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Efficacy of Ozone and Ultrasound for Microbial Reduction in Fruit Juice

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A thesis submitted to Dublin Institute of Technology in fulfilment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

School of Food Science and Environmental Health

College of Sciences and Health

Dublin Institute of Technology

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November 2010

ABSTRACT

Concerns have arisen regarding the microbiological safety of fruit juices due to a number of outbreaks associated with pathogens. Non-thermal technologies for the inactivation of microorganisms are of increasing interest to the food industry for the control of spoilage as well as safety concerns.

The objective of this thesis was to investigate the efficacy of ultrasound and ozone treatments for control of microbial issues associated with fruit juices. Inactivation of *Escherichia coli* (ATCC 25922, NCTC 12900) using power ultrasound was found to be influenced by strain, prior acid adaptation and suspension liquid, but the effect was negated at the higher amplitude levels. Power ultrasound has potential for reducing the microbial load in liquid food systems.

Ozone was another non thermal technology applied to reduce microbial issues associated with fruit juices. The optimum ozone system control parameters of flow rate, temperature and ozone concentration resulted in a 5 log reduction (t_{5d}) in 20 min. These optimum parameters were further used to determine ozone inactivation efficacy in orange juice. The efficacy of ozone for inactivation of two strains of *E. coli* was evaluated as a function of different juice types. Fast ozone inactivation rates of *E. coli* (10^6 CFU/mL) in model orange juice (60 sec) and in orange juice with low pulp content (6 min) indicated that juice organic matter interferes with gaseous ozone efficacy. The effect of prior acid (pH 5.0) exposure of *E. coli* strains resulted in higher inactivation times in some cases by comparison with the control cells. Ozone treatment (33-40 $\mu\text{g/mL}$) of *E. coli* in apple juice achieved t_{5d} within 5 min. A significant pH effect on ozone inactivation of *E. coli* strains in apple juice was also observed. Prior mild acid stress-habituation of *Listeria* strains resulted in higher ozone inactivation times in orange juice. The t_{5d} was achieved within a 5 to 9 min range.

A product specific model was developed and validated under dynamic temperature conditions for determining the growth of *Saccharomyces cerevisiae* in ozonated apple juice. The microbial model developed resulted in accurate predictions when compared with the independent experimental set of dynamic temperatures. In the case of ozone treated apple juice, the shelf life was increased when compared with the controls at higher static storage temperatures (8, 12 and 16°C).

Combining ultrasound and ozone treatment slightly increased the inactivation rate of *E. coli* ATCC 25922 in orange juice compared to ozonation alone.

The effect of gene deletion (*soxR*, *soxS*, *oxyR*, *rpoS*, *dnaK*) towards further understanding of the ozone inactivation mechanism, indicated that mutant *E. coli* strains ($\Delta soxR$, $\Delta soxS$, $\Delta oxyR$, $\Delta rpoS$) were more susceptible to ozone treatment (6 µg/mL), signifying the important role of oxidative stress related genes in protection during ozonation. Cell lysis was not the major mechanism of inactivation observed in this study.

This study demonstrates that the use of ozone as a non-thermal technology is effective for inactivation of *E. coli*, *Listeria* strains and *S. cerevisiae* in fruit juice and could be used as an alternative to traditional thermal pasteurisation. However, the effect of ozone on sensory and nutritional quality retention of liquid foods such as fruit juice should be considered before its use as a preservation technique.

Declaration page

I certify that this thesis which I now submit for examination for the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

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Signature _____ Date _____

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Abbreviations

°C	degrees Celsius
ATP	adenosine triphosphate
CFU/mL	Colony forming unit per millilitre
g	Gram
HCl	hydrochloric acid
K	kelvin
Hz	hertz
L	litre
M	molar
NAD	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
ppm	parts per million
W/mL	watts per milliliter

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Chapter 1 INTRODUCTION

Food spoilage is a major cause of economic loss in the food industry. It may be due to the growth and activity of microorganisms, insects or by the action of food enzymes. Microorganisms are the main agents responsible for food spoilage as well as causing food poisoning. It is estimated that one-third of the food world production is lost by microbial activity, which is an economically significant problem for manufacturers, retailers and consumers (Lund et al., 2000). Because of the particular environmental conditions in a food system, only a small proportion of microorganisms present will be able to grow rapidly and cause spoilage.

Juice is a fluid naturally rendered from plant tissue. Fruit juice consumption in Europe, Australia, New Zealand and the USA has increased in recent years. According to food statistics, consumption of fruit juices in Canada is 52.6 litres, 42.8 litres in the USA, 38.6 litres in Germany, 37.3 litres in Australia and 15.1 litres per person in Ireland per year (Nationmaster.com).

A number of food safety and food spoilage concerns are associated with fruit juices. Fruit juice producers have traditionally relied on the acidity of their products to assure microbiological safety. However, recent outbreaks of foodborne illness related to these products have increased the concerns of public safety authorities.

Escherichia coli is a common organism which is present inside the human gastro intestinal tract. In many cases it is a harmless organism, but some strains such as O157:H7 are pathogenic with potentially fatal health outcomes. This strain produces a powerful toxin that can cause severe illness. Outbreaks of *E. coli* O157:H7 from the consumption of apple cider have been documented; unpasteurised, unpreserved apple cider produced with unwashed apples was the vehicle implicated in an outbreak of *E. coli* O157:H7 causing diarrhoea and haemolytic uremic syndrome. *E. coli* O157:H7 was reported to survive 20 days in refrigerated apple cider (Besser et al., 1993). In 1996, two

outbreaks of haemolytic uremic syndrome from *E. coli* O157:H7 in unpasteurised apple cider were reported (Centre for Disease Control and Prevention (CDC), 1997). Foods that have been identified as sources of contamination include ground beef, venison, sausages, dried (non-cooked) salami, unpasteurised milk and cheese, unpasteurised apple juice and cider (Cody et al., 1999), orange juice, alfalfa and radish sprouts (Breuer et al., 2001), lettuce, spinach, and water (Friedman et al., 1999). In 2001, in response to such outbreaks, the U.S. Food and Drug Administration (FDA) published a final rule requiring fruit juice producers to process juice to achieve a 5-log reduction in critical pathogen levels (USFDA, 2001). Recently, in January 2010, a multistate outbreak of *E. coli* O157:H7 infections associated with beef was reported (<http://www.cdc.gov/ecoli/2010/index.html>).

Yeasts predominate in spoilage of acid food products as they have the ability to exhibit good growth at low pH, high sugar concentration and low water activity and resist inactivation by heat processing which enables them to grow or survive in fruit or fruit products (Put and de Jonge, 1980; Stratford et al., 2000). *Saccharomyces cerevisiae* is one of the most important yeasts causing spoilage of fruit juices and soft drinks (Fleet, 1992; Deak and Beuchat, 1996; Pitt and Hocking, 1997; Barnett et al., 2000). *Alicyclobacillus acidoterrestris* is another spoilage organism associated with fruit juices, in particular apple, pear, orange, peach and white grape juices (Jensen, 2000). This organism has also caused problems with juice blends, fruit juice containing drinks, tomato juice and canned tomatoes.

Heat treatment is the most widely used method for preservation of fruit and vegetable juices due to its effectiveness for microbial inactivation (Tribst et al., 2008) although it has certain disadvantages on product nutritional and organoleptic values (Vachon et al., 2002; Pathanibul et al., 2009). While heating remains the most utilised and effective microbial inactivation processing technique, it has become clear that it would be

valuable with respect to food safety and quality to design alternative non-thermal inactivation technologies/processes that target the elimination of spoilage and food poisoning microorganisms from the most often contaminated foods (Gould, 2001).

There is increasing consumer demand for a wider range of less heavily preserved/processed foods of improved quality which exhibit longer shelf-lives. This demand for minimal changes in the organoleptic and nutritional values has led to increasing interest in non-thermal technologies for the inactivation of microorganisms (Diels et al., 2005).

Novel non-thermal technologies have the ability to inactivate microorganisms at ambient or near ambient temperatures, thereby avoiding the deleterious effects that heat may have on the flavour, colour and nutritive value of foods (Barbosa-Cánovas et al., 1999).

1.1 Non-thermal techniques

The microbial stability and safety as well as the sensory and nutritional quality of most foods are based on application of combined preservative factors that are known as hurdles (Leistner, 2000). The hurdle approach (Leistner, 1978) is used to produce minimally processed food by applying several sub-lethal treatments to achieve microbial stability, rather than focusing only on one lethal preservation method. The microbial stability is achieved by combining the hurdles to increase destruction of the microbial cytoplasmic membrane as well as preventing cell repair of survivors from treatments (e.g. pulsed electric field [PEF]), such as sub-lethally injured cells or bacterial endospores (Leistner, 2000; Galvez et al., 2007). The application of a hurdle approach (PEF and bacteriocins) has been reported by McNamee et al. (2010) to enhance microbial inactivation and preserve orange juice. Similarly, combined ozone and organic acid treatment was reported to be effective for reducing pathogen levels on enoki mushroom (Yuk et al., 2007). Hurdle technology involves the application of a

deliberate and intelligent combination of non-thermal processes, which may also be combined with conventional preservation factors (e.g. pH, temperature, water activity, redox potential) (Leistner, 1999).

A current trend is toward the use of procedures that deliver food products which are less “heavily” preserved, higher in quality, perceived as being more “natural”, contain less additives, and are nutritionally healthier (Leistner and Gould, 2002). Procedures can be bacteriocidal (e.g. ultrasound, high hydrostatic pressure, ozone) or bacteriostatic (e.g. low temperature). However, most of the techniques act by inactivating microbes (e.g., the application of high hydrostatic pressure, high-voltage electric pulses, high intensity laser and noncoherent light pulses) (Sun-Young Lee, 2004). Table 1.1 lists the principle hurdle approaches available.

Table 1.1: Examples of hurdles used to preserve foods

Type of hurdle	Examples
Physical hurdles	<p>electromagnetic energy (microwave, radio frequency, pulsed magnetic fields, high electric fields, ultraviolet radiation, photodynamic inactivation, ionising radiation), high temperatures (blanching, pasteurisation, sterilisation, evaporation, extrusion, baking, frying),</p> <p>low temperature (chilling, freezing), Aseptic packaging, modified atmospheres, packaging films (including active packaging, edible coatings), ultra-high pressures, ultrasonication</p>
Physico-chemical hurdles	<p>Carbon dioxide, ethanol, lactic acid, lactoperoxidase, low pH, low redox potential, low water activity,</p> <p>Maillard reaction products, organic acids, oxygen, ozone, phenols, phosphates, salt, smoking, sodium nitrite/nitrate, sodium or potassium sulphite, spices and herbs, surface treatment agents</p>
Microbially derived hurdles	<p>Antibiotics (ampicillin, tetracycline, ofloxacin, Sulfamethoxazole and trimethoprim), bacteriocins (nisin, pediocin PA-1, lactacin 3147, 481, macedocin, enterocin 4, Lincocin M-18, Piscicolin 126, Leucocin A, Lactocin 705), competitive flora (Lactic acid bacteria flora), protective cultures</p>

(Ahvenainen et al., 2002) *Adapted from Sun-Young Lee (with some modification), 2004*

Non-thermal processing technologies include the application of high-voltage pulsed electric fields (PEF), high hydrostatic pressure, ultraviolet light (UV), high intensity light pulses (HILP) and manothermosonication (MTS) (Butz and Tauscher, 2002; Gould, 2001). Ultrasound and ozone are two promising non-thermal technologies for processing fruit juice and other food systems. These technologies are described in detail in the sections below.

1.2 Ultrasound

Power ultrasounds are pressure waves with a frequency of 20 kHz or more (Butz and Tauscher, 2002). Normally, ultrasound equipment uses frequencies from 20 KHz to 10 MHz (Piyasena et al., 2003). Ultrasound can be classified into low intensity ultrasound with a frequency range of 5-10 MHz and high intensity ultrasound with a range of 20-100 KHz (McClements, 1995; Mason, 1998; Lee et al., 2003). Higher power ultrasound at lower frequencies is referred as power ultrasound (Piyasena et al., 2003) which has been recognised as a promising processing technology to replace or complement conventional thermal treatment of liquids in the food industry. The advantages of ultrasound over heat pasteurisation include: reduction of flavour loss, especially in sweet juices: greater homogeneity; and possible energy savings.

1.2.1 Generation of power ultrasound

A power ultrasound system consists of three basic parts (Mason, 1998):

Generator: this is an electronic or mechanical oscillator that needs to be rugged, robust, reliable and able to operate with and without load.

Transducer: this is a device for converting mechanical or electrical energy into sound energy at ultrasonic frequencies.

Coupler: the working end of a system that helps transfer the ultrasonic vibrations to the substance being treated (usually liquid).

There are three main types of transducer; liquid driven, magnetostrictive and piezoelectric. Liquid driven transducers are effectively a liquid whistle where a liquid is forced across a thin metal blade causing it to vibrate at ultrasonic frequencies: rapidly alternating pressure and cavitation effects in the liquid generate a high degree of mixing (Leadley and Williams, 2006). Magnetostrictive transducers are electrochemical devices that use magnetostriction, an effect found in some ferromagnetic materials which change dimension in response to the application of an electromagnetic field (Leadley and Williams, 2006).

Piezoelectric transducers are electrostrictive that utilise ceramic materials such as lead zirconate titanate or barium titanate and lead metaniobate which are capable of producing piezoelectric vibration when subjected to an alternate electromagnetic field (Leadley and Williams, 2006). Piezoelectric transducers are the most common devices employed for the generation of ultrasound and can be used over the range of ultrasonic frequencies (Mason and Lorimer, 2002).

1.2.2 Mechanism of ultrasound action

Microbial cell inactivation is generally thought to occur due to three different mechanisms: cavitation, localised heating and free radical formation.

Ultrasonic waves propagating in a liquid medium cause cavitation, which has been attributed as the main mechanism responsible for cell disruption (Sala et al., 1995).

Microorganisms can withstand high pressures but are incapable of withstanding the quick alternating pressures produced during cavitation (Earnshaw et al., 1995; Sala et al., 1995). Cavitation is the formation, expansion and implosion of microscopic gas bubbles in the liquid as the molecules in the liquid absorb ultrasound energy. Two different types of cavitation i. e. transient and stable are reported to have different effects. Stable cavitation occurs due to the oscillations of ultrasound waves, which cause tiny bubbles to be produced in the medium. It takes thousands of oscillatory

cycles of the ultrasound waves to cause the bubble to increase in size. As the ultrasonic wave passes through the medium, it causes bubbles to vibrate, causing strong currents to be produced in the surrounding liquid. Other small bubbles attracted to the sonic field add to the creation of micro currents. This effect provides a substantial force, which rubs against the surface of cells, causing them to shear and breakdown without any collapse of the bubbles. The pressures produced on the cell membrane disrupt its structure and causes the cell wall to break down.

During transient cavitation, bubbles rapidly increase in size within a few oscillatory cycles. The larger bubbles eventually collapse causing localised high pressures and temperatures (up to 100 MPa and 5000 K) to be momentarily produced. It is believed that the cellular stress is caused by the cavitation effect, which occurs when the bubble collapses. The pressures produced during bubble collapse are sufficient to disrupt cell wall structures, eventually causing them to break, leading to cell leakage, cell disruption and eventually death or sub-lethal injury (Leadley and Williams, 2006).

Free radical formation is another proposed mode of microbial inactivation. The very high temperatures (several thousand K) and pressures (several hundred atmospheres) of collapsing gas bubbles lead to the thermal dissociation of water vapor into $\cdot\text{OH}$ radicals and $\cdot\text{H}$ atoms (Riesz and Kondo, 1992). The primary target site of free radicals ($\cdot\text{OH}$, $\cdot\text{H}$, hydrogen peroxide) is the DNA in the bacterial cells. The action of the free radicals causes breakage along the length of the DNA and fragmentation occurs where small DNA fragments are produced which are susceptible to attack by the free radicals produced (Leadley and Williams, 2006).

1.2.3 Factors affecting cavitation

The frequency and amplitude of ultrasound waves, as well as temperature and viscosity of the liquid medium influence the degree of cavitation (Sala et al., 1995). The frequency of ultrasound is an important parameter influencing bubble size (Suslick,

1989). At lower frequencies such as 20 kHz, the bubbles produced are larger in size and upon collapse higher energies are produced. At higher frequencies, bubble formation becomes more difficult and at frequencies above 2.5 MHz cavitation does not occur at all (Alliger, 1975). With increasing temperature, cavitation bubble formation increases rapidly, but the intensity of collapse is reduced.

Bacterial cells differ in their sensitivity to ultrasound treatment (Alliger, 1975). The effects of ultrasound on various microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E. coli* have been studied (Scherba et al., 1991). Gram positive cells were reported to be more resistant to ultrasound than Gram negative cells (Drakopoulou et al., 2009). Conversely, other studies reported no significant difference in percentage killing of Gram positive and Gram negative cells by ultrasound (Scherba et al., 1991). Spherical shaped cells (cocci) are more resistant to ultrasound than rod shaped cells (Alliger, 1975).

The characteristics of the food or substrate can influence the effectiveness of the ultrasound treatment applied. Differences in effectiveness may be due to intrinsic effects of the environment on the ultrasound action (cavitation) or due to changes in ultrasound penetration and energy distribution (Leadley and Williams, 2006). It has been found that the resistance of bacteria to ultrasound treatment was higher when treated in real food systems than when treated in microbiological broths (Lee et al., 1989).

1.2.4 Application of ultrasound

Power ultrasound is used as a process intensification tool in a number of unit operations in fruit juice processing such as cleaning, extraction, homogenisation, emulsification, sieving, filtration, crystallisation and pasteurisation (Table 1.2). High energy ultrasound has been applied for degassing of liquid foods, for the induction of oxidation/reduction reactions, for extraction of enzymes and proteins, for enzyme inactivation and for the

induction of nucleation for crystallisation (Roberts, 1993; Thakur and Nelson, 1997; Villamiel and de Jong, 2000).

Due to its effects on cell viability, one of the main applications of ultrasound is to produce a microbial population reduction on its own, with several studies pointing to its suitability: A 4-log reduction in viable cell count was observed when *Salmonella* species were subjected to ultrasound of 160 kHz at a power of 100 W for 10 min in peptone water (Lee et al., 1989). Ugarte- Romero (2006) achieved a 5 log reduction of *E. coli* with power ultrasound in apple cider. Dehghani (2005) investigated the impact of sonication as a disinfection method for determining the effectiveness of ultrasound waves on the inactivation of *E. coli*, and showed a strong influence of ultrasound on the rate of *E. coli* disruption in water. D'Amico et al. (2006) studied the inactivation of microorganisms in milk and apple cider and concluded that ultrasound technology was a promising processing alternative for the reduction of microorganisms in liquid foods. However, ultrasonic treatment (20 kHz and amplitude of 117 μm) at ambient temperature was not very effective against *L. monocytogenes* (Pagan et al., 1999) giving a decimal reduction time of 4.3 min. Hulsen (1999) and Alliger (1975) reported that Gram-negative, rod shape bacteria were more sensitive than Gram-positive, coccus-shaped bacteria.

Lee et al. (2009) concluded the combination of lethal factors (heat and/or sonication, with and without pressurisation) could significantly shorten the treatment time needed to achieve a 5-log reduction in the survival count of *E. coli* K12. Wang et al. (2010) reported that, *Alicyclobacilli* had a higher resistance to ultrasonic treatments in apple juice than in buffer indicating that resistance to ultrasound varied significantly depending on their environment. Adekunle et al. (2010) indicated sonication alone at moderate temperatures can achieve the desired 5 log reductions in yeast cells.

Table 1.2: Effects of power ultrasound

Effect on living cells	Stimulation of activity Sonochemical destruction
Effect on enzymes	Stimulation of activity Controlled denaturation
Effect of 'jet' impact on surface	Improved impregnation Meat processing Crystallisation and freezing
Miscellaneous applications	Emulsification Filtration and drying Rice grain treatment

(Mason et al., 1996)

1.2.5 Effect on food quality

Ultrasound processing has either desirable or detrimental effects on nutritional properties and quality of food. Valero et al. (2007) studied the effects of ultrasonic treatments in orange juice processing and reported no detrimental effects on the quality attributes of orange juice. Cruz et al. (2007) reported an enhancement of green colour in thermosonicated watercress samples. Tiwari et al. (2008) reported a slight increase (1–2%) in the anthocyanin content of sonicated strawberry juice at lower amplitude levels and treatment times. Tiwari et al. (2009) reported a maximum degradation of 5 % in the ascorbic acid content of orange juice when sonicated at high acoustic energy density (0.81 W/mL) and treatment time (10 min). Non enzymatic browning of manothermosonicated orange juice (D'Amico et al., 2006) has been reported. Such darkening of juice has been reported to be influenced by particulate fractions. Ultrasound was reported to inactivate pectin methyl esterase (PME) in tomato juice and

orange juice (López et al., 1998; Vercet et al., 1999; Kuldiloke, 2002; Vercet et al., 2002).

1.3 Ozone

Ozone (O_3) is a triatomic form of oxygen (Figure 1.1) and is characterised by a high oxidation potential that conveys bactericidal and viricidal properties (Burlison et al., 1975; Horvath et al., 1985; Kim et al., 1999).

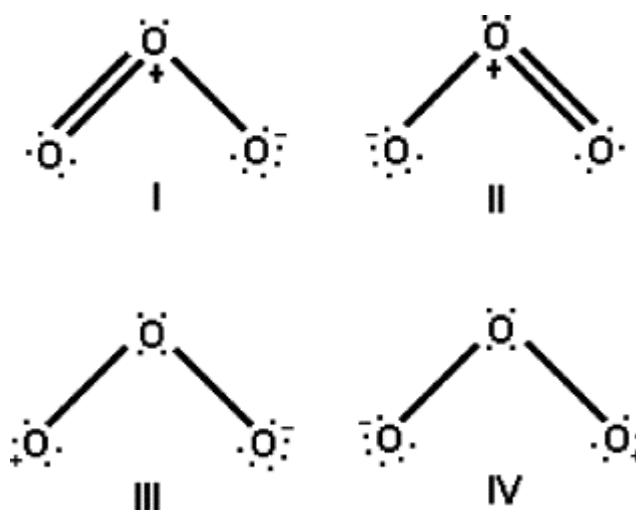


Figure 1.1: Resonance structures of the ozone molecule (Oehlschlaeger, 1978). *Adapted from Guzel-Seydim et al., (2004)*

Ozone results from the rearrangement of atoms when oxygen (O_2) molecules are subjected to high-voltage electric discharge. The product is a bluish gas with pungent odour and strong oxidising properties (Muthukumarappan et al., 2000).

Ozone inactivates microorganisms through oxidation and residual ozone spontaneously decomposes to nontoxic products (i.e. oxygen) making it an environmentally friendly antimicrobial agent for use in the food industry (Kim et al., 1999). The strong biocidal characteristics of ozone are due to a combination of its high oxidising potential and its ability to diffuse through biological membranes (Hunt and Marinas, 1997). The effects of ozone on various microorganisms have received much

attention because of its increasing use in water and sewage disinfection (Katzenelson and Biedermann, 1976; Boyce et al., 1981; Glaze, 1987). Ozone is a powerful broad-spectrum antimicrobial agent that is active against bacteria, fungi, viruses, protozoa and bacterial and fungal spores (Khadre et al., 2001).

Ozone is approved as safe (GRAS) for treatment of bottled water and as a sanitizer for process trains in bottled water plants (FDA, 1995). The affirmation as GRAS triggered broad usage of ozone gas in the food industry. The interest in ozone as an alternative to chlorine and other chemical disinfectants in cleaning and disinfection operations is based on its high biocidal efficacy, wide antimicrobial spectrum, absence of by-products that are detrimental to health and the ability to generate it on demand, in situ, without needing to store it for later use (Pascual et al., 2007).

Such advantages make ozone attractive to the food industry. In 2001, ozone was approved as an antimicrobial agent in foods in the US (USFDA, 2001). This approval of ozone as a direct additive to food triggered interest in ozone applications development, and industry guidelines for apple juice and cider were published by the FDA in 2004, which also highlighted gaps in the scientific knowledge (USFDA, 2004).

1.3.1 Generation of ozone

As ozone is an unstable molecule, it is often generated at the point of application. Ozone is generated by reaction of free oxygen radicals with diatomic oxygen to form triatomic oxygen molecules. Generation of the free oxygen radical occurs by breakage of strong O–O bonds, requiring a significant energy input. UV radiation and corona discharge methods can be used to initiate free radical oxygen formation and thereby generate ozone. In addition to photochemical (UV radiation) and electric discharge methods, ozone can be produced by chemical, thermal, chemonuclear and electrolytic methods (Kim et al., 1999).

1.3.1.1 Electrical (corona) discharge method

In this method, adequately dried air or oxygen is passed between two high-voltage electrodes separated by a dielectric material, which is usually glass. Air or concentrated O_2 passing through an ozonator must be free from particulate matter and dried to a dew point of at least -60°C to properly protect the corona discharge device. The ozone/gas mixture discharged from the ozonator normally contains from 1% to 3% ozone when using dry air, and 3% to 6% ozone when using high purity oxygen as the feed gas (Muthukumarappan et al., 2000).

When a voltage is supplied to the electrodes, a corona discharge forms between the two electrodes, and the O_2 in the discharge gap is converted to ozone. A corona discharge is a physical phenomenon characterised by a low-current electrical discharge across a gas-containing gap at a voltage gradient, which exceeds a certain critical value (Figure 1.2). First, oxygen molecules (O_2) are split into oxygen atoms (O), and then the individual oxygen atoms combine with remaining oxygen molecules to form ozone (O_3) (Muthukumarappan et al., 2008).

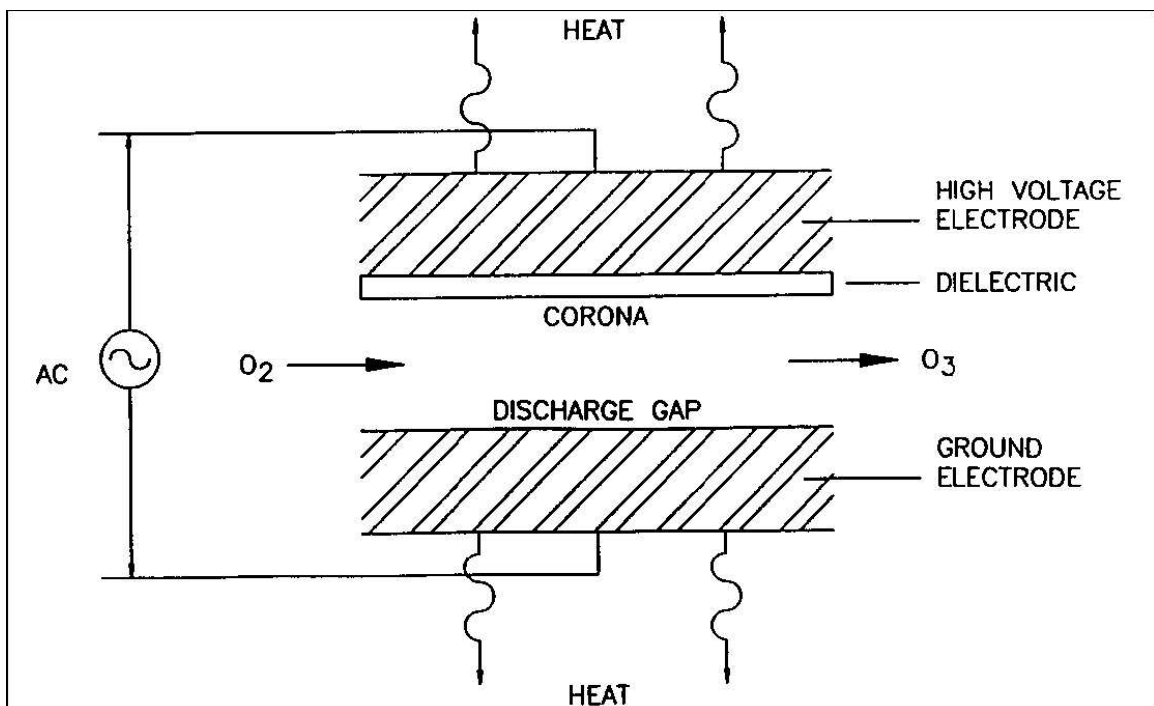


Figure 1.2: Ozone generation by corona discharge method

1.3.1.2 Electrochemical (Cold Plasma) Method

In this method, an electrical current is applied between an anode and cathode in an electrolytic solution containing water and a solution of highly electronegative anions. A mixture of oxygen and ozone is produced at the anode. The advantages associated with this method are the use of low-voltage DC current, no feed gas preparation, reduced equipment size, possible generation of ozone at high concentration and generation in water (Muthukumarappan et al., 2008).

1.3.1.3 Radiochemical ozone generation (RCOG)

High energy irradiation of oxygen can also produce ozone. Although this technique is not yet used commercially in potable or waste water treatment, it is anticipated that in the future this will find much more widespread application (Muthukumarappan et al., 2009).

1.3.1.4 Ultraviolet (UV) Method

In the ultraviolet method of ozone generation, ozone is formed when oxygen is exposed to UV light of 140– 190 nm wavelength. This splits the oxygen molecules into oxygen atoms, which then combine with other oxygen molecules to form ozone (Muthukumarappan et al., 2000).

A wide range of UV bulbs are available covering an appropriate wavelength range from 180 to 254 nm, however, with currently available technology, these bulbs are still not an economical or an efficient way to generate ozone, producing only up to 0.3-0.4% by weight (Sacco, 2009).

1.3.2 Microbial inactivation mechanisms

In general ozone inactivates microorganisms rapidly and at very low ozone concentrations (Poynter et al., 1973). For most planktonic microorganisms (i.e. organisms that are suspended in water, not attached to a surface) the order of disinfectant effectiveness is:

$O_3 > HOCl > ClO_2 > OCl > NH_2Cl > NHCl_2$ (Montgomery, 1985)

Inactivation of bacteria by ozone is a complex process because ozone attacks numerous cellular constituents including proteins, unsaturated lipids and respiratory enzymes in cell membranes, peptidoglycans in cell envelopes, enzymes and nucleic acids in the cytoplasm, and proteins and peptidoglycan in spore coats and virus capsids (Khadre et al., 2001). Ozone is very unstable both in the gaseous phase and in solution, decomposing into hydroxyl ($\cdot OH$), hydroperoxy ($\cdot HO_2$) and superoxide ($\cdot O_2^-$) radicals. The reactivity of ozone is attributed to the oxidising power of these free radicals (Manousaridis et al., 2005), which are responsible for microbial inactivation. Microorganisms are inactivated by disruption of the cell envelope or disintegration leading to cell lysis. Both molecular ozone and the free radicals produced by its breakdown play a part in this inactivation mechanism but there is no consensus on which is more decisive. The resultant disruption or lysis associated with ozone is a faster inactivation mechanism than that of other disinfectants, which require the disinfectant agent to permeate through the cell membrane in order to be effective. Generally with regard to the spectrum of microbial action each microorganism has an inherent sensitivity to ozone. Bacteria are more sensitive than yeasts and fungi. Gram-positive bacteria are more sensitive to ozone than Gram-negative organisms and spores are more resistant than vegetative cells (Pascual et al., 2007).

The inactivation of bacteria by ozone can be considered as an oxidation reaction (Bringmann, 1954). The bacterial membrane seems to be the first site of the attack with proteins and unsaturated lipids in the cell membrane being the primary targets (Christensen and Giese, 1954; Scott and Leshner, 1963; Pryor et al., 1983). Ozone causes alteration in *E. coli* cell membrane permeability leading to leakage of cell contents and eventually causing lysis (Scott and Leshner, 1963). Dave (1999) found that treatment of *S. enteritidis* with aqueous ozone disrupted the cell membranes. Thanomsub et al.

(2002) also supported the proposed mechanism of bacteria inactivation by ozone that caused cell membrane destruction and finally cell lysis reaction. Cell death by ozone is primarily related to cell surface damage (Cho et al., 2010).

Reactions of ozone with proteins and amino acids showed that the susceptibility to oxidation is in the order cysteine = tryptophan = methionine > histidine (Mudd et al., 1969; Pryor et al., 1984). Proteins requiring any of these residues for catalytic activity are inactivated by ozone (Knight and Mudd, 1984; Berlett et al., 1991). Inactivation of enzymes by ozone is probably due to oxidation of sulfhydryl groups in cysteine residues (Chang, 1971).

Vrochinskii (1963) noted that ozone treated bacteria lost their ability to degrade sugars and produce gases. Foegeding (1985) found that *B. cereus* spores with coat proteins removed were rapidly inactivated by ozone, compared to intact spores.

Komanapalli et al. (1997) studied the effect of ozone on metabolic activities of *E. coli* K-12. The cell viability was unaffected with short time ozone exposure. Glyceraldehyde-3-phosphate dehydrogenase showed the greatest susceptibility to ozone followed by oxidation of glutathione, a nonprotein sulfhydryl, and total sulfhydryl compounds. Effects on malate dehydrogenase, lactate dehydrogenase and glutathione disulfide reductase were negligible. Their results also indicated that the mutant (DNA repair deficient) and the wild type strains of *E. coli* were equally sensitive to ozone, suggesting that RecA DNA repair system may not play an important or vital role in ozone resistance.

Fisher et al. (2000) reported both catalase and superoxide dismutase were found to protect listerial cells from ozone attack, with superoxide dismutase being more important than catalase in this protection. Takamoto et al. (1992) observed that ozone decreased enzyme activity in *E. coli* to a greater degree in the case of cytoplasmic β -galactosidase than in case of the periplasmic alkaline phosphatase. Hinze et al. (1987)

studied the effect of ozone on ATP, cytosolic enzymes and permeability of *S. cerevisiae*. They reported the most drastic inactivation for Glyceraldehyde-3-phosphate dehydrogenase and to lesser extents: pyruvate decarboxylase, phosphofructokinase 1 and NAD alcohol dehydrogenase. The accumulation of ATP, NAD and protein in the medium indicated permeabilisation.

Ozone also affects both purines and pyrimidines in nucleic acid (Christensen and Giese, 1954; Scott and Leshner, 1963). Ozone produces single strand breaks in DNA which if unrepaired, cause extensive breakdown of DNA in *E. coli*, resulting in loss of cell viability (Hamelin et al., 1977; 1978). Ito et al. (2005) suggested that ozone caused DNA backbone cleavage via the production of hydroxyl radicals. However, DNA base modifications were mainly caused by ozone itself and the participation of hydroxyl radicals and/or singlet oxygen in base modifications is small, if any. Ishizaki et al. (1987) reported that the plasmid DNA present in *E. coli* cells is converted from closed circular DNA to open circular DNA by ozone.

Komanapalli and Lau (1996) determined the effects of short and long interval ozone exposure on *E. coli* membrane permeability, total proteins and plasmid DNA. They found that short-term exposures of *E. coli* K-12 to ozone gas had no effect on cell viability and tryptophan oxidation. Conversely, a significant leakage of protein followed by malondialdehyde and nucleic acid components in the supernatant indicated membrane permeability but did not affect viability, which progressively decreased with longer exposure. Ozone induces mutations in *E. coli* by penetrating into the cell and genetically altering cytoplasmic contents before destruction of the cell membrane (Hamelin and Chung, 1974).

The mechanism of inactivation of bacteriophage f2 ribonucleic acid (RNA) was studied by Kim et al. (1980). The RNA was released from the phage particles after the phage coat was broken into many protein subunit pieces. They suggested that ozone breaks the

protein capsid liberating RNA and disrupting virus adsorption to the host pili, and that the naked RNA may be secondarily inactivated but at a rate lower than that for the RNA within the intact phage. Ozone inactivated f2 and T4 bacteriophage by attacking the protein capsid, liberating nucleic acid and inactivating the nucleic acid (Sproul et al., 1982).

Shriniki et al. (1988) concluded the ozone inactivation of Tobacco Mosaic Virus (TMV) by posing an inactivation hypothesis that ozone attacks the protein coat and RNA. The damaged RNA crosslinks with amino acids of the coat protein subunits, therefore TMV lost infectivity because of its inability to uncoat. Yoshizaki et al. (1988) found that aqueous ozone caused the coat protein subunits of TMV to aggregate with each other and cross-link with the viral RNA. Ozone exposure caused damage to viral RNA of poliovirus 1 by altering two of the four polypeptide chains in the poliovirus protein coat leading to poliovirus inactivation (Roy et al., 1981).

Enveloped viruses such as hepatitis A virus (HAV) are expected to be much more resistant to ozone compared to nonenveloped viruses such as poliomyelitis (Khadre et al., 2001). A study on the inactivation of HIV by ozone indicated that it could exert several antiviral effects, including viral particle disruption, reverse transcriptase inactivation, and/or a perturbation of the ability of the virus to bind to its receptor on target cells (Wells et al., 1991). Lin and Wu (2006) reported significant effects of inactivation of intracellular EV71 in the case of 45 or 60 min ozone exposure, which correlated with high cell death rates.

Ozone inactivation of viruses occurs primarily in two ways: by lipid peroxidation and by protein peroxidation (Carbonneau et al., 1991; Dianzani, 1993; Friedman and Stromberg, 1993). Murray et al. (2008) studied the inactivation of enveloped and non-enveloped viruses including herpes simplex virus type-1 (HSV-1, strain McIntyre), vesicular stomatitis Indiana virus (VSV), vaccinia virus (VACV, strain Elstree),

adenovirus type-2 (HAdV-2), and the PR8 strain of influenza A virus (FLUAVA/PR/8/34/H1N1; FLUAV) using an ozone-oxygen delivery system. The results of the study showed that ozone exposure reduced viral infectivity by lipid peroxidation and subsequent lipid envelope and protein shell damage.

1.3.3 Factors affecting efficacy of ozone processing

There are different parameters that affect the disinfection ability of ozone in liquid processing treatment. Extrinsic parameters (i.e. flow rate, ozone concentration, temperature) affecting the diffusion and solubility of ozone in the disinfection media, and intrinsic parameters (pH and presence of solid contents or organic matter) that affect the ozone's reactivity are described below.

1.3.3.1 Extrinsic Parameters

Flow rate and bubble size

Depending on the gas flow rate applied for ozone production, different sizes of bubbles are produced. Bubble size has been shown to have an effect on ozone's solubilisation rate and disinfection efficacy. Ahmad and Farooq (1985), reported that ozone mass transfer and disinfection efficacy increased as bubble size decreased (ozone bubble size was varied while all other factors were kept constant). The smaller bubble sizes have a higher interfacial area available for mass transfer which may be responsible for this effect. Decreasing the bubble diameter from 1 cm to 0.1 cm increases the contact area by 32 times (Ogden, 1970). Free suspended bacteria migrate toward the ozone bubbles due to their surface active properties and are preferentially inactivated by comparatively high ozone concentrations at the gas: liquid interface of the bubble (Hill and Spencer, 1974). Gong et al. (2007) showed a nonlinear dependence of the ozone dissolution efficiency on the initial bubble size in a study of the mass transfer process of ozone dissolution in a bubble plume (bubble plumes are an interacting collections of bubbles formed by some event) inside a rectangular water tank. The dissolution efficiency varies

rapidly when the initial bubble size reaches a critical value while the change of efficiency is much slower at other bubble sizes. A simple analysis of the mass transfer inside a bubble plume reveals that the mass transfer process is affected by a number of factors. They are the gross area of the contact surfaces between the gas and liquid phase, the contact time of bubbles with the liquid and the mass transfer rate of individual bubbles and the gross contact area depends on the bubble diameter. At the same void fraction, the contact area increases as the size of the bubble decreases. The contact time, which depends mainly on the bubble diameter increases when the bubble size reduces as smaller bubbles rise slower than big ones. In general, reducing the diameter of ozone bubbles is useful for improving the efficiency of ozone's utilisation. A longer distance or time of rising is important for bigger bubbles to achieve a higher efficiency since they need more time to transfer the dissoluble gas inside the bubbles (Gong et al., 2007).

Concentration

Ozone concentration present or available in the medium is another parameter that determines ozone efficacy. Faster inactivation with a shorter lag time and smaller D-values at the highest ozone concentrations were reported by Steenstrup and Floros (2004) during inactivation of *E. coli* O157:H7 in apple cider by ozone. The concentrations applied were all greater than 1000 ppm.

In a study of ozone disinfection kinetics of *E. coli* in water, increased ozone efficiency was reported at higher ozone concentration tested (Zuma et al., 2009). At a flow rate of 2 L/ min with an ozone concentration of 0.906 mg/L, *E. coli* populations of approximately 1×10^8 cells/mL were reduced by 4 log in 6 min, whereas with a higher ozone concentration of 4.724 mg/L the reduction was much higher, approximately 5 log in 4 min.

Temperature

Ozone solubility in water is 13 times that of oxygen at 0-30°C and it is progressively more soluble in colder water (Rice, 1986). As is common in gases the solubility ratio for ozone increases as the temperature of water decreases (Bablon et al., 1991). As temperature increases ozone becomes less soluble and less stable with an increase in the decomposition rate (Rice et al., 1981). The mass transfer of ozone gas into the liquid phase is also influenced by temperature and pH. The ability of ozone to inactivate bacteria decreases with decreasing temperature (Farooq et al., 1977; Vaughn et al., 1987; Hunt and Marinas, 1997). Driedger et al. (2001) reported decreased ozone inactivation rates of *Cryptosporidium parvum* with decreasing temperature. Increased rates of ozone inactivation with increasing temperature were obtained for *B. Subtilis* spores and *C. parvum* oocysts (Corona-Vasquez et al., 2002; Larson and Mariñas, 2003). Dow et al. (2006) indicated, that increasing temperature (7-22°C) had the strongest influence on the inactivation rate of *B. subtilis* spores in oxidant demand free phosphate buffer and also led to a corresponding decrease in the lag phase and CT₂ log. However, Steenstrup and Floros (2004) reported that the processing times for ozone inactivation of *E. coli* in apple cider varied from 6.5 to 13.4 min, and generally increased with decreasing temperature. As a conclusion from these studies, there is no consensus on the effect of temperature on the biocidal efficacy of ozone, for example a drop in the temperature of the aqueous medium increases ozone solubility and stability, augmenting its availability in the medium and consequently efficacy rises. The simultaneous contribution of these two factors (solubility/stability and reactivity) to ozone efficacy can vary with experimental conditions, making it difficult to predict the influence of temperature on a particular application (Pascual et al., 2007)

1.3.3.2 Intrinsic Parameters

pH

The effect of pH on ozone inactivation is mainly attributed by the fact that ozone decomposition rate changes substantially with changes in pH (Farooq et al., 1977a; Roy et al., 1980). Zuma et al. (2009) reported ozone mediated disinfection kinetics of *E. coli* in water. They investigated the effect of different control parameters and pH on the disinfection rate of *E. coli*. The pH range studied was from 4.93 to 9.16. It was reported that the kinetics of inactivation of *E. coli* was much faster in acidic medium than in basic medium. The inactivation of *E. coli* (10^8 CFU/mL) at a flow rate of 2 L/min with an ozone concentration of 0.906mg/L at $25 \pm 2^\circ\text{C}$ resulted in a higher rate constant of 2.209 min at a pH value of 4.93 than at pH 9.16 with a rate constant of 1.126 min, indicating a 56% decrease in disinfection rate (Zuma et al., 2009). Several other researchers also reported enhanced ozone efficiency at lower pH (Farooq et al., 1977a; Harakeh and Butler, 1984; Vaughn et al., 1987; Lin and Wu, 2006).

Organic matter

Certain organics, inorganics or suspended solids can lead to ozone demand. This effect depends on the types of turbidity rather than the turbidity level. Dissolved organic matter reduces the disinfection activity by consuming ozone to produce compounds with little or no microbicidal activity and reducing the concentration of active species available to react with microorganisms. Inactivation of viruses either in sewage effluent or in secondary effluent was reduced in the presence of increased level of organics or in nonozonated wastewater (Majumdar et al., 1973; Harakeh and Butler, 1984). Williams et al. (2005) studied the inactivation of *E. coli* in orange juice, and found the efficacy of ozonation was reduced in the presence of ascorbic acid and organic matter. In wastewater, proteins, carbohydrates, lipids, and organic amines will elevate the concentration of dissolved organic carbon. Oxidising disinfectants like ozone will lose

bactericidal strength through reaction with organic matter. The reaction products will generally have weak or no bactericidal activity.

Ozone driven inactivation of microorganisms in wastewaters required longer contact times and larger doses than the inactivation in demand free systems. Hunt and Marinas (1999), reported slower rates for the ozone inactivation of *E. coli* in the presence of humic acid than in the absence of natural organic matter due to faster decomposition of dissolved ozone and thus, the lower exposure of *E. coli* to the disinfectant ozone. Farooq et al. (1977a) reported the interference of organic matter present in water with the inactivation of *Candida parapsilosis* by exerting an ozone demand. Hence the applied ozone dosage had to be sufficient to overcome this demand before a free residual concentration was available for the inactivation of the microbial organisms. Schuchmann and Von Sonntag (1989) explained ozone effectiveness in reducing the load of organic matter (added D-glucose) in raw water purification. They found that direct reaction with ozone predominated at high glucose concentration. However, at low glucose concentration and at higher pH levels (9.0), the $\cdot\text{OH}$ pathway played the dominant role (Schuchmann and Von Sonntag, 1989) where $\cdot\text{OH}$ radicals act as an initiator for the decay of ozone. Janex et al. (2000) reported the greatest influence of organic matter on the ozone demand of the effluents during wastewater disinfection by ozone. Dissolved organic or inorganic matter affects the disinfection rate because they compete with the microorganisms for disinfectant. Macauley et al. (2006) stated that the inactivation of bacteria was ineffective up to an ozone dose of 20 and 10 mg/L in wastewater samples obtained from two different swine production facilities, respectively. This implies that at a low ozone dose, the majority of ozone was preferentially consumed by natural organic materials (non-bacterial) present in lagoon samples.

1.3.4 Use of ozone in the food industry

Ozone is used in the food industry for food surface hygiene, sanitation of food plant equipment, waste water treatment, lowering biological oxygen demand (BOD) and chemical oxygen demand (COD) of food plant waste (Rice et al., 1982; Majchrowicz, 1998). Ozone has also been used as a food surface disinfecting agent (Greene et al., 1993; Hampson, 2000; Moore et al., 2000; Taylor and Chana, 2000; Lagrange et al., 2004). Ozone has been used for disinfecting recycled poultry chill water and disinfection of poultry carcasses (Sheldon and Brown, 1986). Greene et al. (1993) proposed the use of ozonated water as a sanitizer for dairy and food plants. Guzel-Seydim et al. (2000) studied the use of ozonated water for dairy equipment. The effectiveness of ozone for disinfecting polystyrene surfaces was reported. Mahfoudh et al. (2010) showed that treating polystyrene surfaces with dry gaseous ozone can denature proteins and inactivate various bacteria and endospores when these are deposited on this surface and kept in contact with it.

Ozonated water has been applied to fresh-cut vegetables for sanitation purposes reducing microbial populations and extending the shelf life of some of these products. Treating fruits and vegetables with ozone has been found to increase shelf-life. Shredded lettuce in water bubbled with ozone gas had decreased bacterial content (Kim et al., 1999). *S. sonnei* counts were reduced by 1.8 log units in lettuce treated with 5 ppm for 5 min (Selma et al., 2007). Ölmez and Akbas (2009) reported that the application of 2 ppm ozonated water treatment for 2 min were found to be the optimum processing conditions for ozone disinfection of green leaf lettuce, in terms of reducing the microbial load and maintaining the sensory quality during cold storage. Klockow and Keener (2009), developed a novel non-thermal ozone processing technology (PK-1) which is capable of generating ozone inside a sealed package at various geometries. This system was capable of reducing *E. coli* O157:H7 (with the largest reductions 3-5

log₁₀CFU/leaf) on prepackaged, ready-to-eat (RTE) spinach leaves; however, with notable colour degradation. Incorporating ozone based sanitization into existing processing practices for fresh produce was effective in reducing *E. coli* O157:H7 populations on spinach (Vurma et al., 2009). Yuk et al. (2007) reported the combined effectiveness of ozone and organic acid in reducing initial population levels of *E. coli* O157:H7 and *L. monocytogenes* on enoki mushrooms.

