Non-Thermal Inactivation of *Escherichia coli* under Conditions Relevant to the Production of Uncooked Comminuted Fermented Meats

By

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Tasmania

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text of this thesis.

C.T. Shadbolt

April 2004

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So much has happened since starting out on the road to the point where I now feel in a position to write this. Arguably the biggest transition was the switch from a full-time doctoral student to a position in the rat race that is Australian public service. Obviously this presented a challenge, considering that my studies were not complete. With the benefit of hindsight, I do not regret leaving my studies at a vulnerable stage. Employment as a Scientific Advisor on all matters relating to food safety at the national level has, and continues to be, a rewarding experience.

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ABSTRACT

Thermal inactivation kinetics of *Escherichia coli* is an area which has received much attention and is relatively well understood. By comparison, inactivation kinetics and mechanisms of cell death are not well described or understood for nonthermal conditions. Since the occurrence of two significant outbreaks associated with uncooked fermented comminuted meats (UCFM) in 1994 and 1995, considerable research effort has been expended to enhance knowledge of acid-tolerant *E. coli* in response to conditions encountered during production of UCFM. The well publicised illness and loss of life resulting from consumption of contaminated mettwurst forced a rethink on the safety of these products and had a dramatic effect on the entire Australian meat industry.

Results of this study, combined with published literature, contradict previously established concepts (the vitalistic and mechanistic[1]) that attempt to describe deviations from exponential inactivation kinetics. Minor variations in the age of inocula harvested at similar cell densities resulted in considerable differences in inactivation and survival. A resistant subpopulation was successfully removed by dilution, proving that all cells do not have a general similarity of resistance. Whilst these observations support the vitalistic concept, it was shown that this was not the main determinant of inactivation.

[1] The vitalistic concept (or hypothesis) states that inactivation may be explained on the basis of differing degrees of resistance within a population to an inimical condition.

The mechanistic concept states that all cells have a general similarity of resistance and population decline is akin to a chemical reaction. Deviations from this reaction are bound to the mechanism of inactivation and not natural variability within a population.

Cells exposed to lethal pH display different inactivation patterns to those exposed to lethal water activity. The latter typically experience biphasic inactivation consisting of a rapid first phase of death followed by a slower more persistent second phase of "tailing". In comparison, cells exposed to lethal pH display triphasic inactivation, with an additional rapid third phase of death. Viable counts on selective media showed that cells exposed to lethal water activity experienced a considerably higher level of injury than those exposed to lethal pH. The latter displayed a consistent, lower level of injury on selective media, but died more rapidly. It was hypothesised that lethal pH placed a considerable energy burden on the cell, which resulted in the rapid third phase of inactivation. Comparison of uptake of a radiolabelled substance indicated that lethal water activity did not place a large energy burden on the cell.

The above knowledge was utilised to determine the effect of variations in the order and timing of lethal stress imposition. Lethal acid stress was found to sensitise cells of *E. coli* to subsequent exposure to lethal water activity. Cells were more sensitive to subsequent stress the longer they were first exposed to low pH. This finding is relevant to food processing where hurdle technology is employed and may lead to novel strategies for the safe manufacture of minimally processed foods.

The requirement for detailed, systematic data on nonthermal inactivation during UCFM processing led to the investigation of the feasibility of modelling death in a broth system. Such a system would allow for reproducible data to be obtained without the need for a complex and expensive meat matrix and smokehouse with appropriate temperature and

relative humidity controls. An anaerobic environment using cooked meat medium was created that was able to mimic the effects of fermentation during manufacture of UCFM. Data from this system were used to refine a predictive model that has influenced Australian food standards legislation and assisted manufacturers to gain a better understanding of their processing effectiveness to reduce pathogens. This demonstrated the value of strategic research on microbial physiology and ecology.

Detailed research using viable count methods is labour intensive and time consuming. The use of flow cytometry as a rapid method for determining the viability of *E. coli* after exposure to lethal pH was investigated. This technique proved to be reliable and rapid over the first 2-Log_{10} reduction with high cell numbers. However, beyond this level of inactivation, the sensitivity of the instrument rapidly decreased and flow cytometry ceased to be an effective method. It is concluded that modern methods and equipment still require considerable effort and design before they become as effective as traditional viable count techniques.

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LIST OF ABBREVIATIONS

%T	per cent transmittance
a_w	water activity
cfu	colony-forming unit(s)
CMM	Cooked Meat Medium
$DiBAC_4(3)$	Bis-(1,3-dibutylbarbituric acid) trimethine oxonol; fluorescent stain
EDTA	ethylenediaminetetraacetic acid
FCM	flow cytometry
HCl	Hydrochloric acid
h	hour(s)
LBB	Luria-Bertani Broth
MilliQ PBS	phosphate buffered saline in MilliQ water
MMM	Minimal Minerals Medium
NA	Nutrient Agar
OD	Optical Density
PI	propidium iodide; fluorescent stain
PW	Peptone water
S.	Section
SLS	Second lethal stress
TGI	Temperature Gradient Incubator
T_0	Time zero (initial experimental time point)
TPABS	Tryptose Phosphate Agar with 0.15% bile salts
TPAP	Tryptose Phosphate Agar with 0.1% pyruvate
TSB	Tryptone Soya Broth

1 MICROBIAL INACTIVATION KINETICS

1.1 INTRODUCTION

Recent years have seen an increase in foodborne disease attributed to stress-tolerant pathogenic bacteria. Pathogens including *Escherichia coli* O157:H7, have been responsible for numerous outbreaks of disease involving contaminated food affecting large numbers of people. The more virulent *E. coli* strains are characterised by their acid tolerance, which enables them to survive food processing and passage through the human gastric acidity barrier to reach the intestine where only a few cells (10's-100's) may be sufficient to cause disease in susceptible populations (Doyle et al., 1997).

The low infectious dose is significant for several reasons. As consumers have become more knowledgeable of health issues relating to (for example) chemical preservatives and their use, there has been a trend towards increased consumption of minimally–processed foods. Whilst the potential risk of long term illnesses, such as cancer or heart disease, may be lowered through consumption of less salt or preservatives there is a greater danger that stress-tolerant pathogens will survive milder food processing conditions and cause infection.

Modern medicine has delivered benefits including extension of the human lifespan. It is estimated that by the year 2051, 25% of the Australian population will be over the age of 65, compared with 12% currently (ACIL, 2000). This will pose a risk management problem

for regulatory authorities when considering standards and practices associated with food safety, as the elderly are among the most susceptible to infection. Many in this age bracket, and those institutions catering for them, will be aware of medical conditions relating to heart disease and high blood pressure from highly processed and preserved foods but may overlook the dangers from ingestion of pathogenic bacteria.

Also of concern is the increasing demand for ready-to-eat meals, arising in part from increased time spent in the workplace and a desire to spend less time on food preparation. This has encouraged the mass-production of pre-packaged foods which are distributed to consumers through large retail chains as opposed to the traditional local corner shop. This centralisation of the food supply can result in a large number of consumers being affected in the event of food contamination, as was the case in salmonellae-contaminated peanut butter (Ng et al., 1996) and orange juice (CDI, 1999). Increased global trade in food has also brought added food safety concerns. While people are able to enjoy a variety of exotic foods, a breakdown in quality assurance from the point of origin or during transport may lead to disease outbreaks occurring in other countries. Examples of this include outbreaks of foodborne disease in Australia and Denmark after contaminated sweets were imported from Turkey (OzFoodNet, 2001a), and consumption of contaminated peanuts from China resulting in salmonellosis both in Australia and the UK (OzFoodNet, 2001b). The current burden of foodborne disease in Australia is estimated at 5.4 million cases of illness per annum with an associated cost of $3.75 billion arising from medical costs and lost productivity (OzFoodNet, 2003, not yet published). Food safety risk factors, including newly emerging pathogens, trends towards minimal processing, an ageing population, and

increased global trade, add to the potential for the current level of foodborne illness to become elevated unless science-based control measures are in place.

Regulatory authorities are now forced to consider such risks on a routine basis, including in food products which were previously thought to be safe due to their methods of manufacture or formulation. Severe outbreaks of disease associated with uncooked comminuted fermented meats (UCFM) in the USA (MMWR, 1995) and South Australia (Cameron et al., 1995) provide examples of the necessity to reconsider the safety of some foods. In both cases acid-tolerant strains of verotoxigenic *E. coli* (VTEC) were found to be the causative agents. Prior to these outbreaks, there was a lack of systematic data regarding the inactivation kinetics of these organisms under conditions relevant to UCFM production. McMeekin et al. (1997) recognised this need. Given the very low infectious dose of VTEC species such as *E. coli* O157:H7 (Doyle et al., 1997), detailed inactivation data for conditions relevant to UCFM processing are essential for the determination of safe manufacturing guidelines and the design of novel strategies for control of these pathogens.

This study seeks to address the lack of knowledge concerning the "nonthermal death" of *E. coli*. Nonthermal death may be defined as inactivation at a temperature which is not, of itself, lethal to the bacterium. Mechanisms of inactivation and variations in cell physiology of *E. coli* under conditions relevant to UCFM production will be examined, and novel processing strategies proposed.

1.2 Escherichia coli as a pathogen and the importance of inactivation kinetics

1.2.1 *Escherichia coli* and pathogenic species

The gram-negative bacterium *Escherichia coli*, a member of the *Enterobacteriaceae* family, predominantly inhabits the gastrointestinal tract of warm-blooded animals (Pelczar et al., 1993). Initially thought to be a harmless commensal organism, four types of pathogenic *E. coli* are now recognised (Doyle and Evans, 2001). As a popular subject for biochemical research (Neidhardt, 1996) and an important pathogen, considerable effort has been expended to determine the growth boundaries of *E. coli*. These are summarised in Table 1.1.

Table 1.1. Biokinetic ranges of *Escherichia coli.*

Growth characteristic	Minimum	Maximum	Reference:
Water Activity (a_w)	0.95	0.999	Troller and Christian, 1978
Temperature (°C)	7.8	48	Shaw et al., 1971
pH	3.9	10	Presser et al., 1998
Undissociated Lactic Acid pH	8-10 mM		Presser et al., 1998

The pH limits in Table 1.1 have been shown to vary, especially between some strains of *E. coli* O157:H7.

Doyle and Evans (2001) categorise the four pathogenic types of *E. coli* as follows: *enteropathogenic* (which includes enterohaemorrhagic or EHEC, and verotoxigenic or VTEC); *enterotoxigenic*; *nontoxigenic*; and *toxigenic*. VTEC has received considerable attention as a foodborne pathogen (Doyle et al., 1997; Faith et al., 1997; Neill, 1997; Ryu et al., 1999).

1.2.2 VTEC

Verotoxigenic *E. coli* (also known as shiga-like toxin producing *E. coli* or STEC), such as O157:H7, have been identified as the causal agent in outbreaks involving contaminated food and water, animal contact, and person-person transmission (Parry and Palmer, 2000). Virulence factors include the *eae* gene, important in the production of attaching and effacing lesions in the intestine, haemolysin-encoding gene (*hly*) and production of SLT-I and/or SLT-II toxins from the *stx1* and *stx2* genes (Neill, 1997; Meng et al., 1998). These toxins directly affect the intestinal epithelium, resulting in diarrhoea (Pelczar, 1993). More serious illnesses, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) are also associated with VTEC (Buchanan and Doyle, 1997). Acute renal failure, anaemia and neurological damage are all characteristics of HUS. Of those affected by HUS, 15% develop chronic kidney failure and the associated mortality rate is 3-5% (Buchanan and Doyle, 1997).

Much attention is focused on O157 VTEC serogroups, which may lead to infections from non-O157 VTEC being overlooked (Bettelheim, 2000). The O157 serotype, to date, has not presented a serious problem in Australia but disease from non-O157 VTEC is more common (Bettelheim, 2000). Examples of the latter include *E. coli* O26 and O111 serogroups. Some pathogenic strains from these groups have been shown to display similar acid tolerance (Grau, 1996) and possess identical virulence factors as the O157 VTEC (Desmarchelier, 1997). This was evident in an outbreak associated with smallgoods in South Australia, 1994, which resulted in twenty-three cases of HUS in children under 16 years of age, thirty cases of bloody diarrhoea, and three adults with thrombocytopenic purpura. Seventy percent of patients required dialysis (Cameron et al., 1995). Tragically, this outbreak also claimed the life of a 4-year-old girl.

Of great concern was the fact that this and other outbreaks occurred in foods which were thought to be safe from microbial hazards due to their method of production and preservation (Buchanan and Doyle, 1997). The low infective dose and associated acid tolerance of VTEC has forced a review of current manufacturing techniques in foods such as uncooked, fermented salami (Nickelson et al., 1996; Faith et al. 1997; Ellajosyula et al. 1998; Vanderlinde, 1999; Duffy et al., 2000; Ross and Shadbolt, 2001). Tilden et al. (1996) estimated that as few as 50 organisms constituted an infective dose in an outbreak attributed to salami in Washington State, 1994. A requirement for detailed knowledge of inactivation kinetics, rather than growth, has become apparent (McMeekin et al., 1997).

While much of the current work focuses on VTEC many of the findings will be applicable to other pathogens that have emerged or may do so in future. Miller et al. (1998) state that "...the timely acquisition of critical research data can transform the hazard from the realm of the unknown to the known, and only the known can be controlled". *E. coli* O157:H7 is but one example of a pathogen that has emerged in the last 20 years. Each change affecting the food chain is hypothesised to create new selective pressures for the emergence of microbial adaptation and resistance to environmental insult (Miller et al., 1998). Developments in food manufacture and handling over the last decade have been rapid indeed, and examples of these are shown in Table 1.2.

Knowledge of bacterial response to challenges encountered in the environment and during food manufacture and subsequent storage may assist in the design of novel processing techniques to counter newly emerged pathogens. Such information is also likely to be of use in determining responses to future emergence of pathogens and the minimisation of human risk (Miller et al., 1998). This knowledge must take the form of physiological behaviour in response to a variety of stress conditions and include detailed data from the resulting inactivation kinetics of bacterial populations. Without such an understanding of how pathogens respond to environmental stimuli, particularly during the process of food production, it will be difficult to design effective measures for their control.

Table 1.2. Factors involved in emergence/re-emergence of pathogens (adapted from Miller et al., 1998).

Factor	Examples
Social	Economic impoverishment War Population demographics (growth or migration)
Technological	Changes in medical technology (devices, immunosuppressive drugs) Industrialised food production and processing (consolidation, preservation technologies) Globalisation of food supplies Genetically modified organisms
Health care and public health infrastructure	Increased organ and tissue transplantation Antibiotic use Increased use of nursing homes Inadequate infectious disease surveillance Insufficient numbers of trained public health personnel Inadequate treatment of potable water supplies and sewage
Demographics	Increase in number of immigrants Increase in number of elderly individuals Increase in number of immunocompromised individuals
Human behaviour	Changes in diet (new foods, fewer meals eaten at home) Shift of women from home to workplace Increased use of childcare (both single parent and dual income families) Increased international business and tourism
Microbial adaptation	Increased gain of virulence factors and/or toxin production Development of antimicrobial resistance Introduction of pathogens into new geographic areas Ability to adapt to new environmental conditions
Vectors	Introduction to new geographic areas (climate change, human migration) Change in land or water ecology

1.3 History of Bacterial Inactivation Kinetics Research

1.3.1 Types of inactivation curves

While the subject of nonthermal microbial inactivation kinetics is (through necessity) receiving more focus, it has been the subject of vigorous debate for over a century. Chick (1908), working with anthrax spores and mercuric chloride, attempted to explain the shape of inactivation curves and proposed a mathematical equation describing the process of observed disinfection. A review of the literature dating back to Chick (1908) provides hypotheses on the subject of microbial inactivation which will be discussed further in [1.3.2-1.3.8].

In reviewing past research, it is first necessary to describe the types of inactivation patterns observed. Examples are shown in Figure 1.1.

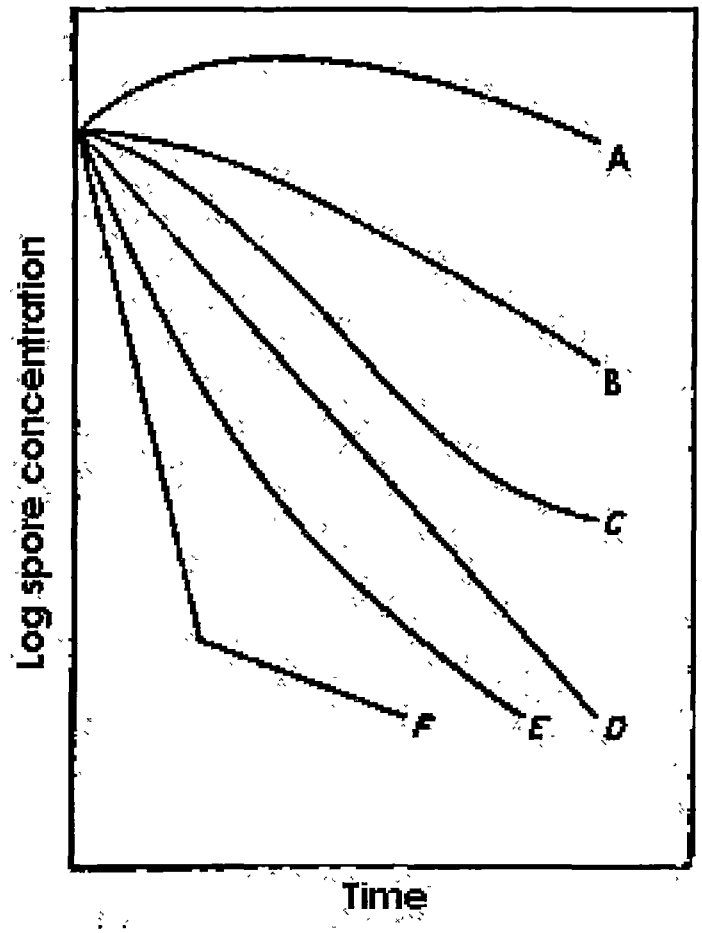

Figure 1.1 Examples of survival curves. A and B, 'shoulder'; C, 'sigmoid'; D, exponential curve; E, 'upward concavity'; F, 'biphasic curve' with a "tail". Reproduced from Cerf (1977).

Inactivation patterns may consist of an initial 'shoulder' (Curves A and B), where cell decline is very slow or negligible, before increasing rapidly. Under certain circumstances (exponential phase cells exposed to very low pH, for example) the inactivation curve may be exponential in nature (Curve D). Shadbolt et al., (1999) observed biphasic inactivation curves (Curve F) when stationary phase cells of *E. coli* were exposed to lethal water activity. Unpublished data from Brown, Mellefont and Shadbolt describe triphasic inactivation patterns, as shown in Figure 1.2. Inactivation patterns of the type shown in Figures 1.1 and 1.2 appear in published literature [1.3.3] and experimental sections ([2], [3.4], and [4.3]) of this dissertation.

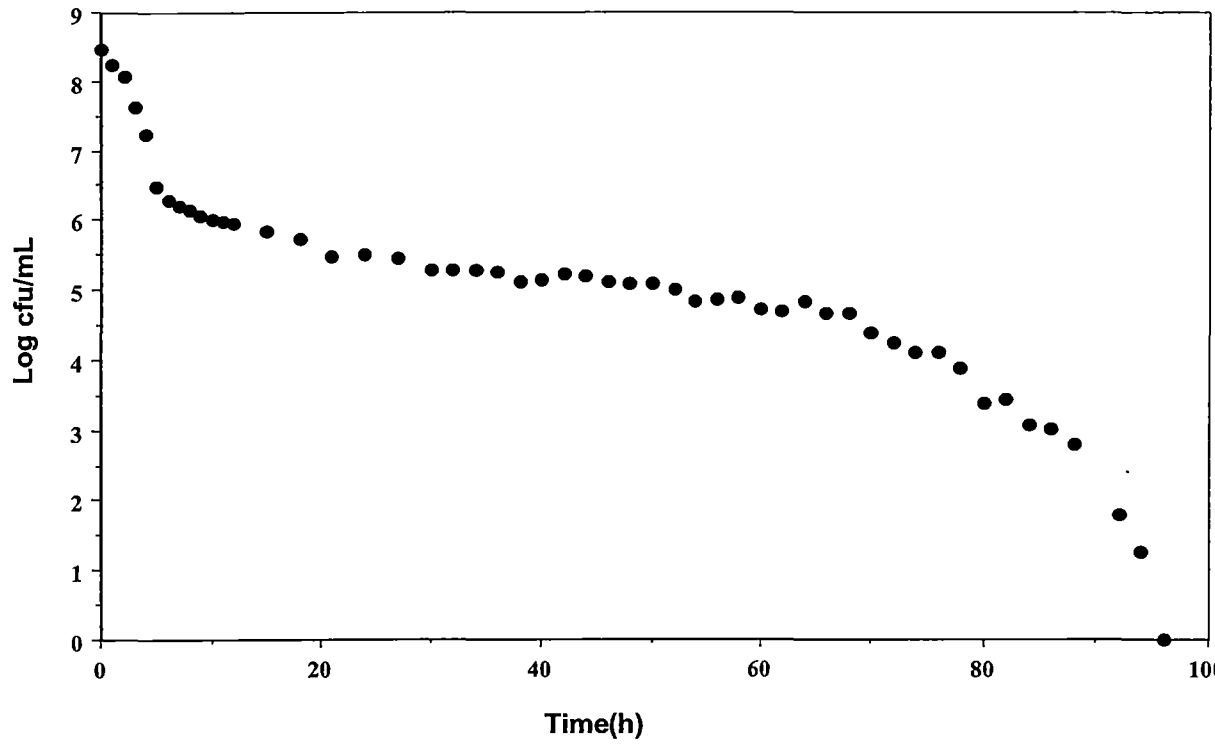

Figure 1.2 Example of triphasic behaviour shown by *E. coli* M23 exposed to pH 3.5 (HCl as acidulant), 35°C, tryptone soya broth (Brown, Mellefont, and Shadbolt, unpublished).

1.3.2 Chick came first: origin of the mechanistic and vitalistic concepts

Chick (1908) was the first worker to comment on inactivation patterns observed in response to the disinfection of *Bacillus anthracis*, *Bacillus paratyphosus*, and *Staphylococcus pyogenes aureus* (old taxonomic classification of *Staphylococcus aureus*) with mercuric chloride, phenol, and silver nitrate. Most of the data obtained by Chick (1908) revealed exponential inactivation. It was stated that "...the course of disinfection, either with mercuric chloride or phenol, proceeds in a perfectly orderly manner...". This was found to

conform with data already published and equation 1 was used to represent the process of disinfection:

$$[1/(t_1 - t_2)].\ [\mathrm{Log}\ (n_1/n_2)] = K \qquad (1)$$

where n_1 and n_2 are the numbers of bacteria surviving after times t_1 and t_2 respectively and K is the rate of "reaction" (inactivation of bacteria in this case). Disinfection was, therefore, described as being analogous to a chemical reaction in which the disinfectant represented one reagent whilst the protoplasm of the bacterium was the second. The "reaction" was suggested to be unimolecular since the disinfectant was considered to be present in such excess that its concentration was effectively unaltered during the course of the experiment. Increased temperature and disinfectant concentration were found to increase the rate of reaction. Chick (1910) states that the "...explanation offered by the physical chemist is that there is no dissimilarity between the molecules, but that at any one time only a proportion of them is in the condition to undergo the dissociation or chemical union". The increased susceptibility of some bacteria, resulting in log-linear (or exponential) inactivation, was due to temporary differences in "internal molecular energies", which was suggested as a possible explanation for temperature increasing the rate of reaction.

Interestingly, however, some inactivation curves of *B. paratyphosus* were found to display upward concavity or tailing as the experiment progressed, regardless of disinfectant type. Chick (1908) hypothesised that the deviation from log-linear kinetics was due to certain cells in the population possessing more resistance to the disinfecting agent. The less

resistant cells were killed in greater proportion during the earlier stages of the reaction and hence the values of K were seen to diminish. That author showed considerable prescience when experiments were carried out to determine if the age of the culture was responsible for the observed tailing (refer to [1.3.4], [1.3.5] and [2.2] for subsequent research on the effect of inoculum age). It was found that young cultures of *B. paratyphosus* grown for 3 hours possessed a higher intrinsic resistance to disinfection than a 24 hour culture. Subsequent authors were to question this finding, but the proffered explanation of permanent variations in resistance in the bacteria under study was the genesis for vigorous and ongoing scientific debate. Lee and Gilbert (1918) cited Chick's work of 1908 as being the basis for two hypotheses of inactivation. The first was termed the "mechanistic concept" and states that "***all cells have a general similarity of resistance and population decline is akin to a chemical reaction***" Lee and Gilbert (1918). Deviations from this reaction are bound to the mechanism of inactivation. The second hypothesis was termed the "vitalistic concept", stating that "***inactivation may be explained on the basis of differing degrees of resistance within a population to an inimical condition***" Lee and Gilbert (1918). These two hypotheses have formed the basis for much scientific argument which has persisted through to the present, with many workers aligning themselves to either the mechanistic or vitalistic concepts. The historical debate surrounding the mechanistic and vitalistic concepts will be discussed in detail subsequently.

1.3.3 Analysis of Chick's observations

Other points of interest arise from close scrutiny of the work of Chick (1908). Figure 1.3 shows the inactivation patterns obtained when *B. paratyphosus* cultures were exposed to a phenol concentration of 0.6% v/v. The experiments were of short duration and low concentrations of cells were used. In several cases, the initial time point was not taken from the beginning of disinfection. Thus, it is difficult to determine the precise pattern of inactivation kinetics. For both cultures grown for 24 hours at 20°C and 21°C, respectively, omission of an initial time point may understate the extent of first phase inactivation. In the case of a culture which was passed through several generations in broth and sub-cultured every 3 hours (taken by Chick (1908) to be a 3rd generation population) it is not clear whether a sharp population decline would have been experienced or if consistent log-linear inactivation kinetics occurred. While Chick (1908) claimed that younger cells showed more resistance than a 24 hour culture, this does not necessarily appear to be the case. A 24 hour culture starting at a higher cell density appeared to experience a very similar inactivation pattern as those grown for 3 hours. These inconsistencies will be discussed further [1.3.4].

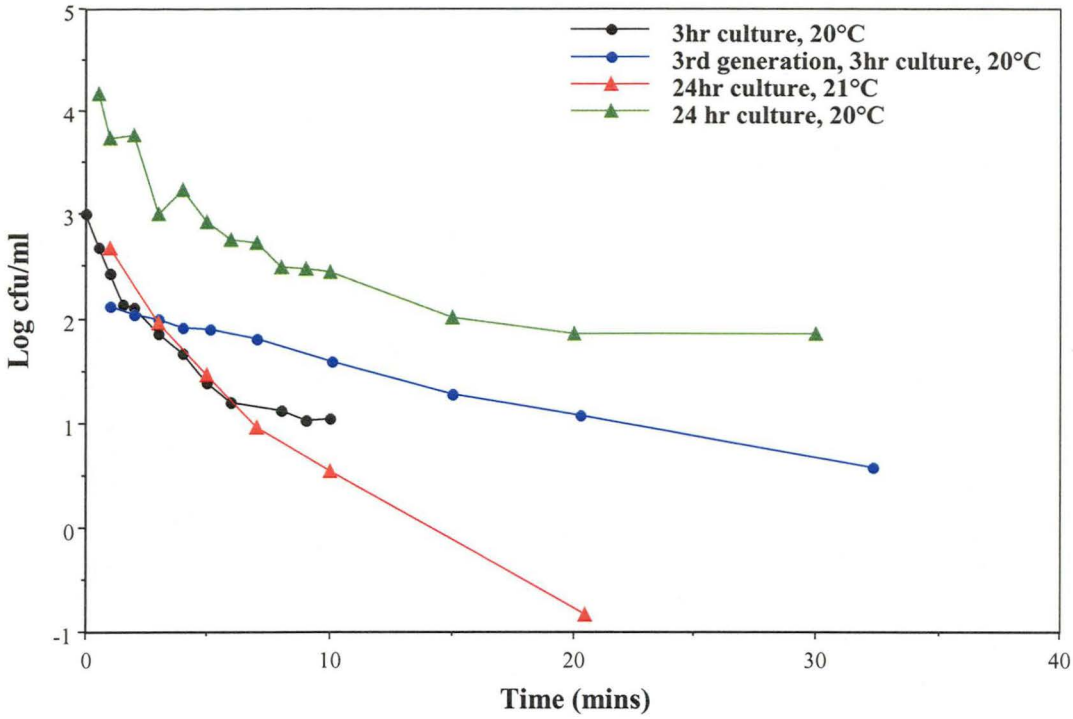

Figure 1.3. Inactivation of *B. paratyphosus* at various ages when exposed to 0.6%v/v phenol. Generated from data of Chick (1908).

With *Staphylococcus py. aureus* (published abbreviation), Chick (1910) observed an initial lag, or "shoulder", before log-linear inactivation kinetics became evident. A comparison was made between two cultures grown at 37 and 42°C for 24 hours. On the basis of results shown in Figure 1.4, reproduced from Chick (1910), Chick stated that bacteria grown at the higher temperature displayed a greatly increased resistance and disinfection was so slow that it was impossible to study it within the time frame of the experiment.

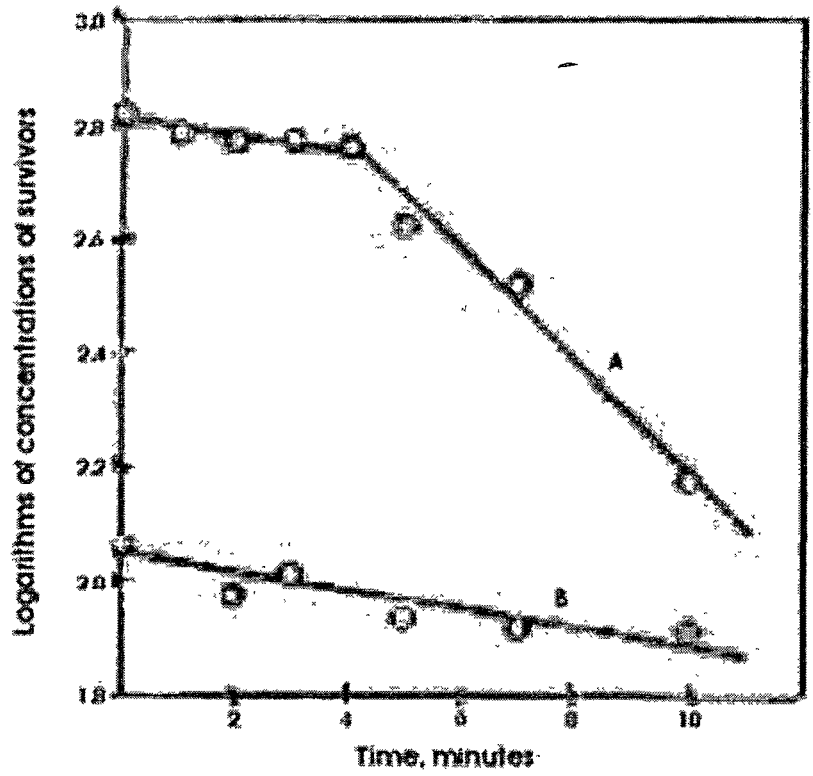

Figure 1.4. Disinfection of *Staphylococcus py. aureus* with 0.6% phenol at 20°C. (A) 24h culture grown at 37°C; (B) 24h culture grown at 42°C. Reproduced from data of Chick, 1910.

Given that the duration of the experiment was only 10 minutes it is interesting to speculate what may have happened subsequently. For example, would the culture grown at 42°C eventually experience a sudden decline and that grown at 37°C commence tailing similar to *B. paratyphosus* grown at 20°C for 24 hours (as shown in Figure 1.3)? Later workers (Sherman and Albus, 1923; Jordan and Jacobs, 1944) criticised the experimental procedures of Chick (1910), arguing that important findings were likely to have been missed due to the short duration of the disinfection investigations.

Chick (1910) speculated that the lag phase of disinfection was a characteristic of *Staphylococcus py. aureus* and was due to slow penetration of the bacterial envelope by

phenol. Chick (1910) stands by her original hypothesis that disinfection is similar to a chemical reaction, stating that deviations from the logarithmic law are minimised by successive sub-culturing of a small amount of broth every 2-3 hours. The deviations shown by a 24 hour culture of *B. paratyphosus* in response to phenol was suggested to be the result of nutrient depletion in the medium in which the bacteria had been grown, causing a lessened state of resistance amongst the majority of cells. Although the author did not acknowledge this herself, it may also be argued that observed deviations from the logarithmic law are due to *variable states of resistance* due to a variety of physiological states within a population of cells and, hence, phenol induced inactivation is not analogous to a chemical reaction at all under the conditions employed.

1.3.4 Mechanistic *versus* vitalistic: the earliest inactivation debate

Phelps (1911) supported the hypothesis of disinfection being similar to a chemical reaction, boldly stating that the rate of inactivation "is unfailingly found to follow the logarithmic curve of the velocity law, if the temperature be constant". That author made no attempt to define any deviation from the logarithmic law. Inactivation rate could be calculated from 50-75% of the initial number "altho [sic] other points serve practically as well if not too near either end". The latter statement is surprising. Despite no explanation of why rate data is not to be calculated from either end of the inactivation curve, this may have been due to deviations from the logarithmic law. To delete observations, especially tailing of a resistant subpopulation, appears scientifically unsound given (for example) the low infective dose of VTEC such as O157 serogroups. And yet, the assumption of log-linear inactivation kinetics

have formed the basis of thermal processing protocols used in the canning industry for decades (Peleg and Cole, 1998). Based on the findings of Chick (1908) and Phelps (1911), Lee and Gilbert (1918) defined the "mechanistic" and "vitalistic" hypotheses to explain deviations from logarithmic reduction. Those authors contend, based on observed inactivation kinetics, that individuals possessing the characteristic of minimum resistance are present in greatest number. This theory, as the authors point out, is accounted for if the hypothesis of "graded" resistance to stress within a population is accepted, rather than the presence of two distinct populations.

