CRANFIELD UNIVERSITY

IOANNIS MYTILINAIOS

MODELLING THE IMPACT OF MILD FOOD PROCESSING CONDITIONS ON THE MICROBIOLOGICAL SAFETY OF FOOD

CRANFIELD HEALTH

PhD THESIS
Academic Years: 2009 - 2013

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

There is significant interest by the food industry in applying milder processing conditions. A major area of research within predictive modelling has been the search for models which accurately predict the effect of combining multiple processes or hurdles. For a mild process, which has temperature as the major microbial injury step, the effect of the other combined hurdles in inhibiting growth of the injured organisms must be understood. The latter means that the inoculum size dependency of the time to growth must also be fully understood. This essentially links injury steps with the potential for growth.

Herein, we have been developing the use of optical density (O.D) for obtaining growth rates and lag times using multiple inocula rather than using the traditional methods which use one single inoculum. All analyses were performed in the Bioscreen analyser which measures O.D. The time to detection (TTD) was defined as the time needed for each inoculum to reach an O.D=0.2 and O.D was related to microbial numbers with simple calibration curves.

Several primary models were used to predict growth curves from O.D data and it was shown that the classic logistic, the Baranyi and the 3-phase linear model (3-PLM) were the most capable primary models of those examined while the *modified* Gompertz and *modified* logistic could not reproduce TTD data. Using the Malthusian approximation of the logistic model the effect of mild temperature shifts was studied. The data obtained showed that for mild temperature shifts, growth rates quickly changed to the new environment without the induction of lags. The growth of *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* was studied at 30°C and/or 37°C, in different NaCl concentrations, pH and their combinations. The classical 3-parameter logistic with lag model was rearranged to provide the theoretical foundation for the observed TTD and accurate growth rates and lag times could be estimated. As the conditions became more unfavourable, the lag time increased while the growth rate decreased. Also, the growth rate was found to be independent from the inoculum size; the inoculum size affected only the TTD. The Minimum

Inhibitory Concentration (MIC_{NaCl} and MIC_{pH}) was calculated using the Lambert and Pearson model (LPM) and also the Growth/No Growth (G/NG) interface was determined using combinations of NaCl and pH. These data were transformed in rate to detection (RTD) and fitted with a response surface model (RSM) which was subsequently compared with the Extended LPM (ELPM). The LPM and the ELPM could analyse results from individual and combined inhibitors, respectively. Following a mild thermal process a lag due to thermal injury was also induced, the magnitude of which was dependent on the organism and environmental conditions; the observed distribution of the lags appeared, in general, to follow the Log-normal distribution. After the lag period due to injury, growth recommenced at the rate dictated by the growth environment present. Traditional growth curves were constructed and compared with the data obtained from the Bioscreen under the same conditions. From the results obtained, it can be suggested that the increased lag times and growth rates obtained from the traditional plate counts compared with the values obtained from the Bioscreen microbiological analyser, might be an artifact of the plating method or may be due to the use of the *modified* Gompertz to study the growth.

In conclusion, O.D can be used to accurately determine growth parameters, to give a better understanding and quantify the G/NG interface and to examine a wealth of phenomena such as fluctuating temperatures and mild thermal treatments. The comparison between the traditional growth curves against the data obtained from the Bioscreen showed that the TTD method is a rapid, more accurate and cheaper method than the traditional plate count method which in combination with the models developed herein can offer new possibilities both to the research and the food industry.

Keywords:

Predictive modelling, food safety, optical density (O.D), time to detection (TTD), growth curve, logistic model, temperature shifts, mild heat injury.

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NOMENCLATURE

List of abbreviations

ADH	Arginine DiHydrolase
AIC	Akaike Information Criterion
API	Analytical Profile Index
ATCC	American Type Culture Collection
C. botulinum	*Clostridium botulinum*
CA	Codex Alimentarius
CAC	Codex Alimentarius Commission
CDC	Centers for Disease Control and prevention
cfu	Colony forming units
CIT	CITrate utilization
CPM	Cardinal Parameter Model
DNA	DeoxyriboNucleic Acid
E. coli	*Escherichia coli*
EC	European Commission
EffeConc	Effective concentration
EFSA	European Food Safety Authority
ELPM	Extended Lambert and Pearson Model
EU	European Union
FAO	Food and Agricultural Organisation
G/NG	Growth/No Growth
GEL	GELatinase
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
GT	Generation Time
H_2S	Hydrogen sulphide (production)
HACCP	Hazard Analysis Critical Control Point
HCl	Hydrochloric acid
ICMSF	International Commission on Microbiological Specifications Foods
IDT	Impedance Detection Time
KOH	Potassium Hydroxide
L. monocytogenes	*Listeria monocytogenes*
LDC	Lysine DeCarboxylase
LogNorm	LogNormal distribution
LPM	Lambert and Pearson Model
MIC	Minimum Inhibitory Concentration
min	Minutes
MPD	Maximum Population Density
MRA	Microbial Risk Assessment
MRV	Microbial Responses Viewer

NaCl	Sodium Chloride
O.D	Optical Density
ODC	Ornithine DeCarboxylase
pH	Potential Hydrogen
QM	Quality Management
QMRA	Quantitative Microbial Risk Assessment
RMSE	Root Mean Square Error
RNA	RiboNucleic Acid
RRTD	Relative Rate To Detection
RSM	Response Surface Model
RTD	Rate To Detection
S. Typhimurium	*Salmonella* Typhimurium
St. aureus	*Staphylococcus aureus*
SD	Standard deviation
sec	Seconds
SS	Sum of squares
SSO	Specific Spoilage Microorganisms
TQM	Total Quality Management
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSBYE	Tryptone Soya Broth Yeast Extract
TSYEA	Tryptic Soy Yeast Extract Agar
TTD	Time To Detection
UGPM	Unified Growth Prediction Model
UK	United Kingdom
URE	UREase
USA	United States of America
VP	Acetoin production (Voges Proskauer)
WHO	World Health Organisation

List of symbols

a	Shape parameter
a_w	Water activity
b	Scale parameter
c	Intercept
CO_2	Carbon dioxide
E_h	Redox potential
$f_{(var)}$	Function of independent variables
Ln	Natural logarithm
Log	Logarithm
M	Maximum population density

m	Gradient
ml	Millilitres
mu	Growth rate
N	Spesific population level
N_D	Detection value
nm	Nanomitres
N_o	Initial inoculum level
O_2	Oxygen
^oC	Degrees celcious
T	Temperature
T_{min}	Minimum temperature for growth
t	Time
λ	Lag time
μ	Population mean
μ	Spesific growth rate
μl	Microlitres
μ_{max}	Maximum specific growth rate
σ	Standard deviation
σ^2	Variance

1 Background

1.1 Microorganisms in food and foodborne disease

1.1.1 Microorganisms and food

Most of the foods we consume cannot be sterile but have a natural flora and can get a transient flora from their environment. In particular, the microorganisms present come from the natural flora of the raw material and a transient flora which derives from harvesting or slaughter, processing, storage and distribution (Adams and Moss, 2008). Important microbial groups in food consist of bacteria, moulds, yeasts and viruses. In some cases microorganisms in food can cause spoilage, food poisoning (foodborne illness) or can transform the food properties in a beneficial way (e.g. food fermentation). Jay *et al*. (2005) have mentioned some of the more significant dates and events in the history of food preservation, food spoilage and food poisoning in the USA.

Food spoilage is an ecological phenomenon and can be defined as any symptom or group of symptoms that occur with changes in odour, the smell (aroma) and the general appearance of the food by microbial activity (Gill, 1986). In other words, food spoilage is the deterioration in the physical, chemical and/or sensory properties of the food resulting in reduction of food quality. The spoilage of the food can be caused by enzymatic (e.g. oxidation) and/or microbial activity. On the other hand food poisoning is any illness caused by bacterial, chemical or biological contamination of food and is related to food safety. The most common cause of food poisoning is cross-contamination which is defined as the transfer of pathogens between food, surfaces and equipment. Food quality refers to the sum of the organoleptic characteristics (properties) of a food which makes it acceptable to consumers while safe food is food that is free of any physical, chemical or biological hazards.

In 1995, the Food and Agricultural Organisation/World Health Organisation (FAO/WHO) defined a foodborne hazard as any biological, chemical or physical factor / property of a food, which can have adverse effects on the

health of the consumer when consumed. The foodborne hazards can be classified as physical (such as wood, stones, metal, plastic), chemical (such as pesticides, insecticides) and biological (bacteria, yeasts, molds, parasites, viruses) hazards.

1.1.2 Foodborne disease

1.1.2.1 Why foodborne disease is important

Foodborne disease is a frequent and serious threat to public health all around the world and it has been defined by the WHO as “any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water”. Foodborne diseases have also been described by FAO/WHO as “a large and growing public health problem”. Griffith (2010) stated that “most countries with systems for reporting foodborne diseases have documented significant increases”. As a result food safety became a greater political, scientific and societal concern (Knowels, 2007; Scholliers, 2008). Most western European countries have suffered at least one major foodborne illness outbreak and the problem seems to be increasing globally (Knowels, 2007). The most recent example was in June 2011, which was an outbreak of an *E. coli* strain (*E.coli* O104:H4) in Germany and France and it was linked to raw sprouted seeds which infected 4,178 people and killed 49. It has been estimated that a new foodborne pathogen is discovered every 16 months (Tauxe, 2009). Often, foodborne illnesses appear to be mild with acute gastrointestinal symptoms such as diarrhoea and vomiting. Sometimes foodborne diseases can be more serious and life-threatening, particularly for young children and elderly people, with sensitive immune system.

Some reported notification zoonoses rates in confirmed human cases in Europe in 2008 are summarised in Figure 1-1. Zoonoses and zoonotic agents have been defined by EFSA (2012) as “any disease and/or infection which is naturally transmissible directly or indirectly between animals and humans (Dir. 2003/99/EC)” and “any virus, bacteria, fungus, parasite or other biological entity which is likely to cause a zoonosis (Dir. 2003/99/EC)”, respectively.

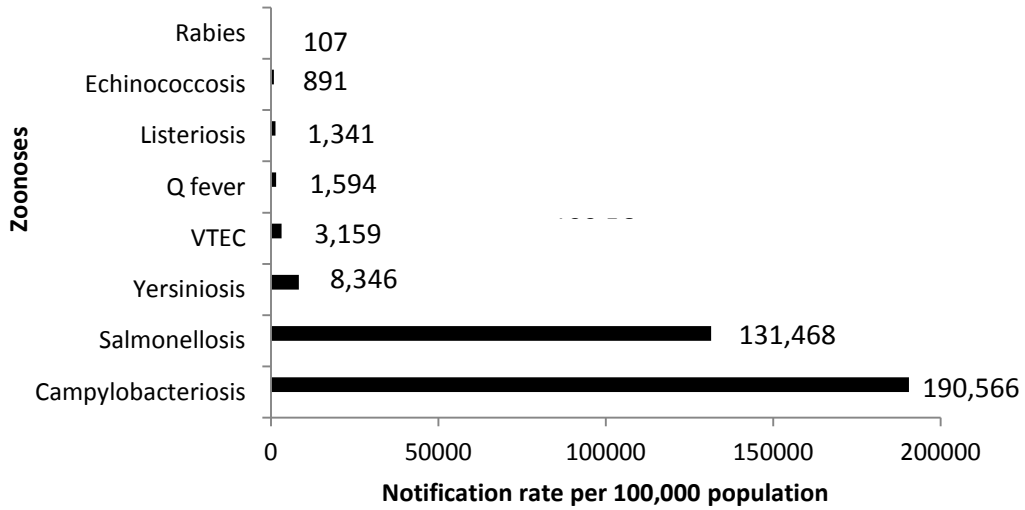


Figure 1-1 Reported notification zoonoses rates in confirmed human cases in the European Union, 2008 (EFSA, 2010)

In 2010, information on the occurrence of zoonoses, zoonotic agents and foodborne outbreaks were submitted by 27 Members States to the European Commission (EC) and the European Food Safety Authority (EFSA). Estimates from the EFSA (2012) reported a total of 5,262 foodborne outbreaks, causing 43,473 human cases, 4,695 hospitalisations and 25 deaths. *Salmonella*, *Campylobacter*, bacterial toxins and viruses caused most of the reported outbreaks. The number of salmonellosis cases in humans decreased by 8.8% compared with 2009 and followed a decreasing trend for 6 consecutive years in the European Union (EU). In foodstuff, it was more often detected in fresh broiler and turkey meat. Human campylobacteriosis has followed an increasing 5-year trend in the EU since 2006, with 212,064 confirmed cases. Human listeriosis decreased slightly with 1,601 cases being reported. A high fatality rate of 17% was reported among the cases as in previous years. A total of 4,000 cases caused by verotoxigenic *Escherichia coli* were reported in 2010 in the EU and showed an increased trend since 2008.

The Foodborne Diseases Active Surveillance Network (FoodNet) of Centers for Disease Control (CDC's) Emerging Infections Program collects data from 10 states in the USA on diseases caused by enteric pathogens commonly transmitted through food. In 2008, the estimated incidence of infections caused by *Campylobacter, Cryptosporidium, Cyclospora, Listeria*, Shiga toxin-

producing *E. coli* (STEC) O157*, Salmonella, Shigella, Vibrio,* and *Yersinia* did not change significantly when compared with the preceding 3 years. For most infections, incidence was highest among children aged less than 4 years, whereas the percentage of people hospitalized and the case fatality rate were highest among people aged over 50 years. During 2008, 1,034 foodborne disease outbreaks were reported, which resulted in 23,152 cases of illness, 1,276 hospitalizations and 22 deaths. In 2008, a total of 18,499 laboratory-confirmed cases of infection in FoodNet surveillance areas were identified. Some reported notification zoonoses rates in confirmed human cases in the USA in 2008 are summarised in Figure 1-2.

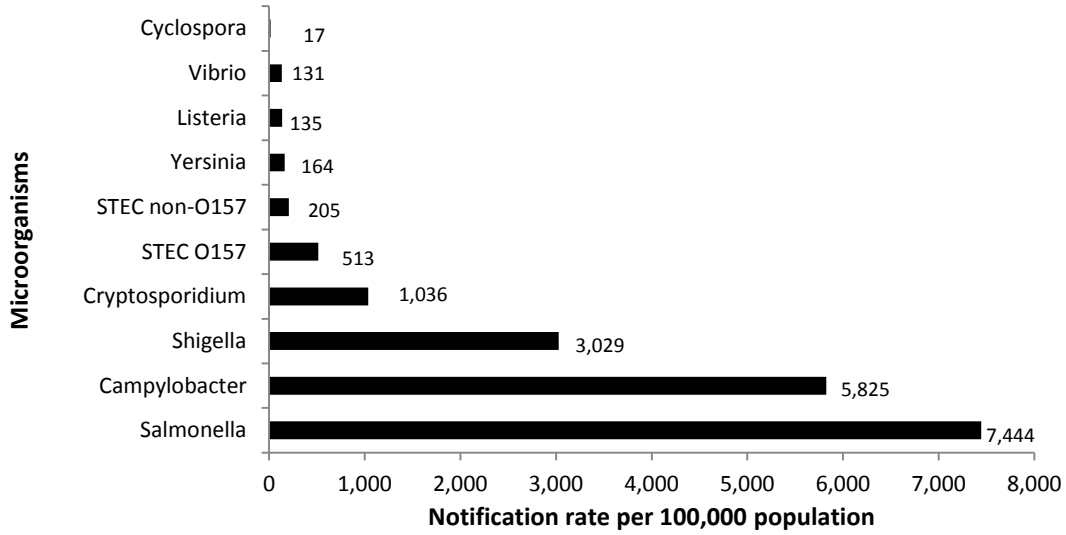


Figure 1-2 Foodborne infections per 100,000 population as reported in the USA (2008)

The CDC estimated that each year 1 in 6 Americans or in other words 17 percent of the Americans (approximately 48 million people) become ill, 128,000 are hospitalized and 3,000 die from foodborne illnesses (CDC, 2011). Also, CDC findings from 2011 showed that reducing foodborne diseases by 10% would prevent 5 million Americans from getting ill.

Apart from human suffering, foodborne disease can be costly as well. The cost of foodborne disease in the developed world can be huge for society, the food industry and food retailers. For less developed countries the consequences of foodborne diseases are even more serious (Adams and

Moss, 2008). Ranking the factors that contribute to outbreaks of foodborne diseases can indicate trends and also differences in the various foodborne pathogens reflecting their association with raw material and physiological properties. Statistics and data indicating the trends in foodborne gastrointestinal infections are very important to monitor foodborne disease but are limited to a few industrialized countries and also there are countries that have no system to collect and report these types of data. The spread of foodborne diseases remains largely unknown and this is not only a problem of the underdeveloped world. Increased awareness of the effects of food hazards on human health and the increasing importance and rapid growth of world trade have prompted regulatory officials and international organisations to consider new and improved strategies to reduce the health risks associated with pathogenic microorganisms in foods. With all these improvements a downward trend in foodborne diseases would be expected (Newell *et al.*, 2010). However, evidence for such a trend is limited. Foodborne pathogens are not static and even well known pathogens can evolve and create new public health challenges. There are also, several unknown foodborne pathogens that are constantly emerging (Newell *et al*., 2010). Food safety is a paramount factor in food quality and it has to do with the consumer's protection. The latter means that food safety is related to food production which will not cause harm to the consumer. It is a legal obligation of the manufacturer and the public authorities and an essential requirement of the consumer. Food safety is a dynamic situation influenced by multiple factors. The complexity of the global food market means that the control of foodborne disease is a joint responsibility and requires action at all levels from the individual to international groups, and at all parts of the supply chain from farm to fork.

1.1.2.2 Trends in foodborne disease

McMeekin and Ross (2002) stated that the changing trends which influence the increased incidence of foodborne diseases in the last 25 years can be categorized as social, demographic, behavioural and technological changes. In particular, the human population continues to grow and according to the current growth rate the global population will reach 9.1 billion by 2050 (United

Nations, 2005). This leads to an ageing population with higher proportions of individuals with a sensitive immune system. Also, the globalization of the food industry may affect the incidence of foodborne disease. The global market for example in fruits, vegetables and ethnic foods can have their origin in countries with inappropriate safety procedures (Newell *et al*., 2010). Moreover, the most recent consumer trend is the demand for more natural, less processed and preserved food. This has resulted in an increase in consumption of fresh fruits and vegetables and the number of outbreaks associated with these types of foods has also increased (Tauxe, 1997). In UK between December 2010 and July 2011, 250 cases of gastrointestinal illnesses were caused by an unusual starin of *E. coli* known as Phage Type 8 (PT8) and the outbreak was associated with soil on vegetables (raw loose leeks and potatoes). This outbreak was not related to the outbreaks in Germany or France earlier this year which were caused by a different strain of *E. coli* called O104 but it was also linked to vegetables (raw sprouted seeds). Another trend which is very common is the increase in eating away from home. This places greater importance on the safe operation of catering establishments for the control of foodborne disease.

During the last century, international travel has also increased. Travellers may become infected by foodborne pathogens that are uncommon in their nation and there is a possibility of transmitting the pathogen further when they return home. International travel is also a vehicle for an increasing demand for international foods in local markets, and this in turn fuels the international trade in foods. Immigration is another factor for the epidemiology of foodborne disease, as some reports of foodborne illnesses involve transmission through foods consumed primarily by immigrant groups. Changes in technology within the food industry (e.g. minimal technologies for food preservation) can also affect the incidence of foodborne disease (McMeekin and Ross, 2002). Tauxe (1997) demonstrated the effect of such changes in the emergence of foodborne pathogens since the 1970s.

1.1.3 Sources of microorganisms in foods

Considering food spoilage and food poisoning it is necessary to examine the possible sources of microorganisms in foods which may contaminate food and cause spoilage or food poisoning. Knowledge of the sources of microorganisms in foods is important to develop methods to control the invasion of some microorganisms in food, develop methods to control their growth and survival in food and determine the microbiological quality and safety of foods and food ingredients (Ray and Bhunia, 2008). Microorganisms occur naturally in the environment, on plants and animals, in the atmosphere, in and on soil and in water. Foods might be contaminated by internal and external sources. Internal sources include plants and animals while external sources are the air, soil, water, waste, feeds, humans and other sources.

The inner tissue of most foods derived from plants is sterile but fruits and vegetables carry on their surface several microorganisms according with the type of soil, the quality of the air and the type of water fertilizers used. Yeasts, moulds, lactic acid bacteria and bacteria from genera *Pseudomonas*, *Bacillus*, *Clostridium* and *Enterobacter* can be present and pathogens especially enteric types (*Salmonella*, *Escherichia coli*, *Shigella* and *Campylobacter*) can be also present if the soil is contaminated from untreated waste (Ray and Bhunia, 2008). A rapid increase in the microbial numbers can be observed with damage to the surface, delay between harvesting and washing and improper storage and transport conditions.

Animals carry many types of microorganisms in their digestive tract but also on skin, hair, and feathers. It is also possible that many of them are carriers of pathogens such as *Salmonella*, *E. coli*, *Listeria monocytogenes* without showing any symptoms. Also, foods from animal origin (milk, meat eggs) can be contaminated by spoilage and pathogenic microorganisms during production and processing. Meat can be cross-contaminated by slaughtering, fish can be contaminated with intestinal contents during processing or milk can be contaminated with faecal materials on the udder surface (Ray and Bhunia, 2008).

In the atmosphere many types of microorganisms are present even although it constitutes a hostile environment for them because of the radiant energy of the sun and the chemical activity of oxygen. Moulds, yeasts, spores of *Bacillus* and *Clostridium* as well as some Gram positive bacteria such as *Micrococcus* can be present in the air. If a specific environment (such as farms) contains a source of pathogens then, it is possible for these pathogens to be transmitted by the air (Adams and Moss, 2008).

Soil in contrast with air is an environment in which microorganisms can multiply and as a result contains many different types of microorganisms in high numbers. Yeasts, moulds and many types of bacteria (*Pseudomonas*, *Enterobacter*, *Bacillus*, *Clostridium*) and also enteric pathogenic bacteria and viruses from soil contaminated with faecal materials can contaminate foods grown from soil (Ray and Bhunia, 2008).

Water is essential for life and has a wide range of uses such as food production, drinking by humans and animals, irrigation of crops, food processing and storage and also washing and sanitation of food and equipment. Surface water may contain different type of microorganisms such as *Flavobacterium* spp. and *Pseudomonas* spp. as well as infectious microorganisms including bacteria (e.g. *Salmonella*, *E. coli* and *Vibrio cholera*e), viruses and protozoa which may be introduced to water sources (WHO, 1993). Different treatments can be applied (e.g. filtration and disinfection) for inactivation of the microorganisms (particularly pathogens) which can be contained in the water. Contaminations of water make any such water dangerous for human health unless treated. If inappropriately treated it can contain spoilage microorganisms such as *Pseudomonas* or pathogenic microorganisms such as *Legionella* and *Aeromonas* (ICMSF, 2005). Potable water is chlorine treated and so it does not contain coliforms or pathogenic microorganisms.

Another source of food contamination is from humans. Foods come in contact with different people from production until consumption. Poor personal hygiene is the major cause of microbial contamination of food from humans. In particular, humans can contaminate foods with spoilage as well as with

pathogenic microorganisms such as *Staphylococcus aureus*, *Salmonella*, *E. coli* and hepatidis A.

Contamination of food can also occur from the equipment used in harvesting, slaughtering, processing, transporting and storing food usually by cross-contamination with *Salmonella*, *Listeria*, *Escherichia*, *Pseudomonas*, *Clostridium*, *Bacillus*, *Lactobacillus*, yeasts and moulds which are the most common contaminants of food from equipment. In addition, there are several other sources of microorganisms which can contaminate such as packaging materials, containers, pets and rodents (Ray and Bhunia, 2008).

1.1.4 Microorganisms in foods

1.1.4.1 Meat and meat products

The carcasses of animals contain several types of microorganisms with an average of 1.55 to 155 cells/ cm^2 (or 10 to 1000 cells/ $inch^2$) (Ray and Bhunia, 2008). Enteric pathogens such as *Salmonella*, *E. coli*, *Yersinia enterolitica*, *Campylobacter jejuni* and *St. aureus* can be found. After boning the meat is chilled and any contaminating microorganisms present might come from the carcasses or the equipment, humans, air and water during processing. Chilled meat contains mesophiles such as *Lactobacillus*, *Bacillus*, *Clostridium*, *Staphylococcus* and other *Enterobacteriaceae* including enteric pathogens. Normally, the meat is stored at low temperatures (-1 to 5ºC), so the major problem is caused by psychotrophs like *Brochothrix thermosphacta*, *Pseudomonas* spp., *Aeromonas* spp. and pathogens like *L. monocytogenes* and *Y. enterolitica*. The packaging (aerobic or anaerobic conditions) also affects the microbial population (ICMSF, 2005).

1.1.4.2 Milk

Milk is rich in proteins and carbohydrates and contains many types of bacteria as predominant microorganisms. The predominant microorganisms present are *Micrococcus*, *Streptococcus* and *Corynobacterium* but if the animal suffers from mastitis *Streptococcus agalactiae*, *St. aureus*, coliforms and *Pseudomonas* can be present. Contaminants from other animals, feeds, soil, water or the equipment used are lactic acid bacteria,, coliforms, *Bacillus*,

Enterococcus, clostridium spores, Gram negative bacteria but also pathogens such as *Salmonella*, *L. monocytogenes*, *Y. enterolitica* and *C. jejuni*. During refrigerated storage psychrotrophs such as *Pseudomonas* and also psychrotrophic pathogens like *L. monocytogenes* and *Y. enterolitica* can grow (ICMSF, 2005).

1.1.4.3 Eggs

The shells of eggs carry many microorganisms such as *Pseudomonas*, *E. coli*, *Enterobacter, Enterococcus*, *Citrobacter* and *Bacillus*, mainly coming from faecal materials, nesting materials, feeds, air and equipment used during processing. *Salmonella*, might be also present from faecal contamination. Eggs have been the most common source linked to *S.* Enteritidis infections. *S.* Enteritidis can be inside of perfectly normal-appearing eggs. Motile Gram negative bacteria can enter the inside of the egg through pores of the eggshells (ICMSF, 2005).

1.1.4.4 Fish and shellfish

The pollution level of the water and the temperature of the water are the main factors which affect the microbial population of fish and shellfish. Freshwater fish can have *Pseudomonas*, *Enterococcus*, *Micrococcus*, *Bacillus* and coliforms while fish and shellfish harvested from marine environments can have halophilic vibrios, *Pseudomonas*, *Alteromonas*, *Enterococcus*, *Micrococcus*, *Bacillus*, coliforms and pathogens like *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Clostridium botulinum* type E. If the water is polluted microorganisms can grow quickly because the high water activity (a_w) and the high pH of the fish tissue. The microorganisms which are present in such cases are *Salmonella*, *Shigella*, *C. perfringens*, *Vibrio cholerae* and hepatitis A (ICMSF, 2005).

1.1.4.5 Fruits and vegetables

Fruits and vegetables are high in carbohydrates and are usually consumed raw or minimally processed. Microorganisms in vegetables and fruits can come from sources described above such as soil, water, air, animals, insects and equipment during processing. Vegetables and fruits have several microorganisms including lactic acid bacteria, *Enterobacter*, *Pseudomonas*,

Proteus, *Micrococcus*, moulds, pathogenic protozoa, parasites and also some enteric pathogens such as *L. monocytogenes*, *Salmonella*, *Shigella*, *E. coli*, *C. botulinum, C. perfringens* and *Campylobacter* from animal wastes and polluted water (ICMSF, 2005).

1.1.5 Factors affecting the growth and survival of microorganisms

The factors that influence the growth of microorganisms which can cause spoilage or food poisoning can be classified in four categories: Intrinsic factors (which encompass the physicochemical properties of the food such as nutrient content, pH, a_w, redox potential and antimicrobials), extrinsic (which encompass storage conditions such as relative humidity, temperature and gaseous atmosphere), implicit factors (which encompass the response of microorganisms to their environment and the interaction between microorganisms present in food) and processing factors (Adams and Moss, 2008). Although, the total number of factors (hurdles) which affect the growth and survival of microorganisms in foods is high, the most widely used and studied hurdles include temperature storage, pH, a_w and heat treatment (Leistner and Gould, 2002). The resistance of bacteria to various environmental hurdles (conditions of storage and / or treatment) is highest when all these factors are at best (optimum) levels. Attention was drawn to *L. monocytogenes*, *S.* Typhimurium and *E. coli* which were the foodborne pathogens used in the studies represented in that manuscript and so some of their major growth characteristics are discussed below.

1.1.5.1 Intrinsic factors

Nutrients

Microbial growth is achieved through the synthesis of cellular components and energy. Microorganisms can use food as a source of the necessary nutrients for growth which include proteins, carbohydrates, lipids, minerals and vitamins. Water is not considered a nutrient but is essential for all the biochemical reactions of cells. Microorganisms can utilize the major components of the foodstuff differently so the nutrient component can affect the growth and survival of microorganisms (Jay *et al*., 2005).

pH

pH shows the hydrogen anion concentration in a system and is equal to the negative logarithm of the hydrogen ion or proton concentration. pH ranges from 0 to 14 with pH=7 corresponding to neutrality. pH values lower than 7 indicate an acidic environment while pH values above 7 are alkaline (Adams and Moss, 2008). pH is inversely related to acidity. In particular, a system with high pH has low acidity and vice versa (Ray and Bhunia, 2008). Also, acids are classified as strong or weak acids. Strong acids (e.g. HCl) dissociate completely while weak acids have a dissociated and an undissociated form. The partial dissociation of the weak acids affects the growth of the microorganisms. Also, even though strong acids have a more significant effect on pH, they are less inhibitory than the weak acids at the same pH. This is because the inhibition from weak acids is related to the concentration of undissociated form of the acid (higher dissociation constant pKa results in more undissociated molecules). The influence of pH on the growth and survival of microorganisms has been used in food preservation of spoilage and pathogenic microorganisms in food.

Listeria grow best in the pH range 6–8, but will grow at a pH between 4.1 and 9.6 (Pearson and Marth, 1990, Tienungoon *et al.*, 2000). The minimum pH that allows growth and survival has been the subject of a large number of studies. In general, the minimum growth pH of a bacterium is a function of temperature of incubation, general nutrient composition of growth substrate, a_w, and the presence and quantity of NaCl and other salts or inhibitors (Buchanan and Klawitter, 1991; Colburn *et al*., 1990). Studies have shown that the organism can survive at pH values below 4.1, especially when exposed to acidic stress before inoculation (acid adaptation) and can present high acid tolerance.

Salmonella can grow in a wide range of pH values. The pH for optimum growth is around neutrality. A minimum growth pH value of 4.05 has been recorded (Chung and Goepfert, 1970). For best growth, *Salmonella* requires a pH between 6.6 and 8.2. Aeration was found to favour growth at the lower pH values. The growth rates are reduced at pH values that deviate from the

optimum. At very extreme pH values it is possible for the microorganism to be killed.

E. coli can grow in a wide range of pH. The pH for optimum growth is around neutrality. The approximate minimum pH values that permit growth for enterohaemorrhagic *E. coli* is 3.9. For best growth, *E. coli* requires a pH between 6 and 7. Studies have shown that is a quite acid tolerant microorganism. The growth rate of *E. coli* slows as pH decline below optimal levels (Presser *et al.*, 1998).

Water activity

Water activity (a_w) can be defined as the available water for microbial growth. In a foodstuff water exists in two forms: free and bound. Bound water is used to hydrate hydrophilic molecules and to dissolve solutes and it is not available for biological functions so it does not contribute to the water activity (Ray and Bhunia, 2008). Water activity is given approximately by the ratio of the number of mols of water to the total number of mols of the aqueous solution and is dependent on the number of molecules or ions. That means compounds which dissociate in more ions (e.g. sodium chloride dissociates in two ions) are more effective than compounds which dissociate in fewer ions (e.g. sucrose dissociates in one ion). Free water is necessary for microbial growth as it can transport nutrients, remove wastes, assist in enzymatic reactions, synthesise cellular materials and help other biochemical reactions. Any reduction in the a_w affects the microbial growth or survival of the microorganisms. The influence of a_w on the growth and survival of microorganisms has been used in food preservation of spoilage and pathogenic microorganisms in food.

L. monocytogenes has a minimum a_w for growth of about 0.90 at 30^{o}C, when glycerol is used and about 0.92 and 0.93 when using NaCl and sucrose, respectively (Farber *et al.*, 1992). Survival, however, has been observed in salami with 0.79 to 0.86 a_w, when it was stored at 4^{o}C (Johnson *et al.*, 1988).

The a_w threshold for the growth of *Salmonella* is 0.94, although it can survive in foods with lower values of a_w. Regarding available moisture, growth inhibition has been reported for a_w values below 0.94 in media with neutral

pH, with higher a_w values being required as the pH is decreased towards growth minima. The water activity of a food product is reduced by the addition of solutes such as sodium chloride and sugars.

The minimum a_w values that permit growth for enterohaemorrhagic *E. coli* is 0.95 (ICMSF, 1996). However, several levels of a_w that prevent growth have been reported (Ryu *et al.*, 1999; Riordan *et al.*, 1998).

1.1.5.2 Extrinsic factors

Relative humidity

Relative humidity and water activity are related but relative humidity refers to the water activity of the gas phase. There is an interaction between the food and the air humidity so that when foods with low water activity are stored in a place with high relative humidity water will transfer from the gas phase to the food. This will result in an increase of water activity and might result in an increase of an existing microbial population which was viable but unable to grow (Adams and Moss, 2008).

Temperature

Microbial growth is carried out through enzymatic reactions. Temperature also affects the enzymatic reaction rates, so it has a key role in microbial growth of food (Jay *et al*., 2005). Microorganisms can be classified regarding their optimum temperature for growth in thermophiles (microorganisms which can grow at high temperatures), mesophiles (microorganisms which grow in ambient temperatures) and psychrophiles (microorganisms which can grow at low temperatures). Mesophilic and psychrophilic microorganisms are of greater importance in food microbiology than thermophilic microorganisms although thermophilic spores of Bacillus and Clostridium can be the source of food contamination (Adams and Moss, 2008).

The optimum temperature for growth of *L. monocytogenes* is 30°C to 37°C, when the pH of the food, is neutral or slightly alkaline. However, the lower limit is about 0°C, where growth is quite slow, with a generation time of 62 to 131 hours. Growth at low temperatures is also influenced by other factors such as

salt (NaCl) concentration, pH and the presence of lactic acid bacteria (Raccach *et al.*, 1989; Berry *et al.*, 1990).

The temperature range for growth of *Salmonella* spp. is from 5.3°C to 45°C with an optimal temperature of 37ºC (ICMSF, 1996). At temperatures below 15°C the growth rate is reduced, while for most strains growth is inhibited at temperatures below 7°C. Particular attention should be given to foods which are kept for long periods in chilling, within the limits of growth, where growth rates are low. Freezing conditions are detrimental for the survival and growth of the organism, but do not guarantee the destruction of the pathogen. Cells have been detected in foods which are stored in low temperatures for years. It seems that some foods provide protection against freezing, especially when the initial population of the pathogen before freezing is high (ICMSF, 1996).

The temperature growth range for *E. coli* is from 7°C to 45°C with an optimal temperature of approximately 37°C. The lowest temperature that allows the growth of enterotoxigenic *E. coli* is 7°C IFT (2001). The environmental limits to growth for *E. coli* are well characterized (Presser *et al.*, 1998; Salter *et al.*, 2000; ICMSF, 1996).

Gaseous atmosphere

Oxygen (O_2) and carbon dioxide (CO_2) have an inhibitory effect on the growth or survival of microorganisms. Oxygen is the most important gas in contact with food and its influence on redox potential (E_h) which is the medium tendency to accept or donate electrons (oxidation or reduction) affect the microbial populations. Carbon dioxide has also an inhibitory effect on the microorganisms and is used by the food industry in modified atmosphere packaging. In general, mould and Gram negative bacteria are more sensitive in carbon dioxide than Gram positive (mostly lactobacilli) bacteria and yeasts which tend to be more tolerant. Also, greater growth inhibition by carbon dioxide is observed under aerobic conditions and decreased temperature (Adams and Moss, 2008).

1.1.5.3 Implicit factors

The responses of the microorganisms in the environment present as well as the interactions between them constitute a third set of factors which affect their growth and survival in food. In particular, the physiological state of the microorganisms can affect their responses to several stresses with the exponential phase cells being more sensitive than the stationary cells. Also, microorganisms can develop adaptation mechanisms to several stresses which results in a decrease of the damaging effect of the adverse conditions (Adams and Moss, 2008). In the literature there are several studies which show microbial adaptation (Hill *et al*., 1995; Belessi *et al*., 2011). Moreover, if several microorganisms are present in a foodstuff the interactions might have an effect in their responses to the applied stresses. Moreover, microorganisms can help other microorganisms to grow or produce a stress response by producing molecules or by removing inhibitory components from the environment. Conversely, these microorganisms might also be antagonistic to each other by producing inhibitory compounds or by consuming essential nutrients such as iron (Adams and Moss, 2008).

1.1.5.4 Processing factors

During food processing, processing factors such as slicing, washing, packing (modified atmospheres or aseptic packaging), use of chemicals, drying, irradiation, high hydrostatic pressure, pulsed electric fields and pasteurization can affect the growth and survival of microorganisms by causing changes in the intrinsic or extrinsic factors or by directly eliminating a portion of the microflora of the food (Adams and Moss, 2008).

1.2 Prevention of foodborne disease

Traditionally, food safety control was based on the inspection of the end product. Under these circumstances, in the case of a positive result (i.e. presence of pathogens) the whole production could be discarded. Further, it was often not possible to identify where the hazard came from because there was often no traceability system. In 1996, the EC stated that “the biological and chemical agents which cause food poisoning are many and varied, but they almost all have one feature in common: they accompany the animal from

stable to table. For this reason, any attempt to maintain a high level of protection of consumer without taking into account of what is happening throughout the whole production chain is doomed to failure" (FVE, 2010). Increased awareness of the effects of food hazards on human health and the increasing importance and rapid growth of world trade have prompted regulatory officials and international organisations to consider new and improved strategies to reduce the health risks associated with pathogenic microorganisms in foods. As a result, and with the increasing incidence of foodborne disease during 1990s food legislation was developed based on the concepts of the Codex Alimentarius Commission (CAC). Also, it has been noted that there was no absolute food safety but that food safety is related to a level of risk that society considers as reasonable (Forsythe, 2000).

1.2.1 International control of microbiological hazards in foods

One of the present trends in foodborne disease is the globalization of food trade. This can cause the dissemination of infectious agents from the original point of production to places miles away. As a result foodborne disease became of significant importance as a global health issue. Food safety measures are not fixed around the world and this leads to trade disagreements between counties. Food safety systems should be established by all (developed and developing) countries together in order to ensure global food safety. The Codex Alimentarius (CA) is a collection of standards, recommendations and guidelines and covers all foods (raw, processed or semi processed). In 1962, the Joint FAO/WHO Committee was established with the CAC as an executive organ. The application of the CA principles will control hazards in foods (Forsythe, 2002).

1.2.2 Food safety (management) tools

The complexity of the global food market means that the control of foodborne disease is a joint responsibility and requires action at all levels from the individual to international groups, and at all parts of the supply chain from farm to fork. The tools used and approaches taken to ensure control require different emphasis, depending on a number of factors such as where food materials have come from, how they have been processed and handled and

how they are stored. The risk of foodborne illness can be reduced by using existing technologies, such as thermal processes, and by adopting some simple precautions such as avoiding cross contamination by separating raw and cooked foods and employing good hygiene practices. The increase in knowledge about foodborne pathogens can provide a focus for effective control measures to help reduce food poisoning.

The microbiological safety of food is guaranteed by the education and training of food handlers and consumers during the whole process from production to consumption, the microbiological testing of food, the implementation of Hazard Analysis Critical Control Point (HACCP) systems, the control at source which includes the prevention of contamination of the raw materials (CAC, 1997) and the product design and process control which encompass the technologies used to prevent foodborne diseases (Forsythe, 2002).

Food safety measures have to be taken over the entire food chain from farm to fork. Forsythe (2002) has discussed the food safety tools required to accomplish this aim. In particular, the food safety tools such as the Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP), HACCP, Microbiological Risk Assessment (MRA), Quality Management (QM) and Total Quality Management (TQM) when integrated appropriately provide a high level of safety assurance.

1.2.2.1 Good Manufacturing Practice and Good Hygiene Practice

GMP is related with the appropriate environment in which a product is produced and it covers all the basic principles and procedures followed to accomplish the production of food of acceptable quality (Forsythe, 2000). GHP encompasses the hygiene practices which all establishments should follow and covers all the field of hygienic design of manufacturing premises, machinery, cleaning, disinfecting and processing procedures (Forsythe, 2000). GMP and GHP have been built up by governments, the CAC on food hygiene (FAO/WHO), the food industry and competent authorities.

1.2.2.2 Hazard Analysis Critical Control Point

In the past, the central challenge of foodborne disease was the suppression of contamination of food but nowadays it is based on the development of proactive, preventative systems of quality control through the adoption of HACCP principles. HACCP is a protocol which defines and controls specific hazards that adversely affect the safety of products and it can be applied from production to the final consumption of the product. GMP and GHP are related with the HACCP systems as these requirements constitute the prerequisites of a HACCP system. The outline of a HACCP system encompasses the prevention of microorganisms from contaminating food by applying hygienic measures and the prevention of microorganisms from growing in food or eliminating them using food preservation processes (Forsythe, 2002).

1.2.2.3 Microbiological Risk Assessment

MRA is a step by step analysis of hazards which can be associated with a product and can give an estimation of the probability of occurrence of adverse effects on the consumer's health (Notermans and Mead, 1996). The MRA should not be confused with the HACCP systems as it is a regulatory activity which is related more with the consumer than with the final product (as the HACCP systems) and can supply valuable information for the development of HACCP systems (Forsythe, 2002). MRA is an approach for understanding and reducing risks where risk has been defined by the CA as "a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food" and consists of risk assessment, risk management and risk communication (FAO/WHO, 1997).

1.2.2.4 Responsibilities of the industry, competent authorities and consumers

The primary role of food industries is to supply the market with safe food by applying the appropriate control measures (e.g. GMP, GHP, TQM and HACCP). The responsibilities for the role of the competent authorities in relation to food safety are specified in Regulations 852/2004, 853/2004 and 178/2002 of the European Parliament and of the Council (EU basic food law). More specifically, the competent authorities are responsible for the approval of

the implementation of HACCP systems and GHP in all food handling and processing facilities, issuing GHP in collaboration with relevant production sectors and approving food processing facilities.

It is clear that in the farm to fork concept, consumers also have certain responsibilities. These responsibilities are related with the way the consumers handle and preserve the products from the time they purchase them until their consumption. However, communication between the industry and the competent authorities and consumers is very important. They have to inform consumers of all the hazards and risks associated with food handling from the time of purchase until the actual consumption. The information has to be simple and understood by everyone to avoid any confusion and panic by the consumers which would result in unreliability to the food industry and the competent authorities.

1.2.3 Hurdle technology

1.2.3.1 Dimensions of hurdle technology

Hurdle technology is a concept which was developed several years ago and is used in food production in industrialized and in developing countries for the mild but effective preservation of foods. Initially, hurdle technology was used exclusively to improve the microbial stability and safety of foods. McKenna (1994) stated that food quality is a broader field and subsequently hurdle technology should be used by researchers as well as the food industry for as many quality improvements as possible. Leistner and Gould (2002) stated that the hurdles used in a food product cannot only affect the microbial stability and safety of the food but also the sensory, nutritional, technological and economical properties of a foodstuff, in a negative or positive way. In particular, regarding the hurdles in a foodstuff, if a hurdle's intensity is too low it should be reinforced while if it impairs the total quality it should be lowered in order to be maintained in an optimum range which will ensure at the same time the safety as well as the quality (Leistner, 1994; 1995a). Other dimensions of the hurdle technology have been suggested such as medical aspects, barriers in food, hurdle technology and enzymes and hurdle technology for sustainable food processing (Leisner and Gould, 2002).

Moreover, the aim of hurdle technology is the deliberate and intelligent combination of different hurdles in order to improve the microbial stability and the total quality of foods (Leistner, 2000; Leistner and Gorris, 1995). Each food has a certain set of hurdles which can ensure its stability and the type of the hurdles depends on the type of the food, the desired stability and safety and the available facilities (Leistner and Gould, 2002). Previously, hurdle technology was used empirically without much quantitative knowledge of the governing principles. In the last 30 years, a wide use of hurdle technology has been observed because the properties of major preservative factors for foods (e.g., temperature, pH, a_w), and their interactions, became better known (Beales, 2004; Adams and Moss, 2008; Brul and Coote, 1999).

1.2.3.2 Mechanisms of microorganisms

In food preservation the hurdles used have to be effective by inhibiting the growth of microorganisms or inactivating them. Microorganisms are in contact with the external environment; changes of the environmental factors cause them to modify their metabolism and to develop mechanisms in order to overcome these environmental changes. In particular, most of those mechanisms are related with homeostasis. In food preservation homeostasis of microorganisms is a very important phenomenon and is the cornerstone for the concept of multi-target preservation (Leistner, 1995a; 1995b). According to Leistner homeostasis ‘’is the tendency to uniformity and stability in the internal status of organisms’’ (Leistner, 1995a). Homeostasis, metabolic exhaustion and stress reactions of microorganisms are all related with hurdle technology. According to Leistner and Gould (2002), homeostatic mechanisms can be classified as active (level of nutrients, pH, a_w, preservatives), passive or refractory (high temperature, hydrostatic pressure, ultrasonication) and population homeostasis (competition from other microorganisms). If homeostasis is disturbed by preservative factors (hurdles), then the microorganisms may not multiply, that is, they cannot proceed from lag phase to exponential phase or they die, before repairing homeostasis mechanisms. In food preservation, the disturbance of homeostasis may be temporary or permanent. Gould (1988; 1995) was the first to refer to the homeostasis of microorganisms and their relation with the preservation of foods. Metabolic

exhaustion of microorganisms is another important phenomenon. Survival mechanisms are the opposite of growth mechanisms. Optimum environmental conditions favour the growth mechanisms of a microorganism. On the other hand, microorganisms in stable hurdle-technology foods use the repair mechanisms for their homeostasis to overcome the hostile environment, that is, use their energy for this purpose and die if they become metabolically exhausted. This leads to an auto-sterilization of such foods (Leistner, 1995b). Also, the stress reactions of the microorganisms under inimical conditions (such as heat, pH, a_w) could increase their resistance or their virulence. For example, Cataldo *et al*. (2007) studied the acid adaptation and survival of *L. monocytogenes* in Italian style soft cheeses. Also, Beales (2004) with his review of the adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH and osmotic stress and Brul and Coote (1999) with their review on the preservative agents in food, gave a better understanding of such mechanisms. Factors such as these could cause problems in the application of hurdle technology.

1.2.3.3 Multi-target preservation

Under stress conditions, bacteria may become more resistant due to the production of stress shock proteins. Several stresses including heat, ethanol and pH, induce the synthesis of these types of proteins. Also, there is the possibility that a microorganism becomes more tolerant to other stresses after the exposure to a single stress (cross – protection). The various responses of microorganisms may create problems to the application of hurdle technology (Leistner, 2000). Exposure of microorganisms to different stresses at the same time may also lead to the synthesis of more protective stress shock proteins which has as result the metabolic exhaustion of the microorganism.

Subsequently, multi-targeted preservation of foods could be the solution in order to avoid the impact of the synthesis of stress shock proteins. Leistner (1995a, 1995b) introduced the concept of multi-target preservation of foods. The concept of multi-target preservation is the intelligent combination of mild hurdles which will have synergistic effect (Leistner, 2000). It has been suspected that the applications of different hurdles in a food, not only have an

additive effect on microbial stability but it might give a synergy too (Leistner, 1978). If different hurdles hit, at the same time, different targets (e.g. DNA, pH, cell membrane, a_w) in the microbial cell and disturb the homeostasis of the microorganism they can achieve a synergistic effect, so the repair of homeostasis will become more difficult (Leistner, 1994). Therefore the hypothesis that it is better to use different preservative factors of low intensity than one of larger intensity is valid, due to the fact that different preservative hurdles could act synergistically (Leistner, 1994).

In the literature there are many studies which have used combined hurdles to inhibit the growth of several microorganisms. In particular, Francois *et al*. (2006) studied the effect of temperature, pH and a_w on the individual lag phase of *L. monocytogenes*. Also, Shadbolt *et al*. (2001) studied the differentiation of the effects of lethal pH and a_w in *E. coli* populations and their implication on food safety. Gabriel and Nakano (2010) used different combinations of pH, a_w and temperature to compare the responses of *E. coli O157:H7*, *L. monocytogenes* ½ c and *S. enteriditis*.