Achen and Yousef (2001) compared the efficacy of ozone against *E. coli* O157:H7 on apples by bubbling of ozone during apple washing and dipping apples in pre-ozonated water. Bubbling was found to be more effective than dipping. Selma et al. (2008), reported that gaseous ozone treatment of 5000 and 20,000 ppm for 30 min reduced total coliforms, *P. fluorescens*, yeast, and lactic acid bacteria recovery from fresh-cut cantaloupe while gaseous ozone (10,000 ppm for 30 min) under partial vacuum resulted in reduction of viable, recoverable Salmonella from inoculated physiologically mature non-ripe and ripe melons.

Akbas and Ozdemir (2008) concluded that, ozonation was found to be effective especially in reduction of vegetative cells (*E. coli*, *B.cereus*) in dried figs and as a promising method for the decontamination of dried figs. Oztekin et al. (2005) reported on the effects of ozone treatment on the micro flora of dried figs, where the application of gaseous ozone at 5 or 10 ppm for 3 to 5 h resulted in significant reductions in total bacteria, coliform and yeast/mould counts. Habibi Najafi and Haddad Khodaparast (2009) concluded that a minimum of 1 h ozone treatment at 5 ppm could be successfully used for reducing both coliform and *S. aureus* populations on date fruits, but that longer exposure times are required for elimination of the total mesophilic bacteria as well as yeasts and moulds.

An increase in the shelf life of apples and oranges by ozone treatment has been attributed to the oxidation of ethylene. Fungal deterioration of blackberries and grapes

was decreased by ozonation of the fruits (Beuchat, 1992). The application of gaseous ozone for control of green and blue mould development on cold stored citrus fruit has also been investigated and showed that sporulation of *P. italicum* was reduced (Palou et al., 2001). Use of ozone has been reported for processing various fruit juices (Steenstrup and Floros, 2004; Williams et al., 2004; Tiwari et al., 2009a).

Antony-Babu and Singleton (2010) demonstrated the use of ozone to reduce spore production in the xerophilic fungus, *Eurotium amstelodami* IS-SAB-01 isolated from naan bread. Khadre and Yousef (2001a) found that ozone was more effective than hydrogen peroxide against foodborne *Bacillus* species spores.

Restaino et al. (1995) determined that ozone effectively killed Gram-positive bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *B. cereus*, *Enterococcus faecalis*, and Gram-negative bacteria including *P. aeruginosa*, and *Yersinia enterocolitica*. Ozone was also reported to be effective against biofilms of *S. aureus* (Cabo et al., 2009).

Guzel-Seydim et al. (2004a) investigated the efficacy of ozone to reduce bacterial populations in food components. Locust bean gum provided an intermediate level of protection, while caseinate solution and whipping cream provided the greatest levels of protection to the bacterial populations. Fan et al. (2007) reported that gaseous ozone effectively inactivated *L. innocua* on solid media at concentrations of 50 and 100 nl/l during short exposure times at both 5 and 20°C.

Ozone has also been used in managing stored grains (Kells et al., 2001). Inan et al. (2007) reported degradation of aflatoxin B₁ in red peppers with no significant variation in colour quality. Tzortzakis et al. (2007) suggested that ozone may constitute a desirable and effective residual free alternative to traditional postharvest fungicide practices.

Ozone also has been shown to be effective against a number of enveloped and non-enveloped viral species (Akey and Walton, 1985; Herbold et al., 1989; Carpendale and Freeberg, 1991; Arimoto et al., 1996; Khadre and Yousef, 2002; Shin and Sobsey, 2003). Ozone is very effective in inactivating feline calicivirus (FCV) and adenovirus type 40 (AD40) in treated water and at low ozone concentrations (Thurston-Enriquez et al., 2005). Other researchers (Kim et al., 1980; Hall and Sobsey 1993) also reported the sensitivity of the bacteriophages MS2, and f2 to ozone. Ozone is highly effective for the inactivation of airborne viruses (Tseng and Li, 2006). A summary table of the application of ozone for inactivation of microorganisms in liquid systems is presented in Table 1.3.

Table 1.3: Inactivation of bacteria, viruses, yeasts and protozoa by ozone

Organism	Percent reduction ^(a) or log ₁₀ reduction	Time (min)	Concentration (mg/L)	pH	Temperature (°C)	Medium	References
<i>Escherichia coli</i>	99.99 ^a	1.67	0.23-0.26	7	24	O ₃ demand free water	Farooq and Akhlaque (1983)
<i>E. coli</i>	17 ^a	19	Initial 0.85 Residual 0	7.5	16	Raw wastewater	Joret et al. (1982)
<i>E. coli</i>	97 ^a	19	Initial 1.4 Residual 0	7.5	16	Raw wastewater	Joret et al. (1982)
<i>E. coli</i> O157:H7 (five strain mixture)	6.0	45	0.9g/h	3.8	50	Apple cider	Williams et al. (2004)
<i>E. coli</i> O157:H7 (five strain mixture)	<5.0	240	0.9g/h	3.8	20	Apple cider	Williams et al. (2004)
<i>E. coli</i> O157:H7 (five strain mixture)	4.8	240	0.9g/h	3.8	4	Apple cider	Williams et al. (2004)
<i>E. coli</i> O157:H7 (five strain mixture)	6.0	75	0.9g/h	3.8	50	Orange juice	Williams et al. (2004)
<i>E. coli</i> O157:H7 (five strain mixture)	<5.0	240	0.9g/h	3.8	20	Orange juice	Williams et al. (2004)
<i>E. coli</i> O157:H7 (five strain mixture)	5.4	240	0.9g/h	3.8	4	Orange juice	Williams et al. (2004)
<i>Salmonella typhimurium</i>	99.995 ^a	1.67	0.23-0.26	7	24	O ₃ demand free water	Farooq and Akhlaque (1983)

<i>Salmonella</i> (five-serovar mixture)	4.8	15	0.9g/h	3.8	50	Apple cider	Williams et al. (2004)
<i>Salmonella</i> (five-serovar mixture)	4.5	240	0.9g/h	3.8	4	Apple cider	Williams et al. (2004)
<i>Salmonella</i> (five-serovar mixture)	<5.0	240	0.9g/h	3.8	20	Apple cider	Williams et al. (2004)
<i>Salmonella</i> (five-serovar mixture)	5.0-6.0	15	0.9g/h	3.8	50	Orange juice	Williams et al. (2004)
<i>Salmonella</i> (five-serovar mixture)	4.2	240	0.9g/h	3.8	4	Orange juice	Williams et al. (2004)
<i>Salmonella</i> (five-serovar mixture)	<5.0	240	0.9g/h	3.8	20	Orange juice	Williams et al. (2004)
<i>Shigella sonnei</i>	3.7	1	1.6ppm			Ozonated deionized water	Selma et al. (2007)
<i>S. sonnei</i>	5.6	1	2.2 ppm			Ozonated deionized water	Selma et al. (2007)
<i>Bacillus cereus</i>	>2.0	5	0.12		28	O ₃ demand free water	Broadwater et al. (1973)
<i>B. cereus</i> spores	>2.0	5	2.29		28	O ₃ demand free water	Broadwater et al. (1973)
<i>Leuconostoc mesenteroides</i>	1.3 to ~7	0.5	0.3-3.8ug/mL	5.9	25		Kim and Yousef 2000
<i>Listeria monocytogenes</i>	0.7 to ~7	0.5	0.2-1.8ug/mL	5.9	25		Kim and Yousef 2000
<i>L. pneumophila</i>	99,997 ^a	20	0.32	7	24	Sterile distilled water	Edelstein et al. (1982)
<i>Mycobacterium fortuitum</i>	90 ^a	1.67	0.23-0.26	7	24	O ₃ demand free water	Farooq and Akhlaque (1983)
Viruses							
CoxSackie virus B5	90 ^a	4.9	0.18	7.2	20	Activated sludge	Harakeh and Butler (1984)

Poliovirus type 1 (Mahoney)	99.7 ^a	1.67	0.23-0.26	7	24	O ₃ demand free water	Farooq and Akhlaque (1983)
Poliovirus type 1	99.99 ^a	2.5	0.40	7.2	20	Activated sludge reactor effluent	Harakeh and Butler (1984)
Poliovirus type 1	99 ^a	9	0.2	4	20	Activated sludge reactor effluent	Harakeh and Butler (1984)
Enteric virus	92 ^a	19	Initial 1.4 Residual 0	7.5	16	Raw wastewater	Joret et al. (1982)
Enteric virus	>98 ^a	19	Initial 4.10 Residual 0.02	7.8	18	Raw wastewater	Joret et al. (1982)
Human rotavirus	80 ^a	10	0.31	7.2	20	Activated sludge	Harakeh and Butler (1984)
H5N1	4 log	10	0.5 (residual ozone level)		22 ± 2	Treated water	Lénès et al. (2010)
H1N1	4 log	10	0.5 (residual ozone level)		22 ± 2	Treated water	Lénès et al. (2010)
H5N1	4 log	10	1.0 (residual ozone level)		22 ± 2	Treated water	Lénès et al. (2010)
H1N1	4 log	10	1.0 (residual ozone level)		22 ± 2	Treated water	Lénès et al. (2010)
Yeasts							
<i>Candida parapsilosis</i>	99.8 ^a	1.67	0.23-0.26	7	24	O ₃ demand free water	Farooq and Akhlaque (1983)
<i>C. tropicalis</i>	99 ^a	0.30-0.08	0.02-1.0	7.2	20	O ₃ demand free water	Kawamura et al. (1986)

Protozoa										
<i>Giardia lamblia</i>	2.0 log	1.1	0.7mg/l	7	5	Water	Wickramanayake et al. (1984)			
<i>G. muris</i>	2.0	2.8	0.5	7	5	Water	Wickramanayake et al. (1984)			
<i>Naegleria gruberi</i>	2.0log	2.1	2.0	7	5	Water	Wickramanayake et al. (1984)			
<i>Cryptosporidium parvum</i>	>1.0	5	1.0	7	25	O ₃ demand free water	Korich et al. (1990)			

*Table modified from Weavers and Wickramanayake (2001)

1.3.5 Effect on food quality

Applying ozone at doses that are large enough for effective decontamination may change the sensory qualities of these products. Some researchers reported no change in chemical composition, sensory quality of food after treatment with ozone or ozonated water (Dock, 1999; Song, 2000; Zhang et al., 2005). Richardson (1994) reported that ozone helps to control odour, flavour and colour while disinfecting wastewater. Zhang et al. (2005) reported no significant difference between ascorbic acid contents for treated and non-treated celery samples. Increase in ascorbic acid levels in spinach, pumpkin leaves, and strawberries were reported in response to ozone exposure (Luwe et al., 1993; Ranieri et al., 1996; Perez et al., 1999). Selma et al. (2008) reported the combination of hot water and gaseous ozone may be an efficient and promising treatment for controlling microbial growth and maintaining sensory quality of melons. Slight decreases in Vitamin C content were reported in lettuce (Beltran et al., 2005). Ozone has been reported for processing of various fruit juices including; apple juice (cider) (Choi and Nielsen, 2005), orange juice (Tiwari et al., 2008a), blackberry juice (Tiwari et al., 2009a) and strawberry juice (Tiwari et al., 2009b). Ozonation of fruit juices rich in anthocyanins such as strawberry and blackberry juice causes a significant reduction in these bioactive compounds. A significant reduction of 98.2% in the pelargonidin-3-glucoside content of strawberry juice was reported at an ozone concentration of 7.8% w/w processed for 10 min (Tiwari et al., 2009b). Similar reductions of >90% in the cyanidin-3-glucoside content of blackberry juice were reported under similar treatment conditions (Tiwari et al., 2009a).

1.4 Microbiological issues pertinent to fresh juice processing

Fruit and vegetables are major components of a healthy diet, but eating fresh uncooked produce is not exempt of risk. Attention has focused on evaluating the microbiological or toxicological risks that may be involved in applying novel preservation processes, and their effect on food safety, in order to obtain “fresh” products that do not present health risks (Esteve and Frígola, 2007).

Outbreaks of foodborne infections associated with the consumption of fresh fruits and vegetables as well as unpasteurised juices contaminated with pathogenic bacteria have been documented (Harris et al., 2003; Greig and Ravel, 2009).

Outbreaks of salmonellosis (CDC 1991, 2002; Moehler-Boetani et al., 1999), and *E. coli* O157:H7 infections (Jackson and Keene, 2003) have been linked to the consumption of cantaloupes. Watermelons have been implicated in outbreaks of salmonellosis (Blostein, 1993; Gaylor et al., 1995) and shigellosis (Fredlund et al., 1987). *E. coli* O157:H7 has been reported to grow on cantaloupe and watermelon cubes (Del Rosario and Beuchat, 1995). *L. monocytogenes* can grow in cantaloupe and watermelon pulp (Penteado and Leitao, 2004). Surface decontamination of melons by treatment with chemical sanitizers (Park and Beuchat, 1999) and heat (Annous et al., 2004; Ukuku et al., 2004) has not been fully successful in eliminating pathogens. *Salmonella* has been linked to outbreaks transmitted by the consumption of unpasteurised orange juice (Hammack et al., 2001). *Listeria*-like infections were attributed to the consumption of raw milk, sour milk, cottage cheese and cream chesse (Ryser, 1999). In the United States 21 juice-associated outbreaks were reported to the CDC between 1995 and 2005; 10 implicated apple juice or cider, 8 were linked to orange juice, and 3 involved other types of fruit juice. These outbreaks caused 1,366 illnesses, with a median of 21 cases per outbreak (range, 2 to 398 cases). Among the 13 outbreaks of known etiology, 5 were caused by *Salmonella*, 5 by *E. coli* O157:H7, 2 by

Cryptosporidium, and one by Shiga toxin-producing *E. coli* O111 and *Cryptosporidium* (Vojdani et al., 2008).

To identify trends of increasing number of foodborne outbreaks associated with fresh produce Sivapalasingam et al. (2004) analyzed data from 1973 to 1997. A produce-associated outbreak was defined as the occurrence of two or more cases of the same illness in which an epidemiologic investigation implicated the same uncooked fruit, vegetable, salad, or juice. A total of 190 produce-associated outbreaks were reported, associated with 16,058 illnesses, 598 hospitalisations, and eight deaths. Among produce-associated outbreaks, the food items most frequently implicated included salad, lettuce, juice, melon, sprouts, and berries. Among 103 (54%) produce-associated outbreaks with a known pathogen, 62 (60%) were caused by bacterial pathogens, of which 30 (48%) were caused by *Salmonella* (Sivapalasingam et al., 2004). Fresh vegetables are of microbiological concern and increasingly being identified as a source of foodborne outbreaks around the world (Lynch et al., 2009). A Number of Shiga toxin producing *E. coli* (STEC) outbreaks associated with the consumption of fresh vegetables such as radish sprout, fresh lettuce, bagged spinach and lettuce were reported (Michino et al., 1999; Söderström et al., 2005; CDC, 2006). Several outbreaks of *L. monocytogenes* infection associated with fresh produce have been reported from various parts of the world (Beuchat, 1996; Gorny, 2006; Meldrum et al., 2009).

While providing a reliable reduction in microbial population, thermal pasteurisation involves high costs in operation, and degrades sensory and nutritional qualities (Splittstoesser et al., 1996).

1.5 Food safety concerns

Microorganisms of concern to fruit juice processors from either spoilage or food safety aspects are listed in Table 1.4.

Table 1.4: Microorganisms of concern

Food spoilage	Food safety
<i>Saccharomyces cerevisiae</i>	<i>Escherichia coli</i> O157:H7
<i>Alicyclobacillus acidoterrestris</i>	<i>Listeria monocytogenes</i>
<i>Pseudomonas fluorescens</i>	
<i>Pseudomonas putida</i>	

1.5.1 *Escherichia coli* O157:H7

E. coli is a Gram negative coco bacillary rod and a commonly occurring inhabitant of the colon of humans and other animals. There are also several pathogenic types of *E. coli*, which cause a variety of human diseases. Based on disease syndromes and characteristics, and also on their effect on certain cell cultures and serological groupings, five virulence groups of *E. coli* are recognised: enteroaggregative (EAaggEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enterotoxigenic (ETEC) (Jay et al., 2005).

Enterohemorrhagic *E. coli* (EHEC), which includes *E. coli* O157:H7 was recognised as a human pathogen for the first time in 1982. Reports of foodborne illnesses caused by *E. coli* have been steadily increasing worldwide (WHO, 1997). Apple juice products were implicated in a disease outbreak caused by *E. coli* O157:H7 in the early 1980's in Canada (Steele et al., 1982), while the frequency of outbreaks has increased over the last decade (Basaran et al., 2004). In 1991, an outbreak of *E. coli* O157:H7 infections and hemolytic uremic syndrome was linked to traditionally pressed apple cider. In

August 2007 an outbreak of *E. coli* O157:H7 occurred in Scotland and was attributed to the consumption of cold cooked meat.

E. coli O157:H7 produces toxins that cause diarrhoea, hemorrhagic colitis, and hemolytic uremic syndrome in humans (Doyle, 1991). The toxins of *E. coli* have been referred to as shiga-like toxins (verotoxin, verocytotoxin). More recent terminology has been applied as Stx1 and Stx2, previously reported as SLT-I and SLT-II. The genes for Stx1 and Stx2 are encoded by temperate bacteriophages in some EHEC strains. Stx1 differs from Stx (Shiga- toxin) by three nucleotides and one amino acid, and is neutralised by antibodies to Stx. All Stx toxins consist of a single enzymatically active A subunit and multiple B subunits. Stx-sensitive cells possess the toxin receptor, globotriaosylceramide (Gb₃), and sodium butyrate appears to play a role in sensitizing cells to Stx (Louise et al., 1995). Stx production occurred at all temperatures that supported cell growth, although less toxin was found when cells were grown at 21°C than at 37°C even though cell numbers were similar. In ground roasted beef slurry, strain O157:H7 was found to produce Stx at either 21°C or 37°C within 24 h (Abdul-Rauof et al., 1995). A wide variety of foods has been implicated as vehicles of *E. coli* O157:H7 infection, including meat (Griffin and Tauxe, 1991), milk (Borczyk et al., 1987), yoghurt (Morgan et al., 1993), fruit juices (Besser et al., 1993), and cheese (Sharp et al., 1995).

Unlike most foodborne pathogens, *E. coli* O157:H7 is tolerant to acidic environments. Survival in apple cider (pH 3.6-4) (Miller and Kaspar, 1994) and mayonnaise (pH 3.6-3.9) has been reported (Wegant et al., 1994). *E. coli* O157:H7 survived buttermilk fermentation (pH 4.4), and drying and storage of fermented sausage (pH 4.5) (Leyer et al., 1995). Acid adaptation and increased resistance to acid stress have been observed in *E. coli* O157:H7 (Buchanan and Edelson, 1996). In 1996, it was discovered that the ability of *E. coli* to survive at pH 2 was dependant on the media that was used for both

growth and acid challenge (Small et al., 1994; Lin et al., 1995; Lin et al., 1996). In a study using apple ciders with pH values between 3.6 and 4.0, an *E. coli* O157:H7 inocula of 10^2 - 10^5 , cells survived for 2-3 days at 25°C (Zhao et al., 1993). Arnold and Kasper (1995) studied a single strain of *E. coli* O157:H7 and concluded that acid tolerance (as determined by exposure to synthetic gastric juice and HCl with pHs of 1.5 and 2.0, respectively) was independent of prior exposure to moderate acid conditions. Studies done by the National Food Processors Association have shown that *E. coli* O157:H7 is able to survive in apple, orange, pineapple, and white grape concentrates and in banana puree at -23°C for at least 12 weeks (Oyarzábal et al., 2003).

These studies concluded that it is important to investigate the response of *E. coli* to processing technologies. The reasons for this are low infectious dose (<100 cells), its increased acid resistance (Lin et al., 1996), and its capacity for extended survival or growth in acidic foods (Berry and Cutter, 2000).

1.5.2 *Listeria monocytogenes*

In the past decades, *L. monocytogenes* has become increasingly important as a food associated pathogen. Most European Union countries have an annual incidence of human listeriosis between two and ten cases per million (Jemmi and Stephan, 2006).

L. monocytogenes is a Gram positive, motile, psychrotrophic pathogen, ubiquitous in the environment and has been found in fruits and vegetables. It is a versatile organism; capable of growth under different conditions. A variety of foods including ready-to-eat foods have been found to be contaminated with *L. monocytogenes* (Zhou and Jiao, 2004). It has a high mortality rate of 20-30% for humans, compared to other pathogens. The presence of *Listeria* on plant materials is likely due to contamination from decaying vegetation, animal faeces, soil, surface, river, and canal waters, or effluents from sewage treatment operations (Beuchat, 1996). It is capable of growing at temperatures ranging from -1 to 45°C, in high salt and acid foods. Its ability to adapt to these

environments suggests that *Listeria* is able to assimilate information about its environment, and process that information quickly in order to adapt to changing conditions (Hill et al., 2002).

Milk and dairy products, various meat and meat products such as beef, pork, fermented sausages as well as fresh produce such as radishes, cabbage, seafood and fish products have been all associated with *Listeria* contamination (Rocourt and Cossart, 1997). The natural flora of raw milk can contain human pathogens including *L. monocytogenes*. Ryser and Marth (1999) observed *L. monocytogenes* in 2 to 4% of raw milk samples. If present in raw milk, it can grow during refrigerated storage (Farber et al., 1990). In 1983, *L. monocytogenes* was epidemiologically linked to 49 cases of listeriosis from pasteurised milk in Massachusetts in which 14 patients died (Fleming et al., 1985). In 1994, 45 people developed listeriosis after consuming pasteurised chocolate milk. The epidemic strain was later recovered from an unopened bottle of chocolate milk at $9 \log_{10}$ CFU/ml. The presence of *L. monocytogenes* at such high levels indicates that the product was temperature abused, allowing the post processing contaminant to grow uninhibited (Dalton et al., 1997). An outbreak of listeriosis in Austria and Germany due to consumption of 'Quargel' cheese in 2009 was reported (Fretz et al., 2010). The number of listeriosis cases reported in Ireland in 2007 (21) was three times that for 2006 (7). No outbreaks involving *L. monocytogenes* in fruit juices have been reported; however this pathogen has been isolated from unpasteurised apple juice (pH 3.78) and apple-raspberry juice blend (pH 3.75) after 1 day storage at 5°C (Sado et al., 1998). Oyarzábal et al. (2003) studied the survival of *L. monocytogenes* and other foodborne pathogens in apple, orange, pineapple, and white grape juice concentrates and showed that these pathogens were recoverable from all concentrates through 12 weeks of storage at -23°C. The low pH of fruit juices plays an important role in survival of foodborne pathogens. The ability of *L. monocytogenes* to respond to low pH conditions

plays an integral role in its survival and resistance to acidic foods (Cotter et al., 2000), thus affecting the food processing and preservation protocols. The organism can become highly resistant to even extremely acidic conditions due to stress hardening (Lou and Yousef, 1997). Some studies have shown that Acid Tolerance Response (ATR) of *L. monocytogenes*, as a consequence of stress hardening, can result in its increased thermal tolerance in apple, orange and white grape juice (Mazzotta, 2001). Caggia et al. (2009) reported that orange juice and minimally processed orange juice slices can support the growth of acid adapted *L. monocytogenes*. In food processing technologies, there is an extensive use of low pH environments (decontamination by acetic acid in beef processing, fermentation etc.) which can result in the alteration of the cellular physiology of the pathogen either by *de novo* protein synthesis or by changes in the fatty acid composition of the cell membrane (Foster, 1991, Phan-Thanh et al., 2000). This can lead to enhanced resistance to any subsequent acid stress which may be part of a processing treatment.

Human listeriosis has been linked to the consumption of fruits and vegetables, and *L. monocytogenes* has been isolated from cucumbers, cabbage, lettuce, celery, tomatoes, and other vegetables (Beuchat, 2002 Table 1.5). *L. monocytogenes* may pose a safety hazard in fruits and vegetables when they are stored under conditions that will permit *Listeria* growth, and when the microbial background which usually competes for nutrients is reduced (Beuchat, 1996). It has been demonstrated that *Listeria* can grow in any type of fruit or vegetable at different temperatures when these were inoculated with *L. monocytogenes*, as long as the background micro flora had been eliminated or inhibited from growing (Bennik et al., 2000).

Table 1.5: Prevalence of *Listeria monocytogenes* in fresh produce in different countries

Produce	Country	% of incidence
Bean sprouts	Malaysia	85
Cabbage	Canada	2.2
	Sri Lanka	33
	USA	1.1
Cucumber	Malaysia	80
	Pakistan	6.7
	USA	2.2
Eggplant	USA	2.2
Lettuce	Malaysia	22.7
	Sri Lanka	50

(Adapted from Beuchat, 2002)

Listeriosis has a long incubation time, which makes it difficult to identify the pathogen and trace the contaminated food. Once the pathogen gains entry into mammalian cells by phagocytosis, they are released from the membrane-bound vacuole and begin to multiply. The pathogen uses actin polymerisation for intracellular movement and cell-to-cell spread infecting a vast range of host tissues, with the liver being the main site of infection (Rocourt and Cossart, 1997). Meningitis, septicemia and other infections of the central nervous system are commonly seen in patients with listeriosis.

Countries differ to their regulatory approach to the presence of *L. monocytogenes* in ready to eat (RTE) food. In the USA there is a “zero tolerance policy” on the presence of *L. monocytogenes* in RTE food. Recent European Union regulations generally permit a count up to 100 CFU/g at the end of shelf life for RTE foods, except those intended for infants and special medical purposes (Lawley et al., 2008).

1.6 Food preservation concerns

1.6.1 Spoilage Microorganisms

Microbial spoilage of foods and beverages is caused by a wide variety of bacteria, moulds and yeasts. Spoilage of fruit and vegetable juices is primarily due to the proliferation of its natural acid tolerant and osmophilic micro flora (Tahiri et al., 2006). The microflora is represented by yeasts, responsible for the fermented taste and carbon dioxide production; lactic acid bacteria, which can produce a buttermilk off-flavour, and moulds which contribute to the spoilage of juices by their surface growth (Tournas et al., 2006). The particular factors which can influence microbial growth are intrinsic (e.g. pH, water activity, salts, sugars) and extrinsic (e.g. temperature, modified atmosphere, heat treatment) factors. Examples of minimum growth requirements for major spoilage organisms are given in Table 1.6 (Betts, 2006) and certain spoilage microorganisms associated with food products are described in Table 1.6.

Table 1.6: Examples of minimum growth requirements for major spoilage organisms

Microorganism	Minimum pH for growth	Minimum a_w ¹ for growth	Minimum growth temperature (°C)	Reference
<i>Pseudomonas</i> spp.	5.0	0.97	0	Tomlins and Ordal, 1976
Lactic acid bacteria	3.5	0.90	4	Tomlins and Ordal, 1976
Yeasts	1.5	0.62	-34	Put and De Jong, 1980
Moulds	1.5	0.61	-12	Hatcher et al., 1979

¹ Water activity (*Adapted from Betts, 2006*)

1.6.1.1 *Saccharomyces* species

Yeasts predominate in spoilage of acid food products as they have the ability to exhibit good growth at low pH, high sugar concentration and low water activity and resist inactivation by heat processing which makes them able to grow or survive in fruit or fruit products (Put and de Jonge, 1980; Stratford et al., 2000). Fruit juices are generally rich in simple carbohydrates and complex nitrogen sources, and hence are ideal substrates for yeasts. More than 110 species of yeasts have been listed as associated with foods, of which a large proportion occur on fruits, and more than 40 in soft drinks (Barnett et al., 2000).

S. cerevisiae is one of the most important yeasts causing spoilage of fruit juices and soft drinks (Fleet, 1992; Deak and Beuchat, 1996; Pitt and Hocking, 1997; Barnett et al.,

2000). Several authors reported that fruit juice concentrates, fruit pulps, packaged fruit juices and soft drinks are particularly prone to fermentative spoilage with *S. cerevisiae*, *S. bayanus* and to a lesser extent *S. pastorianus* (Torok and King, 1991; Deak and Beuchat, 1993, 1993a; Thomas, 1993; Sancho et al., 2000; Stratford et al., 2000; Arias et al., 2002; Heras-Vazquez et al., 2003). Therefore, numerous heat inactivation studies have been conducted with *S. cerevisiae* because of its significance in the spoilage of heat pasteurised fruit juices and carbonated beverages (Fleet, 1992; Thomas, 1993; Stratford and James 2003). *S. cerevisiae* is also important in the spoilage of concentrates, juices and fruit beverages (Gardini and Guerzoni, 1986; Deak and Beuchat, 1993), and in that respect is considered to be the source of most problems associated with processed fruits (Maimer and Busse, 1992).

Growth of yeasts is usually accompanied by formation of carbon dioxide and alcohol. Carbon dioxide gives the product a gassy, frothy appearance and causes a packaged product to swell and explode. In addition, the products develop a distinctive alcoholic, fermentative smell and taste. Spoilage of fruit juice makes it unacceptable for human consumption.

1.6.1.2 *Alicyclobacillus acidoterrestris*

In 1984, *Alicyclobacillus* was reported in aseptically packaged apple juice (Cerny et al., 1984). *B. acidoterrestris* has been reclassified as *Alicyclobacillus*, based on the presence of the ω -alicyclic acids and on DNA sequence evidence (Wisotzkey et al., 1992). *A. acidoterrestris*, isolated from fruit juices and soil (Bevilacqua et al., 2006; Goto et al., 2008; Walker and Phillips, 2008), has an impact in many acidic foods; but was originally regarded as the spoiling microorganism of fruit juices (Bevilacqua et al., 2010).

It is an acid-tolerant and heat resistant bacterium that can spoil heat-treated fruit juices by the formation of taint chemicals. *A. acidoterrestris* spore germination and growth

under acidic conditions was reported in orange juice (Pettipher et al., 1997). This endospore former grows at a pH range of 2.5-6.0 and at temperatures between 25-60°C (Jensen, 1999). The main spoilage characteristic is the medicinal or phenolic off-flavour or odour, caused by guaiacol (Yamazaki et al., 1996; Bahçeci et al., 2005), 2,6-dibromophenol (Borlinghaus and Engel, 1997) and 2,6-dichlorophenol (Jensen, 2000; Jensen and Whitfield, 2003). Fruit juices are generally treated at temperatures of about 95°C for 2 min (Komitopoulou et al., 1999); however spores have been shown to survive such heat treatments (Pontius et al., 1998; Splittstoesser et al., 1998) and surviving spores can germinate and grow at pH <4 (Walker and Phillips, 2008) in fruit juice, leading to spoilage.

A. acidoterrestris has become an important potential spoilage concern, and it is suggested as a target microorganism for the design of a thermal process for fruit juices. In single strength juice these micro-organisms find a favourable environment for germination and growth that, under certain conditions, can lead to product deterioration (Chang and Kang, 2004).

The design of a thermal process should include an optimisation, in order to make sure that the level of inactivation, required for the target microorganism, is satisfied without impairing the quality of the food product (Vieira et al., 2002).

Inactivation of *A. acidoterrestris* spores by using high treatment conditions such as heat or pressure might have potentially detrimental effects on the nutritional and sensory properties of the products (Bae et al., 2009). Non-thermal processing alternatives have been proposed in response to increasing consumer demand for fresh food with maintenance of product quality (Garcia-Gonzalez et al., 2007). Bae et al. (2009) concluded that non-thermal treatment of supercritical carbon dioxide under mild conditions inactivates *A. acidoterrestris* spores in commercial apple juice without affecting the physical-chemical properties of the juice.

1.6.1.3 *Pseudomonas* species

Psychrotrophic microorganisms such as *Pseudomonas* have great importance in products that are stored at low temperatures. They are responsible for the superficial alteration of refrigerated poultry meat since in aerobic atmospheres they can grow and multiply at very low temperatures (4°C). *Pseudomonas* is one of the most important sources in the spoilage of refrigerated foods, causing putrefactive odours and slime (Sofos, 1994).

Pseudomonas species are important spoilage organisms in many chilled food products, such as milk (Reddy et al., 1969), chicken (Pittard et al., 1982), meat (Edwards et al., 1987), and fish (Miller et al., 1973), in which they become the dominant micro flora during chill storage. This dominance is assumed to be attributable exclusively to their rapid growth at chill temperature. Ghenghesh et al. (2005) investigated the microbiological quality of popular fruit juices (146 juice samples) manufactured and sold in Tripoli city and reported that apple, orange, grape, almond juice samples were positive for *P. aeruginosa*.

1.7 Microbial resistance

1.7.1 Microbial resistance response in food environment:

Most of the foodborne microorganisms are able to survive under diverse conditions. When microorganisms are stressed, an adaptive response may follow which can increase the organisms' tolerance to a subsequent similar stress or to a different type of stress.

The stresses that microorganisms have to overcome in order to survive and multiply in the food chain are diverse. They range from establishing themselves in environments with high salts and high in fatty acid/bile, e.g. intestinal tract of animals, to surviving high temperature treatment and low pH (Brul and Wells, 2005). The preservation technology applied can induce the stress response. Bacterial cells have many

mechanisms at their disposal that allow them to adapt to a change in environment. Many bacteria react to stress by inducing the synthesis of various proteins (Herendeen et al., 1979; Jones and Inouye, 1994). Resistance may be conferred by innate structural features of the bacterial strain, mechanism for antibiotic inactivation or by biofilm formation on food processing surfaces as an adaptive response to protect colonies from cleaning and sanitation (Bower et al., 1999). Adaptation enhances tolerance to environmental, chemical and biological stresses and may promote survival or growth in adverse environments. Adaptation to stresses is mediated by changes in the physiology of the organism, including alterations in metabolism and structural changes. These changes can have profound effects on the ability of food spoilage and pathogenic organisms to survive food processing operations and to survive or even grow in normally adverse or harsh food environments (Johnson, 2003). Stress responses would be expected to affect the resistance and survival of pathogens and spoilage organisms through the entire food production chain, from preharvest activities, processing operations, and storage of foods during their shelf-life. Potential stresses encountered that are likely to provoke stress response in foodborne microorganisms at various steps of the food chain have been reported in Table 1.7 (Yousef and Juneja, 2003).

Table 1.7: Potential stresses encountered and responses

Stresses	Relevance to food	Damage	Stress responses, cross responses	Induced gene products
Acidity	Occurs in aquatic environment and in food production	DNA, outer membrane	Acid tolerance, alkali sensitivity, UV resistance, salt tolerance, thermotolerance, H ₂ O ₂ tolerance, resistance to polymixin B	Hyd genes Rpos PhoP Heat shock proteins Lysine or arginine decarboxylase
Heat	During food production, preparation and cooking	DNA, outer membrane, Ribosomes	Thermotolerance, Acid tolerance, Alkali tolerance, UV tolerance, HHP tolerance	Heat shock proteins
Cold	Referigeration	RNA	HHP tolerance	Cold shock proteins
Osmotic stresses (low a _w)	Foods with high level of salt or sugars	outer membrane, transport processes	Osmotolerance Tolerance to oxidative components Thermotolerance HHP tolerance	Heat shock proteins ProP, ProU etc
Starvation	In contaminated waters, if used for food processing	Proteins	Thermotolerance, Acid tolerance, Alkali tolerance, Salt tolerance, H ₂ O ₂ tolerance, Osmotolerance, HHP tolerance	Proteases Heat shock proteins
Irradiation	Preservation	DNA	UV tolerance, Thermotolerance, Acid tolerance, Alkali tolerance	Heat shock proteins UvrA, B,C RecA LexA
High hydrostatic pressure	Preservation	Outer membrane, cytosolic membrane, ribosomes, Proteins	Thermotolerance, Acid sensitisation	Heat shock proteins Cold shock proteins proteases
Pulsed electric field	Preservation	Cytoplasmic Membrane	-	-

(Adapted from Yousef and Juneja, 2003)

The foodborne pathogens *E. coli* O157:H7 and *L. monocytogenes* are among the most important bacteria responsible for food illness. Most enterohaemorrhagic *E. coli* (EHEC) infections in the United States and the UK are caused by *E. coli* O157:H7 (Willshaw et al., 2001; CDC, 2003), and more than 75,000 cases of foodborne diseases attributable to *E. coli* O157:H7 occur every year (Perna et al., 2001).

Acidification is a method commonly used in the food industry to control growth and survival of spoilage as well as pathogenic microorganisms (Brown and Booth, 1991). Acid adaptation and increased resistance to subsequent acid stress have been reported in various foodborne pathogens such as *Listeria* (Kroll and Patchett, 1992; Hill et al., 1995; Koutsoumanis et al., 2003; Koutsoumanis and Sofos, 2004; Caggia et al., 2009), *Salmonella* (Foster, 1991; Foster and Hall, 1990) and *E. coli* O157:H7 (Garren et al., 1997; Skandamis et al., 2007).

1.7.2 Stress responses of *E. coli*

Acid foods such as apple juice, apple cider and hard salami have been reported to be associated with outbreaks of foodborne illness caused by *E. coli* O157:H7 (Steel et al., 1982; Besser et al., 1993; CDC, 1995). Acid adapted *E. coli* O157:H7 had an increased acid resistance and enhanced survival in shredded dry salami (pH 5.0) and apple cider (pH 3.4) (Leyer et al., 1995). Acid adaptation also enhanced the survival of *E. coli* O157:H7 in acidic fruit juice such as mango juice (pH 3.2) and asparagus juice (pH 3.6) (Cheng and Chou, 2001). Under stress conditions, microorganisms utilise signal transduction systems (movement of signals from outside the cell to inside the cell) to sense environmental stresses and to control the coordinated expression of genes involved in cellular defense mechanisms (Kennelly and Potts, 1996). The modification of sigma factors (σ) bound to core RNA polymerase, conferring promoter specificity, is possibly the most important regulatory mechanism in bacterial cells (Abee and Wouters, 1999). The induction of this σ factor can occur in response to starvation, generally when cells enter the stationary phase of growth, and also when exponentially growing cells are subjected to stresses other than starvation (Dodd and Aldsworth, 2002).

Heat stress

The heat shock response in bacteria is a protective mechanism to cope with heat induced damage to proteins by synthesising a specific set of proteins known as heat shock

proteins (HSPs) (Schlesinger, 1990). Sub lethal heat stress (heat shock) or prior exposure to low heat may render *E. coli* O157:H7 more resistant to subsequent heat treatment, which would otherwise be lethal (Juneja et al., 1998). Until now, over 30 proteins associated with the physiological response to heat have been identified, including GroES, GroEL, and DnaK chaperone proteins involved in the folding, repair, and degradation of proteins (Seyer et al., 2003).

Both pathogenic and non-pathogenic *E. coli* exhibit a stress response to sub lethal environmental stresses. These stress responses can result in resistance to a variety of environmental or processing parameters. The survival and growth of *E. coli* in foods depends on the interaction of intrinsic (food related) and extrinsic (environmental) factors such as temperature, pH and water activity (Buchanan and Doyle, 1997).

Acid stress

Acid stress is described as the combined biological effect of H⁺ ion (i.e. pH) and weak acids (organic) in the environment as a result of fermentation or when added as preservatives in foods. The development of acid tolerance can be pH dependant or pH independent or a combination of both types (Lin et al., 1995). *E. coli* O157:H7 with induced acid resistance can remain acid resistant in foods (Castanie-Cornet et al., 1999). Three systems are involved in acid tolerance: an acid induced oxidative system, an acid induced arginine dependant system and glutamate dependent system. The membrane composition *per se* has a prime influence on stress sensitivity. Membrane cyclopropane fatty acid content was a major factor in the acid resistance of *E. coli* (Chang and Cronan, 1999). Acid adapted *E. coli* O157:H7 had an increased resistance to lactic acid, and showed enhanced survival compared to nonadapted cells during sausage fermentation, in shredded dry salami (pH 5.0), and in apple cider (pH 3.5) (Leyer et al., 1995). Acid resistance and survival of pathogens are highly important issues associated with food safety. The ability of non-acid adapted *E. coli* O157 to adapt in short duration

under extreme conditions further results in increased virulence (Beales, 2004). Acid adaptation of *E. coli* O157:H7 and other pathogens enhanced the survival of these microorganisms to thermal treatments in apple, orange and white grape juices (Mazzotta, 2001). Buchanan et al. (2004) reported an increased resistance of *E. coli* O157:H7 to irradiation when cells were previously acid adapted. Benito et al. (1999) studied variation in resistance of natural isolates of *E. coli* O157:H7 to high hydrostatic pressure, mild heat, and other stresses. The results revealed that there were wide differences in pressure resistance among strains and that the most pressure resistant strains were also more resistant to other adverse treatments.

Starvation stress

During starvation conditions many bacterial species show increased resistance to a number of environmental stresses such as low pH, heat and oxidative stress (Watson et al., 1998). Microorganisms respond to starvation stress by arresting all metabolic activity and growth (Chung et al., 2006). They produce many degradative enzymes such as proteases, lipases, and substrate capturing enzymes such as glutamine synthetase and alkaline phosphatase (Kjelleberg et al., 1987; Matin et al., 1989; Siegele and Kolter, 1992).

Cold Stress

Many microorganisms cope with low temperature stress, and are able to survive and grow at low temperatures. This response to cold temperature is mainly due to synthesis of cold shock proteins. They bind RNA without apparent sequence specificity and with low binding affinity (Jiang et al., 1997). These cold shock proteins function as RNA chaperones at low temperatures (Graumann and Marahiel, 1998). Adaptation of microorganisms to low temperature represents a food safety risk since refrigeration is one of the most common methods used to preserve foods (Garcia et al., 2001; Russell, 2002).

Osmotic stress

Osmotic stress induced starvation Pex proteins as well as HSPs in *E. coli* (Schultz et al., 1988). Increased osmolarity in bacterial cells is associated with the inhibition of DNA replication, cell growth and nutrient uptake (Csonka, 1989). Bacterial cells usually use two protective mechanisms to survive hyperosmotic stress; these consist of discharging the excess solutes outside the cells and accumulating compatible solutes or osmolytes (Rodriguez-Romo and Yousef, 2005).

Oxidative stress

An inescapable consequence of life under aerobic conditions is production of reactive oxygen species (ROS), including hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\cdot\text{O}_2^-$), peroxy radical ($\text{ROO}\cdot$), and hydrogen peroxide (H_2O_2), which are formed during respiration and other metabolic reactions (Gonzalez-Flecha and Demple, 1995; Imlay, 2003). Due to high reactivity, oxygen radicals cause a great deal of damage to all biological macromolecules and thus represent a potential threat to living cells, breaking membrane functions, affecting enzyme activities, and causing DNA damage (Imlay, 2003; Jang and Imlay, 2007). Virtually all aerobic organisms have evolved complex antioxidant defense systems, comprising low molecular weight antioxidants, antioxidant enzymes, scavenging ROS, and reparation enzymes, removing oxidative damage of macromolecules (Pomposiello and Demple, 2002).

E. coli respond to oxidative stress by modifying the expression of many genes resulting in induction of proteins with antioxidant functions e. g. superoxide dismutase and catalase. Two redox-responsive transcription regulators (SoxR and OxyR) have been well defined in *E. coli* and serve as paradigms of redox-operated genetic switches. Both proteins have the remarkable ability of directly transducing oxidative signals to genetic regulation. Both proteins are expressed constitutively in an inactive state and are transiently activated in cells under specific types of oxidative stress. The activation of

the OxyR and SoxR proteins results in the transcriptional enhancement of sets of genes (regulons) whose products relieve the stress by eliminating oxidants and preventing or repairing oxidative damage (Pomposiello and Demple, 2001).

SoxR is a member of the MerR family of metal-binding transcriptional factors and it exists in solution as a homodimer with each subunit containing a [2Fe-2S] cluster (Figure 1.3). These clusters are in the reduced state in inactivated SoxR and their oxidation activates SoxR as a powerful transcriptional factor (Gaudu and Weiss, 1996). The SoxR remains tightly bound to its target promoter SoxS in the absence and presence of oxidative stress (Chander et al., 2003; Gaudu and Weiss, 1996). The SoxR protein is produced constitutively at a low level and activates expression of *soxS* gene in response to superoxide generating agents. Oxidation of SoxR induces the transcription of the *soxS* gene. The activity of SoxS is regulated only by its intracellular concentration and the protein binds promoter regions of the target genes to recruit RNA polymerase (Pomposiello and Demple, 2001). These inducible genes (Figure 1.4) constitute the SoxRS regulon, which includes manganese superoxide dismutase (*sodA*), the DNA repair enzyme endonuclease IV (*nfo*), and $\cdot\text{O}_2^-$ resistant isozymes of fumarase (*fumC*) and aconitase (*acnA*). SoxRS activation also leads to increased levels of glucose 6 phosphate dehydrogenase (*zwf*), which increases the reducing power of the cell, and the elevated levels of the Fur repressor (*fur*), which may decrease iron uptake and therefore diminish the formation of $\cdot\text{OH}$. The *tolC* encoded outer membrane protein, the *acrB* encoded drug efflux pump, and the MicF regulatory RNA, which represses the expression of the outer membrane porin, all are likely to exclude redox active compounds that lead to increased $\cdot\text{O}_2^-$ levels. The roles of the SoxRS-induced flavodoxin A (*fldA*) and ferredoxin/ferredoxin-NADP⁺ reductase (*fpr*) are unknown, although they might function to maintain the reduced state of Fe-S clusters (Hidalgo and Demple, 1996; Jamieson and Storz, 1997; Storz and Imlay, 1999).

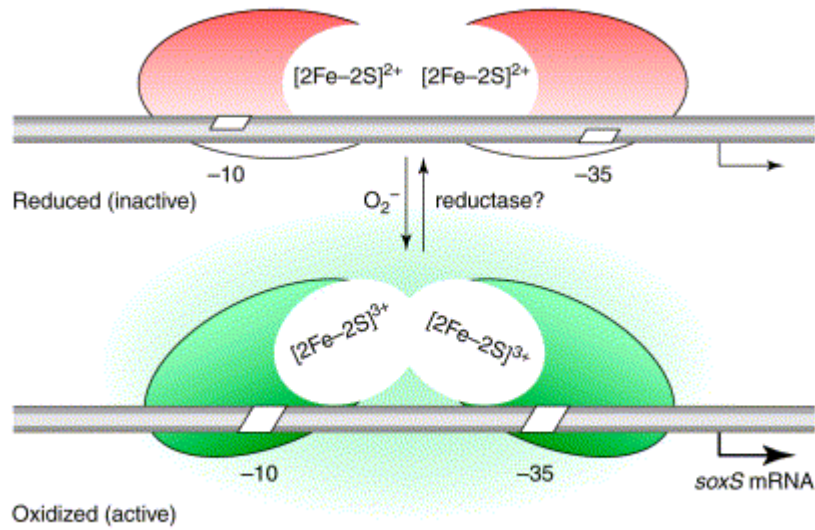


Figure 1.3: Mechanism of SoxR activation by superoxide.

The SoxR dimer can bind DNA in either the reduced or the oxidised form. However, only oxidised SoxR activates transcription of *soxS*. After exposure to superoxide-generating agents, the iron in the Fe-S clusters is oxidized. The model for the activation of *soxS* proposes a conformational change in SoxR that modifies the local DNA topology at the promoter and compensates for a dysfunctional spacing between promoter elements. The oxidation of SoxR is rapid and transient: after cessation of the superoxide stress, SoxR is completely re-reduced in a few minutes. The mechanism of SoxR re-reduction remains elusive.

