**MINISTRY OF AGRICULTURE, FISHERIES AND FOOD**

**CSG 15**

Research and Development

# Final Project Report

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## Executive summary (maximum 2 sides A4)


Minimally processed refrigerated (MPR) fruits and vegetables are fresh, raw fruits and vegetables which are usually processed and sold to consumers ready-to-eat. MPR products support the survival and/or growth of food-borne pathogens and have been implicated as the vehicles of transmission in a number of food poisoning incidents. The increased international sourcing of these products from countries with possibly lower hygiene standards, together with concerns over the use of human and animal waste on agricultural land, has led to increased concern over their safety. However, crops produced in the natural environment cannot be expected to be free of microbiological agents. Where the possibility of contamination cannot be excluded, decontamination practices have to be considered. At present, current cleaning technologies cannot guarantee the microbiological safety of MPR products without compromising their quality. Effective washing and decontamination of fruits and vegetables is difficult. Washing generally involves the immersion of product in cooled sanitised water. Vigorous washing in potable water typically reduces the number of micro-organisms by 10 to 100-fold and is often as effective as treatment with 100 ppm chlorine, the current industry standard. Commercial washing systems are highly variable, there is little data on their decontamination efficiency, removal of dirt and foreign matter is variable, pesticide removal is limited, and shelf-life extension by washing is almost impossible. It is therefore apparent that new and more effective treatments are required.

The research outlined in this report demonstrates the feasibility of applying novel ultrasound (section 3.2) and photodynamic (section 3.3) washing technologies to eliminate the microbiological hazards associated with fruits and vegetables. An assessment of bacterial attachment and fresh produce surface topography was also undertaken to elucidate the possible reasons for the low decontamination efficiency of current fresh produce washing practices (section 3.1). Ultrasound disrupts biological structures and has the potential to cause cell

death when applied with sufficient intensity. Sound waves transfer vibrational energy from one place to another through a series of alternate compression and rarefaction waves in the propagating aqueous medium. In our investigations, ultrasound frequencies between 25 to 70 kHz were studied. At sufficient high power, bubbles or cavities are formed, a process known as cavitation. Many changes can occur to the bubbles during their growth and collapse and it is these changes which lead to a mechanical cleaning action on surfaces.

Singlet oxygen is an extremely reactive, higher energy form of oxygen that is toxic to all living organisms. The interaction of light, photosensitizers, and molecular oxygen mediates the formation of singlet oxygen. The sensitizer Rose Bengal (RB), a xanthene dye, is activated by light at a specific wavelength (550 nm). The energy within this molecule can be dissipated either through fluorescence or it can be transferred to molecular oxygen to produce singlet oxygen. Numerous workers have examined the photodynamic inactivation of a range of microbial pathogens. The biological effects of singlet oxygen have been attributed to photooxidation of membrane lipids and proteins, and damage of a variety of sites at the cellular level.

The research described in this report is the first time that ultrasound and photodynamic technologies have been considered for the washing and decontamination of MPR fruits and vegetables. Current decontamination methods are crude, poorly understood and are thought to be relatively ineffective in terms of pathogen removal. Assessment of bacterial attachment and fresh produce surface topography (section 3.1) highlighted the difficulty in removing and killing attached or entrapped pathogenic bacteria. The surface of fruits and vegetables can offer protection against infection, however pathogens can enter and colonise fresh produce through a variety of natural openings, cut surfaces and wounds. Results from our studies suggested that the decontamination efficiency on fresh produce with cut surfaces is lower than that for the whole uncut products (section 3.1). Also, the release of nutrients and moisture during minimal processing may provide substrates for the growth of psychrotrophic organisms. Scanning electron microscopy studies revealed the different topographical features inherent within fruits and vegetables. Work by other authors have also shown that the common sites for microbial aggregation of leaf surfaces are the veins, trichomes, stomata and cell wall junctions. Current procedures for killing or removing pathogens are focused on surface decontamination and are thought to be ineffective against internalised pathogens. Further research is required to better understand the mechanisms through which pathogens can contaminate MPR fruits and vegetables, either on the surface or in internal tissues. Strict hygiene, good manufacturing practice and use of hazard analysis and critical control point (HACCP) principles during processing will help to minimise the risk of cross contamination and pathogen uptake.

Ultrasound methods focused on process optimisation in terms of microbiological effect whilst ensuring sensory quality acceptability to the consumer. Optimised ultrasound treatment reduced the levels of attached bacteria to the same degree as chlorine treatment. Small scale experiments (2 litre) using chlorine and ultrasound, in combination, offered an additional 10-fold reduction in attached bacteria compared to chlorine or ultrasound alone (section 3.2). The mechanical cleaning action of cavitation appears to remove cells which are attached on the surface of fresh produce, rendering the pathogens more susceptible to sanitizers. However, the decontamination efficiency was less pronounced when the small scale trials were scaled up to 40 litres. A number of factors led to a reduced cavitation efficiency, most notably the treatment temperature, the quality of the wash water and the dissolved gas status of the suspending medium. With a potentially high capital expenditure together with expensive process optimisation and water treatment, it is not known whether the fresh produce industry would be willing to take up this novel technology. The additional reduction does not completely eliminate the risk of pathogens on fresh produce and current methods may be sufficient to ensure “due diligence”.

The main conclusion from the photodynamic technology was the difference in singlet oxygen sensitivity between Gram-positive and Gram-negative bacteria. It was not possible to provide an effective photodynamic catalyst surface area sufficient to kill planktonic *E. coli* and *S.* Typhimurium. Decontamination and inactivation of attached *L. monocytogenes* by the RB catalyst was very much comparable to that reported for chlorine, a 10 to 100-fold reduction at best. However, this technology would not significantly reduce the levels of attached Gram-negative bacteria. Ineffective light penetration coupled with the potentially high organic loading of dirty fresh produce in a large scale photodynamic washer would decrease the effectiveness of singlet oxygen generation. Photodynamic technologies are therefore unlikely to offer the fresh produce industry with a viable alternative to chlorine.

## Scientific report (maximum 20 sides A4)

# 1. Introduction

Minimally processed refrigerated (MPR) fruits and vegetables are fresh, raw fruits and vegetables which are usually processed and sold to consumers in a ready-to-eat or ready-to-use form (Wiley, 1994). The microflora of fruits and vegetables is diverse but is predominantly Gram-negative bacteria (De Roever, 1998). The presence of numerous genera of spoilage bacteria, yeasts and moulds and the occasional pathogen on fresh produce has been recognised for many years (Beuchat, 1998). MPR products support the survival and/or growth of food-borne pathogens and have been implicated as the vehicles of transmission in a number of food poisoning incidents (Nguyen-the & Carlin, 1994; Beuchat, 1998). The increased international sourcing of these products from countries with possibly lower hygiene standards, together with concerns over the use of human and animal waste on agricultural land, has led to increased concern over their safety. At present, current cleaning technologies cannot guarantee the microbiological safety of MPR products without compromising their quality (Seymour, 1999).

Fruits and vegetables can become contaminated with pathogenic micro-organisms whilst growing in fields or orchards, or during harvesting, post-harvest handling, processing and distribution (Beuchat, 1996). Prevention of contamination is probably the most efficient method of ensuring food safety. Protecting the food from the primary sources of contamination may prevent or reduce the risk of food-borne illnesses. This is not always possible since fruits and vegetables are typically grown close to the soil, which will contain many micro-organisms and may be contaminated with human pathogens. Leafy vegetables and herbs have a large surface area and topographical features that harbour the attachment or entrapment of microbes. Crops produced in the natural environment cannot be expected to be free of microbiological agents. Where the possibility of contamination cannot be excluded, decontamination practices have to be considered.

Effective washing and decontamination of fruits and vegetables is difficult. Washing generally involves the immersion of product in cooled sanitised water (Simons & Sanguansri, 1997). Vigorous washing in potable water typically reduces the number of micro-organisms by 10 to 100-fold and is often as effective as treatment with 100 ppm chlorine, the current industry standard (Beuchat, 1998; Seymour, 1999). It is important to remove as much soil as possible from fruits and vegetables prior to sanitation because many of the current surface disinfectants have a high affinity for organic matter (Boyette *et al.*, 1993). Dirty produce contains a large amount of organic matter which "uses up" the available disinfectant and so is not available for inactivation of micro-organisms (Kotula *et al.*, 1997). A recent survey of current industry practice on fruit and vegetable decontamination (Seymour, 1999) raised a number of important points on the effectiveness of washing. Commercial washing systems are highly variable, there is little data on their decontamination efficiency, removal of dirt and foreign matter is variable, pesticide removal is limited, and shelf-life extension by washing is almost impossible (Seymour, 1999). Consequently, current methods for fresh produce decontamination may be relatively ineffective in terms of pathogen removal. It is therefore apparent that new and more effective treatments are required.

Ultrasound (US) is known to disrupt biological structures and has the potential to cause cell death when applied with sufficient intensity. US consists of sound waves with a frequency (pitch) in excess of that for the audible range (20 Hz to 18 kHz). Several theories have been proposed as to the precise mechanism of US cell damage. Sound waves transfer vibrational energy from one place to another through a series of alternate compression

and rarefaction waves in the propagating aqueous medium. Most of the applications for power US use frequencies in the range of 20 to 100 kHz and require only the presence of a liquid medium for power transmission (reviewed by Roberts, 1991; Mason, 1993). At sufficiently high power, bubbles or cavities are formed, a process known as cavitation (Scherba *et al*., 1991). US leads to cavitation at surfaces which is caused by the vapour pressure falling locally below the saturated vapour pressure. Many changes can occur to the bubbles during their growth and collapse and it is these changes which lead to a "cleaning" action on surfaces.

Cavitation can be either stable or transient which produces one of two different effects (Roberts, 1991; Scherba *et al*., 1991). In stable cavitation, small bubbles oscillate during the compression and rarefaction cycles as the ultrasound passes through the liquid. This causes strong eddies to be formed in the surrounding liquid, attracting other small bubbles into the sonic field. These bubbles can form microcurrents around themselves which then spread into the liquid, a process known as microstreaming. This streaming effect provides a large force which effectively "rubs" or "shears" membrane surfaces, causing the cell membrane to break down (Kinsloe *et al*., 1954). In contrast, transient cavitation occurs when the bubble size changes much quicker (within a few oscillatory cycles) causing the bubble to collapse at different intensities (Sala *et al*., 1995). The collapse is thought to generate very high local temperatures (up to 5000°C), pressures (in excess of 1000 atm) and electrical potentials (Raso *et al*., 1998). It has been proposed that these extreme conditions are responsible for the majority of the antimicrobial effects of US treatment. The localised high temperatures and pressures bombard the cell membranes and may be strong enough to disrupt cell wall structures or to remove particles from surfaces (Schett-Abraham *et al*., 1992).

Several workers have examined the effects of US on the inactivation of various bacteria (Ordonez *et al*., 1987; Scherba *et al*., 1991; Raso *et al*., 1998; Pagan *et al*., 1999), spore formers (Sanz *et al*., 1985) and moulds (Idrissi *et al*., 1996). However, these results are difficult to compare due to variation in the physical parameters, such as US frequency, power level, the size and shape of the ultrasonic bath, the depth, volume temperature and nature of the liquid, and treatment time (Jeng *et al*., 1990). Essentially, there are no reliable means of quantifying the cavitation activity and microbiological laboratories must rely on empirical evaluations to evaluate the performance of each individual ultrasonic treatment system (O'Donoghue, 1984). US has recently been proposed for use in food preservation but this purpose has not been readily adopted. This is probably due to the perceived adverse effects on food quality brought about by the high-intensity treatments required to inactivate the most resistant micro-organisms.

Molecular oxygen ($O_2$) is a vital substance for respiratory organisms and is able to form a variety of toxic derivatives due to its high reduction potential. Singlet oxygen ($^1O_2$) is an extremely reactive, higher energy form of oxygen that is toxic to all living organisms. The interaction of light of a specific wavelength, photosensitizers, and molecular oxygen mediates the formation of singlet oxygen (Krinsky, 1977; Bradley & Min, 1992). Over 400 photosensitizing agents have previously been reported (Gallo & Santamaria, 1972). Most of these are fluorescent triheterocyclic compounds which are energised at specific wavelengths (typically greater than 320 nm). For example, Rose Bengal (RB), a xanthene dye, has a peak absorption at 550 nm while the thiazine dye, Methylene Blue (MB), a thiazine dye, absorbs optimally at 665 nm (Houba-Herin *et al*., 1982). In the dark, sensitizer molecules exist in a ground state (S). When the sensitizer is activated by light, the first excited state formed is the singlet species ($^1S$). This species is unstable and has a very short lifetime. The energy within this molecule can be dissipated either through fluorescence or can undergo "intersystem crossing" to form an excited triplet state ($^3S$). The activated triplet state has a much longer lifetime which initiates photochemical reactions by one of two distinct pathways: type I and type II. Type I is the direct interaction of the dye triplet state with various substrates which yields highly reactive free radicals (hydroxyl,

superoxide, hydrogen peroxide) through subsequent autooxidation (Parkin & Lowum, 1990). In contrast, type II involves the transfer of energy from the triplet state to molecular oxygen (quenched) to produce singlet oxygen (Bradley & Min, 1992). Sensitizers such as xanthenes (Rose Bengal, Eosin Y) and thiazines (Methylene Blue) are efficient singlet oxygen producers while some acridines and porphyrins also give good yields (Houba-Herin *et al*., 1982).

The inactivation of micro-organisms with photosensitizer dyes was first described by Rabb in 1900. Since then, numerous workers have examined the inactivation of a range of bacteria (Bezman *et al*., 1978; Banks *et al*., 1985; Dahl *et al*., 1989; Nitzan *et al*., 1989), viruses (Badylak *et al*., 1983; Houba-Herin *et al*., 1982; Lenard *et al*., 1993), yeasts (Bertoloni *et al*., 1989), spores (Abad-Lozano & Rodriguez-Valera, 1984), rat hepatocytes (Yamada, 1991), plant chloroplasts (Percival & Dodge, 1983) and plant leaf tissue damage (Knox & Dodge, 1984). The biological effects of photooxidation include the inactivation of membrane enzymes and transport processes (due to lipid and protein damage), mutagenesis, inhibition of DNA and RNA synthesis, interference with cell metabolism, reproduction and many other processes. Photodynamic (PD) damage therefore affects a variety of sites at the cellular level (reviewed by Ito, 1978). This is hardly surprising since the DNA base guanine is readily oxidised by photosensitizers (Adam *et al*., 1996). However, organisms have developed enzymes that destroy or detoxify certain oxygen products and help to alleviate some of the detrimental effects of free radical formation. These include catalase, peroxidase and superoxide dismutase. Some micro-organisms also contain special protective substances, such as carotenoids, which prevent attack from light-induced singlet oxygen, typically acting as quenching agents.

There are a number of possible applications for PD action. Rose Bengal has been widely used as a food additive in Japan since 1948, and there have been no reports of any toxicity issues to humans (Yamada, 1991). Acra & Ayoub (1997) proposed a novel PD water disinfection technique using Methylene Blue as a sensitising dye. The blue coloration of the dye in the treated wastewater was alleviated by the synergistic action of the "Halosol Process" (super-chlorinated water). PD technologies have also been exploited for the treatment of cancers (MacRobert & Philips, 1992) and new selective media for the enumeration of coliforms (Miyazawa *et al*., 1974).

The aim of this study was to bring together the critical scientific understanding needed to establish fresh produce washing systems based on novel US and PD technologies. This investigation was seen as a potential opportunity to improve the performance of washing/decontamination systems through the addition of new process variables which remove and/or destroy pathogenic bacteria in a cost effective manner and which retain the product quality demanded by the consumer. It was hoped that these working systems would demonstrate benefit in real industrial contexts and provide clear direction and assistance to industry to enable effective uptake of the new technologies. To do this, the relative effectiveness of both technologies were defined in terms of the critical control parameters and operating limits for optimal microbiological effect.

## 2. Materials and Methods

### 2.1 Bacterial strains and growth conditions

The strains used were *Salmonella* Typhimurium NCIMB10248 (Campden Research Association (CRA) 5452), *Escherichia coli* NCTC9703 (CRA 1860), *Escherichia coli* NCIMB 12497 (Ampicillin resistant) and *Listeria monocytogenes* (CRA 1177). Cultures were maintained on glass beads at -80°C in glycerol/water (1:1 v/v). Bacteria were resuscitated by placing one bead in 10 ml of Nutrient Broth (NB) followed by incubation at 37°C

for 24 h. These primary cultures were streaked on Nutrient Agar (NA), incubated at 37°C for 24 h, and stored at 4°C prior to use. Experimental cultures were prepared by inoculating NB (90 ml) with a single colony from a NA plate followed by 24 h incubation at 37°C for *S*. Typhimurium and *E. coli* or 48 h at 30°C for *L. monocytogenes*. For *E. coli* NCIMB 12497 all growth media was supplemented with 50 μg/ml of ampicillin (Sigma).

**2.2 Enumeration of bacteria**

After treatment, fresh produce samples (10 g) were diluted with 90 ml of Maximum Recovery Diluent (MRD; 0.1% peptone + 0.85% NaCl) and then homogenised in a stomacher (Lab-blender 400, Seward, UK) for 120 s. Serial dilutions were prepared in MRD and surviving micro-organisms were enumerated. *Salmonella* spp. were enumerated by spread plating on Xylose Lysine Deoxycholate agar (XLD; Oxoid, CM469) then incubating at 37°C for 24 h. *Listeria* spp. were enumerated by spread plating on *Listeria* Selective Agar [OLSA; Oxford formulation containing OLSA base (Oxoid, CM856) plus *Listeria* selective supplement (Oxoid, SR140E)] then incubating at 30°C for 48h. *E. coli* was enumerated by pour plating on Violet Red Bile Lactose Agar (VRBA; Oxoid, CM485) with overlay then incubating at 37°C for 24 h.

**2.3 Microbiological reference materials**

Fresh produce items (Iceberg lettuce, whole cucumber, cut baton carrot, capsicum pepper, white cabbage, spring onion, strawberries, curly leaf parsley, mint and other herbs) were purchased from a local supermarket for the duration of the project. Reference materials were either left whole (uncut) or trimmed and sliced (cut) by hand using aseptic techniques. All products were stored at 4°C prior to treatment.

**2.4 Preparation of inoculated fruits and vegetables**

Fresh produce items were inoculated with known levels of micro-organisms as described by Zhang & Farber (1996). Approximately 100 g of prepared fruits and vegetables (see section 2.3) were placed into a sterile Stomacher$^{R}$ Lab System plastic bag (Model 400, Seward; 18 cm x 30 cm) and heat sealed. Each bag was inoculated with a 1 ml suspension of culture (approximately $10^{8}$ cfu/ml) and then shaken gently 30 times to ensure an even distribution of the organism in the product. To allow sufficient time for microbial attachment the samples were stored overnight at 4°C before exposing them to various treatments. 10 x 10 g samples were enumerated for each test micro-organism to ensure even mixing throughout the product (results not shown).

**2.5 Chemicals**

Sodium hypochlorite (Sigma) containing 4–20% available chlorine was made up to the correct concentration in sterile distilled water (SDW). The pH was adjusted to pH 7.0 +/- 0.1 with HCl or NaOH. Free residual chlorine was measured using the Palintest$^{R}$ chlorine test kit (section 2.6). The food grade surfactant (wetting agent) dioctyl sodium sulfosuccinate (up to 10 ppm permitted in fruit drinks; Codex Alimentarius, Volume 1A – 1995) was also used in combination with sodium hypochlorite for US trials (section 3.2). Rose Bengal (RB), Methylene Blue (MB) and Eosin Y (EY) (all Sigma) (Figure 1) were made up to the correct concentration in SDW for use in all photodynamic studies (section 3.3).

### 2.6 Chlorine test

The Palintest$^{R}$ Comparator (Model PT 520T, Palintest Ltd, UK) was used to test the active concentration of chlorine. Using different reagents this test detects both total and free chlorine [Chlorine DPD (Comp. 7, Palintest Ltd, UK), 0 – 5.0 mg/l free and total chlorine; Chlorine HR (Comp.8, Palintest Ltd, UK), 0 – 250 mg/l total chlorine].

### 2.7 Assessment of bacterial attachment

100 g samples of inoculated fresh produce items (see section 2.4) were washed for 5 min in 1 litre of sterile tap water in a 5 litre beaker with constant agitation (product to water ratio 1:10). After treatment, samples were removed with the aid of sterile stainless steel forceps and then drained for 1 min. Remaining micro-organisms were enumerated before and after washing to assess bacterial attachment (see section 2.2).

The following method was used to examine the surface topography of the fresh produce items. Small cubes (5-10mm) and cross-sections of each fruit or vegetable were frozen in liquid nitrogen and freeze-dried overnight in an Edwards Modulyo freeze-drier. A small section was cut or fragmented from the freeze-dried food item and mounted onto an aluminium stub using conductive carbon cement. Each sample was then gold coated using an Agar auto sputter coater and examined in a Leica Cambridge 360 Scanning Electron Microscope (SEM).

### 2.8 Photodynamic methods

#### 2.8.1 Immobilisation of photodynamic dyes

Dyes were immobilised onto a nylon support as described by the following method. This involved soaking of 5 $cm^2$ coupons of 100% knitted nylon light-coloured tights (Aristoc 15 denier) overnight in a 2% solution (w/v) of dye in deionised water. The dye was fixed to nylon using potassium dichromate as a mordant. A 4 % solution (w/v) of potassium dichromate was heated to 50°C with constant agitation. The solution was brought to the boil and then simmered for 45 min. The nylon was rinsed in hot water and then autoclaved in deionised water at 121°C for 15 min to remove unbound dye. The nylon was steeped in frequently changed water for 3 days until no further leaching could be detected. An absorbance spectrophotometer was used to analyse the water samples for unbound dye. A standard calibration curve was constructed (results not shown) with known concentrations of each dye (absorbance, 550 nm for RB; 516 nm for EY; 665 nm for MB).

#### 2.8.2 Photodynamic reactor set-up

A fermenter (Electrolab Ltd, UK) with a 5 litre glass vessel, impeller, pH and dissolved oxygen probes, temperature probe and regulator was used as a model photodynamic reactor to monitor and control the experimental parameters. Airflow was controlled at 3.4 litres/min to give 100% air saturation in the liquid medium (approximately 20% oxygen saturation). An impeller speed of 100 rpm was used throughout all treatments to ensure adequate product agitation whilst maintaining product integrity. The temperature was held at 23°C +/- 3°C throughout each experimental run, refrigeration being supplied by a thermocirculating chiller connected to a cold finger. The pH was monitored and controlled at pH 7.0 +/- 0.5. Three 500 W phosphor-mercury blended light bulbs (Mixed Light 31, GE, Belgium) provided the light source for all photodynamic experiments. The bulbs gave a high light emission at 550 nm, the wavelength at which RB absorbs light most

efficiently. A light meter (Foot Candle/Lux meter, Extech Instruments, UK) was used to position the bulbs for optimal light intensity (15,000 lux). Dark (no light) controls were carried out by covering the fermenter in aluminium foil (0 lux). Sterile tap water (STW) was used at all time to mimic industrial conditions.