1.2.3.4 Major hurdles in foods, injury and recovery of bacteria

There are several factors that are used by the food industry in order to preserve food, such as temperature, salt (NaCl), water activity (a_w), and weak acid preservatives. These factors are applied to foods in order to injure or inactivate microorganisms. Different microorganisms have different responses to these stresses and therefore their potential presence in a variety of foods may increase the risk of foodborne illnesses. Leistner and Gould (2002) explained that the microbial stability and safety of foods are actually based on combinations of several factors or hurdles that may individually or additively affect microorganisms. These preservative hurdles can be classified as physical, physicochemical, microbiologically derived and miscellaneous hurdles. However, while the total number of hurdles is very high, the most widely used and studied hurdles include temperature storage, pH, a_w and heat treatment. There are many studies in the literature, which examine the responses of the microorganisms to several physiological parameters. Vermeulen *et al*. (2007) studied the influence of pH, a_w and acetic acid

concentration on *L. monocytogenes* at 7°C. Similarly, McClure *et al.* (1993) studied the growth responses of *L. monocytogenes* in combinations of temperature, hydrogen-ion and NaCl using the Bioscreen analyser.

As mentioned above, bacteria are often subjected to several stresses such as extreme pH, temperatures, a_w, and nutrient limitation. This means that bacteria spend more time in a stressed state than in a non-stressed state. However, instead of this impediment, stressed bacteria remain very important to study due to their ability to retain the ability to contaminate and infect. The injury which results from these stresses may affect the physical properties of the cells or their metabolic reactions (Stephens, 2005). A major physical effect is the damage to the outer membrane of the bacterial cell, resulting in an increase in permeability. External compounds can enter into the cytoplasm and intracellular components may be lost. Furthermore, the increased membrane permeability may lead to the disruption of membrane-based proteins which consequently affects other essential functions such as respiration. Bacterial stress can also lead to ribosome and RNA degradation. Apart from the direct damage of the ribosomes and RNA which is caused from stress, indirect damage can occur through activation of ribonucleases. The concentration of Mg^{2+} is responsible for ribosome and RNA stability. Any change in the concentration of Mg^{2+} leads to injury. DNA can be damaged by using ultraviolet and/or ionising radiation and heat, cold and desiccation stresses (Stephens, 2005).

Mild treatments such as heat, have been shown to be effective in inactivating vegetative cells of bacteria. However, usually they are not able to inactivate completely foodborne pathogens in foods, which results the surviving population being sub-lethally injured (Jasson *et al*., 2007). These microorganisms are not able to grow on selective media if not preceded by repairing such injury with a suitable resuscitation treatment (Mossel and Corry, 1977). Two major factors of determining optimum resuscitation or pre-enrichment treatments are the severity of injury and the repair times of the microorganisms (Mackey and Derrick, 1984).

There are two main considerations on the processing conditions, such as heating which have to be in balance. In particular, the effect of the process on the growth / survival of the microorganisms has to be determined but at the same time it has to be taken into account that this process may change the final quality of the product (texture, taste etc) (McClure, 2000). Food quality and food safety play an essential role in the food industry (Zwietering *et al.*, 1993; Zwietering *et al.*, 1992; Wijtzes *et al.*, 1998; McMeekin *et al.*, 2006).

The food industry uses various processes to control microbial growth. Heat represents a common form of preservation (Gould, 1989). It can cause damage to the cell similar to several other forms of injury and control in the laboratory is relatively easy (Stethens *et al.*, 1997). Rosso *et al.* (1995) have mentioned: “Temperature and pH are the major environmental factors that affect growth which are studied most because of their importance in fundamental research (taxonomy, microbial metabolism) and their practical importance (control of bioprocesses in biotechnology and safe handling of goods, especially in the agriculture and food industries)”. In many food processing systems, heating is used for the reduction of the number of bacteria. That way, safety is enhanced and the shelf life of the products is increased (Esther and Zwietering, 2006). The most important factor for this step is the required time-temperature that is the time which is needed at a particular temperature to achieve the desired result. The D/z concept is a broadly applicable concept which assumes a log-linear inactivation during the heating time. There are two parameters, D and z, which play an important role in the thermal inactivation. D is the time needed to reduce viable numbers by tenfold or one log unit at a specific temperature, and z- is the temperature change needed to cause a tenfold change in D (Mackey *et al.*, 2006). Several strains, products and laboratory media have been studied resulting in numerous D- and z- values for various environmental conditions (ICMSF, 1996; Doyle *et al.*, 2001, van Asselt and Zwietering, 2006). Furthermore, Doyle *et al.* (2001), reported several factors which influence the heat resistance of a pathogen: strain variations, presence of salt or acid, growth phase of the cells, the media which is used etc. McClure (2000) mentioned that heating is one of the most important control measures for *E. coli*. There

are many studies that refer to heat inactivation, for example Chhabra *et al.* (2002) developed a model which evaluated the effect of growth conditions in relation with the thermal inactivation of *L. monocytogenes*.

Bacterial cell components can be damaged by sub-lethal thermal injury. This may lead to increased lag phase duration (LPD) and increased difficulty in isolating and enumerating foodborne pathogens (McKellar *et al.*, 1997). A factor that strongly influences the microbial recovery to thermal treatments is the plating media which are used. If a selective medium is used in order to grow stressed bacteria colonies, then injured cells may not recover and this may lead to an underestimation of the real number of colonies (viable but not countable cells) (Miller *et al.*, 2006). For this reason in many studies non-selective media are used to circumvent this problem. McKellar *et al.* (1997), in order to model the influence of temperature on the recovery of *L. monocytogenes* from heat injury, used Tryptic Soy Yeast Extract Agar (TSAYE) and Tryptic Soy Yeast Extract Agar supplemented with 5% w/v sodium chloride to determine the total cell count and non-injured cell count, respectively. In other studies on the repair times of *S.* Typhimurium, Tryptone Soya Agar (TSA) and Tryptone Soya Agar supplemented with 0.1 % sodium pyruvate was used for the same reason (Mackey and Derrick, 1984; 1982). The reactions and the behaviour of microorganisms in different media must be known (Miller *et al.*, 2009). Moreover, Stephens *et al.* (1997), measured the recovery times of single heat injured *Salmonella* cells using an automated growth analyser.

1.2.3.5 Gamma hypothesis and hurdle concept

The Gamma hypothesis/concept states that the growth of microorganisms is independently affected by combined environmental factors (hurdles) such as temperature, pH, a_w or in other words, that there are no interactions between antimicrobial environmental factors (Zwietering *et al.*, 1992; 1993). Furthermore, Zwietering *et al.* (1993) developed a model which combined qualitative and quantitative information for the prediction of microbial spoilage in foods. It is known, that the different environmental factors (temperature, pH, a_w) influence the growth rate. A growth factor ($\gamma=\mu/\mu_{opt}$) was introduced to

evaluate these effects. As was also stated, that “it is assumed that the growth factor can be calculated by multiplying all $\gamma(x)$ values, with $\gamma(x)$ defined for each of the variables separately, independent of the value of the other variables” (Zwietering *et al.*, 1993). Also, Wijtzes *et al.* (1998), described a method for the prediction of food safety and quality using the same dimensionless growth rate γ. A very important term which has to be mentioned is synergy. In the pharmaceutical area, all attempts to absolutely define synergy have not succeded (Greco *et al.*, 1995). Dufour *et al.* (2003) stated that there was no commonly accepted methodology for the detection or quantification of synergistic interactions. However, editors of known journals have defined synergy e.g. Odds (2003), without any fundamental scientific basis for doing so. The fact that there is no agreement on what constitutes synergy has created many problems (Chou, 2008).

In general, from the literature it can be concluded that there are two different views with respect to the effect of combined antimicrobial environmental factors (hurdles). The first suggests that interactions exist and the second suggests that interactions do not exist. Lambert and Bidlas (2007a) stated that the Gamma hypothesis has been extended in order to include apparent synergistic or interactive effects between hurdles, such as temperature, pH and a_w (Augustin and Carlier, 2000b), temperature, pH and weak acids (Le Marc *et al.*, 2002), and mixed weak acids (Coroller *et al.*, 2005), but that this was a violation of the hypothesis itself.

The Gamma hypothesis is the corner stone for several studies which use time to detection (TTD) for the analysis of multi-factor environmental stresses (hurdles) affecting microorganisms such as pH, weak acids and temperature. Lambert and Bidlas (2007b), investigated the growth of *Aeromonas hydrophila* by challenging it with pH, sodium nitrite ($NaNO_2$) and salt concentrations at 30ºC, based on the Gamma hypothesis. Moreover, Lambert and Bidlas (2007a, 2007c) made a predictive modelling study in which they examined the effect of multi-factor environmental stresses of *Enterobacter sakazakii* and *Aeromonas hydrophila* using a model based on the Gamma hypothesis.

McMeekin *et al.* (1997, 2000) and McMeekin and Ross (2002) characterized predictive microbiology as the quantification of hurdle technology. The concept of hurdle technology, states that combined antimicrobial factors act synergistically (Leistner and Gorris, 1995; Leistner, 2000). Moreover, it has been stated that although hurdles such as temperature, pH and a_w act independently, "it would be expected, however, that interactions must occur between certain hurdles" (Brocklehurst, 2004). On the other hand, the Gamma hypothesis suggests that different antimicrobial environmental hurdles are combined independently (Zwietering *et al.* 1992). Lambert and Bidlas (2007b) stated that this hypothesis may be considered, the foundation of predictive microbiology as it strengthens "the investigation of a supposed synergy over the assumption that it exists".

The interactions or the lack of them between environmental factors affect the Growth/ No Growth (G/NG) boundaries or stability maps. Stability maps are a new trend which predict the probability of growth when the studied population is faced with more than one hurdle (McMeekin and Ross, 2002). Stability maps were described as "the contours of relative growth" by Lambert and Bidlas (2007b). Ratkowksy and Ross (1995) was the first to report a method to define the G/NG boundary of *Shigella flexneri* using different temperature, pH, a_w and nitrite concentrations. Stability maps are very important because they enable product developers and all those who make challenge studies, to find very quickly regions of high and low growth. McMeekin *et al.* (2000) stated that developing the G/NG interface has many practical and scientific implications. As it can be understood, stability maps are based on the Gamma hypothesis. If Gamma hypothesis exists it would mean that a lot of time may be saved by examining inhibitory effects individually, as conventional methods are very time consuming. Given this information, predictive microbiology can become a rapid tool which can benefit the food industry (Lambert and Bidlas, 2007b; Membré and Lambert, 2008).

1.3 Quantitative hurdle technology

1.3.1 Predictive microbiology

Food microbiology covers a very broad field of science including the study of organisms, related to hygiene and food quality. McMeekin and Ross (2002) recognised the mid 1970s as a reference point in food microbiology because of the rapid social, demographic and technological changes which took place and had an impact on the food industry and consumers. Classical microbiological methods include enumeration techniques and identification of specific microorganisms which sometimes are not enough to overcome various problems and difficulties related to the complexity of a food environment, the physiology and behaviour of a microbial population. The collection of adequate data regarding the behaviour of microorganisms, in the food environment requires a large amount of work and increases costs. Despite the description of the behaviour of these microorganisms in food, the information obtained on the effect of various physiological processes that take place in a food and kinetics of a microbial population, is often not sufficient to reach specific conclusions about the likelihood of growth and/or survival of microorganisms.

With predictive microbiology all the knowledge of microbial responses in different environmental conditions is summarized as equations or mathematical models (McMeekin *et al.* 1997). Consequently, predictive microbiology has become a valuable research tool. An alternative term for predictive microbiology is “the quantitative microbial ecology” (Ross and McMeekin, 1995, McMeekin *et al.* 1997, Lambert and Bidlas, 2007c). McMeekin *et al.* (2002) stated that “the concept of predictive microbiology is the detailed knowledge of microbial responses to environmental conditions that enables the objective evaluation of the effect of processing, distribution and storage operations on the microbiological safety and quality of foods”. McMeekin and Ross (2002) and McMeekin *et al*. (2002) suggested that the origin of predictive models for foods was the model developed by Esty and Meyer (1922) to describe the thermal inactivation of *C. botulinum* type A. The re-genesis of predictive microbiology can be traced to the 1970s by Genigiorgis group in the USA and Roberts group in the UK, which used

probability models to solve food poisoning problems. In the 1980s several kinetic models for foodborne pathogens and spoilage organisms were developed and in the 1990s because of the emergence of foodborne pathogens with low infective doses there was a turn in the probabilistic modelling which gives more quantitative information and has practical and scientific implications (McMeekin *et al*., 2000).

The rapid development of microbial models and their ability to predict the growth of microorganisms have made predictive microbiology, a valuable research tool. However, it should be noted that currently, it cannot fully replace the conventional microbiological tests and the experience of trained microbiologists. Also, as it has been suggested by several authors that the results from the models should be used as an indication or as a tool to support decisions and not as absolute numbers or predictions (Wijtzes *et al.*, 1998; Zwietering *et al.*, 1996). The microbial safety or shelf life of the products, the critical points in a process, and in general the optimization of the production can be predicted from the models, as it has been stated by Zwietering *et al.* (1991).

Food hygiene is directly related with the terms of quality and safety. Food quality refers to the sum of the organoleptic characteristics (properties) of a food which make it acceptable for consumers. On the other hand, safe food is food that is free of any physical, chemical or biological hazards. Models which have been developed to ensure food safety are more straightforward while spoilage models are more complicated (Dalgaard *et al*., 2002). Safety models describe the kinetics of particular pathogens under different environmental conditions. On the contrary, the deterioration of food quality may occur due to the metabolic action of a great variety of spoilage bacteria and thus, further studies on the determination of specific spoilage microorganisms (SSO) are required (Dalgaard, 1995).

Moreover, the value of predictive microbiology is increasing and it can be used in order to underpin the quantitative microbial risk assessment (QMRA) and the HACCP systems-plans (Buchanan and Appel, 2010; Membré and

Lambert, 2008; McMeekin *et al.*, 2006; McMeekin *et al.*, 2002; McMeekin and Ross, 2002; Zwietering *et al.*, 1996).

It has been argued by many authors, that predictive microbiology is important to the development of QMRA and HACCP systems-plans (Buchanan and Appel, 2010; McMeekin *et al.*, 2006; McMeekin *et al.*, 2002; McMeekin and Ross, 2002; Zwietering *et al.*, 1996). Mathematical models of predictive microbiology provide valuable information on the behaviour of pathogens in foods and can largely replace the long lasting, traditional enumerating methods. The HACCP is a proactive, preventative system of quality control and is based on a systematic approach to the desired level of food hygiene, which relies on the identification and evaluation of risk factors. When applied, however, there is often a lack of objectivity, which is attributed to the fact that although the HACCP system is expressed quantitatively, it is based on the qualitative assessment of risk factors, due to a lack of available quantitative information.

In particular, predictive microbiology assists HACCP systems by identifying hazards and critical control points and by evaluating limits and is related to the product. The critical limits are strongly associated with probability models. Also, predictive microbiology is providing assessment information, so it is related with QMRA. QMRA requires the use of growth and inactivation models and is related to the consumer. McMeekin *et al*. (2002) stated that: “a dynamic interaction exists between HACCP and QMRA”. The common aim of HACCP systems and risk assessment is to produce safe food, by applying assessment strategies and understanding the potential origin of risk factors and their extent. Risk assessment requires the accurate determination of the potential exposure of the final consumer in a food pathogen, but this information is often not available. These risk factors are strongly associated with the fundamental principle of the use of models in the context of the Gamma concept and the multiple hurdle theory for the development of new product formulations. The effect of mathematically manipulating the levels of hurdles via predictive models in order to develop less processed products (e.g. products of less acidity or salt content) without compromising their safety and quality can be quickly obtained, allowing developers greater flexibility than

was hitherto possible.

1.3.2 Predictive Models

1.3.2.1 What are microbial models?

A microbial model in its simplest form is a simple mathematical description of a process. McMeekin *et al.* (2008), stated that “the model is often a simplified description of relationships between observations of the system (responses) and the factors that are believed to cause the observed responses”. Mathematical equations were used in food microbiology for the first time in the early twentieth century in order to describe the kinetics of pathogen destruction during the heat treatment of foods. However, McMeekin *et al.* (2000) mentioned that the dynamic invasion of predictive microbiology in the area of food lies in the early 1970s, using mathematical models for the identification of the potential toxin production by *C. botulinum*. The use of computers and statistical software programs highlighted the application of mathematical models as a very useful tool in studying the behaviour of microorganisms and predictive microbiology in a separate field of food microbiology.

In the current literature there is a wide range of strategies to develop predictive models in food microbiology. There are different types of problems (toxin production, life expectancy/ spoilage, development of pathogenic bacteria, microbial death kinetics), different types of models (kinetic models, probability models), various methods for data collection (classic method for measuring the microbial load, optical density, conductivity), and several ways to evaluate the models. The various stages of developing a mathematical model as presented by McMeekin *et al.* (1993) are the following:

1) Experimental design
2) Data collection and analysis
3) Mathematical description
4) Evaluation

As it has been stated in the literature, mathematical models should take into account the physiological state of cells through the h_0 or “the work to be done”

concept (Le Marc *et al*., 2010; Baranyi and Roberts, 1994; Robinson *et al*., 1998). Robinson *et al*. (1998) stated that it is more difficult to model the lag time than the growth rate because it is dependent not only on the growth conditions but the physiological state of the cells as well. The effect of sudden shifts in the environment of the bacteria is based on the "the work to be done" approach which is the effort the cells need to undertake to modify to the new environment and the rate at which this effort is accomplished (Belessi *et al*., 2011; Mellefont *et al*., 2003).

In predictive microbiology, a mathematical model is defined as the mathematical expression that describes the growth, survival, destruction or biochemical process that characterize an organism, associated with food. There are different ways of classifying microbial models. However, an absolute type of categorization has not yet been decided. A commonly accepted terminology and classification of patterns into groups that will refer to specific functions would make predictive microbiology more user friendly (Baranyi and Roberts, 1992). Classification of predictive models is based on the population behaviour that they describe and encompasses growth models, limits of growth (interface) models and inactivation models and are important elements in food process (McMeekin and Ross, 2002; Marks, 2008). Peleg (2006) stated that quantitative models can be classified as either empirical, fundamental, probabilistic, phenomenological, or population dynamic models.

Moreover, models can generally be classified as kinetic models or probability models i.e. whether the equation describes the characteristics of the kinetics of growth of the microorganism or studying the possibility of growth under different environmental conditions. Kinetic models determine the time required for a change in the density of the microbial population in relation to environmental factors, such as temperature, pH or a_w (McDonald and Sun, 1999). Kinetic models are also used to predict the kinetics of a microbial population, even under dynamic conditions, where the factors that affect the kinetics of growth of a microorganism vary with time (Zwietering *et al*., 1994). To create a kinetic model, the growth rate can be calculated and can subsequently be used to predict the growth of the microorganism based on the mathematical equation that described the rate. Another approach is to fit a

sigmoidal function to the growth data and the use this model to describe the effects of various environmental factors, using the parameters of the function. The selection of experimental data regarding the changes in the density of the microbial population in relation to specific environmental factors, such as temperature, pH or the value of a_w is important for the development of kinetic models. Kinetic models allow the prediction of the kinetic parameters of the growth curve (lag phase, generation time and growth rate) of a microbial population (Zwietering *et al*., 1991; Van Impe *et al*., 1995).

The probability models can determine the probability of a specific response which can take place under given conditions. These models are based on the relationship and interaction between a microbial population and environmental conditions in which it grows. Probability models do not provide information on the growth characteristics (growth rate) of a microbial population. However, probability changes with time, so probability models are a combination of probability and kinetics and this can make them confusing (McDonald and Sun, 1999).

Studies by Ratkowsky and Ross (1995) were based on a kinetic model for developing a probability model, which defines the limits of microbial growth. This model was developed using logistic regression and could predict the probability and the growth limits of the microbial population in different environmental conditions, which are inhibitory to microbial growth. The combination of probability and kinetics allows the integrated approach of the two types of mathematical models in hurdle technology (Ratkowsky and Ross, 1995).

Models can also be also classified as mechanistic or empirical. The former, describe those with a theoretical basis and the latter describe those without theoretical basis. Empirical models, such as the *modified* Gompertz equation, describe the experimental data as a mathematical relationship. Empirical models are mathematical equations, which are, often, easily implemented and express a process for a specific range of variables. The parameters obtained, in general, do not have biological meaning, however, relevant biological parameters can be defined from them such as generation times and lags. For

all these reasons, many researchers believe that these models do not provide knowledge on the mechanisms that characterise a biological process. In contrast, the development of mechanistic models requires the understanding of biological mechanisms and processes which are the basis of the cellular metabolism. Mechanistic models allow predictions from a simple hypothesis and are considered by most researchers superior to empirical mathematical models, but tend to be more complex (Van Impe *et al*., 1992; Zwietering *et al*., 1993).

1.3.2.2 Primary models

Whiting and Buchanan (1993) classified predictive models as primary, secondary and tertiary. The concept of the primary models is fundamental in predictive food microbiology. Primary models describe the change of population density with time in a specified environment and are depicted as microbial growth or death curves. This change is calculated, either directly by counting the microbial population and quantification of produced toxins or other metabolic products of the microorganism or indirectly by calculating indirect indicators of microbial growth, such as optical density and electrical conductivity measurements. The records of the change of microbial population in relation with time and the production of a growth curve are the experimental data on which the development of a primary model is based. The primary model enables prediction of growth of a particular microorganism and the kinetic parameters such as the generation time, the duration of the lag phase, the growth rate and maximum population density can be obtained (Whiting and Buchanan, 1993; 1994).

Primary models encompass growth models and inactivation models (Marks, 2008). In recent years, many bacterial growth curve models have been developed such as the three-phase linear (3-PLM), *modified* Gompertz, *modified* logistic, the lag-logistic, McKellar and Baranyi models. Also, survival models (classical linear models and nonlinear models) have been developed. McMeekin *et al*. (1993) reviews many growth models, some of the most important primary growth models are being discussed below.

There are several sigmoidal functions (such as the 3-PLM, *modified* Gompertz and Baranyi model) that are used to describe bacterial growth. Baranyi *et al.* (1993a) mentioned that "the typical representation of a bacterial batch culture is to plot the logarithm of the cell concentration against time and in most cases the result is a sigmoid curve". It is known that the bacteria grow exponentially. It is useful to plot the logarithm of the number of the bacterial population against time. The three phases of a typical bacterial growth curve are the lag phase, the exponential phase and the stationary phase (Figure 1-3). These three phases can be described by three parameters: the maximum specific growth rate (μ_{max}) which can be defined as the tangent in the inflection point, the lag time (λ) which is defined as the x- axis intercept of this tangent and the asymptote (A= log(cfu/ml)) which is the maximum value reached. Finally, growth curves may show a decline following the stationary phase which is called the death phase (Zwietering *et al*., 1990).

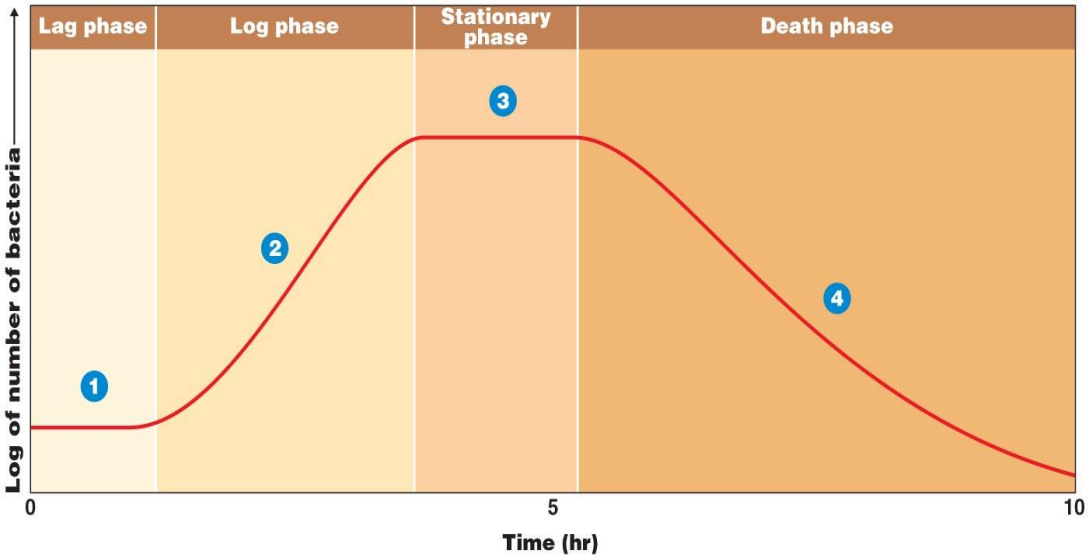


Figure 1-3 Typical representation of the four phases of the general microbial growth (Tortora *et al*., 2010)

The three-phase linear model

The 3-PLM is a simple primary model which divides the bacterial growth curves into three phases: the lag and stationary phases where the specific growth rate is zero (μ=0) and the exponential phase where the logarithm of

the bacterial population increases linearly with time (μ=constant).The death phase is not considered in the model. Figure 1-4 shows the graphic representation of the model.

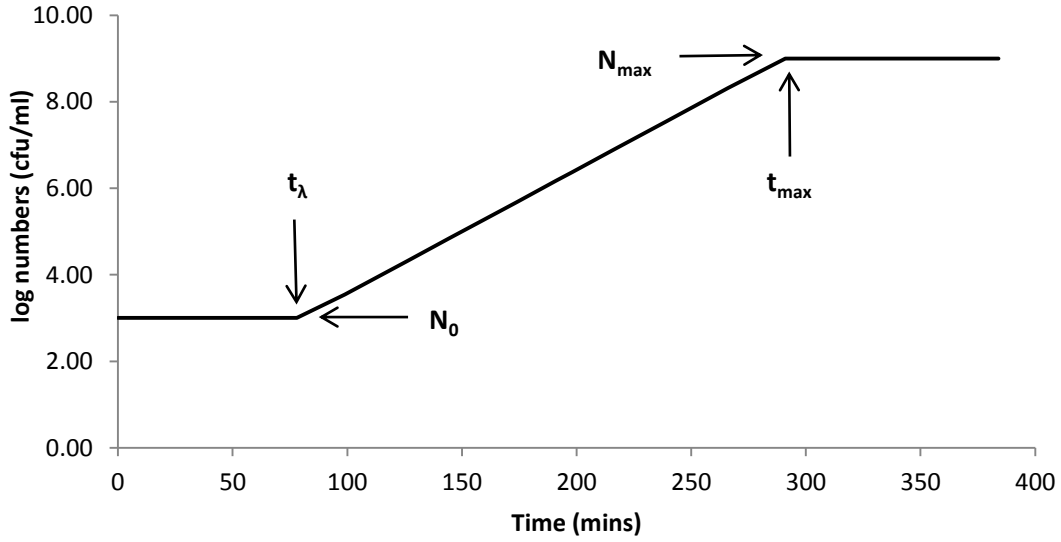


Figure 1-4 Graphic representation of the three-phase linear model (Buchanan *et al.*, 1997)

The 3-PLM is given by:

$$Lag\ phase: For\ t \leq t_\lambda, N_t = N_0$$

$$Exponential\ phase: For\ t_\lambda < t < t_{max}, N_t = N_0 + \mu(t - t_\lambda) \quad \textbf{(1-1)}$$

$$Stationary\ phase: For\ t \geq t_{max}, N_t = N_{max}$$

The model has four parameters: N_0 which is the logarithm of the initial bacterial population density, N_{max} which is the logarithm of the final bacterial population density, t_λ which represents the duration of the lag phase and t_{max} which is the time when the exponential phase ends.

Baranyi and Roberts (1995) mentioned that an equation can be considered as a model and not as an empirical fitting of data only if there is a physiological basis underling the relationship. In the study of Buchanan *et al.* (1997), the lag phase was considered to have two distinct periods. The first period is a period of adaptation and the second period is the metabolic period which is the time needed from the cell to generate energy and start its metabolism. Finally, the

model took into account the biological variability and the subdivision of the lag period and led to the need to reconcile the growth characteristics of bacterial populations.

The Gompertz and *modified* Gompertz model

The *modified* Gompertz curve consists of four phases which may be compared with the four phases of the microbial growth. Furthermore, there is an initial phase where no change occurs (lag phase), followed by a period of accelerating change, a period of decelerating change and finally a stationary period (Gibson *et al.*, 1987).

The *modified* Gompertz curve is given by:

$$L_{(t)} = A + Cexp\left(-exp\left(b(m-t)\right)\right) \quad \textbf{(1-2)}$$

The *modified* Gompertz curve has four parameters: $L_{(t)}$ is the log count of the number of bacteria at time t (in days), A is the asymptotic log count as t decreases indefinitely, C is the asymptotic amount of growth that occurs as t increases indefinitely, and b is the relative growth rate at m, where m is the time at which the absolute growth rate is a maximum (Gibson *et al.*, 1987). The original Gompertz model (Gompertz, 1825) has been used to study the growth of tumours and has a mechanistic basis. The *modified* Gompertz model uses the logarithm of the microbial numbers and is considered to be empirical model (McMeekin *et al*., 1993) since the derivation of the Gompertz cannot be equated with the use of log numbers of microbes. From this equation the growth rate (mu), the lag time (λ) and the generation time (GT) are given by:

$$mu = \frac{bC}{e} \quad \textbf{(1-3)}$$

$$\lambda = m - \frac{1}{b} \quad \textbf{(1-4)}$$

$$GT = \frac{\log(2)e}{bC} \quad \textbf{(1-5)}$$

The *modified* Gompertz model contains mathematical parameters (A, C, b and m) and it was re-parameterised with parameters which have biological

meaning as described by Zwietering *et al*. (1990). The re-parameterised *modified* Gompertz model is given by:

$$L_{(t)} = A\ exp\left(-exp\left(\frac{\mu e}{A}(\lambda - t) + 1\right)\right) \quad \textbf{(1-6)}$$

Where $L_{(t)}$ is the log count of bacteria at time t, A is the asymptotic log count as t decreases indefinitely, λ is the lag time and μ is the maximum specific growth rate. The re-parameterisation allows the direct calculation of the confidence intervals for each of the biologically important, but defined parameters.

The Logistic and *modified* logistic model

The *modified* logistic curve is very similar to the *modified* Gompertz model and was described by Gibson *et al.* (1987). Also, it has been noted by the authors that the only difference with the *modified* Gompertz model is that the *modified* logistic model is symmetric about m (the time when the absolute growth rate is maximum) while the *modified* Gompertz is not.

The *modified* logistic curve is given by:

$$L_{(t)} = A + C/\left(1 + exp\left(-b(t - m)\right)\right) \quad \textbf{(1-7)}$$

Where $L_{(t)}$ is the log count of the number of bacteria at time t (in days), A is the asymptotic log count as t decreases indefinitely, C is the asymptotic amount of growth that occurs as t increases indefinitely, and b is the relative growth rate at m, where m is the time at which the absolute growth rate is a maximum (Gibson *et al.*, 1987). The original logistic model (Jason, 1983) uses microbial numbers and is considered to be a mechanistic model while the *modified* logistic model uses the logarithm of the microbial numbers and is considered to be empirical model (McMeekin *et al*., 1993). From this equation the growth rate (mu), the lag time (λ) and the generation time (GT) are given by:

$$mu = \frac{bC}{4} \quad \textbf{(1-8)}$$

$$\lambda = m - \frac{2}{b} \quad \textbf{(1-9)}$$

$$GT = \frac{\log(2)}{bC} \quad \textbf{(1-10)}$$

The *modified* logistic model contains mathematical parameters (A, C, b and m) and it was re-parameterised with parameters which have biological meaning as described by Zwietering *et al*. (1990). The re-parameterised *modified* logistic model is given by:

$$L_{(t)} = \frac{A}{\left(1+exp\left(\frac{4\mu}{A}(\lambda-t)+2\right)\right)} \quad \textbf{(1-11)}$$

Where $L_{(t)}$ is the log count of bacteria at time t, A is the asymptotic log count as t decreases indefinitely, λ is the lag time and μ is the maximum specific growth rate.

The Baranyi model

The Baranyi model is a mechanistic model which describes the lag as the process of adjustment to the new environment. The model distinguishes the pre-inoculation environment (E1) from the actual (post-inoculation) environment (E2). By these terms, this model describes the lag as the process of adjustment to the new environment. Moreover, the terms “adjustment function” and “potential growth” are introduced and as it is stated that: “two features of our concept are that the definition of lag is independent of the shape of the growth curve and the effect of the previous environment is separated from that of the present environment” (Baranyi *et al.*, 1993a).

The Baranyi model is given by :

$$y_t = y_0 + \mu_{max}A(t) - \frac{1}{m}\ln\left(1 + \frac{e^{m\mu_{max}A(t)}-1}{e^{m(y_{max}-y_o)}}\right) \quad \textbf{(1-12)}$$

Where $y_t=\ln x_t$, is the natural logarithm of the cell concentration, $y_o=\ln x(t_o)$, is the natural logarithm of the cell concentration at $t=t_o$, $y_{max}= \ln x_{max}$, is the natural logarithm of the maximum cell concentration and μ_{max} is the specific growth rate. The parameter m characterizes the curvature before the stationary phase.

The function A_t represents a gradual delay in time and is given by:

$$A_t = t + \frac{1}{\nu}\ln\left(\frac{e^{-\nu t}+q_0}{1+q_0}\right), \qquad \textbf{(1-13)}$$

$$where\ h_0 = \ln\left(1+\frac{1}{q_0}\right) = \mu_{max}\lambda$$

Where h_0 is a transformed physiological state parameter, q_0 is a measure of the initial physiological state of the cells and v is the rate of decrease of the limiting substrate, generally assumed to be equal to μ_{max}. For the curvature parameters Baranyi *et al.* (1995) suggest $v=\mu_{max}$ and m=1. When m=1 the function reduces to the logistic model.

The function A (t) in combination with the conditions after inoculation, allow the prediction of duration of the lag phase. If the specific growth rate follows the environmental changes immediately as they occur, this model can describe the kinetics of microbial growth where factors such as the pH, a_w and the temperature change with time. The modelling systems, which are in a dynamic environment are one of the advantages of this model. The Baranyi model has been used, evaluated and compared in different applications. In many cases compared with other primary models, such as the Gompertz equation, it gave satisfactory results (McDonald and Sun, 1999).

1.3.2.3 Secondary models

Secondary models describe the effect of that various environmental factors such as temperature, pH and a_w have on the kinetic parameters that characterise the growth of a microorganism. Essentially, they indicate the change of the parameters of primary models with respect to changes in the environmental factors such as temperature, storage atmosphere and the intrinsic factors such as pH, a_w and organic acids. Although, secondary models encompass models to explain changes in growth rate and lag time with changes in environmental conditions, secondary models are also available for inactivation and also for models dealing with Growth/No growth boundaries (G/NG), i.e. probability models (McDonald and Sun, 1999; Tienungoon *et al*., 2000; McMeekin *et al*., 1993; Whiting, 1995).

Particular types of models have been described as the square root type models, the Arrhenius models, the Gamma models, polynomial models and the cardinal parameter models. Ratkowsky *et al*. (1982) in order to overcome the problem of the Arrhenius equation which cannot describe the effect of suboptimal temperature on the growth rates of microorganisms introduced the square root type or Ratkowsky type model which uses the theoretical minimum temperature for growth (T_{min}). In 1983 Ratkowsky *et al*. expanded this model to include the entire biokinetic range of temperatures. Since then numerous square root models have been developed using different environmental factors such as different values of a_w (McMeekin *et al*., 1987), pH (Adams *et al*. 1991), temperature, pH and a_w (Wijtzes *et al*., 1995; 2001) and temperature, a_w, pH and lactic acid (Ross *et al*., 2003).

Zwietering *et al*. (1992) introduced the Gamma concept which relies on the idea that the environmental factors which affect the growth rate act independently and that the effect of the growth rate of any factor can be expressed as a fraction of the maximum growth rate (McKellar and Lu, 2003). The Gamma factor (γ) is defined as the fraction of the growth rate at actual environmental conditions to the growth rate at optimal environmental conditions and the combined effect of several factors is then determined by the multiplication of their Gamma factors. Augustin and Carlier (2000) collected and put together in one model literature data and observations of 15 environmental factors in foods that affect the growth rate of *L. monocytogenes*.

The cardinal parameter models (CPMs) are another important group of empirical secondary models which has been used from several authors in the literature (Augustin and Carlier, 2000a; 2000b; Le Marc *et al*., 2002; Pouillot *et al*., 2003). The CPMs use parameters that have a biological or graphical interpretation and the concept which they rely on is that the inhibitory effect of different environmental factors is multiplicative (McKellar and Lu, 2003). In addition, the CPMs encompass a discrete term for each environmental factor, with each term expressed as the growth rate relative to that when that factor is optimal (McKellar and Lu, 2001).

The Arrhenius models are based on reaction kinetics but include terms to account for the observed deviations and are divided into those which are mechanistic modifications based on the hypothesis that there is a single, catalysed, rate limiting reaction in any microorganism (McMeekin *et al*., 1993; Ross and McMeekin, 1994; Ratkowsky *et al*., 1991) and those which are empirical modifications (Davey, 1994; Daughtry *et al*., 1997). Polynomial models have been applied in predictive microbiology as secondary models (McClure *et al*., 1993, Pin *et al*., 2000) and despite the fact that they are easy to fit to data, they lack biological interpretation.

Probability models were first explored in the 1970s (Genigiorgis, 1981) and in the 1990s the need to manage the risk to consumers from certain pathogens led to the re-development of the Growth/No Growth models (McKellar and Lu, 2003). The problem of listeriosis triggered the development of such models (Parente *et al*., 1998; Tienungoon *et al*., 2000) and the logistic regression technique was used to develop those models. The importance of such models for the production of safe and shelf stable food and as a mean of empowering the hurdle concept has been discussed by many authors (Masasa and Baranyi, 2000; McMeekin *et al*., 2000; Ratkowsky and Ross, 1995). In the literature there are several studies regarding the G/NG conditions for several pathogenic bacteria such as *L. monocytogenes* (Ross *et al*., 2000; Tienungoon *et al*., 2000; Koutsoumanis and Sofos, 2005), *E. coli* (Skandamis *et al*., 2007; McKellar and Lu, 2001; Salter *et al*., 2000) and *Salmonella* (Koutsoumanis *et al*., 2004). McKellar and Lu (2003) have summarised some examples of secondary models.

The study of Chorin *et al*. (1997) can be used as an example for primary and secondary modelling. In particular, Chorin *et al*. (1997) modelled the growth of *Bacillus cereus* as a function of temperature, pH and a_w from turbidimetric data. A “calibration model” expressing colony forming units (cfu) in optical density (O.D) was constructed as a function of a_w and the data obtained from their studies were fitted with the *modified* Gompertz equation in order to calculate the growth parameters (primary modelling). A growth rate model and a lag time model (polynomials which cannot be extrapolated outside the

experimental range) were then constructed, taking into account the effect of temperature, pH and a_w (equation (1-14) and (1-15), respectively).

$$\mu = 4.010 - 0.090pH.T + 0.098a_w.pH.T - 4.788{a_w}^2 \quad \textbf{(1-14)}$$

$$\lambda = 1108.6 - 1109.6a_w - 4.6pH.T + 4.6a_w.pH.T \quad \textbf{(1-15)}$$

Where μ is the growth rate in log (cfu) per hour and λ is the lag time in hours.

1.3.2.4 Tertiary models

Tertiary models incorporate primary and secondary models in software, that is, they are application tools such as software packages and expert systems which have the intension of allowing non-specialists in predictive modelling to access data and model predictions. Some major modelling programs are the Pathogen Modelling Programme (USA) which consists of 37 models of growth, survival and inactivation, which is frequently updated and has been available free of charge during the last 15 years, the Growth Predictor (UK) which is based on data previously used in the Food Micromodel software and includes 18 models for growth of pathogenic bacteria and has been available free of charge since 2003 and the ComBase (UK, USA) with information on growth and inactivation of microorganisms (about 48000 growth/inactivation curves) since 2003. In 1999, an extensive decision support system called Sym'Previus started in France which was funded by French ministries, food industries and technical institutes in order to meet food industry needs regarding food safety and quality management. The Sym'Previus includes a database with growth and inactivation responses of microorganisms in foods and predictive models for growth and inactivation of pathogenic bacteria and some spoilage microorganisms and is available online. Also, the Microbial Responses Viewer (MRV) by Koseki (2009), which is a ComBase derived database consisting of G/NG data from 19 microorganisms where their growth rate was modelled as a function of temperature, pH and a_w using a Poisson log-linear model. The Unified Growth Prediction Model (UGPM) by Psomas *et al*. (2011), which applies the Baranyi and Roberts model (1994) combined with a secondary temperature model in order to predict (simulate) the growth of the microorganisms under dynamic and static temperature conditions. In

addition, Dalgaard *et al*. (2002) developed the Seafood Spoilage Predictor (SSP) software which encompasses kinetic models for growth of specific spoilage microorganisms and empirical relative rates of spoilage models. The ultimate tests for predictive microbiology software are comparisons of model predictions with observations of microorganism behaviour in food. To make these comparisons with large data sets, the data recording format must be standardised. Standardisation refers not to the computational platform (such as the type of spreadsheet used) but rather the methodology for classifying and formatting microbiological data. Without this conformity, any attempt to compile data from various sources would result in a data dump rather than a structured database. Furthermore, a uniform system of physical, chemical and biological units and associated terminology must be used to facilitate comparisons among data sets. The aforementioned have made predictive microbiology a powerful tool for food industry and research (Whiting and Buchanan, 1993; 1994). The classification type by Whiting and Buchanan, (1993), into primary, secondary and tertiary is more convenient as the characterization and grouping is allowed for the majority of model types (McDonald and Sun, 1999).

1.3.2.5 Utilisation of microbial models

Marks (2008) discussed some key limitations in the application of microbial models in foods. In particular, it was stated that most of the models used are broth based models which are then applied to real foods and so data from tests conducted in real food may be of limited value. Also, it was stated that the available growth/inactivation data will never be able to cover the whole domain of food safety system. Another key limitation for the use of microbial models is the lack of standard practical methodologies (different treatment protocols, variability of measuring bacterial populations) as well as statistical methodologies for analysing the experimental data (logarithmic transformations before the fitting of models or nonlinear regression techniques).

Moreover, the terms “variability” and “uncertainty” are very important in predictive microbiology. The term “variability” describes the scatter of a

dataset and is commonly measured by three criteria: range, variance and standard deviation. Range is the difference between the largest and the smallest value in the dataset. Variance is the mean of the squares of all the deviation scores for a dataset and represents the amount of deviation of the whole dataset from the mean. The standard deviation is the square root of the variance and shows the deviation from the mean. On the other hand, all the microbial models involve a degree of uncertainty. The original experimental error (variance), uncertainty in the primary model form and regression and uncertainty in the secondary model and fitting procedures, are included in the term uncertainty (Marks, 2008).

Last but not least, the integration from a microbial model into a process model is essential in order to link the microbiological and physical components of process models (Marks, 2008). Validation is an essential part for the applicability of predictive models. This term refers to the comparison of the predictions of the model with the observed responses under conditions encountered in the food chain. In particular, validation aims to build confidence in the model. This is of great importance especially if the model is intended for use by the food industry (Manios *et al.*, 2009).

1.3.3 Optical density versus traditional plate counts and predictive microbiology

The determination of growth rates and lag times has been the subject of many studies within the literature and there are many models which have been developed to determine growth rates and lag times. Additionally, another area within predictive microbiology which has seen a large amount of interest has been the comparison between the traditional plate counts method against rapid methods such turbidimetry which measures growth as a function of optical density (O.D).

1.3.3.1 Development of rapid methods

Monitoring is one of the most important control points in the prevention of diseases by foodborne pathogens. To control foodborne pathogens in food products effective detection and inspection methods are necessary. As it has been mentioned: “Conventional microbiological methods have been a

standard practice for the detection and the identification of pathogens in food for nearly one century and continue to be a reliable standard for ensuring food safety" (Yang and Bashir, 2008). However, the conventional methods build almost exclusively upon the use of specific agar media to isolate and enumerate viable bacterial cells in samples. This method usually includes microbiological culturing and isolation of the pathogen, followed by confirmation with biochemical and/or serological tests, taking up to 5 to 7 days to get a confirmed result for a particular pathogenic organism (Swaminathan and Feng, 1994; Vasavada, 1997). Even if the conventional methods are reliable, they are time consuming and labour intensive and are therefore not suitable for modern food quality assurance to make a timely response to possible risks (Yang and Bashir, 2008). In order to obtain sufficient data using the traditional methods it may take several days of work. The development of rapid, sensitive and specific methods to detect foodborne pathogenic bacteria is a major factor for effective practices which ensure food safety and security. As a result, over the past 25 years numerous novel methods which offer new possibilities, they are cheaper, automated, accurate and most important they are rapid have been developed to reduce the assay time. However, rapid methods have high detection limits and they may exhibit false positive results.

Rasch (2004) reported some examples of these methods, like turbidity, flow cytometry, microscopic methods etc. In particular, the turbidity method measures the O.D of a cell suspension and has been used by many scientists in the area of predictive microbiology for many years (e.g. Monod, 1941). Dalgraard and Koutsoumanis (2001) stated that turbidimetric instruments such as the Bioscreen microbiological analyser might be another way instead of the viable counts in order to study the bacterial growth since O.D measurements give a real time measure of the bacterial population and these machines allow a high throughput. It was also mentioned that despite the high threshold detection of turbidimetric devices which is the most important limitation of this method, the measurements have practical significance when dealing with bacteria in high cell densities. Predictive modelling requires the collection of adequate data. The advantage of these rapid methods is that high numbers of

experiments can be set up in a short period of time conversely with the time-consuming nature of plate counts.

In particular, in food microbiology the Bioscreen microbiological analyser has been used for a number of different applications such as the construction of kinetic models (McClure *et al.,* 1993), the study of the effect of different conditions on growth (McClure *et al.*, 1994; Korkeala *et al.*, 1992; Francois *et al.*, 2005; 2006), the determination of the bacterial growth rates (Dalgaard *et al*., 1994; Dalgaard and Koutsoumanis, 2001), the determination of individual cell lag times (Guiller *et al*., 2006; Guillier and Augustin, 2006; Koutsoumanis, 2008; Dupont and Augustin, 2009; Manios *et al*., 2013), the development of isolating single cells protocols (Francois *et al*., 2003; Standaert *et al*., 2005) the measurement of the recovery times of injured cells (Stephens *et al.*, 1997) as well as the determination of the G/NG boundaries of several foodborne pathogens (Ross *et al*., 2000; Skandamis *et al*., 2007; Koutsoumanis *et al*., 2004; Tienungoon *et al*., 2000). Furthermore, Lambert and Pearson (2000) have developed a simple technique and a method for susceptibility testing. They obtained turbidometric data and produced O.D/time curves. From these data the minimum inhibitory concentration (MIC) of an inhibitor could be found. The technique was based on the comparison of the area under the O.D against time curve of the areas of the test with the control. They observed that "as the amount of preservative in the well increases, the effect on the growth also increases". However, the interpretation of the results is the most important and at the same the most difficult part when using O.D methods.

There are some authors who do not use any type of calibration between O.D and viable counts and directly fit primary models such as the *modified* Gompertz or the logistic model to O.D data (Begot *et al*., 1996; Cheroutre-Vialette *et al*., 1998; Cheroutre-Vialette and Lebert, 2000a; 2000b; Dalgaard and Koutsoumanis, 2001; Cheroutre-Vialette and Lebert, 2002). Also, in the literature there are several methods that can be applied in order to define the relationship between the measured O.D and the viable cell counts. There are authors who have used linear models between O.D and viable counts based on the Lambert and Beer Law (Lack *et al.*, 1999), quadratic models (McClure *et al.*, 1993) as well as cubic models (Stephens *et al.* 1997). Augustin *et al*.

(1999) used polynomials of order 3 and order 1 to calibrate O.D against viable counts. Best fits were obtained from the polynomials of order 3 but using the average values of the parameter estimates at the different temperatures examined, the best fit was obtained from polynomials of order 1. Dalgaard *et al*. (1994) used two equivalent methods for calibration; one in which cells from the stationary-phase were diluted to the appropriate O.D, and the other in which samples for O.D and viable count measurements were taken during growth. A calibration factor was then used for turbidimetric estimates, different for each primary model (logistic or *modified* Gompertz model) and independent of the maximum specific growth rate and the inoculation level. A last method of calibration used in the literature in the logarithmic transformations for both O.D values and the viable counts in order to normalise the variance (Francois *et al.*, 2003; 2005) or the natural logarithmic transformations (Chorin *et al*., 1997).

1.4 Hypothesis and objectives of the project

The hypothesis, on which the work stands, is that the use of micro-titre plates with multiple inocula allow the investigation of a wealth of phenomena - such as the accurate determination of growth parameters, the investigation of mild temperature shifts as well as the evaluation of a mild thermal injury.

In particular, it was hypothesised that O.D data obtained from the Bioscreen microbiological analyser, under different environmental conditions, could be modelled by using existed primary models and thus accurate growth rates and lag times could be obtained from turbidimetric measurements (TTD method). Also, for a mild process which has temperature as the major microbial injury step, the effect of the other combined hurdles in preventing growth of the injured organisms must be understood. The latter means that the inoculum size dependency of the time to growth must also be fully understood. This essentially links injury steps with the potential for growth. Also, it was hypothesised that the TTD method and the traditional plate count method should be considered as two methods describing the same phenomenon of microbial growth, done in a different fashion and not as complementary

methods. To investigate this idea it was also proposed to compare the growth parameters of those two methods.

