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Efficacy of Innovative Anti-microbial Decontamination of Minimally Processed Vegetables

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EFFICACY OF INNOVATIVE ANTI-MICROBIAL DECONTAMINATION OF MINIMALLY PROCESSED VEGETABLES

Submitted by

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Abstract

Chlorine has widespread use commercially as a disinfectant wash for raw vegetables. However, it is an extremely corrosive gas and it may have severe health and environmental implications. The aim of this thesis was to find safe, alternative decontamination treatments for ready-to-eat Iceberg lettuce.

The efficacy of 1% acetic acid, 2% citric acid; calcium lactate concentrations of 0.5%, 1.5%, 2.5% and 3% at wash temperatures of 4, 25 and 50°C, ozone alone (1 mg l^{-1}) and in combination with calcium lactate (2.5%) were compared with chlorine as decontaminating treatments. Microbiological analyses were performed following decontamination treatments using the pour plate method to enumerate coliforms and the spiral plate method for enumeration of mesophile, pseudomonad, psychrophile, Lactic Acid Bacteria and yeast populations. The behaviour of a challenge population of *Escherichia coli* ATCC 25922 on Iceberg lettuce was also investigated for effects of temperature and decontamination treatment. The results reveal that while anti-microbial dipping resulted in significant decontamination, the efficacy of alternatives to chlorine was variable.

Initial investigations of alternative strategies for reduction of the general indigenous microflora using calcium lactate, ozone alone or in combination with calcium lactate, resulted in decontamination efficacies less than chlorine but comparable to or better than washing in water. In a comparison of chlorine with organic acids, acetic acid or citric acid were the most effective, but the use of acetic acid is not recommended due to its negative sensory impact. However, citric acid or calcium lactate treatments were found to be reasonably successful alternatives to chlorine for microbiological decontamination of lettuce.

A lag phase of 5 days was obtained for *E. coli* ATCC 25922 on calcium lactate treated lettuce, indicating its potential as a good alternative decontamination treatment.

Treatment with 3% calcium lactate at 50°C offered the best alternative to chlorine. It appears that the application of a treatment at 50°C improves the microbiological decontamination properties and that the use of calcium lactate would maintain sensory qualities due to the enhancement of tissue firmness as well as contributing to the delay of microbial outgrowth.

I certify that this thesis which I now submit for examination for the award of MPhill 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 an award in any other Institute or University.

The work reported on this thesis conforms to the principles and requirements to the Institute's guidelines for ethics in research.

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Signature _____ Date June - 05

Candidate

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Here is the lyrics of Itaka, one the best songs by the catalan writer Lluis Llach:

Quan surts per fer
el viatge cap Ítaca,
has de pregar que el camí sigui llarg,
ple d'aventures, ple de coneixences.
Has de pregar que el camí sigui llarg,
que siguin moltes les matinades
que entraràs en un port
que els teus ulls ignoraven,
i vagis a ciutats
per aprendre dels que saben.

LIST OF ABBREVIATIONS

ARS	Agricultural Research Service
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
CCP	Critical Control Points
CEMMI	Centre of Excellence in Microbial Modelling and Informatics
CFU	Colony Forming Units
CFU g^{-1}	Colony Forming Units per gram
ERRC	Eastern Regional Research Center
FDA	Food Drug Administration
FMM	Food Micro Model
FSA	Food Standard Agency
FSAI	Food Safety Authority Ireland
g	gram
GAPs	Good Agricultural Practices
GHP	Good Hygienic Practice
GMPs	Good Manufacturing Practices
GRAS	Generally Recognise as Safe
HACCP	Hazard Analysis Critical Control Points
HOCl	Hypochlorous Acid
HUS	Haemolytic Uraemic Syndrome
ICMSF	International Commission on Microbiological Specifications for Foods

IFR	Institute of Food Research
LAB	Lactic Acid Bacteria
LPD	Lag phase duration
LSD	Least Significant Difference
M	Molar
ml	millilitre
mg l^{-1}	milligram per litre
mg ml^{-1}	milligram per millilitre
µm	micrometer
mm	millimetre
MA	Modified Atmosphere
MAFF	UK's Ministry of Agriculture Fisheries and Food
MAP	Modified Atmosphere Packaging
MEA	Malt Extract Agar
min	minute
Mox	Oxidized Organic
MRS	de Man, Rogosa and Sharpe Agar
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
NDSC	National Disease Surveillance Centre
nm	Nanometre
$\cdot O^{2-}$	Superoxide radical
O_3	Ozone
OCl^{-1}	Hypochlorite ion
OH	Hydroxyl Group

·OH	Hydroperoxide radical
PCA	Plate Count Agar
PMP	Pathogen Modelling Program
RTE	Ready-to-Eat
rpm	revolution per minute
Sr	Solubility ratio
spp.	Species (plural)
SPSS	Statistical Package for the Social Science
TSBYE	Tryptone Soya Broth Yeast Extract
TVC	Total Viable Count
USDA	United States Department of Agriculture
WHO	World Health Organization
VRBA	Violet Red Bile Agar
VTEC	Verocytotoxin-producing *Escherichia coli*

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CHAPTER 1: Introduction

The market for minimally processed ready-to-eat (RTE) vegetables has been growing steadily during recent years. The Irish horticultural industry has doubled in value terms since 1990, rising from €205 million in 1990 to €412 million in 2003. Retail sales of fresh fruit and vegetables have increased by over 4% to €844 million in 2003 as more households buy fresh produce in larger volumes and more regularly (Bord Glas, 2004). Such products are typically trimmed, peeled, washed, sliced or shredded vegetables packaged within flexible films or over wrapped in plastic trays and sold through the chill chain. These products are popular with consumers because they eliminate preparation prior to consumption; minimal waste is generated and they are considered a nutritional alternative to cooked meals.