### 2.8.3 Photodynamic inactivation of planktonic and attached bacteria

The fermenter was set up as described in section 2.8.2 and dyes were either added free in solution or immobilised to nylon coupons (section 2.8.1). The fermenter was equilibrated for 30 min prior to the addition of planktonic bacteria (final concentration, $10^6$ cells/ml) or inoculated fresh produce (100 g). Samples of planktonic bacteria were taken at specified times and dispensed into sterile glass containers covered in aluminium foil (to stop the formation of singlet oxygen). Surviving micro-organisms were enumerated as described in section 2.2. Inoculated fresh produce samples (10 g) were removed with the aid of sterile stainless steel forceps and then drained for 1 min. Surviving micro-organisms were enumerated before and after washing to assess the decontamination efficiency (see section 2.2). In order to simulate real industrial conditions expected in fresh produce wash water (high organic loading), Bovine Serum Albumin (BSA, Sigma) was added to a final concentration of 0.3 g/litre ("clean") or 3.0 g/litre ("dirty") as described in British Standard BS EN 1276 : 1997 [Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants used in food, industrial, domestic, and institutional areas – Test method and requirements (phase 2, step 1)].

## 2.9 Ultrasound methods

### 2.9.1 Tests to assess breakdown of foods

Tests were carried out using a range of US tanks (with nominal capacities between 35 and 45 litres) supplied by Kerry Ultrasonics Ltd (an example is shown in Figure 2). Various treatment times were used at frequencies of 25 kHz, 32-40 kHz and 62-70 kHz and a power of 15 W/litre (the highest specified in the research proposal). A single tank assembly comprises of a generator (Neptune Pulsatron 500 MK2, Kerry Ultrasonics Ltd, UK) and a stainless-steel ultrasonic cleaning bath with transducers mounted in the base (KS450, Kerry Ultrasonics Ltd). A 5 litre stainless steel sub bath was used for small scale experiments (section 2.9.2). The Pulsatron US cleaning systems were set up as described in the Operation and Maintenance manual (Kerry Ultrasonics Ltd, Hunting Gate, Wilbury Way, Hitchin, Herts, SG4 0TQ). Ten samples from each batch of food were used and items of food were treated individually.

Alternative high power bar and radial applicators, both operating at 20 kHz, were also used in these studies (FFR Ultrasonics Ltd, UK). The bar applicator (Figure 3) was operated at 450 W in 2 litres of water containing a small sample of food located beneath the bar. The nominal power was 225 W/litre however, the ultrasound field was probably quite localised (as seen from the rippling of the surface of the water near to the bar). Based on the "footprint" of the bar which measured 140 mm by 38 mm, and the height above the base of the container (25 mm), the volume of liquid beneath the bar was 133 ml. Assuming that the ultrasound field is restricted to the liquid directly below the bar (there will certainly be some field around the it), the power input to the liquid and food material would be a maximum of 3,400 W/litre. The radial cell (Figure 4) operated at powers up to 1200 W. Normally, the product flows through the centre of the cell but in these tests the product and water was sealed into the cell using tape. This overcame the need to build a continuous flow system before confirming that the approach would be successful. The cell contained approximately 40 ml of water and a small sample of the food. The power density was approximately 30,000 W/litre.

### 2.9.2 Small scale fresh produce decontamination trials

Small scale (2 litre) decontamination trials were carried out prior to the commencement of large scale (40 litre) investigations. Aseptic conditions were maintained throughout all the experiments to ensure accurate assessment of fresh produce decontamination. A US tank (KS450, Kerry Ultrasonics Ltd) was filled with 40 litres of STW and degassed at an operating frequency of 32-40 kHz (10 W/litre) for 10 min. Expulsion of air from the solution increases the efficiency of acoustic cavitation in ultrasonic cleaning fluids. A 5 litre stainless steel sub bath was placed in the large tank and filled with 2 litres of STW with/without 25 ppm free residual chlorine (pH 7.0 +/- 0.1; section 2.5). 100 g of cut Iceberg lettuce inoculated with $10^6$ cells/g of *S.* Typhimurium (section 2.4) was placed in a stainless steel wire basket and submerged in the sub bath containing the various test solutions (product to water ratio 1:20). The product was washed for 10 min with/without US treatment at 32-40 kHz (10 W/litre). The four treatments are summarised as follows:

(1) tap water dip (control, no agitation)
(2) 25 ppm free chlorine dip
(3) ultrasound only
(4) ultrasound and 25 ppm free chlorine.

Remaining *S.* Typhimurium cells were enumerated before (in triplicate) and after washing (in quintupicate) to assess the decontamination efficiency (see section 2.2).

### 2.9.3 Large scale fresh produce decontamination trials

Large scale (40 litre) decontamination trials were carried out under equivalent batch washer operating conditions to those used by fresh produce processors (Seymour, 1999). Disinfected US tanks (KS450, Kerry Ultrasonics Ltd) were filled with 40 litres of tap water and degassed at the correct operating frequency (25, 32-40, 62-70 kHz; 10 W/litre) for 10 min. Stannard (1997) identified acceptable microbiological limits for raw and prepared vegetables (including salad vegetables). High levels of Aerobic Plate Counts (APC), Enterobacteriaceae and coliforms are probable and may be derived from the soil or poor handling. For the assessment of possible faecal contamination, indicator organisms for example *E. coli*, are thought to be more appropriate. The strain *E. coli* NCIMB 12497 with ampicillin resistance as a selectable marker was used for all large scale decontamination trials (section 2.3). By using this strain in combination with the appropriate selective media the levels of *E. coli* remaining on the fresh produce were routinely monitored (removing high background counts of Enterobacteriaceae and coliforms). As a suitable control, fresh produce items were microbiologically sampled for coliforms using VRBA media with/without 50 μg/ml ampicillin (results not shown). No ampicillin resistant coliforms were found on the VRBA and ampicillin plates, suggesting that the selective medium is a true measure of the inoculated *E. coli* levels.

Samples (200 g) of either cut Iceberg lettuce, curly leaf parsley, strawberries or cabbage were inoculated with $10^6$ cells/g of *E. coli* NCIMB 12497 (section 2.4), placed in a stainless steel wire basket and submerged in the US tank containing 40 litres of the various test solutions. A product to water ratio of 1:200 is not typically used in industry but it was important to present the foodstuffs in the treatment tank as a single layer to enable effective cavitation action. The remaining total and free chlorine levels after washing were monitored (section 2.6). Due to the high product to water ratio, organic loading was minimal and free chlorine levels did not vary significantly throughout treatment (results not shown).

The four products were washed for 10 min using the six treatment regimes summarised below:

(1) tap water dip (control, no agitation)
(2) 100 ppm free chlorine dip (pH 7.0 +/- 0.1)
(3) 100 ppm free chlorine jacuzzi (airflow 3.4 litres/min)
(4) ultrasound only (at 25, 32-40, 62-70 kHz)
(5) ultrasound and 100 ppm free chlorine
(6) ultrasound, 100 ppm free chlorine and 10 ppm dioctyl sodium sulfosuccinate (surfactant).

Remaining *E. coli* cells were enumerated before (in triplicate) and after washing (ten replicates) to assess the decontamination efficiency (see section 2.2). For all four products US treatments (4) to (6) were carried out at three operating frequencies (25, 32-40, 62-70 kHz; 10 W/litre). A chlorine jacuzzi (sparger air applicator) was constructed from a 30 cm stainless steel pipe (1 cm diameter) sealed at one end and drilled with holes at 3 cm intervals along its length. The sparger was connected to an air line with rubber tubing and placed in the bottom of the US tank. Increased product agitation by air (jacuzzi) is thought to enhance the mechanical removal of micro-organisms and soil (a method commonly used by fruit and vegetable processors; Seymour, 1999).

# 3. Results

The Tables and Figures for these results are shown in the Appendices.

### 3.1 Assessment of bacterial attachment and fresh produce surface topography

The attachment efficiency of *S.* Typhimurium on a range of cut and uncut fresh produce items is shown in Table 1. Cut and uncut fruits and vegetables were inoculated with $10^6$ cells/g of *S.* Typhimurium and washed for 5 min in sterile tap water with constant agitation (section 2.7). There was a significant difference in the attachment efficiency of *S.* Typhimurium on different fresh produce types. For example, reductions of 1.03 1.26 and 1.49 logs of *S.* Typhimurium were observed when using uncut cabbage, strawberry and pepper, respectively. However, uncut spring onion and parsley demonstrated less than a 1 log reduction when washed for the same length of time under identical conditions. These results suggest that sufficient numbers of *S.* Typhimurium cells are attaching to the products during the inoculation protocols (section 2.4) to enable accurate assessment of decontamination during washing trials. In general, the presence of cut surfaces significantly increased the levels of bacterial attachment compared to the corresponding uncut products. Experiments were repeated for *E. coli* and *L. monocytogenes* (Iceberg lettuce) but no significant differences in attachment were noted between bacterial species (results not shown).

SEM analysis of a range of fresh produce items are shown in Figures 5 a-j. The surface topography of each fruit or vegetable appear markedly different. For example, the parsley lower surface (Figure 5 d) shows prominent mounds and valleys of surface epidermal cells with numerous cracks and crevices providing sites for bacterial attachment and protection. Microbial biofilms can be seen on the surface and in the crevices of the epidermal cells. The cabbage upper surface (Figure 5 e) shows the presence of a large number of wax crystals while a large number of open and damaged cells can be observed on the baton carrots (Figure 5 j), as a consequence of processing (peeling and slicing). A large number of stomata can also be seen amongst the surface epidermal cells of fruits and vegetables.

### 3.2 Ultrasound studies

#### 3.2.1 Tests to assess breakdown of foods

Investigations were carried out to assess the breakdown of a range different fruit and vegetable products under different US frequency/power/time combinations (Table 2). Even when using the longest treatment time (300 s), only the mint showed signs of damage (a loss of colour very close to the edge of each leaf as shown in Figure 6). All the other products tested were visibly fresh and undamaged. Irrespective of frequency, only the mint showed any signs of breakdown after 150 s (and this was minimal) and none of the foods demonstrated any breakdown after 30 s of treatment. A 600 s treatment was also carried out but no differences between 300 s and 600 s were evident (results not shown). Further tests with each food were carried out using a 35 kHz US system (Kerry Ultrasonics Ltd, UK) operating beyond its design range to achieve a power of 50 W/litre. This could be achieved for only 120 s, without the risk of damage to the equipment. Yet again, mint was the only product to show any signs of breakdown. In conclusion, these tests found no effect of time, frequency or power input on damage to the foods other than mint over the range of the tests originally planned.

Further tests were carried out using materials 4 days after purchase at a treatment time of 300 s. Table 3 shows that the mint exhibited signs of breakdown as in previous tests. Some very slight damage at the edges of lettuce leaves and parsley was also noted. Materials were also treated at (32-40 kHz or 62-70 kHz, 15 W/litre for 300 s) on the day of purchase and were then stored at 5°C for 8 days. Strawberries were held for up to 19 days because, as reported later, they showed clear differences between treated and control samples. Tests were carried out with the water in the tank at 5 or 20°C. Table 4 and the photographs in Figures 7 a & b summarise the results and show the visible changes in the foods during storage. The mint (Figure 7 a) was damaged during the ultrasound treatment and so in practice it was irrelevant in the storage trials. Strawberries (Figure 7 b) treated at the higher frequency (62-70 kHz) exhibited visible mould growth much later and to a lesser extent than untreated strawberries or those treated at the lower frequency (32-40 kHz). However, other tests with strawberries at other times in the year showed variability; sometimes the higher frequency led to an increase in shelf life whilst, in most tests, there was no difference between treated and untreated strawberries. None of the others foods showed any differences between treated and untreated materials during storage (results not shown).

The tests described above demonstrated little or no effect of ultrasound treatment on the breakdown of the foods (with the exception of mint). There was a need to increase the intensity of the ultrasound application to examine if greater effects on the food could be achieved. Reducing the frequency would move the tests into the audible range whilst increasing the frequency would require the development of new systems. Such developments were not warranted as the research had not indicated that increases in frequency would necessarily lead to significant improvements in microbial reductions. Increasing the power input would be expected to have an effect on the food. Substantial increases in power beyond those tested could not be achieved using the tank systems so alternative bar and radial applicators, both operating at 20 kHz, were investigated.

Table 5 and Figures 8 a-c summarise the results from the bar applicator treatment (30, 150 or 300 s). After 30 s, the mint had been affected by the treatment. Also, the “hairs” on very fresh strawberries were removed by such a treatment but no other visible change to the strawberries was apparent. Also, there was no effect on older strawberries. Similar results with mint and strawberries were found after 150 s (Figures 8 a & b respectively), lettuce appeared slightly blotchy (Figure 8 c), while parsley demonstrated slight darkening at the edges. After

300 s treatment, the carrot, pepper and spring onion were the only products to show no visible signs of damage. The foods treated for 150 s were also stored for 15 days at 5°C after US treatment. Apart from the product damage noted at the start of the storage period, no other differences between control and treated samples were observed (results not shown).

Table 6 summaries the results from the radial applicator treatment (1, 2, 5 or 10 s followed by storage for 10 days at 5°C). Figures 9 a-c show photographs of some of the foods after treatment and storage. After 1 s treatment, the extreme edge of the cabbage leaf appeared slightly different (Figure 9 a), the lettuce leaves showed patchy areas (Figure 9 b) while the pepper and spring onion showed no effect (results not shown). A few hairs were dislodged from the surface of the strawberries (Figure 9 c). Increasing the treatment time merely led to more visible changes. After 10 s, the carrot, pepper and spring onion showed no visible change but the water was slightly coloured indicating that some surface damage has occurred. No differences between control and treated samples were found after storage for up to 15 days (results not shown).

### 3.2.2 Tests to assess the effect of water temperature

The optimum water temperature for ultrasound cleaning is thought to be around 60°C. Operating at these temperatures would not be feasible with fresh fruit and vegetables as it would severely affect the food texture and enhance deterioration of sensory qualities. Tests carried out with water at a starting temperature of 5 or 20°C showed no effect on the breakdown of the foods treated for 300 s (Table 4). Significant heating during an US treatment could have deleterious effects on food quality. Table 7 shows the change in water temperature for the tank, bar and radial US systems. For the US tank the water temperature increased by approximately 0.2-0.3°C/min. This compared to a 156°C/min increase for the radial applicator. The rate of change of water temperature was very much dependent on the amount of power (W/litre). The difference between the measured power and nominal (stated) power of the tank systems is generally small. However, the more intense bar and radial applicators became hot and it is unlikely that all of the energy was transferred to the water. All tests were carried out with the water and ultrasound equipment initially at 20°C. Assuming a treatment time of 300 s using a tank system, the temperature rise is small and the results suggest that a cooling system for the water would not be needed. However, in a commercial system, the water would need to be recycled and a filtration and cooling unit would have to be included. A bar or radial applicator would be operated as a continuous flow-through system and cooling of the water would certainly be required.

### 3.2.3 Tests to assess the effect of water composition

The chemical composition of the water in the treatment tank can have an effect on the action of ultrasound. Tests were carried out using a tank system (operating up to 25 W/litre) with tap water from three sources (Clarkes water hardness values of 20, 23.4 and 24.19, respectively), de-aerated water, alcohol in tap water (2% v/v), and tap water with a surfactant (0.05% v/v Triton X-100, Sigma). No difference in the appearance of the foods was found between foods treated in different liquids. Tap water with chemical additives and de-aerated water demonstrated more pronounced wave patterns compared to plain tap water.

### 3.2.4 Microbiological assessment of ultrasound decontamination

#### 3.2.4.1 Small scale fresh produce decontamination

Small scale washing trials were carried out to assess the decontamination efficiency of ultrasound (32-40 kHz) and chlorine, the current industry standard (Table 8). For the water dip control (without agitation), a reduction of 0.73 logs (81% reduction) of *S.* Typhimurium on cut Iceberg lettuce was demonstrated. This result was similar to previous data (section 3.1; Table 1) and demonstrates the repeatability of the inoculation and attachment protocols. Reductions of 1.57 and 1.67 logs of *S.* Typhimurium (97 and 98% reduction, respectively) were observed for ultrasound or chlorine only which suggests that the decontamination efficiencies of both treatments are comparable. These log reductions were significantly different to the water control. In addition, when ultrasound and chlorine were used in combination, there was an additional 1 log reduction of *S.* Typhimurium compared to ultrasound or chlorine alone. This corresponded to a 99.8% reduction in attached bacteria.

#### 3.2.4.2 Large scale fresh produce decontamination

Large scale fresh produce washing trials were carried out to compare the decontamination efficiency of various ultrasound and washing treatments. For statistical analysis, the washing treatments were placed into 12 different groups. Of these, 11 corresponded to three different washing techniques (tap water, chlorine, chlorine and surfactant) with the ultrasound frequency set at four different levels (0, 25, 32-40 and 62-70 kHz); the twelfth treatment was a chlorine jacuzzi. This effectively gives a 4 by 3 grid of results with one value missing. With this structure, the effect of the washing techniques were evaluated as were the effect of changing the ultrasound frequency. However, the treatments were investigated on different days and so each batch of fresh produce had to be evaluated prior to washing in order to establish the quantity of *E. coli* contaminants that had been removed. Since the evaluation process was destructive, i.e. the same sample cannot be washed and then evaluated after the original evaluation has taken place, there is no pairing structure to the data. All of this analysis was repeated on cabbage, Iceberg lettuce, parsley and strawberry (Tables 9 a-d, respectively). The results for the chlorine jacuzzi decontamination trials are shown in Table 9 e.

The mean of the results for each of the treatments was subtracted from the mean of the respective control to effectively give an average log reduction in attached bacteria. These values were then used to perform the final analysis. An analysis of variance (ANOVA) model was fitted to the data in order to detect which of the factors (if any) were having an effect. The four data sets from the different food products were agglomerated. The possibility of a product effect and also the possibilities of interactions were also considered. The tabulated values are those that were earlier referred to as the average log reduction. The tables have been presented separately for the different food products, however the ANOVA was not fitted to them separately but as one data set.

There do appear to be some anomalies in the tables when they are inspected closely. For instance the water only treatment on the strawberry actually increased the microbial loading (Table 9 d). Although this does not seem as though it should be the case there is no reason as to why this data should be omitted from the analysis. The ANOVA model was fitted and the following table summarises the final model and the terms which are significant.

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Product | 3 | 5.0144 | 5.0144 | 1.6715 | 7.82 | 0.000 |
| Treatment | 2 | 6.2133 | 5.6025 | 2.8013 | 13.10 | 0.000 |
| kHz | 3 | 0.3162 | 0.3162 | 0.1054 | 0.49 | 0.690 |
| Error | 35 | 7.4851 | 7.4851 | 0.2139 | | |
| Total | 43 | 19.0290 | | | | |

The main effects of product type, treatment and ultrasound frequency on the efficiency of decontamination are summarised in Figure 10. The different washing techniques gave a systematic difference. The average log reductions in *E. coli* for water, chlorine, chlorine and surfactant, and chlorine jacuzzi were 0.76, 1.45, 1.63 and 1.68, respectively. The treatments and the food products were significantly different (at the 0.1% level) from each other. A Newman-Keulls test indicated that the chlorine and surfactant treatment was significantly better than the tap water only treatment. There were no other differences between the treatments. It was felt that it was not fair to compare the jacuzzi treatment to the other treatments directly since it was only done at 0 kHz. It must be remembered that the jacuzzi treatment was only done once and should therefore not be compared rigorously with the other treatments. Nevertheless, the chlorine jacuzzi treatment was included as a suitable control to mimic current industrial washing practices (Seymour, 1999). Also, it was not feasible to undertake ultrasound treatment in combination with the jacuzzi due to the likely dissipation of acoustic waves. In terms of the differences between the product types, cabbage was the most easily washed (1.75 log reduction) and significantly differed in decontamination efficiency from the other three products. Parlsey was the least easily washed, again significantly different from the other three products (0.81 log reduction).

However, the strawberry and the Iceberg lettuce were not statistically different from each other (1.19 and 1.24 log reduction, respectively). In contrast, the frequency of ultrasound treatment had no significant effect on decontamination efficiency ($p>0.69$). The average log reductions for 25, 32-40 and 62-70 kHz treatment were 1.37, 1.30 and 1.28, respectively. The no ultrasound control (0 kHz) showed a log reduction of 0.95 (0.3 log cycles lower than US treatment).

### 3.3 Photodynamic studies

#### 3.3.1 Photodynamic inactivation of planktonic bacteria: dyes in solution

A range of experiments were carried out to evaluate and optimise the performance of a model photodynamic reactor (5 litre fermenter) using planktonic bacteria. The inactivation kinetics of *S.* Typhimurium, *L. monocytogenes* and *E. coli* were defined for RB, MB and EY in solution (Figures 11-20). The control data sets under dark conditions (0 lux) and intense light (15,000 lux) with no photodynamic dyes are shown in Figures 11 and 12, respectively. There was no appreciable difference in the viability of the three test organisms under the conditions tested. However, dark controls carried out in the presence of $10^{-4}$ M RB led to a 2.7 log reduction of *L. monocytogenes* after 60 min, while *S.* Typhimurium and *E. coli* were unaffected (Figure 13). The dark controls were also repeated using $10^{-4}$ M solutions of MB (Figure 14) and EY (results not shown) but no significant differences in cell viability were noted.

These experiments were repeated for each test organism under illuminated isothermal conditions using RB, MB and EY solutions at concentrations ranging from $10^{-5}$ to $10^{-3}$ M. For *S.* Typhimurium (Figure 15) there was no significant difference in viability after 10 min, at any of the concentrations tested. Reductions of 3.3 and 2.5 logs of *S.* Typhimurium were noted for $10^{-4}$ and $10^{-5}$ M RB, after 60 min treatment. However, there was no

significant change in the survival of *S.* Typhimurium at the highest RB concentration tested ($10^{-3}$ M), even after 60 min. The photodynamic inactivation of *E. coli* by RB was similar to that for *S.* Typhimurium (Figure 16). This corresponded to a 0.4 and 4.8 log reduction after 10 and 60 min, respectively. In contrast, *L. monocytogenes* was an order of magnitude more sensitive to RB than either *E. coli* or *S.* Typhimurium (Figure 15). For example, treatment with $10^{-4}$ M RB and intense light reduced the levels of *L. monocytogenes* by 5.3 logs after 2 min. In addition, $10^{-5}$ M RB led to a 5.2 log reduction after 10 min. The difference in RB sensitivity of the three test organisms is summarised in Figure 17. This corresponds to a 3.31 log reduction of *S.* Typhimurium after 60 min, 4.77 logs for *E. coli* after 60 min, and 5.3 logs for *L. monocytogenes* after 2 min (in order of increasing sensitivity).

Methylene Blue ($10^{-4}$ M) also demonstrated antimicrobial activity in the presence of intense light (Figure 18). However, unlike RB, cell inactivation was the same order of magnitude for all three test organisms. After 60 min, the log reductions were 2.0, 4.4 and 4.8 for *S.* Typhimurium, *L. monocytogenes* and *E. coli*, respectively. In contrast to RB and MB, there were no appreciable differences in the viability of the three test organisms using EY at a concentration of $10^{-4}$ M (Figure 19). Increasing the levels of EY in solution to $10^{-3}$ M, also had no effect on bacterial survival (results not shown). The effects of RB, MB and EY ($10^{-4}$ M) on the inactivation of *S.* Typhimurium, *E. coli* and *L. monocytogenes* are summarised in Figures 20 a-c, respectively. In general, the antimicrobial action of RB was greater than that for MB and EY. It was therefore decided to use only the RB dye for all subsequent trials (section 3.2.2 and 3.3.3).