Specific research objectives associated with the hypothesis of the project are:

1. Obtainment of the growth kinetics of *Listeria monocytogenes, Salmonella* Typhimurium and *Escherichia coli* using the Bioscreen microbiological analyser;
2. Evaluation of the G/NG boundary for a given set of environmental factors common to processed foods;
3. Evaluation of the minimum inhibitory concentrations (MIC) of the microorganisms using different hurdles;
4. Evaluation of mild temperature shifts;
5. Evaluation of a mild heat injury;
6. Comparison between the TTD method against the traditional plate counts;
7. Communication of findings to clients (presentations, reports, manuscripts for evaluation).

2 Materials and methods

2.1 Microbes and identification

The microorganisms used in this study were: *Listeria monocytogenes* 252 (terrine isolate), *Listeria monocytogenes* 271 (ham isolate), *Listeria monocytogenes* 177 (ice-cream isolate), *Listeria monocytogenes* 39 (ScottA-ATCC 49594), (all donated by *Nestlé* Research, Lausanne), *Escherichia coli* ATCC 11229 and *Salmonella enterica* subsp. enterica- *Salmonella* Typhimurium ATCC 53648.

2.1.1 Culture maintenance and preparation

Cultures of the microorganisms were kept in tubes on glass balls at -80°C and new slopes on tryptone soya agar (TSA) were prepared every month. From a previously prepared and stored slope on TSA of the pure culture of the microorganisms, a portion was removed with a sterile loop, transferred into a conical flask containing 80 ml tryptone soya broth (TSB) and incubated with shaking (shaking incubator KS 4000 control, 150 rpm) at 30°C or 37°C overnight. The resulting culture was split into four portions and centrifuged (CENTAUR 2, MSE) at 500 g for 10 min. Two of the resulting pellets were re-suspended in TSB (3 ml) and pooled. The re-suspended culture (1 ml) was transferred into TSB (9 ml) in a universal tube and mixed thoroughly; 1 ml of this suspension was diluted in TSB to obtain a standard optical density (O.D) of approximately 0.5 with a 1 cm path length at 600 nm (M350 Double Beam U.V. Visible Spectrometer). From the standardised culture a series of decimal dilutions were prepared in TSB (labelled 0 to −9).

2.1.2 API identification

L. monocytogenes strains are industrial isolates characterised by *Nestlé* (by ribo print). *E. coli* and *S.* Typhimurium were identified using the Analytical Profile Index (API tests). In particular, API 20E is a standardised identification system for Enterobacteriaceae and other Gram negative bacteria which consists of 21 biochemical tests. A bacterial suspension was prepared in 5ml of sterile distilled water from a pure culture and the strip was inoculated with the bacterial suspension. For the tests CIT (citrate utilisation), VP (acetoin

production) and GEL (gelatinase) the tube and the cupule was filled while for the tests ADH (arginine dihydrolase), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), H_2S (hydrogen sulphide production) and URE (urease) anaerobiosis was created by overlaying with mineral oil. After 24h of incubation at 37°C three tests required the addition of reagents. TDA test required the addition of one drop of TDA reagent, IND test required the addition of one drop of JAMES reagent and VP test required the addition of one drop of VP 1 and one drop of VP 2 reagent. The metabolism produces changes in the colour of the tests which can be characterised as positive or negative using the reading table. According to the reactions (number of positive and negative tests) the identification obtained with the numerical profile using the database (V 4.1) with the api*web*™ identification software (Appendix B).

2.2 Growth curves (Traditional method)

In the literature, the most common way of obtaining growth rates and lag times is the construction of growth curves using the traditional plate counts. Conversely, we have been developing the use of O.D for obtaining growth parameters using time to detection (TTD) data. In order, to assess the rapid method used in this study, traditional growth curves were constructed and compared with the results obtained from the Bioscreen.

In particular, traditional growth curves were made by using 0.5, 3, 6 and/or 9% sodium chloride (NaCl) at 30°C for *L. monocytogenes* 252, *L. monocytogenes* 39, *S.* Typhimurium and *E. coli* using the traditional plate counts. The strains were grown overnight in flasks containing 80 ml TSB shaking at 30°C. The cells were harvested, centrifuged to a pellet at 3000 rpm for 10 min. The resulting cell pellets were resuspended in TSB (3ml). A standard inoculum was produced by diluting the culture to an O.D=0.5 at 600 nm. This standardised culture was then further diluted to produce the starting inoculum of approximately 1×10^5 cfu/ml. Then 1 ml of this inoculum was transferred in flasks which had 99 ml TSB with 0.5, 3, 6 or 9% NaCl, each time. These flasks were then incubated at 30°C and samples were taken at different time intervals and spread onto TSA plates. The plates were then incubated at

30°C, for 24-48 hours. Simultaneously, a Bioscreen experiment was set up under the same conditions, according to the procedure described in paragraph 2.3.5.1.

2.3 Bioscreen analysis

2.3.1 Bioscreen microbiological analyser

The Bioscreen C Reader System (Figure 2-1) is a fully automated instrument which consists of: The Bioscreen C reader which includes an incubator and a measurement unit, a computer, honeycomb plates and the EZE experiment software.

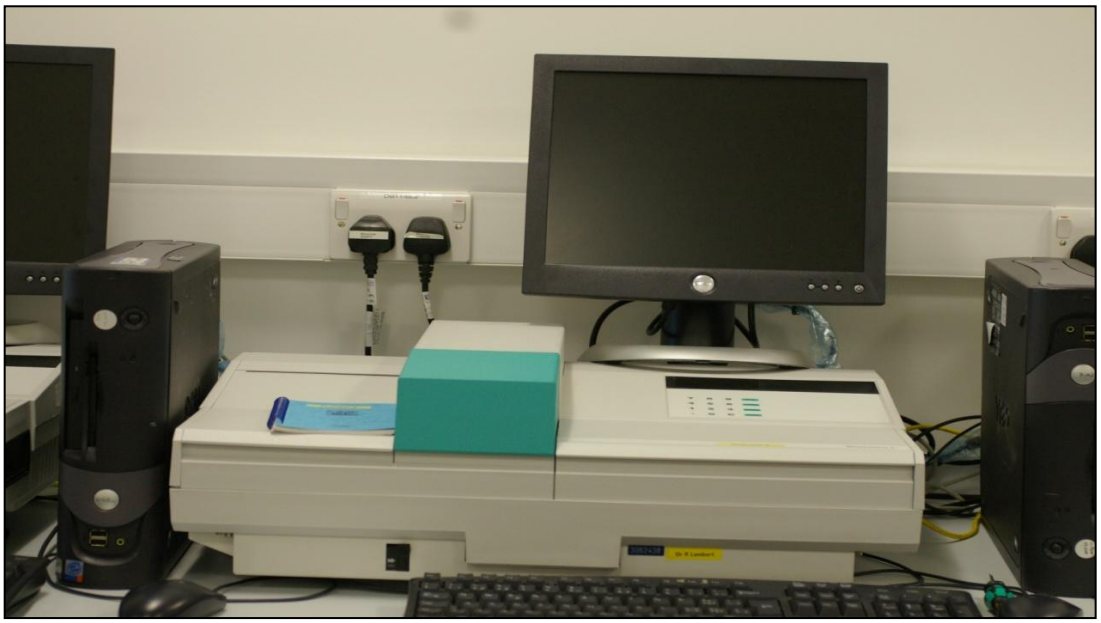


Figure 2-1 The Bioscreen C reader system

The incubation temperature can be set from 1°C to 60°C in steps of 0.1°C. Bioscreen C monitors the growth of microorganisms by measuring the turbidity of liquid growth medium in the well. The measurement is done kinetically using the principle of vertical photometry. In this technique a light beam passes up through the bottom of the plate well, through the sample suspension to the detector. All functions are controlled by computer software according to the parameters entered by the user - (see Appendix A).

2.3.2 Micro-array plates (Honeycomb plates)

Each honeycomb plate consists of 100 wells (Figure 2-2) and the instrument can handle two plates at time, so the maximum capacity is 200 samples per run. Each well of the plate is an individual test vessel, so 200 microbiological growth experiments can be performed in a single run. The plate was designed to both give the most even temperature possible across the whole plate, as well as to eliminate evaporation and condensation, common problems with the conventional 96-well plates.

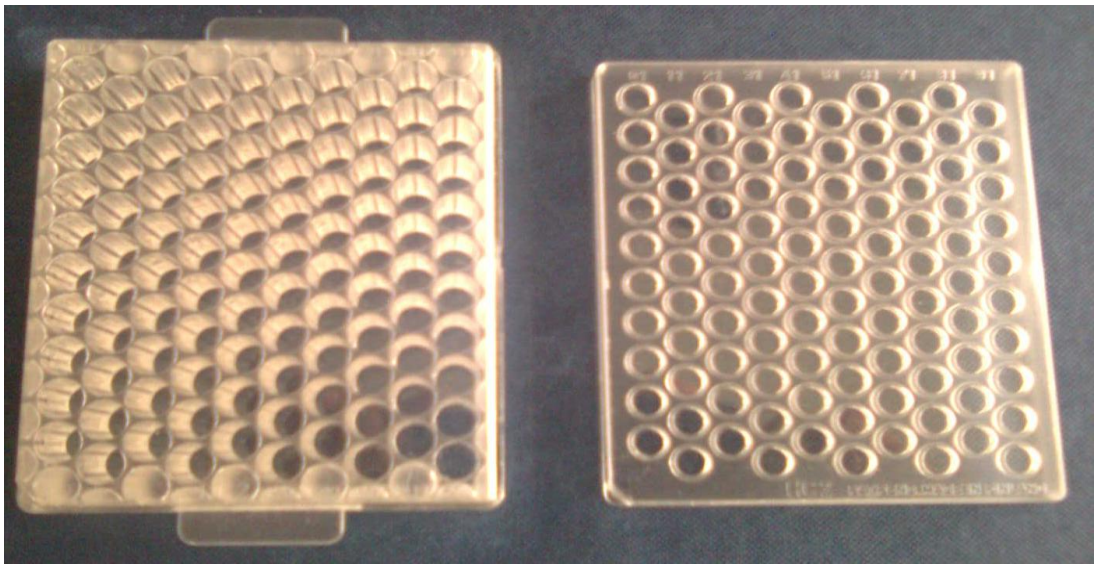


Figure 2-2 The Bioscreen microarray (honeycomb) plate with its lid

2.3.3 General overview of plate filling

Studies on growth rates and lags were carried out using tenfold – half fold dilution or half fold-half fold or tenfold dilution only (to extinction). Studies on the effects of multiple NaCl/pH or on the determination of the MIC_{NaCl} and MIC_{pH} used a single inoculum with the inhibitors diluted in particular ways.

2.3.4 Detailed experimental designs (preparation of micro-array plates)

Each well in the Bioscreen micro-array plates was filled as follow: all wells except column 10 received 200µl of growth broth (TSB). The wells of column 10 were given 400µl of the appropriate serial dilutions, with the highest inoculum (the zero dilution) in well 100. Using a multi-pipette, 200µl were removed from each well of column 10 and transferred into the wells of column

9, mixed by repeated syringing, and then 200µl were removed (using new tips) from the wells of column 9 and transferred to column 8 etc. This was repeated across the plate discarding 200µl after the final mixing in column 1. The O.D of the wells was read at 600nm every ten minutes at 30°C and/or 37°C. Theoretically for an initial inoculum of 1×10^9 cfu/ml, this method will give a range from 9 $\log_{10}$ to −2.7 $\log_{10}$ cfu/ml. The O.D of a sample in the Bioscreen is dependent on the volume used: a standard O.D of 0.5 measured in the spectrophotometer has an O.D of 0.29 at 600 nm for a volume of 200 µl in the Bioscreen. Plates were typically incubated for 1 to 6 days, with the O.D of the wells being read at 600 nm every 10 min. In some experiments, instead of using ten different initial inocula in the last column of the honeycomb plate, five inocula were used (labelled 0 to -4) twice resulting in two replicates at the same plate.

From the -5, -6 and -7 decimal dilutions, 0.1 ml of each was transferred and spread onto previously prepared TSA plates in triplicate and incubated at 30°C or 37°C for 2 days. Plates with 25-300 colonies per plate were counted (Jongerburger *et al*., 2010) and the approximate log number of the initial (zero dilution) culture was calculated.

2.3.5 Inoculum size studies using different hurdles

2.3.5.1 Effect of Sodium chloride

The growth of the microorganisms was studied at 30°C and/or 37°C in 0.5, 3, 6 and/or 9% NaCl. The preparation of the cultures and the fill of the Bioscreen micro-array plates were made as described in paragraphs 2.1.1 and 2.3.4, respectively. The plate was then incubated at the desired temperature (30°C and/or 37°C) for 1-6 days, with the analyser recording the O.D of each well at 600 nm every ten minutes. Experiments were carried out in duplicate.

2.3.5.2 Effect of pH

The growth of *S.* Typhimurium and *E. coli* was studied at 30°C in different pH (6.57, 5.68, 5.10 and 4.58). The pH was adjusted using a pH_meter (HANNA instruments HI 8519N) with hydrochloric acid (HCl, 1M) prior to autoclaving and was checked following sterilisation. The inocula were prepared each time

in the appropriate pH and the Bioscreen plates filled as described in paragraphs 2.1.1 and 2.3.4. The plates were then incubated at the desired temperature (30°C) for 1-5 days, with the analyser recording the O.D of each well at 600 nm every ten minutes. Experiments were carried out in duplicate.

L. monocytogenes 252 was tested in a range of 30 different pH in TSB (pH 7.05 to 3.46 adjusted with filter sterilised HCl (0.01M) in approximately 0.35 pH unit intervals each with 3 replicates per plate and done in duplicate on separate machines against a single inoculum size (approximately 10^5 cfu/ml) at 30°C.

2.3.5.3 Effect of combined NaCl and pH

The growth of *S.* Typhimurium and *E. coli* was studied at 30°C in different NaCl-pH concentrations. The combinations used derived from the studies 2.3.5.1 and 2.3.5.2. The inocula prepared each time in the appropriate NaCl-pH concentration and the Bioscreen plates filled as described in paragraphs 2.1.1 and 2.3.4, respectively. The plates were then incubated at the desired temperature (30°C) for 2-6 days, with the analyser recording the O.D of each well at 600 nm every ten minutes. Experiments were carried out in duplicate.

2.3.6 Calculation of minimum inhibitory concentration using NaCl and pH

2.3.6.1 Minimum inhibitory concentration in NaCl (MIC_{NaCl})

L. monocytogenes strains were examined in a range of 0.5-16.625% NaCl (typical target percentage of NaCl concentrations: 0.5, 1.25, 2, 3.125, 4.25, 5, 6.125, 7.25, 8, 9.125, 9.875, 10.625, 11.375, 12.125, 12.875, 13.625, 14.375, 15.125, 15.875 and 16.625% NaCl). *E. coli* and *S.* Typhimurium were examined in a range of 0.5-9.875% NaCl (typical target percentage of NaCl concentrations: 0.5, 1.25, 2, 2.75, 3.5, 4.25, 5, 5.375, 5.75, 6.125, 6.5, 6.875, 7.25, 7.625, 8, 8.375, 8.75, 9.125, 9.5, and 9.875% NaCl). Two honeycomb plates were used for each experiment. Each column of the honeycomb plate filled with 150µl of the appropriate NaCl concentration resulting in ten replicates for each NaCl concentration. Then 50µl of a particular inoculum (inoculum from dilution labelled -2, approximately 10^7 cfu/ml for *L.*

monocytogenes strains and inoculum from dilution labelled -5, approximately 10^4 cfu/ml for *E. coli* and *S*. Typhimurium) was added in every well resulting in a final volume of 200µl. The plates were then incubated in the Bioscreen at 30°C and/or 37°C for 7 days, with the analyser recording the O.D of each well at 600 nm every ten minutes.

2.3.6.2 Minimum inhibitory concentration in pH (MIC_{pH})

L. monocytogenes strains were examined in a range of pH: 3.46-7.02 (typical target pH were 7.02, 6.95, 6.78, 6.65, 6.32, 6.18, 6.07, 6.03, 5.87, 5.76, 5.65, 5.57, 5.51, 5.35, 5.03, 4.95, 4.88, 4.68, 4.42, 4.35, 4.28, 4.14, 4.05, 3.97, 3.91, 3.87, 3.76, 3.66, 3.54 and 3.46). One plate was used for each experiment. Each well of the honeycomb plate was filled with 200µl of the appropriate pH concentration (three replicates per pH). Then 50µl of a particular inoculum (approximately 10^5 cfu/ml) was added in every well resulting in a final volume of 250µl. The plate was then incubated in the Bioscreen at 30°C for 3 days.

E. coli and *S.* Typhimurium were examined in a range of pH: 3.35-7.14 (typical target pH were 7.14, 6.95, 6.82, 5.58, 6.38, 6.22, 6.07, 5.95, 5.80, 5.64, 5.43, 5.23, 5.01, 4.79, 4.59, 4.38, 4.16, 3.95, 3.75 and 3.35). Two plates were used for each experiment. Each column of the honeycomb plate filled with 180µl of the appropriate pH concentration (ten replicates per pH). Then 20µl of a particular inoculum (inoculum from dilution labelled -3, approximately 10^6 cfu/ml) was added in every well resulting in a final volume of 200µl. The plates were then incubated in the Bioscreen at 30°C for 6 days.

2.3.7 Combined inhibitors (NaCl-pH)

E. coli and *S.* Typhimurium were examined in a range of combinations of pH-NaCl concentrations. Ten solutions with 0.5% NaCl and different pH were prepared (typical target pH were 7.0, 6.50, 6.00, 5.61, 5.31, 5.00, 4.61, 4.31, 4.01 and 3.50). Also, ten solutions with 8% NaCl and different pH were prepared (typical target pH were 7.0, 6.50, 6.00, 5.61, 5.31, 5.00, 4.61, 4.31, 4.01 and 3.50). By combining different volumes of the solutions mentioned above in the Bioscreen plate, 100 different NaCl-pH concentrations were

obtained. Each column of the Bioscreen plate had the same NaCl concentration but different pH and each row had the same pH but different NaCl concentration (typical target percentage of NaCl concentrations were: 0.5, 1.3, 2.2, 3.0, 3.8, 4.7, 5.5, 6.3, 7.2 and 8% NaCl). Each well of the honeycomb plate filled with 180µl of the appropriate pH-NaCl concentration. Then 20µl of a particular inoculum (inoculum from dilution labelled -3, approximately 10^6 cfu/ml) was added in every well resulting in a final volume of 200µl. The plates were then incubated in the Bioscreen at 30°C for 5 days. The experiments were carried out in duplicate.

2.3.8 Mild temperature shifts

The effect of non-isothermal conditions was studied for *L. monocytogenes* 252. The culture preparation and the fill of the Bioscreen plates were made as described in paragraphs 2.1.1 and 2.3.4. For this study, identical plates were placed in different Bioscreens set at particular temperatures (25°C and 37°C). After a given time of incubation the plates were swapped between the machines, without changing the running of the machines (25°C–37°C–25°C–37°C and vice versa). Typical experiments lasted 1 to 2 days. The O.D of the wells was read at 600nm every ten minutes.

One particular effect was noted with Bioscreen data when the plates were removed from a higher incubation temperature to a lower (but not vice-versa); a kink in the O.D/time plot due to the temporary presence of condensation on the underneath of the lid of the Bioscreen plates. In general the condensation took between 30 to 50 minutes to evaporate. Thus, TTD which met the O.D criterion were censored during the 30 to 50 minutes after the transfer.

2.3.9 Heat injury

2.3.9.1 Introduction

The effect of a mild thermal injury was studied using the Bioscreen microbiological analyser, in conjunction with the methods developed for the analysis of the initial inoculum size on the TTD. The time-temperature treatment used was chosen after a series of screening trials. In particular, experiments were conducted by placing the Bioscreen plate in an oven, after

a period of incubation in the Bioscreen, at a set temperature (normally 60, 65 and 70°C) for 5, 10, 12, 15, 20 and 25 minutes, before being placed back into the Bioscreen incubator (data not shown). Heat treatment at 60°C for 25min in the oven injured the existed populations without any microbial reduction. Furthermore, the effect of a mild thermal injury (60°C for 25 minutes in a preheated oven) was studied using the Bioscreen microbiological analyser. The effect of a mild heat treatment for the *L. monocytogenes* strains was studied in different NaCl concentrations (0.5, 3 and 6% NaCl) at 30°C while *S.* Typhimurium and *E. coli* were studied in different NaCl concentrations (0.5, 3 and 6% NaCl), different pH (6.57, 5.68, 5.10 and 4.58) and NaCl-pH combinations (see paragraph 2.3.5.3) at 30°C.

2.3.9.2 Heat injury after initial incubation in the Bioscreen

Duplicate microtitre plates were prepared as described above (see paragraphs 2.1.1 and 2.3.4). Both plates were initially incubated in the Bioscreen at 30°C. After a given time (allowing for up to 1/3rd of the wells to reach the detection limit), one plate was chosen and placed in a preheated oven nominally set at 60°C for 25 minutes and then placed back into the Bioscreen incubator for the remainder of the experiment. The actual thermal treatment given was not examined; the conditions used with the particular oven were obtained through trial and error, to enable a reproducible observation of mild injury without incurring inactivation. Sample was taken from a particular well (well 199) and plated onto TSA plates from both plates (control plate and plate which was thermally injured) in order to examine if there was any microbial reduction after the heat treatment. The TTD (defined as the time to reach an O.D= 0.2 at 600nm in the Bioscreen, after background correction) was obtained for each well.

2.3.9.3 Heat injury before incubation in the Bioscreen

Experiments were conducted in the Bioscreen by filling column 10 of two (10x10) microtitre plates with the decimal dilution series mentioned above (400µl per well). Both plates were incubated at the given temperature (30°C) for approximately 2 hours to ensure the microbes were in exponential phase and then one plate was placed in the preheated oven at 60°C for 25 minutes

to induce a thermal injury. The procedure then followed that described previously, with the injured populations being half–fold diluted across the plate, and subsequently re- incubated in the Bioscreen at 30°C.

2.4 Data analysis and model development

2.4.1 Model the traditional growth curves

The data obtained from the construction of the growth curves using the traditional plate counts were fitted with the *modified* Gompertz equation (Gibson *et al*., 1987) which has been described in paragraph 1.3.2.2 and is given by (1-2).

From this equation the growth rate (mu), the lag time (λ) and the generation time (GT) was calculated by (1-3), (1-4) and (1-5).

2.4.2 Calibration curves

O.D was directly related to microbial numbers for all the examined cases in the Bioscreen (200μl) and in the spectrophotometer (1cm path length) (Appendix D, Table D-1). Also, O.D was related to microbial numbers using simple calibration curves. Cultures of the microorganisms were prepared as described in 2.1.1 and Bioscreen plates were filled as described in 2.3.4 using TSB (0.5% NaCl and pH:7.20). The plates were then incubated in the Bioscreen at 30°C for 1 day. From the honeycomb plate where all the wells had previously reached the maximum O.D, 2ml of TSB were mixed with 0.2ml of inoculum which had reached the maximum O.D. A series of dilutions and/or condensations were made in order to obtain different O.D values. The technique used is shown in Figure 2-3. O.D was measured in the spectrophotometer (1cm path length) and in the Bioscreen (200μl volume). Moreover, 0.1ml of a well which had reached the maximum O.D was plated onto TSA plates in order to obtain the maximum population density (MPD) of the microorganisms. Each of the O.D obtained have been related with a number of microbial counts using the counts calculated from the MPD values but also using the counts from the standardised culture at an O.D=0.5 in the spectrophotometer. The microbial numbers used were calculated from the proportions of volumes used in Figure 2-3.

$$2mlTSB + 0.2ml\ inoculum = O.D1 \qquad (A)$$

$$(A) - 1ml + 1mlTSB = O.D2 \qquad (B)$$

$$(B) - 1ml + 1mlTSB = O.D3 \qquad (B')$$

$$2mlTSB + 0.1ml\ inoculum = O.D4 \qquad (C)$$

$$3mlTSB + 0.1ml\ inoculum = O.D5 \qquad (D)$$

$$5mlTSB + 0.1ml\ inoculum = O.D6 \qquad (E)$$

$$(C) - 1ml + 0.05ml\ inoculum = O.D7 \qquad (C1)$$

$$(D) - 1ml + 0.1ml\ inoculum = O.D8 \qquad (D1)$$

$$(E) - 1ml + 0.1ml\ inoculum = O.D9 \qquad (E1)$$

$$(D1) - 1ml + 0.1ml\ inoculum = O.D10 \qquad (D2)$$

$$(E1) - 1ml + 0.1ml\ inoculum = O.D11 \qquad (E2)$$

$$(E2) - 1ml + 0.5ml\ TSB = O.D12 \qquad (E3)$$

$$(E3) - 1ml + 0.5ml\ TSB = O.D13 \qquad (E3')$$

Figure 2-3 Representation of the technique for constructing calibration curves

2.4.3 Data analysis

From the resulting Bioscreen O.D/time data, the background O.D due to the media was removed from each. A TTD criterion of O.D = 0.2 was then used on the background corrected data: TTD were found using linear interpolation between O.D/time values which straddled the O.D = 0.2 value. That data obtained from the Bioscreen have approximately constant variance until the initial inoculum level is less than 10^2 cfu/ml, below this level the variance increases (Bidlas and Lambert, 2008). To preclude the need of weighted regression or for a data transformation data below this threshold were censored in the regression fits.

2.4.4 Models development for the growth curve prediction from O.D data

2.4.4.1 *Modified* logistic and Gompertz

The re-parameterised *modified* logistic and Gompertz models (Zwietering *et al.*, 1990) were rearranged to equate the initial log inoculum with the time to detection of a known number of microbes per ml (N_D), giving equations (2-1) and (2-2), respectively.

$$TTD = \lambda - \frac{A}{4\mu_m}\left(\ln\left(\frac{A}{\log N_D - \log N_0} - 1\right) - 2\right) \quad \textbf{(2-1)}$$

$$TTD = \lambda - \frac{1}{\mu_m}\left(\frac{A}{e}\right)\left(\ln\left(\ln\left(\frac{A}{\log N_D - \log N_0}\right)\right) - 1\right) \quad \textbf{(2-2)}$$

2.4.4.2 Three phase linear model

The 3-PLM is a simplified model of the growth curve. Its simplicity has been regarded by some as its strength and too simplistic by others (Buchanan *et al*., 1997; Baranyi, 1997; Garthright, 1997). The 3-PLM has been described in paragraph 1.3.2.2.

The parameter t_λ of the model is the duration of lag time. This equation can be rearranged to equate the initial log inoculum with the time to detection of a known number of microbes per ml (N_D),

$$TTD = \lambda + \frac{\log N_D - \log N_0}{\mu_m} \quad \textbf{(2-3)}$$

2.4.4.3 Baranyi and logistic models

$$\log N_D = \log N_0 + \frac{y_1}{\ln(10)} - \frac{y_2}{\ln(10)} \; where$$

$$y_1 = \mu_m t + \ln\left(e^{-\mu_m t} - e^{-\mu_m(t+t_\lambda)} + e^{-\mu_m t_\lambda}\right) \text{ and} \quad \textbf{(2-4)}$$

$$y_2 = \ln\left(1 + 10^{(\log_{10} N_0 - \log_{10} M)}\left(e^{\mu_m(t-t_\lambda)} - e^{-\mu_m t_\lambda}\right)\right)$$

The Baranyi model is a non-autonomous equation. In the absence of lag the Baranyi model defaults to the basic logistic model of growth in which the time to detection for a given number of microbes is given by

$$TTD = \frac{1}{\mu}\ln\left(\frac{\frac{M}{N_0}-1}{\frac{M}{N_D}-1}\right) \quad \textbf{(2-5)}$$

Where M = maximum population density (cfu/ml), N_D = numbers of microbes per ml at the detection value, N_0 = initial inoculum level (cfu/ml) , μ = specific growth rate.

A simple, empirical, approximation to the Baranyi equation when a lag exists is given by:

$$TTD = \lambda + \frac{1}{\mu}\ln\left(\frac{\frac{M}{N_0}-1}{\frac{M}{N_D}-1}\right) \quad \textbf{(2-6)}$$

2.4.5 Logistic with lag model: Estimation of growth rate and lag from time to detection data

For a given set of environmental conditions a plot of the initial inoculum size against the TTD gives a straight line relationship with a growth rate equal to the reciprocal of the gradient. In the absence of a lag this line will intersect the log initial inoculum axis at the detection value for the given O.D criterion used. In this study the TTD was defined as the time to produce an O.D=0.2. This is the methodology described by Cuppers and Smelt (1993).

Theoretical Background: From the classical logistic equation

$$N = \frac{M}{1+\left(\frac{M}{N0}-1\right)e^{-\mu t}} \quad \textbf{(2-7)}$$

Where μ is the specific growth rate and M is the maximum population density (also known as the carrying capacity, cfu/ml), the time taken (TTD_N) to reach a specific population level (N) from a given initial value (N_0, cfu/ml) is given by

$$TTD_N = -\frac{1}{\mu}\ln N_0 + \frac{1}{\mu}\ln\left(\frac{N(M-N_0)}{M-N}\right) \quad \textbf{(2-8)}$$

The TTD_N is defined as the time to reach a given detection threshold (e.g. an optical density of 0.2) for which N_D is the equivalent microbial numbers per ml. If the assumption that $M>>N_0$ is made then this can be approximated by

$$TTD = -\frac{1}{\mu}\ln N_0 + \frac{1}{\mu}\ln\left\{\frac{N_D M}{M - N_D}\right\} \quad \textbf{(2-9)}$$

When N_0 =1, the TTD is given by the right hand expression of (2-9), if $M >> N_D$ then this can be approximated by $\ln N_D/\mu$. Hence, a plot of the initial inoculum against the TTD will give a gradient equal to the negative reciprocal of the growth rate, the TTD intercept at N_0 = 1, is the time taken for one organism to reach the TTD criterion. This expression can be considered as the basis of the methodology of Cuppers and Smelt (1993) described above. In the presence of a lag (2-9) can be supplemented with a lag term (λ). The appropriate rearrangement of the logistic model with lag was used. A simple approximation being:

$$TTD = \lambda + \frac{1}{\mu}\ln\left(\frac{N_D}{N_0}\right) \quad \textbf{(2-10)}$$

2.4.6 Model development for the MIC studies: MIC_{NaCl} and MIC_{pH}

The minimum inhibitory concentration (MIC) in NaCl and pH was defined using the Lambert-Pearson model (Lambert and Pearson, 2000). The LPM of microbial inhibition (equation (2-11)) describes the visual growth of a culture as an exponential decay function of the concentration of the applied inhibitor. A plot of the log concentration against the rate to detection (RTD) which is the reciprocal of the TTD of the test culture, gives a characteristic sigmoid curve, with inflexion at RTD=P_0/exp(1), where P_0 is the RTD of the positive control. A linear extrapolation from this point to the log concentration axis allows the estimation of the MIC (2-12).

If $y_{(x)}$=0 then $RTD = P_0$

If $y_{(x)} < P_1$ then $RTD = P_0 exp\left(-\left(\frac{x}{P_1}\right)^{P_2}\right)$, **(2-11)**

If $y_{(x)} \geq P_1$ then $RTD = \frac{P_0}{e}\left(1 - P_2 \ln\left(\frac{x}{P_1}\right)\right)$

Where RTD is the rate to detection, $y_{(x)}$ is the concentration of the given inhibitor, P_1 is the concentration of inhibitor of 1/e, where e is the exponential

of 1 and P_2 is a slope parameter which can be considered as a measure of the dose response.

From the LPM the MIC which is an important biological parameter could be obtained from the intercept of the maximum plot of RTD against the percentage of NaCl concentration or the ion concentration.

$$\mathrm{MIC} = \mathrm{P}_1 \exp\left(\frac{1}{\mathrm{P}_2}\right) \quad \textbf{(2-12)}$$

The minimum NaCl was calculated using the percentage of NaCl concentration and the minimum pH was calculated using ion concentration and then transformed back to pH. Analyses were done using the JMP Statistical Software (SAS Institute Cary NC USA), using non-linear regression with the minimised sum of squares as the search criterion.

2.4.7 Analysis of combined inhibitors

Three main approaches to modelling the observed data were used: nominal logistic modelling of the Growth/No Growth (G/NG) data, continuous modelling (response surface modelling) and the use of the Extended Lambert and Pearson model (ELPM).

Nominal logistic modelling of the Growth/No Growth boundary

E. coli and *S.* Typhimurium were examined in a range of combinations of pH-NaCl concentrations. Data which showed growth (had an RTD > 0) were degraded to the label “G” and those showing no growth within the period of the experiment were labelled as “NG”. A nominal logistic model was fitted to the data using maximum likelihood:

$$\mathrm{Prob.\,Growth} = 1 - \left(\frac{1}{1+\exp(\mathrm{f}_{(\mathrm{var})})}\right) \quad \textbf{(2-13)}$$

Where $f_{(var)}$ is a function (normally polynomial) of the (independent) variables involved in the experiments. The selection of the most appropriate model was based on the minimum number of parameters required to achieve the highest r^2 whilst still having high statistical significance for each parameter used ($p < 0.05$). Forward elimination was used - in which additional variables (such as cross-products) are added only if they made a significant contribution to the fit.

Nominal logistic modelling was carried out using the appropriate platform in the JMP statistical package (SAS Institute, Cary NC, USA).

Continuous modelling

The TTD were transformed to RTD (reciprocal transformation) and a response surface model (RSM) was produced using the standard least of squares with emphasis on the effect of leverage. Scatter plots 3D, surface plots and contour plots were produced based on the observed and the modelled data. Response surface modelling was carried out using the appropriate platform in the JMP statistical package (SAS Institute, Cary NC, USA).

Extended Lambert and Pearson model

The model used in these studies was developed from a previously published version (Bidlas and Lambert, 2008). The model allows the direct calculation of an absolute G/NG boundary. For two inhibitors in combination, each of which can be modelled by the LPM, the additive effect is given by:

$$\mathrm{RTD} = \begin{cases} & \\ \textit{if} \quad \sum_{i=1}^{n}[x_i] = 0, \quad P_0 & \\ \textit{else} \quad \textit{if} & \\ \quad \ln\{\mathit{EffConc}\} < 0 & \\ \textit{then} & \\ \quad P_0 \exp(-\mathit{EffConc}) & \\ \textit{else} \quad \textit{if} & \\ \quad \frac{P_0}{e}(1 - \ln\{\mathit{EffConc}\}) < 0, \quad 0 & \\ \textit{else} & \\ \quad \frac{P_0}{e}(1 - \ln\{\mathit{EffConc}\}) & \end{cases} \qquad \textbf{(2-14)}$$

Where the effective concentration, *EffConc* is given by:

$$EffConc = \left(\left(\frac{x_1}{P_1}\right)^{\frac{P_2}{P_4}} + \left(\frac{x_2}{P_3}\right) \right)^{P_4} \qquad \textbf{(2-15)}$$

Where the parameters P_i are those obtained from the LPM, and where $P_4 \geq P_2$, $[x_i]$ is the concentration of the i^{th} inhibitor and P_0 is the RTD of the least inhibitory condition.

2.4.8 Model development for the fluctuating temperatures studies

The effect of mild temperature shifts was modelled using the geometric or Malthusian model in conjunction with the methods developed for the analysis of the initial inoculum size on the TTD. Consider two inoculum dependence experiments carried out on the Bioscreen at two different temperatures, where T_1 is at more optimum temperature for growth than T_2. Analysis of the TTD data would give something similar to that shown in Figure 2-4.The gradient observed at T_1 is less than the gradient observed at T_2.

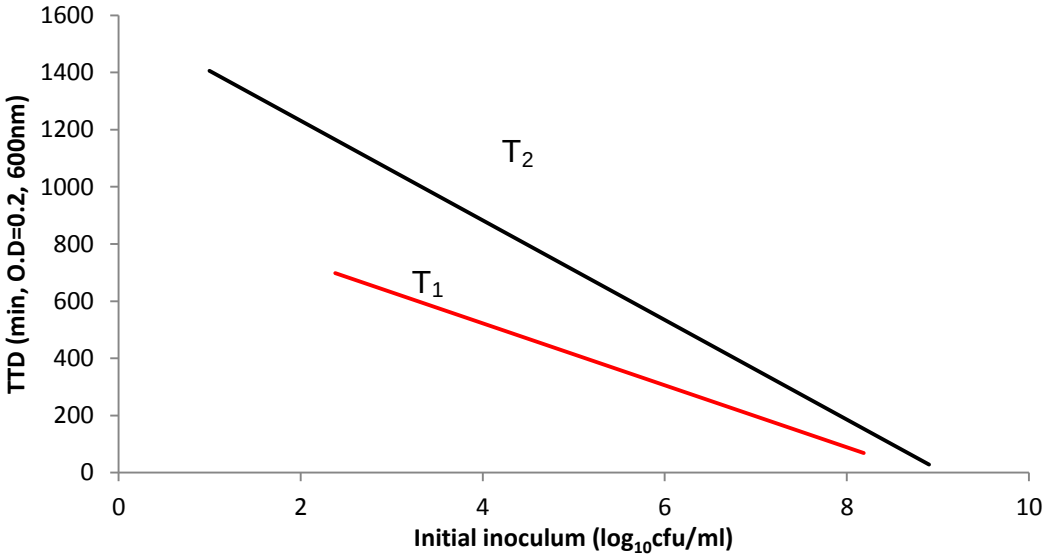


Figure 2-4 Inoculum size dependency on the time to detection (TTD) at two different temperatures, where T_1 is more optimal for growth than T_2

The relationship between the TTD and the initial log inoculum is a linear relationship which was given by:

$$TTD = c - m \log N \quad \textbf{(2-16)}$$

where logN is the log of the initial inoculum size used, m is the gradient and c is the intercept of the slope on the TTD axis. This can also be rewritten as:

$$TTD = -m(\log N - \log N_D) \quad \textbf{(2-17)}$$

where $\log N_D$ is the size of the inoculum for which TTD = 0. If we consider m as a negative value then (2-17) can be rewritten as :

$$TTD = m(\log N - \log N_D) \quad \textbf{(2-18)}$$

If two microtitre plates each containing identically prepared multiple inocula are incubated at different temperatures then each inoculum will grow at a rate dictated by the temperature and media conditions. The difference between the two plates will be governed only by the differential effect of temperature. In particular, if after a period of incubation (t_x) at T_1 the temperature was changed to T_2, then the growth rate will change to accommodate the new temperature. It was hypothesised that this would cause an abrupt change in the slope (a discontinuity), Figure 2-5.

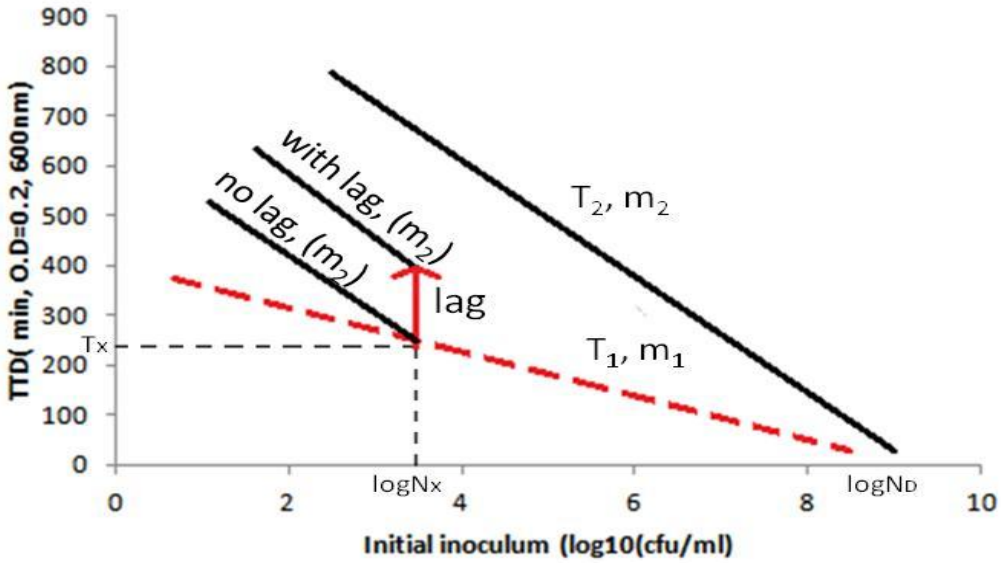


Figure 2-5 The geometrical basis for equation (2-23): for a given rate (m_1), over the time period T_x initial inocula between $\log N_D$-$\log N_x$ will reach the detection threshold of $\log N_D$. If at T_x the rate is changed (m_2) and there is no induction of lag, the TTD now follow the new rate. If a lag is induced a vertical separation at T_x equal to the time of lag will be present before growth recommences

The first part of the TTD/logNo plot should follow the behaviour of the optimal temperature and the second part should follow the behaviour of the less optimal temperature slope. To model the behaviour of the slope it is a simple matter to obtain the value of the log of the initial inoculum size at the detection time given by t_x.

For $t \leq t_x$,

$$\mathrm{TTD} = \mathrm{m}_0 \, (\log N_o - \log N_D) \quad \textbf{(2-19)}$$

Where m_0 is the gradient of the initial condition.

At the time of the first temperature switch, t_1 the $\log N_{t=t1}$ can be calculated by rearranging (2-19) as follows:

$$logN_{t=t1} = logN_D + \frac{t_1}{m_0} \quad \textbf{(2-20)}$$

At the time (t_x) when the temperature shifts, the point ($\log N_x$, t_x) is used to calculate the equation of the second part of the slope

$$(TTD - t_1) = m_1(logN_0 - logN_{t=t1}) \quad \textbf{(2-21)}$$

Rearranging (2-21) and substituting with (2-20) gives:

$$TTD = t_1 + m_1\left(logN_0 - logN_D - \frac{t_1}{mo}\right) \quad \textbf{(2-22)}$$

This model can be extended by using the same method to describe the effect of multiple temperature shifts as follows:

If $t < t_{i+1}$, for i=0, 1, 2, ... (with t_0=0) then:

$$TTD = t_i + m_i\left[logNo - logN_D - \sum_{j=0}^{j=i}\left(\frac{t_j - t_{j-1}}{m_{j-1}}\right)\right] \quad \textbf{(2-23)}$$

For example, for up to two temperature shifts, the overall model can be summarised by:

i=0 $$TTD = m_0[logNo - logN_D] \quad \textbf{(2-24)}$$

i=1 $$TTD = t_1 + m_1\left[logNo - logN_D - \frac{t_1}{m_0}\right] \quad \textbf{(2-25)}$$

i=2 $$TTD = t_2 + m_2\left[logNo - logN_D - \left\{\frac{t_2 - t_1}{m_1} + \frac{t_1 - t_0}{m_0}\right\}\right] \; where \; t_0 = 0 \quad \textbf{(2-26)}$$

If lags are present then at a particular t_i, this value can be modelled as $t_i + lag_i$. Since the models are simple linear models, the majority of the modelling was carried out using Excel (Microsoft) and the data analysis add-in package.

2.4.9 Model of growth following thermal injury

The basis of the model used is that in each well of the Bioscreen a population of organisms exists which results in the observation of the TTD. If these organisms are uninjured then the TTD is given by the model above. Hence the uninjured population will have a TTD given by

$$TTD = \lambda + \frac{1}{\mu}\ln\left(\frac{N_D}{N_0}\right) + Norm(0,\sigma) \quad \textbf{(2-27)}$$

Where Norm(0,σ) is the normal distribution of error about the line. After a thermal insult we hypothesise that the populations present in each well will become injured and present a lag due to the injury before the recommencement of growth. Hence the model

$$TTD = \lambda + \frac{1}{\mu}\ln\left(\frac{N_D}{N_0}\right) + Norm(0,\sigma) + lag_{\ injury} \quad \textbf{(2-28)}$$

Where the distribution of lag due to injury (lag_{injury}) is Log-normal and characterised by scale (μ) and shape parameters (σ), logNorm (μ, σ). The TTD observed is therefore a composite of the growth under the given conditions plus that induced by the need to repair after the thermal insult (the exponential of the logNorm (μ, σ)).

3 Growth curve prediction from optical density data

3.1 Introduction

A fundamental aspect of predictive microbiology is the shape of the microbial growth curve. General population growth can be modelled using the logistic model and variations of this model have been used in many diverse areas such as the analysis of fish stocks, forestry management and human population growth (e.g. Alexandrov, 2008). The general pattern of growth is sigmoidal, with an apparent slow phase followed by a more rapid increase in numbers followed by a slowing down, finally reaching a maximum population level. In most texts it is noted that the growth of bacteria also follows a similar pattern: a lag before replication, followed by exponential growth and then a period of maximum population density eventually followed by the 'death-phase'. A major difference is that the microbial growth curve is depicted in terms of log numbers of microbes. The microbial growth curve (as log numbers) has the characteristic sigmoid shape and the varieties of models which are used to fit the curve reflect this sigmoid character. There are two principal empirical curves used – the symmetric *modified* logistic and the asymmetric *modified* Gompertz ('*modified*' by virtue of using log numbers rather than numbers explicitly). Many models in the microbiological literature are variations on these two themes (Zwietering *et al.*, 1990; Pruitt and Kamau 1993; Li *et al*., 2007).

The Baranyi model, however, is different to the normal growth models in that it is based on the logistic model of growth, but has an additional function which deals with the presence of lag making it a non-autonomous differential equation (Baranyi *et al.*, 1993a; 1993b; Baranyi and Roberts, 1994).

$$\frac{dn}{dt} = a_t \mu_n n \qquad \textbf{(3-1)}$$

Where μ_n is a function of the specific growth rate, n is the numbers of microbes and a_t is termed the adjustment function. The derived equation uses the idea of Michaelis –Menten kinetics to suggest a lag time during which organisms adapt from one environment (the culture) to the test environment. The function used essentially delays the time before growth occurs.

Baranyi and Roberts (1995) in their paper on the fundamentals of mathematics in predictive microbiology stated that rapid methods such as turbidimetry or conductimetry cannot be used directly to obtain growth parameters such as the specific growth rate if the rescaling function employed has a constant other than zero; “if the measured quantity is q, then q=f(x), where f is a linear calibration function: f(x)=ax +b. If b is different from zero then neither q nor log q is linearly proportional to log x. Hence, in a strict sense, the rate of change in q should not be used to estimate the viable count specific growth rate unless the proportionality of q (turbidity, conductance, etc) to the original cell concentration, x, has been established over the complete matrix of environmental variables (temperature, pH, aw). Nor should the viable count models describing x(t) be directly applied to the model q(t). New calibration function, or other considerations, should be taken into account to model q(t) and/or to compare it with the viable count model.”(Baranyi and Roberts, 1995).

Models used to examine the shape of microbial growth generally require four parameters: the initial and final population levels (I_0 and MPD respectively), the maximum specific growth rate and the time at which this occurred. If three pieces of information are available, e.g. the initial population, the MPD and the specific growth rate, then knowledge of the population at a specific time can be used to reproduce the growth curve simply by substituting the values into the equations and solving for the missing parameter. Herein we show that this seemingly simple hypothesis serves as a “consideration” and also has ramifications on the validity of the *modified* empirical growth curves, whilst adding value to the interpretation of the Baranyi equation.

3.2 Materials and methods

The growth of *L. monocytogenes* 252 was studied at 30°C and 37°C in TSB (0.5% NaCl) and in different pH concentrations. The preparation of the cultures and the fill of the Bioscreen micro-array plates were done as described in paragraphs 2.3.5.1 and 2.3.5.2. The data obtained from the Bioscreen were analysed as described in 2.4.3 and the models used to predict the growth curves from O.D data were described in 2.4.4.

3.3 Results

3.3.1 Growth rate of *L. monocytogenes* 252 at 37ºC from O.D data

The O.D/incubation time curves for different initial inocula of *Listeria monocytogenes* 252 in TSB (0.5% NaCl) are shown in Figure 3-1(without background correction). Each individual curve was essentially congruent with all other curves: there is no decrease in the slope with decreasing initial inocula. The average maximum O.D reached was 0.99 (σ = 0.034, se. mean = 0.004). For each curve, the TTD of O.D = 0.2 was found using simple linear interpolation between O.D/time data which straddled the O.D = 0.2 position. The analysis of the TTD of these multiple dilutions of initial inocula (the zero dilution starting culture had a viable count of 1.11 $x10^9$ cfu/ml), showed a simple linear relationship between the initial inoculum and the TTD criterion used (Figure 3-2). The reciprocal of the gradient gives a growth rate of 0.0092 $\log_{10}$ cfu $ml^{-1}min^{-1}$, which equates to a specific growth rate of 1.27 ln cfu ml^{-1} hr^{-1} (Table 3-1). The intercept of 961 minutes corresponds to the time taken for a single organism per ml to reach the TTD= 0.2 criterion; when TTD = 0, the regression line cuts the axis at an initial log inoculum of 8.81 $\log_{10}$ cfu ml^{-1} (95% CI 8.77-8.86), which was statistically equivalent to the $\log_{10}$ cfu ml^{-1} count of the viable count recorded from multiple wells with O.D = 0.2. Hence in this case there was no measurable lag; this was also confirmed from an analysis of Figure 3-1– the highest inocula examined do not show any lag period but immediate growth is observed.

