PIF Powdered infant formula

RAPD Random amplification of polymorphic DNA

RFLP Restriction fragment length polymorphism

rRNA Ribosomal ribonucleic acid

r-MLST Ribosomal Multilocus sequence typing

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel -electrophoresis

SLV Single locus variant

SNP Single nucleotide polymorphism

ST Sequence type

TRs Tandem repeats

TSA Trypticase soy agar

TSB Trypticase soy broth

UV Ultraviolet

VNTR Variable number tandem repeat

VRBLA Violet red bile lactose agar

VRBGA Violet red bile glucose agar

WHO World Health Organization

WGS Whole genome sequencing

XLD Xylose lysine deoxycholate agar

TEMED N,N,N'N'-tetramethyl-ethylenedamine

Chapter 1. General Introduction.

1.1 *Cronobacter* genus taxonomy.

Cronobacter are opportunistic pathogens which can be isolated from a wide range of foods and environmental sources (Iversen et al. 2004; Osaili and Forsythe, 2009; Jackson et al. 2015). This genus is associated with the ingestion of contaminated powdered infant formula (PIF), causing necrotizing enterocolitis, sepsis and meningitis in neonatal infants. The *Cronobacter* genus is composed of Gram-negative, rod-shaped, in general motile, non-spore forming, facultative anaerobic bacteria that belong to the *Enterobacteriaceae* family and to the *Gammaproteobacteria* class. They are related closely to *Citrobacter* and *Enterobacter* genera (Iversen et al. 2008). *Cronobacter* was formerly known as yellow-pigmented *Enterobacter cloacae*. They are catalase positive, oxidase negative and positive for α-D- glucosidase, methyl red negative and able to reduce nitrate to nitrite (Iversen et al. 2008). It was initially designated as a unique species in 1980 (Farmer et al. 1980), and has subsequently been further investigated (Iversen et al. 2007; 2008) to clarify its taxonomy. This is now a new genus consisting of 7 species; *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis, C. condimenti* and *C. universalis* (Figure 1.1) (Iversen et al. 2008; Joseph et al. 2012d).

The initial naming of *Cronobacter* was *Enterobacter sakazakii.* It was distinguished from *E. cloacae* based on DNA-DNA hybridisation (DDH), phenotypic studies, and antimicrobial resistance (Farmer et al. 1980). The name of *E. sakazakii* being derived from the Japanese bacteriologist Riichi Sakazaki. Additional phenotypic studies led to the description of 15 *E. sakazakii* biogroups, and the biotype 1 was the most common (Farmer et al. 1980). Iversen and Forsythe in 2004 using 16s rDNA and *hsp*60 gene sequences analysed the phylogenetic relationship of *E. sakazakii* and *Citrobacte*r. This study identified 4 distinct clusters within *E. sakazakii* each cluster representing a potential novel species (Iversen et al. 2004). Further analysis by Iversen and colleagues in 2006 have studied the relationship between this genotypic clustering and the biogroups identified by Farmer et al (1980). Nevertheless, full taxonomic revision required considerable further analysis for substantiation. Upon detailed analysis, using genotypic analysis such as Amplified Fragment Length Polymorphisms, 16S rDNA sequence analysis, DDH, ribotyping and phenotypic analysis, the *Cronobacter* genus

was defined first in 2007 and revised in 2008 (Iversen et al. 2007; Iversen et al. 2008). Their study identified six *Cronobacter* species, which are *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis* and *C.* genomospecies 1 (Iversen et al. 2008). As a result of the high genetic similarity of *Cronobacter* species, it was difficult to distinguish between *C. sakazakii* and *C. malonaticus* based on the routine 16S sequence analysis. Baldwin and colleagues (2009) described a Multilocus Sequence Typing (MLST) scheme using 7 housekeeping genes (concatenated sequence length of 3036 bp). These 7 genes are include ATP synthase b chain (*atpD*), elongation factor G (*fusA*), glutaminyl tRNA synthetase (*glnS*), glutamate synthase large subunit (*gltB*), DNA gyrase subunit B (*gyrB*), translation initiation factor IF-2 (*infB*) and phosphoenolpyruvate synthase A (*ppsA*) to discriminate species within the *Cronobacter* genus (Baldwin et al. 2009). The 7-loci MLST scheme is more discriminatory and robust than 16S sequence analysis. It has revealed a stable clonal nature of the virulent *C. sakazakii* strains. Moreover, this approach was also used in the later description of the 2 new *Cronobacter* species *C. universalis* and *C. condimenti* (Joseph et al. 2012). The MLST scheme has so far identified more than 470 sequence types in the *Cronobacter* genus across over 1700 strains (http://pubmlst.org/*cronobacter*/). The site is open access and contains the MLST protocols.

Brady and colleagues in 2013 proposed that the 3 new species as *C. pulveris*, *C. helveticus* and *C. zurichensis* should be included into *Cronobacter* species. Follow up studies by Stephan et al (2014) proposed that the 3 new species *C. pulveris*, *C. helveticus* and *C. zurichensis* should constitute 2 new genera which are *Franconibacte*r and *Siccibacter.* Moreover, Forsythe et al (2014) reported that these 3 species do not cluster with other 7 *Cronobacter* species (Figure 1.1).

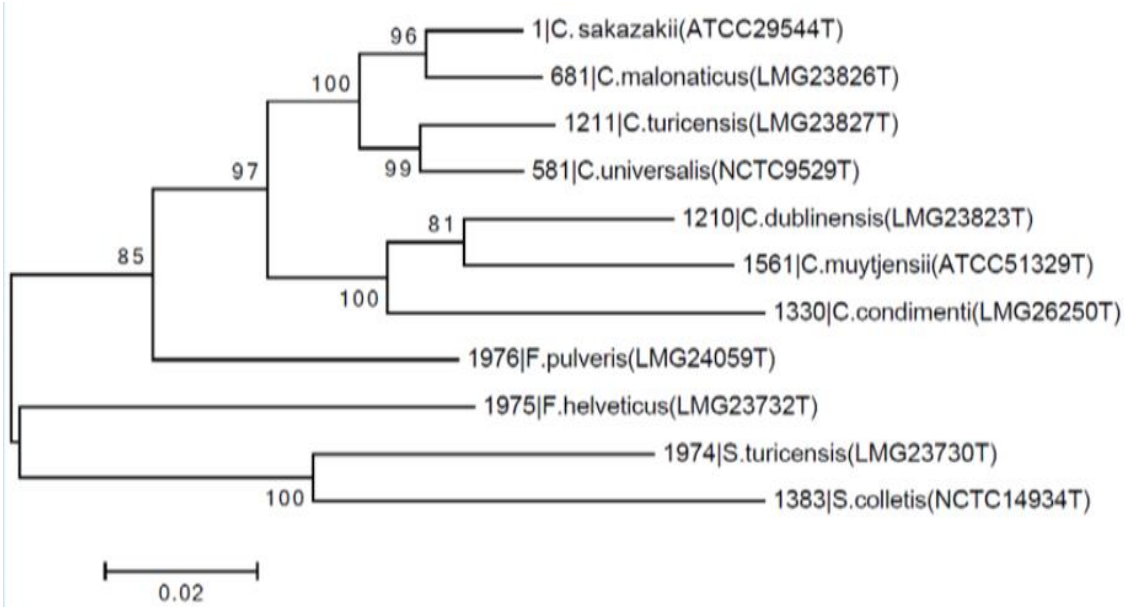


Figure 1.1. Maximum likehood tree of MLST loci (concatenated length 3036 base pair) of *Cronobacter* type strains and related *Enterobacteriaceae* (*Franconibacter* and *Siccibacter*) genera. The tree is drawn to scale using MEGA5.

1.2 Sources of *Cronobacter* spp.

1.2.1 Non-human sources of *Cronobacter* spp.

The genus *Cronobacter* has been isolated from a wide range of food such as PIF, dried milk products, weaning foods, powdered ingredients, processed milk products, many types of spices, herbs, cereals, cheese products, meat, and ready-to-eat products such as vegetables, fruits and salads (Iversen and Forsythe, 2004; Jackson and Forsythe. 2016). In addition, *Cronobacter* spp. are frequently isolated from environmental samples including thermal spring water, soil, grass silage, and dust from households (Iversen et al. 2007; Holy and Forsythe, 2014). Furthermore, this genus has been isolated from the marine environment (Agogue et al. 2005). Moreover, *Cronobacter* spp. has been isolated from food production environments such as PIF manufacturing factories which include floors, bays, roller dryers, tankers and air filters (Hein et al. 2009; Craven et al. 2010; Jacobs et al. 2011; Sonbol et al. 2013).

Cronobacter strains have been isolated from the gut of Mexican fruit flies and wild house flies (Kuzina et al. 2001; Mramba et al. 2006; Butler et al. 2010). In addition, rats and cockroaches may be other sources of contamination (Holy and Forsythe, 2014). The *Cronobacter* MLST database collection has more than 75% isolate as non-clinical strains (http://pubmlst.org/cronobacter//.

1.2.2 Human sources of *Cronobacter* spp.

Cronobacter spp. have been isolated from blood, cerebrospinal fluid (CSF), bone marrow, skin, trachea, urine, saliva, teeth, faeces and breast milk. *Cronobacter* species have also been isolated from wound infections, oral pharynx, pooled plaque and oral rinse (Farmer et al. 1980; Muytjens et al. 1983; Iversen et al. 2006; Caubilla-Barron et al. 2007). In addition, *Cronobacter malonaticus* has been isolated from a breast abscess and found in neonatal enteral feeding tubes in intensive care units (Hurrell et al. 2009b; Jackson et al. 2014).

1.3 *Cronobacter* physiology.

The optimal temperature for *Cronobacter* spp. growth is between 37°C to 39°C. In addition, *Cronobacter* spp. can grow over a wide range of temperatures, the lowest growth temperature is about 5°C and the highest growth temperature is about 47°C (Iversen et al. 2004; Huertas et al. 2015). Huertas and colleagues in 2015 reported that there were variations in heat tolerance between *Cronobacter* strains, and there is no *Cronobacter* species more thermotolerant than another. *Cronobacter* genus is one of the most thermotolerant members of the *Enterobacteriaceae* found in dairy products, and this property contributes to their survival in PIF and dried milk products. Moreover, resistance of heat is an important factor for survival of *Cronobacter* spp. in a wide range of food (Dancer et al. 2009; Huertas et al. 2015). Furthermore, *Cronobacter* spp. has been reported as growing in reconstituted PIF between 6°C to 45°C. Huertas et al (2015) reported that *C. sakazakii* strains can survive for long periods after inoculated reconstituted PIF at varying water temperatures 50, 55, 60, 65 and 70°C, and were capable of proliferating after reconstitution. The WHO (2007) recommended that PIF should be reconstituted with water at temperatures more than 70°C, in order to reduce *Cronobacter* risks. Oriešková and colleagues (2013) reported that some *Cronobacte*r strains contain a genomic island. The thermotolerance marker is encoded by a gene localised on a genomic island, which contains a cluster of conserved genes encoding several stress response protein. The genomic island locus consists of two loci, large version (18 kbp) containing the *thrB-Q* genes and short loci (6 kbp) containing only the *thrBCD* and *thrOP* genes. This locus could be responsible for increased heat tolerance in *Cronobacter* strains (Oriešková et al. 2015; Oriešková et al. 2016).

Overcoming drying stress is very important for the survival of *Cronobacter* species in PIF and dried milk products. Dancer et al (2009) reported that *Cronobacter* species have been recognised to be remarkably resistant to environmental stresses such as osmotic stress and drying when tested in stationary phase (Breeuwer et al. 2003; Dancer et al. 2009; Feeney et al. 2014). Breeuwer et al (2003) stated that the *Cronobacter* species were more resistant to drying than other *Enterobacteriaceae* members. Moreover, Fakruddin et al (2014) reported that *C. sakazakii* strains grown and dried in infant formula showed significantly ($P < 0.05$) better survival during drying than when grown and dried in TSB. Moreover, Kent et al (2015) reported that *Cronobacter* spp. are resistant to desiccation over a varied range of water activities $_{(aw)}$ = 0.25 to 0.86, and were able to survive better in dried formula with a water activity of 0.25-0.30 than at 0.69-0.82 over a one-year storage time. In addition, some of *Cronobacter* strains are able to survive about 2 years dried in infant formula and then grow rapidly on rehydration (Caubilla-Barron et al. 2007; Kent et al. 2015).

Most of *Cronobacter* strains have been reported as moderately resistant to acidic conditions down to pH 3.0. Moreover, Kim and Beuchat (2005) reported that *Cronobacter* strains are able to survive in several acidic vegetables and fruits for up to 48 hours at 25^{o}C (Kim and Beuchat, 2005). However, in recent times acids are key factors in food preservation, many studies were focused on the use of acids as antimicrobial agents against *Cronobacter* species, in particular in baby food products such as milk powder and weaning foods (Back et al. 2009).

The capsule is considered a virulence factor because it enhances the ability of bacteria to cause disease, and may enable the organism to resist desiccation and could facilitate the organism's attachment to plant surfaces (Hurrell et al. 2009). The majority of *Cronobacter* strains are able to produce capsules on milk agar plates. *Cronobacter* species are able to adhere to silicone, stainless steel, plastic surfaces, polycarbonate and glass (Iversen et al. 2004). These materials are usually used for food processing equipment and infant-feeding preparation, which if they are contaminated, may lead to increased risk of infection. Furthermore, Hurrel et al. (2009a) reported that capsule formation is also involved in formation of biofilms which have been detected in enteral feeding tubes of neonates in neonatal intensive care units (NICU),

as well as to contribute to persistence of the pathogen on food contact surfaces (Iversen et al. 2004d).

The polysaccharide capsules (CPS) are majar bacterial virulence factors and environmental fitness traits (Willis et al. 2013; Ogrodzki and Forsythe, 2015). This might explain the survival of the organism in starch, which is a main component of products such as PIF. Capsular polysaccharide genes such as K-capsule type gene cluster of *Cronobacter* consists of three regions. The K-antigen Region 1 including *kpsEDCS* genes, Region 3 including *kpsTM* and a variable Region 2 (K1 and K2) (Ogrodzki and Forsythe, 2015). Another bacterial capsular polysaccharide known as colanic acid (CA) is associated with bacterial protection against desiccation, extreme temperatures and acidic environmental conditions (Navasa et al. 2013). In *Cronobacter* the colanic acid encoding gene cluster was located close to the O-antigen region and separated by the *galF* gene (UDP-glucose pyrophosporylase), two variants in the colanic acid encoding gene cluster were found (CA1 and CA2). CA1 composed of 21 genes, while CA2 is composed of 20 genes, which differ in the presence of *galE* in CA1, and absence in CA2 (Ogrodzki and Forsythe, 2015).

Whole-genome analysis based on the K-antigen and colanic acid encoding genes showed that *C. sakazakii* and *C. malonaticus* strains, isolated from neonatal clinical cases of meningitis were capsular profile K2-CA2. While strains of *C. sakazakii* and *C. malonaticus* associated with less severe clinical cases tended to be capsular profile K1-CA1 (Ogrodzki and Forsythe, 2015).

1.4 *Cronobacter* and the food industry.

The genus *Cronobacter* has come to more attention of the food manufacturers and regulatory authorities because of its association with severe neonatal infections. The majority of neonatal infection cases can be attributed to the ingestion of contaminated reconstituted PIF.

1.4.1 Powdered infant formula.

Neonatal *Cronobacter* infections have been associated with consumption of reconstituted PIF. In many outbreak investigations, *Cronobacter* species have been isolated from PIF that may have been contaminated in the factory and other cases, *Cronobacter* might have contaminated the PIF after it was opened at home or in

another place. Muytjens et al (1988) reported that *Cronobacter* is associated with contaminated PIF, when it was isolated from prepared formula and reconstitution equipment. This report found that about 52.2% (n =141) of PIF samples from 35 countries contained *Enterobacteriaceae*, with about 14% containing *Cronobacter* species. The International Commission for Microbiological Specification for Foods (ICMSF, 2002) has ranked *Cronobacter* spp. as "Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration." Furthermore, after a number of reported cases and outbreaks of *Cronobacter* infections in neonates, the International Commission on Microbiological Specifications for Foods has recognized *Cronobacter* as an emergent issue for the immunocompromised (ICMSF, 2002). However, the Codex Alimentarius Commission (CAC) microbiological criteria of PIF were only changed in 2008 (FAO/WHO, 2008; Chap et al. 2009). The presence/absence testing of *Cronobacter* in 10g of PIF sample is now a CAC requirement.

1.4.2 Other sources related to food industry.

A number of studies reported that *Cronobacter* species can be isolated from different environments such as PIF and milk powder factories including floors, roofs, tanker bays, drying towers, roller dryers, conveyors, and air filters of industrial units (Hein et al. 2009; Craven et al. 2010; Jacobs et al. 2011). *Cronobacter* species can persist in these environments because of its ability to survive spray drying, desiccation and osmotic stress. Caubilla-Baron and Forsythe (2007) reported that *Cronobacter* species can be persist and survive more than 2 years in PIF. In addition, *Cronobacter* spp. have also been isolated from a wide range of foods such as cheese, meat, vegetables, grains, sorghum seeds, rice, herbs, spices, dry powdered, tea and other various food (Iversen et al. 2004). The ingestion of contaminated PIF is the main route of infant infection, and this has led to several studies on improved detection methods for the food industry.

1.5 *Cronobacter* outbreaks.

There are many *Cronobacter* outbreaks that have occurred across different parts of the world. The first outbreak occurred in the Netherlands, and was reported by Muytjens et al (1983). Eight infants had neonatal meningitis in this outbreak; most of them (five

cases) were from the same hospital and the other three cases were from different hospitals. Six of eight infants died, the infections were linked with the ingestion of contaminated infant formula (Muytjens et al. 1983).

The largest outbreak of *Cronobacter* infection occurred in a neonatal intensive care unit (NICU) in France in 1994, which resulted in the death of at least 3 neonates, one due to meningitis and other 2 from NEC. This outbreak was analysed by Caubilla-Barron et al (2007). This analysis covered the phenotypic and genotypic examination of the 31 *Cronobacter* strains.

The third outbreak of *Cronobacter* occurred in a NICU of Belgium in 2001, where 12 infants had NEC. This outbreak was reported by Van Acker and colleagues (2001). It was the first report that linked *Cronobacter* with NEC (Van Acker et al. 2001).

Another *Cronobacter* outbreak occurred in the same year (2001). This outbreak was in a hospital in Tennessee (USA), and was recorded by Himelright and colleagues (2002). As a result of meningitic infant's death, about 49 babies were tested, eight cases were found to be positive for *Cronobacter,* 6 of the 8 infants were asymptomatic, while 2 babies had respiratory illnesses. This outbreak was also linked with formula milk product as a result of which a product recall was announced by the manufacturing company (Himelright et al. 2002).

In 2002, Block and colleagues reported another outbreak of *Cronobacter* in a hospital in Jerusalem. Five babies were positive for *Cronobacter*, 3 infants had bacteraemia and meningitis, and 2 remained asymptomatic. The investigation isolated the organisim from the hospital blender, which was used for preparing the infant formula, this isolate matched with the pulsetype of the infant isolates (Block et al. 2002).

In 2011, the Centres for Disease Control and Prevention (CDC) reported cases of *Cronobacter* species infection from four US states; Florida, Illinois, Missouri and Oklahoma, in which three of the infant cases were meningitic, 2 of which died (CDC, 2011). The investigation of strains showed that the organism was found in an opened PIF tin and opened nursery water bottle, used to prepare the PIF in Missouri, however in Illinois State, the organism was isolated from an opened nursery water bottle (CDC, 2011). These isolates were studied further by our group (Hariri et al. 2013). Fifteen

Cronobacter isolates from the CDC were analysed and found that most (14/15) strains were *C. sakazakii*, and one strain was *C. malonaticus*, and all five CSF isolates were either ST4 or within the ST4 complex, these isolates comprise the ST4 complex. This group included strains from cases during December in Illinois (strain 1577) and in Lebanon, Missouri (strain 1579). Strain isolated from the PIF reconstitution water and associated with the case reported in Illinois was ST111, and other two Illinois strains, 1577 and 1578 belonged to ST110 and ST111.

1.6 Identification and typing methods of *Cronobacter* spp.

1.6.1 Phenotypic and culture methods.

1.6.1.1 Culture methods.

The diversity of sources and high mortality rates of *Cronobacter* spp. has required the improvement and development of reliable detection and identification methods for the bacterium. Standard protocols for the isolation and identification of the bacterium from PIF, based on enrichment methods and biochemical characterization have been approved by the U.S Food and Drug Administration (FDA) (Chen et al. 2004). Many different microbial methods have been used to isolate, identify and distinguish *Cronobacter* species. Microbiological culturing method is normally used to detect and determine the degree of *Cronobacter* contamination in PIF, milk powder samples and in other foods. *Cronobacter* genus are associated with infants and neonatal infections including meningitis, necrotizing enterocolitis and bacteraemia (Hurrell et al. 2009; Hariri et al. 2013) as well as associated with bacteraemia in immunocompromised adults. This led to the development and improvement of specific detection and identification methods for *Cronobacter* spp.

Determining the morphology of colony growth on different media can be an important tool to describe a member of the *Cronobacter* genus. MacConkey agar is a selective culture medium designed to isolate Gram-negative bacteria based on lactose fermentation. Iversen and Forsythe (2003) reported that *Cronobacter* spp. might be of plant origin as a result of their physiological features such as production of a yellow pigment on trypticase soy agar, polysaccharide capsule production, and desiccation resistance. As a result of the unique feature of alpha-D-glucosidase enzyme activity of *Cronobacter* species, a new chromogenic medium (DFI) has been designed by Druggan,

Iversen and Forsythe, in order to differentiate and distinguish *Cronobacte*r species from other *Enterobacteriaceae* members. This media was more accurate when compared with other traditional *Enterobacteriaceae* enumeration agar such as VRBGA (Iversen et al. 2004). *Cronobacter* strains produce blue-green coloured colonies on DFI agar and other Gram-negative bacteria including *Enterobacteriaceae* produced different coloured colonies.

1.6.1.2 *Cronobacter* biochemical characteristics.

Biochemical tests have been used for identification of *Cronobacter* spp., and were also used in the taxonomic reclassification of the genus *Cronobacte*r. Farmer and colleagues (1980) reported that *Cronobacter* species possessed similar biochemical reactions to *E. cloacae* strains, but *Cronobacter* spp. was negative for D-sorbitol and produced yellow-pigmented colonies on TSA after 48h at 25°C. Another distinguishing property for *Cronobacter* species is Tween 80 esterase activity. This test was positive for most *Cronobacter* isolates (97.3%). Muytjens and colleagues in 1984 investigated the enzymatic profiles of 97 *Enterobacter* strains including *E. aerogenes, E. cloacae* and *E. agglomerans* strains, and 129 *Cronobacter* strains and determined the presence of α-glucosidase as a main difference between *Cronobacter* species and other *Enterobacter* species. The more popular biochemical tests kits are API20E, ID32E and VITEK 2.0 (Biomerieux). The biochemical tests are the important for the identification and differentiation of *Cronobacter* species include, indole production, malonate and inositol utilization and α-D- glucoside activity (blue-green colour) (Iversen et al. 2007). Furthermore, sialic acid utilization was stated as a significant biochemical test for the differentiation of *Cronobacter* species (Joseph et al. 2013).

1.6.2 Genotyping methods.

Understanding the molecular characterisation and genetic diversity of the *Cronobacter* genus is essential to ensure reliable detection and control methods are used. It also helps track the source of microbial contamination of PIF and other food.

1.6.2.1 Pulsed-field gel electrophoresis (PFGE).

The PFGE method was developed in 1984 and has become one of the important "gold standard" molecular typing methods. This method is one of the most highly discriminatory typing methods available, and one of the greatest reliable technique for

analysis of diversity of foodborne pathogens. For epidemiological and outbreak investigation, PFGE with two restriction enzymes (Xba1 and Spe1) is one of the most widely used method for subtyping of *Cronobacte*r species (Healy et al. 2008; Holy and Forsythe, 2014). Nevertheless, PFGE is not an ideal method for bacterial surveillance due to the expense of the equipment, requirement of highly trained technicians, time consuming, non-identical strains can give the same profile and limitation with clonal organisms (Holy and Forsythe, 2014). In contrast, previous studies reported that BOX-PCR results yielded 90-95% agreement with PFGE. In addition, RAPD-PCR and BOX-PCR fingerprinting technique allows for the typing of *Cronobacter* strains in a faster and easier way than PFGE (Proudy et al. 2008b). Moreover, the Centres for Disease Control and Prevention (CDC) is changing to bacterial typing using whole genome sequencing (WGS) is an alternative to PFGE, such as the Illumina high-throughput HiSeq, offering simpler workflows, high rate of resolution, and a universally applicable bacterial subtyping method (CDC, 2015).

1.6.2.2 Random amplified polymorphic DNA (RAPD) analysis.

The RAPD is a type of PCR reaction, however the fragments of DNA that are amplified are random. This assay is a powerful tool for genetic studies and is useful as a screening genotyping method (Leal et al. 2004). The RAPD-PCR technique is fast and simple compared with other fingerprinting methods such as PFGE and AFLP (Leal et al. 2004). Blixt and colleagues (2003) reported that the selection of a suitable primer and PCR conditions optimization are the main factor in RAPD-PCR analysis (Blixt et al. 2003). RAPD-PCR technique has been used to determine the genetic diversity of *Cronobacter* species from PIF (Ye et al. 2010). Moreover, RAPD-PCR methods showed a high discriminatory power compared with other typing methods such as ERIC-PCR (Ye et al. 2010).

1.6.2.3 BOX-polymerase chain reaction (BOX-PCR) technique.

The BOX-PCR is a genotyping method which targets the repetitive BOX sequences. It can be used as a genotyping method, in order to identify the points of contamination and investigate clonal persistence (Versalovic et al. 1991, 1994). Proudy et al (2008a) reported that the BOX-PCR genotyping is very useful method for tracing and investigating of *Cronobacte*r in PIF factories and to implement a series of other control measures to reduce the risk of *Cronobacter* species in the final product. Moreover,

Proudy and colleagues (2008b) stated that 92% of the BOX-PCR results were in agreement with PFGE results. Furthermore, the BOX-PCR typing technique is an accurate method for the discrimination of *Cronobacter* spp. and a quick way to identify the source of contamination (Proudy et al. 2008b).

1.6.2.4 16S rRNA gene sequence.

The gene codes for ribosomal ribonucleic acid (rRNA), and this rRNA in turn makes up part of the ribosome. It is present in nearly all bacteria, it is important for the survival of bacterial cells due to its involvement in protein synthesis, the function of the 16S rRNA gene over time has not changed. The 16S rRNA gene is highly conserved between different species of bacteria and archaea (Hillis et al. 1991). Therefore, the 16S rRNA gene amplification and sequencing is extensively used for identification and phylogenetic classification of bacteria species, and families (Stackebrandt and Goebel, 1994). Moreover, 16S rRNA gene sequence analysis has been used for supporting bacterial taxonomic groupings and identification of organism. Therefore, an extensive database called the Ribosomal Database Project has been established and is available for free online (http://rdp.cme.msu.edu/). The full-length of 16S rRNA gene sequence analysis (1500 bp) should be used for phylogenetic bacterial analysis. However, for bacterial strain identification, the main variable region is within the first 528 nucleotides. 16S rRNA gene sequence analysis has been used to differentiate *Cronobacter* from closely related and similar bacteria. Moreover, this gene has been used to distinguish between most of *Cronobacter* species, except closely related species, in particular *C. sakazakii* and *C. malonaticus.*

1.6.2.5 Multilocus sequence typing (MLST).

Due to the high genetic similarity of the genus *Cronobacter*, it was difficult to distinguish between *C. sakazakii* and *C. malonaticus* based on the routine 16S sequence analysis. Therefore, Multilocus Sequence Typing (MLST) scheme using 7 housekeeping genes (concatenated sequence length of 3036 bp) was applied to discriminate the species within *Cronobacter* genus (Baldwin et al. 2009; http://pubmlst.org/*cronobacter*/). This method is more discriminatory and robust than 16S sequence analysis. It has revealed a stable clonal nature of the virulent *C. sakazakii* strains, and was also used in the description of the 2 new *Cronobacter* species including *C. universalis* and *C. condimenti* (Joseph et al. 2012d). The MLST scheme has

so far identified about 470 sequence types in the *Cronobacte*r genus across a database of more than 1700 strains (http://pubmlst.org/cronobacter/).Typing *Cronobacter* isolates to better understand the diversity of the *Cronobacter* genus has led to the development several MLST schemes. The primary scheme used 7-loci (3036bp), however more recently ribosomal protein-MLST (rMLST) using 53 loci and COG-cgMLST using (1865 loci) has been established (Forsythe et al. 2014).

1.6.2.6 O-antigen serotype.

The O antigen forms part of the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria and is one of the most variable constituents on the cell surface. The O-antigen serotyping scheme for *Cronobacter* species has been recently established, and the O-antigen gene clusters and specific primers for these species serotypes have been characterized. Two specific primers have been designed based on the *wehC* and *wehI* genes, in order to identify the *C. sakazakii* serotypes O:2 and O:1, respectively (Jarvis et al. 2011). *C. sakazakii* serotypes; O:3, O:4, O:5, O:6, and O:7, 2 *C. malonaticus* serotypes; O:1 and O:2, 2 *C. turicensis* serotypes; O:1 and O:2, and 1 *C. muytjensii* serotype (O:1) were identified later (Jarvis et al. 2011; Sun et al. 2011, 2012a and 2012b).

The genes, which are involved in O-antigen synthesis, are located in the *rfb* locus between the flanking genes *gnd* and *galF*. The locus differs in size for each serotype based on the composition of sugar and structure complexity. These genes encode for enzymes involved in the synthesis of sugars forming the O-antigen subunit, genes that encode glycosyltransferases and genes such as *wzx* and *wzy* (Figure 1.2). The latter encode for the transporter and polymerase proteins necessary for processing and assembly of the O-antigen from the subunits (Ogrodzki and Forsythe, 2015; Blažková et al. 2015). Both *wzx* and *wzy* genes were found in *C. sakazakii* O:1, O:2 O:3, O:4, O:5, and O:6 serotypes, while only *wzx* gene was found in O:7 (Sun et al. 2012a). However, according to Sun et al (2012), *C. sakazakii* serotype O:4 shared a similar O-antigen gene cluster with *E. coli* O:103 (Figure 1.2).

Blažková et al (2015) tested a total of 82 strains, covering the 7 species of *Cronobacter*. The nucleotide variability of the O antigen gene cluster was determined by restriction fragment length polymorphism (RFLP). These strains were distributed into eleven

serotypes, which were previously published, and six new serotypes. Genomic analysis of strains available in public databases; Genbank and PubMLST *Cronobacter* were used to confirm the 6 new serotypes. These strains were then confirmed using the current serotype-specific PCR probes. The results revealed that the current PCR probes did not always correspond to genomic O-antigen gene cluster variation. Based on this analysis, Blažková et al (2015) proposed that the O-antigen scheme should be expanded, with additional recognition of seven new and two re-assigned serotypes.

A more recent study by Ogrodzki and Forsythe (2015) reported that *galF* (UDP-glucose pyrophosporylase) and *gnd* (6-phosphogluconate) sequences polymorphism analysis could successfully be used as an alternative to RFLP for serotyping. Serogroup profiling using PCR and whole genome sequences is the more common non-MLST profiling method (Ogrodzki and Forsythe, 2015).

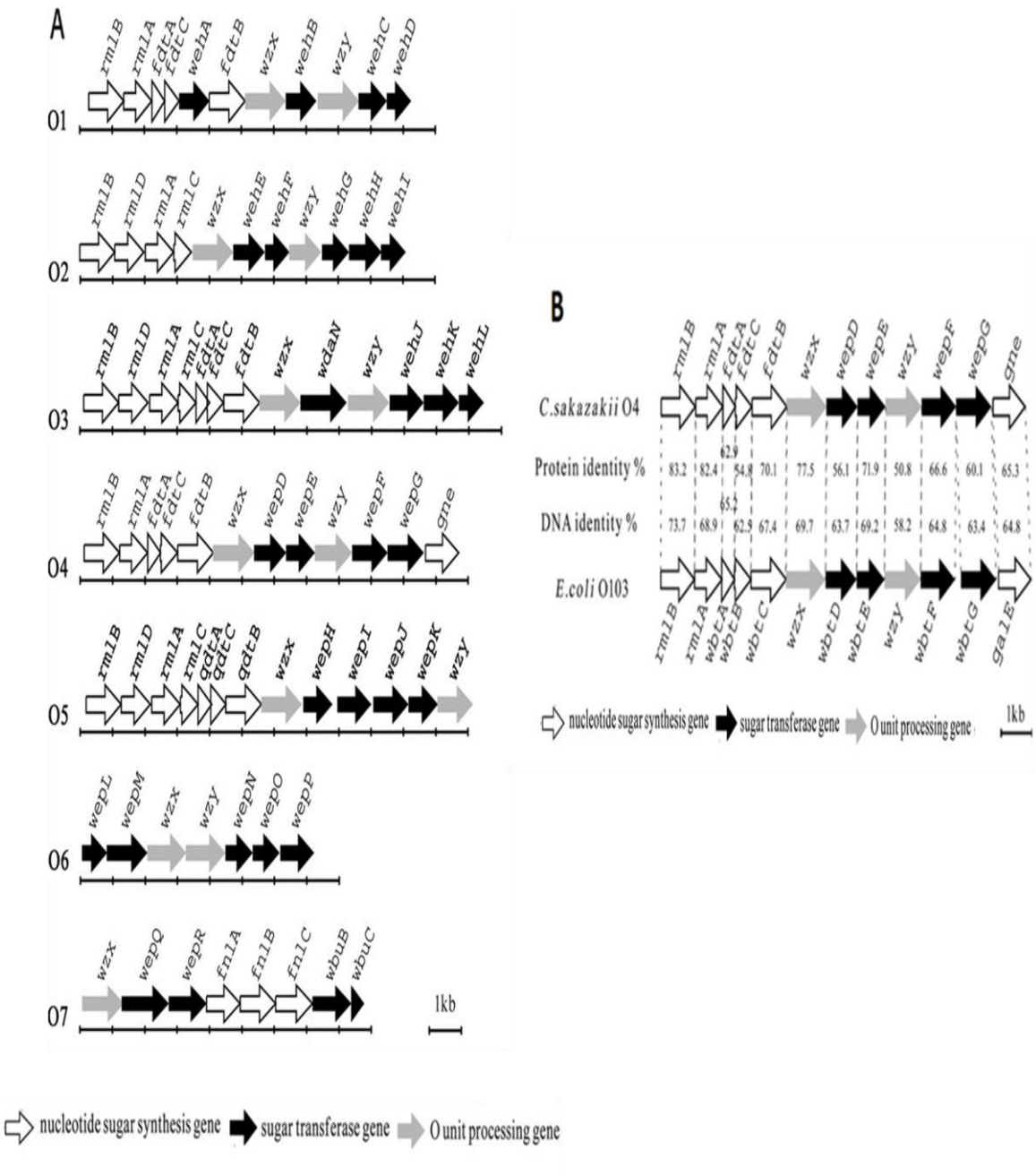


Figure 1.2. (A) Predicted genes of O-antigen gene clusters for *C. sakazakii* O:1 to O:7 serotypes. (B) Comparison of the O-antigen gene clusters of *C. sakazakii* O:4 and *E. coli* O:103. (Sun et al. 2012)

1.6.2.7 Targeted genes and primer design.

1.6.2.7.1 Capsular polysaccharide genes.

The polysaccharide capsules are main bacterial virulence factors and environmental fitness traits (Willis et al. 2013; Ogrodzki and Forsythe, 2015). Capsular polysaccharides of Gram-negative bacteria play a significant role in maintaining the structural integrity of the cell in hostile environments, and as result of polysaccharide capsule diversity within the bacterial species, can be used as taxonomic tools (Aduse-Opoku et al. 2005).

The diversity of capsular material has been the basis for many of the bacterial differentiation methods such as the K-antigen classification scheme of *E. coli* and *Salmonella* serovars serotyping (Whitfield et al. 2003; Ogrodzki and Forsythe, 2015). The O-antigen and K-antigen in Gram-negative bacteria consist of long polysaccharide units, which are covalently linked to lipid A in the outer membrane (Ogrodzki and Forsythe, 2015).

PCR-based methods targeting different bacterial genes are commonly used by different laboratories around the world. In addition, PCR-assay targeting significant bacterial genes including capsular polysaccharide genes such as K-capsule type are widely used, because these are important both pathogenetically and taxonomically. The K-antigen gene cluster of *E. coli* consists of 3 regions. The K-antigen Region 1 including *kpsEDCS* genes, Region 3 including *kpsTM* and a variable Region 2 (Willis and Whitfield, 2013). *E. coli* produce more than 80 different capsular polysaccharide K-antigens. Kaczmarek et al (2014) reported that the K1 antigen is a key virulence determinant of *E. coli* strains and has been associated with meningitis, bacteraemia and septicaemia, particularly in neonatal cases. Neonatal meningitis *Escherichia coli* (NMEC) is a predominant Gram-negative bacterial pathogen associated with meningitis in babies. NMEC are also associated with strains possessing the K1 capsular polysaccharide (Wijetunge et al. 2015). Moreover, a rapid and specific PCR-based assay was used for the detection of group 2 *kpsM* variants in *E. coli* (Johnson and O'Bryan, 2004).

A multiplex PCR targeting a capsular polysaccharide synthesis gene cluster of serotypes K1, K2 and K5 was evaluated using many reference serotype strains of *Klebsiella*, and a panel of clinical isolates subjected previously to conventional serotyping. The PCR assay was highly specific for these serotypes, which are associated with virulence in humans (Turton et al. 2008). Feizabadi et al (2013) suggest that PCR assay is a rapid and reliable method for the identification of *K. pneumoniae* capsular both K1 and K2 serotypes. Nevertheless, the techniques most commonly used for identification of *K. pneumoniae* serotypes K1 and K2 are limited, as a result of costs of various antisera preparation. Therefore, a PCR based method may help to operate *K. pneumoniae* capsular K1 and K2 type identification in routine diagnoses (Feizabadi et al. 2013).

Navasa et al (2009) reported that there is another bacterial capsular polysaccharide known as colanic acid (CA). Colanic acid is associated with bacterial protection against desiccation, extreme temperatures and acidic environmental conditions (Navasa et al. 2009; Navasa et al. 2013). In *Cronobacter,* the colanic acid encoding gene cluster was located close to the O-antigen region and separated by the *galF* gene (Ogrodzki and Forsythe, 2015). Moreover, Ogrodzki and Forsythe (2015) reported that there are two variants in the colanic acid encoding gene cluster. CA1 composed of 21 genes, while CA2 composed 20 genes, which differ in the presence of *galE* in CA1, and absence in CA2.

1.6.2.8 Variable number tandem repeat (VNTR).

Variable number tandem repeat (VNTR) analysis is a genotyping method based on the polymerase chain reaction and sequencing, which distinguishes tandem sequence repeats that vary in copy numbers, and may be useful for tracing and subtyping bacteria due to the simple process, low cost and high-speed. Moreover, VNTR analysis genotyping is becoming an important DNA-based typing method for investigating strains that are related to outbreaks and infection (Mullane et al. 2007). The VNTRA method has been stated to be highly discriminative, quick and inexpensive to perform. Furthermore, VNTR analysis has been applied to several *Enterobacteriaceae* families including *Salmonella* and *E. coli* (Lindstedt et al. 2004; Malorny et al. 2008; Bustamante et al. 2009). This method is now extensively used by European laboratories for *Salmonella* Enteritidis and *Salmonella* Typhimurium cluster detection and investigation of sources of contamination, and has recently been integrated in the European Surveillance System (TESSy), which helps a rapid exchange of data between European laboratories (Hopkins et al. 2011; Lindstedt et al. 2013). Kjeldsen et al (2015) have developed and compared generic VNTR analysis with PFGE for typing of *Salmonella enterica* subsp. *enterica.* They found that the VNTR method showed a high-discriminatory power within most of *Salmonella enterica* subsp. *enterica* serotypes compared with PFGE technique.

1.6.2.9 Clustered regularly interspaced short palindromic repeats (CRISPR)–*cas* loci.

Clustered regularly interspaced short palindromic repeats (CRISPR) are segments of prokaryotic DNA containing short repetitions of base sequences. CRISPRs are found in more than 80% of archeal genomes, and approximately 48% of eubacterial genomes

(Grissa et al. 2007; Ogrodzki and Forsythe, 2016). Several applications have been identified for the CRISPR–*cas* system such as gene editing, evolutionary, phylogenetic studies, and genotyping for epidemiological investigations (Fricke et al. 2011; Makarova et al. 2015; Ogrodzki and Forsythe, 2016). This method has been applied to *Enterobacteriaceae* family such as *Yersinia*, *Salmonella* and *E. coli,* in order to study the phylogenetic analysis, evolutionary and virulence-related (Fricke et al. 2011; Yin et al. 2013).

Ogrodzki and Forsythe (2016) identified the phylogenetic distribution of CRISPR–*cas* loci within the major pathovars of *C. sakazakii* including ST1, ST4, ST8 and ST12. Seventy whole-genome-sequenced isolates were used in this study, they were from 10 different countries and were isolated between 1950–2014. Twenty-six environmental isolates from manufacturing plants in three US states collected within a 6-week period and 20 isolates from an outbreak in a NICU (6-month) were included in the selected isolate cohort, in order to investigate the potential uses of *Cronobacter* CRISPR spacer array profiling for purposes of epidemiology. Twelve CRISPR spacer arrays were identified across *C. sakazakii* ST4, CC1, ST12 and ST8. These contained 32 different direct repeat (DR) sequences and 154 different spacer sequences. Twenty-five ST4 isolates including fourteen strains from NICU *Cronobacter* outbreak in France were analysed. Two groups of CRISPR spacer arrays were found in all isolates. Twenty-five isolates were CRISPR1 and was composed of eight DRs (four sequence variants) and seven unique spacers. CRISPR2 indicated much greater variation through the twenty-five isolates with a total of seven different CRISPR2 profiles. Moreover, the profiles of CRISPR2 showed that no geographic or temporal association occurred as identical combinations were shared between isolates of different country, year and source. All ST4 isolates from NICU *Cronobacter* outbreak in France showed the same designated CRISPR2 profile. Clinical strain (558) isolated from Netherlands in 1983, and (1537) isolated from a Germany milk powder factory in 2009 had also the same profile.

Twenty-nine CC1 isolates from environmental swabs were analysed. Three CRISPR spacer arrays (CRISPR1, CRISPR2 and CRISPR3) were found in all isolates. CRISPR1 and CRISPR2 were found in all twenty-nine isolates. CRISPR3 was found in 7 isolates only, and was found in only 2 out of the 24 US environmental isolates. Three CRISPR arrays were identified from eight ST12 isolates. Moreover, the ST12 lineage showed highly

conserved CRISPR array profiles compared with those within the CC1 and ST4. Eight ST8 isolates were analysed and 4 different CRISPR spacer arrays were identified. This study reported that the CRISPR spacer array profiling has a greater discriminatory power compared with MLST, which will be of use in identifying the source of contamination during *Cronobacter* outbreak investigations (Ogrodzki and Forsythe, 2016).

1.7 *Cronobacter* pathogenicity and virulence.

Infections of *Cronobacter* are associated with neonates and infants in particular those with low birth weight, causing necrotizing enterocolitis (NEC), septicaemia, and meningitis, with high fatality rates (40% - 80%) (Gurtler et al. 2005; Healy et al. 2010; Holy and Forsythe, 2014). However, infections by members of the *Cronobacter* genus are also associated with adults, in particular immunocompromised patients (Holy and Forsythe, 2014; Patrick et al. 2014). Bowen and Braden (2006) reported that neonatal cases of *Cronobacter* meningitis, could lead to severe neurological damage as result of destruction of the brain, and following to death. However, low birth-weight neonates (<1.5 kg) and <28 days in age are riskier compared to more mature infants (Van Acher et al. 2001). Furthermore, groups of adult infections are associated with bacteraemia, wound infections and urosepsis. The *Cronobacter* spp. may be categorised into two groups: clinical group including *C. sakazakii* and *C. malonaticus*, which are isolated from the majority of clinical samples, and less frequently clinical group including *C. turicensis* strains. The others are primarily non-clinical species and maybe have a little clinical significance (Holy and Forsythe, 2014; Jackson et al. 2014). However, to date only *C. sakazakii*, *C. malonaticus*, and *C. turicensis* strains have been associated with neonatal and infants infections (Joseph et al, 2012b). Furthermore, *C. malonaticus* strains seem to be more associated with young and elderly infections (Joseph and Forsythe, 2011). Many *Cronobacter* outbreaks have been reported in neonatal intensive care units (NICU) (Van Acker et al. 2001; Block et al. 2002; Himelright et al. 2002; Friedemann, 2009). The largest outbreak was occurred in a NICU in France that resulted in death of at least 3 neonates. Nevertheless, the first fatal reported *Cronobacter* neonatal cases were in 1958 (Urmenyi and Franklin, 1961). Himelright et al (2002) reported that neonatal Infections have been strongly linked to reconstituted PIF, which may have been contaminated during the preparation or during the

processing. *Cronobacter* strains have been isolated from the tracheae, faeces and urine samples. Moreover, they have been recovered from the feeding tubes of neonates fed breast milk and ready-to-feed formula (Hurrell et al. 2009b). Consequently, many sources of the bacterium during an outbreak need to be investigated and not only the PIF.

1.8 Virulence traits in *Cronobacter* species.

While infections are rare, the organism is of great concern as they have very high mortality rates (40-80%), and the survivors often develop lifelong mental disabilities (Farmer et al. 1980; Iversen et al. 2008; Joseph and Forsythe, 2011). However, there is limited knowledge of the virulence and epidemiology of *Cronobacter* species.

Cronobacter spp. sequenced genomes have identified many virulence factors such as Type VI secretion system, adhesins, outer membrane proteins, efflux systems, iron uptake mechanisms, hemolysins, enterobactin and aerobactin synthesis (Kucerova et al. 2010; Joseph, and Forsythe, 2011; Joseph et al. 2011). Genomic studies of *Cronobacter* also revealed the superoxide dismutase (*sodA*) for macrophage survival, flagella (Cruz et al. 2011), a metalloprotease (Kothary et al. 2007), an enterotoxin (Pagotto et al. 2003), and plasmid-borne virulence factors such as *Cronobacter* plasminogen activator (*cpa*) and type six secretion systems (T6SS) (Franco et al. 2011).

1.8.1 Other virulence traits.

Outer membrane proteins (OMPs). A number of studies reported that outer membrane protein A (*ompA*) contributes to the virulence potential of *Cronobacter* species by invading epithelial and endothelial cells of human and animal origin. Moreover, Mittal et al (2009) stated that OmpA positive strains are able to breach the blood-brain barrier and invade the central nervous system, consequently causing clinical manifestations. Furthermore, *ompX* plays an important role in the invasion, and also the basolateral side of the host cells and can translocate into the deeper organs of rats (Kim et al. 2010).

Utilization of sialic acid. Sialic acid is found in human milk, mucin human brain and infant formulae, which could be utilised by *C. sakazakii* strains as an alternative carbon source. Thus allowing *C. sakazakii* strains to survive and persist in the brain, hence causing brain damage. Joseph and colleague (2013) reported that sialic acid catabolism

genes (*nanAKT*) are essential for exogenous sialic acid utilisation. These genes were identified as virulence factors, and at that time were found only in the *C. sakazakii* strains.

Inositol fermentation. Hamby et al (2011) reported that inositol fermentation is a requirement for pathogenicity in *Cronobacter* genus, or that the genetic locus conferring inositol fermentation was linked to genes conferring pathogenic traits. However, the role of inositol fermentation in virulence of *Cronobacter* species is still not clear (Grim et al. 2013).

Iron acquisition gene system. Iron is an important nutrient for bacterial growth and metabolism and a significant factor for microbial pathogenesis. Franco et al (2011) reported that a homolog of an ABC transport-mediated iron uptake siderophore system gene cluster (*eitCBAD* operon) and a siderophore mediated iron acquisition system gene cluster (*iucABCD/iutA* operon) are found in plasmid pESA3. This characteristic may allow *Cronobacter* species to invade the central nervous system (CNS). Moreover, Grim et al (2012) confirmed that about 98% of the plasmid-harboring *Cronobacter* strains have the aerobactin like siderophore, cronobactin, for transport of ferric iron in *Cronobacter* strains. Cruz et al (2011) have also revealed that *C. sakazakii* isolates harbour siderophore-interacting protein (*sip*) gene.

Lipopolysaccarides (LPS). Lipopolysaccharides (LPSs) are important virulence factors of *Cronobacte*r species. LPS is the main components of the outer surface of Gram-negative bacteria, which interacts with enterocytes respond to LPS through TLR4 inducing NEC in animals (Leaphart et al. 2007). Moreover, the high level of LPSs in serum and stools have been reported in many NEC cases (Singh et al. 2015). Furthermore, Townsend et al (2007) reported that PIF is contaminated with high levels of LPS (endotoxin) from residual bacterial biomass, which could increase the permeability of the host cell membrane, and therefore bacterial translocation from the gut.

1.9 Genome studies.

Many *Cronobacter* genomes have been studied in the last two decades as result of the association of the organism with neonatal infections. The first *Cronobacter* genome sequenced was *C. sakazakii* BAA-894, the genome was sequenced by Genome Centre,

Washington University, USA. This strain was obtained from a case of fatal meningit is associated with NICU outbreak of Tennessee in 2001 (Himelright et al. 2002). The *C. sakazakii* BAA-894 genome sequence was published by Kucerova et al (2010). The genome contains 1 chromosome (4.4 Mb), and 3 plasmids, 2 of 3 plasmids were sequenced which are pESA2 with size 31 kb and pESA3 with size 131 kb (Accession No. NC_009778 – 80). Moreover, the genome sequence was used to construct 384,030 probe oligonucleotides to tile a DNA microarray. Ten isolates belong to 5 species of *Cronobacter* genus (*C. sakazakii, C. dublinensis, C. muytjensii, C. turicensis* and *C. malonaticus*) were included in this analysis. The genome analysis of 5 species showed that the presence of mobile genetic elements and prophage regions increase the diversity among *Cronobacter* species. Additionally, several different virulence associated traits were identified from the genome such as type 6 secretion system (T6SS), fimbrial gene clusters and iron acquisition genes (Kucerova et al. 2010)

Stephan et al (2011) announced the genome of *C. turicensis* z3032. This strain was isolated from a fatal case in a children's hospital in Zurich, Switzerland. The genome (Accession No. NC_013282-85) consisted of a chromosome with size 4.38 Mb and three plasmids of sizes 138 kb, 53kb and 22kb respectively (Stephan et al. 2011). Moreover, Franco et al (2011) have conducted a comparative genomic study between the large plasmid pESA3 of *C. sakazakii* BAA-894 (~ 131 kb) and the large plasmid pCTU3 of *C. turicensis* z3032 (~138 kb). The study showed that both plasmids (pESA3 and pCTU3) have homologous gene content. Furthermore, the plasmids were regarded as virulence plasmids sharing the same virulence associated genes such as plasminogen activator (*cpa*), iron acquisition system and type six secretion systems.

An extensive comparative genomics study of 14 *Cronobacter* genomes was conducted by Joseph et al (2012), which covered 7 species of *Cronobacter.* The study indicated that there was significant variation between genomes. For example, there was a significant variation in the presence of virulence-associated genes like heavy metal resistance genes such as resistance against silver, copper, tellurite, adhesins and genes for T6SS. Moreover, the study found that *C. sakazakii* genomes were unique in that they encoded genes required for the utilization of exogenous sialic acid, and other members of *Cronobacter* genus analysed showed absence of sialic acid utilization genes (Joseph et al. 2012).

Grim and colleagues in 2013, performed comparative genomics on 8 strains of *Cronobacter*, including 6 genomes sequenced in their study; representing six of the seven species, and two strains were previously published, closed genomes. The aim of this study was to identify and characterize the features associated with the core and pan genome of *Cronobacter* species in order to understand the evolution and the genetic content of each species. The genomic analysis was based on average nucleotide identity (ANI), alignment of whole genome and phylogenetic analysis. The study demonstrated a larger core genome containing a 3160 coding sequences. Furthermore, the study identified 84 genomic regions which were found in two or more *Cronobacter* genomes. The study also listed 45 genomic regions which were unique in a subset of the genomes. Moreover, several potentially horizontally transferred genes, including lysogenic prophages, were also identified in this study (Grim et al. 2013).

In another study, Masood et al (2015) analysed the genomic of a *C. sakazakii* isolates, which associated with NICU outbreak in France (1994). The whole genome phylogeny was determined using Mugsy and RaxML analysis. This suggested at least 4 distinct clusters for the sequenced *C. sakazakii* strains, each cluster was consisted of isolates belong to different ST; cluster 1 was ST12, cluster 2 was CC4, cluster 3 was CC13, and cluster 4 was CC1 strains. Cluster 2 was the largest group; three of the clusters (1, 2, 3) were isolated from neonates. Whereas, the group 4 consisted of an only one strain isolated from an unopened can of infant formula.

The result of the whole genome phylogeny was in close agreement with the original PFGE profiles, which previously identified by Caubilla-Barron et al (2007). More interesting is that all the strains within cluster 2 are equivalent to pulsotype 2, and belonged to clonal complex 4 (CC4) which has been associated predominantly with neonatal meningitis. Moreover, all 3 neonates who died were infected by the genotype cluster 2 *C. sakazakii* CC4 strains (Masood et al. 2015). Furthermore, the whole genome phylogeny analysis in this study was an agreement with Forsythe and colleagues study (2014) who revealed that 7-loci MLST, r-MLST and COG-MLST agreed with whole genome phylogeny (Masood et al. 2015). The single nucleotide polymorphism (SNP) analysis covered only strains in clusters 1, 2 and 3 as the genome sequence of only a single strain from cluster 4 was available. SNP analysis was

undertaken to analyse the strain relatedness within each cluster independently of the other clusters. However, SNP analysis of cluster 1, 2 and 3 revealed the likelihood of a recent a common ancestor resident in the hospital environment.

This study concluded that the genomic investigation showed that the outbreak was composed of a heterogeneous population of *C. sakazakii* isolates. The outbreak source was not identified, but maybe as result of environmental and personnel reservoirs resulting in extrinsic contamination of the neonatal feeds. This study also showed that *C. sakazakii* strains from different genotype clusters have the ability to co-infect neonates.

1.10 International surveillance of *Cronobacter* species infections.

In the last three decades, most of *Cronobacter* neonatal infection cases have been caused by strains with *C. sakazakii* sequence type 1, 4, 8 and 12 (Ogrodzki and Forsythe, 2016). These STs have been implicated in many outbreaks associated with meningitis, necrotizing enterocolitis and septicaemia. Recently, genotyping methods are being more and more used as quick and reliable tools to identify the genetic diversity and investigate sources of contamination. Molecular tools have been used more than phenotyping methods in the characterization of *Cronobacter* species. In 2004, a molecular typing scheme was instituted by WHO in order to assist the international control of the *Cronobacter* species infections. Baldwin et al (2009) reported sequencing data from multiple gene loci (MLST) rather than one locus would be better phylogenetic markers and more discriminatory power between strains compared with 16S rDNA sequencing (Baldwin et al. 2009). The MLST scheme is based on 7 housekeeping genes (concatenated sequence length of 3036 bp). These genes have specific functions, including DNA repair, replication, amino acid biosynthesis, and wide distribution across the chromosome. This led to the establishment of the *Cronobacter* PubMLST database (http://pubmlst.org/Cronobacter/). This database containing more than 470 STs in the *Cronobacter* genus across of >1700 strains, and detailed data on specific clonal lineages linked to neonatal meningitis and adult infections.

Joseph and Forsythe (2011) applied the 7-loci MLST to 41 clinical isolates of *C. sakazakii*. About 50% (20/41) of these strains were ST4, and 9 of 12 meningitis isolates were ST4. Furthermore, Forsythe et al (2014) applied the 7-loci MLST scheme to more

than 1000 strains revealing 298 definable STs, they found in *C. sakazakii* clonal complex 4 (CC4) was associated with neonatal meningitis. This lineage has been confirmed using r-MLST (51-loci) and genome-MLST (1865 loci) to analyse 107 whole genomes via the *Cronobacter* PubMLST database. However, *C. sakazakii* ST4 strains seems to be a highly stable clone with a high propensity for neonatal meningitis. This database enables clinical integration, epidemiological, microbiological, and molecular typing data and retrospective analyses of historic cases and outbreaks (Joseph and Forsythe, 2011; Forsythe et al. 2014).

1.11 Limitation of identification methods and lack of understanding of the phenotypic and genotypic diversity of *Cronobacter* spp.

Currently, microbiological criteria require the absence of all 7 *Cronobacter* species in 10g of PIF sample. Consequently, the misidentification of *Cronobacter* species in PIF can lead to substantial losses for manufacturers and may present a risk to neonates (Jackson and Forsythe, 2016). Moreover, the sources diversity and high mortality rates of *Cronobacter* spp. has required the improvement and development of reliable detection and identification methods for the bacterium. The current ISO standard protocol for the identification of *Cronobacter* species in PIF relies on enrichment, chromogenic agar and biochemical methods. This relies on visual detection of colour changes and makes difficult of accurate identification difficult as a result of subjectivity when reading the test results. (Jackson et al. 2014; Jackson and Forsythe, 2016). Jackson and Forsythe (2016) reported that commercially available biochemical test panels are not sufficiently reliable for speciation of *Cronobacter* isolates. Moreover, difficult to identify and distinguish isolates to the sub species level within the *Cronobacter* genus.

Limitation of molecular techniques.

Common methods used to distinguish bacterial species like 16S rRNA gene sequence, do not equally discriminate closely related *Cronobacter* species such as *C. sakazakii* and *C. malonaticus* (Baldwin et al. 2009). 7-loci MLST is used to discriminate species the within *Cronobacter* genus as well as between different STs, but is unable to distinguish strains within the same sequence type. PFGE is generally used for subtyping of *Cronobacte*r species (Holy and Forsythe, 2014). However, this technique is not an

ideal method for bacterial surveillance and epidemiology due to the high clonality of STs and does not discriminate between all unrelated isolates. For example, unrelated *C. sakazakii* ST4 strains isolated from the Czech Republic hospitals from 15 adults between May 2012 and May 2013 showed the same PFGE profile (pulsotype 12), due to the high clonality of ST4 within *C. sakazakii* limiting the discriminatory power of PFGE (Alsonosi et al. 2015). Our current knowledge of understanding the genotypic, clonality and phenotypic diversity of *Cronobacter* spp. is limited, and therefore an improved understanding of the diversity of the genus is warranted.

1.12 Aims and objectives.

The genus *Cronobacter* are food-borne pathogens causing neonatal infections including meningitis, necrotizing enterocolitis. *Cronobacter* infections have been associated with the consumption of contaminated reconstituted PIF. Moreover, this genus has been isolated from a wide range of sources. The diversity of sources and high mortality rates of this organism required the improvement and development of reliable detection and identification methods for the bacterium. Furthermore, understanding the molecular characterisation, clonality and phenotypic diversity of *Cronobacter* species is essential to reduce the source of microbial contamination of PIF and other food.

The overall aim of this research project was to investigate the genetic diversity of the *Cronobacter* species isolated from food and environmental sources, and to link that with their phenotypic and physiological traits.

This study had the following objectives:

I. **To study the diversity of the *Cronobacter* genus from food and environmental sources as revealed by genotyping, clonality and surface structure; Chapter 3.**
   - Studying the genotypic diversity of the *Cronobacter* strains obtained from food and environmental sources, using different molecular subtyping method such as 7-loci MLST clonality analysis, RAPD, BOX-PCR, and O-antigen serotyping.
   - Developing and describing the application of variable numbers of tandem repeats (VNTRA) typing method for *C. sakazakii* ST4 strains.
   - Extraction and characterization of lipopolysaccharide (LPS) profiles from selected strains covering the representatives of four main serotypes (O:1, O:2, O:3 and O:4) and the main sequence types which are associated with clinical significance of the *C. sakazakii* strains (ST1, ST4, ST8, ST12 and ST13) and validate these differences using Bionumerics software (version 7.1).
   - Designing and targeting significant bacterial genes including capsular polysaccharide genes such as K-capsule and colanic acid genes using a multiplex PCR as simple, cheaper, rapid and reliable molecular typing methods.

II. To investigate the phenotypic diversity of *Cronobacter* strains isolated from food and environmental sources and to link these with their molecular characteristics; Chapter 4.

- Investigation of the strains appearance on different media, motility determination, indole and α-D-glucoside production, malonate and inositol metabolism, sialic acid utilization, capsule production, biofilm formation, lipase and protease activity, and to link these with their molecular characteristics.

III. To investigate the *Cronobacter* strains ability to tolerate a variety of environmental stress conditions and to link these with their genetic traits; Chapter 5.

- Investigation of *Cronobacter* species ability to tolerate a variety of environmental stress conditions such as heavy metal, sub-lethal injury during desiccation, long-term drying, heat and acid resistance, and to link these with their genetic traits.

Chapter 2. Material and methods.

2.1 Safety considerations.

All the experiments were conducted according to the Health and Safety Code of practice for microbiology containment level two and appropriate COSHH forms were completed. Handling microbes, media and chemicals according to good laboratory practices. In addition, good laboratory practices were followed for operating laboratory equipment and devices. All the waste and material were disposed according to the recommendations in the material safety data sheets.

2.2 Bacterial strains.

All the *Cronobacter* strains that were used in this study were from the culture collection of *Cronobacter* spp. of Nottingham Trent University (NTU). Strains of *Cronobater* have been used in the present study covered the seven species of *Cronobacter*. Most of these strains (n=79) had been isolated from food, environmental sources and the remaining clinical strains (n=15) were used for comparison purposes. Table 2.1 lists the details of the strains that have been isolated and used in this study.

Table 2.1. *Cronobacter* strains used in this study.

NTU no.	Species	Source	Country	Year	*A	*B	*C	*D
96	*C. universalis*	Spice	UK	2004		√	√	
1435	*C. universalis*	Food	Turkey	2010		√	√	
1883	*C. universalis*	Spice	Czech Republic	2011	√	√	√	
57	*C. turicensis*	Milk powder	UK	2004			√	
92	*C. turicensis*	Herb	UK	2004		√	√	
109	*C. turicensis*	Herb	UK	2004		√	√	
111	*C. turicensis*	Herb	UK	2004			√	
564	*C. turicensis*	Clinical	USA	1970			√	
666	*C. turicensis*	Clinical	USA	1975			√	
1553	*C. turicensis*	Herb	Slovakia	2004		√	√	
1880	*C. turicensis*	Herb	Czech Republic	2011	√	√	√	
1895	*C. turicensis*	Ingredient	Czech Republic	2011	√	√	√	
16	*C. muytjensii*	Spice	UK	2005		√	√	

1371	*C. muytjensii*	Spice	UK	2010		√	√	
1527	*C. muytjensii*	Food	Slovakia	2010		√	√	
1877	*C. muytjensii*	Ingredient	Czech Republic	2011	√	√	√	
35	*C. malonaticus*	Weaning food	USA	2005			√	
93	*C. malonaticus*	Spice	UK	2005		√	√	
101	*C. malonaticus*	Spice	UK	2005		√	√	
507	*C. malonaticus*	Clinical	Czech Republic	1984			√	
510	*C. malonaticus*	Food	Czech Republic	1985		√	√	
512	*C. malonaticus*	Clinical	Czech Republic	1985			√	
893	*C. malonaticus*	Infant formula	Brazil	2007			√	
1369	*C. malonaticus*	Herb	UK	2010		√	√	
1846	*C. malonaticus*	Ingredient	Czech Republic	2011	√	√	√	
1879	*C. malonaticus*	Spice	Czech Republic	2011	√	√	√	
1893	*C. malonaticus*	Ingredient	Czech Republic	2011	√	√	√	
1914	*C. malonaticus*	Clinical	Slovakia	2014			√	
1917	*C. malonaticus*	Clinical	Czech Republic	2012			√	
2045	*C. malonaticus*	Environment	France	Un			√	
2046	*C. malonaticus*	Environment	Un	Un			√	
1330	*C. condimenti*	Food	Slovakia	2010		√	√	
84	*C. dublinensis*	Herb	China	2004		√	√	
582	*C. dublinensis*	Un	UK	Un		√	√	
583	*C. dublinensis*	Environment	UK	1965		√	√	
1130	*C. dublinensis*	Follow up formula	Korea	2008		√	√	
1132	*C. dublinensis*	Follow up formula	Korea	2008		√	√	
1133	*C. dublinensis*	Follow up formula	Korea	2008		√	√	
1134	*C. dublinensis*	Follow up formula	Korea	2008		√	√	
1210	*C. dublinensis*	Environment	Ireland	2004		√	√	
1458	*C. dublinensis*	Food	Korea	2011		√	√	
1460	*C. dublinensis*	Food	Korea	2011		√	√	
1461	*C. dublinensis*	Food	Korea	2011		√	√	
1462	*C. dublinensis*	Food	Korea	2011		√	√	
1463	*C. dublinensis*	Food	Korea	2011		√	√	

1464	*C. dublinensis*	Food	Korea	2011		√	√	
1548	*C. dublinensis*	Clinical	Un	Un		√	√	
1556	*C. dublinensis*	Clinical	USA	1979		√	√	
1560	*C. dublinensis*	Food	Czech Republic	Un		√	√	
1897	*C. dublinensis*	Ingredient	Czech Republic	2011	√	√	√	
25	*C. sakazakii*	Follow up formula	Korea	2004			√	
26	*C. sakazakii*	Follow up formula	Korea	2004			√	
377	*C. sakazakii*	Weaning food	UK	1950		√	√	√
545	*C. sakazakii*	Infant formula	Netherlands	1988			√	
551	*C. sakazakii*	Environment	Netherlands	1988			√	
658	*C. sakazakii*	Non-Infant formula	USA	2001		√	√	√
767	*C. sakazakii*	Clinical	France	1994			√	√
692	*C. sakazakii*	Clinical	France	1994			√	
695	*C. sakazakii*	Clinical	France	1994			√	
705	*C. sakazakii*	Clinical	France	1994			√	√
706	*C. sakazakii*	Clinical	France	1994			√	√
712	*C. sakazakii*	Infant formula	France	1994			√	
713	*C. sakazakii*	Infant formula	France	1994			√	√
890	*C. sakazakii*	Infant formula	Brazil	2007			√	
1105	*C. sakazakii*	milk powder	UK	2008		√	√	√
1106	*C. sakazakii*	Weaning food	UK	2008			√	
1107	*C. sakazakii*	Weaning food	UK	2012		√	√	
1108	*C. sakazakii*	Weaning food	UK	2012		√	√	√
1283	*C. sakazakii*	Food	UK	2010		√	√	√
1480	*C. sakazakii*	Environment	Australia	2007			√	
1533	*C. sakazakii*	Environment	Germany	2006			√	√
1564	*C. sakazakii*	Food	Slovakia	2010			√	
1568	*C. sakazakii*	Infant formula	USA	2011			√	
1570	*C. sakazakii*	Clinical	USA	2011			√	
1579	*C. sakazakii*	Clinical	USA	2011			√	
1843	*C. sakazakii*	Spice	Czech Republic	2011	√	√	√	
1844	*C. sakazakii*	ingredient	Czech Republic	2011	√	√	√	

1845	*C. sakazakii*	Food	Czech Republic	2011	√	√	√	
1847	*C. sakazakii*	Milk powder	Czech Republic	2011	√	√	√	
1881	*C. sakazakii*	ingredient	Czech Republic	2011	√	√	√	
1882	*C. sakazakii*	ingredient	Czech Republic	2011	√	√	√	
1884	*C. sakazakii*	Herb	Czech Republic	2011	√	√	√	
1885	*C. sakazakii*	Herb	Czech Republic	2011	√	√	√	
1886	*C. sakazakii*	Spice	Czech Republic	2011	√	√	√	√
1887	*C. sakazakii*	Food	Czech Republic	2011	√	√	√	√
1888	*C. sakazakii*	Food	Czech Republic	2011	√	√	√	√
1889	*C. sakazakii*	ingredient	Czech Republic	2011	√	√	√	√
1890	*C. sakazakii*	ingredient	Czech Republic	2011	√	√	√	
1906	*C. sakazakii*	Environment	Malaysia	2012	√	√	√	√
1907	*C. sakazakii*	Environment	Malaysia	2012	√	√	√	√
1908	*C. sakazakii*	Environment	Malaysia	2012	√	√	√	√
1990	*C. sakazakii*	Food	UK	2013	√	√	√	
1992	*C. sakazakii*	Food	UK	2013	√	√	√	
2027	*C. sakazakii*	ingredient	UK	2013	√	√	√	

*A – MLST laboratory experiments and sequence data analysis was performed.

