Title   Investigations into the Detection of Injured Salmonella Typhimurium in Foodstuffs

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INVESTIGATIONS INTO THE DETECTION OF INJURED

SALMONELLA TYPHIMURIUM IN FOODSTUFFS

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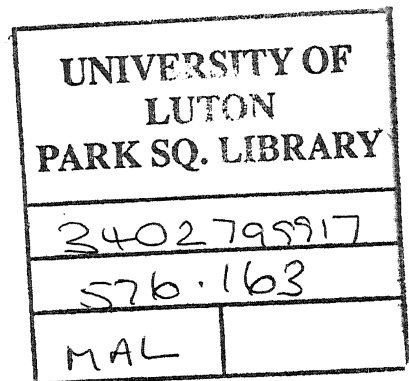
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INVESTIGATIONS INTO THE DETECTION OF INJURED

SALMONELLA TYPHIMURIUM IN FOODSTUFFS

by

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination in any other University.

Michael D. Malactos   31st December 1998

DEDICATION

This thesis is dedicated in the memory of the late **Dr. Colin Merrill.**

ACKNOWLEDGEMENTS

I would like to acknowledge the complete and utter support of my wife. Thank you!

I would also like to give thanks to the friends who supported me most.

GLOSSARY

AOAC	Association of Official Analytical Chemists
$\mathbf{A_x}$	Absorbance at wavelength x
BACLIGHT	Bacterial viability kit
BAM	Bacteriological Analytical Manual
BEP	Bisoxonol - ethidium bromide - propidium iodide triple stain
BGA	Brilliant green agar
BOX	Bisoxonol
BPW	Buffered peptone water
$\mathbf{CCM_{(s)}}$	Conventional culture method$_{(s)}$
CFU	Colony forming units
CRM	Certified reference material
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DMSO	Dimethyl sulphoxide
EB	Ethidium bromide
EDTA	Ethylene di-amine tetra-acidic acid
EIA	Enzyme-bound immunoassay
ELISA	Enzyme linked immunosorbent assay
ES	Enrichment serology
FA	Fluorescent antibody
FACS	Fluorescence activated cell sorting
FALS	Forward angle light scatter
FC	Flow cytometry
FCM	Flow cytometric measurement
FITC	Fluorescein
FOV	Field of view
HE	Hektoen enteric
IMS	Immunomagnetic separation
LL	Lower left
LPS	Lipopolysaccharide
LR	Lower right
MIPA	Magnetic immuno PCR assay
MUCAP	Methyl umbelliferyl caprylate
NALS	Narrow angle light scatter
NCIMB	National Collection of Industrial and Marine Bacteria
PCR	Polymerase chain reaction
PE	Phucoerythrin
PHLS	Public health laboratory services
PI	Propidium iodide
PT	Phage type
RALS	Right angle light scatter
RIA	Radioimmunoassay
RV	Rappaport Vassiliades
SBW	Supplemented buffered water
SS	*Salmonella-Shigella*
TBG	Tetrathionate brilliant green
TE	Tris EDTA
TSA	Tryptone soy agar
TSB	Tryptone soy broth
UL	Upper left
UP	Universal pre-enrichment
UR	Upper right
UV	Ultra violet
VIA	Visual immunoassay
WALS	Wide angle light scatter
XLD	Xylose lysine desoxycholate

Abstract

A chemically defined medium for *Salmonella* growth was developed and optimised using supplements of amino acids, nucleosides, vitamins and different carbon sources. The medium developed was compared to commercially available pre-enrichment media BPW and Salmosyst. Growth of *Salmonella* was significantly higher in BPW and Salmosyst than the medium developed. The amino acid and nucleoside supplement was directly compared with peptone. The results show peptone to be nutritionally superior and promoting better growth of *Salmonella*.

Three different ELISA assays were used to detect *Salmonella* growing in four different media. The ELISA assay sensitivity was determined and a degree of media interference with the immunoassays was established.

Salmonella culture viability was investigated using three different procedures: differential culturing on selective and non-selective media; fluorescence microscopy with *BAC*LIGHT stained cells and flow cytometry analysis of *BAC*LIGHT and BEP stained cells. Flow cytometry was found to be the most consistent, sensitive and rapid procedure for cell viability measurement. Clusters of viable cells unable to grow on solid media and therefore remaining undetectable by cultural methods were identified using flow cytometry.

Severely heat injured *Salmonella* was used to determine media recoverability. The results indicate that media which contain peptone recover injured *Salmonella* better than chemically defined or other media.

Detection of *Salmonella* was performed using PCR assay after sample pre-enrichment. The amplification of *Salmonella* DNA extracted using a crude method resulted in an assay sensitivity of 20 *Salmonella* cells in pure cultures. The specificity of the oligonucleotide primers employed in the PCR assay was confirmed.

Non-*salmonella* organisms present in high numbers interfered with PCR detection of *Salmonella*. Food components also interfered with PCR amplification and reduced the assay sensitivity. Interference by food components and non-*salmonella* DNA was eliminated by the use of a 24 hour pre-enrichment followed by a 3 hour secondary enrichment, a rapid DNA extraction and template preparation.

Using this system it was possible to detect 3 *Salmonella* cells per gram of food in the presence of 10^6 non-*salmonella* cells within 28 hours.

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1.0   GENERAL INTRODUCTION

1.1   Salmonella and the Importance of Detection

Enterobacteria of the genus *Salmonella* belong to a single species, *Salmonella enterica* (Le Minor, 1984). Based on the biochemical reactions of the different *Salmonella* strains, seven subspecies have been recognised and are classified into serotypes based on the diversity of somatic (O) and flagellar (H) antigens. The majority (99%) of the human isolates of *Salmonella* belong to one subspecies (I) which also contains over 60% of the animal isolates including *S. typhimurium*, *S. enteritidis* and *S. virchow* (Palmer and Rowe, 1986; Le Minor, 1988).

Salmonella is an important foodborne pathogen, and accounts for a high percentage of bacterial disease outbreaks world wide at a considerable financial cost. This is due to changes in human diet and the increased consumption of semi-preserved and ready-to-eat foodstuffs. In the USA 51% of all food poisoning cases and 42% of outbreaks reported to the Centers of Disease Control (between 1973 and 1987) were attributed to *Salmonella* (Sockett, 1991). Bean and Griffin (1990) compared data collected between 1985-87 with data collected between 1973-75 and found a 75% increase in the proportion of food poisoning outbreaks due to *Salmonella* and 130% increase in the number of cases due to *Salmonella*. *Salmonella* outbreaks due to beef consumption have significantly decreased since 1981, whereas in the late eighties *Salmonella* outbreaks due to consumption of contaminated chicken and eggs have

increased. Eggs have been found to be one of the major vehicles of *Salmonella* infection, especially *Salmonella enteritidis* outbreaks in the USA (Hedberg *et al.*, 1993; Vugia, D. J. *et al.* 1993; St. Louis, M. E. *et al.*, 1988).

Reports from the UK Public Health Laboratory Services have also shown an increase in food poisoning cases from 1980-1993 in England and Wales. *Salmonella,* and more importantly *Salmonella enteritidis*, is the second most common cause of the reported infections after *Campylobacter* species. Most food poisoning cases are believed to be either unconfirmed or undiagnosed. In 1993 there were over 30,000 positively confirmed *Salmonella* cases in England and Wales, that may represent the tip of the iceberg. The number of human *Salmonella* infections reported in England and Wales remained very high during the period between 1994 and 1997 and *S. enteritidis* (PT 4) is still the most common strain isolated from recorded food poisoning cases (Communicable Disease Report, CDR 8, 15, 1998; CDR 7,50,1997).

Salmonella is widely distributed and has been isolated from poultry, reptiles, livestock, rotents, domestic animals, birds and humans. Most of the 2200 serotypes of this gram negative bacterium are pathogenic to humans and animals. However, some *Salmonella* strains like *Salmonella typhi* and *Salmonella paratyphi* are highly adapted to their human host and do not cause disease to other infected animals (Murray *et al.*, 1990). Ingestion of *Salmonella* by mouth is a prerequisite for the communicable disease of salmonellosis. It is a highly effective opportunistic pathogen such that by consumption of less than 10 cells of *S. typhimurium* can cause an infection (D'Aoust, 1985). It is also believed that cross contamination of food stuffs can occur within one

minute (Bradford *et al.* 1997). Cross contamination is particularly a problem when unhygienic handling of food occurs in badly organised kitchens with inadequate facilities for high volume cooking. Outbreaks of *Salmonella* food poisoning have occurred due to defects in the kitchen design and management, and the incorrect storage or poor handling of foodstuffs (Holtby *et al.*, 1997; Wight *et al.*, 1996).

Symptoms of salmonellosis in humans can be very dangerous and include: diarrhoea, abdominal pain, vomiting and fever, and in severe infections the presence of blood and mucus in stools. In both birds and mammals, symptoms of salmonellosis can include enterocolitis, septicaemia and can lead to death (Smith, 1990). Many reports indicate that **human mortality due to *Salmonella* infections is very high** (Anon., 1993; Levine *et al.*, 1991; St. Louis *et al.*, 1988). The pathogenicity of *Salmonella* begins with the adherence of the bacterium onto intestinal epithelial cells, multiplication of the cells in the intestine, and consequent invasion of the mucosa and the release of a potent toxin which triggers an inflammatory response. Even though recovery can occur within a few days the carrier state can persist for more than a year. An extensive review by D'Aoust (1991) explains the different stages of *Salmonella* pathogenesis from consumption to the pathogen excretion.

1.2 *Salmonella Detection in Food*

Over the last two decades, food industry and regulatory authorities in the UK have substantially increased *Salmonella* surveillance of human and animal food due to the increased number of *Salmonella* cases. Conventional culture methods (CCM) for the

detection and identification of *Salmonella* have been shown to be quite limited and significant efforts are being undertaken to develop alternative methods of detection to increase sensitivity and speed of detection.

Changes in food consumption patterns have resulted in the manufacture of more types of food for point of sale, for example pre-cooked meals and semi-preserved ready-prepared meals for both catering and domestic consumption. **These foodstuffs often undergo preservation treatments that are not guaranteed to be bactericidal.** Treated food can contain dead cells, viable cells and cells in an 'injured state' (Ray, 1979; Litchfield, 1973) of pathogenic bacteria such as *Salmonella*. Cell injury occurs due to several factors such as: low pH, presence of inhibitory substances that are naturally present in foods or have been produced by other competing micro-organisms, prolonged refrigeration, freezing, drying or heating of the foods. The presence of cells that are potentially infective - but inhibited through injury - cause significant difficulties in the methods of detection of *Salmonella* that use cell proliferation as a measure of contamination. Because injured cells are less responsive to promotive conditions, and more sensitive to chemical agents (Mackey and Derrick, 1982; Patil and Parhad, 1986), they may not be adequately detected by the conventional culture methods used for enumeration of bacteria in foods (Ray, 1979).

Currently, detection of *Salmonella* in food is primarily dependent upon the amplification of cell numbers. Amplification is essential since *Salmonella* in foodstuffs occur in very low numbers compared with the presence of high numbers of natural background microflora. Thus in addition to the *Salmonella* cells in food that

are injured due to inadequate treatment or preservation practises by manufacturers their detection is a serious problem to food microbiology laboratories. Unless the *Salmonella* cells are resuscitated and multiply to detectable levels, detection/ identification techniques may lead to unwanted false negative results. Usually, a non-selective pre-enrichment medium enables the resuscitation and proliferation of injured cells to detectable levels. Then, a selective enrichment of *Salmonella* is performed by suppressing any background microflora that may interfere with subsequent detection and identification procedures through growth on selective and differential media (Litchfield, 1973; Beckers *et al.*, 1985).

A conventional culture method with confirmation of *Salmonella* in foodstuffs is illustrated in Fig. 1, indicating how long (4-6 days) and laborious such procedures can be. The method illustrated in Fig. 1 and explained below is a combined outline of *Salmonella* detection as recommended by: the Food and Drug Administration (Bacteriological Analytical Manual, 1992, AOAC International); the International Organisation for Standardisation (ISO 6579:1990); the Campden and Chorleywood Food Research Association (Method 10:1995, Technical Manual No.43); and used by Whitbread Plc. food microbiology department. Homogenisation of foodstuffs allows the release of cells from the complex matrix into the pre-enrichment medium which then allows the cells to resuscitate and proliferate. Enrichment in different selective media leads to the suppression of non-salmonella species in the food samples which may interfere with subsequent *Salmonella* detection procedures. The selective enrichment step is performed in several media since some selective enrichment media may be mildly toxic to different *Salmonella* strains. Furthermore, by using more than

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one selective medium the chance of isolating *Salmonella* from a high background level of microflora is greatly increased. This is particularly important with raw untreated foodstuffs containing a high total microbial load. The presence of *Salmonella* at day 4 is confirmed and typed by enzyme linked immunosorbent assays (ELISA) to determine the presence of specific *Salmonella* antigens. Positive ELISA samples are investigated further by growth of the enriched sample on differential media. Positive testing *Salmonella* strains are then purified on agar plates and other biochemical and immunological tests are curried out to confirm the strain type and to identify the source of contamination.

The conventional method of *Salmonella* detection described above is not favoured by food producers since it results in increased cost due to delayed handling and storage of food during the testing period. It also decreases the shelf life of their products when they are finally released. Consequently a more rapid, sensitive and stringent procedure for *Salmonella* detection in foodstuffs is eagerly sought.

Fig. 1 : Conventional cultural procedure for detection of *Salmonella* in food.

HIGH RISK (RAW) FOOD   LOW RISK (PRE-TREATED) FOOD

DAY 1
am

HOMOGENISE FOOD WITH PRE-ENRICHMENT BROTH
AND INCUBATE 20 - 24 HOURS

DAY 2
am

ENRICHMENT IN
2 SELECTIVE BROTHS
6 - 8 HOURS

ENRICHMENT IN
2 SELECTIVE BROTHS
18 - 24 HOURS

pm

FURTHER ENRICHMENT
IN DIFFERENT BROTH
16 -18 HOURS

DAY 3
am

COMBINE BROTHS
CARRY OUT FURTHER
SELECTIVE ENRICHMENT 7 HOURS

pm

PREPARE A SAMPLE FOR ELISA[1] (20 MINUTES)
KEEP THE REST OF THE SAMPLE FOR FURTHER ANALYSIS

DAY 4
am

RUN ELISA

pm

STREAK POSITIVES ON TWO OR MORE DIFFERENTIAL MEDIA
24 HOURS

DAY 5
pm

PURIFY SUSPECT COLONIES ONTO TSA[2] AGAR 24 HOURS

DAY 6
pm

RUN BIOCHEMICAL AND IMMUNOLOGICAL TESTS FROM TSA PLATE

1. Enzyme linked immunosorbent assay
2. Tryptone soy agar

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The efficiency of sample pre-enrichment and cell repair and multiplication is increased by extracting *Salmonella* cells from the complex food matrix in which they are lodged and placing them into the liquid pre-enrichment medium. Mechanical homogenisation of food samples is the conventional method to remove cells from the food matrix and distribute *Salmonella* homogeneously in a medium. The method introduced by Sharpe and Jackson (1972) was homogenisation using a stomacher, which mashes the food in the liquid medium by the use of two pistons working in a reciprocal way. Stomaching has advantages over conventional homogenisation using a high speed food processor because: (a) the sterile stomaching bags are excellent for taking and transporting samples from factory processing areas to the laboratory, (b) lower running and maintenance costs, (c) it does not require considerable labour as the plastic bags for stomaching are disposable; therefore washing and sterilisation of the homogeniser are not required, (d) no temperature rise is observed with stomaching that may affect stressed bacteria and (e) the initial capital outlay is lower. Homogenisation with a stomacher is carried out in non-selective pre-enrichment media.

1.3 Pre-enrichment

The principal steps of the detection procedure which consumes the majority of time are the **(a)** pre-enrichment and **(b)** selective enrichment incubations. Pre-enrichment is the first step of the detection procedure following homogenisation of the food in a non-selective pre-enrichment medium. Pre-enrichment is the most important step of *Salmonella* detection since it allows low numbers of injured *Salmonella* cells to repair

and proliferate in an optimum environment. Those cells would otherwise remain undetected and present a potential danger to the consumers (D'Aoust, 1981). Injured *Salmonella* cells that repair during pre-enrichment regain resistance to chemical agents present in the latter selective media and can therefore be isolated from background microflora (Clark and Ordal, 1969; Ray, 1979). Van Schothorst and Kampelmacher (1968), Clark and Ordal (1969), and Edel and Kampelmacher (1973) were some of the first investigators to show the need for pre-enrichment of injured cells. They determined that the efficiency of *Salmonella* detection was enhanced when pre-enrichment was used as opposed to direct enrichment of food samples in selective media.

Different foods require different pre-enrichment media for efficient detection of *Salmonella* (D'Aoust, 1981) and liquid media recovery is more advantageous than solid media recovery of injured *Salmonella* cells (Mackey and Derrick, 1982). The lack of a universal pre-enrichment broth for all foods has led to considerable research into the development of different media for recovering injured cells. Differences between the growth requirements of normal and injured bacterial cells became apparent and triggered investigations into cell viability and cell injury, and the mechanisms of damage repair.

Conditions and duration of pre-enrichment vary throughout the laboratories of different countries, depending on the Agency or Group consulted (D'Aoust, 1981). A pre-enrichment step of 18 to 24 hours is standard in most food microbiology laboratories. The most common non-selective pre-enrichment medium used for

Salmonella is buffered peptone water (BPW). A pre-enrichment step between 18-20 hours in BPW was first recommended by Edel and Kampelmacher (1973) as favouring the isolation of *Salmonella* and producing reproducible results. Buffered peptone water has been widely used as a pre-enrichment medium by many workers for the detection of *Salmonella* in seawater samples (Morinigo, 1990), for the resuscitation of injured *Salmonella* in foodstuffs (Bird *et al.*, 1989) and for pre-enrichment of sewage-polluted water (Harvey and Price, 1980). The supplementation of pre-enrichment media like BPW with siderophores ferrioxamine E and G was found to enhance the sensitivity of *Salmonella* isolation from eggs. Siderophores supply *Salmonella* with iron which can be a growth limiting factor especially in the presence of iron-binding proteins such as conalbumin (ovotransferrin) and high pH (Reissbrodt and Rabsch, 1993).

Many other non-selective pre-enrichment media have been developed and used for the resuscitation and recovery of injured *Salmonella*. Bailey and Cox (1992) developed and evaluated the efficiency of a universal pre-enrichment broth for *Salmonella* and *Listeria* in foods. After a 24 hour incubation in the universal broth, ten heat-injured *Salmonella* in different foods multiplied to 10^6 cells ml^{-1} and could be detected. A six hour pre-enrichment in Salmosyst broth (Merck Ltd, Poole, Dorset, UK) allows the repair of heat-injured *Salmonella* and is sufficient for subsequent recovery by selective enrichment (Pignato *et al.*, 1995). Salmosyst resulted in better recovery of *Salmonella* when compared with BPW (Pignato *et al.*, 1995). Previous studies have indicated that a 6 hour pre-enrichment step in BPW was not sufficient for repair of injured *Salmonella* cells and subsequent culturing on selective media, such as

Rappaport Vasiliadis (RV) and Tetrathionate Brilliant Green (TBG) media, after short pre-enrichment in BPW may be toxic to the injured cells (Chen *et al.*, 1993).

1.3.1. Cell Viability Studies

Evaluating pre-enrichment media is carried out by using injured cells in order to imitate real food situations. Investigating bacterial cell viability and evaluating bacterial injury can be carried out by conventional cultural methods or more advanced and accurate cell staining with a new generation of supravital stains. These stains may bind to lipids or nucleic acids and therefore can be used for membrane integrity measurements, or they may be used as metabolic indicators by being metabolically changed into an easily detectable by-product. These stains can be used in combinations and are either detected manually using a fluorescent microscope or using the more rapid and sensitive methodology of flow cytometry. The latter is proposed to be the best currently available technique for cell viability studies and is increasingly being used in microbiology research and development.

1.4 Selective enrichment

Selective enrichment is the step that allows selective growth of target micro-organisms (i.e. *Salmonella*) over the background microflora in the food sample and reach detectable levels. Background microflora are suppressed by chemical components (selective agents) in the media such as novobiocin, sodium thiosulphate, bile salts, iodine, sodium selenite, l-cystine and malachite green.. The majority of

CCMs include selective enrichment as the second step for detection of *Salmonellas* following pre-enrichment. In contrast to clinical specimens, in which high numbers of normal (actively growing) *Salmonella* cells may be present, *Salmonella* cells are present in very low numbers in food specimens in the presence of high numbers of background micro-organisms (Ibrahim and Fleet, 1985). Selective enrichment combined with differential isolation on solid media enhances *Salmonella* detection efficiency (Hine, 1988).

It is common to use several media in parallel for the enrichment of each food sample (Fagerberg and Avens, 1976) and also to use different selective enrichment media for different foodstuffs since no single medium is able to recover all *Salmonellas* under all conditions (Dusch and Altwegg, 1993; Pignato *et al.*, 1995). The incubation conditions can also vary depending on the composition and physical state of the media used for enrichment (D'Aoust, 1981; Mackey and Derrick, 1982; D'Aoust *et al.*, 1990a). Fagerberg and Avens (1976) emphasised the need to optimise each variable in the detection procedure such as the medium, incubation period and temperature and inoculum size used for the pre-enrichment step.

Incubation temperature is probably the most important factor when the incubation period employed is 18-24 hours. Early reports presented evidence that the proportion of *Salmonella* colonies developing on solid media was greater when selective enrichment was carried out at an elevated temperature (i.e. 43° C) instead of 37° C (Gabis and Silliker, 1977). Later researchers have also investigated the possibility of decreasing analysis time, reducing materials required and increasing sensitivity of

Salmonella detection by using direct enrichment (avoiding pre-enrichment) at elevated temperatures (Miller and Koburger, 1984). Work carried out by Vassiliadis (1983) confirmed the advantageous effects of elevated temperature on selective efficiency in Rappaport Vassiliadis (RV) medium. Contrary to the findings of Miller and Koburger (1984) the use of elevated temperature was found particularly effective after pre-enrichment of the sample (Morinigo, 1990). The increased *Salmonella* cell recovery at elevated temperatures is not necessarily due to an increased growth rate, since 43° C approaches the maximum growth temperature for some *Salmonella* species (Miller and Koburger, 1984). However, a greater proportion of *Salmonella* colonies develop on the selective agar because background microflora, which compete for nutrients, are suppressed at this temperature and do not interfere with *Salmonella* detection (Martin and Katz, 1991; D'Aoust, 1981; Gabis and Silliker, 1977). Temperature is thereby acting as a selection mechanism.

1.4.1 Modifications to Enrichment Procedures

Since pre-enrichment and selective enrichment of food samples account for most of the time spent on the whole *Salmonella* detection procedure, it is not surprising that many researchers believe that shortening these first steps could accelerate the detection of *Salmonella* without affecting the efficiency of the identification (Sveum and Hartman, 1977; Bird *et al.*, 1989; Martin and Katz, 1991). However, a further enhancement is to use more sensitive detection methods for *Salmonella* detection in the enriched samples. For example, using a combination of conductance and immunological techniques, Bird *et al.*, (1989) showed that a pre-enrichment step of

only 1-7 hours was required for the repair of injured *Salmonella* cells for subsequent recovery. Others have combined the two enrichment steps. For example, Martin and Katz (1991) developed a pre-enrichment medium which after a six hour incubation period was made selective by addition of either sodium thiosulphate, bile salts and iodine, or sodium selenite and L-cystine. This selective medium was then incubated for 16 hours and the presence or absence of *Salmonella* determined by a *Salmonella*-Tek antibody-based detection kit. This system was noticeably better than the conventional standard method for *Salmonella* detection described in the Bacteriological Analytical Manual (BAM) for naturally contaminated food.

Sveum and Hartman (1977) have also reduced *Salmonella* detection time by combining the pre- and selective enrichment steps into one procedure by adding wax-coated selective agents into the non-selective broth that slowly dissolved during incubation and release the *Salmonella* selective agents. Tetrathionate capsules as well as iodine and selenite capsules were used as *Salmonella* selective agents. Another selective agent used in conjunction with enrichment media is sodium novobiosin which at certain concentrations can increase the efficiency of the medium (Moriñigo *et al.*, 1990). It is also believed to have a synergistic effect with malachite green which is a more efficient selective combination (Moriñigo, 1990).

However, there is some agreement whether short (<8 hours) pre-enrichment steps truly provide suitable conditions for effective resuscitation of injured cells and thereby resulting in unacceptably high numbers of false negative results (D'Aoust and Maishment, 1979; D'Aoust, 1981; D'Aoust, Sewell and Jean, 1990). Chen *et al.*

(1993) determined that after a six hour pre-enrichment in BPW, although injured *Salmonella* were 'completely resuscitated' as determined by reaching the exponential phase of growth in BPW, they were still unable to grow on selective media. This was probably due to the fact that the injured cells regained their ability to divide but not their resistance to the selective agents in the media. These studies probably indicate that selective enrichment has reached its limit in shortening the detection interval.

1.5   Presumptive Identification and Confirmatory Biochemical Tests

Once the sample has been pre- and selectively enriched, differential media are used for the presumptive identification of isolated colonies. Specific differential media have been developed to indicate metabolic characteristics of individual bacterial species (Lithfield 1973). *Salmonella* species identification is performed with the appearance of isolated colonies on solid media and the development of media colour changes. The reliability of detection is increased by employing more than one differential medium in parallel.

In clinical and public health laboratories Salmonella-Shigella (SS) agar, Xylose lysine desoxycholate (XLD) agar and Hektoen enteric (HE) agar are commonly used for the detection of *Salmonella* species (Koneman, 1988). Differential plating media contain selective agents - just like the selective enrichment liquid media - in order to discourage non-*Salmonella* species from growing and swarming the plates, therefore masking typical *Salmonella* colonies. For example, virtually all species of *Salmonella* grow well in the presence of bile salts but other species such as enteric bacteria,

including some of the more fastidious strains of *Shigella* grow poorly in their presence. Thus SS and HE agars contain relatively high concentrations of bile salts and are well adapted for recovering *Salmonella* species from clinical and food samples heavily contaminated with interfering bacteria.

Differential plating media contain substrates which can be metabolised by *Salmonella*, such as lysine in XLD and propylene glycol in Rambach agar, or can not be metabolised, such as lactose and sucrose in XLD, BGA and HE. Using these substrates basic metabolic differences between bacterial species are exploited to distinguish between typical *Salmonella* colonies and non-salmonella colonies in the sample. For example XLD agar contains lactose, sucrose and xylose, and bacteria that ferment these carbohydrates form yellow colonies due to the acidification of the medium whereas bacteria that do not ferment these carbohydrates form colourless colonies. Organisms that produce H_2S, typical to *Salmonella*, form a black coloration due to production of ferrous sulphite beginning in the centre of colonies and gradually diffusing throughout the colonies. XLD agar also contains lysine which is decarboxylated by most *Salmonella* species, forming alkaline amines which results in a light pink halo around the colonies. XLD is one of the media used in this thesis for *Salmonella* detection and identification. Black colonies without a pink halo are more suggestive of an H_2S producing strain of *Proteus* species. The carbohydrates in HE agar are lactose, sucrose and salicin. Bacteria capable of fermenting these carbohydrates also form yellow colonies whereas asaccharolytic strains produce colonies that are translucent or light green. Lactose- and sucrose- negative bacteria

that acidify salicin may produce orange colonies. HE agar also contains ferric salts, thus H_2S-producing colonies appear black. Brilliant green agar (BGA), supplemented with an H_2S indicators such as ferric ammonium citrate and sodium thiosulfate, is an other medium suggested for *Salmonella* identification (Moats and Kinner, 1976).

Differentiation and further identification of typical *Salmonella* colonies on media can be carried out by rapid and simple enzyme detection techniques such as the methyl-umbelliferyl caprylate test (MUCAP; BioLife Italiana S.r. l., Milan, Italy) or biochemical test kits such as API 20E (bioMériéux), Enterotube (Roche), Micro ID (Organon Teknika) and Vitek (bioMériéux).

Furthermore, identification of *Salmonella* isolates can be completed with tests like phage typing, biotyping and antibiograms that can lead to a more comprehensive subdivision of the detected strains. This information supplemented with genetic characterisation of plasmids, recognition of differences in outer membrane protein profiles and analysis of lipopolysaccharide content, is important for investigating *Salmonella* outbreaks and epidemiological studies (Threlfall and Frost, 1990).

1.6 Alternative Methods to Conventional Detection of Salmonella

Even though there is a series of recommended methods for the detection of foodborne *Salmonella*, no ideal method has been devised for the recovery and identification of all *Salmonella* strains from all types of foods. There are many commercial assays

available as kits for identification of *Salmonella* in foods based on fluorescent-antibody staining, Polymerase Chain Reaction (PCR), enrichment serology, radiometric, immunoassays and chromatographic assays (Feng, 1992; Ibrahim, and Fleet, 1985; Cano *et al.,* 1993). However, the availability of kits is often limited to certain manufacturers and are not cost effective (Feng, 1992). Most of the alternative systems for *Salmonella* detection still rely on culture methods for resuscitation of injured cells and selective amplification of *Salmonella* (Blackburn, 1993). This emphasizes the importance of pre- and selective enrichment procedures (D'Aoust, 1984; Rhodes, 1981).

1.6.1 Immunoassays

Immunoassays kits and assays offer rapid and specific identification of micro-organisms utilising antisera specific with certain antigen(s) of the target organism. There are many different approaches to immunoassays including radioimmunoassays (RIA), enzyme-bound immunoassays (EIA), radioimmunoprecipitation tests and enzyme linked immunosorbent assay (ELISA) that is now the most widely accepted method in food microbiology (Ibrahim and Fleet, 1985).

1.6.2 Fluorescent Antibody Staining

One of the first methods to be exploited for the rapid detection of *Salmonella* was fluorescent antibody (FA) staining by Coons *et al.* (1941). This method was later modified by Thomason (1971) who introduced rapid detection of *Salmonella* microcolonies by FA staining. Due to the relatively low sensitivity of the method, FA staining procedures are carried out after selective enrichment of the sample. Although

attempts have been made to shorten the assay development time by reducing or deleting the pre-enrichment and enrichment steps prior to staining, this results in low sensitivity and specificity (i.e. increased non-specific staining) with increased numbers of false positive and negative identifications. Other problems inherent in FA staining include: (a) the requirement of extensive experience to interpret fluorescent cells; (b) presence of autofluorescent cells; (c) doubtful fluorescent intensities and; (d) eye fatigue during examination. Highly contaminated or unprocessed samples are problematic with FA staining. Thomason (1971) recommended that further biochemical and serological tests were carried out to substantiate FA-positive results.

1.6.3   Enrichment Serology

Sperber and Deiberl (1969) introduced enrichment serology (ES) as an accelerated method for the detection of *Salmonella*. This procedure involves pre-enrichment, selective enrichment, elective enrichment in M-broth followed by serological agglutination with polyvalent flagellar antisera in order to detect *Salmonella* antigens. The ES method eliminates the need for selective plating and biochemical confirmation usually required with culture methods and has been very successful in clinical laboratories.

1.6.4   Immunomagnetic Separation of Salmonella from Foodstuffs

Another recently developed technique is the immunomagnetic separation (IMS) that uses magnetic beads coated with antibodies specific to *Salmonella* designed as a tool to selectively remove bacteria from complex food matrices. The specificity of this

method is dependant on the antibody used to coat the magnetic beads. The use of IMS for separation and isolation of *Salmonella* was demonstrated by Blackburn and Patel (1989) and later by Skjerve and Olsvik (1991). However, Skjerve and Olsvik (1991) reported a substantial loss of immunomagnetic beads and recovery of *Salm*onella from certain samples (such as yoghurt and chicken liver) was poor. The sensitivity of IMS was found to be 1-2 cells ml^{-1} and may increase with increasing sample volume. This method has some advantages because it can isolate bacterial cells that can be pre-enriched without interference from competing microflora, and can therefore speed up the *Salmonella* detection.

1.6.5 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a recently developed molecular technique which is used as a major research and identification tool in clinical and food laboratories. PCR amplifies specific and pre-determined sequences of DNA by the use of *in vitro* engineered primers and their subsequent detection by gene probes or visualisation of the stained PCR product, usually with a DNA fluorescent stain such as ethidium bromide, by gel electrophoresis (Mullis, K. B., 1990; Mullis and Faloona, 1987). This technique has been used widely for the detection of pathogens by a number of researchers including Ruano *et al.* (1989), Steffan and Atlas (1991), Widjojoatmodjo *et al.* (1991, 1992), Rahn *et al.* (1992) and Mahon *et al.* (1994).

The PCR reaction involves melting of DNA at a high temperature of 94°C, annealing of the primers to the flanking sites of the target sequence at a lower temperature of 50-

70°C, and extension of the primers using thermostable DNA polymerase (Steffan & Atlas 1991). Even though the procedure was found to be extremely quick and highly specific (Rahn, *et al.*, 1992; Steffan and Atlas, 1991; Mahon, *et al.*, 1994) and sensitive for food and other samples, including water, some problems have been reported and include:

(a) direct application of PCR to complex substrates resulting in no amplification products (Li *et al.*, 1988; Soumet *et al.* 1994) or low sensitivity (Fluit *et al.*, 1993a and b);

(b) amplification of a low number of target template of less than 1000 can be inhibited by the synthesis of "primer dimers" and other spurious products (Ruano *et al.*, 1989);

(c) non-target amplified products of PCR amplification consume the primers and occupy the enzyme, with consequent reduction in target yield (Ruano *et al.*, 1989) and;

(d) the presence of non-viable target cells and their intact amplifiable DNA may yield a false positive PCR result.

PCR has extraordinary potential in food microbiology since it can defeat principal failures associated with other techniques. It can both increase sensitivity and completely reduce the testing interval. It can also selectively identify *Salmonella* species against a high background contaminating microflora. PCR is currently the most sensitive and specific technique available, that can be fully automated to screen large numbers of samples for different pathogens.

The potential of IMS may be used in combination with a very sensitive and rapid detection method such as PCR (Skjerve and Olsvik, 1991). IMS reduces the problem of background chemical components in the food and foreign DNA interfering with subsequent PCR detection. Hence, IMS and PCR together may produce a powerful rapid system for *Salmonella* detection.

1.7 Study Rationale

1.7.1 Defined Medium Development

The most important step of *Salmonella* detection is the pre-enrichment of sample to repair injured cells and allow proliferation of *Salmonella* to detectable levels. Minimal medium recovery suggests that simple media provide a better environment for the resuscitation of injured cells after sub-lethal heat treatment (Hurst, 1984). Based on this phenomenon single amino acids may be more readily available to bacteria than more complex substrates. Therefore, a defined medium containing a mixture of amino acids was proposed to support the growth of heat damaged cells better than a medium containing only peptone - because the cells may need to enzymatically reduce the peptone to its amino acid components and heat damaged

cells would be less able to do this. However, peptone may contain a variety of components which are not found in the defined medium, such as trace elements, metals and biochemical intermediates that the defined media do not intrinsically contain and so further supplementation may be required.

1.7.2   *Evaluation of Cell Viability and Bacterial Injury*

Measuring cell viability or cell injury has been traditionally based on the ability of cells to divide and form colonies on non-selective and selective media. The difference of growth between the two media was considered to represent the level of the population injury. Due to great variations in the culture method this thesis compares the cultural evaluation of cell viability with a combination of bacterial staining with DNA supravital stains, fluorescent microscopy, and flow cytometry. Flow cytometry is the most sensitive technique that indicated the presence of different cell clusters in a heat treated population which can be ultimately used for media evaluation. The efficacy - in terms of recovery - of media can be tested using single cell inoculation with cells of a known viability status. Thereby effecting comparison of different media. Single cell inoculation and media comparisons have been carried out in this thesis. The cells used were from specific clusters obtained with cell staining and flow cytometry.

1.7.3   *Salmonella Detection by ELISA*

ELISA is the most commonly used detection/identification technique in food microbiology. The study rationale comprises a comparison of four different ELISA assay kits used to detect *Salmonella* antigens combined with different pre-enrichment media. Thus it can be determined which of the medium-ELISA systems is best for *Salmonella* detection in cultures, i.e. determine whether any of the media increases *Salmonella* antigen production or whether any of the media interfere with the assays.

1.7.4 Development of Salmonella Detection by PCR

PCR is probably the only method sensitive enough to detect a number of cells as low as 3 *Salmonella* cells per gram of food in the presence of high background microflora in the order of 10^6 cells per gram. The sensitivity and specificity of the oligonucleotide primers for *Salmonella* could make PCR potentially the best detection assay. However, direct detection of *Salmonella* by PCR in food may be inhibited by food components, high concentrations of non-target DNA, and low concentrations of target template DNA. Therefore, apart from pre-enrichment an additional template 'cleaning' or DNA preparation procedure may be required to avoid PCR reaction contamination with food inhibitors.

CHAPTER 2

Development of a Chemically Defined Medium for Salmonella Growth

2.1 Introduction

2.1.1 Media Development

Detection of the presence of *Salmonella* in food initially relies on a period of growth in media designed either for resuscitation, selection or both. Detection, in the absence of such a growth phase seems unlikely, due to the long lag time for the recovery of sub-lethally injured cells and the requirement for enrichment due to the relatively low sensitivity and selectivity of present tests (Blackburn, 1993). Of lesser importance is the problem of false-positives from lethally injured, non-viable cells that direct detection system suffer from.

Most recent commercial detection systems offer increased selectivity, either through the specificity of primers in the polymerase chain reaction (PCR), or antibodies in enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) based detection methods. Such methods also have the advantage of being more rapid, although they are generally more expensive than conventional methods. However, these techniques are still dependant on some form of cultural enrichment.

Different media have been used for the pre-enrichment of *Salmonella,* including: buffered peptone water (BPW) (Edel and Kampelmacher, 1973; Bird, *et al.*, 1989; Moringo,

1990); Salmosyst broth (Pignato, *et al.*, 1995), lactose broth (Sveum and Kraft, 1977) and universal pre-enrichment broth (UP) (Bailey and Cox, 1992). Incubation in these media is known to enhance the efficiency of the subsequent *Salmonella* detection systems. Such media are chemically undefined, being dependant on peptone as a carbon and energy source. However, peptone is commercially produced by the hydrolysis of unspecified proteins, and thus can be highly variable between manufacturers and batches.

Researching the nutrient requirements and uptake of such nutrients inside the cell provide a perfect base on which media design can begin. The knowledge of specific substrate requirements, quantities and conditions of growth may lead in the development of the "perfect" pre-enrichment medium. Optimum growth conditions encourage maximum growth rate of specific microorganisms, and could also provide a good environment for resuscitation of 'injured' cells.

Many researchers studied nutritional requirements of bacteria and nutrient transport in order to provide the optimum conditions for growth (Gutnick *et al.*, 1969, Gomez *et al.*, 1973; Gomez and Sinskey, 1975; Wild *et al.*, 1978; Rao *et al.*, 1980). Nutrients, such as amino acids, vitamins, nucleosides, sugars and peptides are known to support growth of *Salmonella*. However, no report compares directly the nutritional value of mixtures of such nutrients with protein hydrolysates such as peptone. Even so, it is observed that peptides are nutritionally better sources of amino acids for bacteria, than the individual free amino acids (Sokatch, 1969). The complexity of nutrient uptake and multiplicity of transport systems in bacteria is reported to involve specific and general transport systems

that function according to the structural and stereochemical form of the transported nutrients (Naider and Becker, 1975; Rogers *et al.*, 1980).

Competition for growth limiting factors (such as the amino acids arginine, serine, threonine and aspartic acid) between *Salmonella* and other bacterial species in the same environment, can have a suppressive effect on the growth of the former (Ushijima and Seto, 1991). The antagonistic effect of background microflora, for example in food stuffs, on *Salmonella* can be eliminated by the addition of the correct amount of nutrients in pre-enrichment media. Thus, the nutrient requirement becomes a factor directly affecting the detection of the organism in adverse competition with antagonistic bacteria or even adverse environmental conditions such as temperature variations (Liston and Zachariah, 1976; Rao *et al.*, 1980). For example, an elevated temperature (44°C) of incubation was observed to have a negative effect on the growth rate of both *S. weltevreden* and *S. anatum* in the presence of amino acid mixture (Rao *et al.* 1980). Different strains of *Salmonella* appear to have different nutritional requirements, possibly due to differences in their metabolism and biosynthesis, under the same or different growth conditions (Modak *et al.*, 1968).

The production of a chemically defined medium for pre-enrichment of *Salmonella*, that is comparable to media such as BPW is difficult, since peptone is an important factor in cell growth. Recent work however, indicates that significant variation in bacterial growth occurs with different peptones on the market. Replacing the need for peptone with sugars, individual amino acids, nucleosides and vitamins would require the knowledge of

specific requirements and transport mechanisms of such nutrients by the cells. This knowledge would aid in the production of pre-enrichment media that would selectively grow bacteria of specific nutrient requirements.

2.2 Aim

The aim of these studies was to develop a pre-enrichment medium of a defined and reproducible composition, suitable for the growth of *Salmonella* at 37° C and an improvement upon the pre-enrichment of *Salmonella* in BPW and Salmosyst media. The defined medium development was based on Adams (1959) M9 medium, a chemically defined broth originally designed to support the growth of *Escherichia coli*. M9 was used more recently in a modified form as a minimal medium to study recovery of heat treated *Salmonella* (Gomez , 1973; Gomez and Sinskey, 1975; Wilson and Davies, 1976; Mackey and Derrick, 1986). As a chemically defined medium, known to support growth of *Salmonella*, this provided a good starting point from which to develop an optimum, chemically defined medium that is comparable with currently used *Salmonella* pre-enrichment media. There is evidence that a minimal medium may support growth of *Salmonella*, especially injured cells, more than a nutritionally complex medium (Gomez *et al.* 1973; Wilson and Davies, 1976; Mackey and Derrick, 1986).