### 3.3.2 Photodynamic inactivation of planktonic bacteria: immobilised Rose Bengal

Immobilized RB was evaluated for its potential antimicrobial action on the three test organisms in the presence of intense light (Figures 21-23). The original MAFF research plan suggested that ten 5cm squares of nylon mesh would be sufficient to supply an effective photodynamic catalyst surface area. Nevertheless, a photodynamic reactor containing 10 RB nylon coupons had no significant effect on the survivability of *S.* Typhimurium after 60 min (Figure 21). Indeed, when the number of coupons was increased to 20, 50 or 250 there was still no appreciable difference in the viability of *S.* Typhimurium. Furthermore, this was also true for *E. coli*, whereby the addition of 50, 250 or 500 RB coupons had no significant effect on cell numbers (Figure 22). In contrast, 50 RB coupons supplied an adequate photodynamic catalyst surface area, reducing the levels of planktonic *L. monoctogenes* by 4.43 logs after 5 min (Figure 22). The nylon coupons were autoclaved and reused for each experimental run and the same results were obtained for each replicate. This suggests that there is sufficient RB dye immobilised on the nylon coupons to act as a photodynamic catalyst. The rapid inactivation of *L. monocytogenes* by immobilised RB is comparable to that of RB in solution. Reductions of 5.33 (2 min) and 5.15 (10 min) logs of *L. monoctogenes* were observed for $10^{-4}$ and $10^{-5}$ M RB, respectively (Figure 16). The difference in sensitivity of the three test organisms to immobilized RB is summarised in Figure 23.

### 3.3.3 Photodynamic inactivation of attached bacteria: immobilised Rose Bengal

Work carried out in sections 3.3.1 and 3.3.2 involved identification of the critical control limits for optimisation of the PD system. The aim of the next stage in this study was to evaluate the effectiveness of the optimised system under conditions which mimicked real industrial operation (Figures 24-25). Whether RB was used free in solution or immobilised to a nylon support, *S.* Typhimurium and *E. coli* were much less sensitive to photodynamic action than *L. monocytogenes*. In a recent survey of current industry fruit and vegetable washing practices (Seymour, 1999), 81% of those surveyed, maintained contact times of less than 10 min between the product and the wash water. However, effective PD action was only evident after 30-60 min treatment with

both *S.* Typhimurium and *E. coli*. In order to compare the effectiveness of PD systems with chlorine, the current industry standard (Seymour, 1999), washing trials were set up using Iceberg lettuce artificially contaminated with *L. monocytogenes* (Figure 24 and Table 10). After a 60 min wash in water alone (control) the reduction in attached bacteria was only 0.30 logs (50% reduction). Rose Bengal coupons were used in preference to RB free in solution to prevent coloration of the product. Reductions of 1.11, 1.65 and 1.57 logs of attached *L. monoctogenes* were recorded for 50, 100 and 500 RB coupons, respectively (92.4, 97.8 and 97.3 % reductions, respectively). Increasing the number of coupons from 100 to 500 did not significantly increase the decontamination efficiency of PD on Iceberg lettuce. Also, the decontamination efficiencies of chlorine used either alone or in combination with 100 RB coupons, were not significantly different from the samples treated with RB coupons only.

When wash water biocides come into contact with microbes, soil and fruit and vegetable organic matter, there is a tendency for them to become inactivated, and hence they are not available for disinfection. Dirty wash water may possess a high absorbance reading which in turn may reduce light transmittance into the suspending medium. To test this hypothesis, "clean" and "dirty" wash water (Figure 25 a and b, respectively) were simulated by adding Bovine Serum Albumin (BSA; see section 2.8.3). Both "clean" and "dirty" wash water significantly reduced the antimicrobial activity of RB and intense light compared to the the no BSA controls (Figure 17). For example, treatment with $10^{-4}$ M RB and intense light in the presence of 0.3 g/litre of BSA reduced the levels of *L. monocytogenes* by only 2.5 logs after 60 min (Figure 25 a). This compared to 5.3 logs after 2 min in the absence of BSA (Figure 17). "Dirty" conditions (3 g/litre BSA) reduced the antimicrobial activity of RB still further, with log reductions of 1.21 after 60 min for *E. coli* (Figure 25 b).

## 4. Discussion

The aim of this research was to demonstrate the feasibility of applying novel techniques, using the principles of cavitation and photodynamic systems, to eliminate the microbiological hazards associated with vegetables and fruit which are commonly eaten in a raw state. Fresh produce has long been known to act as a vehicle of infection in the transmission of foodborne disease (Beuchat, 1998). Good agricultural practices may protect the food from the primary sources of contamination and may prevent or reduce the risk of food-borne illnesses. The fresh produce processing industry attempts "due diligence" by putting into place suitable decontamination practices when the risk of contamination cannot be avoided. This generally involves washing of the product in cooled sanitised water together with suitable agitation (Simons & Sanguansri, 1997). Even in a fully optimised washing system with correct biocide dosing, monitoring and control, the typical levels of microbial decontamination are 10 to 100-fold (2 log reduction), at best (Beuchat, 1998; Seymour, 1999). A recent Food & Drug Administration (FDA, USA) announcement advised that persons at high risk from severe foodborne disease should avoid eating raw alfalfa sprouts. At present, suitable decontamination methods are not in place to guarantee the safety of these products with respect to pathogens such as *E. coli* O157: H7 and *Salmonella* spp. With the potentially low infective doses of certain pathogens, it is not known whether current cleaning technologies can assure the safety of MPR products without compromising their quality. The processing involved in the manufacture of MPR fruits and vegetables cannot ensure the sterility or microbial stability of these products. There is a need to improve the performance of washing/decontamination systems to enhance the removal and/or destruction of pathogenic bacteria. In order to be economically viable, new technologies must provide decontamination efficiencies at least comparable to, and preferably in excess of, current methods, in a cost effective manner, and which retain the product quality demanded by the consumer.

### 4.1 Bacterial attachment

The surfaces of fruits and vegetables can offer protection against infection, however pathogens can colonise fresh produce through a variety of openings. Cut surfaces, wounds and abrasions (as a result of minimal processing) as well as stem scars and tiny natural openings (stomata, lenticels), provide potential points of entry for pathogens (Boyette *et al*., 1993). Results from our studies suggest that the decontamination efficiency on fresh produce with cut surfaces is lower than that for the whole uncut products (section 3.1; Table 1). Itoh *et al*. (1998) observed that *E. coli* O157:H7 was present not only on the outer surfaces but also the inner tissues of cotyledons and in the stomata of radish sprouts grown from artificially contaminated seeds. The release of high levels of nutrients and moisture from cut or damaged cells during minimal processing may provide adequate substrate levels for microbial growth. Carmichael *et al*. (1999) noticed the presence and development of biofilms on the surface of lettuce, providing a protective environment and reducing the effectiveness of sanitisers and other inhibitory substances. Ineffective hygienic processing, retail and consumer storage (cool chain) may also compromise the integrity of products by providing an ideal environment for microbial growth. SEM studies have highlighted the different topographical features of fruits and vegetables (section 3.1). Work by other authors have shown that the common sites for microbial aggregation of leaf surfaces are the veins, trichomes, stomata and cell wall junctions (reviewed by Carmichael *et al*., 1999). The trimming, peeling and slicing of minimally processed products increases the surface area for microbial attachment and provides a source of entry for microbial colonisation.

The washing and sanitation procedures currently used in the fresh produce industry are thought to be only partially successful in decontaminating micro-organisms from fruit and vegetable surfaces. Our experiments have highlighted the difficulty in removing attached microbial pathogens from both cut and uncut product surfaces. Further research is required to better understand the mechanisms through which pathogens can contaminate MPR fruits and vegetables, either on the surface or in internal tissues. Current procedures for killing or removing pathogens are focused on surface decontamination. However, recent evidence suggests that surface decontamination methods are ineffective against internalised pathogens. It is generally recommended that fresh produce washing should be carried out at less than 5°C (Ahvenainen, 1996). Buchanan *et al*. (1999) examined the effect of temperature differential on *E. coli* O157:H7 infiltration in intact apples and found that warm fruit immersed in cold water occasionally internalised the pathogen. Wei *et al*. (1995) commented that treatment with 100 ppm chlorine for 2 min failed to kill all the *Salmonella* Montevideo bacteria inoculated onto tomato surfaces, including puncture wounds, stem scars and growth cracks. They concluded that these structures provided a better protective environment for bacteria against washing with sanitised potable water rinses. Lin & Wei (1997) indicated that cutting transferred *S.* Montevideo onto the interior surfaces of tomatoes. Zhuang *et al*. (1995) also demonstrated a significantly higher number of *S.* Montevideo cells in the core tissue of tomatoes stored at 25°C and washed at 10°C compared to the number of bacteria taken up when washed at 25°C. The concentration of disinfectant reaching internalised pathogens may be reduced to the point that lethality is substantially diminished (Beuchat, 1998). Since surface decontamination methods cannot guarantee the elimination of internalised pathogens, the temperature of the wash water should ideally be at least 10°C higher than that of the fruits and vegetables. This will ensure that a positive temperature differential is achieved, thereby minimising the uptake of water and pathogens (Zhuang *et al*., 1995). Strict hygiene, good manufacturing practice and use of hazard analysis and critical control point principles may help to minimise the risks of cross contamination and pathogen uptake during processing (Ahvenainen, 1996).

### 4.2 Ultrasound studies

Numerous authors have published data on the germicidal efficacy of ultrasonic energy in aqueous suspensions (see Introduction). However, very few researchers have investigated the use of US technologies for the microbial decontamination of real food products. Lillard (1993) studied the bactericidal effect of chlorine on attached *Salmonella* with and without ultrasound treatment. He found that ultrasound in combination with chlorine was more effective at reducing bacterial counts on poultry than either ultrasound or chlorine alone. The following year Lillard (1994) reviewed the current literature on ultrasound decontamination of poultry, highlighting the inadequacy of current chlorinated washing systems for the reduction of *Salmonella* and *Campylobacter* spp. He concluded that disinfectant solutions were not able to access pathogens attached or entrapped in the skin, but effectively reduced the levels of pathogens in the wash water. However, several researchers confirmed that sonication of *Salmonella*-inoculated broiler skin significantly reduced the levels of attached bacteria in a time-dependent manner.

US has been proposed for use in food preservation on several occasions but has not been readily adopted due to the perceived adverse effects on food quality. The aim of the first part of this investigation was to determine the frequency/power/time/temperature combinations that did not lead to subjective breakdown of a range of fruit and vegetable products (section 3.2.1 – 3.2.3). However, the minimal damage observed on the products during US and storage trials demonstrated the need for microbiological assessment of decontamination by ultrasound (section 3.2.4). Small scale ultrasound trials (section 3.2.4.1) indicated a similar decontamination efficiency to that observed by Lillard (1993) on chicken skin. After 30 min treatment at 20 kHz in combination with chlorine Lillard noted a 2.88 log reduction of attached *Salmonella* spp. This compares favourably with the 2.73 log reduction of *S.* Typhimurium on Iceberg lettuce observed in our studies. However, these results are difficult to compare due to differences in frequency, power level, the size and shape of the ultrasonic bath, the depth, volume, temperature, nature of the liquid, and treatment time. Nevertheless, the underlying trends indicated some similarities in the data. Dewhurst *et al*. (1986) also reported an, at best, 1 log reduction of *Bacillus subtilis* spores on a range of polymer surfaces. In contrast, Sams & Feria (1991) reported no significant changes in aerobic plate counts from chicken drumsticks treated with and without ultrasound. They concluded that the irregular skin surface may provide some level of physical protection for bacteria against cavitation. Results from our microbial attachment studies certainly suggest that this may be the case (section 3.1 and 4.1). The interference of two opposing waves in areas of stationary waves greatly reduces cavitation. Indeed, the food products themselves may provide reflecting surfaces for the creation of stationary waves which will ultimately affect the cavitation frequency (Sams & Feria, 1991).

Attached or entrapped *Salmonella* are not readily accessible to chlorine. However, accessibility to chlorine improved when the lettuce was treated with US. Cavitation appears to detach cells which were attached or entrapped on the surface of the lettuce, making *Salmonella* more susceptible to the sanitiser. With chlorine alone, no *Salmonella* were detected in the wash water after 10 min. In contrast, low levels of *S.* Typhimurium were found in the wash water after US treatment (results not shown). Indeed, no appreciable difference in pathogen viability was noted when aqueous suspensions of *S.* Typhimurium, *E. coli* and *L. monocytogenes* were treated at 20 kHz for 10 min (results not shown). Ordonez *et al*. (1987) commented that not all micro-organisms

show the same sensitivity to cavitation. Generally, small round cells, Gram-positive bacteria and spores are more resistant while protozoa and Gram-negative bacteria are sensitive. Scherba *et al*. (1991) found a small but significant effect of US on the viability of a range of bacteria at a frequency of 26 kHz. The percentage of bacteria killed increased with duration of exposure. For example, *Pseudomonas aeruginosa* displayed an almost 85% kill after a 30 min high intensity treatment (a 90% kill corresponds to a 1-log cycle reduction). However, Ciccolini *et al*. (1997) proved that cavitation at non-lethal temperatures did not display a deactivating action on *Saccharomyces cerevisiae*. Also, Pagan *et al*. (1999) reported very low levels of *L. monocytogenes* inactivation by 20 kHz treatments. It is therefore hardly surprising that no significant differences in viability were evident in our experiments after 10 min. Bacteria detached from the surfaces of fruits and vegetables by cavitation will be released into the wash water and may have the potential to cross-contaminate other fresh produce items. It is evident that ultrasound should be used primarily in combination with disinfectants or biocides in order keep the wash water clean reducing the risks of cross-contamination. In our investigations, the washing treatments that included chlorine were significantly better than the water only treatment (at the 0.1 % level). However, although chlorine and surfactant (dioctyl sodium sulfosuccinate) demonstrated an increased 0.18 log washing efficiency compared to chlorine alone, this difference was not statistically significant. Dewhurst *et al*. (1986) observed an improved recovery of *Bacillus subtilis* spores using a surfactant (Tween 80). Nonetheless, Adams *et al*. (1989) indicated that chlorine and Tween 80 in combination were no more effective at decontaminating lettuce than Tween 80 alone. In fact, the surfactant exerted a chlorine demand on the system and reduced the available chlorine by 30%.

Cavitation appears to enhance the mechanical removal of attached or entrapped bacteria on the surfaces of fresh produce by displacing or loosening particles through a shearing or scrubbing action. However, the cavitation intensity may not be sufficient to kill the bacteria once they are released into solution. Throughout treatment, microstreaming of gas bubbles and strong eddies could be seen, which is typical of stable cavitation. The water temperature increased by approximately 0.2-0.3°C/min in the Kerry Ultrasonics tank with a measured power of 13.9 W/litre (Table 7). Transient cavitation involves bubble collapse releasing short bursts of localised high temperatures and pressures (generating shock waves and highly reactive chemical species). The relative cavitation of the Kerry Ultrasonics tank is optimal at 50 to 60°C. However, our investigations were carried out at room temperature (approximately 20°C). It is not feasible to wash fresh produce at high temperatures due to the potential deleterious effects on their organoleptic quality. Scherba *et al*. (1991) concluded that the physical mechanism of microbial inactivation appeared to be due to transient cavitation. The relatively small increase in wash water temperature during treatment suggests that transient cavitation is minimal and that most of the mechanical cleaning action is a result of stable cavitation (microstreaming).

The decontamination efficiency of cavitation on fresh produce was less pronounced when the small scale 2 litre trials were scaled up to 40 litres (section 3.2.4.2). Addition of chlorine to the wash water significantly increased the decontamination efficiency of *E. coli* contaminants on all the products tested while the product type was also significant (section 4.1). However, there was no significant difference in bacterial removal caused by the frequency of ultrasound treatment, although there was a 0.3 log reduction compared to the no treatment control (0 kHz). The mechanical cleaning efficiency of cavitation in the large scale trials were significantly lower than that for the small scale experiments, for a number of possible reasons. Untreated potable (tap) water was used throughout all the large scale trials to mirror industrial practice. However, the small scale trials used completely degassed autoclaved tap water. There was a tendency for water impurities such as calcium salts (water hardness agents) to precipitate out of solution during the autoclave process. Water hardness and dissolved gases can significantly reduce the cavitation efficiency [as described in the Operation and Maintenance manual (Kerry Ultrasonics Ltd, Hunting Gate, Wilbury Way, Hitchin, Herts, SG4 0TQ)]. Nevertheless, it was important

to carry out the decontamination trials in an industrial context, under conditions that would be experienced in a fresh produce washing line. Degassing and removal of water hardness agents could be a potentially expensive process, and are some of the possible limitations in industrial implementation. Miller (1982) also observed that ultrasonic waves are transmitted most efficiently over flat surfaces. Irregular surfaces either reflected or refracted the waves, creating stationary waves which greatly reduced cavitation. There are no reliable means of quantifying cavitation activity and therefore microbiological studies will be required to assess, validate and optimise the performance of each individual ultrasonic treatment system (O'Donoghue, 1984; Jeng *et al*., 1990).

### 4.2.1 Ultrasound scale up costs

A commercial ultrasound decontamination system will include a pre-wash section and a section with a recirculating water system (filter, cooler, pH and chlorine control). Costs of the ultrasound section were estimated for three different throughputs:

(1) 1 tonne per day over an 8 hour period (small scale processors operating over a limited shift time).
(2) 10 tonnes per day over a 16 hour period (61% of processors handle less than 10 tonnes per day (Seymour, 1999) so this throughout is fairly typical. The operating time represents two 8 hour shifts and an 8 hour cleaning shift).
(3) 100 tonnes per day (6% of processors handle more than 100 tonnes per day (Seymour, 1999) so this represents an upper limit to the throughputs being used).

These throughputs show the amounts of food material being handled. For the ultrasound system to operate the food needs to be in a liquid carrier; in these examples the food material represents approximately 10% of the total throughput. Table 11 shows the estimated capital costs of ultrasound tank systems used at the three throughputs. The figures are based on a 15W/litre treatment applied for 15 minutes. The estimated costs for the 1 tonne per day system are reliable, however, those for the 100 tonne per day system need to be treated with caution. At this high throughput it has been assumed that the system would consist of 10 tanks operating in parallel. This gives an unrealistically high capital cost, which would be reduced in practice due to the scale of the operation. A continuous flow system would probably use 46 mm radial cells which could be used in series or parallel. The capital costs of cells needed to treat 1, 10 and 100 tonnes per day are £20,000, £99,000 and ~£1,000,000, respectively (Table 11). However, if sufficient units were required, the costs of the larger systems could be as much as halved. Compared to a conventional cleaning system, the running cost for the power needed to operate the systems would also have to be taken into account. The wash water would also have to be treated to remove dissolved gases and water hardness agents. Ultrasound is not a replacement for food grade disinfectants because once the bacteria are detached from the fruit and vegetable surfaces they are released into solution and are not killed. To prevent cross-contamination, sufficient levels of biocides would have to be applied to the wash water. To maintain an effective concentration of disinfectant in the wash water it is important to dose, monitor and control the biocide levels and maintain the correct pH (Seymour, 1999). Since the combined use of ultrasound and chlorine can only offer an additional 1 log reduction compared to chlorine alone, it is not known whether the fresh produce industry would be willing to fund the additional capital expenditure (without eliminating the risk).

### 4.3 Photodynamic studies

Although the antimicrobial properties of photosensitising dyes have been known for the last hundred years (see Introduction), the research described in this report is the first time that this technology has been considered as a biocidal agent for the elimination of pathogenic bacteria on food products. A 5 litre photodynamic reactor was constructed and the system was evaluated and optimised in terms of microbiological effect on planktonic and attached pathogenic bacteria (section 3.3). Our results confirm earlier observations by Banks *et al*. (1985) that Rose Bengal has little or no cytotoxic effect on *E. coli* under dark conditions (0 lux). In addition, the viability of *S.* Typhimurium was also found to be unaffected by the presence of $10^{-4}$ M RB. However, a reduction of 2.65 logs of *L. monocytogenes* was observed after 60 min (Figure 13). This result highlights the major differences in RB cytotoxicity between Gram-negative (*E. coli*, *S.* Typhimurium) and Gram-positive (*L. monocytogenes*) bacteria. The results presented in this report suggest that photosensitive dyes may be able to enter Gram-positive cells freely by diffusion and interact with cellular targets.

In the presence of intense light (15,000 lux) both RB (Figure 17) and MB (Figure 18) exhibited more pronounced antimicrobial action than in the dark. However, *L. monocytogenes* was an order of magnitude more sensitive to singlet oxygen than both *E. coli* and *S.* Typhimurium. Out of the three photodynamic dyes tested RB was found to be the best generator of singlet oxygen. Houba-Herin *et al*. (1982) determined the rates of singlet oxygen production (in arbitrary units per minute) for RB (71), MB (70) and Eosin Y (61). All three dyes would have been expected to show comparable singlet oxygen production. The absorption spectra for RB, MB and EY are 550, 665 and 516 nm ($\lambda_{max}$) respectively. This result suggests that the phosphor-mercury blended light bulbs (Mixed Light 31, GE, Belgium) that provided the light source for all photodynamic experiments, were emitting optimally at 550 nm, the peak absorbance for RB. There was a pronounced lag time in exponential death for both *E. coli* and *S.* Typhimurium during which bacteria were not significantly inactivated. However, *L. monocytogenes* was rapidly killed with no viable cells being detected after a 2 min treatment with $10^{-4}$ M RB. The structure of the cell wall therefore plays an important role in the susceptibility of micro-organisms to singlet oxygen. Dahl *et al*. (1989) hypothesised that the outer membrane-lipopolysaccharide portion of the Gram-negative cell wall initially protects the bacteria from extracellular singlet oxygen. The lipopolysaccharide (LPS) coat enables these bacteria to survive in harsh environments, such as those encountered in mammalian intestines. The LPS has previously been shown to present a physical or chemical barrier through which singlet oxygen must pass in order to interact with cellular sites (Banks *et al*., 1985; Dahl *et al*., 1987). For example, membrane lipids undergo peroxidation reactions while integral membrane proteins are damaged by specific reactions with susceptible amino acids. Singlet oxygen is thought to affect nucleic acids by interacting with guanine bases. Banks *et al*. (1985) suggest that the lag in exponential death observed with Gram-negative bacteria may be due progressive photodynamically mediated damage of vital targets. They suggest that a cell only loses viability when a lethal threshold in damage has been exceeded and present evidence of significant intracellular ATP depletion prior to a reduction in viability. In contrast, most Gram-positive bacteria lack a protective LPS layer and outer membrane. The differences in cell wall composition and structure between Gram-positive and Gram-negative bacteria may account for the disparity in RB toxicity. Consistent with this hypothesis, a *Salmonella* mutant (*hisG46 rfa*, derived from LT-2), which is known to allow greater penetration of a range of exogenous agents than its wild type parent (LT-2), was found to be more sensitive than the wild type (Dahl *et al*., 1989).