Fruit and vegetables are an important source of nutrition and a vital component of a healthy balanced diet. A report by the Cardiovascular Health Strategy Group (1999) reiterated the findings of the Nutrition Advisory Group (1995) on dietary guidelines, where at least four servings of fruit and vegetables per day were recommended. The Department of Health and Children, the Food Safety Authority of Ireland (FSAI) and Bord Glas are involved in initiatives aimed at increasing the consumption of fruit and vegetables by the Irish population (FSAI, 2001a).

An increase in fruit and vegetable consumption must be supported by consumer confidence and that requires an exemplary safety record (FSAI, 2001a). Ready-to-eat vegetables are eaten in their raw, uncooked form and it is therefore essential that these commodities are free from contamination, whether chemical or microbiological in nature. The level of awareness of food borne illnesses has risen recently owing to highly publicized outbreaks involving ready-to-eat products such as fruits and vegetables (Barak et *al.*, 2003). These outbreaks will be detailed later in Section 1.6.

Tissue disruption caused by processing results in elevated respiration and transpiration, which can lead to rapid deterioration. In addition, cut tissues release nutrients that support the growth of microflora present on raw produce. Fresh-cut vegetables harbour lower numbers of micro-organisms than do unwashed whole vegetables, as a result of washing in chlorinated water. However, processing procedures, as well as temperature abuse during storage may result in increases in populations of spoilage microflora. Minimal processing does not eliminate the natural microflora nor does it destroy naturally occurring or contaminating pathogens. Minimally processed lettuce is usually washed in cold, chlorinated water before packaging. However, the antimicrobial effectiveness of this treatment is limited. There is a need to improve the efficacy of treatments to kill naturally occurring microflora, thereby increasing the shelf life of fresh-cut lettuce (Li *et al.*, 2001).

The Irish fresh produce industry has not been linked to any outbreak of microbial food poisoning (FSAI, 2001a). However, a large outbreak of a relatively rare strain of the foodborne pathogen, *Salmonella enterica* serovar Newport affected over 350 people (20 hospitalised) in England, Northern Ireland, Scotland and the Isle of Man, in August 2004 (FSAI, 2004). There was statistical epidemiological evidence indicating that the illness was associated with consumption of lettuce from restaurants, fast food and take-away premises. Evidence suggests that the outbreaks were associated with Iceberg lettuce supplied to catering premises only and not to retail traders.

There were no confirmed linked cases of the illness in the Irish Republic, however, Health Boards, Public Health specialists and the National Disease Surveillance Centre (NDSC) increased their surveillance as there was one case of *Salmonella enterica*

serovar Newport in Co. Donegal which may have been associated with the outbreak (FSAI, 2004).

An effective decontamination stage is therefore essential during the preparation of RTE foods. The current industrial use of chlorine as a decontamination treatment is under critical investigation because of reported toxicity and environmental problems. The aim of this study was to examine the efficacy of alternative decontamination treatments on the indigenous microflora and also on inoculated *Escherichia coli* using minimally processed RTE Iceberg lettuce.

1.1 Minimally Processed RTE Vegetables

Minimally processed RTE vegetables are fresh, raw vegetables which undergo as little processing as possible prior to being sold as a ready-to-use product. The vegetables are usually trimmed, peeled, or cut if necessary, washed, and sometimes disinfected. The products are packaged in sealed pouches, or in plastic trays sealed with polymeric films. A shelf life of several days after refrigeration is necessary for feasible transport and retail of final products (Nguyen-the & Carlin, 1994).

Production and consumption of minimally processed (ready-to-eat) lettuce have increased dramatically in recent years. The convenience of shredded, pre-washed, packaged lettuce benefits consumers and provides the industry with considerable savings in transportation, storage and refrigeration costs (Delaquis *et al.*, 1999).

Iceberg lettuce is the leading leafy green vegetable (with *per capita* consumption of 10.6 kg in 1996), although romaine and leaf lettuce have steadily increased in popularity through the 1990s with an average *per capita* consumption of 2.9 kg (McWatters *et al.*, 2002).

Water containing 50 to 200 mg l^{-1} of chlorine is widely used to sanitize whole fruits and vegetables as well as fresh-cut produce on a commercial scale. Treatment of produce with chlorinated water reduces populations of pathogenic and other micro-organisms on fresh produce. Other treatments that have been investigated include warm chlorinated water, electrolyzed oxidizing and acidified chlorinated water, hydrogen peroxide, and low-dose gamma irradiation (McWatters *et al.*, 2002).