A second aim was to determine the nutritional value of different nutrient supplements, including amino acids, nucleosides and vitamins, and sugars and compare these with a plain peptone broth. Nucleosides, amino acids and vitamins have been used as

supplements to minimal media as individual groups of substances. Nucleosides and vitamins present in a solid form of the M9 medium have a positive effect on the survival fraction of heat treated *Salmonella* unlike rich media such as brain heart infusion and tryptone soy yeast extract medium (Gomez *et al.* 1973). The effect of individual amino acids and other compounds on the growth of *Salmonella* has been previously investigated, and approximately 100 out of 600 compounds screened were identified as utilisable sources of carbon and/or nitrogen (Gutnick *et al.*, 1969). Majority of the amino acids and other substrates used in the studies reported here have been identified as metabolisable compounds for *Salmonella* but also have been suggested as growth promoting nutrients (Gomez. *et al.*, 1973; Gomez and Sinskey, 1975; Gutnick *et al.*, 1969). Glucose and mannitol were tested since they are commonly used as carbon sources in non-defined liquid media for *Salmonella* and have both been shown to serve as sole carbon sources for *Salmonella typhimurium* (Gutnick *et al.* 1969). Dulcitol was tested as an alternative sugar alcohol (R.Betts; personal communications) and rhamnose was used due to the enhanced effect it had on *Listeria monocytogenes* (Debbie Ahuga; personal communications).

Therefore, the choice of supplementing compounds used in this study was according to the reported ones.

2.3   Materials and Methods

2.3.1   Modification of M9 base medium

The MM9 base medium used in this study was a modification of the medium described by Adams (1959) and consisted of: $Na_2HPO_4.12 H_2O$, 7.0 g l^{-1}; KH_2PO_4, 3.0 g l^{-1}; NaCl, 1.0 g l^{-1} and $MgSO_4.7H_2O$, 0.25 g l^{-1} in distilled water. The modifications were the omission of glucose and ammonium chloride as carbon and nitrogen sources. The medium was autoclaved at 121° C for 15 minutes in aliquots of 10 ml in universal bottles, before addition of the appropriate supplement under aseptic conditions in a class II laminar flow cabinet. A 50x strength stock solution of each of the mixtures of supplements given below was prepared by dissolving 0.2g of each compound in 400 ml of distilled water. Each of the final mixtures was filter sterilised using a 0.22 μm filter and then stored at -70°C in 20 ml aliquots.

The stock solution used to supplement the MM9 base medium comprised:

(a)   Nucleosides: 10 μg ml^{-1} of each of Adenosine 5'-diphosphate (sodium salt); guanosine 2'+3' monophosphoric acid (free acid); thymidine; cytidine (cytidylic acid); uridine 2'+3' monophosphoric acid.

(b)   Vitamins: 10 μg ml^{-1} of each Thiamine (HCL); riboflavin (diluted in ethanol and added in solution); nicotinic acid (calcium salt); pyridoxine HCL (vitamin B6); folic acid; inositol; choline chloride; biotin; p-aminobenzoic acid; cyanobalamin.

(c) Amino acids: 10 μg ml^{-1} of each L-glutamine; L-alanine; L-arginine HCL; L-aspartic acid; L-cystine; L-glutamic acid (sodium salt); glycine; L-histidine; DL-isoleucine; DL-leucine; L-lycine; DL-methionine; DL-phenylalanine; L-proline; DL-serine; DL-threonine; L-tyrosine; DL-valine; asparagine.

The nomenclature of the MM9 medium modifications used in the studies and other media details are presented in Table 2.1.

The growth of a pure culture of *S. typhimurium* was initially determined in the MM9 base medium. The growth of *S. typhimurium* was then assessed in modified MM9 medium, supplemented with and without 2 g l^{-1} glucose in combination with the above mixtures of nucleosides and amino acids at either 10 μg ml^{-1} (1MM9) or 20 μg ml^{-1} (2MM9) final concentration of each ingredient. Growth was also assessed in media containing glucose and all three supplement mixtures (amino acids, nucleosides and vitamins) at 10 μg ml^{-1} (1MM9v), final concentration.

Then 2MM9 was supplemented with the saccharides Glucose, Mannitol, Dulcitol and Rhamnose to evaluate their nutritional value individually as potential additional carbon/ energy sources at concentrations of 2, 4, 8, and 16 g l^{-1} medium. In all cases saccharides were added to the MM9 base media before autoclaving and the amino acid/nucleoside supplement was added after sterilisation.

The growth of *S. typhimurium* in the most favourable of the above MM9 modifications was compared to that in other media recommended for pre-enrichment of *Salmonella*, namely, buffered peptone water (BPW; CM509, Oxoid, Basingstoke, UK) and Salmosyst (Merck Ltd, Poole, Dorset, UK), prepared as recommended by the manufacturers.

Table 2.1: List of media used in these studies for the growth of *Salmonella typhimurium*.

MEDIA	COMPOSITION
MM9	Base medium prepared as a modification of Adams M9 medium (1959)
1MM9	MM9 supplemented with 10 μg ml^{-1} nucleosides and amino acids plus 2 g l^{-1} glucose
1MM9v	As 1MM9 plus 10 μg ml^{-1} vitamins
2MM9	As 1MM9 with double the concentration of nucleosides and amino acids in 1MM9
2MM9 + Sugar	Supplemented with different concentrations of glucose, dulcitol, mannitol and rhamnose (2, 4, 8, 16 g l^{-1})
BPW	Medium prepared as recommended by the manufacturer (Oxoid)
2SBW	Buffered water prepared as BPW without the peptone and supplemented with 20 μg ml^{-1} of nucleosides and amino acids
SALMOSYST	Medium prepared as recommended by manufacturer (Merck Ltd) for the pre-enrichment of Salmonella

2.3.2 Salmonella Growth in Different Media

Stock cultures of the food isolate *S. typhimurium* (NCIMB 13034) strain, obtained from the microbiology department of Whitbread Plc Research and Development Unit, were kept on porous beads (Microbank™, PRO-LAB Diagnostics) at -70°C. Overnight cultures were grown at 37°C in tryptone soya broth (TSB; CM129, Oxoid) and serial decimal dilutions were made in 9 ml sterile quarter strength ringers solution and then plated and counted on triplicate tryptone soya agar (TSA; CM131, Oxoid) plates. Each 9 ml volume of medium to be tested was inoculated at time zero (t0) with the addition of 1 ml of solution containing 2×10^2 cells, and incubated at 37°C in a static incubator for 10h. Samples were taken from this culture at 2h intervals up to 10h, serial dilutions were prepared, and 0.1 ml spread-plated on triplicate TSA plates, and incubated overnight at 37°C.

Each medium type listed in Table 2.1 was evaluated for *Salmonella* growth in three experiments performed separately. The standard deviation between the triplicate experiments was less than 10% and the data was presented as mean number of cells counted on TSA versus the period of incubation in hours. Standard error of the mean was calculated and plotted for each data point.

It was suggested (Gomez and Sinskey, 1975) that air or oxygen may have adverse effects on the repair of heat treated cells of *S. typhimurium*. To check whether aeration during sampling at each of 2 h interval would affect the level of growth an uninterrupted culture

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was grown in parallel to one sampled at 2h intervals. Thus control culture was inoculated with the same cell concentration and incubated under the same growth conditions. There was no difference between the two cultures after 10h of incubation.

2.3.3 Calculating the Doubling Time (td) of Salmonella

The slope of the growth curves of *S. typhimurium* in each of the media used in these studies was used to calculate the population doubling time (td) for the period of 2 to 8 hours of incubation. The formulae used for the calculations are:

$$\text{Slope} = \text{Specific Growth Rate } (\mu) \, / \, 2.303$$

$$\text{Doubling Time (td)} = \ln(2) \, / \, \mu$$

The time period chosen for these calculations was considered important for sample preenrichment within a working day and also was taken as the most linear section of all the growth curves obtained.

2.3.4 Statistical Analysis

The analysis of variance (*Anova*: single factor test) was used to determine significant differences between the *Salmonella* growth supported by the different media used in these studies at time 10 (t_{10}; after 10 hours of incubation). A Further *T-test* (two-sample equal variance) was carried out between results at t10 obtained from specific media to consolidate *Anova* results.

2.4 *Results*

A control incubation with the initial modification of the M9 media (MM9) was made with omission of both the glucose as a carbon source and ammonium chloride as a nitrogen source. Results indicate that there is minimal growth during 10h of incubation in this nutritionally deficient MM9 medium (Fig. 2.1). Thus any growth observed thereafter could only be due to the additional nutrients.

Supplementation with 2 g l^{-1} glucose, nucleosides and amino acids, both 10 μg ml^{-1} in 1MM9 and 20 μg ml^{-1} in 2MM9 supported substantial growth of *Salmonella* after 10h of incubation at 37° C. This growth was found to be significantly different (P<0.01) from that with MM9 alone (Fig. 2.1). However, doubling the concentration of nucleosides and amino acids, from 10 to 20 μg ml^{-1} (Table 2.2) had a statistically significant (P<0.01) but less than hoped for improvement in *Salmonella* growth.

Addition of vitamins in 1MM9v together with nucleosides and amino acids also produced substantial growth as compared to MM9 alone. However, growth in the presence of vitamins in 1MM9v was significantly lower (P<0.01) than growth obtained in their absence but with amino acids and nucleosides in 1MM9, suggesting that the vitamins used have an inhibitory effect on *Salmonella* proliferation under these experimental conditions.

Fig. 2.1: Growth of *S. typhimurium* in different stages of MM9 development. Illustrates the enhanced growth of *S. typhimuirum* due to the supplement. The lowest td was obtained with the 2MM9 medium. Vitamins in 1MM9v medium resulted reduction in growth.
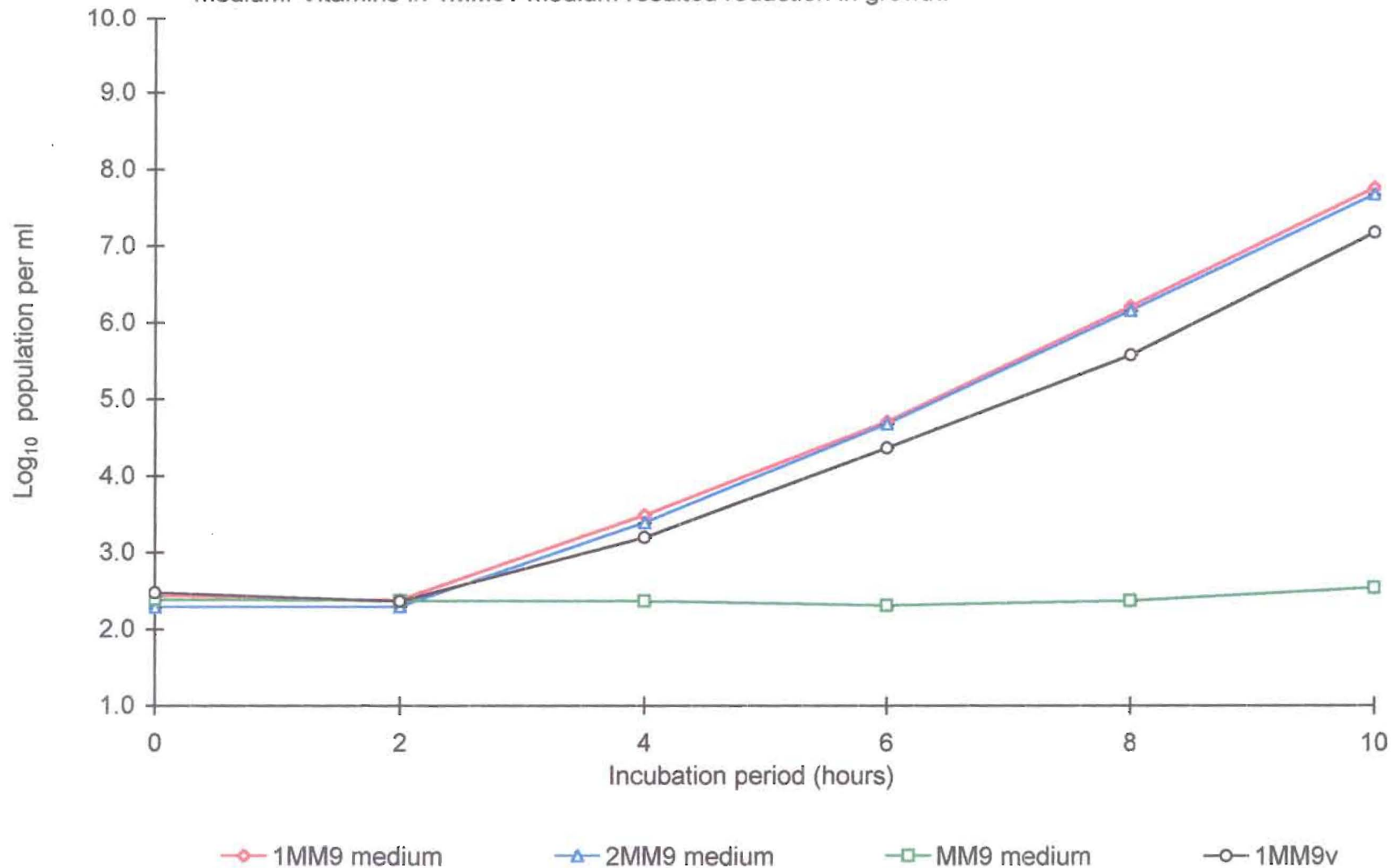
Having developed the basic 2MM9 medium it was important to determine which carbon / energy source and what concentration would result in the lowest doubling time of *Salmonella typhimurium* or a significantly greater *Salmonella* growth. Therefore 2MM9 was thereafter used as the base medium to which the different saccharides were added. The *T-test* results of $P<0.01$ comparing the *Salmonella* growth in the media are thereafter referred to as showing significant differences, either greater or lower.

The concentrations of the saccharides used are 2, 4, 8 and 16 g l^{-1} medium. The growth curves obtained using the four substrates: glucose, dulcitol, mannitol and rhamnose, at those concentrations are illustrated in Figures 2.2 to 2.5.

Four and eight grams per litre glucose (Fig. 2.2) supported significantly greater growth of *S. typhimurium* than 2MM9+2 or 16 gl^{-1} medium. This result was also observed with the calculated doubling time presented in Table 2.2. Four grams per litre glucose in 2MM9 medium was indicated to be an optimum concentration for *S. typhimurium*.

Table 2.2: Doubling times (td) of *S typhimurium* (strain NCIMB 13034) in all media used. The slope of the growth curves was calculated for the time period of 2 to 8 hours of growth. The calculations were done using the following formulae:

Slope = Specific Growth Rate (μ) / 2.303

td = ln (2) / μ

Medium	Doubling Time (minutes)	Medium	Doubling Time (minutes)
MM9	N A	2MM9 + 2g Dulcitol	27.38
1MM9v	33.39	2MM9 + 4g Dulcitol	28.52
1MM9	28.42	2MM9 + 8g Dulcitol	27.66
2MM9	28.06	2MM9 +16g Dulcitol	27.18
2MM9 - No Sugar	32.28		
		2MM9 + 2g Mannitol	27.95
BPW	20.78	2MM9 + 4g Mannitol	27.63
2 SBW	25.40	2MM9 + 8g Mannitol	27.48
Salmosyst	22.68	2MM9 + 16g Mannitol	25.02
2MM9 + 2g Glucose	28.06	2MM9 + 2g Rhamnose	44.38
2MM9 + 4g Glucose	24.19	2MM9 + 4g Rhamnose	34.48
2MM9 + 8g Glucose	24.40	2MM9 + 8g Rhamnose	34.92
2MM9 +16g Glucose	36.89	2MM9 + 16g Rhamnose	47.10

Table 2.2 shows that the commercial media BPW and Salmosyst that supported a significantly greater growth of *Salmonella* achieved the shortest doubling times 20.78 and 22.68 minutes respectively. Based on these results the 2MM9+4g glucose was the next best medium for *S. typhimurium* with a doubling time of 24.19 minutes.

NA = Not applicable to MM9 due to a flat growth curve over the time period tested, therefore no doubling of the population occurred.

Fig. 2.2: Growth of *S. typhimurium* in 2MM9 medium with different glucose concentrations. It is indicated that the media 2MM9+4 or 8 gl⁻¹ supported the best growth. Greater glucose concentrations as with 2MM9+16 gl⁻¹ suppressed the *Salmonella* growth.
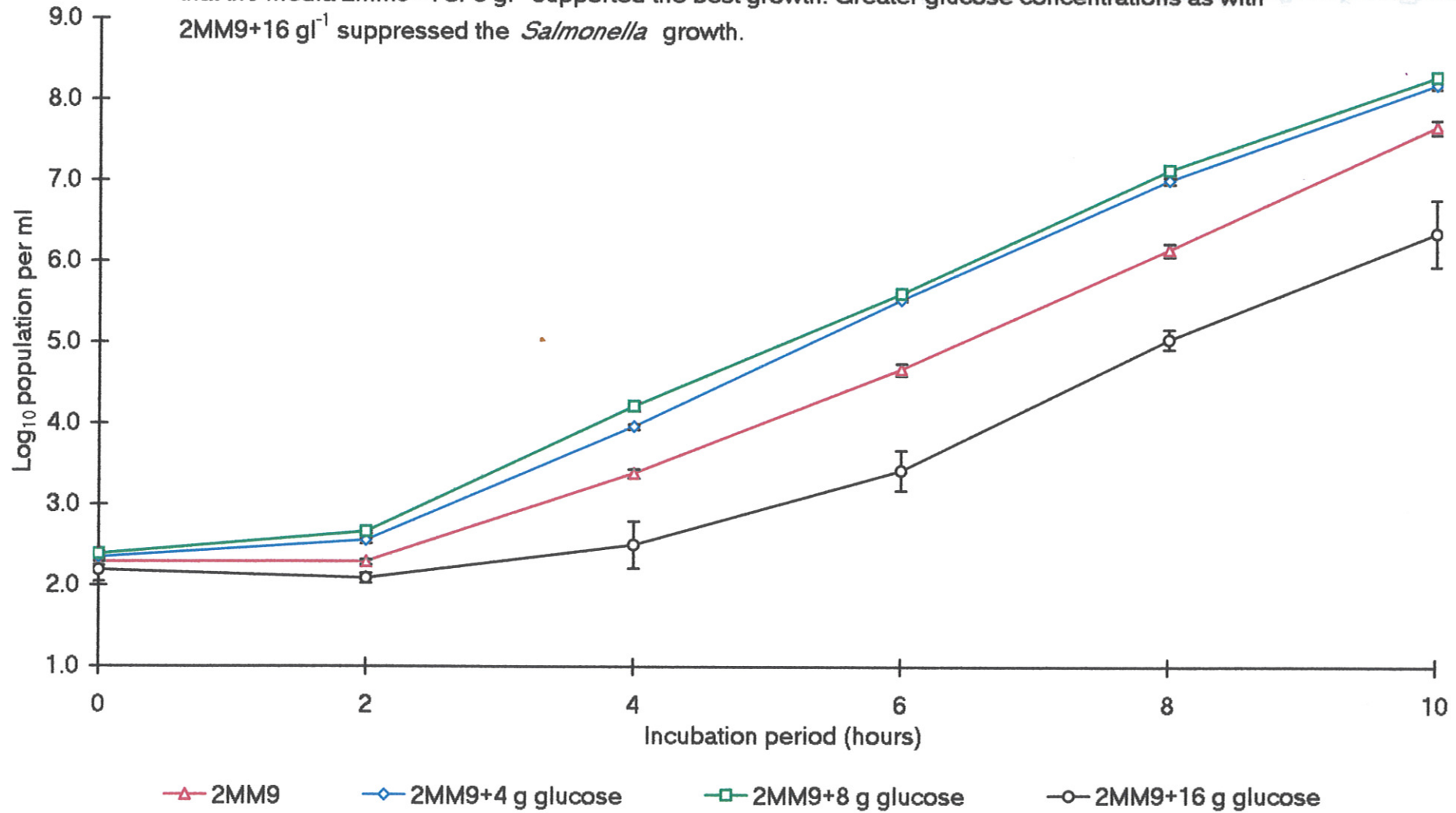
Dulcitol (Fig. 2.3) and mannitol (Fig. 2.4) had a comparable effect, on the growth of *S. typhimurium*. The doubling times obtained from the 2MM9 media supplemented with both dulcitol and mannitol were over 27 minutes. Only the doubling time of 2MM9 medium with $16gl^{-1}$ mannitol was 25 minutes (Table 2.2) which indicated that an increased concentration of mannitol may have supported a better *Salmonella* growth.

Rhamnose used at different concentrations shown in Fig. 2.5 had no stimulatory effect on the growth of *S. typhimurium* in 2MM9 medium. All concentrations resulted in a higher doubling time than that obtained using 2MM9 medium with no sugar substrate (Table 2.2). The maximum growth achieved after 10h incubation with the rhamnose supplemented media is approximately the same as the growth achieved with the 2MM9 medium with no sugar (Fig. 2.6). This indicates that *S. typhimurium* may not be able to utilise rhamnose at all. The addition of $16\ gl^{-1}$ rhamnose resulted in a similar suppressive effect to $16\ gl^{-1}$ glucose.

Fig. 2.3: Growth of *S. typhimurium* in 2MM9 media with different dulcitol concentrations. Illustrates that all dulcitol concentrations used support the same growth of *S. typhimurium* which indicates that 2 gl^{-1} may be an adequate carbon/energy source.
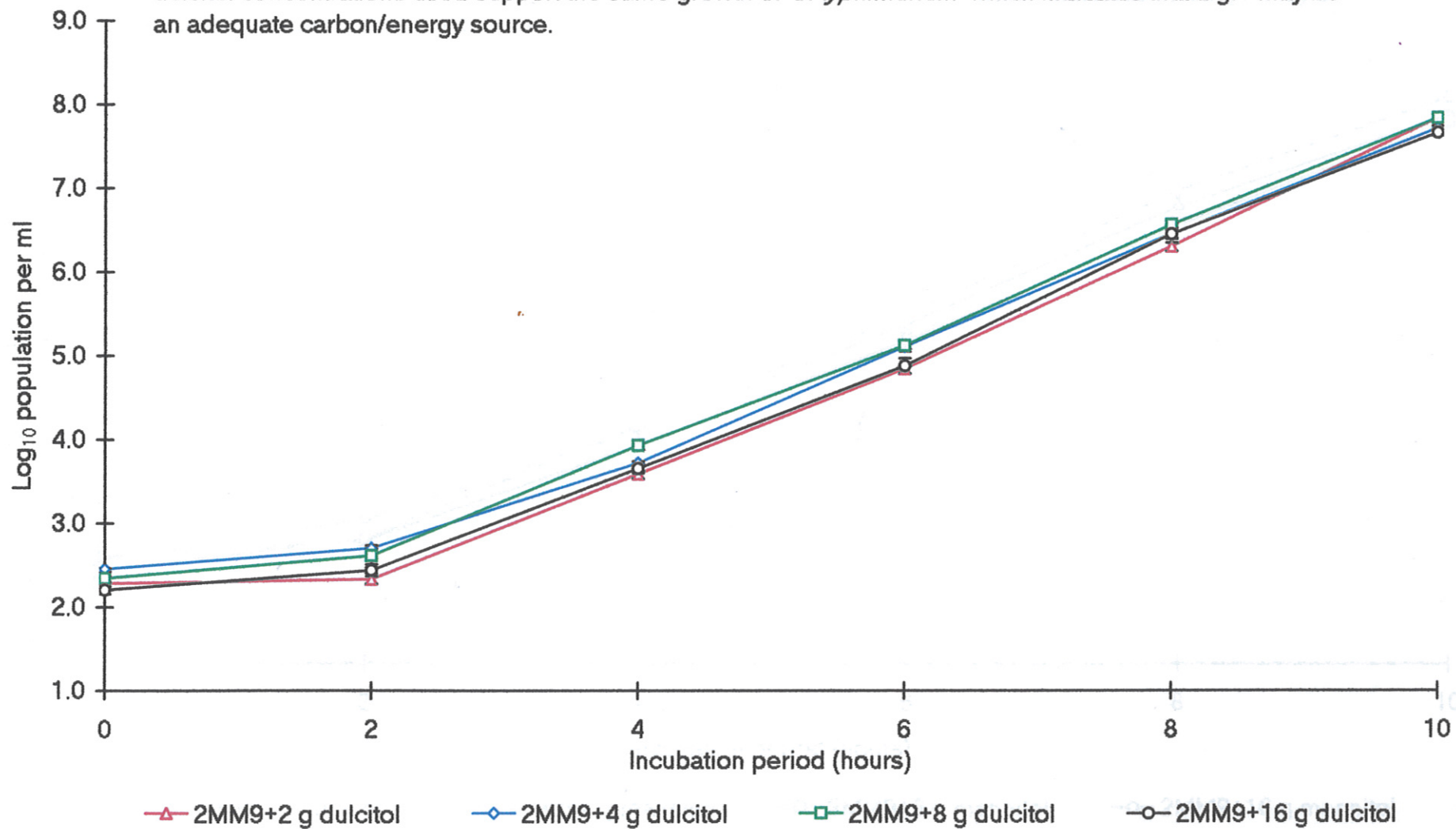
Fig. 2.4: Growth of *S. typhimurium* in 2MM9 media with different mannitol concentrations. Illustates that all mannitol concentrations used support the same growth of *S. typhimurium* which indicates that 2 g l^{-1} may be an adequate carbon/energy source.
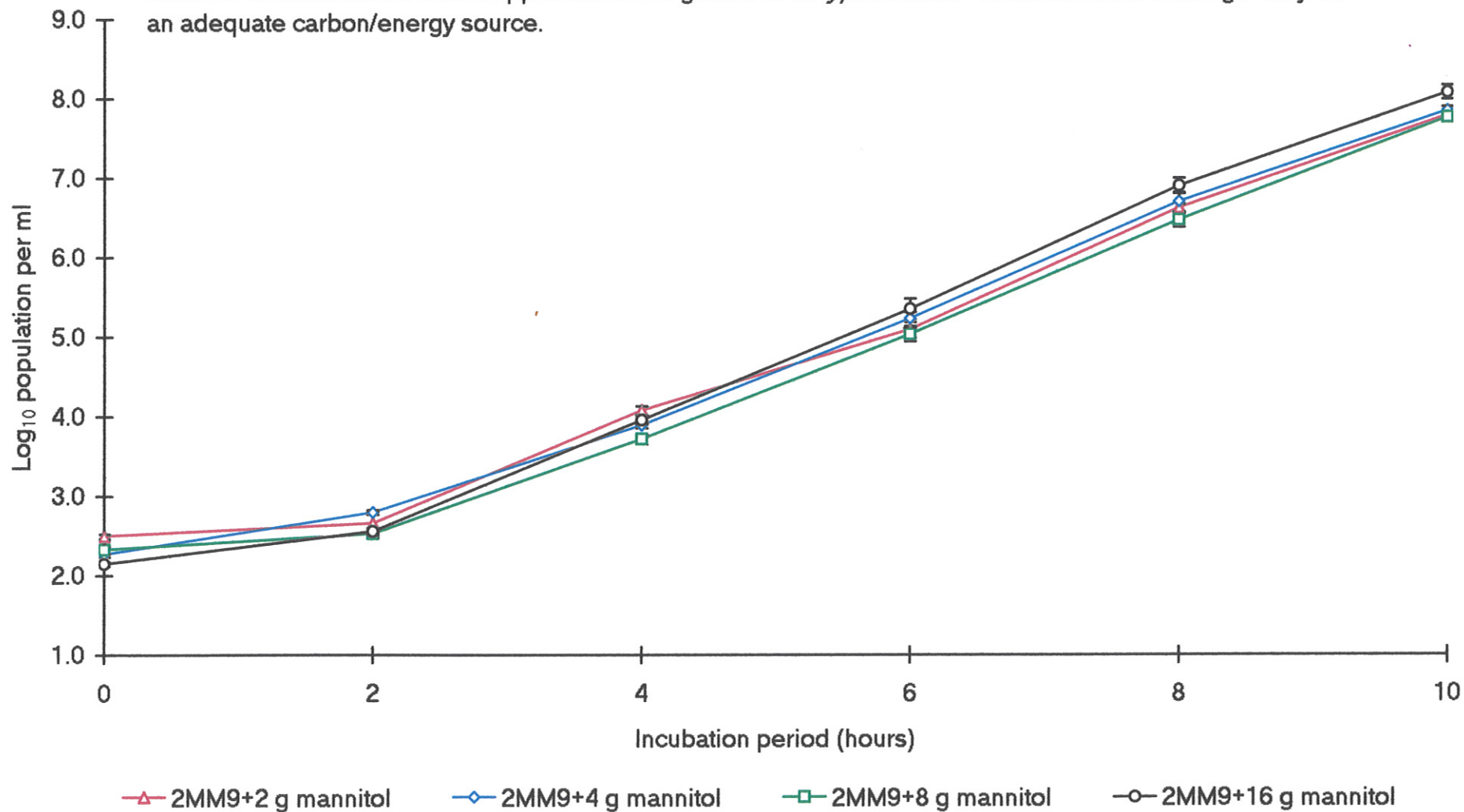
Fig. 2.5: Growth of *S. typhimurium* in 2MM9 medium with different rhamnose concentrations. Illustrates enhanced growth of the organism with increasing concentration of rhamnose from 2 to 4 and 8 gl^{-1} but suppression of growth is indicated with 16 gl^{-1}.
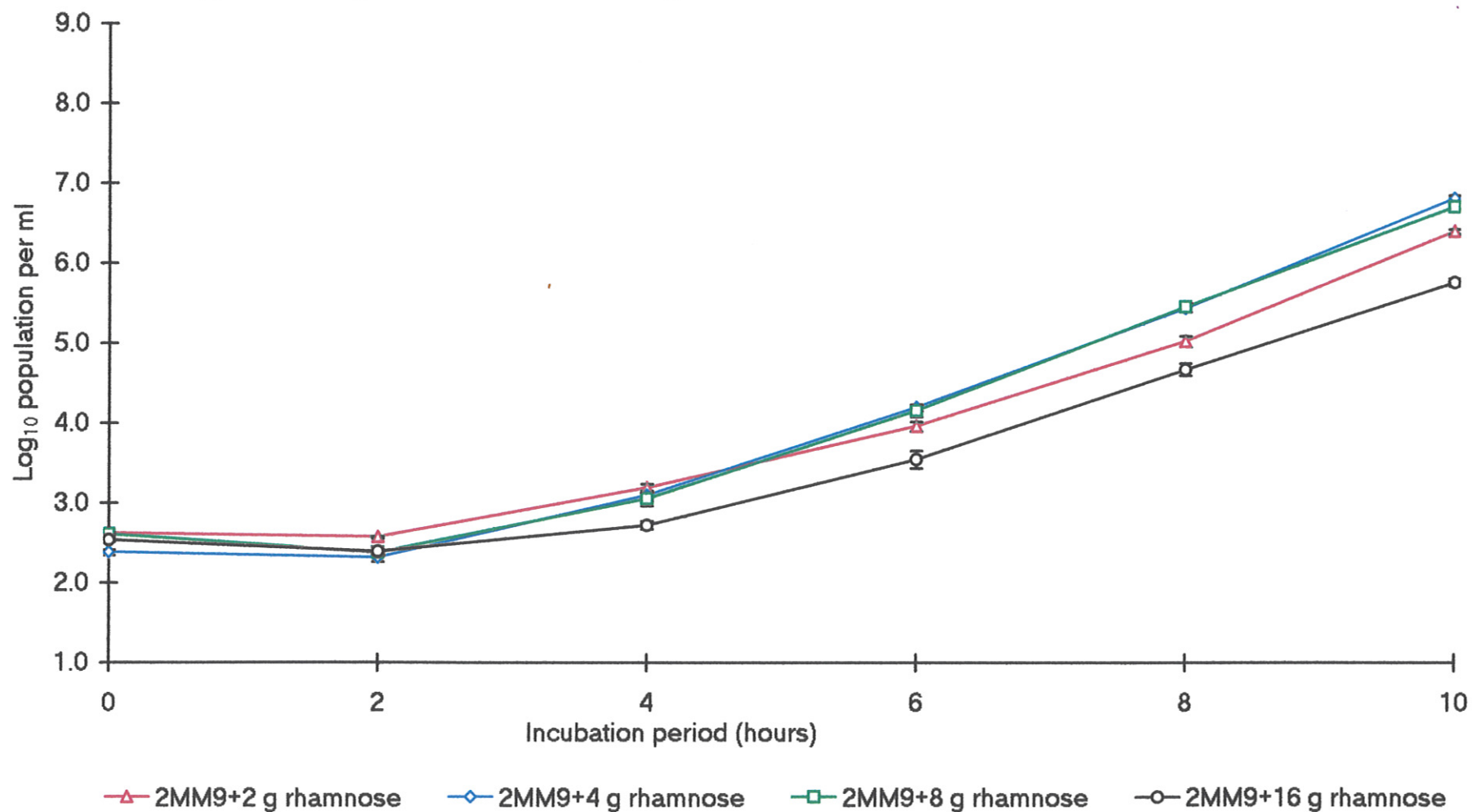
As a final confirmation of the stimulatory effect, on the growth of *S. typhimurium*, of 4 g l^{-1} glucose in the 2MM9 medium an experiment was performed comparing the effects of the 2MM9 medium with and without 4 g l^{-1} glucose shown in Fig. 2.6. The result showed a significant difference between 2MM9 medium with No Sugar and 2MM9 + 4 g l^{-1} glucose medium. This confirms that even though the nucleoside and amino acid supplement allowed a substantial growth of *Salmonella* glucose supported an additional 1 log increase of growth at t10. This increase in growth may be due to the requirement of glucose as an energy source for amino acid accumulation inside bacterial cells. The doubling time (Table 2.2) of *S. typhimurium* in all media from the results from t2-t8 showed that 2MM9 + 4 g l^{-1} glucose was the best defined broth with optimum growth characteristics.

The results of 2MM9 + 4 g l^{-1} glucose medium was then compared to the growth in two of the most widely used nondefined commercial pre-enrichment media, BPW and Salmosyst, shown in Fig. 2.6. The growth in both of the commercial media was significantly better ($P<0.01$) than the one obtained with 2MM9 + 4 g l^{-1} glucose medium.

Fig. 2.6: Illustrates that growth of *S. typhimurium* in BPW and Salmosyst is significantly greater than growth in the 2MM9+4 gl^{-1} glucose. Growth of *S. typhimurium* in 2SBW indicated a superiority of peptone over the supplement since BPW growth is greater.
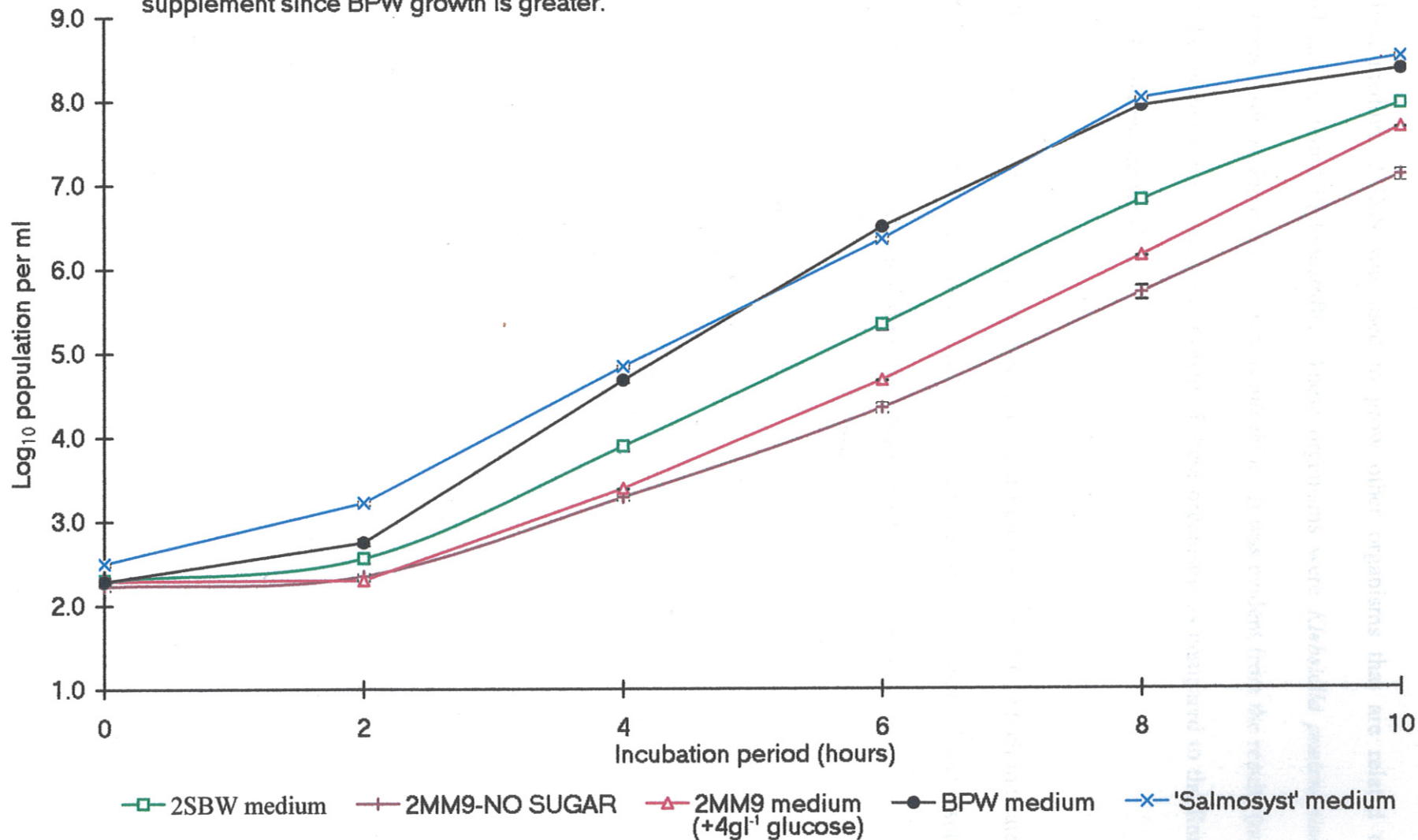
The new defined medium 2MM9 was used to grow other organisms that are related to *Salmonella* and can be found in foodstuffs. These organisms were *Klebsiella pneumoniae, Citrobacter freundii, Escherichia coli and Proteus mirabilis*. It was evident from the results (not presented) that the medium supported good growth of these organisms as compared to the final concentration of *S. typhimurium* at t10.

Note that results obtained from the *Anova* test between the different media at t_{10} which indicated significant differences were also tested using *T-test* of two-sample equal variance. *T-test* results (not presented) confirmed the significant differences obtained with *Anova* tests.

2.5 Discussion

The importance of short pre-enrichment which could be followed by a direct detection of *Salmonella* is widely appreciated. Thus, the knowledge of exact compounds which can enhance the growth of low numbers of cells, and ultimately the resuscitation of injured cells, is of great importance. This thesis reports the use of several supplements which could be used in non-selective media for the recovery of *Salmonella* and how they compare to peptone in non-defined commercial pre-enrichment media.

The stages of development of the new medium indicated the importance of the supplement (1MM9 and 2MM9; Fig. 2.1) for the growth of *Salmonella*. However, the addition of the vitamins (1MM9v) resulted in a decreased growth of *Salmonella*, indicating the vitamins inhibitory effect on the growth of the organism. This contrasts with the observations of Gomez *et al.* (1973) where the addition of vitamins enhanced *Salmonella* growth. However, the vitamin supplement in this report was used in a liquid medium, unlike Gomez *et al.* (1973) who used the vitamins as additional substrates to a solid medium. Therefore, it may be suggested that in a liquid medium chemical constituents such as vitamins are more available to the growing cells and can cause inhibition at lower concentrations than they would do as constituents of solid media. A lower concentration of vitamins may prove more advantageous in a liquid medium such as the 2MM9 broth.

The use of glucose, dulcitol and mannitol as the carbon source for *Salmonella typhimurium* was effective in terms of increasing the growth rate of the cells in the defined medium. Glucose was the optimum substrate at 4 g l^{-1} concentration resulting in a lower doubling time of *Salmonella*

typhimurium (Table 2.2). The higher concentration of glucose (16 g l^{-1}) appears to be inhibitory to the growth of the organism over ten hours. Glucose concentrations of 16 g l^{-1} or more may exert an increased osmotic potential in the medium which may suppress the growth of the cells.

Mannitol may be more effective in liquid media as a carbon source than glucose when present in concentrations higher than 16g l^{-1} medium or 100mM concentration (Fig. 2.4 and Table 2.2). D'Aoust (1978) obtained a high recovery of heat treated *S. typhimurium* with the addition of mannitol (100 and 300 mM) indicating the potential use of this substrate in non selective enrichment media. Rhamnose on the other hand as shown in Fig. 2.5 appears to be unutilisable by *S. typhimurium* and even inhibits growth at high concentrations (16 g l^{-1}) which disagrees with results reported by Gutnick *et al.* (1969).

The difference between the growth of *S. typhimurium* in the new medium and the two non-defined media is not critical but significantly different. Direct replacement of peptone in BPW with the nucleoside and amino acid supplement, termed 2SBW and shown in Fig. 2.6, showed a non-specified protein hydrolysate to be better than the defined media and all the supplements used. This peptone superiority agrees with the findings that peptides are actively transported by bacteria more rapidly than individual amino acids (Sokatch, 1969). Other studies reviewed by Payne and Gilvarg (1978) also indicated that peptides are frequently nutritionally superior to equivalent concentrations of their constituent amino acids and increased cell yield observed on a peptide compared to that on the free amino acid required. These results consolidate the assumption that peptone includes more than just reduced organic compounds that serve as nitrogen, sulphur, carbon and energy sources for bacterial cells. A wider range of utilisable

substrates are available in peptone which may render this substrate as superior growth promoter for both normal but also injured cells.

