CHAPTER 1: INTRODUCTION

1.1 Predictive microbiology

1.1.1 Concept of predictive microbiology

Predictive microbiology aims at predicting responses of a microorganism to environmental conditions (temperature, pH, water activity, gaseous atmosphere, chemical preservatives, either singly or in combination), so that the effect of manufacture, distribution and storage on the extent of microbial proliferation in food can be evaluated (McMeekin et al. 1993, McMeekin et al. 1997, McMeekin et al. 2002). According to McDonald & Sun (1999), a predictive model is a mathematical function or equation in which the growth, survival or inactivation of a bacterium is depicted. McKellar & Lu (2003) defined it as a mathematical model that describes microbial responses to food environments. In order to construct and use a model to estimate the response of the microorganisms in a specific environmental condition, many disciplines can be involved, such as mathematics, chemistry, engineering and microbiology (Whiting 1995, McDonald & Sun 1999).

1.1.2 History of predictive microbiology

An early example of a predictive model for food was developed by Esty and Meyer (1922, cited by McMeekin & Ross 2002) for thermal processing, where a heat process would adequately destroy 10^{12} spores of *Clostridium botulinum* type A. In the1930's, Scott first introduced the concept of predictive microbiology and indicated the potential application of predictive microbiology to describe the growth of microorganisms, which could enable proactive instead of retrospective estimation of microbial safety (cited by McMeekin et al. 2008). The origin of "modern'' predictive microbiology starts in the 1960s and 1970s, when many manuscripts were published, focusing on applying kinetic models to address food spoilage and food poisoning problems. Meanwhile, some probability models based

on toxin production were also developed (McMeekin & Ross 2002). In the 1980s and the large part of 1990s, due to major food poisoning outbreaks and public requirements for a safe and wholesome food supply, substantial advances in predictive microbiology took place, and a range of kinetic models were developed (McMeekin et al. 2002), e.g. for *Staphylococcus aureus* (Broughall et al. 1983), *Salmonella enterica* serovar Typhimurium (Dickson et al. 1992), *Escherichia coli* O157:H7 (Sutherland et al. 1997), and *Clostridium botulinum* (Graham & Lund 1993). Kinetic models are enough to cope with the problem of spoilage. However, it is not adequate when dealing with the emergence of food-borne pathogens with a very low infectious dose, where quantitative information about growth limits may be more valuable (McMeekin et al. 2000). Moreover, because of the increasing awareness of the need for stochastic methods to be applied in quantitative risk assessments, stochastic models were suggested as the main work in the next decade (McMeekin et al. 1997, McMeekin et al. 2002).

1.1.3 Types of predictive model

Several model classification schemes in predictive food microbiology have been proposed.

1.1.3.1 Kinetic and probability models

Traditionally, predictive microbiology is considered to be classified into kinetic and probability models (Ross & McMeekin 1994, McDonald & Sun 1999).

Kinetic models estimate growth rate and the extent of the growth of the bacteria concerned (Ross & McMeekin 1994，Wu et al. 2000). The aim of such models is to interpret the time taken for a growth response with respect to the controlling environmental factors, including temperature, pH, water activity (a_w), gaseous atmosphere, nutrient content and preservatives (McDonald & Sun 1999). The Bělehrádek model (Square Root model), Arrhenius-type model, Polynomial or Response Surface models are examples of kinetic models (Ross & McMeekin 1994).

Probability models estimate the probability of occurrence of a particular event, such as germination of a spore or formation of detectable amount of toxin during a given time, in the specific environment (Ross & McMeekin 1994, McDonald & Sun 1999). In the model construction process, the probability that an individual spore or bacterium will survive and recover could be estimated as the number of spores or bacteria that recover in a culture or food divided by the number of viable spores or bacteria inoculated initially. Then the effects of the combination of preservative factors on this probability are modelled (Lund 1993). Therefore, the response measured by the probability model mentioned above relies on time for the response to become detectable, which depends on germination of spores or lag phase, growth rate and initial inoculum concentration (Ross & McMeekin 1994).

1.1.3.2 Empirical and mechanistic models

Empirical and mechanistic models are the two main types of model (Baranyi & Roberts 1994).

Empirical models are “data-driven” models, which summarize data with a mathematical function under experimental conditions, but importantly, provide little or no insight into the mechanisms defining the observations (McMeekin et al. 1993, McKellar & Lu 2003). These models are easy to use, straightforward and do not require any background knowledge (McDonald & Sun 1999). However, because of lack of understanding of the process, predictions using empirical models are limited to interpolated estimation within the range of environmental conditions used in the experiments (Krist et al. 1998). Nevertheless, empirical models are extensively and relatively successfully used in predictive microbiology (McKellar & Lu 2003). The Gompertz model, the modified Gompertz, Polynomial and Logistic models are examples of empirical models (Baranyi et al. 1993a, 1993b, Ross & McMeekin 1994, McDonald & Sun 1999, Baty et al. 2002, Huang 2011).

A mechanistic model is constructed on a theoretical basis and describes a hypothesis or series of hypotheses which represent the relationship between the independent factors which finally result in the observation (McMeekin et al. 1993, McMeekin et al. 2008). A detailed understanding of the mechanism of the bacterial response to the environment is needed to construct a mechanistic model (Krist et al. 1998). It not only predicts the growth parameters (growth rate and lag phase duration), but also gives an interpretation of the response, with consideration of its underlying mechanisms and provides a good understanding of bacterial growth (McMeekin et al. 1993, Baty et al. 2002, McMeekin et al. 2002, Baty & Delignette-Muller 2004). Mechanistic models have proved to be of more interest, and preferable to, empirical models as they have fewer parameters and each of them is microbiologically relevant. They can give a better foundation for subsequent development and expansion of the model (Draper 1988, McKellar & Lu 2003, Baty & Delignette-Muller 2004) and are claimed to be able to estimate the behaviour of the bacteria beyond the range of the experimental data used to develop the model (Krist et al. 1998). However, none of the models commonly used in predictive microbiology are purely mechanistic (McMeekin et al. 2002). Some of them have a mechanistic basis, e.g. the Baranyi model and the Hills model (McKellar & Lu 2003, Muñoz-Cuevas et al. 2010). It is anticipated that empirical models will become more and more mechanistic when more information about the microorganisms can be included in the model (Box & Draper 1987, cited by Baranyi & Roberts 1995).

1.1.3.3 Deterministic and stochastic models

Deterministic models are those that do not involve any randomness (Griffiths & Oldknow 1993) and where the outcome (the prediction) of the model is estimated using a single 'best guess' value of each independent variable in the model (Vose 2000). Therefore, the outcome is a precise value (Couvert et al. 2010) and uniquely determined by the initial conditions (Edwards & Hamson 1989). Although these models are often easy to understand (Gelman et al. 2010), as they do not take into

consideration variability of strains (Lindqvist 2006) and of individual cells (Baranyi & Pin 2003), they may provide an incomplete or misleading prediction (Lindqvist 2006).

In stochastic models, randomness is present and plays a central role in problem investigation (Edwards & Hamson 1989). Compared with deterministic models, the output of a stochastic model provides information about the distribution of contamination and a probability exceeding a particular critical level (Couvert et al. 2010). Such models are proposed for use when mechanisms are not entirely understood, or are so complex that they cannot be described deterministically (Pin & Baranyi 2006). For example, the first few division times of a large number of cells could be measured using an image system; however, as complicated and unknown intracellular processes occur during lag, use of a stochastic model has been suggested (Pin & Baranyi 2006). Currently, stochastic models are used to deal with single cell lag times (Couvert et al. 2010) and are proving a very useful technique to help understand lag complexity (Baty & Delignette-Muller 2004).

1.1.3.4 Primary, secondary and tertiary models

Although no absolute model classifications have been determined so far (McDonald & Sun 1999), the grouping into primary, secondary and tertiary models proposed by Whiting and Buchanan (1993) is recognized by some microbiologists.

1.1.3.4.1 Primary models

Primary models are first stage models describing response (e.g. change in number) of bacteria to a set of conditionsover time (pH, a_w, preservatives, naturally-occurring organic acids, interactions with other microorganisms, temperature, storage atmosphere) (McDonald & Sun 1999, McKellar & Lu 2003, Kutalik et al. 2005a). The aim of primary models is to describe the kinetic process of the microorganism using as few parameters as possible (parsimony) (McKellar & Lu 2003). The Gompertz, Logistic, Baranyi and

three-phase linear models are examples of primary models (McDonald & Sun 1999) (Table 1.1).

1.1.3.4.2 Secondary models

Secondary models are those that describe how the parameters of primary models (i.e. growth rate, lag time) of a given microorganism vary with the history of the culture and the environmental conditions (i.e. a_w, pH, temperature) (McDonald & Sun 1999, Kutalik et al. 2005a). Response surface equations, square root and Arrhenius functions are usually considered as secondary models (McDonald & Sun 1999; Table 1.1).

1.1.3.4.3 "Tertiary models"

Unlike primary or secondary models, which are a single or a series of mathematical function(s), "tertiary models" are user-friendly software packages, which incorporate one or more secondary models, e.g. Pathogen Modelling Program (PMP) developed by US Department of Agriculture (USDA) and the Food Micromodel (Whiting 1995) (Table 1.1). However, some microbiologists do not recognise "tertiary models" as models at all; considering them to be merely the software that facilitates use of models.

Table 1.1: Examples of primary, secondary and "Tertiary"models (McDonald & Sun 1999)

Primary model	Secondary model	"Tertiary model"
Gompertz function	Bělehrádek model (Square-root model)	USDA PMP
Modified Gompertz	Ratkowsky model (Square-root model)	ComBase
Logistic model	Arrhenius model	*Pseudomonas* Predictor
Baranyi model	Modified Arrhenius model	Expert system
First-order Monod model	Probability models	Food Micromodel
Modified Monod model	Polynomial and response surface model	
D value of thermal inactivation	z value	
Three phase linear model		

Finally, based on behaviour of bacteria, predictive models can be categorized into growth models, growth/no growth (boundary or interface) models and inactivation (e.g. thermal or non-thermal death or survival) models (McMeekin et al. 2002, McMeekin & Ross 2002). The growth/no growth interface model is a model which can estimate the probability of growth when a population of microorganisms is challenged by a single or a number of hurdles impeding growth at the border or limit of being able to grow (McMeekin & Ross 2002).

1.1.4 Applications of modelling

As the original data and models are saved in a database (e.g. ComBase), these can be retrieved and used to estimate the impact of processing and the distribution of growth of microorganisms (McMeekin et al. 1997). A sufficiently validated mathematical model can provide precise information about the population of microorganisms in a food from "farm to fork" in relation to specific product variables, including pH, a_w, cooking/storage

temperature, storage atmosphere and preservatives (Ross et al. 2000). In the past 20 years, predictive microbiology has been used in the food industry for developing new food formulations, shelf life estimation and evaluation of microbiological safety (Dodds 1993, Membré & Lambert 2008). In Hazard Analysis and Critical Control Point (HACCP) systems, modelling provides information on preliminary hazard analysis through showing which organisms can grow in a particular product (growth/no growth model) (McDonald & Sun 1999, McMeekin & Ross 2002) and enable establishment of a critical control point (McDonald & Sun 1999, Membré & Lambert 2008). In addition, predictive modelling could be used to optimize and control processes of food sterilization and fermentation (McMeekin & Ross 1996). Furthermore, predictive modelling has a role in education and training of food industry personnel (e.g. use of ComBase in the MSc Food Science course at London Metropolitan University), as graph generation through modelling can clearly illustrate the response of the microorganism to various conditions (McDonald & Sun 1999).

For government and policy makers, predictive microbiology can provide rational background knowledge for constructing guidelines, criteria and standards with regard to the microbiological status of food (Ross & McMeekin 1994). In risk assessment, predictive microbiology can used to describe microbial responses such as growth, survival and inactivation, which can facilitate an exposure assessment and dose-response assessment (Lammerding & McKellar 2003, Lindqvist 2006).

Finally, for laboratory use, predictive models provide information for analysing performance of media, e.g. by formulation (a_w, pH, addition of selective agents) to maximize growth of target microorganisms in enrichment processes and inhibit overgrowth by background microflora (McMeekin & Ross 1996, Stephens et al. 1997, Dalgaard & Koutsoumanis 2001, Dupont & Augustin 2009, Miled et al. 2011).

1.1.5 The modelling process

Based on the premise that response of microorganisms to a given environment is consistent and reproducible, the modelling procedure involves several steps, as follows (Dodds 1993, McDonald & Sun 1999):

1) Planning: designing experiments according to different requirements of models, including a) exploring the effects of variables on growth of microorganisms, or b) identifying the growth/no-growth boundary for determining preservation conditions. Although not mentioned by Dodds (1993) and McDonald & Sun (1999), the following should also be considered. c) choice of microorganism, d) use of single or mixed strains.

2) Data collection: subjecting the microorganism to various environmental conditions (pH, temperature, a_w), recording the responses and accumulating data.

3) Model construction: fitting raw data with different primary models and choosing the one which best fits the data. This stage usually involves developing primary models to decide the magnitude of the response of interest first, e.g. maximum specific growth rate, lag phase duration, time to achieve a specific level (cell number or concentration of metabolites), or survival/death rate in relevant environments. This is followed by constructing a secondary model relating the dependence of these factors on environmental conditions. Finally, a "tertiary model" may be developed by incorporating these algorithms into a computer software package (McMeekin et al. 2002).

4) Validating the model: Ross (1996) proposed two complementary measures, which are the bias factor and accuracy factor, to evaluate the performance of a model. The bias factor indicates whether there is systematic error in the model (systematic over- or under-prediction) and the accuracy factor measures the difference between the observed and predicted values (McMeekin & Ross

1996). Both provide an indication of the average deviation between the model prediction and observed data (Ross 1996). In some cases, models have been validated through comparison of predictions to observations published for the same organisms, but there are numerous limitations associated with this, e.g. variability of inoculum concentration, different techniques and general lack of information (Sutherland et al. 1994, 1996). In addition, as the main issue is not how well the model fits the data, but how accurately the model mimics the response of the microorganism to the given environment, it is suggested that the performance of the model needs be demonstrated with trials conducted independently ofthe laboratory in which the model was developed (McMeekin et al. 1997) and in real food (McMeekin & Ross 1996, McDonald & Sun 1999).

1.2 Lag phase

1.2.1 Definition of lag phase

Traditionally, growth of a bacterial culture is divided into lag, exponential, and stationary phases (Fig 1.1) (Baranyi 1998, Swinnen et al. 2004). The lag time is often observed when bacterial cells are transferred to a new environment, which is different from the previous growth conditions (Buchanan & Cygnarowicz 1990, Ross & Dalgaard 2003). The exponential growth phase follows lag and bacterial number doubles at a specific time interval, depending on the environment. Due to deficiency of nutrients and accumulation of metabolites, growth of bacteria is then partly inhibited and the bacterial concentration reaches a maximum, which is the stationary phase (Baty & Delignette-Muller 2004). In many cases knowledge of lag time and maximum growth rate is sufficient (Baranyi et al. 1993a, Baty & Delignette-Muller 2004). However, when growth is studied under suboptimum conditions, or when spoilage is studied, knowledge of transition to the stationary phase is necessary (Smelt pers. comm. 2014).

Various definitions of lag have been proposed. Penfold (1914) defined lag phase as the interval between inoculation of the bacterium and the time it reaches its maximum growth rate. It is conventionally measured as the time elapsed from inoculation to the intercept of the slope of the exponential phase with the extension of the initial inoculum number on the lorgarithmic scale (Fig 1.1) (Lodge & Hinshelwood 1943, Robinson et al. 1998). Pirt (1975) gave a similar lag definition: the transition period during which the specific growth rate reaches a maximum, specific to the growth conditions. Currently, this is the most widely used lag definition (Swinnen et al. 2004) and is called the geometrical lag (Métris et al. 2006). However, this definition is relevant only to a stable environment. It is not applicable when the environment changes, as there is no clear distinction between lag and exponential phase (Swinnen et al. 2004). According to the curve fitting procedure used for growth data, Buchanan & Cygnarowicz (1990) suggested lag could be defined as time elapsing from inoculation to when the second derivative of a typical growth curve is at its maximum point.However, it is suggested that for a typical sigmoidal (Gompertz model) growth curve, the difference between these two lags is very small (Fig 1.2; Zwietering et al 1992, Swinnen et al. 2004). Several other definitions of lag with a mechanistic basis have been suggested. Baranyi et al. (1993a) suggested a more mechanistic definition, which is the time required for a critical substrate that is responsible for the "bottleneck" limiting growth accumulates to a threshold amount in a cell. In this definition of lag, the concept of physiological state was introduced to describe the effect of history on the lag and the lag was considered to be a function based on the physiological state ($\alpha(N_0) = e^{-\mu' Lag(N_0)}$, $\alpha(N_0)$ represents the physiological state of N_0 cells; μ' represents the specific growth rate; $Lag(N_0)$ is the population lag) (Baranyi & Roberts 1994, Baranyi & Roberts 1995). Ross & McMeekin (2003) explained it as the period that a bacterium needs to change physiological status in the cell and to optimally exploit the new environment. Robinson et al. (1998) proposed that the lag is dictated by the "work" that needs to be done (h_0) by the cells to enable them to adapt to the new environment and to

prepare to divide, and the rate at which the cells are able to "work" ($h_0 = \mu' Lag(N_0)$， h_0 is work to be done). However, it should be noted that the work to be done (h_0) is actually reparametrization of physiological state. This can be described as 'physiological lag' (Métris et al. 2006).

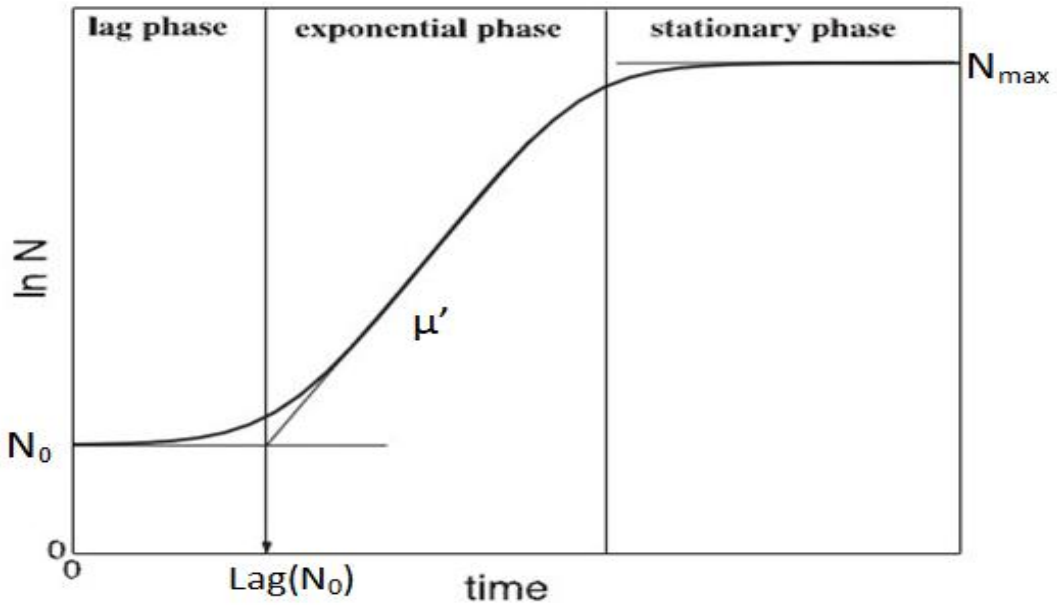

Fig 1.1: The growth curve of bacteria (Swinnen et al. 2004)

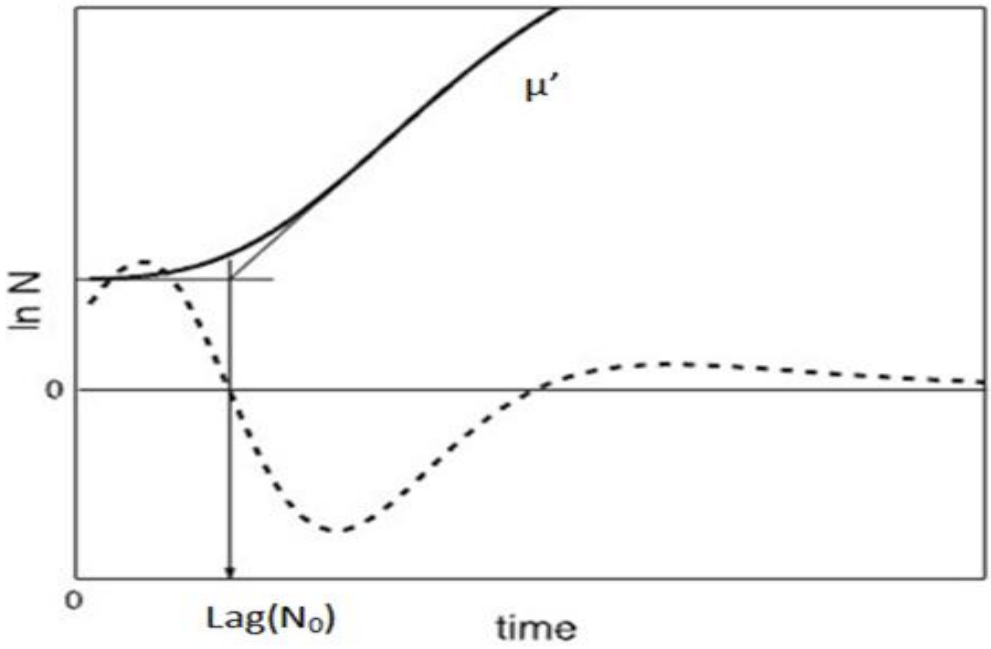

Fig 1.2: Comparison between the lag proposed by Pirt (1975) (solid line) and Buchanan and Cygnarowicz (1990) (dashed line) (Swinnen et al. 2004).

1.2.2 Factors contributing to lag phase

According to Pirt (1975), the fundamental factors that induce lag are as follows:

1) Changes of physical growth conditions (temperature, pH, a_w) and nutrient availability;

2) Existence of an inhibitor;

3) Germination of the spore (entering the vegetative state)

4) Condition of inoculum (e.g. use of exponential/stationary phase cells; stressed cells).

1.2.3 Challenges in predicting lag duration

Growth rate and lag are the two parameters used to characterise the growth curve of microorganisms (Baranyi et al. 1993a, Baty & Delignette-Muller 2004). Maximum population density and total yield of cells may also be modelled (McQuestin et al. 2010). Unlike growth rate, for which numerous models have been published to describe the effect of variables on growth (Guillier et al. 2006), the lag time is difficult to predict (Robinson et al. 1998, 2001) and any such predictions have been poor (McMeekin et al. 2002, Baranyi 2002) and with a high average error (Delignette-Muller et al. 1995).

One of the reasons for prediction of lag being unreliable is that unlike growth rate, which depends solely on the environmental conditions (Niven et al. 2006), lag is affected by the physiological status of the cell, which is a consequence of its prior history (Baranyi & Roberts 1994, McKellar & Lu 2005). And it is suggested that difficulty in predicting lag is not because of lack of an appropriate model, but due to deficiency of information about the physiological status of bacteria (McMeekin et al. 1997, Baty & Delignette-Muller 2004), for instance, cells in the lag phase may be actively increasing in size, or they may be damaged, dead, viable but not culturable, or producing endospores (McMeekin et al. 1997). Furthermore,

it is proposed that variation in the actual definition of lag (i.e. purely geometric, purely mathematical or physiological lag) also makes lag difficult to predict (Baty & Delignette-Muller 2004). In order to facilitate comparison between lag, 'conventional' lag (geometrical lag as in Fig 1.1) is recommended for calculating lag time (Zwietering et al 1992).

1.3 *Cronobacter* spp.

1.3.1 Taxonomy

Cronobacter spp., previously known as *Enterobacter sakazakii*, is a member of the family *Enterobacteriaceae.* Until 1980, *Cronobacter* spp. were referred to "yellow-pigmented *Enterobactercloacae*" and belonged to the genus *Enterobacter*. However, these strains of "yellow-pigmented *Ent.cloacae*" exhibited 83-89% similarity with the strains within this group and only 31-49% with other strains of *Ent. cloacae,* based on DNA-DNA hybridization. Moreover, they were always D-sorbitol negative, produced extracellular deoxyribonuclease and a yellow pigment and were more sensitive to ampicillin and cephalothin compared with *Ent. cloacae*. Consequently, they were reclassified as *Ent. sakazakii* by Farmer et al. (1980). According to ten biochemical tests, 15 biotypes of 57 strains of *Ent. sakazakii* were described, with biotype 1 (the wild type) being the most common.

The taxonomic work by Iversen et al. (2008) has led to reclassification of *Ent. sakazakii* as a new genus, *Cronobacter,* based on detailed fluorescent-amplified fragment length polymorphism (f-AFLP) fingerprinting, full-length 16S rRNA gene sequencing, ribotyping, DNA-DNA hybridization and phenotypic profiles. The new *Cronobacter* genus is still a member of the *Enterobacteriaceae*, comprising at least five species, with a possible sixth species, *Cronobacter* genomospecies 1, which contains only two strains (Iversen et al. 2008, Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) 2008). Joseph et al. (2012) identified a new species (*Cr. condimenti*) and another new species, *Cr. universalis* replaced the original *Cronobacter*

genomospecies 1, based on phenotypic classification, 16S rDNA sequencing, multilocus sequence typing (MLST) and DNA hybridization. With similar methods, recently, three new species *Cr. zurichensis*, *Cr. pulveris*, and *Cr. helveticus* were proposed (Brady et al. 2013).

So, the latest classification scheme of the genus *Cronobacter* based on the proposition of Iversen et al. (2008), Joseph et al. (2012) and Brady et al. (2013) is as follows:

- *Cronobacter sakazakii*;
- *Cronobacter malonaticus*;
- *Cronobacter turicensis*;
- *Cronobacter muytjensii*;
- *Cronobacter dublinensis*;
    - *Cronobacter dublinensis* subsp. d*ublinensis;*
    - *Cronobacter dublinensis* subsp. *lausannensis*;
    - *Cronobacter dublinensis* subsp. *lactaridi*;
- *Cronobacter universalis*;
- *Cronobacter condimenti*;
- *Cronobacter zurichensis;*
- *Cronobacter pulveris;*
- *Cronobacter helveticus.*

In this report, the name *Cronobacte*r spp. is used to represent organisms which were earlier named *Ent. sakazakii*.

1.3.2 Characteristics of *Cronobacter* spp.

Cronobacter spp. are oxidase-negative, catalase positive, faculatively anaerobic, generally motile, peritrichously flagellated, Gram-negative rods (Fig 1.3). They are approximately 3 µm x 1 µm in size (Iversen et al. 2008, Chenu & Cox 2009, Osaili et al. 2009a). Two or more types of colonial morphology of fresh *Cronobacter* isolates are reported, which include a scallop-edged rubbery colony and a typical smooth colony (Fig 1.4). After subculturing, the former morphological appearance converts to the latter

(Farmer et al. 1980, Nazarowec-White & Farber 1997a). Besides the characteristic yellow pigmentation, *Cronobacter* can also produce a gum-like extracellular polysaccharide (Schmid et al. 2009).

Growth of *Cronobacter* spp. occurs between 6 and 45 ^{o}C with an optimal growth temperature between 37 and 43 ^{o}C. Some strains can grow at 47 ^{o}C (Iversen et al. 2004b). In terms of minimum growth temperature, Nazarowec-White & Farber (1997b) reported a temperature range from 5.5 ^{o}C to 8 ^{o}C. Kucerova (2011) reported that *Cr. turicensis* could grow below 5 ^{o}C. The pH range for growth of *Cronobacter* spp. is between 5 and 10, and it grows in sodium chloride concentrations up to 7% (W/V) (Iversen et al. 2008).

There is a dearth of information on virulence and pathogenicity of *Cronobacter* spp. (FAO/WHO 2004a). The study of toxins by Nazarowec-White & Farber (1997a) and Pagotto et al. (2003, 2009a) showed that some strains of *Cronobacter* spp. could produce enterotoxin-like compounds with the suckling mouse assay, but the ability of strains to produce toxin may vary.

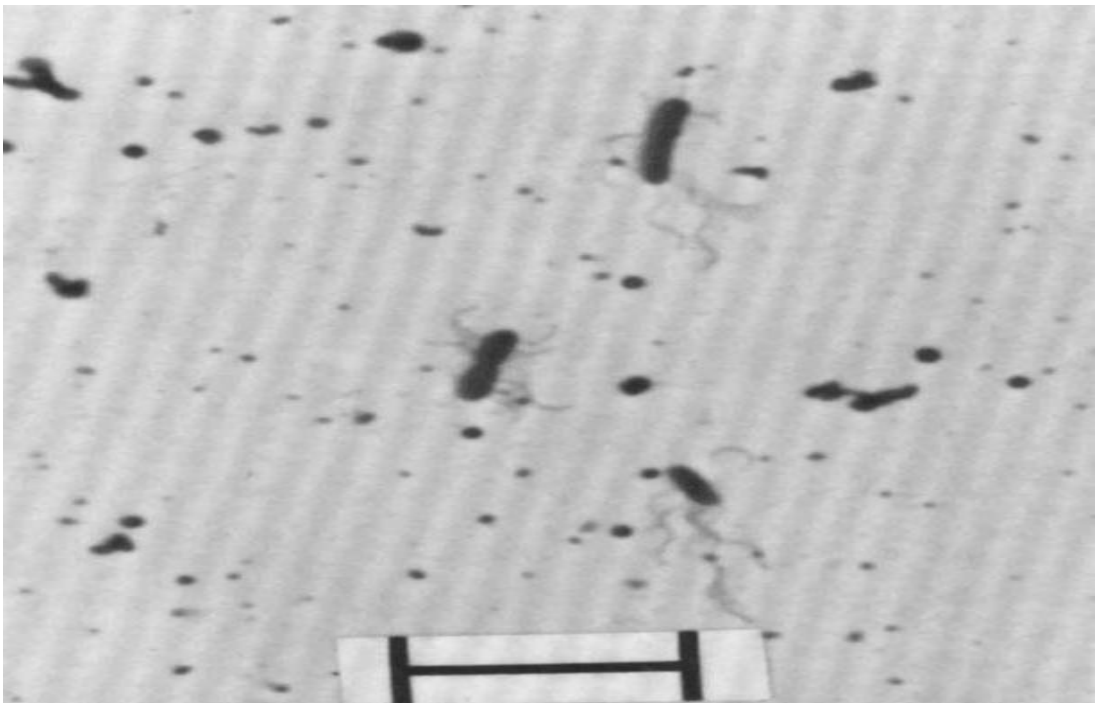

Fig 1.3: Photomicrograph of cells of *Cronobacter* spp. (Bar marker = 10 µm) (Farmer et al. 1980)

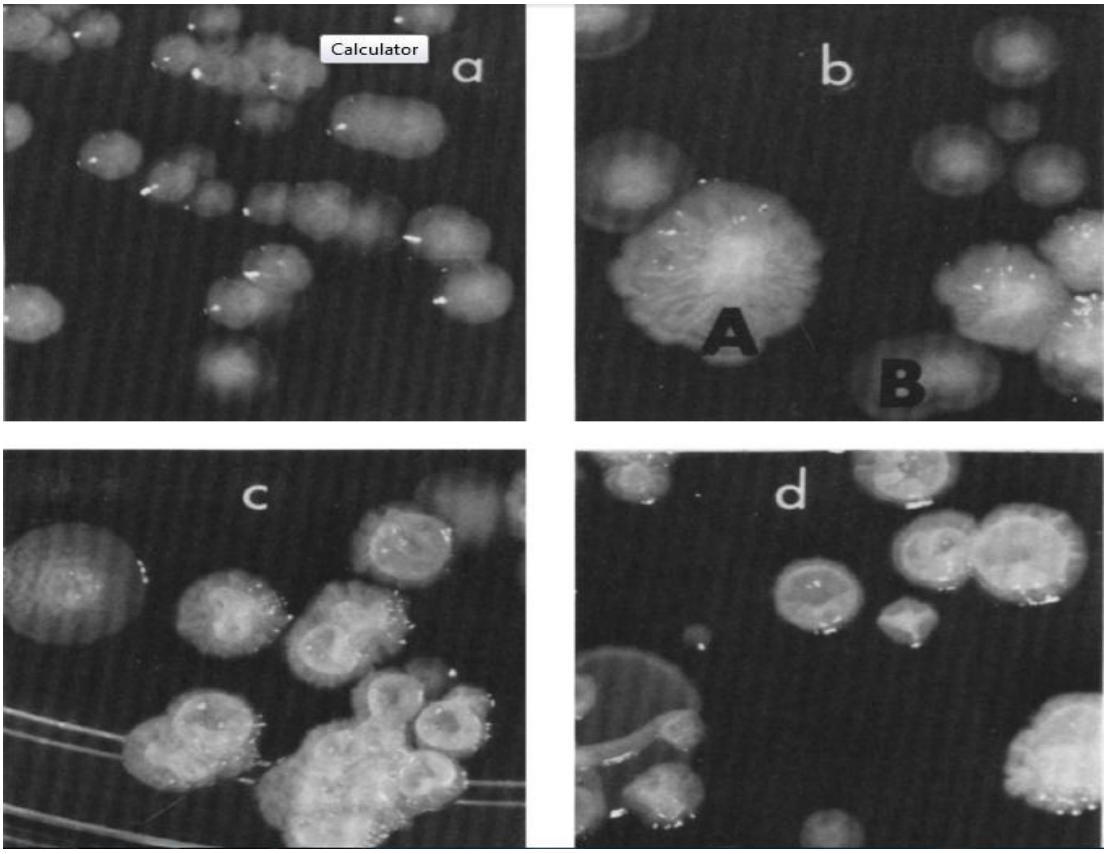

Fig 1.4: Various colonial morphologies of *Cronobacter* spp. (Trypticase soy agar 48 h incubation at 25 ^{o}C) (Farmer et al. 1980)

1.3.3 Clinical manifestation

Cronobacter spp., mostly *Cr. sakazakii, Cr. malonaticus, Cr. turicensis* (Kucerova et al. 2010, 2011), are increasingly regarded as emerging opportunistic pathogens of humans (Farber 2004, Cawthorn et al. 2008) and can infect all age groups (FAO/WHO 2004a, Terrangno et al. 2009).

Due to undeveloped immune systems and lack of competing intestinal flora (Chap et al. 2009, Richardson et al. 2009), infants of less than one year are at particular risk (Pagotto &Farber 2009). This includes specifically low-birth-weight (<2500 g) neonates (Chenu & Cox 2009, Jang& Rhee 2009, Richardson et al. 2009, Kucerova et al. 2010), premature infants (<36 weeks gestational age) to post-term infants (up to 4-6 weeks) (Farber 2004), infants (<28 days) (Mullane et al. 2007a, Chenu & Cox 2009, Richardson et al. 2009), immunocompromised infants and full-term infants hospitalized in level 2 and 3 neonatal intensive care units

(NICU) (Farber 2004). Symptoms related to *Cronobacter* spp. infection in infants include meningitis, necrotizing enterocolitis, septicaemia, bacteraemia and brain abscess (Terrangno et al. 2009, O'Brien et al. 2009, Oonaka et al. 2010) (Fig 1.5a). The sequence type 4 of *Cr. sakazakii* was associated with the majority of severe neonate infections, especially meningitis, identified by Joseph and Forsythe (2011) using MLST. The incidence of *Cronobacter* spp. infection among infants is relatively low, according to a United States FoodNet survey in 2002; an estimated annual rate of 1 per 100,000 infants less than 12 months old (Cawthorn et al. 2008, FAO/WHO 2006, Richardson et al. 2010), and the annual incidence among low birth weight infants is about 8.7 per 100, 000 (FAO/WHO 2006, Yan et al. 2012). However, the illness carries a high fatality rate; the mortality rate of meningitis and necrotizing enterocolitis due to *Cronobacter* spp. infection is 40-80% and 10-55% respectively (Iversen & Forsythe 2003, Kim et al. 2008). The overall mortality rate of the microorganism is 20-80% in infected infants (Hunter et al. 2008). Thus, although the disease is fairly rare, the consequences can be emotionally devastating. The first reported infant infection by *Cronobacter* spp. ("yellow pigmented strain of cloaca group") occurred in Osterhills Hospital (St. Albans City Hospital), UK (Urmenyi & Franklin 1961). From then until 2008, about 162 cases of *Cronobacter* spp. infantile infection cases and at least 29 deaths have been reported worldwide, including UK, USA, Denmark, Iceland, Canada, Greece, Netherlands, Spain, Portugal (FAO/WHO 2008) (Table 1.2). It should be recognised that this number must be under-reported as Muytjens et al. (1983) re-examined 20 *Enterobacter* strains isolated from cerebrospinal fluid in the Netherlands and demonstrated that five strains were in fact *Cronobacter* spp. (cited by Iversen& Forsythe 2003, Healy et al. 2010). The prognosis differs between *Cronobacter* spp. infected infants that develop bacteraemia and those who develop meningitis. Those with bacteraemia generally have a better prognosis (Mullane et al. 2007a). Severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development can occur among 94% of meningitis survivors (Nazarowec-White et al. 1999, Mullane et al. 2007a, Terrangno et al. 2009, Richardson et al. 2010). Thus, along

with *Listeria monocytogenes*, *Clostridium perfringens* types A and Band *Cryptosporidium parvum*, *Cronobacter* spp. are defined as a 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' by the International Commission on Microbiological Specifications for Foods in 2002 (cited by Iversen & Forsythe 2003, Kim & Loessner 2008, Chenu & Cox 2009).

Moreover, *Cronobacter* can also affect immunocompromised adults, especially the elderly (>55 years) who have potential disorders such as malignancy, with 50–67% fatality rate from sepsis and pneumonia (Lai 2001, Gurtler et al. 2005, Hunter et al. 2008). Unusual cases of *Cronobacter* spp. infection among non-immunocompromised elderly or young healthy adults have been reported (Corti et al. 2007, See et al. 2007). The first *Cronobacter* spp. adult infection case with bacteraemia was reported by Jimenez and Gimenez (1982), with an additional 19 cases reported between 1982 and 2010 (cited by Healy et al. 2010). Symptoms related to adult *Cronobacter* spp. infections include abscess, pneumonia, ulcers and wound infections (Healy et al. 2010) (Fig 1.5b). As many clinical laboratories do not test for this microorganism and there is no national or global reporting system in many countries (Farber 2004), infections among adults are often underestimated (Reich et al. 2010).

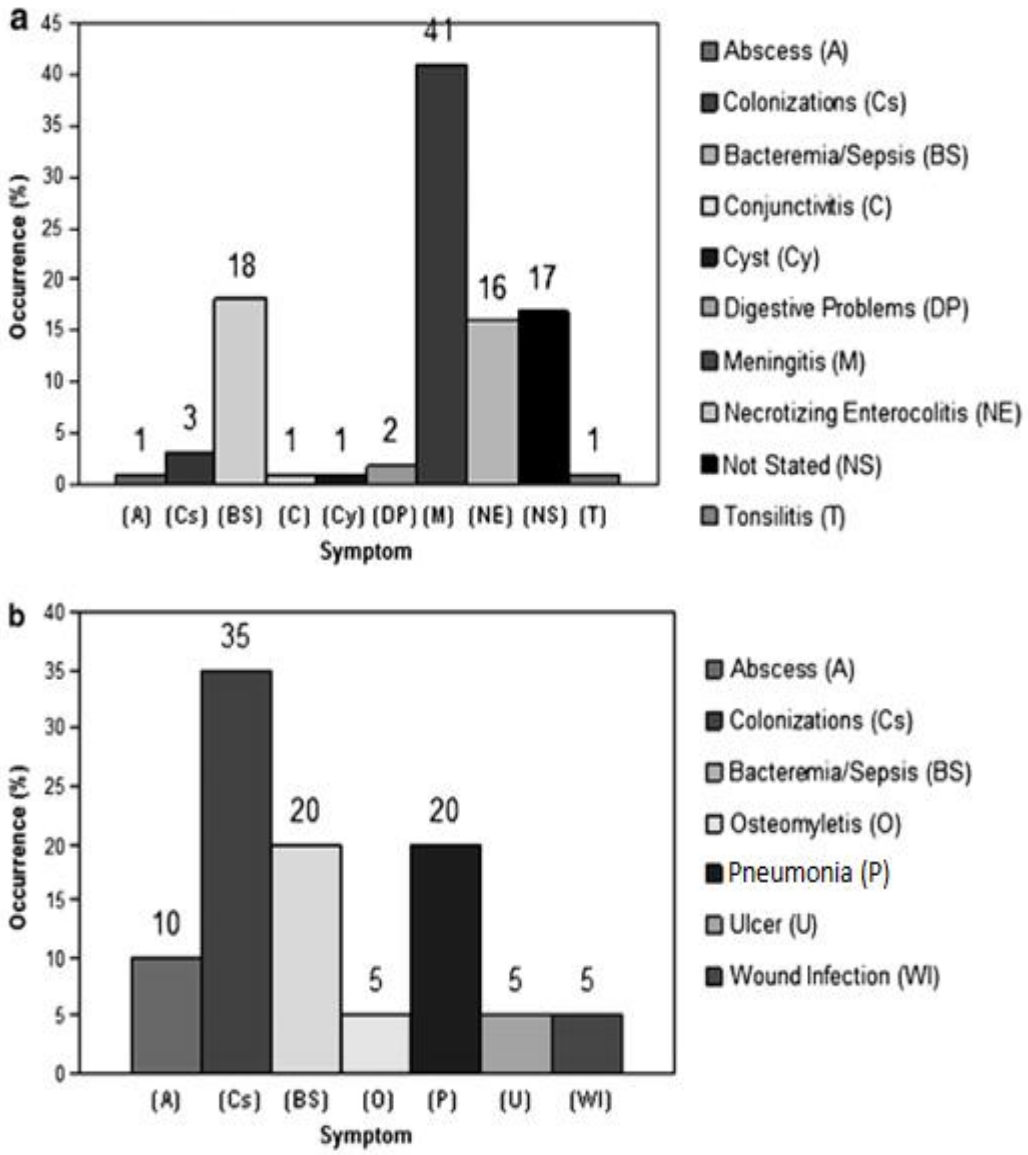

Fig 1.5: Symptoms and incidence of occurrence as a percentage of total *Cronobacter* infection

(a) in children and infants and (b) in adults (Taken from Healy et al. 2010)

Table1.2: Overview of reported cases of *Cronobacter* spp. infection and colonization in infants and young children from 1961 to 2008 (modified based on FAO/WHO 2008)

Year (report)	Country	No of cases	Deaths
1961	UK	2	2
1965	Denmark	1	
1979	USA	1	
1981	USA	2	
1983	Netherlands	8	6
1984	Greece	1	
1985	USA	1	
1987	Greece	11	4
1988	USA	2	
1989	Spain	1	
1989	Ireland	3	1
1989	USA	4	
1989	Portugal	1	1
1990	USA	1	
1991	USA	1	
1994	Germany	1	
1996	Canada	1	
1997	Scotland	1	
2000	Brazil	5	
2000	USA	1	
2001	USA	4	
2001	Belgium	12	2
2001	Israel	5	
2002	USA	1	
2002	USA	5	
2002	USA	1	
2002	USA	1	
2002	USA	1	
2002	USA	1	1
2002	USA	10	1
2002	Israel	3	
2002	Belgium	1	1
2003	Brazil	1	
2003	Hungary	1	
2003	USA	5	1
2004	USA	2	
2004	New Zealand	5	1
2004	Hungary	1	
2005	USA	2	
2005	Hungary	1	
2006	USA	5	
2006	Hungary	1	
2006	France	9	2
2007	France	18	4
2007	USA	9 (possible 10)*	1
2007	Canada	1	
2007	India	2	1
2007	Spain	1	
2008	USA	3	
2008	Japan	1	
	Total	162 (possible 163)	29

*: Represents one case where the illness onset age was 13 months. The illness onset age of other cases was less than 1 year.

1.3.4 Infectious dose

Currently, there is no epidemiological evidence for a specific infectious dose related to *Cronobacter* spp. (Iversen & Forsythe 2003, Healy et al. 2010). Based on the infectious doses of *Esch. coli* O157:H7 and *L. monocytogenes* 4b, Iversen and Forsythe (2003) proposed 1000 *Cronobacter* spp. cells as the approximate infectious dose. In 2004, FAO/WHO suggested 10,000 cells as the infectious dose of *Cronobacter* spp. Research on animal models indicated that infectious dose and lethal dose via oral administration of *Cronobacter* spp. varied significantly, from 10^2 cfu to 10^5 cfu and 10^2 cfu to 10^9 cfu respectively (Table 1.3) (FAO/WHO 2004a). However, animal models can be unreliable predictors of the situation in humans. Although infectious dose and lethal dose vary, based on estimates and experimental results, it is widely accepted that the dose-response of *Cronobacter* spp. is also host dependent (Iversen & Forsythe 2003, Healy et al. 2010).

Table 1.3: Minimum infective and lethal dose of *Cronobacter* spp. for different animal models

Animal model	Oral administration		intraperitoneal administration		Reference
	ID	LD	ID	LD	
Suckling mice	10^5 cfu	-	10^3 cfu	-	Nazarowec-White & Farber 1997a
Suckling mice	-	10^7 cfu	-	10^5 cfu	Pagotto et al. 2003
Neonatal mice	10^2 cfu	10^2 cfu	-	-	Richardson et al. 2009
Non-primate animal*	-	10^9 cfu**	-	-	Pagotto et al. 2009b

ID: Infection dose; LD: lethal dose.
*non-primate animals: include young pigs, chicks, rabbits, guinea pigs and young gerbils
**The dose was lethal only to some gerbils (6/36), no human-type clinical symptoms were observed in any young animals.

1.3.5 Natural reservoirs, food and environmental sources

Because of the ubiquitous nature of *Cronobacter* spp., the primary reservoir remains unidentified (El-Sharoud et al. 2009). *Cronobacter* spp have been detected in various environmental samples including soil (Khan et al. 1998, Sehnem et al. 2010), sewage (Dudley et al. 1980) and mesothermal springs (Mosso et al. 1994). Iversen & Forsythe (2003) suggested that water, soil and plants may be the main reservoir of *Cronobacter* spp.

Although the natural reservoir of *Cronobacter* spp. remains unclear, they have been isolated from a wide spectrum of food, e.g. plant sources including vegetables, rice, bread, tofu, and foods of animal origin such as milk and meats (Chenu & Cox 2009, El-Sharoud et al. 2009), from primary raw food or food ingredients e.g. herbs and spices to final products such as sausages, cheeses and ready-to-eat foods (baked confectionary, fresh salad and sprouted seeds) (Iversen & Forsythe 2004, Friedemann 2007, El-Sharoud et al. 2009, Jaradat et al. 2009, Baumgartner et al. 2009). In addition, even tap water (Lee & Kim 2003), bottled mineral water, glass distilled water, activated carbon-treated drinking water are not free of *Cronobacter* spp. (Friedemann 2007) (Table 1.4).

Furthermore, *Cronobacter* spp. also can occur in food processing plants, hospital equipment, and household environments. A survey carried out by Guillaume-Gentil et al. (2005) found 40% of 192 environmental samples from four powdered infant formula (PIF) plants were contaminated with *Cronobacter*spp. Craven et al. (2010) investigated 298 environmental samples from 100 locations in the non-processing and processing environments of five milk powder factories, and reported 32% of the samples were *Cronobacter* spp. positive, including the samples from external roofs above spray driers, in air treatment areas and where high foot traffic occurs. Besides its presence in PIF or dairy factories, Kandhai (2004) isolated *Cronobacter* spp. from various environmental samples in other food processing environments such as a chocolate plant (25%),

cereal (44%), potato flour (27%) and pasta factories (23%). Masaki et al. (2001) surveyed the Gram-negative bacteria from patients and the hospital environment in a geriatric ward which was specifically subject to infection control against methicillin-resistant *Staph. aureus* (MRSA) and reported that *Cronobacter* spp. were isolated from the air in geriatric wards (agar "settle" plates). Hurrell et al. (2009) investigated 129 neonatal nasogastric enteral feeding tubes used in NICU and found 2% of the tubes were contaminated by *Cr. sakazakii*. In one *Cronobacter* spp. infection, the source of the organism was traced back to the blender used for preparing the PIF (Bar-Oz et al. 2001). In addition, according to the survey conducted by Palcich et al. (2009) in three hospitals in Brazil, *Cronobacter* spp. were found in the residue from one nursing bottle and on the cleaning sponge in the milk kitchen of a maternity ward. Finally, *Cronobacter* spp. have been isolated from household vacuum-cleaner dust (Kandhai 2004, Jaradat et al. 2009, Molloy et al. 2009) and domestic refrigerators (Kilonzo-Nthenge et al. 2008).

1.3.6 *Cronobacter* and powdered Infant formula (PIF)

According to Codex Alimentarius (1981), infant formula is defined as a breast-milk substitute which is particularly designed to meet infants' nutritional requirements without other supplementation during the first months of life up to the introduction of appropriate complementary feeding.

Possibly associated with the ubiquitous nature of *Cronobacter* spp., PIF has been demonstrated to be an important source of *Cronobacter* spp. infection for bottle-fed infants and a transmission vehicle, by using microbiological and molecular typing methods and epidemiological studies (Friedemann 2007, Mullane et al. 2007b, Miled et al. 2010, Ye et al. 2010). The presence of *Cronobacter* spp. in dried milk could be traced back decades. When *Ent*. *sakazakii* was defined as a species in 1980, it was realised that one such strain was recovered from dried milk by Thornley (1960) (cited by Osaili & Forsythe 2009).This strain is now strain NCTC 8155 in the National Collection of Type Cultures. Clark et al. (1990) first confirmed an epidemiological correlation between *Cronobacter* spp.

recovered from patients in two hospitals and PIF with a combination of typing methods (cited by Lehner et al. 2004). Since then, the epidemiological correlation between PIF and *Cronobacter* spp. has been reported in many cases (Van Acker et al. 2001, Himelright et al. 2002).

