Identification of physiological and virulence traits of clinical strains of *Cronobacter malonaticus*

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Declaration

I hereby certify that the work presented herein is the result of my own research work, except where reference has been made to published literature. The thesis has been composed by myself and the work has not been submitted for any other degree or professional qualification. All the work was conducted in the School of Science and Technology at the Nottingham Trent University. You may copy up to 5% of this work for the private study or personal, non-commercial research. Any information used from this thesis should be fully cited.

Abdlrhman Mohamed Alsonosi

Abstract

The *Cronobacter malonaticus* is a member of the genus *Cronobacter* which is considered an opportunistic pathogen. The significance of *C. malonaticus* has recently increased since it was documented to be involved in several serious neonatal infections. In addition, more than 40% of *C. malonaticus* in *Cronobacter* MLST-database was clinical or infant formula isolates. Unlike previous *Cronobacter* projects, this study is mainly focused on *C. malonaticus* and is aimed to investigate its physiological and virulence characteristics that enable this species to survive different stresses and cause adult and neonatal infections.

This project first used a collection of 51 clinical *Cronobacter* isolates which were not speciated. The 51 strains were predominated by *C. sakazakii* ST4 (63 %, 32/51) and *C. malonaticus* ST7 (33 %, 17/51). Interestingly, despite the high clonality of *Cronobacter*, pulsed-field gel electrophoresis (PFGE) profiles differentiated strains within each sequence type into 15 pulsotypes. Furthermore, this study shows the value of applying multilocus sequence typing (MLST) to bacterial population studies with strains from two patient cohorts, combined with PFGE for further discrimination of strains.

Six sequence type 7 (ST7) strains were selected to represent the 5 *C. malonaticus* pulse types of the first part study and an additional 14 *C. malonaticus* strains were chosen from the *Cronobacter* MLST-database to represent different STs. *C. malonaticus* has shown ability to form biofilms, produce capsules, produce cellulose, express curli fimbriae, resist acid condition, survive human serum, produce siderophores, haemolysins, protease, and tolerate the used metals. The antibiotic sensitivity test revealed the sensitivity of *C. malonaticus* to the majority of antibiotics; however, *C. malonaticus* was resistant to tetracycline and some were resistant to chloramphenicol and intermediate resistant to cefotaxime. The presence of associated genes has been confirmed using the genomes of tested strains.

C. malonaticus showed an ability to adhere and invade Caco-2, HBMEC, A549 and T24 cell lines. Moreover, the result shows that certain strains of *C. malonaticus* (including 1827 and 2018) were able to persist in macrophages. However, ST7 strains 1827 and 2018 proved to be the most invasive strains among all used strains. The CDC strain 1569 (ST307) which was isolated from the blood of a fatal neonatal case showed also significant results in this study. The analysis of the genomes of tested strains revealed the presence of several virulence associated genes such as curli fimbrial genes, *apaH*, *ompA*, *ompX*, *ygdP*, *papC*, *ppk1*, *ibeB*, *Hfh*, OatA, Clp proteins and others.

Finally, the results of this project demonstrate the ability of *C. malonaticus* to overcome several stresses that could be faced either in the general environments or in the host body. Moreover, the findings of this study confirm the potential ability of *C. malonaticus* to cause serious infections in neonates or adults such as necrotising enterocolitis, meningitis, bacteremia, pneumonia and urinary tract infection.

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List of abbreviations

A549	Human lung carcinoma cells
AA	Aggregative adherence
ACT	Artemis comparison tool
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
AU	Absorbance units
BBB	Blood brain barrier
BSAC	British Society for Antimicrobial chemotherapy
BLAST	Basic local alignment search tool
bp	Base pairs
BPW	Buffered peptone water
BRIG	BLAST ring image generator
Caco-2	Human colonic carcinoma epithelial cells
CASAD diffusion	Chrome Azurol S agar
CC	Clonal complex
CLB	Cell lysis buffer
CSB	Cell Suspension Buffer
COSHH	Control of substances hazardous to health
CSF	Cerebrospinal fluid
CV	Crystal violet
DA	Diffuse Adherence
DDH	DNA-DNA hybridization
DFI	Druggan Forsythe Iversen medium
DMEM	Dulbecco's modified eagle medium
DSW	Distilled and sterilised water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EE	Enterobacteriaceae enrichment
FAO	Food and Agriculture Organization of the UN
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
HCT-8	Human colon carcinoma cells
HBMEC	Human brain microvascular endothelial cells
HCL	Hydrochloric acid
HDTMA	Hexadecyltrimethylammonium bromide
ICU	Intensive care units
IF	Infant formula
kbp	Kilobase pairs
LA	Localized Adherence
LB	Luria-Bertani
LPS	Lipopolysaccharide
MEM	Minimum Essential Medium
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
MPN	Most probable number
NEC	Necrotising enterocolitis
NICU	Neonatal intensive care unit
NTU	Nottingham Trent University
OD	Optical density

ORF	Open reading frame
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCA	Plate count agar
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PIF	Powdered infant formula
PMA	Phorbol 12-myristate 13-acetate
S	Sensitive
SDS	Sodium dodecyl sulphate
SMS	Skimmed milk solution
SNPs	Single Nucleotide Polymorphisms
Spp.	Species
ST	Sequence type
T24	Human bladder carcinoma cell line
TAE	Tris/acetate/EDTA
TBE	Tris base/ Boric acid / EDTA
TEB	Tris EDTA buffer
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TTC	Triphenyltetrazolium Chloride
U937	Macrophage Cell Line
UPGMA	Unweight pair group method with arithmetic mean
UTI	Urinary tract infection
UV	Ultraviolet
VRBA	Violet red bile agar
VRBGA	Violet red bile glucose agar
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar
α	Alpha
β	Beta

Publications

Alsonosi, A., Hariri, S., Kajsík, M., Oriešková, M., Hanulík, V., Röderová, M., Petrželová, J., Kollárová, H., Drahovská, H., Forsythe, S. and Holý, O. (2015). The speciation and genotyping of *Cronobacter* isolates from hospitalised patients, European Journal of Clinical Microbiology & Infectious Diseases, 34(10), pp. 1979-1988.

Manuscripts in preparation

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Conference presentations (posters)

A. Alsonosi, M. Kajsík, M. Oriešková, V. Hanulík, M. Röderová, J. Petrželová, H. Kollárová, H. Drahovská, S. Forsythe, O. Holý. (2015). Genotyping Hospital Isolates of *Cronobacter* from an Age Profiling Carriage Study. ASM General Meeting New Orleans, Louisiana, USA.

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A. Alsonosi and S. Forsythe. (2016). Investigating the potential pathogenicity of *Cronobacter malonaticus* as a causative agent of urinary and respiratory infections. NSCMID Annual Meeting Rovaniemi, Finland.

Chapter 1 Literature review

1.1 The genus of *Cronobacter*

The *Cronobacter* genus is a member of the large Gram-negative family, *Enterobacteriaceae*. They are Gram-negative rods, facultative anaerobic, non-spore forming and generally motile by peritrichous flagella. They are catalase positive, oxidase negative, methyl red negative, able to reduce nitrate to nitrite, and related closely to the *Citrobacter* and *Enterobacter* genera (Farmer et al., 1980; Iversen et al., 2007a).

In 1980, this organism was placed in the *Enterobacter* genus and named as *Enterobacter sakazakii* (Farmer et al., 1980). This classification was based on genotyping characteristics such as DNA-DNA hybridization (DDH) and phenotyping features such as producing of yellow pigment. The application of more phenotypic analysis revealed that *E. sakazakii* consisted of 15 biogroups. This led to a polyphasic study comprising of fluorescent–Amplified Fragment Length Polymorphisms (f-AFLP), ribotyping, 16S rDNA sequence analysis, DDH and phenotypic characterisation. Consequently, the species of *E. sakazakii* was defined to include 5 genomogroups, which were differentiated according to the division of the 15 *E. sakazakii* biogroups. Accordingly, in 2007 the *Cronobacter* genus was first defined, and this definition was subjected to more revisions in 2008 and 2012. Currently, *Cronobacter* genus consist of 7 species; *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis* and *C. condimenti* (Iversen et al., 2007a; Iversen et al., 2008; Joseph et al., 2011). It is known that *C. sakazakii*, *C. malonaticus* and *C. turicensis* are the only species associated with clinical incidence and particularly with neonatal infections so far (Stephan et al., 2010; Hariri et al., 2013; Asato et al., 2013; Holý et al., 2014).

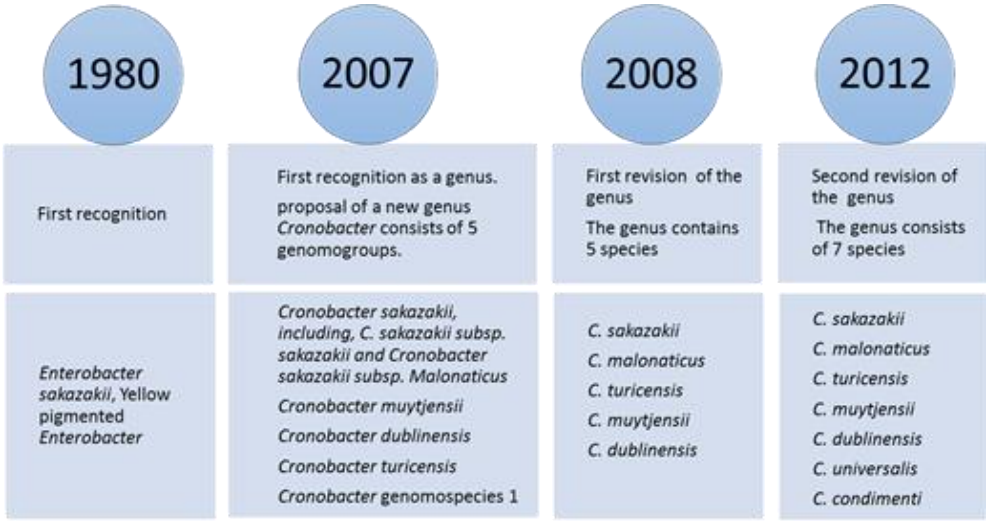


Figure 1-1 a diagram explaining the change of *Cronobacter* taxanomy over 40 years.
C. malonaticus was first recognised as subspecies under *C. sakazakii* spp. and defined as a separate species in 2008.

1.2 Source and transmission

Although plants have been proposed to be the natural habitat of *Cronobacter* spp. (Schmid et al., 2009), this organism has been found to be widely distributed. They have been isolated from environmental sources including hospitals, dust from households and food production factories and soil. In addition, *Cronobacter* has been isolated from several food types such as herbs, spices, cereals, salads, cheese, meat, tea, fruits, vegetables, water and powdered infant formula (PIF) (Iversen and Forsythe, 2004; Friedemann, 2007; Baumgartner et al., 2009). From these food sources, PIF is of most concern as a main infection source for neonatal infections (FAO-WHO, 2004; FAO-WHO, 2006).

Cronobacter has been also isolated from several clinical sources such as faeces, throat, sputum, bone marrow, urine, wound, blood and cerebrospinal fluid (CSF) (Farmer et al., 1980; Jimenez et al., 1982; Muytjens et al., 1983; Gallagher and Ball, 1991; Caubilla-Barron et al., 2007; Asato et al., 2013). According to Forsythe et al. (2014), who analysed the data in *Cronobacter* MLST database, *Cronobacter* has been isolated from 36 different countries and from various sources including environment (35.15%), infant formula (21.6%), clinical specimens (20.4%), food and ingredients (14.2%), other sources (4%) (Table 1.1). At present, the *Cronobacter* PubMLST database shows the source, geographic distribution, clinical presentation and temporal diversity of >1600 isolates of *Cronobacter* genus and contains more than 460 sequence types (ST).

Cronobacter is a foodborne pathogen; therefore, the transmission route for this organism should be via food. However, the way *Cronobacter* contaminates the different foods has not been definitively explained. It could be through people who work in the processing factories or other possible sources such as the use of contaminated water, fruits, vegetables or other plant materials. For PIF, two possible routes of contamination are considered: the first could be caused by poor handling such as the use of equipment contaminated with *Cronobacter* when PIF is reconstituted. The second route is thought to be due to the addition of unscreened raw ingredients during processing, drying or packaging (Healy et al., 2010).

Table 1-1 Summary of *Cronobacter* isolates in the *Cronobacter* PubMLST database by Forsythe et al. (2014).

Species	Number of strains (%)	Number of STs[a]	Number of genomes	Earliest isolate	Countries	Source				
						Clinical	Infant formula	Food and ingredients	Environment	Other
C. sakazakii	726 (72.1)	155	73	1950	33	19.8[b]	23.8	32.8	16.3	7.3
C. malonaticus	136 (13.5)	53	14	1973	17	36.0	16.2	36.8	7.4	3.7
C. dublinensis	59 (5.9)	44	9	1956	11	5.1	13.6	47.5	6.8	27.1
C. turicensis	41 (4.1)	26	6	1970	12	17.1	12.2	36.6	22.0	12.2
C. muytjensii	35 (3.5)	14	2	1988	10	2.9	28.6	45.7	2.9	20.0
C. universalis	9 (0.9)	5	2	1956	6	11.1	0.0	55.6	11.1	22.2
C. condimenti	1 (0.1)	1	1	2010	1	0	0	100	0	0
Total	1007	298	107		36	20.4[c]	21.6	14.2	35.1	8.7

[a]Sequence type.
[b]Percentage of species total.
[c]Percentage of genus total.

1.3 Physiology of *Cronobacter*

In general, *Cronobacter* has several physiological characteristics that enable them to present and persist under different stresses, particularly inside powdered infant formula (PIF). *Cronobacter* produces high quantities of capsular polysaccharides. The production of these capsular materials could enable the bacterium to survive desiccation. Indeed, the involvement of the capsule formation in the bacterial resistance to dissection has been confirmed by Caubilla-Barron and Forsythe, (2007), who demonstrated that capsule forming *Cronobacter* could tolerate the desiccation in dehydrated PIF for more than thirteen months (Caubilla-Barron and Forsythe, 2007). In addition, clinical isolates of *Cronobacter* have been shown to produce profuse capsular materials, and this may contribute in the formation of biofilm (Caubilla-Barron et al., 2007). The contribution of capsule production in the formation of biofilm has also been detected in the neonatal enteral feeding tubes in the neonatal intensive care units (NICU) (Hurrell et al., 2009a).

Cronobacter can grow over a wide temperature range of 6°C to 45°C, with an optimal range of 37°C to 43°C (Iversen et al., 2004). However, different studies have showed a big variation in the ability of different *Cronobacter* species to resist heating (Nazarowec-White and Farber, 1997; Breeuwer et al., 2003; Osaili and Forsythe, 2009). *Cronobacter* has been found to have a thermotolerance ability; therefore, the risk of growth of *Cronobacter* spp. in reconstituted PIF was increased. Consequently, the WHO has reviewed the guidelines for the preparation of PIF in order to minimise this risk (FAO-WHO, 2004; 2006). A study by Gajdosova et al. (2011) reported the upregulating of the 18 kbp region containing 22 open reading frames (ORFs) when *Cronobacter* isolates were

subjected to heat adaptation conditions. The genes within this cluster indicated homologies against recognised bacterial proteins that are associated with stress responses such as heat, acid stress and oxidation (Gajdosova et al., 2011; Jaradat et al., 2014). Orieskova et al. (2016) detected two configurations of the locus for this region; the first one with the size of 18 kbp harbouring the *thrB-Q* genes and a shorter version (6 kbp) only containing the *thrBCD* and *thrOP* genes. They also found that *Cronobacter* strains containing the thermotolerance region survived significantly better at 58°C comparing to a mutant lacking the region. Moreover, *Cronobacter* strains with the longer version of the region, *thrB-Q* genes, were 2–10 times more thermotolerant than those with the *thrBCD* and *thrOP* genes (Orieskova et al., 2016).

The ability of bacteria to tolerate dissection and osmotic stresses is considered as an important characteristic. Comparing with other *Enterobacteriaceae* members which are also found in PIF, *Cronobacter* in general has a high tolerance ability (Breeuwer et al., 2003; Feeney and Sleator, 2011). Caubilla-Barron and Forsythe (2007) demonstrated that these bacteria can resist desiccant conditions in the PIF for long time and grow rapidly when re-constituted. Some genes have been associated with osmoprotection in *Cronobacter* such as trehalose and betaine encoding genes which were described in all *Cronobacter* strains analysed by Joseph et al. (2012a). Trehalose synthesis has been described to be associated with the tolerance to osmotic stresses. It functions as a molecular chaperone and thus protect the cellular membranes and proteins from denaturation. Similar function of trehalose was reported in *E. coli* strains (Breeuwer et al., 2003; Horlacher and Boos, 1997). *Cronobacter* possesses also *ProP* and *OpuC, which* are the homologues of the osmoprotectants, in addition to *rpoS*, which thought to be the global transcriptional regulator (Feeney and Sleator, 2011). Several other genes that associated with desiccation and osmotic stress response have been reported. Genes included the *yihUTRSQVO* gene cluster, which are essential in the metabolism and in transport of the carbohydrates and glucuronide (Grim et al. 2013) have been identified as desiccation-related genes in *C. sakazakii* SP291 (Yan et al., 2013). In addition, the osmoprotectant ABC transporter genes, including *yehZYXW*, have been recognized to contribute in bacterial survival. Yen et al. (2013) have reported other several genes, which are responsible for the regulation of the osmotolerance. These genes included five osmotolerance regulation genes *yiaD*, *osmY*, *ompA*, *aqpZ*, and *glpF*; five genes associated with osmotic stress, namely *osmB* and *osmO*, *yciT*, *yciM*, and *pgpB* and also periplasmic glucan synthesis genes included *mdoC*, *mdoH*, *mdoG*, *mdoD*, *mdoB*, and *opgC*. Furthermore, several other genes such as colonic acid exopolysaccharide genes and cellulose biosynthesis genes might provide resistance against desiccant conditions (Grim et al., 2013).

The high acidity of human stomach could affect the survival of enteropathogenic bacteria. Therefore, the ability of surviving high acidity is an important characteristic for foodborne pathogens to cause infections in neonates and other immuno-compromised individuals. *Cronobacter* has been suggested to be more acid-tolerant than most closely related enteric pathogens and this microorganism was shown to be able to grow at a pH as low as 3.9 (Dancer et al., 2009a). Other studies confirmed the ability of *Cronobacter* to overcome the effect of the acidity and grow under a pH up to 4.2 (Johler et al., 2009; Alvarez-Ordóñez et al., 2014). Another study which was conducted by Edelson-Mammel et al. (2006) showed that *Cronobacter* tested strains were able to survive a pH at 3.5 and some strains showed only <1 log decline in their growth in this condition. Such characteristic may enable *Cronobacter* to establish intestinal infections and even tolerate the low acidity in different foods. *OmpR* gene, which encodes the transcriptional activator protein *OmpR* has been reported to play an essential role in the response of *C. sakazakii* to acid stress (Alvarez-Ordonez et al., 2014). The *ompR* defective transposon mutant showed a defect in the growth of the mutant strain in acidified broth and PIF. This defect was counteracted when this mutant strain was complemented with a functional *ompR* gene (Alvarez-Ordonez et al., 2014).

1.4 Detection, Identification methods and molecular typing

Powdered infant formula has been found to be implicated as a vehicle in the transmission of *Cronobacter* spp. to neonates in the neonatal intensive care unit (NICU) (van Acker et al., 2001; CDC, 2002; Weir, 2002; Chenu and Cox, 2009). In addition, *Cronobacter* has been involved in several serious neonatal infections as well as several adult infections. Therefore, it is important to introduce and develop efficient techniques to detect and identify *Cronobacter* species either from PIF or clinical specimens.

1.4.1 Detection and phenotypic methods

The U.S. Food and Drug Administration (FDA) in 2002 described the first regulatory detection and phenotypic method that requires pre-culturing and culturing steps, this method takes several days. In this method, a pre-enrichment step is required by mixing a specific amount of a powdered milk sample into buffered peptone water (BPW) or sterile distilled water. This is followed by enrichment into *Enterobacteriaceae* enrichment (EE) broth which incubated at 37°C for overnight. After the overnight incubation period, the sample is streaked on Violet Red Bile Glucose (VRBG) agar plates and incubated overnightly at 37°C. The plates were then examined for the presence of purple colonies which confirm the isolation of *Enterobacteriaceae*. Next, five presumptive positive colonies from VRBGA plates are streaked on Tryptone Soya Agar (TSA) plates and incubated at 25°C for 48 to 72 hours. *Cronobacter* form yellow pigmented colonies on TSA, therefore these colonies are

selected for further biochemical investigations. Finally, the API 20E biochemical identification system is used for positive identification of *E. sakazakii*, as *Cronobacter* was called at that time, and the most probable number (MPN) of the bacteria is calculated according to the bacteriological analytical manual (BAM).

The above method had some disadvantages. For example; the method allowed the growing of other *Enterobacteriaceae* members as EE is used; therefore, it was not efficiently selective for *E. sakazakii* (*Cronobacter*). Second, this method could give a false negative identification as not all *Cronobacter* can form yellow pigmented colonies. The method is also time consuming as it takes almost five days. Thus, in 2012 the FDA published another method which addresses all of these drawbacks (Figure 1.1). This method involves a pre-enrichment step in BPW and Incubation for 24 ± 2 h at 36 ± 1 °C, followed by centrifugation of the sample at 3000 g for 10 minutes. The pellet is resuspended in PBS and subjected to be investigated by streaking on the Druggan Forsythe Iversen (DFI) chromogenic agars, ID32E biochemical panel and PCR screening of *Cronobacter*. This method shortens the detection time to 24-48 hours and is more sensitive and specific for the *Cronobacter* detection comparing with the first method (Chen et al., 2010; Chen et al., 2012).

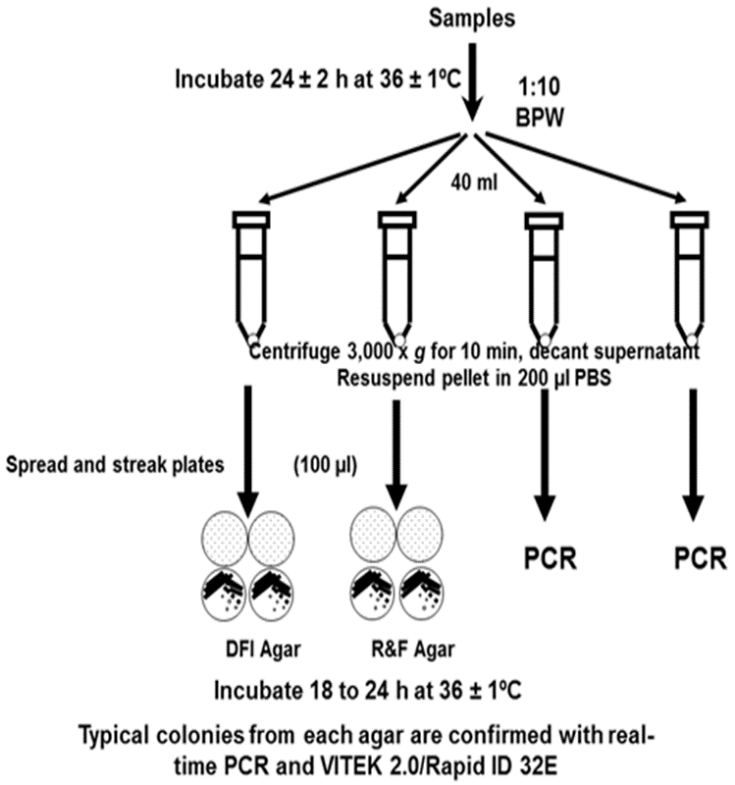


Figure 1-2 Flowchart of the complete procedure for DFI *Cronobacter* detection 2012.
R&F = chromogenic agar for E. sakazakii, VITEK = system for bacterial identification.

The International Organization for Standardization (ISO) had also introduced a specific method for the detection of *Cronobacter* spp. in milk powder and powdered infant formula. In this method, the powdered milk sample is pre-enriched into BPW and incubated at 37°C for 18 hours. For selective enrichment of *Cronobacter* spp., 100 µl of the pre-enrichment milk sample is added to 10 ml of the modified lauryl sulphate (mLST) broth and then incubated at 44°C for 24 hours. A loopful is used to streak the chromogenic *Enterobacter sakazakii* isolation agar (ESIA) plates and incubated at 44°C for 24 hours. Any typical blue green colonies of *Cronobacter* are sub-cultured on the TSA plates and incubated at 25°C for formation of yellow pigmentation and finally the yellow colonies should be further confirmed biochemically (ISO, 2006).

However, the use of biochemical test panels, such as the ID20E and ID32E, in both ISO and the FDA has made these methods insufficient and unreliable for several limitations. Firstly, ID20E and ID32E are subjectively based on visual detection of a color change when reading results and this could make the identification vary between individuals. Secondly, there is a discrepancy between the two schemes. For instance, in a study conducted by Iversen et al. (2007b), the API20E gallery positively identified only 70 % of the "*Cronobacter*" strains to species level (as *E. sakazakii*) while the ID32E gallery identified 90% of the "*Cronobacter*" strains to the species level (as *E. sakazakii*). Finally, the misidentification of *Cronobacter* strains has been continued even after the update of the ID20E and ID32E databases in 2015. For example, when API20E scheme was assessed only 82.3% of "*Cronobacter*" strains resulted in a match with the new version, while 90 % of strains resulted in a match with previous version. The assessment of ID32E revealed also that the previous versions of the ID32E database could identfied 88.9 % of strains produced a match (as *E. sakazakii*); however, when the updated version of the database is used this percentage dropped to only 43.2 % (Jackson and Forsythe, 2016).

1.4.2 Genotypic methods

A number of DNA sequence-based molecular assays have been developed for the identification of *Cronobacter* spp. Some of these methods offer an alternative means for rapid and specific identification. The screening of the partial or the full-length of the 16S rDNA region has been widely used for the identification of *Enterobacteriaceae*. Iversen et al. (2006) applied the 16S rDNA on 282 strains of *Enterobacteriaceae*, including 189 *E. sakazakii* isolates. The analysis of rDNA data showed that *E. sakazakii* strains formed four cluster groups (Iversen et al., 2006). In addition, Iversen et al. (2007a) carried out a phylogenetic analysis study which based on the use of the full-length of rDNA sequence (greater than 1300 bp). The result of this study proposed the reclassification of the *E. sakazakii* group in a new genus (Iversen et al., 2007a). Nevertheless, some limitations were appeared when 16S rDNA technique was applied on *Cronobacter* closely related bacteria such as *E.*

cloacae, *E. hormaechei* and *E. asburiae* (Iversen et al., 2007b). Moreover, 16S rDNA could not distinguish between *C. sakazakii* and *C. malonaticus* as it demonstrated 99.7% resemblance between the two species and accordingly *C. malonaticus* was considered as subspecies of *C. sakazakii* (Iversen et al., 2007a).

RNA polymerase beta-subunit encoding gene (*rpoB*) sequences has been proposed as a technique for universal bacterial identification (Mollet et al., 1997; Adekambi et al., 2009; Stoop et al., 2009). The *rpoB* PCR has been developed by Stoop et al. (2009) in order to cover all the 7 species of *Cronobacter*. The screening of *rpoB* provided more easily and reliable identification than 16S rRNA sequencing (Li et al., 2012). However, the technique was reported to give false-positive identifications as *Cronobacter* during an outbreak (Jackson et al., 2015). The sequencing of *Cronobacter* outer membrane protein A (*OmpA*) which plays an important role in the invasion of the human cells has been used also for identification purpose (Fei et al., 2015). *In silico* analysis of the *ompA* gene using about 187 *Cronobacter* genomes and 20 other genomes represent the closely related bacteria revealed that this method could be sufficient for identifying all *Cronobacter* species (Jackson and Forsythe, 2016).

Other genetic fingerprinting techniques have been used for epidemiological investigations. These include random amplified polymorphic DNA (RAPD), BOX-PCR and pulsed-field gel electrophoresis (PFGE). PFGE is commonly used and considered as a gold standard technique in epidemiological studies of several pathogenic organisms (Olive and Bean, 1999). The technique is based on the use of restriction enzymes such as *XbaI* and *SpeI* to cut the bacterial DNA into several fragments and then separate these fragments into agarose gel electrophoresis (Caubilla-Barron et al., 2007). The discriminating results of the DNA fragments by gel electrophoresis reveal the association of the environmental or food isolates with clinical incidences, and thus allows tracking infection sources. For instance, Caubilla-Barron et al., (2007) used PFGE to investigate thirty *Cronobacter* strains which were isolated from a fatal outbreak that occurred in a neonatal intensive care unit in France during 1994. The result of this investigation revealed that the three fatal *Cronobacter* strains belonged to the same pulse type. In addition, there was no correlation between isolates from the used PIF and the fatal cases (Caubilla-Barron et al., 2007). However, this technique has some limitations such as expense, laborious, time consuming and inability of some strains to generate DNA that is suitable for enzymatic digestion when contained in agarose plugs (Sood et al., 2002).

In Gram-negative bacteria, the outer region of the lipopolysaccharide (LPS) molecules contains O-antigen is used for serological diversity. The serotyping classification technique is based on the differences in O-antigen structures. The O-antigen encoding region which is a highly variable and

located between *galF* and *gnd*. This method has been found to be a useful strategy for the bacterial characterisation, risk assessment of pathogenic strains and is commonly applied for monitoring the outbreaks (Blažková et al., 2015). There have been several molecular serotyping studies which covered the most of *Cronobacter* species (Jarvis et al., 2011; Ogrodzki and Forsythe 2015). Blažková et al. (2015) determined 24 serotypes for the *Cronobacter* genus; of these *C. sakazakii* O2, O1, and O4, *C. turicensis* O1, and *C. malonaticus* O2 serotypes are particularly predominant in the clinical cases (Blažková et al., 2015). However, Ogrodzki and Forsythe, (2015) analyzed 104 whole genome sequenced *Cronobacter* strains and have proposed, based on a presumed O-antigen specific sequences, *wzx* and *wzy* sequences, 38 subgroups of *Cronobacter* as potential serotypes.

Multiple housekeeping genes like those using multilocus sequence typing scheme (MLST) were used for identification of bacteria since 1998 (Maiden, 2006; Lacher et al., 2006). The generated MLST data have been employed in the epidemiological investigations and in the studies of the pathogenicity, population biology, and evolution of bacteria (Maiden, 2006; Joseph and Forsythe, 2012). Baldwin et al. (2009) constructed a specific MLST scheme for *Cronobacter*. This scheme was initially applied for distinguishing between *C. sakazakii* and *C. malonaticus*, the two species which were considered as one species and could not be distinguished by using 16S rDNA. Currently, the MLST scheme covers all the seven *Cronobacter* species and is available online at http://www.pubMLST.org/*cronobacter*. This scheme is based on seven housekeeping genes which are *atpD, fusA, glnS, gltB, gyrB, infB,* and *ppsA*. The selection of these genes was based on several criteria. These genes were considered to be encoding for putative housekeeping products necessary for biological roles in DNA repair, replication and amino acid biosynthesis. In addition, to ensure that each locus was genetically unlinked, thus the selected genes were distributed as much as possible across the bacterial chromosome. Moreover, any genes that could come under greater selective evolutionary pressures such as being putative virulence factors or mobile elements were excluded (Baldwin et al., 2009). The distinguishing between *Cronobacter sakazakii* and *Cronobacter malonaticus,* as they were considered as one species, become obvious after the use of the MLST scheme. Moreover, the 7-loci MLST scheme has been proved to be a reliable and robust after being compared with the 53-loci ribosomal MLST (rMLST) and the 1865-loci core genome MLST (COG-cgMLST) (Forsythe et al., 2014). Therefore, the technique was used to widen the knowledge about *Cronobacter* diversity and evolution (Joseph and Forsythe, 2012; Joseph et al., 2012b).

1.5 Incidences and outbreaks

Clinical species of *Cronobacter* are opportunistic pathogens that can cause rare but severe infections. Elderly and immunocompromised individuals have been found to be infected by *Cronobacter* species and the infections vary from lenient to serious illnesses. This includes wound infection, urine infection, osteomyelitis, bacteraemia and septicaemia (Jimenez et al., 1982; Pribyl et al., 1985; Hawkins et al., 1991; Lai, 2001; Dennison and Morris, 2002; Bhat et al., 2009). However, *Cronobacter* can cause serious infections in adults who are non-immunocompromised (See et al., 2007). Nosocomial infections in adults by *Cronobacter* such as pneumonia, urinary tract infection (UTI) and conjunctivitis are all believed to be due to contact with medical equipment and health care providers who may serve as a possible source of *Cronobacter* (Fridemann, 2009; Flores et al., 2011). Other environmental sources of contamination infections are also plausible as the organism has been isolated from household vacuums, spices and other dried food (Jaradat et al., 2009).

Infections among immunocompromised infants and premature neonates are life-threatening, and have several features such as septicaemia, meningitis and necrotizing enterocolitis (NEC) and thus neurological sequelae can be permanent. Though the incidence of *Cronobacter* infections is rare, the mortality rate is documented to be as high as 40-80 % (Muytjens et al., 1983; Lai, 2001; Iversen et al., 2008; Healy et al., 2010; Joseph and Forsythe, 2011). The FAO-WHO (2006) estimated that in the USA the annual incidence among low birth weight infants was 8.7 per 100000. However, the WHO (2008) report which tracked cases from 1961 to 2008 showed that 120 cases have been recorded among infants and children who were less than three year old. Despite the WHO reports for the incidence of neonatal *Cronobacter* infections, it is believed that the actual number of neonatal *Cronobacter* infections is considered to be far higher (CDC, 2009; Fridemann, 2009; Teramoto et al., 2010). The sources of neonatal infections in neonatal intensive care units due to *Cronobacter* have been epidemiologically strongly linked to contaminated PIF and not supporting the role of the birth canal in the occurrence of these infections (Himelright et al., 2002; Teramoto et al., 2010).

Since 1961 several neonatal *E. sakazakii* (*Cronobacter*) incidences were reported in the UK, Denmark, Georgia and Oklahoma when five cases were detected and four died in all incidences (Urmenyi and Franklin, 1961; Joker et al., 1965; Monroe and Tift, 1979; Adamson and Rodgers, 1981). In 1983 Muytjens et al. reported the detection of eight cases of neonatal meningitis and sepsis due to *E. sakazakii*, six infants died, and PIF was suspected to be the main infection source. Another notable outbreak was reported in Greece in 1984 by Arseni et al. (1987), when 4 infants, out of 11, had clinical signs of severe sepsis and died; however, the infection source was unknown.

In France in 1994, thirty-one *Cronobacter* isolates were taken from 16 infants including sputum, faeces, skin, peritoneal fluid, and conjunctivae in addition to PIF tins. During this outbreak three infected infants died, one due to meningitis and others from necrotizing enterocolitis (NEC) (Caublilla-Barron et al., 2007). Interestingly, the performance of PFGE showed that the fatal *Cronobacter* strains were clustered in the same group (pulsotype 2). This cluster 2 belongs to ST4 *C. sakazakii* or to clonal complex 4 (CC4) which has been associated predominantly with neonatal meningitis (Caubilla-Barron et al., 2007; Masood et al., 2015). In Belgium in 1998, twelve *Cronobacter* infection cases were reported at the NICU of Ziekenhis Vrije University hospital and the infection source was linked to PIF. The twelve infected cases were low birth weight infants who had been fed with PIF and subsequently diagnosed with NEC. This was the first report that highlighted the association of *Cronobacter* with NEC (Van Acker et al., 2001). In 2001, in Tennessee another outbreak was reported when a total of 49 infants were screened due to the death of one neonate who had meningitis. Eight infants were found to be positive for *Cronobacter* colonisation or infection, two had respiratory infections and six were asymptomatic (Himelright et al., 2002).

Another outbreak of *Cronobacter* was reported by Block et al. (2002) in Hadassah University Hospital in Jerusalem. In this outbreak *Cronobacter* strains were isolated from five neonates, three of them were diagnosed with bacteraemia and meningitis and two showed no symptoms. Interestingly, *Cronobacter* strains were isolated from a blender used for preparing infant formula as well as from prepared formula. However, PFGE analysis of these outbreak isolates revealed the presence of a strong correlation between the blender and prepared formula isolates and neonatal isolates (Block et al., 2002). Two outbreaks in 2004 were reported in the New Zealand and France with five and four cases reported respectively. The source behind both outbreaks was found to be PIF. The final outcome showed the death of three neonates one from the New Zealand outbreak and two from the France outbreak (Drudy et al., 2006).

In December 2011 the Centres for Disease Control and Prevention (CDC) reported the incidence of four infant *Cronobacter* infections in four states; Florida, Missouri, Illinois and Oklahoma. Three infants developed meningitis and one had bacteraemia and as a result two died and two recovered (CDC, 2012a). The investigations by CDC in Illinois revealed that *Cronobacter* bacteria were found in an opened container of bottled nursery water used to prepare the infant formula. In Missouri *Cronobacter* bacteria were found in an opened container of powdered infant formula, two prepared bottles of powdered infant formula, and an opened bottle of nursery water used to reconstitute the powdered infant formula given to the infant. The PFGE of the *Cronobacter* strains which were obtained from the infant, the formula, and the used water were indistinguishable from each other (CDC, 2012a).

The *Cronobacter* MLST scheme has been used to analyze and study the diversity of the *Cronobacter* genus (Joseph and Forsythe, 2012; Joseph et al., 2012b). This scheme has been applied by the NTU pathogenic research group on the all available *Cronobacter* strains involved in the previous outbreaks (CDC, 2012a). This has revealed that *C. sakazakii* ST4 is more frequently associated with neonatal infections. However, other *C. sakazakii* STs such as ST1, ST8 and ST12 were also identified, less frequently, as a cause of neonatal infection (Joseph and Forsythe, 2011; Hariri et al., 2013; Masood et al., 2015). Interestingly, one blood isolate was identified as *C. malonaticus* ST307. This strain was isolated from an infant <1 month of age with meningitis who could not survive the infection (Hariri et al., 2013).

In 2014 Patrick and his colleagues investigated the incidence of *Cronobacter* infections in the USA from 2003 to 2009. They identified 544 *Cronobacter* cases from participating laboratories in 6 USA states and the patient age ranged from 1 day–100 years. Urine was the most frequent site of *Cronobacter* infection by 221 isolates (41%). This was followed by wound and respiratory infections; 115 isolates 21% and 87 isolates 16% respectively. The highest percentage of invasive infections occurred among children <5 years of age and the overall incidence rate of *Cronobacter* infection was higher than anticipated, 0.66 cases per 100,000 populations (Patrick et al., 2014).

C. malonaticus particularly *C. malonaticus* ST7 has reported to be more associated with adult infections (Joseph and Forsythe, 2011; Joseph and Forsythe, 2012). However, several severe and fatal neonatal *C. malonaticus* infections have been reported since 2011. The first documented fatal neonatal case caused by *C. malonaticus* was in the USA. The bacteria were isolated from the blood of an infant <1 month of age who was diagnosed with meningitis and could not survive the infection. This fatal *C. malonaticus* strain was identified as *C. malonaticus* ST307 (Hariri et al., 2013). In 2013 Asato and colleagues reported two fatal cases of neonatal infections associated with *C. malonaticus* in Argentina, which occurred between 2009 and 2010. The PFGE analysis of these two *C. malonaticus* isolates showed a genetic relationship between the two isolates (Asato et al., 2013). In Brazil in 2013 an outbreak of three *Cronobacter* cases was reported by Brandão et al. (2013) in a maternity hospital at Teresina. The *Cronobacter* isolates were obtained from the blood of infected neonates diagnosed with bacteraemia and were identified as *C. malonaticus* ST394 and ST440 (Brandão et al., 2013; *Cronobacter* PubMLST database). Recently, another *C. malonaticus* neonatal case has been reported in China. It was a female infant at 11 days of age who was fed by breast milk and supplementary powdered-milk formula. The infection agent was isolated from CSF and identified as *C. malonaticus* ST60. This strain was tested against nine antibiotics and detected to be resistant to six of them (Unpublished data, China).

1.6 Pathogenicity and virulence factors of *Cronobacter*

In spite of the recent increase in research on *Cronobacter* due to the increase of awareness of the infections associated with these bacteria, the precise mechanism of the pathogenicity is still under investigations. Epidemiological and *in vitro* tissue culture studies have shown variable virulence abilities of *Cronobacter* isolates (Caubilla-Barron et al., 2007; Townsend et al., 2007a, 2008; Almajed and Forsythe, 2016). *C. sakazakii*, *C. malonaticus* and *C. turicensis* are the main associated species with neonatal infections (Kucerova et al., 2010). However, several potential virulence factors have been determined, which assist for understanding the pathogenicity of *Cronobacter* (Kucerova et al., 2010; Stephan et al., 2010).

Cronobacter has been confirmed to produce enterotoxins as putative virulence factors. The effect of *Cronobacter* enterotoxins was lethal when tested using a suckling mice assay. In addition to the production of enterotoxins, *Cronobacter* were also able to secrete proteolytic enzymes that cause tissue destruction to the infection site in the same mice (Pagotto et al., 2003). Similar cell damage occurred by a zinc metalloprotease inducing rounding of the Chinese hamster ovary (Kothary et al., 2007). *Cronobacter* endotoxin has shown to enhance the translocation of *C. sakazakii* to the blood brain barrier (BBB). This is likely due to the disruption of tight junction by lipopolysaccharides (LPS) which frequently contaminate PIF (Moriez et al., 2005; Townsend et al., 2007b). This could cause an increase in the permeability of the bacteria to epithelial and endothelial barriers (Kim and Loessner, 2008). Moreover, endotoxin has been also found to inhibit the enterocyte migration and epithelial restitution and this enhances cell adhesiveness which could increase the permeability of the bacteria to the epithelial and endothelial cells (Cetin et al., 2004).

The gastrointestinal tract is thought to be the main colonisation site for *Cronobacter* (Mohan Nair and Venkitanarayanan, 2007). Consequently, the bacteria invade the enterocytes or translocate via the lamina propria to reach the bloodstream and then to other sites like brain cells causing meningitis. One of the factors which could contribute in adhesion is the presence of fimbriae. Fimbriae are the appendages that help pathogenic bacteria to adhere to the surface of host cells and thus helping them with colonisation which is the first and essential step for bacterial pathogenicity (Soto and Hultgren, 1999). *Cronobacter* possess at least ten putative fimbrial clusters. Of these, curli fimbriae have been reported to be involved in *E. coli* adhesion to the host cells. However, curli fimbriae are absent in *C. sakazakii* and present in *C. malonaticus* which indicates that the curli fimbriae could have a role in the pathogenicity of *C. malonaticus* but not *C. sakazakii* (Joseph et al., 2012a).

After the adhesion to the intestinal cells, *Cronobacter* starts to invade using several factors in this process. *OmpA* has been found to play an essential role in the adhesion and invasion of Caco-2 and HBMEC (Franco et al., 2011a). It has been demonstrated that the outer membrane proteins *ompA* and *ompX* are both required for the basolateral invasion of the enterocyte-like human epithelial cells by *Cronobacter* (Kim et al., 2010). In addition, it has also been demonstrated that *ompA* expression is required for the entry of *Cronobacter* into HBMEC. Moreover, it was shown that *ompA* of *Cronobacter* is recognised as a major fibronectin-binding protein that promotes the invasion of HBMEC cells (Mohan Nair et al., 2009). However, the *in vitro* invasion ability of *Cronobacter* into the epithelial and endothelial cells was varied between *Cronobacter* strains which were positive for both *ompA* and *ompX* genes (Townsend et al., 2007a, 2008; Almajed and Forsythe, 2016).

The ability of *Cronobacter* to avoid immune response is crucial for a successful invasion. *Cronobacter* has been found to persist within human macrophages although survival varied between species and even strains within one species (Townsend et al., 2007a, 2008; Almajed and Forsythe, 2016). Additionally, *Cronobacter* plasminogen activator (*cpa*) has been found to be important in the tolerance of *Cronobacter* to human serum and thus *cpa* is overexpressed following the entry of *C. sakazakii* systematically (Franco et al., 2011a; Schwizer et al., 2013). The *cpa* gene is present in *C. sakazakii* but not *C. malonaticus*. Other surface materials such as lipopolysaccharides (LPS), *OmpA* and capsules, which are thought to play important roles in the tolerance of *Cronobacter* to human serum, need more investigations to be clarified (Schwizer et al., 2013).

Several plasmid and chromosomal virulence genes have been identified in *Cronobacter* species. The analysis of *C. sakazakii* ATCC BAA-894 genome revealed the presence of some virulence genes such as haemolysins *(hly)*, siderophore-interacting protein gene (*sip*) (Kucerova et al., 2010; Cruz et al., 2011). The complete efflux system which consists of 4 genes (*cusA. cusB, cusC* and *cusF*) with its regulatory gene (*cusR*) has been proved to be present in some *Cronobacter* strains while absent in others (Kucerova et al., 2010). Franco et al., (2011b) also found that some *Cronobacter* strains have siderophore-mediated iron acquisition system (*iuc*ABCD and *iutA*) and ABC transport-mediated iron uptake, both are involved in systematic survival of *Cronobacter*. Type IV and VI secretion systems also have been identified in *Cronobacter*; however, type VI secretion system thought to have an important role as a virulence factor (Franco et al., 2011b; Joseph et al., 2012a). Other genes such as the putative *sodA* gene which involved in intracellular persistence are found in all *Cronobacter* spp. (Kothary et al., 2007; Townsend et al., 2007a; Joseph et al., 2012a). Sialic acid utilization genes *nanAKT* have been found to present particularly in all *C. sakazakii* and in some *C. turicensis STs* such as ST32, ST35, ST72, ST114, ST118, ST126, ST127, ST342 and ST344 (Joseph et al., 2013, Hariri 2015,

unpublished data). Sialic acid is present in PIF, breast milk and brain, therefore *Cronobacter* pathogenicity could be indirectly enhanced by the present of this substance.

The formation of biofilm by pathogenic microorganisms plays an important role in surviving some environmental stresses such as drying and antimicrobial agents. Many factors could involve in the biofilm formation on the surface of medical equipment or even infected tissue (Annous et al., 2009). In the food industry, the formation of biofilm by bacteria is of high concern as these biofilms could act as reservoirs for spoilage and contamination of the food products (Lehner et al., 2005; Hartmann et al., 2010). Clinically, bacterial biofilm has appeared as a significant clinical problem. Biofilm could increase antibiotic resistance, increase medical device related infections, increase persistence of chronic infection and diminish the host immune function by immunomodulation (Manavathu and Vazquez, 2014). Bacterial capsular polysaccharides (CPS) are considered as major virulence factors and environmental fitness traits. *Cronobacter* produces CPS which facilitate the formation of biofilms on the inert surfaces and subsequently become more resistant to cleaning and disinfectant agents (Hurrell et al.,2009b; Iversen and Forsythe, 2003). Several sources of *Cronobacter* in the hospital environment have been reported include formula preparation equipment, feeding bottles and neonatal enteral feeding tubes (Kim et al.,2006; Hurrell et al., 2009a; Iversen and Forsythe, 2003). The latter might act as a site for neonatal infection in hospitals because of the biofilm formation which make *Cronobacter* resistant to cleaning and disinfectant agents (Hurrell et al., 2009b; Hurrell et al., 2009a).

The capsular profiling of the *Cronobacter* genus by Ogrodzki and Forsythe, (2015), which based on O-antigen, K-antigen (K) and colanic acid (CA), revealed the presence of various capsular polysaccharide structures between *Cronobacter* strains. This variation could be relevant to evade host response mechanisms such as phagocytosis as well as facilitate biofilm formation and desiccation survival. The researchers found that two sequence types *C. sakazakii* CC4 and ST12 which are strongly associated with severe neonatal infections (meningitis and NEC) have the same capsular profile, K2:CA2. In addition, *C. malonaticus* ST307 strain 1569 possesses the same capsular profile, K2:CA2, as *C. sakazakii* CC4. This may be highly significant since strain 1569 was responsible for a fatal meningitis neonatal case (Hariri et al., 2013). In contrast, they have also found that *C. malonaticus* ST7 which is the most frequently isolated *C. malonaticus* sequence type and reported to be more associated with adult infections (Forsythe et al., 2014; Holý et al., 2014) has the capsular profile K1:CA1.

A number of recent potential virulence factors have been proposed using proteomic studies. The production of outer membrane vesicles OMVs could have a role in bacterial pathogenicity.

Alzahrani et al. (2015) identified a total of 18 OMV-associated proteins using mass spectrometry in addition to evaluated the potential pathogenicity roles of these proteins. Their results indicate that *C. sakazakii* OMVs could play a role in pathogenesis by delivering bacterial toxins into host epithelial cells, driving proliferative and proinflammatory responses (Alzahrani et al., 2015).

Another study by Du et al. (2015) identified 89 protein spots; however, their analysis suggested the involvement of 11 proteins in the pathogenesis of *C. sakazakii*. These proteins are; Chaperone Hsp60, GroEL; Molecular chaperone Hsp90, *HtpG*; ATP-dependent chaperone protein, *ClpB*; GTP-binding protein, TypA; Flagellar biosynthesis protein, *FliC*; Putative virulence factor, *SrfC*; Tellurium resistance proteins, *TerADEXZ* (Du et al., 2015). The high expression of these 11 genes was detected in the high virulence strains but not into the low virulence *C. sakazakii* strains. Authors suggested that the genes were absent or present as silent genes in the low virulence *C. sakazakii* strains. However, *terADEXZ* genes which are known as tellurium resistance associated genes were not detected in the *C. sakazakii* isolates which were associated with neonatal meningitis (Joseph et al., 2012a). In a previous study by the same group GroEL was detected at the cell surface of *C. sakazakii* (Wang et al., 2013). This protein has been reported to contribute to both adhesion and invasion of the host cells (Zhu et al., 2013). The heat shock response protein, HtpG, has been also reported to play roles in the pathogenicity of *Edwardsiella tarda*, *Francisella tularensis* and *Leptospira interrogans* (Dang et al., 2011; Weiss et al., 2007; King et al., 2013). Recently, *ClpB* protein has been also suggested to have a pathogenic role in *Leptospira interrogans* isolates (Zeng et al., 2015). In addition, *FliC* gene which encodes flagellin, a main protein of the flagella, plays essential roles in the adhesion and invasion of *E. coli* into host cells (Karam et al., 2013; Duan et al., 2013). Moreover, TypA is a translational GTPase that functions as a virulence regulator in some microorganisms, such as *S. enterica*, *E. coli* and *Pseudomonas aeruginosa* (Sabbagh et al., 2012; Grant et al., 2003; Neidig et al., 2013). Finally, though the exact functions of the putative virulence effector *SrfC* remain unclear the sequence alignment indicated that this protein is widely present in *Cronobacter* (Du et al., 2015).

Another study by Jing et al. (2015) showed that *Cronobacter* alters their gene expression profiles in relation to cellular metabolic processes. Their results indicated that 139 genes were upregulated and 72 genes were downregulated in the adherent *C. sakazakii* ATCC BAA-894 strain on HCT-8 cells. In this study, the expressions of some flagella genes and virulence factors involved in adherence, high osmolarity and osmotic stress-associated genes, genes responsible for the synthesis of lipopolysaccharides and outer membrane proteins, iron acquisition system and glycerol and glycerophospholipid metabolism genes were upregulated. For example, flagellar genes *flgA*, *flgB*, *flgC*, *flgD*, *flgF*, *flgG*, *fliE*, *flgE* and *fliK* were upregulated. Also, lipid metabolism genes such as *glpD*,

encoding glycerol kinase and *glpQ*, encoding glycerophosphoryl diester phosphodiesterase, were upregulated. In addition, lipopolysaccharide and outer membrane associated genes such as *mltD*, encoding membrane-bound lytic murein transglycosylase D, *lpxP*, encoding palmitoleoyl transferase involved in biosynthesis of lipid A, *yidC*, encoding membrane protein insertase *YidC*, *eptB*, encoding phosphoethanolamine transferase involved in biosynthesis of lipid A, and *surA*, encoding peptidyl-prolyl cis-trans isomerase *SurA* were also upregulated. Genes included *fepE* encoding ferric enterobactin transport protein, *entC* encoding isochorismate synthase and iron-regulated, aerobactin operon genes *iucABCD* and *iutA* involved in the biosynthesis of siderophores were also upregulated. Moreover, the osmotic regulator *betB*, encoding betaine-aldehyde dehydrogenase, was also upregulated (Jing et al., 2015). Hence, upregulation of all of these genes which associated with glycerol and glycerophospholipid metabolism, osmotic stress, iron acquisition mechanism, motility, lipopolysaccharide and outer membrane proteins, virulence factor of adherence and invasion to the host cells seems to be important to the pathogenicity of *C. sakazakii* in the human gastrointestinal tract (Jing et al., 2015).

Ye et al. (2015; 2016) have identified more novel potential virulence factors such as adhesion and invasion in *Cronobacter* using proteomic analysis. In 2015, they identified four novel potential virulence membranous proteins including osmolarity sensory histidine kinase *envZ*, LPS-assembly lipoprotein *lptE*, putative multidrug resistance protein *MdtD* and osmotically inducible protein *OsmY*. All of these proteins were upregulated in the virulent *C. sakazakii* isolate but not in the attenuated strain (Ye et al., 2015). In 2016, they also identified more four potential virulence factors, DNA protection during starvation protein Dps, s-ribosylhomocysteinelyase *LuxS*, ATP-dependent Clp protease *ClpC*, and ABC transporter substrate-binding proteins which contribute in the virulence of *C. sakazakii* through enhancing the bacterial adhesion, bacterial invasion, tolerance to environmental stresses and host immune response (Ye et al., 2016). A summary of virulence genes in *C. Sakazakii* and *C. malonaticus* is shown in table 1.2.

Table 1-2 Summary of virulence genes in *C. sakazakii* and *C. malonaticus*.

Gene name	Possible role in virulence	*C. sakazakii*	*C. malonaticus*
Zinc metalloprotease, *zpx*	Protease enzyme	yes	yes
Curli fimbriae region	Adhesion to the host cells	no	yes
Outer membrane protein A, *ompA*	Adhesion and invasion	yes	yes
Outer membrane protein A, *ompX*	Adhesion and invasion	yes	yes
Cronobacter plasminogen activator, *cpa*	Resistance to serum	Yes, not in all	no
Haemolysins, *hly*	Haemolysins	yes	yes
Siderophores-interacting protein gene, *sip*	Involve in systematic infections	yes	yes
Efflux system, *cusC*	Macrophage survival	yes	yes
Iron acquisition system (iucABCD and iutA)	Involve in systematic infections	yes	yes

Type IV secretion systems	Translocate virulence factors into eukaryotic cells	yes	yes
Type VI secretion systems	Transport of proteins or domains directly into target cells	Yes, not in all	no
Superoxide dismutase, *sodA*	macrophage survival	yes	yes
Sialic acid utilization region	Involve in systematic infections	yes	Yes, not complete
Chaperone Hsp60, *GroEL*	Involves in the adhesion and invasion of host cells	yes	yes
Molecular chaperone Hsp90, *HtpG*	Cope with various stress conditions during infection	yes	yes
ATP-dependent chaperone protein, *clpB*	Virulence-related functions	yes	yes
GTP-binding protein, TypA	Virulence regulator	yes	yes
Putative virulence factor, *srfC*	Remain unclear	yes	yes
Flagellar genes	Play roles in the adhesion and invasion into host cells	yes	yes
Lipid metabolism genes, *glpD* and *glpQ*	Involves in the invasion and adherence of host cells	yes	yes
Membrane-bound lytic murein transglycosylase D, *mltD*	Enhances lethality in zebra fish	yes	yes
Palmitoleoyl transferase involved in biosynthesis of lipid A, *lpxP*	Contributes ininvasion across blood-brain barrier	yes	yes
membrane protein insertase, *yidC*	Roles in resistance to host cell antimicrobial responses	yes	yes
Phosphoethanolamine transferase involved in biosynthesis of lipid A, *eptB*	Contributes in invasion across blood-brain barrier	yes	yes
Peptidyl-prolyl cis-trans isomerase, *surA*	Involved in the maturation of *ompA*	yes	yes
Ferric enterobactin transport protein, *fepE*	Involve in systematic infections	yes	yes
Isochorismate synthase and iron-regulated, *entC*	Involve in systematic infections	yes	yes
Osmotic regulator, *betB*	Macrophage survival	yes	yes
Osmolarity sensory histidine kinase, *envZ*	Influence bacterial biofilm formation and flagella motility	yes	yes
LPS-assembly lipoprotein, *lptE*	Immunogenicity with host cells	yes	yes
Putative multidrug resistance protein, *mdtD*	Contributes in resistince to host defences	yes	yes
Osmotically inducible protein, *osmY*	Associated with virulence behavior	yes	yes
DNA protection during starvation protein, *Dps*	Enhance oxidative stress resistance and virulence	yes	yes
s-ribosylhomocysteinelyase, *luxS*	Contributes in the invasion	yes	yes
ATP-dependent Clp protease, *clpC*	Contributes to adhesion and invasion	yes	yes

1.7 Antimicrobial Resistance

Cronobacter is naturally resistant to all macrolides, lincomycin, clindamycin, streptogramins, rifampicin, fusidic acid and fosfomycin. However, they were found to be the most susceptible bacteria comparing with the other *Enterobacteriaceae* (Muytjens and van der Ros-van de Repe, 1986). In 2002, Stock and Weidemann studied some *Cronobacter* strains and found similar results to Muytjens's result when all used strains were susceptible to all β-lactams. Consequently, *Cronobacter* infections were recommended to be treated mainly by ampicillin, gentamicin and /or chloramphenicol. However, in 1988, a combination treatment of ampicillin and gentamicin was advised by Willis and Robinson, (1988).

As other Gram-negative bacteria, antibiotic resistance has been started to be reported among *Cronobacter*. Since 1999, several reports have described the presence of the resistance of *Cronobacter* strains to chloramphenicol and tetracycline (Nazarowec-White and Farber, 1999; Kilonzo-Nthenge et al., 2012; David et al., 2013, Prakash and Sharma, 2013). Resistance to β-lactam antibiotics has been also reported in several studies. Low-level production of β-lactamase by *Cronobacter* has been firstly noticed by Pitout et al. (1997). Lai (2001) also found that *Cronobacter* strains were able to resist ampicillin, cefazolin and extended spectrum penicillins. Thus, β-lactamase activity among *Cronobacter* has been frequently reported by some studies (Pitout et al., 1997; Caubilla-Barron et al., 2007; Zhou et al., 2011; Prakash and Sharma, 2013). A recent study showed that about 10.2% of the tested *Cronobacter* strains were resistant to cefotaxime which is one of the third-generation of cephalosporins, related to penicillin (Pan et al., 2014). Although the overall level of the antibiotic resistance of *Cronobacter* is still considered low comparing with other food-borne pathogens, it needs more consideration. This resistance thought to be due to antibiotic overuse (Lee et al., 2012). Nevertheless, some antibiotics such as gentamicin, kanamycin and ciprofloxacin have demonstrated the effectiveness against *Cronobacter* and therefore they might be used in the treatment of serious *Cronobacter* infections (Al-Nabulsi et al., 2011).

1.8 Genome studies

The importance of *Cronobacter* has increased since it has been found to be involved in serious neonatal infections, such as NEC and meningitis, when the mortality rate ranges from 40 to 80%. *Cronobacter* contaminate the PIF either during processing in PIF factories or during the reconstitution of PIF, and subsequently transfer into the vulnerable infants to cause serious infections. Therefore, many studies, such as genome comparative studies, have been conducted to reveal the molecular physiological and virulence characteristics that enable this genus to survive deferent conditions and cause diseases.

Bacterial genome studies were started 20 years ago when Fleischmann et al. (1995) and Fraser et al. (1995) completed sequencing two genomes. However, after 3 years of introducing the genus of *Cronobacter*, Kucerova et al. (2010) published the first comparative genome analysis of *C. sakazakii* strain BAA-894 and other 9 *Cronobacter* strains. Kucerova et al. (2010) study revealed that gene acquisition and other mobile elements play an essential role in *Cronobacter* diversity. In addition, genes associated with several putative physiological and virulence traits such as desiccation resistance genes, protease activity, type 6 secretion system (T6SS), iron acquisition genes and invasion factors were identified. Moreover, a number of genes associated with neonatal infections that linked to invasion of the blood-brain barrier (BBB) by neonatal meningitic strains of *Escherichia coli* were identified in *C. sakazakii*, *C. malonaticus* and *C. turicensis* (Kucerova et al., 2010).

A year later Joseph et al. (2012a) conducted a comprehensive genome analysis that covers the seven species of *Cronobacter*. This study linked the differences in gene content with the clinical or environmental host of species and sequence types. Moreover, the study investigated the physiological, phenotypic and virulence associated genes in the 7 used species. A clear variation was observed within *Cronobacter* species for the presence of the virulence associated traits such as adhesins, T6SS and heavy metal resistance. However, the study could not find any unique virulence genes for *C. sakazakii* ST4 to indicate its dominance in causing neonatal meningitis.

Another genome analysis study was conducted by Grim et al. (2013), which covered 6 species of *Cronobacter*. The study revealed the divergence of *Cronobacter* into two clusters, the first group included *C. dublinensis* and *C. muytjensii* and the second consisted of *C. sakazakii*, *C. malonaticus*, *C. turicensis* and *C. universalis*. The first group is more associated with environmental host as they contained a number of genomic regions which are important for such survival. The second group, which included *C. malonaticus*, contained numerous virulence genes that enable them to survive the human host.

Several plasmids were recognised in some completed genomes of *Cronobacter*. *C. sakazakii* BAA-894 has been found to harboured two plasmids which are pESA2 (31 kb) and pESA3 (131 kb) (Kucerova et al., 2010). Stephan et al. (2010) also sequenced the clinical *C. turicensis* z3032 strain and revealed that this strain had three plasmids pCTU1 (138kb), pCTU3 (53kb) and pCTU2 (22kb). The associated of *C. sakazakii* BAA-894 and *C. turicensis* z3032 with fatal neonatal cases lead to a genomic study which conducted by Franco et al. (2011b) to compare the two large size plasmids from the two strains. They showed that these plasmids have the homologous gene contents and suggested them as virulence plasmids. However, three plasmids which are pSP291-1 (118.1kb), pSP291-2(52.1kb), and pSP291-3 (4.4kb) were also identified in *C. sakazakii* SP291 (Yan et al., 2013).