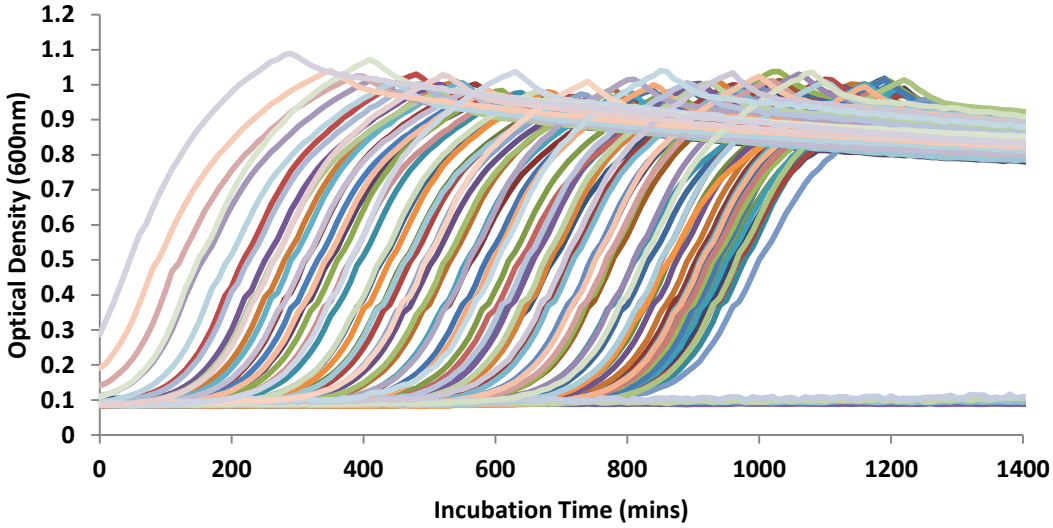


Figure 3-1 Optical density-incubation time plot for the growth of multiple initial inocula of *Listeria monocytogenes* 252 at 37°C in TSB

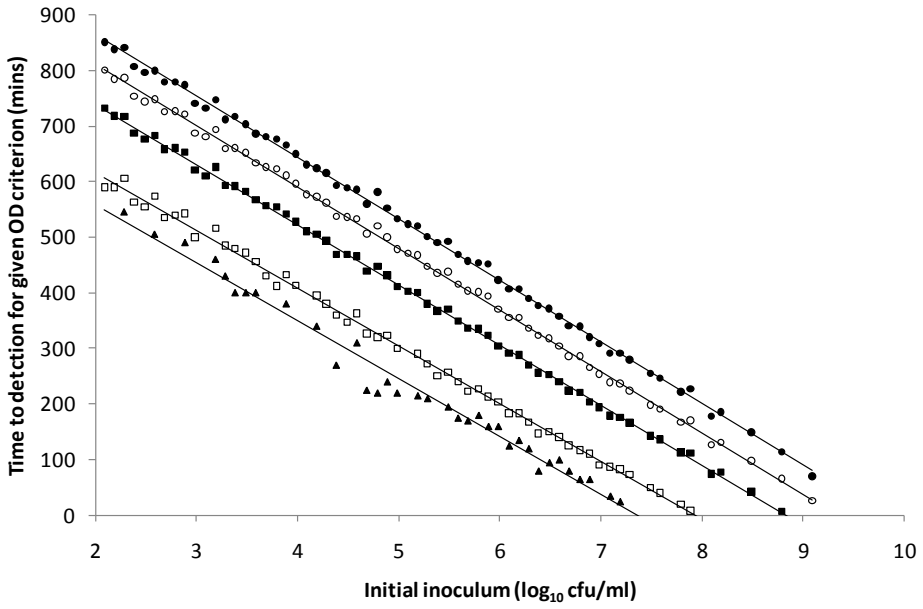


Figure 3-2 The time to detection of multiple initial inocula of *Listeria monocytogenes* 252 at 37°C in TSB. The TTD criterion was set at O.D =0.09 (▲), 0.1 (□), 0.2 (■), 0.4 (○), 0.6 (●). An O.D = 0.2 at 600nm was equal to 8.9 log_{10} cfu ml^{-1}

Table 3-1 Parameter estimates from linear regression fits to TTD data for multiple initial inocula of *Listeria monocytogenes* 252 at 37°C in TSB for different time to detection criteria at 600nm

	Gradient			*Intercept*				
O.D criterion	***Coefficient***	***Lower 95%***	***Upper 95%***	***Coefficient***	***Lower 95.0%***	***Upper 95.0%***	r^2	$log\ N_D^*$
0.09	-104.17	-109.15	-99.19	766.52	740.22	792.81	0.981	7.358
0.1	-104.17	-105.83	-102.50	824.96	816.37	833.54	0.997	7.919
0.2	**-108.49**	**-109.60**	**-107.38**	**956.29**	**950.29**	**962.30**	**0.999**	**8.814**
0.3	-109.20	-110.14	-108.26	996.36	991.23	1001.48	0.999	9.124
0.4	-110.51	-111.54	-109.48	1032.31	1026.56	1038.05	0.999	9.341
0.5	-110.23	-111.31	-109.14	1056.05	1050.08	1062.03	0.999	9.581
0.6	-110.53	-111.63	-109.43	1085.84	1079.78	1091.90	0.998	9.824
0.7	-110.34	-111.75	-108.93	1113.02	1105.24	1120.79	0.997	10.09
0.8	-110.20	-112.44	-107.95	1153.58	1141.18	1165.97	0.994	10.47
0.9	-110.32	-113.38	-107.26	1216.46	1199.52	1233.41	0.988	11.03
0.95	-109.88	-113.23	-106.53	1252.31	1233.60	1271.02	0.987	11.40
1.0	-110.81	-114.12	-107.50	1285.19	1266.28	1304.10	0.993	11.60

*: Theoritical detection values ($logN_D$) calculated from the regression parameters (gradient and intercept).

3.3.2 Fitting the *modified* logistic and Gompertz models to O.D data

From a plate count the MPD of the *Listeria monocytogenes* 252 culture was 9.8 log cfu ml^{-1}, the initial inoculum size for each well was calculated from the plate count of the initial inoculum and the dilution sequence used. From the O.D data, the specific growth rate and lag were obtained, hence all the parameters required to reproduce the growth curve using either the *modified* logistic or Gompertz equations were present. Equations (2-1) and (2-2) were used to calculate the TTD for the given initial inocula using the observed parameters. A plot of the calculated TTD against the initial inocula gave a regression fit of $TTD_{calc} = -125.53\log N_o + 1087.7$ min, $r^2 = 0.999$ and $TTD_{calc} = -144.6\log N_o + 1235$ min, $r^2 = 0.997$ for the *modified* logistic and Gompertz equations, respectively. The gradients were 16% and 33% greater for the *modified* logistic and Gompertz respectively over that observed. In both cases the plot was a curve rather than the observed linear relationship. The sum of squares between the observed TTD and that calculated using the two equations was minimised by regressing the growth rate and lag, this gave growth rates of 0.0107 and 0.0122 $\log_{10}$cfu $ml^{-1}min^{-1}$ with a lag of 17 and 31 mins, respectively, but the gradient of the calculated TTD/ log initial inoculum plots were now equal to the observed (-108.5 $\log_{10}$cfu ml^{-1} min^{-1}). Hence there is a discrepancy between the fit of the *modified* logistic and Gompertz equations with the interpretation of the observed values.

Multiple growth curves were produced *in-silico* using the observed rate, lag and the known initial inocula and MPD. The calculated log numbers were transformed to numbers per ml and plotted against time. If the *modified* Gompertz equation was an adequate descriptor of the observed data then congruent plots should be observed. However, as the initial inocula decreased the modelled curves became shallower, i.e. they do not reflect the observed O.D curves (note a calibrant which transforms the number to an O.D will result in the same conclusion). The analyses performed were also carried out using the *modified* logistic equation, resulting in the same conclusion: the *modified*

logistic model cannot reproduce the congruent shapes of the observed O.D curves shown in Figure 3-1.

3.3.3 Fitting the 3-phase linear model to O.D data

The 3-PLM (2-3) was fitted to the TTD data by minimising the sum of squares between the observed TTD and the modelled; initial values of μ = 0.0092 and lag = 0 mins were used (note the MPD and the log of the detection numbers are fixed values).The fit gave μ = 0.00922 (95% CI: 0.00914 – 0.00930), and a lag = -8.89 mins (95% CI: -12.84 to - 4.93). A plot of the calculated TTD against the initial inocula gave a regression fit of TTD_{calc} = -108.5logNo +965.6, r^2 = 1.00, i.e. the 3-PLM reproduced the observed TTD data and (by definition) was a straight line fit.

Using the full form of the 3-PLM, multiple growth curves were produced *in-silico* using the calculated rate, lag and the known initial inocula and MPD. The calculated log numbers were transformed to numbers per ml and plotted against time. In this case the 3-PLM produced congruent curves and reproduce the initial shape of the O.D curves (Figure 3-3) however, since the model gives only exponential growth until MPD is reached, i.e. there is no slow down in the rate of growth, the discrepancy between the shapes of the observed O.D and calculated numbers quickly increases. In this case the 'simple' is good enough to fit the TTD data but not 'enough' to model the full data (Buchanan *et al*., 1997).

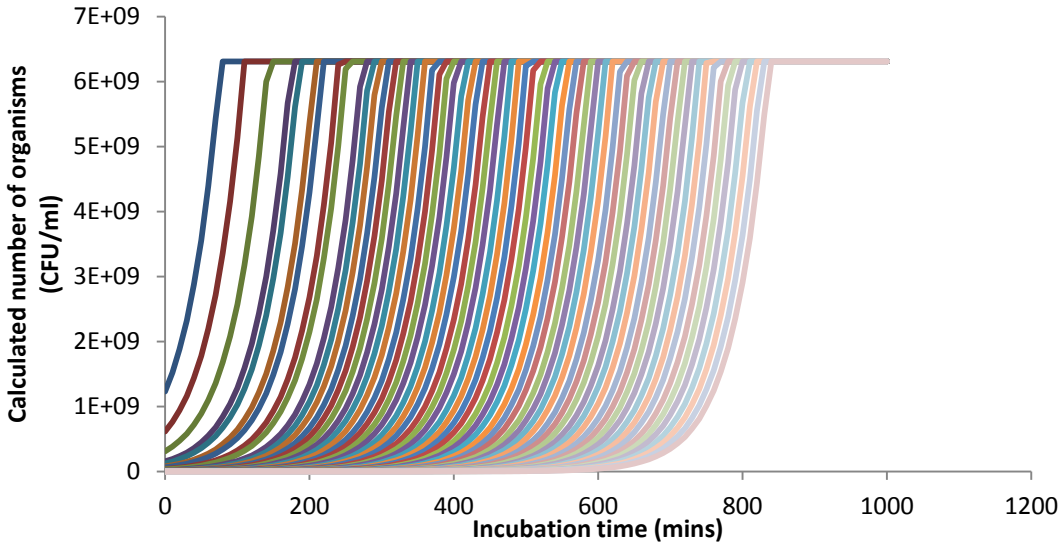


Figure 3-3 Predicted microbial numbers with time from the 3-PLM, with parameters μ=0.00921, Lag = -8.88 mins, MPD = 9.8, with a range of initial inocula

3.3.4 Fitting the Baranyi model to O.D data

The Baranyi model cannot apparently be used to explicitly obtain the TTD for a given set of parameters, although this can be easily solved numerically. To fit the Baranyi model to the observed TTD data, and obtain a growth rate and lag, the observed TTD was used as the independent variable and the model used to fit the difference between the size of the detection inoculum and the initial inoculum. The estimated growth rate was 0.00918 $\log_{10}$ cfu ml^{-1} min^{-1} (95% CI:0.00910 – 0.00927), with a lag of -17.3 mins (95% CI: -21.8 to -12.9).

To compare the calculated TTD value from the Baranyi model, with respect to the observed values a growth rate of 0.00922 $\log_{10}$ cfu ml^{-1} min^{-1} and a lag of zero minutes were used along with the given MPD of 9.8 and the size of the known initial inoculum to produce, *in-silico*, multiple growth curves. The time taken to reach the inoculum detection value of 8.9 for all growth curves was obtained numerically using a simple linear interpolation procedure. A plot of the log initial inoculum against the calculated TTD gave a straight line fit with a regression fit of TTD_{calc} = -108.4$\log_{10}$No+956.49, r^2 = 0.999. The multiple

growth curves obtained are congruent and have the desirable feature of a slow down in the rate of growth as MPD is approached unlike the 3-PLM (Figure 3-4).

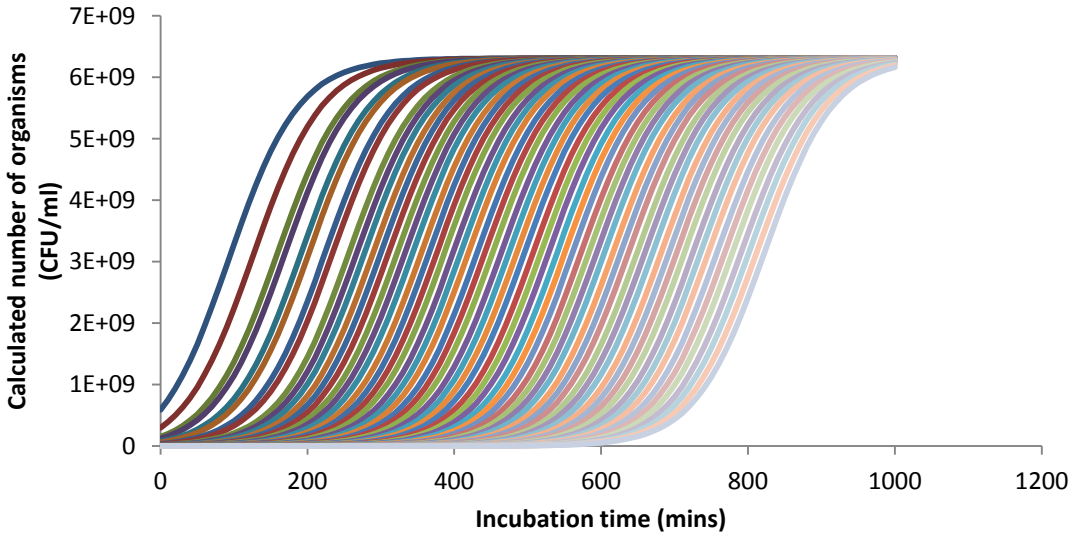


Figure 3-4 Predicted microbial numbers of *Listeria monocytogenes* 252 with time from the Baranyi model, with parameters μ=0.00921, Lag = -8.88, MPD = 9.8, with a range of initial inocula

3.3.5 O.D- Baranyi calibration curve

The Baranyi model and the O.D data are 1:1 up to the maximum O.D. Past the maximum O.D, in the cases studied here, there is a reduction in the O.D with incubation time, whereas the model stays at a constant MPD. This is a failing of the model as it is a purely growth rather than a growth and decay model. The 1:1 nature of the relationship up to the maximum O.D can be used to construct a calibration curve between the O.D at a given time and the number of microbes per ml predicted from the Baranyi model. To construct the calibration curve ten observed O.D curves were chosen and a plot of the O.D (up to a maximum of 0.85) against the equivalent calculated numbers for the observation time constructed. Simple linear regression was applied ((3-2) and (3-3)), Figure 3-5.

$$\text{O.D} = 1.308\text{x}10^{-10}\,(\text{No.}) + 0.0946,\ r^2 = 0.997 \qquad \textbf{(3-2)}$$

$$\text{No.} = 7.625\text{x}10^{9}(\text{O.D}) - 7.172\text{x}10^{8},\ r^2 = 0.997 \quad \textbf{(3-3)}$$

Where No. are the calculated microbial numbers per ml. Data up to an O.D = 0.85 gave a good linear relationship between O.D and the calculated cfu ml^{-1}; at O.D greater than 0.8, the inclusion of the cubic and quadratic terms (No. = $-8.38\text{x}10^{9}(\text{O.D})^3 + 1.23\text{x}10^{10}(\text{O.D})^2 + 2.67\text{x}10^{9}$ (O.D) $-7.049\text{x}10^{8}$, R^2 = 0.999, r^2 =0.997; O.D = $3.217\text{x}10^{-30}$ (No.)^3 - $2.787\text{x}10^{-20}$(No.)^2 +1.896X10^{-10} (No.) + 0.0831, R^2 =0.998) gave better fits.

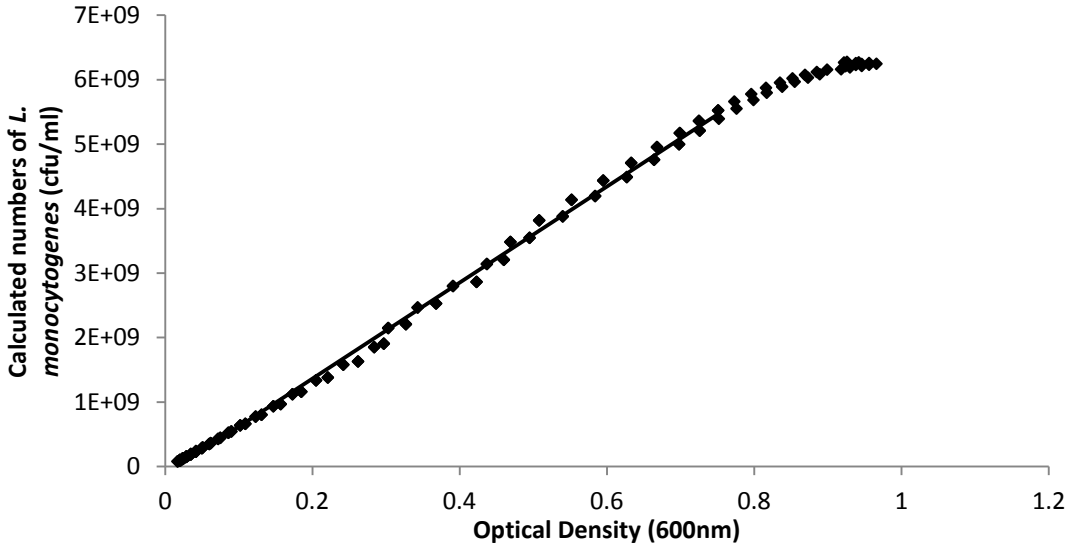


Figure 3-5 Plot of the observed O.D against the calculated numbers/ml from the associated Baranyi equation (diamonds), the solid line is the regression fit used in this study

Using the calibration curve derived from the Baranyi-analysis, the calculated microbial numbers were converted to O.D values. Figure 3-6 shows a direct comparison of the predicted O.D/time curves with the observed for five selected cases.

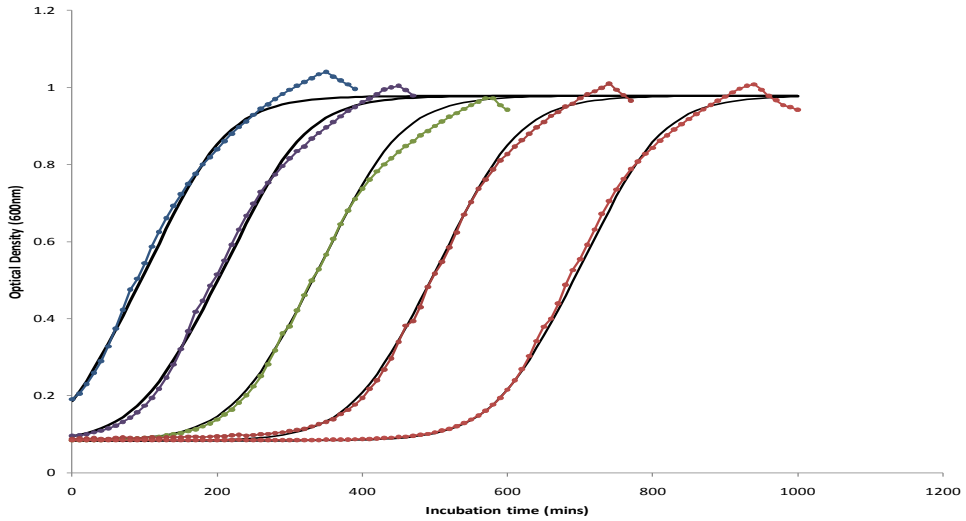


Figure 3-6 Comparison of the observed optical density incubation time plot (symbols) and the calculated (solid lines) for *Listeria monocytogenes* 252 incubated at 37°C with initial log_{10} inocula of (from left to right) 8.789, 7.789, 6.585, 5.09, and 3.284 respectively

3.3.6 Classical population logistic model

Fitting the classical logistic model (2-5) to the TTD data gave a straight line fit of TTD_{calc} = -105.43log_{10}No+943.59, equating to a growth rate of 0.0095 log_{10} cfu ml^{-1} min^{-1} (95% CI: 0.00945 – 0.00955). The addition of a constant lag (2-6) improved the fit giving a straight line fit of TTD_{calc}=-108.48log_{10}No+956.56, equating to a growth rate of 0.00923 log_{10} cfu ml^{-1} min^{-1} (95% CI: 0.00915 – 0.00932). A lag of -14.3 minutes (95% CI: -18.4 to -10.2) was obtained; the correlation between lag and growth rate was -0.90 (negative correlation), a result very similar to the fitting of the Baranyi equation.

3.3.7 Growth rate of *L. monocytogenes* 252 at 30ºC from O.D data

The TTD from a ten-fold dilution series of an initial standardised inoculum of *L. monocytogenes* 252 incubated at 30°C in TSB were obtained. A regression fit gave TTD = -127.09log_{10}No+1121.8, r^2 = 0.999; giving a growth rate of 0.00787 log_{10} cfu ml^{-1} min^{-1}. The intercept of 8.83 log cfu ml^{-1} (95% CI: 8.80 – 8.85) suggests the absence of a lag. Initially the Baranyi model was fitted to

the TTD data using an MPD of 9.8 and a detection inoculum of 8.9. The specific growth rate obtained was 0.00786 log_{10} cfu ml^{-1} min^{-1} (95% CI: 0.00780 – 0.00792) and a lag of -17.5 mins (-22.5 to -12.5 mins). Using the calibration curve found previously ((3-2) and (3-3)) the calculated numbers were transformed to O.D values. An MPD of 9.8 was found, however, to be too low for the maximum O.D observed. The MPD was increased to 9.9 and this gave reproducible O.D curves, Figure 3-7. Changing the MPD left the specific growth rate unchanged but the lag increased to -1.0 min.

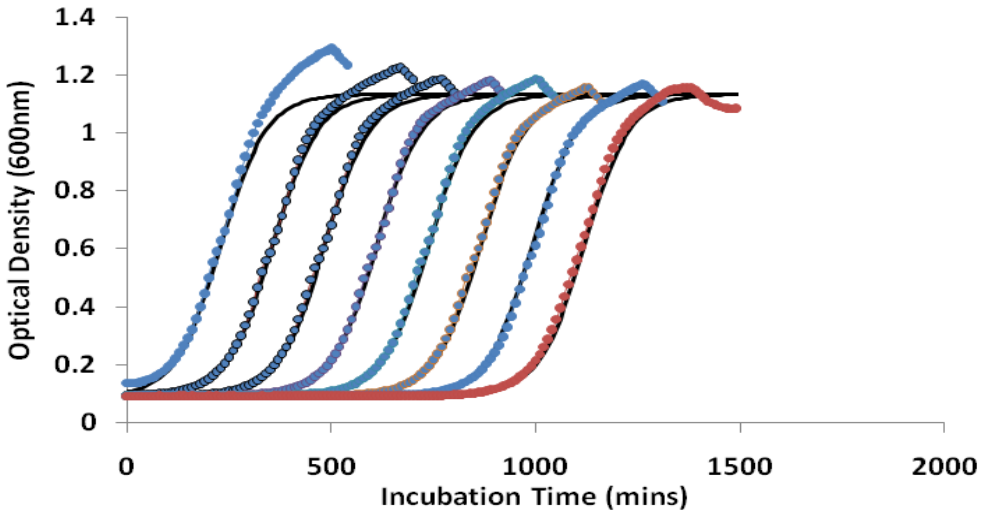


Figure 3-7 Comparison of the observed optical density incubation time plot (symbols) and the calculated (solid lines) for *Listeria monocytogenes* 252 incubated at 30°C with initial log_{10} inocula of (from left to right) 7.97, 6.97, 5.97, 4.97, 3.97, 2.97, 1.97 and 0.97, respectively

3.3.8 Effect of pH

An initial log_{10} inoculum of 5.4 (determined from plate counts) was used to study the effect of a range of pH (7.05 to 3.46) on growth. No visible growth was observed during the 3 day incubation at 30°C at pH 4.42 or less. As the pH was reduced, the O.D maximum was reduced and the rate of change of O.D also decreased. The O.D data at pH 6.95 were fitted with the Baranyi equation in concert with the calibration equation. Although the initial log inoculum size was determined as 5.4 from plate counts, from the TTD/log

initial inoculum calibration curve obtained at 30°C a count of 5.5 was expected. The initial log cfu ml^{-1} was held at 5.5 and the specific growth rate, lag and the MPD were obtained by regressing the calculated O.D against the observed, Figure 3-8 displays these results. Table 3-2 gives the parameters obtained; in no case was a significant value for a lag observed (i.e., in all cases the confidence interval for the calculated lags included zero).

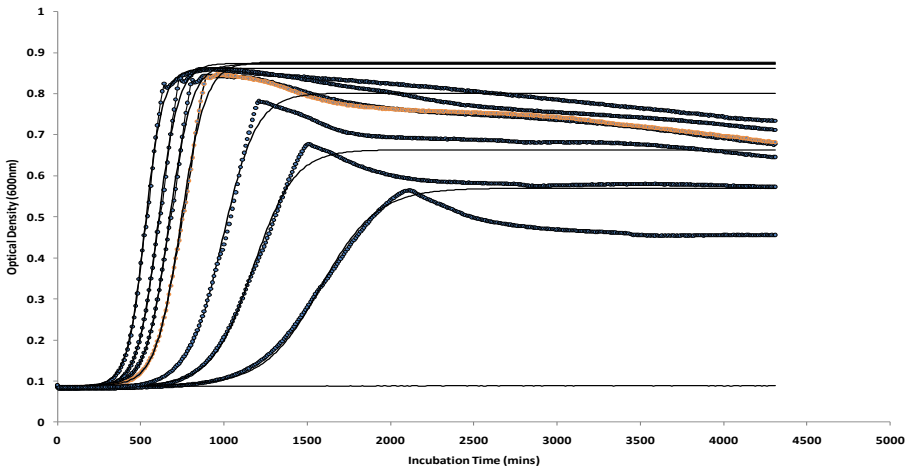


Figure 3-8 Comparison of the observed optical density incubation time plot (symbols) and the calculated (solid lines) for *Listeria monocytogenes* 252 incubated at 30°C with initial log_{10} inocula of 4.97, at pH 6.95, 5.65, 5.51, 5.03, 4.95, 4.88, 4.68 from left to right respectively, pH 4.42 failed to show any visible growth during the period of incubation (constant O.D = 0.088)

Table 3-2 Parameter Estimates for the Baranyi equation fitting of O.D data at various pH values

pH	Mu (ln cfu ml^{-1} min^{-1})	LCL	UCL	MPD	-95% CI	+95% CI
6.95	0.01870	0.01864	0.01875	9.771	9.769	9.773
5.65	0.01630	0.01625	0.01636	9.778	9.776	9.781
5.51	0.01472	0.01467	0.01478	9.777	9.774	9.781
5.03	0.01333	0.01328	0.01338	9.780	9.776	9.784
4.95	0.00984	0.00980	0.00988	9.736	9.732	9.741
4.88	0.00812	0.00808	0.00816	9.643	9.639	9.647
4.68	0.00592	0.00591	0.00594	9.566	9.563	9.569

3.4 Discussion

Cuppers and Smelt (1993) described an observed linear relationship between the log of the initial inoculum size and the time taken for the incubating culture to reach a specified turbidometric detection level due to a $10^{6.4}$ cfu ml^{-1} culture. They modelled the TTD data using a model based on the presence of a lag and the time taken for the initial culture to grow to the threshold value. Hence, the growth rate could be calculated. Essentially this study modelled the underlying growth curve as a 3-phase linear model, ignoring the MPD value.

From the classic logistic equation, the time taken (t_N) to reach a specific population level (N) from a given initial value (N_0) is given by (2-8) where μ is the growth rate and M is the maximum population density (also known as the carrying capacity).

This is almost in the linear form y = mx + c, especially when $M >> N_0$, and a plot of the time to the specific level against the natural log of the initial population number gives a gradient from which the growth rate can be found. When $N_0 = 1$, the intercept is obtained – the time taken for one organism to reach the specified detection number. One important point is that the logistic model as applied here has no lag. When we consider the phenomenon of microbial lag, we could simply state that if t < t(lag) then N(t) = N(0) and change the time function to t-t(lag) to account for the change. Physically this makes sense; the logistic equation is devoid of a lag, microbial lag is caused by an event (or sequence of events) before the onset of growth, hence is not part of the original derivation. Mathematically, however, the resulting logistic with lag equation has some undesirable features: the formula is discontinuous at the end of lag. This was, essentially, the equation reported by Jason (1983) and indeed the linear relationship between the log of the initial inocula (of *E.coli* growth measured using conductivity) and the time to reach a specific value was reported then.

The Baranyi model (Baranyi *et al.*, 1993a; 1993b) can be considered as a well-designed solution to the problem with the application of the Jason model. By invoking a time delay function, based on a firm biological foundation, the model becomes continuous, and remains biologically interpretable. A major feature of the model has been the assignment of the so-called pre-exponential factor which relates the fitness of an organism to thrive in an environment relative to another. If there is no difference between environments then the theory states that there should be no lag if the organism is transferred from one to the other and therefore the basic logistic model should apply – which is the default for the Baranyi model.

The TTD data produced using the multiple inocula technique described could be well fitted using the 3-PLM, the Baranyi and the logistic (with or without lag), the parameters obtained were consistent between models and reflected the observed gradients well. Further, using a simple conversion between O.D and numbers (cfu ml^{-1}), the basic features of the O.D/time plots could be reproduced with these models. The rescaling functions (2-6) and (2-8) overcome the peculiar problem described by Baranyi and Roberts (1995): that direct fitting of viable count data to turbidity or conductivity data or vice-versa should not be considered without additional information being available. The calibration curves used in this work can be used since they are obtained indirectly from pre-knowledge of the initial inoculum size, the maximum population density and the maximum specific growth rate.

The *modified* logistic and *modified* Gompertz equations, however, failed to fit the observed data and could not reproduce the observed O.D/time plots. A simple simulation of growth data with a given μ_{max}, lag and MPD for a number of initial inocula was produced using the *modified* Gompertz (or indeed the *modified* logistic) equation. A plot of the initial log inocula against the TTD ((2-2)) for a given detection number (log N_d) gave approximate straight line fits. The gradients of the line, however, were not the reciprocal of the growth rates used. When TTD were obtained for the same initial conditions, but for differing log N_D then these plots did not have the same gradient as was observed, and as log N_D approached the MPD the plot became increasingly

curved. From a casual glance at equation (2-2), this equation cannot give a simple linear fit with respect to the initial log inoculum used.

The 3-PLM, the Baranyi and the classical population logistic model were the only models examined which were capable of reproducing the straight line fit observed for the plot of the initial log numbers against the TTD. The 3-PLM, however, suffers from the inability to approach the MPD continuously, and although giving the correct TTD for O.D = 0.2 for the cultures, it failed to give the approach to the maximum O.D observed. The Baranyi and the classical logistic models did not have this problem.

Applying the method of fitting the Baranyi model directly to another set of data (30°C), by simply changing the MPD slightly, a good fit to the O.D data was found. Equally, the lack of an apparent lag (either from the fit of the model or from an analysis of the O.D/time plot for large initial inocula), suggested that the basic population logistic model would give an equivalent fit. This was indeed found to be the case.

Several reports have suggested that the O.D technique is limited as it requires high initial inocula (Dalgaard *et al.*, 1994; Dalgaard and Koutsoumanis, 2001; Baty *et al.*, 2002; Perni *et al.*, 2005). The observed data described herein show that this assumption is not valid. If the growth rate of an organism under ideal conditions is obtained using the multiple inoculum dilution method then any subsequent study using non-ideal conditions can use a positive control to set the modelled fit. For example in the study of pH, the growth rate at the ideal growth pH was known. The size of the initial inoculum used could then be either found from the calibration curve (knowledge of the TTD) or from plate counts (or both if confirmation was required). As conditions change (e.g. reducing pH) the fixed parameters of the Baranyi model can be altered to fit the new growth rates and/or lag induction. In the case studied here, the Baranyi model suggested that the growth rate reduced but that lag was not induced over the pH range studied. This result reflects well the conclusions of McKellar *et al.* (2002).

In 2002, McKellar *et al*. produced a study of the effect of pH on the growth of *Listeria monocytogenes* (using the strain Scott A) stating that pH had no affect on the initial physiological state and that the calculated lag was constant. A graph of the initial log inoculum (per well) against the TTD for a range of pH showed multiple linear slopes apparently intercepting the $\log_e$ inoculum axis at 20.086 +/- 1.092: equivalent to a range of 8.25 to 9.2 $\log_{10}$ cfu per well. This range encompasses the 8.37 $\log_{10}$ cfu $well^{-1}$ detection threshold found with this work. Further our study of the effect of pH also suggests that there was no change in lag– only a change in growth rate as described by McKellar. The difference between our interpretation and that of McKellar *et al.* is the presence or not of a lag. However, an important point must be made with respect to the detection value used in McKellar and Knight (2000) and McKellar *et al.* (2002): if the detection threshold is equivalent to $3.5x10^6$ cfu/well, then all the TTD values of wells containing greater than $3.5x10^6$ cfu/well should be zero; from figure 4 of McKellar *et al.* 2002 this is clearly not the case.

In McKellar and Knight (2000) the time of lag was calculated as the difference between observed TTD data and the theoretical TTD plot of the log of the initial inoculum based on a detection limit of $3.5x10^6$ cfu per Bioscreen well. Observed data had the theoretical growth rate but had greater TTD values since a lag was present. The observed data gave a regression fit of TTD_{30^oC}= – 132.84 $\log_{10} N_o$+ 1221, but was quoted in terms of cfu per well, which were filled with 350μl of culture. The growth rate obtained was very similar to that obtained from the data observed in this report (-127.09), but the intercept was higher than was found (in terms of cfu per ml, the intercept was calculated to be 1281 mins, whereas a value of 1122+/- 4.6 was observed in this study).

We suggest the discrepancy between the work reported here and the interpretation of the observations of these other workers may be due to an inadvertent use of the threshold detection value vs. a TTD based on a specific O.D. Figure 3-2 shows the TTD for different criteria: the O.D criteria of 0.09 is slightly above the background, this has a detection value of approximately

$8x10^6$ cfu/well (recalculated for a volume of 350μl per well). If this calibration curve was considered as the threshold curve, then any of the other calibrants would have a constant time delay (i.e. interpreted as lag) between them and this line. This may explain the difference in interpretation between the studies.

From the interpretation of the Baranyi model, the physiological state of the cells, denoted as α_0, at t_0 is a measure of the fitness of the cell in one environment to cope with being placed in a new environment. The negative natural log of α_0 is the product of the maximum specific growth rate and the lag. Hence, if there is no lag then $\ln(\alpha_0) = 0$. Both the study described herein and that of McKellar *et al*. suggest, however, that the automatic link of lag and growth may be globally invalid; growth rates can alter without inducing lags (e.g. the observed data on the change of pH). Conversely, lags can be induced without inducing changes in growth rate (albeit after recovery from injury) as shown by the work of Stephens *et al.* (1997).

3.5 Conclusion

We would simply conclude, therefore, that the Baranyi model is the most capable primary model of those examined (in the absence of lag it defaults to the classic logistic model), but that the *modified* logistic and the *modified* Gompertz should not be used as Primary models as they cannot reproduce observed data.

4 Modelling of bacterial growth with shifts in temperature using automated methods with *Listeria monocytogenes* as an example

4.1 Introduction

The measurement of microbial growth rates, especially its temperature dependency, is of fundamental importance in food microbiology. For many food pathogens growth above 25°C and below 45°C is usually rapid with an optimum around 37°C. Below 5°C only a few (often spoilage, e.g. *Pseudomonas aeruginosa*, but pathogens are also represented e.g. *Listeria monocytogenes*) have growth rates that would give rise to concern. This has been shown, for example, by Thomas and O'Bierne (2000) on the temperature abuse of vegetables with respect to spoilage (lactic acid bacteria) and risk (*L. monocytogenes*).

Within the literature several studies have looked at the effect of non-isothermal conditions on microbial growth using established modelling methods (e.g. Baranyi *et al*., 1995; Bovill *et al*., 2000; Dalgaard *et al*., 2002; Giannakourou *et al*., 2005; Koutsoumanis, 2001; Koutsoumanis *et al*., 2006; Li and Torres, 1993; Taoukis *et al*., 1999; Zwietering *et al*., 1994). The aim of many of these studies was to test the ability of using models based on growth data obtained isothermally to predict growth under non-isothermal conditions. Zwietering *et al*., (1994) concluded that, within the exponential phase, the hypothesis of no lag occurrence was accepted statistically in more than 70% of their experiments for *Lactobacillus plantarum*, however within the lag phase, the hypothesis of additional lag occurrence was accepted statistically in more than 90% of their experiments.

Corradini and Peleg (2005) have eloquently questioned the reasoning and conclusions being drawn from the use of the empirical standard primary and secondary models used to interpret and predict data from isothermal and fluctuating temperature studies. They suggest abandoning specific formats and using, instead, a generalized scheme for both primary and secondary

modelling, “in the absence of a decisively superior theoretical model… [ad hoc empirical models] have the advantage of being simpler mathematically and free of assumptions that require independent verification”.

Automated techniques such as turbidometry tend to come under fire from traditional microbiologists since they cannot directly reproduce the standard microbial growth curve, which the multitude of primary models are fitted to (Augustin *et al*., 1999; Dalgaard *et al*., 1994; McClure *et al*., 1993), yet their very persistence reflects their ease of use, the high quantity and quality of the data obtained and the large savings in consumable costs over that of the traditional (plate-count) methods. It has been shown that the *modified* Gompertz and *modified* logistic models are at odds with the observed TTD data obtained using turbidometry (Mytilinaios *et al*., 2012). The classic logistics models (and by default the Baranyi equation) were the only models used able to reconstruct the observed TTD data. The three parameter model has a firm (if simple) theoretical foundation. Its application to standard microbiological data results in mismatch due to methodological inadequacies (plate counting) and the presence of lag and so is rarely used in its original form. Herein, we further examined the application of the basic logistic model to microbial growth data (obtained as TTD) and use small temperature shifts (or shunts) to examine their effect on the growth rates of *L. monocytogenes 252*.

4.2 Materials and methods

L. monocytogenes 252 was studied using small temperature shifts to examine their effect on the growth rate. The preparation of the cultures and the fill of the Bioscreen micro-array plates were done as described in paragraph 2.3.8. The data obtained from the Bioscreen were analysed as described in 2.4.3 and the model used to fit the data which is based on the Malthusian approximation of the logistic model as described in paragraph 2.4.8.

4.3 Results

4.3.1 Iso-thermal studies

The O.D/time curves for the growth of multiple inocula of *L. monocytogenes* 252 at 25°C and 37°C in TSB (0.5% NaCl) were obtained over a 24 hour period. Figure 4-1 shows the typical results of such experiments: a linear relationship between the log of the initial inoculum size and the time to detection (when O.D = 0.2). Table 4-1 gives the regression parameters obtained.

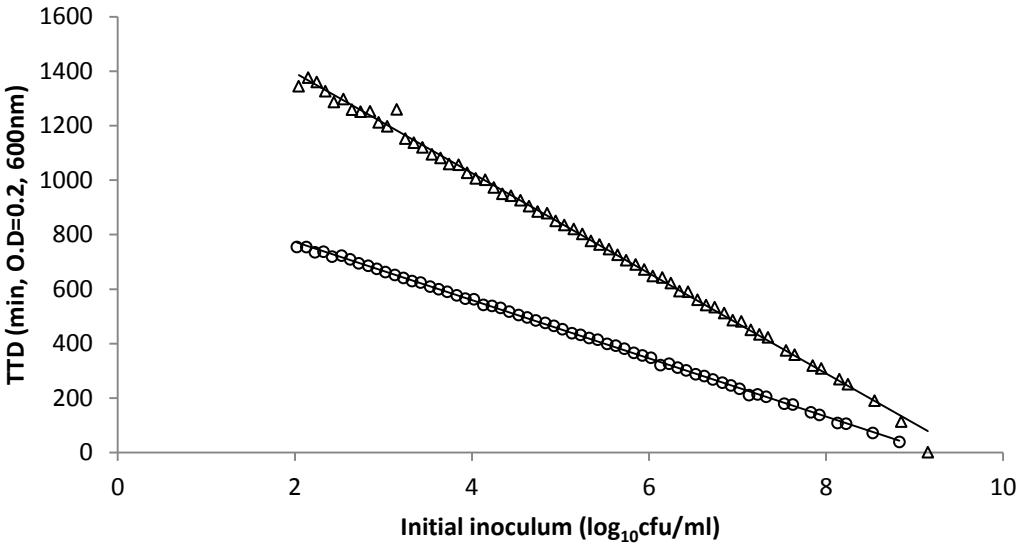


Figure 4-1 Relationship between TTD with the initial populations of *Listeria monocytogenes* 252 at 25ºC (△) and 37ºC (○) in TSB with 0.5% NaCl

Table 4-1 Regression and growth parameters from the inoculum size dependency of *Listeria monocytogenes* 252 on the TTD at 25ºC and 37ºC

Microorganism	Temp (ºC)	Gradient (mins/Log(cfu/ml))	Intercept (mins)	*Specific growth rate (hours^{-1})*	*Generation time (hours)*
***L. monocytogenes* 252**	**25**	-183.40 (-185.94—180.86)	1687.6 (1671.7-1703.6)	0.753	0.920
	37	-109.98 (-110.85—109.10)	999.22 (993.90- 1004.6)	1.256	0.544

4.3.2 Non-isothermal studies

To examine the effect of multiple temperature shifts, two Bioscreens were used; one Bioscreen was set at one particular temperature (37°C), the other machine at a different temperature (25°C). Identically prepared micro-titre plates were placed in each Bioscreen and incubated for a set time. At the set time the plates were swapped between the machines, without stopping the Bioscreens themselves.

Figure 4-2 shows the observed TTD for multiple initial inocula of *L. monocytogenes* 252 undergoing either a 37-25-37-25°C or a 25-37-25-37°C temperature incubation sequence, changing temperatures after 360, 500 and 900 minutes. The observed gradients were -107.9, -179.3,-105.2,-NA for the 37-25-37-25°C sequence and -193.5,-104.7,-172.6,-110.0 for the 25-37-25-37°C sequences respectively. Superimposed, on Figure 4-2 are the predicted values from the model used (lines), the TTD predictions of which are based on the growth rate data given in Table 4-1. From the observed, fitted and predicted data it can be concluded that no induction of lag occurred when moving from the higher to the lower temperatures used: the intercept of the regression lines for each temperature coincide at the time of the temperature shunt, if lags were present this would not occur.

The Geometric model (equation (2-23)) can be either used to predict the outcome of hypothetical experiments –as was done for the multiple temperature shunt with *Listeria* shown in Figure 4-2, or can be used to fit the observed data by minimising the sum of squares of the errors. Another method of using the predictive capacity of the model is to predict the TTD observed from a single Bioscreen incubating at a given temperature, when identical plates are moved in or out of the machine. Figure 4-3 shows a prediction of the pattern of TTD/log initial inocula from the single Bioscreen incubating at 37°C. Using the growth rates described in Table 4-1, in 360 minutes the model predicts that 3.27 logs of growth will occur in this plate, whereas the other plate incubating in the other machine at 25°C will increase

by only 1.97 logs. When the latter plate is placed in the machine at 37°C, if there are no lags then over the next 240 minutes there will be further increase of 2.18 logs in this plate. By calculating the log increase in the numbers of *L. monocytogenes* at 37°C and that incubated at 25°C and then subsequently placed at 37°C, the pattern shown in Figure 4-3 was obtained. The observed data are overlain on the predicted lines.

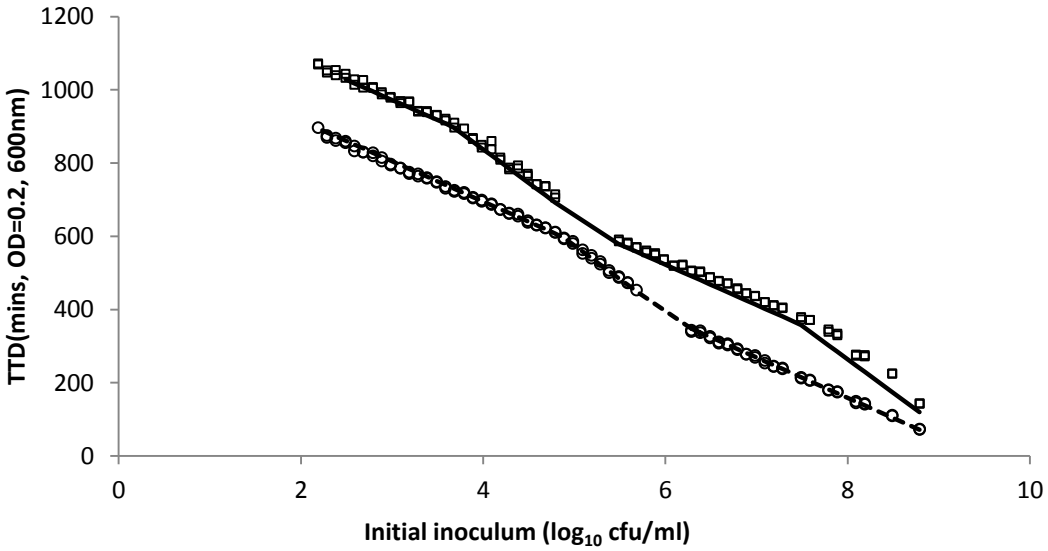


Figure 4-2 Observed TTD data for *L. monocytogenes* 252 incubating at 37-25-37-25ºC (○) or 25-37-25-37ºC (□) with temperature shunts occurring at 360, 600 and 900 minutes. The solid and the dashed lines, respectively, are the predicted values based on the data of Table 4-1, with N_D = 9.4

Table 4-2 Observed regression parameters from the observed TTD from multiple initial inocula of *Listeria monocytogenes* 252 shunted from 37, 25, 37 and 25ºC and concurrently from 25, 37, 25 and 37ºC

Time (min)	Temp (ºC)	Parameter ($logI_D$)	Estimate (9.48)	LCI (9.26)	UCI (9.71)	r^2 (obs)
<360	37	m0	-107.92	-108.36	-106.47	0.998(38)
<600	25	m1	-179.29	-188.04	-170.54	0.992 (17)
<900	37	m2	-104.62	-106	-103.25	0.998(52)
>900	25	m3	-	-	-	no obs

Time (min)	Temp (ºC)	Parameter ($logI_D$)	Estimate (9.58)	LCI (7.98)	UCI (11.52)	r^2 (obs)
<360	25	m0	-193.5	-212.59	-174.4	0.981 (12)
<600	37	m1	-104.73	-106.54	-102.93	0.997(42)
<900	25	m2	-174.69	-189.12	-160.26	0.971 (21)
>900	37	m3	-109.52	-114.69	-104.35	0.984 (33)

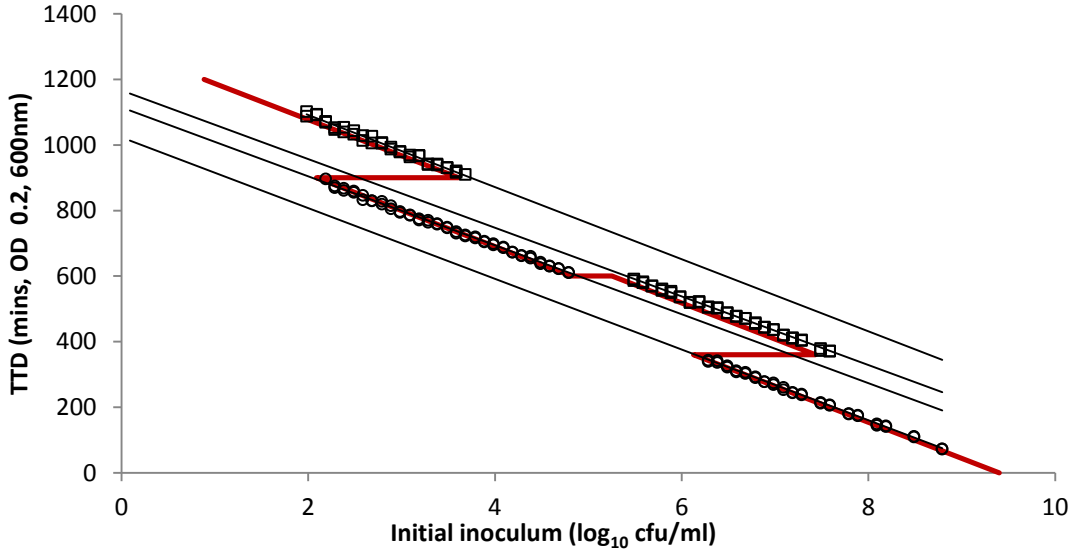


Figure 4-3 Observed TTD data (symbols) and predicted data (solid line) from a single Bioscreen incubating at 37ºC for multiple inocula of *L. monocytogenes* 252 undergoing plate changes to and from another machine incubating at 25ºC (see Figure 4-2). The solid lines are the predicted TTD based on the data of Table 4-1 and the use of the logistic model to calculate the expected increase in numbers in both plates during the periods of incubations at 37ºC and 30ºC. The parallel thin lines are the linear regression fits to the observed data

4.4 Discussion

The simple, classical 3-parameter logistic model can model the TTD data obtained from turbidometric experiments using multiple initial inocula incubated iso-thermally. In all cases studied no lags were observed either from the O.D/incubation time plots or from the plotted or modelled data. Plots of the log initial inoculum against the TTD cut the log N_0 axis at 9.22 $\log_{10}$ cfu/ml (95% CI 9.05 – 9.4 $\log_{10}$ cfu/ml) for *L. monocytogenes 252*. The detection number (N_D) was confirmed by plate counting and from calibration curves of O.D against microbial numbers. In the presence of a lag the plot would fail to cross the axis at the N_D, and a vertical separation equal to the lag between the x-axis and the TTD of the N_D would be present. This was not observed in any of the isothermal studies performed.