The first reported large survey was carried by Muytjens et al. (1988) who analysed 141 PIF samples from 35 countries and found 20 (14%) were contaminated by *Cronobacter* spp. and the levels of contamination were in the range of 0.36 - 66.0 cfu/100 g.After that, a number of surveys were carried out to study prevalence of *Cronobacter* spp. in PIF in different countries (Table 1.5). These revealed a prevalence rate between 2% and 25%, with a contamination level generally less than 1 cfu/100 g.

Due to *Cronobacter* spp. contamination, several high profile recalls of PIF have occurred worldwide. In November 2002 and January 2003, respectively, 1.5 million cans and 3,030 cans of PIF were recalled separately in the United States (Mullane et al. 2007b). In December 2004, a worldwide voluntary recall of PIF, which included Brazil, Hong Kong, Ireland, the Gambia, Gabon and the United Kingdom, happened due to a suspected link to the fatal meningitis outbreak among neonates in France (FAO/WHO 2006, Mullane et al. 2007b, Health Protection Agency (HPA) 2010).

Table 1.4: Examples of food contaminated by *Cronobacter* spp. (from Friedemann 2007, Baumgartner et al. 2009)

Food	Property	Origin
1. Food of vegetable origin		
Rice, rice starch, rice flour	Dried product	USA
Rice seeds	Dried product	Philippines
Adult cereal, infant cereal	Dried product	USA
Herbs and spices	Dried product	UK
Nuts, fruit, almond, pistachio, coconut powder, sesame seeds	Dried products	UK
Soy bean sprouts	Fresh produce	Germany
Mung bean sprouts, alfalfa sprouts	Fresh produce	Norway
Mung bean sprouts, sprout mixture	Fresh produce	Switzerland
Lettuce	Fresh produce	Spain
Mix salad vegetable	Sliced, ready-to-eat	Italy, Germany
Vegetable, laver (red algae)	Ready-to-eat	South Korea
Tofu (soy)	Coagulated soy milk	South Korea
2. Food of animal origin		
Sausages	Cooked, cold cuts	Germany
Meat	Minced	Canada
Meat	Raw	Japan
Cheese		France
Milk		Switzerland
Egg	Egg albumen and yolk	Italy
Fish	Fired, ready-to-eat street food	Ghana
Fish bolus		China
3. Water		
Tap water		Republic of Korea
Activated carbon-treated drinking water		USA
Bottled mineral water		Germany
Glass distilled water		USA

Table 1.5: Surveys of *Cronobacter* spp. in PIF (modified, based on Osaili & Forsythe 2009)

Number of samples	*Cronobacter* spp. (positive)	Enumeration (sample number) (cfu/100 g)	Reference
141	20 (14.2%)	0.36–66	Muytjens et al. 1988
120	8 (6.7%)	0.36	Nazarowec-White and Farber 1997b
101	2 (2%)	ND	Heuvelink et al. 2003 (cited by Osaili & Forsythe 2009)
82	2 (3.9%)	ND	Iversen & Forsythe 2004
98	12 (12.2%)	0.22–1.61	Santos 2006 (cited by Osaili & Forsythe 2009
29	2 (6.9%)	ND	Estuningsih et al. 2006
8	2(25%)	ND	Shaker et al. 2007
25	3 (12%)	ND	Jung & Park 2006
102	7 (6.8%)	ND	Lee et al. 2010
149	9(6.6%)	0.36 MPN/100 g (8) 0.91 MPN/100 g (1)	Oonaka et al. 2010

ND: not done.

1.4 Background to this project

Research on lag is of considerable interest to food microbiologists (Buchanan & Cygnarowicz 1990) and the food industry (Baranyi & Roberts 1994, Baranyi & Pin 1999, Kutalik et al. 2005b, Rasch et al. 2007). The idea of food preservation andprocessing (e.g. heating, use of preservatives) is to prolong the lag phase (Grahame 2000, Stephens et al. 1997, McMeekin et al. 2008), and therefore delay growth of spoilage and/or pathogenic bacteria (Buchanan & Cygnarowicz 1990), thus maintaining safety and quality of the food (Bréand et al. 1997).

Consumers nowadays demand milder heat processes and minimal use of preservatives, since such foods should retain natural flavour and taste for as long as possible (Smelt et al. 2002, Velliou et al. 2011) and are perceived to be more "healthy" (Peck 2006). In European countries, these ready-to-eat foods constitute more than 60% of the average shopping basket (Jackson et al. 2007). In the United Kingdom, the total value of prepared chilled food increased by 81% from 1999 to 2006 with a value of £8,230 million (Chilled Foods Association, no date). The safety and quality of these foods rely solely on refrigeration or a combination of minimal heat treatment with storage at chilled temperature (≤ 8 ^{o}C), sometimes in a modified atmosphere or vacuum pack, as well as intrinsic parameters of the foods (e.g. pH, a_w), with a limited shelf life (Bréand et al. 1997, Peck 2006).

As PIF is not treated with high temperature for sufficient time during processing, the final product is not sterile (Farber 2004). Moreover, intermittent contamination of PIF may occur after the heating process during the addition of heat labile ingredients (Schmid et al. 2009). In order to reduce the bacterial load, FAO/WHO (2006) suggested using hot water at 70 ^{o}C to reconstitute PIF and limiting the time between reconstitution and feeding to less than 2 h (FAO/WHO 2006, Forsythe 2009). However, these recommendations have not been widely adopted and PIF may have instructions recommending a reconstitution temperature as low as 40 ^{o}C (Forsythe 2009, Osaili et al. 2009a, Fang et al. 2012). A mini survey carried out in China showed that among 10 randomly collected PIF samples, 20% (2/10) suggested using room /warm temperature water cooled from boiled water, 20% (2/10) 40 ^{o}C water, 30% (3/10) water at or below 50 ^{o}C, 30% (3/10) 40 ^{o}C-60 ^{o}C water. In addition, only one (10%) suggested feeding within one hour of reconstitution, two (20%) suggested feeding with one hour or refrigerated storage (2-4 ^{o}C) and feed within 24 h (it is not known whether reheating is recommended). There was no holding time limit instruction for the other seven PIF samples (personal observation in China) (Table 1.6).

Table 1.6: Mini survey of preparation instruction of PIF in China (personal observation, 2011)

NO	Temperature of water	Holding time or storage
1	Room temperature water cooled from boiled water	Feed within one hour or refrigerated storage (2-4 ^{o}C) and feeding within 24 h
2	Warm temperature water cooled from boiled water	Feed within one hour or refrigerated storage (2-4 ^{o}C) and feeding within 24 h
3	40 ^{o}C	Feeding within one hour of reconstitution
4	40 ^{o}C	-
5	50 ^{o}C	-
6	≤50 ^{o}C	-
7	≤50 ^{o}C	-
8	40 ^{o}C-60 ^{o}C	-
9	40 ^{o}C-60 ^{o}C	-
10	40 ^{o}C-60 ^{o}C	-

Given their ubiquitous nature (Friedemann 2007), *Cronobacter* spp. may gain access to ready-to-eat food through primary contamination of raw materials, in which they may survive sublethal heating. Alternatively food may be contaminated through secondary contamination during food processing and preparation, e.g. contaminated air particles or dust in the processing area. Such contaminants are unlikely to have been sublethally heated and are therefore present in food in a non-heat stressed condition, although drying will cause stress. *Cronobacter* spp. present in reconstituted PIF also probably survive reconstitution temperatures and may be stressed from sublethal heating. Alternatively, they may be derived from the environment or from cross contamination, in which case they will probably unstressed. In both cases, this may lead to the foods

becoming unsafe, as untreated and mildly heat stressed *Cronobacter* spp present in the foods may have the potential to recover and grow during shelf life (ready-to-eat food) or during the holding time (PIF) (Nazarowec-White & Farber 1997c, Miled et al. 2011). Consequently, if they are able to multiply in the food to an infective level, food borne disease could be triggered (Wu et al. 2000, Baranyi et al. 2009). It is therefore important to determine the potential for untreated and sublethally heat stressed *Cronobacter* spp. to recover and initiate growth.

In reality, pathogens are often present at low levels in food (Francois et al. 2003, Métris et al. 2006). In terms of PIF, most research indicates that the contamination level with *Cronobacter* spp. is <1 cfu/100 g. At such a low level, the inherent biological variability between individual cells will play a more important role in the behaviour of the resulting population than at higher concentrations (Standaert et al. 2007). It has been demonstrated theoretically and experimentally that lag time will be affected by inoculum level when cell number is less than 100 cells/ml, which could illustrate that not all cells are able to divide, or the statistical effect caused by low cell number (Robinson et al. 2001, Métris et al. 2005, 2006).The mean value and variability of individual lag and population lag is not necessarily the same (Métris et al. 2003, D'Arrigo et al. 2006, Manios et al. 2011). Population lag usually represents the behaviour of those cells with shortest lag time and it is less than the mean lag time of the individual cells in the population (Guillier et al. 2006, Niven et al. 2006). In addition, the relationship between individual lag and population lag is not straightforward (Métris et al 2003, D'Arrigo et al. 2006). It is not possible to determine lag times of individual cells and their distribution from the measured population growth curve. However, deterministic population growth can be predicted from the expected value of the individual stochastic growth curve (Baranyi 2002, Kutalik et al. 2005a). Therefore, it is highly important to explore the lag of *Cronobacter* spp. at an individual cellular level.

Currently, some models have been derived for *Cronobacter* spp. Nazarowec-White et al (1999) developed a linear model to describe the thermal inactivation of *Cronobacter* spp. in bovine milk in a high-temperature, short-time pilot scale pasteurizer. Lambert and Bidlas (2007) examined the growth inhibition effect of a range of weak acids (lactic, acetic, propionic, citric, sorbic and benzoic), pH, NaCl and temperature and some of their combinations on *Cronobacter* spp., but found no synergistic interactions between inhibitory factors. A probability model was constructed by Koseki et al. (2009) to predict the inactivation of *Cronobacter* spp. in trypticase soy broth and PIF through processing at high pressures for various times. In the research of Jo et al. (2010), a secondary polynomial model was derived to predict the growth rate of *Cr. sakazakii* in PIF at four different growth temperature profiles. All of these models were focused on predicting growth rate or inactivation by heat treatment or other stresses, rather than lag time. Miled et al. (2011) explored the individual lag distribution of six strains of *Cronobacter* spp. at 25 ^{o}C and 37 ^{o}C. However, the microorganism was subjected to desiccation stress rather than mild heating. Also, to the best of my knowledge, no research exists on determining individual lag variability of *Cronobacter* spp. after sublethal heating damage and subsequent initiation of growth at various temperatures.

This research project therefore addresses the fact that low-level contamination of untreated or sublethally heat stressed *Cronobacter* spp. may be present in food. In addition, as *Cronobacter* spp. can grow at refrigeration temperatures, and temperature is the most important environmental factor that affects the growth of microorganisms (Juneja et al. 2009), the potential ability for these untreated and damaged individual cells to repair and subsequently grow in food at various storage temperatures will be investigated.

CHAPTER 2: Identification and screening of *Cronobacter* spp. with reference to minimum growth temperature

2.1 Introduction

Comparative analysis using small subunit ribosomal RNA (rRNA) gene sequencing was first introduced by Woese in 1970 (cited by Pontes et al. 2007). It was reported that the rDNA sequences of microorganisms of the same genus and species were highly conserved, and were different from those of organisms of other genera and species (Woo et al. 2008). After being amplified by PCR, the amplicons were sequenced in an automatic sequencer. The unknown bacteria could then be identified by comparison of the nucleotide sequence with a library of known sequences (Neonakis et al. 2008).

In bacteria, the 5S, 16S and 23S rDNA are three genes which make the rRNA function. The 16S rRNA gene is the most common PCR target gene used for identifying bacteria, is present in all microorganisms and contains highly conserved regions among various species (Millar et al. 2007, Pontes et al. 2007). A PCR-based method for 16S rDNA sequencing is an efficient procedure which can trace phylogenetic relationships among microorganisms, and identify bacteria from various sources (Mignard & Flandrois 2006).

Many studies have applied 16S rDNA sequence analysis to identify *Cronobacter* spp. After presumptive identification of microorganisms as *Cronobacter* with bioMérieux ID 32E, Chap et al. (2009) used 16S rDNA sequencing to identify 27 *Cronobacter* spp. strains from 91 samples of follow up formula and 199 infant foods and drinks. Pagotto & Farber (2009) used 16S rDNA sequence-based bioinformatics to strengthen the reclassification of 300 strains in the '*Sakazakii*' laboratory database. Fanjat et al. (2007) used 16S rDNA as a reference method to evaluate the performance of commercial biochemical kits bioMérieux ID 32 E for ability

to identify *Cronobacter* spp. Their research indicated that compared with bioMérieux ID 32E version 2.0, which identified 71.4% *Cronobacter* spp., bioMérieux ID 32E version 3.0 exhibited 100% correct identification of the *Cronobacter* spp. tested.

2.1.1 Rationale for research reported in this chapter

In order to confirm the identity of *Cronobacter* spp. provided for this study, the organisms were identified using 16S rDNA sequencing. In addition, due to the ability of *Cronobacter* spp. to grow at 5.5 ^{o}C to 6 ^{o}C and since the main objective of the study is to investigate the ability of *Cronobacter* spp. to multiply at low temperatures, the minimum growth temperatures of the *Cronobacter* spp. were investigated.

2.2 Materials and methods

2.2.1 Bacterial cultures

A total of seven strains of *Cronobacter* spp. were provided. Four *Cr. turicensis* strains (1211, 57, 1325 and 1327) were kindly donated by Professor Stephen Forsythe (Nottingham Trent University) and two strains (*Cr. sakazakii* NCIMB (National Collection of Industrial, Food and Marine Bacteria) 8272, *Cr. sakazakii* NCIMB 5920) were obtained from the Microbiology Research Unit (MRU) culture collection (London Metropolitan University) and *Cr. sakazakii* NCTC 11467 was bought from National Collection of Type Cultures (NCTC). Except *Cr. sakazakii* NCTC 11467, all other cultures were further confirmed using 16S rDNA sequencing.

2.2.2 Confirmation of identity of *Cronobacter* spp.

2.2.2.1 Extraction of DNA

All cultures were streaked on tryptone soya agar (TSA;CM0131; Oxoid, Basingstoke, UK) and incubated at 22 °C for 24-48 h before DNA extraction using InstaGene (732-6030;Bio-Rad,St Alban, UK), following the manufacturer's instructions. The DNA extracts were kept at -20 °C before use.

2.2.2.2 16S rDNA Sequencing

Sequencing of 16S rDNA was carried out according to the method of Ouoba et al. (2008) and Xu et al. (2010). The PCR amplification of a 940 bp portion of 16s rDNA was obtained by using the primer pA (5'-AGA-GTT-TGA-TCC-TGC-CTC-AG-3') and pE (5'-CCG-TCA-ATT-CCT-TTG-AGT-TT-3'), based on a conserved region of this gene. The amplification reaction was carried out in a final 50 µl reaction volume containing 1µl of chromosomal DNA, 5 µl of PCR buffer (with $MgCl_2$) (10x; N808160; Applied Biosystems, Warrington, UK), 5 µl of dNTP (1.25 mmol/l; U1511; Promega, Southampton, UK), 0.5 µl of primer pA (100 µmol/l) and 0.5 µl of primer pE (100 µmol/l), 0.25 µl Taq DNA Polymerase (5 U; N808-0160; Applied Biosystems), 37.75 µl autoclaved distilled water. The amplification was conducted in a GeneAmp PCR 2700 system (Applied Biosystems) using the following procedure: first denaturation at 95 °C for 5 min, then 94°C 1 min, 55 °C 1min, 72 °C 1 min for 35 cycles, finally 72 °C for 5 min. The products were kept at 4 °C before analysis.

In order to check whether the PCR was successful and to estimate the approximate DNA concentration, the PCR products were analyzed by electrophoresis by applying 5 µl of each PCR product with 2 µl of loading dye (AM8556; Applied Biosystems) to 1.2% agarose gel (161-3101; Bio-Rad). DirectLoad wide range DNA molecular marker (D7058; Sigma, Poole, UK) was used as a molecular size marker. The gel was run in 1x TBE buffer (Tris-Borate-EDTA; T4415; Sigma) for 1 h at a constant voltage of 120 V and photographed with a UV transilluminator (UVP, Cambridge, UK) after staining with ethidium bromide (0.5 µg/ml; E1510; Sigma).

The PCR amplicons were purified with the QIAquick PCR purification kit (28106; Qiagen, Manchester, UK) according to the instructions of the manufacturer. Sequencing was carried out to generate 550 bp portion of nucleotides by using the primer pD (5'-GTA-TTA-CCG-CGG-CTG-CTG-3') and ABI Big Dye Terminator v 3.1 Cycle Sequencing Kit (4337455; Applied Biosystems), which was used to stop the reaction. Amplification were carried out in a final 10 µl reaction volume containing 4 µl (30-90 ng)

purified DNA, 2 µl (20 ng/µl) primer pD, and 4 µl ABI Big Dye Terminator Reaction Mix (4337455; Applied Biosystems). Amplification was conducted in a PCR thermal cycler (Applied Biosystems) at the following temperature: 95 °C for 2 min, followed by 35 cycles of 96 °C 15 s, 40°C 1 s, 60°C 4 min.

The extension products were precipitated with 1 µl of 3 mol/l sodium acetate (pH 4.6) and 50 µl 100 % ethanol, followed by rinsing with 250 µl 70% ethanol and air dried. Sequencing was carried out by electrophoresis on a 3730xl DNA Analyser-Titania (Applied Biosystems).

Finally, the sequences of isolates were compared with those available in the GenBank/EMBL/DDBJ/PDB Sequence database using BLAST (Basic Local Alignment Search Tool) and the microorganisms identified.

2.2.3 Minimum growth temperature experiment

2.2.3.1 Culture preparation

The cultures were streaked from TSA stock slopes (stored at 4 ^{o}C) to TSA and incubated at 22 ^{o}C for 24 h. One colony of each culture was subcultured from TSA to 10 ml tryptone soy broth (TSB, CM0129; Oxoid) and incubated at 22 ^{o}C for 24 h.

2.2.3.2 Growth study

Before inoculation, 100 ml volumes of TSB were tempered for 24 h at 4, 5, 6 and 8 ^{o}C in air incubators (LMS, UK) and 7 ^{o}C in a shaking water bath (OLS200; Grant, Cambridge, UK) with a refrigerated immersion cooler (CS200G; Grant). Previous experiments (not herein reported) indicated that in a 24 h stationary phase culture, the maximum population density was 10^{8} cfu/ml. The stationary-phase (24 h) cultures were diluted with TSB to 10^{5} cfu/ml and 1ml of diluted culture was inoculated into 100 ml TSB with a 2 ml sterile syringe (300185; Becton Dickinson, Oxford, UK) fitted with sterilized needle (301750; Becton Dickinson) to give an approximate final concentration of 10^{3}-10^{4} cfu/ml in TSB. The cultures were incubated

at 4, 5, 6, 7 and 8^{o}C for up to 30 days depending on growth of the culture. Temperatures were checked daily (on weekdays) using an equivalent volume (100 ml) of water at each temperature and a calibrated alcohol-in-glass thermometer. The cultures were enumerated at suitable intervals on TSA using the spread plate technique and plates were incubated at 37 ^{o}C for 24 h. The experimental growth data were fitted with the Baranyi model (Baranyi & Roberts 1994) using the DMFit Excel add-in software (www.ifr.ac.uk/safety/DMfit). The experiment was carried out at least twice.

2.3 Results

2.3.1 16S rDNA Sequencing

According to the result of 16S rDNA sequencing, both 1211 and 57 were confirmed as *Cr. turicensis* with respective similarities of 99% and 100%. NCIMB 8272 and NCIMB 5920 were identified as *Cr. sakazakii* with respective similarities of 98% and 99%. The cultures 1325, 1327 were identified as *Erwinia cypripedii* and *Pantoea agglomerans* both with 99% similarity (Table 2.1).

Table 2.1: Confirmation of identity of provided cultures

Culture number	Result	Similarity	Origin
1211	*Cr. turicensis*	99%	Infected neonate
57	*Cr. turicensis*	100%	Milk powder
NCIMB 8272	*Cr. sakazakii*	98%	Dried milk powder 1950
NCIMB 5920	*Cr. sakazakii*	99%	
1325	*Erwinia cypripedii*	99%	
1327	*Pantoea agglomerans*	99%	

2.3.2 Minimum growth temperature experiments

Table 2.2 depicts growth profiles of the five *Cronobacter* spp. at 4, 5, 6, 7 and 8 ^{o}C. At 4 ^{o}C, no cultures grew. At 5 ^{o}C, only *Cr. turicensis* 1211 grew, with a generation time of 59.03 h; at 6 ^{o}C, *Cr. turicensis* 1211 grew with a generation time of 29.29 h, while *Cr. turicensis* 57 was on the borderline of growth and no growth; at 7 ^{o}C, all cultures grew and generation times were between 9.59 h and 60.81 h; at 8 ^{o}C, the generation times of the five *Cronobacter* spp. were within the range of 10.40 h to 21.67 h. This apparent inconsistency is discussed later (section 2.4.3).

Figs 2.1, 2.2, 2.3, 2.4 and 2.5 show typical examples of growth of the cultures at different temperatures fitted with the model of Baranyi using the DMFit Excel add-in software (Baranyi & Roberts 1994). According to Fig 2.1, at 4 ^{o}C, number of *Cr. turicensis* 1211 declined from $10^{4.5}$ cfu/ml to $10^{3.5}$ cfu/ml during the first 200 h, it remained at $10^{3.5}$ cfu/ml for the next 400 h, followed by a drop to $10^{2.5}$ cfu/ml during the final approximate 200 h incubation. At 5 ^{o}C, *Cr. turicensis* 1211 grew gradually from 10^{4} cfu/ml to about 10^{7} cfu/ml during 500 h incubation, while *Cr. turicensis* 57 neither grew nor declined during the first 300 h of incubation; after that it started to decline (Fig 2.2). At 6 ^{o}C, *Cr. turicensis* 1211 took about 500 h to grow from approximately 10^{4} cfu/ml to 10^{8} cfu/ml. *Cr. turicensis* 57 showed growth from about $10^{3.8}$ cfu/ml to 10^{5} cfu/ml for the first 300 h and after that, it declined to 10^{4} cfu/ml during the next about 500 h. Numbers of cfu of the other cultures declined throughout the whole period at this temperature (Fig 2.3). Fig 2.4 depicts the growth profiles at 7 ^{o}C. *Cr. sakazakii* NCIMB 8272 and NCTC 11467 entered stationary phase when cell density attained about 10^{6} cfu/ml. At 8 ^{o}C, all except NCTC 11467 entered stationary phase at 10^{7} cfu/ml and all cultures exhibited relatively good growth at this temperature (Fig 2.5).

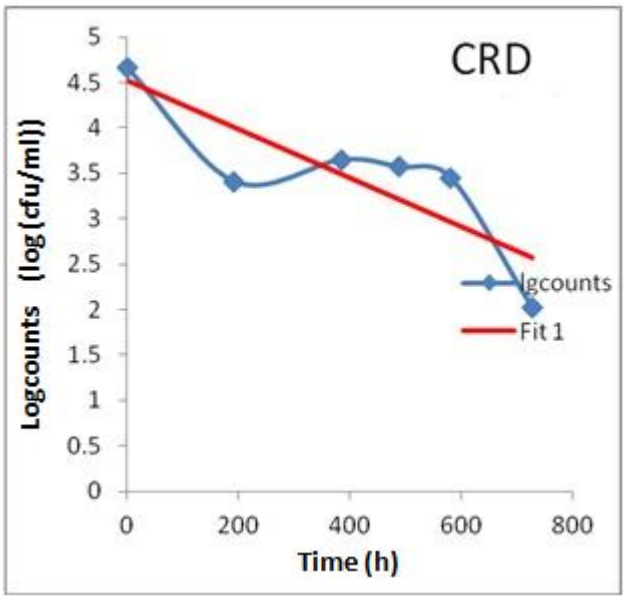

Fig 2.1: Growth profile of *Cr. turicensis* 1211 at 4 ^{o}C

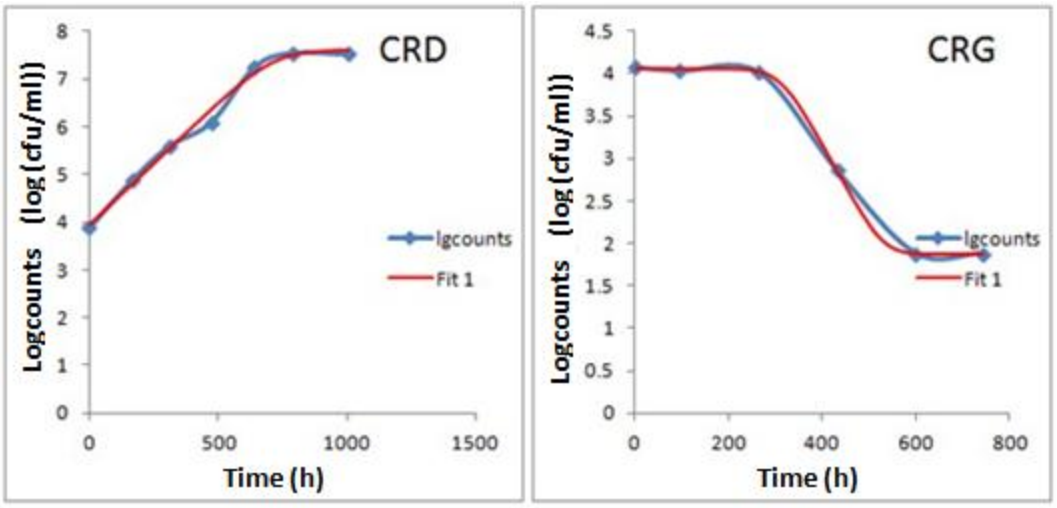

Fig 2.2: Growth profiles of *Cronobacter* spp. at 5 ^{o}C

CRD: *Cr. turicensis* 1211; CRG: *Cr. turicensis* 57; The line joining the blue diamonds represents the observed growth data; the red line represents the data fitted with the Baranyi model.

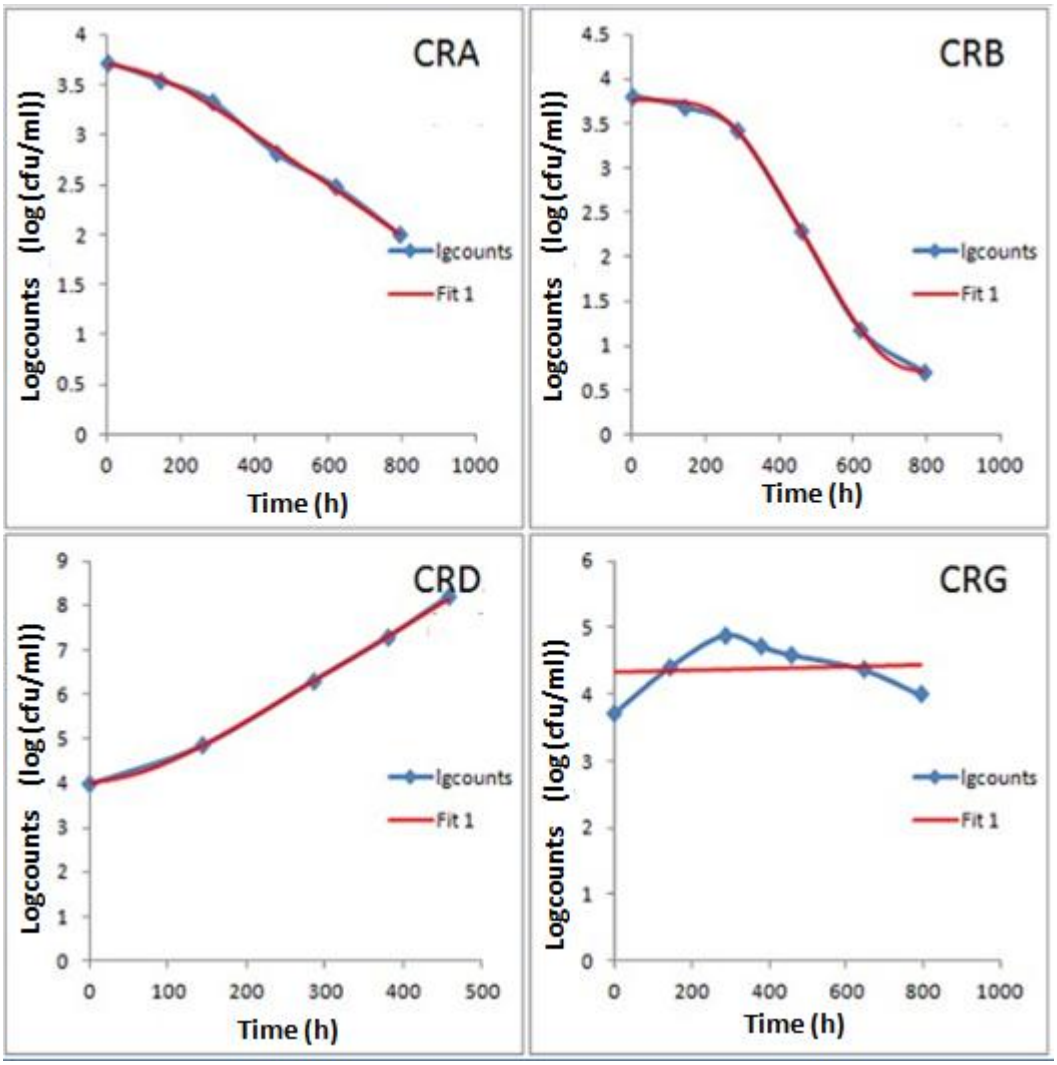

Fig 2.3: Growth profiles of *Cronobacter* spp. at 6 ^{o}C

CRA, *Cr. sakazakii* NICMB 8272; CRB, *Cr. sakazakii* NCIMB 5920; CRD, *Cr. turicensis* 1211; CRG, *Cr. turicensis* 57; The line joining the blue diamonds represents the observed growth data; the red line represents the data fitted with the Baranyi model.

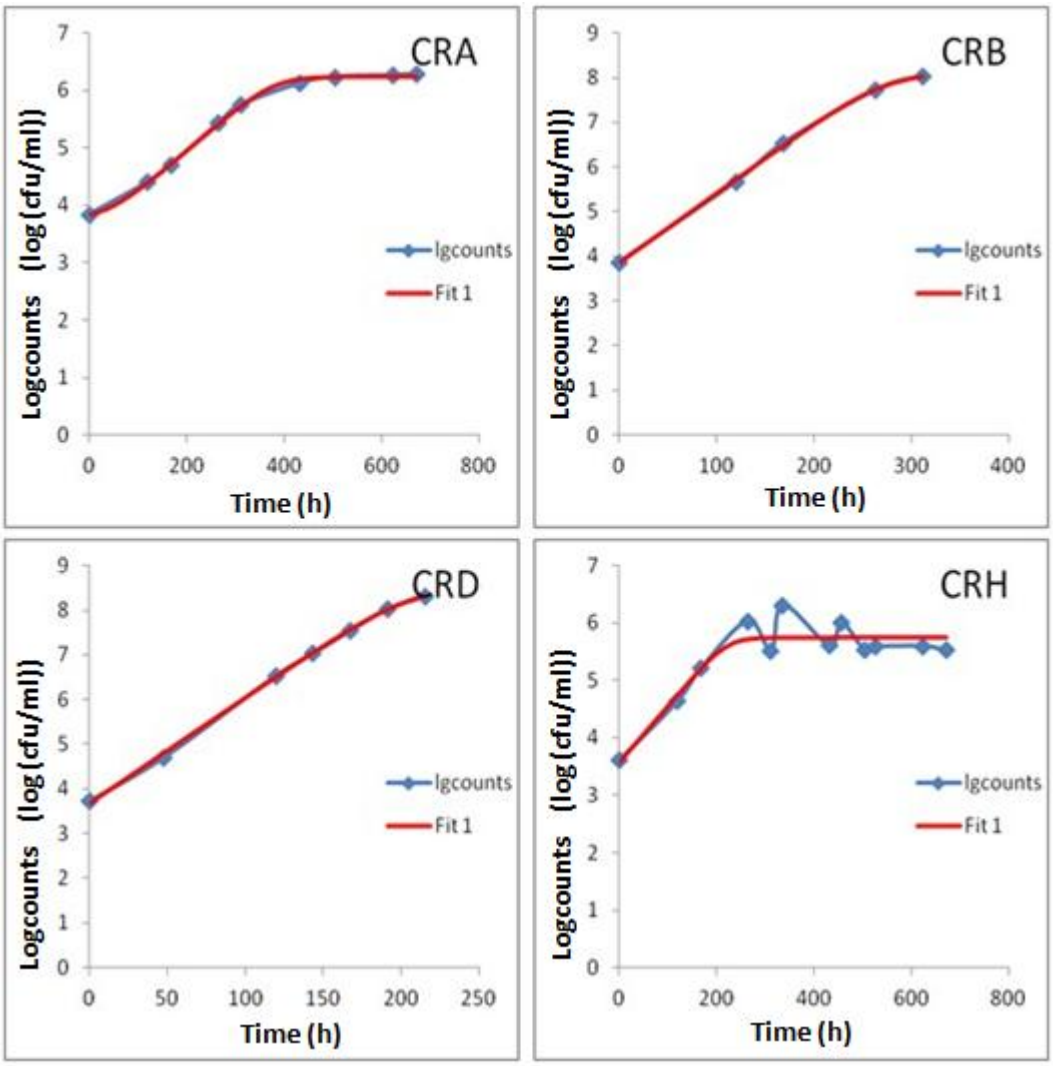

Fig 2.4: Examples of the growth profile of *Cronobacter* spp. at 7 ^{o}C

CRA, *Cr. sakazakii* NICMB 8272; CRB, *Cr. sakazakii* NCIMB 5920; CRD, *Cr. turicensis* 1211; CRH, *Cr. sakazakii* NCTC 11467; The line joining the blue diamonds represents the observed growth data; the red line represents the data fitted with the Baranyi model.

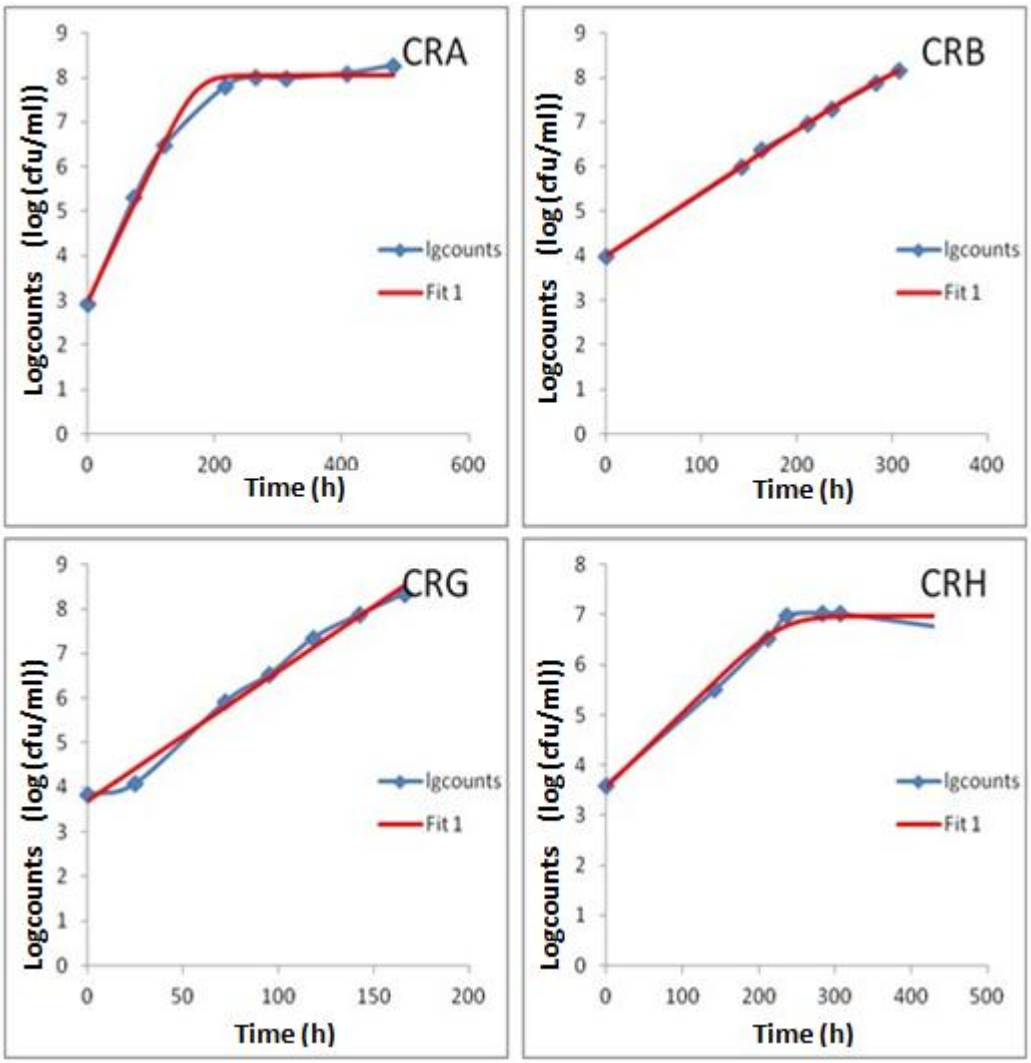

Fig 2.5: Examples of the growth profile of *Cronobacter* spp. at 8 ^{o}C

CRA, *Cr. sakazakii* NICMB 8272; CRB, *Cr. sakazakii* NCIMB 5920; CRG, *Cr. turicensis* 57; CRH, *Cr. sakazakii* NCTC 11467; The line joining the blue diamonds represents the observed growth data; the red line represents the data fitted with the Baranyi model.

Table 2.2: Growth rates of *Cronobacter* spp. at temperatures of 4-8 ^{o}C (including mean and actual results)

	Cronobacter sp.	*Cr. sakazakii* NCIMB 8272	*Cr. sakazakii* NICMB 5920	*Cr. turicensis* 1211	*Cr. turicensis* 57	*Cr. sakazakii* NCTC 11467
4 ^{o}C	Specific growth rate (h^{-1}) Generation time (h) R^2 range	ND	ND	NG	ND	ND
5 ^{o}C	Specific growth rate (h^{-1}) Generation time (h) R^2 range	ND	ND	0.012 (0.012, 0.012) 59.03 (58.59, 59.48) 0.962-0.986	NG	ND
6 ^{o}C	Specific growth rate (h^{-1}) Generation time (h) R^2 range	NG	NG	0.024 (0.022, 0.025) 29.29 (30.87, 27.91) 0.978-0.999	Growth followed by death (Fig 2.3)	NG
7 ^{o}C	Specific growth rate (h^{-1}) Generation time (h) R^2 range	0.013 (0.009, 0.012, 0.017) 57.79 (73.48, 58.32, 41.57) 0.913-0.999	0.026 (0.036, 0.032, 0.019, 0.019) 29.01 (19.29, 21.93, 37.49, 37.34) 0.967-0.999	0.079 (0.102, 0.102, 0.058, 0.054) 9.59 (6.76, 6.83, 12.04, 12.74) 0.983-0.999	0.074 (0.100, 0.088, 0.058, 0.050) 10.09 (6.90, 7.84, 11.91, 13.74) 0.991-0.997	0.016 (0.021, 0.023, 0.006) 60.81 (33.58, 30.74, 118.16) 0.697-0.974
8 ^{o}C	Specific growth rate (h^{-1}) Generation time (h) R^2 range	0.053 (0.068, 0.075, 0.034, 0.033) 15.17 (10.14, 9.27, 20.55, 20.72) 0.982-0.994	0.035 (0.031, 0.038) 19.84 (21.32, 18.35) 0.993-0.999	0.067 (0.067, 0.067) 10.40 (10.41, 10.48) 0.986-0.988	0.065 (0.063, 0.067) 10.66 (10.96, 10.36) 0.979-0.985	0.032 (0.030, 0.034) 21.67 (23.03, 20.31) 0.963-0.977

ND, not done; NG, no growth; Actual results are presented in brackets.

2.4 Discussion

2.4.1 Confirmation of identity of *Cronobacter* spp.

In this study, 16S rDNA sequencing identified most of the cultures tested as *Cronobacter* spp. Cultures NCIMB 8272 and 5920 were identified as *Cr. sakazakii* with 98% and 99% similarity. The cultures 1211 and 57 were identified as *Cr. turicensis* with 99% and 100% similarity respectively. However, two cultures, presumptively identified as *Cr. turicensis* 1325 and *Cr. turicensis* 1327, were confirmed as *Erwinia cypripedii* and *Pantoea agglomerans* respectively. This is consistent with other reported findings that cultures presumptively identified phenotypically as *Cronobacter* spp. appear to belong to different genera and species using molecular methodology (Friedemann 2007, Jaradat et al. 2009, Healy et al. 2010, Joseph et al. 2011). Iversen et al. (2004c) investigated phylogenetic relationships among 126 *Cronobacter* spp. identified using 16S rDNA sequencing and found that several presumptively identified *Cronobacte*r spp. using API 20E and ID32E belonged to *Ent. amnigenus*, *Ent. cloacae* and potentially novel taxa. Drudy et al. (2006) characterized 51 environmental and food *Cronobacter* spp. and six *Cronobacter* spp. type cultures using different biochemical profiles and genotypic methods. One of the presumptively identified *Cronobacter* spp. was identified as *Pantoea agglomerans* with API 20E, but as *Esch. vulneris* with ID32E. This culture was further identified as *Ent. asburiae* with 16S rDNA sequencing. Townsend et al. (2008) re-analysed one of the cultures previously identified as *Cronobacter* spp. during the survey of PIF carried out by Muytjens et al. (1988), and confirmed it as *Ent. hormaechei* using 16S rDNA sequencing.

Reasons for poor correlation between phenotypic and genotypic methods to presumptively identify *Cronobacter* spp. include the fact that some studies report yellow pigment production and commercial biochemical kit results as the confirmatory method to identify *Cronobacte*r spp. For example, in the survey carried out by Muytjens et al. (1988) with 141 milk substitute infant formulas, *Cronobacter* spp. was identified based on

yellow pigment production on TSA, production of extracellular DNase, positive α-glucosidase and API 20E profiles. Iversen and Forsythe (2004) confirmed *Cronobacter* spp. by showing yellow pigment colony on TSA or blue-green colonies on Druggan-Forsythe-Iversen agar (DFI; Iversen et al. 2004a) and by testing using API 20E, in a survey of 82 samples of PIF and 404 other food products. Baumgartner et al. (2009) screened and identified *Cronobacter* spp. from 268 ready-to-eat foods by plating on three chromogenic agars, followed by confirmation using bioMérieux ID 32E test systems. Although a revised method to isolate and enumerate *Cronobacter* spp. in PIF is currently being developed by the Food and Drug Administration (FDA) (Chen et al. 2009), the current protocol of FDA confirms the identity of *Cronobacter* spp. with API 20E (FDA 2002, Chen et al. 2009).

It is reported that biochemical and chromogenic methods lack the necessary discriminatory power to confidently and reliably identify *Cronobacter* spp. (Lehner et al. 2004, Jaradat et al. 2009). Cawthorn et al. (2008) evaluated the accuracy of methods to identify *Cronobacter* spp. based on yellow pigment production, and found that this method has a very low sensitivity, specificity and accuracy (87%, 71% and 74%). In this study, both*Pantoea agglomerans* and *Erwinia cypripedii* produced a yellow pigment on TSA at 22 ^{o}C. Moreover, not all *Cronobacter* spp. produce yellow pigmented colonies on TSA (Jaradat et al. 2009). DFI is a chromogenic agar which contains 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc) and identifies *Cronobacte*r spp based on production of α-glucosidase. Typical *Cronobacte*r spp. show blue-green colonies on it (Iversen et al. 2004a). However, some other *Enterobacteriaceae* are also α-glucosidase positive (Chen et al. 2009), including *Pantoea* spp*., E. vulneris, Citrobacter koseri* (Iversen et al. 2004a), *Ent. aerogenes* and *C. diversus* (Kämpfer et al. 1991). The study of Song (2005, cited by Restaino et al. 2006) indicated that 7% of *Cronobacter* spp. failed to show α-glucosidase activity. In addition, it has been reported that both API 20E and ID 32E kits can give false positive

and false negative results (Iversen et al. 2004a, Restaino et al. 2006, Fanjat et al. 2007, Chen et al. 2009, Jaradat et al. 2009).

As *Cr. malonaticus* and *Cr. sakazakii* are closely related, Baldwin et al. (2009) proposed that 16S rDNA sequencing could not discriminate between these two species. Multilocus sequence typing (MLST) is suggested as a more reliable method to distinguish these two species (Baldwin et al. 2009). MLST is a nucleotide sequence-based typing method proposed by Maiden in 1998 for characterizing *Neisseria meningitides,* to overcome the poor reproducibility between laboratories (Maiden et al. 1998). MLST has been regarded as the 'gold standard' for typing microorganisms (Larsen et al. 2012) and is now widely used for this purpose.

These two cultures, *Cr. sakazakii* NCIMB 5920 and *Cr. sakazakii* NCIMB 8272, were originally bought from NCIMB and both have been confirmed as *Cr. sakazakii* using the MLST method and published on the *Cronobacter* MLST Database (http://pubmlst.org/cronobacter/.) with the accession number 134 and 38 respectively. Consequently, no further identification was carried out and both are regarded as *Cr. sakazakii.*

2.4.2 Minimum growth temperature

Nazarowec-White and Farber (1997b) investigated growth profiles of clinical and food isolates of *Cronobacter* spp. in brain heart infusion (BHI) and reported a minimum growth temperature range between 5.5 ^{o}C and 8 ^{o}C. Iversen et al. (2004b) studied the growth of six clinical and food isolates of *Cronobacter* spp. in PIF and reported that all cultures could grow at 6 ^{o}C. In addition, recent research reported that compared with other species, *Cr. turicensis* has a lower minimum growth temperature, below 5 ^{o}C (Kucerova et al. 2011). In the present study, a minimum growth temperature of 5 ^{o}C of *Cr. turicensis* 1211 was observed, consistent with the findings of Kucerova et al. (2011), and slightly lower than reported by Nazarowec-White and Farber (1997b) and Iversen et al. (2004b). In terms of the other four *Cronobacte*r spp. in this study, the minimum growth

temperature in TSB ranged from approximately 6- 7 ^{o}C for *Cr. turicensis* 57 to 7 ^{o}C for *Cr. sakazakii* NCIMB 8272, *Cr. sakazakii* NCIMB 5920 and NCTC 11467, consistent with previous studies (Nazarowec-White & Farber 1997b, Iversen et al. 2004b).

Although the temperature of domestic refrigerators is recommended to be at or below 4 ^{o}C (FDA 2008), a quantitative survey of 1030 consumers aged between 18 and 64 in the UK suggested that less than 50% of consumers know its correct temperature range and assumed it to be 5-9 ^{o}C (Betts 2002). The Regional Inspectorate for Health Protection in Leeuwarden investigated the temperature of 125 domestic refrigerators in the Netherlands in 1993. They reported that the temperature of 29.6% domestic refrigerators was below 5 ^{o}C, 41.6% between 5 ^{o}C and 7 ^{o}C; 25.6% between 7 ^{o}C and 9 ^{o}C and 3.2% between 9 ^{o}C and 13 ^{o}C (cited by Notermans et al. 1997). In addition, a survey on household refrigerators in France (2001) revealed that only 11% run at the ideal temperature range (at or below 4 ^{o}C) and 25% run with an average temperature above 8^{o}C (cited by Lagendijk et al. 2008). The latest survey carried out by Breen (2006) in the UK shows that the temperature of 33% (8/24) of domestic refrigerators is above 5 ^{o}C. Moreover, Peck et al. (2006) reviewed the various surveys carried out during the last 20 years and concluded that 60%-70% domestic refrigerators worldwide run at an average temperature above 5 ^{o}C. According to this, our results are in accordance with the research of Nazarowec-White and Farber (1997b) which indicates that the commonly encountered temperatures in domestic refrigerators could allow growth of *Cronobacter* spp.

2.4.3 Generation time

The Baranyi model (Baranyi & Roberts 1994) and the modified Gompertz model (Zwietering et al. 1990) have been used to fit the growth curves and estimate generation times (Jo et al. 2010, Hong et al. 2014). In this study, both the Baranyi model and the Zwietering model (for comparison) were fitted to the experimental data for *Cr. turicensis* 1211 generated at temperatures of 4 to 7 ^{o}C. The Baranyi model consistently provided a

lower root mean square (RMS) value, which indicated a better fit, compared with the Zwietering model (results shown in Appendix 2.1). Therefore, the Baranyi model was used to estimate the generation times in this research.