The exclusion of sugars as carbon and energy sources from the medium (Fig. 2.6) determined their requirement to support a higher growth rate of the *S. typhimurium* even in the presence of the amino acids and nucleosides used. This may reflect the utilisation of amino acids by *Salmonella* as both a nitrogen and a carbon source and the preference for a further carbon and energy source that promotes better growth.

This new defined medium, is a non-selective broth that can be used for the pre-enrichment of *Salmonella typhimurium* and further work is required to determine its potential use as a pre-enrichment medium with other strains of *Salmonella* and to determine its recoverability of injured *Salmonella* cells. The new medium (2MM9 + 4 g l^{-1} glucose) potentially supported growth of sufficient *Salmonella* for the reliable subsequent performance of rapid detection techniques such as PCR (Soumet *et al.*, 1994; Fluit *et al.*, 1993) reaching detectable cell number at approximately 6 to 8 hours of incubation.

CHAPTER 3

ELISA Detection of Salmonella in Different Media

3.1 Introduction

3.1.1 ELISA Detection of Salmonella

Many antibody-based assays have been extensively developed for the specific detection of *Salmonella* and other organisms in a wide range of samples including food. Antibody detection systems include plate agglutination, tube agglutination and immuno-electrophoresis (Kumar and Kaushik, 1988), piezoelectric immunosensors (Prusak and Luong, 1990), enzyme-linked amperometric immunosensors (Mirhabibollahi *et al.*, 1990; Brooks *et al.*, 1992), latex agglutination (Metzler and Nachamkin, 1988) and the most widely used method of enzyme linked immunosorbent assay (ELISA). ELISA detection can potentially simplify and accelerate detection of *Salmonella* in foods which is traditionally a laborious procedure depending on sample-screening by cultural techniques (Kerr *et al.,* 1992). For this reason ELISA is an accepted method for the screening of various organisms, including *Salmonella*, in food microbiology (Cheesbrough and Donnelly, 1996).

The sensitivity of immunologically based techniques is primarily dependant upon the number of *Salmonella* cells present, and consequently the level of immunoreactive antigen present in the sample. The incubation period allowed for antibody-antigen interaction also contributes significantly to the assay sensitivity. Most systems

applicable for batch screening of samples within 24 hours detect between 10^6 and 10^7 cells ml^{-1} (Eckner *et al.*, 1986; Kumar and Kaushik, 1988; Metzler and Nachamkin, 1988; Prusak and Luong, 1990; St. Clair and Klenk, 1990). Pre-enrichment steps increase the number of target cells (*Salmonella*) to detectable levels within food samples. Therefore pre-enrichment helps in avoiding the problem of cross reactions with non-*Salmonella* species (Metzler and Nachamkin, 1988; D'Aoust, Daley and Sewell, 1990; St. Clair and Klenk, 1990; Kerr *et al.*, 1992). Selective-enrichment is also essential in many cases, especially when antibodies of low specificity are utilised in the detection techniques. Non-*Salmonella* species are suppressed by selective enrichment and this also increases assay specificity (St. Clair and Klenk, 1990). However, selective enrichment is costly, both in time and money.

The use of specific antibodies after enrichment culture is an established protocol in the detection of *Salmonella* (Rodrigues and Kroll, 1988 and 1989). However, some antibody detection systems lead to a number of false positive results (Thomason, 1971; Ibrahim and Fleet, 1985; Rodrigues and Kroll, 1990).

Antibodies highly specific for *Salmonella* species can significantly reduce the time required for detection. With an 18h pre-enrichment step, the sensitivity of an antibody-capture ELISA is reported to be as low as 10 cells g^{-1} sample (Lee *et al.*, 1989). Lee *et al.* (1990) reported an increase in ELISA sensitivity using a 19 hour pre-enrichment step in an amino acid-rich chemically-defined medium, to 10 cells 25

g^{-1} sample of *Salmonella*. This assay was possible in the presence of a high background microflora : 1 to 10^6 *Salmonella* to non-*Salmonella* ratio.

The detection time of the assay can also be reduced if antigen expression on *Salmonella* cells is increased. Certain media such as M-broth are known to enhance antigen production/expression and can improve *Salmonella* detection (Flowers, 1985). Variations of structural-antigen concentration was reported to cause a variation in ELISA sensitivity (Smith *et al.*, 1979; Ibrahim *et al.*, 1985). The efficiency of antibody detection techniques also depends on the type of antibody or combination of antibodies used for antigen capture (Kocka *et al.*, 1992). For example, ELISA assays detecting the flagellar common structural antigen will fail to detect non-flagellated serotypes such as *Salmonella pullorum* and *Salmonella gallinarum*.

Several workers have reported that monoclonal antibodies (mAb) are more specific compared with polyclonal antibodies (Luk and Lindberg, 1991; Choi *et al.*, 1992). mAb against porin, an outer membrane protein from *Salmonella typhimurium*, has been used in the detection of *Salmonella* (Muthukkumar and Muthukkaruppan, 1992). Cross-reactivity studies using ELISA, with mAbs to *Salmonella* outer membrane proteins, are usually performed using closely related organisms like strains of *Citrobacter, Klebsiella, Serratia, Enterobacter* and *Escherichia* (Kerr *et al.*, 1992). Despite ELISA assays being highly specific, some cross reactivity occurs. *Citrobacter* species, for example, frequently cause false positive reactions with culture procedures due to many biochemical similarities with *Salmonella* (Van der Walt, M.

L. and Steyn, H. C., 1989). These false positive results are eliminated with mAb-based ELISA (Kerr *et al.* 1992).

3.1.2 Evaluation of Antibody Based Assays

A summary of reported studies on detecting *Salmonella* species using antibody capture systems are listed below (Table 3.1). Assay evaluation studies concentrate on determining assay sensitivity with samples from different origins and on determining the antibody specificity with low numbers of antigen/target cells, in the presence of high levels of background interference.

Despite ELISA being accepted by most laboratories as a reliable and rapid method for the screening of *Salmonellae*, further confirmation of ELISA results is required (Cheesbrough and Donnelly, 1996). Traditionally this involves sub-culturing the ELISA positive samples on more than one selective differential medium followed by biochemical and serological testing. This slows down the detection procedure.

To improve the speed with which the confirmation of ELISA positive samples can be determined, a system named Spectate® was developed (Rhône-Poulenc Diagnostics Ltd, West Scotland Science Park, Glasgow) as a fast way of detecting *Salmonella* antigen: specific to serotypes B, C, D, E, G and to Vi antigen. Unlike the ELISA system, Spectate® provides presumptive negative rather than presumptive positive results. The test is reported to have a sensitivity of 10^7 cells ml^{-1}, and when combined

with ELISA produced a 90% confidence limits of a true positive result (Cheesbrough and Donnelly, 1996).

Table 3.1: Some of the studies carried out with antibody based *Salmonella* detection systems and their findings.

Antibody-based system	Reference	Advantages	Disadvantages
FA staining	Thomason (1971); Ibrahim & Fleet (1985); Rodrigues & Kroll (1990).	Relatively sensitive and specific to *Salmonella*.	False positive results obtained.
Antibody capture ELISA	Lee *et al.* (1989)	High *Salmonella* specificity and sensitivity when combined with 19h pre-enrichment in an amino acid-rich defined medium	
Latex agglutination (Colour *Salmonella* test)	Kocka *et al.* (1992)	High *Salmonella* specificity	
BacTrace™ ELISA	D'Aoust, Daley and Sewell (1990)	High sensitivity	High cross reactivity (with *C. freundii, E. coli, P. mirabilis*)
Spectate® antibody capture	Cheesbrough & Donnelly (1996)	Rapid confirmation procedure specific to *Salmonella*.	Low sensitivity (10^7 cells ml^{-1})
Salmonella-Tek ELISA	Kerr *et al.* (1992); St. Claire & Klenk (1990); Van Poucke (1990).	High sensitivity (10^5 cells ml^{-1}). High specificity when combined with selective enrichment (e.g. in Salmosyst broth)	High number of false positives
LOCATE ELISA	Gangar *et al.* (1998)	High sensitivity and specificity after pre-, selective- and post-enrichment.Comparative to BAM/AOAC methods. Visual and automated assessment. Rapid and simple to use.	Very few false negatives with some food samples.
TECRA *Salmonella* Immunoassay	Lambiri *et al.* (1990)	High sensitivity and specificity after pre-, selective- and post-enrichment. Agrees with conventional methods. Rapid and simple.	Very few false negatives and false positives depending on selective medium.

3.2 Aims

The objectives of these studies were to:

a. Determine the number of *S. typhimurium* cells required to provide a positive reaction with three different commercially available ELISA assays (TECRA® *Salmonella* Visual Immunoassay, LOCATE® *Salmonella* Screening Test and *Salmonella*-TEK™ ELISA test) and

b. Investigate the compatibility of four pre-enrichment media (BPW, Salmosyst, M-broth and MM9s'2 + 4g glucose) with the above ELISA kits. Possible media interference with ELISA should be detected.

3.3   Materials and Methods

A comparison of three ELISA kits was made using *S. typhimurium* grown in each of four liquid enrichment media: BPW (OXOID CM509); Salmosyst broth (Merck Ltd UK); M-broth (DIFCO Bacto M Broth); and 2MM9 + 4g glucose (as described in 2.3).   The media were prepared as recommended by the manufacturers.   The 2MM9+4g glucose medium was prepared as in Chapter 2 (2.3).

20ml of each medium was inoculated with about 10^3 cells of *S. typhimurium* and incubated for 24 h at 37°C. A 1ml sample was taken in 1.5ml Eppendorf tubes after 4, 5, 6, 8, 10 and 24 hours of incubation for ELISA analysis. A second 1 ml sample was taken at each of the intervals for viable counts on TSA plates. A volume of 100μl of each sample was spread on triplicate TSA plates which were incubated at 37° C for 24 h and colonies were counted.

Each 1 ml sample in the Eppendorf tubes was prepared for ELISA analysis according to manufacturers recommendations. Sample preparation generally involve immersion in a boiling waterbath for 15 to 20 minutes to lyse and disrupt cells followed by cooling to room temperature before storage at 4° C. Storage was for up to 3 days before use in the assays.   The three ELISA assays were then performed by the standard manufacturers recommendations. These assays are detailed below in section 3.4.1 to 3.4.3.

3.3.1 The Salmonella-TEK™ ELISA Assay

The *Salmonella-TEK™ ELISA* kit was stored at 4°C prior to use. The samples were prepared for analysis before removal of the kit from its refrigeration. Sample preparation comprised: boiling 1ml culture in Eppendorf tubes in a waterbath for 20 minutes and then cooling at room temperature to about 25°C. This was used for the assay which followed.

The microwell stripholder was fitted with the appropriate Microelisa wells for the samples, plus three more wells for controls. 100μl of each of the boiled samples was pipetted into assigned wells, including two negative and one positive control samples for each assay. The plate with the Microelisa wells was covered with an adhesive sealer and incubated at 37°C for 30 minutes. Each well was washed three times with wash solution and 100 μl conjugate was added in all the wells. The plate was covered again with the sealer and incubated for a further 30 minute period at the same temperature. Each well was finally washed six times with wash solution and 100 μl TMB substrate was added. Substrate incubation was at room temperature 25°C for 30 minutes. The reaction was terminated on addition of 10μl of 'stop' solution. The colour development was determined as an absorbance at 450 nm measured with a microplate reader. The cut-off absorbance value above which samples were positive was calculated as suggested by the manufacturers.

3.3.2   The TECRA Salmonella Visual Immunoassay

The sample preparation for TECRA *Salmonella* VIA differed from the *Salmonella*-TEK™ only in the boiling period of the cultures in the waterbath which required only 15 minutes.

The microwell holder was fitted with the required number of wells for the samples, allowing two more wells for positive and negative controls. 200μl of each of the boiled samples was pipetted in the assigned wells, including the negative and positive control. The plate with the Microelisa wells was covered with a an adhesive sealer and incubated at 37°C for 30 minutes. Each well was then washed three times with wash solution as suggested by the manufacturer and 200 μl conjugate was added in each well. The plate was covered again and incubated for a further 30 minute period at the same temperature. Each well was washed four times with wash solution and 200 μl substrate was added. Substrate incubation was at room temperature 25°C for 30 minutes. If the positive control reading measured with a plate reader at 414 nm was over 1.0 after 10 minutes of incubation, then the reaction continued to the maximum 30 minute period. The reaction was finally terminated on addition of 20μl of 'stop' solution. In order to get an even colour in the wells, the sides of the well holder were tapped gently and the absorbance was measured at 450 nm. The cut-off value is indicated by the manufacturers to be an absorbance of 0.3 at 414±10 nm.

3.3.3   The LOCATE® Salmonella Screening Test

As with the *Salmonella*-TEK preparation the 1ml samples were boiled for 20 minutes in a waterbath and then cooled down to room temperature before analysis.

The microwell plate holder was fitted with the required amount of Microelisa wells for the samples, plus two more wells for controls. 100μl of each of the prepared samples was pipetted in the assigned wells, including a negative and a positive control per assay. The tray with the Microelisa wells was incubated at room temperature for 30 minutes. Each well was then washed four times with 250 μl working wash buffer per well and once with distilled water before adding the 100 μl conjugate to each sample and control wells. The tray was incubated again for a further 30 minute period at room temperature. Each well was washed exactly as previously with wash buffer and 100 μl TMB substrate was added. Any extraneous substrate was blotted from the top of the wells with absorbent paper before incubating in the dark at room temperature for 30 minutes. The reaction was terminated on addition of 100μl of 'stop' solution per well. The absorbance was measured with a microplate reader at 450 nm within 5 minutes. The cut-off value suggested by the manufacturers was 0.3 below which the samples were considered to be negative.

3.4 Results

After the initial inoculation of the four media with *S. typhimurium* cells proliferation was monitored by spread plating on TSA medium and obtaining the total viable count every two hour period (Table 3.1-3.3). In order to illustrate the sensitivity of the ELISA assays the colony counts obtained on the triplicate TSA plates from each medium were plotted against the absorbance values obtained from the corresponding assays (Fig. 3.1-3.3). The variability of the assays is indicated by the standard error bars on each data point on the graphs.

3.4.1 Results of Salmonella TEK™

The results of the *S. typhimurium* growth in the four different media and *Salmonella* detection using the *Salmonella*-TEK™ assay are presented in Fig. 3.1. It appeared that the lowest cell concentration yielding a positive A_{450} reading was approximately 2×10^6 cfu ml^{-1} (Table 3.2). Proliferation of *S. typhimurium* in both BPW and M-broth was determined to be quicker than growth in Salmosyst and 2MM9+4g Glucose. Positive detection of Salmonella in BPW medium was possible after only 4h of incubation in at least one of the samples tested while the other samples were marginally negative, being <0.02 below the cut-off value of the assay. The cell concentration of the positive BPW sample was 4×10^4 cfu ml^{-1}. Using the other assays (Table 3.3 and 3.4) it was not possible to detect such a low number of *Salmonella*. However, the positive BPW result after only 4 hours of incubation was unreliable since the negative control of the medium was also positive when tested twice. After 6 h of incubation in M-broth *Salmonella*-TEK™ assay resulted a positive detection

with a minimum concentration of 1.6×10^6 cfu ml^{-1}. Salmosyst and 2MM9+4g glucose media resulted in a positive A_{450} 2h later at t8.

Fig. 3.1: *Salmonella* TEK[TM] absorbance readings (mean of 3 replicates) at 450 nm, with *S. typhimurium* growing in four different non-selective enrichment media over a period of 24 hours. Readings were taken at 4, 6, 8, 10 and 24 hours of incubation.

Table 3.2: *Salmonella*-TEK™ results of *Salmonella typhimurium* growing in different non-selective enrichment broths at different time periods of incubation.

Time (h) of sampling	Number of cfu *Salmonella* ml⁻¹ sample (mean of three replicate plates) and ELISA positive (+) or negative (-) results obtained per sample							
	BPW		Salmosyst		M-Broth		2MM9+4g Gluc.	
Inoculation	146		146		159		159	
4	4.2×10^4	+	1.1×10^4	-	5.3×10^4	-	1.5×10^4	-
	4.1×10^4	-	1.8×10^4	-	3.5×10^4	-	1.1×10^4	-
	5.8×10^4	-	2.5×10^4	-	6.5×10^4	-	2.0×10^4	-
6	2.0×10^6	+	3.3×10^5	-	2.5×10^6	+	3.4×10^5	-
	1.7×10^6	+	4.1×10^5	-	1.6×10^6	+	1.3×10^5	-
	6.1×10^5	+	7.0×10^5	-	2.0×10^6	+	2.3×10^5	-
8	4.8×10^7	+	1.4×10^7	+	7.8×10^7	+	7.8×10^6	+
	6.8×10^7	+	4.7×10^6	+	6.6×10^7	+	6.4×10^6	+
	5.8×10^7	+	1.3×10^7	+	1.0×10^8	+	9.4×10^6	+
10	1.9×10^8	+	2.0×10^8	+	1.2×10^9	+	1.1×10^8	+
	2.1×10^8	+	1.3×10^8	+	1.1×10^9	+	6.7×10^7	+
	1.8×10^8	+	2.3×10^8	+	1.2×10^9	+	1.3×10^8	+
24	2.0×10^9	+	3.2×10^9	+	4.4×10^9	+	1.2×10^9	+
	1.8×10^9	+	3.1×10^9	+	3.8×10^9	+	1.6×10^9	+
	2.5×10^9	+	3.0×10^9	+	4.4×10^9	+	1.1×10^9	+
CONTROL		+		-		-		-

3.4.2 Results of TECRA Salmonella Immunoassay

The performance of **TECRA** *Salmonella* **Immunoassay** results are shown in (Fig. 3.2) and show great similarity to those obtained with the *Salmonella*-**TEK**™ assay. The number of *Salmonella* cells resulting in positive detection was >1.6×10^6 cfu ml^{-1} which was achieved after six hours of incubation in BPW, Salmosyst and M-Broth media (Table 3.3).

However, the first positive result obtained with 2MM9+4g glucose medium was after eight hours of growth when cell concentration reached 1.1×10^7 cfu ml^{-1}. Two of the three samples of t8 with cell concentrations of 5.1×10^6 and 6.9×10^6 cfu ml^{-1} appeared to give a negative result (Table 3.3) which was unlike the trend with the other three media.

Fig. 3.2: TECRA *Salmonella* VIA absorbance readings (mean of 3 replicates) at 414 nm, with *S. typhimurium* growing in four different non-selective enrichment media over a period of 24 hours. Readings were taken at 4,5,6,8,10 and 24 hours of incubation.
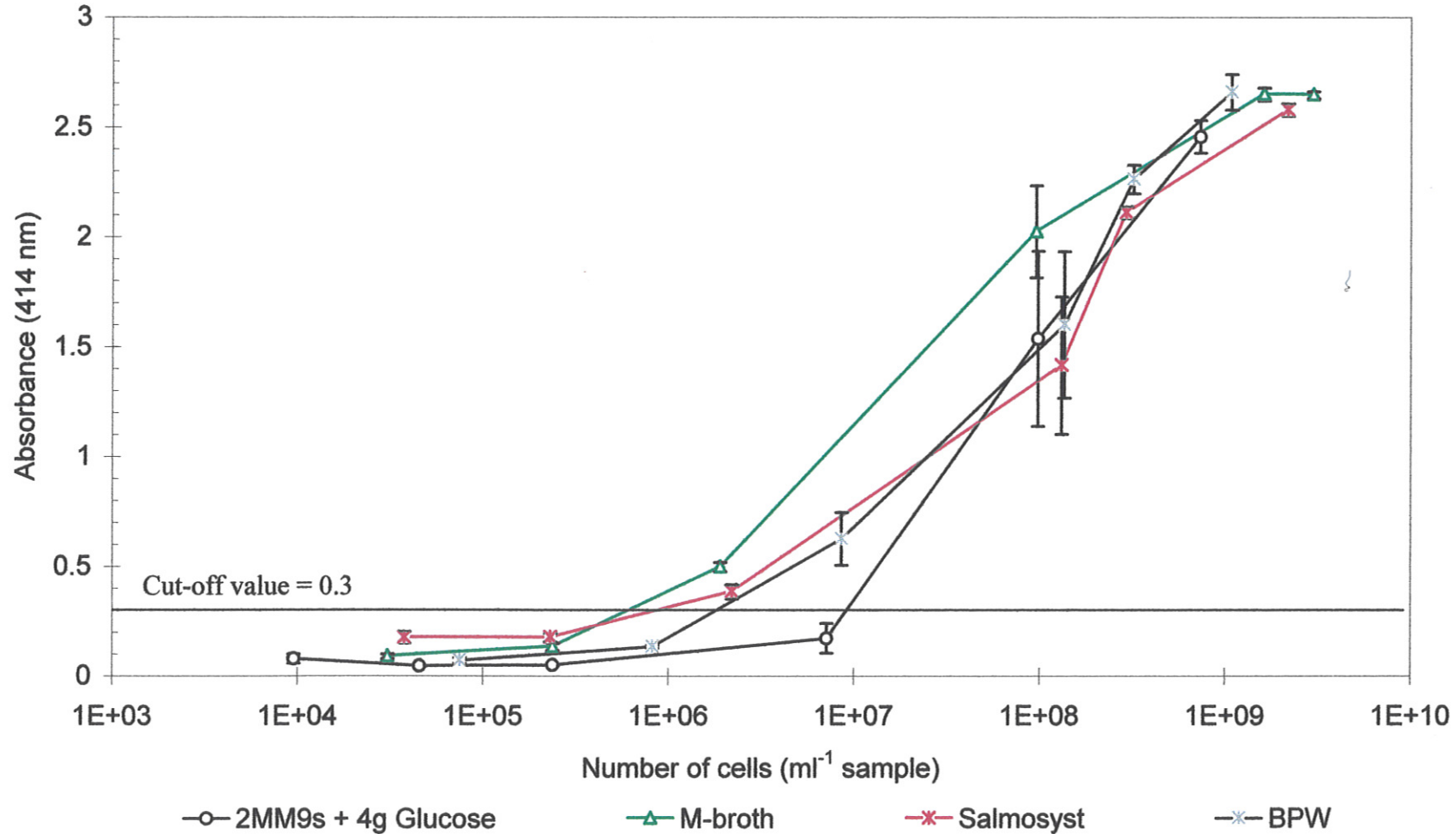
Table 3.3: TECRA *Salmonella* Immunoassay results of *Salmonella typhimurium* growing in different non-selective enrichment broths at different time periods of incubation.

Time (h) of sampling	Number of cfu *Salmonella* ml^{-1} sample (mean of three replicate plates) and ELISA positive (+) or negative (-) results obtained per sample							
	BPW		Salmosyst		M-Broth		2MM9+4g Gluc.	
Inoculation	90		75		90		75	
4	6.8×10^4	-	3.9×10^4	-	3.1×10^4	-	9.3×10^3	-
	6.4×10^4	-	3.9×10^4	-	3.1×10^4	-	9.7×10^3	-
	9.3×10^4	-	3.5×10^4	-	3.0×10^4	-	2.7×10^3	-
5	8.8×10^5	-	2.1×10^5	-	2.9×10^5	-	7.0×10^4	-
	7.8×10^5	-	2.7×10^5	-	2.4×10^5	-	3.3×10^4	-
	7.9×10^5	-	2.1×10^5	-	1.8×10^5	-	2.0×10^4	-
6	6.5×10^6	+	2.7×10^6	+	2.3×10^6	+	3.7×10^5	-
	6.5×10^6	+	1.8×10^6	+	1.6×10^6	+	1.7×10^5	-
	1.3×10^7	+	2.1×10^6	+	1.8×10^6	+	1.6×10^5	-
8	1.3×10^8	+	1.9×10^8	+	9.3×10^7	+	1.1×10^7	+
	1.3×10^8	+	1.1×10^8	+	1.0×10^8	+	5.1×10^6	-
	1.5×10^8	+	9.7×10^7	+	9.3×10^7	+	6.9×10^6	-
10	2.7×10^8	+	3.1×10^8	+	1.6×10^9	+	8.0×10^7	+
	3.2×10^8	+	2.9×10^8	+	1.5×10^9	+	1.1×10^8	+
	3.7×10^8	+	2.7×10^8	+	1.7×10^9	+	7.3×10^7	+
24	1.1×10^9	+	2.0×10^9	+	3.0×10^9	+	7.3×10^8	+
	1.1×10^9	+	2.2×10^9	+	3.7×10^9	+	7.3×10^8	+
	1.0×10^9	+	2.2×10^9	+	2.2×10^9	+	5.3×10^8	+
CONTROL		-		-		-		-

3.4.3 Results of LOCATE Salmonella Screening Test

LOCATE *Salmonella* screening test detected as low as 3.7×10^5 cfu *Salmonella* ml^{-1} growing in 2MM9+4g glucose medium (Table 3.4). When *Salmonella* concentration in this medium reached between 10^6 to 10^7 cfu ml^{-1} the assay resulted in a positive detection whereas similar concentrations of the organism in either BPW or M-broth media could not be detected by the assay.

LOCATE *Salmonella* assay is less sensitive and only detects *Salmonella* at concentrations exceeding 10^7 cfu ml^{-1} when growing in BPW and M-Broth (Fig. 3.3, Table 3.4). Six hours of incubation in these two media promoted the growth of *Salmonella* to a cell concentration of 10^6 to 10^7 cfu ml^{-1} but was still undetectable by the LOCATE assay. The results were positive only when the organism reached concentrations of approximately 10^8 cfu ml^{-1} (Table 3.4).

The **LOCATE *Salmonella*** assay failed to detect *S. typhimurium* in Salmosyst medium even when cell number reached detectable levels of the organism growing in other media (Fig. 3.3). *Salmonella* growth in Salmosyst broth appeared to be completely undetected by the assay even at 2.2×10^9 cfu ml^{-1} (Fig. 3.3).

Fig. 3.3: LOCATE *Salmonella* Screening absorbance readings (mean of 3 replicates) at 450nm, with *S. typhimurium* growing in four different non-selective enrichment media over a period of 24 hours. Readings were taken at 4,5,6,8,10 and 24 hours incubation.
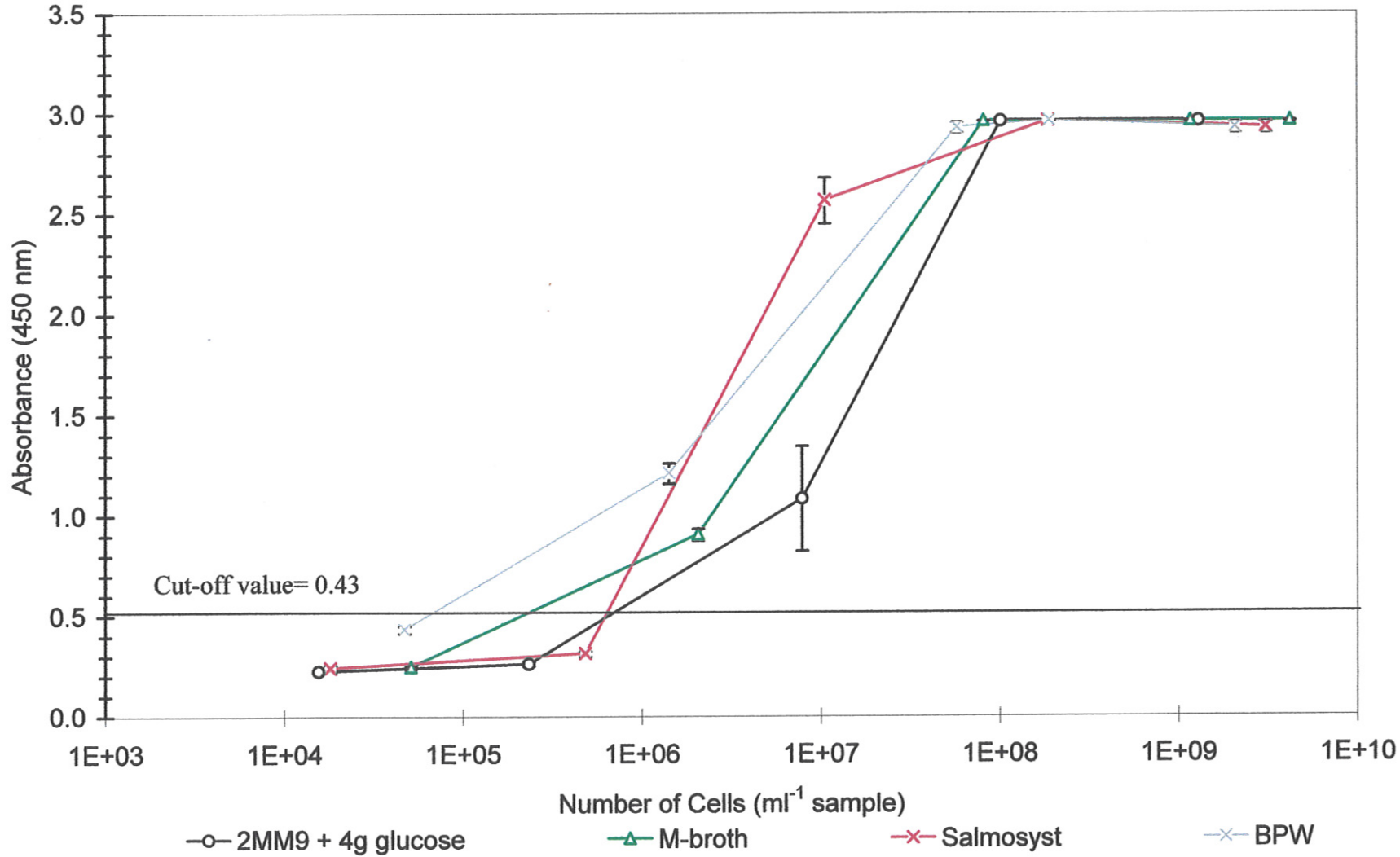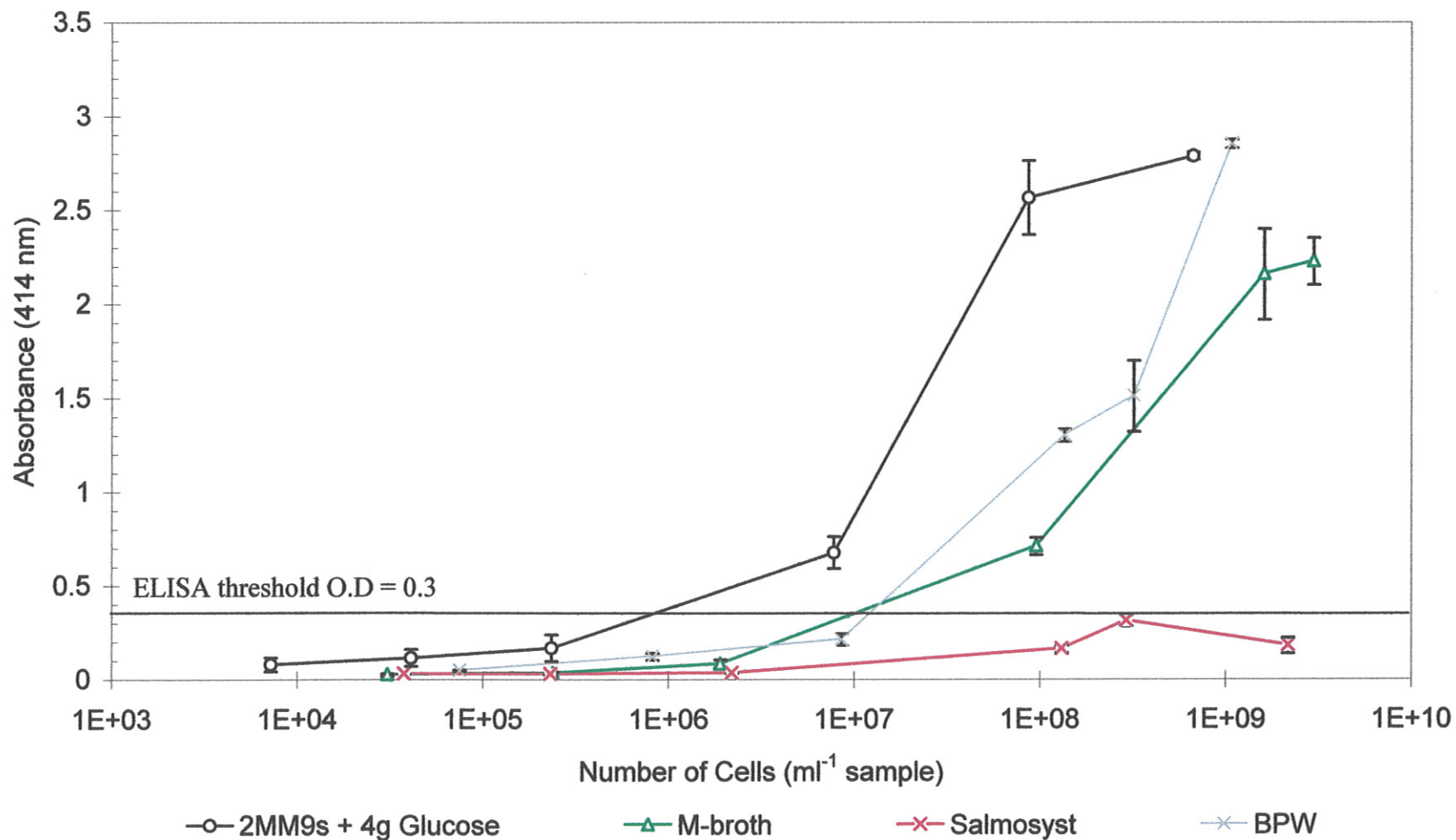
Table 3.4: LOCATE *Salmonella* Screening Test results of *Salmonella typhimurium* growing in different non-selective enrichment broths at different time periods of incubation.

Time (h) of sampling	Number of cfu *Salmonella* ml^{-1} sample (mean of three replicate plates) and ELISA positive (+) or negative (-) results obtained per sample							
	BPW		Salmosyst		M-Broth		2MM9+4g Gluc.	
Inoculation	90		75		90		75	
4	6.8×10^4	-	3.9×10^4	-	3.1×10^4	-	9.3×10^3	-
	6.4×10^4	-	3.9×10^4	-	3.1×10^4	-	9.7×10^3	-
	9.3×10^4	-	3.5×10^4	-	3.0×10^4	-	2.7×10^3	-
5	8.8×10^5	-	2.1×10^5	-	2.9×10^5	-	7.0×10^4	-
	7.8×10^5	-	2.7×10^5	-	2.4×10^5	-	3.3×10^4	-
	7.9×10^5	-	2.1×10^5	-	1.8×10^5	-	2.0×10^4	-
6	6.5×10^6	-	2.7×10^6	-	2.3×10^6	-	3.7×10^5	+
	6.5×10^6	-	1.8×10^6	-	1.6×10^6	-	1.7×10^5	-
	1.3×10^7	-	2.1×10^6	-	1.8×10^6	-	1.6×10^5	-
8	1.3×10^8	+	1.9×10^8	-	9.3×10^7	+	1.1×10^7	+
	1.3×10^8	+	1.1×10^8	-	1.0×10^8	+	5.1×10^6	+
	1.5×10^8	+	9.7×10^7	-	9.3×10^7	+	6.9×10^6	+
10	2.7×10^8	+	3.1×10^8	-	1.6×10^9	+	8.0×10^7	+
	3.2×10^8	+	2.9×10^8	+	1.5×10^9	+	1.1×10^8	+
	3.7×10^8	+	2.7×10^8	-	1.7×10^9	+	7.3×10^7	+
24	1.1×10^9	+	2.0×10^9	-	3.0×10^9	+	7.3×10^8	+
	1.1×10^9	+	2.2×10^9	-	3.7×10^9	+	7.3×10^8	+
	1.0×10^9	+	2.2×10^9	-	2.2×10^9	+	5.3×10^8	+
CONTROL		-		-		-		-

3.5 Discussion

It has been suggested that using the ELISA assays used in the present study and other commerscial assays, a cell concentration of approximately 10^6 cfu ml^{-1} is required for a reliable positive ELISA result (Eckner *et al.*, 1986; Metzler and Nachamkin, 1988; Prusak and Luong, 1990; St. Clair and Klenk, 1990; Van Poucke, 1990; Lambini *et al.*, 1990; Hold *et al.* 1995). The reported sensitivity values tend to agree with the results obtained in these studies which only investigated the detection of pure cultures of *S. typhimurium* analysed directly after incubation in the four non-selective media.

All but two medium-ELISA combinations resulted in detection levels of less than 10^6 cfu *Salmonella* ml^{-1} sample. The first low-number *Salmonella* detection being the combination of BPW with the *Salmonella*-TEK™ kit which resulted a positive reaction with 4.2×10^4 cfu ml^{-1} (Table 3.2). However, BPW control also gave a positive reaction on duplicated assays which indicated a possible medium-assay interaction that may yield false positive results.

The second low-number positive detection was achieved using the LOCATE *Salmonella* screening test after incubation in the 2MM9+4g glucose medium (Table 3.4). The importance of this result was that 2MM9+4g glucose medium gave a positive LOCATE detection with ten times less cells than the positive results obtained with the BPW and M-broth media. The first positive result with the 2MM9+4g glucose medium was obtained after 6 hours of incubation at a cell concentration of

3.7×10^5 cfu ml^{-1}, the same time at which the cell concentration in the other media was approximately ten times higher but the LOCATE reactions were negative. This result shows that certain media, in this case 2MM9+4g glucose medium, may improve antigen production by the cells and hence improve detection by ELISA. Flowers (1985) results also showed this effect with M-broth and serological detection, such as EIA, of *Salmonella*. Therefore, the present study results indicate that the *Salmonella* antigens that LOCATE assay detects are being promoted better in the 2MM9+4g glucose medium than in the other media tested.

In the present study results with the *Salmonella*-TEK™ ELISA kit assay contradict results of Van Poucke (1990) who detected 10^5 cfu *Salmonella* ml^{-1} sample. A cell concentration of approximately 10^5 cfu ml^{-1} was not detected with any of the media tested using the *Salmonella*-TEK™ ELISA kit assay in this study.

The results show that the minimum incubation period for a positive ELISA detection depends upon the non-selective enrichment medium used and the antibody detection kit employed. It should be pointed out that this study investigated detection of *Salmonella* in pure cultures alone without the complications of background microflora interference with assay specificity (St Claire and Klenk, 1990; D'Aoust *et al.*, 1990). Such complications would most likely increase the required *Salmonella* number before a positive detection can be obtained and selective enrichment should be considered (Flowers, 1985). The results presented in this study clearly indicate that the positive assay detection is almost always after 6 hours of incubation in the media

used under the reported assay conditions. As soon as the sensitivity of the assay is not

hindered by the media in any way, then cell concentrations above 10^6 cfu ml^{-1} were

generally sufficient to give a positive reaction with both *Salmonella* TEK™ and

TECRA assay kits but not with LOCATE.

Slow growth of *Salmonella* in an enrichment medium, such as the 2MM9+4g glucose

broth, is also expected to increase the incubation period required for a positive

detection. However, if the medium promotes production of the antigens detected by

the specific immunoassay then a slower growth rate may be counteracted.

Medium interference with ELISA assays is expected to affect detection whatever the

incubation period. This appeared to be the case with the Salmosyst medium and the

LOCATE assay. The LOCATE assay failed to detect levels of *S. typhimurium*

reaching 10^9 cfu ml^{-1} (Fig. 3.3). In addition, detection of *S. typhimurium* using the

Salmonella TEK™ assay after incubation in BPW was hindered and false positive

results (Fig. 3.1) were obtained even with no *Salmonella* in the control medium. This

suggested interference of the BPW medium with the *Salmonella* TEK™ assay

antibodies.

This study showed that the medium which resulted in no interference with any of the

ELISA assays tested and leads to a steady cell multiplication over time appears to be

the M-Broth. M-Broth promotes a similar growth of *S. typhimurium* to BPW and

Salmosyst media, and it does not interfere with the assays. Contradictory to the results of Flowers (1985) the results did not indicate that M-broth promote a better antigen production than the other media tested.

The importance of the pre-enrichment medium for a consequent *Salmonella* detection by ELISA assays is highlighted by the results of this study. The results have shown that: **(a)** media used for sample enrichment in the *Salmonella* detection may interfere with ELISA assays and lead to false positive or false negative results, and **(b)** compatibility of certain media with specific ELISA assays may be due to an enhanced promotion of antigen production and therefore may lead to a positive detection with a lower number of target Salmonella cells, thereby reducing the time period for sample enrichment.

CHAPTER 4

Studies on Salmonella Heat Injury and Media Recoverability

4.1   Introduction

4.1.1   Bacterial Viability

Foods have always contained injured micro-organisms, which may not be detected by standard environmental microbiological monitoring procedures. Sublethally injured cells may fully recover within the human host, and therefore present a health hazard if they are not adequately detected in food matrices (Smith *et al.*, 1994; Nychas and Tassou, 1996). Insufficient treatment of semi preserved foodstuffs increases the risk of injured pathogen contamination (Palumbo *et al.*, 1995). Whether or not this has previously presented major dangers to the consumer, or affected the shelf life of preserved food is unclear; but, given the potential lethality of *Salmonella* and the frequency of food preservation, it is unacceptable not to improve *Salmonella* testing to a point that effectively guarantees that food remains uncontaminated beyond all reasonable doubt. It is imperative that *Salmonella* detection in foodstuffs includes stages that allow resuscitation of sublethally injured *Salmonella* (Mossel and Ratto, 1970). Consequently, research into *Salmonella* contamination and bacterial injury is still actively pursued, and much of it is originated in food microbiology laboratories.