*B – Physiological and phenotyping study as part of this project.

*C – Genotypic analysis or genome study as part of this project.

*D– Capsule isolation and sugar analysis as part of this project.

USA: United States of America; UK: United Kingdom; Un: Unknown.

2.3 Bacterial storage and culture.

All strains of *Cronobacter* were stored long term in TSB-glycerol (80%) at -20°C and -80°C. Cells were recovered from frozen stock, subcultured on Tryptone Soya Agar (TSA) and incubated under aerobic conditions at 37°C for 24 h. All culture strains were stored at 4°C for short periods.

2.4 Preparation of media and buffers.

2.4.1 Culture media.

2.4.1.1 Trypticase soy agar (TSA).

According to the manufacturer's instruction one litre of distilled water was used to re-suspend 40 grams of TSA (Thermo Fisher, UK) base. The solution was then autoclaved at 121°C for 15 minutes. The agar was allowed to cool to a temperature of 50°C. When the agar had cooled, approximately 15-20 ml of media was poured in each sterile Petri dish. The plates were dried and then stored for up to three weeks at 4°C.

2.4.1.2 Trypticase soy broth (TSB).

Trypticase soy broth (TSB) was prepared in the accordance with the manufacturer's instructions. Fifteen grams of TSB (CM0129, Oxoid Thermo Fisher; UK) was added to 500 ml of distilled water. The solution was then autoclaved at 121°C for 15 minutes. The broth was then stored in a dark and cool place at 4°C until required.

2.4.1.3 Druggan-Forsythe-Iversen (DFI).

Five hundred millilitre of distilled water was used to re-suspend 21.5 grams of DFI agar base (HAL013, Lab M Limited; UK). The components were mixed well and then autoclaved at 121°C for 15 minutes. Media was cooled to 50°C and then about 15-20 ml of media was poured into each sterile Petri dish. These plates were then stored at 4°C until required.

2.4.1.4 Luria-Bertani agar (LBA).

The manufacturer's directions were followed to prepare Luria-Bertani Agar (Merck KgaA, Darmstad, Germany; 1102830). Thirty-seven grams was dissolved in 1 L of water, mixed and then sterilized for 15 min at 121°C.

2.4.1.5 Brain heart infusion broth (BHI).

One litre of distilled water was used to re-suspend 37 grams of BHI (CM1135, Oxoid Thermo Fisher; UK). The mixture was then heated with mixing until the BHI was completely dissolved. The solution was then dispensed into universal bottles in 20 ml volumes, and autoclaved at 121°C for 15 minutes. The bottles were stored in the refrigerator at 4°C until required.

2.4.1.6 Milk agar.

To prepare milk agar, four hundred milliliter of distilled water was used to re-suspend 10 grams of Plate Count Agar. The re-suspended solution was then autoclaved at 121°C for 15 minutes. Agar was allowed to cool to a temperature of 50°C. The mixture was combined with 100 ml of warm (55°C) sterile liquid infant formula (Cow & Gate Premium 1). When the agar had cooled about 15 - 20 ml of media was poured in each sterile Petri dish. The plates were then stored at 4°C.

2.4.1.7 Xylose lysine deoxycholate agar (XLD).

Xylose lysine deoxycholate agar medium was prepared in the accordance with the manufacturer's instructions; 26.5 grams of XLD were added to 500 ml of distilled water. The solution was then heated with frequent agitation until the medium boiled. The medium was transferred immediately to a water bath at 50°C for 10 min; approximately 15-20 ml of media was dispensed into sterile petri dishes. The plates were stored for about 3 weeks at 4°C.

2.4.1.8 Violet red bile glucose agar (VRBGA).

According to the manufacturer's instructions. One litre of distilled water was used to re-suspend 38.5 grams of Violet Red Bile Glucose Agar. The solution was then heated with frequent agitation until the media boiled. Media was transferred immediately to a water bath at 50 °C for 10 min. When the agar had cooled, 15-20 ml of media was dispensed into sterile petri dishes.

2.4.1.9 Violet red bile lactose agar (VRBL).

To prepare one litre of Violet Red Bile Lactose media, 38.5 grams of VRBLA were added to one litre of distilled water. The solution was then heated with frequent agitation until the media boiled. Media was transferred immediately to a water bath at 50°C for 10 min, approximately 15-20 ml of media was dispensed into sterile petri dishes.

2.4.1.10 MacConkey agar.

To prepare one litre of MacConkey agar, fifty-two grams of media (Oxoid Thermo Fisher; UK) were suspend in one litre of distilled water. The solution was autoclaved at 121°C for 15 minutes. Media was transferred to a water bath at 50°C for 10 min; approximately 15-20 ml of media was dispensed into sterile petri dishes.

2.4.1.11 Congo red medium.

Congo red agar was prepared by dissolved of 5 grams of tryptone, 2.5 grams yeast extract and 7.5 grams of technical agar (LP0013 Agar No 3; Oxoid Thermo Fisher; UK) in 500 ml of distilled water. It was mixed until completely dissolved and autoclaved at 121°C for 15 minutes. Then it was cooled to 50°C. Thirty-two milligrams of Congo red dye (C6277, Sigma) were dissolved in 8 ml of distilled water. The 500 ml of agar was combined with 5 ml of Congo red dye, and 20 mg/ml of Coomassie Brilliant Blue R pure (Scientific Laboratory Supplies, B7920-50G), when the agar had cooled about 15 - 20 ml of media was poured in each sterile Petri dish. The plates were then stored in dark and cool place for up to 2 weeks at 4°C.

2.4.1.12 Calcofluor media.

Calcofluor media was prepared by dissolved of 5 grams of tryptone, 2.5 grams yeast extract and 7.5 grams of technical agar (LP0013 Agar No 3; Oxoid Thermo Fisher; UK) in 500 ml of distilled water. It was mixed until completely dissolved and autoclaved at 121°C for 15 minutes. Then it was cooled to 50°C. The agar was supplement by 25ml of Calcofluor White Stain (18909 Sigma, UK), and about 15 - 20 ml of media was poured in each sterile Petri dish. The plates were then stored in dark and cool place for up to 2 weeks at 4°C, followed by incubation 18 h at 37°C and 48h at 30°C.

2.4.1.13 Motility medium.

To prepare the motility medium. Four grams of Luria-Bertani Agar (LBA) (CM0129, Oxoid Thermo Fisher; England) and 0.8 grams of technical agar (LP0013 Agar No 3; Oxoid Thermo Fisher; UK) were added to 200 ml of distilled water. One millilitre of TTCS (Triphenyl-tetrazolium chloride, solution) was added to the solution. This was mixed well and after that autoclaved at 121°C for 15 minutes. The media was then cooled to 50°C and approximately 15-20 ml of media was poured into each sterile Petri dish, then left at room temperature to allow to solidify and then stored at 4°C.

2.4.1.14 10% Skimmed milk (protease activity).

Ten percent of skimmed milk powder (Oxoid Thermo Fisher; UK) were used to prepare the skimmed milk solution (SMS) and then autoclaved for 5 minutes at 121°C. Plate count agar (PCA) was prepared by adding 22.5 grams to 1 liter of distilled water and

autoclaved at 121°C under 15-psi pressure for 15 minutes. Twenty millilitre of 10 % SMS was aseptically mixed with 980ml of PCA and then dispensed into sterile Petri dishes. These plates were stored at room temperature for 2 days or for a few weeks at 4°C.

2.4.1.15 Tributyrin agar, base (Lipase activity).

The lipase activity assay was performed by using Tributyrin agar (pre-prepared Oxoid, UK). The pre-prepared agar was rehydrated and then poured into petri dishes. The petri dish plates were left at room temperature to dry for two to three days and after that, strains were streaked on tributyrin agar and incubated for 72 h at 37^{o}C. Each strain was evaluated for lipase activity by visual observation. A positive result was defined as a clear zone around the bacterial colonies.

2.4.2 Stock reagents and buffers.

2.4.2.1 Peptone water.

Fifteen gram of peptone water (Sigma Aldrich, UK) were dissolved into 1 litre of distilled water and autoclaved at 121°C for 15 minutes then stored at room temperature.

2.4.2.2 Phosphate buffered saline (PBS).

Five tablets of PBS (Sigma Aldrich, UK) were dissolved in 500 ml of distilled water and autoclaved at 121°C for 15 minutes. The phosphate buffered saline was used during the work with tissue culture.

2.4.2.3 Saline solution (0.85 %).

One tablet of saline (Fisher Scientific, UK) was dissolved into 500 ml of distilled water and autoclaved. This was distributed into glass bottles in 50 ml amounts.

2.4.2.4 1X Tris-acetate-EDTA (TAE) buffer.

Fifty millilitre of 5X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK) were dissolved in 450 ml of distilled water.

2.4.2.5 Triton X-100 (1%).

One millilitre of Triton X-100 (Fisher Scientific, UK) was added to 99 ml of dH_2O and autoclaved at 121°C for 15 minutes then stored at room temperature.

2.4.2.6 Lipopolysaccharide PAGE with silver stain.

Lysis buffer regent and SDS-PAGE gel are summarised in table 2.2 and table 2.3

Table 2.2. Lysis buffer regent.

Reagent	In 1L
Sodium dodecyl sulfate (SDS)	2g
2-Mercaptoethanol	4mL
Bromophenol Blue	0.003g
1M tris-Cl pH6.8	to 100ml

Table 2.3. SDS-PAGE gel.

15% separating gel	5% stacking gel
100 ml 30% protoGel	2ml 30% protoGel
50 ml resolving buffer	5 ml protoGel stacking buffer
68 ml deionized water	12 ml deionized water
6µl TEMED	3µl TEMED
150µL 10% APS	30µL 10% APS

2.4.2.6.1 SDS-PAGE ruinning buffer.

One hundred millilitre of 10X Tris/Glycine/SDS (0.025M Tris- 1.92M Glycine- 1% SDS) (Geneflow, UK) was added to 900 ml of distilled water.

2.4.2.7 Preparation of stock solution for heavy metals resistance assay.

The 1M stock solution of nickel chloride (Fisher Scientific, UK), sodium arsenate (Sigma-Aldrich, UK), cobalt (II) nitrate (Sigma-Aldrich, UK), silver nitrate (Alfa Aesar, UK), sodium tellurite (Sigma-Aldrich, UK) zinc sulphate (BDH chemicals England), copper (II) sulphate (Sigma-Aldrich, UK), and cadmium carbonate was prepared by dissolving 159.60g, 180.03g, 129.60g, 182.94g, 169.87g, 161.47g, 221.58g and 172.42g respectively into 1L of distilled water (molecular weight equivalent of the substance into 1L of distilled water). Further dilutions of 0.1M, 0.01M and 0.001M were prepared from 1M stock solution.

2.5 Methods.

2.5.1 *Cronobacter* genus colony morphology types.

2.5.1.1 Appearance of *Cronobacter* genus on different media (TSA, DFI, XLD, VRBGA, VRBLA and MacConkey agar)

Colony appearance of *Cronobacter* spp. was investigated by using different detection media such as TSA, XLD, VRBGA, VRBLA, DFI and MacConkey. The colony morphology was recorded after overnight period incubation at 37°C.

2.5.1.2 Congo red morphotypes.

Congo red morphotypes were examined by inculcating three microliters overnight culture on Congo Red agar plates as described previously in section 2.4.1.11, and incubated overnight at 37°C and for 48h at 28°C.

2.5.1.3 Colony morphology on calcofluor.

Cellulose production was investigated by streaking the bacterial strains on supplemented LBA medium with calcofluor white stain as described previously in section 2.4.1.12, and incubated overnight at 37°C and for 48h at 28°C. The cell morphology was recorded at 24h and 48 h, production of cellulose was detected through monitoring the fluorescent signal observed at 366 nm under UV light.

2.5.1.4 Motility assay.

The motility assay of the *Cronobacter* strains was performed by inoculating one to two colonies from each strain into five ml of TSB at 37°C with shaking incubator at 200 rpm. Three microliter of each overnight culture broth was inoculated into the centre of motility medium agar as described above in section 2.4.1.13, and incubated overnight at 37°C. The diameters of motility zones were measured and interpreted according to the diameters of zones. *S.* Enteritidis and *E. coli* K12 were used as positive and negative control respectively.

2.5.2 *Cronobacter* genus metabolism.

2.5.2.1 Biochemical test profiling.

2.5.2.1.1 API32E.

Strains were examined using the API32E kit following the manufacturer's recommendations (bioMerieux, Montalieu-Vercieu, France).

2.5.2.2 Capsule Production.

The capsule production of the *Cronobacter* isolates was performed as follows; see also sections 2.4.1.6, 2.4.1.7, 2.4.1.8, 2.4.1.9. Strains were streaked on milk agar, XLD Agar (Oxoid Thermo Fisher; UK), Violet Red Bile glucose agar (VRBGA) and Violet Red Bile lactose (VRBLA) agar and incubated overnight at 37°C and for 48h at room temperature.

2.5.2.3 Biofilm formations assay.

Cronobacter spp. isolates were grown on TSA plates at 37°C for 24 h. A single colony from a purity TSA plate was inoculated into 5 ml in sterile liquid infant formula (Cow & Gate Premium 1) and incubated for 6h at 37°C in a shaking incubator. The suspension was diluted in to OD 600 nm = 0.3. Then 200 µl of cell suspension was transferred to each well of a 96 microtiter plates, and then incubated statically at 25°C and 37°C for 24 h. After incubation, the microtiter plates were gently washed 2 times with sterile distilled water (SDW) and dried at room temperature. Each well was stained with 200 µl of 1% crystal violet solution. After 30 minutes staining, the plates were washed 3 times with SDW before addition of 0.2 ml ethanol and incubation for 15 min at room temperature. Finally, the extracted dye was quantified by measuring the absorbance at 600 nm with a microplate reader. All the experiments were repeated three times.

2.5.2.4 Sialic acid utilization (M9 Minimal Medium).

To prepare 5X M9 Minimal medium, 220g of 1.25% agar technical (Oxoid, UK), 25ml of 5X M9 medium, 500µl 1M of $MgSO_4$ and 25µl of 1M $CaCl_2$.50µl 1M $CaCl_2$, 1.25ml 10% casein hydrolysate, 500ml dH_2O. All solutions were filtered if needed and mixed in a total volume of 250ml after autoclaved at 121°C for 15 minutes. To prepare sialic acid media, 250 mg of sialic acid (Sigma, UK) was added to the mixture and the total volume 250ml. The media was poured separately into plates and were stored at room

temperature for 2 days or for a few weeks at 4°C. Each strain was carefully streaked on the media and all plates were incubated at 37°C for overnight.

2.5.2.5 Malonate utilization.

The ability of *Cronobacter* spp. to utilize malonate was performed by using malonate PPA Broth (Thermo Fisher, UK). One to two pure colonies from an overnight TSA plate was used to inoculate this broth which was incubated at 37°C for 24h. A positive malonate test is indicated by the development of a blue or blue green colour in the medium. A negative malonate test is indicated by the media remaining green or turning yellow.

2.5.2.6 Lipase activity.

Lipase activity was examined by streaking strains on Tributyrin agar plates as described previously in section 2.4.1.15, and incubated for 72h at 37°C.

2.5.2.7 Protease activity.

The strains were normally streaked on the plates as described previously in section 2.4.1.14, and incubated at 37°C for 72h and monitored every 24h. Each strain was evaluated for protease activity by visual observation.

2.5.3 *Cronobacter* strains stress responses.

2.5.3.1 Metal resistance assay.

The resistance of *Cronobacter* spp. to 7 different concentration of heavy metal including; nickel chloride, zinc sulphate, copper (II) sulphate, silver nitrate, sodium tellurite, cadmium carbonate and cobalt(II) nitrate were studied. Filter paper disks were used in this test. About 5 colonies of fresh TSA culture were suspended in 3ml of sterile distilled water and the OD adjusted to be equivalent to 0.5 McFarland standard. Suspensions were swabbed onto the TSA with sterile swab and then the filter paper disks were applied to the surface of TSA plates. Seven microliters from different concentration of heavy metals (1M, 0.1M, 0.01M, and 0.001M) were pipetted onto the filter paper disks. The plates were incubating at 37°C for 24 hours. The diameters of zones of inhibition were measured and interpreted according to the diameters of zones of inhibition.

2.5.3.2 Determination of sublethal injury during the desiccation of *Cronobacter* species in dehydrated powdered infant formula (PIF).

All *Cronobacter* strains were grown on TSA plates overnight at 37°C. A single colony from a purity TSA plate was inoculated into 5 ml in sterile liquid infant formula (Cow & Gate Premium 1) and incubated overnight at 37°C. After that the cell density was adjusted to approximately 10^{11} CFU/ml. Aliquots (0.2 ml) of the suspension were transferred into six-well plates and air dried overnight in a class II cabinet at room temperature (20 to 25°C) as described by Caubilla-Barron and Forsythe (2007). After desiccation, the test strains were re-suspended in 2 ml of sterile water and viable counts were determined in triplicate on TSA and VRBGA. Plates were incubated overnight at 37°C. The sublethally injured cells are defined as the difference between the viable counts obtained on VRBGA (selective agar) and those obtained on TSA (nonselective agar).

2.5.3.3 Resistance of *Cronobacter* spp. to drying (90 days).

According to the procedure described by Breeuwer et al (2003) the ability of the *Cronobacter* isolates to withstand drying was tested. In brief, 50 µl aliquots of 24 h culture in sterile liquid infant formula (Cow & Gate Premium 1) were placed in 12-well sterile polystyrene tissue culture plates (Corning Inc., Corning, NY, USA) and allowed to air dry in a 30°C incubator. The original culture was enumerated and reported as CFU ml^{-1} on day 0. On intervals up to 90 days, the inoculum dried in the incubator was reconstituted in 1 ml of sterile peptone water, and appropriate dilutions were plated on TSA to determine the number of survivors

2.5.3.4 Thermotolerance.

Thermal resistance of *Cronobacter* strains was investigated at 58°C. The thermotolerance of *Cronobacter* isolates were determined by suspending 1 ml overnight culture in 20 ml of temperature equilibrated sterile liquid infant formula (Cow & Gate Premium 1) in a water bath 58°C. At timed intervals, 0.1 ml aliquots were transferred to 2 ml TSB at room temperature and the number of survivors determined. The number of survivors at this temperature was plotted against time. The best fit-line was extrapolated and the D values were determined (-1/slope of the regression line). Each

single number is an average of three replicate experiments. The standard deviations of the D value were calculated.

2.5.3.5 Acid sensitivity.

The effect of acid on *Cronobacter* spp. viability was performed to determine the survival of *Cronobacter* in low level of pH according to investigation by Edelson-Mammel et al (2005). One to two colonies from a purity TSA plate were used to inoculate into 5 ml of TSB and incubated overnight at 37°C. The pH of liquid infant formula (Cow & Gate Premium 1) was adjusted to a pH value of 3.5 units with hydrochloric acid molarity. This was used to mimic the stomach acid. Three millilitres of overnight culture were inoculated into 12 ml of the acidified infant formula, and were then were distributed into three sterile tubes and incubated in a water bath at 37°C. Viable cells were enumerated after 0, 15, 30, 60, 90 and 120 min. At known time intervals of incubation 200µl was transferred from each tube, diluted in normal saline and plated in triplicate on TSA plates. Plates were incubated overnight at 37°C for after those viable cells were enumerated. All experiments were performed in triplicate from separate overnight cultures.

2.5.4 Genotyping and surface structure methods.

2.5.4.1 Multilocus sequence typing (MLST) analysis.

2.5.4.1.1 Genomic DNA extraction.

Genomic DNA was prepared according to the instructions of the manufacturer, 1.5 ml of culture grown overnight in TSB using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, UK). The purity and concentration of the extracted DNA was measured by using nanodrop 2000 (Thermo Scientific, UK). For polymerase chain reaction, the purity of DNA samples with minimum 260/280 nm values of 1.8 and concentration with minimum 260/230 nm values of 15ng were used, otherwise DNA was extracted again.

2.5.4.1.2 PCR amplification of MLST loci.

Amplification and nested sequencing primers for the MLST loci (Table 2.4) were described by Baldwin et al (2009). Each 25 µl amplification reaction mixture composed, 10 ng chromosomal DNA, 20 pmol forward and reverse primer (Sigma-Aldrich, UK), 1× PCR buffer (Promega, UK) complemented with 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide

triphosphates (dntps) and 1.25 U Taq polymerase (Promega, UK). The reaction condition of all the primers were as follows, Initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 2 min; followed by a final extension step of 72°C for 5 min.

Table 2.4. Primers sequences for the amplification and sequencing of the seven loci.

Gene	Putative Gene Product	Amplification primers	Sequencing primers	Product Size (bp)
atpD	ATP synthase β chain	CGACATGAAAGGCGACAT TTAAAGCCACGGATGGTG	CGAAATGACCGACTCCAA GGATGGCGATGATGTCTT	390
fusA	Elongation factor	GAAACCGTATGGCGTCAG AGAACCGAAGTGCAGACG	GCTGGATGCGGTAATTGA CCCATACCAGCGATGATG	438
glnS	Glutaminyl-tRNA synthetase	GCATCTACCCGATGTACG TTGGCACGCTGAACAGAC	GGGTGCTGGATAACATCA CTTGTTGGCTTCTTCACG	363
gltB	Glutamate synthase large subunit	CATCTCGACCATCGCTTC CAGCACTTCCACCAGCTC	GCGAATACCACGCCTACA GCGTATTTCACGGAGGAG	507
gyrB	DNA gyrase B	TGCACCACATGGTATTCG CACCGGTCACAAACTCGT	CTCGCGGGTCACTGTAAA ACGCCGATACCGTCTTTT	402
infB	Translation initiation factor IF-2	GAAGAAGCGGTAATGAGC CGATACCACATTCCATGC	TGACCACGGTAAAACCTC GGACCACGACCTTTATCC	441
Pps	Phosphoenol-pyruvate Synthase	GTCCAACAATGGCTCGTC CAGACTCAGCCAGGTTTG	ACCCTGACGAATTCTACG CAGATCCGGCATGGTATC	495

2.5.4.1.3 Agarose gel electrophoresis.

Agarose gel was used to visualize and size the amplified PCR products. The gel was prepared as follows: 1% (w/v) agarose (Fisher Scientific, UK) was dissolved in 1X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK). The solution was heated in a microwave oven to dissolve the agarose. 0.1 µ/ml (v/v) of SYBR® Safe DNA gel stain (Life Technologies – Invitrogen, UK) was added and dissolved well in the agarose solution. The gel was then dispensed into the tray of gel and left for 20 to 30 minutes to cool. After making wells, 10 µl of 1kb ladder (Promega, UK) was loaded as marker into two wells and 10 µl PCR products were loaded into each of the well. Gel electrophoresis was performed for forty minutes at one hundred V in 1X TAE buffer. The gel was then visualized under ultraviolet (UV) light. The DNA bands were observed using the InGenius® gel documentation system (Syngene, UK).

2.5.4.1.4 Purification of PCR product.

The amplified products were purified using the MinElute PCR Purification Kits (Qiagen, UK) according to manufacturer's protocol. The concentration and purity of the samples was checked using the Nanodrop 2000 (Thermo Scientific, UK). The purity of DNA samples with minimum 260/280 nm values of 1.8 and concentration with minimum 260/230 nm values of 15 ng were used. The products were finally eluded in 50 µl of molecular biology grade water (Fisher Scientific, UK).

2.5.4.1.5 DNA sequencing.

Samples were sequenced by using the ABI 3730XL sequencing machines by Eurofins MWG Operon (London, UK) and source Bioscience (Nottingham, UK). The nucleotide sequences were determined on each DNA strand using the nested sequencing primers.

2.5.4.2 Random amplified polymorphic DNA (RAPD-PCR).

2.5.4.2.1 RAPD-PCR amplification.

Amplification for the RAPD-PCR was described by Ye et al (2010). A 25-µL amplification reaction mixture contained of 50 ng of DNA template, 2.5 U of Taq polymerase, 10× buffers (2.5 µL), 200 µmol/mL of each deoxynucleoside triphosphate, 0.5 µmol/mL primer (CGCGTGCCAG), and water to get the volume to 25µL. The RAPD-PCR reaction condition were as follows: 95°C for 5 min, 36°C for 1 min, 72°C for 4 min, 35 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 4 min.

2.5.4.2.2 Agarose gel electrophoresis.

1.5% (w/v) agarose (Fisher Scientific, UK) was dissolved in 1X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK). 0.1 µ/ml (v/v) of SYBR® Safe DNA gel stain (Life Technologies – Invitrogen, UK) was added and dissolved well in the agarose solution. Eight microliter of 1kb ladder (Promega, UK) was loaded as marker into 3 wells and 8 µl PCR products were loaded into each of the well. Gel electrophoresis was performed for 1.5 h at 100 V in 1X TAE buffer. The gel was then visualized under UV light. The DNA bands were observed using the gel documentation system (Syngene, UK).

2.5.4.3 BOX- PCR amplification.

The BOX-PCR reaction condition was as follows, in the table 2.5

Table 2.5. The BOX-PCR reaction condition.

Step	Temperature (°C)	Duration	Number of cycles
Initial denaturation	94	120 sec	1
Denaturation	94	30 sec	30
Annealing	55	60 sec	30
Extension	72	480 sec	30
Final extension	72	480 sec	1
BOX Primer	5'-CTACGGCAAGGCGACGCTGACG-3'		

2.5.4.4 Variable number of tandem repeats analysis (VNTRA).

Identification of VNTRs. Potential tandem repeats (TRs) were first extracted from the genome of *C. sakazakii* SP291/ (ST4) using *in silico* simulation of molecular biology experiments program and the http://insilico.ehu.es/ website. Primers flanking STRs were designed using the Primer 3.0 software and were ordered from Eurofins MWG Operon company (London, UK). The primer name, primer sequence and repeat size for each locus corresponding to *Cronobacter sakazakii* SP291 are summarized in table 2.6.

Table2.6. Primers for PCR amplification of selected VNTR used in this study.

Primer name	Primer sequence	SSR motif	Repeat size	Product size (bp)
Tr1-F	AGGCGCCTGGATAATTCC	TGCGCCC	7	197
Tr1-R	TTCGGCCACCGTGACTAT			
Tr2- F	CACCAGTGGCGTATGCTG	CTGCGGT	7	239
Tr2-R	CTCCGAAGACGGGTGATG			
Tr3-F	GGCATGCTGAGCGTAGGT	GCTCCG	6	237
Tr3-R	CAGAACCACGACGGAACC			
Tr4- F	TTACCAGAATTTGGGCGG	GGCGTATCGGTCGGCGTATCCGTC	24	203
Tr4-R	CCGATACGCCAACGGATA			
Tr5- F	CGTATCGGTCGGCGTATC	GTATCGGTTGGC	12	248
Tr5-R	TTTTACCGCTGCCTCTGC			

Tr6-F	CGTATCGGTCGGCGTATC	TATCCGTTGGCG	12	248
Tr6-R	TTTTACCGCTGCCTCTGC			

2.5.4.4.1 VNTRA-PCR amplification.

A 25 µl final reaction volume contained, 0.1 µmol/ml dNTPs, 0.2 µmol/ml primers, 0.5 U Taq DNA polymerase and water to get the volume to 25 µL. The VNTR-PCR reaction condition was as follows: 10 min at 95˚C, followed by 30 cycles of three temperatures (15 secs at 95˚C, 1 min at 55~60˚C, 1 min at 72˚C) and then 10 min at 72˚C.

2.5.4.4.2 Agarose gel electrophoresis.

PCR products (5µl) were loaded in 1.5% agarose gels in 1X TAE buffer) at a voltage of 6 V/cm for ~3 h. The gels were stained in 0.1 µ/ml (v/v) of SYBR® Safe DNA gel stain (Life Technologies – Invitrogen, UK). The gel was then visualized under ultraviolet (UV) light. Then the DNA bands can be observed using the InGenius® gel documentation system (Syngene, UK). The 50 bp DNA Ladder was loaded in 3 wells of the gels to determine the size of the DNA fragments. The bands (STRs) were identified to be polymorphic if large differences between their PCR product sizes in the agarose gel electrophoresis were observed.

2.5.4.5 Molecular serotyping of *Cronobacter* O-antigens.

Serotype of *Cronobacter* spp. O-antigens was determined using multiplex PCR amplification with two sets of primers listed in table 2.7. The multiplex PCR was performed by mixing seven primers (table 2.7) in a final volume of 50 µl containing the following components: 1x DreamTag buffer; 2,5mM $MgCl_2$; 400µM concentrations (each) of dATP, dCTP, dGTP, and dTTP; 0,06 to 0,10 µM primer; and 1 U of DreamTaq DNA polymerase and DNA template (200 ng).

The following PCR conditions were used for amplification: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s (55°C for *C. turicensis* and *C. malonaticus* set of primers), and 72°C for 1 min, with a final extension at 72°C for 8 min. Five microliter of the PCR products were loaded in 1.5% agarose gel in 1X TAE buffer) at a voltage of 75V for ~2 h (Jarvis et al., 2011; Sun et al. 2012a).

Table 2.7. O-antigens PCR primers used in this study.

Spieces	serotype	Target gene	Primer Sequence	Product size (bp)	Reference
C. sakazakii	O:1	*wzy*	CCCGCTTGTATGGATGTT	364	Sun et al (2012)
			CTTTGGGAGCGTTAGGTT		
	O:2	*wzy*	ATTGTTTGCGATGGTGAG	152	
			AAAACAATCCAGCAGCAA		
	O:3	*wzy*	CTCTGTTACTCTCCATAGTGTTC	704	
			GATTAGACCACCATAGCCA		
	O:4	*wzy*	ACTATGGTTTGGCTATACTCCT	890	
			ATTCATATCCTGCGTGGC		
	O:5	*wzy*	GATGATTTTGTAAGCGGTCT	235	
			ACCTACTGGCATAGAGGATAA		
	O:6	*wzy*	ATGGTGAAGGGAACGACT	424	
			ATCCCCGTGCTATGAGAC		
	O:7	*wzx*	CATTTCCAGATTATTACCTTTC	615	
			ACACTGGCGATTCTACCC		
C. malonaticus	O:2	*wzx*	TGGCCCTTGTTAGCAAGACGTTTC	394	Jarvis et al (2011)
			ATCCACATGCCGTCCTTCATCTGT		
	O:1	*wzx*	AGGGGCACGGCTTAGTTCTGG	323	
			CCCGCTTGCCCTTCACCTAAC		
C. muytjensii	O:1	*wzx*	TGGCTGTCATGGTTTTCTTGC	258	
			TAGTTGGCACCATCAACGCC		
C. turicensis	O:1	*wzx*	AGGGGCACGGCTTAGTTCTGG	323	
			CCCGCTTGCCCTTCACCTAAC		
C. dublinensis	O:1	*wzm*	TCGTTTTGATGCTCTCGCTGCG	435	
			ACAAATCGCGTGCTGGCTTGAA		
	O:2	*wzm*	CTCGGTTCATGGATTTGCGGC	227	
			CAGCGTGAAAACAGCCAGGT		

2.5.4.6 Lipopolysaccharide profiling (LPS).

2.5.4.6.1 Extraction of lipopolysaccharide.

2.5.4.6.1.1 Cell lysis and proteinase K digestion methods.

Extraction and characterization of lipopolysaccharide, lysed cells and proteinase K digestion were performed with 12 strains as described previously by Yan et al (2015) with some modifications. In brief, overnight cultures were subcultured after 18 h into fresh prewarmed tryptone soy broth (TSB) at 37°C, bacterial suspensions (10^8 colony-forming units/mL) were centrifuged at 10,000×g for 5 min. The pellets were washed three times with phosphate-buffered saline (PBS) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The bacterial pellets were resuspend in 200µL Lysis buffer (100 ml, 1 M Tris-HCl [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate [SDS], 4 ml 2-mercaptoethanol and 0.003g bromophenol blue). The suspensions were incubated at 100°C for 10 mins, followed by adding 3 µL 20 mg/ml Proteinase K (Roche, Mannheim, Germany) and incubating at 60°C for 1h. The digested product could be stored at -20°C if required.

2.5.4.6.2 SDS-PAGE and silver staining.

Preparations of proteinase K digests were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 4% (wt/vol) stacking and a 15% (wt/vol) separating gel. Twenty-five microliters of extracted LPS were loaded in the gel under reducing condition at 60 mA for 2 hr using mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, California, USA). Molecular weight marker (precision plus proteinTM Dual color standards, BIO-RAD) was loaded in 3 wells of the gels to determine the size of the LPS fragments. Following electrophoresis, LPS was visualized by silver staining using silver stain kit (Thermo scientific, UK).

2.5.4.7 *C. sakazakii* K-capsule profiling.

K1 and K2 primers were designed based on capsular gene (*kpsS*) and were identified from the sequence information of *C. sakazakii* 658 (kpsS1) and 6 (kpsS2) respectively. Primers flanking K1and K2 were designed using the Primer 3.0 software, and were ordered from Eurofins MWG Operon company (London, UK). The primer name, primer sequence and product size are summarized in table 2.8.

Table 2.8. K-capsule type primer name, target gene, primer sequence and product size

K-type	Target gene	primer sequence	T_m	Product Size (bp)	Reference
K1	*kpsS1*	F- CGTATCACACCCTCGCTACT	59	248	This study
		R- TTAAACACCTGAAGCACCGC			
K2	*kpsS2*	F- TTATATGCAGTTGCCCGACA	59	120	This study
		R-TATGAACGATGATACGCCCA			

2.5.4.7.1 K-capsule -PCR amplification.

The multiplex PCR was performed by mixing two primers (K1 and K2) in a final volume of 50 µl containing the following components: 1x DreamTag buffer; 2,5mM MgCl2; 400µM concentrations (each) of dATP, dCTP, dGTP, and dTTP; 0,06 to 0,10 µM primer; and 1 U of DreamTaq DNA polymerase and DNA template (200 ng). The following PCR conditions were used for amplification: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30s, and 72°C for 1 min, with a final extension at 72°C for 8 min. Five microliters of the PCR products were loaded in 1.5% agarose gel in 1X TAE buffer) at a voltage of 70V for ~60 min.

2.5.4.8 *C. sakazakii* colonic acid profiling, CA-type

Primers were designed based on *galE* gene sequence (966 bp) using the Primer 3.0 software, and were ordered from Eurofins MWG Operon company (London, UK); table 2.9.

Table 2.9. CA-type primer name, target gene, primer sequence and product size.

CA-type	Target gene	primer sequence	T_m	Product Size (bp)	Reference
CA1	*galE*	F- GTCGGCACCAGACTGATTGA	60	429	This study
		R-TAAAGAACGGGTATCCGGCG			

2.5.4.8.1 Colanic acid CA-PCR amplification.

The PCR conditions were used for amplification: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final

extension at 72°C for 8 min. Five microliter of the PCR products were loaded in 1.5% agarose gel in 1X TAE buffer) at a voltage of 75V for ~60 min.

2.5.5 Role of the capsule in the virulence potential of *Cronobacter.*

2.5.5.1 Capsule isolation.

Bacterial cultures were grown overnight at 37°C and for a 48h at 21°C on agar plates containing infant formula ready to feed. Bacterial growth was then scraped into centrifuge tubes and sterile normal saline was added to balance the tubes and then centrifuged at 5000 rpm for 5 minutes. The supernatant was then discarded and the thick mucoid layer was transferred to weighed watch glasses, which were put into a drying oven overnight and then re-weighed. The dry mass was collected and stored at room temperature.

2.5.5.2 Sugar analysis.

The dry mass of capsule materials were analysed at the Faculty of Chemistry, Gdansk University, Poland. Capsule composition was analysed using carbohydrate nuclear magnetic resonance (NMR) spectroscopy. This method used to elucidate structure of monosaccharides, oligosaccharides, polysaccharides and other carbohydrate derivatives from synthetic and natural sources.

2.5.6 Whole genome study and comparative genomic analysis.

Genome comparisons were performed using WebACT comparative tool, artimes comparative tool (ACT) for genome alignment, which has been developed by Carver et al. (2005). In addition, BLAST searches were performed using *Cronobacter* BLAST research facility at (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_cronobacter_isolates&page=plugin&name=BLAST).

2.5.7 Statistical analysis.

Statistical analysis has been undertaken using unpaired t-test and ANOVA one way (GraphPad Prism Program Version 5.0) of the independent experiments of the data. The P value $p < 0.05$ assigned significantly to the significance variation results. Furthermore, those $p < 0.01$ and $p < 0.001$ were considered highly significant, and very highly significant respectively.

Chapter 3. Diversity of the *Cronobacter* genus from food and environmental sources as revealed by genotyping, clonality and surface structure.

3.1 Introduction.

Cronobacter species have been isolated from plant material such as wheat, rice, herbs, and spices and a wide range of environments, such as water, soil, food production environments, and many food products. More importantly, members of *Cronobacter* spp. have been isolated from the PIF, milk powder and milk powder production factories. Moreover, this genus is associated with infant and neonatal infections. Members of the genus *Cronobacter* can cause severe neonatal infections through the ingestion of contaminated PIF and milk powder, particularly in low-birth-weight infants (Holy and Forsythe, 2014).

In general, biochemical profiling can differentiate and identify bacterial species. However, molecular techniques are becoming popular as robust and reliable tools to study genomic diversity of bacteria and to track sources of infection and contamination. *Cronobacter* is a ubiquitous organism, and typing schemes are required both for epidemiological and environmental investigation. According to Iversen et al (2006) *Cronobacter* were defined with 15 biogroups, and biogroup 1 being the most common. The biotyping approach is now regarded as unreliable for speciation due to the initial use of biotype index strains that were attributed to incorrect *Cronobacter* species (Baldwin et al. 2009).

Understanding the molecular characterisation and genetic diversity of the *Cronobacter* genus is essential to confirm reliable detection and control methods are used. It also helps track the source of microbial contamination of food.

16S rRNA gene sequence analysis has been used for supporting bacterial taxonomic groupings and identification of organism. Therefore, an extensive database called the Ribosomal Database Project has been established and is available for free online (http://rdp.cme.msu.edu/) (Holy and Forsythe, 2014). However, due to the high genetic similarity of the genus *Cronobacter*, it is difficult to distinguish between *C. sakazakii* and

C. malonaticus based on the routine 16S sequence analysis. The development of a Multilocus Sequence Typing (MLST) scheme using 7 housekeeping genes (concatenated sequence length of 3036 bp) was applied to discriminate the species within the *Cronobacte*r genus (Baldwin et al. 2009; http://pubmlst.org/cronobacter/). The scheme is more discriminatory and robust than 16S sequence analysis. It has revealed a stable clonal nature of the virulent *C. sakazakii* strains and was also used in the description of the 2 new *Cronobacter* species: *C. universalis* and *C. condimenti* (Joseph et al. 2012d). The MLST scheme has so far identified 470 sequence types in the *Cronobacter* genus across a database of above 1700 strains (http://pubmlst.org/cronobacter/). The site is open access and contains the MLST protocols. Typing *Cronobacter* isolates to better understand the diversity of the *Cronobacter* genus has led to the development of many MLST schemes. The primary scheme used 7-loci (3036bp), however more recently ribosomal protein-MLST (rMLST) using 53 loci and COG-cgMLST using 1865 loci have been established (Forsythe et al. 2014).

The O antigen forms part of the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria and is one of the most variable constituents on the cell surface. The O-antigen serotyping scheme for *Cronobacter* species has been recently established, and the O-antigen gene clusters and specific primers for these species serotypes have been characterized. Serogroups profiling using PCR and whole genome sequences is the more common non-MLST profiling method (Jarvis et al. 2013; Sun et al. 2012; Ogrodzki and Forsythe, 2015). For epidemiological and outbreak investigation pulsed-field gel electrophoresis (PFGE) with two restriction enzymes (Xba1 and Spe1) is the most common method (Holy and Forsythe, 2014). The PFGE technique is one of the most widely used method for molecular typing and epidemic surveillance of bacteria. Nevertheless, PFGE is not an ideal method for bacterial surveillance due to the expense of the equipment, requirement of highly trained technicians, time requrred, limitation with clonal organisms and non-identical strains can give the same profile (Holy and Forsythe, 2014). The Centres for Disease Control and Prevention (CDC) is changing to alternative typing methods. Bacterial typing using whole genome sequencing (WGS) is an alternative to PFGE, such as the Illumina high-throughput HiSeq, offering simpler

workflows, high rate of resolution, and a universally applicable bacterial subtyping method (CDC, 2015).

Variable Number Tandem Repeat (VNTR) analysis a genotyping method based on polymerase chain reaction and sequencing, which distinguishes tandem sequence repeats that vary in copy numbers, may be useful for tracing and subtyping bacteria due to the simple process, cost and high-speed. Moreover, multi-locus variable number tandem repeat analysis genotyping is becoming an important DNA-based typing method for investigating strains that are related to outbreaks and infection (Mullane et al. 2007).

Clustered regularly interspaced short palindromic repeats (CRISPR) are segments of prokaryotic DNA containing short repetitions of base sequences. CRISPRs are found in more than 80% of archeal genomes and approximately 48% of eubacterial genomes (Grissa et al. 2006; Ogrodzki and Forsythe, 2016). This method has been applied to *Enterobacteriaceae* family such as *Yersinia, Salmonella* and *E. coli* in the for virulence-related, analysis phylogenetic and evolutionary (Fricke et al. 2011; Yin et al. 2013). Ogrodzki and Forsythe, (2016) identified the phylogenetic distribution of CRISPR–cas loci within the major pathovars of *C. sakazakii* including ST1, ST4, ST8 and ST12. This study reported that the CRISPR spacer array profiling have a greater discriminatory power compared with MLST, which will be of use to identify the source of contamination during *Cronobacter* outbreak investigations (Ogrodzki and Forsythe, 2016).

PCR-based methods targeting different bacterial genes are commonly used by different laboratories around the world. Moreover, PCR-assay targeting significant bacterial genes including capsular polysaccharide genes such as K-capsule type is widely used, because they are important both pathogenetically and taxonomically. *E. coli* produce more than 80 different capsular polysaccharide K-antigens. Kaczmarek et al (2014) reported that K1 antigen is a key virulence determinant of *E. coli* strains and has been associated with meningitis, bacteraemia and septicaemia, particularly neonatal cases. Furthermore, a rapid and specific PCR-based assay was used for the detection of group 2 *kpsM* variants in *E. coli* (Johnson and O'Bryan, 2004). Furthermore, a multiplex PCR targeting a capsular polysaccharide synthesis gene cluster of serotypes K1, K2 and K5 was evaluated using many of reference serotype strains of *Klebsiella*, and a panel of clinical isolates

subjected previously to conventional serotyping. The PCR assay showed a highly specific for these serotypes (Turton et al. 2008). Feizabadi et al (2013) suggest that PCR assay is a rapid and reliable method for identification of *K. pneumoniae* capsular both K1 and K2 serotypes.

The present study presented in this chapter will compare different molecular subtyping method and propose the most suitable method to determine the genetic relationship of *Cronobacter* isolates for the purpose of surveillance and outbreak investigation.

3.2 Results.

3.2.1 Multilocus sequence typing (MLST) clonality analysis.

The 7-loci multilocus sequence typing (MLST) scheme was applied in this study. In total 26 strains were analysed including three collections of food and environmental strains that had not previously been profiled. These strains were originated from the Czech Republic, Malaysia and the UK (Table 3.1).

The sequence types (ST) obtained for 26 strains are given in table 3.1. Twenty strains from the Czech Republic had been collected during 2011, they were isolated from food, ingredients, spice, herb and milk powder. Thirteen out of twenty isolates were *C. sakazakii*, three strains were *C. malonaticus,* two strains were *C. turicensis,* one strain was *C. muytjensii* and a single strain was *C. dublinensis.* Thirteen strains of *C. sakazakii* were identified as belonging to the 13 different sequence type (STs); ST4, ST8, ST13, ST20, ST23, ST42, ST64, ST198, ST233, ST245, ST263, ST405 and ST406. Three strains of *C. malonaticus* were identified as belonging to the three STs; ST60, ST139 and ST289, two strains of *C. turicensis* were identified as belonging to the two STs; strain 1880 as ST344 and strain 1895 as ST252, one *C muytjensii* strain 1877 was ST407 and one strains of *C. dublinensis* 1897 was ST213.

Three strains from the UK had been collected from food during 2013. These strains were *C. sakazakii*, and were identified as belonging to 3 different sequence types (ST), strain 1990 was ST264, strain 1992 was ST136 and strain 2027 was ST406.

Three environmental strains from Malaysia had been collected during 2012. These strains were *C. sakazakii,* and were identified as belonging to two different STs. Strains 1907 and 1908 were ST4 and strain 1906 was ST8 (Table 3.1).

Table 3.1. *Cronobacter* stains used in this part and their MLST profiles.

Strain	Species	Source	Country	Year	*atpD*	*fusA*	*glnS*	*gltB*	*gyrB*	*infB*	*pps*	ST	CC
1880	*C. turicensis*	Herb	Cz	2011	47	5	4	63	116	150	150	344	-
1895	*C. turicensis*	Ingredient	Cz	2011	86	22	104	120	117	116	152	252	-
1877	*C. muytjensii*	Ingredient	Cz	2011	35	62	36	180	45	114	217	407	-
1846	*C. malonaticus*	Ingredient	Cz	2011	12	7	8	8	10	16	43	60	-
1879	*C. malonaticus*	Spice	Cz	2011	13	13	64	74	72	23	88	139	-
1893	*C. malonaticus*	Ingredient	Cz	2011	57	40	100	125	61	115	151	289	-
1843	*C. sakazakii*	spice	Cz	2011	20	18	16	10	3	20	27	23	23
1844	*C. sakazakii*	Ingredient	Cz	2011	123	37	9	179	114	65	148	405	-
1845	*C. sakazakii*	Food	Cz	2011	10	37	9	3	115	56	126	233	-
1847	*C. sakazakii*	Milk powder	Cz	2011	16	8	9	32	21	113	149	245	-
1881	*C. sakazakii*	Ingredient	Cz	2011	16	8	13	40	15	15	10	64	64
1882	*C. sakazakii*	Ingredient	Cz	2011	16	14	24	17	25	33	25	20	20
1884	*C. sakazakii*	Herb	Cz	2011	16	18	13	119	88	73	25	263	263
1885	*C. sakazakii*	Herb	Cz	2011	16	1	13	178	21	5	21	406	264
1886	*C. sakazakii*	Spice	Cz	2011	5	1	3	3	5	5	4	4	4
1887	*C. sakazakii*	Food	Cz	2011	15	14	15	13	22	5	16	13	13
1888	*C. sakazakii*	Food	Cz	2011	1	8	7	5	8	15	10	8	8
1889	*C. sakazakii*	Ingredient	Cz	2011	3	8	3	3	18	46	127	198	52
1890	*C. sakazakii*	Ingredient	Cz	2011	48	17	10	69	71	5	81	42	-
1897	*C. dublinensis*	Ingredient	Cz	2011	23	63	78	102	83	102	114	213	-
1906	*C. sakazakii*	Environment	Malaysia	2012	1	8	7	5	8	15	10	8	8

1907	*C. sakazakii*	Environment	Malaysia	2012	5	1	3	3	5	5	4	4	4
1908	*C. sakazakii*	Environment	Malaysia	2012	5	1	3	3	5	5	4	4	4
1990	*C. sakazakii*	Food	UK	2013	16	1	13	39	21	5	21	264	264
1992	*C. sakazakii*	Food	UK	2013	16	18	9	5	3	73	87	136	-
2027	*C. sakazakii*	Ingredient	UK	2013	16	1	13	178	21	5	21	406	264

MLST typing technique involving seven loci, each isolate is given a combination of 7 allelic profile numbers. Every distinct combination of these profile numbers denotes a sequence type (ST) for the specific isolate.

Cz= Czech Republic. UK= United Kingdom. CC= Clonal complex.

A total of 26 strains identified as *Cronobacter* spp. have been clustered into 21 sequence type (STs), according to *Cronobacter* Multilocus Sequence Typing (MLST) database (http://pubmlst.org/cronobacter/), 14 of these sequence types had been previously reported, while 9 new STs were also identified; ST213, ST233, ST245, ST263, ST264, ST289, ST344, ST406 and ST407 (Figure 3.1).

3.2.1.1 Phylogenetic relationships.

Using the Maximum-Likelihood algorithm in MEGA 6, the phylogenetic tree based on the concatenated sequences of the seven housekeeping genes (*atpD, fusA, glnS, gltB, gyrB, infB,* and *ppsA*; 3036 bp) was constructed to reveal the connection between the different STs of *Cronobacter* spp. (Figure 3.1). The phylogenetic tree in figure 3.1 shows a clear phylogenetic relationship of *Cronobacter* spp. consisting of 26 *Cronobacter* strains isolated from food and environmental sources. The 26 strains were clearly divided into five groups. 19 *C. sakazakii* strains were clustered into the same group, these strains were divided into 15 clusters. Strains with the same ST were clustered together such as 1886, 1907 and 1908 (ST4), 1888 and 1906 (ST8), and 1885 and 2027 (ST406). Strains 1990 (ST264), and 1885 and 2027 (ST406) were closely related STs were belonged to the same clonal complex 264 (CC264). Only one allele (*gltB*) was different between ST406 and ST264 (16-1-13-178-21-5-21 and 16-1-13-39-21-5-21). Consequently, they are regarded as single locus variants (Table 3.1).

Three *C. malonaticus* strains 1846 (ST60), 1879 (ST139) and 1893 (ST289) were divided into 3 clusters, and two *C. turicensis* strains were divided into 2 groups based on their ST. *C. malonaticus* strains were closer to *C. sakazakii* strains than the other 3 species in the phylogenetic relationship (Table 3.1).

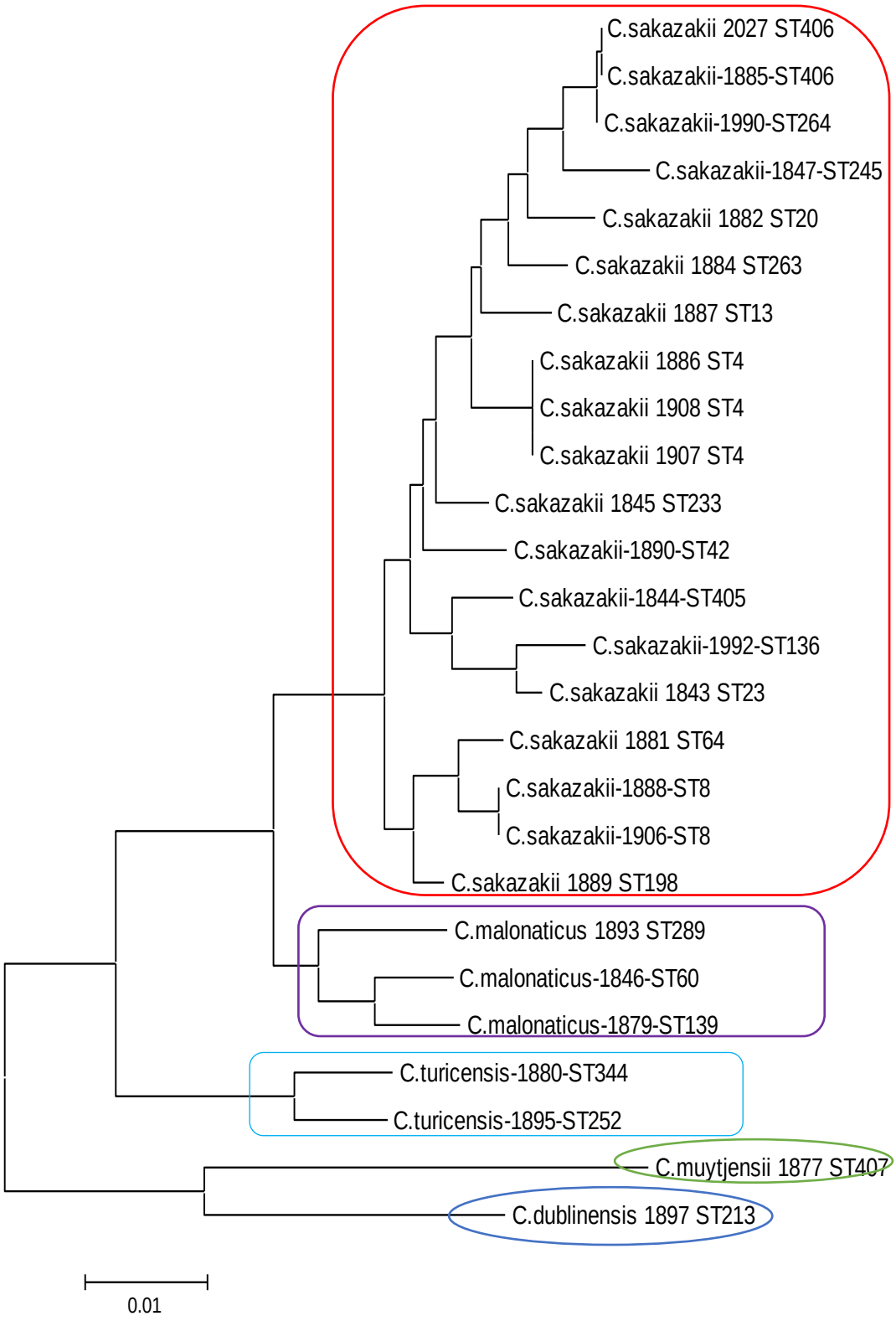


Figure 3.1. Maximum-Likelihood tree of the seven housekeeping genes (3036 base pair) for the *Cronobacter* genus. The STs is indicated at the leaf of each branch. MEGA6 with 1000 bootstrap replicates have been used to assess the quality of the tree produced.

3.2.2 RAPD-PCR patterns and genomic cluster analysis.

Sixty-one *Cronobacter* isolates were used in this study for both RAPD-PCR and BOX-PCR fingerprinting, these isolates covering 45 different sequence type (STs). These include 27 *C. sakazakii* strains (15 STs), nine *C. malonaticus* strains (6 STs), seven *C. dublinensis* strains (7 STs), nine *C. turicensis* strains (6 STs), four *C. muytjensii* strains with (4 STs), and five *C. universalis* strains (5 STs). These strains were chosen based on geographic diversity, date and source of isolation. Strains had been isolated from environment, water, food sources, and clinical. Strains were from 10 different countries (Netherlands, Brazil, United Kingdom, Australia, Slovakia, Czech Republic, Korea, Turkey, Malaysia and United States). The strains were isolated between 1950–2013 (Figure 3.2).

Molecular typing was performed by random amplified polymorphic DNA (RAPD) as previously described (Nazarowec-White and Farber, 1999). Amplified products were visualized following electrophoresis through a conventional 1.5% (w/v) agarose gel stained with ethidium bromide. The RAPD-PCR fingerprints of 61 *Cronobacter* isolates were shown in figure 3.2. All were analyzed by RAPD and a quantitative assessment of the genomic relationships was made based on the BioNumerics software (7.1) output. Each isolate produced banding patterns consisting of 3 to 11 amplified products, ranging in size from approximately 300 to 2700bp. In the RAPD-PCR fingerprints profiles, a 90% similarity cut-off was used to determine the strains clonality clustering.

The RAPD-PCR fingerprints of 61 isolates were grouped into 41 major clusters based on their sequence type (STs). Twenty-seven *C. sakazakii* strains were divided into 17 groups, strains with the same STs were grouped in the same cluster such as ST3, ST8 and ST406, the exception were *C. sakazakii* ST4 strains; 377, 551, 1907, 1908 and 1886 which divided into 2 groups, these strains were from the UK, Netherlands, Malaysia (1907 and 1908) and Czech Republic, respectively. Group 1 was strains 377, 551, 1907 and 1908; these strains were isolated from milk powder (377) and environmental sources (551, 1907 and 1908). The second group was a lone strain 1886, and was isolated from spice. The four *C. sakazakii* ST3 strains; 25, 26, 545 and 1106 were clustered together, strains (25 and 26) were isolated from follow up formula in 2004 from Korea, strain 545 from the Netherlands, and was isolated from infant formula (1988), and strain 1106 was isolated from weaning food in 2008 from the UK. *C. sakazakii* ST8 strains; 1906, 1283

and 1888 have been isolated from different sources and different country, these isolates produced identical RAPD profile. *C. sakazakii* strains 1885 and 2027 (ST406) and 1990 strain ST264 were closely related strains, only one band different was observed between these STs (figure 3.2). Other *C. sakazakii* strains were divided based on their sequence type.

The nine *C. malonaticus* strains; 35, 893 and 510 (ST7), 93 (ST29), 1369 (ST69), 101 and 1846 (ST60), 1879 (ST139), and 1893 (ST289) were isolated from different sources, countries and different date. These strains were clustered together based on their sequence type, except *C. malonaticus* ST7 strains (35, 893 and 510) which dived into 2 clusters, cluster 1 were strains; 35 and 893 and strain 510 was cluster 2. Moreover, the 9 *C. turicensis* strains*;* 57, 564, 566 and 109 (ST5), 92 (35), 1880 (ST344), 111 (ST24), 1553 (ST72) and 1895 (ST552) were isolated from different sources and different countries. These strains formed a distinct cluster based on their sequence type. However, C. *turicensis* ST5 strains (57, 564, 566 and 109) were separated into two clusters. Cluster 1 were strains 57, 564 and 566, strain 57 has been isolated from milk powder from the UK in 2004, while 564 and 566 strains have been isolated from clinical sources in 1975 (USA). Cluster 2 was a lone strain (109), this strain was isolated from herb (Figure 3.2).

The 7 *C. dublinensis* strains; 1458 (ST74), 1460 (ST76), 1461 (ST77), 1462 (ST70), 1463 (ST95), 1464 (ST78) and 1897 (ST213) all were divided into 6 groups in the basis of their sequence type, the exception were strains 1460 (ST76) and 1464 (ST78) which clustered together. Similarly, the 5 *C. universalis* strains, 1435 (ST51), 581 and 1905 (ST54), 1883 (ST137) and 96 (ST48) were cluster into four cluster, cluster one was strain 1435 (ST51). Cluster 2 was strain 96 (ST48), these two strains were more closely related compared to other *C. universalis strains.* Cluster 3 was strains 1883 (ST137), while cluster 4 was 581 and 1905 (ST54) strains, it was notable that these two strains formed a unique outgroup to the rest of the *C. sakazakii* strains 1881 (ST64) and 1882 (ST20). The 4 *C. muytjensii* strains; 1371 (ST403), 1877(ST407), 16 (ST247), and 1527 (ST75) formed a distinct cluster based on their sequence type (Figure 3.2). There is no correlation between the RAPD profile and the source, date and place of isolation was observed

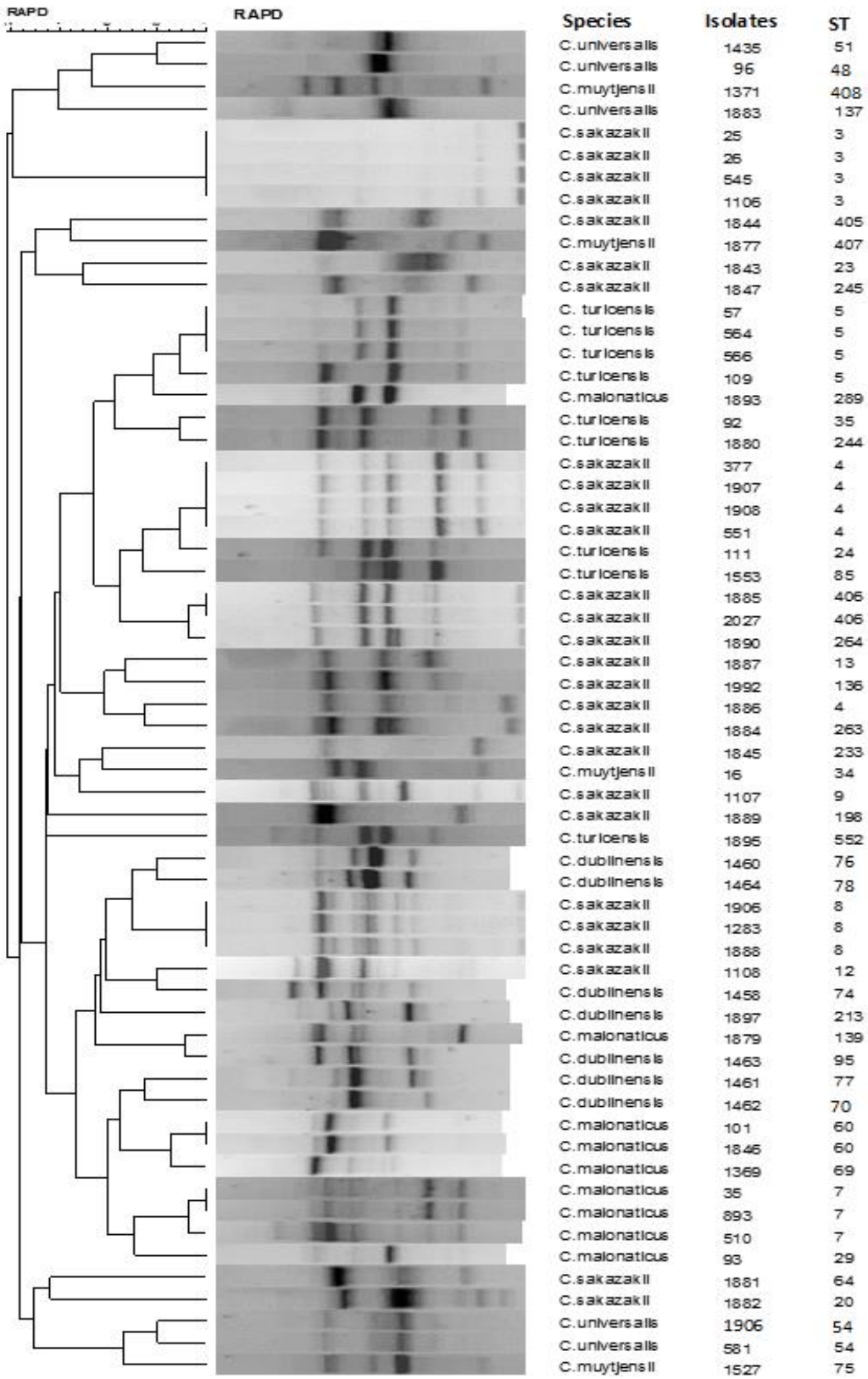


Figure 3.2. The agarose gel was analysed using BioNumerics software, version 7.1. Dice coefficient, unweight pair group method with arithmetic mean (UPGMA) for cluster analysis of

the RAPD-PCR fingerprints profiles of *Cronobacter* spp. The RAPD-PCR fingerprints of 61 isolates were grouped into 41 major clusters based on their sequence type (STs).

3.2.3 BOX-PCR fingerprinting.

Sixty-one *Cronobacter* isolates were typed by the BOX-PCR method. These isolates covering 6 species and 45 different sequence type. The fingerprints were composed of about 12 main bands with sizes ranging from approximately 200 bp to 2000 bp. BOX-PCR genotyping was carried out using BOX primer. The BOX-PCR fingerprints and dendrogram of 61 studied *Cronobacter* isolates are shown in Figure 3.3. In the BOX-PCR genotyping profiles, a 90% similarity cut-off was used to determine the strains clonality clustering. The result showed that fingerprints of 61 strains of *Cronobacter* were divided into 39 major clusters.