In terms of generation time, the five *Cronobacter* spp. examined show a wide variability when grown at 7 ^{o}C. At 7 ^{o}C, *Cr. turicensis* 1211 shows the shortest generation time (9.59 h), followed by *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 with, respectively, 10.09 h and 29.01 h. *Cr. sakazakii* strains NCIMB 8272 and NCTC 11467 grew the slowest at this temperature, with very similar generation times of 57.79 h and 60.81 h respectively. The difference between the generation time of the quickest growing culture, *Cr. turicensis* 1211 and the slowest, *Cr. sakazakii* NCTC 11467, was almost six times. At 8 ^{o}C, the generation times of the five *Cronobacter* spp. were more similar. At this temperature, both *Cr. turicensis* 1211 and *Cr. turicensis* 57 grew most rapidly with a very similar generation time (10.40 h and 10.66 h respectively), followed by *Cr. sakazakii* NCIMB 8272 and *Cr. sakazakii* 5920 with very close generation times (15.17 h and 19.84 h). Again, NCTC 11467 is the slowest-growing culture with a generation time of 21.67 h, almost twice as long as *Cr. turicensis* 1211. However, these results contradict those of Ghassem et al. (2011). In their study, albeit carried out at higher temperatures, the growth characteristics of three *Cr. sakazakii* strains and one *Cr. muytjensii* strain in PIF and TSB at 10, 25, 37 and 45^{o}C were investigated, and no significant difference was found between the generation times among organisms at each temperature.

Currently, to the best of my knowledge, there is little information concerning generation times of *Cronobacter* spp. below 10 ^{o}C. Nazarowec-White and Farber (1997b) reported the average generation time for clinical and food isolates of *Cronobacter* spp. in reconstituted PIF was 4.98 h at 10 ^{o}C. Ghassem et al. (2011) reported that the mean generation times of four *Cronobacter* spp. in PIF and TSB at 10 ^{o}C were 3.64 h and 3.98 h in the respective media. Iversen et al. (2004b)

investigated growth profiles of *Cronobacter* spp. in PIF and found a mean generation time of 13.7 h at 6 ^{o}C. In comparison, the generation time of 19.29 h of *Cr. turicensis* 1211 at 6 ^{o}C in TSB in this study, is longer than those reported by Iverson et al. (2004b).

Cr. turicensis 1211 and *Cr. turicensis* 57, for which the mean generation times at 7 ^{o}C (9.59 h, 10.09 h) were slightly shorter than those at 8^{o}C (10.40 h, 10.66 h). This may be caused by different instruments used in the experiment. A shaking water bath was used in the 7 ^{o}C experiment, while an airincubator without shaking was used to provide 8 ^{o}C, and the shaking mode may cause this slightly shorter generation time, due to enhanced aeration of the culture.This explanation can be confirmed by the estimation of 13.17 h for the generation time of *Cr. turicensis* 1211 (specific growth rate 0.053 h^{-1}) at 7 ^{o}C in TSB in an air incubator reported in Chapter 5. Regarding the other cultures, since they grew very slowly at 7 ^{o}C, with a long generation time, shaking may not make such a noticeable difference. The second atypical growth feature was that both *Cr. sakazakii* strains NCIMB 8272 and NCTC 11467 achieved stationary phase when the cell concentration was only 10^{6}-10^{7} cfu/ml at 7 ^{o}C and 8 ^{o}C (normally 10^{8} cfu/ml would be expected after 24 h incubation at 22 ^{o}C in TSB). This is probably due to the environmental temperature, as these temperatures are the minimum, or close to minimum for growth of both strains and it has been reported that the maximum population density can be affected by the temperature (McQuestin et al. 2010).

2.5 Conclusions

Using 16S rDNA sequencing, NCIMB 8272 and NCIMB 5920 were confirmed as *Cr. sakazakii* and 1211 and 57 were confirmed as *Cr. turicensis.* Two cultures presumptively identified as *Cr. turicensis* 1325 and *Cr. turicensis* 1327 were confirmed as *Erwinia cypripedii*and *Pantoea agglomerans.*

Regarding observations of growth at 4-8 ^{o}C: firstly, *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 grew faster than *Cr.*

sakazakii strains NCIMB 8272 and NCTC 11467 at 7 ^{o}C; secondly, *Cr. sakazakii* strains NCIMB 8272 and NCTC 11467 showed atypical growth profiles at 7 ^{o}C and 8 ^{o}C (entering stationary phase at low cell density); finally, both *Cr. sakazakii* and *Cr. turicensis* are recognised as being pathogenic (Kucerova et al. 2010, 2011), *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 were selected for thermal inactivation experiments in the next stage.

CHAPTER 3: Identification of suitable sublethal heat treatments for selected *Cronobacter* spp.

3.1 Introduction

3.1.1 Sublethal heat treatment

A sublethal heat treatment is defined as a heating process that may cause damage but no loss of viability of cells and all of them are able to recover after exposure to the stress (Métris et al. 2008, Niven et al. 2008, Wesche et al. 2009). When microorganisms are subjected to a sublethal heat treatment, the stress causes repairable damage (Martin et al. 1976), which includes damage to the outer membrane of Gram negative microorganisms (Mackey 2000), cytoplasmic membrane, DNA, RNA and specific enzymes (Gould 1989). The injured microorganism becomes more fastidious in nutritional requirements for growth (Andrew & Martin 1979).

3.1.2 Recovery methods for stressed cells

It is reported that the viable count of bacterial subject to stress will be affected by unsuitable enumeration methods (Özkanca et al. 2009). In order to prevent underestimating microbial numbers in samples, especially stressed cells, a lot of recovery approaches have been investigated (Stephens & Mackey 2003). The most probable number (MPN) method is a technique used to estimate populations of microorganisms in samples (Brewer 1977). As it can be carried out with a variety of liquid media, it enables stressed cells to be recovered (Varughese et al. 2007). Bredie & de Boer (1992) evaluated the MPN, Anderson-Baird-Parker, Petrifilm *Esch. coli* and Fluorocult ECD methods for routine enumeration of *Esch. coli* in naturally contaminated food of animal origin, and reported that the MPN method had the highest recovery capability and is the most efficient method for enumerating low numbers of microorganisms. Yu and Fung

(1993) compared survival of sublethally heated and freeze injured *L. monocytogenes* in cooked and chopped ham stored at 5 ^{o}C for five weeks, using the five tube MPN containing TSB and direct plating methods on TSA. The results showed that the MPN method could enumerate *L. monocytogenes* in food at both low and high numbers. In addition, due to the inactivation of catalase of the cell during mild heat stress (Baird-Parker & Davenport 1965, Andrew & Martin 1979, Gould 1989, Mackey 2000, Besse et al. 2002) and its further decline during the recovery period (Andrew & Martin 1979), accumulation of hydrogen peroxide (H_20_2) during aerobic metabolic processes, which has pronounced bactericidal effects, could result in reduced recovery of injured microorganisms (Baird-Parker & Davenport 1965, Andrew & Martin 1979). Baird-Parker & Davenport (1965) first suggested addition of sodium pyruvate to media to improve recovery of heat stressed *Staph. aureus*. Brewer et al. (1977) investigated the effect of addition of pyruvate on recovery of heat stressed *Staph. aureus* in TSB with 10% NaCl using an MPN method. This research showed that, compared with enumeration in TSB with 10% NaCl, addition of pyruvate dramatically increased recovery of all heat stressed *Staph. aureus* strains. McDonald et al. (1983) reported that addition of sodium pyruvate at 0.33%, 0.66% and 1.2% to tryptone glucose extract agar and violet red bile agar significantly improved recovery of heat or freeze injured *Esch. coli*, with variable results according to medium used.

3.1.3 Method to estimate injury percentage

As the outer membrane of Gram-negative bacteria, which is responsible for protecting cells against bile salts and certain antibiotics, could be injured when the cells are subjected to heat (Mackey 2000), one of the manifestations of injured cells is that they become more sensitive to selective media (Ray & Speck 1973, Stephens & Mackey 2003, Jasson et al. 2007, Özkanca et al. 2009). As the sublethally injured cells become more exacting in their growth requirements and fail to grow on the selective media normally used in their isolation, the difference between two counts from the same sample on the optimum medium and the

selective medium could give some indication of the proportion of injured cells in a population (Mackey 2000). Stephens et al. (1997) used this method to quantify numbers of heat injured cells by estimating the difference in number of cells unable to grow on heart infusion agar (HIA) with added 2.5% w/v NaCl, but able to grow on HIA without added NaCl and found that the proportion of uninjured cells estimated based on enumeration matched with that estimated based on the growth curve generated by the Bioscreen. Pascual et al. (2001) plated sublethally heated *L. monocytogenes* on TSA and *Listeria* selective agar (LSA), and estimated that ca. 90% of the cell population was injured.

3.1.4 Rationale for research reported in this chapter

There is little research investigating recovery ability of different media for heat stressed *Cronobacter* spp. Gurtler and Beuchat (2005) compared recovery of a heat stressed cocktail of four *Cronobacter* spp. on seven media, which included TSA supplemented with 0.1% sodium pyruvate (0.1%TSAP), Leuschner, Baird, Donald, and Cox (LBDC) agar (a differential, non-selective medium), Oh and Kang agar (OK), fecal coliform agar (FCA), DFI agar, violet red bile glucose agar (VRBGA) and *Enterobacteriaceae* enrichment (EE) agar. The result showed that 0.1% TSAP performed better than all other media for recovering stressed cells. Forsythe (2009) compared recovery of heat stressed *Cr. muytjensii* and *Cr.sakazakii* onTSA and 0.1%TSAP, and found no significant difference between recovery on TSA and 0.1%TSAP. No investigations address different recovery abilities between MPN in TSB, TSA and TSAP for injured *Cronobacter* spp.

In this chapter, first, in order to avoid underestimating numbers of heat stressed *Cronobacter* spp., differences in ability of TSA, 0.1%TSAP, 1%TSAP and the MPN method to recover heat damaged *Cronobacter* spp. was evaluated. Then, as this study is focused on response of *Cronobacter* spp. after sublethal heat treatment, the appropriate sublethal heat treatment for each *Cronobacter* spp. was determined. Finally, the degree of injury for each sublethal condition applied to *Cronobacter* spp. was

estimated according to the numbers of sublethally heated organisms recovered on non-selective and selective media.

3.2 Materials and methods

3.2.1 Bacterial cultures

A total of three *Cronobacter* cultures were used: *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920.

3.2.2 Culture preparation

One colony of *Cronobacter* spp. was subcultured from TSA to10 ml TSB followed by incubating at 22 ^{o}C for 24 h. The culture was diluted in TSB and finally inoculated into 30 ml TSB to give approximately 1000 cells/ml. The culture was then incubated at 22 ^{o}C for 48 h to stationary phase.

3.2.3 Thermal inactivation experiment

The thermal inactivation experiments were carried out according to the method of Métris et al. (2008). Stationary phase culture was centrifuged (11,000 x g, 15 min at 4 ^{o}C), and the pellet resuspended in 2 ml of TSB. Tubes (the caps of which contained a rubber septum) containing 10 ml TSB were preheated to the required temperature (48, 49, or 50^{o}C) by submersion in a shaking water bath (SS40-A2; Grant, Cambridge, UK) for about 1h before inoculation. Tubes were vented with a sterile needle through the septum to release pressure. A volume of 100µl of the prepared suspension was injected via the septum directly under the surface of the TSB using a sterile precision syringe (81027; Hamilton, Bonaduz, Switzerland) fitted with a long sterile needle (90138; Hamilton). Tubes were removed from the bath at pre-defined intervals and cooled in ice water immediately.

3.2.4 Recovery of heat stressed cells using various recovery methods

Only *Cr. turicensis* 1211 was used for this experiment. The thermal inactivation experiment at 49 ^{o}C was carried out as described above. Heated samples were serially diluted in maximum recovery diluent (MRD; CM0733; Oxoid) and appropriate dilutions enumerated on TSA, TSA with 0.1% (w/v; 0.1%TSAP) or 1% (w/v; 1%TSAP) sodium pyruvate (P5280; Sigma, Poole, UK) and with the three tube MPN method. Sodium pyruvate was added to the agars both before autoclaving (TSAPA) and after autoclaving (TSAPF). For sodium pyruvate added after autoclaving, 0.1%TSAPF was prepared by adding 5 ml of 10% (w/v) aqueous sodium pyruvate to 500 ml autoclaved TSA tempered at 50 ^{o}C, before pouring the plates. The 1%TSAPF was prepared by adding 10 ml of 50% (w/v) aqueous sodium pyruvate to 500 ml TSA. Both 10% and 50% sodium pyruvate solutions were filtersterilized with a 0.2 µm filter (190-2520; Fisher Scientific, Loughborough, UK) before adding to TSA. The three-tube MPN technique was performed by adding 1 ml of each decimal dilution into each of three tubes containing 9 ml of TSB. The incubation temperature for recovery in all cases was 22 ^{o}C for 48 h~72 h. The number of tubes that showed growth at each dilution was counted and the MPN estimated from the probability table of de Man (1983). The experiments were carried out in duplicate on three independent days, i.e. a total of six estimations.

3.2.5 Identification of sublethal heating conditions for each organism

For all three *Cronobacter* spp., thermal inactivation experiments were carried out as described in section 3.2.3. Heated samples were serially diluted in MRD and appropriate dilutions were enumerated on TSA incubated at 22 ^{o}C (48 h~72 h). The experiment was done in duplicate on three different days, i.e. a total of six estimations.

3.2.6 Heat resistance parameters

The $\log_{10}$ of number of survivors of each *Cronobacter* spp. after heat treatment was plotted against heating time for each temperature. Traditional log-linear inactivation kinetics model was used to describe the survival curve obtained at various temperatures. The D value of each *Cronobacter* spp. at various temperatures was calculated as the negative reciprocal of the slope of linear regression of the $\log_{10}$ survivor as a function of heating time. The z value was determined as negative reciprocal of the slope of linear regression for the $\log_{10}$ D-values against the heating temperature tested (Iversen et al. 2004b).

3.2.7 Determination of percentage of sublethal injury

The microorganisms were thermally treated as described (section 3.2.3), but tubes were removed from the bath at intervals based onthe results of sublethal heating conditions for each culture (Table 3.6) and cooled in ice water immediately. Moreover, 100 µl suspension was injected into 10 ml TSB at room temperature, which was used as an unheated control. The sublethally heated culture and the control were diluted in MRD and enumerated on TSA and violet red bile glucose agar (VRBGA; CM485B; Oxoid). The plates were incubated at 22 ^{o}C for 48 h~72 h. Sublethally injured cells were considered to be those that could grow on TSA (non-selective agar) but not on VRBGA (selective agar) (Besse et al. 2000). The proportion of sublethally injured cell was calculated according to equation (3.1). The experiment was carried out in duplicate on three independent days, i.e. a total of six estimations.

$$\%\boldsymbol{injury} = \frac{(\boldsymbol{count\ on\ TSA - count\ on\ VRBGA})}{\boldsymbol{count\ on\ TSA}} \times \mathbf{100}\% \qquad (3.1)$$

3.2.8 Statistical analysis

Data for recovered cells after thermal inactivation experiments were subjected to parametric or non-parametric method (when the data does not follow a normal distribution and variance is not equal) (SPSS 11.0). The significance level was set at 5%.

3.3 Results

3.3.1 Recovery of heat stressed cells using various recovery methods

The viable counts of *Cr. turicensis* 1211 after heat treatment in TSB using TSA, 0.1%TSAPA, 1%TSAPA, 0.1%TSAPF, 1%TSAPF and MPN is depicted in Table 3.1 and Fig 3.1. Among six samples, two showed higher recovery with the MPN method ($p<0.05$). For TSA supplemented with sodium pyruvate, there was no significant difference between recoveries on TSA, 0.1%TSAPA, 1%TSAPA, 0.1%TSAPF, 1%TSAPF.

Table 3.1: Log recovery of *Cr. turicensis* 1211 after heat treatment at 49 ^{o}C

Time (min)	TSA	0.1%TSAPA	1%TSAPA	0.1%TSAPF	1%TSAPF	MPN
0	8.22±0.11	8.15±0.11	8.21±0.07	8.16±0.10	8.18±0.12	8.60±0.30*
5	8.15±0.16	8.18±0.10	8.11±0.13	8.09±0.13	8.15±0.12	8.55±0.30*
10	8.14±0.15	8.11±0.13	8.12±0.16	8.14±0.13	8.10±0.17	8.18±0.41
20	7.66±0.30	7.72±0.41	7.60±0.31	7.65±0.30	7.67±0.37	7.91±0.37
30	7.15±0.39	7.12±0.38	7.14±0.44	7.09±0.38	7.03±0.39	7.28±0.34
40	6.81±0.36	6.73±0.44	6.72±0.38	6.75±0.38	6.68±0.35	7.03±0.11

*: representsa significant difference from the other two methods using ANOVA and the Mann-Whitney U method ($p<0.05$).

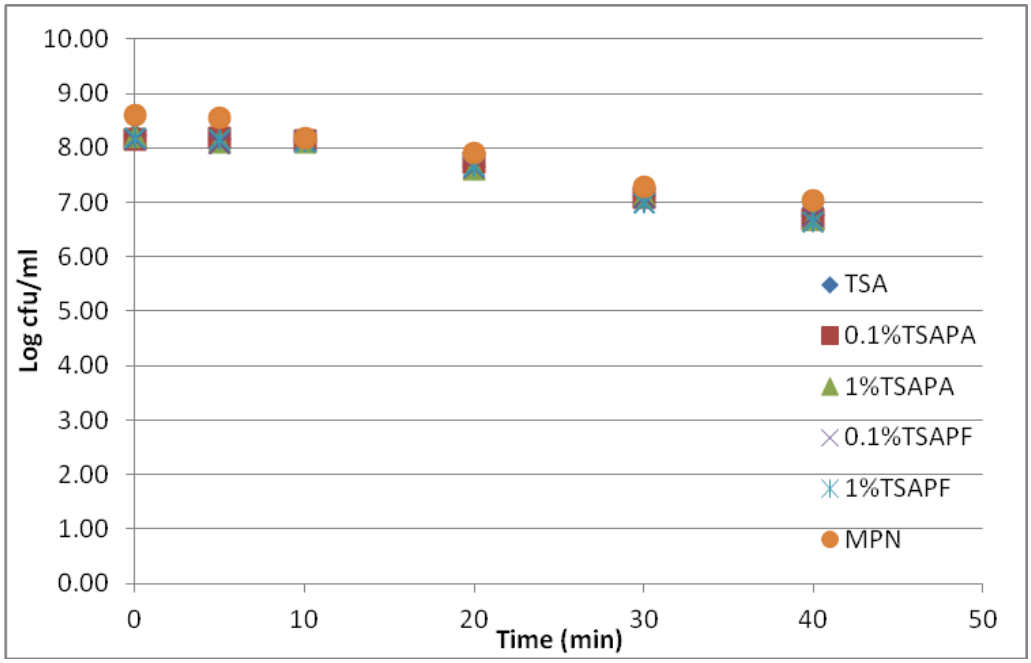

Fig 3.1: Comparison of recovery methods for heat stressed *Cr. turicensis* 1211 at 49 ^{o}C

3.3.2 Identification of sublethal heating condition for each culture

Table 3.2 and Fig 3.2 describe survival of *Cr. turicensis* 1211 after heating at 48, 49 and 50 ^{o}C. Using the plate count technique for enumeration, it was found that at 48 ^{o}C, the cell number reduced very slightly within 40 min; at 49 ^{o}C, cells decreased slowly during the whole 30 min; and finally at 50 ^{o}C, the cell number declined a little faster than at 49 ^{o}C during 30 min. Observation of the results suggested little difference between recovery during the whole heating period at 48 ^{o}C and 49 ^{o}C, and during the first 15 min heating at 50 ^{o}C. However, based on statistical evaluation, at 48 ^{o}C, no significant reduction in cell number was recorded during the whole 40 min. At 49 ^{o}C, there was no significant difference between the initial and first 7 min enumeration results. After this, there was a significant decline, so the heating was considered lethal, rather than sublethal. At 50 ^{o}C, the p value was 0.05 between initial cell number and number surviving after the first 5 min heating. As the p value was marginal for significance, it was difficult to determine whether there was a significant difference between them.

Table 3.2: Log (cfu/ml) numbers of surviving *Cr. turicensis* 1211 after heating (mean±SD) (six replicates)

Time (min)	48 °C	49 °C	50 °C
0	8.09±0.13	8.01±0.11	8.07±0.11
5	8.05±0.13	7.98±0.05	7.88±0.14**
7	-	7.91±0.12	-
10	8.08±0.13	7.87±0.12*	7.66±0.16*
15	-	-	7.26±0.21*
20	8.00±0.11	7.59±0.10*	6.86±0.20*
30	7.94±0.13	7.37±0.19*	6.30±0.14*
40	7.91±0.15	-	-

* significant difference between survival numbers using ANOVA ($p<0.05$).
** p value is 0.05 using ANOVA.

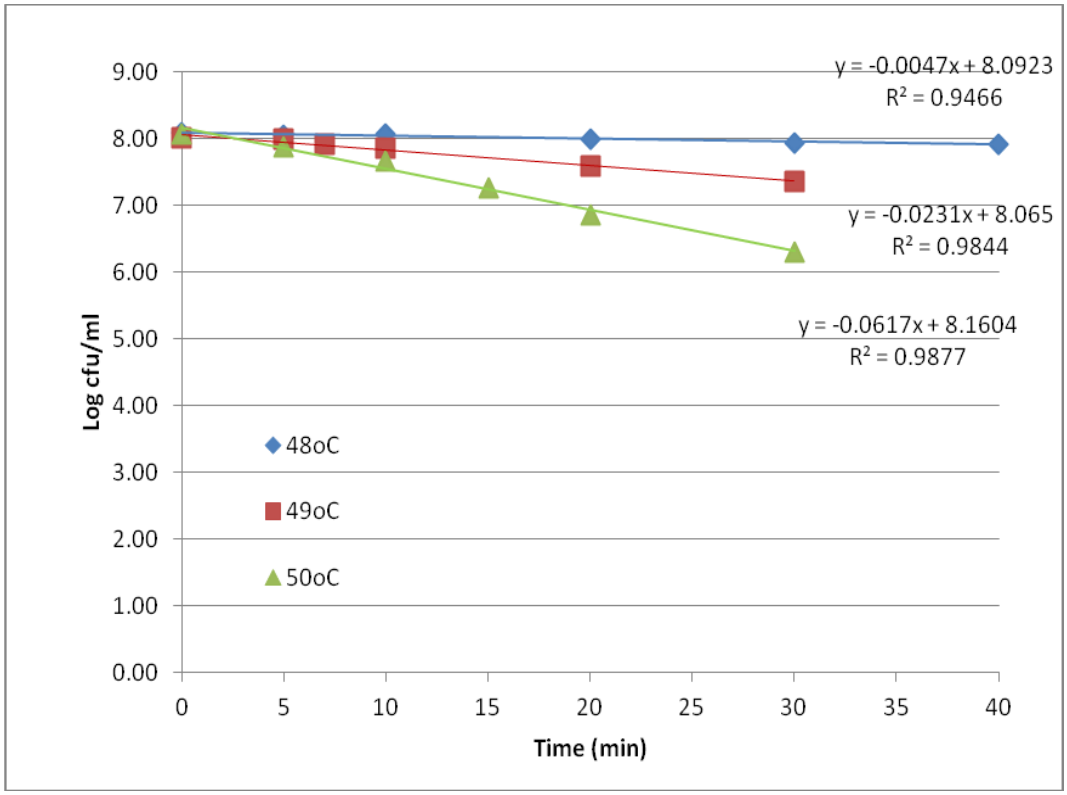

Fig 3.2: Survival curves of *Cr. turicensis* 1211 at 48, 49 and 50 °C

Regarding *Cr. turicensis* 57, Table 3.3 and Fig 3.3 show the cell number reduced slightly during the whole 40 min at 48 ^{o}C; at 49 ^{o}C, the cell number reduced noticeably, while at 50 ^{o}C, the cell number decreased considerably. By inspection, no major difference was observed between recoveries during the whole heating period at 48 ^{o}C and 49 ^{o}C, and the first 10 min heating at 50 ^{o}C. Based on statistics, at 48 ^{o}C, there was no significant difference between the cell number initially and after 10 min heating. But after 10 min, the cell number reduced significantly. At 49 ^{o}C, there was no significant difference between the initial cell number and the surviving number after heating 3.5 min, but after that the cells reduced significantly. At 50 ^{o}C, there was a significant difference in cell number between each interval throughout the heating period.

Table 3.3: Log(cfu/ml) numbers of surviving *Cr. turicensis* 57 after heating (mean±SD)

Time (min)	48 ^{o}C	49 ^{o}C	50 ^{o}C
0	8.13±0.07	8.03±0.07	8.00±0.13
2.5	-	-	7.88±0.08**
3.5	-	7.97±0.09	-
5	8.14±0.10	7.90±0.10**	7.72±0.10**
10	8.10±0.08	7.83±0.06**	7.55±0.08**
20	8.00±0.06*	7.64±0.07**	6.91±0.16**
30	7.96±0.05*	7.34±0.13**	5.94±0.32**
40	7.88±0.06*		

* Significant difference between the survival numbers using ANOVA ($p<0.05$). ** Significant difference between survival numbers using ANOVA and Mann-Whitney U method ($p<0.05$).

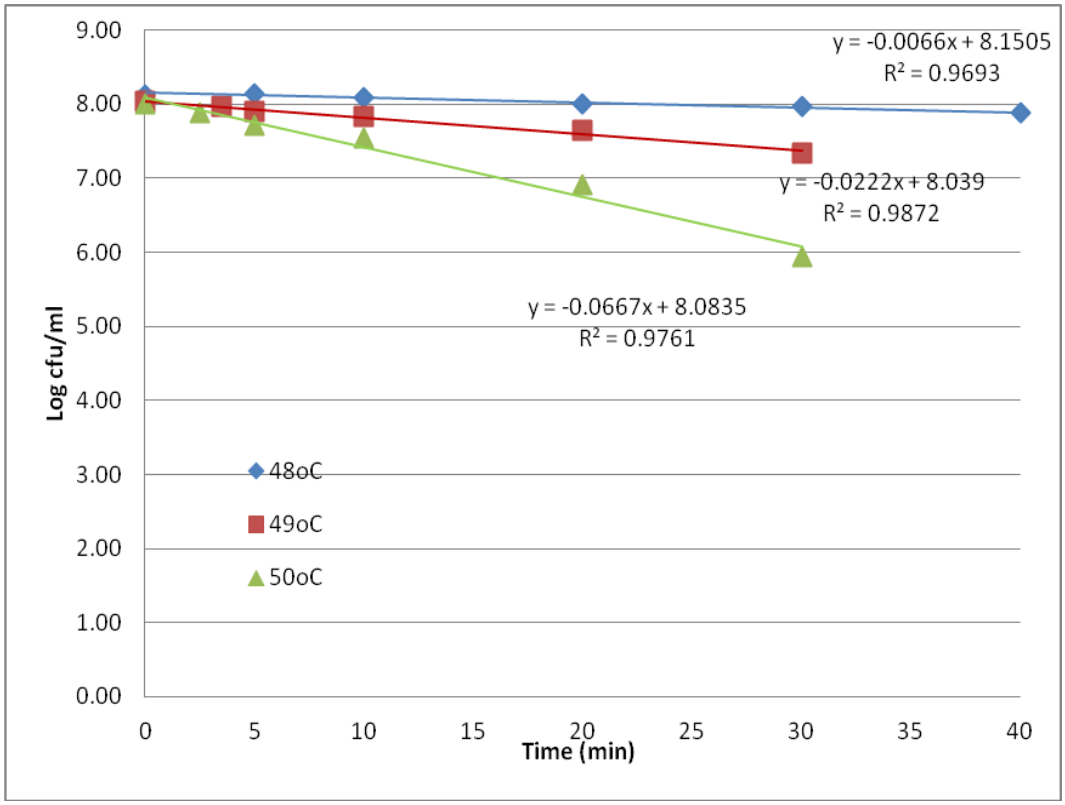

Fig 3.3: Survival curves of *Cr. turicensis* 57 at 48, 49 and 50 ^{o}C

Table 3.4 and Fig 3.4 describe survival of *Cr. sakazakii* NCIMB 5920 after heating at 48, 49 and 50 ^{o}C. Observation of the data at 48 ^{o}C and 49 ^{o}C suggests cell numbers appeared unchanged during the whole heating period; at 50 ^{o}C, the cell number decreased gradually after 10 min heating.Based on statistical evaluations, at 48 ^{o}C, there was no significanct difference between surviving cell numbers throughout the whole 40 min; at 49 ^{o}C, for the first 20 min, there was no significant difference between the enumerations. After that, surviving cells decreased significantly. At 50 ^{o}C, during the first 5 min, there was no significant difference between the surviving cell numbers, but subsequently there was a significant difference between the surviving cells at each time point.

Table 3.4: Log (cfu/ml) numbers of surviving *Cr. sakazakii* NCIMB 5920 after heating (mean±SD)

Time (min)	48 ^{o}C	49 ^{o}C	50 ^{o}C
0	8.07±0.04	8.11±0.06	8.09±0.07
3	-	-	8.07±0.04
5	8.05±0.04	8.09±0.05	8.02±0.06
7	-	8.11±0.05	7.94±0.07**
10	8.04±0.03	8.08±0.06	7.91±0.05**
20	8.05±0.05	8.06±0.07	7.72±0.15**
30	8.04±0.04	7.95±0.07*	-
40	8.04±0.04	-	-

* Significant difference between the survival numbers using ANOVA ($p<0.05$). ** Significant difference between survival numbers using ANOVA and Mann-Whitney U method ($p<0.05$).

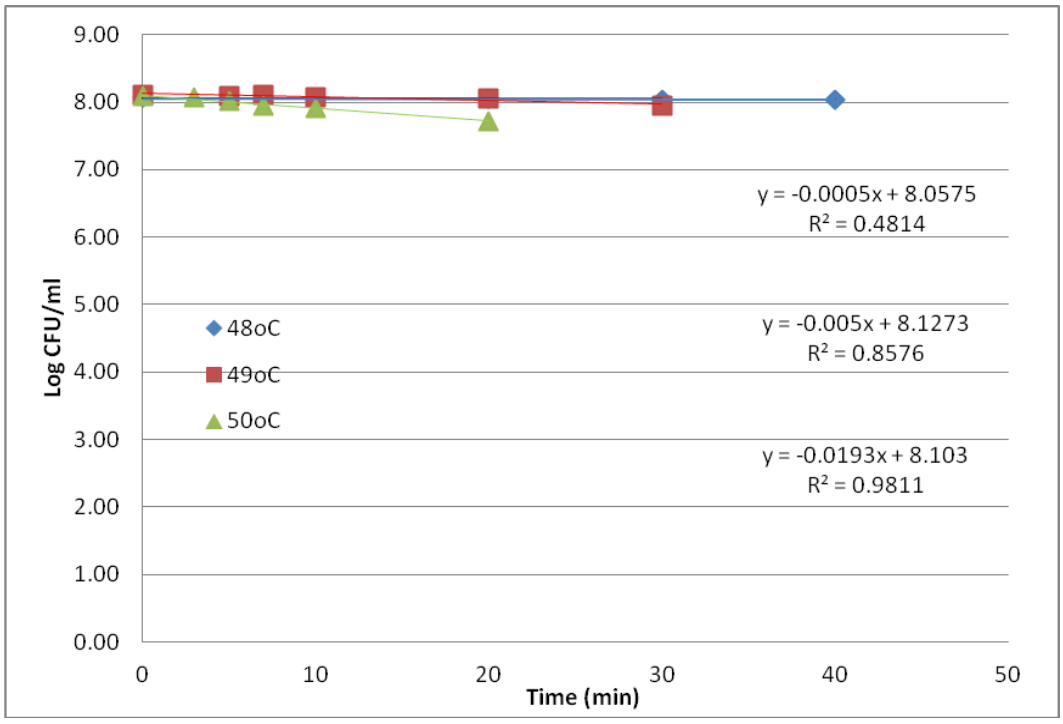

Fig 3.4: Survival curves of *Cr. sakazakii* NCIMB *5920* at 48, 49 and 50 ^{o}C

As summarised in Table 3.5, sublethal conditions (that may cause damage but no loss of viability) for *Cr. turicensis* 1211 were >40 min at 48 ^{o}C, 7 min at 49 ^{o}C; for *Cr. turicensis* 57, they were 10 min at 48 ^{o}C and 3.5 min at 49 ^{o}C and for *Cr. sakazakii* NCIMB 5920, they were >40 min at 48 ^{o}C, 20 min at 49 ^{o}C and 5 min 50 ^{o}C. The other conditions were lethal.

Table 3.5: Summary of sublethal conditions for *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920

Temp	Overall sublethal condition (min)		
	Cr. turicensis 1211	*Cr. turicensis* 57	*Cr.sakazakii*NCIMB 5920
48 ^{o}C	>40	10	>40
49 ^{o}C	7	3.5	20
50 ^{o}C	-	-	5

Temp: temperature.

3.3.3 Estimation of heat resistance parameters

According to Figs 3.2, 3.3 and 3.4, it is clear that linear regression fits most of the survival data for the three *Cronobacter* cultures at three temperatures (R^2>0.9). However, the coefficients of determination for linear regression for *Cr. sakazakii* NCIMB 5920 at 48 ^{o}C and 49 ^{o}C are quite low, at only 0.4814 and 0.8576.

According to Table 3.6, for *Cr. turicensis* 1211 and *Cr. turicensis* 57, the D values were 223.47 and 153.65 min at 48 ^{o}C; 45.28 and 46.84 min at 49 ^{o}C and 16.3 and 15.13 min at 50 ^{o}C. In terms of *Cr. sakazakii* NCIMB 5920, only the Dvalue at 50 ^{o}C was estimated, which is 62.12 min.

Cr. sakazakii NCIMB 5920 showed the highest thermal resistance of the three strains, while the thermotolerance of *Cr. turicensis* 1211 and *Cr. turicensis* 57 was quite close at 49 ^{o}C and 50 ^{o}C. However at 48 ^{o}C, based

on the mean of the D value, *Cr. turicensis* 1211 was more heat tolerant than *Cr. turicensis* 57, but the high SD of the D value of *Cr. turicensis* 1211 cannot be readily explained.

Table 3.6: Heat resistance (D value in min) of *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920

HRP	Temp	*Cr. turicensis* 1211	*Cr. turicensis* 57	*Cr.sakazakii* NCIMB 5920
D value (mean±SD) (min)	48 ^{o}C	223.47±101.82	153.65 ± 20.34	-
	49 ^{o}C	45.28±10.78	46.84± 10.22	-
	50 ^{o}C	16.3±0.9	15.13±2.04	62.12±24.4
z value (^{o}C)		1.79	1.98	-

HRP: heat resistance parameters; Temp: temperature. D and z values were estimated from data that follow death through <2 log cycles.

Linear regression was used to fit the $\log_{10}$ D value versus temperature (Fig 3.5). The coefficients of determination (R^2) for *Cr.turicensis* 1211 and *Cr.turicensis* 57 are 0.9814 and 0.9992 respectively. According to Table 3.7, the z values of *Cr.turicensis* 1211 and *Cr.turicensis* 57 were 1.79 ^{o}C and 1.98 ^{o}C.

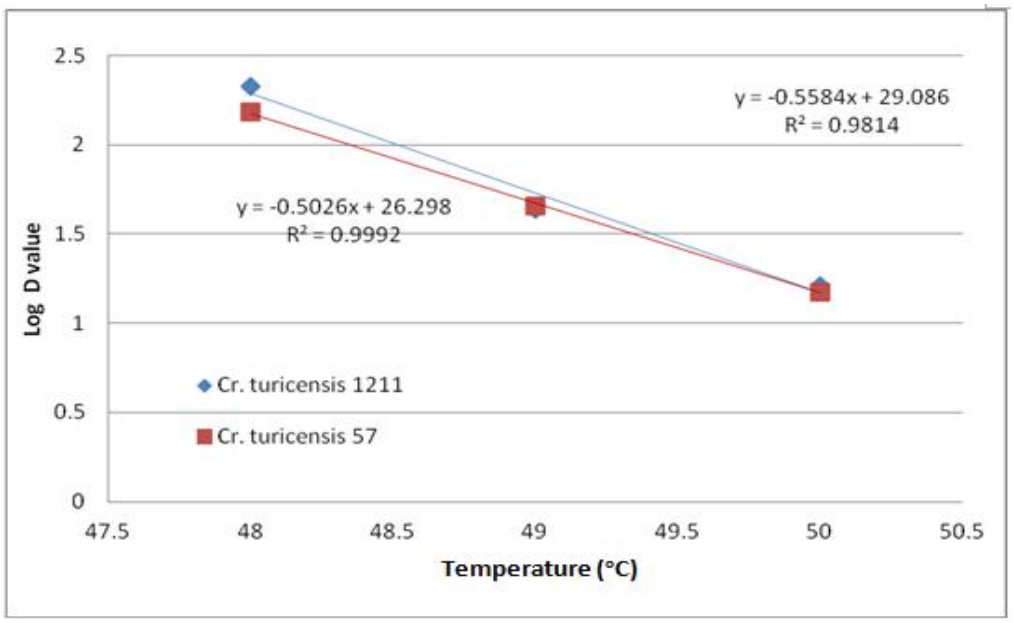

Fig 3.5: z value for *Cr. turicensis* 1211 and *Cr. turicensis* 57

3.3.4 Evaluation of degree of sublethal injury

Table 3.7 depicts the injured cell percentage in each sublethally heat stressed culture. For *Cr. turicensis* 1211, the percentage of injured cells in the whole population after sublethal heating at 48 ^{o}C and 49 ^{o}C were 86.64% and 94.04% respectively. For *Cr. turicensis* 57, the percentages of injured cells were 84.39% at 48 ^{o}C and 95.04% at 49 ^{o}C. The percentage of injured cells in each sublethally heated culture of *Cr. sakazakii* NCIMB 5920 was between 75.75% and 96.54%.

According to Table 3.8, there were 55.93%, 71.04% and 48.81% injured cells present in untreated (control) stationary phase cultures of *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 respectively. To distinguish the relevant degree of sublethal injury due to the imposed heat stress, an approach described by Jasson et al. (2007) was used. A threshold is defined and estimated as one standard deviation (SD) above the mean percentage of injured cells in an untreated culture. Above this threshold, the percentage of sublethal injury of the cells is due to the imposed heat stress. The threshold values for *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 were calculated to be 69.71%, 80.04% and 63.79% respectively. Based on this, the percentage

of injured cells resulting from heat treatment for each culture was estimated. For *Cr. turicensis* 1211, 16.93% of the cells were injured due to heating at 48 ^{o}C for 40 min; 24.33% due to heating at 49 ^{o}C for 7 min. For *Cr. turicensis* 57, 4.34% of the cells were injured due to heating at 48 ^{o}C for 10 min; 15.00% due to heating at 49 ^{o}C for 3.5 min. Finally, for *Cr. sakazakii* NCIMB 5920, it was 11.96%, 35.11% and 32.75% caused by heating at 48 ^{o}C for 40 min, 49 ^{o}C for 20 min heating and 5 min for 50 ^{o}C heating, respectively.

Table 3.7: Comparison of recovery procedures and determination of percentage injury for *Cronobacter* spp. surviving sublethal heat treatments (mean±SD)

Cronobacter sp.	Type of sublethal heat treatment	Counts on TSA (log cfu/ml)	Counts on VRBGA (log cfu/ml)	Percentage of injured cells (%)	Percentage of injured cells (%) due to heat treatment
Cr. turicensis 1211	SPC*	8.25±0.13	7.88±0.17	55.93±13.78	-
	48 ^{o}C/ 40 min	7.81±0.22	6.92±0.30	86.64±3.38	16.93
	49 ^{o}C/ 7 min	8.05±0.05	6.65±0.38	94.04±6.06	24.33
Cr. turicensis 57	SPC*	8.21±0.07	7.66±0.14	71.04±9.00	-
	48 ^{o}C/ 10 min	8.10±0.12	7.25±0.17	84.39±6.77	4.34
	49 ^{o}C /3.5 min	7.93±0.19	6.57±0.19	95.04±2.74	15.00
Cr. sakazakii NCIMB 5920	SPC*	8.22±0.04	7.91±0.13	48.81±14.98	-
	48 ^{o}C/40 min	8.01±0.04	7.40±0.07	75.75±3.44	11.96
	49 ^{o}C /20 min	7.86±0.14	5.83±0.26	98.90±0.55	35.11
	50 ^{o}C/ 5 min	8.11±0.08	6.63±0.11	96.54±1.26	32.75

SPC* stationary phase cells, unheated (control)

3.4 Discussion

Although exponential phase cells are more homogeneous and likely to provide more consistent heat injury profiles (Stephens et al. 1997), such

cells are usually more fragile and more susceptible to stress than stationary phase cells (Bréand et al. 1998, Mackey 2000, Robinson et al. 2001, Pascual et al. 2001, Arroyo et al. 2009, McMeekin et al. 2011). In addition, compared with exponential phase cells, stationary phase cells are more predominant in normal environments (Bréand et al. 1998, Skandamis et al. 2008). Moreover, stationary phase cells also provided reproducible results (Robinson et al. 1998), so stationary phase cultures were used for thermal inactivation experiments and to identify sublethal heating conditions for each culture at various temperatures. Growth experiments undertaken, but not herein reported, revealed that *Cr. turicensis* 1211 enter stationary phase after 14 h incubation at 22 ^{o}C.

3.4.1 Recovery of heated stress cells using various recovery methods

A substantial body of research suggests that possibly due to inhibitors in agar or desiccation effects, stressed cells recover better in liquid media than on solid (Mackey et al. 1994, Stephens et al. 1997, Mackey2000, Niven et al. 2008, Özkanca et al. 2009). Meanwhile, Özkanca et al. (2009) evaluated the use of different recovery methods for enumeration of *Esch. coli* subjected to heat stress and light induced oxidative stress, and found no significance difference in recovery efficiency between the MPN and plate count methods. In this study, two out of six samples of *Cr. turicensis* 1211 showed significantly higher recovery using the MPN method. However, as in the findings of Özkanca et al. (2009), four samples showed no significant difference between the MPN method and the plate method for recovery of *Cr. turicensis* 1211. Nevertheless, Özkanca et al. (2009) reported that the MPN method may enhance recovery of chlorine stressed bacteria.

Regarding sodium pyruvate, few papers mention procedures for adding sodium pyruvate. Among these papers, autoclaved (Brewer et al. 1977, McDonald et al. 1983) and filter sterilized sodium pyruvate (Iandolo &Ordal 1965, D'Aoust 1978) were used. So, in this study, the recovery ability of both methods was investigated.

Earlier research demonstrated that addition of sodium pyruvate could improve recovery of heat stressed microorganisms on non-selective media. The research of Martin et al. (1976) shows that, compared with TSA, both uninjured and heat injured *Staph. aureus* show a higher recovery on TSA supplemented with sodium pyruvate. Rayman et al. (1978) resuscitated heat stressed *S. enterica* serovar Senftenberg in phosphate buffer at 56 ^{o}C for 20 and 30 min and found that recovery on TSA supplemented with 1% sodium pyruvate was 90 to 315 times higher than that on non-supplemented TSA. In the research of McDonald et al. (1983), *Esch. coli* was enumerated on non-selective tryptone-glucose extract agar (TGE) with and without supplementation with sodium pyruvate after being heat stressed at 57 ^{o}C for 12 min. Numbers of stressed *Esch. coli* increased at least 2,384% more than those on non-supplemented TGE. Moreover, addition of sodium pyruvate (1% or 0.1%) to non-selective media to improve recovery of heat stressed *Cronobacter* spp. has been widely used in recent years (Breeuwer et al. 2003, Edelson-Mammel &Buchanan 2004, Gurtler & Beuchat 2005, Osaili et al. 2008, Shaker et al. 2008, Al-Holy et al. 2009). However, in this project, whether using TSAPA (autoclaved) or TSAPF (filter sterilized) such improvements were not observed. Moreover, the results herein reported indicated no statistically significant difference between recovery on TSA compared with TSAP (1% or 0.1%), which is in agreement with the finding of Forsythe (2009).

In this study, first, although two samples showed significantly higher recovery with the MPN method, most samples did not demonstrate significant improvement in recovery with this method; second, the MPN method proved extremely costly in terms of time and materials; third, as there was no significant difference between the recovery on TSA and TSAP, direct plating on TSA without added pyruvate was chosen as the recovery method for heat stressed cells.

3.4.2 Selection of appropriate culture and sublethal heating regime

According to Table 3.6, for *Cr. turicensis* 1211 and 57, two sublethal conditions for each strain were obtained; while for *Cr. sakazakii* NCIMB

5920, three sublethal conditions were achieved. If all recoveries of single cells of these three organisms in optimal and suboptimal conditions at various temperatures after sublethal heating were to be investigated, the project would not finish in three years. So, further experiments to evaluate sublethal injury of each organism were carried out to make a further selection of a single culture and sublethal condition. Basically, the strain with the highest thermal tolerance and injury rate was preferred.

VRBGA is one of the selective media for *Cronobacter* spp. based on the FDA detection method (FDA 2002, Cawthorn et al. 2008, Lampel & Chen 2009). Heat damage to the Gram-negative outer membrane causes the cells to become sensitized to bile salts present in VRBGA (Mackey 2000). Moreover, VRBGA is cheaper, so, based on these considerations, VRBGA was used as a selective medium and the enumeration result compared with that of TSA. The proportion of injured cells could therefore be estimated for each sublethal condition.

Jasson et al. (2007) verified performance of selective media by plating unstressed *L. monocytogenes*, *Esch. coli* O157:H7 and *Campylobacter jejuni* on non-selective media and various selective media. They reported that for unstressed *Esch. coli* O157:H7, there was a significant difference between the numbers obtained on tryptone soy agar supplement with yeast extract (non-selective) and sorbitol MacConkey agar supplemented with cefixime tellurite selective supplement (selective). Gurtler and Beuchat (2005) investigated performance of five selective media for supporting recovery of heat, freeze, acid, alkaline and desiccation stressed cells of *Cronobacter spp*. Based on enumeration on TSAP and VRBGA, 63.69% of injured cells were estimated to exist in the unstressed stationary phase population of *Cronobacter* spp. In addition, a range of 46.30% to 84.51% of injured cells in unstressed stationary phase *Cronobacter* spp. was calculated, depending on the selective media used (Gurtler and Beuchat 2005). In this study, a very high percentage of injured cells in the untreated *Cronobacter* spp. population was observed, i.e. 55.93%, 71.04% and 48.81% for *Cr. turicensis* 1211, *Cr. turicensis* 57

and *Cr. sakazakii* 5920 respectively (Table 3.7). This observation is consistent with the studies of Jasson et al. (2007) and Gurtler and Beuchat (2005). Moreover, our results also indicate that a false negative result may occur when using the current FDA method to recover *Cronobacter* spp., because a minimum of approximately 50% (48.81%) of untreated *Cronobacter* spp. grew on TSA while they could not grow on VRBGA. Therefore, more studies should be carried out to choose an appropriate selective agar to improve the performance of the current FDA method. Alternatively, a resuscitation or pre-enrichment step could be investigated, that could be used prior to plating onto VRBGA.

According to Tables 3.5 and 3.6, among all cultures, *Cr. sakazakii* NCIMB 5920 showed the highest thermal resistance. In addition, after heating at 49 ^{o}C for 20 min (Table 3.7), this culture showed 98.90% of the cells were injured and within this percentage, 35.11% of the injury was attributed to the heating. These percentages represent the highest percentage of injury in all sublethal heating conditions. So ideally, it would appear that *Cr. sakazakii* NCIMB 5920 and the sublethal heating condition (49 ^{o}C for 20 min) should be chosen for further study. However, the main interest of this study is to explore the ability of individual cells of untreated and heat stressed *Cronobacter* spp. to recover and multiply at low temperatures. The minimum growth temperature experiments showed that growth of *Cr. sakazakii* NCIMB 5920 in TSB at 7 ^{o}C is very slow (Chapter 2) and when an individual cell was inoculated into microtitre plate, the culture does not become turbid until it forms colony (deposit of cells) at the bottom of the well in the microtitre plate (preliminary experimental results). Consequently, another organism which has a relatively high thermal tolerance and a good ability to initiate growth at 7 ^{o}C, was preferred. For *Cr. turicensis* 1211 and *Cr. turicensis* 57, because there is insufficient thermal injury at 48 ^{o}C to consider this temperature for further experiments, only 49 ^{o}C was considered. Since the thermal tolerance of *Cr. turicensis* 1211 and *Cr. turicensis* 57 are quite similar at 49 ^{o}C (Table 3.6), while *Cr. turicensis* 1211 has a high percentage of injury, in both the entire stressed population (94.04%) and due to the imposed sublethal heating (24.33%) at

49 ^{o}C (Table 3.7), with a very good ability to grow at 7 ^{o}C. Therefore, this organism and a sublethal heating regime of 49 ^{o}C for 7 min was chosen for further research.

3.4.3 Determination of thermal inactivation rates of *Cronobacter* spp.

Research investigating kinetics of thermal inactivation assumes that viable counts of the microorganism reduce more or less exponentially with heating time. Based on this assumption, the D value is estimated (Métris et al. 2008). The D value is defined as the time needed to result in a 90% reduction in the number of bacteria at a specific temperature, while the z value is defined as the temperature change required to cause a 10 fold alteration in D value (Forsythe 2009).

Determination of thermal inactivation rates of *Cronobac*ter spp. is not part of the project. However, since thermal inactivation experiments were carried out, and most of the survival curves of *Cronobacter* spp. followed first order kinetics, evidenced by an acceptable coefficient of determination (R^2>0.9), it was of interest to calculate the D value and z values of each species. It should be pointed out that in this study, as the coefficients of determination of the log-linear model for *Cr. sakazakii* 5920 at 48 ^{o}C and 49 ^{o}C were very low, about 0.4814 and 0.8576 respectively, so for *Cr. sakazakii* 5920, only the D value at 50 ^{o}C was estimated.