Adapted from Pomposiello and Demple, 2001

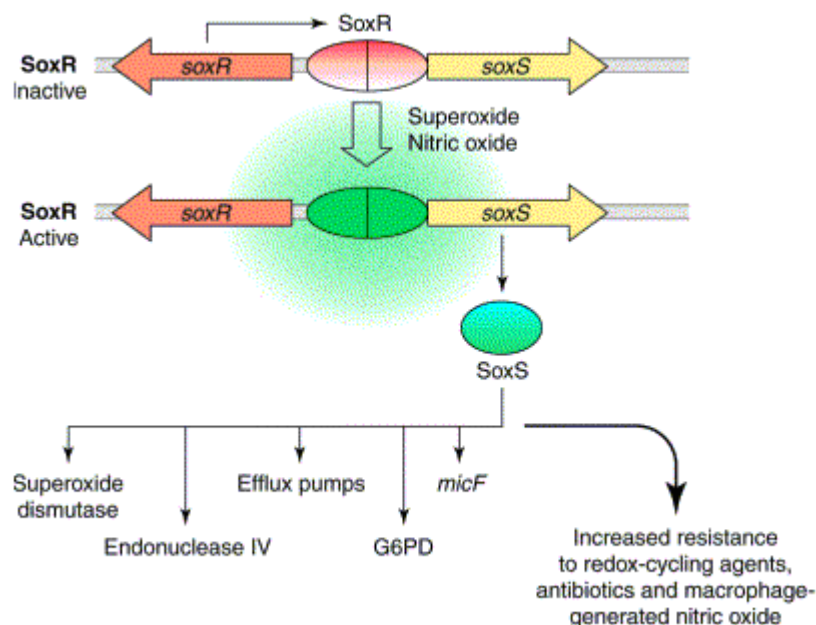


Figure 1.4: The soxRS regulon.

The soxRS locus is composed of the divergently transcribed *soxR* and *soxS* genes. The SoxR protein is produced constitutively and is activated upon exposure to superoxide-generating agents or nitric oxide (NO). The oxidised form of SoxR enhances the transcription of the *soxS* gene, the product of which is also a transcriptional activator. The SoxS protein activates transcription of genes that increase the resistance to oxidants. Additionally, activation of the SoxS-regulated genes increases the resistance to antibiotics and macrophage-generated NO. Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

Adapted from Pomposiello and Demple, 2001

With *E. coli*, in response to an increased flux of H_2O_2 a set of genes is induced, part of which are under the control of transcriptional regulator OxyR and include hydroperoxidase I (catalase, *katG*), the two subunits of alkylhydroperoxide reductase (*ahpCF*), glutathione reductase (*gor*), glutaredoxin 1 (*grxA*), iron uptake repressor Fur (*fur*), and many others (Hidalgo and Demple, 1996; Jamieson and Storz, 1997; Tao, 1997; Zheng et al., 1998, Zheng et al., 2001). Several other transcriptional regulators in addition to SoxR and OxyR modulate the expression of antioxidant genes, illustrating the extensive connectivity between the SoxRS/OxyR regulons and other regulatory networks (Storz and Imlay, 1999).

The *rpoS*-encoded σ^S has been shown to regulate the expression of several antioxidant genes including hydroperoxidase II (*katE*), exonucleaseIII (*xthA*), Copper-zinc superoxide dismutase (*sodC*) (Gort et al., 1999; Loewen et al., 1998). The SoxRS regulated *pqi5* gene and the OxyR regulated *katG*, *gorA* and non specific DNA binding protein (Dps) genes are also part of the σ^S (Loewen et al., 1998). Heat shock protein DnaK has also been shown to be induced by the treatment with H₂O₂, nalidixic acid, UV irradiation and starvation (Farr and Kogoma, 1991).

1.7.3 Stress responses of *L. monocytogenes*

Acid stress

L. monocytogenes encounters a low-pH environment in acidic foods, during gastric passage and in the phagosome of the macrophage (Cotter and Hill, 2003). The pathogen responds to and survives in these low-pH environments by utilising a number of stress adaptation mechanisms. Phan-Thanh and Mahouin (1999) studied the expression of proteins by exposing cells to a lethal acidic pH (acid stress) and a non-lethal acidic pH (acid adaptation). The protein GroEL that showed increased synthesis during the growth of *Listeria* at low temperature was also induced under acid stress. Other proteins induced were ATP synthase and various transcriptional regulators. Acid-adapted *L. monocytogenes* (pH 5.2, 2 h) had increased resistance to heat shock (52°C), osmotic shock (25–30%NaCl) and alcohol stress, suggesting that acid adaptation also provides cross-protection against other stress factors (Phan-Thanh et al., 2000). The cross-resistance of acid adapted cells to other stresses has important implications for the food industry, particularly since foods commonly encounter sublethal acidic treatments during processing (van Schaik et al., 1999).

L. monocytogenes is a facultative anaerobic bacterium that may use both processes for pH homeostasis (Shabala et al., 2002). *L. monocytogenes* utilises the glutamate decarboxylase (GAD) system which is vital for the acid resistance and to successfully

pass through the gastric environment and infect the small intestine (Cotter et al., 2001). The GAD system is composed of three genes *gadA*, *gadB*, *gadC*. The *gadA* and *gadB* genes encode two glutamate decarboxylase and the *gadC* encodes a glutamate/ γ -aminobutyrate antiporter (Cotter et al., 2001). Wiedmann et al. (1998) conducted studies to determine the role of general stress transcription factor σ B on the acid resistance of *L. monocytogenes*. Their findings suggested that the survival of *L. monocytogenes* upon exposure to an acidic environment is dependent on the expression of σ B-dependent proteins. The survival and increased resistance of log-phase *L. monocytogenes* cells to gastric fluid following exposure to mild acidic conditions were partially dependent on σ B (Ferreira et al., 2003). In addition to the regulation of genes for survival under acid stress conditions, the stress-responsive factor σ B also regulates virulence gene expression in this foodborne pathogen (Kazmierczak et al., 2003). The signal transduction systems can sense changes in the environment such as low pH, oxidative and ethanol stresses via a membrane-associated histidine kinase, and the response regulator enables the cell to respond by altering gene expression (Cotter et al., 1999). This study showed that LisRK signal transduction system is involved in response to stresses such as low pH and regulation of virulence gene expression in *Listeria*. Gahan et al. (1996) used acid-adapted and non-adapted *L. monocytogenes* to compare their survival in a variety of acidic food products. The acid-adapted strain demonstrated enhanced survival compared to the non-adapted culture. Faleiro et al. (2003) showed that acid-adapted strains of *L. monocytogenes* isolated from cheese showed enhanced survival during salt stress (20% NaCl) compared to the non-adapted strains. Caggia et al. (2009) recorded the highest acid tolerance response of *L. monocytogenes* OML 45 strain, after 3 h treatment in TSB adjusted to pH 5.7, thus concluding that cells adapted to acidic environments can grow in normally lethal pH conditions. Although large numbers of studies have reported acid adaptation and increased survival of pathogens,

there remains a gap in the literature with respect to studying the effect of this phenomenon on efficacy of food processing technologies.

Cold stress

L. monocytogenes survival and growth at refrigeration temperatures (2–4°C) make the control of this foodborne pathogen difficult (Rocourt and Cossart, 1997). A high proportion of iso and anteiso, odd-numbered, branched-chain fatty acids characterise the cell membrane of *Listeria* (Annous et al., 1997). Annous et al. (1997) also showed that changing the growth temperature from 20°C to 5°C led to fatty acid shortening (a decrease in C17:0) and a switch from iso to anteiso branching (i-C15:0 to a-C15:0). The shortening of fatty acid chain length decreases the carbon–carbon interaction between neighboring chains in the cell membrane and this helps maintain the optimum degree of membrane fluidity for growth at low temperatures (Beales, 2004). *L. monocytogenes* produces cold shock proteins (Csps) in response to a temperature down shock, and cold acclimation proteins (Caps) are synthesised during balanced growth at low temperatures (Bayles et al., 1996). Liu et al. (2002) identified RNAs that are synthesised at higher levels when *L. monocytogenes* is grown at 10°C in comparison to 37°C. Increased expression of mRNA for chaperone proteases such as GroEL, ClpP and ClpB indicates that these enzymes may be involved in the degradation of abnormal or damaged polypeptides that arise due to growth at low temperatures (Liu et al., 2002).

Osmotic stress

The ability of *L. monocytogenes* to accumulate compatible solutes such as glycine betaine and carnitine and the role of these compounds as cryoprotectants has been widely studied (Angelidis and Smith, 2003; Wemekamp-Kamphuis et al., 2004).

The general stress sigma factor σ_B is stimulated in response to temperature downshift and the sigB mutant fails to accumulate solutes such as betaine and carnitine in *L.*

monocytogenes (Becker et al., 2000). This leads to accumulation of cryoprotectants as one of the functions of σ during growth at low temperature.

The response of microorganisms to osmotic stress involves both physiological changes and variations of gene expression patterns and is called osmoadaptation (Hill et al., 2002). Ctc protein was induced in response to salt stress (Duche et al., 2002). The *ctc* gene was involved in the resistance of *L. monocytogenes* to high osmolarity in the absence of osmoprotectants such as glycine betaine and carnitine in the medium (Gardan et al., 2003). Bayles and Wilkinson (2000) showed that glycine betaine, proline betaine, acetyl carnitine, carnitine, butyrobetaine and 3-dimethylsulphoniopropionate function as osmoprotectants in *L. monocytogenes*. The cells take up osmolytes from the external environment as a response to osmotic stress, which helps to regain the osmotic balance within cells (Bayles and Wilkinson, 2000). The general stress sigma factor σ^B in *L. monocytogenes* is important for the utilisation of betaine and carnitine as osmoprotectants (Becker et al., 1998). Kallipolitis and Ingmer (2001) identified response regulators that are a part of the two-component signal transduction system and involved in the osmotic stress response.

1.8 Objectives of the study:

Based on the review of the literature, a number of investigations were prioritised and designed for this study. The overall aim was to comprehensively investigate the efficacy of ultrasound and ozone treatments for reduction of microbial issues associated with fruit juices.

In order to fulfil this aim the following objectives were addressed:

- To achieve the overall aim, the controlling parameters were determined and optimised to:
 - Investigate the effect of intrinsic and extrinsic parameters on ultrasound inactivation rate in a liquid system.

- Investigate the effect of extrinsic critical control parameters on ozone inactivation rate in a bubble column.
- To further optimise applications in real food or beverage systems, a sequential approach was used to:
 - Determine bactericidal effects of ozone in model media.
 - Investigate the efficacy of continuous gaseous ozone treatment for reduction of *E. coli* strains in orange and apple juice in optimised conditions.
- It was also important to determine any effect of the matrix of the product itself, therefore the objective was to:
 - Determine the effect of intrinsic parameters (pulp, pH) on ozone inactivation efficacy.
- As described in the introduction, a number of important pathogens can survive in fruit juices and their survival can be closely related to environmental exposure and stresses encountered prior to and during processing, therefore the objectives were to:
 - Investigate the efficiency of ozone for inactivation of *Listeria* spp. in orange juice under optimised conditions.
 - Study the effect of prior acid stress conditions on inactivation efficacy of the applied non-thermal techniques.
- To fully investigate and challenge the applicability of ozone from a practical perspective, a full shelf life study was warranted to:
 - Investigate the application of ozone to extend the shelf life of apple juice.
- Finally, in order to progress the optimised application of any novel or emerging technology, it is important to understand as completely as possible, how and why the technology works against the target microorganisms. This is required to

facilitate optimal application. Therefore to advance knowledge in the area regarding mechanism of action, the objective included in the study was to:

- Investigate the mechanism of action of ozone on microbial populations during the treatment of liquid systems.

Chapter 2 MATERIALS AND METHODS

2.1 Microbial strains, growth conditions and preparation of cell suspensions

The microorganisms, treatment media and non-thermal techniques used in this study are listed in Table 2.1

Table 2.1: List of microorganisms, treatment media and non-thermal techniques used

Microorganisms	Treatment media	Non-thermal technique applied
<i>Escherichia coli</i> ATCC 25922* (generic strain)	Tryptic soy broth (TSB), Model apple juice (MAJ), Model Orange juice (MOJ)	Ultrasound
<i>E. coli</i> NCTC 12900** (non-toxicogenic O157:H7)	TSB, MOJ	Ultrasound
<i>E. coli</i> ATCC 25922*	TSB, Phosphate buffered saline (PBS), MAJ, MOJ, Orange juice, Apple juice	Ozone
<i>E. coli</i> NCTC 12900**	MOJ, Orange juice, Apple juice	Ozone
<i>E. coli</i> ATCC 25922*	Orange juice	Ultrasound and ozone
<i>E. coli</i> BW 25113 (Parent strain) ***	Saline	Ozone
<i>E. coli</i> JW 5437 ($\Delta rpoS$) ***	Saline	Ozone
<i>E. coli</i> JW 4024 ($\Delta soxR$) ***	Saline	Ozone
<i>E. coli</i> JW 4023 ($\Delta soxS$) ***	Saline	Ozone
<i>E. coli</i> JW 3933 ($\Delta oxyR$) ***	Saline	Ozone
<i>E. coli</i> JW 0013 ($\Delta dnaK$) ***	Saline	Ozone
<i>Listeria monocytogenes</i> NCTC 11994*	Orange juice	Ozone
<i>L. monocytogenes</i> ATCC 7644*	Orange juice	Ozone
<i>L. innocua</i> NCTC 11288*	Orange juice	Ozone
<i>Saccharomyces cerevisiae</i> ATCC 9763*	Apple juice	Ozone

* Strains were obtained from microbiology stock culture, DIT

** Strain was obtained from National Collection of Type Cultures, Health Protection Agency, London, UK.

*** Strains were obtained from National BioResource Project (NIG), Japan

Escherichia coli and *Listeria* strains were selected as challenge microorganisms for studying the efficacy of the applied non-thermal treatments. *Saccharomyces cerevisiae* was also chosen to study the growth dynamics in treated apple juice stored under static and dynamic temperature conditions and to study the effect of the applied treatments on the extension of shelf life of fruit juice. The specific microbial strains, growth conditions and preparation of cell suspensions are described below.

2.1.1 *Escherichia coli*

Two strains of *E. coli* (ATCC 25922 and NCTC 12900) were used to investigate the inactivation kinetics for the applied non-thermal treatments. *E. coli* NCTC 12900 (non pathogenic strain of *E. coli* O157:H7) does not produce toxin although it is phenotypically very similar to toxigenic strains of *E. coli* O157:H7. These two representative strains were used for inactivation studies to ensure the possible useful effects of the applied technologies (ultrasound and ozone) against key pathogens of concern to fruit juice processors.

In a separate but related study, to determine the effect of deletion of certain genes (*soxR*, *soxS*, *oxyR*, *rpoS*, *dnaK*) on the survival of *E. coli* during ozone treatment, six different *E. coli* strains were used (Baba et al., 2006) (Table 2.1).

Bacterial strains were maintained as frozen stocks at -70°C in the form of protective beads (Technical Services Consultants Ltd, UK), which were plated onto tryptic soy agar (TSA, Scharlau Chemie) and incubated at 37°C to obtain single colonies before storage at 4°C.

A single colony of *E. coli* was inoculated either into tryptic soya broth with glucose (TSB+G 0.25%, Scharlau Chemie, Barcelona, Spain) or into tryptic soya broth without glucose (TSB-G) and incubated overnight at 37°C. The bacterial cell density was determined by measuring absorbance at 550 nm using McFarland standard (BioMérieux, Marcy -l'Etoile, France) and a working inoculum corresponding to $1.0 \times$

10^8 CFU/mL was prepared. Cells were then adjusted to a density of 1.0×10^6 CFU/mL in the treatment medium; either TSB, phosphate buffered saline (PBS), fresh fruit juices (orange juice, apple juice) or model fruit juices. In some cases, overnight grown culture with a cell density of 10^9 CFU/mL was suspended directly in treatment media.

2.1.1.1 Acid exposure of *E. coli* cells

In order to investigate the impact of prior acid stress or acid adaptation on treatment efficacy, *E. coli* cells were exposed to acid conditions for three time periods (depending on the objective of the study); 1 h, 4 h and 18 h by employing the following procedure (Leyer et al., 1995; Cheng et al., 2003): Working cultures were grown overnight in TSB+G, 0.25% at 37°C. Cells were then harvested by centrifugation (SIGMA 2K15, Bench Top Refrigerated Ultracentrifuge, AGB scientific LTD) at 10,000 rpm for 10 min at 4°C. The cell pellet was washed twice with sterile phosphate buffered saline (PBS, Oxoid, UK), re-suspended in 10 mL TSB (pH5.0, adjusted with HCl, at ambient temperature) and incubated at 37°C either for 1 h or 4 h. For a 18 h acid exposure, bacterial strains were grown directly in TSB+G, 0.25% (pH 5.0) at 37°C. After incubation, cultures were diluted in maximum recovery diluent (MRD, Scharlau Chemie) (pH 5.0) to yield approximately 10^7 CFU/mL, with further dilution in appropriate liquid media to a final concentration of 10^6 CFU/mL.

2.1.2 *Listeria* strains

Three strains of *Listeria* were used to investigate the ozone inactivation kinetics in orange juice (Table 2.1). Two strains of *L. monocytogenes* were used for inactivation studies to ensure the potential useful effects of the applied technologies (ultrasound and ozone) against this key pathogen of concern to food processing industries. *L. innocua* is closely related to *L. monocytogenes* hence it was also used for inactivation studies to determine the inactivation kinetics of this organism.

Bacterial strains were maintained as frozen stocks at -70°C in the form of protective beads (Technical Services Consultants Ltd, UK), which were plated onto tryptic soy agar (TSA, Scharlau Chemie) and incubated at 37°C to obtain single colonies before storage at 4°C.

To obtain a non acid stressed control population, cells were grown in TSB without glucose (TSB-G) at 37°C.

2.1.2.1 Mild acid stress and acid stress habituation of *Listeria* cells

In order to obtain mild acid stressed cells, a single isolated colony of each strain was inoculated separately in TSB+G, 0.25% and was incubated overnight at 37°C. Cells were then harvested by centrifugation as described above in section 2. 1.1.1 and the bacterial density was determined by using McFarland standard. For each investigation, the cell concentration was further diluted to yield a final concentration of 10⁶ CFU/mL when suspended in orange juice.

To obtain a mild acid stress-habituated cells, two acid stress-habituating conditions were imposed, i.e., 1 h and 18 h. For the 1 h habituation environment, working cultures were grown overnight in TSB+G, 0.25% at 37°C (thus creating a mild acid stress environment). Cells were then harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cell pellet was washed twice with sterile PBS, re-suspended in 10 mL TSB adjusted to pH 5.5 (using lactic acid), and incubated at 37°C for 1 h (Caggia et al., 2009). To prepare 18 h habituated cells, bacterial strains were grown directly in TSB+G, 0.25% (pH 5.5) at 37°C. The mild acid stress-habituated cells were further diluted in orange juice to a final concentration of 10⁶ CFU/mL.

To obtain a population habituated in orange juice, the cells were grown in TSB+G, 1.25% leading to acid stressed cells which were then habituated in orange juice for 90 min at 37°C. This was performed to produce a more acid stressed population, as described by Buchanan and Edelson (1996) with some modifications. The pH of the

culture following overnight incubation was measured using a pH meter (Orion model, UK) with a glass electrode and was found to be in the range of 4.4-4.6. Cultures were then centrifuged as described above and the cell pellet was resuspended directly in 10 mL orange juice (pH 3.5-3.7) and incubated at 37°C for 90 min. Cultures were further diluted in orange juice to yield an approximate final concentration of $10^6 - 10^7$ CFU/mL. Cells prepared under this range of conditions were then treated with ozone in orange juice.

2.1.3 *Saccharomyces cerevisiae*

Saccharomyces strain was maintained as frozen stocks at -70°C in the form of protective beads, which were then plated onto potato dextrose agar (PDA, Scharlau Chemie) and incubated at 30°C for 48 h to obtain colonies before storage at 4°C.

Working cultures were prepared by inoculating a single colony into malt extract broth (MEB, Scharlau Chemie) and incubating at 30°C for 24 h.

2.1.3.1 Preparation of cell suspensions

S. cerevisiae cells grown for 24 h were harvested by centrifugation as described in section 2.1.1.1 and the cell pellet was re-suspended in PBS. The yeast density was determined by measuring absorbance at 550 nm using McFarland standard. The inoculum was then diluted in apple juice to obtain approximately 10^6 CFU/mL. The cell concentration was further diluted in apple juice to yield a final concentration of 10^3 CFU/mL. Apple juice samples inoculated with *S. cerevisiae* were then treated with ozone for 8 min.

2.2 Liquid systems under investigation

Different liquid systems were used in this study. General liquid media; TSB and PBS were used to investigate the efficacy of the applied non-thermal treatments. Others included saline (0.85%) solution, fresh orange juice, fresh apple juice, model orange juice and model apple juice.

2.2.1 Fresh orange juice and model orange juice

2.2.1.1 Fresh orange juice unfiltered

Oranges (*Citrus sinensis* variety: Balady, Egypt) were purchased from a local market, washed with tap water and halved. Juice was extracted with a fruit juicer (Rowenta NEO type 8332). All juice preparations were stored at 4°C. The pH was measured using a pH meter with a glass electrode and was in the range of 3.75 ± 0.35 .

2.2.1.2 Fresh orange juice filtered (without pulp)

Juice without pulp was prepared as above but with an additional centrifugation at 13000 rpm for 10 min, followed by filtering the juice through Whatman No.1 filter paper, giving a 75% yield in terms of filtrate.

2.2.1.3 Fresh orange juice with reduced pulp content

Juice with reduced pulp was prepared as above and submitted to a finishing process by passing through sieves (Laboratory test sieve, Retsch, Germany) to further reduce the pulp content. Two different sieve sizes were employed to obtain juice with different pulp levels using a sieve size of 500 μm (mesh no.35) and a sieve size of 1mm (mesh no.18).

2.2.1.4 Preparation of model orange juice

Model orange juice (MOJ) with a pH of 3.0 was prepared as per the method described by Shinoda et al. (2004). The composition of MOJ per 100 mL was as follows: sucrose: 5.0 g; glucose: 2.5 g; fructose: 2.5 g; citric acid: 1.0 g; ascorbic acid: 30 mg; L-serine: 7.0 mmol (735.63 mg); L-asparagine: 5.4 mmol (713.39 mg), L-alanine: 1.9 mmol (169.27 mg); L-arginine: 0.75 mmol (130.65 mg); L-glutamic acid: 0.54 mmol (79.45 mg); L-proline: 0.42 mmol (48.35 mg).

2.2.2 Apple juice and model apple juice

2.2.2.1 Unclarified and clarified apple juice

Unpasteurised apple (*Malus domestica*) juice (unclarified) (10.7°Brix) free of added preservatives was purchased from a local processor (Artizan Food Company, Dublin, Ireland), dispensed in 20 mL sterile centrifuge tubes under aseptic conditions and stored at -20°C. Stored apple juice samples were thawed in cold water prior to use. The pH of apple juice was measured using a pH meter with a glass electrode and was approximately 3.2.

Unclarified apple juice was centrifuged at 13000 rpm for 10 min and the resulting clarified juice was dispensed in 20 mL sterile centrifuge tubes under aseptic conditions and stored at -20°C.

2.2.2.2 Model apple juice

Model apple juice (MAJ) was prepared in the laboratory as per the method described by Reinders et al. (2001). The composition of MAJ per 1000 mL was as follows: fructose: 66 g; glucose: 22 g; sucrose: 27 g; sorbitol: 6.0 g; malic acid: 6.0 g; sodium citrate: 0.07 g; K₂HPO₄·3H₂O: 2 g.

2.2.2.3 Apple juice of different pH levels

To determine the effect of pH on the inactivation efficacy of the applied treatments, five different pH levels i.e., 3.0, 3.5, 4.0, 4.5 and 5.0 were studied. The unadjusted pH level of apple juice was measured as 3.13 using a pH meter with a glass electrode. The pH of apple juice was adjusted from 3.0 to 5.0 by addition of either 10% malic acid or 1 N NaOH.

2.3 Ultrasound treatment

2.3.1 Experimental set-up

Liquid samples (50 mL) were sonicated in a 100 mL glass beaker using a VC750 ultrasound generator (Sonics and Materials, Inc., Newtown, Conn., U.S.A.) fitted with

an autoclavable 13 mm diameter ultrasound probe attached to an ultrasound transducer (Figure 2.1). Samples were processed at a constant frequency of 20 kHz. The measurement of the amplitude as an indication of the ultrasonic cavitation is reported to be a reliable method for indication of the ultrasound power (Tsukamoto et al., 2004). Before and after each experiment, the ultrasound probe was sterilised by washing with Virkon (DuPont, Ireland), followed by thorough rinsing with sterile water.



Standard Probe

Figure 2.1: Sonics VC-750 Sonicator for liquid processing with a standard probe (13mm diameter) encased in a soundproof box

2.3.2 Experimental design

Experiments were performed with non-acid exposed control cultures as well as a range of acid exposed cultures of *E. coli*. Treatment media (TSB, MAJ, MOJ) inoculated either with *E. coli* ATCC 25922 or NCTC 12900 were treated with ultrasound at amplitude levels of 0.4 μm , 7.5 μm and 37.5 μm with pulse durations of 5 sec on and 5 sec off for up to 15 min. The sample beaker was placed in an ice bath to dissipate the heat generated during ultrasound treatment, and processing temperatures were always maintained below 30°C. Samples (1 mL) were removed for analysis at 3 min intervals. All experiments were carried out in duplicate.

2.3.3 Microbiological analysis

Treated samples were serially diluted in MRD. Aliquots (0.1 mL) of appropriate dilutions were plated on TSA in duplicate and plates were incubated at 37°C for 24 h. Results were reported as $\text{Log}_{10}\text{CFU/mL}$.

2.4 Ozone treatment

2.4.1 Experimental set up

Ozone was produced by a corona discharge generator (Model OL80, OzoneLab™, Ozone services, Canada). Pure oxygen was supplied via an oxygen cylinder (Air Products Ltd., Dublin, Ireland) and the flow rate was controlled using an oxygen flow regulator. Experiments were carried out in a 100 mL glass bubble column or in a 250 mL glass bubble column with heating jacket (Figure 2.2). Ozone concentration was recorded using an ozone analyser (built in ozone module OL80A/DLS, Ozone services, Burton, Canada). Temperature was controlled in the 250 mL bubble column by circulating water at the appropriate temperature through a heating jacket. Steady state ozone production was achieved prior to sample treatment by passing ozone through sterile distilled water for 10 min at the required flow rate and temperature. To prevent excess foaming, 10-20 μL sterile anti-foaming agent (Antifoam B emulsion, Sigma

Aldrich Ireland Ltd., Dublin, Ireland) was added to the medium before each ozone treatment.

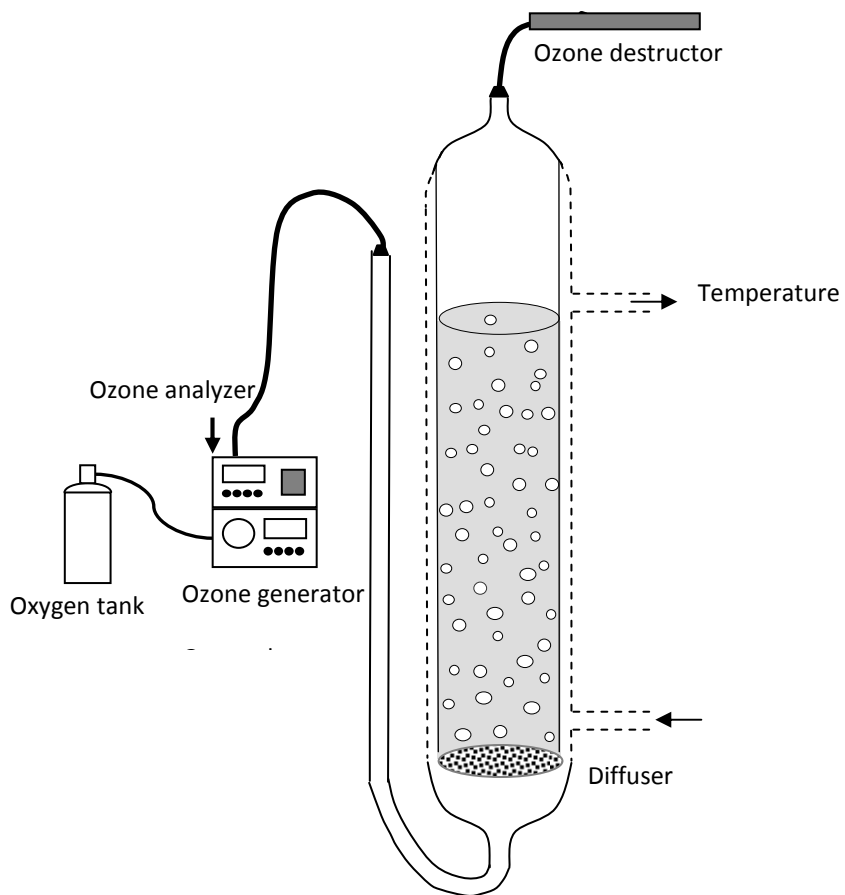


Figure 2.2: Schematics of ozone processing equipment

2.4.2 Experimental design

2.4.2.1 Determination of the effect of different extrinsic control parameters

A working concentration of 1×10^6 CFU/mL *E. coli* ATCC 25922 inoculum was prepared in TSB and treated with ozone to determine the effect of different extrinsic parameters on the inactivation efficacy of ozone.

Experiments were conducted to determine the effect of gas flow rate (0.03, 0.06, 0.12, 0.25, 0.5 and 0.75 L/min), effect of different ozone concentrations (in the range of 28-120 $\mu\text{g/mL}$ and 17-75 $\mu\text{g/mL}$) at two different flow rates i. e. 0.06 L/min and 0.12 L/min (Table 2.2) and the effect of temperature on ozone treatment efficacy (12-15°C, 20°C, 25°C and 30°C) utilising the pre-determined optimum flow rate (0.12 L/min). Samples were treated with ozone for up to 30 min and 1 mL samples were removed at 5 min intervals. All experiments were carried out in duplicate and replicated at least twice.

Table 2.2: Ozone concentrations at two selected gas flow rates

Levels of ozone concentration	Flow rate 0.06 L/min concentration ($\mu\text{g/mL}$)	Flow rate 0.12 L/min concentration ($\mu\text{g/mL}$)
Level 1 (Regulator setting 2)	28-49	17-28
Level 2 (Regulator setting 4)	60-70	33-40
Level 3 (Regulator setting 6)	82-87	46-56
Level 4 (Regulator setting 8)	103-114	67-70
Level 5 (Regulator setting 10)	115-120	72-75

2.4.2.2 Ozone inactivation of *E. coli* ATCC 25922 in phosphate buffered saline (PBS) and model apple juice (MAJ)

Cells were grown overnight in TSB+G, 0.25% at 37°C and then harvested as described in section 2.1.1.1. The cell concentration adjusted to a density of 10^6 CFU/mL either in PBS or MAJ (pH 3.0) and then treated with different flow rates (0.03, 0.06, 0.12, 0.25, and 0.5 L/min) for 2 min. Samples were removed for analysis at 30 sec intervals.

2.4.2.3 Investigation of ozone inactivation efficacy in model orange juice (MOJ) and fresh orange juice

A previously determined optimum flow rate of 0.12 L/min with an ozone concentration of 72-75 $\mu\text{g/mL}$ was applied for each treatment. Two strains of *E. coli* (ATCC 25922 and NCTC 12900) were investigated for their response to ozone treatment. MOJ was treated for 180 sec with sampling at 30 sec intervals.

Ozone inactivation experiments in fresh orange juice were performed with non-acid exposed control cultures as well as a range of acid exposed cultures; namely 1 h, and 18 h acid exposed cultures. Unfiltered juice was treated for 30 min with samples (1 mL) removed at 3 min intervals. All other juices (juice without pulp and juice with reduced pulp content) were treated for 6-7 min with sampling at 1 min intervals. All experiments were carried out in duplicate and replicated at least twice.

2.4.2.4 Evaluation of effect of ozone inactivation of *E. coli* in apple juice

Two strains of *E. coli* (ATCC 25922 and NCTC 12900) were investigated for their response to ozone treatment. A working concentration of 1×10^6 CFU/mL was prepared in apple juice (either in unclarified or clarified juice).

Ozone treatment was carried out in a 100 mL glass bubble column using a flow rate of 0.12 L/min and ozone concentration of 33-40 $\mu\text{g/mL}$. The operating conditions were selected in such a way that a limited production of ozone was required for a previously optimised flow rate. Experiments were performed with non-acid exposed control cells,

mild acid stressed and with acid exposed cells (1 h, and 18 h acid exposed cells). The treatment of the inoculated apple juice samples (clarified and unclarified) was carried out for 5-6 min with sampling intervals of 30 sec.

To evaluate the effect of different pH levels (3.0-5.0) on ozone inactivation efficacy, the apple juice samples of varying pH were treated at identical ozone treatment conditions (0.12 L/min, 33-40 µg/mL) for 5-18 min with sampling at 30 sec or 1 or 2 min intervals depending on the pH of apple juice.

The surviving population after ozone treatment was determined by the agar overlay method as described in section 2.4.4. All experiments were carried out in duplicate and replicated at least twice.

2.4.2.5 Investigation of the effect of acid environment (low pH) and residual ozone on *E. coli*

To determine if there was an independent effect of the lowest pH on the microbial inactivation (Table 2.3), control experiment with no ozone treatment was conducted at pH 3.0. Untreated apple juice samples were inoculated with *E. coli* ATCC 25922 (10^6 CFU/mL) and samples were removed for analysis at 1 min time intervals up to 6 min. Samples were serially diluted in MRD, and *E. coli* ATCC 25922 population density was determined by agar overlay method.

To determine any possible effect of residual ozone on inactivation of *E. coli* in apple juice an additional experiment was conducted. Apple juice samples of unadjusted pH were inoculated with *E. coli* ATCC 25922 (10^6 CFU/mL) and ozonation was carried out at identical ozone treatment conditions (0.12 L/min, 33-40 µg/mL) for 2 min. The treatment time selected was to ensure a detectable reduction of *E. coli* levels during this post processing analysis. After the 2 min of ozonation, apple juice was stored at 4°C for 3 h. Samples were removed every 15 min, diluted in MRD and microbiological analysis was carried out by agar overlay technique. Non-ozonated samples were also evaluated

for reduction of *E. coli* after 3 h storage at 4°C. Consequently, the potential antimicrobial effect of residual ozone or its breakdown products could be measured by comparison with untreated control samples stored for the same period.

Table 2.3: Time of ozone exposure of *E. coli* in apple juice of different pH

pH level of apple juice	Ozone treatment time (min)
3.0	5.0
3.5	6.0
4.0	9.0
4.5	12.0
5.0	18.0

2.4.2.6 Effect of ozone inactivation of *Listeria* strains in orange juice

Three strains of *Listeria* (Table 2.1) were investigated for their response to ozone treatment in orange juice submitted to a finishing process by passing through a sieve of 1 mm diameter (mesh no. 18) to reduce the pulp content. A working concentration of 1×10^6 CFU/mL was prepared in orange juice. Experiments were conducted with non-acid stressed control cells, mild acid stressed cells, acid stress-habituated cells (1 h, and 18 h acid stress-habituated) and cells habituated in orange juice.

Ozone treatment was applied in a 100 mL glass bubble column at an optimum flow rate of 0.12 L/min with a concentration of 72-75 µg/mL.

The treatment of the inoculated orange juice samples was carried out for 7-8 min with sampling intervals of 1 min. All experiments were carried out in duplicate and replicated at least twice.

2.4.3 Calculation of ozone concentration

Ozone concentration (ozone flux) was calculated from the flow rate of oxygen gas supplied (L/min), ozone concentration recorded on the analyser at a particular flow rate ($\mu\text{g}/\text{mL}$) and the volume of the reactor (mL) used.

e.g. when the flow rate applied was 0.12 L/min, ozone concentration was determined as follows:

$$\frac{\text{Gas flow rate} \times \text{ozone concentration}}{\text{Volume of the reactor}} \quad (2-1)$$
$$= \frac{0.12 \text{ L/min} \times 74 \mu\text{g/mL}}{90 \text{ mL}}$$

= 0.099 mg of ozone/min/mL of reactor

2.4.4 Microbiological analysis

The efficacy of treatments was determined in terms of reduction in viable counts over time. To quantify the effects of extrinsic control parameters, 1 mL samples were removed in duplicate at 5 min intervals, serially diluted in MRD, and 0.1 mL aliquots of appropriate dilutions were plated on TSA in duplicate, incubated at 37°C for 24 h and counted. Control experiments were performed where the working inoculum was exposed to oxygen only.

Surviving populations of *E. coli* cells in orange juice were determined by plating onto TSA and selective media, Sorbitol MacConkey agar (SMAC, Scharlau Chemie, Barcelona, Spain) respectively. Samples (1 mL aliquots) were withdrawn from treated juice at specific time intervals, serially diluted in MRD and 0.1 mL aliquots of appropriate dilutions were surface plated on TSA and SMAC in duplicate. Plates were then incubated at 37°C for 24 h and then counted.

To determine surviving populations of *E. coli* in apple juice, the agar overlay method was employed. Populations of aerobic, indigenous microflora were determined by plating onto TSA and selective medium SMAC. Samples (1 mL) were withdrawn

from the treated juice, serially diluted in MRD and 0.1 mL appropriate dilutions surface plated onto the selected media in duplicate. In order to obtain low microbial detection limits, 1 mL of the treated sample was spread onto 3 plates as described by EN ISO 11290-2 method (ISO 11290-2, 1998). Populations of challenge organisms were determined by the overlay method, where, 100 μ L of dilution or 1 mL of treated sample was surface-plated onto pre-poured TSA plates, followed by incubation at 37°C for 2 h. The plates were then overlaid with SMAC, incubated at 37°C for 22 h and then counted.

Surviving populations of *Listeria* strains were determined by plating onto TSA and selective media (Palcam), respectively. Samples (1 mL aliquots) were withdrawn from treated juice at specific time intervals, serially diluted in MRD and 0.1 mL aliquots of appropriate dilutions were surface plated on TSA and Palcam agar in duplicate. Plates were incubated at 37°C for 48 h and then colony forming units were counted. The non-selective medium TSA was expected to support the growth of both uninjured and ozone injured cells, whereas the selective medium, Palcam agar, was expected to support growth of uninjured populations. The difference from selective to non-selective media gives an indication of cell injury during the ozone treatment. Percent injury was calculated using Eq. (2-2) (Hansen and Knochel, 2001). The time intervals of samples which resulted in colony formation on both media were used.

$$\% \text{ Injured Cells} = \frac{N_{\text{TSA}} - N_{\text{PALCAM}}}{N_{\text{TSA}}} \times 100\% \quad (2-2)$$

A recovery index was defined as the t_{5d} (time required to obtain a 5 log reduction) determined from the counts on the Palcam divided by t_{5d} determined from the counts on TSA (adapted from Hansen and Knochel, 2001).

2.5 Storage studies

Packages (1 L) of sterile, commercially prepared apple juice were obtained from a local retailer. Samples (100 mL) were then inoculated with *S. cerevisiae* cells to yield a final concentration of 10^3 CFU/mL (as described in section 2.1.3.1). This sample served as an untreated control 1. Similarly, untreated control 2 was prepared by inoculating *S. cerevisiae* cells with an inoculum level of 10^1 CFU/mL (in order to commence with a level similar to that attained after 8 min of ozone treatment). The third sample was the ozonated (flow rate of 0.12 L/min and concentration of 33-40 μ g/mL) apple juice.

2.5.1 Static storage study

Unprocessed control samples of apple juice and ozone processed apple juice samples (45 mL each) were stored in incubators (LMS cooled incubators, Dublin, Ireland) at temperatures of 4, 8, 12, and 16°C for a period of up to 30 days. Aliquots of unprocessed and ozone processed samples were taken every day.

2.5.2 Dynamic storage study

For the dynamic storage study, apple juice samples were stored in an incubator (Medical Supply Company Ltd, Dublin, Ireland) where the initial and final temperatures were set to 4 and 16°C. The temperature was programmed to fluctuate according to a profile consisting of 4°C for 12 h followed by ramped increase from 4 to 16°C and maintained at 16°C for 12 h. These temperature profiles were recorded by a temperature sensor connected to a data logger (Grant 1000 series Squirrel meter/data logger, UK). The actual temperature profiles were recorded every 10 min.

2.5.3 Microbiological analysis

Yeast populations were determined by plating onto PDA. Samples (1 mL aliquots) were withdrawn each day from ozone processed and unprocessed juice stored at each temperature, serially diluted in MRD and 0.1 mL aliquots of appropriate dilutions were

surface plated on PDA in duplicate. Plates were incubated at 30°C for 48 h and colony forming units were counted. Results were reported as Log₁₀CFU/mL.

2.6 Evaluation of efficacy of combined ultrasound and ozone treatment

To investigate the combined effectiveness of ultrasound and ozone, *E. coli* ATCC 25922 cells were grown overnight in TSB + G, 0.25% at 37°C and then harvested as described in section 2.1.1.1. A working concentration of 1×10^6 CFU/mL was prepared in fresh orange juice with reduced pulp content (juice passed through sieve size of 1 mm). The inoculated juice sample was then treated simultaneously with ultrasound at amplitude level of 7.5 μ m and ozone at a flow rate of 0.12 L/min with a concentration of 72-75 μ g/mL for up to 10 min with sampling at 2 min intervals. The surviving culturable population was determined on TSA as described in section 2.4.4.

2.7 Assessing the microbial oxidative stress mechanism of ozone treatments through the responses of *Escherichia coli* mutants

For this investigation, parent *E. coli* BW 25113 and a range of mutated *E. coli* strains were used as described in Table 2.1. The activities of regulators that have been knocked out in mutated *E. coli* strains are described in Table 2.4.

Table 2.4: Activities of the regulators knocked out in mutated *E. coli* strains

<i>E. coli</i> strain	Regulator	Gene	Activities knocked out
$\Delta rpoS$	σ^S	fumC, acnA, sodB, sodC, katG, katE, xthA, gorA, dps	Fumarase C, Aconitase A, Iron superoxide dismutase, Copper zinc superoxide dismutase, Hydroperoxidase I, Hydroperoxidase II, Exonuclease III, Glutathione reductase, Non-specific DNA binding protein
$\Delta soxR$	soxRS	sodA, fumC, acnA, zwf, fur, micF, acrAB, tolC, fpr, fldA, nfo	Manganese superoxide dismutase, Fumarase C, Aconitase A, Glucose-6-phosphate dehydrogenase, Ferric uptake repressor, RNA regulator of ompF, Multidrug efflux pump, Outer membrane protein, Ferredoxin reductase, Flavodoxin, Endonuclease IV
$\Delta soxS$			
$\Delta oxyR$	oxyR	katG, ahpCF, gorA, grxA, dps, oxyS, fur	Hydroperoxidase I, Alkyl hydroperoxidase reductase, Glutathione reductase, Glutaredoxin 1, Non-specific DNA binding protein, Regulatory RNA, Ferric uptake repressor
$\Delta dnaK$	HS	dnaK	Heat shock protein

(Table modified from Storz and Imlay, 1999)

2.7.1 Preparation of cell suspension

Cells were grown overnight in TSB-G at 37°C and harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was washed twice with sterile saline solution (0.85%) and re-suspended in 10 mL saline. The bacterial cell density was determined by measuring absorbance at 550 nm using McFarland standard and a working inoculum corresponding to 1.0×10^8 CFU/mL was prepared in 100 mL saline solution.

2.7.2 Experimental design

E. coli cells (1.0×10^8 CFU/mL) inoculated in saline solution were treated with ozone at a flow rate of 0.06 L/min with an ozone concentration of 6 µg/mL using an air cylinder for generation of ozone. Conditions were chosen so as to collect complete kinetic responses in order to characterise accurately the microbial resistance of the studied strains. Ozone treatment of each *E. coli* strain was carried out for 4 min with sampling intervals of 30 sec. All experiments were performed in triplicate.

2.7.3 Microbiological analysis

The effect of the ozone treatment on the microbial inactivation studies was determined in terms of changes in the culturable count change over time. Populations of each *E. coli* strain were determined by plating onto TSA. Samples (1 mL aliquots) were withdrawn from the treated saline, serially diluted in MRD and 0.1 mL aliquots of appropriate dilutions were surface plated onto TSA in duplicate. In order to obtain low microbial detection limits, 0.1 mL or 1 mL of the treated sample was spread onto TSA plates as described by EN ISO 11290-2 method (ISO 11290-2, 1998). Plates were incubated at 37°C for 24 h and then counted. Results were reported as Log₁₀ CFU/mL.

2.7.4 Determination of cell membrane integrity

The effect of ozone treatment on *E. coli* membrane integrity was determined by measuring the absorbance of supernatant (release of cell components) at 260 nm and 280 nm.

Ozone treated samples of different time intervals were subjected to centrifugation at 15,000 rpm for 20 min at 4°C. Two hundred microlitres of supernatant of each sample was added to the wells of a 96-well plate (UV-transparent flat-bottom microplates, Corning-Costar Cat. No. 3635; Fisher Scientific, Ireland), and absorbance values at 260 nm and 280 nm were recorded using a UV spectrophotometer (Synergy HT; Bio-Tek). Controls included (i) *E. coli* in saline and (ii) saline only. Triplicate samples were analysed and each experiment was performed in triplicate. The absorbance values (for cell free supernatant of untreated and ozone treated samples) were subtracted with the simultaneously recorded value of cell free supernatant of saline sample.

2.7.5 Determination of membrane permeability based on 1-N Phenyl-naphthylamine (NPN)

The effect of ozone treatment on *E. coli* membrane permeability was determined by using NPN, a hydrophobic fluorescent probe. The quantum yield of NPN is greatly

enhanced in a glycerophospholipid environment compared to an aqueous environment (Träuble and Overath, 1973).

2.7.5.1 Preparation of NPN solution

A 100 mM stock solution of NPN in acetone was prepared. This stock solution was diluted to a concentration of 100 μ M in saline. NPN solution (100 μ M) was added to the test sample to obtain a required end concentration of 20 μ M.

2.7.5.2 Fluorescence measurements

Ozone treated *E. coli* cultures (160 μ L) were pipetted into microtitre plate wells (black; Nunc) to which 40 μ L of 100 μ M NPN (Sigma-Aldrich, Dublin, Ireland) was added, yielding an end concentration of 20 μ M NPN. Immediately after mixing, plates were read on a Bio Tek Synergy HT fluorescence plate reader (excitation wavelength, 360/40 nm and an emission wavelength, 460/40 nm). Controls included (i) *E. coli* in saline with NPN and (ii) saline with NPN. Triplicate samples were analysed and each experiment was performed in triplicate. The fluorescence values were subtracted with the simultaneously recorded value of untreated cell sample (control sample not exposed to ozone) in the presence of 20 μ M NPN.