Finally, p1 (126.5 Kb) and p2 (56 Kb) plasmids were also detected in *C. malonaticus* CMCC45402 (Zhao et al., 2014).

Yan et al. (2013) profiled the plasmid content of *C. sakazakii* and *C. turicensis* strains and accordingly they categorised them into two groups. However, based on the similar criteria, Yan et al. (2015) included *C. malonaticus* plasmids P1 and P2, and performed further analysis to categorise the above mentioned plasmids. Plasmid group one includes plasmids pESA3 from *C. sakazakii* BAA-894, pSP291-1 from *C. sakazakii* SP291, pCTU1 from *C. turicensis* z3032 and p1 from *C. malonaticus* CMCC45402. This group was found to harbour two arsenic resistance genes and several putative virulence genes, including two genetic loci encoding iron acquisition systems, namely an ABC transporter gene cluster and an aerobactin or cronobactin siderophore receptor gene cluster identified as *eitCBAD* and *iucABCD*/*iutA*, respectively (Yan et al., 2015). Plasmid group two contains plasmids pSP291-2 from *C. sakazakii* SP291, pCTU3 from *C. turicensis* z3032 and p2 from *C. malonaticus* CMCC45402. All plasmids in this group broadly shared 15 metal (copper, cobalt, zinc, cadmium, lead, and mercury) resistance genes, an osmosensitive K+ channel histidine kinase gene *kdpD* and a virulence associated gene *vagC* (Yan et al., 2015). In addition, membrane proteins, suppressor of copper-sensitivity (*scsA* and *scsB*) were shared among pESA3, pSP291-1 and p1 plasmids, seven arsenic resistance genes were shared between pCTU3 and p2. Accordingly, authors suggested that unique virulence determinants are present in these three species (Yan et al., 2015).

1.9 Prevention and public health concerns

Although *Cronobacter* causes rare infections, these infections are usually devastating among neonates. The neonatal infections are often accompanied by complications and severe neurologic impairment and death. These bacteria appear to have a low infectious dose and short period of incubation (Muytjens et al., 1983; Lai, 2001; Iversen et al., 2008; Healy et al., 2010; Joseph and Forsythe, 2011). However, over 100 outbreaks of *Cronobacter* infections, which mostly occured in neonatal intensive care units, have been reported (Friedemann, 2009). *Cronobacter* infections are more likely to be underrecorded due to misidentifications. Several paediatric *Cronobacter* infections have been linked with the presence of PIF as an infection source. This product is consumed by millions of infants every day. The fact that PIF is not a sterile product makes it a potential health risk if contaminated by *Cronobacter*, combined with the very high consumption rate (Odeyemi and Sani, 2016). Indeed, *Cronobacter* has been isolated from a considerable number of unopened PIF tins (Iversen and Forsythe, 2004; Flores et al., 2011; Fei et al., 2015). Also, a remarkable number of PIF *Cronobacter* isolates, mainly *C. sakazakii* and *C. malonaticus*, is shown in the *Cronobacter* PubMLST database. Neonatal *Cronobacter* infections have been documented to

occur in hospitalized and non-hospitalized infants (Jason, 2012). Moreover, this organism has been recovered from important environmental places which are used for processing or preparation of PIF such as household kitchens, PIF preparation areas in NICUs and PIF factories (Friedemann, 2009; Craven et al., 2010; Kilonzo-Nthenge et al., 2012).

All of the above risks to public health have been considered and used for recommending appropriate strategies for controlling the occurrence of the neonatal *Cronobacter* infections. The WHO has strongly recommended that infants should be fed on breast milk and when infant formula is needed for some reasons the preparation guidelines should be applied to minimise the chance of the occurrence of *Cronobacter* infections (FAO-WHO, 2007). *Cronobacter* infections could be diminished by the application of several measures. A list of recommendations for hospitals and homes regarding preparation of PIF for infants has been established by the WHO. They recommend cleaning personnel hands and preparation areas, cleaning equipment as well as discarding warmed unfed formula. In addition, the prepared formula must be consumed within half an hour; however, the prepared milk could be stored in a refrigerator for no more than 24 hours (FAO-WHO, 2007; Pastore, 2010). The WHO preparation guidelines also recommended that water which is used in the reconstitution of PIF should be boiled and cooled for up to 30 minutes before adding to PIF to achieve a reconstitution temperature of 70°C. This temperature is determined to prevent the growth of *Cronobacter* (FAO-WHO, 2007). However, some organisations have raised some issues regarding the use of such high temperatures. For example, it causes potential loss of heat-sensitive nutrients, changing the physical characteristics of some formula, harm to personnel preparing formula and is inadequate for destruction of bacteria (Jason, 2012)

Certainly, all the WHO published meetings and guidelines helped to reduce the incidence of neonatal *Cronobacter* infections (FAO-WHO, 2004; FAO-WHO, 2006; FAO-WHO, 2007; FAO-WHO, 2008). However, like health care professionals, parents need to be educated regarding the appropriate way of safe handling and storage of PIF. In addition, they need to be provided with accurate information about neonatal enteric infections including *Cronobacter* infections. Moreover, the exclusive use of breast milk or other alternative commercially sterile products such as ready-to-feed formula should be encouraged. As a part of the prevention strategy, research on *Cronobacter* has covered several aspects such as the development of identification methods and classification, cultural variations of *Cronobacter* species, the possibility of cross contamination to different sources and bacterial response to environmental stresses. However, further research should be conducted on improving isolation methods, identification of molecular mechanisms of pathogenicity and survival under environmental stress, development of alternative control

methods during processing and preparation of PIF, and identifying of other possible contamination sources.

1.10 Aim and objectives

Most of the research conducted on *Cronobacter* has been focused on *C. sakazakii*. This due to the responsibility of this species for the majority of severe and fatal *Cronobacter* neonatal infections. Recently, *C. malonaticus* has been also reported to be involved in the several neonatal infections, and some of these infections were fatal. Therefore, research also needs to be directed toward the investigation of pathogenicity and physiological characteristics of *C. malonaticus*. This study is the first research focused on *C. malonaticus*; it presents new knowledge about physiological and virulence traits of *Cronobacter* genus in general and *C. malonaticus* in particular.

Holý et al. (2014) and Patrick et al. (2014) reported an age profile of *Cronobacter* carriage and infections respectively. These two studies confirmed the predominance of *Cronobacter* presence and infection in the adult population. However, none of these age profiling studies speciated or genotyped the *Cronobacter* isolates. *C. malonaticus* has been reported to be more associated with adult infections. This part of the study aims to address this lack of knowledge and reveal the predominant species using the collection of 51 clinical *Cronobacter* strains, which included those from the study of Holý et al. (2014). Consequently, a number of phenotypic and genotypic identification techniques have been applied to answer some questions such as 1) is *C. malonaticus* the predominant species among this collection, 2) is there any link between age group and a specific species, 3) is there any relatedness between strains, and finally 4) the study will find the effectiveness of each technique.

Representative *C. malonaticus* strains from the 51 clinical *Cronobacter* strains that mentioned above will be selected according to PFGE groups. Other 14 clinical *C. malonaticus* strains were selected from the *Cronobacter* PubMLST database to cover the available STs. Hence, a total of 20 *C. malonaticus* strains from different STs, sources (e.g. blood, sputum, faeces) and clinical outcomes (e.g. meningitis, abscess, unknown) were subjected to a range of physiological and virulence related assessments. The aim of this part is to investigate the physiological and virulence traits that enable *C. malonaticus* to survive different stresses and facilitate several virulence machanisms.

C. malonaticus has been recently reported to be involved in several devastating neonatal infections, though it is hypothesised that this species is more associated with adult infections. Nevertheless, there is no known study investigating the virulence potential and pathogenicity of *C. malonaticus*. The same 20 clinical *C. malonaticus* isolates, which were selected for physiological and virulence studies, will be used to assess the virulence potential and pathogenicity of *C. malonaticus* clinical

strains. The first step to achieve that is studying the organism's ability to adhere and invade through Caco-2 and HBMEC cell lines, and survive phagocytosis. This will confirm the ability of *C. malonaticus* to cause NEC, bacteremia and meningitis. This part also aims to examine the ability of *C. malonaticus* to cause UTI and pneumniae by studying the organism's ability to adhere, invade the T24 and A549 cell lines.

The 20 selected *C. malonaticus* strains were subjected to complete genome sequence in order to use these genomes for further molecular investigations such as finding genes that underlie the physiological and virulence traits, identification of the genes associated with attachment and invasion of human cells as well as survival in macrophages.

Chapter 2 Materials and Methods

2.1 Safety consideration

Health and safety code of practice for microbiology level two containment laboratories at Nottingham Trent University was considered before carrying out any experiments and protocols in this project. The procedural COSHH forms were also completed and considered. Category 2 organisms and materials were disposed of according to the recommended instructions. For tissue culture laboratories health and safety regulations, hepatitis B antibodies and vaccination were assessed before starting any tissue culture experiments.

2.2 Sterilisation and aseptic techniques

All buffers, solutions, media, and equipment were decontaminated by autoclave sterilisation at 121°C under 15 psi pressure for 15 minutes, 121°C for 5 minutes, filtered using 0.2 µm pore size filters (Thermo Fisher Scientific, UK) or sprayed with 70% ethanol, as appropriate.

2.3 Bacterial storage

All the *Cronobacter*, and any additional positive and negative control strains were stored at -80°C and -20°C in TSB/ glycerol (80%) (Thermo Fisher Scientific, UK) for a long term. When required, bacteria were recovered from frozen stock and subcultured on Tryptone Soya Agar (TSA) and incubated aerobically at 37°C for 18 h. For short periods of use, bacteria were streaked on TSA and stored at 4°C.

2.4 General stock reagents and buffers

2.4.1 Phosphate buffered saline (PBS)

The PBS solution was prepared by dissolving two tablets of PBS (Sigma Aldrich, UK) into 400 ml of distilled water and autoclaved at 121°C for 15 minutes.

2.4.2 Saline Solution (0.85 %)

For preparing the 0.85% saline solution, one tablet of saline (Thermo Fisher Scientific, UK) was dissolved into 500ml of distilled water and autoclaved at 121°C for 15 minutes.

2.4.3 Hydrochloric acid (HCl)

One molar of hydrochloric acid (HCl) was prepared by adding 86ml of 37% HCl (Thermo Fisher Scientific, UK) to 914 ml of distilled water. The mixture was gently mixed and used to adjust the pH of solutions used in this project.

2.4.4 TRITON X-100 (1%)

In order to prepare 1% Triton X-100, 1 ml of TRITON X-100 (Thermo Fisher Scientific, UK) was added to 99ml of distilled water, next autoclaved and then stored at room temperature to be used.

2.4.5 1 M Tris-HCl (pH8)

For preparing 1 M Tris-HCL, 60.55 g of Tris base (Thermo Fisher Scientific, UK) was dissolved into 400 ml of distilled water and then the mixture was adjusted to pH 8 by adding HCl and measured by pH meter (HANNA, USA). The volume was completed with distilled water to be 500 ml and then autoclaved at 121°C for 15 minutes.

2.4.6 0.5 M EDTA (Ethylenediamine tetra-acetic acid, sodium hydroxide) (pH8)

The preparation of 0.5 EDTA was obtained by dissolving 93 g of EDTA (Sigma Aldrich, UK) into 400 ml of distilled water and the mixture then was adjusted to pH 8 by adding sodium hydroxide pellets (NaOH) (Sigma Aldrich, UK) and the pH was measured by a pH meter. The total volume of the mixture was completed to be 500 ml and then it was autoclaved at 121°C for 15 minutes.

2.4.7 10 X TBE Buffer (Tris base, boric acid and EDTA buffer)

The preparation of 10X TBE buffer was obtained by dissolving 108 g Tris base (Thermo Fisher Scientific, UK), 55 g boric acid (Thermo Fisher Scientific, UK) into 800 ml of distilled water. Once the chemicals were completely dissolved, 40 ml of 0.5 EDTA pH 8 was added and the mixture volume was adjusted to 1000 ml by adding distilled water. The prepared mixture then autoclaved at 121°C for 15 minutes.

2.4.8 1 X TAE buffer (1 X Tris-acetate-EDTA buffer)

In order to prepare 1X TAE buffer, 20ml of 50X TAE buffer (National Diagnostics, UK) was diluted with 980 ml of distilled water. The 1X TAE diluted buffer was used for preparing agarose gel and filling the gel electrophoresis tanks.

2.4.9 GLYCEROL (80 %)

The preparation of 80% of glycerol was completed by adding 80 ml of glycerol (Thermo Fisher Scientific, UK) to 20 ml of distilled water. The mixture was autoclaved and stored at room temperature for using.

2.4.10 Giemsa stain (5%)

Five millilitres of Giemsa stain (Life Technologies, UK) was added into 95 ml of sterile distilled water and stored at room temperature for one week.

2.4.11 IRON III SOLUTION

This solution was prepared by dissolving 0.0027 g of $FeCl_3$-6 H_2O (Sigma Aldrich, UK) into 10 ml of 10 mM HCl.

2.4.12 Chrome Azurol Sulphate (CAS) SOLUTION

CAS solution was prepared by mixing 0.06 g CAS (Sigma Aldrich, UK) with 50ml of sterile distilled water.

2.4.13 Hexadecyltrimethylammonium bromide (HDTMA)

Exactly 0.1458 g of HDTMA (H6268, Sigma Aldrich) was dissolved in 80 ml of distilled water.

2.4.14 Sodium hydroxide solution

This solution was prepared by dissolving ten gram of NaOH into 20 ml distilled water.

2.4.15 M9 Minimal medium

To prepare M 9 Minimal Medium, 50 ml of 5XM9 medium, 1ml 1M $MgSO_4$, 5ml 20% glucose , 50 µl 1 M $CaCl_2$ were mixed in 500 ml distilled and sterilised water (DSW) and then autoclaved at 121°C for 15 minutes.

2.5 General culture media

2.5.1 Tryptone Soya Agar (TSA)

According to manufacturer's instruction of TSA (Thermo Fisher Scientific, UK) the media was prepared by dissolving 40 grams of TSA agar in 1L of distilled water. The mixture was boiled to completely melt the agar and then autoclaved at 121°C for 15 minutes. The media was cooled to 50°C and then dispensed into petri dishes about 20 ml each. These plates were dried and stored at 4°C to be ready for using.

2.5.2 Trypticase soy broth (TSB)

Exactly 15 grams of TSB (Thermo Fisher Scientific, UK) were dissolved into 500 ml of distilled water. This mixture was dispensed to 100 ml in separate bottles. These 100 ml bottles were autoclaved at 121°C for 15 minutes and then stored at room temperature.

2.5.3 DRUGGAN-FORSYTHE-IVERSEN (DFI) AGAR Formulation

One litre of distilled water was used to suspend 43 grams of DFI agar (Lab M, UK) and then autoclaved at 121°C for 15 minutes. The medium was cooled to 50°C and directly dispensed into petri dishes about 20ml each. The plates were finally stored at 4°C to be used.

2.5.4 MacConkey agar

To prepare 1 litre, 51.5 grams of MacConkey agar (Thermo Fisher Scientific, UK) were added to 1 litre of distilled water. The mixture was boiled to completely dissolve the powder and then autoclaved at 121°C for 15 minutes after which it was cooled to 50°C and dispensed into petri dishes and stored at 4°C until needed.

2.5.5 Violet Red Bile Glucose Agar and Violet Red Bile Lactose Agar

The medium was prepared by dissolving 38.5 grams of agar (Thermo Fisher Scientific, UK) into 1 litre of distilled water. The medium was boiled then cooled to 50°C to be dispensed into petri dishes.

2.5.6 XLD Medium

Fifty-three grams of XLD (Thermo Fisher Scientific, UK) agar was added to 1 litre of distilled water and heated with frequent agitation until the mixture boiled. The medium was transferred immediately to a 50°C water bath and then poured into petri dish plates.

2.5.7 Iso-Sensitest agar

Exactly 31.4 g were suspended in 1 litre of distilled water and next the mixture was boiled to dissolve the agar and then autoclaved at 121°C for 15 minutes. The medium was cooled to 50°C and dispensed into petri dishes.

2.6 Identification methods and molecular typing

2.6.1 Bacterial strains

Fifty-one clinical *Cronobacter* strains were used in Chapter 3. The strains had been collected from patients from two hospitals (Olomouc and Prostějov), during a 6-year period from May 2007 to August 2013. This includes strains isolated in the previous study by Holý et al. (2014). Patient information such as age, sex, clinical presentation, isolated site and date of isolation are given in Table 2.1.

Table 2-1 Source of *Cronobacter* strains used in this study

Strain number	Hospital	Department	Patient age (y)	Patient sex	Isolation date	Isolation site
1830	Olomouc	Paediatrics	<1	Male	09/05/2007	Throat swab
1829	Olomouc	Paediatrics	1	Male	04/06/2007	Throat swab
1828	Olomouc	Paediatrics	2	Male	12/10/2007	Nose swab
1831	Olomouc	Paediatrics	3	Male	06/06/2007	Throat swab
1832	Olomouc	Paediatrics	3	Female	27/03/2009	Throat swab
1999	Olomouc	Paediatrics	3	Male	30/01/2013	Throat swab
2020	Olomouc	Paediatrics	5	Female	26/05/2013	Stool
1835	Olomouc	Paediatrics	6	Male	30/03/2012	Throat swab
2015	Olomouc	Paediatrics	7	Female	16/08/2013	Throat swab
2014	Olomouc	Paediatrics	8	Male	08/04/2013	Throat swab
1917	Olomouc	Paediatrics	15	Male	28/10/2012	Throat swab
1834	Olomouc	Paediatrics	16	Male	31/05/2010	Throat swab
2004	Olomouc	Paediatrics	17	Female	02/03/2013	Throat swab
1827	Olomouc	Internal Medicine III	76	Female	09/10/2007	Cannula
1833	Olomouc	CMP	5	Male	11/01/2010	Stool
1838	Olomouc	AICU	63	Female	10/04/2012	Sputum
1998	Prostějov	Internal Medicine (A)	49	Female	22/01/2013	Sputum
2008	Prostějov	Internal Medicine (A)	68	Male	12/03/2013	Sputum
2011	Prostějov	Internal Medicine (A)	68	Male	31/03/2013	USC
2006	Prostějov	Internal Medicine (A)	70	Female	28/02/2013	Sputum
2007	Prostějov	Internal Medicine (A)	70	Female	06/03/2013	Sputum
2022	Prostějov	Internal Medicine (A)	70	Female	06/03/2013	Sputum
1842	Prostějov	Internal Medicine (A)	72	Female	27/06/2012	Sputum
2005	Prostějov	Internal Medicine (A)	73	Female	24/02/2013	Sputum
2021	Prostějov	Internal Medicine (A)	76	Female	07/04/2013	Sputum
1841	Prostějov	Internal Medicine (A)	79	Female	18/06/2012	Sputum
2003	Prostějov	Internal Medicine (A)	83	Male	20/02/2013	Sputum
1915	Prostějov	Internal Medicine (A)	84	Female	18/10/2012	Sputum
1996	Prostějov	Internal Medicine (A)	84	Female	14/01/2013	Sputum
2010	Prostějov-	Internal Medicine (A)	84	Female	12/03/2013	Throat swab
2019	Prostějov	Internal Medicine (A)	87	Male	10/05/2013	Sputum
2001	Prostějov	Internal Medicine (B)	68	Male	29/01/2013	SOC
2000	Prostějov	Internal Medicine (B)	71	Male	03/02/2013	Rectal Swab
2002	Prostějov	Internal Medicine (B)	77	Male	19/02/2013	Sputum
1916	Prostějov	Internal Medicine (B)	84	Male	06/11/2012	Sputum
2013	Prostějov	Internal Medicine (B)	91	Female	04/04/2013	Sputum
2012	Prostějov	Internal Medicine (C)	70	Male	04/04/2013	Sputum
2009	Prostějov	Internal Medicine (C)	77	Female	16/03/2013	Tongue swab
1903	Prostějov	Internal Medicine - ICU	59	Male	24/08/2012	Sputum
1902	Prostějov	Internal Medicine - ICU	69	Male	21/08/2012	Sputum
1901	Prostějov	Internal Medicine - ICU	82	Male	15/08/2012	Sputum
1997	Prostějov	ICU	65	Male	21/01/2013	Sputum
1839	Prostějov	ICU	73	Female	12/06/2012	SPEG
1840	Prostějov	ICU	80	Female	19/06/2012	Sputum
1836	Prostějov	Surgery	63	Male	23/05/2012	Wound swab
1837	Prostějov	Surgery	85	Female	25/05/2012	Wound swab
1914	Prostějov	Infectious Diseases	69	Male	02/10/2012	Sputum
2018	Prostějov	Infectious Diseases	72	Male	05/05/2013	Sputum
2016	Prostějov	AICU	27	Male	18/04/2013	Sputum
2017	Prostějov	AICU	27	Male	22/04/2013	Sputum
1995	Prostějov	Out patient	50	Male	10/01/2013	Sputum

SPEG= Smear from area of percutaneous endoscopic gastrostomy, USC= Urine suction catheter, SOC= swab of the oral cavity, CMP = Clinical and Molecular Pathology, AICU= Anaesthesiology and Intensive Care Unit.

2.6.2 Phenotyping

2.6.2.1 Appearance on different media

Five different media were used to observe the appearance of the *Cronobacter* colonies. These media were TSA, DFI, XLD, VRBGA and MacConkey agar.

2.6.2.2 API ID32E

Cronobacter isolates were phenotyped using the ID 32E kit (bioMérieux), according to the manufacturer's instructions. The resultant phenotypic profiles were compared to the bioMérieux online database, version 3.0, at https://apiweb.biomerieux.com.

2.6.3 Genotyping

2.6.3.1 Multilocus sequence typing (MLST) and *rpoB* gene screening

2.6.3.1.1 DNA extraction

DNA was extracted from the target strains using the GeneEluteTM kit (NA2110-1KT, Sigma Aldrich, UK). The instructions were followed as explained by the manufacturer. The DNA concentration was confirmed by using a NanoDrop® ND-2000 UV-Vis spectrometer (Thermo Scientific, UK), and the DNA in accepted concentration was stored at -20°C for 6 months.

2.6.3.1.2 Polymerase chain reaction amplification

Multilocus sequence typing (MLST) was performed as described by Baldwin et al. (2009) and with consideration of the additional information in *Cronobacter* PubMLST database http://www.pubmlst.org/*cronobacter*. Seven housekeeping genes of *Cronobacter* were amplified using the primers; ATP synthase beta 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*), phosphoenolpyruvate synthase A (*ppsA*) as showed in table 2.2. Polymerase chain reaction (PCR) amplification product was performed using PCR machine (Techne, UK). A total volume of PCR reaction was of 20 µl composed of GoTaq® Green Master Mix (Promega, UK) which diluted with 50% SDW, 2% of primers showed in table and 1 µl of bacterial DNA. Reaction condition for all the MLST primers was 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. For screening of *rpoB* gene, Stoop, et al. (2009) developed and evaluated the conventional PCR *rpoB* for identification of *Cronobacter* species and the screening of *rpoB* was performed as described by them. PCR machine was used for amplification of *rpoB* DNA. GoTaq® Green Master Mix (Promega, UK) was diluted to 50% with sterile distilled water and 2% of primers showed in table 2 was added. One microliter of DNA of each isolate was added into 19 µl of master mix with 2% primer and the PCR condition was as following: initial

denaturation at 94°C for 90 second; 40 cycles of denaturation at 94°C for 10 second; primer annealing at 55°C for 20 second; extension at 72°C for 50 second; followed by a final extension step of 72°C for 5 min.

2.6.3.1.3 Confirmation of PCR products by agarose gel electrophoresis

The PCR products were visualised by using 1% agarose gel electrophoresis. The agarose gel was prepared by dissolving 1% agarose (Thermo Fisher Scientific, UK) in 1X Tris-acetate-EDTA (TAE) buffer (National Diagnostics, UK). To completely dissolve the agarose in TAE, the mixture was microwaved until no crystals were seen, the mixture then cooled to 50°C when 0.1 µl/ml of SYBR® Safe DNA gel stain (Life Technologies – Invitrogen, UK) was added to agarose solution before poured into a gel tray. Agarose solution into the tray was left to set at room temperature and then placed in a Cell GT tank (Geneflow; UK) containing 1x TAE buffer. Five microliter of PCR samples were loaded into well gel beside a 100bp DNA ladder (Promega, UK) to determine the PCR product size. A run condition of the gel was at 90V for 45 minutes and DNA bands were visualised using ultraviolet (UV) InGenius® gel documentation system (Syngene, UK).

2.6.3.1.4 Purification and Sequencing of PCR products

To purify the PCR products from other PCR component reactions, MinElute PCR Purification Kits (Qiagen, UK) were used and the purification was performed as instructed by manufacturer. The concentration and purity of purified DNA was checked by using Nanodrop 2000 (Thermo Scientific, UK). The purified DNA for the 7 housekeeping genes, *rpoB* gene and primers were adjusted to be in appropriate concentrations for sequencing procedure. Twelve microliter of purified DNA of each sample for forward and reverse were sent to be sequenced by The Source BioScience http://www.lifesciences.sourcebioscience.com.

2.6.3.1.5 Analysis of DNA sequence:

After sequencing, the reaction samples were received as FASTA files and GENtle 2.0 beta version software was used to check the sequence chromatograms. The sequences of both strands of a given locus were assembled as a contigs by using CAP3 program (Huang and Madan, 1999), and then the contigs were checked into *Cronobacter* PubMLST database. The *rpoB* (637 bp) was aligned with additional sequences from the *Cronobacter* PubMLST database in MEGA (Molecular Evolutionary Genetics Analysis) software version 5.2 using the ClustalW algorithm. *RpoB* alleles were allocated numbered profiles according to the *Cronobacter* PubMLST database. For multilocus sequence analysis (MLSA), concatenated sequences of the 7 loci MLST (3036 bp total length) were aligned in MEGA version 5.2 using the ClustalW algorithm. All samples were analysed and checked by using *Cronobacter* PubMLST database http://www.pubmlst.org/*cronobacter* in order to determine their

order numbers for each gene and consequently to recognise the ST of each isolate. The phylogeny analysis of the isolates based on the seven housekeeping genes or *rpoB* gene was performed by using the MEGA6 (6140226) software to construct and analyse the phylogenetic relationship between the studied strains.

Table 2-2 Primers used for MLST, *rpoB* and serotyping

Primer name		Prime sequences		PCR product size
		PCR	Sequencing	
atpD	F	CGACATGAAAGGCGACAT	CGAAATGACCGACTCCAA	390 bp
	R	TTAAAGCCACGGATGGTG	GGATGGCGATGATGTCTT	
fusA	F	GAAACCGTATGGCGTCAG	GCTGGATGCGGTAATTGA	438 bp
	R	AGAACCGAAGTGCAGACG	CCCATACCAGCGATGATG	
glnS	F	GCATCTACCCGATGTACG	GGGTGCTGGATAACATCA	363 bp
	R	TTGGCACGCTGAACAGAC	CTTGTTGGCTTCTTCACG	
gltB	F	CATCTCGACCATCGCTTC	GCGAATACCACGCCTACA	507 bp
	R	CAGCACTTCCACCAGCTC	GCGTATTTCACGGAGGAG	
gyrB	F	TGCACCACATGGTATTCG	CTCGCGGGTCACTGTAAA	402 bp
	R	CACCGGTCACAAACTCGT	ACGCCGATACCGTCTTTT	
infB	F	GAAGAAGCGGTAATGAGC	TGACCACGGTAAAACCTC	441 bp
	R	CGATACCACATTCCATGC	GGACCACGACCTTTATCC	
ppsA	F	GTCCAACAATGGCTCGTC	ACCCTGACGAATTCTACG	495 bp
	R	CAGACTCAGCCAGGTTTG	CAGATCCGGCATGGTATC	
CsO1	F	CCCGCTTGTATGGATGTT		364 bp
	R	CTTTGGGAGCGTTAGGTT		
CsO2	F	ATTGTTTGCGATGGTGAG		152 bp
	R	AAAACAATCCAGCAGCAA		
CsO3	F	CTCTGTTACTCTCCATAGTGTTC		704 bp
	R	GATTAGACCACCATAGCCA		
CsO4	F	ACTATGGTTTGGCTATACTCCT		890 bp
	R	ATTCATATCCTGCGTGGC		
CsO5	F	GATGATTTTGTAAGCGGTCT		235 bp
	R	ACCTACTGGCATAGAGGATAA		
CsO6	F	ATGGTGAAGGGAACGACT		424 bp
	R	ATCCCCGTGCTATGAGAC		
CsO7	F	CATTTCCAGATTATTACCTTTC		615 bp
	R	ACACTGGCGATTCTACCC		
CmaO1	F	AGGGGCACGGCTTAGTTCTGG		323 bp
	R	CCCGCTTGCCCTTCACCTAAC		
CmaO2	F	TGGCCCTTGTTAGCAAGACGTTTC		394 bp
	R	ATCCACATGCCGTCCTTCATCTGT		
CmuO2	F	TGGCTGTCATGGTTTTCTTGC		258 bp
	R	TAGTTGGCACCATCAACGCC		
rpoB	F	AACCAGTTCCGCGTTGGCCTGG		637 bp
	R	CCTGAACAACACGCTCGGA		

F = forward. R = reverse. Cs = *C. sakazakii*, Cm = *C. malonaticus*, Cu = *C. muytjensii*.

2.6.3.2 Molecular serotyping of *Cronobacter* O-antigens

Cronobacter serotypes were determined using the multiplex polymerase chain reaction (PCR) assay as described by Jarvis et al. (2011) and Sun et al. (2012). All used primers are shown in table 2.2. In brief, DNA was extracted as previously described, primers were selected from earlier mentioned studies and PCR condition was as following; an initial denaturing step at 94°C for 30 s, followed by 35 cycles of 94°C for 10 s, 65°C for 35 s, and 68°C for 15 min, followed by a final 10 min extension step at 72°C.

2.6.3.3 Pulsed-field Gel Electrophoresis (PFGE)

PFGE was used in order to determine whether there was a relationship between the strains which were collected from two hospitals and the sites or departments. After slight modification, PFGE of *Enterobacteriaceae* was used as described by the PulseNet USA protocol for molecular subtyping of *Escherichia coli* O157:H7, non-typhodial *Salmonella* serotypes, and *Shigella sonnei* CDC (2004) and also by Ribot et al. (2006). In this experiment fifty-one strains of *Cronobacter* collected from different clinical sites and at different department of two hospitals, Olomouc and Prostejov hospitals in the Czech Republic were used. These had been collected and donated by Ondřej Holy and his group.

2.6.3.3.1 Tris EDTA Buffer (TEB)

TEB was prepared aseptically by diluting 10 ml of 1M Tris pH8 and 2ml of 0.5 M EDTA pH 8 with 988ml distilled water. This buffer was used for making and washing the PFGE plugs.

2.6.3.3.2 Cell Suspension Buffer (CSB)

The preparation of CSB was obtained aseptically by diluting 10 ml of 1 M Tris pH8 and 20 ml of 0.5 M EDTA pH8 with 70ml of distilled water.

2.6.3.3.3 Cell Lysis Buffer (CLB)

CLB was prepared aseptically by diluting 2.5 ml of 1 M Tris pH8 and 5 ml of 0.5 M EDTA pH8 with 42.5 ml distilled water. 1% Sarkosyl NL (N-Dodecanoyl-N-methylglycine sodium salt) (Sigma Aldrich, UK) was dissolved into the mixture before adding 20 mg/mL of proteinase K (Sigma Aldrich, UK).

2.6.3.3.4 DNA preparation in agarose plugs

Fifty-one *Cronobacter* strains and a marker strain *Salmonella* enterica serovar Typhimurium reference standard H9812 (NTU 732) were screened in this project. About two full loops of overnight culture on TSA of the isolates and reference strain were individually suspended into cell suspension buffer and centrifuged at 7000 rpm for 4 minutes. The previous step was repeated two times and then, using same buffer, an optical density (OD) was adjusted to be between 1.35-1.5 at

610 nm. Exactly 400 µl of bacterial suspension was allocated into a 1.5 ml Eppendorf tube and while they were incubated in water bath at 37°C for 10 minutes, 1% agarose gel in TEB was microwaved for melting and 0.5% SDS was added and the gel was left in 55°C water path. Each Eppendorf tube was mixed with 25 µl proteinase K and then mixed with 400 µl of TEB agarose and immediately about 100 µl of mixture was dispensed into five well plug molds. The plugs were transferred into 15 ml falcon tube contain 5 ml of CLB and incubated in a shaking water bath at 50°C for 2 hours. After the period of incubation, the plugs were washed twice with 15 ml of warmed sterile distilled water and incubated in shaking water bath at 50°C for 15 minutes. Finally, the DNA plugs were washed three times into 15 ml warmed TEB in shaking water bath at 55°C for 15 minutes and stored in 5 ml of TEB at 4°C until needed.

2.6.3.3.5 Digestion and Electrophoresis

Two restriction enzymes *XbaI* and *SpeI* (Promega, UK) were used for digestion. About 2 millimetres of each plug was transferred into 2 ml Eppendorf tube contain 2 µl BSA, 20 µl 10X buffer D and 178 µl sterile distilled water and incubated in water bath 37°C for 15 minutes. After the incubation, the content of tubes was removed and digestion was performed on each plug by adding 2 µl BSA, 20 µl 10X buffer D, 5 µl *XbaI* enzyme and 173 µl sterile distilled water or 2 µl BSA, 20 µl 10X buffer B, 3 µl *SpeI* enzyme and 175 µl and incubated in water bath at 37°C for 4 hours. 1% agarose gel was prepared in 100 ml of 0.5XTBE buffer and kept in 55°C water bath. The restricted plugs were then loaded on a 15-tooth comb (BIO-RAD Laboratory; Belgium). The comb was inserted in the gel tray and the 1% agarose gel was carefully poured into the gel tray. After the gel solidified, the comb was removed, it was placed in an electrophoresis cell and covered with 2400 ml of 0.5X TBE buffer. The used system for PFGE was CHEF-DR II system (BIO-RAD, Belgium) and it was performed at 14°C, 6V for 20 hours and initial and final switch was 5 and 50 seconds respectively.

2.6.3.3.6 Staining and Analysis:

The gel was stained with ethidium bromide (Sigma Aldrich, UK) 0.1 µg /ml for 45 minutes, visualised under UV light and photographed using InGenius® gel documentation system (Syngene, UK). The DNA band profiles were analysed using BioNumerics software version 7.1 (Applied Maths, Belgium).

2.7 Physiological experiments

2.7.1 Bacterial strains

Following PFGE analysis, six representative strains of the different C*. malonaticus* pulsetypes were chosen for further analysis. Another 14 clinical *C. malonaticus* strains were selected from the *Cronobacter* PubMLST database to cover the available STs. Hence a total of 20 *C. malonaticus* strains were used in this Chapter as explained in table 2.3.

Table 2-3 *C. malonaticus* strains used in Chapter 4 and 5

Strain No.	ST	rpoB	O-antigen	Source	Country	Year of isolation	Comment
565	7	18	CmalO2	Faecal isolate	USA	1973	CPMD
681	7	18	CmalO2	Breast abscess isolate	USA	1977	CPMD
688	7	18	CmalO2	Sputum	Czech Republic	2004	CPMD
983	7	18	CmalO2	Infant formula	Brazil	2007	CPMD
1558	7	18	CmalO2	Faecal isolate	Czech Republic	Unknown	CPMD
1827	7	18	CmalO2	Cannula (Blood)	Czech Republic	2007	Table 2.1
1830	7	18	CmalO2	Throat swab	Czech Republic	2007	Table 2.1
1833	7	18	CmalO2	Faecal isolate	Czech Republic	2010	Table 2.1
1835	7	18	CmalO2	Throat swab	Czech Republic	2012	Table 2.1
2018	7	18	CmalO2	Sputum	Czech Republic	2013	Table 2.1
2020	7	18	CmalO2	Faecal isolate	Czech Republic	2013	Table 2.1
507	11	20	CmalO3	Faecal isolate	Czech Republic	1984	CPMD
512	11	20	CmalO3	Clinical	Czech Republic	1983	CPMD
514	11	20	CmalO3	Clinical	Czech Republic	1983	CPMD
15	60	39	CmalO1	Faecal isolate	Czech Republic	2003	CPMD
687	60	39	CmalO1	Sputum	Czech Republic	2004	CPMD
689	60	39	CmalO1	Faecal isolate	Czech Republic	2005	CPMD
1545	84	18	CmalO2	Faecal isolate	Czech Republic	Unknown	CPMD
685	129	39	CmalO2	Blood isolate	USA	1977	CPMD
1569	307	39	CmalO1	Blood isolate	USA	2011	CPMD

ST: sequence type. CPMD: *Cronobacter* PubMLST database.

2.7.2 Utilization of malonate

In this assay malonate PPA Broth (Oxiod, UK) was used. One colony from an overnight TSA plate was used to inoculate this broth, which was then incubated at 37°C for 24h. A positive result was interpreted by changing the colour of broth from green to blue or blue green and negative result showed no colour change.

2.7.3 Utilization of sialic acid

Three types of media were prepared for this experiment; 5X M9 minimal medium (Sigma Aldrich, UK) without carbon source, minimal medium with glucose and minimal medium with sialic acid. 5X M9 Minimal medium was prepared by mixing 220 of 1.25% agar technical (Thermo Fisher Scientific, UK), 25 ml of 5X M9 medium, 500 µl 1 M of $MgSO_4$ and 25 µl of 1 M $CaCl_2$. All solutions were filtered if needed and then autoclaved at 121°C for 15 minutes before mixed together in total volume of 250 ml. In order to prepare glucose and sialic acid media, 5 ml of 20% glucose or 250 mg of sialic acid (Sigma Aldrich, UK) was added to the mixture and total volume was completed to be 250 ml. The three media were dispensed separately into 6 well plates and the plates left for a day to set. Each strain was carefully streaked on the three wells with the different media and all plates were incubated at 37°C for 24h.

2.7.4 Motility Test

Twenty grams of Luria Bertani (LB) broth (Sigma Aldrich, UK) and 0.4% of agar technical (Thermo Fisher Scientific, UK) were dissolved in 1000 ml of distilled water and then 5 ml of 1% TTC solution (Triphenyl-tetrazolium chloride) (Fluka, UK) was added, the mixture was autoclaved at 121°C under 15 psi pressure for 15 minutes. After autoclaving, the media was dispensed into petri dishes 30 ml in each one and left 48 h in room temperature. One colony of fresh TSA culture was inoculated into 5ml of TSB and incubated in shaking incubator at 37°C for 18-20 h. Three microliter of each overnight culture broth was inoculated into the centre of the petri dish and incubated overnight at 37°C. *S*. Enteritidis and *K. pneumoniae* were used as positive and negative control respectively.

2.7.5 Biofilm formation

Eighteen hour TSB cultures were used to inoculate 5 ml of TSB and adjusted to O.D = 0.3 reading at 600nm. In three 96 plates, 600 µl of each inoculated TSB was dispensed into 3 wells (200 µl into each well) of each 96 well plate. Also liquid infant formula (Cow & Gate Premium 1) was used instead of TSB to detect and compare the ability of *C. malonaticus* of forming biofilm in both media. After measuring the O.D of TSB culture the milk was adjusted to a cell density of an O.D = 0.3. Loading plates were incubated for 24h at 25°C and 37°C. After 24 hours of incubation the plates were emptied from the TSB or the milk and washed twice with sterile distilled water (SDW) the plates then left for 10 minutes at room temperature to dry. Two hundred microliter of 1% crystal violet (CV) was added to each well and left for 30 minutes after which all the wells were washed three times with SDW. Finally, 200 µl of absolute ethanol was added to each well and after 15 minutes the content of the plates was transferred into new plates and read using ELx800 absorbance microplate reader (BioTek, UK) at absorbance 600nm.

2.7.6 Acid Resistance

The effect of acid at low pH on *C. malonaticus* was performed as described by Edelson-Mammel et al. (2006). One colony from a fresh TSA plate was used to inoculate 5 ml of TSB and incubated in shaking incubator for overnight at 37°C. To mimic the stomach acid, the pH of a liquid infant formula (Cow & Gate Premium 1) was adjusted to pH value of 3.5 units with 1 M of hydrochloric acid. Four millilitres of overnight culture were inoculated into 15 ml of the acidified infant formula, and distributed into five sterile tubes and incubated in water bath at 37°C. The 5 tubes were used after 0, 15, 30, 60, 90 and 120 minutes to determine viable cells. At specific time, 200 µl of each tube was transferred and serial diluted in normal saline and plated on TSA plates using the Miles Misra technique. The TSA plates were incubated at 37°C for overnight before enumeration of cells.

2.7.7 Metal Resistance

For dilutions 1 M, 0.1 M, 0.01 M and 0.001 M of eight metals were used in this assay. The metals were copper II sulphate (Sigma Aldrich, UK) , sodium arsenate (Sigma Aldrich, UK), silver nitrate (Alfa Aesar, UK), sodium tellurite (Sigma Aldrich, UK), nickel chloride (Thermo Fisher Scientific, UK), zinc sulfate heptahydrate (BDH chemicals, England), cobalt II nitrate (Sigma Aldrich, UK) and cadmium carbonate (Harrington, USA). A TSB overnight culture of strains were used to inculate TSA agar by swabing the agar surface using a tight zig- zag pattern. Four paper discs were placed on each plate and 7 µl of each dilution was pipetted on a specific disc in the same plate. All plates were incubated at 37°C for overnight and any inhibtion zone was measured.

2.7.8 Congo red morphotype

To prepare LB agar without salt, 5 g of tryptone (Thermo Fisher Scientific, UK), 2.5 g of yeast extract (Melford laboratories, UK) and 7.5 g agar (Thermo Fisher Scientific, UK) were mixed in 500 ml distilled water and then autoclaved. Congo red solution dye was prepared by dissolving 32 mg of Congo red powder (Sigma Aldrich, Uk) in 8 ml SDW and the solution was filtered with 0.20µm pore filter. After this, LB agar without salt was cooled to 55°C, 5 ml of filtered congo red solution was added, gently mixed and dispensed into petri dishes.

2.7.9 Cellulose production

LB agar without salt was prepared as mentioned in 2.7.8 and supplemented with 200 µg/ml calcofluor white stain (Fluka, UK). The medium was poured in petri dishes and left to dry for 2 days. The *C. malonaticus* strains were streaked and incubated either at 37°C or 30°C for 24 h and then the cellulose production was visualised by using ultraviolet light at 366 nm.

2.7.10 Desiccation stress and determining of sublethaly injured *C. malonaticus* cells

One colony of each *C. malonaticus* strain was inoculated into 5 ml of liquid infant formula (Cow & Gate Premium 1) and incubated in shaking incubator at 37°C for 20 h. The overnight bacterial growth was diluted to 1:10 using same milk and adjusted a cell density of approximately 10^{11} CFU/ml and 200 µl of the suspension were transferred into 6 well plates. The plates then left for overnight in a class II cabinet at room temperature. After desiccation, the content of each well was resuspended in 2 ml of SDW. The viable counts were determined using Miles and Misra method on VRBGA as a selective medium and TSA as non-selective medium to define the sublethally injured cells. The differences in recovery on TSA and VRBGA after desiccation reflect the number of cells that were sublethally injured during overnight desiccation.

2.8 Virulence experiments

2.8.1 Bacterial strains

See Section 2.7.1.

2.8.2 Haemolysis on blood agar

TSA was prepared as it was explained in Section 2.5.1; however, when TSA medium cooled to 50 °C, 5% of horse blood (Sigma Aldrich, UK) was added and thoroughly mixed and then dispensed into petri dishes after which they were stored at 4°C until needed. *Staphylococcus aureus* NCTC 10788 was used as a positive control for β haemolysis, *Streptococcus pneumoniae* was used as a positive control for α haemolysis and *Staphylococcus epidermidis* was used as a negative control for λ haemolysis. All examined strains were streaked on the horse blood agar and all plates were incubated at 37°C for 24 h.

2.8.3 Iron siderophore detection

Siderophore detection assay was performed as described by Shin et al. (2001). Two solutions were used to prepare the Chrome azurolsulphate (CAS) agar. The first solution which is a dark blue liquid was prepared by using 10 ml of iron III solution Section 2.4.11, 50 ml of CAS solution Section 2.4.12, and 40 ml of HDTMA Section 2.4.13 and then autoclaved at 121°C for 15 minutes, (the total volume of dark solution is 100 ml). The second solution was prepared by mixing 900 ml of DW, 15 g agar, 30.24 g PIPES (Sigma Aldrich, UK) and 12 g NaOH and then autoclaved at 121°C for 15 minutes. After autoclaving, the first solution was mixed with the second solution and then the media was dispensed into the petri dishes. Immediately before use, 5mm diameter holes were punched into the agar using 5mm diameter gel plug cutter. The bacterial suspension was prepared by inoculating five colonies from TSA culture into 10 ml TSB broth containing 200 µM of 2,2′-dipyridyl (Sigma Aldrich, UK) and incubated at 37°C in shaking incubator at 200 rpm for 20 h. Then the samples were centrifuged at 5000 rpm for 10 minutes and 70 µl of the supernatant was placed into a specific hole. The agar was incubated at 37^{o}C for up to 8 hours and observed for the presence of an orange zone around the hole which indicated that the strain is positive for siderophore production. Y*ersinia enterocolitica* strain 1880 and PBS were used as positive and negative controls respectively.

2.8.4 Capsule formation

Three types of media were used for detecting the capsule production, VRBGA, VRBA and milk agar. VRBGA and VRBA were prepared as given in Sections 2.5.5. For preparing milk agar, 800 ml of PCA (Merck, Germany) was made according to the manufacture instructions. The PCA was cooled in water bath to 50°C before 200 ml of liquid infant formula (Cow & Gate Premium 1) was added and

gently mixed and the media then dispensed into petri dishes. All plates were left at room temperature for drying. The tested strains were streaked on the VRBGA, VRBA and milk agar and incubated at 37°C for 24 h. *C. sakazakii* strains 2 and 1 were used as positive and negative controls respectively.

2.8.5 Protease activity assay

Ten percent skimmed milk powder (Thermo Fisher Scientific, UK) was used to prepare the skimmed milk solution (SMS) and autoclaved for 5 minutes at 121°C. PCA plate count agar was prepared by adding 22.5 grams to 1 litre of distilled water and autoclaved at 121°C under 15 psi pressures for 15 minutes. Twenty milliliter of 10 % SMS was aseptically mixed with 980 ml of PCA and then dispensed into Petri dishes to be stored at room temperature for 2 days. The tested strains were streaked on the dried plates and incubated at 37°C for 72 h and monitored every 24 h. *Bacillus cereus* was used as a positive control while *E. coli* DH5α NTUCC407 was used as a negative control.

2.8.6 Serum resistance

The assay was conducted as described by Hughes et al. (1982) with a slight modification. A single colony from a fresh bacterial culture on TSA was grown overnight at 37°C with shaking in 5 ml TSB broth. This was then diluted 1:10 in new 5 ml TSB broth and thus grown in the same previous condition for 2 hours to approximately 10^6 cfu/ml. Forty microliters of culture broth were added to 360 µl of 50% human serum (Sigma Aldrich, UK). Tested strains were compared with positive and negative control which are *Salmonella* Enteritidis and *Escherichia coli* K-12 respectively. The viable bacterial cells were gained and counted using Miles and Misra technique. All strains were tested in triplicate and in three independent experiments. Each strain was tested in triplicate on TSA plates, and the mean results were stated as percent survival of inoculum.

$$\%\ Survival\ in\ serum = \frac{\text{The total number of survived bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \text{ X } 100$$

2.8.7 Plasmid profiling using plasmid alkaline lysis method.

SOLUTION I:

In 80 ml of SDW, 50 mM glucose (Sigma Aldrich, UK), 10mM EDTA (Sigma Aldrich, UK), 25 mM Tris pH 8.0 (Sigma Aldrich, UK) and 50 µg/ml of RNAse (Sigma Aldrich, UK) were mixed and the volume was added to be 100 ml, next filtered with 0.20 µm pore filter. The solution I was freshly prepared and stored at 4°C.

SOLUTION II:

Together 1% SDS and 0.2 M NaOH were mixed with total volume of 100 ml of SDW. This solution was freshly prepared in the same day of the experiment.

SOLUTION III:

To prepare high salt solutions 3 M of potassium acetate, 147.25 g of potassium acetate (Sigma Aldrich, UK) were dissolved in approx. 250 ml of SDW. Glacial acetic acid was added to lower the pH to 5.5. The final volume was brought to 500 ml with SDW.

SOLUTION IV:

TE pH 7.5 solution was prepared from 10mM of Tris (from 1 M pH 7.5 stock) and 1mM EDTA (from 0.5 M, pH 8.0 stock).

One colony of each strain was inoculated into 3 ml of TSB and incubated in shaking incubator at 37°C for 18-20 h. Next day, 1.5 ml of overnight cultures was placed into Eppendorf tubes and centrifuged at maximum speed for 5 mins. The supernatant media was carefully aspirated without touching the pellet and tubes were inverted and briefly tapped on paper tissue to dry. Twenty hundred microliter of cold 4°C solution I were added and tubes were vortexed until the pellet cells were completely resuspended and there were no clumps of cells remain. From fresh solution II, 200 µl were added and tubes were inverted about 6 times to lyse the cells, in this stage the lysate should become relatively clear. Same as previous step 200 µl from cold 4°C solution III were added and tubes were inverted about 6 times to thoroughly mix the content of tubes, in this stage a dense fluffy white precipitate should form. Then all tubes were centrifuged at 6000 rpm for 5 min. Five hundred microliter of supernatant were transferred into fresh tubes to be used for the next step. One millilitre of cold (-20°C) absolute alcohol was added and tubes were well mixed by inverting and brief vortexing. To increase the final yield all tubes were incubated at -20°C for 30 mins before centrifuging the tubes at maximum speed for 15 mins, in this stage a pellet should be readily observable. The tubes were all decanted from supernatant, inverted and tapped gently on paper tissue. A hundred microliter of cold (-20°C) 70% alcohol were added to tubes and centrifuged at maximum speed for 2 mins. All of the 70% ethanol supernatant was carefully removed from pellet and tubes were left opened for about 5 mins to dry. Finally, the pellet was dissolved in 25 µl of TE pH 7.5. To detect the plasmid DNA, 0.8% agarose gel was prepared by dissolving agarose (Thermo Fisher Scientific, UK) into 1X TAE buffer (National Diagnostics, UK) and about 8 µl of each sample was mixed with 2 µl of loading dye and the sample was then loaded into the agarose gel alongside the 1Kb DNA ladder (Promega, UK) to determine the size of plasmid. A run condition of the gel was

at 120v for 120 minutes, the agarose gel was stained with ethidium bromide (Sigma Aldrich, UK) 0.1 µg /ml for 45 min and the DNA bands were visualised using ultraviolet (UV) InGenius® gel documentation system (Syngene, UK).

2.8.8 Antimicrobial Susceptibility

The assay was performed by using disk diffusion method following the instructions in guidelines, version 12 – May 2013, described by British Society for Antimicrobial Chemotherapy (BSAC, 2013). The antimicrobial susceptibility test disks (MAST Group Ltd, UK) included ampicillin 10 µg, amoxicillin/clavulanic acid 30 µg, cefotaxime 5 µg, ceftazidime 30 µg, ceftriaxone 30 µg, cefuroxime 30 µg, amikacin 30 µg, chloramphenicol 30 µg, ciprofloxacin 5 µg, gentamycin 10 µg, imipenem 10 µg, meropenem 10 µg, nalidixic acid 30 µg, tetracycline 10 µg, trimethoprim/sulfamethoxazole 25 µg, nitrofurantoin 200 µg. About 5 colonies of fresh TSA culture were suspended in 3 ml of SDW and the OD adjusted to be equivalent to 0.5 McFarland standard (BioMerieux; France) and distilled 1:10 in distilled water. The suspensions were then swabbed onto the entire surface of Iso Sensitest agar (Thermo Fisher Scientific, UK) with sterile swab after which the antibiotic disks were applied onto the surface of ISA plates. *E. coli* 13353 and *E. coli* 10418 were used as reference strains. The plates were incubated at 37°C for 20 hours and the diameters of zones of inhibition were measured and interpreted according to guidelines, version 12 – May 2013, BSAC.

2.9 Investigation of pathogenicity using tissue culture

2.9.1 Bacterial strains

The same *C. malonaticus* strains as shown in table 2.3 were used in Chapter 5. *Salmonella* Entertidis strain number NCTC 3046 358, *Citrobacter koseri* strain number SMT319 48, *Klebsiella pneumoniae* strain ATCC43816, *Escherichia coli* strain UTI89 were all used as positive controls for Caco-2, HMBEC, A549 and T24 cell lines respectively and *E. coli* K12 MG1655 was the negative control for the all cell lines. A single colony of each tested and control strains was inoculated into 5 ml of TSB and incubated in shaking incubator at 200 rpm at 37°C for 18 h. Next day, 120 µl of overnight growth were added to 5 ml of appropriate infection culture media as explained in Section 2.9.3 and incubated as previous condition for about 2 h to reach the OD of 0.3-0.5 at 600 nm using the spectrophotometer (JENWAH, UK). The infection medium growth was diluted to obtain a number of $4x10^6$ cfu/ml which is multiplicity of infection (MOI) 1:100.

2.9.2 Human cell lines

Five human eukaryotic cell lines were used to determine the bacterial adhesion and invasion ability as shown in table 3. All cell lines were stored in the liquid nitrogen. When a cell line is required, a vial of an appropriate cell line was taken from the liquid nitrogen and thawed quickly. The content

of the cell line tube was mixed into 6ml of suitable pre-warmed growth medium, as explained in Section 2.9.3, in 15 ml falcon tube. The tube was then centrifuged at 1200 rpm for 5 minutes to harvest the cells. After discarding the supernatant, the cell pellet was re-suspended into 6ml pre-warmed growth medium and transferred into 25 cm^3 tissue culture flask. Finally, the flask was incubated for 48 hours at 37°C with the presence of 5% CO_2. After the cell line achieved confluent monolayer, the medium was decanted out and the cells were detached using 5 ml of TrypLe™ express (Life Technologies, UK). The cell suspension was mixed with 5 ml pre-warmed growth medium and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in pre-warmed growth medium and then transferred into a new 75 cm^3 tissue culture flask containing 20 ml of pre-warmed growth medium. The cell lines were maintained and split routinely twice a week to keep continuity of growth.

Table 2-4 Cell lines used in this study.

Cell line	Reference code	Source
Human colonic carcinoma epithelial cells (Caco-2)	ECACC #86010202	European Collection of Cell Cultures
Human brain microvascular endothelial cells (HBMEC)	#P10354	Inooprot, Spain
Macrophage cell line (U937)	ATCC#CRL-1593.2	American Type Culture Collection
Human lung carcinoma epithelial cells (A549)	ATCC# CCL-185	American Type Culture Collection
human bladder carcinoma epithelial cells (T24)	ATCC#HTB-4	American Type Culture Collection

2.9.3 Culture Media for Caco-2, HBMEC, A549 and T24 cell lines

All media were obtained from Sigma Aldrich, UK. Growth medium for Caco-2 cells was Minimum Essential Medium (MEM), (Sigma Aldrich, UK) with 10% fetal bovine serum (FBS) (Sigma Aldrich, UK), 1% non-essential amino acid (Sigma Aldrich, UK) solution and 1% Penicillin-Streptomycin (Sigma Aldrich, UK). HBMEC cells and A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, UK) supplemented with 10% FBS (Sigma Aldrich, UK), 1% non-essential amino acid (Sigma Aldrich, UK) solution and 1% Penicillin-Streptomycin (Sigma Aldrich, UK). T24 cells was grown in McCoy's 5A Medium (Sigma Aldrich, UK) with 10% FBS (Sigma Aldrich, UK), 1% non-essential amino acid (Sigma Aldrich, UK) solution and 1% Penicillin-Streptomycin (Sigma Aldrich, UK). The infection medium for all the above mentioned cell lines was same as the growth medium but lacking the 1% Penicillin-Streptomycin.

2.9.4 *C. malonaticus* attachment to human cells

For Caco-2 and HBMEC the cells were grown as described in Section 2.9.2. The adhesion assay was conducted as displayed by Townsend et al. (2008). The cells were seeded into 24-well plates

(Sarstedt, Germany) at a concentration of $4x10^4$ cell/well in growth medium and incubated at 37°C under 5% CO_2 for 48 h to achieve a confluent monolayer. For T24 cell line, the cells were seeded in 24-well plates at a concentration of $5x10^4$ cells/well and incubated as previous condition for 24h. For A549 cell line the cells were seeded at a concentration of $3x10^5$ cells/well and incubated in same condition for 16 h to achieve a confluent monolayer. After achieving the confluent monolayer, *C. malonaticus* and control strains were grown as described in Section 2.9.1. Bacterial suspension was added to the wells at a concentration of $4x10^6$ cfu /well which is MOI 1:100 and 24-well plates were incubated at 37°C with 5% CO_2 for 2 h. After the incubation period, the plates were washed 3 times with PBS (Sigma Aldrich, UK) and bacterial cells were released by lysing the human cells with 1% Triton X-100 (Thermo Fisher Scientific, UK). Serial dilution and Miles Misra method on TSA were performed to determine the overall of viable attached bacterial cells. Finally, the percentage efficiency of attachment was counted as shown in the following equation.

$$\%\ efficiency\ of\ attachment = \frac{\text{The total number of attached bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} X\ 100$$

2.9.5 *C. malonaticus* invasion of human cells

The preparation of cell lines and bacterial infection dose were done as described in the attachment Section 2.9.4. However, after 3 times washing with PBS the wells were filled with 500 µl infection media supplemented with gentamicin at a concentration of 125 µg/ml and the plates were incubated in same condition for 1 h to kill any extracellular bacteria. The plates were washed 3 times with PBS (Sigma Aldrich, UK) and bacterial cells were released by lysing the human cells with 1% Triton X-100 (Thermo Fisher Scientific, UK). Serial dilution and Miles Misra method on TSA were performed to determine the overall of viable invaded bacterial cells. Finally, the percentage efficiency of invasion was counted as shown in the following equation.

$$\%\ efficiency\ of\ invasion = \frac{\text{The total number of invaded bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} X\ 100$$

2.9.6 Uptake and persistence of *C. malonaticus* into macrophage cell line U937

2.9.6.1 Culture media

The macrophage cell line U937 was grown in RPMI medium containing 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (Sigma Aldrich, UK). The growth medium was supplemented with 10% FBS (Sigma Aldrich, UK), 1% non-essential amino acid (Sigma Aldrich, UK) solution and 1% Penicillin-Streptomycin (Sigma Aldrich, UK) while infection medium was supplemented just with 10%

FBS (Sigma Aldrich, UK) and 1% non-essential amino acid (Sigma Aldrich, UK) solution. The condition of incubation was at 37°C under 5% CO_2 for 24 h.

2.9.6.2 *C. malonaticus* uptake and persistence in human macrophages

The experiment was performed as described by Townsend et al. (2007a). The macrophage cells were seeded into four 24-well plates (Sarstedt, Germany) at a concentration of $4x10^4$ cell/well in growth medium and incubated at 37°C under 5% CO_2 for 72 h to achieve a confluent monolayer. However, before seeding the cells into 24-well plates, the macrophage cell suspension was supplemented with phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, UK) at a concentration of 0.1 µg/ml to ensure cell adhesion. The seeded plates were washed 3 times with PBS before infected with the determined infection dose. All tested and control strains were prepared as described previously in Section 2.9.1; however, the infection dose was at a concentration of $4x10^5$ cfu/ml which is MOI 10. The plates were incubated at 37°C under 5% CO_2 for 1h and the bacterial infection medium was replaced with infection medium supplemented with gentamicin at a concentration of 125 µg/ml and incubated in the same condition for further one hour. Three plates were washed 3 times with PBS and infection medium with gentamicin at a concentration of 50 µg/ml was added into the wells and incubated for intervals period 24 h, 48 h and 72 h. The infection medium with gentamicin was changed every day. In different time point 0h, 24 h, 48 h and 72 h, each plate washed 3 times with PBS and treated with 1% Triton X-100 to lyse the macrophage cells. Serial dilution and Miles Misra method on TSA were performed to determine the overall of viable intracellular bacterial cells. Finally, the percentage efficiency of uptake and persistence was counted as shown in the following equation.

$$\%\ uptake\ and\ persistence = \frac{\text{The total number of up taken/persisted bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \text{ X } 100$$

2.9.7 Giemsa staining

Visualisation of adhered *C. malonaticus* cells was performed in 8-well tissue culture slides (BD Falcon, UK). The cell lines and bacterial strains were prepared as described in Sections 2.9.1 and 2.9.4. After 2 hours of incubation the slides were washed 3 times with PBS and fixed with absolute methanol for 5 min after which the slides were left in the air to dry. The slides then stained with 5% of Giemsa stain (Life Technologies, UK) for 15 min. After staining the slides were washed once with PBS and dried in the air. Finally, the slide was visualised using immersion oil lens of the light microscope.

2.10 Genome studies

2.10.1 Genome sequencing

Bacterial DNA was extracted from 1-day old cultures using GenElute bacterial genome kit (Sigma Aldrich, UK) using the manufacturer's protocol. A total of 18 *C. malonaticus* genomes were sequenced during this project using different versions of illumina (GAIIx, HiSeq and MiSeq) sequencing technology. All of the strains were sequenced by Exeter Sequencing Service, Wellcome Trust Biomedical Informatics Hub, University of Exeter, UK.

2.10.2 Prokka genome annotation

The genome annotation refers to the identification and labelling of all the features on a sequenced genome (Richardson & Watson, 2013). In this study, the genome annotation of the sequenced *C. malonaticus* isolates was undertaken using Prokka; a command line software tool which takes preassembled genomes as input and requires the BLAST+ to be preinstalled on the Unix system (Seemann, 2014). Naqash Masood, who is a member of our pathogen research group, has kindly performed this work.

2.10.3 Genome screening of *C. malonaticus* for virulence and physiological associated traits

The screening of the presence and absence of interested genes and genome comparison was performed to reveal the presence of unique genes or regions that may contribute with physiological or virulence features. Artemis Comparison Tool (ACT) (Carver et al., 2005) was used for comparative analysis. The nucleotide sequence of any gene of interest was obtained in FASTA format and the sequence genes were checked against 20 *C. malonaticus* genomes using BLAST genome search in *Cronobacter* PubMLST database http://pubmlst.org/perl/bigsdb/bigsdb.pl?page=plugin&name=BLAST&db=pubmlst*cronobacter* isolates.

