R&D REPORT
NO. 97

High pressure resistance of *Salmonella typhimurium* and *Listeria monocytogenes*: effects of altered pH, temperature and water activity

1999

Campden BRI

Campden BRI

Chipping Campden
Gloucestershire
GL55 6LD, UK

Tel: +44 (0)1386 842000
Fax: +44 (0)1386 842100
www.campden.co.uk

R&D Report No. 97

High pressure resistance of *Salmonella typhimurium* and *Listeria monocytogenes*: effects of altered pH, temperature and water activity

N Beales, GD Betts and NJ Russell

1999

SUMMARY

The aim of this study was to determine the mode of action of high pressure (HP) treatments on *Salmonella typhimurium* and *Listeria monocytogenes* and the effects of altered pH, temperature and water activity on the HP resistance of these pathogens. Several different analyses were used to determine the mode of action of the HP treatment: assessment of cell death/injury; level of UV-absorbing materials leaking from the cells; activity of membrane-bound ATPase and ability to maintain internal pH homeostasis.

The level of cell inactivation increased with increasing severity of HP treatment, as did the level of leakage of UV-absorbing materials. There was minimal cell death or injury for *L. monocytogenes* or *S. typhimurium* treated at 200MPa, whilst at 400MPa there was a high level of cell death, up to a 9-log reduction, within a 5 minute treatment. At 300MPa, there were high levels of both cell death and sublethal injury. Although the cells were injured by application of 300MPa, they were able to recover rapidly and then grow at the same rate as healthy non-treated cells.

Growth of either organism at low temperature prior to HP treatment enhanced the degree of inactivation observed. With respect to pH and water activity, both had a marked effect on inactivation characteristics. Cells grown at lower pH showed increased inactivation when high pressure treated, whereas cells grown at lowered water activity appeared to show greater survival when high pressure treated.

The biggest effect on high pressure treatment appeared to be due to the pH of the suspending medium during treatment. The food trial studies further indicated that the pH of the food substrate used affected the sensitivity of the microorganisms to high pressure. Whilst resistance of both microorganisms did vary depending on the nature of the food substrate in which they were treated, generally the greatest inactivation and injury was seen at the lowest pH value for any substrate tested.

It is also apparent that the lack of correlation between the inhibition of H^+-ATPase activity, cell viability and UV leakage for HP treatment indicates that the membrane may not be a major site of inactivation for high pressure treatment.

CONTENTS

1. INTRODUCTION

As the search continues for novel, commercially viable, non-thermal food processing techniques, attention is focused on the possibility of using high pressure (HP) treatment. The realisation that high pressure could feasibly be used for food processing and preservation of food is not new, and was first demonstrated by Bert Hite, a chemist at the Agricultural Experiment Station in West Virginia in 1899. Using a high pressure machine that he had assembled with a maximum working load of 680MPa, he carried out experiments which showed that the microbial count in milk could be reduced with a pressure treatment of 650MPa for 10 minutes (reported by Hoover *et al.*, 1989).

Later, in 1914, Hite *et al.* published further data on the use of HP to sterilise and preserve bananas, elderberries, plums, pears and peaches. They found that pressure was more detrimental to cells at acid pH than neutral pH, cells pressurised at 5 or 40^{O}C were more sensitive to pressure than those pressurised at room temperature and cells treated in distilled water were more susceptible to pressure than those in buffer.

In 1970, over fifty years after Hite's group published its last paper on the subject, renewed interest into the possibility of applying high pressure for the purpose of microbial killing was reported. Data was published on spore germination and inactivation. For example, spores of *Bacillus* and *Clostridium* spp. were inactivated by pressures of up to 800MPa and were more readily inactivated at high temperatures (>50^{O}C) (Gould, 1970). Despite these findings, it was not until the late 1980's when the Japanese set up the Research and Development Association for High Pressure Technology in the food industry, to explore the practical applications of the technology (Farr, 1990), that the importance of this method became clear .

At the beginning of 1990s, the world's first pressure sterilised food products were launched by the Japanese Meidi-Ya company in Japan (Eley, 1992). These products were a range of jams that had been treated at 400 to 500 MPa after packing into flexible, sealed plastic packs. It was reported that all the products were of excellent quality and that they had retained the flavour and colour of fresh fruit, not normally found in conventional heat processed jams. Finally in 1992, European research activities began. The ability of high pressure to inactivate microorganisms and food quality enzymes, while leaving other quality attributes intact, encouraged Japanese industries and recently an American food company to introduce high-pressure preserved foods on the market (Mermelstein, 1997).

Over the last fifteen years, scientists have been very active in this field. It is now established that the response of microorganisms to high pressure is varied (Hoover, 1993) and the kinetic nature of the high pressure inhibition and inactivation is significantly different to phenomena caused by elevated temperature and other food processing methods. Thus microorganisms which are particularly sensitive to temperature may not necessarily be sensitive to high pressure (Earnshaw, 1995).

Variables that influence the effects of pressure on microorganisms are the magnitude and duration of treatment, growth stage of the microorganism, and the processing medium composition. It is also known that environmental factors such as a_w and pH, in which the cells are grown or treated, will affect the observed inactivation (Pandya *et al.*, 1995).

Pandya et al. (1995) showed that mild heat and acidity contributed to the effectiveness of the inactivation of yeast by high pressure; lowering the pH from 5 to 3 enhanced lethality up to 2-log cycles at 225MPa and increasing temperature from 25 to 45^{O}C at 200MPa (pH 3.0) increased inactivation of yeast by 6-log cycles.

Oxen and Knorr (1993) also showed that high pressure inactivation of the yeast, *Rhodotorula rubra* was reduced at room temperature when the a_w was below 0.94. A 7-log cycle reduction in cell number occurred at a_w 0.96; however, there was less than 2-log inactivation at a_w 0.94 and no inactivation at an a_w of 0.91. This indicated that increased resistance to HP treatment occurred as the water activity was reduced.

A wide range of pathogens and spoilage organisms are inactivated by high pressure and it also appears that there is a link between cell shape, size and cell wall structure, and the effectiveness of pressure treatment (Hoover *et al.* 1989). For example, yeast cells are more sensitive than bacteria and inactivation begins at 200MPa. Gram negative rods (e.g. *Escherichia coli* and *Pseudomonas aeruginosa*) are the next sensitive group where greater than 350MPa may be required to cause injury or death. The Gram positive bacteria are more resistant to pressure than Gram negative bacteria, requiring greater than 400 MPa (e.g. *Listeria monocytogenes*) and Gram positive cocci such as *Staphylococcus aureus* may require pressures greater than 450MPa to cause inactivation. Finally vegetative cells are more susceptible than spores which require greater than 600MPa, usually in cycles (e.g. 6 cycles of 600MPa at 70^{O}C), to achieve spore inactivation (Smelt, 1998).

The effect of HP treatment is not well defined but there are data to substantiate the theory that the major site of damage is the cell membrane with associated leakage and membrane-bound ATPase inactivation (Smelt, 1993; Smelt, 1995; Isaacs and Chilton, 1995). The cytoplasmic membrane is one site within the cell that can be damaged when the cell undergoes injury. The cytoplasmic membrane consists of a continuous double layer of lipid molecules, which are phospholipids, glycolipids and cholesterol, within which various proteins are found. The lipid bilayer is fluid which means that individual lipids are able to move and diffuse quickly within their own layer. The cytoplasmic membrane surrounds the cell and protects it from the environment and it also acts as a semi-permeable barrier that controls the entry of nutrients and exit waste products. If the cell is exposed to a physical agent such as HP and the cell membrane is damaged and no injury occurs, cellular death is likely to happen (Alberts *et al.*, 1994).

A better understanding of the effects of pressure processes on the cell, especially when combinations are applied, including other physical treatments such as temperature and pH, is essential for formulation of effective processes.

The aim of this investigation was to determine the high pressure (HP) resistance of *Salmonella typhimurium* and *Listeria monocytogenes,* the mode of action of HP treatment and the effects of altered pH, temperature and water activity. Particular attention was given to the kinetic aspects of cell death and the processes by which cells can either protect themselves against damage or facilitate repair.

2. MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

The two bacteria used were *Listeria monocytogenes* (NCTC 11994) and *Salmonella typhimurium* (Campden Research Association (CRA) 1005). Cultures were maintained on beads at -80^{O}C in a cryoprotective fluid (glycerol/water 1:1, v/v). When required, bacteria were revived by placing one bead in 10ml of Tryptone Soya Broth (Oxoid, CM131) containing 0.6% (w/v) added yeast extract (Oxoid, L21) (TSBYE), and incubating at 37^{O}C for 24 h. These primary cultures were streaked on Tryptone Soya Agar (Oxoid, CM131) with 0.6% added yeast extract (TSAYE), incubated at 37^{O}C for 24 h, and used as inocula for experimental cultures.

Experimental cultures were prepared by inoculating TSBYE (500ml) with a single colony from a TSAYE plate, followed by incubation at 37^{O}C for 18hrs. Preliminary studies (data not shown) indicated that this procedure yielded cultures in the late logarithmic to early stationary phase of growth. The bacteria were harvested by centrifugation (10,000g for 10 min; RC5C, Sorvall UK, Stevenage, UK), and washed twice in 10mM Tris-maleate buffer, pH 7.4.

L. monocytogenes and *S. typhimurium* were treated in five suspending media: distilled water (pH 6.25) (SDW), a model beef broth (0.7% w/v, pH 7.05, BBL 97531) (MBB) and 10mM Tris-maleate buffer (pH 7.4, 5.5 and 4.4) (TMB). Each medium had a final concentration of approximately 1 x 10^{10} bacteria/ml. The cell suspensions were mixed thoroughly and cooled on ice until they reached a temperature of 10^{O}C.

To establish the effects of modified growth environments on membrane damage, the choice of medium included TSBYE at reduced water activity (a_W) and TSBYE at reduced pH. The a_w for HP treatment included 0.95 a_w (NaCl 5% w/v) for *S. typhimurium* and 0.93 a_w (NaCl 10% w/v) for *L. monocytogenes*.

The reduced pH media included pH 5.5 for *L. monocytogenes* and pH 4.5 for *S. typhimurium*. The pH of the buffers were adjusted using HCl. The temperature at which the TSBYE broth was incubated also varied; *L. monocytogenes* was grown at 4, 15, and 37^{O}C, and *S. typhimurium* was grown at 10, 37 and 45^{O}C.

2.2 Treatment by high pressure

The bacterial cell suspensions (~5ml) were transferred to cryovials (Fisher CRY-100-035R) and filled to capacity. Hydrostatic pressure was applied at ambient temperature using a high pressure unit (National Forge Europe, Industriepark-Noord, Belgium) for holding periods of 1, 3, 5 and 10 minutes. For all resuspended media, the samples were treated at 200, 300, and 400MPa. The high pressure unit had a high pressure cylinder with an internal bore diameter of 250mm and length 750mm, and an internal volume of approximately 37 litres.

Within the high pressure cylinder, the samples were submerged in distilled water containing soluble oil which acted as the pressurising medium. After each treatment, the samples were stored on ice until required. In each case, an untreated sample acted as a control. This procedure was carried out on three separate occasions for each combination of pressure and holding time for both *L. monocytogenes* and *S. typhimurium*.

2.3 Enumeration of treated cells

After treatment using HP, appropriate decimal dilutions were prepared from each sample in peptone diluent (0.1% peptone + 0.85% NaCl). The number of *L. monocytogenes* survivors was enumerated by spotting duplicate 0.1ml volumes onto TSAYE, TSAYE+NaCl (3% w/v) and Listeria selective agar [OLSA; Oxford formulation containing OLSA base (Oxoid CM856) plus *Listeria* selective supplement (Oxoid SR140E)].

The number of *S. typhimurium* survivors was enumerated by spotting duplicate 0.1ml volumes onto TSAYE, TSAYE+NaCl (3% w/v) and Xylose Lysine Deoxycholate (XLD; Oxoid CM469).

All plates were incubated for 48h at 37°C. The number of sublethally injured bacteria was taken as the difference between counts obtained on TSAYE and those obtained on the selective media (TSAYE+3%NaCl, OLSA, and XLD).

2.4 Assessment of membrane damage

In order to assess the effects of HP on bacterial membrane damage, four techniques were used: analysis of UV leakage, assessment of ATPase inactivation, evaluation of cell death and injury, and analysis of internal pH homeostasis. In addition, the effects of HP on the growth rate of surviving cells during subsequent storage was evaluated.

2.4.1 Leakage of UV absorbing substances

Samples (2.8ml) of cells treated by HP were centrifuged (Sorvall, RC5C) at 10,000g for 10 minutes. The upper 1ml of the supernatant was removed and the UV absorbance measured at a wavelength of 280nm (CE 2020, Cecil Instruments Ltd., Cambridge, UK). The remainder of the supernatant was discarded and the pellet was resuspended in 1ml of 10mM Tris-maleate buffer (50mM, pH 7.4) and retained for measurement of ATPase activity.

2.4.2 ATPase Assay

2.4.2.1 Membrane isolation

Lysozyme (0.1%, Sigma L6876) was added to each 1ml sample derived from the UV assay (2.4.1) and the suspensions were incubated at 37°C for 30 mins. In the case of *S. typhimurium*, 0.1ml of EDTA (100mM, pH7.0) was also added to the suspension prior to incubation. Samples were sonicated (power setting 4; 50% pulsed duty cycle; Ultrasonics Processor, Heat Systems-Ultrasonics Inc., Farmingdale, NY, USA) for 3 min in a cuphorn containing iced water. Treated samples were centrifuged at 10,000g for 10 min to remove intact bacteria and large fragments of bacterial debris. The supernatant was removed and recentrifuged at 100,000g for 1 h (OTD-combi Ultracentrifuge, Sorvall UK) to recover membrane fragments.

Membrane pellets prepared from *L. monocytogenes* and *S. typhimurium* samples were resuspended in 0.3 and 3.0ml of Tris-maleate buffer (100mM, pH 7.4) respectively. Samples were stored at -80^{O}C until required. Preliminary studies (data not shown) indicated that ATPase activity remained unaffected by storage at -80^{O}C for several months.

2.4.2.2 Production of inorganic phosphate

ATPase activity was assayed by measuring the release of inorganic phosphate from ATP using the method of Fiske and SubbaRow (1925). Membrane suspensions (100 µl; previously thawed on ice) were transferred to polyethylene tubes containing 50µl of $MgCl_2$ (50mM) and 300µl of Tris-maleate buffer (100mM , pH7.4). The samples were preincubated at 55^{O}C (preliminary experiments indicated that ATPase activity was optimal at 55^{O}C) for 5 min in a water bath, before the reaction was started by the addition of 50µl of ATP (30mM; Sigma A7699). After 20 min, the reaction was stopped by adding 250µl of trichloroacetic acid (10% w/v).

2.4.2.3 Quantification of release of inorganic phosphate (Pi)

Distilled water (3.85ml) was added to the samples prior to centrifugation at 2,500g for 20 min (BS400, Denley Instruments Life Sciences U.K. Ltd., Basingstoke, Hampshire, UK). The supernatant was transferred to a clean tube. Ammonium molybate (200µl, 5%w/v (Sigma 76H3669)) and Amidol reagent (200µl, 0.5g of 2,4-diaminophenol in 20%, w/v, sodium sulphite) were added and the samples mixed thoroughly. Samples were incubated at 80^{O}C for 2 min in a waterbath and cooled on ice for 3 min. The absorbance was measured at 830nm.

Membrane protein determinations were carried out on each sample by following the method of Markwell *et al.* (1978), which is a modification of the method of Lowry *et al.* (1951). Bovine serum albumin (Sigma, A3059) was used as a protein standard.

ATPase-specific activity was calculated as nmol inorganic phosphate released from ATP per mg of protein, and the results were expressed as a percentage activity of an untreated control.

2.4.3 Measurement of internal pH

The effect of HP on the internal pH of *L. monocytogenes* was measured following the method of Breeuwer *et al.* (1996).

2.4.3.1 Loading of fluorescent probe

Immediately after treatment with HP, 150µl of sample was added to 1ml of 50mM potassium phosphate buffer, pH 7.0 (PPB) in an Eppendorf tube. The bacteria were washed and resuspended once more in 1ml PPB, before being incubated at 37°C for 5 min in the presence of 1.0 µM 5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester (cFDASE; Molecular Probes Europe, Leiden, Netherlands). Samples were washed and resuspended in PPB. Non-conjugated 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) was eliminated by incubating the cells with glucose (10mM) for 20 min at 37°C in a water bath. The bacteria were resuspended in fresh PPB and the glucose incubation step was repeated once more. The bacteria were washed twice, resuspended in 1 ml of PPB and kept on ice until required.

2.4.3.2 Measurement of internal pH

Cell suspension (150µl) was added to 3 ml of PPB in a glass cuvette and placed in a thermostatically controlled cuvette holder (held at 37°C) in a spectrofluorimeter (Model 3000 fluorescence spectrometer, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK). The intensity of fluorescence of the samples was measured at two excitation wavelengths, 440 and 500nm, by rapidly switching the monochromator between them at approximately 5 second intervals. The emission wavelength was held at 530nm and the excitation and emission slit widths were 5 and 10nm, respectively. Prior to the addition of each sample to the cuvette, the background signal was measured at excitation wavelengths of both 440 and 500nm, and the values obtained were subsequently subtracted from sample fluorescence signals.

After measurement of internal pH, the extracellular fluorescence signal (arising from the leakage of non-conjugated probe) was determined by passing the sample through a membrane filter (0.22 μm, Nalgene, Rochester, NY, USA) and measuring the fluorescence of the filtrate at 440nm. The extracellular fluorescence signal was found to vary from 10 to 15% of the total sample fluorescence at an excitation wavelength of 440nm.

2.4.3.3 Calibration

Calibration curves were obtained in a range of buffers (50mM glycine, 50mM potassium phosphate, 50mM citrate) with pH values of between 4.0 and 10.0. The pH of the buffers was adjusted using HCl or NaOH. Internal pH and external pH were equilibrated by the addition of the ionophores valinomycin (1mM; Sigma, V0627) and nigericin (1mM; Sigma, N-7143) to the samples in the cuvette. Calibration curves were obtained by plotting the ratio of fluorescence (500:440nm) against buffer pH.

2.5 Growth rate studies

To correlate membrane damage with cell survival and subsequent growth rate following HP treatment, cultures of *L. monocytogenes* and *S. typhimurium* following various pre-treatment conditions (i.e. 10^{O}C or 37^{O}C and pH 5.5, 4.5 or 7.0 in TSBYE) were prepared (as described in preparation of inocula). The samples were then HP treated for holding times of 3, 5, and 10 min at 300MPa. For *L. monocytogenes,* treatment at 400MPa was also carried out. After treatment, each sample was added to 100ml of TSBYE and 100ml TSBYE+NaCl (3% w/v) to give a final concentration of approximately 1 x 10^2 bacteria/ml. Counts were immediately carried out as described in enumeration of survivors (2.3), onto TSAYE and TSAYE+3%NaCl, and the broths were incubated under various post treatment conditions (i.e. 10^{O}C or 37^{O}C and pH 5.5, 4.5 or 7.0). Counts from the two broths were then performed at various times during incubation in order to obtain a growth curve.

2.6 Model food system trials

Experimental cultures of *L. monocytogenes* and *S. typhimurium* were prepared as described earlier (2.1) and grown at 10 or 37°C. Sterile food homogenates were prepared from chicken, carrots and strawberries. The criterion used in the formulation of these homogenates was to produce final media which contained a minimal amount of water but were of a suitable viscosity to allow uniform mixing of product during high pressure treatment. The percentage of water added to each food was different but the final homogenates were of similar consistency. Orange juice was used without any further pre-treatment.

2.6.1 Chicken

Samples of fresh chicken breast fillets (raw) were obtained from a local retail outlet. The raw chicken was macerated for 1.5 minutes and distributed throughout several autoclavable bags to an approximate thickness of 1cm in each. The chicken was cooked by autoclaving at 110°C for 5 minutes, then macerated for 1.5 minutes with sterile distilled water in the ratio of 2 chicken : 1 water. The pH of the samples was adjusted to pH 4.4, 5.5 or 7.4 using 10M NaOH or 10M HCl; unadjusted samples were also prepared. Samples of the homogenate were transferred to sterile universal bottles and autoclaved at 110°C for 5 minutes.

Fifteen grams of each sample were placed in stomacher bags. The samples were then inoculated with 0.5ml of the bacterial suspension and heat sealed into individual sachets after expelling as much air as possible. A sample of the final homogenate was streaked onto a prepoured TSAYE plate and incubated aerobically at 30°C for 72 hours to ensure that it was sterile. In each case the procedure was carried out in duplicate.

2.6.2 Strawberries

Fresh strawberries were sliced into quarters, then macerated for 1.5 minutes. Samples (15g) were dispensed, pH adjusted, autoclaved, and checked for sterility, as for the chicken. No additional water was required.

2.6.3 Carrot

Raw carrots (Class 1 British carrot) were sliced into rings (approximate thickness of 3mm) and autoclaved at 110^{O}C for 15 minutes. The carrots were then macerated for 1.5 minutes with sterile distilled water in the ratio of 3 carrot : 1 water. Samples (15g) were dispensed, pH adjusted, autoclaved, and checked for sterility, as for the chicken.

2.6.4 Orange juice

Fresh orange juice in a sterile container was obtained from a local retailer. Fifteen grams of duplicate samples were placed in stomacher bags. The samples were then inoculated with 0.5ml of the bacterial suspension and heat sealed into individual sachets after expelling as much air as possible. Prior to inoculation, the samples were checked for sterility as for the chicken. The pH of the orange juice was also taken.

2.6.5 Pressure treatment

Hydrostatic pressure was applied at room temperature for holding times of 3, 5, or 10 minutes at 3Kbar (4Kbar was also used for *L. monocytogenes*). After processing, the content of each sachet was aseptically transferred into individual sterile stomacher bags and a 1 : 10 dilution performed using peptone diluent (0.1% peptone + 0.85% NaCl). Appropriate decimal dilutions in peptone diluent were prepared as previously described in enumeration of survivors. Untreated samples were used as controls. Prior to HP treatment, some chicken samples were also inoculated with *S. typhimurium* and *L. monocytogenes* and then chilled at 5^{O}C for 24 hours.

3. RESULTS

The Figures relating to these results are contained in the Appendices to this report.

3.1. Cell inactivation and injury

3.1.1. Effect of treatment and medium used during HP treatment

As a general observation, increasing the pressure and length of treatment resulted in increasing inactivation of both *S. typhimurium* and *L. monocytogenes*, as measured by viable plate counts (Fig. 1 and 2). A marked difference between the survival counts was obtained on the non selective medium (TSAYE) compared with the two selective media (TSAYE+3%NaCl, OLSA or XLD).

Little difference was observed between the two selective media used for *S. typhimurium* and *L. monocytogenes*. Thus it was decided to carry out further investigations using only one selective medium (TSAYE+3%NaCl).

Treatment at 2 Kbar (200MPa) had very little effect on the survival of either S. ty*phimurium* or *L. monocytogenes*. Both strains remained unaffected by this pressure even after an exposure of 10 min. This was found to be the case for all resuspending media tested, i.e. distilled water (pH 6.25), 10mM Tris-maleate buffer (pH 7.4, 5.5 and 4.4) and model beef broth (0.7% w/v) (data only shown for 10mM Tris-maleate (pH 7.4) (Figures 1 and 2)).

Treatment in 10mM Tris-maleate (pH 7.4), at 3Kbar, resulted in an approximate one-log reduction in *L. monocytogenes* after a 10 min holding time and a greater than two-log reduction in the numbers of *S. typhimurium.* In both cases, there was 30% injury observed after 10 minutes. Similar results were observed with the model beef broth where there was high survival and low injury.

In contrast, for both distilled water and 10mM Tris-maleate at pH4.4 treated at 3Kbar, high injury and high inactivation was observed (Figures 3 and 4). For *L. monocytogenes,* a greater than three-log decrease after 10 minutes holding time was observed with approximately 30-40% injury after 3 minutes. For *S. typhimurium* a four to five-log reduction after a 5 minute holding time was observed with 50-60% injury after 3 minutes. For 10mM Tris-maleate (pH 5.5) the results were between 10mM Tris-maleate pH7.4 and 4.4, indicating some injury (40% injury for both organisms) and some inactivation (Figures 3 and 4).

Treatment at 4 Kbar (400MPa) showed increased inactivation of both *L. monocytogenes* and *S. typhimurium* and cells were found to be more susceptible to sublethal injury with 80% injury observed after a treatment time of 1 minute. This was found to be the case for all resuspended media used (data only shown for 10mM Tris-maleate (pH 7.4) in Figures 1 and 2). Following these initial investigations, it was decided to use 3Kbar for the majority of the experiments as it achieved some inactivation of cells plus a large proportion of cell injury.

3.1.2 Effect of growth temperature

Generally, for both *L. monocytogenes* and *S. typhimurium,* the level of inactivation and injury was increased for cells grown at sub-optimal temperatures.

For *L. monocytogenes*, the rates of cell inactivation and cell injury increased as the growth temperature decreased. For cells grown at $4^{O}C$, there was an approximate five-log reduction after 5 minutes holding time compared to a three- and one-log reduction for cells grown at $15^{O}C$ and $37^{O}C$ respectively in 10mM Tris-maleate (pH 7.4) (Figure 5a).

Further log reductions were seen after treatment with 10mM Tris-maleate (pH 4.4). Total inactivation was observed within 5 minutes when grown at $4^{O}C$, compared to five- and two-log reductions when grown at $15^{O}C$ and $37^{O}C$ after five minutes, and a six- to seven-log reduction and 3-log reduction at $15^{O}C$ and $37^{O}C$, respectively after 10 minutes holding time (Figure 5b).

For *S. typhimurium*, growth at $45^{O}C$ or $10^{O}C$ appeared to cause increased sensitivity to HP. There was an approximate three- to four-log reduction in *S. typhimurium* with approximately 50-60% injury after a 10 min holding time at $10^{o}C$ and $45^{O}C$ compared to a two-log reduction and 30% injury within 10 minutes for cells grown at $37^{O}C$ and treated in 10mM Tris-maleate (pH 7.4) (Figure 6a).

These results were enhanced after treatment with 10mM Tris-maleate (pH 4.4), where total inactivation was observed after 5 minutes` treatment at $10^{O}C$ and a seven-log reduction found at $45^{O}C$, compared to a three-log reduction within 10 minutes for cells grown at $37^{O}C$ (Figure 6b).

3.1.3 Effect of growth a_w

For both microorganisms, there appeared to be greater survival after growth at reduced a_w compared with growth at normal a_w. For *L. monocytogenes*, there was a one-log reduction for cells grown at normal a_w after 10 minutes, compared to no reduction when grown at reduced a_w and treated in 10mM Tris-maleate (pH 7.4) (Figure 7a).

For those treated in 10mM Tris-maleate (pH 4.4), the results were enhanced with a three-log reduction at normal a_w and a one-log reduction after 10 minutes at reduced a_w (Figure 7b).

For *S. typhimurium*, there was a two-log reduction for cells grown at normal a_w after 10 minutes compared to half a log reduction when grown at reduced a_w and treated in 10mM Tris-maleate (pH 7.4). For those treated in 10mM Tris-maleate (pH 4.4), the results were enhanced as found with *L. monocytogenes* (Figure 8 a & b).

3.1.4 Effect of growth pH

L. monocytogenes and *S. typhimurium* grown at reduced pH and treated in Tris-maleate (pH7.4), showed increased inactivation and injury compared to cells grown at pH 7 (Figures 9a and 10a). When treated in Tris-maleate (pH 4.4), the cells grown at reduced pH showed less injury (approximately 20% injury after 3 minutes) than those grown at pH 7 (60-70% injury after 3 minutes). This indicates that when the microorganisms were grown and treated at reduced pH, the microorganisms had adapted to the low pH environment. However, the levels of inactivation were greater by a factor of one-log at reduced pH than at pH7, where only slight inactivation was noted (Figures 9b and 10b).

3.2 ATPase measurement

Generally there was an inconsistent effect of pressure on ATPase activity for both *L. monocytogenes* and *S. typhimurium* and no trends were evident (Figures 11 and 12). It is concluded therefore that membrane-bound ATPase may not be a site of inactivation for HP treated cells.

3.3 Leakage of UV-absorbing substances (UV leakage)

3.3.1 Effect of treatment and medium used during HP treatment

Generally, increasing the pressure and length of treatment resulted in increasing levels of leakage of UV-absorbing substances from both microorganisms when treated in 10mM Tris-maleate (pH 7.4). Similar results were found after treatment in distilled water. However, when treated with 10mM Tris-maleate (pH 4.4) and Model Beef Broth, the levels of leakage of UV-absorbing materials remained at a similar level for each treatment time.