The linear approximation (equation (2-17)) to the logistic expression (equation (2-8)) assumes that $M>>N_0$ and also that $M>>N_D$; when $N_0 = N_D$, TTD = 0. If $M < 10N_D$ then curvature of the observed TTD occurs, if $M < 3N_D$ then this curvature is substantial and the mismatch between equation (2-17) and equation (2-8) becomes significant. In all the cases studied here, $M > 10N_D$ and this curvature was not observed. If the detection threshold is increased, e.g. use of a higher O.D threshold, then curvature is observed. Conversely, lowering the threshold would reduce any observed curvature. Equation (2-17) does not require the estimation of the MPD and in the absence of a lag is a two parameter model. Rearranging equation (2-17) results in the Malthusian approximation of biological growth – i.e. growth without limit. The value of M is, however, used in the full form of the logistic model. M can be obtained through plate count, from dilutions of the MPD culture to produce a calibration curve using O.D or from using the phenomenon of curvature discussed above.

When a temperature shunt was applied to growing bacteria, the cultures reduced or increased their growth rate commensurate with the incubation temperature. When cultures were shunted from a lower temperature to a higher temperature there was no evidence of an induced lag and growth continued at the rate dictated by the new temperature. These observations

are in accordance with the general conclusion of Zwietering *et al*., (1994). When cultures were shunted from a higher to a lower temperature condensation on the inside of the plate lid occurred and this led to unusable data for a period after the shunt (the period depended on the temperature difference). From the observed, fitted and predicted data it can be concluded that no induction of lag occurred when moving from the higher to the lower temperatures used: the intercept of the regression lines for each temperature coincide at the time of the temperature shunt, if lags were present this would not occur (e.g. Figure 4-2).

The traditional method of examining growth using plates can be considered to be a repeated measures experiment following the growth of an initial inoculum with time, whereas the method used here is a multiple inoculum experiment with a single time measurement (the TTD) per inoculum. These methods should be considered not as complementary but methods describing the same phenomenon of microbial growth, done in a different fashion. However, the models used to extract the growth rate data from the two methods are not consistent: the *modified* Gompertz and *modified* logistic model cannot reproduce the observed TTD data (Mytilinaios *et al*., 2012). This has implications when such models are used to examine data obtained from turbidimetry.

4.5 Conclusion

Using micro-titre plates with multiple inocula allows the investigation of a wealth of phenomena - such as the temperature shifts investigated here. From our modest results, we would conclude that for small temperature shifts, for *L. monocytogenes* 252, growth rates quickly changed to the new environment without the induction of lags and conclude that the classic logistic model is an adequate descriptor and theoretical model for TTD data obtained from turbidimetry.

5 Modelling the effect of sodium chloride, pH and their combinations on the growth of *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* using a rapid optical density method

5.1 Introduction

Listeria monocytogenes is a dangerous foodborne pathogen especially for the vulnerable members of the society with high mortality rates up to 30-40% (Datta, 2003). *L. monocytogenes* can grow in low pH and a_w concentrations as well as at temperatures lower than 0°C (Tienungoon *et al*., 2000). The aforementioned in conjunction with the intracellular characteristic of *L. monocytogenes* have made that pathogen a main concern of food safety. *Salmonella* Typhimurium and *Escherichia coli* are also of much concern within the food industry as they have been responsible for many outbreaks (EFSA, 2010). *Salmonella* has been reported as one of the most common pathogens which cause foodborne diseases in Europe in 2010 (EFSA, 2012). *S*. Typhimurium has been considered as one of the most commonly associated serovars with human infections (EFSA, 2010). The low infective dose of *E. coli* in conjuction with the most recent consumer trend for less preserved and processed food have increased the need for collecting growth data on these microorganisms (Shadbolt *et al*., 1999).

Obtaining growth rates and lag times is fundamental in food microbiology. There are many studies within the literature which have looked at the growth parameters of several microorganisms (Perni *et al*., 2005; Augustin and Carlier, 2000; McKellar and Knight, 2000). Also, the so called inoculum effect and the increased variability in the low inoculum sizes have been the subject of many studies (Pin and Baranyi, 2006; Metris *et al*., 2006; Masana and Baranyi, 2000; Koutsoumanis and Sofos, 2005). With predictive microbiology all the knowledge of the microbial responses in different environmental conditions is summarised as mathematical models or equations (McMeekin *et al*., 1997). The collection of data regarding the behaviour of the

microorganisms in different condition requires a large amount of work. The development of rapid, automated, accurate and cheap methods (such as the optical density method) which offer new possibilities is essential in predictive food microbiology.

The Bioscreen microbiological analyser is a machine which measures O.D and it has been used in food microbiology for several applications such as the determination of growth rates (Dalgaard *et al.*, 1994; Dalgaard and Koutsoumanis, 2001). The use of the Bioscreen can give a large amount of data in a very short period of time. However, the interpretation of the results is the most important but also the most difficult part when using O.D methods.

In the literature, there are some authors who do not use any type of calibration between O.D and viable counts and directly fit primary models such as the *modified* Gompertz or the logistic model to O.D data (Dalgaard and Koutsoumanis, 2001; Cheroutre-Vialette and Lebert, 2002). Also, there are several methods that have been used in order to define the relationship between the measured O.D and the viable cell counts. There are authors who have used linear models based on the Lambert and Beer law (Lack *et al.*, 1999), quadratic models (McClure *et al.*, 1993), cubic models (Stephens *et al.* 1997) as well as logarithmic transformations for both O.D values and the viable counts in order to normalise the variance (Francois *et al.*, 2003; 2005) or the natural logarithmic transformations (Chorin *et al*., 1997).

The evaluation of the Growth/No Growth (G/NG) interface is of particular interest in terms of the food safety where a possible contamination of the food with foodborne pathogens resulting in bacterial growth and subsequently the risk for a foodborne illness would increase (Tienungoon *et al*., 2000).

Herein, we have been developing the use of O.D for obtaining growth rates and lag times using multiple inocula, of *L. monocytogenes* strains in different NaCl concentrations and incubation temperatures. Also, *S.* Typhimurium *and E. coli* were studied in different NaCl concentrations, different pH and their combinations. All analyses were performed in the Bioscreen microbiological analyser and the classic logistic model was rearranged to fit TTD data. The

Lambert and Pearson model (LPM) was used to calculate the MIC_{NaCl} and/or MIC_{pH} of all *L. monocytogenes* strains, *S.* Typhimirium and *E. coli*. In addition, the G/NG boundaries using pH and NaCl as hurdles were determined from O.D measurements based on nominal logistic regression and a response surface model (RFM) was produced and compared with the Extended Lambert and Pearson model (ELPM) from the continuous data which transformed in rate to detection (RTD). The results obtained showed that the rearranged logistic model with lag could give accurate growth rates and lag times and that the inoculum size did not affect the growth rates but affected only the TTD. The LPM and ELPM can also analyse results from individual and combined inhibitors, respectively.

5.2 Materials and methods

Calibration curves were made as described in 2.4.2 for four *L. monocytogenes* strains (252, 271, 177 and ScottA), *S.* Typhimurium and *E. coli*. The growth of the *L. monocytogenes* strains was studied at 30°C and 37°C in 0.5, 3, 6 and/or 9% NaCl as described in 2.3.5.1 while the growth of *S.* Typhimurium and *E. coli* was studied at 30°C in 0.5, 3 and 6% NaCl as described in 2.3.5.1, in different pH as described in 2.3.5.2 and in their combinations (NaCl-pH) as described in 2.3.5.3. The data obtained from the Bioscreen were analysed as described in 2.4.3 and the rearranged logistic model with lag was used to fit the data as described in 2.4.5. Also, the MIC_{NaCl} and the MIC_{pH} was calculated for all microorganisms as described in 2.3.6.1 and 2.3.6.2, respectively and modelled as described in 2.4.6 with the LPM model. Finally, the G/NG boundaries were determined using combinations of NaCl-pH as described in 2.3.7 and modelled using three approaches as described in 2.4.7.

5.3 Results

5.3.1 Construction of calibration curves

In the literature there are several methods used to define the relationship between O.D and microbial numbers. O.D was directly related to microbial numbers by measuring different O.D in the Bioscreen microbiological analyser

(volume 200µl) and in the spectrophotometer (1cm path length) and then plate samples onto TSA plates. The results obtained are shown in Table D-1 (Appendix D).

O.D was also related to microbial numbers by accomplishing a series of dilutions and condensations following two protocols. In the first, O.D was related to microbial numbers using as a basis the plate counts obtained from a culture which had previously reached the maximum O.D. In the second, O.D was related to microbial numbers using as a basis the average plate counts calculated from the standardised cultures at an O.D=0.5 from each individual experiment carried out into the Bioscreen (see 2.1.1, culture maintenance and preparation). The relationship between O.D and microbial numbers was linear and Figure 5-1 shows the calibration curves constructed using the average counts from the standardised culture (O.D=0.5), for *S.* Typhimurium in the Bioscreen (200µl volume) and in the spectrophotometer (1cm path length). Similar figures obtained for all cases.

The detection limits calculated from the three protocols, for an O.D=0.2 which is the criterion used for all studies carried out, were similar (Data not shown). However, we have used the detection limits calculated from the calibration curves using the counts measured from the standardised cultures at an O.D=0.5 because the measurements we have got are much greater and it was considered as more accurate. The parameters describing the relationship between the O.D against the microbial populations calculated from the average value (microbial numbers) of the standardised culture at an O.D=0.5 are shown in Table 5-1.

The TTD was defined as the time for each inoculum to reach an O.D=0.2, which is the O.D criterion used in all studies performed. The detection values (N_D) for an O.D=0.2 are shown with their confidence intervals in Table 5-1. We would conclude that the detection values (N_D) found to be 9.16, 9.15, 9.31, 9.22, 8.69 and 8.78 logs for *L. monocytogenes* ScottA, *L. monocytogenes* 252, *L. monocytogenes* 271, *L. monocytogenes* 177, *E. coli* and *S.* Typhimurium, respectively (Table 5-1).

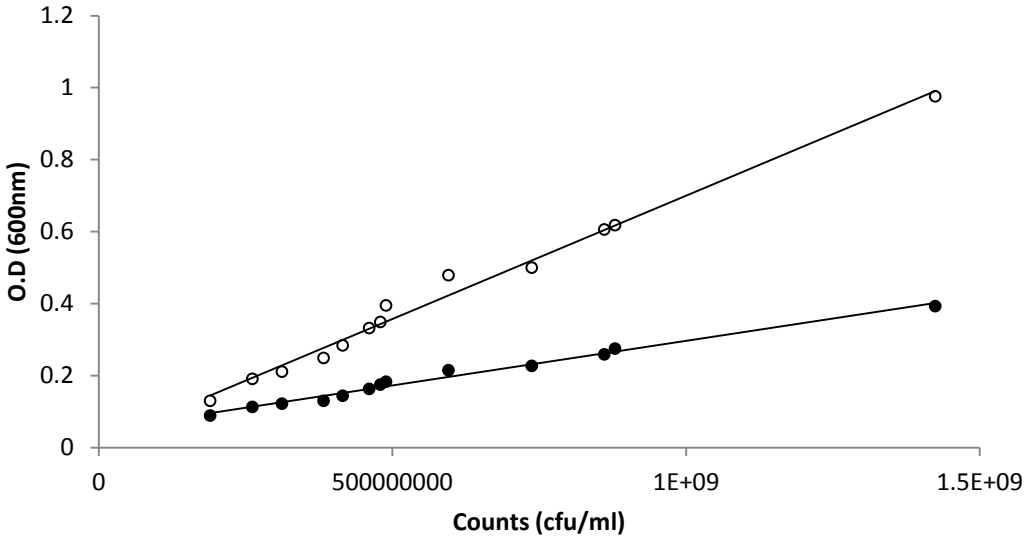


Figure 5-1 Relationship between Optical Density (O.D) against the microbial numbers of *Salmonella* Typhimurium in the spectrophotometer (○, 1cm path length) and in the Bioscreen (●, 200µl volume).The counts used were calculated from the standardised culture at an O.D= 0.5

Table 5-1 Parameters describing the linear relationship between O.D against the microbial populations of *L. monocytogenes* 252, *L. monocytogenes* 39, *L. monocytogenes* 271 and *L. monocytogenes* 177, *S.* Typhimurium and *E. coli* as measured in the Bioscreen (200µl volume) and in the spectrophotometer (1cm path length) using the average counts from the standardised culture at an O.D=0.5

Microorganism	Spectrophotometer		Bioscreen		
	Gradient (*10^{10})	Intercept (*10^{2})	Gradient (*10^{10})	Intercept (*10^{2})	Detection value (N_D)
***L. monocytogenes* 252**	3.88 (3.62-4.14)	0.03 (-1.0-1.07)	1.42 (1.33-1.52)	-0.004 (-0.39-0.38)	9.15 (9.11-9.19)
***L. monocytogenes* 39**	3.52 (3.24-3.80)	0.97 (-0.1-2.05)	1.35 (1.24-1.45)	0.29 (-0.1-0.7)	9.16 (9.12-9.21)
***L. monocytogenes* 271**	2.87 (2.66-3.08)	-0.7 (-1.7-0.4)	0.99 (0.89-1.08)	-0.08 (-0.5-0.4)	9.31 (9.26-9.36)
***L. monocytogenes* 177**	3.32 (2.95-3.69)	0.7 (-0.6-2.1)	1.19 (1.01-1.37)	0.03 (-0.6-0.7)	9.22 (9.15-9.32)
E. coli	10.3 (9.49-11.1)	3.9 (-0.8-8.6)	4.21 (3.43-4.99)	-1.0 (-5.5-3.5)	8.69 (8.52-8.87)
***S*. Typhimurium**	6.86 (6.36-7.35)	1.4 (-1.8-4.7)	2.48 (2.29-2.66)	4.9 (3.6-6.1)	8.78 (8.72-8.85)

5.3.2 Estimation of growth rates and lag times from TTD data

The effect of sodium chloride (0.5%, 3%, 6% and/or 9% NaCl) on the growth of the four *L. monocytogenes* strains*, E. coli* and *S.* Typhimurium was studied at 30°C and/or 37°C using the Bioscreen microbiological analyser. Figure 5-2 depicts the relationship between the TTD against the initial populations of *L. monocytogenes* ScottA at 30°C in different NaCl concentrations (0.5, 3, 6 and 9% NaCl). The relationship is linear and the observed TTD were modelled with the rearranged with lag logistic model. As the NaCl concentration increased, the gradient and the intercept of the rearranged logistic model increased. Similar findings were observed for all microorganisms.

The growth parameters (including their confidence intervals) obtained from the model at 30°C for the *L. monocytogenes* strains are shown in Table 5-2 while for *E. coli* and *S.* Typhimurium are shown in Table 5-4. With increasing NaCl concentration the growth rate decreased while the lag time increased for all cases. Also, the root mean square error (RMSE) increased as the NaCl concentration increased. An increased RMSE suggests that the differences between the observed and the modelled values are also increased.

The incubation temperature also affected the results. The results obtained at 30°C (Table 5-2) showed a higher effect on the growth rates and lag times than at 37°C (Table 5-3) which is what was expected as 37°C is more optimal temperature than 30°C. In particular, at 37°C the growth rates were higher while the lag times were shorter than at 30°C. *Listeria* strains were examined in 9% NaCl only at 30°C because at 37°C and in 9% NaCl concentration cell clumping was observed at the bottom of the honeycomb plates and O.D measurements were not reliable. *S.* Typhimurium and *E. coli* did not grow in 9% NaCl.

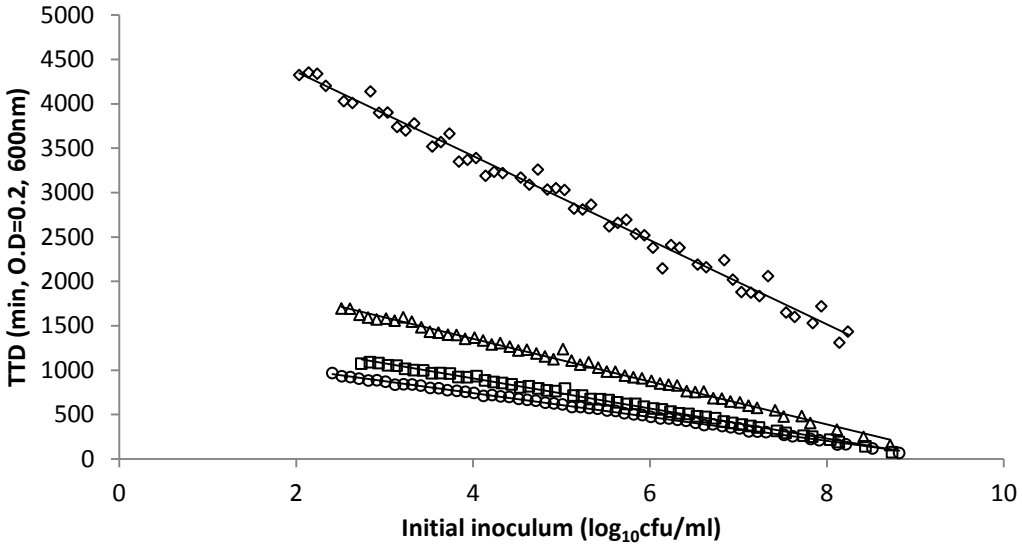


Figure 5-2 Relationship between TTD with the initial populations (logNo(cfu/ml)) of *Listeria monocytogenes* 39 in TSB with 0.5% NaCl (○), 3% NaCl (□), 6% NaCl (△) and 9% NaCl (◊) at 30°C

Table 5-2 Parameters describing the growth kinetics of *L. monocytogenes* 252, *L. monocytogenes* 39, *L. monocytogenes* 271 and *L. monocytogenes* 177 in different NaCl concentrations as calculated from data fitted with the rearranged logistic model at 30°C, including their confidence intervals, the root mean square error with the number of observations and the r-squared

			Obtained parameters			
Organism	**Temp (°C)**	**NaCl (%)**	**SGR (lncfu/h)**	**Lag (h)**	**RMSE (obs)**	**r^2**
***L. monocytogenes* 252**	30	0.5	1.07 (1.06-1.08)	0.28 (0.21-0.35)	6.91 (61)	0.999
N_D 9.15	30	3	0.91 (0.91-0.92)	0.65 (0.57-0.73)	7.95 (61)	0.999
	30	6	0.62 (0.61-63)	0.98 (0.79-1.18)	18.5 (60)	0.998
	30	9	0.30 (0.29-0.31)	12.69 (11.66-13.71)	84.5 (51)	0.989
***L. monocytogenes* 39 (ScottA)**	30	0.5	1.02 (1.01-1.03)	0.85 (0.73-0.96)	11.9 (63)	0.998
N_D 9.16	30	3	0.83 (0.81-0.84)	0.75 (0.59-0.92)	16.2 (61)	0.997
	30	6	0.58 (0.57-0.59)	2.00 (1.67-2.32)	30.2 (60)	0.995
	30	9	0.29 (0.28-0.30)	16.14 (15.08-17.19)	92.6 (53)	0.988
***L. monocytogenes* 271**	30	0.5	1.03 (1.02-1.04)	-0.43 (-0.54—0.32)	9.77 (60)	0.998
N_D 9.31	30	3	0.86 (0.85-0.87)	-0.08 (-0.19-0.02)	9.67 (61)	0.999
	30	6	0.59 (0.58-0.60)	0.33 (0.15-0.50)	15.6 (60)	0.999
	30	9	0.30 (0.28-0.33)	20.06 (17.23-22.89)	197 (49)	0.935
***L. monocytogenes* 177**	30	0.5	0.95 (0.94-0.95)	-0.40 (-0.47—0.34)	7.79 (98)	0.999
N_D 9.22	30	3	0.79 (0.78-0.79)	-0.41 (-0.50—0.32)	8.03 (60)	0.999
	30	6	0.59 (0.58-0.59)	0.54 (0.35-0.73)	17.0 (60)	0.998
	30	9	0.29 (0.28-0.31)	10.05 (8.74-11.36)	102 (52)	0.985

Table 5-3 Parameters describing the growth kinetics of *L. monocytogenes* 252, *L. monocytogenes* 39, *L. monocytogenes* 271 and *L. monocytogenes* 177 in different NaCl concentrations as calculated from data fitted with the rearranged logistic model at 37°C, including their confidence intervals, the root mean square error with the number of observations and the r-squared

			Obtained parameters			
Organism	**Temp (^{0}C)**	**NaCl (%)**	**SGR (lncfu/h)**	**Lag (h)**	**RMSE (obs)**	**r^2**
***L. monocytogenes* 252**	37	0.5	1.29 (1.28-1.30)	0.30 (0.24-0.35)	5.66 (63)	0.999
N_D 9.15	37	3	1.08 (1.07-1.09)	0.06 (-0.02-0.14)	7.26 (60)	0.999
	37	6	0.67 (0.66-0.68)	0.10 (-0.18-0.39)	28.2 (61)	0.995
***L. monocytogenes* 39**	37	0.5	1.26 (1.24-1.28)	0.58 (0.48-0.69)	10.7 (63)	0.997
N_D 9.16	37	3	1.03 (1.02-1.04)	0.39 (0.27-0.51)	11.6 (61)	0.998
	37	6	0.68 (0.67-0.69)	1.17 (0.93-1.41)	22.3 (60)	0.996
***L. monocytogenes* 271**	37	0.5	1.25 (1.24-1.26)	0.58 (0.51-0.64)	6.01 (63)	0.999
N_D 9.31	37	3	1.02 (1.00-1.04)	-0.20 (-0.40-0.00)	18.6 (61)	0.995
	37	6	0.67 (0.66-0.68)	-0.22 (-0.45-0.00)	20.1 (60)	0.997
***L. monocytogenes* 177**	37	0.5	1.07 (1.06-1.08)	0.34 (0.29-0.39)	4.99 (63)	0.999
N_D 9.22	37	3	0.87 (0.85-0.89)	-1.01 (-1.27--0.75)	23.2 (60)	0.994
	37	6	0.62 (0.61-0.63)	-0.288 (-0.59-0.03)	28.2 (60)	0.995

Table 5-4 Parameters describing the growth kinetics of *Salmonella* Typhimurium and *Escherichia coli* in different NaCl concentrations (0.5, 3 and 6% NaCl) as calculated from data fitted with the rearranged logistic model at 30°C, including their confidence intervals, the root mean square error with the number of observations and the r-squared

			Obtained parameters			
Organism	**Temp (°C)**	**NaCl (%)**	**SGR (lncfu/h)**	**Lag (h)**	**RMSE (obs)**	**r^2**
E. coli	30	0.5	1.42 (1.40-1.43)	-0.006 (-0.07-0.06)	6.34 (58)	0.999
N_D 8.69	30	3	1.08 (1.07-1.09)	0.69 (0.63-0.76)	6.82 (59)	0.999
	30	6	0.36 (0.34-0.38)	4.85 (3.43-6.27)	132 (56)	0.964
***S.* Typhimurium**	30	0.5	1.21 (1.19-1.24)	1.06 (0.89-1.22)	16.6 (59)	0.994
N_D 8.78	30	3	0.95 (0.93-0.96)	1.93 (1.79-2.07)	14.3 (59)	0.997
	30	6	0.32 (0.31-0.33)	4.14 (3.25-5.03)	82.0 (53)	0.988

E. coli and *S.* Typhimurium were also studied in different pH (6.57, 5.68, 5.10 and 4.58) as well as in different combinations of NaCl-pH at 30°C. The relationship between the TTD against the initial populations of the microorganisms was linear and as the conditions became harsher (decreased pH or increased NaCl and decreased pH), the gradient and the intercept increased. Figure 5-3 shows the observed TTD against the initial populations of *S.* Typhimurium at 30°C in different pH. Similar figures were obtained for all cases.

The observed TTD were modelled with the rearranged logistic model and the parameters obtained in different pH at 30°C are shown in Table 5-5 while the parameters obtained in different combinations of NaCl-pH at 30°C are shown in Table 5-6. With decreasing pH (or decreasing pH and increasing NaCl) the growth rate decreased while the lag time increased in all cases. The r^2 was high in all cases which suggest a high correlation between the model and the observed data while the RMSE increased as the conditions became harsher. Under the more extreme NaCl-pH combinations used (6% NaCl-pH: 5.17, 3% NaCl-pH: 4.58 and 6% NaCl-pH: 4.58) *E. coli* did not grow.

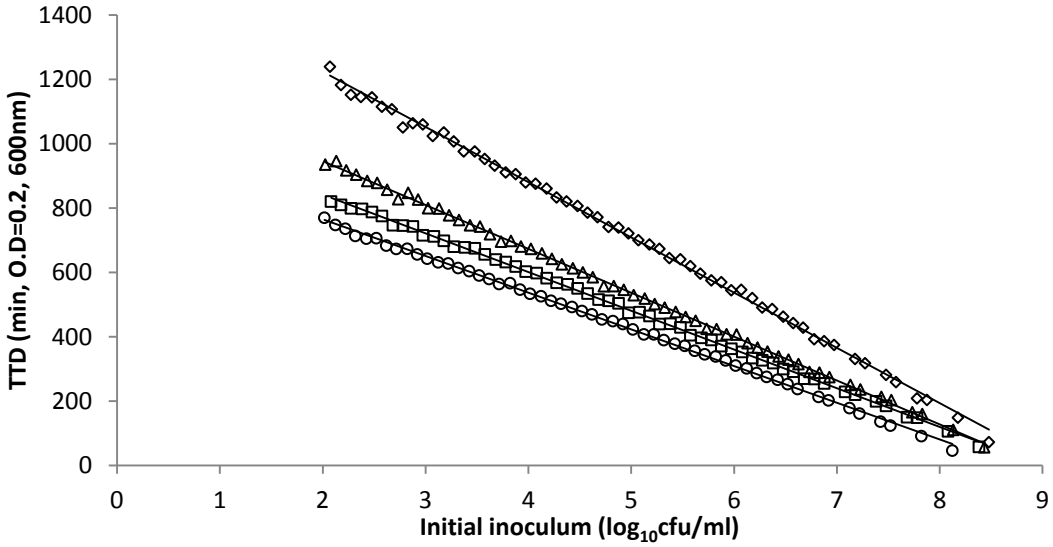


Figure 5-3 Relationship between TTD with the initial populations (logNo(cfu/ml)) of *Salmonella* Typhimurium in TSB with pH:6.57 (○), pH:5.68 (□), pH:5.10 (△) and pH:4.58 (◊) at 30°C fitted with the rearranged logistic model (continuous lines)

Table 5-5 Parameters describing the growth kinetics of *Salmonella* Typhimurium and *Escherichia coli* in different pH (6.57, 5.68, 5.10 and 4.558) as calculated from data fitted with the rearranged logistic model at 30°C, including their confidence intervals, the root mean square error with the number of observations and the r-squared

			Obtained parameters			
Organism	**Temp (°C)**	**pH**	**SGR (lncfu/h)**	**Lag (h)**	**RMSE (obs)**	**r^2**
E. coli	30	6.57	1.45 (1.43-1.46)	-0.25 (-0.31—0.20)	4.34 (52)	0.999
N_D 8.69	30	5.68	1.48 (1.47-1.50)	0.31 (0.24-0.38)	6.40 (57)	0.998
	30	5.10	1.29 (1.28-1.30)	0.45 (0.38-0.51)	6.00 (56)	0.999
	30	4.58	0.99 (0.97-1.02)	0.89 (0.66-1.12)	21.3 (57)	0.992
***S.* Typhimurium**	30	6.57	1.21 (1.20-1.22)	-0.07 (-0.15-0.01)	6.71 (55)	0.999
N_D 8.78	30	5.68	1.15 (1.14-1.16)	0.49 (0.43-0.56)	6.08 (57)	0.999
	30	5.10	1.01 (1.00-1.02)	0.43 (0.36-0.50)	6.79 (58)	0.999
	30	4.58	0.80 (0.79-0.81)	1.08 (0.95-1.21)	12.6 (58)	0.998

Table 5-6 Parameters describing the growth kinetics of *Salmonella* Typhimurium and *Escherichia coli* in different combinations of NaCl-pH as calculated from data fitted with the rearranged logistic model at 30°C, including their confidence intervals, the root mean square error with the number of observations and the R-squared

				Obtained parameters			
Organism	**Temp (°C)**	**NaCl (%)**	**pH**	**SGR (lncfu/h)**	**Lag (h)**	**RMSE (obs)**	**r^2**
E. coli	30	3	6.53	1.06 (1.05-1.07)	0.38 (0.32-0.44)	6.82 (96)	0.999
N_D 8.69	30	6	6.51	0.38 (0.37-0.39)	11.24 (10.48-11.99)	86.9 (86)	0.975
	30	3	5.80	1.08 (1.07-1.09)	0.61 (0.56-0.65)	5.72 (96)	0.999
	30	6	5.75	0.34 (0.32-0.36)	10.30 (9.10-11.50)	137 (80)	0.951
	30	3	5.17	0.90 (0.89-0.91)	0.22 (0.14-0.30)	9.64 (96)	0.998
***S.* Typhimurium**	30	3	6.53	0.77 (0.76-0.778)	1.34 (1.23-1.45)	13.7 (98)	0.998
N_D 8.78	30	6	6.51	0.30 (0.29-0.31)	3.91 (2.94-4.88)	117 (93)	0.973
	30	3	5.80	0.96 (0.95-0.97)	1.33 (1.28-1.38)	6.63 (98)	0.999
	30	6	5.75	0.35 (0.34-0.36)	3.26 (2.65-3.88)	79.9 (98)	0.985
	30	3	5.17	0.89 (0.87-0.90)	1.29 (1.22-1.36)	9.28 (98)	0.999
	30	6	5.15	0.39 (0.37-0.40)	5.31 (4.58-6.04)	91.9 (96)	0.976
	30	3	4.58	0.71 (0.70-0.72)	1.48 (1.40-1.57)	10.7 (98)	0.999
	30	6	4.59	0.25 (0.23-0.28)	8.92 (5.62-12.22)	413 (99)	0.824

5.3.3 Calculation of the Minimum Inhibitory Concentration using NaCl and pH

The MIC in different NaCl concentrations (MIC_{NaCl}) and different pH (MIC_{pH}) for all the microorganisms were calculated from O.D measurements. *L. monocytogenes* was tested in a range of NaCl and pH, incubated at 30°C and/or 37°C and the TTD were obtained. The TTD were transformed to RTD and the data were fitted with the LPM model (Lambert and Pearson, 2000). Figure 5-4 shows the relationship between the RTD against the percentage of NaCl concentration of *L. monocytogenes* Scott A at 30°C and 37°C and the fit of the LPM. Also, Figure 5-5 shows the relationship between the RTD against the different pH of *L. monocytogenes* Scott A at 30°C and the fit of the LPM. Similar results were obtained for all strains. The parameters obtained from the LPM (including their confidence intervals) for all cases are shown in Table 5-7. The relative rate to detection (RRTD) was also calculated at 30°C and 37°C to examine the relative effect of temperature. Figure 5-6 shows the plot of RRTD of *L. monocytogenes* ScottA at 30°C and 37°C against the percentage of NaCl concentration. The parameter P_0 of the LMP was dependent on the temperature while the parameters P_1 and P_2 were not. The results obtained for the LPM parameters from Table 5-7 and Figure 5-6 showed that temperature did not have any effect on the determination of the MIC.

Figure 5-7 shows the relationship between O.D with the incubation time of *L. monocytogenes* Scott A in the range of NaCl concentrations tested. That figure represents the different shape of the O.D curves in the different NaCl concentrations. As the concentration of NaCl increased the maximum O.D decreased and also fluctuations in the O.D measurements occurred as the maximum O.D was reached. This might be due to cell clumping which was observed at the bottom of the Bioscreen plate under the most inimical conditions. Similar findings were observed for all cases.

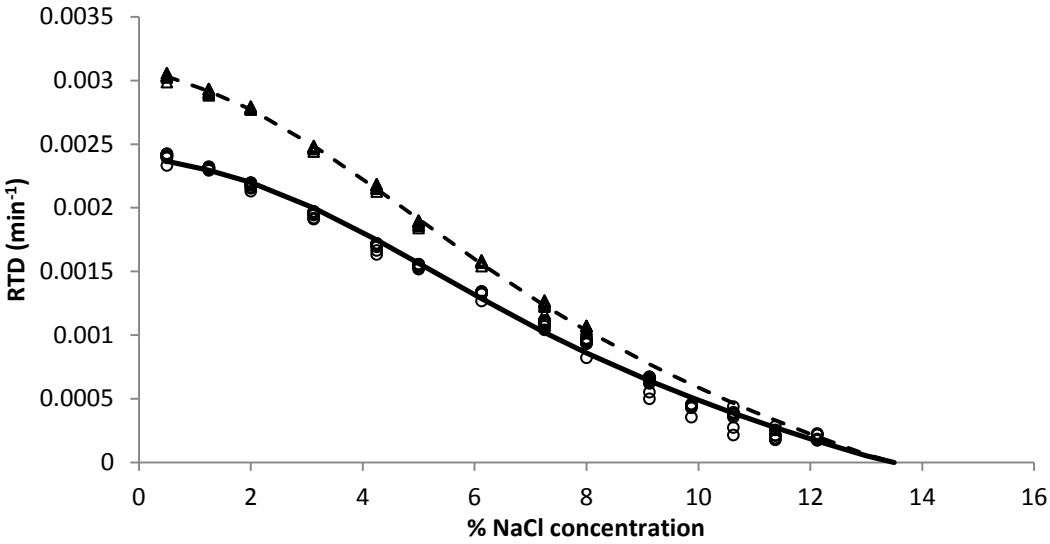


Figure 5-4 Relationship between the rate to detection (RTD) against the percentage of NaCl concentration of *Listeria monocytogenes* 39. The symbols represent the observed data (○, △), the lines the fitted Lambert-Pearson model (continuous, dashed) at 30°C and 37°C, respectively

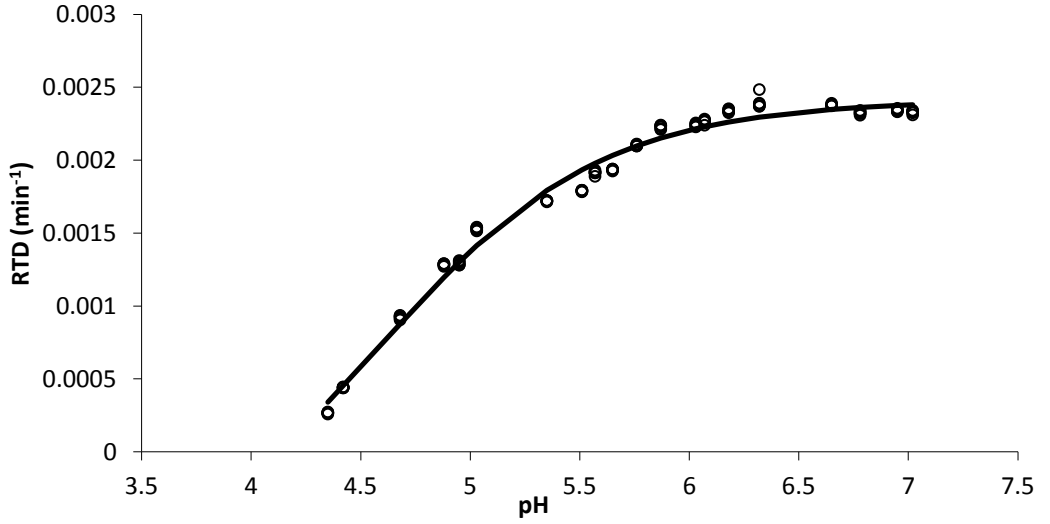


Figure 5-5 Relationship between rate to detection (RTD) against pH of *Listeria monocytogenes* Scott A at 30°C. The opened symbols represent the observed data and the solid line the fitted Lambert-Pearson model

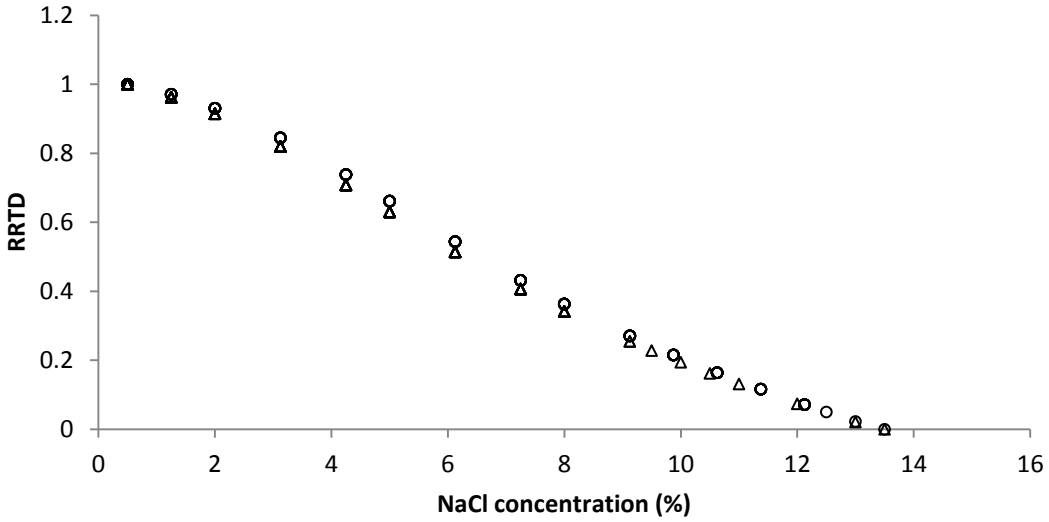


Figure 5-6 Relationship between the relative rate to detection (RRTD) against the percentage of NaCl concentrations of *Listeria monocytogenes* Scott A at 30°C (○) and 37°C (△), respectively

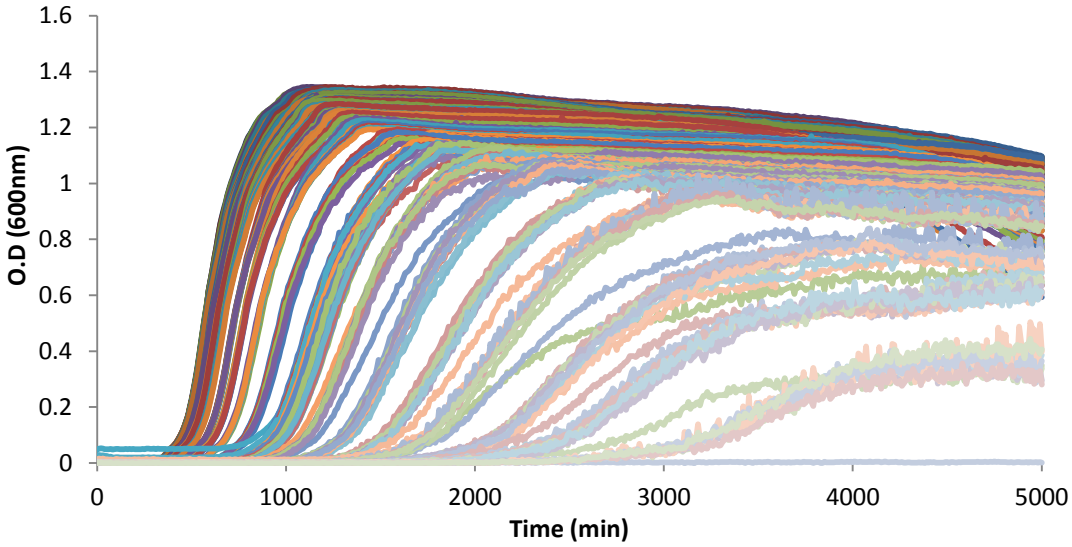


Figure 5-7 Relationship between O.D-Time of *Listeria monocytogenes* Scott A at 37°C, in a range of 0.5-16.625% NaCl

The MIC_{NaCl} and the MIC_{pH} were also calculated for *S.* Typhimurium and *E. coli* from O.D measurements. The microorganisms were tested in a range of NaCl and pH at 30°C and the TTD obtained. The TTD were transformed to RTD and the data fitted with the LPM (Lambert and Pearson, 2000). The relationship between the RTD against the percentage of NaCl concentration of *S.* Typhimurium *and E. coli* at 30°C was similar to that described above. Figure 5-8 shows the relationship between the RTD against the pH of both microorganisms at 30°C and the fit of the LPM. The parameters obtained from the LPM (with their confidence intervals) for all cases (NaCl and pH) are shown in Table 5-8. The results obtained showed that both the MIC_{NaCl} and MIC_{pH} of *E. coli* and *S.* Typhimurium are lower than those obtained for the *Listeria* strains suggesting that *E. coli* and *S.* Typhimurium are more sensitive to NaCl and pH than the *Listeria* strains.

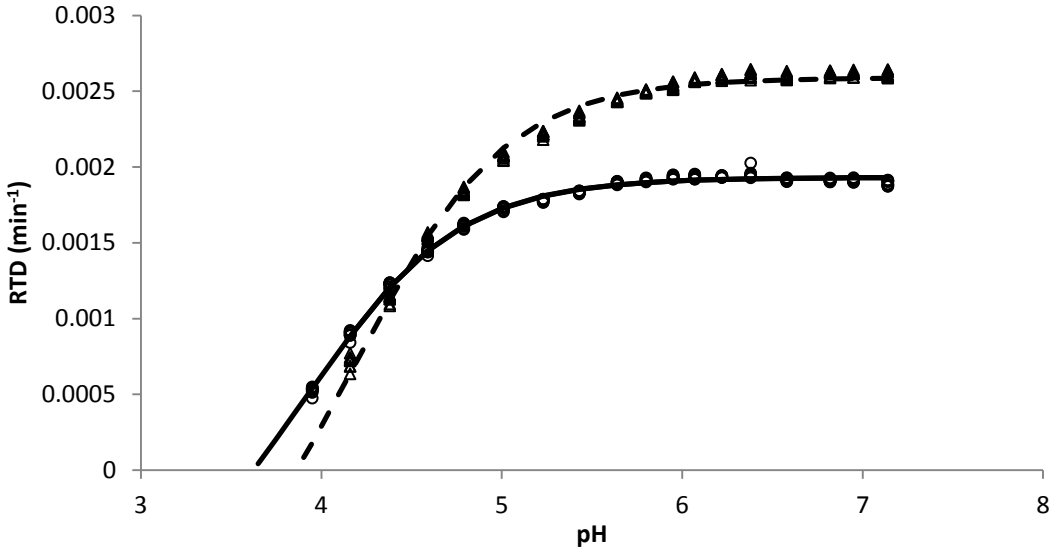


Figure 5-8 Relationship between RTD against pH of *Salmonella* Typhimurium and *Escherichia coli* at 30ºC. The symbols represent the observed data (○, △) and the lines the fitted Lambert-Pearson model (continuous, dashed) of *S.* Typhimurium and *E. coli,* respectively

Table 5-7 Parameters describing the Lambert and Pearson model, the MIC_{NaCl} and the MIC_{pH} calculated for all *Listeria monocytogenes* at 30°C and/or 37°C with their confidence intervals

Microorganism	T (°C)	NaCl/pH	P_0	P_1	P_2	MIC_{NaCl}/MIC_{pH}
***L. monocytogenes* 252**	30°C	NaCl	0.00255 (0.00253-0.00257)	8.023 (7.970-8.078)	2.063 (2.012-2.116)	13.03 (12.96-13.10)
***L. monocytogenes* 252**	37°C	NaCl	0.00319 (0.00317-0.00320)	7.598 (7.553-7.644)	1.857 (1.815-1.899)	13.02 (12.94-13.11)
***L. monocytogenes* 252**	30°C	pH	0.00229 (0.00226-0.0023)	$1.88*10^{-5}$ ($1.79*10^{-5}$-$2*10^{-5}$)	0.976 (0.897-1.062)	4.28 (4.22-4.34)
***L. monocytogenes* 39**	30°C	NaCl	0.00237 (0.00234-0.00240)	7.931 (7.845-8.023)	1.897 (1.836-1.967)	13.44 (13.34-13.53)
***L. monocytogenes* 39**	37°C	NaCl	0.00304 (0.00303-0.00306)	7.676 (7.627-7.725)	1.774 (1.732-1.817)	13.49 (13.39-13.58)
***L. monocytogenes* 39**	30°C	pH	0.00241 (0.00238-0.00245)	$2.06*10^{-5}$ ($1.98*10^{-5}$-$2.1*10^{-5}$)	0.795 (0.755-0.835)	4.14 (4.09-4.18)
***L. monocytogenes* 271**	30°C	NaCl	0.0038 (0.00378-0.00382)	7.706 (7.663-7.749)	2.092 (2.052-2.132)	12.43 (12.38-12.47)
***L. monocytogenes* 271**	37°C	NaCl	0.00494 (0.00493-0.00496)	7.336 (7.311-7.362)	1.935 (1.909-1.962)	12.30 (12.26-12.35)
***L. monocytogenes* 271**	30°C	pH	0.00225 (0.00223-0.00228)	$2.16*10^{-5}$ ($2.0*10^{-5}$-$2.3*10^{-5}$)	0.869 (0.797-0.947)	4.16 (4.09-4.23)
***L. monocytogenes* 177**	30°C	NaCl	0.00395 (0.00392-0.00397)	7.762 (7.717-7.7807)	2.045 (2.008-2.083)	12.66 (12.62-12.70)
***L. monocytogenes* 177**	37°C	NaCl	0.00484 (0.00479-0.00489)	7.486 (7.408-7.566)	1.911 (1.842-1.985)	12.63 (12.52, 12.75)
***L. monocytogenes* 177**	30°C	pH	0.00219 (0.00216-0.00222)	$1.8*10^{-5}$ ($1.7*10^{-5}$-$1.9*10^{-5}$)	0.815 (0.762-0.871)	4.22 (4.16-4.27)

Table 5-8 Parameters describing the Lambert and Pearson model, the MIC_{NaCl} and the MIC_{pH} calculated for *Escherichia coli* and *Salmonella* Typhimurium at 30°C, with their confidence intervals

Microorganism	T	NaCl/pH	P_0	P_1	P_2	MIC_{NaCl}/MIC_{pH}
E. coli	30°C	NaCl	0.00174 (0.00172-0.00176)	5.240 (5.195-5.285)	2.445 (2.361-2.534)	7.89 (7.84-7.93)
E. coli	30°C	pH	0.00263 (0.00262-0.00264)	$5.2*10^{-5}$ ($5.1*10^{-}5$-$5.3*10^{-5}$)	0.851 (0.837-0.865)	3.78 (3.77- 3.78)
***S.* Typhimurium**	30°C	NaCl	0.00145 (0.00144-0.00146)	5.335 (5.312-5.359)	2.226 (2.185-2.268)	8.36 (8.33-8.39)
***S.* Typhimurium**	30°C	pH	0.00193 (0.00192-0.00194)	$8.8*10^{-5}$ ($8.7*10^{-5}$-$9.0*10^{-5}$)	1.011 (0.991-1.031)	3.62 (3.62- 3.63)

5.3.4 Combined inhibitors (NaCl-pH)

E. coli and *S.* Typhimurium were examined in a range of multiple combinations of pH-NaCl concentrations. The range of NaCl was 0.5-8% NaCl while the range of pH was 3.5-6.9 and the incubation time was 7000 min (5 days). Figure 5-9 and Figure 5-10 show the observed data (in duplicate) obtained from the Bioscreen of *E. coli* and *S.* Typhimurium, respectively. As the pH decreased and the NaCl increased, higher TTD were obtained. It was also suggested, that increasing the percentage of NaCl concentration, increased the minimum pH allowing growth. An interesting overall trend is that the TTD's obtained, increased both at high and low pH's for different NaCl concentrations, resulting in parabolic curves instead of monotone trends as it would be expected.