2.10.4 *In silico* plasmid profiling by alignment of known size plasmids with *C. malonaticus* genomes

Plasmid pESA3 (131 kb) which is regarded as virulence plasmid (Franco et al., 2011a), the large plasmid (118 kb) of *C. sakazakii* SP291 (ST4) (pSP291-1) (Power et al., 2013), and the large plasmid (~126 Kb) of *C. malonaticus* CMCC45402 ST7 (pCMCC 45402-1) (Zhao et al., 2014) were selected as reference plasmids. *C. sakazakii* (NTU6) was used as a reference (plasmid-less strains). These plasmids were aligned against 20 *C. malonaticus* genome using Blast Ring Image Generator (BRIG).

2.11 Statistical analysis

All the conducted experiments in this study have been repeated at least twice. The statistical analysis was carried out using unpaired t-test and One-way and Two-way ANOVA (GraphPad Prism Software Version 5.0) to obtain the consistence of the independent experiments. A *P* value of <0.05, <0.01 and < 0.001 was considered statistically significant, high significant and very high significant respectively.

Chapter 3 Phenotyping and Genotyping Profiles

3.1 Introduction

Cronobacter has been frequently isolated from PIF which has been recognised as a source of infection in several cases of neonatal *Cronobacter* outbreaks. *Cronobacter* has been also isolated from different clinical sites in hospital laboratories. The number of reported *Cronobacter* clinical isolates might not reflect the correct number of infections in hospitals as the major risk has been just linked to PIF (CDC, 2012b; Kalyantanda et al., 2015; Healy et al., 2010). Several phenotypic and genotypic methods have been introduced for detection and identification of *Cronobacter* spp. from different sources. Currently, there are numbers of phenotypic and genotypic techniques which have been applied for the detection and identification of *Cronobacter* spp. (Baldwin et al., 2009; Fei et al., 2015; Jackson et al., 2016).

The appearance of bacterial colonies on differential or selective media can give an initial presumptive identification as colonies exhibit a colour change that provides information about their identity. MacConkey agar which has been used since 1881 was designed for the isolation of Gram-negative enteric bacteria, differentiate between lactose fermenting and non-fermenting organisms and was used for the isolation of coliforms and other intestinal pathogens in dairy products and clinical specimens (Schauer, 2007). In 1962 Mossel et al. modified the MacConkey agar by adding glucose which is fermented by all of *Enterobacteriaceae* family. This alternative agar which is known as Violet Red Bile Glucose Agar (VRBGA) has been recommended for examination of food and enumeration of *Enterobacteriaceae*. Xylose lysine deoxycholate agar (XLD agar) is another agar was developed by Taylor in 1965 for isolation, differentiation and identification of enteric pathogens. The XLD agar was found to be a selective medium for *Salmonella* and *Shigella* which produce red black colonies and colourless colonies respectively.

For *Cronobacter* spp., Patrick Druggan designed the Druggan Forsythe Iversen (DFI) agar which is a chromogenic agar based on the ability of *Cronobacter* to produce α-glucosidase. This medium was compared with the traditional *Enterobacteriaceae* enumeration agar VRBGA by Iversen and Forsythe, (2004). The results showed that the detection of *Cronobacter* using DFI medium is more accurate as the use of VRBGA, the general *Enterobacteriaceae* selective medium, has some limitations. DFI agar is now used as a part of the conventional microbiology isolation method which involves pre-enrichment in BPW, enrichment in EE broth and finally plating onto DFI agar. The formation of blue green colonies on DFI indicates the organism is more likely *Cronobacter* spp.; however, this presumptive identification needs to be confirmed by other methods.

Presumptive identifications for enteric pathogens have been applied for a long time and several biochemical investigations are still used (Farmer et al., 1985). For *Cronobacter* spp. the use of these identification methods such as API20E or API ID32E are no longer regarded as reliable in distinguishing of *Cronobacter* from other genus. Of these biochemical test, Ivy et al. (2013) and Jackson et al. (2016) have subjected *Cronobacter* strains to the API 20E and ID32E schemes. Both studies showed the lack of sufficient robustness for the identification of *Cronobacter* genus and call for the need of applying other reliable identification methods. It is worthy to mention that *C. malonaticus* was initially considered as a subspecies within *C. sakazakii* and was differentiated from *C. sakazakii* subsp. sakazakii by malonate utilisation test. It was thought that *C. sakazakii* cannot utilise malonate while *C. sakazakii subsp. malonaticus* can (Iversen et al., 2007). However, in 2008, Iversen and colleagues revealed that a small portion of *C. sakazakii* strains utilize malonate.

To date, numerous genotypic methods have been developed for the identification and genotyping of *Cronobacter* spp. Of these methods 16S rRNA which is used for identification of different bacterial species (Iversen et al., 2008), RNA polymerase beta-subunit encoding gene (*rpoB*) sequences which is proposed as a technique for universal bacterial identification (Adekambi et al., 2009; Mollet et al., 1997; Stoop et al., 2009), and O-antigen typing which is used to divide species in different serotypes (Jarvis et al., 2011; Sun et al., 2012; Blazkova et al., 2015). Baldwin et al. (2009) introduced also a multilocus sequence typing scheme (MLST) which is 7 loci-based screening for *Cronobacter* genus. The scheme was initially created to distinguish between closely related species *C. sakazakii* and *C. malonaticus* which could not be distinguished using 16S rRNA. The concatenated of 7 loci *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB* and *ppsA* together give 3036 nucleotides long sequence which can distinguish between species and separate one species into several sequence types (STs). The MLST scheme has been proved to be a reliable and robust after being compared with whole genome analysis (Forsythe et al., 2014). Therefore, the technique was used to widen the knowledge about *Cronobacter* diversity and evolution (Joseph and Forsythe, 2012; Joseph et al., 2012b).

Other genetic fingerprinting techniques have been used for epidemiological investigations. These include random amplified polymorphic DNA (RAPD), BOX-PCR and pulsed-field gel electrophoresis (PFGE). PFGE is commonly used and considered as a gold standard technique in epidemiological studies of several pathogenic organisms (Olive and Bean, 1999). The technique is based on the use of restriction enzymes such as *XbaI* and *SpeI* to cut the bacterial DNA into several fragments and then separate these fragments into agarose gel electrophoresis (Caubilla-Barron et al., 2007). The discriminating results of DNA fragments by gel electrophoresis reveal the association of environmental or food isolates with clinical incidences, and thus allow to track the infection sources.

In this chapter, 51 *Cronobacter* strains which were collected from two hospitals of the Czech Republic during a 6-year period were studied phenotypically and genotypically to examine their appearance on different media, test the biochemical responses, identify their species using *rpoB* and MLST and finally discriminate them by PFGE. The results would reveal the proportion of *C. malonaticus* among this clinical collection. Thus, parts of this chapter have been already published.

3.2 Materials and methods

The methods for this chapter were described previously in Chapter 2 Materials and Methods.

3.3 Results

3.3.1 Appearance on different media

The results of using different culture media as a detection technique are shown in table 3.1. All the tested strains but one was able to produce the yellow pigment on TSA agar after incubation for 24 hours at 35°C, the exception was strain 2012 which showed light yellow colonies. Colonies of all strains on MacConkey agar had typical pink colour, and majority of them had mucoid pink colonies as demonstrating in table 3.1. All tested strains produced yellow colonies on XLD agar and most of them were mucoid colonies. After growing the strains on VRBGA for 24h at 37°C they all produced dark pink violet colonies. The same strains which gave mucoid colonies on MacConkey agar and XLD agar had mucoid colonies on VRBGA agar as well. On DFI agar all strains showed blue green colonies after 24h at 37°C.

3.3.2 Api32E

The biomedical identification result of strains using Api32E ID is showing in table 3.1. The majority of strains were identified as *Enterobacter sakazakii*; however, 48 strains were a 99.9% match to *E. sakazakii* and one strain had a 99.4% match to *E. sakazakii*. Two strains (1838 and 1841) had different profile identifications. Strain 1838 had a 99.6 match to *Pantoea* spp. and strain 1841 had match to *Enterobacter cloacae* with unacceptable profile.

3.3.3 Malonate utilisation test

In this assay the malonate broth was changed to blue-green colour by all *C. malonaticus* strains which indicate the ability of these strains to use malonate as a carbon source. Interestingly, malonate broth was also changed to blue colour by *C. sakazakii* 1995 (ST64), and the remaining *C. sakazakii* strains showed no ability of using malonate. When bacteria utilize malonate as a carbon source and ammonium sulfate as a nitrogen source from malonate broth, they produce sodium hydroxide which thereby results in an alkaline reaction and changes the pH indicator from its original green colour to blue. However, the formation of the blue green colour by some strains

might indicate the light production of sodium hydroxide due to weak utilisation of carbon and nitrogen sources.

Table 3-1 Phenotypic characterisation of *Cronobacter* isolates using cultural detection methods and api32EID version 3.0.

NTU	Apl32E	ID%	Colony appearance				
			TSA 37°C /72h	MacConkey 37°C /24h	XLD 37°C /24h	VRBGA 37°C /24h	DFI 37°C /24h
1827	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1828	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1829	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1830	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1831	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1832	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1833	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1834	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1835	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1836	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1837	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1838	*Pantoea spp1*	99.6%	yellow	pink	yellow	dark pink violet	Blue green
1839	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1840	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1841	*E. cloacae*	NA	yellow	pink M	yellow M	dark pink violet M	Blue green
1842	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1901	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1902	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1903	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1914	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1915	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1916	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1917	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1995	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1996	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1997	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
1998	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
1999	*E. sakazakii*	99.4%	yellow	pink	yellow	dark pink violet	Blue green
2000	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2001	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2002	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2003	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2004	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
2005	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2006	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2007	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2008	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green

2009	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2010	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2011	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2012	*E. sakazakii*	99.9%	light yellow	pink M	yellow M	dark pink violet M	light green
2013	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2014	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
2015	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
2016	*E. sakazakii*	99.9%	yellow	pink M	yellow	dark pink violet	Blue green
2017	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet M	Blue green
2018	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green
2019	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
2020	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
2021	*E. sakazakii*	99.9%	yellow	pink	yellow	dark pink violet	Blue green
2022	*E. sakazakii*	99.9%	yellow	pink M	yellow M	dark pink violet M	Blue green

M= mucoid.

3.3.4 *RpoB*, MLST and serotyping

Using the *fusA* sequence analysis and comparison with the *Cronobacter* PubMLST database identified the 51 strains as primarily *C. sakazakii* (33/51), followed by *C. malonaticus* (17/51) and one *C. muytjensii* strain. The strains were then further genotyped using the 7-loci MLST scheme. This supported the species identification-based *fusA* sequence analysis, and further subtyped the isolates (Table 3.2). The *C. sakazakii* strains were from two sequence types; ST4 (32/51, 63 %) and ST64 (1/51, 2 %). All the *C. malonaticus* strains were ST7 (17/51, 33 %) and the single *C. muytjensii* isolate was ST28 (2 %) (Figure 3.1 and 3.2).

The identification of strains using *rpoB* sequence analysis and comparison with *rpoB* sequences in the *Cronobacter* PubMLST database agreed with species designation using *fusA* allele sequence analysis (Table 3.2). There were four different *rpoB* profiles, 1, 18, 35 and 36, which correlated with their 7-loci sequences types (Figure 3.2 and 3.3). All *C. sakazakii* ST4 and ST64 strains were *rpoB* profiles 1 and 35 respectively. The *C. malonaticus* ST7 strains were *rpoB* profile 18 and *C. muytjensii* ST28 was *rpoB* profile 36. See table 2 and Figure 3.2 and 3.3 for more information.

Comparison with serotyping profiling showed a strong correlation between some sequence types and serotypes. O-serotype *C. sakazakii* O:2 corresponded with *C. sakazakii* ST4. The association was not exclusive; however, as *C. sakazakii* ST64 (strain 1995) was also serotype *C. sakazakii* O:2. In addition, the serotype of all (n = 17) *C. malonaticus* ST7 strains corresponded with the two designated serotypes *C. malonaticus* O:2 according to the schemes of Jarvis et al. and Sun et al.

respectively (Jarvis et al., 2011 and Sun et al., 2012). Based on *fusA* speciation, *C. malonaticus* O:2 was given as the serotype for these strains (Table 3.2). No serotype could be determined for the *C. muytjensii* strain as no PCR products were obtained with either PCR serotyping scheme.

Table 3-2 Speciation and genotyping of *Cronobacter* spp. from two hospitals

Strain	Hospital	Species	Pulsotype	*rpoB* allele	*FusA* allele	Serotype	Sequence type
1995	Prostějov	*C. sakazakii*	15	35	8	CS O:2	ST64
1836	Prostějov	*C. sakazakii*	14	1	1	CS O:2	ST4
1839	Prostějov	*C. sakazakii*	13	1	1	CS O:2	ST4
2021	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2022	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1901	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1915	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1996	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1837	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1841	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1842	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2003	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2005	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2007	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2010	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2016	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2019	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
2017	Prostějov	*C. sakazakii*	12	1	1	CS O:2	ST4
1902	Prostějov	*C. sakazakii*	11	1	1	CS O:2	ST4
1997	Prostějov	*C. sakazakii*	11	1	1	CS O:2	ST4
1903	Prostějov	*C. sakazakii*	10	1	1	CS O:2	ST4
1998	Prostějov	*C. sakazakii*	10	1	1	CS O:2	ST4
2006	Prostějov	*C. sakazakii*	10	1	1	CS O:2	ST4
2008	Prostějov	*C. sakazakii*	10	1	1	CS O:2	ST4

2012	Prostějov	*C. sakazakii*	9	1	1	CS O:2	ST4
2013	Prostějov	*C. sakazakii*	8	1	1	CS O:2	ST4
1916	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
1840	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
2000	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
2001	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
2002	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
2009	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
2011	Prostějov	*C. sakazakii*	7	1	1	CS O:2	ST4
1828	Olomouc	*C. malonaticus*	5	18	7	CMa O:2	ST7
1829	Olomouc	*C. malonaticus*	5	18	7	CMa O:2	ST7
1830	Olomouc	*C. malonaticus*	5	18	7	CMa O:2	ST7
1831	Olomouc	*C. malonaticus*	5	18	7	CMa O:2	ST7
1832	Olomouc	*C. malonaticus*	5	18	7	CMa O:2	ST7
1917	Olomouc	*C. malonaticus*	4	18	7	CMa O:2	ST7
1999	Olomouc	*C. malonaticus*	4	18	7	CMa O:2	ST7
2004	Olomouc	*C. malonaticus*	4	18	7	CMa O:2	ST7
2015	Olomouc	*C. malonaticus*	4	18	7	CMa O:2	ST7
2014	Olomouc	*C. malonaticus*	4	18	7	CMa O:2	ST7
2020	Olomouc	*C. malonaticus*	4	18	7	CMa O:2	ST7
1833	Olomouc	*C. malonaticus*	3	18	7	CMa O:2	ST7
1834	Olomouc	*C. malonaticus*	3	18	7	CMa O:2	ST7
1835	Olomouc	*C. malonaticus*	3	18	7	CMa O:2	ST7
1827	Olomouc	*C. malonaticus*	2	18	7	CMa O:2	ST7
1914	Prostějov	*C. malonaticus*	1	18	7	CMa O:2	ST7
2018	Prostějov	*C. malonaticus*	1	18	7	CMa O:2	ST7
1838	Olomouc	*C. muytjensii*	6	36	24	No PCR product	ST28

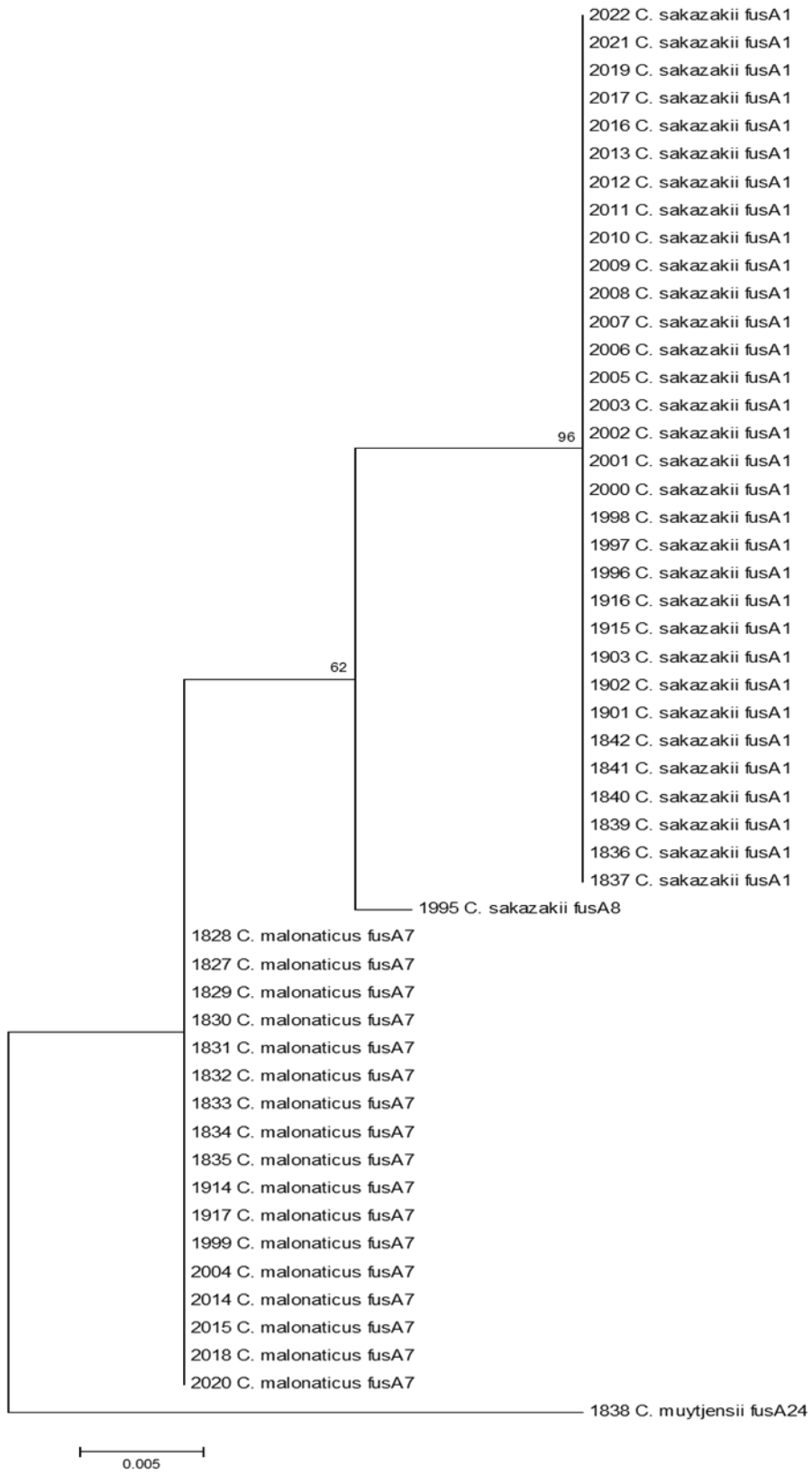


Figure 3-1 Maximum likehood tree of *fusA* gene (438 bp) of *Cronobacter* strains.
The tree was drawn to scale using MEGA6. Five fuseA profiles are shown; fusA1 and fusA8 for *C. sakazakii*, fusA7 for *C. malonaticus*, and fusA24 for *C. muytjensii*.

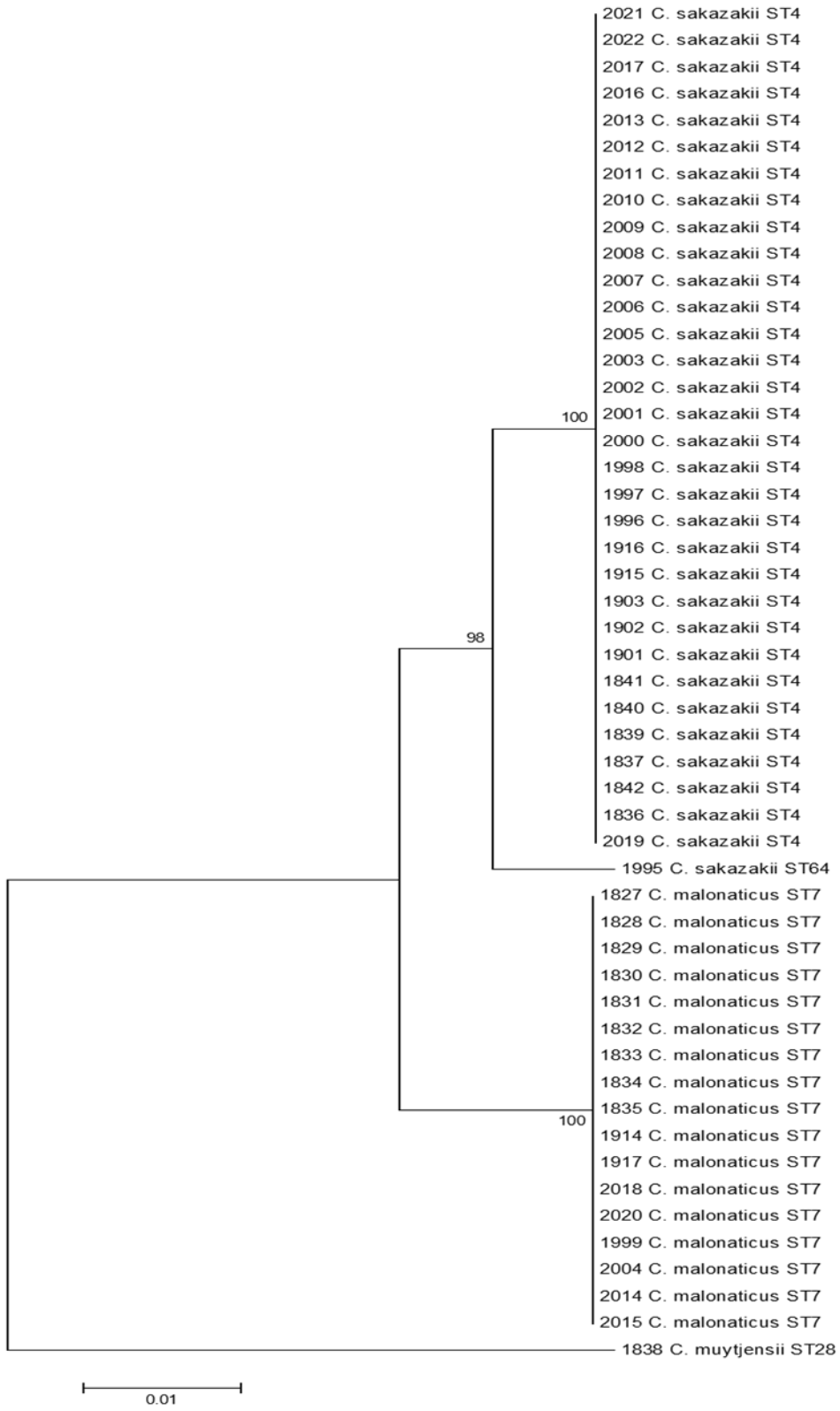


Figure 3-2 Maximum likehood tree of sequence types (STs) of *Cronobacter* strains.
This tree based on the concatenated sequences (3,036 bp) of the seven MLST loci and was drawn to scale using MEGA6. Five ST profiles are shown; ST4 and ST64 for *C. sakazakii*, ST7 for *C. malonaticus*, and ST28 for *C. muytjensii*.

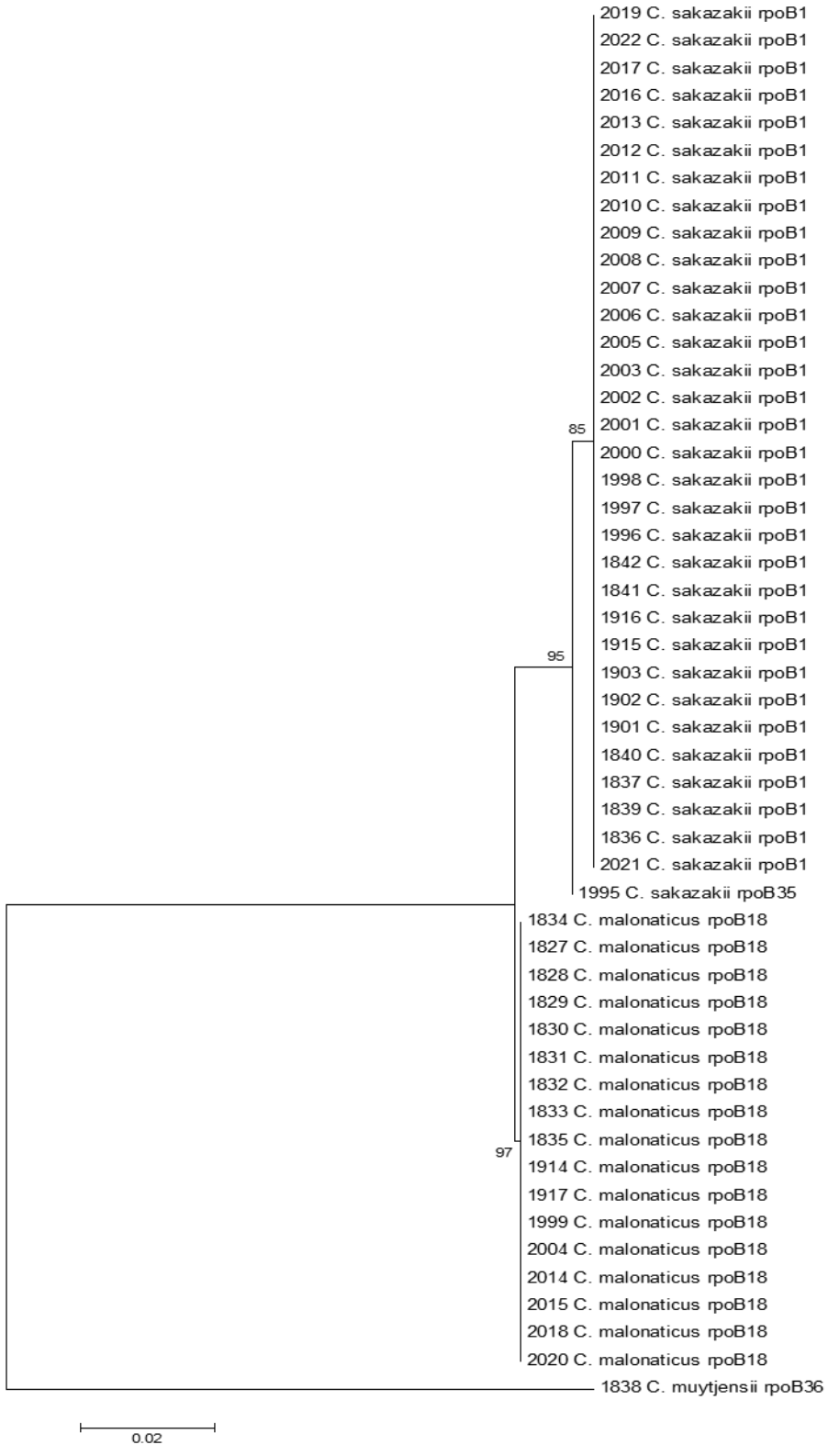
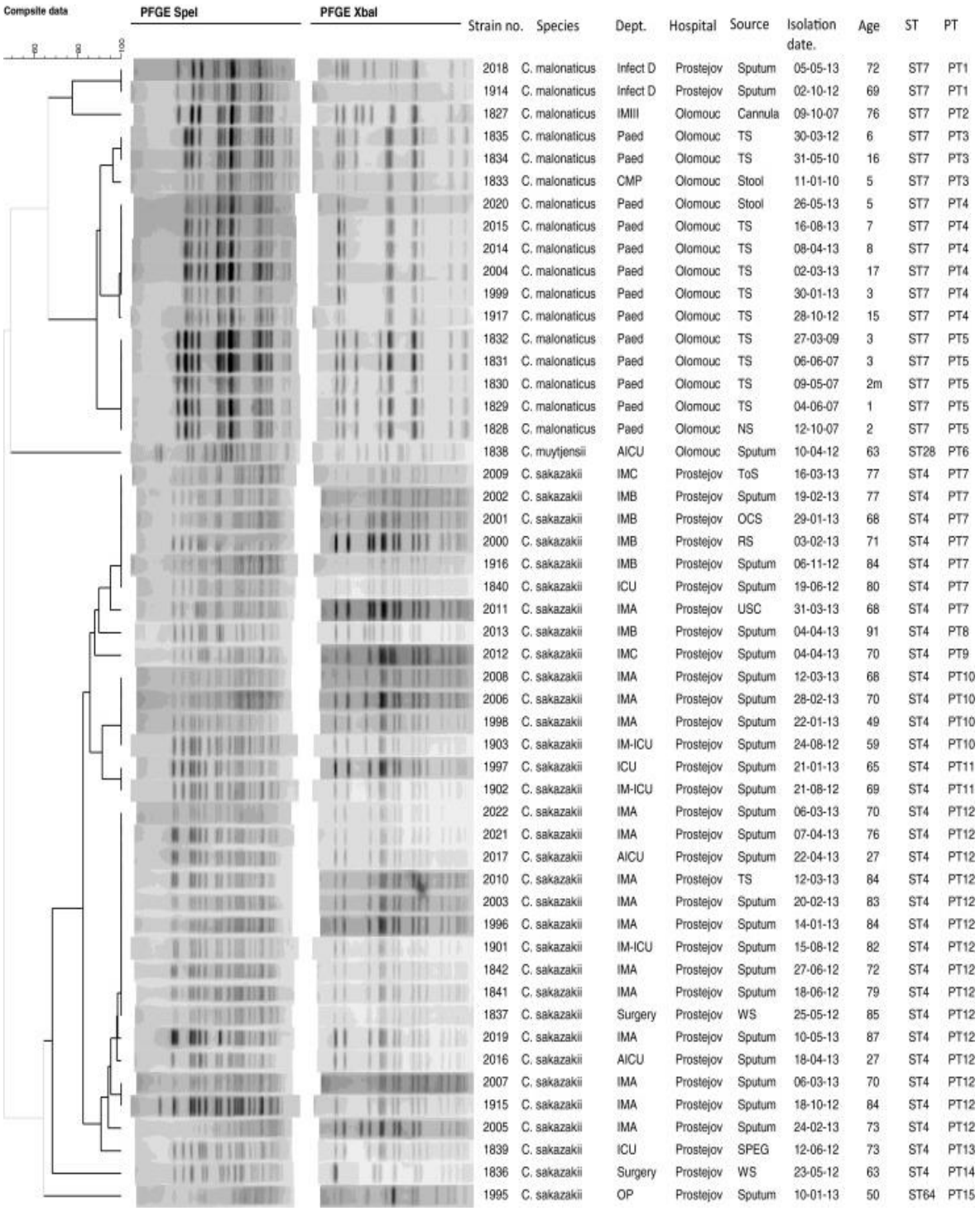


Figure 3-3 Maximum likehood tree of *rpoB* gene (637 bp) of *Cronobacter* strains.
The tree was drawn to scale using MEGA6. Five *rpoB* profiles are shown; *rpoB*1 and *rpoB*35 for *C. sakazakii*, *rpoB*8 for *C. malonaticus*, and *rpoB*36 for *C. muytjensii*.

3.3.5 Pulsed field gel electrophoresis (PFGE) of *Cronobacter* isolates

PFGE analysis of *Cronobacter* isolates was performed by using the two restriction enzymes *XbaI* and *SpeI* (Promega, UK). The bands were separated using a CHEF-DR II system (BIO-RAD, Belgium) at 14°C, 6V for 20h with initial and final switch of 5 and 50 secs respectively. The DNA band profiles were analysed using BioNumerics software version 7.1 (Applied Maths, Belgium). Comparison of the pulsetypes obtained from the PFGE for *XbaI* and *SpeI* was made by Composite Data combined compression with average of experiment by the unweighted-pair group method using arithmetic averages (UPGMA). Less than 95 % of band similarity value was used to consider the isolates to be non-clonal (Tenover et al., 1995).

PFGE was used to ascertain whether the strains in each sequence type (i.e. *C. sakazakii* ST4 and *C. malonaticus* ST7) could be further distinguished and whether this could be used to profile the strains from the two hospitals. Using the restriction enzyme *XbaI*, *C. sakazakii* strains gave 12 to 17 DNA fragments per strain, whereas *C. malonaticus* strains gave 8 to 10 bands (Figure 3.4). Comparable numbers of fragments were obtained using *SpeI*, 14 to 17 bands for *C. sakazakii* strains and 14 to 16 bands for *C. malonaticus* strains. The *XbaI* restriction enzyme separated the collection into 16 pulsotypes: ten for *C. sakazakii*, five for *C. malonaticus* and one for *C. muytjensii*, while the *SpeI* restriction enzyme divided the collection into 14 pulsotypes: eight for *C. sakazakii*, five for *C. malonaticus* and one for *C. muytjensii*. Combining the PFGE profiles generated with the restriction enzymes *XbaI* and *SpeI* grouped the 51 strains into a total of 15 pulsotypes: nine for *C. sakazakii*, five for *C. malonaticus* and one for *C. muytjensii*. Strains of the same sequence type from different hospital departments were distinguishable by PFGE and are considered in more detail below.



Strain no.	Species	Dept.	Hospital	Source	Isolation date.	Age	ST	PT
2018	C. malonaticus	Infect D	Prostejov	Sputum	05-05-13	72	ST7	PT1
1914	C. malonaticus	Infect D	Prostejov	Sputum	02-10-12	69	ST7	PT1
1827	C. malonaticus	IMIII	Olomouc	Cannula	09-10-07	76	ST7	PT2
1835	C. malonaticus	Paed	Olomouc	TS	30-03-12	6	ST7	PT3
1834	C. malonaticus	Paed	Olomouc	TS	31-05-10	16	ST7	PT3
1833	C. malonaticus	CMP	Olomouc	Stool	11-01-10	5	ST7	PT3
2020	C. malonaticus	Paed	Olomouc	Stool	26-05-13	5	ST7	PT4
2015	C. malonaticus	Paed	Olomouc	TS	16-08-13	7	ST7	PT4
2014	C. malonaticus	Paed	Olomouc	TS	08-04-13	8	ST7	PT4
2004	C. malonaticus	Paed	Olomouc	TS	02-03-13	17	ST7	PT4
1999	C. malonaticus	Paed	Olomouc	TS	30-01-13	3	ST7	PT4
1917	C. malonaticus	Paed	Olomouc	TS	28-10-12	15	ST7	PT4
1832	C. malonaticus	Paed	Olomouc	TS	27-03-09	3	ST7	PT5
1831	C. malonaticus	Paed	Olomouc	TS	06-06-07	3	ST7	PT5
1830	C. malonaticus	Paed	Olomouc	TS	09-05-07	2m	ST7	PT5
1829	C. malonaticus	Paed	Olomouc	TS	04-06-07	1	ST7	PT5
1828	C. malonaticus	Paed	Olomouc	NS	12-10-07	2	ST7	PT5
1838	C. muytjensii	AICU	Olomouc	Sputum	10-04-12	63	ST28	PT6
2009	C. sakazakii	IMC	Prostejov	ToS	16-03-13	77	ST4	PT7
2002	C. sakazakii	IMB	Prostejov	Sputum	19-02-13	77	ST4	PT7
2001	C. sakazakii	IMB	Prostejov	OCS	29-01-13	68	ST4	PT7
2000	C. sakazakii	IMB	Prostejov	RS	03-02-13	71	ST4	PT7
1916	C. sakazakii	IMB	Prostejov	Sputum	06-11-12	84	ST4	PT7
1840	C. sakazakii	ICU	Prostejov	Sputum	19-06-12	80	ST4	PT7
2011	C. sakazakii	IMA	Prostejov	USC	31-03-13	68	ST4	PT7
2013	C. sakazakii	IMB	Prostejov	Sputum	04-04-13	91	ST4	PT8
2012	C. sakazakii	IMC	Prostejov	Sputum	04-04-13	70	ST4	PT9
2008	C. sakazakii	IMA	Prostejov	Sputum	12-03-13	68	ST4	PT10
2006	C. sakazakii	IMA	Prostejov	Sputum	28-02-13	70	ST4	PT10
1998	C. sakazakii	IMA	Prostejov	Sputum	22-01-13	49	ST4	PT10
1903	C. sakazakii	IM-ICU	Prostejov	Sputum	24-08-12	59	ST4	PT10
1997	C. sakazakii	ICU	Prostejov	Sputum	21-01-13	65	ST4	PT11
1902	C. sakazakii	IM-ICU	Prostejov	Sputum	21-08-12	69	ST4	PT11
2022	C. sakazakii	IMA	Prostejov	Sputum	06-03-13	70	ST4	PT12
2021	C. sakazakii	IMA	Prostejov	Sputum	07-04-13	76	ST4	PT12
2017	C. sakazakii	AICU	Prostejov	Sputum	22-04-13	27	ST4	PT12
2010	C. sakazakii	IMA	Prostejov	TS	12-03-13	84	ST4	PT12
2003	C. sakazakii	IMA	Prostejov	Sputum	20-02-13	83	ST4	PT12
1996	C. sakazakii	IMA	Prostejov	Sputum	14-01-13	84	ST4	PT12
1901	C. sakazakii	IM-ICU	Prostejov	Sputum	15-08-12	82	ST4	PT12
1842	C. sakazakii	IMA	Prostejov	Sputum	27-06-12	72	ST4	PT12
1841	C. sakazakii	IMA	Prostejov	Sputum	18-06-12	79	ST4	PT12
1837	C. sakazakii	Surgery	Prostejov	WS	25-05-12	85	ST4	PT12
2019	C. sakazakii	IMA	Prostejov	Sputum	10-05-13	87	ST4	PT12
2016	C. sakazakii	AICU	Prostejov	Sputum	18-04-13	27	ST4	PT12
2007	C. sakazakii	IMA	Prostejov	Sputum	06-03-13	70	ST4	PT12
1915	C. sakazakii	IMA	Prostejov	Sputum	18-10-12	84	ST4	PT12
2005	C. sakazakii	IMA	Prostejov	Sputum	24-02-13	73	ST4	PT12
1839	C. sakazakii	ICU	Prostejov	SPEG	12-06-12	73	ST4	PT13
1836	C. sakazakii	Surgery	Prostejov	WS	23-05-12	63	ST4	PT14
1995	C. sakazakii	OP	Prostejov	Sputum	10-01-13	50	ST64	PT15

Figure 3-4 Combined *XbaI* and *SpeI* PFGE profiles of fifty-one *Cronobacter* strains.
InfectD = Infection Disease, IMIII = Internal Medicine III, Paed Paediatric, CMP = Clinical and Molecular Pathology department, AICU = Anaesthesiology and Intensive Care Unit, IMA IMB IMC = Internal Medicine ABC, ICU = Intensive Care UnitOP = Out-Patient, TS = Throat Swab, NS, = Nasal Swab, ToS = Tongue Swab, OCS = Oral Cavity Swab, RS = Rectal Swab, USC= Urine suction catheter, WS = Wound Swab SPEG = Smear of Percutaneous Endoscopic Gastrostomy.

The isolates from Olomouc hospital formed four distinguishable *C. malonaticus* pulsotypes (PT2 to 5) and one *C. muytjensii* pulsotype (PT6), which were recovered from different age groups of patients from four hospital departments. PT2 was one *C. malonaticus* ST7 strain (1827) isolated in the Internal Medicine Department from the intravenous cannula of a 76-year-old patient in 2007. PT3 was composed of three *C. malonaticus* ST7 strains (1834, 1835, 1833), two of which were isolated from the Paediatric Department and one was from the Clinical and Molecular Pathology Department. The three PT3 strains had been isolated over a 2-year period from throat and stool samples of patients under 16 years of age. The six isolates in PT4 were all *C. malonaticus* ST7 strains. Five had been isolated from the Paediatric Department over a 10-month period from throat swabs and one from a stool sample. The patient ages ranged from 3 to 17 years old. The majority (4/5) of PT5 strains were isolated from the throat and one from nose from the same Paediatric Department. These strains were also *C. malonaticus* ST7 and had been collected over a period of 2 years. The patient ages ranged from 2 months to 3 years. *C. muytjensii* ST28 strain 1838 was in a unique pulsotype (PT6). This strain was isolated in 2012 at the Anaesthesiology and Intensive Care Unit, from the sputum of a 63-year-old patient.

The isolates from Prostějov hospital were recovered from seven departments and were clustered in ten distinguishable *Cronobacter* pulsotypes (Table 3.2 and 3.3). PT1 was the only *C. malonaticus* pulsotype (strains 1914 and 2018). These were both *C. malonaticus* ST7 strains which were isolated from patients' sputum at the Infectious Disease Department. The collection was over a 7-month period, and the patients were 69 and 72 years in age. All the remaining isolates were strains of *C. sakazakii*, which formed nine pulsotypes (PT7 to 15). Eight of these pulsotypes (PT7 to 14) were composed of 32 strains of *C. sakazakii* ST4. PT15 was composed of one *C. sakazakii* ST64 strain (1995). Most of the 15 *C. sakazakii* ST4 strains in PT12 were isolated from sputum except strains 1837 and 2010, which were isolated from a wound swab and throat swab, respectively. This pulsotype was collected over period of about 1 year and the patients' ages ranged from 27 to 87 years. In PT12, 12 isolates were collected from the Internal Medicine Department, two from the Anaesthesiology and Intensive Care Unit and one from the Surgery Department. PT13 and PT14 each contained single *C. sakazakii* ST4 strains; 1839 and 1836, respectively. PT15 contained a single *C. sakazakii* ST64 strain (1995). These strains were isolated from a percutaneous endoscopic gastrostomy smear ICU, wound surgery and the sputum of an outpatient, respectively. The isolations were over a 7-month period and the patient ages ranged from 50 to 73 years. PT7 consisted of seven *C. sakazakii* ST4; strains 1840, 1916 and 2002 were isolated from sputum, strain 2000 from rectal swab, strain 2001 from oral cavity swab, strain 2009 from tongue swab and strain 2011 from section catheter. Six of the isolates were collected from the Internal Medicine

department, and strain 1840 was isolated from an Intensive Care Unit patient (Table 3.2, Table 3.3). The collection was over a 7-month period and all patients were over 68 years of age. PT8, 9, 10 and 11 consisted of eight *C. sakazakii* ST4 strains. All these strains except one (1997) were isolated from sputum at the Internal Medicine Department, whereas strain 1997 was collected from the Intensive Care Unit. The PT8 strain was isolated in 2013 from a 91-year-old patient. PT9 was isolated in 2013 from a 70-year-old patient. PT10 was collected over a roughly 8-month period and the patient ages were between 49 and 70 years old. The two strains in PT11 were collected in 2012 and 2013 and the mean patient age was 67 years (Table 3.4).

Table 3-3 number of isolated *Cronobacter* strains from various hospitals departments.

Hospital	Department	Number *Cronobacter* strains isolated
Olomouc	Paediatrics	13
	Internal Medicine	1
	AICU	1
	Pathology	1
Prostějov	Internal Medicine	22
	Internal Medicine - ICU	3
	Surgery	2
	ICU	3
	Infectious Diseases	2
	AICU	2
	Outpatient	1

Table 3-4 Distribution of *Cronobacter* species and genotype according to hospital and patient details.

Cronobacter species	Sequence type	No of isolates (%)	Pulsotype (n)	Hospital	Period of isolation	Age (y)	Sex		Source (n)
							Male	Female	
C. sakazakii	ST4	32 (63)	12 (15), 7 (7) 10 (4), 11 (2) 8 (1), 9 (1), 13 (1), 14 (1)	Prostějov	12/06/12-10/05/13	>27	16	16	Sputum (24), Wound swab (2), Section catheter (1), Tongue swab (1) Throat swab (1), Oral cavity (1), Rectal swab (1), SPEG (1)
C. sakazakii	ST64	1 (2)	15 (1)	Prostějov	10/01/2013	50	1	0	Sputum (1)
C. malonaticus	ST7	17 (33)	4 (6), 5 (5) 3 (3), 2 (1)	Olomouc	06/05/07-16/08/13	<1-76	12	5	Throat swab (11), Fecal material (2), Cannula (1), Nasal swab (1)
			1 (2)	Prostějov	2/10/2012 & 5/05/2013	69 & 72	2	0	Sputum (2)
C. muytjensii	ST28	1 (2)	6 (1)	Olomouc	10/04/2012	63	0	1	Sputum (1)
Total		51			6 years		29	22	

GoeBURST analysis showed the range of patient ages and sources with *Cronobacter* species (Figure 3.5). *C. sakazakii* ST4 strains were predominantly sputum samples from adults >70 years in age, whereas *C. malonaticus* ST7 were from throat swabs of children <6 years old.

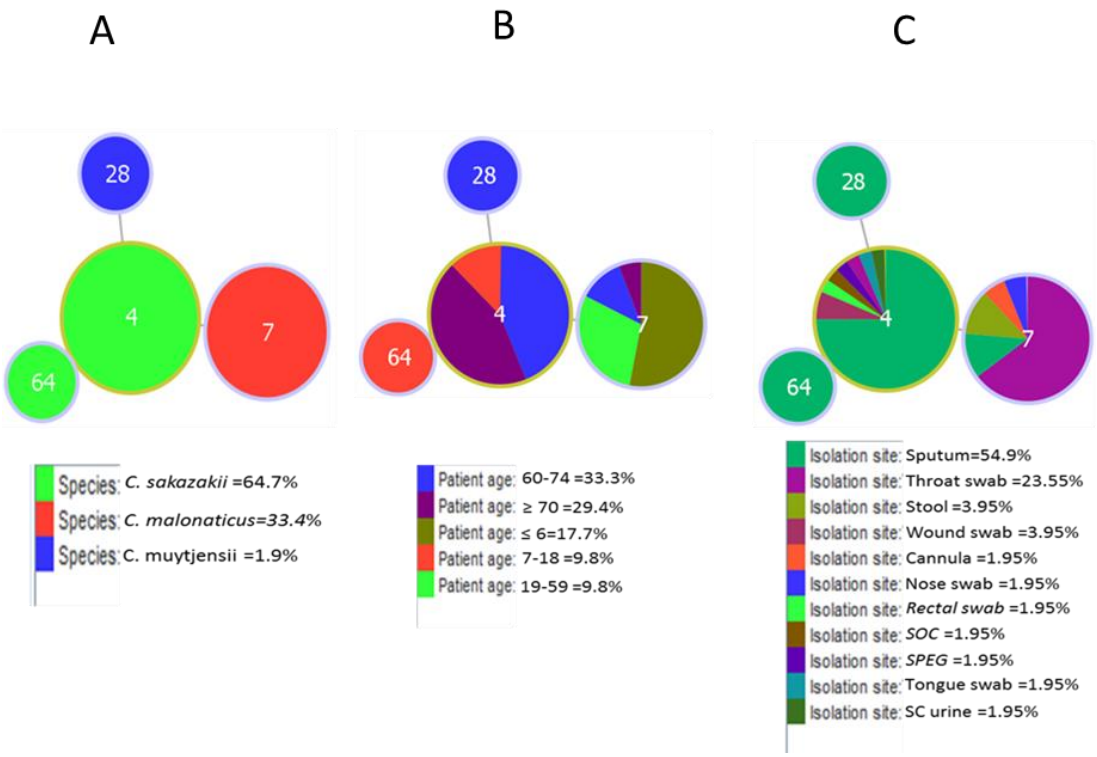


Figure 3-5 GoeBURST analysis of *Cronobacter* strains.
A; shows the percentage of each identified spicies, each color represent a spcicies (*C. sakazakii* is green, *C. malonaticus* is red, *C. muytjensii* is blue), and the dominant STs are represented by the circles with larger diameters. **B**; shows the percentage of different patient ages. Each color represent a group of age within each ST. **C**; shows the percentage of different clinical isolation sites. Each color represent an isolation source within each ST.

3.4 Discussion

Rapid detection methods help in reducing the risk of the occurrence of the infection incidences by applying an appropriate treatment strategy and recognising the bacterial source, transmission route and thus lead to set steps of control measures (Adamsson et al., 2000; Almuneef et al., 2001). Some phenotypic methods using growth media give a quick presumptive identification of the infection causes as the bacteria grow with different morphology aspects. Other growth media help in the identification by inhibiting the growth of some bacterial groups. This preliminary identification is still confirmed biochemically in some laboratories; however, this has been proved to be no longer reliable especially with *Cronobacter* species. Therefore, the using of molecular typing methods is important to achieve high-accuracy identification (Iversen et al., 2007; Baldwin et al., 2009; Cetinkaya et al., 2013).

The reported *Cronobacter* infections have primarily concerned with the infants and many of these cases have been linked to contaminated reconstituted infant formula (van Acker et al., 2001; CDC, 2002; Weir, 2002; Chenu and Cox, 2009); however, other routes appear to exist, as infections occur in breast-fed infants as well (Bowen and Braden, 2006; Stoll et al., 2004; Ravisankar et al., 2014). The carriage of the organism by adults (Holý et al., 2014) and the high incidence of UTIs (Patrick et al., 2014) indicate that the exposure routes to this microorganism still require further elucidation. The first process of isolation the microorganism from different sources is the use of basic technique as culture media, these basic techniques are still required before applying the molecular level techniques as these culture media assist in minimising the anticipated bacteria. In order to have a wider perspective on the exposure and the identification of *Cronobacter*, this study phenotyped and genotyped *Cronobacter* strains from age-profiled clinical isolates, and extended the previous study by Holý and his co-authors who reported the incidence of *Cronobacter* from >45,000 patients (Holý et al., 2014).

In this study, the colony morphology of all 51 *Cronobacter* strains was investigated using four different media. Appearance of the tested colonies on MacConkey agar was in pink colour indicated the fermentation of lactose in this medium. MacConkey agar is considered as an alternative enrichment medium to VRBGA as it can differentiate the majority of *Enterobacteriaceae* family (Druggan and Iversen, 2009) and is one of widely used media in clinical laboratories. VRBGA is a selective and differentiation medium that inhibit the growth of Gram positive bacteria and differentiate the Gram negative to glucose fermenters and non-fermenters organisms. This medium is used in the enrichment step of DFA *Enterobacteriaceae* isolation methods (Druggan and Iversen, 2009; Mossel and Ratto, 1970). The *Cronobacter* strains in this study expressed typical pink colonies

surrounded with purple halo. However, majority of colonies were mucoid while strains 1828 to 1835, 1902, 1914, 1915, 1917, 1997, 1999, 2004, 2014, 2015, 2016, 2019, 2020 and 2021 were non-mucoid. The production of mucoid colonies might be associated to utilisation of carbon sources result in production of exopolysaccharides (EPS). The production of EPS relies on the use of carbohydrates as carbon sources, which allow for high productivities and yields. The most commonly used substrates are sucrose and glucose; however, lactose, xylose and galactose are less frequently used as many bacteria are unable to use them which result in reduced the production of EPS (Roca et al., 2015). For example, *Streptococcus mutans* use sucrose to produce a sticky, extracellular, dextran-based polysaccharide (Busuioc et al., 2009). *Cronobacter* also secrete EPS colanic acid which is composed of glucuronic acid, D-glucose, D-galactose, D-fucose and D-mannose (0–0.8%) (Ogrodzki and Forsythe, 2015). Finally, one strain which is 1838 grew very poorly on VRBGA, similar poor growth of some strains on VRBGA has been reported by Iversen and Forsythe (2007).

On XLD agar all tested strains formed yellow colonies and colonies that were mucoid on VRBGA were mucoid on XLD as well. The selective agent in XLD agar is sodium deoxycholate, which inhibits the growth of gram-positive organisms. The carbohydrate source is xylose which is fermented by most enterics except for *Shigella* species, and these colonies appear red on this medium as a result. A second differential mechanism for *Salmonella* is employed by the addition of lysine. Lysine decarboxylation reverts the pH of the medium to an alkaline condition. To avoid this reversal to a *Shigella* reaction, lactose and sucrose are added in excess. The addition of sodium thiosulfate and ferric ammonium citrate as a sulfur source and indicator, respectively, allows hydrogen sulfide forming organisms to produce colonies with black centers, under alkaline conditions. The XLD agar is a selective agar for *Salmonella* and *Shigella*; however, it can distinguish some of other enteric pathogens such as *Citrobacter* and *Escherichia coli* by xylose fermentation which leads to the formation of yellow colonies. On TSA agar the colonies that produce yellow pigment is presumably identified as *Cronobacter* spp.; however, different factors such as temperature, light could affect the expression of this pigment making it an unreliable feature (Druggan and Iversen, 2009; Farmer et al., 1980; Johler et al., 2009). Nevertheless, all 51 *Cronobacter* strains were able to produce the yellow pigment after 24h at 37°C with light yellow colour for strain 2012. *Cronobacter* form yellow pigment on TSA because they possess a gene cluster which contained seven genes (*crtE-idi-crtXYIBZ*). This cluster is responsible for biosynthesis of carotenoids which involve in the pigment's carotenogenic nature (Lehner et al., 2006). The 51 *Cronobacter* strains formed clear blue green colonies on DFI agar due to α-glucosidase activity**.** The appearance of the blue-green colonies on DFI indicates that colonies are more likely to be *Cronobacter* spp.; however, other bacteria such as

Buttauxiella nokiae and *Escherichia hermanii* could form same blue-green colonies which may give false identification that need further confirmation (Jackson et al., 2014). Although the use of these media in hospital laboratories does not give a complete reliable identification, it is still essential for growing and isolation of different bacteria and also for minimising the possible identification of microorganism in this level.

This study biotyped the 51 *Cronobacter* strains using API ID32E. The API scheme is aimed to speciate the organisms using group of biomedical tests and online database to identify the species. When this study was undertaken the database did not show the *Cronobacter*, consequently the positive result was displayed as *E. sakazakii*. Nevertheless, all but two of 51 strains were identified as *E. sakazakii* with high percentage matching >99.94, the exception was strain 1838 and strain 1841 which were identified as *Pantoea* spp. and *E. cloacae* respectively. These strains were genotypically confirmed later as *Cronobacter* spp. This false identification was reported also by other studies used API ID32E scheme (Ivy et al., 2013; Jackson et al., 2014.).

It was expected that all *C. malonaticus* strains utilise malonate; however, *C. sakazakii* ST64 showed also ability of utilising malonate. Iversen et al. (2006) extended the *E. sakazakii* biogroups, which intilally created by Farmer et al. (1980), to 16 biogroups. They reported that 7 biogroups out of 16 were able to utilise malonate and 4 of these 7 biogroups contain *C. sakazakii*. However, there was no more details about which ST of *C. sakazakii* could utilise this malonate. This lead to test all of *C. sakazakii* ST64 strains, which belong to biogroup 5, that available in the *Cronobacter* MLST Databases. The results confirmed that all tested *C. sakazakii* ST64 strains were able to utilise malonate (Data not shown). *Cronobacter* PubMLST database provide to the researchers several facilities such as BLAST genome search as it contains more than 300 *Cronobacter* genome. Thus, the malonate utilisation associated genes have been searched and these genes were detected in all *C. malonaticus* strains, *C. sakazakii* ST64 and also in *C. sakazakii* ST233 and *C. sakazakii* ST245. ST64 and ST233 belonged to biogroup 5 which contain also different STs of *C. malonaticus*. These results confirmed that malonate utilisation feature is shared between both species.

The phenotypic methods, which have been applied in this study, showed some limitations that they could not be used for correct identifications and could not speciate the strains. Therefore, genotypic methods are necessary and important in order to achieve a high level of identification. The 51 *Cronobacter* strains were speciated and genotyped using *rpoB* gene sequence analysis, 7-loci MLST, O-antigen typing and PFGE. The results were further analysed using provided information and the goeBURST software.

PFGE analysis of isolates revealed that the 35 strains isolated at Prostějov hospital could be divided into three groups. The majority (32/35) of strains belonged to *C. sakazakii* ST4 and were serotype *C. sakazakii* O:2. These strains were isolated from various hospital departments during 2012–2013. Two other group isolates were also recovered from patients in this hospital. These were two strains of *C. malonaticus* ST7 and were serotype *C. malonaticus* O:2, and were the only strains isolated from the Department of Infectious Diseases. The remaining strain was *C. sakazakii* ST64 serotype O:2, which was isolated from sputum of an outpatient who is 50 year old.

In contrast, fifteen *Cronobacter* strains isolated from patients at Olomouc hospital were identified as *C. malonaticus* and one isolate was identified as *C. muytjensii*. The *C. malonaticus* strains belonged to the identical sequence type 7 and identical serotype *C. malonaticus* O:2. With two exceptions, all these strains were from patients at the Department of Paediatrics who had an age range of 0–18 years. There were two strains from adults, one *C. malonaticus* from an intravenous cannula and another which was *C. muytjensii* from sputum.

Despite the greater discrimination of strains using PFGE than MLST, isolates from patients for whom there were no known links could not be further differentiated. For example, the *C. sakazakii* ST4, pulsotype 12 strains were isolated from 15 adults (aged 27–85 years) between May 2012 and May 2013. This could be due to the reported high clonality of sequence types within *C. sakazakii* and *C. malonaticus* limiting the discriminatory power of PFGE (Joseph et al., 2012b; Forsythe et al., 2014). This study shows the value of applying MLST to bacterial population studies with strains from two patient cohorts, combined with PFGE for further discrimination of strains.

The result of this study showed the presence of just one *C. malonaticus* ST and confirmed the high clonality between *C. malonaticus* strains. Therefore, this project was amended as there was not enough *C. malonaticus* STs or diversity between strains. Consequently, six *C. malonaticus* strains (2018, 1827, 1830, 1835, 1833, 2020), representing the 5 *C. malonaticus* PTs (PT1 to 5), were selected from this collection for the PhD stage parallel with 14 *C. malonaticus* strains from the *Cronobacter* PubMLST database (Table 3.5).

Table 3-5 *C. malonaticus* strains used in Chapter 4 and 5

Strain No.	ST	rpoB	O-antigen	Source	Country	Year of isolation	Comment
565	7	18	CmalO2	Faecal isolate	USA	1973	CPMD
681	7	18	CmalO2	Breast abscess isolate	USA	1977	CPMD
688	7	18	CmalO2	Sputum	Czech Republic	2004	CPMD
983	7	18	CmalO2	Infant formula	Brazil	2007	CPMD
1558	7	18	CmalO2	Faecal isolate	Czech Republic	Unknown	CPMD
1827	7	18	CmalO2	Cannula (Blood)	Czech Republic	2007	Table 2.1
1830	7	18	CmalO2	Throat swab	Czech Republic	2007	Table 2.1
1833	7	18	CmalO2	Faecal isolate	Czech Republic	2010	Table 2.1
1835	7	18	CmalO2	Throat swab	Czech Republic	2012	Table 2.1
2018	7	18	CmalO2	Sputum	Czech Republic	2013	Table 2.1
2020	7	18	CmalO2	Faecal isolate	Czech Republic	2013	Table 2.1
507	11	20	CmalO3	Faecal isolate	Czech Republic	1984	CPMD
512	11	20	CmalO3	Clinical	Czech Republic	1983	CPMD
514	11	20	CmalO3	Clinical	Czech Republic	1983	CPMD
15	60	39	CmalO1	Faecal isolate	Czech Republic	2003	CPMD
687	60	39	CmalO1	Sputum	Czech Republic	2004	CPMD
689	60	39	CmalO1	Faecal isolate	Czech Republic	2005	CPMD
1545	84	18	CmalO2	Faecal isolate	Czech Republic	Unknown	CPMD
685	129	39	CmalO2	Blood isolate	USA	1977	CPMD
1569	307	39	CmalO1	Blood isolate	USA	2011	CPMD

ST: sequence type. CPMD*: Cronobacter* PubMLST database.

Chapter 4 Physiological and virulence traits

4.1 Introduction

C. malonaticus is an opportunistic pathogen that is usually linked to adult infections. However, recent reports have shown the involvement of this species with neonatal infections (Joseph et al., 2012b; Hariri et al., 2013; Asato et al., 2013; Holý et al., 2014; Brandao et al., 2015; unpublished data China). *C. malonaticus* is a foodborne pathogen that is present in several types of dry food including PIF, powdered milk and spices. To cause a disease, foodborne pathogens first need to tolerate diverse conditions such as desiccation, high temperature and high pH in the food and water. In addition, once these pathogens are ingested, they must be able to survive the exposure to several host conditions such as stomach acid, bile and mechanisms of the immune system (Boor, 2006).

In this chapter, the experimental work will be focused on investigation of physiological and virulence traits that might contribute to the pathogenicity of *C. malonaticus*. In this study 20 strains of different sequence types of *C. malonaticus* were used. Six ST7 strains were selected to represent the 5 pulse types of the previous study in Chapter 3 and an additional 14 *C. malonaticus* strains were chosen from the *Cronobacter* PubMLST database from different countries to represent different STs (Table 3.5, Chapter 3). Utilisation of sialic acid was tested among used *C. malonaticus* strains. In addition, several resistance traits such as acid resistance, heavy metal tolerance, susceptibility to antibacterial agents and human serum resistance were conducted. Moreover, biofilm formation, recovery of sublethally dry-injured bacterial cells and production of capsule, cellulose, haemolysins, siderophore, protease and motility were investigated.

C. malonaticus has been isolated from PIF (*Cronobacter* PubMLST database) which is considered as the source of some incidences of *Cronobacter* neonatal infections. In addition, *Cronobacter* can tolerate the desiccation even more than other *Enterobacteriaceae* (Breeuwer et al., 2003). During food processing, bacterial cells could be injured due to exposure to different stresses such as drying. The injured bacterial cells can survive diverse conditions; however, some cellular changes of the cell wall, cytoplasmic membrane or inner membrane, ribosomes, DNA, RNA, tricarboxylic-acid-cycle enzymes may occur. Nevertheless, injured cells could repair themselves and multiply in the reconstituted milk resulting in a restoration of their virulence activity and thus causing illness (Caubilla-Barron and Forsythe, 2007; Wesceie et al., 2009). Moreover, some sublethally injured bacteria fail to grow on detection media, which enable them to evade detection and consequently threaten the health of consumers. Caubilla-Barron and Forsythe, (2007) investigated the generation of sublethally injured cells during desiccation in PIF using *Enterobacteriaceae* strains including *Cronobacter* strains. However, none of the *Cronobacter* strains used were recognised as *C.*

malonaticus. Therefore, in this study 20 strains of *C. malonaticus* will be used to examine the generation of sublethally injured cells during desiccation in PIF.