Some research reported that heat resistance of *Cronobacter* spp. varied widely (Dancer et al. 2009). Gurtler and Beuchat (2005) found a nearly 4-fold difference in heat resistance between the most and the least thermally resistant strain in PIF, while Al-Holy et al. (2009) found a 10 times difference in the same conditions and Edelson-Mammel &Buchanan (2004) found a nearly 20 times difference between the strains. Inthis study, at 50 ^{o}C, a 4.1 times difference was recorded between the heat resistance of *Cr. sakazakii* NCIMB 5920 and *Cr. turicensis* 57, similar to the result of Gurtler and Beuchat (2005).

Many factors affect heat resistance of microorganisms, e.g. strain or species of culture studied, physiological state of bacteria, growth

temperature of inoculum, different heating media and recovery methods (Nazarowec-White and Farber 1997c, Arroyo et al. 2009, Osaili et al. 2009b). All these elements make it difficult to compare published D values (Nazarowec-White and Farber 1997c). Dancer et al. (2009) reported D values between 8.6 and 85.5 min at 50 ^{o}C in phosphate bufferfor *Cronobacter* spp. The D values of *Cr. sakazakii* NCIMB 5920, *Cr. turicensis* 1211, *Cr. turicensis* 57 at 50 ^{o}C obtained in this study were 62.12, 16.3 and 15.13 min respectively, which is within the range reported by Dancer et al. (2009). Kim and Park (2007) studied the thermal characteristics of Korean isolates of *Cronobacter* spp. in saline, rehydrated infant formula and dried baby food. The D values for *Cronobacter* spp. they obtained in saline and dried baby food at 52 ^{o}C ranged from 12.0 to 16.2 min and 14.2 to 17.5 min respectively, quite close to the D values of *Cr. turicensis* 1211 and *Cr. turicensis* 57 at 50 ^{o}C obtained in this study.

So far, most of the z values of *Cronobacter* spp. published are between 4 ^{o}C and 6 ^{o}C (Nazarowec-White and Farber 1997c, Iversen et al. 2004b, Shaker et al. 2008, Osaili et al. 2009b) (Table 3.8), which is within the usual range for most non-sporeforming bacteria (Nazarowec-White and Farber 1997c). Also, however, there are some studies which report a z value out of this range. Breeuwer et al. (2003) reported z values of 3.1 ^{o}C and 3.6 ^{o}C for a *Cronobacter* sp. in phosphate buffer. Forsythe (2009) reported a 2.1 ^{o}C z value for *Cr. sakazakii* in whey-based PIF and Dancer et al. (2009) reported z values for *Cronobacter* spp. in phosphate buffer of 6.3-10.9 ^{o}C. Kim & Park (2007) published z values of *Cronobacter* spp. in saline of 6.3-8.1^{o}C, in PIF of 8.2-10.4 ^{o}C and in baby food of 9.2-11.3 ^{o}C. In this study, the z values of *Cr. turicensis* 1211 and *Cr. turicensis* 57 are 1.79 and 1.98 ^{o}C respectively, much lower compared to these previous publications. This may be due to the different temperature range used to estimate z values. In this study, the z values were estimated for the temperature range between 48 ^{o}C and 50 ^{o}C, which is just above the maximum growth temperature, while in most other studies, z values were estimated for the temperature range 52 ^{o}C to 58 ^{o}C (Table 3.8). It also may be due to the fact that the D and z values estimated in this study from

the data following death through <2 log cycles. However, the D and z values determined in this circumstance may be of limited validity.

3.5 Conclusion

Only a limited number of heat stressed samples (two out of six) showed a higher recovery with the MPN method than on solid media, and there was no significant difference between recovery on TSA and TSAP. Therefore, also taking time and cost into consideration, direct plating on TSA without added pyruvate was selected as the recovery method for heat stressed cells.

Based on thermal inactivation experiments, sublethal heating conditions for each culture were identified. For *Cr. turicensis* 1211 heating for >40 min at 48 ^{o}C and for 7 min at 49 ^{o}C were sublethal. In the case of *Cr. turicensis* 57, heating for 10min at 48 ^{o}C and 3.5 min at 49 ^{o}C were sublethal, while for *Cr. sakazakii* NCIMB 5920, sublethality occurred at times beyond 40 min at 48 ^{o}C, 20 min at 49 ^{o}C and 5 min at 50 ^{o}C. Other conditions were lethal.

The heat resistance of *Cronobacter* spp. varied widely for a given temperature among the organisms examined. The D values of *Cronobacter* spp. were between 153.65 and 223.47 min at 48 ^{o}C, between 45.28 and 46.84 min at 49 ^{o}C and between 15.13 and 62.12 min at 50 ^{o}C.

Since the strain *Cr. turicensis* 1211 showed a high percentage of injury at 49 ^{o}C and a good ability to grow at 7 ^{o}C, this strain and the sublethal heating condition of 49 ^{o}C for 7 min was chosen for further research. The recovery of single cells of untreated and sublethally heat stressed *Cr.turicensis* 1211 under refrigeration and at room temperature will be further investigated in the following chapter.

Table 3.8: D value and z value of *Cronobacter* spp. (Modified based on Iversen and Forsythe 2003)

Substrate	$D_{50(}$min$_{)}$	$D_{52(}$min$_{)}$	$D_{54(}$min$_{)}$	$D_{55(}$min$_{)}$	$D_{56(}$min$_{)}$	$D_{58(}$min$_{)}$	$D_{60\ (}$min$_{)}$	$D_{62(}$min$_{)}$	Z(^{o}C) value	Reference
Citrate-phosphate buffer						2.0	0.9	0.2	4.5	Arroyo et al. 2009
IFM				1.5-14.8			0.2-2.7		3.8-10.1	Al-Holy et al. 2009
Phosphate buffer	8.6-85.5			3.3-17.1			1.1-2.2		6.3-10.9	Dancer et al. 2009
Reconstituted milk		15.3-22.1	4.8-7.5		1.5-2.7	0.5-0.7			4.0-4.4	Osaili et al. 2009b
Reconstituted feeding formula		17.2-19.6	5.4-6.1		2.3-2.5	0.6-0.7			4.2-4.3	
PIF		15.3±2.2	4.5±0.6		2.0±0.4	0.5±0.03			4.2±0.2	Shaker et al. 2008
PIF		16.4± 0.12	5.3±0.01		2.1± 0.1	0.6± 0.01			4.1±0.03	Osaili et al. 2008
Saline		12.0-16.2			3.4-4.7		0.9-1.2		6.3-8.1	
Infant formula		16.4-20.1			3.9-4.7		2.1-2.8		8.2-10.4	Kim & Park 2007
Baby food		14.2-17.5			5.2-6.3		2.1-2.8		9.2-11.3	
TSB			14.9±0.7 10.2±3.6[a]		2.7±0.1 1.2±0.01[a]	1.3±0.3 1.7±0.4[a]	0.9±0.2 0.2±0.1[a]	0.4±0.1 0.2±0.13[a]	5.6±0.1 5.6±0.5[a]	Iversen et al. 2004b
IFM			16.4±0.7 11.7±5.8[a]		5.1±0.3 3.9±0.1[a]	2.6±0.5 3.8±1.9[a]	1.1±0.1 1.8±0.8[a]	0.3±0.1 0.2±0.1[a]	5.8±0.4 5.7±0.1[a]	
PIF					21.1±2.7	0.5-10.4	4.4±0.4		5.6	Edelson-Mammel & Buchanan 2004
Phosphate buffer			6.4-7.1		1.1-2.4	0.3-0.5			3.1-3.6	Breeuwer et al. 2003
PIF with highest fat content (3.8g/100ml)		54.8-54.8	18.6-36.7		10.9- 9.8	5.5-3.4	3.1-2.2		5.6-6.0	Nazarowec-White and Farber 1997c

a: Capsulated strain

CHAPTER 4: Modelling variability of single-cell lag times for untreated and sublethally heat stressed *Cronobacter* spp.

4.1 Introduction

Direct and indirect methods have been proposed to investigate the lag times of single cells. Direct methods are based mainly on microscopic imaging systems by which the time to first division of the individual cell immobilized on an agar slide is determined (Wu et al. 2000, Niven et al. 2006, Rasch et al. 2007, Niven et al. 2008). This method allows direct observation of the first cell division and is often regarded as the 'gold standard' for studying individual cell behaviour (Niven et al. 2008). However, the limitation of this method is that the cells with short lag will overgrow those cells dividing later (with a longer lag) (Métris et al. 2006, Niven et al. 2006). An alternative way is mounting the microscope image system with a flow chamber; the sheer force of the flow can flush the daughter cells away and enable monitoring of the consecutive divisions of the individual cells (Elfwing et al. 2004, Kutalik et al. 2005b). However, while this method works with cells that attach well to the surface, e.g. *Esch. coli*, it is less useful for others, such as *Listeria* spp., which are easily detached from surfaces (Métris et al. 2006).

Regarding indirect methods, lag times of single cells can be estimated through optical density (OD) measurements using a specialised OD reader incorporating an incubator, such as the Bioscreen (Labsystems, Vantaa, Finland), in which growth of single cells inoculated into a maximum of 200 wells can be followed concurrently (Guillier et al. 2006, Niven et al. 2008). If each well is inoculated with N_0 cells, and assuming that, after the lag, the cells grow at a constant specific growth rate (μ') until the cell concentration reaches N_{det} at detection time (T_{det}), then the lag ($Lag(N_0)$) can be calculated with equation 4.1. This is only valid if the culture is still in the

exponential phase (the maximum growth rate is still extant) at the T_{det} (Métris et al 2003).

$$\mathrm{Lag}(\mathrm{N}_0) = \mathrm{T}_{\mathrm{det}} - \frac{\ln(\mathrm{N}_{\mathrm{det}}) - \ln(\mathrm{N}_0)}{\mu'} \qquad (4.1)$$

The estimated lag based on this method is the geometrical lag (Métris et al 2006; see also Chapter 1). If the starting number is exactly one cell, i.e. if the new population develops from one single cell, then distribution of detection times for a number of single cells should be reflected as a shifted distribution of individual lag phase (Métris et al 2003).

It is not easy to deliver exactly one individual cell into each of the wells of standard microtitre plates or the specialised 100-well “honeycomb” plates used in the Bioscreen. One choice is to use a flow cytometer (Smelt et al. 2002). A sampling bias against cells with short lag could possibly be introduced as the cells are sorted according to size (Métris et al. 2006, Baranyi et al. 2009). Serial dilution is an alternative way to obtain approximate single cells (Francois et al. 2003). This results in a random number of cells per well which follow a Poisson distribution (Baranyi et al. 2009). So, from the equation 4.1, the distribution of the detection time is a convolution of the distribution of the initial cell numbers and the single cell lag time (Métris et al. 2003).

Early researches used a linear method to relate individual lag time to detection time, which substitutes the random variable N_0 (equation 4.1) with its mean (Francois et al. 2005a, 2005b, Li et al. 2006, McKellar & Hawke 2006). However, perfect serial dilution techniques are needed with this method. After dilution, the average initial cell number in each well in the honeycomb plate should not be too high. It has been demonstrated that this linear method is reliable when the average initial cell number in the well is less than two. When it is between two and three, the relative difference of SD between the true single cell lag and the convolution single cell lag (estimated by the linear method) can be as high as 40-50% depend on the generation time (Baranyi et al. 2009). Conversely, the initial cell count must not be too low, since although a sufficiently high dilution

can be used to make sure that most of the wells in the honeycomb plate containzero or one cell and confirm an accurate estimation of individual lag, it will cause too few results for each experiment (many wells will be negative for growth). It is reported that for a statistically robust distribution, a minimum of about one hundred datasets should be obtained (Métris et al. 2003, BACANOVA, 2005). Therefore, the experiment has to be repeated several times under the same condition to obtain sufficient data. When cells are in a stressed condition, the reproducibility is poor and some appropriate standardization procedures are required (Métris et al. 2003, Métris et al. 2006), e.g. Guillier et al. (2005) used the detection time of exponential cells to standardize the variability of detection time of stressed cells between the replicated experiments.

A method to estimate individual geometrical lag from the detection time was developed by Métris et al. (2006). The individual lag time wasassumed to follow a gamma distribution and the parameter of the distribution was estimated based on the physiological state of the culture. Moreover, this method also takes into account the fact that the initial count israndom and is valid when the initial cell number is between one and three (Métris et al. 2006). Using this method, Métris et al. (2006) investigated the individual lag of *L. innocu*a cells at different acetic acid concentrations, compared the population lag estimated from individual lag distribution with the population lag obtained experimentally from a plate count, and reported that all of the predicted population lag times estimated fell within 95% confidence interval of the plate count experiment.

4.1.1 Rationale for research in this chapter

First, cells do not enter the exponential phase immediately at the end of lag; they need a few generations to reach their maximum specific growth rate (Kutalik et al. 2005a). The variability of geometrical lag based on the detection time depends not only on the variability of the lag time, but it is also affected by the variability of the first few generation times (Rasch et al. 2007). With regard to risk assessment of foods, the main concern is the concentration of pathogenic bacteria in the food when consumed. So, the

geometrical lag variability is the most important parameter (Métris et al. 2006). Second, despite the fact that a gamma distribution was assumed for individual lag time, it has been demonstrated to be a generally good fit for the lag time (Francois et al. 2005b, Guillier et al. 2005, Kutalik et al. 2005b, Niven et al. 2006). Third, compared with thedirect linear method, the method of Métris et al. (2006) takes the randomness of initial cell number into consideration.Therefore, the geometrical lag (based on the assumption that the single-cell lag times follow a gamma distribution) was used in the work described in this chapter to investigate the response to various recovery temperatures of individual untreated and sublethally heat stressed *Cronobacter* spp. A primary model was developed to describe the distribution of individual lag for cells of untreated and heat stressed *Cr. turicensis* 1211 at different recovery temperatures. A secondary model was constructed to relate the recovery conditions to the parameters of the primary model.

4.2 Materials and methods

4.2.1 Culture preparation

The culture of *Cr. turicensis* 1211 was prepared as described in section 3.2.2.

4.2.2 Thermal inactivation experiment

The thermal inactivation experiments for *Cr. turicensis* 1211 at 49 ^{o}C were carried out as described in section 3.2.3. However, in this experiment, the tubes were removed from the bath after being heated for 7 min and serial dilutions prepared immediately.

4.2.3 Growth ability of individual cells of untreated and heat stressed *Cr. turicensis* 1211 at various temperatures

Untreated and heat stressed stationary phase cultures were serially diluted to 15 cell/ml and 50 µl of diluted culture were added to 56 wells of a 96-well microtitre plate which already contained 230 µl TSB per well. A

total of three microtitre plates were inoculated from the same culture. The microtitre plates were placed in closed plastic containers, in which wetted paper towel was present to prevent the plates drying out, and incubated in 7, 12 and 12 ^{o}C incubators respectively for an appropriate period (Table 4.1). Wells exhibiting turbidity were considered positive for growth. Wells showing no growth were counted and the average cell number for each plate calculated. The experiment was carried out six times.

Table 4.1: Incubation periods for untreated and heat stressed individual cells of *Cr. turicensis* 1211 in microtitre plates incubated at 7, 12 and 22 ^{o}C

Condition of cells	7 ^{o}C	12 ^{o}C	22 ^{o}C
Untreated	4 weeks	4 weeks	1 week
Heat stressed	4 weeks	4 weeks	4 weeks

4.2.4 OD measurement

Sublethally heated and untreated stationary phase cultures were serially diluted to give approximately 15 cells/ml, and 50 µl of diluted culture were added to 190 wells of two 100-well honeycomb plates (Thermo Fisher Scientific, Basingstoke, UK), which already contained 350 µl TSB per well. This should give an average of one cell per well, although some wells will contain no cells. The other 10 wells (randomly selected) of each plate contained 400 µl of TSB and served as blanks. The filled plates were incubated in the reading chamber of Bioscreen C (FP-1100-C; Labsystems, Finland) at 7, 12 and 22 ^{o}C for up to one month (Table 4.2). The Bioscreen was pre-stabilised in a laboratory refrigerator (LKPv6520; Liebherr, Austria) or a constant temperature room at the appropriate temperature (Table 4.2) to provide a stable environment. The reading chamber of the Bioscreen was pre-heated to a set-point temperature one day before the experiment to allow equilibration. The growth of *Cr.*

turicensis was monitored by reading the OD of the wells at a wavelength of 600 nm at regular intervals (Table 4.2). The single cell growth kinetics were developed by plotting the OD of the suspensions minus the OD of the average of the ten blanks against the time of incubation. For each experiment, the culture in 20 positive wells from the honeycomb plate was randomly collected and streaked onto 20 TSA plates to check the purity of *Cr. turicensis* 1211. The experiment was done twice.

Table 4.2: Monitoring intervals and incubation times in the Bioscreen for untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

Temperature		Time	Untreated	Heat stressed
Bioscreen	R/CR			
7 ^{o}C	4 ^{o}C	Monitoring interval	Every 3 h	Every 3 h
		Incubation time	1month	1month
12 ^{o}C	9 ^{o}C	Monitoring interval	Every 1 h	Every 1 h
		Incubation time	2 weeks	1 month
22 ^{o}C	19 ^{o}C	Monitoring interval	Every 10 min	Every 10 min
		Incubation time	48 h	2 weeks

R/CR: Temperatures in refrigerator and constant temperature room.

4.2.5 OD calibration curve

Calibration curves were constructed at 7 ^{o}C and 22 ^{o}C based on the method of Métris et al. (2006). The 48 h incubation culture (mentioned in 4.2.1) was diluted to contain 10^6 and 10^5 cell/ml. The 400 µl of each diluted culture were added to 95 wells of a honeycomb plate. The other 5 wells, randomly chosen in four positions with the fifth in the centre of the plate, served as blanks, to which were added 400 µl of sterile TSB. The plate was incubated in a Bioscreen at 7 ^{o}C and 22 ^{o}C and the OD values were measured at a wavelength of 600 nm at the same time intervals as described in Table 4.2. A volume of 100 µl of samples from wells which had achieved the appropriate OD value (between 0.01 and 0.4; see 4.1)

was delivered into 9.9 ml of MRD (10^{-2} dilution). Samples (20 µl) of this were decimally diluted and the viable count estimatedthrough plating on TSA followed by incubation at 22 ^{o}C for 48 h. Each sample was taken immediately after the OD was measured. The OD measurement was corrected by subtracting the average of the blanks. The experiment was done twice.

4.2.6 Determination of the detection point

The detection point is the point where a population of cells reaches a detectable OD, while the culture is still in the exponential phase (Métris et al. 2003).

The OD range corresponding to the exponential phase was determined according to the method described by Augustin et al. (1999). Procedurally, $\frac{dOD}{dt}$ is plotted against OD, and if the cells are in the exponential phase, there will be a good linear relationship between $\frac{dOD}{dt}$ and OD. The value of dOD can be estimated by the difference between two consecutive OD readings and d_t is the time interval between two OD readings.

Ten series of datasets of untreated and sublethally heat stressed individual OD growth curves in each experiment were randomly chosen to identify the detection point, which is in the exponential phase.

4.2.7Specific growth rate

4.2.7.1Estimation of the specific growth rate with OD detection time method

The specific growth rate was estimated based on the OD detection time prescribed by Wu et al. (2000). Serial dilutions (10^5, $\frac{1}{2}10^5$, 10^4, $\frac{1}{2}10^4$, 10^3, $\frac{1}{2}10^3$, 10^2 and $\frac{1}{2}10^2$) were prepared from a 48 h, 22 ^{o}C incubation culture. Volumes of 400µl of diluted culture were added to 3, 5, 10 or 15 wells, depending on dilution (Table 4.3). Meanwhile, 400 µl of TSB was added to

five wells of each honeycomb plate and served as blanks. Before incubating the honeycomb plate, the Bioscreen was tempered at the appropriate temperature (7, 12 or 22^{o}C) for 24 h. Growth was monitored by measuring the turbidity at appropriate times (Table 4.2) for up to fifteen days (depending on incubation temperature) until all wells showed growth. Growth curves were prepared by plotting OD of suspensions minus the mean OD of the blanks against time of incubation. The detection time was considered to be the time for the microbial population to generate a 0.035 increase from the initial OD baseline. The initial concentration for each dilution was estimated through serial dilution and plating on TSA, followed by incubation at 22 ^{o}C for 48 h. A linear regression was made between the detection time and the natural logarithm of the initial cell concentration. The specific growth rate was calculated as the negative reciprocal of the slope of the regression line. The experiment was done at least three times.

Table 4.3: Well inocula with corresponding dilutions

Concentration (cfu/ml)	Number of wells	Concentration (cfu/ml)	Number of wells
10^5	3	$\frac{1}{2}$ 10^5	3
10^4	3	$\frac{1}{2}$ 10^4	3
10^3	3	$\frac{1}{2}10^3$	5
10^2	10	$\frac{1}{2}10^2$	15

4.2.7.2 Polynomial growth model for specific growth rate

A polynomial model (4.2) was constructed based on the specific growth rate estimated using the OD detection method to describe the influence of the temperature on the growth rate of *Cr. turicensis* 1211.

$$\ln\mu' = a_1 + a_2 T + a_3 T^2 \qquad (4.2)$$

Where μ' is the specific growth rate (h^{-1}); a_1, a_2 and a_3 are parameters; T is the temperature (^{o}C).

4.2.8 Estimation of the individual cell lag time

The individual lag estimation is based on amethod combining those of Métris et al. (2006) and Baranyi et al (2009). Here it is assumed that the single cell lag time follows the shifted Gamma distribution with a fixed shape parameter; the initial cell number follows the Poisson distribution; the specific growth rate and the detection level are same for the cells in each well of the honeycomb plate.

4.2.8.1 The physiological state

A concept of physiological state was introduced to quantify the suitability of a cell to the new environment (equation 4.3) (Baranyi & Roberts 1995, Baranyi 1998).

$$\alpha(1) = e^{-\mu' Lag(1)} \qquad (4.3)$$

$\alpha(1)$ represents the physiological state of single cell; μ' represents the specific growth rate (h^{-1}); $Lag(1)$is the single cell lag time. According to equation 4.3, when the value of the physiological state is 1, there is no adjustment time for a cell (because there is no requirement for it to adjust); while the lag of the cell is infinitely long when the value is zero.

In order to relate the population lag and individual lag, a relationship between the physiological state of the population and the physiological state of the individual cells was deduced (Baranyi 1998, Baranyi & Pin 1999, 2001) (equation 4.4). For inocula containing N_0 cells, the population physiological state is the arithmetic average of individual physiological states.

$$\alpha(N_0) = e^{-\mu' Lag(N_0)} = \frac{\sum_{i=1}^{N_0} \alpha_i(1)}{N_0} \qquad (4.4)$$

Assume S_α is the sum of the physiological states of the individual cells in a well. Then equation 4.5 can be deduced based on equations 4.1 and 4.4.

$$S_\alpha = \sum_{i=1}^{N_0} \alpha_i(1) = N_0 \times \alpha(N_0) = N_0 \times e^{-\mu' Lag(N_0)} = N_{det} \times e^{-\mu' T_{det}} \quad (4.5)$$

4.2.8.2 Experimental estimation of the mean and variance of S_α for one experiment

According to equation4.5, if the N_{det}, μ' and T_{det} are known, then the value of S_α in each well could be estimated. Consequently, for one experiment, the mean and variance of S_α can be calculated as function 4.6, 4.7.

$$E(S_\alpha)_{experiment} = \frac{\sum_{j=1}^{n}\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right)}{n} \qquad (4.6)$$

$$Var(S_\alpha)_{experiment} = \frac{\sum_{j=1}^{n}\left[\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right) - E(S_\alpha)_{experiment}\right]^2}{n-1} \qquad (4.7)$$

Where j=1…..n numbers of the observation in one experiment.

4.2.8.3 Theoretical estimation of the mean and variance of S_α for one experiment

4.2.8.3.1 The distribution of N_0

As the cell number in a well follows the Poisson distribution, the probability mass function of N_0 can be described as in equation 4.8.

$$P_k = P(N_0 = k) = \exp(-\rho)\frac{\rho^k}{k!} \quad (k=0, 1, 2, 3\ldots\ldots) \qquad (4.\ 8)$$

And the parameter ρ can be estimated by the numbers of empty wells which are as equation (4.9).

$$\rho = -\ln\frac{w_0}{w} \qquad (4.9)$$

W_0 represents the number of negative wells and W is the total well number.

As growth only occurs in the wells that contain cells, the cell number in the positive well follows a zero truncated Poisson distribution, with the average cell number (ρ^+) and variance estimated by equations (4.10) and (4.11) (Métris et al. 2006).

$$E(N_0|N_0 > 0) = \rho^+ = \frac{\rho}{1-e^{-\rho}} \quad (4.10)$$

$$Var(N_0|N_0 > 0) = \rho^+ - (\rho^+)^2 e^{-\rho} \quad (4.11)$$

4.2.8.3.2 The shifted Gamma distribution assumption

Assume for a single cell, the lag time *Lag(1)* follows a shifted Gamma distribution (4.12). T_{shift} is a constant time shift and it is the same for all cells in one experiment. For untreated cells, T_{shift} can compensate for the randomness of the first few generations; for stressed cells, it could be interpreted as a 'compulsory repair time' during which cells can repair the damage caused by stress (in this case, it is the sublethal heating treatment). τ is the time that the cell needs to adjust itself to the new environment, which varies randomly according to the Gamma distribution with a scale parameter θ and a shape parameter β. The mean value and SD of τ can be estimated as $\beta\theta$, $\sqrt{\beta\theta^2}$(Baranyi et al. 2009).

$$Lag(1) = T_{shift} + \tau \sim \text{Gamma}(\beta, \theta) \quad (4.12)$$

4.2.8.3.3 Theoretical estimationthe mean and square of$e^{-\mu'\tau}$

As τ follow the gamma distribution, then $E\left(e^{-\mu'\tau}\right)$ and $E\left(e^{-2\mu'\tau}\right)$ can beestimated (4.13, 4.14).

$$E\left(e^{-\mu'\tau}\right) = \frac{1}{(1-\theta(-\mu'))^{\beta}} = (1+\mu'\theta)^{-\beta} \quad (4.13)$$

$$E\left(e^{-2\mu'\tau}\right) = \frac{1}{(1-\theta(-2\mu'))^{\beta}} = (1+2\mu'\theta)^{-\beta} \quad (4.14)$$

4.2.8.3.4 Theoretical estimationthe mean and variance of$\alpha(1)$

Based on equation 4.13 and 4.14, combined with equations 4.3 and 4.12,$E[\alpha(1)]$ and $Var[\alpha(1)]$ can be estimated as follows (4.15, 4.16).

$$E[\alpha(1)] = E\left[e^{-\mu' Lag(1)}\right] = E\left[e^{-\mu'(T_{shift}+\tau)}\right] = e^{-\mu' T_{shift}} \times (1+\mu'\theta)^{-\beta} \quad (4.15)$$

$$Var[\alpha(1)] = Var\left[e^{-\mu' Lag(1)}\right] = Var\left[e^{-\mu'(Tshift+\tau)}\right] = e^{-2\mu' T_{shift}} \times \left[(1+2\mu'\theta)^{-\beta} - (1+\mu'\theta)^{-2\beta}\right] \quad (4.16)$$

4.2.8.3.5 Relationship ofthe mean and variance of $\alpha(1)$ with the mean and varianceof S_α for one experiment

As $S_\alpha = \sum_{i=1}^{N_0} \alpha_i(1)$ (4.5), therefore, mean and variance of S_α can be mathematically deduced as equation 4.17 and 4.18, which are$E(S_\alpha)_{theoretically}$ and $Var(S_\alpha)_{theoretically}$.

$$E(S_\alpha)_{theoretically} = E\left[\sum_{i=1}^{N_0} \alpha_i(1)\right] = E(N_0|N_0 > 0) \times E[\alpha(1)] \quad (4.17)$$

$$Var(S_\alpha)_{theoretically} = Var\left(\sum_{i=1}^{N_0} \alpha_i\right) = Var(N_0|N_0 > 0) \times E[\alpha(1)]^2 + E(N_0|N_0 > 0) \times Var[\alpha(1)] \quad (4.18)$$

By replacing $E(N_0|N_0 > 0)$, $Var(N_0|N_0 > 0)$, $E[\alpha(1)]$ and $Var[\alpha(1)]$ in equations 4.17and 4.18 with their respective equations (4.10, 4.11, 4.15 and 4.16), then the function of 4.19 and 4.20 could be obtained.

$$E(S_\alpha)_{theoretically} = \rho^+ e^{-\mu' T_{shift}}(1 + \mu'\theta)^{-\beta} \quad (4.19)$$

$$Var(S_\alpha)_{theoretically} = e^{-2\mu' T_{shift}} \times \left[\rho^+(1 + 2\mu'\theta)^{-\beta} - [\rho^+]^2 e^{-\rho}(1 + \mu'\theta)^{-2\beta}\right] \quad (4.20)$$

4.2.8.4 Estimation of the parameters T_{shift}, θ of *Lag*(1)

In a given experiment, the theoretically estimated mean and variance of S_α, the sum of the physiological states of all cells in a well, should equal the mean and variance of S_α obtained experimentally, therefore, equations 4.21, 4.22 could be obtained.

$$\frac{\sum_{j=1}^{n}\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right)}{n} = \rho^+ e^{-\mu' T_{shift}}(1 + \mu'\theta)^{-\beta} \quad (4.21)$$

$$\frac{\sum_{j=1}^{n}\left[\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right) - E(S_\alpha)_{experiment}\right]^2}{n-1} = e^{-2\mu' T_{shift}} \times \left[\rho^+(1 + 2\mu'\theta)^{-\beta} - (\rho^+)^2 e^{-\rho}(1 + \mu'\theta)^{-2\beta}\right] \quad (4.22)$$

Where j=1…..n are the numbers of observations in each experiment.

In equations 4.21 and 4.22, the specific growth rate μ', N_{det} at the detection level in a well, T_{det} can be determined experimentally; for one experiment, ρ and ρ^+ can be estimated from the number of the wells that did not show growth with functions 4.9 and 4.10. Moreover, as the fixed shape parameter β is assumed (for untreated individual lag time, β equal 4.8; for heat stressed *Cr. turicensis* 1211 single cell lag time, it is 1.1), θ and T_{shift} can be solved using equations 4.21, 4.22.

4.2.9 Data analysis

ANOVA was used to determine whether the different recovery temperatures had a significant impact on the ability of untreated and sublethally heat stressed *Cr. turicensis* 1211 individual cells to initiate growth in TSB; analysis of covariance was applied to decide whether the temperature has a significant effect on the calibration. Both tests were carried out in SPSS 11.0. The significance level was set at 5%. Other data analyses were carried out in Microsoft Excel spreadsheets (Microsoft Excel 2007), using its regression functions and the Solver add-in.

4.3 Results

4.3.1 Growth ability of individual cells of untreated and heat stressed *Cr. turicensis* 1211 at various temperatures

Table 4.4 describes the number of the wells that showed growth, the total wells inoculated, and the estimated average cell number (ρ^+) in positive wells for six repeat experiments for untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C. According to Table 4.4, the average cell number (ρ+) of untreated *Cr. turicensis* 1211 among the positive wells in each microtitre plate incubated at 7, 12 and 22 ^{o}C were 1.51, 1.45, and 1.52, respectively. For heat stressed *Cr. turicensis* 1211, the average cell number (ρ^+) in each well for plates incubated at 7, 12 and 22 ^{o}C were 1.50, 1.72, and 1.77. The statistical results demonstrated that there was no significant difference between the average cell number in the positive

wells incubated at various temperatures (for untreated cells, ANOVA, P=0.390; for heat stressed cells, ANOVA, P=0.531).

Table 4.4: Numbers of wells showing growth, total wells inoculated and average cell number (ρ+) in positive wells for untreated and heat stressed *Cr. turicensis* 1211 in microtitre plates incubated at 7, 12 and 22 ^{o}C

Experiment		7 ^{o}C		12 ^{o}C		22 ^{o}C	
		W/W_{total}	ρ+	W/W_{total}	ρ+	W/W_{total}	ρ+
U	1	31/56	1.46	27/56	1.36	39/56	1.71
	2	33/56	1.51	31/56	1.46	32/56	1.48
	3	31/56	1.46	29/56	1.41	29/56	1.41
	4	35/56	1.57	27/56	1.36	28/56	1.39
	5	32/56	1.48	35/56	1.57	33/56	1.51
	6	35/56	1.57	34/56	1.54	37/56	1.64
	Mean cells	-	1.51	-	1.45	-	1.52
H	1	45/56	2.03	51/56	2.65	50/56	2.50
	2	28/56	1.39	37/56	1.64	34/56	1.54
	3	36/56	1.60	40/56	1.75	41/56	1.80
	4	31/56	1.46	32/56	1.48	35/56	1.57
	5	32/55	1.50	37/55	1.66	42/55	1.89
	6	6/56	1.06	12/56	1.13	24/56	1.31
	Mean cells	-	1.50	-	1.72	-	1.77

U: untreated cells; H: heat stressed cells; W/W_{total}: wells showing growth/ total wells inoculated.

4.3.2 Calibration curve

Figure 4.1 demonstrates the relationship between OD and cell density when OD is between 0.003 and 0.4. It is clear that for four experiments, the OD reading showed two linear phases. When the OD reading was

below ca. 0.1, the cell number increased quickly. After OD reached 0.1, cell numbers tended to become stable.

The calibration curves for *Cr. turicensis* 1211 at 7 and 22 ^{o}C were constructed in the OD ranges of 0.011~0.066 and 0.012~0.071 respectively. The OD datasets, along with the cell counts, were fitted with a linear regression. According to Fig 4.2, the calibration curves demonstrated good R^2 values (0.945 and 0.959; Fig 4.2).

Based on the statistics, there was no significant difference between the slopes and the intercepts between the calibration curves at 7 and 22 ^{o}C (analysis of covariance, P>0.05). The overall calibration curve for *Cr. turicensis* 1211 was constructed by merging the 7 ^{o}C and 22 ^{o}C datasets and fitting with a linear regression. Figure 4.3 and equation 4.23 illustrate the calibration curve with a coefficient of determination of 0.952. The calibration curve is valid when the cell count is between about $10^{6.5}$ cfu/ml and $10^{7.4}$ cfu/ml.

$$OD = 3 \times 10^{-9} \times N + 0.0025 \quad (4.23)$$

N: represents the cell number.

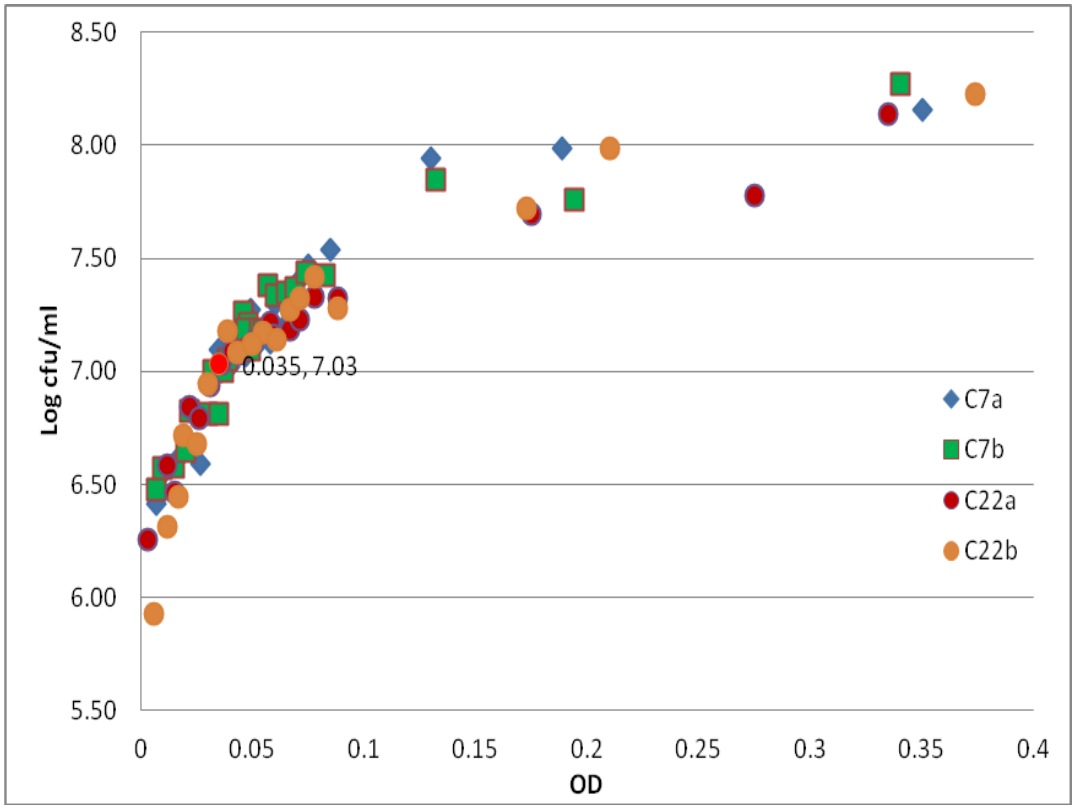

Fig 4.1: Relationship between cell number and OD of *Cr. turicensis* 1211 at 7 and 22 ^{o}C

The bright red point indicates the approximate location of the detection point. C7a, c7b represent two calibration curves at 7 ^{o}C; c22a, c22b are two calibration curves at 22 ^{o}C.

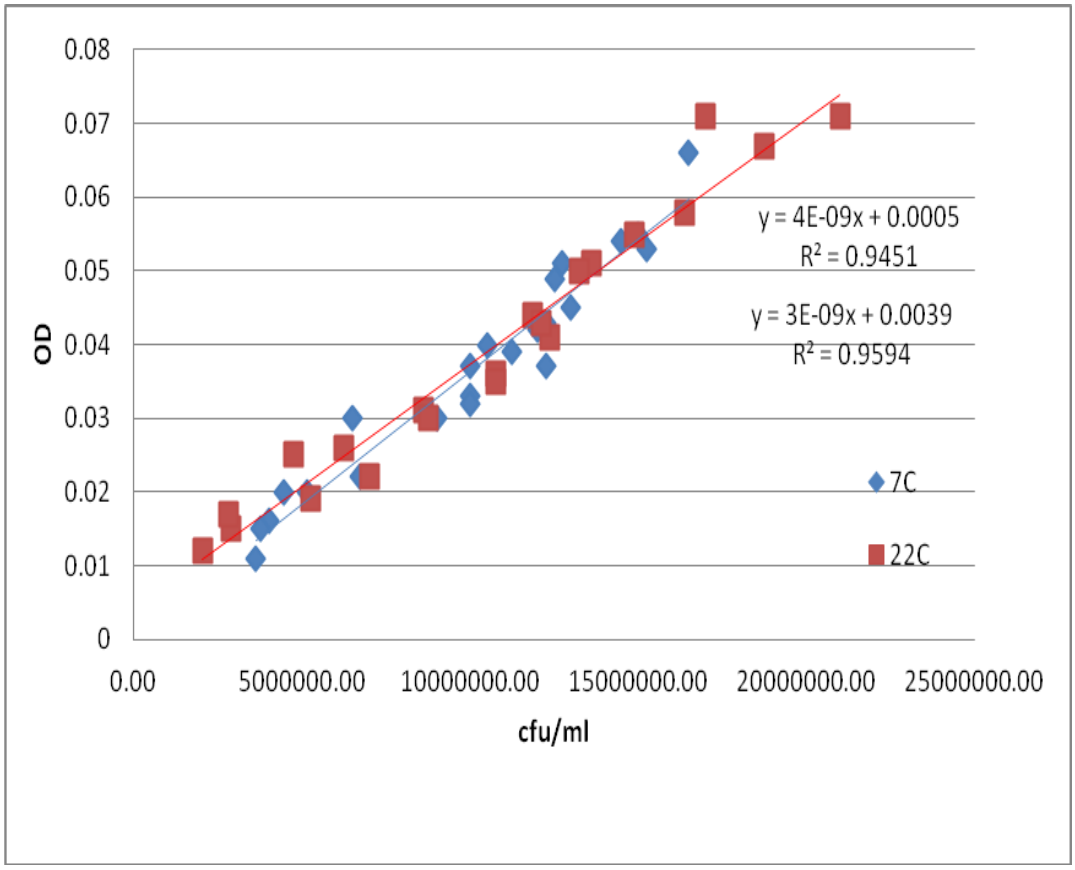

Fig 4.2: Calibration curves of *Cr. turicensis* 1211 at 7 °C (OD range 0.011~0.066) and 22 °C (OD range 0.012~0.071)

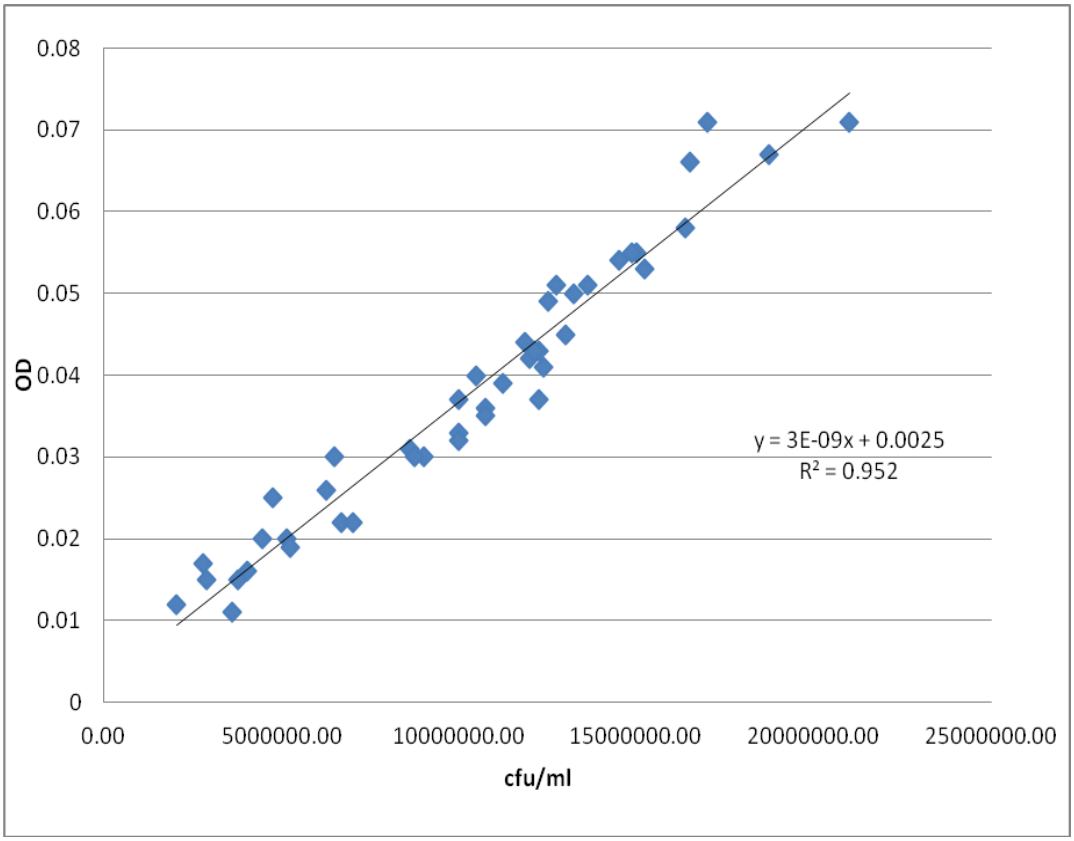

Fig 4.3: Calibration curve of *Cr. turicensis* 1211 between 7 °C and 22 °C when OD reading is between 0.011 and 0.071

4.3.3 Determination of the detection point of growth

As mentioned in 4.2.6, in order to estimate the OD range corresponding to the exponential growth phase, $\frac{dOD}{dt}$ was plotted against OD. It could be considered that when cells are in exponential phase, there is a linear correlation between $\frac{dOD}{dt}$ and OD.

Among sixty randomly chosen OD datasets showing growth of untreated single cells (each of the six experiments provided ten OD datasets), three datasets showed a linear relationship between $\frac{dOD}{dt}$ and OD when OD values were below 0.035 with a coefficient of determination (R^2) between 0.874 and 0.978; sixteen datasets exhibited a linear relationship when OD was below 0.04 and one dataset showed a slightly low R^2 value (0.744); except for one dataset with a 0.783 R^2 value, the other forty datasets showed linearity when the OD was below 0.05.

Among the sixty OD data series for sublethally heated cells, one dataset showed a linear relationship when OD was below 0.035; when OD was below 0.04 (apart from one dataset which exhibited a slightly low R^2 value of 0.783), the other twelve datasets showed linearity with an acceptable R^2 value. Among the remaining forty six datasets, in which the OD reading was between 0.01 and 0.05, seven showed a linear relationship with R^2 range between 0.730 and 0.785, while the other thirty nine datasets had acceptable R^2 values for the linear relationship between $\frac{dOD}{dt}$ and OD (Table 4.5).

Fig 4.4 shows three examples of relationships between OD growth rate ($\frac{dOD}{dt}$) and the OD of untreated and sublethally heated cells of *Cr. turicensis* 1211. As all 120 randomly chosen untreated and sublethally heat stressed cell OD datasets show a linear relationship between $\frac{dOD}{dt}$ and OD when OD is below 0.035, it is considered that the cells were still in the exponential phase when the OD value was below 0.035.

Table 4.5: The OD range and regression coefficient showing linear relationship between $\frac{dOD}{dt}$ and OD for untreated and sublethally heat stressed *Cr. turicensis* 1211

Untreated cells			Sublethally stressed cells		
DS	OD range	R^2 range	DS	OD range	R^2 range
3/60	0.01-0.035	0.874-0.978	1/60	0.01-0.035	0.828
1/60	0.01-0.04	0.744	1/60	0.01-0.04	0.783
15/60	0.01-0.04	0.876-0.994	12/60	0.01-0.04	0.815-0.987
1/60	0.01-0.05	0.783	7/60	0.01-0.05	0.730-0.785
40/60	0.01-0.05	0.804-0.986	39/60	0.01-0.05	0.812-0.990

DS: Datasets

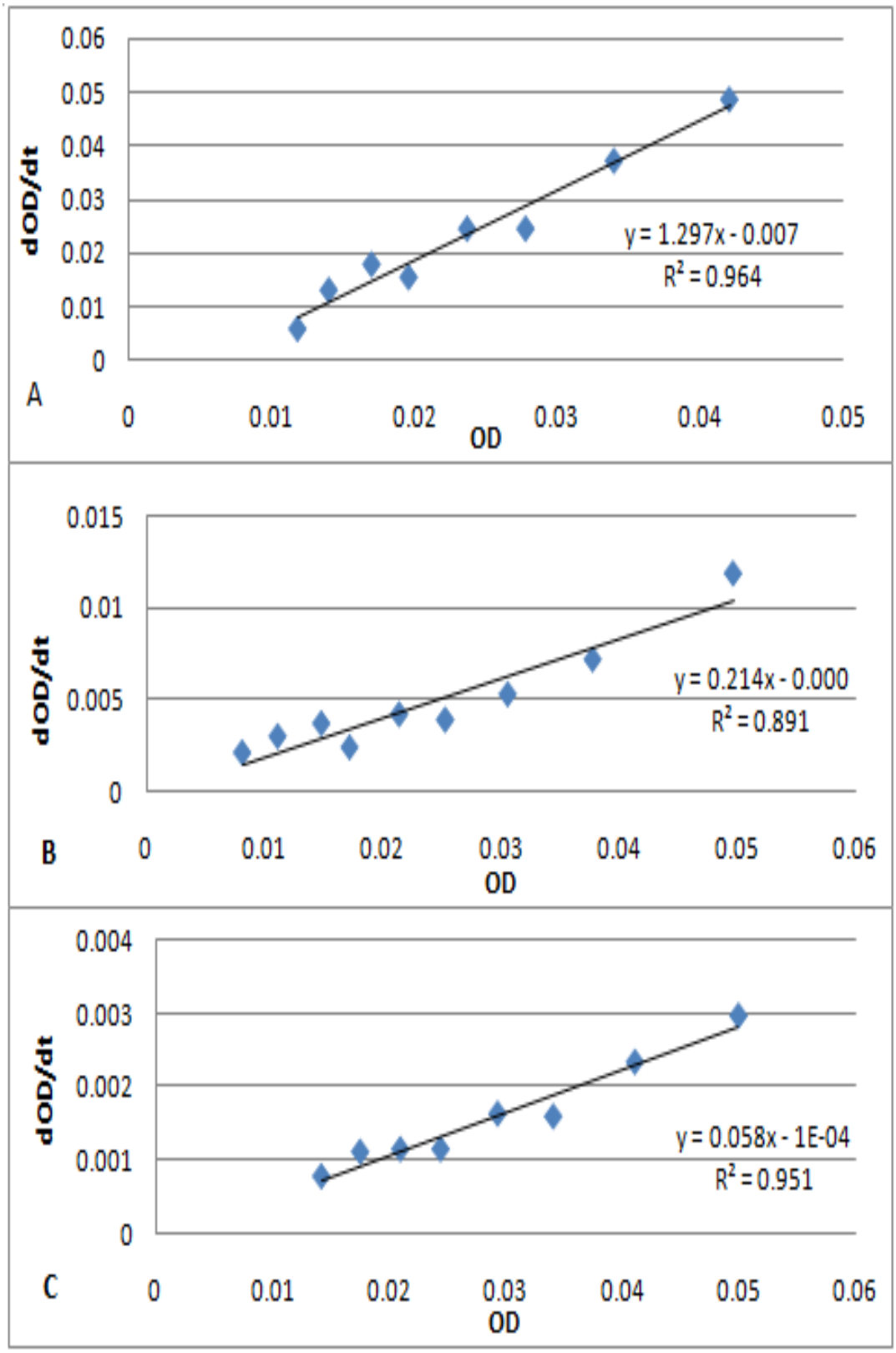

Fig 4.4: Examples of relationship between $\frac{dOD}{dt}$ and OD of *Cr. turicensis* 1211

(A) untreated cells at 22 ^{o}C (B) sublethally stressed cells at 12 ^{o}C (C) sublethally stressed cell at 7 ^{o}C

4.3.4 Determination of the initial cell number

Based on equations 4.9 and 4.10, the average cell number in positive well (ρ^+) in each individual lag experiment was estimated (Table 4.8). The average cell number in positive wells (ρ^+) for untreated *Cr. turicensis* 1211 individual lag experiment at 7, 12 and 22 ^oC were between 1.33 and 1.68 cell/well. For sublethally heat stressed *Cr. turicensis* 1211 culture at 7, 12 and 22 ^oC, the average cell number in positive wells (ρ^+) were between 1.13 and 2.04 cell/well.