E. coli did not give growth at any pH with 8% NaCl and at any NaCl concentration with pH 3.5. The most inimical condition where growth was observed was the combination 1.3% NaCl with pH 4.05. *S.* Typhimurium did not growth at any NaCl concentration with pH 3.5 and the most inimical condition where growth was observed was the combination 8% NaCl with pH 4.67. The results obtained from these studies are in agreement with the results obtained from the individual inhibitor studies (determination of the MIC_{NaCl} and MIC_{pH}).

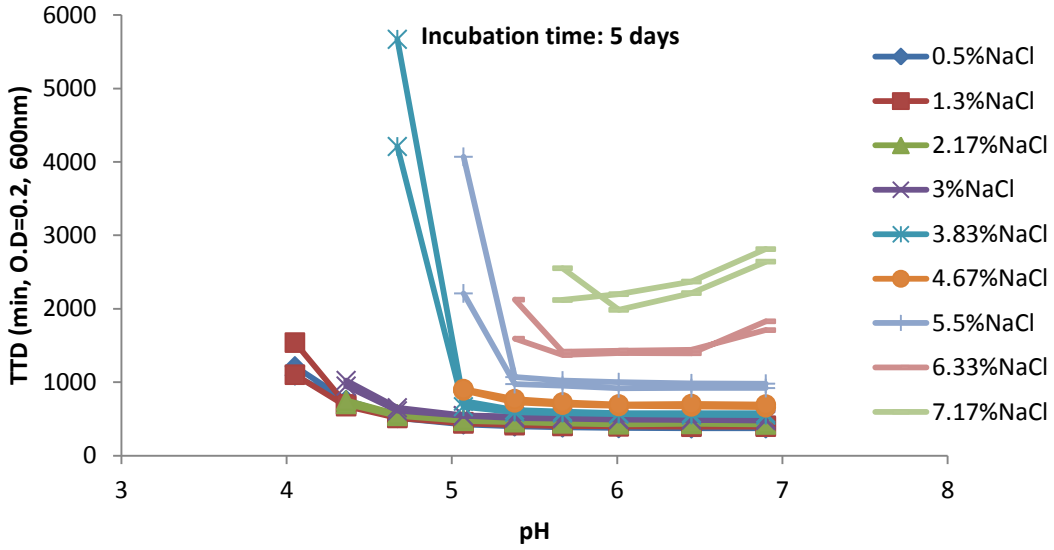


Figure 5-9 Relationship between the TTD with a particular inoculum size (≈10^5 cfu/ml), in different pH (3.5, 4.05, 4.36, 4.67, 5.07, 5.38, 5.67, 6.01, 6.45 and 6.9) and different NaCl concentration (legend), of *Escherichia coli* at 30ºC, in duplicate

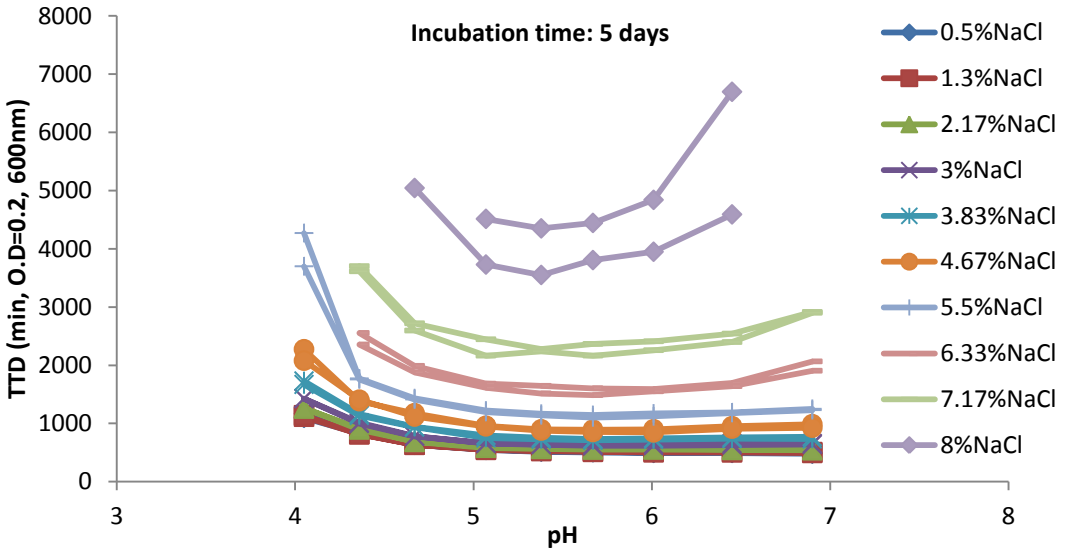


Figure 5-10 Relationship between the TTD with a particular inoculum size (≈10^5 cfu/ml), in different pH (3.5, 4.05, 4.36, 4.67, 5.07, 5.38, 5.67, 6.01, 6.45 and 6.9) and different NaCl concentration (legend), of *Salmonella* Typhimurium at 30ºC, in duplicate

Three main approaches to modelling the observed data were used: nominal logistic modelling of the G/NG data, continuous modelling and the use of the ELPM.

5.3.4.1 Probabilistic modelling

The determination of G/NG boundaries is dependent on time. The incubation time of the combined inhibitor experiments was 5 days. The TTD data from these experiments were analysed after 1 day, 3 days and 5 days of incubation at 30°C, in order to define the G/NG boundaries at different time intervals. The TTD data where transformed to nominal G/NG data (TTD=0 means no growth observed experimentally within the time frame studied and was labelled as NG) and nominal logistic regression was used to fit these data. The nominal logistic models produced for each day of the two microorganisms are summarised in Table 5-9 and in all cases the model produced was linear described by the intercept and two parameters (NaCl and H^+).

The most likely G/NG data were also obtained and the contingency analysis of G/NG by the most likely G/NG were obtained for *E. coli* after 1 day of incubation as shown in Table 5-10 as well as the mosaic plot as shown in Figure 5-11. Similar findings were obtained for all days tested and for *S.* Typhimurium.

Using the nominal logistic models described in Table 5-9 the G/NG interface of *E. coli* and *S. Typhimurium* using combinations of NaCl-pH were determined after 1 day, 3 days and 5 days at 30°C (Figure 5-12). With increasing incubation time the G/NG interface changed in both cases.

Table 5-9 Nominal logistic models after 1 day, 3 days and 5 days of incubation for *Escherichia coli* and *Salmonella* Typhimurium at 30°C

Microorganism	Day 1	Day 3	Day 5
Escherichia coli	$f_{(NaCl,H^+)}$=17.43-(2.86*NaCl)-(167615.63*H^+)	$f_{(NaCl,H^+)}$=13.70-(1.94*NaCl)-(136116.60*H^+)	$f_{(NaCl,H^+)}$=18.35-(2.55*NaCl)-(178309.54*H^+)
***Salmonella* Typhimurium**	$f_{(NaCl,H^+)}$=468.36-(75.41*NaCl)-(2119597.78*H^+)	$f_{(NaCl,H^+)}$=45.10-(5.53*NaCl)-(140489.21*H^+)	$f_{(NaCl,H^+)}$=41.10-(4.83*NaCl)-(133994.63*H^+)

Table 5-10 Contingency table of *Escherichia coli* after 1 day of incubation at 30°C resulted from the contingency analysis of G/NG by most likely G/NG

	G/NG		
Most Likely G/NG	Total count %	G	NG
	G	100 50.00 94.34 94.34	6 3.00 6.38 5.66	106 53.00
	NG	6 3.00 5.66 6.38	88 44.00 93.62 93.62	94 47.00
		106 53.00	94 47.00	200

Figure 5-11 Mosaic plot of *Escherichia coli* after 1 day of incubation at 30°C resulted from the contingency analysis of G/NG by most likely G/NG

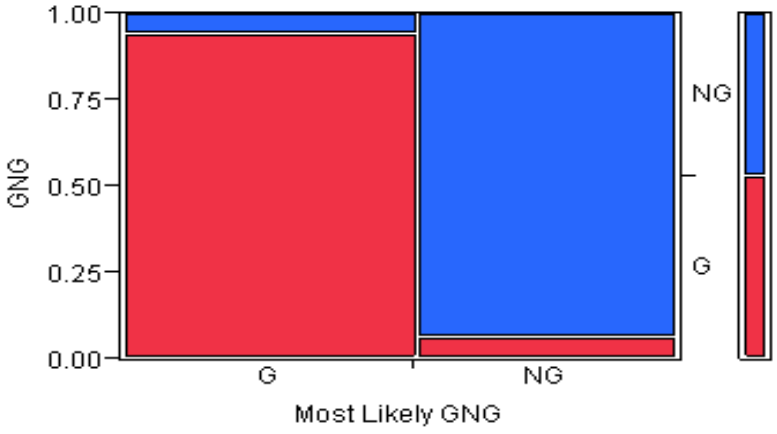
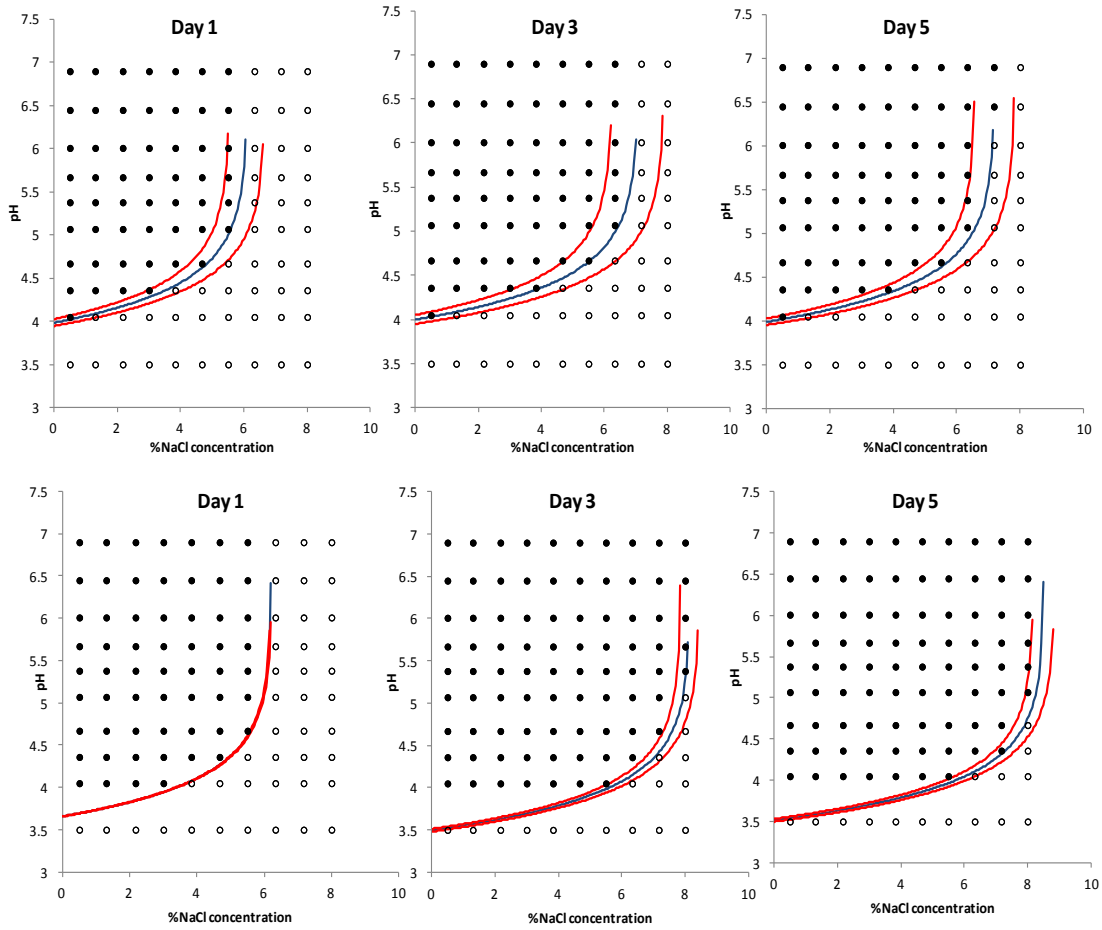


Figure 5-12 Growth/No Growth interface of *Escherichia coli* (top) and *Salmonella* Typhimurium (bottom) after 1 day, 3 days and 5 days of incubation at 30°C using combinations of NaCl and pH. The closed symbols represent the growth data while the opened symbols represent the no growth data. Also, the blue lines represent the nominal logistic models while the red lines the confidence intervals of the models.



5.3.4.2 Response surface modelling (Continuous modelling)

The TTD data obtained from the combined inhibitor experiments were also transformed to RTD. Using the standard least squares with emphasis to the effect of leverage a response surface model (RSM) was produced for each day and for both microorganisms at 30°C. In this type of modelling data where TTD=0 have been excluded from the analysis. In all cases the best RSM was described by 5 parameters (intercept, NaCl, pH, $NaCl^2$ and pH^2). Table 5-11 summarises the parameters of the RSM with the summary of fits obtained from JMP 8.

Figure 5-13 shows the scatter plots 3D of the observed data in relation with the two inhibitors (NaCl and pH) and the RSM in relation with the two inhibitors of *E. coli* after 1 day at 30°C. Similar findings were obtained for each day and both microorganisms. Figure 5-14 shows the surface plot of the RSM in relation with the two inhibitors, obtained for *E. coli* after 1 day of incubation at 30°C. Similar plots were obtained for each day and both microorganisms.

Table 5-11 Parameters of the response surface models (RSM) of *Escherichia coli* and *Salmonella* Typhimurium with the summary of fits at 30°C for 1 day, 3 days and 5 days of incubation

Microorganism	Day 1			Day 3			Day 5		
Escherichia coli	**Term**	**Estimate**	**Std error**	**Term**	**Estimate**	**Std error**	**Term**	**Estimate**	**Std error**
	Intercept	0.00093	7.3e-5	Intercept	0.001	8.9e-5	Intercept	0.0008	0.0001
	NaCl	-0.00029	6.3e-6	NaCl	-0.0003	6.5e-6	NaCl	-0.0003	8.1e-6
	pH	0.00035	0.00001	pH	0.0003	1.5e-5	pH	0.0004	1.9e-5
	(NaCl-2.85) * (NaCl*2.85)	-4.1e-5	3.4e-6	(NaCl-3.31) * (NaCl*3.31)	-2.9e-5	3.1e-6	(NaCl-3.35) * (NaCl*3.35)	-2.6e-5	3.8e-6
	(pH-5.67)* (pH-5.67)	-0.0003	1.5e-5	(pH-5.70)* (pH-5.70)	-0.0003	1.8e-5	(pH-5.69)* (pH-5.69)	-0.0003	2.3e-5
	Summary of fit			**Summary of fit**			**Summary of fit**		
	R^2	0.968161		R^2	0.96418		R^2	0.945868	
	RMSE	0.000102		RMSE	0.000132		RMSE	0.000168	
***Salmonella* Typhimurium**	**Term**	**Estimate**	**Std error**	**Term**	**Estimate**	**Std error**	**Term**	**Estimate**	**Std error**
	Intercept	0.00069	5.3e-5	Intercept	0.00086	5.7e-5	Intercept	0.00091	5.7e-5
	NaCl	-0.00021	5.1e-6	NaCl	-0.00022	3.9e-6	NaCl	-0.00023	3.8e-6
	pH	0.00028	9.5e-6	pH	0.00025	1.0e-5	pH	0.00024	1.0e-5
	(NaCl-2.87) * (NaCl*2.87)	-0.00003	3.4e-6	(NaCl-3.91) * (NaCl*3.91)	-1.3e-5	1.9e-6	(NaCl-4.03) * (NaCl*4.03)	-1.2e-5	1.8e-6
	(pH-5.48)* (pH-5.48)	-0.00023	1.1e-5	(pH-5.43)* (pH-5.43)	-0.00021	1.2e-5	(pH-5.44)* (pH-5.44)	-0.00021	1.2e-5
	Summary of fit			**Summary of fit**			**Summary of fit**		
	R^2	0.957697		R^2	0.959644		R^2	0.960449	
	RMSE	8.862e-5		RMSE	0.000113		RMSE	0.000114	

Figure 5-13 3D Scatter plot of the rate to detection (RTD) of the observed data in relation with the two inhibitors (NaCl and pH) (left) and 3D scatter plot of the Response Surface Model (RSM) in relation with the two inhibitors (right) of *Escherichia coli* after 1 day at 30°C

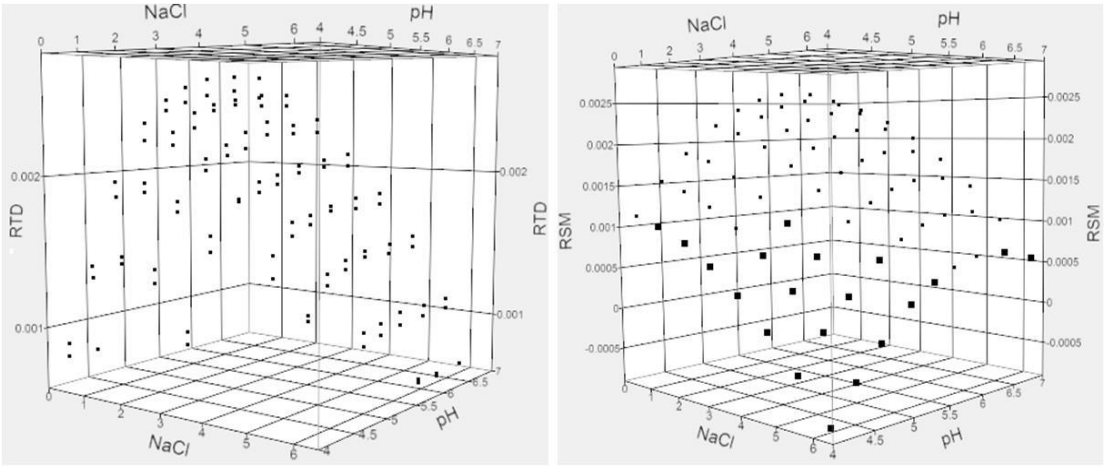
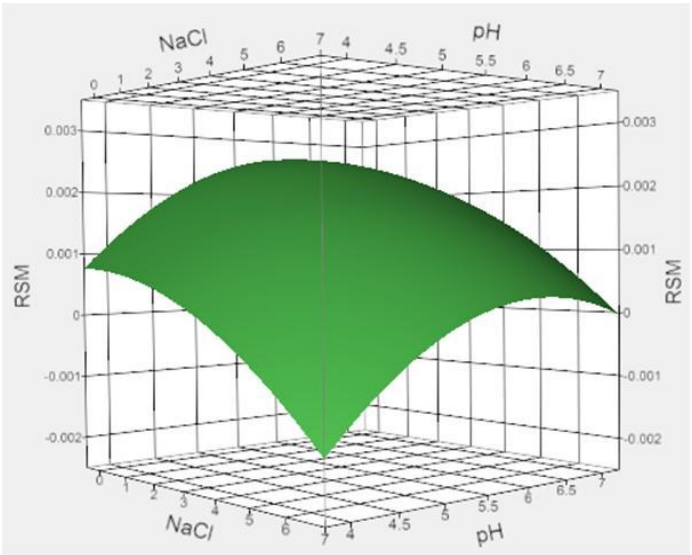


Figure 5-14 Surface plot of the Response Surface Model (RSM) in relation with the two inhibitors (NaCl and pH), obtained for *Escherichia coli* after 1 day of incubation at 30°C



5.3.4.3 Extended Lambert and Pearson modelling

The continuous data obtained from these experiments were also modelled with the Extended Lambert and Pearson model (ELPM), equation (2-14) and (2-15) and the parameters describing this model are shown in Table 5-12 for *E. coli* and *S.* Typhimurium at 30°C after 1 day, 3 days and 5 days.

Figure 5-15 shows the contour plots of *E. coli* after 1 day of incubation at 30°C, derived from the actual data (RTD), the RSM and the ELPM. Similar plots obtained for *S.* Typhimurium.

Figure 5-15 Contour plots of *Escherichia coli* after 1 day of incubation at 30°C, derived from the actual rate to detections (RTD), the Response Surface Model (RSM) and the Extended Lambert and Pearson model (ELPM), produced using JMP 8

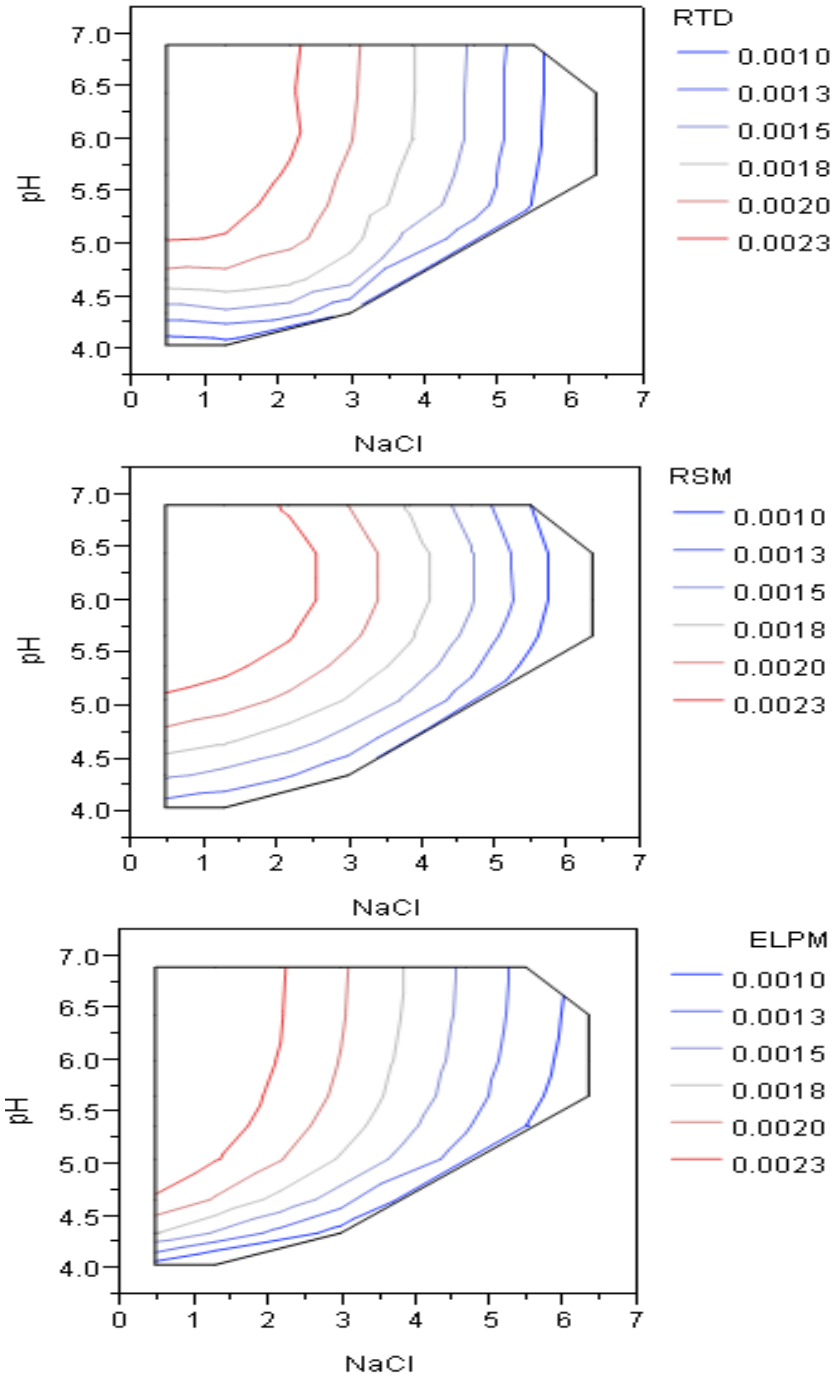


Table 5-12 Parameters describing the Extended Lambert and Pearson model used to fit experimental data from *Escherichia coli* and *Salmonella* Typhimurium at 30°C

Microorganism		Day 1			
Escherichia coli	**Parameter**	**Estimate**	**Lower CI**	**Upper CI**	**RMSE**
	P_1	6.22	6.04	6.41	0.0001146
	P_3	0.000097	8.9e-5	0.0001072	
	P_4	2.049	1.829	2.291	
	P_2	0.774	0.683	0.873	
	P_0	0.00257	0.00250	0.00264	
***Salmonella* Typhimurium**	**Parameter**	**Estimate**	**Lower CI**	**Upper CI**	**RMSE**
	P_1	6.72	6.47	6.99	0.000091
	P_3	0.000125	0.000113	0.000141	
	P_4	1.555	1.388	1.734	
	P_2	0.838	0.740	0.946	
	P_0	0.00209	0.00202	0.00216	
		Day 3			
Escherichia coli	**Parameter**	**Estimate**	**Lower CI**	**Upper CI**	**RMSE**
	P_1	5.99	5.84	6.16	0.000138
	P_3	0.000101	9.2e-5	0.000112	
	P_4	2.455	2.217	2.714	
	P_2	0.728	0.640	0.826	
	P_0	0.00251	0.00244	0.00258	
***Salmonella* Typhimurium**	**Parameter**	**Estimate**	**Lower CI**	**Upper CI**	**RMSE**
	P_1	6.20	6.04	6.37	0.0001035
	P_3	0.000143	0.000128	0.000162	
	P_4	1.790	1.657	1.931	
	P_2	0.867	0.754	0.995	
	P_0	0.00203	0.00198	0.00210	
		Day 5			
Escherichia coli	**Parameter**	**Estimate**	**Lower CI**	**Upper CI**	**RMSE**
	P_1	6.04	5.84	6.26	0.0001692
	P_3	0.000102	0.000091	0.000117	
	P_4	2.576	2.283	2.901	
	P_2	0.650	0.560	0.751	
	P_0	0.00251	0.00243	0.00260	
***Salmonella* Typhimurium**	**Parameter**	**Estimate**	**Lower CI**	**Upper CI**	**RMSE**
	P_1	6.16	6.00	6.33	0.0001086
	P_3	0.000144	0.000129	0.000164	
	P_4	1.837	1.703	1.979	
	P_2	0.881	0.765	1.012	
	P_0	0.00202	0.00196	0.00208	

5.4 Discussion

We have been developing the use of O.D for obtaining growth rates and lag times using multiple inocula rather than using the traditional methods which use one single inoculum. McKellar *et al*. (2002) and McKellar and Knight (2000) have suggested a method for the analysis of lag time using the same methodology employed in our laboratory. O.D was directly related to microbial numbers with simple calibration curves. Calibration curves showed that a direct relationship between O.D and cfu/ml existed and that a specific O.D was equivalent to a specific number of organisms per ml.

The analysis of the data obtained from the inoculum size experiments in elevated NaCl concentrations, in different pH and in their combinations (NaCl and pH) showed that the growth rate was independent of the inoculum size. The inoculum size affected only the time to reach the TTD, where the higher inocula needed less time to reach the TTD criterion (e.g. O.D = 0.2) compared with lower inocula. However, the literature does show some controversy over the so called 'inoculum effect'. For example, there is the assumption that the inoculum size does not have any effect on the microbial growth rate parameters (Buchanan *et al*., 1993a,b; Bhaduri *et al*., 1994). In contrary, there are observations that the inoculum size, could have an effect on the duration of the lag phase (Pin and Baranyi, 2006; Metris *et al*., 2006; Francois *et al*., 2005; Guillier *et al*. 2005; Augustin *et al*., 2000; Robinson *et al*., 2001) or that the inoculum size may affect microbial growth (Masana and Baranyi, 2000; Koutsoumanis and Sofos, 2005). An increased variance was observed as the inoculum size decreased which might be due to the fact that as the inocula are diluted across the plate, the probability of obtaining a well in the honeycomb plate with precisely the expected number of microbial cells decreases. This leads to an increased variability in the TTD as the cell density decreases (Bidlas and Lambert, 2008).

The classic 3 parameter logistic model was rearranged to provide the theoretical foundation for the observed TTD and it was able to fit the TTD data obtained from turbidometric experiments using multiple inocula incubated iso-

thermally. In all cases, with increasing incubation temperature (30°C and 37°C) the growth rate increased hence the lag time decreased which shows that 37°C is a more optimal temperature. The parameters obtained suggest that as the conditions became more inimical (increased NaCl concentration, low pH or combinations of NaCl-pH) the growth rate decreased while the lag time increased. A higher lag time was observed for *S.* Typhimurium and *E. coli* in 6% NaCl and in pH 4.58 while for the *L. monocytogenes* strains a higher lag time was observed in 9% NaCl. Under the more extreme NaCl-pH combinations used (6% NaCl-pH: 5.17, 3% NaCl-pH: 4.58 and 6% NaCl-pH: 4.58) *E. coli* did not grow which suggests that it is more sensitive under these conditions from *S.* Typhimurium. Also, the minimum and maximum values of NaCl and pH for growth found from this study for *E. coli* and *S.* Typhimurium, were in agreement with the corresponding values suggested by the ICMSF (1996) and were reported in paragraph 1.1.5.1.

There are studies in the literature which suggest morphological changes occur when the growth conditions becoming more unfavourable. Bereksi *et al*. (2002) reported that some *L. monocytogenes* strains may change their adhesion properties due to modification of their surface properties under high NaCl concentrations. Similar, Giotis *et al*. (2007) observed filament formations or elongated chain forms of *L. monocytogenes* in sub-lethal alkaline environment. Isom *et al*. (1995) observed increased filament formations of *L. monocytogenes* with increasing NaCl concentration and in low or alkaline pH adjusted using NaOH. It was also observed that no morphological changes occurred when the media were acidified using HCl. However, no gross morphology changes were observed microscopically under the most inimical conditions used in this work. In some cases cell clumping was observed at the bottom of the Bioscreen plate which resulted in non reliable O.D measurements.

McMeekin *et al*. (2000) stated the importance of the G/NG interface modelling by mentioning its practical and scientific implications. Probabilistic modelling using logistic regression has been used extensively in the literature. Lopez-

Malo *et al*. (2000) predicted the G/NG boundary of *Saccharomyces cerevisiae* under the effect of aw, pH and potassium sorbate. Also, Gysemaans *et al*. (2007) based on a case study with monoculture and mixed strain culture data compared the ordinary logistic regression model with the nonlinear logistic regression model derived from a square root type kinetic model and concluded that the first one performed slightly better than the square root type model.

E. coli and *S.* Typhimurium were examined in a range of combinations of pH-NaCl concentrations. The TTD data obtained from the Bioscreen where transformed in G/NG data and using nominal logistic regression to the data after 1 day, 3 days and 5 days of incubation the G/NG interfaces were determined and were dependent on the time. The data from these experiments were also transformed to RTD and were fitted with RSM as well as with the ELPM. In all cases examined the RMSE of the RSM was lower than the RMSE of the ELPM, plus both models had an equivalent number of parameters (5), which suggest a better fit of the data by the RSM over the ELPM. However, the differences between the RMSE are not statistically significant. Also, the results obtained from the ELPM can be extrapolated while the results from the probabilistic modelling or the response surface modelling can not be extrapolated. This advantage of the ELPM in conjuction with the low RMSE makes it an effective and accurate model of analysing results of combined inhibitors.

5.5 Conclusion

The rearranged logistic with lag model could give accurate growth rates and lag times from O.D measurements. The growth rate was found to be independent from the inoculum size; the inoculum size affected only the TTD. Also, the LPM and the ELPM can analyse results for individual and combined inhibitors.

6 Modelling microbial growth after a mild thermal injury: An analysis using optical density

6.1 Introduction

Foodborne disease is a threat to public health and as the trend towards more natural and less preserved and processed foods continues, the threat is not diminishing. Heat is a basic and common form of preservation (Gould, 1989); reducing the severity of the thermal process increases the acceptability of a product but can also increase the risk of foodborne illness. It is not a balancing act between safety and palatability – safety first and always is the food producer's mantra. One way of assessing the impact of a control strategy is to model the effects of the various processes on the growth or inactivation of microbes present in the foods. Modelling, however, is normally carried out in conjunction with actual testing of the process – a 2-phase process with the modelling, the cheaper alternative, given the availability of a model (Zwietering *et al*., 1992; Koutsoumanis *et al*., 2006) guiding the more expensive on-site testing/validation.

A successful model will allow the prediction of the impact of varying process and product conditions on the microbes present in the foodstuff. However, the model can either be highly specific – can only be used with predefined conditions or more general but less precise with respect to the particular foodstuff.

Predictive models are often based, initially, on the growth of a particular species in a laboratory growth media. Typically for a given set of conditions a growth curve is constructed to which a standard 'primary' model such as the *modified* Gompertz is fitted (Li *et al*., 2007). From the fitted parameters the growth rate, lag, and maximum population density are found for a given initial inoculum. Changing the conditions alters these parameters, and these changes are modelled themselves with respect to the environmental conditions (secondary modelling). The standard methodology using plate counts is both tedious and expensive but is also very time consuming and large experimental designs can be prohibitive – and become more the realm

of the large research grant than the ability of an industrial microbiological laboratory.

Recently further developments of obtaining growth rates from O.D data was published (Mytilinaios *et al*., 2012). It was shown using this technique that the standard *modified* Gompertz equation was unable to model O.D data and that the basic logistic model fitted the data extremely well. Further, in the experiments carried out no lag was observed when grown under the conditions used. It was hypothesised that in the systems studied no lag was observed because there was no injury present nor did the conditions used induce a lag (i.e. the pre-exponential factor was 1). These conditions were useful since the fitting of the logistic model was facile. In the presence of conditions that induced a lag, it was envisaged we would have to invoke the logisitic with lag or the Baranyi model to give cogent fits to the data (Jason, 1983; Baranyi *et al*., 1993a). To investigate this idea we also proposed to study the effect of a mild thermal insult against *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* during the growth process, in the presence of NaCl, pH and their combinations – all known to induce a lag before the onset of growth (Stephens *et al*., 1997), and to couple this with the O.D technique developed to study growth rates.

6.2 Materials and methods

The effect of a mild thermal injury on the growth of four *L. monocytogenes* strains (252, 39 or Scott A, 271 and 177), *S.* Typhimurium and *E. coli* was studied in different NaCl concentrations at 30°C and the procedure followed was described in paragraph 2.3.9.1 . Also, the effect of a mild thermal injury on the growth of *S.* Typhimurium and *E. coli* was studied at 30°C in different pH and pH-NaCl concentrations, as described in paragraphs, 2.3.9.2 and 2.3.9.3, respectively. The model used to fit the data before and after the thermal injury was described in paragraph 2.4.5 while the model used to simulate the observed TTD data was described in paragraph 2.4.9.

6.3 Results

6.3.1 Selection of time-temperature treatment

The effect of a mild thermal injury was studied using the Bioscreen microbiological analyser, in conjunction with the methods developed for the analysis of the initial inoculum size on the TTD. In these studies the Bioscreen plate was incubated in an oven, after a period of incubation in the Bioscreen, at 60°C for 25 minutes, before being placed back into the Bioscreen incubator. This time-temperature treatment was chosen after a series of screening trials using *L. monocytogenes* 252 at 60°C for 5, 10, 15 and 20 min, at 65°C for 5 and 10 min and at 70°C for 5 min (Data not shown). Also, *L. monocytogenes* 252 was examined in TSB with 0.5% NaCl at 37°C, with placement in a preheated oven at 60°C for 25, 30, 35 or 45 min (Figure 6-1). An increased variability was observed when the time of heat treatment was increased (Table 6-1). Heating for more than 25 minutes resulted in reduction of the microbial counts (data not shown) and thus, the populations which should be used for those treatments (Figure 6-1), are different from the values used. Heat treatment at 60°C for 25 min in a preheated oven injured the existing populations without any microbial reduction. Table D-2 (Appendix D) shows the plate counts from a specific well (well 199 and 299) before and after the mild heat treatment respectively at 60°C for 25 min. The plates counts before and after the heat treatment, were not significantly different and so it was concluded that the chosen heat treatment induced an injury without any microbial reduction.

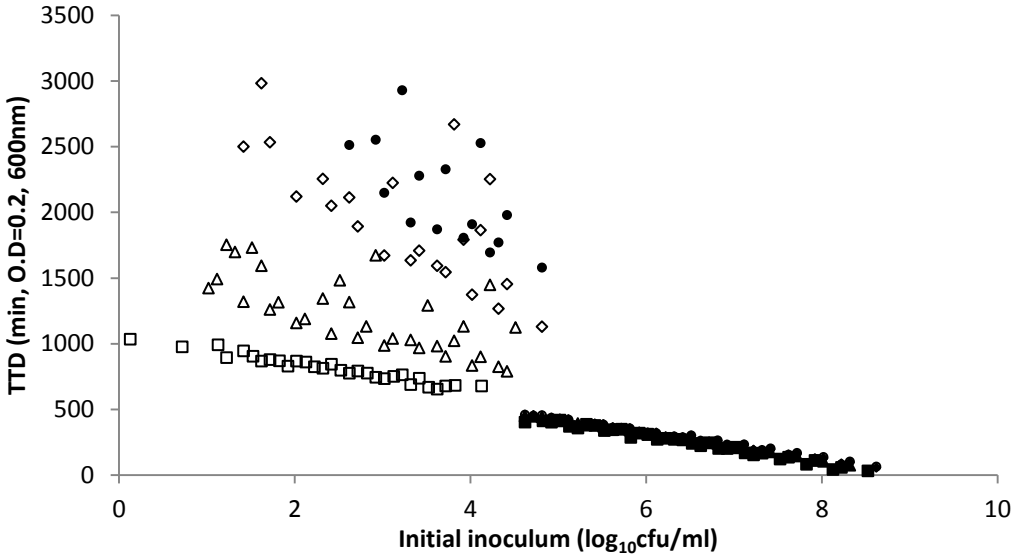


Figure 6-1 Relationship between TTD with the initial populations of *Listeria monocytogenes* 252, grown in TSB with 0.5% NaCl at 37°C. The symbols (■, ▲, ◆, ○) represent the observed data before the heat injury and the symbols (□, △, ◇, ●) represent the observed data after the heat injury in a preheated oven at 60°C for 25, 30, 35 and 45 min, respectively

Table 6-1 Parameters describing the linear relationship between the TTD against the initial populations of *L. monocytogenes* 252 at 37°C in TSB 0.5% NaCl, before and after the heat treatment at 60ºC for 25, 30, 35 or 45 min

Micoorganism	Heat treatment	Gradient (h/Log(cfu/ml))	Intercept (h)	Growth rate (hours $^{-1}$)
L. monocytogenes 252	Before	1.72	15.29	0.58
	After 25min	1.69	18.06	0.59
	Before	1.72	15.61	0.58
	After 30min	3.03	28.81	0.33
	Before	1.69	15.66	0.59
	After 35min	5.77	50.84	0.17
	Before	1.66	15.42	0.60
	After45min	6.82	60.69	0.15

6.3.2 Thermal injury in different NaCl concentrations, different pH and different NaCl-pH combinations

The effect of a mild thermal injury in a preheated oven at 60°C for 25 min, on the growth of four strains of *L. monocytogenes*, *S.* Typhimurium and *E. coli* was studied in different NaCl concentrations at 30°C incubation temperature. At 30°C, *E. coli* in TSB with 0.5%, 3% or 6% NaCl grew with an observed SGR = 1.358, 1.082 and 0.319/h respectively, with an induction of an apparent lag in 6% NaCl (lag=4.89h). After 400-1300 mins of incubation the micro titre-plates were placed in an oven at 60°C for 25 mins, and then subsequently placed back in the Bioscreen to continue the incubation at 30°C. The resulting TTD of the thermally treated plate showed a significant step, with an apparent larger variance compared to the control TTD; Figure 6-2 shows the observed data for the thermal treatment (before and after the heat treatment) of *E. coli* at 3% TSB. A regression line through the thermally treated TTD data gave a gradient (and so a growth rate) approximately equal to that of the control, however the fit was poor, Table 6-2.

The observed TTD for multiple initial inocula of *S.* Typhimurium at 30°C in TSB (0.5, 3 or 6% NaCl) showed the presence of a short lag before the start of growth in all cases (Table 6-2). This was manifested in curvature close to the detection threshold. After the thermal treatment, the resulting TTD gave similar results to those of *E. coli*: a definite step between the expected, untreated control TTD and the observed, with the latter showing a large variance, Table 6-2.

The four strains of *L. monocytogenes* examined were more tolerant of the thermal insult than either of the strains of *S*. Typhimurium or *E. coli* used. In all cases an apparent lag was observed in 6% NaCl (Table 6-2). After the thermal insult, a lag was induced and the growth rate was approximately the same as in pre-treatment.

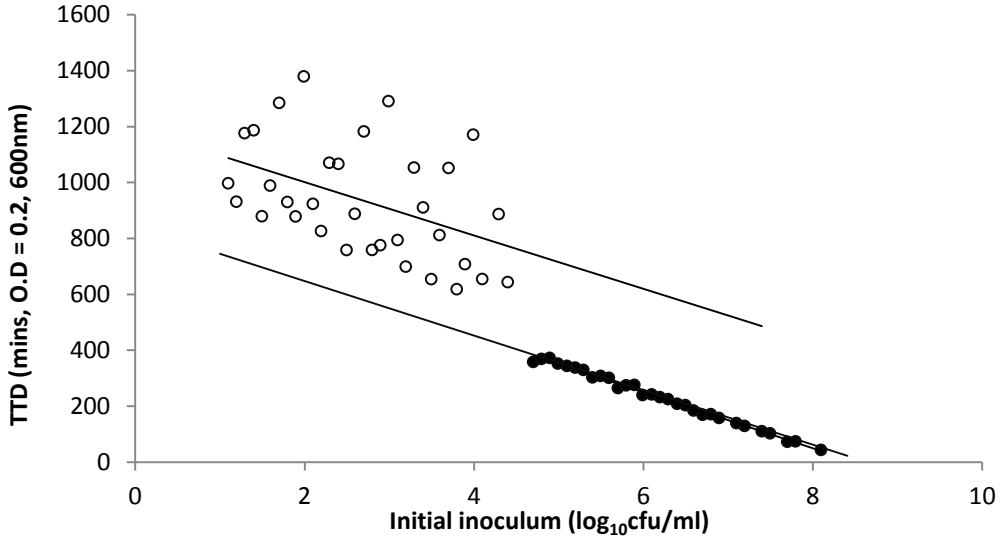


Figure 6-2 The observed effect of a small thermal insult (60°C for 25 mins) after 400 mins of growth at 30°C on the TTD against the initial populations of *E. coli* in 3% NaCl. The closed symbols represent the observed data before the heat treatment while the open symbols represent the observed data after the heat treatment

The effect of a mild thermal injury on the growth of *S.* Typhimurium and *E. coli* was also studied at 30°C in different pH and different combinations of NaCl-pH. The relationship between the initial inoculum sizes against the TTD was the same as described previously. The results obtained from the Bioscreen before the treatment showed that with decreasing pH or decreasing pH and increasing NaCl concentration the growth rate decreased while the lag time increased and were similar and reproducible with the results obtained from the inoculum size studies under the same conditions. Following the heat treatment, the TTD suggested that the growth rate of the data was essentially the same as that before the heat treatment (Table 6-4 and Table 6-6) but with a higher degree of variability. The observed discontinuity after a period of thermal injury was interpreted as a heat induced lag before growth recommenced. Similar results were obtained for all cases and for both microorganisms.

The differences in time between the observed thermally treated TTD and the expected TTD for the given initial inoculum without treatment were calculated (lag due to thermal injury, lag_{injury}) and tested with the Log-normal, Gamma and Weibull distribution. These distributions are characterised by 2 parameters (scale and shape parameter) and they were compared using the -2*LogLikelihood ratio test and the Akaike Information Criterion (AIC) test to examine which distribution best fits the distribution of lag_{injury}. From the results obtained, it was observed the distribution of lag_{injury} was best described by the Log- normal distribution in most cases (Table 6-2, Table 6-4, and Table 6-6) with $p>0.05$. From the studies performed in different NaCl concentrations, only for *E. coli* in 3% NaCl and for *L. monocytogenes* 39 in 0.5% NaCl was the lag_{injury} distribution best described by the Gamma distribution (Table 6-3) but even in this case, the fit of the Log-normal distribution was very good with $p>0.05$. From the studies performed in different pH, only the lag_{injury} of *S.* Typhimurium at pH=6.50 was better fitted with the Gamma model but still the Log-normal distribution could describe the data well (Table 6-5) while from the studies in different combinations of NaCl-pH, only in two cases of *S.* Typhimurium in 3% NaCl-pH=5.84 and in 3% NaCl-pH=5.16 the Gamma distribution found to fit the lag_{injury} better but the Log-normal distribution fit the data well with $p>0.05$ (Table 6-7).

TTD data were simulated using equation (2-28), with the lag given by the fit of the Log-normal distribution; Figure 6-3 gives a resulting simulation for the experimental data described in Figure 6-2 for *E. coli* at 3% NaCl. A similar result was obtained for all the microorganisms and in all the conditions tested.

In all cases there were no significant correlations between the difference of the TTD following injury and the TTD calculated for the untreated control and the initial inoculum.