The interest in cell injury and differential nature of cell viability has arisen because of many of the early studies showed that viable cells often did not grow and multiply under normal conditions, and therefore eluded traditional culture detection. Postgate

(1967) defined viable bacteria as those capable of *'dividing to form one or more live daughter cells when provided with a favourable environment'*. However, this definition is based upon direct enumeration of distinct colonies on solid media. Since sublethally injured cells have been defined as those that are *'unable to multiply and form colonies on media that would otherwise normally support growth'* (Busta, 1976), there is something of a dichotomy for the microbiologist.

The dichotomy was summarised by Ray (1979), and lies in assessing the success of food treatments: bacteria become sublethally injured when exposed to adverse environmental or physical conditions like extremes of temperature, irradiation or chemical treatment. All these are used as methods of anti-microbial treatments in many food processing industries. Ray indicated that assessment was difficult because: (a) injured cells become temporarily sensitive to many chemical -selective- agents in media; (b) injured cells developed sensitivity due to cell membrane damage; (c) injury is reversible and can be repaired in a nutritionally rich non-selective medium; repaired cells regain their resistance to the selective compounds and their ability to multiply; (d) the presence of selective agent inhibit cell repair and/or multiplication; (e) resuscitation of injured cells in pre-enrichment media is required for subsequent detection and enumeration; and (f) the surviving population contains both uninjured and injured cells.

The mechanism of sublethal injury results in one or more alterations in normal cell structure and function, which can be either transient or permanent (Gilbert, 1984; Russell, 1984). The injuries bacterial cell accumulate include: increased permeability

of membranes; loss of essential intracellular nutrients for growth; damage and/or loss of nucleic acids; impaired protein production, and inactivation of cell repair mechanisms. Although injured cells often possess some metabolic activity and retain partial membrane function, it is very difficult to culture these cells under normal growth conditions.

The above principles, itemised by Ray, make characterisation of cell injury difficult. Injured cells are conspicuously sensitive to their external environment with respect to: temperature, osmotic potential, chemical concentration, change of diluent and the mechanical stresses of shear and vibration. These make handling of the sampled cell culture a problem. Thus, even the reproducibility of work carried out with stressed cells can be hindered when the same analytical protocol is followed.

4.1.2 Evaluating Injury and Cell Viability

4.1.2.1 Culture Methods

Evaluation of injury is frequently carried out by growing the stressed culture on two different media namely a non-selective plating medium, which would theoretically allow the colony formation of both normal and injured cells, and a medium that contains selective agents or chemicals that allows the growth of normal, but not injured cells (Ray et al., 1972; Gomez et al., 1976; D'Aoust, 1978; Mackey, 1984; Alexandrou et al., 1995). The difference of growth between the two media is presented as being the percentage injury of the culture (D'Aoust, 1978). More details

on cellular injury and cell viability status (than simple growth differences on selective and non-selective media) can be detected in liquid media by other techniques such as capacitance monitoring (Alexandrou *et al.*, 1995).

4.1.2.2 *Use of Fluorescent Stains for Cellular Viability*

The primary measurement of viability is the presence of reproductive cells that proliferate rapidly, and in the case of microbiology, can lead to the appearance of viable colonies of cells from a single mother cell (Nebe-von Caron and Badley, 1995). Traditional assessment of viability by colony counts only shows reproductive ability, whilst direct single-cell investigation using differential staining can distinguish cells of different viability states such as: (a) reproductive viable cells which grow under laboratory conditions, and can be detected using traditional plate culture methodology; (b) vital cells which possess metabolic activity or display cellular response to outside conditions; (c) intact cells demonstrate selective permeability or cell wall integrity; and (d) dead cells are particles with genetic information, but without an intact membrane, any signs of metabolic activity, or growth, they are freely exposed to environmental conditions and inevitably decompose.

Recently, methods have been developed that measure directly the viability status of cells by direct counting of viable cells using differential staining with specific fluorescent stains or fluorochromes, and the use of an epifluorescent UV microscope (Rodriquez *et al.*, 1992; Roszak and Colwell, 1987).

The technique of epifluorescent microscopy has been combined, in principal, with biochemical methods into a new to microbiology technique, flow cytometry which can analyse thousands of cells per second and has the potential to measure many parameters. With this recent introduction of flow cytometry in microbiology (Shapiro, 1990; Edwards *et al.*, 1994) in combination with a new generation of cellular stains it is apparent that a high proportion of viable cells are not culturable on solid media. This observation confirmed the existence of a 'viable non-culturable' group of cells, and provided evidence for the presence of different stages of cell injury. The latter is more obvious when employing multistaining flow cytometry with stressed cultures (Nebe von-Caron, personal communication). Flow cytometry offers the advantage of evaluating cell viability status by measuring metabolic and enzymatic activity, membrane integrity and polarisation, as well as other parameters that do not require cell proliferation.

4.1.2.3   Flow Cytometry

Flow cytometry is the technique by which cells are presented singly into an intense and focused light beam by injection into a pressurised stream of fluid, the sheath fluid. As the cells pass through the excitation beam light will be scattered and this can be detected by closely aligned photomultiplier tubes. If the cells are stained with fluorochromes and pass through the incident beam of correct excitation wavelength, each cell will emit fluorescence as it passes through the beam, the intensity of which can be quantified by fluorescence detectors. Most instruments have two light scatter

detectors. One measures the light scattered at the surface of the cell which will be a reflection of size, and is known as the narrow angle light scatter (NALS). The other measures light that is scattered after passage through the cells and is potentially a reflection of internal structure as reflectality, and is known as wide angle light scatter (WALS). The fluorescence intensity is converted into electric signals which can be displayed as event histograms that represent the range of light intensities as a number of channels (Edwards *et al.*, 1994).

The majority of dyes used by workers now fall into two groups: a) fluorescent dyes which accumulate intracellularly due to metabolic activity, and b) colourless dye conjugates which are cleaved to release a fluorescent molecule when taken up into the cell, and is only retained by live cells with intact membranes (Deere *et al.*, 1995).

Cytometry is a fast analytical system, allowing many cells to be enumerated in a short time. The advantage of counting more than 5000 cells in a few seconds reduces counting errors to below 1%, and reduces the time required by cultural procedures for proliferation (Nebe-von Caron and Badley, 1995). The percentage viability of a culture determined by flow cytometry shows good correlation with a standard cultured cell viability count. However, in natural environments direct counts of viable bacteria using epifluorescent microscopy or flow cytometry are reported to exceed the viable counts on media considerably (Roszak and Colwell, 1987; Diaper and Edwards, 1994). This indicates the presence of viable but non-culturable cells present in environmental samples due to cell stress and injury.

Differential cell viability can be revealed with great sensitivity using multistaining flow cytometry (Nebe-von Caron and Badley, 1995 and 1996). More than one fluorescent dye can be used to stain a single sample to deduce more information about the cells present. Multiparameter measurements were reviewed by Nebe-von Caron and Badley (1996).

The combination of redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) with the DNA-binding fluorochrome 4',6-diamidino-2-phenylindole (DAPI) allows the concurrent determination of both actively respiring or metabolically active cells and the total number of cells in environmental samples (Rodriguez *et al.*, 1992). Staining variations of a fraction of respiring bacteria can be seen using these dyes.

Direct single-cell measurement by the rapid flow cytometry is superior to viability measurements based on selective growth. Fluorometric techniques are now so versatile that cells can be followed through their recovery with characteristically different clusters of events. Kaprelyants and Kell (1992) showed flow cytometry was capable of the distinction of several physiological states of bacteria into 'viable', 'non-viable' and 'non-viable but resuscitable' cell types.

'Dormant' or 'viable non-culturable' bacterial cells can be successfully studied and characterised according to their respiratory activity or membrane potential and integrity using flow cytometric measurements (Kaprelyants and Kell, 1993; Roszak

and Colwell, 1987). These cells are morphologically intact but with undetectable metabolic activity as measured by respiration and membrane potential measurements were concerned (Kaprelyants and Kell, 1993). Actively growing cells have been shown to exist in populations of starved 'dormant' cells which present potential problems in resuscitation. Flow cytometry is a powerful tool for microbiologists, allowing the observation of real time resuscitation of the dormant cells increasing in size and regaining metabolic activity and culturability.

Direct counting of viable cells can also be carried out by differential staining of cells with a particular fluorescent stain or fluorochrome under an epifluorescent microscope (Roszak and Colwell, 1987; Rodriguez et al., 1992).

4.1.2.4   Membrane Integrity Measurement

The measurement of membrane integrity is a sensitive way of determining cell viability. Dyes with intracellular binding sites which are normally impermeable to an intact cell membrane are used as vital stains, to exploit dye retention or exclusion characteristics (Nebe-von Caron and Badley, 1996). Some dyes show disadvantages through quenching intracellularly by pH changes or superconcentration, or are actively removed by membrane pumps, but generally their cellular retention or exclusion is proportional to the cell viability.

Demonstration of selective permeability based on dye retention or dye exclusion measurements is an important feature of cells in a 'compromised' state. Since

membrane bilayers prevent the uncontrolled passage of intracellular dyes, especially large molecules and hydrophilic dyes, their penetration indicates cell damage and at the extreme, cell death. Ethidium bromide (EB) produces an orange fluorescence, and propidium iodide (PI) a red fluorescence, and are both nucleic acid stains that differ in their permeability through the cell membrane. Intact membranes do not allow nucleic acid or DNA staining with PI whereas EB penetrates and stains intact bacteria with time, unless pumped out of the cell (Davies, 1991; Nebe-von Caron and Badley, 1995). Therefore, when both stains are used together to stain a mixture of exponentially growing cells and heat fixed cells the different populations are differentiated by dye exclusion.

PI can be used in combination with other vital probes phycoerythrin (PE) and fluorescein (FITC) to distinguish intact cells from dead cells, which stain positively with PE and FITC. (Sasaki *et al.*, 1987). Interference of flow cytometric measurements by non-nucleated particles is eliminated by the use of EB which differentiates stained cells from depris and micelles (Nebe-von Caron and Badley, 1996).

4.1.2.5   *Metabolic Activity and Membrane Potential Measurements*

Metabolic activity is an other determinant of cell viability, that can be detected by measurements of membrane potential, intracellular pH and enzyme activities (Shapiro,

1988; Diaper *et al.*, 1992; Yen, 1992; Nebe-von Caron and Badley, 1996). Membrane potential is probably the most explored measure of metabolic activity.

Membrane potential measurements reflect active and passive transport mechanisms across the membrane resulting in an electrochemical gradient. For this, the distribution of anionic or cationic dyes, which are lipophilic and charged, freely penetrate and distribute inside the cells according to the transmembrane potential. Depolarisation of the cell membrane causes the release of the indicator dye from the cell into the medium, whereas hyper-polarisation causes additional take up of dye from the medium inside the cell. When inside the cells, the dyes may bind to lipid compounds therefore allowing more molecules to accumulate without being discharged by the cell (Shapiro, 1988; Nebe-von Caron and Badley, 1995).

The membrane potential dependent staining can be lost with high concentrations of lipophilic dyes. These distributional dyes, or 'probes' as they are termed, are slow in their response time but have the advantage of high signal intensity which is perfect for flow cytometric analysis of bacterial cells (Nebe-von Caron and Badley, 1996). Most membrane potential dyes have a high photodynamic toxicity (become highly toxic under light) to the cell. Therefore care must be taken to avoid light and heat damage to the stained cells which can be caused by prolonged illumination of the sample (Shapiro, 1988). Photodynamic damage is also applicable to the dye itself.

Typical charged lipophilic dyes are: carbocyanines ($DiOC_5$), rhodamine 123 or oxonols, safranins, acridines, pyronines and other classes of dyes. Depending on the charge (anionic [$DiBAC_4$ or otherwise known as BOX]; cationic [rh 123]) the dyes accumulate in depolarised or polarised cells (respectively) according to the Nernst equation (Shapiro, 1988).

Oxonols, which are negatively charged, bind the cell membrane but do not accumulate inside the cell; this may be the main reason why oxonols are much less toxic than other membrane potential dyes. The highly lipophilic character of oxonols causes them to stick to everything inside the injured cells which leads to bright staining of damaged cells (Shapiro, 1988). Unlike dyes like rhodamine 123 (rh 123) the oxonol dyes are lipophilic anions. Their accumulation in bacterial cells are favoured by the reduction of membrane potential and are therefore expected to concentrate inside dead bacteria. The discriminatory ability, between live and dead cells, and the sensitivity of the lipophilic anionic BOX ($diBA-C_4(3)^-$) is confirmed by Deere et al. (1995). The authors followed the viability of injured bacteria (treated by heat or an antibiotic over a period of time) using both BOX and viable counts on solid media. An appreciable delay was observed following loss of culturability before the membrane potential was dissipated to levels where the cells stained BOX positive (green fluorescence). When BOX is used to stain heat-fixed depolarised and permeabilised cells simultaneously with PI, the cells fluoresce intensively in the green and red region, unlike the normal cells which remain unstained (Nebe-von Caron and Badley, 1996).

4.1.3 Resuscitation of Injury

Repair or resuscitation treatment in non-selective liquid media can be used to recover stressed or damaged cells, and they can then be detected using standard procedures.

Sometimes it is impossible to resuscitate injured cells in non-selective media even after prolonged incubation and the addition of chemical additives (van Schothorst and van Leusden, 1972; Gomez *et al.*, 1973; Gomez and Sinskey, 1975; Mossel *et al.*, 1980). In certain circumstances, a medium may offer an advantage over another in terms of cellular resuscitation, even without the addition of essential additives (Clark and Ordal, 1969; Wilson and Davies, 1976; Park *et al.*, 1977).

Additives that enhance cell resuscitation, and hence recovery, include catalase and pyruvate, both of which deactivate the toxic effects of H_2O_2 that accumulates in or around the cells (Martin *et al.*, 1976; Rodrigues and Kroll, 1989; Leyer and Johnson 1992). Inorganic phosphate (with and without Mg^{2+}), citrate (in the presence $MgSO_4$), and complex organic nutrients (such as peptone), also support repair of injured cells (Borst and Slater, 1961; Mahler and Cordes 1966; van Schothorst and van Leusden, 1972).

Studying the resuscitation of injured cells in different media is important for identifying cell requirements and optimising conditions for growth. It is, however, important to be able to discriminate between true resuscitation of injured cells and multiplication of non-injured (normal) cells. Many researchers agree that studies on resuscitation make more sense when severely stressed populations are examined (van Schothorst and van Leusden, 1975; Mossel and Corry, 1977; Mossel *et al.*, 1980).

The results, of such studies, are more indicative of true repair of injured cells. Many reports point out that in a surviving population of injured cells, which differ in their site and degree of damage, uninjured cells also exist (Ray, 1979; Mossel *et al.*, 1980; Kaprelyants and Kell, 1993). These uninjured cells may start multiplying long before the injured cells recover and start dividing under resuscitation conditions, therefore giving a false count of recovered cells.

The assessment of bacterial detection procedures would be more accurate and could be standardised if certified reference materials (CRF) were used, including a set number of cells of the same viability status. Single cell inoculations using fluorescently activated cell sorting (FACS) can accurately assess media recoverability. This technique is advantageous because the viability status of each inoculated cell is known, therefore a more uniform assessment of recoverability can be obtained.

4.1.4 Certified Reference Materials

Despite carefully described standardised methods such as ISO procedures for isolation of *Salmonella* from foodstuffs, success of *Salmonella* detection varies between laboratories (Van Leusden *et al.*, 1982). Standard methods can be influenced by 'human factors' such as skill, dexterity, as well as by laboratory-associated factors, such as the preparation and storage of media and temperature control.

Homogeneous and biologically stable certified reference material (CRM capsules) have been developed by RVM (Netherlands) and can be used as positive controls to evaluate the performance of standard methods of bacterial detection/identification. Certified reference material consists of spray-dried milk contaminated with a known number of *Salmonella typhimurium* or other strains and contained in gelatine capsules. During spray-drying *S. typhimurium* cells are sub-lethally injured, reflecting a more realistic or natural *Salmonella* contamination of processed food (Beckers *et al.*, 1985). However, the exact nature of the cellular injury is unknown and therefore spray-dried cells may be unrepresentative of injured cells in foodstuffs.

4.2   *Aim*

The aim of the studies in this Chapter was to investigate the effect of 54°C heat treatment of *Salmonella* using three different methods for the determination of cell viability. Each method was an indirect measure of viability, and were: a) a standard culture technique, b) a flow cytometry procedure using three fluorescent stains, and c) the *BA*CLIGHT kit of vital staining coupled to a flow cytometry and fluorescent microscopy measurement of viability. The procedures are defined as 'indirect' since none of them ensures that the cells can be simultaneously cultured under normal conditions and possess a functional membrane.

The aim of these studies was to compare the sensitivity and reproducibility of each procedure in evaluating cellular viability and injury, and to assess the efficacy of different media to recover severely injured *Salmonella*.

4.3   Materials and Methods

All glassware used for fluorescence microscopy and flow cytometry experiments were washed in cell free water and dried for a minimum of 2 hours in a 150°C oven. All the media and distilled water used for these experiments were filter sterilised in order to eliminate the possible presence of any dead cells or fluorescent particulates.

4.3.1   Obtaining a Standard Growth Curve

20ml of TSB medium in universal bottles was inoculated with about 100 cells of *Salmonella typhimurium* and incubated at 37° C in a static incubator for 36 hours. Samples were taken at different time intervals and spread plated onto TSA to obtain total viable counts after 24 hours at 37° C. The number of colony forming units per millilitre (cfu ml^{-1}) was plotted against time in hours in order to determine the growth phase of the specific strain at certain incubation periods.

4.3.2   Heat Treatment of Salmonella Cultures

Protocol 1:

1. 10 ml of pre-warmed (at 37°C) TSB medium was inoculated with a coated bead of *S. typhimurium* from the stock culture stored at -70° C. The medium was incubated at 37°C in a static incubator for 24 hr or until a concentration of approximately 10^{9} cfu ml^{-1} was reached.

2. A total viable count was carried out by means of serial decimal dilutions in sterile 9ml TSB in universal bottles and spread plated 100 µl onto 3 x TSA and 3 x XLD plates.

3. The remainder of the 9ml TSB culture was immersed for 60 minutes in a water bath at 54°C and sampled every 10 minutes for viability. Samples were taken without removing the culture from the water bath.

4. Samples were cooled in ice water and stored on ice, for use within two hours.

5. In addition, 1ml samples of the heat treated TSB culture were taken every 10 min and were diluted in filter sterilised TSB and plated on 3 x TSA and 3 x XLD plates.

The percentage injury of the particular strain was calculated using the formula:

$$\% \text{ injury} = \frac{(\text{CFU of treated cells on TSA} - \text{CFU of treated cells on XLD}) \times 100}{\text{CFU of treated cells on TSA}}$$

The percentage survival of the treated cultures was calculated using the formula:

$$\% \text{ survival} = \frac{(\text{CFU of treated cells on TSA / XLD at } t_x) \times 100}{\text{CFU of treated cells on TSA / XLD at } t_0}$$

Protocol 2:

All the stages are identical to the Protocol 1 with the exception of:

3. The 10^{-5} dilution of the culture containing about 10^4 cells ml^{-1} was immersed for 60 or 180 minutes in a water bath at 54° C and sampled every 10 minutes for the first hour and after that at 90, 120 and 180 minutes of heat treatment.

Results were plotted as cfu on TSA and XLD after 24h-incubation, against sampling time to follow the survival of the treated organism.

4.3.3 Staining Salmonella with the BACLIGHT™ Bacteria Viability Kit and Fluorescence Microscopy

The *BAC*LIGHT Viability kit (L-7007; Molecular Probes Europe BV) provides a two-colour fluorescence assay of bacterial viability. It contains a mixture of nucleic acid stains that differ in their spectral characteristics and in their ability to penetrate healthy bacterial cell membranes: the SYTO® 9 (Reagent A) green fluorescent stain and the Propidium Iodide (PI; Reagent B) red fluorescent stain. When used alone SYTO® 9 labels all bacteria in a population, with intact and damaged membranes. In contrast PI labels bacteria with damaged membranes which stain red. Therefore when both stains are used together in an appropriate mixture, bacteria with intact cell membranes stain fluorescent green while bacteria with damaged membranes stain fluorescent red. Under certain conditions injured bacteria which may be able to recover and reproduce may stain variably.

20 ml filter sterilised TSB broth was inoculated with a *Salmonella typhimurium* coated bead from stock culture and was incubated overnight at 37° C. The appropriate dilution, either 10^{-5} dilution containing about 10^4 cfu ml^{-1} in sterile cell free TSB, or concentrated 12h-old culture was used for the heat treatment.

The culture in the universal bottle was immersed in a water bath at 54° C for 60 minutes. Samples of the heat treated culture were taken every 10 minutes and the percentage viability of the culture was determined for each sampling period (i.e. t_0, t_{10}, t_{20}, t_{30}, t_{40}, t_{50} and t_{60}) by fluorescence microscopy.

4.3.4 Sample Preparation for Fluorescence Microscopy

50 µl of the heat treated bacterial culture were added to 1 ml of sterile, 0.2 µm sterile-filtered water in a microfuge tube. The cells were concentrated by centrifugation for 5 minutes in a micro-centrifuge at 10,000 g and the supernatant was discarded. The cell pellet was resuspended in 1 ml of sterile, 0.2 µm sterile-filtered distilled water. This wash step was repeated twice more to ensure no medium carryover during staining, which interferes with the staining ability of BACLIGHT.

Equal volumes of Reagent A and Reagent B were combined in a microfuge tube, mixed thoroughly and 3 µl ml^{-1} of this solution was added to the bacterial suspension. The solution was mixed thoroughly and incubated at room temperature in the dark for 15 minutes.

1ml of the stained cell suspension was then filtered using 0.2 µm polycarbonate filters; Molecular Probes Europe (MPE BV) recommend black polycarbonate, 0.2 µm, for membrane filtration of the sample. The filtration apparatus was rinsed with 100ml filter-sterile water while the vacuum pump was on. A pair of forceps was used to remove the filter onto a piece of tissue to dry. One drop of non-fluorescent immersion

oil was added onto the centre of a microscope slide and the filter was placed onto the oil drop. Another drop of oil was placed onto the centre of the membrane, with a cover slip pressed firmly to remove any trapped air and to reduce the thickness of the oil layer.

The prepared slide was positioned under the fluorescent microscope equipped with standard fluorescein longpass filter(s) set O-5717 and the red/orange and green cells in each field of view were counted. 20 x fields of view were counted per sample and the mean of red/orange and green cells per sample was calculated. The distribution of the fields was random and selected from the entire area of the filter.

The number of red/orange and green cells counted separately, expressed as a percentage of the mean of the total number of cells counted under each of the 20 x fields of view, determines the percentage of dead and live cells in the cell suspension for each time period: t_0, t_{10}, t_{20}, t_{30}, t_{40}, t_{50} and t_{60}. This is given as the number of green 'live' cells as a percentage of green and red/orange cells x 100%.

Finally, the comparison was made between the *BAC*LIGHT percentage viability and the equivalent percentage viability obtained from the cultural experiments onto TSA and XLD.

4.3.5 Staining with PI, EB, BOX and Flow Cytometry

Stock solutions of ethidium bromide (EB) and propidium iodide (PI) were both prepared at 1 mg ml^{-1} in sterile distilled water and were kept refrigerated at 4°C. Bis-oxonol (BOX) stock solution was 100 µg ml^{-1} in solution of 0.3% Tween-20 in 1% dimethyl sulphoxide (DMSO). All stains were filter sterilised in order to exclude particulate interference with flow cytometry. 200µl of a 12h-old *S. typhimurium* culture were diluted 1:10 in filter-sterilised BPW (OXOID) and then placed in an ultrasonic bath for 10 seconds. Bacteria counts using flow cytometry were found to be most effective when a single-cell suspension was obtained by a sonication step in the "hot-spot" of an ultrasonic waterbath (Nebe-von Caron and Badley, 1995). This helps to disaggregate the cells without causing any considerable side effect such as cell damage (Nebe-von Caron and Badley, 1996).

The diluted cells in BPW were stained by adding 2 µl of each BOX, EB, PI (BEP), mixed well using a vortex and incubated at room temperature 25°C in the dark for a minimum of 10 minutes. The flow cytometric measurements were made using a Coulter EPICS Elite flow cytometer equipped with an Autoclone, an air-cooled argon ion laser at 488 nm at 15 mW and a 76 µm sort-sense flow tip. The optical filter setting used (Fig. 4.0) was as indicated by Nebe-von Caron and Badley (1995). The instrument set-up and method validation was carried out by Nebe-von Caron (Unilever Research, Colworth Laboratory, Shamprook, Bedfordshire). The parameters measured with flow cytemtry include: narrow angle light scatter (NALS) reflecting cell size; wide angle light scatter (WALS) reflecting of internal structure of cells; red,

orange and green fluorescence. Cells were gated on the basis of light scatter and their position in orange versus green fluorescence. Data analysis was performed using WinMDI software which allowed colour gating.

A detailed account on molecular and other staining, buffers, media as well as flow cytometry is reviewed by Nebe-von Caron and Badley (1995). The absence of nucleic acids from interfering particles proved very effective in targeting bacteria with the use of the three stains (PEB). EB is used to differentiate stained cells from interfering depris and micelles found in a solution. PI is used to measure membrane integrity, after sonication. (Nebe-von Caron and Badley, 1996).
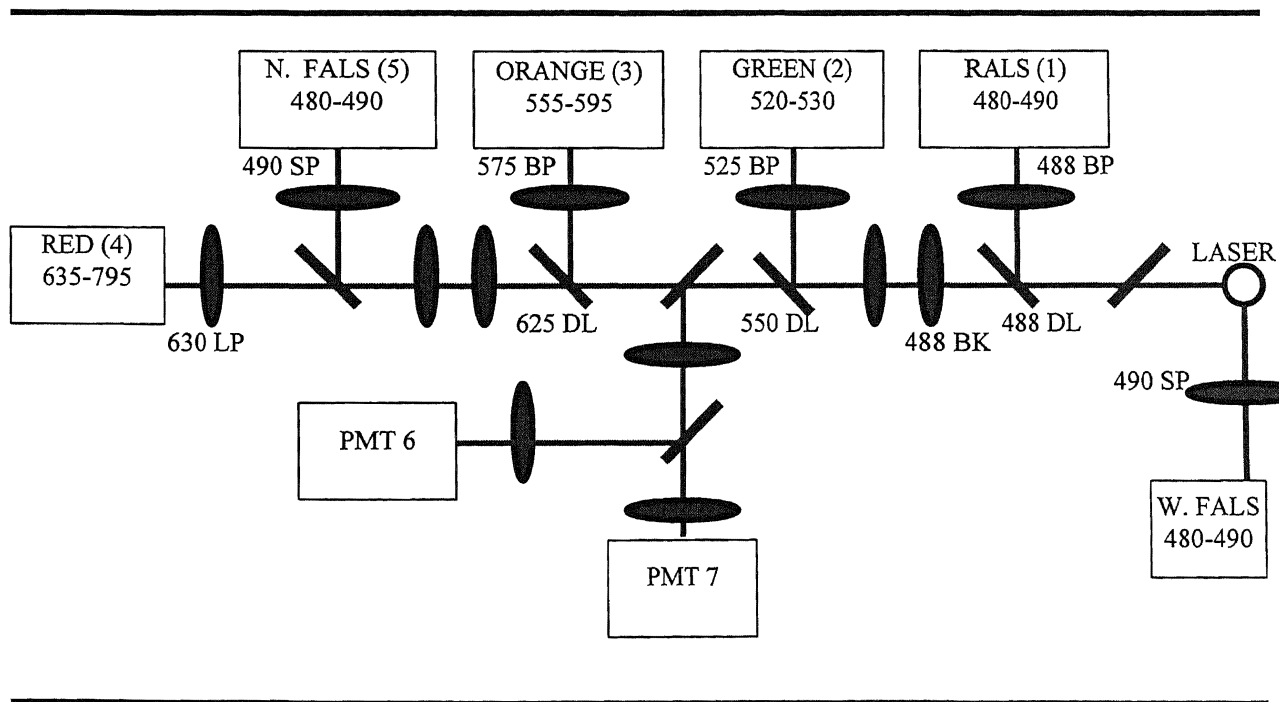
Fig. 4.0: Optical filter arrangement of the Coulter EPICS Elite®. BP= band pass, LP= long pass, SP= short pass, DL= dichroic long pass, BK= blocker, FALS= forward angle light scatter (Narrow, Wide), RALS= right angle light scatter. The N. FALS signal in detector is fed via a fibre optic and picked up on the optical bench. Diagram reproduced as per Nebe-von Caron and Badley (1995).

4.3.6 Fluorescence Activated Cell Sorting (FACS) of Cell Clusters

A 12h-old *S. typhimurium* culture was heat treated using Protocol 1 for 60 minutes at 54° C, and stained using the BEP and *BAC*LIGHT viability stains. Single cell sorting of differentially stained cells in 200μl liquid media in 96-well microtitre plates was obtained using a Coulter Autoclone unit attached to a Coulter Epics Elite flow cytometer. Five microtitre plates per culture medium were each inoculated with cells extracted from the different clusters of both BEP and BACLIGHT stained heat treated cultures.

Single cells from cell clusters were gated and sorted on the basis of their light scatter and their cluster position in orange versus green, or red versus green fluorescence. The clusters were classified as: (1) actively pumping cells: not stained; (2) de-energised cells: intact but non-pumping; stained only with EB; (3) depolarised cells; positively stained with BOX and EB; (4) permeabilised cells: positively stained with PI and BOX; and (5) ghosts or non-nucleated cells: only stained by BOX.

*BAC*LIGHT stained cells were differentiated in three clusters: (1) viable cells: green cluster; (2) dead or permeabilised cells: red and green cluster; and (3) intermediate / transitional cells: orange cells, were sorted into three liquid media. Recovery was assessed by an increase in the absorbance (A) of the media during incubation at 37°C, and measured using a spectrophotometer at 620 nm (A_{620}).

Single cell sorting was carried out in: a) BPW, b) L-broth and c) 2MM9+4g glucose chemically defined medium, and the cells were incubated at 37° C for up to 108 h. The recovery and growth of the cells was followed with A_{620} readings being taken at different time periods after inoculation: 12h, 15h, 18h, 21h, 24h, 36h, 39h, 42h, 48h, and 108h.

Cells being capable of recovery were assessed as those reaching an optical density over the threshold A value which was calculated using the formula:

$$\text{Threshold } A_{620} = \text{Control Mean } A_{620} + (3 \times \text{Standard Deviations})$$

where:

Control Mean A_{620} = mean A_{620} reading of uninoculated media at t_0
Standard Deviation = standard of the control A_{620} measurements at t_0

4.3.7 Recovery of Heat Treated S. typhimurium

To evaluate recovery of injured cells, a 3h heat treated culture of *S. typhimurium* from a 12h-old culture, was inoculated into the six different liquid media: BPW (OXOID CM509); 2SPW (OXOID CM9) with added supplement as with 2MM9 medium; TSB (OXOID CM129); Lactose Broth (OXOID CM137); M-broth (DIFCO Bacto M Broth); and 2MM9 + 4g glucose (as described in 2.3).

The heat treatment was performed with Protocol 1, with the difference that: for the period immediately after three hours of heat treatment the culture was diluted in TSB, to obtain a solution containing minimum culturable cells. The final cell concentration of culturable *Salmonella* on TSA plates was 0.09 cfu ml^{-1} on TSA plates.

200µl aliquots of each of the sic media, in duplicate 96-microwell plates, were inoculated with 25µl of the injured-cell dilution, equating to $P < 0.00225$ chance for inoculation with a viable culturable cell. The plates were then inoculated at 37° C for 24h and A_{620} readings were taken after 10h, 18h, and 24h of incubation. Cells capable of recovery were assessed as all wells reaching an A_{620} over the threshold absorbance value: mean A_{620} + (3 x Standard Deviation) at t_0, after 18 h incubation.

4.4 Results

In this chapter are described a series of studies using three indirect systems of measurement of survival/viability of *S. typhimurium* after sublethal heat treatment at 54°C. These systems were: rich and selective agar culture, fluorescence microscopy of *BAC*LIGHT stained heat-treated cells, and flow cytometry of heat-treated cells stained with either *BAC*LIGHT or triple stained with BEP.

For the flow cytometry studies, the three vital fluorescent stains used - Bisoxonol (BOX), Ethidium Bromide (EB) and Propidium Iodide (PI) - represent a cell membrane function. The stains generally represent the membrane integrity in terms of a functional permeability barrier. This barrier selectively excludes stains such as EB, PI and BOX when it is in a fully functional condition but fails to do so when injury alters transmembrane potential and energy level, and changes the permeability of the cell membrane. Detecting the presence of the vital stains inside the cells using flow cytometry shows cell damage.

Although the vital stains do not directly represent the capability of the cells to grow and survive, the results of the triple-staining do correlate with the results of *BAC*LIGHT viability stains.

However, neither vital triple-staining nor *BAC*LIGHT staining correlate well with agar culture studies. The culture studies were expected to show lower viability than the vital

staining with either flow cytometry or fluorescence microscopy. The results substantiate this expectation.

4.4.1 Survival of heat-treated S. typhimurium on TSA and XLD Agar

The objective of this study was to determine the heat sensitivity of *S. typhimurium* at different stages of growth. The survival of *S. typhimurium* using protocol 1 of heat treatment, was investigated. The results are presented in Figures 4.2 and 4.2a, Figures 4.3 and 4.3a, and Figures 4.4 and 4.4a.

Salmonella typhimurium at different growth phases were used in this study investigating survival of heat treaded cultures on both TSA and XLD media. The cultures used were 6h, 12h and 24h-old grown in TSB which corresponded to the middle of the exponential phase, the start of stationary phase and approximately 12 hours in the stationary phase, respectively (Appendix - Fig. 4.1).

Fig. 4.2 : Survival curves of *S. typhimurium* (6h-old undiluted culture) heat treated at 54°C and plated on triplicate TSA and XLD agar.

Fig. 4.2a : Survival curves of *S. typhimurium* (6h-old undiluted culture) heat treated at 54° C and plated on triplicate TSA and XLD agar.

Fig. 4.3 : Survival curves of *S. typhimurium* (12 h-old undiluted culture) heat treated at 54°C and plated on triplicate TSA and XLD agar.
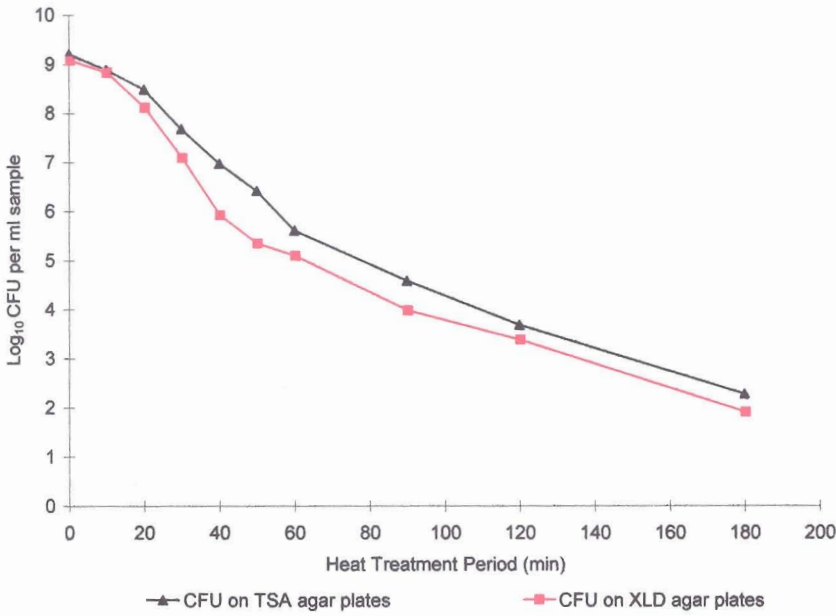
Fig. 4.3a : Survival curves of *S. typhimurium* (12 h-old undiluted culture) heat treated at 54°C and plated on triplicate TSA and XLD agar.
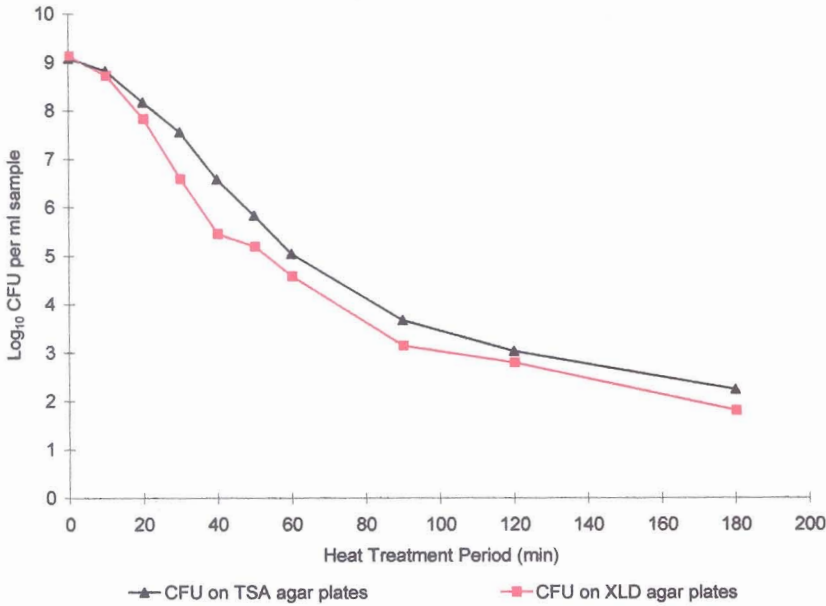
Fig. 4.4 : Survival curves of *S. typhimurium* (24h-old undiluted culture) heat treated at 54°C and plated on triplicate TSA and XLD agar.
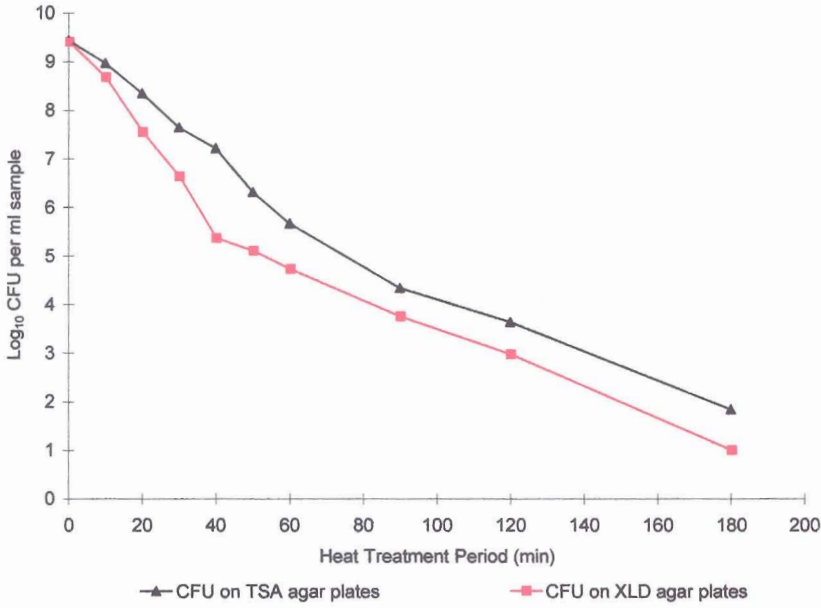
Fig. 4.4a : Survival curves of *S. typhimurium* (24h-old undiluted culture) heat treated at 54°C and plated on triplicate TSA and XLD agar.
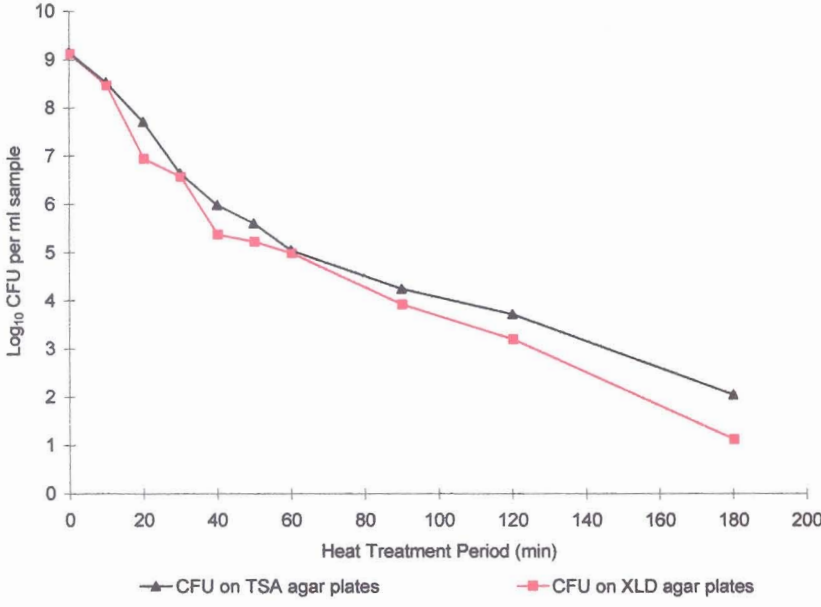
The percentage injury is the difference between the two lines (growth on TSA and XLD) which typically increases with time of treatment reaching maximum levels between 30 to 60 minutes. It appears that the survival of the cells on the two media decreases concomitantly. The results also indicate that 6h-old cells are inherently the most sensitive cells to the selective agents in XLD resulting in as much as 60% failure to form colonies on XLD before the heat treatment began (Appendix, Table 4.1). The results presented here are typical of those collected from our study.

In order to further examine the sensitivity of the different aged cultures they were diluted down to a starting population of approximately 10^4 cfu ml^{-1} (using protocol 2). This was carried out in order to avoid the long decimal serial dilutions carried out before the treated culture was spread plated onto TSA and XLD. Avoiding many of the serial decimal dilutions may decrease potential diluent stress on the already injured cells. After 40 minutes of heat treatment 200 µl rather than 100 µl of the cultures were spread plated to double the chances of obtaining colony forming units on either of the two media. Results are presented in Figures 4.5, 4.6 and 4.7.