For all the pathogenic bacteria tested, $10^{-4}$ M RB was the most effective in terms of antimicrobial action. In order to improve singlet oxygen generation for inactivation of Gram-negative bacteria the RB concentration was increased 10-fold from $10^{-4}$ M to $10^{-3}$ M. However, no photodynamic inactivation of *S.* Typhimurium was

evident, even after 60 min treatment (Figure 15). At the highest concentration the RB solution was dark red in colour and it may be postulated that insufficient light was penetrating the suspending medium. There will also be a corresponding decrease in singlet oxygen production but this is difficult to measure experimentally. Light transmittance into the wash water is a major control parameter in terms of microbiological effect and very much determines the operating limits of a photodynamic system.

In order to assess the commercial application of photodynamic technologies it is essential to minimise the levels of dye residues remaining on fresh produce after washing. Photodynamic dyes are highly coloured, and if used free in solution, the finished product would possess sensory qualities unacceptable to the consumer. In light of that matter, investigations were carried out to determine the effects of immobilised RB (nylon mesh support) on the inactivation of planktonic (section 3.3.2) and attached bacteria (section 3.3.3). Fifty 5cm squares of nylon mesh supplied a photodynamic catalyst surface area sufficient to inactivate *L. monocytogenes*. The effects on this Gram-positive bacteria was the same order of magnitude when RB was used either free in solution or immobilised to nylon. Leaching of immobilised RB from the nylon support was monitored in the wash water. However, spectrophotometric analysis indicated that the levels of RB in the were less than $10^{-7}$ M for the duration of the treatments (results not shown). In contrast to *L. monocytogenes*, both *E. coli* and *S.* Typhimurium viability were unaffected by the presence of fifty RB coupons. Increasing the number of coupons to 250 or 500 still had no significant effect on the inactivation of Gram-negative bacteria. It was not possible to generate sufficient catalyst surface area and singlet oxygen to inactivate Gram-negative bacteria. Most of the investigations by other researchers have studied the photodynamic inactivation of micro-organisms on a small scale (test tube). However, our studies have demonstrated a scaled up illuminated reactor system (up to a 5 litre capacity). The 4.77 log reduction of planktonic *E. coli* by $10^{-4}$ M RB (in solution) observed in our experiments compared favourably to the 4 log reduction of *E. coli* B/r by 2 x $10^{-4}$ M RB reported by Banks *et al*. (1985).

The final part of this study involved evaluation of the optimised photodynamic system under conditions which mirrored real industrial operation (section 3.3.3). The photodynamic reactor was previously shown to be ineffective for the inactivation of Gram-negative bacteria using immobilized RB. It was therefore decided to assess the photodynamic system for decontamination of real produce items as a means of demonstrating the technology. In these studies, Iceberg lettuce was inoculated with known levels of *L. monocytogenes* contaminants and the decontamination efficiency of immobilised RB was compared to 100 ppm free chlorine, the current industry standard (Seymour, 1999). Decontamination and inactivation of attached *L. monocytogenes* by the RB catalyst was very much comparable to that reported for chlorine, a 1 to 2 log reduction at best. Using RB coupons and chlorine in combination did not offer a significant increase in washing efficiency compared to RB or chlorine alone (Figure 24). Accessibility of attached bacteria to disinfectants is a well known problem in the fresh produce industry and it is hardly surprising that singlet oxygen has a similar decontamination efficiency to that of chlorine. Many of the disinfectants used extensively for fresh produce washing are oxidising agents, and most of them have a particular affinity for organic matter (Boyette *et al*., 1993). Dirty produce contains a large amount of organic matter which "uses up" the available disinfectant much faster than relatively clean produce. The more organic matter in the washing tank (fruit, leaves, dirt etc), the more disinfectant is lost (Kotula *et al*., 1997). The effects of organic matter on the efficiency of singlet oxygen as an antimicrobial agent was simulated by using Bovine Serum Albumin under "clean" (Figure 25 a) and "dirty" conditions (Figure 25 b). Under both organic loading regimes the effectiveness of photodynamic inactivation was significantly lower than the no BSA control (Figure 17). It is likely that the organic matter is "using up" the available singlet oxygen, which is then unavailable for antimicrobial action. The BSA also made the wash water go cloudy and may possibly decrease the transmittance of light into the photodynamic system. This is another

factor which will significantly reduce the effectiveness of an industrial scale photodynamic washer. This, together with the poor inactivation of Gram-negative bacteria, reduces the likely uptake of photodynamic technologies by the fresh produce industry. Over 50% of fresh produce washing systems currently used in the UK have a capacity greater than 1000 litres (Seymour, 1999). The potential problems of effective light penetration coupled with a high organic loading in a large scale washer are likely to significantly reduce the levels of generated singlet oxygen. Photodynamic technologies are therefore unlikely to offer the fresh produce industry with a viable alternative to chlorine.

# 5. Conclusions

The research described in this report is the first time that ultrasound and photodynamics have been investigated as viable technologies for the washing and decontamination of MPR fruits and vegetables. Current decontamination methods are crude, poorly understood and relatively ineffective. Assessment of bacterial attachment and fresh produce surface topography (section 3.1) highlighted the difficulty in removing and killing attached or entrapped pathogenic bacteria. Even with a fully optimised washing system with excess disinfectant the best levels of microbial reduction are 10 to 100-fold at best.

Ultrasound methods focused on process optimisation in terms of microbiological effect whilst ensuring organoleptic quality acceptability to the consumer. In combination, chlorine and ultrasound offered an additional one log reduction in attached bacteria compared to chlorine or ultrasound alone (small scale; section 3.2). The mechanical cleaning action of cavitation appears to remove cells which are attached on the surface of fresh produce, rendering the pathogens more susceptible to the sanitiser. However, the decontamination efficiency was less pronounced when the small scale 2 litre trials were scaled up to 40 litres. A number of factors led to a reduction in the efficiency of cavitation, most notably the treatment temperature, the wash water quality and the dissolved gas status of the suspending medium. With the potentially high capital expenditure together with expensive process optimisation and water treatment, it is not known whether the fresh produce industry would be willing to take up this novel technology. An additional one log reduction does not completely eliminate the risk of pathogens on fresh produce and current methods may be sufficient to ensure “due diligence”.

The main conclusion from the photodynamic methods was the difference in singlet oxygen sensitivity between Gram-positive and Gram-negative bacteria. It was not possible to provide an effective photodynamic catalyst surface area sufficient to kill planktonic *E. coli* and *S.* Typhimurium. Decontamination and inactivation of attached *L. monocytogenes* by the RB catalyst was very much comparable to that reported for chlorine, a one to 2 log reduction at best. However, this technology is unlikely to significantly reduce the levels of attached Gram-negative bacteria. Ineffective light penetration coupled with the potentially high organic loading of dirty fresh produce in a large scale photodynamic washer may decrease the effectiveness of singlet oxygen generation. Photodynamic technologies are therefore unlikely to offer the fresh produce industry with a viable alternative to chlorine.

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# Appendices

**Table 1. Assessment of *S*. Typhimurium attachment on cut and uncut fresh produce.**

| | Uncut | | | Cut | | |
|---|---|---|---|---|---|---|
| | $log_{10}$ reduction (cfu/g) | SD | % reduction | $log_{10}$ reduction (cfu/g) | SD | % reduction |
| **Food** | | | | | | |
| Baton carrot | n/a | n/a | n/a | 0.57 | 0.11 | 73 |
| Cabbage | 1.03 | 0.2 | 91 | 0.34 | 0.13 | 54 |
| Cucumber | n/a | n/a | n/a | 0.38 | 0.12 | 58 |
| Iceberg lettuce | 1.48 | 0.46 | 97 | 0.59 | 0.15 | 74 |
| Parsley | 0.21 | 0.29 | 37 | 0.34 | 0.13 | 54 |
| Pepper | 1.49 | 0.36 | 97 | 0.68 | 0.15 | 76 |
| Spring onion | 0.89 | 0.2 | 87 | 0.32 | 0.14 | 52 |
| Strawberry | 1.26 | 0.2 | 94 | 0.66 | 0.28 | 78 |

**$log_{10}$ reduction = $log_{10}$ (unwashed control – washed)**
**SD = standard deviation**
**n/a = not applicable (not tested)**

**Table 2. Summary of damage studies on nine types of food treated in ultrasound tanks for 300 s.**

| **Power** | 15 W/litre | 15 W/litre | 15 W/litre | 25 W/litre |
|---|---|---|---|---|
| **Frequency** | 25 kHz | 32-40 kHz | 62-70 kHz | 35 k Hz |
| **Time** | 300 s | 300 s | 300 s | 300 s |
| **Food** | | | | |
| Baton Carrot | No damage | No damage | No damage | No damage |
| Cabbage | No damage | No damage | No damage | No damage |
| Cucumber | No damage | No damage | No damage | No damage |
| Mint | Loss of colour near to edge of the leaf | Loss of colour near to edge of the leaf | Loss of colour near to edge of the leaf | Loss of colour near to edge of the leaf |
| Iceberg lettuce | No damage | No damage | No damage | No damage |
| Parsley | No damage | No damage | No damage | No damage |
| Pepper | No damage | No damage | No damage | No damage |
| Spring onion | No damage | No damage | No damage | No damage |
| Strawberry | No damage | No damage | No damage | No damage |

**Table 3. Summary of damage studies on nine types of food held for 4 days (at 5°C) after purchase and then treated in ultrasound tanks for 300 s.**

| **Power** | 15 W/litre | 15 W/litre | 15 W/litre | 25 W/litre |
|---|---|---|---|---|
| **Frequency** | 25 kHz | 32-40 kHz | 62-70 kHz | 35 k Hz |
| **Time** | 300 s | 300 s | 300 s | 300 s |
| **Food** | | | | |
| Cabbage | No damage | No damage | No damage | No damage |
| Baton Carrot | No damage | No damage | No damage | No damage |
| Cucumber | No damage | No damage | No damage | No damage |
| Mint | Loss of colour near to edge of the leaf | Loss of colour near to edge of the leaf | Loss of colour near to edge of the leaf | Loss of colour near to edge of the leaf |
| Iceberg lettuce | No damage | Very slight loss of colour near to the edge of the leaf | Very slight loss of colour near to the edge of the leaf | Very slight loss of colour near to the edge of the leaf |
| Parsley | No damage | Very slight loss of colour near to the edges | Very slight loss of colour near to the edges | Very slight loss of colour near to the edges |
| Pepper | No damage | No damage | No damage | No damage |
| Spring onion | No damage | No damage | No damage | No damage |
| Strawberry | No damage | No damage | No damage | No damage |

**Table 4. Comparison of nine types of food treated on the day of purchase with ultrasound and then stored for 15 days or left untreated (control) and stored for 15 days.**

| **Power** | 15 W/litre | 15 W/litre | 15 W/litre | 25 W/litre |
|---|---|---|---|---|
| **Frequency** | 25 kHz | 32-40 kHz | 62-70 kHz | 35 k Hz |
| **Time** | 300 s | 300 s | 300 s | 300 s |
| **Initial water temperature** | 5°C | 5°C | 20°C | 20°C |
| **Food** | | | | |
| Baton Carrot | No difference | No difference | No difference | No difference |
| Cabbage | No difference | No difference | No difference | No difference |
| Cucumber | No difference | No difference | No difference | No difference |
| Mint | Loss of colour near to edge of the leaf directly after treatment | Loss of colour near to edge of the leaf directly after treatment | Loss of colour near to edge of the leaf directly after treatment | Loss of colour near to edge of the leaf directly after treatment |
| Iceberg lettuce | No difference | No difference | No difference | No difference |
| Parsley | No difference | No difference | No difference | No difference |
| Pepper | No difference | No difference | No difference | No difference |
| Spring onion | No difference | No difference | No difference | No difference |
| Strawberry | No difference | Less mould on treated samples but dependent on supply of strawberries | No difference | Less mould on treated samples but dependent on supply of strawberries |

**No difference = No visual difference between treated and control samples**

**Table 5. Summary of damage to nine types of food treated with an ultrasound bar applicator.**

| **Power** | 225 W/litre (nominal) | | |
|---|---|---|---|
| **Frequency** | 20 kHz | | |
| **Time** | 30 s | 150 s | 300 s |
| **Food** | | | |
| Baton Carrot | No damage | No damage | No damage |
| Cabbage | No damage | No damage | Slight damage at the edges if the food was not very fresh |
| Cucumber | No damage | No damage | Slight damage |
| Mint | Darkened edges | Becoming darker | Black everywhere |
| Iceberg lettuce | No damage | Slight blotches | Slight blotches |
| Parsley | No damage | Darkened edges | Darkened edges |
| Pepper | No damage | No damage | No damage |
| Spring onion | No damage | No damage | No damage |
| Strawberry | 'Hairs' removed from very fresh strawberries. No damage to older strawberries. | 'Hairs' removed from very fresh strawberries. No damage to older strawberries. | 'Hairs' removed from very fresh strawberries. No damage to older strawberries. |

**Table 6. Summary of damage to nine types of food treated with an ultrasound radial cell applicator.**

| **Power** | 30,000 W/litre (nominal) | | | |
|---|---|---|---|---|
| **Frequency** | 20 kHz | | | |
| **Time** | 1 s | 2 s | 5 s | 10 s |
| **Food** | | | | |
| Baton Carrot | No visible damage but the water became coloured | No visible damage but the water became coloured | No visible damage but the water became coloured | No visible damage but the water became coloured |
| Cabbage | Slight change at the edges | Slight change at the edges | Slight change at the edges | Slight change at the edges |
| Cucumber | No damage | No damage | No damage | Slight damage |
| Mint | Clearly visible edge effects | Edge effects increasing with time | Edge effects increasing with time | Edge effects increasing with time |
| Iceberg lettuce | Patchy areas | Patchy areas | Patchy areas | Patchy areas |
| Parsley | Darkened edges | Edge effect increasing with time | Edge effect increasing with time | Edge effect increasing with time |
| Pepper | No visible damage but the water became slightly coloured | No visible damage but the water became coloured | No visible damage but the water became coloured | No visible damage but the water became coloured |
| Spring onion | No visible damage but the water became slightly coloured | No visible damage but the water became coloured | No visible damage but the water became coloured | No visible damage but the water became coloured |
| Strawberry | No damage | ‘Hairs’ dislodged from surface | More ‘hairs’ dislodged | More ‘hairs’ dislodged |

**Table 8. Small-scale decontamination trials to investigate the washing efficiency of ultrasound and chlorine.**

| | | | |
|---|---|---|---|
| **Power** | 10 W/litre | | |
| **Frequency** | 32-40 kHz | | |
| **Time** | 10 min | | |
| **Food** | Iceberg lettuce | | |
| **Chlorine** | 25 ppm free residual chlorine, pH 7.0 +/- 0.1 | | |
| **Organism** | *S.* Typhimurium | | |
| **Treatment** | $\log_{10}$ reduction (cfu/g) | SD | % reduction |
| Water control | 0.73 | 0.24 | 81 |
| Ultrasound | 1.51 | 0.18 | 97 |
| Chlorine | 1.67 | 0.51 | 98 |
| Ultrasound + chlorine | 2.73 | 0.58 | 99.8 |

**Table 9 a. Large-scale cabbage decontamination trials to investigate the washing efficiency of ultrasound and chlorine treatments.**

| | Average $\log_{10}$ reduction in attached *E. coli* | | | |
|---|---|---|---|---|
| Ultrasound | Water only | Chlorine | Chlorine and Surfactant | Mean |
| 0 kHz | 0.65 | 1.41 | --- | 1.85 |
| 25 kHz | 0.91 | 1.74 | 2.91 | 2.01 |
| 32 kHz | 1.61 | 2.05 | 2.38 | 0.78 |
| 70 kHz | 1.40 | 1.93 | 2.31 | 1.88 |
| Mean | 1.14 | 1.76 | 2.53 | 1.81 |

**Table 9 b. Large-scale Iceberg lettuce decontamination trials to investigate the washing efficiency of ultrasound and chlorine treatments.**

| | Average $\log_{10}$ reduction in attached *E. coli* | | | |
|---|---|---|---|---|
| Ultrasound | Water only | Chlorine | Chlorine and Surfactant | Mean |
| 0 kHz | 0.38 | 0.72 | --- | 0.55 |
| 25 kHz | 0.97 | 1.26 | 2.11 | 1.45 |
| 32 kHz | 0.19 | 1.93 | 1.88 | 1.33 |
| 70 kHz | 0.97 | 1.59 | 1.12 | 1.23 |
| Mean | 0.63 | 1.38 | 1.70 | 1.19 |

**Table 9 c. Large-scale parsley decontamination trials to investigate the washing efficiency of ultrasound and chlorine treatments.**

| | Average $\log_{10}$ reduction in attached *E. coli* | | | |
|---|---|---|---|---|
| Ultrasound | Water only | Chlorine | Chlorine and Surfactant | Mean |
| 0 kHz | 0.85 | 1.56 | --- | 1.20 |
| 25 kHz | 0.77 | 0.58 | 1.21 | 0.85 |
| 32 kHz | 0.29 | 0.84 | 0.11 | 0.41 |
| 70 kHz | 0.49 | 1.08 | 1.08 | 0.88 |
| Mean | 0.60 | 1.01 | 0.80 | 0.80 |

**Table 9 d. Large-scale strawberry decontamination trials to investigate the washing efficiency of ultrasound and chlorine treatments.**

| | Average $log_{10}$ reduction in attached *E. coli* | | | |
|---|---|---|---|---|
| Ultrasound | Water only | Chlorine | Chlorine and Surfactant | Mean |
| 0 kHz | -0.09 | 2.12 | --- | 1.02 |
| 25 kHz | 1.38 | 1.37 | 1.20 | 1.31 |
| 32 kHz | 0.77 | 1.58 | 1.96 | 1.44 |
| 70 kHz | 0.63 | 1.50 | 1.25 | 1.13 |
| Mean | 0.67 | 1.64 | 1.47 | 1.26 |

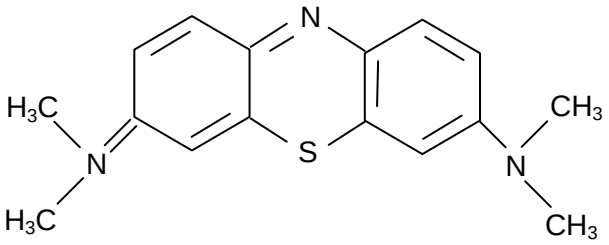
**Table 9 e. Large-scale fresh produce decontamination trials to investigate the washing efficiency of a chlorine jacuzzi treatment.**

| | Average $log_{10}$ reduction in attached *E. coli* |
|---|---|
| ***Food*** | Chlorine Jacuzzi |
| Cabbage | 2.58 |
| Iceberg lettuce | 0.89 |
| Parsley | 1.48 |
| Strawberry | 1.77 |
| Mean | 1.68 |

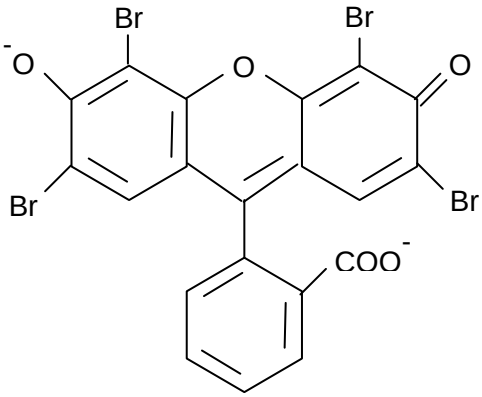
**Table 10. The survival of *L. monocytogenes* on Iceberg lettuce after washing for 60 min in the presence of Rose Bengal Coupons, 100 ppm free chlorine and Intense light (15,000 lux).**

| **Treatment** | $log_{10}$ reduction (cfu/g) | SD | % reduction |
|---|---|---|---|
| Water only | 0.3 | 0.08 | 50.4 |
| 50 coupons | 1.11 | 0.12 | 92.4 |
| 100 coupons | 1.65 | 0.18 | 97.8 |
| 500 coupons | 1.57 | 0.33 | 97.3 |
| Chlorine | 1.77 | 0.06 | 98.3 |
| 100 coupons + chl | 1.52 | 0.04 | 97.0 |

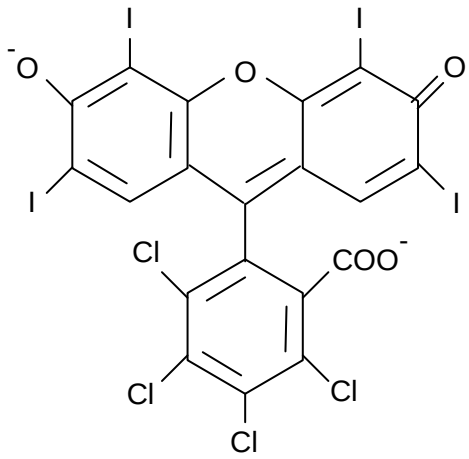
**Figure 1. Structure, formula and molecular weight of the sensitizing dyes (from Houba-Herin *et al.*, 1982).**




**Methylene blue (MB)**
$C_{16}H_{17}N_3SHCl.3H_2O$ (mol.wt 373.9)




**Eosin Y (EY)**
$C_{20}H_6O_5Br_4$2Na (mol. wt 691.9)




**Rose bengal (RB)**
$C_{20}H_2O_5Cl_4I_4$2Na (mol. wt 1017.7)

**Figure 2. Example of an ultrasound treatment tank (provided by Kerry Ultrasonics Ltd).**

**Figure 3. Photograph of an ultrasound bar applicator (20 kHz).**

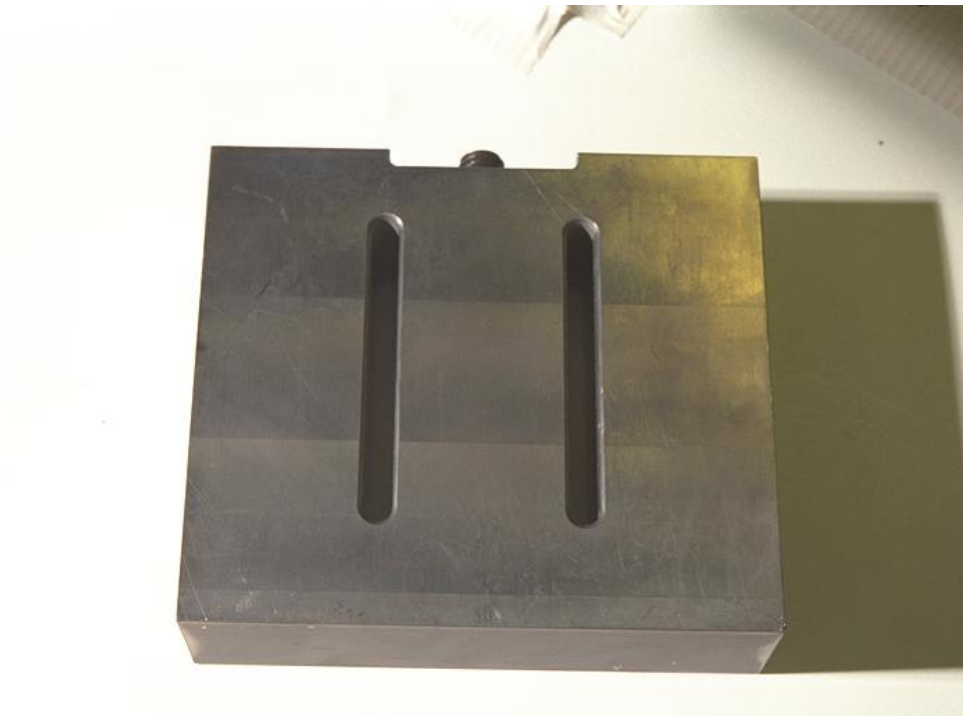


**Figure 4. Photograph of an ultrasound radial cell applicator (20 kHz).**

**Figure 5 a. SEM view of green pepper surface. Note the ribbed appearance of the anticlinal walls, due to epidermal cell collapse.**