C. sakazakii strains (n=27) were divided into 15 groups, strains with the same STs were clustered together such as ST3 strains (25,26, 545 and 1106), ST4 strains (377,551,1886, 1907 and 1908), ST64 (1881), ST9 (1107), ST23 (1843), ST13(1847), ST13 (1887), ST233 (1845), ST263 (1884), ST12 (1108), ST198 (1889) and ST405 (1844). Nevertheless, in some cases strains with different STs were clustered in the same cluster, for instance strains 1906, 1283 and 1888 (ST8), 1885 and 2027 (ST406) and 1990 (ST264) were clustered together. *C. malonaticus* strains (n=9); 35, 893 and 510 (ST7), 93 (ST29), 1369 (ST69), 101 and 1846 (ST60), 1879 (ST139), and 1893 (ST289) were isolated from different sources, countries and different date. These strains were clustered together based on their sequence type, the exception was C. *malonaticus* ST7 strains (35, 893 and 510) which dived into two clusters, cluster 1 was strains; 35 and 893 and cluster 2 was strain 510.

C. turicensis strains (n=9)*;* 57, 564, 566 and 109 (ST5), 92 (35), 1880 (ST344), 111 (ST24), 1553 (ST72) and 1895 (ST552) formed a distinct cluster based on their sequence type, except strains 1553 (ST72) and 1880 (ST344) produced identical BOX profile (Figure 3.3). *C. dublinensis* strains (n=7); 1458 (ST74), 1460 (ST76), 1461 (ST77), 1462 (ST70), 1463 (ST95), 1464 (ST78) and 1897 (ST213) were divided into 2 groups. Group 1 were strains 1458 (ST74), 1460 (ST76), 1461 (ST77), 1462 (ST70), 1464 (ST78) and 1897 (ST213). While, group 1 was 1460 (ST76) strains, it was notable that this strain formed a unique outgroup to the rest of the *C. sakazakii* ST4 strains. *C. universalis* strains (n=5); 1435

(ST51), 581 and 1905 (ST54), 1883 (ST137) and 96 (ST48) were clustered into five clusters based on their sequence type. Moreover, *C. muytjensii* strains; 1371 (ST403), 1877(ST407), 16 (ST247), and 1527 (ST75) formed a distinct cluster based on their sequence type.

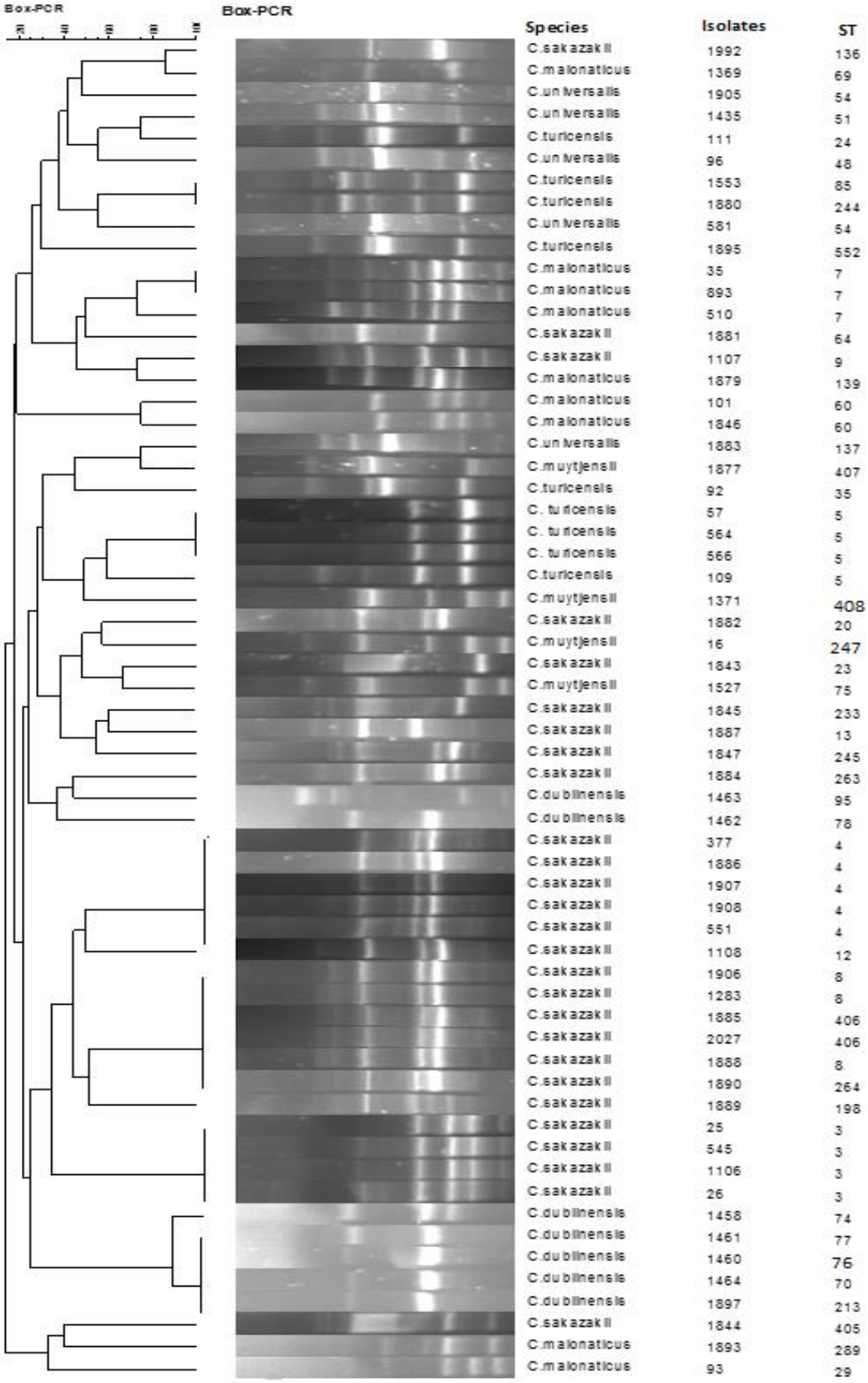


Figure 3.3. The agarose gel was analysed using BioNumerics software, version 7.1. Dice coefficient, unweight pair group method with arithmetic mean (UPGMA) for cluster analysis of the BOX-PCR fingerprints profiles of *Cronobacter* spp. The BOX-PCR fingerprints of 61 *Cronobacter* strains were divided into 39 major clusters.

3.2.4 Variable number of tandem repeats analysis (VNTRA).

C. sakazakii sequence type 4 (ST4) is strongly associated with meningitis and necrotizing enterocolitis in neonates and infants (Joseph and Forsythe, 2011; Hariri et al. 2013; Masood et al. 2015). Genetic diversity within *C. sakazakii* ST4 and tracking of infection sources needs a simple and rapid process, in order to reduce the risk infection in neonatal and infant cases. We were therefore interested in applying the VNTRA method to the *C. sakazakii* ST4 strains to find out more about the genetic diversity within the sequence type 4 (ST4). Nineteen strains of *C. sakazakii* were used in this study; these strains were chosen based on geographic diversity, date and source of isolation. The ST4 strains were isolated from 10 different countries (Ireland, Netherlands, France, Brazil, United Kingdom, Australia, Slovakia, Czech Republic, Malaysia and United States). These strains were isolated between 1950–2012 (Table 3.2). Only one strain 658 (ST1) was used for the purpose of comparison.

Table3.2. *C. sakazakii* strains used in Variable Number of Tandem Repeats Analysis (VNTRA).

Isolate number	Source	Country	Date of isolate	ST	Serotype
Sp291	Environment	Ireland	Unknown	4	O:2
377	Milk powder	UK	1950	4	O:2
551	Environment	Netherlands	1988	4	O:2
692	Clinical	France	1994	4	O:2
695	Clinical	France	1994	4	O:2
705	Clinical	France	1994	4	O:2
706	Clinical	France	1994	4	O:2
712	Clinical	France	1994	4	O:2
890	Infant formula	Brazil	2007	4	O:2
1105	Food	UK	2008	4	O:2
1480	Environment	Australia	2007	4	O:2
1564	Food	Slovakia	2010	4	O:3
1568	Clinical	USA	2011	4	O:2
1570	Clinical	USA	2011	4	O:2
1579	Clinical	USA	2011	4	O:2
1886	Food	Czech Republic	2011	4	O:3
1907	Environment	Malaysia	2012	4	O:4
1908	Environment	Malaysia	2012	4	O:4
658	Non-infant formula	USA	2001	1	O:1

3.2.4.1 Identification of VNTRs.

Potential short tandem repeats (STRs) were first extracted from the genome of *C. sakazakii* SP291 (ST4) using *in silico* simulation of molecular biology experiments program and the http://insilico.ehu.es website. A large number of STRs were identified, and a selection of these STRs were initially assessed for their potential value in a typing scheme. PCR primers flanking the selected STRs were designed using the Primer 3 software (Table 2.6).

3.2.4.2 Sequencing of loci.

In order to confirm that any length polymorphism of fragment was due to variations in the VNTR copy number, the purified PCR products amplified from selected strains were sequenced. The numbers of tandem repeats in each allele were recorded in table 3.3.

VNTRA-PCR genotyping was carried out using six VNTRA primers. Eighteen strains of *C. sakazakii* were used in this study, and first genome sequence of *C. sakazakii* strains 658 (ST1) was used for the purpose of comparison. These strains were isolated from a range of sources including; clinical, food and environmental sites.

VNTRA analysis for 18 *C. sakazakii* ST4 isolates and ST1 comparator.

The VNTRA-PCR fingerprints of 19 *C. sakazakii* strains are shown in table 3.3. The reference genome used was *C. sakazakii* SP291. Strains were grouped into 16 distinct groups (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O and P) based on the number of tandem repeats of 6 VNTR loci (Table 3.3).

VNTR analysis of the five *C. sakazakii* ST4 NICU strains.

Five strains (692, 695, 705, 706 and 712) were selected from the largest outbreak of *C. sakazakii* which occurred in a neonatal intensive care unit (NICU) in France (1994). These strains had been isolated between 24th May and 17st Jun, and were previously defined as pulsotype 2 (Caubilla-Barron et al. 2007). These strains had been isolated from different neonatal sites including trachea, stools, and one strain (712) has been isolated from prepared formula. The 4 strains (692, 695, 705,706) had been isolated from NEC cases at different stages, strain 695 was from a fatal case, of particular interest (Caubilla-Barron et al. 2007).

Short tandem repeats in loci 1 (STR1) was identical across all isolates and was composed of 2 tandem repeats, only strain 695 was composed of 1 tandem repeat. These strains divided into 2 groups based on STR2. Strains 705, 706 and 712 were composed of 5 tandem repeats, while 6 tandem repeats were found in strains 692 and 695. STR3 was identical across all 5 isolates and was composed of 5 tandem repeats. The five strains were divided into 2 groups based on STR4, one tandem repeat was found in strains 705 and 706, while no tandem repeats were noted in strains 692 695 and 712. Regarding STR5, no tandem repeats were noted in all strains, except strain 695, which composed of 1 tandem repeat. Strains divided into 2 groups based on STR6. One tandem repeat was noted in strains 705, 706 and 712, while 2 tandem repeats were recorded in strains 692 and 695.

In general, these strains were divided into 4 groups (B, C, D and E) based on the number of short tandem repeats of six VNTR loci (Table 3.3); group B was strain 695 with VNTRA profile (1-6-5-0-1-2). Group C was strain 692 with VNTRA profile (2-6-5-0-1-2), group D was strain 712 with VNTRA profile (2-5-5-0-0-1), and group E was strains 705 and 706 with VNTRA profile (2-5-5-1-0-1).

VNTR analysis of the three *C. sakazakii* ST4 CDC strains.

Three strains (1568, 1570 and 1579) from the Centres for Disease Control and Prevention (CDC) were collected during 2011. Strain 1568 associated with *Cronobacter* infection in Ohio, and has been isolated from opened PIF; strain 1570 has been isolated from CSF and was from Minnesota. Strain 1579 associated with *Cronobacter* infection in Missouri, and was from fatal case.

These strains were identical based on the number of tandem repeats of 6 VNTR loci (STR1, STR2, STR3, STR4, STR5 and STR6), and were composed of 2, 8, 5, 0, 0 and 2 tandem repeats respectively.

VNTR analysis of the *C. sakazakii* ST4 environmental strains.

Environmental strains; SP291, 551, 1480, 1907 and 1908 had been isolated from different countries. These strains were divided into 3 groups based on STR1. One tandem repeat was noted in strains 551, 1907 and 1908, and 2 tandem repeats was

noted in strain 1480. While five tandem repeats were found in strain SP291. Regarding STR2, 6 tandem repeats were recorded in strains 1480 and 1908, and 8 tandem repeats were recorded in strains 551 and 1907, while 5 tandem repeats were recorded in strain SP291. STR3 was identical across all environmental strains and were composed of 5 tandem repeats, only strain SP291 was composed of 4 tandem repeats. Strains were divided into 3 groups based on STR4, 2 tandem repeats were found in strains 1480, 1907 and 1908, and 3 tandem repeats were found in strain SP291, however no tandem repeats were noted in strain 551.

All strains were identical according to STR5 and was composed of 4 tandem repeats, except strain 1480 was composed of 5 tandem repeats. Regarding STR6, 3 tandem repeats were recorded in strains 1480, SP291 and 551, and 1 tandem repeat was recorded in strains 1908 and 1907. Strains were divided into 2 groups based on STR6, 3 tandem repeats were found in strains 551, 1480 and SP291, while 1 tandem repeat was found in strains 1907 and 1908.

Strains 1907 and 1908 were more closely related, and were identical for all 6 VNTR loci, the exception was STR2 which showed different profile; 1907 (1-8-5-2-4-1) and strain 1908 (1-6-5-2-4-1).

VNTR analysis of the 5 *C. sakazakii* ST4 food strains.

Strains (377, 890, 1105, 1564 and 1886) had been isolated from different sources of food including milk powder, Infant formula and other food as well as from different countries (Table 3.2). These strains were divided into 4 groups based on STR1 in loci 1, strains 890 was composed of 1 tandem repeat, 2 tandem repeats were recorded in strain 1105, and 4 tandem repeats were found in strains 1886 and 1564. While 5 tandem repeats was found in the earliest ST4 strain 377 which was isolated in 1950. Regarding STR2, 6 tandem repeats were recorded in strains 890 1105 and 1564, and 5 tandem repeats were recorded in strain 1886, while 4 tandem repeats was noted in strain 377. STR3 was identical across all five-food isolates and was composed of five tandem repeats. The 5 strains were divided into 2 groups based on short tandem repeats in loci 4; strains 377, 1564 and 1886 were composed of 3 tandem repeats. While, no tandem repeats were noted in strains 890 and 1105. According to STR in loci 5, strains 377 and

1886 were composed of 5 tandem repeats, and 4 tandem repeats was recorded in strain 1564. Whereas, no tandem repeats were noted in strains 890 and 1105. Moreover, these strains were divided into 3 groups based on STR6 in loci 6; 2 tandem repeats were found in strains 890, 1564 and 377, and 3 tandem repeats were found in strain 1886, nonetheless no tandem repeats was noted in strain 1105.

Strain 658 (ST1) showed a significantly different cluster (0-2-5-0-0-0) compared with *C. sakazakii* ST4 strains. STR2 and STR1, being the most variable VNTRs respectively (Figure 3.4, 3.5).

STR2 was the main contributor to the separation of the *C. sakazakii* sequence type 4 (ST4) strains, while STR3 showed less variation between *C. sakazakii* strains (ST4); Figure 3.4, 3.5, 3.6. The predicted band size of short tandem repeats (STR1, STR2 and STR3) on gel electrophoresis was nearly in agreement with the final VNTRA profile (sequences of PCR products).

Table 3.3 **.** Diversity for six variable number of tandem repeats (VNTRs) in *C. sakazakii* ST4 strains. Strains were sorted based on Final VNTRA profile.

NTU	Source	Country	Date of isolate	ST	O-antigen	Variable number of tandem repeats						Final VNTRA profile	VNTRA Cluster
						STR1	STR2	STR3	STR4	STR5	STR6		
658	Non- infant formula	USA	2001	1	O:1	0	2	5	0	0	0	0-2-5-0-0-0	A
695	Clinical	France	1994	4	O:2	1	6	5	0	1	2	1-6-5-0-1-2	B
692	Clinical	France	1994		O:2	2	6	5	0	0	2	2-6-5-0-0-2	C
712	Clinical	France	1994		O:2	2	5	5	0	0	1	2-5-5-0-0-1	D
705	Clinical	France	1994		O:2	2	5	5	1	0	1	2-5-5-1-0-1	E
706	Clinical	France	1994		O:2	2	5	5	1	0	1	2-5-5-1-0-1	
551	Environment	Netherlands	1988		O:2	1	8	5	0	4	3	2-8-5-0-4-3	F
1908	Environment	Malaysia	2012		O:4	1	6	5	2	4	1	1-6-5-2-4-1	G
1907	Environment	Malaysia	2012		O:4	1	8	5	2	4	1	1-8-5-2-4-1	H
890	Infant formula	Brazil	2007		O:2	1	6	5	0	0	2	1-6-5-0-0-2	I
1480	Environment	Australia	2007		O:2	2	6	5	2	5	3	2-6-5-2-5-3	J
1105	Food	UK	2008		O:2	2	6	5	0	0	0	2-6-5-0-0-0	K
1568	Clinical	USA (CDC)	2011		O:2	2	8	5	0	0	2	2-8-5-0-0-2	
1570	Clinical	USA (CDC)	2011		O:2	2	8	5	0	0	2	2-8-5-0-0-2	L
1579	Clinical	USA (CDC)	2011		O:2	2	8	5	0	0	2	2-8-5-0-0-2	
1886	Food	CZ	2011		O:3	4	5	5	3	5	3	4-5-5-3-5-3	M
1564	Food	Slovakia	2010		O:3	4	6	5	3	4	2	4-6-5-3-4-2	N
377	Milk powder	UK	1950		O:2	5	4	5	3	5	3	5-4-4-3-5-3	O
Sp291	Environment	Ireland	Unknown		O:2	5	5	4	3	4	3	5-5-4-3-4-3	P

VNTRA-PCR genotyping was carried out using six VNTRA primers. Eighteen strains of *C. sakazakii* were used in this study, and first genome sequence of *C. sakazakii* strains 658 (ST1) was used for the purpose of comparison. The numbers of tandem repeats in each allele were recorded in this table.

ST: sequence type. STR: short tandem repeats. VNTRA: Variable number of tandem repeats analysis.

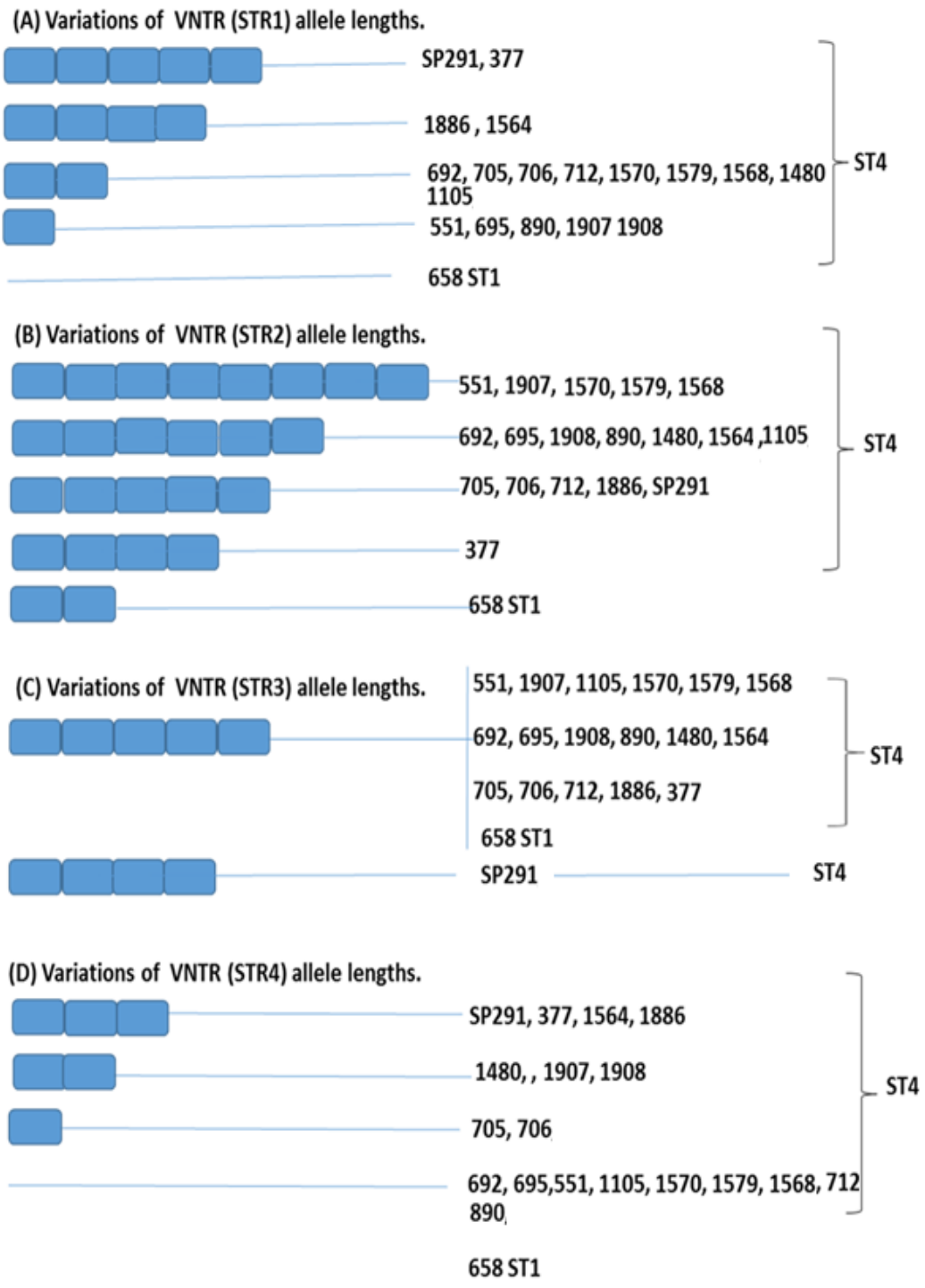


Figure 3.4. Variations of four VNTR allele lengths in 19 individuals (strains).
STR1, STR2, STR3 and STR4. (A) Variations of VNTR allele lengths (STR1, TGCGCCC) in 19 individuals (B) Variations of VNTR allele lengths (STR2, CTGCGGT) in 19 individuals. (C) Variations of VNTR allele lengths (STR3, GCTCCG) in 19 individuals. (D) Variations of VNTR allele lengths (STR4, GGCGTATCGGTCGGCGTATCCGTC) in 19 individuals.

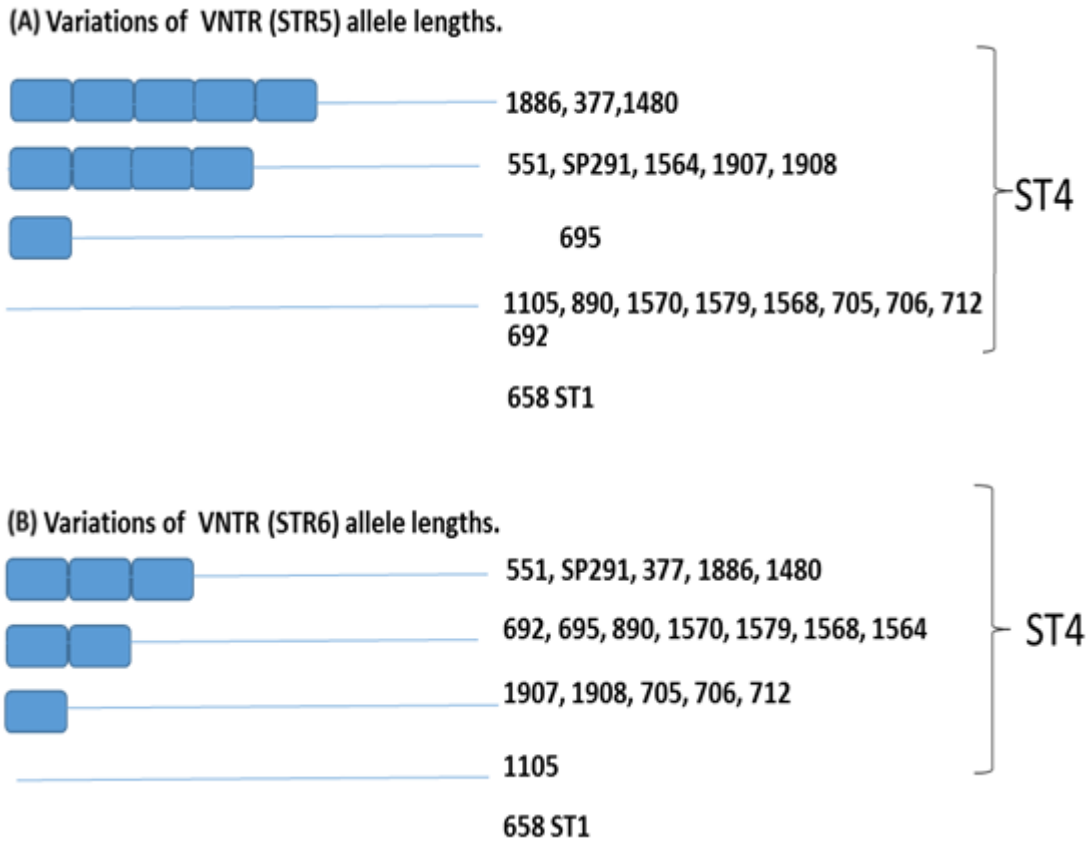
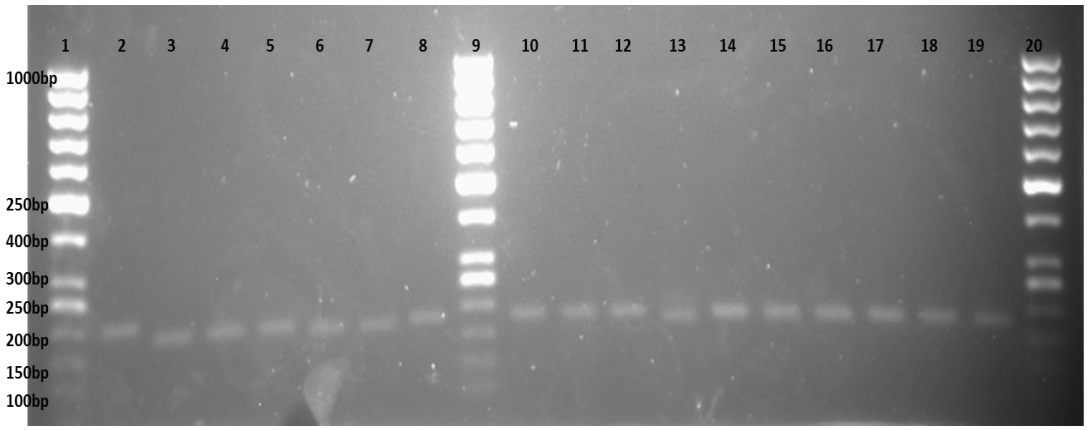
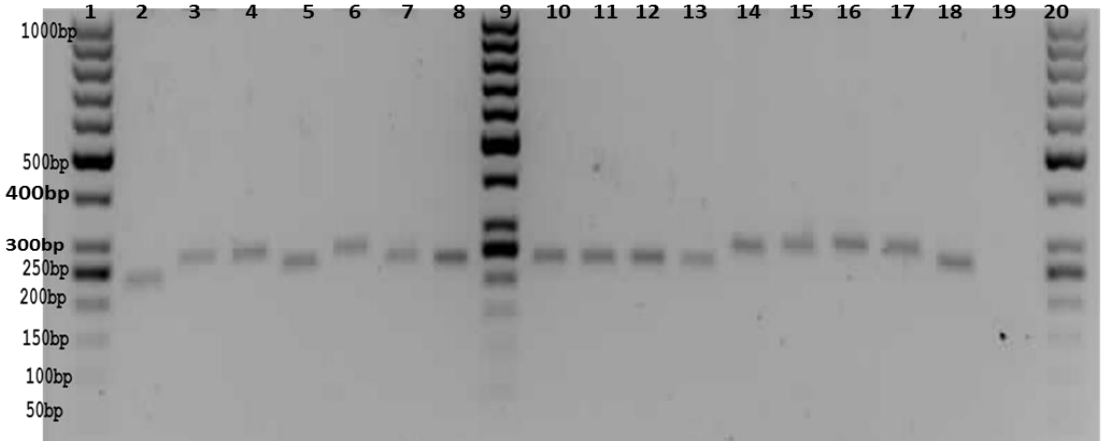


Figure 3.5. Variations of 2 VNTR allele lengths in 19 individuals (strains).
STR5 and STR6. (A) Variations of VNTR allele lengths (STR5, GTATCGGTTGGC) in 19 individuals. (B) Variations of VNTR allele lengths (STR6, TATCCGTTGGCG) in 19 individuals.

A. STR1



B. STR2



C. STR3

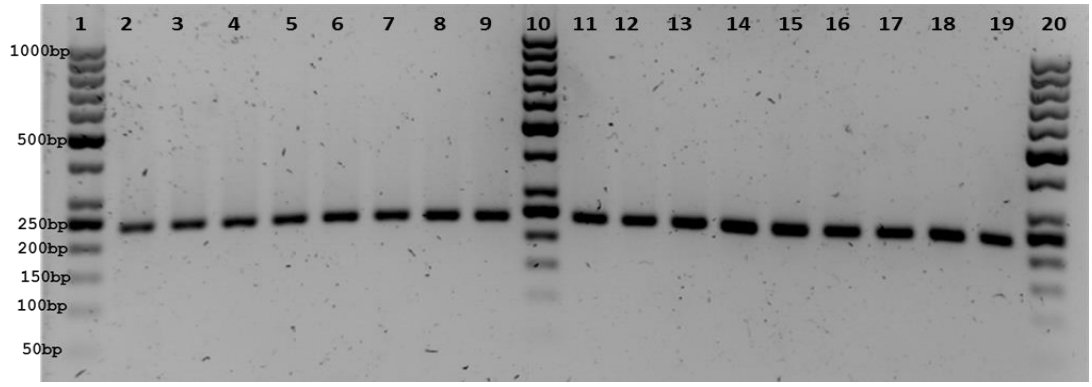


Figure 3.6. The agarose gel electrophoresis for PCR products of three VNTR loci (STR1, STR2 and STR3).

(A) The PCR products of STR1 Lanes: left to right, 50bp DNA Ladder Marker, 377, 1105, 1564, 1586, 1907, 1908, 50bp DNA Ladder Marker, 705, 706, 712, 692,695, 1570, 1568, 1579, 551, 890, 1480 and 50bp DNA Ladder Marker. (B) The PCR products of STR2 Lanes: left to right, 50bp DNA Ladder Marker, 377, 1105, 1564, 1886, 1907, 1908, 705, 50bp DNA Ladder Marker, 706, 712, 692,695, 1570, 1568, 1579, 551, 890, negative control and 50bp DNA Ladder Marker. (C) The PCR products of STR3 Lanes: left to right, 50bp DNA Ladder Marker, 377, 1105, 1564, 1886, 1907, 1908, 705, 50bp DNA Ladder Marker, 706, 712, 692,695, 1570, 1568, 1579, 551, 890, negative control and 50bp DNA Ladder Marker.

3.2.4.3 Phylogenetic tree for 18 *C. sakazakii* ST4 strains.

Using the Maximum-Likelihood algorithm in MEGA 6, the phylogenetic tree based on the concatenated sequences of the 6 VNTR loci (STR1, STR2, STR3, STR4, STR5 and STR6) was constructed to reveal the connection between the *C. sakazakii* ST4 strains. The reference strain used was *C. sakazakii* SP291. The 18 (ST4) strains were clearly divided into 15 groups. The VNTRA phylogenetic tree of *C. sakazakii* ST4 (Figure 3.7) indicated that most of the *C. sakazakii* ST4 (n=18) strains clustered relatively close to the reference strain. Strains 705 and 706 were clustered into the same cluster; these strains were isolated from the same baby. Moreover, CDC strains 1568, 1570 and 1579 were clustered together. Strains 1907 and 1908 were more closely related, and were identical for all 6 VNTR loci, the exception was STR2 which showed different profile; 1907 (1-8-5-2-4-1) and strain 1908 (1-6-5-2-4-1). These strains are notable as it is serotype O:4 unlike other ST4 isolates which are O:2 and O:3.

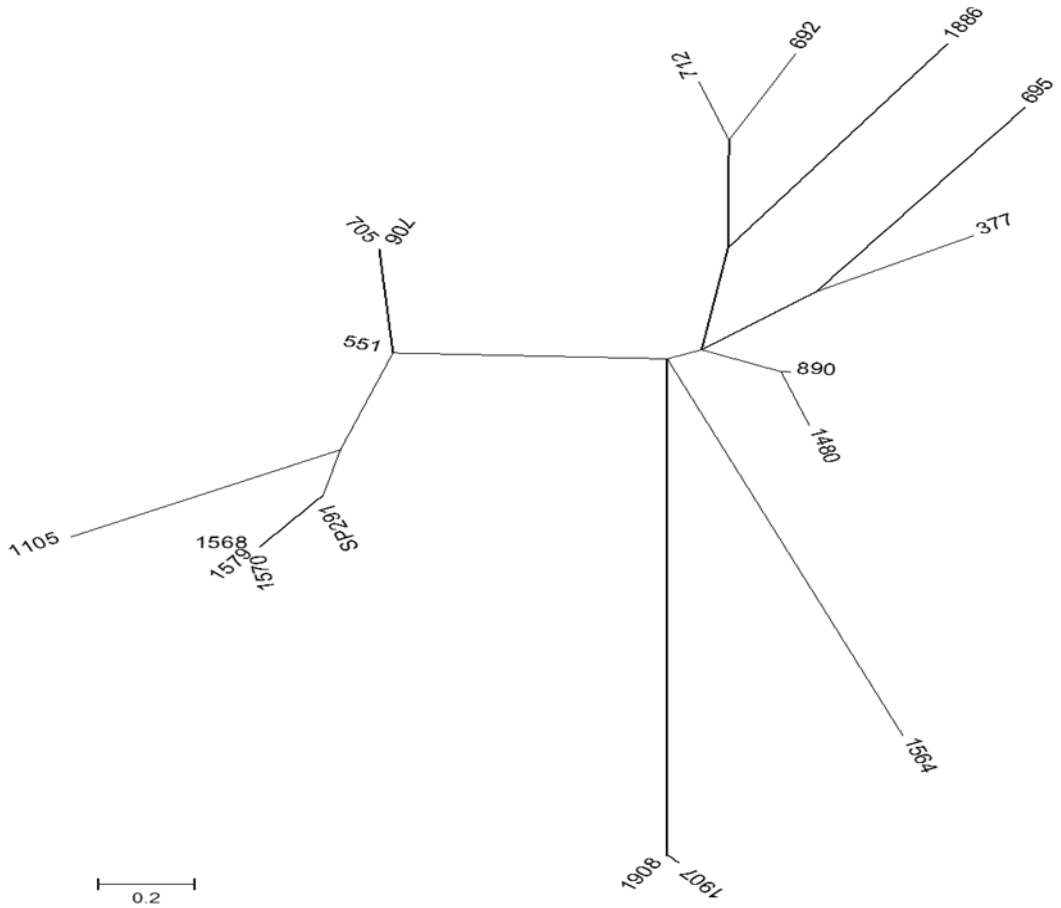


Figure 3.7. The VNTRA based phylogeny of 18 ST4 strains.
Phylogenetic tree for 18 strains constructed by the software MEGA 6.0 based on 6 VNTR loci.

3.2.5 *Cronobacter* O-antigen diversity.

The O-antigen is a major surface antigen. Genes involved in O-antigen synthesis are in the *rfb* locus between the flanking genes *gnd* and *galF,* this locus contains between six and nineteen genes, and varies between 6–20 kb. The locus varies in size for each serotype according to the sugar composition and complexity of structure. Ogrodzki and Forsythe (2015) stated that *gnd* (6-phosphogluconate) and *galF* (UDP-glucose pyrophosporylase) sequences polymorphism analysis could be successfully used as an alternative to RFLP for serotyping. Therefore, the sequence polymorphisms in the O-antigen flanking genes *gnd* and *galF* were evaluated for *Cronobacter* strain typing.

Serotyping PCRs. Fifty-one *Cronobacter* isolates were used in this study including, 25 *C. sakazakii*, 7 *C. malonaticus,* 8 *C. dublinensis*, 4 *C. muytjensii*, 6 *C. turicensis* strains, and the single *C. condimenti* strain (Table 2.1). Strains were determined using the previous PCR methods (Jarvis et al. 2011; Sun et al. 2012a). The results of the O-antigen serotyping for 51 strains are included in table 3.4, 3.9.

Genomic investigation. *In silico* analyses were carried out using (n=41) *Cronobacter* genomes accessible at the PubMLST *Cronobacter* database (www.pubmlst.org/*cronobacter*/). The *Cronobacter* genomes included in the study were *C. sakazakii* strains; 1844, 1882, 1992, 1886, 1888, 1906, 1105, 377, 658, 1885, 2027, 1890, 1847, 1845, 1881, 1887, 1283, 1889, 1108 and 1908. Four *C. malonaticus* strains; 101, 510, 1846 and 1879. Four *C. turicenesis* strains; 92, 1553, 1880 and 1895, seven *C. dublinensis* strains; 1210, 1458, 1460, 1461, 1462, 1463 and 1897. Four *C. muytjensii* strains; 16, 1371, 1877 and 1527, and the single *C. condimenti* strain 1330 (Table 2.1).

Serotyping of *C. sakazakii* strains, 25 strains divided into four O-antigen serotypes O:1, O:2, O:3 and O:4. Three strains (1847, 1888 and 1906) were O:1, thirteen strains (377, 1105, 1107, 1843, 1845, 1881, 1884, 1885, 1887, 1890, 1990, 1992 and 2027) were O:2. Five strains (1283, 1564, 1844, 1882 and 1886) were O:3, and four strains (1108, 1889, 1907 and 1908) were O:4. Serotype O:2 was the most dominant serotype in *C. sakazakii* strains (13 strains, 52%). However, *C. sakazakii* O:5, O:6 and O:7 specific primers gave negative results for all tested strains.

Serotyping of *C. malonaticus* strains, seven strains separated into two O-antigen serotypes O:1, O:2. Five strains; 93, 101, 1369, 1846 and 1893 were CM O:1 and two strains; 510 and 1879 were CM O:2. Serotype CM O:1 was the most dominant serotype in *C. malonaticus* strains (five strains,71.4%).

Serotyping of *C. dublinensis* strains, eight strains were divided into 2 O-antigen serotypes. Strains 1210, 1458, 1461, 1462 and 1463 were Cdub O:1 (62.5%), while three strains 1460, 1464 and 1879 were Cdub O:2.

Serotyping of *C. muytjensii* strains, three O-antigen serotypes were recorded in five *C. muytjensii* strains. Strains; 1527 and 1877 were Cmuy O:1 serotype (50%), and strain 1371 was Cmuy O:2 serotype (25%); while strain 16 was Cmuy O:3 (25%).

Serotyping of *C. turicenesis* strains, six strains were separated into two O-antigen serotypes Ctur O:1 and O:3. Three strains 92, 1553 and 1880 were CturO:3 (50%), one strain 1895 was CturO:1, and other 2 strains 109 and 111 (non-available genome) showed negative result with *C. turicenesis* O-antigen PCR assays (not determined).

Genomic investigation. According to Ogrodzki and Forsythe (2015), the flanking genes *galF*and *gnd* were selected to investigate the diversity of the O-antigen region in *Cronobacter* species.

3.2.5.1 Profiling of *galF* and *gnd* locus.

According to the table (3.4), a total of 37 alleles were observed at the *gnd* locus. While, 33 alleles were observed at the *galF* locus. Sixteen and thirteen alleles were observed at the *gnd* and *galF* locus respectively within 25 *C. sakazakii* strains, these covering 17 STs and 4 serotypes (O:1, O:2, O:3 and O:4), and only 1 allele was observed at both *gnd* and *galF* locus within 2 STs of 4 *C. sakazakii* O:4 strains (*gnd*14-*galF*13). Four alleles were observed at both *gnd* and *galF* locus within 7 *C. malonaticus* strains, covering 6 STs and 2 serotypes (O:1 and O:2). While, 8 and 7 alleles were observed at the *gnd* and *galF* locus respectively within 8 *C. dublinensis* strains, covering 6 STs and 2 serotypes (O:1 and O:2). Moreover, 4 alleles were observed at both *gnd* and *galF* locus within 4 *C. muytjensii* strains, covering 4 STs and 3 serotypes (O:1, O:2 and O:3), also 4 alleles were observed at both *gnd* and *galF* locus within 6 *C. turicensis* strains,

covering 4 STs and 2 serotypes (O:1 and O:3). However, the single *C. condimenti* strain showed profile (*gnd* 9 and *galF* 34) (Table 3.4).

In general, there is a variation in *gnd* and *galF* alleles profiling within the same sequence type and within the same serotype, except strains in serotype O:4 (1108-ST12, 1889-ST198 and 1908-ST4) which showed the same *gnd* and *galF* alleles profile (*gnd* 14 and *galF* 13). This variation might be related to the sugar composition and complexity of structure or the gene order in the O-antigen gene cluster (Figure 1.2, 3.9). Therefore, the LPS of 12 *C. sakazakii* strains belonging to the main serogroups, O:1, O:2, O:3 and O:4, were extracted and subjected to SDS-PAGE, and the gel was analysed using BioNumerics software (version 7.1), in order to investigate the correlation between the sequence type and the variation of O-antigen serotype (within the LPS profiling). This will be further discussed in the lipopolysaccharide profiling of this study (section 3.3.6).

Table 3.4. Distribution of *Cronobacter* serogroups among 51 non-clinical strains.

Species	Serotype	*galF* alleles	*gnd* alleles	*C. sakazakii*	*C. malonaticus*	*C. dublinensis*	*C. muytjensii*	*C. turicnesis*	*C. condimenti*	Number of STs	Total of strains
C. sakazakii	O:1	2,4,45	1,3,58	3	-	-	-	-	-	3	
	O:2	3,25,26,33,47, 50	2,28,62,37,23,47 ,61	13	-	-	-	-	-	11	
	O:3	4,48,21	45,46,3,60,23	5	-	-	-	-	-	4	
	O:4	13	14	4	-	-	-	-	-	2	
	O:5	-	-	-	-	-	-	-	-	-	
	O:6	-	-	-	-	-	-	-	-	-	
	O:7	-	-	-	-	-	-	-	-	-	
C. malonaticus	O:1	24,6	27,40	-	5	-	-	-	-	4	
	O:2	5,59	18,39	-	2	-	-	-	-	2	
	O:3	-	-	-	-	-	-	-	-	-	
C. dublinensis	O:1	12,39,38,40,43	12,48,44,52,57,5	-	-	5	-	-	-	5	
	O:2	29,52	50,51	-	-	3	-	-	-	3	
C. muytjensii	O:1	44,51	53,55	-	-	-	2	-	-	2	
	O:2	37	43	-	-	-	1	-	-	1	
	O:3	10	10				1		-	1	
C. turicnesis	O:1	49	49	-	-	-	-	1	-	1	
	O:2	-	-	-	-	-	-	-	-	-	
	O:3	20,27,30	22,30,34	-	-	-	-	3	-	3	
C. condimenti	O:1	34	9	-	-	-	-	-	1	1	
ND	-	-	-	0	0	0	0	2	0	-	
Total		33	37	25	7	8	4	6	1	41	51

Serotyping PCRs. Fifty-one *Cronobacter* isolates were used in this study, *In silico* analyses were carried out using (n=41) *Cronobacter* genomes accessible at the PubMLST *Cronobacter* database (www.pubmlst.org/cronobacter/). ND, not determined (negative with *Cronobacter* PCR assays).

3.2.6 Lipopolysaccharide (LPS) profiling.

Lipopolysaccharide is a main component of the outer membrane of Gram-negative bacteria. LPS consist of many oligosaccharide units (individual O units containing of between 3 and 6 sugars) with usually between 10 and 13 repeats. It is a virulence factor produced by many bacteria and released by the organism during an infection. In this study, a total of 12 clinically significant strains, covering the four main serotypes and the main sequence types which are associated with clinical cases of the *C. sakazakii* strains were studied for their LPS profile. This was silver staining of LPS extracts, and then the SDS- PAGE gel was analysed using BioNumerics software, version 7.1 (Table 3.5).

Table 3.5. *C. sakazakii* isolates used in lipopolysaccharide (LPS) profiling.

NTU:NO	Country	Source	Year	Serotype	*galF* allele	*gnd* allele	ST	Clonal complex
658	USA	Non-infant formula	2001	O:1	2	1	1	CC1
1888	Czech Republic	Food	2011	O:1	4	3	8	CC8
1906	Malaysia	Environmental	2012	O:1	4	3	8	CC8
377	UK	Milk powder	1950	O:2	3	2	4	CC4
1105	UK	Weaning food	2008	O:2	3	2	4	CC4
713	France	Infant formula	1994	O:2	25	28	13	CC13
1564	Slovakia	Food	2010	O:3	50	61	4	CC4
1886	Czech Republic	Spice	2011	O:3	50	61	4	CC4
520	Czech Republic	Clinical	1983	O:3	22	24	12	-
1108	UK	Weaning food	2008	O:4	13	14	12	-
1907	Malaysia	Environmental	2012	O:4	13	14	4	CC4
1908	Malaysia	Environmental	2012	O:4	13	14	4	CC4

3.2.6.1 Strain's screening and selection.

Strains of *C. sakazakii* were selected based on the diversity of their sequence type (ST), which associated with clinical significance including; ST1, ST4, ST8, ST12 and ST13, type of O-antigen serotype (O:1, O:2, O:3 and O:4), date of isolation and geographic diversity, in order to investigate the correlation between the sequence type and O-antigen serotype within the LPS profiling. SDS-PAGE gel was analysed using BioNumerics software (version 7.1). To the best of our knowledge, this is the first study to validate these differences using Bionumerics software. Table 3.5 lists the details of the strains that have used in this part of study.

3.2.6.2 *C. sakazakii* LPS analysis.

LPS of 12 *C. sakazakii* strains belonging to 4 serogroups, O1, O2, O3 and O4, were extracted and subjected to SDS-PAGE. The corresponding banding patterns were visualized by silver staining, and the profile of LPS molecules were analysed by comparison with sequence type profile (ST) and serogroups (O-antigen). The structure of *C. sakazakii* LPSs showed different molecular-mass bands. In order to clarify this variation, SDS- PAGE gel was analysed using BioNumerics software (version 7.1).

The result demonstrated that LPS fingerprints were composed of several bands (18 to 24 bands), and the 12 *C. sakazakii* strains were split into 4 groups based on the correlation coefficient of 90 %. As shown in figure 3.10, the LPS profile of 12 strains formed four clusters based on their O-antigen serotype (O:1, O:2, O:3 and O:4), and each cluster was sub-divided into 2 branches based on the *galF* and *gnd* allele profile (Figure 3.8).

3.2.6.3 Correlation between the LPS profile and serotypes.

LPS analysis of *C. sakazakii* serotyping O:1 cluster. Strains belong to serogroup O:1; 1888, 1906 and 658 were sub-divided into 2 clusters based on *galF* and *gnd* profile. The first group was strains; 1888 and 1906 (ST8-CC8) with profile *galF*4-*gnd*3, and the second group was strain 658 (ST1-CC1) with profile *galF*2-*gnd*1.

LPS analysis of *C. sakazakii* serotyping O:2 and O:3 clusters. The LPS analysis of three strains belong to serogroup O:2 (377, 1105 and 713) were also sub-divided into 2 branches; branch 1 was strains 377 and 1105 (ST4) with profile (*galF*3-*gnd*2), and branch 2 was strain 713 (ST13) (*galF*25-*gnd*28). As well as serogroup O:3 strains (1886, 1564 and 520) were sub-divided into 2 groups; group1 were ST4 strains 1886 and 1564 (*galF*50-*gnd*61) and group2 was strain 520 ST12 (*galF*22-*gnd*24), figure 3.8, 3.10 and 3.11).

LPS analysis of *C. sakazakii* serotyping O:4 cluster. The LPS analysis of serogroup O:4 strains; 1907 and 1908 (ST4), and 1108 (ST12) were nearly grouped in the same cluster, and all shared the same *galF* and *gnd* profile (*galF*13-*gnd*14).

Using BioNumerics software, the LPS of 12 selected strains were compared with their assigned serogroups and sequence type. The analysis showed that there was no

correlation between sequence type (ST), clonal complex (CC) and the LPS profiling. For example, six *C. sakazakii* belong to ST4 strains; 1886, 1564, 1908, 1907, 377 and 1105 were divided into 3 groups according to their LPS profile on the basis of their O-antigen profile. This was also noted within 2 *C. sakazakii* ST12 strains. In contrast, a strong correlation between the LPS profiling and type of serotype was observed (Figure 3.8). Moreover, each serogroup were sub-divided into 2 groups based on the *gnd* and *galF* profile. This suggests that PCR-based serotype method can be further sub divided.

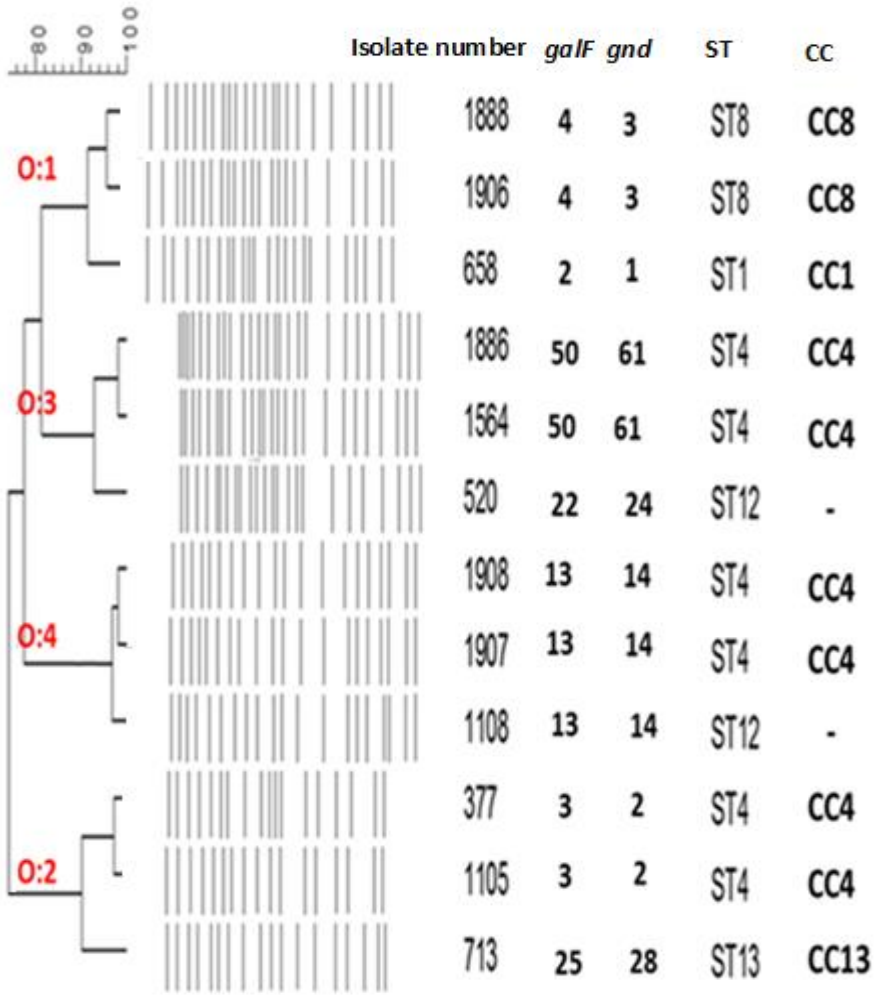


Figure 3.8. LPS profiles of twelve selected *C. sakazakii* strains.
Dendrogram obtained from cluster analysis by Bionumerics software, version 7.1, Dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis.

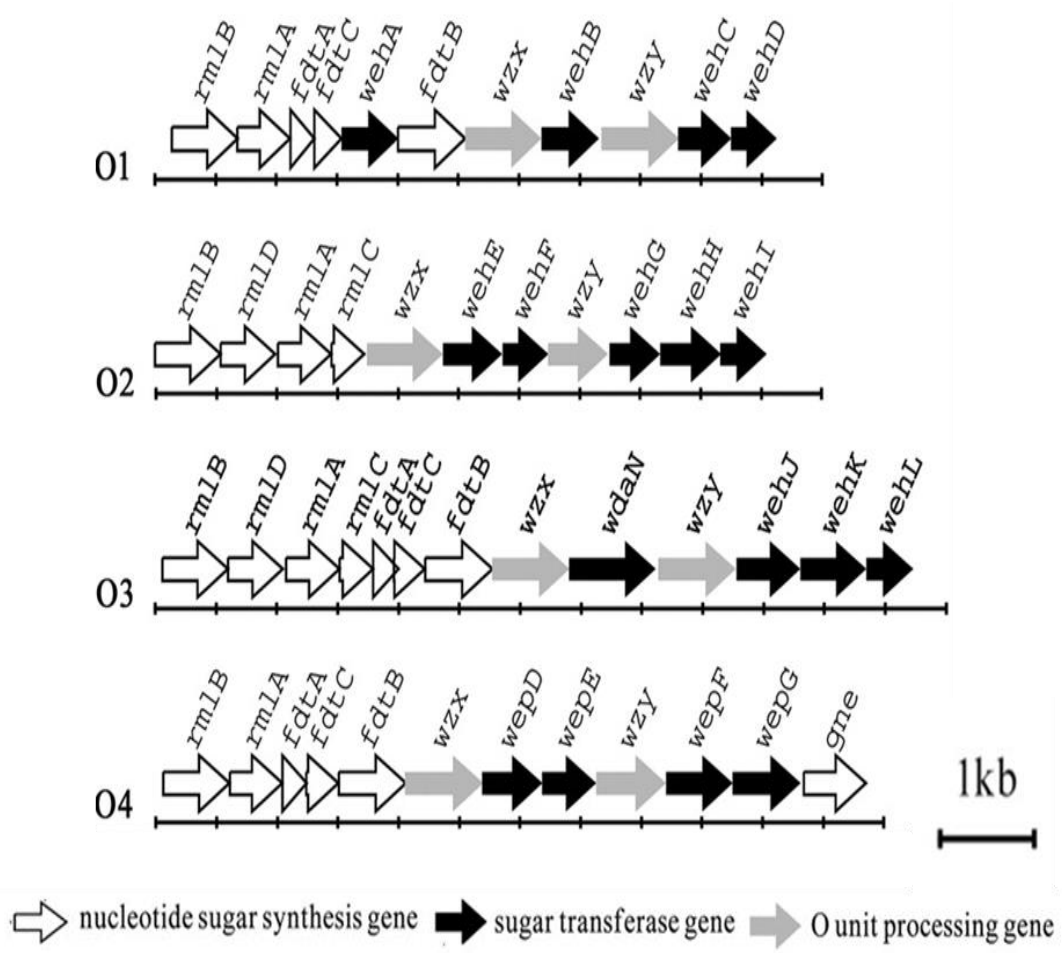


Figure 3.9. Predicted genes of O-antigen gene clusters for *C. sakazakii* O1 to O4 serotypes. (Sun et al. 2012).

3.2.6.4 Phylogenetic tree of *gnd* and *galF* allele sequences

According to the LPS profiling and phylogenetic tree of *gnd* and *galf* allele sequences, strains belong to serogroup O:1 and O:3 were more closely related than other serotype strains (Figure 3.8, 3.10 and 3.11). In general, there is a variation in *gnd* and *galF* alleles profiling within the same sequence type and the same serotype, except strains in serotype O:4 (1108-ST12,and 1907, 1908-ST4) which clustered together with the same *gnd* and *galF* alleles profile (*gnd* 14 and *galF* 13). These strains were clustered outgroup to the rest of the *Cronobacter* strains.

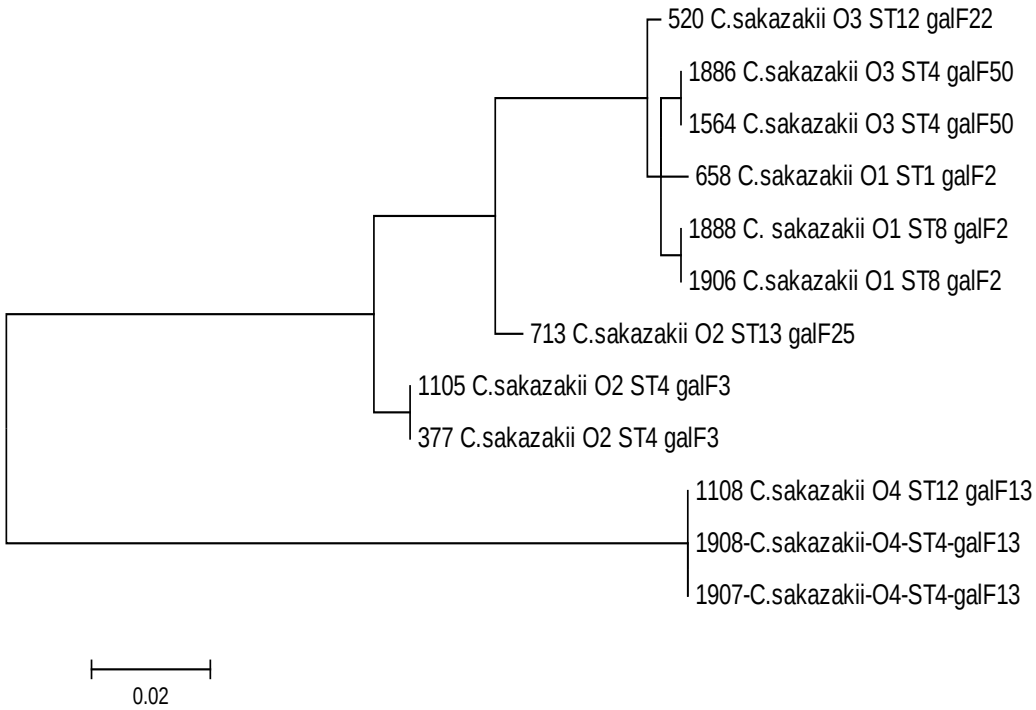


Figure 3.10. The phylogenetic tree of *C. sakazakii* strains, based on nucleotide sequences of *galF* gene (890 bp). The tree was drawn using MEGA 6.

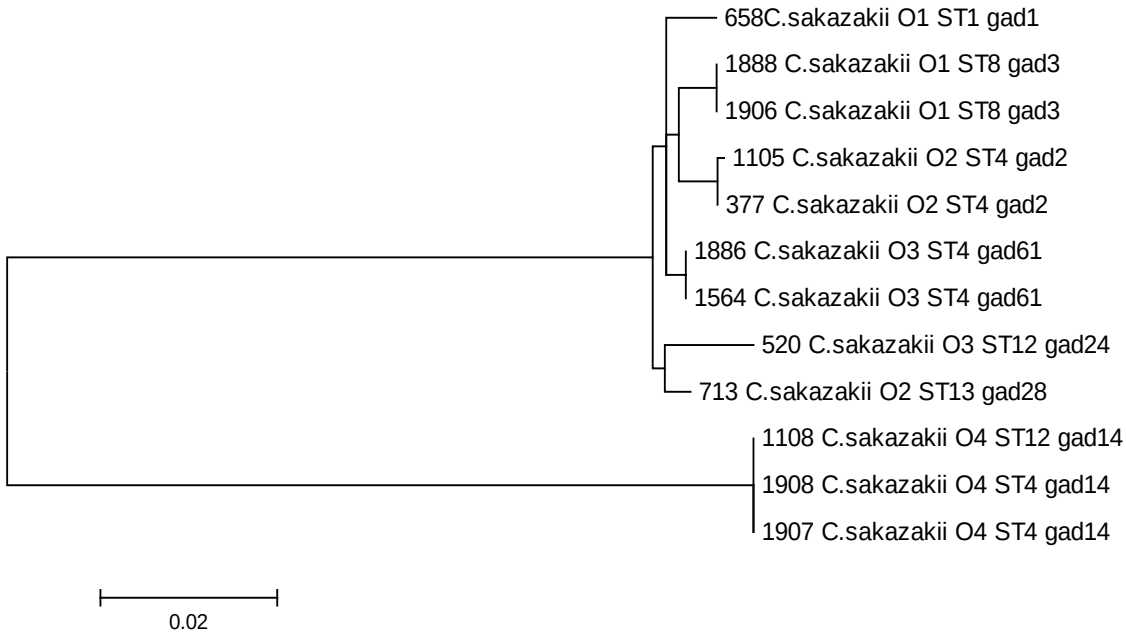


Figure 3.11. The phylogenetic tree of *C. sakazakii* strains, based on nucleotide sequences of *gnd* gene (1400 bp). The tree was drawn using MEGA 6.

3.2.7 K-capsule and colanic acid (CA) profiling of *C. sakazakii* strains.

Diversity of capsular compnents has been the basis for the many of bacterial differentiation methods such as the K-antigen classification scheme of *E. coli* and *Salmonella* serovars serotyping (Whitfield et al. 2003). PCR-based methods targeting different bacterial genes are frequently used by different laboratories. Moreover, PCR-assay targeting significant bacterial genes including capsular polysaccharide genes such as the K-capsule are widely used, because these are important both pathogenetically and taxonomically. Furthermore, Turton et al (2008) reported that the PCR assay was highly specific for *Klebsiella* polysaccharide synthesis gene cluster of serotypes K1, K2 and K5, which are associated with virulence in humans. Moreover, Feizabadi et al (2013) suggest that PCR assay is a rapid and reliable method for identification of *K. pneumoniae* capsular both K1 and K2 serotypes.

The aim of this part of study was to develop and apply a multiplex PCR assay targeting capsular polysaccharide genes such as *kpsS* (K1 and K2) and *galE* (CA1 and CA2) for the specific detection, rapid and simple identification of K-capsule and colanic acid type respectively.

3.2.7.1 K-capsule profiling.

Twenty-six strains of *C. sakazakii* were used in this study (Table 3.6). These strains were isolated from food and environmental sources, covering different STs (n=18) and 4 main serotypes (O:1, O:2, O:3 and O:4).

3.2.7.1.1 Targeted genes and primer design.

According to Ogrodzki and Forsythe (2015), the K-capsule encoding region in *Cronobacter* spp. is composed of three regions. The genomic study indicated that the variations between K1 and K2 capsule type were attributed to the Region 2 and within the *kpsS* gene (encoding for the capsular polysaccharide transport protein) of the Region1 (Figure 3.12). Therefore, K1 and K2 primers were designed based on capsular gene (*kpsS*) and were identified from the sequence information of *C. sakazakii* 658 ($kpsS_1$) and 6 ($kpsS_2$) respectively (Figure 3.14, 15).

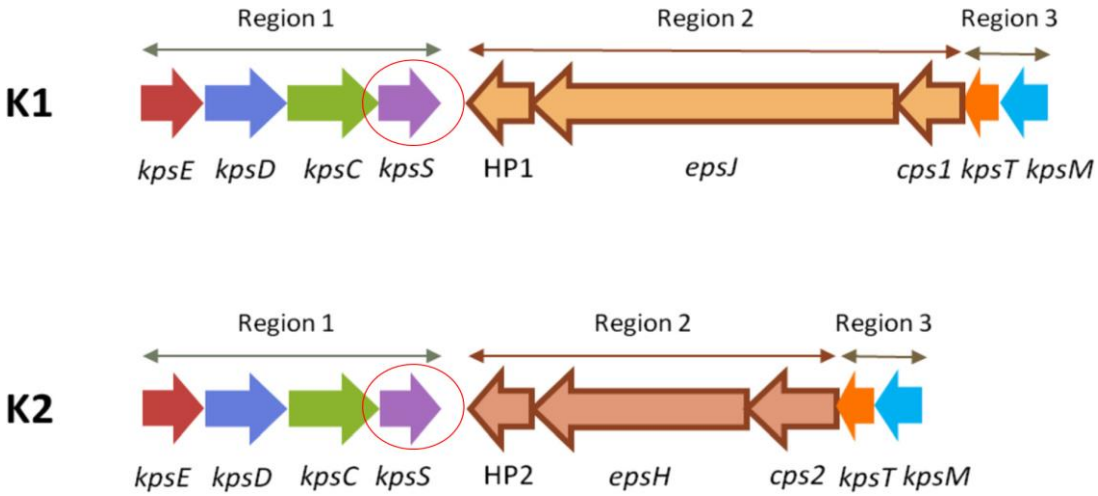


Figure 3.12. *Cronobacter* spp. K1 and K2 Region 1–3 kps genes.
K1 and K2 primers were designed based on capsular gene (*kpsS*) (Ogrodzki and Forsythe, 2015).

3.2.7.1.2 The multiplex polymerase chain reaction.

The multiplex PCR was performed by mixing 2 K-capsule type primers (K1 and K2). PCR amplification of K1 and K2 for *C. sakazakii* strains is shown in figure 3.13. The K1 capsular type was noted in *C. sakazakii* strains in ST1, ST8, ST20, ST23, ST64, ST198, ST263, ST264 and ST408, these strains produced bands of the expected size 248bp. Whereas, K-capsule type 2 was primarily found in *C. sakazakii* sequence types ST4, ST9, ST12, ST13, ST136, ST233, ST245 and ST405, and were with expected product size 120 bp (Figure 3.13).