It is important for foodborne pathogens to cope with the effect of the acidity of the human stomach and intestines. *Cronobacter* has been proven not just to overcome the effect of acidity but also to grow at low pH up to 4.2 (Johler et al., 2009; Alvarez-Ordóñez et al., 2014). Furthermore, *Cronobacter* spp. have been proven to possess the heavy metal resistance genes, which usually encoded on the same plasmid with antibiotic resistance genes (Kucerova et al., 2010; Joseph et al., 2012a); however, limited knowledge is known about this activity. The fact that several of metals such as copper, silver, zinc, nickel, cobalt cadmium and arsenic are affecting the bacterial growth by either their toxic action inside or outside the macrophage cells make the investigation of the ability of *C. malonaticus* to tolerate or adapt heavy metals important (Osman and Cavet, 2011).

Gram negative bacteria which cause systemic infections need to overcome the action of complement in human serum (Schwizer et al., 2013); however, *Cronobacter* spp. vary with respect to their ability to survive the human serum (Townsend et al., 2007a; Almajed et al., 2016; Sonbol, 2015 unpublished data). Utilisation of sialic acid is an important virulence trait as it enables pathogenic bacteria to survive more in body sites where sialic acid exist. *C. sakazakii* has been proven to be able to utilise sialic acid. Such trait enhance the ability of *C. sakazakii* to cause systemic infections (Joseph et al., 2013). The antibiotic resistance due to antibiotic overuse has become a major clinical and public health problem; however, the overall level of *Cronobacter* resistance is considered low compared with other food-borne pathogens (Lee et al., 2012). Yet this issue needs more consideration. In this study the ability of *C. malonaticus* strains to tolerate acid, tolerate heavy metals and resist human serum and antibacterial agents will be studied as they are factors associated with bacterial pathogenicity.

As a part of this study all strains have been completely sequenced. Therefore, further molecular investigation will be performed using the 20 genomes of *C. malonaticus* strains to support the laboratory results. Two stress response genes which are *ompR* (which encodes the transcriptional activator protein *OmpR*), and *rpoS* (which encodes the alternative sigma factor S, a subunit of RNA polymerase), are found to be associated with acid stress response and general stress response respectively (Stasic et al., 2012; Alvarez-Ordonez et al., 2014). The investigation included also several factors such as the outer membrane protease *cpa*, the major outer membrane protein *OmpA*, plasmid-encoded proteins *TraT*, regulator of colanic acid biosynthesis *rcsA*, siderophore-encoding gene *viuB* and envelope stress regulators *rcsB*, *cpxR*, which have been reported to be involved in the bacterial ability to survive the killing activity of human serum (Allen et al., 1987;

Bogard and Oliver, 2007; Franco et al., 2011a; Miajlovic et al., 2013; Phan et al., 2013). *Nan* sialic acid utilisation genes, which have been recognised in *Cronobacter* by Joseph et al. (2012a), will be also investigated. Finally, genes, which are associated with metal and antibiotic resistance, will be investigated as well.

The formation of biofilm by pathogenic microorganisms plays an important role in surviving several stresses such as drying and antimicrobial agents. Many factors could involve in biofilm formation on the surface of medical equipments or even infected tissue (Annous et al., 2009). The production of extracellular materials such as cellulose is reported to play a clear role in biofilm formation as well as cell-cell aggregation (Hu et al., 2015). Capsular polysaccharides are also found to be involved in several bacterial activities include biofilm formation, escaping from phagocytosis and adhesion (Pluschke et al., 1983; Willis and Whitfield, 2013; Jaradat et al., 2014). In addition, expression of curli fimbriae has been found to facilitate the formation of biofilm and adhesion to host cells (Kim et al., 2012). Moreover, bacterial flagella which are responsible for motility are an important physical and virulence factor. Bacterial flagella are thought to have a role in the formation of biofilm and also involved in other pathogenic activities such as adhesion and invasion (Cruz-Cordova et al., 2012; Haiko and Westerlund-Wikström, 2013).

Several genes that have been found to be associated with some traits are reported. *Cronobacter* possess about forty flagellar genes, and these genes will be studied to check the absent and present of these genes in the used strains (Kucerova et al., 2010, Joseph et al., 2012a). Of these flagellar genes three genes, *flhE*, *fliD* and *flgJ*, were associated with biofilm formation (Hartmann et al., 2010). Other genes such as ESA_00281 and ESA_00282, which are hypothetical proteins, were also shown to be associated with biofilm formation (Hartmann et al., 2010). In addition, genes associated with the production of curli fimbriae (*csgA-g* and Ctu_16230), cellulose (*bcs*) and capsule (K-antigen cluster and colonic acid genes) will be investigated in this study (Joseph et al., 2012a; Ogrodzki and Forsythe, 2015).

The study in this chapter was extended to examine other virulence factors that are hypothesised to be involved in the *Cronobacter* pathogenicity. These factors included bacterial haemolysin, protease and siderophore. Bacterial haemolysin is a toxin that damages cell membranes, including the red blood cells (RBCs), while bacterial protease has the potential to destroy the structural and functional proteins in host tissue (Goebel et al., 1988; Lantz, 1997; Joseph et al., 2012a; Kucerova et al., 2010). Microorganisms use iron as an essential element for bacterial metabolism, survival and bacterial pathogenicity (Negre et al., 2004). *Cronobacter* is reported to harbour plasmids

containing two iron acquisition systems which might enable *Cronobacter* to cause systemic infections (Franco et al., 2011b).

Six putative genes associated with haemolytic activity (ESA_00102, ESA_00432, ESA_00643, ESA_02810, ESA_02937 and ESA_03540) have been described in *Cronobacter* (Joseph et al., 2012a). In addition, the outer membrane protease *zpx* and hydrogenase 3 maturation protease *hycI* are associated with the proteolytic activity. Two iron acquisition operons, *eitCBAD* and *iucABCD/iutA* have been revealed in previous genome studies (Franco et al., 2011b; Joseph et al., 2012a). Grim et al. (2012) reported also that *Cronobacter* possess two transport systems to acquire iron. These systems are the *feoABC* and *efeUOB* systems for acquisition of ferrous and Cronobactin siderophores (*iucABCD-iutA*), which encoded on the larger plasmid of *C. sakazakii* and *C. turicensis*, for ferric iron. In addition, five siderophores receptors (*FhuA*, *YncD*, *FoxA*, *FhuE*, and *PfeA*) were also reported into clinical *C. sakazakii* and *C. malonaticus* isolates (Grim et al., 2012). Plasmid profiling will be conducted to determine the number and size of plasmid that could be harboured by tested strains. *In silico* plasmid investigation has been conducted in several studies and will be performed in this study as well (Kucerova et al., 2010; Franco et al., 2011b; Joseph et al., 2012a; Yan et al., 2015).

4.2 Materials and methods

The methods for this chapter were described previously in Chapter 2 of Materials and Methods.

4.3 Results

4.3.1 Utilisation of sialic acid

All *C. malonaticus* strains were unable to grow on minimal medium M9 with sialic acid as the only carbon source. The *C. sakazakii* strain 658, which was used as a positive control in this assay, was able to grow on minimal medium M9 with sialic acid which means it was capable to utilize sialic acid as a carbon source. All strains grew on minimal medium M9 with glucose as the sole carbon source while there was no growth on minimal medium M9 without any carbon sources.

Further investigations to determine the presence or the absence of the key genes were conducted by applying genome comparison tools such as BLAST search genome and Artemis comparison tool (ACT). Genes required for utilisation of sialic acid were reported in *Cronobacter* spp. (Kucerova et al., 2010; Joseph et al., 2012a). All tested *C. malonaticus* strains lack a cluster of essential genes for sialic acid utilisation. This cluster consists of *yhch*, *nanA*, *nanK*, *nanT* and *nanR*. Locus *nanC* which also involve in the metabolic of sialic acid was absent in all *C. malonaticus* strains. However, genes essential for sialic acid utilisation were present in the positive strain which is *C. sakazakii* 658 (Table

4.1). Other genes associated with utilization of sialic acid such as *nanE*,*nagA*, *nagB*, *nagC*, *siaP*, *siaQ* and *siaM* were present in all tested strains (Table 4.1). The pathway of sialic acid starts with the uptake of sialic acid by *NanC,* in addition to *NanT* which is a major facilitator superfamily (MFS) protein in the inner membrane. As soon as the sialic acid enters the cell, it is catabolised by *nanA* and converted into phosphoenolpyruvate (PEP) and N-acetylmannosamine (ManNAc). ManNAc is also converted to N-acetylmannosamine-6-phosphate (ManNAc-6-P) by *NanK* which is an ATP-dependent kinase. In this stage, epimerase (*NanE*) will act on ManNAc-6-P and converted to N-acetylglucosamine-6-phosphate (GlcNAc-6-P). This is followed by conversion of GlcNAc-6-P by glucosamine-6-P deaminase (*NagB*) and GlcNAc-6-P deacetylase (*NagA*) to form fructose-6-phosphate which is used and consumed in the glycolytic pathway. Nevertheless, the activity of these genes is regulated by *NanR* which is basically a repressor (Severi et al., 2007; Vimr 2013).

Table 4-1 Sialic acid utilization associated genes.

NTU	ST	Sialic acid utilisation genes												
		Yhch	*nanA*	*nanK*	*nanT*	*nanR*	*nanC*	*nanE*	*nagA*	*nagB*	*nagC*	*siaP*	*siaQ*	*siaM*
565	7	-	-	-	-	-	-	+	+	+	+	+	+	+
681	7	-	-	-	-	-	-	+	+	+	+	+	+	+
688	7	-	-	-	-	-	-	+	+	+	+	+	+	+
893	7	-	-	-	-	-	-	+	+	+	+	+	+	+
1558	7	-	-	-	-	-	-	+	+	+	+	+	+	+
1827	7	-	-	-	-	-	-	+	+	+	+	+	+	+
1830	7	-	-	-	-	-	-	+	+	+	+	+	+	+
1833	7	-	-	-	-	-	-	+	+	+	+	+	+	+
1835	7	-	-	-	-	-	-	+	+	+	+	+	+	+
2018	7	-	-	-	-	-	-	+	+	+	+	+	+	+
2020	7	-	-	-	-	-	-	+	+	+	+	+	+	+
507	11	-	-	-	-	-	-	+	+	+	+	+	+	+
512	11	-	-	-	-	-	-	+	+	+	+	+	+	+
514	11	-	-	-	-	-	-	+	+	+	+	+	+	+
15	60	-	-	-	-	-	-	+	+	+	+	+	+	+
687	60	-	-	-	-	-	-	+	+	+	+	+	+	+
689	60	-	-	-	-	-	-	+	+	+	+	+	+	+
1545	84	-	-	-	-	-	-	+	+	+	+	+	+	+
685	129	-	-	-	-	-	-	+	+	+	+	+	+	+
1569	307	-	-	-	-	-	-	+	+	+	+	+	+	+
658	1	+	+	+	+	+	+	+	+	+	+	+	+	+

All used strains are *C. malonaticus* except strain 658 which is *C. sakazakii*. ST sequence type, + Present. – Absent.

4.3.2 Biofilm formation

The ability of *C. malonaticus* strains to form biofilms on plastic surfaces was studied using two different media, TSB and infant formula (IF), at two temperatures; 25°C and 37°C. Figure 4.1 and Figure 4.2 show the biofilm formation of the 20 strains of *C. malonaticus*.

Figure 4.1 shows the biofilm formation of the 20 *C. malonaticus* in TSB medium. In general, all strains showed an ability to form biofilm in this medium particularly at 25°C comparing with the control; however, a clear variation between strains was noticed. Some *C. malonaticus* ST7 strains such as 1827, 1830, 2018 and 2020 showed more ability to form biofilm than other *C. malonaticus* strains in both used temperatures. Strain 507, which belongs to ST11, showed also high ability to form biofilm in both temperatures. When the medium was replaced with infant formula, the results were completely changed. As shown in figures 4.1 and 4.2 the preferred temperature for biofilm formation when TSB was used was not the same when PIF milk was used. One-way ANOVA test was performed to compare the effect of temperatures. This showed significantly higher biofilm formation at 25°C ($p < 0.05$) compared to 37°C when TSB was used. However, when the infant formula was used biofilm formation at 37°C was dramatically higher ($p < 0.001$) compared to 25°C. According to figure, 4.2 all strains showed an ability to form biofilm at 37°C in the infant formula comparing with the control. Seven strains which are 681, 688, 1827, 2018 (ST7), 507 (ST11), 685 (ST129) and 1569 (ST307) showed the highest amount of biofilm formation above 2 absorbance units (AU) in IF medium (Figure 4.2). Some strains such as 565, 893 which are ST7 and 687 which is ST60 formed good values of biofilm in IF when the absorbance was just under 2 AU. The remaining strains showed modest ability comparing with the high values. At 25°C, though all strain still showed an ability to form biofilm, there was a variation between strains. Strains 1558, 685 and 1569 formed the highest values at this temperature (Figure 4.2). Interestingly, in TSB the maximum absorbance was around 0.5 AU, whereas in IF was up to 3.0 AU.

Two hypothetical proteins (ESA_00281 and ESA_00282) and about three flagellar genes *flhE*, *fliD* and *flgJ* were reported to have a role in surface adhesion (Hartmann et al., 2010). Genome search revealed that all of these factors are present in all used *C. malonaticus* strains.

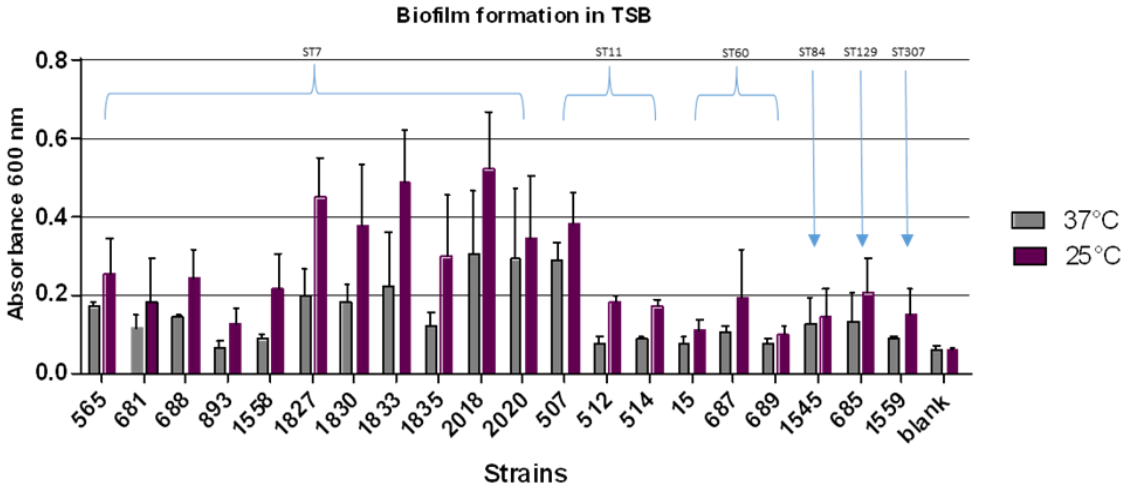


Figure 4-1 Biofilm formation at 25°C and 37°C temperatures in TSB.
Prefered temperature is 37°C. ST7 strains such as 1827, 1830, 2018, 2020 and strain 507, which belongs to ST11 showed more ability to form biofilm than other strains.

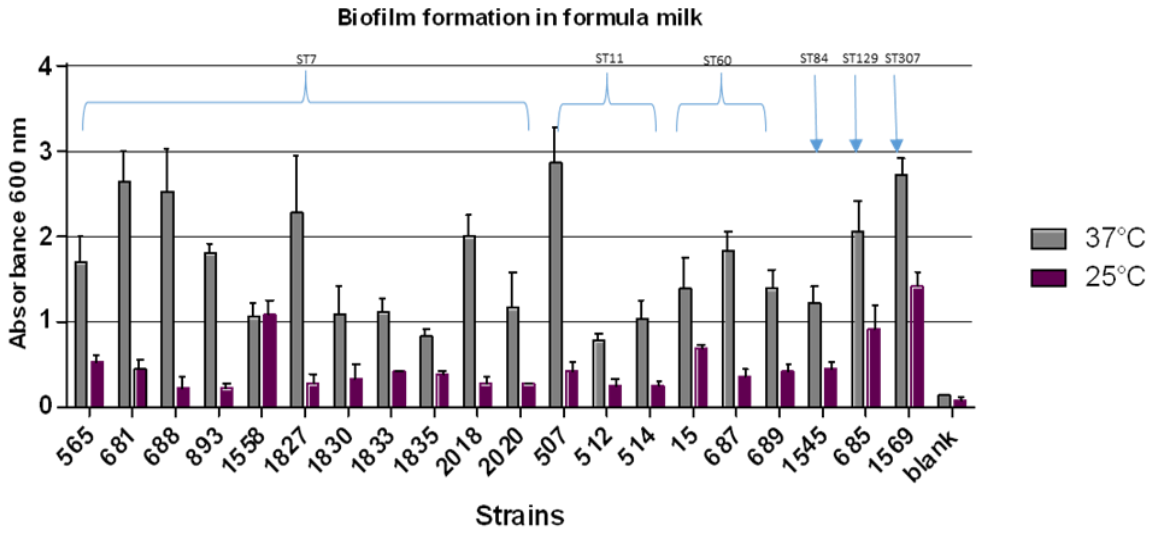


Figure 4-2 Biofilm formation at 25°C and 37°C temperatures in infant formula.
Prefered temperature is 37°C. Seven strains which are 681, 688, 1827, 2018 (ST7), 507 (ST11), 685 (ST129) and 1569 (ST307) showed the highest amount of biofilm formation above 2 absorbance units (AU) in IF medium.

4.3.3 Motility

The motility of *C. malonaticus* strains was demonstrated by their grown on LB broth supplemented with 0.4% agar and 0.5% TTC solution, and the diameter of the bacterial growth after overnight incubation was measured. Figure 4.3 shows the results of this assay, *C. malonaticus* strains 565, 893, 1827, 2018, 681, 507,512, 514, 15, 685, and 1569 showed wide range of motility zone diameter. The highest motility was above 72mm and was shown by *C. malonaticus* strains 507 (ST11), 893, 681 (ST7), 685 (ST129) and 1569 (ST307). *C. malonaticus* strains 1830, 1833, 1835 and 2020 (ST7) demonstrated the lowest motility exactly 4mm which indicates that these strains are non-motile. The remaining strains of *C. malonaticus* showed different zone diameter between 19mm and 42mm (Figure 4.3).

Additional information regarding the presence of flagellar genes that are responsible for flagella expression and motility was obtained by genomic study (Kucerova et al., 2010, Joseph et al., 2012a). All strains had the 40 flagellar genes; however, just one gene which is *flhC*, flagellar regulatory gene, was found to have different sequences among used strains. As shown in figure 4.3 four strains (1830, 1833, 1835 and 2020) that were non-motile by motility test have similar sequence of *flhC*. These four strains showed to be clustered together in Figure 4.4. This indicates that there might be Single Nucleotide Polymorphism (SNP) changes at fewer positions across the sequences of *flhC* gene. Indeed, SNP analysis revealed the presence of nucleotide difference among the gene sequence (Figure S1). The DNA sequence of *flhC* gene was translated using MEGA6 software and the result showed that the non-motile strains share same translated amino acid sequence (Figure 4.5 and Figure S.4)

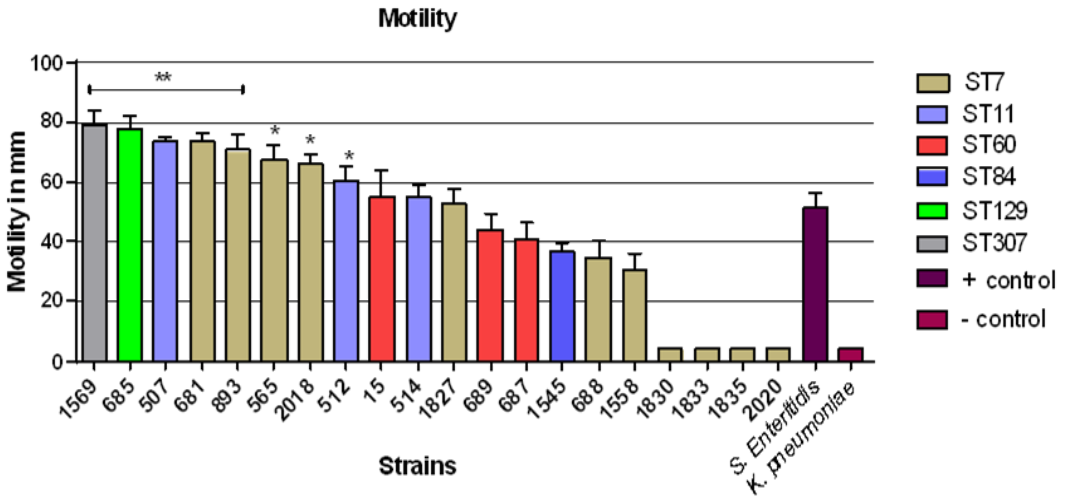


Figure 4-3 Motility of *C. malonaticus* strains at 37°C, incubated for 18h.
The diameter for the motility zone was measured in millimetres. Four ST7 strains (1830, 1833, 1835 and 2020) are nonmotile.

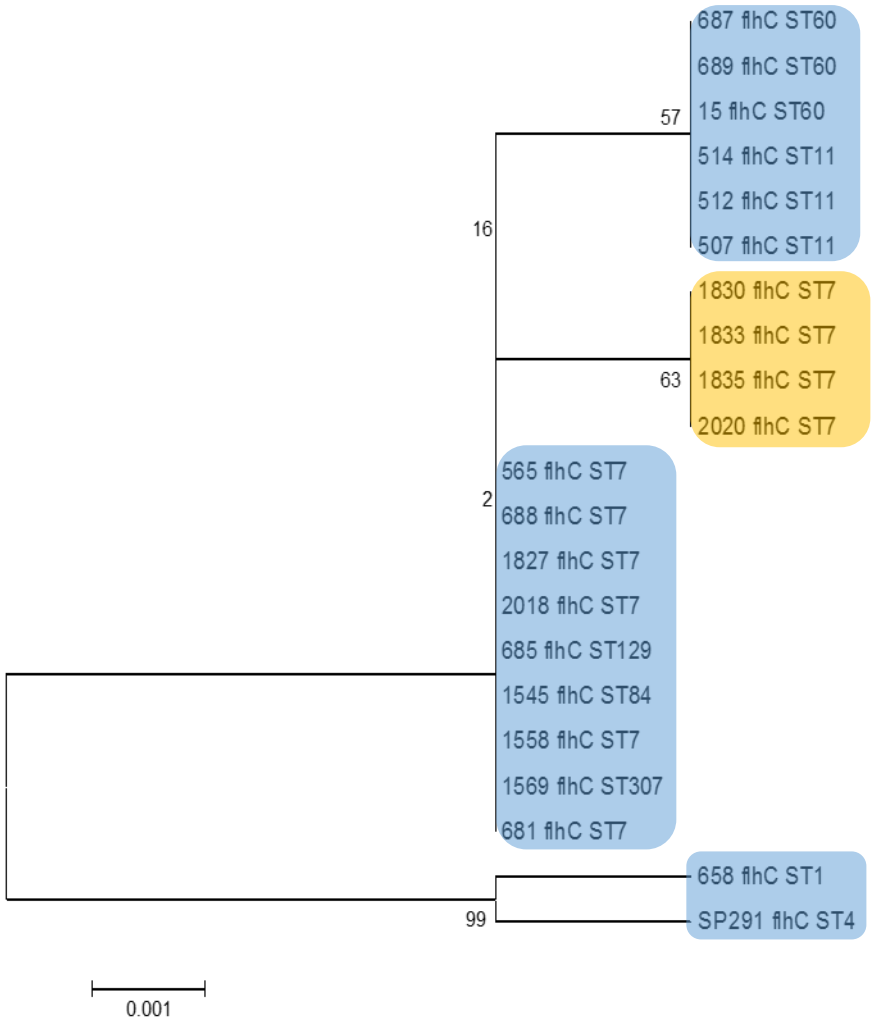


Figure 4-4 shows a maximum likelihood tree for DNA *flhC* gene sequence (582bp).
FlhC one of 40 flagellar genes, for *C. malonaticus* strains. *C. sakazakii* 658 and SP291 were added for comparison purpose. The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. Motile strains are highlighted in blue color, while the nonmotile strains are highlighted in orange color.

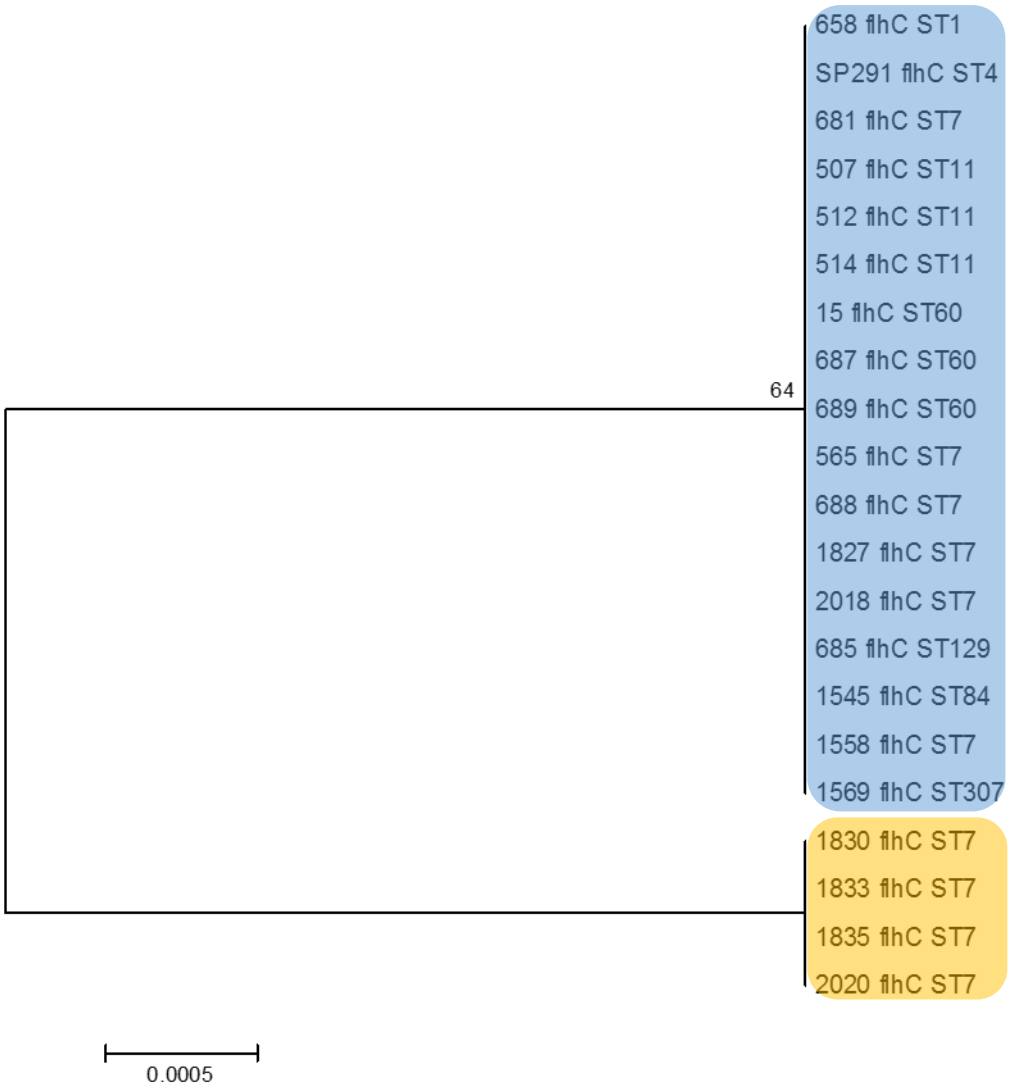


Figure 4-5 shows a maximum likelihood tree for *flhC* amino acid sequence for *C. malonaticus* strains. *C. sakazakii* 658 and SP291 were added for comparison purpose. The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. Motile strains are highlighted in blue color, while the nonmotile strains are highlighted in orange color.

4.3.4 Congo red morphotype

Colony morphology of *C. malonaticus* strains was studied using Congo red binding assay and their results are shown in table 4.2. Three colony morphologies were determined; red and dry (RAD), brown and smooth (BAS) and red and smooth (RAS). The expression of RAD colonies was considered positive for the Congo red dye binding assay, colonies which expressed RAS was considered as a weak binding to the Congo red dye and BAS did not show any binding to the Congo red dye (Yan et al., 2015). The colonies that formed by strains 681, 688, 1558, 1833, 685, 1545 and 1569 were considered as RAD colonies, while strains 893, 1835 and 2020 formed RAS colonies, and the remaining strains expressed BAS colonies which indicate there was no binding to the Congo red dye.

4.3.5 Cellulose formation

Calcofluor binding assay was used to detect the expression of cellulose by C*. malonaticus* strains and results are shown in table 4.2. In general, results of this assay indicated that all used strains produced cellulose. However, the ability of cellulose production varies between strains. Strains 681, 893, 1558, 1833, 685 and 1569 grew on calcofluor agar exhibited a strong fluorescence at 366 nm, while strains 688 and 2018 exhibited a weak fluorescence and the remaining strains exhibited moderate fluorescence.

4.3.6 Capsule formation

Table 4.2 shows the results of capsule production in three different media VRBGA, VRBA and infant formula agar. The results clearly showed a variation in the production of capsule within the tested strains. Therefore, a comparative scales of high, medium, low and non-capsule production are represented by +++, ++, + and – respectively. As shown in table 4.2 some strains were able to produce capsular materials on the all three used media; however, there was a variation in the amount of materials that produced on the used media from some strains. The highest amount of capsular materials was produced by strains 893, 1827, 2018 (ST7), 507, 514 (ST11) and 685 (ST129) in all used media whereas strains such as 1830, 1833, 1835, 2020 (ST7), 15, 687, 689 (ST60) and 1569 (ST307) were not able to produce any capsular materials on any of the used media. Strains 565 and 1545 produced medium amount of capsules. The production of capsular materials vary between growth media. For example, strain 512 (ST11) produced less capsule materials on VRBGA while the capsule production was remarkably enhanced on the other two types of media. In addition, some *C. malonaticus* strains showed to be not able at all to form capsule on some media whereas on others they produced high amount of capsules. For instance, strains 681, 688 and 1558 (ST7) were not able to produce capsular materials on VRBGA and VRBA while the capsule production was observed on milk agar.

Curli fimbriae associated genes have been searched into the genomes of used *C. malonaticus* strains. All the analysed genomes have been found to harbour curli fimbriae region which contain *CsgA-G* and Ctu_16230 genes. Similarly, cellulose associated genes were searched and the investigation confirmed the presence of all cellulose biosynthesis genes (*bcs* genes) in all used strains. In addition, the genes for capsular polysaccharide assembly and export (ESA_03350-59) that reported by Joseph et al. (2012a) were investigated using the *C. malonaticus* genomes (Joseph et al., 2012a). However, when BLAST analysis was performed to check these ten genes using sequence of *C. sakazakii* BAA-894 genes, four strains were found to lack four genes (ESA_03354-57) of the ten genes. These strains are 15, 687, 689 (ST60) and 1569 (ST307). Nevertheless, Ogrodzki and Forsythe (2015) have found that the ten genes are homologous to the previously well described K-antigen gene cluster of *E. coli*. They found also that this K-antigen gene cluster categorised to three regions. Region 1 and 3 are conserved across the *Cronobacter* genus, while there are two variants of region 2. The two variants of region 2 are not identical and differed in their length and GC% content. Therefore, the capsular profiling scheme, which includes K-antigen and introduced by Ogrodzki and Forsythe (2015), was also performed in this study. All the ST7 strains showed profile K-antigen 1 (K1) same profile that reported in the mentioned study. Similarly, the colanic acid (CA) type among used ST7 strains was CA1 (Table 4.2). ST60 and ST307 strains showed K2 and CA2, while ST11 strains showed K1 and CA2. Interestingly, ST60 and ST307 strains, which showed K2 and CA2 capsular profile, did not give any mucoid colonies on the used media including milk agar (Table 4.2).

Table 4-2 Colony morphology, capsule formation of *C. malonaticus* on different media and capsule profiles. Congo red agar, calcofluor agar, VRBGA, VRBA and milk agar were used.

NTU	ST	Congo red agar	Calcofluor agar	Capsule production			Capsular profiles			
				VRBGA	VRBA	Infant Formula	O-antigen type	K-antigen type	Colanic acid type	Cellulose *bcs* genes
565	7	BAS	++	++	++	++	O2	K1	CA1	+
681	7	RAD	+++	-	-	++	O2	K1	CA1	+
688	7	RAD	+	-	-	+	O2	K1	CA1	+
893	7	RAS	+++	++	+++	+++	O2	K1	CA1	+
1558	7	RAD	+++	-	-	++	O2	K1	CA1	+
1827	7	BAS	++	+++	+++	+++	O2	K1	CA1	+
1830	7	BAS	++	-	-	-	O2	K1	CA1	+
1833	7	RAD	+++	-	-	-	O2	K1	CA1	+
1835	7	RAS	++	-	-	-	O2	K1	CA1	+
2018	7	BAS	+	+++	+++	+++	O2	K1	CA1	+
2020	7	BAS	++	-	-	-	O2	K1	CA1	+
507	11	BAS	++	++	+++	+++	O3	K1	CA2	+
512	11	BAS	++	+	+++	+++	O3	K1	CA2	+
514	11	BAS	++	++	+++	+++	O3	K1	CA2	+
15	60	BAS	++	-	-	-	O1	K2	CA2	+
687	60	BAS	++	-	-	-	O1	K2	CA2	+
689	60	BAS	++	-	-	-	O1	K2	CA2	+
1545	84	RAD	+++	++	++	++	O2	K1	CA1	+
685	129	RAD	+++	++	+++	+++	O2	K1	CA1	+
1569	307	RAD	+++	-	-	-	O1	K2	CA2	+

ST= sequence type. Colony morphology on Congo red; BAS = brown and smooth (no binding of curli fimbriae to the Congo red agar), RAD = red and dry (strong binding of curli fimbriae to the Congo red agar), RAS = red and smooth (less strong binding of curli fimbriae to the Congo red agar). Capsule production; - = non-capsule production, + = low, ++ = medium, +++ = high.

4.3.7 Acid resistance

Twenty strains of *C. malonaticus*, including eleven strains ST7, three strains ST11, three strains ST60, one strain ST84, one strain ST129 and one strain of ST307 were investigated for their tolerance to pH 3.5. Figure 4.6 shows the results for the different *C. malonaticus* strains. In general, the initial viable count for all strains was between 5.69 $\log_{10}$ CFU/ml and 6.26 $\log_{10}$ CFU/ml. Over the 2-hour period of the experiment the *C. malonaticus* strains showed a variation in their ability to resist acid at pH3.5. All *C. malonaticus* strains ST7, ST11, ST84 and ST307 were able to survive this level of acid over the 2-hour period. The three strains of ST60 showed resistance to the acid in the first hour of the time; however, after 60 minutes the viable count cells decreased by about 2 logs at 120 minutes. Interestingly**,** the viable count of the strain 685, which is ST129, started to decrease after 30 minutes of the experiment and continued to decrease to about 3.5 $\log_{10}$ CFU/ml at 60 minutes and at 90 minutes this strain could not survive this level of acidity.

Alvarez-Ordonez et al. (2014) reported that the gene *ompR*, which encodes the osmolarity response regulator *ompR*, is a key player in the response of *C. sakazakii* to acid stress. In this study all tested strains were found to harbour this gene. The sequence of the *ompR* gene of the 20 *C. malonaticus* strains and 2 *C. sakazakii* strains was extracted from their genomes and aligned using MEGA6 software to find any differences in the sequences of this gene. The result showed that the sequence of *ompR* gene for strain 685 differs from other *ompR* sequence of other strains. As shown in figure 4.7 strain 685 showed to cluster alone indicating the presence of difference in nucleotide sequence between the *ompR* gene of this strain and others. Strain 685 showed to be sensitive to acid at pH 3.5 after 30 minutes. Further SNP analysis for the sequences of this gene revealed the presence of the difference in some nucleotide positions (Figure S2).

Alvarez-Ordonez et al. (2012) reported also the contribution of *rpoS* sigma factor in the ability of *Cronobacter* to tolerate several stresses. This study suggested a significant positive correlation between *rpoS* activity and the ability of bacteria to tolerate the lethal acid, alkaline, osmotic, and oxidative stress conditions. *C. sakazakii* strain ATCC BAA-894 (NTU 658) was one of the two sensitive strains in Alvarez-Ordonez et al. (2012) study. These strains returned their ability to resist acid, osmotic, and oxidative when complemented with a functional *rpoS* gene. Loss-of-function mutation was found in *C. sakazakii* strain NTU658. Therefore, the sequence of *rpoS* gene of *C. malonaticus* strains and *C. sakazakii* 658 were aligned using MEGA6 software and a phylogeny tree was constructed. Interestingly, as shown in figure 4.8, strain 685 which was shown as acid sensitive strain in this study clustered together with *C. sakazakii* 658 which showed to have the lowest capacity to withstand the stress conditions in Alvarez-Ordonez et al. (2012) study. The two sequences of the *rpoS* gene of these two strains shared same SNP at position 552. This might

indicate that the loss-of-function mutation of the *rpoS* in *C. malonaticus* strain 685 could be also present.

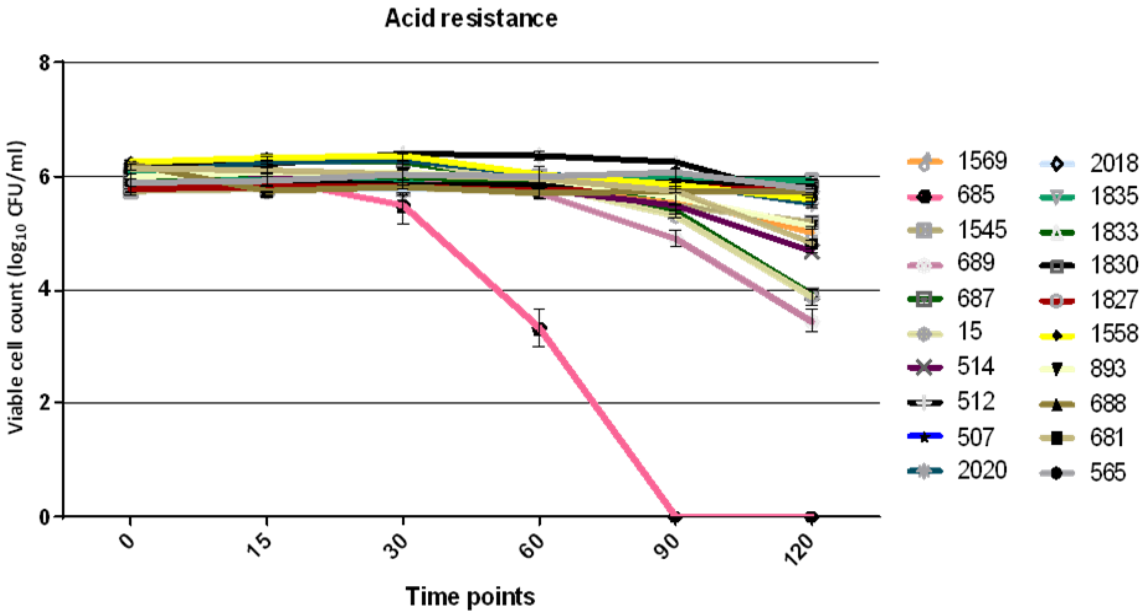


Figure 4-6 Survival of *C. malonaticus* after the exposure to pH 3.5.
The survival was measured for up to 2 h at 0, 15, 30, 60, 90 and 120 minutes. NTU numbers of *C. malonaticus* strains with their representative line are shown in the right of this figure. Strains 15,687 and 689 showed a decrease in the viable count cells by about 2 logs at 120 minutes. Strain 685 started to decrease after 30 minutes, continued to decrease to about 3.5 log10 CFU/ml at 60 minutes, and could not survive at 90 minutes.

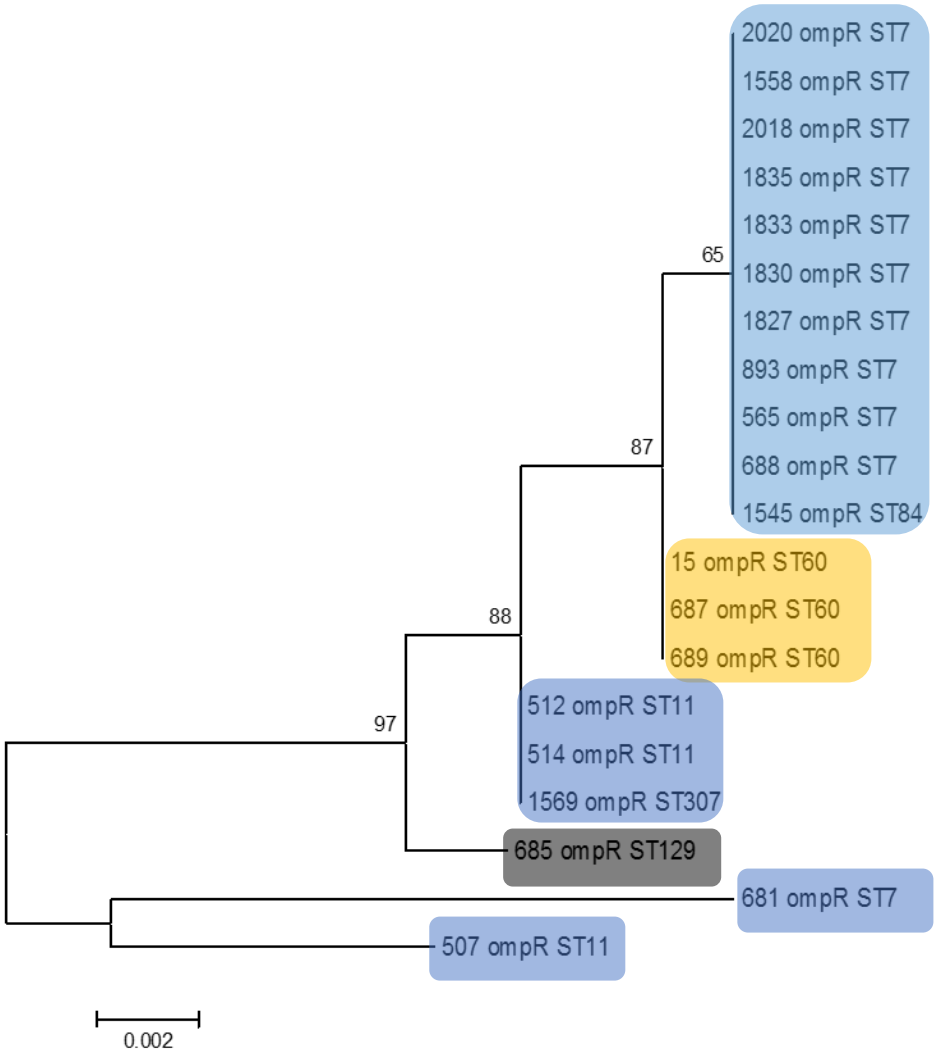


Figure 4-7 shows a maximum likelihood tree for *ompR* gene (720 bp).
OmpR gene is a proposed acid tolerance gene, *for C. malonaticus* strains. The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. Purpal color = can survive during 2 h, yellow color = decreased by about 2 logs at 120 minutes, Gray color = could not survive. The blue highlighted strains survived the acid in very good level over 2 h period. The orange highlighted strains showed resistance to the acid in the first hour; however, after 60 minutes the viable count cells decreased by about 2 logs at 120 minutes. The grey highlighted strain could not survive the acid directly after 30 mins.

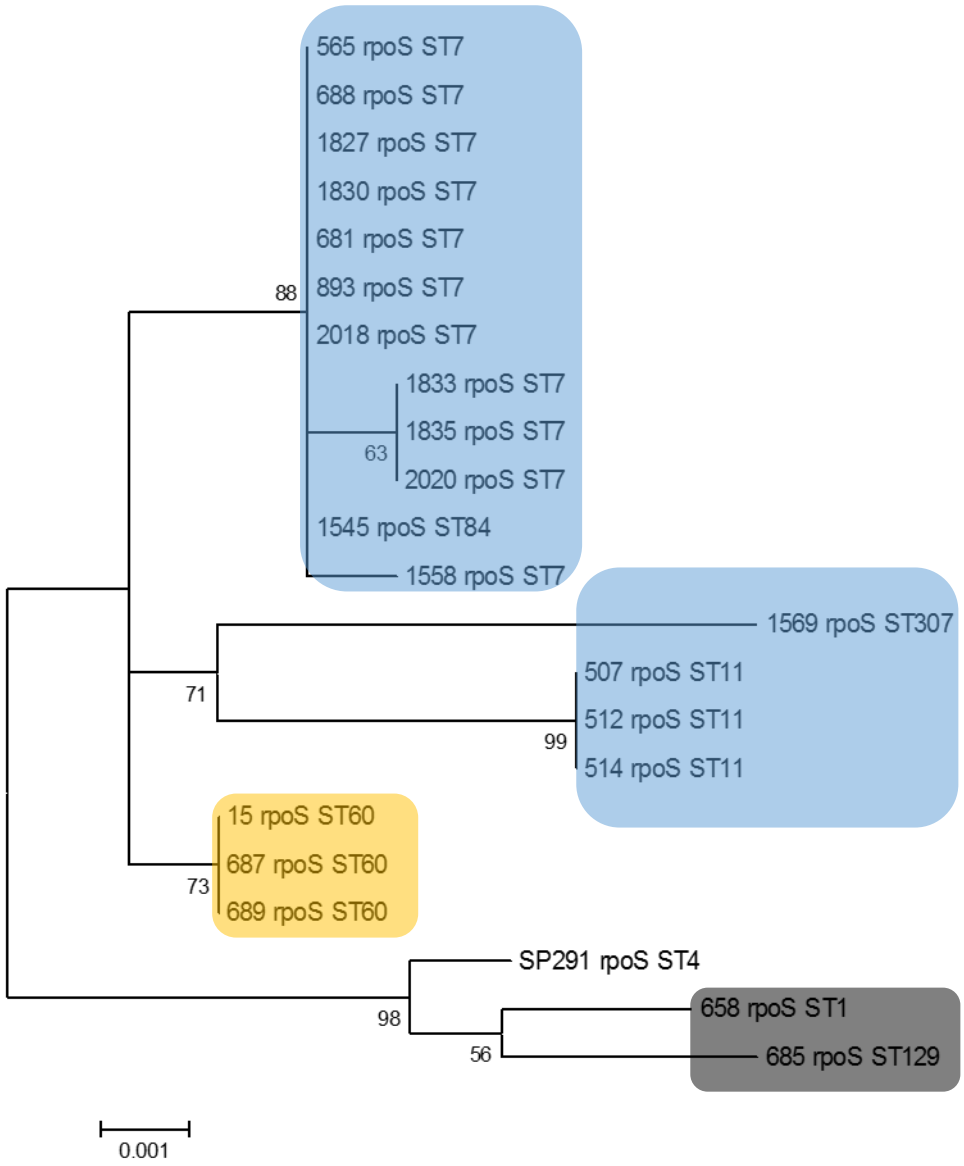


Figure 4-8 shows a maximum likelihood tree for *rpoS* sequence gene (993 bp).
RpoS gene is a proposed stress gene, for *C. malonaticus* strains. *C. sakazakii* 658 and SP291 were added for comparison purpose. The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. The blue highlighted strains survived the acid in very good level over 2 h period. The orange highlighted strains showed resistance to the acid in the first hour; however, after 60 minutes the viable count cells decreased by about 2 logs at 120 minutes. The grey highlighted strains, strain 658 could not survive the acid directly after 30 mins and strain 658, which is *C. sakazakii* strain, was one of the two acid sensitive strains in Alvarez-Ordonez et al. (2012) study.

4.3.8 Sublethal Injury and desiccation

Sublethally injured cells of *C. malonaticus* strains were generated upon desiccation. Figure 4.9 showed the differences between viable cell counts on TSA and VRBGA. The inoculum for tested strains was around 8 $\log_{10}$ CFU/ml. However, all the strains displayed a greater recovery on TSA ($P<0.05$) than on VRBGA agar (Figure 4.9). On TSA the cell recovery ranged from 5.9 to 6.8 $\log_{10}$ CFU/ml with the lowest recovery was showed by strain 685. The cell recovery on VRBGA ranged from 2.5 to 6.3 $\log_{10}$ CFU/ml. Strains 1827 and 893 showed the highest cell recovery rate at about 6.3 $\log_{10}$ CFU/ml while strain 685 showed the lowest recovery rate at 2.5 $\log_{10}$ CFU/ml. The remaining strains showed recovery ranged from 4 to 5.9 $\log_{10}$ CFU/ml on VRBGA.

On the other hand, Figure 4.10 showed the differences between dead cells that were not detected on TSA and sublethally injured cells that were not detected on VRBGA. The number of dead cells that were not detected on TSA agar was nearly same among the used strains, which was around 1 $\log_{10}$ CFU/ml and strain 685 was around 2 $\log_{10}$ CFU/ml. However, on VRBGA agar the number of undetected cells (sublethally injured cells and dead cells) varied between the used strains. The highest number of undetected cells (sublethally injured cells and dead cells) was showed by strain 685 at 5.5 $\log_{10}$ CFU/ml followed by strain 565 at 4 $\log_{10}$ CFU/ml. The lowest number of undetected cells was showed by strain 1827 at 1.5 $\log_{10}$ CFU/ml and the number of undetected cells for the remaining strains was ranged from 2 to around 3 $\log_{10}$ CFU/ml.

The differences in recovery on TSA and VRBGA after desiccation reflect the number of cells that were sublethally injured during overnight desiccation. As shown in table 4.3 the highest number of sublethal injured cells (3.37 $\log_{10}$ CFU/ml) was shown by strain 685 (ST129) followed by strain 565 (ST7) which showed 2.85 $\log_{10}$ CFU/ml. In contrast, strain 1827 (ST7) showed the lowest number of sublethal injured cells which is 0.36 $\log_{10}$ CFU/ml (Table 4.3). There was a clear variation among ST7 strains regarding the number of injured bacterial cells; however, less variation was noticed among ST11 and ST60 (Table 4.3).

Several genes that associated with desiccation and osmotic stress response have been investigated in the used strains. Genes included the *yihUTRSQVO* gene cluster, which are essential in metabolism and in the transport of carbohydrates and glucuronide have been identified as desiccation-related genes in *C. sakazakii* SP291 (Grim et al., 2013; Yan et al., 2013). They reported also the osmoprotectant ABC transporter genes, including *yehZYXW*, which play roles in bacterial survival. Yen et al. (2013) have reported other several genes, which are responsible for the regulation of osmotolerance. These genes included five osmotolerance regulation genes *yiaD*, *osmY*, *ompA*, *aqpZ*, and *glpF*; five genes associated with osmotic stress, namely *osmB* and *osmO*, *yciT*, *yciM*, and *pgpB*

and also periplasmic glucan synthesis genes included *mdoC*, *mdoH*, *mdoG*, *mdoD*, *mdoB*, and *opgC*. The results of BLAST search of 20 sequenced *C. malonaticus* showed that all of the strains had all of these genes.

RpoS is reported to be required for the tolerance to desiccation among *E. coli* (Stasic et al., 2012). In *Cronobacter*, Alvarez-Ordonez et al. (2012) provided an evidence that this gene is important for the tolerance of *C. sakazakii* under stress conditions. Strain 685 showed the lowest capacity to withstand desiccation stress in this study. This strain could have, as shown in the previous section (4.3.7), loss-of-function mutation of the *rpoS*.

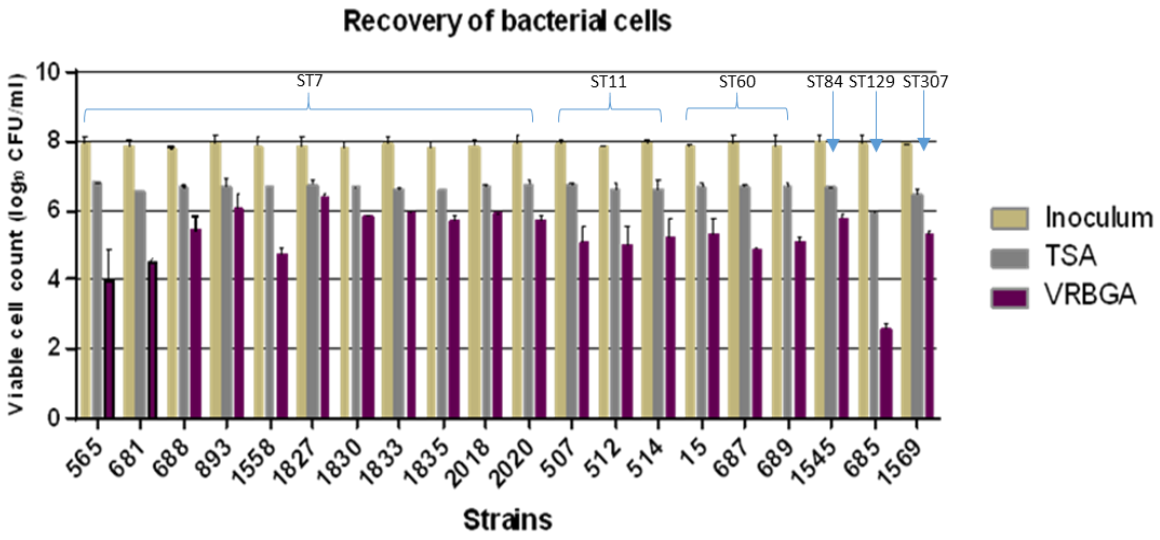


Figure 4-9 Viable cell counts for *C. malonaticus* on TSA and VRBGA after desiccation and then reconstitution in IF. Strains displayed a greater recovery on TSA than on VRBGA agar. The lowest recovery was showed by strain 685 on both media.

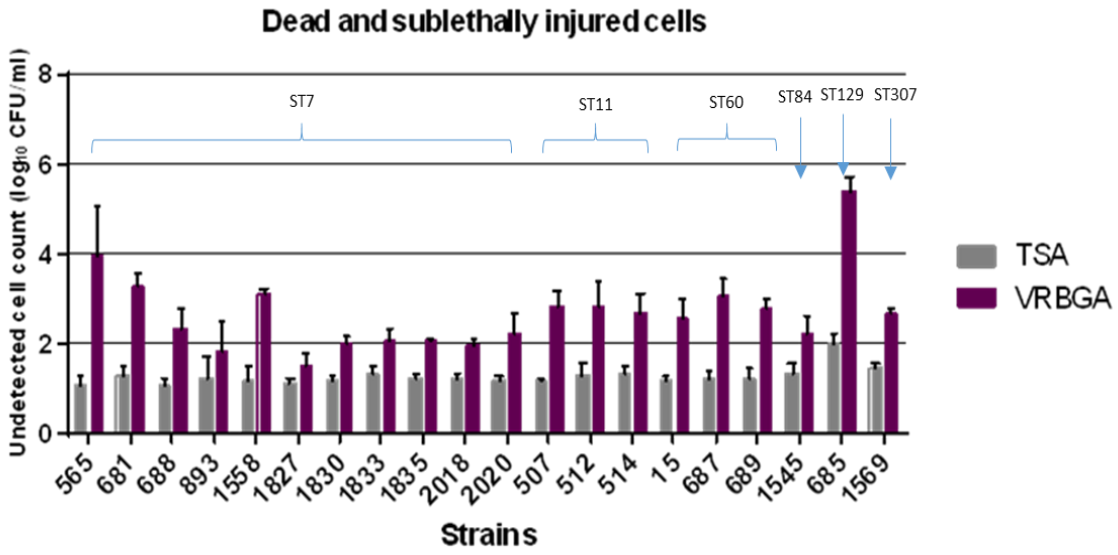


Figure 4-10 The count of undetected bacterial cells on TSA and VRBGA after desiccation and then reconstitution in IF. The highest number of undetected cells (sublethally injured cells and dead cells) was showed by strain 685 followed by strain 565.

Table 4-3 Comparison of viable counts for *C. malonaticus* on TSA and VRBGA after desiccation and then reconstitution in infant formula

NTU	ST	Dead and injured cells on VRBGA in log CFU/ml	Dead cells on TSA in log CFU/ml	Sublethal injured cells in log CFU/ml
685	129	5.38	2.01	3.37
565	7	3.93	1.08	2.85
681	7	3.30	1.27	2.02
1558	7	3.10	1.18	1.92
687	60	3.06	1.21	1.86
507	11	2.84	1.19	1.65
689	60	2.79	1.20	1.59
512	11	2.85	1.28	1.57
15	60	2.55	1.17	1.38
514	11	2.69	1.32	1.37
688	7	2.32	1.06	1.26
1569	307	2.65	1.45	1.20
2020	7	2.22	1.19	1.02
1545	84	2.20	1.32	0.88
1835	7	2.07	1.20	0.87
1830	7	1.97	1.18	0.79
2018	7	1.94	1.20	0.74
1833	7	2.03	1.30	0.73
893	7	1.82	1.21	0.62
1827	7	1.48	1.12	0.36

4.3.9 Metal Resistance

The *C. malonaticus* strains were subjected to 4 different concentrations; 1M. 0.1M, 0.01M and 0.001M of 8 metals, table 4.4 shows the results of this experiment. All *C. malonaticus* strains were sensitive to 1M copper II sulphate while they were able to resist up to 0.1M concentrations of this heavy metal. Similar results to copper tolerance were shown with exception of two strains when same strains were tested against sodium arsenate. The exception strains are 1827 and 15 which were also sensitive at a concentration of 0.1M. *C. malonaticus* showed to be sensitive to silver nitrate at the high 3 different concentrations (1M. 0.1M, 0.01M); however, at the lowest concentration (0.001M) only 3 strains which are 512, 514, and 15 remained sensitive. For cobalt II nitrate, the results indicated that all of the strains were able to resist cobalt II nitrate at low concentrations of up to 0.01M. For nickel chloride, although all strains were able to resist at low concentrations of up to 0.01M, only about 50% of the used strains showed resistance at a concentration of 0.1M. These strains are 688, 1558, 1827, 1833, 1835, 512, 514, 15, and 685. The majority of tested strains were sensitive to zinc sulfate heptahydrate at concentrations 1M and 0.1M, and as shown just strains 565, 688, 893, 512, 514, 15, 689 and 1569 were resistant at 0.1M. *C. malonaticus* showed to be resistant to cadmium carbonate at all used concentrations. Finally, without any exception all tested *C. malonaticus* strains were shown to be sensitive to sodium tellurite at all concentrations.

The genomes of 20 *C. malonaticus* strains were screened for detecting the presence of the two copper and silver resistance associated regions; *cusABCFR*/*silABCER* and *pcoABCDR* (Kucerova et al., 2010; Joseph et al., 2012a). As shown in table 4.5 five strains were missing both regions, these five strains showed same susceptibility against copper and silver as other strains which harbour the both region. Therefore, other associated genes which are *cueR*, *cueO*, *copA*, and *cutC* that reported in *Salmonella* were further screened into the 20 *C. malonaticus* strains. These four genes were detected in all tested strains, which might indicate that the genes *cueR*, *cueO*, *copA*, and *cutC* could play an important role in the control of these two copper and silver. All strains were screened also for the presence of tellurite resistance associated genes; *terA*, *terC*, *terD*, *terY* and *terZ*. The screening covered also other tellurate resistence gene *tehB*, which is reported in *C. sakazakii SP291* (Yan et al., 2013). However, all tested *C. malonaticus* strains, which showed sensitivity to tellurite metal, lack the presence of tellurite resistance associated genes, *terACDYZ*. These genes were reported to be present in *C. sakazakii* BAA-894 (Joseph et al., 2012a). Nevertheless, *tehB* was present in all *C. malonaticus* strains.

Other genes that have been reported to be associated with arsenic resistance among *C. sakazakii* such as the regulatory gene *arsR*, gene including the arsenic efflux pump protein (*arsB*) and gene

encoding the arsenate reductase (*arsC*) were screened (Masood 2015, unpublished data). The three genes were detected in all used strains except strain 565 which lack the regulatory gene *arsR*; however, the tolerance to arsenic at the highest concentration (1M) was varied. For nickel/cobalt resistance, gene *rcnA*, which is encoded into a nickel/cobalt efflux system, was screened, thus just one *C. malonaticus* strain which is 1558 was found to harbour this gene. However, all tested strains showed nearly same tolerance pattern. This indicates that this gene is not involved in the resistance to nickel or cobalt and this trait could be controlled by involvement of other genes. Moreover, two genes *zntA* and *cadA* genes, which were reported to involve in zinc and cadmium resistance (Perez et al., 2006; Lutsenko, 2000), were detected in all tested strains (Table 4.5). These two genes could contribute in the tolerance of *C. malonaticus* to both zinc and cadmium as this finding is consistent with the result of laboratory work (Table 4.4)

Table 4-4 Heavy metal susceptibility in *C. malonaticus*.