The figures show clearly that below a certain volume of inoculation the survival of the cells falls more rapidly than the survival of the undiluted concentrated cultures. The percentage injury and survival of the concentrated and pre-diluted cultures are presented in Table 4.1 and 4.2 (Appendix).

Fig. 4.5 : Survival curves of *S. typhimurium* (pre-diluted 6h-old culture) heat-treated at 54° C and plated on triplicate TSA and XLD agar.
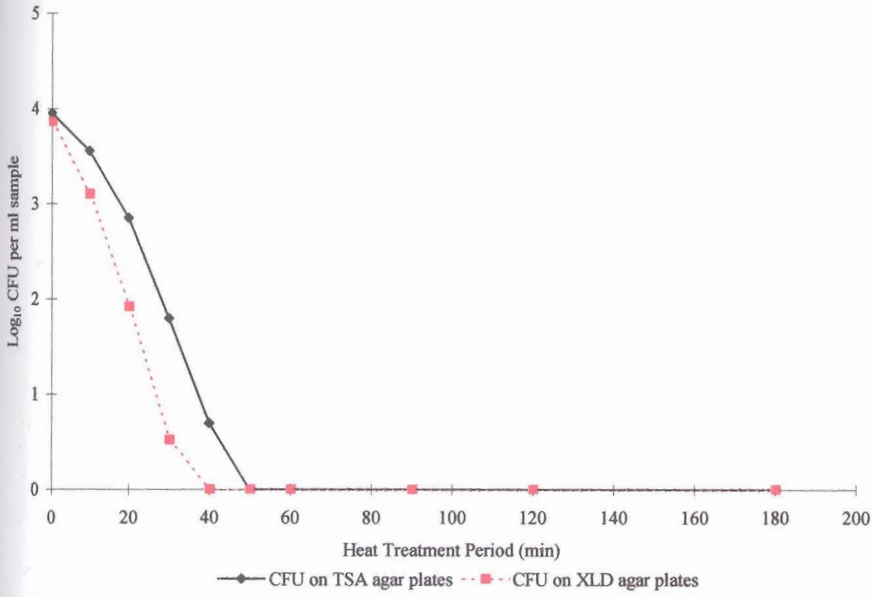
Fig. 4.6 : Survival curves of *S. typhimurium* (pre-diluted 12h-old culture) heat-treated at 54° C and plated on triplicate TSA and XLD agar.
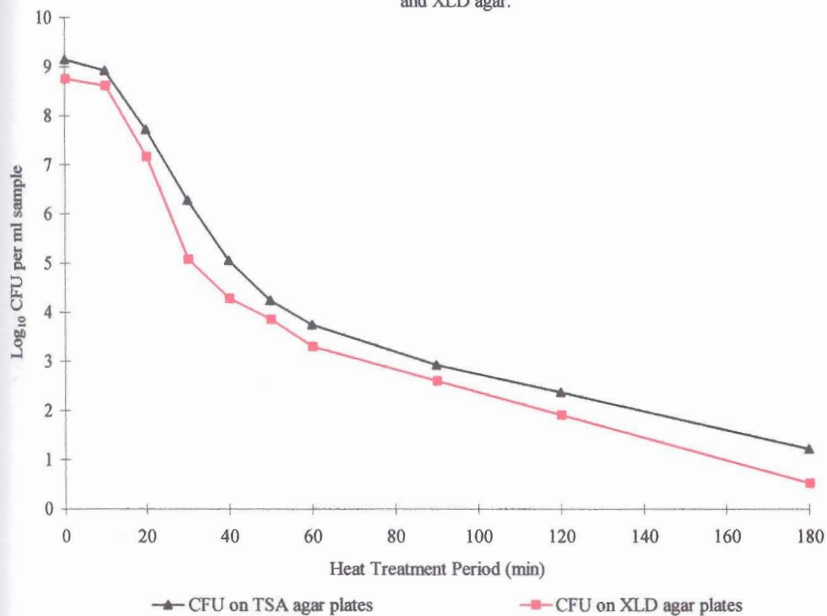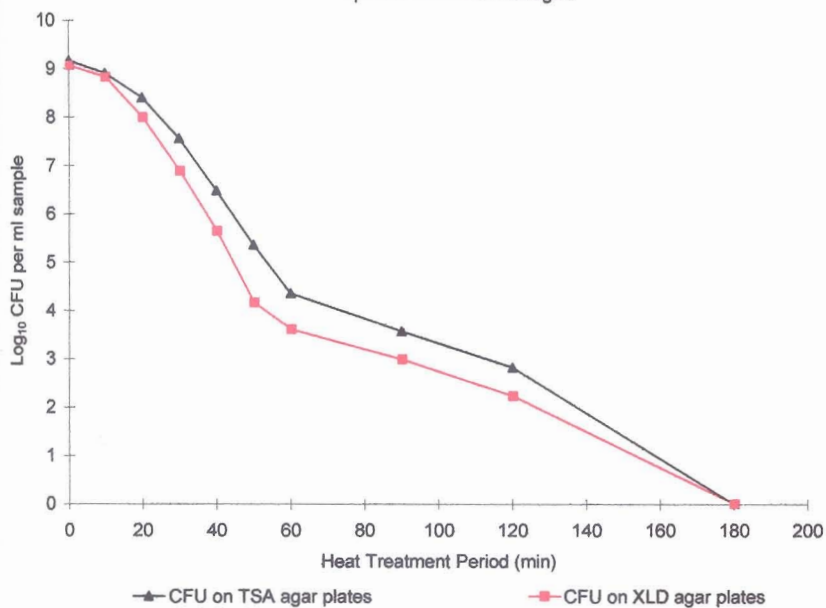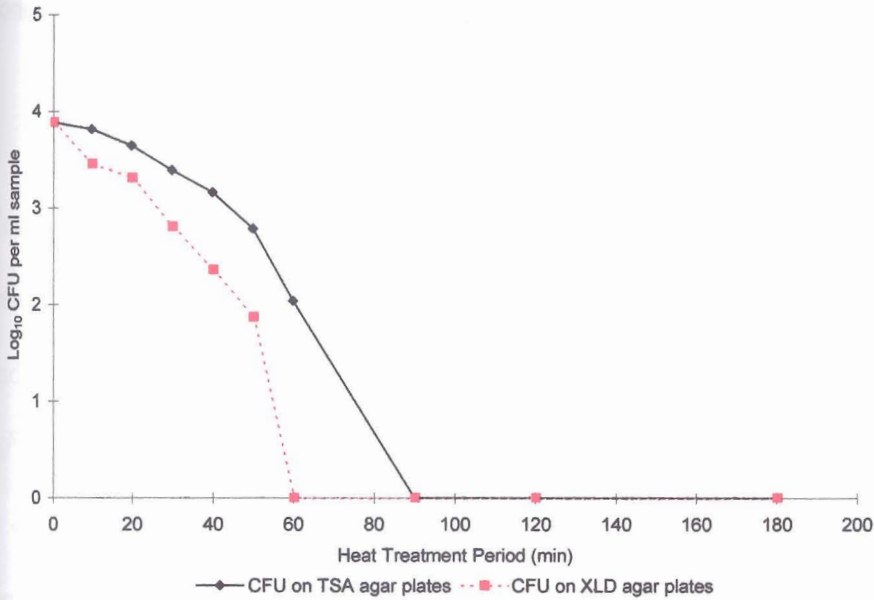
Fig. 4.7 : Survival curves of *S. typhimurium* (pre-diluted 24h-old culture) heat-treated at 54° C and plated on triplicate TSA and XLD agar.
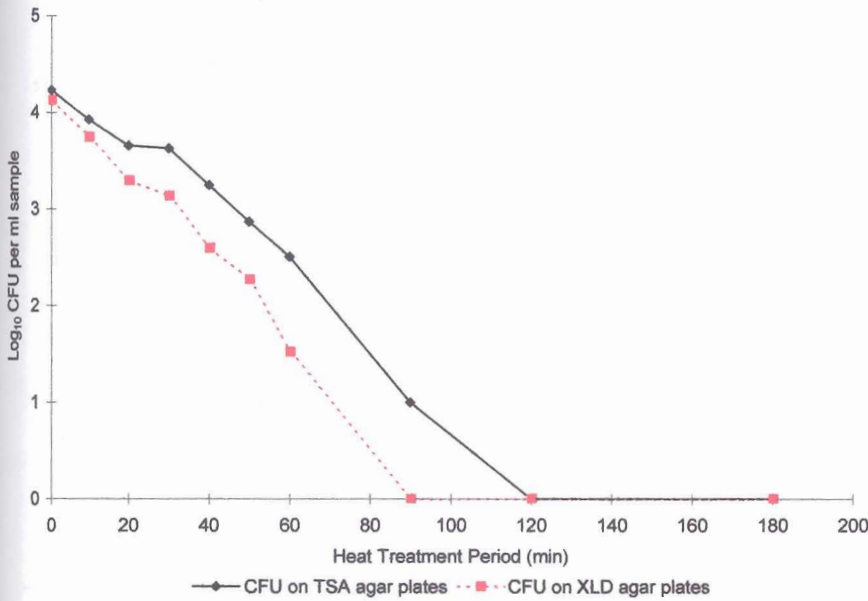
The percentage injury of each of the cell growth stages with diluted and undiluted concentrated cultures are presented in Figures 4.8 and 4.9.

Concentrated cultures shown in Fig. 4.8 are generally much less sensitive to heat treatment than the diluted cultures shown in Fig. 4.9 at all stages of growth (i.e. 6h-, 12h-, and 24h-old cells). With the initial cell concentration used in Protocol 1 which was about 10^9 cfu ml^{-1}, the results show considerable variation and a pattern of heat sensitivity that is only partially correlated to the length of heat treatment. However, there is a clear tendency for older cells to be more resistant to heat treatment than early-phase cells.

Fig. 4.8 : Percentage Injury of heat-treated *S. typhimurium* (6, 12, and 24-h old, undiluted cultures) as evaluated by cultural methodology over three hours. Duplicate experiments are presented for each culture age.

Exp.2 / 6h   Exp. 1 / 6h   Exp. 1 / 12h   Exp.2 / 12h   Exp.1 / 24h   Exp. 2 / 24h
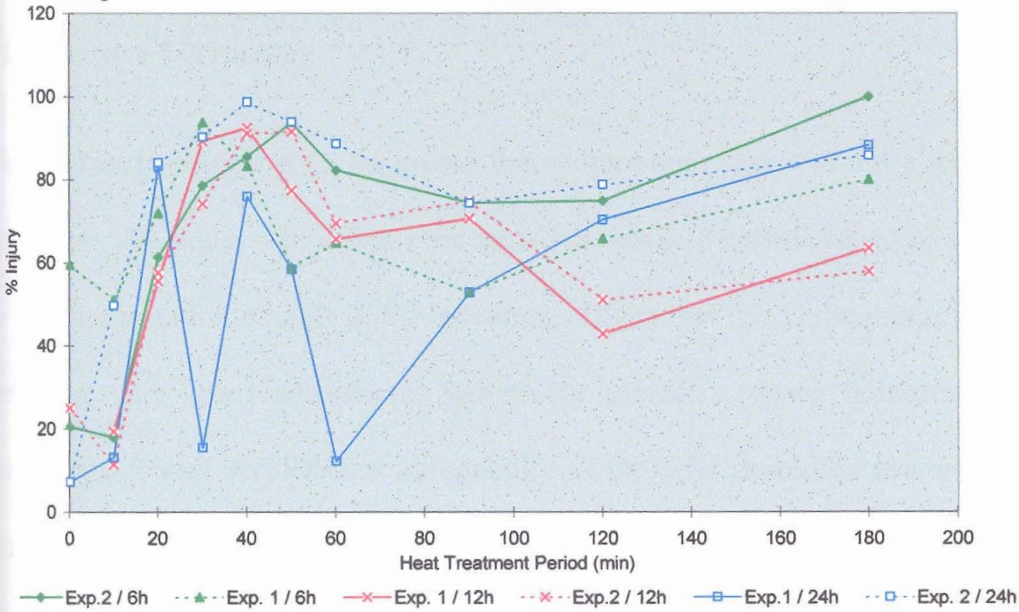
Fig. 4.9 : Percentage Injury of heat-treated *S. typhimurium* (6, 12, and 24h-old, diluted cultures) as evaluated by cultural methodology over three hours. Duplicate experiments are presented for each culture age.

Exp. 1 /6h   Exp. 2 / 6h   Exp. 1 / 12h   Exp. 2 / 12h   Exp. 1 / 24h   Exp. 2 / 24h
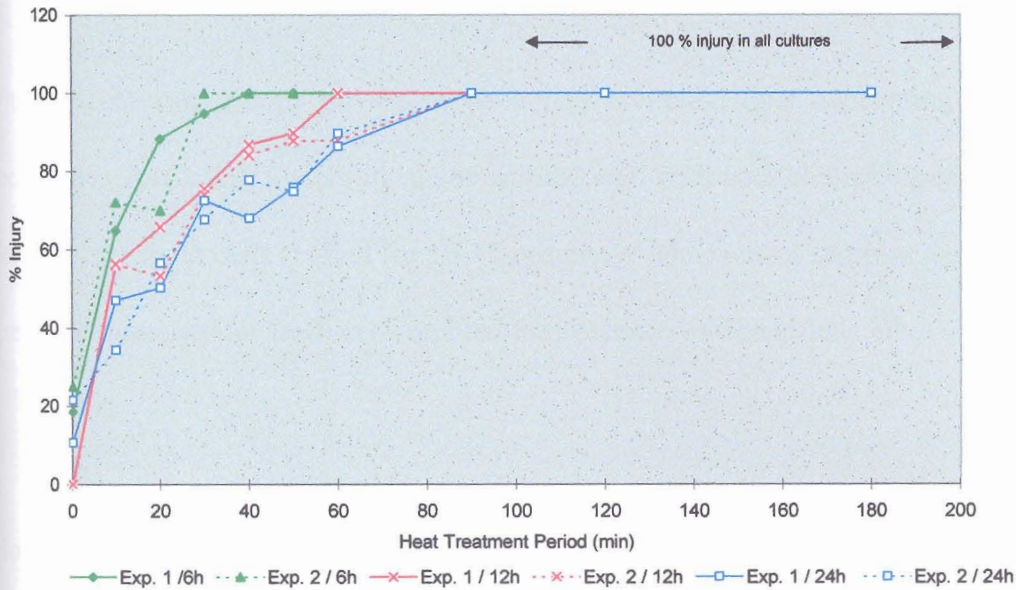
4.4.2 Survival of S. typhimurium Assessed by BACLIGHT Staining with Fluorescence Microscopy

The studies in section *4.4.1* suggested that culture onto agar was not a reproducible or consistent technique. The differences shown in culture detection/recovery were sensitive to the concentration of cells in the inoculum. Given that this is the primary difficulty in detecting very low numbers of *Salmonella* against a dense background of other microflora, it was decided that the viability of the cells should be evaluated using the fluorescent dyes supplied as part of the *BAC*LIGHT *kit* viability dyes; to perhaps use the *BAC*LIGHT system to discriminate between the differential survival of cells from different phases of growth.

Fluorescence microscopy proved to be a very complex, time consuming and difficult technique. *BAC*LIGHT viability stains worked well with both control log-phase cells and control heat-fixed cells (boiled for 10-15 minutes). The control samples displayed bright green fluorescence for fresh cells and red fluorescence for the killed cells.

However, when a number of cells were counted in a field of view (FOV) the fluorescence was poor. Despite the cells showing bright images in the first 10-15 seconds of UV illumination the fluorescence of the stained cells faded and reduced so the cells were difficult to distinguish from the bright-field background illumination. As previously

reported, phototoxicity, not just to the cells but also the dyes themselves, may take place and destroy the fluorescent characteristics of the cell bound dye.

In addition to the photodynamic fading of the dyes, the filters used to hold the treated cells after staining (black polycarbonate filters, Ø25mm, 0.2μm), appeared to hold part of the stain and fluoresce when irradiated under the microscope. Nylon and other PTFE filters also appeared to have a background fluorescent after staining. Rinsing the filters with distilled water, methylated spirits, ethanol or propan-2-ol, did not eradicate background fluorescence.

The variability of staining injured cells was true as expressed by the Kit manufacturer. A proportion of the cells were dual stained with parts of the same cells fluorescing both orange and green. When injured cells were stained and examined under the microscope, without filtering but just held under the cover-slip, some of the orange cells showed motility. The heat fixed populations of dead control cells were bright red and motility was not observed. The actively growing live control cells fluoresced bright green and were highly motile (Plate 4.1; photograph of *S. typhimurium* culture stained with *BA*CLIGHT).

Plate 4.1: Illustrates *Salmonella typhimurium* cells stained with *BAC*LIGHT viability stains and fluorescing under the fluorescence microscope. Red cells (a) represent the dead permeabilised cells which allow the diffusion of both PI and SYTO9 stains; green cells (b) represent the viable intact cells which allow the diffusion of SYTO9 alone; and orange cells (c) represent the cells in the intermediate state which are partially permeabilised and allow some diffusion of PI.
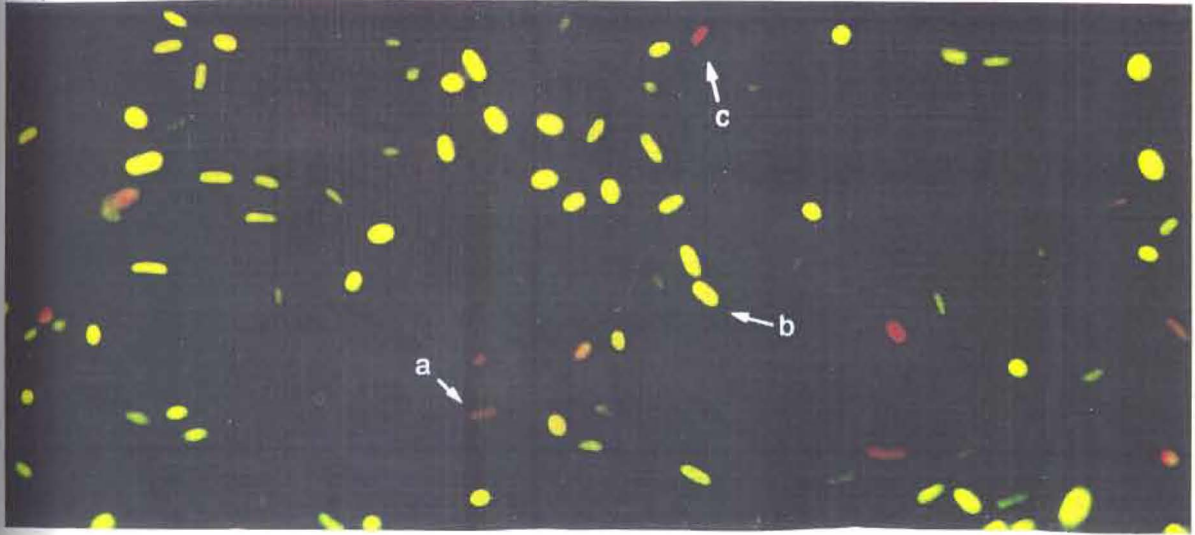
For the purpose of obtaining results which could be compared with those obtained above, with culturability of treated *S. typhimurium* on rich and selective media, the orange and red cells were counted as one group of cells, and considered to be dead cells, with green cells counted as the other group of live/viable cells. By doing this, the percentage of dead cells in the samples may have been over-estimated. This over-estimation was assumed not to have an effect on the final result / conclusion of the study.

When diluted cultures of *S. typhimurium* were stained with *BA*CLIGHT as indicated in the methodology, no fluorescent cells could be seen when checked under the fluorescence microscope. It appeared that the washing and centrifugation steps of the samples had eliminated the stained cells which were in low concentrations. A culture containing more cells of about 10^8-10^9 cfu. ml^{-1} was used for following experiments.

A substantial difference in the detected survival of the 24h-old culture method and detected viability of the *BA*CLIGHT viability assay under the fluorescence microscope was observed (Figures 4.3 and 3a and Fig. 4.10, Table 4.1).

These results agree with previous reports and confirm the presence of "viable but non-culturable" cells in the heat injured population. The size of this "viable but non-culturable" population, according to the *BA*CLIGHT stained "live" population could be

approximately 30% of the initial population after 60 minutes of heat treatment; probably

higher if we consider the over-estimation of the "dead" population.

Both the results of *BAC*LIGHT viability measurements with fluorescence microscopy and

the survival evaluation of heat treated *Salmonella* using the culture technique on both

TSA and XLD show that the rate of survival reduction reduces between the periods of 40

to 60 minutes. This may reflect the presence of a small population of heat resistant cells

as a result of their position in their growth cycle or due to a genetic superiority.

Fig. 4.10 : Percentage Viability of *S. typhimurium* (undiluted 24h-old culture) heat treated at 54° C (for 60 min.), as evaluated by the use of *BAC* LIGHT viability stains and fluorescent microscopy.

—x— Percentage viability evaluated by BACLIGHT viability stains and fluorescent microscopy
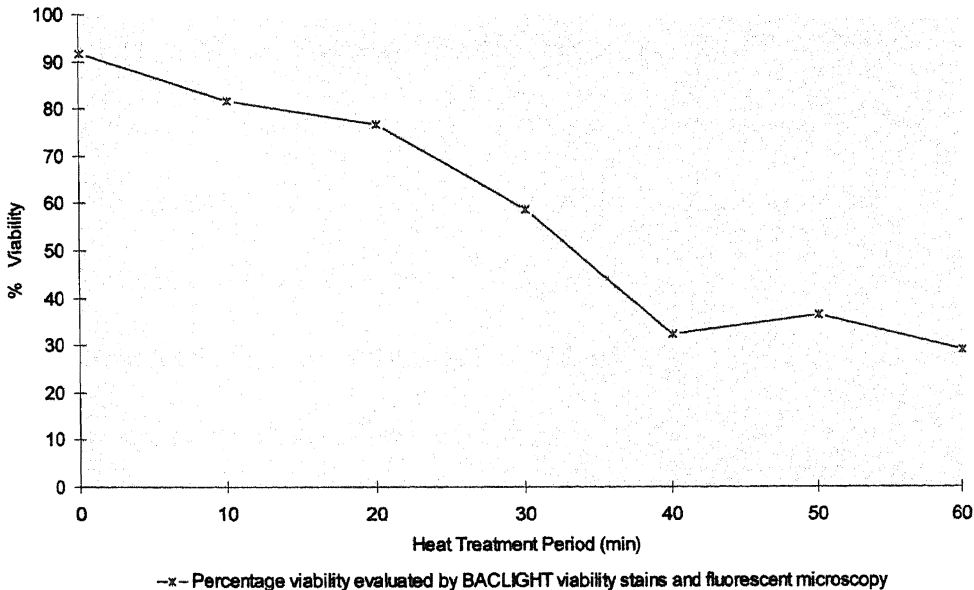
4.4.3 *Flow Cytometry and Heat Treated S. typhimurium*

The difficulties of studying stained heat-treated cells with fluorescent probes under a fluorescence microscope were simplified with the use of a flow cytometer. The large number of cells and the number of parameters that can be measured with a flow cytometer increases the capability of distinguishing between the different groups/clusters of cells in the heat treated population.

The time for sample preparation and analysis is also reduced from that of microscopic analysis. This is a huge advantage of the technique in routine analysis. Phototoxicity and other factors, such as chemical composition of the dyes and temperature, may still influence the ratio of dead, alive and the cells in an intermediate viability status, but the very short exposure of the cells to the laser illumination effectively reduces this possibility below that of a concern. The observation above showed the UV exposure neede >10 seconds for a phototoxic effect to become evident, and the UV exposure of the cells in the FC is less than milliseconds.

Control populations of actively growing cells and heat fixed cells were stained with *BAC*LIGHT and flow cytometric measurements (FCM) determined the two highly distinct clusters of cells (Fig. 4.11). These clusters are widely and specifically separated when both red and green parameters are plotted.
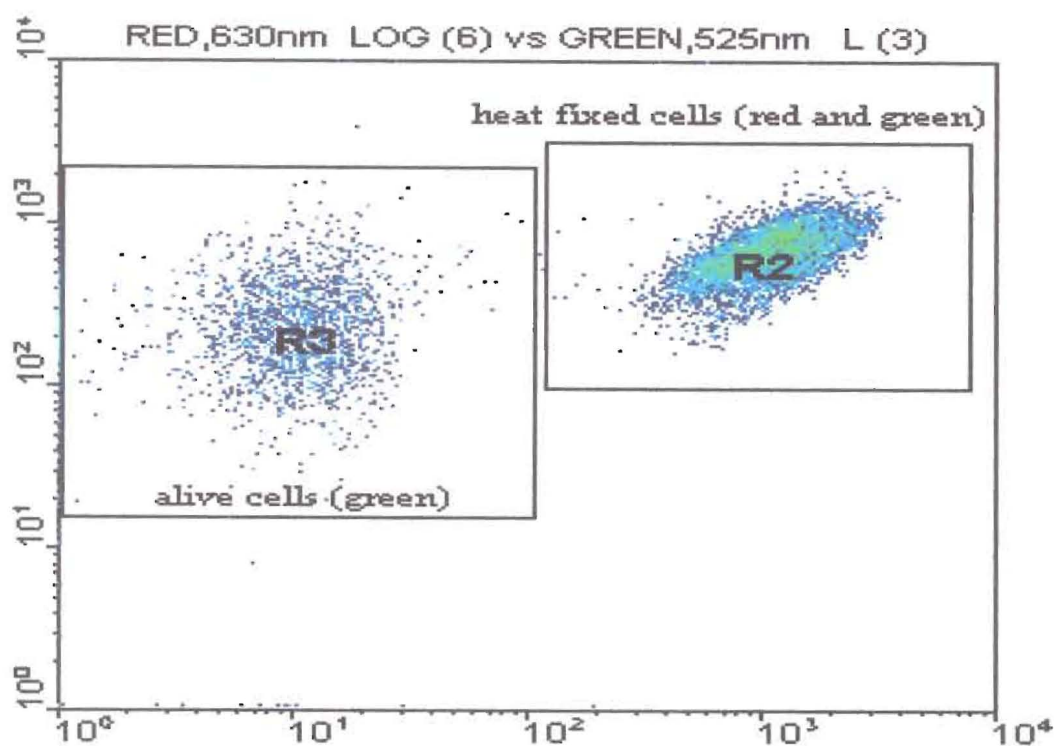
Fig. 4.11 : Control live and dead cells stained with *BAC*LIGHT show the expected positions of the two clusters in a Coulter counter 2D plot of cell clusters. The live cells appear as a green cluster due to the diffusion of the green dye SYTO9 diffusing into cell membrane. PI is selectively excluded from the cells. Dead cells appear both red and green being unable to exclude SYTO9.

Note that fluorescence microscopy is unable to produce this discrimination due to red quenching of the green in the dead cells. The position of dead and live cells on the red versus green fluorescence plots shows PI does not quench the green fluorescence of SYTO9 in the cells which are permeable to both stains.

Each point represents 5 cells.

After 60 and 120 minutes of heat treatment, shown in Figures 4.12 A and 4.12B respectively, of the 12h-old *S. typhimurium* culture was stained again with *BAC*LIGHT and FC examined. The results show a shift of the viable population into different clusters throughout the heat treatment. After 60 minutes of treatment, the otherwise green population of the actively growing culture, has split in almost three equal fractions of

alive green, dead red and green, and a transitional stage which most likely represent injured cells. This transitional stage may relate to the two component survival curves seen earlier in this Chapter (Figures 4.3 and 4.3a). The transitional cells appear between the previous two clusters and are marked as region C (Fig. 4.12 A).
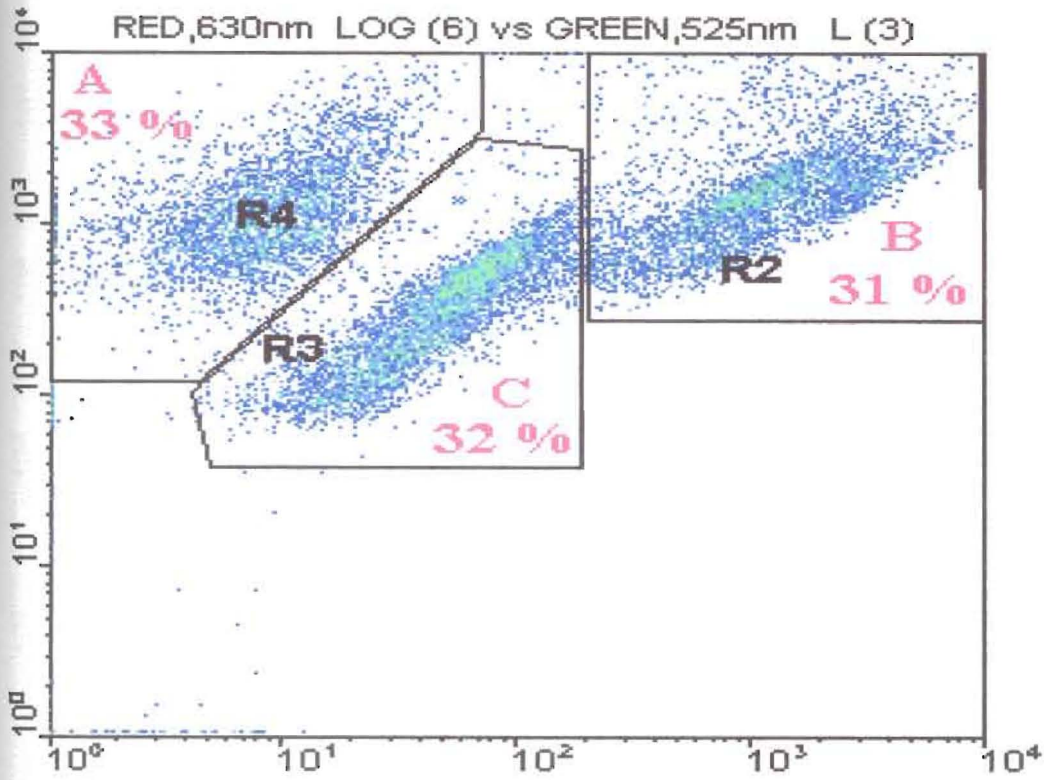
Fig. 4.12 A : *S. typhimurium* culture after 60 minutes of heat treatment at 54° C and stained with *BA*CLIGHT viability stains. There are three cell clusters: region A representing live cells; region B representing dead cells; region C is a cluster of cells representing an injured population which appear as dual stained equivalent to the orange-stained cells under a fluorescence microscope.

With increasing length of exposure to 54°C the injury shifts the cells from cluster A through C to B. Compare this with Fig. 4.9 B in which the majority of cells are shifted in cluster B.

A two hour heat treatment of the *S. typhimurium* culture has reduced population A of the live cells to 9% of the total count/gated events and reduced the injured population to 23%. The cells are dying and moving into cluster B which increased to 64% after 120 minutes (Fig. 4.9 B). The surviving population A was detected by flow cytometry from about 20,000 gated events after staining. The number of cells used in FC to estimate viability are vastly superior to the visual or plating techniques which are only able to examine a smaller fraction of the population.
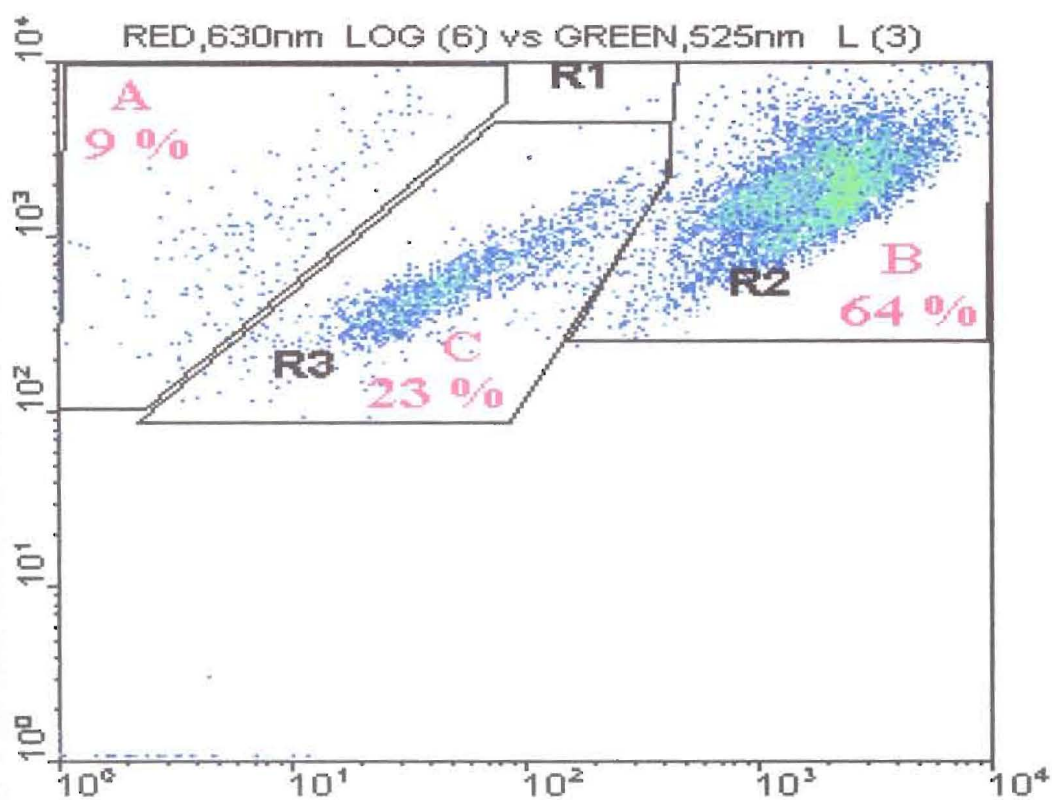
Fig. 4.12 B : *S. typhimurium* culture after 120 minutes of heat treatment at 54° C and stained with *BAC*LIGHT viability stains. The results show a shift of cells from cluster A through C to cluster B. Cluster B represents the dead cells in the heat treated sample and has increased compared to the 60 minute heat-treated population. Cluster C is a transitional state of viability between the live and dead cells.

Note that the percentage of viable cells in cluster A is higher than that evaluated by culture procedure (Table 4.1, undiluted cultures; Appendix).

The results obtained using *BAC*LIGHT viability staining with FC show a shift of cells from the viable population to the dead population, and the presence of a transitional stage between the dead and live clusters.

Further investigations on the viability of heat injured *S. typhimurium* were carried out using flow cytometry and staining with three fluorescent dyes BOX, EB and PI concurrently. Unlike *BAC*LIGHT staining which allows the determination of cell integrity, the combination of the three dyes which was termed BEP staining can differentiate between clusters of cells according to membrane integrity and membrane potential.

Note that BEP staining and FC of the heat treated *S. typhimurium* do not show neither nucleic acid damage nor the cells ability to grow on media forming visible colonies. Cell sorting of the cell clusters on specific media is the only way to determine cell culturability.

BEP staining was primarily carried out with a control culture of actively growing 12h-old culture alone. As mentioned above in the introduction, these actively pumping cells do not allow the diffusion and accumulation of dyes within the cell envelope, and are therefore non-stained (Fig. 4.13). BOX is excluded from the intact polarised cells due to

its charge. PI is selectively excluded by the membrane barrier. EB diffuses through the membrane slowly but is actively pumped by the cells dye extrusion mechanism which only works with energised intact cells that can provide the ATP for the pumps to operate.
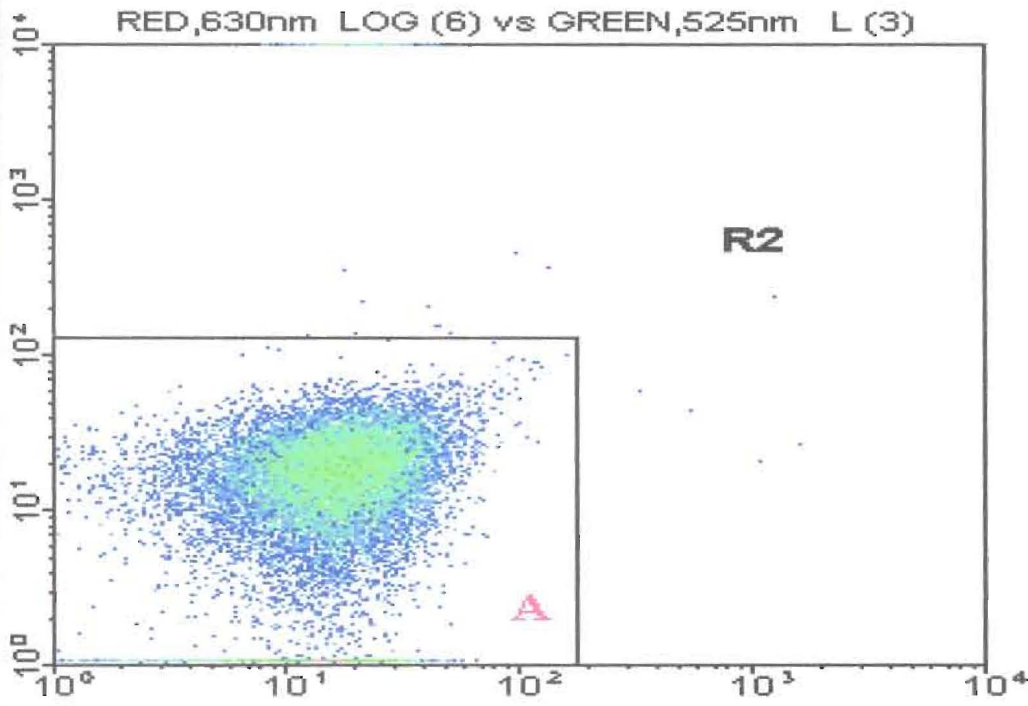
Fig. 4.13 : Control population of actively growing 12h-old *S. typhimurium* stained with BEP result in one cell cluster. Live cells are not stained by the fluorescent dyes since PI and BOX stains are excluded, and EB is actively pumped out of the cells. Therefore, cluster A appears non-stained as shown on the red versus green fluorescence plot.

The control live population was also mixed with a heat fixed sample of *S. typhimurium* in order to observe the BEP staining differences between the two cell states. In order to delineate more information about the two populations more than one combination of measured parameters in terms of wavelengths emitted from the stained cells was plotted shown in three different plots A, B and C in Fig. 4.14.

ORANGE,575nm L (4) vs RED,630nm LOG (6)

PLOT A

B

R8

R9

A

GREEN,525nm L (3) vs RED,630nm LOG (6)

PLOT B

B

R4

R2

A
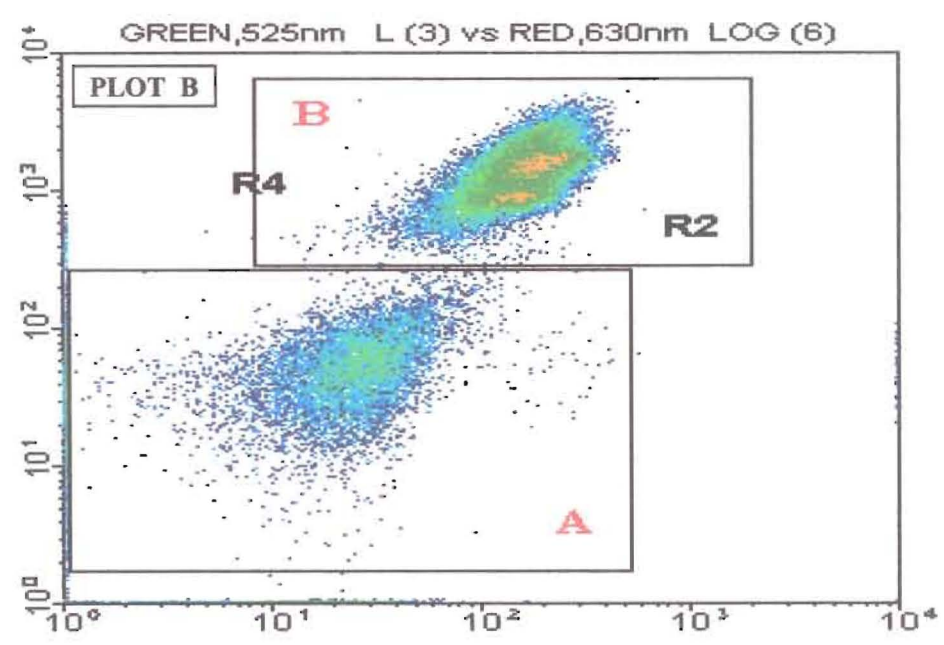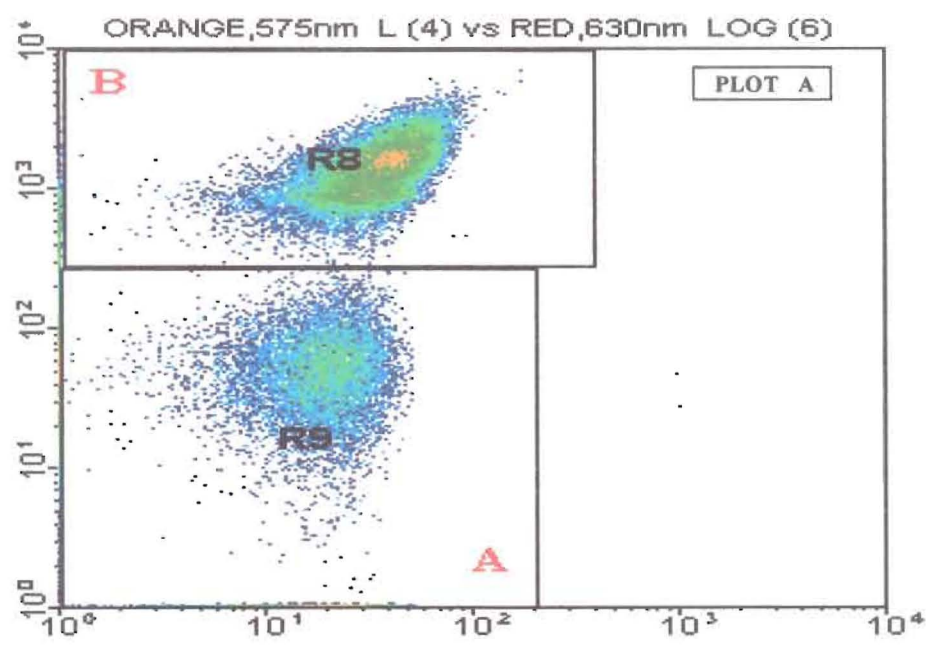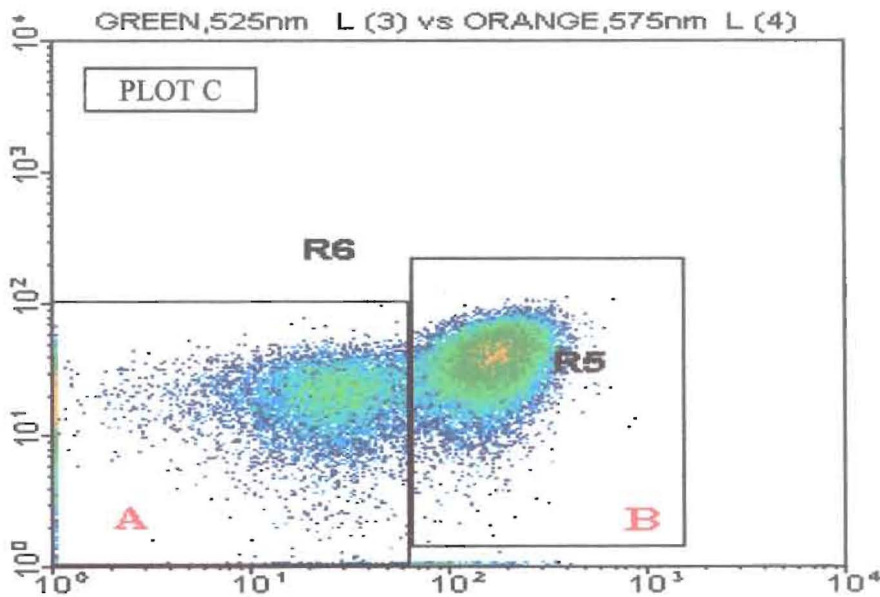
Fig. 4.14 : Results of the mixed populations of actively growing and heat-fixed *S. typhimurium* after staining with BEP. The actively pumping non-stained population A has the same position on the different plots indicating the homogeneity of its characteristic fluorescent emission (EB⁻ PI⁻ BOX⁻). The heat fixed or permeabilised population B is shown to emit in the green and red (BOX⁺ PI⁺) region.