The shelf life of minimally processed products can be extended by modified atmosphere packaging (MAP) with a reduced O_2 and/or elevated-CO_2 modified atmosphere (MA) and the use of appropriate antibrowning agents, and refrigeration (Gunes & Hotchkiss, 2002). Typical storage life achieved with these products under proper refrigeration is approximately 6-8 days (Bourke & O'Beirne, 2004). The extension of a product's shelf life could theoretically provide time for *E. coli* O157:H7 to multiply without adversely affecting the organoleptic quality of the product and therefore could increase the risk of disease, especially if the product has been temperature abused (Gunes & Hotchkiss, 2002).

Physiological changes or injuries incurred during processing and storage lead to the appearance of defects that limit the shelf life of packaged, ready-to-eat lettuce. Loss of quality attributes can include tissue discoloration ranging from yellow to brown, texture loss, softening, exudation, and the development of off-odours and flavour defects. Current processing schemes for ready-to-eat lettuce normally include a washing step in cold chlorinated water, water removal by centrifugation, and packaging. Low oxygen levels are considered desirable for the reduction of enzymatic browning and can be achieved passively, by tissue respiration, or by the injection of gas mixtures into the impermeable film bags. Despite these measures, the

shelf life of ready-to-eat lettuce is limited, and processing schemes that reduce or delay physiological damage are under investigation (Delaquis et *al.*, 2002).

1.2 Microflora of Minimally Processed RTE Vegetables

Micro-organisms form part of the epiphytic flora of fruits and vegetables and many will be present at time of consumption. The majority of bacteria found on the surface of plants are Gram-negative and belong either to the pseudomonad group or to the family Enterobacteriaceae (Lund, 1992). Many of these organisms are normally non-pathogenic for humans. The numbers of bacteria present will vary depending on seasonal and climatic variation and may range from 10^4 to 10^8 colony forming units per gram (CFU g^{-1}). The inner tissues of fruits and vegetables are usually regarded as sterile (Lund, 1992). However, bacteria can be present in low numbers as a result of the uptake of water through certain irrigation or washing procedures. If the water is contaminated with human pathogens these may also be introduced (European Commission, 2002).

About two thirds of the spoilage of fruits and vegetables are caused by moulds (ICMSF, 1998). Members of the genera *Penicillium, Aspergillus, Sclerotinia, Botrytis* and *Rhizopus* are commonly involved in this process. The spoilage is usually associated with cellulolytic or pectinolytic activity, which causes softening and weakening of plant structures. These structures are important barriers to prevent growth in the products by contaminating microbes (European Commission, 2002).

The survival or growth of contaminating micro-organisms is affected by intrinsic, extrinsic and processing factors. Factors of importance are nutrient composition, pH, presence of scales and fibres, redox potential, temperature and gaseous atmosphere.

Mechanical shredding, cutting and slicing of the produce opens the plant to microbial attack (European Commission, 2002).

The relationship between human pathogens and the native microflora, including postharvest spoilage organisms on produce, is of interest for two reasons. Firstly, it has been suggested that reducing/controlling the native microbial populations by washing and sanitizing or by controlled atmosphere storage can allow human pathogens to flourish on produce surfaces (FDA, 2001).

Concern has been expressed that reductions in surface populations reduces competition for space and nutrients thereby providing growth potential for pathogenic contaminants. In theory, this scenario can result in an unspoiled product that is unsafe for consumption. Berrang *et al.* (1989a, b) showed that pathogens grow to higher levels on produce stored under atmospheres designed to extend shelf life than on traditionally stored produce. While the cut salad industry traditionally uses natural spoilage as a food safety control measure, lengthening product shelf life would not be desirable if it increases the risk that pathogens would grow before spoilage is detectable. Secondly, a proliferation of postharvest spoilage organisms may compromise peel integrity and alter product pH thereby enhancing the survival and growth of human pathogens (FDA, 2001).

Most fresh fruits and vegetables are grown in fields and orchards that are non-sterile environments. Growers have less control over conditions in the field compared to an enclosed processing facility. Occasionally, low level, sporadic contamination of produce with human pathogens may occur. Usually, such contamination is not of public health significance. For example, the pathogen may not survive until harvest; harvest workers may be instructed to avoid harvesting produce with obvious contamination, such as bird droppings; or post-harvest treatments, such as washing,

commercial manipulation (e. g. washing, cutting, slicing, packaging) of fresh produce offer additional opportunities for product to become contaminated with pathogens or for pathogens on products to grow (FDA, 2001).

The commonly encountered microflora of fruits and vegetables include *Pseudomonas* spp., *Erwinia herbicola, Flavobacterium* spp., *Xanthomonas* spp., *Enterobacter agglomerans*, Lactic Acid Bacteria (LAB) such as *Leuconostoc mesenteroides* and *Lactobacillus* spp., moulds and yeasts (Nguyen-the & Carlin 1994; Zagory 1999). Although this microflora is largely responsible for the spoilage of fresh produce, it can vary greatly for each product and storage conditions. Temperatures can play a large role in determining the outcome of the final microflora found on refrigerated fruits and vegetables, leading to selection of psychrotrophs and a decrease in the number of mesophilic micro-organisms (FDA, 2001).