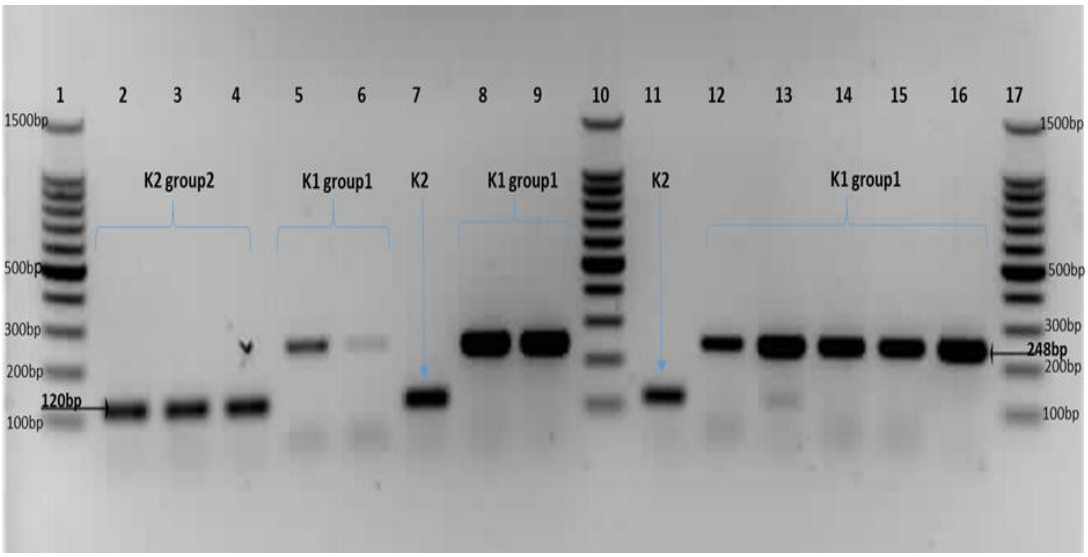


Figure 3.13. PCR amplification of K1-capsule and K2-capsule type.
Lane 1: 100bp DNA Ladder marker. Lane 2: strain 1908. Lane 3: strain 1107. Lane 4: strain 1564. Lane 5: strain 1906. Lane 6: strain 1888. Lane 7: strain 1105. Lane 8: strain 1283. Lane9: strain 1884. Lane 10: 100bp DNA Ladder marker. Lane11: strain 377. Lane 12: strain 1990. Lane 13: strain1884. Lane 14: strain 1885. Lane 15: strain 1843. Lane 16: strain1882. Lane 17: 100bp DNA Ladder marker.

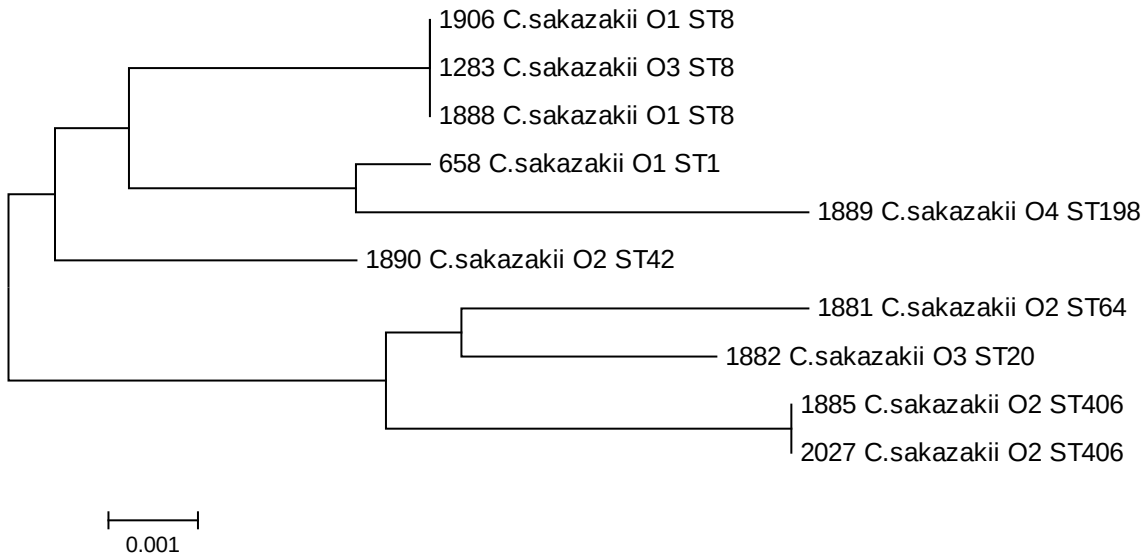


Figure 3.14. The phylogenetic tree of *C. sakazakii* strains, based on nucleotide sequences of *kpsS*1 gene (1198bp) (K1). The tree was drawn using MEGA 6.

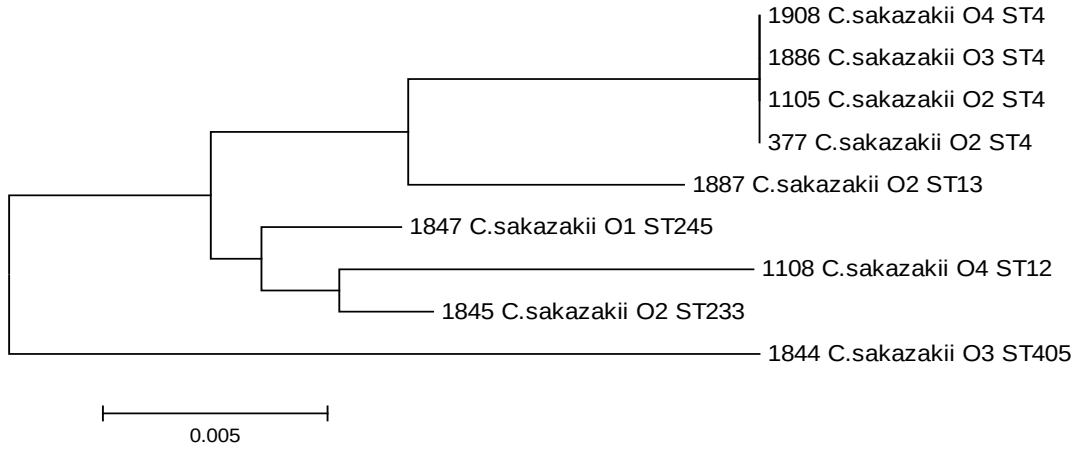


Figure 3.15. The phylogenetic tree of *C. sakazakii* strains, based on nucleotide sequences of *kpsS*2 gene (1230bp) (K2). The tree was drawn using MEGA 6

3.2.7.1.3 Phylogenetic analysis of *kpsS*1 (K1) and *kpsS*2 (K2) locus.

The phylogenetic analysis of *C. sakazakii* strains (n=11) with *kpsS*1 (K1), divided into 8 clusters based on their STs, including ST8, ST42, ST64, ST20 and ST264 strains (Figure 3.14). However, the phylogenetic analysis of *kpsS*2 (K2) strains (n=9) was also divided into 6 groups based on the sequence type, this include strains with ST4, ST13, ST245, ST12, ST233 and ST405 (Figure 3.15).

3.2.7.2 Colanic acid (CA) profiling, CA-type.

3.2.7.2.1 Targeted gene and primer design.

Ogrodzki and Forsythe (2015) reported that the colanic acid (CA) encoding gene cluster was located close to the O-antigen region and started by the *galF* gene. Moreover, two variants were found, CA1 and CA2 composed of 21 and 20 genes respectively, which differ in the presence of *galE* in CA1 (21 genes, Figure 3.16, 3.17), and absence in CA2 (20 genes). Consequently, primers were identified from the sequence information of *C. sakazakii* 658 (CA1) and was designed based on *galE* gene sequence (presence of *galE*).

3.2.7.2.2 Polymerase chain reaction for colanic acid type.

PCR amplification of CA1 for *C. sakazakii* strains were shown in figure 3.18. Colanic acid type 1 (CA1) were found in majority *C. sakazakii* sequence types such as ST1, ST8, ST9, ST20, ST245 and ST405 with PCR product size 429bp. While, CA2 was mainly found in *C. sakazakii* sequence types; ST4, ST12, ST13, ST23 and ST64, and these strains showed no PCR products as result of absence of *galE* gene (Figure 3.16). Table 3.6 shows the agreement between the PCR determination and genome investigation for CA-type of studied strains.

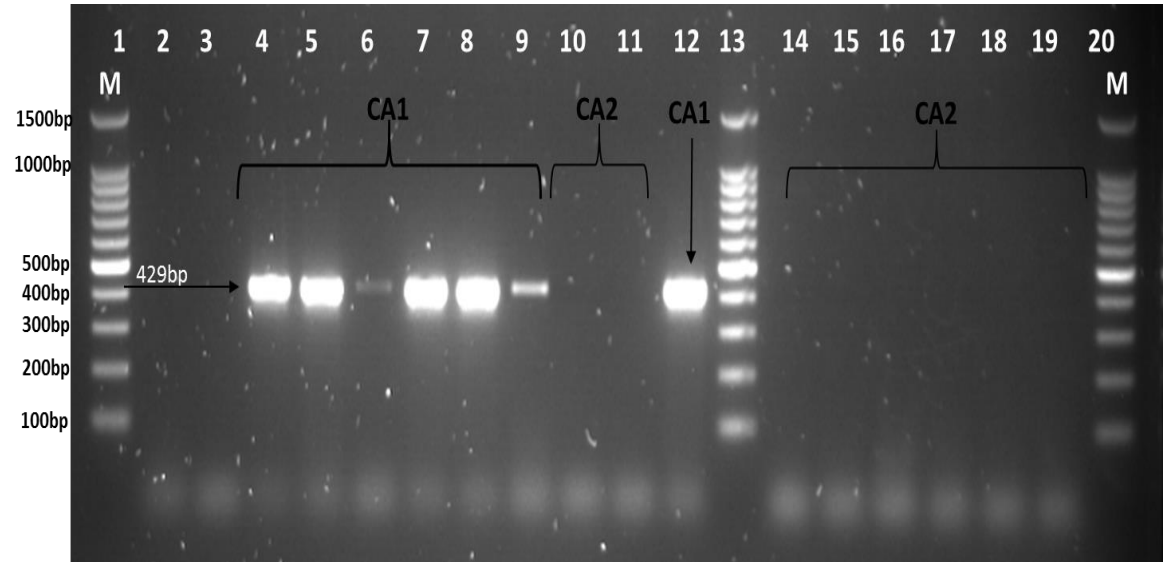


Figure 3.16. PCR amplification of colanic acid (CA1) type.
Lane 1: 100bp DNA Ladder Marker. Lane 2: the negative control (with no bacterial DNA). Lane 3: strain 377 as positive control for CA2 (no PCR product). Lane 4: strain 1107. Lane 5: strain 1283. Lane 6: strain 1888. Lane 7: strain 1906. Lane 8: strain 1844. Lane 9: strain 1882. Lane 10: strain 1105. Lane 11: strain 1564. Lane 12: strain 1 as positive control for CA1. Lane 13: 100bp DNA Ladder Marker. Lane 14: strain 1843. Lane 15: strain 1884. Lane 16: strain 1907. Lane 17: strain 1908. Lane 18: strain 1990. Lane 19: strain 1886. Lane 20: 100bp DNA Ladder Marker.

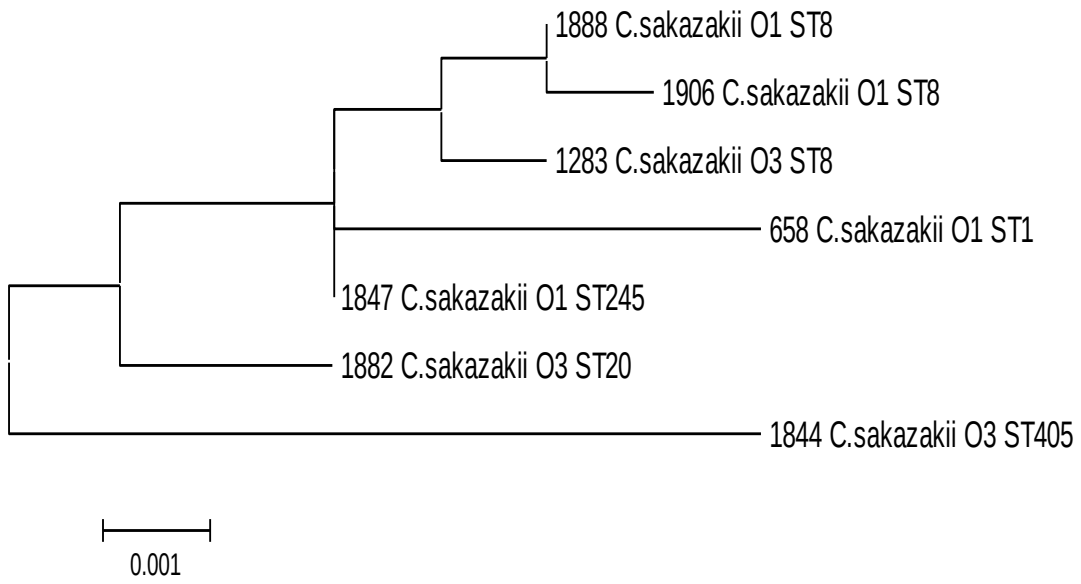


Figure 3.17. The phylogenetic tree of *C. sakazakii* strains, based on nucleotide sequences of *galE* gene (996 bp) gene (CA1). The tree was drawn using MEGA 6. CA1 strains (presence of *galE* gene).

3.2.7.2.3 Phylogenetic analysis of *galE* (CA1)

Based on the genome invesitication, 7 of 26 strains showed the presence of the *galE* gene within the colanic acid gene cluster (CA1). These strains were 1888, 1906 and 1283 (ST8) , 658 (ST1), 1847 (ST245), 1882 (ST20) and 1844 (ST405). These divided into 5 clusters accodrding to their STs. ST8 strains were sub-divided into 2 branches based on their serotype (Fgure 3.17).

3.2.7.3 Comparison between PCR amplification result and the genomic investigation.

The capsular type 1 (K1) strains produced bands of the expected size 248bp. Whereas, K-capsule type 2 (K2) strains produced bands of the expected product size 120 bp (Figure 3.13). The PCR product size of K-capsule type (K1 and K2) was compared with the genome investigation of studied strains (Region 2) as shown in table 3.6. The comparison showed an agreement between PCR amplification result and the genomic study of K-capsule Region 2.

According to genome investigation, two variants were found within colanic acid (CA) cluster, CA1 and CA2 composed of 21 and 20 genes respectively, which differ in the presence of *galE* in CA1 (21 genes, Figure 3.16, 3.17), and absence in CA2 (20 genes). Therefore, primers were designed based on *galE* gene sequence (presence of *galE,* CA1).

The colanic acid type 1 (CA1) strains produced PCR product size 429bp. While, CA2 strains showed no PCR products as result of absence of *galE* gene (Figure 3.16). Table 3.6 showed also an agreement between the PCR determination and genome investigation for CA-type of studied strains (presence/ absence *galE* gene).

Table 3.6**.** K-capsule type and colanic acid type (CA) profiling of *C. sakazakii* strains by PCR determination and genome investigation.

NTU NO:	ST	O-antigen	K-capsule. PCR assay	K-capsule. G	Colanic acid (CA). PCR assay	Colanic acid (CA). G
658	1	O:1	K1	K1	CA1	CA1
1906	8	O:1	K1	K1	CA1	CA1
1888	8	O:1	K1	K1	CA1	CA1
1843	23	O:2	K1	NG	CA2	NG
1890	42	O:2	K1	K1	CA2	CA2
1881	64	O:2	K1	K1	CA2	CA2
1884	263	O:2	K1	NG	CA2	NG
1990	264	O:2	K1	NG	CA2	NG
1885	406	O:2	K1	K1	CA2	CA2
2027	406	O:2	K1	K1	CA2	CA2
1283	8	O:3	K1	K1	CA1	CA1
1882	20	O:3	K1	K1	CA1	CA1
1889	198	O:4	K1	K1	CA2	CA2
1847	245	O:1	K2	K2	CA1	CA1
1105	4	O:2	K2	K2	CA2	CA2
377	4	O:2	K2	K2	CA2	CA2
1107	9	O:2	K2	NG	CA1	NG
1887	13	O:2	K2	K2	CA2	CA2
1992	136	O:2	K2	K2	CA2	CA2
1845	233	O:2	K2	K2	CA2	CA2
1564	4	O:3	K2	NG	CA2	NG
1886	4	O:3	K2	K2	CA2	CA2
1844	405	O:3	K2	K2	CA1	CA1
1907	4	O:4	K2	NG	CA2	NG
1908	4	O:4	K2	K1	CA2	CA2
1108	12	O:4	K2	K2	CA2	CA2

This table shows the comparison between PCR amplification result and the genomic investigation of 26 *C. sakazakii* strains

ST: sequence type. G: genome investigation. NG: no genome available

3.3 Discussion.

Typing of bacterial pathogens, or distinguishing bacteria at the strain level, is very important for diagnosis, treatment, and tracking of bacterial infection sources. *Cronobacter* genus is ubiquitous, hence typing schemes are required both for epidemiological and environmental investigation. Particularly, the *Cronobacter* genus are associated with neonatal infections causing meningitis, necrotizing enterocolitis (NEC), bacteraemia and widely isolated PIF (Hurrell et al. 2009; Hariri et al. 2013). In addition, this genus is associated with bacteraemia in immunocompromised adults. Furthermore, *Cronobacter* species are frequently isolated from environmental samples, dry powdered foods, agricultural product such as wheat, rice, herbs, spices and other foods (Holy and Forsythe, 2014). One of the key source of infant infection is the ingestion of contaminated PIF, and this has led to numerous investigations on the enhanced detection methods for the food industry. This thesis focused on non-clinical isolates of *Cronobacter* to balance the more frequent analysis of clinical strains.

Multilocus Sequence Typing (MLST)

MLST is a robust and reliable molecular method that was applied recently for analysing the *Cronobacter* species. The MLST scheme was subsequently applied to differentiate and discriminate the species within the *Cronobacter* genus (Baldwin et al. 2009; http://pubmlst.org/*cronobacter*/). The 7-loci MLST is based on an analysis of 7 housekeeping genes: *atpD, fusA, glnS, gltB, gyrB, infB* and ppsA, which are essentially required by the bacterium, these genes are constitutively expressed and independent of any selective conditions (Baldwin et al. 2009).

In this part of study, the 7-loci MLST was used to analyse previously un-profiled *Cronobacter* strains that were isolated from food and environmental sources. Twenty-six strains that had not previously been profiled were used in this study (Table 3.1). These strains were originated from the Czech Republic (n=20), Malaysia (n=3) and the UK (n=3), and they had been collected between 2011-2013. The species of 26 strains were determined and their sequence type (STs) were defined. This study also constructed a phylogenetic tree to analyse these strains based on the MLST data, and the tree revealed

the diversity of species in the *Cronobacter* genus as well as the major STs found in this collection (Figure 3.1).

Twenty-six strains of *Cronobacter* spp. that were isolated from food and environmental sources, were divided into 21 sequence type (STs) in five out of seven species. The most of these strains that were identified, 19 strains were *C. sakazakii* (73%), followed by *C. malonaticus* (n=3, 11.5%), *C. turicensis* (n=3, 7.6%), *C. dublinensis* (n=1, 4%) and *C. muytjensii* (n=1, 4%) (Table 3.1). In terms of sequence type, these strains were divided into 21 sequence type (STs), 3 strains were *C. sakazakii* ST4, 2 strains were *C. sakazakii* ST8, 2 strains were ST406, and other strains showed different ST (individual ST). ST264 (1990) and ST406 (1885 and 2027) were closely related STs, shared the same clonal complex (CC264), and were nearly clustered together. Only one allele (*gltB*) was different between ST406 and ST264 (16-1-13-178-21-5-21 and 16-1-13-39-21-5-21)((Figure 3.1). Consequently, they are regarded as single locus variants. Nine new STs were identified, which had not been previously reported; ST213, ST233, ST245, ST263, ST264, ST289, ST344, ST406 and ST407 (Figure 3.1). This result revealed that *Cronobacter* strains isolated from food and environmental sources were highly diverse with respect to their ST, in particular those from different sources of food.

RAPD-PCR and BOX-PCR fingerprinting

Pulsed field gel electrophoresis (PFGE) is one of the most widely used method for subtyping of *Cronobacter* species (Healy et al. 2008). Nevertheless, this method is expensive and time-consuming. In contrast, previous studies reported that BOX-PCR results yielded (90-95%) agreement with PFGE. In addition, RAPD-PCR and BOX-PCR fingerprinting techniques allowed for the typing of *Cronobacter* strains in a faster and easier way than the PFGE technique (Proudy et al. 2008).

In this study, RAPD and BOX-PCR were used as molecular tools to characterize 61 *Cronobacter* strains isolated from environment, water, foods, and clinical, and were compared that with their sequence type. These isolates covered 6 species of *Cronobacter* and 45 different STs. These include 27 *C. sakazakii* strains, 9 *C. malonaticus* strains, 7 *C. dublinensis* strains, 9 *C. turicensis* strains, 4 *C. muytjensii* strains and 5 *C.*

universalis strains. These strains were chosen based on geographic diversity, date and source of isolation (Figure 3.2).

Molecular typing was performed by random amplified polymorphic DNA (RAPD) as previously described by Nazarowec-White and Farber (1999). Nazarowec-White and Farber (1999) characterized 18 *Cronobacter* isolates from food and clinical sources using ribotyping, RAPD, and PFGE. Their study is the only published comparison of typing methods and concluded that PFGE and RAPD gave greater discriminatory power compared to ribotyping.

The RAPD-PCR fingerprints of 61 *Cronobacter* isolates were shown in figure 3.2. All were analysed by RAPD and a quantitative assessment of the genomic relationships was made based on the BioNumerics software (7.1) output. The RAPD analysis showed that these isolates could be grouped into 40 major clusters based on the correlation coefficient of 90 % on the bases of their sequence type (STs). Twenty-seven *C. sakazakii* isolates were discriminated into 17 genotypes, 9 *C. malonaticus* strains were discriminated into 6 genotypes, 9 *C. turicensis* strains were discriminated into 7 genotypes. The 7 *C. dublinensis* strains were divided into 6 groups in the basis of their sequence type, the exception were strains 1460 (ST76) and 1464 (ST78) which clustered together, five *C. universalis* strains discriminated into 4 cluster genotypes. The 4 *C. muytjensii* strains formed a distinct cluster based on their sequence type (Figure 3.2).

Our findings indicate that RAPD is capable of discriminating amongst a collection of *Cronobacter* based on their sequence type. Moreover, RAPD-PCR fingerprint has been successfully used to discriminate closely related STs such as ST406 and ST264 (CC264). According to the 7 loci-MLST scheme (3036 bp), these 2 STs were more closely related STs compared with other STs, and only one allele (*gltB*) was found different between them (CC=264) (Figure 3.1 and Table 3.1). Furthermore, in some cases this technique is able to distinguish between strains within the same ST such as *C. sakazakii* ST4 strains, *C. malonaticus* ST7 strains and *C. turicensis* ST5 strains (Figure 3.2).

In general, a significant correlation was observed between the sequence type and the molecular profiles obtained from RAPD fingerprinting of the *Cronobacter* isolates. However, no correlation between the RAPD profile and the source, date and place of

isolation was observed (Figure 3.20). Nevertheless, RAPD is a useful molecular tool providing rapid analysis of isolates in a local context.

In this study, BOX-PCR was also evaluated against the 7 loci-MLST scheme (3036 bp) as a genotyping method to discriminate the 61 *Cronobacter* strains with 45 different STs. The BOX-PCR fingerprinting technique, which targets the repetitive BOX sequences has been used for the first time to differentiate between 27 *Cronobacter* strains from clinical and environmental sources. The discriminatory power of BOX-PCR techniques was compared with PFGE. BOX-PCR results yielded 92% agreement with PFGE results (Proudy et al. 2008).

The result showed that fingerprints of 61 strains of *Cronobacter* were divided into 39 major clusters based on the correlation coefficient of 80 %. *C. sakazakii* strains (n=27) were divided into 15 groups, strains with the same STs were clustered together. However, in some cases strains with different STs were clustered together, for instance strains 1906, 1283 and 1888 (ST8), 1885 and 2027 (ST406), and 1990 (ST264) were clustered together. *C. malonaticus* strains (n=9) were clustered together based on their sequence type, the exception was *C. malonaticus* ST7 strains which dived into two clusters. Moreover, *C. turicensis* strains formed a distinct cluster based on their sequence type, except strains 1553 (ST72) and 1880 (ST344) which produced identical BOX profile (Figure 3.3).

C. dublinensis strains (n=7) were divided into 2 groups. *C. universalis* strains (n=5) were clustered into 5 clusters based on their sequence type. Moreover, the 4 *C. muytjensii* strains formed a distinct cluster based on their sequence type (Figure 3.2). However, compared with the phylogenetic analysis of the 7 loci-MLST scheme (3036 bp), BOX-PCR fingerprints showed less ability to cluster the same *Cronobacter* sequence type together. In addition, the discriminatory power of BOX-PCR techniques was compared with the RAPD-PCR fingerprints. The RAPD-PCR had slightly higher discriminatory power than BOX-PCR (Figure 3.2, 3.3).

Variable numbers of tandem repeats (VNTR).

MLST has become a frequently used method for genotyping *Cronobacter* isolates, particularly as it both speciates and indicates the pathovar or clonal lineage. Nevertheless, the 7-loci-MLST and PFGE are not able to distinguish strains within the same STs; it occurs in *Cronobacter* in particular *C. sakazakii* ST4 strains. Therefore, for epidemiological purposes, it is important to investigate more discriminatory methods as a simple, rapid process, given lowering costs and the increasing availability. Variable numbers of tandem repeats (VNTR) is one such approach. Due to the clinical significance of *C. sakazakii* ST4 strains, this sequence type was the focus of study.

Eighteen *C. sakazakii* ST4 strains were used in this study, and only one strain 658 (ST1) was used for the purpose of comparison. These strains had been chosen based on geographic diversity (10 countries), date and source of isolate, also widely temporally (1950–2012) (Table 3.2).

VNTR analysis is a genotyping method based on polymerase chain reaction which may be useful for tracing and subtyping bacteria with a simple procedure, cost effectively and with high-speed. Furthermore, this genotyping method is becoming an important DNA-based typing method for investigating bacterial strains that are related to outbreaks and infection. Mullane et al (2008) have described the development and application of this method to subtype *Cronobacter* spp. This study focused on *C. sakazakii* ST4 strains for the first time, using *in silico* analysis of sequenced strains. The VNTR analysis was used to investigate if further subdivision occurs within this important sequence type.

In this study, we have described the development and application of the VNTRA typing method for the important *C. sakazakii* ST4. A set of six discriminatory STRs markers were identified and used in the development of the typing scheme. Strains (n=19) were grouped into 16 distinct groups (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O and P) based on the number of tandem repeats of 6 VNTR loci (Table 3.2), which were not geographically or temporally associated (Table 3.3).

C. sakazakii neonatal meningitis (ST4) strains from a NICU outbreak (692, 695, 705,706 and 712), all of the isolates were previously defined as pulsotype 2 (Caubilla-Barron et al. 2007). These isolates were divided into 4 groups (B, C, D and E) based on the number of tandem repeats of 6 VNTR loci (Table 3.2); group B was strain 695, group C was strain

692, group D was strains 712, and group E was strains 705 and 706. This observation is in agreement with Caubilla-Barron et al (2007), where these strains formed a sub-cluster (differing by 1 to 3 band) within the pulsotype 2. Moreover, according to Masood et al (2015), these strains divided into 2 groups against the earliest isolate of this cluster *C. sakazakii* 701 isolated on 7th of April 1994, based on single nucleotide polymorphisms (SNPs) analysis. Group 1 was strains (692 and 695), shown a small number of SNP differences; a maximum of 15 SNP differences against strain 701. Strain 712 differed by only 6 SNPs from the reference strain 701. Group 2 was (705 and 706) isolates shown over 300 SNP differences from the reference strain 701. Moreover, these strains differed from each other by a maximum of 16 SNPs; since these 2 isolates were isolated from the same patient (neonate B) at different time points. The result of NICU outbreak strains was in agreement with the single nucleotide polymorphisms (SNPs) analysis (Masood et al. 2015). Furthermore, Ogrodzki and Forsythe (2016) reported that isolates from the 1994 NICU outbreak (692, 695, 705,706 and 712); all had the same designated CRISPR2 profile based on CRISPR–*cas* loci profiling analysis. In this study however, these strains were divided into 4 groups based on the VNTR analysis.

The three CDC ST4 strains (1568, 1570 and 1579) were isolated from different US States, and were collected during 2011 to 2012. These strains show the same VNTRA profile. However, the presence of identical VNTR profile across this isolates, has demonstrated an overlap in genetic diversity.

Food and environmental strains (377, 890, 1105, 1480, 1564, 1886, 551, 1480, 1907 and 1908) were isolated from different countries and different years. These strains all had the different designated VNTRA profiles. Strains 1907 (1-8-5-2-4-1) and 1908 (1-6-5-2-4-1) had been isolated from the same hospital environment in Malaysia during 2012, and they were more closely related strains. These strains differed from each other only in STR2; strains 1907 showed 8 tandem repeats in locus 8, while 6 tandem repeats was recorded in strain 1908 (Figure 3.5). However, these two strains showed the same genotyping profile including RAPD and BOX-PCR fingerprint, capsule profile (K-type and CA-type), serotype (*galF* and *gnd*) and rpoB profile.

STR2, STR1, STR4 and STR6 were the most variable VNTRs respectively. STR2 was the main contributor to the separation of the *C. sakazakii* sequence type 4 (ST4) strains, while STR3 showed less variation between *C. sakazakii* strains (ST4).

The VNTRA profiles were not geographically or temporally associated as identical combinations were shared between isolates of different source, year, and country. For instance, *C. sakazakii* neonatal meningitis (ST4) strains from an NICU *Cronobacter* outbreak (692, 695, 705, 706 and 712), and environmental strains (1907 and 1908), which were isolated from the same country and same year, show different VNTRA profile. In contrast, other isolates, which were isolated from different country, years and sources had the different VNTRA profile (Individual profile). The exception was the 3 CDC strains which showed identical VNTRA profile, this might be reflected an overlap in genetic diversity across these isolates.

The result revealed that VNTRA technique has a greater-discriminatory power within the *C. sakazakii* sequence type 4 strains. It was concluded that VNTRA profiling could contribute to further understanding of *C. sakazakii* ST4 diversity and tracking of infection sources, and could be useful for tracking strains rather than the specific VNTR profile being of clinical significance.

***Cronobacter* O-antigen diversity.**

The O antigen forms part of the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria and is one of the most variable constituents on the cell surface (Jarvis et al. 2011; Sun et al. 2012a; Ogrodzki and Forsythe, 2015). Serotyping is a useful method for characterization of Gram-negative bacteria. Many studies focused on the O-antigen classification within *Cronobacter* genus have been reported including molecular based methods and structural studies (Blažková et al. 2015). Ogrodzki and Forsythe (2015) stated that *galF* and *gnd* sequences polymorphism analysis can successfully be used as an alternative to RFLP for serotyping. Therefore, the sequence polymorphisms in the O-antigen flanking genes *gnd* and *galF* were evaluated for *Cronobacter* strain typing.

Fifty-one *Cronobacter* isolates were used in this study including, 25 *C. sakazakii,* 7 *C. malonaticus, 8 C. dublinensis, 4 C. muytjensii,* 6 *C. turicensis* strains, and the single *C.*

condimenti strain (Table 3.4). Strains were determined using the previous PCR methods (Jarvis et al. 2011; Sun et al. 2012a). *In silico* analyses were carried out using (n=41) *Cronobacter* genomes accessible at the PubMLST *Cronobacter* database www.pubmlst.org/cronobacter/). The results of the O-antigen serotyping are included in table 3.4, 3.9.

C. sakazakii serotyping. Strains (n=25) divided into four O-antigen serotypes O:1, O:2, O:3 and O:4. Serotype O:2 was the most dominant serotype in *C. sakazakii* strains (13 strains, 52%), followed by serotypes O:3 (20%), less often were *C. sakazakii* O:1 (12%) and O:4 (12%). However, *C. sakazakii* O:5, O:6 and O:7 specific primers gave negative results for all tested strains. The O-antigen structures have been determined for *C. sakazakii* serotypes O:1 to O:7. The 7 serotypes contain between 5 and 7 saccharides in each O-antigen unit. The *C. sakazakii* O:1 and O:2 have 2 saccharides in the branching fragment of the O antigen, while the *C. sakazakii* O:3 to O:7 have only one saccharide (Blažková et al. 2015). There is a variation in *gnd* and *galF* alleles profiling within the same sequence type and the within same serotype, except strains in serotype O:4 which possessed the same *gnd* and *galF* alleles profile (*gnd* 14 and *galF* 13). According to Sun et al (2012), *C. sakazakii* serotype O:4 shared a similar O-antigen gene cluster with *Escherichia coli* O103 (Figure 1.2). This may explain why this group clustered outgroup to the rest of the *Cronobacter* strains. However, this variation might be related to the sugar composition and complexity of structure or the gene order in the O-antigen gene cluster (Figure 1.2).

C. malonaticus serotyping. Strains (n=7) separated into 2 O-antigen serotypes CM O:1 and O:2. Serotype CM O:1 was the most dominant serotype in *C. malonaticus* strains (five strains, 71.4%). Two strains were serotype CM O:1 (28.5%). This variation was supported by the phylogenetic analysis of *galF* and *gnd* locus, *C. malonaticus* strains divided into 2 clusters according to their serotype.

C. dublinensis strains were divided into 2 O-antigen serotypes. Five strains were serotype Cdub O:1 (62.5%), and three strains were Cdub O:2. Jarvis et al (2013) have already reported these two serotypes. *C. dublinensis* subsp. *lactaridi* strains 1461 (ST77), 1462 (ST70), and *C. dublinensis* subsp. *dublinensis* strains 1463 (ST95), 1210 (ST106) and

1458 (ST74) were Cdub O:1. While, *C. dublinensis* subsp. *lausanensis* strains 1460 (ST76) and 1897 (ST213) were serotype Cdub O:2. This observation was also supported by the phylogenetic analysis of *galF* and *gnd* locus, *C. dublinensis* subsp. *lactaridi*, and *C. dublinensis* subsp. *dublinensis* strains (O:1) were more closely related than *C. dublinensis* subsp. *lausanensis* strains (O:2). This variation might be related to gene order of O-antigen gene cluster.

Five *C. muytjensii* strains were divided into 3 O-antigen serotypes (O:1, O:2and O:3), two strains were Cmuy O:1 serotype (50%), one strain was Cmuy O:2 serotype (25%) and one strain was Cmuy O:3 (25%). However, *C. muytjensii* strains (4) clustered together in both *galF* and *gnd* gene sequences, no significant variation was noted between 3 O-antigen serotypes based on *galF* and *gnd* phylogenetic analysis.

Serotyping of *C. turicensis* strains, it contains at least 3 serotypes. Six *C. turicensis* strains were separated into 2 O-antigen serotypes O:1 and O:3. three strains were CturO:3 (50%), one strain was CturO:1, and other 2 strains (non-available genome; 109 and 111) showed negative result with *C. turicenesis* O-antigen PCR assays (not determined) (Table 3.4). The O-antigen structure of 3 C. *turicensis* strains have been published by Czerwicka et al (2013). According to their results, the new serotype *C. turicensis* O:4 should be recognized. This O-antigen is specified by the reference strain *C. turicensis* 564. Therefore, these two strains (109 and 111) could be *C. turicensis* O:4. However, the *C. turicensis* strains (n=5) all varied in their *galF* and *gnd* sequences. The single *C. condimenti* strain was Cuni O:1 with allele profile (*galF*34-*gnd*9).

In general, strains within the same sequence type showed different serotypes. Moreover, there is a variation in *gnd* and *galF* alleles profiling within the same sequence type and within the same serotype, except strains in serotype O:4 which clustered together with the same *gnd* and *galF* alleles profile (*gnd* 14 and *galF* 13). Therefore, the LPS of selected *C. sakazakii* strains (n=12) belonging to the representatives of four main serotypes (O1, O2, O3 and O4), and clinically significant STs were extracted and subjected to SDS-PAGE, and the gel was analysed using BioNumerics software (version 7.1). In order to investigate the variation of O-antigen serotype within the LPS profiling and linked that with the the ST (Table 3.5).

Lipopolysaccharides (LPSs) profiling.

The lipopolysaccharides (LPSs) that form part of the outer membrane of the Gram-negative bacteria has been linked with virulence in numerous studies. It is a virulence factor produced by many bacteria and released by the organism during an infection. Bacterial lipopolysaccharides generally consist of three parts: lipid A, an oligosaccharide core, and an O-antigen. The O-antigen is a polysaccharide (O-PS) that extends from the cell surface and consists of repetitive oligosaccharide units (O units) generally composed of 3 to 6 sugars (Mullane et al. 2008). In this study, extraction and characterization of lipopolysaccharide from selected *C. sakazakii* strains were performed by using proteinase K digestion methods were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and LPS fragments were visualized by silver staining.

The LPS patterns were determined for 12 selected *C. sakazakii* strains, covering the representatives of four main serotypes (O:1, O:2, O:3 and O:4) and the main STs which are associated with clinical significance of the *C. sakazakii* strains (ST1, ST4, ST8, ST12 and ST13); table 3.5.). In order to investigate the correlation between the sequence type and the O-antigen serotype variation within the LPS profiling, SDS-PAGE gel was analysed using BioNumerics software, version 7.1 (Figure 3.8). To the best of our knowledge, this is the first study to validate these differences using Bionumerics software (version 7.1).

The result demonstrated that LPS profile of 12 strains were clearly divided into four groups based on the serotype. A 90 % similarity cut-off was used to determine the strains clonality, and each group belonging to the same serogroups were clustered into 2 clusters based on their *galF* and *gnd* allele profile (Figure 3.10, 3.11).

Analysis of the LPS profiling using BioNumerics software, version (7.1) showed great ability to discriminate between strains within the same serotype. There was no correlation between sequence type (ST), clonal complex (CC) profiling and the LPS profiling (Figure 3.8). The LPS profile was in close agreement with the O-antigen serotype profile (sequence polymorphisms in the O-antigen flanking genes *gnd* and *galF*). This suggests that these two (*gnd* and *galF*) alleles are important for determination of

LPS oligosaccharide units (O units) type, and PCR-based serotype could be further subdivided.

K-capsule and colanic acid (CA) profiling of *C. sakazakii* strains.

Gram-negative bacteria species can produce different structures of capsular polysaccharides (CPSs) and these help distinguish isolates by serotyping, as is the case with *Escherichia* coli K antigens. PCR-assay targeting significant bacterial genes including capsular polysaccharide genes such as K-capsule type is widely used, because of those are important both pathogenetically and taxonomically. The K-capsule of *E. coli* is consisting of three regions, Region 1 (*kpsFEDUCS*) and Region 3 (*kpsMT*), and a variable Region 2 (Willis and Whitfield, 2013).

The previous genomic study by Ogrodzki and Forsythe (2015) reported that K-capsule type of *Cronobacter* genus is composed of 3 Regions and the variations between K1 and K2 capsule type were attributed to the Region 2 and within the *kpsS* gene of the Region1 (Figure 12, 13, 14, 15). Therefore, the aim of this part of study was to develop and validate a multiplex PCR assay targeting capsular polysaccharide genes such as *kpsS* (K1 and K2) and *galE* (CA1 and CA2) for the specific detection, rapid and simple identification of K-capsule and colanic acid type respectively.

Twenty-Six *C. sakazakii* strains were used in this study, these strains were isolated from food and environmental sources, covering different STs (n=18) and 4 main serotypes (O:1, O:2, O:3 and O:4). K1 and K2 primers were designed based on *KpsS* genes Region1, using primer 3 software. A multiplex PCR was used to determinate the K-capsule type (K1 and K2). Amplification products of 248bp were observed for the capsular type1 (primer *kpsS1,* K1), and a product of 120 bp was observed for the capsular type2 (primer *kpsS*2, K2). No cross-reactions were observed between the specific two primers of capsular type K1 and K2 (Figure 3.13). The K1 capsular type was noted in *C. sakazakii* strains with STS; ST1, ST8, ST20, ST23, ST64, ST198, ST263, ST264 and ST406. Whereas, K2 was primarily found in *C. sakazakii* sequence types ST4, ST9, ST12, ST13, ST136, ST233, ST245 and ST405. The PCR product size of K-capsule type (K1 and K2) was compared with genome investigation of studied strains as shown in table 3.6. The

comparison showed an agreement between PCR amplification result and genomic study of K-capsule Region 2.

Additional exopolysaccharide, called colanic acid (CA) is insecurely bound to the cell. CA may also be secreted into the environment and it might be play a role in bacterial biofilm structure. The colanic acid gene cluster (CA) was located close to the O-antigen region, and there are two variants were found, CA1 and CA2 composed of 21 and 20 genes respectively, which differ in the presence of *galE* (Ogrodzki and Forsythe, 2015) (Figure 3.16, 3.17). Therefore, CA1 primer was identified from the sequence information of *C. sakazakii* 658 (CA1) and was designed based on *galE* gene sequence (presence of *galE*). Colanic acid type 1 (CA1) were found in *C. sakazakii* sequence types such as ST1, ST8, ST9, ST20, ST245 and ST405 with PCR product size 429bp. While, CA2 was found in *C. sakazakii* sequence types; ST4, ST12, ST13, ST23, ST42, ST64, ST136, ST198, ST233, ST263, ST264 and ST406, these strains showed no PCR products as result of absence of *galE* gene. Table 3.6 shows the agreement between the PCR determination and genome investigation for CA-type of studied strains. Moreover, this observation was in agreement with Ogrodzki and Forsythe (2015).

The techniques most commonly used for identification of K capsule type (K1 and K2) and colanic acid type (CA-type) are limited, because of costs of genome investigation. It is suggesting that methods based on PCR amplification might be used to determine the K-capsule type and colanic acid type in *Cronobacter* spp. as simple, cheaper, rapid and reliable methods.

In general, table (3.7) has summarised the molecular characterization and genotyping diversity of *Cronobacter* spp. isolate from food and environmental sources. According to this table (3.7) *C. sakazakii* strains were divided into 4 groups based on their capsular profile (K-capsule and CA-type); K1-CA1, K2-CA2, K1-CA2 and K2-CA1. All strains in ST12, ST13, ST136, ST233 and ST4 had the capsular profile K2-CA2. Sequence types 4 (ST4) and ST12 are strongly associated with severe neonatal infections including meningitis and necrotizing enterocolitis (Joseph and Forsythe, 2011; Hariri et al. 2013; Forsythe et al. 2014). *C. sakazakii* strains ST1, ST8 and ST20 have the capsular profile K1-CA1. On the other hand, strains in ST23, ST42, ST64, ST198, ST263, ST264 and ST406 had the capsular

profile K1-CA2. *C. sakazakii* ST64 is isolated from PIF and the environment of manufacturing plants in many countries around the world (Sonbol et al. 2013; Forsythe et al. 2014; Fe et al. 2015). *C. sakazakii* strains ST245 and ST405 had the capsular profile K2-CA1.

Table 3.7. Molecular characterization and genotyping diversity of *Cronobacter* spp. isolates from food and environmental sources.

NTU	Species	Country	Source	Year	ST	Serotype	K-capsule	CA	*galF*	*gnd*	*rpoB*
658	*C. sakazakii*	USA	Non-infant formula	2001	1	Csak O:1'	K1	CA1	2	1	22
377		UK	Milk powder	1950	4	Csak O:2	K2	CA2	3	2	1
1105		UK	Weaning food	2008	4	Csak O:2	K2	CA2	3	2	1
1564		Slovakia	Food	2010	4	Csak O:3	K2	CA2	ND	ND	1
1886		Czech Republic	Spice	2011	4	Csak O:3	K2	CA2	4	45,46	1
1907		Malaysia	Environmental	2012	4	Csak O:4	K2	CA2	13	14	1
1908		Malaysia	Environmental	2012	4	Csak O:4	K2	CA2	13	14	1
1283		UK	Food	2010	8	Csak O:3	K1	CA1	4	3	21
1888		Czech Republic	Food	2011	8	Csak O:1	K1	CA1	4	3	21
1906		Malaysia	Environmental	2012	8	Csak O:1	K1	CA1	4	3	21
1108		UK	Weaning food	2008	12	Csak O:4	K2	CA2	13	14	24
1887		Czech Republic	Food	2011	13	Csak O:2	K2	CA2	25	28	44
1882		Czech Republic	Food ingredient	2011	20	Csak O:3	K1	CA1	48	60	59
1843		Czech Republic	Spice	2010	23	Csak O:2	K1	CA2	ND	ND	ND
1890		Czech Republic	Food ingredient	2011	42	Csak O:2	K1	CA2	26	62	62
1881		Czech Republic	Food ingredient	2011	64	Csak O:2	K1	CA2	26	37	35
1992		UK	Food	2013	136	Csak O:2	K2	CA2	33	23	40
1889		Czech Republic	Food ingredient	2011	198	Csak O:4	K1	CA2	13	14	63
1845		Czech Republic	Food	2010	233	Csak O:2	K2	CA2	47	47	66
1847		Czech Republic	Milk powder	2010	245	Csak O:1	K2	CA1	45	58	56
1884		Czech Republic	Herb	2011	263	Csak O:2	K1	CA2	ND	ND	ND
1990		UK	Food	2013	264	Csak O:2	K1	CA2	ND	ND	ND
1844		Czech Republic	Food ingredient	2010	405	Csak O:3	K2	CA1	21	23	64
1885		Czech Republic	Herb	2011	406	Csak O:2	K1	CA2	50	61	65
2027		UK	Food	2013	406	Csak O:2	K1	CA2	50	61	65
101	*C. malonaticus*	Unknown	Spice	2005	60	Cmal O:1	K2	CA2	24	27	39

510		Czech Republic	Food	1985	7	Cmal O:2	K1	CA1	5	4	18
1369		UK	Herb	2010	69	Cmal O:1	K1	CA2	6	40	39
1846		Czech Republic	Food ingredient	2010	60	Cmal O:1	K2	CA2	24	27	39
1879		Czech Republic	Spice		139	Cmal O:2	K1	CA1	6	59	39
1893		Czech Republic	Food ingredient	2011	289	Cmal O:1	K2	CA2	ND	ND	ND
93		Unknown	Spice	2005	29	Cmal O:1	K2	CA2	ND	ND	ND
1210	*C. dublinensis*	Ireland	Environmental	2004	106	Cdub O:1b	K1	CA1	12	12	7
1458		Korea	Food	2011	74	Cdub O:1	K1	CA1	39	48	7
1463		Korea	Food	2011	95	Cdub O:1	K1	CA1	38	44	54
1460		Korea	Food	2011	76	Cdub O:2	K1	CA2	29	51	58
1461		Korea	Food	2011	77	Cdub O:1	K1	CA1	40	52	67
1462		Korea	Food	2011	70	Cdub O:1	K1	CA1	43	57	5
1464		Korea	Food	2011	78	Cdub O:2	K1	ND	ND	ND	ND
1897		Czech Republic	Food ingredient	2011	213	Cdub O:2	K1	CA2	52	50	68
1330	*C. condimenti*	Slovakia	Food	2010	98	Cuni O:1	K1	CA2	34	9	4
96	*C. universalis*	UK	Spice	2004	48	ND	K2	CA1	36	39	53
1435		Turkey	Food	2010	51	ND	K2	ND	ND	ND	ND
1883		Czech Republic	Spice		137	ND	K2	CA2	46	54	2
92	*C. turicensis*	UK	Herb	2004	35	Ctur O:3	K2	CA2	20	22	42
109		Unknown	Herb	2004	5	ND	K2	CA2	7	6	25
111		UK	Herb	2004	24	ND	K2	CA2	55	42	70
1553		Slovakia	Unknown	-	72	Ctur O:3	K1	CA2	27	30	45
1880		Czech Republic	Herb	2011	344	Ctur O:3	K1	CA2	30	34	47
1895		Czech Republic	Food ingredient	2011	252	Ctur O:1	K2	CA2	49	49	60
16	*C. muytjensii*	Unknown	Spice	2005	347	Cmuyt O:3	K1	CA1	10	10	51
1371		Unknown	Spice	2010	403	Cmuyt O:2	K1	CA2	37	43	26
1527		Slovakia	Food	2010	411	Cmuyt O:1	K1	CA1	44	53	57
1877		Czech Republic	Herb	2011	407	Cmuyt O:1	K1	CA2	51	55	61

This table summarised the molecular characterization of *Cronobacter* spp. isolate from food and environmental sources. This include sequence type, serotype, capsule type, Colanic acid, *galF,gnd* and *rpoB* profile, ND: not determined. K: capsule type. CA: Colanic acid

C. malonaticus strains were divided into 3 groups based on K-capsule, CA-type and serotype profile. Majority of the *C. malonaticus* strains had the capsular profile K2-CA2-O:1; including; ST29, ST60 and ST289. Strains in ST69 were K1-CA2-O:1. While, *C. malonaticus* strains ST7 and ST139 have the capsular profile K1-CA1-O:2. *C. malonaticus* ST7 is associated with adult infections, and is the most frequently isolated *C. malonaticus* sequence type (Forsythe et al. 2014; Alsonosi et al. 2015). Most of *C. dublinensis* strains had the capsular profile K1-CA1-O:1 such as ST70, ST74, ST77, ST95 and ST106. However, strains in ST76 and ST213 had the profile K1-CA2-O:2. *C. turicensis* strains in ST5, ST524, ST35 and ST252 had the capsular profile K2-CA2, and minority of them had the profile K1-CA2 such as ST72 and ST344. *C. universalis* strains had two capsular profile; K2-CA1 in ST48 strains and K2-CA2 in ST137 strains. *C. muytjensii* strains were divided into 2 groups; the first group (ST347 and ST411) had the profile K1-CA1, and the second group (ST403 and ST407) have the profile K1-CA2, a single *C. condimenti* strain in ST98 also had a profile K1-CA2.

Genomic study of *rpoB* gene showed the discriminatory power between species within the *Cronobacter* genus. However, in some cases, it showed less ability to discriminate between STs within the same species such as *C. malonaticus* strains in ST60, ST69 and ST139, these strains have the same *rpoB* profile (*rpoB*39), as well as *C. dublinensis* strains in ST70 and ST106 had the same *rpoB* profile (*rpoB*7).

Chapter 4. Phenotypic diversity of *Cronobacter* genus from food and environmental sources.

4.1 Introduction.

Cronobacter species have been isolated from PIF, milk powder, weaning food, formula constituents, a wide range food and from environments such as milk powder production factories and other manufacturing plants (Forsythe, 2004; Sonbol et al. 2013). Many different microbial methods have been used to isolate, identify and distinguish *Cronobacter* species. Microbiological culturing method is normally used to detect and determine the degree of *Cronobacter* contamination in PIF, milk powder samples and in other foods. *Cronobacter* genus are associated with infants and neonatal infections including meningitis, necrotizing enterocolitis and bacteraemia (Hurrell et al. 2009; Hariri et al. 2013) as well as associated with bacteraemia in immunocompromised adults. This lead to the development and improvement of specific detection and identification methods for *Cronobacter* spp.

Determining the morphology of colony growth on different plate culture media can be an important tool to describe and identify members of the *Cronobacter* genus. MacConkey agar is a selective culture medium designed to isolate and identify Gram-negative bacteria based on lactose fermentation. Iversen and Forsythe (2003) stated that *Cronobacter* species might be of plant origin as a result of their physiological features such as yellow pigment production on TSA, polysaccharide capsule production, and its desiccation resistance, and this led to the early description of 'yellow-pigmented Enterobacter *cloacae'*. Violet Red Bile Glucose Agar (VRBGA) is selective for the *Enterobacteriaceae* family, and it is based on acid production from glucose. *Cronobacter* species can produce red-pink colonies on VRBGA (Iversen and Forsythe, 2007). Xylose lysine deoxycholate agar (XLD) has been recommended for the identification and isolation of *Enterobacteriaceae* from water, foods and dairy products (Chadwick, 1974).

As a result of the unique feature of alpha-D-glucosidase enzyme activity of *Cronobacter* species, a new chromogenic medium (DFI) has been designed by Druggan, Iversen and Forsythe, in order to differentiate and distinguish *Cronobacter* species from other *Enterobacteriaceae* members. This media was more accurate when compared with other traditional *Enterobacteriaceae* enumeration agar such as VRBGA (Iversen et al, 2004).

Cronobacter strains produce blue-green coloured colonies on DFI agar and other Gram-negative bacteria including *Enterobacteriaceae* produced different coloured colonies.

Biochemical tests that the important for the identification and differentiation of *Cronobacter* species include, indole production, malonate and inositol utilization and α-D- glucoside activity (blue-green colour) (Iversen et al. 2007). Furthermore, sialic acid utilization was stated as a significant biochemical test for the differentiation of *Cronobacter* species (Joseph et al. 2013).

The capsule is considered a virulence factor because it enhances the ability of bacteria to cause disease, and may enable the organism to resist desiccation and could facilitate the organism's attachment to plant surfaces. In addition, Hurrel et al. (2009a) illustrated that capsule formation is also involved in formation of biofilms which have been detected in enteral feeding tubes of neonates in neonatal intensive care unit (NICU), as well as contribute to persistence of the pathogen on food contact surfaces (Iversen et al. 2004d).

In this chapter, *Cronobacter* strains isolated from food and environmental sources were studied phenotypically to investigate their appearance on different media, motility determination, indole and α-D- glucoside production, malonate and inositol metabolism, sialic acid utilization, capsule production, biofilm formation, lipase and protease activity, and to link these with their molecular characteristics.

4.2 Results

4.2.1 *Cronobacter* colony morphology types.

4.2.1.1 Appearance of *Cronobacte*r genus on different media (TSA, DFI, XLD, VRBGA, VRBLA and MacConkey agar).

Colony appearance of *Cronobacter* spp. was investigated by using different detection media such as TSA, XLD, VRBGA, VRBLA, DFI and MacConkey agar. Colonies of all isolates on TSA had typical yellow colours after incubation for 24h at 37°C. Strain 1907 (ST4-O:4) showed yellow colour with rough colonies, while strain 1908 (ST4-O:4) was yellow with slimy colonies (Table 4.1). All strains but four showed yellow colour with mucoid appearance on XLD agar, the exception were strain *C. turicensis* 92 (ST35), and *C. sakazakii* strains in ST8 (1283, 1888 and 1906) which showed yellow colour with dry colonies.

All strains were able to grow on VRBGA, and showed pink/purple colour and mucoid colony appearance, except one strain 1888 (ST8) which was pink/purple colour with dry appearance. On VRBLA and MacConkey agar, all strains had a typical pink colour with red halo, except two strains; *C. sakazakii* 1888 (ST8) and *C. turicensis* 109 (ST5) which showed light pink colure with red halo. All strains produced typical blue-green colonies on DFI media after incubation for 24h at 37°C (Table 4.1).

Table 4.1. Appearance of *Cronobacter* spp. on different media; TSA, XLD, VRBGA and VRBLA.

NTU	Species	TSA	XLD	VRBGA	VRBLA
96	*C. universalis*	Yellow/ SM	Yellow/M	Pink/purple/M	Pink/H
1435		″	″	″	″
1883		″	″	″	″
92	*C. turicensis*	″	Yellow/D	″	″
109		″	Yellow/M	″	L/Pink/H
111		″	″	″	″
1553		″	″	″	″
1880		″	″	″	″
1895		″	″	″	″
16	*C. muytjensii*	″	″	″	″
1371		″	″	″	″
1527		″	″	″	″
1877		″	″	″	″
93	*C. malonaticus*	″	″	″	″
101		″	″	″	″
1846		″	″	″	″
510		″	″	″	″
1369		″	″	″	″
1879		″	″	″	″
1893		″	″	″	″
1330	*C. condimenti*	″	″	″	″
1210	*C. dublinensis*	″	″	″	″
1458		″	″	″	″
1460		″	″	″	″
1461		″	″	″	″
1462		″	″	″	″
1463		″	″	″	″
1464		″	″	″	″
1897		″	″	″	″
377	*C. sakazakii*	″	″	″	″
1105		″	″	″	″
1564		″	″	″	″
1886		″	″	″	″
1907		Yellow/ R	″	″	″
1908		Yellow/ S	″	″	″
1283		Yellow/ SM	Yellow/D	″	″
1888		″	″	Pink/purple/D	L/Pink/H
1906		″	″	Pink/purple/M	Pink/H
1107		″	Yellow/M	″	″
1108		″	″	″	″
1887		″	″	″	″
1885		″	Yellow/M	Pink/purple/M	Pink/H
1990		″	″	″	″
2027		″	″	″	″
1843		″	″	″	″
1844		″	″	″	″
1845		″	″	″	″
1847		″	″	″	″
1881		″	″	″	″
1882		″	″	″	″
1884		″	″	″	″
1889		″	″	″	″
1890		″	″	″	″
1992		″	″	″	″

Colony appearance of *Cronobacter* strains were investigated by using different detection media such as TSA, XLD, VRBGA, VRBLA, DFI and MacConkey agar.

S= slimy. R= rough. M= mucoid. D= dry. L= light. H= halo. SM= smooth

4.2.1.2 Appearance on Congo red agar.

Fifty-four strains were incubated on LB agar plates supplemented with Congo red. The colony morphology and colour were recorded as an indication of the binding of the curli fimbriae with Congo red dye after incubation at 37°C for 24 and 48 hours, and at 25°C for 72 hours and 5 days (Table 4.2). *Salmonella enterica* 358 and *E.coli*-K12 were used as positive and negative controls respectively. Five morphotypes were recorded, as demonstrated in table 4.2. Including, pink, dry, and rough (PDAR); brown, dry, and rough (BDAR); pink and smooth (PAS); brown and smooth (BAS); red and smooth (RAS). The positive control was characterized as PAS after incubation at 37°C for 24 and 48 hours and characterized as PDAR after incubation at 25°C for 72 hours and 5 days.

The most common morphotype among the *Cronobacter* tested strains after incubation at 37°C for 24 and 48 hours was BAS, as noted for 23 isolates (41.8%), 16 isolates (29%) that were identified as PAS, followed by 13 isolates (23.6%) with the BDAR morphotype, and 4 isolates (7.2%) with the PDAR morphotype. The majority of tested strains were the same morphotype (BAS) after incubation at 37°C for 24 and 48 hours, the exception were strains *C. malonaticus* (1879), four *C. dublinensis* strains (1210, 1458 and 1462) and four *C. sakazakii* strains (1886, 1845, 1889 and 1890) which showed BDAR morphology after incubation for 48 hours at 37°C (Table 4.2). Regarding incubation period at 25°C, BAS morphotype was the most common morphotype among the tested strains, as noted for 24 isolates (42.6 %) followed by 17 isolates (31.4%) with BDAR morphotype. However, most strains showed different morphotype after incubation at 25°C for 72h compared with incubating at 25°C after 5 days. Two *C. malonaticus* strains (93 and 1893), and one *C.sakazakii* strain (377) exhibited a RAS morphotype (5.5 %), and were considered to exhibit reduced binding of the Congo red dye, while those with the BAS morphotype (56.5%) did not show any binding of the Congo red dye.

4.2.1.2.1 Curli fimbriae genes.

Curli-encoding genes are clustered in two operons: *csgBAC* and *csgDEFG* (Brombacher et al, 2006). Joseph et al (2012b) reported that a curli fimbrial gene cluster has been found in the genome of *C. turicensis* z3032. Curli fimbriae homologues were found in *C. malonaticus*, *C. universalis* strains and the single *C. condimenti* strain. However, none of the curli fimbriae homologues were noted in any of the *C. sakazakii* and *C. muytjensii*

genomes analysed in this study. On the other hand, *C. dublinensis* strains were divided into two groups based on presence or absences of these genes. Strains *C. dublinensis* subsp *C. dublinensis* (LMG 23823, 1210, 1458 and 1463) showed presence of this gene cluster, whereas strains *C. dublinensis* ssp. *lausannensis* (LMG 23824, 583 and 1460) and *C. dublinensis* ssp. *lactaridi* (LMG 23825, 1461 and 1462) showed absences of these genes. Strains (LMG 23823, LMG 23824 and LMG 23825) *C. dublinensis* subsp *C. dublinensis*, *C. dublinensis* subsp. *lausannensis*, and *C. dublinensis* subsp. *lactaridi*, were used as a references for *C. dublinensis* subspecies strains respectively. Furthermore, *C. turicensis* strains were also divided into groups, this cluster was found in strains *C. turicensis* (1211, 1880 and 1895), however none of these genes were noted in 92, 1553 and 1554 strains. Gene clusters *csgBAC* and *csgDEFG* do not seem to be necessary for production pink colonies. This observation shows that no clear correlation exists between the phenotypic detection of curli fimbriae by Congo red staining and the genotypic detection of the curli fimbriae genes.

4.2.1.3 Appearance of *Cronobacter* species on Calcofluor media.

Production of cellulose was investigated by streaking the *Cronobacter* strains on supplemented LBA medium with Calcofluor White stain. The production of cellulose and cell appearance were visualised using ultra-violet (366nm) fluorescence. Binding of any cellulose produced by the *Cronobacter* isolates was observed based on the presence of a blue colony under UV light; figure 4.1. The majority of tested strains (19 isolates, 35%) showed moderate fluorescent signal (++). These include *C. sakazakii* strains; 377 and 1907 (ST4), 1887 (ST13), 1885 and 2027 (ST406), 1990 (ST264), 1845 (ST233), 1881(ST64), 1882 (ST20), 1884 (ST263), 1890 (ST42), three *C. malonaticus* strains; 510 (ST7), 1846 (ST60) and 1369 (ST69), three *C. muytjensii;* 1371 (ST403), 1527 (ST411) and 1877 (ST407). *C. turicensis* strain 1895 (ST252) was also showed moderate fluorescent signal (++). Followed by 13 isolates (24%) with the strong fluorescent signal (+++). These include three *C. sakazakii* strains; 1886 (ST4), 1107 (ST9) and 1844 (ST405), three *C. malonaticus* strains; 93 (ST29), 101 (ST60) and 1893 (ST289), and two *C. turicensis* strain; 92 (ST35) and 1880 (ST344).

The strong fluorescence was noted also in 3 *C. universalis* strains; 96 (ST48), 1435 (ST51) and 1883 (ST137), as well as was noted in the single *C. condimenti* strain 1330 (ST98).

Eight strains (14.8%) had a low fluorescent signal (+). These include six *C. sakazakii* strains; 1105 and 1908 (ST4), 1843 (ST23), 1847 (ST245), 1889 (STST198) and 1992 (ST136), one *C. malonaticus* strain 1879 (ST139) and one *C. turicensis* strain 111 (ST24). However, 14 isolates (25.9%) were negative for this phenotype (Figure 4.1). These including, one *C. turicensis* strain 109 (ST5), all of *C. dublinensis* strains (n=8); 1210 (ST106), 1458 (ST74), 1460 (ST76), 1461 (ST77), 1462 (ST70), 1463 (ST95), 1464 (ST78) and 1897 (ST213), and five *C. sakazakii* strains; 1564 (ST4), 1283, 1888 and 1906 (ST8) and 1108 (ST12); table 4.2.

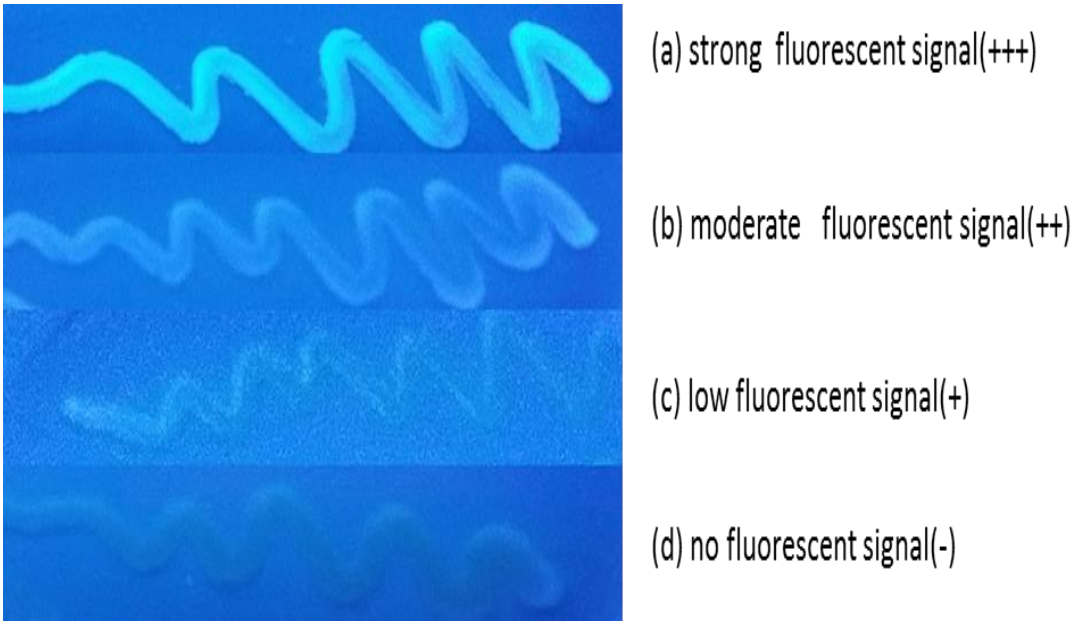


Figure 4.1. Cell morphology on Calcofluor media. (a) Strong fluorescent signal (+++), (b) moderate fluorescent signal (++), (c) low fluorescent signal (+) and (d) no fluorescent signal (-).

4.2.1.3.1 Cellulose gene cluster

Ogrodzki and Forsythe (2015) stated that the cellulose gene cluster consist of nine genes (*bcsCZBAQEFG* and *yhjR*). This cluster was present in nearly all *Cronobacter* strains, the exceptions were *C. sakazakii* strain 1882 (ST20, CC20) and the single *C. condimenti* 1330 strain (ST98). No obvious correlation between the phenotypic detection of cellulose production by Calcofluor white staining and the genotypic detection of the cellulose gene cluster was observed.