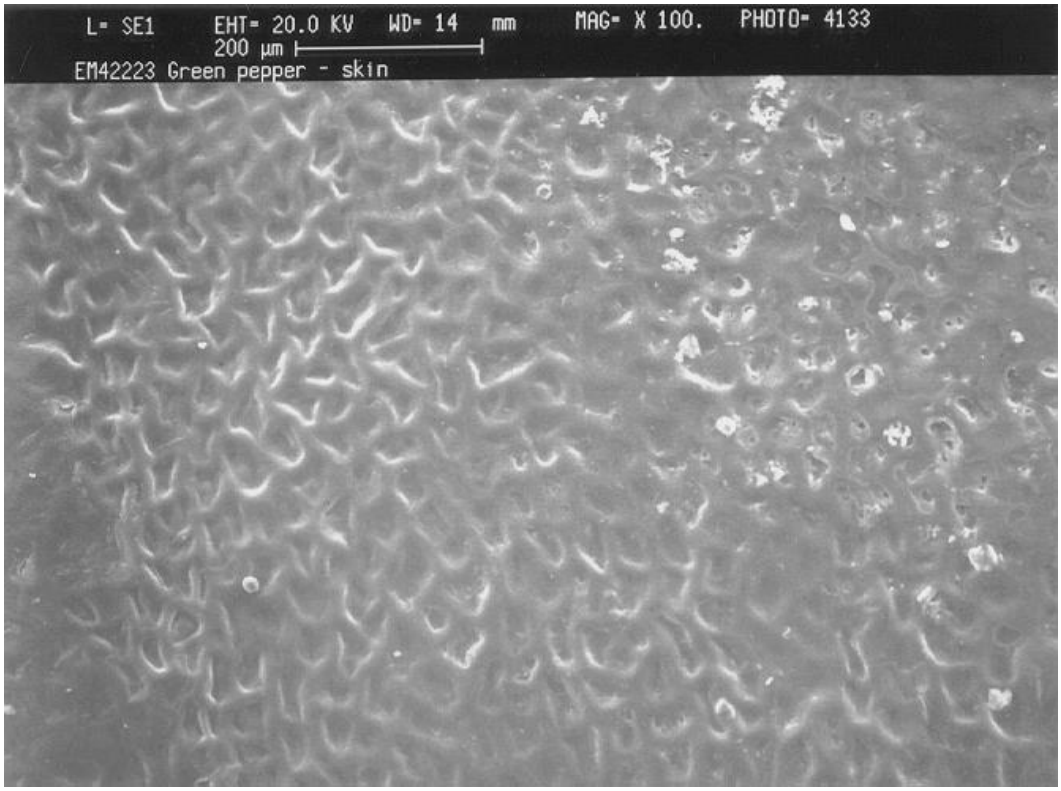



**Figure 5 b. SEM view of spring onion surface. Note the files of parallel cells typical of a monocotyledon.**

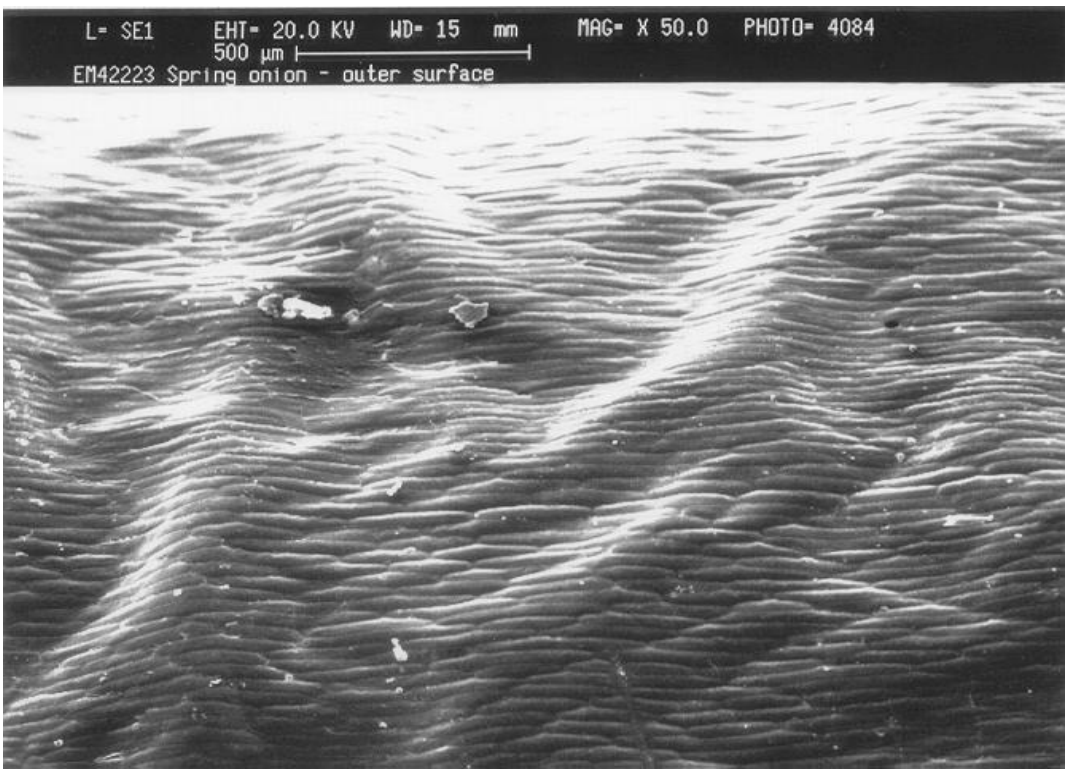


**Figure 5 c. SEM view of a strawberry seed. Note the turgid epidermal cells, where the anticlinal walls are marked by valleys, in the lower half of the picture.**

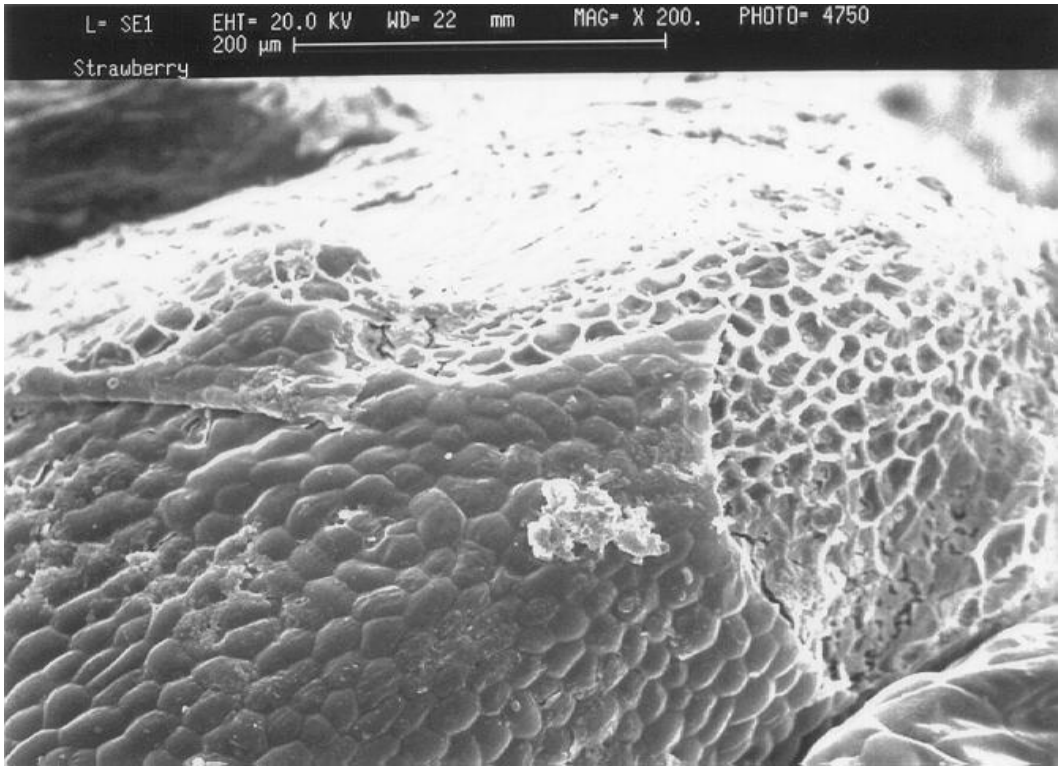



**Figure 5 d. SEM view of parsley lower surface. Note the natural bacteria present and the stoma at the lower centre of the picture.**

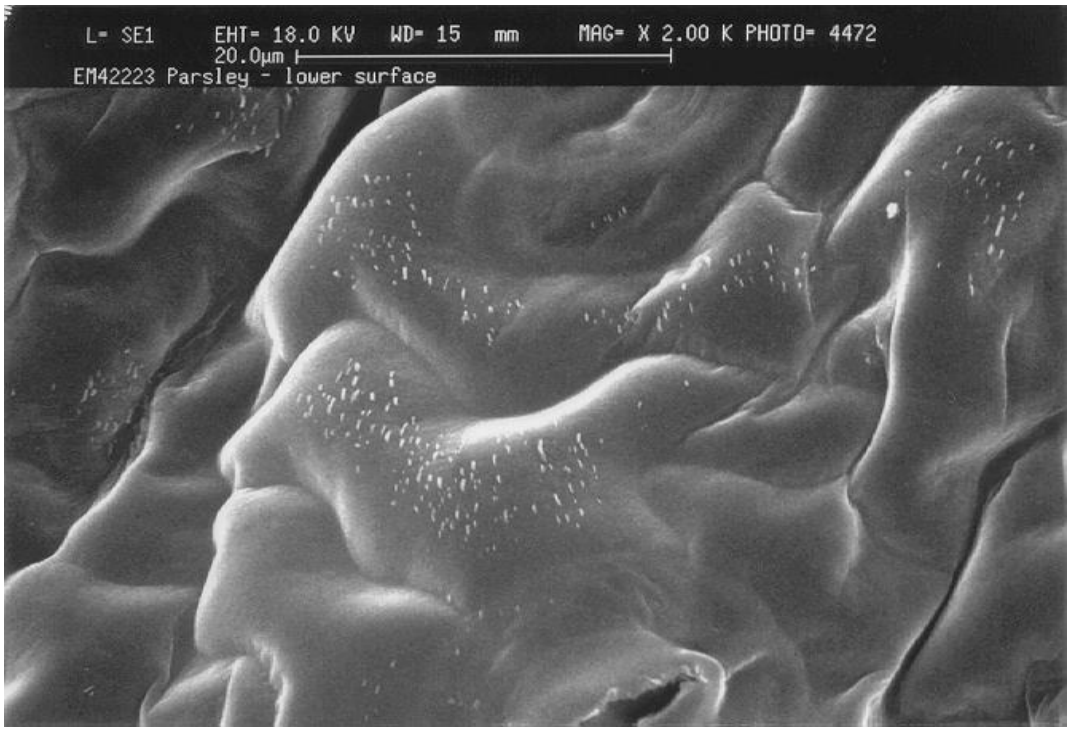


**Figure 5 e. SEM view of cabbage upper surface. Note the wax crystals on the surface, which is typical of cabbage.**

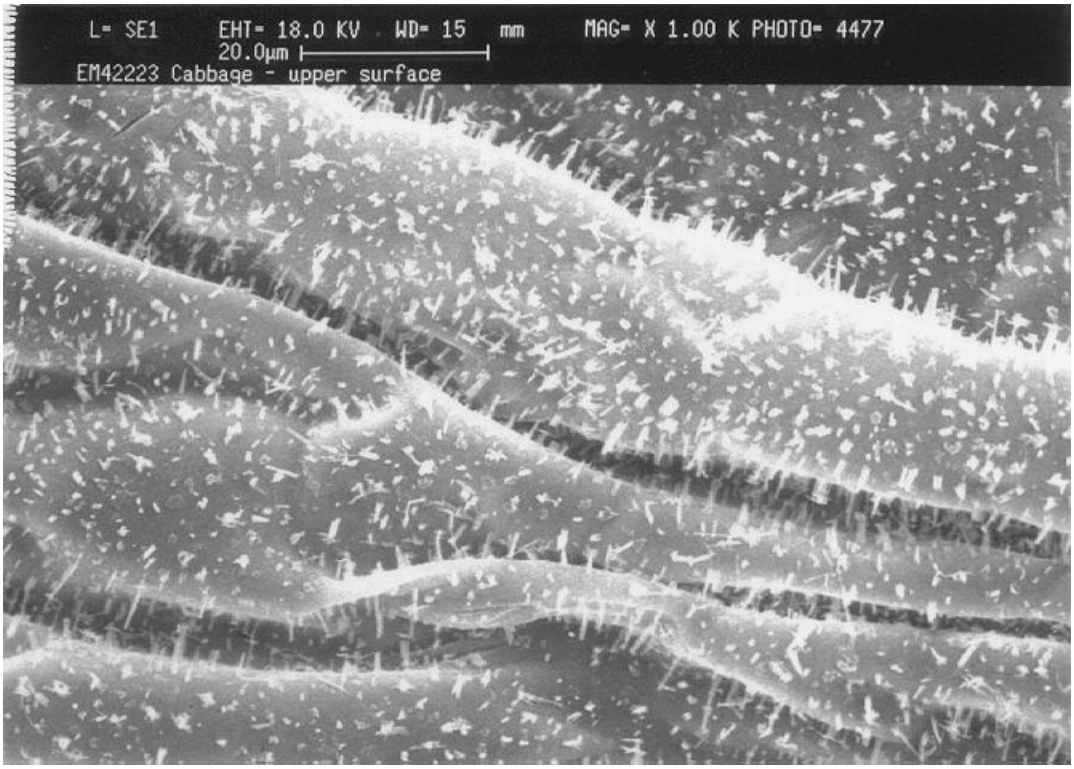



**Figure 5 f. SEM view of lettuce lower surface. Note the stomata and the wavy lines denoting anticlinal walls.**

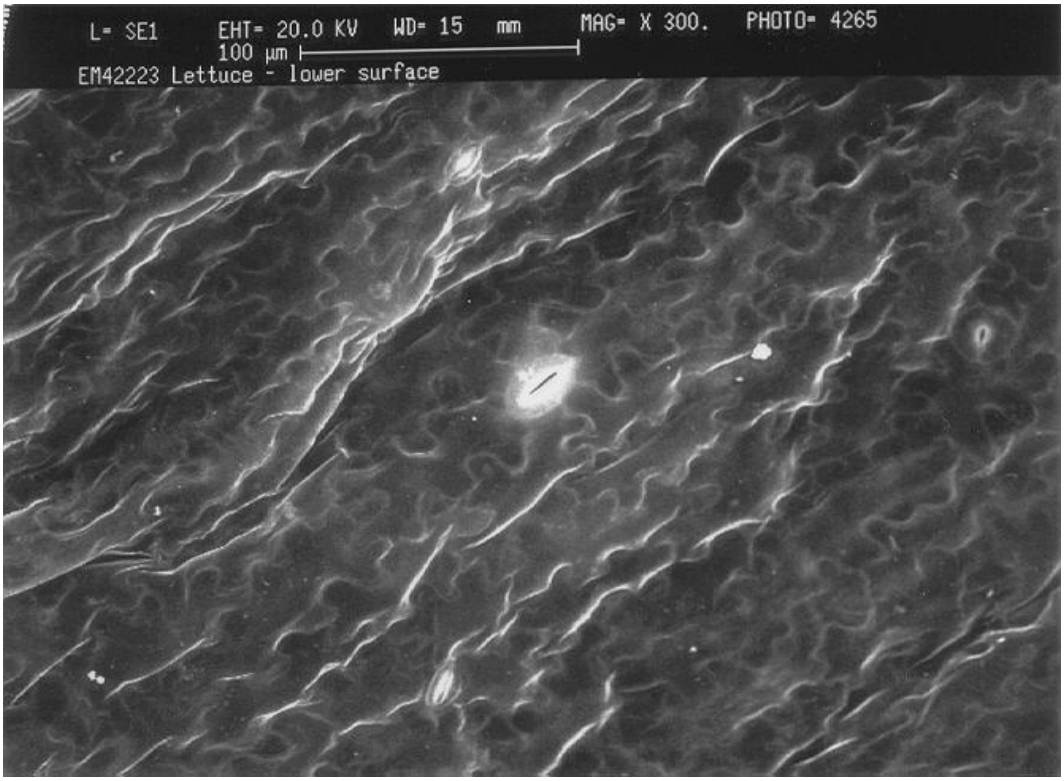


**Figure 5 g. SEM view of cucumber outer surface. Note the stomata and the anticlinal walls of the collapsed epidermal cells.**

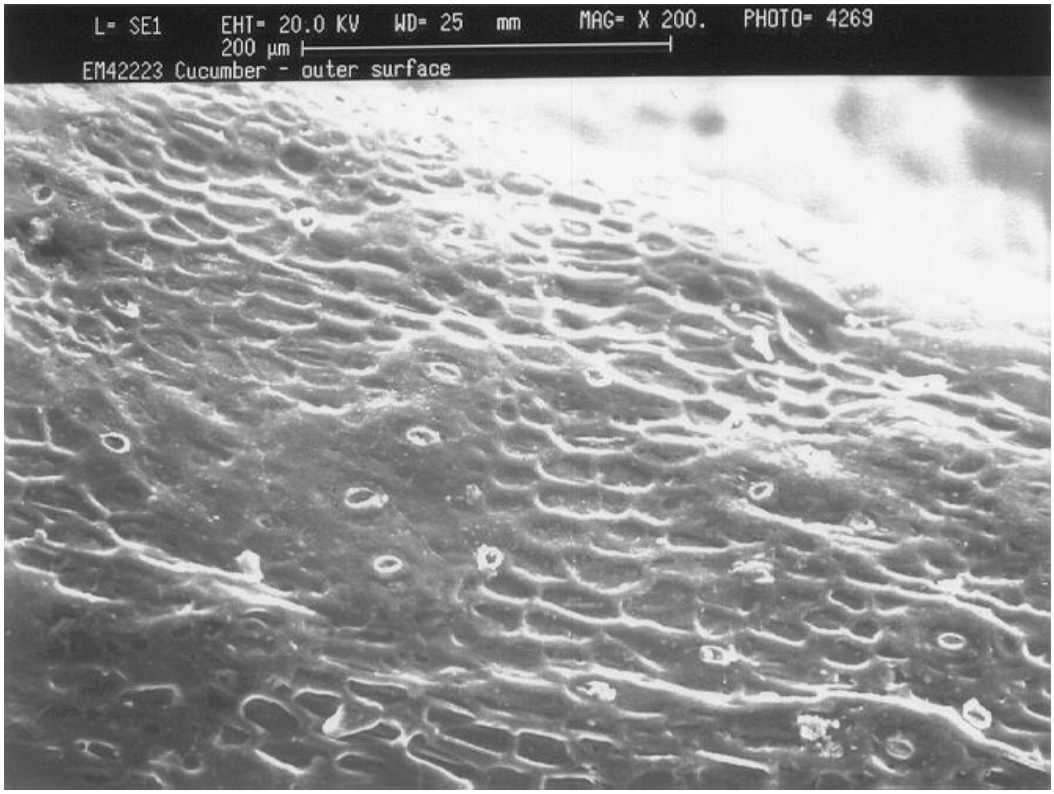



**Figure 5 h. SEM view of cucumber in trans-section showing two seeds embedded in the cucumber tissue.**

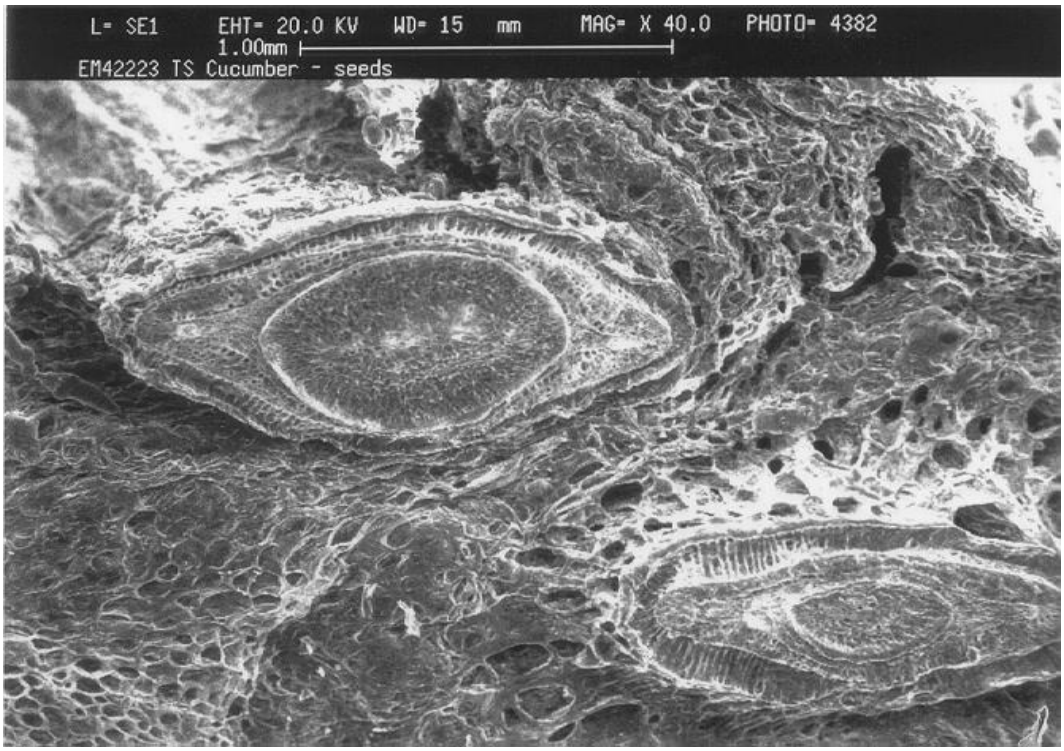


**Figure 5 i. SEM view of carrot peeled surface. Note the large number of open cells exposed, readily colonized by bacteria and difficult to clean.**