2.7.6 Preparation of SEM (Scanning Electron Microscope) samples

Samples for SEM were prepared according to the procedure employed by Thanomsub et al. (2002) with minor modification. During ozone treatment of each *E. coli* strain (parent strain BW 25113, $\Delta oxyR$ and $\Delta soxR$) in saline, samples were collected at time 0 and at 30 sec. Immediately samples were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the cells were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer pH 7.4 for 2 h. After first fixation, cells were then washed with the 0.05 M sodium cacodylate buffer (pH 7.4) three times and were then fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate for 2 h at 4°C. Cells were then washed once with the 0.05 M sodium cacodylate buffer (pH 7.4) followed by three

washes with distilled water. Samples were then dehydrated in progressively increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95% and 99.5% ethanol). The dehydrated samples were then freeze dried (Labconco; FreeZone 6, Mason Technology, Dublin, Ireland). Samples were mounted on stubs using double sided carbon tape, and sputter coated with Au, using a Emitech K575X Sputter coating unit, to prevent surface charging by the electron beam. Samples were sputter coated at a vacuum of 5×10^{-3} mbar around 30 sec resulting in a coating of 10 nm. Samples were then examined using a FEI Quanta 3D FEG Dual Beam (FEI Ltd, Hillsboro, USA) at 5KV.

2.8 Mathematical modeling

2.8.1 Primary modelling

For all the studies performed, a number of different kinetic models were investigated. These included log-linear, Weibull, Shoulder log-linear type and biphasic model.

2.8.1.1 Linear regression

The log-linear-death kinetic model (Geeraerd et al., 2005) was considered to perform regression analysis of microbial inactivation data obtained from ultrasound treatment of bacterial cells.

$$\log_{10}(N) = \log_{10}(N(0)) - \frac{t}{D} \quad (2-3)$$

$$= \log_{10}(N(0)) - \frac{k_{\max} t}{\ln(10)} \quad (2-4)$$

Where N represents the microbial population, $N(0)$ initial microbial population, $k_{\max}[\text{min}^{-1}]$ the first order inactivation constant and $D[\text{min}]$ the decimal reduction time.

The slope of the regression line of the $\log_{10}(N)$ vs time was used to calculate a D-value over 1 log cycle reduction in the numbers of bacteria. The D-value (decimal reduction time) is the amount of time required for the number of surviving microorganisms to decrease 1 decimal logarithmic unit. D-values were calculated for the full range of

experimental conditions outlined for ultrasound treatment. D-values were calculated using linear regression of the survivor curves for each ultrasound treatment.

2.8.1.2 Non linear regression

The ozone inactivation kinetics of *E. coli* and *Listeria* strains in orange juice and apple juice showed a characteristic non-linear behaviour. The Weibull model was used to describe this behaviour. The shoulder-log linear model was also used to describe the ozone inactivation kinetics of *E. coli* in apple juice.

The Weibull distribution corresponds to a concave upward survival curve if $p < 1$ and concave downward if $p > 1$ (Van Boekel, 2002). The Weibull model was used to model the experimental death curves, which displayed a downward concave trend. This model has been used previously to describe the inactivation kinetics for ozone (Bialka et al., 2008). The model used was (Mafart et al., 2002):

$$\log_{10}\left(\frac{N}{N_0}\right) = \left(\frac{t}{\delta}\right)^p \quad (2-5)$$

where δ [min] (time for the first decimal reduction) and p [-] are parameters related to the scale and shape of the inactivation curve, respectively.

The time required to obtain a 5 log reduction (t_{5d}) was calculated from model parameters by employing Eq. (2-6) (Van Boekel, 2002).

$$t_{5d} = \delta \times (5)^{\frac{1}{p}} \quad (2-6)$$

The shoulder-log linear model (Geeraerd et al., 2000) was also used to study the survival kinetic data:

$$\log_{10}(N) = \log_{10}(N_0) - \frac{k_{\max} \cdot (t)}{\ln(10)} + \frac{\log_{10} e^{(k_{\max} \cdot S_l)}}{(1 + e^{(k_{\max} \cdot S_l)} - 1)} \cdot e^{(-k_{\max} \cdot t)} \quad (2-7)$$

where, S_l is the shoulder length [min] and k_{\max} the inactivation rate [min⁻¹].

The numerical values of S_l , $\log_{10} (N_0)$ and k_{max} were used to calculate a desired log reduction. The t_{5d} was calculated using Eq. (2-8) (Valdramidis et al., 2005).

$$t_{5d} = S_l + (5) \times \frac{\ln(10)}{k_{max}} \quad (2-8)$$

The ozone inactivation kinetics of *E. coli* strains in saline showed a characteristic non-linear curve. This behaviour was described by the biphasic model and is given by Eq. (2-9) (Cerf, 1977). The biphasic Eq. (2-9) was selected based on preliminary statistical comparison of the different inactivation models available at GInaFiT. The parameterisation of the biphasic equation employed was:

$$\log_{10}(N) = \log_{10}(N_0) + \log_{10}\left(f \times e^{(-k_{max1} \times t)} + (1-f) \times e^{(-k_{max2} \times t)}\right) \quad (2-9)$$

where, f is the fraction of the initial population in a major subpopulation, k_{max1} and k_{max2} are the parameters that determine the inactivation rate.

The numerical values of f , k_{max1} and k_{max2} were used to calculate the time required to achieve a reduction by 5 log cycles (t_{5d}) using the Solver in Microsoft excel 2003 (Microsoft Corporation, USA) by equalizing $\log_{10} (N_0) - \log_{10} (N) = 5$.

The GInaFiT tool was employed to perform the regression analysis of the microbial inactivation data described by the Weibull model and shoulder log-linear model (Geeraerd et al., 2005). Other non linear regressions were performed using the R statistical software libraries (R Core Development Team, 2007).

2.8.2 Modeling the growth of *S. cerevisiae*

2.8.2.1 Parameter identification under static conditions

S. cerevisiae growth data in ozone processed apple juice stored under SST conditions were fitted to the explicit version of the Baranyi and Roberts (1994) model (Eq.2-10 to 2-12). Regression was performed by using the DMFit Excel add-in software, version 2.1(www.ifr.ac.uk/safety/DMFit). The model reads as follows

$$N(t) = N(0) + \mu_{\max} A(t) - \ln \left(1 + \frac{e^{\mu_{\max} A(t)} - 1}{e^{(N_{\max} - N(0))}} \right) \quad (2-10)$$

$$\text{with } A(t) = t + \frac{1}{\mu_{\max}} \ln \left(\frac{e^{(-\mu_{\max} t)} + q(0)}{1 + q(0)} \right) \quad (2-11)$$

$$\text{and } \lambda = \ln \left(\frac{1 + \frac{1}{q(0)}}{\mu_{\max}} \right) \quad (2-12)$$

The kinetic parameters of maximum specific growth rate (μ_{\max}) (1/days), lag phase (λ) (days), initial microbial population ($N(0)$) ($\text{Log}_{10}\text{CFU/mL}$) and maximum population density (N_{\max}) ($\text{Log}_{10}\text{CFU/ml}$) have then been estimated. $q(0)$ (-) denotes the concentration of substance critical to the microbial growth and is related to the physiological state of the cells.

2.8.2.2 Model validation under dynamic storage temperature conditions

The validation of yeast growth model was performed under dynamic storage temperature conditions based on the time temperature profile of apple juice samples during storage (control and ozone processed), in conjunction with the square root model Eq (2-17). The predictions were performed with the set of ordinary differential equations of Baranyi and Roberts model (Eq. (2-13), (2-14)):

$$\frac{dN(t)}{dt} = (b(T(t) - T_{\min})^2) \left(\frac{q(t)}{q(t) + 1} \right) \left(1 - \frac{N(t)}{N_{\max}} \right) N(t) \quad (2-13)$$

$$\frac{dq(t)}{dt} = (b(T(t) - T_{\min})^2) q(t) \quad (2-14)$$

The Runge-Kutta method (ode23s, Matlab, The Mathworks) was applied for the approximation of these equations with the appropriate initial conditions (at $N(0) = \text{average}(N(0))$, $q(0) = q(0)$, $N_{\max} = \text{average}(N_{\max})$ in all cases as estimated from the

corresponding static conditions) and together with a linear interpolation of the measured temperature data for the simulation of the complete temperature profile (interp1, Matlab, The Mathworks).

2.8.2.3 Calculation of the Q_{10} value

The temperature quotient (Q_{10}) was also calculated from the information obtained in Section 2.8.2.1 (parameter identification under static conditions). Q_{10} shows the effect of temperature on the shelf-life and it is given as follows

$$Q_{10} = \frac{\text{shelf life at } T^{\circ}\text{C}}{\text{shelf life at } (T + 10^{\circ}\text{C})} \quad (2-15)$$

This parameter was developed for a zero order reaction when the influence of temperature on the reaction rate is described by using the Arrhenius relationship (Man and Jones, 2000).

Nevertheless this approach is proposed and applied for the current microbial kinetic study as an alternative method to assess the efficacy of the ozonated juice.

This Q_{10} value can be easily calculated by performing a regression between the ln shelf life (days) versus the temperature which yields a straight line. Consequently, $Q_{10} = \exp(10 \cdot m)$ with m the slope of the regression line. The estimation of the time of the shelf-life (t_s) was calculated considering that a microbial level $> 10^6$ CFU/mL resulted in a failure (spoilage) of the product (Al-Kadamany, et al., 2002). The shelf-life time, t_s , was obtained by solving Eq. (2-10 to 2-12) (*solve* command in Matlab, The Mathworks) for the estimated parameters of the two controls and the ozonated growth kinetics when $\log N(t_s) = 6 \log(\text{CFU/mL})$.

2.8.3 Secondary modelling

To characterise the dependence of the Weibull parameters (δ and P) on the ozone processing variables, the following steps were taken:

1. Individual nonlinear regressions of all experiments with Eq. (2-5) were performed.
2. Inspection of the variation of δ and p with processing temperature (T) and ozone flow rate per reactor volume (fO_3) (mg /min/mL).
3. Experiments at each processing condition were averaged.
4. Polynomial relationships of $\ln(\delta)$ and p with the ozone flow rate and the temperature were proposed.
5. Additional terms to the model were investigated and compared with the base model using a log-likelihood ratio test.

In the case of the effect of different pH levels of apple juice on ozone inactivation efficacy, a correlation was established between the time required to obtain a 5 log reduction and the different adjusted pH levels of apple juice. The equation developed was validated against a similar microbial reduction achieved in natural apple juice where pH was unadjusted (i.e., pH 3.13).

$$t_{5d} = a \cdot e^{k \cdot pH} \quad (2-16)$$

where a and k are the parameters, t_{5d} is the time required for a 5 log reduction.

The maximum specific growth rates of *S. cerevisiae* estimated under static storage conditions were further modelled as a function of storage temperature by using the Square root model (Ratkowsky et al., 1982; Ratkowsky et al., 1983):

$$\mu_{max} = \left\{ b(T_{(t)} - T_{min})^2 \right\} \quad (2-17)$$

where b is a constant, T is the storage temperature (°C), T_{min} is the theoretical minimum temperature for the growth of the organism. Eq. (2-17) has been used without the commonly applied square root transformation of the μ_{max} value. This required the performance of a non-linear regression which is available from the DMFit software. A (geometric) mean value for $h_0 = \lambda * \mu_{max}$ for each of the experimental set-ups (Control 1, Control 2, Ozonated) was estimated from the individual growth curves, considering

that the parameter is constant, independent of the storage temperature (Fu et al., 1991; Baranyi and Roberts, 1994; Le Marc et al., 2009) and the fact that the resulting h_0 was derived from the 3 levels of temperatures. $q(0)$ is related to the parameter h_0 by the following equation (Baranyi and Roberts, 1994):

$$q(0) = \frac{1 - e^{-h_0}}{e^{-h_0}} \quad (2-18)$$

2.9 Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, U.S.A).

All microbial data were pooled and average values and standard deviations determined.

Means were compared using ANOVA followed by LSD testing at $p < 0.05$ level.

The main criteria to discriminate between nonlinear models used was the likelihood ratio test (in the case of nested models to determine secondary models), the determination coefficient R^2 and the determination coefficient adjusted to the degrees of freedom (R^2_{adj}). The R^2 and R^2_{adj} represent the relative predictive power of a linear model. Although it is not considered the most appropriate criteria for nonlinear models, the lack of other simple alternatives for nonlinear models and the intuitive understanding motivated choosing this indicator. R^2_{adj} is a bound between 0 and 1. The closer it is to one, the better the model is.

Root mean squared error (RMSE) is considered to be the appropriate criteria for nonlinear models. The fitting ability of the tested models was evaluated by calculating the RMSE (Neter et al., 1992).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n+} (y_{\text{exp } i} - y_{\text{pre}})^2}{n_t - n_p}} \quad (2-19)$$

Where $y_{\text{exp } i}$ are experimental observations, y_{pre} are model predictions, n_t are number of data points and n_p are number of estimated model parameters.

Additionally, the error propagation technique was employed to calculate the standard error of the computed results of t_{5d} (Skoog et al., 1996; Skoog et al., 1998).

Chapter 3 POWER ULTRASOUND PROCESSING OF LIQUIDS

3.1 Inactivation of *Escherichia coli* using power ultrasound

Two strains of *E. coli* were used in this study: *E. coli* ATCC 25922 (generic strain) and *E. coli* NCTC 12900 (non-toxigenic strain). Inactivation of *E. coli* in liquids was carried out using power ultrasound. Parameters examined included amplitude levels (0.4µm, 7.5 µm, 37.5 µm), treatment time, cell condition (non acid exposed cells, acid exposed cells), liquid media (TSB, MOJ and MAJ) and *E. coli* strain (ATCC 25922, NCTC 12900).

The objectives of this study were to optimise power ultrasound with regard to the control parameters of amplitude level and treatment time for the inactivation of *E. coli*. Due to the reported survival of *E. coli* O157:H7 within acid environments, the effects of prior acid adaptation/exposure on the efficacy of sonication was also evaluated for both generic and non-toxigenic *E. coli* O157:H7.

3.1.1 Validation of equipment

Preliminary experiments were carried out with different initial microbial loads of *E. coli* ATCC 25922. Microbial populations of 10^9 CFU/mL and 10^6 CFU/mL were treated with varying amplitude levels (µm) for a specific time period.

The initial microbial load of 10^9 CFU/mL was reduced by approximately 7.8 log cycles and 4.8 log cycles with 37.5 µm and 7.5 µm ultrasound amplitude levels, respectively (Figure 3.1a) within 21 min of treatment time. However, initial *E. coli* concentrations of 10^6 CFU/ml were reduced by approximately 5.9 log cycles and 4.4 log cycles with 37.5 µm and 7.5 µm ultrasound amplitude levels, within 15 min of ultrasonication, respectively (Figure 3.1b).

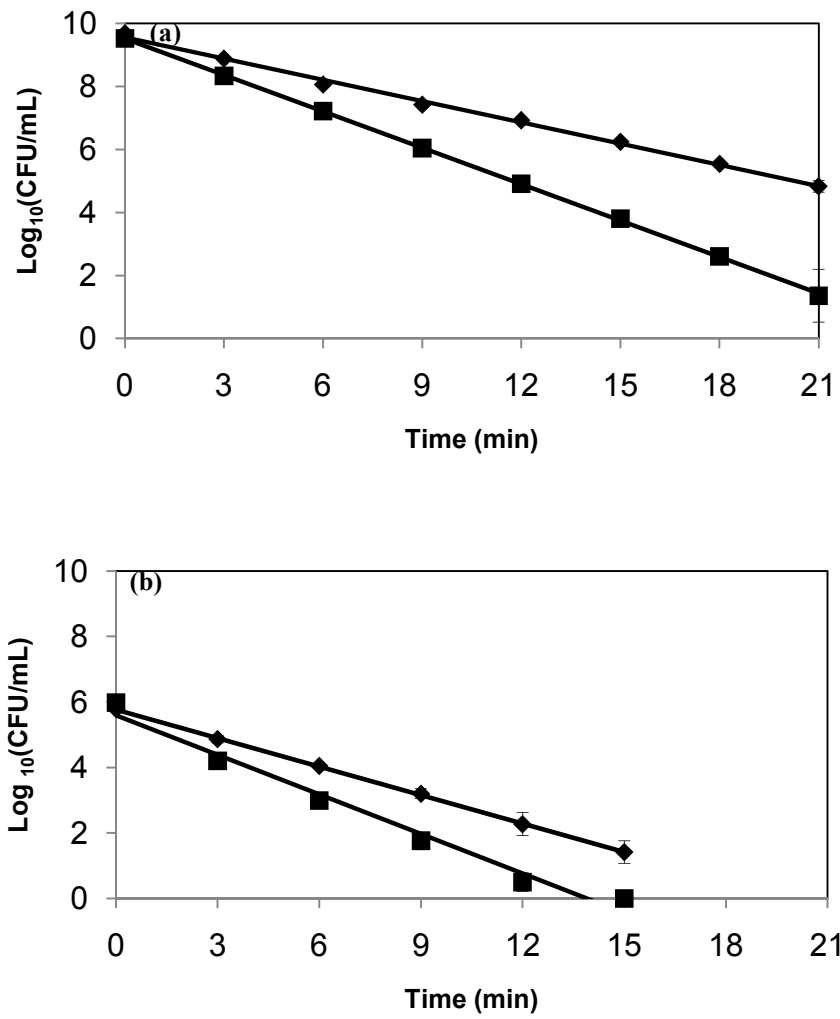


Figure 3.1: Effect of amplitude for the ultrasound treatment of *E. coli* ATCC 25922
(a) 10^9 CFU/mL, (b) 10^6 CFU/mL

◆ 7.5 μm ■ 37.5 μm

3.1.2 Effect of ultrasound amplitude level on inactivation of *E. coli* strains

The inactivation of both *E. coli* strains was found to be dependant on the amplitude levels ($p < 0.05$). During ultrasound treatment, a linear response of the decimal logarithm of the microbial population with exposure time was observed. Total inactivation of *E. coli* was achieved using 37.5 μm amplitude after 15 min (Figure 3.2). Both strains of *E. coli* studied (*E. coli* ATCC 25922, *E. coli* NCTC 12900) were found to be sensitive to sonication ($p < 0.05$). An amplitude of 0.4 μm reduced *E. coli* ATCC 25922 by 1.2 log cycles (Figure 3.2a) and *E. coli* ATCC 12900 by 1.1 log cycles (Figure 3.2b) within 15 min. Ultrasonication for 15 min at 7.5 μm amplitude resulted in reduction of *E. coli* ATCC 25922 by 4.4 log cycles (Figure 3.2a). Similarly, strain NCTC 12900 was reduced by 4.7 log cycles after ultrasound treatment of 15 min at 7.5 μm (Figure 3.2b). D-values decreased with increasing levels of ultrasound amplitude ($p < 0.05$). At 0.4 μm amplitude the D-value of *E. coli* NCTC 12900 was higher than that of strain ATCC 25922. The time required to achieve inactivation by 5 log cycles (t_{5d}) for strain 25922 were 68.6 min, 17.2 min and 11.1 min at 0.4 μm , 7.5 μm and 37.5 μm amplitude levels, respectively. For strain NCTC 12900 the t_{5d} values were 76.3 min, 15.2 min and 13.8 min at 0.4 μm , 7.5 μm and 37.5 μm amplitude levels, respectively. Both strains responded similarly to increasing amplitude levels, but, as it can be noted in Figure 3.2, at 37.5 μm amplitude level there was a significant difference between D-values of the two strains ($p < 0.05$).

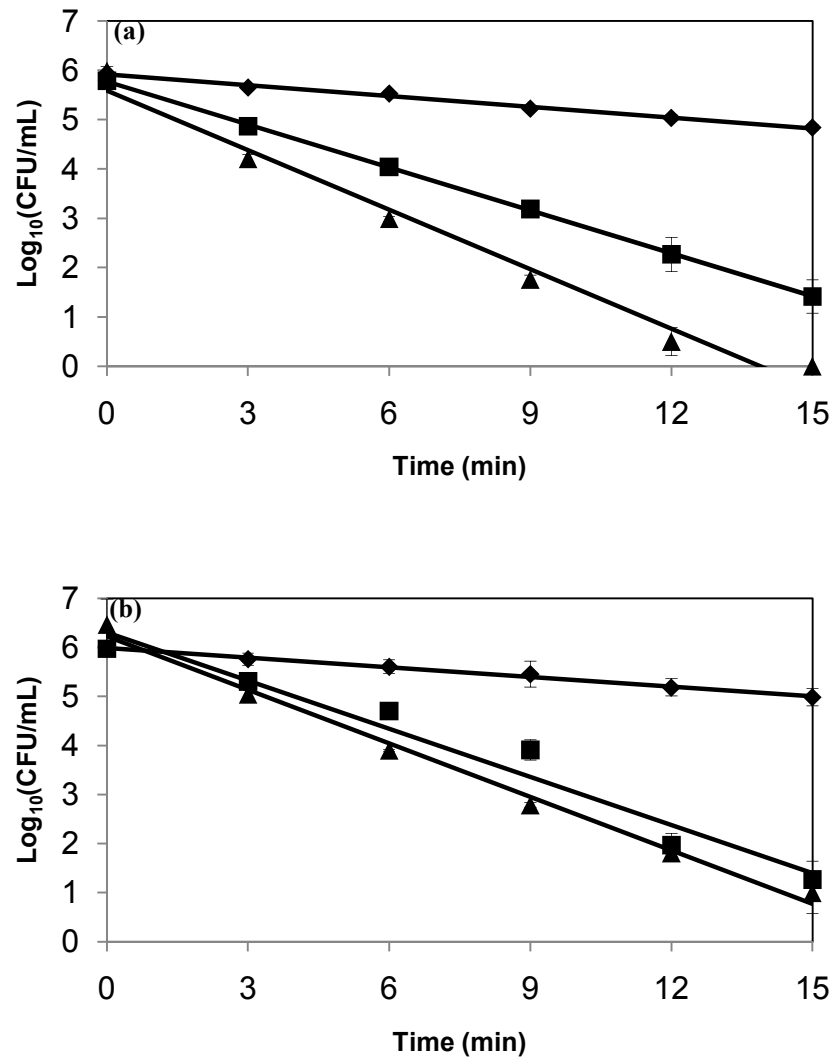


Figure 3.2: Effect of amplitude levels on inactivation of *E. coli* strains

(a) *E. coli* ATCC 25922

(b) *E. coli* NCTC 12900

◆ 0.4 μm ■ 7.5 μm ▲ 37.5 μm

3.1.3 Effect of acid exposure on inactivation of *E. coli* strains

Ultrasound treatment (37.5 μm amplitude) for 15 min of *E. coli* ATCC 25922 exposed to acid for 1 h, 4 h or 18 h resulted in 5.7, 4.8 and 4.9 log cycle reductions respectively (Figure 3.3 a,b,c). Strain NCTC 12900 had a similar response with 5.9, 5.8 and 5.5 log cycle reductions with 37.5 μm amplitude for the different conditions respectively (Figure 3.4 a,b,c). Ultrasound treatment with 7.5 μm amplitude showed a maximum reduction by 4.7 and 3.7 log cycles, with 1 h acid exposed *E. coli* ATCC 25922 and NCTC 12900, respectively. During 15 min treatment of ultrasound with 0.4 μm amplitude, the 1 h acid exposed strains of *E. coli* ATCC 25922 and *E. coli* NCTC 12900 in TSB was reduced by 1.71 and 1.14 log cycles, respectively. In general, regardless of acid exposure time, the D-values of *E. coli* decreased as the amplitude level was increased. D-values of the non acid exposed control and acid exposed *E. coli* cultures are outlined in Tables 3.1 and 3.2. At 0.4 μm amplitude, 1 h acid exposure of *E. coli* 25922 resulted in lower D-values compared to the control ($p < 0.05$). However, at longer acid exposure times of 4 h and 18 h, this effect was not evident in *E. coli* ATCC 25922 (Table 3.1). At 7.5 μm amplitude, there was no significant effect of acid exposure condition compared with control cultures. At 37.5 μm amplitude, prior acid exposure of *E. coli* ATCC 25922 for 1 h or 4 h did not significantly affect the D-value, however, with 18 h acid exposed cells, the D-value increased, yielding a seeming increased resistance to ultrasound treatment. In the case of *E. coli* NCTC 12900 there were no significant differences in the inactivation of *E. coli* with regard to prior acid exposure at 0.4 μm amplitude. However, at 7.5 μm amplitude, increased duration of acid exposure was associated with higher D-values (Table 3.2). The t_{5d} values for 1 h, 4 h and 18 h acid exposed *E. coli* 25922 were in the range of 44.1-70.8min, 16-16.7 min and 10.6-14.9 min at 0.4 μm - 37.5 μm amplitude, respectively. For 1 h, 4 h and 18 h acid exposed *E. coli* 12900 the t_{5d} values were in the range of 67.4-12.8 min, 78.9-13 min and 67.4-

13.5 min, at 0.4 μm - 37.5 μm amplitude, respectively. Generally ultrasound treatment with 7.5 μm and 37.5 μm amplitude resulted in greater inactivation levels than with 0.4 μm amplitude indicating an increased inactivation efficacy at higher amplitude levels.

Table 3.1: D values and R² values for ultrasound treatment of acid exposed *E. coli* ATCC 25922

Amplitude (μm)	Control		1 h		4 h		18 h	
	D value	R ²	D value	R ²	D value	R ²	D value	R ²
0.4	13.7 \pm 0.9 ^a	0.99	8.83 \pm 0.03 ^b	0.99	12.46 \pm 0.10 ^a	0.97	14.2 \pm 1.0 ^a	0.97
7.5	3.44 \pm 0.03 ^c	0.99	3.2 \pm 0.2 ^c	0.98	3.29 \pm 0.10 ^c	0.99	3.34 \pm 0.03 ^c	0.99
37.5	2.23 \pm 0.10 ^d	0.99	2.12 \pm 0.16 ^d	0.98	2.4 \pm 0.3 ^d	0.96	2.98 \pm 0.17 ^c	0.98

Different letters indicate a significant difference at the 0.05 level

Table 3.2: D values and R² values for ultrasound treatment of acid exposed *E. coli* NCTC 12900

Amplitude (μm)	Control		1 h		4 h		18 h	
	D value	R ²	D value	R ²	D value	R ²	D value	R ²
0.4	15.26 \pm 0.10 ^a	0.99	13.47 \pm 0.12 ^a	0.99	15.7 \pm 1.5 ^a	0.98	13.5 \pm 1.1 ^a	0.97
7.5	3.1 \pm 0.3 ^b	0.95	4.0 \pm 0.2 ^c	0.99	4.15 \pm 0.08 ^{cd}	0.99	4.48 \pm 0.09 ^{de}	0.99
37.5	2.75 \pm 0.10 ^f	0.99	2.55 \pm 0.09 ^f	0.98	2.60 \pm 0.09 ^f	0.99	2.69 \pm 0.09 ^f	0.99

Different letters indicate a significant difference at the 0.05 levels

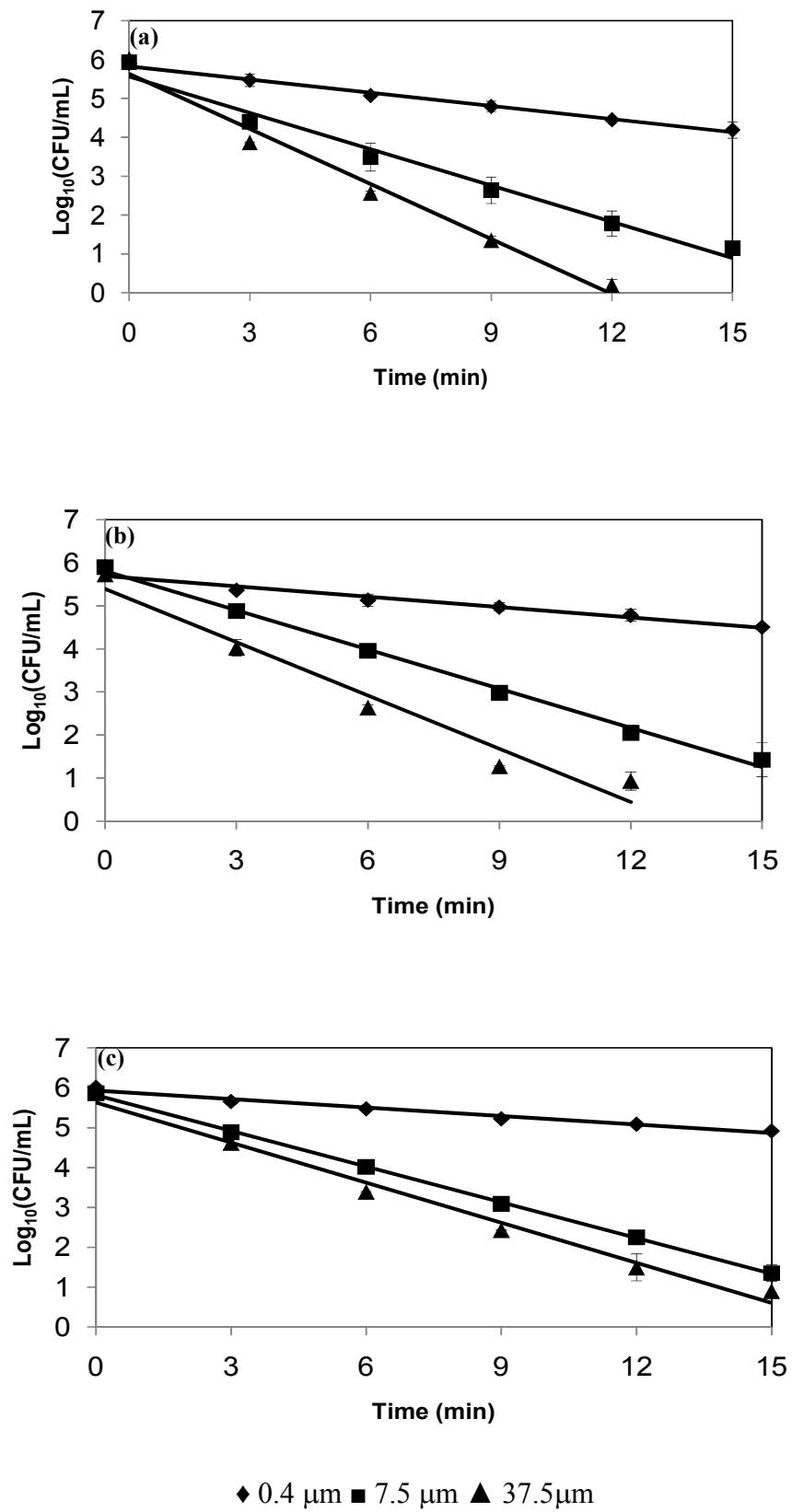


Figure 3.3: Effect of amplitude levels on acid adapted *E. coli* ATCC 25922
 (a) 1 h acid adaptation
 (b) 4 h acid adaptation
 (c) 18 h acid adaptation

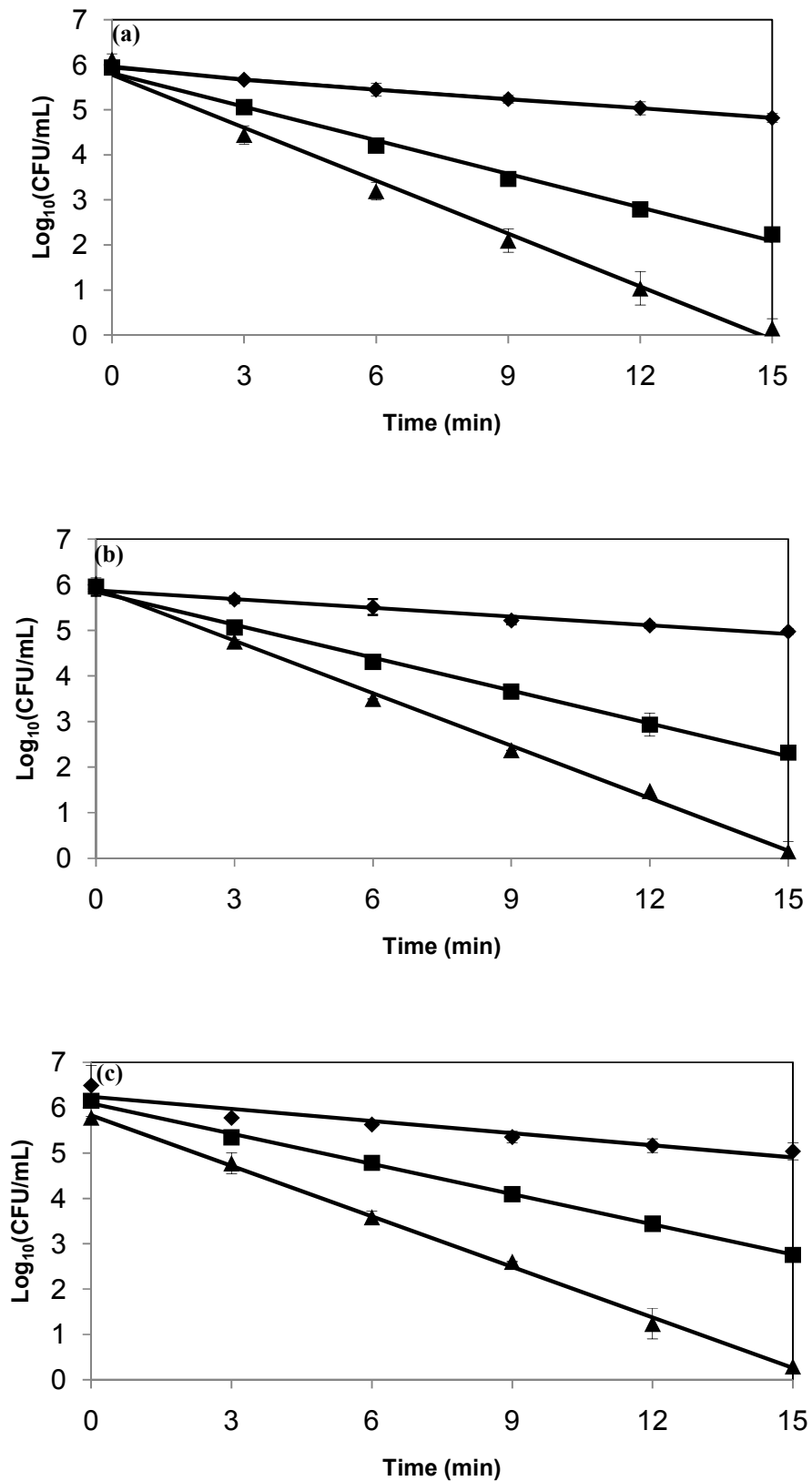


Figure 3.4: Effect of amplitude levels on acid adapted *E. coli* NCTC 12900
 (a) 1 h acid adaptation
 (b) 4 h acid adaptation
 (c) 18 h acid adaptation

3.1.4 Ultrasound inactivation of *E. coli* strains in model orange juice (MOJ)

Following analyses using TSB, model orange juice (MOJ) was used as a test system to more closely mimic real conditions (the processing of a complex food system). Ultrasound inactivation of both *E. coli* strains in MOJ was dependant on the level of amplitude applied ($p < 0.05$) with increasing amplitudes resulting in larger reductions in the microbial population. As with TSB, ultrasound treatment in MOJ gave a linear response with exposure time. Ultrasound amplitudes of 7.5 μm and 37.5 μm caused total inactivation of *E. coli* ATCC 25922 within 15 min. However, in the case of *E. coli* NCTC 12900, amplitudes of 7.5 μm and 37.5 μm resulted in a 2.5 log reduction and a 2.7 log reduction respectively. Both strains of *E. coli* studied (*E. coli* ATCC 25922, *E. coli* NCTC 12900) were found to be sensitive to ultrasonication within MOJ ($p < 0.05$). Using 0.4 μm amplitude, *E. coli* ATCC 25922 and *E. coli* ATCC 12900 were similarly reduced by 1 log cycle. D-values for both strains at all amplitudes in MOJ are shown in Tables 3.3 and 3.4. D-values decreased with increasing levels of ultrasound amplitude ($p < 0.05$). In the case of *E. coli* ATCC 25922, there were no significant differences observed between D-values obtained in TSB and MOJ. However, for *E. coli* NCTC 12900, there were significant differences between D-values obtained in TSB and MOJ at all level of amplitudes.

Table 3.3: D-values and R² values for ultrasound treatment of *E. coli* ATCC 25922 in TSB and MOJ

Amplitude (μm)	TSB		MOJ	
	D-value	R ²	D-value	R ²
0.4	13.7 \pm 0.9	0.99	14.85 \pm 0.10	0.94
7.5	3.44 \pm 0.03	0.99	2.9 \pm 0.7	0.90
37.5	2.2 \pm 0.1	0.99	2.4 \pm 0.7	0.93

Table 3.4: D-values and R² values for ultrasound treatment of *E. coli* NCTC 12900 in TSB and MOJ

Amplitude (μm)	TSB		MOJ	
	D-value	R ²	D-value	R ²
0.4	15.26 \pm 0.10	0.99	6.6 \pm 0.3	0.92
7.5	3.1 \pm 0.3	0.95	6.14 \pm 0.10	0.99
37.5	2.8 \pm 0.1	0.99	5.4 \pm 0.2	0.97

3.1.5 Ultrasound inactivation of *E. coli* ATCC 25922 in model apple juice (MAJ)

In this study MAJ was used as a test system. *E. coli* cells previously grown in TSB were re-suspended in MAJ and treated with varying amplitude levels. Ultrasound treatment at 0.4 μm amplitude resulted in a 2.7 $\log_{10}\text{CFU/mL}$ reduction of cells with a corresponding D value of 5.3 min (Figure 3.5). When the amplitude was increased to 7.5 μm or 37.5 μm , inactivation was achieved within 6 and 3 min respectively. Therefore, inactivation of *E. coli* only at lowest (0.4 μm) amplitude in MAJ is shown in Figure 3.5. The effect of media on inactivation of *E. coli* ATCC 25922 at lowest amplitude level tested is shown in Figure 3.5.

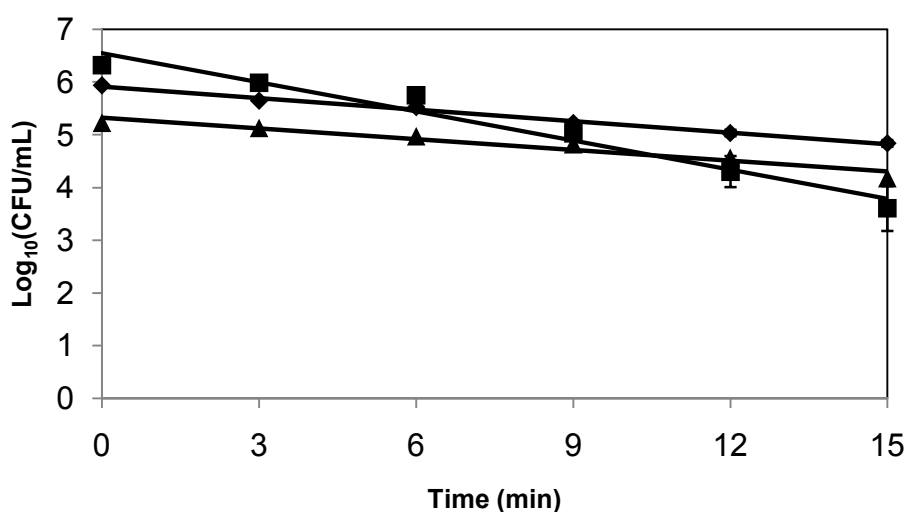


Figure 3.5: Effect of media on *E. coli* ATCC 25922 inactivation using 0.4 μm amplitude

◆ TSB, ■ Model apple juice and ▲ Model orange juice

3.1.6 Discussion

Ultrasound has been identified as one possible non-thermal technology to meet the required microbial log reduction and to maintain the textual and sensory characteristics of fresh food products. Several authors reported the effectiveness of ultrasound for microbial inactivation (Furuta et al., 2004; Cameron et al., 2008; Koda et al., 2009; Adekunle et al., 2010; Rahman et al., 2010) in different treatment media. The present study also showed a greater than 5 log reduction of both *E. coli* strains with increasing level of amplitude in 15 min or less. For this work, the level of amplitude employed was taken as an indication of the ultrasonic power intensity. Ultrasound treatment with 7.5 μm or 37.5 μm amplitude displayed a strong influence on the rate of *E. coli* inactivation in TSB, as shown in Figure 3.2a and b.

It has also been previously reported by several investigators (Villamiel and de Jong, 2000; Baumann et al., 2005) that ultrasound processing of liquids is most effective in combination with mild heating. Lee et al. (2009) concluded that, the combination of lethal factors such as heat and/or sonication, with and without pressurisation has shown to significantly shorten the treatment time required to achieve a 5-log reduction in the survival count of *E. coli* K-12 and it might also provide a practical alternative to the traditional thermal pasteurisation. However, in this study an ice bath was used to dissipate the heat generated during treatment in order to evaluate the inactivation effects of ultrasound alone. This is very relevant to the processing of fruit juice as it is desirable to maintain low processing temperature to retain the quality characteristics of fresh juice, and to maintain energy efficiency. At 37.5 μm amplitude, *E. coli* ATCC 25922 was reduced by 5.9 log cycles and *E. coli* NCTC 12900 by 5.6 log cycles within 15 min of ultrasound treatment. This inactivation results from a combination of physical and chemical mechanisms which occur during cavitation. At higher amplitude levels, corresponding to higher ultrasound intensities, the inactivation rate was enhanced in

both *E. coli* strains, in accordance with previous studies that found that increasing the acoustic energy density, another indication of ultrasonic power intensity, increased the inactivation of foodborne pathogens (Hua and Thompson, 2000, Ugarte-Romero et al., 2007). There was only a marginal increase in the efficacy of ultrasound at 37.5 μm amplitude levels when compared to 7.5 μm level. Thus, in a processing context, it may be desirable to use 7.5 μm amplitude, from the energy efficiency point of view and as it was shown previously that the quality parameters of orange juice change might be affected by exposure at high amplitude levels (Tiwari et al., 2008b).

It has been reported that acid adaptation prolongs the survival of *E. coli* O157:H7 in various food systems, including apple cider, sausages (Leyer et al., 1995) and acidic fruit juice (Hsin-Yi and Chou, 2001). Acidic conditions such as those found in fruit juices could render the microorganism more resistant to food processing techniques. Buchanan and Edelson (1999) reported pH dependant stationary phase acid resistance of *E. coli* O157:H7 increased pathogen's tolerance to heat. Rowe and Kirk (1999) reported that strains of *E. coli* exhibit cross-protection when subjected to combinations of acid, salt and heat stresses. The strain, type of acid and acid adaptation or acid shocking was also shown to affect acid resistance of *E. coli* O157:H7 in TSB, orange juice and apple cider (Ryu and Beuchat, 1998). Acid adaptation of *E. coli* O157:H7 was also reported to provide cross protection against irradiation, sodium lactate, sodium chloride, lactoperoxidase activation and sublethal heat treatments in milk (Garren et al., 1998; Buchanan et al., 1999; Parry-Hanson et al., 2009).

Although extensive information on acid stress of *E. coli* has been reported, there is not enough information available to understand the cross protective effect of acid adapted *E. coli* towards stresses applied in food processing and preservation. Also understanding the relation between acid adaptation and resistance of microorganisms towards processing techniques is of importance for food safety issues. Understanding the level

of the possible microbial adaptation when applying innovative technologies is of importance in order to achieve microbiologically safe products (Yousef and Courtney, 2003). Therefore, it was necessary to determine the response of acid adapted *E. coli* to ultrasound treatment. There is a gap in the scientific knowledge with respect to acid adaptation and whether this phenomenon confers resistance to ultrasound treatment. Thus, the effect of prior acid adaptation on ultrasound effectiveness was assessed and this study was conducted to determine efficiency of ultrasound alone to inactivate acid adapted *E. coli* cells. Acid adaptation responses of foodborne pathogens at different pH conditions were previously examined and pH 5.0-5.5 lead to the highest level of acid resistance for *E. coli* O157:H7 (Koutsoumanis and Sofos, 2004). Consequently, in this study both *E. coli* strains were subjected to prior acid adaptation at pH 5.0 to examine for any effects on the efficacy of ultrasound treatment. When *E. coli* ATCC 25922 was acid adapted for 18 h, an increased resistance to ultrasound treatment at 37.5 μm amplitude was observed. However, the non-adapted control strain showed sensitivity to treatment at 7.5 μm and 37.5 μm amplitude, thus indicating that the longer acid adaptation of 18 h increased the resistance to ultrasound treatment. All prior acid adaptation treatments of *E. coli* NCTC 12900 increased the resistance of the organism to ultrasound treatment at 7.5 μm amplitude but no effect was evident at the other amplitudes. Acid adaptation involves changes in protein expression profiles (Huang et al., 2007) and membrane lipid composition (Yuk and Marshall, 2004). This could alter the physiological state of the cells enabling them to withstand longer exposures to the cavitation effect than the control cells. For both strains, there was a dominant effect where increasing the levels of amplitude (7.5 μm and 37.5 μm) of the ultrasound treatment negated any cell condition effects.

Ultrasound inactivation of bacteria has been found to be dependent upon the solution which is under study. Salleh-Mack and Roberts, (2007) investigated the effect of

varying concentrations of soluble solids on the efficacy of ultrasound inactivation of *E. coli* ATCC 25922, and found that solutions with higher soluble solids required a longer time to achieve a higher inactivation. In this study, this effect was not found for *E. coli* ATCC 25922 as the D-values for TSB, a complex media, were similar to the D-values for MOJ. However, in *E. coli* NCTC 12900 a longer time was required to achieve a higher inactivation in MOJ at all amplitude levels examined. So, differences in the two *E. coli* strains seem to impact the efficacy of ultrasound treatment in MOJ. Similarly, little effect of sonication was noted in *Salmonella* spp inactivation when broiler drumstick skin or egg was used as a medium suggesting physical shielding provided to microbes due to irregular skin surface or high viscosity of the product (Sams and Feria, 1991; Wrigley and Llorca, 1992). Cavitation is known to be reduced with increased viscosity (Hulsen, 1999). In the current study, the difference in inactivation of two strains seemed to depend on the type of organism and medium type. Therefore, type of strain, influence of food properties such as viscosity, particle size on microbial inactivation by ultrasound should always be considered to design process parameters. The survival of the non-toxicogenic strain of *E. coli* O157:H7 used in this study was greater than that for the generic strain of *E. coli* used and this trend was enhanced following acid adaptation for 18 h. Nonetheless, although the non-toxicogenic strain of *E. coli* O157:H7 had greater survival capabilities, the application of power ultrasound resulted in a > 5 log reduction within 15 min. From the present study and based on the different inactivation responses observed to ultrasound treatment, it was observed that the response of non-toxicogenic strain of *E. coli* O157:H7 should be considered to determine the effectiveness of sonication in a food processing environment. Temperatures employed in this study were maintained below 30°C so as to utilise lower processing conditions than those used for thermal pasteurisation. The findings of the present work might have practical applications with respect to food safety issues during

processing. Acidic pH of fruit juice may induce acid tolerance response in microorganisms which could give protection to a subsequent processing treatment. The present work showed that sonication was effective for inactivation of acid adapted *E. coli*. Thus, ultrasound could be used for inactivation of microorganisms in liquid systems.

Chapter 4 OZONE PROCESSING OF LIQUIDS

4.1 Extrinsic control parameters for ozone inactivation of *E. coli* using a bubble column

In order to determine the efficacy of ozone, the first step was to optimise the processing parameters. This preliminary study investigated the effect of extrinsic control parameters for ozone inactivation of *E. coli* in a bubble column. Ozone inactivation of *E. coli* ATCC 25922 in TSB was examined. The parameters studied included temperature (ambient, 20, 25 and 30°C), exposure time (up to 30 min), gas flow rate (0.03, 0.06, 0.12, 0.25, 0.5, and 0.75 L/min) and ozone concentration level (5 different levels). In 2001, the FDA approved use of ozone as a direct additive to food and in 2004, issued guidelines for the use of ozone in liquid systems (US FDA, 2004). These guidelines also highlighted gaps in the literature for ozonation of liquid foods. This study provides useful information regarding optimum extrinsic control parameters for *E. coli* inactivation in liquid media using a bubble column to ensure microbiological safety.