Microbial decay of shredded lettuce is mainly due to the growth of micro-organisms acquired in the field (Delaquis *et al.*, 1999). During cutting, the product surface is also exposed to possible contamination with bacteria, yeasts and moulds. In the case of minimally processed lettuce, which falls into the low-acid category of horticultural products (pH 5.8-6.0), high humidity and the large number of cut surfaces can provide ideal conditions for the growth of micro-organisms (Ihl *et al.*, 2003). The nature of the microflora that develops over time is influenced by temperature, and to a lesser extent, the composition of the storage atmosphere. Mesophilic and psychrotrophic counts tend to be similar in stored shredded lettuce, while the LAB, yeast and mould counts generally remain low. Bacterial populations are usually dominated by psychrotrophic *Pseudomonas* spp. (Delaquis *et al.*, 1999). The mesophilic bacterial counts on samples of RTE vegetables were found to be highly variable, ranging from 10^3 to 10^9 CFU g^{-1} (Table 1.1).

Product quality is often acceptable, despite such high counts. Some procedures may nevertheless increase the number of mesophilic bacteria: shredding and slicing were found to increase counts from 10^3 to 10^6 CFU g^{-1} for a range of vegetables and from 10^4 to 10^6 CFU g^{-1} for lettuce and chicory salads (Nguyen-the & Carlin., 1994).

Table 1.1 Number of micro-organisms (CFU g^{-1}) found in minimally processed RTE vegetables sampled in industrial locations or in commercial display units (adapted from Nguyen-the & Carlin, 1994).

Products	Mesophilic microflora	Lactic Acid Bacteria (LAB)	Coliforms	Yeasts & Moulds	Pectinolytic microflora
Mixed vegetables from retail outlet: lettuce, cabbage, pepper, cress, sweet corn, onion.	10^8-10^9	10^5-10^9	10^5-10^7	10^4-10^8	$<10^6$-10^7
Ready to eat salads: lettuce, mixed green salads with tomato & radish, coleslaw	10^5-10^7 for 90% of samples		<10-10^2 for 60% of samples	3×10^2	
Cut lettuce (No storage)	6×10^3		10^5-10^7		
Prepared lettuce for caterers	4×10^5		8×10^3		10^4-10^5

This study by Nguyen-the & Carlin. (1994) found that in a sample of 50 pouches of shredded carrots of industrial origin stored at 10°C, spoilage after 14 days was always associated with high numbers of LAB, identified as *Leuconostoc mesenteroides,* but not with the mesophilic bacterial population generally. Spoiled samples contained high concentrations of lactic acid, acetic acid and ethanol; while CO_2 accounted for

25% or more of the atmosphere of the pouches. The accumulation of those compounds could have been the result of the lactic heterofermentative metabolism of *Leu. mesenteroides*. In contrast, these fermentation products did not accumulate (or only slightly) in shredded chicory salads, and LAB were not identified in shredded Iceberg lettuce, suggesting a limited role of fermentative micro-organisms in spoilage of this product (Nguyen-the & Carlin, 1994).

Ready-to-eat fruits and vegetables are normally stored at refrigeration temperatures that select for psychrotrophic micro-organisms. In shredded Iceberg lettuce, counts of mesophilic and psychrotrophic bacteria were identical. Nevertheless, psychrotrophic bacterial counts could represent less than 1% of the mesophilic bacteria counts in some samples initially (Nguyen-the & Carlin, 1994).

Sources of microbial pathogens on fresh produce at the preharvest stage include faeces, irrigation water, inadequately composed manure, soil, and human handling. Postharvest sources of contamination can result from tainted harvest equipment, wash and rinse water, transport vehicles and cross-contamination, as well as improper storage, processing and packaging. Furthermore, new processing techniques that increase shelf life by retarding the growth of competitive spoilage micro-organisms also enhance the risks associated with the growth of pathogens on the modified microenvironment of produce (Endley *et al.*, 2002).

Ready-to-use vegetable salads generally contain few or no antimicrobial additives, and rely on refrigeration as the main preservation factor. However, psychrotrophic pathogens have been isolated from a wide variety of raw or processed vegetables, in the absence of sensory defects. Vegetable products have been implicated in at least two outbreaks of listeriosis (Gomez *et al.*, 2002). Outbreaks of human gastroenteritis associated with consumption of contaminated vegetables have been attributed to a

wide range of other microbes, including *Salmonella*, *Shigella*, *Escherichia coli*, and *Campylobacter*, as well as several viruses and protozoan parasites. Endley *et al.* (2002) reported a possible mechanism for the contamination of leaf lettuce postharvest. He suggests that unsanitary produce-handling practice while the lettuce was immersed in a common water bath was possibly responsible for contamination with *E. coli* O157:H7.

1.3 Preservation of Minimally Processed RTE Vegetables

Fresh processed mixed vegetable salad is a perishable product. Current techniques used by the fresh processing vegetable industry have improved the overall quality and extended the shelf life of these products, but safety is still an issue of concern (Allende *et al.*, 2002). The main preservation technologies applied to RTE vegetables are refrigeration and MAP. The application of these technologies in the form of "hurdles" is designed to ensure the microbiological safety of these products. Other important sub-inhibitory hurdles applied prior to packaging include good quality produce and effective decontamination strategies.