The characteristics of the two populations were clearly different as they were either EB⁻PI⁻BOX⁻ (cluster A/ live cells) or EB⁺PI⁺BOX⁺ (cluster B/ dead cells). During the heat treatment of the cells at 54°C, samples were diluted in BPW after 1h, 2h and 3h, sonicated for at least 10s and stained using BEP dyes. Unlike the control population shown in Fig. 4.13, the 1h and 2h heat treated culture resulted in additional clusters to the A and B populations representing live and dead cells respectively (Fig. 4.15 and Fig. 4.16).

Triple staining (BEP) of 1hour heat treated cells and FC distinguished between at least four different clusters of cells (Fig. 4.15). It appears that the actively pumping cells in the lower left (LL) quadrant of Fig. 4.15A are gradually losing energy and their dye extrusion mechanism or pumping ability is diminished resulting in a gradual accumulation of EB inside the cell. Therefore, the population of non-pumping intact cells or de-energised cells in the LR quadrant of Fig. 4.15A increases and becomes more orange moving gradually along the X axis. Depolarised cells allow the diffusion of EB as well as the charged lipophilic dye BOX through the outer cell membrane which still acts as a selectivity barrier towards PI. Therefore, depolarised cells appear as green and orange emitting events in the UR quadrant of Fig. 4.15A. Non-nucleated events or ghosts which represent cell debris are stained only with BOX and appear fluorescent green. Permeabilised or dead cells allow the diffusion of all three dyes and the representative cluster emit strongly in the red and green region. PI red fluorescence quenches the EB orange fluorescence and BOX stains the cell lipids fluorescent green.

After two hours of heat treatment the clusters shift again into more compromised positions showing a worse state of injury than before. This is explained by the dramatically reduced number of events in the non-stained and de-energised clusters which appear in the LL and LR quadrants respectively of Fig. 4.16.
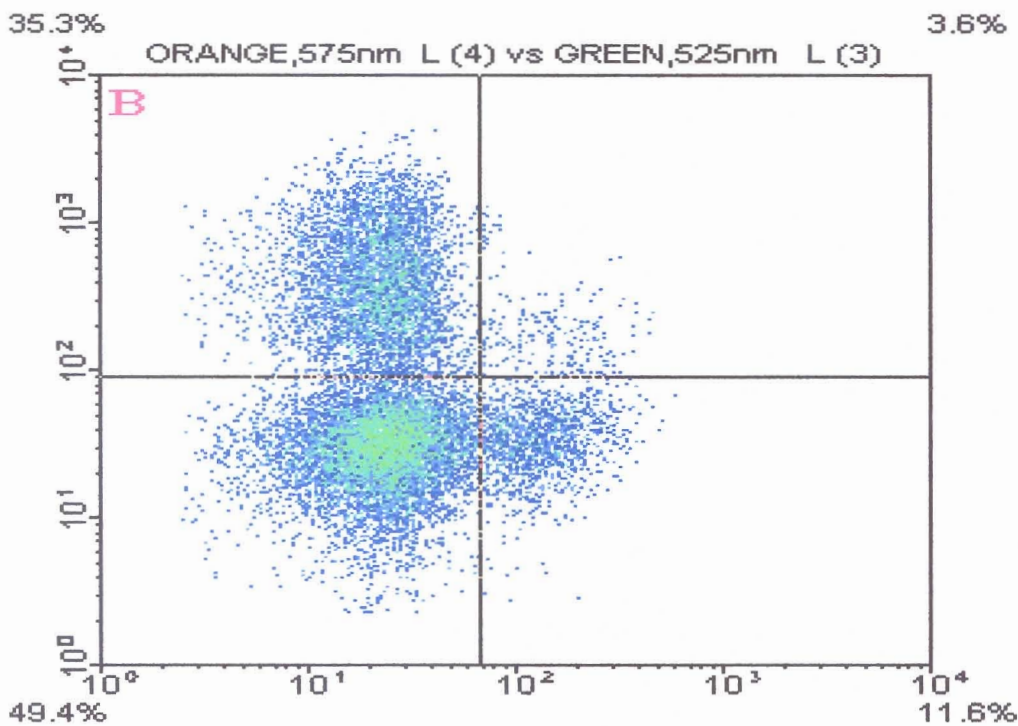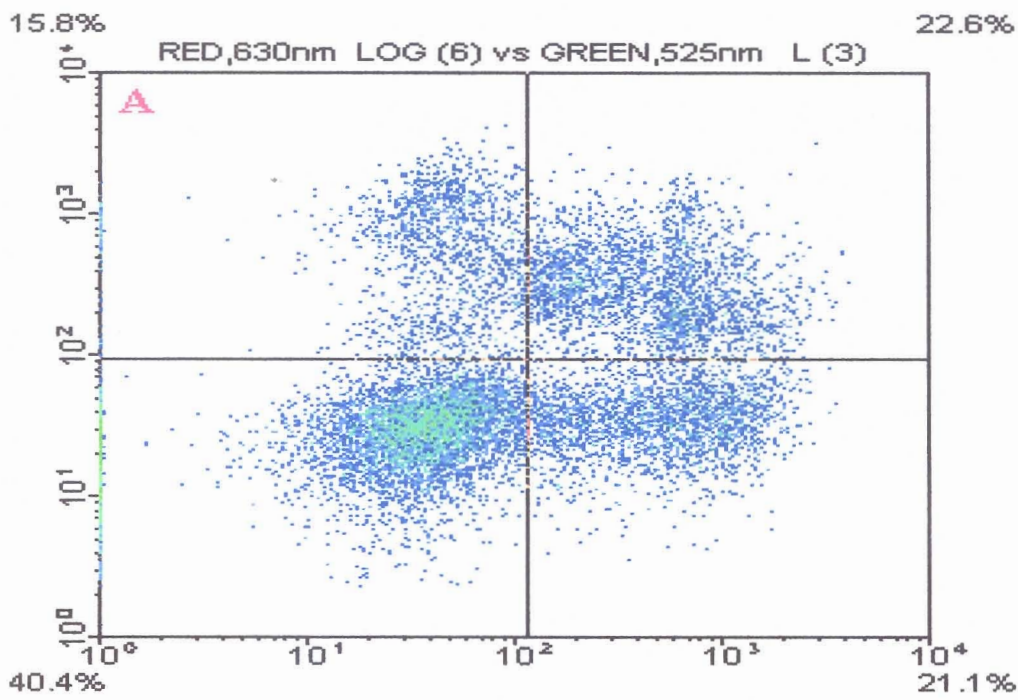
Fig. 4.15 : FC results of the 1h heat-treated *S. typhimurium* cells after staining with BEP. The different plots show the presence of more cell clusters and are therefore required in order to get more information out of the triple stained population. The LL and LR quadrants of the two plots represent the actively pumping intact cells (EB⁻PI⁻ BOX⁻) and the non-pumping intact or de-energised cells (EB⁺PI⁻BOX⁻) and represent 61%of the total number of gated cells. The clusters that appear in the upper quadrants represent the depolarised cells (EB⁺PI⁻BOX⁺), the permeabilised cells (EB⁺PI⁺BOX⁺) and ghosts or non-nucleated events (EB⁻PI⁻BOX⁺).
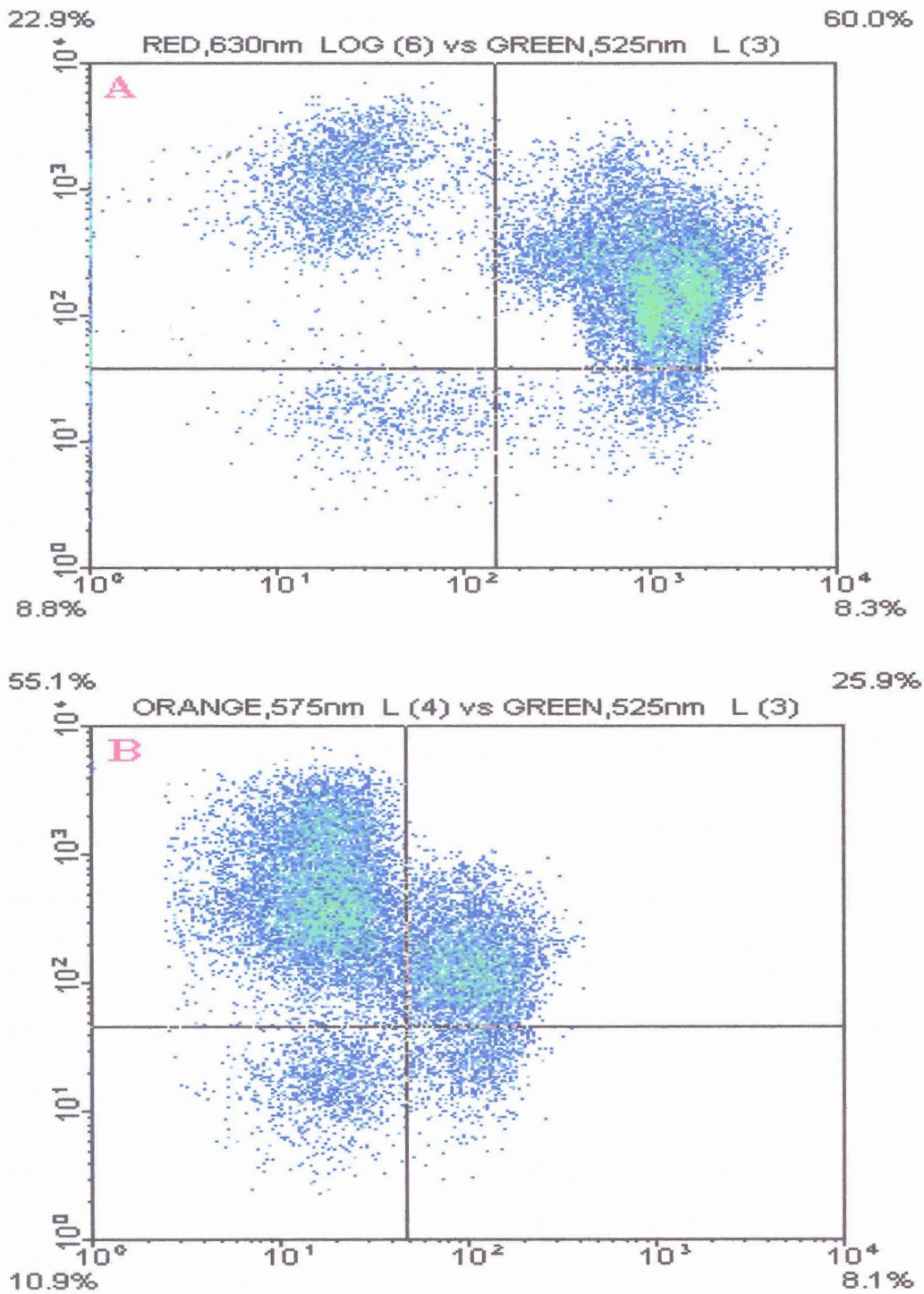
Fig. 4.16 : FC results of the 2h-heat treated *S. typhimurium* cells after staining with BEP. The actively pumping and the non-pumping de-energised cell clusters, that appeared above with the 1h-heat-treated population (LL and LR quadrants of both A and B plots) have been reduced significantly to <20% of the total gated events. The cells have moved predominantly into the depolarised state of injury which allows the diffusion of BOX and EB accumulation thereby fluorescing both green and orange as shown in UR(B) quadrant and part of the UR(A) quadrant. Red and green events (EB⁻ PI⁺BOX⁺) in the UR(A) quadrant are permeabilised cells which are less green than the non-nucleated ghost cells. Possibly due to the quenching effect by the PI fluorescence.

Cultures of *S. typhimurium* heat treated for three hours resulted in no BEP staining pattern. No clusters could be obtained with the usual plotting of red (630nm) or orange (575nm) versus green (525nm) emission characteristics. When the events were plotted as wide forward angle light scatter (WIDE FALS) versus right angle light scatter (RALS) it was indicated that the gated events were spread in all directions and were most likely too fractured or structurally damaged for triple staining to yield meaningful results.

4.4.4   *Cell Sorting of Salmonella Clusters in Three Different Liquid Media*

Cell sorting was carried out as indicated above in this Chapter (4.3.6) with cells from the different clusters separated by their emission characteristics and the results are presented as percentage recovery of the cells in the different media (Table 4.3). This type of sorting is termed Fluorescence Activated Cell Sorting (FACS). The control actively pumping cells and intact de-energised cells stained with BEP grew very well in all media as expected, however, the cells of the same clusters which have been heat treated and then BEP stained appeared to be largely unrecoverable, with a maximum recovery percentage in the 2MM9 medium (16.3%).

Depolarised and then permeabilised cells were apparently the worse viability states of the control untreated samples of *S. typhimurium* which did not recover well in any of the media. Only the depolarised cells recovered relatively well in L-broth which resulted in a 21.9% recovery. Hardly any of the depolarised and permeabilised cells

recovered with heat treated cultures. Surprising was the fact that what were considered as "ghost" cells or non-nucleated events in the control sample appeared to recover and grew very well in all media.

*BAC*LIGHT stained dead or permeabilised cells which allow the diffusion of PI as well as SYTO9 through the cell membrane did not show any growth in the media tested. The green cells representing the live population recovered poorly in the media. Poor recovery was also shown with injured cells, which were possibly stained orange with *BAC*LIGHT and represented the transitional state between the dead and live clusters.

Table 4.3: Results of single cell sorting of *BAC*LIGHT and BEP viability stained *S. typhimurium* culture (12h-old) heat treated at 54° C for 60 minutes.

	Cell Clusters and % of Recoverable Cells (108 h incubation)				
	Intact Actively Pumping	Intact De-energised	Depolarised	Permeabilised	Ghosts
Control Culture (BEP stained)					
BPW	93.8	100	3.1	0	87.5
Lactose Broth	93.8	87.5	21.9	12.5	100
2MM9	93.8	93.8	12.5	12.5	75
Heat Treated Culture (BEP stained)					
BPW	11.3	0	2.5	0	0
Lactose Broth	1.3	1.3	0	0	0
2MM9	16.3	6.3	0	0	0

	"Pumping" Transitional State	"Alive" (Green +ve)	"Dead" (Red & Green +ve)
Heat Treated Culture (*BAC*LIGHT stained)			
BPW	4.7	3.1	0
Lactose Broth	7.8	3.1	0
2MM9	6.3	1.6	0

4.4.5 *Recovery of heat-treated S. typhimurium in Six Different Liquid Media*

The FACS results indicated poor recoverability of the stained cells in the three media. It was decided that further experiments on injured cell recoverability would be carried out to determine whether recovery of highly injured cells of a 12h-old culture of *S. typhimurium*, heat-treated for three hours at 54° C, in different media is possible and to identify which of the liquid media is/are most suitable for recovery.

As explained previously earlier in this Chapter (4.3.7) each microtitre well of a total 192 wells per medium was inoculated with 25µl of the diluted heat treated culture with a calculated chance of 0.00225 of each well being inoculated with a normal culturable cell. The absorbance readings at 620nm were calculated to give a recovery percentage as explained above. The results in Table 4.4 show that three hours of heat treatment at 54°C was not enough to render the culture non-viable. Surviving cells in the unculturable but undetermined state of viability in terms of BEP or *BACLIGHT* staining, appear to recover very well (100%) within 18h in BPW and Peptone Water with the amino acid and nucleoside supplement of 2MM9 (2SPW). Recoverability of BPW, 2SPW and TSB was over 99% within 24h. Very good recoverability of 93.8% was achieved using TSB medium. The chemically defined 2MM9+4g glucose medium proved to be a very poor medium in terms of recovering the heat injured cells whereas M-broth recovered approximately 46%.

Table 4.4 : Recovery of *Salmonella typhimurium* in different media after heat treatment at 54°C for three hours. Each medium was tested in two microwell plates[1] which contained 200 ml broth per well (96x). These were inoculated with 25ml of the diluted heat-treated culture, and approximately 1 per 400 wells was inoculated with a culturable cell.

	Incubation period	Media tested					
		BPW	2SPW	TSB	Lactose broth	M-broth	2MM9
Recovery (%)[2]	18 hours	100	100	98.4	62	40.1	11.5
	24 hours	100	100	99.5	93.8	45.8	18.2

[1] Absorbance of the plates was read at 620 nm.

[2] Recovery was considered to be the increase in absorbance above the threshold value set. Threshold was the mean reading of the control plates plus three times the standard deviation of the mean.

4.5   Discussion

The results of this study show a discrepancy between the measurements of *Salmonella* culture viability after a period of heat treatment at 54°C using the three different viability measurement methods.   There is a correlation between the results of fluorescence microscopy and flow cytometry measurements using *BAC*LIGHT and triple staining with BEP.   However, these results do not correlate with cultural evaluation of viability of the heat treated culture.

Cultural evaluation of bacterial viability which is based upon the culturability -ability to divide and form colonies- of the cells on a particular medium is often underestimating the actual viability of a culture. However, it is not clear how to truly evaluate viability without over- or under-estimating the proportion of non-viable cells in a culture.

Being able to clearly distinguish between the non-viable and viable culturable cells is what initially pointed people to the group of cells termed viable but non-culturable. The importance of this group of cells derives from two main factors: **a.** their potential pathogenicity, had they recovered the ability to multiply and invade, and **b.** the chance they pass through pathogen detection techniques unnoticed. To avoid the chance of presenting food consumers with this 'silent' potential danger, it would be best if the detection procedures used in food microbiology laboratories were evaluated using non-culturable cells or severely damaged cells.

It was initially the aim of the study to evaluate different pre-enrichment media using injured cells created by sublethal heat treatment and evaluated using cultural methodology. Variations observed with culture methodology lead to the investigation of different techniques used to evaluate bacterial viability. The ultimate goal was to obtain the worst state of injured cells reproducibly, and these cells were to be used for media evaluation based on cell recoverability.

Using the culture viability procedure, the age of the *Salmonella* culture appears to be significant in viability measurements of heat treated population since the survival of stationary cells in 24h-old cultures appears to be more heat resistant than younger earlier phase cells as shown in Figures 4.5-4.7, and Table 4.2 (Appendix). This finding consolidates previous reports on bacterial injury and heat resistance (Ellison *et al.*, 1991; Henge-Aronis *et al.*, 1991; Dodd *et al.*, 1997; Kaur *et al.*, 1998).

The culture viability procedure is also cell concentration sensitive. The effect of heat treatment on *S. typhimurium* is more clearly demonstrated when the culture is pre-diluted as indicated by the survival curves shown in Figures 4.2 - 4.7. It appears that calculating the percentage injury using the formula mentioned earlier in this Chapter (4.5.2) is greatly affected by the cell concentration and can be highly sensitive. When the cell concentration is high there is great variability of results (Fig. 4.8) and when the cell concentration is low the culture technique is not sensitive enough to determine percentage viability or percentage injury (Fig. 4.9). An other drawback of the culture procedure for viability measurement is the fact that all cultures used showed variable resistance to the selective medium previous to heat treatment at t0, therefore, presenting another variable to the whole evaluation of viability.

With these findings alone, it can be deduced that: a) culture evaluation of cell viability is inaccurate because it only refers to culturable cells on the media used; b) it can be highly variable; and c) it can only be used with a narrow range of cell concentrations.

The so called percentage injury calculated as the difference of the growth on the two media may only represent the increased sensitivity to the specific selective medium. The culture procedure does not take into account the number of cells which lose the ability to divide and form colonies on either selective and non-selective media but are not yet dead. This number of cells increases almost linearly with increasing heat treatment up to the first 40-60 minutes of heating.

Based on these observations media should not be evaluated using injured cells as described by the culture viability evaluation procedure.

Using *BACLIGHT* viability staining and fluorescence microscopy shows the considerable amount of cells in the *S. typhimurium* heat treated culture unable to form colonies on media, and therefore, being undetected by the culture procedure. As presented in the results (Fig. 4.10) after 60 minutes of heat treatment of the 24h-old concentrated culture, there are approximately 30% viable cells in the culture based on their functional permeability barrier / membrane integrity. Even though 30% may be an underestimated viability percentage due to the counting of red and orange cells as dead, it is still far more than the estimated survival fraction calculated using the culture procedure (Table 4.1; Appendix).

Fluorescence microscopy and *BAC*LIGHT staining was probably more accurate in estimating culture viability than the culture procedure, without taking in consideration the reproductive ability of the cells. This can be based on the assumption that membrane integrity along with metabolic activity of the cells may be required but are not necessary for reproductive viability. Therefore, intact and metabolically active cells that are stained green with *BAC*LIGHT are not necessarily able to form colonies, but they may recover that ability if they resuscitate.

Having determined the distinct difference between cultural viability and *BAC*LIGHT viability of heat-treated *S. typhimurium* it was decided to carry out further work on cell viability using more sensitive methodology. Fluorescence microscopy proved to be a very time consuming and laborious procedure. The stains used under the fluorescent microscope appeared to be UV or temperature sensitive and faded away while examining the slides under the microscope, making the distinction of the differentially stained cells very difficult against a fluorescing background. Also, there is a limitation to the amount of cells one can count manually in a set time period before extraneous factors start having an effect on the culture viability.

Flow cytometry is a much more sensitive, rapid, and accurate technique regarding the differentiation of a very high number of stained cells in a few seconds, than any other technique used so far including the cultural one. Control populations of live and dead cells stained both with *BAC*LIGHT and BEP (Fig 4.11 and Fig. 4.13 to 4.14 respectively) indicate the clear positions of the two clusters of cells when plotted accordingly (X vs Y).

One hour heat treatment of the *S. typhimurium* culture and *BACLIGHT* staining clearly increased the number of cell clusters. An additional cluster C appeared in the population as shown in Fig. 4.12A. Increasing cell injury in the population due to increased heat treatment appears as an increased cell membrane permeability to the otherwise impermeable PI. The third cluster may represent the number of orange cells appearing under the fluorescence microscope (Plate 4.1).

The percentage of viable cells in the population shown in Fig. 4.12A (cluster A) correlates with the fluorescent microscopy results which is approximately 30% but does not correlate with the cultural viability results presented in Table 4.1 (Appendix) which show that after 60 minutes of heat treatment there is only a minute fraction of cells (<0.1%) able to divide on solid media. This can lead to the conclusion that a common characteristic of the three clusters is the inability to reproduce due to possible DNA damage, and protein damage critically-important for cell division (Rees *et al.*, 1995). Inability to multiply can potentially give rise to elongated cells which may possess metabolic activity and membrane integrity. Such cells were visualised in the *S. typhimurium* culture after only 20 minutes of heat treatment using transmission electron microscopy of phosphotungstic acid stained *Salmonella* cells (Plate 4.2.3, Appendix). Elongated cells were not observed in control cultures where cells appeared much shorter than the cells which presumably lost the ability to divide (Plate 4.2.1 - 4.1.2, Appendix).

After two hours of heat treatment the shift of populations between the live cell cluster (A) and the semi-permeable / transitional cluster (C) to the dead cell cluster (B) was apparent (Fig. 4.12B). This is solid evidence that the membrane permeability of the

cells was disappearing with increasing heat treatment. It was also determined that between the two clusters A and C there was approximately 30% of the population which had the potential to recover the reproductive ability. At least 9% of that population possessed functional membranes.

More details of the heat treated population were obtained by triple staining (BEP) and FC. Apart from the membrane integrity indicated by the exclusion of PI, the ability of the cell membrane to actively pump out EB was indicated, based on energy available to pumping mechanisms, as well as the presence of a transmembrane potential difference or membrane polarisation was indicated by the exclusion of BOX (Figures 4.15 and 4.16).

After one hour of heat treatment (Fig. 4.15A and B) a high proportion of the cells (between 40-50 %) appear to be actively pumping cells unstained by BEP (LL quadrants) therefore showing membrane energisation and integrity. This is a higher percentage than the one obtained by *BAC*LIGHT staining as green cells (Fig. 4.12A). However, there is a correlation between the total BEP stained population percentage of actively pumping (LL quadrant) and de-energised cells (LR quadrant) reaching approximately 61% (Fig. 4.15) and the *BAC*LIGHT stained clusters A and C (Fig. 4.12A) reaching approximately 65%. This may indicate that cluster C includes de-energised cells which have just began to allow the permeability of PI.

One hour of heat treatment also gave rise to de-energised cells which allow the accumulation of EB (Fig. 4.15A, LR quadrant), which gradually become depolarised and allow permeability of bisoxonol and move gradually into the UR quadrant shown

in Fig. 4.15A. Then there is the permeabilised cluster which represents the cells that allow permeability of PI as well as BOX and ghost cells or non-nucleated events that appear more green in the UL quadrant. The ghost cells could also be viewed under the transmission electron microscope and appeared as empty see-through cells with seemingly intact rigid membranes (Plate 4.2.4 to 4.2.5, Appendix).

Two hours of heat treatment and BEP staining of the *S. typhimurium* culture resulted in a more complex but clearer picture of cell viability than previous results. Actively pumping BEP unstained cells (Fig. 4.16, LL quadrants) appear to correlate with the *BAC*LIGHT stained green cells (Fig. 4.12B, cluster A) which are considered as live cells and represent approximately 10% of the population.

Similarly to the *BAC*LIGHT results (Fig. 4.12A and B), there is a shift of populations from the actively pumping cell cluster (LL quadrant) towards the de-energised cell cluster (LR quadrant) and into the depolarised and permeabilised clusters in an anticlockwise manner (Fig. 4.15 and Fig. 4.16). With these results, of BEP stained 2h-heat treated *Salmonella*, a progression of cellular injury is indicated and can be viewed with the FC as a real time event.

More specifically an increasing population of cells appear to have lost membrane potential (depolarised), i.e. taking up EB and BOX (Fig. 4.16A and B, UR quadrant), an increasing number of cells appear to be permeabilised, i.e. taking up BOX and PI (Fig. 4.16A, UR quadrant), and an increasing number of non-nucleated events appears to be stained only with BOX (Fig. 4.16A, UL quadrant). The results of FC measurements of the BEP and *BAC*LIGHT stained heat treated cells are supported by

the recent report of Nebe-von Caron *et al.* (1998) who improved the procedure used in this study which was at its infancy.

All these cell clusters must represent the structural changes which go on due to the physical stress sustained by the organism. Lipopolysaccharide changes of the *Salmonella* outer membrane which can alter the membrane fluidity or protein function (Brãnes, *et al.*, 1981), conformational changes and packing arrangements of membrane proteins which may take place due to LPS changes (Gmeiner and Schlecht, 1979), the possible removal of the divalent cationic bridges (Mg^{2+} and Ca^{2+}) which stabilise the outer membrane onto the peptidoglycan and/or lipoprotein layer (Russel, 1984) and other changes can alter functional properties of the membrane as a permeability barrier (Gilbert, 1984). These cellular changes due to physical stress, such as heat, may be paralleled with the actual ability of the cells to grow and multiply in media. By obtaining this parallelism we can determine how the culture viability and staining viability correlate. In order to achieve this, individual cells from the different cell clusters have been sorted into different media.

Fluorescently activated cell sorting, as described earlier (4.3.6) was at the time in the early developmental stages and results may reflect that. In general, the results shown in Table 4.3 indicate that control unheated stained cultures are more recoverable than the heat treated BEP or BACLIGHT stained cultures.

The actively pumping and de-energised BEP stained cells in the control sample recovered well (>90%) on all three media unlike the corresponding clusters of the heat treated populations. This may reflect the effect of heat on the cells ability to recover

and divide in liquid media. Depolarised heat treated cells that allowed the diffusion of EB and BOX through the cell membrane, also appeared largely unrecoverable. However, the depolarised cluster of the control population appears to contain cells that can recover (maximum of 21.9% recovery in lactose broth). This agrees with recent findings (Nebe-von Caron *et al.* 1998).

Permeabilised (PI$^+$ BOX$^+$), ghost (BOX$^+$) and dead (BACLIGHT- PI$^+$ SYTO9$^+$) cells in the heat treated population did not recover in any of the media which strongly suggests that PI is indeed a stain permeable to severely damaged unrecoverable and dead cells. The latter statement could be questioned due to the minute (approximately 5-7%) recoverability observed with the PI and SYTO9 stained cells (cluster C, Fig. 4.12A and B).

Most surprising was the recovery of what was considered the control ghost population (BOX positive green cluster), and therefore questioning the validity of the true identity of the cluster. This result indicated the possibility that a large part of the control "ghost" cluster is probably intact cells which allow the diffusion of the lipophilic stain BOX probably due to the presence of neutral molecules of the stain being able to diffuse through the membrane disregarding the membrane potential or the metabolic energy state of the structure. Efficient dye extrusion mechanisms and therefore energetically active membranes pump out the EB, however are unable to pump out BOX which binds strongly to lipids when inside the cell. It is also possible that the events in the cluster which recovered were in fact actively pumping cells stuck to the ghosts and passed through the detector as green events.

Clearly this area of flow cytometry and cell sorting, based on triple staining of injured cells with BEP, needs further improvement before the technique becomes an excellent way of evaluating media based on their ability to recover single cells of a know viability status.

Until then, this study attempted to evaluate the recoverability of six different media with 3h-heat treated *S. typhimurium* cells at 54° C, diluted to extinction (4.3.7). The 3h heat-treatment period was decided based on the results obtained from all the culture experiments mentioned in this study and the FC measurements of 3h heat-treated populations. The 0.00225 chance of a single well being inoculated with a viable cell is based on the culturability of the heat treated population on solid medium.

Results presented in Table 4.4 show that 100% recovery of the heat treated cells can be achieve after 18h of non-selective enrichment in BPW or 2SBW media. A very good recovery (99.5%) was observed with TSB medium as well after 24h enrichment. The first two media include the same concentration of peptone (10g l^{-1}) and the third medium is a very rich containing both soya peptone and tryptone. Lactose broth which also contains peptone (5g l^{-1}) recovers 93.8% of the population after 24h enrichment. The poorer recovery after 24h enrichment was obtained with 2MM9 and then M-broth which contain no peptone. It appears that peptone is the common factor and indeed comprises of everything the injured cells require for repair and recovery.

These results of injured *Salmonella* repair in the different media are in agreement with recent findings showing great differences/variability between media recoverability of injured cells which is probably due to the peptone content and/or peptone source of the individual medium (Stephens *et al.* 1997). In addition the findings of this study agree with the results of Stephens *et al.* in that recovery (100%) of injured *Salmonella* can occur within 24h of enrichment in certain media.

The difference between the two studies lies in the extend of injury induced to the *Salmonella* population used for assessing media recoverability. As mentioned earlier, the results in this study show that the difference between non-selective and selective media do not represent the percentage injury of a heat treated culture. However, the culture method was used by Stephens *et al.* (1997) to calculate 97% injury after only 15 minutes of heat treating the culture at 53.5° C. The injured cells used in the current study have been heat treated for 180 minutes at 54° C and include a more adversely injured population. Studies which use such severely stressed cells have been suggested to result in more indicative true repair of injury (van Schothorst and van Leusden, 1975; Mossel and Corry, 1977; Mossel et al. 1980; Kaprelyants and Kell, 1993).

The difference of growth of sublethaly heat treated cells on different media can be due to a metabolic imbalance caused by heat that disrupts growth, and results in the self-destruction of the population through free-radical attack (Dodd *et al.*, 1997). The

difference between the direct destructive effect of the heat-treatment and bacterial self-destruction is critical in food microbiology.

This difference may be reduced when longer periods of heat-treatment at sub-lethal temperatures are employed. Therefore, cell viability studies and media evaluation studies may be more indicative of reality if cell populations used undergo such prolonged heat treatment and are severely injured.

CHAPTER 5

Detection of Salmonella using PCR

5.1 Introduction

5.1.1 The Development of Polymerase Chain Reaction

In 1983, Kary Mullis, a nucleotide chemist (Cetus Corporation, California), developed a cyclic procedure by which a single sequence of nucleic acids could be multiplied a billion fold over a few hours (Mullis, 1990). Polymerase chain reaction (PCR) is a method of amplifying a specific DNA or RNA sequence so that the presence of a specific sequence can be determined with extraordinary sensitivity. The success of PCR is dependent on a detailed knowledge of a target sequence to be amplified from which oligonucleotide primers can be synthesized that specifically anneal to the 3' and 5' terminals of the target sequence. The sequence initially needs to be present as a discrete molecule or part of a larger molecule, such as a single gene in human DNA (Mullis and Faloona, 1987). The human gene of β-globin was one of the first DNA sequences to be amplified by PCR (Saiki *et al.*, 1985).

PCR is a method comprising three stages: **a)** a source of DNA including the desired sequence is denatured by heat at around 94°C in the presence of a large molar excess of two specific oligonucleotide primers and the four deoxyribonucleoside triphosphates (dNTPs); **b)** the oligonucleotide primers are complementary to different strands of the sequence to be amplified. They hybridize / anneal between 50 to 70° C onto the homologous positions at each end of the sequence so that the DNA

polymerase extension product of one can serve as a 'template' for the other when denatured, and vice versa (Mullis and Faloona, 1987); c) during the first few cycles of the reaction extension / polymerisation of the nucleic acid sequence at around 70° C of each oligonucleotide primer on the original template produces long single stranded DNA molecules that act as templates for successive primers. However, during the second primer extension, the polymerization is terminated at the 5' end of the primer on the long single stranded DNA template. The produced molecule is of a distinct length and is a copy of the desired target sequence. By changing the temperature of the reaction adjusted for each stage of the procedure and with the use of a thermostable DNA polymerase, a chain reaction can be sustained which accumulates a specific double stranded DNA product called the amplicon, at an exponential rate relative to the number of cycles (Saiki et al., 1988).

The high sensitivity and superb specificity of PCR amplification assays have made the technique fundamental to many areas of biological research including molecular biology, biotechnology, medicine research and environmental microbiology (Bej et al., 1991; Steffan and Atlas, 1991). It has been used extensively in microbiology for the bacterial detection and identification, as PCR is more sensitive than other methods such as ELISA, DNA hybridisation and immunofluorescence (Rahn et al., 1992). PCR is an extremely sensitive tool in diagnostic microbiology and is recommended for the rapid detection of pathogens in a range of samples, including food, by many researchers (Ruano et al., 1989; Steffan and Atlas, 1991; Widjojoatmodjo et al., 1991 and 1992; Rahn et al., 1992; Mahon et al., 1994). The high specificity of PCR depends on the genus-specific oligonucleotide primers, which can specifically anneal

to the target template, even when the target DNA is highly homologous with DNA from many other bacterial species (Bej *et al.*, 1992; Cohen *et al.* 1993).

5.1.2 PCR and Assay Evolution

Variations of normal PCR amplifications utilising one set of primers and standard cycle conditions have been developed in order to increase specificity and sensitivity of the assay. Nested and multiplex PCR assays are both highly specific and sensitive assays, detecting as low as 10 cells. These assays utilise two or more oligo-nucleotide primer sets for the detection of *Salmonella* and other pathogens in food, clinical and environmental samples (Niederhauser *et al.*, 1992; Song *et al.*, 1993; Way *et al.*, 1993; Mahon *et al.*, 1994).

Magnetic immuno PCR assay (MIPA) was developed to avoid sample interference with PCR amplification, and to increase the specificity of *Salmonella* detection. This procedure involves the extraction of target cells from complex food or clinical matrices, using magnetic particles coated with monoclonal antibodies targeted to *Salmonella*. The PCR amplification step then uses the mAb bound cells as templates (Widjojoatmodjo *et al.*, 1991 and 1992; Fluit *et al.*, 1993a and b). Better specificity is attributed to mAb-based cell extraction. MIPA is an extraordinarily sensitive assay detecting between 10-100 cfu of *Salmonella* g^{-1} food (Widjojoatmodjo *et al.*, 1991). A sensitivity level of 0.1 cfu g^{-1} *Salmonella* in food is possible when the sample is pre-enriched for 24h prior to MIPA (Fluit *et al.*, 1993). In addition, MIPA allows the

detection of 10^3 cfu of *Salmonella* in the presence of 10^7 cfu of non-salmonellas with no detectable interference (Fluit *et al.*, 1991).

Problems have been identified with PCR including false positive detection and identification due to amplicon contamination (Persing, 1991). Physical separation of the pre- and post-PCR working areas is an absolute requirement, as well as the use of dedicated and or disposable supplies and pipetting devices for reaction preparation to eliminate contamination and amplicon carryover. Contamination between samples is a problem primarily because the large number of manipulations of the sample prior to PCR amplification (Widjojoatmodjo *et al.*, 1992; Soumet *et al.*, 1994). Reducing the number of manipulations prior to amplification during sample preparation, contamination problems can be minimised. Therefore, DNA extraction, template preparation and assay preparation should be separated.

Synthesis of primer-dimers and other non-target amplification products also hinders PCR amplification and consume the stock of primers and enzyme in the reaction mixture. This occurs when there is a low number of template DNA copies <1000, and consequently causes a reduction in the yield of target product (Ruano *et al.*, 1989). The problem of low template concentrations and primer-dimer production can be overcome by using "Booster" PCR. This is a biphasic variation of PCR amplification that uses: a) an initial primer concentration lower than standard, equivalent to about 10^7-fold molar excess to DNA template during the first stage of PCR; b) the primer concentration is then increased to standard PCR levels of around 0.1 μM at the beginning of the second stage. Booster PCR amplification is of a particular

advantage when the number of template copies is between five and ten (Ruano *et al.*, 1989).

Booster PCR has been used for the detection of bacterial DNA in complex samples, such as faecal specimens (Saulnier and Andremont, 1992; Cohen *et al.*, 1994a). Compared to traditional culture procedures for the detection of *Salmonella*, "booster" PCR is more sensitive, and can be used to detect 1cfu g^{-1} faeces, compared to standard culture when samples containing 100 cfu g^{-1} sample remain negative (Cohen *et al.*, 1994a).

5.1.3   *Salmonella Detection in Foods using PCR*

PCR amplification is inhibited when it is applied directly to complex matrices. It has been shown that no amplification product, poor assay sensitivity and high numbers of false positive results may be obtained with direct PCR amplification of both food and environmental samples (Li *et al.*, 1988; Fluit *et al.*, 1993; Soumet *et al.*, 1994; Tsen *et al.*, 1994; Cohen *et al.* 1994b). Direct detection of *Salmonella* in oyster and chicken meat, is not possible even when the concentration of *Salmonella* is as high as 10^2 cfu g^{-1} sample or even 10^7 cfu g^{-1} sample, respectively (Bej *et al.*, 1994; Fluit *et al.*, 1993b). Foodstuffs such as mayonnaise-based chicken salad and soft cheeses like brie and blue-veined brie considerably inhibit PCR amplification detection of *Salmonella* probably due to low DNA recovery or interference by components of the food sample (Rossen *et al.*, 1992). In cheese samples these components may be proteinases, which inhibit the polymerase activity, concentrated protein or hydrolysed protein in the PCR

mixture (Rossen *et al.*, 1992). PCR sensitivity for food can be improved through sample enrichment prior to PCR amplification. With reasonable cell numbers and a sufficient enrichment step the PCR system can be more reliable and sensitive, even in the presence of high numbers of background microflora. PCR sensitivity range is from 1 to 10 cfu of *Salmonella* g^{-1} oyster meat after a 3h enrichment; 1 cfu of *Salmonella* 25g^{-1} of chicken meat after 8 to 10h pre-enrichment and between 1-10 cfu *Salmonella* g^{-1} beef meat in the presence of 4.3x10^4 cfu non-salmonella microflora g^{-1} sample, after 24h enrichment (Bej *et al.*, 1994; Soumet *et al.*, 1994; Tsen *et al.*, 1994).