It was also noted that the levels of UV-absorbing materials for *L. monocytogenes* were low, especially with the suspending medium 10mM Tris-maleate (pH 7.4) and distilled water. Here, the level of UV-absorbing substances was found to be approximately 5-6 times less than that observed for *S. typhimurium* after 5 minutes treatment (Figures 13 and 14).

3.3.2 Effect of growth temperature

The level of leakage of UV-absorbing substances for both microorganisms increased for cells grown at sub-optimal temperatures. This was more pronounced when cells were treated in 10mM Tris-maleate (pH 7.4) compared to 10mM Tris-maleate (pH 4.4) (Figures 16 and 17). With *S. typhimurium*, the level of leakage of UV-absorbing substances after growth at $10^{o}C$ was five times greater than after growth at $37^{o}C$ or $45^{o}C$ when treated in both 10mM Tris-maleate pH 7.4 and 4.4. However, the actual optical density (OD) reading at $10^{o}C$ when treated in 10mM Tris-maleate (pH 4.4) (Figure 16b) was approximately six to seven times less than the OD reading obtained using 10mM Tris-maleate (pH 7.4) (Figure 16a). Although the levels of leakage of UV-absorbing substances did increase at sub-optimal temperatures for *L. monocytogenes* the results were again less pronounced (Figure 15 a and b).

3.3.3 Effect of growth a_w

The level of UV-absorbing substances from both microorganisms, when grown at normal a_w, were slightly higher than at reduced a_w. However, when treated in 10mM Tris-maleate (pH 4.4 or pH 7.4) the trends were very similar (Figures 17 and 18).

3.3.4 Effect of growth pH

The level of UV leakage from both microorganisms was greater after growth at reduced pH than after growth at normal pH (Figures 19 and 20). However, as with the growth temperature and growth a_w, *S. typhimurium* showed the greatest difference, especially in the suspending medium 10mM Tris-maleate (pH 7.4) (Figures 19a and 20a).

3.4 pH homeostasis (ΔpH)

The ability of *L. monocytogenes* to maintain pH homeostasis diminished with increasing treatment time when treated at 3Kbar in distilled water and to a greater extent when treated in Tris-maleate (pH 4.4). However, when *L. monocytogenes* was grown at reduced a_w and then HP treated at 3Kbar, better internal pH control was observed (Figure 21).

3.5 Growth rate following HP

Various pre- and post-treatment growth conditions were investigated (Table 1). Of these, only two showed evidence of some effect on subsequent growth rates due to sublethal injury (Figures 22 and 23).

When *S. typhimurium* was grown at 10°C, pH 7.0 before and after HP treatment (3Kbar), some differences in lag time and growth rate of healthy cells could be seen. No lag period was observed with TSBYE but a lag period of twenty-five hours and fifty hours was observed in TSBYE+3%NaCl on TSAYE and TSAYE+3%NaCl respectively (Figure 22a). When HP treated for 3 and 5 minutes holding times, the lag period observed in the TSBYE +3%NaCl on TSAYE and TSAYE+3%NaCl was double that observed in the untreated cells, 50 and 100 hours respectively (Figures 22 b and c).

Similar results were observed with *L. monocytogenes* grown at 4°C, pH 7.0 and subjected to 4Kbar. Following holding times of 5 and 10 minutes, lag periods of 10 and 20 hours were seen in TSBYE+3%NaCl on TSAYE and TSAYE+3%NaCl respectively (Figures 23a-c).

Although the starting levels of cells were different when *S. typhimurium* was grown at 37°C, pH 7.0 then HP treated (3Kbar) and then grown at 10°C, pH 7.0, the shapes of all the curves were the same. The TSBYE broths also showed a small rise in numbers of 0.5 log in the first 20 hours that was then followed by a stable lag period of about 175 hours. This initial increase was not seen in TSBYE+3%NaCl (Figures 24a-b).

When *S. typhimurium* was grown at pH 4.5 at 10°C either as a pre- or post-treatment, the conditions were found to be too severe to allow any cells to grow. Therefore no injury was evident (Figures 25a-b).

Table 1. Growth rate of *S.typhimurium* and *L. monocytogenes* following HP treatment and pre- and post-treatment

Organism	Pre-HP treatment conditions	HP treatment	Post-HP treatment conditions	Effects on growth rate	Lag time
S. typhimurium	10°C, pH 7	3Kbar 0,1,3 min	37°C, pH 7	Optimum conditions	0 hours
S. typhimurium	37°C, pH 7	3Kbar 0,1,5 min	37°C, pH 7	Optimum conditions	0 hours
S. typhimurium	10°C, pH 7	3Kbar 0,3,5 min	10°C, pH 7	Some injury effects. Conditions favourable for healthy cells and inhibitory to injured cells	50-100 hours
S. typhimurium	37°C, pH 7	3Kbar 0,3,5 min	10°C, pH 7	TSBYE broths show small rise in cell numbers within 20hrs followed by stable lag phase	stable lag period ~175 hrs
S. typhimurium	10°C, pH 7	3Kbar 0,3,5 min	10°C, pH 4.5	The post-treatment conditions too severe to allow growth of any cells	>14 days
S. typhimurium	10°C, pH 4.5	3Kbar 0,3,5 min	10°C, pH 4.5	Little growth following 2 months of pre-treatment condition	N/A
L. monocytogenes	37°C, pH 7	3Kbar 0,5,10 min	37°C, pH 7	Optimum conditions	0 hours
L. monocytogenes	10°C, pH 7	3Kbar 0,5,10 min	10°C, pH 5.5	Optimum conditions	0 hours
L. monocytogenes	10°C, pH 7	3Kbar 0,5,10 min	10°C, pH 7	Optimum conditions	0 hours
L. monocytogenes	37°C, pH 7	3Kbar 0,5,10 min	10°C, pH 7	Optimum conditions	0 hours
L. monocytogenes	10°C, pH 5.5	3Kbar 0,5,10 min	10°C, pH 5.5	Optimum conditions	0 hours
L. monocytogenes	4°C, pH 7	4Kbar 0,5,10 min	4°C, pH 7	Some injury effects	20hours
L. monocytogenes	10°C, pH 7	4Kbar 0,5,10 min	37°C, pH 7	Optimum conditions	0 hours
L. monocytogenes	37°C, pH 7	4Kbar 0,5,10 min	10°C, pH 7	Injured cells repaired quickly	stable lag period 30 hours

3.6 Model food studies

For both microorganisms, the extent of inactivation and injury at 300MPa was different for each food substrate used for the food trials. However, the results gained were similar to those seen with 10mM Tris-maleate at varying pH values during treatment, where increased inactivation and injury was observed at pH 4.4, compared to pH 5.5 and pH 7.4 (Figures 26-32).

Generally, the extent of inactivation was found to be slightly higher with the food samples than with the buffer 10mM Tris-maleate. For *L. monocytogenes*, the level of inactivation was greater in strawberries at pH 4.4, with a seven log reduction, compared to a five log and four log reduction seen with carrots and chicken substrates, respectively, and a three log reduction seen with Tris-maleate (pH 4.4) (Figures 26a, 27a and 28a).

For *S. typhimurium*, similar results were obtained. For strawberries at pH 4.4, total inactivation was observed after 10 minutes holding time, compared to a six log reduction with carrots and chicken substrates at pH 4.4 and a five log reduction with Tris-maleate (pH 4.4) (Figures 26b, 27b and 28b). When the trial used naturally acidic products such as orange juice (pH 3.02) and strawberries (pH 3.54), total inactivation was seen after a one minute holding time for *S. typhimurium* (Figures 28b and 29b) and after 5 minutes treatment for *L. monocytogenes* (Figures 28a and 29a). This could be due to different acid types or due to natural organic acids.

When chicken slurry samples at varying pH values were inoculated then chilled at 5^{O}C for 24 hours prior to high pressure treatment, the level of inactivation increased for *S. typhimurium* by a factor of one to two logs (Figure 30a). However for *L. monocytogenes,* the resistance to HP appeared to increase, as seen by the decreased inactivation by a factor of half a log and decreased injury (Figure 30b).

For *L. monocytogenes*, the most effective treatment was found at 400MPa, where a further reduction of 1 to 2 logs was seen compared to 300MPa (Figure 31). For both microorganisms, however, as with the 10mM Tris-maleate, further reductions in terms of inactivation were seen when the microorganisms were grown at a reduced temperature (10^{O}C), prior to HP treatment (Figure 32 a and b).

4. DISCUSSION

It has been demonstrated previously that, when a similar technique was applied for the enumeration of high pressure treated *L. monocytogenes* cells, the percentage of survivors sustaining sublethal injury increases with increasing severity of HP treatment (Simpson and Gilmour, 1997). The results obtained for *S. typhimurium* and *L. monocytogenes* in the present series of experiments concur with these observations and also suggest that whilst some of the population is inactivated, there is a population of cells that are sublethally injured by HP treatment.

Survival was much higher on the rich non-selective media (TSAYE) than on the selective media containing inhibitory ingredients (XLD, OLSA or TSAYE+3%NaCl). This indicates that there is a proportion of microorganisms, which after pressurisation can repair damage caused and thus reproduce, whereas the added stress caused by culturing on a selective media inhibits the repair processes.

Similar results following high pressure treatment have also been observed in other studies (Isaacs and Chilton, 1995; Simpson and Gilmour, 1997). Therefore the possibility exists that microorganisms may fail to initiate growth when plated out immediately after pressurisation, whereas given the right conditions, they may be capable of regeneration if repair mechanisms remain intact.

It is important to ascertain the importance of recovery since a food manufacturing process must be able to rely on a definite kill and these sublethally injured cells may be capable of recovery and possibly subsequent growth (Isaacs and Chilton, 1995). However, the fact that pressure can cause injury may be used to advantage when the process is combined with other preservation treatments such as heat, organic acids, pH, preservatives or processing with CO_2 (Patterson *et al.*, 1995).

It was suggested by Ray (1979) that the resistance of microorganisms to selective chemical inhibitors such as the antibiotics contained in OLSA and XLD and the salt contained in TSAYE+3%NaCl, is due to the inability of these compounds to enter the cells through the cell membrane. Therefore loss of tolerance to these chemicals indicates that the injured cells have sustained membrane damage.

In the present study, it would appear from the growth rate trial studies that there are no major effects on subsequent growth rate due to sublethal injury. Two exceptions may be for *S. typhimurium* following HP (300MPa) and *L. monocytogenes* following HP (400MPa) with pre- and post-HP growth conditions of 10 ^{o}C, pH 7 for *S. typhimurium* and 4^{o}C, pH 7 for *L. monocytogenes*. In both these cases, the difference in lag time for treated cells compared to untreated cells may due to injured cells taking time to repair. Also it can be seen that the growth rate of the cells in the TSBYE+3%NaCl on TSAYE+3%NaCl is slightly lower and the final numbers are lower. This is likely to be due to the stress of the two sets of 3% salt on all cells, as any injured cells that repaired during lag phase should grow as normal once repair has occurred. Thus these conditions are fairly favourable for growth of healthy cells, but inhibitory to injured cells. This could increase the lag time and thus the shelf life of the product.

With respect to the growth rate of *S. typhimurium* following HP (300MPa) and pre-HP treatment of 37°C, pH 7 and post-HP treatment of 10°C, pH 7, there was no evidence of any effects on growth rate due to injured cells. The feature of a small rise in numbers within the first 20 hours followed by a stable lag period, seen with the TSBYE broths, indicates that any injured cells present repair quickly. This initial rise was not seen in TSBYE+3%NaCl, where the repair is slower and occurs during the entire lag period. Thus generally, optimum growth conditions were available and no injury was evident.

When *S. typhimurium* was grown at 10°C at pH 4.5, these conditions were found to be too severe to allow growth of any cells whether treated or not, due to the effect of the reduced pH and temperature in combination.

The species of bacteria used can have a significant effect on the degree of inactivation achieved (Smelt, 1998). In the present study, *S. typhimurium* (CRA 1005) was more sensitive to HP treatment than *L. monocytogenes* (NCTC 11994), at levels in excess of 200MPa, irrespective of the medium in which the bacteria was treated and the growth conditions used. It has been postulated that as the cell structure is less complex in Gram positive bacteria, it could be less susceptible to environmental changes caused by pressure treatment (Shigehisa *et al.*, 1991). The cell wall of Gram positive bacteria consists mostly of peptidoglycan linked with teichoic acids, whereas in the Gram negative bacteria, the peptidoglycan forms a separate layer and is linked by weak covalent bonds to elongated lipoprotein molecules. This layer is in between the cytoplasmic membrane and cell wall. The cell wall consists of phospholipids, lipopolysaccharides and proteins (Neidhart *et al.*, 1990)

One aim of this study was to determine whether bacterial membranes were the site of action for HP. One measure of membrane damage is leakage of materials from within the cell which is indicative of physical damage having occurred.

Increasing levels of leakage of UV-absorbing substances were detected when both microorganisms were treated in 10mM Tris-maleate (pH 7.4); however, this medium showed the least amount of cell injury and inactivation, therefore leakage materials were not essential for continued cell function.

With regard to the other media used to treat the cells, the model beef broth and Tris-maleate (pH 4.4) showed the least amount of UV leakage, whilst cells treated in sterile distilled water (pH 6.25) showed a medium amount of UV leakage. The UV leakage effects with Tris-maleate (pH 7.4) were not so pronounced with *L. monocytogenes*; this may be due to the Gram positive cell structure being less susceptible to damage.

The loss of ΔpH observed when cells were HP treated in SDW appeared to correlate with the increase in UV leakage. With Tris-maleate (pH 4.4), there were signs of the pH gradient diminishing over increasing treatment time as with SDW, which appeared to correlate with the high inactivation rate observed with this medium. However, when grown at reduced water activity, *L. monocytogenes* showed better pH control which correlated with the protective effect against inactivation and the low level of UV leakage. Loss of the pH homeostatic mechanisms with increased HP treatment time is indicative of some membrane sited mode of action as mechanisms for maintaining cell internal pH are based in the membrane.

It is known that growth pH, temperature and water activity affect the inactivation rate of microorganisms following HP treatment (Pandya, *et al.*, 1995). In the present study, both microorganisms were more sensitive to inactivation following growth at sub optimal temperatures and reduced pH values.

From this investigation, it is apparent that water activity can have a protective effect and enable microorganisms to become more resistant to high pressure treatments. It has been suggested that this increased resistance may be attributed to shrinkage of the cell and suppression of cell growth and it appears possible that a_w values critical for inhibition of growth of organisms are also critical for resistance to high pressure (Knorr, 1993).

Other workers have shown that microorganisms are more resistant to high pressure when dry than when moist (Kowalshi *et al.*, 1992). Electrostriction of free water during pressurisation is thought to facilitate microbial killing via changes in enzyme activity (Hui Bon Hoa *et al.*, 1992) and protein stability (Smelt, 1995). It is possible that reduction in water activity alters the electrostriction effect.

In this investigation, the biggest effect on high pressure treatment appeared to be due to the pH during treatment, where the cells that had undergone HP treatment had increased sensitivity to acidic environments. Even though reduced a_w offered protection to the cells, there was still an obvious effect of pH in cells grown at reduced a_W. This is seen throughout the investigation with all the different growth treatments used. Reducing the pH (pH 4.4) during treatment caused more inactivation and injury but little leakage of UV-absorbing materials and at near neutral pH (pH 7.4), there is less inactivation and injury but increased leakage of UV-absorbing material.

The pH of the medium under pressure has long been known to affect microbial growth (Hite *et al.*, 1914) and in some cases, pH is thought to enhance high pressure effects (Kuhne and Knorr, 1990; Pandya *et al.*, 1995). The results obtained for *S. typhimurium* and *L. monocytogenes* in the present series of experiments concur with these observations. The application of high pressure can also alter the pH of a medium, as well as progressively narrow the pH range of growth (Marquis, 1976).

Marquis (1984) suggested that these effects may be due to the pressure effect on membrane ATPase with the proton and cation translocational function of the membrane ATPase being inhibited. However, in this investigation there was an inconsistent effect on ATPase activity in both *L. monocytogenes* and *S. typhimurium*. It is thought that the injury and death effects seen with pH 4.4 may not be due to membrane damage but to the antimicrobial effects on the intracellular components.

The food trial studies carried out also showed that the pH of the food substrate used affected the sensitivity of the microorganisms to high pressure. Whilst resistance of both microorganisms did vary depending on the nature of the food substrate in which they were treated, generally the greatest inactivation and injury was seen at the lowest pH value. The differences in inactivation and injury seen with the various food substrates and buffer could be attributed to the differences in the amount of nutrients available affecting the recovery of the pressure damaged cells. Such an occurrence has been reported when heat injured bacteria are deprived of nutrients where lack of nutrients in the buffer prevented recovery of the damaged cells (Iandolo and Ordal, 1966; Patterson *et al.*, 1995).

As with the buffers, the level of inactivation and injury in the chicken substrate was generally increased when the microorganisms were grown at reduced temperature, then inoculated into the food sample, compared to when grown at 37^{O}C. It was also found that chilling the inoculated *S. typhimurium* food samples at 5^{O}C for 24 hours prior to high pressure treatment caused increased inactivation. However, with *L. monocytogenes*, although the levels of this organism decrease during storage at 5^{O}C, resistance to high pressure increases, as any cold shock proteins (Berry and Foegeding, 1997) may confer resistance to HP.

From the model food trials, the most likely application of high pressure processing is to preservation of acid foods with a pH of less than 4.6. Several other studies have reported that in foods with pH values of between 2.5 and 4.5 HP increased inactivation of yeasts, moulds and vegetative bacteria by 5 logs or more (Kuhne and Knorr, 1990). Other studies have stated that sterilisation of low acid foods pH greater than 4.6 is likely to rely on combination of high pressure processing and other mild treatments. For both pasteurisation and sterilisation processes, combined pressure temperature treatments are frequently regarded as most appropriate (Farr, 1990; Patterson *et al.*, 1995). Effective combinations to counteract the protective effect observed with reduced water activity could include the use of pressure and heat, pressure and organic acids or pressure and preservatives. Palou *et al.* (1997) showed that HP treatmentof cells at an a_w of 0.98 or 0.95 was more effective when combined with potassium sorbate.

In conclusion, it is apparent that the lack of correlation between the inhibition of H^+-ATPase activity, cell viability and UV leakage for HP treatment indicates that the membrane may not be a major site of inactivation in high pressure treatment.

5. ACKNOWLEDGEMENTS

The authors are grateful to the Ministry of Agriculture, Fisheries and Food, UK for financial support.

6. REFERENCES

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1994). The membrane structure. In *Molecular Biology of the Cell* (Third Edition). Garland Publishing Inc., London, pp 477-506.

Berry, E.D. and Foegeding, P.M. (1997). Cold temperature adaption and growth of microorganisms. *Journal of Food Protection*, **60**(12), 1583-1594.

Breeuwer, P., Drocourt, J.L., Rombouts, F.M., and Abee, T. (1996). A novel method for the continuous determination of intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. *Applied and Environmental Microbiology*, **62**, 178-183.

Earnshaw, R.G. (1995). High pressure microbial inactivation kinetics. In *High Pressure Processing of Foods*. (Eds. Ledward, D.A., Johnston, D.E., Earnshaw, R.G., & Hasting, A.P.M.). Nottingham University Press, pp. 37-46.

Eley, E. (1992). Under Pressure. *Food Processing*, **61**(April), 23-25.

Farr, D. (1990). High pressure technology in the food industry. *Trends in Food Science and Technology*, **1**(1), 14-16.

Fiske, C.H. and SubbaRow, Y. (1925). The colorimetric determination of phosphorus. *Journal of Biological Chemistry*, **66**, 375-400.

Gould (1970). Inactivation of bacterial spores by high hydrostatic pressure. *Journal of General Microbiology*, **60**, 323-334.

Hite, B.H. Giddings, N.J. and Weakly, C.E. (1914). The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. Bulletin No. 146, pp. 1-67. West Virginia Agricultural Experiment Station, Morgantown Virginia.

Hoover, D.G., Metrick, C., Papineau, A.M., Farkas, D.F., and Knorr, D. (1989). Biological effects of high hydrostatic pressure on food microorganisms. *Food Technology*, **43**(3), 99-107.

Hoover, D.G. (1993). Pressure effects on biological systems. *Food Technology*, **47**(6), 150-155.

Hui Bon Hoa, G., Di Primo, C., and Douzou, P. (1992). The role of electrostriction in the control of reaction volumes and biochemical processes. In *High Pressure and Biotechnology*. (Eds. Balny, C., Hayashi, R., Heremans, K. and Masson, P.). London. John Libby Eurotext. pp. 815-826.

Isaacs, N.S. and Chilton, P. (1995). Studies on the inactivation by high pressure of microorganisms. In *High Pressure Processing of Foods*. (Eds. Ledward, D.A., Johnston, D.E., Earnshaw, R.G., & Hasting, A.P.M.). Nottingham University Press, pp. 65-79.

Iandolo, J.J. and Ordal, Z.J. (1966). Repair of thermal injury in *Staphylococcus aureus. Journal of Bacteriology*, **91**, 134-142.

Knorr, D. (1993). Effects of high hydrostatic pressure on food safety and quality. *Food Technology*, **47**(6), 156.

Kowalshi, E., Ludwig, H., and Tauscher, B. (1992). High hydrostatic pressure for sterilisation of foods. Application to Pepper *(Piper nigrum L.). Deutsche Lebensmittel Rundschau*, **88**(3), 74-76.

Kuhne, K. and Knorr, D. (1990). Effect of high pressure carbon dioxide on the reduction of microorganisms in fresh celery. ZFL, *International Zeitschrift fer Lebensmittel-technologie and Verfahrenstechnik,* **41**, 55-57.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.

Markwell, M.A.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, **87**, 206-210.

Marquis, R. E. (1976). High pressure microbial physiology. *Advances in Microbial Physiology*, **14**(1), 159.

Marquis, R.E. (1984). Reversible actions of hydrostatic pressure and compressed gases on microorganisms. In *Repairable Lesions in Microorganisms*. (Eds. Hurst, A. and Nasim A.) Academic Press, New York, p273.

Mermelstein, N.H. (1997). High pressure processing reaches the U.S. market. *Food Technology*, **51**(6), 95-96.

Neidhart, F.C., Ingraham, J.L. and Schaeter, M. (1990). Physiology of the Bacterial Cell: A Molecular Approach. Sinauer Associates Inc. Publishers, Sunderland, Massachusettes, USA.

Oxen, P. and Knorr, D. (1993). Baroprotective effects of high solute concentrations against inactivation of *Rhodotorula rubra*. Lebensmittel Wissenschaft, und Technologie, **26**(3), 220-223.

Palou, E., López-Malo, A., Barbosa-Canovas, G.V., Welti-Chanes, J. and Swanson, B.G. (1997). High hydrostatic pressure as a hurdle for *Zygosaccharomyces baillii* inactivation. *Journal of Food Science*, **62**(4), 855-857.

Pandya, Y., Jewett, F.F., and Hoover, D.G. (1995). Concurrent effects of high hydrostatic pressure, acidity and heat on the destruction and injury of yeasts. *Journal of Food Protection*, **58**(3), 301-304.

Patterson, M.F., Quinn, M., Simpson, R. and Gilmour, A. (1995). Effects of high pressure on vegetative pathogens. In *High Pressure Processing of Foods*. (Eds. Ledward, D.A., Johnston, D.E., Earnshaw, R.G., and Hasting, A.P.M.). Nottingham University Press, pp47-63.

Ray, B. (1979). Methods to detect stressed microorganisms. *Journal of Food Protection*, **42**(4), 346-355.

Shigehisa, T., Ohmori, T., Saito, A., Taji, S. and Hayashi, R. (1991). Effects of high pressure on the characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. *International Journal of Food Microbiology*, **12**, 207-216.

Simpson, R.K. and Gilmour, A. (1997). The resistance of *Listeria monocytogenes* to high hydrostatic pressure in foods. *Food Microbiology*, **14**, 567-573.

Smelt, J.P.P.M. (1993). High pressure inactivation of microorganisms: possible mechanisms of inactivation. EC AAIR Progress Report, Brussels.

Smelt, J.P.P.M. (1995). Some mechanistic aspects of inactivation of bacteria by high pressure. In: Proceedings of European Symposium Effects of high pressure on foods. University of Montpellier, France, 16th and 17th February.

Smelt, J.P.P.M. (1998). Recent advances in the microbiology of high pressure processing. *Trends in Food Science and Technology*, **9**, 152-158.