As previously shown in Table 1.1, the numbers of microorganims in processed and minimal processed lettuce can be as high as 10^9 CFU g^{-1}. Several reports have indicated that bacterial counts in unprocessed and minimally processed lettuce usually range from 10^5 to 10^7 CFU/g. Therefore, the food industry still needs alternative processing technologies in order to meet the consumer expectations of safer and fresher vegetable products. There has been an increasing development in new technologies which can be applied to fresh produce by the food industry, such as novel MAP or the use of ozone (Allende *et al.*, 2003).

The safe shelf-life of refrigerated RTE vegetables depends on temperature conditions throughout the entire chill-chain, as the integrated effects of the time and temperature may allow for the proliferation of pathogenic and spoilage organisms (Willocx *et al.*, 1994). Retail display cabinets and domestic refrigerators are known to be critical control points in the chill-chain of minimally processed vegetables, with temperatures above 10°C reported in both (Willocx *et al.*, 1994). Unlike frozen foods, chilled foods are easily temperature abused, enhancing the importance of maintaining good temperature control throughout the chill chain (Gormley, 1990).

Increasing temperature speeds up spoilage of RTE products, and fluctuating temperatures may cause in-pack condensation which can also stimulate spoilage.

Temperature abuse of MAP-RTE vegetables has economic implications; if packs are exposed to temperature abuse, there is potential for rapid proliferation of mesophilic organisms resulting in earlier spoilage and consequent reduction in the shelf-life (Manvell & Ackland, 1986). Garg *et al.*, (1993) reported that storage of packaged spinach, cauliflower florets and carrot sticks at 15°C resulted in significantly higher total aerobic counts, compared with those stored at 3.3°C. Upward shifts in storage temperature and consequent changes in gas atmosphere towards more anaerobic, high CO_2 conditions, may alter the microbial ecology of RTE vegetables, favouring LAB, accelerated by the inhibitory effects of CO_2 upon competitive, aerobic Gram-negative bacteria (Manvell & Ackland, 1986; Willocx *et al.*, 1994).

1.4 Production of RTE Vegetables

Physiological changes or injuries incurred during processing and storage lead to the appearance of defects that limit the shelf life of packaged, ready-to-eat lettuce (Delaquis *et al.*, 2002). Unit operations applied in fresh-cut lettuce processing usually include trimming, core removal, cutting or slicing, washing, drying and packaging (Delaquis et *al.*, 2004). A typical flow diagram of the production stages involved in the production of minimally processed RTE vegetables is shown in Figure 1. 1. Each of these processing steps is briefly discussed.

1.4.1 Raw Material

The selection of the vegetables for minimal processing is the initial step in the processing procedure. It is an important stage in the process as the quality of the raw material is a reflection of the expected quality of the end product. The vegetables intended for minimally processing must therefore be of first class quality and they must be easily washable and peelable (FSAI, 2001a).