C. malonaticus	ST	copper II solphate				sodium arsenate				silver nitrate				cobalt II nitrate				nickel chloride				zinc sulfate heptahydra				cadmium carbonate				sodium tellurite			
		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	1M	0.1	0.01	0.001
565	7	10	0	0	0	13	0	0	0	14	12	12	0	18	12	0	0	17	8	0	0	12	0	0	0	0	0	0	0	46	36	29	10
681	7	10	0	0	0	9	0	0	0	12	13	10	0	19	7	0	0	17	7	0	0	14	8	0	0	0	0	0	0	45	39	32	22
688	7	10	0	0	0	20	0	0	0	11	12	12	0	20	8	0	0	16	0	0	0	19	0	0	0	0	0	0	0	46	36	33	20
893	7	10	0	0	0	12	0	0	0	10	11	10	0	21	12	0	0	18	8	0	0	13	0	0	0	0	0	0	0	47	40	36	18
1558	7	10	0	0	0	11	0	0	0	12	13	10	0	20	7	0	0	17	0	0	0	12	8	0	0	0	0	0	0	47	40	32	22
1827	7	11	0	0	0	33	20	0	0	10	9	8	0	20	7	0	0	17	0	0	0	13	7	0	0	0	0	0	0	43	36	29	21
1830	7	11	0	0	0	23	0	0	0	11	12	8	0	19	11	0	0	18	7	0	0	17	8	0	0	0	0	0	0	43	30	20	15
1833	7	12	0	0	0	13	0	0	0	10	11	9	0	19	9	0	0	18	0	0	0	18	7	0	0	0	0	0	0	45	36	30	15
1835	7	11	0	0	0	22	0	0	0	11	11	9	0	20	7	0	0	17	0	0	0	17	7	0	0	0	0	0	0	40	35	20	17
2018	7	12	0	0	0	19	0	0	0	11	11	9	0	20	8	0	0	16	7	0	0	15	7	0	0	0	0	0	0	44	38	29	20
2020	7	11	0	0	0	25	0	0	0	10	11	7	0	19	11	0	0	18	7	0	0	16	8	0	0	0	0	0	0	41	27	16	11
507	11	11	0	0	0	19	0	0	0	12	13	10	0	20	10	0	0	19	7	0	0	12	7	0	0	0	0	0	0	43	37	27	22
512	11	13	0	0	0	25	0	0	0	13	13	14	10	22	12	0	0	16	0	0	0	25	0	0	0	0	0	0	0	48	45	36	25
514	11	13	0	0	0	30	0	0	0	13	13	12	11	22	11	0	0	17	0	0	0	23	0	0	0	0	0	0	0	50	40	35	25
15	60	11	0	0	0	25	10	0	0	14	13	14	11	19	10	0	0	15	0	0	0	25	0	0	0	0	0	0	0	50	42	35	25
687	60	12	0	0	0	21	0	0	0	12	13	10	0	19	8	0	0	19	9	0	0	14	7	0	0	0	0	0	0	45	37	28	22
689	60	9	0	0	0	26	0	0	0	12	10	9	0	19	11	0	0	17	7	0	0	26	0	0	0	0	0	0	0	50	46	40	25
1545	84	11	0	0	0	14	0	0	0	13	13	11	0	22	10	0	0	19	7	0	0	15	8	0	0	0	0	0	0	43	38	28	22
685	129	12	0	0	0	22	0	0	0	14	14	11	0	18	8	0	0	17	0	0	0	18	10	0	0	0	0	0	0	44	38	31	21
1569	307	10	0	0	0	13	0	0	0	11	11	8	0	19	8	0	0	16	7	0	0	12	0	0	0	0	0	0	0	42	36	30	13

The numbers indicate the diameter zone of growth inhibition in mm.

Table 4-5 Resistance associated genes for copper and silver, tellurite, arsenic, nickel and cobalt, zinc and cadmium

NTU	ST	copper and silver resistance associated regions in *Cronobacter*		Other copper associated genes from *Salmonella*				Tellurite						Arsenic			Nickel and cobalt	Zinc and cadmium	
		cusABCFR/silABCER	*pcoABCDR*	*cueR*	*cueO*	*copA*	*cutC*	*terA*	*terC*	*terD*	*terY*	*terZ*	*tehB*	*arsB*	*arsC*	*arsR*	*rcnA*	*zntA*	*cadA*
565	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+
681	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
688	7	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
893	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
1558	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
1827	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
1830	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
1833	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
1835	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
2018	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
2020	7	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
507	11	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
512	11	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
514	11	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
15	60	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
687	60	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
689	60	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
1545	84	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
685	129	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
1569	307	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+
658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+

(+) = present, (-) = absent.

4.3.10 Iron Siderophore Detection

After the incubation period (up to 8 hours) the orange zones, which formed around the wells, were measured and the results were represented in millimetre (mm) as shown in figure 4.11. All *C. malonaticus* strains showed an ability to produce siderophores. According to the measured halo zones, strains 681, 1558, 1833, 1835, 2018 2020 507, 687, 689, 1569 produced the highest siderophore levels above 20mm diameter. The diameter of remaining strains ranged from 12 to 18 mm.

Cronobacter has been reported to possess two transport systems to acquire iron in both structures, ferric and ferrous. These systems are the *feoABC* and *efeUOB* systems for acquisition of ferrous and Cronobactin siderophores (*iucABCD-iutA*) for ferric iron. In addition, five siderophores receptors (*FhuA*, *YncD*, *FoxA*, *FhuE*, and *PfeA*) were also reported into clinical *C. sakazakii* isolates (Grim et al., 2012). In this study, the tested *C. malonaticus* strains, which showed ability to produce siderophores, harbour also the siderophore acquisition systems that reported in Grim et al. study.

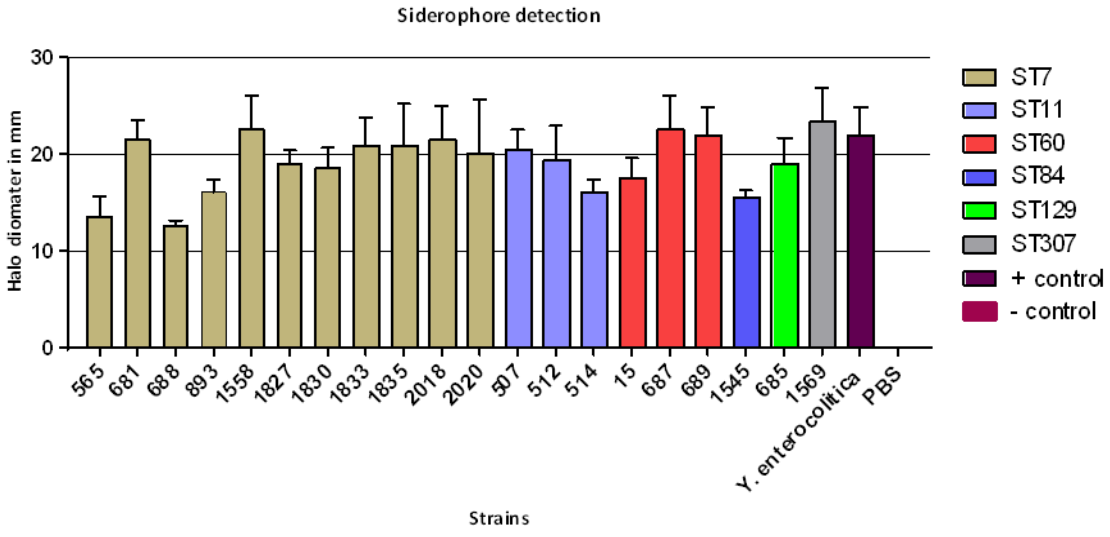


Figure 4-11 Iron siderophore production by *C. malonaticus* using the CASAD assay.
After the incubation period (up to 8 hours), the orange zones were measured and the results were represented in millimetre (mm).

4.3.11 Serum Resistance

Serum resistance for the all investigated strains was determined at four time points 0h, 1h, 2h and 3h. The results of this experiment are shown in Figure 4.12. There was a variation between *C. malonaticus* strains in their response to the human serum. *C. malonaticus* ST7 strains 1827 and 2018 demonstrated a high level of resistance to the human serum over the 4 time points comparing with other strains, while strains 688, 893 (ST7), 512, 514 (ST11), 15, 687, 689 (ST60), 685 (ST129) and 1569 (ST307) showed ability to survive over the 4 time points at lower levels. Other strains such as 565, 681 (ST7), 507 (ST11) demonstrated moderate levels of resistance and were able to survive over the first 3 time points of incubation. Interestingly, *C. malonaticus* ST60 strain 687 showed a decrease in the number of recovered viable cells during the first and second hours of incubation; however, this number was increased by 2 logs at the last time point. All the remaining *C. malonaticus* strains showed no ability to tolerate human serum after one hour comparing with negative and positive controls.

A study by Franco et al. (2011a) showed that the outer membrane protease *Cpa* contributes in the serum resistance activity among *Cronobacter* spp. However, all *C. malonaticus* strains which used in this study showed absence of this factor in their genomes. Nevertheless, some studies stated that several mechanisms contribute in the resistance to the bactericidal activity of serum complement (Miajlovic et al., 2013; Phan et al., 2013). Indeed, several studies reported the involvement of other factors in the bacterial ability to survive the killing activity of human serum. These factors are the major outer membrane protein *OmpA*, plasmid-encoded proteins *TraT*, regulator of colanic acid biosynthesis *rcsA*, siderophore-encoding gene *viuB* and envelope stress regulators *rcsB*, *cpxR* (Allen et al., 1987; Bogard and Oliver, 2007; Miajlovic et al., 2013; Phan et al., 2013). Screening for these genes in *Cronobacter* BLAST confirmed that all the tested strains in this study possess these factors regardless of their response against human serum.

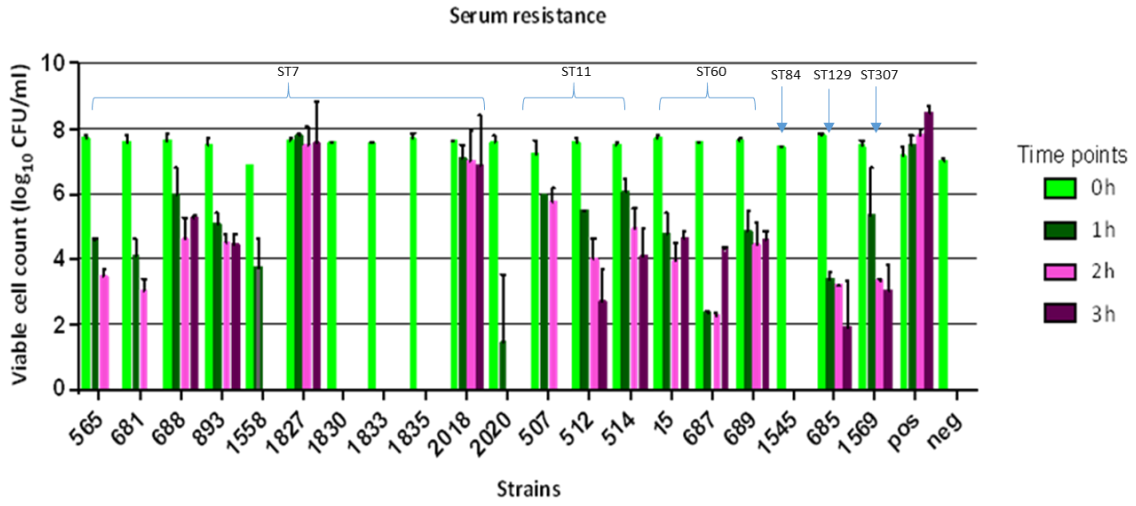


Figure 4-12 Sensitivity of *C. malonaticus* to human serum over 3 h of incubation at 37°C.
S. Enteritidis and *E. coli* K12 were used as positive and negative controls respectively. ST7 strains 1827 and 2018 demonstrated a high level of resistance to the human serum over 3 hours of incubation. While strains 1830, 1833, 1835, 1545 and 2020 were not able to survive directly after being taken up.

4.3.12 Haemolysis on blood agar

The haemolytic activity for *C. malonaticus* strains was confirmed using TSA agar supplemented with 5% horse blood and the results of this assay showed that all tested strains produced β haemolysis on TSA horse blood agar.

4.3.13 Protease production

After 72 hours of incubation period at 37°C all *C. malonaticus* strains demonstrated a positive result for production of protease activity as a clear proteolysis was detected.

The ability of *C. malonaticus* to lyse blood and proteins was confirmed also by the presence of several haemolysin and protease associated genes. Six putative genes associated with haemolytic activity (ESA_00102, ESA_00432, ESA_00643, ESA_02810, ESA_02937 and ESA_03540) have been detected in the used genome of the all tested *C. malonaticus* strains (Joseph et al., 2012a). In addition, the outer membrane protease *zpx* that has a role in proteolytic activety was detected in the all genomes of *C. malonaticus* strains.

4.3.14 Antimicrobial Susceptibility

Seven antibiotic groups were used to screen the susceptibility of the *C. malonaticus* strains. These antibacterial groups were; Penicillins (ampicillin, augmentin), Cephalosporins (cefuroxime, cefotaxime, ceftriaxone and ceftazidime) Quinolones (ciprofloxacin and nalidixic acid), Aminoglycosides (gentamicin, amikacin), carbapenems (imipenem and meropenem), Tetracycline

(tetracycline) and Miscellaneous (chloramphenicol, co-trimoxazole and nitrofurantoin) (Table 4.6). As shown in the table 4.6, all of the screened strains were resistant to tetracycline. In contrast, all *C. malonaticus* strains were susceptible to the remaining antibiotics except to two of them. *C. malonaticus* strains 681, 688, 893, 2020, 687, 689, 685 and 1569, which are sequence types 7, 7, 7, 7, 60, 60, 129 and 307 respectively, all showed resistance to chloramphenicol while others showed susceptibility to the same antibiotic. An intermediate response was detected also against cefotaxime the third generation of cephalosporin group. The strains that showed an intermediate response to cefotaxime are 681, 688, 507, 15, 687, 689 and 1569 which are sequence types 7, 7, 11, 60, 60, 60 and 307 respectively.

In addition to the laboratory antimicrobial susceptibility test, multiple drug resistance operons, *mar*, was also searched into the genome of *C. malonaticus* strains. All strains were found to harbour this multiple antibiotic resistance operon *mar*, though strains showed susceptibility to the majority of antimicrobial agents. The presence of the multidrug efflux pump *AcrAB*, acridine resistance proteins AB, was also detected among used genomes. The efflux pump *AcrAB* has been linked with the ability of *E. coli* to expel both tetracycline and chloramphenicol (Sun et al., 2014). Therefore, the detection of resistance to tetracycline and chloramphenicol could be due to the presence of *AcrAB* pump system. A summary of the previous results in this chapter is provided in the table Table 4.7 below.

Table 4-6 Susceptibility of *C. malonaticus* strains to the agents of antibiotic.

Antibiotic groups	Antibiotic	Disc content (μg)	565	681	688	893	1558	1827	1830	1833	1835	2018	2020	507	512	514	15	687	689	1545	685	1569
			C. malonaticus	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*	*C. malonaticus*
			ST7	ST7	ST7	ST7	ST7	ST7	ST7	ST7	ST7	ST7	ST7	ST11	ST11	ST11	ST60	ST60	ST60	ST84	ST129	ST307
Penicillins	Ampicillin	10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Augmentin	20/10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cephalosporins	Cefotaxime	30	S	I	I	S	S	S	S	S	S	S	S	I	S	S	I	I	I	S	S	I
	Ceftazidime	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Ceftriaxone	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Cefuroxime	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Quinolones	Ciprofloxacin	5	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Nalidixic acid	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Aminoglycosides	Gentamicin	10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Amikacin	30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Carbapenems	Imipenem	10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Meropenem	10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Tetracyclline	Tetracycline	10	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Miscellaneous	Chloramphenicol	30	S	R	R	R	S	S	S	S	S	S	R	S	S	S	S	R	R	S	R	R
	Co-trimoxazole	1.25/23.75	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Nitrofurantoin	200	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

S = susceptibility, OR = resistance = intermediate resistance.

Table 4-7 Results summary of sialic acid utilization, biofilm formation, motility, congo red, cellulose production, capsule production on milk agar, acid resistance, desiccated injured cells, production of siderophores, serum resistance, haemolysins, protease, in Chapter 4.

NTU	ST	Source	Sialic acid utilization	Biofilm Formation in IF	Motility	Congo red	Cellulose production	Capsule production on milk agar	Acid resistance	Desiccated injured cells in logs	Production of siderophores	Serum resistance	Haemolysins, protease
565	7	Faecal isolate	no	moderate	yes	BAS	++	++	yes	2.85	+	Low moderate	+
681	7	Breast abscess isolate	no	high	yes	RAD	+++	++	yes	2.02	+	Low moderate	+
688	7	Sputum	no	high	yes	RAD	+	+	yes	1.26	+	moderate	+
893	7	Infant formula	no	moderate	yes	RAS	+++	+++	yes	0.62	+	moderate	+
1558	7	Faecal isolate	no	low	yes	RAD	+++	++	yes	1.92	+	sensitive	+
1827	7	Cannula (Blood)	no	low	yes	BAS	++	+++	yes	0.36	+	high	+
1830	7	Throat swab	no	low	no	BAS	++	-	yes	0.79	+	sensitive	+
1833	7	Faecal isolate	no	low	no	RAD	+++	-	yes	0.73	+	sensitive	+
1835	7	Throat swab	no	low	no	RAS	++	-	yes	0.87	+	sensitive	+
2018	7	Sputum	no	high	yes	BAS	+	+++	yes	0.74	+	high	+
2020	7	Faecal isolate	no	low	no	BAS	++	-	yes	1.02	+	sensitive	+
507	11	Faecal isolate	no	high	yes	BAS	++	+++	yes	1.65	+	Low moderate	+
512	11	Clinical	no	low	yes	BAS	++	+++	yes	1.57	+	moderate	+
514	11	Clinical	no	low	yes	BAS	++	+++	yes	1.37	+	moderate	+
15	60	Faecal isolate	no	low	yes	BAS	++	-	yes	1.38	+	moderate	+
687	60	Sputum	no	moderate	yes	BAS	++	-	yes	1.86	+	moderate	+
689	60	Faecal isolate	no	low	yes	BAS	++	-	yes	1.59	+	moderate	+
1545	84	Faecal isolate	no	low	yes	RAD	+++	++	yes	0.88	+	sensitive	+
685	129	Blood isolate	no	high	yes	RAD	+++	+++	no	3.37	+	moderate	+
1569	307	Blood isolate	no	high	yes	RAD	+++	-	yes	1.20	+	moderate	+

ST = sequence type. IF = infant formula. + = able to produce. - = not able to produce. BAS = brown and smooth. RAD= red and dry. RAS= red and smooth. Capsule and cellulose production; - = non-capsule production, + = low, ++ = medium, +++ = high.

4.3.15 Plasmid profiling

Plasmid profiling was conducted on the 20 *C. malonaticus* strains to determine the presence of the plasmids. *C. sakazakii* BAA-894 which contains two known size plasmids (pESA2 31 kb and pESA3 131 kb) and *C. sakazakii* 6 which is plasmid-less strain were used as a reference marker and a negative control respectively. All *C. malonaticus* strains have been found to harbour at least one plasmid (Figure 4.13). The reference strain, which reported to harbour 2 plasmids (pESA2 31 kb and pESA3 131 kb), shows to have four bands. Strain 6 was used as a negative control as it is a plasmid-less strain.

In silico plasmid profiling was performed in parallel with laboratory plasmid profiling. Plasmid pESA3 (131 kb) which is regarded as virulence plasmid (Franco et al., 2011a), the large plasmid (118 kb) of *C. sakazakii* SP291 (ST4) (pSP291-1) and the large plasmid (~126 Kb) of *C. malonaticus* CMCC45402 ST7 (pCMCC 45402-1) were selected as reference plasmids. The whole genome of *C. sakazakii* strain NTU6 was also used in this part as a negative control. The reference plasmids were aligned against 20 *C. malonaticus* genomes using Blast Ring Image Generator (BRIG) (Figure 4.14-A, 4.14-B and 4.14-C). It was important to screen *C. malonaticus* genomes analysed in this study for the presence of the content of reference plasmids and to observe if there is any variation among C*. malonaticus* regarding the presence of full-length plasmid or present of any missing regions.

The BRIG alignment outcome in figure 4.14-A, 4.14-B and 4.14-C showed that the content of the three reference plasmids was totally absent in the *C. sakazakii* 6 which used as a negative control. This finding is consistent with the result that showed in figure 4.13. In addition, *C. malonaticus* 688 contained only partial length of pESA3, pSP291-1 and pCMCC 45402-1 plasmids. It showed homology with only about 65 kb of pESA3 and pSP291-1 plasmids and about 80 Kb of pCMCC 45402-1 plasmid. Two *C. malonaticus* strains which belong to clonal complex 7 (CC7), 1558 (ST7) and 1545 (ST84) showed to contain a full length of pCMCC 45402-1 plasmids which harboured by ST7 strain. Approximately 10 kb region that harboured by pESA3, consisting of 10 hypothetical proteins and known as the T6SS system clusters was found to be mostly missing in all of the *C. malonaticus* strains Figure (4.14-A). Moreover, *Cronobacter* plasminogen activator (*cpa*) and a starvation sensing protein encoded by the *rspA* gene which harboured by both pESA3 and pSP291-1 plasmids were absent in all *C. malonaticus* strains (Figure 4.14-A and 4.14-B). Interestingly, a complete plasmid-borne operon for aerobactin synthesis (*iucABCD*) and arsenic resistance genes which harboured by the three reference plasmids were found to be present in all *C. malonaticus* strains as shown in Figure 4.14. However, the location of the aerobactin synthesis operon and arsenic resistance genes was not same in the three reference plasmids.

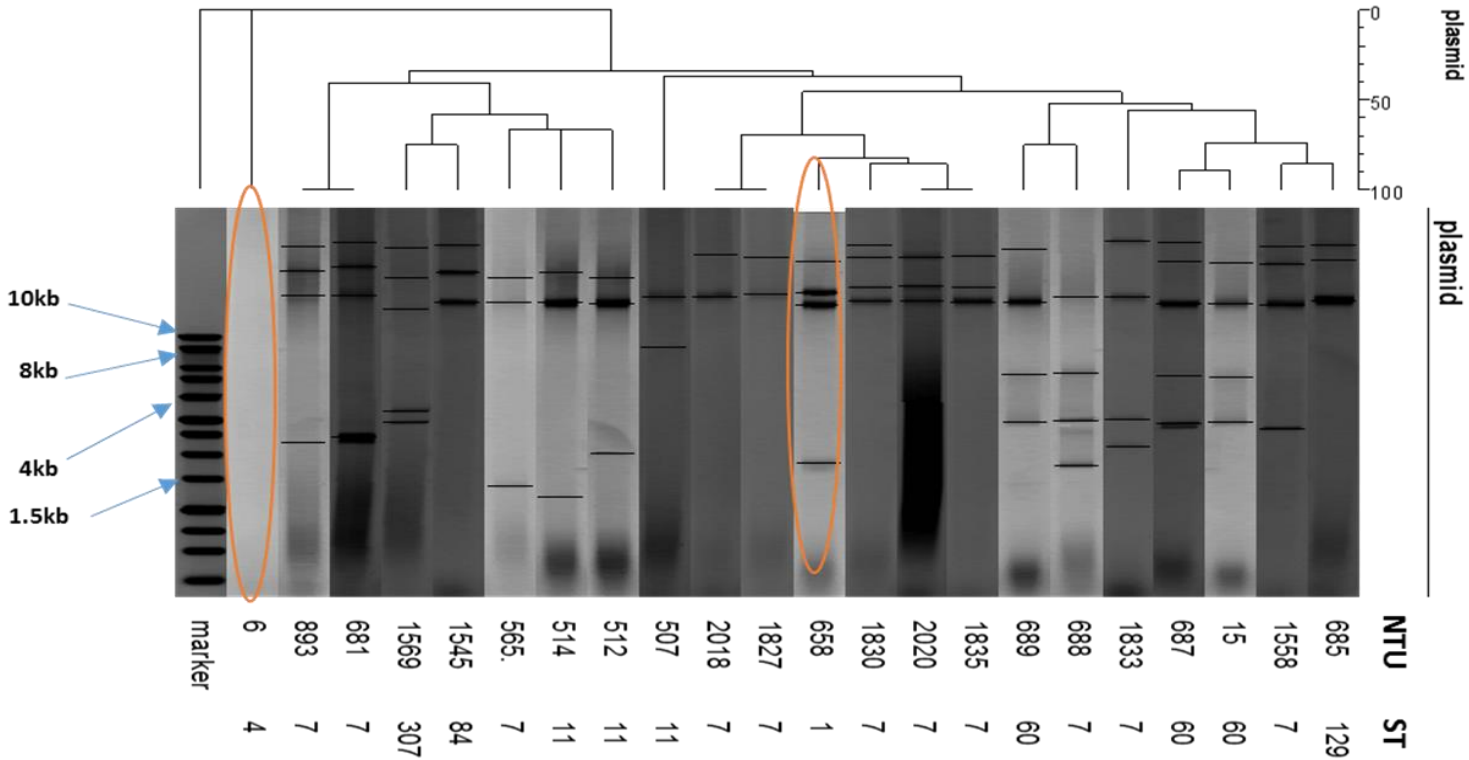


Figure 4-13 The plasmid profiles of *C. malonaticus* strains.
The strain *C. sakazakii* BAA-894 and *C. sakazakii* 6 which indicated by the red circles were used as reference strains. The plasmid sizes of *C. sakazakii* BAA-894 had been determined (Kucerova et al., 2010; Stephan et al., 2011) and strain 6 is plasmid-less strain.

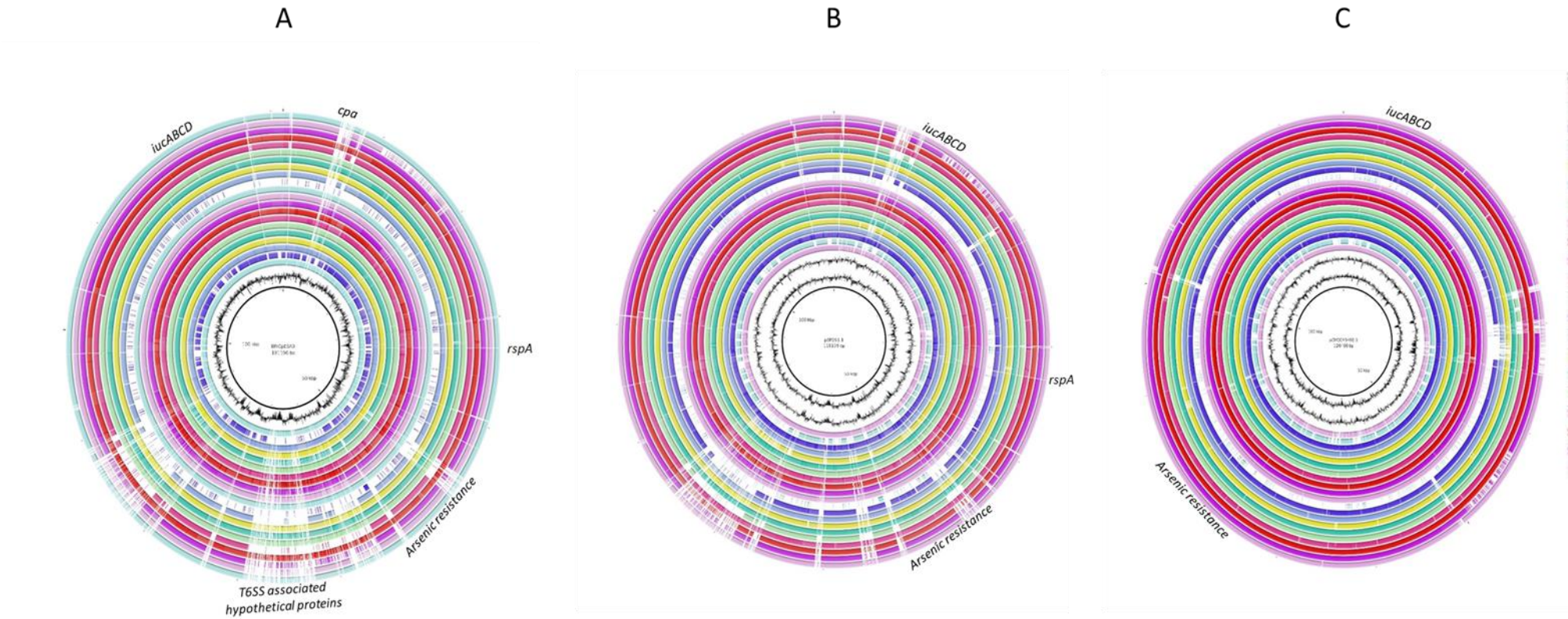


Figure 4-14 BRIG alignment of the pESA3 plasmid, pSP291-1 plasmid and pCMCC 45402-1 plasmid with *C. malonaticus* genomes.
(A) The alignment of the pESA3 plasmid (131 kb) with 20 *C. malonaticus*. (B) The alignment of the pSP291-1 plasmid (120 kb) with 20 *C. malonaticus*. (C) The alignment of the pCMCC 45402-1 plasmid (126 kb) with 20 *C. malonaticus*. The order is from inside to outside as following; rings from 1 to 11 are ST7 (565, 688, 681, 893, 1558, 1827, 1830, 1833, 1835, 2018 and 2020); ring number 12 is *C. sakazakii* 6 (negative control) ; rings 13 to 15 are ST11 (507, 512 and 514); rings 16 to 18 are ST60 (15, 687 and 689); Ring 19 is ST84 (1545); Ring 20 is ST129 (685) and ring 21 is ST307 (1569).

4.4 Discussion

Cronobacter, which is a member of Enterobacteriaceae family, has been occasionally reported to cause severe infections in human. However, these infections sometimes are fatal among neonatal group. *C. sakazakii*, *C. malonaticus* and *C. turicensis* are the only species that have been linked to the neonatal infections. Recently, *C. malonaticus* has been reported more to be an opportunistic pathogen that can cause life threatening infections among infants (Joseph et al., 2012b; Hariri et al., 2013; Asato et al., 2013; Holý et al., 2014; Brandao et al., 2015; unpublished data China). To cause infections, bacteria need to have several characteristics that enable them to overcome many stresses and possess other virulence factors which could facilitate the cause of infections. To date, the majority of studies related to physiological and virulence traits of *Cronobacter* spp. have focused on *C. sakazakii* and other species were usually used for a comparative purpose. In this study, 20 *C. malonaticus* strains, from various STs, were used to assess their ability to survive different conditions as well as investigate some of their virulence traits. The putative responsible genes for all the mentioned traits in this chapter were searched using the available genomes of the 20 strains.

Sialic acid is a carbon source that involve in the pathogenicity of bacteria (Almagro-Moreno and Boyed, 2009). Sialic acid is present in the important human body sites such as intestine and brain and thought to play an important role in ability of bacteria to grow in such places where the access to carbon source such as sialic acid is available and thereby result in causing NEC and meningitis (Joseph et al., 2013). The utilization of sialic acid has been detected in both clinical and non-clinical of *C. sakazakii* strains. Recently some *C. turicensis* have been recognised as sialic acid utilised (Joseph et al., 2013; Grim et al., 2013; Hariri 2015, unpublished data). However, all tested *C. malonaticus* were not able to utilise this source of carbon. This might limit the ability of *C. malonaticus* of causing infections in nervous system like *C. sakazakii*. However, the finding that *C. malonaticus* is not able to utilize sialic acid may not affect the potential of *C. malonaticus* to cause disease if other carbon sources present in the same place were the sialic acid is.

Further investigations to determine the presence or the absence of the key genes were conducted by applying genome comparisons tools such as BLAST search genome and Artemis comparison tool (ACT). Genes required for utilisation of sialic acid were reported in *Cronobacter* spp. (Kucerova et al., 2010; Joseph et al., 2012a). *C. malonaticus* strains lack a cluster of essential genes for sialic acid utilisation. This cluster consists of *yhch*, *nanA*, *nanK*, *nanT* and *nanR*. Locus *nanC* which is also involved in the metabolic of sialic acid was absent in all *C. malonaticus* strains. However, these genes which are essential for sialic acid utilisation were present in the positive strain which is *C.*

sakazakii 658 (Table 4.1). The absence of the essential genes for sialic acid utilisation is consistent with laboratory experiment finding which showed *C. malonaticus* was not able to utilise sialic acid.

In biofilm formation bacterial cells act more as a co-operative living system than as individual cells. They become more resistant to environmental stresses and antimicrobial agents. This biofilm could be formed on the surface of medical equipment or even infected tissue (Annous et al., 2009). In this study, the ability of *C. malonaticus* strains to form biofilms on plastic surfaces was investigated using two different media at two temperatures; 25°C and 37°C. The effect of temperature and other factors such as O_2, pH, osmolarity and hydrodynamics on biofilm formation were studied (Romeo, 2008). Indeed, temperature was a crucial factor in biofilm formation as shown in Figure 4.1 and 4.2. The optimal temperature for biofilm formation was changed according to the used medium. When TSB was used the optimal temperature was at 25°C, whereas when IF medium was used the optimal temperature was changed to be at 37°C. The quantity of biofilm that was detected on the two types of media was higher in IF medium compared with TSB medium among all tested strains ($p < 0.001$) (Figure 4.1 and 4.2). These observations indicate that the nutrient type present in milk is important to enhance the formation of biofilm. Milk nutrients helped the *C. malonaticus* strains to form a very big amount of biofilm comparing with TSB nutrients. Indeed, Hood and Zottola, (1997) showed that nutrients and other components effect the ability of bacteria to adhere to different surfaces. The ability of *C. malonaticus* to form biofilm in different conditions make these bacteria become more resistant to environmental stresses and antimicrobial agents. This will allow *C. malonaticus* to survive and expose to human resulting in establishing infection if the bacteria possess mechanisms to do so.

Different genes have been reported to be associated with biofilm formation. Two *C. sakazakii* hypothetical proteins (ESA_00281 and ESA_00282) and about three flagellar genes *flhE*, *fliD* and *flgJ* were described to contribute to surface adhesion (Hartmann et al., 2010). Although the ability of strains to form biofilm varied, the genome search revealed the presence of these factors in the all used *C. malonaticus* strains. This indicates that some of the detected genes might be inactivated and not expressed or that the biofilm formation by *C. malonaticus* strains is influenced by other factors. Certainly, biofilm formation is affected by some bacterial traits such as motility, curli fimbriae and production of cellulose and capsular materials (Kim et al., 2012; Hu et al., 2015; Pluschke et al., 1983; Willis and Whitfield, 2013; Jaradat et al., 2014; Cruz-Cordova et al., 2012; Haiko and Westerlund-Wikström, 2013). These traits were investigated in this study to find if there is any correlation between such traits and the ability of *C. malonaticus* to form biofilm. In addition, the study of some of the above traits was performed also to find any other contributions in the pathogenicity of *C. malonaticus*.

Traditionally, flagella have only been linked to the motility function; however, these organelles are found to has a role in other important functions such as adhesion to and invasion of host cells even in indirect way (Haiko and Westerlund, 2013). In *Cronobacter*, Cruz-Córdova et al. (2012) found that the flagellated *Cronobacter* strains have ability to adhere and invade the human cells as well as elicit the host immune response. In our study, most of *C. malonaticus* strains that were used were motile except for strains 1830, 1833, 1835 and 2020 which are ST7 (Figure 4.3). These four strains were isolated at same hospital from different departments in different years. However, the results indicate that this motility was due to the presence and the expression of flagella in motile strains. *Cronobacter* harbour about forty flagellar genes, all of them were found in the all used strains. However, the gene by gene investigation of the forthy flagellar genes revealed the presence of a difference in the nucleotide sequence of *flhC*, encoding a regulator of flagellar gene expression, across used strains. Motility in *E. coli* is controlled by a master regulator encoded by *flhD* and *flhC*, which contain the *flhDC* operon (Gauger et al., 2007). Indeed, a study by Monday et al. (2004) has found that *E. coli* O157:H−nonmotile strains have been shown to contain a deletion in *flhC* sequence. Interestingly, strains 1830, 1833, 1835 and 2020 which were non-motile harboured same *flhC* gene sequence (Figure 4.4). This indicates that there might be SNP changes at fewer positions across the sequences of *flhC* gene. Indeed, SNP analysis revealed the presence of nucleotide difference among the gene sequence (Figure S.1). Such SNP change could cause a variation in the resulting structure of the protein, which may affect their functional characteristics. The DNA sequences of this gene from the 20 used strains were translated using MEGA6 software to check if they have synonymous or non-synonymous SNPs. The result showed a difference in the produced amino acid sequences when all motile strains showed transclation of same amino acid sequence while non-motile strains share same amino acid sequence (Figure 4.5 and Figure S.4). These results suggest the importance of the *flhC* gene in the motility of *C. malonaticus* and might contribute also in their pathogenicity.

The relationship between motility and biofilm formation is complicated to interprete. The inhibition of motility is believed to be an important event in biofilm formation; motility is required early, but inhibited later when biofilm is formed (Guttenplan and Kearns, 2013). However, seven strains, which are 565, 681, 893, 2018 (ST7), 507 (ST11), 685 (ST129) and 1569 (ST307), out of eight that showed highest range of motility were high biofilm producers in PIF at 37°C. The motility could be used at the beginning of biofilm formation and inhibited in the advanced stages of biofilm formation.

The morphology of the capsular materials that produced by the selected *C. malonaticus* strains was assessed by colony appearance using three different media, VRBGA, VRBA and IF agar. In general, the production of capsular materials was enhanced when IF was used. For instance, strains 681, 688, 1558 (ST7) did not produce any capsular materials on VRBGA and VRBA; however, their ability

to produce capsular materials was obvious when streaked on IF agar (Table 4.2). In addition, strain 893 (ST7), strain 685 (ST129) and all ST11 strains produced less capsular materials on VRBGA; however, the production was enhanced by using VRBA and IF agar. These findings may confirm the effect of ingredients of different medium on production of bacterial exopolysaccharides (EPS). VRBGA medium contains glucose, VRBA contains lactose and IF medium contains lactose and whey. Therefore, lactose and whey could play an important role in the production of EPS by *C. malonaticus*. Indeed, *Cronobacter* can produce optimal EPS under nitrogen-limited growth conditions and this is influenced by milk components (Dancer et al., 2009b; Scheepe-Leberkühne and Wagner, 1986). The organism has shown an ability to produce a notable extensive mucoid appearance on milk agar which causes the growth to drip on to the inverted lid (Caubilla-Barron et al., 2007).

There was no correlation between capsule production and biofilm formation for tested strains as shown in Table 4.2 and Figure 4.1-2. For instance, strains 688 and 1569 produced less or none capsules respectively, nevertheless those strains were produced more biofilm in IF (Table 4.2 and Figure 4.2). Similarly, Hurrell et al. (2009a) showed that strains which were not able to produce any capsular materials produced more biofilm. Although there was no obvious correlation between capsule production and biofilm formation, capsules are known to play an important role in resistance to desiccation, serum activity and contribute in avoiding phagocytic killing (Guerry and Szymanski, 2008; Willis and Whitfield, 2013; Ogrodzki and Forsythe, 2015). Among ST7 strains four strains, which are 1830, 1833, 1835 and 2020 were not able to produce any capsular materials on all media, showed no ability to survive human serum (Figure 4.12), or invade and survive different used cell lines in Chapter 5.

The application of capsular profiling scheme which based on O-antigen, K-antigen and colanic acid and introduced by Ogrodzki and Forsythe, (2015) revealed the presence of relationship between a capsular profile and production of capsular materials. For example, strains 15, 687, 689 and 1569 which had O-antigen type O1, K-antigen type K2 and colonic acid type CA2 were not able to produce any capsular materials on media including milk agar (Table 4.2). These strains seem also to lack four (ESA_03354-57) of ten capsular polysaccharide assembly and export genes (ESA_03350-59). Nevertheless, Ogrodzki and Forsythe, (2015) have found that the ten genes are homologous to the previously well described K-antigen gene cluster of *E. coli*. They found also that this K-antigen gene cluster categorised to three regions. Regions 1 and 3 are conserved across the *Cronobacter* genus, while there are two variants of region 2. Interestingly, the two variants of region 2 differed in their length and GC% content. Four other strains (1830, 1833, 1835 and 2020) with capsular profile O2, K1 and CA1 were not able also to produce any capsular materials; however, other strains with same

capsular profile showed ability of producing capsular materials particularly on milk agar. This might indicate that other genes might contribute in the capsule formation.

Cellulose is another extracellular material produced by bacteria. It has been shown to play a role in interaction between individual bacterial cells, and between bacteria and host cells. This component is produced by bacteria for the formation of biofilm which impact the effect of chemical and mechanical action on bacteria (Hu et al., 2015). All tested *C. malonaticus* strains produced cellulose in different amounts (Table 4.2). Consistently, all used *C. malonaticus* strains harboured the *bcs* genes which are associated with cellulose synthesis. Although cellulose is one of the major components of the biofilm matrix in *Salmonella* and *E. coli*, (Da Re and Ghigo, 2006), there was no correlation between the ability of *C. malonaticus* to form biofilm and the amount of cellulose that produced by strains**.** Curli fimbriae are also extracellular fibres that expressed by bacteria and involved in the formation of biofilm and mediate the adhesion to host cells (Kim et al., 2012). Congo red is used to supplement nutrient agar as a selection medium to differentiate between curli-producing bacteria and non-curliated bacteria (Reichhardt et al., 2015). In this study, the Congo red binding assay was conducted to assess the expression of curli fimbriae. All *C. malonaticus* strains were confirmed to have curli fimbriae region which consist of *CsgA-G* and Ctu_16230 genes. When bacteria express curli and cellulose, they display red and dry colony morphology on Congo red agar plates (Bokranz et al., 2005; Jonas et al., 2007). Accordingly, not all *C. malonaticus* strains were able to express the curli fimbriae. ST11 (507, 512, 514), ST60 (15, 687, 689) strains produced BAS colonies while ST84, ST129 and ST307 produced RAD colonies. The morphology of ST7 colonies vary in three forms which are BAS, RAS and RAD. Nevertheless, the finding showed no association between the morphology of *C. malonaticus* colonies on Congo red and biofilm formation. A study by Kim et al. (2012) using RT-PCR showed a strong correlation between the expression of curli fimbriae and biofilm formation among *Enterobacter* strains. Such technique could be performed in the future to confirm whether the same phenomena is present among *Cronobacter* or not.

When the food borne pathogen such as *Cronobacter* is ingested with food, they are exposed to the stomach acid which could affect the survival of the ingested pathogens. The human stomach acid ranges between pH 1.5 and 6 with consideration to the type of ingestion food (Hurrell et al., 2009b). To mimic this environment of the stomach acid the IF media was adjusted to 3.5 pH. The majority of *C. malonaticus* strains showed ability to survive IF at 3.5 pH over 2 hours of incubation (Figure 4.6), with the exception of strain 685 which could not survive after 30 minutes. All *C. malonaticus* ST7 and ST11 and ST84 and ST307 showed ability of surviving this condition. A previous study confirmed that *Cronobacter* has a great ability to survive at low pH values of about 3.5 (Edelson-Mammel et al., 2006). Only ST60 strains declined by more than 2 logs at 2 hours. This was shown in

a study by Edelson-Mammel et al. (2006) when some of *Cronobacter* strains declined by one log. However, just one strain which belongs to ST129 showed no ability to survive directly after 30 minutes of incubation. The results of this study indicate the potential of *C. malonaticus* strains, which showed ability to survive the stomach acidity, to invade intestinal cells and cause diseases. *OmpR* is a gene known to play an important role in the response of *C. sakazakii* to acid stress (Alvarez-Ordonez et al., 2014). This gene was detected in all used *C. malonaticus* strains; however, strain 685, which was sensitive to acid after 30 mins, has a unique sequence of this gene (Figure 4.6). The analysis of *ompR* genes showed the presence of SNP difference in 685 gene, such SNP differences may alter the functional characteristics of this gene (Figure S.3). However, the *in silico* trascription of the DNA sequence showed that these SNPs are synonymous changes. This required further laboratory investigation by using protein analysis techniques to confirm wiether this gene is translated or not. *RpoS* sigma factor, which is suggested to play an important role in the tolerance of *Cronobacter* to acid, osmotic, and oxidative stresses (Alvarez-Ordonez et al., 2012) is also detected in all *C. malonaticus* strains. Interestingly, strain 685 which showed sensitivity to acid effect directly after 30 minutes could lose the function of *rpoS* gene due to the presence of SNP change that shared with *C. sakazakii* strain NTU658 (Figure 4.8, S.4). *C. sakazakii* NTU658 has been showed to lose the function of *rpoS* gene and therefore it become a sensitive strain to different stresses include acid (Alvarez-Ordonez et al., 2012). Alvarez-Ordonez et al. (2012) suggested a significant positive correlation between *rpoS* activity and the bacterial tolerance to lethal acid, alkaline, osmotic, and oxidative stress conditions. Similarly, there could be a strong correlation between this defect in the sequence of *rpoS* gene and the sensitivity of *C. malonaticus* strain 685 to the effect of acid.

PIF has been found to be one of the most important *Cronobacter* infection source among neonatal age. Some bacteria are hardly detected from PIF when they are sublethally injured due to exposure to dry stress. Therefore, to protect the consumers from this product, it is important to study the detection of sublethal injury bacteria. In this study, 20 *C. malonaticus* strains were subjected to dry stress and then recovered using two type media, VRBGA and TSA. The study showed the effects of two microbiological media, TSA and VRBGA, on cell recovery after desiccation. As shown in Figure 4.9 the recovery of all *C. malonaticus* strains showed decrease comparing with the number of inoculum bacteria. However, the number of undetected, dead and sublethal injured bacterial cells, from VRBGA was higher than from TSA ($P<0.05$) (Figure 4.10). Interestingly, strain 685 showed the highest number of sublethal injured cells (3.37 log10 CFU/ml) (Table 4.3). Exceptionally, this strain was not able to survive when it was exposed to acid stress (Figure 4.6). In contrast, strain 1827 (ST7) showed the lowest number of sublethal injured cells which is 0.36 log10 CFU/ml. In general, there

was a clear variation among ST7 strains regarding the number of injured bacterial cells; however, less variation was noticed among ST11 and ST60**.**

Desiccation can damage bacterial cell membrane during drying which could reduce the viable cell count of the recovered bacteria (Gardiner et al., 2000; Ramos et al., 2001). Many factors affect the recovery of bacteria after desiccation such as media composition and capsule formation. The PIF, which is used during desiccation experiment in this study, contains different substances such as lactose, milk fat, and proteins. Such components could provide bacteria with protection during milk drying (Lian et al., 2002; Caubilla-Barron and Forsythe, 2007). In addition, during the formulation process of PIF, osmotic protection could be also provided by milk components to the present bacteria and this will enhance the ability of bacteria to survive desiccation (Lian et al., 2002; Caubilla-Barron and Forsythe, 2007; Osaili and Forsythe, 2009). Moreover, sublethal injured bacterial cells can repair cellular damage and recover their normal characteristics including virulence factors (Wesche et al., 2009). Thus sublethal injured *C. malonaticus* strains in this study could repair their normal traits which allow them to cause infections. In addition, the findings in this study confirm the possibility of false negative results in the detection of *C. malonaticus* in PIF or other dry food, which is potentially dangerous to the health of consumers. However, to overcome such drawback, BPW is used in the first step when *Enterobacteriaceae* is detected in PIF or other foods to repair and proliferate the sublethal damaged cells.

Several genes that reported by Yen et al. (2013) for their association with desiccation and osmotic stress response have been detected in the all *C. malonaticus* strains. These genes included the *yihUTRSQVO* gene cluster, the osmoprotectant ABC transporter genes, including *yehZYXW*, which play roles in bacterial survival, osmotolerance regulation genes *yiaD*, *osmY*, *ompA*, *aqpZ*, and *glpF*, osmotic stress genes, *osmB* and *osmO*, *yciT*, *yciM*, and *pgpB* and also periplasmic glucan synthesis genes included *mdoC*, *mdoH*, *mdoG*, *mdoD*, *mdoB*, and *opgC* (Yen et al., 2013).

RpoS is also reported to be required for the tolerance to desiccation among *E. coli* (Stasic et al., 2012). In addition, Alvarez-Ordonez et al. (2012) showed the importance of this gene in the tolerance of *Cronobacter* to acid, osmotic, and oxidative. This gene was found in all used *C. malonaticus* strains; however, the sequence of this gene in strain 685 which showed the lowest capacity to withstand desiccation stress in this study was different from the other *rpoS* sequences in other strains (Figure 4.8). As shown in section 4.3.7 and discussed previously a SNP change has been detected and shared in both *rpoS* sequences of *C. malonaticus* 685 and *C. sakazakii* NTU658. Such SNPs may alter the functional characteristics of the *rpoS* genes. Therefore, this difference could be a default which makes this gene as an inactive gene resulting to make this strain to be a

vulnerable to desiccation. However, the variation of the used strains in their ability to tolerate desiccation could be controlled by other genes such as cellulose biosynthesis and colanic acid exopolysaccharide genes which could also play a role in resistance against dry and desiccant conditions (Grim et al., 2013).

Bacteria have an ability to control the metal homeostasis which could help the survival of pathogens into macrophages (Reva and Bezuidt, 2012). Some metals such as copper, zinc, iron, nickel and cobalt, manganese are essential in the structure of bacterial proteins, nonetheless these heavy metals could be toxic for bacteria even at low concentrations (Osman and Cavet, 2011). In this study the tolerance of C*. malonaticus* to eight heavy metals that might contribute in the bacterial virulence abilities or the bacterial growth was investigated. All tested strains showed an ability to tolerate or adapt the majority of metals at different concentrations (Table 4.4). Nevertheless, *C. malonaticus* strains were sensitive to sodium tellurite at all used concentrations, which indicates that tested strains do not have an efficient mechanism for adaptation or tolerance of this metal. Indeed, the BLAST searches revealed the absence of tellurite resistance associated genes *terACDYZ* in all *C. malonaticus* strains (Table 4.5). This indicates that *terACDYZ* genes are involved in the resistance to tellurite in *C. malonaticus*. In contrast, all of tested strains were able to tolerate cadmium at all used concentrations (Table 4.4). The *cadA* gene, which was reported to be involved in cadmium resistance (Perez et al., 2006; Lutsenko, 2000), was detected in the all tested strains (Table 4.5).

Pathogenic bacteria have an ability to control heavy metal homeostasis which help them to survive the killing action of macrophages (Reva and Bezuidt, 2012). The control of heavy metals such as copper and zinc are involved in the ability of the pathogenic microorganisms to survive into macrophages. Copper levels are reported to increase and zinc levels decrease in macrophages that exposed to cytokines and other mediators of inflammation. Therefore, bacteria need to have mechanisms for sensing and rapidly adapting the flux of metals and ensure the delivery of the exact metal to metal-requiring proteins (Osman and Cavet, 2011). In this study, *C. malonaticus* strains showed an ability to adapt these two metals in three concentrations (0.001, 0.01 and 0.1) and could not adapt them in the highest concentration (1 M) (Table 4.4). This indicates the presence of homeostasis and resistance mechanisms for copper and zinc in *C. malonaticus* strains which could improve the ability of *C. malonaticus* to survive the actions of macrophages. The genome study reveals the presence of the responsible genes for the copper and zinc resistance in the all used strains (Table 4.5). *C. malonaticus* showed also, in this study, the ability to control other metals such as arsenate, silver, nickel and cobalt. These metals have been reported to have roles in virulence of pathogenic bacteria such as *Salmonella* and *Yersinia* (Vasishta et al., 1991; Neyt et al., 1997; Tan et

al., 2006). The ability of *C. malonaticus* to control the homeostasis and adaptation of such toxic metals could enable them to survive different killing actions such as bactericidal activity of macrophages. Indeed, metal resistance genes for arsenate, silver, nickel and cobalt are present in the all *C. malonaticus* strains (Table 4.5). Arsenic resistance genes were also detected in the big plasmids of the used strains as shown in figure 4.14. Noticeably, not all strains that showed the ability to resist or adapt heavy metals were able to survive macrophages in Chapter 5. This is more likely due to contribution of different factors in such activity.

Iron is an essential element that incorporates in the composition of the bacterial proteins and in several bacterial activities (Negre et al., 2004). In the human body, the availability of iron is limited by iron-binding proteins. This iron-sequestering property is associated with the prevention of the growth of pathogenic bacteria (Rooijakkers et al., 2010). In addition, the stimulation of the immune response during infections, resulting in removal of the iron from the blood circulation and human cells (Collins, 2008). Moreover, in aerobic conditions, where oxygen is present, the availability of iron is extremely limited for bacteria. To acquire iron in such environments, microorganisms secrete high-affinity iron chelating compounds such as siderophores which help bacteria to survive. In this study, as shown in figure 4.11, all C*. malonaticus* strains were able to produce siderophores which indicates that *C. malonaticus* could acquire iron in iron-limited environments in human body. Grim et al. (2012) reported that *Cronobacter* possess two transport systems to acquire iron, ferric and ferrous. These systems are the *feoABC* and *efeUOB* systems for acquisition of ferrous and Cronobactin siderophores (*iucABCD-iutA*) which is encoded on the larger plasmid of *C. sakazakii* and *C. turicensis* for ferric iron. In addition, five siderophores receptors (*FhuA*, *YncD*, *FoxA*, *FhuE*, and *PfeA*) were also reported into the clinical *C. sakazakii* and *C. malonaticus* isolates (Grim et al., 2012). In this study, all of these iron transport systems and receptors have been detected in the used *C. malonaticus* strains. Moreover, the *in silico* analysis of plasmid profiling, as shown in figure 4.14, confirmed the presence of siderophore genes (*iucABCD*). These iron acquisition systems are more likely to be responsible for such trait in all *C. malonaticus* strains in this study.

The Gram negative bacteria which cause systemic infections need to overcome the mechanisms of killing by serum components and the action of human complement in human serum. In this study some *C. malonaticus* showed the ability of surviving human serum. For example, two ST7 strains, 1827 and 2018, demonstrated a high level of resistance to human serum (Figure 4.12). In addition, other *C. malonaticus* strains that belong to several STs displayed different levels of serum resistance. The involvement of bacterial outer membrane proteins and protease in resistance to human serum has been confirmed (Lambris et al., 2008; Schwizer et al., 2013). For *Cronobacter* spp., Franco et al. (2011a) suggested that *cpa* (plasminogen activator) may have an important role in serum resistance.

However, a recent study showed a variation in the ability of some *C. sakazakii* strains, which possess the *cpa*, to survive in human serum (Almajed and Forsythe, 2016). Another recent study used *C. sakazakii* strains by Hana Sonbol did not find a clear correlation between the presence of *cpa* and serum resistance. In her study, two *C. sakazakii* strains, which were serum resistant, were also lacked *cpa* gene (Sonbol, 2015 unpublished data). Such findings indicate the contributions of other bacterial outer membrane structures and secretions such as *OmpA*, LPS, capsules and others in the survival of bactericidal activity of human serum. Indeed, several studies reported the involvement of other factors such as the major outer membrane protein *OmpA*, plasmid-encoded proteins *TraT*, regulator of colanic acid biosynthesis *rcsA*, siderophore-encoding gene *viuB* and envelope stress regulators *rcsB* in the bacterial ability to survive the killing activity of human serum (Allen et al., 1987; Bogard and Oliver, 2007; Miajlovic et al., 2013; Phan et al., 2013). It was found that all tested strains in this study possess these factors regardless of their response against human serum. However, some genes could be present as a silent gene without any expression. Moreover, four ST7 strains (1830, 1833, 1835 and 2020) showed no ability to survive human serum. These strains were non-motile and not able to produce any capsular materials (Table 4.2), which indicates that capsule and flagella might play important roles in resistance to human serum. Certainly, the expression of exopolysaccharide colanic acid and the K2 capsule in *E. coli* showed a protective role against the effect of human serum (Buckles et al., 2009; Miajlovic et al., 2013). In addition, serum resistance and expression of the bacterial surface such as phase I flagellin (*fliC*) have been found to be facilitated by O-antigen capsule of *S*. Typhimurium. This was confirmed when the constructed O-antigen capsule-deficient mutants in *S*. Typhimurium showed reduced resistance to the bactericidal activity of human serum (Marshall and Gunn, 2015).

Interestingly, Schwizer et al. (2013) reported that strains not expressing the highly immunogenic type 1 fimbriae may survive during exposure to human serum. It is worth mentioning that all tested *C. malonaticus* strain in this study lack the presence of type 1 fimbriae; however, these structures are important for pathogenicity of *E. coli* (Teng et al., 2005). In general, the ability of some *C. malonaticus* strains to survive the human serum in this study indicates that this species can cause bacteraemia or systemic infections. Strains such as 688, 893, 1827, 2018, 512, 514, 15, 687, 689, 685, 1569 were able to survive human serum up to 3 hours. Three of these strains (685, 1569 and 1827) were isolated from blood and one of these three (1569) was associated with a fatal meningitis case. This indicates that *C. malonaticus* could cause bacteraemia and systemic infections.

Other virulence factors such as bacterial haemolysins and protease have been detected among the tested *C. malonaticus* strains. These activities are important virulence factors in bacterial disease. Bacterial haemolysin is a toxin that can lyse red blood cells (RBCs) and there are three different

feature effects by haemolysins, which are β haemolysis, α-haemolysis and γ-haemolysis (Goebel et al., 1988). While bacterial protease, such as zinc-containing metalloprotease encoded by *zpx*, has potential to destroy the structural and functional proteins in host tissue, cell damage was occurred by *Cronobacter* zinc metalloprotease which noticed to induce the rounding of the Chinese hamster ovary (Lantz, 1997; Kothary et al., 2007). All tested strains were able to lyse the RBCs of horse blood; the activity was determined as type β haemolysis. In addition, the strains also degraded the milk protein which was used in this study. Consistently, six putative genes associated with haemolytic activity (ESA_00102, ESA_00432, ESA_00643, ESA_02810, ESA_02937 and ESA_03540) have been detected in the used genome of the tested *C. malonaticus* (Joseph et al., 2012a). In addition, the presence of proteolytic activity associated gene such as *zpx* was confirmed. These genes could play important roles in the previous mentioned traits. The presence of such virulence traits indicates the possible occurrence of serious destruction in human tissue during *C. malonaticus* infections.

Antimicrobial susceptibility was performed against eight antibacterial agents for all tested strains (Table 4.6). All strains showed susceptibility to quinolones, aminoglycosides and carbapenems groups though *C. malonaticus* strains harbour multiple drug resistance operon, *mar*. In contrast all the tested strains were resistant to tetracycline and 40% of them were resistant to chloramphenicol (Table 4.6). This resistance against tetracycline and chloramphenicol among *Cronobacter* was reported previously (Nazarowec-White and Farber, 1999; Kilonzo-Nthenge et al., 2012; David et al., 2013). This resistance could be due to the presence of the multidrug efflux pump *AcrAB* which was detected among used genomes. The efflux pump *AcrAB* has been linked with the ability of *E. coli* to expel both tetracycline and chloramphenicol (Sun et al., 2014). In contrast to Lai, (2001) results who reported the presence of ampicillin resistance among *Cronobacter*, the results in this study have not detected any resistance to ampicillin. However, about 35% of *C. malonaticus* strains showed intermediate resistance to cefotaxime which is one of the third-generation of cephalosporins, related to penicillin. Thus, in 2014 Pan and co-authors reported that about 10.2% of the tested *Cronobacter* strains in their study were resistant to cefotaxime (Pan et al., 2014). These results raised concerns about the possible emerging resistance against β-lactamase antibiotic group.

Kucerova et al. (2010) reported the first genome sequence of *Cronobacter* strain which is *C. sakazakii* BAA-894 with the presence of two plasmids, pESA3 (131 kb) and pESA2 (31 kb). The pESA3 and another large plasmid pCTU1 (138 kb) of *C. turicensis* z3032 were studied more by Franco et al. (2011b) who found that these two plasmids contain similar virulence genes such as iron acquisition systems. Some genes of pESA3 and pCTU1 plasmids were screening among 229 *Cronobacter* strains from different species, resulting in the presence of these genes in 97% of screened strains. Other large plasmids were reported in *C. sakazakii* SP291 and *C. malonaticus* CMCC4502 with size 118 kb

and 126.5 kb respectively (Power et al., 2013; Zhao et al., 2014). In this study plasmid profiling was performed on 20 *C. malonaticus* strains including two *C. sakazakii* strains BAA-894 and 6 which used as a reference marker and a negative control respectively.

As shown in figure 4.13, all of the strains contain at least one plasmid. Although, the figure shows the appearence of more than one band in the same lane for the majority of lanes, these bands could be present as supercoiled and open circle bands. Therfore, because of the limitations of the used simple method, it was impossible to exactely specify how many plasmids are contained by the srains, or detect an accurate size of the plasmids. The used strains could contain more than one plasmid as this was confirmed in *Cronobacter* in previous studies (Kucerova et al., 2010; Power et al., 2013; Zhao et al., 2014), and some of these plasmids are very large. Such large plasmids were detected previously in *C. malonaticus* CMCC45402 by Zhao and colleagues when found that two circular plasmids sized ~ 126,5 kb and ~ 55,9 kb were harboured by this strain (Zhao et al., 2014). large plasmids with size around 126,5 kb, are believed to harbour two arsenic resistance genes and several putative virulence genes, including two genetic loci encoding iron acquisition systems, namely an ABC transporter gene cluster and an aerobactin or cronobactin siderophore receptor gene cluster identified as *eitCBAD* and *iucABCD*/*iutA*, respectively (Franco et al., 2011b; Yan et al., 2015). In addition, plasmids with size around 50 kb reported to harbour metal resistance traits such as pCTU3 (53.8 kb) (Joseph et al., 2012a).

The contents of the large plasmids of *C. malonaticus* strains were more investigated by *in silico* method. Three known large plasmids which are plasmid pESA3 (131 kb) that is regarded as a virulence plasmid (Franco et al., 2011), the large plasmid (118 kb) of *C. sakazakii* SP291 (ST4) (pSP291-1) and the large plasmid (~126 Kb) of *C. malonaticus* CMCC45402 ST7 (pCMCC 45402-1) were used as reference plasmids. The whole genome of *C. sakazakii* strain 6 was also used in this part as a negative control. As shown in figure 4.14 the content of the three reference plasmids was totally absent in the negative control. This finding is consistent with the laboratory result that showed in figure 4.13. Interestingly, *C. malonaticus* 688 contained only partial length of pESA3, pSP291-1 and pCMCC 45402-1 plasmids; It showed homology with only about 65 kb of pESA3 and pSP291-1 plasmids and about 80 Kb of pCMCC 45402-1 plasmid (Figure 4.14). In contrast, two *C. malonaticus* strains, which belong to clonal complex 7 (CC7), 1558 (ST7) and 1545 (ST84) showed to contain a full length of pCMCC 45402-1 plasmid which harboured by ST7 strain (Figure 4.14-C).

Approximately 10 kb region that harboured by pESA3 plasmid, consisting of 10 hypothetical proteins and known as the T6SS system clusters was missed in all *C. malonaticus* strains (Figure 4.14-A). This region was missed also in the majority of *C. sakazakii* strains which were used by

another research (Masood, 2015 unpublished data). Moreover, *Cronobacter* plasminogen activator (*cpa*) and a starvation sensing protein encoded by the *rspA* gene which harboured by both pESA3 and pSP291-1 plasmids were absent in all *C. malonaticus* strains (Figure 4.14-A and 4.14-B). Interestingly, a complete plasmid-borne operon for aerobactin synthesis (*iucABCD*) and arsenic resistance genes which harboured by the three reference plasmids were detected in all *C. malonaticus* strains as shown in Figure 4.14. However, the location of the aerobactin synthesis operon and arsenic resistance genes was not same in the three reference plasmids.