4.1.1 Effect of gas flow rate

Survival curves of *E. coli* ATCC 25922 in TSB treated with ozone (0.045-0.170 mg/min/mL) at the selected flow rates are shown in Figure 4.1. The inactivation of *E. coli* ATCC 25922 was found to be dependant on gas flow rate. Each curve shows a noticeable lag time prior to reduction, due primarily to ozone demanding substances present in the TSB medium. Regardless of the lag time, after 25 min, complete inactivation was achieved using the following flow rates: 0.06, 0.12, 0.25 and 0.5 L/min. However, complete inactivation was not achieved with flow rates of 0.03 and 0.75 L/min, even after 30 min treatment. The optimum flow rate was 0.12 L/min, with a

t_{5d} value of 20 min (Table 4.1). Exposure of *E. coli* to pure oxygen for 30 min yielded no detectable inactivation.

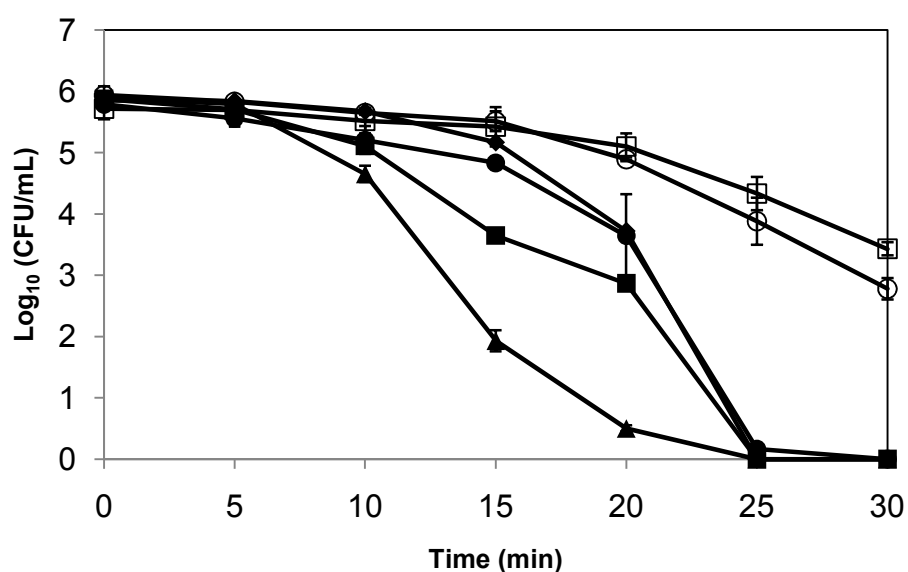


Figure 4.1: Ozone inactivation of *E. coli* at different flow rates in TSB

(□ 0.03 L/min, ◆ 0.06 L/min, ▲ 0.12 L/min, ■ 0.25 L/min, ● 0.5 L/min, ○ 0.75 L/min)
Error bars represent standard deviation.

4.1.2 Effect of ozone concentration

The optimum flow rate of 0.12 L/min was chosen to further investigate the effect of ozone concentration on inactivation. A second flow rate of 0.06 L/min was used in this study as this flow rate was found to incur the least amount of colour degradation for freshly squeezed orange juice (Tiwari et al., 2008c).

Five different levels of ozone concentration were investigated ranging from 28-120 $\mu\text{g/mL}$ ozone in the case of 0.06 L/min, and from 17-75 $\mu\text{g/mL}$ ozone in the case of 0.12 L/min (Figure 4.2). For both flow rates, the highest concentration (level 5) was the most effective to inactivate *E. coli* ATCC 25922 (Figure 4.2 a, b). The flow rate of 0.06 L/min yielded a t_{5d} of 24.2 min and the 0.12 L/min flow rate yielded a t_{5d} value of 20 min. From the graph (Figure 4.2 a, b) it is evident that there is a stepwise decrease in the

efficiency of *E. coli* inactivation at the lower concentration levels (1 and 2), where inactivation was insufficient to allow for the calculation of t_{5d} values.

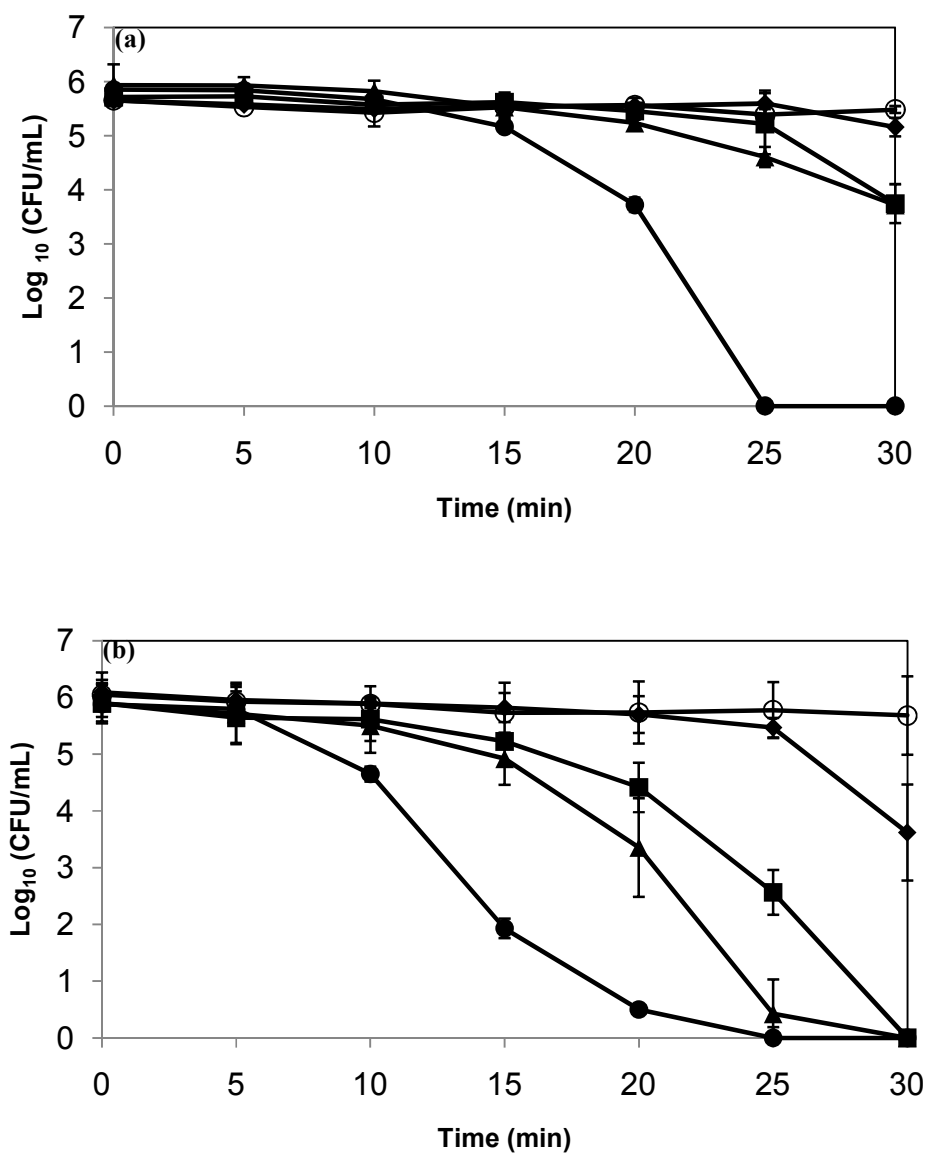


Figure 4.2: Effect of ozone concentration on inactivation of *E. coli* ATCC 25922 in TSB

(a) At 0.06 L/min flow rate ozone concentrations (○ 38 $\mu\text{g/mL}$, ◆ 65 $\mu\text{g/mL}$, ■ 84.5 $\mu\text{g/mL}$, ▲ 108.5 $\mu\text{g/mL}$, ● 117.5 $\mu\text{g/mL}$)
 (b) At 0.12 L/min flow rate ozone concentrations (○ 28 $\mu\text{g/mL}$, ◆ 36.5 $\mu\text{g/mL}$, ■ 51 $\mu\text{g/mL}$, ▲ 68.5 $\mu\text{g/mL}$, ● 73.5 $\mu\text{g/mL}$)

4.1.3 Effect of temperature

Temperature effects on the efficacy of ozone inactivation on *E. coli* ATCC 25922 were examined using the optimum inactivation flow rate of 0.12 L/min. Four different temperatures were investigated: ambient temperature (12-15°C), 20°C, 25°C and 30°C. Survival curves are shown in Figure 4.3. Ambient temperature gave the best inactivation levels with a t_{5d} value of 20 min (Table 4.1).

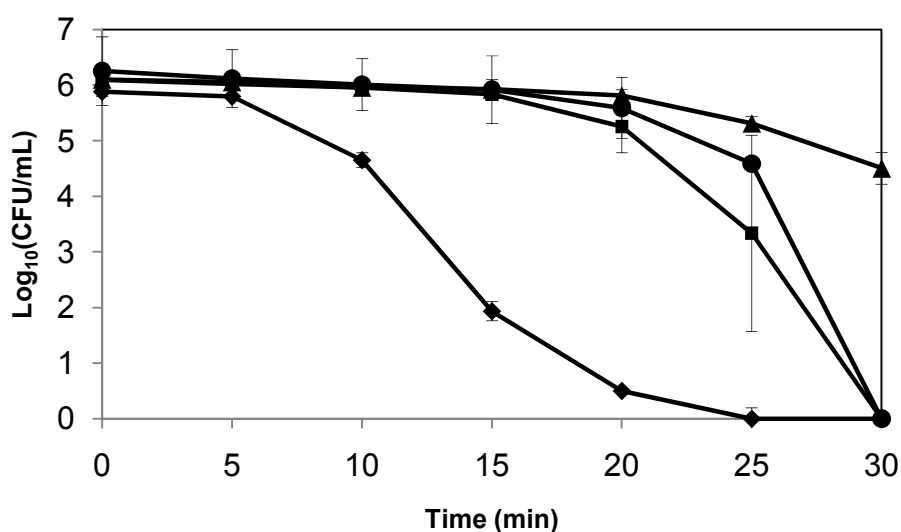


Figure 4.3: Effect of temperature on the efficacy of ozone inactivation of *E. coli* in TSB

(♦ Ambient, ■ 20°C, ● 30°C, ▲ 25°C)
Error bars represent standard deviation

4.1.4 Modelling of the inactivation kinetics and assessment of the process

Each individual experiment was fit to a Weibull model. The Weibull model adequately described the microbial inactivation (Figure 4.4). The relationship between the model parameters (δ, p), and processing parameter of ozone flux (fO_3) in mg of O_3 per min per mL of reactor and temperature (T) are shown in Figure 4.5. The characteristic time relationship with the ozone flux through the reactor showed an increase in the inactivation rate as ozone flux increased until reaching a flux of ca. 0.1 mg O_3 per

processing min per mL of reactor volume. The dependence of the characteristic time with temperature showed that lower processing temperatures achieved higher inactivation rates. The shape parameter p showed no clear dependence with fO_3 , however, a sharp increase of p was found with increasing temperature. At higher processing temperatures the concavity of the Weibull curve increased and the lag time prior to inactivation was found to be shorter. This effect was opposite to the effect that temperature had on the $\log(\delta)$, however, in the present study the temperature effect on $\log(\delta)$ prevailed, consequently, at higher temperatures (up to 25°C) there was less inactivation.

Considering the evidence for curvature on the $\log(\delta)$ and p values with ozone and temperature from Figure 4.5, a model with quadratic effects of ozone and temperature on the characteristic time and shape parameter was built. Additional parameters of dependence of the $\log(\delta)$ with the ozone concentration and the flow rate were found to be significant. Table 4.2 shows the estimated parameters from the Weibull model fit (all parameters were significant at $p < 0.05$).

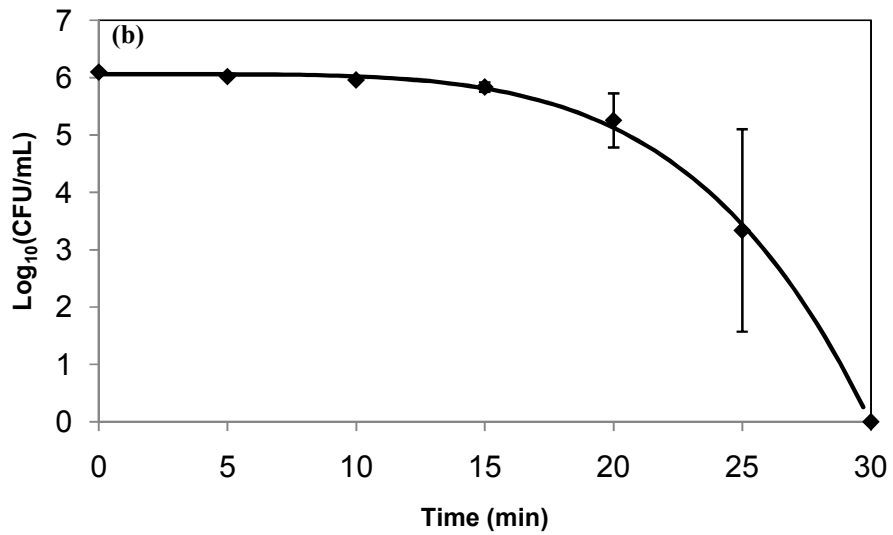
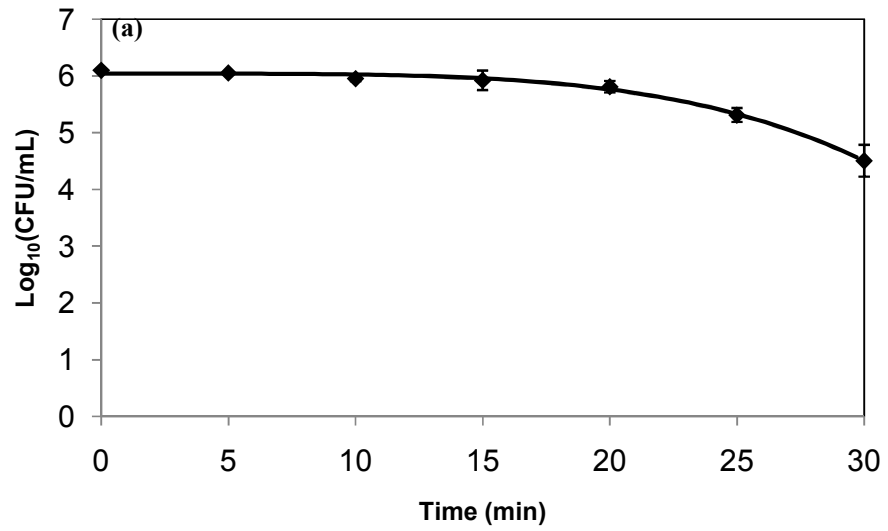


Figure 4.4: Typical Weibull model fit for ozone inactivation of *E. coli*

(a) 0.098 mg Ozone/min/mL at 25°C.

(b) 0.098 mg Ozone/min/mL at 20°C.

Error bars represent standard deviation

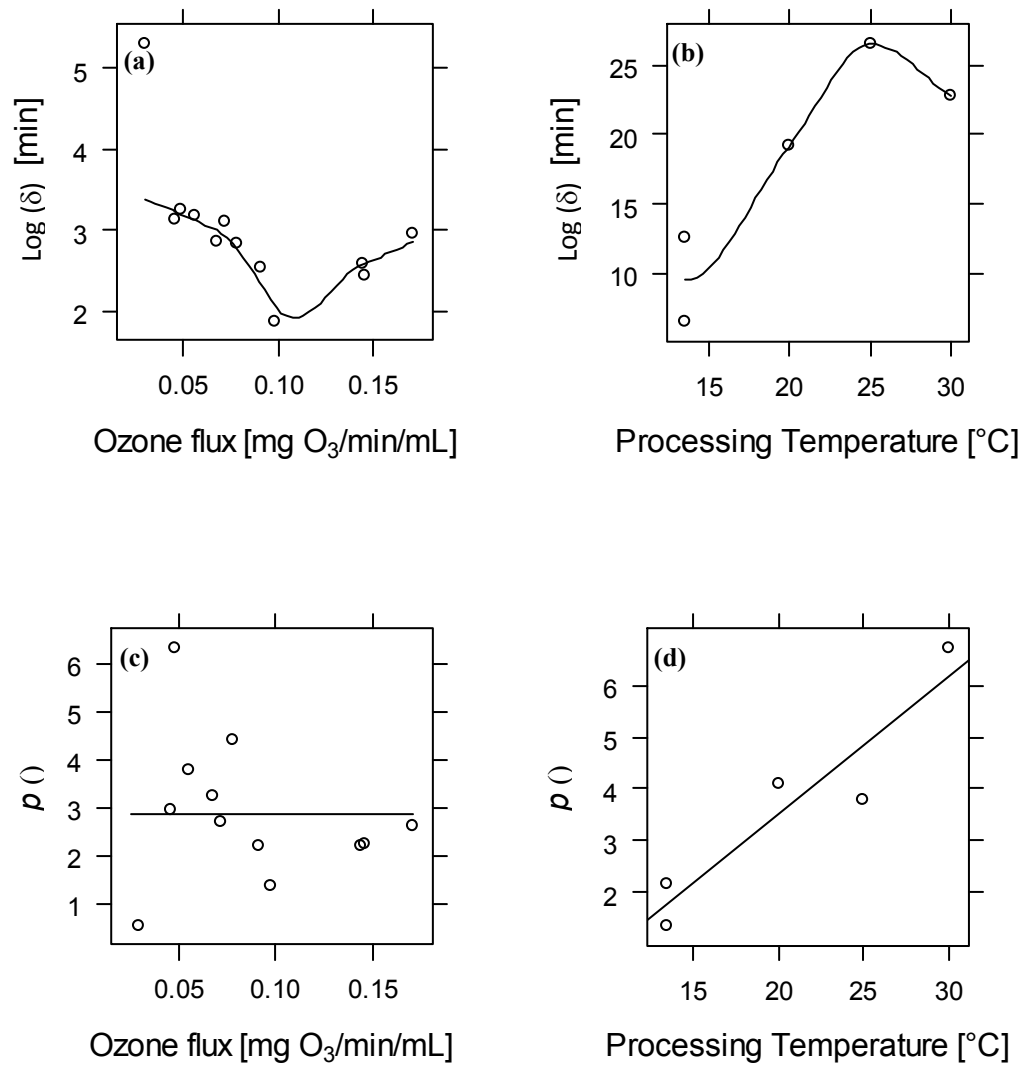


Figure 4.5: Relationship of the log of the characteristic time δ (a) with the ozone processing conditions at ambient temperature and (b) with the processing temperature at 0.09 mg O_3 /min/mL and the shape parameter relationship p with (c) the ozone processing conditions at ambient temperature and (d) the temperature at 0.09 mg O_3 /min/mL of reactor volume.

The continuous line represents a smoothing of the estimates.

Table 4.1: Parameters of the Weibull model and extrinsic control parameters for ozone inactivation of *E. coli*

Extrinsic parameters	fO ₃ [mg O ₃ min ⁻¹ ml ⁻¹]	δ (min) ± STE	p ± STE	RMSE	t _{5d} (min) inactivation time
Gas flow rate (L min⁻¹)					
0.03	0.045	22.98±0.51	3.07±0.23	0.064	38.82±1.75
0.06	0.078	16.88±0.20	4.48±0.13	0.057	24.2±0.38
0.12	0.098	5.53±2.62	1.25±0.4	0.786	20±12.55
0.25	0.145	11.65±1.67	2.27±0.4	0.363	23.7±4.50
0.5	0.144	16.60±0.81	3.83±0.44	0.802	29.5±2.02
0.75	0.170	15.31±0.67	2.28±0.19	0.090	31±2.27
Temperature(°C)					
Ambient	0.098	5.53±2.62	1.25±0.4	0.786	20±12.55
20	0.087	19.18±0.93	4.08±0.13	0.094	28.5±1.42
25	0.087	27.09±0.37	4.22±0.42	0.056	39.7±1.60
30	0.087	23.6±0.45	7.46±0.48	0.152	29.3±0.69
Concentration (levels)					
0.06 L/min flow rate					
1	0.025	-	-	-	-
2	0.043	-	-	-	-
3	0.056	25.68±0.71	2.38±0.38	0.087	50.54±5.63
4	0.072	22.40±0.39	2.54±0.22	0.040	42.2±2.42
5	0.078	16.88±0.20	4.48±0.13	0.057	24.18±0.38
Concentration (levels)					
0.12 L/min flow rate					
1	0.03	-	-	-	-
2	0.048	37.01±4.28	1.52±0.39	0.124	107.04±31.60
3	0.068	18.3±0.61	3.70±0.37	0.110	28.27±1.54
4	0.087	15.50±0.34	3.51±0.16	0.669	24.5±0.74
5	0.098	5.53±2.62	1.25±0.40	0.786	20±12.55

STE: Standard error
 -: not determined
 R²: Coefficient of determination

Table 4.2: Final candidate model for the inactivation of *E. coli* with ozone bubbling.

Parameter	Estimates
Log δ	$1.7_{(0.3)} - 47_{(5)} \times fO_3 + 192_{(23)} \times fO_3^2 + 0.281_{(0.02)} \times T - 0.0053_{(0.0005)} \times T^2 + 1.1_{(0.2)} \times \text{flow.rate} + 0.006_{(0.001)} \times [\text{Ozone}]$
p	$-5.2_{(1.1)} + 0.52_{(0.08)} \times T$
Model Fit	
RMSE*	0.732
SSQ	172.81
R^2	0.91
R^2_{adj}	0.90
n	115

All parameters significant at $p < 0.05$ level.
Standard error of the parameter estimates are represented as subscripts.

*RMSE: Root Mean Square error
SSQ: Sum of squared residual
 R^2 : Coefficient of determination
 R^2_{adj} : Adjusted R-square
n: Number of experiments

4.1.5 Ozone treatment of *E. coli* ATCC 25922 in PBS and model apple juice (MAJ)

This part of the study investigated ozone inactivation efficacy of *E. coli* in different liquid media, PBS and MAJ. Ozone treatment at different flow rates (0.03, 0.06, 0.12, 0.25 and 0.5 L/min) resulted in rapid inactivation of challenge microorganisms. Microbial populations of 10^6 CFU/mL in PBS and MAJ were inactivated after 30 sec and 2 min treatment times respectively, regardless of the ozone flow rate employed.

4.1.6 Discussion

The extrinsic parameters investigated had significant influence on the efficacy of ozone. Five log reductions were achieved in TSB, depending on the flow rate applied to the cell suspension. Other researchers have reported on the effects of intrinsic factors on ozone inactivation including medium type and organic matter content (Chen et al., 1992; Williams et al., 2005). Ozone has a high oxidation potential, reacting well with microorganisms and other particles and compounds if placed in an environment such as food systems rich in organic matter (Kim et al., 1999). The survival curves of *E. coli* in TSB treated with ozone showed a noticeable lag time prior to cell death. Lag times were also observed in other studies, Chen et al. (1992) reported ca. 10 min lag time in 0.8% saline solution when *E. coli* was treated with 5 mg O₃/L at a flow rate of 0.1 L/min. Williams et al. (2005), studied the inactivation of *E. coli* in orange juice, and found that ozonation efficacy was reduced in the presence of ascorbic acid and organic matter. A defined lag time was also observed in the current work when treatment was carried out in TSB. However, in the present study, the absence of organic matter resulted in rapid inactivation of *E. coli* in PBS and MAJ compared to TSB. The ozone flow rate was an important factor for inactivation, which may be related to the effect of gas flow rates on bubble size and therefore to the transport properties of the gas dissolution process. A possible explanation could be that the bubble size generated at the higher flow rates was

too large; reducing the area/volume ratio available for transport and reducing the effective transport compared to lower flow rates. At high flow rates a small number of large bubbles were produced, which rose to the liquid surface quickly, thereby escaping the medium quickly. The resulting poor gas dissolution reduced the concentration of ozone in solution together with the contact time of ozone with the microorganisms, leading to a lower inactivation rate. At low flow rates, small bubbles were produced and transit of the bubble was slow, allowing for the transport of ozone to the solution to be effective. However, as the amount of ozone applied was low, the corresponding inactivation was slow. Some authors indicated the dependence of ozone disinfection efficiency with generated bubble size (Ogden, 1970; Ahmad and Farooq, 1985). Gong et al. (2007) reported that reducing the diameter of ozone bubbles was useful for improving the efficiency of ozone's utilisation. A longer distance or time of rising is important for bigger bubbles to achieve higher transport efficiency, as they need more time to transfer the dissolvable gas inside the bubbles. The results reported in section 4.1.2 indicated that the highest ozone concentrations for two different flow rates were most effective for inactivation of *E. coli* ATCC 25922 in TSB. Similarly, Zuma et al. (2009) also recorded higher reduction of *E. coli* of approximately 5 log in 4 min at the higher ozone concentration (4.724 mg/L) in their study.

Lag times tended to increase with increasing temperature. Herbold et al. (1989) showed that ozone inactivation of hepatitis A virus and *E. coli* was faster at 10°C than at 20°C. Ozone solubility in water is 13 times that of oxygen at 0-30°C (Rice, 1986). The solubility ratio for ozone increases as the temperature of water decreases (Bablon et al., 1991). Ozone decomposition is faster at higher water temperatures (Rice et al., 1981). As the temperature increases, ozone becomes less soluble and less stable with an accompanying increase in the decomposition rate. Achen and Yousef (2001) treated *E. coli* contaminated apples with ozone at 4, 22 and 45°C, and observed that counts of the

bacterium on the surface decreased by 3.3, 3.7 and 3.4 log₁₀-units, respectively. Steenstrup and Floros (2004) studied the effect of temperature (5–20°C) at 860 ppm (v/v) ozone and different gaseous ozone concentrations above 1,000 ppm on inactivation of *E. coli* O157:H7 in apple cider and reported D values ranging from 1.5 to 0.6 min at 5°C and 20°C, respectively. An increased rate of ozone inactivation with increasing temperature was obtained for *B. subtilis* spores and *Cryptosporidium parvum* oocysts (Corona-Vasquez et al., 2002; Larson and Mariñas, 2003). Dow et al. (2006) indicated that raising the temperature over a range of 7-22°C had the strongest influence on the inactivation rate of *B. subtilis* spores in oxidant demand free phosphate buffer and also led to corresponding decreases in the lag phase and CT-2 log (CT value is the exposure value obtained by the multiplication of the disinfectant concentration (C) in mg/L and contact time (T) in min to deactivate the organism, CT_{2 log}= 99% deactivation). Driedger et al. (2001) reported decreased ozone inactivation rate of *C. parvum* with decreasing temperature. The temperatures employed in this study ranged from ambient to 30°C. In the current study, a reduction > 5 log cycles was achieved at ambient temperature (12-15°C) within 25 min. However, increased temperatures of 20 and 30°C achieved similar log reduction in 30 min. But a reduction of only 1.6 log cycles was obtained at 25°C. In general, the antimicrobial activity of ozone decreased with increasing temperatures.

This result showed that the efficacy of ozone inactivation depended on the medium type and organic matter content. Based on these investigations, to optimise the use of ozone for liquid systems, temperatures should be maintained at low to ambient (12-18°C), the maximum obtainable ozone concentration should be employed and the flow rate should be selected to maximise solubility thus minimising gas escape from the free surface. Tiwari et al. (2008a; 2009a; 2009b) recently studied the effects of ozone on quality and nutritional parameters for a range of fruit juices, highlighting significant losses in

nutritional quality, which were dependent on ozone control parameters of ozone concentration and gas flow rate. However, achieving rapid microbial inactivation using optimised control parameters may mitigate losses in nutritional quality. Balance of the parameters governing ozone inactivation process is important. Process optimisation (optimum parameters) is vital to achieve the required log reduction to attain microbial safety. Therefore, the balance of parameters should be considered as to which takes priority in determining an optimised process, thereby assuring microbial safety without loss of quality parameters. Ozone inactivation efficacy will be dependent on the food system employed; therefore various fruit juices should be investigated further.

The ozone inactivation kinetics of *E. coli* was well described by the Weibull model. The inactivation of *E. coli* displayed a downward concavity, with a p parameter > 1 , indicating the susceptibility of cells to the ozone treatment (van Boekel, 2002).

The fitting of the microbial inactivation parameters with the process variables yielded a model that presented a dependence not only on the amount of ozone introduced per min, but also a dependence on the total gas flux and the ozone concentration in the bubble. The final model points to a compromise region of intermediate ozone flow rates (around 0.1 mg O₃/min/mL) at ambient temperatures.

4.2 Inactivation of *E. coli* in orange juice using ozone

This second ozone based study investigated the efficacy of gaseous ozone at constant parameters of the optimum flow rate of 0.12 L/min with an ozone concentration of 72-75 µg/mL for the inactivation of *E. coli* ATCC 25922 and NCTC 12900 strains in orange juice. Variable parameters included orange juice composition and prior stress exposure of the challenge organisms. The range of orange juice types included model orange juice (MOJ), fresh unfiltered juice, juice without pulp, and juice filtered through 500 µm or 1 mm sieves.

The objectives of this study were (i) to determine the efficacy of continuous gaseous ozone treatment for reduction of two different strains of *E. coli* at ambient temperature in orange juice, (ii) to evaluate how inactivation was affected by the orange juice pulp content and (iii) to investigate if prior acid exposure of the challenge microorganism significantly impacted on treatment efficacy.

4.2.1 Effect of ozone inactivation of *E. coli* in model orange juice (MOJ)

Ozone inactivation of both *E. coli* strains in MOJ was rapid in this low pH medium. Ozone treatment at the optimum flow rate of 0.12 L/min with an ozone concentration of 72-75 µg/mL resulted in a 6.0 log cycle reduction within 60 sec.

4.2.2 Effect of ozone on inactivation of *E. coli* in orange juice

The inactivation of *E. coli* in orange juice was fitted using the Weibull model, which provided estimations of microbial inactivation in terms of processing time required. The Weibull parameters δ and p are shown in Table 4.3. In the present study, the shape parameter p showed downward concavity for both *E. coli* strains (Figures 4.6 and 4.7); p values >1 indicate the susceptibility of the cells to the treatment (van Boekel, 2002). The R^2 values of 0.93 and above (Figures 4.6 and 4.7) and the RMSE values of the regression analysis (Tables 4.3 and 4.4), showed that the Weibull model was a good fit for the experimental data analysed.

The efficacy of ozone was found to depend both on the juice type and the bacterial strain ($p < 0.05$). Both strains of *E. coli* studied (*E. coli* ATCC 25922, *E. coli* NCTC 12900) were sensitive to ozone ($p < 0.05$). In unfiltered juice, ATCC 25922 and NCTC 12900 were completely inactivated after 18 and 15 min respectively (Figures 4.6 and 4.7) as determined on TSA and SMAC. However, ozone treatment of ATCC 25922 in orange juice without pulp and juice passed through the 500 µm sieve, resulted in complete inactivation within 5 min (Figure 4.6). The population of *E. coli* 25922 in juice passed through sieve of 1 mm diameter decreased by 6.0 log cycles in 6 min

treatment time (Figure 4.6). Similarly, ozone treatment of NCTC 12900 in orange juice without pulp and juice passed through the 500 μm sieve resulted in complete inactivation in 5 and 6 min, respectively (Figure 4.7). NCTC 12900 decreased by 4.6 and 6.0 log cycles after 6 min treatment time in juice passed through the 1 mm sieve as determined on TSA and SMAC, respectively.

The t_{5d} for both *E. coli* strains in the different juice types are shown in Table 4.3. The t_{5d} values were lower as the amount of pulp present in the orange juice decreased ($p < 0.05$). The inactivation of *E. coli* strains in unfiltered juice showed the highest t_{5d} values.

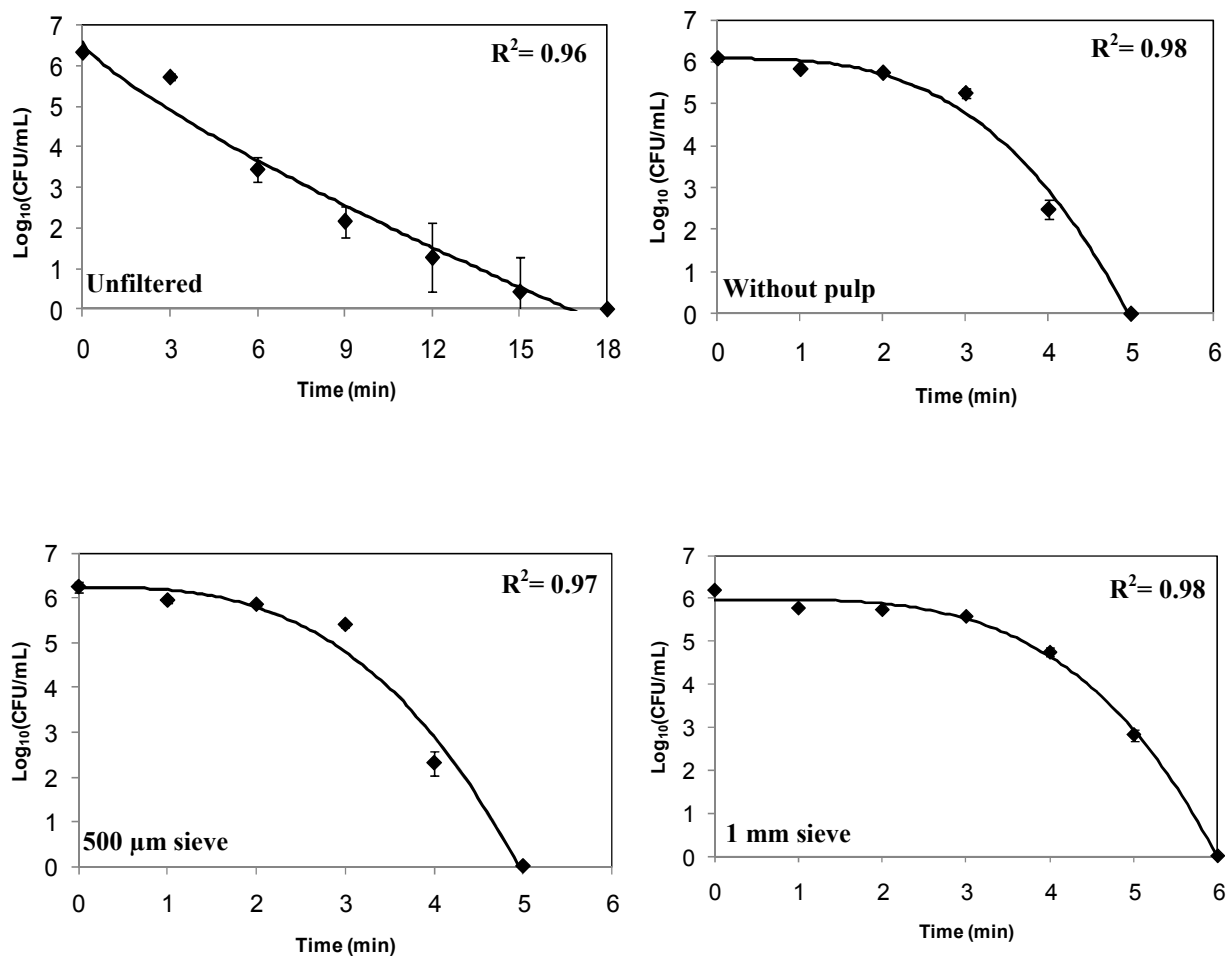


Figure 4.6: Microbial survival curve of *Escherichia coli* ATCC 25922 for the different orange juice types.

(Data points were obtained on TSA agar)

Curves were fitted using the Weibull model.

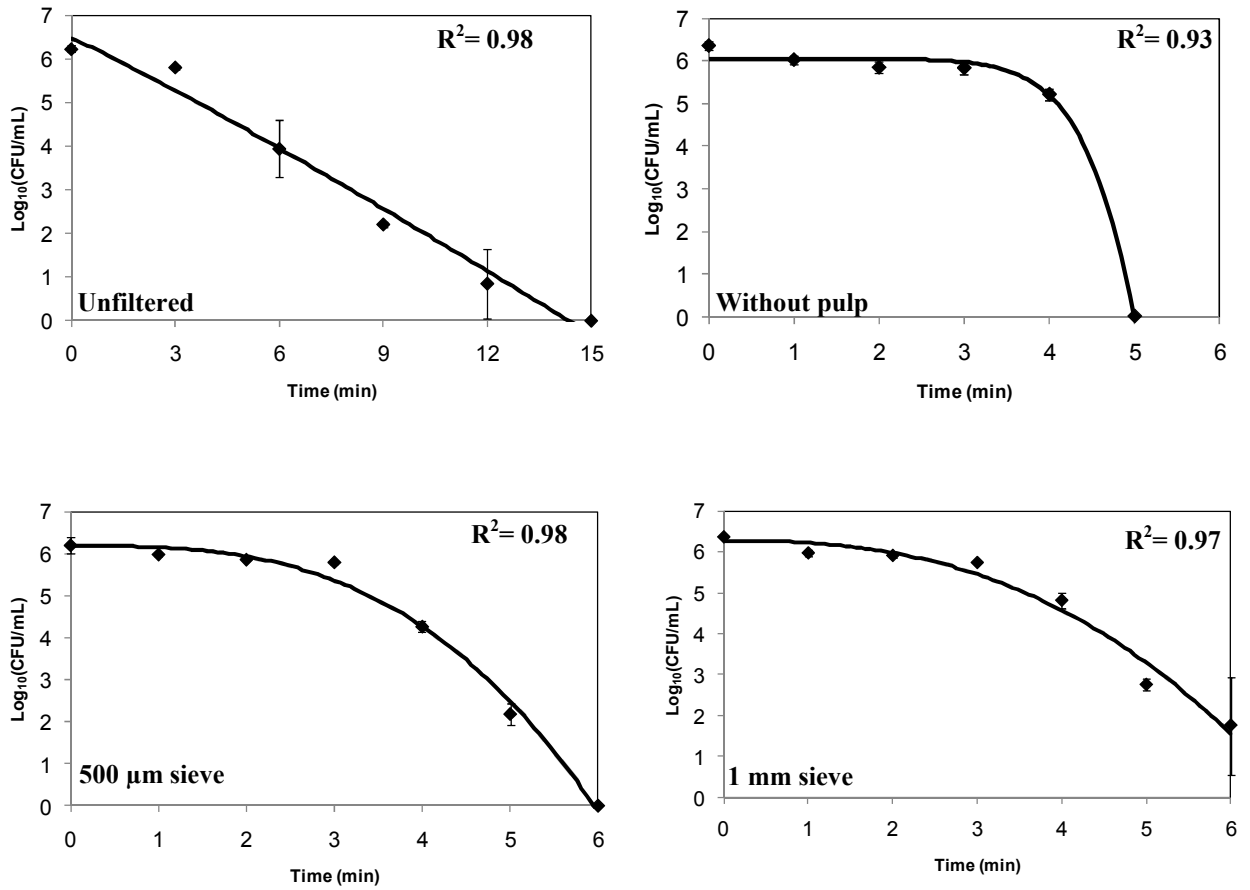


Figure 4.7: Microbial survival curve of *E. coli* NCTC 12900 for the different orange juice types.

(Data points were obtained on TSA agar)

Curves were fitted using the Weibull model.

Table 4.3: Parameters of the Weibull model and the time required to reach a 5 log reduction for *E. coli* strains in orange juice after treatment with ozone

Microorganism	Juice type	Condition	δ (min) \pm SE	$p \pm$ SE	RMSE	t_{5d} (min)
<i>E. coli</i> ATCC 25922	Unfiltered	control	1.58 ^a \pm 0.84	0.80 ^a \pm 0.16	0.54	11.86 ^a
	1 mm sieve	control	3.28 ^b \pm 0.33	2.98 ^b \pm 0.49	0.17	5.62 ^b
	500 μ m sieve	control	2.77 ^c \pm 0.39	3.14 ^b \pm 0.73	0.51	4.63 ^c
	Without pulp	control	2.91 ^d \pm 0.35	3.26 ^b \pm 0.69	0.40	4.76 ^c
<i>E. coli</i> NCTC 12900	Unfiltered	control	2.55 ^a \pm 0.91	1.08 ^a \pm 0.21	0.46	11.30 ^a
	1 mm sieve	control	3.24 ^a \pm 0.43	2.52 ^b \pm 0.52	0.36	6.14 ^b
	500 μ m sieve	control	3.12 ^a \pm 0.26	2.81 ^{bc} \pm 0.35	0.28	5.53 ^{cd}
	Without pulp	control	3.41 ^a \pm 0.55	4.44 ^c \pm 1.81	0.22	4.90 ^c

(Different letters indicate a significant difference at the 0.05 level between each type of juice and each strain).
* SE: standard error

4.2.3 Effect of acid exposure on treatment efficacy

The effect of acid exposure on ozone treatment efficacy was evaluated using orange juice passed through a 1 mm sieve. Ozone inactivation curves for acid-exposed *E. coli* cells at the different acid exposure conditions are shown in Figure 4.8. For acid exposed *E. coli* strains the shape parameter p showed downward concavity. The p values for 1 h acid exposed cells were lower by comparison with both the 18 h acid exposed and control populations (Table 4.4), indicating a lower susceptibility to the ozone treatment with a short period of acid adaptation.

Ozone treatment of 1 h acid exposed *E. coli* ATCC 25922 resulted in a reduction of 4.8 and 5.5 log cycles after 7 min treatment time on TSA and SMAC, respectively. However, ozone treatment of 1 h acid exposed *E. coli* NCTC 12900 reduced an initial count of log 6.28 CFU/mL to below detectable levels after 7 min treatment time on TSA and SMAC, respectively. With the 18 h acid exposed cells, populations of *E. coli* ATCC 25922 and *E. coli* NCTC 12900 were decreased by 6.0 and 5.3 log cycles respectively within 7 min as determined by using TSA. Similar trends were observed using SMAC where 18 h acid exposed *E. coli* ATCC 25922 and *E. coli* NCTC 12900 decreased by 5.8 and 5.1 log cycles, respectively. The t_{5d} values of the acid exposed *E. coli* strains are shown in Table 4.4. There was a strain difference observed between acid exposed and control populations. The estimated time for a 5 log reduction of control (non-acid exposed) *E. coli* NCTC 12900, was 6.14 min, while the estimate for the generic strain *E. coli* ATCC 25922 was 5.62 min. When the strains were subjected to a 1 h acid exposure, the estimated time required for a 5 log cycle reduction in *E. coli* ATCC 25922 increased to 6.46 min, while there was no similar increase for *E. coli* NCTC 12900. Conversely, following 18 h acid exposure, the estimated time required for a 5 log cycle reduction in *E. coli* NCTC 12900 increased to 6.84 min, while the estimated time for *E. coli* ATCC 25922 was similar to that recorded for the control cells. However, there was

a significant difference observed for *E. coli* ATCC 25922 between 1 h acid exposed population compared to the control and 18 h acid exposed population ($p>0.05$); whereas there was no significant difference observed between control population of *E. coli* NCTC 12900 and those exposed to acid conditions for 1 h or 18 h.

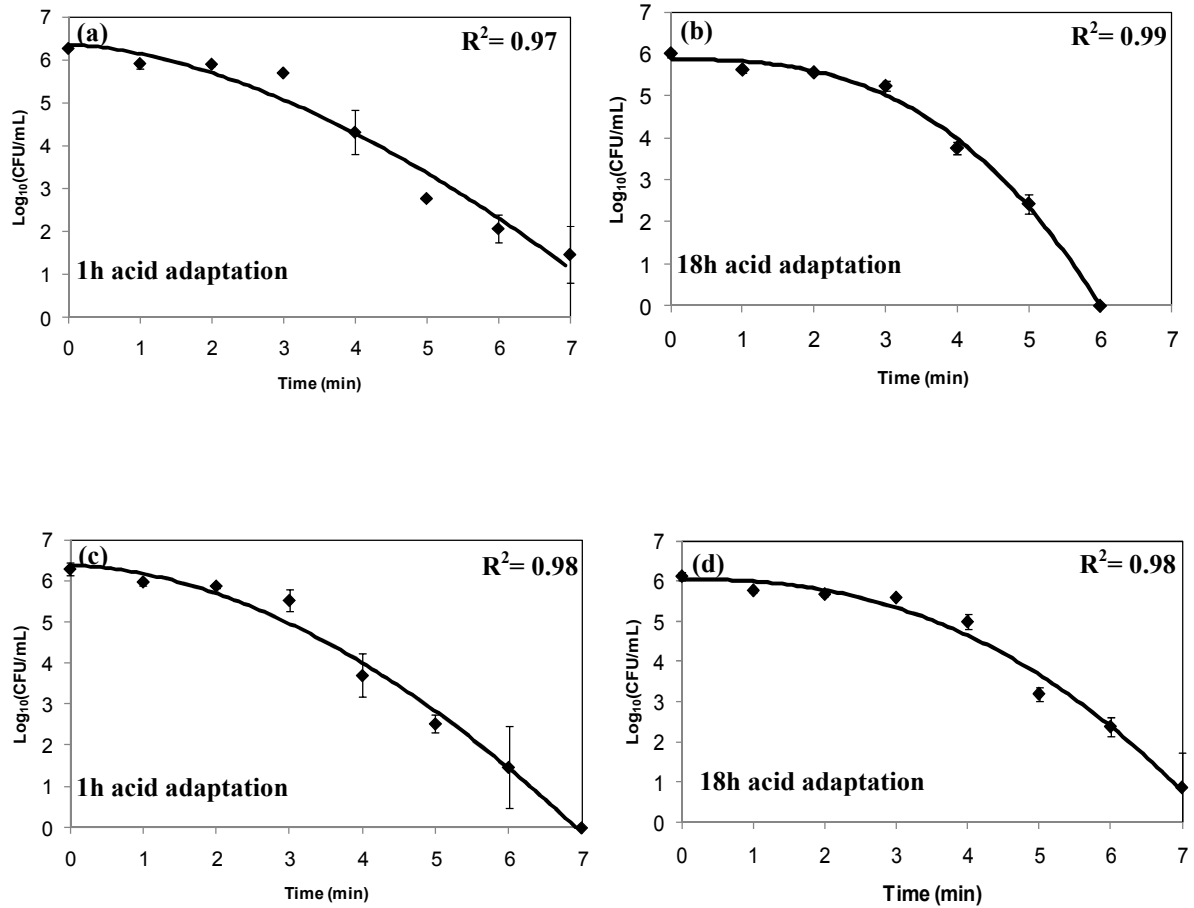


Figure 4.8: Microbial survival curve of acid exposed *Escherichia coli* strains of the reduced pulp orange juice (1mm sieve size).

(Data points were obtained on TSA agar)

Curves were fitted using the Weibull model.