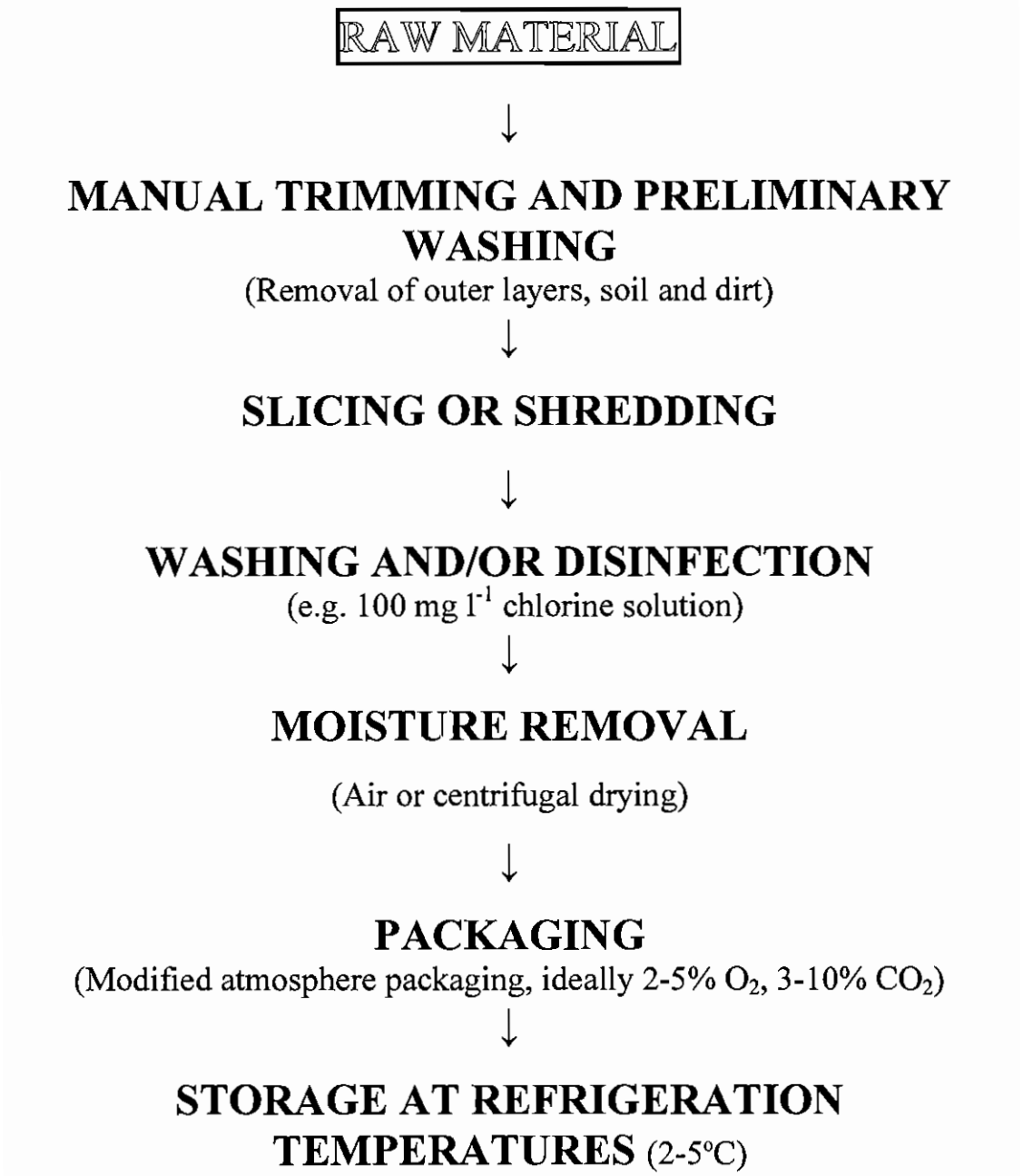

Figure 1.1 Flow diagram for the production of minimally processed vegetables

(Source: Francis et al., 1999)

1.4.2 Manual Trimming and Preliminary Washing

The first step in reducing contamination of the raw vegetables is removal of outer layers or surface dirt (Francis *et al.*, 1999). Washing of the produce reduces the chances of micro-organisms and chemicals remaining on the surface. This is an essential step since most contaminants are on the surface of fruit and vegetables and can also contaminate surrounding produce, thus spreading the hazard. The washing process must be sufficient to remove soil chemicals, micro-organisms and foreign bodies (FSAI, 2001a).

1.4.3 Slicing or Shredding

Depending on the relevant produce and its application, various shredding or size reduction machines can be used. These are, in almost all instances, constructed of good quality stainless steel (grade 316 or better) but a very well organised competent cleaning programme must be put in place to ensure that thorough cleaning is undertaken and actually achieved on each machine every day (FSAI, 2001a).

Cutting lettuce leaves can induce biochemical reactions that cause deterioration of sensory quality (Li *et al.*, 2001). The preparation of vegetables prior to packaging leads to cell and tissue damage. As a consequence of such damage, vegetable spoilage is accelerated and colour and texture changes, as well as the generation of off-odours, may occur (Sanz *et al.*, 2003).

Cutting is a critical operation, leading to produce tissue lesions, increased respiratory activity, and leaching of cellular fluids capable of sustaining growth of resident micro-organisms. These are mainly Gram-negative psychrotrophic rods, belonging to

the genus *Pseudomonas* with strong pectinolytic activity, and among the fermentative forms, *Erwinia carotovora* is the species most frequently found (Riva *et al.*, 2001).

1.4.4 Washing and/or Disinfection

Washing is a very critical part of produce preparation process especially if raw, processed fresh produce is sold as "ready-to-eat". Washing serves three purposes and the correct washing process must be accurately designed, controlled, and applied to each type of produce. Washing should:

- Remove pieces of actual dirt and debris.
- Reduce the microbiological and chemical load on the produce.
- Reduce the temperature of the finished product to help enhance shelf-life (FSAI, 2001a).

Chlorination is used extensively throughout Western Europe and North America. It is an effective way of getting significant micro-organism reduction. Free chlorine concentrations of 50-100 mg l^{-1} with a contact time of 1-3 minutes are recommended (FSAI, 2001a). A good three stage produce washing process should include a final tank stage using non-chlorinated rinse water which has been chilled to 1-2°C. This has the effect of removing traces of chlorine, giving the product a final wash but also very importantly, reducing the product temperature, so that the washed product leaves the wash tank at a temperature below 5°C, thus increasing shelf life (FSAI, 2001a).

1.4.5 Moisture Removal

On removal from the wash tank, excess water must be separated from the washed produce (depending on the produce item). This can be achieved by simple draining for the necessary time period or alternatively where leafy bulky products are involved, by spin drying or drying in fluidised drying tunnels. Care must be taken to ensure the washed material is treated appropriately, that proper stock rotation is achieved and that the air temperature within the drying area room is maintained as low as possible (1-5°C). If produce is left wet and micro-organisms are present, these may then increase in number, accelerating spoilage or making the produce unsafe (FSAI, 2001a).

1. 4. 6. Packaging

Many different packing formats are possible. Processors must remember that the product contained therein is still alive and respiring, so for some produce items with high respiration rates e.g. mushrooms, or broccoli, a packaging medium with a high permeability must be used. Permeability is the rate at which various packaging materials allow carbon dioxide and oxygen to pass through their walls and the selection of packaging material with suitable permeability is critical to good processing and in some aspects critical to food safety standards (FSAI, 2001a).