Chapter 5 Pathogenicity of *C. malonaticus*

5.1 Introduction

Cronobacter is an emergent opportunistic pathogen that causes infections in both infants and adults. In infants, it can cause necrotising enterocolitis (NEC), bacteraemia and meningitis with mortality rates ranged from 40% to 80 % and survivors often suffer from neurological disorders (Bowen and Braden, 2006; Caubilla-Barron et al., 2007; Mullane et al., 2007; van Acker et al., 2001; Hariri et al., 2013; CDC, 2016). In adults particularly who are immunocompromised and elderly, *Cronobacter* causes bacteraemia, pneumoniae, urinary tract infections, wound infections, osteomyelitis and splenic abscess (Tamigniau et al., 2015; See et al., 2011; Jimenez and Gimenez, 1982; Healy et al., 2010). Figure 5.1, which obtained from Healy et al. (2010), confirms that *Cronobacter* spp. is bacterial etiologic agents that can cause neonatal incidence such as meningitis, bacteraemia and necrotizing enterocolitis. In addition, other clinical symptoms include pneumoniae, sepsis; foot ulcers, wound infections, osteomyelitis, and splenic abscesses were reported in adults (Fig. 1b).

It is known that *C. sakazakii*, *C. malonaticus* and *C. turicensis* are the only species associated with clinical incidence and particularly with neonatal infections so far (Stephan et al., 2010; Holý et al., 2014). Recently *C. malonaticus* has been more reported to be responsible for several neonatal infections in different countries, and some of these infections end with a fatal outcome. These reports have put *C. malonaticus* to be the second agent of neonatal infection risks among the genus of *Cronobacter* after *C. sakazakii* (Hariri et al., 2013; Asato et al., 2013; Brandao et al., 2015; unpublished data China).

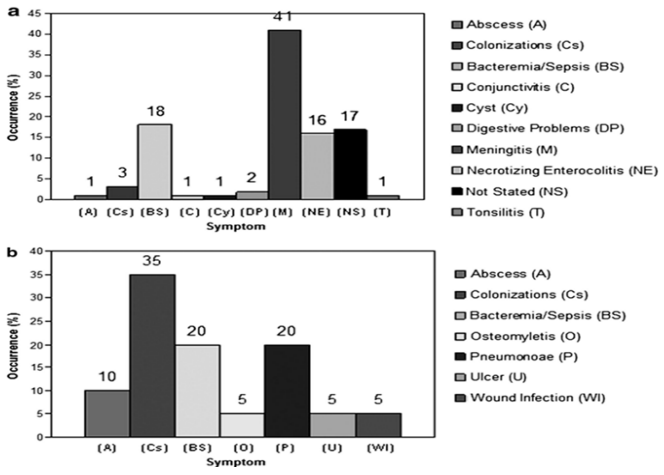


Figure 5-1 Colonisations, symptoms, and incidence of occurrence as a percentage of total *Cronobacter* infections.
(a) in children and infants (36 cases) and (b) cases in adults (19 cases). Data collected from 3 studies from. Numbers are presented in percentages. From Healy et al. (2010).

To cause disease, pathogenic bacteria utilise a number of mechanisms in human hosts. Adhesion, invasion and surviving of the host cells are shown to be some of these machnisms (Wilson et al., 2002). Pathogens of *Enterobacteriaceae* family such as *Salmonella*, *Shigella* and *E. coli* have proved to be able to adhere, invade and survive within host cells and subsequently cause several of infections (Formal et al., 1983; Torres, 2004). For *Cronobacter* the ability to adhere and invade the host cells such as different epithelial and brain endothelial cells is an essential pathogenic factor for causing infectious diseases (Almajed and Forsythe, 2016).

Cronobacter is considered as a food-borne pathogen that firstly colonises and invades the intestinal cells from where it can disseminate and cause systemic infections. *C. sakazakii* was used to examine the ability of this microorganism to adhere and invade intestinal cells and brain cells as well as to survive the macrophages. This was studied using Caco-2, HBMEC and macrophages U937 cell lines (Mange et al., 2006; Townsend et al., 2007a; Townsend et al., 2008; Giri et al., 2012). Recently Almajed and Forsythe, (2016) studied the virulence potential and pathogenicity of *C. sakazakii* clinical isolates and their ability to overcome host physical barriers and evade host immune response. The study showed that *C. sakazakii* clinical isolates were able to invade and translocate through Caco-2 and HBMEC cell lines and to persist and multiply within macrophages and microglial cells (Almajed and Forsythe, 2016).

Recent reports have shown that the majority of *Cronobacter* strains isolated from humans were from urinary tract and respiratory systems (Patrick et al., 2014; Chapter 3). However, to our knowledge, no *in vitro* research exists confirming the ability of *Cronobacter* to cause infections in such sites. Mammalian tissue culture assays are appropriate methods for investigating the bacterial adhesive and invasive abilities. Human urinary T24 cell line and human respiratory A549 cell line have been used to investigate the adhesive and invasive properties. Some studies on *E. coli* (the most common causative pathogen in adult UTI) have shown the capability of this pathogen to invade T24 cell line (Meier et al., 1996; Croxall et al., 2010). Similarly, other studies investigated the ability of some respiratory pathogens to invade the A549 cells when they confirmed the ability of these pathogens to invade this cell line (Giannouli et al., 2013; Pracht et al., 2005; Ahmed et al., 2014). Nevertheless, there is no known previous study investigating the ability of *Cronobacter* to adhere or invade these cell lines.

A number of virulence factors have been identified in *Cronobacter*. These virulence factors have an essential role in the adhesion, invasion and distribution of the organism in the human host. Bacterial fimbriae or pili are filamentous appendages which help bacteria to adhere to the surface of the host cells thus allowing them to colonise and establish successful infections (Soto and Hultgren, 1999).

Joseph et al. (2012a) described about ten putative fimbrial gene clusters in *Cronobacter* (Joseph et al., 2012a). It has been also confirmed that the outer membrane protein A (*OmpA*) of *Cronobacter* contributes in the colonisation of the gastrointestinal tract (GIT) (Kim et al., 2010). Moreover, it was demonstrated in *C. sakazakii* that the outer membrane proteins ompA and ompX involved in the basolateral invasion of enterocyte like human epithelial cells (Kim et al., 2010). The entry of *Cronobacter* into HBMEC requires the expression of *ompA* and depends on microtubule condensation in these cells (Singamsetty et al., 2008). This could facilitate the invasion of human intestinal cells and invasion of the brain endothelial cells to cause meningitis (Franco et al., 2011a).

Several intramacrophage survival associated genes have been described in *Cronobacter* and other pathogen bacteria. The *PhoP/PhoQ* regulatory system of *S*. Typhimurium and *Y. pestis* and their regulated genes including *mgtB* and *pmrABE* were shown to play an essential role for survival in macrophage phagosomes (Ernst et al., 1999; Grabenstein et al., 2006). In addition, *gsrA* of *Y. enterocolitica* has been found to provide protection against oxidative stress killing by macrophages (Yamamoto et al., 1996). *SodA* gene encoding for superoxide dismutase has been also reported to be associated with macrophage survival in *Cronobacter* (Townsend et al., 2007a). This gene is involved in resistance to the early oxygen-dependent bactericidal mechanisms of phagocytes in *Salmonella* (Tsolis et al., 1995; Cox et al., 2003). Two other genes, *betB,* encoding betaine-aldehyde dehydrogenase, and the sensor *rcsC*, which act as a senser of *RcsC*/*YojN*/*RcsB* regulatory system, have been also found to be associated with macrophage survival in *Brucella* and *Salmonella* respectively (Lee et al., 2014; Mouslim et al., 2004).

In this chapter, the adhesive and invasive abilities of 20 clinical *C. malonaticus* strains (Page 63, Table 3.5) to human intestinal epithelial cell line, Caco-2, human brain microvascular endothelial cell line, HBMEC, human lung epithelial cell line, A549 and human bladder epithelial cell line, T24 as well as the ability of these strains to survive the U937 macrophages were investigated. The experiments were conducted to help shed further light on the pathogenicity of *C. malonaticus*.

5.2 Materials and methods

The methods for this part were described previously in Chapter 2 of materials and methods.

5.3 Results

5.3.1 Attachment to Caco-2 Intestinal Epithelial Cells

A standard adhesion assay was conducted to assess the adherence ability of *C. malonaticus* strains to epithelial Caco-2 cells. Figure 5.2 below shows the attachment ability of the selected *C. malonaticus* strains. *S*. Enteritidis and *E. coli* K12 were used as positive and negative controls

respectively for comparative purpose. All strains showed the ability to adhere to Caco-2 cells; however, there was a variation between strains. Strain 688, which is ST7, demonstrated the greatest ability to adhere to Caco-2 cells ($P<0.05$). In contrast, strain 565 which is also ST7 showed the lowest ability to adhere to this cell line. The ability of adherence to Caco-2 cells was confirmed using Giemsa stain protocol, and three different patterns of adhesion were determined. Figure 5.3 shows three representative strains for the three different patterns of adhesion.

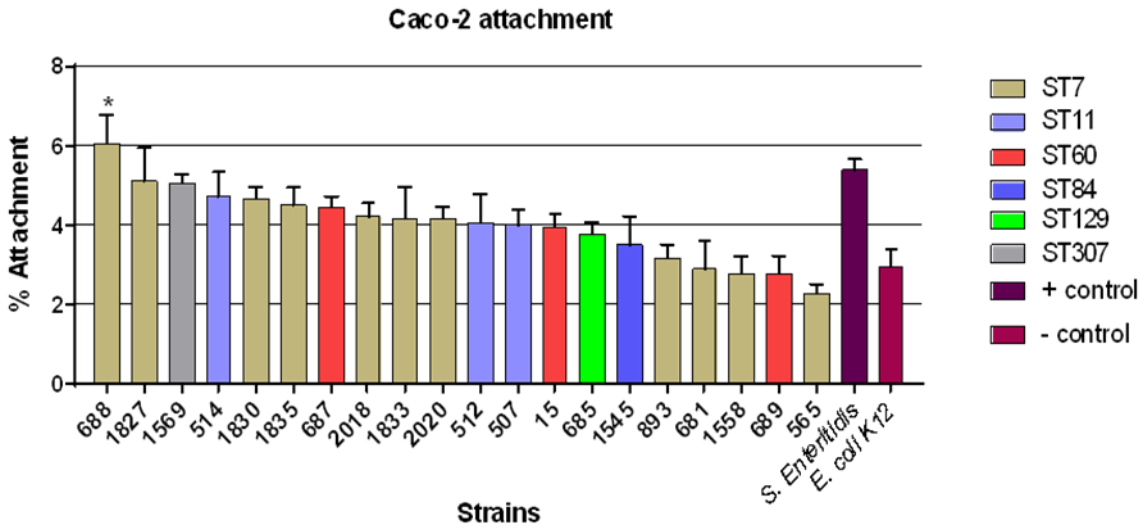


Figure 5-2 *C. malonaticus* attachment assay using Caco-2 cells, after 2-h of incubation.
S. Enteritidis and *E. coli* K12 were used as positive and negative controls, respectively. The attachment is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive, negative and tested strains in this experiment ($P<0.05$).

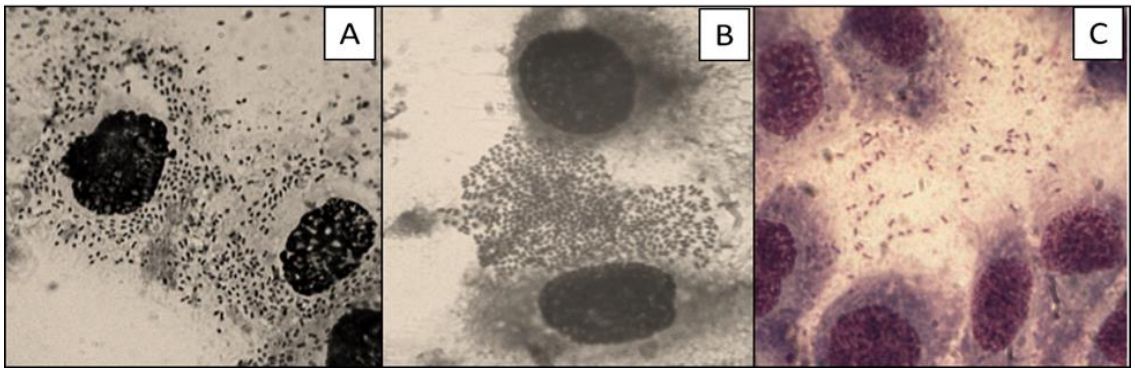


Figure 5-3 Three representative *C. malonaticus* strains show the three patterns of adhesion to Caco-2 cells.
(A) Shows aggregative adherence where the bacteria covered all around the cells (strain 685). (B) Localised adherence where the bacterial clusters attached to the cells in specific locations (strain 688) which demonstrated the highest attachment level. (C) Non-specific adherence pattern (strain 565).

5.3.2 Invasion into Caco-2 Intestinal Epithelial Cells

The selected *C. malonaticus* strains, as shown in page 63 table 3.5, were further examined for their ability to invade the Caco-2 cells and gentamicin protection assay was applied. The majority of strains were able to invade the Caco-2 cells; however, there was a clear variation between strains in their ability to invade the Caco-2 cells (Figure 5.4). Strains 2018, 1827 and 1569 showed the most significant invasion among the tested strains (P<0.05), whereas strains 1830, 1833, 1835 and 2020 did not invade these cells. Some strains; such as 514, 565, 893 and 512; were moderate and the rest were low.

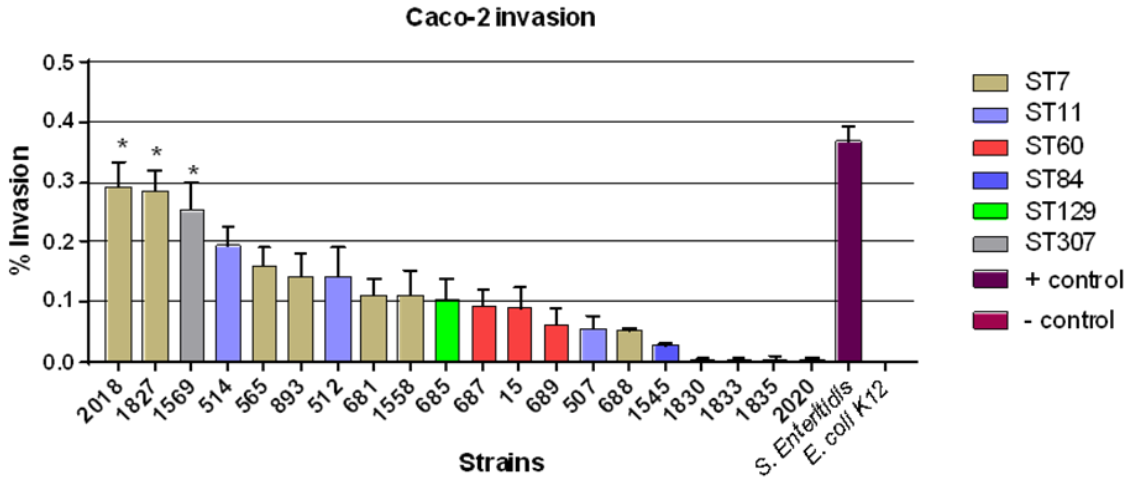


Figure 5-4 *C. malonaticus* invasion assay using Caco-2 cell line over 3 h of incubation.
S. Enteritidis and *E. coli* K12 were used as positive and negative controls respectively. The invasion is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive, negative and tested strains in this experiment (P<0.05).

5.3.3 Attachment to Human Brain Microvascular Endothelial Cells (HBMEC)

Figure 5.5 shows the adherence ability of the selected *C. malonaticus* strains to HBMEC cell line. In this assay *C. koseri* and *E. coli* K12 were used as reference strains for the cell line and for comparative purposes. Comparing to the negative control, all strains were able to adhere to this endothelial cell line. The strain, which demonstrated the greatest adherence, was strain 685, which belongs to ST129. Strains 1827, 2018 and 688, which are ST7, also demonstrated high level of adhesion to this cell line. In addition, strains 1545, 1558, 2020 and 514 showed a moderate adherence level and the rest of used strains were similarly low in their ability to adhere the HBMEC cells line. The adherence ability of *C. malonaticus* strains to HBMEC was confirmed by microscopic

examination. Subsequently, four patterns of adhesion by used *C. malonaticus* strains were detected. Figure 5.6 shows four representative strains for the four different patterns of adhesion.

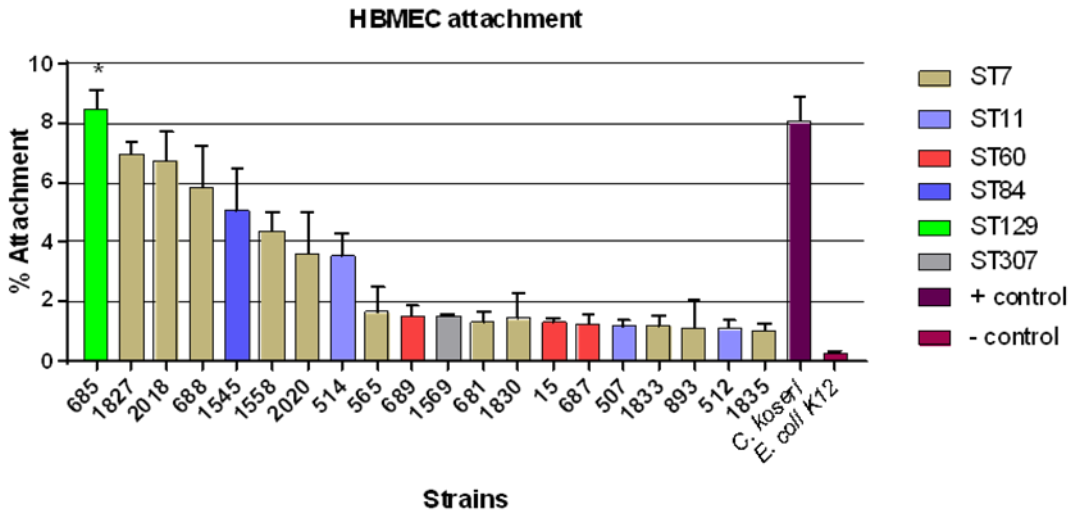


Figure 5-5 *C. malonaticus* attachment assay using HBMEC cell, after 2 h of incubation.
C. koseri and *E. coli* K12 were used as positive and negative controls respectively. The attachment is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive and tested strains in this experiment (P<0.05).

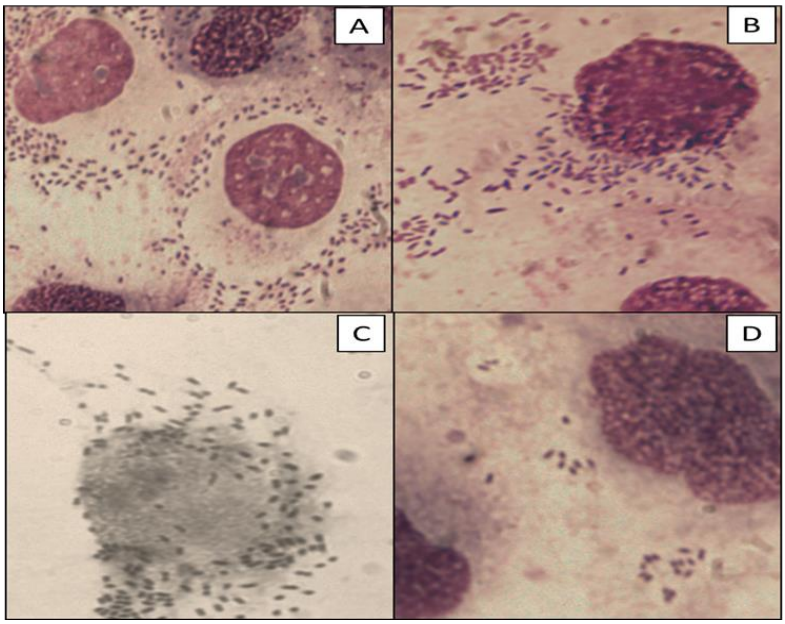


Figure 5-6 Four representative *C. malonaticus* strains show the four patterns of adhesion to HBMEC cells.
(A) Shows aggregative adherence where the bacteria covered all around the cells (strain 685) which demonstrated the highest attachment level. (B) Localised adherence where the bacterial clusters attached to the cells in specific locations (strain 2018). (C) Diffused adherence where the bacterial cells surround the cell (strain 1558). (D) Non-specific adherence pattern (strain 15).

5.3.4 Invasion into Human Brain Microvascular Endothelial Cells (HBMEC)

As *C. malonaticus* strains showed the ability to adhere to HBMEC cells, this could indicate the ability of used strains to invade these human blood brain barrier endothelial cells. Consequently, the gentamicin protection assay to kill all extracellular bacteria was conducted to confirm the ability of bacterial cells to invade after being attached. *C. koseri* and *E. coli* K12 were used as positive and negative controls respectively to compare them with tested strains. Figure 5.7 below shows the ability of the 20 *C. malonaticus* strains with the positive and negative controls to invade the HBMEC cells. Four of five strains which showed an obvious ability to invade these cells belong to ST7 and one belongs to ST307 which was isolated from a fatal neonatal case. Comparing with reference strains, strains 1558, 1827 and 2018 which are ST7 showed significantly the highest invasion levels ($P<0.05$). Strains 1569 (ST307) and 565 (ST7) showed also high level of invasion. In contrast, four ST7 strains which are 1830, 1833, 1835 and 2020 showed no ability to invade the HBMEC cells, whereas strain 893 which is ST7 showed very low ability of invasion. The remaining strains showed moderate ability to invade the HBMEC cell line.

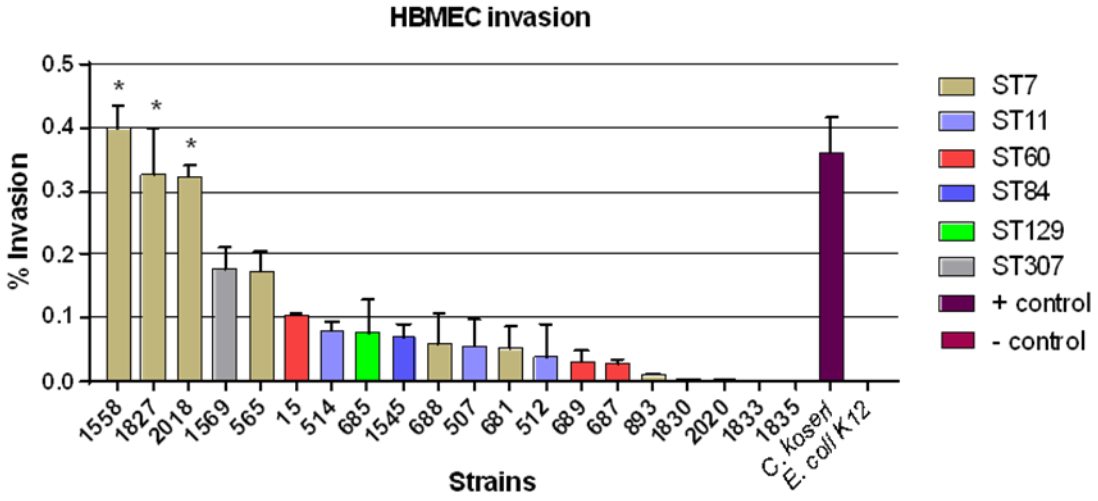


Figure 5-7 *C. malonaticus* invasion assay using HBMEC cell line over 3 h of incubation.
C. koseri and *E. coli* K12 were used as positive and negative controls respectively. The invasion is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive, negative and tested strains in this experiment ($P<0.05$).

5.3.5 Attachment to A549 Lung Epithelial Cells

Although *C. malonaticus* has been linked to adult infections such as pneumonia, no evidence has been shown to confirm this hypothesis. Therefore, the human lung epithelial cells A549 were used to investigate the ability of *C. malonaticus* to adhere these lung cells. In addition, *K. pneumoniae* and *E. coli* K12 which are positive and negative controls respectevely were used in this assay. Figure 5.8 below shows the result of this assay when the used strains showed an ability to adhere to A549 cells. Remarkably, six *C. malonaticus* strains (688, 1558, 685, 1827, 2018 and 1835) showed higher adhesion levels than *K. pneumoniae* which is the positive control for this cell line ($P<0.05$). Five of these six high adhesive strains belong to ST7 and one belongs to ST129. Two other ST7 strains which are 1833 and 2020 strains showed also high levels of adhesion comparing to the reference strains, while all the rest of the tested strains displayed moderate or low ability of adherence to this cell line. The Giemsa stain assay was used here to confirm the adherence ability of tested cells to A549 cells (Figure 5.9). Three different patterns of adhesion were displayed as shown in figure 5.9 by three representative strains.

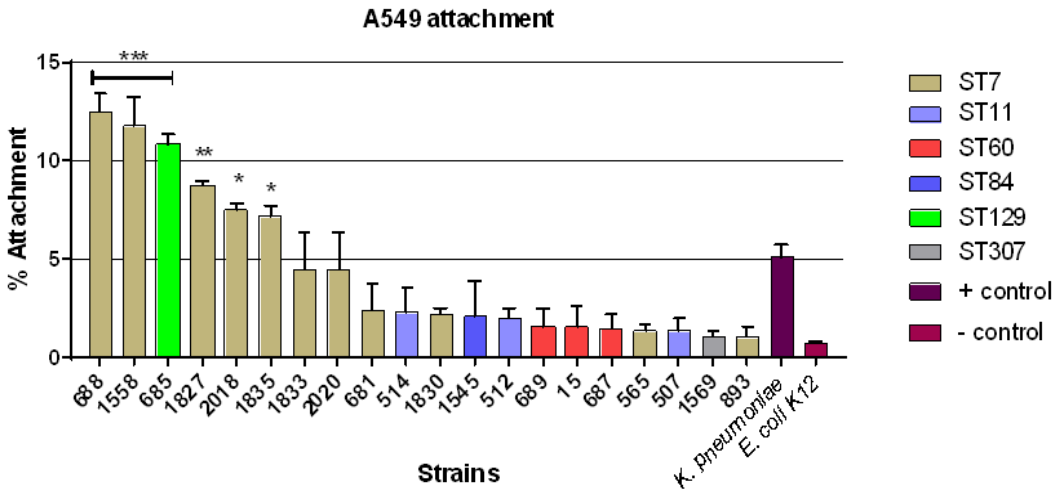


Figure 5-8 *C. malonaticus* attachment assay using A549 cell line after 2-hour incubation.
K. pneumoniae and *E. coli* K12 were used as positive and negative controls respectively. The attachment is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive, and tested strains in this experiment ($P<0.05$).

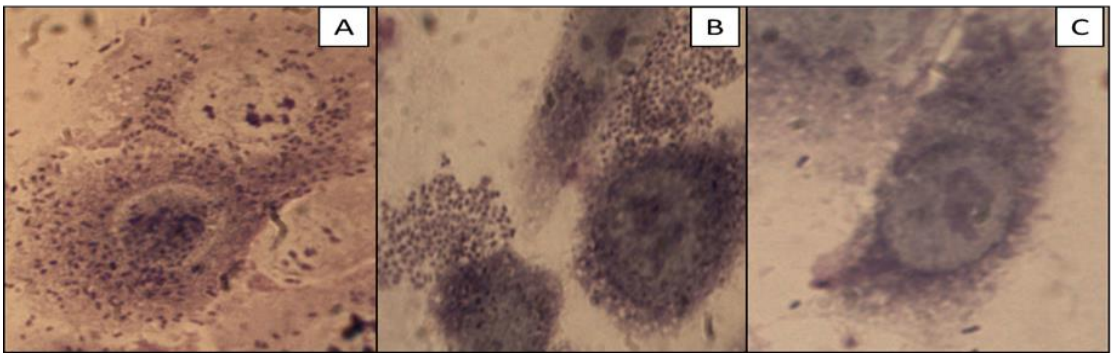


Figure 5-9 Three representative *C. malonaticus* strains show the three patterns of adhesion to A549 cells.
(A) Shows aggregative adherence where the bacteria covered all around the cells (strain 685) which demonstrated high attachment level. (B) Localised adherence where the bacterial clusters attached to the cells in specific locations (strain 688) which demonstrated high attachment level. (C) Non-specific adherence pattern (strain 15).

5.3.6 Invasion into A549 Lung Epithelial Cells

Invasion assay is an important examination to confirm the ability of *C. malonaticus* strains to invade the human lung cells A549. Gentamicin protection assay was used to kill any extracellular bacteria and find the number of invading bacteria into this cell line. Same reference strains that used in attachment assay were also used in this experiment. Interestingly, four ST7 strains (565, 1827, 1558 and 2018) showed very high ability to invade A549 cells, when one was similar and three were higher than positive control ($P<0.05$) (Figure 5.10). Two ST11 strains (507 and 514) showed high invasion level comparing to the reference strains. Once again, four ST7 strains (1830, 1833, 1835 and 2020) were shown as not able to invade this cell line comparing with non-invasive control. The rest of tested strains showed moderate levels of invasion which is considered as a modest level comparing to the highest invasive *C. malonaticus* strains (1827, 1558 and 2018).

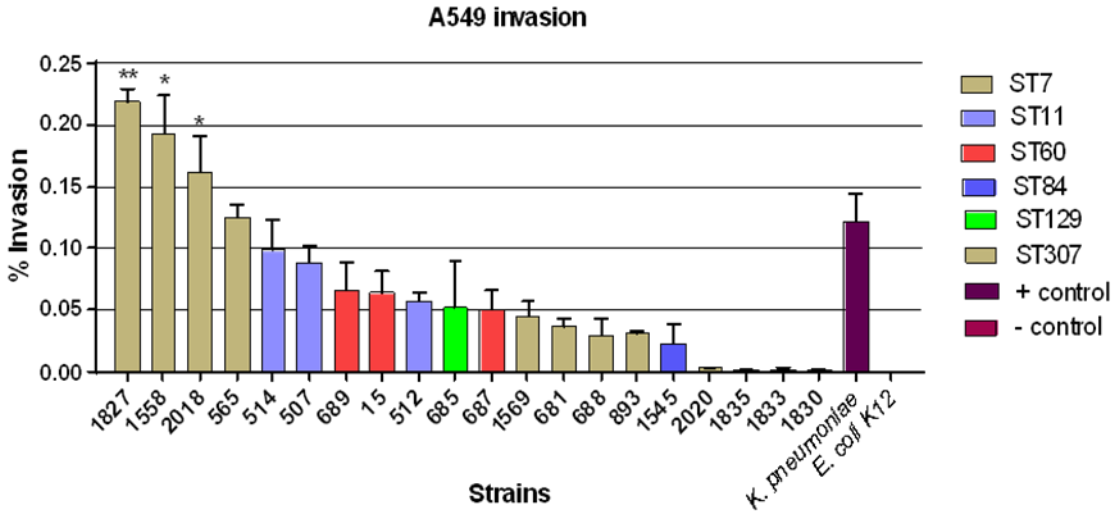


Figure 5-10 *C. malonaticus* invasion assay using A549 cell line over 3 h of incubation.
K. pneumoniae and *E. coli* K12 were used as positive and negative controls respectively. The invasion is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive, negative and tested strains in this experiment (P<0.05).

5.3.7 Attachment to T24 Urinary Bladder Epithelial Cells

In this assay T24 human bladder cells were used to investigate the ability of selected *C. malonaticus* to adhere to these cells. *E. coli* UTI89 and *E. coli* K12 were used as positive and negative controls respectively in this assay. Figure 5.11 shows the adhesion ability of 20 *C. malonaticus* strains with the reference strains. Five strains from which one belongs to ST129 (685) and four belong to ST7 (688, 1558, 1827 and 2018) showed the highest levels of adherence comparing to other used *C. malonaticus* strains (P<0.05). A moderate level of attachment showed by ten tested strains, three of them are ST7 (1833, 1835 and 681), three are ST11 (507, 512 and 514), two ST60 (687 and 15), one ST307 (1569) and one ST84 (1545). The remaining strains showed low attachment levels comparing to the reference strains. Microscopic examination of *C. malonaticus*-infected T24 cell line was applied to confirm the adherence ability of the organism (Figure 5.12). Consequently, three different patterns of the adhesion are displayed, which are shown in figure 5.12 with three representative strains.

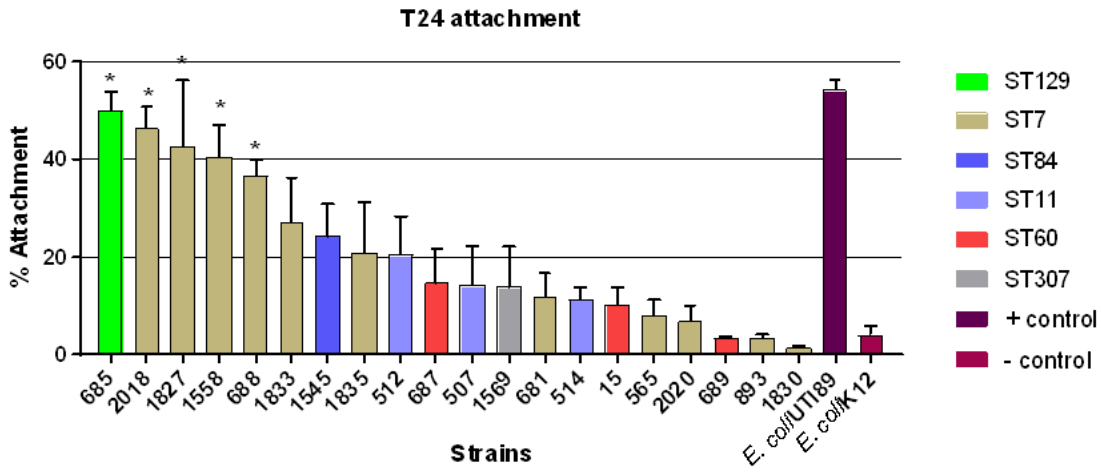


Figure 5-11 *C. malonaticus* attachment assay using T24 cell line after 2-hour incubation.
E. coli UTI89 and *E. coli* K12 were used as positive and negative controls, respectively. The attachement is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive, negative and tested strains in this experiment (P<0.05).

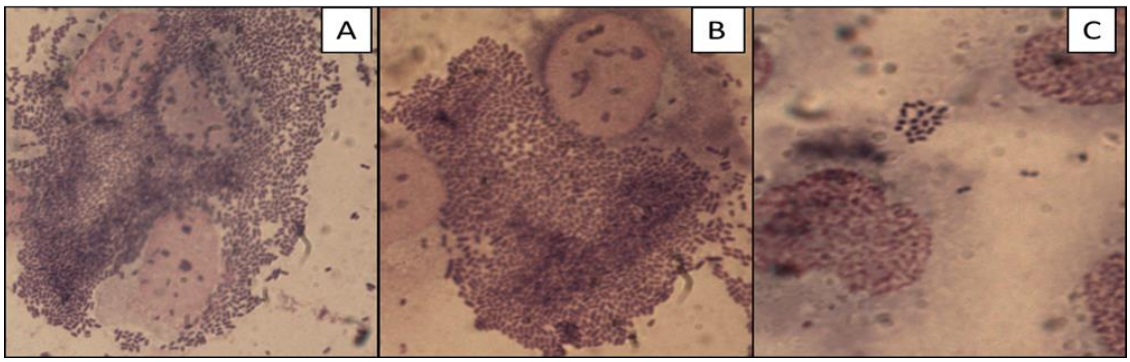


Figure 5-12 Three representative *C. malonaticus* strains show the three patterns of adhesion to T24 cells.
(A) Shows aggregative adherence where the bacteria covered all around the cells (strain 685) which demonstrated high attachment level. (B) localised adherence where the bacterial clusters attached to the cells in specific locations (strain 2018) which demonstrated high attachment level. (C) Non-specific adherence pattern (strain 15).

5.3.8 Invasion into T24 Urinary Bladder Epithelial Cells

The investigation of the ability of the selected *C. malonaticus* strains to invade the human bladder T24 cells was accomplished by using the gentamicin protection assay. Figure 5.13 shows the results of this assay. The majority of tested strains showed an ability to invade the bladder cells. Thirteen strains showed the ability to invade these bladder cells similar or even higher than the positive control. Three ST7 strains (1558, 1827 and 2018) displayed the highest invasion levels among all used strain (P<0.001). In addition, 10 strains that belong to ST7 (681 and 565), ST11 (507, 512 and 514), ST60 (15, 687 and 689), ST84 (1545) and ST307 (1569) invaded this cell line similar or higher than reference strain (P<0.05). The same strains that showed no ability to invade previous cell lines showed no ability to invade this cell line as well, and strains 688, 685 and 893 were able to invade in moderate levels compering to other tested strains.

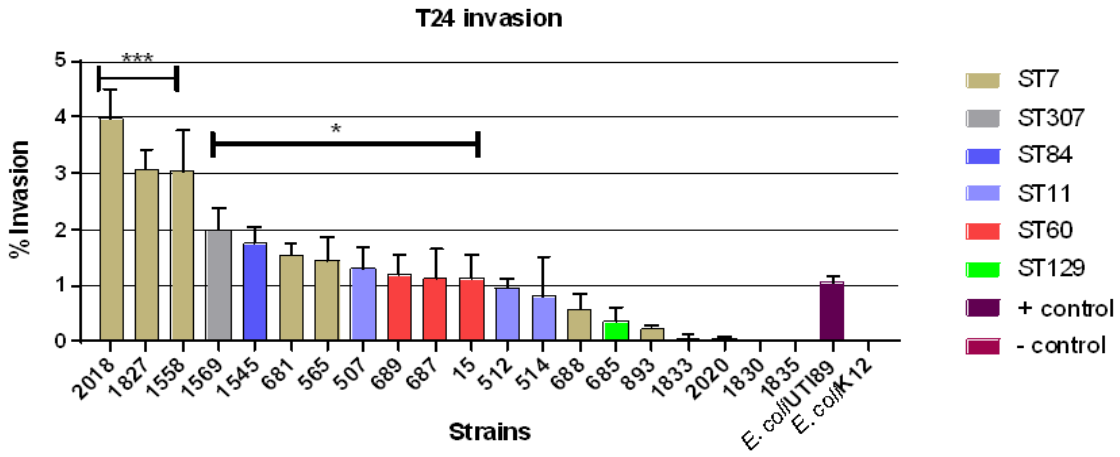


Figure 5-13 *C. malonaticus* invasion assay using T24 cell line over 3 hours of incubation.
*E. coliUTI*89 and *E. coli* K12 were used as positive and negative controls respectively. The invasion is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the positive and tested strains in this experiment (P<0.05).

5.3.9 Adhesion associated genes

Bacterial fimbriae or pili are filamentous appendages which help bacteria to adhere to the surface of the host cells thus allowing them to colonise and establish a successful infection (Soto and Hultgren, 1999). Joseph et al. (2012a) described about ten putative fimbrial gene clusters in *Cronobacter* (Joseph et al., 2012a). The genomes of the 20 strains were screened using Web ACT

and *Cronobacter* BLAST. C*. malonaticus* strains possess 7 of 10 regions as complete regions and 3 regions were partially present (Table S1, S2). In *E. coli* curli fimbriae have been associated with adhesion to the host cells (Doran et al., 1993; Olsen et al., 1993; Cordeiro et al., 2016). A complete curli fimbrial gene cluster (ctu_16160-230) that was reported in the genome of *C. turicensis* z3032 (Joseph et al., 2012a), is harboured by *C. malonaticus* strains.

5.3.10 Invasion associated genes

Numbers of invasion-associated genes have been searched across the selected genomes. These genes are *ompA*, *ompX*, *apaH*, *ygdP*, *ppk1*, *ibeB* and *Hfq*. All of these genes have been detected in the used strains regardless of their invasion ability. Recently, some studies have proposed several virulence associated genes in *Cronobacter*. These genes were upregulated during the intraction of *Cronobacter* with host cells. Table 5.1 showed a list of the proposed virulence genes and more details regarding these genes are providing in the discussion. As there was a clear variation regarding the invasion ability of used strains and this variation was detected even among the same STs such as ST7. Representative strains of high invasive and low invasive of ST7 strains were selected for further genome investigations. Strains 565, 1558, 1827 and 2018 were selected to represent the high invasive ST7 strains and strains 1830, 1833, 1835 and 2020 were selected to represent the low invasive strains (Table 5.2). The genome comparison study using the genome of the selected strains revealed the presence of 93 gene differences between the selected strains. Four genes have been detected in the hight invasive strains but not in the low invasive strains. These genes are, Oacetyltransferase_*OatA* (Cmal_1827_00922) and more three hypothetical_proteins (Cmal_1827_00918, Cmal_1827_00919, Cmal_1827_00007). More investigation using gene-by-gene analysis among selected genomes revealed further significant findings.

Fifty-five recognised genes and 17 hypothetical proteins were detected to have different sequences between high and low invasive strains**.** The comparison of the sequence of these genes exposed some Single Nucleotide Polymorphism (SNP) changes at fewer positions across the sequence of the genes. In the most of these genes, the high invasive strains (656, 1558, 1827, 2018) possess same SNPs change in their genes and similarly the low invasive strains (1830, 1833, 1835, 2020) share same SNPs change. The DNA sequences of virulence related genes were translated using MEGA6 software to check if they have synonymous or non-synonymous SNPs. The analysis revealed that two putative virulence genes which are Betaine aldehyde dehydrogenase (*BetB*) and a protein that specifically targets *CsrB* and *CsrC* (*CsrD*) have non-synonymous SNPs. Figures 5.14, 5.15 and S.5, S.6 showed the DNA sequences and amino acid sequences of *BetB* respectively while figures 5.16, 5.17 and S.7, S.8 showed the DNA sequences and amino acid sequences of *CsrD* respectively. However,

strain 685 showed to have a shorter size of *BetB* gene. Therefore, it was excluded from translational analysis.

Table 5-1 Proposed virulence genes in *Cronobacter* in recent studies.

Gene	Function of gene	Possible role in virulence	Reference
Chaperone Hsp60, *GroEL*	heat shock protein	Involves in the adhesion and invasion of host cells	Du et al., 2015
Molecular chaperone Hsp90, *HtpG*	heat shock protein	Cope with various stress conditions during infection	Du et al., 2015
ATP-dependent chaperone protein, *ClpB*	part of stress-induced multi-chaperone system	Virulence-related functions	Du et al., 2015
GTP-binding protein, TypA	interaction with the ribosomes in a GTP dependent manner	Virulence regulator	Du et al., 2015
Flagellar biosynthesis protein, *FliC*	major protein of bacterial flagella	Plays crucial roles in the adhesion and invasion into host cells	Du et al., 2015
Putative virulence factor, *SrfC*	putative virulence factor	Remain unclear	Du et al., 2015
glpD, encoding glycerol kinase	lipid metabolism genes	Involves in the invasion and adherence of host cells	Jing et al., 2015
glpQ, encoding glycerophosphoryl diester phosphodiesterase	lipid metabolism genes	Involves in the invasion and adherence of host cells	Jing et al., 2015
mltD, encoding membrane-bound lytic murein transglycosylase D	lipopolysaccharide and outer memberance associated genes	Enhances lethality in zebra fish	Jing et al., 2015
lpxP, encoding palmitoleoyl transferase involved in biosynthesis of lipid A	lipopolysaccharide and outer memberance associated genes	Contributes in invasion across blood-brain barrier	Jing et al., 2015
yidC, encoding membrane protein insertase *YidC*	lipopolysaccharide and outer memberance associated genes	Roles in resistance to host cell antimicrobial responses	Jing et al., 2015
eptB, encoding phosphoethanolamine transferase involved in biosynthesis of lipid A	lipopolysaccharide and outer memberance associated genes	Contributes in invasion across blood-brain barrier	Jing et al., 2015
surA, encoding peptidyl-prolyl cis-trans isomerase *SurA*	lipopolysaccharide and outer memberance associated genes	Involved in the maturation of *ompA*	Jing et al., 2015
Osmolarity sensory histidine kinase, *EnvZ*	membranous proteins	Influence bacterial biofilm formation and flagella motility	Ye et al., 2015
LPS-assembly lipoprotein, *LptE*	membranous proteins	Immunogenicity with host cells	Ye et al., 2015
putative multidrug resistance protein, *MdtD*	membranous proteins	Contributes in resistince to host defences	Ye et al., 2015
Osmotically inducible protein *OsmY*	membranous proteins	Associated with virulence behavior	Ye et al., 2015
DNA protection during starvation protein, *Dps*	protection during starvation	Enhance oxidative stress resistance and virulence	Ye et al., 2016
S-ribosylhomocysteinelyase, *LuxS*	synthesis of autoinducer 2 (AI-2)	Contributes in the invasion	Ye et al., 2016
ATP-dependent Clp protease, *ClpC*	stress tolerance	Contributes to adhesion and invasion	Ye et al., 2016
ABC transporter substrate-binding proteins	substrate-binding proteins	Increases virulence gene expression	Ye et al., 2016

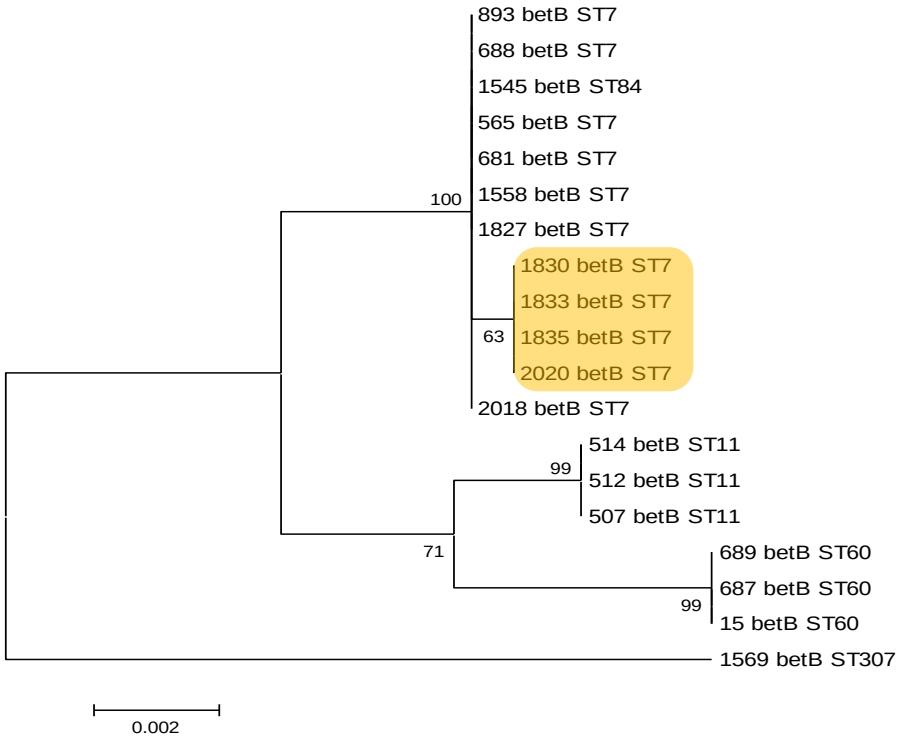


Figure 5-14 A maximum likelihood tree for DNA *betB* gene sequence (1473 bp) for *C. malonaticus* strains.
The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. The four highlighted strains 1830, 1833, 1835 and 2020 are non-invasive strains. The non-highlighted strains invade the used cell lines in different levels.

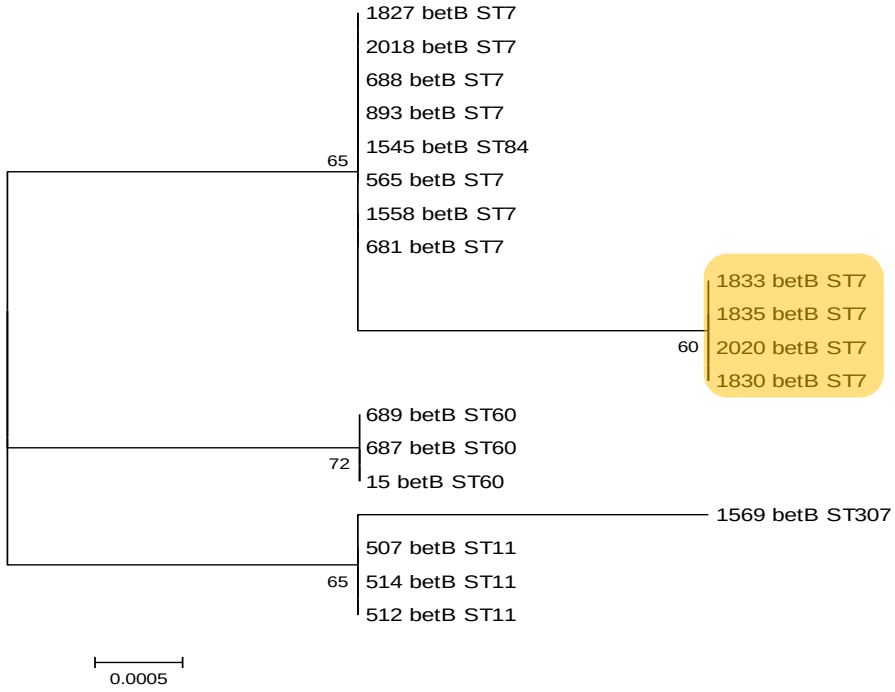


Figure 5-15 Maximum likelihood tree for *betB* amino acid sequence for used strains.
The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. The four highlighted strains 1830, 1833, 1835 and 2020 are non-invasive strains. The non-highlighted strains invade the used cell lines in different levels.

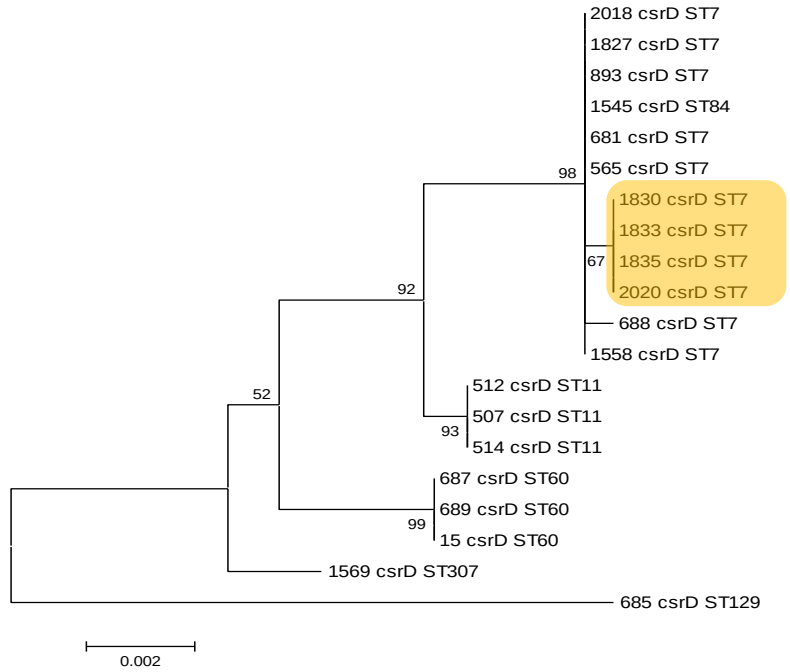


Figure 5-16 Maximum likelihood tree for DNA *csrD* gene sequence (1941 bp) for *C. malonaticus* strains.
The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. The four highlighted strains 1830, 1833, 1835 and 2020 are non-invasive strains. The non-highlighted strains invade the used cell lines in different levels.

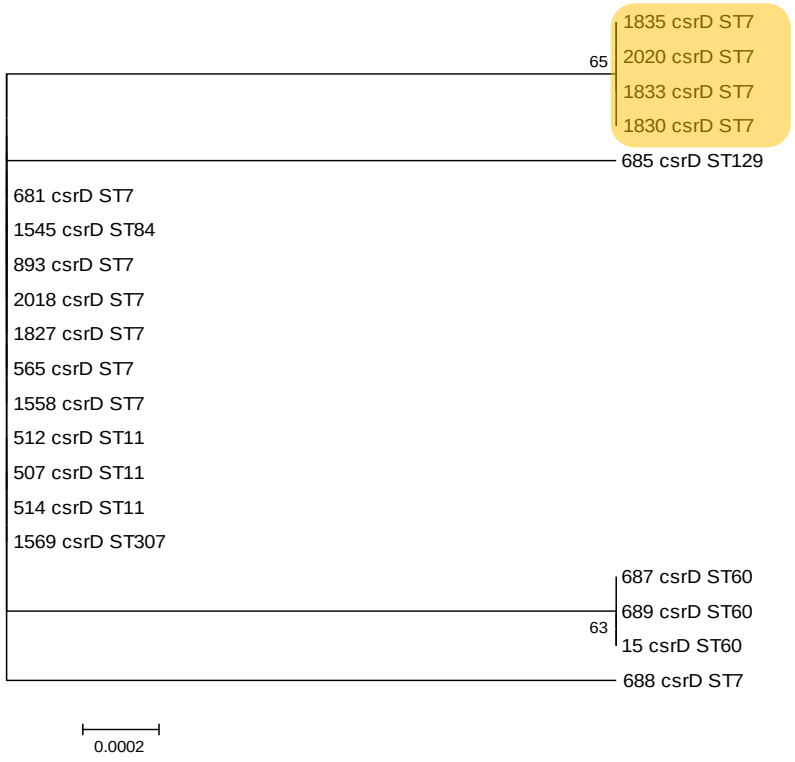


Figure 5-17 a maximum likelihood tree for *csrD* amino acid sequence for *C. malonaticus* strains.
The NTU strains IDs, name of gene and sequence type ST number are shown. The alignment was constructed using MEGA6. The four highlighted strains 1830, 1833, 1835 and 2020 are non-invasive strains. The non-highlighted strains invade the used cell lines in different levels.

5.3.11 Uptake and Persistence into Macrophage Cell Line (U937)

The human macrophages are part of the immune system. They were used to examine the capability of the selected *C. malonaticus* strains to survive, persist and replicate after being engulfed by the phagocytic cells. *C. koseri* and *E. coli* K12 were used as positive and negative controls respectively. After one hour of incubation the bacterial cells were taken up by macrophage cells; however, the uptake was not in the same levels for all tested strains (Figure 5.18). After 24 hours of incubation, five ST7 strains (2018, 1827, 688, 681, and 1558), one ST60 strain (687), one ST128 strain (685) and one ST307 strain (1569) showed an obvious ability to survive and replicate within the U937 cells. After 48 hours of incubation, the survival and replication statuses were not same as after 24 hours, just five of the used strains continued to replicate. The majority of these strains belong to ST7 (2018, 1827, 688 and 681) and one strain belongs to ST307 (1569) ($P<0.05$). Also the ST60 strain (687) showed to be able to persist in the macrophages in the same level as after 24 hours. Strain 685 (ST129) and 1558 (ST7) which showed a dramatic increase after 24 hours to around 40%, decreased also dramatically after 48 hours to less than 20%. One more ST7 strain (893) showed a modest ability to survive and replicate into the U937 cells over the 48 hours. Two more ST60 strains (15 and 689) also showed modest abilities to persist within the macrophage cells over 48 hours of incubation. The rest of used strains were not able to persist or replicate when they were either not taken up or killed. A summary of the results in this chapter is shown in the Table 5.2.

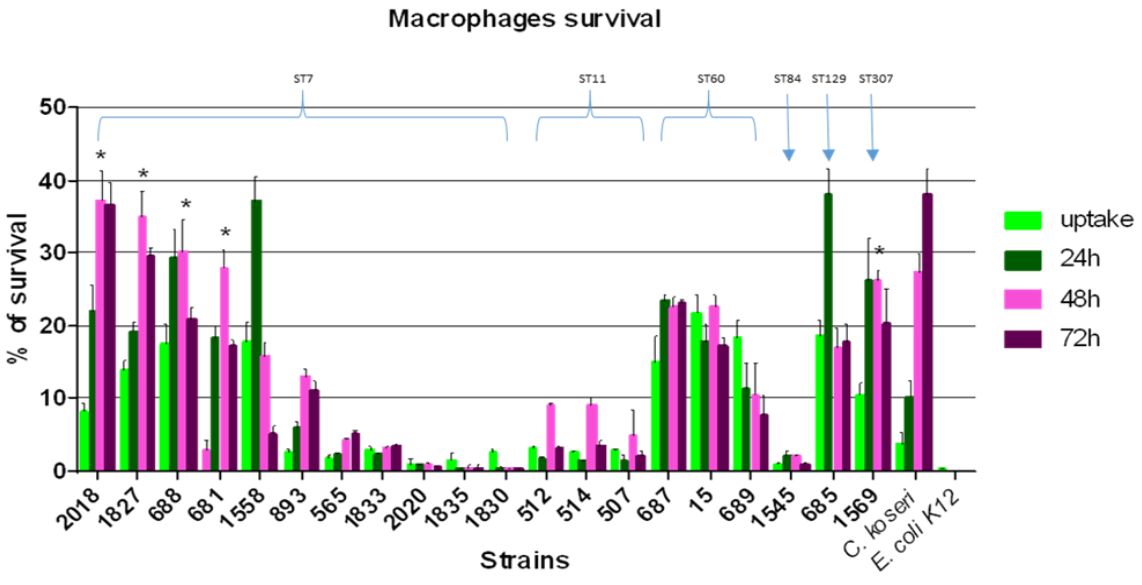


Figure 5-18 Uptake, persistence and replication of *C. malonaticus strains* in U937 cells over 72 hours of incubation. *C. koseri* and *E. coli* K12 were used as positive and negative controls respectively. The survival is shown as % of the initial inoculum. The asterisks above the bars indicate statistically significant differences between the strains in this experiment ($P<0.05$).

5.3.12 Macrophage survival associated genes

A group of genes were found to be associated with bacterial survival into macrophages. The *PhoP*/*PhoQ* regulatory system of *S*. Typhimurium and *Y. pestis* and their regulated genes including *mgtB* and *pmrABE* were shown to play an essential role for survival in the macrophage phagosomes (Ernst et al., 1999; Grabenstein et al., 2006). In addition, *gsrA* of *Y. enterocolitica* has been found to provide protection against oxidative stress killing by macrophages (Yamamoto et al., 1996). *SodA* gene encoding for superoxide dismutase has been also reported to be associated with macrophage survival in *Cronobacter* (Townsend et al., 2007a). This gene involved in resistance to the early oxygen-dependent bactericidal mechanisms of phagocytes in *Salmonella* (Tsolis et al., 1995; Cox et al., 2003). All of these genes have been detected in all used *C. malonaticus* strains regardless of their ability to survive into macrophages.

Four other phagocytosis resistance-associated genes have been investigated in this study. One of these is *YjcC* gene which regulates the oxidative stress response in *Klebsiella pneumoniae* (Huang et al., 2013). In addition, Lu et al. (2010) demonstrated that (d)NTP pyrophosphohydrolase-encoding *mazG* is a potent NTP-PPase and this activity is required to keep the full capacity of the mycobacteria to respond to oxidative stress. Moreover, *betB* gene, which is highly expressed during infections, has been suggested to affect the phagocytic pathway in human phagocytes (Lee et al., 2014). Finally, Mouslim et al. (2004) confirmed the role of *rcsC* gene in macrophage survival for *Salmonella* when they found that its mutant was phagocytized less efficiently by macrophages and it was defective for invasion of non-phagocytic cells and survival within macrophages. All of these genes were also detected in the 20 used strains and more detailes are provided in discussion section.

Table 5-2 Results summary of attachment, invasion and phagocytosis survival of Caco-2, HBMEC, A549, T24 and U937 cell lines.

Strain	ST	Source	Caco-2		HBMEC		A549		T24		Survival in macrophages	Assessment of pathogenicity
			Attachment	Invasion	Attachment	Invasion	Attachment	Invasion	Attachment	Invasion		
1827	7	blood	high	high	high	high	high	very high	high	very high	persisted + replicated	high
2018	7	sputum	high	high	high	high	high	very high	high	very high	persisted + replicated	high
1569	307	blood	high	high	low	moderate	low	moderate	moderate	high	persisted + replicated	high
1558	7	faeces	moderate	low	moderate	high	high	very high	high	very high	persisted	moderate
565	7	faeces	moderate	moderate	low	moderate	low	very high	low	high	low persisted	moderate
681	7	breast abscess	moderate	low	low	low	moderate	moderate	moderate	high	persisted + replicated	moderate
688	7	sputum	high	low	high	low	high	moderate	high	moderate	persisted + replicated	moderate
507	11	faeces	high	low	low	low	low	high	moderate	high	low persisted	low
512	11	clinical	high	moderate	low	low	moderate	moderate	moderate	high	low persisted	low
514	11	clinical	high	moderate	moderate	low	moderate	high	moderate	high	low persisted	low
15	60	faeces	high	low	low	low	low	moderate	moderate	high	persisted	low
687	60	sputum	high	low	low	low	low	moderate	moderate	high	persisted	low
689	60	faeces	moderate	low	low	low	low	moderate	low	high	low persisted	low
1545	84	faeces	high	low	moderate	low	moderate	moderate	moderate	high	low persisted	low
685	129	blood	high	low	high	low	high	moderate	high	moderate	persisted	low
893	7	infant formula	moderate	moderate	low	very low	low	moderate	low	low	persisted	low
1830	7	throat swab	high	not able	low	not able	moderate	not able	low	not able	killed	very low
1833	7	faeces	high	not able	low	not able	high	not able	moderate	not able	low persisted	very low
1835	7	throat swab	high	not able	low	not able	high	not able	moderate	not able	killed	very low
2020	7	faeces	high	not able	moderate	not able	high	not able	low	not able	killed	very low

5.4 Discussion

Cronobacter is an opportunistic pathogen that causes different diseases in all age groups. The ability of bacteria to cause infections depends on the contribution of a number of virulence factors such as attachment, invasion and evading the immune system. In this chapter, the potential virulence of 20 *C. malonaticus* strains was investigated. Five human cell lines, which are Caco-2 cells (representative of intestinal site), HBMEC cells (representative of BBB), U937 cells (representative of immune system), A549 cells (representative of lower respiratory tract), and T24 cells (representative of urinary tract) were used. The first three cell lines were used to investigate the potential ability of *C. malonaticus* to cause NEC, bacteraemia and meningitis. The fourth and fifth cell lines were utilised to investigate the potential ability of *C. malonaticus* to cause pneumonia and UTI, these infections might also lead to systemic infections.