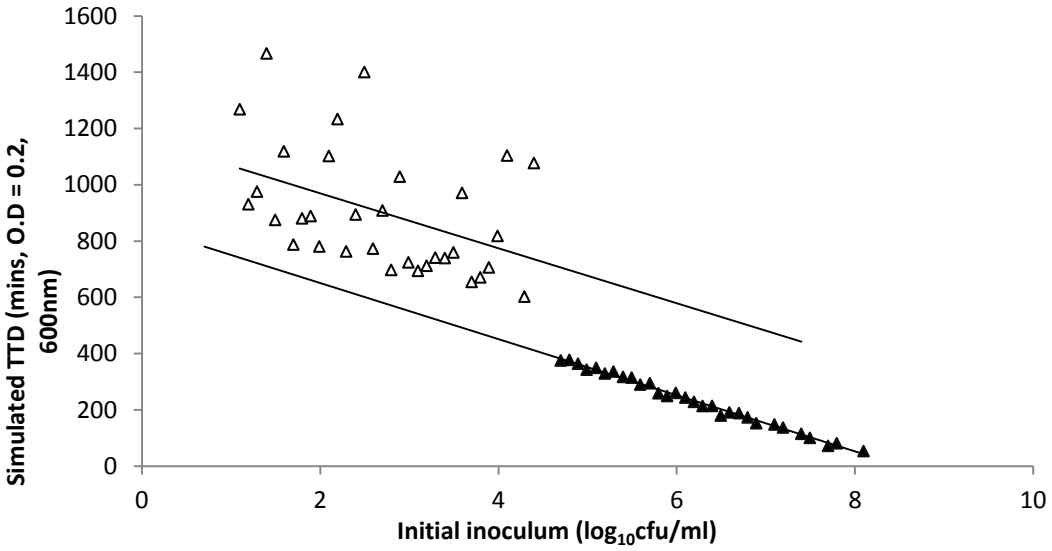


Figure 6-3 The simulated effect of a small thermal insult (60°C, 25mins) after 400 mins of growth at 30°C on the TTD of *E. coli* in 3% NaCl. The closed symbols represent the simulated data before the heat treatment while the open symbols represent the simulated data after the heat treatment. The simulation was based on equation (2-28) with intercept = 1144.5 mins, gradient = -127.8/min, lag = exp(LogNorm(6.46,0.260))

Table 6-2 Growth parameters calculated from the regression lines before and after the heat treatment for all the microorganisms

			Pre Thermal				Post Thermal				Post Thermal lag parameters (Log-Normal distribution)		
Organism	**Temp (ºC)**	**Salt (%)**	**SGR /h**	**Lag (h)**	**RMSE (obs)**	**r^2**	**SGR /h**	**Lag (h)**	**RMSE (obs)**	**r^2**	**Scale**	**Shape**	**P**
E. coli	30	0.5	1.358	-0.21	7.43 (56)	0.998	1.446	6.12	186 (33)	0.206	1.647	0.504	>0.05
N_D 8.69	30	3	1.082	0.69	6.82 (60)	0.999	1.029	11.07	169 (34)	0.406	2.361	0.260	>0.05
	30	6	0.319	4.89	179 (96)	0.937	0.186	18.06	736 (61)	0.433	3.537	0.263	>0.05
***S.* Typhimurium**	30	0.5	1.212	1.17	14.5 (56)	0.995	0.943	3.44	113 (35)	0.705	1.200	0.500	>0.05
N_D 8.78	30	3	0.912	1.68	13.3 (59)	0.998	0.803	12.3	295 (41)	0.334	2.343	0.394	>0.05
	30	6	0.285	2.81	192 (94)	0.938	0.332	24.58	464 (47)	0.425	3.511	0.195	>0.05
***L. monocytogenes* 252**	30	0.5	1.073	0.28	6.91 (61)	0.999	1.040	2.32	75.8 (40)	0.817	0.633	0.474	>0.05
N_D 9.15	30	3	0.915	0.65	7.95 (61)	0.999	0.903	2.62	51.8 (44)	0.940	0.498	0.412	>0.05
	30	6	0.616	1.29	18.9 (98)	0.997	0.569	2.02	77.9 (51)	0.892	0.542	0.590	>0.05
***L. monocytogenes* ScottA**	30	0.5	1.025	0.851	11.9 (63)	0.998	1.043	2.95	36.6 (40)	0.948	0.356	0.376	>0.05
N_D 9.16	30	3	0.810	1.10	13.5 (62)	0.998	0.895	4.04	33.0 (39)	0.967	0.466	0.377	>0.05
	30	6	0.571	1.15	34.9 (99)	0.993	0.574	2.70	89.5 (54)	0.874	0.278	0.674	>0.05
***L. monocytogenes* 271**	30	0.5	1.029	-0.433	9.77 (60)	0.998	1.020	1.18	36.4 (30)	0.934	0.379	0.396	>0.05
N_D 9.32	30	3	0.875	-0.153	9.79 (98)	0.999	0.833	2.07	69.2 (59)	0.859	0.811	0.445	>0.05
	30	6	0.566	0.335	25.9 (98)	0.996	0.536	1.47	146 (58)	0.768	0.267	0.999	>0.05
***L. monocytogenes* 177**	30	0.5	0.948	-0.403	7.79 (98)	0.999	0.907	0.68	29.2 (49)	0.959	0.369	0.300	>0.05
N_D 9.33	30	3	0.812	0.042	6.67 (98)	0.999	0.833	1.98	59.7 (58)	0.891	0.314	0.550	>0.05
	30	6	0.606	0.765	25.8 (98)	0.995	0.542	1.05	94.9 (42)	0.830	0.872	0.595	>0.05

Table 6-3 Comparison tests between the Log-normal, Weibull and Gamma distribution in different NaCl concentrations

			Lognormal distribution		Weibull distribution		Gamma distribution	
Organism	**Temp (^oC)**	**Salt (%)**	**-2*LogLikelihood**	**AIC_C**	**-2*LogLikelihood**	**AIC_C**	**-2*LogLikelihood**	**AIC_C**
E. coli	30	0.5	426.4	430.8	430.8	435.2	428.2	432.6
N_D 8.69	30	3	**442.9**	**447.3**	**443.5**	**447.9**	**442.6**	**447.0**
	30	6	893.9	898.1	902.5	906.7	894.2	898.4
***S.* Typhimurium**	30	0.5	420.4	424.8	423.6	428.0	421.2	425.6
N_D 8.78	30	3	566.9	571.2	576.4	580.8	569.8	574.1
	30	6	693.5	697.8	698.3	702.6	693.7	697.9
***L. monocytogenes* 252**	30	0.5	431.0	435.3	445.3	449.7	437.6	442.0
N_D 9.15	30	3	450.0	454.3	463.5	467.8	455.1	459.4
	30	6	576.3	580.5	578.0	582.2	576.4	580.7
***L. monocytogenes* 39**	30	0.5	**394.8**	**399.1**	**397.5**	**401.9**	**394.5**	**398.9**
N_D 9.16	30	3	382.7	387.0	389.2	393.5	384.2	388.5
	30	6	508.6	512.8	511.0	515.2	508.7	512.9
***L. monocytogenes* 271**	30	0.5	316.8	321.2	320.8	325.3	317.9	322.3
N_D 9.32	30	3	649.9	654.1	658.2	662.4	652.1	656.3
	30	6	600.1	604.3	607.9	612.1	607.9	611.4
***L. monocytogenes* 177**	30	0.5	457.6	461.9	470.0	474.3	459.4	463.7
N_D 9.33	30	3	605.7	609.9	618.0	622.2	610.5	614.7
	30	6	409.6	413.9	410.7	415.1	409.8	414.2

Table 6-4 Growth parameters calculated from the rearranged logistic model for TTD data for *E. coli* and *S.* Typhimurium at 30°C and in different pH (6.50, 5.88, 5.16 and 4.58) and the parameters describing the lag_{injury} distribution

			Pre Thermal				Post Thermal				Post Thermal lag parameters (Log-normal distribution)		
Organism	**Temp (°C)**	**pH**	**SGR /h**	**Lag (h)**	**RMSE (obs)**	**r^2**	**SGR /h**	**Lag (h)**	**RMSE (obs)**	**r^2**	**Scale**	**Shape**	**P**
E. coli	30	6.50	1.396	-0.288	7.64 (98)	0.998	1.240	3.937	146 (47)	0.329	1.649	0.404	>0.05
N_D 8.69	30	5.88	1.482	0.31	6.40 (57)	0.998	1.344	4.381	109 (41)	0.328	1.539	0.347	>0.05
	30	5.16	1.292	0.45	6.00 (56)	0.999	1.141	7.387	250 (43)	0.142	1.918	0.477	>0.05
	30	4.58	0.886	1.073	11.3 (98)	0.998	0.914	9.810	483 (48)	0.077	1.736	0.891	>0.05
***S.* Typhimurium**	30	6.50	1.212	-0.07	6.71 (55)	0.999	1.067	7.736	169 (32)	0.205	2.031	0.353	>0.05
N_D 8.78	30	5.88	1.147	0.49	6.08 (57)	0.999	1.039	9.618	216 (27)	0.129	2.201	0.347	>0.05
	30	5.16	1.013	0.43	6.79 (58)	0.999	0.734	11.42	379 (20)	0.044	2.598	0.422	>0.05
	30	4.58	0.804	1.08	12.6 (58)	0.998	0.785	11.85	405 (41)	0.101	2.301	0.543	>0.05

Table 6-5 Comparison tests between the Log-normal, Weibull and Gamma distribution in different pH

			Lognormal distribution		Weibull distribution		Gamma distribution	
Organism	**Temp (°C)**	**pH**	**-2*LogLikelihood**	**AIC_C**	**-2*LogLikelihood**	**AIC_C**	**-2*LogLikelihood**	**AIC_C**
E. coli	30	6.50	587.0	591.3	596.2	600.4	589.9	594.2
N_D 8.69	30	5.88	490.6	494.9	498.1	502.4	492.2	496.5
	30	5.16	574.4	578.7	584.9	589.2	578.0	582.3
	30	4.58	683.8	688.1	691.2	695.5	689.7	693.9
***S.* Typhimurium**	30	6.50	**415.2**	**419.6**	**416.0**	**420.4**	**415.1**	**419.5**
N_D 8.78	30	5.88	358.4	362.9	363.8	368.3	359.8	364.3
	30	5.16	288.9	293.6	290.6	295.3	289.4	294.1
	30	4.58	589.7	594.0	596.6	600.9	592.8	597.1

Table 6-6 Growth parameters calculated from the rearranged logistic model for TTD data for *E. coli* and *S.* Typhimurium at 30°C and in different NaCl-pH combinations and the parameters describing the lag_{injury} distribution (Log-normal distribution)

				Pre Thermal				Post Thermal				Post Thermal lag parameters		
Organism	**Temp (^{0}C)**	**pH**	**NaCl (%)**	**SGR /h**	**Lag (h)**	**RMSE (obs)**	**r^2**	**SGR /h**	**Lag (h)**	**RMSE (obs)**	**r^2**	**Scale**	**Shape**	**P**
E. coli	30	6.45	3	1.060	0.381	6.82 (96)	0.999	1.073	8.198	207 (52)	0.240	1.998	0.411	>0.05
N_D 8.69	30	6.42	6	0.379	11.24	86.9 (86)	0.975	0.373	35.69	393 (64)	0.541	3.176	0.240	>0.05
	30	5.84	3	1.021	0.625	7.55 (98)	0.999	0.953	7.317	157 (63)	0.481	1.982	0.334	>0.05
	30	5.81	6	0.339	10.30	137 (80)	0.951	0.336	33.35	309 (59)	0.731	3.134	0.209	>0.05
	30	5.16	3	0.831	0.294	15.9 (98)	0.997	0.802	11.87	284 (52)	0.258	2.446	0.376	>0.05
***S.* Typhimurium**	30	6.45	3	0.926	1.323	11.4 (98)	0.998	0.885	12.85	275 (42)	0.156	2.358	0.391	>0.05
N_D 8.78	30	6.42	6	0.345	3.767	82.5 (98)	0.984	0.341	23.05	464 (43)	0.346	2.998	0.353	>0.05
	30	5.84	3	0.966	1.330	6.63 (98)	0.999	0.804	14.19	268 (48)	0.237	2.538	0.317	>0.05
	30	5.81	6	0.378	3.789	85.2 (94)	0.978	0.365	23.97	738 (32)	0.102	2.953	0.557	>0.05
	30	5.16	3	0.918	1.453	11.6 (98)	0.998	0.819	13.40	285 (44)	0.184	2.487	0.376	>0.05
	30	5.15	6	0.386	5.312	91.9 (96)	0.976	0.283	29.91	877 (19)	0.132	3.539	0.382	>0.05
	30	4.61	3	0.751	1.420	13.4 (98)	0.998	0.664	15.24	401 (35)	0.115	2.695	0.423	>0.05
	30	4.60	6	0.255	8.919	413 (99)	0.824	0.169	17.90	729 (42)	0.471	3.069	0.526	>0.05

Table 6-7 Comparison tests between the Lognormal, Weibull and Gamma distribution in different NaCl-pH combinations

				Lognormal distribution		Weibull distribution		Gamma distribution	
Organism	**Temp (^{0}C)**	**pH**	**NaCl (%)**	**-2*LogLikelihood**	**AIC_C**	**-2*LogLikelihood**	**AIC_C**	**-2*LogLikelihood**	**AIC_C**
E. coli	30	6.45	3	687.7	691.9	695.8	700.0	689.5	693.8
N_D 8.69	30	6.42	6	928.6	932.8	949.0	953.2	932.8	937.0
	30	5.84	3	805.3	809.5	813.8	818.0	806.6	810.8
	30	5.81	6	834.5	838.8	847.7	852.0	836.2	840.5
	30	5.16	3	725.0	729.2	730.8	735.1	725.6	729.8
***S.* Typhimurium**	30	6.45	3	498.1	502.4	500.2	504.5	498.2	502.5
N_D 8.78	30	6.42	6	641.5	645.8	646.7	651.0	642.7	647.0
	30	5.84	3	**537.3**	**541.6**	**538.9**	**543.1**	**537.1**	**541.4**
	30	5.81	6	503.3	507.8	505.6	510.0	504.0	508.4
	30	5.16	3	**616.9**	**621.2**	**618.4**	**622.7**	**616.8**	**621.1**
	30	5.15	6	306.4	311.1	308.6	313.4	306.8	311.5
	30	4.61	3	513.3	517.6	514.6	519.0	513.4	517.7
	30	4.60	6	665.9	670.2	668.9	673.2	666.9	671.2

6.3.3 Growth rate of inocula following repair and relationship between the MaxO.D and initial populations

From the observed TTD after the thermal insult, although the parameters for the best fit of equation (2-28) were obtained, it was hypothesised that the growth rate was equivalent to that of the control for each observation. Once injury had been repaired each inoculum grew at the rate governed by the imposed environmental conditions of temperature and NaCl, however, only one point (the TTD) was available. To test the hypothesis two methods were used: 1. shape of the O.D/time curve post-treatment compared to an equivalent control curve; 2. performing a serial dilution on a heat treated inoculum prior to the onset of growth.

Figure 6-4 gives the observed O.D/time curves for three control inocula and those from the corresponding wells of the heat treated plate for the growth of *E. coli* in TSB with 3% NaCl. The calculated lags for the three control wells were 499, 680 and 880 mins for the initial inocula of 5.03, 3.63 and 1.94, respectively. By translating these control curves by 495, 700 and 980 minutes respectively, the O.D curves coincided to a high degree with those of the heat treated, Figure 6-5. This implied that the growth rates of the inocula following the heat treatment, once recovered from injury, were the same as the uninjured wells. This phenomenon was observed for all analyses conducted.

Figure 6-6 shows results from several half-fold dilutions of a known inoculum of *S*. Typhimurium in 3% TSB following the heat injury procedure. The plate was prepared using five initial inocula serially diluted from a known amount and these were added to the first column in duplicate; and incubated at 30°C for 2 hours prior to the heat injury step to ensure that the inocula were in exponential phase. Equation (2-28) was fitted to the control TTD data giving a lag of 2.01 h (1.93 – 2.10) and a SGR = 0.912/h (0.903 – 0.920) with 98 observations used. After the thermal treatment, data from the serial dilutions with an initial inoculum of 7.771 $\log_{10}$ cfu/ml gave a lag of 15.45 h (15.3 – 15.6) and a SGR of 0.975/h (0.947 – 1.004); from an initial inoculum of 4.771 $\log_{10}$ cfu/ml a lag of 15.4 h (13.1 – 17.7), and a SGR of 0.898/h (0.772 –

1.076) were observed; two rows with an initial inoculum of 6.771 $\log_{10}$ cfu/ml gave lags of 16.8 h (16.1 – 17.5) and 20.4 h (18.3 – 22.5) and SGR of 0.94/h (0.865 – 1.026) and 0.800/h (0.659 – 1.019) respectively. Data from the serial dilution of the largest initial inoculum used shows a substantial curvature as the inocula approaches the detection threshold of 8.78.

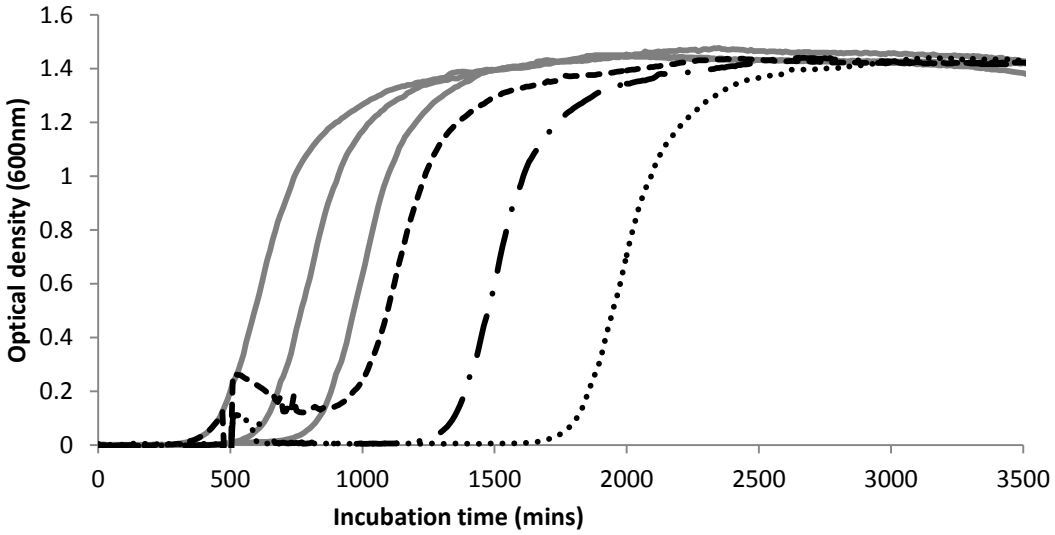


Figure 6-4 Displays three optical density /incubation time curves of *E. coli* in 3% NaCl at 30°C from identical well numbers in the control (grey solid lines, from left to right with initial inoculum 5.032, 3.635, 1.936 $\log_{10}$ cfu/ml) and from the heat treated wells (symbols; dash, 5.032 $\log_{10}$ cfu/ml; dash-dot, 3.635 $\log_{10}$ cfu/ml; dots, 1.936 $\log_{10}$ cfu/ml)

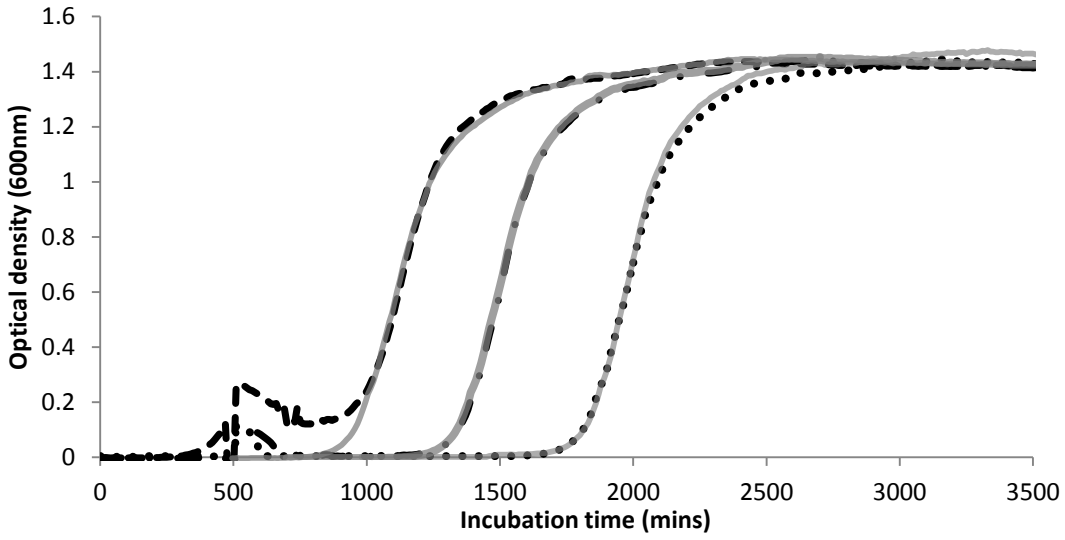


Figure 6-5 Gives the resulting displacement to the right for the three control curves of *E. coli* in 3% NaCl at 30°C by 495, 700 and 980 mins respectively; the observed TTD (O.D = 0.2) for the heat treated wells relative to the untreated controls were 970, 1388 and 1856 minutes, respectively

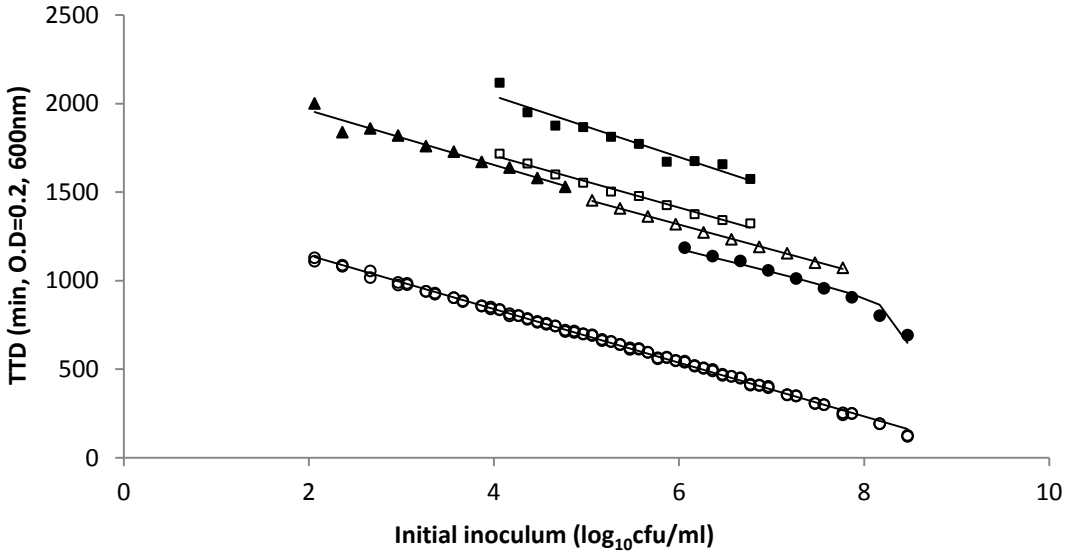


Figure 6-6 *Salmonella* Typhimurium (3% NaCl, 30°C); control (○), SGR = 0.91/h, lag = 2.01 h; selected initial inocula from a heat treated plate –treated after 120 mins incubation at 30°C- and subsequently half-fold diluted across the plate : log_{10} Initial inocula, 8.470 (●); 7.771 (△) SGR = 0.975, lag = 15.4 h; 6.771 (■), 6.771 (□), 4.771 (▲), SGR = 0.898/h, lag = 15.4 h

By plotting the maximum O.D against the initial populations of the microorganisms before and after the heat treatment, the effect of the thermal treatment on the max O.D (which corresponds to the maximum population density) was observed. In particular, Figure 6-7 shows the maximum O.D with respect with the initial populations of *L. monocytogenes* 252 incubated at 30^{o}C, in TSB with 3% NaCl, before and after the heat treatment. At an inoculum size of approximately 10^6 cfu/ml, a decrease in the O.D was observed after the heat treatment. A hypothesis for this observation is that inocula with lower populations than 10^6 cfu/ml, have enough nutrients after the heat treatment to grow and reach the maximum O.D, while inocula with higher populations, have already reached the maximum O.D, before the heat treatment. Samples with inoculum sizes of approximately 10^6 cfu/ml, had not reached the maximum O.D before the treatment, however, the nutrients had already been consumed prior to injury and therefore these populations could not reach the maximum O.D because the available nutrients were used to repair the injury. Similar findings were observed in all NaCl concentrations for all the *L. monocytogenes* strains with the maximum O.D being decreased as the NaCl being increased, e.g from max O.D=0.9 at 0.5% NaCl to max O.D=0.7 at 6% NaCl for *L. monocytogenes* 252. On the contrary, *E. coli* and *S.* Typhimurium did not show such an effect on the max O.D. The max O.D remained the same before and after the heat treatment in all NaCl concentrations.

When the maximum O.D was plotted against the initial populations of *E. coli* in the different pH and different combinations of NaCl-pH studied, showed the same effect as described previously e.g a reduction of the max O.D after the heat treatment compared with the max O.D before the heat treatment. On the other hand, this effect was not observed in any of the pH or NaCl-pH combinations studied for S. Typhimurium, which means that the max O.D before and after the heat treatment remained the same. Figure 6-8 show the maximum O.D with respect with the initial populations of *S.* Typhimurium incubated at 30^{o}C, in TSB with pH=4.58 before and after the heat treatment while Figure 6-9 show the maximum O.D with respect with the initial

populations of *S.* Typhimurium incubated at 30°C, in TSB with 3% NaCl and pH=5.88 before and after the heat treatment. As it can be observed there were no differences between the max O.D before and after the thermal treatment.

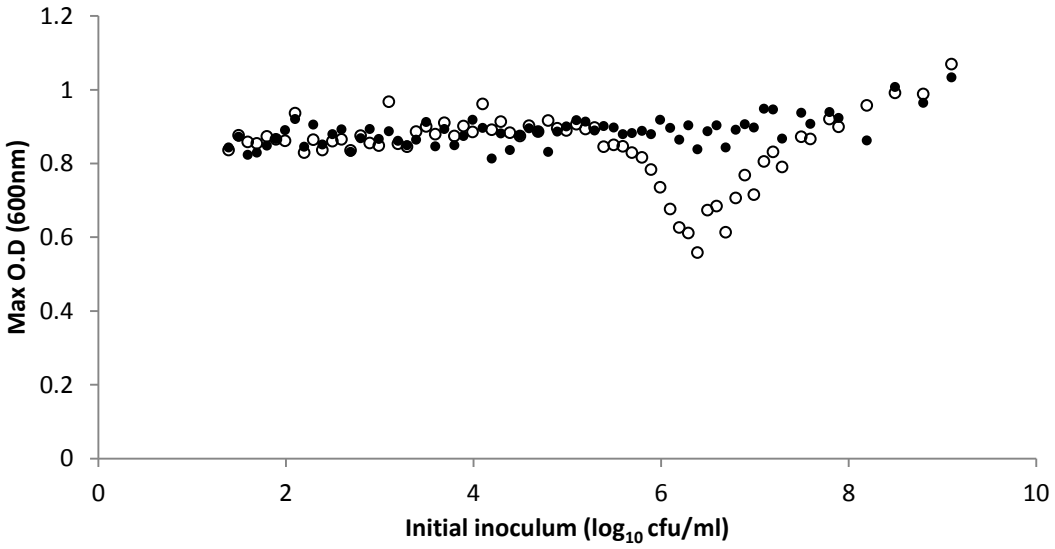


Figure 6-7 Relationship between maximum optical density with the initial populations of *Listeria monocytogenes* 252, grown in TSB with 3% NaCl at 30°C, before (closed symbols) and after heat treatment (opened symbols) at 60°C for 25 min

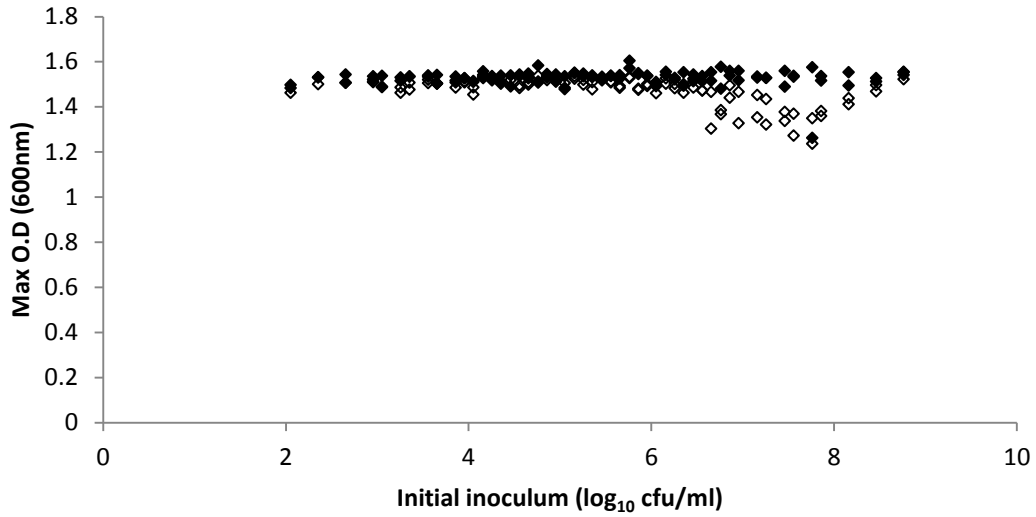


Figure 6-8 Relationship between maximum optical density with the initial populations of *Salmonella* Typhimurium, grown in TSB with pH=4.58 at 30°C, before (closed symbols) and after heat treatment (opened symbols) at 60°C for 25 min

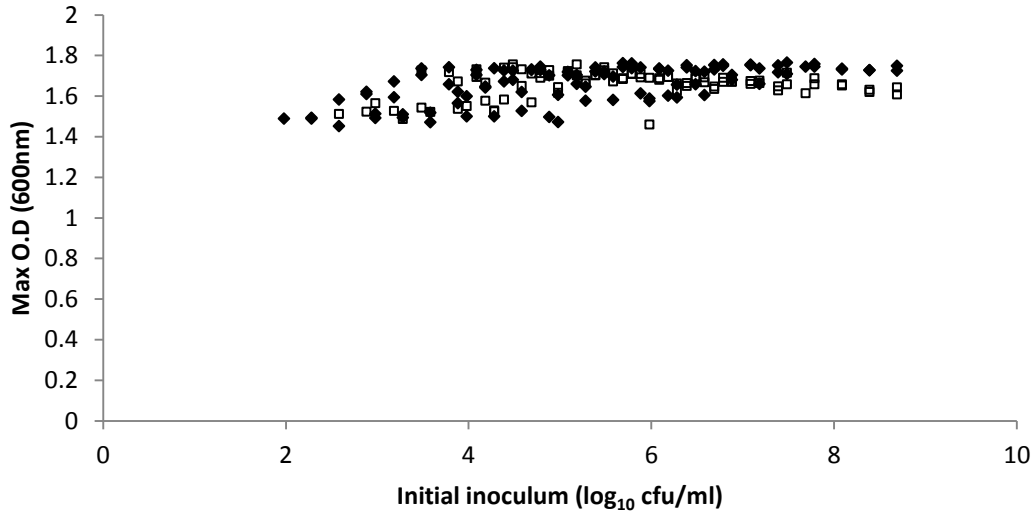


Figure 6-9 Relationship between maximum optical density with the initial populations of *Salmonella* Typhimurium, grown in TSB with 3% NaCl and pH=4.58 at 30°C, before (closed symbols) and after heat treatment (opened symbols) at 60°C for 25 min

6.4 Discussion

The standard lag and growth rate can be easily obtained from TTD experiments when modelled using the rearranged logistic with lag model (equation (2-10)). Growth rates, (and lags) change accordingly when the environment is more amenable for growth (e.g. move to more optimal temperature) or less cordial (e.g. increasing salt concentration). In the studies performed here, *L. monocytogenes* strains were the most recalcitrant to the effect of increasing NaCl concentration, whereas *S*. Typhimurium and *E. coli* were quite sensitive. *L. monocytogenes* strains were not studied in different pH or combinations of NaCl-pH because cell clumping was observed under these conditions and so O.D measurements were not reliable.

When a small thermal insult (nominally 60°C for 25mins) was applied to the Bioscreen plate, whilst the organisms were in exponential phase, *E. coli* and *S*.Typhimurium showed a significant response relative to *L. monocytogenes*. The observed TTD data showed a step between the control (no thermal injury) and the treated wells, the size of the step appeared correlated with the growth conditions and was dependent on the species under observation. There was also a large increase in the variance of the data following the thermal process, again the magnitude of which appeared to be dependent on the environmental conditions and the species under test.

The distribution of the thermally induced lags was found to be Log-normal for the majority of experiments; only in some cases was the Gamma distribution found to fit the lags due to injury better than the Log-normal but even in those cases the Log-normal distribution had a good fit with $p>0.05$. McKellar and Hawke (2006) assessed several distributions for fitting lag times of individual cells using six strains of *E. coli* O157:H7 and suggested that the Log-normal distribution can be used successfully to characterise the individual cell lag times, a result similar with our observations.

Data were simulated (using equation (2-28)) using the hypothesis that the organisms undergoing a thermal insult have an induced lag the size of which

is randomly given by the Log-normal distribution, but when repair is over (the time of lag) growth occurs at the same rate as before, dictated by the environment. However, although the simulations appear to give credible reconstructions of the observed data, there was no-way to obtain the growth rate data from a single observation of the TTD. However, each well provides a wealth of O.D data, at values other than the set O.D criterion.

The shape of the O.D-incubation time curve is a reflection of the lag, the growth rate and the MPD attained. Different inocula with the same lag and growth rate will have congruent O.D/incubation time curves, but shifted up or down the time axis depending on whether the initial inoculum is greater or less than a given value. If the shapes for a series of control experiments are known, from which the growth rates have been calculated, then any test inoculum having the same growth rate will have the same O.D/time shape, i.e. it will show congruence with the controls. It is known that as growth conditions become more inimical, growth rates and lags increase and the shape of the O.D/incubation curve become shallower relative to a positive control (e.g. grown under optimal conditions). In the experiments carried out here, the thermally treated inocula had congruent O.D/time curves after the injury had been dealt with (lag due to injury). Indeed, Figure 6-4 and Figure 6-5 show that the control and treated inocula grew identically, but after the mild thermal injury, the treated inoculum showed no growth for the period of lag, before the onset of growth recommenced at the same rate as the control.

To further show that the treated inocula grew at the same rate once recovery was complete, it was hypothesised that if an initial inoculum which underwent the mild thermal process was then subsequently serially diluted, then the distribution of injury occurring to the population in the initial well would be identical to those diluted across the plate. Once recovery was achieved the wells would show a linear relationship, if the lag is a function of the injury distribution, with a gradient equal to that of the untreated control. Such experiments carried out on all the organisms studied confirmed this hypothesis. Figure 6-6 shows the results from an experiment with *S*. Typhimurium, all plots show a linear relationship with the log of the initial

inoculum, with gradient approximately equal to that of the control. The curvature of the highest inoculum used is due to the approach to the detection threshold; equation (2-28) suffers from the same problem that the logistic with lag growth model has – no growth until the end of lag. Figure 6-4, Figure 6-5 and Figure 6-6 show that, as predicted by the Baranyi equation, growth occurs prior to the end of lag, as the organisms adapt to the new environment.

Stephens *et al.* (1997) conducted work on thermally injured *S.* Typhimurium cells using multiple dilutions to examine injury with single cells. In their work lag times for single cells undergoing a mild thermal process was highly variable with lag times commonly greater than 20 h, but with longer than 30 h observed. From the data given in Table 6-2, a model for the TTD of a single *S.* Typhimurium (grown at 30^{o}C, in TSB with 3% NaCl), can be constructed, and for such a model the mild thermal process carried out here gives lags between between approximately 1700 and 2900 mins (approx. range between the 2.5 and 97.5 quantiles for multiple simulations of the TTD for a single cell, according to equation (2-28). The calculation suggests that for high inoculum densities (e.g. 10^{8} cfu/ml) the distribution of injury is also wide; a similar calculation to that done for single cells gives an interquantile range (2.5 to 97.5%) of approx 500 to 1700 min, whereas the uninjured have a TTD = 213 min.

It would be interesting to conclude that the examination of lags from single cell studies can be extrapolated from using larger initial inocula, however, this requires further study, as, for example, the effect of a definitive log reduction, e.g. a 3 log reduction has yet to be added to the simple model discussed here.

When the maximum O.D before and after the thermal injury in different NaCl concentrations, was plotted against the initial populations of the *L. monocytogenes* strains showed a decrease of the max O.D after the heat treatment at an inoculum size of approximately 10^{6} cfu/ml because the available nutrients were used to repair injury. With large numbers of microbes the available pool of nutrients is reduced significantly, hence the maximum O.D observed in untreated cases cannot be attained. On the other hand, the

max O.D of *S.* Typhimurium and *E. coli* was the same before and after the thermal injury. This suggests a problem or that there were enough nutrients available to be used from the microorganisms and reach the maximum O.D, at all inoculum sizes. Also, the plots mentioned above were obtained for *S.* Typhimurium and *E. coli* when studied in different pH and combinations of NaCl-pH. *E. coli* showed a decrease of the max O.D after the heat treatment while *S.* Typhimurium did not.

6.5 Conclusion

Optical density can be used to determine accurate growth rates and lags. Following a mild thermal process a lag is induced, the magnitude of which is dependent on the organism and environmental conditions; the observed distribution of the lags appears, in general, to follow the Log-normal distribution. After the lag period due to injury, growth recommences at the rate dictated by the growth environment. The examination of lags from single cell studies might be extrapolated from using larger initial inocula according to the studies accomplished herein.

7 Traditional growth curves for different NaCl concentrations compared with the Bioscreen technique using *Listeria monocytogenes* strains, *Salmonella* Typhimurium and *Escherichia coli*

7.1 Introduction

The development of rapid, sensitive and specific methods to detect foodborne pathogenic bacteria is a major factor for effective practices which ensure food safety and security. Monitoring is one of the most important control points in the prevention of diseases by foodborne pathogens. To control foodborne pathogens in food products effective detection and inspection methods are necessary. “Conventional microbiological methods have been a standard practice for the detection and the identification of pathogens in food for nearly one century and continue to be a reliable standard for ensuring food safety”(Yang and Bashir, 2008). However, the conventional methods build almost exclusively upon the use of specific agar media to isolate and enumerate viable bacterial cells in samples. These methods usually include microbiological culturing and isolation of the pathogen, which is followed by confirmation with biochemical and or serological tests, taking up to 5-7 days to get a confirmed result for a particular pathogenic organism (Swaminathan and Feng, 1994; Vasavada, 1997). Even if the conventional methods are reliable, they are time consuming and labour intensive and are therefore not suitable for modern food quality assurance to make a timely response to possible risks (Yang and Bashir, 2008). In order to obtain sufficient data using the traditional methods it may take several days of work. As a result, over the past 25 years numerous novel methods which offer new possibilities, are cheaper, automated, accurate and most importantly they have been developed to reduce the assay time.

However, rapid methods often have high detection limits and they may exhibit false positive results (e.g. ELISA). Rasch (2004) reported some examples of these methods, like turbidity, flow cytometry, microscopic methods etc. In particular, the turbidity method measures the optical density (O.D) of a cell

suspension and has been used by many scientists in the area of predictive microbiology. Dalgraard and Koutsoumanis (2001) stated that turbidimetric methodologies such as the use of Bioscreen microbiological analyser which measures O.D might be another way (instead of the traditional viable counts method) of studing the bacterial growth since O.D measurements give a real time measure of bacterial population. It has also been stated that, despite the high threshold detection of turbidimetric devices which is often the most important limitation of this method, the measurements have practical significance when dealing with bacteria at high cell densities. Predictive modelling requires the collection of adequate data. The advantage of the turbidimetric methods is that large numbers of experiments can be set up in a short period of time conversely with the time-consuming nature of plate counts and thus these methods constitute a valuable tool for predictive modelling.

Herein, traditional growth curves using the plate count method were constructed and compared with the results obtained from the Bioscreen microbiological analyser under the same conditions. The results suggested that the growth parameters (growth rate and lag time) obtained from the traditional plates counts are higher than the growth parameters obtained from the TTD method which could be explained as an artifact of the plating method or may be due to the use of the *modified* Gompertz model to study the growth.

7.2 Materials and methods

Growth curves of *L. monocytogenes* strains (252 and 39), *S.* Typhimurium and *E. coli* were constructed at different NaCl concentration (0.5, 3, 6 and/or 9% NaCl) at 30^{o}C using the traditional plate counts as described in paragraph 2.2. Simultaneously, a Bioscreen experiment was set up under the same conditions as described in paragraph 2.3.5.1. The data obtained from the traditional growth curves were fitted with the *modified* Gompertz equation as described in paragraph 2.4.1 while the data obtained from the Bioscreen were fitted with the rearranged logistic with lag model as described in paragraph 2.4.5.

7.3 Results

7.3.1 Traditional growth curves

Growth curves were made with the traditional plating method in TSB with 0.5%, 3%, 6% and/or 9% NaCl at 30°C for *L. monocytogenes* 252, *L. monocytogenes* 39, *E. coli* and *S.* Typhimurium. Figure 7-1 to Figure 7-4 show the growth curves (Log (cfu/ml)/time) for the microorganisms examined in different NaCl concentrations. The data obtained were fitted with the *modified* Gompertz model and using JMP 8, the parameters (A, C, b and m) of the model determined.

The growth parameters (growth rate and lag time) as well as the maximum population density (MPD) were calculated from the parameters of the *modified* Gompertz model and are shown in Table 7-1. The *modified* Gompertz model uses the logarithm of the microbial numbers and thus the growth rates calculated are expressed in Log cfu/hours. The growth rates were also expressed in Ln cfu/hours (Table 7-1), in order to be able to directly compare them with the growth rates obtained from the experiments conducted in the Bioscreen microbiological analyser under the same conditions but calculated from the rearranged lag logistic model which was used to fit the O.D data.

As the NaCl concentration increased, the growth rate decreased while the lag time increased in all cases. *L. monocytogenes* 252 and *L. monocytogenes* 39 had a high lag time (19.83 and 15.32 hours, respectively) and a low growth rate (0.392 and 0.345 Ln cfu/hour, respectively) in 9% NaCl and were more salt tolerant than *S*. Typhimurium or *E. coli* which did not grow at 9% NaCl. *S*. Typhimurium and *E. coli* had high lag time (8.08 and 14.88 hours, respectively) and a low growth rate (0.368 and 0.391 Ln cfu/hour, respectively) in 6% NaCl and *E. coli* found to be the most salt sensitive microorganism from those examined. Also, with increasing NaCl concentration, a decrease in the MPD was observed in all cases.

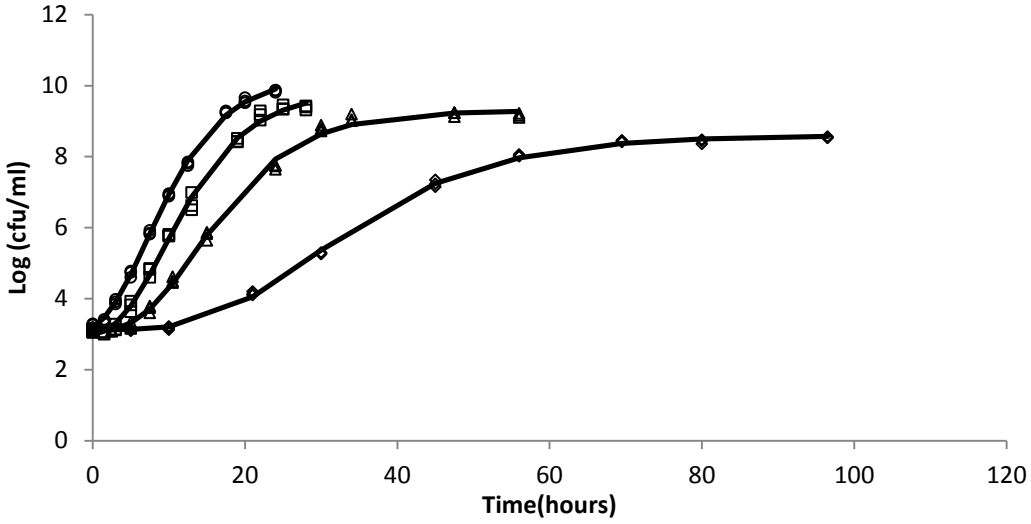


Figure 7-1 Growth curves of *Listeria monocytogenes* 39 in TSB with 0.5% NaCl (○), 3% NaCl (□), 6% NaCl (△) and 9% NaCl (◊) at 30°C, fitted with the *modified* Gompertz equation

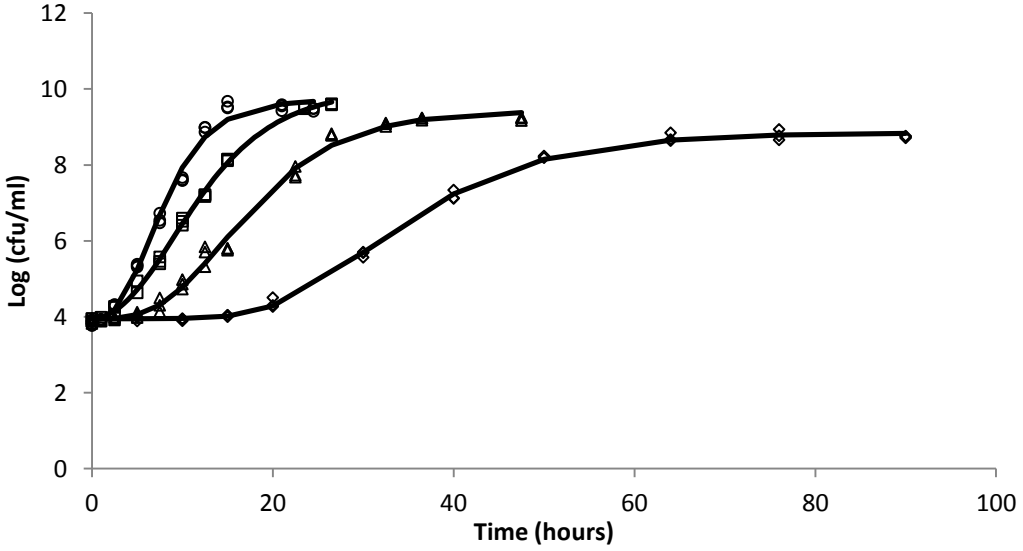


Figure 7-2 Growth curves of *Listeria monocytogenes* 252 in TSB with 0.5% NaCl (○), 3% NaCl (□), 6% NaCl (△) and 9% NaCl (◊) at 30°C, fitted with the *modified* Gompertz equation

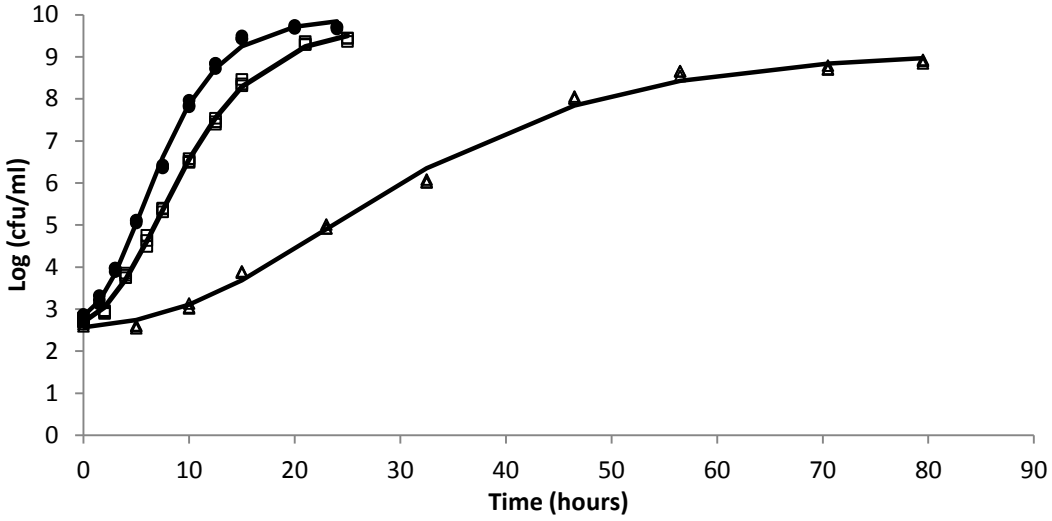


Figure 7-3 Growth curves of *Salmonella* Typhimurium in TSB with 0.5% NaCl (○), 3% NaCl (□) and 6% NaCl (△) at 30°C, fitted with the *modified* Gompertz equation

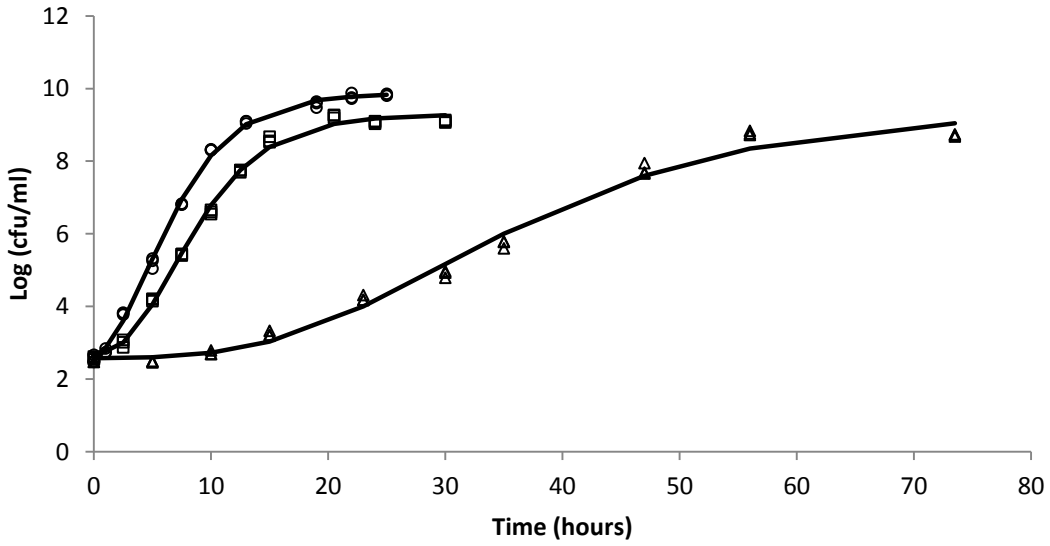


Figure 7-4 Growth curves of *Escherichia coli* in TSB with 0.5% NaCl (○), 3% NaCl (□) and 6% NaCl (△) at 30°C, fitted with the *modified* Gompertz equation

Table 7-1 Parameters obtained from the *modified* Gompertz equation for *Listeria monocytogenes* 252, *Listeria monocytogenes* 39, *Escherichia coli* and *Salmonella* Typhimurium in TSB with 0.5% NaCl, 3% NaCl, 6% NaCl and/or 9% NaCl at 30°C

Microorganism	NaCl (%)	Growth rate (Log cfu/hours)	Growth rate (Ln cfu/hours)	Lag time (hours)	MPD (log cfu/ml)
***Listeria monocytogenes* 252**	0.5	0.59 (0.54-0.66)	1.37 (1.24-1.52)	2.75 (1.91-3.46)	9.9
	3	0.37 (0.35-0.40)	0.86 (0.81-0.91)	2.97 (2.18-3.62)	9.7
	6	0.29 (0.26-0.31)	0.66 (0.60-0.72)	7.39 (6.24-8.42)	9.4
	9	0.17 (0.16-0.18)	0.39 (0.37-0.42)	19.8 (18.8-20.9)	8.9
***Listeria monocytogenes* 39**	0.5	0.47 (0.45-0.49)	1.08 (1.04-1.12)	1.37 (0.77-1.89)	10.3
	3	0.42 (0.39-0.45)	0.96 (0.89-1.02)	3.31 (2.42-4.04)	9.8
	6	0.31 (0.29-0.33)	0.71 (0.67-0.75)	6.09 (5.27-6.85)	9.3
	9	0.15 (0.14-0.16)	0.35 (0.34-0.37)	15.4 (14.5-16.2)	8.6
***Salmonella* Typhimurium**	0.5	0.65 (0.62-0.68)	1.50 (1.43-1.57)	1.37 (0.76-1.89)	9.9
	3	0.50 (0.48-0.52)	1.16 (1.12-1.20)	1.91 (1.38-2.37)	9.7
	6	0.16 (0.15-0.17)	0.37 (0.34-0.40)	8.08 (5.40-10.3)	9.1
Escherichia coli	0.5	0.72 (0.68-0.75)	1.65 (1.57-1.73)	0.76 (0.09-1.30)	9.9
	3	0.58 (0.55-0.62)	1.34 (1.26-1.42)	2.49 (1.88-3.04)	9.3
	6	0.17 (0.15-0.19)	0.39 (0.35-0.45)	14.9 (11.5-17.8)	9.2

7.3.2 Performing tests in the Bioscreen

A Bioscreen experiment was set up under the same conditions in order to compare the growth parameters obtained from the two methods. Figure 7-5 shows the relationship between the initial log inocula against the TTD of *S.* Typhimurium at 30°C in different NaCl concentrations (0.5, 3 and 6% NaCl). Similar figures were obtained for all analyses conducted.