In Modified Atmosphere Packaging (MAP), the product-package compatibility is important. It is important to carry out shelf-life testing to ensure that pathogenic micro-organisms cannot exploit the storage conditions within the package. Certain pathogenic micro-organisms can survive and grow without oxygen and at low

temperatures over extended storage periods. In addition, MAP designed to prevent produce spoilage may inadvertently remove a natural barrier to the growth of pathogenic micro-organisms, namely competitive spoilage micro-organisms (FSAI, 2001a).

1.4.7 Storage at Refrigeration Temperatures

Bacteria will multiply slowly at temperatures below 5°C. Their rate of growth becomes even slower at temperatures approaching 0°C. Temperatures just below 0°C will cause chill/freeze damage to products. In general most produce items will achieve maximum shelf life at temperatures between 1-3°C. During preparation of high quality produce it is essential to control the air temperatures of the various storage areas very carefully. Following processing, storage temperatures should be as close to a 1-3°C temperature target as possible (FSAI, 2001a).

However, refrigerated storage alone cannot be relied upon to prevent growth of pathogenic micro-organisms on produce. Populations of *Listeria monocytogenes* may remain constant or grow on a variety of whole and cut produce stored at refrigerated temperatures (FDA, 2001; Parish *et al.*, 2003).

1.5 Decontamination Strategies for Minimally Processed RTE Vegetables

Because minimally processed vegetables can be contaminated by pathogens and because there is no heating or kill step involved in the processing of these vegetables which are typically consumed raw, the need for intervention methods to maintain the safety of minimally processed vegetables is very important. In addition, there is increasing demand for "natural" and "additive-free" products. Therefore, it is desirable to preserve food by natural means (Schuenzel *et al.*, 2002).

Several commercially available disinfectants have been shown to be effective against food spoilage and pathogenic bacteria in the food industry (Jean *et al.*, 2003).

There are a variety of methods used to reduce populations of micro-organisms on whole and fresh-cut produce. Each method has distinct advantages and disadvantages. The best method to ensure produce is pathogen free is to prevent contamination in the first place. However, this is not always possible and the need to wash and sanitise many types of produce remains of paramount importance to prevent disease outbreaks (FDA, 2001; Parish *et al.*, 2003). The food industry is currently in need of innovative processing technologies in order to meet consumers' demand for fresher and safer ready-to-eat products (Khadre *et al.*, 2001).

Traditional methods of reducing microbial populations on produce involve chemical and physical treatments. Control of contamination requires that these treatments be applied to equipment and facilities as well as to produce. It is important to ensure that water used for washing and sanitizing purposes is clean so that it does not become a vehicle for contamination (FDA, 2001; Parish et *al.*, 2003). Currently used, alternative and novel decontamination strategies are described below.

1.5.1 Chlorine

Currently, there is no processing method that will totally inactivate pathogens on fresh produce without compromising sensory quality. The Food and Drug Administration (FDA) proposed that treatments of fruits, vegetables and commercial fresh juices should be capable of reducing pathogen loads by a minimum of 5.0 logs (FDA, 2001).

Chlorine has been used for sanitation purposes in food processing for several decades and is perhaps the most widely used sanitizer in the food industry. Chemicals that are chlorine based are often used to sanitize produce and surfaces within produce processing facilities, as well as to reduce microbial populations in water used during cleaning and packing operations. At the foodservice and household levels, chlorine remains a convenient and inexpensive sanitizer for use against many foodborne pathogens. Safety concerns about the production of chlorinated organic compounds, such as trihalomethanes, and their impact on human and environmental safety have been raised in recent years, and alternatives to chlorine have been investigated (FDA, 2001; Parish *et al.*, 2003). Chlorine washes may leave surface residues of chlorinated compounds on lettuce, therefore this process is banned in organic production. Some chlorinated compounds are known to be cancer causing, but there appears to be little research on those left on food treated with high doses of chlorine (Lawrence, 2004). Some advantages and limitations of chlorine are shown in Table. 1. 2.

Table 1.2 Advantages and limitations of chlorine (adapted from Parish *et al.*, 2003).

Advantages	**Limitations**
Long history of use	Potential adverse health effects
Easy to use	Corrosive to equipment
Very low costs	Sensitive to temperature, light, air, metals and organic materials
Very effective killing bacteria	pH dependent
	Some resistance by bacterial spores and protozoan oocysts.
	Toxic for the environment
	Adverse ecological health effects

The most common forms of free chlorine include liquid chlorine and hypochlorites, chlorine dioxide and acidified sodium chlorite. Liquid chlorine and hypochlorites are generally used in the 50 to 200 mgl^{-1} concentration range with a contact time of 1 to 2 minutes to sanitize produce surfaces and processing equipment. Higher concentrations have been investigated for use on seeds for sprout production. Hypochlorous acid (HOCl) is the form of free available chlorine that has the highest bactericidal activity against a broad range of micro-organisms. In aqueous solutions, the equilibrium between hypochlorous acid (HOCl) and the hypochlorite ion (OCl^-) is pH dependent with the concentration of HOCl increasing as pH decreases. Typically, pH values between 6.0 and 7.5 are used in sanitiser solutions to minimize corrosion of equipment while yielding acceptable chlorine efficacy. HOCl concentration is also significantly affected by temperature, presence of organic matter, light, air, and metals. For example, increasing levels of organic matter decreases HOCl concentration and overall antimicrobial activity. Maximum solubility in water is observed near 4°C; however, the World Health Organization has suggested that the

temperature of processing water should be maintained at least 10°C higher than that of produce items in order to reduce the possibility of microbial infiltration caused by a temperature-generated pressure differential (FSAI, 2001a). The opportunity of infiltration of micro-organisms is also minimized when the sanitary condition of the water is maintained (FDA, 2001; Parish et *al.*, 2003).