4.3.5 The specific growth rate

4.3.5.1 The specific growth rate at 7, 12 and 22 ^oC

Table 4.6 depicts the specific growth rates of *Cr. turicensis*1211 at 7, 12 and 22 ^oC with the OD detection time method; they are 0.076±0.002 h^{-1}, 0.221±0.010 h^{-1} and 0.857±0.014 h^{-1} respectively.

Table 4.6: The specific growth rates of *Cr. turicensis* 1211 determined by the OD detection method

Exp	7 ^oC		12 ^oC		22 ^oC	
	μ'	R^2	μ'	R^2	μ'	R^2
1	0.076	0.980	0.214	0.988	0.867	0.977
2	0.075	0.986	0.217	0.985	0.840	0.990
3	0.075	0.986	0.218	0.993	0.862	0.989
4	0.080	0.988	0.235	0.990	-	-
Average	0.076		0.221		0.857	
SD	0.002		0.010		0.014	
SE	0.001		0.005		0.007	
Relative Accuracy (SE/Average)	1.45%		2.18%		0.83%	

SD: Standard Deviation; SE: Standard Error

4.3.5.2 Polynomial growth rate model

Using polynomial regression in Excel, a secondary model for describing the effect of temperature on the specific growth rates of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C was constructed, which is shown in equation 4.24 and Figure 4.5.

$$Ln(\mu') = -0.0051T^2 + 0.3100T - 4.4915 \qquad (4.24)$$

Where μ' represent specific growth rate (h^{-1}), T is the temperature between 7 ^{o}C and 22 ^{o}C.

Based on this secondary model, the specific growth rates at 7, 12 and 22 ^{o}C were calculated to be 0.076, 0.221 and 0.869 h^{-1}. These specific growth rates were used to construct individual lag primary model.

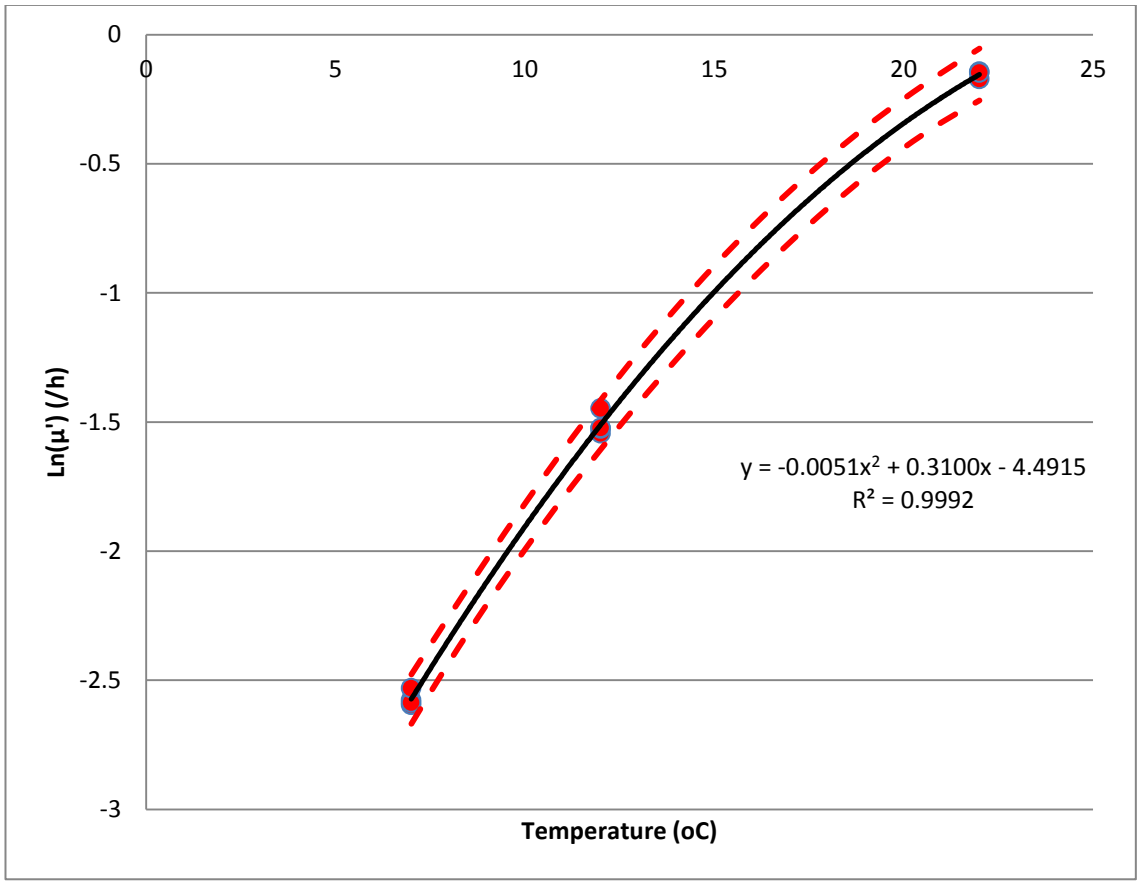

Fig 4.5: Polynomial model (solid black line) of the growth rate of *Cr. turicensis* 1211 with 95% confidence limits (dashed red line)

4.3.6 Detection time of untreated and heat stressed *Cr. turicensis* 1211

4.3.6.1 The mean and standard deviation of the detection time of untreated and heat stressed *Cr.turicensis* 1211

The mean and SD of the detection time for untreated and heat stressed *Cr.turicensis* 1211 cells at 7, 12 and 22 ^{o}C are shown in Table 4.7. For untreated *Cr. turicensis* 1211 cells, the mean and SD of the detection time of two separate experiments at 7, 12 and 22 ^{o}C are 263.32±10.59 h and 258.08±10.99 h, 75.87±2.93 h and 76.86±2.84 h, 19.76±0.74 h and 19.99±0.72 h at the respective temperatures. For heat stressed *Cr. turicensis* 1211 cells, the mean and SD of the detection time of two experiments are 294.88±33.22 h and 352.06±75.21 h, 85.54±9.24 h and 96.44±22.92 h, 24.12±6.02 h and 27.13±5.67 h at the respective temperatures 7, 12 and 22 ^{o}C.

4.3.6.2 The histogram of the detection time of untreated and heat stressed *Cr. turicensis* 1211

Fig 4.6 shows the histogram of detection times of untreated *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C. For untreated *Cr. turicensis* 1211, the range of detection time of two separate experiments at 22 ^{o}C is between 18.30 and 22.07 h, and it shifted right to a range of 230.82 - 325.33 h when the recovery temperature was lowered to 7 ^{o}C. For heat stressed *Cr. turicensis 1211*, the same phenomenon was observed (Fig 4.7). The range of detection time at 22 ^{o}C was 18.47-60.16 h, and it shifted right to 260.21-567.44 h when it was a 7 ^{o}C recovery temperature. The lower the recovery temperature, the more the histogram of the detection time shifts to the right and the more the distribution was spread for both untreated and heat stressed *Cr. turicensis* 1211.

When analysing the histogram of the detection time of the untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C by considering Fig 4.6, Fig 4.7 and Table 4.7, it is clear that the detection time ranges of untreated

Cr. turicensis 1211 at 7, 12 and 22 ^{o}C are respectively 230.82-325.33 h, 66.88-84.81 h and 18.30-22.07 h; after heat stress, the histogram of detection time at the respective recovery temperatures shifted right to 260.21-567.44 h, 71.91-170.96 h and 18.47-60.16 h. Although at 22 ^{o}C the shift is less pronounced, generally, heat stress results a rightward shift and an increasing scatter of the histogram of the detection time of *Cr. turicensis* 1211 cells at 7, 12 and 22 ^{o}C.

Table 4.7: Mean, standard deviation (SD), minimum value and maximum value of the detection time of untreated and heat stressed individual *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

Cells	RT	Mini T_{det} (h)	Max T_{det} (h)	Mean (h)	SD
U	7 ^{o}C	230.82	325.33	263.32	10.59
		234.51	282	258.08	10.99
	12 ^{o}C	66.88	82.86	75.87	2.93
		69.23	84.81	76.86	2.84
	22 ^{o}C	18.30	21.49	19.76	0.74
		18.69	22.07	19.99	0.72
H	7 ^{o}C	260.21	499	294.88	33.22
		277.05	567.44	352.06	75.21
	12 ^{o}C	71.91	131.62	84.54	9.24
		74.02	170.96	96.44	22.92
	22 ^{o}C	18.47	60.16	24.12	6.02
		20.67	53.27	27.13	5.67

U: untreated; H: heat stressed at 49^{o}C for 7min; RT: recovery temperature

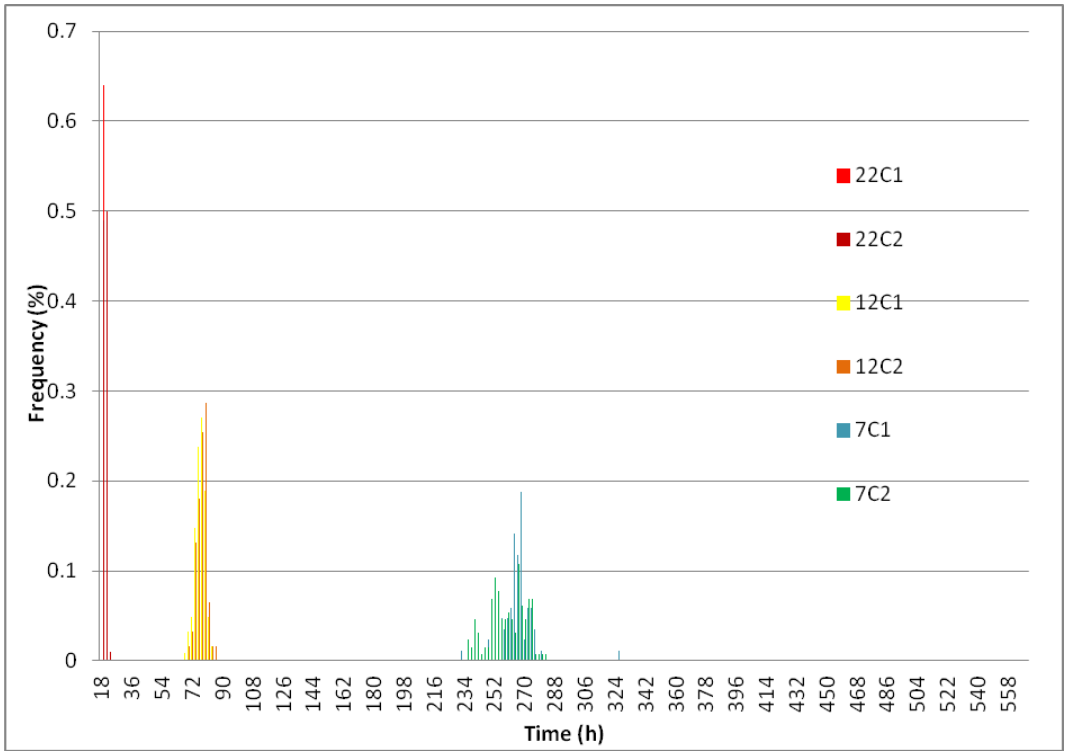

Fig 4.6: Distribution of detection times of two separate experiments of untreated *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

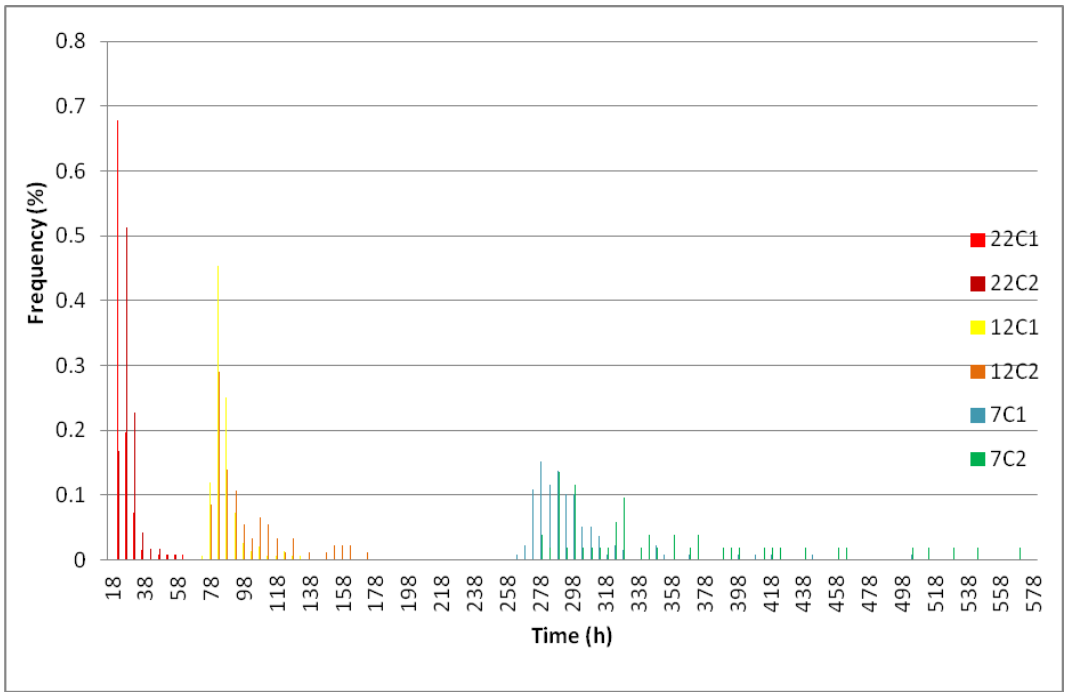

Fig 4.7: Distribution of detection time of two separate experiments of heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

7C1, 7C2 represent experiments 1 and 2 carried out at 7 ^{o}C; 12C1, 12C2 represent experiments 1 and 2 carried out at 12 ^{o}C; 22C1, 22C2 represent experiments 1 and 2 carried out at 22 ^{o}C.

4.3.7 Estimation of single cell lag times for untreated and sublethally heated cells

4.3.7.1 Individual lag time primary model

4.3.7.1.1 Estimation of the shape parameter of the Gamma distribution

As the average cell number (ρ) per well of most of individual lag experiments was lower than 1.6, the linear method is used to provide a preliminary estimate of 'individual lag', which is actually the shifted detection time. According to the mean and SD of the shifted detection time, a constant coefficient of variation (CV) with a value of 0.46±0.084 was observed for untreated cells at 7, 12 and 22 ^{o}C, and it increased to 0.98±0.08 after heat stress. For the Gamma distribution, the CV is equal to $\frac{1}{\sqrt{\beta}}$, so a constant β, with value of 4.8 for untreated cells and 1.1 for heat stressed cells, was estimated.

It should be mentioned that for heat stressed *Cr. turicensis* 1211, the CV was estimated based on the experiments with an average cell number (ρ) between 0.68 and 1.31. In one of the experiments with heat stressed *Cr. turicensis* 1211 recovered at 12 ^{o}C, the average cell number was 1.65 (ρ) with an average cell number in positive wells of 2.04 (ρ^{+}), which was not sufficiently reliable to reflect the individual lag. In another experiment for heat stressed *Cr. turicensis* 1211 recovered at 7 ^{o}C, only 52 positive data were obtained, which were not sufficient to allow a reliable estimate of the shape parameter. Therefore, these two datasets were omitted from the preliminary estimate of the shape parameter for individual cellular lag of heat stressed *Cr. turicensis* 1211. Despite this, however, the estimated shape parameter of shifted detection time for both experiments was still close to 1.1, since the individual values were 1.8 for the 12 ^{o}C experiment and 0.99 for the 7 ^{o}C experiment.

4.3.7.1.2 Estimation of the shift and scale parameter of the Gamma distribution

Table 4.8 describes the parameters (T_{shift}, β and θ) of the primary model of the individual lag of untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C. According to Table 4.8, for individual lag times of untreated *Cr. turicensis* 1211 cells, the T_{shift} parameter of two separate experiments at 7, 12 and 22 ^{o}C are estimated as 30.69 and 32.18 h, 2.13 and 2.82 h, 1.36 h and 1.75 h respectively; the scale parameter θ *values* are 9.09 h and 7.37 h, 2.18 h and 2.55 h, 0.47 h and 0.40 h, respectively. For heat stressed single cell lag times of *Cr.turicensis* 1211, the T_{shift} parameters of two repeat experiments at 7, 12 and 22 ^{o}C are 65.07 h and 76.92 h, 8.67 h and 7.6 h, 2.68 h and 3.72 h respectively. For the θ parameter, they are 82.6 h and 63.96 h, 34.34 and 35.17 h, 9.65 h and 21.79 h.

Table 4.8: Parameters for primary model (shifted Gamma distribution) and estimation of individual and population lag time for untreated and heat stressed cells

Cells	RT	W/W_{total}	ρ	$\rho+$	T_{shift} (h)	β	θ (h)	*Lag(1)* (h) (mean±SD)*	$Lag(N_0)$ (h) (mean)*
U	7 °C	85/190	0.59	1.33	30.69	4.8	9.09	48.90±16.01	41.69
		130/190	1.15	1.68	32.18	4.8	7.37		
	12 °C	122/190	1.03	1.60	2.13	4.8	2.18	17.37±6.13	14.39
		123/190	1.04	1.61	2.82	4.8	2.55		
	22 °C	115/190	0.93	1.54	1.36	4.8	0.47	3.42±0.89	3.14
		100/190	0.75	1.42	1.75	4.8	0.40		
H	7 °C	138/189	1.31	1.79	65.07	1.1	82.61	123.00±70.22	70.39
		52/190	0.32	1.13	76.92	1.1	63.96		
	12 °C	152/190	1.65	2.04	8.67	1.1	34.34	57.54±41.75	23.41
		94/190	0.68	1.38	7.6	1.1	35.17		
	22 °C	138/190	1.3	1.8	2.68	1.1	9.65	18.71±14.76	6.20
		119/189	0.99	1.58	3.72	1.1	21.79		

U: untreated; H: heat stressed at 49 °C for 7 min; RT: recovery temperature; W/W_{total}: wells showing growth/ total wells inoculated; * estimated value from the secondary model

4.3.7.1.3 Individual lag primary model at various recovery temperatures

Fig 4.8 depicts the primary model for untreated and heat stressed individual lag times at various recovery temperatures and their relevant shifted detection time, calculated with the linear method. As the shifted detection time is estimated by assuming that the initial cell number is the average cell number of positive wells, which is the truncated Poisson distribution parameter, it is not the same as the individual lag distribution.It

is actually a convolution distribution of the Poisson distribution (the initial cell number) and Gamma distribution (individual lag).

Vertical analysis of Fig 4.8 for untreated cells shows the primary model of individual lag is concentrated on the left side, with a lag time less than approximately 19.5 h at 22 ^{o}C, and it shifted the right, demonstrating longer individual lag times (maximum about 125 h) and more scatter as the recovery temperature was reduced. For heat stressed cells, the primary model of individual lag is in the range of 0h to 45 h at 22 ^{o}C and again it moves right, to between 60 h and 360 h, with increasing dispersion of the distribution as recovery temperature declined.

Moreover, when the Fig 4.8 was analysed on horizontally, it is clear that at 22 ^{o}C the primary model of untreated individual lag times of *Cr. turicensis* 1211 was at the extreme left side, almost on the y axis, while after heat stress, it moved right with the individual lag times scattered within the range of approximately 0 h to 45 h. A similar trend was observed for untreated and heat stressed primary models at 12 ^{o}C and 7 ^{o}C.

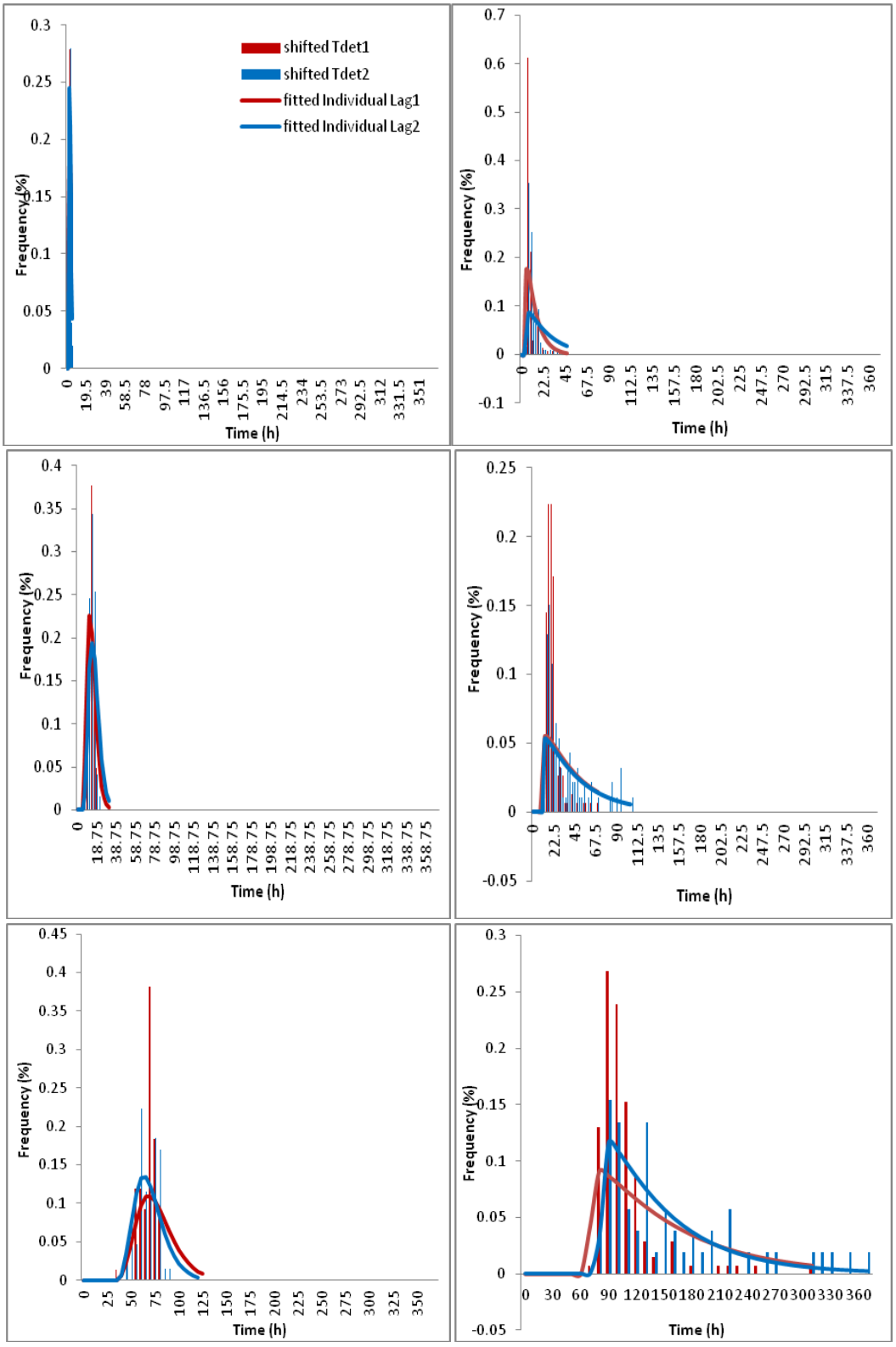

Fig 4.8: Primary model of individual lag of untreated and heat stressed *Cr.turicensis* 1211 (continuous line) and the shifted detection time (bars)

Untreated cells are shown on the left, heat stressed cells on the right. The recovery temperatures from bottom to top are 7, 12 and 22°C, respectively.

4.3.7.2 Individual lag time secondary model

4.3.7.2.1 T_{shift} parameter and recovery temperature

Fig 4.9 shows the T_{shift} parameter of the primary model of untreated and heat stressed individual lag times of *Cr. turicensis* 1211. The natural logarithm of the T_{shift} of both untreated and heat stressed individual lag timesof *Cr. turicensis* 1211 show an exponential relationship with the recovery temperature between 7 ^{o}C and 22 ^{o}C (for untreated cells, R^2=0.837; for heat stressed cells, R^2=0.923). Therefore, for untreated *Cr. turicensis* 1211 individual lag, equation 4.25 is used to model the T_{shift} parameter.

$$T_{shift} = \exp(6.525 \times \exp(-0.13T)) \quad (4.25)$$

And for sublethally heat stressed *Cr. turicensis* 1211 individual lag, function 4.26 is used to model T_{shift} parameter.

$$T_{shift} = \exp(6.826 \times \exp(-0.08T)) \quad (4.26)$$

T in equations 4.25 and 4.26 represents the recovery temperature between 7^{o}C and 22^{o}C.

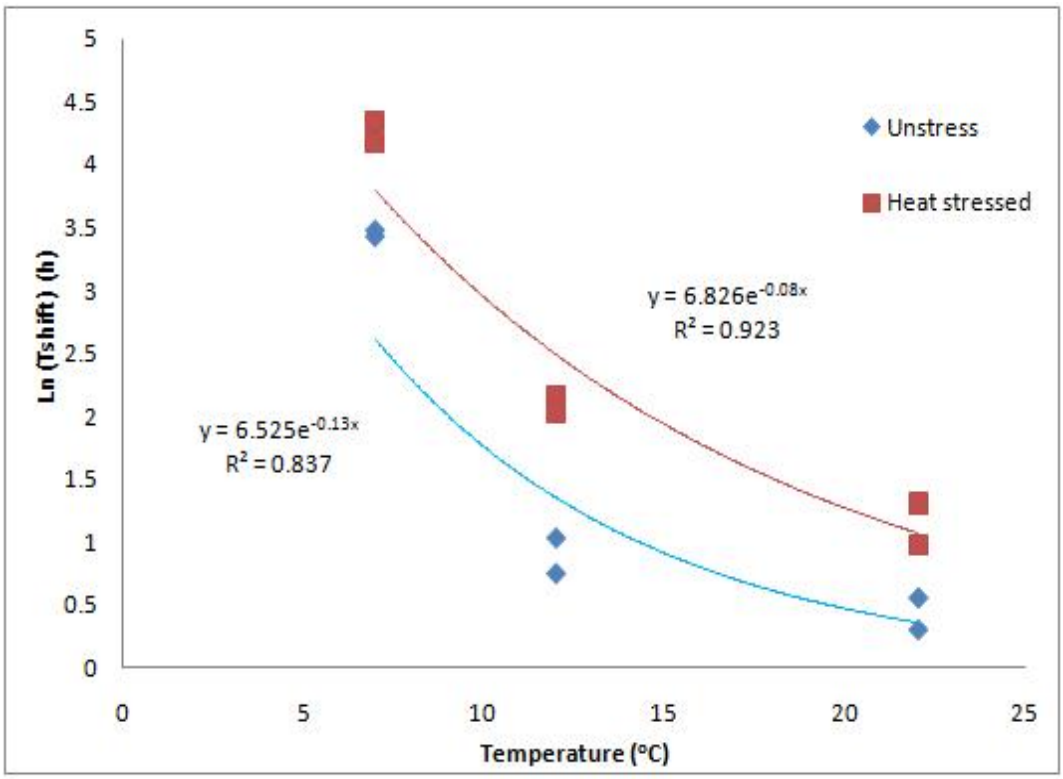

Fig 4.9: Natural logarithm of T_{shift} of primary model of untreated and heat stressed *Cr. turicensis*1211 individual lag time as a function of recovery temperature

4.3.7.2.2 The parameter (θ) of Gamma distribution and recovery temperature

Fig 4.10 describes the θ parameter of the shifted Gamma distribution of untreated and heat stressed individual lag times of *Cr. turicensis* 1211. According to Fig 4.10, both the natural logarithm of scale parameter (θ) of the primary model of untreated and the heat stressed individual lag times of *Cr. turicensis* 1211 exhibit a linear relationship with recovery temperature between 7 ^{o}C and 22 ^{o}C (for untreated cells, R^2=0.984; for heat stressed cells, R^2=0.859).

Consequently, for untreated *Cr. turicensis* 1211 individual lag times, the θ parameter of the shifted Gamma distribution could be modelled by function 4.27.

$$\theta = \exp(-0.192T + 3.333) \qquad (4.27)$$

And for sublethally heat stressed *Cr. turicensis* 1211 individual lag times, equation 4.28 is used to model the θ parameter of the shifted Gamma distribution.

$$\theta = \exp(-0.104T + 4.932) \qquad (4.28)$$

T in both equations 4.27 and 4.28 represents the recovery temperature between 7 ^{o}C and 22 ^{o}C.

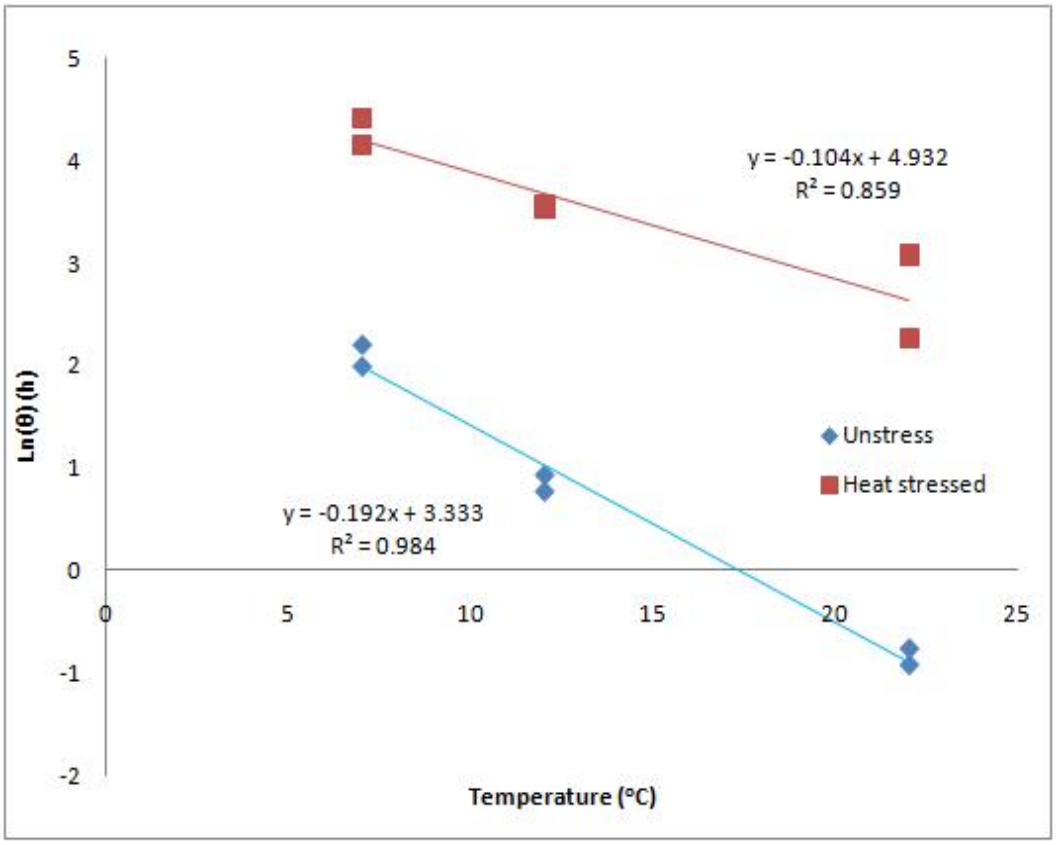

Fig 4.10: Natural logarithm of θ of primary model of untreated and heat stressed *Cr. turicensis* 1211 individual lag time as a function of recovery temperature

4.3.7.3 Overall model for untreated and heat stressed *Cr. turicensis* 1211 individual cell lag times

Combining equations 4.12, 4.25 and 4.27, the model of individual lag times of untreated *Cr. turicensis* 1211 is derived (equation 4.29).

$$Lag(1) = \exp(6.525 \times \exp(-0.13T)) + Gamma(4.8, \exp(-0.192T + 3.333))$$ (4.29)

And equation 4.30 could be used to model the individual lag of sublethally heat stressed (49 ^{o}C for 7 min) *Cr.turicensis* 1211.

$$Lag(1) = \exp(6.826 \times \exp(-0.08T)) + Gamma(1.1, \exp(-0.104T + 4.932))$$ (4.30)

T in both equations 4.29 and 4.30 represents the recovery temperature between 7 ^{o}C and 22 ^{o}C.

4.3.7.4 Estimation of mean and SD of individual lag times for untreated and heat stressed cells

Based on the secondary model, T_{shift}, θ at 7, 12 and 22 ^{o}C could be calculated. As $\mathrm{Lag}(1) = \mathrm{T_{shift}} + \tau$(4.12) and τ~Gamma (β, θ), the mean ($\mathrm{T_{shift}} + \beta\theta$) and SD ($\sqrt{\beta\theta^2}$) of individual lag of untreated and sublethally heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C were estimated. According to Table 4.8, for untreated *Cr. turicensis* 1211, the individual lag times at 7, 12 and 22 ^{o}C are 48.9±16.01 h, 17.37±6.13 h and 3.42±0.89 h respectively; for heat stressed *Cr. turicensis* 1211, they are 123±70.22 h, 57.43±41.75 h and 18.71±14.76 h.

4.3.8 Estimation of the equivalent population lag times for untreated and heat stressed cells

Based on equation 4.4 and 4.15, the population lag ($Lag(N_0)$) of untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C could be estimated. According to Table 4.8, the mean values of population lag of untreated *Cr. turicensis* are 41.69 h, 14.39 h and 3.14 h at 7, 12 and 22 ^{o}C respectively. And it increased to 70.39 h, 23.41 h and 6.20 h at those temperatures after heat stress.

4.4 Discussion

4.4.1 Ability of individual untreated and heat stressed cells of *Cr. turicensis* 1211 to initiate growth under suboptimal conditions

It is reported that under some unfavourable conditions, e.g. under high osmotic/pH stress or after mild heat stress, not all cells are able to initiate growth and multiplication commences after an initial viable count decrease (Jackson & Woodbine 1963, Bréand et al. 1997, Dupont & Augustin 2009, Zhou et al. 2011). Thus when cells are inoculated into a very unfavourable environment, it is necessary to increase inoculum concentration to an effective level which will ensure that at least one cell can initiate growth (Pascual et al. 2001, Koutsoumanis & Sofos 2005, Francois et al. 2006a). Koutsoumanis (2008) reported that in order to get four cells of *S. enterica* serovar Enteritidis to grow and form colonies on TSA with 8% NaCl at 30 ^{o}C, an initial population of 10^{6} cells were required. To study the variability of distribution of detection time of very few *L. monocytogenes* in TSB with various NaCl concentrations, Robinson et al. (2001) inoculated 1.2, 5.0 and 2000 cells into TSB with 0, 1.2 M and 1.6 M NaCl at 37 ^{o}C to achieve an average of 0.7 viable cells per well in each case. Pascual et al. (2001) investigated the ability of *L. monocytogenes* cells to initiate growth under suboptimal conditions of NaCl and pH, and reported that an approximate inoculum of $10^{5.5}$ cells per well in TSB at pH 4.5 resulted in only about 5% of the wells of microtitre plate showing growth at 37 ^{o}C. Moreover, a dramatic reduction in growth ability of sublethally heat stressed *L. monocytogenes* under suboptimal conditions was also observed in that research. Approximately 100 sublethally heat stressed *L. monocytogenes* were required to achieve growth in 50% of wells containing TSB with 0.8 M NaCl.

For this study, there was no information as to whether untreated or heat stressed individual cells of *Cr. turicensis* 1211 could initiate growth at suboptimal temperature. Consequently, it was not known if inoculum size should be increased to find a single cell to initiate growth. Therefore, before investigating individual lag times of untreated and heat stressed

cells of *Cr. turicensis* 1211, the ability of such cells to initiate growth and multiply at various temperatures was determined.

According to the results, neither a decline in average cell number of untreated individual cells, nor a decrease of growth ability of sublethally heat stressed cells under suboptimal temperature conditions (7 and 12 ^{o}C) was observed. The average cell number of both untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C in six experiments was quite similar (Table 4.4). The results of this study are therefore inconsistent with those of Pascual et al. (2001), Robinson et al. (2001) and Koutsoumanis (2008) (above). This may be because 7 ^{o}C is not the minimum growth temperature of *Cr. turicensis* 1211 (a minimum growth temperature of 5 ^{o}C was reported in Chapter 2, section 2.3.2). Alternatively, it may be due to the fact that this study described in this thesis examined suboptimal temperature, whereas the previous studies investigated low a_w or pH. Since both untreated and heat stressed *Cr. turicensis* 1211 individual cells have the same ability to initiate growth at 7, 12 and 22 ^{o}C, it is therefore unnecessary to increase inoculum concentration for low recovery temperatures to achieve an effective single cell inoculum in each well.

4.4.2 Calibration curve

4.4.2.1 Culture

After heat shock, bacterial cells shrink in size by about 10% (Kutalik et al. 2005b), so stressed cells are smaller than unstressed ones. This will affect the relationship between turbidity and concentration (Métris et al. 2003). In this study, approximately one damaged cell was inoculated into each well of the honeycomb plate and all of the daughter cells were assumed to be healthy cells. The untreated culture was therefore used to develop the calibration curve.

4.4.2.2 Deciding the appropriate OD range

In this study, the OD range of 0.011-0.071 was selected to construct the calibration curve. The reasons for selecting this range were: firstly, in published literature, the detection point usually set at a 0.05 increase in the initial base line value of the OD value (McKellar & Knight 2000, Guillier et al. 2005, Miled et al. 2011), so an OD range with 0.05 approximately in the middle is preferred. Secondly, the linear calibration curve calculated based on this OD range gave a good R^2 value (0.952; Fig 4.3). Thirdly, the cell density ($10^{6.5}$-$10^{7.4}$ cfu/ml) corresponding to the OD range chosen for the calibration curve is consistent with Beer-Lambert law, which states that at higher cell densities (10^{6}-$10^{7.5}$ cfu/ml), the absorption of light is proportional to the cell concentration (Francois et al. 2003, Métris et al. 2003).

4.4.2.3 Relationship between OD and viable count

A good calibration curve relating OD and cell concentration is very important to accurately estimate growth parameters, e.g. lag time. In the published literature, different kinds of calibration curve have been applied. In order to stabilize (normalize) the variance, a logarithmic or natural logarithmic transformation is often used to construct a linear regression calibration (Francois et al. 2005a, Gaillard et al. 2005). Moreover, OD has been calibrated against cell concentration with a quadratic equation (McClure et al. 1993) or cubic equation (Stephens et al. 1997). Meanwhile, some studies use direct linear regression to summarize the relationship between OD and viable count based on the Beer-Lambert law ($OD=kN$, where k is the coefficient and N the cell concentration)(Begot et al.1996, Augustin et al. 1999, Muñoz-Cuevas et al. 2010, Mytilinaios et al. 2012). First, a good coefficient of determination was obtained (0.952) based on simple linear regression (Fig 4.3). Second, only by using this simple linear calibration curve ($OD=kN$), can the exponential phase of the cells be deduced from the relationship between $\frac{\mathrm{dOD}}{\mathrm{dt}}$ and OD (equation 4.33, below).

This enables location of the detection point (see section 4.4.3). So, in this study, direct linear regression was preferred.

4.4.2.4 Effect of temperature on relationship between OD and cell count

It is reported that cell morphology can be affected by environmental stressors e.g. temperature, pH and osmotic stress (Francois et al. 2004). Cells of *Esch. coli* and *Salmonella* have been demonstrated to become longer when temperature is near the minimum for growth (7 ^{o}C and 8 ^{o}C) (Jones et al. 2003, Mattick et al. 2003). Moreover, cell elongation of *L. monocytogenes* at low (4 ^{o}C) and high temperature (42 ^{o}C and 45 ^{o}C) has been described by Geng et al. (2003), Li et al. (2003) and Zaika and Fanelli (2003). Therefore, the relationship between OD and cell concentration at sub-optimal temperatures can be significantly affected by cell elongation (Francois et al. 2005a).

Research investigating the relationship between OD and cell concentration at different temperatures has been carried out. Muñoz-Cuevas et al. (2010) developed a calibration curve to relate OD and cell concentration at 37 ^{o}C and 15 ^{o}C for *L. monocytogenes* in a Bioscreen. It was demonstrated that the temperature shift did not affect the relationship between OD and cell concentration. Francois et al. (2005a) studied the effect of temperature (2-30^{o}C) on the relationship between OD (at 600nm) and bacterial concentration for *L. monocytogenes* and reported that the calibration curve at 4 ^{o}C showed no statistically significant difference from that at optimal temperature (30 ^{o}C). At 2 ^{o}C, a parallel shift of the calibration was observed, but the slope did not change significantly. In this study, there was no significant difference between the calibration curves at 7 ^{o}C and 22 ^{o}C, which is consistent with the research of Muñoz-Cuevas et al. (2010). However, compared with the findings of Francois et al. (2005a), no shift was observed. Maybe again this is due to the fact that 7 ^{o}C is not the minimum growth temperature for *Cr. turicensis* 1211 (Chapter 2, section 2.3.2) and cells did not lose viability at this temperature. It was reported that the lower cell viability *of L. monocytogenes* in more stringent stress

conditions is the main reason for the shift in the calibration curve (Francois et al. 2005a).

4.4.3 Identification of the detection point in the exponential phase

That the detection point is in the exponential phase is one of the important assumptions made in order to estimate geometrical lag according to equation 4.1, which ensures that the maximum specific growth rate is still valid at this detection point. Many previous studies set the detection point based on various assumptions. For example, some research has set the detection point at the time when the microbial population generates a 0.05 increase of the initial OD baseline (McKellar & Knight 2000, Dalgaard & Koutsoumanis 2001, Guillier et al. 2005, Li et al. 2006, Miled et al. 2011). Métris et al. (2008) set the detection time at the point at which OD of the bacteria reaches 0.11. Muñoz et al. (2010) set the detection time at the time when the OD of population increases to 0.15 from the start of incubation. D'Arrigo et al. (2006) assumed the detection time to be the time needed for the OD to reach 0.2. Stephens et al. (1997) estimated the detection point to be the point at which the OD of 200 population growth curves in the Bioscreen showed significantly higher readings compared with the initial OD baseline. All these assumptions or methods make sure that the OD is above the detection threshold of the Bioscreen. However, they do not confirm that the cells are in the exponential phase.

In this study, according to the method of Augustin et al. (1999), the detection point was estimated in a way which ensured that the cells were in the exponential phase. The theory that when cells are in exponential phase, there is a linear correlation between $\frac{\mathrm{dOD}}{\mathrm{dt}}$ and OD is deduced as follows:

Rewrite the function of (4.1), equation (4.31) could be obtained.

$$\mathrm{N} = \mathrm{N}_0 \mathrm{e}^{\mathrm{u}'(\mathrm{t}-\mathrm{Lag}(\mathrm{N}_0))} \qquad (4.31)$$

Substitute equation (4.23) into (4.31) and equation (4.32) is obtained:

$$\frac{OD-0.0025}{3\text{x}10^{-9}} = \text{N}_0\text{e}^{\text{u}'(\text{t}-\text{Lag}(\text{N}_0))} \quad (4.32)$$

Derived from equation 4.32, equation 4.33 is obtained:

$$\frac{dOD}{dt} = (3\text{x}10^{-9})N_0u'e^{u'(t-\text{Lag}(\text{N}_0))} = (3\text{x}10^{-9})u'N = u'(OD - 0.0025) \quad (4.33)$$

Based on equation 4.33, when the cell is in the exponential phase, the specific growth rate μ' is constant, therefore there is a linear relationship between $\frac{\text{dOD}}{\text{dt}}$ and OD.

It has been demonstrated that for untreated and heat stressed *Cr. turicensis* 1211, the cells were in the exponential phase when OD was below 0.035 (section 4.3.2). In addition, Fig 4.1 shows a clear increase in OD reading before the OD reached 0.035, which is mainly because of the culture growth rather than variability of instrumental readings. So a detection level at the OD reading of 0.035 was set. The cell density of $10^{7.03}$ cfu/ml at the detection level was estimated from the calibration curve.

4.4.4 Initial cell number in each experiment

As mentioned above, the linear method is reliable when the average cell number in the well is very low. When it is between two and three (ρ>2), the SD of real individual lag will be different from the SD of single cell lag estimated by the linear method (Baranyi et al. 2009). Moreover, D'Arrigo et al. (2006) studied the effect of the average number of cells on the lag time distribution using simulation and reported that there was a significant difference between the distribution of lag time simulated from exactly one cell and the distribution from the culture initiated with cell number with a Poisson parameter (ρ) more than 1.6±0.08. In this study, the average cell number in the untreated individual lag experiment was between 0.59 and 1.15 (ρ) cells per well. Based on the threshold proposed by D'Arrigo et al. (2006) (ρ<1.6±0.08) and Baranyi et al. (2009) (ρ<2), the linear method is valid for estimating the individual lag of untreated individual cells. For heat stressed *Cr. turicensis* 1211, the average cell number in most experiments was between 0.32 and 1.31(ρ) (Table 4.8). Therefore, the linear method is

valid for most of the heat stressed individual lag experiments. However, in one such experiment, with recovery at 12 ^{o}C, the average cell number was 1.65 (ρ) (Table 4.8). Based on the limiting cell number set by Baranyi et al. (2009), the linear method is still acceptable. When compared with the threshold proposed by D'Arrigo et al. (2006) ($\rho<1.6\pm0.08$), the initial cell number is on the border of acceptability. Nevertheless, as the moments-based method (Métris et al. 2006) is valid in a scenario where the average initial cell per well is between 1 and 3, and for reasons of consistency, the moments-based method (mean and variance) was applied to investigate the recovery of individual cells of *Cr. turicensis* 1211.

4.4.5 Specific growth rate of *Cr. turicensis* 1211 at 7, 12 and 22^{o}C

4.4.5.1 OD detection method to estimate the maximum specific growth rate

Many methods based on use of OD to estimate growth rate have been reported. Early research estimated growth rate by fitting non-linear regression functions (e.g. Gompertz model, Exponential model) to OD, or its transformed equivalent viable count, with a calibration curve (Begot 1996, Chorin et al. 1997, Stephens et al. 1997, Dalgaard & Koutsoumanis 2001). Augustin et al. (1999) set up a two-fold dilution series and defined generation time as the time between two successive curves (derived from two successive doubling dilutions) within a certain OD range. In addition, other research used the linear relationship between detection time and natural logarithm of the initial cell concentration and estimated specific growth rate with the negative reciprocal of the slope (McKellar & Knight 2000, Muñoz-Cuevas et al. 2010). The OD is detectable only when bacteria have achieved a relatively high concentration (10^{6} cfu/ml), which indicates that the cells are in the last part of exponential phase and close to the stationary phase (Standaert et al. 2007). Therefore, it is not appropriate to estimate growth rate by fitting the Gompertz and Exponential models to OD, or to the transformed viable count, as these models estimate the specific growth rate as the slope of the growth curve (Dalgaard & Koutsoumanis 2001). Meanwhile, although the maximum

specific growth rate estimated with the half-dilution series method is the same as that estimated with the viable count method, it shows that the time to separate two successive growth curves is very variable (Augustin et al. 1999). The growth rate obtained using the OD detection time method (based on the linear relationship between detection time and initial cell concentration) is similar to that estimated by viable count incubated in the Bioscreen (Dalgaard & Koutsoumanis 2001). Consequently, the detection time method based on OD was chosen to estimate the growth rate.

4.4.5.2 Relative accuracy of the specific growth rate

It is proposed that the relative accuracy (standard error of the estimate divided by the estimate itself) of the specific growth rate determined by traditional plate counts is about 5% in optimal conditions for bacterial growth. However, when the relative accuracy increases to 10%, it is not sufficiently accurate to estimate individual lag times (Métris et al. 2006). Calculation of relative accuracy in the experiments, using the detection method herein reported, results in values of 1.45%, 2.18% and 0.83% at 7, 12 and 22 ^{o}C respectively; consequently the accuracy of the growth rate is considered acceptable. However, it should be mentioned that because the growth rate experiments based on the OD detection method were carried out concurrently (i.e. are biological replicate experiments) instead of on different days. The variability of the Bioscreen reading was not taken into consideration, the relative accuracy of the growth rate here, may be underestimated.