- a) 1 h acid exposed *Escherichia coli* ATCC 25922
- b) 18 h acid exposed *Escherichia coli* ATCC 25922
- c) 1 h acid exposed *Escherichia coli* NCTC 12900
- d) 18 h acid exposed *Escherichia coli* NCTC 12900

Table 4.4: Parameters of the Weibull model and the time required to reach a 5 log reduction for acid exposed *E. coli* strains in orange juice after treatment with ozone

Microorganism	Juice type	Condition	δ (min) \pm SE	$p \pm$ SE	RMSE	t_{5d} (min)
<i>E. coli</i> ATCC 25922	1 mm sieve	Control	3.28 ^a \pm 0.33	2.98 ^a \pm 0.49	0.17	5.62 ^a
	1 mm sieve	1h acid adaptation	2.41 ^b \pm 0.52	1.63 ^b \pm 0.31	0.44	6.46 ^b
	1 mm sieve	18 h acid adaptation	3.08 ^a \pm 0.20	2.63 ^c \pm 0.25	0.20	5.67 ^a
<i>E. coli</i> NCTC 12900	1 mm sieve	Control	3.24 ^a \pm 0.43	2.52 ^a \pm 0.52	0.36	6.14 ^a
	1 mm sieve	1h acid adaptation	2.49 ^a \pm 0.36	1.81 ^a \pm 0.25	0.34	6.06 ^{ab}
	1 mm sieve	18 h acid adaptation	3.47 ^a \pm 0.37	2.37 ^a \pm 0.35	0.31	6.84 ^{ac}

(Different letters indicate a significant difference at the 0.05 level)

* SE: standard error

4.2.4 Discussion

The direct application of ozone was found to be effective for the inactivation or reduction of *E. coli* in orange juice (Figures 4.6, 4.7 and 4.8), but the rate was dependant on the juice type used. Complete inactivation in unfiltered juice was achieved after 15-18 min treatment time by comparison with a time of 60 sec or 5 min in MOJ or juice with low pulp content, respectively. This may be attributed to the organic compounds present in orange juice, including sugars, fibre and ascorbic acid which could act as protective barriers for *E. coli* cells against ozone. The organic load present within the medium is known to decrease the effectiveness of ozone for the inactivation of microorganisms. Williams et al. (2005), observed a reduced efficacy of ozonation for inactivation of *E. coli* in orange juice compared to apple cider and Mielcke and Ried (2004), also reported that a high and persistent level of organic substances will have a negative impact on the ozone disinfection rate. The effectiveness of ozone against microorganisms has been reported to depend not only on the amount applied, but also on the residual ozone in the medium, various environmental factors such as medium pH, temperature, humidity, additives (surfactants, sugars, etc.), and the amount of organic matter surrounding the cells (Pascual et al., 2007).

The type of organic material may impact ozone efficacy more than the amount of organic material present (Restaino et al., 1995). This is in agreement with Guzel-Seydim et al. (2004a), who observed that the presence of caseinate in whipping cream provided a high level of protection to the bacterial populations against ozone treatment, whereas locust bean gum resulted in an intermediate level of protection. Komanapalli and Lau (1998) also found that the cidal activity of ozone was greatly affected by the dose applied, the presence of ozone-quenching proteins, and the type of challenge microorganisms. In the present study, fast inactivation rates were achieved in the MOJ and the filtered juices which may be attributed to the absence of high ozone demanding

substances. Similarly, the antimicrobial activity of ozone was reported to be affected by the presence of organic matter which resulted in slower inactivation rate of challenge microorganisms with increased ozone demand (Farooq et al., 1977a; Hunt and Marinas, 1999; Janex et al., 2000). Therefore, it is necessary that the applied ozone concentration should be sufficient to overcome this demand so that a free residual ozone concentration will be available for inactivation of microorganisms. Williams et al. (2004) reported *E. coli* O157:H7 was inactivated in orange juice after a 75 min ozone treatment applied at ambient temperature, while in the present study faster inactivation rates within a period of 6 to 18 min were achieved. The antibacterial efficacy of ozone was greater when target microorganisms were suspended in pure water or simple buffers than in complex systems (Khadre et al., 2001). Decreasing pH and temperature are associated with increasing stability of ozone molecules (Kim et al., 1999).

When microorganisms are stressed, an adaptive response may follow which can increase the organisms' tolerance to the same or to a different type of stress (Yousef and Courtney, 2003). Many bacteria react to stress by inducing the synthesis of various proteins (Herendeen et al., 1979; Jones and Inouye, 1994). Buchanan and Edelson (1999a), reported a cross protective effect of acid shock and acid adaptation of enterohaemorrhagic *E. coli* (EHEC) against heat or other stresses but also observed that the determination of survival of EHEC in acidic foods should consider the strain and its ability to induce stress responses. The resistance or adaptation of microorganisms to acid conditions can have implications for food safety. Additionally, Johnson (2003) observed that challenge studies in food systems are required to adequately assess growth or survival of pathogens. In this study both *E. coli* strains were subjected to acid exposure at pH 5.0 to examine the effect of prior acid exposure on the efficacy of ozone treatment in orange juice. Increased inactivation time of acid exposed *E. coli* cells of both strains to ozone treatment over the control cells was observed. The t_{5d} values of

acid exposed *E. coli* cells were higher than the t_{5d} values of control cells in some cases. Treatment of *E. coli* O157:H7 with acid has been reported to increase acid resistance after exposure to moderate acid environments (Kroll and Patchett, 1992; Leyer et al., 1995) and was also shown to confer cross resistance to salt and heat (Rowe and Kirk, 1999). In beef processing, prior acid adaptation negatively influenced the efficacy of a 2% acetic acid decontamination treatment for reduction of *E. coli* O157:H7 on carcasses (Berry and Cutter, 2000) and acid adaptation prolonged the survival of *E. coli* O157:H7 in various food systems, including apple cider, sausages (Leyer et al., 1995) and acidic fruit juice (Hsin-Yi and Chou, 2001).

Acid habituation of pathogens may enhance survival in acidic food (e.g. fruit juice) or in the stomach and subsequently cause infection after ingestion (Goodson and Rowbury, 1989). In an environment with changing pH, acid sensitive *E. coli* O157 cultures can become acid-resistant within 17 min (de Jonge et al., 2003). Acid resistance and survival of pathogens have significant implications for food safety and the virulence of pathogenic microorganisms and the ability of non-acid adapted *E. coli* O157 to adapt within a very short period under extreme conditions further contribute to their virulence (Beales, 2004). The results of the present study also showed that the extent of increased acid resistance varied with the strain and acid exposure conditions. When *E. coli* ATCC 25922 was acid exposed for 1 h, an increased resistance to ozone treatment was observed. In the case of *E. coli* NCTC 12900 only the longer acid exposure time (18 h) showed an increased t_{5d} value compared to the control cells. However, while increased resistance of acid stressed *E. coli* cells to ozone treatment was observed, 5 log cycle reductions in populations were still achieved in less than 7 min. Buchanan et al. (1999) also reported that while pH during exposure had little effect on survival of *E. coli* O157:H7, acid-resistance consistently enhanced radiation resistance. Therefore, acid

resistance should be considered when determining t_{5d} values or other similar processing parameters in foods.

4.3 Ozone inactivation of *E. coli* in apple juice (unclarified)

This research investigated the efficacy of gaseous ozone (at a flow rate of 0.12 L/min and ozone concentration of 33-40 $\mu\text{g/mL}$) on the inactivation of *E. coli* ATCC 25922 and NCTC 12900 strains in apple juice, using an ozone bubble column.

The objectives of this study were (i) to determine the efficacy of continuous gaseous ozone treatment for reduction of two strains of *E. coli* at ambient temperature in apple juice, and (ii) to investigate if prior acid exposure of the challenge microorganism significantly impacted on treatment efficacy and (iii) to evaluate the effect of apple juice pH on the efficacy of gaseous ozone treatment.

4.3.1 Ozone inactivation of control non acid exposed and mild acid stressed/exposed *E. coli* cells

Populations of indigenous background microflora in unpasteurised apple juice were generally in the range of 4.3-4.8 log CFU/mL.

Initial populations of *E. coli* inoculated in apple juice were approximately between 6.2-6.7 log CFU/mL. The inactivation of *E. coli* in apple juice was fitted using the Weibull and the shoulder log-linear model, which provided estimations of microbial inactivation in terms of processing time required. The low RMSE values (Table 4.5) showed that both models gave a good fit for the experimental data analysed. The estimated t_{5d} values are also shown in Table 4.5. Both strains of *E. coli* studied (*E. coli* ATCC 25922, *E. coli* NCTC 12900) were sensitive to ozone. Ozone treatment of control *E. coli* ATCC 25922 resulted in reduction of 6.0 log cycles in 4 min of treatment time as determined on TSA-SMAC by overlay method and SMAC, respectively (Figure 4.9). However, within the same 4 min treatment time, control *E. coli* NCTC 12900 was reduced by 4.98 and 5.25

log cycles as determined on TSA-SMAC by overlay method and SMAC, respectively (Figure 4.9).

Ozone treatment of mild acid stressed ATCC 25922 in apple juice resulted in complete inactivation within 5 min of treatment time (Figure 4.10). However, treatment of mild acid stressed *E. coli* NCTC 12900 for 5 min yielded 4.52 and 4.54 log cycle reductions as determined on TSA-SMAC and SMAC respectively (Figure 4.10). The t_{5d} value for control and mild acid stressed population of NCTC 12900 was higher when compared with ATCC 25922 (Table 4.5).

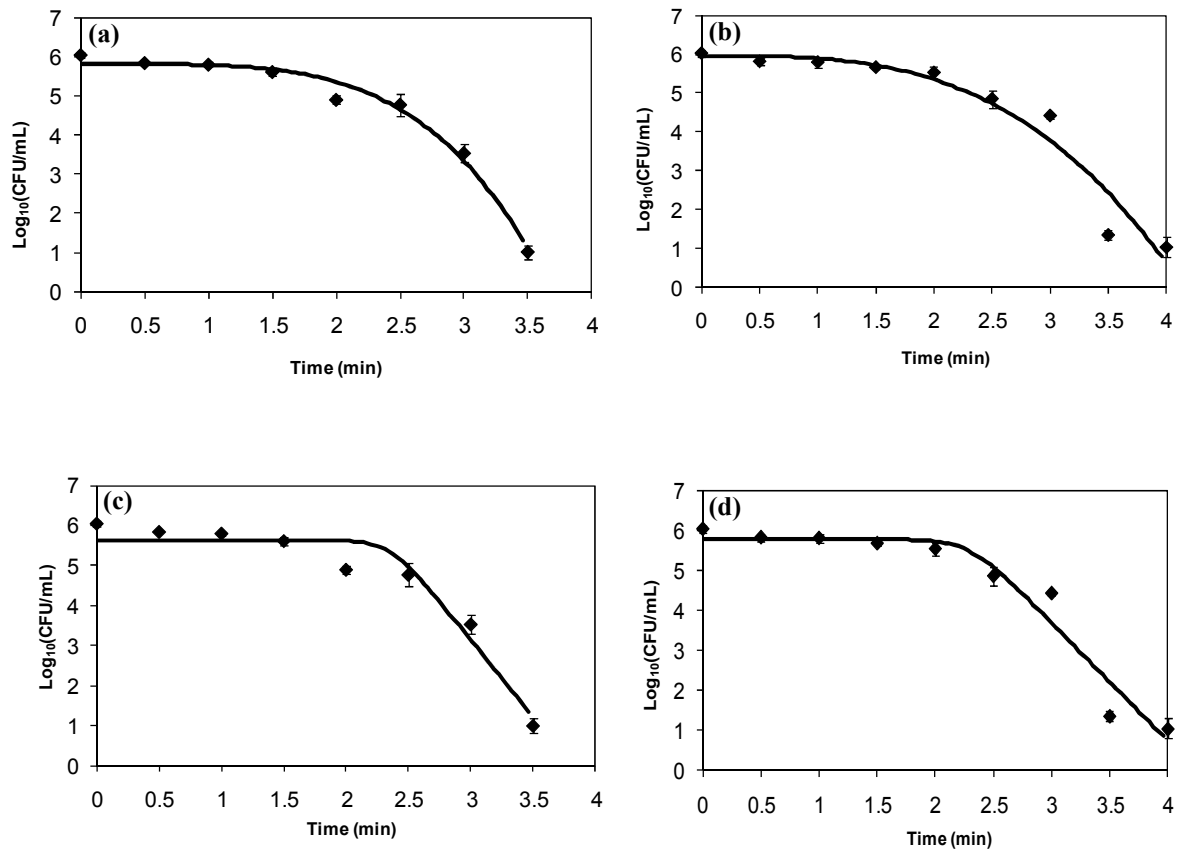


Figure 4.9: Ozone inactivation of control non acid exposed cells of *E. coli* in apple juice

(Data points were obtained on TSA-SMAC agar overlay)

- a) *E. coli* ATCC 25922 (Weibull model)
- b) *E. coli* NCTC 12900 (Weibull model)
- c) *E. coli* ATCC 25922 (Shoulder log linear model)
- d) *E. coli* NCTC 12900 (Shoulder log linear model)

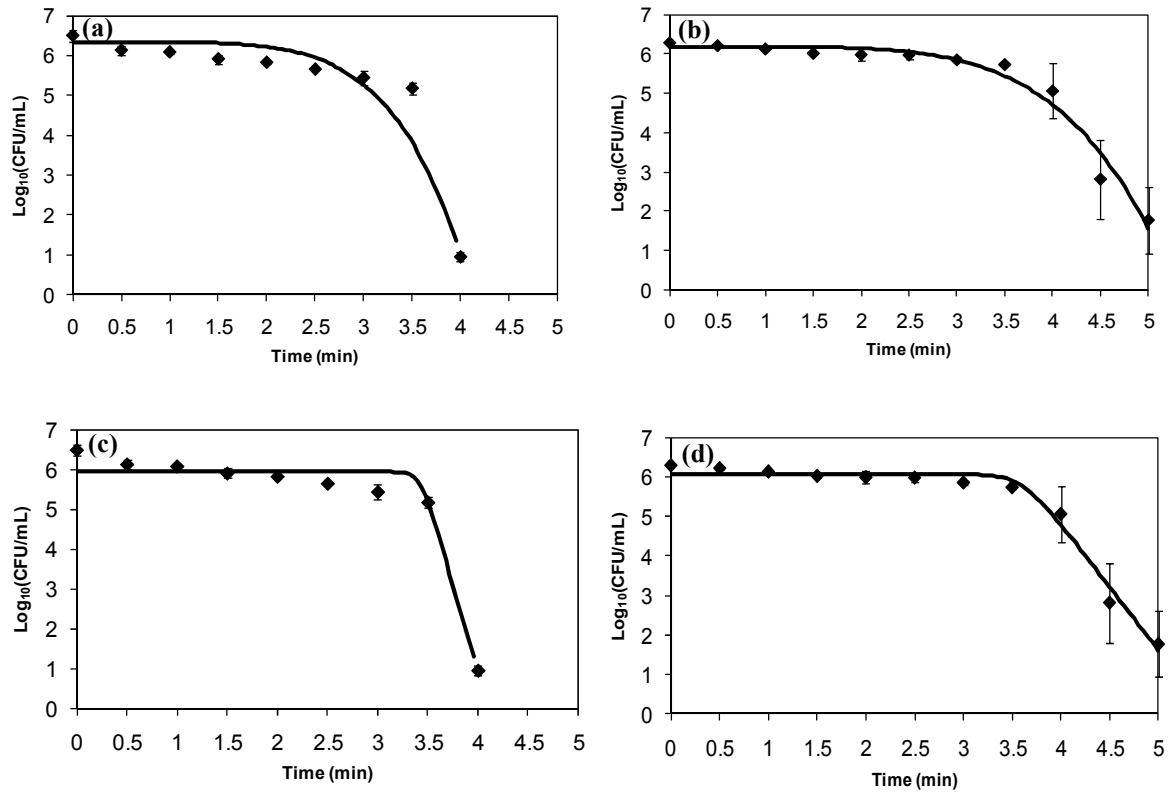


Figure 4.10: Ozone inactivation of mild acid stressed cells of *E. coli* in apple juice
(Data points were obtained on TSA-SMAC agar overlay)

- a) *E. coli* ATCC 25922 (Weibull model)
- b) *E. coli* NCTC 12900 (Weibull model)
- c) *E. coli* ATCC 25922 (Shoulder log linear model)
- d) *E. coli* NCTC 12900 (Shoulder log linear model)

4.3.2 Ozone inactivation of acid exposed *E. coli* cells

Ozone inactivation curves for acid exposed *E. coli* cells at the different acid exposure conditions are shown in Figures 4.11 and 4.12. The t_{5d} values for 18 h acid exposed cells of ATCC 25922 were higher by comparison with the control, 1 h acid exposed and mild acid stressed cells (Table 4.5), indicating a greater susceptibility to ozone treatment with a shorter period of acid exposure. However, the t_{5d} values for NCTC 12900 mild acid stressed cells were higher by comparison with control 1 h and 18 h acid exposed cells (Table 4.5). Ozone treatment of 1 h acid exposed *E. coli* ATCC 25922 resulted in a reduction of 6.3 and 6.0 log cycles after 5 min treatment time as determined on TSA-SMAC and SMAC, respectively. However, 1 h acid exposed *E. coli* NCTC 12900 cells treated with ozone decreased by 5.9 and 6.0 log cycles after 5 min treatment as determined on TSA-SMAC and SMAC, respectively. On the other hand, with the 18 h acid exposed cells, populations of *E. coli* ATCC 25922 and *E. coli* NCTC 12900 were reduced by 5.0 and 5.6 log cycles respectively within 5 min as determined by using TSA-SMAC overlay. Similar trends were observed using SMAC where 18 h acid exposed *E. coli* ATCC 25922 and *E. coli* NCTC 12900 were decreased by 4.99 and 5.3 log cycles, respectively.

The estimated time for a 5 log reduction of mild acid stressed *E. coli* NCTC 12900, the non-toxicogenic *E. coli* O157:H7 strain, determined by both models, was higher by comparison with the generic strain ATCC 25922. However, when *E. coli* ATCC 25922 cells were subjected to a 1 h and 18 h acid exposure, the estimated time required for a 5 log cycle reduction was higher as determined by both models. There was a significantly higher t_{5d} value ($p < 0.05$) recorded for *E. coli* ATCC 25922 for 18 h acid exposed cells compared with control, mild acid stressed and 1 h acid exposed cells; whereas, for *E. coli* NCTC 12900 there was agreement between both models applied, but only where

mild acid stressed *E. coli* NCTC 12900 had higher t_{5d} value ($p < 0.05$) than control populations.

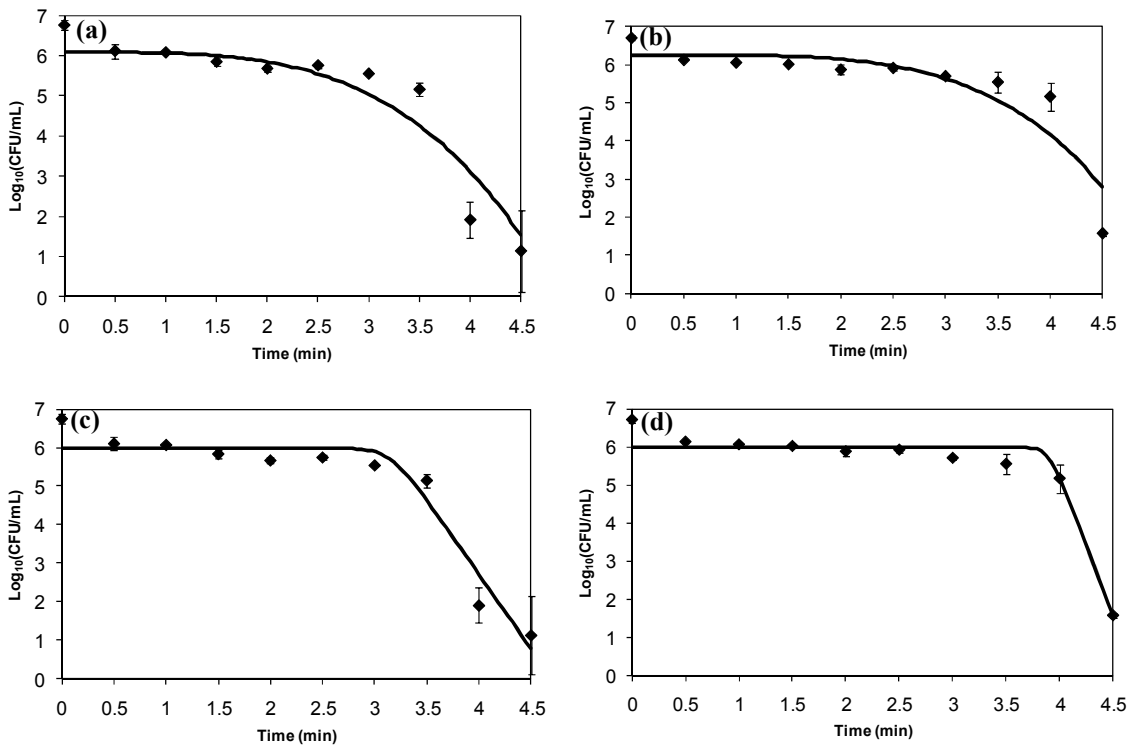


Figure 4.11: Ozone inactivation of 1 h acid exposed *E. coli* in apple juice
(Data points were obtained on TSA-SMAC agar overlay)

- a) *E. coli* ATCC 25922 (Weibull model)
- b) *E. coli* NCTC 12900 (Weibull model)
- c) *E. coli* ATCC 25922 (Shoulder log linear model)
- d) *E. coli* NCTC 12900 (Shoulder log linear model)

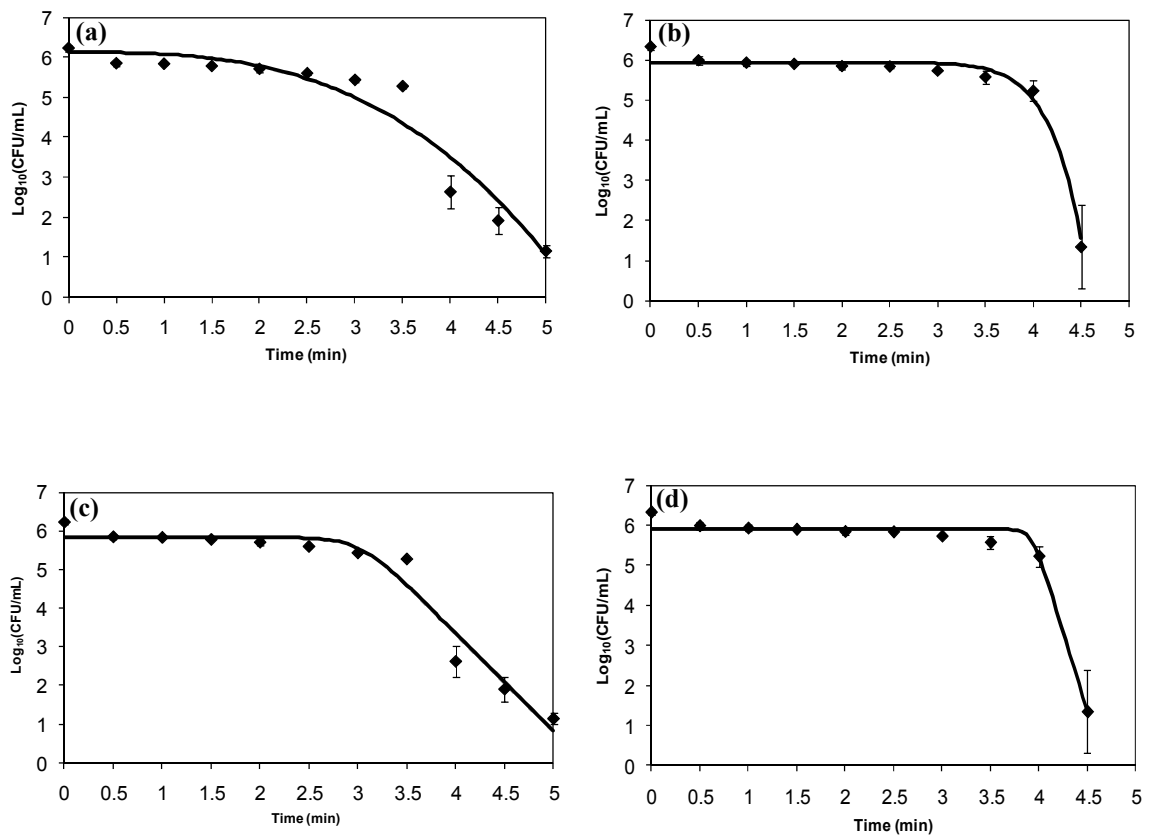


Figure 4.12: Ozone inactivation of 18 h acid exposed *E. coli* in apple juice
(Data points were obtained on TSA-SMAC agar overlay)

- a) *E. coli* ATCC 25922 (Weibull model)
- b) *E. coli* NCTC 12900 (Weibull model)
- c) *E. coli* ATCC 25922 (Shoulder log linear model)
- d) *E. coli* NCTC 12900 (Shoulder log linear model)

Table 4.5: Time required to achieve a 5 log reduction (t_{5d}) for *E. coli* strains in apple juice

Strain	Condition	t_{5d} (min) (Weibull model, Eq. (2-6))	RMSE	t_{5d} (min) (Shoulder log- linear model, Eq. (2-8))	RMSE
<i>E. coli</i> ATCC 25922	Control	3.55 ^A ± 0.25	0.27	3.65 ^A ± 0.29	0.46
	Mild acid stressed	3.96 ^B ± 0.48	0.31	4.00 ^{AB} ± 0.09	0.34
	1h acid exposure	4.62 ^C ± 0.95	0.52	4.44 ^B ± 0.25	0.59
	18 h acid exposure	4.98 ^D ± 0.89	0.51	5.00 ^C ± 0.33	0.41
<i>E. coli</i> NCTC 12900	Control	3.92 ^A ± 0.72	0.55	3.98 ^A ± 0.35	0.50
	Mild acid stressed	5.08 ^B ± 0.33	0.31	5.17 ^B ± 0.18	0.22
	1h acid exposure	4.91 ^B ± 1.05	0.31	4.58 ^{AB} ± 0.12	0.34
	18 h acid exposure	4.55 ^B ± 0.12	0.20	4.56 ^{AB} ± 0.07	0.21

Different letters indicate the significant difference at the 0.05 level between each type of condition

RMSE: root mean squared error for the regression

±: standard error

4.3.3 Ozone inactivation of *E. coli* in clarified apple juice

Populations of indigenous background microflora in clarified apple juice were generally in the range of 2.88-3.0 log CFU/mL.

Initial populations of *E. coli* inoculated in apple juice were between 6.0-6.2 log CFU/mL, with the limit of detection at 1.0 log CFU/mL.

Both strains of *E. coli* studied (ATCC 25922, NCTC 12900) were sensitive to ozone.

Ozone treatment of ATCC 25922 in clarified apple juice resulted in reduction by 6.2 log cycles in 240 sec of treatment time as determined on TSA-SMAC by overlay method and SMAC, respectively. However, more than 4.6 log cycles of this reduction was obtained between 180 to 210 sec of treatment time. *E. coli* NCTC 12900 decreased by 6.0 log cycles in 240 sec of treatment time as determined on TSA-SMAC and SMAC respectively and conversely, more than 5.1 log cycles of this reduction was obtained from 210 to 240 sec. This abrupt microbial reduction did not allow for a nonlinear

regression analysis of the inactivation data, as the experiments wouldn't contain information to estimate the model parameters appropriately.

4.3.4 Ozone inactivation of *E. coli* in apple juice of different pH

This study investigated the efficacy of gaseous ozone for inactivation of *E. coli* strains in apple juice over a range of pH levels (3.0- 5.0), using an ozone bubble column.

Initial populations of *E. coli* inoculated in apple juice were between 6.5-6.7 log CFU/mL, and the limit of detection was 1.0 log CFU/mL.

The ozone sensitivity of both strains studied (*E. coli* ATCC 25922, *E. coli* NCTC 12900) was found to be dependent on the pH of the apple juice ($p < 0.05$). The inactivation was fitted using the Weibull and the shoulder log linear model, which provided estimations of microbial inactivation parameters. The RMSE values of the regression analysis (Table 4.6) showed that both models gave good fits for the experimental data analysed. The shoulder log linear model was considered for the rest of the study taking into account its better performance at most pH levels for both strains studied (Table 4.6).

Ozone treatment of ATCC 25922 and NCTC 12900 in apple juice of pH 5.0 resulted in a reduction of 5.24 and 5.07 log cycles within 18 min, respectively (Figures 4.13a and 4.14a). In apple juice of pH 4.5, ATCC 25922 and NCTC 12900 were reduced by 5.6 and 5.3 log cycles in 11 min, respectively (Figures 4.13b and 4.14b). *E. coli* ATCC 25922 and NCTC 12900 were reduced by > 5 log cycles in apple juice of pH 4.0 within 8.5 min and 9 min, respectively (Figures 4.13c and 4.14c). The strong effect of lower pH on ozone inactivation rate was observed for both strains. In apple juice of pH 3.5 and 3.0, both strains of *E. coli* were reduced by ≥ 5 log cycles in ≤ 6.0 or ≤ 5.0 min, respectively (Figures 4.13d, 4.14d, 4.13e and 4.14e). The calculated t_{5d} for both *E. coli* strains in apple juice of different pH are shown in Table 4.6. The t_{5d} value for ATCC 25922 in apple juice of pH 5 was 18.15 min and that for NCTC 12900 was 18.16 min.

However, at lower pH (3.0), the t_{5d} values for ATCC 25922 and NCTC 12900 were 4.01 min and 4.88 min, respectively. There was no significant difference between the microbial inactivation of the two strains of *E. coli* in apple juice at the pH values studied ($p>0.05$) (Table 4.6). There was a significant effect of pH level on the calculated t_{5d} values for both strains, where lower pH level was associated with more rapid inactivation. Figure 4.15 represents the correlation between the t_{5d} values for both strains of *E. coli* and pH of the apple juice. This correlation was described by an exponential equation (Eq. 2-16).

The RMSE values of 1.10 and 1.5 for ATCC 25922 and NCTC 12900, respectively, prove a good fitting performance of the selected models (Figures 4.13 and 4.14). A validation step of the equations developed was performed as presented in Figure 4.15. The estimated t_{5d} value of the pH unadjusted apple juice (i.e., pH 3.13), lies within the confidence and prediction bounds of the developed exponential equation.

The confirmatory experiments evaluating the effect of acid environment (low pH) and residual ozone had shown no significant effects of these factors on the microbial responses. On one hand, the experiments for *E. coli* in untreated apple juice conducted at pH 3.0, showed no significant changes in cell population, thus confirming that the lowest pH level within the range evaluated played no significant independent role in the inactivation of *E. coli* after exposure to acidic apple juice. On the other hand, the experiments conducted to determine any effect of residual ozone on the inactivation of *E. coli* in apple juice showed that there was no antimicrobial effect of residual ozone or its breakdown products as the microbial levels during storage (at 4°C for 180 min) of the treated juice were similar to those of the controlled samples.

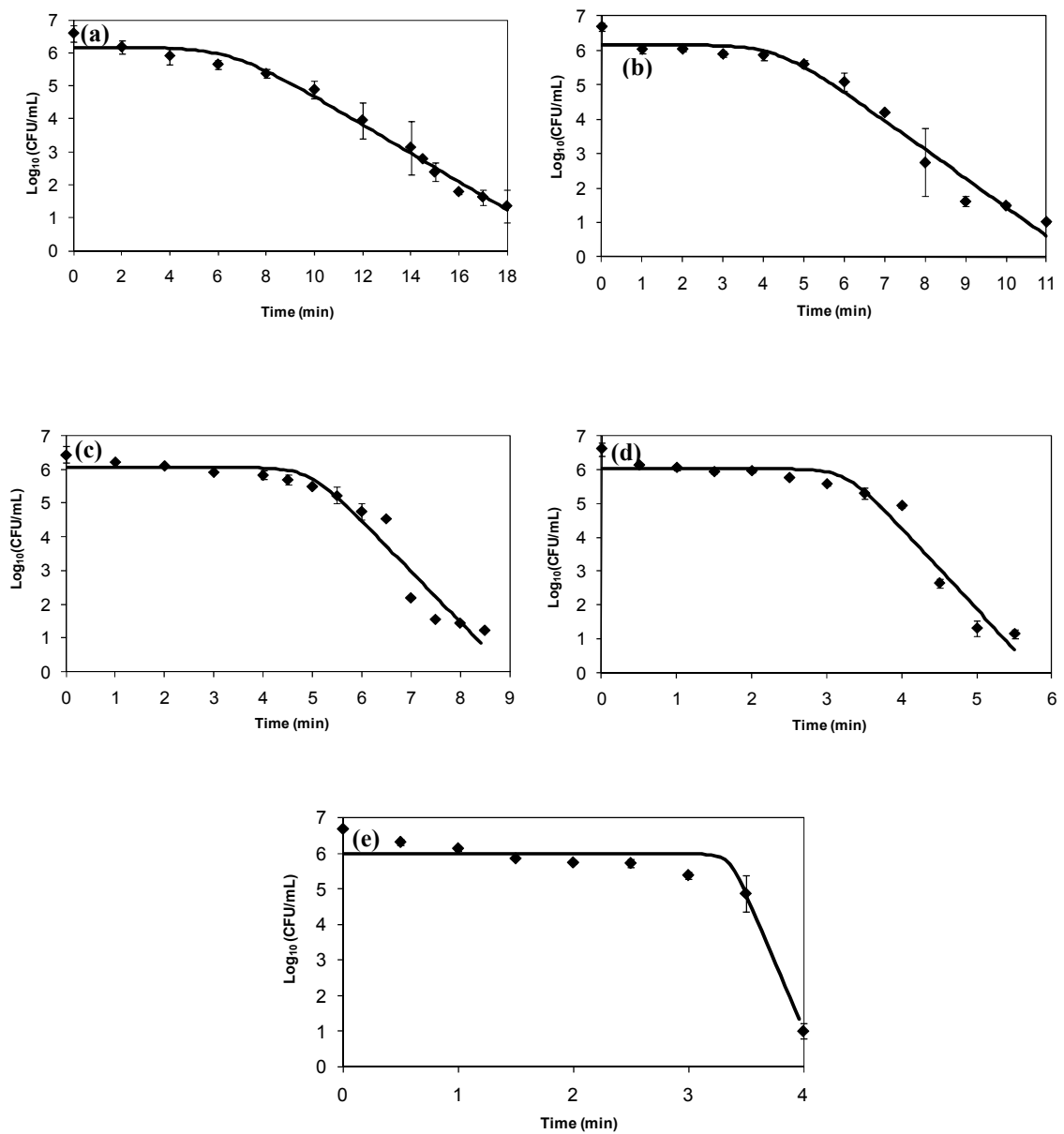


Figure 4.13: Ozone inactivation of *E. coli* ATCC 25922 in apple juice of different pH

(Data points were obtained on TSA-SMAC agar overlay)

The solid lines represent the individual fit of the shoulder log-linear model.

- (a) pH 5.0
- (b) pH 4.5
- (c) pH 4.0
- (d) pH 3.5
- (e) pH 3.0

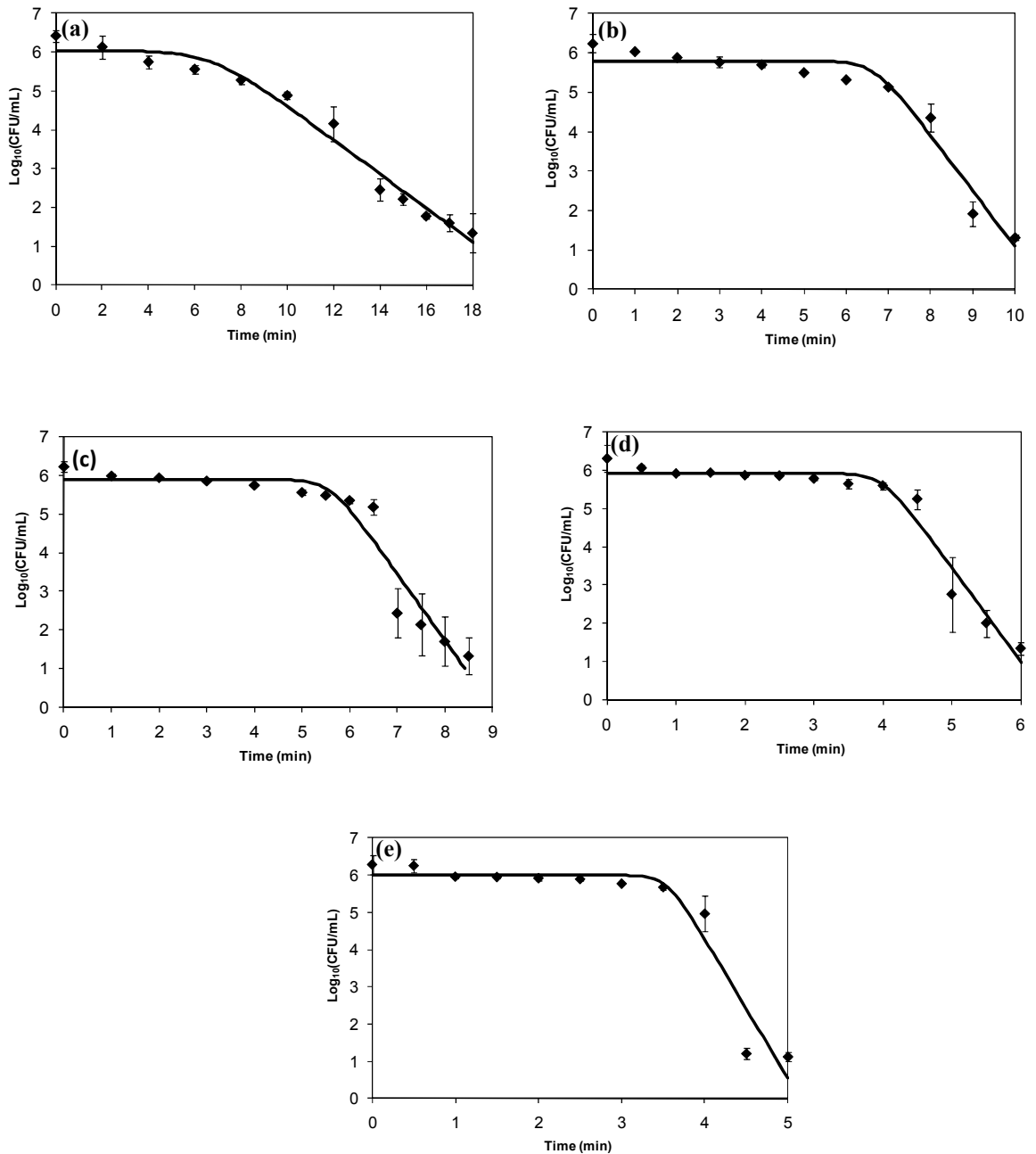


Figure 4.14: **Ozone inactivation of *E. coli* NCTC 12900 in apple juice of different pH**

(Data points were obtained on TSA-SMAC agar overlay)

The solid lines represent the individual fit of the shoulder log-linear model.

- (a) pH 5.0
- (b) pH 4.5
- (c) pH 4.0
- (d) pH 3.5
- (e) pH 3.0

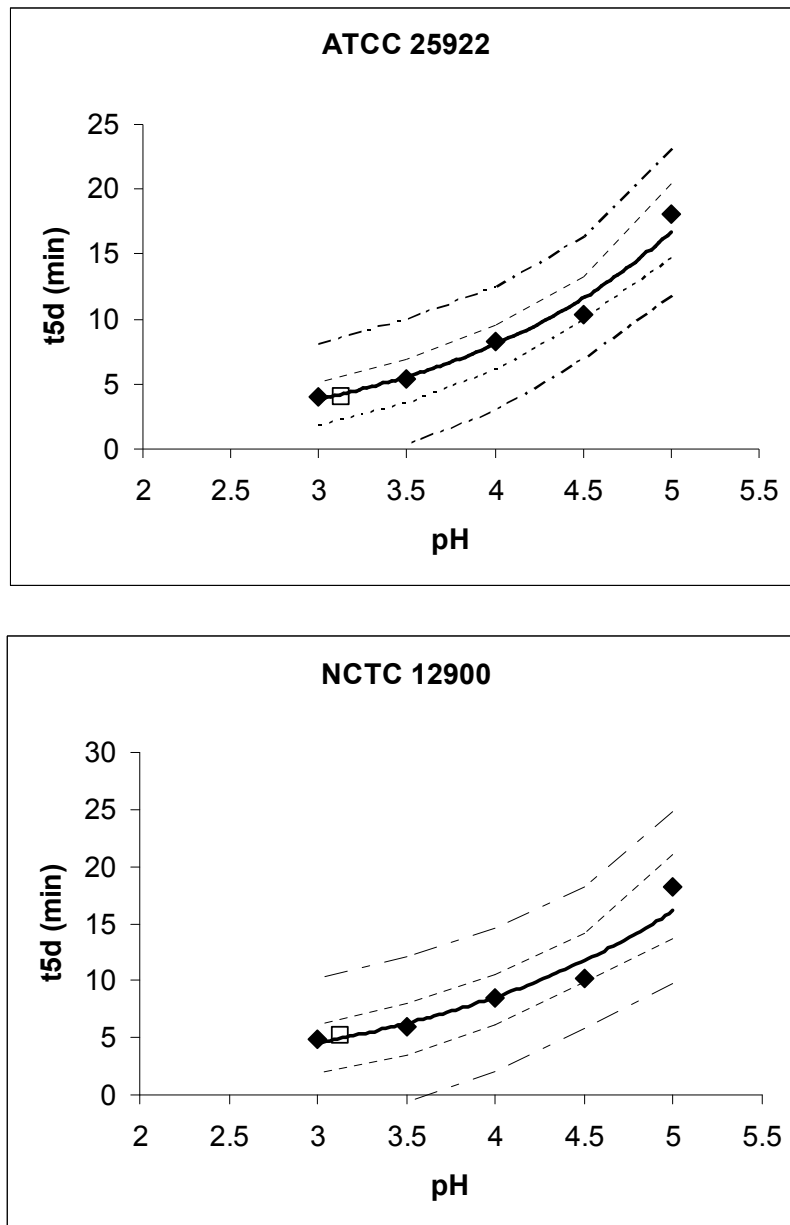


Figure 4.15: t_{5d} values of *E. coli* ATCC 25922, *E. coli* NCTC 12900 in apple juice of different pH

as described by

$$t_{5d} = 0.42 \times e^{0.74 \times pH} \text{ and } t_{5d} = 0.69 \times e^{0.63 \times pH}, \text{ respectively. (-): fit of}$$

Equation 15, (-): 95 % prediction bounds, (---): 95% confidence bounds.

(□) Validation point for apple juice of pH 3.13, (♦) Observed data values at controlled experimental conditions.

Table 4.6: t_{5d} values for *E. coli* strains in apple juice of different pH.

Strain	pH	Weibull model	RMSE	Shoulder log linear	RMSE
ATCC 25922	5.0	17.37 ± 1.92 ^{a*}	0.20	18.15 ± 0.71 ^{a*}	0.23
	4.5	10.07 ± 2.17 ^b	0.46	10.34 ± 0.57 ^b	0.37
	4.0	8.10 ± 1.11 ^c	0.53	8.28 ± 0.55 ^c	0.46
	3.5	5.27 ± 0.70 ^d	0.49	5.36 ± 0.74 ^d	0.44
	3.0	4.30 ± 0.99 ^d	0.74	4.01 ± 2.93 ^d	0.43
NCTC 12900	5.0	17.41 ± 3.03 ^a	0.31	18.16 ± 0.96 ^a	0.31
	4.5	10.37 ± 1.84 ^b	0.47	10.21 ± 0.65 ^b	0.37
	4.0	8.31 ± 1.18 ^c	0.62	8.48 ± 0.69 ^c	0.50
	3.5	5.99 ± 0.67 ^d	0.46	6.03 ± 0.69 ^d	0.35
	3.0	4.88 ± 0.66 ^e	0.68	4.88 ± 1.62 ^e	0.55

*Different letters indicate the significant difference at the 0.05 level between pH values
± : Standard error calculated based on error propagation technique
RMSE: Root mean square error

4.3.5 Discussion

The direct application of ozone was found to be effective for the inactivation or reduction of *E. coli* in apple juice. In the present study, t_{5d} values calculated showed that the inactivation of *E. coli* populations by 5 log cycles was achieved in less than 6 min treatment time. However, a strong effect of pH was observed on the ozone inactivation

efficiency and the time required to achieve reductions by 5 log cycles varied between 4.01 to 18.16 min.

Microbial adaptation to a stressful environment results in enhanced tolerance to either a similar type of stress or to a different stress condition. In the present work it was observed that the extent of increased acid resistance induced varied with the strain and acid exposure conditions. Applying acid exposure for a longer time (18 h) actually increased the ozone treatment time required for a 5 log reduction for *E. coli* strain 25922 by comparison with the control, mild acid stress and 1 h acid exposure.

Applying a mild acid stress also increased the ozone treatment time required for a 5 log reduction for *E. coli* NCTC 12900 by comparison with the control population. Leistner (2000) reported that simultaneous exposure of bacteria to different stress factors requires increased energy consumption and leads bacteria to cellular death through metabolic exhaustion. Acid exposure of NCTC 12900 either for 1 h or 18 h did not increase t_{5d} values.

The direct application of ozone was effective for the inactivation or reduction of *E. coli* in clarified apple juice, with fast inactivation of *E. coli* populations observed after 180 sec of ozone treatment. This could be due to presence of suspended solids in apple juice which reduced the inactivation efficacy of ozone initially and then direct contact of bacterial cells with ozone led to rapid inactivation. As mentioned earlier, the effectiveness of ozone against microorganisms depends not only on the amount applied, but also on the amount of organic matter surrounding the cells (Pascual et al., 2007).

The direct application of ozone for the inactivation of *E. coli* strains in apple juice was found to be dependent on the product pH (Figures 4.13 and 4.14). Bacteria can be classified by the pH ranges in which they grow. The internal pH of the cell remains neutral, and thus an organism's tolerance to fluctuations in pH reflects the capacity of the membrane pump to maintain that pH (Zuma et al., 2009). The pH range for *E. coli*

growth is from a minimum level of 4.4 to a maximum level of 9.0. However, this organism can survive and adapt to acidic conditions of fruit juice during processing and preservation.

In this work, it was observed that ozone inactivation of *E. coli* was much faster at lower pH rather than higher pH values (see section 4.3.4). Similar results by Zuma et al. (2009) reported ozone mediated disinfection kinetics of *E. coli* in water. They investigated the effect of different control parameters and pH (from 4.93 to 9.16) on the disinfection rate of *E. coli*. The inactivation of *E. coli* (10^8 CFU/mL) at a flow rate of 2 L/min with an ozone concentration of 0.906 mg/L at $25 \pm 2^\circ\text{C}$ resulted in a higher rate constant of 2.209 min at a pH value of 4.93 than at pH 9.16 with a rate constant of 1.126 min, indicating a 56% decrease in disinfection rate (Zuma et al., 2009). In the present work, the t_{5d} required for inactivation of both strains at higher pH (5.0) was increased four times relative to the time required at pH 3.0, suggesting additive or synergistic potential of ozone inactivation with acidic pH. Lin and Wu (2006), studied the potential of ozone on inactivation of intra- and extracellular enterovirus 71 (EV71) at either various ozone flow rates of 100, 80 or 60 mg/h or a constant flow rate of 80 mg/h, given to culture medium (pH 6.7 ± 0.2) or various pH culture media (pH 3, 5, 7, 9 and 11). The results demonstrated that EV71 inactivation by ozone was related to the kinetics of ozone solubility. However, the inactivation was dependent on the ozone input flow rate and was positively enhanced at acidic pH. Vaughn et al. (1987) and Harakeh and Butler (1984), also reported that the efficacy of ozone seems to decrease at alkaline pH for rotavirus and Poliovirus type 1 in ozone-demand-free phosphate carbonate buffer and effluent, respectively. Farooq et al. (1977a) reported increased survival rate of *Mycobacterium fortuitum* during ozone treatment when pH was increased. The authors ascribed this increased survival to a smaller ozone residual as the pH of water increased. The effect of pH on ozone inactivation is mainly attributed to the fact that the ozone

decomposition rate changes substantially with changes in pH. At high pH, the chain reactions of ozone decomposition results in the formation of numerous radical species with high oxidative capabilities.