Similar to Chick (1908), Lee and Gilbert (1918) used small numbers of cells in experiments of short duration. Data from identical experiments, using cultures "six to twelve hours old" of *B. paratyphosus* exposed to 0.2% phenol at 37°C, shows two different patterns of inactivation, the first being log-linear whilst the other is clearly biphasic. Lee and Gilbert (1918) claim, however, that both patterns are examples of a definite logarithmic relationship between bacteria and the action of the disinfectant with time. This is shown graphically by *omission* of an initial time point for one of the curves. In the same paper, the authors lend support for the vitalistic theory by stating that a decreased rate of disinfection (tailing) is to be expected if factors such as age of the culture and temperature of incubation are taken into account. That statement undermines the mechanistic hypothesis and casts doubt on the conclusions of those authors regarding exponential inactivation.

Sherman and Albus (1923) supported the vitalistic hypothesis as the explanation for deviations from exponential inactivation. It was demonstrated that actively growing

cultures were more susceptible to a variety of stress types (cold shock, heat, phenol and NaCl) than populations cultured for 24 hours or more which have entered the stationary phase. This is in contrast to Chick (1908) whose methodology was questioned by Sherman and Albus (1923), stating that the 3rd generation cells used by her may have been considerably older than the 3 hours claimed. Thus Sherman and Albus (1923) were the first to conclusively demonstrate that the age of a culture had a significant effect on the kinetics of inactivation.

Falk and Winslow (1926) argued for the mechanistic hypothesis, but stated that deviations from the logarithmic law were to be expected due to the complex nature of physical and chemical systems involved in reactions between bacteria and toxic agents. A decrease in the rate of inactivation over time was consistently observed, and the authors commented on the tendency of previous workers to minimise observations of tailing. Based on their own work and that of others, Falk and Winslow (1926) stated that the death of bacteria in water followed monomolecular inactivation kinetics very closely, deviated slightly in the presence of standard disinfectants, and showed considerable differences in the presence of NaCl and $CaCl_2$. Such variations, according to those authors, were to be expected since it appeared very unlikely that all of the above destructive processes should be of the same chemical nature. While the logarithmic rate was, at best, generally found to express the rate of disinfection it was proposed that a series of reactions were taking place during inactivation. Deviations from the monomolecular theory were then explained as the result of multimolecular reactions.

1.3.5 An attempt to find middle ground: the work of Rahn

Rahn (1929) attempted to find a compromise between the vitalistic and mechanistic groups, declaring that "whenever two groups of thorough research workers hold such opposed views as in this case, it is fairly safe to assume that some essentially new principle is involved which none of the two parties realised". Finding such a compromise was said to be difficult "given that mechanists do not pay sufficient attention to deviations from the logarithmic law, while vitalists largely refuse to acknowledge facts". Rahn (1930) agreed that deviation from logarithmic inactivation kinetics was most common and special conditions had to be employed to achieve otherwise. Rahn (1929) believed that a special group of molecules existed in all bacteria, the destruction of which was lethal for the cell. In some cases, multiples of this molecule may exist and result in the multimolecular order of death hypothesis. Such a simplistic explanation is likely to be at odds with the variations observed in destructive processes by Falk and Winslow (1926). This hypothesis may actually be of more support to the vitalists, in that multiples of some critical molecule suggests the existence of a variation of resistances within a cell population. Two further points are worthy of note. Rahn (1929) states that much of the data of Chick (1908) is invalid due to the omission of the initial time point in many of her figures. The second is Rahn's paper of 1930, in which a 5.5 hour culture of *B. paratyphosus* displayed a decreasing death rate, indicating an "inhomogeneous mixture of quite resistant (old) and quite sensitive (young) cells". These observations were summarised by Rahn (1930) as:

- the exponential order of death is explained by the assumption that each cell contains one or several very sensitive molecules and destruction of one of these prevents multiplication
- a decreasing death rate is accounted for by the assumption of a variation in the resistance of cells
- increasing death rate (convex survivor curve) is indicative of a different cell structure requiring destruction of 2 or 3 molecules before inactivation is possible.

With these three hypotheses Rahn (1930) was the first to argue for and against various points of both the mechanistic and vitalistic hypotheses.

1.3.6 Criticism of Rahn and progression of the inactivation debate

Despite attempts to find a compromise, additional evidence for both hypotheses continued to be presented. Watkins and Winslow (1932) experimented with various concentrations of NaOH and heat against *E. coli* of different ages. Inactivation rate was found to increase with higher temperature and alkali concentration. The age of the inoculum was varied and young cells found to possess the least resistance, confirming the earlier work of Sherman and Albus (1923). It was speculated that young cells lacked the power to form a protective substance. Also of interest was their observation that the concentration of the inoculum affected the rate of inactivation. A higher inoculum experienced slower death rates. Watkins and Winslow (1932) were among the first to suggest that dead cells may protect the living by shielding them from inimical agents and heat treatments. This particular hypothesis is the only suggestion from those authors that would support the mechanistic

hypothesis. Withell (1942) was very critical of the mechanistic hypothesis and statements by Rahn (1929, 1930) regarding the number of molecules of bactericide calculated to be reacting with one organism, quoting others who stated that "the monomolecular theory...has therefore very little solid evidence in its support and its application leads to conclusions that are so absurd they are difficult to discuss". Withell observed considerable variation in the shape of the inactivation curve with duplicated experiments, and suggested that previous workers had largely ignored similar findings. In short, Withell (1942) concluded that different rates of microbial inactivation were determined essentially by variations in the distribution of resistance amongst a bacterial population.

Earlier methodologies that formed the basis for many of the mechanistic theories were also criticised by Jordan and Jacobs (1944). They recognised that experiments should not be too brief [see methodologies of Chick, (1908); Lee and Gilbert (1918)] or changes in the death rate may be missed; that the concentration of the biocide should not be too high; and adequate numbers of bacteria must be used. Jordan and Jacobs (1944) stated that there had been a tendency to use too few organisms and high concentrations of germicide, which would lead to an overstatement of the process of inactivation following the logarithmic law. Jordan and Jacobs (1944) observed variations in the death rate with differing concentrations of phenol. They observed an initial inactivation lag (shoulder) which increased in length with decreasing phenol concentration. This was followed by a constant rate of decline and there also appeared to be a slower second phase of inactivation, or tailing, but this was not commented on further. Based on their methods those authors stated that there was a normal distribution of resistance present, supporting the vitalistic hypothesis. Jordan et al. (1947a)

also disputed the mechanistic assumptions that all cells are essentially identical in resistance and that only a single vital molecule in each cell needs to be inactivated to prevent cultivation.

1.3.7 Recognition of the practical significance of bacterial inactivation kinetics

From a practical standpoint, Jordan et al. (1947b) recognised that tailing of subpopulations may have important ramifications in the pasteurisation of milk. Resistant bacteria may survive the pasteurisation process and lead to infection if consumed. Those authors were also amongst the first to speculate on the origin of resistant cells, hypothesising that such cells may arise through sub-lethal exposure to stress or that resistance was purely due to normal distribution within a population. Fresh cultures were generated from persistent subpopulations to determine whether their progeny were also resistant to the effects of heat stress. Jordan et al. (1947b) claimed to have limited success with their experiment, but successive generations beyond the second displayed no unusual tolerance to stress. Rahn (1943) also reported a previous attempt to carry out this experiment, noting that no unusual resistance was found. Hence Jordan et al. (1947b) stated that the results of their study were inconclusive.

In their examination of the heat resistance of *Bacillus cereus* spores, Vas and Proszt (1957) observed that a constant fraction of 1 in 10^7 or 1 in 10^8 cells possessed unusual resistance. They observed that dilution of their concentrated spore suspension reduced the extent of

tailing to the point where it was no longer visible. Parent cultures of resistant cells did not produce heat tolerant offspring, supporting the results of Rahn (1943) and Jordan et al (1947b). This led the authors to endorse earlier findings that tailing of survival curves was not due to tolerance arising from genetic mutation. They concluded the non-exponential inactivation was instead caused by the presence of a definite small proportion of cells with heat resistance manifested through natural biological variability.

Moats et al. (1971), expressed the firm opinion that the vitalistic theory explained deviation from log-linear inactivation kinetics, presenting new data to support this conclusion and critically reviewing previous work. Earlier reports of 1 in 10^{6-8} cells displaying unusual tolerance to heat were confirmed. Previous investigators may have missed tailing, as inactivation curves were often not followed for long enough to observe any deviation. Moats et al. (1971) commented that such results are very important where near total destruction of microorganisms is required. This heat resistance was found to be physiological rather than genetic, since offspring adopted the heat resistance of their parent culture. Thus, while Moats et al. (1971) thought it possible, there was no contemporary evidence to support genetic mutation through heat adaptation. No protective effects were observed by the addition of dead cells to the medium. The possibility of cells clumping together to result in tailing was also discounted, as dilution and direct microscopy proved otherwise. The assumptions of the mechanistic theory that (i) populations of single strains of bacteria are homogenous with regard to heat resistance and (ii) thermal death of bacteria is unimolecular and occurs from inactivation of a single molecule or critical site were countered. Based on changes in resistance due to age of cultures and examination of

different temperatures of inactivation the first assumption was seldom true and only achieved through rigorous cycling of cultures to achieve populations of pure exponential cells. Assumption (ii) did not agree with evidence for sublethal injury. If death was due to inactivation of a single site, populations should not display any difference in numbers between culture on standard or selective media, which was found not to be the case.

1.3.8 Reviews of the early bacterial inactivation kinetics research

Reviews by Dean and Hinshelwood (1966) and Cerf (1977) showed the considerable benefit of hindsight. The former authors stated that the precise form of the survival curve depends upon the criteria used to assess viability, the previous history of the culture (including exposure to mutagenic influences), the length and conditions of the survival test itself, and the possible incidence of adaptive processes. In addition, the role of cyclical processes such as lysis and repair against irreversible injury may also be expected to vary. Hence it was not considered surprising that a wide variety of survival curves had been observed. In the review of Cerf (1977), it was pointed out that many curves previously labelled as logarithmic, showed considerable deviation from log-linearity. Consideration of evidence for both mechanistic and vitalistic hypotheses provided no definite conclusion except that more research was needed. Cerf (1977) remarked that microbial inactivation research was of practical significance and should not be neglected, reinforcing the view of Jordan et al. (1947b).

The pioneering studies of bacterial inactivation kinetics in the very early-mid twentieth century set the scene for vigorous debate. This strong scientific argument continues to the present day. Current research is extending earlier bacterial inactivation kinetics and yielding important findings. These will be discussed in the following section.

1.4 Current Knowledge of Bacterial Inactivation Kinetics

1.4.1 Motivations for enhanced knowledge of bacterial inactivation kinetics

The last two decades have seen a rapid rise in the amount of research pertaining to microbial growth inhibition and survival under stressful conditions. There can be little doubt that emerging pathogens with enhanced survival and low infectious dose, development of antibiotic resistance, globalisation of the food trade, and changes in eating habits are motivating factors for the rise in increased scientific output in this field. Few studies specifically address the subject of microbial inactivation kinetics but contain typical examples of the various survivor curves shown in Figure 1.1. Where authors provide comment on patterns such as tailing, rarely is it discussed in terms of a "mechanistic" or vitalistic" hypothesis. Strong scientific argument currently exists, however, concerning the viable-but-non-culturable state of bacteria, which has led to much disagreement in terms of when a cell may be considered "dead" (Kell et al., 1998). From a practical viewpoint this is particularly important when determining safe microbial levels in foods which may be

contaminated with a virulent pathogen such as VTEC. A review of selected research from the last two decades follows the above historical description of the debate on microbial inactivation kinetics. The work discussed in the remainder of [1.4] was selected on the basis that it describes microbial inactivation kinetics and the appearance of non-linear survivor curves.

1.4.2 Current inactivation kinetics research areas

With advances in technology there are now a variety of methods available for the treatment and sterilisation of foods. In an effort to counter emerging pathogens and cope with consumer demands for minimally processed, ready-to-eat products, research has been conducted to develop knowledge of bacterial physiology and design novel processing protocols. Table 1.3 lists some areas of physiological research in which non-linear inactivation kinetics have been observed. Few of the authors listed have attempted to describe the observed survivor curves. Despite an increased understanding of bacterial physiology and genetics, detailed knowledge of many of the mechanisms relating to microbial inactivation are not available. Without such information it remains difficult to address questions relating to bacterial viability and microbial inactivation kinetics.

Table 1.3. Studies since 1980 in which non-linear microbial inactivation have been observed

Physiological aspect(s)	Author(s)
Acid-induced death, pH tolerance	Mendonca et al., 1994 Benjamin and Datta, 1995 Brown et al., 1997 Buchanan and Edelson, 1999 Jordan et al., 1999 Ryu et al., 1999 Xiong et al., 1999 Wilde et al., 2000 Tetteh and Beuchat, 2001
Influence of temperature (thermal, nonthermal, chilling)	Kirby and Davies, 1990 Teo et al., 1996 Schuman et al., 1997 Humpheson et al., 1998 Kaur et al., 1998 Kudva et al., 1998 Yang et al., 2001
Osmotic pressure	Heller et al., 1998 Shadbolt et al., 1999
Hydrostatic pressure	McClements et al., 2001
Antibiotic, chemical	Moore, 1982 Gustafson et al., 1998 Appendini and Hotchkiss, 1999 Lambert and Johnston, 2000
Irradiation (gamma and ultra-violet)	Doudney and Rinaldi, 1989 Buchanan et al., 1999
Combined treatments	Shadbolt et al., 2001 Uyttendaele et al., 2001

1.4.3 A modern contribution to the vitalistic concept

One of the few recent papers to focus on non-exponential kinetics is that by Peleg and Cole (1998) discussing the heat inactivation of bacteria. It was recognised that thermal death data from which regulatory guidelines are often developed rarely cover more than 5 or 6 log reductions, and that deviations in exponential inactivation kinetics are often observed beyond this level of inactivation. From a practical perspective Peleg and Cole (1998) suggested that some of these guidelines might need to be reassessed. Deviations from log-linear kinetics were explained on the basis that the survival curve was the cumulative form of a temporal distribution of lethal events, where each organism is inactivated at a certain time. The shape of the curve, whether upward or downward concavity, was determined by different distributions in the heat resistance of the population having a different mode, variation and skewness and not mortality kinetics of different orders [see Falk and Winslow (1926) and Rahn (1929)]. Although not specifically acknowledged, this would support the vitalistic hypothesis from the perspective that inactivation is *explained on the basis of differing degrees of resistance within a population to an inimical condition* [1.3.2].

Using published data on inactivation of pathogens with heat alone and in combination with pH or high pressure, Peleg and Cole (1998) described survival patterns with different Weibull distributions. Changes in growth or environmental conditions were considered to shift the distribution of resistances. It was stated that the Weibull distribution did not take into account specific mechanisms of inactivation but was consistent with the theory that

destruction of a critical system is a probabilistic process and due, in part, to natural variability that exists within a bacterial population.

1.4.4 Support for the mechanistic concept

Disputing the above theory is one of the few modern studies to address inactivation in terms of the vitalistic and mechanistic concepts, through use of an Intrinsic Quenching hypothesis (Lambert and Johnston, 2000). It was suggested that non-linear inactivation was due to quenching of the biocide to which bacteria were exposed. Previous assumptions that disinfection was analogous to a chemical reaction with the biocide present in excess quantities were questioned on the basis of bacterial target molecules. Inactivation using a quaternary ammonium compound (QAC) against the microbial lipid membrane was used as an example. For a 0.01% solution of QAC in a 1 x 10^8 cells/ml culture there are approximately 2 x 10^9 molecules of biocide per cell (Lambert and Johnston, 2000). The biocide would thus appear to be several orders of magnitude in excess, but for every cell of *E. coli* there are 2.2 x 10^7 lipid and 1.2 x 10^6 lipopolysaccharide molecules respectively. The amount of QAC required to dissolve each molecule was questioned. Lambert and Johnston (2000) developed the following model that described well the inactivation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in response to chemical exposure:

$$\mathrm{Log}R = k_0/Q.(1 - e^{-Qt}) \qquad (2)$$

Where k_0 is the independent rate constant, $Q = k_1 n$ (quenching Q, governed by a concentration independent rate constant k_1, and a dilution coefficient, n) and log *R* (log reduction) = log (N_0/N), N_0 is the initial number of organisms per ml. On the basis of model fit it was stated that the ability of microbes to "intrinsically quench" the bulk concentration of the biocide resulted in non-linear survival kinetics. In criticising the vitalistic hypothesis, Lambert and Johnston (2000) appear to have misunderstood its underlying assumptions. According to those authors, the vitalistic hypothesis states that the distribution of resistance is permanent and independent of cell concentration, hence identical survivor curves should be obtained from different sizes of inocula (the mechanistic hypothesis). Changes in the cell concentration resulted in different inactivation kinetics, which Lambert and Johnston (2000) interpreted as the bacteria altering the concentration of disinfectant. This conclusion is flawed, since earlier authors (Vas and Proszt, 1957; Moats et al., 1971) had already shown that dilution of a culture removed a fraction of stress tolerant cells and reduced the extent of tailing. Additional to this is the work showing that age of cells also has an impact on the inactivation kinetics [refer to 1.3.4].

1.4.5 Evidence for both mechanistic and vitalistic concepts

Humpheson et al., (1998) and Mattick et al., (2001) investigated the biphasic thermal inactivation kinetics of two strains of *Salmonella*. The latter authors described a model that showed agreement between observed and predicted survival curves at temperatures between 55 and 80°C. It was concluded that survival under such conditions could, therefore, be predicted without assumption of any mortality kinetics. Humpheson (1998)

observed tailing at a level of 1 in 10^{4-5} cells, but this was only reproducible in populations above 10^7cfu/ml. As the temperature was reduced from 60°C to 49°C, the initial death rate was found to decrease and survival curves became gradually more linear. This suggested the presence of two discrete populations. Cells cultured from a 60°C heat challenge showed no difference in thermotolerance from their parent population. A fresh population inoculated into a previously challenged medium displayed almost identical inactivation as the previous culture. Hence genotypical differences and protective effects from dead cells were discounted as reasons for tailing, in agreement with the work of Rahn (1943), Jordan et al., (1947b), and Moats et al., (1971). Cultures of various ages grown in a chemostat were exposed to 60°C and their resistance found to vary, a characteristic also reported by earlier researchers (Sherman and Albus, 1923; Falk and Winslow, 1926; Withell, 1942).

The findings of Humpheson et al. (1998) discussed thus far support the vitalistic hypothesis. An exception is their observation that chloramphenicol reduced levels of tailing which suggested de novo synthesis of heat shock proteins (HSP). This lends support to the hypothesis that tailing is bound to the mechanism of resistance, one of the mechanistic concepts listed by Lee and Gilbert (1918), and Cerf (1977). Wang and Doyle (1998) observed similar results when they examined the effect of heat shock on acid tolerance of *E. coli* O157:H7. Sublethally shocked cells (10 minutes at 48°C) demonstrated enhanced acid resistance. This was conferred by two outer-membrane HSP's synthesised shortly after the sublethal stress. Addition of chloramphenicol prior to the heat shock blocked their synthesis and resulted in decreased acid-tolerance. Further evidence was provided by Shadbolt et al. (1999) who observed that addition of chloramphenicol to a culture exposed

to low water activity also reduced the extent of tailing. Brown (2002) observed chloramphenicol had no effect on the rate of inactivation or tailing when *E. coli* was exposed to a lethal pH stress. A variable effect of chloramphenicol in response to different types of stress is consistent with the hypothesis that resistance is dependent on the mechanisms of inactivation, further supporting the mechanistic concept.

1.4.6 Modelling cell death and the requirement for more detailed data

Few of the studies listed in Table 1.3 involve detailed, systematic research of microbial inactivation kinetics. Most reports aim to present novel information regarding the magnitude of cell death in response to a particular treatment applied to an individual food commodity. Such pathogen-commodity studies may generate novel results, but not in the form of adequate kinetic data from which inactivation models may be formulated. The absence of detailed kinetic data limits an understanding of the underlying mechanisms of cell death. Commodity-treatment studies are useful as a short-term measure for describing the inactivation of a pathogen in a particular food, however, the results are not easily transferable to other food systems (refer to the development of a predictive model in [3.3]) and may require re-evaluation with changes to food processing parameters, product formulation, or the emergence of new pathogens.

Detailed kinetic research into the underlying mechanisms of inactivation will provide valuable information that may be used to control pathogens in a variety of foods, and allow the development of predictive models that are able to account for variations in food

processing (McMeekin and Ross, 2002). The development and use of predictive food microbiology models within the food industry is expanding (Sumner and Krist, 2002). McMeekin et al. (1993) describes the various types of predictive models used and their construction. Critical to model formulation is the accumulation of comprehensive and reproducible data relevant to the conditions likely to be encountered by bacteria in a given environment, such as temperature, pH, a_w or redox potential. Bacterial growth rates and the growth/no-growth interface has been successfully modelled (Presser et al., 1998; Salter et al., 1998; Salter et al., 2000) but there has been limited application to nonthermal inactivation kinetics due to lack of data. As mentioned by Peleg and Cole (1998), thermal inactivation models have been in use for a considerable period. More attention is being focused on nonthermal inactivation in response to outbreaks from emerging pathogens (Membré et al., 1997; Buchanan et al., 1997; Takumi et al., 2000; Pond et al., 2001; Duffy and Schaffner, 2001).

1.5 Physiology of Cell Death

This section reviews current knowledge of physiology influencing bacterial survival under lethal pH and water activity constraints. Such information is crucial to the development of experimental protocols for examining inactivation kinetics.

1.5.1 Water activity

When placed into an environment of high osmolality (upshock), *E. coli* initiates several responses to cope with the new stress, depending on the composition of the medium. Sudden exposure causes rapid water loss and reduction in turgor pressure followed by shrinkage of the cell membrane (Cayley et al., 1991). To survive, the organism must be able to restore the osmotic equilibrium across the membrane. Figure 1.5 shows some of the biochemical pathways involved in osmoregulation. In response to a loss of turgor pressure, the most rapid response is uptake of potassium ions, K^+ (Csonka, 1989; Cayley et al., 1991; Csonka and Epstein, 1996). Membrane proteins sense loss of turgor and are likely to provide the first signal for potassium ion uptake. K^+ is the most prevalent cation in the cytoplasm of *E. coli*, and hence one of the major intracellular osmolytes responsible for maintaining turgor pressure. Accumulation of potassium ions and organic solutes in the cytoplasm builds up internal osmotic strength and prevents diffusion of water out of the cell (Landfald and Strøm, 1986). An increase in salinity has been shown to result in an accumulation of intracellular potassium (Galinski, 1995). Therefore, it is assumed that uptake of K^+ is largely responsible for initial osmotic balance across the membrane.

In addition to being an important early response solute, K^+ is also believed to act as a second messenger that activates other osmotic responses (Csonka and Epstein, 1996; Ingraham and Marr, 1996). Evidence for this comes from the dependence of other osmotic responses on K^+ uptake. An important secondary response is the accumulation and/or

synthesis of other solutes or osmoprotective compounds. In minimal medium it has been observed that other anions, such as glutamate, are accumulated to regulate cytoplasmic osmolality. Uptake of these anions however, may inhibit the function of certain enzymes. To circumvent this problem, *E. coli* will accumulate electrochemically neutral solutes, termed compatible solutes, which do not interfere with biochemical processes at high concentrations (Ingraham and Marr, 1996). Accumulation of compatible solutes may extend the growth limits of *E. coli* under otherwise inhibitory osmolalities (Galinski, 1995). In minimal medium the bacterium synthesises a neutral solute, trehalose, which protects against dehydration. Synthesis occurs via two enzymes encoded by the *ots*AB operon. During periods of osmotic stress, this operon is induced 5- to 10-fold (Csonka and Epstein, 1996).

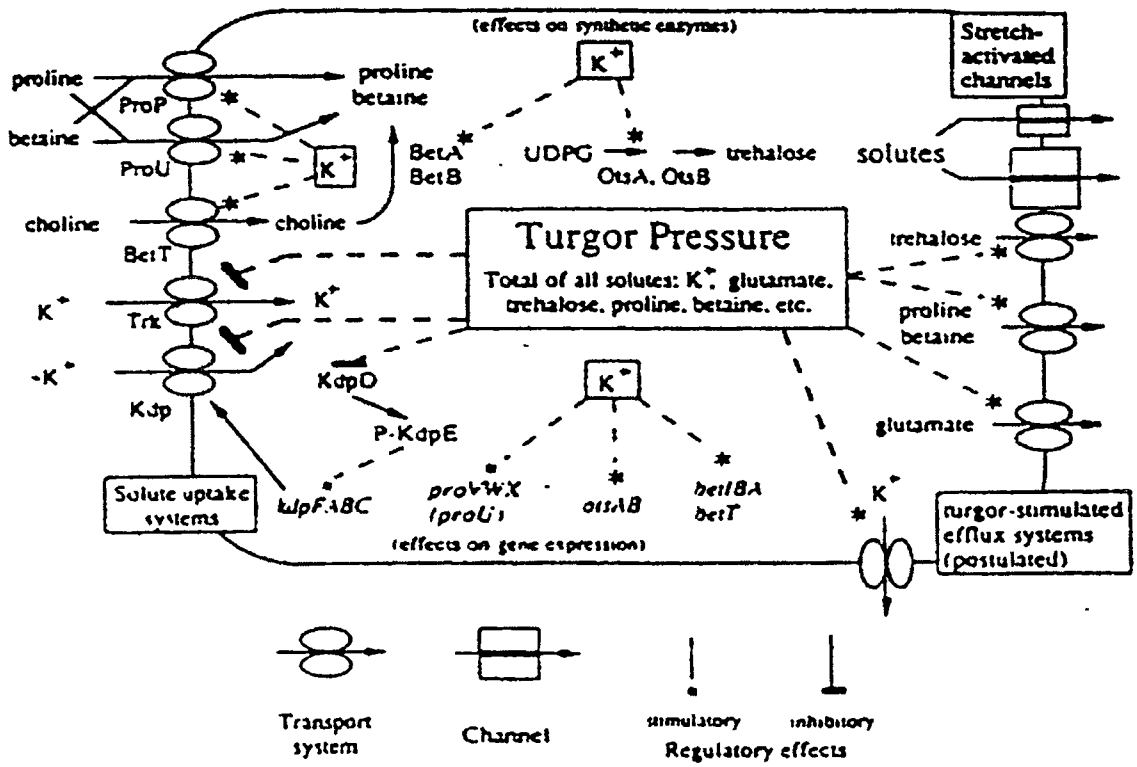

Figure 1.5. Model of systems active in osmoregulation. Reproduced from Csonka and Epstein, (1996).

K^+ may also stimulate the uptake of other osmoprotectants, such as glycine betaine (more commonly referred to as betaine), choline or proline (Ingraham and Marr, 1996). Uptake occurs via a transport system regulated by two permeases, ProP and ProU. Replacing ions with neutral osmoprotectants negates the inhibition that high ionic strength places on enzymatic reactions. Energy required for uptake of osmoprotectants may be less than that required for K^+ accumulation and glutamate synthesis. It is believed that osmoprotectants stabilise and protect enzymes by being excluded from the protein surface (Csonka and Epstein, 1996). The ProP system transports betaine, proline and ectoine across the cell membrane, and is greatly enhanced by increased medium osmolality. The ProU system also transports the above osmoprotectants, but has a very high affinity for betaine in comparison to proline and ectoine. This is due to the binding protein component of the ProU system, which has a preference for betaine. Transcription of proteins utilised by the ProU system may be induced to greater than 100-fold when exposed to high osmolality (Csonka and Epstein, 1996).

Betaine may also be synthesised from exogenous choline (Landfald and Strøm, 1986). Choline uptake can occur via the ProU system, although preferentially occurs via the BetT transport route. Under aerobic conditions, conversion of choline to betaine occurs via two steps through the action of choline and glycine betaine-aldehyde dehydrogenase respectively. According to Landfald and Strøm (1986), anaerobic conditions prevent conversion of choline to betaine, indicating that the response is oxygen-dependent. The same authors reported a 30- to 40-fold increase in the two enzymes responsible for

conversion of choline to betaine compared with activity in unstressed cells. Csonka and Epstein (1996) report a greater than 10-fold induction under osmotic stress for the same process.

1.5.2 Acid (pH) homeostasis

Bacterial cells maintain pH homeostasis over a narrow range (Slonczewski and Foster, 1996). Intracellular pH must be maintained above some inhibitory level, since below this level intracellular proteins become denatured (Montville, 1997). Under acidic conditions, protons are pumped from the cell. If the flow of protons from the cell becomes uncontrolled, the internal pH change will be severe enough that synthesis of cellular components and cell division are inhibited (Brown and Booth, 1991). The degree of acid sensitivity of *E. coli* varies, depending upon factors such as physiology and the type of acid used. It has been shown that strong acids inhibit bacterial cells in a different manner to organic or weak acids in undissociated form. Strong acids, such as HCl, are completely dissociated in solution into H^+ and Cl^- ions. Rowbury and Goodson (1993) determined that anions attack the outer membrane of *E. coli* and prolonged exposure resulted in cell death.

Glass et al. (1992) found that organic acids were more inhibitory to *E. coli* than HCl at any pH. In undissociated form, weak acids may freely cross the bacterial membrane and act against intracellular contents, i.e. enter the cell; dissociate; and reduce intracellular pH (Cherrington et al., 1991; Benjamin and Datta, 1995; Ingraham and Marr, 1997). Rate of death in the presence of organic acids increases with increasing undissociated acid

concentration (Przybylski and Witter, 1979). The type of organic acid also influences lethality. Abdul-Raouf et al. (1993) found that the order of effectiveness of three acids against bacterial survival was acetic acid > lactic acid > citric acid. This is most likely due to the ease of entry into the bacterial cell. Acetic acid has the lowest molecular weight, and hence is more easily capable of traversing the bacterial membrane. Transmission electron microscopy of *E. coli* cells exposed to organic acids shows no damage to the outer membrane or leakage of intracellular contents (Cherrington et al., 1991). Once inside the cytoplasm, dissociation of weak acids is followed by efflux of the anion due to the effects of membrane potential (Booth and Kroll, 1989). This causes inward movement of protons and dissipation of the proton motive force (PMF). Hence the cytoplasmic pH is lowered to that of the medium and cell death is most likely caused by denaturation of acid-labile proteins and DNA (Cherrington et al., 1991).

1.5.3 *rpoS* mediated resistance and cross protection

The *rpoS* gene controls the expression of proteins essential for survival during periods of starvation and stress (Hengge-Aronis, 1993). Entry into the stationary phase results in production of several proteins that provide resistance to chemical and physical challenge. In addition to protein synthesis, other genes or operons are regulated by activation of *rpoS* (Rees et al., 1995). Such genes include those responsible for osmotic protection and thermotolerance (*osmA*, *osmB*), as well as DNA repair and protection (*dps*, *xthA*). Glycogen synthesis is another feature of stressed cells in stationary phase. Molecules such as glycogen and polyphosphates may be stored for later use if not immediately required

(Hengge-Aronis, 1993). This process is enhanced by *rpoS* and provides the cell with a carbon and energy source in the absence of other nutrients or during starvation. Induction of one response to environmental stress leads to synthesis of proteins specific for other inimical conditions. Starvation of cells leads to osmotic cross-protection (Iyer et al., 1994; Jenkins et al., 1990). Synthesis of many heat-shock proteins is activated by a variety of environmental stresses (Clark and Parker, 1984; Jenkins et al., 1988; Jenkins et al., 1990). Clark and Parker (1984) observed that while osmotic stress resulted in the induction of heat stress proteins, some of these were only expressed transiently, while the osmotic stress proteins were induced for longer. Kilstrup et al. (1997) also recognised that heat shock proteins are induced by osmotic stress, contradicting older reports that this was not the case. Givskov et al. (1994) found that exposure to sublethal osmotic stress resulted in cross-protection to oxidative stress, heat and ethanol, but starvation induced a higher level of protection than sublethal treatments. Acid shock induces many of the recognised heat-stress proteins (Heyde and Portalier, 1990).

1.6 Physiology of inactivation and further study

Literature relating to the study of inactivation kinetics yields one certainty - there is considerable debate over the exact nature of cell death, whether mechanistic or vitalistic in nature, which can only be settled by more systematic collection of precise, descriptive data and physiological characteristics. There is evidence to suggest that the vitalistic theory holds, since tolerance to stress varies with age and resistant tail populations may be removed by dilution, thus producing linear inactivation kinetics. Conversely, previous

studies also suggest that aspects of the mechanistic theory are true, with evidence that cells may be actively resisting a lethal environment through the production of stress proteins. The work presented in Chapter 2 aims to provide a clearer perspective on the nature of cell death under nonthermal conditions, particularly in the context of the mechanistic and vitalistic concepts. In doing so, novel aspects of cell physiology and inactivation will be addressed.