Table 4.2. Cell morphology of *Cronobacter* spp. on Congo red and Calcofluor media.

strain	species	Congo red				Calcofluor media	
		37°C/(24h)	37°C/(48h)	25°C/(72h)	25°C/(5days)	28°C/(48h)	37°C/(24h)
96	*C. universalis*	BAS	BAS	BAS	PDAR	+++	+++
1435		PAS	PAS	PDAR	PDAR	+++	+++
1883		BDAR	BDAR	BDAR	PDAR	+++	+++
92	*C. turicensis*	PAS	PAS	BAS	PAS	+++	+++
109		PAS	PAS	BAS	PAS	-	+(-)
111		BAS	BAS	BAS	BAS/H	+	+
1553		BDAR	BDAR	BAS	BAS	++	++
1880		PAS	PAS	BAS	PAS	++	+++
1895		PDAR	PDAR	BAS	PAS	++	++
16	*C. muytjensii*	PDAR	PDAR	PDAR	PDAR	++	+++
1371		PAS	PAS	BAS	BAS	+	++
1527		PAS	PAS	BAS	BAS	+	++
1877		PAS	PAS	BAS	PDAR	++	++
93	*C. malonaticus*	PAS	PAS	PDAR	RAS	+++	+++
101		PAS	PAS	PAS	PAS	+++	+++
1846		BAS	BAS	PDAR	PDAR	++	++
510		PAS	PAS	BAS	BAS	++	++
1369		BAS	BAS	BAS	PDAR	++	++
1879		BAS	BDAR	BAS	BDAR	+	+
1893		PDAR	PDAR	RAS	RAS	+++	+++
1330	*C. condimenti*	BAS	BAS	BAS	BDAR	+++	+++
1210	*C. dublinensis*	BAS	BDAR	BDAR	BDAR	-	+(-)
1458		BAS	BDAR	BDAR	BDAR	-	+(-)
1460		BDAR	BDAR	BDAR	BDAR	-	+(-)
1461		BAS	BAS	BAS	BAS	-	+(-)
1462		BAS	BDAR	BAS	BAS	-	+(-)
1463		BAS	BAS	BDAR	BDAR	-	+(-)
1464		BDAR	PDAR	BDAR	BDAR	-	+(-)
1897		BAS	BAS	BDAR	BDAR	-	+(-)
377	*C. sakazakii*	PAS	PAS	PAS	RAS	++	++
1105		BAS	BAS	PAS	PAS	+	+
1564		BAS	BAS	BAS	BAS	-	-
1886		BAS	BDAR	BDAR	PDAR	+++	+++
1907		BDAR	BDAR	BDAR	PDAR	++	++
1908		BDAR	BDAR	BDAR	PDAR	++	+
1283		PAS	PAS	BAS	BAS	+(-)	-
1888		PDAR	PDAR	BAS	BAS	+(-)	-
1906		PAS	PAS	BAS	BAS	+(-)	-
1107		PAS	PAS	PAS	RAS	+++	+++
1108		BDAR	BDAR	BDAR	BDAR	-	-
1887		BAS	BAS	PAS	PAS	++	++
1885		BAS	BAS	BAS	BAS	++	++
1990		BAS	BAS	BAS	BAS	++	++
2027		BAS	BAS	BDAR	PDAR	++	++
1843		BDAR	BDAR	BDAR	PDAR	+	+
1844		BAS	BAS	BDAR	PDAR	+++	+++
1845		BAS	BDAR	PDAR	PDAR	++	++
1847		BDAR	BDAR	PAS	PAS	+	+
1881		BDAR	BDAR	PDAR	PDAR	++	++
1882		BDAR	BDAR	BDAR	PDAR	++	++
1884		BDAR	BDAR	BAS	PDAR	++	++
1889		BAS	PDAR	BDAR	PDAR	+	+
1890		BAS	BDAR	BDAR	PDAR	++	++
1992		BAS	BAS	BAS	PDAR	+	+
1230	*E.coli-K12*	BDAR	BDAR	BAS	BDAR	-	-
358	*salmonella*	PAS	PAS	PDAR	PDAR	++	++

(1) Cell morphology observed with Congo red dye binding. (a) pink, dry, and rough (PDAR); (b) brown, dry, and rough (BDAR); (c) pink and smooth (PAS); (d) brown and smooth (BAS)., red and smooth (RAS). (2) Cell

morphology observed with Calcofluor media (a) strong fluorescent signal (+++),(b) moderate fluorescent signal(++) , (b) low fluorescent signal(+) and no fluorescent signal(-)

4.2.2 Motility of the *Cronobacter* genus.

The mobility of *Cronobacter* spp. strains through the motility medium at 37°C after the incubation for 18h are shown in figures 4.2 and figure 4.3. *S. enterica* 358 and *E.coli* K12 strains were used as positive and negative controls respectively. Strains showed a wide-range of motility zone diameter. Most of *C. sakazakii* strains were motile, except 1847 (ST245) and 1906 (ST8). The largest motility zone was over 75 mm (diameter) and was shown by *C. sakazakii* strains 1887 (ST13), 1845 (ST233), 1990 (ST264), 1885 and 2027 (ST406). However, the smallest motility zone was 45 mm and was shown by *C. sakazakii* ST4 strains 377, 1564 and 1908, 1108 (ST12).

All *C. universalis, C. turicensis, C. muytjensii, C. malonaticus, C. dublinensis* strains and single *C. condimenti* strain were motile. These strains showed different motility zone diameters, between 28 to 77 mm. For example, the largest motility zone for these strains were *C. turicensis* strain 1880 (ST344), *C. malonaticus* strain 93 (ST29), *C. universalis* 96 (ST48), *C. muytjensii* 1527 (ST411), *C. dublinensis* 1210 (ST106) and the single C. *condimenti* strain 1330 (ST98) were 79 mm, 78.5 mm, 78.5 mm and 72 mm, 67 mm,33mm respectively. While, the smallest motility zone were *C. universalis* strain 1435-ST51 (71mm), *C. turicensis* strain 111 (ST72, 28mm), *C. muytjensii* strain 16 (ST34, 45mm), *C. malonaticus* strain 1879 (ST139, 63mm) and *C. dublinensis* strain 1897 (ST213, 38mm). Generally, for comparison data of all strains, *C. sakazakii* strains in ST8 and ST4 were less motile than other *C. sakazakii* STs. Overall, there was no significant difference when the motility of seven species were compared to each other (Figures 4.2 and Figure 4.3).

4.2.2.1 Flagellar genes.

Kucerova et al (2010) and Joseph et al (2012b) reported that *fli* genes that are linked to flagella expression and movement in *Cronobacter* species. A BLAST genome search was applied to investigate the presence of these genes. All strains that have their genomes sequenced for both motile and non-motile strains showed the presence of *fliA-Z* gene cluster. This, however suggests that these motility genes might be not expressed or unrecognised mutation could have occurred in non-motile strains.

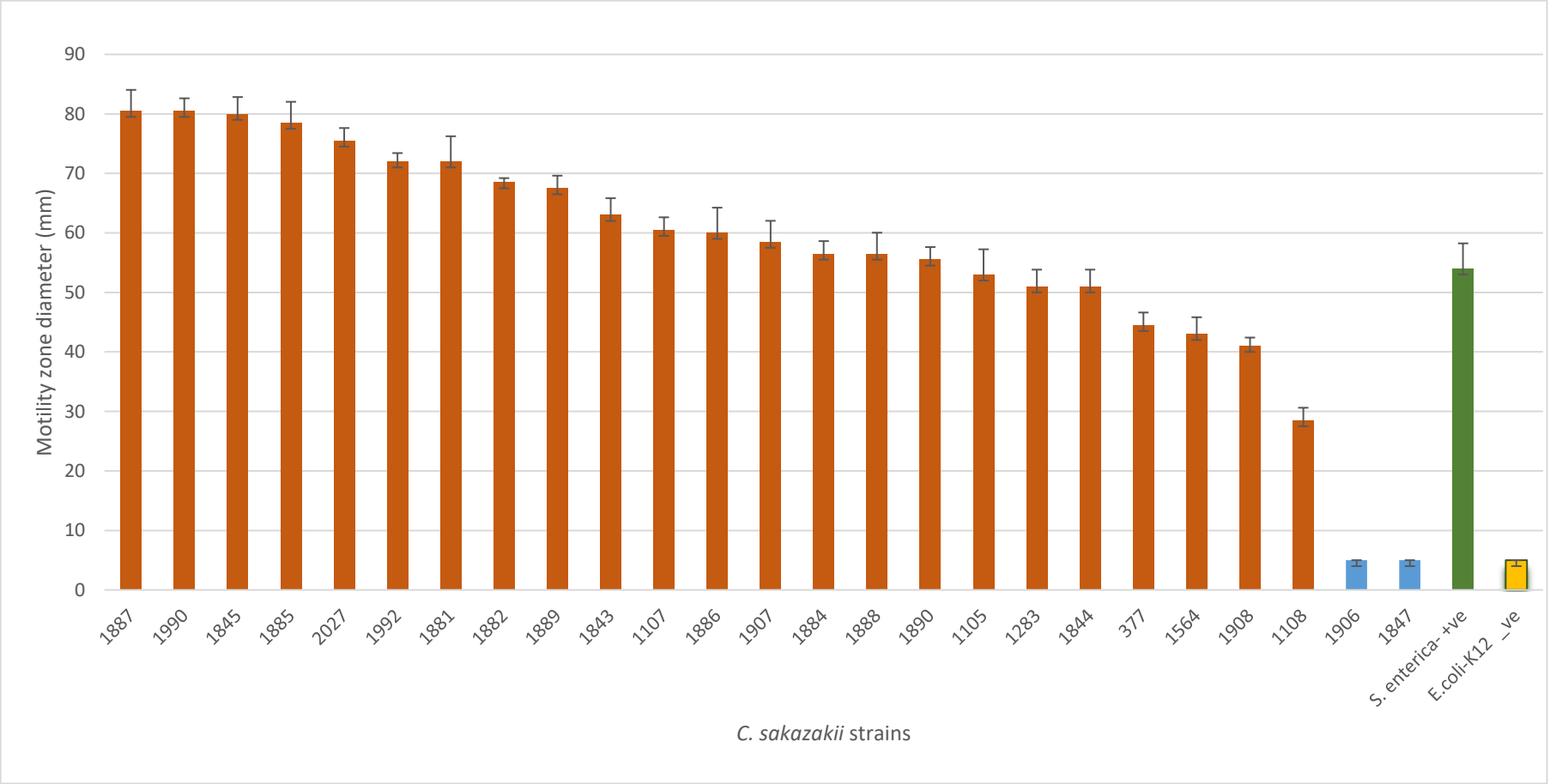


Figure 4.2. Motility of *C. sakazakii* strains through the motility medium at 37°C after the incubation for 18h. The diameter for the motility zone was measured in millimetres. Standard deviation represents the error bars, experiments were assayed in three independent assays.

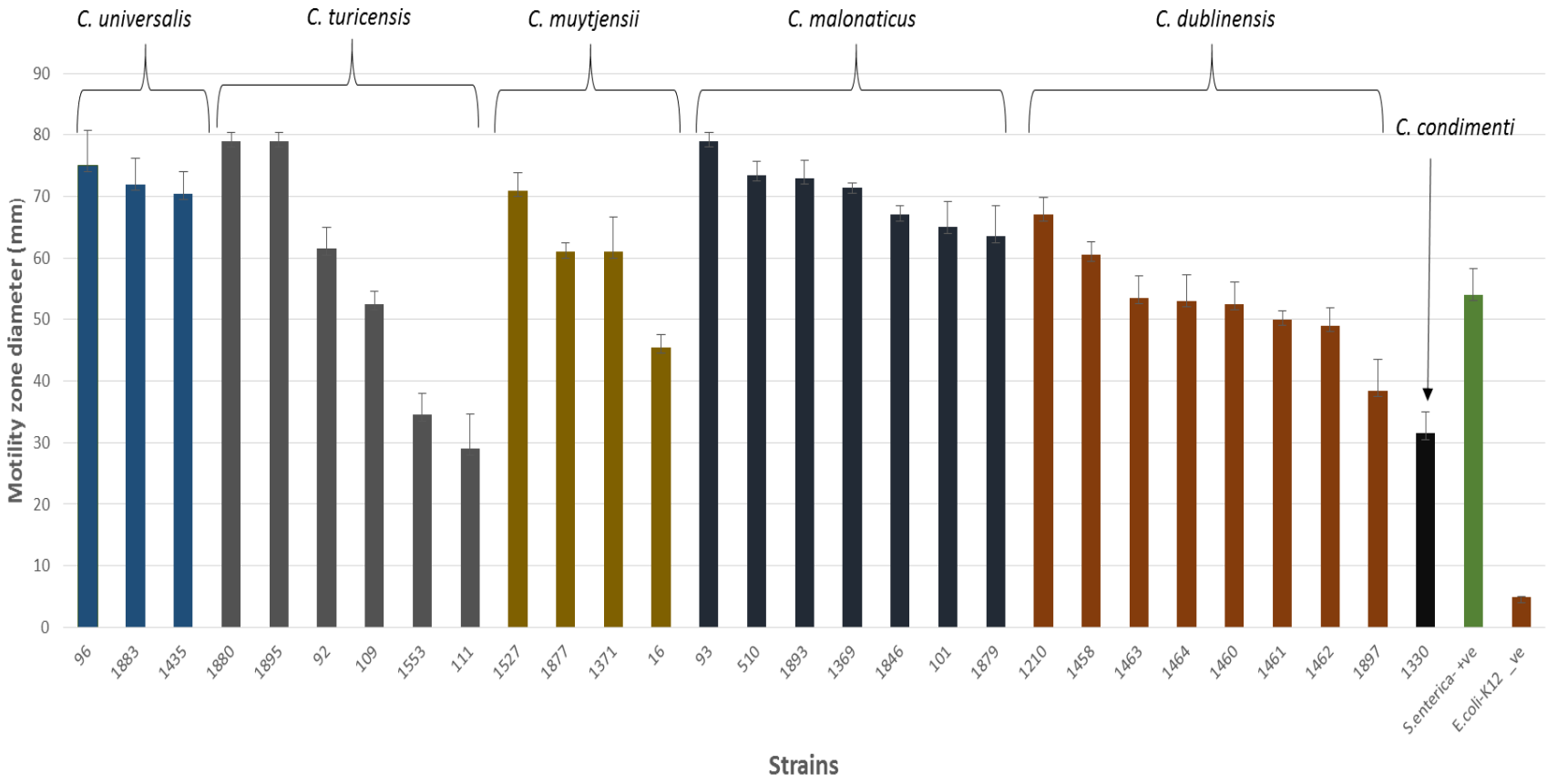


Figure 4.3. Motility of *C. universalis*, *C. turicensis*, *C. muytjensii*, *C. malonaticus, C. dublinensis* strains and single *C. condimenti* strain through the motility medium at 37°C after the incubation for 18h. The diameter for the motility zone was measured in millimetres. Standard deviation represents the error bars, experiments were assayed in three independent assays.

4.2.3 *Cronobacter* genus metabolism.

4.2.3.1 Biochemical test profiling.

Biochemical differentiation between *Cronobacter* species. According to Iversen et al (2007), the important biochemical tests for the differentiation of *Cronobacter* species were indole production, malonate, inositol utilization and production of methyl-α-D-glucoside. Moreover, sialic acid utilization was reported as a significant biochemical test for the differentiation of *Cronobacter* species, In particularly closely related species such as all *C. sakazakii* and *C. malonaticus* (Joseph et al. 2013). These relevant biochemical tests were applied to all strains. Indole production, inositol utilization and α- glucoside activity were measured by using the API32E kit (bio- Mérieux). Malonate PPA Broth (Thermo Fisher, UK) was used to determine malonate utilization. Minimal medium containing sialic acid was used to determine sialic acid utilization. The biochemical test profiling of strains is shown in table 4.5.

4.2.3.1.1 Indole production.

The indole test is a biochemical test performed to detect the ability of *Cronobacter* species to degrade the amino acid tryptophan and produce indole. All *C. muytjensii*, *C. dublinensis* strains and the single *C. condimenti* strain were able to produce indole. However, all *C. sakazakii, C. malonaticus, C. turicensis* and *C. universalis* strains were negative for indole production test (Table 4.5).

4.2.3.1.1.1 Indole production genes.

A *tnaA* gene encodes for the tryptophanase/L-cysteine desulfhydrase, which degrades L-tryptophan to indole (Li and Young, 2013). This gene was found in all *C. dublinensis,* and *C. muytjensii* strains as well as the single *C. condimenti* strain (1330), and was absent in all *C. sakazakii, C. malonaticus, C. turicensis* and *C. universalis* strains.

According to the phylogenetic tree of *Cronobacter* strains, based on nucleotide sequences of *tnaA* gene (728 bp), the seven *C. dublinensis* strains clustered into 3 clusters based on sub species level (Figure 4.4). Group 1 were *dublinensis* subsp. *dublinensis* strains 1463 (ST95), 1210 (ST106) and 1458 (ST74), group 2 was *C. dublinensis* subsp. *lausanensis* strains 1460 (ST76) and 1897 (ST213), and group 3 was *C. dublinensis* subsp. *lactaridi* strains 1461 (ST77) and 1462 (ST70).

C. muytjensii strains were divided into 2 groups groups, the first group was strain 1371 (ST403), and the second group was strains 16 (ST347), 1527 (ST411), and 1877 (ST407). The single *C. condimenti* strain clustered nearest the *C. muytjensii* strains (Figure 4.4).

All strains were positive for *tnaA* gene except for the non-indole production strains (negative strains); *C. sakazakii, C. malonaticus, C. turicensis and C. universalis* strains that lacked this gene. This suggests that this gene is important for indole production, and might be used to identify and distinguish between *Cronobacter* species.

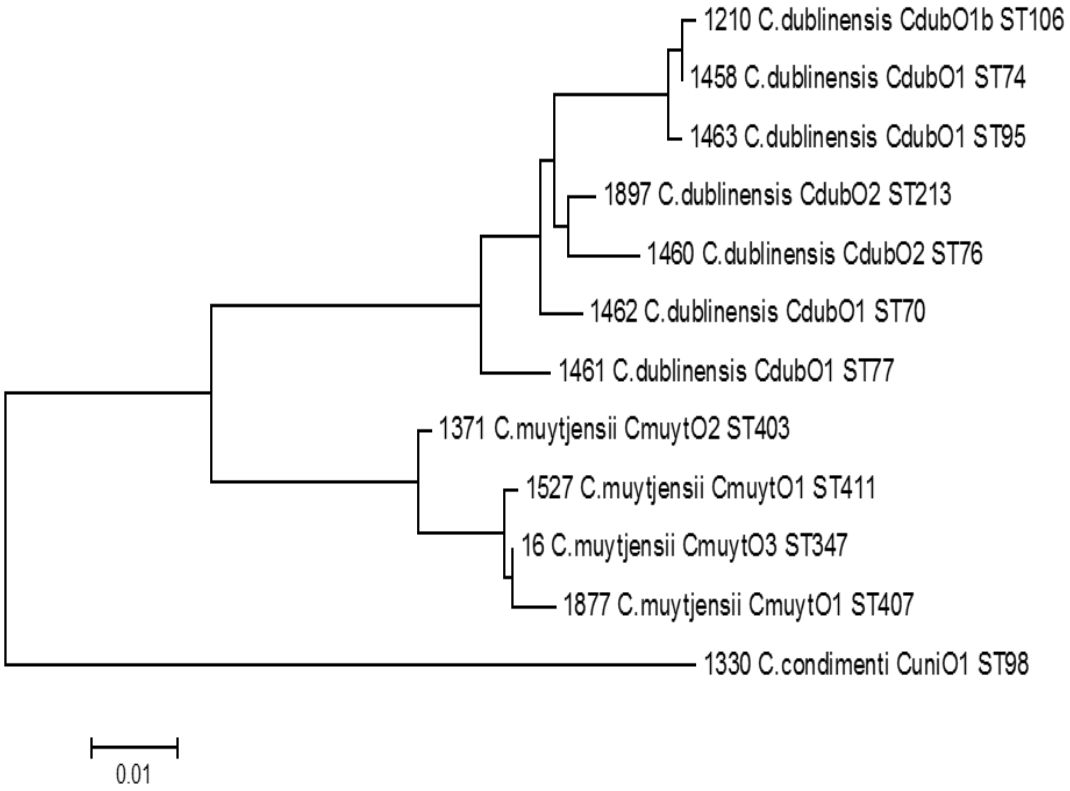


Figure 4.4. The phylogenetic tree of *Cronobacter* strains, based on nucleotide sequences of *tnaA* gene (728 bp). The tree was drawn using MEGA 6.

4.2.3.1.2 α-glucosidase activity (blue-green colour production).

Alpha-glucosidase activity is one of the most important biochemical features, which distinguishes and differentiates *Cronobacter* species from other *Enterobacteriaceae* members (Lehner et al. 2006; Iversen et al. 2007). All *Cronobacter* strains were able to produce the blue-green colour as result of α-glucosidase activity (Table 4.5).

4.2.3.1.3 Inositol utilization.

The inositol utilization test of 54 strains was determined using the API32E kit (bio-Mérieux). Most *C. sakazakii* strains showed the ability to utilize inositol, the exception were strains 377 (ST4), 658 (ST1), 1844 (ST405), 1845 (ST233), 1847 (ST245) and 1992 (ST136). All *C. muytjensii, C. universalis, C. turicensis* strains and the single *C. condimenti* strain were able to utilize inositol. All *C. malonaticus* strains were able to utilize inositol, but one strain 510 (ST7) was unable to utilize inositol. This observation led to an expanded the study concerning *C. malonaticus* species (18 strains; table 4.3), in particular sequence type 7, because this sequence type is associated with adult infections, and the most frequently isolated *C. malonaticus* sequence type (Forsythe et al. 2014; Holy et al. 2014; Alsonosi et al. 2015).

C. dublinensis strains were divided into 2 separate groups based on inositol utilization. The first group showed ability to utilize inositol; including strains 1210, 1458, 1463, 1461 and 1462, and the second group was unable to utilize inositol (1460).

4.2.3.1.3.1 Inositol utilization genes.

Genome analyses using BLAST genome search was applied to investigate the presence of inositol genes that are linked with inositol utilization. Regarding the laboratory result, particularly the variation between the *C. malonaticus* ST7 and other *C. malonaticus* STs, and the variation within *C. dublinensis* strains based on inositol utilization. Available genome sequences of *Cronobacter* spp. were used in this study (Table.3.1). The gene cluster (*iolD, iolE, iolB, iolT, iolG* and *iolC*) encoding for inositol utilization were present in all *C. sakazakii* strains except for 6 strains; 377 (ST4), 658 (ST1), 1844 (ST405), 1845 (ST233), 1847 (245) and 1992 (ST136).

All *C. muytjensii, C. universalis, C. turicensis* strains and the single *C. condimenti* strain that have their genomes sequenced showed the presence of *iolD, iolE, iolB, iolT, iolG* and *iolC* genes. *C. dublinensis* genome sequences (n=11) were divided into 2 groups, the first group showed presence of inositol utilization genes including strains; *C. dublinensis* subsp *dublinensis;* LMG 23823 and 1210 (ST106), 1458 (ST740, 1463 (ST95), and *C. dublinensis* subsp. *lactaridi* strains LMG 23825 (ST 79) 1461 (ST77) and 1462 (ST70). The second group showed absences of these genes including; *C. dublinensis*

subsp. *lausanensis* strains; LMG 23824 (ST80), 583 (ST346), 1460 (ST76) and 1560 (ST341).

All STs of *C. malonaticus* strains were positive for *iolD, iolE, iolB, iolT, iolG* and *iolC*, the exception were ST7 strains (510, 681, 665, 688, 893,1830, 1835, 1835, 1914 and LMG23826; non-inositol utilization strains) which showed the absence of these genes. In general, strains, which are positive for inositol utilization, showed the presence of inositol gene cluster and negative strains showed absences of this gene cluster (Table.4.3). This suggests that these genes are essential for inositol utilization, and might be used to identify and distinguish of *Cronobacter* strains to the subspecies level.

Table 4.3. Presence/absence of inositol utilization genes in sequenced genomes of *Cronobacter* strains.

Species	NTU	ST	O-antigen	*iolD*	*iolE*	*iolB*	*iolT*	*iolG*	*iolC*
C. dublinensis	1210	106	Cdub O:1	+	+	+	+	+	+
	LMG 23823	106	Cdub O:1	+	+	+	+	+	+
	1458	74	Cdub O:1	+	+	+	+	+	+
	1463	95	Cdub O:1	+	+	+	+	+	+
	1461	77	Cdub O:1	+	+	+	+	+	+
	1462	70	Cdub O:1	+	+	+	+	+	+
	LMG 23825	79	Cdub O:1	+	+	+	+	+	+
	LMG 23824	80	Cdub O:2	-	-	-	-	-	-
	1560	341	Cdub O:2	-	-	-	-	-	-
	583	346	Cdub O:2	-	-	-	-	-	-
	1460	76	Cdub O:2	-	-	-	-	-	-
C. malonaticus	101	60	Cmal O:1	+	+	+	+	+	+
	1846	60	Cmal O:1	+	+	+	+	+	+
	507	11	Cmal O:3	+	+	+	+	+	+
	512	11	CsakO:5	+	+	+	+	+	+
	1369	69	Cmal O:1	+	+	+	+	+	+
	1569	307	Cmal O:1	+	+	+	+	+	+
	1879	139	Cmal O:2	+	+	+	+	+	+
	2045	302	Cmal O:2	+	+	+	+	+	+
	2046	302	CsakO:2	+	+	+	+	+	+
	510	7	Cmal O:2	-	-	-	-	-	-
	681	7	Cmal O:2	-	-	-	-	-	-
	665	7	Cmal O:2	-	-	-	-	-	-
	688	7	Cmal O:2	-	-	-	-	-	-
	893	7	Cmal O:2	-	-	-	-	-	-
	1830	7	Cmal O:2	-	-	-	-	-	-
	1835	7	Cmal O:2	-	-	-	-	-	-
	1914	7	Cmal O:2	-	-	-	-	-	-
	LMG23826	7	Cmal O:2	-	-	-	-	-	-
C. condimenti	1330	98	Cuni O:1	+	+	+	+	+	+
C. muytjensii	3 strains	3 STs	O:1, O:2, O:3	+	+	+	+	+	+
C. universalis	2 strains	2 STs	-	+	+	+	+	+	+
C. turicensis	5 strains	4 STs	O:3	+	+	+	+	+	+
C. sakazakii	377	4	sakaO:2	-	-	-	-	-	-
	658	1	sakaO:1	-	-	-	-	-	-
	1844	405	sakaO:3	-	-	-	-	-	-
	1845	233	sakaO:1	-	-	-	-	-	-
	1847	245	sakaO:1	-	-	-	-	-	-
	1992	136	sakaO:2	-	-	-	-	-	-
	20 strains	18 STS	most	+	+	+	+	+	+

Genome analyses using BLAST genome search was applied to investigate the presence of inositol genes that are linked with inositol utilization. + = gene presence. - = gene absence

4.2.3.1.4 Sialic acid and malonate utilization by *Cronobacter* spp.

This study investigated whether all *Cronobacter* species can use sialic acid as a carbon source. Sialic acid is found in the human body, such as breast milk, intestinal mucin, gangliosides in the brain and infant formula (Joseph et al. 2013). Therefore, this metabolism could be a significant virulence factor. The present study showed that all *C. malonaticus*, *C. muytjensii C. universalis*, *C. dublinensis* strains and single *C. condimenti* strain were unable to grow on minimal medium M9 with sialic acid as the only carbon source. All *C. sakazakii* strains and some of *C. turicensis* strains (92, 1553 and 1880) were able to grow on minimal medium M9 with sialic acid which means they were capable to utilize sialic acid as a carbon source. Conversely, *C. turicensis* strains 109, 111, and 1895 were not able to grow on minimal medium M9 with sialic acid; table 4.5.

4.2.3.1.4.1 Sialic acid utilization genes.

The *NanKTAR* gene cluster encodes for the proteins involved in the uptake and utilization of sialic acid. These genes were only found in the genomes of *C. sakazakii* strains and some of the *C. turicensis* strains; 92, 1553 and 1880. However, this gene cluster was absent from the genomes of all other *Cronobacter* species. Artemis comparison was used to compare between isolate *C. sakazakii* and *C. turicensis*, which are positive for sialic acid utilization test against *C. turicensis*, isolate which negative to utilize sialic acid, in order to investigate if the sialic acid utilization cluster genes were found or missing in *Cronobacter* as a result of genome evolution. Figure 4.5 showed the comparison between genomic structure of *NanKTA* gene cluster in *C. sakazakii* and *C. turicensis* isolates positive to utilize sialic acid against *C. turicensis* isolate which negative for sialic acid utilization (*NanKTA* cluster encoding for the proteins involved in the uptake and utilization of sialic acid).

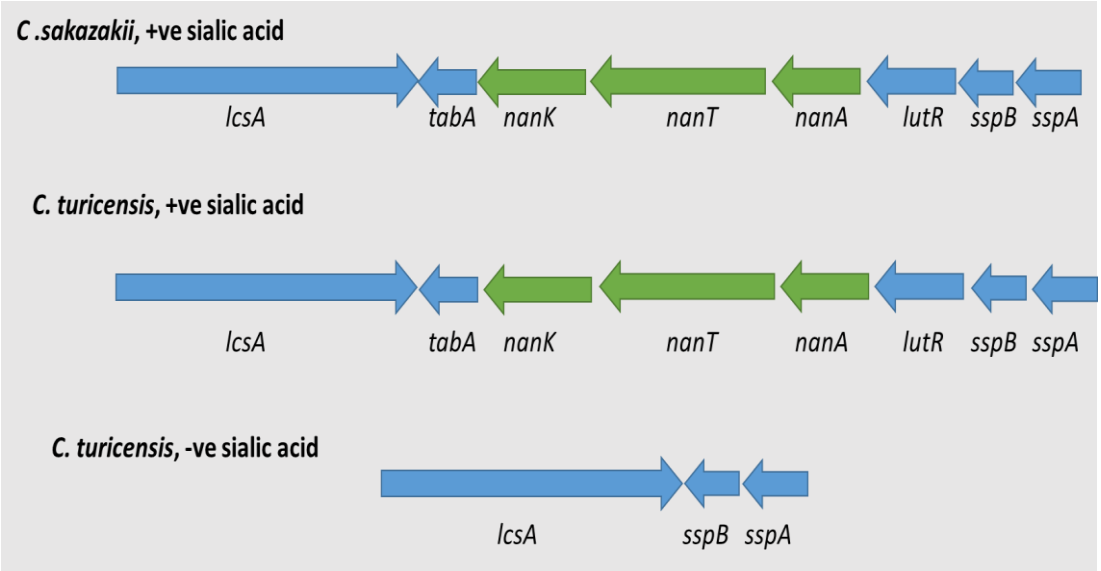


Figure 4.5. Genomic structure of cluster *nanKTA* in the genomes of *C. sakazakii, C. turicensis* (positive for sialic acid utilization) and genome of *C. turicensis* (negative to utilize sialic acid).

4.2.3.1.5 Utilization of malonate.

Malonate is an organic acid that some bacteria can use for carbon and energy. Malonate utilization is an important differential trait. This study demonstrated that most of *C. sakazakii* strains were unable to utilize malonate, except strains 1845 (ST233) and 1881 (ST64). All strains of *C. malonaticus*, *C. universalis*, *C. turicensis* and *C. muytjensii* strains were able to utilize malonate. The single *C. condimenti* strain was also able to growth on malonate broth. On the other hand, *C. dublinensis* strains were divided into two separate groups based on malonate utilization (Table 4.5). Three of eight strains were able to utilize malonate; 1458 (ST74), 1463 (ST95) and 1210 (ST106). Whereas, five strains (1460, 1461, 1462, 1464 and 1897) were not able to utilize malonate (Table 4.5).

4.2.3.1.5.1 Malonate utilization genes.

Malonate utilization, is an important biochemical test for the differentiation of *Cronobacter* species. Malonate metabolism is encoded by the *mauR, madA, citG, mdcC, madC, madD, mdcG* and *fabD* genes. Presence and absence of malonate utilization genes were investigated by using BLAST genome search. Available genome sequences of *C. malonaticus*, *C. muytjensii*, *C. universalis, C. turicensis* strains and single genome of *C. condimenti* strain showed the presence of the genes which are responsible for

malonate utilization; *mauR, madA, citG, mdcC, madC, madD, mdcG* and *fabD*. However, these genes were absent in all *C. sakazakii* sequence types, but two strains in ST64 and ST233 showed presences of these genes. As a result of this variation, all available genome sequences of ST64 (3 strains) and ST233 (2 strains) in *Cronobacter* MLST databases were used in this part of study, in order to investigate if these genes are found or missing in all *C. sakazakii,* in these two STs (Table 4.4). Moreover, Artemis comparison tool was used to consider if the malonate utilization cluster genes were acquired or lost in *C. sakazakii* strains as a result of genome evolution (Figure 4.6).

C. dublinensis strains were divided into two separate groups based on presence or absences of these genes, *C. dublinensis* subsp. dublinensis strains; LMG 23823, 1210, 1458 and 1463, which malonate positive showed presences of the malonate gene cluster. While, *C. dublinensis* subsp*. lausannensis*, and *C. dublinensis* subsp. *lactaridi* which malonate negative strains showed absences of this gene cluster. *C. dublinensis* subsp. *dublinensi*s (LMG 23823) strain, *C. dublinensis* subsp*. lausannensis* (LMG 23824), and *C. dublinensis* subsp. *lactaridi* (LMG 23825)*,* were used as a references for *C. dublinensis* subspecies strains (Table 4.4).

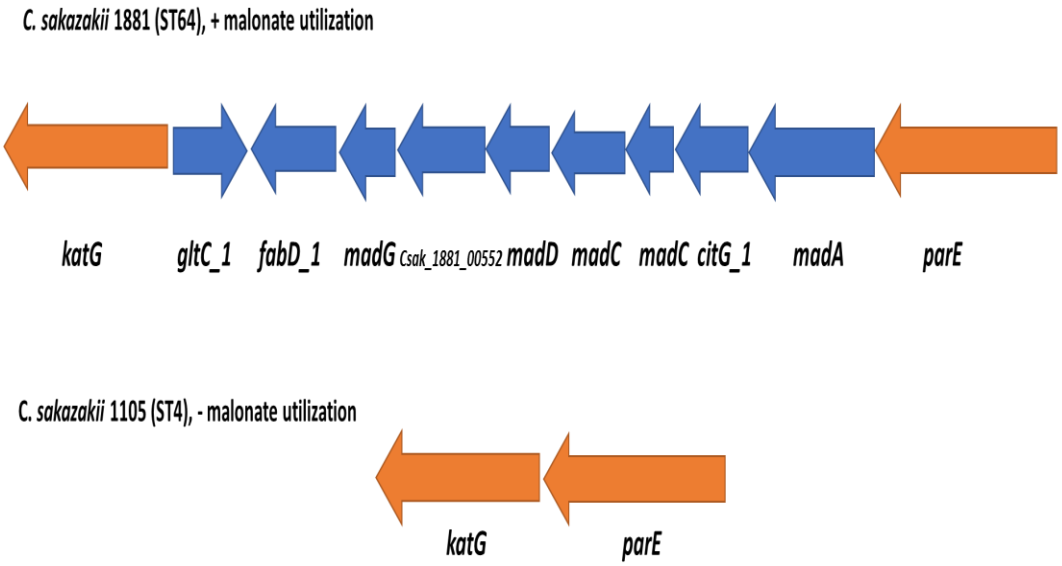


Figure 4.6. Comparison between genomic structure of malonate genes cluster *madGDCA,fadD* and *citG* in the genome of *C. sakazakii* 1881 (ST64), positive for malonate utilization and genome of *C. sakazakii* 1105 (ST4), negative to utilize malonate.
Malonate metabolism is encoded by the *mauR, madA, citG, mdcC, madC, madD, mdcG* and *fabD* genes. Artemis comparison tool was used to consider if the malonate utilization cluster genes were acquired or lost in *C. sakazakii* strains.

Table 4.4. Distribution of the malonate and other related genes across the available sequenced genomes of seven *Cronobacter* species.

Species	Available genome sequences information			Malonate utilization 37°C/24h	*mauR*	*madA*	*citG*	*mdcC*	*madC*	*madD*	*mdcG*	*fabD*
	NTU	ST	O-antigen									
C. dublinensis	1210	106	Cdub O:1	+	+	+	+	+	+	+	+	+
	LMG 23823	106	Cdub O:1	+	+	+	+	+	+	+	+	+
	1458	74	Cdub O:1	+	+	+	+	+	+	+	+	+
	1463	95	Cdub O:1	+	+	+	+	+	+	+	+	+
	1460	76	Cdub O:2	-	-	-	-	-	-	-	-	-
	1461	77	Cdub O:1	-	-	-	-	-	-	-	-	-
	1462	70	Cdub O:1	-	-	-	-	-	-	-	-	-
	LMG 23824	80	Cdub O:2	-	-	-	-	-	-	-	-	-
	LMG 23825	79	Cdub O:1	-	-	-	-	-	-	-	-	-
C. sakazakii	1845	233	Csak O:2	+	+	+	+	+	+	+	+	+
	FDA00008004	233	Csak O:2	+	+	+	+	+	+	+	+	+
	2051	64	Csak O:2	+	+	+	+	+	+	+	+	+
	1881	64	Csak O:2	+	+	+	+	+	+	+	+	+
	Chcon-8	64	Csak O:2	+	+	+	+	+	+	+	+	+
	23 strains	17 STs	O:1, O:2 O:3, O:4	-	-	-	-	-	-	-	-	-
C. condimenti	1330	98	Cuni O:1	+	+	+	+	+	+	+	+	+
C. muytjensii	3 strains	3 STs	O:1, O:2, O:3	+	+	+	+	+	+	+	+	+
C. malonaticus	6 strains	5 STs	O:1, O:2	+	+	+	+	+	+	+	+	+
C. universalis	2 strains	2 STs	-	+	+	+	+	+	+	+	+	+
C. turicensis	5 strains	5 STs	O:3	+	+	+	+	+	+	+	+	+

Presence and absence of malonate utilization genes were investigated by using BLAST genome search. + = gene presence. - = gene absence

Table 4.5. Biochemical tests profiling of *Cronobacter* species used in this study.

NTU	Species	ST	O-antigen	Production of		Utilization of		
				α-glucosidase	Indole	Inositol	Malonate	Sialic acid
96	*C. universalis*	48	ND	+	-	+	+	-
1435		51	ND	+	-	+	+	-
1883		137	ND	+	-	+	+	-
92	*C. turicensis*	35	Ctur O:3	+	-	+	+	+
109		5	ND	+	-	+	+	-
111		24	ND	+	-	+	+	-
1553		72	Ctur O:3	+	-	+	+	+
1880		344	Ctur O:3	+	-	+	+	+
1895		252	Ctur O:3	+	-	+	+	-
16	*C. muytjensii*	34	CmuyO:3	+	+	+	+	-
1371		71	CmuyO:2	+	+	+	+	-
1527		75	CmuyO:1	+	+	+	+	-
1877		288	CmuyO:1	+	+	+	+	-
93	*C. malonaticus*	29	Cmal O:1	+	-	+	+	-
101		60	Cmal O:1	+	-	+	+	-
1846		60	Cmal O:1	+	-	+	+	-
1893		289	Cmal O:1	+	-	+	+	-
510		7	Cmal O:2	+	-	-	+	-
1369		69	Cmal O:2	+	-	+	+	-
1879		139	Cmal O:2	+	-	+	+	-
1330	*C. condimenti*	98	CuniO:1	+	+	+	+	-
1210	*C. dublinensis*	106	Cdub O:1	+	+	+	+	-
1458		74	Cdub O:1	+	+	+	+	-
1463		95	Cdub O:1	+	+	+	+	-
1461		77	Cdub O:1	+	+	+	-	-
1462		70	Cdub O:1	+	+	+	-	-
1460		76	Cdub O:2	+	+	-	-	-
1464		78	Cdub O:2	+	+	+	-	-
1897		213	Cdub O:2	+	+	+	-	-
377	*C. sakazakii*	4	Csak O:2	+	-	-	-	+
1105		4	Csak O2	+	-	+	-	+
1564		4	Csak O:3	+	-	+	-	+
1886		4	Csak O:3	+	-	+	-	+
1907		4	Csak O:4	+	-	+	-	+
1908		4	Csak O:4	+	-	+	-	+
1283		8	Csak O:3	+	-	+	-	+
1888		8	Csak O:1	+	-	+	-	+
1906		8	Csak O:1	+	-	+	-	+
1107		9	Csak O:2	+	-	+	-	+
1108		12	Csak O:4	+	-	+	-	+
1887		13	Csak O:2	+	-	+	-	+
1885		406	Csak O:2	+	-	+	-	+
1990		264	Csak O:2	+	-	+	-	+
2027		406	Csak O:2	+	-	+	-	+
1843		23	Csak O:2	+	-	+	-	+
1844		405	Csak O:3	+	-	-	-	+
1845		233	Csak O:2	+	-	-	+	+
1847		245	Csak O:1	+	-	-	-	+
1881		64	Csak O:2	+	-	+	+	+
1882		20	Csak O:3	+	-	+	-	+
1884		263	Csak O:2	+	-	+	-	+
1889		198	Csak O:4	+	-	+	-	+
1890		42	Csak O:2	+	-	+	-	+
1992		136	Csak O:2	+	-	-	-	+

+ = gene presence. - = gene absence ND= not defined

4.2.3.2 Lipase and protease activity.

The lipase activity assay was performed using tributyrin agar. After 3 days of incubation at 37°C. *C. sakazakii* strains 1844 (ST405), 1845 (ST233) and 2027 (406), *C. malonaticus* strain 1846 (ST60), *C. muytjensii* strain 1371 (ST403) and *C. dublinensis* strain 1463 (ST95) showed positive result for production of lipase as a strong clear inhibition zone around the colony.

C. sakazakii strains; 377, 1105, 1886, 1907 and 1908 (ST4), 1107 (ST9), 1108 (ST12), 1283, 1888 and 1906 (ST8), 1843 (ST23), 1847 (ST245), 1881 (ST64), 1882 (ST20), 1884 (ST263), 1885 (ST406), 1887 (ST13), 1889 (ST198), 1890 (ST42), 1990 (ST264) and 1992(ST136), 6 *C. malonaticus* strains; 93 (ST29), 101 (ST60), 510 (ST7), 1369 (ST690, 1879 (ST139) and 1893(ST289) showed positive results for production of lipase as a clear inhibition zone around colony. Moreover, clear inhibition zone around colony was also noted in 6 *C. dublinensis* strains; 1210 (ST106), 1458 (ST74), 1460 (ST76), 1461 (ST77), 1462 (ST70) and 1464 (ST78), 2 *C. muytjensii* strains; 16 (ST347) and 1877(ST407). In addition to 3 *C. universalis* strains; 96 (ST48), 1435 (ST51) and 1883(ST137), and 6 *C. turicensis* strains, 92 (ST35), 109 (ST5), 111 (ST24), 1553 (ST72), 1880 (ST344) and 1895 (ST252) showed positive result for production of lipase as a clear inhibition zone around colony. Furthermore, *C. muytjensii* strain 1527 (ST411), *C. dublinensis* strain 1897 (ST213) and the single *C. condimenti* strain 1330(ST98) showed positive result for production of lipase as a weak inhibition zone around colony. The proteolytic activity on skim milk agar was also clearly demonstrated by all *Cronobacter* strains; table 4.6.

4.2.3.2.1 Genes responsible for lipase activity.

The *lipA*/*lipB* genes encode a secreted lipase and are responsible for the lipase activity (lipolytic phenotype) of bacteria (Beven et al. 2001). These two genes were present in all *Cronobacter* genomes.

Table 4.6. Lipase and protease activity of *Cronobacter* strains.

NTU	Species	Source	ST	O-antigen	Lipase activity 37°C/(72h)	Protease activity 37°C/(72h)
96	*C. universalis*	Spice	48	ND	++	+
1435		Food	51	ND	++	+
1883		Spice	137	ND	++	+
109	*C. turicensis*	Herb	5	ND	++	+
111		Herb	24	ND	++	+
92		Herb	35	Ctur O:3	++	+
1880		Herb	344	Ctur O:3	++	+
1553		Herb	72	Ctur O:3	++	+
1895		Ingredient	252	Ctur O:1	++	+
1371	*C. muytjensii*	Spice	403	Cmuyt O:3	+++	+
1527		Food	411	Cmuyt O:2	+	+
16		Spice	347	Cmuyt O:1	++	+
1977		Ingredient	407	Cmuyt O:1	++	+
1879	*C. malonaticus*	Spice	139	Cmal O:2	++	+
1369		Herb	69	Cmal O:1	++	+
101		Spice	60	Cmal O:1	++	+
93		Spice	29	Cmal O:1	++	+
510		Food	7	Cmal O:2	++	+
1846		Ingredient	60	Cmal O:1	+++	+
1893		Ingredient	289	Cmal O:1	++	+
1210	*C.dublinensis*	Food	106	Cdub O:1	++	+
1458		Food	74	Cdub O:1	++	+
1460		Food	76	Cdub O:2	++	+
1463		Food	95	Cdub O:1	+++	+
1461		Food	77	Cdub O:1	++	+
1462		Food	70	Cdub O:1	++	+
1464		Food	78	Cdub O:2	++	+
1897		Herb	213	Cdub O:2	+	+
1330	*C. condimenti*	Food	98	CuniO:1	+	+
377	*C. sakazakii*	Food	4	Csak O:2	++	+
1105		Weaning food	4	Csak O:2	++	+
1564		Food	4	Csak O:3	++	+
1886		Spice	4	Csak O:3	++	+
1907		Environment	4	Csak O:4	++	+
1908		Environment	4	Csak O:4	++	+
1107		Weaning food	9	Csak O:2	++	+
1108		Weaning food	12	Csak O:4	++	+
1283		Food	8	Csak O:3	++	+
1888		Food	8	Csak O:1	++	+
1906		Food	8	Csak O:1	++	+
1843		Spice	23	Csak O:2	++	+
1844		Ingredient	244	Csak O:3	+++	+
1845		Food	233	Csak O:2	+++	+
1847		Milk powder	245	Csak O:1	++	+
1881		Ingredient	64	Csak O:2	++	+
1882		Ingredient	20	Csak O:3	++	+
1884		Herb	263	Csak O:2	++	+
1887		Food	13	Csak O:2	++	+
1889		Ingredient	198	Csak O:4	++	+
1890		Ingredient	42	Csak O:2	++	+
1992		Food	136	Csak O:2	++	+
1885		Herb	406	Csak O:2	++	+
1990		Food	264	Csak O:2	++	+
2027		Ingredient	406	Csak O:2	+++	+

The lipase activity assay was performed using tributyrin agar and the proteolytic activity was performed using skim milk agar. + = weak. ++= moderate. +++ = strong.

4.2.4 Capsule production on different media.

Capsule production by 54 strains of *Cronobacter* species was determined by colony appearance on different media including milk agar, XLD, VRBLA and VRBGA; table 4.7. There was a notable variation between strains in their ability to produce capsules; table 4.7 shows the relative results. Strains were divided into four groups according to the relative scale of high, medium, low and no mucoid production are represented by +++, ++, + and - respectively. The majority of *C. sakazakii* strains had the most mucoid appearance indicating capsular material production. However, less capsular material was produced by the *C. sakazakii* strains with serotype profile O:1 and O:4 such as 1906 (ST8-O:1), 1847 (ST245-O:1), 1907 and 1908 (ST4-O:4), 1108 (ST12-O:4) and 1889 (ST189-O:4). The dry colony appearance of the *C. sakazakii* strain 1888 (ST8-O:1) indicated no capsular material was produced. Significant capsular material was produced by *C. sakazakii* strains with serotype profile O:2 and O:3. *C. sakazakii* strains with the same ST and with different serotype profile showed different capsular profile, for example strains with profile (ST8-O:1) and (ST4-O:4) had a lower levels of capsule production. In contrast, strains with profile (ST8-O:3), (ST4-O:2) and (ST4-O:3) were able to form high levels of capsular materials (Table 4.8). On the other hand, considerable capsular material was produced by the other *Cronobacter* species*; C. malonaticus, C. turicensis, C. universalis, C. muytjensii, C. dublinensis* strains*,* and the single *C. condimenti* strain (Table 4.7, 4.8).

4.2.4.1 Capsule profiling.

A BLAST genome search was used to investigate the correlation between capsule profiling (O-antigen type, K- capsule type and CA-type) and the amount of capsular material (Table 4.8). *C. sakazakii* with capsular profile (O-antigen type, K-capsule and CA-type); O:1-K1-CA1, O:1-K2-CA1, O:4-K1-CA2 and O:4-K2-CA2 showed low level of capsule production. Whereas, the great value of capsular material was produced by *C. sakazakii* strains with capsular profile O:2-K1-CA2, O:2-K2-CA2, O:3-K1-CA1 and O:3-K2-CA1. No correlation between capsule profiling and the amount of capsular material in other *Cronobacter* species was noted.

Table 4.7 Capsule production on different media; XLD, VRBGA, VRBLA and milk agar

NTU	Species	ST	Mucoid production 25°C /(48h)				Mucoid production at 37°C /(24h)			
			XLD	VRBGA	VRBLA	Milk agar	XLD	VRBGA	VRBLA	Milk agar
96	*C. universalis*	48	+	++	++	+++	++	++	++	+++
1435		51	++	++	++	++	++	++	++	+++
1883		137	++	++	++	+++	++	+++	++	+++
92	*C. turicensis*	35	+(-)	++	++	++	+	++	++	++
109		5	+	++	++	++	++	++	++	++
111		24	++	++	++	+++	+++	++	+++	+++
1553		72	++	+++	++	+++	+++	+++	++	+++
1880		344	++	+++	+++	+++	+++	+++	+++	+++
1895		252	++	+++	+++	+++	+++	+++	+++	+++
16	*C. muytjensii*	347	+(-)	++	++	++	+	++	++	++
1371		403	+++	++	+++	+++	+++	+++	+++	+++
1527		411	++	++	++	+++	++	+++	+++	+++
1877		407	++	++	++	+++	++	+++	+++	+++
93	*C. malonaticus*	29	+++	+++	+++	+++	+++	+++	+++	+++
101		60	+++	+++	+++	+++	+++	+++	+++	+++
1846		60	+++	+++	+++	+++	+++	+++	+++	+++
510		7	++	+++	++	+++	+++	+++	+++	+++
1369		69	+++	+++	+++	+++	+++	+++	+++	+++
1879		139	++	+++	++	+++	+++	+++	+++	+++
1893		289	+++	+++	+++	+++	+++	+++	+++	+++
1330	*C. condimenti*	98	++	++	++	++	++	++	++	+++
1210	*C. dublinensis*	106	++	+++	+++	+++	+++	+++	+++	+++
1458		74	++	+++	+++	+++	+++	+++	+++	+++
1460		76	++	++	+++	+++	+++	+++	+++	+++
1461		77	++	+++	+++	+++	+++	+++	+++	+++
1462		70	++	+++	+++	+++	+++	+++	+++	+++
1463		95	++	+++	+++	+++	+++	+++	+++	+++
1464		78	++	++	+++	+++	+++	+++	+++	+++
1897		213	++	++	+++	+++	+++	+++	+++	+++
1906	*C. sakazakii*	8	_	_	+	+	_	_	+	+
1888		8	_	_	_	_	_	_	_	_
1847		245	+	+	+	++	+	+	++	++
1907		4	+	+	+	+	+	+	+	+
1908		4	_	+	+	+	_	+	+	+
1889		198	+	+	+	+	+	+	+	+
1108		12	+	+	+	+	+	+	+	++
1882		20	+++	+++	+++	+++	+++	+++	+++	+++
1283		8	++	++	++	+++	++	+++	+++	+++
1844		405	+++	++	++	+++	+++	++	++	+++
1564		4	++	++	++	+++	++	++	+++	+++
1886		4	+++	+++	+++	+++	+++	+++	+++	+++
1487		4	+++	+++	+++	+++	+++	+++	+++	+++
1884		263	+++	+++	+++	+++	+++	+++	+++	+++
1885		406	+++	+++	+++	+++	+++	+++	+++	+++
1990		264	+++	+++	+++	+++	+++	+++	+++	+++
2027		406	+++	+++	+++	+++	+++	+++	+++	+++
1881		64	+++	+++	+++	+++	+++	+++	+++	+++
1890		42	+++	+++	+++	+++	+++	+++	+++	+++
1843		23	+++	+++	+++	+++	+++	+++	+++	+++
1992		136	++	+++	++	+++	+++	+++	+++	+++
1105		4	+++	+++	+++	+++	+++	+++	+++	+++
377		4	+++	++	+++	+++	+++	++	+++	+++
1107		9	+	++	++	+++	+	++	++	+++
1845		233	++	+++	++	+++	+++	+++	+++	+++
1887		13	+	+	++	++	++	+	++	++

Capsule production of *Cronobacter* strains was determined by colony appearance on different media including milk agar, XLD, VRBLA and VRBGA +++= High mucoid production. ++= Moderate mucoid production. += Low mucoid production. -= No mucoid production

Table 4.8. The correlation between capsule profiling (O-antigen type, K- capsule type and CA-type) and the amount of capsular material.

Isolates	species	ST	CC	Serotype	K-type	CA-type	Mucoid production 25C/(48h)				Mucoid production 37C/(24h)			
							XLD	VRBGA	VRBLA	Milk agar	XLD	VRBGA	VRBLA	Milk agar
1906		8	8	Csak O1	K1	CA1	_	_	+	+	_	_	+	+
1888		8	8	Csak O1	K1	CA1	_	_	_	_	_	_	_	_
1847		245	ND	Csak O1	K2	CA1	+	+	+	++	+	+	++	++
1907		4	4	Csak O4	K2	CA2	+	+	+	+	+	+	+	+
1908		4	4	Csak O4	K2	CA2	_	+	+	+	_	+	+	+
1889		198	52	Csak O4	K1	CA2	+	+	+	+	+	+	+	+
1108		12	ND	Csak O4	K2	CA2	+	+	+	+	+	+	+	++
1882		20	20	Csak O3	K1	CA1	+++	+++	+++	+++	+++	+++	+++	+++
1283		8	8	Csak O3	K1	CA1	++	++	++	+++	++	+++	+++	+++
1844		405	ND	Csak O3	K2	CA1	+++	++	++	+++	+++	++	++	+++
1564		4	4	Csak O3	K2	CA2	++	++	++	+++	++	++	+++	+++
1886		4	4	Csak O3	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1487	*C. sakazakii*	4	4	Csak O3	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1884		263	263	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1885		406	264	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1990		264	264	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
2027		406	264	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1881		64	64	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1890		42	ND	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1843		23	23	Csak O2	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1992		136	ND	Csak O2	K2	CA2	++	+++	++	+++	+++	+++	+++	+++
1105		4	4	Csak O2	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
377		4	4	Csak O2	K2	CA2	+++	++	+++	+++	+++	++	+++	+++
1107		9	3	Csak O2	K2	CA2	+	++	++	+++	+	++	++	+++
1845		233	ND	Csak O2	K2	CA2	++	+++	++	+++	+++	+++	+++	+++
1887		13	13	Csak O2	K2	CA2	+	+	++	++	++	+	++	++

93	*C. malonaticus*	29	ND	Cmal O1	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
101		60	ND	Cmal O1	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1369		69	ND	Cmal O1	K1	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1846		60	ND	Cmal O1	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
1893		289	ND	Cmal O1	K2	CA2	+++	+++	+++	+++	+++	+++	+++	+++
510		7	7	Cmal O2	K1	CA1	++	+++	++	+++	+++	+++	+++	+++
1879		139	ND	Cmal O2	K1	CA1	++	+++	++	+++	+++	+++	+++	+++
1210	*C. dublinensis*	106	ND	Cdub O1	K1	CA1	++	+++	+++	+++	+++	+++	+++	+++
1458		74	74	Cdub O1	K1	CA1	++	+++	+++	+++	+++	+++	+++	+++
1463		95	162	Cdub O1	K1	CA1	++	++	+++	+++	+++	+++	+++	+++
1461		77	ND	Cdub O1	K1	CA1	++	+++	+++	+++	+++	+++	+++	+++
1462		70	ND	Cdub O1	K1	CA1	++	+++	+++	+++	+++	+++	+++	+++
1460		76	78	Cdub O2	K1	CA2	++	+++	+++	+++	+++	+++	+++	+++
1464		78	ND	Cdub O2	K1	ND	++	++	+++	+++	+++	+++	+++	+++
1897		213	ND	Cdub O2	K1	CA2	++	++	+++	+++	+++	+++	+++	+++
1330	*C. condimenti*	98	ND	Cuni O1	K1	CA2	++	++	++	++	++	++	++	+++
96	*C. universalis*	48	ND	ND	K2	CA1	+	++	++	+++	++	++	++	+++
1435		51	ND	ND	K2	ND	++	++	++	++	++	++	++	+++
1883		137	ND	ND	K2	CA2	++	++	++	+++	++	+++	++	+++
1895	*C. turicensis*	252	ND	Ctur O1	K2	CA2	++	+++	+++	+++	+++	+++	+++	+++
92		35	ND	Ctur O3	K2	CA2	+	++	++	++	+	++	++	++
109		5	5	ND	K2	CA2	+	++	++	++	++	++	++	++
111		24	24	ND	K2	CA2	++	++	++	+++	+++	++	+++	+++
1553		72	72	Ctur O3	K1	CA2	++	+++	++	+++	+++	+++	++	+++
1880		344	ND	Ctur O3	K1	CA2	++	+++	+++	+++	+++	+++	+++	+++
16	*C. muytjensii*	347	ND	CmuyO3	K1	CA1	+	++	++	++	+	++	++	++
1371		403	ND	CmuyO2	K1	CA2	+++	++	+++	+++	+++	+++	+++	+++
1527		411	ND	CmuyO1	K1	CA1	++	++	++	+++	++	+++	+++	+++
1877		407	ND	CmuyO1	K1	CA2	++	++	++	+++	++	+++	+++	+++

ST= sequence type. CC= clonal complex. ND= not determined. +++= high mucoid production. ++= moderate mucoid production. += low mucoid production. -= no mucoid production

4.2.4.2 The correlation between the capsular polysaccharide composition (CPS) of *C. sakazakii* strains and their genetic traits such as ST, O-antigen, K-capsule and colonic acid type (CA).

4.2.4.2.1 Strains screening and selection.

A 13 *C. sakazakii* strains were used in this study. These strains were selected based on the diversity of their sequence type (ST) which associated with clinical significance including; ST1, ST4, ST8, ST12 and ST13, O-antigen serotype (O:1, O:2, O:3 and O:4) and capsule type (K-type and CA-type), in order to investigate the correlation between the amount of capsule production, capsule compositions (monosaccharides) and their genetic traits. To the best of our knowledge, this is the first study that focused on the association between the capsule compositions (monosaccharides), amount of capsule production and linked that with molecular characterisation. Two clinical strains 767 (ST4) and 2107 (ST12) were used for the purpose of comparison. Table 4.9 lists the details of the strains that have used in this part of study.

Table 4.9. Details of the *C. sakazakii* strains used in this part.

NTU NO:	Sources	Country	Year	ST	O-serotype	Capsule profile
658	Non-infant formula	USA	2001	1	O:1	K1-CA1
767	Clinical	France	1994	4	O:2	K2-CA2
377	Milk powder	UK	1950	4	O:2	K2-CA2
1105	Weaning food	UK	2008	4	O:2	K2-CA2
1533	Environmental	Germany	2006	4	O:2	K2-CA2
1886	Spice	Czech Republic	2011	4	O:3	K2-CA2
1908	Environmental	Malaysia	2012	4	O:4	K2-CA2
1283	Food	UK	2010	8	O:3	K1-CA1
1888	Food	Czech Republic	2011	8	O:1	K1-CA1
1906	Environmental	Malaysia	2012	8	O:1	K1-CA1
2107	Clinical	Belgium		12	O:4	K2-CA2
1108	Weaning food	UK	2008	12	O:4	K2-CA2
1887	Food	Czech Republic	2011	13	O:2	K2-CA2

4.2.4.2.2 Mucoid production on milk agar.

Production of capsule by thirteen strains of *C. sakazakii* was determined by colony appearance on milk agar; table 4.10. There was a notable variation between strains in their ability to produce capsules. Strains were divided into four groups according to the relative scale of high, medium, low and no mucoid production are represented by +++, ++, + and – respectively. Less capsular material was produced by the *C. sakazakii* strains with serotype profile O:1 and O:4 such as 658 (ST1-O:1-K1-CA1), 1908 (ST4-O:4-K2-CA2), 1108 (ST12-O:4-K2-CA2) and 2107 (ST12-O:4-K2-CA2). Moreover, *C. sakazakii* strains 1888 and 1906 (ST8-O:1-K1-CA1) showed dry colony appearance. While, significant capsular material was produced by *C. sakazakii* strains with serotype profile O:2 and O:3. These strains were 377, 767, 1105 and 1533, 1886 (ST4-O:3-K2-CA2), 1887 (ST13-O:2-K2-CA2), and 1283 strain (ST8-O:3-K1-CA1); table 4.10.

Table 4.10 The correlation between the amount of mucoid production and the genetic traits of *C. sakazakii* strains.

strain	Sources	Genetic traits			Mucoid production/IF	
		ST	O-serotype	Capsule profile	21C/(48h)	37C/(24h)
658	Non-infant formula	1	O:1	K1-CA1	+	++
1888	Food	8	O:1	K1-CA1	-	-
1906	Environmental	8	O:1	K1-CA1	-	-
1908	Environmental	4	O:4	K2-CA2	+	+
2107	Clinical	12	O:4	K2-CA2	+	+
1108	Weaning food	12	O:4	K2-CA2	+	++
377	Milk powder	4	O:2	K2-CA2	+++	+++
767	Clinical	4	O:2	K2-CA2	+++	++
1105	Weaning food	4	O:2	K2-CA2	+++	+++
1533	Environmental	4	O:2	K2-CA2	++	++
1887	Food	13	O:2	K2-CA2	++	++
1886	Spice	4	O:3	K2-CA2	+++	+++
1283	Food	8	O:3	K1-CA1	++	+++

IF= infant formula. - = no mucoid production. += low mucoid production. ++ = moderate mucoid production. +++ = high mucoid production.

4.2.4.2.3 Isolation of the mucoid layer.

The mucoid layer of thirteen *C. sakazakii* strains was isolated, in brief bacterial cultures were grown overnight at 37°C and 48h at 21°C on agar plates containing infant formula ready to feed. Bacterial growth was then scraped into centrifuge tubes and sterile normal saline was added to balance the tubes and then centrifuged at 5000 rpm for 5 minutes (Figure 4.7, 4.8). The saline/supernatant was then discarded and the thick mucoid layer was transferred to weigh watch glasses, which were put into a drying oven overnight and then re-weighed (Figure 4.9). The dry mass was collected and stored at room temperature.