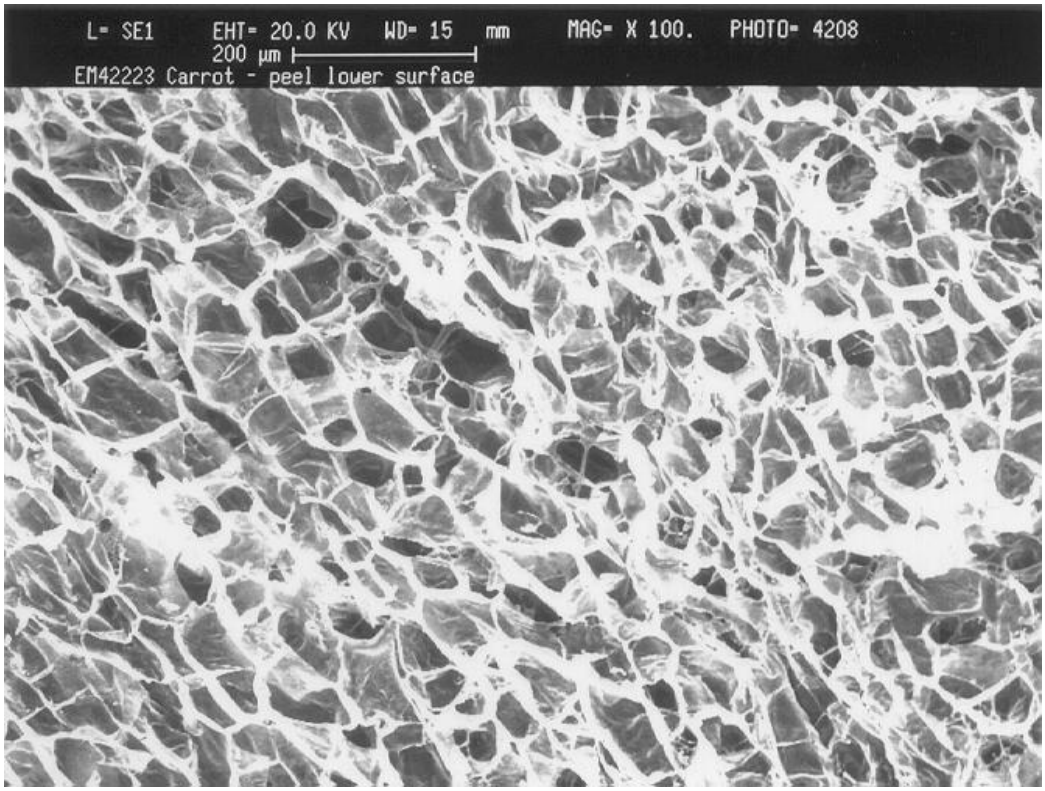



**Figure 5 j. SEM view of carrot in trans-section. Note the large number of open cells and the xylem vessel at the centre extreme left of the picture.**

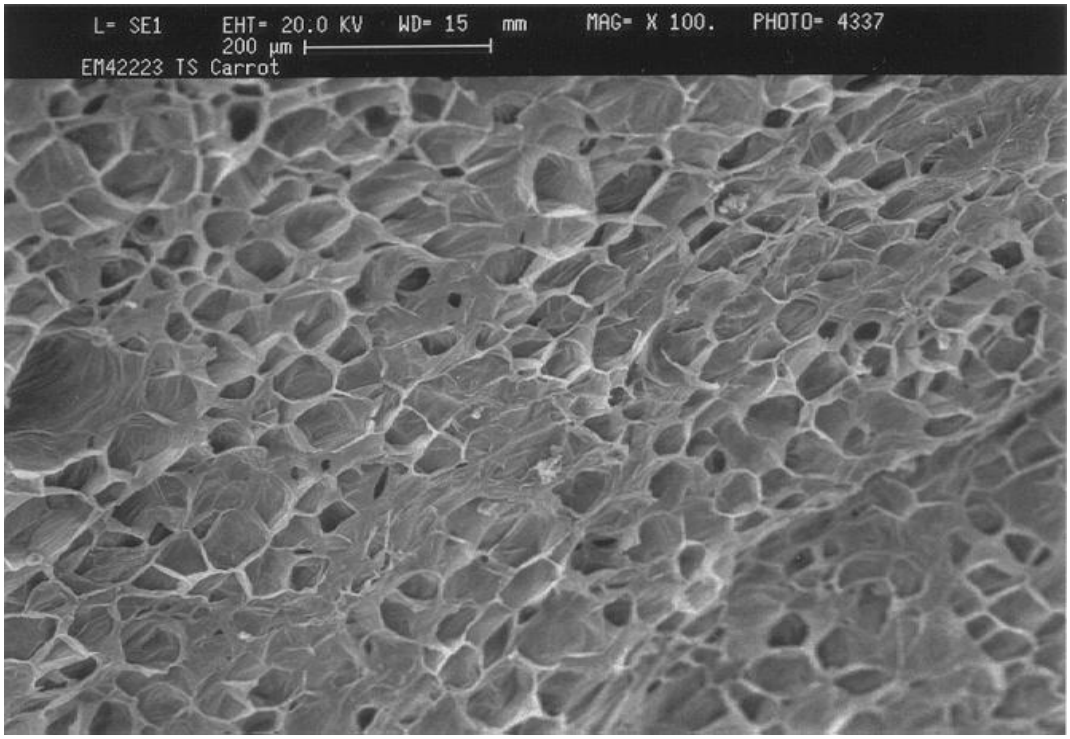


**Figure 6. Photograph of mint after 300 s treatment in an ultrasound tank operating at 15 W/litre and 32-40 kHz. Slight damage is seen as loss of colour near to the edge of the leaf.**

**Figure 10. Summary of the effects of the main washing treatments.**

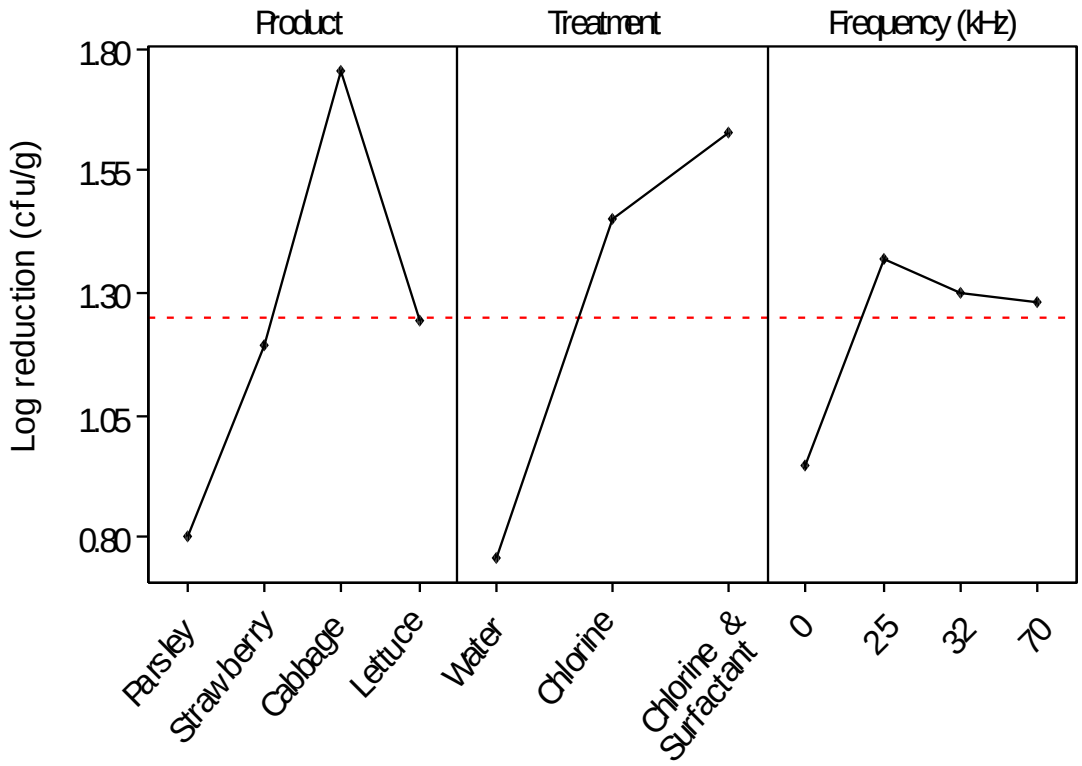


**Figure 11. Dark controls (0 Lux).**

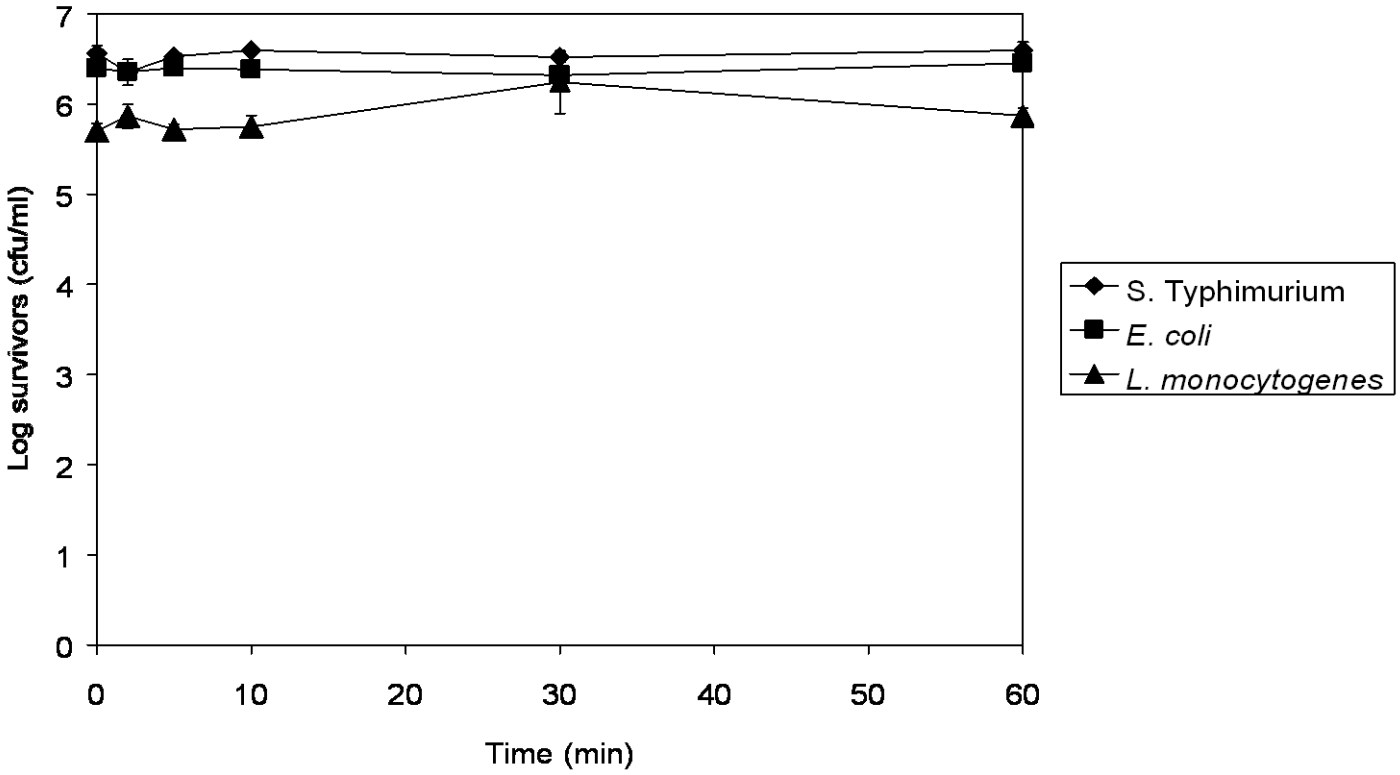



**Figure 12. Intense light controls (15,000 Lux).**

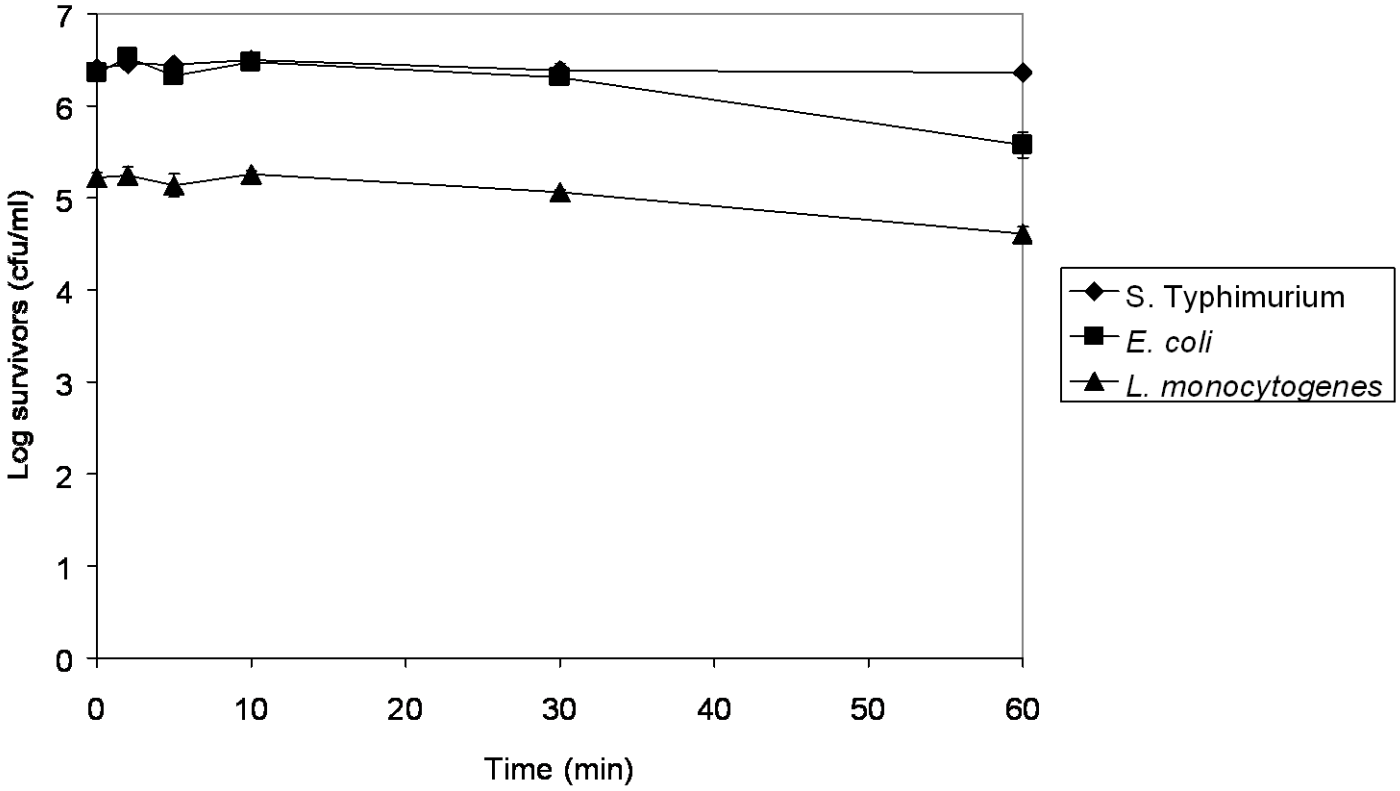


**Figure 13. Dark controls in the presence of $10^{-4}$ M Rose Bengal.**

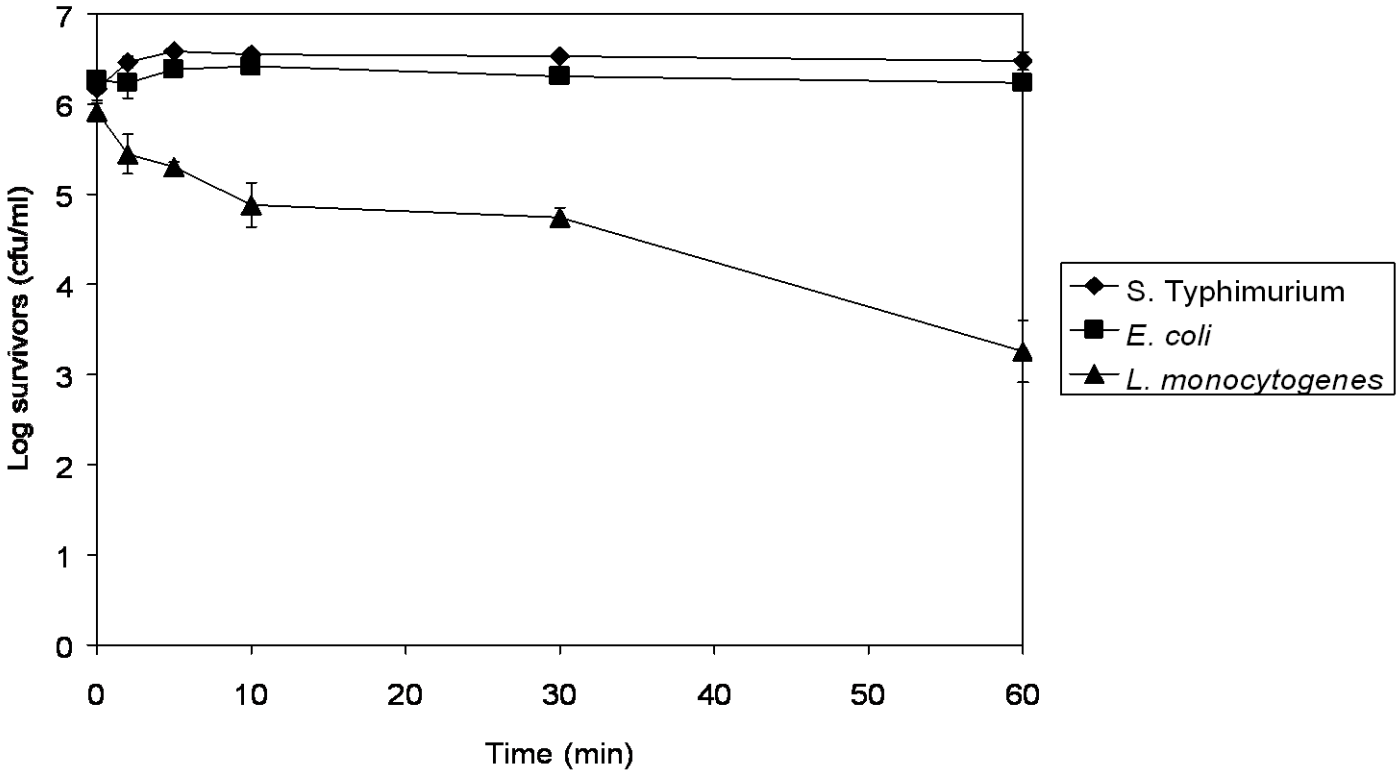



**Figure 14. Dark controls in the presence of $10^{-4}$ M Methylene Blue.**

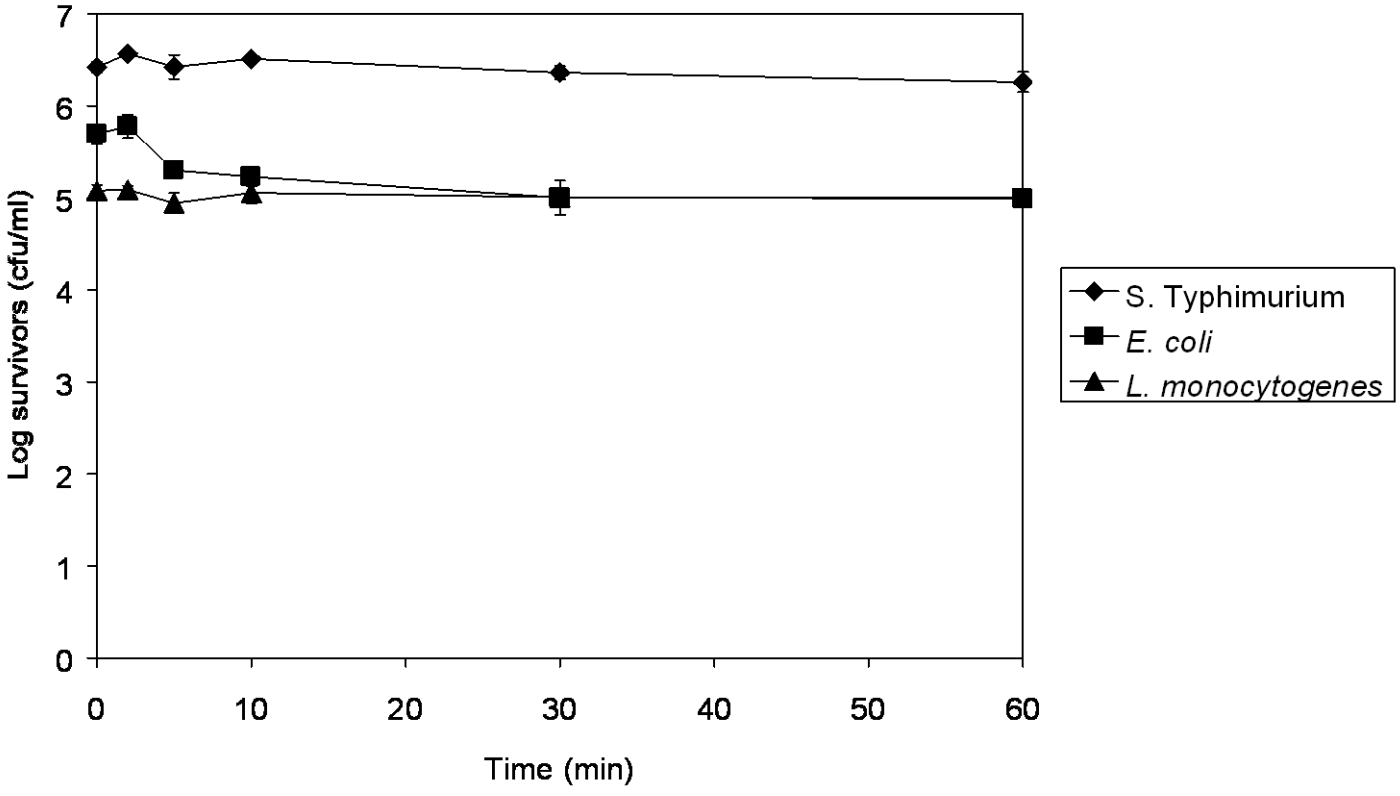


**Figure 15. The survival of *S*. Typhimurium in the presence of Rose Bengal (RB) and intense light (15,000 Lux).**

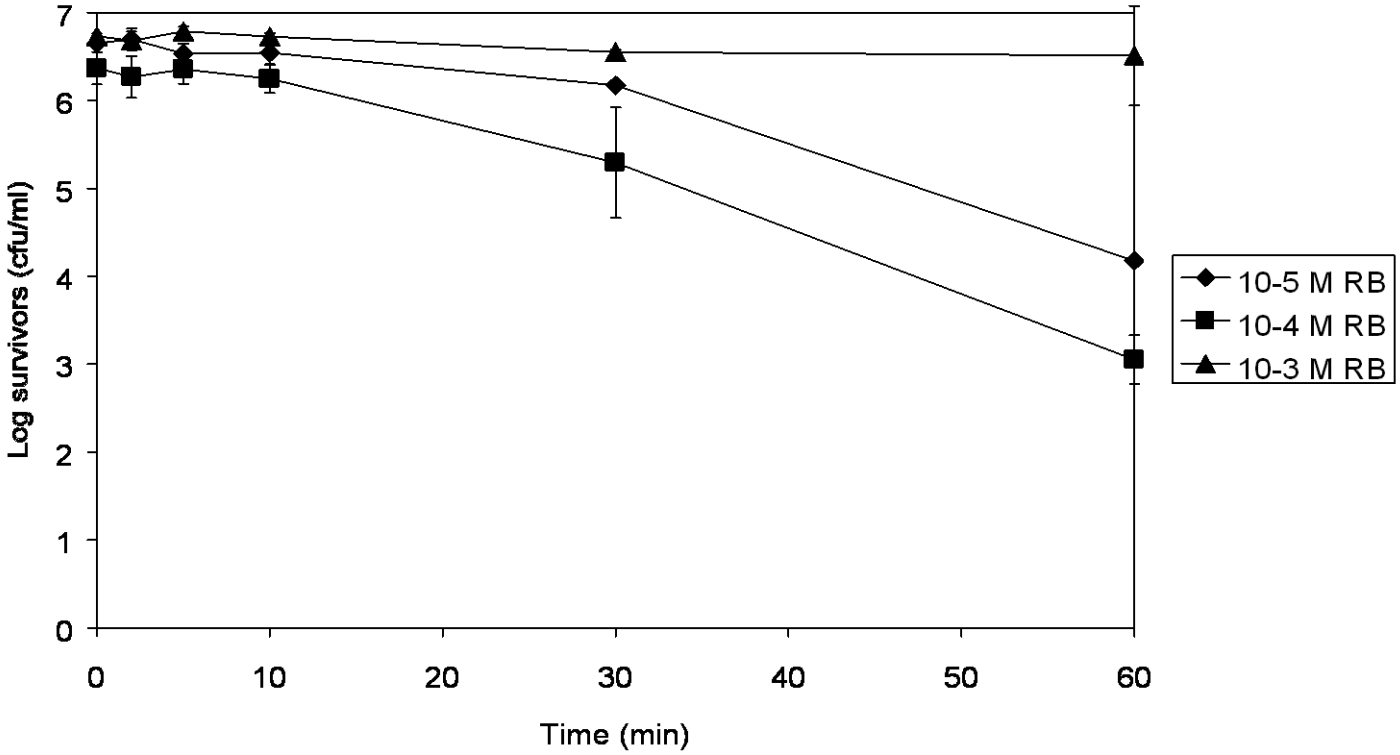



**Figure 16. The survival of *L. monocytogenes* (L. m) and *E. coli* (E. c) in the presence of Rose Bengal (RB) and intense light (15,000 Lux).**