It has not been well established whether molecular ozone or the radical species are responsible for inactivation of microorganisms. Some researchers have suggested that direct reaction with molecular ozone is the predominant mechanism of inactivation of microorganisms (Finch et al., 1992; Labatiuk et al., 1994; Hunt and Marinas, 1997), while others suggest indirect reactions with radicals to be responsible for inactivation (Dahi, 1976; Bancroft et al., 1984). It is likely that the relative importance of direct and indirect reactions with ozone in determining microbial inactivation responses will vary between microorganisms (Blatchley and Nimrata, 2002).

The validation results in apple juice for the developed exponential Eq.(2-16) also showed a good degree of correlation which will be useful in the fruit juice processing industry to design treatment time periods in order to achieve a desired level of reduction of a foodborne pathogen present in apple juice of different acidity levels.

4.4 Ozone inactivation of *Listeria monocytogenes* and *Listeria innocua* in orange juice using a bubble column

In this study, orange juice was inoculated with *Listeria monocytogenes* ATCC 7644 and NCTC 11994 strains and *Listeria innocua* NCTC 11288 strain (10^6 CFU/ml) as a challenge microorganism prior to ozonation. The objectives of this study were to investigate (i) the efficacy of gaseous ozone treatment for reduction of *Listeria* spp. at ambient temperature in orange juice, (ii) the effect of a mild acid stress on treatment efficacy (iii) the effect of mild acid stress-habituation on treatment efficacy and (iv) the inactivation kinetics during ozonation of acid stressed *Listeria* strains that are habituated in orange juice.

4.4.1 Inactivation of *Listeria monocytogenes* NCTC 11994

The inactivation kinetics of *Listeria* in orange juice were fitted using the Weibull model, which provided estimations of microbial inactivation parameters in terms of the processing times required. The resulting Weibull parameters δ and p are shown in Table 4.7. The shape parameter p indicated a downward concavity for the kinetic curves of all the *Listeria* strains. p values of >1 indicates a greater susceptibility of microorganisms to the treatment (van Boekel, 2002).

The inactivation curves of *L. monocytogenes* NCTC 11994 are shown in Figure 4.16. Ozone treatment of non acid-stressed cells reduced populations by 5.25 log cycles in 6 min (Figure 4.16a). The mild acid stressed populations also decreased by 5.14 log cycles after 7 min of ozone treatment (Figure 4.16b). However, significant differences were observed in the recovery index as well as the time required to achieve reduction by 5 log cycles (t_{5d}) between the different test conditions in spite of the similar reduction in log cycles obtained on TSA ($p<0.05$) (Table 4.7). In the case of acid stressed cells habituated in orange juice, a 6.0 log cycle reduction was achieved after 8 min (Figure 4.16c). Ozone treatment of 1 h habituated cells yielded a 6.4 log cycle reduction after 8 min (Figure 4.16d). However, with 18 h habituated cells populations declined by only 4.0 log cycles after 8 min, and this population also showed the highest time required for achieving t_{5d} (Figure 4.16e) (Table 4.7). The recovery index for acid stress-habituated cells was significantly lower and t_{5d} values were significantly higher compared to the control non acid stressed cells ($p<0.05$). The t_{5d} value for non-acid stressed cells was 5.78 min ($p<0.05$). For all test conditions except for acid stressed cells habituated in orange juice, $\geq 95.9\%$ injury was observed (Table 4.7). In the case of cells habituated in orange juice, the estimation of the uninjured vs. the injured population counts on the Palcam agar was recorded for a period up to 6 min of ozone treatment in which the detection limit was not reached for both the media used.

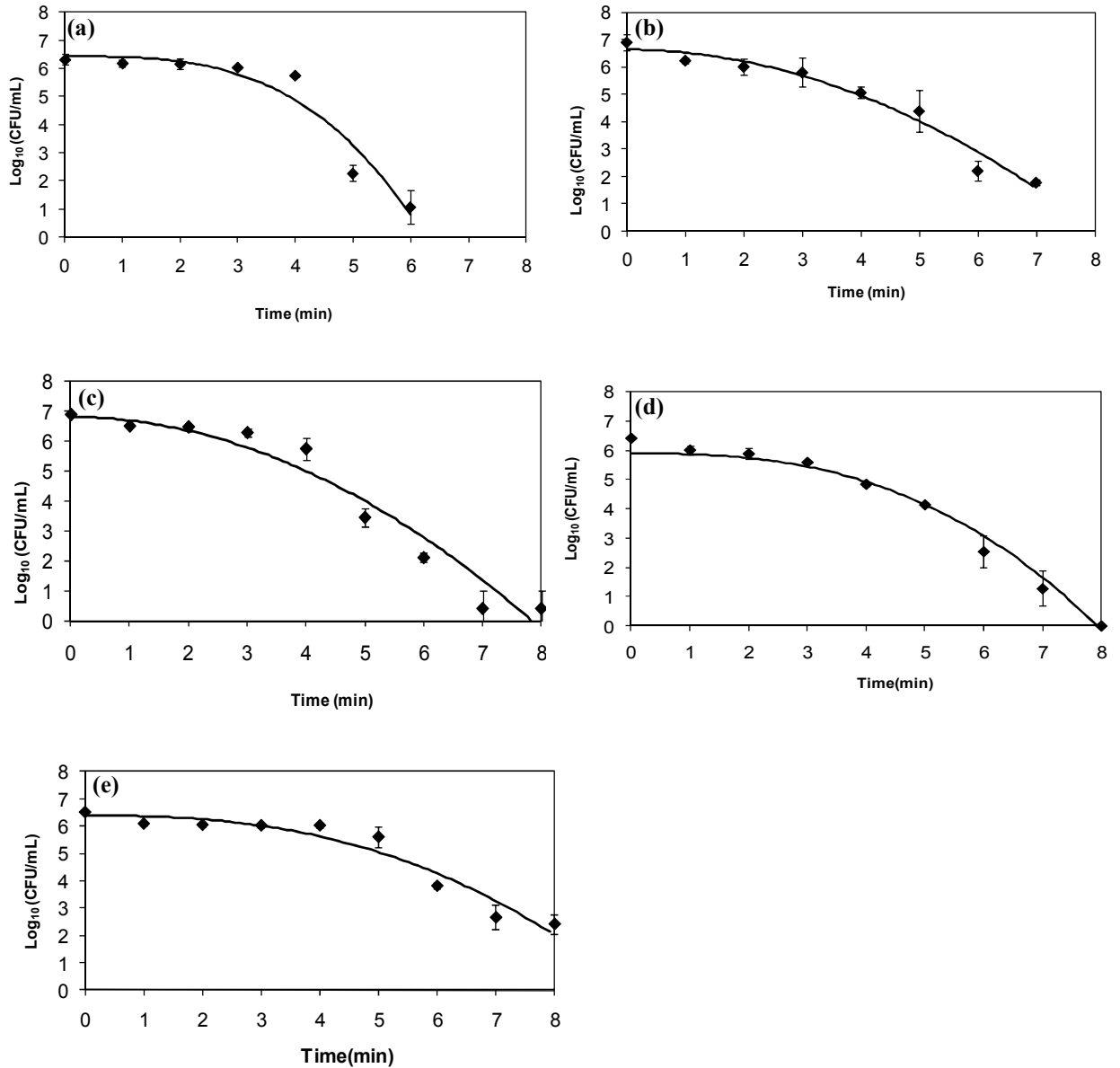


Figure 4.16: Ozone inactivation of *Listeria monocytogenes* NCTC 11994 in orange juice at flow rate of 0.12 L/min and concentration 72-75 $\mu\text{g/mL}$.

(Data points were obtained on TSA agar)

- (a) Non acid-stressed cells
- (b) Mild acid-stressed cells
- (c) Habituated cells in orange juice
- (d) 1 h acid stress-habituated cells
- (e) 18 h acid stress-habituated cells

4.4.2 Inactivation of *Listeria monocytogenes* ATCC 7644

Survivor curves for *Listeria* strain ATCC 7644 are presented in Figure 4.17. Non acid-stressed and mild acid-stressed cells were reduced by 6.12 and 6.4 log cycles in 6 min (Figure 4.17a and b). Ozone treatment of acid stressed cells habituated in orange juice resulted in reduction by 6.3 log cycles after 8 min (Figure 4.17c). However, there were no significant differences reported with respect to recovery index and t_{5d} values between all three conditions investigated. Ozone treatment of 1 h or 18 h habituated *L. monocytogenes* ATCC 7644 resulted in 6.0 and 5.3 log reductions respectively by 8 min (Figure 4.17d and e). In the case of the acid stress-habituated population, significant differences were observed in t_{5d} values compared to three other test conditions ($p < 0.05$). However, at all test conditions, $\geq 91.6\%$ injury was observed, indicating the generation of an ozone injured population after a specific time period for different conditions (Table 4.7).

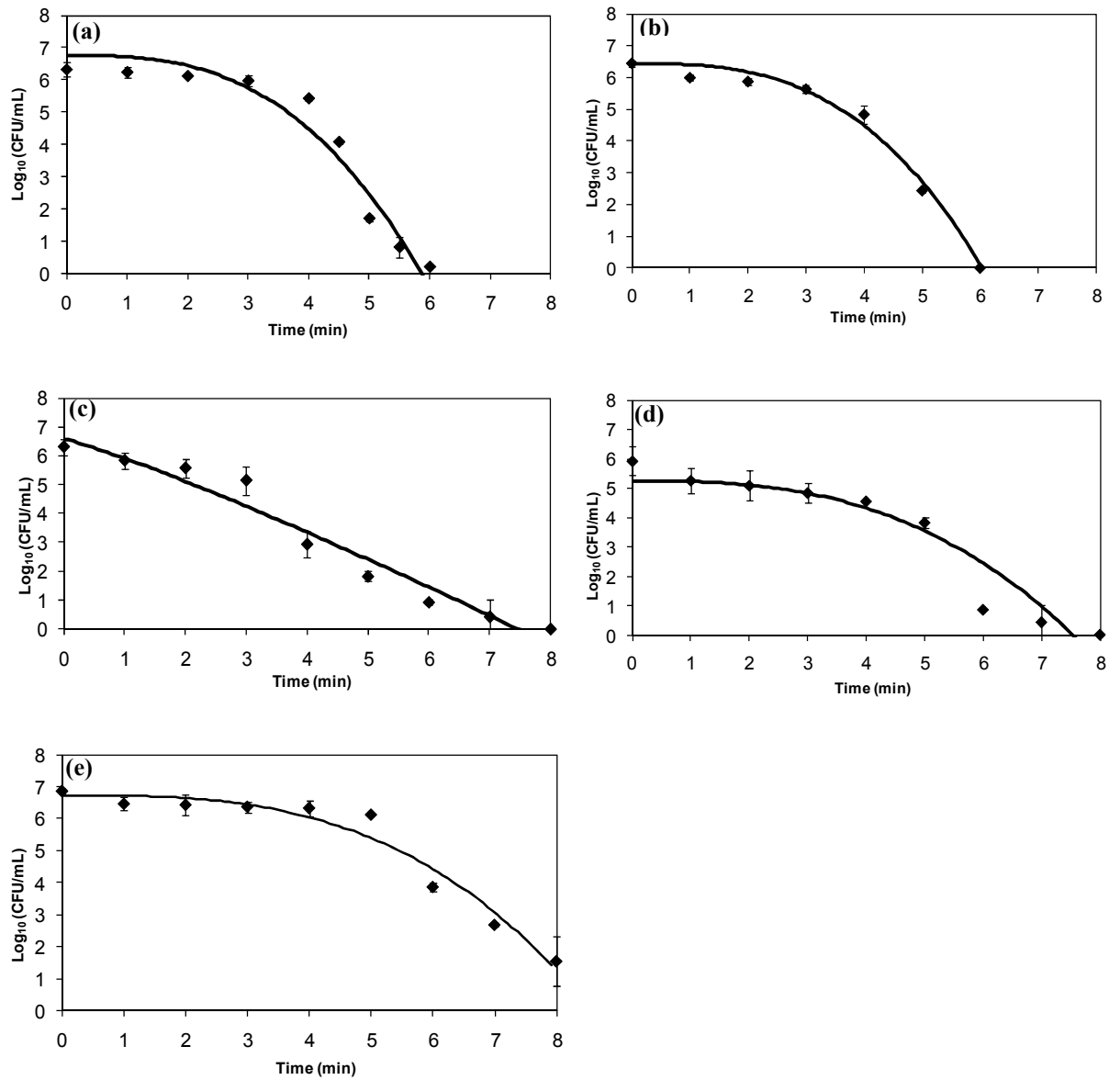


Figure 4.17: Ozone inactivation of *Listeria monocytogenes* ATCC 7644 in orange juice at flow rate of 0.12 L/min and concentration 72-75 $\mu\text{g/mL}$.

(Data points were obtained on TSA agar)

- (a) Non acid-stressed cells
- (b) Mild acid-stressed cells
- (c) Habituated cells in orange juice
- (d) 1 h acid stress-habituated cells
- (e) 18 h acid stress-habituated cells

4.4.3 Inactivation of *Listeria innocua* NCTC 11288

Ozone inactivation curves of *L. innocua* cells for different test conditions are shown in Figure 4.18. The control non acid-stressed and mild acid stressed cells were reduced by 5 log cycles in a short treatment time (Figure 4.18a and b) with no significant differences reported for t_{5d} values (Table 4.7). Ozone treatment of the cells habituated in orange juice (Figure 4.18c) and 18 h mild acid stress-habituated cells resulted in reduction of > 5 log cycles in 8 min (Figure 4.18e) whereas 1 h mild acid stress-habituated cells were reduced in 6 min of treatment time (Figure 4.18d). The longer acid stress-habitation (18 h) led to a significantly higher t_{5d} value compared to other test conditions investigated ($p < 0.05$) (Table 4.7). The lower % of injury observed for acid stressed cells habituated in orange juice after 7 min ozone treatment underlines the importance of investigating the efficacy of ozone in real product formulations in addition to simulated stress conditions in model media.

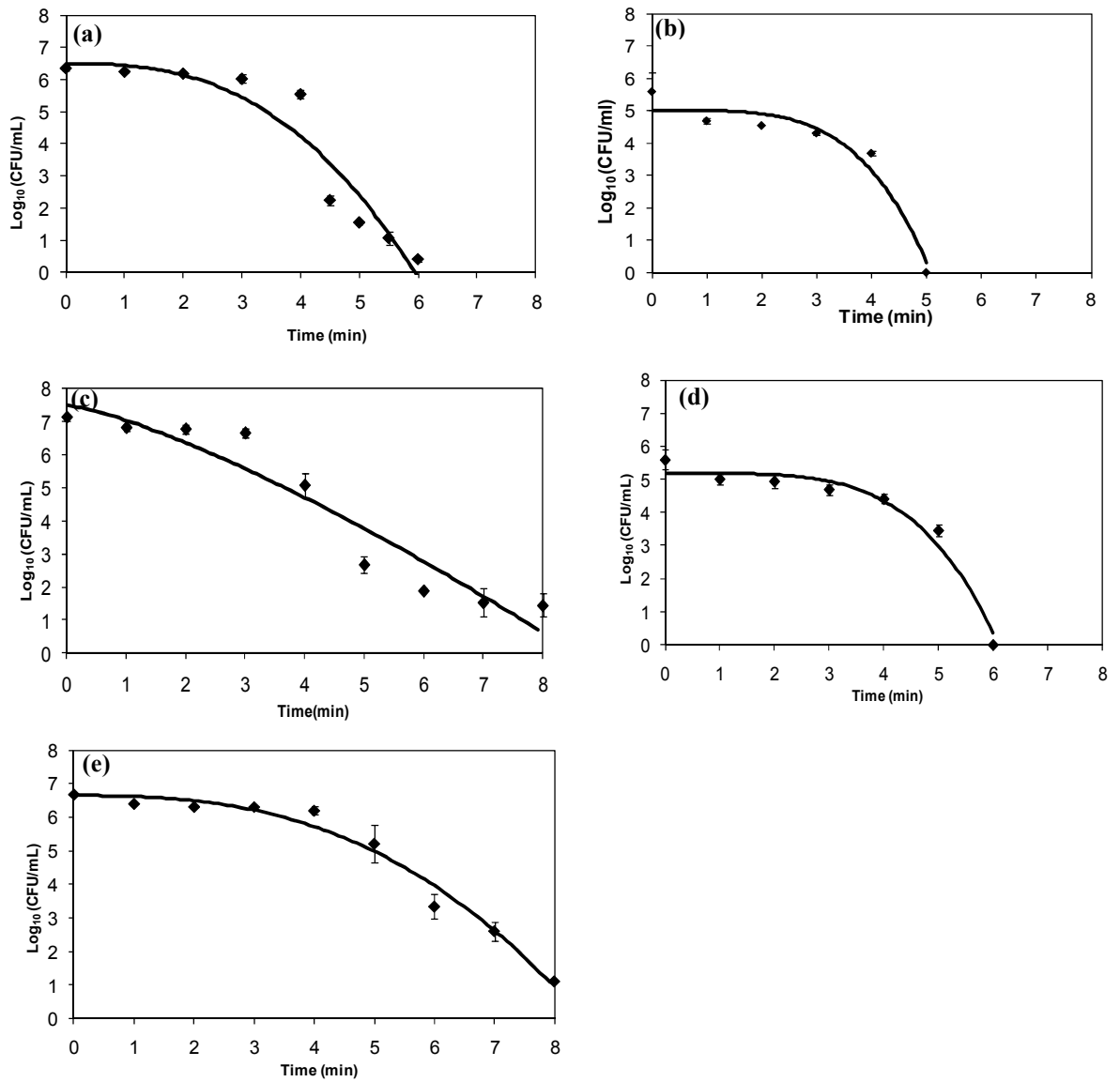


Figure 4.18: Ozone inactivation of *Listeria innocua* NCTC 11288 in orange juice at flow rate of 0.12 L/min and concentration 72-75 µg/mL.

(Data points were obtained on TSA agar)

- (a) Non acid-stressed cells
- (b) Mild acid-stressed cells
- (c) Habituated cells in orange juice
- (d) 1 h acid stress-habituated cells
- (e) 18 h acid stress-habituated cells

Table 4.7: Parameters of the Weibull model and the time required to reach a 5 log reduction for *Listeria* strains in orange juice

Microorganism	Condition	$\delta(\text{min}) \pm \text{STE}$	$p \pm \text{STE}$	RMSE	$t_{5d}(\text{min})$	Recovery index	% injury
<i>L. monocytogenes</i> NCTC 11994	Control non-acid stressed	3.48±0.64	3.17±1.04	0.70	5.78 ^a	0.99 ^k	95.9
	mild-acid stressed cells	3.07±0.55	1.97±0.41	0.41	6.95 ^b	0.76 ^l	99.7
	1h acid stress-habituation	4.05±0.40	2.64±0.38	0.36	7.45 ^c	0.79 ^l	97.8
	18 h acid stress-habituation	4.45±0.69	2.52±0.65	0.47	8.44 ^d	0.60 ^{lm}	99.9
	Habituated cells in orange juice	2.96±0.73	1.97±0.48	0.70	6.69 ^{ab}	0.89 ^k	76.6
<i>L. monocytogenes</i> ATCC 7644	Control non-acid stressed	2.99±0.47	2.84±0.64	0.73	5.27 ^e	0.98 ⁿ	91.6
	mild-acid stressed cells	3.17±0.30	2.89±0.42	0.34	5.53 ^e	1.00 ⁿ	99.8
	1h acid stress-habituation	4.12±0.90	2.74±0.89	0.84	7.41 ^f	0.75 ^o	99.3
	18h acid stress-habituation	4.54±0.52	3.00±0.60	0.46	7.77 ^f	0.80 ⁿ	99.2
	Habituated cells in orange juice	1.43±0.56	1.14±0.24	0.60	5.87 ^e	0.86 ⁿ	97.4
<i>L. innocua</i> NCTC 11288	Control non-acid stressed	2.94±0.66	2.66±0.82	0.86	5.38 ^h	0.96 ^p	74.6
	mild-acid stressed cells	3.44±0.47	4.14±1.45	0.57	5.08 ^h	1.0 ^q	99.8
	1h acid stress-habituation	4.17±0.34	4.33±0.96	0.39	6.05 ⁱ	0.85 ^{pr}	98.4
	18h acid stress-habituation	4.12±0.42	2.62±0.40	0.36	7.60 ^j	0.80 ^r	89.5
	Habituated cells in orange juice	1.82±0.88	1.30±0.40	0.84	6.26 ⁱ	0.83 ^r	66.7

(Different letters indicate a significant difference at the 0.05 level between each type of condition).

- δ : time for the first decimal reduction
 STE: standard error
 p : parameters related to the scale and shape of the inactivation curve
 R^2 : coefficient of determination
 % injury: calculated using equation 1
 Recovery index: t_{5d} determined on Palcam divided by t_{5d} determined on TSA

4.4.4 Discussion

The direct application of ozone was found to be effective for the inactivation or reduction of *Listeria* in orange juice. In the present study, t_{5d} values calculated showed that the inactivation of control populations of *L. monocytogenes* by 5 log cycles was achieved in less than 6 min treatment time. Literature reports on the efficiency of ozone for inactivating *Listeria* in food products vary. Rodgers et al. (2004) reported about 5 log CFU/g reductions in *L. monocytogenes* counts on lettuce as a result of 3 ppm ozone treatment for 5 min whereas Yuk et al. (2006), found that application of 5 ppm ozone for 5 min did not affect *L. monocytogenes* counts in shredded iceberg lettuce, but significantly ($p < 0.05$) reduced *E. coli* O157:H7 counts. Ölmez and Akbas (2009) concluded that *L. monocytogenes* ATCC 7644 populations inoculated on lettuce leaves decreased by 1.45 log CFU/g at 26°C using 1 ppm ozone for 2.0 min. Vaz-Velho et al. (2006) reported that ozone treatment with 0.32 mg/L had no significant effect on *L. innocua* counts on fish samples. Ölmez and Akbas (2009) stated that the efficiency of ozone treatment can be related to the delivery method. The direct application of ozone using the bubble column as in the current study, proved the best delivery tool for the efficient decontamination of orange juice. However, there were some significant effects of bacterial cell pre-treatment and condition observed. The pre-treatments and conditions employed were designed to mimic the environment that a contaminating population could be exposed to in orange juice and other food processing scenarios.

Applying a mild acid stress actually increased the ozone treatment time required for a 5 log reduction for both strains of *L. monocytogenes* by comparison with the control population. However, in the case of *L. innocua*, applying a mild acid stress did not significantly effect the ozone treatment time required by comparison with the control. This

could be due to the applied mild stress which did not result in any added protection against ozone and subsequent ozone stress resulted in rapid inactivation.

Bacteria are exposed to severe acid stress in all links of the food chain, and are capable of surviving a range of different stresses and in many cases sub-populations can survive normally injurious or lethal conditions. Foodborne bacteria encounter organic and inorganic acids in foods or in the gastrointestinal tract and cells of the host (Yousef and Courtney, 2003). Adaptation of *L. monocytogenes* to sublethal stresses has been demonstrated to protect the pathogen to a variety of normally lethal conditions present in certain foods (Lou and Yousef, 1997). The resistance or adaptation of microorganisms to acid conditions can have implications for food safety. In this study, all three *Listeria* strains were subjected to acid stress-habituation at pH 5.5 to examine the effect of this stress on the efficacy of ozone treatment in orange juice. Acid stress-habituated *Listeria* cells had an increased resistance to ozone treatment and also recorded the highest time estimated for achieving 5 log (t_{5d}) reductions, i.e., 8.44 min for *L. monocytogenes* NCTC 11994. Similar findings of significantly increased resistance of *L. monocytogenes* to heat were reported by Mazzotta (2001) after acid adaptation of *Listeria* in single strength apple, orange and white grape juices adjusted to pH 3.9. Caggia et al. (2009) recorded the highest acid tolerance response of *L. monocytogenes* OML 45 strain, after 3 h treatment in TSB adjusted to pH 5.7, thus, concluding that cells adapted to acidic environments can grow in normally lethal pH conditions. Acid adaptation increased survival of *L. monocytogenes* in acidic foods and during milk fermentation (Gahan et al., 1996).

It has been reported that the heat and acid resistance of *L. monocytogenes* are strain dependant (Skandamis et al., 2008). Reliable assessment of the effectiveness of food processes for microbiological control should be based on studies evaluating the most

resistant strain or combinations of strains. Phan-Thanh et al. (2000) reported the lowest pH value which *L. monocytogenes* could resist was dependant on the strain and the kind of acid used. The present results also showed that the extent of increased acid resistance varied with the strain and acid stress conditions. Strain NCTC 11994 was the most resistant strain independent of the applied conditions. However, the % injury (89.5-99.9%) reported for the mild acid stressed and acid stress-habituated *Listeria* populations for all strains was greater than that recorded for the respective control population.

In orange juice production, low acidic conditions are present prior to pasteurisation and may induce an acid tolerance response that can result in increased thermal tolerance (Caggia et al., 2009). In this study, *Listeria* cells grown in TSB +1.25% glucose and then incubated in orange juice for 90 min showed lower t_{5d} values compared to 18 h habituated populations. Although lower t_{5d} values were reported for cells habituated in orange juice compared to 18 h acid stress-habituated cells, a lower % injury was recorded for strains NCTC 11994 (76.6%) and NCTC 11288 (66.7%) when habituated in orange juice. This indicated that exposure to sequential acid stressors such as a prior acid stress followed by an acid environment in the product, may result in cross protection to a subsequent processing treatment as observed here, where *Listeria* cells habituated in orange juice had a generally lower % injury following ozone treatment. In the case of all 18 h acid stress-habituated populations, the highest t_{5d} values were estimated, however, lower recovery indices were reported (0.60-0.80), where greater recovery of cells was evident on non-selective media by comparison with selective media. The applied acid stress did not promote recovery on selective medium (Palcam) at the same rate of the recovery on non-selective medium (TSA), however the injured sub-population may have a greater resistance to ozone. Therefore, to mimic the stresses encountered in food processing environments,

conditions like acid stress-habitation and habituation in actual orange juice should be considered for determining inactivation parameters (e.g., t_{xd} , %injury, recovery index) and process design in foods.

From the present study and based on the different inactivation responses to ozone treatment it was also observed that inactivation responses of *L. innocua* NCTC 11288 were closer to those of *L. monocytogenes* ATCC 7644 than *L. monocytogenes* NCTC 11994.

4.5 Quantitative evaluation of the shelf life of ozonated apple juice

In this work, apple juice inoculated with *S. cerevisiae* ATCC 9763 (10^3 CFU/mL) was treated with ozone at a flow rate of 0.12 L/min and a concentration of 33-40 μ g/mL for 8 min. The objective was to study the growth dynamics of *S. cerevisiae* in untreated and ozone treated apple juice and to investigate the efficacy of ozone to extend the shelf life of apple juice at a range of static (isothermal) storage temperature conditions of 4, 8, 12 and 16°C for up to 30 days. The growth kinetic parameters were determined by primary model of Baranyi and Robert's and the maximum specific growth rate was further modelled as a function of temperature by Ratkowsky type model. The developed model was then validated for the growth of the control and ozonated samples during dynamic storage temperature of periodic changes from 4 to 16°C.

4.5.1 Model development

Growth of *S. cerevisiae* in untreated and ozone treated apple juice was assessed and representative growth curves of the yeast population are shown in Figure 4.19. In the case of untreated control samples 1 and 2 (i.e., initial inoculum level of 3.0 and 1.30 \log_{10} CFU/mL, respectively) the lag phase was not present when the juice was stored under high static storage temperatures (12°C and 16°C) (Figure 4.19a and b). However, a typical

growth pattern of *S. cerevisiae* was observed in the ozone treated apple juice stored at these temperatures, consisting of an initial lag phase, an exponential growth phase followed by a stationary phase (Figure 4.19c).

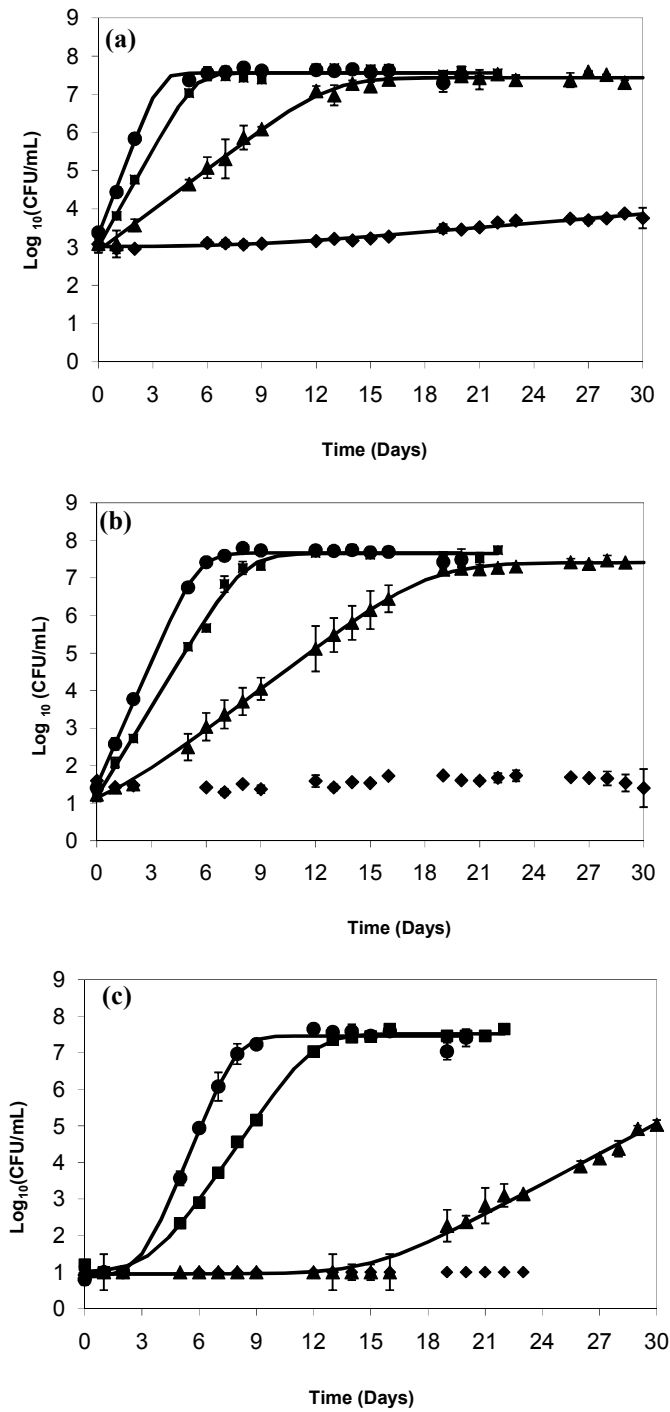


Figure 4.19: Growth curves of *Saccharomyces cerevisiae* in untreated and ozone treated apple juice stored at different static storage conditions

(\blacklozenge 4°C, \blacktriangle 8°C, \blacksquare 12°C, \bullet 16°C) (a) Untreated control 1 (b) Untreated control 2

(c) Ozone treated

The estimated kinetic parameters and statistical indices resulting from the regression of the microbial data using the Baranyi and Roberts model are shown in Table 4.8. The values of μ_{max} and λ varied according to the storage temperature. The μ_{max} of the untreated control samples increased from 0.35 log CFU /day to 1.23 log CFU /day with a similar trend observed for ozone treated apple juice concomitant with increasing temperature from 8 to 16°C. However, the lag phase for ozone treated apple juice decreased from 15.07 days at 8°C to 2.84 days at 16°C. For both untreated and ozone treated apple juice, the maximum population density (N_{max}) was found to be unaffected under high static storage temperatures. The effect of storage temperature on μ_{max} was further modelled as a function of temperature using the secondary square root model and estimated parameters are shown in Table 4.9. The model described satisfactorily the effect of temperature on the growth of *S. cerevisiae*. The estimated value for the theoretical minimum temperature of growth in ozone treated apple juice was 0.28°C. The h_0 values obtained for the static environments were 0.336 ± 0.14 , 0.671 ± 0.085 and 3.417 ± 0.48 for untreated control 1, untreated control 2 and ozone treated apple juice samples, respectively.

Table 4.8: Parameters of the Baranyi and Robert's model for the growth of *Saccharomyces cerevisiae* in untreated and ozone treated apple juice under static storage conditions

Temperature (°C)	Sample type	μ_{max} (1/days)	λ (1/days)	N_{max}	SE of fit	RMSE
4	Untreated control 1	0.039±0.003	8.48±1.64	-	0.060	0.063
8	Untreated control 1	0.354±0.010	-	-	0.108	0.121
	Untreated control 1	0.367±0.008	1.10±0.29	7.43±0.031	0.069	0.075
	Ozone treated	0.275±0.013	15.07±0.55	7.41±0.025	0.156	0.175
12	Untreated control 1	0.846±0.035	-	-	0.104	0.094
	Untreated control 1	0.798±0.020	-	7.56±0.029	0.127	0.116
	Ozone treated	0.762±0.031	3.48±0.214	7.65±0.041	0.100	0.090
16	Untreated control 1	1.227±0.085	-	7.51±0.038	0.119	0.100
	Untreated control 1	1.103±0.033	-	7.66±0.034	0.109	0.091
	Ozone treated	1.27±0.104	2.84±0.303	7.45±0.060	0.165	0.137

Table 4.9: Parameters of the square root type model for the effect of temperature on the maximum specific growth rate of *Saccharomyces cerevisiae*

Sample type	Parameter	Estimated value	RMSE
Untreated control-1	b	0.0043±0.0011	0.093
Untreated control-1	T _{min} (°C)	-1.121±0.0215	
Untreated control	b	0.00276±0.0010	0.088
Untreated control	T _{min} (°C)	-4.2439±0.1455	
Ozone treated	b	0.0052±0.0010	0.033
Ozone treated	T _{min} (°C)	0.2799±0.00370	

4.5.2 Validation under dynamic temperature conditions

The model developed under static storage conditions was validated under dynamic temperature conditions by using a periodically changing temperature profile and performing predictions with Eq. (2-13), (2-14). As the maximum population density was independent of the storage temperature it was fixed at 7.5 logs CFU/mL (average of N_{max} estimated during isothermal conditions for which microbial stationary phase was reached). For the initial concentration $N(0)$, the nominal value was taken from the measured plate count result, i.e., 3.02 (for control 1), 1.32 (for control 2), 1.24 (for ozonated) log (CFU/mL) respectively. Finally, the nominal values for $q(0)$ were 2.49, 1.05 and 0.03,

respectively, calculated by Eq. (2-18) and after estimation of the h_o from the parameters derived under static environmental conditions. The comparison between the predicted and observed growth of *S. cerevisiae* in untreated and ozone treated apple juice is shown in Figure 4.20. The performance of the model was evaluated statistically by the calculation of the bias (B_f) and accuracy (A_f) factors. The values of B_f and A_f indicated good agreement between observed data and predicted data points, while in the case of control 2 some discrepancy was evident (Table 4.10).

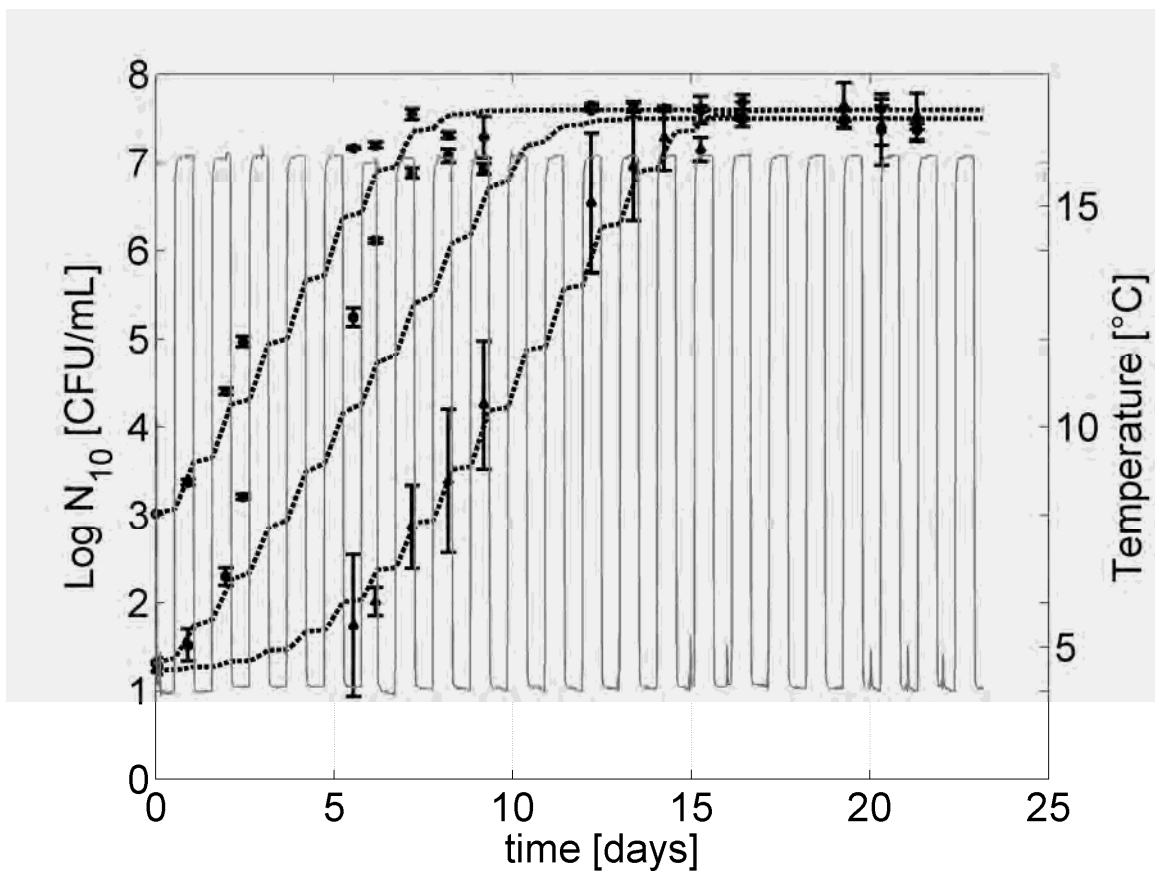


Figure 4-20: Comparison between observed (points) and predicted (dotted lines) growth of *Saccharomyces cerevisiae* in untreated and ozone treated apple juice under dynamic temperature conditions
 (● untreated control 1, ■ untreated control 2, ▲ ozone treated).

Table 4.10: Accuracy (A_f) and bias (B_f) values between the predictions of the model tested

	A_f	B_f
Control 1	1.9907	1.2227
Control 2	4.7422	2.4866
Ozonated	1.7575	1.0187

Two further characteristic parameters were evaluated, the Q_{10} and the time of spoilage of the product under static temperature conditions (Figure 4.21). At 4°C no spoilage occurred, either for untreated or ozone treated apple juice. However, with increasing static storage temperature, product spoilage was observed in 9.45, 3.78, and 2.35 days for untreated control 1 at 8, 12 and 16°C, respectively. For untreated control 2, the spoilage was reached after 15.08, 6.30 and 4.29 days at 8, 12 and 16°C, respectively. In the case of ozone treated apple juice, the shelf life was increased when compared with the controls and resulted in 34.26, 10.34 and 7.08 days at 8, 12 and 16°C, respectively. Finally the Q_{10} was found to be 7.17 in the case of ozonated juice, which is much higher than that of the controls, i.e., 5.68, 4.81, thus indicating the efficiency of ozonation for extension of apple juice shelf-life by affecting the subsequent microbial growth during storage.

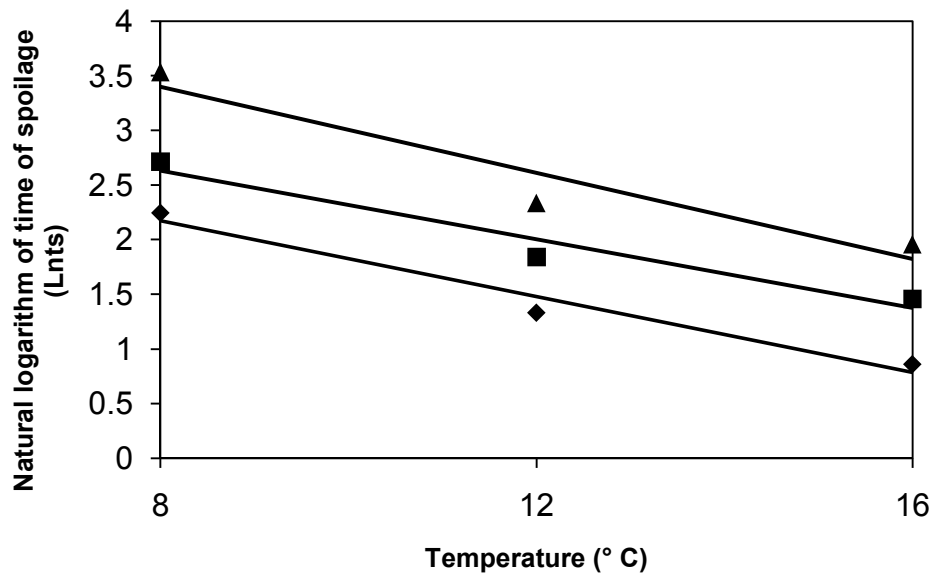


Figure 4.21: Shelf life (natural logarithm of the time to spoilage (Lnts) in days) for untreated control (1 and 2) and ozone treated apple juice samples
 (◆ untreated control 1, ■ untreated control 2, ▲ ozone treated at different storage temperatures tested).

4.5.3 Discussion

From the present work it is evident that ozone as a non-thermal technology is suitable for extending the shelf life of apple juice.

S. cerevisiae ATCC 9763 was able to grow in apple juice stored within the temperature range of 8 to 16°C. The Baranyi and Roberts model and the square root model described the growth of yeast populations in untreated and ozone treated apple juice. Based on the static data, a model was developed that described well the growth of *S. cerevisiae* population in untreated and ozone treated apple juice under dynamic conditions which simulated temperature abuse conditions. At 4 and 8°C static storage, the longer lag phase indicated that more environmental adaptation was required by the yeast population.

However, at higher storage temperatures this effect was not seen, indicating the ability of yeasts to grow at these temperatures without any noticeable lag time. In the case of ozone treated apple juice stored at temperatures within the range of 8 to 16°C, the lag phase (λ) increased, indicating the effect of temperature and applied stress on growth of *S. cerevisiae* populations. Panagou et al. (2009) reported a very short lag phase in different fruit juices even at the lowest temperatures of storage suggesting that yeasts' adaptation time was not affected by these temperatures (4, 8, 12 and 16°C). However, in this study the lag phase was present in all the ozone treated samples. This could be due to the strong oxidising action of the applied ozone treatment that exerts additional stress on the yeast population. Ozone has been reported to inactivate cytosolic enzymes with the most drastic inactivation for glyceraldehyde3-phosphate dehydrogenase. It also reduces the quantity of ATP and other nucleoside triphosphates by about 50% (Hinze et al., 1987).

Different technologies have been applied for inhibiting the growth of spoilage microorganisms in fruit juices. Patrignani et al. (2009) evaluated the potential of high pressure homogenization (HPH) for inactivation of *S. cerevisiae* 635 inoculated in apricot and carrot juice and their shelf life extension. They reported that refrigerated storage (4°C) indicated satisfactory extension of shelf life of HPH treated juices. Qin et al. (1995) reported over 3 weeks extension of shelf life with pulsed electric field (PEF) treated apple juice stored at 4°C and 25°C. Some authors reported the efficacy of high pressure carbon dioxide (HPCD) and ultra high pressure homogenization to produce juices either with extension of shelf life and safety or with an equivalent shelf life to pasteurised juice (Ferrentino et al., 2009; Suarez-Jacobo et al., 2009). Valdramidis et al. (2009) found that no spoilage of apple juice was evident when stored at 4, 8 and 12°C for 36 days after treatment with high hydrostatic pressure at 500 MPa and 550 MPa. In this study, the present results

proved an increase of the shelf-life of the ozonated product that varied between 2.79 to 24.81 days depending on the storage temperatures when compared with the control samples.

The present study has established the ability of ozone to extend the shelf life and the shelf life related parameters that are affected by the process, as well as the main factors that may reduce the ozonation effectiveness. Finally, validation of the developed modelling approaches could be expanded based on the suggestions by Pin et al. (1999). The effect of shelf life arising from competition of inoculated *S. cerevisiae*, pathogenic microorganism with the naturally occurring microflora could be incorporated in future model developments while comparative studies between ozonated and other non-thermal technologies could be applied.

4.6 Inactivation of *E. coli* in fresh orange juice by combined ultrasound and ozone treatment

Ultrasound and ozone, both non-thermal technologies showed effectiveness for inactivation of microorganisms in liquid system. Hence, in this study sonication and ozonation were applied simultaneously under optimised conditions. The objective of this study was to investigate the efficacy of combined ultrasound and ozone treatment against *E. coli* ATCC 25922 in orange juice passed through sieve size of 1 mm.

4.6.1 Results

Orange juice inoculated with *E. coli* ATCC 25922 (10^6 CFU/mL) was treated with ultrasound (7.5 μm amplitude level) and ozone (0.12 L/min flow rate with an ozone concentration of 72-75 $\mu\text{g/mL}$) simultaneously under optimised conditions. Combined treatment with ozone and sonication resulted in increased inactivation rate. Ozone treatment alone decreased the population of *E. coli* 25922 in juice passed through sieve of 1 mm

diameter by 6.0 log cycles in 6 min of treatment time. However, combined ozone and sonication reduced the population in juice by 6.0 log cycles in 4 min of treatment time. Conversely, ≥ 5 log cycles of this reduction was obtained from 2 to 4 min. This abrupt microbial reduction did not allow for a nonlinear regression analysis of the inactivation data, as the experiments wouldn't contain information to estimate appropriately the model parameter.

4.6.2 Discussion

In the present study, it was observed that simultaneous ultrasound and ozone treatment was effective for inactivation of *E. coli*.

In the literature, combined use of sonication and ozone has been reported for various applications. Ultrasound and ozone assisted biological degradation of thermally pretreated and anaerobically pre-treated distillery wastewater was reported (Sangave et al., 2007). Xu et al. (2010) reported that, combined ultrasound and ozone was an effective technology for improving solubilisation and anaerobic biodegradability of waste activated sludge. Jyoti and Pandit (2004) concluded that hybrid techniques (combination of hydrodynamic cavitation, acoustic cavitation and hydrogen peroxide or ozone) were far superior compared to any individual physical treatment techniques for water treatment. Burleson et al. (1975) reported complete inactivation of bacteria in PBS after 15 sec of treatment with ozone alone or with simultaneous treatment with sonication and ozone. However, microorganisms suspended in secondary effluent required longer contact times with ozone for complete inactivation. Simultaneous treatment with sonication and ozonation resulted in a synergistic effect. Similarly, in the present study simultaneous sonication and ozonation increased the inactivation rate of *E. coli*. This could be due to the reason that sonication enhances the inactivation of bacteria by ozone, by breaking up clusters of bacteria making them

susceptible to the oxidising power of ozone (Dahi, 1976; Burlison et al., 1975). Cavitation produced by sonication reduces the high surface tension caused by organic material increasing the inactivation rate. Boucher et al. (1967) reported that sonication accumulates a large amount of vibrational energy at microorganisms' solid gas interphase, thus enhancing the diffusion process allowing more rapid penetration of gas into the microorganism. Sonication has been demonstrated to increase the mass transfer of ozone to solution via increasing volumetric mass transfer coefficient in combined ultrasound and ozone reaction process (Xu et al., 2010). Free radicals are generally produced during ozone decomposition. Ultrasound treatment also increased the ozone decomposition rate thus increasing free radicals concentration that led to rapid microbial inactivation (Dahi, 1976). These different factors of combined process increase the inactivation rate of microorganisms. However, the ozone demand exerted by organic material (juice pulp) increases the treatment time slightly to attain microbial inactivation by ozone alone. Combined ultrasound and ozone process slightly increased the inactivation efficacy.