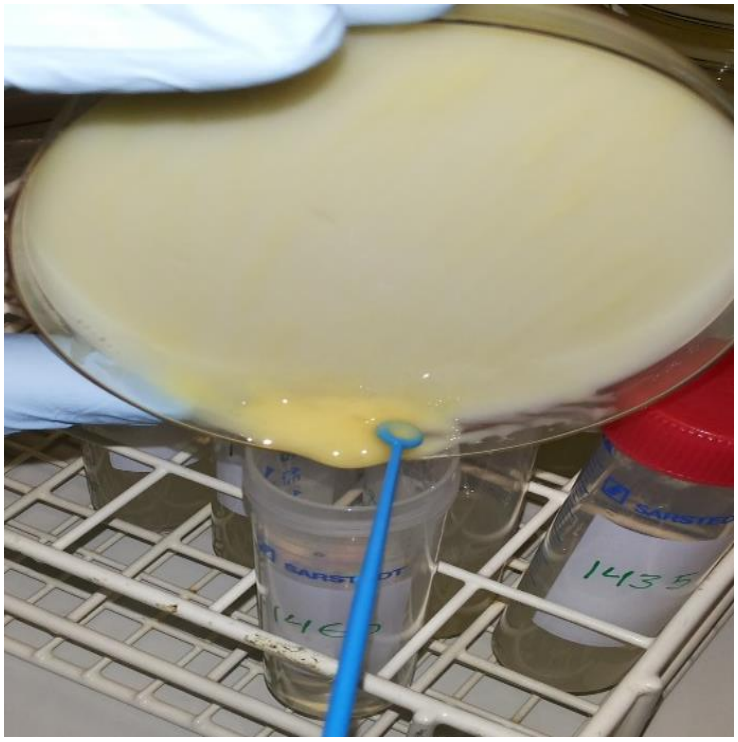


Figure 4.7 . Isolation of capsule materials.
Bacterial growth was then scraped into centrifuge tubes and sterile normal saline was added to balance the tubes

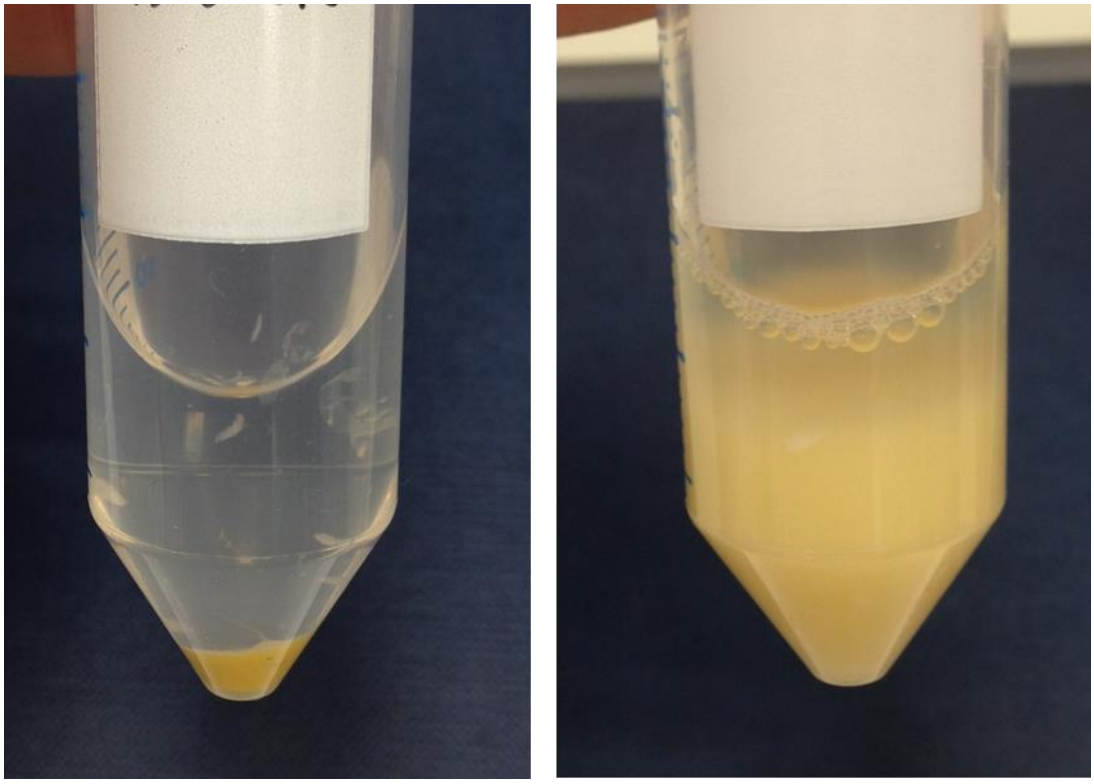


Figure 4.8. The mucoid layer after centrifugation at 5000 rpm for 5 minutes
There was a notable variation between strains in their ability to produce capsules

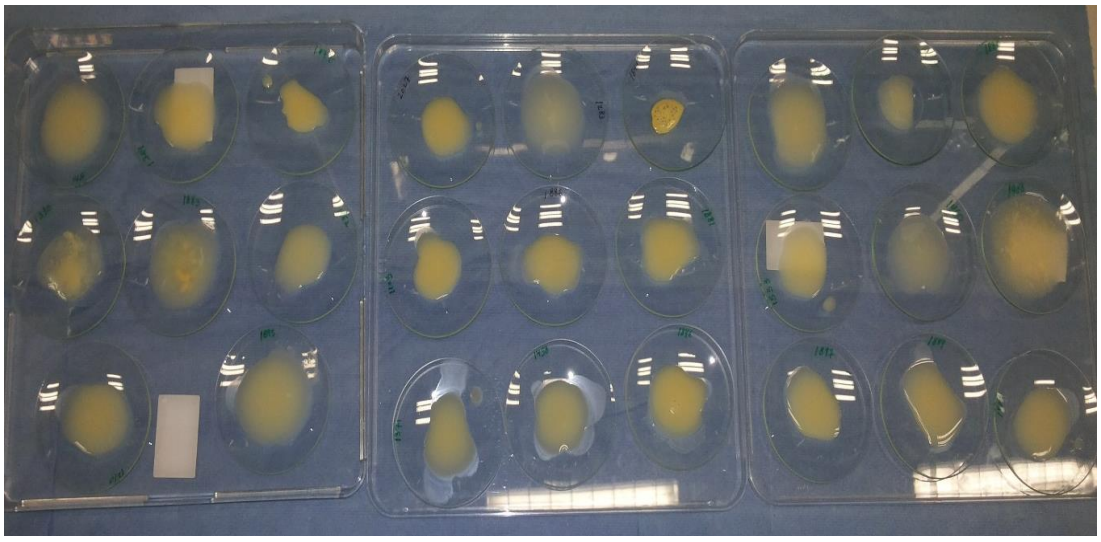


Figure 4.9. Oven overnight drying of the thick mucoid layer using watch glasses.
The dry mass was collected and stored at room temperature.

4.2.4.2.4 Sugar analysis of capsule polysaccharides.

The dry mass of capsule materials were analysed at the Faculty of Chemistry, Gdansk University, Poland. The materials were analysed using carbohydrate nuclear magnetic resonance (NMR) Spectroscopy. The analysis of capsule polysaccharides of 13 selected *C. sakazakii* strains showed they were composed of four different sugars; galactose (Gal), glucose (Glc), fucose (Fuc) and rhamnose (Rha).

The monosaccharides in 658 (ST1-O:1) sample were galactose (Gal), glucose (Glc) and fucose (Fuc), with total amount 7.82, 15.85 and 7.33 mg at 21°C and 18.05, 32.02 and 15.23 mg at 37°C respectively. *C. sakazakii* ST8 strains (1888, 1906 and 1283) were divided into 2 groups based on their sugar composition (monosaccharides), 1888 and 1906 (ST8-O:1) samples composed only one type of sugar which is glucose at both temperatures (21°C and 37 °C). Whereas, strain 1283 (ST8-O:3) showed a different sugar composition (monosaccharides), it composed of 3 type of sugars which are (Gal), (Glc) and (Fuc) with different amount at different temperatures (21°C and 37 °C). Moreover, monosaccharides in *C. sakazakii* ST4 samples 377, 767, and 1533 (O:2) were (Gal), (Glc), (Fuc) and rhamnose (Rha) with different amount at different temperatures (21 °C and 37 °C).

The monosaccharides in 1908 sample (ST4-O:4) were (Gal), (Glc) and (Fuc), while strain 1886 (ST4-O:3-K2-CA2) had the same ratio of sugars, but with less amount of monosaccharides. The monosaccharides in *C. sakazakii* ST12 samples 1108 and 2107 (O:4) were also containing a (Gal), (Glc) and (Fuc) with different amount at different temperatures (21 °C and 37 °C), however strain 2107 showed lack of (Fuc) at 21 °C. Furthermore, capsule materials of 1887 sample (ST13-O:2) composed of 4 sugars including (Gal), (Glc) , (Fuc) and (Rha) at 21 °C, and was lacked galactose (Gal) and fucose (Fuc) at 37°C. This observation shows that there is a strong correlation between the amount of mucoid production, type and ratio of monosaccharides production and the genetic traits, in particular O-antigen serotype (Table 4.11 and Figure 4.9).

4.2.4.2.5 Rhamnose biosynthesis genes.

Rhamnose sugar is commonly found in Gram-negative and Gram-positive bacteria. This sugar is generally distributed in O-antigens of Gram-negative bacteria. Moreover, this sugar is frequently found in capsular polysaccharides, which are covalently bound to the cell wall (Boels et al. 2004). More Interestingly, L-rhamnose is often essential for bacterial virulence and viability (Maki and Renkonen, 2004; Mistou et al, 2016). Genes *rmlA* and *rfbBCD* encode the biosynthesis of dTDP-l-rhamnose from glucose 1-phosphate in *E.coli* and *Salmonella* strains (Silva et al. 2005). Genes (*rmlA* and *rfbB*) were found in all tested *C. sakazakii* strains. More interestingly, *rfbCD* genes were found in ST4 stains 377, 767, 1105 and 1533 (O:2-K2-CA2) and ST13 strain 1887 (O2-K2-CA2), and were absent in strains 658 ST1 strain (O:1-K1-CA1), ST12 strains 1108 and 2107 (O:4-K2-CA2), ST8 strains 1888, 1906 (O:1-K1-CA1) and 1283 (O:3-K1-CA1), ST4 strain 1908 (O4-K2-CA2) and 1886 (O3-K2-CA2). Furthermore, Artemis comparison tool was used to consider if the rhamnose biosynthesis cluster genes were acquired or lost in *C. sakazakii* strains as a result of genome evolution (Figure 4.10).

All strains were positive for *rfbCD* genes except for the non-rhamnose production strains (negative strains); 658, 1108, 2107, 1888, 1906, 1283, 1908 and 1886 that lacked these genes (Table 4.12). This suggests that these genes (*rfbCD*) are important for rhamnose biosynthesis in *C. sakazakii* strains, and might be used to predict the capsule polysaccharides composition in *Cronobacter* species.

4.2.4.2.6 Fucose utilization genes.

Obadia et al (2007) reported that the capsular polysaccharide synthesis gene cluster In *E. coli* K-12, termed the *wca* operon, controls the biosynthesis of the exopolysaccharide, colanic acid. This gene cluster controls the biosynthesis of the exopolysaccharide colanic acid containing fucose and glucuronic acid. Moreover, Pacheco et al (2012) reported that L-fucose utilization in *E.coli* requires the *fuc* genes (*fucPIKUR*), and their activator (*FucR*). Fucose utilization genes (*fucPIK)* were absent in all strains, while *fucUR* were found in all strains (Table 4.12). This observation shows that there is no correlation between the ability of *C. sakazakii* strains to produces

fucose sugar as a part of capsule polysaccharides and the genotypic detection of the Fucose utilization genes (*fucPIKUR*).

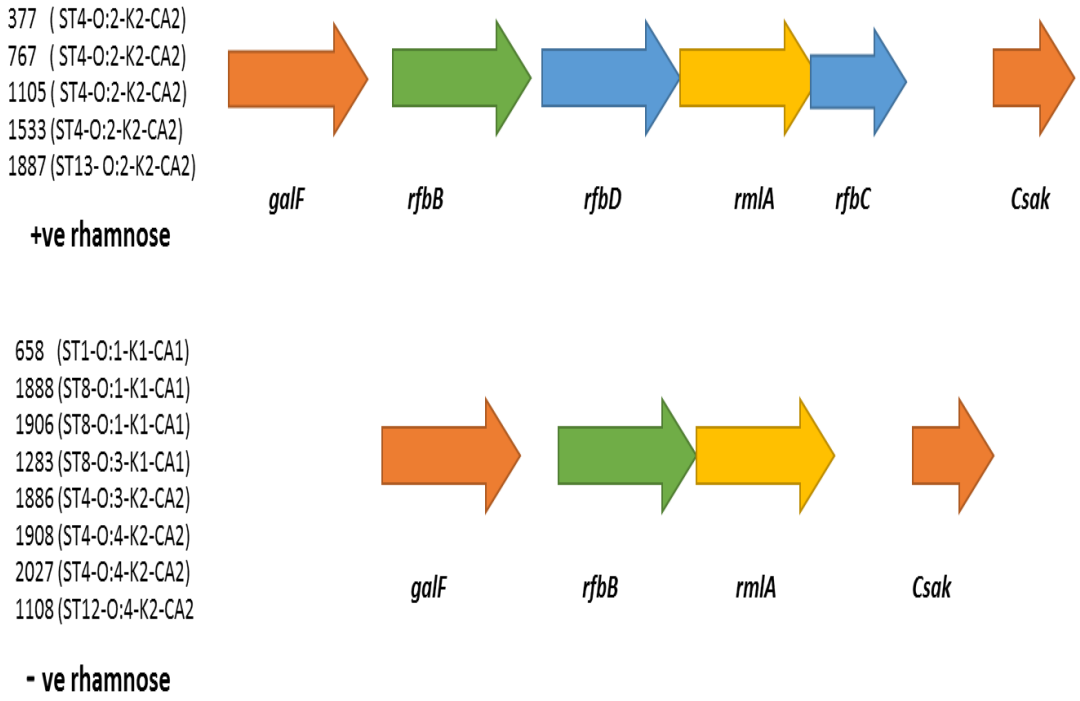


Figure 4.10. Diagrammatic representation of putative or demonstrated operonic organization of dTDP-l-rhamnose-synthesizing genes from different *C. sakazaki* strains.
rmlA, glucose-1-phosphate thymidylyltransferase; *rfbB*, dTDP-glucose-4,6-dehydratase; *rfbC*, dTDP-4-keto-l-rhamnose-3,5-epimerase; and *rfbD*, dTDP-l-rhamnose synthase.

Table 4.11. The correlation between the composition of capsule polysaccharide of *C. sakazakii* strains and their genetic traits.

strains	ST	O-serotype	Capsule profile	Medium	Temp (°C)	Mucoid appearance	Dry weight of sample (mg)	Analysed part (mg)	Total amount of monosaccharides in the sample [umol]			
									Gal	Glc	Fuc	Rha
658	1	O:1	K1-CA1	IF	21	+	63.4	10.2	7.82	15.85	7.33	N
					37	++	110.1	30.2	18.05	32.02	15.23	N
377	4	O:2	K2-CA2	IF	21	+++	174.6	11.1	41.08	62.17	46.83	5.37
					37	+++	N	N	N	N	N	N
767	4	O:2	K2-CA2	IF	21	+++	86.4	13.6	17.69	33.48	19.02	1.86
					37	++	74.4	12.8	11.47	25.53	13.15	2.0466
1105	4	O:2	K2-CA2	IF	21	+++	54.8	7.3	10.57	31.33	8.94	4.39
					37	+++	24.7	5.5	N	13.99	N	1.0
1533	4	O:2	K2-CA2	IF	21	++	55.8	7	13.48	30.01	16.25	7.776
					37	++	101.5	5.6	N	N	N	N
1886	4	O:3	K2-CA2	IF	21	+++	43.9	7.9	7.52	19.15	4.99	N
					37	+++	65.5	14.4	0.83	26.54	0.95	N
1908	4	O:4	K2-CA2	IF	21	+	70.7	6.4	8.28	39.47	7.85	N
					37	+	47.3	7	8.03	22.54	6.78	N
1906	8	O:1	K1-CA1	IF	21	-	69.4	5.6	N	28.81	N	N
					37	-	88.4	9.8	N	N	N	N
1888	8	O:1	K1-CA1	IF	21	-	53.5	8.8	N	36.97	N	N
					37	-	80.3	8	N	79.35	N	N
1283	8	O:3	K1-CA1	IF	21	++	84	8.7	6.88	18.47	6.46	N
					37	+++	18.4	7.9	1.02	3.17	0.68	N
1108	12	O:4	K2-CA2	IF	21	++	85.1	14.4	12.64	48.12	10.87	N
					37	+	86.1	6.1	2.04	9.3	0.8	N
2107	12	O:4	K2-CA2	IF	21	+	59.7	7.4	2.86	25.01	N	N
					37	+	58.2	9.3	3.31	34.12	2.25	N
1887	13	O:2	K2-CA2	IF	21	+++	174.3	8.6	39.62	56.22	19.32	8.326
					37	+	52.5	13.4	N	29.49	N	3.276

The analysis of capsule materials of 13 selected *C. sakazakii* strains showed they were composed of four different sugars; galactose (Gal), glucose (Glc), fucose (Fuc) and rhamnose (Rha). The materials were analysed using carbohydrate nuclear magnetic resonance (NMR) Spectroscopy IF= infant formula. - = no mucoid production. += low mucoid production. ++ = moderate mucoid production. +++ = high mucoid production. N= no product.

Table 4.12. Presence /absence of rhamnose and fucose genes in *C. sakazakii* strains.

strains	Genetic traits			Capsule production/IF		Rhamnose genes			Fucose genes	
	ST	O-serotype	Capsule profile	21C/(48h)	37C/(24h)	*rmlA rfbB*	*rfbC*	*rfbD*	*fucPIK*	*fucUR*
658	1	O:1	K1-CA1	+	++	+	-	-	-	+
1888	8	O:1	K1-CA1	-	-	+	-	-	-	+
1906	8	O:1	K1-CA1	-	+	+	-	-	-	+
377	4	O:2	K2-CA2	+++	+++	+	+	+	-	+
767	4	O:2	K2-CA2	+++	++	+	+	+	-	+
1105	4	O:2	K2-CA2	+++	+++	+	+	+	-	+
1533	4	O:2	K2-CA2	++	++	+	+	+	-	+
1887	13	O:2	K2-CA2	++	++	+	+	+	-	+
1886	4	O:3	K2-CA2	+++	+++	+	-	-	-	+
1283	8	O:3	K1-CA1	++	+++	+	-	-	-	+
1908	4	O:4	K2-CA2	+	+	+	-	-	-	+
2107	12	O:4	K2-CA2	+	+	+	-	-	-	+
1108	12	O:4	K2-CA2	+	++	+	-	-	-	+

Presence and absence of rhamnose and fucose utilization genes were investigated by using BLAST genome search. + = gene presence. - = gene absence.

4.2.5 Biofilm formation of seven *Cronobacter* species.

The crystal violet staining method was employed to examine the biofilm-forming abilities of the 54 *Cronobacter* spp. isolates. Iversen et al (2003) reported that *Cronobacter* species are able to adhere to different materials such as silicon, latex, polycarbonate and stainless steel. Moreover, the *Cronobacte*r genus has been reported to form a biofilm on glass and polyvinyl chloride (Lehner et al. 2005). These materials are normally used for infant feeding and food manufacturing equipment. Consequently, contamination of these materials may increase the infection risk. The ability of 54 *Cronobacter* strains isolated from food and environmental sources to form biofilms on plastic surfaces using liquid infant formula (Cow & Gate Premium 1) is shown in figure 4.11 and figure 4.12. Strains were categorised into three groups, high biofilm formation (OD600 >1.0), intermediate biofilm formation (0.75 < OD600 < 1.0),

and low biofilm formation (OD600 < 0.75). All strains showed the ability to form biofilm compared with the control, and there was variation between strains. Most of studied strains were able to form considerable amount of biofilm at 37°C. Moreover, this variation was observed even within *C. sakazakii* strains. Strains in ST4, ST8, ST245, ST198, ST263 and ST264 showed the ability to form great volumes of biofilm compared with other *C. sakazakii* STs. However, the lowest value of biofilm formation were recorded by *C. sakazakii* strains; 1882 (ST20), 1844 (ST405) and 1843 (ST23), *C. malonaticus* strain 1846 (ST60) and *C. turicensis* strain 92 (ST35). The incubation temperature affected the ability of tested strains to form biofilm. Figure 4.11 and figure 4.12 showed that 37°C was the optimal temperature condition for most strains, which were able to form high values of biofilm. Nonetheless, the minority of tested strains were able to form considerable amount of biofilm at incubation temperature 25°C.

4.2.5.1 Genes involved in biofilm formation

Hartmann et al (2010) reported that some flagellar genes *flhE, fliD* and *flgJ* and cellulose genes; *bcsB, bcsE, bcsF* and *bcsZ* (encoding for cellulose biosynthesis) involve in biofilm formation are present in *Cronobacter* strains. Most strains were positive for *flhE, fliD, flgJ, bcsB, bcsE, bcsF* and *bcsZ*. Interestingly, absence of *flhE* gene and cellulose biosynthesis genes (*fliD, flgJ bcsB, bcsE, bcsF* and *bcsZ*) in genomes was observed in *C. sakazakii* strain1882 (ST20), and this strain showed lowest value of biofilm formation (OD600 < 0.75) for both incubation temperatures (25°C and 37°C).

4.2.5.2 Poly-Nacetylglucosamine (PNAG) polymer (polysaccharide capsule).

Poly acetyl-D-glucosamine (PNAG) is a significant component of the biofilm structure made by most pathogenic bacteria such as *Bordetella*, *Yersinia*, *Staphylococcus*, *Escherichia coli* and *Pseudomonas fluorescens*. This polysaccharide material is a major virulence factor, and is produced by many pathogenic bacteria (Fitzpatrick et al. 2005). Moreover, a recent study presented that the majority of *E. coli* strains isolated from urinary tract and neonatal bloodstream infections possess the *pga* locus required for PNAG biosynthesis, and almost all of these isolates produce immunologically detectable levels of PNAG. This material is encoded by *pgaABCD* in *E. coli*, under the

control of *csrA*, the carbon starvation protein (Amini et al, 2009). BLAST search revealed that these genes *pgaABCD* were not found in nearly all *Cronobacter* species, but was found only in *C. sakazakii* strains in ST198 (1889 and CFSAN019572). This observation indicates that unlike other pathogenic bacteria, *Cronobacter* pathogenicity does not require poly acetyl-D-glucosamine (PNAG).

Table 4.13, 4.14 has summarised the characterisation of the *Cronobacter* strains used in this study, according this table phenotypic differences were noted when comparisons were made between 7 species of *Cronobacter*, including differences in bacterial motility, biofilm formation, capsule production, and biochemical test differences such as Indole production, inositol, malonate and sialic acid utilization, and protease and lipase activity.

Figure 4.11. Biofilm formation of *C. sakazakii* strains on plastic surfaces using liquid infant formula, at 25°C and 37°C temperatures for 24h.

Biofilms were stained with 0.01% (w/v) crystal violet, which was analysed at 600 nm. Experiments were assayed in three independent assays. Error bars represented using standard error.

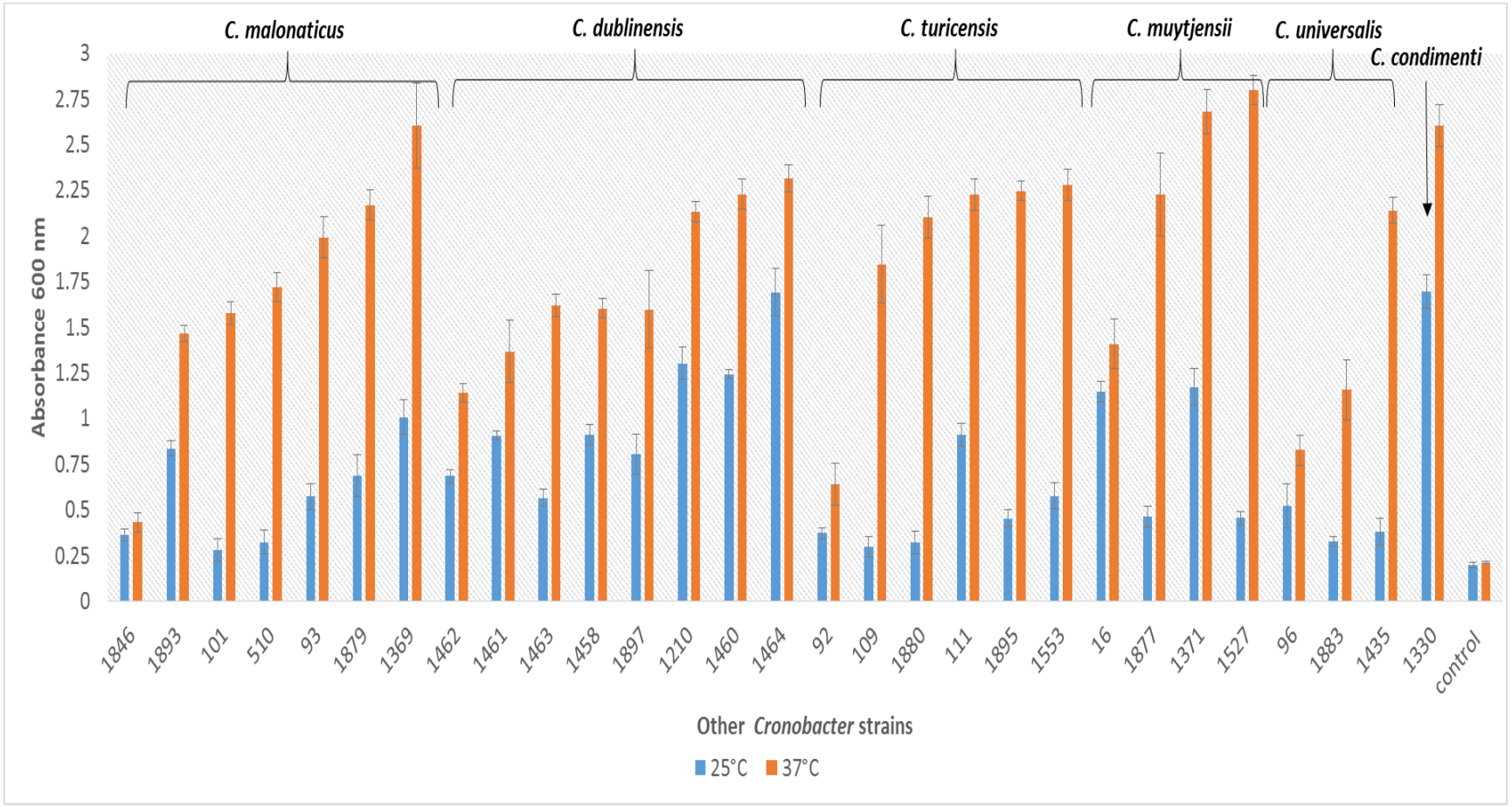


Figure 4.12. Biofilm formation of other *Cronobacter* species*; C. malonaticus, C. turicensis, C. universalis, C. muytjensii, C. dublinensis* strains, and single C. *condimenti* strain on plastic surfaces using liquid infant formula, at 25°C and 37°C temperatures for 24h. Biofilms were stained with 0.01% (w/v) crystal violet, which was analysed at 600 nm. Experiments were assayed in three independent assays. Error bars represented using standard error.

Table 4.13. Characterisation of *C. universalis, C. turicensis, C. muytjensii, C. malonaticus, C. dublinensis* strains and *C. condimenti* strain used in this study.

NTU No:	Species	ST	O-antigen	K-capsule	CA-type	Capsule production	Biofilm formation	Motility	37°C / (24h) Sialic acid utilisation	37°C / (24h) Malonate utilization	37°C / (24h) Indole production	37°C/(72h) Inositol utilization	37°C/(72h) Protease activity	37°C/(72h) Lipase activity
96	*C. universalis*	48	ND	K2	CA1	+++	moderate	+	-	+	-	+	+	++
1435		51	ND	K2	ND	+++	High	+	-	+	-	+	+	++
1883		137	ND	K2	CA2	+++	High	+	-	+	-	+	+	++
92	*C. turicensis*	35	Ctur O:3	K2	CA2	++	low	+	+	+	-	+	+	++
109		5	ND	K2	CA2	++	High	+	-	+	-	+	+	++
111		24	ND	K2	CA2	+++	High	+	-	+	-	+	+	++
1553		72	Ctur O:3	K1	CA2	+++	High	+	+	+	-	+	+	++
1880		344	Ctur O:3	K1	CA2	+++	High	+	+	+	-	+	+	++
1895		252	Ctur O:1	K2	CA2	+++	High	+	-	+	-	+	+	++
16	*C. muytjensii*	34	CmuyO:3	K1	CA1	++	High	+	-	+	+	+	+	+++
1371		71	CmuyO:2	K1	CA2	+++	High	+	-	+	+	+	+	+
1527		75	CmuyO:1	K1	CA1	+++	High	+	-	+	+	+	+	++
1877		288	CmuyO:1	K1	CA2	+++	High	+	-	+	+	+	+	++
93	*C. malonaticus*	29	Cmal O:1	K2	CA2	+++	High	+	-	+	-	+	+	++
101		60	Cmal O:1	K2	CA2	+++	High	+	-	+	-	+	+	++
1846		60	Cmal O:1	K2	CA2	+++	Low	+	-	+	-	+	+	+++
510		7	Cmal O:2	K1	CA1	+++	High	+	-	+	-	-	+	++
1369		69	Cmal O:1	K1	CA2	+++	High	+	-	+	-	+	+	++
1879		139	Cmal O:2	K1	CA1	+++	High	+	-	+	-	+	+	+
1893		289	Cmal O:1	K2	CA2	+++	High	+	-	+	-	+	+	++
1330	*C. condimenti*	98	Cuni O:1	K1	CA2	+++	High	+	-	+	+	+	+	+
1210	*C. dublinensis*	106	Cdub O:1	K1	CA1	+++	High	+	-	+	+	+	+	++
1458		74	Cdub O:1	K1	CA1	+++	High	+	-	+	+	+	+	++
1460		76	Cdub O:2	K1	CA2	+++	High	+	-	-	+	-	+	++
1461		77	Cdub O:1	K1	CA1	+++	High	+	-	-	+	+	+	++
1462		70	Cdub O:1	K1	CA1	+++	High	+	-	-	+	+	+	++
1463		95	Cdub O:1	K1	CA1	+++	High	+	-	+	+	+	+	+++
1464		78	Cdub O:2	K1	ND	+++	High	+	-	-	+	+	+	++
1897		290	Cdub O:2	K1	CA2	+++	High	+	-	-	+	+	+	+

Table 4.14. Characterisation of *C. sakazakii* strains used in this study.

NTU NO:	Species	ST	O-antigen	K-capsule	CA-type	37°C / (24h)							37°C / (72h)	
						Capsule production	Biofilm formation	Motility	Sialic acid utilisation	Malonate utilization	Indole production	Inositol utilization	Protease activity	Lipase activity
1105	*C. sakazakii*	4	O:2	K2	CA2	+++	High	+	+	-	-	+	+	++
1564		4	O:3	K2	CA2	+++	High	+	+	-	-	+	+	++
1886		4	O:3	K2	CA2	+++	High	+	+	-	-	+	+	++
1907		4	O:4	K2	CA2	+	High	+	+	-	-	+	+	++
1908		4	O:4	K2	CA2	+	High	+	+	-	-	+	+	++
1283		8	O:3	K1	CA1	+++	High	+	+	-	-	+	+	++
1888		8	O:1	K1	CA1	-	High	+	+	-	-	+	+	++
1906		8	O:1	K1	CA1	+	High	-	+	-	-	+	+	++
1107		9	O:2	K2	CA1	+++	High	+	+	-	-	+	+	++
1108		12	O:4	K2	CA2	++	moderate	+	+	-	-	+	+	++
1887		13	O:2	K2	CA2	++	moderate	+	+	-	-	+	+	++
1885		406	O:2	K1	CA2	+++	moderate	+	+	-	-	+	+	++
1990		264	O:2	K1	CA2	+++	High	+	+	-	-	+	+	++
2027		406	O:2	K1	CA2	+++	moderate	+	+	-	-	+	+	++
1843		23	O:2	K1	CA2	+++	Low	+	+	-	-	+	+	++
1844		405	O:3	K2	CA1	+++	Low	+	+	-	-	-	+	+++
1845		233	O:2	K2	CA2	+++	moderate	+	+	+	-	-	+	+++
1847		245	O:1	K2	CA1	++	High	-	+	-	-	-	+	++
1881		64	O:2	K1	CA2	+++	moderate	+	+	+	-	+	+	++
1882		20	O:3	K1	CA1	+++	Low	+	+	-	-	+	+	++
1884		263	O:2	K1	CA2	+++	High	+	+	-	-	+	+	++
1889		198	O:4	K1	CA2	+	High	+	+	-	-	+	+	++
1890		42	O:2	K1	CA2	+++	moderate	+	+	-	-	+	+	++
1992		136	O:2	K2	CA2	+++	moderate	+	+	-	-	+	+	+++

Phenotypic study of 7 *Cronobacter* species, including differences in bacterial motility, biofilm formation, capsule production, Indole production, inositol, malonate, sialic acid utilization, and protease and lipase activity. + = positive. - = negative.

4.3 Discussion.

Cronobacter species have been isolated from a wide range of food including, PIF, milk powder, weaning food, dry foods and other foods, in addition this genus have isolated from environments sources such as milk powder production factories and other manufacturing foods (Sonbol et al. 2013; Holy and Forsythe, 2014). This led to the development and improvement of specific detection and identification methods for *Cronobacter* spp. A variety of methods have been used to phenotypically identify *Cronobacter* species, including a conventional identification scheme such as colony morphology on different growth media and biochemical tests (Iversen et al. 2007).

Phenotypic identification and biochemical profiling give a rapid presumptive identification to *Cronobacter* species. According to current ISO standard (2006), detection and identification of *Cronobacter* in PIF relies on cultural and biochemical methods. However, some of these tests showed a lack of sufficient robustness for this diverse *Cronobacter* genus (Joseph and Forsythe, 2012; Joseph et al. 2013; Jackson and Forsythe, 2016). Therefore, this study focused on the phenotypic diversity of *Cronobacter* isolates from food and environmental sources and linked these with their molecular characteristics, in order to find out more about the diversity of the *Cronobacter* genus.

Colony appearance.

In this study, colony appearance and morphology of 54 *Cronobacter* strains were investigated by using different detection media such as TSA, XLD, VRBGA, VRBLA, DFI and MacConkey agar (Table 4.1). Colony appearance of 54 strains showed yellow colonies on TSA, strain 1907 showed yellow colour with rough colonies. While strain 1908 was yellow with slimy colonies. This criterion, which has been recommended for the presumptive identification of *Cronobacter* genus (Iversen and Forsythe, 2007). The pigments have important functions in protection against photooxidative damage as well as play a role in the fitness of the organisms to persist in harsh environment (Lehner et al. 2006). However, many factors such as light and temperature could affect the expression of the yellow pigment making it an unreliable feature (Jackson et al. 2014).

MacConkey agar, a selective culture medium was designed to isolate and identify Gram-negative bacteria based on lactose fermentation (Druggen and Iverson, 2009). On VRBLA and MacConkey agar, all strains in this study had pink colour with halo, except two strains, *C. sakazakii* strain 1888 (ST8) and *C. turicensis* strain 109 (ST5) showed light pink colour with a red halo on VRBLA, pink colour indicated the fermentation of lactose in these medium.

VRBGA is selective for the *Enterobacteriaceae* family, and it is based on acid production from glucose (Iversen and Forsythe, 2007). All of the strains had pink/purple colour and were mucoid on VRBGA, except one strain 1888 (ST8) was pink/purple colour and dry. Mucoid production could be linked to carbon source utilization as a result of polysaccharide production. All strains, but four showed yellow colour with mucoid on XLD agar, the exception were *C. turicensis* strain 92 (ST35), and *C. sakazakii* strains in ST8 (1283, 1888 and 1906) which showed yellow colour with dry colonies. Production of yellow colour indicated the fermentation of xylose in this media. This medium has been recommended for the identification and isolation of *Enterobacteriaceae* from water, foods and dairy products (Chadwick, 1974). Chromogenic media (DFI) has been designed by Druggan, Iversen and Forsythe, in order to differentiate and distinguish *Cronobacter* species from other *Enterobacteriaceae* members. All strains produced typical blue green colonies on DFI media after incubation for 24h at 37°C. This media was more accurate compared with other traditional *Enterobacteriaceae* enumeration agar such as VRBGA (Iversen et al. 2004).

The abilities of a bacterium to bind Congo red dye are known to be determinants related to virulence. The Congo red phenotypes of 7 species of *Cronobacter* was linked with their molecular characteristics such as STs and curli fimbriae genes. The colony morphology and colour were recorded as an indication of the binding of the Congo red dye with curli fimbriae after incubation at 37°C for 24 and 48 hours, and at 25°C for 72 hours and 5 days (Table 4.2).

In this study 5 morphotypes were recorded including, pink, dry, and rough (PDAR); brown, dry, and rough (BDAR); pink and smooth (PAS); brown and smooth (BAS); red

and smooth (RAS). The majority of tested strains were the same morphotype (BAS) after incubation at 37°C for 24 and 48 hours. The exceptions were *C. malonaticus* 1879 (ST139), four *C. dublinensis* strains 1210 (ST106), 1458 (ST74) and 1462 (ST70) and four *C. sakazakii* strains 1886 (ST4), 1845 (ST233), 1889 (ST198) and 1890 (ST42) which showed BDAR morphology after incubation for 48 hours at 37°C. Regarding incubation period at 25°C, BAS morphotype was the most common morphotype among the tested strains, as noted for 24 isolates (42.6 %) followed by 17 isolates (31.4%) with BDAR morphotype. However, most of tested strains showed different morphotype after incubation at 25°C for 72h compared with incubation period at 25°C after 5 days. Only strains defined by either the RDAR or BDAR morphotypes were considered positive for the Congo red dye binding assay. Two *C. malonaticus* strains 93 (ST29) and 1893 (ST289) and one *C. sakazakii* strain 377 (ST4) exhibited a RAS morphotype (1.8%) were considered to exhibit reduced binding of the Congo red dye, while those with the BAS morphotype (56.5%) did not show any binding of the Congo red dye. However, in this study no correlation between the Congo red phenotypes of 7 species of *Cronobacter* and their STs was noted.

Curli fimbriae genes (*csgBAC* and *csgDEFG*) were found in *C. malonaticus*, *C. universalis* strains and the single *C. condimenti* strain (1330). However, none of the curli fimbriae homologues was noted in *C. sakazakii* and *C. muytjensii* genome analysed in this study. *C. dublinensis* were divided into two groups based on presence or absences of this gene cluster. *C. dublinensis* spp. *C. dublinensis* strains LMG 23823 (ST106), 1210 (ST106), 1458 (ST74) and 1463 (STST95) showed the presence of these genes. While, *C. dublinensis* ssp. *lausannensis* LMG 23824 (ST80), 583 (ST346) and 1460 (ST76) and *C. dublinensis* ssp. *lactaridi* strains LMG 23825 (ST79), 1461 (ST77) and 1462 (ST70) showed the absences of this gene cluster. Furthermore, *C. turicensis* strains were also divided into groups, this gene cluster was found in *C. turicensis* strains 1211 (ST19), 1880 (ST344) and 1895 (ST252), and was absent in strains 92 (ST35), 1553 (ST72) and 1554 (ST342). Gene clusters *csgBAC* and *csgDEFG* do not seem to be necessary for production pink colonies. The present study showed that no clear correlation between the phenotypic detection of curli fimbriae by Congo red staining and the genotypic detection of the curli fimbriae genes.

Calcofluor binding assays were used to investigate colony morphotypes and the expression of cellulose (Table 4.2). The binding of any cellulose produced by the *Cronobacter* isolates were observed based on the presence of blue florescent colonies under UV light (Figure 4.1). The majority of tested strains (19/54 isolates, 35%) showed a moderate fluorescent signal (++), followed by 13 isolates (24%) with the strong fluorescent signal (+++) and 8 isolates (14.8%) were low fluorescent signal (+), while 14 isolates (25.9%) were negative for this phenotype. These including, one *C. turicensis* strain 109 (ST5), all of *C. dublinensis* isolates 1210 (ST106), 1458 (ST74), 1460 (ST76), 1461 (ST77), 1462 (ST70), 1463 (ST95), 1464 (ST78), 1897(ST290) and five *C. sakazakii* strains; 1564 (ST4), 1283, 1888 and 1906 (ST8), and 1108 (ST12). The phenotypic detection of cellulose production was nearly the same in all studied strains for both 28°C/ (48h) and 37°C/(24h) incubation period (Table 4.2). The cellulose gene cluster consisted of nine genes: *bcsCZBAQEFG* and *yhjR* (Ogrodzki and Forsythe, 2015). These genes were present in nearly all *Cronobacter* strains, the exceptions were *C. sakazakii* strain 1882 (ST20, CC20) and the single *C. condimenti* strain 1330 (ST98). However, no obvious correlation between the phenotypic detection of cellulose production by Calcofluor white staining and the genotypic detection of the cellulose gene cluster was observed.

Cronobacter spp. strains motility.

Flagella are bacterial organelles which are responsible for motility. In addition, flagella have been proposed as an essential requirement for biofilm formation as well as adhesion and invasion of host cells. The mobility of 54 *Cronobacter* spp. strains through the motility medium at 37°C after the incubation for 18h are shown in figures 4.2 and 4.3. *S. enterica* 358 and *E. coli* K12 strains were used as positive and negative controls respectively. *Cronobacter* species are motile (Iversen et al. 2008). Strains showed a wide-range of motility zone diameters. Most strains were motile except for 2 *C. sakazakii* strains; 1906 (ST8) and 1847 (ST245). In general, for comparison data of all strains, *C. sakazakii* strains in ST8 and ST4 were less motile than other *C. sakazakii* STs. Overall, no significant different was obtained when the motility of seven species were compared to each other. All strains that have their genomes sequenced for both

motile and non-motile strains showed the presence of *fliA-Z* gene cluster. This suggest that these motility genes might be not expressed or an unrecognised mutation could occurrent in non-motile strains

Biochemical test profiling.

Iversen et al (2007) reported that the important biochemical tests for the differentiation of *Cronobacter* species were indole production, malonate and inositol utilization and production of α-glucosidase. In addition, Joseph et al (2013) stated that utilization of sialic acid was a significant biochemical test for the differentiation of *Cronobacter* species. However, these relevant biochemical tests were applied to all strains and were linked with their genetic traits.

Indole production.

All *C. muytjensii*, *C. dublinensis* strains and the single *C. condimenti* strain were able to produce indole, but *C. sakazakii*, *C. malonaticus*, *C. turicensis, C. universalis* and *C. universalis* strains were negative for indole production test. The *tnaA* gene, encoding for the tryptophanase/L-cysteine desulfhydrase, PLP-dependent, which primarily degrades L-tryptophan to indole, pyruvate, and ammonia (Li and Young, 2013). This gene was found in all *C. dublinensis*, *C. muytjensii* strains and the single *C. condimenti* strain, and was missing in all *C. sakazakii*, *C. malonaticus*, *C. turicensis and C. universalis* strains (Figure 4.4). All strains were positive for *tnaA* gene except for the non-indole production strains (negative strains); *C. sakazakii*, *C. malonaticus*, *C. turicensis* and *C. universalis* strains that lacked this gene. The result revealed that not all *Cronobacter* species are able to produce indole as a result of tryptophan degradation, this is due to a lack of the *tnaA* gene, which primarily degrades L-tryptophan to indole. Thus, this characteristic could be used to distinguish between 7 species of *Cronobacter*.

Alpha-glucosidase activity.

Alpha-glucosidase activity is one of the most important biochemical features, which distinguishes *Cronobacter* species from other *Enterobacteriaceae* members (Lehner et al. 2006; Iversen et al. 2007). All *Cronobacter* strains showed the ability to produce a

blue-green colour as result of α- glucosidase activity. The gene cluster (ESA_02709-14) encodes for maltose utilisation and including α–glucosidase (Joseph et al. 2012). These genes were found in all *Cronobacter* strains. Hence, DFI medium was designed based on the α-glucosidase reaction as result of presence of these genes. *Cronobacter* genus hydrolyses 5-bromo-4-chloro-3-indolyl α-D-glucopyranoside substrate to an indigo pigment, producing blue-green colonies on this medium (Iversen et al. 2004).

Inositol utilization.

The inositol and inositol-monophospohate are included in infant formula, *myo*-inositol (MI) compounds are necessary for infant development (Koletzko et al. 2013). *C. muytjensii, C. universalis, C. turicensis* strains and single strain of *C. condimenti* showed ability to utilize inositol. Most *C. sakazakii* strains were able to utilize inositol, except strains 377 (ST4), 658 (ST1), 1844 (ST405), 1845 (ST233), 1847 (ST245) and 1992 (ST136). All *C. malonaticus* strains were able to utilize inositol, but one strain 510 (ST7) was unable to utilize inositol, and this led to more detailed study of *C. malonaticus* species (14 strains), in particular sequence type 7, because this sequence type is linked to adult infections, and the most frequently isolated *C. malonaticus* sequence type (Forsythe et al. 2014; Alsonosi et al. 2015). Strains of *C. dublinensis* were divided into groups, this variation led to an expanded study concerning *C. dublinensis* species. Hamby et al (2011) reported that inositol fermentation is a requirement for pathogenicity. In this study, genome analyses using BLAST genome search was applied to investigate the presence of inositol genes that are linked with the inositol utilization. Inositol metabolism encoded by the gene cluster (*iolD, iolE, iolB, iolT, iolG* and *iolC*). These genes were found in all *C. muytjensii*, *C. universalis*, *C. turicensis, C. sakazakii* strains and the single *C. condimenti* strain, the exceptions were 6 *C. sakazakii* strains; 377 (ST4), 658 (ST1), 1844 (ST405), 1845 (ST233), 1847 (ST245) and 1992 (ST136).

C. dublinensis strains were divided into 2 groups, the first group showed the presence of inositol utilization genes including *C. dublinensis* spp. *dublinensis* strains LMG 23823 (ST106), 1210 (ST106), 1458 (ST74) and 1463 (ST95) and *C. dublinensis* ssp. *lactaridi* strains LMG 23825 (ST79) , 1461 (ST77) and 1462 (ST70), and the second group showed lack of these genes including *C. dublinensis* ssp. *lausannensis* strains LMG

23824 (ST80), 583 (ST346), 1460 (ST76) and 1560 (ST341). This could help to distinguish of *C. dublinensis* strains to the subspecies level. All *C. malonaticus* strains were positive for this gene cluster, the exception were ST7 strains (510, 681, 665, 688, 893, 1830, 1835, 1835, 1914 and LMG23826 (non-inositol utilization strains) which showed absence of these genes. This suggests that inositol utilization test might be used to distinguish pathogenic ST7 strains from other *C. malonaticus* STs.

Overall, strains which are positive for inositol utilization, showed the presence of inositol gene cluster and negative strains showed absence of this gene cluster (Table 4.3). A recent study by Grim et al (2013) proposed that inositol fermentation is a marker of pathogenicity for *Cronobacte*r strains. However, in this study inositol utilization genes were found in the majority of *Cronobacter* strains isolated from the environment and food. Whereas, also being absent in genomes of pathogenic strains such as *C. malonaticus* ST7 and some of *C. sakazakii* ST4 . Consequently, its role in virulence is still unclear at present.

A clear correlation between the phenotypic detection of inositol production and the genotypic detection of the inositol utilization gene cluster was observed (Table 4.5). This suggests that these genes are necessary for inositol utilization, and might be used to distinguish of *Cronobacter* strains to the subspecies level.

Sialic acid utilization.

Sialic acid is found in the human body, such as breast milk, intestinal mucin, gangliosides in the brain and infant formula (Joseph et al. 2013). *Cronobacter* spp. could use sialic acid as carbon and energy source. Therefore, this metabolism could be a significant virulence factor. However, the present study showed that all *C. malonaticus*, *C. muytjensii C. universalis, C. dublinensis* strains and the single *C. condimenti* strain were unable to use sialic acid as the only carbon source. Joseph et al (2012) reported that *C. sakazakii* was the only member of *Cronobacter* genus, which can utilize sialic acid as a source of carbon. However, in this study all *C. sakazakii* strains and some of *C. turicensis* strains were capable to utilize sialic acid as a carbon source. This observation was in agreement with the previous study by our group, which indicated that all *C. sakazakii* and some *C. turicensis* strains were unique in the

Cronobacter genus in utilization of exogenous sialic acid as a carbon source (Sumyya Hariri 2015, unpublished data).

Artemis comparison tool was used to compare between isolates of *C. sakazakii* and *C. turicensis*, which are positive for sialic acid utilization against a *C. turicensis* isolate which is un able to utilize sialic acid (Carver et al. 2005) in order to investigate if the sialic acid utilization cluster genes were found or missing in *Cronobacter* as a result of genome evolution. The comparison showed that the genomic structure of *nanKTA* gene cluster were found in isolates which were positive for sialic acid and missing of these genes in negative strains (Figure 4.5). This might reflect the evolutionary history of members of the *Cronobacter* genus.

Malonate utilization.

Malonate is an organic acid that some bacteria can use for carbon and energy, and reflects the plant-association of the *Cronobacter* genus (Forsythe et al. 2014). Malonate utilization of 54 *Cronobacter* strains was determined using malonate PPA Broth (Table 4.3, 4.5). All *C. malonaticus, C. universalis, C. turicensis* and *C. muytjensii* strains were able to utilize malonate. All *C. sakazakii* strains were negative for malonate utilization test, but two strains 1845 (ST233-O:2) and 1881 (ST64-O:2) were able to utilize malonate. *C. dublinensis* strains were divided into two separate groups based on malonate utilization. Three of eight strains were positive for malonate utilization; 1458 (ST74), 1463 (ST95) and 1210 (ST106). However, other strains 1460 (ST76), 1461 (ST77), 1462 (ST70), 1464 (ST78) and 1897(ST290) were negative for malonate utilization test. The single *C. condimenti* strain 1330 (ST98) was also able to grow on malonate broth. Additionally, malonate metabolism encoded by the *mauR, madA, citG, mdcC, madC, madD, mdcG* and *fabD* genes. These genes were found in all *Cronobacter* strains which were positive for malonate utilization test (*C. malonaticus, C. universalis*, *C. turicensis*, *C. muytjensii* strains and the single *C. condimenti*). However, all *C. sakazakii* and *C. dublinensis* strains were negative for this cluster, the exception were *C. sakazakii* strains in ST1, ST64 and ST233 as well as *C. dublinensis* spp. *dublinensis* strains ST106, ST74 and ST95 (Table 4.3, 4.5 and Figure 4.6). Therefore, this test cannot be used solely for the purpose of identifying and distinguishing of

Cronobacter species, particularly between *C. sakazakii* and *C. malonaticus* strains. This distribution of genes may reflect gene loss or acquires at during the genus evolution and adaption.

Description of *Cronobacter* strains.

***C. muytjensii, C. turicensis, C. universalis* strains and the single *C. condimenti* strain.**

According to the above biochemical tests (Table 4.5). *C. muytjensii* strains were positive for malonate, indole, and inositol, but were negative for sialic acid. *C. universalis* strains were positive for malonate and inositol tests, but were negative for indole and sialic acid test. Single *C. condimenti* strain showed positive for all biochemical tests; malonate, indole, and inositol, the exception was the sialic acid test. *C. turicensis* strains were positive for malonate and inositol test, but were negative for indole test, and some strains were positive for sialic acid test (50%).

***C. dublinensis* strains.**

The relevant biochemical tests showed great ability to characterize and distinguish *C. dublinensis* strains to the subspecies level. *C. dublinensis* ssp. *dublinensis* (1210, 1458 and 1463) strains were positive for indole, inositol, malonate test, but were negative for sialic acid utilization test. *C. dublinensis* ssp. *lausannensis* (583 and 1460) strains were positive for indole, but were negative for inositol, sialic acid and malonate utilization test. While, *C. dublinensis* ssp. *lactaridi* (1461 and 1462) strains were positive for inositol and indole tests, but were negative for sialic acid and malonate utilization tests. This observation was supported by genomic investigation of 14 *C. dublinensis* genome strains (Table 4.15). According to this table, *C. dublinensis* ssp. *dublinensis* strains were positive for malonate gene cluster (*madACD, mdcCG* and *mauR*), inositol gene cluster (*iolTEGDCB*) and Curli fimbriae genes (*csgABCDEFG).* Moreover, these strains were O:1 serotype, K-capsule type 1 (K1) and colonic acid type1 (CA1).

C. dublinensis ssp. *lactaridi* strains showed presence of the inositol gene cluster (*iolTEGDCB).* Nonetheless, the malonate gene cluster (*madACD, mdcCG* and *mauR*) and Curli fimbriae genes (*csgABCDEFG*) were absent. These strains showed the same

genetic profile of *C. dublinensis* ssp. *dublinensis* strains (O:1-K1-CA1). *C. dublinensis* ssp. *lausannensis* strains were negitive for malonate gene cluster, inositol gene cluster and Curli fimbriae genes. This sub species showed different capsule profile (O:2-K1-CA2) compared with other *C. dublinensis* strains(Table 4.15).

Table 4.15. Distribution of some genes related to some physiological and virulence traits across the sequenced genomes of *C. dublinensis* species

C. dublinensis strains				malonate cluster	inositol cluster	Curli fimbriae genes	Colanic acid cluster(*galE*)	K-capsule type
	Strain NO:	ST	O-antigen	*madACD, mdcCG, mauR*	*iolTEGDCB*	*csgABCDEFG*	CA1 / CA2	K1/ K2
C. dublinensis spp. dublinensis	LMG-23823	106	O1	+	+	+	CA1	K1
	1210	106	O1	+	+	+	CA1	K1
	1458	74	O1	+	+	+	CA1	K1
	1463	95	O1	+	+	+	CA1	K1
C. dublinensis . ssp. lactaridi	LMG-23825	79	O1	-	+	-	CA1	K1
	1461	77	O1	-	+	-	CA1	K1
	1462	70	O1	-	+	-	CA1	K1
	CFSAN022296	388	O1	-	+	-	CA1	K1
	CFSAN022297	388	O1	-	+	-	CA1	K1
C. dublinensis ssp. lausannensis	LMG-23824	80	O2	-	-	-	CA2	K1
	582	80	O2	-	-	-	CA2	K1
	1556	80	O2	-	-	-	CA2	K1
	583	346	O2	-	-	-	CA2	K1
	1460	76	O2	-	-	-	CA2	K1

Presence and absence of some genes related to physiological and virulence traits genes were investigated by using BLAST genome search. + = Present - = Absence

Closely related species; *C. sakazakii* and *C. malonaticus* strains.

More importantly these four tests, in particular sialic acid and malonate test showed a greater-discriminatory power between two closely related species, which are *C. sakazakii*, and *C. malonaticus* strains. Iversen et al (2008) reported that the *C. sakazakii* type strain (ATCC 29544^T) was positive for utilization of inositol and negative for utilization of malonate. Whereas, *C. malonaticus* type strain, (LMG 23826^T) was positive for utilization of malonate and negative for inositol utilization.

In this study, all *C. sakazakii* strains were positive for sialic acid and most of them were positive for inositol (85%), but were negative for indole, and negative for malonate except strains in ST1, ST64, and ST233 (11%). In contrast, *C. malonaticus* strains were negative for sialic acid and indole test. Nevertheless, these strains were positive for malonate, and inositol test, except ST7 strains. This suggests that inositol utilization test might be used to distinguish *C. malonaticus* ST7 strains from other *C. malonaticus* STs. Particularly ST7 is the most frequently isolated *C. malonaticus* sequence type, and is associated with adult infections (Holy et al. 2014; Alsonosi et al. 2015).

Therefore, for this dataset it is possible to differentiate and distinguish the seven species of *Cronobacter* as well as to characterize and distinguish of *C. dublinensis* strains to the subspecies level using genomic clusters and sialic acid, malonate, indole, and inositol tests.

Capsule production

The capsule is considered a virulence factor because it increases the ability of bacteria to cause disease, and may enable the organism to resist desiccation and could facilitate the organism's attachment to plant surfaces. Capsule formation is also involved in formation of biofilms which have been detected in enteral feeding tubes of neonates in neonatal intensive care unit (NICU) (Hurrel et al. 2009a, Kim et al. 2006), as well as contribute to persistence of the pathogen on food contact surfaces (Iversen et al. 2004d). This study focused on the mucoid appearance of 54 *Cronobacter* strains on different media, and linked these with their molecular characteristics such as ST, O-antigen , K-capsule type and CA-type, in order to find out more about the diversity of the *Cronobacter* genus. The colony appearance on different media including milk agar, XLD, VRBLA and VRBGA was used as the main parameter to compare capsule production between *Cronobacter* strains (Table 4.7). The majority of strains had a very mucoid appearance indicating capsular material production. However, less capsular material was produced by the *C. sakazakii* strains with serotype profile O:1 and O:4 such as 1906 (ST8-O:1), 1847 (ST245-O:1), 1907 (ST4-O:4), 1908 (ST4-O:4), 1108 (ST12-O:4) and 1889 (ST189-O:4). One *C. sakazakii* strain 1888 (ST8-O:1) showed a dry colony appearance in all media indicating no capsular material was produced. Significant

capsular material was produced by *C.sakazakii* strains with serotype profile O:2 and O:3. Strains of *C. sakazakii* with the same sequence type (ST) and different serotype profile showed different capsular material, for example strains with ST8-O:1 and ST4-O:4 had a lower amount of capsular material. In contrast, strains with ST8-O:3, ST4-O:2 and ST4-O:3 were able to form high amount of capsular material.

The study indicated that there were different levels of mucoid production in the same sequence types. In addition, there was no correlation between the amount of capsular material and genotypic detection including sequence type (ST), K-capsule type and CA-type. Conversely, a strong correlation between type of serotype (O-antigen) and amount of capsular material was noted (Table 4.8). The O-antigen is a polysaccharide consists of repetitive oligosaccharide units (O units), and usually consist of three to six sugars (Blažková et al, 2016). The repetition and variations in oligosaccharide units (O-units) may play an important role for production of capsular material amount.

Capsule composition.

In Gram-negative bacteria, capsular polysaccharides (CPS) are responsible for bacterial virulence factors and environmental fitness traits (Willis et al. 2013). In addition, Ogrodzki and Forsythe (2015) reported that the CPS vary considerably between bacteria strains, and even between strains of the same species. The O-antigen is a polysaccharide (O-PS) that extends from the cell surface and consists of repetitive oligosaccharide units (O units) generally composed of 3 to 6 sugars (Mullane et al. 2008). Diversity of the capsule has been referred to by many of bacterial differentiation methods such as O-antigen serotyping of *Salmonella* serovars and the K-antigen (k-capsule) classification scheme of *E. coli* (Whitfield et al. 2006). Therefore, the correlation between the composition capsule of *C. sakazakii* strains and their genetic traits including ST, O-antigen, K-capsule type and colonic acid type (CA) were studied in this part (Table 4.11). To the best of our knowledge, this is the first study that focused on the association between the capsule compositions (monosaccharides), amount of capsule production and linked that with genetic traits.

C. sakazakii strains (n=13) were selected based on the diversity of their sequence type (ST) which associated with clinical significance including; ST1, ST4, ST8, ST12 and ST13,

O-antigen serotype (O:1, O:2, O:3 and O:4) and capsule profiling (K-type and CA-type). Production of capsule by thirteen strains of *C. sakazakii* was determined by colony appearance on milk agar. The dry mass of capsule materials were analysed using carbohydrate nuclear magnetic resonance (NMR) Spectroscopy. The analysis of capsule of 13 selected *C. sakazakii* strains showed they were composed of four different sugars; galactose (Gal), glucose (Glc) and fucose (Fuc) and rhamnose (Rha).

The correlation between the capsule composition and ST, O-antigen, K-capsule type and colonic acid type (CA).

The monosaccharides in 658 ST1 (O:1-K1-CA1) sample were galactose (Gal), glucose (Glc) and fucose (Fuc-Gal-Glc). *C. sakazakii* ST8 strains were divided into 2 groups based on their sugar composition (monosaccharides), 1888 and 1906 (ST8-O:1-K1-CA1) samples contains only one type of sugars which is (Glc) at both temperatures (21°C and 37 °C). Whereas 1283 (ST8-O:3-K1-CA1) sample was consist of 3 type of sugars which are (Gal-Glc-Fuc) with different amount at different temperatures (21°C and 37 °C). More interestingly, these two groups showed different mucoid production on milk agar and different O-antigen profile, but same capsule profile. Strains 1888 and 1906 (O:1) showed dry colony appearance, while significant capsular material was produced by strain 1283 (O3). Whitfield et al (2006) reported that diversity of capsular referred to bacterial differentiation in O-antigen serotyping or the K-antigen (K-capsule) classification scheme. It seems to be that the amount of capsule production and type of O-antigen serotyping are linked with capsule composition (monosaccharides) in ST8 strains.

The monosaccharides in *C. sakazakii* ST4 samples; 377, 767, and 1533 (O:2-K2-CA2) composed of 4 sugars; galactose, glucose, fucose and rhamnose (Gal-Glc-Fuc-Rha), these strains produced strong mucoid production on milk agar. However, monosaccharides of 1908 ST4 strain (O:4-K2-CA2) contained only 3 sugars (Gal-Glc-Fuc), this strain showed low capsule production on milk agar. Strain 1886 (ST4-O:3-K2-CA2) had the same ratio of sugars, but with less amount of monosaccharides. Rhamnose was only found in ST4 strains with serotype O:2, and was lack in ST4 strains

in serotype O:3 and O:4. This sugar is often essential for bacterial virulence and viability (Maki and Renkonen, 2004; Mistou et al. 2016).

The monosaccharides in *C. sakazakii* ST12 samples 1108 and 2107 (O:4-K2-CA2) were also composed of 3 monosaccharides (Gal-Glc-Fuc) with different amount at different temperatures (21°C and 37°C). However, strain 2107 showed a lack of (Fuc) at 21°C, and produced less capsular materials on milk agar compared with strain 1108. The monosaccharides of *C. sakazakii* strain 1887 ST13 (O:2-K2-CA2) were the same sugars of *C. sakazakii* ST4 (O2:). It was composed of 4 sugars (Gal-Glc-Fuc-Rha) at 21 °C, and lacked (Gal) and (Fuc) at 37°C. This strain showed high mucoid production on milk agar at 37°C, and weak mucoid production was observed at 21°C.

It seems to be that colanic acid consist of galactose-glucose-fucose in the ratio 1:1:1, and the additional glucose is owing to cellulose. This observation shows that there is a strong correlation between the amount of mucoid production, type and ratio of monosaccharides production and the genetic traits, in particular O-antigen serotype (Table 4.11). Moreover, this study indicated that rhamnose is the main sugar in *C. sakazakii* strain in serotype O:2. However, no clear correlation was observed between the amount of mucoid production, type and ratio of monosaccharides production and the capsule profiling (K-type and CA-type) .

Rhamnose genes.

Rhamnose sugar is commonly found in Gram-negative and Gram-positive bacteria. This sugar generally distributed in O-antigens of Gram-negative bacteria. Moreover, this sugar is frequently found in capsular polysaccharides (CPS), which are covalently bound to the cell wall (Boels et al. 2004). Interestingly, L-rhamnose is often essential for bacterial virulence and viability (Maki and Renkonen, 2004; Mistou et al. 2016). Silva et al (2005) reported that genes (*rmlA* and *rfbBCD*) are responsible for the biosynthesis of dTDP- l-rhamnose from glucose 1-phosphate in *E.coli* and *Salmonella* strains (Silva et al, 2005). These genes (*rmlA* and *rfbB*) were found in 13 selected *C. sakazakii* strains.

More interestingly, *rfbCD* genes were found only in *C. sakazakii* strain with serotype O:2 such as ST4 stains; 377, 767, 1105 and 1533 (O:2) and ST13 strain 1887 (O:2). However, these genes were absent in other *C. sakazakii* serotypes (O:1, O:3 and O:4) including, 658 ST1 strain (O:1), ST12 strains 1108 and 2107 (O:4), ST8 strains 1888, 1906 (O:1) and 1283 (O:3), ST4 strain 1908 (O4) and 1886 (O3). In general, all strains were negative for *rfbCD* genes, except for the rhamnose production strains (positive strains); ST4 strains 377, 767, 1105 and 1533 (O:2) and 1887 ST13 strain (O:2) that found these gene (Table 4.12 and Figure 4.9). Rhamnose sugar was only found in *C. sakazakii* strains with O-antigen (O:2), and was absent in other O-antigen serotypes (O:1, O:3 and O:4) (Figure 4.10). This suggests that these genes (*rfbCD*) are important for rhamnose biosynthesis, and might be used to predict the capsule polysaccharide composition in *Cronobacter* species.

Biofilm formation

Biofilm formation gives bacteria a competitive benefit due to its protection of the bacteria from shear forces and low pH environments such as in the stomach. In addition, this makes them more resistant to environmental stresses such as extreme temperature, desiccation, disinfections, antibiotics and heavy metals (Scher et al. 2005; Kim et al. 2006; Dancer et al. 2009). The *Cronobacter* genus has been reported to form biofilm on glass and polyvinyl chloride, silicon, latex, polycarbonate and stainless steel (Lehner et al. 2005; Iversen et al. 2003). These materials are normally used for infant feeding and food manufacturing equipment. In this study, the ability of 54 *Cronobacter* strains to form biofilms on plastic surfaces in PIF, using two incubation temperatures (25°C and 37°C) were evaluated. Strains were able to form a biofilm at both the human body temperature (37°C) and the room temperature (25°C), and a variation in the degree of biofilm was observed at these temperatures. The result presented that 37°C were the optimal temperature condition for the most strains, which were able to form high values of biofilm (Figure 4.11, 4.12). Moreover, *C. sakazakii* strains in ST4, ST8, ST245 and ST198 showed the ability to form great values of biofilm compared with other *C. sakazakii* STs (Figure 4.11).

Strains of *C. sakazakii* in ST4 and ST8 are association with infant infections and were predominantly clinical isolates (Joseph and Forsythe, 2011; Joseph et al. 2012; Hariri et al. 2013; Masood et al. 2015). These two STs; ST4 and ST8 showed the ability to form great volumes of biofilm compared with other *C. sakazakii* STs, this may increase their ability to cause infection, and creating a food safety risk. Iversen et al (2004) stated that *Cronobacter* capsulated strains produced biofilms of a higher cell density than those produced by a non-capsulated strains. In this study however, there was no correlation between values of biofilm formation, and the amount of capsule material on milk agar for the 54 strains studied (Table 4.13, 4.14). For example, *C. sakazakii* strains 1888 (ST8) produce dry mucoid on milk agar plates, but produced more biofilm on plastic surfaces than some capsulated *C. sakazakii* strains such as 1283 (ST8), 1885 (ST406), 2027 (ST406), 1890 (ST42), 1881 (ST64), 1845 (ST233), 1844 (ST405) and 1882 (ST20). Similarly *C. sakazakii* strains 1907 (ST4), 1908 (ST4) and 1847 (ST245) produce a weak capsule mucoid, but produced more biofilm than most capsulated *C. sakazakii* strains (Figure 4.11).

Chapter 5. *Cronobacter* strains stress responses.

5.1 Introduction.

Cronobacter species have been isolated from a wide range of sources such as PIF, dried milk products, weaning foods, powdered ingredients, processed milk products, and food production environments (Osaili and Forsythe, 2009; Holý and Forsythe, 2014; Jackson et al. 2015). The resistance of *Cronobacter* species to environmental stresses is a main factor responsible for its survival and behaviour in infant formula and other foods. *Cronobacter* shows a high resistance to heat, drying, and acid stress compared to other *Enterobacteriaceae* species (Nazarowec-White and Farber, 1997; Breeuwer et al. 2003; Dancer et al. 2009). In addition, the introduction of heavy metals, in many forms in the environment, can cause substantial modifications in the function and structure of bacterial communities (Yao et al. 2016). Most of heavy metals are essential micronutrients for organisms since they are incorporated into enzymes and cofactors. Nevertheless, Nies (2003) stated that at high concentrations heavy metals can be toxic for the bacterial cells because of binding to enzymes and DNA, and by production of oxygen radicals through the Fenton reaction.