2 Analysis of Nonthermal Inactivation Kinetics: validity of the vitalistic and mechanistic hypotheses

As discussed in Chapter 1, considerable debate exists regarding microbial inactivation kinetics and the cause of deviations from exponential cell decline (refer to [1.3-1.4]). This chapter examines the two hypotheses for microbial inactivation kinetics proposed by Lee and Gilbert (1918), under nonthermal conditions lethal to *E. coli*. Briefly, the hypotheses are:

- the "mechanistic concept" which states that ***all cells have a general similarity of resistance and population decline is akin to a chemical reaction***, and
- the "vitalistic concept", where ***inactivation may be explained on the basis of differing degrees of resistance within a population to an inimical condition.***

Each hypothesis will be tested experimentally to determine whether it is able to account for nonthermal inactivation kinetics observed during production of UCFM.

Appendix A gives details of bacterial strains, media preparation, and equipment used. Brown et al. (1997) found that *E. coli* M23 OR.H- responds similarly to acid stress as some of the most virulent strains of pathogenic *E. coli*. Under growth-permitting conditions, Salter et al. (1998) found little difference in the growth rate and response between *E. coli* M23 OR.H- and pathogenic strains, such as *E. coli* O157:H7, to salt and temperature. Hence the use of a non-pathogenic strain M23 OR.H- was deemed suitable for this study.

Note that error bars were not included on figures due to close agreement between duplicate cultures. Figure 2.1 shows an example of this close agreement with error bars.

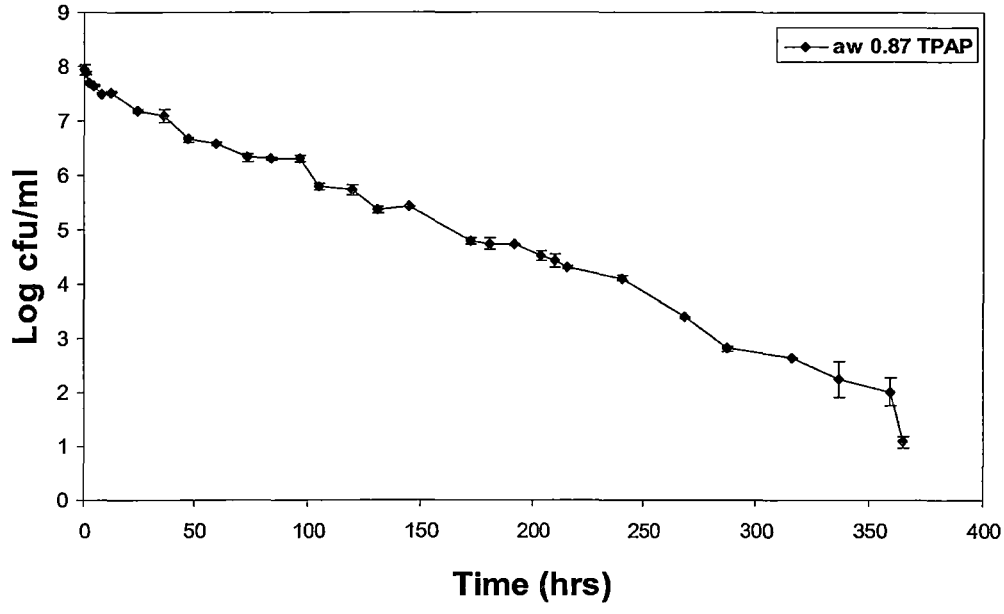

Figure 2.1. Inactivation of stationary phase *E. coli* M23 OR.H- due to lethal a_w (0.87 - NaCl as humectant) in TSB, 25°C. Cells were recovered on TPAP at 37°C.

2.1 Investigation of deviations as an experimental artefact

2.1.1 Introduction

Cerf (1977) suggested experimental artefacts as a possible reason for deviations from exponential inactivation kinetics. Clumping of bacterial spores in suspension was provided as an example. Cerf (1977) stated that clumping during a lethal treatment could account for extended multiphasic inactivation as the biocide would take time to penetrate through the

layers of external cells. Such a hypothesis is at odds with both the mechanistic and vitalistic concepts, and must be discounted before these can be studied further.

Direct microscopy of treatment flasks was sufficient to discount clumping as an artefact in the work of this dissertation. However, additional evidence that tailing may be an artefact under nonthermal conditions of pH and water activity was provided from the results of Mellefont and Ross (2003), who examined the effects of a sublethal a_w shift from 0.997 to 0.962 on the growth kinetics of *Klebsiella oxytoca* using both selective and non-selective recovery media at 37°C. Immediately after the shift, cells were recovered on Brain Heart Infusion Agar supplemented with 0.1% pyruvate (BHIAP) and standard MacConkey (MAC) agar. Substantial injury was produced by the a_w shift as inferred by differences in viable count on selective *versus* non-selective media. After an initial lag period, rapid recovery was observed. On the basis of comparison to a number of predictive models, the increase in counts on MAC was considered too fast to be due to growth alone, as shown in Figure 2.2.

From the work of Mellefont and Ross (2003) the candidate postulated that tailing of survivor curves under nonthermal conditions may be artefactual. Immediately after imposition of a lethal stress a large degree of injury is expected due to shock (Shadbolt et al., 1999). When exposed to lethal pH under nonthermal conditions, *E. coli* M23 OR.H- displays characteristic triphasic inactivation kinetics ([1.3.1], Figure 1.2) with a rapid first phase of inactivation, followed by a second phase of tailing. A third final phase is observed with a similar rate of decline to phase 1. Given that phases 1 and 3 are similar, it was

considered that phase 2 may be an artefact that does not represent a "true" rate of death. What is observed during phase 2 may be a mix of healthy and injured cells (recovering the ability to form colonies on media) that distort the inactivation pattern as shown by Figure 2.3. To investigate the possibility of this occurring, cells of *E. coli* M23OR.H- were exposed to a lethal pH of 3.5 at 25°C.

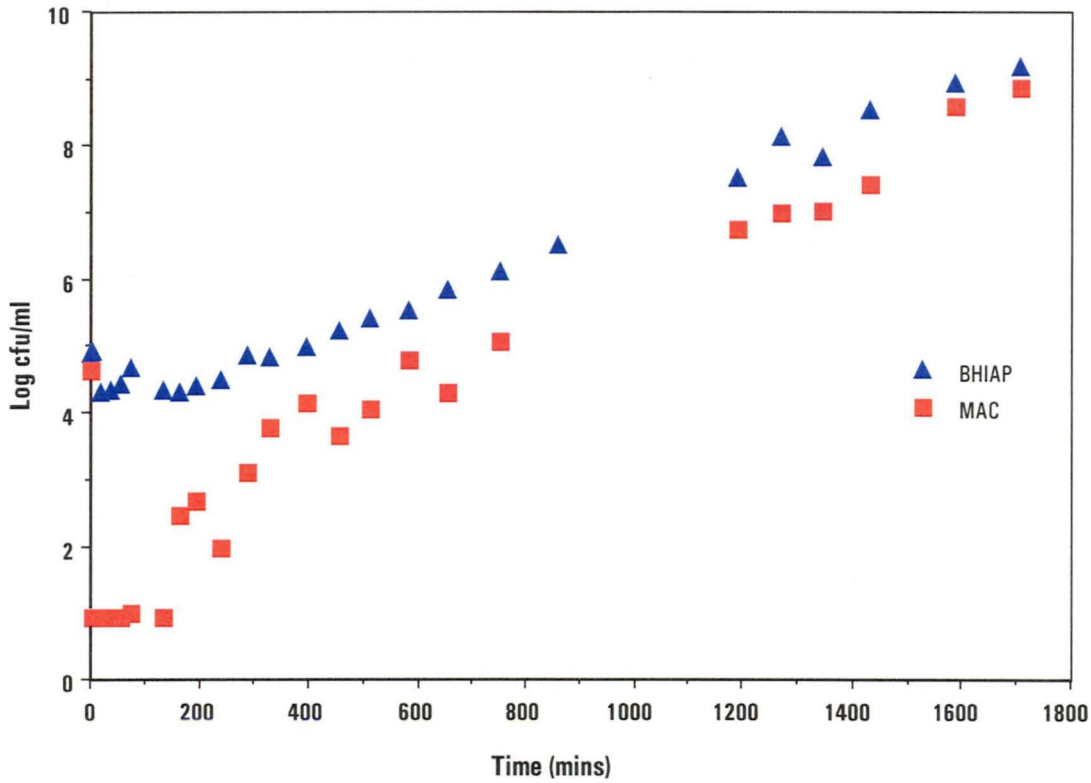

Figure 2.2. Growth of cells in Brain Heart Infusion Broth after a_w shift from 0.995 to 0.962, enumerated on Brain Heart Infusion Agar with 0.1% pyruvate (BHIAP) and MacConkey agar (MAC) (Adapted from Mellefont and Ross, 2003).

For the hypothesis to hold that phase 2 is an artefact, a rapid period of recovery (excluding growth), similar to that in Figure 2.2, should be observed when cell aliquots are removed from a lethal environment and placed into fresh media. Following the rapid recovery, resumption of a normal sigmoidal growth pattern is expected.

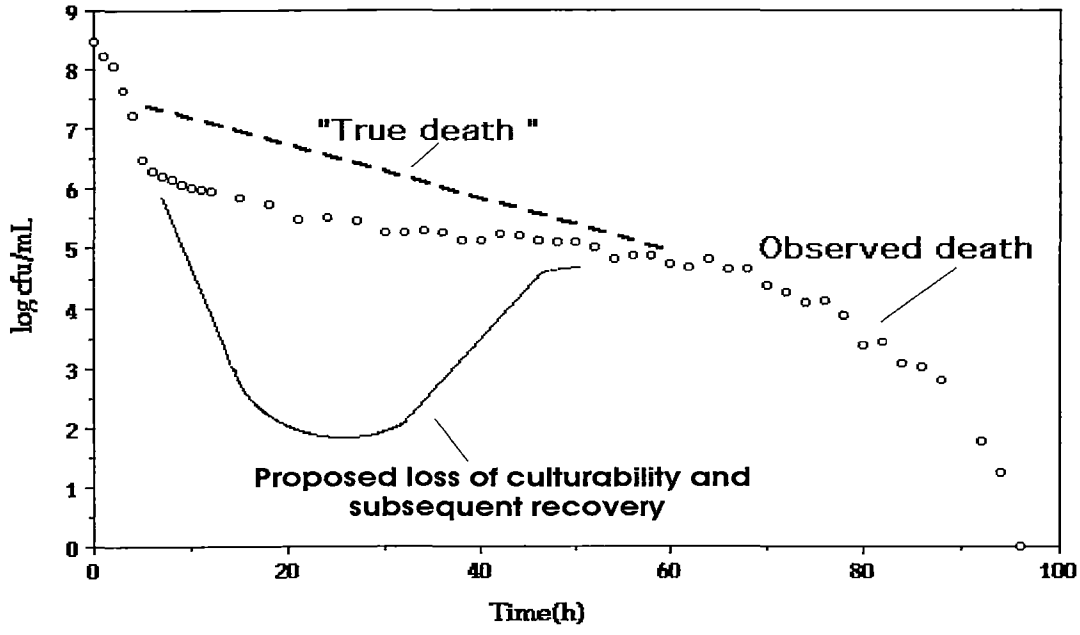

Figure 2.3. Proposed "true death" rate of *E. coli* in response to acid stress of pH 3.50 (HCl as acidulant), 25°C, TSB. Extent of sublethally injured cells is predicted to distort the apparent inactivation kinetics.

2.1.2 Experimental procedure

E. coli M23 OR.H- was recovered from a Nutrient Agar slope and grown on TPAP at 37°C for 18 hours. A standard loopful of this culture was used to inoculate 100ml of TSB in a 250ml Erlenmeyer flask with sidearm for optical density (OD) measurements. This broth was previously temperature equilibrated to 25°C and kept shaking in a water bath. The culture was grown to early stationary phase (25% Transmittance, %T). This was taken as T_0 for the death curve and a 100μl aliquot was removed for spiral plating on TPAP. Immediately following this T_0 sample the pH was lowered to 3.50 using concentrated HCl.

For investigation of a rapid recovery of culturability, aliquots were removed from the acidified broth and pipetted into fresh 100ml TSB in 250ml Erlenmeyer flasks equipped with sidearms for OD measurement. The volume of acidified broth that was required to achieve an inoculum size of *ca* 1 x 10^4 cells/ml for each rapid recovery/growth curve was determined from previous experiments (Brown, unpublished data, 1999). The OD of outgrowth curves was measured at regular intervals and aliquots were removed for plating on TPAP.

All plates were incubated at 37°C for 18 hours before enumeration using an image scanner and software.

2.1.3 Results

The results of the experiment are shown in Figure 2.4. The number of surviving bacteria described by the inactivation curve corresponds to the scale on the y-axis. The same scale is used to represent Log Units of growth for the rapid recovery/growth curves from the point of sampling rather than actual cell numbers in cfu/ml. Aliquots removed for analysis showed no indication of a rapid increase in cell number due to recovery, as proposed by the hypothesis proposed in 2.1.1. It must be noted that from 24 hours onwards, points on the inactivation plot were not obtained by regular plating methods, but were inferred from starting populations of each growth curve. Aliquots removed for enumeration of the inactivation curve contained too many viable cells and were not able to be counted. As

shown in Figure 2.5, lag time increased rapidly over the first 10 hours, consistent with the first phase of inactivation. From 10-40 hours the lag time remained constant, before consistently increasing over the last 8 hours. At 32 hours an unexpectedly long lag time was observed.

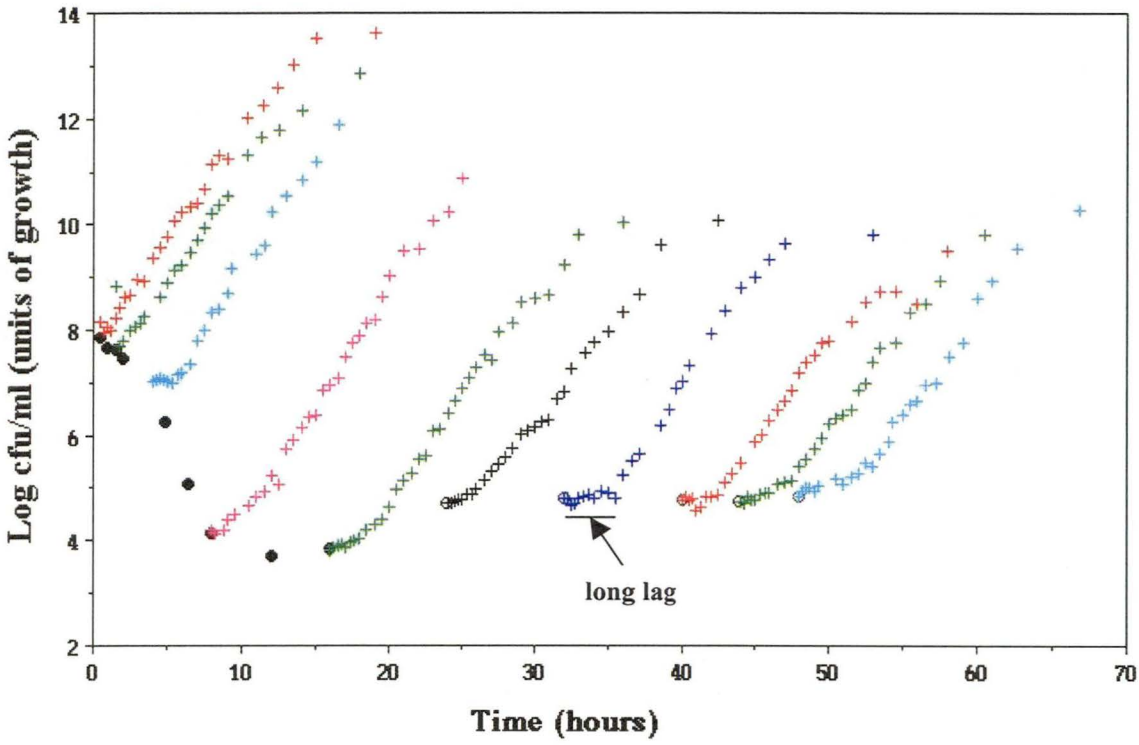

Figure 2.4. Growth of cells removed from low pH (pH 3.5 - HCl as acidulant) TSB broth at 25°C and inoculated into fresh TSB broth, pH 7, 25°C. Cells were enumerated on TPAP at 37°C. Closed circles (●) represent points on the death curve determined by viable count; open circles (○) at 24h onwards indicate points on the death curve that were inferred from starting populations of growth curves of recovered cells; recovery/growth curves indicated by crosses (+)[2].

[2] This experiment was a collaborative effort with colleagues (Drs J. Brown, L. Mellefont, and T. Ross). Intensive preparation and sampling was required on a scale not achievable by the author alone.

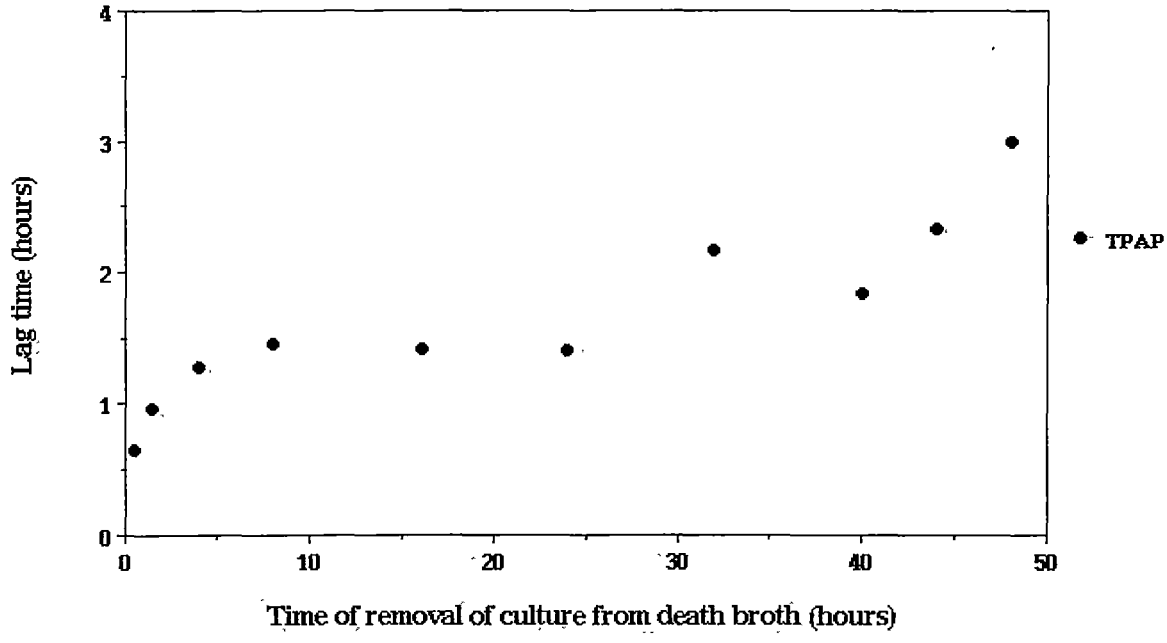

Figure 2.5. Lag time of *E. coli* M23 OR.H- after removal from low acid (pH 3.5 - HCl as acidulant) TSB broth at 25°C and inoculation into fresh TSB broth at 25°C. Cells were enumerated on TPAP at 37°C.

2.1.3 Discussion

From the results shown in Figure 2.4, the observed second phase tailing under conditions of lethal pH was not an artefact caused by recovery of sublethally injured cells. Unlike the results of Mellefont and Ross (2003), no rapid increase in numbers was observed when cells were transferred to fresh TSB under growth permissive conditions. Aside from studies reviewed by Cerf (1977) there is little information to suggest that multiphasic inactivation curves are artefactual. Addition of compounds to block protein synthesis (Humpheson et al., 1998; Wang and Doyle, 1998; Shadbolt et al., 1999), effects of inoculum age [2.2.1], and removal of a resistant subpopulation by dilution [2.2.3] all provide evidence that

inactivation curves are real phenomena which may be manipulated by varying experimental conditions.

The death curve (Figure 2.4) was not well described by viable count. After 20 hours the death curve data points were inferred from starting inoculum sizes obtained from outgrowth curve data (Figure 2.4). At 24 hours a 1-Log unit increase in cell counts occurred. Occasional peaks in death curves are not unusual and may be due to sampling inconsistencies, but have also been attributed to cryptic growth where compounds released from dead cells are utilised by survivors to enable their recovery (Postgate, 1976). However, recovery to a constant level is unusual based on previous inactivation studies involving pH and a_w (Brown, 2002; Shadbolt et al., 1999).

Evidence for a change in cell physiology during the course of the experiment was observed in the form of increasing lag times (Figure 2.5) of recovered populations. The lag time increased rapidly over the first 10 hours, which corresponded with the first phase of inactivation. Between approximately 10 and 24 hours, the lag time was constant, and corresponded with the onset of a second, slower, phase of inactivation. At 32 hours a doubling of the lag time occurred. No explanation other than experimental variation is offered for this. From 40 - 48 hours the lag time steadily increased to a maximum. This may reflect an increase in the extent of injury as a result of exposure to lethal pH, from which the cell requires additional time to recover before initiating growth. Based on the results obtained in [2.3.1], it would seem unlikely that an increase in injury is a valid hypothesis. Cells exposed to pH 3.50 do not appear to experience high levels of injury. A

more plausible reason for the extended lag may be the lack of available energy to initiate growth. Exposure to acid requires the constant removal of H^+ ions from the protoplasm (Ingraham and Marr, 1996). An alternative explanation is that increased carry-over of liquid from the treatment broth after prolonged pH 3.50 exposure may also result in the increased lag times. To obtain a sufficient number of cells for initiation of proposed rapid-recovery curves, larger aliquots were removed from the treatment flask over the course of the experiment to allow for loss of cell viability with time. These aliquots may have altered the pH and introduced detrimental compounds into the fresh TSB medium to result in increased lag times from 40 hours onwards.

Despite unresolved questions relating to the extent of injury and increasing lag time duration, it would appear likely that the tailing observed under lethal pH is not an artefact caused by recovery of culturability of a sublethally injured, and viable but non-culturable population. Discussion will now focus on the mechanistic and vitalistic concepts, with analysis of the novel information on bacterial inactivation kinetics presented in [2.2-2.3].

2.2 Examination of the Vitalistic Hypothesis

2.2.1 Effect of Inoculum Age on Inactivation

2.2.1.1 Introduction

Previous research has supported the vitalistic hypothesis in describing a variation in resistance with the age of the inoculum (Sherman and Albus, (1923); Watkins and

Winslow, (1932); Moats et al., (1971)). As noted in [2.1], difficulty was experienced in enumerating points on the inactivation curve, despite apparently identical preparation procedures being employed to generate previous curves. To investigate the effect of slight variations in inoculum age, inocula were harvested from early stationary phase populations of *E. coli* M23 OR.H- at four different optical densities but with similar cell numbers.

2.2.1.2 Experimental procedure

E. coli M23 OR.H- was recovered from agar slopes and grown on TPAP as described above [2.1.1]. Standard loopfuls of bacteria were inoculated into duplicate 250 Erlenmeyer flasks equipped with sidearms for OD measurement, containing 100ml TSB. Each flask was previously temperature equilibrated to 25°C and kept shaking in a waterbath. Inocula were prepared by growth to OD of 30%T, 24%T, 20%T and 15%T (corresponding to densities between 8.1 Log cfu/ml [30%T] and 8.3 Log cfu/ml [15%T]). At each density, a 100μl aliquot was removed and spiral-plated on TPAP, corresponding to T_0 for the experiment. Immediately following this sample the pH of each broth was lowered to 3.50 using HCl. Broths were sampled at intervals and *E. coli* enumerated as described above [2.1.1].

2.2.1.3 Results

The results are shown in Figure 2.6. At the termination of the experiment a culture grown to 30%T was found to display a similar pattern of inactivation to that grown to a higher density of 24%T. However, after 36 hours the culture harvested at 30%T demonstrated 0.5 Log cfu/ml better survival. A culture grown to 20%T was found to display better survival during the first phase of inactivation than a culture harvested at 15%T. Both cultures experienced similar decline over the second phase of inactivation, and after 36 hours the population grown to 20%T displayed a 0.5 Log cfu/ml higher viable count.

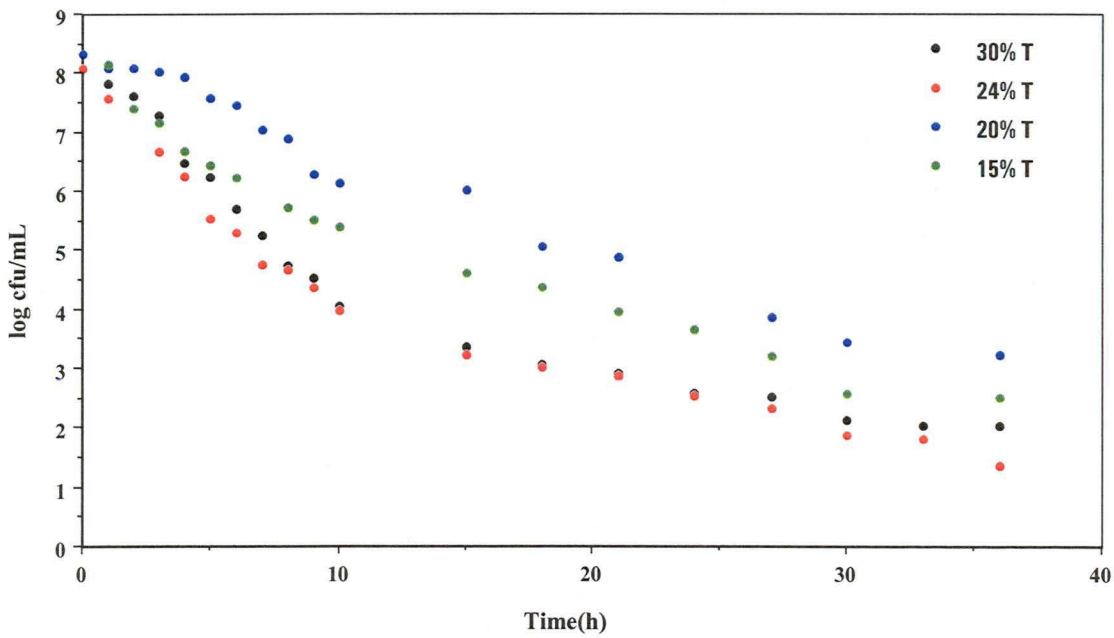

Figure 2.6. Effect of inoculum age on the acid-induced inactivation kinetics of *E. coli* M23 OR.H- , TSB pH 3.50 (HCl as acidulant) 25°C, from inocula harvested at four different cell densities. Cells were enumerated on TPAP at 37°C[3].

[3] This experiment was a collaborative effort with colleagues (Drs J. Brown, L. Mellefont, and T. Ross). Intensive preparation and sampling was required on a scale not achievable by the author alone.

2.2.1.4. Discussion

As noted in [2.1], viable count data were not available to describe fully the latter stages (24-68 hours) of the inactivation curve in Figure 2.4. While identical conditions had been used previously to generate a nonthermal death curve, it was apparent that inactivation processes can be highly variable. This is evident from in Figure 2.6 which shows that minor adjustments to inoculum preparation had considerable impact on the kinetics of inactivation. Cells in that experiment were harvested at a similar density (difference of approximately 0.2 Log cfu/ml from 30%T – 15%T) with a period of several minutes only between the desired optical density of the initial and final inoculum.

It was initially hypothesized that, as the optical density (%T) declined, cells would move further into stationary phase and their tolerance to inimical conditions would increase. This proved not to be the case. An alternative explanation for the differences is now proposed. The inoculum used for all four densities was a stationary phase culture. The culture harvested at 30%T included cells in stationary phase. Hence this population displayed better survival at pH 3.50 than the culture harvested at 24%T, in which more cells had entered the exponential phase. A culture harvested at 15%T was found to decline marginally faster than one grown to 20%T. It was speculated that the former possessed more detrimental metabolic by-products as a result of cellular respiration which contributed to the faster decline.

The difference in survival between a culture grown to 30%T compared with that grown to 24%T might be analogous to the results observed by Chick (1908), as discussed in [1.3.3]. Chick (1908) observed that a culture of *Bacillus* spp grown for 3 hours survived better than one grown for 24 hours. This would be feasible if there was a larger spore fraction present in the culture. Work by Brown (2002) demonstrated the reproducibility of minor variations in inoculum density that affect cellular resistance to stress.

These results illustrate two key points. The first is the need for researchers to be rigorous in their replication of experimental conditions when deriving inactivation data, particularly with inoculum preparation. The second point is that a variation in resistance from minor deviations in optical density provides evidence for the vitalistic hypothesis. A minor change in the cell density of the inoculum altered the distribution of resistance to acid stress.

2.2.2 Effect of Dilution on Resistance of Stationary Phase *Escherichia coli* M23 OR.H- at Low a_w

2.2.2.1 Introduction

Brown (2002) observed that when a stationary phase culture of *E. coli* was diluted 100-1000 fold the first phase of inactivation usually observed was eliminated resulting in an extended period of survival or "shouldering". Similarly, a culture prepared by the candidate and incubated statically at 37°C for 22 hours to a late stationary phase density of *ca* 1 x 10^9

cells/ml, displayed greater tolerance to acid stress and no rapid first phase of inactivation when diluted 10-fold prior to the inimical treatment (refer also to [4], Fig 4.5). Brown (2002) attributed this pattern of inactivation to cell density and challenged several established concepts regarding enhanced resistance of bacteria in the stationary phase. The first of these challenged concepts is that bacteria grown to maximum population density (MPD), enter stationary phase due to nutrient limitations or inhibition by toxic metabolic byproducts. Brown (2002) was able to grow *E.* coli to normal MPD levels in spent TSB broth, challenging the conventional wisdom that stationary phase is entered as a consequence of nutrient depletion or accumulation of toxic metabolites. Brown (2002) proposed that live bacteria at a density of 1 x 10^9 were able to sense neighbouring cells in close proximity and produce an unknown signal factor which prevented replication, but was not lethal in itself. However, when combined with a lethal pH stress this signal factor resulted in a rapid 1000-fold inactivation, the "first phase". Bacteria at lower population densities were able to persist longer before a final period of rapid decline. At a lower density of approximately 10^6 cells/ml, it was hypothesised the signal factor was not present in sufficient quantity to result in rapid bacterial destruction.

To examine the applicability of this concept to cells exposed to lethal a_w stress , a stationary phase culture of *E. coli* M23 OR.H- was grown to *ca* 1 x 10^9 cells/ml and exposed to a_w 0.90 at dilutions of 10^8, 10^7 and 10^6 cells/ml. At the latter two dilutions, inocula were added as washed or unwashed aliquots of the parent stationary phase culture.

2.2.2.2 Experimental procedure

The inoculum for all flasks was prepared as described in Appendix A. TSB (45.0ml) was placed into a 250ml Erlenmeyer flask and 9.60g NaCl was added so that addition of 5.0ml stationary phase inoculum yielded a final a_w 0.90. The flask was placed into a shaking water bath (80rpm) for several hours at 25°C to temperature-equilibrate before inoculation.

To achieve cell densities of 10^7 and 10^6cells/ml respectively, 1.00ml and 100µl aliquots of the parent inocula were combined with 4.00ml and 4.90ml of fresh TSB respectively before addition to the a_w-adjusted flasks. Washed inocula at the same cell density were prepared by centrifugation (8000*g*, 10 min) and resuspension in fresh TSB. Aliquots were removed for enumeration at intervals as described in Appendix A.

2.2.2.3 Results

The effects on *E. coli* M23 OR.H- at three different stationary phase cell densities exposed to a lethal a_w 0.90 is shown in Figure 2.7. All cultures experienced similar first and second phase inactivation kinetics. No differences were observed between washed or unwashed cultures at the two inoculum levels examined.