ANNEX

Figure 1. Survival of *L. monocytogenes* (NCTC 11994) treated with high pressure in 10mM Tris-maleate (pH7.4)

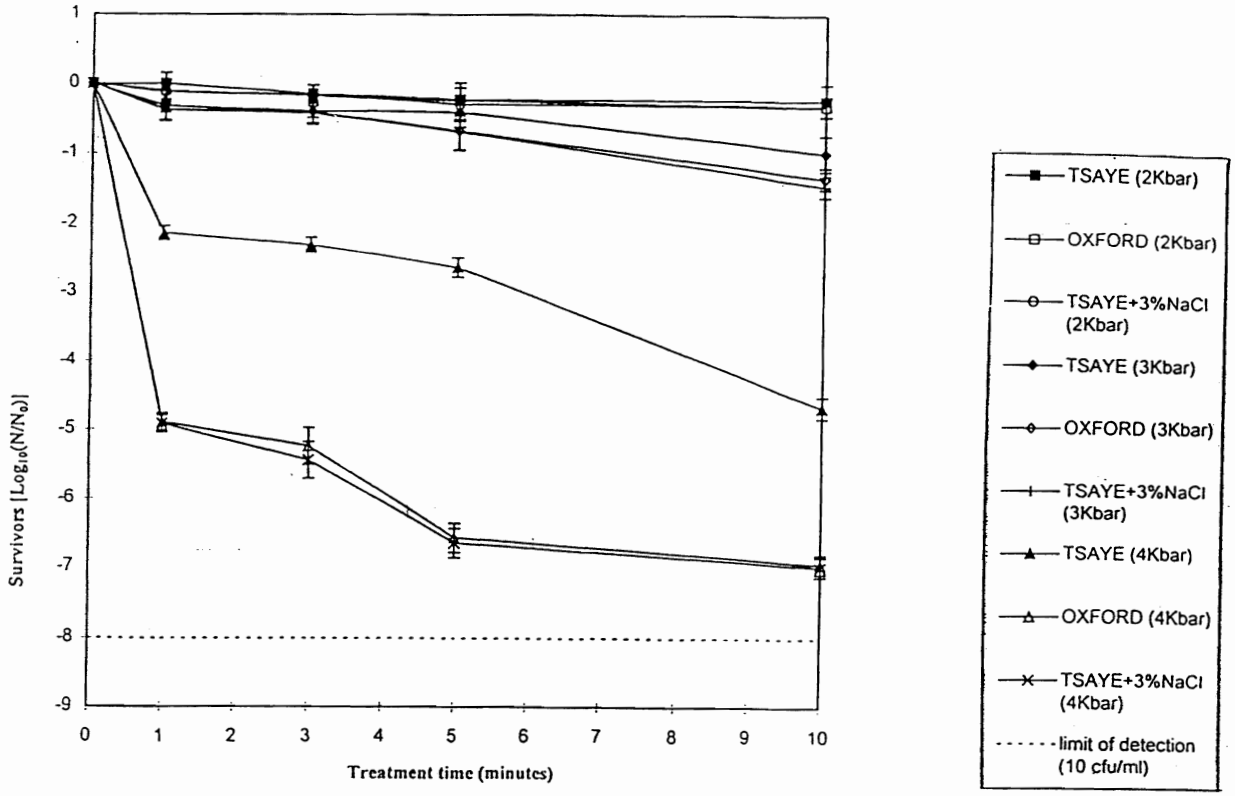

Figure 2. Survival of *S. typhimurium* (CRA 1005) treated with high pressure in 10mM Tris-maleate (pH 7.4)

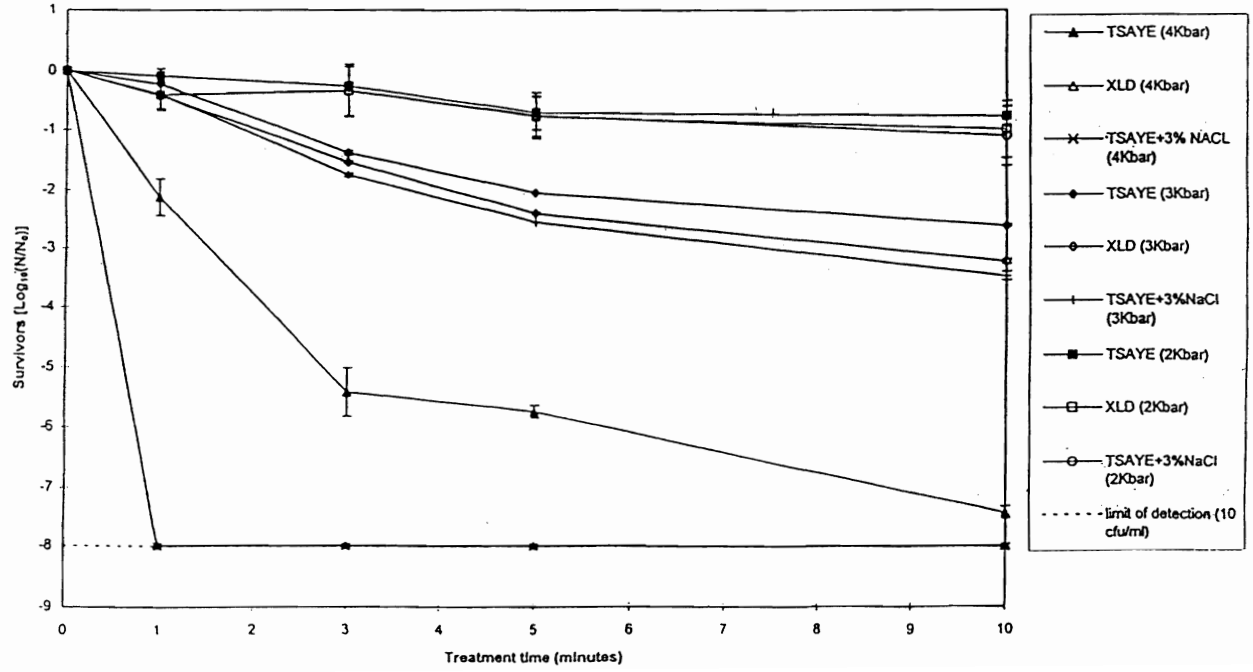

Figure 3. Survival of *L. monocytogenes* grown at 37°C and high pressure (3Kbar) treated in Distilled Water (SDW), Model Beef Broth (MBB) and 10mM Tris-maleate (TMB)

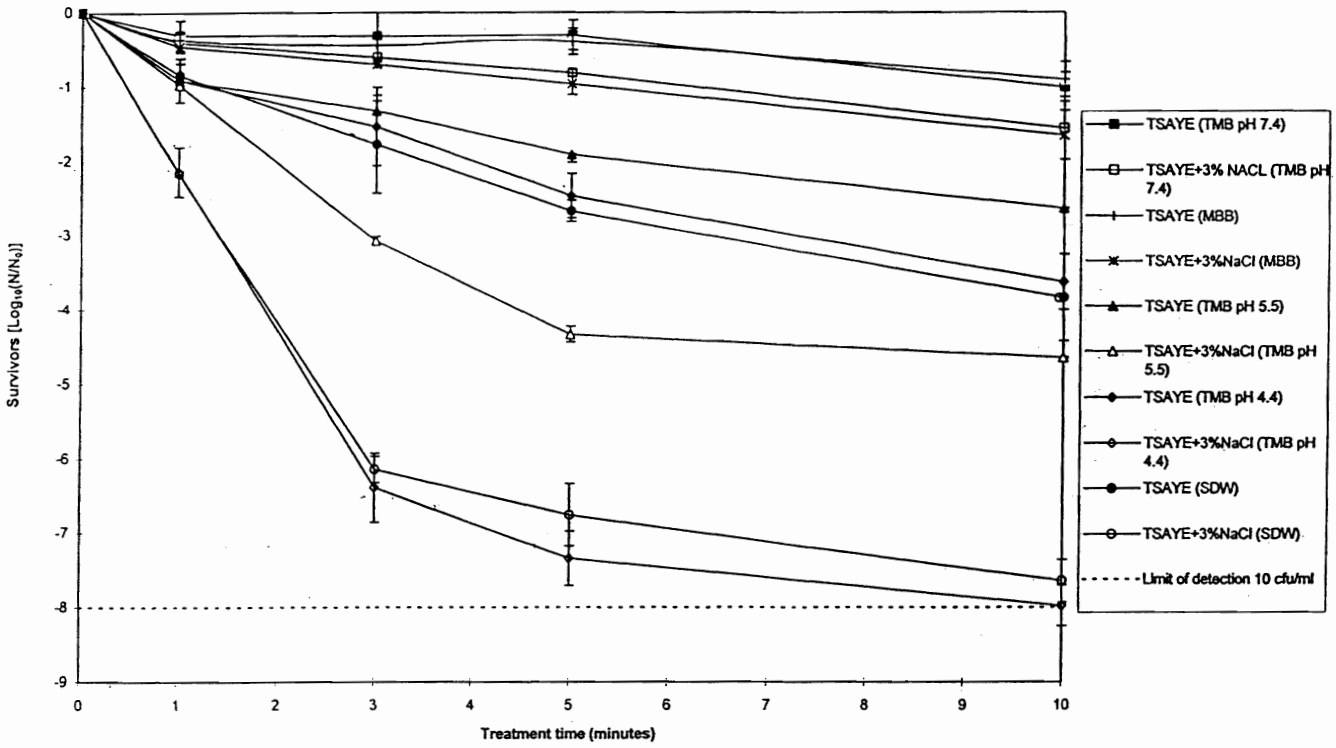

Figure 4. Survival of *S. typhimurium* grown at 37°C and high pressure treated in Distilled Water (SDW), Model Beef Broth (MBB), and 10mM Tris-maleate (TMB)

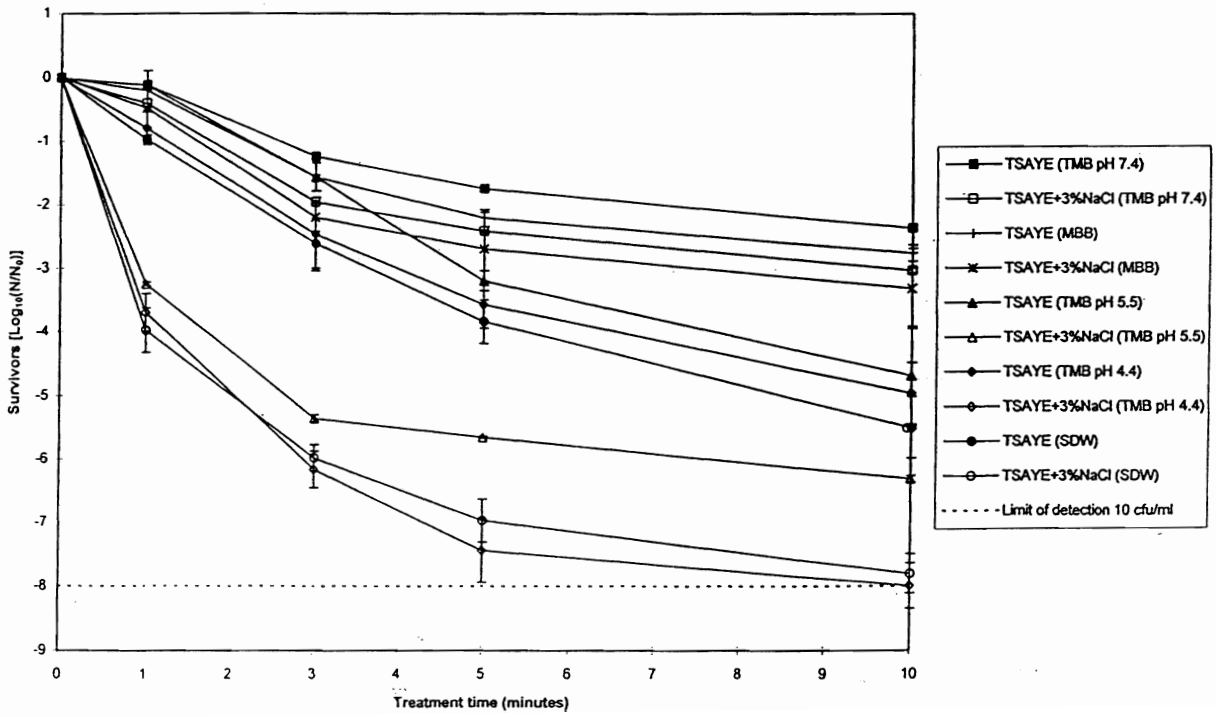

Figure 5a. Survivors of *L. monocytogenes* (NCTC 11994) grown at 4°, 15° or 37°C and treated with high pressure in 10 mM Tris maleate (pH 7.4)

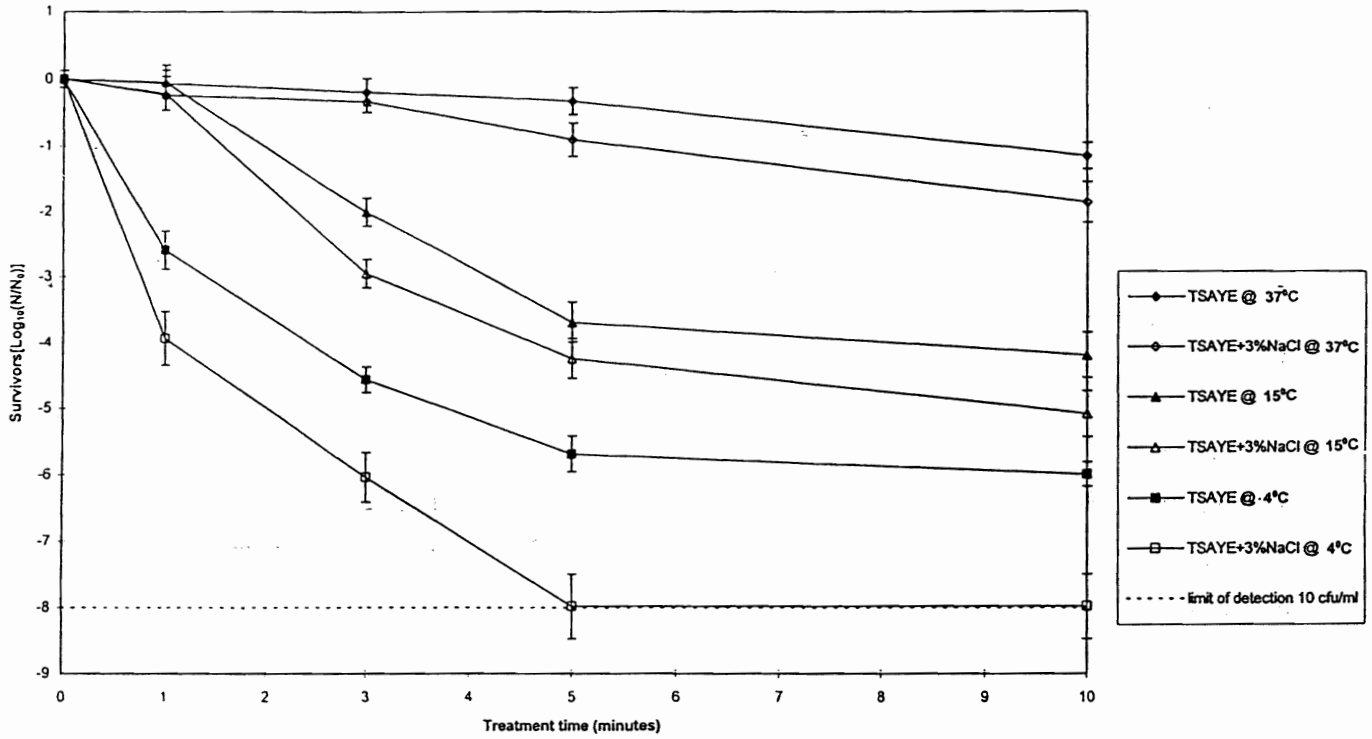

Figure 5b. Survivors of *L. monocytogenes* (NCTC 11994) grown at 4°, 15° or 37°C and treated with high pressure in 10 mM Tris maleate (pH 4.4)

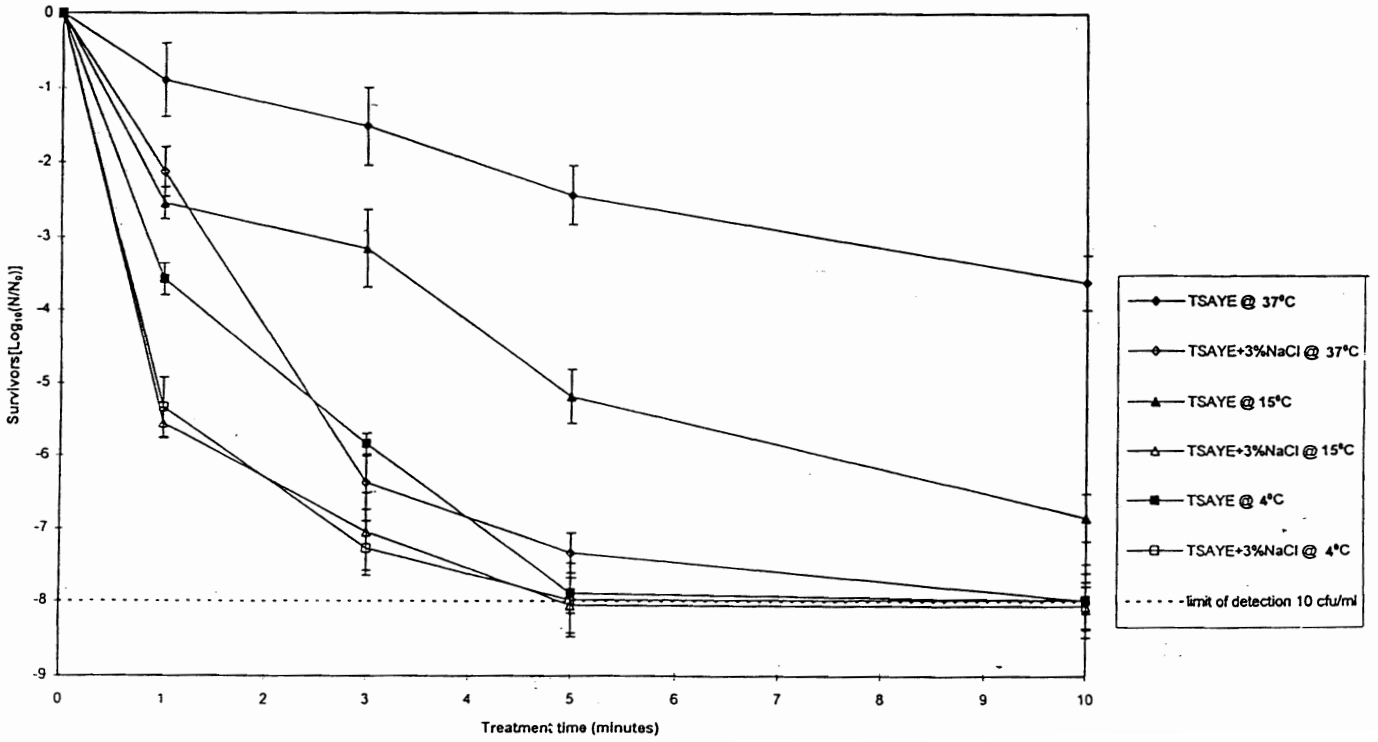

Figure 6a. Survivors of *S. typhimurium* (CRA 1005) grown at 10°, 37° or 45°C and treated with high pressure in 10mM Tris maleate (pH 7.4)

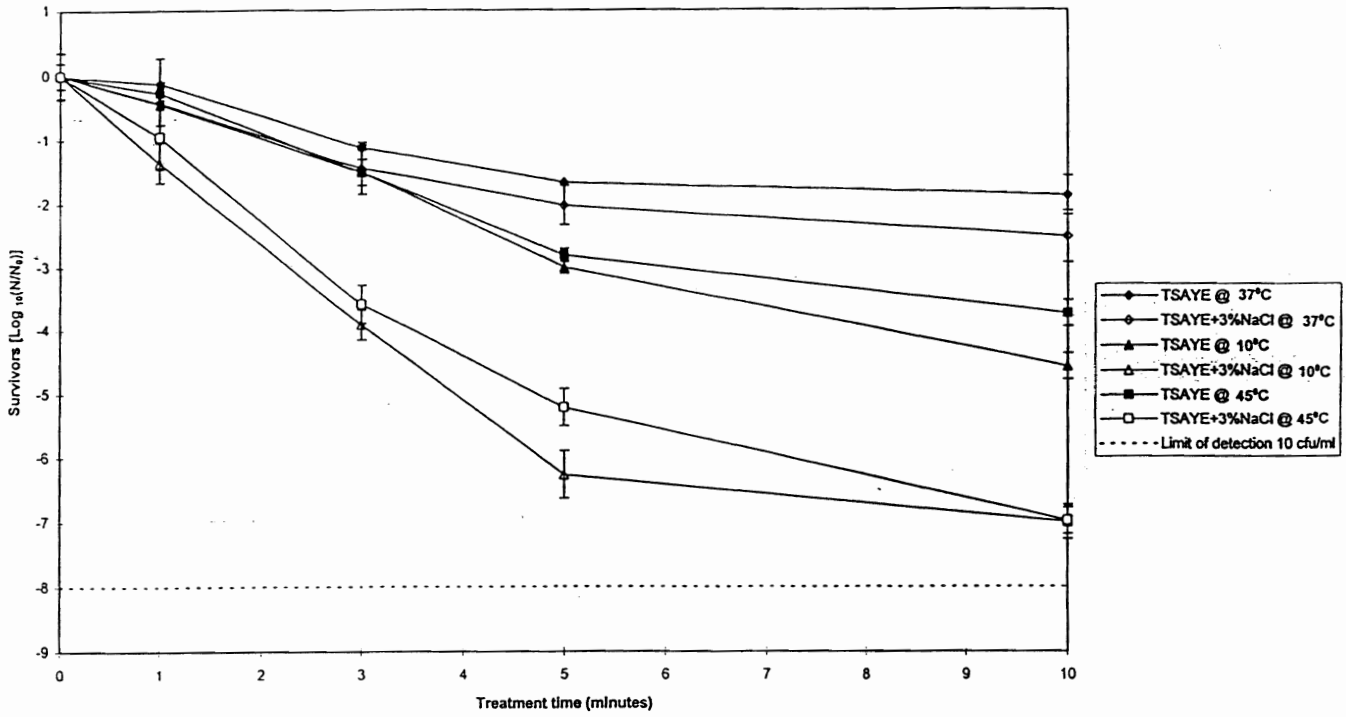

Figure 6b. Survivors of *S. typhimurium* (CRA 1005) grown at 10°, 37° or 45°C and treated with high pressure in 10mM Tris-maleate (pH 4.4)

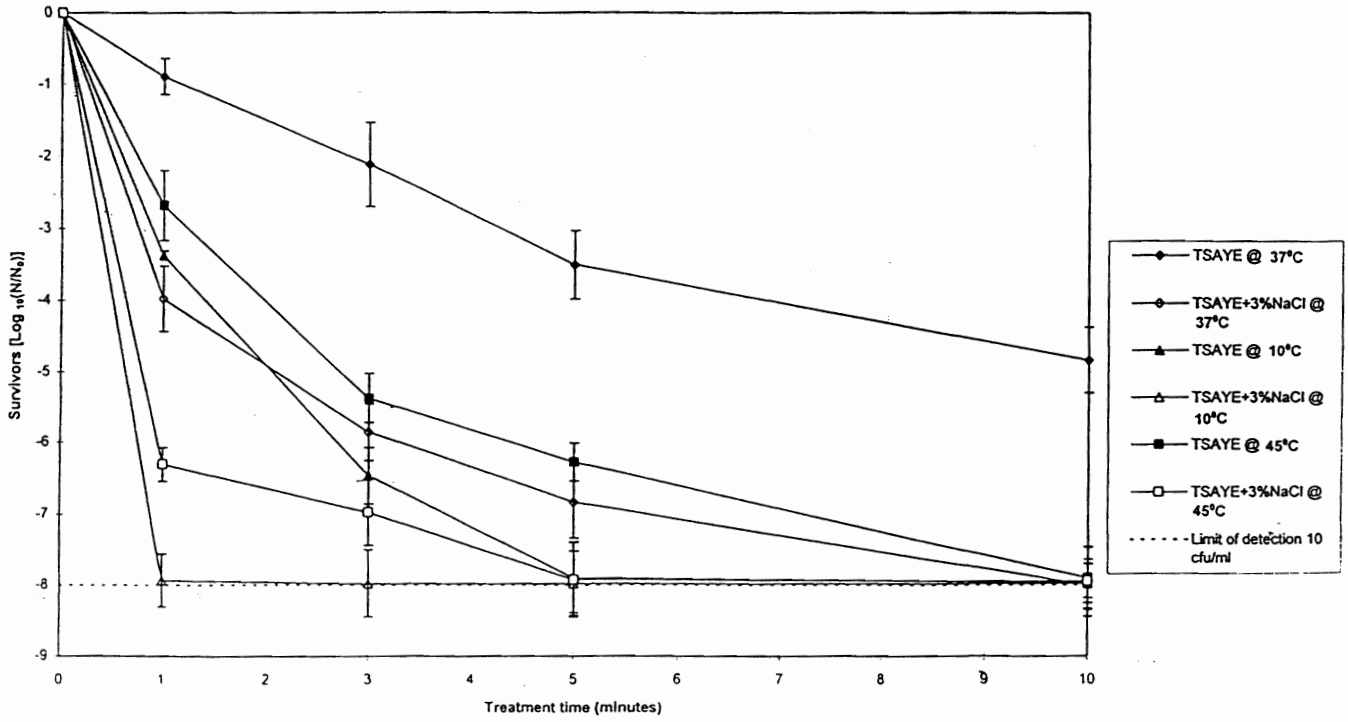

Figure 7a. Survival of *L. monocytogenes* grown at normal (a_w 0.98) or reduced water activity (a_w 0.93) and high pressure treated (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

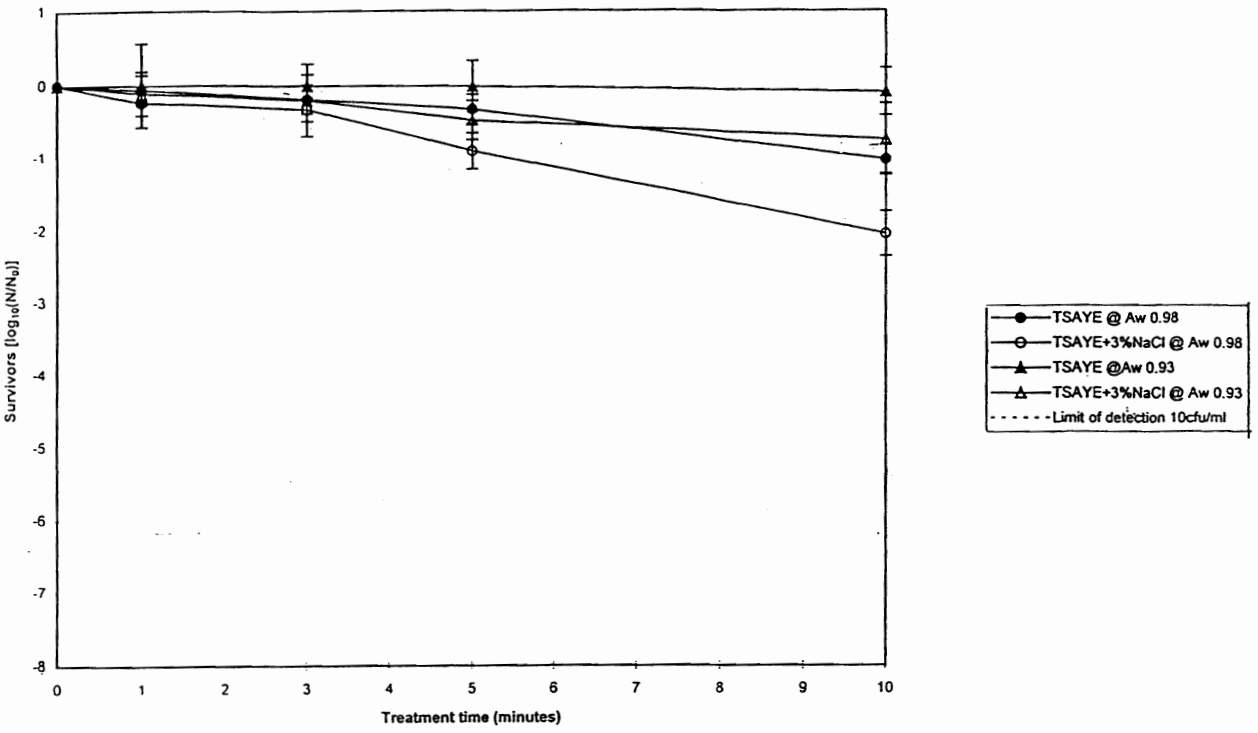

Figure 7b. Survival of *L. monocytogenes* grown at normal (a_w 0.98) or reduced water activity (a_w 0.93) and high pressure treated (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

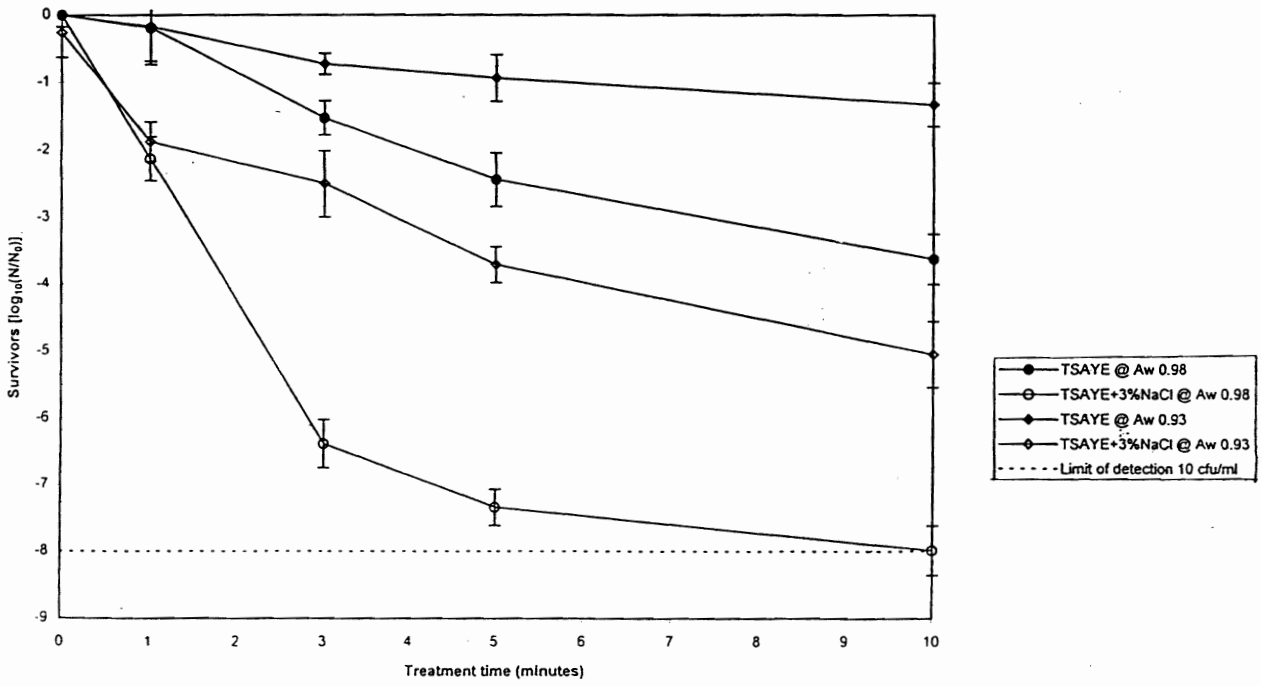

Figure 8a. Survival of *S.typhimurium* grown at normal (a_w 0.98) or reduced water activity (a_w 0.95) and high pressure treated (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