4.7 Assessing the microbial oxidative stress mechanism of ozone treatments through the responses of *Escherichia coli* mutants

The general mechanism by which ozone can induce cell damage and eventually cell death has been reported but there is no available information about the effect of oxidative stress on regulated knockout genes and the protection or sensitivity of these microbial mutants against ozone or ozone generated radicals. In the present study, the separated effects of *soxR*, *soxS*, *oxyR*, *rpoS* and *dnaK* deleted genes on the microbial resistance, cell membrane properties and cell morphology changes during ozone treatment was investigated towards interpreting the role of these systems in the mechanism of action of ozone and the potential cascade of bactericidal effects.

Ozone treatment of *E. coli* strains was carried out in a saline solution at a flow rate of 0.06 L/min and ozone concentration of 6 µg/mL for up to 4 min.

4.7.1 Effect on microbial inactivation kinetics

There was a significant effect of ozone exposure on cell viability observed (Figure 4.22). The parent *E. coli* BW 25113 and the $\Delta dnaK$ mutant were comparatively less susceptible to ozone treatment than $\Delta soxR$, $\Delta soxS$, $\Delta oxyR$ $\Delta rpoS$ mutants as illustrated from the estimation of the t_{5d} parameter ($p < 0.05$) (Table 4.11). All the mutants were susceptible to ozone treatment. No significant difference was recorded for the ozone inactivation rate of mutants ($\Delta soxR$, $\Delta soxS$, $\Delta oxyR$ and $\Delta rpoS$ mutants). However, for all strains tested, the reduction by 5 log cycles was achieved in 4 min of treatment time (Table 4.11).

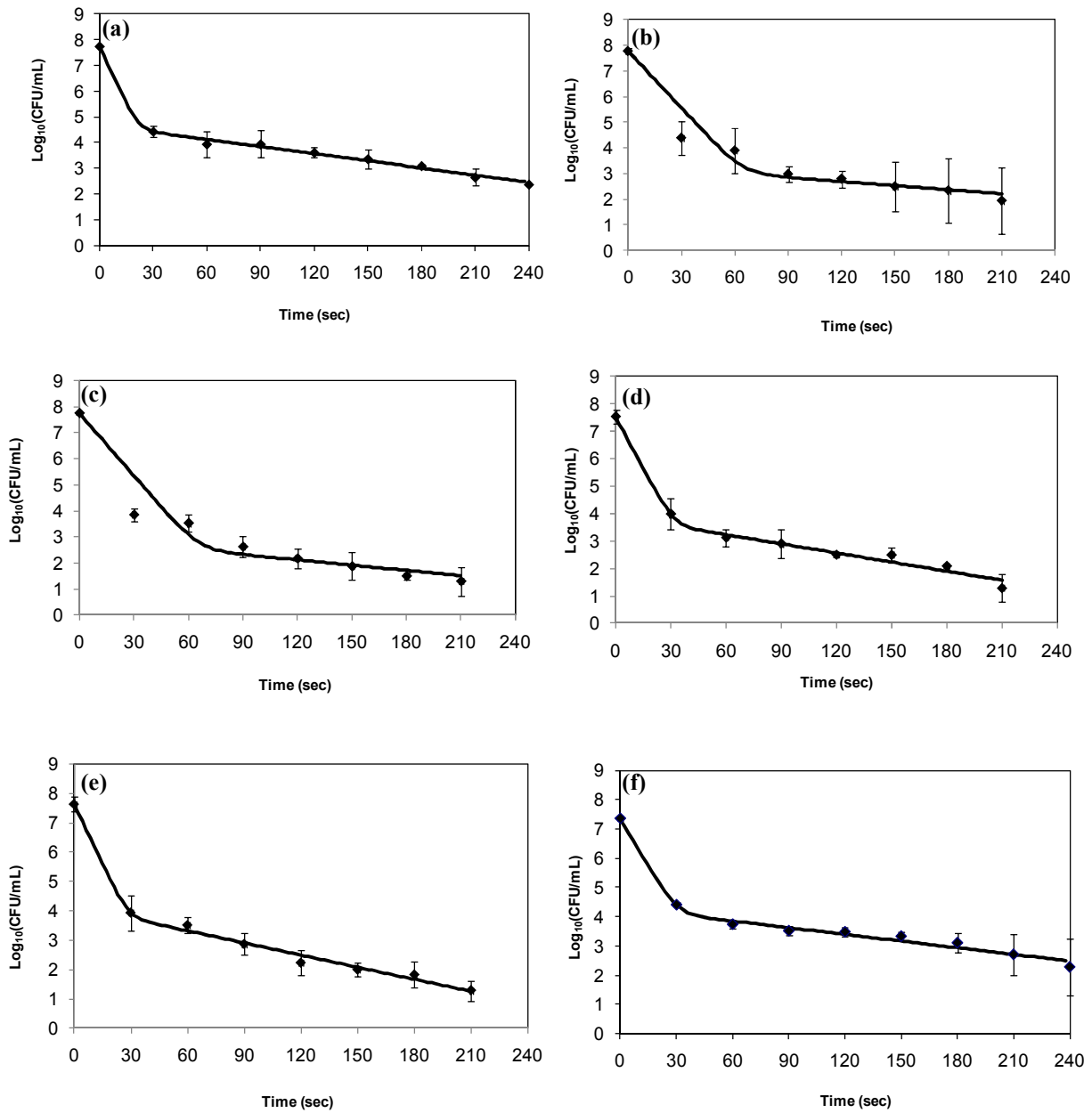


Figure 4.22: Effect of ozone on microbial inactivation kinetics of different *E. coli* strains

(a) *E. coli* BW 25113 (parent strain) (b) $\Delta rpoS$ (c) $\Delta soxR$ (d) $\Delta soxS$ (e) $\Delta oxyR$ (f) $\Delta dnaK$

The solid line represents the fit of the data to a biphasic model (Eq. 2-9)

4.7.2 Effect on cell membrane integrity and membrane permeability

When *E. coli* strains were treated with ozone, the absorbance at 260 nm increased immediately after 30 sec of ozonation irrespective of the parent strain or mutant strains studied (Figure 4.23). The maximum release for intracellular components was noticeable for $\Delta oxyR$ and $\Delta rpoS$ mutants. $\Delta oxyR$ mutant showed significantly higher absorbance values at 260 nm ($p < 0.05$) after 30 and 60 sec of ozone treatment compared to parent and other mutant strains studied. Ozone treatment of 90 sec showed significantly higher absorbance values ($p < 0.05$) at 260 nm for $\Delta oxyR$ and $\Delta rpoS$ mutants. Ozonation for 240 sec showed significantly lower absorbance values for parent, $\Delta soxR$, $\Delta soxS$ and $\Delta dnaK$ mutants compared to $\Delta oxyR$ and $\Delta rpoS$ mutants. The absorbance at 280 nm increased after 30 sec of ozone treatment (Figure 4.23) but it was less than that at 260nm. The $\Delta oxyR$ mutant showed significantly higher absorbance values at 280 nm ($p < 0.05$) after 30 sec of ozone treatment compared to the parent and the rest of the mutants studied. Ozone treatment (240 sec) of $\Delta oxyR$, $\Delta rpoS$ and $\Delta soxS$ mutants showed significantly higher absorbance values at 280 nm ($p < 0.05$).

The uptake of NPN by *E. coli* strains after 30 sec of ozone treatment is shown in Table 4.11. For all *E. coli* strains, ozone treatment of 30 sec resulted in increased NPN uptake. Further exposure to ozone did not result into further significant increase in fluorescence.

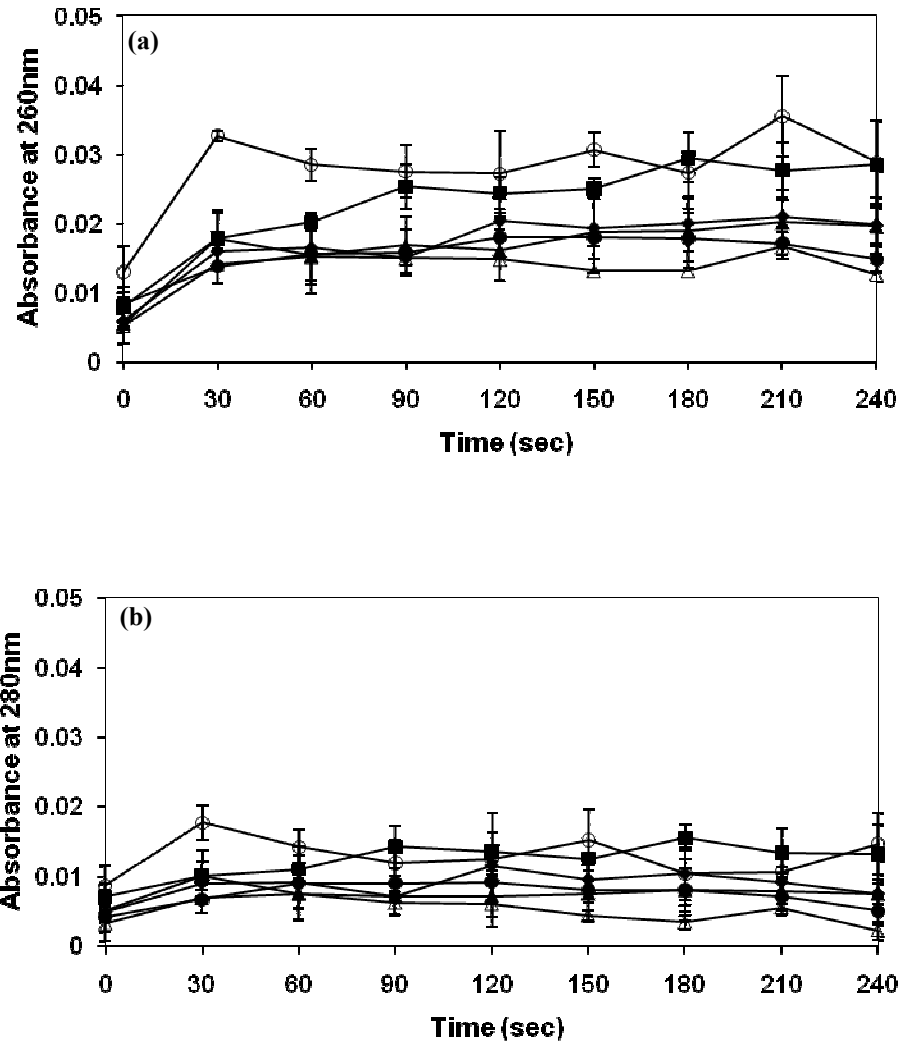


Figure 4.23: Release of cell materials absorbing at a) 260 nm and b) 280 nm from *E. coli* cells treated with ozone

◆- parent strain BW 25113; ■- $\Delta rpos$; ▲- $\Delta soxR$; △- $\Delta soxS$; ○- $\Delta oxyR$; ●- $\Delta dnaK$

Table 4.11: The t_{5d} (time to achieve 5-log reduction) values and relative fluorescence values for *E. coli* strains

<i>E. coli</i> Strain	k_{max1}	k_{max2}	RMSE	t_{5d} (sec)	Relative NPN fluorescence (after 30 sec of ozone treatment)
BW 25113 (Parent strain)	0.3403±0.150	0.0214±0.001	0.11	204.96 ^a	44±8.71
$\Delta rpoS$	0.2915±0.080	0.0332±0.004	0.33	128.08 ^b	40±3.60
$\Delta soxR$	0.3436±0.079	0.0345±0.003	0.22	98.64 ^b	53.33±12.4
$\Delta soxS$	0.2928±0.040	0.0283±0.003	0.23	120.06 ^b	45 ±7.5
$\Delta oxyR$	0.3168±0.041	0.0314±0.002	0.16	108.18 ^b	40±5.40
$\Delta dnaK$	0.2478±0.027	0.0175±0.002	0.15	258.61 ^a	38.9±9.8

(Different letters indicate a significant difference at the 0.05 level between each type of strain).

4.7.3 SEM examination of ozone treated *E. coli*

All *E. coli* strains showed rapid population reductions and release of intracellular components following even 30 sec of ozone treatment. Therefore, for SEM analysis 0 and 30 sec samples were chosen and the analysis was mainly performed for parent strain BW 25113 and sensitive mutant strains; $\Delta oxyR$ and $\Delta soxR$. Detailed observation of *E. coli* cells after SEM analysis showed slightly altered cell surface structure and damage to the cell

surface but to a lesser extent compared to the untreated cells (Figure 4.24). The surface of ozone treated *E. coli* appeared to be slightly rough compared to the non ozonated cells.

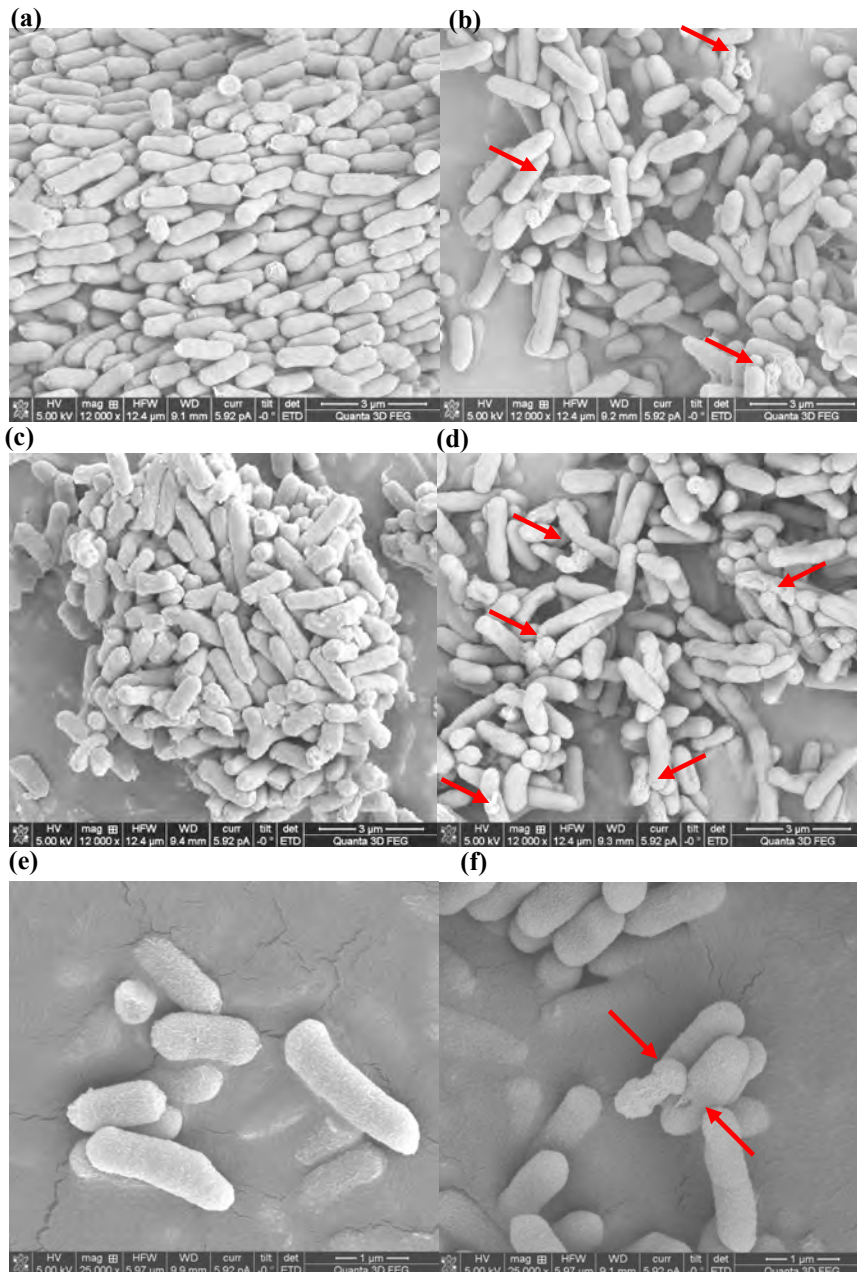


Figure 4.24: Scanning electron micrograph of untreated and ozone treated *E. coli*

- (a) *E. coli* BW 25113- untreated
- (b) *E. coli* BW 25113- ozone treated (30 sec)
- (c) $\Delta oxyR$ - untreated
- (d) $\Delta oxyR$ - ozone treated (30sec)
- (e) $\Delta soxR$ - untreated
- (f) $\Delta soxR$ - ozone treated (30 sec)

4.7.4 Discussion

This work attempted for the first time to get information regarding the nature and the main cellular targets of ozone treatment. Interestingly, the same intensity of ozone treatment had different effects on mutants with deleted genes which have previously been shown to play an important role in oxidative stress. These could lead to some interesting conclusions regarding ozone treatment such as sensitivity of mutants highlighting the importance of oxidative stress related genes in providing protection against ozone or ozone generated radicals.

Ozone treatment of parent *E. coli* BW 25113 and $\Delta dnaK$ mutant showed a requirement for higher inactivation times compared to the other mutants studied. DnaK has previously been shown to play an important role in the resistance of *E. coli* cells against H₂O₂ (Delaney, 1990; Rockabrand et al., 1995) mainly through the protection of proteins from oxidative damage (Echave et al., 2002). However, in this work DnaK seemed to confer no protection against ozone-derived oxidative stress. It is possible that the specific radical species being produced during ozone treatment do not exert their lethal effects through protein denaturation. In that case a cellular mechanism like DnaK, protecting the proteins from denaturation would not enhance the survival of the wild type in comparison to a $\Delta dnaK$ mutant. Another explanation could be that the generated radical species and the intensity of the treatment led to the rapid inactivation of DnaK in the wild type. In this case the DnaK would be inactive in both the wild type and $\Delta dnaK$ mutant, resulting in similar inactivation for both strains. Inactivation of DnaK by severe oxidative stress at high temperatures has been previously demonstrated by Winter et al. (2005) and it was suggested that severe oxidative stress rendered DnaK thermolabile at high temperatures rather than causing its inactivation *per se*. However, there was no temperature increase during the ozone treatment

in these experiments. The above results suggest that additional studies are required to further identify the nature of ozone treatment, its cellular targets and the role of DnaK, if any, against specific radicals.

The lowest t_{5d} reported for $\Delta soxR$, $\Delta soxS$, and $\Delta oxyR$ and $\Delta rpoS$ mutants highlight the importance of oxidative stress related genes for their protection against the ozone environment. The SoxRS regulon (superoxide response regulon) has previously been shown to play an important role in protection against ozone treatment in *E. coli* (Jimenez-Arribas et al. 2001). However, this reported study involved the use of dried cells on a disk in an attempt to investigate the response of bacteria to city pollutants which would contribute an additional stress. Several genes which belong to this regulon (e.g. *sodA* and *nfo*) are directly responsible for the removal of superoxide anions or repair of superoxide damaged macromolecules and mainly DNA. These could explain why the mutant in *soxR* was sensitive to ozone treatment. In addition, it provides strong evidence that one of the main cellular targets of ozone treatment is DNA. Previous studies also reported ozone causes damage to DNA which if unrepaired, cause extensive breakdown of DNA in *E. coli*, resulting in loss of cell viability (Hamelin and Chung, 1974; Hamelin et al., 1977; 1978). The damage to chromosomal DNA might be one of the reasons for inactivation of *E. coli* by ozone (Ishizaki et al., 1987). In the case of $\Delta soxS$ mutant, the continued presence of SoxR regulates its own promoter, thus providing a certain extent of protection against ozone mediated stress. This hypothesis is based on the previous studies indicating that in *Pseudomonas*, SoxR directly regulated the expression of superoxide regulated genes (Kobayashi and Tagawa, 2004). Moreover, SoxR acts directly on target promoters without the involvement of SoxS (Kobayashi and Tagawa, 2004; Park et al., 2006). Eiamphungporn et al. (2006), reported on the physiological function of *Agrobacterium tumefaciens* SoxR in

superoxide stress protection, which directly regulated its own promoter and promoters of other genes in its regulon. This explains that in $\Delta soxS$ mutant, the protection to a certain extent against ozone mediated stress was provided by SoxR.

OxyR is another transcriptional regulator required for the induction of hydrogen peroxide (H_2O_2) inducible genes like *katG*, *ahpCF*, *grxA* and many others (Hidalgo and Demple, 1996; Jamieson and Storz, 1997; Tao 1997; Zheng et al., 1998, Zheng et al., 2001). The interaction of H_2O_2 with iron localized along the phosphodiester backbone of nucleic acids leads to cell death upon exposure to H_2O_2 (Storz and Imlay, 1999). Strains with *oxyR* deletions are unable to induce this regulon and are hypersensitive to H_2O_2 (Christman et al., 1989). Hence, the absence of the H_2O_2 inducible gene activator in $\Delta oxyR$ mutant resulted in increased sensitivity to ozone.

One of the most important regulators of stress genes involved in general stress resistance is RpoS. The RpoS subunit of RNA polymerase is the master regulator of general stress response in *E. coli*, positively regulating more than 500 (10%) genes (Hengge, 2009). RpoS is known to regulate the expression of genes which are important against oxidative stress (*katP*, *ahpCF*). However, until now it has not been shown if it plays any role in protecting the cells against ozone treatment. The results obtained demonstrated for the first time that RpoS plays an important role in the resistance of *E. coli* against ozone. This result opens the door for further work which could identify the specific genes of the RpoS regulon which are important for protection against ozone treatment.

The estimation of oxidative stress intensity is a complicated task due to different macromolecules such as proteins and lipids which can have different susceptibilities to oxidative damage within a range of time scales (Semchyshyn et al. 2005). Release of intracellular components after ozonation was observed for all *E. coli* strains studied.

Continuous increase of release of intracellular components from ozone damaged *E. coli* was previously reported (absorbance at 260 nm and protein content) (Komanapalli and Lau, 1996; Curtiellas et al., 2005). However, in the present study, the absorbance observed at 260 nm was 10 times less than that reported previously (Komanapalli and Lau, 1996; Curtiellas et al., 2005). These results indicated that the cell membrane might not be the main target of ozone.

In the presence of ozone, the macromolecules released from the cell could be further cleaved and oxidized, resulting in much smaller molecules (White, 1999). Cho et al. (2010) concluded that proteins degrade due to the reaction with disinfectant in the order of ozone, chlorine dioxide, free chlorine and UV and the amount of proteins released from *E. coli* inactivation would be underestimated if the degradation reaction is not considered. Another possibility of comparatively low release of intracellular material in this study could be due to further interaction of intracellular materials with ozone resulting in significant degradation of proteins and nucleic acids.

In Gram negative bacteria, the outer membrane maintains the bacterial structure and is a permeability barrier to large molecules such as proteins and hydrophobic molecules. The lipopolysaccharides (LPS) in the outer membrane form specific contacts with integral outer membrane proteins (Omp), such as porins (Alexander and Rietschel, 2001; Bos and Tommassen, 2004). Porins allow diffusion of hydrophilic molecules less than 700 Da through the membrane that allows the passage of metabolites and small hydrophilic antibiotics. The outer membrane is held together by divalent cation (Mg^{2+} and Ca^{2+}) linkages between phosphates on LPS molecules and hydrophobic interactions between LPS and proteins. Disruption of the outer membrane weakens the bacteria and allows the permeability of large, hydrophobic molecules (Murray et al., 2009). NPN is a hydrophobic

probe normally excluded by Gram negative bacteria. The parent and mutant *E. coli* strains showed increased uptake of NPN followed by ozonation. Disruption of the outer membrane weakens the bacteria and allows permeability to large, hydrophobic molecules like NPN indicating damage to the outer membrane (Helander and Mattila-Sandholm, 2000). However, comparatively less membrane damage was observed in the present study in contrast to membrane damage reported by using chitosan (Liu et al., 2004) and lactic acid (Alakomi et al., 2000) as antimicrobial agents.

In the current study, from SEM analysis, it was observed that cell lysis was not the main mechanism of inactivation and the ability of ozone to diffuse through the membrane could be damaging the cell constituents, thereby negatively impacting on metabolic activity leading to final inactivation of the cells. The ability of ozone to diffuse through the membrane and react with cell constituents was indicated by several authors (Ingram and Haines 1949; Hamelin and Chung, 1974; Hamelin et al., 1977, 1978; Ishizaki et al., 1987). Ingram and Haines (1949) found ozone treatment results in a destruction of dehydrogenating enzyme systems in *E. coli* and proposed that death of the cell may result from a damaged respiratory system. Similarly, Perich et al. (1975) concluded that cell lysis was not the main mechanism for *E. coli* inactivation and that the cells remained morphologically intact after inactivation. Several mechanisms or hypothesis were proposed for characterizing the ozone mode of action. Many studies indicated that ozone attacks the cell membrane, resulting in membrane deterioration, leakage of cell contents into the medium and ultimately cell lysis (Scott and Lesher, 1963; Mudd et al., 1969; Scott, 1975; Hamelin et al., 1978; Hunt and Marinas, 1999; Thanomsub et al., 2002). However, the present work indicated cell lysis was not the major inactivation mechanism but that ozone

penetration through the cell and the resulting damage to the vital cell components brought about by oxidation may be responsible for cell death.

Chapter 5 OVERALL DISCUSSION

Fruit juices are important sources of bioactive compounds and are becoming an important part of the modern diet in many communities. Historically, unpasteurised juice was considered non-hazardous due its acidic nature; but it is known that *Salmonella* spp., *L. monocytogenes* and especially *E. coli* O157 can survive for extended periods of time in low pH foods (Gahan et al., 1996; Oyarzábal et al., 2003; Duffy et al., 2006). Many foodborne pathogens including *Vibrio cholerae*, *Shigella* spp., *L. monocytogenes* and *E. coli* have an acid tolerance response that is induced at moderately acidic pH which may allow the bacteria to subsequently survive severe acidic pH conditions (Foster, 1999; Merrell and Camilli, 2002; Smith, 2003). Human infection by *E. coli* O157:H7 has increased worldwide since the early 1980s. In the Republic of Ireland, 148 VTEC cases were notified up to the end of August 2008, compared to 70-90 confirmed cases reported in 2006 and 2007. In the case of *E. coli* O157:H7, a very small number of bacteria can cause serious illness.

L. monocytogenes is a Gram positive facultative intracellular pathogen that is the causative agent of listeriosis and can be found in a wide variety of raw and processed foods. It has not been reported with juice associated outbreaks so far, but is an important foodborne pathogen to be considered while developing food preservation process and has been isolated from unpasteurised fruit juice (Sado et al., 1998).

Food spoilage is a serious problem for the food industry as it renders products unacceptable for human consumption with the accompanying economic loss. Thermal pasteurisation is one of the main conventional techniques used for food preservation, which extends shelf life with assured safety, but may have detrimental effects on aroma and flavour. To facilitate the preservation of unstable or thermo labile nutrients, many juice processors have investigated alternatives to thermal pasteurisation, including un-pasteurised short shelf life

juices with high retail value. Consumer demand for healthy minimally processed, preservative free foods with a useful shelf-life is high and on the rise. Thus, a key goal for the juice industry is to seek ways to improve processing technology to address the combined requirements of guaranteed food safety, minimising microbial and chemical spoilage, whilst maintaining nutritional quality.

In the present study, two non-thermal technologies i.e. ultrasound and ozone were employed to determine their effectiveness for inactivation of microorganisms in liquid systems. The challenge microorganisms were *E. coli*, *L. monocytogenes* and *L. innocua*. The use of non-thermal techniques for microbial inactivation can omit or reduce the use of heat, which is useful from the point of view of product quality. The advantages presented by non-thermal technologies for pasteurisation of liquid foods such as fruit juices are many, including the ability to inactivate microorganisms at near ambient temperatures by avoiding the deleterious effects that heat may have on the flavour, colour and nutritive value of foods (Barbosa-Cánovas et al., 1999) and consequently preserving the sensory and nutritional quality of fresh-like food products (Pereira and Vicente, 2009). Novel non-thermal technologies can provide not only energy savings but also water savings, increased reliability, high product quality (Masanet et al., 2008) and consequently, less impact on environment (Pereira and Vicente, 2009).

Food acidification is a common food preservation method. Foodborne bacteria usually encounter drastic pH variations in the environment, and are exposed to acid conditions while present in foods, during processing, and when they invade the gastrointestinal tract of animals and humans (Sharma et al., 2003). In nature, microbial cells have developed strategies to respond to acid stress by inducing a protective, acid tolerance response (ATR) (Foster, 2000). Microorganisms develop ATR when exposed to moderately low pH levels;

resulting in the induction of proteins that can then protect the cells against subsequent extremely acidic conditions. In addition, cells respond to acid environments by modifying their membrane composition, increasing proton efflux and amino acid catabolism, and by synthesising enzymes involved in DNA repair (Yousef and Courtney, 2003; Beales, 2004). Hsin-Yi and Chou (2001) indicated that acid-adapted *E. coli* O157:H7 survived longer than the non adapted bacterium in fruit juices under refrigeration. Ravishankar et al. (2000) reported acid adapted *L. monocytogenes* exhibiting cross protection against an activated lactoperoxidase system in tryptic soya broth. Although extensive information on acid stress of *E. coli* and *L. monocytogenes* have been reported, there is a gap in the information available on the cross protective effect of acid adapted bacteria towards stresses applied in food processing and preservation. Indeed as new technologies emerge, it is important that they should be tested with appropriate microbiological risks, including stressed or adapted populations to ensure true challenges can be overcome. The cross protective responses are of importance when evaluating the efficacy of intervention strategies to achieve food safety and to preserve the quality of food products (Rodriguez-Romo and Yousef, 2005). There is a gap in the scientific literature concerning the prior acid adaptation effect on inactivation efficacy of ultrasound and ozone. Understanding the relation between acid adaptation and resistance of microorganisms towards processing techniques is of great importance for food safety issues in industry. Therefore, the effect of prior exposure to acid stress or acid adaptation of microorganisms on inactivation efficiency of processing treatment was investigated as a priority.

The effectiveness of ultrasound in combination with mild heating has been shown (Villamiel and de Jong, 2000; Baumann et al., 2005) but in the present study only ultrasound treatment (7.5 μm or 37.5 μm amplitude) displayed a strong influence on the

rate of *E. coli* inactivation in TSB. This is of high significance for fruit juice processing where maintaining a low processing temperature is important for retention of quality characteristics of fresh juice as well as maintaining energy efficiency. Prior acid adaptation of the *E. coli* cells studied resulted in increased treatment time for inactivation, reinforcing that the acid adaptation phenomenon should be considered when designing process parameters. Nonetheless, the work presented showed that ultrasound treatment could be effective for inactivation of *E. coli*, even a population that has been exposed to prior acid stress or adaptation, such as those encountered in acidic products such as fruit juices.

Ozone was the second non-thermal technology investigated, considering its broad usage in industry for decontamination. An important advantage of using ozone is its rapid decomposition into non toxic products thus leaving no residues. The FDA's approval of ozone as a direct additive to food in 2001 triggered interest in the development of ozone applications, and industry guidelines for apple juice and cider were published by the USFDA in 2004 (US FDA, 2004). However, these guidelines highlighted gaps with respect to lack of knowledge regarding the critical control parameters for ozone inactivation in liquid systems. The main considerations addressed in this work were with respect to process parameter optimisation, where the balance of the parameters governing inactivation process was important. The effect of extrinsic control parameters on ozone inactivation of *E. coli* in liquid system was investigated and optimised controlled conditions of direct ozone were then applied to further investigate its efficacy against challenge microorganisms of concern in orange juice and apple juice. An increased ozone inactivation time was attributed to the presence of organic matter (orange juice, apple juice). Similar observations of reduced ozone efficacy in presence of organic matter were reported previously (Restaino et al., 1995; Guzel-Seydim et al., 2004a; Mielcke and Ried, 2004;

Williams et al., 2005; Macauley et al., 2006), but overall the present work showed that ozone treatment was effective for inactivation of challenge microorganisms in orange and apple juice. Again, as with ultrasound, prior exposure of challenge microorganisms to acid stress resulted in increased inactivation times. Previous studies also reported increased resistance of bacteria after acid adaptation (Mazzotta, 2001; Caggia et al., 2009; Leyer et al., 1995). Therefore, in the current work, prior acidic pH exposure (juice) and its effect on processing was considered in more detail. Different acid exposure or stress conditions were applied to determine any effects on the survival rate against ozone treatment. These conditions were carefully selected to mimic the environments to which a contaminating population could be exposed in fruit juice and other food processing scenarios. An increased inactivation time of acid exposed *E. coli* cells of both strains to ozone treatment, over the control cells was observed. Similarly, acid stress-habituated *Listeria* cells showed an increased resistance to ozone treatment. The extent of the enhanced acid resistance varied with the strain and the conditions applied. A number of studies have previously reported induction of cross protective responses in foodborne pathogens and other microorganisms as a result of previous exposure to acid (Ryu and Beuchat, 1998; Duffy et al., 2000; Ravishankar et al., 2000; Rowe and Kirk, 2001; Parry-Hanson et al., 2009). The acid tolerance response has a great impact on food processing and the virulence of bacteria, as exposure to acidic conditions not only increases survival at lethal acidic pH but also provides protection against other challenges, such as heat, ethanol, oxidative and osmotic stresses (Gahan et al., 1996; Lou and Yousef, 1997; Conte et al., 2000). A prior acid stress followed by an acid environment in the product (fruit juice), may result in cross protection to a subsequent processing treatment, as observed in this work where *Listeria* cells habituated in orange juice generally had a lower % injury following ozone treatment.

Therefore, in food processing conditions where prior acid exposure, acid stress-habituation and habituation in actual product can occur, along with the influence of food properties such as viscosity, particle size, effect of various environmental factors such as medium pH, temperature etc. should be considered for determining inactivation parameters and process design in foods.

Prevention of food spoilage is also important along with retention of food safety. There was also a gap observed in the scientific literature with regard to shelf life of ozonated fruit juice. Therefore, ozonation was applied to investigate the growth behaviour of the spoilage yeast, *S. cerevisiae*, in both untreated and ozonated apple juice. Feasible conditions of ozonation for extension of apple juice shelf life were shown. An increased shelf life of ozonated apple juice was achieved when compared with the untreated apple juice (controls), which could be due to the strong oxidising action of the applied ozone treatment exerting an additional stress on the yeast population. This is advantageous as ozonation was carried out at ambient temperature, thus mitigating against the possible degradation of sensory or nutritional quality losses that can occur with thermal processes. However, these aspects relating to fruit juice quality and sensory analysis during the shelf life of processed juice should be considered in detail.

The combined effectiveness of two technologies was also studied under optimised controlled conditions. A synergistic effect of ultrasound and ozone was observed for inactivating *E. coli* ATCC 25922 in orange juice. However, the efficacy of combined treatment was only slightly increased compared to treatment with ozone alone. Cost plays a vital role in selection of a disinfection technique; hence it is necessary to evaluate the energy efficiency of the combined process before it can be applied for processing. Further

studies are necessary to optimise the process parameters of simultaneous or sequential ultrasound and ozone treatment.

Ozone destroys microorganisms by the progressive oxidation of vital cellular components. Many research groups have studied mechanisms of microbial inactivation by ozone. The bacterial cell surface has been suggested as the primary target of ozonation. Ozone oxidises sulfhydryl groups and amino acids of enzymes, peptides and proteins to shorter peptides and oxidises polyunsaturated fatty acids to acid peroxides (Victorin, 1992). Oxidation reactions are caused by either dissolved molecular ozone or free radical species formed during auto-decomposition of ozone (Hunt and Marinas, 1997). The mechanism by which ozone induces the cell damage and eventually cell death has been reported but there was previously no available information about the effect of oxidative stress on regulated knockout genes and the protection or sensitivity of these microbial mutants against ozone or ozone generated radicals. In this study, a strong role of oxidative stress related proteins in protection against oxidative stress by ozone was observed. Cells lacking any of the oxidative stress related protein showed sensitivity to ozone treatment. Similar observations were reported by Jimenez-Arribas et al. (2001) and Fisher et al. (2000). An ozone process is always based on the effect of direct and indirect reaction mechanisms. Depending on various factors such as temperature, pH, organic matter, one kind of reaction will occur. Both molecular ozone and the free radicals produced by its breakdown play a part in the inactivation mechanism but there is no consensus on which is more decisive (Khadre et al., 2001). It has not been well established whether molecular ozone or the radical species are responsible for inactivation of microorganisms (Blatchley and Nimrata, 2002) or if there is a prevalence which may be effected by other intrinsic or extrinsic parameters. In the present work, during the ozonation process, there could be generation of radicals such as

superoxide radicals and hydroxyl radicals which had a stronger inactivation effect on strains with deleted genes.

Ascorbic acid may be a key reactant for ozone reactivity in juices, as in plants and human respiratory tract (Thiele et al., 1997). Fast ozone inactivation of *Cryptosporidium parvum* oocysts in orange juice due to the high amount of ascorbic acid was reported (Kniel, 2002). Various radicals were produced due to reaction of ozone with ascorbic acid and it was hypothesised that oocyst cell wall proteins were oxidised by the reactive oxygen species generated from the decomposition of ozone (Kniel, 2002). At high pH, the chain reactions of ozone decomposition result in the formation of numerous radical species with high oxidative capabilities.

During ozone processing of orange juice or apple juice, inactivation of challenge microorganisms could be accomplished either by the direct action of molecular ozone or an indirect action of ozone. However, juice contains large amount of organic matter, hence it is difficult to determine the stability of ozone and it could be decomposed, thus generating different radicals that also may have a strong inactivation effects on microorganisms. Therefore, the possibility of interaction of ozone with the various types of organic material present in a food product which may result in different radical production profiles should be studied.

Hence, looking at the results it was observed that direct ozone treatment was effective for inactivating challenge microorganisms and for increasing shelf life of ozonated fruit juice and could be used as a potential non-thermal technology for fruit juice processing.

Chapter 6 CONCLUSIONS AND FUTURE RECOMMENDATIONS

Conclusions

- ❖ Power ultrasound treatment alone was effective for inactivation of *E. coli* populations exposed to prior acid stress or adaptation, conditions that are commonly encountered in acidic products such as fruit juices.
- ❖ Although a higher level of ultrasound amplitude negated the enhanced survival of the acid adapted non-toxigenic strain of *E. coli* O157:H7, it remains important to take the higher D-values observed into account during process design.
- ❖ Inactivation of greater than the 5 log level reduction occurred without the use of extra heating which is very relevant to the processing of fruit juice.
- ❖ The efficacy of ozone treatment was found to be a function of treatment time and flow rate ($p < 0.05$). Inactivation of *E. coli* was rapid at ambient temperature, which presents the advantage of lower energy consumption than traditional thermal pasteurisation processes, while minimising the effects on quality parameters. When the efficacy of ozone was assessed in MOJ and PBS, treatment times were significantly reduced.
- ❖ The optimised critical control parameters applied to inactivate *E. coli* in orange juice show the effectiveness of direct ozone treatment. The efficacy of ozone treatment was found to be a function of juice type, strain of *E. coli* and duration of acid exposure conditions.
- ❖ Whilst ozone treatment was also found to be effective for inactivation of the two *E. coli* strains studied in apple juice, processors employing direct ozone for fruit juice treatment should take food product characteristics for example prior acid exposure into consideration when designing processing protocols. Fast ozone inactivation of

E. coli strains in clarified apple juice was recorded. The efficacy of ozone treatment was found to be a function of pH of apple juice.

- ❖ Direct ozone treatment was effective to inactivate *L. monocytogenes* and *L. innocua* in orange juice, where efficacy was found to be a function of strain and duration of acid stress-habituation conditions. Adaptive stress responses should be taken into account for process design or method development for the inactivation of *L. monocytogenes*.
- ❖ The shelf life of ozonated apple juice with respect to control of *S. cerevisiae* was increased compared to untreated samples. The model developed described accurately the growth behaviour of *S. cerevisiae* in untreated and ozone treated apple juice under both static and dynamic storage temperature conditions.
- ❖ Simultaneous application of ultrasound and ozone for inactivation of *E. coli* in orange juice slightly increased the inactivation rate compared to ozonation alone.
- ❖ The absence of oxidative stress related genes resulted in increased susceptibility of *E. coli* cells to ozone treatment, indicating that there could be generation of radicals such as superoxide radicals and hydroxyl radicals during the process. Cell lysis was not identified as the major mechanism of inactivation in this study.
- ❖ Direct ozone diffusion treatment could be used for control of *E. coli*, *Listeria* populations in fruit juices or other liquid foods. It can also be employed for preventing juice spoilage by *S. cerevisiae* and increasing the shelf life of processed juice sample.

Future recommendations

Ultrasound was effective for inactivation of *E. coli* in liquid systems. Further studies are warranted to determine ultrasound efficacy in real food products such as fruit juice and an expanded range of microbial spp and conditions. The influence of food properties such as fruit juice type, presence of pulp, soluble solid content and viscosity should be determined which will have an important effect on the inactivation rate and treatment time to achieve the desired log reduction. The effects on quality parameters should also be determined. Further studies are merited to investigate the mechanism of resistance of acid adapted cells to ultrasound treatment.

Direct ozone treatment was also effective for inactivation of different strains of *E. coli*, *L. monocytogenes*, *L. innocua* in fruit juices. Applied ozone treatment also extended the shelf life of apple juice. However, the effect of ozonation on important quality parameters also needs to be determined before it can be considered by processors as a preservation technology in liquid food applications. Application of ozone at higher doses that effectively inactivates bacteria may change sensory qualities of food. Hence, the possible impact of ozone treatment on the sensory quality of ozonated food products warrants further study. These should focus on defining the failure (spoilage) parameters of treated fruit juice based on the effect of ozone on quality, nutritional parameters and sensory evaluation.

The present study investigated the role of oxidative stress related proteins in *E. coli* survival during ozonation. However, the role of DnaK as well as the identification of genes from the RpoS regulon which play a role in ozone treatment requires further investigation. Elucidation of sequential events that take place when *E. coli* cells are exposed to ozone would be useful in tracking down key events, therefore facilitating further process

optimisation. The possibility that interaction of ozone with different types of organic material present in the food product could result into various radical production profiles should be studied as this could also enhance understanding of how to optimise the process. Further research is required to ascertain the interaction of food constituents with ozone and the role of resulting compounds in the inactivation process.

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APPENDIX

Peer reviewed publications

- Patil S, Cullen PJ, Kelly B, Frias JM, Bourke P. 2009. Extrinsic critical control parameters for ozone inactivation of *Escherichia coli* using a bubble column. *Journal of Applied Microbiology* 107(3):830-837.
- Patil S, Bourke P, Frias JM, Tiwari BK, Cullen PJ. 2009. Inactivation of *Escherichia coli* in orange juice using ozone. *Innovative Food Science and Emerging Technologies* 10 (4): 551-557.
- Patil Sonal, Bourke Paula, Kelly Bridget, Frias Jesus M, Cullen PJ. 2009. The effects of acid adaptation on *Escherichia coli* inactivation using power ultrasound. *Innovative Food Science and Emerging Technologies* 10 (4): 486-490.
- Patil S, Valdramidis VP, Cullen PJ, Frias J, Bourke P. 2010. Inactivation of *Escherichia coli* by ozone treatment of apple juice at different pH levels. *Food Microbiology* 27 (6):835-840.
- Patil S, Valdramidis VP, Cullen PJ, Frias J, Bourke P. 2010. Ozone inactivation of acid stressed *Listeria monocytogenes* and *Listeria innocua* in orange juice using a bubble column. *Food Control* 21 (12): 1723-1730.
- Patil S, Torres B, Tiwari BK, Wijngaard HH, Bourke P, Cullen PJ, O'Donnell CP, Valdramidis VP. 2010. Safety and quality assessment during the ozonation of cloudy apple juice. *Journal of Food Science* 75 (7):M437-M443.
- Cullen PJ, Valdramidis VP, Tiwari B, Patil S, Bourke P, O'Donnell CP. 2010. Ozone Processing For Food Preservation: An Overview on Fruit Juice Treatments. *Ozone: Science and Engineering* 32(3):166-179.

- Patil S, Valdramidis VP, Tiwari BK, Cullen PJ, Bourke P. 2010. Quantitative assessment of the shelf-life of ozonated apple juice. *European Food Research and Technology* 232 (3): 469-477.
- Patil S, Valdramidis VP, Karatzas Kimon AG, Cullen PJ, Bourke P. 2010. Assessing the microbial oxidative stress mechanism of ozone treatments through the responses of *Escherichia coli* mutants. *Journal of Applied Microbiology* (Accepted).

Congress and Conferences

- Patil S, Valdramidis VP, Tiwari BK, Cullen PJ, Frias JM, Bourke P. 2010. Quantitative evaluation of the shelf life of ozonated apple juice. The 22nd International ICFMH symposium, FoodMicro 2010, Copenhagen, Denmark, August 30- September 3, 2010.
- Patil S, Valdramidis VP, Frias JM, Cullen PJ, Bourke P. 2010. Ozone inactivation of acid-stressed *Listeria monocytogenes* and *Listeria innocua* in orange juice. IAFP's Sixth European Symposium on Food Safety held at Dublin, Ireland, 9-11 June, 2010.
- Patil S, Valdramidis VP, Frias JM, Cullen PJ, Bourke P. 2009. Evaluating the microbial responses of *Escherichia coli* during ozonation of apple juice. CD-ROM, 3d congress of European Microbiologists, FEMS 2009 [Gothenburg (Sweden), 28 June - 2 July, 2009].
- Valdramidis VP, Patil S, Frias J, Borke P, Cullen PJ. 2009. Towards a quantitative approach for evaluating ozone processing of liquid food systems. Congress proceedings, 69th IFT Annual Meeting, Anaheim, CA, USA, 6–9 June, 2009.

- Tiwari BK, Patil S, Valdramidis V, O'Donnell CP, Cullen PJ. 2009. Ozonation of apple juice: A quality and microbial inactivation study. EFFOST conference, Budapest, 11-13 November, 2009.
- Tiwari BK, Torres B, O'Donnell CP, Valdramidis VP, Patil S, Bourke P, Cullen PJ. 2009. Effect of ozonation on the quality parameters of fruit juices. 19th World Congress and Exhibition, Tokyo, Japan, 31 August - 3 September, 2009.
- Cullen PJ, Patil S, Valdramidis VP, Bourke P, Tiwari BK, O'Donnell C.P. 2009. Inactivation of pathogenic microorganisms in fruit juices during ozone processing. 19th World Congress and Exhibition, Tokyo, Japan, 31 August - 3 September, 2009.
- Patil Sonal, Kelly Bridget, Bourke Paula, Cullen, PJ. 2008. Efficacy of ultrasound for inactivation of *Escherichia coli*. The 21st International ICFMH Symposium on “Evolving microbial food quality and safety” held at Aberdeen, Scotland, 1-4 September, 2008.
- Patil S, Kelly B, Cullen PJ, Frias J, Bourke P. 2007. The application of ultrasonication as a non-thermal technology for quality and safety enhancement of fresh fruit juices. sfam summer conference on “ Microbiology of fresh produce” held Cardiff, UK, 2 -5 July 2007.

Oral Presentation

- Valdramidis VP, Patil S, Frias JM, Cullen PJ, Bourke P. 2010. Ozone treatment of apple juice at different pH levels; Effect on the microbial responses of *Escherichia coli*. IAFP's Sixth European Symposium on Food Safety held at Dublin, Ireland, 9-11 June, 2010.