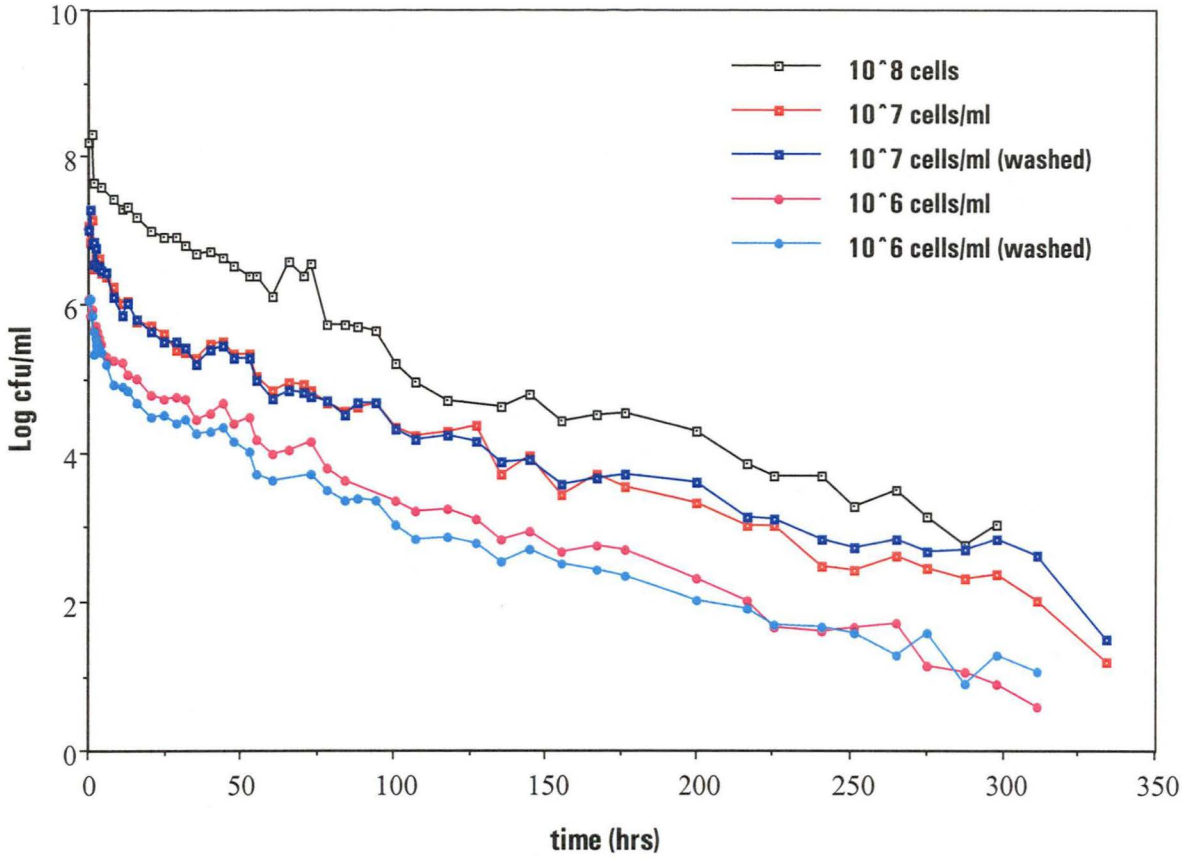

Figure 2.7. Effect of inoculum dilution and washing on the inactivation kinetics of *E. coli* M23 OR.H- , a_w 0.90 (NaCl as humectant) 25°C, TSB. Cells were enumerated on TPAP at 37°C.

2.2.2.4 Discussion

Dilution of stationary phase cultures to 10^{6-8}cells/ml was observed to have no effect on the inactivation kinetics of *E. coli* M23 OR.H-. Washing cells to remove any possibility of the detrimental signal factor proposed by Brown (2002) prior to lethal a_w exposure also had no effect on the survival curves (Figure 2.7). Diluted and washed cultures displayed almost identical first and second phase rates of inactivation to untreated controls. Unlike exposure

to lethal pH, dilution did not result in a shoulder period where the first phase of inactivation was eliminated. This would indicate that the cell density theory discussed in [2.2.2.1] is not applicable for a_w stress. This finding suggests that the mechanisms of a_w and pH mediated cell death are fundamentally different and aspects of their respective inactivation patterns are unique. In terms of inactivation hypotheses, it would appear that the vitalistic concept is inadequate under these conditions. If the observed inactivation patterns were simply due to a variation in the states of resistance it would be expected that similar kinetics would be shown for a_w and pH. Elimination of first phase inactivation by dilution of stationary phase populations and the presence of a distinct third phase appear characteristic for acid stress. Such trends are not observed with exposure to lethal water activity. This implies that physical or chemical mechanisms (not biological variability) are responsible for deviation from log-linear inactivation kinetics.

There are, however, aspects of these results that also support the vitalistic concept. These findings suggest that a resistant fraction of cells may be removed by dilution and, hence, that not all cells have similar resistance. If this were not the case then multiphasic inactivation kinetics should be observed at any cell density, according to the mechanistic concept. This aspect is considered in [2.2.3].

2.2.3 Effect of Dilution on Resistance of Late-Exponential Phase Escherichia coli *M23 OR.H- at Low pH (3.5)*

2.2.3.1 Introduction

Cells in stationary phase are inherently more resistant to many stresses than those in exponential phase due to induction of the Rpos factor (Rees et al., 1995). Examination of the inactivation kinetics of exponential phase cells exposed to pH 3.50 at 25°C revealed a small subpopulation of resistant bacteria (Brown, 2002). To examine the effect of dilution on the acid resistance of this population at pH 3.50, *E. coli* M23 OR.H- inocula were prepared according to the method of Brown (2002) and serially diluted before exposure to a pH stress. This resulted in a bacterial population with all cells in exponential phase.

2.2.3.2 Experimental procedure

A TPAP plate was inoculated with a standard loopful of culture from a NA slope stored at 4°C. The TPAP plate was incubated at 37°C for 14h. A standard wire loop of culture from the TPAP plate was used to inoculate 8.00ml TSB in a 15ml centrifuge tube adjusted to pH 7.0. The centrifuge tube was incubated statically for 12h at 37°C. From the centrifuge tube, 60μl of TSB was transferred into 60ml of temperature-equilibrated (25°C) TSB, adjusted to pH 7.0, in a 250ml Erlenmeyer flask shaking in a waterbath.

The culture was incubated for 5.5h with shaking at 25°C. From the Erlenmeyer flask, 60µl was transferred into a fresh 60ml TSB broth prepared as above (equilibrated at 25°C, adjusted to pH 7.0). This was incubated for 7h with shaking at 25°C. The final cell density was *ca* 1 x 10^8 cells/ml.

A 100µl aliquot was removed for enumeration (T_0) and the pH of the broth was then adjusted to 3.50. Surviving bacteria were enumerated at appropriate intervals using the method described in Appendix A.

The inoculum prepared as described above was diluted 100- and 1000- fold into fresh 60ml temperature-equilibrated (25°C), TSB adjusted to pH 7.0, in 250ml Erlenmeyer flasks that were shaking in a waterbath. The pH of the broths was then adjusted to 3.50 and surviving bacteria enumerated as described above.

2.2.3.3 Results

The response of non-dilute and dilute exponential phase cultures of *E. coli* M23 OR.H- at 25°C to an acid stress of pH 3.50 is shown in Figure 2.8. The culture with a starting density of 10^8cells/ml displayed a rapid 6-Log decline in number before tailing occurred. No cells were recovered after 24 hours. A culture diluted to 10^6cells/ml decreased to less than 0.5 Log cfu/ml within 4 hours and was non-detectable within 9 hours. The culture diluted to 10^5 cells/ml displayed linear inactivation kinetics and declined below the level of detection after 3 hours.

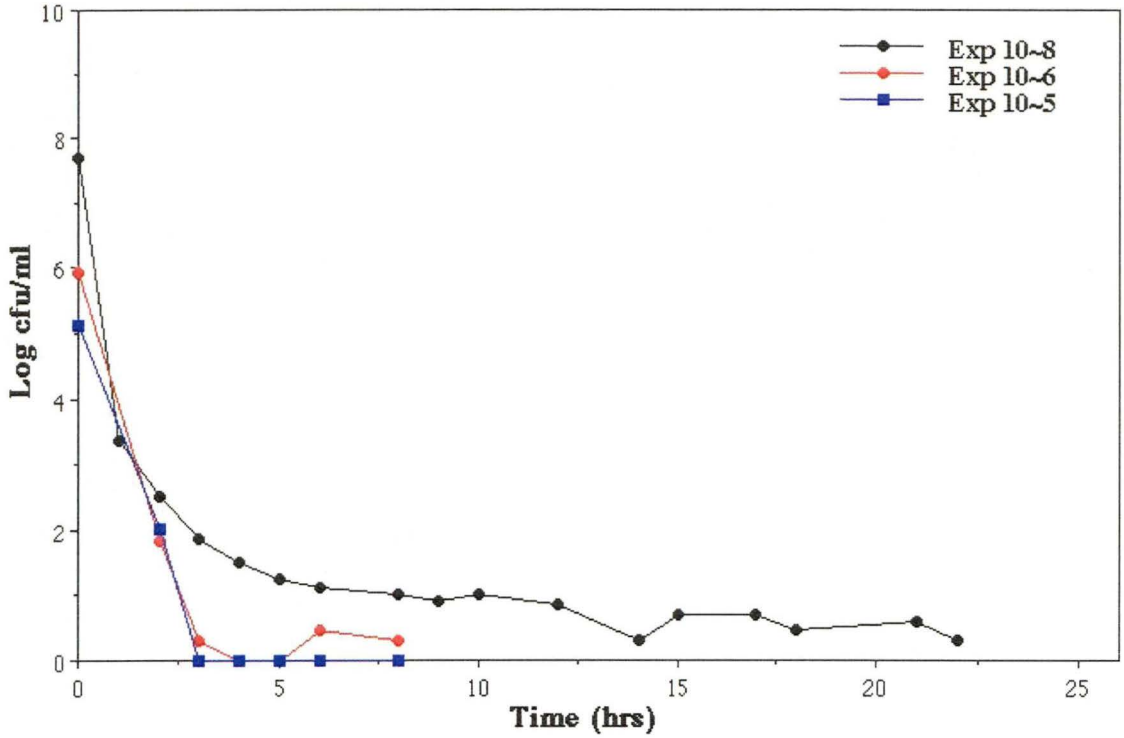

Figure 2.8. Inactivation kinetics in response to dilution and acid-injury (pH 3.50, HCl as acidulant) of exponential phase cells of *E. coli* M23 OR.H- , 25°C, TSB. Cells were recovered on TPAP at 37°C.

2.2.3.4 Discussion

Additional support for the vitalistic hypothesis may be derived from the results shown in Figure 2.8. An exponential phase population of 10^8cells/ml experienced a 6-Log decline in response to a stress of pH 3.50. If the mechanistic hypothesis was solely responsible for the tailing observed above, exponential phase populations should display similar kinetics at any dilution. When the 10^8cfu/ml population was diluted to 10^6 and 10^5cfu/ml, the observed inactivation patterns were not similar. No tailing was evident in the population of lowest density. Markedly reduced tailing was observed in the culture with 10^6cells/ml initially.

These findings indicate that a resistant population was reduced by simple dilution of the parent culture. This supports the observations of Vas and Proszt (1957) and Moats et al. (1971) and provides evidence for the vitalistic hypothesis.

2.3 Examination of the Mechanistic Hypothesis

2.3.1 Comparison of Stationary Phase pH and aw Injury

2.3.1.1 Introduction

To compare the effects of injury caused by pH and a_w stress, stationary phase cultures of *E. coli* M23 OR.H- were exposed to lethal conditions of pH 3.50 and a_w 0.87. Two different methods of inoculum preparation were used for each stress. This will affect the inactivation kinetics (refer to [2.2.1]) but still provide comparisons of injury to stationary phase cells. The most practical method of preparing stationary phase inocula for exposure to a_w stress was that described in [2.2.2.2] above. Briefly, 5.00ml of late stationary phase cells grown statically for 22 hours at 37°C (*ca* 1 x 10^9cells/ml) was added to 45.0ml TSB. The inoculum to be acid-stressed was prepared according to the method of Brown (2002) in order to yield a high-density culture and to facilitate comparison with her results.

2.3.1.2 Experimental procedure

A TPAP plate was inoculated with a standard loopful of culture from a NA slope stored at 4°C. The TPAP plate was incubated for 14h at 37°C. A standard wire loop of culture was removed from the TPAP plate and used to inoculate 8.00ml TSB in a 15ml centrifuge tube adjusted to pH 7.0. The centrifuge tube was incubated statically for 12h at 37°C. From the centrifuge tube, 60μl TSB was transferred into 60ml of temperature-equilibrated (25°C), fresh TSB adjusted to pH 7.0, in a 250ml Erlenmeyer flask shaking in a waterbath. The Erlenmeyer flask was incubated for 5.5h with shaking at 25°C. From the Erlenmeyer flask, 60μl TSB was transferred into a fresh 60ml TSB broth prepared as above. This was incubated for 24h with shaking at 25°C to a cell density of *ca* 10^9cells/ml. A 100μl aliquot was removed for enumeration (T_0) and the pH of the broth lowered to 3.50. Surviving bacteria were enumerated at appropriate intervals using the method described in Appendix A.

For both treatments, bacteria were plated on TPAP and TPABS to determine the extent of injury.

2.3.1.3 Results

Examination of the difference in injury levels resulting from the a_w and pH treatments applied indicated that the former stress results in considerably more damage to the bacterial

cell (Figure 2.9). Inactivation data for cells exposed to a_w 0.87 revealed a 0.5 Log cfu/ml first phase decline for bacteria recovered on TPAP against a 1.8 Log cfu/ml decline for those plated on TPABS. Both first phases were of a similar rate. The extent of a_w injury increased as the experiment progressed, with no recovery observed on TPABS after 132 hours. At this time there was a 5.5 Log cfu/ml population of injured cells from an initial level of 8 Log cfu/ml (calculated by difference in recovery on TPAP and TPABS). The rate of second phase inactivation for cells enumerated on TPABS was approximately 2.6 fold higher than those enumerated on TPAP.

E. coli M23 OR.H- cells exposed to a pH of 3.50 displayed a markedly different inactivation pattern to that of a_w stressed bacteria. Instead of an increasing level of damage, pH-treated stationary phase cells showed an approximate level of 90% injury to the surviving population throughout the experiment. Acid-induced inactivation rates were similar for cells enumerated on both TPAP and TPABS, and the results comparable with those of Brown (2002) (not shown). After 150 hours a rapid third phase of inactivation occurred, and no cells were recoverable on TPABS and TPAP at 185 and 194 hours respectively.

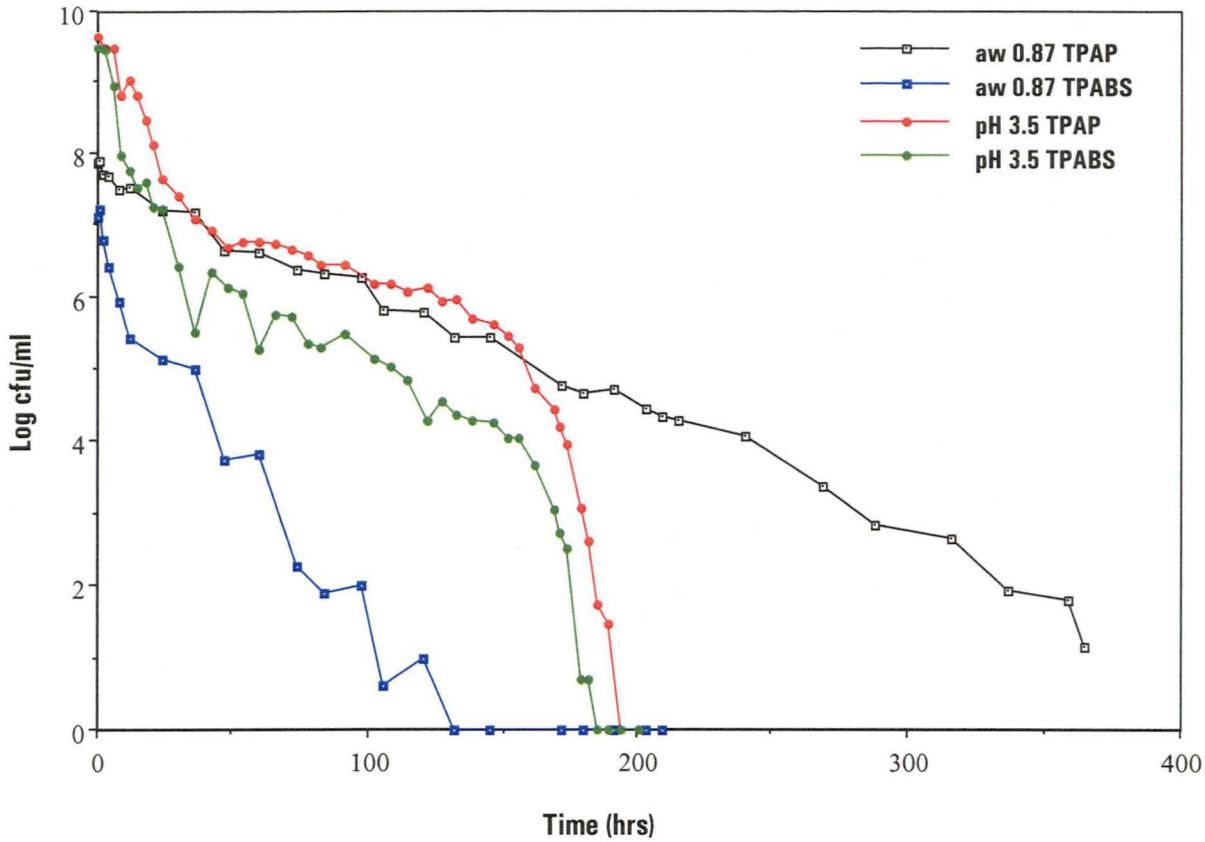

Figure 2.9. Comparison of injury to stationary phase cultures of *E. coli* M23 OR.H- due to lethal a_w (0.87 - NaCl as humectant) and lethal pH injury (3.50 - HCl as acidulant) in TSB, 25°C. Cells were recovered on TPAP or TPABS (to quantify injury) at 37°C.

2.3.1.4 Discussion

Results from this experiment demonstrate the importance of following inactivation curves to the limit of detection, as suggested by Jordan and Jacobs (1944) and Moats et al., (1971). After 50 hours cultures exposed to both types of stress had a similar population density. At this time, counts on TPAP under both types of stress were equivalent and remained so for a further 5 days. After this period the acid-stressed culture commenced a characteristic third

phase of inactivation whilst the cells subjected to low a_w continued to decline at a constant rate. Termination of the experiment at this time could have led to the conclusion that a_w and pH inactivation was identical under the conditions (based on TPAP counts) imposed. Use of this erroneous assumption in the generation of models for the food industry would lead to overestimation of the time required for inactivation of pathogens under the influence of the low pH under the conditions employed.

Based on the conditions employed for this experiment pH was more stressful than a_w. Once the third phase of inactivation commenced cultures exposed to lethal pH remained viable for a period of 20 hours. In contrast, bacteria experiencing lethal a_w stress persisted for a further 200 hours.

On the basis of injury alone pH appears to be the least stringent environmental parameter than water activity at the levels imposed. Figure 2.10 shows the comparison of a_w to pH injury. The *rate* of cells becoming *injured* at a_w 0.87 was 5-fold higher than that at pH 3.50. Injury under the a_w stress reached a maximum of 5.3 Log units at 145 hours, after which no cells were recoverable on TPABS and hence all remaining bacteria were considered as damaged. Acid-induced injury rose slowly to approximately 1.5 Log units at 170 hours, although several peaks above this were apparent. After 170 hours the third phase of inactivation commenced, in which injury was observed to peak at 2.4 Log units before rapidly decreasing with cell viability.

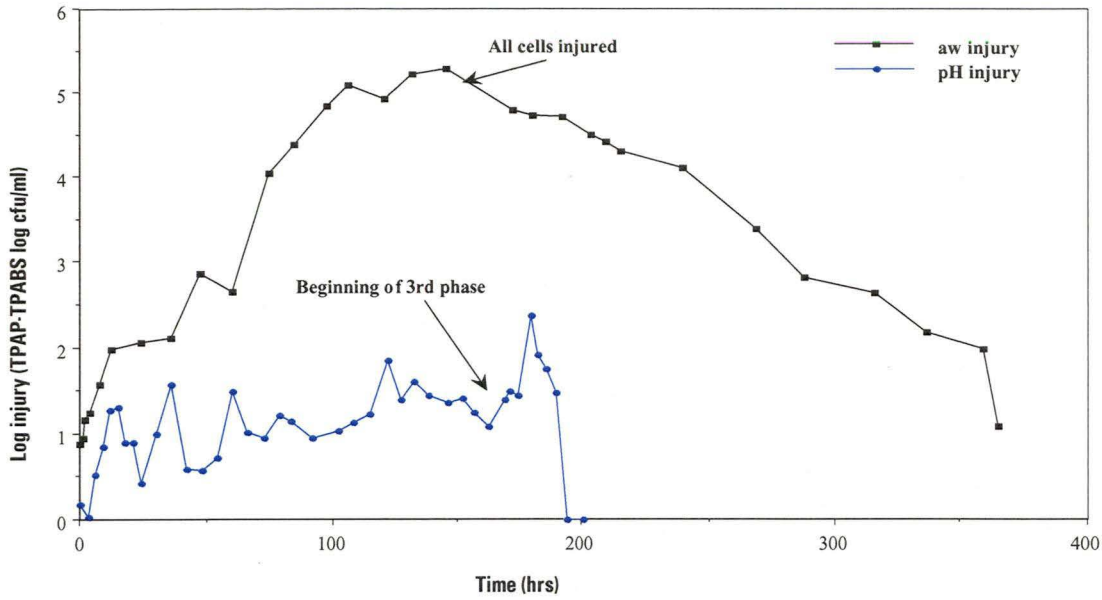

Figure 2.10. Comparison of stationary phase a_w (0.87 - NaCl as humectant) and pH injury (3.50 - HCl as acidulant) of *E. coli* M23 OR.H- in TSB, 25°C.

These results provide further evidence for different mechanisms of inactivation influencing the shape of pH-induced non-linear survivor curves compared to a_w-induced inactivation. They also suggest that the hypothesis proposed in [2.1.] was incorrect. For the hypothesis to hold, a rapid recovery of a large population (in the order of 3-Log cfu/ml based on Figure 2.2) of sublethally injured cells would have been expected. The results shown in Figure 2.10 indicate that this was not possible given that pH (at the level imposed) does not cause substantial injury. The hypothesis put forward in [2.1] is not applicable to lethal a_w stress as no third phase of inactivation is observed under such conditions. Regardless that the hypothesis in [2.1] was not supported there is sufficient proof to show that multiphasic inactivation curves are not due to experimental artefacts. In this case (figure 2.9) the shape of the curves varied, with the effects of water activity and pH appearing to result from different mechanisms of inactivation.

These results also indicate the need to define more accurately the use of the terms 'injury' and 'stress', particularly in the context of combined lethal parameters. At the levels imposed, water activity caused the greatest *injury* to bacteria based on differential viable counts from TPABS. In terms of lethality, pH imposed the greater *stress* on the cell. However, if stress is equated with cell injury, in this instance water activity is more *stressful* but pH is the more *lethal* parameter. Care must, therefore, be taken when describing conditions as "stressful" to bacteria. Henceforth in this dissertation, 'stress' will be equated with lethality whenever two lethal constraints are present.

2.3.2 Effect of Lethal Hurdle Sequence

The influence of a second lethal stress (SLS) on inactivation kinetics was investigated by exposing populations of *E. coli* M23 OR.H- to either a lethal low water activity of 0.90 or a pH of 3.50, and then applying a subsequent second lethal environmental stress. Inactivation rates were examined in response to variations in the order and timing of imposing the SLS.

2.3.2.1 Water activity and pH shock treatments

Initial water activity shock

TSB (45.0ml) was placed into a 250ml Erlenmeyer flask and 9.60g NaCl added so that addition of 5.00ml stationary phase inoculum yielded a final a_w 0.90. The flask was placed

into a shaking water bath at 80rpm for several hours at 25°C to temperature-equilibrate before inoculation. The broth was inoculated with a stationary phase inoculum of *E. coli* M23 OR.H- prepared as described in Appendix A. After 24 hours a SLS of pH 3.50 was imposed by addition of concentrated HCl.

A control prepared in identical fashion was also monitored but with no second lethal stress treatment.

Initial pH shock

A 250ml Erlenmeyer flask containing 45.0ml TSB was prepared, placed in a shaking water bath and allowed to equilibrate to 25°C as described above. A stationary phase inoculum (prepared as described in Appendix A) was added and the pH adjusted to 3.50 using concentrated HCl. After 24h a SLS was imposed by adding NaCl to achieve a final a_w 0.90.

A pH control was prepared similarly to the treatment flasks, but no SLS was imposed. A further treatment consisted of a culture in which both stresses were applied at the commencement of the experiment by addition of a 5.00ml stationary phase culture of *E. coli* M23 OR.H- to 45.0ml TSB broth so that at T_0 the a_w and pH were 0.90 and 3.50, respectively. All experiments were conducted with duplicate cultures. Cultures were sampled at intervals and *E. coli* surviving enumerated.

2.3.2.2 Variation in timing of SLS imposition

An identical experiment was prepared as described in [2.3.2.1] with the exception that the SLS was introduced after 12 hours. A further experiment was conducted in which an initial pH shock of 3.50 was followed with a SLS of a_w 0.90 after 2, 12 and 24 hours exposure in separate flasks from a single inoculum. All experiments were conducted with duplicate cultures.

2.3.2.3 Results

The decline of *E. coli* M23 OR.H- in response to the various a_w and pH stresses imposed is shown in Fig. 2.11. All test cultures experienced a rapid 1 log cfu/ml decline when first exposed to lethal a_w or pH stress, before exhibiting a slower inactivation rate or tailing for approximately 20 hours. The control culture (A5 - both a_w 0.90 and pH 3.50 imposed at zero time) displayed similar inactivation kinetics to the pH control (A3) throughout the course of the experiment. The a_w control (A1) displayed better survival than either the pH or combined pH + a_w controls and experienced a 2-Log cfu/ml decline in comparison with a 7-Log cfu/ml decrease in the latter treatments after 74 hours.

The survival of *E. coli* M23 OR.H- in the water activity control treatment closely followed that of the treatment first exposed to a_w before addition of the SLS (A2). When the pH of the treatment was lowered to 3.50 after 24 hours, a second rapid inactivation of 3-Log cfu/ml was observed over approximately 3 hours, followed by the emergence of a

subpopulation which experienced a 4-Log cfu/ml reduction in the next 50 hours, at a rate similar to that of the pH 3.50 treatment.

The *E. coli* M23 OR.H- exposed to an initial acid shock also displayed similar inactivation kinetics as the pH control before the SLS was imposed (A4). However, when the a_w was lowered to 0.90 the treatment culture experienced a rapid 6-Log cfu/ml decrease over approximately 5 hours and survivors were below the limit of detection 7 hours later. It was also noted that the culture initially exposed to lethal pH had declined by over 2-Log units before addition of the SLS, while those exposed to low a_w first experienced a 1-Log decrease in number.

Figure 2.12 shows the inactivation kinetics after imposition of a SLS at 12 hours. Inactivation kinetics for cultures that experienced an initial a_w shock followed by pH were also similar. The culture experiencing a SLS at 24 hours (A2) showed slightly higher rates and magnitudes of inactivation than that (B2) which was exposed to an SLS at 12 hours, showing that a_w stress was less lethal than the pH stress under the conditions imposed.

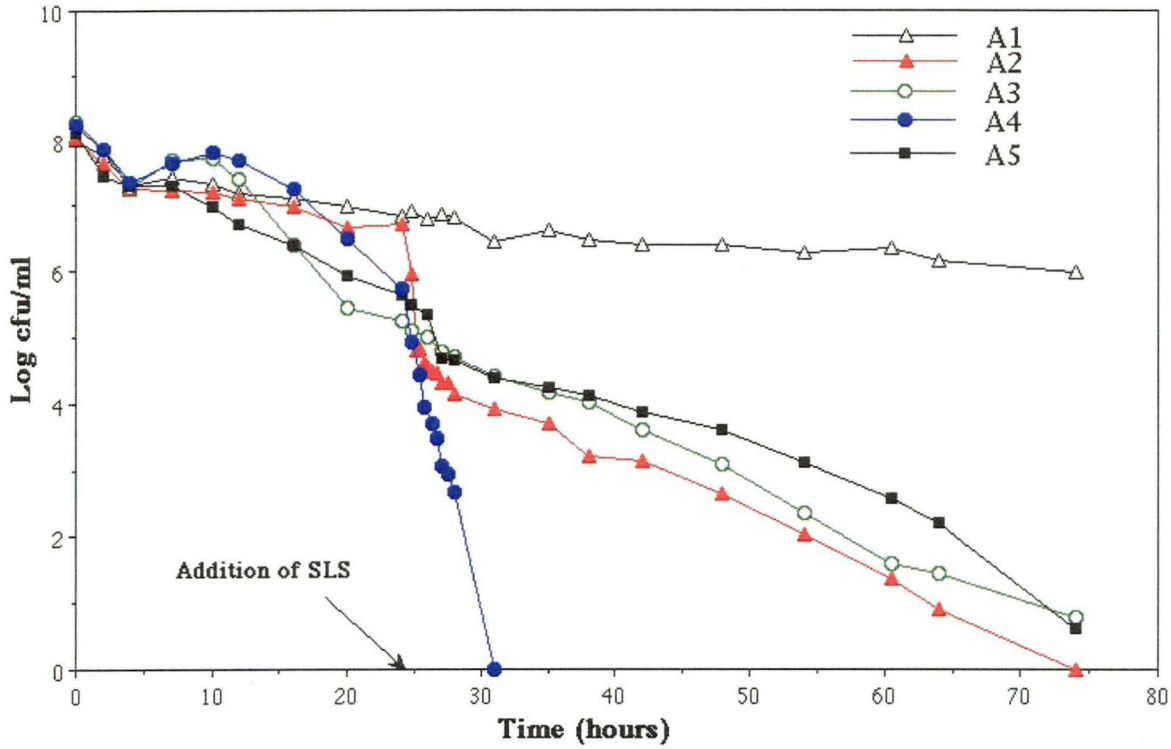

Figure 2.11. Inactivation of *E. coli* M23 OR.H- in response to lethal a_w (NaCl as humectant) and pH (HCl) stress at 25°C, TSB. SLS added at 24h mark. Cells were recovered on TPAP at 37°C. Cultures exposed to a_w 0.90 (Δ), a_w 0.90 followed by SLS pH 3.50 (▲), pH 3.50 (○), pH 3.50 followed by SLS a_w 0.90 (●), a_w 0.90 and pH 3.50 from T_0 (■).

Inactivation kinetics for a culture exposed to an initial pH shock followed by a water activity stress was considerably different at 12 (B4) and 24 hours (A4). While both cultures showed a similar rate of decline after imposition of the SLS, the culture exposed to a SLS at 12 hours declined by approximately 4-Log units before a change in the rate of decline was observed at 18 hours. This is in contrast with a 6-Log unit decline shown by the 24 hour culture (Figure 2.11) where no cells were recoverable on TPAP 7 hours after the SLS was imposed. In Figure 2.12 it can be seen that a culture with a SLS imposed after 12 hours persisted for a further 16 hours before declining below a detectable level. The slower rate of decline following imposition of the SLS was approximately 8 hours in duration. After this a

final period of faster decline was observed. Comparison of inactivation rate data for the a_w control cultures (A1, B1) reveals similar rates and magnitudes of decline. The pH controls (A3, B3) displayed near identical rates and magnitudes of decline for both experiments. The combined a_w and pH controls (A5, B5) displayed similar rates of inactivation, although the culture exposed to a SLS at 12 hours exhibited an additional 1-Log cfu/ml first phase decline.

Figure 2.13 describes the inactivation patterns obtained after three SLS treatments to acid-stressed cultures at 2, 12 and 24 hours. All treatments were prepared from the same inoculum. Prior to addition of a SLS, the culture exposed to an initial pH stress for 24 hours had declined by 3-log units, while the other two populations remained almost unchanged, exhibiting a prolonged shoulder period. Addition of the a_w shock resulted in phases of rapid inactivation, increasing in magnitude as cultures were exposed to acid stress for a longer duration. The 12 hour culture displayed a similar pattern of decline as that in the analogous experiment (Figure 2.12), with a short phase of tailing after the SLS was added. The culture to which the SLS was imposed after 2 hours showed a very different pattern of decline. A 4-Log cfu/ml population decline occurred after the SLS. A slower rate of inactivation was observed for another 30 hours before the experiment was terminated. Comparing this culture to control cultures (see Figs. 2.11, 2.12) in which the combined a_w and pH stress began at T_0, the 2 hour SLS treatment exhibited a greater initial rate and magnitude of decline after imposition of the stress. After 30 hours the latter was also 1-2 Log units lower in number than the control cultures.

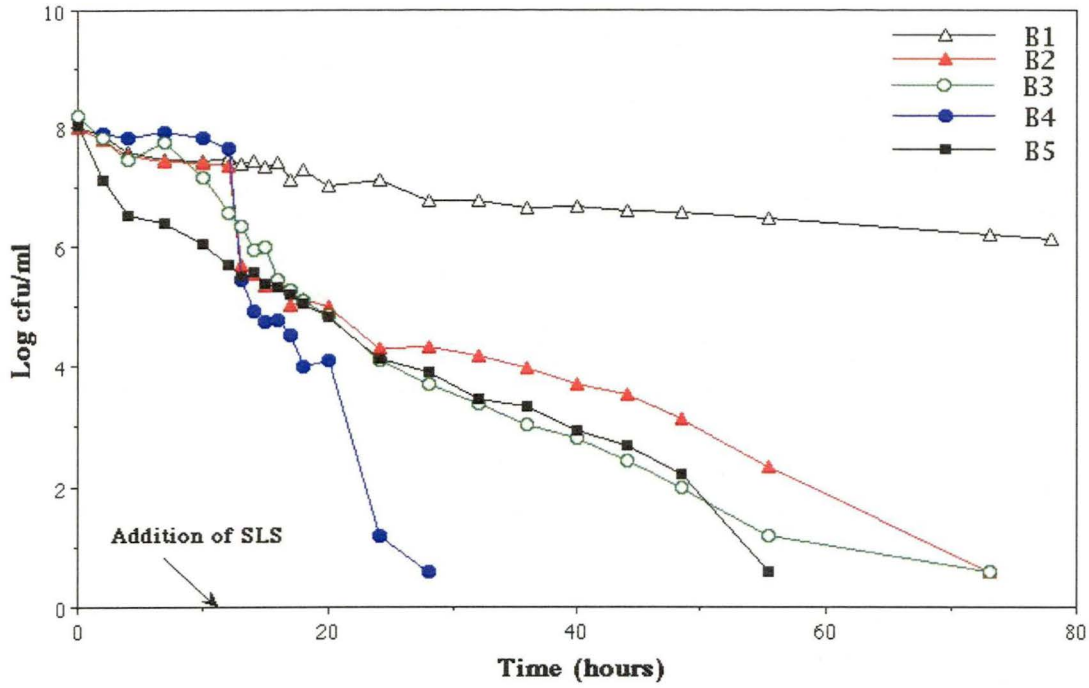

Figure 2.12. Inactivation of *E. coli* M23 OR.H- in response to lethal a_w (NaCl as humectant) and pH (HCl) stress at 25°C, in TSB. SLS imposed after 12h. Cells were recovered on TPAP at 37°C. Cultures exposed to a_w 0.90 (Δ), a_w 0.90 followed by SLS pH 3.50 (▲), pH 3.50 (○), pH 3.50 followed by SLS a_w 0.90 (●), a_w 0.90 and pH 3.50 from T_0 (■).