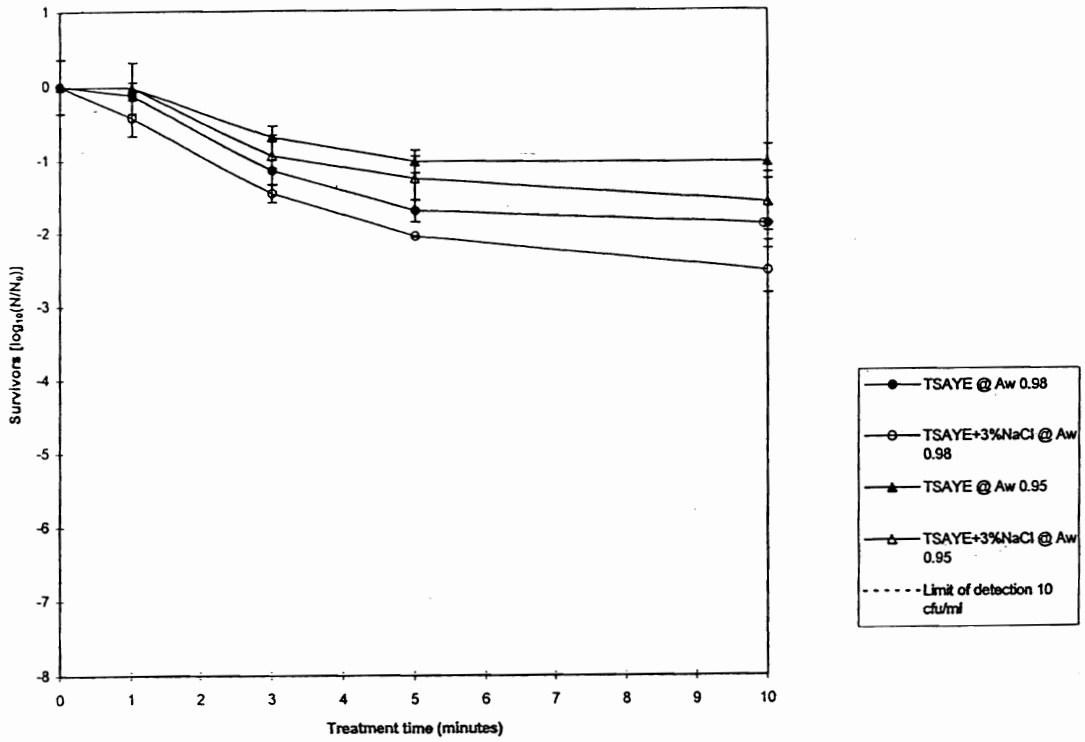

Figure 8b. Survival of *S.typhimurium* grown at normal (a_w 0.98) or reduced water activity (a_w 0.95) and high pressure treated (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

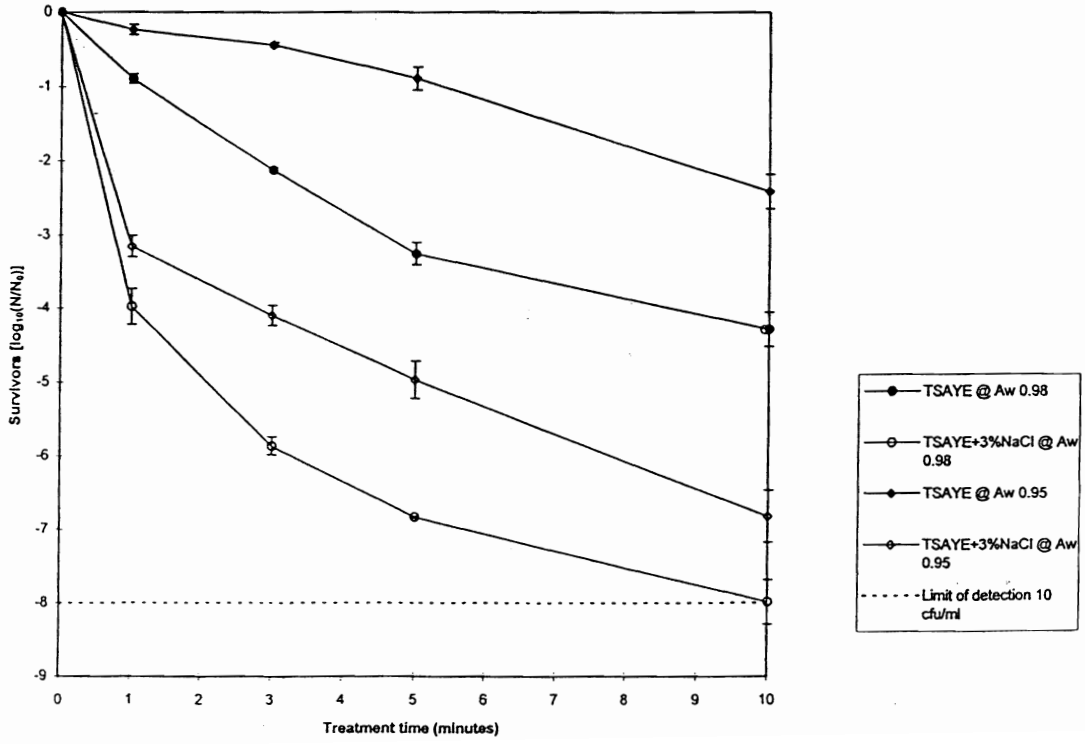

Figure 9a. Survival of *L.monocytogenes* grown at pH 5.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

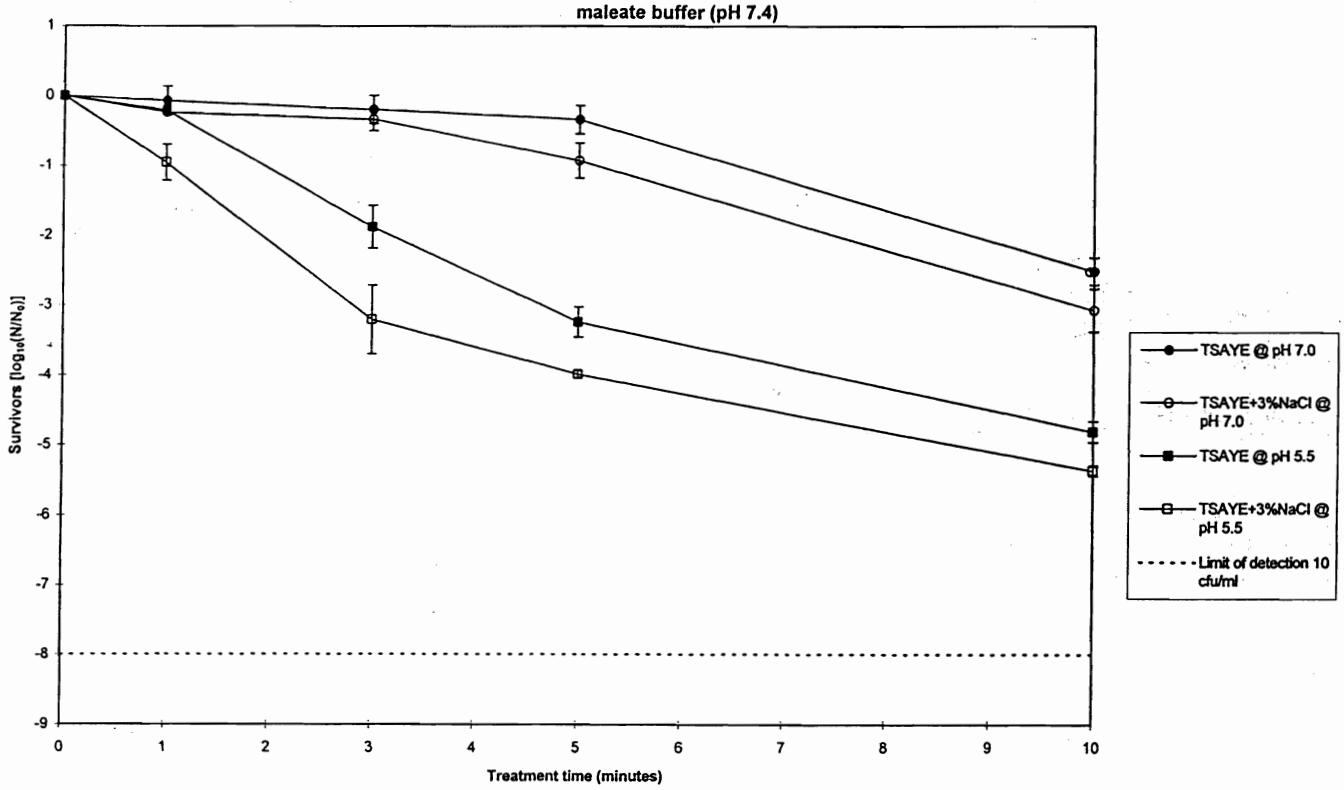

Figure 9b. Survival of *L.monocytogenes* grown at pH 5.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

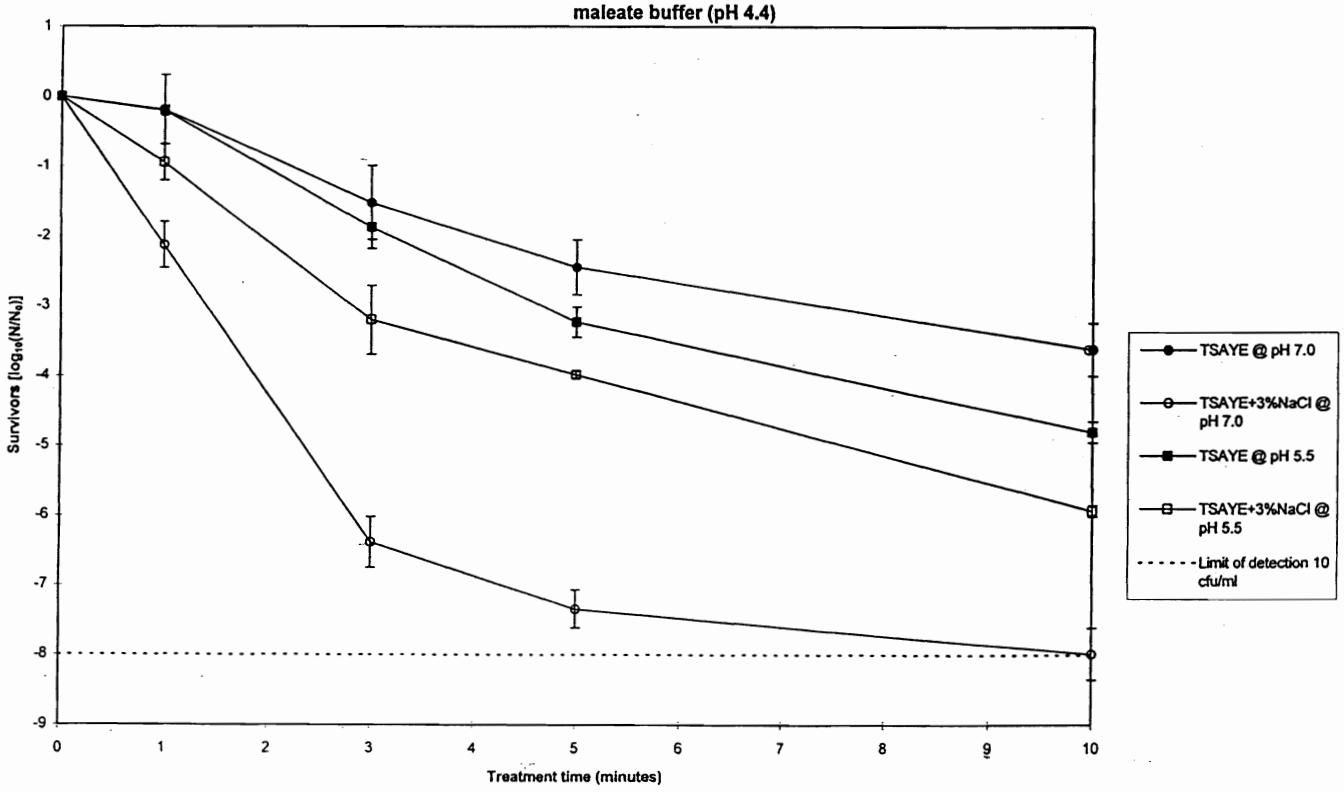

Figure 10a. Survival of *S. typhimurium* grown at pH 4.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

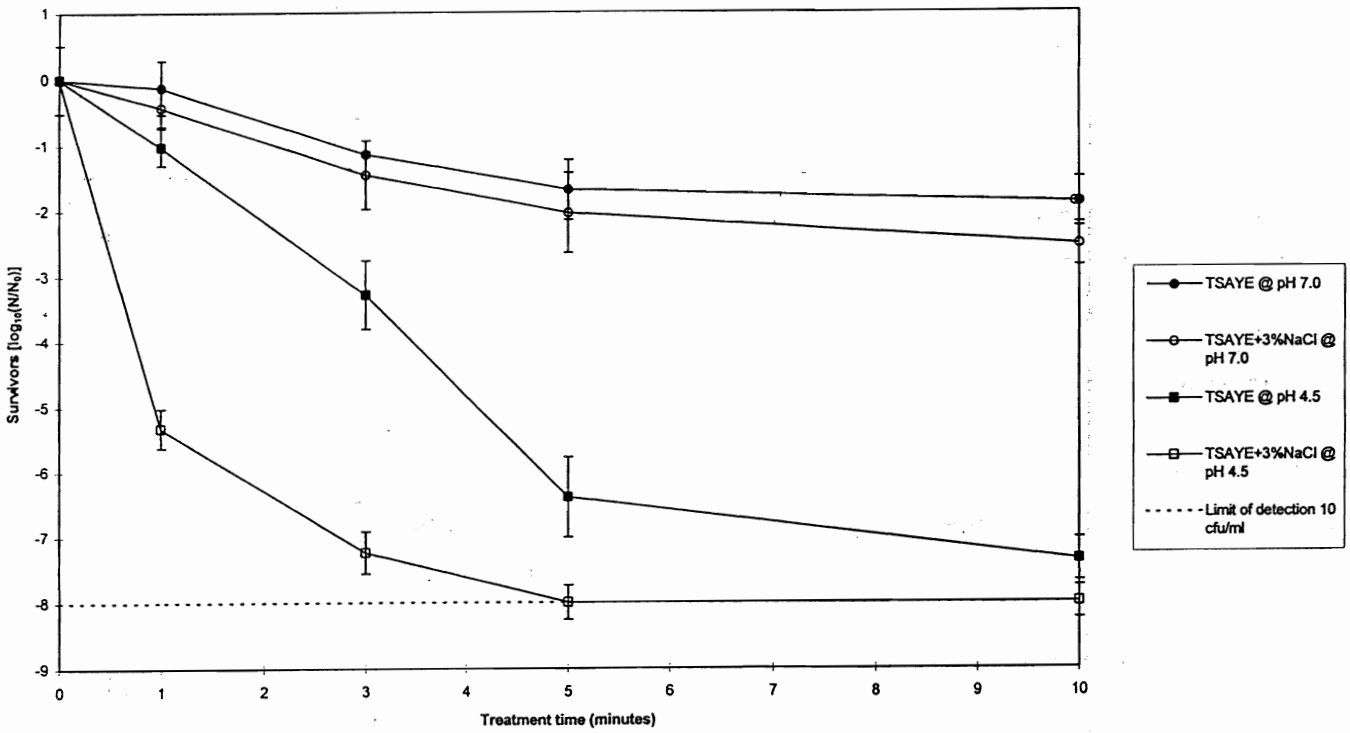

Figure 10b. Survival of *S. typhimurium* grown at pH 4.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

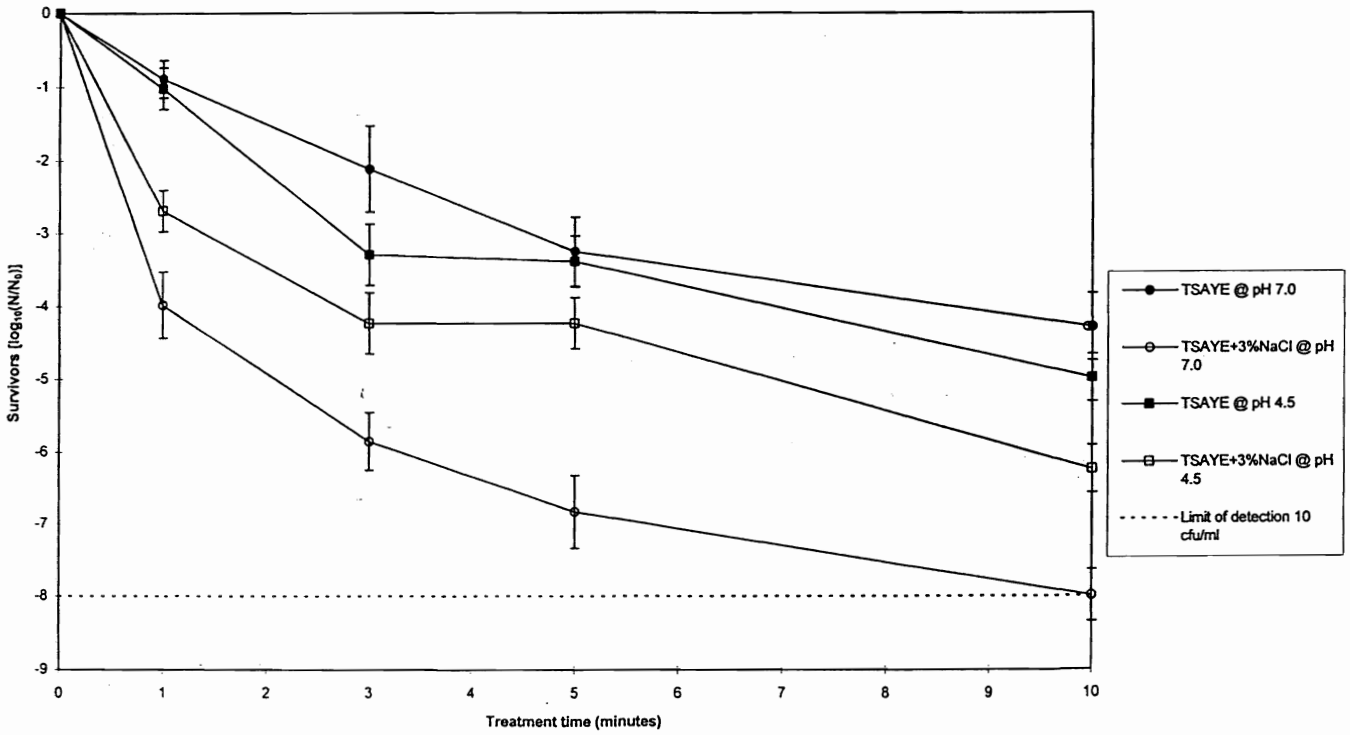

Figure 11. Effect of HP on ATPase activity of *L. monocytogenes* in 10mM Tris-maleate (pH 7.4)

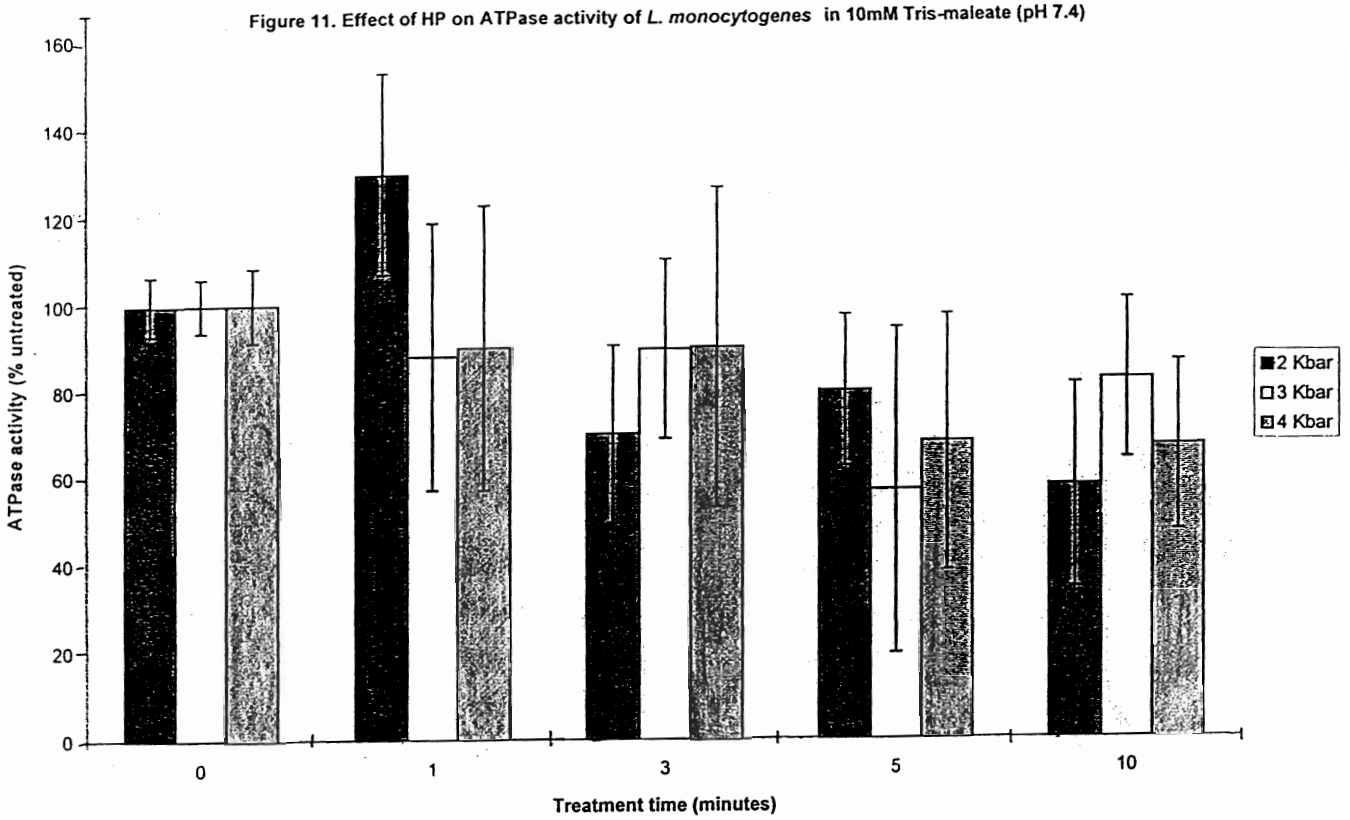

Figure 12. Effect of HP on ATPase activity of *S. typhimurium* in 10mM Tris-maleate (pH 7.4)

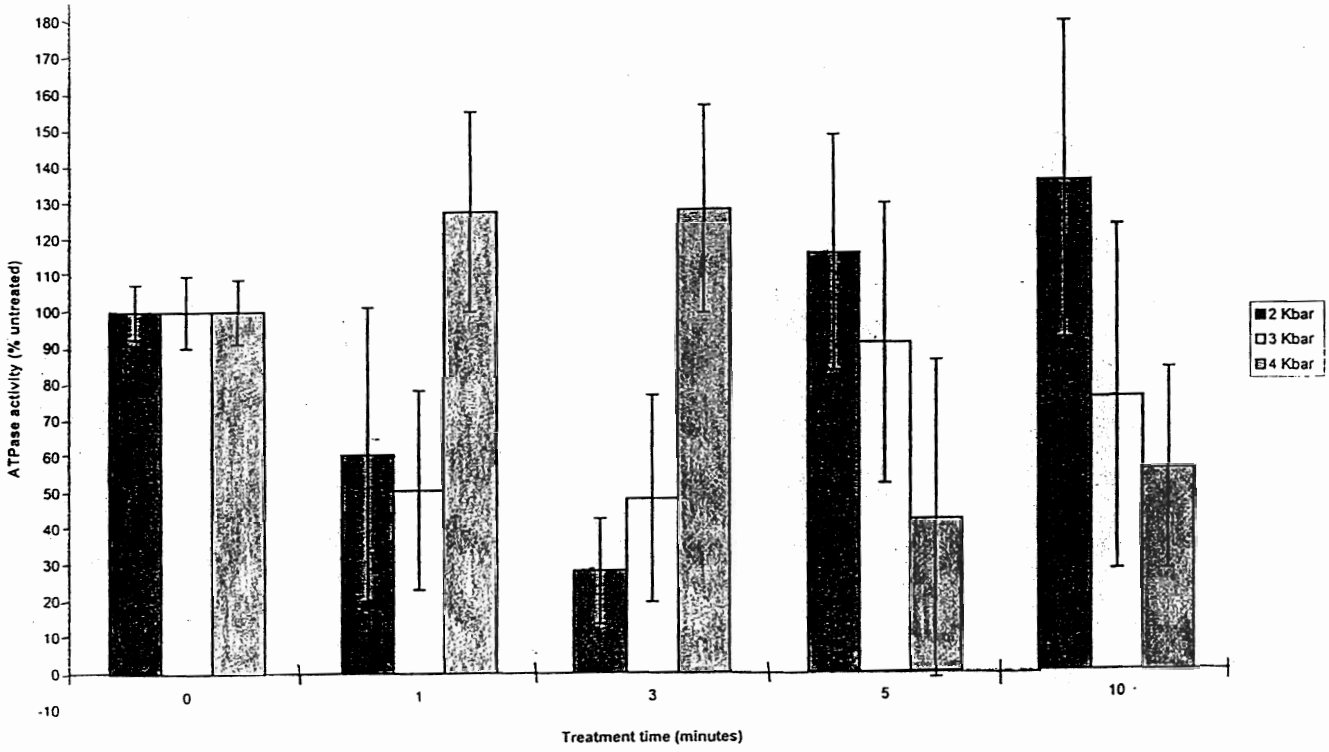

Figure 13. Leakage of UV-absorbing substances from *L. monocytogenes* grown at 37°C and high pressure treated (3Kbar) in Distilled Water (SDW), Model Beef Broth (MBB) and 10mM Tris-maleate (TMB).

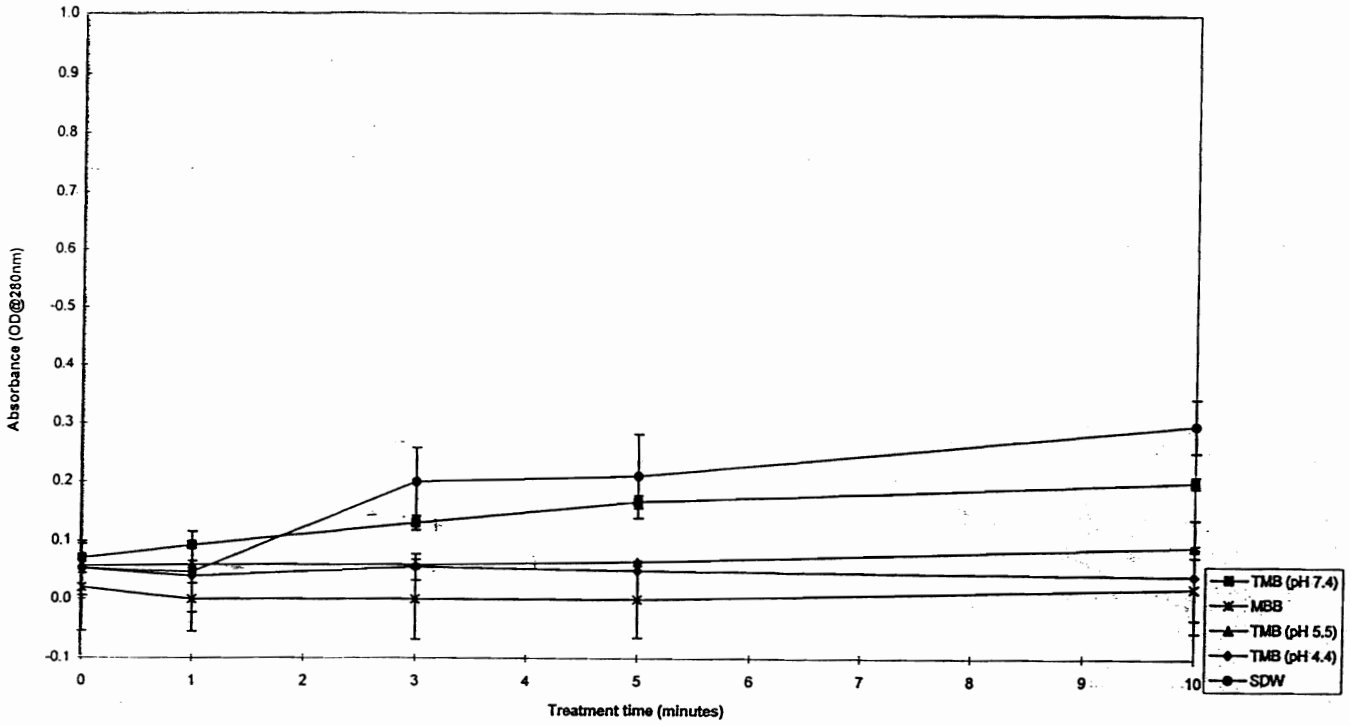

Figure 14. Leakage of UV-absorbing substances from *S. typhimurium* grown at 37°C and high pressure treated (3Kbar) in Distilled Water (SDW), Model Beef Broth (MBB) and 10mM Tris-maleate (TMB).