Overcoming drying stress is important for the survival and persistence of *Cronobacter* spp. in PIF and dried milk products. Dancer et al (2009) reported that *Cronobacter* species have been recognised to be remarkably resistant to environmental stresses such as osmotic stress and drying, when tested in stationary phase (Breeuwer et al. 2003; Dancer et al. 2009; Fakruddin et al. 2014; Feeney et al. 2014). The *Cronobacter* genus is one of the most thermotolerant members of the *Enterobacteriaceae* found in dairy products, and this property contributes to their survival in infant formula milk, dried milk products and other food. Furthermore, resistance to heat is an important factor for survival of *Cronobacter* species in food (Dancer et al. 2009; Huertas et al. 2015). Huertas et al (2015) reported that heat resistance varies widely between *Cronobacter* strains. The *D*-values of *Cronobacter* strains in PIF and other laboratory media ranged from 8.58 to 85.50 and 0.12 to 15 at 50 °C and 58 °C, respectively (Dancer et al, 2009; Nazarowec-White and Farber, 1997; Osaili et al. 2009; Huertas et al. 2015; Yang et al. 2015). The D value is a measure of the heat resistance of a

microorganism. It is the time in minutes at a given temperature required to destroy 1 log cycle (90%) of the target microorganism. Yan et al (2013) stated that in many bacteria, ABC transporters such as *yehZYXW*, *proP*, *proU*, and opu*CABCD*, are involved in bacterial osmoprotection (Finn et al. 2013; Yan et al. 2013). Moreover, the osmotolerance genes in *C. sakazakii* play an important role in the resistant to environmental stress in the human stomach and in the environment (Feeney and Salter, 2011). *Cronobacter* that are ingested via contaminated PIF are exposed to the highly acidic environment of the stomach. Resistance to acid is an important physiological characteristic that has been widely studied in *Cronobacter* species. Dancer et al (2009) have shown the ability of *Cronobacter* spp. to survive under highly acidic conditions. It was stated that at least 80% of the tested strains were able to survive and grow at a pH as low as 3.9 (Dancer et al, 2009). Alvarez-Ordones et al (2014) reported that the *ompR* gene is an important factor in the adaptive response of *Cronobacte*r spp. to highly acidic conditions, in particular *C. sakazakii* strains.

In this chapter, *Cronobacter* strains isolated from food and environmental sources were studied physiologically to investigate their ability to tolerate a variety of environmental stress conditions such as heavy metal, sub-lethal injury during desiccation, long-term drying, heat and acid resistance, and to link these with their genetic traits.

5.2 Bacterial strains used in this study.

Fifty-four *Cronobacter* spp. strains were used in the stress responses study (Chapter 2, Section 2.5.3)

5.3 Results.

5.3.1 Heavy metal resistance.

The resistance of *Cronobacter* species to 7 different concentration of heavy metal including; nickel chloride, zinc sulphate, copper (II) sulphate, silver nitrate, sodium tellurite, cadmium carbonate and cobalt(II) nitrate were studied. Each isolate was exposed to 4 different concentrations of 1M, 0.1M, 0.01M and 0.001M of each heavy metal.

The sensitivity of 54 *Cronobacter* strains to the heavy metals, which are mentioned above, were investigated (Table 5.1 and 5.2). All *Cronobacter* isolates were sensitive to sodium tellurite and silver nitrate for 4 different concentrations of 1M, 0.1M, 0.01M and 0.001M. In addition, most of these isolates were sensitive to the high concentration of heavy metals (1M and 0.1M), except the cadmium. Most of strains were resistance to cadmium, except *C. turicensis* strain 1553 (ST72), four *C. muytjensii* strains 1527 (ST75), 16 (ST43), 1371 (ST71), 1877 (ST288), three *C. dublinensis* strains 1460 (ST76), 1461 (ST77), 1462 (ST70), two strains of *C. sakazakii* (ST4) 377 and 1907, two *C. universalis* strains 96 (ST48) and 1883 (ST137), and the single *C. condimenti* strain 1330 (ST98) which showed sensitivity to the high concentration (1M, 0.1M). All strains were sensitive to the high concentration (1M) of the zinc sulphate, as well as some of them were sensitive to concentration (0.1M). These later strains were *C. sakazakii* 377, 1105, 1564, 1907 and 1908 (ST4), 1108 (ST12), 1107 (ST9), 1990 (ST264), 2027(ST406), 1843 (ST23), 1844 (ST405), 1845 (ST233) and 1887 (ST13), *C. turicensis* 1893 (ST252), *C. universalis* strains 96 (ST48), 1435 (ST51) and 1883 (ST137), *C. muytjensii* strains 1371 (ST71) and 1877 (ST288), and *C. dublinensis* strains 1458 (ST74), 1460 (ST76) and 1462 (ST70).

Table 5.1. Sensitivity of *C. sakazakii* strains to heavy metal

NO:	ST	Copper (II) sulphate				Silver nitrate				Sodium tellurite				Nickel chloride				Zinc sulphate				Cadmium carbonate				Cobalt(II) nitrate			
		1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001
377	4	15	8	0	0	12	11	11	10	58	51	49	41	24	10	8	0	39	25	0	0	10	8	0	0	26	16	0	0
1105	4	10	8	0	0	12	10	8	8	56	46	43	36	20	0	0	0	36	20	0	0	0	0	0	0	25	8	0	0
1564	4	11	8	0	0	14	11	10	10	55	44	40	34	16	0	0	0	40	20	0	0	0	0	0	0	20	8	0	0
1886	4	14	8	0	0	12	19	9	8	55	46	37	24	17	8	0	0	45	22	12	0	0	0	0	0	27	10	0	0
1907	4	13	8	0	0	14	13	10	10	55	47	42	35	20	8	0	0	38	9	0	0	11	8	0	0	23	8	0	0
1908	4	10	0	0	0	13	13	11	9	54	46	41	34	19	8	0	0	40	18	0	0	0	0	0	0	24	8	0	0
1283	8	15	8	0	0	12	11	10	9	54	44	39	32	22	8	0	0	12	0	0	0	0	0	0	0	25	8	0	0
1888	8	14	8	0	0	15	11	10	8	50	42	35	26	18	0	0	0	12	0	0	0	0	0	0	0	23	8	0	0
1906	8	11	8	0	0	13	12	11	9	54	43	40	33	16	8	0	0	9	0	0	0	0	0	0	0	23	8	0	0
1107	9	22	8	0	0	13	13	11	9	56	45	41	36	20	8	0	0	36	15	0	0	0	0	0	0	24	8	0	0
1108	12	11	8	0	0	14	12	11	9	55	44	38	31	22	8	0	0	34	14	0	0	0	0	0	0	23	8	0	0
1887	13	15	8	0	0	15	14	13	11	54	46	39	29	20	8	0	0	38	14	0	0	0	0	0	0	21	8	0	0
1882	20	14	9	0	0	12	11	10	8	52	46	43	33	16	0	0	0	29	0	0	0	0	0	0	0	22	9	0	0
1843	23	11	0	0	0	14	13	12	10	53	48	39	34	19	8	0	0	33	19	0	0	0	0	0	0	24	8	0	0
1890	42	13	9	0	0	15	12	12	10	54	47	38	32	18	8	0	0	28	17	0	0	0	0	0	0	23	8	0	0
1881	64	10	8	0	0	14	11	10	8	53	47	40	34	17	8	0	0	18	0	0	0	0	0	0	0	22	8	0	0
1992	136	15	8	0	0	12	11	9	9	57	48	43	37	19	0	0	0	33	0	0	0	0	0	0	0	24	9	0	0
1889	189	14	8	0	0	14	13	12	10	56	48	42	36	20	0	0	0	32	0	0	0	0	0	0	0	28	10	0	0
1845	233	11	8	0	0	11	10	10	8	54	49	41	36	21	0	0	0	36	18	0	0	0	0	0	0	20	9	0	0
1847	245	18	8	0	0	13	11	10	8	52	45	38	30	20	8	0	0	10	0	0	0	0	0	0	0	29	11	0	0
1884	263	11	0	0	0	14	12	11	8	55	46	39	30	17	0	0	0	31	0	0	0	0	0	0	0	22	8	0	0
1990	264	21	9	8	0	13	13	11	9	55	48	37	31	21	8	0	0	13	13	0	0	0	0	0	0	21	9	0	0
1844	405	14	0	0	0	11	11	10	9	55	48	40	35	19	0	0	0	36	12	0	0	0	0	0	0	23	9	0	0
1885	406	16	8	0	0	13	9	8	8	49	43	34	25	15	0	0	0	30	0	0	0	0	0	0	0	26	8	0	0
2027	406	13	8	0	0	13	11	10	8	53	46	35	30	17	0	0	0	36	14	0	0	0	0	0	0	22	8	0	0

The resistance of *Cronobacter* strains to 7 different concentration of heavy metal including; nickel chloride, zinc sulphate, copper (II) sulphate, silver nitrate, sodium tellurite, cadmium carbonate and cobalt(II) nitrate were studied. Each isolate was exposed to four different concentrations of 1M, 0.1M, 0.01M and 0.001M of each heavy metal. The diameters of zones of inhibition were measured and interpreted according to the diameters of zones of inhibition. Experiments were assayed in three independent assays.

Table 5.2**.** Heavy metal sensitivity in *C. malonaticus, C. muytjensii, C. turicensis, C. universalis and C. dublinensis* strains and the single *C. condimenti* strain.

Strain	Species	ST	Copper (II) sulphate				Silver nitrate				Sodium tellurite				Nickel chloride				Zinc sulphate				Cadmium carbonate				Cobalt(II) nitrate			
			1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001	1M	0.1	0.01	0.001
101		60	15	7	0	0	12	11	9	0	58	48	39	36	20	0	0	0	27	0	0	0	0	0	0	0	24	7	0	0
93		29	16	8	0	0	11	11	10	0	54	46	37	33	21	0	0	0	25	0	0	0	0	0	0	0	23	7	0	0
510		7	14	9	0	0	12	11	10	0	53	48	44	38	20	9	0	0	27	0	0	0	0	0	0	0	24	10	0	0
1846	*C. malonaticus*	60	15	9	0	0	13	12	11	11	55	50	47	39	20	8	0	0	25	0	0	0	0	0	0	0	25	11	0	0
1369		69	15	8	0	0	13	12	11	10	56	46	40	30	18	7	0	0	26	0	0	0	0	0	0	0	22	9	0	0
1879		139	16	8	0	0	12	11	11	9	57	51	48	43	22	9	0	0	22	0	0	0	0	0	0	0	24	10	0	0
1893		289	17	7	0	0	14	12	11	9	57	47	36	33	18	0	0	0	44	30	0	0	0	0	0	0	24	8	0	0
1210		106	16	8	0	0	16	15	14	11	56	47	40	32	20	8	8	0	31	0	0	0	0	0	0	0	22	9	0	0
1458		74	15	8	0	0	13	13	13	12	55	45	38	31	13	7	0	0	30	19	0	0	0	0	0	0	22	7	0	0
1460		76	15	8	0	0	15	13	12	11	56	48	39	32	20	8	0	0	48	30	0	0	9	7	0	0	21	9	0	0
1461	*C. dublinensis*	77	15	8	7	0	14	13	12	12	58	49	40	35	20	8	7	0	9	0	0	0	9	8	0	0	20	9	0	0
1462		70	14	8	7	0	15	15	14	13	57	48	39	34	19	8	8	0	48	38	0	0	13	11	0	0	22	9	0	0
1463		95	21	9	8	0	14	14	13	12	58	51	42	39	19	8	8	0	28	10	0	0	0	0	0	0	26	13	0	0
1464		78	22	9	8	0	15	13	12	11	58	49	41	38	18	8	8	0	43	12	0	0	0	0	0	0	28	14	0	0
1897		213	18	8	7	0	14	12	12	11	57	47	42	38	18	8	7	0	44	14	0	0	0	0	0	0	25	12	0	0
1883		137	14	7	0	0	14	13	12	9	58	50	42	38	19	7	0	0	41	12	0	0	10	8	0	0	25	10	0	0
96	*C. universalis*	48	21	8	0	0	12	11	11	9	54	48	41	37	18	7	0	0	37	18	0	0	10	0	0	0	22	0	0	0
1435		51	20	7	0	0	15	13	12	10	52	47	40	37	18	8	0	0	29	16	0	0	0	0	0	0	21	9	0	0
1330	*C. condimenti*	98	12	7	0	0	13	12	10	7	58	49	44	36	13	0	0	0	40	24	0	0	15	10	0	0	26	8	0	0
92		35	22	7	0	0	14	13	11	10	55	44	40	36	18	7	0	0	28	0	0	0	0	0	0	0	24	9	0	0
109		5	16	7	0	0	13	13	12	0	58	48	46	38	20	7	0	0	7	0	0	0	0	0	0	0	24	8	0	0
111	*C. turicensis*	24	14	7	0	0	13	12	11	9	57	48	44	38	22	7	0	0	30	0	0	0	0	0	0	0	23	8	0	0
1880		344	14	7	0	0	13	12	12	10	59	49	47	40	21	7	0	0	26	0	0	0	0	0	0	0	29	9	0	0
1553		72	14	7	0	0	13	12	12	10	60	50	47	41	17	7	0	0	33	0	0	0	13	11	0	0	30	25	0	0
1895		252	15	7	0	0	12	11	10	9	58	48	37	34	19	7	0	0	12	0	0	0	0	0	0	0	13	8	0	0
16		34	16	8	0	0	14	12	10	0	59	50	41	37	17	0	0	0	48	26	0	0	10	9	0	0	21	7	0	0
1371	*C. muytjensii*	71	20	8	0	0	13	13	12	10	58	50	48	43	16	0	0	0	48	32	9	0	8	0	0	0	27	14	0	0
1527		75	16	8	0	0	12	12	11	9	59	53	49	46	20	10	0	0	19	0	0	0	12	9	0	0	23	9	0	0
1877		288	19	8	0	0	13	12	10	9	54	49	44	38	20	0	0	0	41	24	0	0	11	0	0	0	25	10	0	0

The resistance of *Cronobacter* strains to 7 different concentration of heavy metal including; nickel chloride, zinc sulphate, copper (II) sulphate, silver nitrate, sodium tellurite, cadmium carbonate and cobalt(II) nitrate were studied. Each isolate was exposed to four different concentrations of 1M, 0.1M, 0.01M and 0.001M of each heavy metal. The diameters of zones of inhibition were measured and interpreted according to the diameters of zones of inhibition. Experiments were assayed in three independent assays.

5.3.1.1 Copper and silver resistance genes.

Copper and silver are important for bacterial survival and fitness. However, in inceased concentrations, it is toxic for all living bacteria (Bonderczuk et al. 2013; Randall et al. 2015). Copper and silver resistance associated regions; (*cusABCFR*/*silABCER*) and (*pcoABCDR)* have been reported in *C. sakazakii* (Kucerova et al. 2010; Joseph et al.2012b; Yan et al. 2013). The genomes of 41 *Cronobacter* strains were screened for the presence of these two regions using BLAST tools. The genomic analysis shown that two copper and silver resistance associated regions (*cusABCFR/silABCER* and *pcoABCDR*) were absent in the majority of strains, except two *C. malonaticus* strains 510 (ST7) and 101 (ST60), two *C. turicensis* strains 1553 (ST72) and 1895 (ST252), one *C. muytjensii* strain 1877 (ST288), and one *C. dublinensis* strain 1463 (ST95), in addition to *C. sakazakii* strains belong to (ST4, ST8 and ST64) which are associated with neonatal infection and powder infant formula, ST8 strains (1283, 1888 and 1906), three ST4 strains (377, 1105 and 1886), 1881 (ST64) and 1847 ST245 (isolated from milk powder) (Table 5.3 and 5.4). The presence of the copper/silver resistance regions from the significant STs suggest that these regions may be essential for the virulence of *Cronobacter* in general or *C. sakazakii* strains in ST4, ST8, ST64, and *C. malonaticus* (ST7) strains in particular.

5.3.1.2 Nickel and cobalt resistance gene, *rcnA* (*yohM*).

Nickel and cobalt are required as trace elements in bacterial metabolism. Nonetheless, high intracellular concentrations of these two metals are toxic. One of the strategies evolved by bacteria to prevent damage is to export excess metal by efflux systems (Rodrigue et al. 2005). The inactivation of the *yohM* gene induces sensitivity to nickel and cobalt to *E. coli* cells. Therefore, they proposed the new de-nomination for the *yohM* gene that is *rcnA* (Rodrigue et al. 2005). The results showed that all of the strains were able to resist nickel chloride and cobalt (II) nitrate at low concentrations (0.01 and 0.001M). While, most the strains were sensitive to cobalt (II) nitrate at high concentrations (1M and 0.1M). However, *rcnA* gene was absent in all *Cronobacter* studied strains (Table 5.3 and 5.4).

5.3.1.3 Tellurite resistance genes (*terACDYZ*) (*tehB*).

Tellurite resistance genes including homologues of tellurite cluster gene (*terACDYZ*) was located in the loci ESA_01775–ESA_01804 of *C. sakazakii* BAA-894 (Joseph et al. 2012). Moreover, tellurite resistance-encoding gene *tehB*, matches a 593 bp hypothetical protein in *C. sakazakii* ATCC BAA-894 (Yan et al. 2013). All strains were sensitive to sodium tellurite. The genome study presented that the tellurite resistance genes (*terACDYZ*) were absent in all *Cronobacter* strains studied. However, *tehB* gene was found in all strains (Table 5.3 and 5.4).

5.3.1.4 Zinc and cadmium resistance genes (*cadAR/zntAR/znuSBC*).

The *CadA* gene is responsible for conferring resistance to cadmium and zinc. *cadR* is a gene in *Pseudomonas aeruginosa* similar to *zntA* in *E. coli* and encodes the transcriptional regulatory protein, CadR *(*Lee et al. 2001). These genes were found in all *C. sakazakii* strains, except strain 1882 (ST20-O:3). *C. muytjensii*, *C. malonaticus* strains and *C. universalis* strain 1883 (ST48) showed the presence of these genes. While, *C. turicensis, C. dublinensis* strains*, C. universalis* strain 96 *(*ST137*)* and the single *C. condimenti* strain showed the absence of these genes (Table 5.3 and 5.4).

Table 5.3. Presence/absence of heavy metal genes in sequenced genomes of *C.sakazakii* strains.

NTU	Source	ST	O-antigen	Copper and silver resistance genes			Nickel and cobalt resistance gene	Tellurite resistance genes	Zinc and cadmium resistance genes	
				cusABCFR	*silABCER*	*pcoABCDR*	*rcnA (yohM)*	*terACDYZ*	*cadAR/ zntAR*	*znuSBC*
377	Food	4	Csak O:2	+	+	+	-	-	+	+
1105	Weaning food	4	Csak O:2	+	+	+	-	-	+	+
1886	Spice	4	Csak O:3	+	+	+	-	-	+	+
1908	Environment	4	Csak O:4	-	-	-	-	-	+	+
1283	Food	8	Csak O:3	+	+	+	-	-	+	+
1888	Food	8	Csak O:1	+	+	+	-	-	+	+
1906	Food	8	Csak O:1	+	+	+	-	-	+	+
1107	Weaning food	9	Csak O:2	-	-	-	-	-	+	+
1108	Weaning food	12	Csak O:4	-	-	-	-	-	+	+
1887	Food	13	Csak O:2	-	-	-	-	-	+	+
1882	Ingredient	20	Csak O:3	-	-	-	-	-	-	-
1843	Spice	23	Csak O:2	-	-	-	-	-	+	+
1890	Ingredient	42	Csak O:2	-	-	-	-	-	+	+
1881	Ingredient	64	Csak O:2	+	+	+	-	-	+	+
1844	Ingredient	244	Csak O:3	-	-	-	-	-	+	+
1845	Food	233	Csak O:2	-	-	-	-	-	+	+
1847	Milk powder	245	Csak O:1	+	+	+	-	-	+	+
1884	Herb	263	Csak O:2	-	-	-	-	-	+	+
1889	Ingredient	198	Csak O:4	-	-	-	-	-	+	+
1992	Food	136	Csak O:2	-	-	-	-	-	+	+
1885	Herb	406	Csak O:2	-	-	-	-	-	+	+
1990	Food	264	Csak O:2	-	-	-	-	-	+	+
2027	Ingredient	406	Csak O:2	-	-	-	-	-	+	+

The Presence/absence of heavy metal genes in *C. sakazakii* strains were screened using BLAST tools. + = gene presence. - = gene absence

Table 5.4. Presence/absence of heavy metal genes in sequenced genomes of *C. universalis, C. turicensis, C. muytjensii, C. malonaticus, C. dublinensis* strains and the single *C. condimenti strain*.

NTU	Species	Source	ST	O-antigen	Copper and silver resistance genes			Nickel and cobalt resistance gene	Tellurite resistance genes	Zinc and cadmium resistance genes	
					cusABCFR	*silABCER*	*pcoABCDR*	*rcnA (yohM)*	*terACDYZ*	*cadAR/ zntAR*	*znuSBC*
96	*C. universalis*	Spice	48	ND	-	-	-	-	-	-	-
1883		Spice	137	ND	-	-	-	-	-	+	+
92	*C. turicensis*	Herb	35	Ctur O:3	-	-	-	-	-	-	-
1880		Herb	344	Ctur O:3	-	-	-	-	-	-	-
1553		Herb	72	Ctur O:3	+	+	+	-	-	-	-
1895		Ingredient	252	Ctur O:1	+	+	+	-	-	-	-
1371	*C. muytjensii*	Spice	403	Cmuyt O:3	-	-	-	-	-	+	+
1527		Food	411	Cmuyt O:2	-	-	-	-	-	+	+
16		Spice	347	Cmuyt O:1	-	-	-	-	-	+	+
1977		Ingredient	407	Cmuyt O:1	+	+	+	-	-	+	+
1879	*C. malonaticus*	Spice	139	Cmal O:2	-	-	-	-	-	+	+
1369		Herb	69	Cmal O:1	+	+	+	-	-	+	+
101		Spice	60	Cmal O:1	-	-	-	-	-	+	+
510		Food	7	Cmal O:2	+	+	+	-	-	+	+
1846		Ingredient	60	Cmal O:1	-	-	-	-	-	+	+
1210	*C. dublinensis*	Food	106	Cdub O:1	-	-	-	-	-	-	-
1458		Food	74	Cdub O:1	-	-	-	-	-	-	-
1460		Food	76	Cdub O:2	-	-	-	-	-	-	-
1463		Food	95	Cdub O:1	+	+	+	-	-	-	-
1461		Food	77	Cdub O:1	-	-	-	-	-	-	-
1462		Food	70	Cdub O:1	-	-	-	-	-	-	-
1897		Herb	213	Cdub O:2	-	-	-	-	-	-	-
1330	*C. condimenti*	Food	98	CuniO:1	-	-	-	-	-	-	-

The Presence/absence of heavy metal genes in other *Cronobacter* strains were screened using BLAST tools. + = gene presence. - = gene absence. .

5.3.2 Determination of sublethal injury to cells during the desiccation.

Fifty-four desiccated *Cronobacter* strains, including twenty-five *C. sakazakii,* seven *C. malonaticus,* eight *C. dublinensis,* three *C. universalis*, six *C. turicensis* and three *C. muytjensii* strains, and a single *C. condimenti* strain were recovered after overnight incubation at room temperature on selective (VRBGA) and nonselective (TSA) media. Furthermore, all of the strains showed greater recovery on TSA than on VRBGA (Table 5.3 and 5.6). The differences in recovery after desiccation reflect the number of cells that were sublethally injured during the desiccation procedure.

There was significant difference in the recovery of *Cronobacter* spp. between TSA and VRBGA (Table 5.5 and 5.6). There were significantly fewer sublethally injured cells generated for high capsulated than for non-capsulated and low capsulated strains of *C. sakazakii*: 6.5% to 18.4%, 18.2% to 36.4% differences, respectively. *C. sakazakii* strains in serotype O:1 and O:4 had high numbers of sublethally injured cells; 18.2% to 36.4%, 25% to 33.2%, respectively. While, strains with serotype O:2 and O:3 showed low numbers of sublethally injured cells; 7% to 15.6%, 6.5% to 18.36%, respectively. Strains, 1887 (ST13-O:2), 1890 (ST42-O:2) 1845 (ST233-O:2), 1107 (ST9-O:2), 1843 (ST23-O:2), 1990 (ST264-O:2), 377 (ST4-O:2), and 1283 (ST8-O:3) showed better recovery on selective and non-selective media (TSA and VRBGA) suggesting higher resistance to desiccation than the other strains (Figure 5.1). However, *C. sakazakii* strains, 1906 (ST8-O:1), 1888 (ST8-O:1), 1847 (ST245-O:1), 1108 (ST12-O:4), 1907 (ST4-O:4), 1908 (ST4-O:4) and 1889 (ST198-O:4), showed highly reduced cell counts (sensitive to desiccation).

C. malonaticus sublethally injured cells were generated upon desiccation, the difference between the percentage of sublethally injured cells on TSA and VRBGA ranged from 7.72% to 40.86 % between *C. malonaticus* strains, the lowest sublethally injured cells was noted in strains in ST60; 101(10.86%) and 1846 (7.72%). The other *Cronobacter* spp. strains were also injured during desiccation. The lowest sublethal injury cells (0.83% to 2.48%) were obtained for *C. universalis* strain 96 (ST48), *C. muytjensii* strain 16 (ST34), and *C. dublinensis* strains 1460 (ST76) and 1464 (ST78). Whereas, the higher sublethal injury cells (33% to 40% differences) were obtained for *C. turicensis* strain 111 (ST24), *C. muytjensii* strain 1527 (ST75), *C. malonaticus* strains

1369 (ST69), 510 (ST7), 93 (ST29), 1879 (139) and the single *C. condimenti* strain 1330 (ST98). All of the strains were capsulated. No obvious correlation between the sublethally injured cells during the desiccation and the genetic diversity including sequence type (ST) and serotype (O-antigen) was observed in other *Cronobacter* species (Table 5.3, 5.6, and Figure 5.1).

Table 5.5. Comparison of viable counts percentage for *C. sakazakii* strains on TSA and VRBGA after desiccation and then reconstitution in infant formula.

Strains	Sources	ST	O-antigen	Capsule formation	TSA (%)	VRBGA (%)	Sublethal injury (%)
1906	Environment	8	O:1	+	2.36	0.86	36.44
1888	Food	8	O:1	-	4.97	1.01	21.96
1847	Milk powder	245	O:1	++	3.79	0.69	18.20
1907	Environment	4	O:4	+	4.33	1.44	33.25
1908	Environment	4	O:4	+	5.18	1.92	29.34
1108	Weaning food	12	O:4	+	3.94	1.14	28.93
1889	Ingredient	198	O:4	+	5.23	1.31	25.04
1564	Food	4	O:3	+++	7.77	0.51	6.56
1886	Spice	4	O:3	+++	8.98	0.78	8.68
1283	Food	8	O:3	+++	12.14	2.23	18.36
1882	Ingredient	20	O:3	+++	9.32	1.58	16.95
1844	Ingredient	405	O:3	+++	5.89	0.63	10.69
1105	Weaning food	4	O:2	+++	9.761	1.11	11.37
377	Food	4	O:2	+++	10.12	1.34	13.24
1107	Weaning food	9	O:2	+++	11.39	1.96	17.2
1887	Food	13	O:2	+++	26.86	4.3	15.4
1843	Ingredient	23	O:2	+++	10.9	0.76	7
1890	Ingredient	42	O:2	+++	15.66	1.5	9.57
1881	Ingredient	64	O:2	+++	7.59	0.87	11.46
1992	Food	136	O:2	+++	8.88	1.06	11.93
1845	Food	233	O:2	+++	15.33	1.21	7.89
1884	Herb	263	O:2	+++	7.69	0.57	7.41
1990	Food	264	O:2	+++	10.42	1.12	10.74
1885	Herb	406	O:2	+++	9.22	0.98	10.62
2027	Ingredient	406	O:2	+++	11.97	1.87	15.62

+++= high mucoid production. ++= moderate mucoid production. += low mucoid production. -= no mucoid production

Table 5.6. Comparison of viable counts percentage for *C. universalis, C. turicensis, C. muytjensii, C. condimenti, C. malonaticus, C. dublinensis* strains on TSA and VRBGA after desiccation and then reconstitution in infant formula.

Strains	Species	Source	ST	Serotype	TSA (%)	VRBGA (%)	Sublethal injury (%)
96	*C. universalis*	Spice	48	ND	18.79	0.15	0.83
1435		Food	51	ND	15.15	2.24	14.66
1883		Spice	137	ND	15.66	4.33	27.71
109	*C. turicensis*	Herb	5	ND	10.69	1.05	9.34
111		Herb	24	ND	21.05	6.97	33.12
1553		Herb	72	Cturi O:3	3.22	0.37	11.62
92		Herb	35	Cturi O:3	7.76	0.32	4.125
1880		Herb	127	Cturi O:3	5.52	1.21	22.12
1895		Ingredient	252	Cturi O:1	16.96	2.72	16.07
1527	*C. muytjensii*	Food	75	CmuyO:1	28.25	10.04	35.39
1977		Ingredient	288	CmuyO:1	76.66	8.69	11.34
1371		Spice	71	CmuyO:2	64.28	7.82	12.17
16		Spice	34	CmuyO:3	30.54	0.58	1.92
93	*C. malonaticus*	Spice	29	CmalO:1	31.03	12.66	40.86
101		Spice	60	CmalO:1	4.621	0.52	10.86
1369		Herb	69	CmalO:1	42.07	15.09	35.87
1846		Ingredient	60	CmalO:1	40.93	3.16	7.72
1893		Ingredient	289	CmalO:1	38.57	10.14	25.92
510		Food	7	CmalO:2	18.41	6.82	37.06
1879		Spice	139	CmalO:2	6.18	2.23	36.09
1210	*C. dublinensis*	Environment	106	CdubO:1	14.65	1.025	6.82
1458		Food	74	CdubO:1	7.52	1.75	23.33
1460		Food	76	CdubO:2	28.91	0.71	2.48
1463		Food	95	CdubO:1	12.04	2.12	17.70
1461		Food	77	CdubO:1	16.97	1.46	8.63
1462		Food	70	CdubO:1	7.76	1.61	20.75
1464		Food	78	CdubO:2	21.42	0.31	1.48
1897		Herb	290	CdubO:2	10.71	1.53	14.33
1330	*C. condimenti*	Food	98	CuniO:1	13.66	5.55	40.65

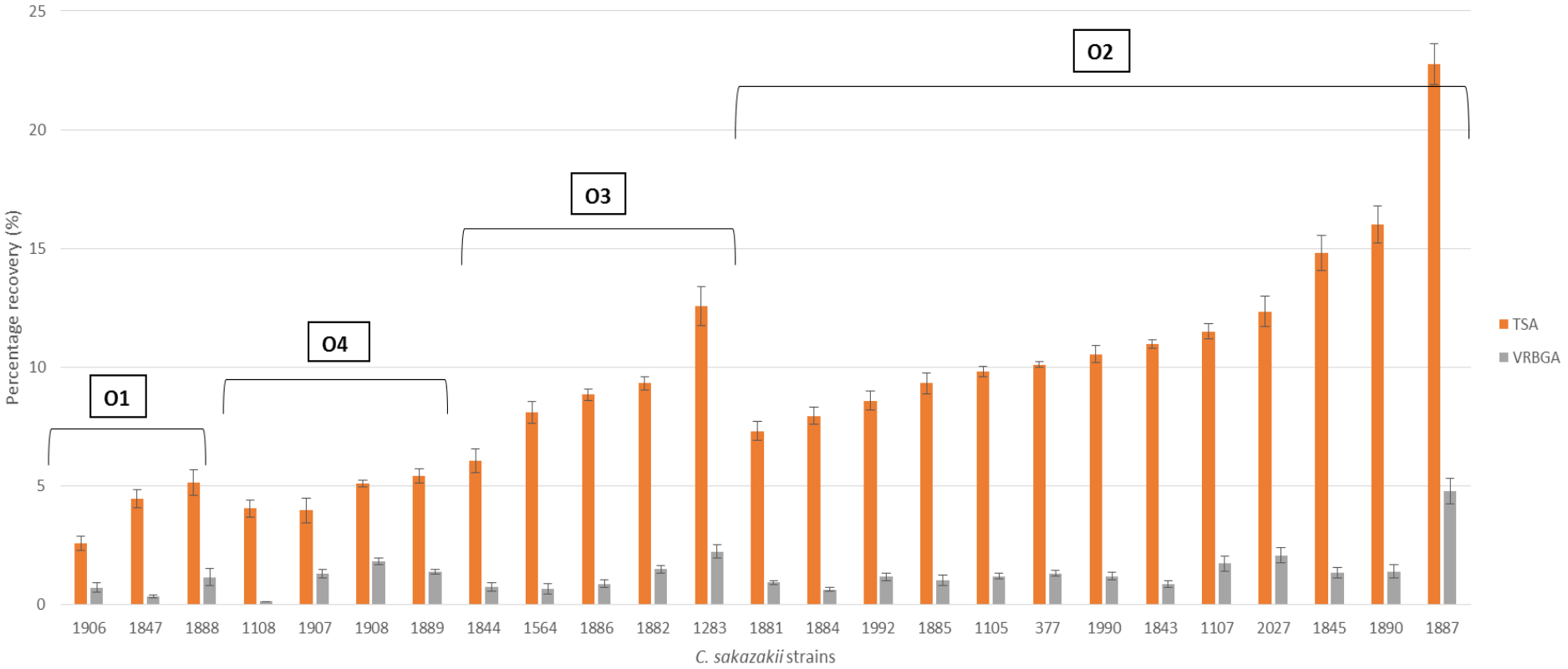


Figure 5.1. The correlation between the sublethally injured cells during the desiccation and type of serotype (O-antigen).
Determination of sublethal injury to cells during the desiccation experiments were assayed in three independent assays. Error bars represented using standard error

5.3.3 Resistance of *Cronobacter* species to drying (3 months).

The ability of the *Cronobacter* strains to withstand long-term drying (90 days) was tested according to the procedure described by Breeuweret al (2003). In brief, fifty-microliter aliquots of overnight culture in sterile liquid infant formula (Cow & Gate Premium 1) were placed in 12-well sterile polystyrene tissue culture plates and allowed to air dry in a 30°C incubator. The original culture was enumerated and reported as CFU ml^{-1} on day 0. At intervals up to 90 days, the inoculum dried in the incubator was reconstituted in 1 ml of sterile peptone water, and appropriate dilutions were plated on TSA to determine the number of cell survivors.

5.3.3.1 Survival of *C. sakazakii* strains after the exposure to drying.

Desiccation of *C. sakazakii* (n=25) resulted in an initial 3-log decrease in viability from approximately 10^{11} to 10^{8} CFU/ml. Subsequently, for up to 90 days, there was a slow decline in viability (Figure. 5.2). There was variation in the viability between *C. sakazakii* strains in terms of resistance to drying (90 days). Strains 1105 (ST4), 1564 (ST4), 1886 (ST4) and 1906 (ST8) lost viability faster than did the other *C. sakazakii* strains, and were no longer detectable after 60 days. Strains 377 (ST4), 1907 (ST4), 1908 (ST4), 1283 (ST8), 1888 (ST8), 1990 (ST264), 2027 (ST406), 1843 (ST23), 1844 (ST244), 1845 (ST233), 1847 (ST245), 1881 (ST64) and 1890 (ST42) were no longer detectable after 90 days. However, strains 1107 (ST9), 1108 (ST12), 1884 (ST263), 1885 (ST406), 1889 (ST198) and 1887 (ST13) were able to survive after 90 days.

In general, *C. sakazakii* strains in ST4 were more sensitive to drying than other *C. sakazakii* STs. *C. sakazakii* ST64 and ST8 are isolated from PIF and the environment of manufacturing plants in many countries around the world, theses strains showed moderate recovery after the exposure to drying (90 days). In contrast, strains 1887 (ST13), 1890 (ST42), 1108 (ST12), 1107 (ST9), 1884 (ST263) and 1885 (ST406), showed better recovery after the exposure to drying (90 days) suggesting higher resistance to draying than the other *C. sakazakii* strains (Figure 5.2).

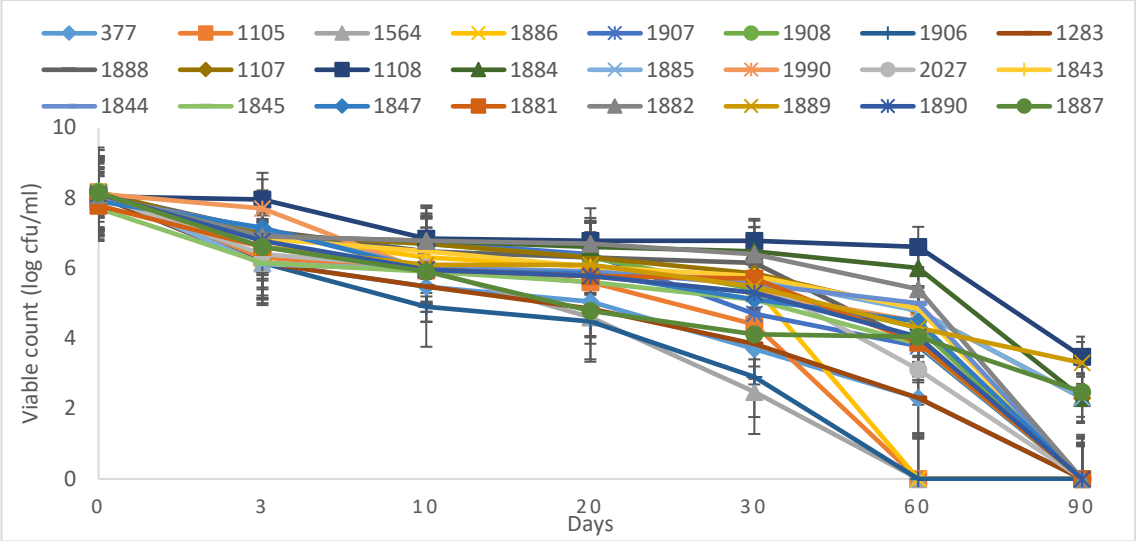


Figure 5.2. Resistance of *C. sakazakii* strains to drying. The survival was measured at 0, 3, 1 0, 20, 30, 60 and 90 days.
Recovery was measured on TSA at 37°C. The data are presented as means ± standard deviation of triplicate counts of at least two independent experiments. The initial inoculum was ~10^{10} CFU/ml for the desiccated culture.

5.3.3.2 Survival of *C. malonaticus* and *C. dublinensis* strains after the exposure to drying.

The viability of the seven *C. malonaticus* strains decreased similarly during the 30 days of storage (Figure.5 .3). All of these strains (93, 101, 510, 1369, 1846, 1879 and 1893) were no longer detectable after 60 days. *C. dublinensis* strains were divided into 2 groups based on their resistance to drying, the first group (1460, 1461, and 1462) were detectable after 60 days. Whereas, the second group (1210, 1458, 1463, 1464 and 1879) were no longer detectable after 60 days (Figure5.3).

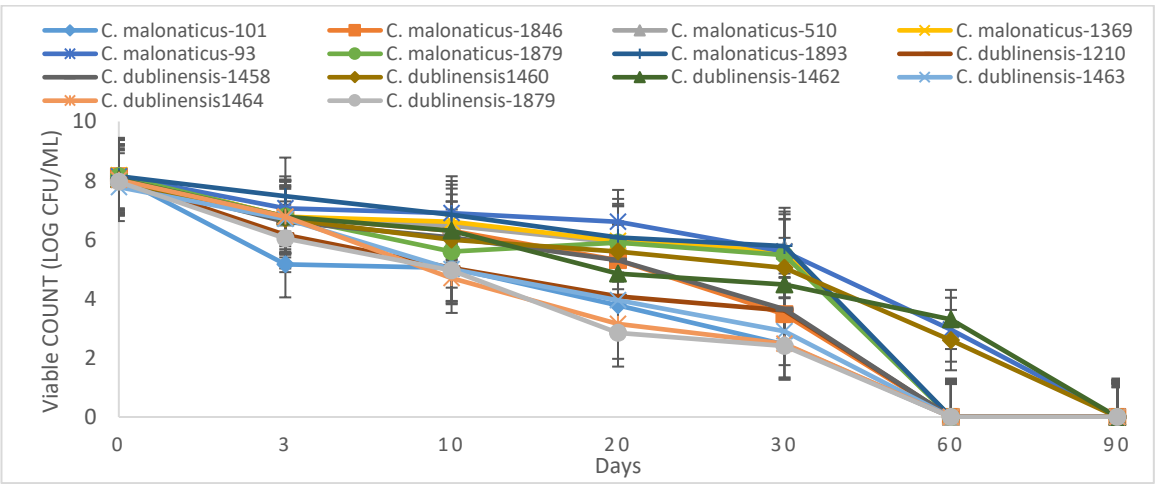


Figure 5.3. Survival of *C. malonaticus* and *C. dublinensis* strains after the exposure to drying. The survival was measured at 0, 3, 1 0, 20, 30, 60 and 90 days.
Recovery was measured on TSA at 37°C. The data are presented as means ± standard deviation of triplicate counts of at least two independent experiments. The initial inoculum was ~10^{10} CFU/ml for the desiccated culture.

5.3.3.3 Survival of *C. universalis*, *C. turicensis*, *C. muytjensii* strains and single *C. condimenti* strain after the exposure to drying.

C. turicensis strains had a low survival rate after the exposure to drying. Most *C. turicensis* strains (92, 109, 111, 1553 and 1880) were no longer detectable after 20 days, but strain 1895 was able to survive until 60 days. Moreover, a *C. universalis* strains (96, 1435 and 1883) were no longer detectable after 60 days, the single *C. condimenti* strain was also no longer detectable after 60 days. However, *C. muytjensii* strains (16, 1371, 1527 and 1877) were no detectable after 90 days (Figure 5.4).

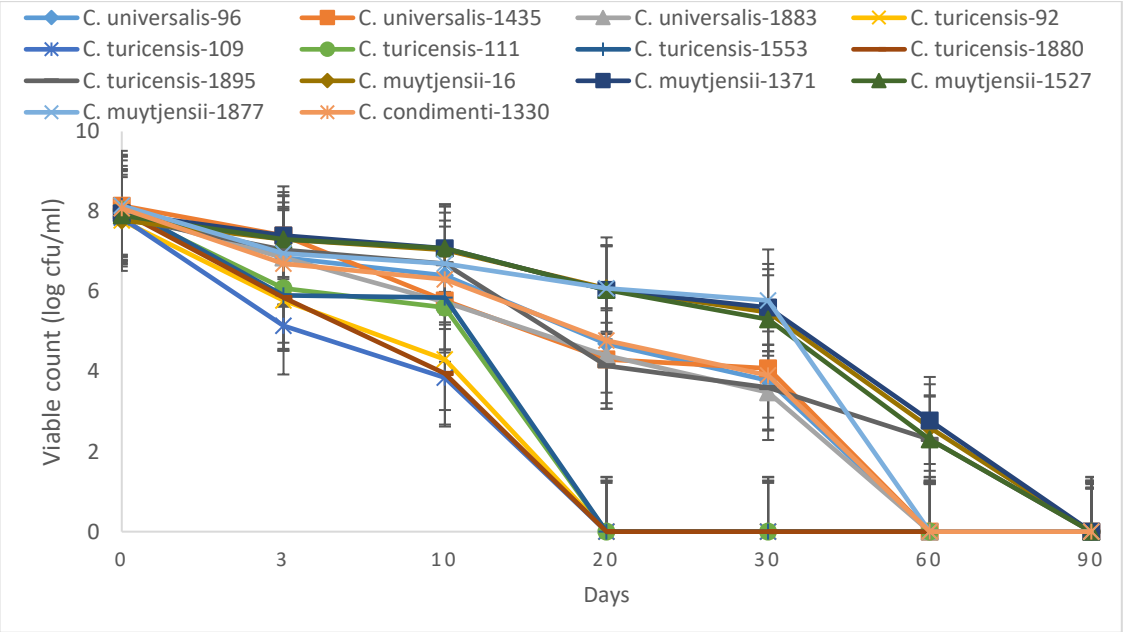


Figure 5.4. Resistance of *C. universalis, C. turicensis, C. muytjensii* strains, and the single *C. condimenti* strain to drying. The survival was measured at 0, 3, 1 0, 20, 30, 60 and 90 days. Recovery was measured on TSA at 37°C. The data are presented as means ± standard deviation of triplicate counts of at least two independent experiments. The initial inoculum was ~10^{10} CFU/ml for the desiccated culture.

Overall, *C. sakazakii* strains showed a high survival rate after the exposure to drying (90 days) compared with other *Cronobacter* species. However, the lowest survival rate after the exposure to drying was noted by *C. turicensis* strains (no longer detectable after 20 days).

5.3.3.4 Desiccation-related genes.

Many genes linked to desiccation and osmotic stress conditions were identified in the genome of *C. sakazakii* SP291 (Yan et al. 2013), which included the *yihUTRSQVO* genes; this gene cluster plays roles in metabolism and in the transport of carbohydrates and

glucuronide, and *yehZYXW*, which play roles in bacterial survival. Many other genes associated with the osmotic stress response were identified, including the osmotolerance regulation genes *yiaD, osmY, ompA, aqpZ*, and *glpF* and osmotic stress genes including, *osmBO*, *yciTM*, and *pgpB (*Grim et al. 2013; Yan et al. 2013). A recent study by Alvarez-Ordonez and Hill (2016) reported that in *C. sakazakii rpoS* activity confers protection against a range of stress conditions, including acid, alkaline, osmotic and oxidative stress (Alvarez-Ordonez et al. 2012; Alvarez-Ordonez et al. 2013; Alvarez-Ordonez and Hill. 2016). Moreover, Lalaouna et al (2012) reported that the *mutS-rpoS* genomic region may contribute to survival in particular environments.

The genomes of 41 C*ronobacter* strains were screened for the presence of these genes using BLAST tools (Table 5.3). The results showed that most of strains had the Osmoprotectant ABC, transporter genes (*yehXWYZ*) and desiccation-related genes (*yihTQVWPAOS*), except *C. dublinensis* strains 1210 (ST106), 1458 (ST74), 1460 (ST76), 1461 (ST77), 1463 (ST95) and 1897(ST213). Osmotic stress related genes *osmBOY, yiaD, ompA, aqpZ, glpF, yciTM* and *pgpB* were found in the most of the strains, except *aqpZ* gene which was absent in *C. sakazakii* strains 1845 (ST233), 1847 (ST245), 1890 (ST42), and 1908 (ST4), *C. malonaticus* strains 101 (ST60), 1369 (ST69), 1879 (ST139) and 1893 (ST289) and *C. muytjensii* strains 16 (ST347), 1371 (ST403), 1527 (ST411) and 1877 (ST407). Moreover, the genomic analysis shown that one desiccation-related gene (*yihR*) was found in *C. sakazakii, C. turicensis* and *C. malonaticus* strains, and was absent in *C. universalis, C. muytjensii, C. dublinensis* strains and the single *C. condimenti* strain (Table 5.7 and 5.8).

Enteric bacteria contain different genes in the *mutS-rpoS* genomic region, this could contribute to virulence or survival (Lalaouna et al. 2012). This region was found in all *Cronobacter* strains, and contained the same seven genes between *mutS* and *rpoS* (Figure 5.5).

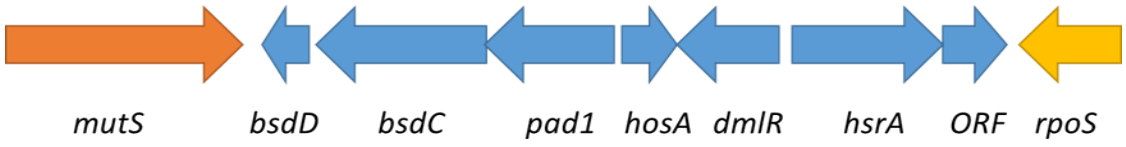


Figure 5.5. The *mutS-rpoS* genomic region in the genomes of 41 *Cronobacter* strains.

Table 5.7**.** The presence /absence of genes involved in desiccation survival in *C. sakazakii.*

NTU.	ST	Osmoprotectant ABC, transporter genes	Desiccation-related genes		Osmotic stress related genes								
		yehXWYZ	*yihTQVWPAOS*	*yihR*	*osmBOY*	*yiaD*	*ompA*	*aqpZ*	*glpF*	*yciTM*	*pgpB*	*rpoS*	*mutS*
377	4	+	+	+	+	+	+	+	+	+	+	+	+
1105	4	+	+	+	+	+	+	+	+	+	+	+	+
1886	4	+	+	+	+	+	+	+	+	+	+	+	+
1908	4	+	+	+	+	+	+	-	+	+	+	+	+
1283	8	+	+	+	+	+	+	+	+	+	+	+	+
1888	8	+	+	+	+	+	+	+	+	+	+	+	+
1906	8	+	+	+	+	+	+	+	+	+	+	+	+
1107	9	+	+	+	+	+	+	+	+	+	+	+	+
1108	12	+	+	+	+	+	+	+	+	+	+	+	+
1887	13	+	+	+	+	+	+	+	+	+	+	+	+
1882	20	+	+	+	+	+	+	+	+	+	+	+	+
1843	23	+	+	+	+	+	+	+	+	+	+	+	+
1890	42	+	+	+	+	+	+	-	+	+	+	+	+
1881	64	+	+	+	+	+	+	+	+	+	+	+	+
1844	244	+	+	+	+	+	+	+	+	+	+	+	+
1845	233	+	+	+	+	+	+	-	+	+	+	+	+
1847	245	+	+	+	+	+	+	-	+	+	+	+	+
1884	263	+	+	+	+	+	+	+	+	+	+	+	+
1889	198	+	+	+	+	+	+	+	+	+	+	+	+
1992	136	+	+	+	+	+	+	+	+	+	+	+	+
1885	406	+	+	+	+	+	+	+	+	+	+	+	+
1990	264	+	+	+	+	+	+	+	+	+	+	+	+
2027	406	+	+	+	+	+	+	+	+	+	+	+	+

The Presence/absence of desiccation-related and Osmotic stress related genes in *C. sakazakii* strains were screened using BLAST tools. + = gene presence. - = gene absenc

Table 5.8. The presence /absence of genes involved in desiccation survival in other *Cronobacter* species.

NTU	Species	ST	Osmoprotectant ABC, transporter genes	Desiccation-related genes		Osmotic stress related genes								
			yehXWYZ	*yihTQVWPAOS*	*yihR*	*osmBOY*	*yiaD*	*ompA*	*aqpZ*	*glpF*	*yciTM*	*pgpB*	*rpoS*	*mutS*
96	*C. universalis*	48	+	+	-	+	+	+	+	+	+	+	+	+
1883		137	+	+	-	+	+	+	+	+	+	+	+	+
92	*C. turicensis*	35	+	+	+	+	+	+	+	+	+	+	+	+
1880		344	+	+	+	+	+	+	+	+	+	+	+	+
1553		72	+	+	+	+	+	+	+	+	+	+	+	+
1895		252	+	+	+	+	+	+	+	+	+	+	+	+
1371	*C. muytjensii*	403	+	+	-	+	+	+	-	+	+	+	+	+
1527		411	+	+	-	+	+	+	-	+	+	+	+	+
16		347	+	+	-	+	+	+	-	+	+	+	+	+
1977		407	+	+	-	+	+	+	-	+	+	+	+	+
1879	*C. malonaticus*	139	+	+	+	+	+	+	-	+	+	+	+	+
1369		69	+	+	+	+	+	+	-	+	+	+	+	+
101		60	+	+	+	+	+	+	-	+	+	+	+	+
510		7	+	+	+	+	+	+	+	+	+	+	+	+
1846		60	+	+	+	+	+	+	+	+	+	+	+	+
1893		289	+	+	+	+	+	+	-	+	+	+	+	+
1210	*C. dublinensis*	106	+	-	-	+	+	+	+	+	+	+	+	+
1458		74	+	-	-	+	+	+	+	+	+	+	+	+
1460		76	+	-	-	+	+	+	+	+	+	+	+	+
1463		95	+	-	-	+	+	+	+	+	+	+	+	+
1461		77	+	-	-	+	+	+	+	+	+	+	+	+
1462		70	+	+	-	+	+	+	+	+	+	+	+	+
1897		213	+	-	-	+	+	+	+	+	+	+	+	+

1330	*C. condimenti*	98	+	+	-	+	+	+	+	+	+	+	+	+

+ = gene presence. - = gene absence,

5.3.4 Heat tolerance.

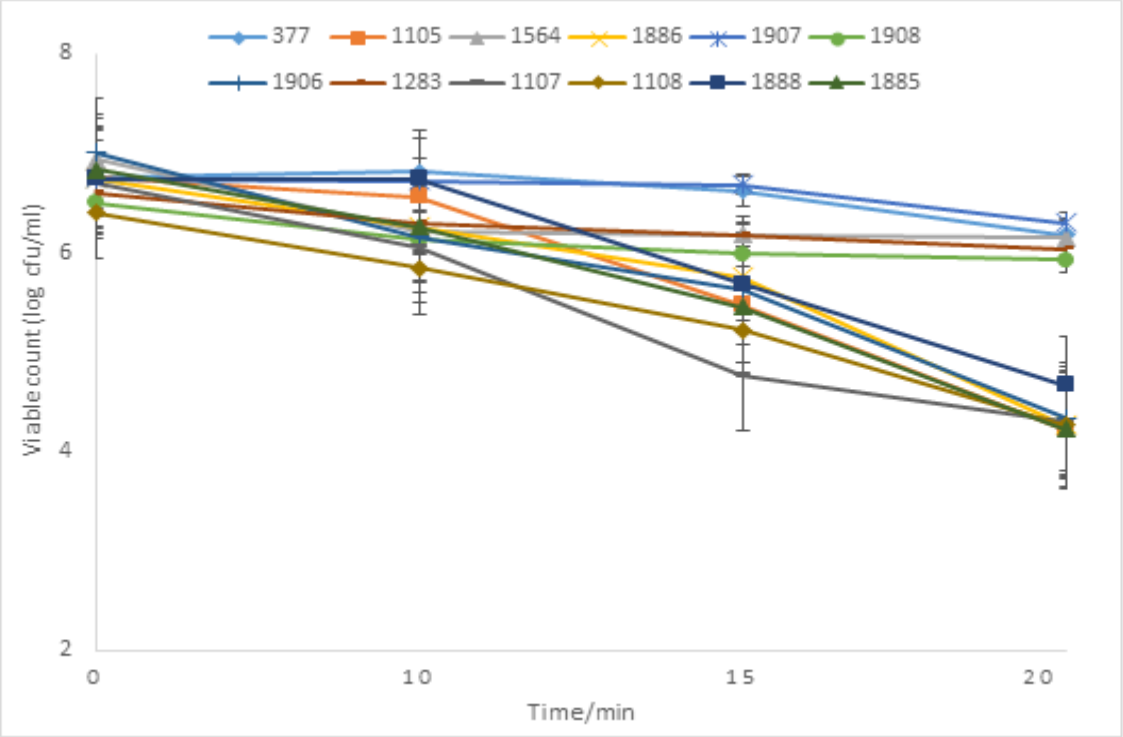
The heat tolerance of *Cronobacter* strains was determined as described by Iversen et al (2004). Briefly, 1 ml overnight culture was suspended in 20 ml of temperature equilibrated sterile liquid infant formula (Cow & Gate Premium 1) in water baths (58°C). At timed intervals, 100 µl aliquots were transferred to 2 ml TSB at room temperature and the number of survivors determined. The number of survivors at 58°C was plotted against time. The best fit-line was extrapolated and the decimal reduction time was determined (−1/slope of the regression line). Strains were classified into three groups, high heat resistance (D_{58} >25), moderate (10< D_{58}< 25), and heat sensitive (D_{58} < 10).

5.3.4.1 Survival of *C. sakazakii* strains in liquid infant formula at 58°C.

C. sakazakii strains 377, 1105, 1564, 1886, 1907 and 1908 (ST4), 1283 (ST8), 1843 (ST23), 1889 (ST198), 1845 (ST233), 1847 (245), 1890 (ST42), 1844 (ST405), 1990 (ST264) and 1887 (ST13) showed high heat resistance compared with other *C. sakazakii* STs. However, *C. sakazakii* strains; 1107 (ST9), 1108 (ST12), 1906 and 1884 (ST263) and 1885 (ST406) showed sensitivity to heat and subsequently had lower D_{58} ranged between 8 to 10 min (Table 5.9). Other *C. sakazakii* strains; 1888 (ST8), 1881 (ST64), 1882 (ST20), 1992 (ST136) and 2027 (ST406) showed moderate heat tolerance, the D-values was ranged from 10 to 25 min.

The *D*-values of *C. sakazakii* ST4 strains ranged from 25 to 50 min, heat resistance. As shown in table 5.9 *C. sakazakii* environmental strains 1907 and 1908 (ST4-O:4) were more heat resistant (58°C) than the other ST4 *C. sakazakii* strains. They had *D*-values ranging from 36 to 50 minutes at 58°C as presented in table 5.9. The variation in the viability of *C. sakazakii* ST8 strains; ST8 (O:1) and ST8 (O:3) isolates at 58°C was interesting. The *D*-values of *C. sakazakii* strains 1888 and 1906 (ST8-O:1) ranged from 8 to 13 min (heat sensitive), while *C. sakazakii* strain 1283 (ST8-O:3) showed high heat resistance and the viability decline was linear in liquid infant formula, with D_{58} = 35 minute (Table 5.9 and Figure 5.6 A, B).

A



B

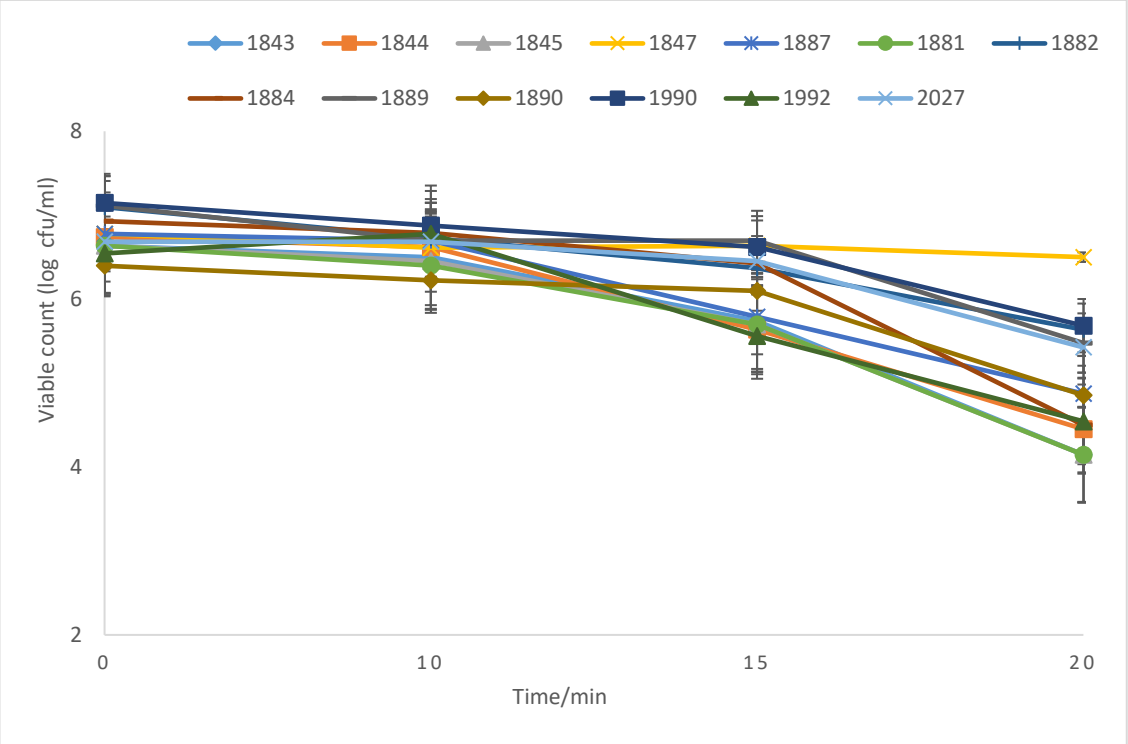


Figure 5.6. **A, B.** Survival of *C. sakazakii* strains in liquid infant formula after exposure to the heat at 58°C for 20 mintus.

Data are reported as the means ± standard deviation of the number of recovered cells in three independent experiments.

Table 5.9. D-value for *C. sakazakii* strains.

Strain	Source	ST	O-antigen	K-type	CA-type	D-value (min)	Category
377	Food	4	O:2	K2	CA2	34	R
1105	Weaning food	4	O:2	K2	CA2	25	R
1564	Food	4	O:3	K2	CA2	27	R
1886	Spice	4	O:3	K2	CA2	28	R
1907	Environment	4	O:4	K2	CA2	50	R
1908	Environment	4	O:4	K2	CA2	36	R
1906	Food	8	O:1	K1	CA1	8.18	S
1888	Food	8	O:1	K1	CA1	13	M
1283	Food	8	O:3	K1	CA1	35	R
1107	Weaning food	9	O:2	K2	CA2	8	S
1108	Weaning food	12	O:4	K2	CA2	10.2	S
1887	Food	13	O:2	K2	CA2	26	R
1882	Ingredient	20	O:3	K1	CA1	18	M
1843	Spice	23	O:2	K1	CA2	41	R
1890	Ingredient	42	O:2	K1	CA2	40	R
1881	Ingredient	64	O:2	K1	CA2	25	R
1992	Food	136	O:2	K2	CA2	21	M
1889	Ingredient	198	O:4	K1	CA2	30	R
1845	Food	233	O:2	K1	CA2	32	R
1847	Milk powder	245	O:1	K2	CA1	45	R
1884	Herb	263	O:2	K1	CA2	9.7	S
1990	Food	264	O:2	K1	CA2	41	R
1844	Ingredient	405	O:3	K2	CA1	42	R
1885	Herb	406	O:2	K1	CA2	10	S
2027	Ingredient	406	O:2	K1	CA2	24	M

The number of survivors at 58°C was plotted against time. The best fit-line was extrapolated and the decimal reduction time was determined (−1/slope of the regression line). *C. sakazakii* strains were classified into three groups, high heat resistance (D58 >25), moderate (10< D58< 25), and heat sensitive (D58 < 10).

R = resistance. M = moderate . S = sensitive

5.3.4.2 Survival of *C. malonaticus* strains in liquid infant formula at 58°C.

There was variation in thermotolerance between *C. malonaticus* strains. *C. malonaticus* strains were categorised into three groups based on their capability of heat resistance. Strain 510 (ST7-O:2) showed high thermoresistance in liquid infant formula with D_{58} = 45 minutes, while strain 1879 (ST139-O:2) showed moderate heat resistance with D_{58} = 15 minutes. In contrast, *C. malonaticus* strains 1369 (ST69-O:1), 1846 (ST60-O:1) and 1893 (ST289-O:1) showed heat sensitivity at 58°C in liquid infant formula. These strains had a *D*-value ranging from 9.2 to 10 minutes at 58°C (Figures 5.7 and Table 5.10).

5.3.4.3 Survival of *C. dublinensis, C. turicensis* and *C. muytjensii* strains and the single *C. condimenti* strain in liquid infant formula at 58°C.

C. dublinensis, *C. turicensis and C. muytjensii* strains were heat sensitive. They had D-values ranging from 3 (± 2) to 10 (± 2.1) minutes at 58°C as presented in figures 5.8 and table 5.10. Two of three *C. universalis* strains 96 (ST48) and 1883 (ST137) showed heat sensitivity, with D-value= 4.2, 6.5 min respectively. However, *C. universalis* strain 1435 (ST51) showed high thermotolerance (resistance), with *D*-value = 39 minute. The single *C. condimenti* strain 1330 (ST98) was also heat resistant at 58°C in liquid infant formula, *D*-value = 46 min (Figures 5.8 and Table 5.10).

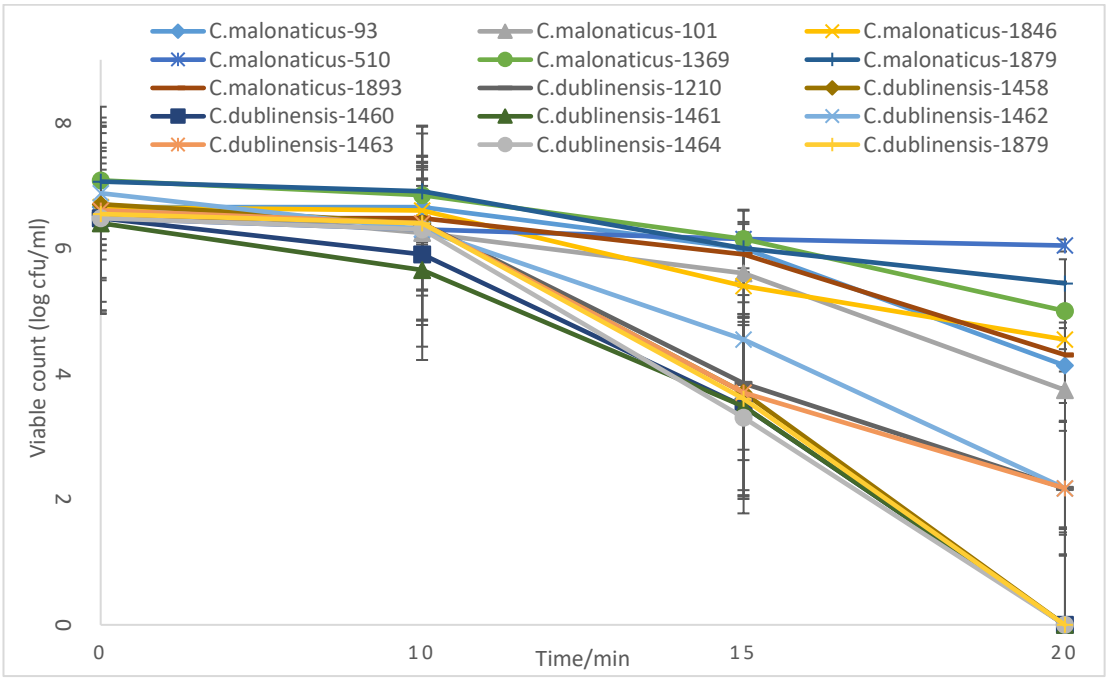


Figure 5.7**.** Survival of *C. malonaticus* and *C. dublinensis* strains in liquid infant formula after exposure to the heat at 58°C for 20 mintus.