The relationship between the initial populations of the microorganisms against the TTD was linear and the observed TTD were modelled with the rearranged lag logistic model. As the NaCl concentration increased, the gradient and intercept increased. The growth parameters obtained showed an increased lag time and a decreased growth rate as the NaCl concentration increased (Table 7-2). Also, the results obtained from the studies herein were similar and thus reproducible, with the results obtained from the inoculum size studies accomplished in the Bioscreen under the same conditions in Chapter 5.

Comparing the growth parameters obtained from the fit of the *modified* Gompertz model to the data obtained from the traditional growth curves (Table 7-1) against the growth parameters obtained from the fit of the rearranged logistic with lag model to the data obtained from O.D measurements (Table 7-2), it can be observed that these results are significantly different from each other. In particular, the lag times obtained from the fit of the *modified* Gompertz model, were significantly higher than those obtained from the TTD technique while the growth rates obtained from the fit of the *modified* Gompertz model were, in most cases, higher than those obtained from the TTD technique.

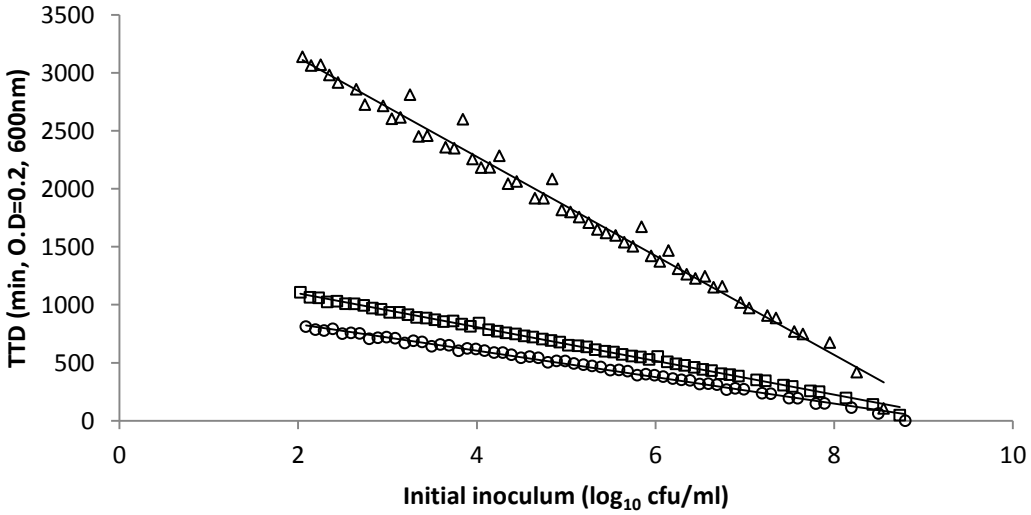


Figure 7-5 Relationship between TTD with the initial populations of *Salmonella* Typhimurium in TSB with 0.5% NaCl (○), 3% NaCl (□) and 6% NaCl (△) at 30°C

Table 7-2 Parameters describing the growth kinetics of 3 different microorganisms (with their confidence intervals), as calculated from data obtained from the rearranged logistic with lag model at 30°C

Microorganism	NaCl (%)	Growth rate (Ln cfu/hours)	Lag time (hours)
***Listeria monocytogenes* 252**	0.5	1.073 (1.065-1.081)	0.279 (0.207-0.351)
	3	0.915 (0.908-0.922)	0.653 (0.571-0.735)
	6	0.616 (0.609-0.623)	0.985 (0.787-1.184)
	9	0.300 (0.291-0.309)	12.69 (11.66-13.71)
***Listeria monocytogenes* 39**	0.5	1.043 (1.036-1.050)	1.269 (1.204-1.334)
	3	0.848 (0.838-0.858)	2.060 (1.923-2.198)
	6	0.614 (0.605-0.624)	4.239 (3.991-4.487)
	9	0.295 (0.286-0.304)	16.14 (15.08-17.19)
***Salmonella* Typhimurium**	0.5	1.264 (1.251-1.277)	0.583 (0.505-0.660)
	3	1.535 (1.522-1.548)	0.948 (0.896-1.001)
	6	0.295 (0.290-0.299)	2.568 (2.077-3.059)
Escherichia coli	0.5	1.506 (1.496-1.517)	-0.02 (-0.06-0.022)
	3	1.076 (1.070-1.082)	0.212 (0.164-0.260)
	6	0.352 (0.345-0.360)	6.078 (5.534-6.622)

7.4 Discussion

The growth data obtained at 30°C, in different NaCl concentrations using the traditional plate method were fitted with the *modified* Gompertz model and compared directly with the growth data obtained from the Bioscreen which were fitted with the rearranged lag logistic model for TTD data. In the literature, one of the most common used models to fit growth data is the *modified* Gompertz model (McClure *et al*., 1994; Linton *et al*., 1995a; 1995b; Li *et al*., 2007). However, it has been previously shown that the *modified* Gompertz model (as well as the *modified* logistic model) was not able to fit TTD data (Mytilinaios *et al*., 2012). In contrary, the classical 3 parameter logistic model (and by default the Baranyi model) was able to give accurate growth rates and lag times from O.D data.

The traditional method of examining growth using plates can be considered to be a repeated measures experiment following the growth of an initial inoculum with time, whereas the method used here is a multiple inoculum experiment with a single time measurement (the TTD) per inoculum. These methods should be considered not as complementary but methods describing the same phenomenon of microbial growth, done in a different fashion. However, the growth rates and the lag times obtained from the two methods were significantly different. In particular, the growth rates and lag times obtained from the *modified* Gompertz model which was used to fit the data from the traditional growth curves were significantly higher in comparison with the growth parameters obtained from the classical logistic model which was used to fit the TTD data from O.D measurements.

There are studies within the literature which report differences between the parameters obtained from O.D data compared with those from the plate counts (Augustin *et al*., 1999; Baty *et al*., 2002). Francois *et al.* (2005) stated that these discrepancies may be explained because of the underestimation that may occur from the plate counts as the condition becoming harsher and subsequently the estimation of the growth parameters is less accurate. Also, Farber *et al*. (1996) studied the effect of temperature, pH and carbon dioxide

on the growth or survival of five strain mixture of *L. monocytogenes* and used two primary models (*modified* Gompertz and Baranyi) to fit the data. They stated that the Baranyi model appeared to fit the data better than the *modified* Gompertz model and that the *modified* Gompertz model predicted longer lag times.

Furthermore, the discrepancies mentioned above could be an artifact of the plating method and could be explained from the mitotic process. In particular, if we consider a single cell which starts growing and duplicating its DNA (interphase), it appears on the agar plate as one colony. As the mitosis begins the cell starts dividing itself into two identical cells (daughter cells), even at the end of the telophase, on the plate it appears as a single colony. Only at the final phase (cytokinesis) where the cell is completely divided in two identical cells, on the plate appears as two colonies. On the other hand with the Bioscreen microbiological analyser the growth of the microorganisms is monitored continuously and any difference in the size of the cells results in a higher O.D. The aforementioned details could be the explanation for the differences on the growth data obtained from the two methods compared.

It is also noteworthy to mention the differences in the quantity of the materials used for conducting each experiment with the two methods as well as the differences in time needed to accomplish each experiment. Figure 7-6 illustrates the differences in the materials used with the two methods studied. It is obvious that the materials used for the traditional plate counts are much more than the materials used to set up a Bioscreen experiment and thus the costs are increased. Also, the time consumed to set up a Bioscreen experiment was approximately 2 hours hence with the traditional method measurements needed to be taken every 2-5 hours for 1-5 days. This is also important for modern food quality assurance to make a timely response to possible risks (Yang and Bashir, 2008) and for effective practices which ensure food safety and security.

Figure 7-6 Materials used to set up a Bioscreen experiment (left) and to obtain a growth curve with the traditional plate counts (right)

7.5 Conclusion

The increased lag times and growth rates obtained from the traditional plate counts compared with the values obtained from the Bioscreen microbiological analyser, might be an artifact of the plating method or may be due to the use of the *modified* Gompertz to study the growth. This is an empirical model which we have already shown to be incapable of describing the results of TTD experiments. It is also possible that the *modified* Gompertz is not as applicable to plate data as thought. The study herein suggests that growth parameters can be obtained from TTD data faster, cheaper and more accurately compared with the so far used plate count method.

8 General discussion

Microorganisms in foodstuffs can cause spoilage, food poisoning or can affect their properties in a beneficial way (food fermentation). Food poisoning is defined as any illness caused by bacterial, chemical or biological contamination of food and is related to food safety. Foodborne disease is a serious threat to public health and it seems to be increasing globally (Knowels, 2007). Foodborne pathogens are dynamic and even the well known ones can evolve and create new public health challenges but also there are several unknown foodborne pathogens constantly emerging (Newell *et al*., 2010). It is clear that the prevention of foodborne disease is important for food safety. Traditionally, food safety control was based on the inspection of the end product but nowadays, new strategies and technologies have been developed like the food safety tools (GMP, GHP), the implementation of HACCP systems/plans and the MRA which all together constitute a proactive and preventative concept of assuring food safety.

The multi-target hurdle technology is a concept developed several years ago in food production for the mild but effective preservation of foods (Leistner, 1995 a; 1995 b). Also, with predictive microbiology the knowledge of the microbial responses in different environmental conditions is summarised as mathematical models or equations and therefore has become a valuable research tool. To control foodborne pathogens in food products the effective detection and inspection methods are necessary. Conventional methods built almost solely upon the use of agar plates, are time consuming, labour intensive and costly. On the other hand, the TTD method (measure of O.D) is a rapid, cheap and reliable method which has been used for many applications in food microbiology (e.g. McClure *et al.,* 1993; Francois *et al.*, 2005; Dalgaard and Koutsoumanis, 2001; Guiller *et al*., 2006; Standaert *et al*., 2005; Stephens *et al.*, 1997).

We have been developing the use of O.D for obtaining growth rates and lag times using multiple inocula rather than using the traditional methods which use one single inoculum. In particular, all analysis were performed in the

Bioscreen microbiological analyser which measures O.D and the TTD was defined as the time which each inoculum needs to reach an O.D=0.2 (TTD were found using linear interpolation between O.D/time values which straddled the O.D = 0.2 value).

The first part of this project examined the use of the Bioscreen to predict growth curves from O.D data. Models used to examine the shape of microbial growth generally require four parameters: the initial and final population levels (I_0 and MPD respectively), the maximum specific growth rate and the time at which this occurred. If three pieces of information are available, e.g. the initial population, the MPD and the specific growth rate, then knowledge of the population at a specific time can be used to reproduce the growth curve simply by substituting the values into the equations and solving for the missing parameter.

The TTD data produced using the multiple inocula technique described could be well fitted using the 3-PLM, the Baranyi and the logistic (with or without lag), the parameters obtained were consistent between models and reflected the observed gradients well. Further, using a simple conversion between O.D and numbers (cfu ml^{-1}), the basic features of the O.D/time plots could be reproduced with these models and thus the peculiar problem described by Baranyi and Roberts (1995): that direct fitting of viable count data to turbidity or conductivity data or vice-versa should not be considered without additional information being available could be overcome. The *modified* logistic and *modified* Gompertz equations, however, failed to fit the observed data and could not reproduce the observed O.D/time plots. Several reports have suggested that the O.D technique is limited as it requires high initial inocula (Dalgaard *et al.*, 1994; Dalgaard and Koutsoumanis, 2001; Baty *et al.*, 2002; Perni *et al.*, 2005). The observed data described herein showed that this assumption is not valid. If the growth rate of an organism under ideal conditions is obtained using the multiple inoculum method then any subsequent study using non-ideal conditions can use a positive control to set the modelled fit.

The measurement of microbial growth rates, especially its temperature dependency, is of fundamental importance in food microbiology. Within the literature several studies have looked at the effect of non-isothermal studies conditions on microbial growth using established modelling methods (Baranyi *et al*., 1995; Bovill *et at*., 2000; Dalgaard *et al*., 2002; Giannakourou *et al*., 2005; Koutsoumanis, 2001; Koutsoumanis *et al*., 2006; Taoukis *et al*., 1999; Zwietering *et al*., 1994). The aim of these studies was to test the ability of using models based on growth data obtained isothermally to predict growth under non-isothermal conditions.

Using micro-titre plates with multiple inocula allowed the investigation of a wealth of phenomena such as small temperature shifts using *L. monocytogenes* 252 as an example. The model used to fit the data obtained from the Bioscreen microbiological analyser was based on the Malthusian approximation of the logistic model. The results obtained from the non-isothermal studies showed that when a temperature shunt was applied to growing bacteria, the culture reduced or increased its growth rate commensurate with the incubation temperature. When the culture was shunted from a lower temperature to a higher temperature there was no evidence of an induced lag and growth continued at the rate dictated by the new temperature. When the culture was shunted from a higher to a lower temperature condensation on the inside of the plate lid occurred and this led to unusable data for a period after the shunt (the period depended on the temperature difference). From the observed and the fitted data it can be concluded that no induction of lag occurred when moving from the higher to the lower temperatures used: the intercept of the regression lines for each temperature coincide at the time of the temperature shunt, if lags were present this would not occur.

Our better understanding from these studies regarding the O.D curves (TTD method) and the way they can be fitted, led to the use of the rearranged logistic with lag model. O.D was directly related to microbial numbers with simple calibration curves. Calibration curves showed that a direct relationship between O.D and cfu/ml existed and that a specific O.D was equivalent to a

specific number of organisms per ml. McKellar *et al*. (2002) and McKellar and Knight (2000) have suggested a method for the analysis of lag time using the same methodology employed in our laboratory. The classic 3-parameter logistic model was able to fit the TTD data obtained from turbidometric experiments using multiple inocula incubated iso-thermally. In all cases, with increasing incubation temperature (30-37°C) the growth rate increased hence the lag time decreased which shows that 37°C is a more optimal temperature. Also, the parameters obtained suggested that as the conditions became more inimical (increased NaCl concentration, low pH or combinations of NaCl-pH) the growth rate decreased while the lag time increased. The analysis of the data obtained from the inoculum size experiments showed that the growth rate was independent of the inoculum size. The inoculum size affected only the time to reach the TTD, where the higher inocula needed less time to reach the TTD criterion (e.g. O.D = 0.2) compared with lower inocula. The MIC_{NaCl} and MIC_{pH} were obtained from O.D measuremenets using the LPM (Lambert and Pearson, 2000). The results obtained were in agreement with the inoculum size experiments.

When the microorganisms were thermally injured, a lag (due to thermal injury) was also induced (lag_{injury}). The standard lag and growth rate can be easily obtained from TTD experiments when modelled using the logistic with lag equation. Growth rates, (and lags) change accordingly when the environment is more amenable for growth (e.g. move to more optimal temperature) or less cordial (e.g. increasing salt concentration). When a small thermal insult (nominally 60°C for 25mins) was applied to the Bioscreen plate, whilst the organisms were in exponential phase, *E. coli* and *S*.Typhimurium showed a significant response relative to *L. monocytogenes*. The observed TTD data showed a step between the control (no thermal injury) and the treated wells, the size of the step appeared correlated with the growth conditions and was dependent on the species under observation. There was also a large increase in the variance of the data following the thermal process, again the magnitude of which appeared to be dependent on the environmental conditions and the species under test.

The distribution of the thermally induced lags was found to be Log-normal for the majority of experiments; in some cases the Gamma distribution had a slightly better fit from the Log-normal distribution but even in those cases the fit of the Log-normal distribution was quite well with $p>0.05$.

Data were simulated using the hypothesis that the organisms undergoing a thermal insult have an induced lag the size of which is randomly given by the Log-normal distribution, but when repair is over (the time of lag) growth occurs at the same rate as before, dictated by the environment. However, although the simulations appear to give credible reconstructions of the observed data, there was no-way to obtain the growth rate data from a single observation of the TTD. However, each well provides a wealth of O.D data, at values other than the set O.D criterion.

The shape of the O.D-incubation time curve is a reflection of the lag, the growth rate and the maximum population attained. Different inocula with the same lag and growth rate will have congruent O.D/incubation time curves, but shifted up or down the time axis depending on whether the initial inoculum is greater or less than a given value. If the shapes for a series of control experiments are known, from which the growth rates have been calculated, then any test inoculum having the same growth rate will have the same O.D/time shape, i.e. it will show congruence with the controls. It is known that as growth conditions become more inimical, growth rates and lags increase and the shape of the O.D/incubation curve become more shallow relative to a positive control (e.g. grown under optimal conditions). In the experiments carried out here, the thermally treated inocula had congruent O.D/time curves after the injury had been dealt with (lag due to injury).

To further show that the treated inocula grow at the same rate once recovery is complete, it was hypothesised that if an initial inoculum which underwent the mild thermal process was then subsequently serially diluted, then the distribution of injury occurring to the population in the initial well would be identical to those diluted across the plate. Once recovery was achieved the wells would show a linear relationship, if the lag is a function of the injury

distribution, with a gradient equal to that of the untreated control. Such experiments carried out on all the organisms studied confirmed this hypothesis. All plots showed a linear relationship with the log of the initial inoculum, with gradient approximately equal to that of the control. The curvature of the highest inoculum used is due to the approach to the detection threshold. The model used after the heat treatment suffers from the same problem that the logistic with lag growth model has – no growth until the end of lag.

It would be interesting to conclude that the examination of lags from single cell studies can be extrapolated from using larger initial inocula, however, this requires further study, as, for example, the effect of a definitive log reduction, e.g. a 3 log reduction has yet to be added to the simple model discussed here.

Finally, the growth curves obtained at 30°C, in different concentrations of salt (3, 6 or 9% NaCl) using the traditional plate method, compared directly with the Bioscreen method. The growth rates and the lag times obtained from the two methods were significantly different. Additionally, there are studies that report differences between the parameters obtained from O.D data compared with those from the plate counts (Augustin et al., 1999; Baty et al. 2002). Francois *et al.* (2005) mentioned that these discrepancies may be explained because of the underestimation that may occur from the plate counts as the condition becoming harsher and subsequently the estimation of the growth parameters is less accurate. Similarly, the study herein suggests that growth parameters can be obtained from TTD data faster, cheaper and more accurately compared with the so far used plate count method.

9 General conclusions

The conclusions which can be derived from this study are:

- Optical density can be used to determine accurate growth rates and lag times;
- The Baranyi model is the most capable primary model of those examined (in the absence of lag it defaults to the classic 3 parameter logistic model), but the *modified* logistic and the *modified* Gompertz should not be used as primary models as they cannot reproduce observed (TTD) data;
- Studies of the mild temperature shifts using the Malthusian approximation of the logistic model suggested that there were no indications of induced lags when the plates were exchanged from one temperature to the other;
- The classic 3-parameter logistic model (with a lag term) was rearranged to provide the theoretical foundation for the observed TTD.
- Inoculum size studies showed that as the conditions became more inimical the growth rate decreased while the lag time increased. Also, the growth rate was independent of the inoculum size. The inoculum size affected only the time to reach the TTD;
- The Lambert and Pearson model (LPM) and the Extended Lambert and Pearson model (ELPM) can analyse results from individual and combined inhibitors, respectively;
- A heat injury induced a lag due to inhury (lag_{injury}), the magnitude of which is dependent on the organism and environmental conditions; the observed distribution of the lags appears, in general, to follow the Log-normal distribution. After the lag period due to injury, growth recommences at the rate dictated by the growth environment;
- Growth rates can alter without inducing lags or lags can be induced without inducing changes in growth rate;
- The increased lag times and growth rates obtained from the traditional plate counts compared with the values obtained from the Bioscreen,

might be an artifact of the plating method or may be due to the use of the *modified* Gompertz to study the growth. The study herein suggests that growth parameters can be obtained from TTD data faster, cheaper and more accurately compared with the so far used plate count method;

- The aforementioned in combination with the models developed herein can offer new possibilities both to the research and food industry.

10 Future Work

Based on the findings of the present study further work could include:

- The use of the methodology and models developed from the mild temperature shifts studies in order to examine more extreme temperature shifts (e.g. shifts between refrigeration temperatures and ambient temperatures) which can be considered as a more accurate simulation of the temperature shifts that foods could undergo after purchased from the consumers;
- The use of the methodology and the models developed to fit and to simulate the data obtained from the mild thermal injury studies in order to examine the effect of a definitive log reduction, e.g. a 3-log reduction, which is one of the most common processing factors used by the food industry to control microbial growth as well as the examination of the effect of refrigeration temperatures in combination with a (mild) thermal injury;
- The use of flow cytometry in order to have a better understanding of the thermal injury applied by quantifing the number of healthy and dead cells;
- The use of other methodologies such as the flow cytometry and/or molecular methods in conjunction with the Bioscreen technique would supplement this rapid and cheap method with physiological and molecular information which would result in a more integrated methodology for the effective application of predictive microbiology;
- The better understanding and the wider knowledge obtained from the studies accomplished herein regarding the TTD method, can be used as the basis for applying the same methodologies in real food such as milk or other food products which can be used using the Bioscreen microbiological analyser.

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APPENDICES

Appendix A Bioscreen microbiological analyser

The Bioscreen C Reader System (Figure A-1) is a fully automated instrument developed to perform a wide range of microbiology experiments. The system consists of:

- Bioscreen C reader which includes an incubator and a measurement unit
- Computer
- EZE experiment software
- Honeycomb plates

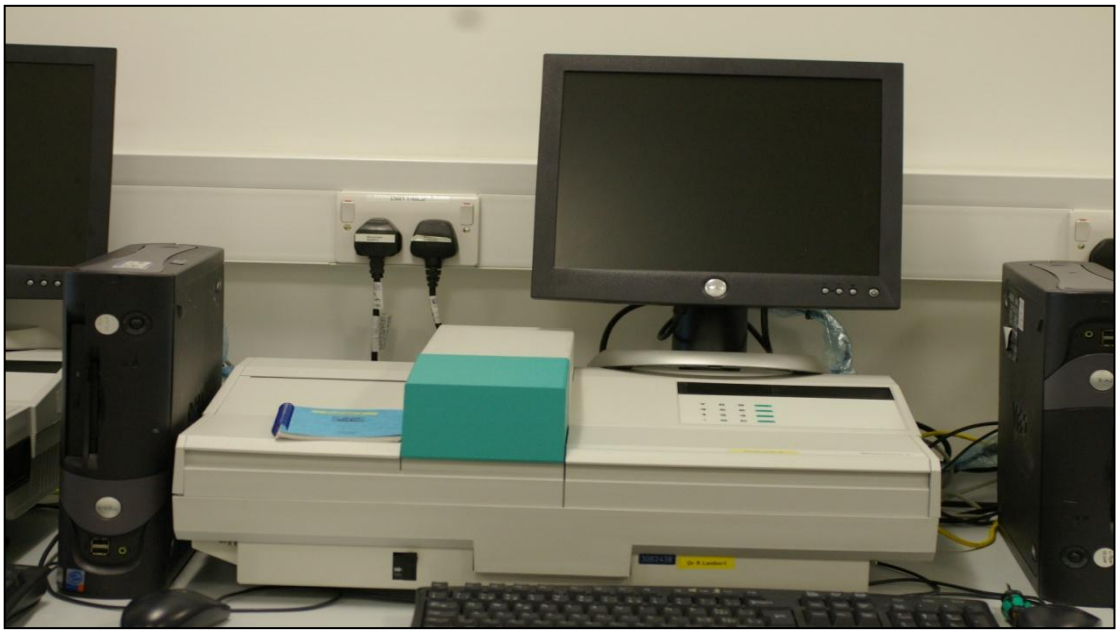

Figure A-1 The Bioscreen C reader system

The incubation temperature can be set from 1 to 60°C in steps of 0.1°C. The maximum temperature of 60°C is reached when the lid is 60°C and the cassette holding the samples has reached 59°C. Bioscreen can reach 6°C below and 30°C above ambient temperature. If lower temperatures are desired, the whole Bioscreen must be placed in a cooled room. The measurement is done kinetically using the principle of vertical photometry. In

this technique a light beam passes up through the bottom of the plate well, through the sample suspension to the detector. All functions are controlled by computer software according to the parameters entered by the user. Each honeycomb plate consists of 100 wells and the instrument can handle two plates at time, so the maximum capacity is 200 samples per run. Each well of the plate is an individual test vessel, so 200 microbiological growth experiments can be performed in a single run. The plate was designed to give the most even temperature possible across the whole plate, as well as to eliminate evaporation and condensation, common problems with the conventional 96-well plates. Bioscreen C monitors the growth of microorganisms by measuring the turbidity of liquid growth medium in the well. These measurements are done kinetically and recorded as optical density (O.D) measurements. These values are recorded by the controlling PC. Bioscreen C can measure growth of any organism that will cause turbidity in its growth medium such as bacteria, yeasts and fungi.

The reader includes three interrelated systems:

- Mechanical transport
- Incubator and
- An optical system

These three systems work in a coordinated way to provide automated heating or cooling sample indexing and O.D readings.

Mechanical transport

The incubator tray assembly holds the honeycomb plates in the correct position. The assembly shuttles left from the plate loading section into the measurement compartment, where light is passed through each well of the plate and the detector makes the O.D readings.

Incubator

The incubator consists of the incubator tray itself and the incubator tray cover. The incubator has a liquid circulation heat exchanger which gives constant temperature to all wells and at the selected value. This is essential for

developing high quality “growth curves”. It should be noted that the curves obtained from the Bioscreen is a curve which relates the O.D in relation with the time (O.D/Time curves) at a particular wavelength. This is not a growth curve i.e. a curve which relates the number of microorganisms in relation with the time. The latter means that what we can see from that curve as an apparent lag phase, is in fact the time taken for a culture, with an initial inoculum below the detection threshold, to reach the detection capability of the Bioscreen. Further, the apparent linear phase of the O.D curve is not exponential growth but it is due to the linear increase in microbial numbers.

Optical system

A halogen lamp produces light which then passes through the chopper wheel. The light path is turned 90 degrees by a mirror. The light then passes through the filter wheel. The correct filter is chosen by the user, by making the appropriate entry during the experiment’s set up. After that, the filtered light moves through an optical fiber to the lens assembly in the measurement compartment, below the honeycomb plate(s). The light passes through the bottom of each well and the results are collected from the detector. The detector is on a retractable arm which moves into the right places above the plate. The optical system is shown in Figure A-2.

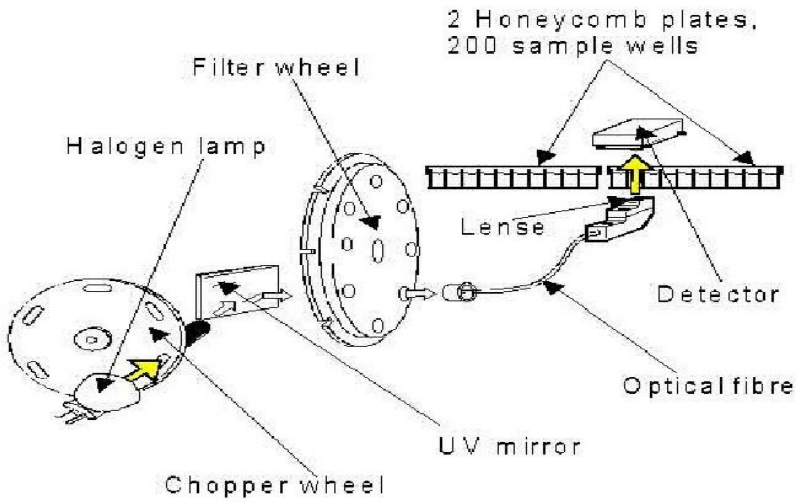

Figure A-2 The optical system of the Bioscreen C

Some of the applications of the Bioscreen C in microbiology are mentioned below:

- General research (Skytta *et al.*, 1993);
- Food microbiology research (Stephens *et al.*, 1997; Francois *et al.*, 2005; Bidlas and Lambert, 2008; Lambert and Bidlas, 2007a, 2007b, 2007c; Carlos *et al.*, 2009);
- Dairy applications (Mattila and Alivehmas, 1987);
- Food QC for measuring total counts (Mattila, 1987);
- Veterinary microbiology (Mattila *et al.*, 1988);
- Estimating the effects of chemicals on microorganisms (Adams and Hall, 1988).

Appendix B API 20E test

API 20E is a standardised and rapid identification system for Enterobacteriaceae and other Gram negative bacteria. Moreover, the API 20E strip consists of 21 miniaturised biochemical tests (Table B-1). The microtubes in the strip contain dehydrated substrates. The principle on which these tests are based is that after inoculation of the tests with the bacterial suspension, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. A single colony from an isolation plate was removed and resuspended in a tube with 5ml sterile distilled water. The incubation box (tray and lid) was prepared by distributing 3-5ml of distilled water into the wells of the tray to create a humid atmosphere. The test microtubes on the strip consist of the tube and the cupule. With a Pasteur pipette the bacterial suspension is distributed into the tubes of the strip. The tip of the pipette should be placed on the side of the cupule in order to avoid the formation of bubbles. For the tests CIT, VP and GEL the tube and the cupule was filled while for the tests ADH, LDC, ODC, H_2S and URE anaerobiosis was created by overlaying with mineral oil. Then the incubation box must be closed with the lid and then be incubated at 37^{o}C for 24 hours. After 24h of incubation at 37^{o}C three tests required the addition of reagents. TDA test required the addition of one drop of TDA reagent, IND test required the addition of one drop of JAMES reagent and VP test required the addition of one drop of VP 1 and one drop of VP 2 reagent. The metabolism produces changes in the colour of the tests which can be characterised as positive or negative using the reading table. According to the reactions (number of positive and negative tests) the identification obtained with the numerical profile using the database (V4.1) with the api*web*^{TM} identification software.

Table B-1 Reading table for the API 20E

Tests	Active ingredients	Reactions/Enzymes
ONPG	2-nitrophenyl-βD-galactopyranoside	β-galactosidase
ADH	L-arginine	Arginine dihydrolase
LDC	L-lysine	Lysine Decarboxylase
ODC	L-ornithine	Ornithine Decarboxylase
CIT	Trisodium citrate	Citrate utilisation
H2S	Sodium triosulfate	H2S production
URE	Urea	Urease
TDA	L-tryptophane	Tryptophane Deaminase
IND	L-tryptophane	Indole production
VP	Sodium pyruvate	Acetoin production
GEL	Gelatin (bovine origin)	Gelatinase
GLU	D-glucose	Fermentation/Oxidation Glucose
MAN	D-mannitol	Fermentation/Oxidation Mannitol
INO	Inositol	Fermentation/Oxidation Inositol
SOR	D-sorbitol	Fermentation/Oxidation Sorbitol
RHA	L-rhamnose	Fermentation/Oxidation Phamnose
SAC	D-sucrose	Fermentation/Oxidation Saccharose
MEL	D-melibiose	Fermentation/Oxidation Melibiose
AMY	Amygdalin	Fermentation/Oxidation Amygdalin
ARA	L-arabinose	Fermentation/Oxidation Arabinose

Appendix C Mathematical modelling of growth data

C.1 Non-linear and linear regression

Non-linear regression is used to fit data to a model which defines y as a function of x. y must be a continuous variable. If y is a binomial outcome then logistic regression should be used instead. Linear regression can be characterized as a special case of non linear regression. A linear function is described by:

$$y = ax + b \quad \textbf{(C-1)}$$

Where a is the slope and b is the intercept.

If the slope is positive y increases as x increases while if slope is negative y decreases as x increases.

Regression is done by minimizing the sum of squares (SS) of the vertical distances of the data from the line or the curve. The SS is the sum of the squares of the vertical distances of the points from the curve. Non linear regression minimizes the sum of the square of the vertical distances of the data points from the curve. On the other hand, linear regression is modelling data in a straight line. When modelling data it is not only necessary to obtain the parameters of the model which give the best fit but also an indication of how good a fit the model gives. Linear regression uses the idea that we minimise the sum of squares of the differences between the modelled and the observed data, by changing values of the intercept and gradient until the minimum values are reached. We then state that this minimised sum of squares is a measure of the inherent variability of the data, i.e. the 'stochastic' part of the data that we cannot model directly. The difference between linear and nonlinear regression is that non linear regression is an iterative or cyclical process while linear regression needs only one single calculation to get the lowest SS required (Brown, 2001).

The aim of regression is to find the best fit values for the parameters of the model. The assumptions where non linear regression is based, are that x values are known, all the error is in y values, the variability of y values follow the Gaussian distribution, the standard deviation of the residuals is the same all the way along the curve and that the observations are independent. The assumptions where linear regression is based are that all x values are known, and that the entire scatter is in the assessment of the dependent y values.

C.2 Weighted regression

One of the assumptions of linear and non linear regression is that the standard deviation of the error is constant over all values. In cases where the standard deviation (SD) of the error is not consistent, minimizing the SS would be inappropriate. Data points with large deviations from the curve would have a large impact on the SS value whilst data points with small deviations from the curve would have little impact. This is undesirable as we want all the data points to have the same influence to the goodness of fit. In order to accomplish this weighted least squares can give each data point the proper amount of influence over the parameter estimates. One method to do this is to use the reciprocal of the variance found for replicates as the weighting regime.

C.3 Logistic regression

If an experiment gives two possible outcomes – e.g. live or dead, like growth/no growth experiments, then the data is considered to be either categorical or nominal if you can assign a number to it. Such data are dichotomous - they have two possible values or outcomes and are also known as binary variables. In a particular situation the values may be dependent on a multiple set of explanatory variables.

The Odds of some event occurring is the probability of an event occurring relative to the probability that it will not.

$$Odds\ (E) = \frac{P(E)}{1 - P(E)} \qquad \textbf{(C-2)}$$

The probability of success can have values between 0 and 1 only. A linear model of the variables can take any value. We need to link the two systems by a transformation of the model to the probability. This is called the link function, and often the logistic function can be used to achieve this.

When probabilities are plotted against a variable a sigmoid curve is obtained. There are lots of functions which can reproduce a sigmoid shape, however, one of the most common is

$$P(x) = \frac{1}{1 + \exp\left(-\beta_0 - \beta_1 x\right)} \qquad \textbf{(C-3)}$$

The parameters β_0 and β_1 determine the slope and the spread of the curve. The function is symmetric about the point $x=-\beta_0/\beta_1$, and at this point P(x) = 0.5. If logs are taken, then we obtain:

$$P(x)(1 + \exp(-\beta_0 - \beta_1 x)) = 1$$

$$\exp(-\beta_0 - \beta_1 x) = \frac{1 - P(x)}{P(x)}$$

$$\exp(\beta_0 + \beta_1 x) = \frac{P(x)}{1 - P(x)}$$

$$\beta_0 + \beta_1 x = ln\left(\frac{P(x)}{1 - P(x)}\right) \qquad \textbf{(C-4)}$$

The expression on the RHS is known as the logit. The expression is now linear in the x-variable. The ratio P(x)/(1-P(x)) is the ratio between the probability of success and the probability of failure. Hence the RHS is the log of the odds of success. The log odds ratio is the difference between the probability of success when x = x=1 and when x=x; and this is simply β_1. The odds ratio is therefore $e^{\beta 1}$, also known as the odds multiplier.

Regression modelling

The relationship between a dichotomous variable Y and n explanatory variables $x_1,x_2,\ldots x_n$ is described by the logistic regression model. Consider a series of observations, let p_i be the mean probability of the observations, then

$$p_i = \frac{1}{1 + \exp\left(-\beta_0 - \sum_{j=1}^{k} \beta_j \, x_{i,j}\right)} \quad \textbf{(C-5)}$$

Applying the logit transformation gives the logit form of the model

$$logit(p_i) = \beta_0 + \sum_{j=1}^{k} \beta_j \, x_{i,j} \quad \textbf{(C-6)}$$

In general, when the explanatory variables are quantitative, each of the regression parameters $x_1,x_2, \ldots, x_k$ can be interpreted as log odds ratios for the corresponding explanatory variable, when all other explanatory variables are held fixed. That is, the odds multiplier for x_i is equal to $e^{\beta i}$. When the explanatory variable x_i is increased by 1 unit, and all other explanatory variables are held constant, the odds of success is increased by a factor $e^{\beta i}$. (Note that, if β_i is negative, then $e^{\beta i} < 1$, so the odd of success is actually reduced by that factor.

Significance of explanatory variables is tested in a different way to normal regression because the response variables are Bernoulli distributed as they are binary. In logistic regression the parameters are obtained by using maximum likelihood estimation.

C.4 Distributions

C.4.1 Normal distribution

The normal distribution which is also known as the Gaussian distribution is a continuous probability distribution that has a bell shaped probability density function. The distribution is given by

$$f_{(x;\mu,\sigma^2)} = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{1}{2}\left(\frac{x-\mu}{\sigma}\right)^2} \quad \textbf{(C-7)}$$

Where μ is the mean (continuous location parameter) and σ (continuous scale parameter) is the standard deviation and σ^2 is the variance, with domain $-\infty \leq x \leq +\infty$. When μ=0 and σ=1, the distribution is called standard normal distribution.

C.4.2 Log-Normal distribution

The log normal distribution is a continuous probability distribution of a variable whose logarithm is normally distributed. The distribution is given by:

$$f_{(x;\mu,\sigma)} = \frac{1}{x\sigma\sqrt{2\pi}} e^{-\frac{1}{2}\left(\frac{\ln x-\mu}{\sigma}\right)^2} \quad \textbf{(C-8)}$$

Where μ which is the mean and σ which is the standard deviation, are continuous parameters (μ>0 and σ>0) with domain $0 \leq x \leq +\infty$.

C.4.3 Weibull distribution

The Weibull distribution is given by:

$$f_{(x;\mathrm{a},\sigma)} = \frac{\mathrm{a}}{\sigma}\left(\frac{x}{\sigma}\right)^{\mathrm{a}-1} e^{-\left(\frac{x}{\sigma}\right)^{\mathrm{a}}} \quad \textbf{(C-9)}$$

Where the variable x and the parameters a (shape parameter) and σ (scale parameter) are all positive real numbers. The parameter σ is a scale parameter and the variable $y = \frac{x}{\sigma}$ has the distribution given by:

$$g_y = \mathrm{a}y^{\mathrm{a}-1}e^{-y^{\mathrm{a}}} \quad \textbf{(C-10)}$$

C.4.4 Gamma distribution

The Gamma distribution is given by:

$$f_{(x;\mathrm{a},b)} = \mathrm{a}(\mathrm{a}x)^{b-1}e^{-\mathrm{a}x}/\Gamma_b \quad \textbf{(C-11)}$$

Where the variable x and the parameters a (shape parameter) and b (scale parameter) are all positive real quantities and Γ_b is the Gamma distribution.

C.4.5 Comparison between distributions

In statistics a widely accepted test for evaluating the goodness of fit of models is the likelihood ratio test. In particular the loglikelihood test compares two models, a null model and an alternative, of which the first one is a special case of the other one. The test is based on the likelihood ratio which represents how many times more likely the data are under one model than the other. Most of the times the logarithm of the likelihood test is used (loglikelihood) to calculate the p-value. The model ($\delta=2\log\Lambda$) is given by:

$$\Lambda = \frac{\max\left(L_0(null\ model/data)\right)}{\max\left(L_1\ (alternative\ model/\ data)\right)} \quad \textbf{(C-12)}$$

Where L_0 is the likelihood under the null hypothesis and L_1 is the likelihood under the alternative hypothesis.

Another way of comparing models is the Akaike Information Criterion (AIC). The AIC is given by:

$$AIC = -2\ln L + 2n \quad \textbf{(C-13)}$$

Where L is the maximum value of the likelihood function for a model and n is the number of the parameters of the model. Small AIC values indicate better models. Also, the AIC does not test a null hypothesis but it is a good tool-measure of how good a fit of a model is but imposes a penalty of unnecessary parameters.

Appendix D Additional information for the studies accomplished

Table D-1 Relationship between optical density in the Bioscreen (200µl volume) and in the spectrophotometer (1cm pathway) with microbial numbers of *L. monocytogenes* 252, *L. monocytogenes* 39, *S.* Typhimurium and *E. coli*.

Microorganism	O.D (Bioscreen, 200µl, 600nm)	O.D (Spectrophotometer, 1ml, 600nm)	Log (cfu/ml)	N_D value for an O.D=0.2
***L. monocytogenes* 252**	0.373	1.01	9.27	N_D=8.99
	0.373	1.01	9.24	
	0.325	0.735	9.12	
	0.325	0.735	9.03	
	0.253	0.56	9.08	
	0.253	0.56	9.02	
	0.194	0.435	8.87	
	0.194	0.435	9.00	
	0.152	0.34	8.84	
	0.152	0.34	8.87	
	0.125	0.265	8.61	
	0.125	0.265	8.67	
	0.067	0.169	8.57	
	0.067	0.169	8.44	
***L. monocytogenes* 39**	0.382	0.987	9.53	N_D=9.17
	0.382	0.987	9.5	
	0.306	0.768	9.44	
	0.306	0.768	9.32	
	0.237	0.597	9.25	
	0.237	0.597	9.28	
	0.186	0.465	9.08	
	0.186	0.465	9.2	
	0.15	0.36	9.02	
	0.15	0.36	9.04	
	0.133	0.282	8.77	
	0.133	0.282	8.79	
	0.076	0.17	8.62	
	0.076	0.17	8.64	
***S.* Typhimurium**	0.424	1.021	9.27	N_D=8.38
	0.424	1.021	9.28	
	0.379	0.811	9.08	
	0.379	0.811	9.11	
	0.314	0.64	8.93	

	0.314	0.64	8.83	
	0.263	0.507	8.5	
	0.263	0.507	8.65	
	0.227	0.41	8.8	
	0.227	0.41	8.5	
	0.192	0.326	8.38	
	0.192	0.326	8.35	
	0.125	0.225	8.02	
	0.125	0.225	8.00	
E. coli	0.530	1.044	9.2	N_D=8.30
	0.530	1.044	9.16	
	0.470	0.842	9.13	
	0.470	0.842	9.1	
	0.407	0.739	9.06	
	0.407	0.739	9.07	
	0.335	0.575	8.84	
	0.335	0.575	8.83	
	0.292	0.425	8.69	
	0.292	0.425	8.71	
	0.260	0.357	8.31	
	0.260	0.357	8.23	
	0.231	0.264	7.64	
	0.231	0.264	7.75	

Table D-2 Plate counts from a particular well (well 199) from the control plate and from the plate after the heat treatment (well 299) at 60ºC for 25min in the oven

Organism	NaCl	pH	LogN (Control plate)	LogN (Heat treated plate)
	0.5	7.2	9.34	9.24
	3	7.2	9.06	8.91
	6	7.2	8.74	8.36
	0.5	6.50	9.11	8.93
	0.5	5.88	9.19	9.07
E. coli	0.5	5.16	9.0	8.93
N_D 8.69	0.5	4.58	9.83	9.48
	3	6.45	8.9	8.61
	6	6.42	7.86	7.56
	3	5.84	8.9	8.6
	6	5.81	7.54	7.24
	3	5.16	8.60	8.20
	0.5	7.2	9.38	9.20
	3	7.2	9.18	8.90
	6	7.2	8.58	8.33
	0.5	6.50	9.53	9.16
	0.5	5.88	9.64	9.24
	0.5	5.16	9.74	9.53
***S.* Typhimurium**	0.5	4.58	9.67	9.34
N_D 8.78	3	6.45	9.05	8.85
	6	6.42	8.64	8.3
	3	5.84	9.16	8.97
	6	5.81	8.56	8.45
	3	5.16	9.02	8.88
	6	5.15	8.20	8.10
	3	4.61	8.87	8.84
	6	4.60	8.01	7.7
***L. monocytogenes* 252**	0.5	7.2	9.65	9.61
N_D 9.15	3	7.2	9.48	9.40
	6	7.2	9.26	9.34
***L. monocytogenes* 39**	0.5	7.2	9.5	9.3
N_D 9.16	3	7.2	9.58	9.41
	6	7.2	9.45	9.36
***L. monocytogenes* 271**	0.5	7.2	9.63	9.54
N_D 9.31	3	7.2	9.49	9.14
	6	7.2	9.24	9.20
***L. monocytogenes* 177**	0.5	7.2	9.60	9.62
N_D 9.22	3	7.2	9.62	9.56
	6	7.2	9.28	9.30

Appendix E Activities and publications

E.1 Peer reviewed publications

- I. Mytilinaios, M. Salih, H.K. Schofield and R.J.W. Lambert (2012). Growth curve prediction from Optical Density Data. International Journal of Food Microbiology 154, 169-176.

International Journal of Food Microbiology 154 (2012) 169–176

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Growth curve prediction from optical density data

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ABSTRACT

A fundamental aspect of predictive microbiology is the shape of the microbial growth curve and many models are used to fit microbial count data, the modified Gompertz and Baranyi equation being two of the most widely used. Rapid, automated methods such as turbidimetry have been widely used to obtain growth parameters, but do not directly give the microbial growth curve. Optical density (OD) data can be used to obtain the specific growth rate and if used in conjunction with the known initial inocula, the maximum population data and knowledge of the microbial number at a predefined OD at a known time then all the information required for the reconstruction of a standard growth curve can be obtained.

Using multiple initial inocula the times to detection (TTD) at a given standard OD were obtained from which the specific growth rate was calculated. The modified logistic, modified Gompertz, 3-phase linear, Baranyi and the classical logistic model (with or without lag) were fitted to the TTD data. In all cases the modified logistic and modified Gompertz failed to reproduce the observed linear plots of the log initial inocula against TTD using the known parameters (initial inoculum, MPD and growth rate). The 3 phase linear model (3PLM), Baranyi and classical logistic models fitted the observed data and were able to reproduce elements of the OD incubation-time curves. Using a calibration curve relating OD and microbial numbers, the Baranyi equation was able to reproduce OD data obtained for *Listeria monocytogenes* at 37 and 30 °C as well as data on the effect of pH (range 7.05 to 3.46) at 30 °C.

The Baranyi model was found to be the most capable primary model of those examined (in the absence of lag it defaults to the classic logistic model). The results suggested that the *modified* logistic and the *modified* Gompertz models should not be used as Primary models for TTD data as they cannot reproduce the observed data.

- M. Salih, I. Mytilinaios, H. K. Schofield and R.J.W Lambert (2012). Modelling of Bacterial Growth with Shifts in Temperature Using Automated Methods with *Listeria monocytogenes* and *Pseudomonas aeruginosa* as examples. International Journal of Food Microbiology 155, 29-35.

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Modelling of bacterial growth with shifts in temperature using automated methods with *Listeria monocytogenes* and *Pseudomonas aeruginosa* as examples

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ABSTRACT

Time to detection (TTD) measurements using turbidometry allow a straightforward method for the measurement of bacterial growth rates under isothermal conditions. Growth rate measurements were carried out for *Listeria monocytogenes* at 25, 30 and 37 °C and for *Pseudomonas aeruginosa* over the temperature range 25 to 45 °C. The classical three-parameter logistic model was rearranged to provide the theoretical foundation for the observed TTD. A model was subsequently developed for the analysis of TTD data from non-isothermal studies based on the Malthusian approximation of the logistic model. The model was able to predict the TTD for cultures of *L. monocytogenes* or *P. aeruginosa* undergoing simple temperature shunts (e.g. 25 to 37 °C and vice versa), and for a multiple temperature shunt for *L. monocytogenes* (25–37–25–37 °C and 37–25–37–25 °C) over a period of 24 h. In no case did a temperature shunt induce a lag.

E.2 Oral presentations

- 7th International conference (2011). Predictive Modelling of Food quality and Safety. Growth of *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* in the presence of sodium chloride following a mild thermal process (Ioannis K. Mytilinaios, Ronald J.W. Lambert).
- FSA workshop (2011). Modelling the impact of mild food processing conditions on the microbiological safety of food (Ioannis K. Mytilinaios, Ronald J.W. Lambert).

- Cranfield Health postgraduate conference (2012). Modelling the impact of mild food processing conditions on the microbiological safety of food (Ioannis K. Mytilinaios, Ronald J.W. Lambert).

E.3 Poster presentations

- Unilever symposium (2011). Growth of *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* in the presence of sodium chloride following a mild thermal process (Ioannis K. Mytilinaios, Ronald J.W. Lambert).
- Cranfield workshop (2011). Growth of *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* in the presence of sodium chloride following a mild thermal process (Ioannis K. Mytilinaios, Ronald J.W. Lambert).