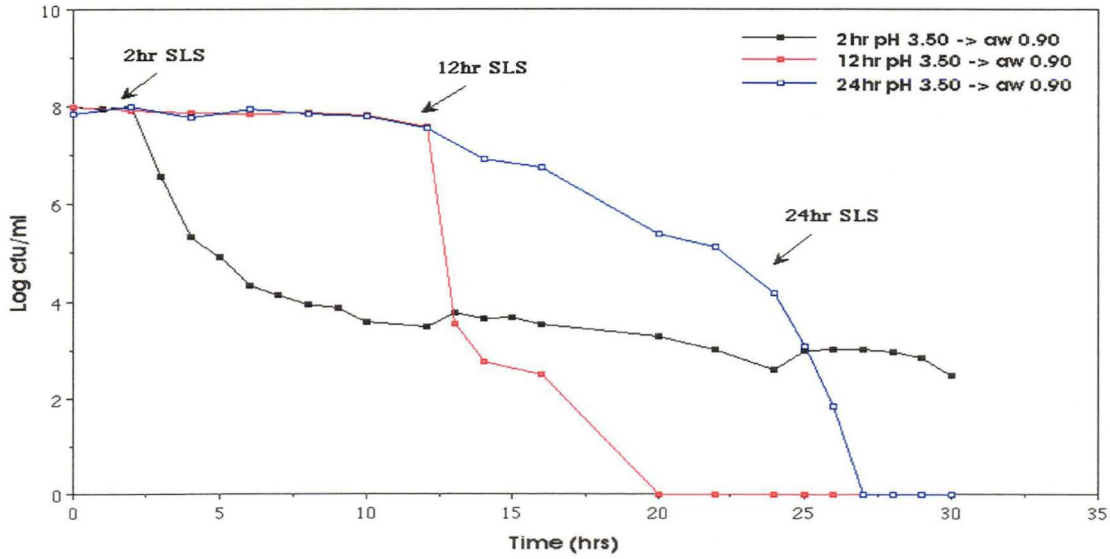

Figure 2.13. Inactivation of *E. coli* M23 OR.H- in response to lethal a_w 0.90 (NaCl as humectant) and pH (HCl) stress at 25°C, TSB. HCl added as SLS at 2, 12, and 24 h. Cells were recovered on TPAP at 37°C.

2.3.2.4 Discussion

From the control curves in Figures 2.11 and 2.12 it is apparent that the pH (3.50) stress is more severe than the imposed osmotic stress (a_w 0.90). It is widely considered that a combination of parameters can result in a synergism that enhances microbial stasis or inactivation (Leistner, 1994). However, when salt and acid stresses were combined at the start of the experiment, the inactivation kinetics closely resembled death due to low pH alone and no synergism was apparent.

The treatment first exposed to low a_w displayed a rapid 3-Log cfu/ml decrease when the SLS (pH) was added. Following this, survivors displayed a similar rate of inactivation as the pH control. Addition of the acid shock may have selected for a population of physiological variants with high levels of regulatory and repair proteins (Peleg and Cole 1998), which resulted in the nearly identical pattern shown for the pH control.

In contrast, a culture exposed initially to acid shock displayed markedly different inactivation kinetics after imposition of the SLS (a_w). No resistant subpopulation emerged, and no cells were recovered after 32 hours (limit of detection). Acid stress is energetically very demanding and requires continuous removal of H^+ ions by efflux pumps (Ingraham and Marr 1996). A_w stress is also considered to be a homeostatic burden (Csonka 1989), although the results of Krist et al. (1998) suggest that this interpretation may be incorrect. In Krist et al (1998), growth rate and cell yield experiments were performed in MMM with

glucose as the sole carbon source in the presence of high and low NaCl concentration. Growth rate declined with decreasing temperature and water activity, but cell yield was not substantially affected until close to water activity levels at which growth ceased. Since the glucose was converted to the same amount of biomass, those authors hypothesised that the stress imposed by suboptimal water activity is not highly energetically demanding. Under pH stress (pH 6.94 – 3.77 with HCl as the acidulant), cell yield was shown to substantially decline in the pH range of 5.2 – 3.9, compared to growth at pH>5.2.

The inactivation patterns observed with introduction of a SLS after 12 hours (Figure 2.11) are consistent with the above observations and interpretations. Little change was evident between a_w stressed cultures at 12 and 24 hours which then experienced a SLS (pH). In contrast cells exposed to lethal pH for 12 hours followed by a SLS (a_w) survived for longer than those which experienced an initial 24 hour pH stress. The lower magnitude of decline after imposition of the SLS and reduction in inactivation rate after 8 hours may indicate that a 12 hour culture possesses more energy to cope with the demands of additional stress.

This theory was further tested by exposing a single inoculum to a SLS (a_w) at 2, 12 and 24 hours (Figure 2.12). The inactivation kinetics after introduction of a SLS at 12 and 24 hours were identical to previous experiments. A culture experiencing a SLS (a_w) at 2 hours demonstrated a decreased rate and magnitude of inactivation compared to one exposed to a SLS (a_w) at 12 and 24 hours. The persistence of a tail 28 hours after exposure to a SLS (a_w) at 2 hours is consistent with the hypothesis that a larger proportion of cells possessed more available energy to cope with the demands of an added a_w burden.

These experiments provide further evidence that pH 3.50 is a greater *stress* on the bacterial cell than a_w 0.90 and consequently provides additional support to the mechanistic hypothesis. From a practical perspective, the order and timing of processing parameters, such as water activity and pH, is clearly an important consideration. It is hypothesised that energy demand as a result of acid stress greatly sensitises the cell to successive treatments, such as water activity stress. This finding is of relevance to the fermented meat industry where hurdles of pH and water activity are imposed in the form of fermentation and a drying step, respectively (Leistner 1994b). Further work must be done to determine which conditions and parameters deliver the optimum reduction in microbial load. This may lead to novel food processes which ensure the aesthetic qualities of foods whilst ensuring safety from microbial hazards.

2.3.3 Investigation of Metabolic Activity under Lethal Conditions by Measuring Uptake of Radiolabelled Substrates

2.3.3.1 Introduction

Comparisons of inactivation kinetics under conditions of lethal a_w and pH presented and discussed in this dissertation thus far are summarised in Table 2.1.

Table 2.1. Characteristics of nonthermal inactivation of stationary phase *E. coli* M23 OR.H- with lethal pH and water activity.

Lethal a_w (NaCl as humectant)	**Lethal pH (HCl as acidulant)**
Biphasic inactivation with rapid first phase - not affected by dilution	Triphasic inactivation - rapid first phase is eliminated by 100-1000 fold dilution
Large degree of injury apparent	Small amount of injury
Chloramphenicol reduces tailing	Chloramphenicol has no effect on tailing
Addition of pH as SLS results in an initial rapid decline followed by tailing. Rates not greatly increased with addition over a 24h time period.	Addition of a_w as SLS results in a rapid decline from which cells are increasingly non-recoverable the longer cells are held at the initial low pH.

To further examine differences in cell physiology in response to a_w and pH stress, stationary phase inocula were washed by centrifugation and resuspension in Minimal Minerals Medium (MMM) with acetic acid–2–^{14}C (Sigma) as the sole carbon source. Differences in utilisation of the labelled substrate under pH or a_w stress were studied and compared.

2.3.3.2 Experimental procedure

2.3.3.2.1 Uptake of labelled acetate on the growth boundary

E. coli M23 OR.H- was grown to late stationary phase (*ca* 1 x 10^9 cfu/ml) in 80ml MMM with 1% glucose and incubated statically at 37°C for 23h in a 250ml Erlenmeyer flask. Aliquots (1.00ml) of this stationary phase culture were removed and washed twice in

MMM without glucose by centrifugation (8000*g*, 10min). After resuspension in MMM, a 1.00ml aliquot was then placed into a 50ml conical flask containing 9.00ml MMM, NaCl, and acetic acid-2-^{14}C so that addition of the inoculum gave a final a_w 0.955 (growth permissive boundary for *E. coli* M23 OR.H-) and 1.25μCi/ml of labelled-isotope. The 50ml flask was temperature-equilibrated at 37°C in a shaking water bath prior to inoculation.

At appropriate intervals 100μl aliquots were removed from the flask. Aliquots were filtered through a 0.2μm cellulose-acetate membrane filter and washed in MMM without glucose to remove unincorporated label. Each filter was placed into a vial for assessment of metabolic activity by scintillation count. Utilisation of the labelled substrate on the growth boundary for *E. coli* M23 OR.H- (a_w 0.955, Salter et al., (2000)) was compared against growth permissive conditions (a_w 0.995) as a control.

2.3.3.2.2 Exposure of a culture pre-labelled with ^{14}C-acetate to lethal a_w stress

E. coli M23 OR.H- inocula were prepared as described in 2.3.3.2.1 above. A 1.00ml aliquot was then placed into a 50ml conical flask containing 9.00ml MMM and acetic acid-2-^{14}C so that addition of the inoculum gave 1.25μCi/ml of labelled-isotope. This population was then grown to late exponential phase with a final scintillation count of approximately 93,000 disintegrations/min and diluted into 9.00ml MMM and NaCl so that addition of the inoculum gave a final a_w 0.90. The scintillation count after dilution was approximately

6,700 disintegrations/min. 50ml flasks were temperature-equilibrated to 37°C in a shaking water bath prior to inoculation.

Metabolic activity was measured by scintillation count as described in [2.3.3.2.1].

2.3.3.2.3 Uptake of labelled acetate under lethal a_w and pH

E. coli M23 OR.H- inocula were prepared as described in [2.3.3.2.1] above. Two 1.00ml aliquots were then placed into separate 50ml conical flasks containing 9.00ml MMM, NaCl, and acetic acid-2-^{14}C so that addition of the inoculum gave a final a_w 0.90 or 0.83 and 1.25μCi/ml of labelled-isotope. A third aliquot was placed into an identical 50ml flask containing 9.00ml MMM, 1.25μCi/ml of labelled-isotope and the pH lowered to 3.60 using concentrated HCl. All 50ml flasks were temperature-equilibrated in a shaking water bath prior to inoculation.

At appropriate intervals 2 x 100μl aliquots were removed from each flask. One aliquot was diluted as required in 0.900ml MMM without glucose and enumerated on TPAP (refer to Appendix A). The second aliquot was used to assess metabolic activity by scintillation count as described [2.3.3.2.2].

2.3.3.2.4 Effect of a SLS on uptake of labelled acetate

Lethal a_w and pH broths were prepared as described in [2.3.3.2.3] above, except that the pH was set to 3.50. After 12 hours the low a_w broth was subjected to a SLS of pH 3.50. Similarly, a SLS of a_w 0.90 was imposed on the pH 3.50 broth. At appropriate intervals 100µl aliquots were removed for analysis of metabolic activity by scintillation count.

Experiments were also performed with tritiated (^3H) thymidine to determine if any uptake under lethal conditions was due to growth. Incorporation of thymidine into molecules other than DNA is negligible and use of the tritiated compound, therefore, is an indicator of cell growth (Mathews and van Holde, 1990). Background incorporation with both tritiated (^3H) thymidine and acetic acid-2-^{14}C was determined with UV-killed cells of *E. coli* M23 OR.H-

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2.3.3.3 Results

The uptake of labelled acetic acid-2-^{14}C by *E. coli* M23 OR.H- when exposed to various water activities is shown by Figure 2.14. In the absence of any water activity stress *E. coli* M23 OR.H- were able to utilise and incorporate the acetate as a growth substrate as measured by scintillation count.

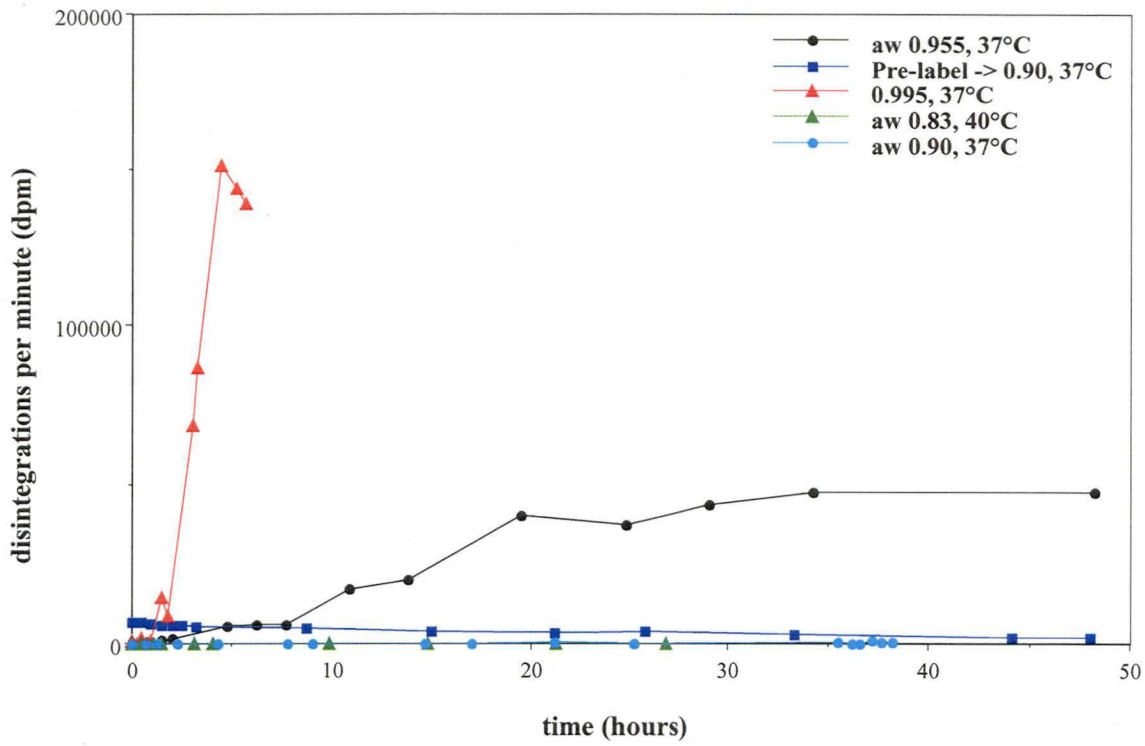

Figure 2.14. Acetic acid-2-^{14}C (sole carbon source) uptake of *E. coli* M23 OR.H- under growth permissive/non-growth permissive a_w (NaCl as humectant) in MMM.

Near the water activity limit for growth (a_w 0.955) the rate of uptake of ^{14}C-acetate was lower and appeared to reach a peak after 35 hours. Over 48 hours, the viable count slowly declined from 7.7 to 7.1 Log cfu/ml (data not shown).

A pre-labelled late exponential phase culture showed no uptake when exposed to lethal a_w 0.90. *E. coli* M23 OR.H- survivor counts declined over 48 hours at a linear rate from 6.7 to 3.5 Log units (data not shown), while the number of disintegrations per minute also showed a linear decline from approximately 6,700 to 2000 dpm.

Two cultures exposed to lethal conditions of a_w 0.90 and 0.83 did not appear to take up the labelled substrate. Scintillation counts for these cultures varied and it was difficult to

determine whether apparent uptake was due to background radio-isotope or metabolic activity. The incorporation levels observed were comparable with that obtained by UV-killed cells (data not shown) used as a control.

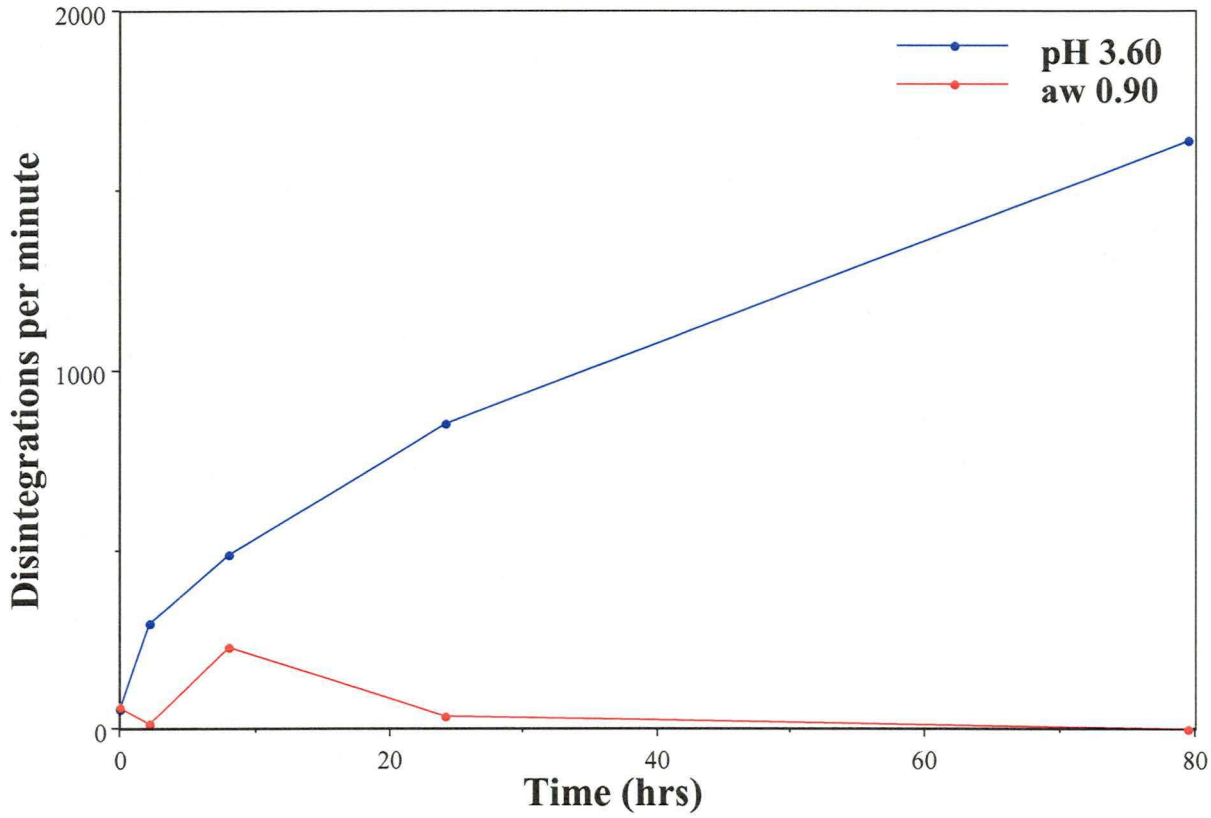

Figure 2.15. Uptake of acetic acid-2-^{14}C by *E. coli* M23 OR.H- exposed to a_w 0.90 (NaCl as humectant) or pH 3.60 (HCl as acidulant) stress at 25°C, MMM.

The incorporation of acetic acid-2-^{14}C by cultures under lethal a_w (0.90) and pH (3.50) stress in MMM is shown by Fig. 2.15. Cells at a_w 0.90 displayed little uptake of the isotope suggesting minimal metabolic activity. A peak in uptake was observed at 8h, before declining again to a low level. This was in contrast to *E. coli* M23 OR.H- at pH 3.60. Over

an 80h period the measured level of isotope uptake continually increased. The decline in cell numbers (Figure 2.16) for pH 3.60 and a_w 0.90 was very similar.

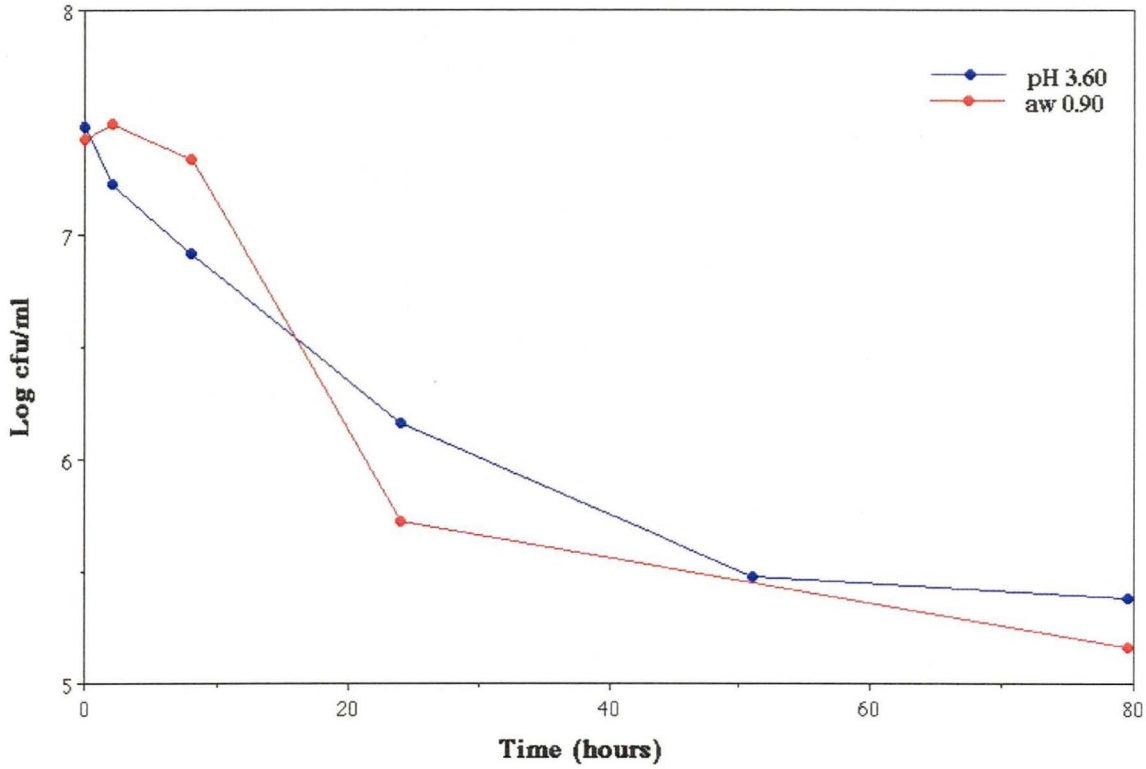

Figure 2.16. Viability of *E. coli* M23 OR.H- in response to lethal a_w 0.90 (NaCl as humectant) and pH 3.60 (HCl as acidulant) stress at 25°C, MMM.

The effects of a SLS on uptake of labelled acetate (Log (disintegrations per minute)) are shown in Figure 2.17. Addition of a pH stress to the culture with an initial lethal a_w caused a sudden rise in label incorporation to a level that remained constant for the remainder of the experiment. The culture with an initial pH shock ceased uptake of acetate after imposition of a_w stress.

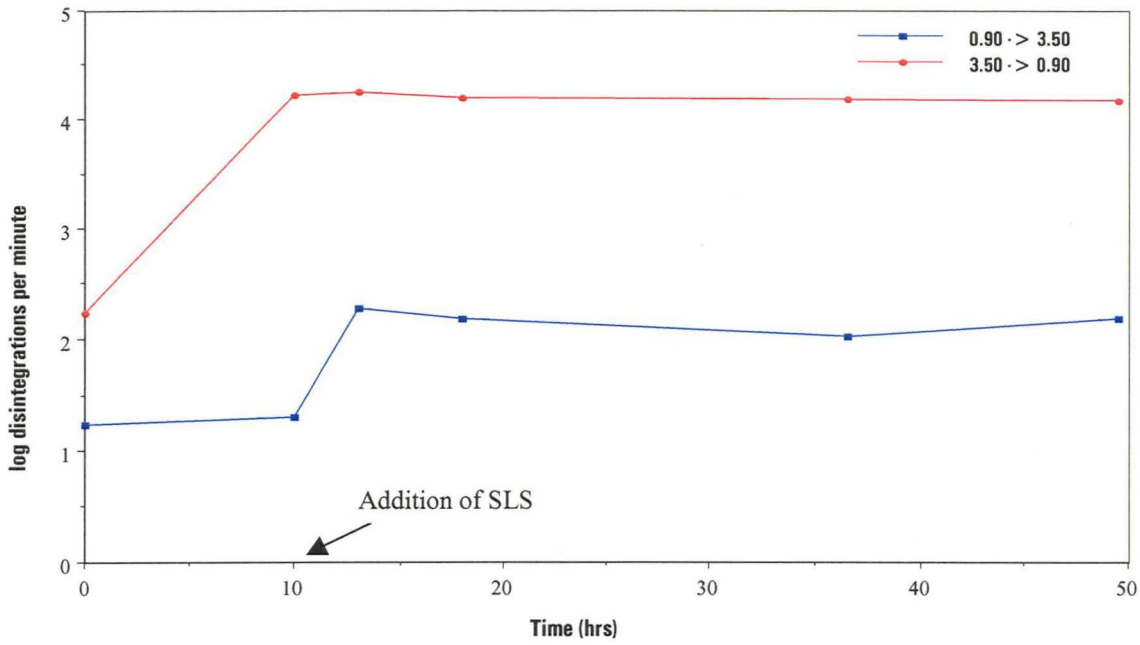

Figure 2.17. Examination of *E. coli* M23 OR.H- uptake of acetic acid-2-^{14}C in response to lethal a_w 0.90 (NaCl as humectant) and pH (HCl) stress at 25°C, MMM. SLS at 12 h.

2.3.3.4 Discussion

Controls indicated that *E. coli* M23 OR.H- were capable of utilising acetic acid-2-^{14}C as a growth substrate (Figure 2.14). Cells close to the a_w growth/no-growth boundary (a_w 0.955) demonstrated metabolic activity through uptake of the labelled compound. Under lethal conditions ($<a_w$ 0.95), no uptake was observed. This may be interpreted as cells under these conditions not actively resisting the inimical conditions by utilising external energy substrates. Minor peaks were observed which may be due to synthesis of an osmoprotectant before returning to a lowered state of metabolic activity. In many cases, however, it was difficult to distinguish these peaks from background scintillation counts.

It was apparent that cells exposed to pH stress (Figure 2.14) actively incorporated labelled acetate and displayed a much higher metabolic activity than those exposed to lethal a_w. This may be the result of a need to offset the continuous depletion of ATP as result of active efflux of H^+ ions, a characteristic of pH stress (Ingraham and Marr, 1996; Shabala et al., 2002). This result also supports the hypothesis of [2.3.2.4] that there is an additional energy demand on the cell after exposure to acid stress that may sensitise it to other stresses.

The effects on acetate incorporation in response to a SLS at 12 hours (Figure 2.16) showed that a culture initially exposed to a lethal a_w stress and subsequently subjected to a pH stress rapidly increased acetate uptake, before reaching a constant rate of incorporation. By contrast, cells actively metabolising in response to a pH stress were observed to cease uptake when a SLS (a_w) was added. Based on the inactivation curves obtained in Figures 2.10-2.12, addition of NaCl results in sudden loss of viability and a cessation of the ability to cope with acid stress. Addition of HCl as a SLS caused a slight increase in metabolic activity before remaining constant.

These results further support the mechanistic theory by displaying differences in the mechanisms of pH and a_w inactivation.

2.4 Conclusion

This body of work builds on the considerable effort expended during earlier investigations into inactivation kinetics over the past century. The results of this study and previous research reviewed in [1.3], show that the observed inactivation kinetics are not artefactual, but a true reflection of physiological changes experienced by cell populations. The shape of inactivation curves were found to be determined by:

- age of the inoculum
- inoculum history
- order, timing and type of the applied stress(es)

The observed inactivation kinetics were markedly different for a_w and pH stress, and the mechanisms also suggested to be different. It is not possible to state with certainty that either the vitalistic concept or the mechanistic concept, as proposed by Lee and Gilbert (1918), were the dominant hypotheses responsible for inactivation. Based on the above observations it is simplistic and unrealistic to suggest that one concept is mutually exclusive of the other. This is certainly the case for conditions of nonthermal inactivation relevant to uncooked comminuted fermented meat (such as salami or mettwurst) production, where non-lethal temperatures and pH levels are combined with lethal water activity.

Hence, while the inactivation mechanism hypothesised by Rahn (1929) [see 1.3.5] may have been incorrect, it appears that his statement:

> "whenever two groups of thorough research workers hold such opposed views as in this (mechanistic *versus* vitalistic) case, it is fairly safe to assume that some essentially new principle is involved which none of the two parties realised"

was not without justification.

Aside from novel theoretical developments, this research lends itself to commercial application. Sequence and timing of differing physiological stresses may be an effective method of food preservation or disinfection without addition of large quantities of preservatives. Further work should focus on the influence of other stresses, such as organic acids, and in various combinations. The hypothesis of a cell density dependent signal factor, as hypothesised by Brown (2002), should also be further investigated. If such a compound were detected, synthesis of this and combination with an acid stress may be an effective tool for pathogen reduction.

These studies of nonthermal inactivation kinetics have provided insight into mechanisms of inactivation and strategies for control of acid-tolerant pathogens such as *Escherichia coli*. In the following section, this knowledge will be utilised to investigate the feasibility of modelling the death of pathogens in UCFM through the development and use of a broth system.

3 INACTIVATION OF *Escherichia coli* IN UNCOOKED COMMINUTED FERMENTED MEAT

3.1 Introduction

The manufacture of uncooked comminuted fermented meats (UCFM), including salami, has been practiced for several centuries (Lücke, 1985). European sausages have existed since the Middle Ages and are still very popular. In the USA, large quantities of pepperoni are consumed in the form of pizza topping (Hinkens et al., 1996) while Chinese-style sausage, with pork as the main ingredient, is also common (Yu and Chou, 1997). These food products were thought of as safe from any pathogenic bacteria initially present within the food matrix due to the inimical conditions established by their fermentation to a low pH and subsequent drying. However, outbreaks arising from contamination with acid-tolerant *Escherichia coli* forced a major "rethink". Twenty cases of food-poisoning, attributed to consumption of commercial dry-cured salami contaminated with *E. coli* O157:H7, were reported from 16 November to 21 December 1994 in Washington State, USA (MMWR, 1995). Three patients required hospitalisation, including a 2-year-old boy with haemolytic uraemic syndrome (HUS). Three additional cases were subsequently identified in northern California. A brand of salami sold from local delicatessens was identified as the disease vector. Environmental investigations found no errors in food handling at the point of sale or during manufacture.

In December 1994 to January 1995 contaminated mettwurst, produced by a smallgoods company in South Australia, led to a large outbreak caused by *E. coli* O111 (Cameron et al., 1995). Twenty-three children were hospitalised with HUS and, tragically, a 4-year-old girl died. In addition, a further 120 people suffered effects of severe food poisoning. As a result of this outbreak, the smallgoods company was forced to shut down and the directors were charged with manslaughter due to improper manufacturing practices. However, the effects of this outbreak were not confined to the company. Consumer confidence in the entire meat industry fell markedly, with sales dropping nationwide (FSANZ, 2002).

In June 1995, the National Food Standards Council (comprised of Health Ministers from all Australian States and Territories) recognised the need for changes to Australia's food hygiene regulations to prevent further outbreaks from occurring (ANZFA, 1996). These changes resulted in a shift away from traditional, prescriptive, end-product testing regulations towards a system based on food safety outcomes, where there was a requirement for the production process to be consistently monitored. To comply with the regulations introduced in 1996, UCFM manufacturers were required to:

- store meat below 5°C before use
- employ a lactic-acid starter culture
- monitor and record levels of *E. coli* in raw ingredients and the end-product
- use a process capable of achieving a 99.9% reduction (3 Log units) in the number of *E. coli* initially present through fermentation or any other process

These regulations were the subject of a review by Ross and Shadbolt (2001). Those authors determined that many processes used in Australia and internationally to manufacture UCFM were not able to achieve the required 3-Log reduction. The few processes that were able to conform to the introduced regulations usually employed extended processing times and/or high temperatures (above 40°C) and were not commonly used in Australia. Another outcome of that review was the recommendation that research be undertaken to develop a better predictive model to assess *E. coli* inactivation during production. A distinct lack of descriptive, systematic data on the inactivation kinetics of *Escherichia coli* observed in UCFM made it difficult to predict the safety of some processes.

This study seeks to address the above lack of data through development of a model broth system to simulate inactivation of pathogens during processing. This will enable better assessment of the inactivation process and assist in the design of safe UCFM manufacture.