Enrichment of food samples, and detection of amplification products non-detectable prior to sample enrichment, also demonstrates the presence of viable bacterial cells in food samples. The proliferation of target cells ensures that DNA detected is not a remnant of dead bacteria or a false interpretation due to DNA contamination, i.e. reduces risks of false positives (Bej *et al.*, 1994). False positive PCR results due to the presence of dead *Salmonella* cells can occur with cold seawater or other environmental samples in which DNA can persist for several weeks without degradation (Josephson *et al.*, 1993; Dupray *et al.*, 1997).

The advantage of combining sample enrichment with PCR amplification, whether enrichment is selective or non-selective, is the **increase in the number of target templates**. An additional advantage of selective enrichment is the **increased ratio of target to non-target DNA sequences** (Steffan and Atlas, 1991). However, selective enrichment of samples has two major disadvantages: a) selective media can be toxic to injured *Salmonella* cells that need to be resuscitated in non-selective pre-enrichment

media in order to proliferate; and b) carryover of some selective media can severely inhibit PCR amplification, while non-selective media do not interfere with PCR amplification (Rossen et al., 1992). Non-target DNA sequences do not interfere with PCR amplification, unless they are present in very high concentrations, like 0.4mg DNA 100µl^{-1} reaction mixture (Rossen et al., 1992).

5.1.4 DNA Template Preparation for PCR

The procedure of DNA extraction and template preparation is important for a successful PCR amplification (Rossen et al., 1992; Jones et al., 1993; Soumet et al., 1994; Tsen et al., 1994). Template preparation can eliminate PCR-inhibitory components from the sample, and produces a concentrated target DNA suitable for the amplification assay (Soumet et al., 1994). However, inhibitors may become concentrated along with the DNA and interfere with PCR amplification. Therefore, it is important that remnants of chemical inhibitors from the DNA extraction / preparation process are not carried over to the PCR reaction mixture (Weyant et al., 1990; Rossen et al., 1992).

Typical DNA extraction/preparation involves digestions of the bacterial cells using incubation steps with lysozyme and proteinase K, phenol and phenol-chloroform extraction / purification steps, and ethanol DNA precipitation steps in order to obtain an amplifiable DNA template (Walsh et al., 1991; Tsen et al., 1994). While the methods utilising these steps are successful in obtaining PCR grade DNA, the lengthy and multistep manipulations involved make them less desirable for routine analysis of

high numbers of samples, and more likely to cause cross contamination (Walsh *et al.*, 1991). Thus, short and simple DNA extraction procedures are preferred to complex and time consuming procedures which also use a number of PCR inhibitory components (Rossen *et al.*, 1992).

Guanidium thiocyanate DNA extraction is a rapid method, that avoids the proteinase and phenol:chloroform treatment steps, and produces high purity, high molecular mass DNA for PCR amplification (Pitcher *et al.*, 1986). However, it is a multistep procedure.

Crude DNA extraction procedures could be compared with guanidium thiocyanate DNA extraction for PCR amplification. Crude unpurified DNA extracts, by boiling of cells for 15 minutes in sterile distilled water or Tris-EDTA (TE) buffer, are successfully used for DNA amplification by PCR (Gibson and McKee, 1993; Hilton *et al.*, 1997). PCR amplification using crude DNA extraction and template preparation can result in almost identical results to those generated from a purified template (Hilton *et al.*, 1997). However, crude DNA extracts can suffer from the presence of thermostable nucleases that digest nucleic acids, even when extracted DNA is refrigerated over 24h at 4°C, and therefore render the extracted DNA unsuitable for PCR amplification (Walsh *et al.*, 1991; Gibson and McKee, 1993; Hilton *et al.*, 1997). However, this is not a major problem since DNA can be protected from thermostable nuclease activity by the addition of EDTA (Walsh *et al.*, 1991; Gibson and McKee, 1993). The advantages offered by crude exractions are,

primarily the reduction of sample manipulations, and secondly both the reduction of time and costs.

5.1.5 Detection of PCR Products

The amplification products obtained by PCR are traditionally detected by gel electrophoresis of the stained DNA with ethidium bromide. This type of UV visualisation of the products can be very tedious and cumbersome when a high number of sample products are to be tested.

Other techniques such as DNA hybridization and fluorescence detection can be used for PCR product detection, and can be 1-2 orders of magnitude more sensitive than traditional gel electrophoresis (Cano *et al.*, 1993; Rusmussen *et al.*, 1994). Even though the sensitivity of such detection techniques is higher than gel electrophoresis the latter remains a more cost effective option.

5.2 Aim

In view of the exciting PCR detection of *Salmonella* the aim of this project was to develop a procedure for *Salmonella* detection in food using PCR directly after pre-enrichment of the samples. A rapid, simple and cost effective DNA extraction procedure will be used. The ultimate objective of the study is to rapidly detect very low numbers of injured *Salmonella* cells in foodstuffs, in the presence of high cell numbers of closely related species.

5.3   Materials and Methods

5.3.1   Preparation of Pure Salmonella Cultures

20 ml of BPW, 20 ml of Salmosyst and 2MM9+4g glucose media in universals were inoculated with stock cultures of the organisms and incubated for 24h at 37° C in a static incubator. CRM capsules of *S. typhimurium* and *S. panama* were removed from -20°C storage and kept at room temperature 25°C under sterile conditions for 20-30 minutes before use.

5.3.2   Food Spiking

The food samples: country pate, breaded brie cheese, raw chicken breast and chocolate mousse were obtained from Whitbread plc and were stored at -20°C sealed in their original package. These food samples were chosen because they are common *Salmonella* carriers and they are all, with the exception of the chicken sample, complex matrices that present problems to the conventional culture *Salmonella* detection procedure. 2g food samples were sterilised by autoclaving in universal bottles, at 121° C for 15 minutes. The sterilised food samples in the universal bottles were stored refrigerated for a maximum of 30 days before use.

Artificially contaminated food samples were prepared by homogenising the 2g food samples by stomaching in 18 ml of the required medium, in sterile stomacher bags for 2 minutes. 1ml of pure or pre-diluted *Salmonella* culture was added to the homogenised

sample in the pre-enrichment medium. Homogenised food samples in 18ml media were also inoculated with 1 CRM capsule when required.

5.3.3 Guanidium Thiocyanate DNA Extraction

A guanidium thiocyanate DNA extraction procedure was used in these studies and was based on the procedure of Pitcher et al., (1986). Broth cultures of Salmonella were harvested from the end of the exponential phase of growth and centrifuged at 3000 rpm for 15 min using a Denley BS400 centrifuge. The cell pellet was resuspended in 100 ml TE buffer (10mM Tris pH 8.0, 1mM ethylene di-amine tetra-acidic acid (EDTA; Analar, Sigma)). The cells were lysed with 0.5 ml of GES reagent (5 mol l^{-1} guanidium thiocyanate (Sigma), 100 mmol/l EDTA and 0.5% v/v sarkosyl (Sigma)) which was mixed well with cell suspension by vortex on a whirlimixer for about 5 to 10 minutes. The lysate was then cooled on ice, and 0.25 ml cold 7.5 mol l^{-1} ammonium acetate was added with mixing, and the samples held on ice for a further 10 minutes. 500μl of chloroform:2-pentanol 24:1 mixture was added and the phases mixed thoroughly, transferred with a wide-bore Pasteur pipette to a 1.5 ml Eppendorf tube and centrifuged for 10 minutes using the Eppendorf centrifuge 5412 at maximum speed. Supernatant was transferred to Eppendorf tubes and 0.54 volumes of cold 2-propanol added. The tubes were mixed gently for 1 minute to mix the solutions and the fibrous DNA precipitate was pelleted by centrifugation using the Eppendorf centrifuge 5412, at maximum speed for 20 seconds. DNA pellets were washed five times in 70% ethanol by resuspension and

centrifugation, and then dried under vacuum. The dry DNA samples were re-dissolved overnight in 100 µl of sterile deionized water at 4°C.

The GES reagent was prepared as follows:

60g of guanidium thiocyanate was added to 20ml 0.5 mol l^{-1} EDTA at pH 8 and 20ml deionized water and was heated to 65°C with mixing until dissolved. After cooling to 25°C 5ml of 10% v/v sarkosyl was added, and the solution made up to 100ml with deionized water, filtered through a 0.2 µm Nalgene filter (BDH Ltd) and stored at room temperature.

5.3.4   Crude DNA Extraction and Template Preparation

DNA extraction was carried out in three stages to determine possible interference by the extraction components to consequent PCR amplification. The first stage is termed the crude DNA extraction, and the second and third stages are termed template preparation.

Crude DNA extraction -

Stage 1: Cells from pre-enriched broth cultures were harvested at the end of the exponential phase by centrifugation at 3000 rpm in a Denley BS 400 centrifuge. The cell pellet was resuspended in 100µl TE buffer (10mM Tris pH 8.0, 1mM EDTA) and boiled for 15 minutes in a water bath and cooled on ice.

Template preparation -

Stage 2: 250μl of cold ammonium acetate (7.5 mol l⁻¹) was added with mixing, and held on ice for about 10 minutes. 0.5 ml chloroform : 2-pentanol 24:1 mixture was added and mixed by vortex and transferred with Pasteur pipette to a 1.5ml Eppendorf tube. This was centrifuged for 10 minutes using an Eppendorf 5412 centrifuge at maximum speed.

Stage 3: Supernatant was transferred to fresh Eppendorf tubes and 0.54 volumes of cold 2-propanol were added with gentle mixing, and the DNA was precipitated by centrifugation for 20 seconds. The DNA pellet was washed once in 100μl of 70% v/v ethanol, dried under vacuum and then resuspended in 100μl sterile deionised water. The resuspended DNA was stored refrigerated in 0.5ml Eppendorf tubes when necessary.

The samples obtained after crude extraction, *stage 1*, were either used for PCR directly or further sample preparation, *stage 2 & 3*, were carried out when required.

5.3.5   *Assay Reagents and Conditions for PCR*

The PCR amplification reagents and conditions were those of Tsen *et al.* (1994). The *Salmonella* specific primers used for annealing a *Salmonella* specific 1.8 Kb *Hind* III fragment, were identified by Tsen *et al.* (1991a and b). The primers used in this study are **TS11** 5' GTCACGGAA GAAGAGAAATCCGTACG 3' 26-mer, Base 26-51 in the 1.8

Kb *Hind* III fragment, and **TS5** 5' GGGAGTCCAGGTTGACGGAAAATTT 3' 25-mer,

reversal and complementary sequence for base 376-400 in the 1.8 Kb *Hind* III fragment.

The PCR reaction mixture consisted of: 2µl sample DNA extracted and prepared

according to the above methodology; 2µl of each dNTPs (10mM mix, Sigma D7295);

10µl reaction buffer (10x; 750 mM Tris Hcl, pH 9.0, 200 mM $(NH_4)_2SO_4$, 0.1% w/v

Tween, Advanced Biotechnologies, UK); 6µl $MgCl_2$ (25 mM, Advanced

Biotechnologies, UK); 0.5µl each of the primers (TS11 / TS5; 200 pmol µl⁻¹, Genosys,

UK); 0.5 µl DNA polymerase (5 U µl⁻¹; AB-0192/A, Advanced Biotechnologies, UK);

80µl PCR grade sterile water (Sigma); and 50µl overlay of sterile mineral oil.

The cyclic conditions used for the PCR amplification, using Techne PHC-1A Dri Block

Thermocycler, were: 1.5 minutes at 94°C   DNA denaturation;   1.5 minutes at 66°C,

primer annealing; and 3.0 minutes at 72°C, DNA polymerization/ primer extension. The

reaction was performed for 30 cycles, after which 5µl of product was analysed by agarose

gel electrophoresis. PCR products were run on 1.5% agarose gel (Sigma, A9539; w/v in

TBE buffer; 89 mmol l⁻¹ Tris, 89 mmol l⁻¹ boric acid, 2 mmol l⁻¹ EDTA, pH 8.3), at 50 v

cm⁻¹ for approximately 30 minutes. A PCR marker / DNA ladder was also run along side

the PCR products on the agarose gel to determine the position of the 375bp PCR product

expected for a *Salmonella* positive result. The PCR marker (Sigma, P9577) comprised of

the following bands: 2.0 Kb, 1.5 Kb, 1.0 Kb, 750bp, 500bp, 300bp, 150bp, and 50bp.

After electrophoresis the agarose gel was then soaked for 20-30 minutes in TE buffer

with 0.5 mg ml^{-1} ethidium bromide, and DNA bands were observed and photographed by Polaroid on a UV transilluminator (New Branswick Scientific).

5.3.6 Sensitivity and Specificity Assurance of the PCR Assay

The sensitivity of the amplification assay was determined by detecting fresh DNA extracts of a known number of target *S. typhimurium* or *S. enteritidis* cells. Also PCR assay sensitivity was determined with decimal serial dilutions of calculated DNA concentrations using absorbance measurements (section 5.3.7).

The specificity of the assay was also determined, even though the primers were reported to be *Salmonella*-specific by Tsen *et al.* (1994). Crude DNA extracts of pure cultures of *C. freundii*, *E. coli*, *E. feacalis*, *P. mirabilis* and *K. pneumoniae* were used to test for possible non-specific primer annealing.

Cross reactivity and interference of non-salmonellas with PCR amplification was tested. PCR assay was performed to detect *Salmonella* growing in three pre-enrichment media, BPW, Salmosyst, and 2MM9+4g glucose. These assays were performed using pure *Salmonella* cultures or *Salmonella* in mixed with high numbers of related bacteria. The appropriate serial dilutions of pure cultures of *S. typhimurium*, *E. coli*, *K. pneumoniae*, *C. freundii*, *E. feacalis* and *P. mirabilis* growing in TSB were mixed together and pre-enriched in the different media at 37°C for 8 h.

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To obtain uniformity throughout the work for this thesis, the number and the state of viability of *Salmonella* cells in the initial sample inoculum were standardised by the use of Certified Reference Materials - CRM capsules - containing 6 cfu of *S. typhimurium* or *S. panama*. 20ml of each media in universal bottles or 2g of the homogenised food samples in 18 ml broth, were inoculated with one CRM capsule and incubated for 24 h at 37° C. 1ml samples were taken for DNA extraction at time periods t_4, t_6, t_8, t_{10}, and t_{24} during enrichment. Negative controls were taken at t_0 before inoculation of the sample.

5.3.7   *DNA Concentration Measurements*

The DNA concentrations in the extracts of the crude extraction and template preparation procedure were measured using the absorbance reading in 1cm light path in a quartz cuvette at 260nm and 280nm using a UNICAM spectrophotometer. One unit of absorbance at 260nm, in a 1cm light path, is equivalent to a concentration of 50 μg ml^{-1} of double stranded DNA, or 40 μg ml^{-1} of single stranded DNA and RNA. The DNA purity can be determined by calculating the ratio of the absorbance at 260nm to the one at 280nm, which should be 1.8 for DNA and 2.0 for RNA. Ratios lower than this indicate protein contamination and accurate determination of the nucleic acid concentrations will not be obtained (Slater, 1986).

5.4 Results of PCR Detection of Salmonella

PCR is probably the only method sensitive and specific enough to detect <10 cfu of injured *Salmonella* g^{-1} foodstuffs in the presence of 10^6 cfu g^{-1} of non-salmonellas. This chapter shows how this was achieved using two-stage pre-enrichment and a rapid crude DNA extraction and template preparation system.

Figure 5.1 shows the effectiveness of a crude DNA extraction procedure for subsequent PCR amplification and *Salmonella* detection. The DNA extraction procedure used was separated into three stages. *Stage 1* was termed the crude extraction; *stages 2* and *3* were termed the template preparation or "cleaning" step. DNA extracts at the end of each stage were successfully amplified by PCR and target 375bp-products were obtained as shown in Fig. 5.1, lanes a to f , comparable to the amplification of control genomic DNA extracted using the guanidium thiocyanate procedure as shown in Fig. 5.1, lane C.
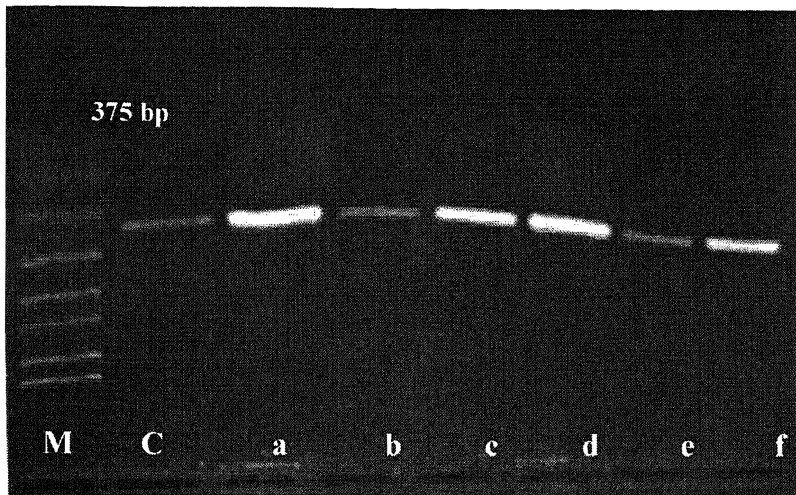
Fig. 5. 1 : PCR amplification of *Salmonella typhimurium* (a,b,c), and *S. enteritidis* (d,e,f) DNA extracted using a crude extraction procedure. Control genomic *Salmonella* DNA (C) was extracted using a modification of the Pitcher *et al.* (1989) quanidium thiocyanate procedure. Lanes a-f, indicate the presence of the amplified product, a *Salmonella* specific 375bp sequence, which appears between the bands 5-6 of the PCR marker (M) used. Samples (2 μl) were taken at different stages of crude extraction in order to determine the template DNA suitability for PCR, and compared with the guanidium thiocyanate extraction. Lanes a and d are samples taken at *stage 1*; lanes b and e are samples taken at *stage 2*; and lanes c and f are samples taken at *stage 3*. Successful PCR amplification occurs with all samples and *Salmonella* detection appears to be possible with samples after the first stage of the extraction procedure.

Figure 5. 2 shows the sensitivity of the amplification assay, as determined with fresh DNA crude extracts of known cell concentration. The sensitivity was found to be twenty cells of *S. typhimurium* and *S. enteritidis* in pure TSB cultures.
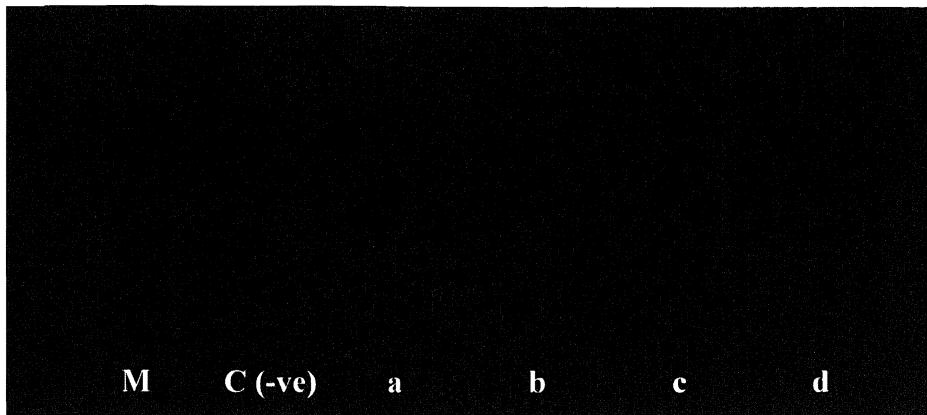
Fig. 5. 2 : PCR amplification products of different cell numbers of pure cultures of *Salmonella* growing in TSB medium. DNA was extracted using the crude extraction. Lane M is the PCR marker; C is the negative control which included TSB medium before inoculation with *Salmonella*; lane a (20 cells); lane b (100 cells); lane c (6.5×10^3 cells); and lane d (460×10^3 cells).

Figure 5. 3 shows the sensitivity of PCR detection with calculated DNA concentrations to be 40-50pg, after crude extraction and template preparation. However, this is an underestimated sensitivity of the actual PCR assay since the ratio of A_{260}/A_{280} of the extract indicated protein contamination. Therefore, higher absorbance values should be expected in the presence of protein that would over-estimate the DNA concentration in the extract.
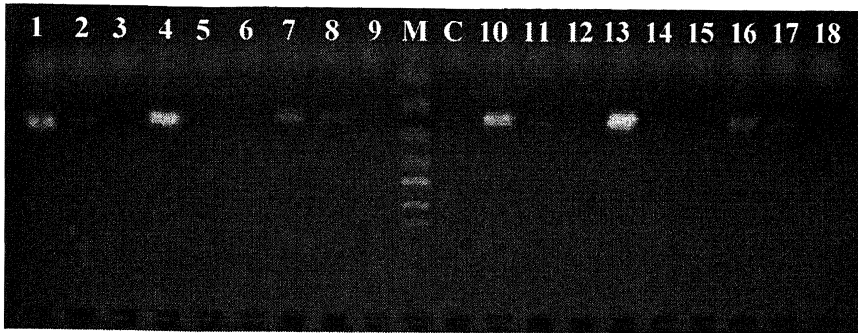
Fig. 5. 3 : PCR sensitivity is demonstrated in terms of DNA concentration (as opposed to number of cells), which was calculated using spectrophotometric measurements of the extracted DNA at 260 and 280 nm. Lanes 1-6 and 10-15 represent PCR amplifications with *S. typhimurium*, and lanes 7-9 and 16-18 with *S. enteritidis*. Lanes 1+10 (41ng DNA), lanes 2+11 (41pg DNA), lanes 3+12 (41fg DNA), lanes 4+13 (30ng DNA), lanes 5+14 (30pg DNA), lane 6+15 (30fg DNA), lane 7+16 (49ng DNA), lanes 8+17 (49 pg DNA), and lanes 9+18 (49fg DNA). Lanes 2, 8, 11 and 17 show a faint positive 375bp product.

Figure 5. 4 shows that the primers used in the assay were *Salmonella*-specific and agree with the results of Tsen *et al.* (1994). Target PCR product was only obtained with *S. typhimurium*. Fresh DNA crude extracts from pure 24h old cultures of *Salmonella* related and sometimes interfering species, such as *C. freundii*, *E. coli*, *E. feacalis*, *P. mirabilis* and *K. pneumoniae*, resulted in no PCR products.
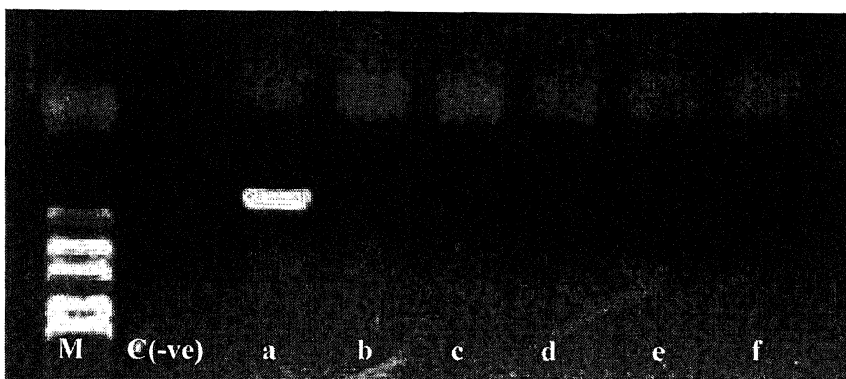
Fig. 5. 4 : PCR amplification specificity (using primers TS5/TS11) is demonstrated by the detection of amplified *Salmonella* DNA product (lane a; *S. typhimurium*) and no amplification product in lanes b (*C. freundii*), c (*E. coli*), d (*E. faecalis*), e (*P. mirabilis*), and f (*K. pneumoniae*). All DNA used for PCR amplification was extracted, using the crude extraction procedure, from 24h-old culture samples (1 ml) of each organism.

When the crude DNA extracts were kept refrigerated at 4°C for 24h or more, no PCR amplification product could be obtained. This indicated the possible presence of thermostable nucleases in the crude extracts degrading the DNA to a non-amplifiable state. Figure 5. 5 shows that further cleaning of the crude extracts, using template preparation procedure (*stages 2 and 3*), proved effective in eliminating DNA degrading enzymes and resulted in PCR amplification of 48h old extracts.
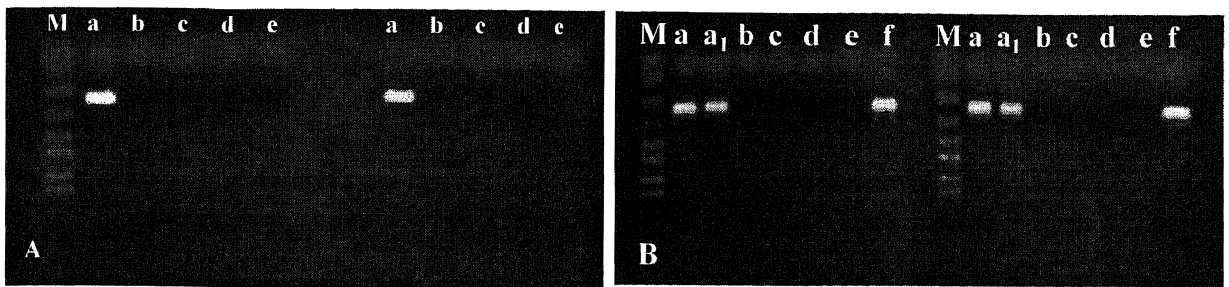
Fig. 5. 5 : PCR amplification of crude DNA extract after template preparation. The pure cultures used are: lane a (*S. typhimurium*), lane a_1 (*S. enteritidis*), lane b (*C. freundii*), lane c (*E. coli*), lane d (*P. mirabilis*), lane e (negative control), and lane f (*S. typhimurium*, 48h-old refrigerated DNA). Replicates are presented on both A and B plates. No chemical interference with PCR amplification was obvious from the DNA-cleaning compounds, and the assay specificity was not affected.

Figure 5. 6 shows that a 24h incubation period is required for a positive PCR detection of *Salmonella* when media were inoculated with 1 CRM capsule of *S. typhimurium*. BPW and Salmosyst media were inoculated with 1 *S. typhimurium* CRM capsule, incubated for 24 hours, and crude extraction of DNA was performed on the samples taken. PCR amplification of the crude extracts resulted in a positive detection of the organism after 24 h, when the cell concentration was 10^9 cfu ml^{-1}. The 375bp PCR product in lane 5 shows that 24h pre-enrichment was required for 6 injured *Salmonella* cells in the spray-dried milk of the CRM capsules (in't Veld *et al.*, 1996) to reach detectable levels. *Salmonella* concentration reached detectable levels 2.2x10^9 cfu ml^{-1} at t_{24} in Salmosyst medium. However, no amplification product was obtained indicating the possible presence of PCR inhibitors in the medium. Therefore, PCR *Salmonella* detection in Salmosyst broth may require the performance of DNA template preparation to eliminate PCR-inhibitory effects of the medium.
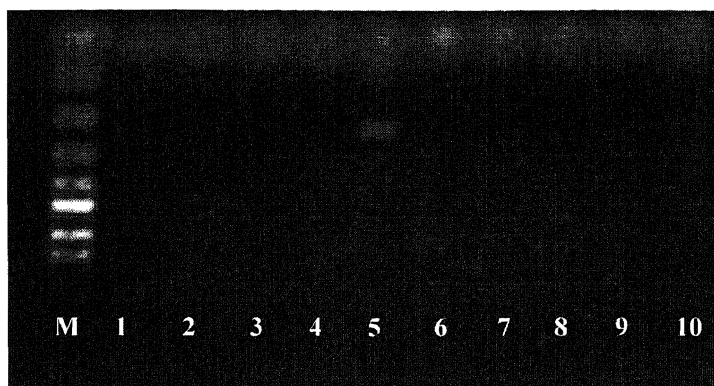
Fig. 5. 6 : PCR amplification of pure cultures of *S. typhimurium* growing in BPW (lanes 1-5) and Salmosyst (lanes 6-10) media. Each medium (20 ml) was inoculated with one CRM capsule, incubated for 24 h at 37° C, and sampled at t_4, t_6, t_8, t_{10}, and t_{24}. Crude DNA extractions were carried out with no template preparation. Lane 1-5 represent the samples $t_{4, 6, 8, 10, 24}$ in BPW; and lanes 6-10 represent the samples $t_{4, 6, 8, 10, 24}$ in Salmosyst broth.

Figure 5. 7 shows that inoculation of 2MM9+4g glucose medium with 1 *Salmonella typhimurium* CRM capsule resulted in a positive PCR *Salmonella*-detection after a 24h incubation period. PCR amplification was performed using crude extracts, after template preparation. Even though the number of cells reached PCR detectable levels by t_{10} with 10^3 cfu ml^{-1}, detection of target DNA product was not possible.

It may be suggested that 10^3 cfu of *Salmonella* ml^{-1} sample remain undetected, in BPW (Fig. 5. 6) and 2MM9 (Fig. 5. 7), due to the inhibitory effect of gelatine surrounding the capsule or the amount of spray-dried milk in the capsule.
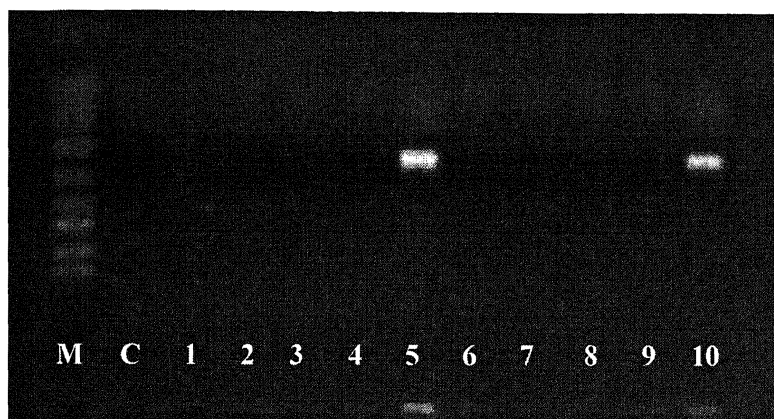
Fig. 5. 7 : PCR amplification of *S. typhimurium* growing in 2MM9+4g glucose medium inoculated with 1 CRM capsule and incubated for 24h at 37° C. Samples were taken after 4, 6, 8, 10, and 24h after inoculation, and DNA was extracted using the crude extraction and template preparation procedure. Lanes 1-5 represent samples t_4, t_6, t_8, t_{10} and t_{24}; these samples are duplicated in lanes 6-10. 24 h of incubation in 2MM9+4g glucose is required for a positive PCR amplification, when inoculated with a CRM capsule.

Figure 5. 8 shows that BPW is probably the most efficient medium, for sample enrichment with high numbers of background microflora, for subsequent *Salmonella* detection using a PCR assay. Three media: BPW, Salmosyst and 2MM9+4g glucose, were inoculated with different concentrations of *S. typhimurium* in the presence of high numbers of 5 non-salmonella species, incubated for 8h at 37° C, and PCR amplification was carried out after crude DNA extraction and template preparation. BPW was the only medium which resulted a positive PCR detection of *Salmonella*, after 8 hours of incubation. BPW promotes a better *Salmonella* growth and the PCR detection is probably due to a higher number of *Salmonella* after 8h incubation compared to the other media (Fig. 5. 8).

These studies show that it was possible to detect <10 cells of *Salmonella* in the presence of around 1.3 x 10^6 of non-salmonellas, and approximately 20 cells of *Salmonella* in the presence of around 13 x 10^6 non-salmonellas. This shows the high specificity and sensitivity of the PCR assay for the detection of *Salmonella* and the potential use of the assay as a routine analysis tool for pathogen detection in the presence of non-pathogenic species.
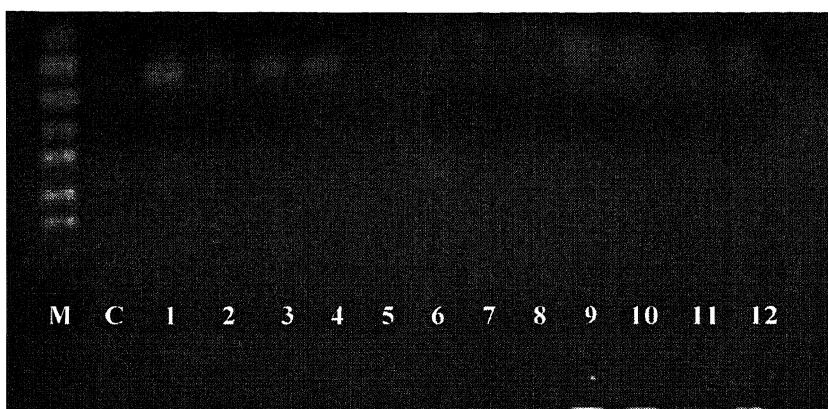
Fig. 5. 8 : PCR detection of *Salmonella* in inoculated BPW (lanes 1-4), Salmosyst (lanes 5-8) and 2MM9 (lanes 9-12) media with low numbers of *S. typhimurium* cells and high numbers of *E. coli*, *E. faecalis*, *K. pneumoniae*, *P. mirabilis* and *C. freundii*. The media were incubated at 37° C for 8 h, and DNA extraction and template preparation was carried out. Fresh extracts were used for PCR. BPW appeared to be the better medium which can bring the number of *Salmonella* to detectable levels, after 8h incubation, without interfering with the DNA amplification assay.

Lanes 1, 5, 9: media inoculated with <10 *S. typhimurium* cells, 300x10^3 cells of each *E. coli*, *K. pneumoniae* and *C. freundii*, and 200x10^3 cells of each *E. feacalis* and *P. mirabilis*.

Lanes 2, 6, 10: media inoculated with <10 *S. typhimurium* cells, 3x10^6 cells of each *E. coli*, *K. pneumoniae* and *C. freundii*, and 2x10^6 cells of each *E. feacalis* and *P. mirabilis*.

Lanes 3, 7, 11: media inoculated with approximately 20 *S. typhimurium* cells, 300x10^3 cells of each *E. coli*, *K. pneumoniae* and *C. freundii*, and 200x10^3 cells of each *E. feacalis* and *P. mirabilis*.

Lanes 4, 8, 12: media inoculated with approximately 20 *S. typhimurium* cells, 3x10^6 cells of each *E. coli*, *K. pneumoniae* and *C. freundii*, and 2x10^6 cells of each *E. feacalis* and *P. mirabilis*.

Figure 5. 9 shows that PCR can detect 5 *S. typhimurium* cells in breaded cheese, country pate and chicken breast after a 24 hour pre-enrichment period in BPW, whereas pre-enrichment of chocolate mousse sample resulted in no PCR detection. The foods were homogenised in BPW, artificially inoculated with around 5 *S. typhimurium* cells, and incubated for 24h at 37°C. Samples were taken at different time periods during incubation, at t_4, t_6, and t_{24}, and crude DNA extraction and template preparation was performed.
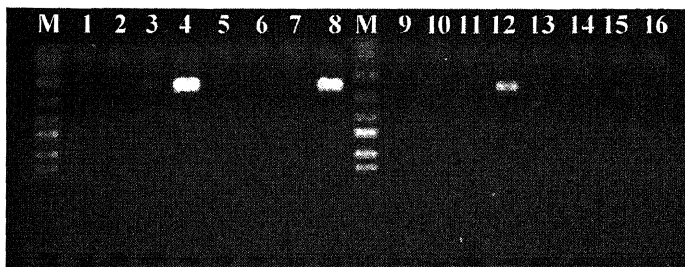
Fig. 5. 9 :   PCR detection of *S. typhimurium* DNA extracted from artificially inoculated foodstuffs, such as breaded brie cheese, country pate, chicken breast, and chocolate mousse. The food homogenates in BPW medium were inoculated with approximately 5 *S. typhimurium* cells of a 24 h-old culture, and incubated for 24 h at 37°C. Samples were taken after 4, 6, and 24h of incubation (t_4, t_6 and t_{24}) and controls were taken at t_0 before inoculation.
Lanes 2-5: Brie cheese samples at t_0, t_4, t_6 and t_{24}; **lanes 5-8**: Country pate samples at t_0, t_4, t_6 and t_{24}; **lanes 9-12**: Chicken samples at t_0, t_4, t_6 and t_{24}; and **lanes 13-16**: Chocolate mousse samples at t_0, t_4, t_6 and t_{24}.

Figure 5. 10 shows that 24 hour incubation is also required for PCR Salmonella-detection when food samples were inoculated with 1 CRM capsule of *S. typhimurium* and pre-enriched in 2MM9+4g glucose medium. The chocolate mousse sample resulted in no PCR product, while *Salmonella*-detection with PCR was possible with all the other food samples after 24h incubation. Both BPW and 2MM9 medium

appear to be suitable for subsequent PCR detection of *Salmonella* in foodstuffs, when the samples are artificially contaminated with no background microflora.
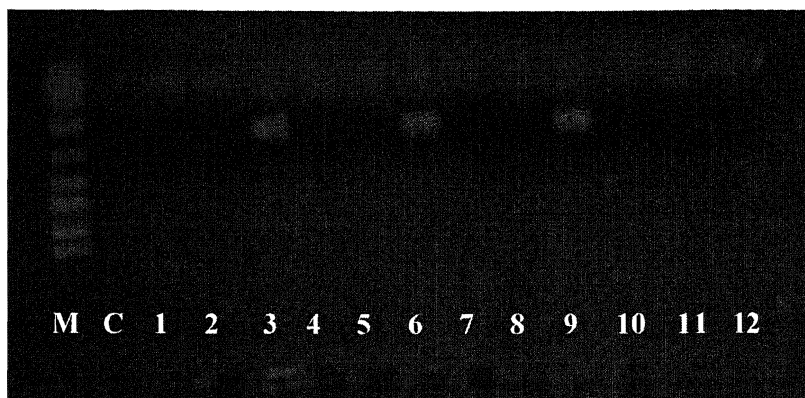
Fig. 5. 10 : PCR amplification of *S. typhimurium* DNA, extracted from food samples (2g) such as chicken (lanes 1-3), country pate (lanes 4-6), breaded brie cheese (lanes 7-9), and chocolate mousse (lanes 10-12), which were inoculated with one CRM capsule of *S. typhimurium*. The samples were pre-enriched in 2MM9 medium for up to 24h and samples were taken for crude DNA extraction and template preparation at t_4 (lanes 1, 4, 7 and 10), t_6 (lanes 2, 5, 8 and 11), and t_{24} (lanes 3, 6, 9 and 12). All samples resulted in positive PCR amplification detection, except from the chocolate mousse sample, after 24h incubation.

Figure 5. 11 shows that PCR detection of *Salmonella* was not possible in food samples inoculated with 1 CRM capsule and high numbers of non-salmonellas after a 24 hour pre-enrichment period in BPW. The food samples were homogenated in BPW, inoculated with 1 CRM *Salmonella* capsule and between 13×10^3 to 1.3×10^6 cells of 5 non-salmonella species. Performance of PCR assay with the 24h pre-enriched samples lead to false negative results. This was either: a) due to interference of high concentrations of non-target DNA since the same samples resulted in positive detection when the non-salmonella species were absent from the foods (Figure 5.9 and 5.10), or b) due to the suppressed growth of injured *Salmonella* in the CRM capsule due to nutrient competition. The chocolate mousse sample was PCR negative as with previous results shown in Figure 5.s 8 and 9.

Plate 1   **Plate 2**

Plate 3

Fig. 5. 11 : PCR detection of *Salmonella* in homogenized foodstuffs (2g of each country pate, breaded brie, chicken and chocolate mousse), in BPW medium, artificially contaminated with 1 CRM capsule of *Salmonella* and high numbers of *E.coli*, *E. feacalis*, *K. pneumoniae*, *P. mirabilis* and *C. freundii*. The foodstuffs were incubated for 24h at 37° C and samples were taken for crude DNA extraction and template preparation for PCR. Lanes M, +, and − represent the PCR marker, positive and negative controls of *Salmonella*, respectively. Samples in lanes 1-4 are duplicated in lanes 5-8 (in all plates presented). Lanes 1 and 5 are samples of country pate; lanes 2 and 6 are breaded-brie cheese samples; lanes 3 and 7 are chicken breast samples; and lanes 4 and 8 are chocolate mousse samples.

It shows that a low number of *Salmonella* of about 3cfu g^{-1} foodstuff in the presence of high numbers of total background bacterial microflora, approximately 13×10^3-1.3×10^6 cells, is not detectable in food stuffs after a 24h incubation in BPW.

In **plate 1** the samples were inoculated with 1 CRM capsule of *S. panama*, *E. coli* (2.6×10^3 cells), *E feacalis* (1.9×10^3 cells), *K. pneumoniae* (3.1×10^3 cells), *P. mirabilis* (2.3×10^3 cells), and *C. freundii* (3.1×10^3 cells).

In **plate 2** the samples were inoculated with 1 CRM capsule of *S. typhimurium*, *E. coli* (26×10^3 cells), *E feacalis* (19×10^3 cells), *K. pneumoniae* (31×10^3 cells), *P. mirabilis* (23×10^3 cells), and *C. freundii* (31×10^3 cells).