Recent reports have confirmed the involvement of *C. malonaticus* in the neonatal infections and some of these infections were fatal (Hariri et al., 2013; Asato et al., 2013; Brandao et al., 2015; unpublished data China). NEC and meningitis are the most common *Cronobacter* serious clinical features among neonates (van Acker et al., 2001; Hariri et al., 2013; CDC, 2016). The majority of the previous studies were focused on *C. sakazakii* to investigate the ability of *Cronobacter* to cause these infections (Mange et al., 2006; Townsend et al., 2007a; Townsend et al., 2008; Almajed and Forsythe, 2016). However, Giri et al. (2012) used 23 *Cronobacter* strains to investigate their ability to attach and invade the human intestinal INT407 and Caco-2 cells and HBMEC cells, two of these strains were environmental isolates of *C. malonaticus*. In this study 20 clinical *C. malonaticus* strains with different STs were selected to investigate the ability of this species to be potentially able to cause NEC and meningitis. All but one of the used strains were isolated from clinical sites such as blood (685, 1569, 1827), breast abscess (681), upper and lower respiratory tracts (687, 688, 1830, 1835, 2018), faeces (15, 507, 565, 689, 1545, 1558, 1833, 2020) and unknown clinical source (512, 514). The exceptional is ST7 strain 893, which was isolated from PIF and was selected to represent the PIF *C. malonaticus* isolates (Page 63, table 3.5).

The adhesion to the human host cells is an essential step for *Cronobacter* pathogenicity. The ability of 20 *C. malonaticus* strains to adhere to Caco-2 and HBMEC cell lines are shown in Figures 5.2 and 5.5. All *C. malonaticus* strains showed the ability to adhere to the Caco-2 and HBMEC cells, indicating that these strains have virulence potential. The majority of strains adhered to Caco-2 cells at high levels and five strains were moderate in their ability to adhere to this cell line. Some strains such as 688, 1827, 2018 (ST7) and 685 (ST129) showed higher levels of adhesion to HBMEC cells ($P<0.05$). These four strains demonstrated also high levels of attachment to Caco-2 cell line

indicating that these strains could have potential to invade the both cell lines. The obtained results for Caco-2 in this study agreed with the results found by a previous PhD student who used a group of *C. sakazakii* strains (Almajed 2015, unpublished data). However, Townsend et al. (2008) confirmed also the ability of *C. sakazakii* strains to adhere the Caco2, but the adherence levels of these strains were slightly lower than the adherence level that showed by *C. malonaticus* strains in this study. Almajed confirmed also the ability of the *C. sakazakii* strains to adhere to the HBMEC cell line, but the attachment levels for these strains were also slightly lower when compared with *C. malonaticus* attachment level in this study (Almajed 2015, unpublished data). This difference in the adherence ability could be due to the possessing of *C. malonaticus* to some adhesion factors such as curli fimbriae.

Seven fimbrial regions have been detected in the used *C. malonaticus* strains. A curli fimbrial gene cluster (ctu_16160-230) is one of these regions. Curli fimbriae are reported to have an important role for adhesion to the host cells in *E. coli* (Doran et al., 1993; Olsen et al., 1993; Cordeiro et al., 2016). Therefore, this region could be associated with the adhesion to host cells in *C. malonaticus* as well. The ability of *C. malonaticus* to adhere to used cell lines has been confirmed in this study (Figure 5.2, 5.5, 5.8, 5.11) although, there was a variation between tested strains. This variation indicates that not only curli fimbriae was associated with the adhesion activity and there might be other bacterial traits were involved, or some of fimbrial genes were not expressed making the adhesion function of this region not active in low adhesive strains. This required transcriptomic studies to confirm whether these genes are expressed or not.

Attachment is the first step of the interaction of pathogens with the host surfaces after which bacteria can colonise and cause disease by toxin secretion and host cell invasion (Wilson et al., 2002). Therefore, the *C. malonaticus* strains were further investigated for their ability to invade the Caco-2 and HBME cell lines. The ability of *C. sakazakii* to invade the Caco-2 and HBMEC cell lines was assessed in some studies (Townsend et al., 2008; Giri et al., 2012; Almajed and Forsythe, 2016). Their results confirmed the ability of *C. sakazakii* strains to invade these cell lines in different levels. The invasion of neonatal intestinal cells by bacteria such as *Cronobacter* could lead to the occurrence of NEC which is a leading cause of morbidity and mortality in premature infant (Remon et al., 2015). Moreover, once *Cronobacter* cross the human intestinal epithelial cells, they will have access to the blood stream and the central nervous system (CNS) which leads to the occurrence of neonatal bacteraemia and meningitis (Mittal et al., 2009; Lee et al., 2011).

The majority of *C. malonaticus* strains showed ability to invade the Caco-2 cells in different levels (Figure 5.4 and Table 5.2). Although, strains 1830, 1833, 1835 and 2020 which are ST7

demonstrated high level of adhesion to this cell line (Figures 5.2), they were not able to invade this cell line (Figures 5.4). This suggested that there was no correlation between the adherence and invasion ability. Strains 1830 and 1835 were isolated from upper respiratory tract while strains 1833 and 2020 were isolated from faeces. The highest invasion levels were showed by strains 1827 and 2018 (ST7) and strain 1569 (ST307) ($P<0.05$). Interestingly, these high invasive strains 1827, 2018 and 1569 demonstrated same adherence pattern which is localised adherence (LA). This suggests that the adherence pattern might play an important role in the ability of the pathogenic bacteria to invade host cells. Strains 1827 and 2018 were isolated from blood and sputum respectively and were noticed to cluster together in the PFGE analysis in figure 3.4, Chapter 3. Strain 1569 is a fatal *C. malonaticus* strain which was isolated from neonatal blood in the USA. The route of the infection of this strain was proposed to be through contaminated water which could be used in the reconstitution of PIF that fed the CDC fatal case with. Thus the high invasion level into Caco-2 that showed by this strain in this study supports the story that this strain could be ingested with milk and started its infection journey from the intestines. Similar to this strain, strains 1827 and 2018 (ST7) which showed a very high level of invasion into Caco-2 could cause the same infection with the same outcome if they ingested by such infant. Strains 565, 1558 (ST7), 512 and 514 (ST11) showed moderate invasion levels, whereas the other strain showed low invasion levels. There is a strong agreement between the invasion results in this study with the invasion results that obtained by Townsend et al. (2008) and Almajed and Forsythe, (2016) who used *C. sakazakii* strains. This agreement indicates that *C. malonaticus* has ability to invade intestinal cells similarly like *C. sakazakii*.

With regard to the assessment of the ability of *C. malonaticus* strains to invade the HBMEC cells, same strains (1830, 1833, 1835, 2020) that were unable to invade the Caco-2 cells, were also incapable to invade the HBMEC cells (Figure 5.7 and Table 5.2). Noticeably, only one strain, 2020, showed high ability of adherence to HBMEC though it could not invade this cell line. Similar to the invasion result of the Caco-2 cells, same ST7 strains 1827 and 2018 showed very high invasion levels into HBMEC cells ($P<0.05$). In addition, strain 1558 (ST7) showed the highest invasion level among all tested strains ($P<0.01$). This strain displayed intermediate level for Caco-2 invasion and was isolated from faeces. Noticeably, strain 1558 was the only strain displayed completely different adherence pattern which is diffused adherence (DA) to HBMEC, though it showed locolised patteren (LA) pattern to Caco-2 cells. In addition, ST7 strains (1827 and 2018) demonstrated same adherence pattern that shown with Caco-2 cells, LA. Two strains 1569 (ST307) and 565 (ST7) demonstrated also high levels of invasion into HBMEC; however, these two strains showed low adhesion levels to HBMEC cells (Figure 5.4 and Table 5.2). The adherence pattern of CDC 1569 and

656 strain was also LA pattern either to HBMEC or Caco-2 cells. The ability of CDC strain 1569 to invade Caco-2 and HBMEC cells and to tolerate human serum confirms the responsibility of this strain for a fatal case of meningitis in <1-month infant in Wisconsin, the USA. In this study, other *C. malonaticus* strains such as 565, 1558, 1827 and 2018 which belong to ST7 were evidently able to invade both Intestinal cells (Caco-2) and human brain cells (HBMEC). This suggested that these strains can cause similar infections as CDC 1569 strain did. Strains 565 and 1558 were isolated from faeces of unknown age patients and they could cause serious infections especially if they were isolated from infants. Strains 1827 and 2018 were isolated from 76 and 72-year old patients respectively; however, the outcome of them was not reported. These particular two strains, 1827 and 2018, demonstrated a very high ability of invasion into Caco-2 and HBMEC cells as well as an ability to tolerate human serum. This indicates that they could cause serious infections among neonates, particularly who is suffering from impaired immunity. The rest of tested strains showed low levels of invasion into HBMEC. Moreover**,** the invasion results of HBMEC assay were compared to the results obtained by Almajed and Forsythe, (2016) who used *C. sakazakii* strains; however, some *C. malonaticus* strains in this assay showed similar high invasion levels that showed by *C. sakazakii* strains in the comparative study. This indicates that *C. malonaticus* can cause also serious infections such as meningitis similarly like *C. sakazakii*.

Cronobacter has involved in many other infections such as pneumonia and UTI (Lai, 2001; Bhat et al., 2009; Healy et al., 2010). However, the ability of *Cronobacter* to adhere and invade the respiratory and urinary cells was not investigated before. The A549 respiratory cell line and the T24 urinary cell line were used to investigate the ability of 20 *C. malonaticus* to adhere and invade these cells for the first time in this study. Figure 5.8 and 5.11 showed the assessment of the adherence abilities of *C. malonaticus* strains to A549 and T24 cells. All tested strains showed ability to adhere A549 cells in different levels (Figure 5.8). Six strains showed adherence abilities higher than positive control ($P<0.05$), five of these strain are ST7 (688, 1558, 1827, 1835, 2018) and one strain is ST129 (685). Three of these strains (688, 1835 and 2018) were isolated from lower respiratory tract, while two strains (1827 and 685) were isolated from blood and one strain (1558) was isolated from faeces. The majority of these six strains displayed LA pattern to A549 cells, strain 685 showed AA pattern and strain 1835 showed NSA pattern. Other two ST7 strains which are 1833 and 2020 showed high level of adhesion comparing with reference strains, whereas the other strains displayed moderate levels of adherence. With referencing to the adhesion to the T24 cells, all tested strains demonstrated an ability to attach to this cell line (Figure 5.11). Strains 685 (ST129), 688, 1558, 1827, 2018 (ST7) showed the highest levels of adhesion among *C. malonaticus* strains ($P<0.05$). These five strains showed also very high adhesion levels in A549 adhesion assay. Interestingly, the same

adherence patterns were demonstrated by these high adhesive strains in both cell lines. Strain 685 ST (129) showed AA pattern while strains 688, 1558, 1827 and 2018 (ST7) showed LA pattern. Low levels of attachment were showed by strains 565, 893, 1830, 2020 (ST7) and 689 (ST60), whereas the rest of strains were moderate in their adhesion levels. Interestingly, the values of adhesion to T24 cells, which derived from human bladder, was very high comparing with A549 cells. This was noticed also in a study by Martinez et al. (2000) when *E. coli* adhere very well to bladder epithelial cells (about 50 %). The invasion values were also very high comparing with other used cell lines. *E. coli* and *C. freundii* showed very high invasion values when bladder cell lines were used; however, same bacteria showed also very low values of invasion, comparing to T24 cells, when Caco-2 cells were used (Meier et al.,1996; Martinez et al., 2000).

As the tested strains showed a clear ability to adhere the A549 and T24 cells, the gentamicin protection assay was conducted to assess the invasion ability of same strains. Not all strains that highly attached were highly invasive and vice versa. As shown in figure 5.10, four ST7 strains (1830, 1833, 1835 and 2020) were not able to invade the A549 cells, these strains showed same ability in the previous invasion assays using Caco-2 and HBMEC cells. In contrast, other four ST7 strains (1558, 1827, 2018, 565) showed the highest levels of invasion when the invasion levels were equal or even higher than the positive control ($P<0.05$). Interestingly, strains 1558, 1827, 2018 and 656 demonstrated LA adhesion pattern to A549 cells, suggesting once again that the type of adherence might affect the invasion abilities. It is obvious that strain 565 which showed high invasion level was moderate in its ability to attach the A549 cells. In addition, strains 507 and 514 which belong to ST11 showed high invasion level, though their attachment levels were moderate. Other strains such as strains 685 and 688 attached to A549 cells at high level; however, their invasion levels were moderate. These observations confirm again that there was no direct correlation between adhesion and invasion abilities. The remaining strains showed moderate invasion levels. These results confirm the ability of *Cronobacter* in general and *C. malonaticus* in particular to cause pneumonia. According to this, lower respiratory isolates such as 687, 688 and 2018 might be isolated from patients who were infected with pneumonia. However, the clinical presentations and the final outcomes for these patients are not available. Also of importance is that, the ability of *C. malonaticus* to adhere and invade the respiratory cells A549 in this study is consistent with the reports that have already showed the responsibility of *Cronobacter* for a considerable number of pneumonia cases (Healy et al., 2010; Patrick et al., 2014). Also in Chapter 3, figure 3.5, about 78 % of the used clinical collection was isolated from upper and lower respiratory tract and about 55% was isolated from the lower respiratory site.

Although, none of tested strains were isolated from urinary tract, the majority of these strains showed the ability to invade the T24 urinary cell line (Figure 5.13). The urinary cell line T24 was used based on the fact that the UTI is the most common infection caused by *Cronobacter* and these infections count more than 60% among elderly people (Patrick et al., 2014). Nevertheless, the same four ST7 strains (1830, 1833, 1835 and 2020) that were not able to invade the previous cells showed no ability to invade T24 cells too. In contrast, three ST7 strains (1558, 1827, 2018) which showed very high level of invasion into A549 showed also to be the highest invasive strains into T24 cell line ($P<0.01$). These three strains showed also same adhesion pattern which is LA. This pattern of adhesion, LA, was demonstrated by the majority of high invasive strains into all used cell lines, these results indicate there could be a relationship between the adhesion patterns and invasion of the epithelial cells. Certainly, Livrelli et al. (1996) have found that localized-adhesion isolates were involved in severe infections. All strains which belong to ST11, ST60, ST84 and ST307 showed high ability to invade the T24 cells when compared with reference strains ($P<0.05$). Similarly, ST7 strains 681, 565, 688 showed high level of invasion into the T24 cells ($P<0.05$), whereas strain 893 showed moderate levels of invasion. *C. malonaticus* strains clearly demonstrated an ability to adhere and invade the T24 cells which indicates that they are causative agents of UTI if they have an opportunity to colonise urinary tract.

A group of invasion-associated genes such as *ompA*, *ompX*, *apaH*, *ygdP*, *ppk1*, *ibeB* and *Hfq* were examined for their presence in the used *C. malonaticus* genomes. Kim et al. (2010) proposed that outer membrane protein A (*ompA*) and outer membrane protein X (*ompX*) could play a crucial role in the invasion of the human intestinal cells (Kim et al., 2010). *OmpA* protein has been found also to promote the invasion ability to HBMEC *in vitro* (Singamsetty et al., 2008; Mohan Nair et al., 2009). The association of *ygdP* and *apaH* in bacterial invasion was tested by Ismail et al. (2003). The disruption of these genes individually or together in *S.* Typhimurium clearly reduced the ability of the intracellular invasion of human HEp-2 epithelial cells (Ismail et al., 2003). Furthermore, *ygdP* gene in *E. coli* K1 suggested to has a role in the invasion of the HBMEC cells (Badger et al., 2000). Polyphosphate kinase 1 (PPK1), which is an enzyme that catalyse the polymerisation and degradation of polyphosphate chains resulting in producing energy in the form of ATP (Achbergerova and Nahalka, 2014) is another gene involve in bacterial invasion. It has been found that the deletion of *PPk1* in *E. coli* K1 led to a decrease in the attachment and invasion of HBMEC (Peng et al., 2012). *IbeB*, which is synonymous to *cusC* in *Cronobacter*, has been found to contribute in the BBB invasion in *E. coli* K1 (Wang et al., 2012). Kucerova et al. (2010) have identified the complete cation efflux system *cusA*, *cusB*, *cusC*, *cusF*, and its regulatory gene *cusR* in *Cronobacter* isoltaes that involved in neonatal infections (Kucerova et al., 2010). The role of *Hfq*, which is known

as an RNA chaperone, in the invasion ability of pathogenic bacteria is also confirmed in several studies. The deletion of this gene reduced the invasion ability in *E. coli* and *Salmonella* (Simonsen et al., 2011; Sittka et al., 2007). In addition, Kim et al. (2015) investigated the role of *hfq* in *C. sakazakii*, their result confirmed the role of this gene in the invasion ability of *C. sakazakii* into Caco-2 cells. In the absence of *Hfq*, the invasion, survival and hydrogen peroxide resistance ability of *C. sakazakii* was attenuated. Therefore, it was suggested that *hfq* participates in the regulation of several genes that involve in the virulence of *C. sakazakii* (Kim et al., 2015). All *C. malonaticus* strains in this study (n=20) were confirmed positive for *ompA*, *ompX*, apaH, *ygdP*, *ppk1*, *ibeB* and *Hfq* though these strains vary in their invasion ability into Caco-2, HBMEC, A549 and T24 cells (Figure 5.4, 5.7, 5.10, 5.13). This variation indicates that these genes may not contribute in invasion ability of *C. malonaticus* or that some of these genes are silent in some strains.

Interestingly, a recent study by Du et al. (2015) used proteomic analysis identified about 89 proteins; however, their analysis suggested the involvment of 11 proteins in the pathogenicity of *C. sakazakii*. Six of these proteins are detected in the *C. malonaticus* strains in this study (Table 5.1). These proteins are; chaperone Hsp60, GroEL; molecular chaperone Hsp90, *HtpG*; ATP-dependent chaperone protein, *ClpB*; GTP-binding protein, TypA; Flagellar biosynthesis protein, *FliC*; Putative virulence factor, *SrfC* and *TerADEXZ* (Du et al., 2015). The *TerADEXZ* which are known as tellurium resistance associated genes, were not detected in the *C. sakazakii* isolates that were associated with neonatal meningitis (Joseph et al., 2012a), which indicates that these genes may not essential in pathogenicity of *Cronobacter*. Another study by Jing et al. (2015) determined the transcriptome profile of *C. sakazakii* ATCC BAA-894 after being interacted with HCT-8 human colorectal epithelial cells (Jing et al., 2015). In this study, lipid metabolism genes such as *glpD*, encoding glycerol kinase and *glpQ*, encoding glycerophosphoryl diester phosphodiesterase, were upregulated (Table 5.1). In addition, lipopolysaccharide and outer memberance associated genes such as *mltD*, encoding membrane-bound lytic murein transglycosylase D, *lpxP*, encoding palmitoleoyl transferase involved in biosynthesis of lipid A, *yidC*, encoding membrane protein insertase *YidC*, *eptB*, encoding phosphoethanolamine transferase involved in biosynthesis of lipid A, and *surA*, encoding peptidyl-prolyl cis-trans isomerase *SurA* were also upregulated (Jing et al., 2015). More novel potential virulence factors that inviolve in pathogenicity, of *Cronobacter*, such as adhesion and invasion have been identified using proteomic analysis by Ye et al. (2015;2016). In 2015, membranous proteins such as *envZ*, *lptE*, *MdtD* and *OsmY* (Table 5.1), were upregulated in the virulent *C. sakazakii* isolate but not in the attenuated strain (Ye et al., 2015). In 2016, they also identified more four potential virulence factors (Table 5.1), DNA protection during starvation protein *Dps*, S-ribosylhomocysteinelyase *LuxS*, ATP-dependent Clp protease *ClpC*, and ABC transporter substrate-

binding proteins, which are thought to involve in the virulence of *C. sakazakii* through enhancing bacterial adhesion, invasion as well as tolerance to environmental stresses and host immune response (Ye et al., 2016). The upregulated genes that mentioned in Du et al. (2015), Jing et al. (2015) and Ye et al. (2015,2016) studies have been also detected in the *C. malonaticus* strains in this study. These genes could be also upregulated during the interaction of *C. malonaticus* with the used cell lines and subsequently contribute in thier pathogenicity, particularly adhesion and invasion. Therefore, proteomic analysis using *C. malonaticus* strains from this study are required to reveal more facts regarding the pathogencity of *C. malonaticus*.

More interesting is that the variation of invasion ability of used strains was noticed even among same STs. For example, among ST7 strains, strains 565, 1558, 1827 and 2018 showed to be high invasive strains into all cell lines (Caco-2, HBMEC, A549 and T24). In contrast, ST7 strains 1830, 1833, 1835 and 2020 were not able to invade the same cell lines. Therefore, these ST7 strains were selected for further genomic investigations in order to explore any difference in their virulence genes.

The genome search of the selected strains revealed the presence of about 93 gene differences between the high and low invasive strains. Four genes have been detected in the highest invasive strains but not in low invasive strains. These genes are, O-Acetyltransferase_*OatA* (Cmal_1827_00922) and more three hypothetical_proteins (Cmal_1827_00918, Cmal_1827_00919, Cmal_1827_00007). The role of *OatA*, in bacterial pathogenicity have been investigated in several studies. *OatA*, a Peptidoglycan O-Acetyltransferase, was confirmed to have a role in bacterial pathogenicity such as limiting innate immune responses and promoting bacterial survival in the infected host (Aubry et al., 2011). These four genes could play an important role in the pathogenicity of the *C. malonaticus* strains and they could have played an essential role in the ability of ST7 high invasive strains to invade the used cell lines.

The gene by gene investigations among selected genomes revealed further significant findings. Fifty-five recognised genes and 17 hypothetical proteins were detected to have different DNA sequences inside high and low invasive strains. The comparison of the sequence of these genes exposed some Single Nucleotide Polymorphism (SNP) changes at fewer positions across the sequences of the genes. In the most of these genes, the high invasive strains (656, 1558, 1827, 2018) possess same SNPs change in their genes and similarly the low invasive strains (1830, 1833, 1835, 2020) share same SNPs change. These SNPs can cause a variation in the resulting structure of the protein which may affect their functional characteristics. In this study, such alteration in functional properties of a protein may enhance the virulence potential of the high invasive *C. malonaticus*

strains. The DNA sequences of virulence related genes were translated using MEGA6 software to check if they have synonymous or non-synonymous SNPs. The analysis revealed that two putative virulence genes which are betaine aldehyde dehydrogenase (*BetB*) and protein that specifically targets *CsrB* and *CsrC* (*CsrD*) have non-synonymous SNPs (figures 5.14 - 5.17, S.6, S.8).

Betaine aldehyde dehydrogenase (*BetB*) is an efficient osmotic regulator which contributes in catalyzing the oxidation of betaine aldehyde to glycine betaine (Kempf and Bremer, 1998). Some studies have suggested the role of *BetB* during invasion, intracellular trafficking, and multiplication in the host cells (Velasco-García et al., 2005; Lee et al., 2014). The upregulation of this gene has been detected in *C. sakazakii* after the intraction of this organism with human intestinal cells HCT-8 (Jing et al., 2015). In this study *BetB* might be also upregulated by high invasive strains and played a role during the intraction of *C. malonaticus* with used cell lines.

The activity of carbon store regulator A (*csrA*), the global regulatory protein that involve in regulation of several genes encluding virulence genes, is regulated by two noncodons RNAs, *CsrB-C*. However, in *E. coli* the global regulatory RNAs *CsrB-C* require GGDEF-EAL protein (*CsrD*) for their decay through an RNase E-mediated pathway. It is likely that the *CsrD*–RNase E-mediated decay pathway operates in many Gram-negative bacteria and broadly influences metabolism, motility, quorum sensing, interactions with host cells, and virulence factor expression (Suzuki et al., 2006; Vakulskas et al., 2015). Therefore, *CsrD* could also play similar role in *C. malonaticus* which is resulting in affecting of several traits including its pathogenic characteristics. Thus the difference in the sequence of these genes, *betB* and *csrD*, between high and low invasive strain could have a role in the difference of their ability to invade the used cells.

The majority of *C. malonaticus* strains were able to persist within human macrophages over 72 hours of incubation (Figure 5.18 and Table 5.2). Four ST7 *C. malonaticus* strains (1830, 1833, 1835 and 2020) were either not taken up or they were rapidly killed following macrophage uptake. These strains showed also no ability to invade Caco-2, HBMEC, A549 and T24 cell lines. Potential pathogenic bacteria show ability to survive and multiply within macrophages. ST7 strains 681, 688, 1827 and 2018 in addition to 1569 (ST307) were able to survive and multiply significantly during the 48 hours compared to other *C. malonaticus* strains ($P<0.05$). These five strains were isolated from breast abscess, sputum, blood, sputum and blood respectevily. This suggests that these particular strains possess virulence mechanisms to tolerate the bactericidal activities within human macrophage cells and use them as a vehicle to invade the other organs of human body. Our results particularly for strains 1827, 2018 and 1569 correlate the virulence potential of these strains with the attachment and invasion, as they were invasive strains for both Caco-2 and HBMEC cell lines.

This suggests that these strains could be able to establish a successful infection and has the required tools for this process including overcoming phagocytic killing. Interestingly, strain 1569 is a clinical strain that was responsible for a fatal meningitis infection in the USA (Hariri et al., 2013). The macrophage survival findings in this study are the first confirmation of the ability of *C. malonaticus* to survive and multiply within macrophages. However, previous studies investigated and confirmed the ability of *C. sakazakii* strains to survive and replicate within human macrophages (Townsend et al., 2007a; Townsend et al., 2008; Almajed and Forsythe, 2016).

The survival and multiplication of *C. malonaticus* within macrophages help these bacteria to use phagocytic cells as a vehicle to reach other body organs such as the human brain. Phagocytic cells are attracted by some of cytokines which are secreted from infected tissue accordingly the infected tissue become permeable. This allows the phagocytic cells, which contained the invading organism, to migrate to the infection site and subsequently help to increase the number of bacteria. Moreover, *C. malonaticus* strains 1827, 2018 and 1569, which showed ability to invade Caco-2 and HBMEC celles and showed ability to survive and multiply within macrophages, were also serum resistant (Figure 4.12). The presence of these traits could enhance the ability of these strains to avoid the host immune response and cause bacteraemia, which finally could help them to migrate through the BBB endothelial cells and cause meningitis.

Several intramacrophage survival associated genes have been detected in the genome of the 20 *C. malonaticus* strains. The *PhoP*/*PhoQ* regulatory system of *S.* Typhimurium and *Y. pestis* and their regulated genes including *mgtB* and *pmrABE* were shown to play an essential role for survival in macrophage phagosomes (Ernst et al., 1999; Grabenstein et al., 2006). *PhoP*/*PhoQ* belong to a family of regulatory proteins which is affected by the environmental change to activate the transcription of genes through phosphotransfer machanism. Thus *PhoQ* is an environmental sensor while *PhoP* is a transcriptional activator. In the low Mg^{2+} concentration environment, *PhoQ* promotes the phosphorylated state of the *PhoP* protein which directly regulates the transcription of several genes that are necessary for the pathogenicity of *Salmonella* including macrophage survival and other physiological activities in *Salmonella* (Vescovi et al., 1997; Groisman, 2001). Genes such as *mgtA* and *mgtB* which are encoding Mg^{2+} transporters are also regulated by *PhoP*/*PhoQ* system which allow bacteria to grow at limited concentrations of Mg^{2+} (Soncini et al., 1996). At low pH the PhoQ-mediated *PhoP* phosphorylation is also occured, and transcription levels of the *PhoP*-activated genes are increased in acidic conditions (Perez and Groisman, 2007; Prost et al., 2007). Moreover, antimicrobial peptides promote also the expression of a subset of the *PhoP*-regulated genes through the *PhoP* and *PhoQ* proteins (Bader et al., 2005). In *Salmonella*, the *pmrA*/*B*, which are *PhoP*-regulated genes, play also a role in the modification of the outer

membrane structure by changing the structure of the lipid A of LPS to provide protection against the effect of antimicrobial peptides (Ernst et al., 1999).

Y. pestis require also the *PhoP*-regulated genes for modification of LPS and adaptaion of low Mg^{2+} levels which mediates the survival into macrophages. These genes involve also in preventing the delivery of cathepsin D, an acid-acting macrophage lysosomal protease that kill bacteria, to its vacuole in macrophages (Bewley et al., 2011). In addition, it has been shown that *gsrA* (global stress requirement) of *Y. enterocolitica* provide protection against oxidative stress killing by macrophages (Yamamoto et al., 1996). It is a stress protein that act to prevent the accumulation of the abnormal stress peptides which form toxin effect in the periplasmic space. It has been shown that the mutation of the *gsrA* gene in *Y. enterocolitica* made this organism unable to survive within macrophages due to being sensitive to the oxidative stress actions into macrophages (Yamamoto et al., 1996). Moreover, superoxide dismutase, *SodA,* gene is involved in protection of bacteria against phagocytosis when the first contact occurs at the surface of the phagocyte (Tsolis et al., 1995; Cox et al., 2003).

For *C. malonaticus*, it could be proposed that the actvation of *PhoQ* after exposure to low Mg^{2+} concentration as a phagocytic defense mechanism, oxidative stress and low pH could lead to the phosphorylation of *PhoP*. This will activate the expression of *PhoP*-regulated genes such as *pmrABE* and *mgtB*. In this stage *C. malonaticus* will have an opportiunity to acquire Mg^{2+} and avoid the killing action of antimicrobial peptides. This will be accompanied by more protection against oxidative stress killing action as *C. malonaticus* harbour the *gsrA* and *sodA* which are responsible for superoxide dismutase production. Such complete protection mechanism might be used by *C. malonaticus* to survive the phagocytic killing action of macrophages. However, in this study all tested strain were positive for the genes that are required for the intramacrophage survival. Nontheless, some strains were killed directly after being uptaken and displayed low survival and replications within macrophages. These strains might have contained genes which are not functional genes or other genes could contribute in the intramacrophage survival. Therefore, the previous proposed mechanism required further investigation particularly at the functional genome level by using transcriptomic studies to confirm whether these genes are expressed or not.

Four other phagocytosis resistance-associated genes have been investigated in this study. One of these is *YjcC* gene which regulates the oxidative stress response in *Klebsiella pneumoniae* (Huang et al., 2013). In addition, Lu et al. (2010) demonstrated that (d)NTP pyrophosphohydrolase-encoding *mazG* is a potent NTP-PPase and this activity is required to keep the full capacity of the mycobacteria to respond to oxidative stress. Bacterial susceptibility to oxidative stress could

contribute to reduced virulence and intramacrophage survival of *Cronobacter* (Townsend et al., 2007a**).** However, deletion of *mazG* in *Mycobacterium tuberculosis* reduced survival in macrophages as well as in the spleen of infected mice (Lyu et al., 2013). Moreover, *betB* gene, which is highly expressed during infections, has been suggested to affect the phagocytic pathway in human phagocytes (Lee et al., 2014). Transcriptome analysis of *C. sakazakii* ATCCBAA-894 by Jing et al. (2015) revealed the upregulation of *betB* after the ineraction with intestinal epithelial cell line HCT-8. Finally, Mouslim et al. (2004) confirmed the role of *rcsC* gene in macrophage survival for *Salmonella* when they found that its mutant was phagocytized less efficiently by macrophages and it was defective for invasion of non-phagocytic cells and survival within macrophages. Accordingly, the *mazG*, *BetB* and *yjcC* could be present as active genes in strains 681, 688, 1569, 1827 and 2018, whereas *rcsC* gene which attenuated the virulence of *Salmonella* could be present as a silent gene in these strains.

Chapter 6 Conclusions and future directions

6.1 Conclusion

Cronobacter malonaticus is a member of the genus *Cronobacter* which is considered an opportunistic pathogen. *C. malonaticus* has been recognised to be more associated with adult infections, whereas the closely related species *C. sakazakii* has been reported to be responsible for several serious neonatal infections. Therefore, research has been focused on *C. sakazakii* to investigate its physiological and virulence traits. However, the significance of *C. malonaticus* has recently increased since it was documented to be involved in several severe neonatal infections. Unlike previous *Cronobacter* studies, this study is mainly focused on *C. malonaticus* to investigate its physiological and virulence characteristics that enable this species to survive different stresses and cause adult and neonatal infections.

- In the first part of this study a unique clinical collection of *Cronobacter* strains were used. The collection has been isolated from about eight different departments in two hospitals of the Czech Republic and isolates were collected from different clinical sites over a 6-year period. As the majority of the collected strains were isolated from adults, it was hypothesised that most of these strains could possibly be *C. malonaticus*. Also some strains were collected from the same department, thus it is worthy to study if there is any relatedness between strains. Therefore, a number of phenotypic and genotypic identification techniques have been applied to answer some questions such as 1) is *C. malonaticus* the predominant species among this collection, 2) is there any link between age group and a specific species, 3) is there any relatedness between strains, and finally 4) the study will find the effectiveness of each technique.

  The use of some media such as TSA, VRBGA and DFI in laboratories does not give a complete reliable identification; however, they are still essential for growing and isolation of different bacteria and also for minimising the possible identification of microorganism in this level. Other phenotypic methods which have been applied in this study such as API ID32E showed also some limitations that confuse the user to reach a correct identification and could not speciate the strains. Therefore, genotypic methods such as *rpoB*, MLST, O-antigen and PFGE were applied. The 51 strains were predominated by *C. sakazakii* ST4 (63 %, 32/51) and *C. malonaticus* ST7 (33 %, 17/51). These had been isolated from throat and sputum samples of all age groups, as well as rectal and faecal swabs. There was no apparent relatedness between the age or gender in patients and the *Cronobacter* species. There was almost

complete agreement between O-antigen typing and *rpoB* gene sequence analysis and MLST profiling. The majority (42/51) of strains were from the respiratory system (i.e. throat swabs and sputum samples) and only three were from faeces, two from wound, one from blood and one from urine. Despite the high clonality of *Cronobacter*, PFGE profiles differentiated strains within each sequence type into 15 pulsotypes. This study shows the value of applying MLST to bacterial population studies with strains from two patient cohorts, combined with PFGE for further discrimination of strains.

- The second part of the study focused on comparative physiological and virulence related tests of 20 strains of different sequence types of *C. malonaticus*. Six ST7 strains were selected to represent the 5 *C. malonaticus* pulse types of the first part of this study and an additional 14 *C. malonaticus* strains were chosen from the *Cronobacter* PubMLST database from different countries to represent different STs.

As shown in figure 4.1 and 4.2 temperature was a crucial factor in biofilm formation, in addition to nutrient type present in milk is important in enhancement of biofilm formation. Capsules, cellulose and curli fimbriae were not confirmed to have a role in biofilm formation. Just four *C. malonaticus* strains, which are 1830, 1833, 1835 and 2020 were not able to show any movement activity. These strains share same DNA nucleotides and amino acid sequences for *flhC* gene.

In general, though there was a variation in capsule production between used strains, production of capsule was enhanced when IF was used. These findings may confirm the effect of ingredients of different medium on production of bacterial exopolysaccharides. The production of such capsular materials could be important in desiccation persistence, serum resistance and macrophage evasion (Ogrodzki and Forsythe, 2015). All *C. malonaticus* showed an ability of producing cellulose in different amounts. Although *C. malonaticus* possesses curli fimbriae associated genes not all *C. malonaticus* strains were able to express the curli fimbriae.

The majority of *C. malonaticus* strains showed the ability to survive low pH (3.5 pH) over 2 hours of incubation. Just ST60 strains declined by more than 2 logs at the last time point of incubation. While just one strain, 685, which belongs to ST129 showed no ability to survive directly after 30 minutes. Although the *ompR* and *rpoS* genes were detected in all *C.*

malonaticus, strain 685 showed to have different nucleotide sequence of these two genes. This difference could effect the function of these genes and thus this strain become less tolerant to the acidic environment.

The recovery of sublethally injured *C. malonaticus* 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. Interestingly, the lowest recovery of injured cells was showed by strain 685. Exceptionally, this strain was not able to survive acidic stress after 30 minutes. This strain was shown to have different nucleotides sequence of two genes, *ompR* and *rpoS*, that associated with stress response. In general, *C. malonaticus* has been frequently isolated from PIF and other dry and desiccated environments (*Cronobacter* MLST Pub Databases) which suggest that *C. malonaticus* can survive the dry conditions. Such a trait could make this microorganism a potential pathogen from dry sources such as PIF.

Some metals such as copper, zinc, iron, nickel and cobalt, manganese are essential in the structure of bacterial proteins; nonetheless these heavy metals could be toxic for bacteria even at low concentrations (Osman and Cavet, 2011). All tested strains showed an ability to tolerate or adapt the majority of the used metals at different concentrations. Nevertheless, *C. malonaticus* strains were sensitive to sodium tellurite at all used concentrations. This susceptibility was explained when all strains were confirmed in this study to lack the genes associated with tellurite resistance, *terACDYZ*. Pathogenic bacteria have an ability to control heavy metal homeostasis which help them to survive the killing action of macrophages. The control of heavy metals such as copper and zinc are involved in the ability of pathogenic microorganisms to survive into macrophages (Reva and Bezuidt, 2012). Noticeably, not all strains that showed ability to resist or adapt heavy metals were able to survive macrophages. This is more likely due to contribution of different factors in such activity. The iron acquisition system encoded in the genome of the sequenced of the *C. malonaticus* strains, along with the ability of these strains to produce iron siderophores *in vitro*, are important to maintain its growth *in vitro*. This might also enhance its growth *in vivo,* and enable *C. malonaticus* to acquire iron in iron-limited environments in the human body which will support its growth and pathogenicity.

Gram-negative bacteria which cause systemic infections need to overcome the mechanisms of killing by serum components and the action of the human complement in the human serum. In this study, despite the fact that some *C. malonaticus* strains were sensitive to human serum as well as rapidly killed by phagocytes, some other strains such as 688, 1827, 2018 (ST7) and 1569 (ST307) were serum tolerant and were able to survive and multiply within macrophages in the laboratory assays. This allows *C. malonaticus* to enhance their ability to grow in the bloodstream and thus this could be an advantage of causing bacteremia. Moreover, *C. malonaticus* was able to produce haemolysins and protease. The presence of such virulence factors indicates the possible occurrence of serious destruction in the human tissue during infections.

Though *C. malonaticus* strains harbour multiple drug resistance operon, *mar*, *C. malonaticus* strains showed susceptibility to quinolones, aminoglycosides and carbapenems groups. In contrast, all strains were resistant to tetracycline and 40 % of them were resistant to chloramphenicol. About 35% of *C. malonaticus* strains showed intermediate resistance to cefotaxime which is one of the third-generation of cephalosporins, related to penicillin.

Plasmid profiling was performed, using simpe experiment, on 20 *C. malonaticus* strains including two *C. sakazakii* strains BAA-894 and 6, which used as a reference marker and a negative control respectively. All of the strains contain at least one plasmid. However, because of the limitations of the experiment, it was impossible to determine the numbers and sizes of the plasmids.

- The third part of this study concentrated on the pathogenicity of the same strains that were used in the second part. The results in Figures 5.4, 5.7, 5.18 and Table 5.2 confirm that *C. malonaticus* is potentially capable of causing serious infections such as NEC, bacteraemia and meningitis. Two ST7 strains which are 1827 and 2018 were always high in their ability to invade and survive the Caco-2, HBMEC and macrophage cells (Table 5.2). Moreover, these two strains were resistant to human serum as shown in Chapter 4, resulting in bacteremia. These strains were isolated from blood and sputum respectively and were clustered together when PFGE was performed in Chapter 3. The CDC strain 1569 (ST307) which was isolated from the blood of a fatal neonatal case showed also significant results when it was able to invade Caco-2 at high level, invade HBMEC at moderate level and

survive the human macrophages over the 72 hours of incubation and replicate during the 48 hours (Table 5.2). This strain showed also moderate levels of serum resistance in Chapter 3. The result of the CDC strain 1569 confirm its responsibility for the fatal neonatal case in the USA.

Table 5.2 showed a summary of the ability of the *C. malonaticus* strains to adhere and invade the A549 respiratory and the T24 urinary cells. The two ST7 strains 1827 and 2018 which showed significant results when tested against Caco-2, HBMEC and macrophages showed also notable invasion results when they tested against A549 and T24. In addition, strain 1558 (ST7) showed also remarkable invasion levels into A549 and T24 cells (Table 5.2). However, majority of the tested strains showed higher ability to invade respiratory and urinary cells than other used cells. This might explain why *C. malonaticus* more associated with adult infections than neonatal infections. It's noteworthy that four ST7 strains (1830, 1833, 1835 and 2020), which were not motile and not able to produce any capsular materials, were always not able to invade the used cell lines or survive the macrophages, although they were isolated from throat and faeces. This indicates that *C. malonaticus* could colonise the human intestine and throat without having abilities to cause infections, presuming that they inhabit the human body as normal flora.

Finally, the results of this study proved the ability of *C. malonaticus* to overcome several stresses that could be faced either in the general environments such dry and acids or in host body such as acids, action of serum and toxic effects of metals. In addition, *C. malonaticus* showed a great ability to invade and survive different human cell lines. These findings, taken together, confirm the potential of *C. malonaticus* to cause serious infections in neonates or adults.

6.2 Future directions

This study is the first research focused on *C. malonaticus*; it has extended our knowledge about physiological and virulence traits of *Cronobacter* in general and provides a new knowledge about *C. malonaticus* in particular. However, more researches should be continued on this species include the following areas.

- Performing gene expression study such as RNA-Seq transcriptome analysis of some genes that thought to be responsible for physiological and virulence traits and to confirm the presence of silent genes.

- Performing proteomic studies to investigate the synthesis of virulence proteins.

- Detection of pro-inflammatory IL-1β, TNF-α, IL-6, and IL-8 and anti-inflammatory IL-10 and IL-4 cytokines, and NF-KB production by Caco-2 and HBMEC cells on high and low invasive *C. malonaticus* exposure.

- Assessment of the ability of the high invasive *C. malonaticus* to translocate through different cell lines such as Caco-2 and HBMEC.

- Study of apoptosis in vitro due to *C. malonaticus*, as this is an important mechanism contributing to the development of diseases.

- Epidemiological studies in the hospital to detect the real number of *C. malonaticus* infections.

References

Achbergerová, L. and Nahálka, J. (2014). PPK1 and PPK2 - which polyphosphate kinase is older? Biologia, 69(3), pp.263-269.

Adamson, D. and Rogers, J. (1981). *Enterobacter sakazakii* meningitis with sepsis. Clinical Microbiology Newsletter, 3(3), pp.19-20.

Adamsson, I., Edlund, C., Seensalu, R. and Engstrand, L. (2000). The use of AP-PCR and flaA-RFLP typing to investigate treatment failure in *Helicobacter pylori* infection. Clinical Microbiology and Infection, 6 (5), pp.265-267.

Adékambi, T., Drancourt, M. and Raoult, D. (2009). The *rpoB* gene as a tool for clinical microbiologists. Trends in Microbiology, 17(1), pp.37-45.

Ahmed, G., Elkhatib, W. and Noreddin, A. (2014). Inhibition of *Pseudomonas aeruginosa* PAO1 adhesion to and invasion of A549 lung epithelial cells by natural extracts. Journal of Infection and Public Health, 7(5), pp.436-444.

Allen, P., Hart, C. and Saunders, J. (1987). Isolation from *Klebsiella* and characterization of two *rcs* genes that activate colanic acid capsular biosynthesis in *Escherichia coli*. Microbiology, 133(2), pp.331-340.

Almagro-Moreno, S. and Boyd, E.F. (2009). Insights into the evolution of sialic acid analysis. *In Silico* Biology, 3, pp. 287-300.

Almajed, F. and Forsythe, S. (2016). *Cronobacter sakazakii* clinical isolates overcome host barriers and evade the immune response. Microbial Pathogenesis, 90, pp.55-63.

Almuneef, M.A., Baltimore, R.S., Farrel, P.A., Reagan-Cirincione, P. and Dembry, L.M. (2001). Molecular typing demonstrating transmission of gram-negative rods in a neonatal intensive care unit in the absence of a recognized epidemic. Clinical Infectious Diseases, 32(2), pp. 220-227.

Al-Nabulsi, A., Osaili, T., Elabedeen, N., Jaradat, Z., Shaker, R., Kheirallah, K., Tarazi, Y. and Holley, R. (2011). Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. International Journal of Food Microbiology, 146(2), pp.137-143.

Alvarez-Ordonez, A., Begley, M. and Hill, C. (2012). Polymorphisms in *rpoS* and stress tolerance heterogeneity in natural isolates of *Cronobacter sakazakii*. Applied and Environmental Microbiology, 78(11), pp. 3975-3984.

Alvarez-Ordóñez, A., Cummins, C., Deasy, T., Clifford, T., Begley, M. and Hill, C. (2014). Acid stress management by Cronobacter sakazakii. International Journal of Food Microbiology, 178, pp.21-28.

Alzahrani, H., Winter, J., Boocock, D., De Girolamo, L. and Forsythe, S.J. (2015). Characterization of outer membrane vesicles from a neonatal meningitic strain of *Cronobacter sakazakii*. FEMS Microbiology Letters, 362(12), pp. fnv085-fnv085.

Annous, B., Fratamico, P. and Smith, J. (2009). Quorum sensing in biofilms: why bacteria behave the way they do. Journal of Food Science, 74(1), pp.1-14.

Arseni, A., Malamou-Ladas, E., Koutsia, C., Xanthou, M. and Trikka, E. (1987). Outbreak of colonization of neonates with *Enterobacter sakazakii*. Journal of Hospital Infection, 9(2), pp.143 - 150.

Asato, V., Vilches, V., Pineda, M., Casanueva, E., Cane, A., Moroni, M., Brengi, S. and Pichel, M. (2013). First clinical isolates of *Cronobacter* spp. (*Enterobacter sakazakii*) in Argentina: Characterization and subtyping by pulsed-field gel electrophoresis. Revista Argentina de Microbiología, 45(3), pp.160 -164.

Aubry, C., Goulard, C., Nahori, M., Cayet, N., Decalf, J., Sachse, M., Boneca, I., Cossart, P. and Dussurget, O. (2011). OatA, a peptidoglycan o-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence. Journal of Infectious Diseases, 204(5), pp.731-740.

Bader, M.W., Sanowar, S., Daley, M.E., Schneider, A.R., Cho, U., Xu, W., Klevit, R.E., Le Moual, H. and Miller, S.I. (2005). Recognition of antimicrobial peptides by a bacterial sensor Kinase. Cell, 122(3), pp. 461- 472.

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C. and Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. BMC Microbiology, 9(1), pp.223 -231.

Baumgartner, A., Grand, M., Liniger, M. and Iversen, C. (2009). Detection and frequency of *Cronobacter* spp. (*Enterobacter sakazakii*) in different categories of ready-to-eat foods other than infant formula. International Journal of Food Microbiology, 136(2), pp.189 -192.

Bewley, M.A., Pham, T.K., Marriott, H.M., Noirel, J., Chu, H.., Ow, S.Y., Ryazanov, A.G., Read, R.C., Whyte, M.K.B., Chain, B., Wright, P.C. and Dockrell, D.H. (2011). Proteomic evaluation and

validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*. Molecular and Cellular Proteomics, 10(6), pp. M111.008193-M111.008193.

Bhat, G., Anandhi, R., Dhanya, V. and Shenoy, S. (2009). Urinary tract infection due to *Enterobacter sakazakii*. Indian Journal of Pathology and Microbiology, 52(3), pp. 430-431.

Blažková, M., Javůrková, B., Vlach, J., Göselová, S., Karamonová, L., Ogrodzki, P., Forsythe, S. and Fukal, L. (2015). Diversity of O antigens within the genus *Cronobacter*: from disorder to order. Applied and Environmental Microbiology, 81(16), pp.5574-5582.

Block, C., Peleg, O., Minster, N., Bar-Oz, B., Simhon, A., Arad, I. and Shapiro, M. (2002). Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. European Journal of Clinical Microbiology and Infectious Diseases, 21(8), pp.613-616.

Bogard, R. and Oliver, J. (2007). Role of iron in human serum resistance of the clinical and environmental *Vibrio vulnificus* genotypes. Applied and Environmental Microbiology, 73(23), pp.7501-7505.

Bokranz, W., Wang, X., Tschape, H. and Romling, U. (2005). Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. Journal of Medical Microbiology, 54(12), pp.1171-1182.

Boor, K. (2006). Bacterial Stress Responses: What doesn't Kill them can make them stronger. PLoS Biology, 4(1), pp. e23-e25.

Bowen, A. and Braden, C. (2006). Invasive *Enterobacter sakazakii* disease in infants. Emerging Infectious Diseases, 12(8), pp.1185-1189.

Brandão, M., Umeda, N., Carvalho, K. and De Filippis, I. (2015). Investigação de um surto causado por *Cronobacter malonaticus* em um hospital maternidade em Teresina, Piauí: caracterização e tipificação por eletroforese em gel de campo pulsado. Vigilância Sanitária em Debate, 0(0).

Breeuwer, P., Lardeau, A., Peterz, M. and Joosten, H. (2003). Desiccation and heat tolerance of *Enterobacter sakazakii*. Journal of Applied Microbiology, 95(5), pp.967-973.

Buckles, E., Wang, X., Lane, M., Lockatell, C., Johnson, D., Rasko, D., Mobley, H. and Donnenberg, M. (2009). Role of the K2 Capsule in *Escherichia coli* Urinary Tract Infection and Serum Resistance. The Journal of Infectious Diseases, 199(11), pp.1689-1697.

Busuioc, M., Mackiewicz, K., Buttaro, B. A. and Piggot, P. J. (2009). Role of Intracellular Polysaccharide in persistence of *Streptococcus mutans*. Journal of Bacteriology, 191(23), pp.7315-7322.

Carver, T., Rutherford, K., Berriman, M., Rajandream, M., Barrell, B. and Parkhill, J. (2005). ACT: the Artemis comparison tool. Bioinformatics, 21(16), pp.3422-3423.

Caubilla-barron, J. and Forsythe, S. (2007). Dry stress and survival time of *Enterobacter sakazakii* and other *Enterobacteriaceae*. Journal of Food Protection, 70(9), pp.2111- 2117.

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., Prere, M. and Forsythe, S. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. Journal of Clinical Microbiology, 45(12), pp.3979-3985.

CDC. (2002). *Enterobacter sakazakii* infections associated with the use of powdered infant formula--Tennessee, 2001. MMWR Morbidity and Mortality Weekly Report, 51(14), pp.297-300.

CDC. (2004). One-Day (24-28 h) Standardized laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by pulsed field gel electrophoresis (PFGE). [online] Available at: http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf. [Accessed 30 June. 2013].

CDC. (2009). *Cronobacter* species isolation in two infants - New Mexico, 2008. Morbidity and Mortality Weekly Report, 58(42), pp.1179-1183.

CDC. (2012a). Investigation of *Cronobacter* infections *Cronobacter* CDC. [online] Available at: http://www.cdc.gov/*cronobacter*/investigation.html. [Accessed 20 Aug. 2016].

CDC. (2012b). Centres for disease control and prevention. *Cronobacter* Infection. [online] Available at: http://www.cdc.gov/features/*cronobacter/*. [Accessed 30 Mar. 2016].

CDC. (2016). Centres for disease control and prevention. *Cronobacter* infection. [online] Available at: http://www.cdc.gov/features/*cronobacter/*. [Accessed May. 2016].

Cetinkaya, E., Joseph, S., Ayhan, K. and Forsythe, S. (2013). Comparison of methods for the microbiological identification and profiling of *Cronobacter* species from ingredients used in the preparation of infant formula. Molecular and Cellular Probes, 27(1), pp.60-64.

Cetin, S., Ford, H., Sysko, L., Agarwal, C., Wang, J., Neal, M., Baty, C., Apodaca, G. and Hackam, D. (2004). Endotoxin inhibits intestinal epithelial restitution through activation of Rho-GTPase and increased focal adhesions. Journal of Biological Chemistry, 279(23), pp.24592-600.

Chen, Y., Noe, K., Thompson, S., Elems, C., Brown, E., Lampel, K. and Hammack, T. (2012). Evaluation of a revised U.S. food and drug administration method for the detection of *Cronobacter* in powdered infant formula: a collaborative study. Journal of Food Protection, 75(6), pp.1144-1147.

Chen, Y., K.Y. Song, E.W. Brown and K.A. Lampel. (2010). Development of an improved protocol for the isolation and detection of *Enterobacter sakazakii* (*Cronobacter*) from powdered infant formula. Journal of Food Protection, 73(6), pp.1016-1022.

Chenu, J. and Cox, J. (2009). *Cronobacter* (*Enterobacter sakazakii*): current status and future prospects. Letters in Applied Microbiology, 49(2), pp.153-159.

Cordeiro, M., Werle, C., Milanez, G. and Yano, T. (2016). Curli fimbria: an *Escherichia coli* adhesin associated with human cystitis. Brazilian Journal of Microbiology, 47(2), pp.414-416.

Cox, G., Harrison, T., McDade, H., Taborda, C., Heinrich, G., Casadevall, A. and Perfect, J. (2003). Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. Infection and Immunity, 71(1), pp.173-180.

Craven, H., McAuley, C., Duffy, L. and Fegan, N. (2010). Distribution, prevalence and persistence of *Cronobacter* (*Enterobacter sakazakii*) in the nonprocessing and processing environments of five milk powder factories. Journal of Applied Microbiology, 109(3), pp.1044-1052.

Croxall, G., Weston, V., Joseph, S., Manning, G., Cheetham, P. and McNally, A. (2010). Increased human pathogenic potential of *Escherichia coli* from polymicrobial urinary tract infections in comparison to isolates from monomicrobial culture samples. Journal of Medical Microbiology, 60(1), pp. 102-109.

Cruz-Córdova, A., Rocha-Ramírez, L., Ochoa, S., Gónzalez-Pedrajo, B., Espinosa, N., Eslava, C., Hernández-Chiñas, U., Mendoza-Hernández, G., Rodríguez-Leviz, A., Valencia-Mayoral, P., Sadowinski-Pine, S., Hernández-Castro, R., Estrada-García, I., Muñoz-Hernández, O., Rosas, I. and Xicohtencatl-Cortes, J. (2012). Flagella from five *Cronobacter* species induce pro-inflammatory cytokines in macrophage derivatives from human monocytes. PLoS ONE, 7(12), pp. e52091-e52103

Cruz, A., Xicohtencatl-Cortes, J., González-Pedrajo, B., Bobadilla, M., Eslava, C. and Rosas, I. (2011). Virulence traits in *Cronobacter* species isolated from different sources. Canadian Journal of Microbiology, 57(9), pp.735-744.

Dancer, G., Mah, J., Rhee, M., Hwang, I. and Kang, D. (2009a). Resistance of *Enterobacter sakazakii* (*Cronobacter* spp.) to environmental stresses. Journal of Applied Microbiology, 107(5), pp.1606-1614.

Dancer, G.I., Mah, J. and Kang, D. (2009b). Influences of milk components on biofilm formation of *Cronobacter* spp. (*Enterobacter sakazakii*). Letters in Applied Microbiology, 48(6), pp718-725.

Dang, W., Hu Y.H. and Sun, L. (2011). HtpG is involved in the pathogenesis of *Edwardsiella tarda*. Veterinary Microbiology, 152(3-4), pp. 394-400

Da Re, S. and Ghigo, J. (2006). A CsgD-independent pathway for cellulose production and biofilm formation in *Escherichia coli*. Journal of Bacteriology, 188(8), pp.3073-3087.

David, O., Falegan, C., and Oluyege, O. (2013). Pattern of breastfeeding and occurrence of *Cronobacter sakazakii* in infant formula sold in Ekiti State, Nigeria. Shiraz E-Medical Journal, 14(1), pp. 21-34.

Dennison, S.K. and Morris, J. (2002). Multi-resistant *Enterobacter sakazakii* wound infection in an adult. Infections and Medicine, 19(11), pp.533-535.

Doran, L., Collinson, K., Burian, J., Sarlos, G., Todd, D., Munro, K., Kay, M., Banser, A., Peterkin, P. and Kay, W. (1993). DNA-based diagnostic tests for *Salmonella* species targeting *agfA*, the structural gene for thin, aggregative fimbriae. Journal of Clinical Microbiology 31(9), pp.2263–2273.

Drudy, D., Mullane, N., Quinn, T., Wall, P. and Fanning, S. (2006). *Enterobacter sakazakii*: an emerging pathogen in powdered infant formula. Clinical Infectious Diseases, 42(7), pp.996-1002.

Druggan, P. and Iversen, C. (2009). Culture media for the isolation of *Cronobacter* spp. International Journal of Food Microbiology, 136(2), pp.169-178.

Du, X., Han, R., Li, P. and Wang, S. (2015). Comparative proteomic analysis of *Cronobacter sakazakii* isolates with different virulences. Journal of Proteomics, 128, pp. 344-351.

Duan, Q., Zhou, M., Liang, H., Zhu, X., Guo, Z., Li, Y., Hardwidge, P.R. and Zhu, G. (2013). Contribution of flagellin subunit *FliC* to piglet epithelial cells invasion by F18ab *E. Coli*. Veterinary Microbiology, 166(1-2), pp. 220-224.

Edelson-Mammel, S., Porteous, M. and Buchanan, R. (2006). Acid resistance of twelve strains of *Enterobacter sakazakii*, and the impact of habituating the cells to an acidic environment. Journal of Food Science, 71(6), pp.M201-M207.

Ernst, R., Guina, T. and Miller, S. (1999). How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. The Journal of Infectious Diseases, 179(s2), pp. S326-S330.

FAO-WHO. (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula: meeting report. microbiological risk assessment series 6. [online] Available at: http://www.fao.org/3/a-y5502e.pdf [Accessed 16 Aug. 2016].

FAO-WHO. (2006). WHO) *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: meeting report. microbiological risk assessment series 10. [online] Available at: http://www.who.int/foodsafety/publications/micro/mra10/en/index.htm [Accessed 16 Aug. 2016].

FAO/WHO. (2007). Safe preparation, storage and handling of powdered infant formula: guidelines stored in a refrigerator (to prevent growth of *E. sakazakii*) [online] Available at: http://www.who.int/foodsafety/publications/micro/pif_guidelines.pdf [Accessed 16 Aug. 2016].

FAO-WHO. (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae: meeting report. microbiological risk assessment series 15 [online] Available at: http://www.who.int/foodsafety/publications/micro/MRA_followup.pdf [Accessed 17 Aug. 2016].

Farmer, J.J., Asbury, M.A., Hickman, F.W. and Brenner, D.J. (1980). *Enterobacter sakazakii*: a new species of "*Enterobacteriaceae*" isolated from clinical specimens. International Journal of Systematic Bacteriology, 30(3), pp. 569-584.

Farmer, J., Davis, R., Hickman-Brenner, W., McWhorter A., Huntley- Carter, A., Asbury, A., Riddle, C., Wathen-Grady, G., Elias, C., Fanning, R., Steigerwalt, G., O'Hara, M., Morris, K., Smith, P. and Brenner D.J. (1985). Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. Journal of Clinical Microbiology 21(1), pp.46-76.

FDA. (2002). Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. [online] Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm114665.htm [Accessed 15 Aug. 2016].

Feeney, A. and Sleator, R. (2011). An *in silico* analysis of osmotolerance in the emerging gastrointestinal pathogen *Cronobacter sakazakii*. Bioengineered Bugs, 2(5), pp.260-270.

Fei, P., Man, C., Lou, B., Forsythe, S., Chai, Y., Li, R., Niu, J. and Jiang, Y. (2015). Genotyping and source tracking of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula

and an infant formula production factory in China. Applied and Environmental Microbiology, 81(16), pp.5430-5439.

Fleischmann, R., Adams, M., White, O., Clayton, R., Kirkness, E., Kerlavage, A., Bult, C., Tomb, J., Dougherty, B., Merrick, J. and al., e. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science, 269(5223), pp.496-512.

Flores, J., Medrano, S., Sánchez, J. and Fernández-Escartín, E. (2011). Two cases of hemorrhagic diarrhea caused by *Cronobacter sakazakii* in hospitalized nursing infants associated with the consumption of powdered infant formula. Journal of Food Protection, 74(12), pp.2177-2181.

Formal, S., Hale, T. and Sansonetti, P. (1983). Invasive enteric pathogens. Clinical Infectious Diseases. 5(4), pp. S702-S707.

Forsythe, S., Dickins, B. and Jolley, K. (2014). *Cronobacter*, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age; MLST and whole genome sequence analysis. BMC Genomics, 15(1), pp.1121-1134.

Franco, A., Kothary, M., Gopinath, G., Jarvis, K., Grim, C., Hu, L., Datta, A., McCardell, B. and Tall, B. (2011a). *Cpa*, the outer membrane protease of *Cronobacter sakazakii*, activates plasminogen and mediates resistance to serum bactericidal activity. Infection and Immunity, 79(4), pp.1578-1587.

Franco, A., Hu, L., Grim, C., Gopinath, G., Sathyamoorthy, V., Jarvis, K., Lee, C., Sadowski, J., Kim, J., Kothary, M., McCardell, B. and Tall, B. (2011b). Characterization of putative virulence genes on the related RepFIB plasmids harboured by *Cronobacter* spp. Applied and Environmental Microbiology, 77(10), pp.3255-3267.

Fraser, C., Gocayne, J., White, O., Adams, M., Clayton, R., Fleischmann, R., Bult, C., Kerlavage, A., Sutton, G., Kelley, J., Fritchman, J., Weidman, J., Small, K., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T., Saudek, D., Phillips, C., Merrick, J., Tomb, J., Dougherty, B., Bott, K., Hu, P. and Lucier, T. (1995). The minimal gene complement of *Mycoplasma genitalium*. Science, 270(5235), pp.397-404.

Friedemann, M. (2007). *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). International Journal of Food Microbiology, 116(1), pp.1-10.

Friedemann, M. (2009). Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. European Journal of Clinical Microbiology and Infectious Diseases, 28(11), pp.1297-1304.