3.2 Uncooked Comminuted Fermented Meat Manufacture

3.2.1 Introduction

Leistner (Leistner, 1994; Leistner, 1995) and Lücke (1985) have written extensively on the topic of fermented meat manufacture. Figure 3.1 outlines the production process for UCFM. Specific hurdles at each step help to prevent growth and facilitate inactivation of undesirable microorganisms. The initial stage is preparation of the batter. Meat is first chilled before grinding to prevent growth of microorganisms and fat smearing. Depending on the type of mix, curing salt and carbohydrates are added. Fatty tissue is broken down while frozen and added to the mixture. The size of the fat particles largely determines the product type (Lücke, 1985). Curing salt, such as nitrite, is supposedly the first hurdle utilised at this stage in production. According to Leistner (1995) nitrite inhibits salmonellae which may be present in the mix at this stage. However other authors state that the primary roles of nitrite are the formation of characteristic colour, flavour, texture, and inhibition of *Clostridium botulinum* growth and toxin formation (Mossel et al., 1995; Davidson, 1997). In association with other factors such as NaCl and low pH, nitrite exerts a concentration-dependent antimicrobial effect on the outgrowth of spores from *Clostridium botulinum* and other clostridia (Davidson, 1997). The effect of nitrite against gram-negative bacteria appears to be uncertain, but Yu and Chou (1997) demonstrated that low concentration had little effect on the death of *E. coli*. The effectiveness of nitrite is observed to be pH dependent, and Gibson and Roberts (1986) found limited inhibition of *E. coli* at levels used in fermented meats with various NaCl

concentrations and pH. Thus the role of nitrite as a significant hurdle for enteric pathogens in salami manufacture may be overstated.

During preparation of the batter, oxygen enters the mixture. It is important to remove as much of this as possible since it interferes with the formation of colour, flavour, and results in a high redox potential (E_h) (Lücke, 1985). Addition of ascorbic acid or sugar reduces E_h and enables growth of lactic acid bacteria to commence fermentation. Lactic acid bacteria, such as *Pediococcus acidolacti* or *Lactobacillus* sp, are inoculated onto the batter prior to fermentation. The process of fermentation may take 15-48 hours and continues until some target pH is reached; typically in the range of pH 4.6 - 5.8 (Leistner, 1995; Calicioglu et al., 1997). Fermentation temperature for dry salami varies from 15-26°C, while for semi-dry sausages the range is around 32.5-38.1°C (Ricke and Keeton, 1997). The pH hurdle is important in preventing growth of food spoilage organisms, particularly in quick-ripened products. Such foods have a high moisture/protein ratio, and hence a high a_w, which supports bacterial proliferation (Leistner, 1995). The effects of fermentation may be enhanced by addition of glucono-δ-lactone or smoking. Phenols and other carbonyl compounds from smoke have antimicrobial properties that inhibit growth post-fermentation, although these effects are minimal and mainly enhance flavour (Ricke and Keeton, 1997). In the production of some semi-dry sausages, such as that typically seen in the USA, the temperature is rapidly increased after a short fermentation period. The meat is held at elevated temperature for up to 1 hour before further drying or chilling and packaging. The aim of this is to inactivate the inoculum and destroy any pathogens

present. This process is not strictly non-thermal, and the heated product is often classified as low-cooked (Calicioglu et al., 1997).

The final hurdle in UCFM production to be considered is the water activity. As the pH is lowered during fermentation, protein binding occurs to remove some water. The water activity further decreases during ripening and is dependent on the levels of ingredients in the sausage (NaCl for example), fermentation temperature, relative humidity of the drying room, and time (Leistner, 1995). Drying can be a fairly rapid process, particularly if the pH is below 5.3 since protein solubility is low and a gel forms around meat and fat particles (Lücke, 1985). This facilitates the removal of water and lowering of a_w, preventing growth of pathogens. Depending upon the type of sausage, final a_w may vary from <0.85 - 0.91 for dry sausages and 0.90 - 0.94 for semi-dry sausages (Calicioglu et al., 1997; Ricke and Keeton, 1997).

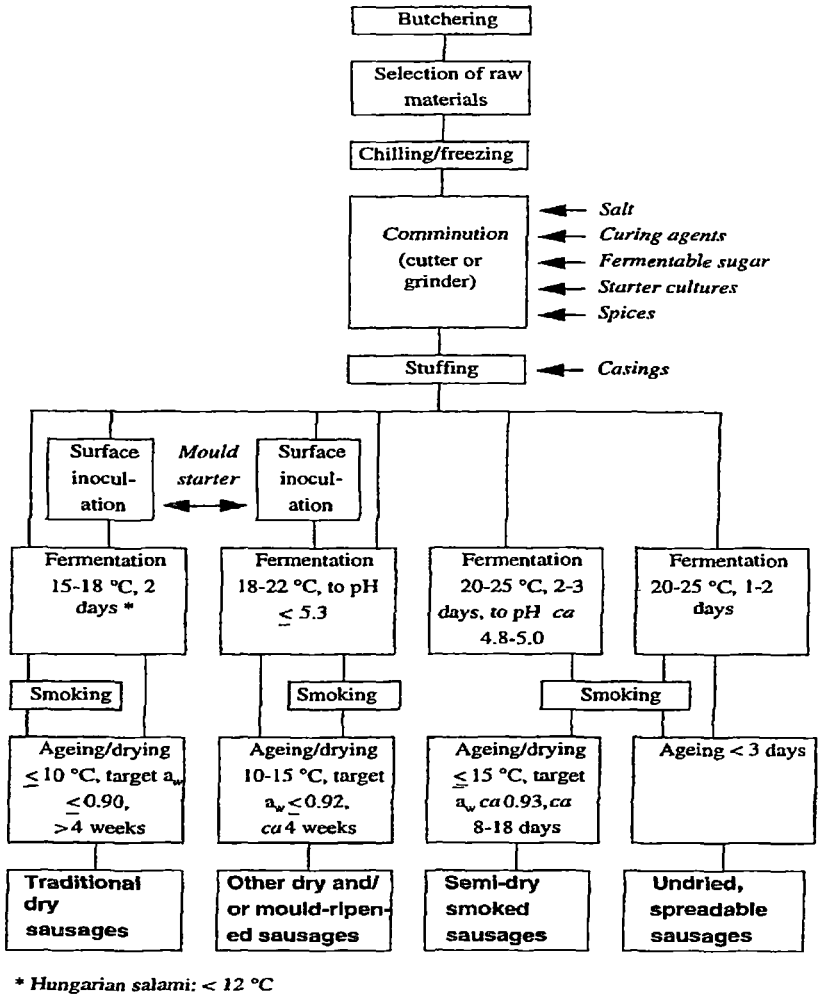

Figure 3.1. Diagrammatic representation of UCFM production (reproduced from Lücke, 1995).

3.2.2 Characteristics of Non-Thermal Inactivation Associated with UCFM Production

In contrast to thermal inactivation kinetics, mechanisms of non-thermal death are not well understood, possibly due to a lack of systematic research in this field [refer to 1.4]. Brown (2002) and Shadbolt et al. (1999) examined the effects of lethal pH and water activity over a range of temperatures that were non-lethal to *E. coli*. Those authors

observed triphasic and biphasic inactivation curves in response to lethal pH and water activity (a_w), respectively. When the stress was first imposed, a rapid phase of death was observed initially, which became more apparent with increased severity of the lethal agent. The size of the first phase kill appeared proportional to the magnitude of applied stress. Following the first phase of inactivation, a significant tailing effect was observed. This second slower death rate persisted for extended periods and appeared to be independent of the lethal agent. Other workers have also made reference to biphasic or multiphasic inactivation patterns (Gustafson et al., 1998; Humpheson et al., 1998). Increased temperature resulted in higher levels of inactivation over all phases of death. It appeared that the duration of tailing was influenced more by temperature than the type of stress.

Studies of inactivation during UCFM production have demonstrated synergistic or hurdle effects between environmental parameters such as pH, a_w, organic acid concentration and temperature (Leistner, 1995; Ellajosyula et al., 1998; Casey and Condon, 2000). Results from Shadbolt et al. (2001) and [2.3.2] indicate that the order and timing in which different types of stress are applied is critical for achieving the maximum possible inactivation rate.

3.2.3 Effectiveness of Current Processing

Many studies have shown that standard fermentation and drying steps used in UCFM manufacture are insufficient to deliver a 3-Log reduction in pathogen numbers (Glass et

al., 1992; Grau, 1996; Nickelson et al., 1996; Sauer et al., 1997; FSA, 1999). Faith et al., (1997) reported that fermentation at 36°C for up to 16 hours resulted in a 1 Log decrease of *E. coli* O157:H7. This level of decline is similar to others reported throughout the literature (Hinkens et al., 1996; Calicioglu et al., 1997; Faith et al., 1998a; Faith et al., 1998b; Riordan et al., 1998; FSA, 1999; Casey and Condon, 2000). Exceptions where a greater inactivation has been achieved involves the use of higher fermentation temperatures, increased amounts of preservatives, lower pH levels, and longer time. Grau (1996) reported a 2.1-Log reduction with 161ppm nitrite, 3.05% NaCl, and fermentation to pH 4.6 at 26°C for 72 hours. Petchsing and Woodburn (1990) reported a 2.5-Log reduction in *E. coli* after fermentation at 30°C for 72-96 hours to a pH of around 4.6, with 200ppm nitrite and 3% NaCl. The safety of this product (Thai-style fermented sausage) is questionable, since no drying step is employed and the *E. coli* bacterium used in this study was not of an acid-tolerant variety. Riordan et al. (1998) were able to demonstrate a 3.36-Log decrease in *E. coli* O157:H7 after the fermentation step of pepperoni manufacture. However, this was achieved in the presence of increased levels of preservatives (300ppm nitrite), NaCl (3.3%) and low pH (4.4) at a high temperature of 38°C. Those authors later stated that increasing the amount of preservatives in fermented products was likely to result in unacceptable organoleptic changes. This is especially true given the current trend toward minimally processed foods.

Drying (or 'ripening', or 'maturation') is considered to be the next important step for reducing numbers of foodborne pathogens in salami production. However, most literature studies report pathogen reductions from around 0.5 - 0.90-Log cfu/g after drying (Grau,

1996; Faith et al., 1997; Faith et al., 1998a; Faith et al., 1998b). Combined with typical reductions after fermentation it is obvious that most processes cannot reliably guarantee a 3-Log reduction after manufacture.

3.2.4 Australian Manufacture and Effectiveness

Ross and Shadbolt (2001) analysed data concerning UCFM (predominantly salami and mettwurst) produced in Australia and found that many manufacturing processes were unlikely to achieve a 3-Log reduction in the presence of acid-tolerant pathogens such as *E. coli* O157:H7 or *E. coli* O111:NM (and, therefore, unable to comply with 1996 Australian regulations). Some products also suffered from manufacturing flaws, such as the addition of alcohol with starter cultures that may have inhibited their ability to produce lactic acid. Other products did not have sufficient fermentation time, or adequate drying (none at all for some products). Average production characteristics for the two most common types, salami and mettwurst, are tabulated below:

Table 3.1. Averge characteristics of Australia UCFM processes and products (reproduced from Ross and Shadbolt, 2001).

Sausage Characteristics	Salami (variation)	Mettwurst (variation)
Composition (lean%:fat%)	80.4:19.6 (70-90:10-30)	83.25: 16.75 (70-96:4-30)
NaCl (%)	2.45 (2.0 - 3.3)	2.02 (1.3 - 2.8)
Nitrite (ppm)	284.3 (144.5 - 490)	211 (35 - 490)
Fermentation time (h)	48.9 (24 - 72)	41.6 (18 - 72)
Fermentation temp. (°C)	23.3 (18 - 28)	28.6 (17 - 40)
pH	4.72 (5.0 - 4.4)	4.66 (4.8 - 4.4)
Ripening time (days)	14.8 (1 - 30)	5.3 (0 - 28)
Ripening temp. (°C)	14.1 (4 - 32)	18 (0 - 40)

Both product types experience a weight loss of around 20% after manufacture and vary markedly in nitrite levels and ripening time. "Salami" also tends to be fermented for slightly longer, although at a lower temperature than "mettwurst". However, major production differences existed within each product and these variations resulted in many types of salami and mettwurst being potentially unsafe. A confidential survey of UCFM manufacturing processes in Australia, carried out by the Australia New Zealand Food Authority (now Food Standards Australia New Zealand, the agency responsible for drafting food standards in Australia and New Zealand), indicated that only one salami product was capable of complying with a 3-Log reduction (A. Naco, FSANZ, pers comm., 2000). This product possessed a high level of preservatives (NaCl 2.65%, nitrite 392ppm), long fermentation and ripening times (72 hours, 21 days respectively), and was fermented at 28°C. Mettwurst products were only found to be safe if a heating step was employed after fermentation or ripening. Heating was also effective in the manufacture of pepperoni if the product was heated after ripening to a core temperature of 65°C for 15-20 minutes, depending upon the diameter of the sausage. Fermented sausage without any ripening, such as kabanossi or braunschweiger, was deemed to be unsafe. The latter are particularly hazardous since they are exceptionally moist (often marketed as 'soft spreadable' sausage) and employ a very short fermentation (24 hours at 24°C).

The National Risk Validation Project (FSA and MEC 2002) recommended that Hazard Analysis and Critical Control Points (HACCP)-based food safety programs be mandatory for UCFM. Such regulations ensure that businesses maintain adequate records, monitor

their process, and maintain equipment. However, safe food production will be facilitated by accurate data concerning the reduction of pathogens during processing. This will provide manufacturers and regulators with a greater understanding of the effectiveness of a particular process, and subsequent variations in UCFM formula or processes.

3.2.5 Alternatives for Safe Production

Literature studies which are comparable to Australian formulations (Petchsing and Woodburn, 1990; Grau, 1996; Sauer et al., 1997; Faith et al., 1998; FSA, 1999; Casey and Condon, 2000) indicate that current manufacturing processes cannot guarantee a 3-Log reduction without high levels of preservatives and long production times, or the addition of a heating step. Given the inability of many processes to achieve a 3-Log reduction, alterations to processes or additional hurdles have been considered.

Addition of heat or a mild pasteurisation step (54°C for 30-60min) results in a demonstrated 3 - 5 Log or greater kill (Hinkens et al., 1996; Calicioglu et al., 1997; Ellajosyula et al., 1998). Calicioglu et al. (1997) and Ellajosyula et al. (1998) both studied the effect of a lethal heating step after fermentation without any subsequent period of drying. From an initial starting population density of 7.99 Log cfu/g of *E. coli* O157:H7 in the batter, Calicioglu et al. (1997) reported reductions in the level of *E. coli* to <1.0 Log cfu/g. This was achieved when an internal temperature of 54°C was reached by heating at 40°C for 1h, 54°C for 1h, 60°C for 1h and 66°C for 35min at RH 60% to a pH of 4.6. A 3-Log cfu/g decrease was achieved if the product was fermented to pH 5.0,

but a greater than 5-Log cfu/g decrease was not attained until the product was held at an internal temperature of 54°C for 60min. Similar results were reported by Ellajosyula et al. (1998). Fermentation alone was not sufficient to destroy more than 2-Log cfu/g, but heating to 46°C for 5 hours or 49°C was sufficient to deliver a 5-Log cfu/g kill or better. Hinkens et al. (1996) used a heating step after fermentation and drying to achieve a ≥5 Log cfu/g decline in cell number. Before heating, a reduction of 1.2-Log cfu/g was achieved. Heating to an internal temperature of 63°C or 53°C and holding for 60min were sufficient to decrease the population by at least 5-Log cfu/g. However, while addition of a mild heat step is effective in reducing levels of *E. coli* in UCFM, it is not suitable for all products in this category. Heating to the levels described above will cause undesirable organoleptic changes in products with high fat or moisture content.

The best alternative to high levels of preservatives or a heating step is associated with storage of the final product after manufacture (Faith et al., 1997; Faith et al., 1998a; Faith et al., 1998b; Ihnot et al., 1998). Faith et al. (1997) showed that fermentation and drying caused less than a 3-Log cfu/g decrease, but storage at ambient temperatures for an extended period after manufacture was effective in reducing acid-tolerant *E. coli* to low levels. After standard processing the population of *E. coli* O157:H7 had declined by 2.9-Log cfu/g. Post-drying storage at 21°C in air resulted in a further decrease of 4-Log cfu/g after 14 days. After storage at 28 days the population was less than 1.0 cfu/g. By contrast, storage at 4°C or under vacuum packaging slowed inactivation and bacteria were able to persist for considerably longer. Faith et al. (1998b) also showed that manipulation of the batter before fermentation enhanced the rate of inactivation. In that study, a meat batter

was first tempered (2h at 13°C), frozen (3d at –20°C) and then thawed (3d at 4°C) before fermentation. Manufactured salami was tested following storage at 21°C in air. Compared with batter which had only been refrigerated for 8h, the manipulated product demonstrated a greater decline during fermentation and drying (2.6-Log cfu/g decrease compared to 1.1 Log cfu/g) and experienced a more rapid decrease during storage (3.0-Log cfu/g after 7 days compared to 1.8 Log cfu/g decrease). Grau (1996) also noted that freezing cells in meat before manufacture increased the level of death compared with bacteria which were added just prior to the fermentation step. Hence, manipulation of the batter prior to fermentation and drying provides an effective measure for control of foodborne disease-causing bacteria. Vanderlinde (1999) recommended that fermentation occur at 32°C and 88%RH for 3d, followed by normal maturation to a_w <0.89. The above storage protocols enable rapid fermentation techniques and lower temperatures to be used, hence negating any requirement for a heating step or very high levels or preservatives.

3.2.6 Broth and Meat Studies

Research on inactivation of *E. coli* within a real UCFM matrix presents numerous experimental problems, not least of which is the presence of background microflora (Grau, 1996). Some authors have attempted to address the requirement for more data relating to UCFM production by studying inactivation in broth systems (Grau, 1996; Tomicka et al., 1997; FSA, 1999; Casey and Condon, 2000; Duffy et al., 2000). Tomicka et al. (1997) attempted to measure the survival of *E. coli* O157:H7 during fermentation

using "American style" (37°C, 1d) and "European style" (22°C, 3d) conditions. After fermentation, broths were stored at 10°C and monitored for several days. No values for *E. coli* inactivation in cfu/ml were shown, but this may be academic since the additives in each broth are unlikely to provide a true reflection of conditions in salami. Only 2% NaCl and 0.8% dextrose was added which would not adequately reflect the true a_w in the absence of any drying or moisture loss. The pH in the "American style" model went from 7.2 to 4.2 and remained at this very low level for the remainder of the study. Hence it is difficult to draw meaningful conclusions from the work of Tomicka et al. (1997).

The study of Casey and Condon (2000) is likely to be far more representative of conditions encountered in salami. Those authors examined the effects of varying nitrite levels in Tryptic Soya Broth (TSB) modified with 40g/l NaCl, 13.5g/l sucrose, 0.8g/l ascorbate and pediococcal starter culture. The pH decreased to 4.5 from an initial value of 5.8 within 24 hours. In the presence of 300ppm nitrite, levels of *E. coli* O157:H45 (a verotoxin-negative relative of *E. coli* O157:H7; no comparative survival data provided) decreased by 3-Log over 2 days. A similar result was obtained at 200ppm, but in the presence of 100ppm nitrite bacterial levels declined by only 1-Log over the same period. A comparative study by the same authors was done with a laboratory-scale fermented sausage (37°C fermentation for 2 days, ripening at 15°C for 12 days). Sausages prepared in the absence of nitrite were reported to be an unpleasant green colour. Addition of 50ppm nitrite resulted in the characteristic pink colouration. The pH fell from 5.8 to approximately 4.7 within the first 2 days. At levels of 300 and 200ppm nitrite, numbers of *E. coli* declined by around 1.5- and 1.3-Log respectively. After 12 days of drying, the

total decline for both 300 and 200ppm was around 3.5-Log, whilst for 100 and 50ppm the reduction was approximately 2.5-Log.

Riordan et al. (1998) state that sodium nitrite, stable in laboratory broth, is rapidly inactivated by sodium ascorbate which is present in many commercial meat products. Grau (1996) reinforces this finding. Duffy et al. (2000) agreed with previous reports on the protective nature of fat in salami. Products with a higher fat content show lower levels of pathogen reduction. Ross and Shadbolt (2001) questioned this, observing that the presence of fat was not important from a microbiological perspective in UCFM production, except that higher levels contributed to a lower water activity and actually increased the rate of inactivation.

Inoculum history is an important factor in determining the effectiveness of manufacturing protocols (refer to [2.2.1] and Brown, 2002). Conner et al. (1997) state that practices such as carcass-washing with organic acids, resulting in a small decrease in *E. coli* O157:H7, may not be an effective solution for decontamination of meat surfaces due to activation of acid tolerance mechanisms in surviving cells. These cells may then survive processing and passage through the human gastric barrier, leading to infection.

Equivalent comparisons of inactivation in broth with that observed in fermented meats are yet to be published in the scientific literature. A model describing inactivation of *E. coli* in UCFM, based on accumulated data from broth and meat systems was presented in Ross and Shadbolt (2001). This model has enabled Australian regulators and producers to

gain a better understanding of the effectiveness of UCFM process parameters (D. Miles, NSW Food Authority, pers comm., 2003). However, as the model was constructed from numerous independent published and unpublished studies relating to non-thermal inactivation its accuracy and universality has been questioned. Ross and Shadbolt (2001) stated that there was a need for further systematic research to enhance their model describing inactivation in UCFM. To facilitate the collection of data required to enhance the model, it was considered that the potential for a broth-based system should be explored. Such a system would have advantages in terms of simplicity and cost effectiveness over trials using actual UCFM. The following section focuses on the development of a broth-based system for modelling inactivation of *E. coli* in UCFM.

3.3 MATERIALS AND METHODS

Appendix A describes preparation of material and equipment used. All treatments were performed in duplicate.

3.3.1 Investigation of Inactivation under Anaerobic, Lethal Conditions

Overview

To create a model broth environment that would simulate conditions in UCFM during processing, L-tubes containing cooked meat medium (CMM, Oxoid CM81) were pH and a_w adjusted to desired levels and made anaerobic.

3.3.1.1 Experimental procedure

A flow chart describing the experimental procedure is shown in Figure 3.2. An oxygen-reductant solution of 0.2M sodium thioglycollate was prepared by adding 1.14g thioglycollate to 50ml distilled H_2O in a 100ml serum-bottle. The bottle was then stoppered, crimped and autoclaved. Thioglycollate was added to CMM in a ratio of 7.5ml thioglycollate: 1L CMM where specified.

In a 1L Schott bottle, 750ml of CMM was autoclaved with 67.5g NaCl so that the final a_w was 0.95. This stock solution was allowed to cool under N_2. A slow rate of N_2 flow was essential to prevent the mixture from bubbling over. After cooling, a 100ml aliquot was removed and pipetted into a 300ml Erlenmeyer flask under N_2. Duplicate 19.9ml aliquots were removed from the Erlenmeyer flask and pipetted (anaerobically by flushing the pipette tip in the headspace of the CMM stock solution) into L-tubes containing 2g dehydrated CMM pellets under N_2. Thioglycollate (150µl of 0.2M thioglycollate solution) was added to each tube before it was stoppered, crimped, and autoclaved.

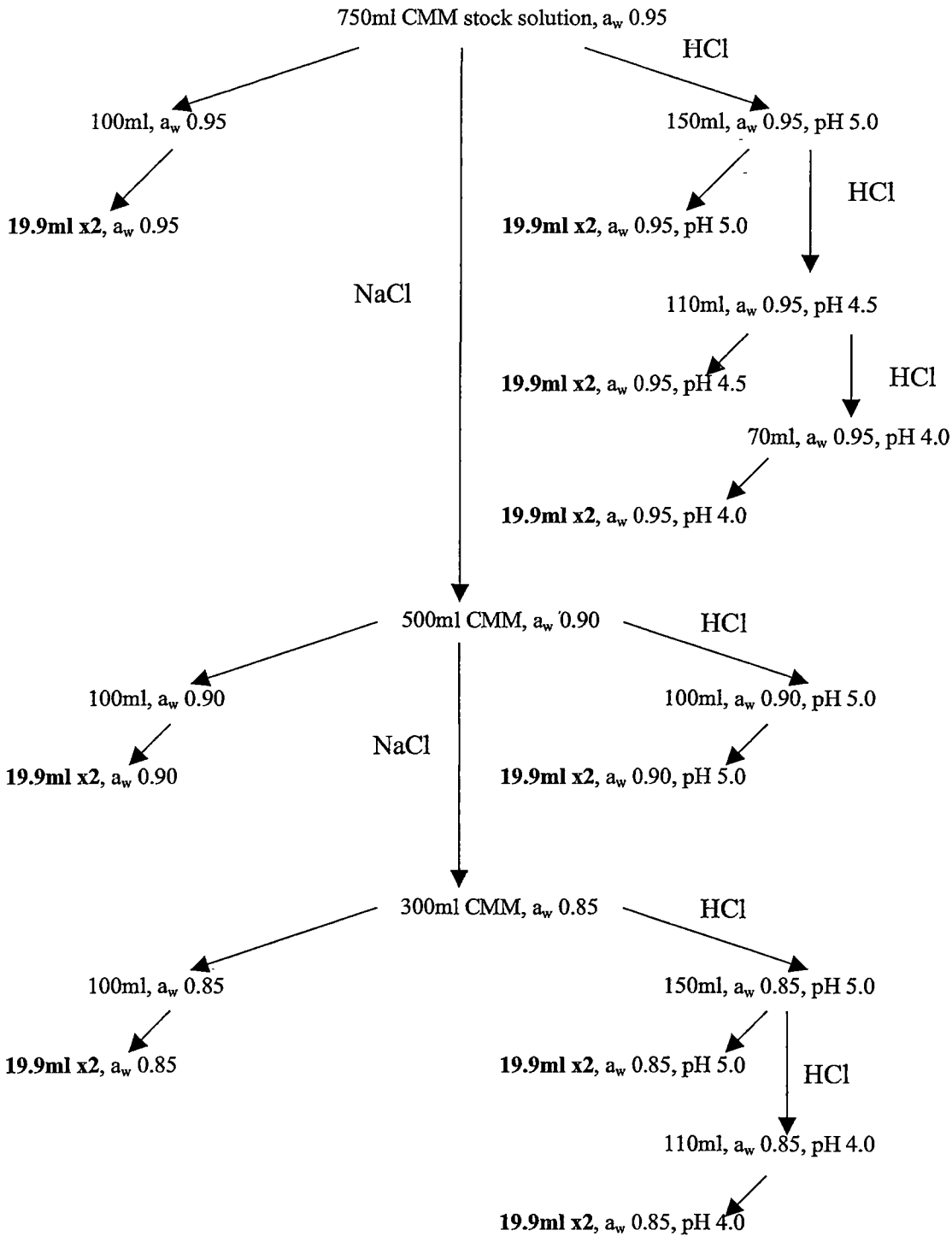

Figure 3.2. Diagrammatic representation of experimental procedure for preparation of UCFM broth model in CMM. All vessels were flushed with N_2 prior to, and during, addition of CMM broth, NaCl and HCl. All 19.9ml volumes were dispensed into glass L-tubes in duplicate.

From the stock solution a further 150.0ml CMM at a_w 0.95 was pipetted into another 300ml Erlenmeyer flask under N_2. This stock solution was adjusted to pH 5.0 using concentrated HCl. Duplicate 19.9ml aliquots were removed anaerobically as described above and placed into L-tubes containing 2g dehydrated CMM pellets under N_2. Thioglycollate (150µl, 0.2M) was added as described above before each tube was stoppered, crimped, and autoclaved. The remaining 110ml CMM in the Erlenmeyer flask was lowered to pH 4.50, and two more 19.9ml aliquots were removed anaerobically and placed into L-tubes containing 2g CMM as described above. This procedure was repeated after the remaining CMM solution in the Erlenmeyer flask was lowered to pH 4.0.

The remaining 500ml CMM stock solution was lowered to a_w 0.90 by addition of 35.0g NaCl. A 100ml aliquot was removed and pipetted into a 300ml Erlenmeyer flask under nitrogen. Duplicate 19.9ml aliquots were removed from the Erlenmeyer flask and pipetted (anaerobically) into L-tubes containing 2g dehydrated CMM pellets under N_2. Thioglycollate was added as described before each tube was stoppered, crimped, and autoclaved. A separate 100ml aliquot was removed and again pipetted into a 300ml Erlenmeyer flask under nitrogen. The pH of this solution was lowered to pH 5.0 and two 19.9ml aliquots removed for addition to L-tubes, as described above. This step was repeated after the pH in the Erlenmeyer flask was lowered to pH 4.5.

The final 300ml of original CMM stock solution was lowered to a_w 0.85 by addition of 27.0g NaCl. Identical steps were followed to that described above to result in duplicate aliquots at a_w 0.85; a_w 0.85 and pH 5.0; a_w 0.85 and pH 4.5.

A stationary-phase inoculum of *E. coli* M23 OR.H- was prepared as described in Appendix A. Each L-tube was inoculated with a 100µl aliquot of the stationary phase inoculum and the water activity and pH monitored. Prior to inoculation it was found that the 2g CMM in each L-tube altered the water activity and pH. The measured starting water activity/pH combinations are listed in Table 3.2.

Table 3.2. Water activity/pH combinations after autoclaving and inoculum addition

Water Activity	pH			
0.93	6.03	6.31	6.62	7.14
0.89		6.13	6.46	7.17
0.82		6.33	6.53	7.16

Before commencing the experiment all L-tubes were temperature-equilibrated to 35°C in a temperature-gradient incubator set to gentle shaking. Aliquots of 100µl were removed for enumeration by hypodermic syringe. Each syringe was flushed with N_2 before aliquots were removed. Samples were withdrawn for pH measurement at every enumeration time, and water activity was monitored at regular intervals. For each volume of broth removed from the L-tube, an equivalent volume of N_2 was inserted to maintain constant pressure.

The pH was lowered throughout the experiment by addition of concentrated HCl from syringes that were previously flushed with N_2. Enumeration was performed as described

in Appendix A. After 24 hours the temperature was lowered from 35°C to 15°C, typical of the maturation temperature employed in UCFM production.

3.3.2 Investigation of Inactivation during Simulated Fermentation of UCFM Manufacture

The conditions employed in this experiment were intended to simulate the fermentation stage employed during semi-dry UCFM manufacture as described in [3.2].

3.3.2.1 Experimental procedure

The experimental protocol employed was identical to that of [3.3.1.1] with the exception that acidification of broths did not commence until 3 hours into the experiment. The following water activity/acidulant combinations (Table 3.3) were used, taking into account effects of the dehydrated CMM on final a_w values.

Table 3.3. Water activity/acidulant combinations for UCFM fermentation simulation

Water Activity	pH
0.963	HCl
0.963	Lactic
0.956	HCl
0.943	HCl
0.937	HCl
0.935	Lactic
0.900	HCl
0.821	HCl

The higher water activity values of 0.963 are close to the initial values observed in UCFM formulations. Sampling and enumeration methods are identical to that described in [3.3.1.1]. Three hours after inoculation the pH of cultures was gradually lowered using concentrated HCl (as described in [3.3.1.1]). At 24 hours no further HCl was added to the broths and the temperature was lowered to 15°C. This was designed to mimic the fast-fermentation stage of semi-dry UCFM production, followed by the temperature shift which occurs upon commencement of the maturation phase.

Water activity and pH were monitored throughout the experiment. Each treatment was conducted in duplicate.

3.3.3 Comparison of Aerobic and Anaerobic Inactivation in CMM

Ross and Shadbolt (2001) noted that previous inactivation rate data obtained from studies in laboratory broth was faster than that observed in comparable UCFM formulations. It was hypothesised that the anaerobic environment during UCFM production may account for this. This hypothesis was investigated by examination of the inactivation kinetics of cultures exposed to varying water activity/pH conditions in CMM in parallel under aerobic or anaerobic conditions.

3.3.3.1 Experimental procedure

Erlenmeyer flasks (250ml) were prepared with 49.0ml CMM and NaCl so that addition of 1.00ml stationary phase inoculum of *E. coli* M23 OR.H- yielded final water activities of 0.94, 0.92 and 0.89. Prior to inoculation each flask was temperature equilibrated to 35°C in a shaking water bath. Inocula were prepared as described in Appendix A. After 3 hours the pH of each culture was gradually lowered throughout the course of the experiment. Samples were periodically removed for pH and a_w monitoring. Each treatment was conducted in duplicate. Cultures were enumerated as described in Appendix A.

3.4 RESULTS

3.4.1 Investigation of Inactivation under Anaerobic, Lethal Conditions

Figure 3.3 is representative of the results obtained when *E. coli* M23 OR.H- was exposed to pH and a_w adjusted CMM broths under anaerobic conditions at 35°C. Inactivation curves generated with additional a_w and pH combinations are shown in Figure 3.5. The inactivation curves in Figure 3.3 exhibited exponential decline for 24 hours after the experiment commenced. At this time, the a_w 0.93 culture had declined by over 2 Log cfu/ml. The cultures exposed to a_w 0.89 and a_w 0.82 both declined by 4 Log cfu/ml after 24 hours. After 24 hours when the temperature was lowered to 15°C, there was little difference in population size between cultures exposed to a_w 0.89 and a_w 0.82 after 172

hours (difference of 0.5 Log cfu/ml), when both appeared to experience a more rapid decline. There was no further decrease in cell number at a_w 0.93 once the temperature had been lowered.