Data are reported as the means ± standard deviation of the number of recovered cells in three independent experiments

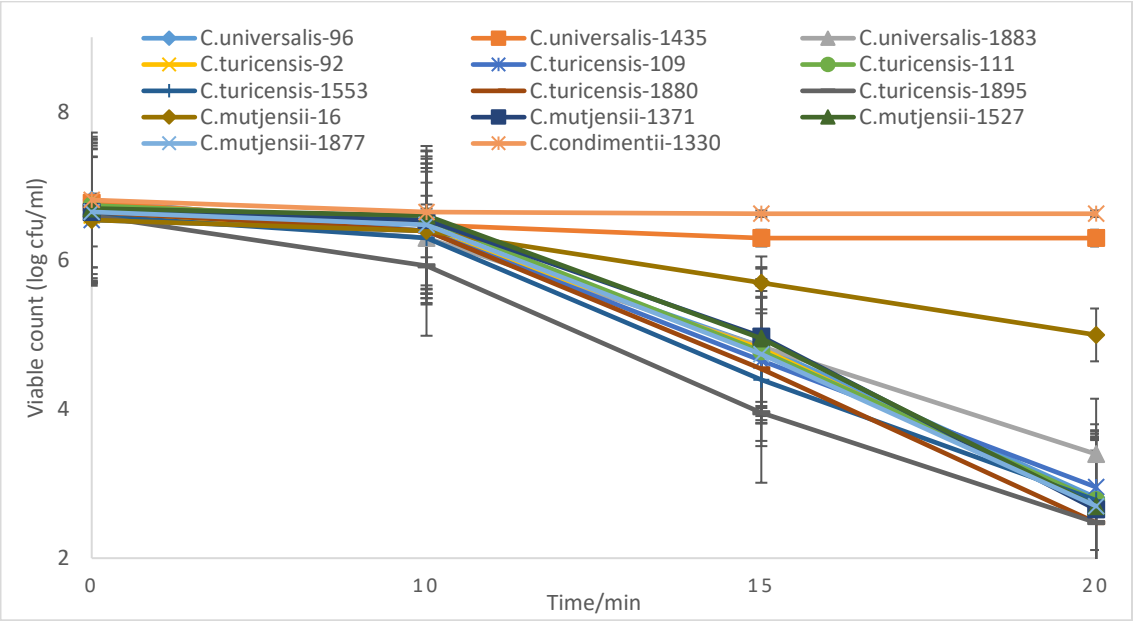


Figure 5.8. Survival of *C. turicensis*, *C. muytjensii* and *C. universalis* strains and the single *C. condimenti* strain in liquid infant formula after exposure to the heat at 58°C for 20 mintus at 58°C.

Data are reported as the means ± standard deviation of the number of recovered cells in three independent experiments.

Table 5.10. D-value for *C. malonaticus, C. dublinensis, C. universalis, C. turicensis, C. muytjensii* strains and the single *C. condimenti strain*.

strain	Species	Source	ST	O-antigen	K-type	CA-type	D-value (min)	Category
1369	*C. malonaticus*	Herb	69	O:1	K2	CA2	10	S
101		Spice	60	O:1	K2	CA2	9.5	S
1846		Ingredient	60	O:1	K2	CA2	9.2	S
1893		Ingredient	289	O:1	K2	CA2	9.8.	S
510		Food	7	O:2	K1	CA1	45	R
1879		Spice	139	O:2	K1	CA1	15	M
1210	*C. dublinensis*	Food	106	O1	K1	CA1	4	S
1458		Food	74	O1	K1	CA1	3	S
1463		Food	95	O1	K1	CA1	4.4	S
1461		Food	77	O1	K1	CA1	3.3	S
1462		Food	70	O1	K1	CA1	3.4	S
1460		Food	76	O2	K1	CA2	3.2	S
1464		Food	78	O2	K1	ND	3.2	S
1897		Herb	213	O2	K1	CA2	3.3	S
1330	*C. condimenti*	Food	98	Cuni O1	K1	CA2	46	R
96	*C. universalis*	Spice	48	ND	K2	CA1	4.2	S
1435		Food	51	ND	K2	ND	39	R
1883		Spice	137	ND	K2	CA2	6.5	S
1895	*C. turicensis*	Ingredient	252	O1	K2	CA2	5	S
92		Herb	35	O3	K2	CA2	5.5	S
109		Herb	5	ND	K2	CA2	6.21	S
111		Herb	24	ND	K2	CA2	5	S
1553		Herb	72	O3	K1	CA2	5.5	S
1880		Herb	344	O3	K1	CA2	5	S
16	*C. muytjensii*	Spice	347	O3	K1	CA1	10	S
1371		Spice	403	O2	K1	CA2	5.5	S
1527		Food	411	O1	K1	CA1	5	S
1877		Ingredient	407	O1	K1	CA2	6	S

The number of survivors at 58°C was plotted against time. The best fit-line was extrapolated and the decimal reduction time was determined (−1/slope of the regression line). Strains were classified into three groups, high heat resistance (D58 >25), moderate (10< D58< 25), and heat sensitive (D58 < 10).

R = resistance. M = moderate . S = sensitive

5.3.4.4 Role of the thermotolerance genomic island to increased thermal tolerance in *Cronobacter* spp.

Oriešková et al (2016) reported that some of *Cronobacter* strains contain a genomic island, which might be responsible for increased thermotolerance. The genomic island consist of two loci, the first one containing the *thrB-Q* genes with a size of 18 kbp, and the second locus containing the *thrBCD* and *thrOP* genes (6 kbp). A BLAST genome search was applied to investigate the presence of the genomic island genes and correlate that with increase in thermal tolerance in *Cronobacter* spp. The long island locus (*thrB-Q;* 18 kbp) was found in *C. sakazakii* strains 1283 and 1888 (ST8) and 1908 (ST4), and *C. malonaticus* strain 510 (ST7). The short island locus (*thrBCD* and *thrOP)* was only found in two *C. sakazakii* strains which are 377 (ST4) and 1847 (ST245). However, other *C. sakazakii* and C. *malonaticus* strains showed an absence of the genomic island genes (Table 5.11 and 5.12). All *C. dublinensis, C. universalis, C. turicensis, C. muytjensii* strains and the single *C. condimenti* strain showed the absence of the genomic island in both short genomic island and long genomic island loci (Table 5.12).

Table 5.11**.** Presence of the thermotolerance genomic island in 44 *Cronobacter* sequenced genomes

Species	Number of strains	Short genomic island *thrBCDOP* (6 kbp).	Long genomic island *thrB-Q* (18 kbp)
C. sakazakii	21	2	3
C. dublinensis	7	-	-
C. malonaticus	5	-	1
C. muytjensii	3	-	-
C. turicensis	4	-	-
C. universalis	3	-	-
C. condimenti	1	-	-
Total	44	2	4

A BLAST genome search was applied to investigate the presence of the genomic island genes and correlate that with increase in thermal tolerance in *Cronobacter* spp.

Table 5.12. Presence/ absence of the thermotolerance island genes in *C. sakazakii* and *C. malonaticus* sequenced genomes.

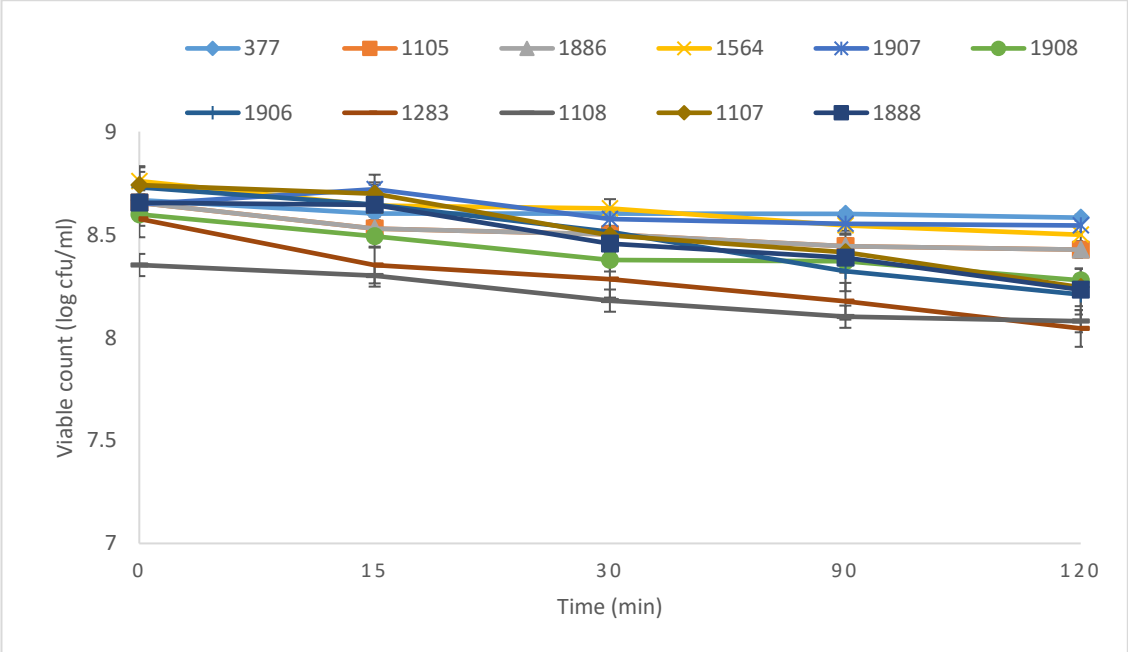
Genes			*thrB*	*thrC*	*thrD*	*thrF*	*thrH*	*thrI*	*thrJ*	*thrK*	*thrL*	*thrM*	*thrN*	*thrO*	*thrP*	*thrQ*
Function			DNA binding protein	Small heat shock protein	ATP-dependent Clp protease, ClpK	N-terminal fragment of ATP-dependent metallopeptidase FtsH	Small heat shock protein	Conservative protein, yfdX family	Conservative protein, yfdX family	Conservative transmembrane protein	Conservative hypothetical protein	Thioredoxin III	Sodium/hydrogen exchanger of KefC family	Phosphate-starvation-inducible E family protein	Zn-dependent protease, M48 family	DegQ peptidase of HtrA/DegP family
NTU:NO	Species	ST														
377		4	+	+	+	-	-	-	-	-	-	-	-	+	+	-
1105		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1886		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1908		4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1906		8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1888		8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1283		8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1108		12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1887		13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1882		20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1890	*C. sakazakii*	42	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1881		64	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1992		136	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1889		198	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1845		233	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1847		245	+	+	+	-	-	-	-	-	-	-	-	+	+	-
1884		263	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1990		264	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1844		405	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1885		406	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2027		406	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1369		69	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101		60	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1846	*C. malonaticus*	60	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1879		139	-	-	-	-	-	-	-	-	-	-	-	-	-	-
510		7	+	+	+	+	+	+	+	+	+	+	+	+	+	+

5.3.5 Growth of *Cronobacter* strains at pH 3.5.

The acid tolerance of 54 *Cronobacter* strains including seven species was determined by treatment at pH 3.5. The experiment was conducted in order to better understand which of these strains could survive a pH resembling that of the human stomach and acidified food. Strains were exposed to acidified infant formula (pH 3.5) for 120 minutes at 37°C. The initial viability of the strains ranged from 8.1 to 8.75 log_{10} CFU/ml (Figures 5.9, 5.10, and 5.11).

Cronobacter strains showed variety in their ability to survive at pH 3.5, with reductions in viability ranging from 0.02 to 1.02 log_{10} CFU/ml. *C. sakazakii* strains ST4; 377, 1105, 1564, 1886, 1907 and 1908 showed slight reduction, with reductions of about 0.08 log_{10} CFU/ml. *C. sakazakii* strains in ST8 (1283, 1888, and 1906), 1881 (ST64) and 1843 (ST23) showed the greatest decreases, with reductions of about 0.52 log_{10} CFU/ml (Figure 5.9 a, b). While The lowest decreases was noted in strains 1889 (ST198), 1105 (ST4) and 1887 (ST13) with reductions .07, 0.08 and 0.17 log_{10} CFU/ml respectively.

(A).



(B).

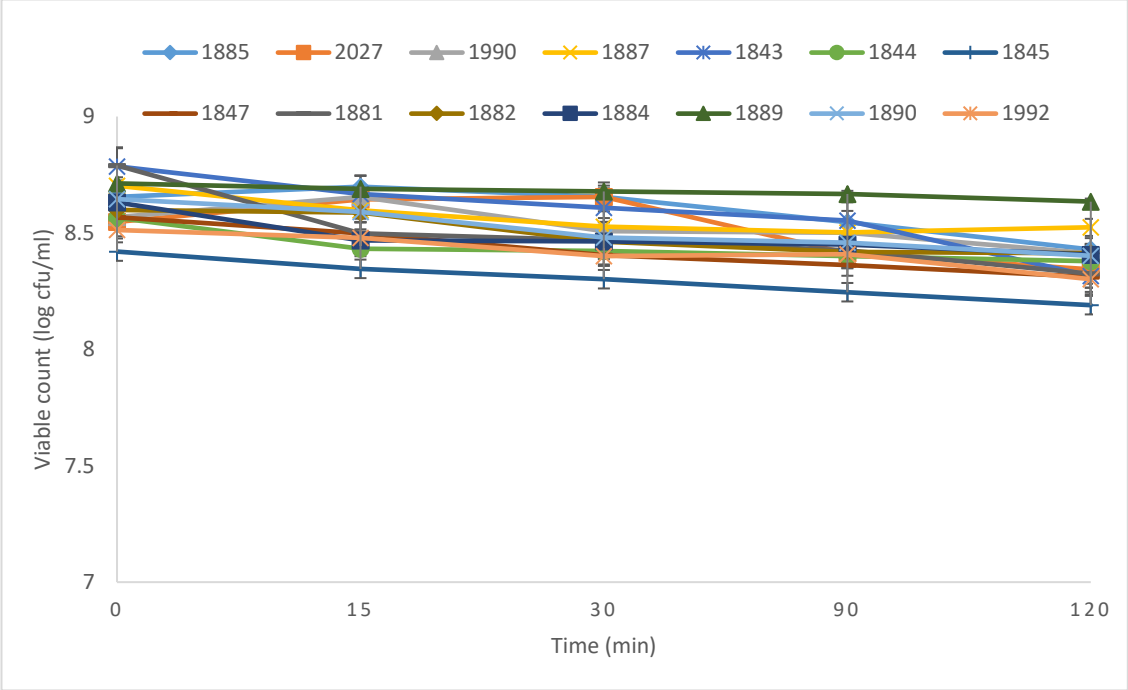


Figure 5.9. A, B. The survival of *C. sakazakii* strains at pH 3.5. The survival of bacteria was measured at 0, 15, 30, 90 and 120 minutes.
Viability was measured at 0, 15, 30, 90 and 120 minutes. Most of the *C. sakazakii* strains showed acid tolerance, with little variation among the strains. The number of recovered cells was determined in triplicate in two independent experiments. The data are reported as means ± standard deviation

Growth of *C. malonaticus, C. dublinensis C. turicensis C. muytjensii* , *C. universalis* strains and the single *C. condimenti* strains on pH 3.5. Figure 5.10 shows the viability of the *C. malonaticus* and *C. dublinensis* strains after 120 minutes of incubation at pH 3.5. There was a reduction of 0.01 to 0.49 $\log_{10}$ CFU/ml after 120 minutes of exposure to pH 3.5. *C. dublinensis* strains, 1458 (ST74) and 1460 (ST78) and *C. malonaticus* strain 1846 (ST60) showed the greatest decreases, with reductions of about 0.41, 0.49 and 0.32 $\log_{10}$ CFU/ml (Figure 5.5, 5.6). Two *C. malonaticus* strains 510 (ST7- O:2- K1) and 1879 (ST139 - O:2-K1) were highly resistant to acid, which showed slightly increased in viability of about 0.02 to 0.06 $\log_{10}$ CFU/ml, respectively (Figure 5.10). The single *C. condimenti* strain 1330 (ST98) was also resistant to acid. *C. turicensis* C. *muytjensii* and *C. universalis* strains showed only slight declines in viability after 60 minutes at pH 3.5, these strains showed reductions in viability of 0.02 to 0.01 $\log_{10}$ CFU/ml (Figure 5.11). In general, strains were nearly resistant to two 120 minutes exposure to pH 3.5 in

liquid infant formula. There was a correlation between equences type of *C. sakazakii* strains and their acid resistance, in particular ST4, ST8 and ST13. These STs are of particular clinical significance.

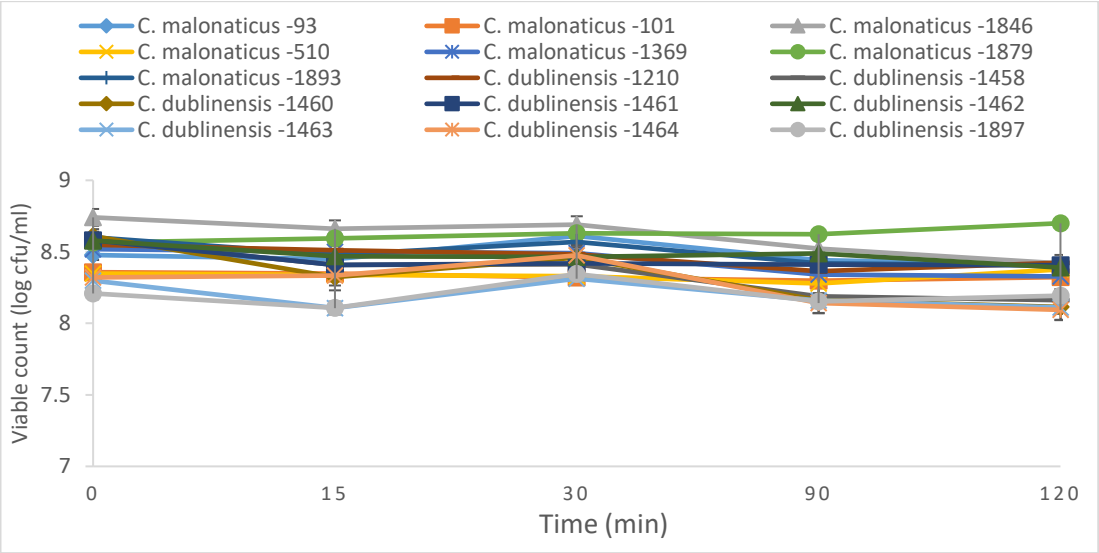


Figure 5.10. The survival of *C. malonaticus* and *C. dublinensis* strains after the exposure to pH 3.5.

Viability was measured at 0, 15, 30, 90 and 120 minutes. Most of the *C. malonaticus and C. dublinensis* strains showed acid tolerance, with little variation among the strains. The number of recovered cells was determined in triplicate in two independent experiments. The data are reported as means ± standard deviation.

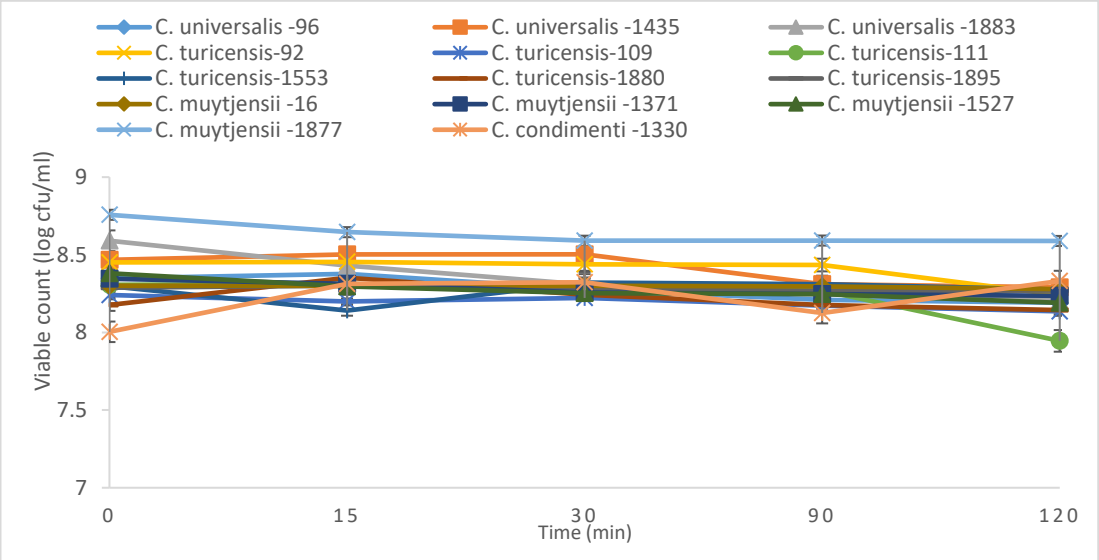


Figure 5.11. The survival of *C. turicensis C. muytjensii and C. universalis* strains and the single *C. condimenti* strain after the exposure to pH 3.5.Viability was measured at 0, 15, 30, 90 and 120 minutes. Most of the strains showed acid tolerance, with little variation among the strains. The number of recovered cells was determined in triplicate in two independent experiments. The data are reported as means ± standard deviation

5.3.5.1 Phylogenetic analysis of the Acid tolerance associated gene *ompR*.

Alvarez-Ordonez et al (2014) reported that the *ompR* gene play a key role in response of *C. sakazakii* against acid resistance. This gene is a homologue of the gene ESA_04334 encoding for osmolarity response regulator (*ompR*). The genomes of 41 *Cronobacter* strains were screened for the presence of the *ompR* gene using BLAST tools. The genomic analysis shown that *ompR* was present in all *Cronobacter* strains. The phylogenetic tree was constructed, using MEGA 6.0 (Figure 5.12). There were variations observed within *Cronobacter* isolates based on the phylogenetic tree of *ompR*. The *ompR* phylogeny showed that *C. sakazakii* strains were nearly divided into 3 groups (Figure 5.12). Moreover, *C. malonaticus* strains were divided into 3 different branches between *C. sakazakii* clusters, *C. turicensis* strains were also divided into 2 clusters. *C. dublinensis* strains had been located on the same cluster; however, the cluster was divided onto different branches. *C. muytjensii* and *C. universalis* strains and the single *C. condimenti* strain were separated into individual clusters. *C. malonaticus* strains were closer to *C. sakazakii* strains than the other species in the phylogenetic relationship. This observation shows that no clear correlation between the growth of strains on pH 3.5 and the phylogenetic tree of *Cronobacter* strains based on nucleotide sequences of *ompR* gene (Figure 5.12).

In general, table 5.13 and 5.14 has summarised the ability of 7 *Cronobacter* species isolated from food and environmental sources to tolerate a variety of environmental stress conditions, and correlated these with their genetic traits.

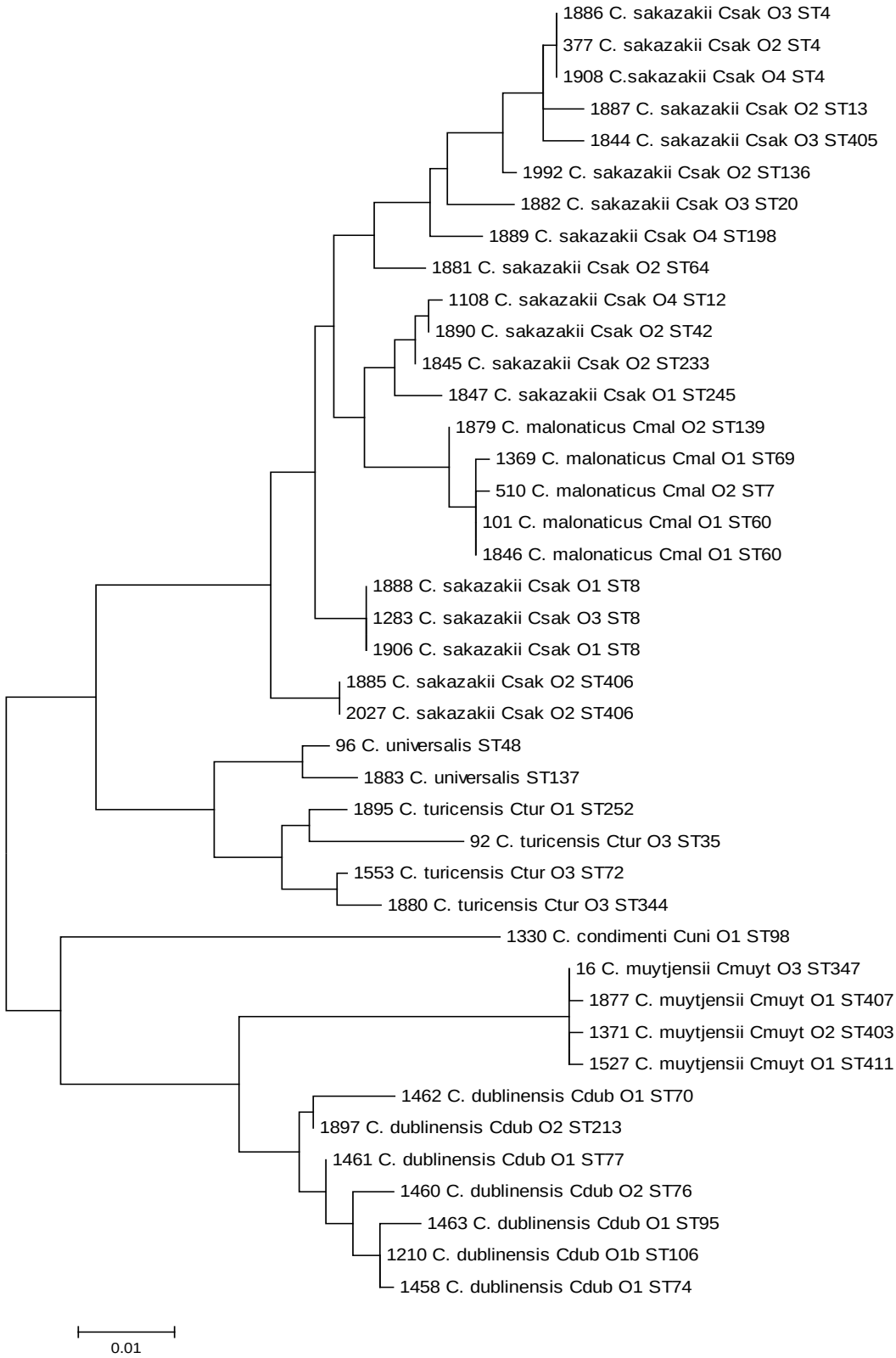


Figure 5.12. The phylogenetic tree of *Cronobacter* strains, based on nucleotide sequences of *ompR* gene (720bp). The tree was drawn using MEGA 6.

The NTU strain IDs are shown at the top of each branch, *ompR* tree analysis revealed 4 distinct clusters based on the level of nucleotide sequence similarity.

Table 5.13. The ability of *C. sakazakii* strains and the single *C. condimenti* strain to tolerate a variety of environmental stress conditions, and correlated these with their molecular characteristics.

Strains	Species	Sources	ST	O-antigen	Capsule formation	Desiccation	Resistance to drying (90 days)	Heat tolerance	Acid sensitivity
					Milk agar 37°C/(24h)	Sublethally injured cells (%)	(Detectable day)	(58°C)	(pH 3.5)
377	*C. sakazakii*	Food	4	O:2	+++	13.24	90	R	R
1105		Weaning food	4	O:2	+++	11.37	60	R	R
1564		Food	4	O:3	+++	6.56	60	R	R
1886		Spice	4	O:3	+++	8.68	60	R	R
1907		Environment	4	O:4	+	33.25	90	R	R
1908		Environment	4	O:4	+	29.34	90	R	R
1283		Food	8	O:3	+++	18.36	90	R	M
1888		Food	8	O:1	-	21.96	90	M	M
1906		Environment	8	O:1	+	36.44	60	S	M
1107		Weaning food	9	O:2	+++	17.2	90	S	R
1108		Weaning food	12	O:4	+	28.93	After 90	S	R
1887		Food	13	O:2	+++	15.4	After 90	R	R
1882		Ingredient	20	O:3	+++	16.95	90	M	R
1843		Ingredient	23	O:2	+++	7	90	R	M
1890		Ingredient	42	O:2	+++	9.57	90	R	R
1881		Ingredient	64	O:2	+++	11.46	90	M	M
1992		Food	136	O:2	+++	11.93	90	M	R
1889		Ingredient	198	O:4	+	25.04	After 90	R	R
1845		Food	233	O:2	+++	7.89	90	R	R
1847		Milk powder	245	O:1	++	18.2	90	R	R
1884		Herb	263	O:2	+++	7.41	After 90	S	R
1990		Food	264	O:2	+++	10.74	90	R	R
1844		Ingredient	405	O:3	+++	10.69	90	R	R
1885		Herb	406	O:2	+++	10.62	After 90	S	R
2027		Ingredient	406	O:2	+++	15.62	90	M	R
1330	*C.condimenti*	Food	98	CuniO:1	+++	40.65	60	R	R

R= Resistance. M= Moderate. S= sensitive. +++= high mucoid production. ++= moderate mucoid production. += low mucoid production. -= no mucoid production

Table 5.14. The ability of *C. universalis, C. turicensis, C. muytjensii, C. malonaticus* and *C.dublinensis* strains to tolerate a variety of environmental stress conditions, and correlated these with their molecular characteristic.

NTU: NO	Species	Sources	ST	O-antigen	Capsule formation	Desiccation	Resistance to drying (90 days)	Heat tolerance	Acid sensitivity
					Milk agar 37°C/(24h)	Sublethally injured cells (%)	Detectable day	(58°C)	(pH 3.5)
96	*C. universalis*	Spice	48	ND	+++	0.83	60	S	R
1435		Food	51	ND	+++	14.66	60	R	R
1883		Spice	137	ND	+++	27.71	60	S	R
109	*C. turicensis*	Herb	5	ND	+++	9.34	20	S	R
111		Herb	24	ND	+++	33.12	20	S	R
92		Herb	35	Ctur O:3	+++	4.125	20	S	R
1880		Herb	127	Ctur O:3	+++	22.12	20	S	R
1553		Herb	72	Ctur O:3	+++	11.62	20	S	R
1895		Ingredient	252	Ctur O:1	+++	16.07	60	S	R
1527	*C. muytjensii*	Food	75	CmuyO:1	+++	35.39	90	S	R
1977		Ingredient	288	CmuyO:1	+++	11.34	90	S	R
1371		Spice	71	CmuyO:2	+++	12.17	90	S	R
16		Spice	34	CmuyO:3	+++	1.92	90	S	R
1369	*C. malonaticus*	Herb	69	Cmal O:1	+++	35.87	60	S	R
101		Spice	60	Cmal O:1	+++	10.86	60	S	M
93		Spice	29	Cmal O:1	+++	40.86	60	S	M
1893		Ingredient	289	Cmal O:1	+++	25.92	60	S	M
1846		Ingredient	60	Cmal O:1	+++	7.72	60	S	M
510		Food	7	Cmal O:2	+++	37.06	60	R	R
1879		Spice	139	Cmal O:2	+++	36.09	60	M	R
1210	*C.dublinensis*	Environment	106	Cdub O:1	+++	6.82	60	S	R
1458		Food	74	Cdub O:1	+++	23.33	60	S	R
1460		Food	76	Cdub O:1	+++	2.48	90	S	R
1461		Food	77	Cdub O:1	+++	8.63	90	S	R
1462		Food	70	Cdub O:1	+++	20.75	90	S	M
1463		Food	95	Cdub O:1	+++	17.7	60	S	R
1464		Food	78	Cdub O:2	+++	1.48	60	S	M
1897		Herb	290	Cdub O:2	+++	14.33	60	S	M

Discussion.

Cronobacter species have been isolated from a wide range of sources such as PIF, dried milk products, weaning foods, powdered ingredients, processed milk products, and food production environments. The resistance of *Cronobacter* species to environmental stresses is a main factor, which is responsible for its survival and behaviour in milk powder plants, factory environments and other foods. Many studies reported that *Cronobacter* spp. persist for a long time in food (Iversen et al. 2008; Jung et al. 2013). Nevertheless, our understanding of the survival mechanisms and persistence of *Cronobacter* species in environmental stresses is still limited (Forsythe, 2014). Therefore, the objective of this chapter was to investigate the ability of 7 *Cronobacter* species to tolerate a variety of environmental stress conditions such as heavy metal toxicity, sub-lethal injury during desiccation, resistance to long-term drying, heat and acid resistance, and to link these with their molecular characteristics.

Heavy metals.

The introduction of heavy metals, in many forms in the environment, can cause substantial modifications in the function and structure of bacterial communities (Yao et al. 2016). Most heavy metals are essential micronutrients for organisms since they are incorporated into enzymes and cofactors. However, resistance of bacterial to heavy metals and antibiotics is an increasing problem in today's society. Heavy metals used in industry are, along with antibiotics creating a selective pressure in the environment that leads to the mutations in microorganisms that will allowed them better survive and multiply. A correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to antibiotics and heavy metals might be located closely together on the same plasmid in bacteria and are hence more likely to be transferred together in the environment (Nies, 2003).

The sensitivity of *Cronobacte*r spp. to 7 different concentration of heavy metal were studied. These included; nickel chloride, zinc sulphate, copper (II) sulphate, silver nitrate, sodium tellurite, cadmium carbonate and cobalt(II) nitrate. To the best of our knowledge, this is the first study to validate the sensitivity of seven species of *Cronobacter* to heavy metals and linked that with their molecular characteristics. The 54 *Cronobacter* strains were sensitive to sodium tellurite and silver nitrate for 4 different concentrations of 1M, 0.1M, 0.01M and 0.001M. Moreover, majorty of these isolates were sensitive to the high concentration of heavy metals (1M and 0.1M), except the cadmium. Most strains were resistance to cadmium, except *C. turicensis* strain 1553 (ST72), *C. muytjensii* strain 1527 (ST75), 3 *C. dublinensis* subsp. *dublinensis* strains 1210 (ST106), 1458 (ST74), 1463 (ST95), two strains of *C. sakazakii* ST4 (377 and 1907) and the single *C. condimenti* strain 1330 (ST98) which showed sensitivity to the high concentration (1M, 0.1M). All strains were sensitive to the high concentration (1M) of the zinc sulphate, as well as some of them were sensitive to concentration (0.1M). Nies (2003) stated that high concentrations of heavy metals could be toxic for the bacterial. However, this study was in close agreement with the previous study by Nies (2003).

The copper and silver resistance associated regions (*cusABCFR/silABCER* and *pcoABCDR*) were absent in majority of strains, except 2 *C. malonaticus* strains; 510 (ST7) and 101 (ST60), 2 *C. turicensis;* 1553 (ST72), 1895 (ST252), *C. muytjensii* strain 1877 (ST288), *C. dublinensis* 1463 (ST95), in addition to *C. sakazakii* strains belong to ST4, ST8 and ST64 which are associated with neonatal infection and powder infant formula and 1847 (ST245) strain which isolated from milk powder (Table 5.3 and 5.4). The presence of the copper/silver resistance regions (*cusABCFR/silABCER* and *pcoABCDR*) in the significant STs suggest that these regions may be essential for the survival and the virulence of *Cronobacter* strains in particular *C. sakazakii* ST4, ST8 and ST64 strains and *C. malonaticus* ST7 strains

Nickel and cobalt are required as trace elements in bacteria metabolism, nonetheless high intracellular concentrations of these two metals are toxic. One of

the strategies evolved by bacteria to prevent damage is to export excess metal by efflux systems (Rodrigue et al. 2005). Furthermore, Rodrigue et al (2005) reported that inactivation of the *yohM* gene induces sensitivity in nickel and cobalt to *E. coli* cells, therefore they proposed the new de-nomination for the *yohM* gene that is *rcnA*. The results indicated that all of the strains were able to resist nickel chloride and cobalt (II) nitrate at low concentrations (0.01 and 0.001M), while most of them were sensitive to cobalt (II) nitrate at high concentrations (1M and 0.1M). However, by sequence search *rcnA* gene was absent in all *Cronobacter* sequenced strains. The present study indicated that there was no obvious correlation between the phenotypic detection of nickel and cobalt resistance and the genotypic detection of the *rcnA* (*yohM*) (Table 5.3 and 5.4). Therefore, *rcnA* gene may not be the only gene responsible for nickel/cobalt resistance or efflux in *Cronobacter* species.

Cronobacter isolates were sensitive to sodium tellurite for 4 different concentrations of 1M, 0.1M, 0.01M and 0.001M. Joseph et al (2012) reported that homologues of tellurite cluster gene (*terACDYZ*) was located in the loci ESA_01775–ESA_01804 of *C. sakazakii* BAA-894. The results indicated all *Cronobacter* strains were sensitive to sodium tellurite. In addition, the tellurite resistance genes (*terACDYZ*) were absent in all *Cronobacter* strains studied (Table 5.3 and 5.4). This suggests that these genes are may necessary for tellurite resistance in *Cronobacter* species. In general, no clear correlation between the sensitivity of seven species of *Cronobacte*r to 7 heavy metals and molecular characterisation including ST and type of O-antigen was observed.

Sublethally injured cells during desiccation.

The *Cronobacter* genus has been isolated from a wide range of food, including PIF, milk powder and dry powdered foods (Iversen and Forsythe 2004; Friedemann, 2007). The *Cronobacter* genus, in particular *C sakazakii* strains have been associated with serious neonatal infections, whereas *C. malonaticus* strains have been associated with infections of immunocompromised adult patient. The presence of

Cronobacter spp. during the manufacture of PIF and other food production environments is an indicator of production hygiene (Caubilla-Barron and Forsythe, 2007).

Desiccation is the process of extreme drying, desiccation refers to the drying out of a living organism. Bacteria cannot grow and divide when desiccated, but can survive for certain periods of time, depending on their features. They may no longer be able to form colonies and therefore cannot be detected by conventional growth tests even though the bacteria are still alive. Once more favorable growth conditions are available, the bacteria can recover from their inactive state and begin to divide. This can be a critical concern in the food packaging industry for storage of dried foods such as PIF, powdred milk, spices, grains and other packaged foods. Therefore, sublethally injured bacterial cells may be generated during the production of PIF and other dry foods. These cells are more sensitive to the bile salts and dyes used in selective media such as VRBGA and consequently cannot grow in these media (Caubilla-Barron and Forsythe, 2007).

In this study, the generation of sublethally injured cells during desiccation of 54 strains were examined, and linked with their genetic traits. Fifty-four desiccated *Cronobacter* strains were recovered after 24h at 37°C on selective (VRBGA) and nonselective (TSA) media (Caubilla-Barron and Forsythe, 2007). All of the strains showed greater recovery on TSA than on VRBGA (Table 5.3 and 5.6). The differences in recovery after desiccation reflect the number of cells that were sublethally injured during the desiccation procedure.

There were significantly fewer sublethally injured cells generated for highly capsulated than for non-capsulated (1888) and low capsulated strains of *C. sakazakii* (1906, 1907, 1908, 1108, 1889). This finding was in close agreement with result obtained from Caubilla-Barron and Forsythe (2007). The present study also revealed that *C. sakazakii* strains in serotype O:1 (1888, 1906 and 1847) and O:4 strains (1108, 1907, 1908 and 1889) had high numbers of sublethally injured cells;

18.2% to 36.4%, 25% to 33.2%, respectively. While, strains in serotype O:2 and O:3 showed low numbers of sublethally injured cells; 7% to 15.6%, 6.5% to 18.36%, respectively. This variation might be related to the sugar composition and complexity of structure in the capsule LPS.

There was a variation among other *Cronobacter* species strains in their response to desiccation-induced injury. The lowest sublethal injury cells (0.83% to 2.48%) were obtained for *C. universalis* strain 96 (ST48), *C. muytjensii* strain 16 (ST34), *C. dublinensis* strains 1460 (ST76) and 1464 (ST78). Whereas, the higher sublethal injury cells (33% to 40% differences) were obtained for *C. turicensis* strain 111 (ST24), *C. muytjensii* strain 1527 (ST75), *C. malonaticus* strains 1369 (ST69), 510 (ST7), 93 (ST29), 1879 (139) and single *C. condimenti* strain 1330 (ST98). All of the strains were capsulated. No obvious correlation between the sublethally injured cells during the desiccation and the genetic diversity including sequence type (ST) and serotype (O-antigen) was observed in other *Cronobacter* species (Table 5.3 and 5.6 and Figure 5.1). This difference may be another example of the diversity within the *Cronobacter* species in response to environmental stress.

Resistance of *Cronobacter* spp. to drying (90 days).

Breeuwer et al (2003) reported that *Cronobacter* strains were more resistant to dying than other members of *Enterobacteriaceae*. Moreover, Fakruddin et al (2014) stated that *C. sakazakii* isolates grown and dried in infant formula showed significantly ($P < 0.05$) better survival during drying than grown and dried in TSB. However, the aims of this part of the current work were to investigate the ability of 7 *Cronobacte*r species to survive in drying conditions (90 days), and to determine whether *C. sakazakii* strains are more resistant than other species to drying, and correlated that with their molecular characteristic such as ST, O-antigen and K-capsule type. The present study demonstrated that there was a variation in the viability between *C. sakazakii* strains in terms of resistance to drying. *C. sakazakii* ST64 and ST8 are isolated from PIF and the environment of manufacturing plants in

many countries around the world, these strains showed moderate recovery after the exposure to drying (90 days). In contrast, strains 1887 (ST13), 1890 (ST42), 1108 (ST12), 1107 (ST9), 1884 (ST263) and 1885 (ST406), showed better recovery after the exposure to drying (90 days) suggesting higher resistance to drying than the other *C. sakazakii* strains (Figure 5.2). Strains with the same sequence type and same clonal complex such as ST406 and ST264 (CC264) showed different ability to survive in drying conditions.

Several genes linked to desiccation and osmotic stress conditions were identified (Yan et al. 2013), which included the *yihUTRSQVO, yehZYXW* genes. Many other genes linked with the osmotic stress response were identified, including the osmotolerance regulation genes *yiaD, osmY, ompA, aqpZ*, and *glpF* and osmotic stress genes including, *osmBO, yciTM,* and *pgpB* (Grim et al. 2013; Yan et al. 2013). The genomes of 41 *Cronobacter* strains were screened for the presence of these genes using BLAST tools. The results demonstrated that most of strains had the osmoprotectant ABC, transporter genes (*yehXWYZ*) and desiccation-related genes (*yihTQVWPAOS*), except *C. dublinensis* strains. Osmotic stress related genes *osmBOY, yiaD, ompA, aqpZ, glpF*, *yciTM* and *pgpB* were found in most of the strains, except *aqpZ* gene which was lacked in *C. sakazakii* strains 1845 (ST233), 1847 (ST245), 1890 (ST42), and 1908 (ST4), *C. malonaticus* strains 101 (ST60), 1369 (ST69), 1879 (ST139) and 1893 (ST289) and *C. muytjensii* strains 16 (ST347), 1371 (ST403), 1527 (ST411) and 1877 (ST407). Moreover, the genomic analysis shown that one desiccation-related gene (*yihR*) was found in *C. sakazakii, C. turicensis* and *C. malonaticus* strains, and was absent in *C. universalis, C. muytjensii, C. dublinensis* strains and the single *C. condimenti* strain (Table 5.7 and 5.8).

A recent study by Alvarez-Ordonez and Hill (2016) reported that in *C. sakazakii rpoS* activity confers protection against a range of stress conditions, including acid, alkaline, osmotic and oxidative stress. Moreover, Lalaouna et al (2012) reported that the *mutS-rpoS* genomic region may help the enteric bacteria to survive in the harsh environments. In this study, the genomic study of 41 *Cronobacter* strains

showed that the *mutS-rpoS* genomic region was found in all *Cronobacter* strains, and contained the same seven genes between *mutS* and *rpoS* (Figure 5.5). Nonetheless, it has been suggested that the genes in the *mutS-rpoS* region did not seem to correlate with the results of the environmental stress assay, in particular desiccation and drying stress.

According to the above data, *C. sakazakii* strains showed a higher survival rate after the exposure to drying (90 days) than other *Cronobacter* species (Figure 5.2, 5.3, 5.4 and Table 5.13). This might explain why *C. sakazakii* strains are associated with PIF, milk powder and dry powdered foods, and more frequently isolated than other species of *Cronobacter*.

Heat resistance.

Heat resistance is an important factor for survival of *Cronobacter* spp. in PIF and other foods (Dancer et al. 2009; Huertas et al. 2015; Oriešková et al. 2016). It has been reported that *Cronobacte*r strains differ in their tolerance to heat stress. A study by Oriešková et al (2016) stated that some of *Cronobacter* strains contain a genomic island, which might be responsible for increased thermotolerance. Therefore, the objectives of this part of the work were to (i) evaluate the heat resistance of 7 *Cronobacter* species (ii) linked that with their molecular characteristics (iii) compare the thermotolerance genomic island composition in 41 genome sequenced *Cronobacter* strains and (iv) to correlate the genomic differences with their phenotypes for heat tolerance.

The heat tolerance of 7 species of *Cronobacter* strains were determined in liquid infant formula at 58°C. Strains were classified into three groups, high heat resistance (D_{58} >25), heat moderate (10< D_{58}< 25), and heat sensitive (D_{58} < 10). *C. sakazakii* strains 377, 1105, 1564, 1886, 1907 and 1908 (ST4), 1283 (ST8), 1843 (ST23), 1889 (ST198), 1845 (ST233), 1847 (245), 1890 (ST42), 1844 (ST405), 1990 (ST264) and 1887 (ST13) showed high heat resistance compared with other *C. sakazakii* STs. However, *C. sakazakii* strains; 1107 (ST9), 1108 (ST12), 1906 and 1888

(ST8), 1884 (ST263) and 1885 (ST406) showed sensitivity to heat and subsequently had lower D_{58} ranged between 8 to 10 min (Table 5.9). Other *C. sakazakii* strains showed moderate heat tolerance, the D-values ranged from 10 to 25 min. As shown in table 5.9 *C. sakazakii* environment strains 1907 and 1908 (ST4-O:4) were more heat resistant (58°C) than the other ST4 *C. sakazakii* strains. They had a D-value ranged from 36 to 50 minutes at 58°C as presented in table 5.9. The variation in the viability of *C. sakazakii* ST8 strains ST8 (O:1) and ST8 (O:3) isolates at 58°C was interesting. The D-values of *C. sakazakii* strains 1888 and 1906 (ST8.O:1) ranged from 8 to 10 min (heat sensitive), while *C. sakazakii* strain 1283 (ST8-O:3) showed high heat resistance and the viability decline was linear in liquid infant formula, with D_{58} = 35 minute (Table 4.9 and Figure 5.6 a, b).

The result demonstrated that *C. malonaticus* strains differ in their tolerance to heat stress (58°C). These strains were categorised into three groups based on their capability of heat resistance. Strain 510 (ST7-O:2-K1-CA1) showed high thermoresistance with D_{58} = 45 minutes, whereas strain 1879 (ST139-O:2-K1-CA1) showed moderate heat resistance with D_{58} = 15 minutes. In contrast, *C. malonaticus* strains 1369 (ST69-O:1-K1-CA2), 1846 (ST60-O:1-K2-CA2) and 1893 (ST289-O:1-K2-CA2) showed heat sensitivity at 58°C, D-value ranged from 9.2 to 10 minutes at 58°C (Figures 5.7 and Table 5.10). There was an obvious correlation between the heat resistance and the capsule profile of *C. malonaticus* strains, in particular O-antigen type and colanic acid capsule type (CA). It has been suggested that the O-antigen serotype type and colanic acid capsule type (CA) may increase the ability of *C. malonaticus* strains to the heat resistance. However, other *Cronobacter* species; *C. dublinensis, C. turicensis, C. universalis* and *C. muytjensii* strains were heat sensitive (D_{58} = 3 to 10 min), except *C. universalis* strain 1435 (ST51) showed high thermotolerance (resistance, with D_{58}= 39 min). The single *C. condimenti* strain 1330 (ST98) was also heat resistance at 58°C in liquid infant formula, D-value = 46 (Figures 5.8 and Table 5.10).

Oriešková et al (2013) reported that some *Cronobacter* strains contain a genomic island. The genomic island locus consist of two loci, a longer version (18 kbp) containing the *thrB-Q* genes and a shorter loci (6 kbp) containing only the *thrBCD* and *thrOP* genes. This locus could be responsible for increased heat tolerance in *Cronobacter* strains (Oriešková et al. 2015; Oriešková et al. 2016). The genomic island locus was found to be present in only 5/21 C. *sakazakii* strains and in 1/5 *C. malonaticus* strains. The long island locus (*thrB-Q*) were found in *C. sakazakii* strains 1283 and 1888 (ST8) and 1908 (ST4), and *C. malonaticus* strain 510 (ST7). The short island locus (*thrBCD* and *thrOP*) was only found in two *C. sakazakii* strains which are 377 (ST4) and 1847 (ST245). However, other *C. sakazakii* and *C. malonaticus* strains showed absences of the genomic island genes (Table 5.11 and 5.12). All *C. dublinensis*, *C. universalis*, *C. turicensis*, *C. muytjensii* strains and the single *C. condimenti* strain lacked both short genomic island and long genomic island loci (Table 5.12). In general, strains containing the thermotolerance genomic island (1283, 1888, 377, 1847, 1908 and 510) tended to survive better at 58°C compared to other strains (Table 5.11 and 5.12).

Acid tolerance.

Dancer et al (2009) have shown the ability of *Cronobacter* spp. to survive under high acidic conditions. It was stated that at least 79.2% of the tested strains were able to survive and grow at a pH as low as 3.9 (Dancer et al. 2009). Moreover, Alvarez-Ordones et al (2014) reported that the *ompR* gene is an important factor in the adaptive response of *Cronobacter spp*. to highly acidic conditions, in particular *C. sakazakii* strains. Therefore, the aims of the study presented in this chapter were to investigate the ability of 7 *Cronobacter* species to survive in pH 3.5, and whether *C. sakazakii* strains are more resistant than other species to high acid conditions, and linked that with their molecular characteristic such as sequence type, O-antigen serotye and *ompR* gene profile.

The acid tolerance of 54 *Cronobacter* strains including seven species was determined by treatment at pH 3.5. Strains were exposed to acidified infant formula (pH 3.5) for 120 minutes at 37°C. In general, all strains were nearly resistant to two 120 minutes exposure to pH 3.5 in liquid infant formula. There was a correlation between sequence type of *C. sakazakii* strains and their acid resistance, in particular ST4, ST8 and ST13, these STs are of particular clinical significance. The genomic analysis showed that the *ompR* gene was present in all *Cronobacter* strains, and there were variations within *Cronobacter* isolates based on the phylogenetic tree of *ompR* gene. This observation shows that no clear correlation between the growth of strains at pH 3.5 and the phylogenetic tree of *Cronobacte*r strains based on nucleotide sequences of the *ompR* gene (Figure 5.12). These observations suggests that another factor might contribute to acid resistance.

Chapter 6. Conclusions.

The genus *Cronobacter* are food-borne pathogens causing neonatal infections including meningitis and necrotizing enterocolitis. *Cronobacter* infections in neonates have been associated with the consumption of contaminated reconstituted PIF. However, a large number of infections are reported in the adult population, where the sources are unknown. The diversity of sources and high infant mortality rates of this organism required the improvement and development of reliable detection and identification methods for the bacterium. Furthermore, understanding the molecular characterisation and phenotypic diversity of *Cronobacter* species is essential to reduce the source of microbial contamination of PIF and other food. The studies described in this thesis aimed to investigate the genetic diversity of the *Cronobacter* species isolated from food and environmental sources, and to link that with their phenotypic and physiological traits. Therefore, the phenotypic and physiological characteristics of the bacteria were analysed and the genomes were screened for genetic traits that were suggested previously to be associated with the phenotypic and physiological traits of *Cronobacter* species.

- The aim of the first part of this study was to study the diversity of the *Cronobacter* genus from food and environmental sources as revealed by genotyping, clonality and surface structure.

The 7-loci MLST scheme was applied in this study (**Section 3.3.1**). The strains (n=26) were divided into 21 sequence types (STs), 12 of these STs had been previously reported, and 9 new STs were defined (Table 3.1 and Figure 3.1). Consequently, *Cronobacter* strains isolated from food and environmental sources are highly diverse, in particular the isolates which are obtained from different sources of food.

In this study, RAPD and BOX-PCR fingerprints were used as molecular tools to characterize 61 *Cronobacter* strains isolated from environment, water, foods and clinical, and compared that with their sequence type (**Section 3.3.2, 3.3.3**). Our findings indicated that RAPD-PCR is capable of discriminating among a collection of *Cronobacter* based on their sequence type **(Section 3.3.2)**. Moreover, RAPD-PCR

fingerprint has been successfully used to discriminate closely related STs such as ST406 and ST264 (CC264). Furthermore, in some cases this technique is able to distinguish between strains within the same ST. However, BOX-PCR fingerprints showed less ability to cluster the same *Cronobacter* STs together (Figure 3.3) (**Section 3.3.3)**. In addition, the discriminatory power of BOX-PCR techniques was compared with the RAPD-PCR fingerprints. The RAPD-PCR had slightly higher discriminatory power than BOX-PCR. Nevertheless, RAPD might be a useful molecular tool providing rapid analysis of isolates in a local context.

The screening of the O-antigen serotype of fifty-one *Cronobacter* strains showed that **(Section 3.3.5)**, four O-antigen serotypes were assigned in 25 *C. sakazakii* strains, CSO:1, O:2, O:3 and O:4. Serotype CSO:2 was the most dominant serotype in *C. sakazakii* strains (13 strains, 52%), followed by serotype CSO:3 (20%). Two O-antigen serotypes were assigned in 7 *C. malonaticus* strains CMO:1 and CMO:2. Serotype CM O:1 was the most dominant serotype in *C. malonaticus* strains (five strains, 71.4%). *C. dublinensis* strains were divided into 2 O-antigen serotypes (CDO:1 and O:2). *C. dublinensis* ssp. *dublinensis* and *C. dublinensis* ssp. *lactaridi* strains were CDO:1 serotype (62.5%). While *C. dublinensis* ssp. *lausannensis* strains were CDO:2 serotype(Table 3.4). This suggests that screening of the O-antigen serotype might be used to distinguish *C. dublinensis* strains to the subspecies level.

The analysis of LPS pattern using BioNumerics software, version (7.1) discriminated between strains within the same serotype (**Section 3.3.6)**. The results demonstrated that the 12 LPS profiled *C. sakazakii* strains were clearly divided into four clusters based on their type of serotype, and each cluster belonging to the same serogroups were branched into two branches based on their *galF* and *gnd* allele profile (Figure 3.10, 3.11). Therefore, PCR-based serotypes can be further subdivided.

C. sakazakii ST4 strains are strongly associated with meningitis and necrotizing enterocolitis in neonates and babies. Therefore, more discriminating typing of ST4 is very important for outbreak detection and tracing outbreak sources. The VNTR analysis was used to investigate it and was able to subdivide within this important

sequence type. This study is the first to describe the development and application of VNTRA typing method for the major pathovar, *C. sakazakii* ST4 strains **(Section 3.3.4)**. A total of nineteen *C. sakazakii* ST4 strains, which were widely distributed geographically, temporally and origin of source were profiled. The study revealed the VNTRA technique has a greater-discriminatory power within the *C. sakazakii* ST4 strains. It was concluded that VNTRA profiling could contribute to further understanding of pathovar *C. sakazakii* ST4 diversity and tracking of infection sources.

The present study developed and applied a multiplex PCR assay targeting capsular polysaccharide genes *kpsS* (K1 and K2) and *galE* (CA1 and CA2) for the specific detection of K-capsule and colanic acid type respectively (Figure 3.12, 3.16). It is suggesting that methods based on PCR amplification might be used to determine the K-capsule type and CA-type in *Cronobacter* spp. as simple, cheaper, rapid and reliable methods **(Section 3.3.7)**.

- The second part of the study focused on investigation of the phenotypic diversity of *Cronobacter* strains. Phenotypic identification and biochemical profiling give a rapid presumptive identification for *Cronobacter* species. However, some of these tests showed lack sufficient robustness for this diverse *Cronobacter* genus (Jackson and Forsythe, 2016). Therefore, this study focused on phenotypic diversity of *Cronobacter* isolates from food and environmental sources and linked these with their molecular characteristics, in order to find out more about the diversity of the *Cronobacter* genus.

The strains in this study showed variable motility rates **(Section 4.3.2)**. Most strains were motile except for 2 *C.sakazakii* strains; 1906 (ST8) and 1847 (ST245). All genome sequenced for both motile and non-motile strains showed the presence of the *fliA-Z* gene cluster. This suggests that these motility genes might be not expressed or that unrecognised mutation could have occurred in non-motile strains. The main biochemical tests for the differentiation of *Cronobacter* species were indole production, malonate, inositol utilization, production of methyl-α-D-

glucoside and sialic acid utilization (Iversen et al. 2007; Joseph et al. 2013). These relevant biochemical tests were applied to 54 strains, and were linked with their genetic traits, for the purpose of differentiation and distinguishing the 7 species of *Cronobacter* as well as to characterize and distinguish *C. dublinensis* strains to the subspecies level **(Section 4.3.3.1)**.

According to this observation, the present study recommends that.

1- Using the malonate and sialic acid utilization test can be useful tools for the differentiation of and distinguishing between closely related species, *C. malonaticus* and *C. sakazakii.*
2- The inositol utilization test could be able to distinguish pathogenic ST7 from other *C. malonaticus* STs.
3- Combination of malonate, inositol utilization test, capsule profile (O-antigen, K-capsule type and CA-type) and curli fimbriae gene cluster detection can be useful tools for the differentiation and distinguishing of *C. dublinensis* strains to the subspecies level. This observation was supported by a genomic investigation of 14 *C. dublinensis* genome strains (Table 4.15).

Overall, this part suggests that a combination of genomic clusters and specific biochemical tests including indole production, malonate, inositol, and sialic acid utilization can be useful tools for the differentiation and distinguishing of seven *Cronobacter* species, distinguishing pathogenic ST7 from other *C. malonaticus* STs as well as to characterize and distinguish *C. dublinensis* strains to the subspecies level.

There was a notable variation between strains in their ability to produce capsules on different media **(Section 4.3.4)**. The majority of *C. sakazakii* strains had the most mucoid appearance indicating capsular material production. *C. sakazakii* strains in serotype O:1 and O:4 produced less capsular material compared with *C.sakazakii* strains in serotype O:2 and O:3. The study indicated that there was no clear correlation between the amount of capsular material and genotypic detection such as ST, K-capsule type and CA-type. Conversely, a strong correlation between serotype (O-antigen) and amount of capsular material was noted.

The analysis of capsule polysaccharides of 13 selected *C. sakazakii* strains showed they were composed of four different sugars; galactose (Gal), glucose (Glc) and fucose (Fuc) and rhamnose (Rha). Rhamnose sugar was only found in strains with serotype O:2, and was absent in serotype O:1, O:3 and O:4 strains. Moreover, genes (*rfbCD*) are responsible for the biosynthesis of dTDP- l-rhamnose from glucose 1-phosphate. These genes *rfbCD* were found only in *C. sakazakii* strains serotype O:2 and were absent in other *C. sakazakii* serotypes; O:1, O:3 and O:4. This suggests that these genes *rfbCD* might be used to predict the capsule polysaccharides composition in *Cronobacter* species, in particular rhamnose sugar. The observation also shows that there is a strong correlation between the amount of mucoid production, type and ratio of monosaccharide production and the genetic traits, in particular O-antigen serotype. Moreover, this study indicated that rhamnose is the main sugar in *C. sakazakii* serotype O:2 strain.

The incubation temperature affected the ability of tested strains to form a biofilm; 37°C was the optimal temperature condition for most strains **(Section 4.3.5**. Moreover, in this study of 54 strains, there was no correlation between the amount of biofilm formation, and the amount of capsule material on milk agar.

This section suggests that the repetition and variations in oligosaccharide units (O-units) may play an important role for production of capsular material amount.

- The objective of the third part was to investigate the ability of *Cronobacter* strains to tolerate a variety of environmental stress conditions such as sub-lethal injury during desiccation, long-term drying, heat and acid resistance, and linked these with their genetic traits.

The resistance of *Cronobacter* species to environmental stresses is a major factor, which is responsible for its survival and behaviour in milk powder plants, factory environments and other foods.

The recovery of sublethally injured *Cronobacter* strains was decreased comparing with the number of inoculum bacteria. However, the number of non-detected bacteria on VRBGA was higher than on TSA. This reflects the differences in

composition between the two culture media. Moreover, there were significantly fewer sublethally injured cells generated during desiccation for highly capsulated than for non-capsulated and low capsulated strains of *C. sakazakii* **(Section 5.4.2)**. The present study revealed also that *C. sakazakii* strains in serotype O:1 and O:4 had high numbers of sublethally injured cells compared with strains in serotype O:2 and O:3 (Figure 5.1). This might be linked with the sugar composition and structure complexity or the gene order in the O-antigen gene cluster.

C. sakazakii strains showed a high survival rate after the exposure to drying (90 days) compared with other *Cronobacter* species **(Section 5.4.3)**. The *mutS-rpoS* genomic region may help enteric bacteria to survive in harsh environments (Lalaouna et al, 2012). In the present study the *mutS-rpoS* genomic region was found in all *Cronobacter* strains, and contained the same seven genes between *mutS* and *rpoS* (Figure 5.5). It has been suggested that the genes in the *mutS-rpoS* region did not seem to correlate with the results of the environmental stress assay, in particular desiccation and drying stress.

There was variation in thermotolerance between *Cronobacter* strains **(Section 5.4.4)**. The variation was also observed within the same STs. Generally, *C. sakazakii* strains were more heat resistant than other *Cronobacter.* Orieškova et al (2016) reported that some of *Cronobacter* strains contain a genomic island, which might be responsible for increased thermotolerance. The genomic island locus was found to be present in only 5/21 *C. sakazakii* strains and in 1/5 *C. malonaticus* strains. The result indicated that strains containing the thermotolerance genomic island (1283, 1888, 377, 1847, 1908 and 510) tended to survive better at 58°C comparing to other strains (Table 5.11 and 5.12).

This section noted that *C. sakazakii* strains are more resistant to environmental stress conditions than other *Cronobacter* species. This might explain why *C. sakazakii* strains are associated with PIF, milk powder and dry powdered foods and more frequently isolated than other species of *Cronobacter.*

Key novel findings of the current PhD study.

- The Multilocus Sequence Typing (MLST) analyses suggested that *Cronobacter* strains isolated from food and environmental sources were highly diverse. This was particularly notable for the isolates, which were obtained from different foods.
- The present study developed and applied the VNTRA typing method for *C. sakazakii* ST4 strains. This technique revealed a greater discriminatory power within the *C. sakazakii* ST4 strains.
- Analysis of the LPS profiling using BioNumerics software version (7.1) revealed the ability to discriminate between *C. sakazakii* strains within the same serotype based on their *galF* and *gnd* allele profile. Therefore, PCR-based serotype can be further subdivided
- The present study developed and applied a multiplex PCR assay targeting the *kpsS* (K1 and K2) and *galE* (CA1 and CA2) genes for the specific detection and rapid identification of K-capsule type and colanic acid type respectively in *C. sakazakii* strains.
- The observation shows that there is a strong correlation between the amount of mucoid production, type and ratio of monosaccharides production and the genetic traits, in particular O-antigen serotype. Moreover, rhamnose is the main sugar in *C. sakazakii* serotype O:2 strain.
- There was variation among other *Cronobacter* species strains in their response to desiccation-induced injury. *C. sakazakii* strains with serotype O:1 and O:4 had high numbers of sublethally injured cells, while serotype O:2 and O:3 strains showed low numbers of sublethally injured cells.
- In general, *C. sakazakii* strains were more resistant than other *Cronobacter* species to environmental stresses. This might explain why *C. sakazakii* strains are associated with PIF, milk powder and dry powdered foods, than other *Cronobacter* species.

Finally, the results of this study indicated multiple methods for typing *Cronobacter* at the species and strain level, however with different discriminatory powers. The

present study also showed that *C. sakazakii* strains are more resistant to environmental stress conditions than other *Cronobacter* species. This might explain why *C. sakazakii* strains are more frequently isolated than other species of *Cronobacter*, and are associated with PIF and dry powdered foods, and. These findings are an important contribution to the understanding of the diversity and characteristics of the *Cronobacter* genus using different typing methods which is essential to reduce the risk of contaminations for the food products, in particular food babies such as PIF, milk powder and weaning food.

Future work.

This study is the first research focused on phenotypic diversity of *Cronobacter* genus and their ability to tolerate a variety of environmental stress conditions and linked that with their genetic traits; it has extended our knowledge about physiological and phenotypic traits of *Cronobacter* in general and about *C. sakazakii* in particular. However, more researches should be continued on this genus include the following areas.

I. Applying the VNTRA typing method for other *Cronobacte*r clinically significant STs, particularly *C. sakazakii* ST1, ST8, ST12, ST13, ST64 as well as *C. malonaticus* ST7 strains.
II. Applying a multiplex PCR assay targeting the *kpsS* (K1 and K2) and *galE* (CA1 and CA2) gene for the specific detection and rapid identification of K-capsule type and colanic acid type respectively in other *Cronobacter* species strains in general and *C. malonaticus* strains in particular.
III. Studying the role of capsule profiling (O-antigen, K-capslue and colanic acid type) and capsule composition (monosaccharides) in the virulence potential of *C. sakazakii* strains including serum resistance, adhesion and invasion to different human cell lines, cytotoxicity, and macrophage survival.

IV. Investigate the role of capsule profiling, amount of capsule production and capsule composition (monosaccharides) in the diversity of other *Cronobacter* species in general and *C. malonaticus* strains in particular.

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