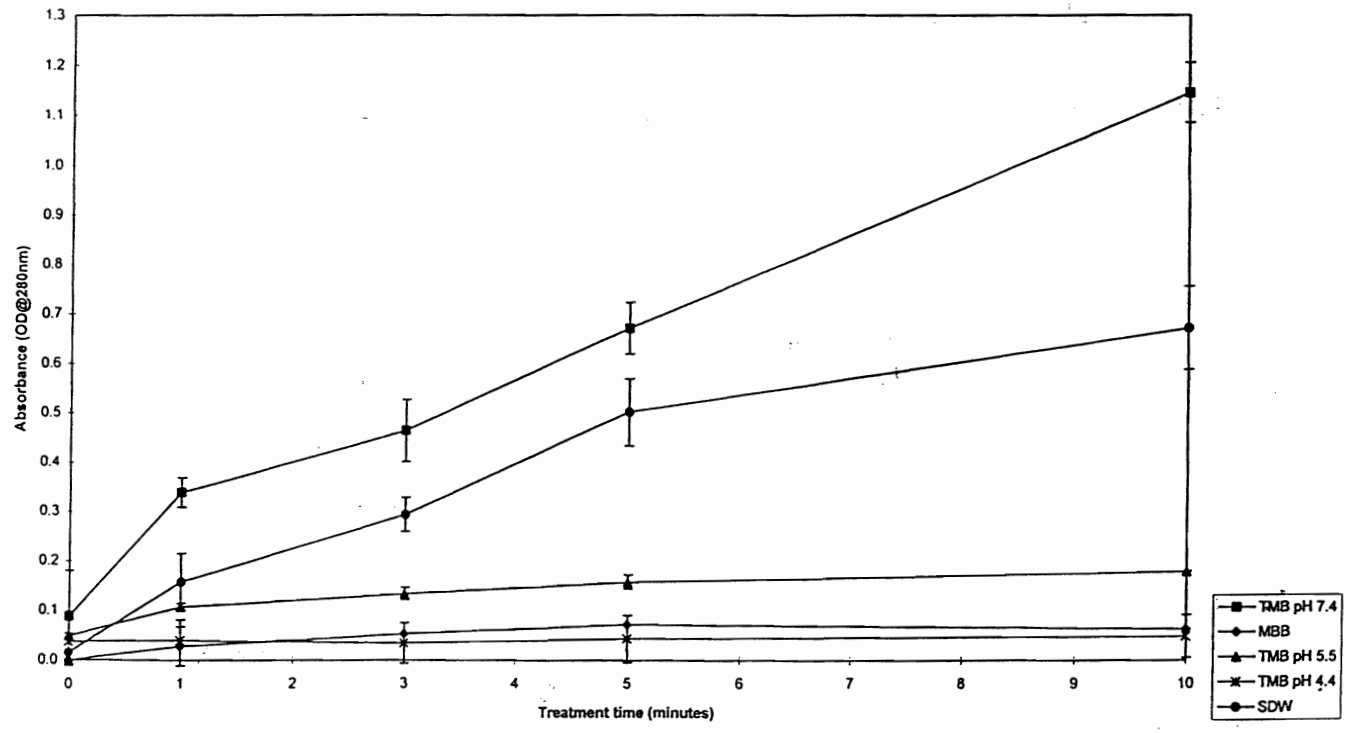

Figure 15a. Leakage of UV-absorbing substances from *L. monocytogenes* grown at 4°, 15°, or 37°C and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

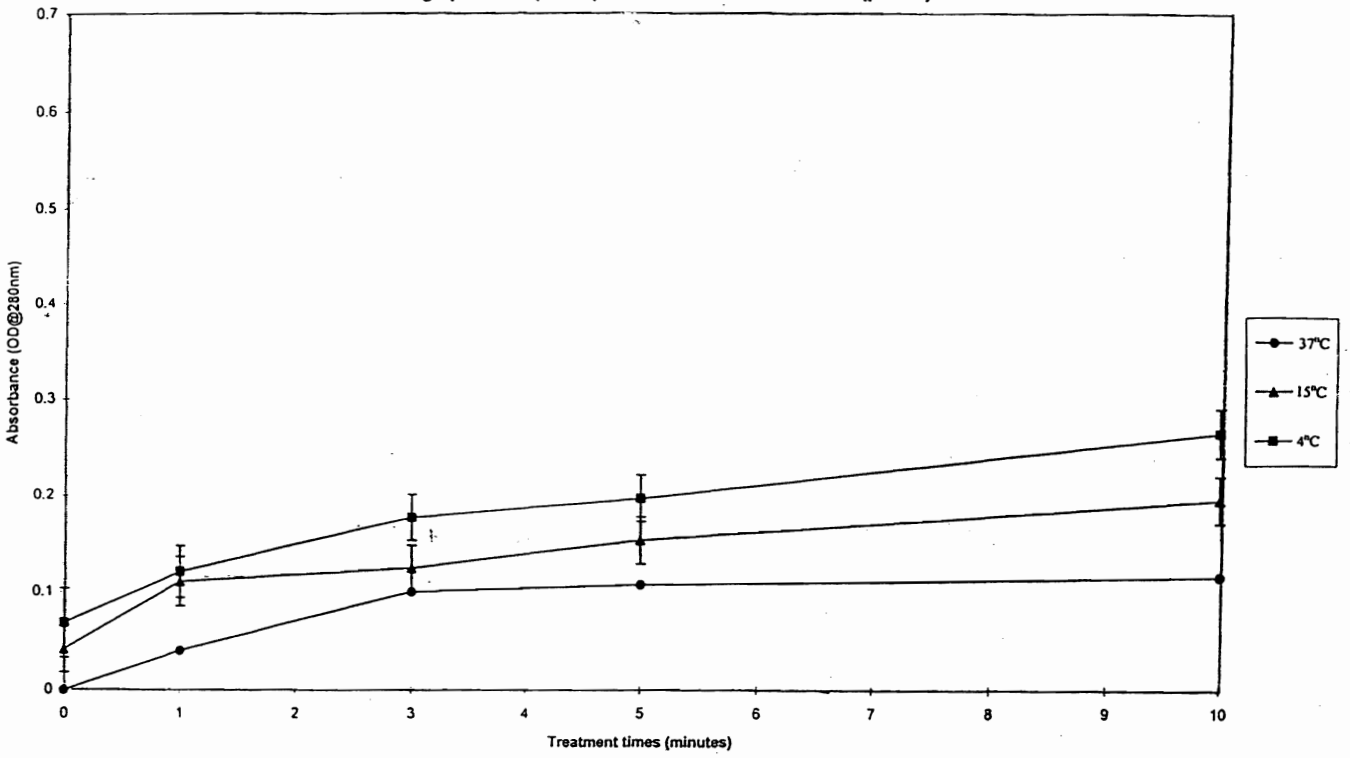

Figure 15b. Leakage of UV-absorbing substances from *L. monocytogenes* grown at 4°, 15°, or 37°C and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

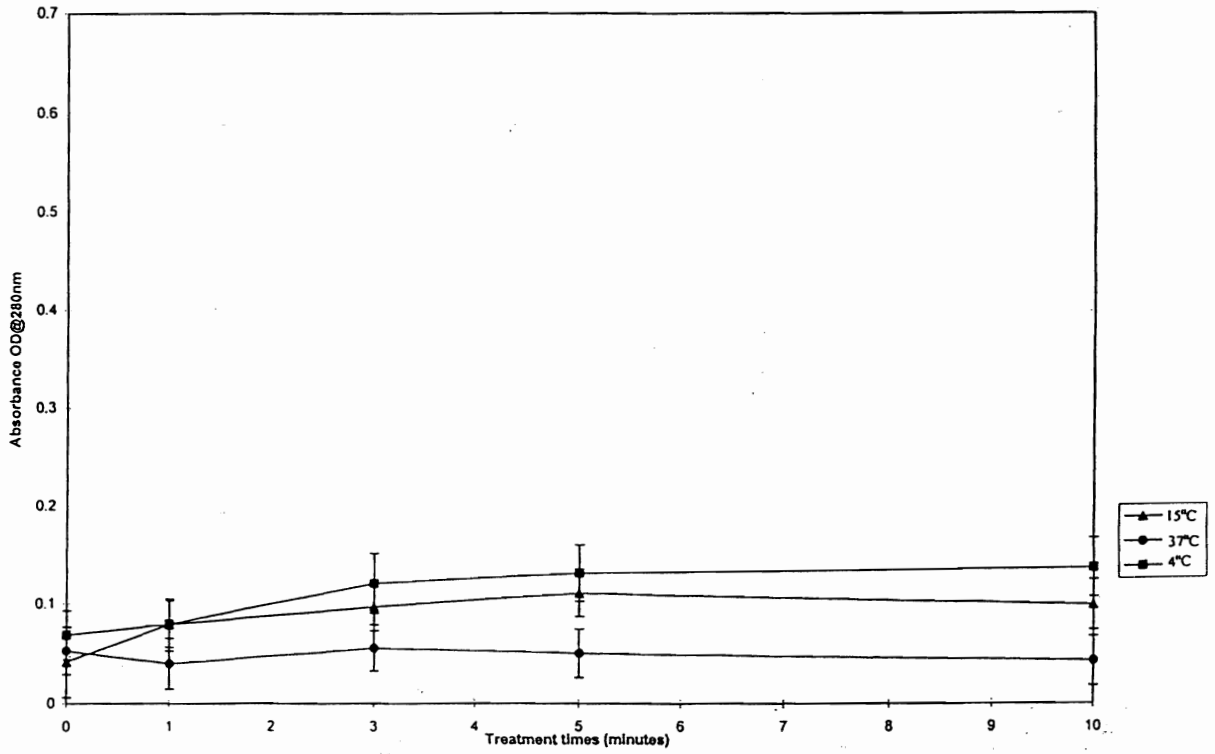

Figure 16a. Leakage of UV-absorbing substances from *S. typhimurium* grown at 10°, 37°, or 45°C and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

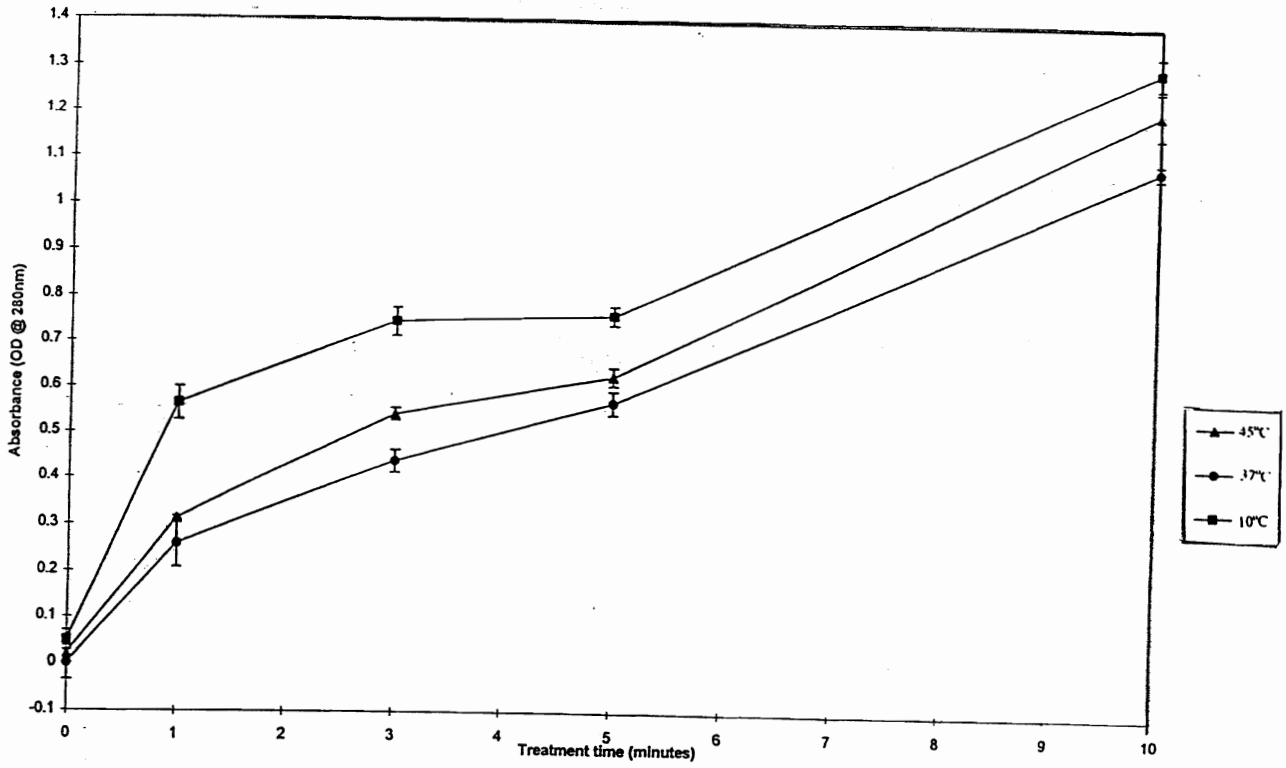

Figure 16b. Leakage of UV-absorbing substances from *S. typhimurium* grown at 10°, 37°, or 45°C and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

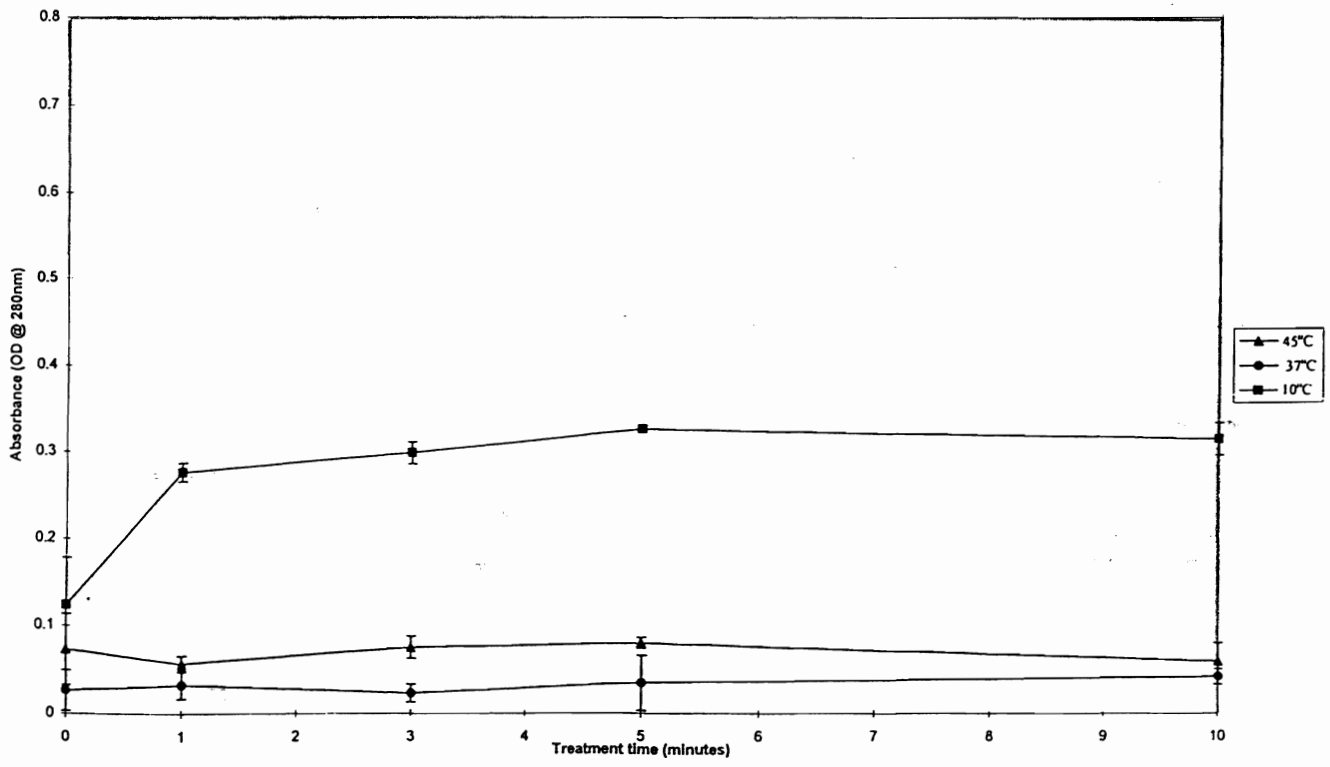

Figure 17a. Leakage of UV-absorbing substances from *L. monocytogenes* grown at normal (a_w 0.98) or reduced water activity (a_w 0.93) and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

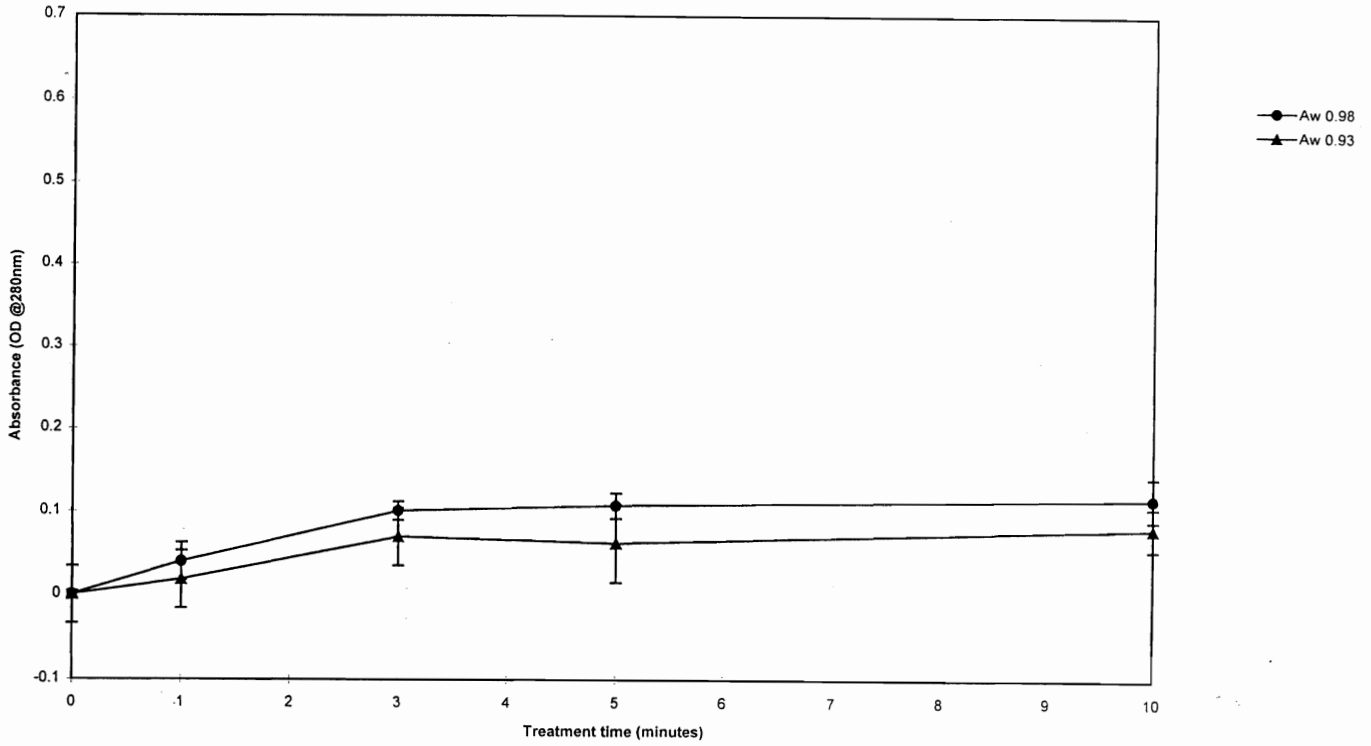

Figure 17b. Leakage of UV-absorbing substances from *L. monocytogenes* grown at normal (a_w 0.98) or reduced water activity (a_w 0.93) and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

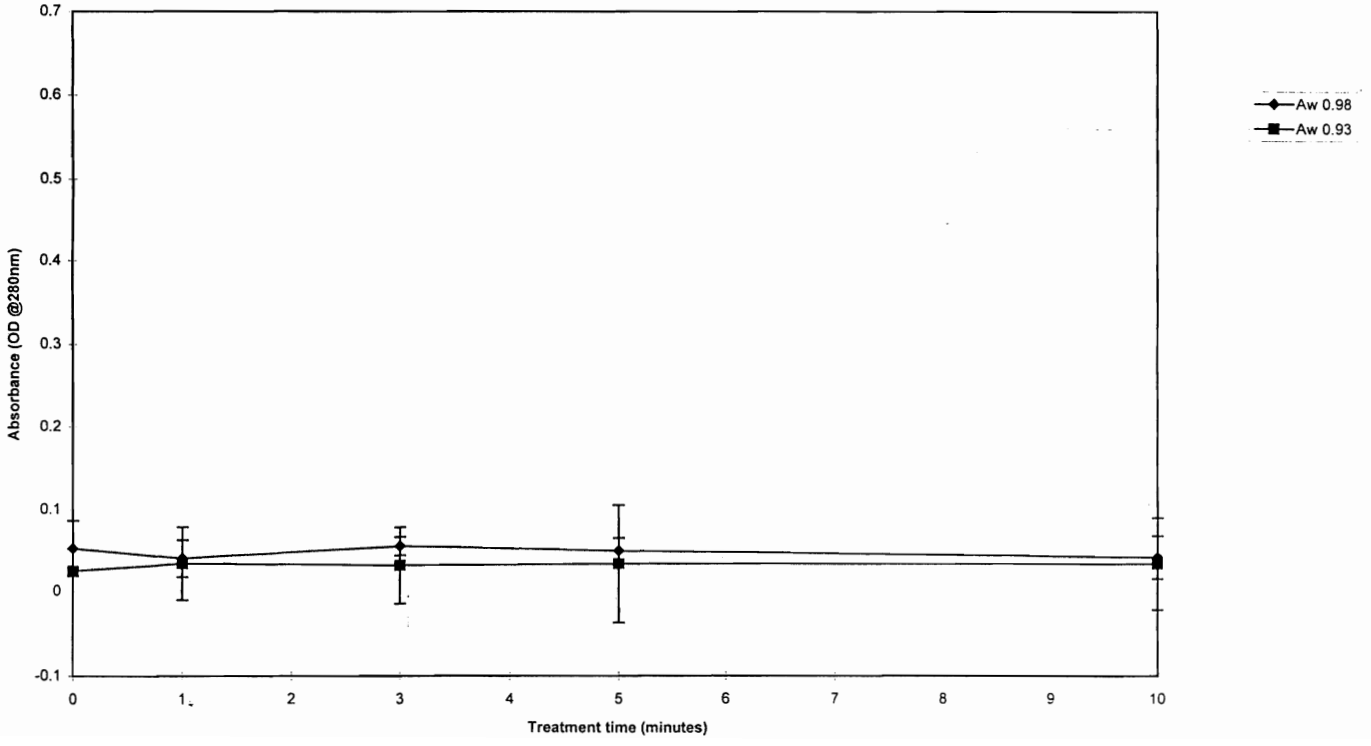

Figure 18a. Leakage of UV-absorbing substances from *S. typhimurium* grown at normal (a_w 0.98) or reduced water activity (a_w 0.95) and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

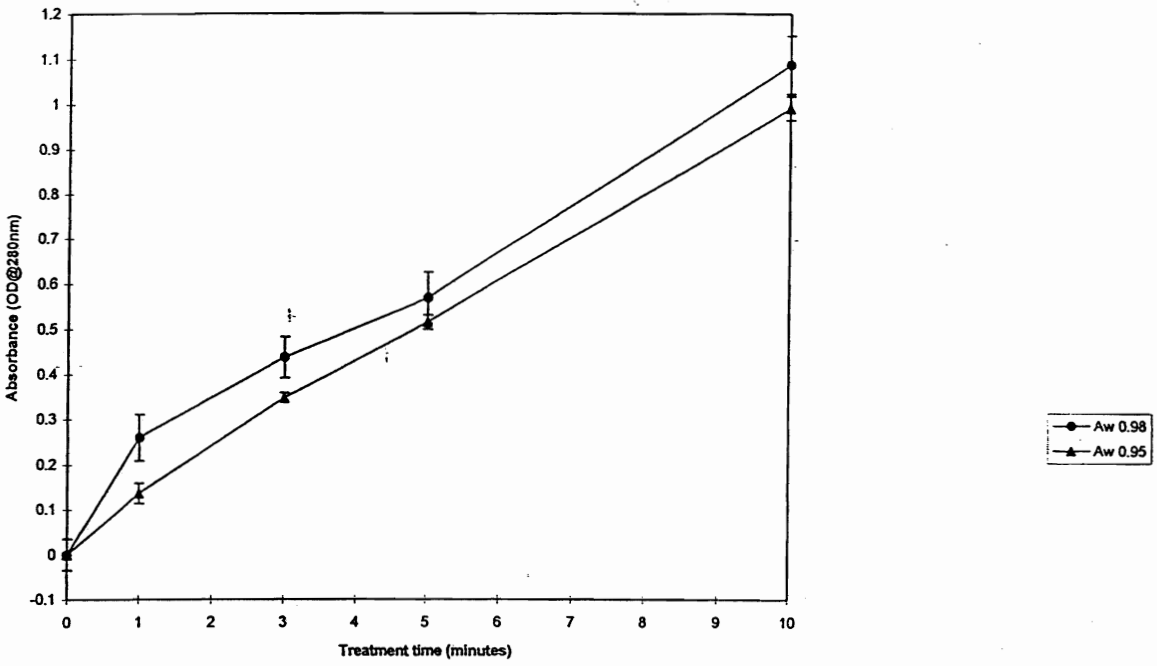

Figure 18b. Leakage of UV-absorbing substances from *S. typhimurium* grown at normal (a_w 0.98) or reduced water activity (a_w 0.95) and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

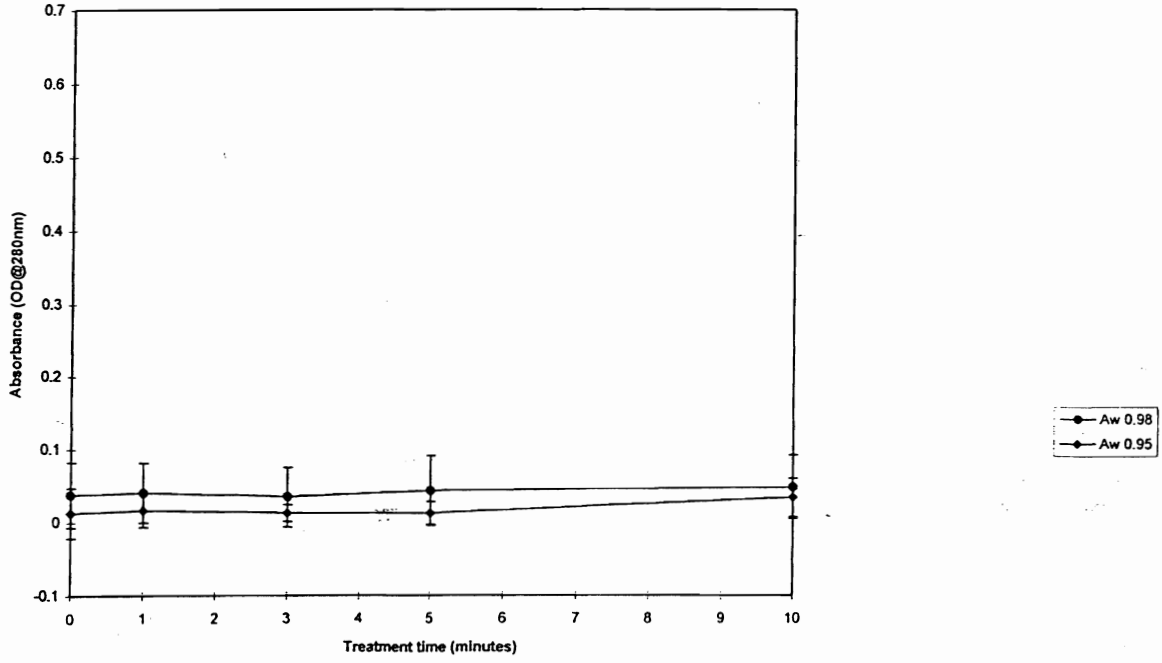

Figure 19a. Leakage of UV-absorbing substances from *L.monocytogenes* grown at pH 5.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