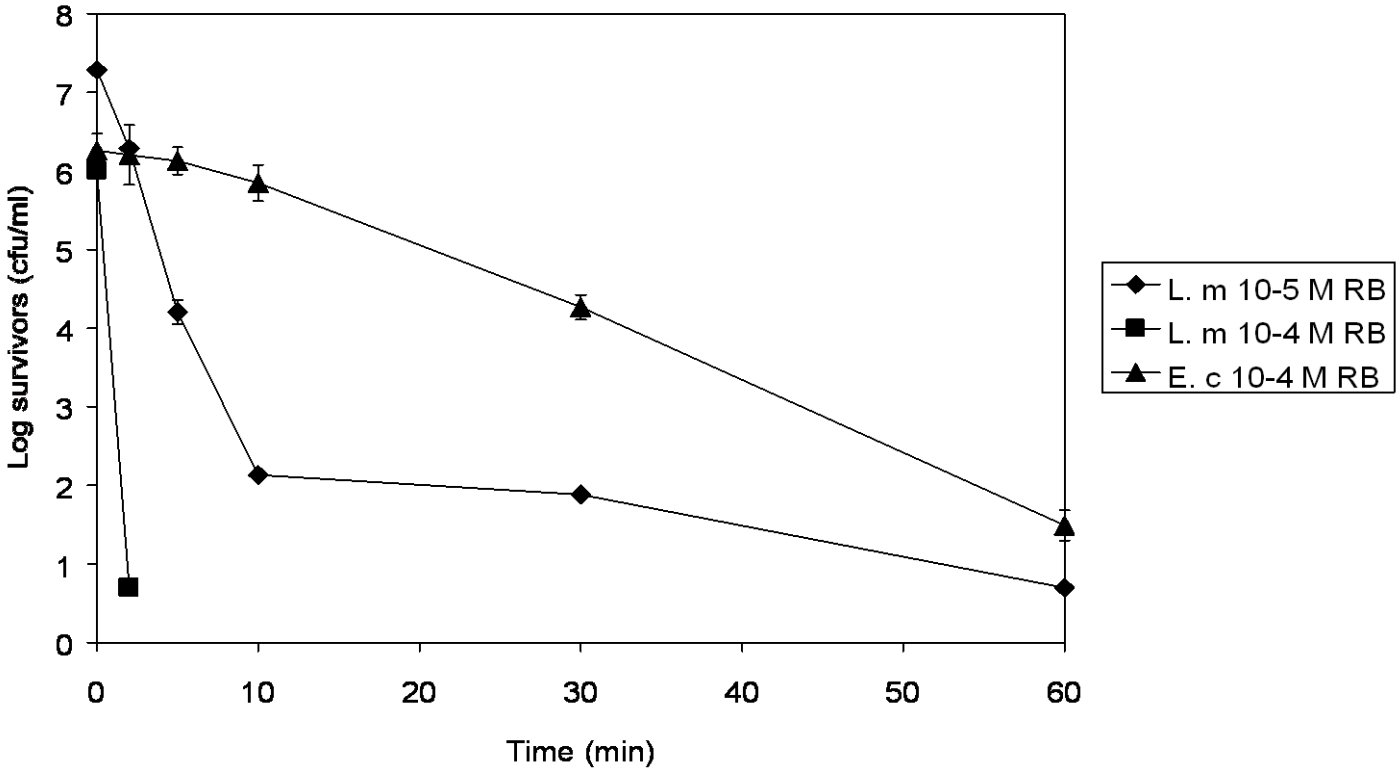


**Figure 17. The survival of pathogenic bacteria in the presence of $10^{-4}$ M Rose Bengal and intense light (15,000 Lux).**

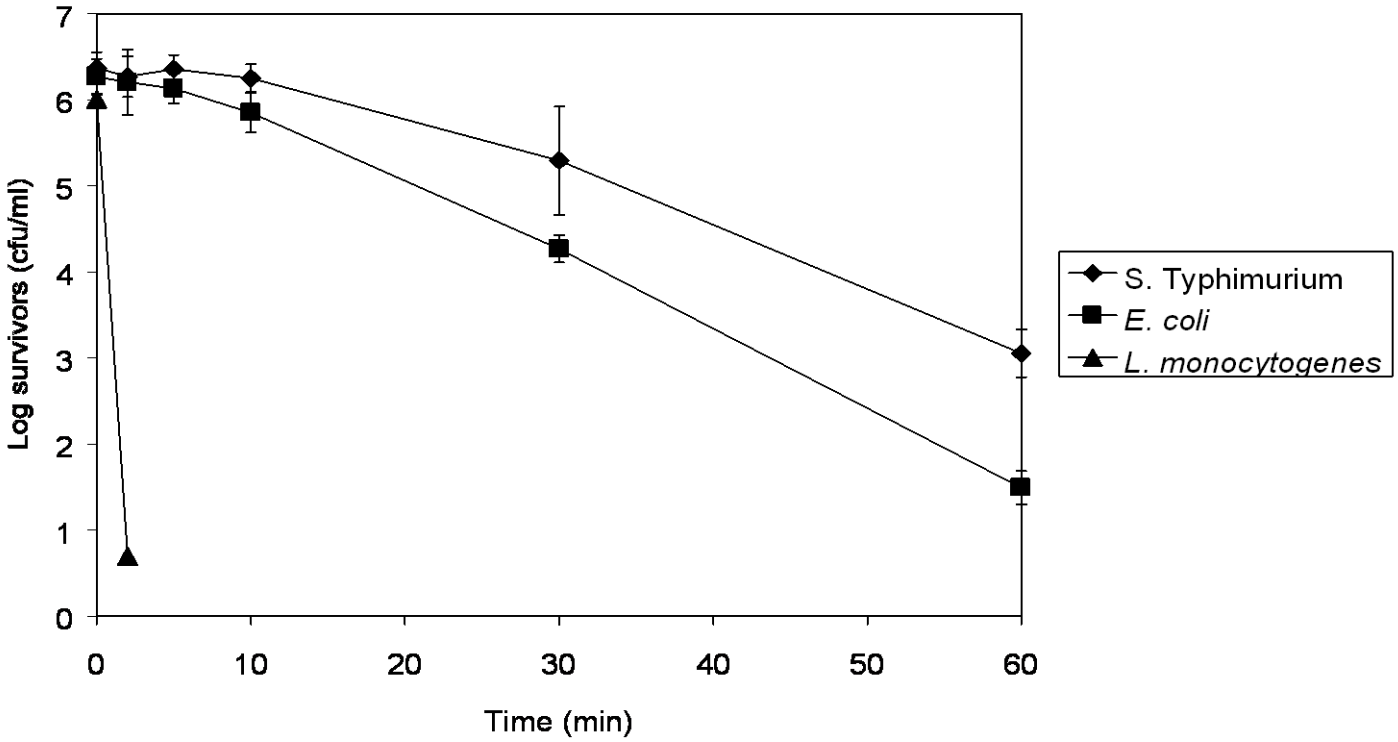



**Figure 18. The survival of pathogenic bacteria in the presence of $10^{-4}$ M Methylene Blue and intense light (15,000 Lux).**

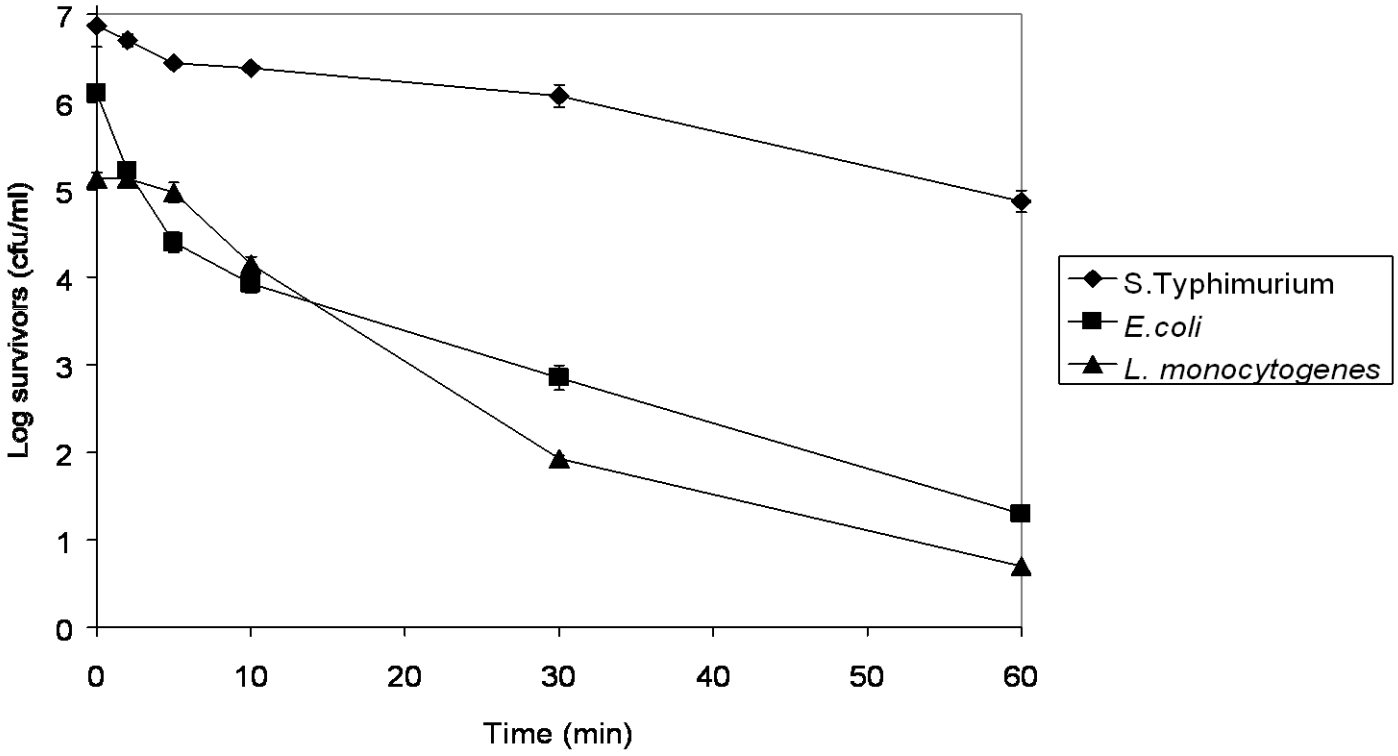


**Figure 19. The survival of pathogenic bacteria in the presence of $10^{-4}$ M Eosin Y and intense light (15,000 Lux).**

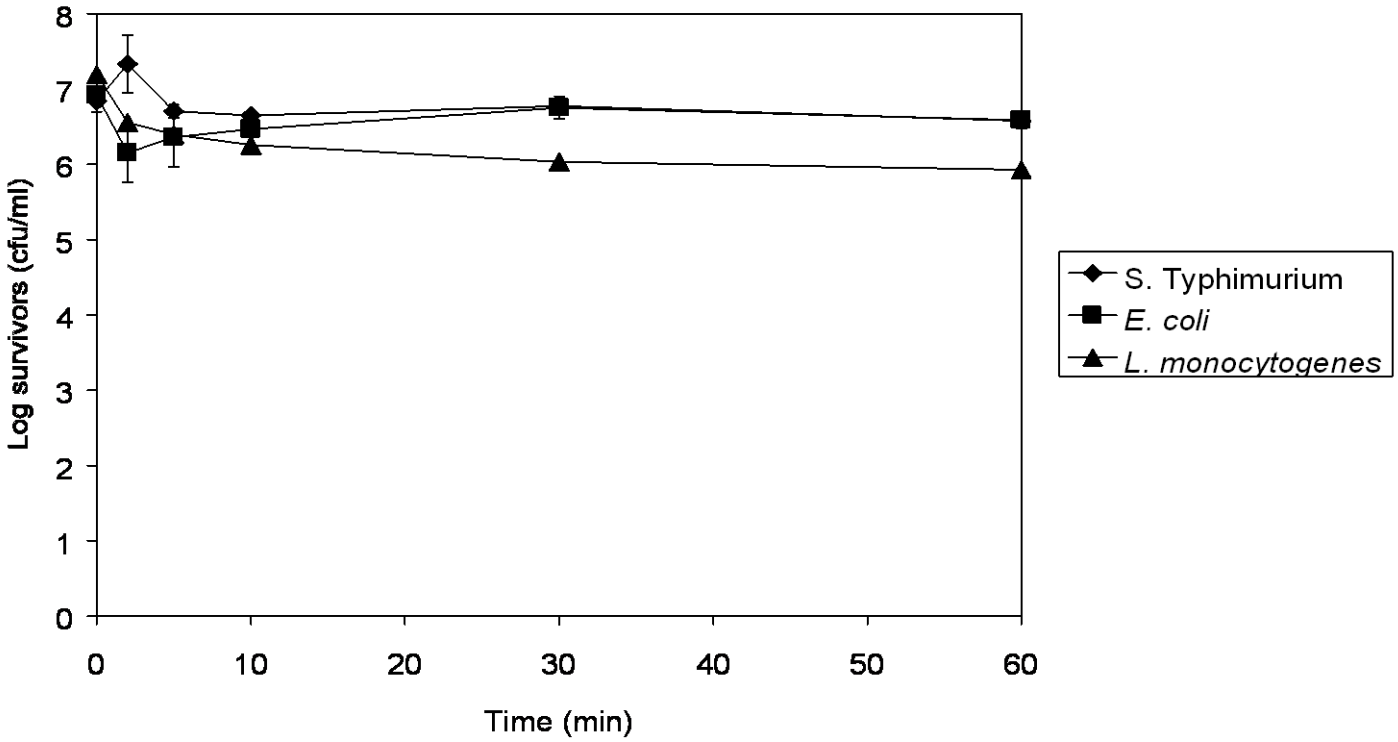



**Figure 20 a. The survival of *S*. Typhimurium in the presence of photoreactive dyes and intense light (15,000 Lux).**

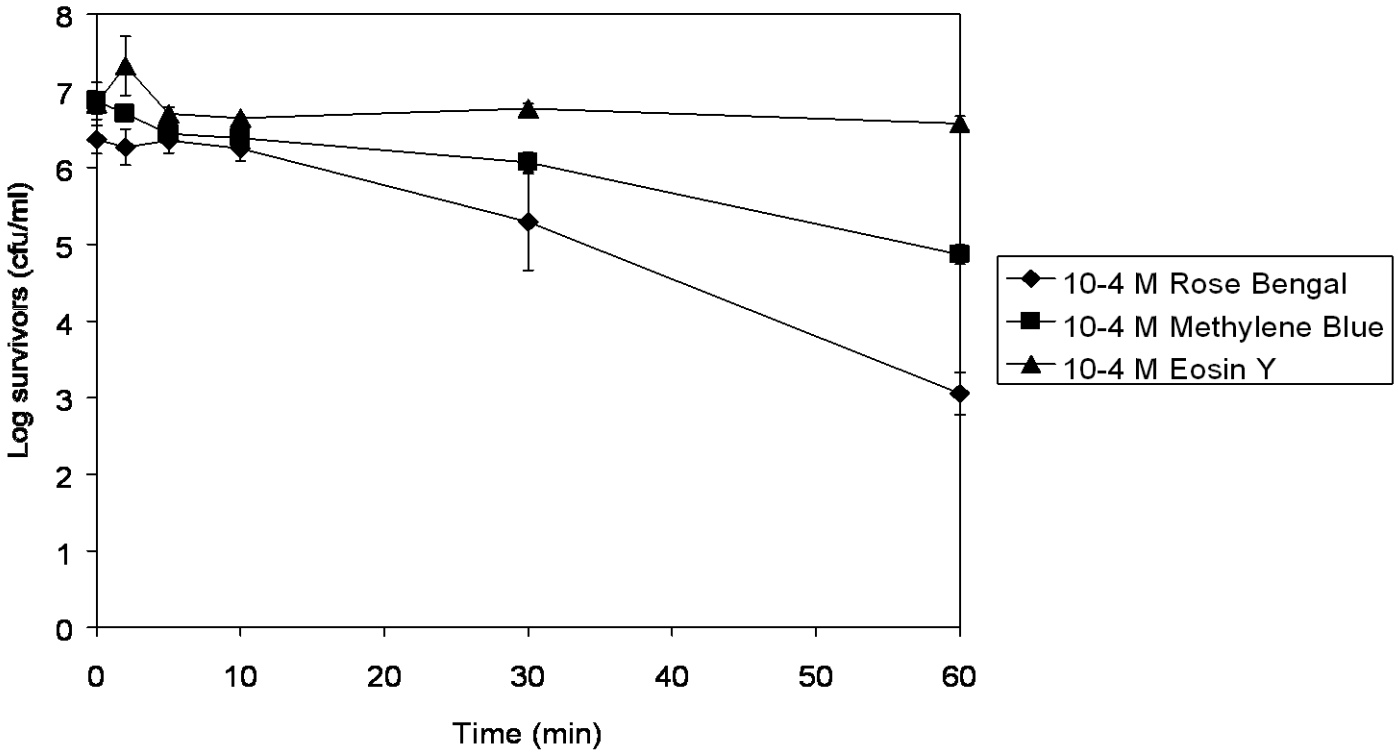


**Figure 20 b. The survival of *E. coli* in the presence of photoreactive dyes and intense light (15,000 Lux).**

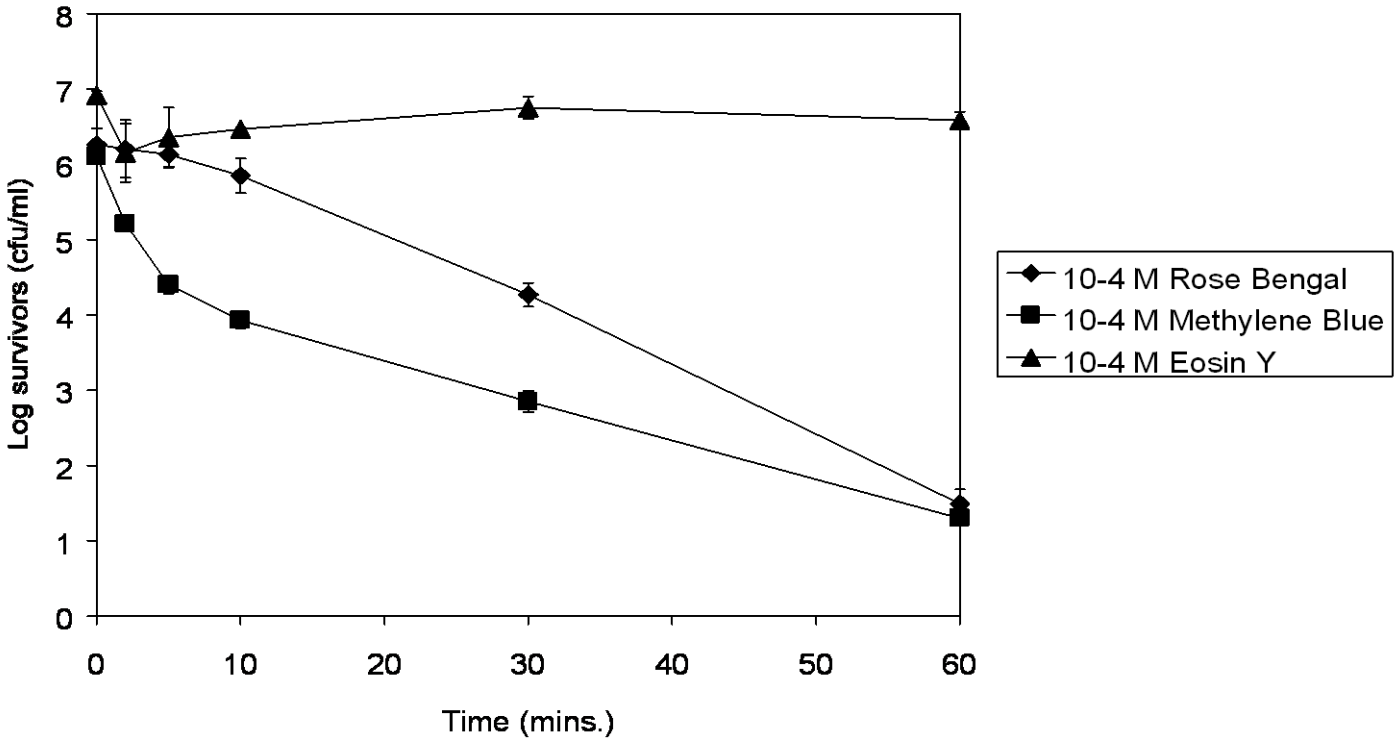



**Figure 20 c. The survival of *L. monocytogenes* in the presence of photoreactive dyes and intense light (15,000 Lux).**