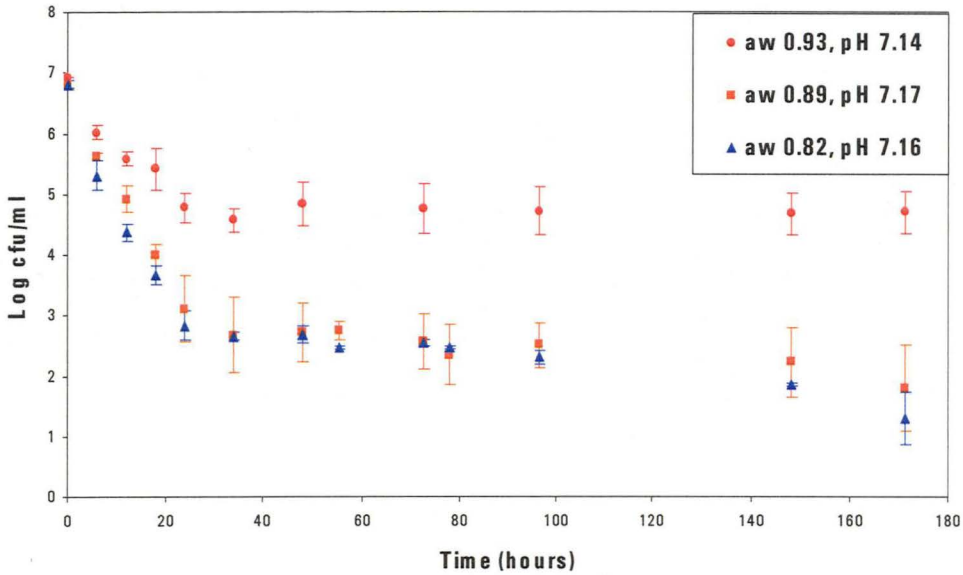

Figure 3.3. Anaerobic decline of *E. coli* M23 OR.H-, CMM medium at three water activities (NaCl as humectant), neutral starting pH, initial temperature of 35°C reduced to 15°C after 24 hours. Cells were enumerated on TPAP at 37°C.

Analysis of Figure 3.4 shows that the pH dropped rapidly over 24 hours to below pH 5.0 for the cultures at a_w 0.93 and a_w 0.82, and below pH 5.5 for the culture at a_w 0.89. At the termination of the experiment, pH levels for the cultures at a_w 0.82 and a_w 0.89 had risen by approximately 0.5 of a pH unit. Similar trends were observed with other water activity/pH trials (Figure 3.6)

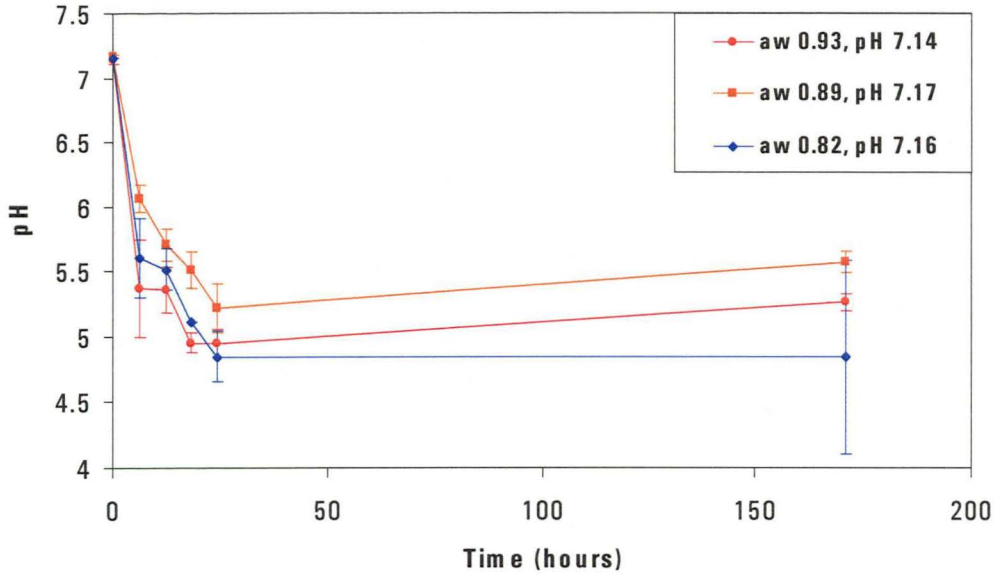

Figure 3.4. Change in pH of *E. coli* M23 OR.H-, CMM medium at three water activities (NaCl as humectant), neutral starting pH, initial temperature of 35°C reduced to 15°C after 24 hours.

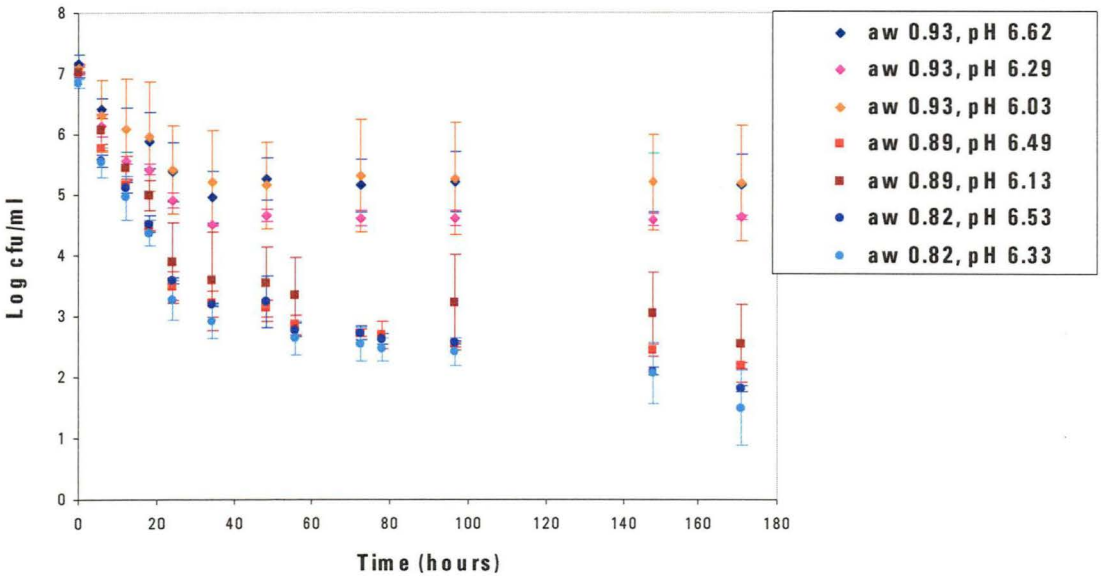

Figure 3.5. Anaerobic decline of *E. coli* M23 OR.H-, CMM medium at seven water activity/pH combinations (NaCl as humectant), initial temperature of 35°C reduced to 15°C after 24 hours. Cells were enumerated on TPAP at 37°C.

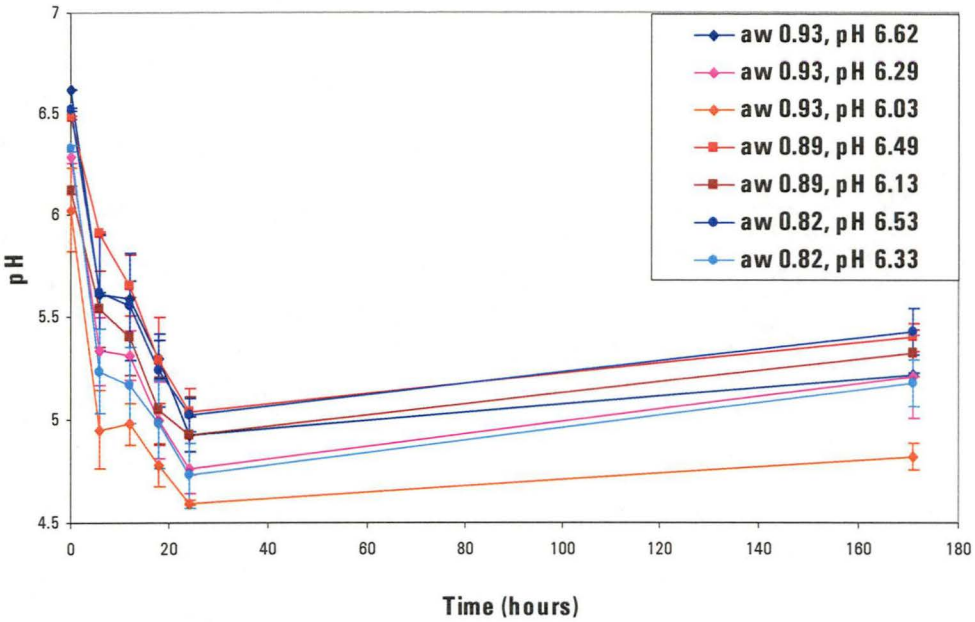

Figure 3.6. Change in pH of *E. coli* M23 OR.H-, CMM medium at seven water activity/pH combinations (NaCl as humectant), initial temperature of 35°C.

3.4.2 A Model System for Inactivation during Fermentation

Water activities more representative of conditions in UCFM prior to, and after fermentation, were used to generate the inactivation curves shown in Figure 3.7. In contrast to Figure 3.3, biphasic inactivation kinetics were observed during the first 24 hours, despite the continual pH decline for 21 hours after the experiment commenced. Figure 3.8 shows fluctuations in the pH of all treatments. These fluctuations appeared to result in a temporary 0.5 Log cfu/ml decline for cultures at a_w 0.956 and a_w 0.963 (with lactic acid). Addition of lactic acid to cultures did not appear to have an appreciable effect on survival when compared to those exposed to HCl at identical water activities.

A gradual decline in all populations was observed between 24-48 hours, when the temperature was 15°C and acidification had ceased (Figure 3.7). At the termination of the experiment, the maximum population decline of any culture was approximately 1.4 Log cfu/ml (at a_w 0.956), while the minimum was approximately 0.8 Log cfu/ml (a_w 0.943). Similar to cultures described by data in Figures 3.4 and 3.6, the pH of all cultures had increased by approximately 0.5 units at the conclusion of the experiment.

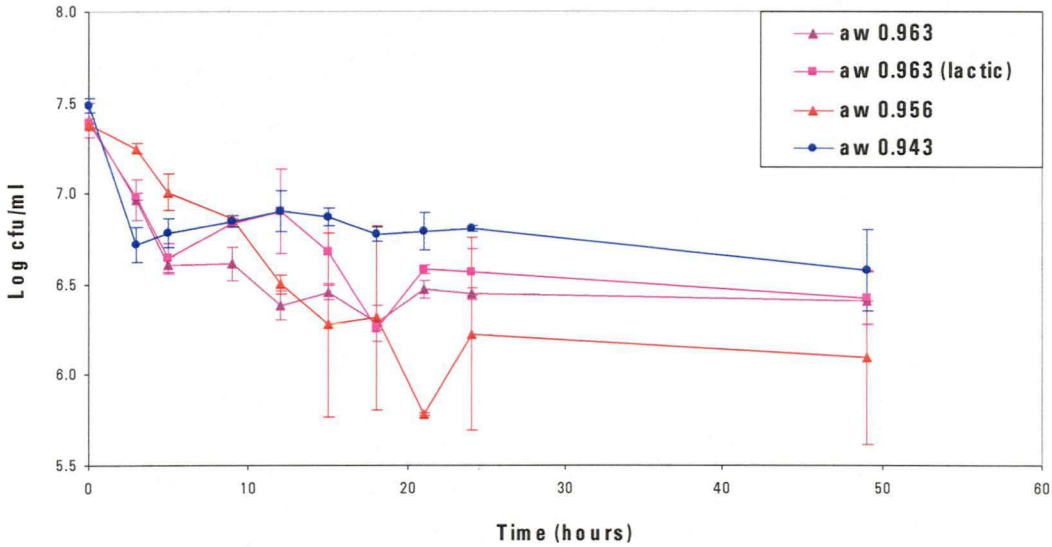

Figure 3.7. Anaerobic decline of *E. coli* M23 OR.H-, CMM medium at three water activities (NaCl as humectant), initial pH 7.0, start temperature of 35°C reduced to 15°C after 24 hours. Cells were recovered on TPAP at 37°C.

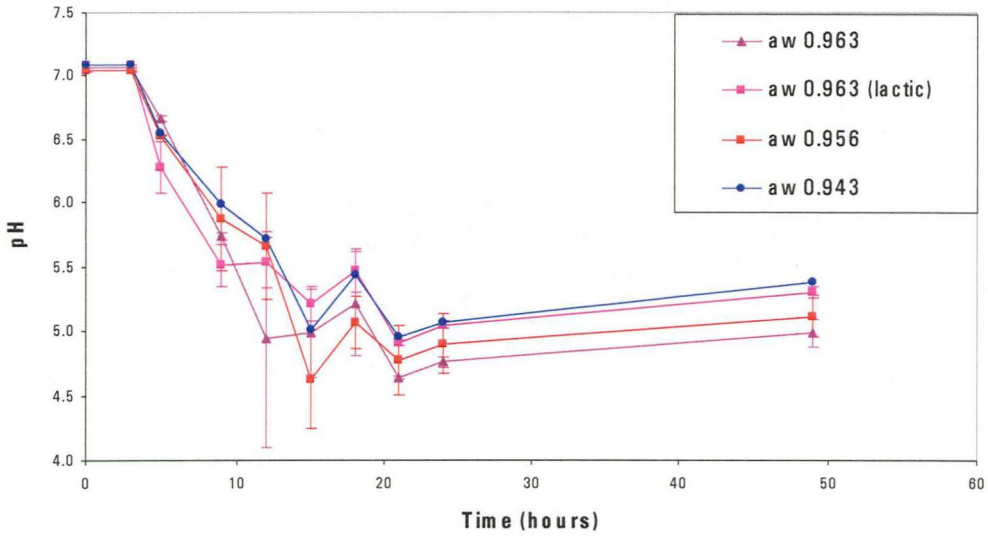

Figure 3.8. Change in pH of *E. coli* M23 OR.H-, CMM medium at three water activities (NaCl as humectant), initial pH 7.0, start temperature of 35°C reduced to 15°C after 24 hours.

3.4.3 Comparison of Aerobic and Anaerobic Inactivation in Cooked Meat Medium

The decline of cultures in CMM under aerobic conditions is shown in Figure 3.9. Inactivation of approximately 1 Log cfu/ml, which increases with decreasing water activity, is apparent over the first 3 hours of the experiment. Addition of HCl after 3 hours did not result in an increased rate of death. Cultures exposed to a_w 0.943 and a_w 0.924 reached maximum population declines of approximately 2.5 and 3.5 Log cfu/ml respectively, after 18 hours. After 3 hours, the culture exposed to a_w 0.897 experienced a steady decline in cell number. The fall in pH experienced by all cultures showed some fluctuation, but was more stable than that shown in Figure 3.10.

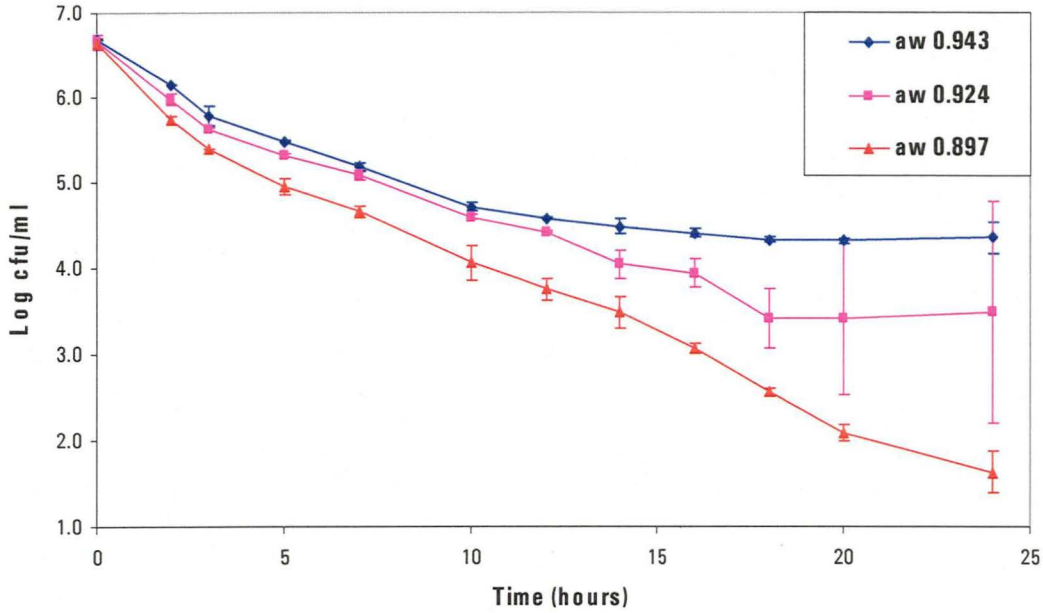

Figure 3.9. Aerobic decline of *E. coli* M23 OR.H-, CMM medium at three water activities (NaCl as humectant), neutral starting pH, 35°C. Cells were recovered on TPAP at 37°C.

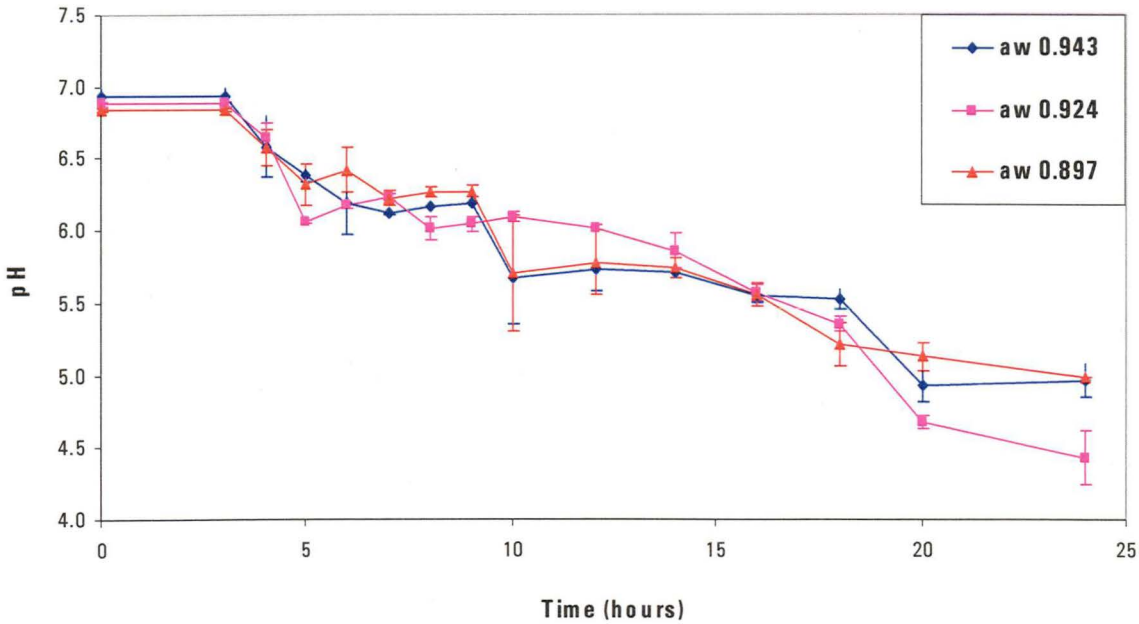

Figure 3.10. Change in pH of *E. coli* M23 OR.H-, CMM medium at three water activities (NaCl as humectant), neutral starting pH, 35°C.

In comparison, anaerobic cultures demonstrated better survival than those under aerobic conditions and exposed to similar water activities (Figure 3.11). Accounting for the higher starting cell numbers in both anaerobic cultures, it appears that populations in these flasks experienced an almost identical initial level of inactivation. The inactivation rate for the aerobic culture exposed to a_w 0.943 slowed after 3 hours and tailing commenced after 15 hours. In contrast, the anaerobic culture at a_w 0.943 did not display any further reduction in cell number after 3 hours. Anaerobic and aerobic cultures exposed to a_w 0.90 displayed similar inactivation characteristics over the first 7 hours, before diverging. Allowing for different starting densities, there was approximately a 2-Log cfu/ml difference in cell number between anaerobic and aerobic populations after 24 hrs.

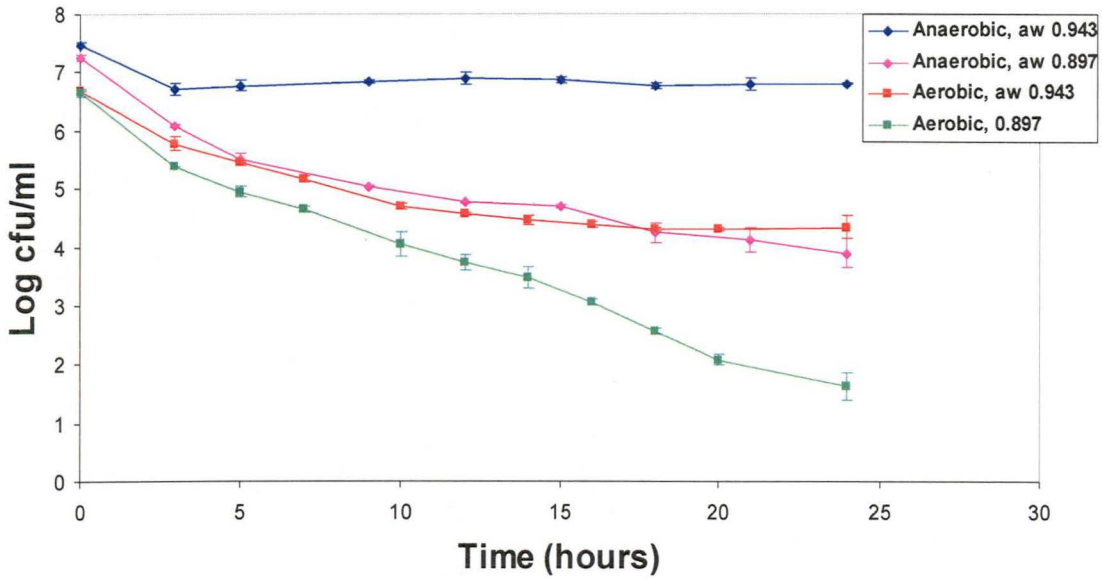

Figure 3.11. Comparison of population decline between aerobic and anaerobic cultures of *E. coli* M23 OR.H-, CMM medium at two comparable water activities (NaCl as humectant), neutral starting pH, 35°C. Cells were recovered on TPAP at 37°C.

The water activities in Figure 3.11 were chosen on the basis of their similar declines in pH. This is illustrated in Figure 3.12, which describes comparable pH decline in cultures exposed to a_w 0.897 and a_w 0.900.

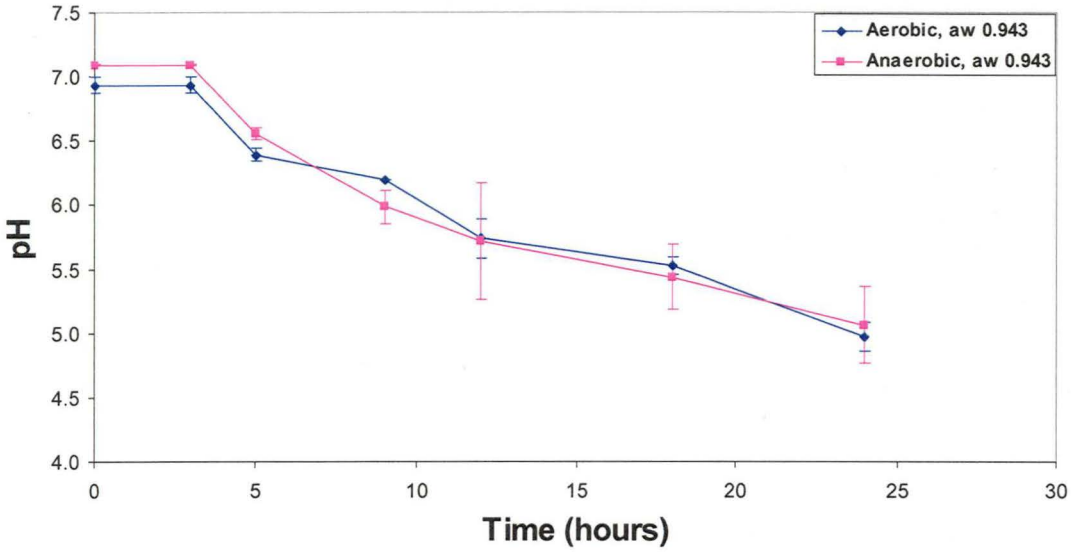

Figure 3.12. Comparison of pH change in aerobic and anaerobic cultures of *E. coli* M23 OR.H-, CMM medium at a_w 0.943 (NaCl as humectant), neutral starting pH, 35°C.

3.5 DISCUSSION

3.5.1 Inactivation under Anaerobic Conditions

The development of a system using CMM under anaerobic conditions to model the inactivation of *E. coli* in UCFM during fermentation appeared to be successful. Problems were encountered in maintaining control of pH. Fluctuations were observed in many of

the pH *versus* time curves. As the CMM has some effect on absorption of [H^+], efficient use of the model broth system will require an element of operator expertise and familiarity in order to simulate accurately the pH change observed in UCFM production. Despite the rapid rate of pH change over the first 12 hours, final levels (e.g. Figure 3.7) and the time taken to reach them, were representative of semidry sausage produced in the USA (Ross and Shadbolt, 2001). Such products are characterised by fermentation of short duration (15-20h) and high temperature (27-41°C).

Of more importance is the comparability of inactivation rates with those observed in the literature and, hence, how well the broth system is able to model the effects of fermentation in UCFM. After 24 hours, the magnitude of inactivation shown in Figure 3.7 varied from approximately 0.8 Log cfu/ml to 1.4 Log cfu/ml. These rates are similar to reported literature values for reduction of *E. coli* O157:H7 after fermentation in UCFM as reviewed by Ross and Shadbolt (2001). Table 3.4 tabulates inactivation rates from this study and unpublished data obtained using a similar broth-model system designed to replicate the effects of UCFM production (L. McQuestin, University of Tasmania, pers. Comm, 2004). Higher inactivation rates were obtained at water activities of 0.956 and 0.963, however, the pH imposed on these treatments was more severe than those which L McQuestin used.

Table 3.4. Inactivation rates obtained with UCFM broth-model systems, 35°C, NaCl as humectant, HCl as acidulant. * Denotes results from this study.

Water activity	PH	Inactivation Rate [1/(Log cfu/ml).h^{-1}]
0.893	5.76	0.067
0.894	5.97	0.0893
0.910	6.00	0.073
0.927	6.05	0.0404
0.941	6.13	0.0258
0.943*	5.1*	0.0261*
0.956*	4.8*	0.0595*
0.963*	4.7*	0.0552*

This suggests that the model CMM-broth system employed can simulate the fermentation stage in other types of UCFM processes, including those typically observed in Australian production which are fermented for over 40 hours (Ross and Shadbolt, 2001).

3.5.2 Comparison between Anaerobic and Aerobic Conditions

Ross and Shadbolt (2001) reported that comparisons between broth models and actual UCFM studies indicated that inactivation rates tended to be faster in the former. This is reinforced by the 2-Log cfu/ml difference between aerobic and anaerobic cultures shown

in Figure 3.11. It remains to be elucidated why this difference occurs, although considerable information exists in the literature concerning the formation of reactive oxygen species (ROS) within aerated systems and their bactericidal effects (Dodd et al., 1997). Bloomfield et al. (1998) also proposed that some of the resistance to inimical processes shown by stationary phase cells was explained by a switch from respiration to substrate-level phosphorylation. This had the effect of lowering the rate of metabolism and hence ROS generation. Shadbolt (1998) observed that a lowered metabolism also reduced the rate of inactivation. Cells grown to stationary phase in a Minimal Minerals Medium and exposed to lethal water activity did not experience the same magnitude of first phase death as cells cultured in a rich medium which were then exposed to the same water activity conditions. It was speculated that cells had more time to adapt to the lethal conditions with a lowered state of metabolism.

The faster rates of inactivation under aerobic conditions in this study may, in part, be artefactual as Erlenmeyer flasks were employed for this work, rather than L-tubes which were used for the anaerobic studies. Erlenmeyer flasks were chosen for their ease of use, but the increased aeration may exaggerate the effects of ROS in this study. Nevertheless, the basic principle of ROS formation remains the central hypothesis regarding increased inactivation under aerobic conditions, and why past broth model systems have largely been inadequate for simulating *E. coli* death in UCFM.

3.5.3 Future Research and Applicability of Models to UCFM Production

Assuming that it is possible to model the extent of microbial inactivation during fermentation, further investigation should focus on the drying phase of UCFM production. This may be achievable by slow addition of sterile, NaCl-saturated CMM broth over a period representative of the maturation phase. Given the observed pH fluctuations, some expertise and operator familiarity will be necessary to obtain inactivation rates that accurately reflect that occurring in real UCFM formulations.

However, the CMM broth system used in this study has several advantages over full-scale UCFM trials, such as:

- less expense due to greater cost of meat, additives and processing equipment needed for UCFM;
- no requirement for fermentation chamber with temperature/relative humidity controls;
- sampling procedure less intrusive and easier to perform (can do multiple samples from a single broth, where separate UCFMs are needed for each sampling time);
- no interference from background microflora; and
- more homogeneity than a meat matrix which facilitates better distribution of *E. coli*.

The UCFM model in Ross and Shadbolt (2001) was able to predict inactivation with an accuracy of ± 0.5 – 1.0 Log cfu/ml. Despite the wide confidence intervals this model has been used extensively in Australia by regulators and industry to gain an understanding of their processing effectiveness against a 3-Log reduction, as required by 1996 legislation (D. Miles, NSW Food Authority, pers comm., 2003).

However, as highlighted by Ross and Shadbolt (2001), a 3-Log reduction is rarely achievable without a heating step. This information was considered by Food Standards Australia New Zealand and in late 2001 an amendment to the Australia's *Food Standards Code* was drafted. The draft amendment was accepted into legislation in September 2003. The new standard for UCFM production reflects a shift away from prescriptive processing guidelines by removing the mandatory 3-Log reduction. Manufacturers are now required to adopt HACCP-based food safety programs and food safety objectives. This gives manufacturers more flexibility in how they meet their food safety objective, instead of compliance with a prescriptive 3-Log reduction which was found to be unachievable. The broth system described in this study has generated additional data to refine the UCFM model described in Ross and Shadbolt (2001) (McQuestin, pers comm, 2003). This will facilitate more accurate prediction of *E. coli* inactivation, ensuring that manufacturers can produce a safe UCFM, and determine the effect of variations in product composition.

This is a practical example of detailed systematic studies providing a better understanding of pathogen inactivation from which to better understand pathogen responses in food

during processing and storage. This understanding has led to changes in national legislation, and provided industry with flexibility to develop a range of process variables that meet food safety needs. Without such knowledge of inactivation kinetics, industry and regulators are forced to adopt a conservative approach (such as a mandatory 3-Log reduction) which restricts the range of available products and requires strict enforcement.

The following chapter investigates the potential for the use of modern analytical methods to automate analysis of bacterial viability and quantification. Given that generation of inactivation curves using agar-based viable count methods is a laborious process, use of an automated method would facilitate rapid collection of quantitative inactivation data essential for development of predictive models, such as that in Ross and Shadbolt (2001).

4 RAPID ANALYSIS OF *Escherichia coli* VIABILITY USING FLOW CYTOMETRY

4.1 INTRODUCTION

Survival characteristics and tolerance to inimical conditions have been the focus of many studies on pathogenic *E. coli* species in recent years as a result of an increased incidence in foodborne disease [refer to 1.4.1 and 3.2.6]. Disease outbreaks associated with foods such as salami, previously thought to have been microbiologically safe due to the method of production, resulted in a heightened awareness of the dangers from acid-tolerant vero-toxigenic *E. coli* (VTEC) (Cameron et al., 1995; MMWR, 1995).

With the increasing incidence of foodborne disease the need for detailed, systematic data describing the inactivation kinetics of pathogens during processing has become apparent (McMeekin et al., 1997). To design novel, yet safer, manufacturing methods the survival characteristics of pathogens must be described in detail. Traditional plate count methods of enumeration are tedious and time consuming. In the absence of differential agar combinations that quantify cell injury it is only possible to obtain single parameter measurements of bacterial viability. Epi-fluorescent microscopy may be used to obtain additional information, such as respiration or viability status of cells (Mason et al., 1986; Monfort and Baleux, 1992; Millet and Lonvaud-Funel, 2000). However such techniques are also time–consuming and tend to be subjective due to operator fatigue (Seo et al.,

1998). The use of rapid, automated methods for examination of bacterial viability is gaining increasing attention.

Much has been written about the value of flow cytometry (FCM) as a rapid method for detection of bacteria and examination of cellular heterogeneity and viability (Kell et al., 1991; Kaprelyants et al., 1996; Nebe-von Caron et al., 1998; Seo et al., 1998; Turner et al., 2000). In combination with fluorescent staining it is possible to detect bacterial populations and differentiate between healthy, injured and dead cells. The ability to quantify rates of injury and heterogeneity rapidly under simulated processing conditions would be an invaluable tool for the microbiologist in the design of novel food processing techniques.

The experiments described in this chapter were specifically intended to differentiate cell viability and cell culturability. This is a critical question in the interpretation of non-thermal inactivation data, which is of particular relevance to *E. coli* inactivation during production of uncooked comminuted fermented meat products. The objective was to assess the potential of flow cytometry coupled with viability staining techniques to detect injured but non-culturable cells. This has presented something of a problem for interpretation of survival studies using traditional viable count methods (Nebe-von Caron et al., 1998; Turner et al., 2000). In addition, the potential for flow cytometry to be used as a rapid method of bacterial enumeration during inactivation studies was assessed.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and growth conditions

E. coli M23 OR.H-, was maintained on Luria Bertani (LB, 10.0g tryptone/LabM; 10.0g NaCl/Sigma; 5.0g Yeast Extract/Oxoid; 14.0g tech agar) agar at 4°C. Experimental inocula were cultured from plates and grown at 37°C in 80ml LB broth (per litre MilliQ H_2O, 10.0g tryptone/LabM; 10.0g NaCl/Sigma; 5.0g Yeast Extract/Oxoid) in an Erlenmeyer flask with shaking incubation (Gellenkamp, Germany) for 23 hours at pH 7.35 ± 0.05 to a stationary phase population *ca* 1 x 10^9 cfu/ml.