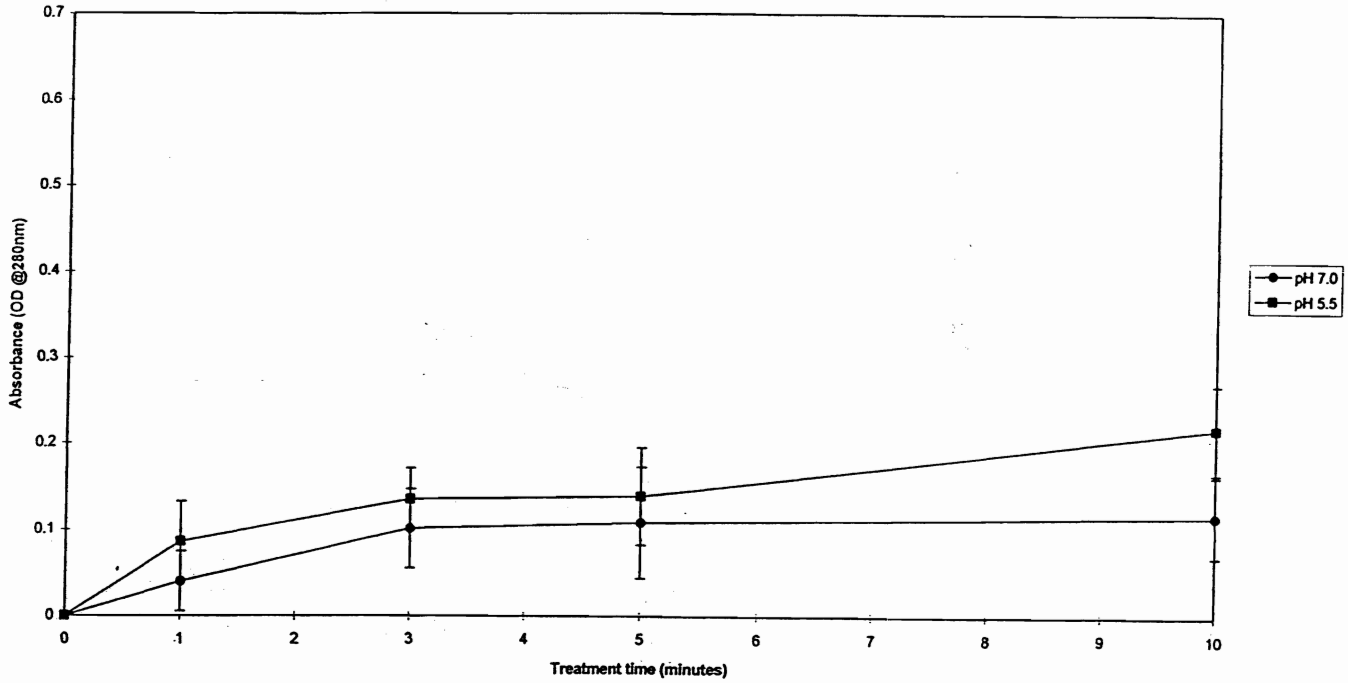

Figure 19b. Leakage of UV-absorbing substances from *L.monocytogenes* grown at pH 5.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

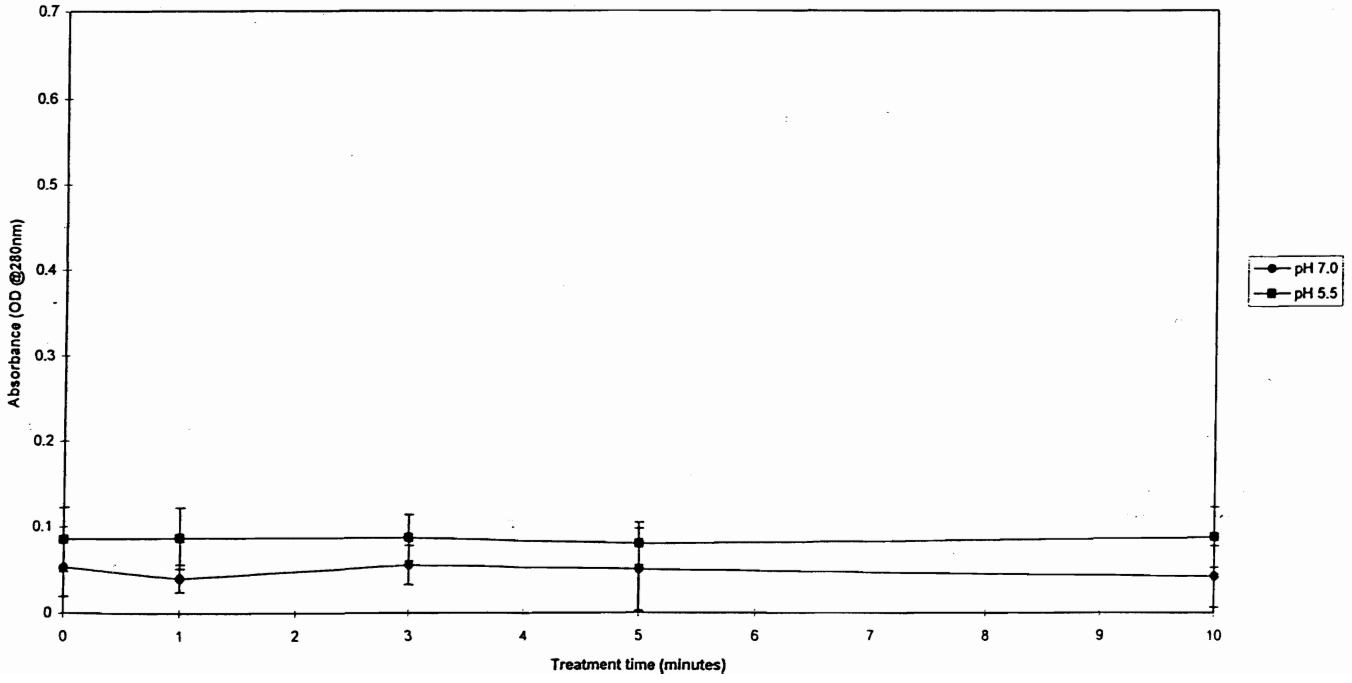

Figure 20a. Leakage of UV-absorbing substances from *S. typhimurium* grown at pH 4.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 7.4)

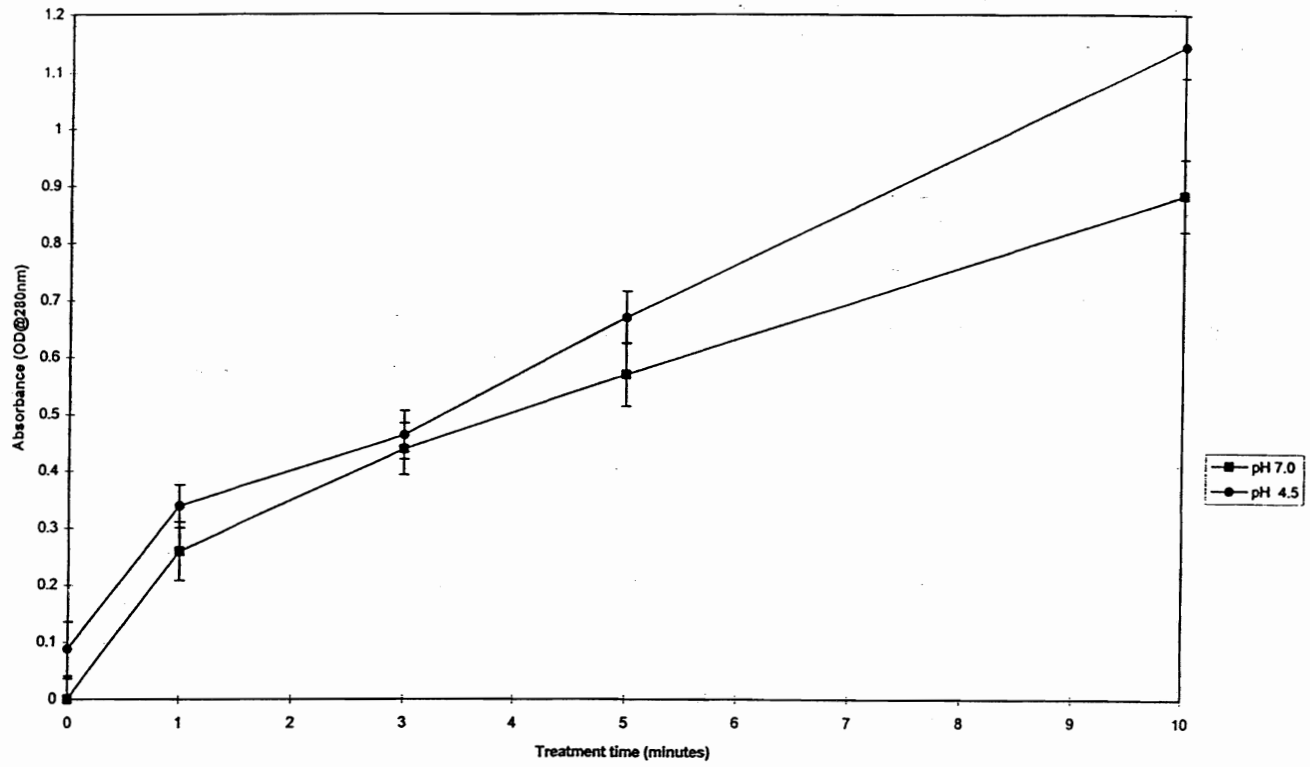

Figure 20b. Leakage of UV-absorbing substances from *S. typhimurium* grown at pH 4.5 and 7.0 and treated with high pressure (3Kbar) in 10mM Tris-maleate buffer (pH 4.4)

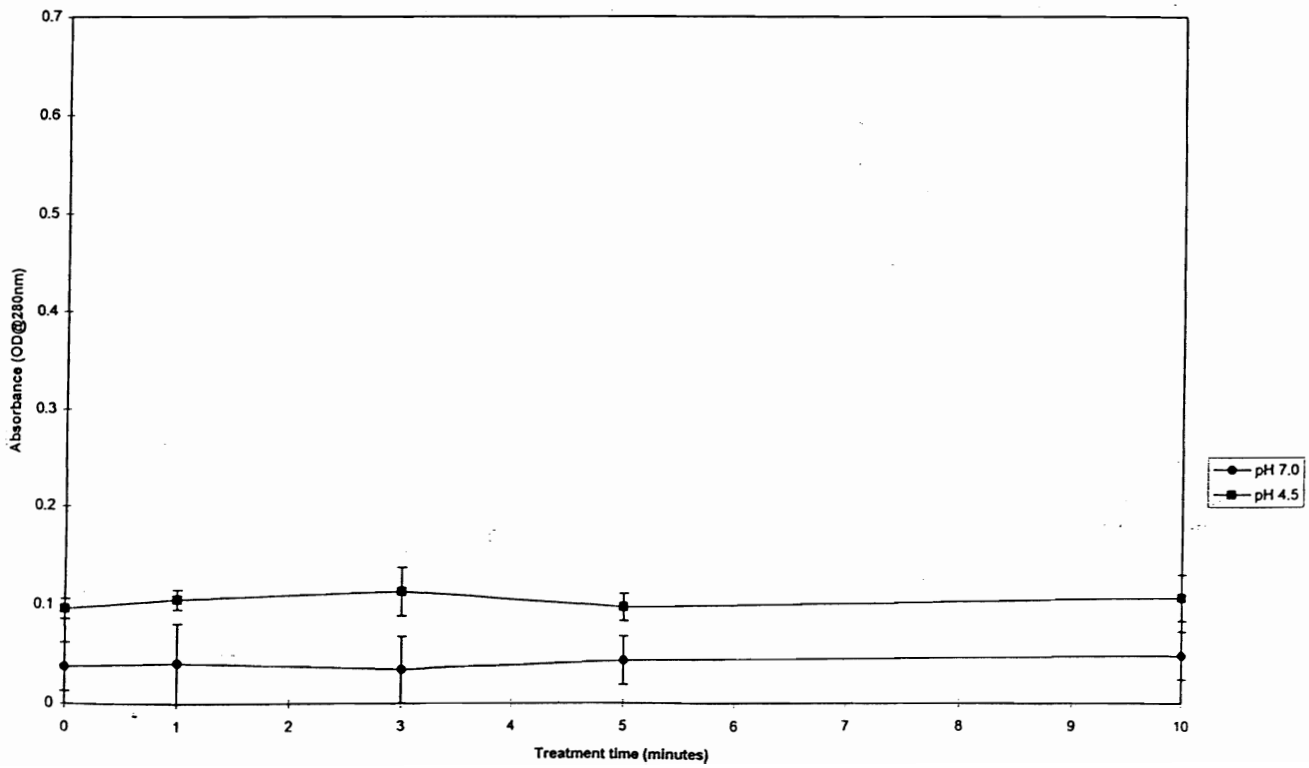

Figure 21. Effect of high pressure (3Kbar) on Δ pH, the difference between internal and external pH, in *L. monocytogenes* (NCTC 11994)

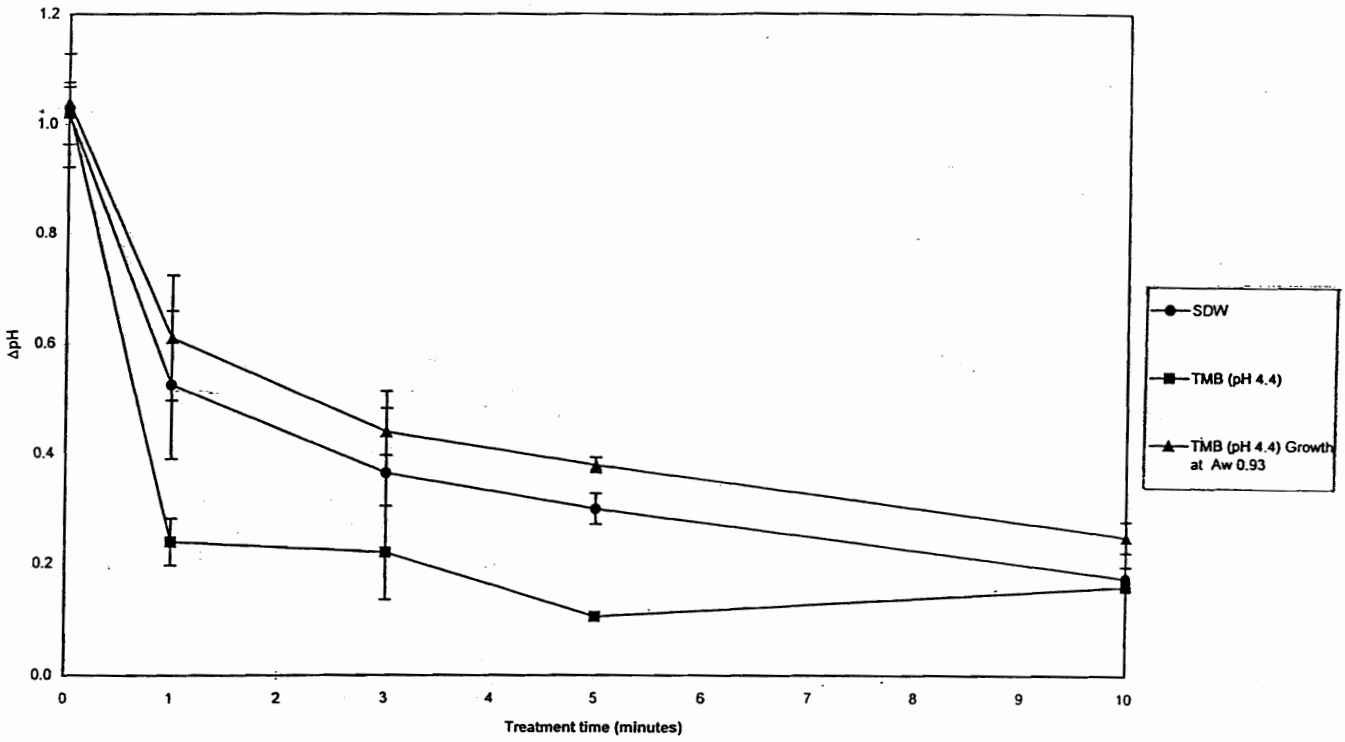

Figure 22a. Growth rate of *S. typhimurium* @ 10°C after pre-treatment at 10°C pH 7.0 (untreated samples)

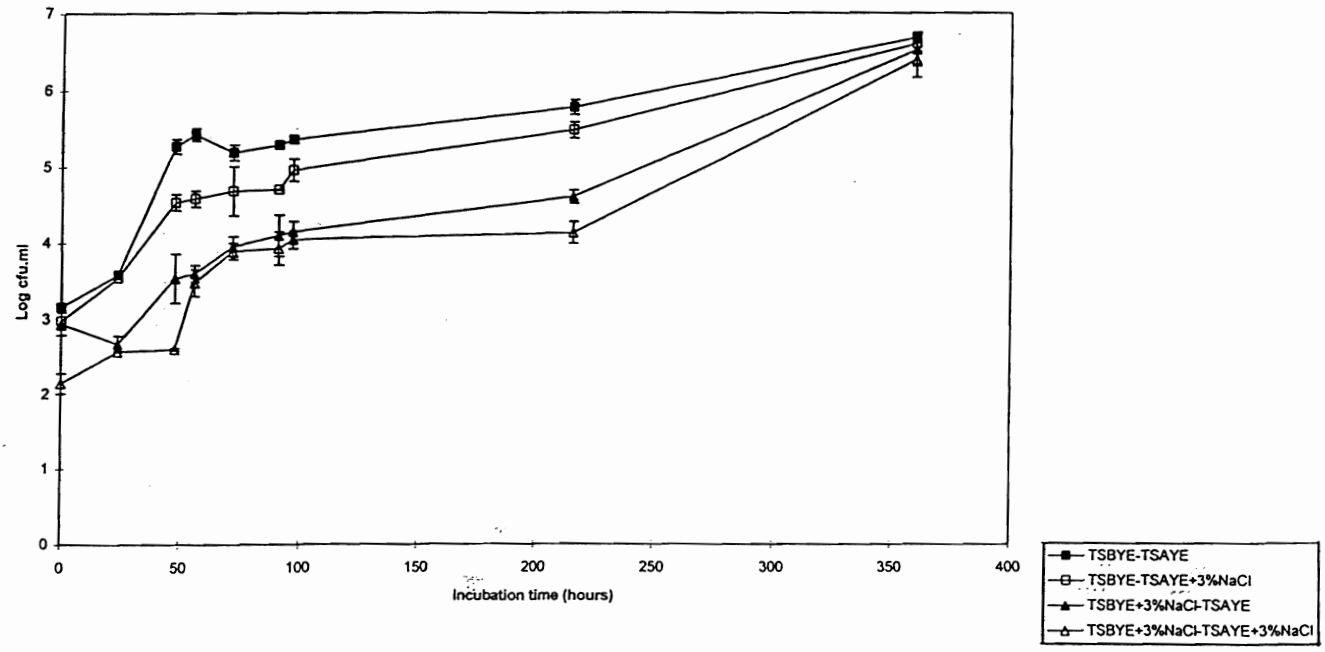

Figure 22b. Growth rate of *S.typhimurium* grown @ 10°C after pre-treatment at 10°C pH 7.0 (3 minute HP treatment 3KBar)

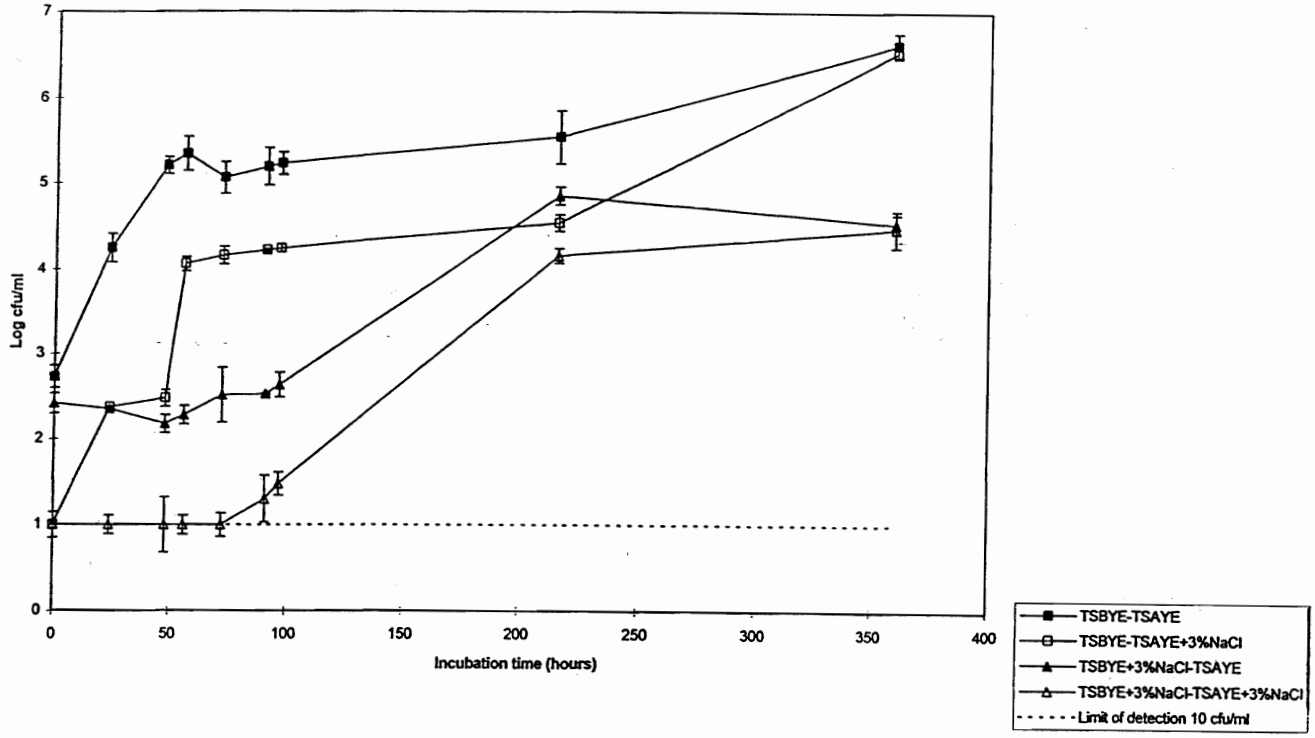

Figure 22c. Growth rate of *S. typhimurium* grown @ 10°C after pre-treatment at 10°C and pH 7.0 (5 minute HP treatment 3KBar)

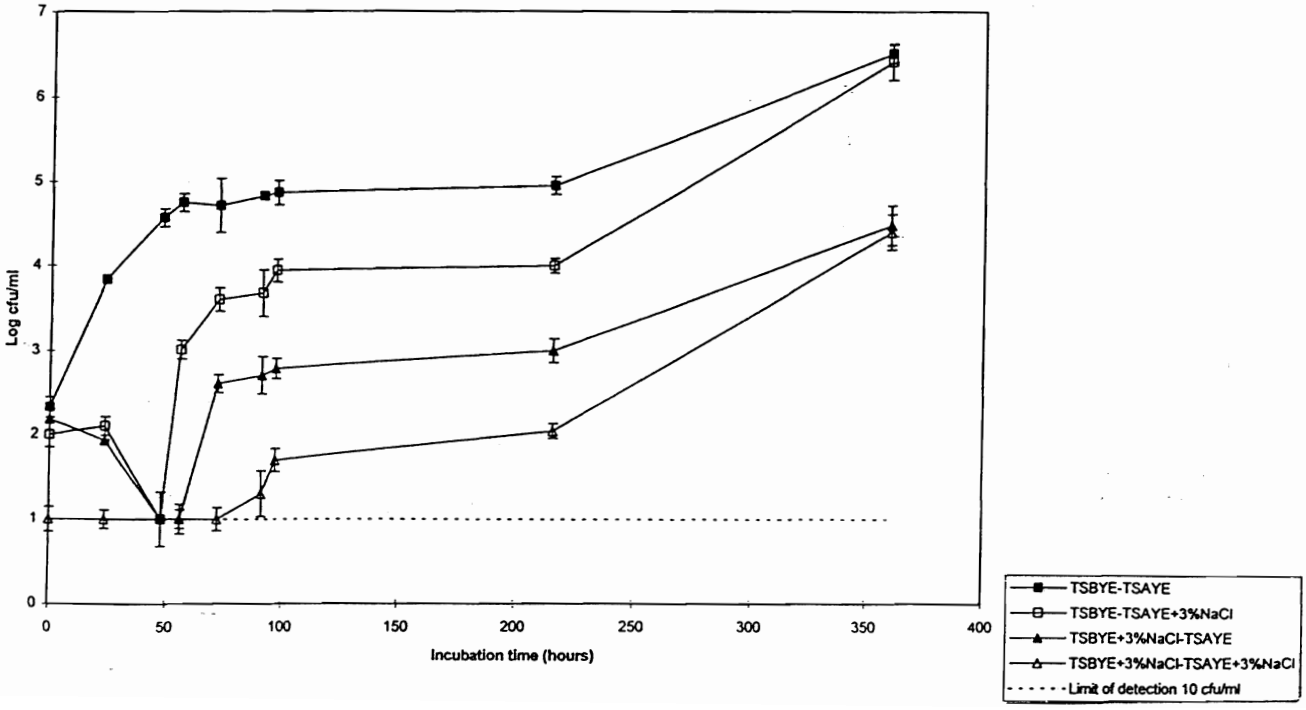

Figure 23a. Growth rate of *L. monocytogenes* @ 4°C after pre-treatment at 4°C pH 7.0 (untreated samples)

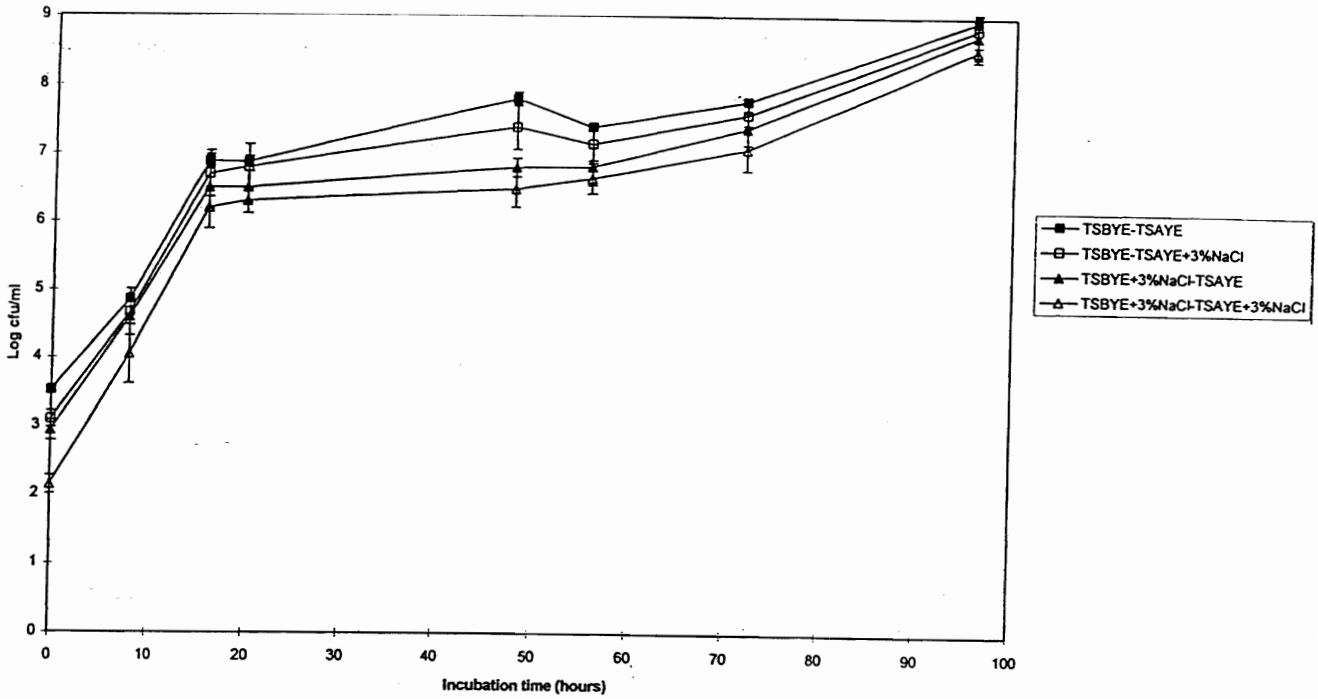

Figure 23b. Growth rate of *L. monocytogenes* grown @ 4°C after pre-treatment at 4°C pH 7.0 (3 minute HP treatment 3KBar)