4.4.5.3 Generation time at 7, 12 and 22 ^{o}C

In this study, using the OD detection method, the specific growth rates of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C in TSB were estimated to be 0.076±0.002 h^{-1}, 0.211±0.010 h^{-1} and 0.857±0.014 h^{-1}, which represent generation times of 9.10±0.26 h, 3.14±0.13 h and 48.56±0.82 min respectively. As the generation time of *Cr. turicensis* 1211 at 7 ^{o}C has been discussed in Chapter 2 (section 2.4.3), only the generation time at 12 ^{o}C and 22 ^{o}C will be discussed here. Nazarowec-White and Farber (1997b)

evaluated generation time of a mixture of ten *Cronobacter* spp. in reconstituted PIFat 10 ^{o}C and 23 ^{o}C and recorded average generation times of 4.98 h and 40 min respectively. Iversen et al. (2004b) studied the growth rate of six clinical and food isolates of *Cronobacter* spp. and found a generation time of 1.7 h in PIF at 21 ^{o}C. Ghassem et al. (2011) investigated generation times of four *Cronobacter* spp. in PIF and TSB at four temperatures (10, 25, 37 and 45^{o}C) and reported mean generation times of 3.98 h and 36 min in TSB at 10 ^{o}C and 25 °C. Miled et al. (2011) estimated the maximum specific growth rate of *Cr. turicensis* in BHI at 25 ^{o}C and reported an approximate 42 min generation time (0.99 h^{-1} maximum specific growth rate). Compared with these studies, the generation times at 12 ^{o}C and 22 ^{o}C in this study are generally consistent with most of the previously reported generation times (Nazarowec-White & Farber 1997b, Ghassem et al. 2011, Milled et al. 2011). However, the generation time at 22 ^{o}C seems slightly shorter than that reported by Iversen et al. (2004b), which may be attributable to species or strain differences.

4.4.6 Modelling individual lag of untreated and stressed *Cr. turicensis* 1211 cells at 7, 12 and 22 ^{o}C

4.4.6.1 Assumption of the Gamma distribution

Various distributions have been used to describe the individual cell lag time. Wu et al. (2000) investigated individual lag times of *L. monocytogenes* cells using the OD method and reported that they follow a normal distribution, while Li et al. (2006) studied the individual lag times of starvation, heat and acid stressed *Esch. coli* O157:H7 and suggested that overall the log-normal distribution was the best fit to describe the individual lag for both unstressed and stressed cells. Miled et al. (2011) analysed the single cell lag time of six desiccation stressed *Cronobacter* spp. at 25 ^{o}C and 37 ^{o}C and reported that, in general, the 12 observed distributions of individual cell lag times followed the extreme value type II distribution, with a shape parameter fixed to 5 (EVIIb). Furthermore, Francois et al. (2005b) investigated the effect of different temperature and pH values on

individual lag times of *L. monocytogenes* cells and reported that the Gamma distribution was best for moderately stressed individual cells, while if the stress became harsher, the Weibull became the best distribution.

In the methods reported herein, a shifted Gamma distribution of individual lag time was assumed at the very beginning. The reasons for selecting the Gamma distribution are as follows: First, theoretically, individual lag could be regarded as the sum of the time required by a cell to carry out β "tasks", given that each task occurs randomly, with a mean time between tasks of θ, which can be modelled using the Gamma (β, θ) distribution (Vose 2000, Kutalik et al. 2005b, Métris et al. 2006). These tasks may include repair of macromolecular damage to components such as DNA, lipid and protein that accumulated during the stationary phase (Dukan & Nyström 1998); biosynthesis or accumulation of cellular components necessary for growth which include lipopolysaccharide, fatty acids, manganese, calcium and iron; induction and up-regulation of the genes involved in translation and cell division (Rolfe et al. 2012). If the cell was sublethally heated, some extra work is required for the cell to repair damaged components, such as the 30S ribosome, which will restore outer membrane integrity in heat-injured Gram negative bacteria (Métris et al. 2008). Second, practically, although the Gamma distribution is not always the best fit and various other distributions have been suggested for individual lag (mentioned above), it has been demonstrated to be a generally satisfactory fit, regardless of whether the OD method or imaging method is used (Francois et al. 2005b, Guillier et al. 2005, Kutalik et al. 2005b, Niven et al. 2006). Third, previous studies showed that the Gamma distribution is adequate to describe the individual lag of heat treated cells of other bacteria (Métris et al. 2008). Finally, because of various physiological impacts of different stresses and the fact that stressed cells are extremely sensitive to small changes in their growth conditions, it is claimed to be improbable that the lag will consistently follow only one mathematical model (Niven et al. 2008, Guillier et al. 2005). It is probably better to have a 'good enough' model with a reasonable theoretical basis, rather than try

to use various empirical models to fit the experimental data (Niven et al. 2008).

4.4.6.2 The assumption of fixed shape parameter

It is over simplified to assume the β parameter of Gamma distribution is constant when there are three unknown variables (β, θ and T_{shift}) and two equations (4.21 and 4.22). However, in this study, the β parameter is estimated based on the shifted detection time. Moreover, as previous research has indicated that the CV could be assumed to be constant in some experiments, the β parameter could also be assumed to be constant (D'Arrigo et al. 2006, Guillier & Augustin 2006, Métris et al. 2006, 2008). Consequently, the shape parameter of Gamma distribution (β) was fixed at 4.8 for untreated cells and 1.1 for heat stressed.

A constant CV has been reported in many studies. Niven et al. (2008) investigated the distribution of time to first division of pre-inoculation stressed (heat at 50 ^{o}C for 0, 20, 40, 60, 70 and 80 min; recovered on TSA at 20 ^{o}C) and post-inoculation stressed *Esch. coli* (grown on TSA with 0.5%, 2.5%, 3% and 4% NaCl). Using a digital image analysis system, they reported a constant CV (about 0.2) of the time to first division in all cases. D'Arrigo et al. (2006) investigated individual lag of unstressed and heat stressed (55 ^{o}C for 45 min, 62 ^{o}C for 2 min heating) *L. innocua* in dairy products incubated at 4 ^{o}C and in paté at 15 ^{o}C (55 ^{o}C for 25 min, 62 ^{o}C for 81 s and 65 ^{o}C for 20 s heating) and found the CV in all conditions was around 0.2. Métris et al. (2008) reported that the CV of individual lag time varied little between experiments (0.68±0.08). In this study, a constant CV of 'individual lag' was observed at different recovery temperatures for untreated and heat stressed cells, which were 0.46±0.084 and 0.98±0.08 respectively; the CV increased after heat stress.

4.4.6.3 Data for modelling individual lag

According to Table 4.8, it is clear that most experiments produced sufficient positive data (85-152) which enabled construction of a robust individual lag distribution of untreated and heat stressed cells of *Cr.*

turicensis 1211 at 7, 12 and 22 ^{o}C. However, in one experiment for estimating heat stressed *Cr. turicensis* 1211 individual lag at 7 ^{o}C, only 52 positive data points were obtained, not enough to estimate the shape of a distribution. According to the BACANOVA final report (2005), a sample number of around 60 is enough to estimate the mean and SD, therefore these data were still used to estimate the T_{shift} and θ.

4.4.6.4 Individual lag primary model

4.4.6.4.1 The effect of pre inoculation stress

The effect of pre-inoculation stress on the distribution of individual lag times has been reported by various workers. Stephens et al. (1997) quantified individual lag times of healthy and heat stressed *S. enterica* serovar Typhimurium and observed the distribution of the individual lag shifted right and become more spread after heat stress. Similar results were found by Smelt et al. (2002), who analysed the impact of sublethal heat stress on the subsequent duration of lag time of *Lactobacillus plantarum* single cells and showed a rightward shift and more spread in the distribution of individual lag. After observing a large number of unstressed and sublethally heat stressed individual cells of *L. innocua* during a long period of cell growth using an image analysis system, Elfwing et al. (2004) reported that that after heat stress, both mean and variance of the individual lag increased. Li et al. (2006) analysed the effect of environment stressors (starvation, heat and acid) on the distribution of individual lag of *Esch. coli* O157:H7 and demonstrated that, after stress, all distributions of individual lag shifted to the right and become more spread. In this study, Fig 4.8 clearly shows that the heat stress resulted in a rightward shift and an increasing scatter of the distribution of *Cr. turicensis*1211 individual cell lag at 7, 12 and 22 ^{o}C, in accordance with observations from other research indicated above.

4.4.6.4.2 The effect of post inoculation stress

Publications exist concerned with the effect of post-inoculation stress on the distribution of individual cell lag times. Métris et al. (2006) studied the

distribution of individual lag times of *L. innocua* cells in different acetic acid concentrations (0-2000 ppm) at 22 ^{o}C, and found that higher acetic acid concentrations resulted in longer individual lag and a more spread distribution. Rasch et al. (2007) studied the effect of reuterin on individual lag of *L. innocua* on solid agar at room temperature using a microscope and image analysis system and observed that with increasing reuterin concentration, both individual lag and its variance increased. Niven et al. (2008) investigated time to first division of individual *Esch. coli* cells growing on TSA containing 0.5%, 2.5%, 3% and 4% NaCl (w/v) at 20 ^{o}C using digital image analysis and reported that the stress resulted in an increased mean time to first division and a wider distribution. Correspondingly, in this study, for both untreated and heat stressed cells, a lower recovery temperature was accompanied by longer individual lag and a wider distribution (Fig. 4.8), which is in agreement with those previous studies.

4.4.6.5 Individual lag secondary model

Few secondary models for individual lag have been reported to date. Metris et al. (2006) studied the effect of acetic acid concentration on single cell kineticsof *L. innocua,* based on assuming a gamma distribution of individual cell lag. With a fixed shape parameter (β=4), they reported a linear trend between the natural logarithm of the scale parameter (θ) and acetic acid concentration. After investigation of lag of individual *L.monocytogenes* cells at different combinations of the temperature (2 to 30^{o}C), pH (4.4 to 7.4) and a_w (0.947 to 0.995), Standaert et al (2007) proposed an integrated modelling approach which separated lag into “zero” and “non-zero” lag. A logistic model was applied to model the probability of “zero” lag occurring under different conditions, while a hyperbolic model was used to model the mean and variance of the individual cell lag with various combinations of temperature, pH and a_w. The mean and variance of single cell lag enabled identification of the parameters of the two parameter Weibull distribution, which represents the “non-zero” lag distribution. Guiller and Augustin (2006) characterised the individual cell

lag of *L. monocytogenes* for 54 combinations of 22 physiological states, 18 growth conditions and 11 strains, and reported the natural logarithm of the mean of individual lag linearly linked with the natural logarithm of the population lag, and SD of the individual lag. Based on this, the parameters could be estimated of the extreme value type II distribution with a shape parameter fixed at 5, which represents the primary model of individual lag. Métris et al. (2008) studied the variability of individual lag of *L. innocua cells* after sublethal and lethal heat treatment based on the subdividing the individual lag into repair time (T_{shift}) and adjustment time (~Gamma (β, θ)). They found a linear relationship between the natural logarithm of T_{shift} and the logarithm of heating time for different temperatures, with the intercept linearly related to the heating temperature; Moreover, the shape parameter of Gamma distribution varied little and was independent of the severity of the heat treatment. The mean of adjustment time ($\beta\theta$) was fitted with an asymptotic model, which increased with duration of the heat treatment, but stabilized as the heat treatment became lethal. In this study, the natural logarithm of T_{shift} of both untreated and heat stressed *Cr. turicensis* 1211 individual lag showed an exponential relationship with the recovery temperature, while a linear relationship was found between the natural logarithm of θ of the Gamma distribution of individual lag and the recovery temperature. The scale parameter in this study shows some similarities to the results of Métris et al. (2006); however, it is important to note that in their research the scale parameter was related to acetic acid concentration and no shift was assumed in the lag.

4.4.7 The mean of individual lag of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

Jo et al. (2010) investigated growth of *Cronobacter* spp. in reconstituted PIF and reported population lag times of 63.86±7.46 h and 8.33±0.68 h at 10 ^{o}C and 20 ^{o}C. Kandhai et al. (2006) studied the effect of pre-culturing conditions on lag time of *Cronobacter* spp. in reconstituted PIF and estimated avalue of 83.3±18.7 h for population lag at 10 ^{o}C. Nazarowec-White and Farber (1997b) investigated population lag times of ten

Cronobacter spp. in reconstituted infant formula at 10 ^{o}C and 23 ^{o}C and reported lag times of 37.20±11.12 h and 2.75±0.67 h for clinical isolates and 28.56±7.44 h and 2.76±0.51 h for food isolates. In this research, based on the secondary model, the mean and SD of individual lag of untreated *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C were estimated, to be 48.9±16.01 h, 17.37±6.13 h and 3.42±0.89 h respectively. The mean value of the untreated individual lag at 7 ^{o}C in this study is consistent with the population lag reported by Nazarowec-White and Farber (1997b). The difference, which was expected, may be due to a lower incubation temperature in this work, or to differences in initial cell number. However, when comparing with the data reported by Kandhai et al. (2006) and Jo et al. (2010), the lag (48.9±16.01 h) obtained at 7 ^{o}C is much shorter than the 83.3±18.7 h and 63.86±7.46 h they reported at 10 ^{o}C. This was not expected, because the temperature (7 ^{o}C) in this work was 3 ^{o}C lower than that used by Kandhai et al. (2006) and Jo et al. (2010) (10 ^{o}C), and also a relatively low initial cell number was used in this study, while in the research cited above, a relatively high initial cell number (10^{2}-10^{4} cfu/ml) was inoculated. It is reported that the mean of individual lag is usually larger than the population lag (Baranyi 2010). Possibly the most important reason for this discrepancy might be again different species used (Miled et al. 2011), since in the study of Kandhai et al. (2006), *Cronobacter* spp. were used, while *Cr. sakazakii* was studied in the research of Jo et al. (2010); alternatively, different modelling methods used to estimate the lag might also be a reason. Both Kandhai et al. (2006) and Jo et al. (2010) used the Gompertz model as the primary growth model to estimate population lag. In contrast, in this study, a geometrical individual lag was estimated which was quite close to that estimated with the three-phase linear model. Buchanan et al. (1997) reported that the Gompertz model estimates a longer lag compared with the three phase linear model. At 22 ^{o}C, the lag time estimated for untreated cells in this study was comparable to the results from previous studies (Nazarowec-White and Farber 1997b, Jo et al. 2010).

4.4.8 Population lag of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

It should be admitted that determining the population lag of *Cr. turicensis* 1211 at various temperatures was not the main objective of this study. However, since Baranyi & Pin (1999) proposed a formula linking the distribution of individual lag with the population lag (equation 4.4), for general interest, the population lag of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C was estimated based on the parameters (T_{shift}, ϑ) estimated from secondary model. Compared with the population lag obtained by the studies above (Table 4.8), a similar phenomenon to individual lag was observed. The population lag calculated from equation 4.4 at 7 ^{o}C (41.69 h) in this study is consistent with the population lag observed by Nazarowec-White and Farber (1997b) at 10 ^{o}C, although it is shorter than the population lags at 10 ^{o}C observed by Kandhai et al. (2006) and Jo et al. (2010). Reasons that might explain this difference are as explained above (section 4.4.7). In terms of the population lag at 22 ^{o}C (3.14 h), the result estimated in this study was in accordance with those observed by Nazarowec-White and Farber (1997b) and Jo et al. (2010).

4.5 Conclusion

A primary model for untreated and sublethally heat stressed *Cr. turicensis* 1211 individual cell lag at various recovery temperatures was developed, based on the shifted Gamma distribution with a fixed shape parameter (β). For untreated cells the value of β was 4.8; for heat stressed cells β was 1.1. The results show clearly that the distribution of individual lag of untreated *Cr. turicensis* 1211 shifted rightward and became more spread, both when the recovery temperature decreased and after application of heat stress.

In addition, a secondary model for both untreated and heat stressed *Cr. turicensis* 1211 individual lag was developed. For the parameter T_{shift}, an exponential relationship between the natural logarithm of T_{shift} (of both untreated and heat stressed *Cr. turicensis* 1211) of individual lag and recovery temperature was observed; while a linear relationship was found

between the natural logarithm of θ and the recovery temperature. The secondary model will be evaluated in TSB and “first milk” (commercial reconstituted PIF) in the next chapter.

CHAPTER 5: Single cell variability of *Cronobacter* spp. grown in food: comparing challenge test data with Monte Carlo simulation

5.1 Introduction

5.1.1 Monte Carlo simulation

Monte Carlo simulation was first proposed by Metropolis & Ulam in 1940. It is a calculation which uses a few randomly collected samples to discover the outcome of a system (Sawilowsky 2003, Szalay & Rohoncyz 2010). The main principles of Monte Carlo simulation are as follows: assuming *y* is a function of a random set of variables *x*; x_i represents a random variable which is collected from the specified probability distribution of *x*; each value of x_i yields a corresponding value y_i; a set of *y* values (y_1, y_2, y_3….y_n) will be yielded after repeatedly collecting values of x_i; therefore, the statistical characteristics (mean, variance, distribution) of y can be estimated based on the set of data for values of y (y_1, y_2, y_3….y_n) (Poschet et al. 2003). Monte Carlo simulation has been suggested as a technology that could provide probability information of growth or inactivation of bacteria under a certain condition (FAO/WHO 2004b, Muñoz et al. 2010). Although it is often claimed as an approximate method, theoretically the level of precision can be increased if enough simulations are carried out (Vose 2000).

5.1.2 Using a challenge test to validate single cell lag times

It is assumed that the initial cell number in the food is a random variable ($ln(N_0)$), which follows a 'vertical' distribution (Fig 5.1). After the lag phase, which could be considered as a 'horizontal' distribution, each cell enters the exponential phase. The natural logarithm of the cell number increases linearly. Assuming $ln(N(t_c))$ is the cellular concentration at time t_c, then according to Fig 5.1, the 'vertical' distribution $ln(N(t_c))$ is affected by the

'vertical' distribution of initial cell number ($ln(N_0)$), the 'horizontal' distribution of *Lag(1)* and the specific growth rate. It is seldom that the distribution of the cell number $ln(N(t_c))$ can be estimated by algebraic calculation; however, it can be estimated through Monte-Carlo simulation (Baranyi et al. 2009).

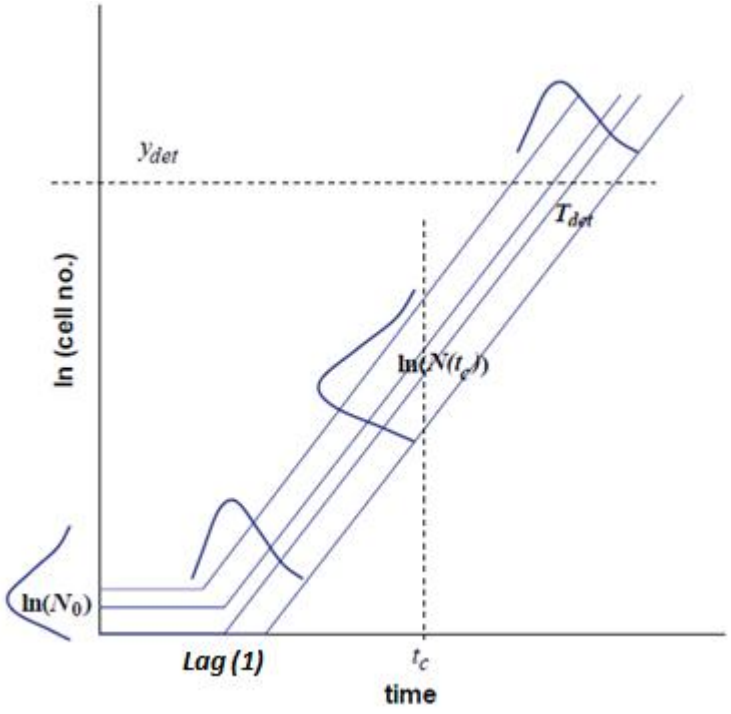

Fig 5.1: Graphic illustration for the 'horizontal' and 'vertical' distributions (Baranyi et al. 2009)

Monte Carlo simulation has been used by many studies in predictive microbiology to link single cell lag times to the distribution of cell numbers at a given time. Koutsoumanis & Lianou (2013) estimated the kinetic parameters (lag and maximum specific growth rate) by monitoring the colonial growth of single cells of *S. enterica* serovar Typhimurium with time-lapse microscopy videos. By assuming that the individual lag follows a log normal distribution, the maximum specific growth rate follows a logistic distribution, which was obtained based on observation, with a simple exponential growth model including lag phase, they reported that after 8h incubation at 25 ^{o}C, the cell number originating from a single cell

will reach between 7 (1%) and 2324 (99%), with a most probable number of 314. Pouillot & Lubran (2011) carried out a quantitative risk assessment of listeriosis considering the growth of *L. monocytogenes* on smoked salmon during home storage. By assuming the initial $\log_{10}$ contamination level of *L. monocytogenes* on cold-smoked salmon followed a normal distribution, the storage duration followed an exponential distribution with a maximum 28 days, the individual lag of *L. monocytogenes* followed the extreme value type II distribution, the storage temperatures of domestic refrigerators follow a normal distribution, using Monte Carlo simulation, Pouillot & Lubran (2011) predicted that $\log_{10}$ of the mean of the final concentration at the time of consumption per contaminated serving was 5.42.

5.1.3 Rationale for research in this chapter

As the main issue is not how well the model fits the data, but how accurately the model represents the response of the microorganism to the given environment, the performance of the model needs to be demonstrated with trials conducted independently of the laboratory experiments from which the model was developed (McMeekin et al. 1997) and confirmed in real food (McMeekin & Ross 1996, McDonald & Sun 1999). Therefore, in the present study, the first objective was to validate the model prepared using microbiological media (TSB) by setting up a separate experiment in TSB. The second objective was to evaluate the applicability of the model to real food such as 'first milk' (pre-prepared, ready-to-feed, sterilized PIF), available from supermarkets and chemists. The vertical and horizontal distributions combining Monte Carlo simulation were applied to estimate the growth of *Cr. turicensis* 1211 initiated from untreated and heat stressed single cells in TSB and first milk.

5.2 Materials and methods

5.2.1 Ready to feed first milk

Two packages each containing 15 x 200 ml of ready to feed first milk from the same batch (Aptamil 21/2/2013) were bought from the local supermarket. Before use, two samples of milk from each package were withdrawn and the pH and microbiological status of the first milk were both determined for each sample.

The pH value of the milk for both samples was determined and means and standard deviations were calculated.

Aerobic and anaerobic plate counts and detection of *Cronobacter* were carried out. The aerobic plate count and *Cronobacter* detection were based on the method described by Chap et al. (2009). In order to achieve a detection limit of 1cfu/ml, 1ml of first milk was taken aseptically from the sample and spread over five plates each of nutrient agar (NA; CM003; Oxoid), and blood agar (BA; 5% horse blood (SR050; Oxoid) in blood agar base (CM055; Oxoid)). The plates were incubated overnight at 37 °C. Colonies on NA and BA were counted to determine aerobic plate count. For the anaerobic plate count, 1ml of milk was plated on a further five blood agar plates and incubated at 37 °C in an anaerobic jar (AG0025; Oxoid) with an AnaeroGen sachet (AN0025; Oxoid) for 5 days. The colonies were counted and reported as the anaerobic plate count.

For *Cronobacter* detection by enrichment, 25 ml of first milk were added to 225 ml buffered peptone water (BPW; CM509; Oxoid) and incubated at 37 °C as a pre-enrichment step. After overnight incubation, a 10 ml volume of BPW was transferred to 90 ml EE broth (CM0317; Oxoid) and incubated at 37 ^{o}C overnight, following which the broth was streaked on DFI agar (CM1055; Oxoid) and incubated at 37 °C for 18 h. Blue-green colonies on DFI agar (presumptive *Cronobacter*) were selected for further identification with 16S rDNA sequence analysis.

5.2.2 Culture preparation

The culture of *Cr. turicensis* 1211 was prepared as described in section 3.2.2.

5.2.3 Thermal inactivation experiment

The thermal inactivation experiments for *Cr. turicensis* 1211 at 49 ^{o}C were carried out as described in section 3.2.3. However, in this experiment, the tubes were removed from the bath after being heated for 7 min and serial dilutions prepared immediately.

5.2.4 Specific growth rate

5.2.4.1 Estimation of specific growth rate with viable count method

Traditional plate count methods based on the method described by Métris et al. (2006) were used to estimate the specific growth rate of *Cr. turicensis* 1211 at different temperatures. A 100 ml volume of TSB or first milk was tempered in incubator (LMS, Kent, UK) at the appropriate temperature for 24 h (Table 5.1). The temperature of 100 ml distilled water in a separate Duran bottle held in the same incubator was periodically checked using a calibrated alcohol in glass thermometer. The 48 h stationary phase cultures were diluted with TSB to 10^5 cfu/ml, and 1 ml of diluted culture was inoculated into 100 ml TSB or first milk to give an approximate final 10^3-10^4 cfu/ml concentration, using a 2 ml syringe (BD, 300185) fitted with a sterilized needle. The cultures thus prepared in TSB or first milk were incubated at 7, 12, 18 and 22 ^{o}C in air incubator for appropriate times (7 ^{o}C 2 weeks; 12 ^{o}C 1 week; 18 ^{o}C 48 h and 22 ^{o}C 24 h). Samples were taken at regular intervals, serially diluted and enumerated on plates of TSA incubated at 22 ^{o}C for 48 h. The experimental growth data were fitted with the DMFit Excel add-in programme (Baranyi&Roberts 1994; www.ifr.ac.uk/safety/DMfit). The experiment was done three times.

Table 5.1: The incubation temperature and time for TSB or first milk samples that inoculated one or two untreated and heat stressed *Cr. turicensis* 1211 cells

Validation		7 ^{o}C	12 ^{o}C	18 ^{o}C	22 ^{o}C
In TSB	U	2 weeks	48 h	18 h	8 h
	HS	2 weeks	96 h	36 h	24 h
In first milk	U	2 weeks	48 h	-	8 h
	HS	2 weeks	96 h	-	24 h

U: untreated; HS: heat stressed

5.2.4.2 Polynomial growth model for specific growth rate

A polynomial model (5.1) was fitted to the logarithm of the specific growth rate generated by traditional viable count method as described in section 4.2.7.2.

$$ln\mu' = a_1 + a_2T + a_3T^2 \quad (5.1)$$

Where μ' is the specific growth rate (h^{-1}); a_1, a_2 and a_3 are parameters; T is the temperature (^{o}C).

5.2.5 Maximum cell density in TSB and first milk

The maximum cell density of *Cr. turicensis* 1211 in both TSB and first milk at each temperature (Table 5.1) was determined. The stationary-phase (48 h) culture was diluted with TSB to 10^5 cfu/ml and 10 µl of diluted culture was inoculated to 1 ml of TSB or first milk in eppendorf tubes tempered at appropriate temperature (Table 5.1) 24 h before experiment, to give an approximate final 10^3-10^4 cfu/ml concentration. After incubation for 24 h to 2 weeks depending on temperature, the samples in TSB or first milk were enumerated on TSA using the spread plate technique and

plates were incubated at 22 ^{o}C for 48 h. The colonies on TSA were counted. The experiment was repeated three times.

5.2.6 Challenge test with one to two cells in TSB and first milk

The untreated and sublethally heated stressed stationary phase cultures of *Cr. turicensis* 1211 were serially diluted to 15 cell/ml and 50 µl of diluted culture were inoculated into 1 ml TSB or 1 ml milk which were contained in Eppendorf tubes and tempered at appropriate temperature (Table 5.1) 24 h before the experiment. This should give an inoculum of approximately one cell. For TSB, a total of 200 samples were inoculated and incubated at 7, 12, 18 and 22 ^{o}C; for first milk, a total of 150 samples were inoculated and incubated at 7, 12 and 22 ^{o}C, with 50 samples at each temperature. The incubation time ranged from 8 to 336 h, depending on the incubation temperature and whether the inoculated cells had been heat stressed (Table 5.1). At each pre-determined sampling time, samples were withdrawn and put into ice immediately to stop growth. The samples were then serially diluted and the viable count determined through plating on TSA and incubation at 22 ^{o}C for 48 h. The lowest detection limit of this enumeration method was 5 cfu/ml. In addition, in order to estimate the initial average cell number in the Eppendorf tubes, 50 µl of the same diluted culture were added simultaneously to 50 wells of microtiter plate, which contained 220 µl of TSB in each well. The microtiter plate was then incubated at 22 ^{o}C for appropriate time (untreated cells 48 h, heat stressed cells 2 weeks). The wells that failed to show growth were used to estimate the cell number.

5.2.7 Monte Carlo simulation study

The observed levels of *Cr. turicensis* 1211 from the challenge tests were compared to the Monte Carlo simulation based on equation 5.2. Three sources of variability were considered: the initial contamination level (N_0), variability of specific growth rate (μ') and the individual lag (*Lag(1)*). The initial cell number in TSB or first milk was assumed to follow the Poisson distribution with the average cell number estimated by microtitre plates,

parallel with the inoculated sample.The natural logarithm of specific growth rate was assumed to follow a normal distribution (Kahane 2000, Miled et al. 2011) with the mean value estimated using the polynomial model for each environmental condition. The SD was estimated as the SD of residual between the natural logarithm of the observed and predicted growth rates.The lag time of a single cell *Lag(1)* was assumed to follow the shifted gamma distribution ($T_{shift}+\tau$, and τ~Gamma (β, ϑ)). The parameters (T_{shift}, β, ϑ) were estimated by the secondary model developed in Chapter 4. Each cell was assumed able to initiate growth. A total of 1000 simulation runs were carried out by random sampling in the distribution describing the variability of the parameters (Table 5.2). The maximum cell density was set based on the results of the maximum cell concentration experiment.

$$lnN_t = lnN_0 + \mu'\left(t - T_{shift} + \frac{1}{\mu'} ln \frac{\sum_{i=1}^{N_0} e^{-\mu\tau}}{N_0}\right) \qquad (5.2)$$

5.2.8 Statistical data processing

Analysis of covariance was used to compare the growth rate in first milk and TSB using SPSS 11.0; the Chi-square (χ^2) test was applied in Excel (2007) to compare the distribution of cell concentration estimated by simulation and from challenge tests. The significance level was set at 5%.

Table 5.2: Parameters to simulate growth of untreated and heat stressed cells of *Cr. turicensis* 1211 in TSB and first milk

	Temp	Distribution of the parameter			
		N_0	$Ln\mu'$ (h^{-1})	T_{shift}(h)	τ
UTSB	7 °C	Pois(0.65) [a]	N(Ln(0.050), 0.107)[b]	13.47	Gamma(4.8, 7.31)[c]
	12 °C		N(Ln(0.170), 0.107)	3.85	Gamma(4.8, 2.79)
	18 °C		N(Ln(0.481), 0.107)	1.84	Gamma(4.8, 0.88)
	22 °C		N(Ln(0.745), 0.107)	1.43	Gamma(4.8, 0.41)
HTSB	7 °C	Pois(1.08)	N(Ln(0.050), 0.107)[b]	44.32	Gamma(1.1, 66.95)
	12 °C		N(Ln(0.170), 0.107)	12.08	Gamma(1.1, 39.81)
	18 °C		N(Ln(0.481), 0.107)	4.50	Gamma(1.1, 21.33)
	22 °C		N(Ln(0.745), 0.107)	2.93	Gamma(1.1, 14.07)
Umilk	7 °C	Pois(0.78)	N(Ln(0.048), 0.039)	13.47	Gamma(4.8, 7.31)
	12 °C		N(Ln(0.155), 0.039)	3.85	Gamma(4.8, 2.79)
	22 °C		N(Ln(0.803), 0.039)	1.43	Gamma(4.8, 0.41)
Hmilk	7 °C	Pois(1.61)	N(Ln(0.048), 0.039)	44.32	Gamma(1.1, 66.95)
	12 °C		N(Ln(0.155), 0.039)	12.08	Gamma(1.1, 39.81)
	22 °C		N(Ln(0.803), 0.039)	2.93	Gamma(1.1, 14.07)

UTSB: TSB inoculated with untreated cells; HTSB: TSB inoculated with heat stressed cells; Umilk: first milk inoculated with untreated cells; Hmilk: first milk inoculated with heat stressed cells; [a]Pois(ρ): Poisson distribution with a mean value (ρ); [b]N(m, s): Normal distribution with mean value (m) and SD (s); [c]Gamma(β, θ): Gamma distribution with shape parameter β and scale parameter θ

5.3 Results

5.3.1 Microbiological analysis of first milk and pH evaluation

In total, four samples were examined from two packages of first milk. The pH of the samples was 6.9 ± 0.013. There was no growth on any plates of NA, BA (aerobic), BA (anaerobic) and DFI.

5.3.2 Specific growth rate

5.3.2.1 Specific growth rate in TSB and milk

Table 5.3 describes the specific growth rate of *Cr. turicensis* 1211 in TSB and milk. In TSB, the specific growth rate of *Cr. turicensis* 1211 ranged from 0.053±0.004 h^{-1} to 0.703±0.007 h^{-1} from 7 ^{o}C to 22 ^{o}C. In milk, it is from 0.048±0.003 h^{-1} to 0.803±0.004 h^{-1} between 7 ^{o}C and 22 ^{o}C.

Table 5.3: Specific growth rate (h^{-1}) (mean±SD) of *Cr. turicensis* 1211 in TSB and first milk

	Temperature	mean±SD
TSB*	7 ^{o}C	0.053±0.004
	12 ^{o}C	0.157±0.018
	18 ^{o}C	0.532±0.003
	22 ^{o}C	0.703 ±0.007
Milk*	7 ^{o}C	0.048 ±0.003
	12 ^{o}C	0.155 ±0.002
	22 ^{o}C	0.803±0.004

*: *Cr. turicensis* 1211 shows significantly different behaviour in TSB and first milk at 7 ^{o}C to 22 ^{o}C (analysis of covariance, p=0.019).

5.3.2.2 Polynomial model for specific growth rate in TSB and milk

The polynomial growth rate model *of Cr. turicensis* 1211 in TSB and first milk are described by equation 5.3 and 5.4 with the R^2 equal to 0.989, 0.999 separately (Fig 5.2).

$$ln\mu' = -0.0064T^2 + 0.3643T - 5.2263 \qquad (5.3)$$

$$ln\mu' = -0.0046T^2 + 0.3208T - 5.0492 \qquad (5.4)$$

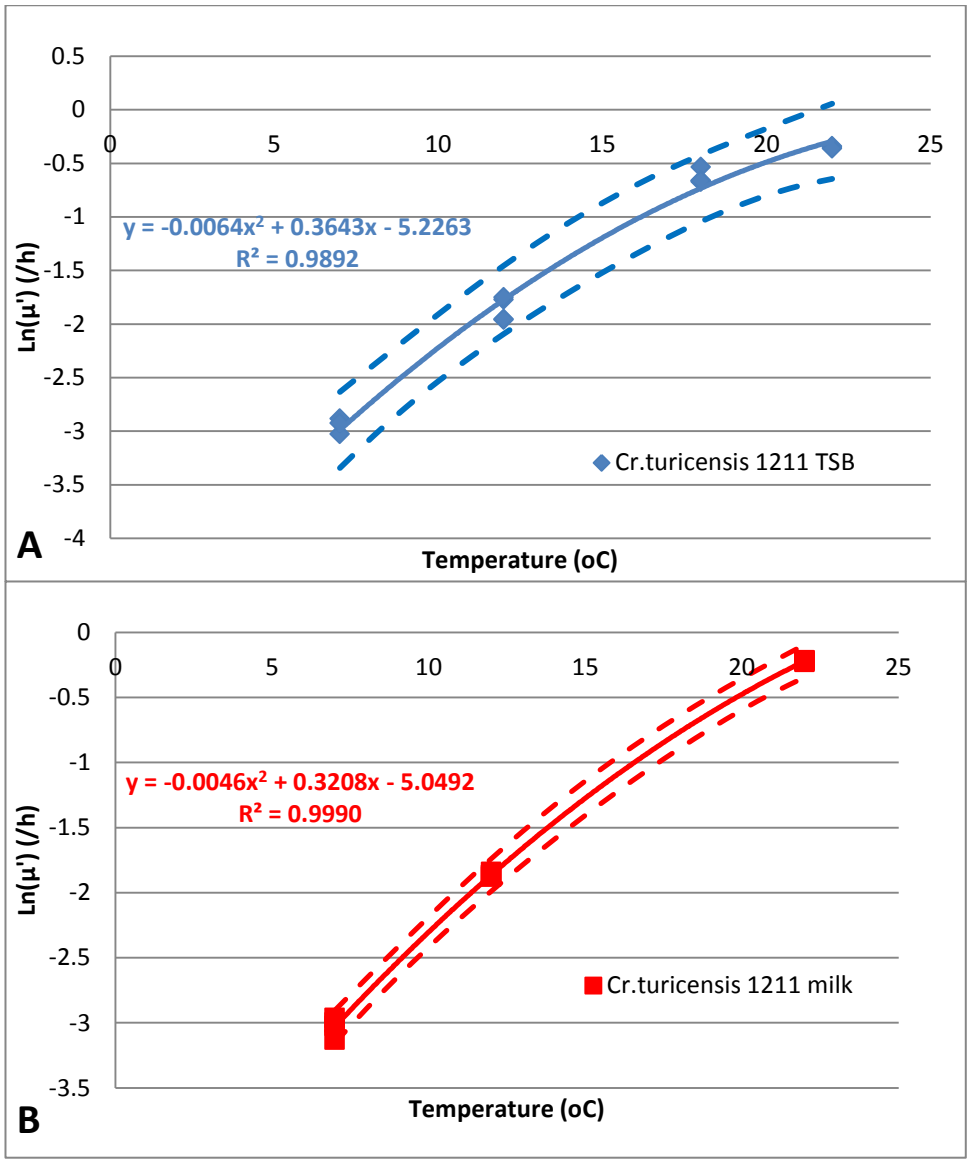

Fig 5.2: Polynomial model for specific growth rate (solid lines) of *Cr. turicensis* 1211 in TSB (A) and first milk (B) with 95% confidence limits (dashed lines)

5.3.3 Maximum cell density

Table 5.4 describes the maximum cell concentration of *Cr. turicensis* 1211 in 1ml TSB and 1ml first milk at each temperature. In TSB, the maximum cell concentration of *Cr. turicensis* 1211 is from 8.26±0.09 to 9.09±0.09 log between 7 ^{o}C and 22 ^{o}C; in first milk, it is from 8.13±0.09 to 8.96±0.25 log. As the maximum cell concentration in all conditions is more than 8 log, therefore, 8 log (the end of the exponential phase) was set as the maximum cell concentration for both strains in these conditions for Monte Carlo simulation.

Table 5.4: Maximum cell concentration (mean±SD) of *Cr. turicensis* 1211 in 1ml TSB and first milk at different temperatures

	Temperature	Maximum cell concentration of *Cr. turicensis* 1211(log(cfu/ml))
TSB	7 ^{o}C	8.26±0.09
	12 ^{o}C	8.52±0.04
	18 ^{o}C	9.09±0.09
	22 ^{o}C	9.01±0.16
First milk	7 ^{o}C	8.13±0.09
	12 ^{o}C	8.17±0.21
	22 ^{o}C	8.96±0.25

5.3.4 Single cell inoculation in TSB and first milk

Based on growth of the 50 parallel samples in TSB incubated at 22 ^{o}C, which were inoculated with the same culture used in the challenge tests in TSB/first milk, the average cell number for each experiment was estimated. Table 5.5 describes the estimated average cell number in each challenge test and the numbers of positive samples obtained in both challenge tests and the 50 parallel TSB samples. The average cell number (ρ) in each

challenge test is around 0.65 to 1.61 cells. The positive sample numbers of *Cr. turicensis* 1211 in each test is around 14-35.

Table 5.5: Numbers of positive samples and estimated average cell number inoculated in each challenge test in TSB and first milk

		7 °C	12 °C	18 °C	22 °C	parallel 50 samples	Average cell number (ρ)
UTSB	SN	50	50	50	50	50	
	OPS	22	29	29	35	24	
	%	44.0%	58.0%	58.0%	70.0%	48.00%	0.65
HTSB	SN	50	50	50	49	50	
	OPS	33	35	37	35	33	
	%	66.0%	70.0%	74.0%	71.4%	66.00%	1.08
Umilk	SN	50	50	-	49	50	
	OPS	29	29	-	28	27	
	%	58.0%	58.0%		57.1%	54.0%	0.78
Hmilk	SN	50	50	-	50	50	
	OPS	14	22	-	30	40	
	%	28.0%	44.0%		60.0%	80.0%	1.61

UTSB: TSB inoculated with untreated cells; HTSB: TSB inoculated with heat stressed cells; Umilk: first milk inoculated with untreated cells; Hmilk: first milk inoculated with heat stressed cells. SN: sample number; OPS: observed positive sample

5.3.5 Validation and applicability of models

5.3.5.1 Validation of models in TSB

5.3.5.1.1 Untreated cells: comparing challenge test results with Monte Carlo simulations

Table 5.6 and Fig 5.3 describe the observed and predicted cell concentrations in TSB inoculated with approximately one untreated *Cr. turicensis* 1211 cell incubated at 7, 12, 18 and 22 ^{o}C for 336, 48, 18 and 8 h respectively.

Based on Table 5.6, the predicted concentration of *Cr. turicensis* 1211 in TSB includes all observed cell numbers at each temperature. For example, at 12 ^{o}C, the observed concentration of *Cr. turicensis* 1211 in TSB ranged from a minimum value of 1.18 log to a maximum of 3.97 log, while the predicted cell concentration range was from a minimum of 0.57 to a maximum of 4.64 log. In terms of the mean value, when the temperature was above 12 ^{o}C, the observed mean value of number of *Cr. turicensis* 1211 was close to the mean estimated by Monte Carlo simulation. For example, at 18 ^{o}C, the observed average cell concentration of untreated *Cr. turicensis* 1211 in TSB after 18 h incubation was 2.80 log and the predicted mean value was 2.61 log. However, at low temperatures, the difference between the mean of the observed and simulated cell concentration increased. For example, at 7 ^{o}C, the observed average cell density (7.40 log) is almost 1log higher than the simulated mean (6.48 log) (Table 5.6). Fig 5.3 also confirms that the observed cell density fell within the range of the simulated cell concentration. In terms of the distribution of the cell density at each temperature, through observation, at high temperature (22 ^{o}C and 18 ^{o}C) seem to show good agreement between the distributions from challenge tests and simulations; when the temperature lowered to 12 ^{o}C and 7 ^{o}C, the observed results of challenge tests suggest that the cells in reality grow faster than the prediction. However, the statistical analysis shows that there is a significant difference

between the observed and simulated distribution (χ^2 test, $p<0.05$) at all temperatures.

Table 5.6: Predicted and observed concentrations of *Cr. turicensis* 1211 in TSB and first milk inoculated with one or two untreated and heat stressed cells at various temperatures

		simulated log cfu/ml				observed log cfu/ml			
	Temp	Mini	Max	Mean	SD	Mini	Max	Mean	SD
UTSB	7°C	4.28	9.17	6.48	0.75	5.07	8.04	7.40	0.65
	12°C	0.57	4.64	2.40	1.74	1.18	3.97	3.28	0.48
	18°C	1.04	4.10	2.61	0.51	2.38	3.20	2.80	0.21
	22°C	0.43	2.57	1.63	0.37	1.24	2.49	1.87	0.34
HTSB	7°C	0.01	8.63	5.24	1.33	4.04	7.42	6.32	0.80
	12°C	0.003	7.29	4.36	1.60	2.65	6.76	5.62	0.90
	18°C	0.04	8.07	4.16	1.76	2.95	5.48	4.65	0.61
	22°C	0.03	9.71	5.01	1.87	4.88	7.16	6.39	0.47
Umilk	7°C	4.73	7.28	6.22	0.45	6.14	7.31	6.75	0.29
	12°C	0.44	3.21	2.20	0.45	2.51	3.31	2.94	0.20
	22°C	0.33	2.53	1.73	0.35	1.00	2.38	1.58	0.35
Hmilk	7°C	0.23	7.09	5.12	1.10	4.80	5.72	5.26	0.27
	12°C	0.04	6.41	4.24	1.27	1.68	5.92	4.41	1.16
	22°C	0.02	7.88	4.99	1.82	3.00	6.01	4.77	0.90

UTSB: TSB inoculated with untreated cells; HTSB: TSB inoculated with heat stressed cells; Umilk: first milk inoculated with untreated cells; Hmilk: first milk inoculated with heat stressed cells; Mini: minimum value; Max: maximum value.

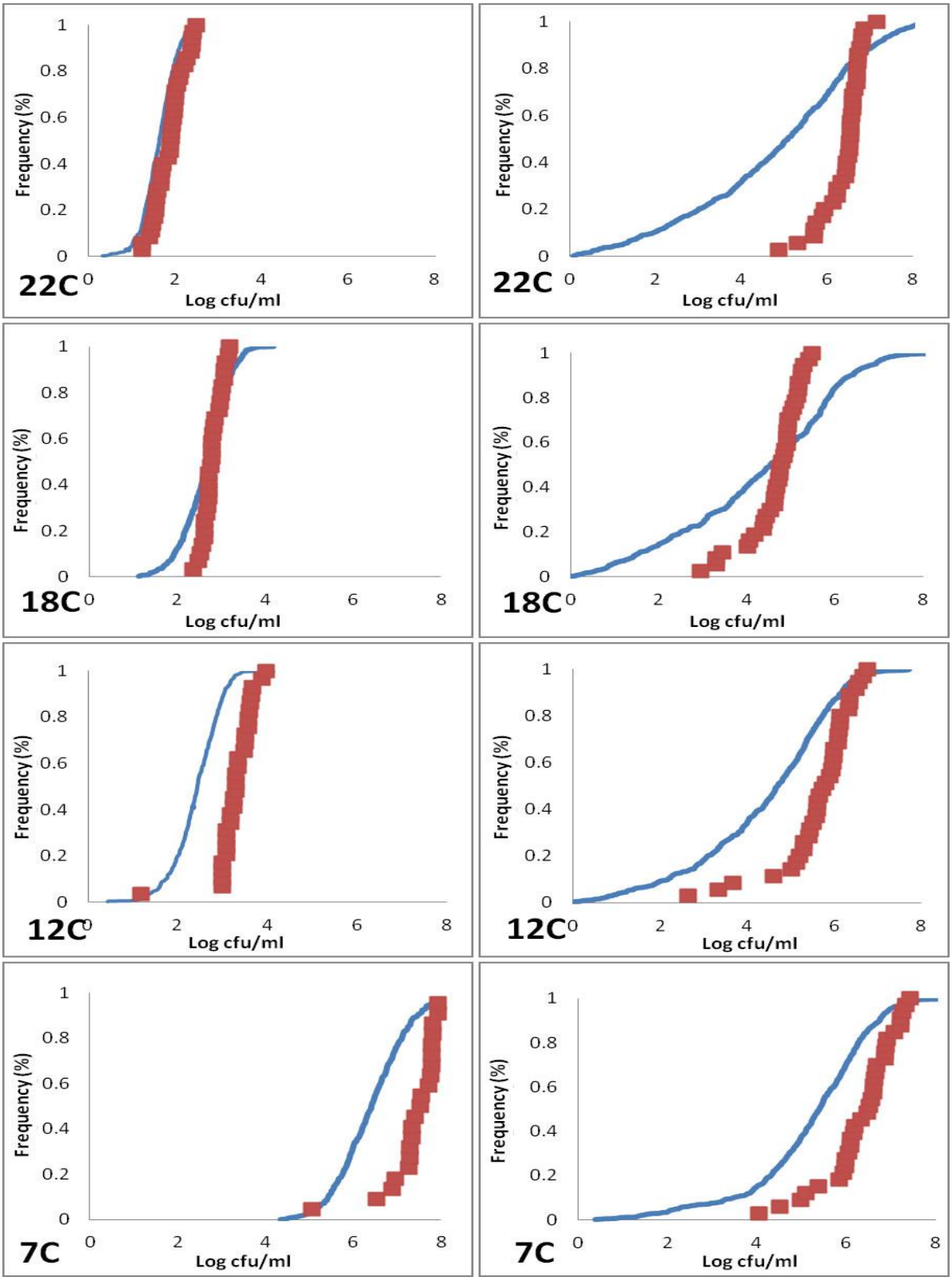

Fig 5.3: Observed (squares) and predicted cell concentration (lines) of *Cr. turicensis* 1211 in TSB inoculated with untreated and heat stressed single cells at 7, 12, 18 and 22 ^{o}C.

untreated cells on the left, heat stressed cells on the right

5.3.5.1.2 Heat stressed cells: comparing challenge test results with Monte Carlo simulations

For TSB inoculated with heat stressed individual cells, according to Table 5.6, the simulated cell density encompasses all the observed results from challenge tests. For example, at 7 ^{o}C, the simulated minimum and maximum cell density covers a wide range, from 0.01 to 8.63 log, whereas the observed concentration of *Cr. turicensis* 1211 in the challenge test range from 4.04 to 7.42 log, i.e. the observed results are included within the broad range of the simulated results. In terms of mean value, in most cases, the observed cell density is at least 1 log higher than the simulated cell density (except at 18 ^{o}C). For example, at 12 ^{o}C, the simulated cell density is 4.36 log, about 1.3 log lower than the observed average cell concentration (5.62 log). Fig 5.3 confirms that the concentrations of cells observed in the challenge tests occurred within the broader range covered by the simulations. However, through observation, the simulations show a large variability with a tail on the left, compared with the results from challenge tests and in most cases (7, 12 and 22 ^{o}C), the cells from the challenge test grew faster than the simulation. According to statistical evaluation, the distributions from challenge tests show significant differences from those obtained by simulation (χ^2 test, $p<0.05$).