4.2.2 pH stress treatments and determination of heterogeneity

Late-stationary phase cultures were diluted ten-fold to *ca.* 1 x 10^8 cells/ml and exposed to a pH range of 2.5 - 3.5 in shaking LB broth (as above) at 37°C. Aliquots were removed at appropriate intervals for analysis of viability by FCM. Viable (plate) counts were performed by spread plating 100µl aliquots on LB agar and incubating for 24 hours at 37°C. Total cell counts using an improved Neubauer counting chamber were also performed.

4.2.3 Fluorescent Staining

Fluorescent dyes were obtained from Molecular Probes (Eugene, Oregon, USA). Samples were stained according to the following protocol based on Hewitt et al. (1999). $DiBAC_4(_3)$, an anionic dye that binds to lipid-components of the cell membrane in bacteria with depolarised plasma membranes, was used an indicator of injury. Propidium iodide (PI), accumulates intracellularly and binds to DNA of cells with compromised membrane integrity, and was used as an indicator of bacterial death. One ml aliquots of *E. coli* M23 OR.H-, prepared as described above were removed from flasks, centrifuged and washed in 900μl of 0.1M MilliQ phosphate-buffered saline (PBS). Fluorescent stains were added to the washed cell suspensions at concentrations of 10μg/ml. Staining was assisted by addition of 50μl 0.1M EDTA to enhance membrane permeability. Cell suspensions were incubated for 15min in darkness at room temperature. Excess stain was removed by centrifugation and washing in 0.1M MilliQ PBS. Cytometry percentage compositions (heterogeneity - incorporating live, injured, or dead cells) of cell populations according to their physiological status were estimated using WinMDI version 2.8 software (Joseph Trotter, The Scripps Research Institute, Cal, USA).

4.2.4 Flow cytometry instrumentation

A Partec PAS-III flow cytometer (Partec GmbH, Germany) equipped with air-cooled, argon-ion (488nm and 25mW) and red helium-neon lasers (635nm and 25mW) was used

to measure the effects of cells exposed to lethal pH treatments. Light scattering and fluorescence were triggered by side scatter (SSC) signals. Filter blocks were set according to the manufacturers instruction for argon and red diode lasers. $DiBAC_4(_3)$ and PI fluorescence were detected in the FL2 and FL3 photomultiplier tubes (PMT) at wavelengths of 575nm and >645nm respectively. Optimum PMT voltages were as follows: forward scatter (FSC) 200V; SSC 150V; FL2 400V; FL3 460V. Minimum sample acquisition was $2x10^4$ events with a flow rate of 300-600 cells/sec. Equipment calibration was performed using 3.5μm fluorescent beads. When required, cell sorting was performed using a Coulter EPICS Elite (Coulter Electronics, Hialeah, FL, USA) configured as described in Kaprelyants et al. (1996) with an argon laser at 488nm and measurements gated on forward scatter and 3 cells/well used to inoculate 96-well plates containing LB broth. Uninoculated wells were used as controls.

4.3 RESULTS

4.3.1 Comparison of plate count and flow cytometry viability data at lethal pH

Results from plate count data and FCM were collated and compared. Figure 4.1 shows the relationship between colony-forming unit viable count and FCM data at pH 3.20, 37°C. Total cell counts remained *ca* $1x10^8$ cfu/ml over the time period shown. Viable counts (based on membrane permeability) derived from FCM were consistently higher than colony-forming assays on LB agar.

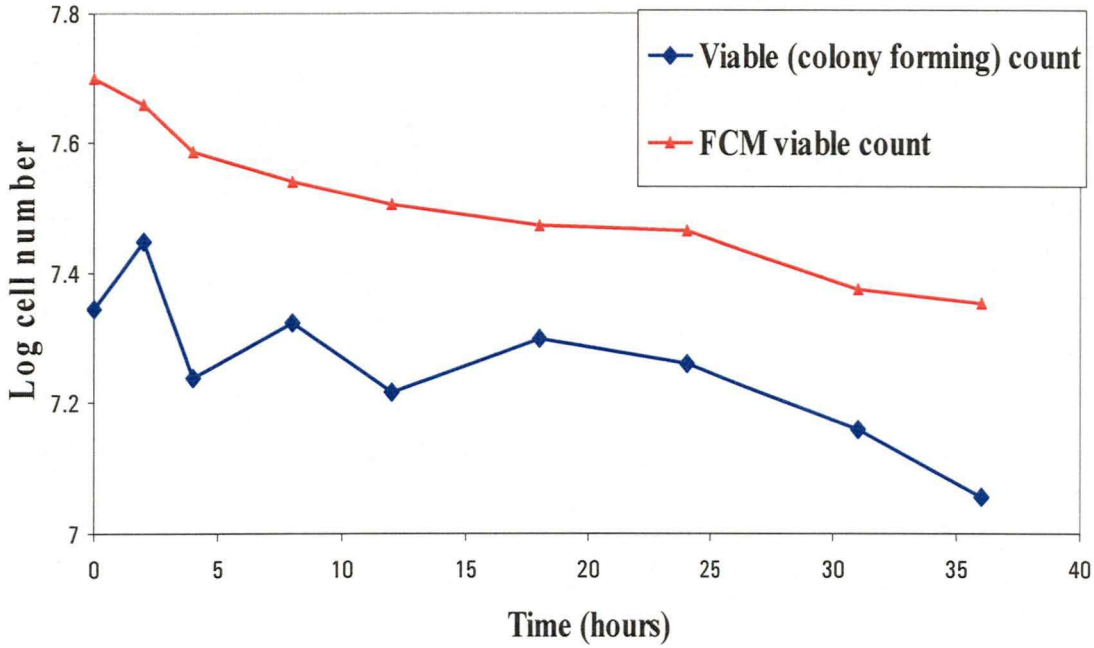

Figure 4.1. Comparison of direct cell count, and viability of *E. coli* M23 OR:H- with inactivation over 35h, 37°C, pH 3.2 (HCl as acidulant), LB broth, as determined by FCM and viable count. Cells recovered on LB agar at 37°C.

4.3.2 Changes in heterogeneity with acid-stress

Figure 4.2 describes the change in microbial heterogeneity under identical conditions to those described in Figure 4.1. Based on the amount of PI and $DiBAC_4(_3)$ uptake cells were grouped into three categories corresponding to their physiological status, these being (i) healthy, (ii) injured, and (iii) dead. As the experiment progressed, the number of dead cells increased at approximately the same rate as decline of culturable cells measured by viable count, also shown by Table 4.1. The number of cells showing little or no staining (healthy) over 30hrs dropped to around 0.5-1% of the total bacterial

population. Injured cells sorted into LB broth all showed growth after 24 hours (data not shown).

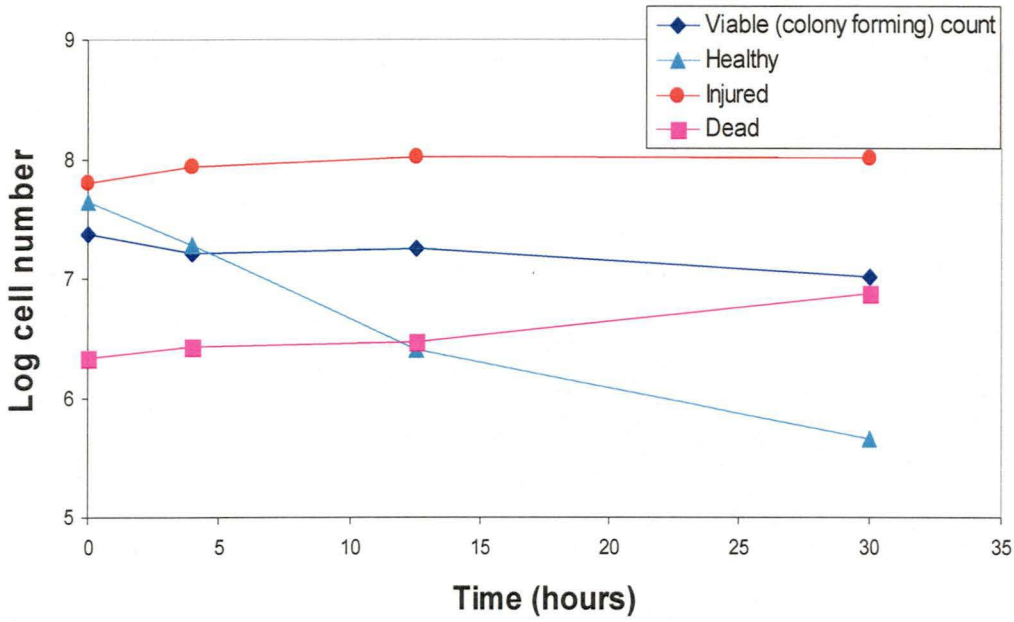

Figure 4.2. Heterogeneity of *E. coli* M23 OR.H- determined by FCM during inactivation in pH 3.20 (HCl as acidulant), 37°C, LB broth, showing change in physiological status over 30 hrs and comparison with data derived from viable count. Cells recovered on LB agar at 37°C.

Table 4.1. Change in heterogeneity of *E. coli* M23 OR:H- with prolonged exposed to pH 3.2 in LB broth at 37°C.

Time (hrs)	Viability on LB agar (log cfu/ml)	FCM Heterogeneity (log count/ml)		
		Healthy	Injured	Dead
0	7.38	7.65	7.80	6.34
4	7.22	7.28	7.94	6.44
12.5	7.25	6.41	8.02	6.48
30	7.02	5.66	8.01	6.88

4.3.3 Heterogeneity at two acid levels

The magnitude and duration of the imposed stress influenced the extent of heterogeneity. Figure 4.3 shows the comparison of staining intensity between cultures held at pH 2.50 and 3.20 immediately before exposure to acid stress and exposure after 2 hours, with $DiBAC_4(_3)$ on the y-axis against PI staining on the x-axis. At T_0 both populations exhibit minimal $DiBAC_4(_3)$ staining, indicating healthy cells. After 2 hours at pH 2.50 a greater proportion of cells were heavily stained with PI (and hence "dead") compared to a population at pH 3.20. At pH 3.20 there appears to be a greater distribution of physiological states of *E. coli* cells. A large dead fraction was observed at pH 2.50 after 2 hours with few injured cells relative to that observed at pH 3.20.

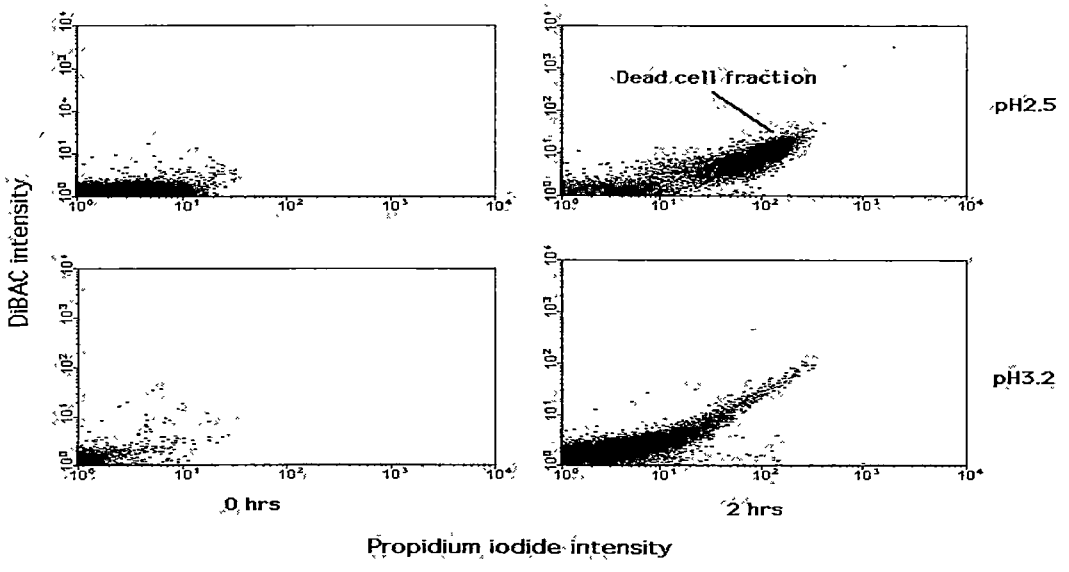

Figure 4.3. Comparison in change of *E. coli* M23 OR:H- heterogeneity at pH 2.5 and 3.2 (HCl as acidulant), 37°C, LB broth, as determined by differences in fluorescent stain uptake.

4.3.4 Heterogeneity of a control culture

A control culture with no acid stress was monitored. Figure 4.4 describes the change in heterogeneity of this culture with time. The number of healthy cells determined by FCM compared well with viable count data (not shown). At 12 hours the culture had entered stationary phase, which corresponded with an approximate 1 Log increase in injury. As fractions of the total population, however, numbers of injured and dead cells declined with an increase in total cell number (Figure 4.4) due to growth of new, healthy cells.

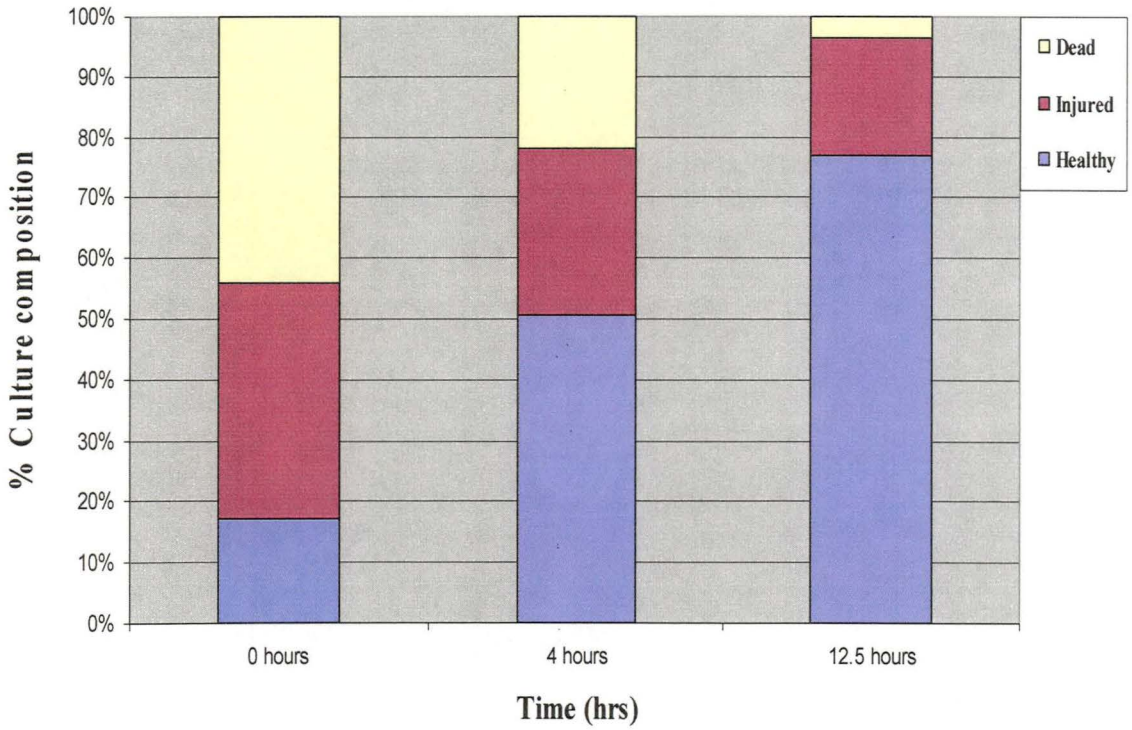

Figure 4.4. Change in total percent composition of cells in a control culture, pH 7.0, 37°C, LB broth, 12.5 hours.

4.3.5 Sensitivity limits of flow cytometry

Totals viable counts by culture and as measured by FCM were found to correlate well over the first 1-2 Log units of cell death (Figure 4.1). After a prolonged period of exposure to acid stress, however, the level of viable cells determined by FCM did not appear to fall below 0.5% of the original population and actually increased in contrast to viable count on agar plate (Figure 4.5). Total cell counts as determined by direct microscopy remained *ca* $1x10^8$ cfu/ml over the course of the experiment (data not shown).

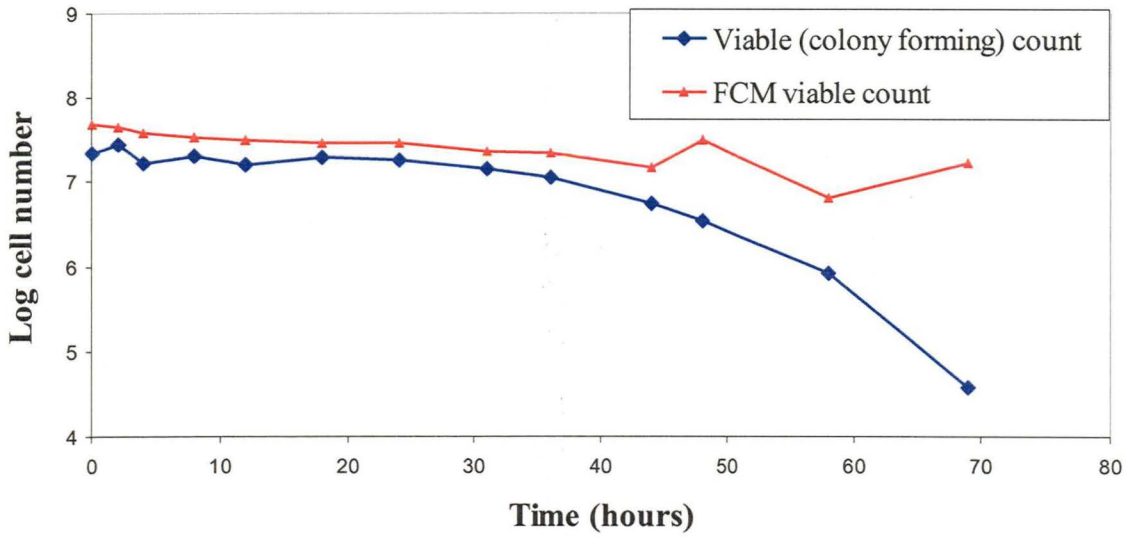

Figure 4.5. Comparison of viable (colony-forming unit) count, and FCM-derived viability assessment of *E. coli* M23 OR.H- over 70h, at 37°C, pH 3.2 (HCl as acidulant), LB broth, conditions inimical to *E. coli*. Cells enumerated on LB agar at 37°C.

4.4 DISCUSSION

4.4.1 Viability and heterogeneity of Escherichia coli determined by flow cytometry at lethal pH

It has been stated that standard, cultural (agar-based), measurements of viability are not sufficient for recovery of severely injured cells which may later regain culturability if conditions enable them to repair themselves (Kaprelyants and Kell, 1992; Otero et al., 1998; Suller and Lloyd, 1999). The data in Figure 4.1 and from other experiments (not shown) support this. Viability determined by FCM was approximately 0.1-0.2 Log units higher than that determined by standard plate counts over the first 44 hours at pH 3.20. The initial number of injured cells shown in Figure 4.2 may be due to the use of a late stationary phase inoculum. The recovery of injured cells sorted into LB broth (data not shown) suggests that these bacteria were able to recover following exposure to stress. This reinforces the view that agar-based counts are inadequate for determining total number of viable cells, and highlights the possibility of a foodborne disease outbreak if injured and non-culturable pathogenic bacteria are provided with means of recovery.

4.4.2 Heterogeneity at two acid levels

As shown in Figure 4.3, more stringent stress may reduce the extent of heterogeneity by selecting for an acid-tolerant subpopulation of bacteria. At pH 2.50 a clearly defined subpopulation is visible. At the milder pH there is a broad spectrum apparent in the

resistance of cells to acid-stress. Thus, it has been demonstrated that over a defined period of time it is possible to visualise cell heterogeneity and subpopulations which otherwise are not detectable by conventional plate counting methods using differential/selective media. However the staining technique was found to have limited sensitivity as discussed below [4.4.3].

4.4.3 Flow cytometric analysis of control cultures

The level of dead cells in a control culture with no acid-stress imposed (Figure 4.4) appeared to fall slightly. This may be due to injured cells wrongly interpreted as dead. The results of the present study indicate that the use of EDTA to enhance stain uptake as described by Hewitt et al. (1999) may lead to incorrect interpretation of the data. Enhanced membrane permeability is likely to produce an artificially high injury count. Fuller et al. (2000) also questioned the use of EDTA, noting that it resulted in lower fluorescence when staining with 5-(and 6–)-carboxyfluorescein diacetate, succinimidyl ester (CFDA/SE). Based on data already shown and cell sorting into LB broth, the total viable population determined by flow cytometry and viability staining under conditions favouring maximal recovery is likely to be the sum of healthy and injured cells. Similar to Figure 4.1, this population is higher than the level indicated by plate counts.

4.4.4 Sensitivity limits of flow cytometry

While FCM has proven useful for examining cellular heterogeneity in this study, there are several drawbacks as commented on by other authors. Some have noted that instrument costs are prohibitive (Davey and Kell 1996; Hewitt et al., 1999). In terms of research however, it may be argued that cytometers are widespread enough for studies to be undertaken collaboratively, thus eliminating the requirement to purchase a flow cytometer. Rather, the largest obstacles may be the lack of reliable validation available since methods and results differ between users, and also the inadequacy of commercially available instruments for precise microbial measurements on rare (below 1% of total population) cell populations (Shapiro, Purdue Cytometry Mailing List, May 2001).

There is little agreement (and much conjecture) on an optimum method for fluorescent staining, other than it is case specific for each organism and strain (Davey et al., 1999; Suller and Lloyd, 1999; Nebe-von Caron et al., 2000; Turner et al., 2000). The membrane structure of gram negative bacteria makes them particularly difficult to stain, leading to disagreement amongst workers in this field over the best methods (Porter et al., 1995; Nebe-von Caron et al., 2000; Shapiro, 2000).

Nebe-von Caron et al. (1998) describes many of the practical problems and limitations of fluorescent staining. Results from the current study were limited to a resolution of approximately 1% of the total starting population (1% sensitivity). As shown in Figure 4.5, after prolonged exposure to acid stress, the proportion of viable counts as determined

by FCM actually increased. This may be attributed to loss of DNA from permeabilised cells, which therefore do not show as being stained by PI. Nebe-von Caron et al. (1998) and Brul et al. (2000) refer to the existence of these "ghost" cells. The latter (Brul et al. 2000) also observed a lack of correlation between viable (plate) counts and PI uptake in yeast cells. Variable results with staining and a lack of correlation between dye uptake and *E. coli* viable (plate) count have been observed in other studies (Lisle et al. 1999, Wickens et al. 2000). In terms of the protocol used in this study, centrifugation and washing may also remove cells of interest and distort the results. Diaper and Edwards (1994) observed large differences between plate counts and FCM viability measurements, and speculated that cells were lost during sample preparation.

Reviews (Otero et al., 1998; Abee and Wouters, 1999) have suggested the use of flow cytometry as a rapid method for analysis of viability of bacterial cells in meat products and minimally processed foods. From the perspective of describing and understanding the kinetics of non-thermal inactivation the value of a sensitivity limit of 1% of all cells in FCM must be questioned. For example, regulations governing commercial salami manufacture in the USA are currently based on a 5 Log cfu/ml reduction in levels of *E. coli* potentially present in the batter. Nebe-von Caron et al. (2000) state that in order to distinguish the cell sample signal from background noise and debris it is desirable to select at least 100 cells of interest (100 viable cells of *E. coli* for the purpose of this dissertation). Thus, after a 3 Log reduction (0.1% of starting number or sensitivity in this case) 100,000 events must be analysed to be certain that no more than 0.1% of the remaining cells are viable. Under (absolutely) ideal conditions of low signal-noise ratio

and using a slow flow rate such an assay may require up to 10 minutes of sample acquisition time. If one considers now the analysis of a 5 log reduction in viable cell number, it becomes apparent that under such conditions flow cytometry is no longer a rapid method, as over 16 hours would be required to collect a single sample and the cells must not undergo any physiological change during this time. In terms of minimally processed foods, the main consideration is whether or not a population of pathogenic *Escherichia coli* constituting an infective dose will survive manufacture. As stated previously [1.2.2] the infective dose for VTEC is believed to be very low and detection of such small numbers, while possible with the use of specific antibodies, will be difficult. Determination of viability of those cells with FCM is impossible with current limits in instrument sensitivity.

Many authors promote FCM for multi-parameter bacterial viability measurements and as a rapid method (Diaper and Edwards, 1994; Lopez'Amoros et al., 1997; Tanaka et al., 2000; Turner et al., 2000). However, the precise definition of a viable cell will be the subject of considerable debate for some time as indicated by the extensive review of Kell et al. (1998). The suitability of stains as viability indicators will also depend on the method of inactivation. Villarino et al. (1999) observed that ultra-violet killed *Escherichia coli* cells retained metabolic activity for 2 hours after death, while Mackey (pers comm., 1999) observed that membrane integrity persisted for up to 3 weeks. Clearly, the protocol used in this study is unsuitable for analysis of UV-treated cultures. From a food microbiology perspective, FCM examination of *Escherichia coli* viability provides a rapid qualitative output, but a limited amount of quantitative data required for

the detailed and systematic analysis of inactivation kinetics. Application of flow cytometry is becoming widespread in microbiology, but few reports have discussed the technological limitations. Davey et al. (1999), however, provides a comprehensive discussion of viability analysis and evaluation of protocols. Those authors consider that culturability is the only true indicator of viability, a view shared by Bogosian et al. (2000).

FCM has been effective for the analysis of yeasts and many gram positive bacteria, but physiology restricts measurements on microbes such as *E. coli*. Steen (2000) and Shapiro (Purdue Cytometry Mailing List, May 2001) consider that flow cytometry has yet to be fully utilised in a microbiological sense and requires the construction of purpose built instruments for the dedicated analysis of bacteria. Despite years of research and several publications, manufacturers are yet to assemble such a flow cytometer. Until this occurs, quantitative viability measurements of bacteria, especially gram negatives, will remain limited.

5 CONCLUSION

The genesis for this research was an outbreak of foodborne disease associated with an uncooked comminuted fermented meat (UCFM) product in South Australia, 1995. The outbreak was severe enough to generate a high level of media interest and public outrage, resulting in a large economic downturn for the Australia smallgoods industry. Regulatory authorities at that time were challenged by the lack of knowledge regarding the survival of acid-tolerant *Escherichia coli* during the UCFM process. Hence the need for detailed, systematic data on the conditions of nonthermal inactivation employed during manufacture of UCFM products was realised.

A literature review revealed that research into bacterial inactivation kinetics had commenced in the early twentieth-century. Of note was the scientific debate concerning deviations from log-linear inactivation kinetics and the hypotheses that this was due to:

- the mechanism of inactivation conditions employed,
- or variability of resistance within a given cell population.

Under the variable water activity and pH conditions at nonthermal inactivation temperatures employed in this study, neither hypothesis was determined to be solely responsible for the observed kinetics. Minor variations in inoculum preparation and age of an *E. coli* M23 OR.H- culture were found to affect the resistance of cells to pH and water activity stress. Regardless of inoculum preparation, it was determined that cells exposed to lethal pH display different inactivation kinetics to those exposed to lethal water activity. Based on selective media counts, the latter stress generated a higher level of injury compared to cells at lethal pH. However, it was observed that cells exposed to lethal pH died more rapidly. It was

hypothesised that pH stress placed a large energy demand on the cell, resulting in the faster level of inactivation.

The above knowledge was utilised to determine the effect of variations in the order and timing of lethal stress imposition. Lethal acid stress was found to sensitise cells of *E. coli* to subsequent exposure to lethal water activity. Cells were more sensitive to subsequent stress the longer they were first exposed to low pH.

The requirement for detailed, systematic data on nonthermal inactivation during UCFM processing led to the investigation of the feasibility of modelling death in a broth system. An anaerobic environment using cooked meat medium was created that was able to mimic the effects of fermentation during manufacture of UCFM. Data from this system were used to refine a predictive model that has influenced Australian food standards legislation and assisted manufacturers to gain a better understanding of their processing effectiveness to reduce pathogens. This demonstrated the value of strategic research on microbial physiology and ecology.

Traditional viable-count methods used to generate detailed systematic, inactivation data is time consuming and labour intensive. The use of flow cytometry as a rapid method for determining the viability of *E. coli* (and hence simplifying the process of systematic data collection) after exposure to lethal pH was investigated. This technique proved to be reliable and rapid over the first 2-Log_{10} reduction with high cell numbers. However, beyond this level of inactivation, the sensitivity of the instrument rapidly decreased and flow cytometry ceased to be an effective method. It is concluded that modern methods and equipment still require

considerable effort and design before they become as effective as traditional viable count techniques.

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APPENDIX A

STRAINS

Escherichia coli M23 OR.H-

Non-pathogenic strain from cryogenic culture, courtesy of Dr K Sanderson, University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001.Unless otherwise indicated, *E. coli* M23 was maintained on Nutrient Agar slopes at 4°C. Inocula were prepared by placing a standard loop of culture in a 250ml Erlenmeyer flask containing 80ml TSB with static incubation at 37°C for 22 hours.

STANDARD PROCEDURES

Preparation of media

All media were sterilised by autoclaving under conditions of 121°C for 15min unless noted otherwise.

Enumeration of bacteria

Aliquots (0.100ml) were removed from the test cultures at appropriate intervals and serially diluted in 0.1% bacteriological peptone (Oxoid/L37) containing 0.85% NaCl. Samples were surface plated using a spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD, USA) onto tryptose phosphate agar with pyruvate (TPAP) containing 29.5 g l^{-1} Tryptose Phosphate Broth (Oxoid/CM283); 1 g l^{-1} sodium pyruvate (Sigma, P-2256); and 14 g l^{-1} J3 agar (Leiner Davis Gelatin). Plates were incubated for 18h at 37°C. Colonies were enumerated using an image scanner and analysis software (CASBA™4, Spiral Biotech).

MEDIA

Cooked Meat Medium, Oxoid CM81 (CMM)

Suspend 10g in 100ml distilled H_20. Prepare according to manufacturer's instructions.

Labelling studies

acetic acid–2–^{14}C (sodium salt, 250μCi/ml, Sigma, Melbourne, VIC, Australia)

Peptone Water (PW): 0.1% peptone with 0.85% NaCl

Bacteriological Peptone (Oxoid/L37)	1.00g
NaCl	8.50g
Distilled Water	1000mL

Minimal Minerals Medium

Minimal Broth Davis w/o Dextrose (Difco; Detroit, MI, USA)	10.6g
Distilled Water	1000ml

Minimal Minerals Medium with 1% glucose

Minimal Broth Davis w/o Dextrose (Difco; Detroit, MI, USA)	10.6g
Distilled Water	1000ml
Filter sterilised 10% glucose in distilled water	10.0ml

Note: Filter sterilised glucose was added aseptically after other media had been autoclaved and cooled.

Nutrient Agar slopes (NA)

Nutrient Broth (Oxoid/CM1;Sydney, NSW, Australia)	13.0g
J3 Agar (Leiner Davis Gelatin; Crown Sci, Kingston, TAS, Aust)	15.0g
Distilled Water	1000mL

Tryptose Phosphate Agar with bile salts (TPABS)

Tryptose phosphate broth (Oxoid/CM283)	13.0g
J3 (Leiner Davis Gelatin)	15.0g
Bile salts No. 3 (Oxoid/L56)	1.50g
Distilled Water	1000mL

Tryptose Phosphate Agar with 0.1% pyruvate (TPAP)

Tryptose phosphate broth (Oxoid/CM283)	13.0g
J3 (Leiner Davis Gelatin)	15.0g
Sodium pyruvate (P-2256,Sigma)	1.00g
Distilled Water	1000mL

Tryptone Soya Broth at various a_w (TSB/a_w)

TSB (Oxoid CM225)	37.0g
NaCl	%w/w (3 decimal places)
Distilled Water	1000mL

EQUIPMENT

Optical Density measurement

• Spectrophotometer Spectronic 20+, Milton Roy Co., USA

pH measurement

• pH Meter and flat tip probe Orion pH meter 250A (Orion Research Inc., Boston, MA 02129, USA)

Note: The instrument was calibrated on each occasion before use by reference to buffers at pH 4 and pH 7.

Uptake of radiolabelled isotopes

- Scintillation counter (Beckman Coulter LS6500 Multi–Purpose Scintillation Counter, Fullerton, CA, USA).

Spread-plating and enumeration

- Spiral Plater- Autoplate 4000, Spiral Biotech Inc., 7830 Old Georgetown Rd, Bethesda, MD 20814 USA
- Image scanner CASBA™4, Spiral Biotech
- Analysis software CIA-BEN, version 2.2, Spiral Biotech

Temperature controlled water bath

- Water bath (±0.1 °C) Ratek SWB20D shaking waterbaths, Ratek, Instruments Pty. Ltd., Unit 1/3 Wadhurst Drive, Boronia, VIC, Australia, 3155

Temperature Gradient Incubator

Terratec Asia-Pacific Pty., Ltd. Lot 7, Patriarch Dr, Kingston, Tasmania, 7054, Australia

Water activity measurement

- Water Activity Meter: Aqualab CX-2 (Decagon Devices, Inc., PO Box 835, Pullman, Washington 99163, USA).