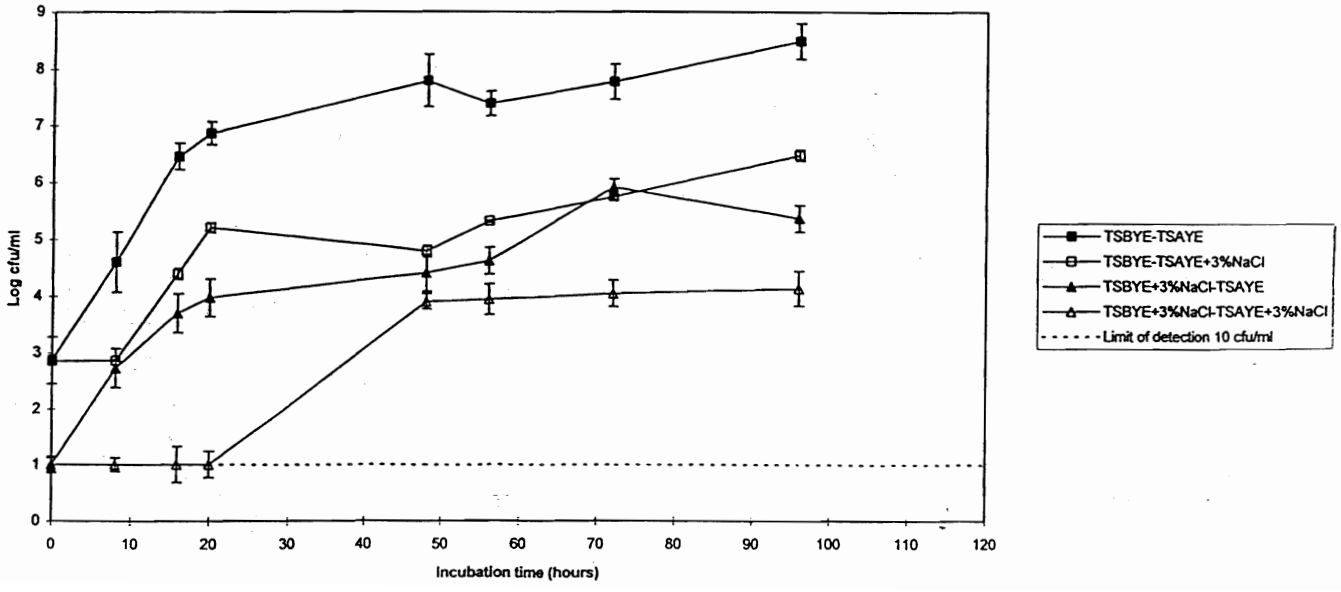

Figure 23c. Growth rate of *L. monocytogenes* grown @ 4°C after pre-treatment at 4°C and pH 7.0 (5 minute HP treatment 3KBar)

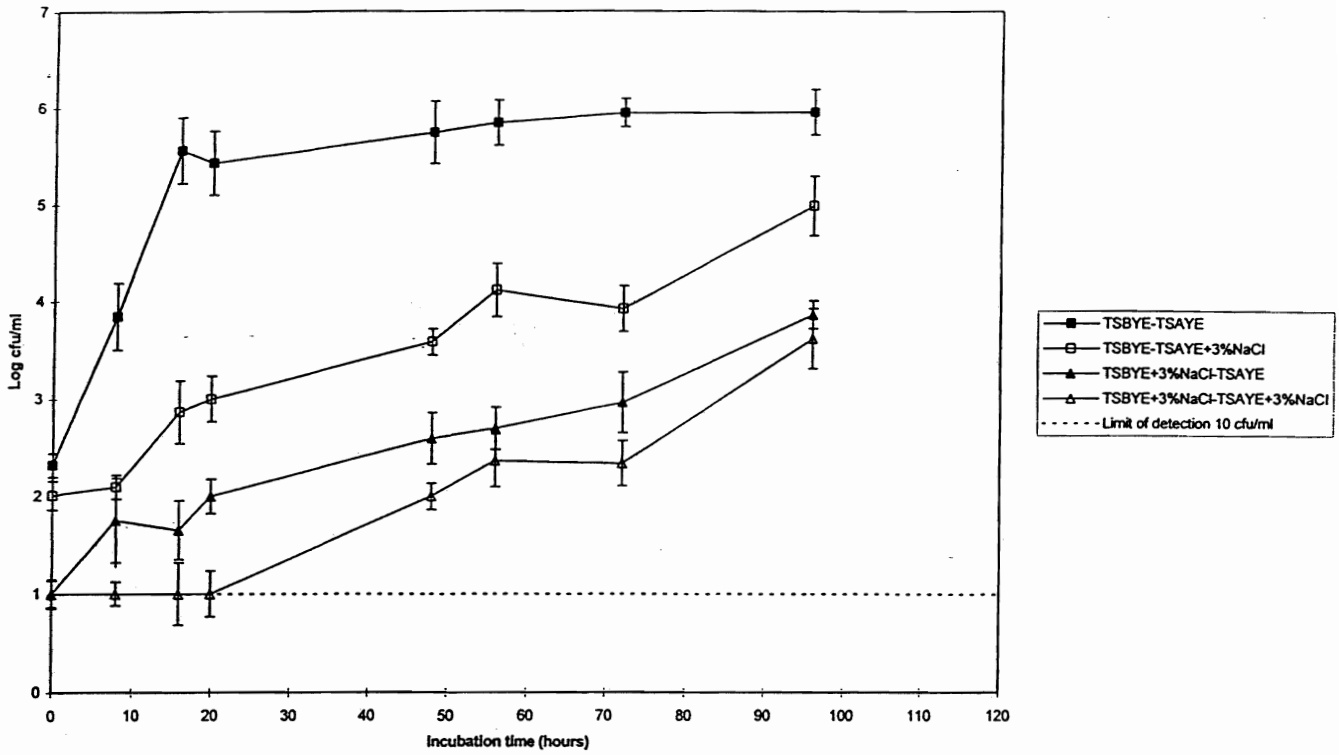

Figure 24a. Growth rate of *S. typhimurium* @ 10°C pH 7.0 after pre-treatment of 37°C pH 7.0 (untreated samples)

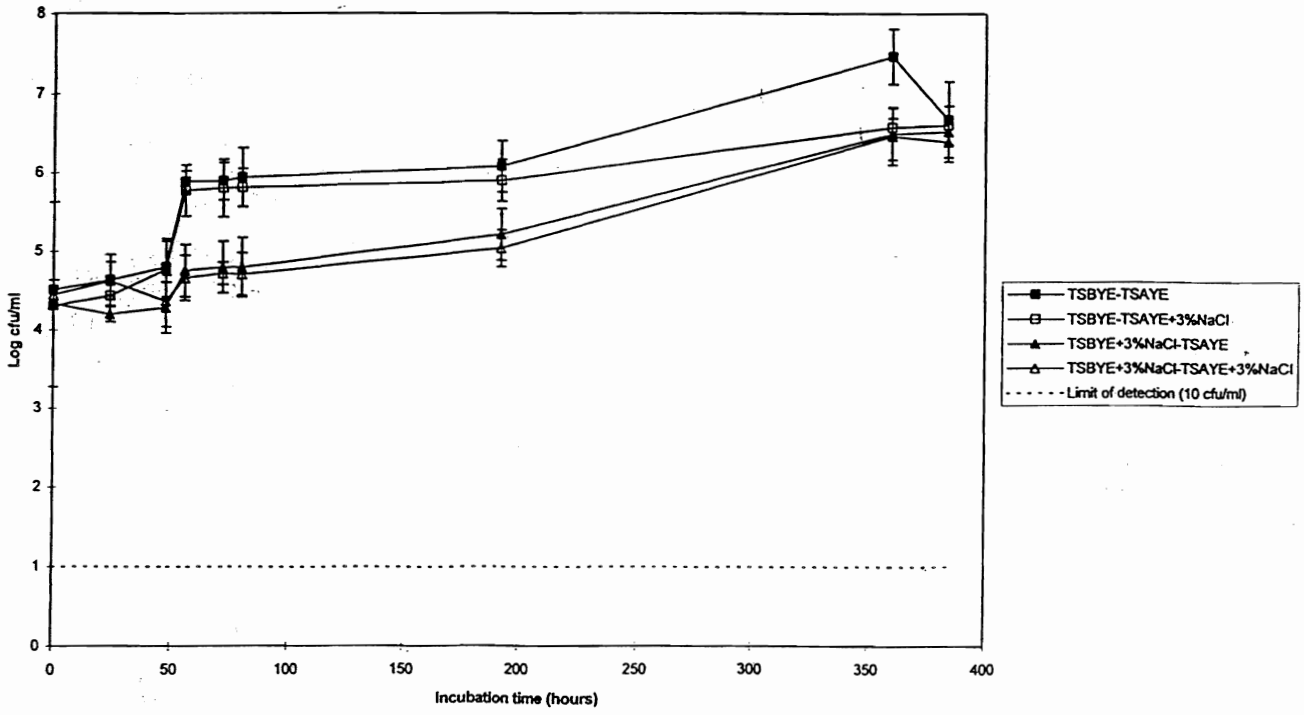

Figure 24b. Growth rate of *S. typhimurium* @ 10°C pH 7.0 after pre-treatment at 37°C pH 7.0 (5 minute HP treatment (3KBar))

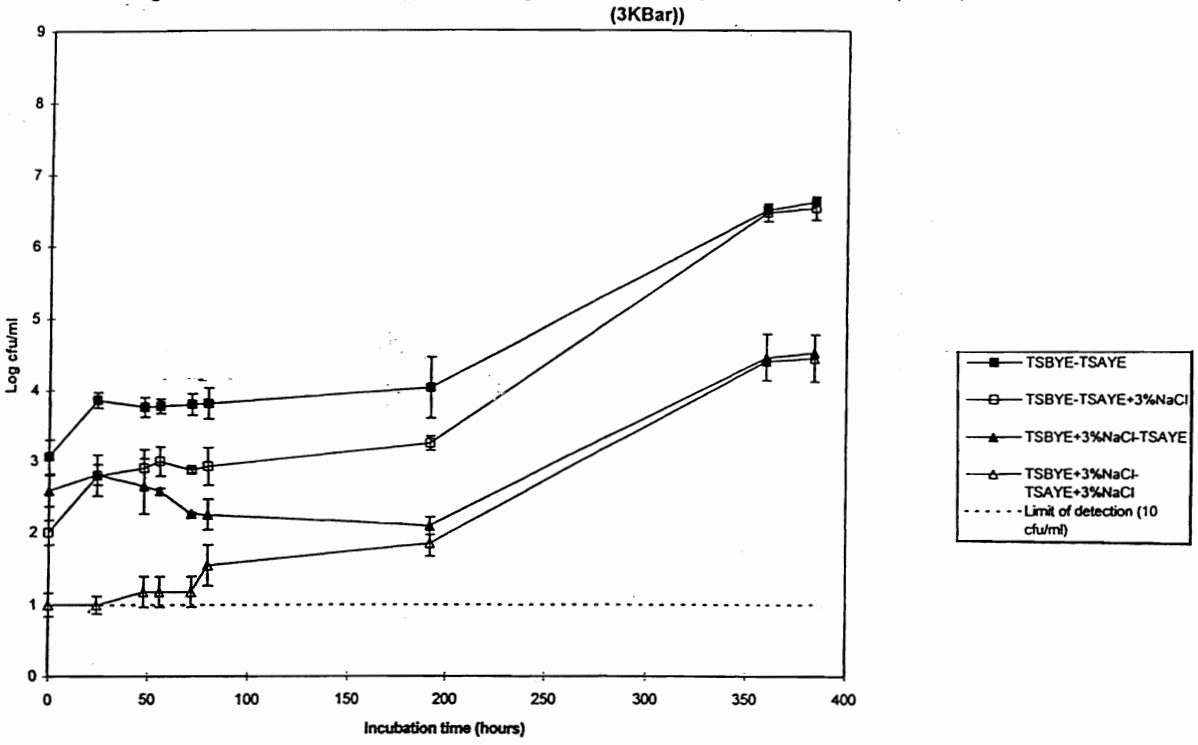

Figure 25a. Growth rate of *S. typhimurium* @ 10°C pH 4.5 after pre-treated at 10°C pH 7.0 (untreated samples)

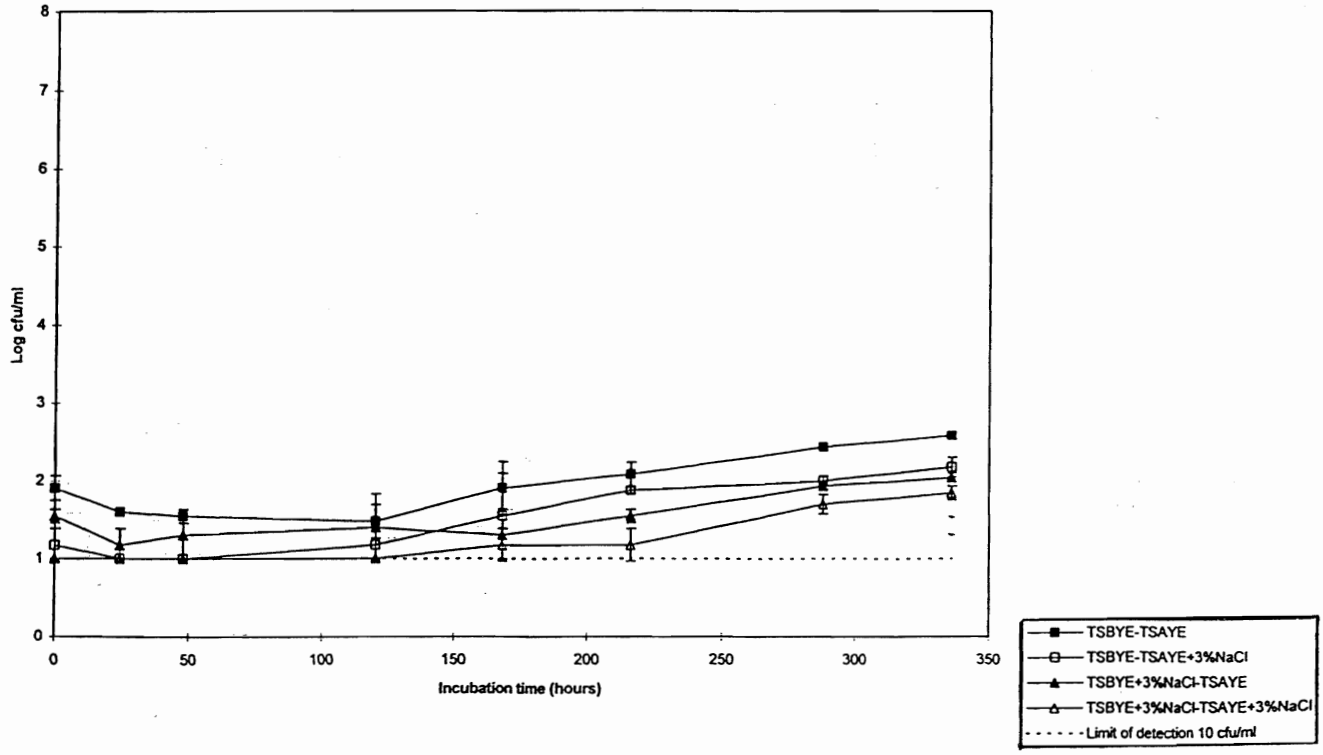

Figure 25b. Growth rate of *S. typhimurium* @ 10°C pH 4.5 after pre-treatment of 10°C pH 7.0 (5 minute HP treatment 3KBar)

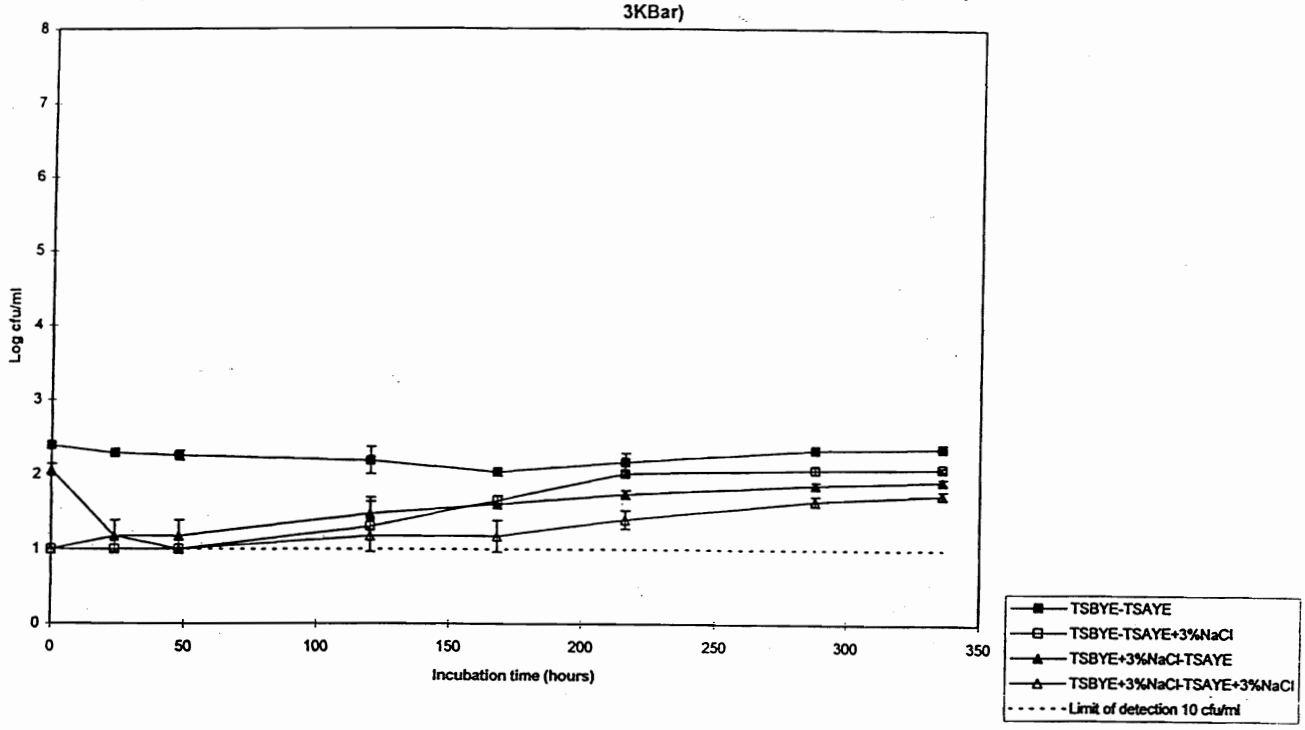

Figure 26a. Survival of *L. monocytogenes* grown at 37°C and treated with high pressure (3Kbar) in chicken slurry

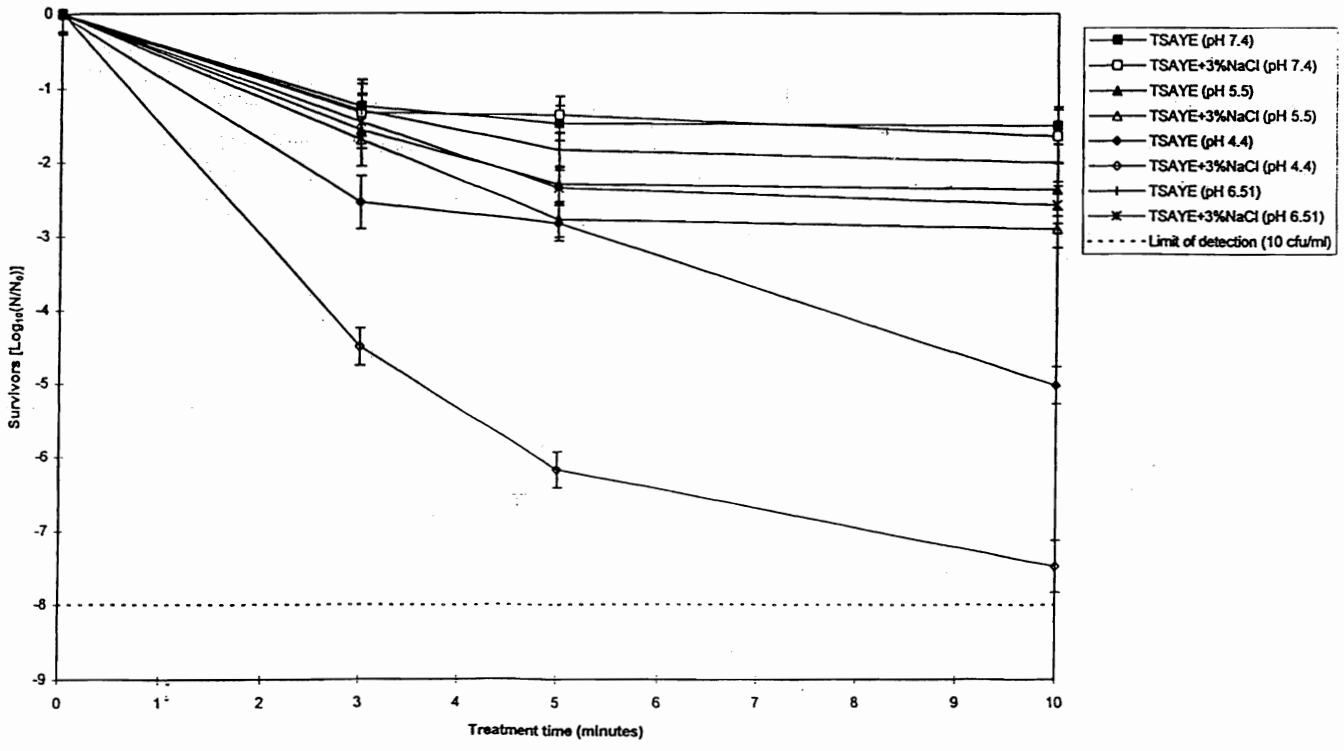

Figure 26b. Survival of *S. typhimurium* (CRA 1005) grown at 37°C and treated with high pressure (3Kbar) in chicken slurry

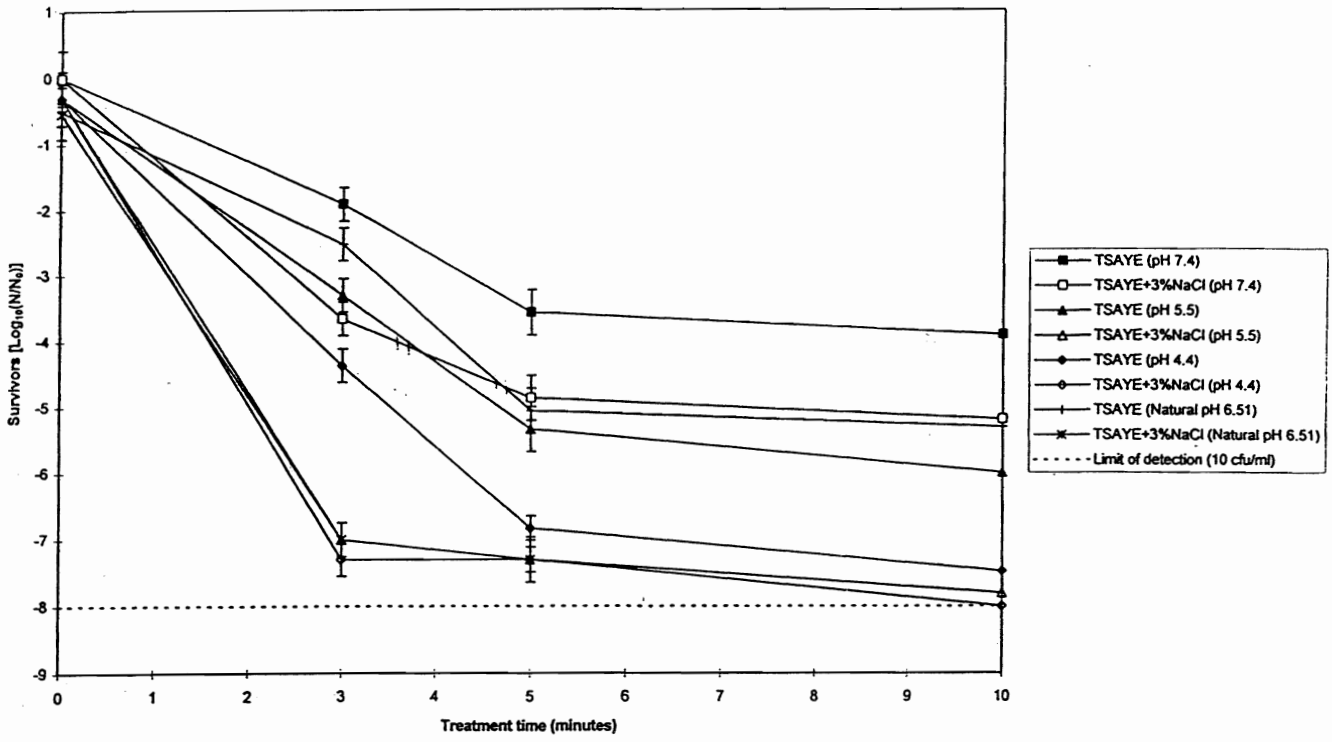

Figure 27a. Survival of *L. monocytogenes* (NCTC 11994) treated with high pressure (3Kbar) in carrots

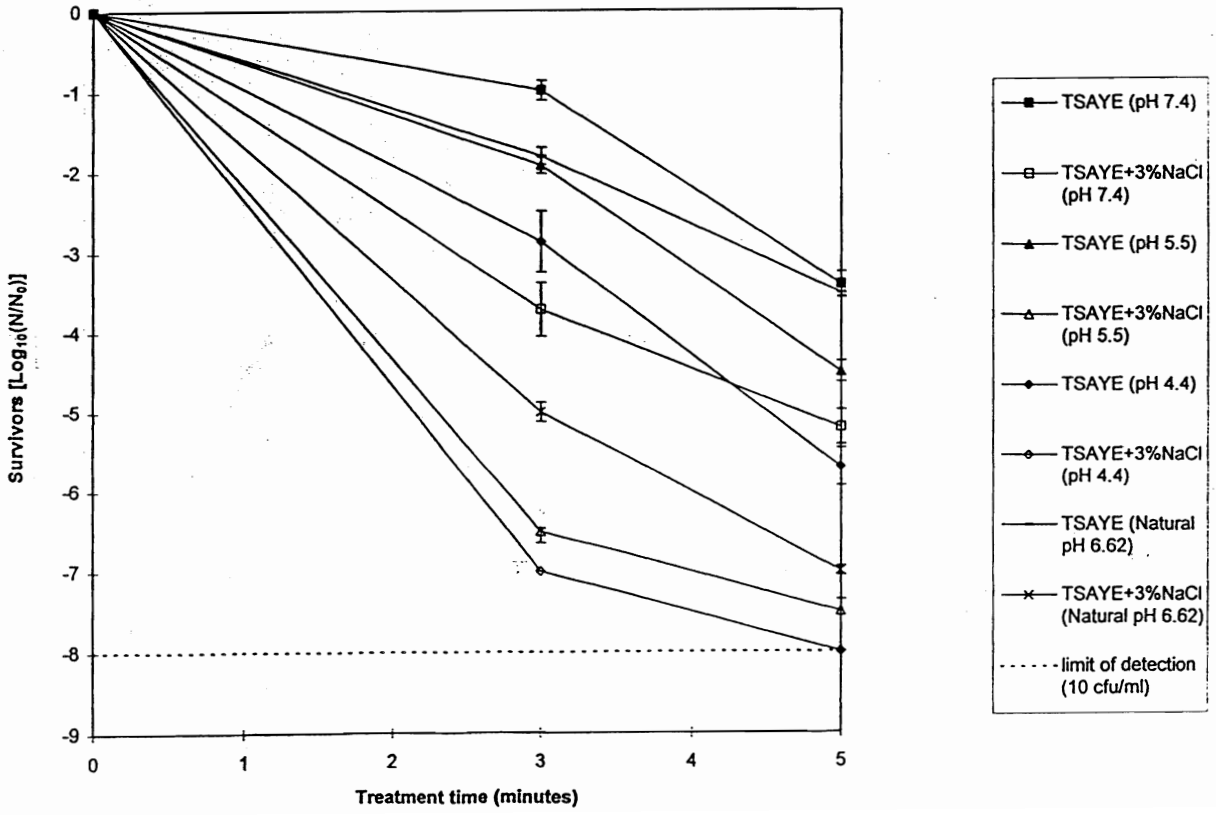

Figure 27b. Survival of *S. typhimurium (CRA 1005)* treated with high pressure (3Kbar) in carrots