Gajdosova, J., Benedikovicova, K., Kamodyova, N., Tothova, L., Kaclikova, E., Stuchlik, S., Turna, J. and Drahovska, H. (2011). Analysis of the DNA region mediating increased thermotolerance at 58°C

in *Cronobacter* spp. and other enterobacterial strains. Antonie van Leeuwenhoek, 100(2), pp.279-289.

Gallagher, P. and Ball, W. (1991). Cerebral infarctions due to CNS infection with *Enterobacter sakazakii*. Pediatric Radiology, 21(2), pp.135-136.

Gardiner, G.E., O'Sullivan, E., Kelly, J., Auty, M.A.E., Fitzgerald, G.F., Collins, J.K., Ross, R.P. and Stanton, C. (2000). Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. Salivarius* strains during heat treatment and spray drying. Applied and Environmental Microbiology, 66(6), pp. 2605-2612.

Gauger, E., Leatham, M., Mercado-Lubo, R., Laux, D., Conway, T. and Cohen, P. (2007). Role of motility and the *flhDC* operon in *Escherichia coli* MG1655 colonization of the mouse intestine. Infection and Immunity, 75(7), pp.3315-3324.

Giannouli, M., Antunes, L., Marchetti, V., Triassi, M., Visca, P. and Zarrilli, R. (2013). Virulence-related traits of epidemic *Acinetobacter baumannii* strains belonging to the international clonal lineages I-III and to the emerging genotypes ST25 and ST78. BMC Infectious Diseases, 13(1), pp.282-292

Giri, C., Shima, K., Tall, B., Curtis, S., Sathyamoorthy, V., Hanisch, B., Kim, K. and Kopecko, D. (2012). *Cronobacter* spp. (previously *Enterobacter sakazakii*) invade and translocate across both cultured human intestinal epithelial cells and human brain microvascular endothelial cells. Microbial Pathogenesis, 52(2), pp.140-147.

Goebel W1, Chakraborty T, Kreft J. (1988). Bacterial haemolysins as virulence factors. Antonie Van Leeuwenhoek, 54(5), pp.453-63.

Grabenstein, J., Fukuto, H., Palmer, L. and Bliska, J. (2006). Characterization of phagosome trafficking and identification of *PhoP*-regulated genes important for survival of *Yersinia pestis* in macrophages. Infection and Immunity, 74(7), pp.3727-3741.

Grant, A.J., Farris, M., Alefounder, P., Williams, P.H., Woodward, M.J. and O'Connor, C.D. (2003). Co-ordination of pathogenicity island expression by the *BipA* GTPase in enteropathogenic *Escherichia coli* (EPEC). Molecular Microbiology, 48(2), pp. 507-521.

Grim, C., Kothary, M., Gopinath, G., Jarvis, K., Beaubrun, J., McClelland, M., Tall, B. and Franco, A. (2012). Identification and characterization of *Cronobacter* iron acquisition systems. Applied and Environmental Microbiology, 78(17), pp.6035-6050.

Grim, C., Kotewicz, M., Power, K., Gopinath, G., Franco, A., Jarvis, K., Yan, Q., Jackson, S., Sathyamoorthy, V., Hu, L., Pagotto, F., Iversen, C., Lehner, A., Stephan, R., Fanning, S. and Tall, B. (2013). Pan-genome analysis of the emerging foodborne pathogen *Cronobacter* spp. suggests a species-level bidirectional divergence driven by niche adaptation. BMC Genomics, 14(1), p.366-382.

Groisman, E.A. (2001). The pleiotropic two-component regulatory system *PhoP-PhoQ*. Journal of Bacteriology, 183(6), pp. 1835-1842.

Guerry, P. and Szymanski, C. (2008). *Campylobacter* sugars sticking out. Trends in Microbiology, 16(9), pp.428-435.

Guttenplan, S. and Kearns, D. (2013). Regulation of flagellar motility during biofilm formation. FEMS Microbiology Reviews, 37(6), pp.849-871.

Haiko, J. and Westerlund-Wikström, B. (2013). The Role of the Bacterial Flagellum in Adhesion and Virulence. Biology, 2(4), pp.1242-1267.

Hariri, S., Joseph, S. and Forsythe, S. (2013). *Cronobacter sakazakii* ST4 strains and neonatal meningitis, United States. Emerging Infectious Diseases, 19(1), pp.175-177.

Hartmann, I., Carranza, P., Lehner, A., Stephan, R., Eberl, L. and Riedel, K. (2010). Genes involved in *Cronobacter sakazakii* biofilm formation. Applied and Environmental Microbiology, 76(7), pp.2251-2261.

Hawkins, R.E., Lissner, C.R. and Sanford, J.P. (1991). *Enterobacter sakazakii* bacteraemia in an adult. Southern Medical Journal, 84(6), pp.793-795.

Healy, B., Cooney, S., O'Brien, S., Iversen, C., Whyte, P., Nally, J., Callanan, J. and Fanning, S. (2010). *Cronobacter* (*Enterobacter sakazakii*): an opportunistic foodborne pathogen. Foodborne Pathogens and Disease, 7(4), pp.339-350.

Himelright, I., Harris, E., Lorch, V. and Anderson, M. (2002). *Enterobacter sakazakii* infections associated with the use of powdered infant formula -Tennessee, 2001. Journal of the American Medical Association, 287, pp.2204-2205.

Holý, O., Petrželová, J., Hanulík, V., Chromá, M., Matoušková, I. and Forsythe, S.J. (2014). Epidemiology of *Cronobacter* spp. isolates from patients admitted to the Olomouc university hospital (Czech Republic). Epidemiology Microbiology Immunology, 63(1), pp.69-72.

Hood, S. and Zottola, E. (1997). Adherence to stainless steel by foodborne microorganisms during growth in model food systems. International Journal of Food Microbiology, 37(2-3), pp.145-153.

Horlacher, R. and Boos, W. (1997). Characterization of *TreR*, the major regulator of the *Escherichia coli* trehalose system. Journal of Biological Chemistry, 272(20), pp.13026-13032.

Hu, L., Grim, C., Franco, A., Jarvis, K., Sathyamoorthy, V., Kothary, M., McCardell, B. and Tall, B. (2015). Analysis of the cellulose synthase operon genes, *bcsA*, *bcsB*, and *bcsC* in *Cronobacter* species: prevalence among species and their roles in biofilm formation and cell–cell aggregation. Food Microbiology, 52, pp.97-105.

Huang, C., Wang, Z., Huang, H., Huang, H. and Peng, H. (2013). *YjcC*, a c-di-GMP phosphodiesterase protein, regulates the oxidative stress response and virulence of *Klebsiella pneumoniae* CG43. PLoS ONE, 8(7), pp. e66740-e66754.

Huang, X. and Madan, A. (1999). CAP3: a DNA sequence assembly program. Genome Research, 9(9), pp.868-877.

Hughes, C., Phillips, R. and Roberts, A. (1982). Serum resistance among *Escherichia coli* strains causing urinary tract infection in relation to O type and the carriage of hemolysin, colicin, and antibiotic resistance determinants. Infection and Immunity, 35(1), pp.270-275.

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J. and Forsythe, S. (2009a). Biofilm formation on enteral feeding tubes by *Cronobacter sakazakii*, *Salmonella serovars* and other *Enterobacteriaceae*. International Journal of Food Microbiology, 136(2), pp.227-231.

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., Hilton, A., Armstrong, R., Smith, C., Grant, J., Shoo, S. and Forsythe, S. (2009b). Neonatal enteral feeding tubes as loci for colonisation by members of the *Enterobacteriaceae*. BMC Infectious Diseases, 9(1), pp.146-154.

Ismail, T., Hart, C. and McLennan, A. (2003). Regulation of dinucleoside polyphosphate pools by the *YgdP* and *ApaH* hydrolases is essential for the ability of *Salmonella enterica* serovar Typhimurium to invade cultured mammalian cells. Journal of Biological Chemistry, 278(35), pp.32602-32607.

ISO. (2006). ISO/TS 22964:2006 (IDF/RM 210: 2006) - Milk and milk products - Detection of *Enterobacter sakazakii*. [online] Available at: http://www.iso.org/iso/catalogue_detail.htm?csnumber=41258. [Accessed 15 Aug. 2016].

Iversen, C. and Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. Trends in Food Science and Technology, 14(11), pp.443-454.

Iversen, C. and Forsythe, S. (2004). Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. Food Microbiology, 21(6), pp.771-777.

Iversen, C., Lane, M. and Forsythe, S. (2004). The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Letters in Applied Microbiology, 38(5), pp.378-382.

Iversen, C., Waddington, M., Farmer, J. and Forsythe, S. (2006). The biochemical differentiation of *Enterobacter sakazakii* genotypes. BMC Microbiology, 26(6), pp.94-100.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. and Joosten, H. (2007a). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. BMC Evolutionary Biology, 7(1), pp.64-74.

Iversen, C., Lehner, A., Mullane, N., Marugg, J., Fanning, S., Stephan, R. and Joosten, H. (2007b). Identification of "*Cronobacter*" spp. (*Enterobacter sakazakii*). Journal of Clinical Microbiology, 45(11), pp.3814-3816.

Iversen, C., Mullane, N., McCardell, B., Tall, B., Lehner, A., Fanning, S., Stephan, R. and Joosten, H. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. dublinensis subsp. nov., *Cronobacter dublinensis* subsp. lausannensis subsp. nov. and *Cronobacter dublinensis* subsp. lactaridi subsp. nov. International Journal of Systematic and Evolutionary Microbiology, 58(6), pp.1442-1447.

Ivy, R., Farber, J., Pagotto, F. and Wiedmann, M. (2013). International life science institute north America *Cronobacter* (formerly *Enterobacter sakazakii* liolate set. Journal of Food Protection, 76(1), pp.40-51.

Jackson, E., Sonbol, H., Masood, N. and Forsythe, S. (2014). Genotypic and phenotypic characteristics of *Cronobacter* species, with particular attention to the newly reclassified species *Cronobacter helveticus*, *Cronobacter pulveris*, and *Cronobacter zurichensis*. Food Microbiology, 44, pp.226-235.

Jackson, E., Flores, J., Fernández-Escartín, E. and Forsythe, S. (2015). Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. Journal of Food Protection, 78(6), pp.1191-1196.

Jackson, E. and Forsythe, S. (2016). Comparative study of *Cronobacter* identification according to phenotyping methods. BMC Microbiology, 16(1), pp.146-155.

Jaradat, Z., Ababneh, Q., Saadoun, I., Samara, N. and Rashdan, A. (2009). Isolation of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic assays, PCR and 16S rRNA sequencing. BMC Microbiology, 9(1), pp.225-235.

Jaradat, Z., Al Mousa, W., Elbetieha, A., Al Nabulsi, A. and Tall, B. (2014). *Cronobacter* spp. opportunistic food-borne pathogens. A review of their virulence and environmental-adaptive traits. Journal of Medical Microbiology, 63(Pt_8), pp.1023-1037.

Jarvis, K., Grim, C., Franco, A., Gopinath, G., Sathyamoorthy, V., Hu, L., Sadowski, J., Lee, C. and Tall, B. (2011). Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. Applied and Environmental Microbiology, 77(12), pp.4017-4026.

Jason, J. (2012). Prevention of invasive *Cronobacter* infections in young infants fed powdered infant formulas. Paediatrics, 130(5), pp.1076-1084.

Jiménez, E. and Giménez, C. (1982). Septic shock due to *Enterobacter sakazakii*. Clinical Microbiology Newsletter, 4(4), p.30.

Jing, C., Du, X., Li, P. and Wang, S. (2015). Transcriptome analysis of *Cronobacter sakazakii* ATCC BAA-894 after interaction with human intestinal epithelial cell line HCT-8. Applied Microbiology and Biotechnology, 100(1), pp. 311-322.

Johler, S., Stephan, R., Hartmann, I., Kuehner, K. and Lehner, A. (2009). Genes involved in yellow pigmentation of *Cronobacter sakazakii* ES5 and influence of pigmentation on persistence and growth under environmental stress. Applied and Environmental Microbiology, 76(4), pp.1053-1061.

Joker, R.N., Norholm, T. and Siboni, K.E. (1965). A case of neonatal meningitis caused by a yellow *Enterobacter*. Danish Medical Bulletin, 12(5), pp.128-30.

Jonas, K., Tomenius, H., Kader, A., Normark, S., Römling, U., Belova, L. and Melefors, Ö. (2007). Roles of curli, cellulose and *BapA* in *Salmonella* biofilm morphology studied by atomic force microscopy. BMC Microbiology, 7(1), pp.70-79.

Joseph, S. and Forsythe, S. (2011). *Cronobacter sakazakii* sequence type 4 in neonatal infections. emerging infectious diseases, 17(9), pp.1713-1715.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. and Forsythe, S. (2011). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter* universalis sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. International Journal OF Systematic and Evolutionary Microbiology, 62(Pt 6), pp.1277-1283.

Joseph, S. and Forsythe, S. (2012). Insights into the emergent bacterial pathogen *Cronobacter* spp., generated by multilocus sequence typing and analysis. Frontiers in Microbiology, 3(397), p.1-11.

Joseph, S., Desai, P., Ji, Y., Cummings, C., Shih, R., Degoricija, L., Rico, A., Brzoska, P., Hamby, S., Masood, N., Hariri, S., Sonbol, H., Chuzhanova, N., McClelland, M., Furtado, M. and Forsythe, S. (2012a). Comparative analysis of genome sequences covering the seven *Cronobacter* species. PLoS One, 7(11), p. e49455-e49467.

Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M. and Forsythe, S. (2012b). Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. Journal of Clinical Microbiology, 50(9), pp.3031-3039.

Joseph, S., Hariri, S., Masood, N. and Forsythe, S. (2013). Sialic acid utilization by *Cronobacter sakazakii*. Microbial Informatics and Experimentation, 3(1), pp.3-13.

Kalyantanda, G., Shumyak, L. and Archibald, L. (2015). *Cronobacter* species contamination of powdered infant formula and the implications for neonatal health. Frontiers in Pediatrics, 3(65), pp.1-5.

Karam, M.R.A., Oloomi, M., Mahdavi, M., Habibi, M. and Bouzari, S. (2013). Assessment of immune responses of the flagellin (*FliC*) fused to *FimH* adhesin of uropathogenic *Escherichia coli*. Molecular Immunology, 54(1), pp. 32-39.

Kempf, B. and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Archives of Microbiology, 170(5), pp. 319-330.

Kilonzo-Nthenge, A., Rotich, E., Godwin, S., Nahashon, S. and Chen, F. (2012). Prevalence and antimicrobial resistance of *Cronobacter sakazakii* isolated from domestic kitchens in middle Tennessee, United States. Journal of Food Protection, 75(8), pp.1512-1517.

Kim, H., Ryu, J. and Beuchat, L.R. (2006). Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. Applied and Environmental Microbiology, 72(9), pp. 5846–5856.

Kim, K. P. and Loessner, M. J. (2008). *Enterobacter sakazakii* invasion in human intestinal Caco-2 cells requires the host cell cytoskeleton and is enhanced by disruption of tight junction. Infection and Immunity, 76(2), pp.562-570.

Kim, K., Kim, K., Choi, J., Lim, J., Lee, J., Hwang, S. and Ryu, S. (2010). Outer membrane proteins A (*OmpA*) and X (*OmpX*) are essential for basolateral invasion of *Cronobacter sakazakii*. Applied and Environmental Microbiology, 76(15), pp.5188-5198.

Kim, S., Lee, H., Choi, Y., Kim, S., Lee, J., Lee, Y., Seol, S., Cho, D. and Kim, J. (2012). Involvement of curli fimbriae in the biofilm formation of *Enterobacter cloacae*. The Journal of Microbiology, 50(1), pp.175-178.

Kim, S., Hwang, H., Kim, K., Yoon, H., Kang, D. and Ryu, S. (2015). *Hfq* plays important roles in virulence and stress adaptation in *Cronobacter sakazakii* ATCC 29544. Infection and Immunity, 83(5), pp.2089-2098.

King, A.M., Pretre, G., Bartpho, T., Sermswan, R.W., Toma, C., Suzuki, T., Eshghi, A., Picardeau, M., Adler, B. and Murray, G.L. (2013). High-temperature protein G is an essential virulence factor of *Leptospira interrogans*, Infection and Immunity, 82(3), pp. 1123-1131.

Kothary, M., McCardell, B., Frazar, C., Deer, D. and Tall, B. (2007). Characterization of the zinc-containing metalloprotease encoded by zpx and development of a species-specific detection method for *Enterobacter sakazakii*. Applied and Environmental Microbiology, 73(13), pp.4142-4151.

Kucerova, E., Clifton, S., Xia, X., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K., Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W., Hallsworth-Pepin, K., Wilson, R., McClelland, M. and Forsythe, S. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. PLoS ONE, 5(3), p. e9556-e9565.

Lacher, D., Steinsland, H., Blank, T., Donnenberg, M. and Whittam, T. (2006). Molecular evolution of typical *enteropathogenic Escherichia coli*: clonal analysis by multilocus sequence typing and virulence gene allelic profiling. Journal of Bacteriology, 189(2), pp.342-350.

Lai, K.K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children and adults. Medicine, 80(2), 113-122.

Lambris, J., Ricklin, D. and Geisbrecht, B. (2008). Complement evasion by human pathogens. Nature Reviews Microbiology, 6(2), pp.132-142.

Lantz, M. (1997). Are bacterial proteases important virulence factors? Journal of Periodontal Research, 32(1), pp.126-132.

Lee, H., Hong, S., Park, H., Kim, H. and Kim, O. (2011). *Cronobacter sakazakii* infection induced fatal clinical sequels including meningitis in neonatal ICR mice. Laboratory Animal Research, 27(1), pp.59-62.

Lee, Y., Park, J. and Chang, H. (2012). Detection, antibiotic susceptibility and biofilm formation of *Cronobacter* spp. from various foods in Korea. Food Control, 24(12), pp.225-230.

Lee, J., Kim, J., Kim, D., Kim, D., Simborio, H., Min, W., Rhee, M., Lim, J., Chang, H. and Kim, S. (2014). Characterization of betaine aldehyde dehydrogenase (*BetB*) as an essential virulence factor of *Brucella abortus*. Veterinary Microbiology, 168(1), pp.131-140.

Lehner, A., Riedel, K., Eberl, L., Breeuwer, P., Diep, B., and Stephan, R. (2005). Biofilm formation, EPS production and cell-to-cell signalling in various *Enterobacter sakazakii* strains: aspects promoting environmental persistence. Journal of Food Protection, 68(11), pp.2287-2294.

Lehner, A., Grimm, M., Rattei, T., Ruepp, A., Frishman, D., Manzardo, G.G.G. and Stephan, R. (2006). Cloning and characterization of *Enterobacter sakazakii* pigment genes and in situ spectroscopic analysis of the pigment. FEMS Microbiology Letters, 265(2), pp. 244–248.

Li, Y., Cao, L., Zhao, J., Cheng, Q., Lu, F., Bie, X. and Lu, Z. (2012). Use of *rpoB* gene sequence analysis for phylogenetic identification of *Cronobacter* species. Journal of Microbiological Methods, 88(2), pp.316-318.

Lian, W. (2002). Survival of bifidobacteria after spray-drying, International Journal of Food Microbiology, 74(1-2), pp. 79-86.

Livrelli, V., De Champs, C., Di Martino, P., Darfeuille-Michaud, A., Forestier, C. and JOLY, B. (1996). Adhesive properties and antibiotic resistance of *Klebsiella*, *Enterobacter*, and *Serratia* clinical isolates involved in nosocomial infections. Journal of Clinical Microbiology, 34 (8), pp.1963-1969.

Lu, L., Sun, Q., Fan, X., Zhong, Y., Yao, Y. and Zhao, G. (2010). Mycobacterial *MazG* Is a novel NTP pyrophosphohydrolase involved in oxidative stress response. Journal of Biological Chemistry, 285(36), pp.28076-28085.

Lutsenko, M. (2000). Expression of *ZntA*, a Zinc-transporting P 1 -type ATPase, is specifically regulated by zinc and cadmium. IUBMB Life (International Union of Biochemistry and Molecular Biology: Life), 49(4), pp.297-302.

Lyu, L. D., Tang, B. K., Fan, X. Y., Ma, H. and Zhao, G. P. (2013). Mycobacterial *MazG* safeguards genetic stability via housecleaning of 5-OH-dCTP. PLoS Pathogens, 9(12), pp. e1003814-e1003824.

Maiden, M. (2006). multilocus sequence typing of bacteria. Annual Review of Microbiology, 60(1), pp.561-588.

Manavathu EK, and Vazquez JA. (2014). Biofilms: emerging importance in infectious diseases. Journal of Multidisciplinary Pathology, 1(2), pp.1-13.

Mange, J., Stephan, R., Borel, N., Wild, P., Kim, K., Pospischil, A. and Lehner, A. (2006). Adhesive properties of *Enterobacter sakazakii* to human epithelial and brain microvascular endothelial cells. BMC Microbiology, 26(6), pp.58-67.

Marshall, J.M. and Gunn, J.S. (2015). The O-antigen capsule of *salmonella* enterica Serovar Typhimurium facilitates serum resistance and surface expression of FliC. Infection and Immunity, 83(10), pp. 3946–3959.

Martinez, J.J. (2000). Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. The EMBO Journal, 19(12), pp. 2803-2812.

Masood, N., Moore, K., Farbos, A., Paszkiewicz, K., Dickins, B., McNally, A. and Forsythe, S. (2015). Genomic dissection of the 1994 *Cronobacter sakazakii* outbreak in a French neonatal intensive care unit. BMC Genomics, 16(1), pp.750-758.

Meier, C., Oelschlaeger, T., Merkert, H, Korhonen, T. and Hacker, J. (1996). Ability of *Escherichia coli* isolates that cause meningitis in newborns to invade epithelial and endothelial cells. Infection and Immunity, 64(7), pp.2391-2399.

Miajlovic, H., Cooke, N., Moran, G., Rogers, T. and Smith, S. (2013). Response of extraintestinal pathogenic *Escherichia coli* to human serum reveals a protective role for *Rcs*-regulated exopolysaccharide colanic acid. Infection and Immunity, 82(1), pp.298-305.

Mittal, R., Wang, Y., Hunter, C., Gonzalez-Gomez, I. and Prasadarao, N. (2009). Brain damage in newborn rat model of meningitis by *Enterobacter sakazakii*: a role for outer membrane protein A. Laboratory Investigation, 89(3), pp.263-277.

Mohan Nair, M. and Venkitanarayanan, K. (2007). Role of Bacterial OmpA and Host Cytoskeleton in the Invasion of Human Intestinal Epithelial Cells by *Enterobacter sakazakii*. Pediatric Research, 62(6), pp.664-669.

Mohan Nair, M., Venkitanarayanan, K., Silbart, L. and Kim, K. (2009). Outer membrane protein A (*OmpA*) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. Foodborne Pathogens and Disease, 6(4), pp.495-501.

Mollet, C., Drancourt, M. and Raoult, D. (1997). *RpoB* sequence analysis as a novel basis for bacterial identification. Molecular Microbiology, 26(5), pp.1005-1011.

Monday, S.R., Minnich, S.A. and Feng, P.C.H. (2004). A 12-base-pair deletion in the flagellar master control gene *flhC* causes nonmotility of the pathogenic German sorbitol-fermenting *Escherichia coli* O157: H- strains, Journal of Bacteriology, 186(8), pp. 2319-2327.

Monroe, P.W. and Tift, W.L. (1979). bacteraemia associated with *Enterobacter sakazakii* (yellow pigmented *Enterobacter cloacae*). Journal of Clinical Microbiology, 10(6), pp.850-851.

Moriez, R., Salvador-Cartier, C., Theodorou, V., Fioramonti, J., Eutamene, H. and Bueno, L. (2005). Myosin light chain Kinase is involved in lipopolysaccharide-induced disruption of colonic epithelial barrier and bacterial translocation in rats, The American Journal of Pathology, 167(4), pp. 1071-1079.

Mossel D. A. A., Mengerink W. H. J., and Scholts H. H. (1962). Use of a modified MacConkey agar medium for the selective growth and enumeration of *Enterobacteriaceae*. Journal of Bacteriology, 84(2), p.381.

Mossel DA. and Ratto MA. (1970). Rapid detection of sublethally impaired cells of *Enterobacteriaceae* in dried foods. Journal of Applied Microbiology, 20(2), pp.273-275.

Mouslim, C., Delgado, M. and Groisman, E. (2004). Activation of the *RcsC*/*YojN*/*RcsB* phosphorelay system attenuates *Salmonella* virulence. Molecular Microbiology, 54(2), pp.386-395.

Mullane, N., Iversen, C., Healy, B., Walsh, C., Whyte, P., Wall, P., Quinn, T. and Fanning S. (2007). *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. Minerva Pediatrica, 59(2), pp.137-48.

Muytjens, H. and van der Ros-van de Repe, J. (1986). Comparative *in vitro* susceptibilities of eight *Enterobacter* species, with special reference to *Enterobacter sakazakii*. Antimicrobial Agents and Chemotherapy, 29(2), pp.367-370.

Muytjens, H.L., Zanen, H.C., Sonderkamp, H.J., Kollee, L.A., Wachsmuth, K. and Farmer, J.J. (1983). Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. Journal of Clinical Microbiology, 18(1), pp.115-120.

Nazarowec-White, M. and Farber, J. (1997). Incidence, survival, and growth of *Enterobacter sakazakii* in infant formula. Journal of Food Protection, 60(3), pp.226-230.

Nazarowec-White, M. and Farber, J. (1999). Phenotypic and genotypic typing of food and clinical isolates of *Enterobacter sakazakii*. Journal of Medical Microbiology, 48(6), pp.559-567.

Negre, V., Bonacorsi, S., Schubert, S., Bidet, P., Nassif, X. and Bingen, E. (2004). The siderophore receptor IroN, but not the high-pathogenicity island or the hemin receptor *ChuA*, contributes to the bacteremic step of *Escherichia coli* neonatal meningitis. Infection and Immunity, 72(2), pp.1216-1220.

Neidig, A., Yeung, A.T., Rosay, T., Tettmann, B., Strempel, N., Rueger, M., Lesouhaitier, O. and Overhage, J. (2013). TypA is involved in virulence, antimicrobial resistance and biofilm formation in *Pseudomonas aeruginosa*. BMC Microbiology, 13(1), pp.77-86.

Neyt, C., Iriarte, M., Thi, V. and Cornelis G. (1997). Virulence and arsenic resistance in *Yersinia*. Journal of Bacteriology, 179(3), pp.612-619.

Odeyemi, O. and Sani, N. (2016). The prevention of *Cronobacter* infections in hospital neonatal intensive care units. Journal of Infection and Public Health, 9(1), pp.110-112.

Ogrodzki, P. and Forsythe, S. (2015). Capsular profiling of the *Cronobacter* genus and the association of specific *Cronobacter sakazakii* and *C. malonaticus* capsule types with neonatal meningitis and necrotizing enterocolitis. BMC Genomics, 16(1), pp.758-772.

Olive DM1, Bean P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. Journal of Clinical Microbiology, 37(6), pp.1661-1669.

Olsen, A., Arnqvist, A., Hammar, M. and Normark, S. (1993). Environmental regulation of curli production in *Escherichia coli*. Infectious Agents and Disease 2(2), pp.272–274.

Orieskova, M., Kajsik, M., Szemes, T., Holy, O., Forsythe, S., Turna, J. and Drahovska, H. (2016). Contribution of the thermotolerance genomic island to increased thermal tolerance in *Cronobacter* strains. Antonie van Leeuwenhoek, 109(3), pp.405-414.

Osaili, T. and Forsythe, S. (2009). Desiccation resistance and persistence of *Cronobacter* species in infant formula. International Journal of Food Microbiology, 136(2), pp.214-220.

Osman D. and Cavet J.S. (2011). Metal sensing in *Salmonella*: implications for pathogenesis. Advances in Microbial Physiology, 58, pp.175-232.

Pagotto, F., Nazarowec-White, M., Bidawid, S. and Farber, J. (2003). *Enterobacter sakazakii*: infectivity and enterotoxin production *in vitro* and *in vivo*. Journal of Food Protection, 66(3), pp.370-375.

Pan, Z., Cui, J., Lyu, G., Du, X., Qin, L., Guo, Y., Xu, B., Li, W., Cui, Z. and Zhao, C. (2014). Isolation and molecular typing of *Cronobacter* spp. in commercial powdered infant formula and follow-up formula. Foodborne Pathogens and Disease, 11(6), pp.456-461.

Pastore, R. (2010). Are you preparing your baby's bottles correctly? AAP news, [online] 31(10), pp.37-37. Available at: http://www.aappublications.org/content/31/10/37.5. [Accessed 16 Aug. 2016].

Patrick, M., Mahon, B., Greene, S., Rounds, J., Cronquist, A., Wymore, K., Boothe, E., Lathrop, S., Palmer, A. and Bowen, A. (2014). Incidence of *Cronobacter* spp. infections, United States, 2003–2009. Emerging Infectious Diseases, 20(9), pp.1536-1539.

Peng, L., Luo, W., Zhao, T., Wan, C., Jiang, Y., Chi, F., Zhao, W., Cao, H. and Huang, S. (2012). Polyphosphate kinase 1 is required for the pathogenesis process of meningitic *Escherichia coli* K1 (RS218). Future Microbiology, 7(3), pp.411-423.

Pérez, J., Pradeñas, G., Navarro, C., Henríquez, D., Pichuantes, S. and Vásquez, C. (2006). Geobacillus stearothermophilus LV *cadA* gene mediates resistance to cadmium, lead and zinc in zntA mutants of *Salmonella enterica* serovar Typhimurium. Biological Research, 39(4), pp. 661-668.

Perez, J.C. and Groisman, E.A. (2007). Acid pH activation of the *PmrA*/*PmrB* two-component regulatory system of *Salmonella enterica*. Molecular Microbiology, 63(1), pp. 283-293.

Phan, M., Peters, K., Sarkar, S., Lukowski, S., Allsopp, L., Moriel, D., Achard, M., Totsika, M., Marshall, V., Upton, M., Beatson, S. and Schembri, M. (2013). The serum resistome of a globally disseminated multidrug resistant uropathogenic *Escherichia coli* clone. PLoS Genetics, 9(10), pp. e1003834-e1003851.

Pitout, J., Moland, E., Sanders, C., Thomson, K. and Fitzsimmons, S. (1997). Beta-lactamases and detection of beta-lactam resistance in *Enterobacter* spp. Antimicrobial Agents and Chemotherapy, 41(1) pp.35-39.

Pluschke G, Mayden J, Achtman M, Levine RP. (1983). Role of the capsule and the O antigen in resistance of O18:K1 *Escherichia coli* to complement-mediated killing. Infection and Immunity, 42(3), pp.907-913.

Power, K., Yan, Q., Fox, E., Cooney, S. and Fanning, S. (2013). Genome sequence of *Cronobacter sakazakii* SP291, a persistent thermotolerant isolate derived from a factory producing powdered infant formula. Genome Announcements, 1(2), pp.e00082-13-e00082-13.

Pracht, D., Elm, C., Gerber, J., Bergmann, S., Rohde, M., Seiler, M., Kim, K., Jenkinson, H., Nau, R. and Hammerschmidt, S. (2005). *PavA* of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. Infection and Immunity, 73(5), pp.2680-2689.

Prakash, A. and Sharma, G. (2013). Susceptibility of *Cronobacter sakazakii* to plant products, antibiotics, and to lactic acid bacteria. International Journal of Nutrition, Pharmacology, Neurological Diseases, 3(3), pp. 263-268.

Pribyl, C., Salzer, R., Beskin, J., Haddad, R., Pollock, B., Beville, R., Holmes, B. and Mogabgab, W. (1985). Aztreonam in the treatment of serious orthopaedic infections. The American Journal of Medicine, 78(2), pp.51-56.

Prost, L.R., Daley, M.E., Le Sage, V., Bader, M.W., Le Moual, H., Klevit, R.E. and Miller, S.I. (2007). Activation of the bacterial sensor Kinase *PhoQ* by acidic pH. Molecular Cell, 26(2), pp.165-174

Ramos, J.L., Gallegos, M.-T., Marqués, S., Ramos-González, M.-I., Espinosa-Urgel, M. and Segura, A. (2001). Responses of gram-negative bacteria to certain environmental stressors. Current Opinion in Microbiology, 4(2), pp. 166-171.

Ravisankar, S., Syed, S., Garg, P. and Higginson, J. (2014). Is *Cronobacter sakazakii* infection possible in an exclusively breastfed premature neonate in the neonatal intensive care unit? Journal of Perinatology, 34(5), pp.408-409.

Reichhardt, C., Jacobson, A., Maher, M., Uang, J., McCrate, O., Eckart, M. and Cegelski, L. (2015). Congo red interactions with curli-producing *e. coli* and native curli amyloid fibers. PLOS ONE, 10(10), p. e0140388-e0140397.

Remon, J., Amin, S., Mehendale, S., Rao, R., Luciano, A., Garzon, S. and Maheshwari, A. (2015). Depth of bacterial invasion in resected intestinal tissue predicts mortality in surgical necrotizing enterocolitis. Journal of Perinatology, 35(9), pp.755-762.

Reva, O. and Bezuidt, O. (2012). Distribution of horizontally transferred heavy metal resistance operons in recent outbreak bacteria. Mobile Genetic Elements, 2(2), pp.96-100.

Ribot, E., Fair, M., Gautom, R., Cameron, D., Hunter, S., Swaminathan, B. and Barrett, T. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathogens and Disease, 3(1), pp.59-67.

Roca, C., Alves, V. D., Freitas, F. and Reis, M. A. M. (2015). Exopolysaccharides enriched in rare sugars: Bacterial sources, production, and applications. Frontiers in Microbiology, 6(288), pp.1-7.

Romeo, T. (2008). Bacterial biofilms. Berlin: Springer-Verlag Berlin and Heidelberg GmbH & Co. K.

Rooijakkers, S., Rasmussen, S., McGillivray, S., Bartnikas, T., Mason, A., Friedlander, A. and Nizet, V. (2010). Human transferrin confers serum resistance against *Bacillus anthracis*. Journal of Biological Chemistry, 285(36), pp.27609-27613.

Sabbagh, S.C., Lepage, C., McClelland, M. and Daigle, F. (2012). Selection of *Salmonella enterica* Serovar Typhi genes involved during interaction with human macrophages by screening of a transposon mutant library. PLoS ONE, 7(5), p. e36643-e36655.

Scheepe-Leberkühne M. and Wagner, F. (1986). Optimization and preliminary characterization of an exopolysaccharide synthezised by *Enterobacter sakazakii*. Biotechnology Letters, 8(10), pp. 695-700.

Schmid, M., Iversen, C., Gontia, I., Stephan, R., Hofmann, A., Hartmann, A., Jha, B., Eberl, L., Riedel, K. and Lehner, A. (2009). Evidence for a plant-associated natural habitat for *Cronobacter* spp. Research in Microbiology, 160(8), pp.608-614.

Schwizer, S., Tasara, T., Zurfluh, K., Stephan, R. and Lehner, A. (2013). Identification of genes involved in serum tolerance in the clinical strain *Cronobacter sakazakii* ES5. BMC Microbiology, 13(1), pp.38-45.

See, K., Then, H. and Tang, T. (2007). *Enterobacter sakazakii* bacteraemia with multiple splenic abscesses in a 75-year-old woman: a case report. Age and Ageing, 36(5), pp.595-596.

Severi, E., Hood, D. and Thomas, G. (2007). Sialic acid utilization by bacterial pathogens. Microbiology, 153(9), pp.2817-2822.

Simonsen, K., Nielsen, G., Bjerrum, J., Kruse, T., Kallipolitis, B. and Møller-Jensen, J. (2011). A role for the RNA chaperone *Hfq* in controlling adherent-invasive *Escherichia coli* colonization and virulence. PLoS ONE, 6(1), pp. e16387-e16399.

Shin, S.H., Lim, Y., Lee, S.E., Yang, N.W. and Rhee, J.H. (2001). CAS agar diffusion assay for the measurement of siderophores in biological fluids. Journal of Microbiological Methods, 44(1), pp. 89-95.

Singamsetty, V., Wang, Y., Shimada, H. and Prasadarao, N. (2008). Outer membrane protein A expression in *Enterobacter sakazakii* is required to induce microtubule condensation in human brain microvascular endothelial cells for invasion. Microbial Pathogenesis, 45(3), pp.181-191.

Sittka, A., Pfeiffer, V., Tedin, K. and Vogel, J. (2007). The RNA chaperone *Hfq* is essential for the virulence of *Salmonella typhimurium*. Molecular Microbiology, 63(1), pp.193-217.

Soncini, F.C., García Véscovi, E., Solomon, F. and Groisman, E.A. (1996). Molecular basis of the magnesium deprivation response in *Salmonella* typhimurium: identifi-cation of PhoP-regulated genes. Journal of Bacteriology, 178(17), pp. 5092–5099.

Sood, S., Peters, T., Ward, L. and Threlfall, E. (2002). Combination of pulsed-field gel electrophoresis (PFGE) and single-enzyme amplified fragment length polymorphism (SAFLP) for differentiation of multiresistant *Salmonella enterica* serotype typhimurium. Clinical Microbiology and Infection, 8(3), pp.154-161.

Soto, G. and Hultgren, S. (1999). Bacterial adhesins: common themes and variations in architecture and assembly. Journal of Bacteriology, 181(4), pp.1059-1071.

Stasic, A., Wong, A. and Kaspar, C. (2012). Osmotic and desiccation tolerance in *Escherichia coli* O157:H7 requires *rpoS* (σ38). Current Microbiology, 65(6), pp.660-665.

Stephan, R., Lehner, A., Tischler, P. and Rattei, T. (2010). Complete genome sequence of *Cronobacter turicensis* LMG 23827, a food-borne pathogen causing deaths in neonates. Journal of Bacteriology, 193(1), pp.309-310.

Stock, I. and Wiedemann, B. (2002). Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae* and *Enterobacter sakazakii* strains. Clinical Microbiology and Infection, 8(9), pp.564-578.

Stoop, B., Lehner, A., Iversen, C., Fanning, S. and Stephan, R. (2009). Development and evaluation of *rpoB* based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. International Journal of Food Microbiology, 136(2), pp.165-168.

Stoll, B., Hansen, N., Fanaroff, A. and Lemons, J. (2004). *Enterobacter sakazakii* is a rare cause of neonatal septicaemia or meningitis in vlbw infants. The Journal of Paediatrics, 144(6), pp.821-823.

Sun, Y., Wang, M., Wang, Q., Cao, B., He, X., Li, K., Feng, L. and Wang, L. (2012). Genetic analysis of the *Cronobacter sakazakii* O4 to O7 O-antigen gene clusters and development of a PCR assay for identification of all *C. sakazakii* O serotypes. Applied and Environmental Microbiology, 78(11), pp.3966-3974.

Sun, J., Deng, Z. and Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochemical and Biophysical Research Communications, 453(2), pp.254-267.

Suzuki, K., Babitzke, P., Kushner, S.R., and Romeo, T. (2006). Identification of a novel regulatory protein (*CsrD*) that targets the global regulatory RNAs *CsrB* and *CsrC* for degradation by RNase E. Genes and Development, 20(18), pp. 2605-2617.

Tamigniau, A., Vanhaecke, J. and Saegeman, V. (2015). *Cronobacter sakazakii* bacteraemia in a heart transplant patient with polycystic kidney disease. Transplant Infectious Disease, 17(6), pp.921-925.

Tan, B., Meinken, C., Bastian, M., Bruns, H., Legaspi, A., Ochoa, M., Krutzik, S., Bloom, B., Ganz, T., Modlin, R. and Stenger, S. (2006). Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. The Journal of Immunology, 177(3), pp.1864-1871.

Teng, C.., Cai, M., Shin, S., Xie, Y., Kim, K.., Khan, N.A. and Di Cello, F. (2005). *Escherichia coli* K1 RS218 interacts with human brain microvascular endothelial cells via type 1 fimbria bacteria in the fimbriated state. Infection and Immunity, 73(5), pp. 2923-2931.

Tenover, F.C., Abreit, R.D., Goering, R.V., Michelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. Journal of Clinical Microbiology, 33(9), pp.2233-2239.

Teramoto, S., Tanabe, Y., Okano, E., Nagashima, T., Kobayashi, M. and Etoh, Y. (2010). A first fatal neonatal case of *Enterobacter sakazakii* infection in Japan. Paediatrics International, 52(2), pp.312-313.

Torres, G. (2004). Current aspects of *Shigella* pathogenesis. Revista latinoamericana de microbiología, 46(3-4), pp. 89-97.

Townsend, S., Hurrell, E., Gonzalez-Gomez, I., Lowe, J., Frye, J., Forsythe, S. and Badger, J. (2007a). *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages

influencing cytokine secretion and induces severe brain pathology in the neonatal rat. Microbiology, 153(10), pp.3538-3547.

Townsend, S., Caubillabarron, J., Loccarrillo, C. and Forsythe, S. (2007b). The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat. Food Microbiology, 24(1), pp. 67-74.

Townsend, S., Hurrell, E. and Forsythe, S. (2008). Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. BMC Microbiology, 8(1), pp.64-72.

Tsolis, M., Bäumler, J. and Heffron, F. (1995). Role of *Salmonella typhimurium* Mn-superoxide dismutase (*SodA*) in protection against early killing by J774 macrophages. Infection and Immunity, 63(5), pp.1739-1744.

Urmenyi, A. and White Franklin, A. (1961). Neonatal death from pigmented coliform infection. The Lancet, 277(7172), pp.313-315.

Vakulskas, C.A., Potts, A.H., Babitzke, P., Ahmer, B.M.M. and Romeo, T. (2015). Regulation of bacterial virulence by *Csr* (*Rsm*) systems. Microbiology and Molecular Biology Reviews, 79(2), pp. 193-224.

van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. and Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. Journal of Clinical Microbiology, 39(1), pp.293-297.

Vasishta, R., Saxena, M. and Chhibber, S. (1991). Contribution of silver ion resistance to the pathogenicity of *Pseudomonas aeruginosa* with special reference to burn wound sepsis. Folia Microbiologica, 36(5), pp.498-501.

Velasco-García, R., Villalobos, M.A., Ramírez-Romero, M.A., Mújica-Jiménez, C., Iturriaga, G. and Muñoz-Clares, R.A. (2005). Betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa*: cloning, over-expression in *Escherichia coli*, and regulation by choline and salt, Archives of Microbiology, 185(1), pp. 14-22.

Vescovi, E.G., Ayala, Y.M., Di Cera, E. and Groisman, E.A. (1997). Characterization of the bacterial sensor protein *PhoQ*: evidence for distinct binding sites for mg^{2+} and ca^{2+}. Journal of Biological Chemistry, 272(3), pp. 1440-1443.

Vimr, E. (2013). Unified theory of bacterial sialometabolism: how and why bacteria metabolize host sialic acids. ISRN Microbiology, 2013, pp.1-26.

Wang, J., Du, X.-J., Lu, X.-N. and Wang, S. (2013). Immunoproteomic identification of immunogenic proteins in *Cronobacter sakazakii* strain BAA-894. Applied Microbiology and Biotechnology, 97(5), pp. 2077-2091.

Wang, S., Shi, Z., Xia, Y., Li, H., Kou, Y., Bao, Y., Dai, J. and Lu, C. (2012). *IbeB* is involved in the invasion and pathogenicity of avian pathogenic *Escherichia coli*. Veterinary Microbiology, 159(3-4), pp.411-419.

Weiss, D.S., Brotcke, A., Henry, T., Margolis, J.J., Chan, K. and Monack, D.M. (2007). *In vivo* negative selection screen identifies genes required for *Francisella* virulence. Proceedings of the National Academy of Sciences, 104(14), pp. 6037-6042.

Wesche, A.M., Gurtler, J.B., Marks, B.P. and Ryser, E.T. (2009). Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. Journal of Food Protection, 72(5), pp.1121-1138.

Willis, J. and Robinson, J. (1988). *Enterobacter sakazakii* meningitis in neonates. The Paediatric Infectious Disease Journal, 7(3), pp.196-199.

Willis, L. and Whitfield, C. (2013). Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. Carbohydrate Research, 378, pp.35-44.

Wilson, J. (2002). Mechanisms of bacterial pathogenicity. Postgraduate Medical Journal, 78(918), pp.216-224.

Yamamoto, T., Hanawa, T., Ogata, S. and Kamiya, S. (1996). Identification and characterization of the *Yersinia enterocolitica gsrA* gene, which protectively responds to intracellular stress induced by macrophage phagocytosis and to extracellular environmental stress. Infection and Immunity, 64(8), pp.2980-2987.

Yan, Q., Power, K., Cooney, S., Fox, E., Gopinath, G., Grim, C., Tall, B., McCusker, M. and Fanning, S. (2013). Complete genome sequence and phenotype microarray analysis of *Cronobacter sakazakii* SP291: a persistent isolate cultured from a powdered infant formula production facility. Frontiers in Microbiology, 2(4), pp.256-275.

Yan, Q. and Fanning, S. (2015). Strategies for the identification and tracking of *Cronobacter* species: an opportunistic pathogen of concern to neonatal health. Frontiers in Paediatrics, 3(38), pp.1-9.

Ye, Y., Gao, J., Jiao, R., Li, H., Wu, Q., Zhang, J. and Zhong, X. (2015). The membrane proteins involved in virulence of *Cronobacter sakazakii* virulent G362 and attenuated L3101 isolates. Frontiers in Microbiology, 9(6), pp.1238-1244.

Ye, Y., Li, H., Ling, N., Han, Y., Wu, Q., Xu, X., Jiao, R. and Gao, J. (2016). Identification of potential virulence factors of *Cronobacter sakazakii* isolates by comparative proteomic analysis, International Journal of Food Microbiology, 217, pp. 182-188.

Zeng, L. B., Zhuang, X.-R., Huang, L.-L., Zhang, Y.-Y., Chen, C.-Y., Dong, K., Cui, Z.-L., Ding, X.-L., Chang, Y.-F., Guo, X.-K. and Zhu, Y.-Z. (2015). Comparative subproteome analysis of three representative *Leptospira interrogans* vaccine strains reveals cross-reactive antigens and novel virulence determinants. Journal of Proteomics, 112, pp. 27-37.

Zhao, Z., Wang, L., Wang, B., Liang, H., Ye, Q. and Zeng, M. (2014). Complete genome sequence of *Cronobacter sakazakii* strain CMCC 45402. Genome Announcements, 2(1), pp.e01139-13-e01139-13.

Zhu, H., Lee, C., Zhang, D., Wu, W., Wang, L., Fang, X., Xu, X., Song, D., Xie, J., Ren, S. and Gu, J. (2013). Surface-associated GroEL facilitates the adhesion of *Escherichia coli* to macrophages through lectin-like oxidized low-density lipoprotein receptor-1. Microbes and Infection, 15(3), pp. 172-180.

Appendix

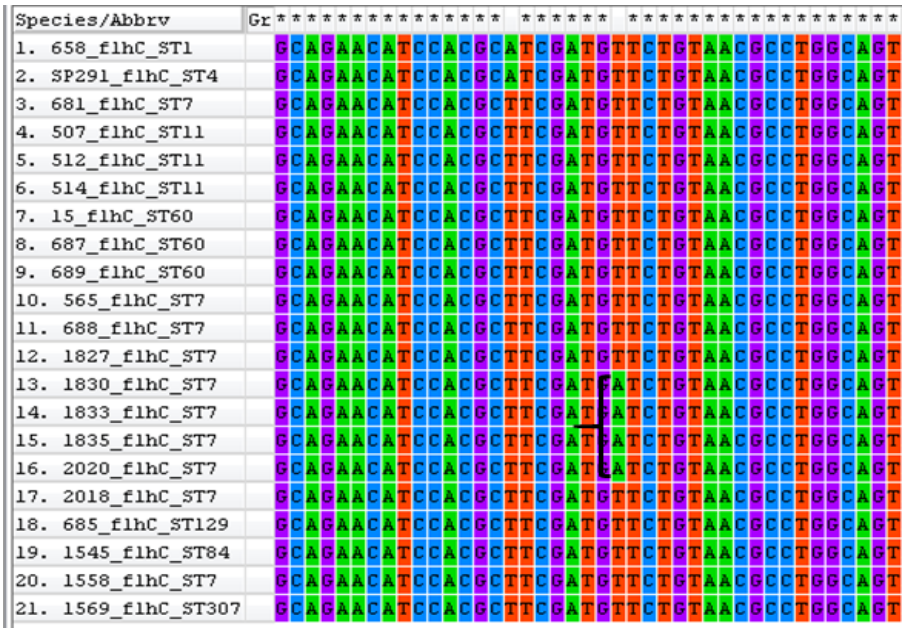


Figure S.1 Manual curation of the *flhC* gene sequence alignment. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *flhC* gene sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

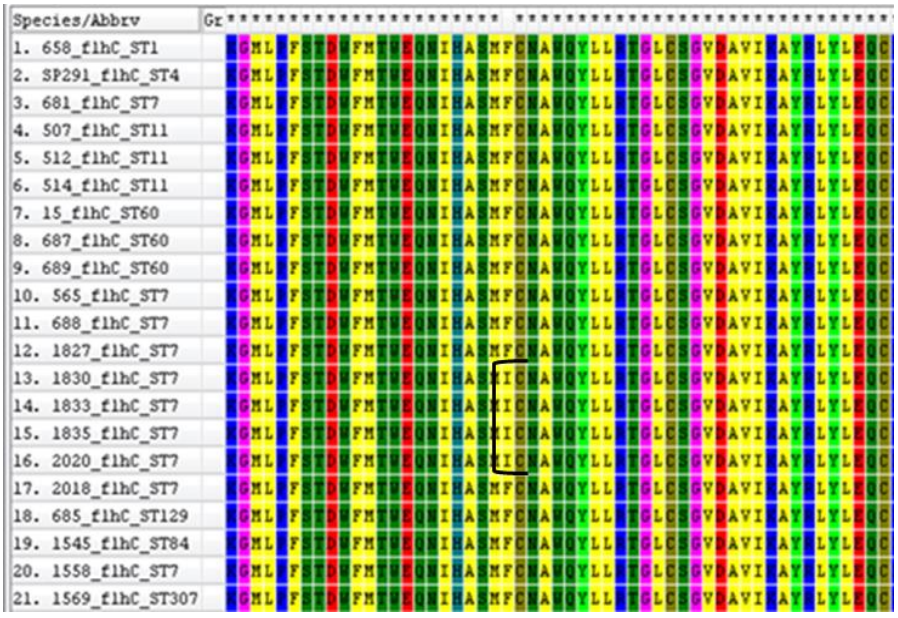


Figure S.2 Manual curation of the translated protein sequence of *flhC* gene. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *flhC* protien sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

Figure S.3 Manual curation of the *ompR* gene sequence alignment. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the ompR gene sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

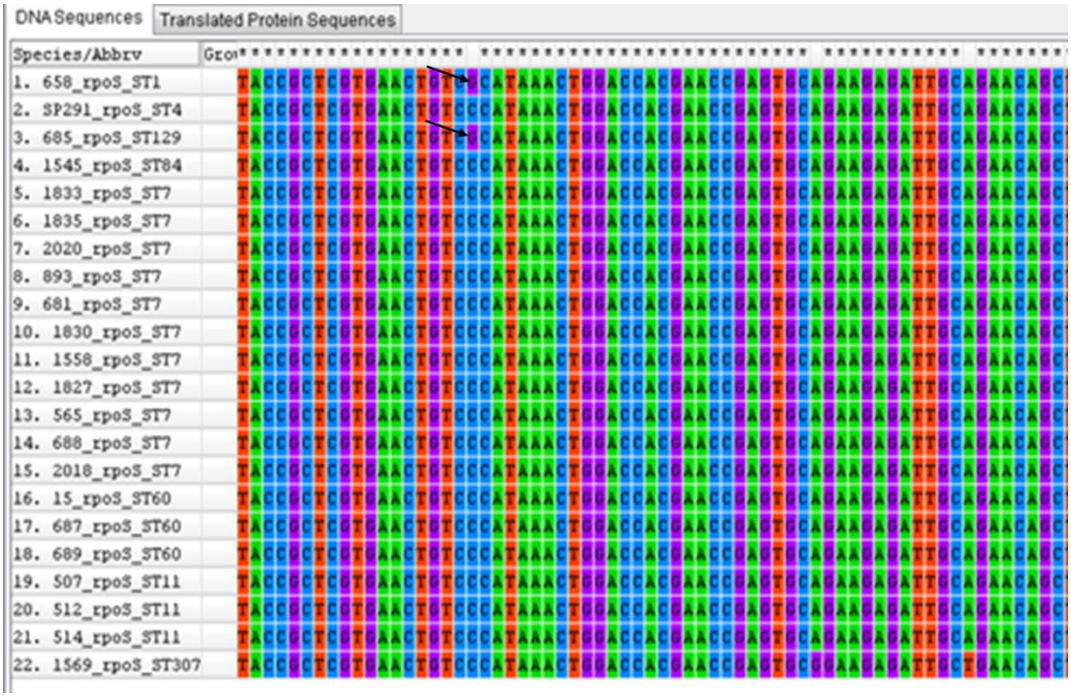


Figure S.4 Manual curation of the *rpoS* gene sequence alignment. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *rpoS* gene sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes that shared between *C. sakazakii* strain NTU658 and *C. malonaticus* strain 685.

Figure S.5 Manual curation of the *betB* gene sequence alignment. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *betB* gene sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

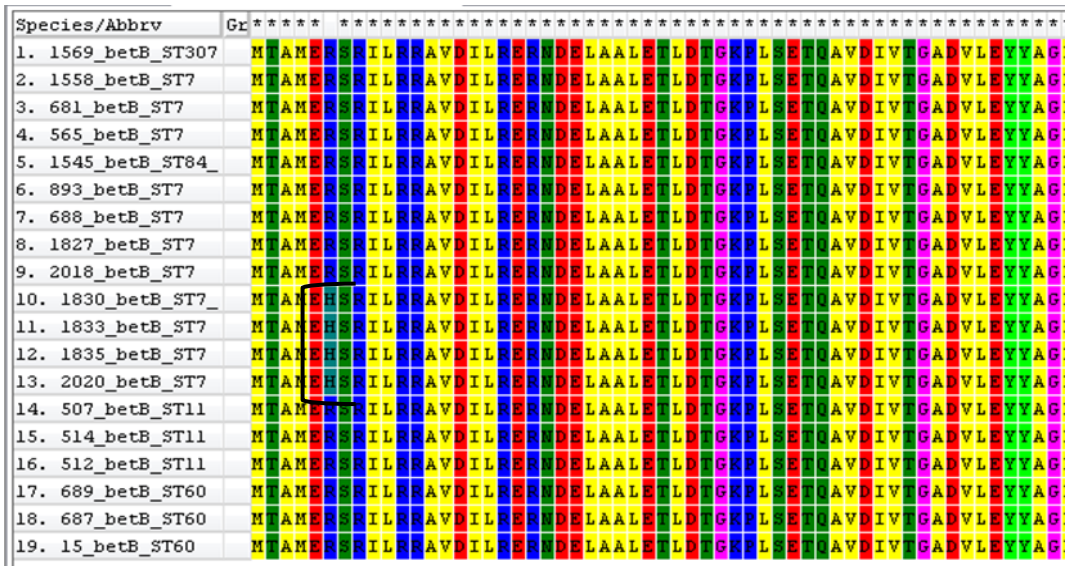


Figure S.6 Manual curation of the translated protein sequence of *betB* gene. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *betB* protien sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

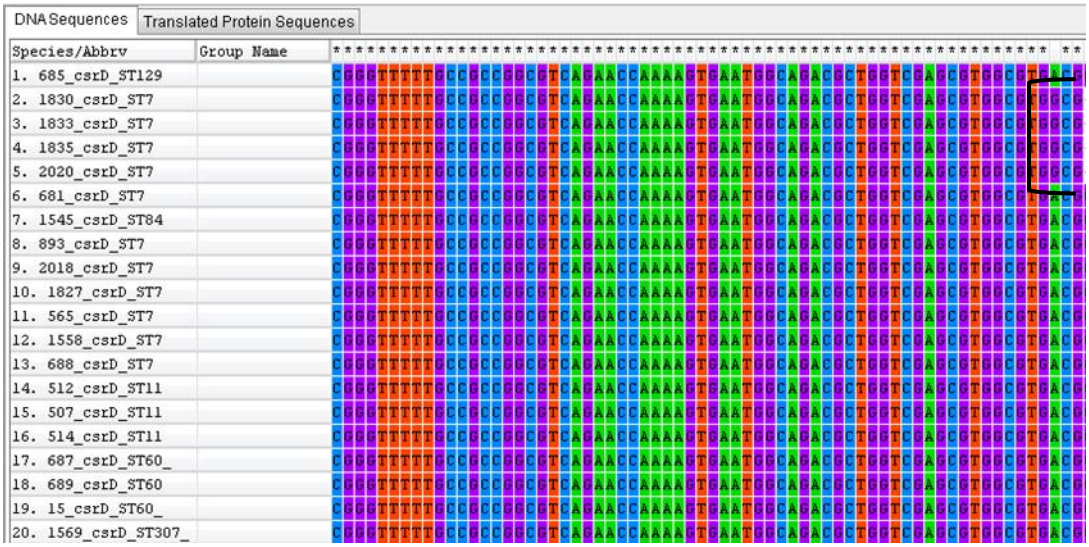


Figure S.7 Manual curation of the *csrD* gene sequence alignment. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *csrD* gene sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

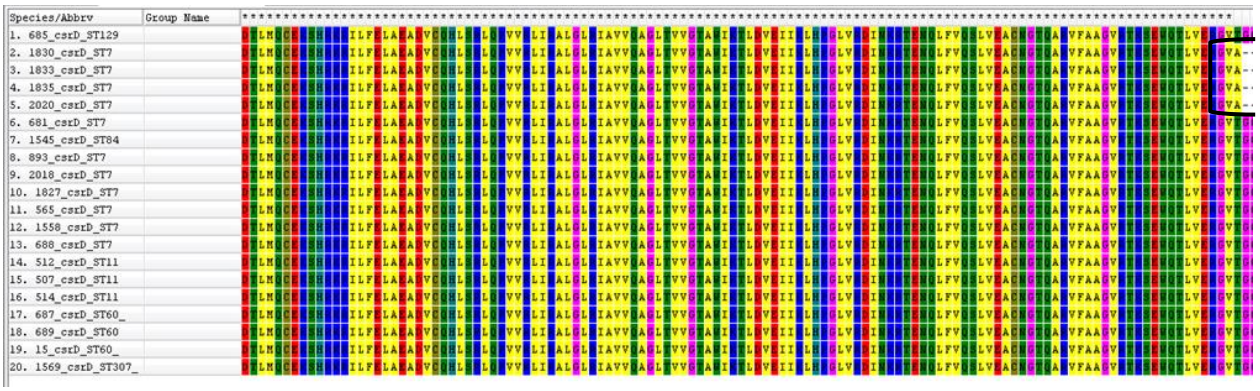


Figure S.8 Manual curation of the translated protein sequence of the *csrD*. The figure above represents the cropped segments of the screen shots combined as one image showing only the sections where variations in the *csrD* protien sequence was observed. The black arrows point towards the Single Nucleotide Variations (SNP) changes in *C. malonaticus* strains.

Table S.1 Fimbrial regions in *C. malonaticus*.

NTU	ST	Region 1				Region 2	Region 3		Region 4	Region 5
		ESA_01970-73	ESA_01974	ESA_01975	ESA_01976	ESA_02342-45	ESA_02538-41	ESA_02542	(ESA_02795-99	(ESA_03512-20
565	7	-	p	p	-	+	+	+	+	-
681	7	-	p	p	-	+	+	-	+	-
688	7	-	p	p	-	+	+	+	+	-
893	7	-	p	p	-	+	+	+	+	-
1558	7	-	p	p	-	+	+	+	+	-
1827	7	-	p	p	-	+	+	+	+	-
1830	7	-	p	p	-	+	+	+	+	-
1833	7	-	p	p	-	+	+	+	+	-
1835	7	-	p	p	-	+	+	+	+	-
2018	7	-	p	p	-	+	+	+	+	-
2020	7	-	p	p	-	+	+	+	+	-
507	11	-	p	-	-	+	+	+	+	-
512	11	-	p	p	-	+	+	+	+	-
514	11	-	p	p	-	+	+	+	+	-
15	60	-	p	p	-	+	+	+	+	-
687	60	-	p	p	-	+	+	+	+	-
689	60	-	p	p	-	+	+	+	+	-
1545	84	-	p	p	-	+	+	+	+	-
685	129	-	p	p	-	+	+	+	+	-
1569	307	-	p	p	-	+	+	+	+	-
658	1	+	+	+	+	+	+	+	+	+
SP291	4	-	-	-	-	+	+	+	+	+

Table S.2 Fimbrial regions in *C. malonaticus*.

NTU	ST	Region 6				Region 7	Region 8	Region 9			Region 10	
		ESA_04067-8	ESA_04069	ESA_04070-2	ESA_04073	ESA)_03812-15	CTU_36390-450	ESA_03231	ESA_03232	ESA_03233	CsgA-G	Ctu_16230
565	7	-	p	+	-	+	+	+	+	+	+	+
681	7	-	p	+	-	+	+	+	p	+	+	+
688	7	-	p	+	-	+	+	+	+	+	+	+
893	7	-	p	+	-	+	+	+	+	+	+	+
1558	7	-	p	+	-	+	+	-	-	+	+	+
1827	7	-	p	+	-	+	+	+	+	+	+	+
1830	7	-	p	+	-	+	+	+	+	+	+	+
1833	7	-	p	+	-	+	+	+	+	+	+	+
1835	7	-	p	+	-	+	+	+	+	+	+	+
2018	7	-	p	+	-	+	+	+	+	+	+	+
2020	7	-	p	+	-	+	+	+	+	+	+	+
507	11	-	p	+	-	+	+	+	+	+	+	+
512	11	-	p	+	-	+	+	+	+	+	+	+
514	11	-	p	+	-	+	+	+	+	+	+	+
15	60	-	p	+	-	+	+	+	+	+	+	+
687	60	-	p	+	-	+	+	+	+	+	+	+
689	60	-	p	+	-	+	+	+	+	+	+	+
1545	84	-	p	+	-	+	+	+	p	+	+	+
685	129	-	p	+	-	+	+	+	+	+	+	+
1569	307	-	p	+	-	+	+	+	+	+	+	+
658	1	+	+	+	+	+	-	+	+	+	-	-
SP291	4	+	+	+	+	+	+	+	+	+	-	-