5.3.5.2 Applicability of models to first milk

5.3.5.2.1 Comparing challenge test results for untreated cells with Monte Carlo simulations

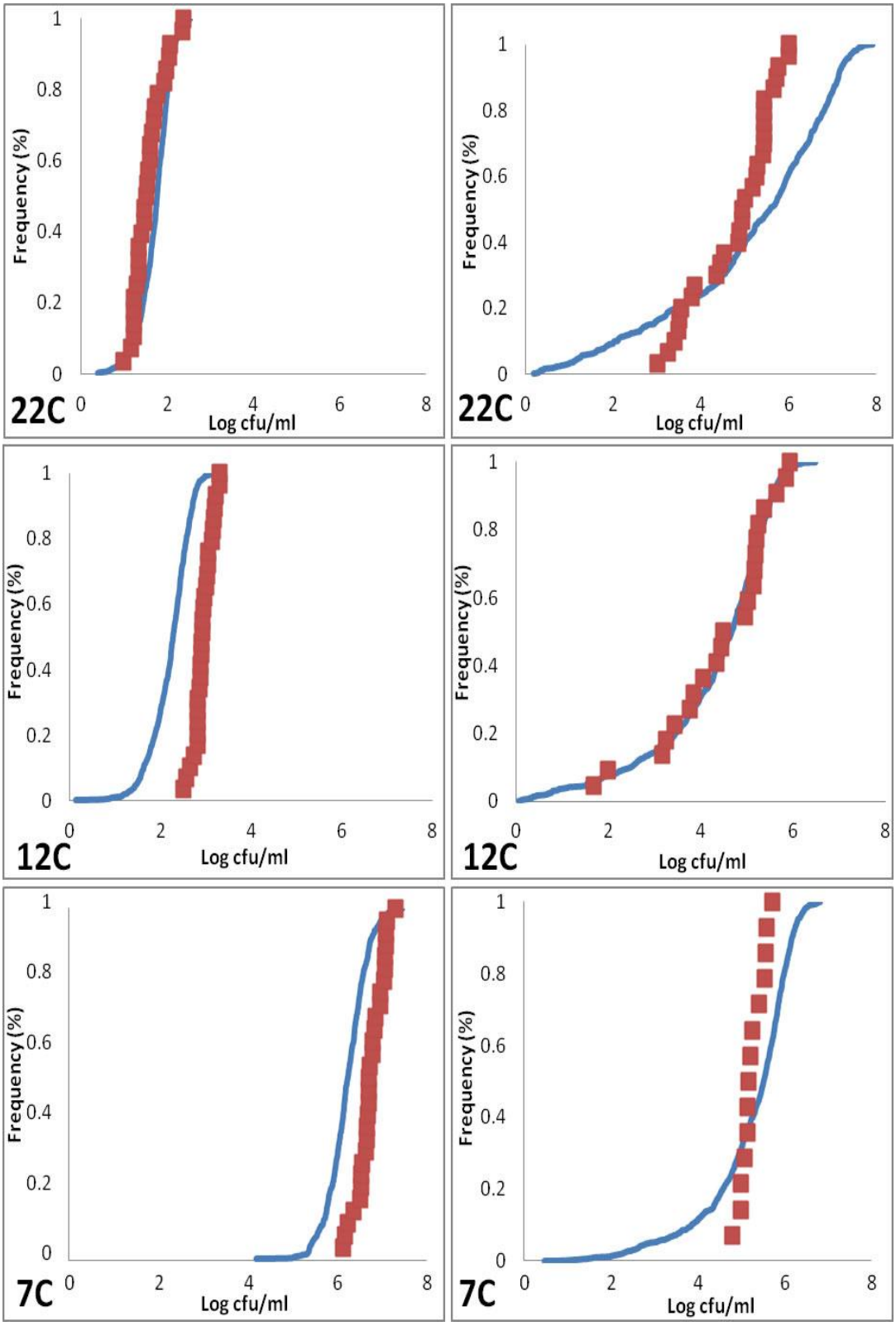

Fig 5.4: Observed (squares) and predicted (lines) cell concentrations of *Cr. turicensis* 1211 in first milk inoculated with untreated and heat stressed single cells at 7, 12 and 22 ^{o}C.

Untreated cells on the left, heat stressed cells on the right

Table 5.6 and Fig 5.4 show the predicted and observed number of cells of *Cr. turicensis* 1211 in first milk inoculated with approximately one individual untreated cell which was incubated at 7, 12 and 22 ^{o}C for 336 h, 48 h and 8 h. Table 5.6 shows that at 22 ^{o}C the range of predicted cell densities (0.33~2.53 log) included the range of observed results (1~2.38 log), with very close similarity between the mean value of the challenge test (1.58 log) and the predicted mean (1.73 log). At 7 and 12 ^{o}C, compared with 22 ^{o}C, the difference between the observed and simulated mean cell density is slightly higher and the simulated maximum cell density is slightly lower than in the challenge test. For example, at 12 ^{o}C, the simulation predicted a maximum 3.21 log *Cr. turicensis* 1211 concentration would develop in first milk, while the observed maximum cell concentration was 3.31 log and the difference between the observed and simulated mean cell concentration at 12 ^{o}C was 0.74 log. Fig 5.4 further confirms that at 22 ^{o}C, the simulation encompasses all results observed in the challenge test and the distribution between the simulation and observation look similar. However, at lower temperatures (7 and 12 ^{o}C), the simulation slightly underestimated the observed level of growth, in terms of both the maximum value and the distribution. The χ^2 test showed that at 22 ^{o}C, there was no significant difference between the distributions obtained from the challenge tests and simulations ($p>0.05$); while at 12 ^{o}C and 7 ^{o}C, the distribution between simulation and observation was significantly different ($p<0.05$).

5.3.5.2.2 Comparing challenge test results for heat stressed cells with Monte Carlo simulations

In terms of the applicability of the model for heat stressed cells in first milk, Table 5.6 indicates that the range of cell density (maximum and minimum values) predicted for *Cr. turicensis* 1211 encompassed the range of the observed data obtained from the challenge test, with very close mean values. For example, at 12 ^{o}C, the mean result of the challenge test was 4.41 log, with minimum and maximum values 1.68 and 5.92 log respectively. The predicted average number was 4.24 log, with a 0.04 log

minimum value and a 6.41 log maximum value. Although the simulation tends to estimate slightly faster growth than that observed, as can be seen in first milk (22 ^{o}C; Fig 5.4) and there is a long tail on the left in the simulation (22 ^{o}C and 7 ^{o}C), generally there is a good similarity between simulation and challenge test data. According to the χ^2 test, except at 22 ^{o}C ($p<0.05$), there was no significant difference between the distribution of simulation and the observed results from the challenge test at 7 ^{o}C and 12 ^{o}C ($p>0.05$). However, it should be mentioned that at 7 ^{o}C, due to the limited sample size (14 samples), it might difficult to obtain a very powerful statistical result.

5.4 Discussion

5.4.1 The choice of ready-to-feed first milk

PIF has been demonstrated to be an important infection source of *Cronobacter* spp. for bottle-fed infants and a transmission vehicle (Friedemann 2007, Mullane et al. 2007b, Miled et al. 2010, Ye et al. 2010). Ideally, therefore, PIF should be used to examine the applicability of the model in food. However PIF is not sterile (Farber 2004). To avoid the problem of pre-existing microflora, sterilized ready-to-feed liquid milk, which is another type of infant formula (Forsythe 2009), was selected in preference to PIF.

Aptamil ready-to-feed first milk is a commercial UHT sterilized first infant milk (Aptaclub, no date) and it is one of the brands with the largest market share in the UK in 2009 (Crawley & Westland, 2011, 2013). Therefore Aptamil ready-to-feed first milk was used for validation experiments.

5.4.2 Specific growth rate

5.4.2.1 Specific growth rate of *Cr. turicensis* 1211 in first milk

In this chapter, the specific growth rate of *Cr. turicensis* 1211 in both TSB and first milk at various temperatures was studied. As the specific growth rate for *Cr. turicensis* 1211 in TSB has already been discussed in

Chapters 2 and 4, the focus of this section will be the growth rate of *Cr. turicensis* 1211 in first milk at 7, 12 and 22 ^{o}C.

Kandhai et al. (2006) determined the growth of *Cronobacter* spp. in reconstituted infant formula between 10 ^{o}C and 37 ^{o}C and reported a 0.12 h^{-1} specific growth rate at 10 ^{o}C (generation time 5.8 h). Nazarowec-White et al. (1997b) reported the mean generation time of *Cronobacter* spp. was 4.98 h and 40 min at 10 ^{o}C and 23 ^{o}C respectively. Jo et al. (2010) studied the growth rate of *Cronobacter* spp. in reconstituted PIF at a similar temperature range and reported 0.0263 h^{-1} and 0.2166 h^{-1} specific growth rates at 10 ^{o}C and 20 ^{o}C respectively, which equal 26.4 h and 3.2 h generation times. Moreover, Ghassem et al. (2011) reported mean generation times of 3.64 h and 0.50 h at 10 ^{o}C and 25 ^{o}C respectively for four *Cr. sakazakii* strains in reconstituted PIF. In the current study, specific growth rates of 0.048±0.003 h^{-1}, 0.157±0.018 h^{-1} and 0.803±0.004 h^{-1} at 7, 12 and 22 ^{o}C were estimated, corresponding to generation times of 14.32 h, 4.43 h and 52 min respectively. Compared with previous studies, generation times obtained in first milk in this study are consonant with the results reported by Nazarowec-White et al. (1997b), Kandhai et al. (2006) and Ghassem et al. (2011), but were shorter than those reported by Jo et al. (2010). Jo et al. (2010) estimated the generation time by the Gompertz model, which may underestimate generation time by 13% (Baranyi et al. 1993a, McKellar & Lu 2003). However, this was probably not the reason for the difference between these results and those of Jo et al. (2010) since generation timesin this study herein reported were shorter. Because both studies used the same technique (plate count method) to estimate generation time, the differences may be attributable to use of different species. In this study, *Cr. turicensis* 1211 was used, while *Cr. sakazakii* 5920 KCTC 2949 was used by Jo et al. (2010).

5.4.2.2 Polynomial growth rate model

Both square root and polynomial models have been widely used to study the impact of temperature on growth rate. Using a second order polynomial model, Sutherland et al. (1996) investigated growth rate and

lag time of *Bacillus cereus* as influenced by temperature, pH value, sodium chloride and carbon dioxide concentrations and reported that the model predictions gave realistic estimations of growth rate for a range of foods as published in the literature. Huang (2010) studied the effect of storage temperature on growth of *Esch. coli* O157:H7 in beef and reported that the traditional Ratkowsky square-root model may underestimate the minimum growth temperature of mesophilic bacteria, while the modified Ratkowsky square root model gave a more realistic estimation of minimum growth temperature. Lobacz et al. (2013) determined the effect of temperature (3 to 15 ^{o}C) on growth rate of *L. monocytogenes* in Camembert and blue cheese using both square root and polynomial models and reported that the polynomial model predicted effect of temperature on growth rate more accurately. In the study herein reported, first, the 95% confidence limits of the predicted specific growth rate in TSB and first milk using the polynomial model at low temperature (7 and 12 ^{o}C) were closer to the observed value, compared with use of the square root model (Appendix 5.1). Second, an underestimation of specific growth rate by 15.1% to 22.9% in TSB and first milk at low temperature (7 ^{o}C) was observed using the square root model (Appendix 5.1). Therefore the polynomial growth rate model was used. This underestimation at low temperature also occurred in the study of Lobacz et al. (2013), which shows a figure describing impact of temperature on growth of *L. monocytogenes* using square root and polynomial models. The figure demonstrates an underestimation of growth rate in Camembert cheese at refrigeration temperature using the square root model.

In addition, when growth rate in TSB and first milk were compared throughout the temperature range (7-22 ^{o}C) using analysis of covariance, it showed that *Cr. turicensis* 1211 grew significantly differently in TSB and first milk throughout the whole temperature range. Therefore, in this section, two separate growth models for *Cr. turicensis* 1211 in TSB and in first milk were developed instead of constructing an overall model.

5.4.3 Estimation of average cell number in validation experiments

In order to run simulations, the average cell number inoculated into each TSB and first milk sample (ρ) in Eppendorf tubes must be known. Francois et al. (2006b) estimated the average cell number and likelihood of inoculating single cells into liver pâté and cooked ham using a simulation model which described the experimental process used to isolate single cells (Standaert et al. 2005). D'Arrigo et al. (2006) estimated the average cell number of heat stressed *L. innocua* per food portion able to grow at 4 ^{o}C in a dairy product, based on the proportion of samples without growth compared with the total number inoculated. Manios et al. (2011) estimated the cell number inoculated in salad and cabbage by actually measuring the cell numbers. In this study, as suggested by Francois et al. (2006b), at some enumeration times possibly not all samples will show growth above the detection level (5 cfu/ml). Some cells may remain in lag phase without initiating growth, or growth may be below the detection limit. This may result in underestimation of cell numbers if estimates are based on actual growth in each of the 50 samples inoculated for validation experiments. Therefore, in order to avoid underestimating the initial cell number, the initial average cell number of *Cr. turicensis* 1211 inoculated into TSB/first milk for validation experiments was estimated, based on growth of *Cr. turicensis* 1211 in a separate, parallel, experiment involving inoculation of the same culture into 50 TSB samples in microtitre plates.

Inevitably, some variability between the number of positive samples in the validation experiment and the parallel 50 TSB samples in microtitre plates was expected. For example, in the validation experiment for heat stressed *Cr. turicensis* 1211 in TSB, a total of 33 samples showed growth in the parallel 50-well TSB experiment, while in the TSB in Eppendorf tubes, 33 to 37 positive samples were observed at 7 ^{o}C to 22 ^{o}C. However, a very low positive sample number was observed in the validation experiment for heat stressed *Cr. turicensis* 1211 in first milk at 7 ^{o}C (14 samples) and 12 ^{o}C (22 samples), compared with the number of positive samples in the parallel 50-well TSB experiment (40 samples). This would not contribute

to the reasons that the cell was still in the lag or below the detection limit. The reason for this is that since validation experiments for heat stressed cells were carried out in both TSB and first milk, an identical protocol was used and similar initial cell numbers were inoculated into both media; they would be expected to give a similar number of positive samples at each temperature (7 ^{o}C and 12 ^{o}C). The results suggested that at lower temperatures, the individual sublethally heat stressed *Cr. turicensis* 1211 cells inoculated intofirst milk may not be able to initiate growth as competently as in TSB; they may enter a viable but nonculturable condition (Wesche et al. 2009). Or they may die at low incubation temperatures, e.g. Labots (1959) reported that *Esch. coli* which survived sublethal heat treatment died slowly when subsequently incubated in both raw and preheated milk (85 ^{o}C for 5 sec) at 4 ^{o}C, while unheated *Esch. coli* inoculated in preheated milk (74 ^{o}C for 5 sec) neither multiplied nor died at the same temperature. It should be noted that in 1959, the concept of the viable but nonculturable condition was not recognised.

5.4.4 Validation and application of the models

In this chapter, in order to validate the model describing the effect of recovery temperature between 7 and 22 ^{o}C for the individual cellular lag of untreated and heat stressed *Cr. turicensis* 1211, four temperature points (7, 12, 18 and 22 ^{o}C) were initially chosen to validate the performance of the model in TSB. Later, three temperature points (7, 12 and 22 ^{o}C) were selected to evaluate the model in first milk. In this way, the validation addresses not only the whole temperature range used to develop the model, but also can give some indication about the performance of the model in first milk.

Currently, to the best of my knowledge, few papers have been published to validate individual lag models. Moreover, no standard method has been proposed. Francois et al. (2006b) studied variability of growth of a single cell of *L. monocytogenes* in 15-26 liver pâté samples (pH 6.1, a_w 0.966) at 7 ^{o}C and compared the observed growth to Monte Carlo simulations, based on the model they developed which described the individual and

combined effects of temperature (2-30 ^{o}C), pH (4.4-7.4) and a_w (0.947-0.995) on individual cell lag (Francois et al. 2006a, Standaert et al. 2007). Through visual observation, they concluded that although slightly greater variability was observed in challenge tests and the simulation results slightly over- predicted the growth, there was good similarity between challenge test results and simulations. Miled et al. (2011) validated the applicability of a model to *Cr. sakazakii* which characterised the individual lag distribution of *L. monocytogenes* for 54 combinations of 22 initial physiological states, 18 growth conditions and 11 strains. They compared the simulated and observed distributions of *Cr. sakazakii* concentration initiated from approximately 4 dry stressed cells from 40 samples of 10 g and 100 g of PIF at 37 ^{o}C and reported good agreement between simulation and observed resultsby observation. Moreover, in the paper of D'Arrigo (2006), it is not real 'model validation', it is a test of the applicability of the method; however it can give some indication about validating the model. In the paper, they compared the distribution of detection times for *L. innocua* obtained in a Bioscreen with the distribution of detection times in TSB estimated using log count distribution from 120 to 160 positive samples among 200. Using the χ^2 test, they found no significant difference between the distribution of detection times (in Bioscreen) and that estimated from log counts. Therefore, the log count method could be used to estimate the individual lag in food.

Taking into consideration these varying approaches, in this study, the model was evaluated in TSB and first milk in two steps. First, the simulated range of cell concentrations were compared with the observed result to determine whether the range of simulated cell concentrations encompassed the full range of results obtained from challenge tests. Second, since Monte Carlo simulation allows quantification of the probability distribution of the microbial load at a certain time instant (Poschet et al. 2003), the second step of the model validation used a χ^2 test to check the distribution between the simulation and the observed challenge test.

5.4.4.1 Validation of the models in TSB

For both untreated and heat stressed single cell models, comparison of the simulated range with the observed range of results from challenge tests indicated clearly that simulation from both models could encompass the cell densities from challenge tests in TSB. When visually comparing the distributions, in a few cases (22 ^{o}C untreated cells, Fig 5.3), the simulation apparently showed a similar distribution to that from the challenge tests. However, the χ^2 test demonstrated that in all cases in TSB, there was in fact a significant difference between the simulations and the actual observations from challenge tests. In addition, in most cases, both models underestimated the actual cell growth (Fig 5.3).

This underestimation may be because: first, the assumptions made in developing or validating the model are not valid. For example the gamma distribution assumption may be not appropriate. The shape parameter, fixed during model development, may change with temperature. The individual lag times of *Cr. turicensis* 1211 in TSB in the Bioscreen were different from that in the incubator. Since in most cases in TSB, the simulation based on both models underestimated the actual growth from challenge tests in Eppendorf tubes, individual lag times of *Cr. turicensis* 1211 cells in TSB in Eppendorf tubes in an incubator might be shorter compared with those in the Bioscreen. This may be the result of differences in degree of aerobiosis in TSB in the wells of Bioscreen plates compared with Eppendorf tubes. The environment in the wells of Bioscreen plates may be more aerobic compared with that in the closed Eppendorf tubes. It has been reported that stressed cells recover better anaerobically than aerobically (George & Peck 1998, Stephen & Mackey 2003). However it should be noted that, since the plate count method was used in those studies to compare the recovery ability anaerobically and aerobically, the plate count was measuring the ability to actually form a colony, rather than the length of lag. Therefore a study to investigate effect of oxygen on the individual lag might be required to confirm this possible reason. Moreover, it cannot be assumed that “the work to be done”

(h_0=μ'*Lag(1)*) for bacteria in the Bioscreen is as same as that in an incubator, because from the experiments, it was found that the specific growth rate in the Bioscreen was higher than in an incubator (Appendix 5.2). If the work to be done in the Bioscreen was the same as that in an incubator, an even longer *Lag(1)* in Eppendorf tubes would be used in the simulations, which would cause a further underestimation of simulated results. A second reason for the underestimation may be due to the limited data used in developing and validating the model. For example, three temperature points may not be enough to develop a robust secondary model, especially at low temperatures. Moreover, since only 14 - 35 samples grew in the challenge tests, from the 50 samples inoculated. It is suggested that a minimum of 100 data points are required to construct a robust distribution (BACANOVA, 2005). Moreover, Vose (2008) showed that a limited number of samples (20) randomly drawn from a normal distribution (100, 10) are not enough to reproduce the same distribution. The third possible reason for the underestimation may be because ofsome uncertainty involved in the experiment during the transition from TSB in theBioscreen to Eppendorf tubes in an incubator. Therefore, in order to decrease the uncertainty associated with the simulations, more data would be needed and more conditions (combinations of heat treatment-recovery temperature) need to be tested.

5.4.4.2 Applicability of the models to first milk

When the applicability of the untreated cell model was determined in first milk, comparison of the range of cell densities at 22 ^{o}C suggested that the model could provide an appropriate prediction which included the range of observed microbial numbers. However, at lower temperatures, the simulations slightly underestimated the actual growth of the cells from challenge tests (Table 5.6). Comparison of the distributions further confirmed that at 22 ^{o}C, the untreated cell model provided an appropriate prediction for actual growth of the cells in first milk; while at lower temperature, the cells in first milk appeared to grow faster than the simulation and the distributions between the simulation and actual growth

were significantly different (Fig 5.4). This apparent faster growth in first milk may be due to invalid assumptions or the limited data, as mentioned above (section 5.4.4.1); or it might be attributable to some uncertainty affecting growth of the bacteria at low temperature in first milk. In addition, it highlights the necessity for validation of the model throughout the whole temperature range, not only for optimal conditions.

Regarding the heat stressed cell model, as in previous situations, the range of simulations from the model encompassed the range of results from the challenge tests. Based on the distribution, although at 22 ^{o}C the model slightly overestimated the real growth (Fig 5.4) and in some cases, large variability was observed in the simulation (7 ^{o}C, 22 ^{o}C), possibly due to the limited sample numbers in the challenge tests; overall, the predicted distribution seems reasonably close to the observed distribution. Moreover, because of the observed phenomenon, that growth of sublethally heat stressed *Cr. turicensis* 1211 may be inhibited in first milk at lower temperatures, another simulation based on actual growth in first milk at 7 and 12 ^{o}C was performed. Based on those results (Appendix 5.3), the simulated range of cell densities again encompassed the range of data from the challenge tests, and also no significant difference was observed between the simulation and the observation. Consequently, it can be assumed that the heat stressed cell model developed in TSB might be applied to first milk and provide a foundation for quantitative microbiological risks assessments for food safety issues related to*Cronobacter* spp. in first milk.

It should be noted that in the case of both models, because a limited number of samples (less than 30) were obtained from the challenge tests, they may not be enough to construct robust distributions (BACANOVA 2005, Vose 2008). More samples are required to truly verify the applicability of the models to first milk and other foods.

5.4.5 Physiological state of the cells

Baranyi & Roberts (1994) proposed that for cells with the same pre-inoculation history, the product of the lag parameter and the maximum specific growth rate is relatively constant and independent of current growth conditions. Muñoz et al. (2010) studied the physiological states of individual unstressed and heat stressed *L. monocytogenes* cells at two pH values and three concentrations of eugenol and reported two different patterns of physiological state, depending on pH. When the pH was 7.4, a value equal to or higher than 5.4 for the physiological state was observed. However, when the pH was 5.0, the value of physiological state ranged between 2.7 and 4.8. George et al. (2008) investigated growth of *L. innocua* in various concentrations of undissociated organic acids and reported a linear relationship between the physiological states with concentration of undissociated organic acid.

In the study herein reported, a long tail on the left side of the cumulative distribution of simulation results from the heat stressed cell model was observed (Figs 5.3, 5.4), while it appeared in only some enumeration results in TSB or first milk inoculated with approximately one single cell. This is possibly due to the limited sample numbers obtained from challenge tests (14-35 samples, from 50 inoculated), which were not enough to establish that tail. It also may be indicated that although cells have the same pre-inoculation history, the product of lag time and growth rate in samples in the Bioscreen and in samples in Eppendorf tubes are different. The specific growth rate of samples in the Bioscreen was higher than the growth rate in incubated samples (in both TSB and first milk) (Appendix 5.2), and the long tail on the left side in the simulation results (Figs 5.3, 5.4) indicated that those cells have a long lag in the Bioscreen. Therefore, for some cells, the product of lag and specific growth rate is higher in the Bioscreen samples than the incubated samples.

5.4.6 Application of the validation results

In this chapter, the growth of untreated and heat stressed single cells of *Cr. turicensis* 1211 in first milk incubated at different temperatures was examined. Besides validation of the model, growth of *Cr. turicensis* in first milk also can provide some indication about the potential risk to infants. For example, when PIF is prepared for consumption by infants away from home, in order to minimise the risk, WHO (2007) suggested transportation of freshly prepared PIF in a cool bag and feeding within two hours. However, in reality, parents are reported to feed babies with pre-prepared feeds which have been in a cool bag for up to four hours, or even not chilled at all (Forsythe 2009). Although in this study growth of *Cr. turicensis* 1211 in first milk which initiated from approximately one untreated cell was analysed after 8 h incubation at 22 ^{o}C (rather than 4 h), it represents the worst case scenario: that parents prepare the PIF in the morning and feed the baby in the later afternoon. Assuming that for each feed, 100 ml of reconstituted PIF is needed, and if it is contaminated with one *Cr. turicensis* 1211 cell from the environment, then after eight hours (according to this study) the concentration of *Cr. turicensis* 1211 in one feed will increase to a mean value of 1.58 log, with a minimum and maximum cell concentration of 1 log and 2.38 log respectively (Table 5.6). The average cell number in one feed will increase to 3,828 cells, with a minimum and maximum value 1,000 and 23,768 cells respectively, which equals or exceeds the proposed infectious dose of 1,000 cells. Therefore, when PIF is prepared for use away from home, parental behaviour in feeding the baby with PIF prepared earlier and not refrigerated, may present a potential risk to the baby.

Moreover, as ready to eat food is also a concern in this study, the growth of single untreated and heat stressed *Cr. turicensis* 1211 cells at 7 ^{o}C for two weeks, which is an approximate shelf life of ready to eat food, was also calculated. Assuming that one portion of ready to eat food is 100ml, then after two weeks' incubation at 7 ^{o}C, the average concentrations of untreated and heat stressed cells in first milk (as an example of ready to

eat food) will reach 6.75 log and 5.26 log (Table 5.6), substantially exceeding the proposed infectious dose. This might be a potential risk, especially to immuno-compromised people. However, it should be noted that these risk assessments are based on growth of *Cr. turicensis* 1211 in first milk. For other foods, the nutrient content, structure and background microflora, may all affect growth of bacteria (Manios et al. 2011, Koseki et al. 2011, Ohkochi et al. 2013). Growth of Cr. *turicensis* may not reach such a high density and further study of the growth of *Cronobacter* spp. in food is required.

5.5 Conclusion

In this chapter, the performance of both untreated and heat stressed cell models were tested. The distribution of cell concentrations obtained from low inoculation levels in TSB and first milk at given times were measured experimentally and compared with simulations.

It was found that in TSB, the range of data derived from simulations of cell concentration for both models always encompassed the entire range of data estimated by challenge testing; however in most cases, the simulated distribution based on both models underestimated the actual growth in TSB. This might be a consequence of invalid assumptions made during model development and validation. Alternatively, it might be attributable to the limited data generated in both developing and validating the models, Finally, it might be because of some uncertainty arising during the transition from the Bioscreen to Eppendorf tubes in an air incubator.

In first milk, it was shown that for the untreated cell model at 22 ^{o}C, simulations showed good agreement with measured growth (in terms of both cell density range and distribution). However, at lower temperatures, simulations based on the untreated cell model slightly underestimated the actual growth (maximum cell density and distribution); while for the heat stressed cell model, predictions included all results from challenge tests and the simulated distribution generally showed a good representation of the actual growth. The results suggest that the heat stressed cell model in

TSB might provide a basis for quantitative microbiological risk assessments concerning *Cronobacter* spp. in first milk.

Both simulated and observed results showed that when PIF is prepared for use away from home, parental behaviour in feeding the baby PIF prepared earlier and not refrigerated, may present a potential risk to the baby. In addition, cell concentrations of both untreated and heat stressed *Cr. turicensis* 1211 in first milk after two weeks' incubation at 7 ^{o}C were well above the reported infectious dose, which suggests ready-to-eat foods might be a potential risk, to immuno-compromised people in particular. However, this research refers to growth in first milk and the different physico-chemical factors associated with other foods will affect the growth of bacteria. Consequently, additional studies of growth of *Cronobacter* spp. in food are required.

CHAPTER 6: GENERAL DISCUSSION

6.1 General framework of the study

6.1.1 Individual cellular lag variability

Considering the fact that in practice, contamination of food by pathogens usually occurs at a very low number (Koutsoumanis 2008) and the lag time is affected by inoculum size (Augustin et al. 2000, Robinson et al. 2001, Coleman et al. 2003), a lot of research into variability of individual lag has been published. Kamdem et al. (2008) evaluated the effect of free fatty acids on first division time of single cells of *Staph. aureus* and reported a longer first division time with a greater variability was observed in the presence of capric, lauric and α-linolenic acid compared with the control. Li et al. (2006) investigated the effect of starvation, heat and acid stress on individual lag time of *Esch. coli* O 157:H7 and reported that single cell lag of either untreated or stressed cells followed the lognormal distribution. Rasch et al. (2007) explored the individual lag time of *L. innocua* growing on a solid agar surface with different concentrations of reuterin, singly and in combination with different pH values and sodium chloride concentrations. The results showed that addition of reuterin increased both the mean and the SD of the individual lag times of *L. innocua.* Reuterin combined with both low pH and NaCl inhibited cell division more efficiently.

In contrast to substantial studies on individual lag for *L. monocytogenes*, *Esch. coli* and *Staph. aureus,* few data are available on variability of single cell lag times of *Cronobacter*spp. Given the ubiquitous nature of *Cronobacter* spp., the organism may be present in ready-to-eat food and reconstituted PIF in either an unstressed or sublethally heat stressed condition. If this pathogen can recover and initiate growth during shelf life or holding time to an infective level, food borne disease may be triggered.

Therefore, in this study, the fact was addressed that low-level contamination of untreated or sublethally heat stressed *Cronobacter* spp. may be present in food and can grow at refrigeration temperatures; consequently the potential ability of untreated and sublethally heat stressed individual cells of *Cronobacter* spp. to recover at different temperatures was studied.

In order to achieve the overall aim, the studies were carried out in three steps. In the first step, an appropriate species of *Cronobacter* was selected and its relevant sublethal heating conditions determined; Then, individual lag times of untreated and sublethally heat stressed *Cronobacter* spp. cells in TSB at different recovery temperatures were studied and a model that described the effect of recovery temperature on variability of individual lag of both types of cell was developed. Finally, the model was validated and its applicability in a suitable food (first milk) was tested.

6.1.2 *Cronobacter* spp. selection and determination of sublethal heating conditions

As the main interest of the project was to study the ability of untreated and sublethally heat stressed *Cronobacter* spp. to recover and grow at low temperature, a *Cronobacter* sp. which had good growth ability at refrigeration temperature (7 ^{o}C) and the highest level of thermotolerance would be ideal for further study. In order to achieve this, three main experiments were carried out, which were: identificationof *Cronobacter* spp.; minimum growth temperature experiments (Chapter 2) and thermal inactivation experiments (Chapter 3).

A total of seven *Cronobacter* spp. were acquired for this study. Except *Cr. sakazakii* NCTC 11467, which was bought directly from NCTC, the identities of the other six strains belonging to two species were confirmed with 16S rDNA sequencing. Based on 16S rDNA sequencing, NCIMB 8272 and NCIMB 5920 were confirmed as *Cr. sakazakii*, while 1211 and 57 were confirmed as *Cr. turicensis.* However, as in the studies of

Townsend et al. (2008) and Jaradat et al. (2009) (also working with *Cronobacter* spp. but not specifically *Cr. turicensis*), in the current study the cultures 1325 and 1327, presumptively identified as *Cr. turicensis,* were found to belong to completely different genera: *Erwinia cypripedii* and *Pantoea agglomerans.*

Based on the growth experiment, the minimum growth temperatures of the *Cronobacter* spp. (*Cr. sakazakii* NCIMB 8272, *Cr. sakazakii*NCIMB 5920, *Cr. sakazakii* NCTC 11467, *Cr. turicensis* 1211*, Cr. turicensis* 57) ranged from 5 ^{o}C to 7 ^{o}C, which is consistent with the study of Nazarowec-White and Farber (1997b). As most home refrigerators run at an average temperature above 5 ^{o}C (Peck et al. 2006), the present study further confirmed that *Cronobacter* spp. can grow at domestic refrigeration temperatures. In contrast to the observation of Ghassem et al. (2011), who reported no significant differences between the growth rates of four *Cronobacter* spp. at temperature between 10 ^{o}C and 45 ^{o}C, in the present study, a high variability between the generation times of *Cronobacter* spp. at 7 ^{o}C was observed and this variability decreased when the temperature was increased to 8 ^{o}C (refer to Chapter 2, Table 2.2). This discrepancy may be due to the higher minimum temperature used by Ghassem et al. (2011) to estimate generation time.Most reports, however, suggest increasing variability of growth rate/generation time at low temperature. Fehlhaber and Krüger (1998) studied the generation times of 45 *S. enteritidis* strains over a temperature range from 7 ^{o}C to 42 ^{o}C, and observed the generation time variability among strains increased remarkably as the minimum growth temperature (7 ^{o}C) was reached. De Jesús and Whiting (2003) studied growth rates of 21 *L. monocytogenes* strains at 5 and 35 ^{o}C and reported a significant difference among strains at low temperature, which was not observed at high temperature. Coleman et al. (2003) reported that strain variability is more apparent at the growth/no growth boundary of the growth condition, e.g. at a combination of low pH and low temperature. As *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 showed the fastest growth rates at 7 ^{o}C, these three strains were selected for testing thermotolerance.

Use of the MPN method and addition of pyruvate to media have been suggested to aid in the recovery of stressed cells to avoid underestimating the viable count (Al-Holy et al. 2008, Métris et al. 2008, Osaili et al. 2008). However, the results of the present study (Chapter 3, Table 3.1) show that the MPN method has limited recovery ability compared with the surface plating method and no difference between the recovery ability of TSA with/without addition of pyruvate, which is in agreement with the observations of Özkanca et al. (2009) and Forsythe (2009). Therefore, TSA was selected as the recovery medium for heat stressed *Cronobacter* spp. Based on the thermal inactivation experiment, the sublethal heating conditions for *Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920 were determined. For *Cr. turicensis* 1211, heating for >40 min at 48 ^{o}C and 7 min at 49 ^{o}C were sublethal; for *Cr. turicensis* 57, 48 ^{o}C for 10 min and 49 ^{o}C for 3.5 min were sublethal heating conditions and for *Cr. sakazakii* NCIMB 5920, sublethal heating occurred at >40 min at 48 ^{o}C, 20 min at 49 ^{o}C and 5 min at 50 ^{o}C (Chapter 3, Table 3.5). Due to time and instrument limitations, it was impossible to study individual lag times of all three strains at different recovery temperatures and under all these sublethal heating conditions. Experiments to evaluate the percentage of injured cells at each sublethal heat condition were carried out. Unexpectedly, the results showed that approximately 50% of untreated *Cronobacter* spp. cells could not be recovered on VRBGA (Chapter 3, Table 3.7). It may be because the untreated cells used in current study were in late stationary phase and according to Juck et al. (2012), they are actually nutritionally starved. It also indicates that a false negative result might be achieved when using the current FDA method to recover *Cronobacter* spp., as VRBGA is listed in the FDA methods (FDA 2002, Osaili et al. 2010) as a selective medium to recover *Cronobacter*spp. Although *Cr. sakazakii* NCIMB 5920 was the most heat resistant among the three cultures (*Cr. turicensis* 1211, *Cr. turicensis* 57 and *Cr. sakazakii* NCIMB 5920) and it had the highest percentage of injured cells after being heated at 49 ^{o}C for 20 min (Chapter 3, Table 3.7), taking into consideration growth ability at 7 ^{o}C, *Cr. turicensis* 1211, which had a high injury

percentage after heating at 49 ^{o}C for 7 min was chosen for further study (Chapter 3).

6.1.3 Individual lag time variability of untreated and heat stressed *Cr. turicensis* 1211 and development of a model

In Chapter 4, the distribution of individual lag times of untreated and sublethally heat stressed *Cr. turicensis* 1211 at different recovery temperatures was determined. A model was developed to describe the effect of recovery temperature on the variability of individual untreated and heat stressed *Cr. turicensis* 1211.

Due to being rapid, automatic and relatively inexpensive (Dalgaard&Koutsoumanis 2001, Lindqvist 2006), the optical density method, in which serial dilutions of cells are made to obtain single cells, has been the favoured indirect method to investigate individual cellular lag time (Guillier et al. 2006). However, most research estimates individual lag based on substituting the random initial cell number with its mean value (Métris et al. 2003, Francois et al. 2005b, 2006a, Guillier et al. 2005, Guillier& Augustin. 2006, Miled et al. 2011). In order to reduce the impact of more than one cell on the individual lag distribution, the culture used to inoculate the honeycomb plate (for the Bioscreen) is usually diluted to a very low cell number and consequently results in an extremely low positive well percentage for a given experiment. Among these positive wells, a high percentage containing only 1 cell would be expected, e.g. Guillier et al. (2005) diluted the suspension such that only 35% of the wells showed growth (70 among 200 wells). However, 80% of these positive wells contained 1 cell and the remaining 20% contained more than 1 cell. The experiment has to be repeated several times and under stress conditions, and reproducibility is poor. In addition, appropriate standardization procedures are required (Métris et al. 2006).

Based on an assumption of individual lag following the Gamma distribution, Métris et al. (2006) estimated the parameter of distribution of individual lag with the mean and variance of the sum of physiological states. The

principle of this method is that with the equation 4.1 (Chapter 4) and the relation between the physiological state of a single cell and the population (equation 4.4), the sum of physiological states in a well on the plate could be estimated (equation 4.5). For one experiment, the average and variance of the sum of physiological states in a well could further be determined experimentally (equation 4.6, 4.7). On the other hand, assuming the initial cell number follows the Poisson distribution and the individual lag follows the Gamma distribution, for one experiment, the average and variance of the sum of physiological states in a well also could be calculated theoretically (equations 4.19, 4.20). In a given experiment, since the theoretical and experimental estimations of the mean and variance of the sum of the physiological states in a well should be the same, then the parameter of the Gamma distribution could be estimated (equation 4.21, 4.22). First, this method can take into consideration not only the randomness of initial cell number, but also is valid even whenthe initial cell number is as high as three cells. Second, the Gamma distribution has proved to be an appropriate distribution to describe the individual lag (Francois et al. 2006a, Métris et al. 2006, 2008, Muñoz et al. 2010), therefore, the method of Métris et al. (2006) was used to model the potential for individual cells of untreated and sublethally heat stressed *Cronobacter* spp. to recover and initiate growth.

With the assumption that, for a single cell of *Cr. turicensis* 1211, the lag follows a shifted Gamma distribution ($T_{shift} + Gamma(\beta, \theta)$), and with the assumption that the shape parameter (β) of the Gamma distribution is fixed, the individual lag of untreated and sublethally heat stressed cells of *Cr. turicensis* 1211 was investigated. It was found that the distribution of individual lag of untreated and heat stressed *Cr. turicensis* 1211 cells become more spread and shifted to the right when the recovery temperature decreased from 22 ^{o}C to 7 ^{o}C. This was particularly evident after heat stress. This is consistent with observations from other studies (Stephens et al. 1997, Smelt et al. 2002, Li et al. 2006). The natural logarithm of T_{shift} was found have an exponential relationship with the recovery temperature. And the natural logarithm of θ has alinear

relationship with the recovery temperature. A similar relationship between θ with acetic acid was reported in the research of Métris et al. (2006).

Another point of interest which should be mentioned here is that, unlike Chapters 1 and 5, in which specific growth rate was estimated based on plate count techniques from samples stored in an incubator, in Chapter 4 specific growth rate was determined using the time to detection point based on readings from theBioscreen ($\mu' = -\frac{1}{slope}$). Although the specific growth rate estimated by viable count also has been used to determine individual lag with OD measurements (Métris et al. 2008), in the current study, a lower specific growth rate was estimated with the plate count method from samples stored in an incubator compared with that estimated using the time to detection method in the Bioscreen. Moreover, the lower the temperature, the greater the difference (Appendix 5.2), which will have a big impact on individual lag, especially at low temperature. The difference between the specific growth rates estimated with OD and plate count technique has already been reported (Augustin et al. 1999). The possible reasons contributing to this difference are that in the various growth models used, the OD detection limit is in the growth rate decreasing range, and the nonlinear relationship between OD and cell number (Lindqvist 2006). However, in this case, the specific growth rate was estimated using the time to detection point method, and it is therefore reasonable to consider that the cells were still in the exponential phase at the detection point when OD reached 0.035 (Chapter 4, Table 4.5), so these sources of bias are less likely to be the possible reasons. In addition, Lindqvist (2006) and Dalgaard and Koutsoumanis (2001) already confirmed that if the growth rate was measured in the same system, the detection time method yielded similar specific growth rates to those from theplate count method. So, in this case, the difference between the specific growth rate estimated by time to detection point method and plate count method may be due to the two different incubation systems (Bioscreen vs. air incubator) used. Both the greater aeration of the culture in the shallow wells of the honeycomb plate (Bioscreen) compared to 100ml universal bottle in the incubator, and the agitation caused by each

OD reading in the Bioscreen could contribute to this difference in the growth rate. Coleman et al. (2003) estimated the slope of the linear regression of exponential phase of *Esch. coli* O157:H7 with or without agitation at 10, 19 and 37 ^{o}C and found significant differences between the slopes at 10 and 19 ^{o}C due to agitation.

6.1.4 Single cell variability of *Cronobacter* spp. grown in TSB and food: comparing challenge test data with Monte Carlo simulation

It is claimed that for a model, the main issue is not how well it fits the data, but how accurately it can describe the behaviour of the microorganism in a given environment (McDonald & Sun 1999). Therefore, in Chapter 5, the performance of the model developed in Chapter 4 was assessed in TSB and infant first milk, independently from the data used in constructing the model.

For validation purposes, the distribution of the cell concentrations obtained from low inoculation levels at a given time were measured experimentally and compared with simulations (Baranyi et al., 2009).After dilution, approximately one or two cells of untreated and heat stressed *Cr. turicensis* 1211 were inoculated into 200 Eppendorf tubes, which contained 1ml TSB and 150 Eppendorf tubes, which included 1ml first milk. These samples were incubated as follows: TSB: 7, 12, 18 and 22 ^{o}C; first milk: 7, 12 and 22 ^{o}C. At each temperature, there were 50 samples. After appropriate incubation times, samples were enumerated by plating on TSA. The recorded concentrations of cells were compared with the Monte Carlo simulations based on the stochastic model developed in Chapter 4. In this way, the model was validated not only throughout the whole temperature range, but also in TSB and food (first milk).

Currently, published literature about validation of the individual cellular lag models is limited. No agreed validation protocol has been developed and different methods have been used, e.g. comparing distributions from challenge tests with simulations through visual observation (Francois et al. 2006b, Miled et al. 2011); comparing mean values (Muñoz et al. 2010,

Muñoz-Cuevas et al. 2013); and evaluating the bias and accuracy factors (Standaert et al. 2007).

In this study, the validation was carried out in two steps: first, the simulated range of cell concentrations were compared with data from challenge tests; then the χ^2 test was used to compare the distributions between the simulated and observed cell concentrations.

The validation of untreated and heat stressed models in TSB shows that the range of cell concentrations for the simulation based on both models encompassed the range of cell concentrations obtained from challenge tests in TSB (Chapter 5, Table 5.6). However, when it comes to comparison of the distributions, in most cases, both models underestimated the actual growth of the cells in TSB (Chapter 5, Fig 5.3). This might be because of invalid assumptions made during model development and validation. Alternatively, it may be because of limited sample data involved in development and validation. Finally, it might be due to the uncertainty involved in the experiment in Bioscreen and in transition to Eppendorf tubes in incubator. Consequently, more data would be needed and more conditions (combinations of heat treatment-recovery temperature) tested to decrease the uncertainty associated with the simulations.

The applicability of both models to food was analysed in first milk. For the untreated cell model at 22 ^{o}C, the model provides appropriate predictions close to the observations from the challenge tests, in terms of both cell number range and distribution. At 7 ^{o}C and 12 ^{o}C, the model gave a slight underestimation (Chapter 5, Table 5.6, Fig 5.4). This may be due to the invalid assumption or limited data (as mentioned above), or to some unknown uncertainty affecting growth of the bacteria in first milk at lower temperatures, which was not taken into consideration. In addition, these discrepancies highlight the importance of validating the model throughout the whole temperature range. Although at 22 ^{o}C the heat stressed cell model slightly overestimates the real growth, generally the simulations based on the model show good agreement with observed results from the

challenge tests, both in terms of range of the cell density and the distribution (Chapter 5, Table 5.6, Fig 5.4). Consequently, it can reasonably be assumed that the heat stressed model developed in TSB provides a basis for quantitative microbiological risk assessments for safety issues related to *Cronobacter* spp. in first milk.

6.2 Strengths of this study

Cronobacter as a new genus was proposed in 2008 (Iversen et al. 2008) and a lot of research on this bacterium has been undertaken. However, the identification of the natural reservoir and its presence in food, developing detection methods, investigations of environmental persistence of the bacterium, pathogenicity and virulence factors and genome studies are active research areas for this microorganism (Chenu & Cox 2009, Norberg et al. 2009, Kucerova et al. 2011). Only limited data have been published to describe the growth rates and lag times of *Cronobacter* spp. Most of them have been in conditions above 10 ^{o}C, around 20-30 ^{o}C, and also on a population level (Nazarowec-White and Farber 1997b, Iversen et al. 2004b, Kandhai et al. 2006, Lenati et al. 2008, Jo et al. 2010, Ghassem et al. 2011, Fang et al 2012). In addition, although many studies have been carried out to investigate the thermotolerance of *Cronobacter* spp., most of them were performed between 52 ^{o}C to 62 ^{o}C (Table 3.9, Chapter 3). Research reporting survival of *Cronobacte*r spp. at temperatures just above the maximum growth temperature (47 ^{o}C) is limited. This temperature is not recommended but is often used to reconstitute PIF in reality (Forsythe 2009).

In this study, some of these problems have been addressed. First, survival of *Cronobacter* spp. at a temperature range of 48 ^{o}C to 50 ^{o}C was studied. It was found that the heat resistance of *Cronobacter* spp. varied widely between strains. The D values of *Cronobacter* spp. are between 153.65 and 223.47 min at 48 ^{o}C, between 45.28 and 46.84 min at 49 ^{o}C and between 15.13 and 62.12 min at 50 ^{o}C (Chapter 3, Table 3.7). Second, as the microorganism which has a minimum growth temperature of 5.5-8 ^{o}C (Nazarowec-White and Farber 1997b), the growth of five strains of

Cronobacter spp. at refrigeration temperature was investigated. At 7 ^{o}C, generation times of *Cronobacter* spp. varied between 9.59 h and 60.81 h; at 8 ^{o}C, generation time was 10.40 h to 21.67 h and higher variability among strains was observed at the boundary of the growth conditions (Chapter 2, Table 2.2). Third, variability of individual lag of untreated and heat stressed *Cronobacter* spp. at the temperature range between 7 ^{o}C and 22 ^{o}C was investigated. For untreated *Cr. turicensis* 1211, the mean value of individual lag ranged between 3.42±0.89 h and 48.9±16.01 h at 7 ^{o}C to 22 ^{o}C; for sublethal heat stressed *Cr. turicensis* 1211, the mean value of individual lag varied between 18.71±14.76 h and 123±70.22 h at 7 ^{o}C to 22 ^{o}C (Chapter 4, Table 4.8). The individual lag time distribution shifted to the right and became more spread when the recovery temperature was reduced and after heat stress (Chapter 4, Fig 4.8); finally, in the validation model part, the growth of approximate one or two untreated and heat stressed cells of *Cr. turicensis* 1211 in milk at different temperatures was briefly explored. Parents may prepare PIF in the home prior to going out with the baby. The feed may remain unrefrigerated for several hours before consumption and in this situation, could present a potential risk to the baby. So this study contributes original information about survival, growth and variability of individual lag concerning *Cronobacter* spp., thereby contributing to quantification of potential risks.

6.3 Suggestions for future research

First, more data are needed for both model development and model validation. In the current study, for model development (Chapter 4), only three points were used to link the parameter of individual lag (T_{shift} and ϑ) with the temperature. This definitely limits development of a robust model. For model validation, 14 to 35 samples were obtained from each challenge test (Chapter 5, Table 5.5). However, as mentioned in Chapter 4, in order to construct a robust distribution, 100 data points are required (BACANOVA 2005). Moreover, the studies of Vose (2000, 2008) also show that limited sample numbers (20 samples) may not be enough to develop the same distribution as the original distribution where these

samples were collected from. Therefore, in order to achieve an exact idea of the performance of the model, more samples from challenge tests are required.

Second, more *Cronobacter* species and strains should be considered. In the current study, only *Cr. turicensis* was studied. As *Cronobacter* is a genus which includes *Cr. sakazakii, Cr. malonaticus*, *Cr. turicensis*, *Cr. muytjensii*, *Cr. dublinensi*s, *Cr. universalis*, *Cr. condiment, Cr. zurichensis*, *Cr. pulveris*, and *Cr. helveticus,*there is a total of ten species which should be considered (Iversen et al. 2008, Joseph et al. 2012, Brady et al., 2013). Other studies (Edelson-Mammel&Buchanan 2004, Gurtler & Beuchat 2005, Al-Holy et al. 2009, Dancer et al. 2009), as well as this study have demonstrated that thermal tolerance of *Cronobacter* spp. varies widely (Chapter 3, Table 3.7). Moreover, to the best of my knowledge, although no publications report variability of individual lag among different *Cronobacter* spp., the study of Lenati et al. (2008) shows significant differences exist in the population lag in *Cronobacter* spp. from food, clinical and environment sources. Therefore, in order to get a clear picture of the potential ability of individual untreated and sublethally heat stressed *Cronobacter* spp. cells to recover at various temperatures, more studies of different species are required, especially *Cr. sakazakii and Cr. malonaticus,* because as well as *Cr. turicensis,* these are a significant cause of neonatal infections (Kucerova et al. 2010).