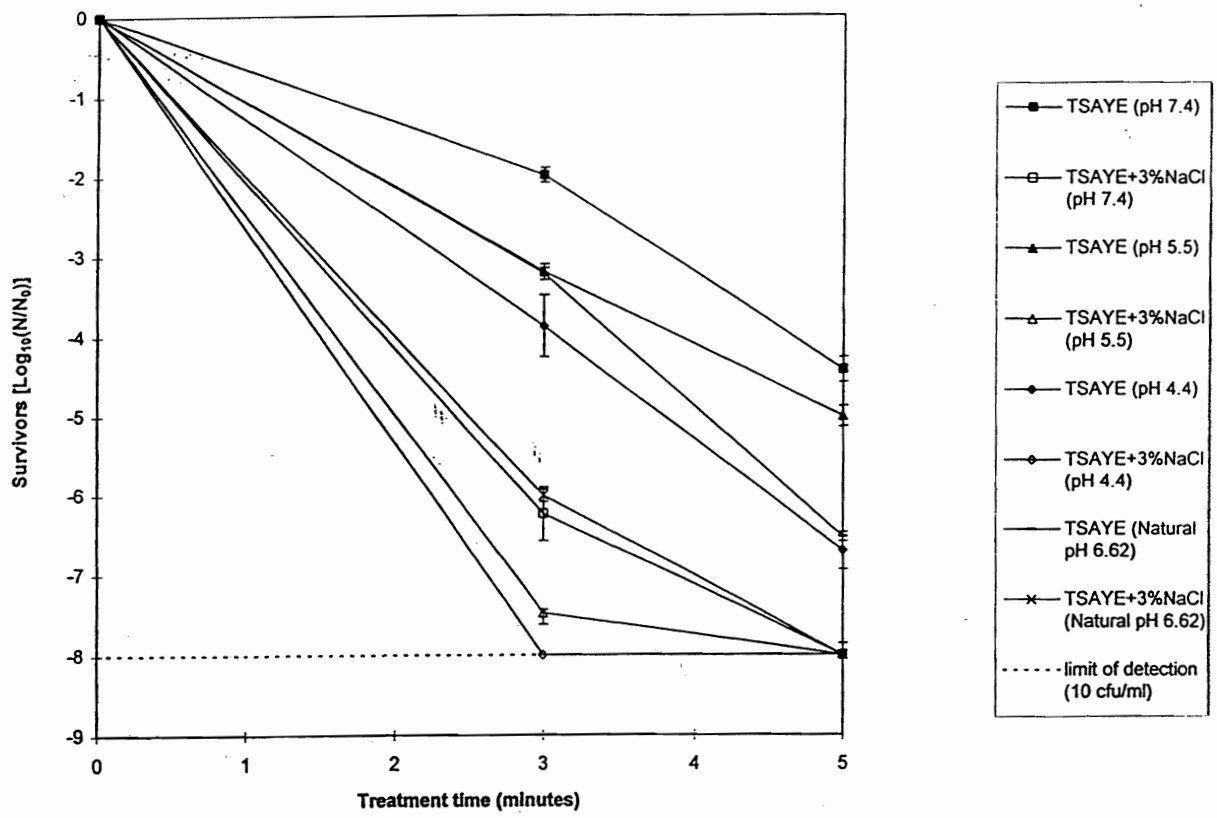

Figure 28a. Survival of *L. monocytogenes (NCTC 11994)* treated with high pressure (3Kbar) in strawberries

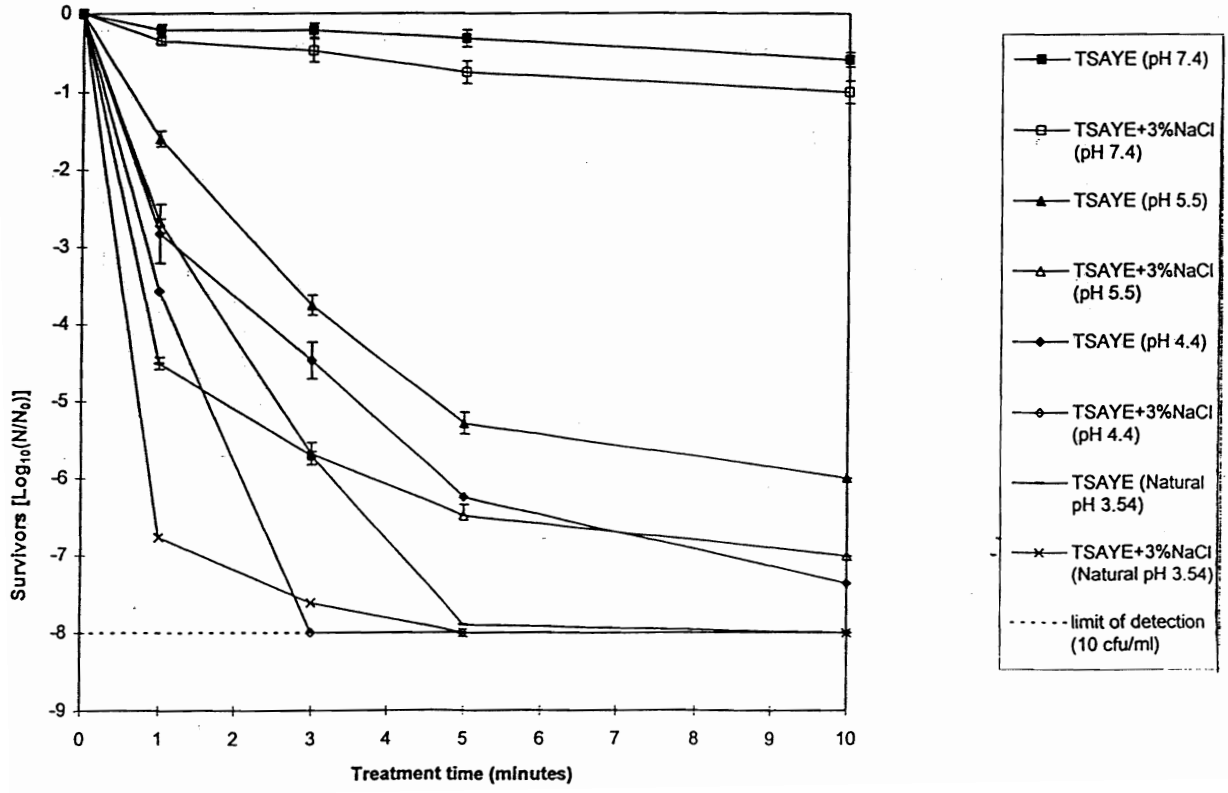

Figure 28b. Survival of *S. typhimurium (CRA 1005)* treated with high pressure (3Kbar) in strawberries

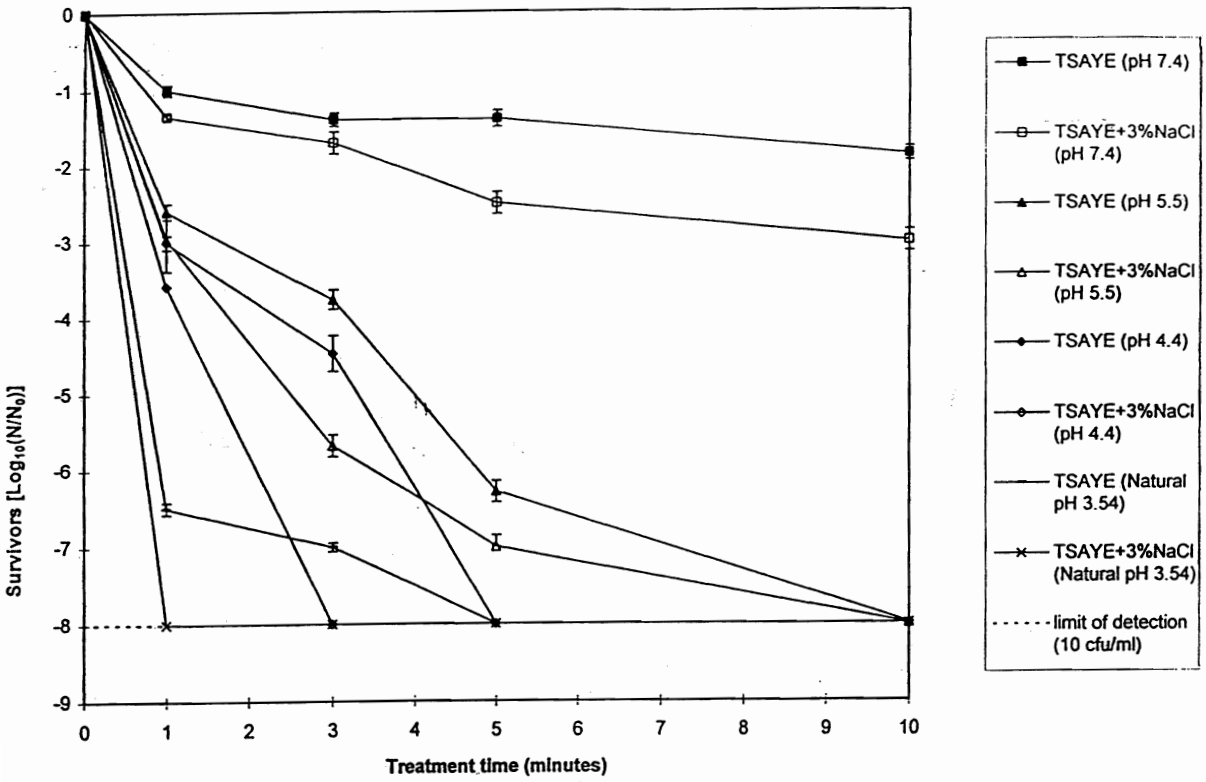

Figure 29a. Survival of *L. monocytogenes* (NCTC 11994) treated with high pressure (3Kbar) in Pure Orange juice (pH 3.02)

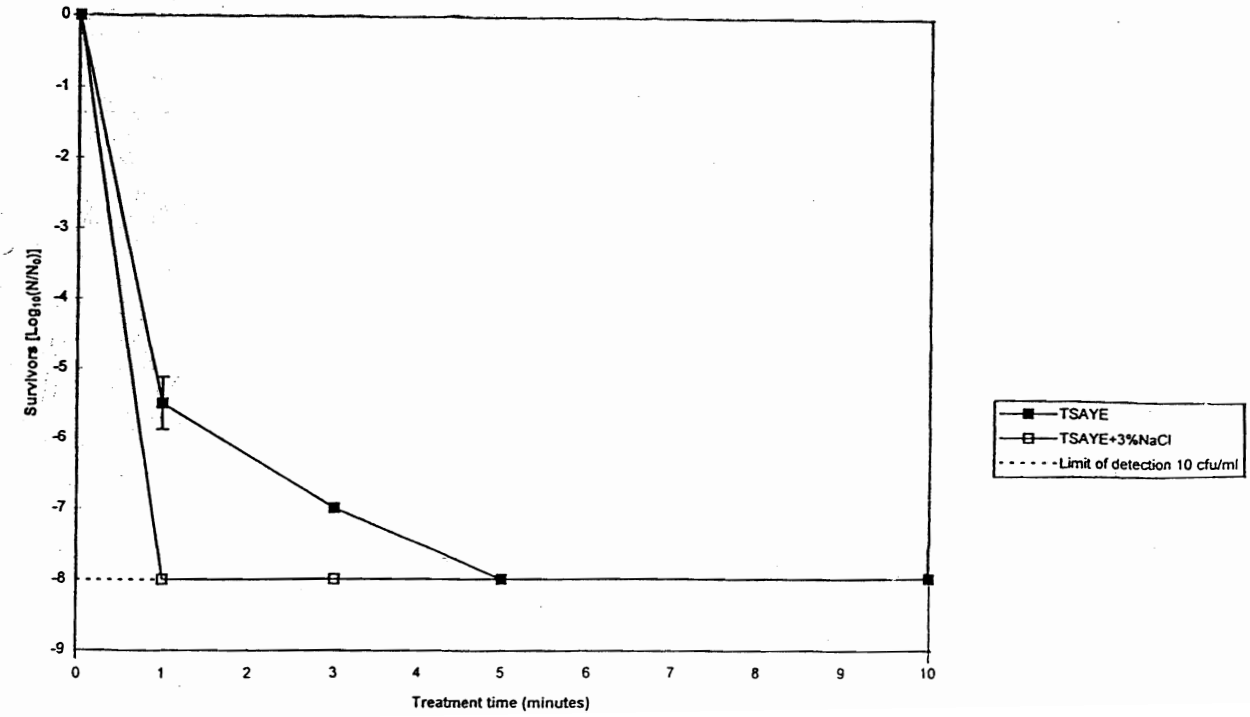

Figure 29b. Survival of *S.typhimurium* (CRA1005) treated with high pressure (3Kbar) in Pure Orange juice (pH 3.02)

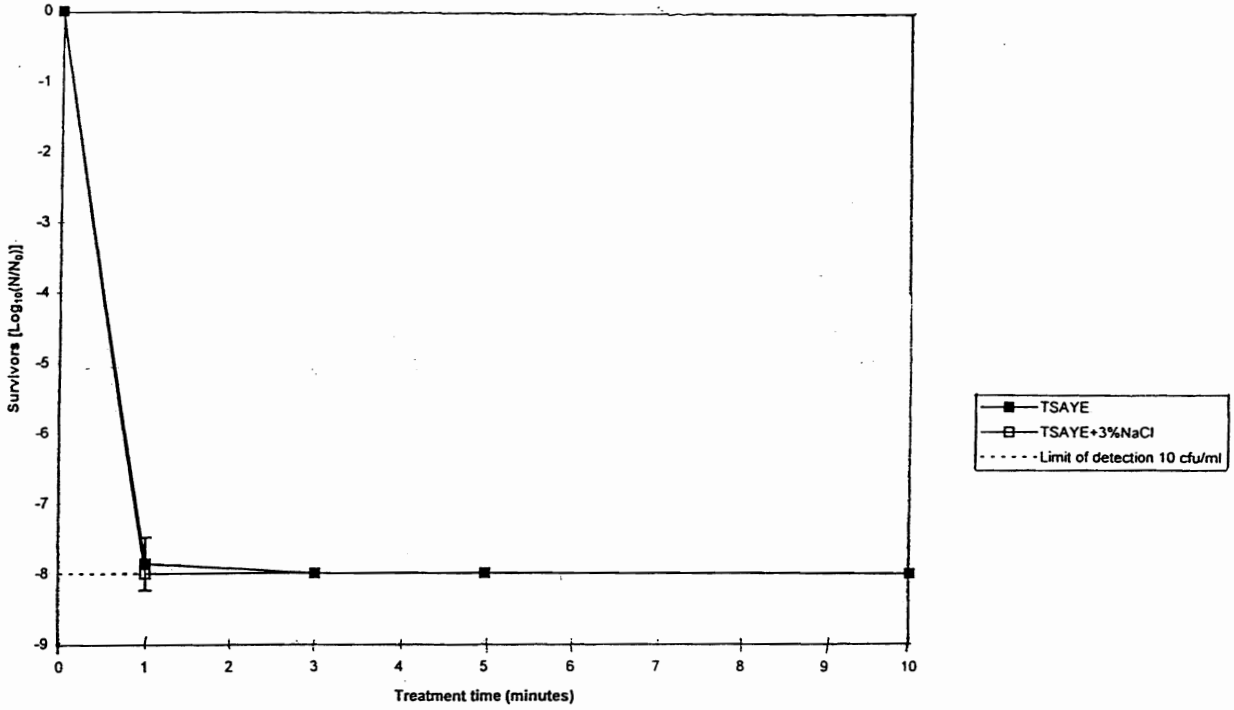

Figure 30a. Survival of *L. monocytogenes* grown at 37°C treated in chicken slurry and chilled @ 5°C prior to HP treatment

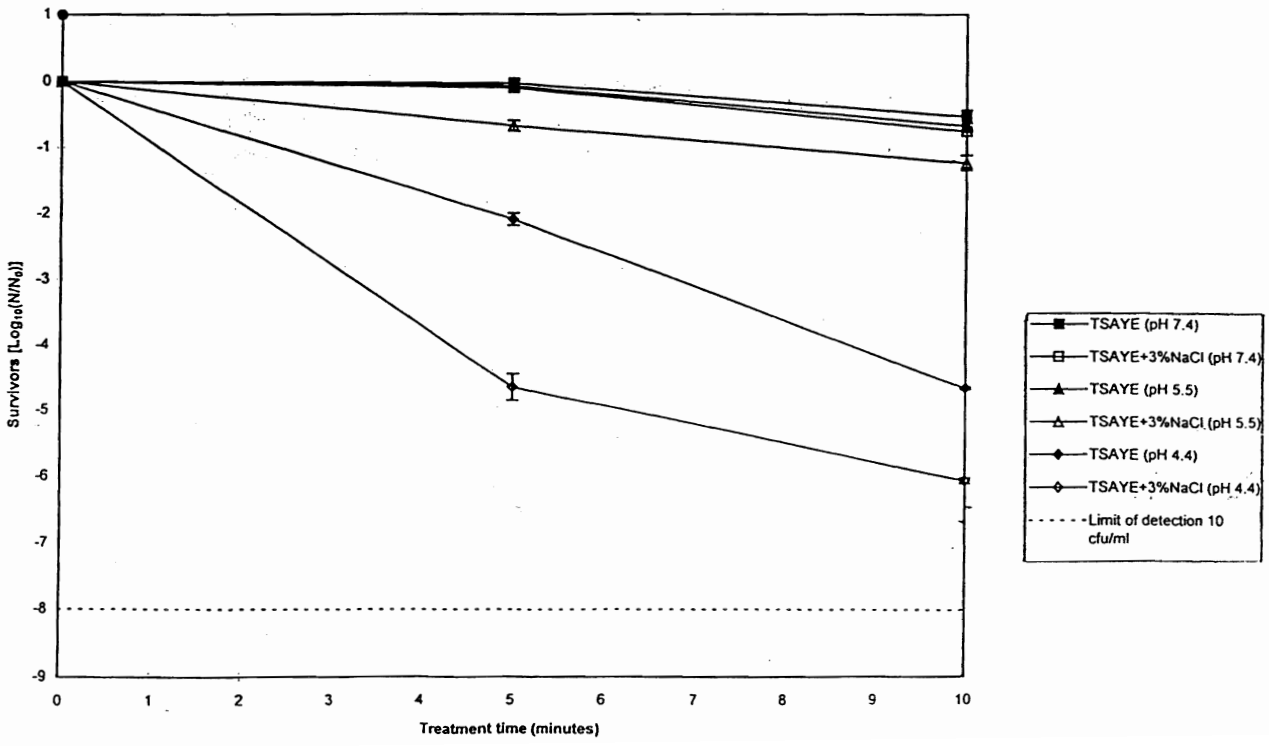

Figure 30b. Survival of *S. typhimurium* grown at 37°C and inoculated and treated in chicken slurry after being chilled at 5°C prior to HP treatment (3 Kbar)

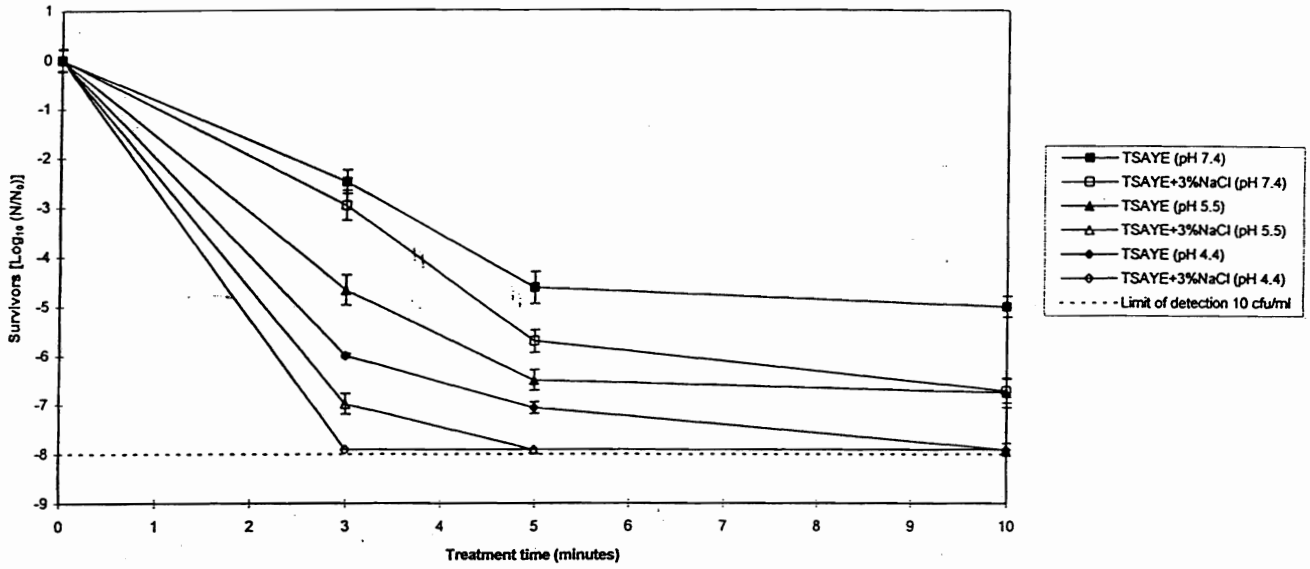

Figure 31. Survival of *L. monocytogenes* grown at 37°C and high presssure treated (4Kbar) in chicken slurry

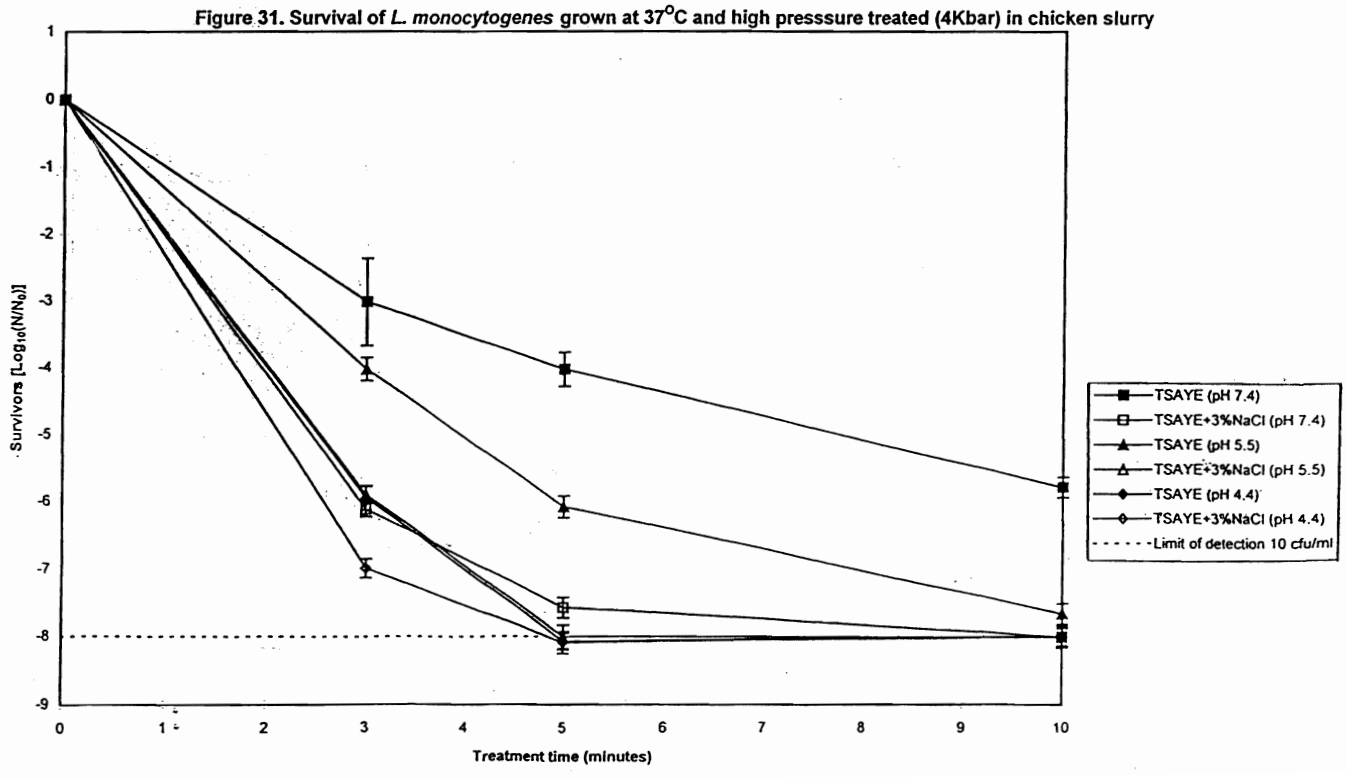

Figure 32a. Survival of *L. monocytogenes* grown at 10°C and high pressure treated (4 Kbar) in chicken slurry

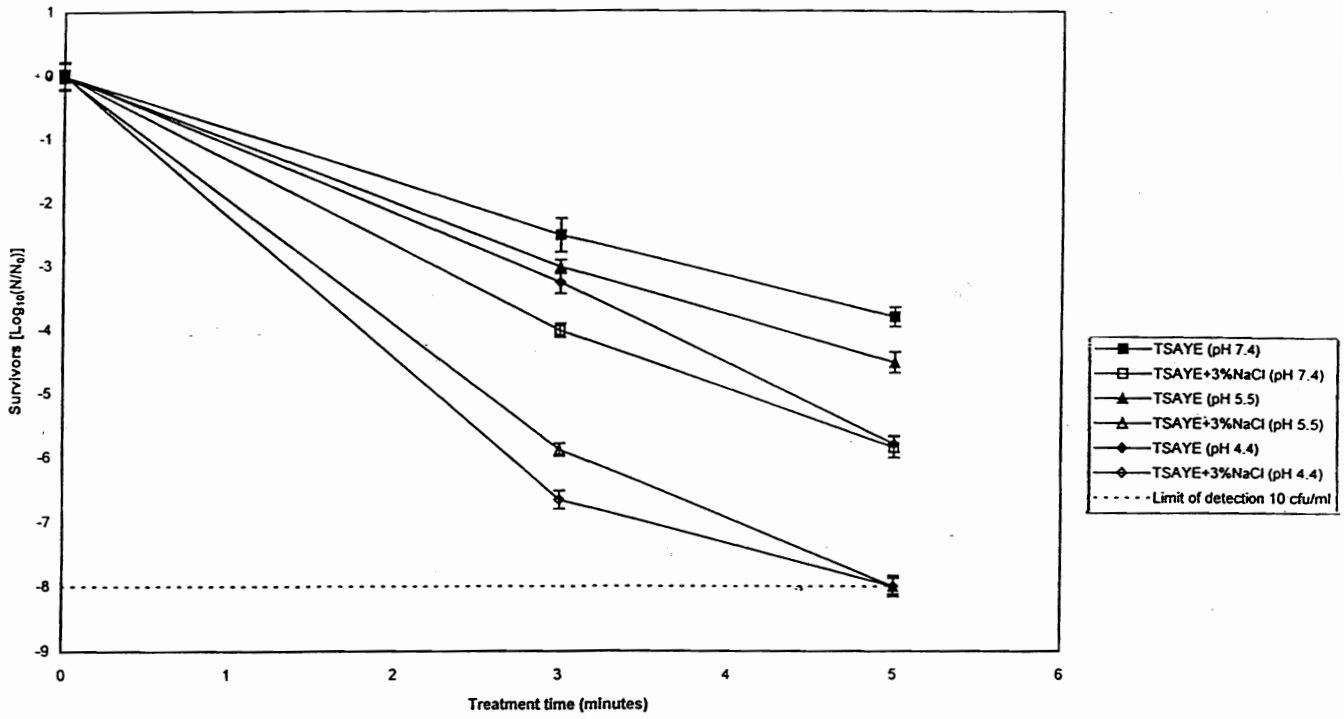

Figure 32b. Survival of *S. typhimurium* grown at 10°C and high pressure treated (3Kbar) in chicken slurry