In **plate 3** the samples were inoculated with 1 CRM capsule of *S. typhimurium*, *E. coli* (260×10^3 cells), *E feacalis* (190×10^3 cells), *K. pneumoniae* (310×10^3 cells), *P. mirabilis* (230×10^3 cells), and *C. freundii* (310×10^3 cells).
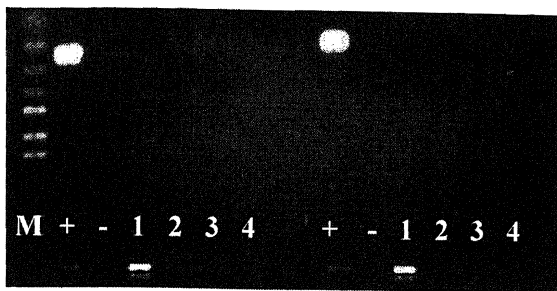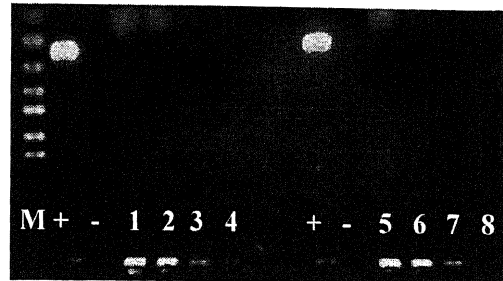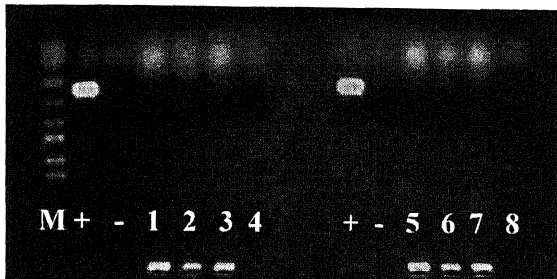
Plate 1

Plate 2

Plate 3

Fig. 5. 12 : PCR detection of *Salmonella* in 2g homogenized foodstuffs: country pate, breaded brie, chicken and chocolate mousse in BPW medium, inoculated with 1 CRM capsule of *Salmonella* and high numbers of *E.coli*, *E. feacalis*, *K. pneumoniae*, *P. mirabilis* and *C. freundii* cells. The foodstuffs were incubated for 24h at 37° C and then 1 ml of each pre-enriched sample was transferred to 20ml fresh BPW medium and incubated for a further 3h. 1ml samples were taken for crude DNA extraction and template preparation for PCR. Lanes M, +, and – represent the PCR marker, positive and negative controls of *Salmonella*, respectively. Samples in lanes 1-4 are duplicated in lanes 5-8 in all plates presented. Lanes 1 and 5 are samples of country pate; lanes 2 and 6 are breaded-brie cheese samples; lanes 3 and 7 are chicken samples; and lanes 4 and 8 are chocolate mousse samples.

It shows that a low number of *Salmonella* of approximately 3cfu g^{-1} foodstuff, in the presence of about 1.3×10^6 cells total background bacterial microflora, is detectable in food stuffs after a primary 24h incubation in BPW and a secondary 3h incubation in fresh medium.

In **plate 1** the samples were inoculated with 1 CRM capsule of *S. panama*, *E. coli* (2.6×10^3 cells), *E feacalis* (1.9×10^3 cells), *K. pneumoniae* (3.1×10^3 cells), *P. mirabilis* (2.3×10^3 cells), and *C. freundii* (3.1×10^3 cells).

In **plate 2** the samples were inoculated with 1 CRM capsule of *S. typhimurium*, *E. coli* (26×10^3 cells), *E feacalis* (19×10^3 cells), *K. pneumoniae* (31×10^3 cells), *P. mirabilis* (23×10^3 cells), and *C. freundii* (31×10^3 cells).

In **plate 3** the samples were inoculated with 1 CRM capsule of *S. typhimurium*, *E. coli* (260×10^3 cells), *E feacalis* (190×10^3 cells), *K. pneumoniae* (310×10^3 cells), *P. mirabilis* (230×10^3 cells), and *C. freundii* (310×10^3 cells).

Figure 5. 12 shows the extraordinary sensitivity and specificity of the PCR assay detecting 6 injured *Salmonella* cells in foods with 1.3×10^6 cells of 5 non-salmonella species after a 24h primary enrichment and a 3h secondary enrichment in BPW. The successful PCR *Salmonella*-detection followed a rapid DNA extraction and template preparation procedure.

In order to avoid the inhibitory effect of the high numbers of non-salmonella cells shown in Figure 5. 11, the 24h pre-enriched 1ml-samples were further diluted in fresh 20ml BPW and incubated for 3 hours. This secondary enrichment for 3h was carried out to increase the number of *Salmonellas* present and dilute PCR inhibitors to non-inhibiting concentrations. Secondary enrichment resulted in the PCR detection of *Salmonella* not only in the breaded brie, the chicken breast and the country pate, but also the chocolate mousse samples (Fig. 5. 12).

5.5 Discussion

The results in these studies show that detection of 3 *Salmonella* cells g^{-1} food in the presence of high background microflora, of about 10^6 cells of 5 non-salmonella species, is possible with PCR after the primary enrichment of the sample for 24h, and a secondary enrichment in the same pre-enrichment medium (BPW) for 3h. It is implied that the secondary 3h enrichment is essential for elimination of PCR-inhibitors in the food samples and for promoting *Salmonella* growth to reach detectable numbers. This result shows that PCR is an extremely sensitive and specific assay that can be used successfully as a rapid routine procedure for the detection of *Salmonella* in foods.

A rapid <2h crude DNA extraction and template preparation procedure was employed successfully to get amplifiable DNA from the food samples following the secondary enrichment. The foods tested with the PCR detection procedure included chicken breast, country pate, breaded brie cheese and chocolate mousse.

PCR detection of *Salmonella* using fresh crude DNA extracts of all three stages of extraction and template preparation shown earlier in Fig. 5. 1, clearly indicated PCR inhibitors are absent from samples. All stages of the extraction produced amplifiable DNA in subsequent PCR. Assay sensitivity and specificity were found to be satisfactory for fresh *stage 1*-extracted DNA from pure *Salmonella* cultures. Also when the crude extracts were stored refrigerated for 24h no further amplification product was observed. This agrees with the results of Gibson and McKee (1993) and is likely to be due to the presence of heat stable enzymes that degrade extracted DNA

to fragments that can not be amplified. The DNA degrading enzymes present a problem when the extracted DNA samples can not be used for PCR in the same working day. Template preparation, *stages 2* and *3*, of the crude extracts eliminated the loss of sensitivity due to DNA degradation. Template preparation allows deproteinization and precipitation of fibrous DNA from the cell debris present in the crude extracts. PCR specifically detected *Salmonella* in 48h-old refrigerated extracts obtained with crude extraction and template preparation (Fig. 5. 5).

PCR Sensitivity

As low as 20 cells of *S. typhimurium* and *S. enteritidis* could be detected in pure TSB cultures following the rapid crude DNA extraction without template preparation (Fig. 5. 2). This level of sensitivity is comparable to reported PCR sensitivity from pure cultures (Tsen *et al.*, 1994; Stone *et al.*, 1994; Soumet *et al.*, 1994). When the DNA concentration was measured, after crude extraction, the assay sensitivity was determined to be approximately 40-50 pg of DNA (Fig. 5. 3). However, protein contamination of the crude extract was indicated, which means that the actual assay sensitivity is higher than the calculated one (i.e. < 40-50 pg DNA should be detected).

PCR Specificity

The TS11/TS5 primers employed by PCR were found to be highly specific to *Salmonella* (Figure 5.s 4 and 5). No amplification product was observed with other species including *C. freundii*, *E.coli*, *E. faecalis*, *P. mirabilis* and *K. pneumoniae*. The specificity agrees with studies of Tsen *et al.* (1994, 1991a and 1991b) for PCR

amplification and DNA hybridization. Tsen designed the primers and tested their specificity with many organisms including Enterobacteriaciae.

PCR Interference and Detection of Salmonella in Pure Cultures

Rossen *et al.* (1994) reported that components of media used for food enrichment were found to interfere with PCR amplification. Selective enrichment media such as Rapaport Vassiliadis and tetrathionate broths, or components of other media were previously been reported to be inhibitory to PCR (Rossen *et al.*, 1994; Stone *et al.*, 1994). The suitability of three non-selective pre-enrichment media BPW, Salmosyst and 2MM9+4g glucose medium, with PCR was tested. The media were inoculated with 6 *Salmonella* cells using 1 CRM capsule per medium. CRM capsules of *S. typhimurium* and *S. panama* were used to standardise media inoculation since they contain a known number of cells of the same viability status. The results shown in Fig. 5. 6 imply that Salmosyst broth interferes with PCR and no PCR target-product was observed even when the *Salmonella* concentration reached 2.2×10^9 cfu ml^{-1} after a 24 hour incubation. Template preparation of the crude DNA extract may be necessary to eliminate PCR-inhibitors in the sample. Inhibition of PCR by Salmosyst broth has not been previously reported.

Even though the cell concentration in both BPW and 2MM9+4g glucose media at t_{10} reached detectable levels for PCR, about 10^3 cfu ml^{-1}, PCR could not detect *Salmonella* target-DNA. BPW and 2MM9+4g glucose medium resulted in a positive detection of *Salmonella* only after 24h of incubation as shown in Figure 5. 6 and 7. It

was recently reported that milk, specifically the Ca^{2+} ions in milk, inhibit the PCR assay (Bickley *et al.*, 1996). Therefore, the PCR failure to detect *Salmonella* at t_{10} could be due to the presence of inhibitory substances such as the gelatine surrounding the capsules or the amount of spray-dried milk in the capsule. The effect of PCR inhibitors are probably accumulative. Inhibitors in Salmosyst together with Ca^{2+} ions in spay dried milk and the gelatine surrounding the capsules made detection of *Salmonella* at t_{24} impossible (Fig. 5. 6). It was concluded that a 24 h pre-enrichment in BPW or in 2MM9+4g glucose broth is essential for a positive PCR detection of *Salmonella*, when inoculated with a CRM capsule in the absence of other microflora.

PCR Detection of Salmonella in Mixed Cultures and Food Samples

The inoculation of the three media with serial dilutions of <10 cfu *Salmonella* ml^{-1} of pure cultures, instead of CRM capsules, in the presence of high numbers of background microflora led to a positive PCR detection with a shorter pre-enrichment period (Fig. 5. 8). This shows that the earlier failure of PCR to detect *Salmonella* in media inoculated with a CRM capsule was due to components of the capsule and possibly an accumulative effect with other media components interfering with the assay. An 8 h incubation in BPW was adequate for the detection of <10 cells of *Salmonella* in the presence of approximately 1.3×10^6 cells of non-salmonella species, and approximately 20 cells of *Salmonella* in the presence of approximately 1.3×10^7 of non-salmonella species (Fig. 5. 8). The previous failure of PCR to detect *Salmonella* in BPW at t_{10} (Fig. 5. 6) or indeed in 2MM9 at t_{10} (Fig. 5. 7) inoculated with CRM capsules may be due to either: (a) the presence of PCR inhibitors in the capsule; or (b) cells in the capsule are injured and therefore need more time to

resuscitate and reach detectable level in the presence of high numbers of competing background microflora. Pre-enrichment in Salmosyst broth and 2MM9+4g glucose medium did not result in *Salmonella* detection as shown in Fig. 5. 8, presumably due to: (a) inhibitors in the Salmosyst broth and/or components of the CRM capsules; and (b) poor promotion of *Salmonella* growth and therefore low number of target cells in the media at t_8. It is apparent PCR detection of *Salmonella* in foodstuffs relies upon high numbers of the organism which are best achieved by using BPW as the pre-enrichment medium. Therefore, BPW was preferred for pre-enrichment of artificially contaminated foodstuffs and PCR detection of *Salmonella*.

PCR detected about 5 cells of *S. typhimurium*, after 24h incubation in BPW, in all artificially contaminated food samples analysed apart from the chocolate mousse (Fig. 5. 9). Given the fast growth of *Salmonella* in BPW, the low assay sensitivity must be due to PCR inhibitors in the chocolate mousse which remain in the sample after crude extraction and template preparation (Fig. 5. 9). This finding agrees with those of other workers detecting *Salmonella* in chocolate by PCR (Duran-Torres, et al., 1997; Bennett, A. R., 1996 personal communication).

Soft cheese, like brie, was found to inhibit PCR detection of *Salmonella* (Wang *et al.*, 1992; Rossen *et al.*, 1992) but brie did not inhibit PCR in this study. This may be due to a more effective elimination of interfering components in cheese, like the proteinase enzymes, high fat content and Ca^{2+} ions, by the employed (in this study) DNA extraction and template preparation procedure. The steps of the DNA extraction and template preparation used in this study achieve: a) deproteinisation and nucleic

acid stabilisation by the use of ammonium acetate; b) removal of insoluble protein and cell debris by the use of ammonium acetate and choloform:2-pentanol mixture; c) inactivation of DNA digestive enzymes with the use of EDTA; and d) high molecular mass DNA precipitation with the use of cold 2-propanol.

Positive PCR detection of *Salmonella* in chicken breast, country pate and breaded brie cheese artificially contaminated using one *S. typhimurium* CRM capsule was also possible when the samples were pre-enriched in 2MM9+4g glucose for 24 hours as shown in Fig. 5. 10. PCR failed once more to detect *Salmonella* in chocolate mousse at t_{24}. Fluit *et al.* (1993) reported earlier that food components can reduce PCR sensitivity to about 10^7 cfu g^{-1} food. The poor resuscitation of injured cells in the 2MM9+4g glucose medium combined with food and CRM capsule components which may interfere with PCR, reduce the sensitivity of the assay at t_6. The results imply that 24 hours of pre-enrichment of CRM *Salmonella*-inoculated foods are essential for cell resuscitation and proliferation in 2MM9+4g glucose medium for PCR detection.

BPW and 2MM9 medium appear to be suitable for subsequent PCR amplification of *Salmonella* in foodstuffs, when the samples are artificially contaminated with no background microflora. However, since BPW was earlier found to be superior to 2MM9+4g glucose medium in recovering injured *Salmonella,* as shown in Chapter 4, it was suggested that BPW would be more appropriate as a pre-enrichment medium for contaminated foodstuffs with injured *Salmonella* cells and high numbers of background microflora.

Subsequently, BPW was used for the pre-enrichment of artificially contaminated foodstuffs with one CRM capsule, about 3 cfu *Salmonella* g^{-1} food, in the presence of high background microflora of about $13x10^3$ to $1.3x10^6$ cfu g^{-1}. Figure 5. 11 shows that PCR failed to detect *Salmonella* in all foodstuffs even after a 24 h pre-enrichment in BPW. Since the only difference from the previous successful PCR detection of *Salmonella* shown in Fig. 5. 10 is the competing bacteria added in high numbers, it is concluded that PCR failure is due to the very high concentration of non-target DNA. The competing bacteria outgrow the injured *Salmonella* cells and mask the signal of target DNA. This agrees with previous reports of PCR interference due to high levels of non-target bacteria (Niederhauser *et al.*, 1992; Rossen *et al.*, 1992).

Niederhauser *et al.* (1992) reported that one way of avoiding the effect of PCR inhibitors in the samples is diluting the inhibitors in the sample by using short secondary enrichment of the already pre-enriched samples, thereby enhancing the ratio of target to non-target DNA as well as diluting PCR-inhibitors in the sample to ineffective concentrations. Secondary enrichment was adopted in this study.

Results in Fig. 5. 12 show that a low number of *Salmonella* of about 3 cfu g^{-1} food in the presence of about 10^6 cells of 5 non-salmonella species, is detectable in food stuffs using PCR after a 24 hour primary enrichment in BPW with a further 3 hour secondary enrichment in fresh BPW. It is concluded that the secondary enrichment eliminated PCR inhibition, whether this was exerted by components in foodstuffs, components of the CRM capsule, or the high concentration of non-target DNA. PCR

detection of *Salmonella* in chocolate mousse samples, as shown in Fig. 5. 12, implies that the secondary enrichment achieved the elimination of PCR-inhibitory effect of components in the chocolate mousse.

The first available *Salmonella* PCR detection commercial kit, the *BAX*™ system (Qualicon L.L.C.) also employs a secondary enrichment step in brain heart infusion broth (Bennett *et al.*, 1998). The ability of the *BAX*™ system to detect *Salmonella* in meat, poultry and dairy products was reported to be comparable to International and British Standard cultural procedures. The sensitivity of the system was found to be as low as 1.0 to 4.9 x 10^2 cfu ml^{-1} with some *Salmonella* isolates in pure BPW cultures (Bennett *et al.*, 1998). The sensitivity level of PCR detection of *Salmonella* in foodstuffs determined in this study is certainly comparable to reported PCR sensitivity for *Salmonella* or other pathogens in foodstuffs (Bennett *et al.*, 1998; Marsiglia *et al.*, 1998; Bej *et al.*, 1994; Wang *et al.*, 1992; Rossen *et al.*,1991). However, others did not report PCR sensitivity of 3 cfu of injured *Salmonella* g^{-1} food in the presence of as high numbers of mixed background microflora as used in this study. Moreover, no report to date show PCR detection of *Salmonella* in a chocolate product like the one tested in this study.

PCR Findings

The results of this study show:

a) The rapid crude DNA extraction procedure carried out with pure cultures of *Salmonella* compared favourably with the guanidium thiocyanate extraction procedure.

b) The need of a further DNA purification / template preparation step was determined when crudely extracted DNA was stored refrigerated or *Salmonella* DNA extraction was carried out with food samples.

c) Using a short template preparation step the interference of possibly thermostable DNA degrading enzymes in the DNA extracts, or the interference of unknown food components was eliminated.

d) The enrichment of food samples for 24 hours, artificially contaminated with CRM capsules of *Salmonella* for standardisation purposes, was found to be essential for PCR detection of *Salmonella*.

e) Direct PCR detection of *Salmonella* in artificially contaminated foodstuffs with high numbers of cells was not possible.

f) BPW proved to be a more suitable medium for sample pre-enrichment, than Salmosyst and 2MM9+4g glucose media, for PCR detection of *Salmonella* in foods.

g) A secondary enrichment, of three hours in the same pre-enrichment medium BPW, proved an invaluable step for diluting food inhibitors in chocolate and other food samples, and increasing the number of target template DNA for PCR *Salmonella* detection in the presence of high background microflora.

CHAPTER 6

General Discussion

Detection of the presence of *Salmonella* in food initially relies on a period of growth in liquid culture designed either for resuscitation or selection or both. This liquid culture growth phase is essential for the recovery of injured cells which are present in treated foods (Ray 1979; Litchfield, 1973) and the proliferation of these cells to reach detectable levels (D'Aoust, 1984; Blackburn, 1993). Injured cells are sensitive to cultural enrichment (Chen *et al.*, 1993) and chemical agents (Mackey and Derrick, 1982; Patil and Perhed, 1986). They can be pathogenic upon resuscitation and can revert to their normal cell state.

The broad objective of the present study was to investigate the use of PCR amplification assay for detection of very low numbers of injured *Salmonella* in the presence of high numbers of competing and interfering background microflora in foodstuffs. A short, <8 hours, pre-enrichment step in a non-selective medium precedes the PCR assay.

In this study the development of a chemically defined medium with supplements of several amino acids, nucleosides and the use of different carbon/energy sources was performed. It was considered that a simple defined medium with the supplements used would provide a better nutritional environment for the promotion of *Salmonella* than the peptone containing commercial media (Gomez *et al.*, 1973 and 1975; Mackey

and Derrick, 1986; Hurst, 1984). It was concluded that peptone containing media provide a better nutritional environment for growth of both normal and severely heat-injured *Salmonella* which was heat treated at 54°C for 180 minutes. This peptone superiority to the list of amino acids, nucleosides and sugars used as nitrogen and carbon sources has not been previously reported for the growth of both normal and heat injured *Salmonella* cells. It agrees with the general observation made earlier by Sokatch (1969) that peptides are better nutritional sources of amino acids for bacteria than the individual free amino acids. Peptone is considered as a complex nutritional product of protein hydrolysis. The observed superiority over the supplements used in this study implies that it consists of a multitude of unspecified utilisable substrates. However, to standardise the nutritional value and recoverability of pre-enrichment media, peptone manufacturing needs to be reproducible. This is unlikely to happen due to high costs, therefore leading to a variable bacterial recovery as shown recently by Stephens *et al.* (1997).

ELISA is one of the most common *Salmonella* detection/identification tools used in food microbiology laboratories. Different commercial kits were evaluated in this study for direct detection of *Salmonella* grown in different media. Flowers (1985) concluded that growth of *Salmonella* in M-broth enhanced Salmonella flagellar antigen production compared with *Salmonella* growing in gram negative broth. Results in this study show that the compatibility of a media-ELISA assay system is variable and some media used for sample enrichment can interfere with subsequent ELISA detection of *Salmonella*. M-broth was found to result in the most consistent *Salmonella* detection with the ELISA kits used. No antigen production enhancement

was indicated by M-broth results. Growth of *Salmonella* in the 2MM9+4g glucose medium and performance of LOCATE *Salmonella* screening test showed that an enhanced production of the antigens detected using this assay may have occurred. This 2MM9 medium in combination with the LOCATE ELISA kit resulted in the most sensitive detection of *Salmonella* when very high cell numbers, 3.7×10^7 to 10^8 cfu ml^{-1}, were required for LOCATE ELISA to detect *Salmonella* in the other media. *Salmonella* TEC™ was found to be incompatible with BPW enrichment, and LOCATE was incompatible with Salmosyst enrichment. The ELISA studies show that the assays used are not sensitive enough for direct detection of *Salmonella* in foods after pre-enrichment.

The evaluation of media for injured cell recovery led to the studies of bacterial viability. The ultimate test for pre-enrichment media is the resuscitation of severely injured cells (van Schothorst and van Leusden, 1975; Mossel and Corry, 1977; Mossel *et al.*, 1980). Inoculating media with a mixed population of injured and normal cells (Ray 1979; Mossel *et al.*, 1980; Kaprelyants and Kell, 1993) will result in fast cell proliferation of normal uninjured cells, thereby giving a false count of recovery.

The viability studies in this thesis showed that cultural evaluation of *Salmonella* viability by differential growth as used by several workers (Ray *et al.*, 1972; Gomez *et al.*, 1976; D'Aoust, 1978; Mackey, 1984) does not correlate with viability measured using supravital fluorescent stains in conjunction with fluorescence microscopy or flow cytometry. It is concluded that the loss of culturability of heat injured cells does not imply either cell death nor does it reflect severe cellular damage. De-energised or

depolarised cells detected by Nebe-von Caron *et al.*(1998) using BEP staining and flow cytometry were unable to form colonies on solid media but were largely resuscitated in liquid media.

The determination of several cell clusters of heat-treated viable *Salmonella* stained with BEP has major implications on media evaluation studies. The results strongly suggest that injury resuscitation and cell recovery studies are best performed using severely injured cells. Media, that have been evaluated to date, based on the recovery of sub lethally injured *Salmonella* heated for <60 minutes, are likely to cause recovery of mildly-damaged viable cells. Mildly damaged cells would recover faster than severely injured cells, therefore, a short pre-enrichment step would be sufficient for injury resuscitation and detection of pathogens in foods. This is a misleading situation disregarding the presence of severely damaged cells in food which are potentially pathogenic upon resuscitation. The results suggest that the best media evaluation should be carried out using cells from the same degree of injury obtained by differential staining using supravital stains like BEP and flow cytometry.

The studies of PCR detection of *Salmonella* in foods shows that the assay is extremely sensitive and specific. PCR was found to be inhibited by food components and high non-salmonella background microflora. This apparent inhibition may be caused either by suppression of Salmonella growth which fails to reach detectable levels or by direct interference of non-salmonella DNA and food components with PCR reaction. These two factors which reduce PCR performance may exert a cumulative inhibition on the assay reaction.

DNA extraction and template preparation were found to be extremely important for subsequent PCR detection of *Salmonella*. Food components and other interfering compounds in the sample can be eliminated or deactivated by certain steps of the DNA preparation. It is important to note that such steps can enhance PCR sensitivity by simply concentrating DNA in the sample. Care should be taken in avoiding the use of PCR inhibiting compounds during DNA preparation because carryover of these compounds in the PCR reaction mixture would have a detrimental effect on the assay performance.

The results also showed that PCR sensitivity can be increased using a 24 hour pre-enrichment and a subsequent 3 hour secondary enrichment of the sample. These two steps achieve both cell proliferation of the target organism and a dilution of PCR inhibitors to non-effective concentrations.

The performance of a 24 hour pre-enrichment followed by a 3 hour secondary enrichment, a rapid DNA extraction and template preparation were found to increase the assay sensitivity to 3 injured *Salmonella* cells g^{-1} food in the presence of approximately 10^6 cells of non-salmonella species. This extraordinary sensitivity and specificity of the assay suggests that PCR is the best assay to be employed in food microbiology. PCR detection of *Salmonella* in foodstuffs would be more cost effective than conventional *Salmonella* detection because time saved in the laboratory would be added to the products' shelf life, which is the ultimate purpose of the producers. The potential for PCR automation would be very advantageous as more food samples would be processed in routine screening of food for *Salmonella*.

FUTURE WORK

The findings and the problems encountered during this project indicated that further work is required to optimise the detection of injured *Salmonella typhimurium* and other *Salmonella* strains in food in the presence of high numbers of background microflora. The successful detection of extracted *Salmonella* DNA by PCR following a primary and secondary pre-enrichment of food samples could be tuned to higher specificity and sensitivity when combined with *Salmonella* specific separation techniques such as immunomagnetic separation (IMS).

The 24h primary food pre-enrichment step can be significantly shortened by using IMS. IMS would selectively separate the *Salmonellas* from the pool of organisms in food samples thereby increasing the specificity of the detection procedure tremendously. Immunomagnetic particles from the first step could then be used as the inoculum for the secondary pre-enrichment step. This would be an extremely advantageous step diluting down food ingredients and other PCR interfering components of the primary pre-enrichment. IMS combined with PCR would also allow the use of a higher volume of DNA sample in the PCR reaction mixture due to the elimination of PCR inhibitors. By simply increasing the sample volume to possibly 10 µl the sensitivity of the assay would be increased by a factor of 5 or even more depending on the type of food and the efficiency of the DNA extraction and template preparation procedure.

Further work is also required in order to obtain knowledge on the specific injuries sustained by the cells during food treatment and preparation. In addition to the

cellular permeability injury, metabolic and nucleic acid damage could be evaluated in order to create a more holistic picture of true cell injury. Such knowledge would play an important role in the development of more efficient pre-enrichment media but also it could contribute to the design of more efficient anti-bacterial treatments. Flow cytometry in combination with a multitude of biological stains is probably the most sensitive and effective tool to carry out work on cellular viability and bacterial injury.

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APPENDICES

A. Fig. 4.1: Growth curve of *S. typhimurium* in TSB

B. Table 4.1: Calculated % injuries and % survivals of heat treated *S. typhimurium* (6h, 12h and 24h-old) over 3 hours of injury

C. Table 4.2: Calculated % injuries and % survivals of heat-treated *S. typhimurium* (6h, 12h and 24h-old, pre-diluted to about 10^4 cfu ml^{-1}) over 3h of injury

D. Plate 4.2: Electron micrographs of *S. typhimurium* (24h-old cultures) growing in TSB medium and negative stained with phosphotangstic acid

Fig. 4.1 : Growth curve of *S. typhimurium* in TSB
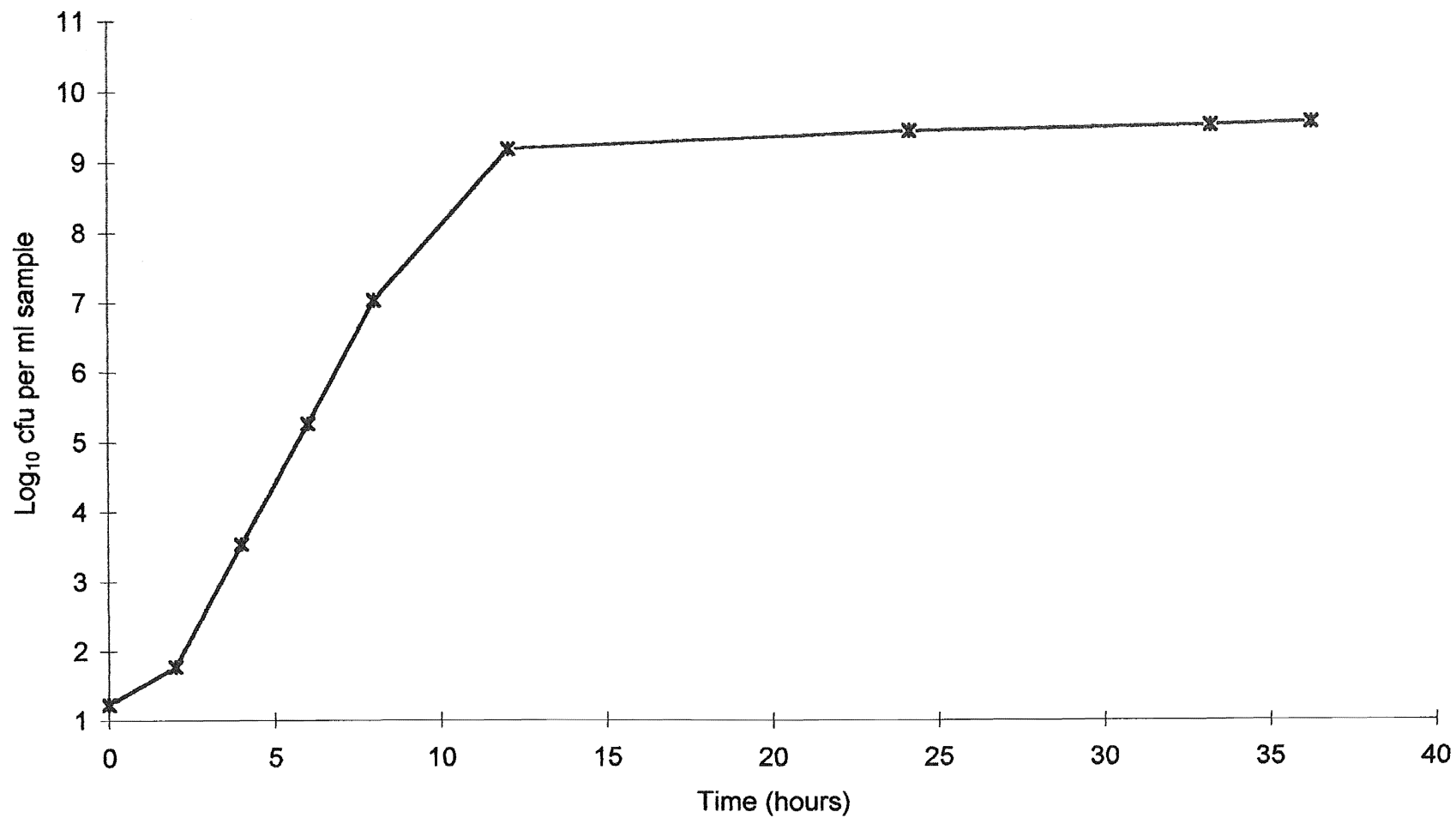
Table 4.1 : Calculated % injurie and % survival of heat-treated undiluted *S. typhimurium* (6h, 12h, and 24h-old) over three hours of injury.

Time	6h-old treated cells			12h-old treated cells			24h-old treated cells		
(min)	% Injury	% Survival on TSA	% Survival on XLD	% Injury	% Survival on TSA	% Survival on XLD	% Injury	% Survival on TSA	% Survival on XLD
0	59.5	100.00	100.00	25.0	100.00	100.00	7.2	100.00	100.00
10	51.0	60.238	72.941	11.4	47.500	56.111	49.6	34.217	18.571
20	71.8	3.7143	2.5882	57.6	19.1667	10.833	84.0	8.0723	1.3896
30	93.7	0.1357	0.0212	74.1	2.9792	1.0278	90.2	1.6024	0.1688
40	83.2	0.0081	0.0034	91.1	0.5833	0.0694	98.6	0.6024	0.0091
50	58.8	0.0012	0.0013	91.5	0.1625	0.0183	93.9	0.0747	0.0049
60	64.7	0.0004	0.0004	69.4	0.0252	0.0103	88.6	0.0169	0.0021
90	52.8	0.0001	0.0001	74.8	0.0024	0.0008	74.2	0.0008	0.0002
120	65.7	0.0000	0.0000	51.0	0.0003	0.0002	78.6	0.0002	0.0000
180	80.0	0.0000	0.0000	57.9	0.0000	0.0000	85.7	0.0000	0.0000

The results show that after 90 minutes of heat treatment the survival % of the culture is >0.001 on either media used for plating. Results of concentrated cultures show high variability.

Table 4.2: Calculated % injuries and % survivals of heat-treated *S. typhimurium* (6h, 12h and 24h-old, pre-diluted to approximately 10^4 cfu ml^{-1}) over 3h of injury.

| Time	6h-old treated cells			12h-old treated cells			24h-old treated cells		
	% Injury	% Survival on TSA	% Survival on XLD	% Injury	% Survival on TSA	% Survival on XLD	% Injury	% Survival on TSA	% Survival on XLD
(min)									
0	18.5	100.0	100.0	0.0	100.0	100.0	21.6	100.0	100.0
10	64.8	40.00	17.27	56.3	85.65	37.39	34.4	49.608	41.500
20	88.3	7.889	1.136	53.4	57.826	26.957	56.6	26.667	14.750
30	94.7	0.704	0.045	74.0	32.304	8.391	67.7	24.902	10.250
40*	100.0	0.056	0.000	84.2	18.935	3.000	77.7	10.392	2.950
50*	ND	0.000	0.000	87.8	8.022	0.978	74.8	4.353	1.400
60*	ND	0.000	0.000	87.9	1.435	0.174	89.6	1.882	0.250
90*	ND	0.000	0.000	ND	0.000	0.000	100.0	0.059	0.000
120*	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000
180*	ND	0.000	0.000	ND	0.000	0.000	ND	0.000	0.000

* : 0.2 ml of the treated culture was spread plated on the agar plates.
ND : no colony forming units detected.

The results show that after 90 minutes of heat treatment the survival % of the pre-diluted culture is >0.001 for all growth phases except the 24h old cells. Even though the culturable cells disappear early in the treatment more information is obtained from diluted cultures than concentrated cultures.

Plate 4.2: Electron micrographs of *S. typhimurium* (24h-old cultures) growing in TSB medium and negative stained with phosphotungstic acid. Plate 4.2.1-2 present control *S. typhimurium* cells. Plate 4.2.3-5 indicates physical changes during heat-treatment (HT) at 54° C for up to 60 min. As the HT proceeds, the appearance of elongated cells becomes more apparent (Plate 4.2.3). After 60 min of HT, ghost cells (or seemingly intact cells membranes) with hardly any contents appear to be numerous.

Plate 4.2.1: Control *S. typhimurium* (25,000X)

Plate 4.2.2: Control *S. typhimurium* (40,000X)
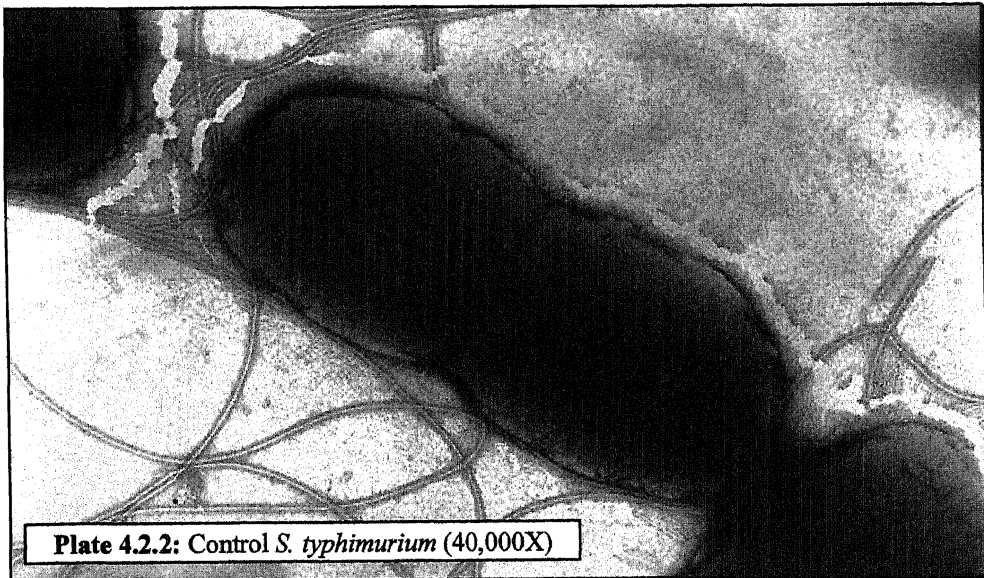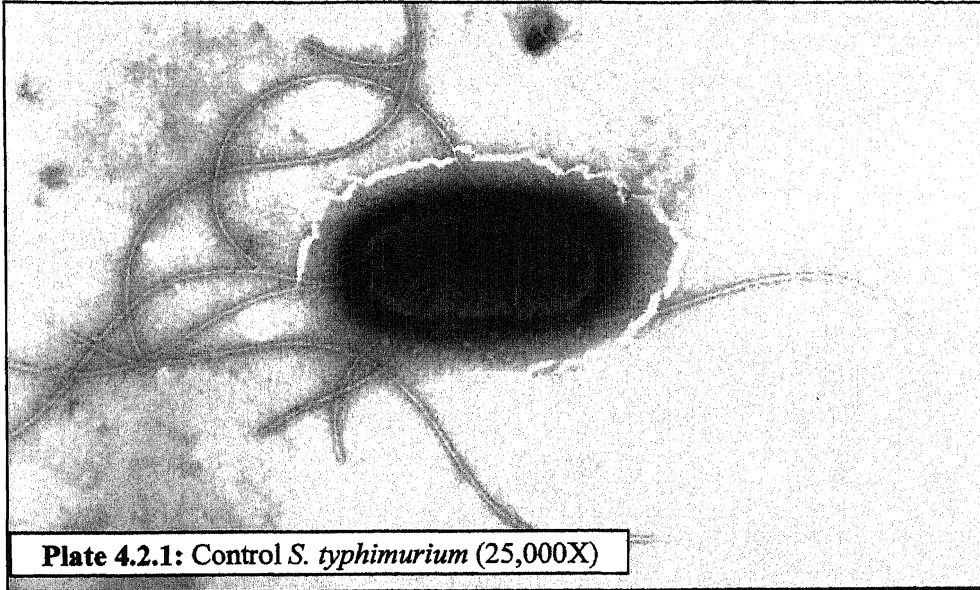
Plate 4.2.3: *S. typhimurium* HT for 20 minutes (25,000X).
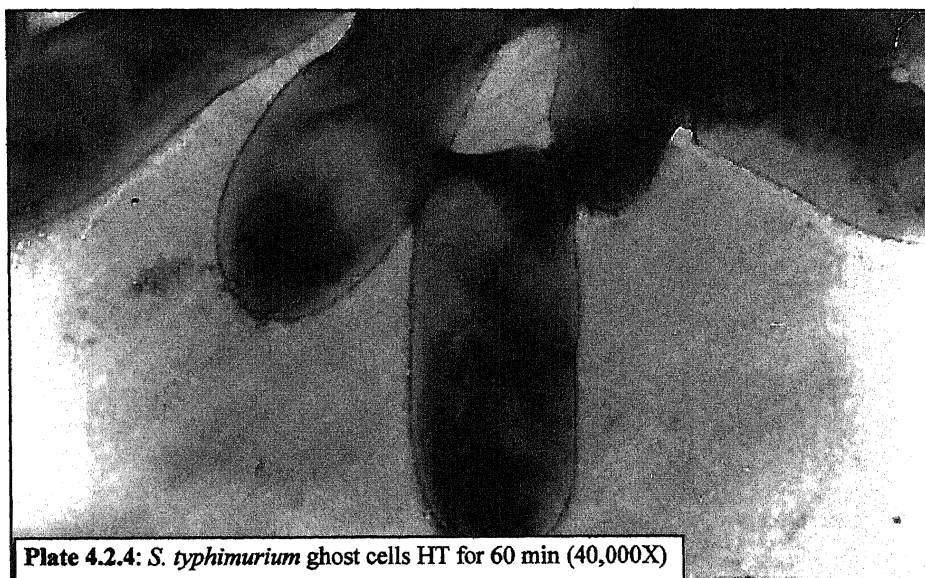

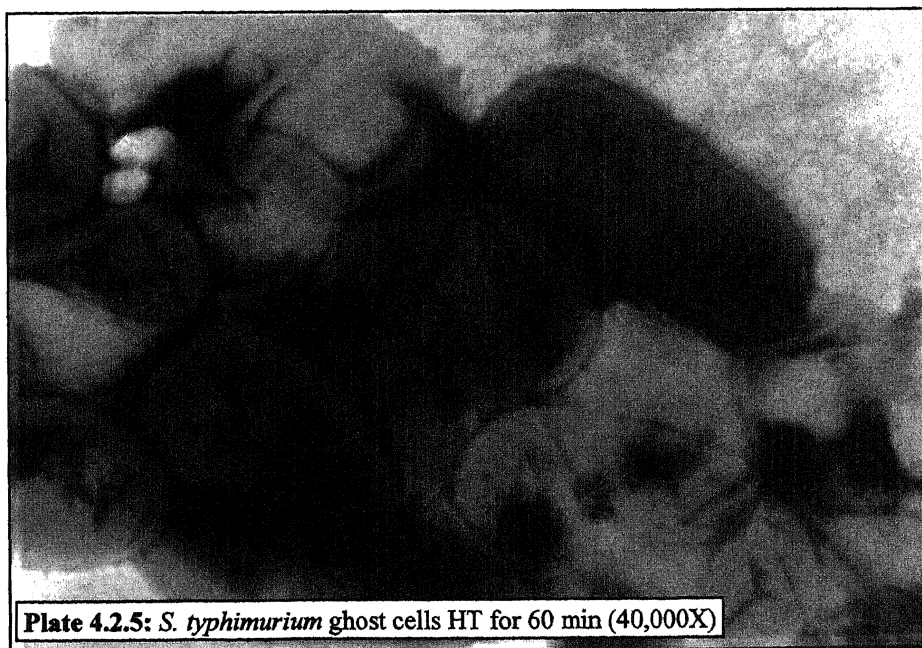**Plate 4.2.4**: *S. typhimurium* ghost cells HT for 60 min (40,000X)

Plate 4.2.5: *S. typhimurium* ghost cells HT for 60 min (40,000X)