Finally, more factors need to be taken into consideration. In this study, some simplifications were applied. For example, the sublethal heating conditions were determined in TSB (Chapter 3). However, various studies have demonstrated that heat resistance of bacteria could be affected by the content of media or food such as the content of fat, carbohydrate and protein (Juneja&Eblen 2000, Jay 2000, Kim & Park 2007, Osaili et al. 2009b). Furthermore, during model development (Chapter 4), the effect of recovery temperature only on individual lag of untreated and heat stressed *Cr.turicensis* 1211 was studied. However, other factors including a_w, pH and antimicrobial compounds will also affect variability of individual lag

(Francois et al. 2006a, Métris et al. 2006, Rasch et al. 2007, Koutsoumanis 2008，Muñoz et al. 2010). Moreover, to validate the model in food (Chapter 5), commercial sterile infant milk was employed. Factors like competitive background flora, structure of food and packaging of food were (of necessity) excluded from the study. However, all these factors will affect variability of log cell count in challenge tests (Francois et al. 2006b, Miled et al. 2011, Manios et al. 2011, Sant'Ana et al. 2013). Therefore, for future work, these additional factors might be considered in designing experiments to target more specific problems and in testing to what extent the model is valid and applicable.

CHAPTER 7: CONCLUSION

Research on bacterial lag is of considerable importance to the food industry because it provides information which will contribute to understanding the safety of foods and preparation of risk assessments. The lag phase depends on both the previous history of the cells and on inoculum size.

Cronobacter spp. are increasingly regarded as emerging opportunistic pathogens and are associated with illness among infants following consumption of PIF. Immuno-compromised adults and the elderly are also at risk. Apart from PIF, *Cronobacter* spp. can be recovered from a wide range of foodsand are present in the general environment.

Given the ubiquitous nature of *Cronobacter* spp., the organism may gain access to foods or to reconstituted PIF, in either a non-heat stressed or sublethally heat stressed condition, generally at a low concentration. If the cells are able to multiply in the food to an infective level, food borne disease may be triggered. It is therefore important to determine the potential for untreated and sublethally heat stressed individual *Cronobacter* spp. cells to recover and grow.

The present study investigated the distribution of individual lag times of untreated and heat stressed *Cronobacter* spp. at various recovery temperatures. Assuming that the individual lag follows a Gamma distribution with a fixed shape parameter, the effect of recovery temperature onthe individual cellular lag of untreated and sublethally heat stressed *Cronobacter* spp. was modelled. Both models were validated in TSB and their applicability to food (first milk) was tested. In order to select an appropriate strain for study, the minimum growth temperature and the thermal resistance of *Cronobacter* spp. to mild heat treatment was studied. The strain *Cr. turicensis* 1211, which showed a relatively high injury

percentage at 49 ^{o}C, and a good ability to grow at 7 ^{o}C, was selected as the test organism in this project.

It can be concluded that the mean and SD of the distribution of the individual cellular lag of *Cr. turicensis* 1211 increased when the recovery temperature decreased and also after sublethal heat treatment. For both untreated and heat stressed individual lag times of *Cr. turicensis* 1211 cells, the natural logarithm of the shift parameter (T_{shift}) shows an exponential relationship with the recovery temperature, while the natural logarithm of the scale parameter (θ) of the Gamma distribution was linearly related to recovery temperature.

Validation of the models in TSB showed that, in most cases, both models underestimated the measured growth of individual cellsof *Cr. turicensis* 1211 in challenge tests. It indicated that the assumptions made during model development and validation may not be valid. This may be due to the limited data obtained during construction and validation of the model, or other uncertainties involved during transition from incubation in the Bioscreen to Eppendorf tubes. Therefore, more data and more conditions (combinations of heat treatment-recovery temperature) could decrease the uncertainty associated with the simulations.

Evaluation of the applicability of both models in infant first milk showed that for the untreated cell model at high temperature (22 ^{o}C), the predictions of the model showed good agreement with the observed growth of individual *Cr. turicensis* 1211 cells. However, at lower temperatures (7 and 12 ^{o}C), the untreated cell model gave a slight underestimation. This may be because of invalid assumptions or limited data as mentioned above or some unknown uncertainty which affects growth of untreated individual cells of *Cr. turicensis* 1211 in first milk at low temperatures that was not taken into consideration. In addition, it highlighted the importance of validating the model throughout the whole temperature range. However, predictions from the heat stressed model seem toshow good agreementwith the results from challenge tests.

Therefore, the heat stressed cell model developed in TSB may be used for quantitative risk assessment for *Cronobacter* spp. in infant milk.

This study increases the knowledge of certain *Cronobacter* spp. in terms of tolerance to mild heat treatment and growth profile at 7 ^{o}C (refrigeration temperature). Moreover, it contributes to the understanding and modelling of the variability of individual cell lag times of untreated and sublethally heat stressed *Cr. turicensis* 1211 under normal and low temperature stress conditions.

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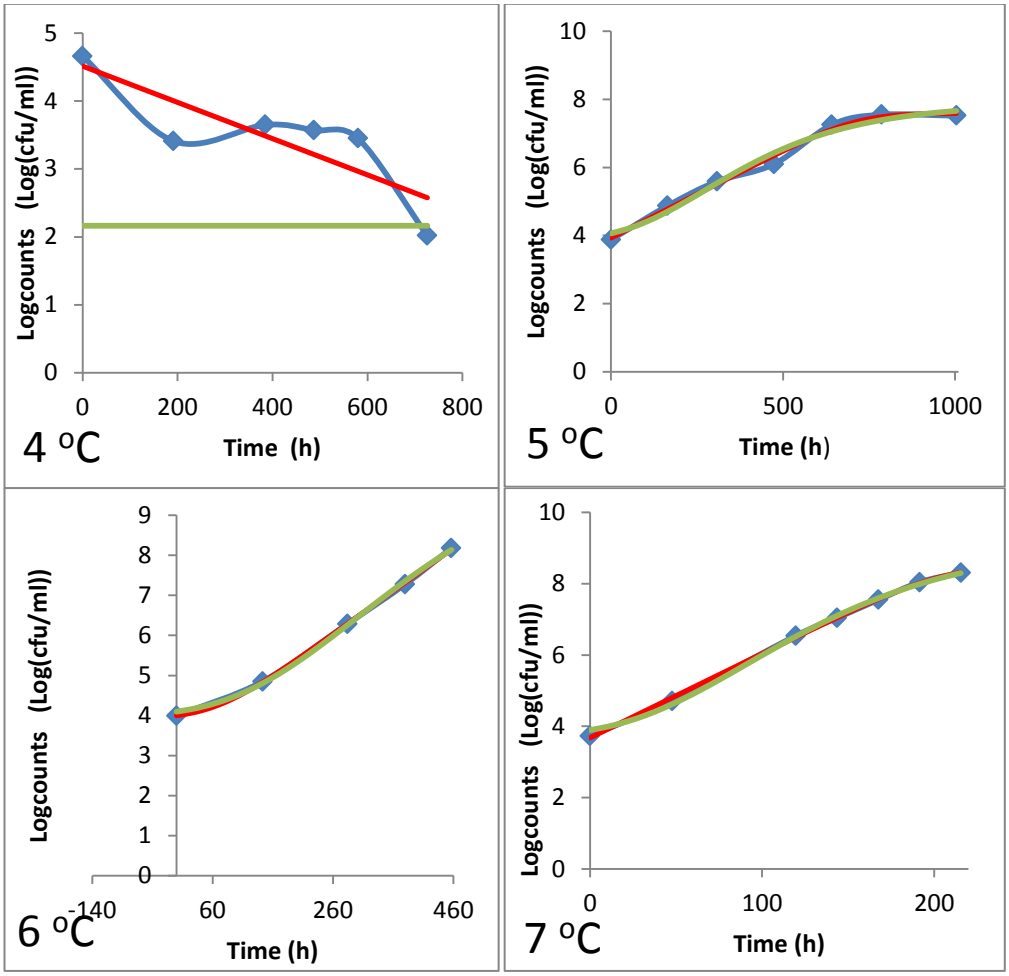
Appendix 2.1: Comparison of the Baranyi model and the modified Gompertz model



Comparison of the fitting of the Baranyi (red line) and the modified Gomperatz model (green line) developed by Zwietering et al. (1990) applied to the growth/death data (blue diamonds) of *Cr. turicensis* 1211 at 4, 5, 6 and 7 °C

Root mean square values achieved with the Baranyi and modified Gompertz models (Zwietering et al. 1990)

Temperature	Baranyi model	Modified Gompertz model
4 ^{o}C	0.42	1.51
5 ^{o}C	0.13	0.19
6 ^{o}C	0.02	0.07
7 ^{o}C	0.05	0.12

The root mean square (RMS) value was determined as described by Buchanan et al. (1997), with the following function.

$$RMS = \sqrt{\frac{\sum_{i}^{n}\left(y_{i\ predicted} - y_{i\ observed}\right)^{2}}{n}}$$

Where $y_{i\ predicted}$ is the predicted log cell concentration, $y_{i\ observed}$ is the observed log cell concentration and n is the number of data points.

Appendix 4.1: Development of the dilution method for untreated and heat stressed *Cr. turicensis* 1211 culture to get a single cell

Develop the dilution method for untreated *Cr. turicensis* 1211

	30ml untreated culture (cfu/ml)	10 f	10 f	10 f	10 f	10 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	Estimated inoculated cell number 50 µl Enumeration results *	Estimated inoculated cell number microtitre plate 22 °C 1week (ρ) *
1	1548816619	2E+08	2E+07	2E+06	2E+05		77441	38720	19360.2	9680.1	4840	2420	1210	605	303	151.3	75.6	37.8	1.88	1.37
2	1621810097	2E+08	2E+07	2E+06	2E+05		81091	40545	20272.6	10136	5068	2534	1267	633.5	317	158.4	79.2	39.6	2.13	2.44
3	1513561248	2E+08	2E+07	2E+06	2E+05		75678	37839	18919.5	9459.8	4730	2365	1182	591.2	296	147.8	73.9	37	1.38	1.49
4	1905460718	2E+08	2E+07	2E+06	2E+05		95273	47637	23818.3	11909	5955	2977	1489	744.3	372	186.1	93	46.5	1.00	1.75
5	1862087137	2E+08	2E+07	2E+06	2E+05		93104	46552	23276.1	11638	5819	2910	1455	727.4	364	181.8	90.9	45.5	3.00	3.33
6	1621810097	2E+08	2E+07	2E+06	2E+05		81091	40545	20272.6	10136	5068	2534	1267	633.5	317	158.4	79.2	39.6	2.50	2.51
7	1862087137	2E+08	2E+07	2E+06	2E+05		93104	46552	23276.1	11638	5819	2910	1455	727.4	364	181.8	90.9	45.5	2.00	2.23
8	2344228815	2E+08	2E+07	2E+06	2E+05		1E+05	58606	29302.9	14651	7326	3663	1831	915.7	458	228.9	114	57.2	2.38	2.98
9	2454708916	2E+08	2E+07	2E+06	2E+05		1E+05	61368	30683.9	15342	7671	3835	1918	958.9	479	239.7	120	59.9	2.88	2.71
10	1905460718	2E+08	2E+07	2E+06	2E+05	19055	9527	4764	2381.83	1190.9	595	297.7	149	74.43	37.2				1.88	1.09
11	1698243652	2E+08	2E+07	2E+06	2E+05	16982	8491	4246	2122.8	1061.4	531	265.4	133	66.34	33.2				1.63	1.78
12	1548816619	2E+08	2E+07	2E+06	2E+05	15488	7744	3872	1936.02	968.01	484	242	121	60.5	30.3				1.00	1.07
13	1621810097	2E+08	2E+07	2E+06	2E+05	16218	8109	4055	2027.26	1013.6	507	253.4	127	63.35	26.4	(5/12 fold)			1.00	1.78
14	1659586907	2E+08	2E+07	2E+06	2E+05	16596	8298	4149	2074.48	1037.2	519	259.3	130	64.83	27	(5/12 fold)			1.00	1.07

10f, 2f: represent: 10 fold and 2 fold; *: no significant difference observed between the average cell number enumerated from 50ul of final dilution (8 replicates) and from microtitre plate (ρ) (t-test, p>0.05).

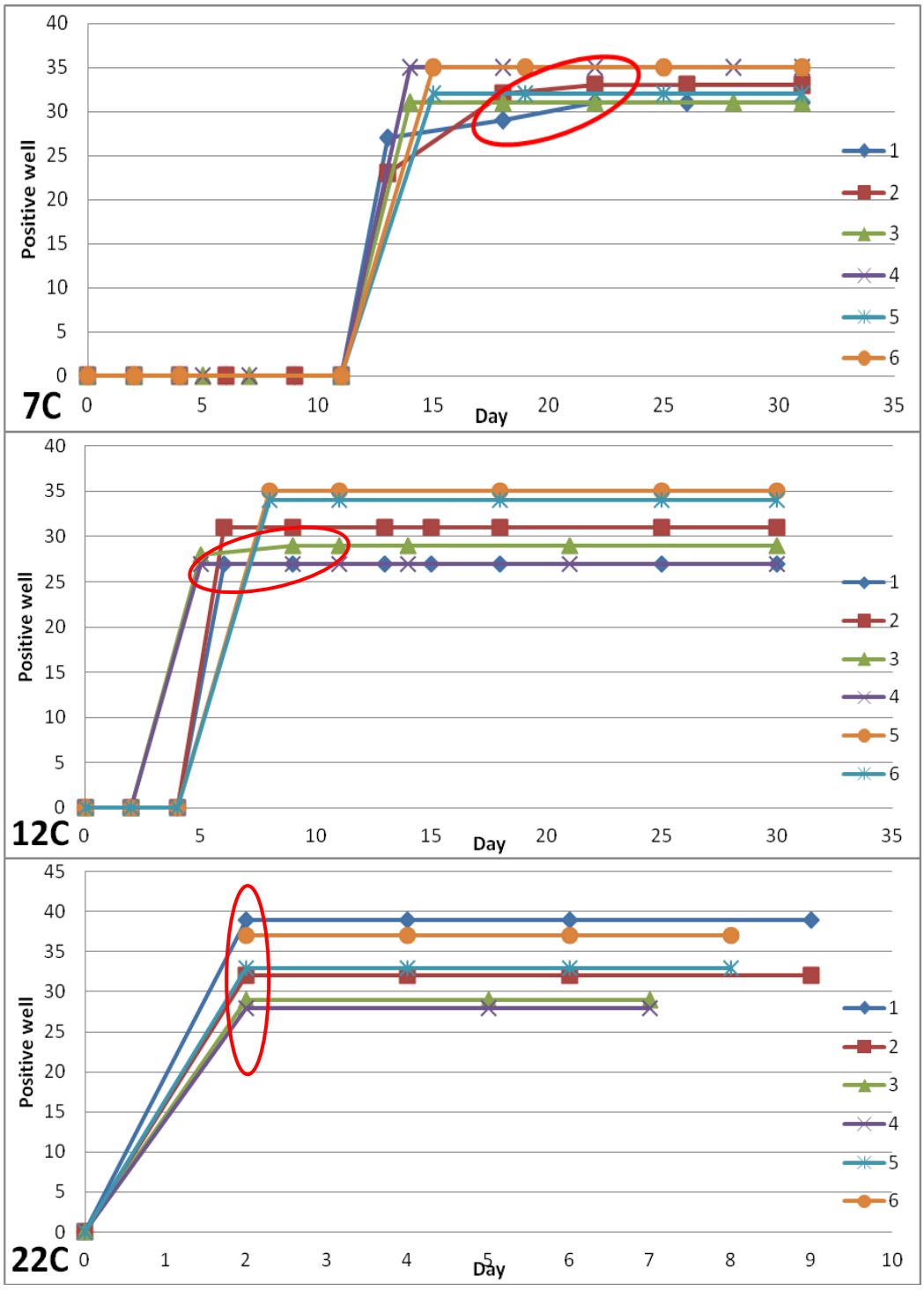
Develop the dilution method for sublethal heat stressed *Cr. turicensis* 1211

	10ml heat stressed culture (cfu/ml)	10 f	10 f	10 f	10 f	2 f	2 f	2 f	2 f	2 f	2 f	2 f	4/12 or 5/12 fold	Estimated inoculated cell number	
														50 µl enumeration results*	from microtitre plate 22 °C 2 weeks (ρ)*
1	102500000	1E+07	1E+06	102500	10250	5125	2563	1281.25	640.6	320.31	160.16	80.08	40.04	4.00	3.89
2	95000000	1E+07	1E+06	95000	9500	4750	2375	1187.50	593.7	296.88	148.44	74.22	37.11	3.25	3.89
3	62500000	6E+06	6E+05	62500	6250	3125	1563	781.25	390.6	195.31	97.66	48.83	24.41	0.88	1.17
4	77500000	8E+06	8E+05	77500	7750	3875	1938	968.75	484.3	242.19	121.09	60.55	30.27	0.75	1.29
5	92500000	9E+06	9E+05	92500	9250	4625	2313	1156.25	578.1	289.06	144.53	72.27	36.13	0.88	0.99
6	70000000	7E+06	7E+05	70000	7000	3500	1750	875.00	437.5	218.75	109.38	54.69	27.34	1.38	1.49
7	77500000	8E+06	8E+05	77500	7750	3875	1938	968.75	484.3	242.19	121.09	60.55	30.27	12.61	1.5	1.33
8	122500000	1E+07	1E+06	122500	12250	6125	3063	1531.25	765.6	382.81	191.41	95.70	47.85	19.94	1.5	2.02
9	90000000	9E+06	9E+05	90000	9000	4500	2250	1125.00	562.5	281.25	140.63	70.31	35.16	14.65	0.75	1.52
10	95000000	1E+07	1E+06	95000	9500	4750	2375	1187.50	593.7	296.88	148.44	74.22	37.11	15.46	1.75	1.24
11	135000000	1E+07	1E+06	135000	13500	6750	3375	1687.50	843.7	421.88	210.94	105.47	52.73	17.58	1.5	1.81
12	107500000	1E+07	1E+06	107500	10750	5375	2688	1343.75	671.8	335.94	167.97	83.98	41.99	14.00	1	1.12

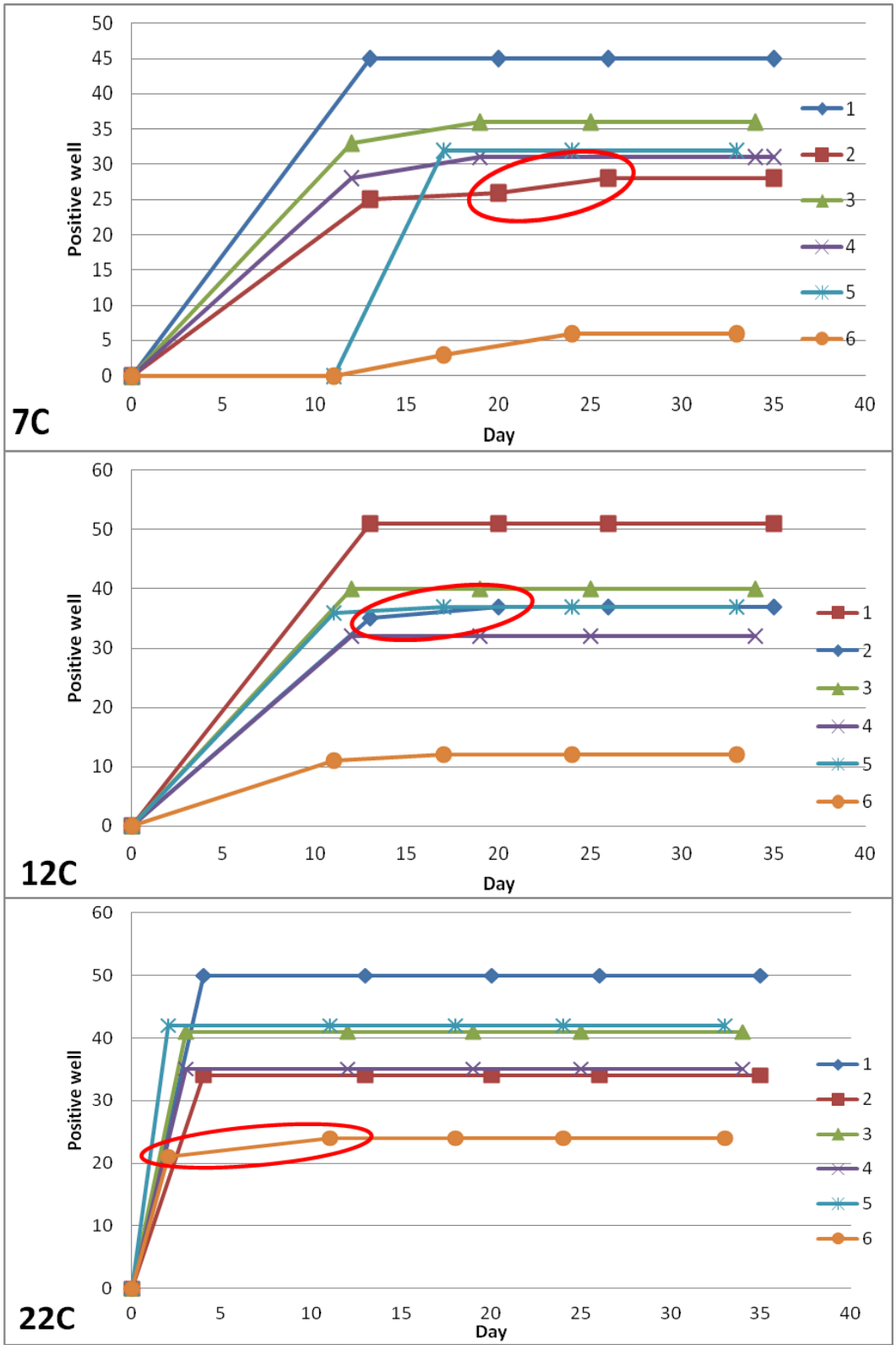
10f, 2f: represent: 10 fold and 2 fold; *: no significant difference observed between the average cell number enumerated from 50ul of final dilution (8 replicates) and from microtitre plate (ρ) (t-test, p>0.05; Mann-Whitney U test, p>0.05).

Appendix 4.2: The increase in positive wells of untreated and heat stressed *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C

This part is carried out based on method 4.2.3.



Growth of untreated individual cells of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C. Lines 1 to 6 represent six replicates at each temperature. The red ring indicates that this was the last stage between 2 points on the graph where growth was observed, suggesting a guideline for the incubation period using the Bioscreen.



Growth of heat stressed individual cell of *Cr. turicensis* 1211 at 7, 12 and 22 ^{o}C. Lines 1 to 6 represent six replicates at each temperature. The red ring indicates that this was the last stage between 2 points on the graph where growth was observed, suggesting a guideline for the incubation period using the Bioscreen.

Appendix 4.3: The principle of the method to identify the T_{shift} and θ of the individual lag distribution.

The main principle of this method is based on the S_α, the sum of the physiological states of the cells in a well. For one experiment, the mean and variance of S_α experimentally obtained should EQUAL the mean and variance of S_α theoretically obtained.

- $$S_\alpha = \sum_{i=1}^{N_0} \alpha_i(1) = N_0 \times \alpha(N_0) = N_0 \times e^{-\mu' Lag(N_0)} = N_{det} \times e^{-\mu' T_{det}} \quad (4.5)$$

For an experiment, if j wells show growth, S_α, sum of the physiological states in each well could be calculated based on the equation $S_\alpha = N_{det} \times e^{-\mu' T_{det}}$ (4.5). $E(S_\alpha)_{experiment}$ is the mean of the S_α of j wells $\frac{\sum_{j=1}^{n}\left(S_\alpha^j\right)}{n}$ (4.6)

$$E\left(e^{-\mu'\tau}\right) = (1+\mu'\theta)^{-\beta} \quad (4.13)$$

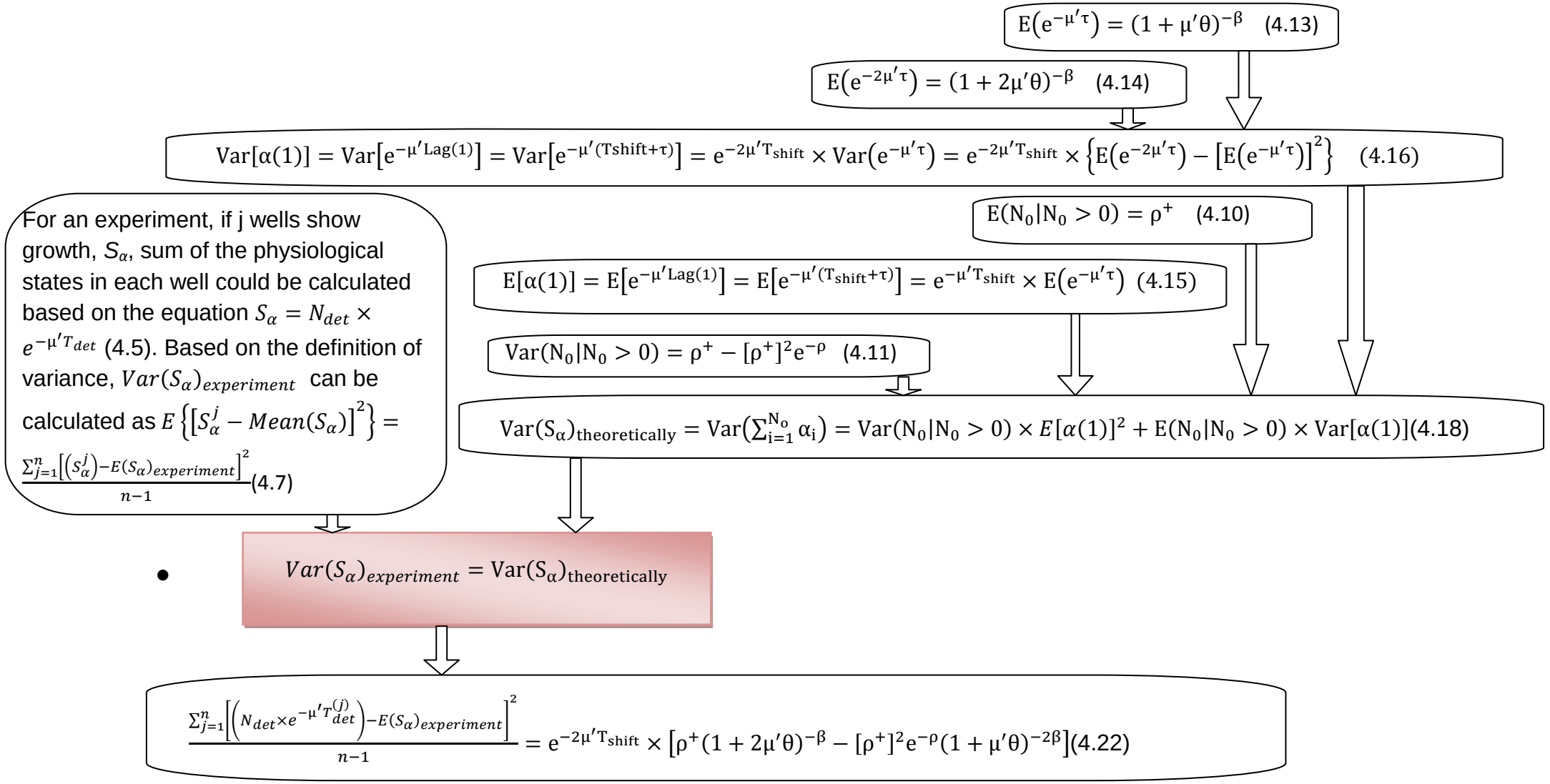
$$E[\alpha(1)] = E\left[e^{-\mu' Lag(1)}\right] = E\left[e^{-\mu'(T_{shift}+\tau)}\right] = e^{-\mu' T_{shift}} \times E\left(e^{-\mu'\tau}\right) \quad (4.15)$$

$$E(N_o | N_o > 0) = \rho^+ \quad (4.10)$$

$$E(S_\alpha)_{theoretically} = E\left[\sum_{i=1}^{N_0} \alpha_i(1)\right] = E(N_0 | N_0 > 0) \times E[\alpha(1)] \quad (4.17)$$

- $$E(S_\alpha)_{experiment} = E(S_\alpha)_{theoretically}$$

$$\frac{\sum_{j=1}^{n}\left(N_{det} \times e^{-\mu T_{det}^{(j)}}\right)}{n} = \rho^+ e^{-\mu' T_{shift}} (1+\mu\theta)^{-\beta} \quad (4.21)$$


$E(e^{-\mu'\tau}) = (1+\mu'\theta)^{-\beta}$ (4.13)
$E(e^{-2\mu'\tau}) = (1+2\mu'\theta)^{-\beta}$ (4.14)
$Var[\alpha(1)] = Var[e^{-\mu' Lag(1)}] = Var[e^{-\mu'(Tshift+\tau)}] = e^{-2\mu' T_{shift}} \times Var(e^{-\mu'\tau}) = e^{-2\mu' T_{shift}} \times \{E(e^{-2\mu'\tau}) - [E(e^{-\mu'\tau})]^2\}$ (4.16)
$E(N_0|N_0>0) = \rho^+$ (4.10)
$E[\alpha(1)] = E[e^{-\mu' Lag(1)}] = E[e^{-\mu'(T_{shift}+\tau)}] = e^{-\mu' T_{shift}} \times E(e^{-\mu'\tau})$ (4.15)
$Var(N_0|N_0>0) = \rho^+ - [\rho^+]^2 e^{-\rho}$ (4.11)
$Var(S_\alpha)_{theoretically} = Var(\sum_{i=1}^{N_o} \alpha_i) = Var(N_0|N_0>0) \times E[\alpha(1)]^2 + E(N_0|N_0>0) \times Var[\alpha(1)]$(4.18)
For an experiment, if j wells show growth, S_α, sum of the physiological states in each well could be calculated based on the equation $S_\alpha = N_{det} \times e^{-\mu' T_{det}}$ (4.5). Based on the definition of variance, $Var(S_\alpha)_{experiment}$ can be calculated as $E\left\{\left[S_\alpha^j - Mean(S_\alpha)\right]^2\right\} = \frac{\sum_{j=1}^{n}\left[\left(S_\alpha^j\right) - E(S_\alpha)_{experiment}\right]^2}{n-1}$(4.7)
$Var(S_\alpha)_{experiment} = Var(S_\alpha)_{theoretically}$
$\frac{\sum_{j=1}^{n}\left[\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right) - E(S_\alpha)_{experiment}\right]^2}{n-1} = e^{-2\mu' T_{shift}} \times \left[\rho^+(1+2\mu'\theta)^{-\beta} - [\rho^+]^2 e^{-\rho}(1+\mu'\theta)^{-2\beta}\right]$(4.22)

Appendix 4.4: The procedure of function deduction.

4.2.8.1 The physiological state

$$S_\alpha = \sum_{i=1}^{N_0} \alpha_i(1) = N_0 \times \alpha(N_0) = N_0 \times e^{-\mu' Lag(N_0)} = N_0 \times e^{-\mu'\left(T_{det} - \frac{\ln N_{det} - \ln N_0}{\mu'}\right)} = N_0 \times e^{-\mu' T_{det} + \ln N_{det} - \ln N_0} = N_0 \times e^{-\mu' T_{det}} \times e^{Ln N_{det}} \times e^{\ln \frac{1}{N_0}} = N_{det} \times e^{-\mu' T_{det}} \quad (4.5)$$

4.2.8.2 Experimental estimation the mean and variance of S_α for one experiment

For one experiment, among 200 wells, only some wells show growth, some will not. Therefore, for an experiment, if j wells (from 1 to n) show growth, S_α^j, sum of the physiological states in j^{th} well could be calculated based on the equation $S_\alpha^j = N_{det} \times e^{-\mu' T_{det}^j}$ (4.5). The mean of S_α, and the variance of S_α for one experiment can be estimated with function (4.6) and (4.7).

$$E(S_\alpha)_{experiment} = \frac{\sum_{j=1}^{n} S_\alpha^j}{n} = \frac{\sum_{j=1}^{n}\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right)}{n} \quad (4.6)$$

$$Var(S_\alpha)_{experiment} = \frac{\sum_{j=1}^{n}\left[\left(S_\alpha^j\right) - E(S_\alpha)_{experiment}\right]^2}{n-1} = \frac{\sum_{j=1}^{n}\left[\left(N_{det} \times e^{-\mu' T_{det}^{(j)}}\right) - E(S_\alpha)_{experiment}\right]^2}{n-1} \quad (4.7)$$

Where j=1…..n numbers of the observation in one experiment.

4.2.8.3 Theoretical estimation the mean and variance of S_α for one experiment

If X follow the Poisson distribution truncated at zero, then, $E(N_0|N_0 > 0) = \frac{\rho}{1-e^{-\rho}}$, $E(N_0^2|N_0 > 0) = \frac{\rho(1+\rho)}{1-e^{-\rho}}$ (Haight 1967).

As growth only occurs in the wells that contain cell, the cell number in the positive well follows a zero truncated Poisson distribution. Therefore the mean and variance of the zero truncated Poisson distribution could be estimated as function 4.10, 4.11 (Métris et al. 2006).

$$E(N_0|N_0 > 0) = \rho^+ = \frac{\rho}{1-e^{-\rho}} \quad (4.10)$$

$$Var(N_0|N_0 > 0) = E(N_0^2|N_0 > 0) - [E(N_0|N_0 > 0)]^2 = \frac{1}{1-e^{-\rho}} \times (\rho + \rho^2) - \frac{1}{(1-e^{-\rho})^2} \times \rho^2 = \rho^+ - (\rho^+)^2 e^{-\rho} \quad (4.11)$$

4.2.8.3.3 Theoretical estimation the mean and square of $e^{-\mu'\tau}$

If X ~ Gamma (β, θ), β is the shape parameter, and θ is the scale parameter. The moment generation function of X could be written as $\mathbf{M_X(t) = E(e^{tX}) = \frac{1}{(1-\theta t)^{\beta}}}$, $\mathbf{t < \frac{1}{\theta}}$ (Virtual Laboratories in Probability and Statistics, no date, Kobayashi et al. 2012)

As τ follow the gamma distribution, then $E\left(e^{-\mu'\tau}\right)$and $E\left(e^{-2\mu'\tau}\right)$can beestimated (4.13, 4.14) (Baranyi et al. 2009).

$$E\left(e^{-\mu'\tau}\right) = \frac{1}{(1-\theta(-\mu'))^{\beta}} = (1+\mu'\theta)^{-\beta} \quad (4.13)$$

$$E\left(e^{-2\mu'\tau}\right) = \frac{1}{(1-\theta(-2\mu'))^{\beta}} = (1+2\mu'\theta)^{-\beta} \quad (4.14)$$

4.2.8.3.4 Theoretical estimation the mean and variance of $\alpha(1)$.

Based on equation 4.13 and 4.14, combined with equation 4.3 and 4.12, $E[\alpha(1)]$ and $Var[\alpha(1)]$can be estimated as follows (4.15, 4.16).

$$E[\alpha(1)] = E\left[e^{-\mu' Lag(1)}\right] = E\left[e^{-\mu'(T_{shift}+\tau)}\right] = E\left(e^{-\mu' T_{shift}} \times e^{-\mu'\tau}\right) = e^{-\mu' T_{shift}} \times E\left(e^{-\mu'\tau}\right) = e^{-\mu' T_{shift}} \times (1+\mu'\theta)^{-\beta} \quad (4.15)$$

$$\mathrm{Var}[\alpha(1)] = \mathrm{Var}\left[e^{-\mu'\mathrm{Lag}(1)}\right] = \mathrm{Var}\left[e^{-\mu'(\mathrm{Tshift}+\tau)}\right] = \mathrm{Var}\left(e^{-\mu'T_{shift}} \times e^{-\mu'\tau}\right) = e^{-2\mu'T_{shift}} \times \mathrm{Var}\left(e^{-\mu'\tau}\right) = e^{-2\mu'T_{shift}} \times \left\{E\left(e^{-2\mu'\tau}\right) - \left[E\left(e^{-\mu'\tau}\right)\right]^2\right\} = e^{-2\mu'T_{shift}} \times \left[\left(1+2\mu'\theta\right)^{-\beta} - \left(1+\mu'\theta\right)^{-2\beta}\right] \quad (4.16)$$

4.2.8.3.5 Relationship of the mean and variance of $\alpha(1)$ with the mean and variance of S_α for one experiment

If X_1, X_2, X_3….is a series of independent identically distributed random variables, and N is also a nonnegative integer-valued random variable, independent from the X_j, then for $S = \sum_{j=1}^{N} X_j$, $E(S) = E(X) \times E(N)$; $Var(S) = Var(N) \times [E(X)]^2 + E(N) \times Var(X)$ (Ross 2002, Métris et al. 2006).

$$\mathrm{Var}(S_\alpha)_{\text{theoretically}} = \mathrm{Var}\left(\sum_{i=1}^{N_o} \alpha_i\right) = \mathrm{Var}(N_0|N_0>0) \times E[\alpha(1)]^2 + E(N_0|N_0>0) \times \mathrm{Var}[\alpha(1)] \quad (4.18)$$

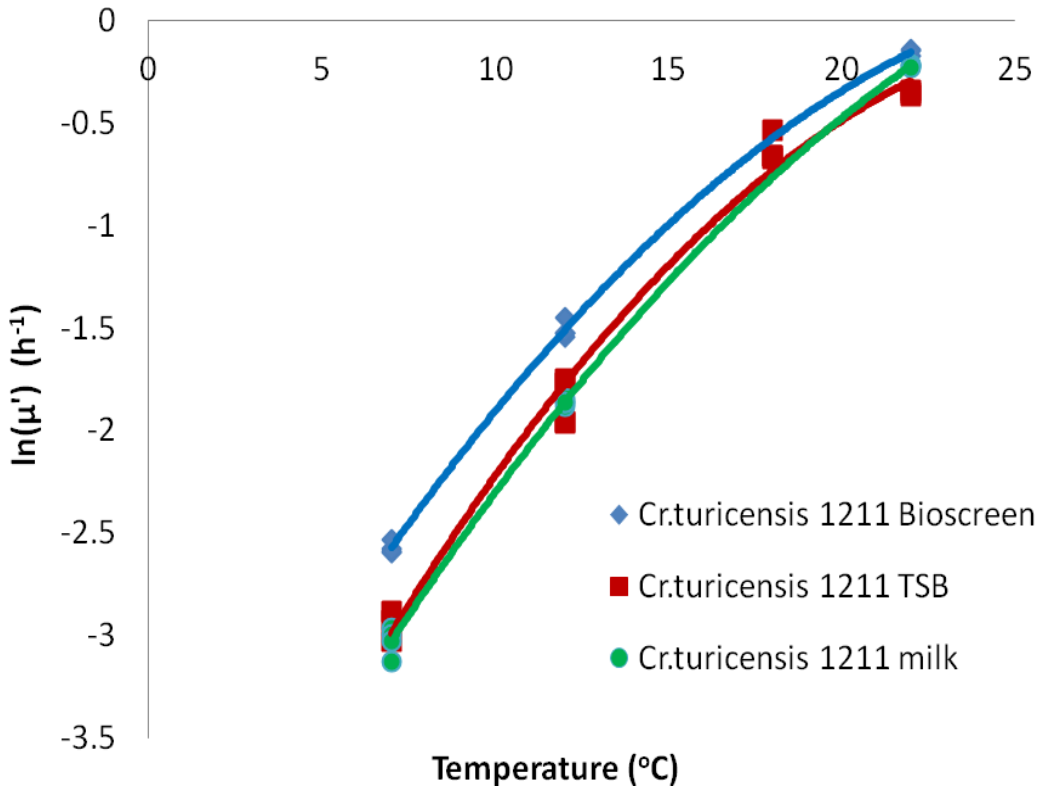
$$\mathrm{Var}(S_\alpha)_{\text{theoretically}} = \mathrm{Var}\left(\sum_{i=1}^{N_o} \alpha_i\right) = \mathrm{Var}(N_0|N_0>0) \times E[\alpha(1)]^2 + E(N_0|N_0>0) \times \mathrm{Var}[\alpha(1)] = [\rho^+ - (\rho^+)^2 e^{-\rho}] \times \left[e^{-\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-\beta}\right]^2 + \rho^+ \times e^{-2\mu'T_{shift}} \times \left[\left(1+2\mu'\theta\right)^{-\beta} - \left(1+\mu'\theta\right)^{-2\beta}\right] = [\rho^+ - (\rho^+)^2 e^{-\rho}] \times e^{-2\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-2\beta} + \rho^+ \times e^{-2\mu'T_{shift}} \times \left(1+2\mu'\theta\right)^{-\beta} - \rho^+ \times e^{-2\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-2\beta} = \cancel{\rho^+ \times e^{-2\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-2\beta}} - (\rho^+)^2 e^{-\rho} \times e^{-2\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-2\beta} + \rho^+ \times e^{-2\mu'T_{shift}} \times \left(1+2\mu'\theta\right)^{-\beta} \cancel{- \rho^+ \times e^{-2\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-2\beta}} = \rho^+ \times e^{-2\mu'T_{shift}} \times \left(1+2\mu'\theta\right)^{-\beta} - (\rho^+)^2 e^{-\rho} \times e^{-2\mu'T_{shift}} \times \left(1+\mu'\theta\right)^{-2\beta} = e^{-2\mu'T_{shift}} \times \left[\rho^+\left(1+2\mu'\theta\right)^{-\beta} - (\rho^+)^2 e^{-\rho}\left(1+\mu'\theta\right)^{-2\beta}\right] \quad (4.20)$$

Appendix 5.1

The specific growth rate (h^{-1}) of untreated cells of *Cr. turicensis* 1211 was estimated with the polynomial model and square root model and compared to the observed values. The 95% confidence limits of the predicted specific growth rates using both models, compared to the observed values, are shown.

	Temperature	Observed (mean±SD)	Polynomial model		Square root model	
			mean	95% confidence limit	mean	95% confidence limit
TSB	7 °C	0.053±0.004	0.050	0.035-0.072	0.045	0.011-0.105
	12 °C	0.157±0.018	0.170	0.124-0.234	0.182	0.110-0.278
	18 °C	0.532±0.003	0.481	0.352-0.656	0.469	0.349-0.621
	22 °C	0.703 ±0.007	0.745	0.525-1.059	0.734	0.568-0.934
First milk	7 °C	0.048 ±0.003	0.048	0.043-0.055	0.037	0.015-0.075
	12 °C	0.155 ±0.002	0.155	0.137-0.176	0.175	0.128-0.246
	22 °C	0.803±0.004	0.803	0.708-0.910	0.753	0.651-0.928

Appendix 5.2



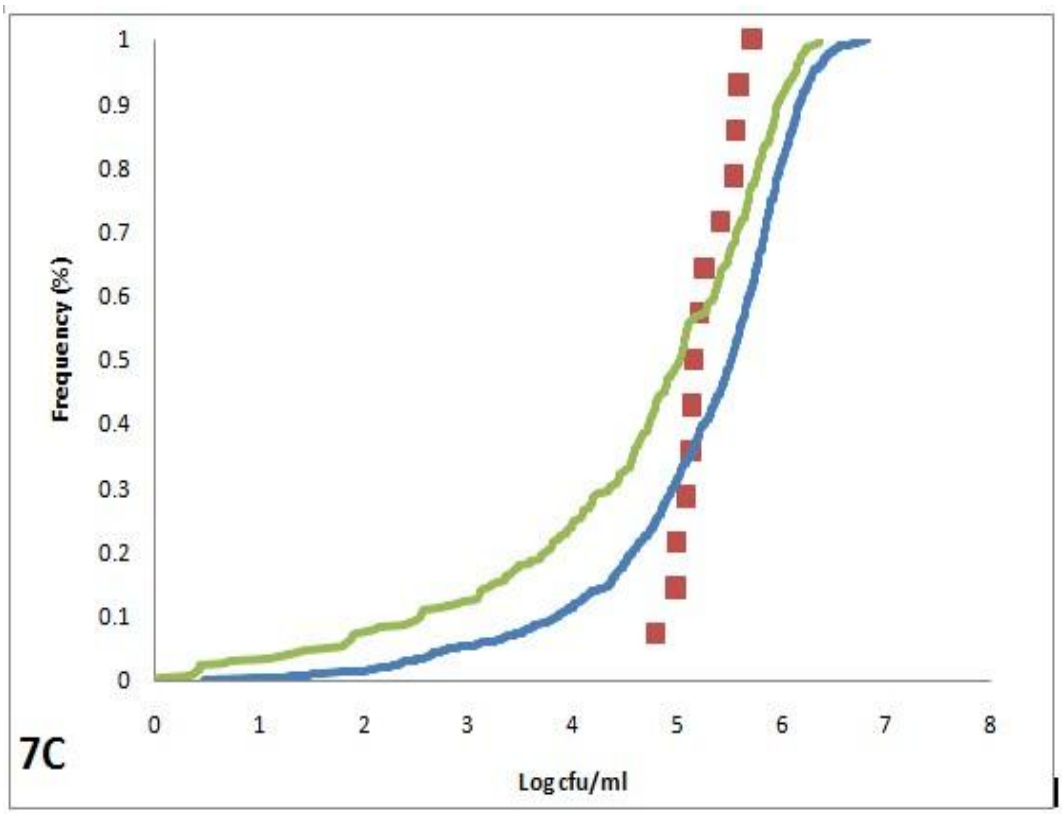
Comparison of the specific growth rate estimated by Bioscreen detection method with that in TSB and first milk in incubator.

Appendix 5.3

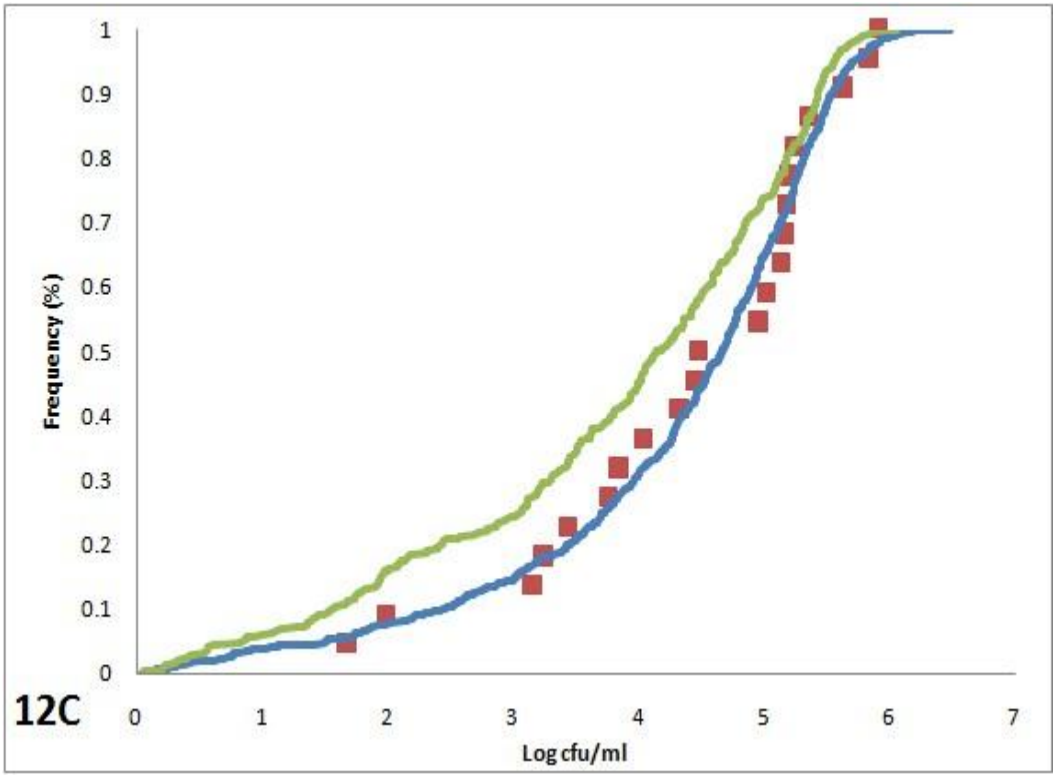
Comparison between simulation based on the growth of 50 parallel samples, simulation based on the real growth of *Cr. turicensis* 1211 and observed contamination level of *Cr. turicensis* 1211 which initiate growth from single heat stressed cell in milk at 7 ^{o}C and 12 ^{o}C

Temp	Cell number	Mini	Max	Mean	SD	P value of χ^2 test
7 ^{o}C	observed	4.80	5.72	5.26	0.27	
	Simulation (ρ=1.61)	0.23	7.09	5.12	1.10	0.29
	Simulation (ρ=0.33)	0.43	6.70	4.70	1.26	0.75
12 ^{o}C	observed	1.68	5.92	4.41	1.16	
	Simulation (ρ=1.61)	0.04	6.41	4.24	1.27	0.92
	Simulation (ρ=0.58)	0.01	6.16	4.00	1.37	0.19

Temp: Temperature



Comparison between simulation based on the growth of 50 parallel samples (blue line), simulation based on the real growth of *Cr. turicensis* 1211 (green line) and observed contamination level of *Cr. turicensis* 1211 (red square) which initiate growth from single heat stressed cell in milk at 7 ^{o}C.



Comparison between simulation based on the growth of 50 parallel samples (blue line), simulation based on the real growth of *Cr. turicensis* 1211(green line) and observed contamination level of *Cr. turicensis* 1211 (red square) which initiate growth from single heat stressed cell in milk at 12 ^{o}C.