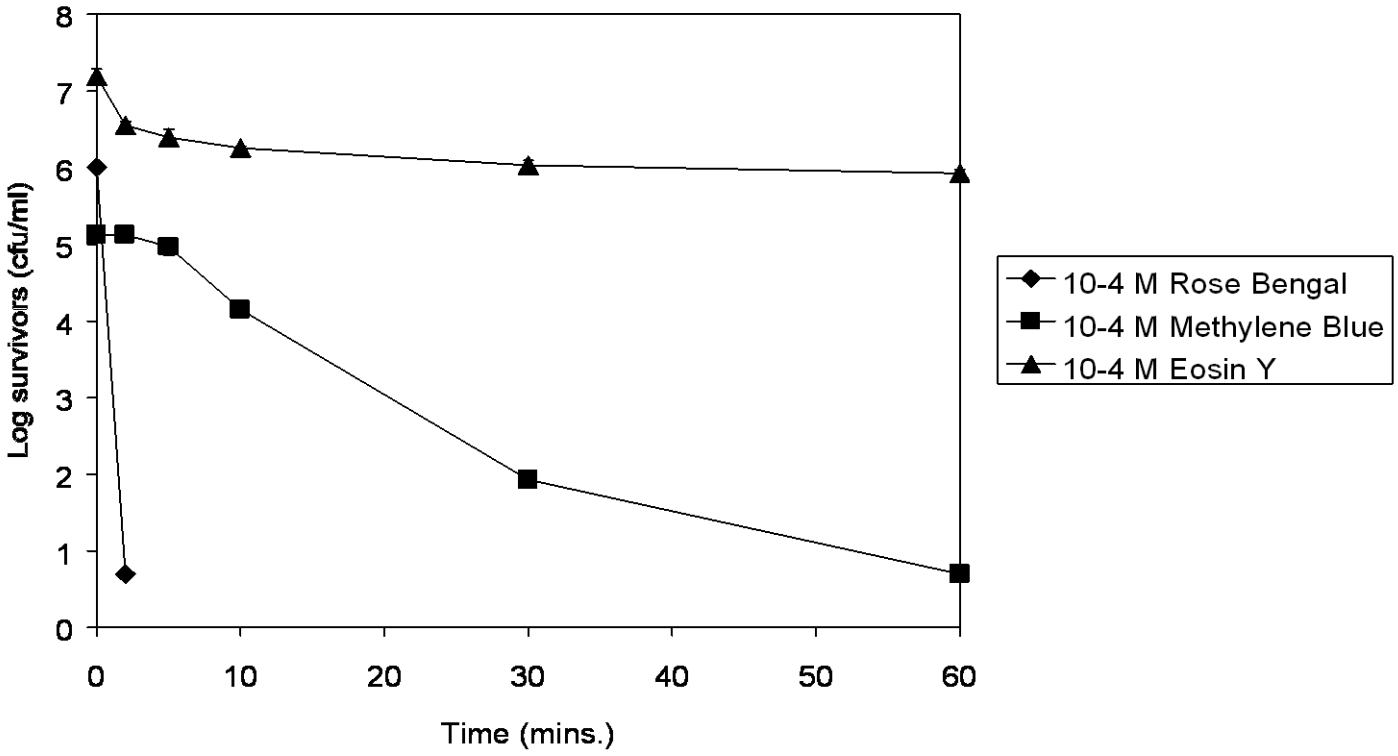


**Figure 21. The survival of *S*. Typhimurium in the presence of Rose Bengal coupons and intense light (15,000 Lux).**

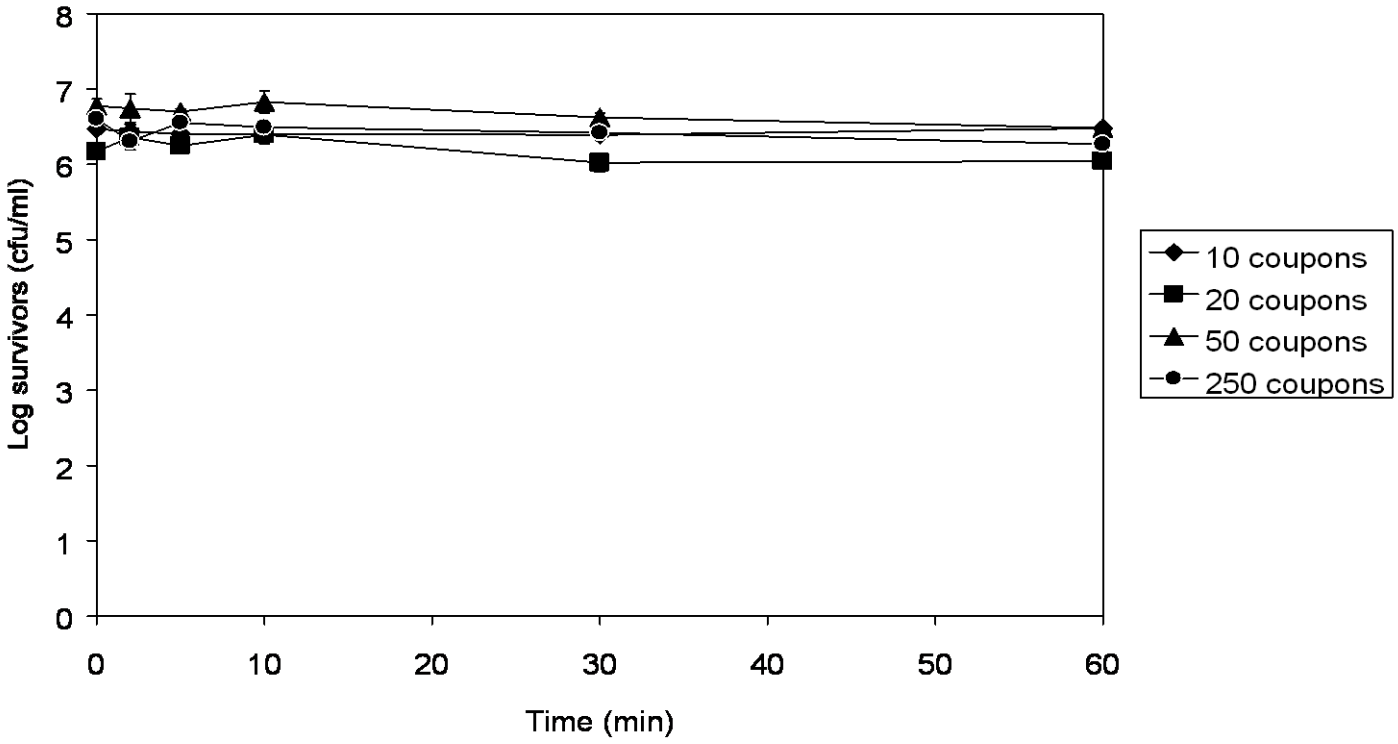



**Figure 22. The survival of *E. coli* (E. c) and *L. monocytogenes* (L. m) in the presence of Rose Bengal coupons and intense light (15,000 Lux).**

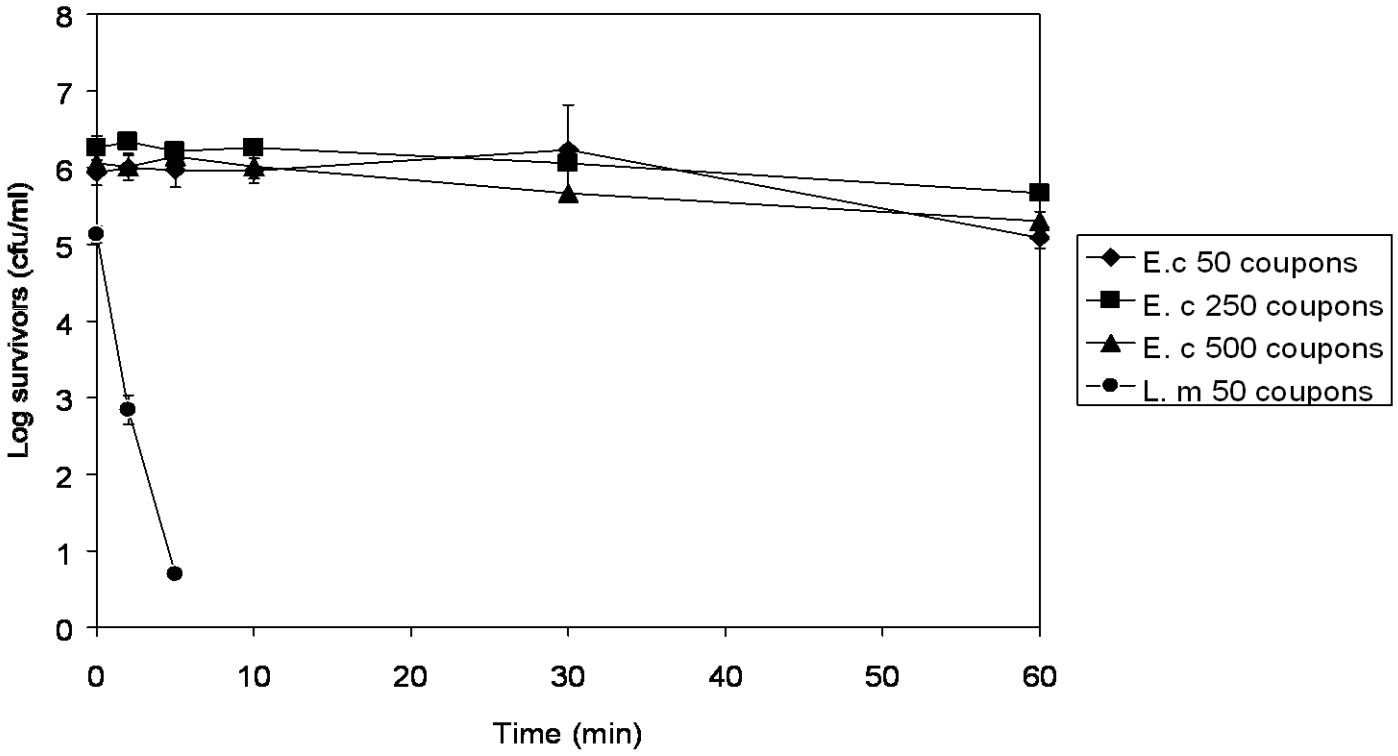


**Figure 23. The survival of pathogenic bacteria in the presence of Rose Bengal coupons and intense light (15,000 Lux).**

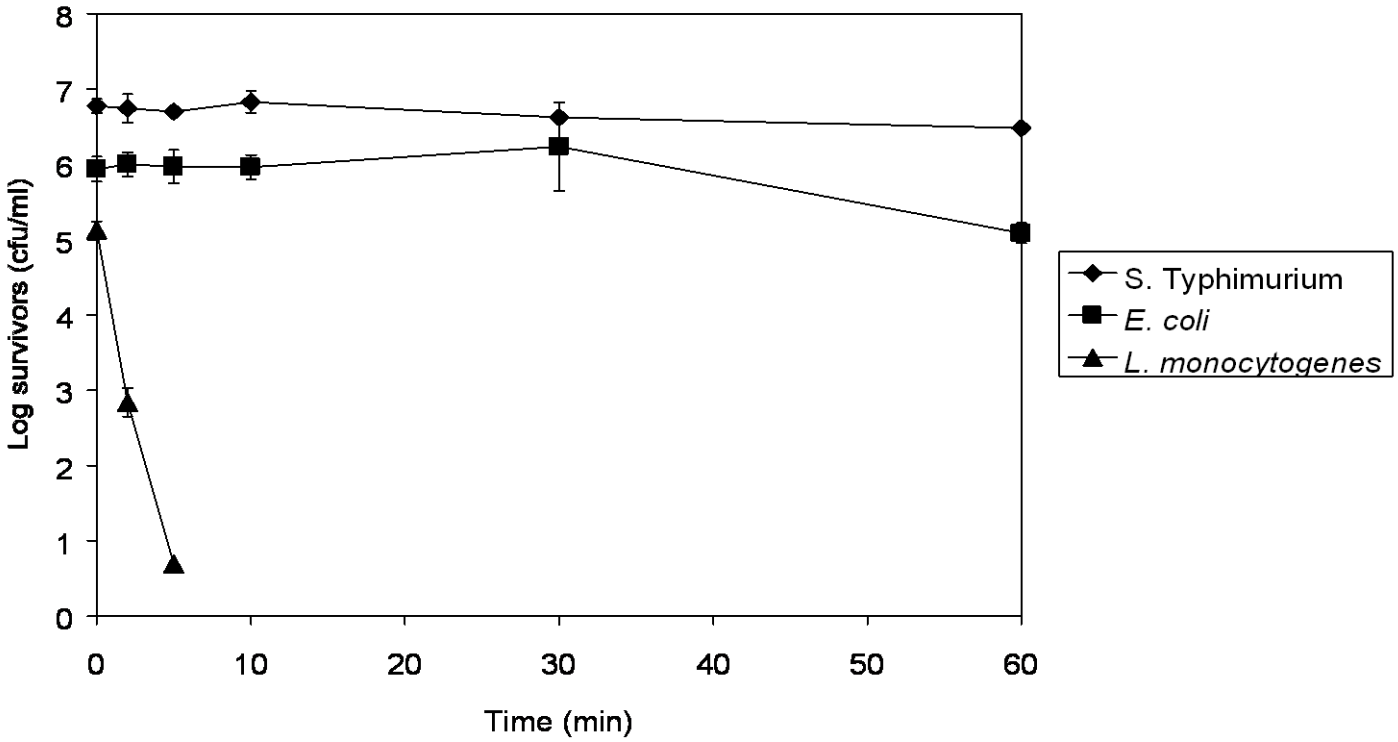



**Figure 24. The survival of *L. monocytogenes* on Iceberg lettuce in the presence of Rose Bengal coupons, 100 ppm free chlorine and intense light (15,000 Lux).**

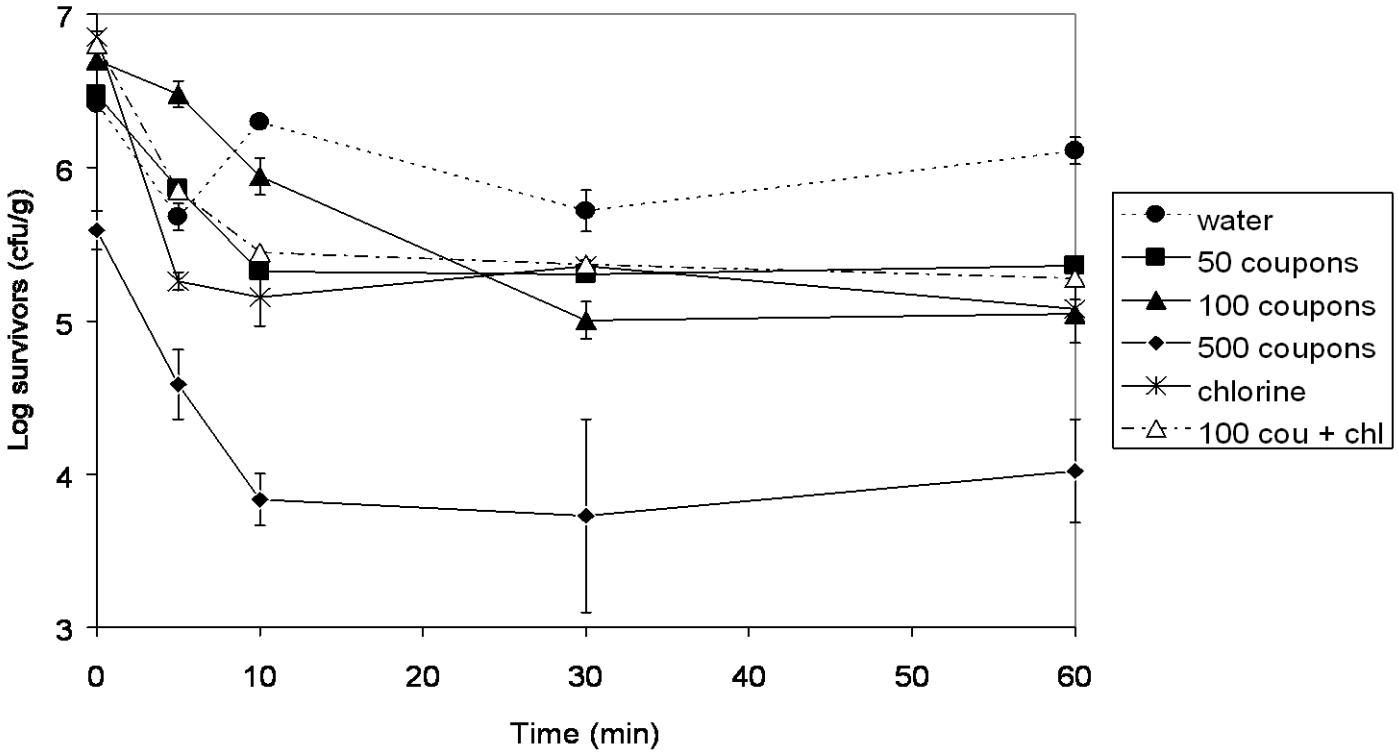


**Figure 25 a. The survival of pathogenic bacteria in the presence of $10^{-4}$ M Rose Bengal, 0.3 g/litre BSA ("clean") and intense light (15,000 Lux).**

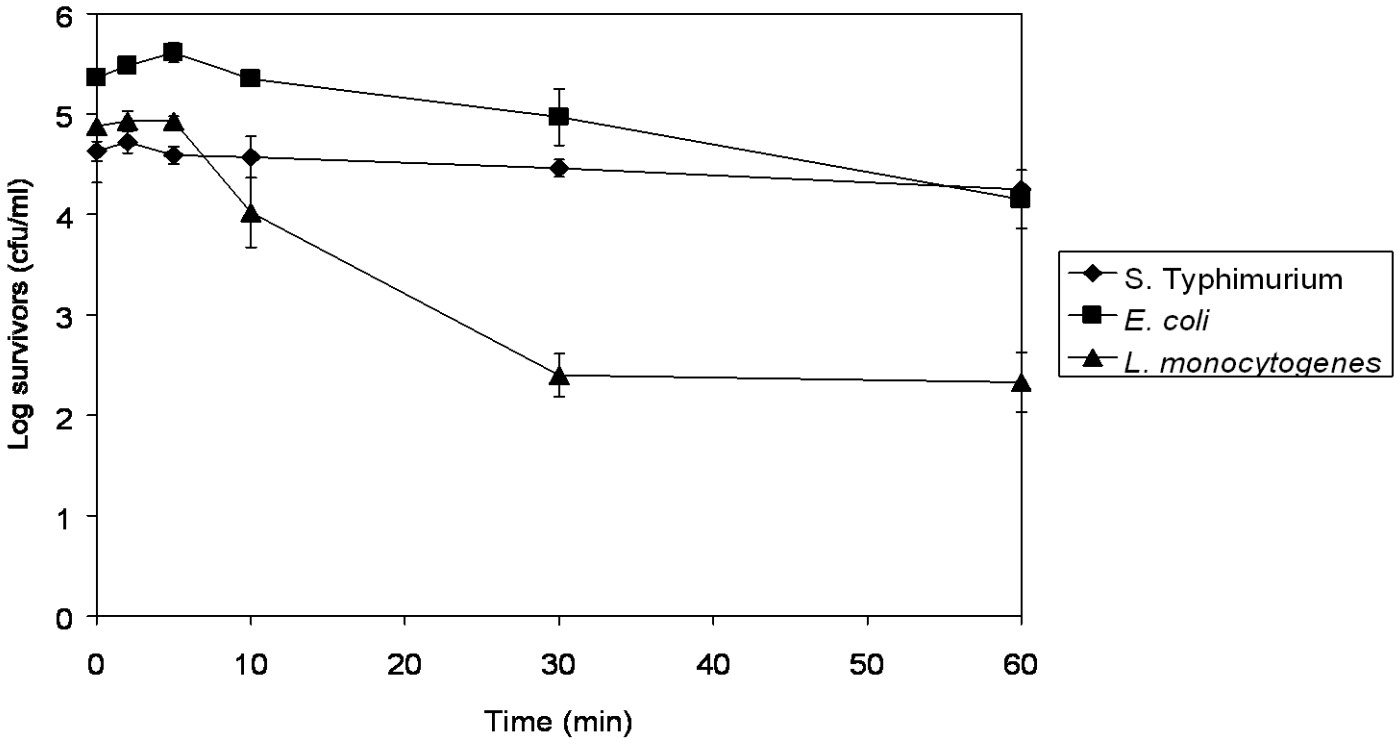
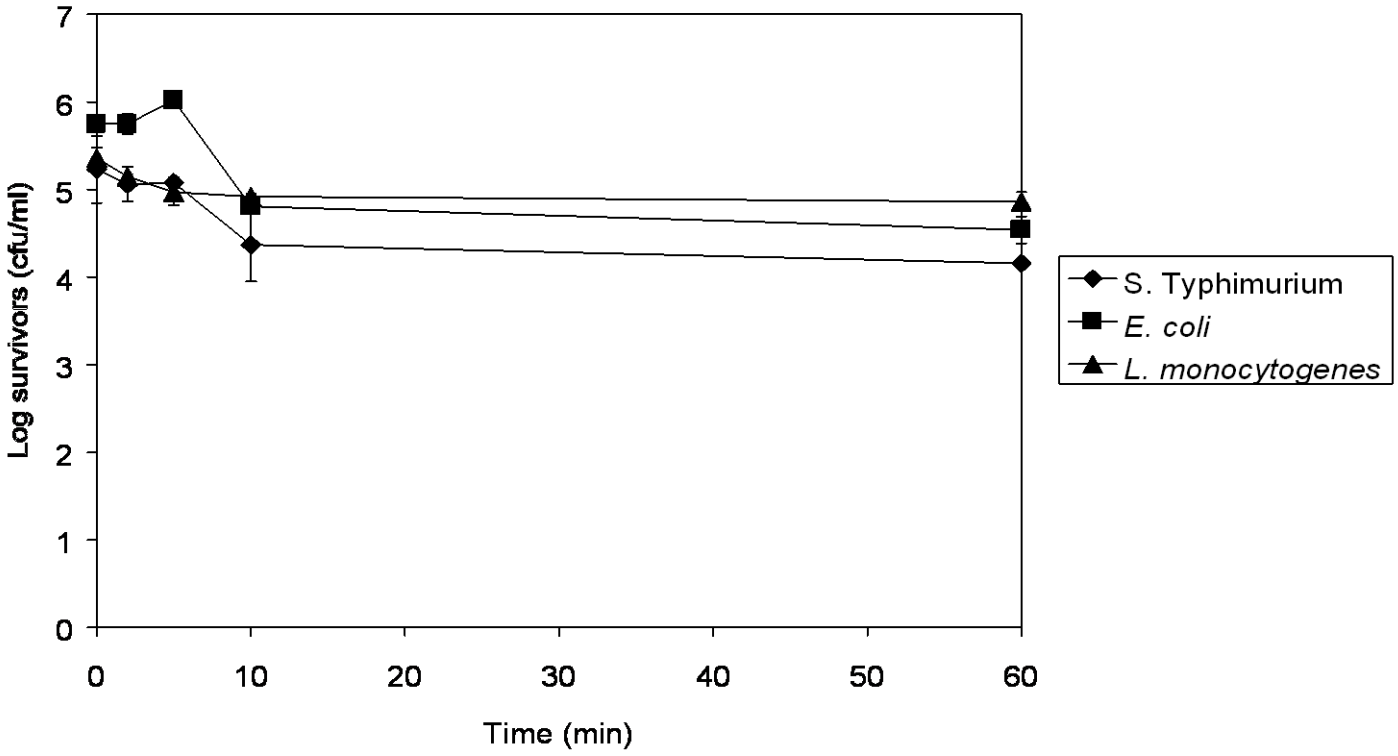



**Figure 25 b. The survival of pathogenic bacteria in the presence of $10^{-4}$ M Rose Bengal, 3.0 g/litre BSA ("dirty") and intense light (15,000 Lux).**



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