The effects of chlorine on bacterial pathogens inoculated onto produce have been investigated, with mixed results. Studies indicate that chlorine concentrations traditionally used with produce (<200 mg l^{-1}), are not particularly effective at reducing microbial populations on lettuce. Survival of *E. coli* O157:H7 on cut lettuce pieces after submersion for 90 seconds in a solution of 20 mg l^{-1} chlorine at 20 or 50°C was not significantly different from the non-chlorine treatment (Li *et al.*, 2001). Spray treatment of lettuce with 200 mg l^{-1} chlorine was no more effective at removing *E. coli* O157:H7 than treatment with deionised water (Beuchat, 1999). Increasing the exposure time from 1 to 5 minutes did not result in an increased kill. Likewise, Adams *et al.*, (1989) indicated that a standardized washing procedure for lettuce leaves with inclusion of 100 mg l^{-1} chlorine was only a slight improvement over tap water alone. Although a reduction of pH of the chlorine solution to between 4.5 and 5.0 increased lethality up to 4-fold, longer wash times (from 5 to 30 minutes) did not result in increased removal of micro-organisms (FDA, 2001; Parish *et al.*, 2003).

Since chlorine reacts with organic matter, components leaching from tissues of cut produce surfaces may neutralize some of the chlorine before it reaches microbial cells, thereby reducing its effectiveness. Additionally, crevices, cracks, and small fissures in produce, along with the hydrophobic nature of the waxy cuticle on the surface of many fruit and vegetables, may prevent chlorine and other sanitizers from reaching the micro-organisms. Surfactants, detergents, and solvents, alone or coupled

with physical manipulation such as brushing, may be used to reduce hydrophobicity or remove part of the wax to increase exposure of micro-organisms to sanitizers. However, such treatments may cause deterioration of sensory quality, thereby limiting their usefulness to applications just prior to consumption (Adams *et al.*, 1989; Zhang & Farber, 1996). The major advantages of chlorine dioxide over HOCl include reduced reactivity with organic matter and greater activity at neutral pH; however, stability of chlorine dioxide is a problem. A maximum of 200 mg l^{-1} of chlorine dioxide is allowed for sanitizing of processing equipment and 3 mg l^{-1} maximum is allowable for contact with whole produce. A 1 mg l^{-1} maximum is permitted for peeled potatoes. Treatment of produce with chlorine dioxide must be followed by a potable water rinse (FDA, 2001; Parish *et al.*, 2003).

Water containing 50-200 mg l^{-1} of chlorine is widely used in food processing plants to sanitize whole fruits and vegetables as well as fresh-cut produce, but this treatment was found to result in a reduction of bacterial populations of less than 2 logs (Singh *et al.*, 2002). Zhang & Farber (1996) reported that chlorine dioxide solution treatment (5 mg l^{-1}, 10 min) at 4 and 22°C resulted in 1.1 and 0.8 log reductions of *L. monocytogenes*, respectively on cut lettuce.

Inclusions of 100 mg l^{-1} of free chlorine in water used to wash lettuce leaves has been reported to reduce populations of aerobic mesophiles by more than 98% as compared to a 93% reduction using tap water without chlorine (Adams *et al.*, 1989). Increasing the washing time in chlorine solution from 5 to 30 minutes did not significantly decrease microbial numbers further, whereas extended washing in tap water resulted in a reduction comparable to that obtained with chlorinated water. Maximum log reduction of *Listeria monocytogenes* on fresh-cut lettuce and cabbage treated with 200 mg l^{-1} of chlorine was reported to be 1.3 to 1.7 logs CFU g^{-1} and 0.9 to 1.2 logs

CFU g^{-1}, respectively (Zhang & Farber, 1996). Dipping Brussels sprouts in water reduced the viable population by 1 log (Beuchat *et al.*, 1998), however Brackett (1987) reported a 2 log reduction with chlorine (200 mg l^{-1}). The efficacy of chlorine in removing *Salmonella* from tomatoes has been studied. Populations on the surface and in the stem core tissue were significantly reduced by dipping tomatoes in solutions containing 60 or 110 mg l^{-1} of chlorine, respectively; however, treatment with 320 mg l^{-1} of chlorine did not result in complete inactivation (Zhuang *et al.*, 1995). The ineffectiveness of 100 mg l^{-1} of chlorine against *Salmonella* inoculated into cracked skin of tomatoes has been demonstrated (Wei *et al.*, 1995, Beuchat *et al.*, 1998). Chlorine in concentrations may cause taste and odour defects in treated products. Due to these problems, there is a great interest in developing alternative sanitizers for washing (Singh *et al.*, 2002).

1.5.2 Novel Alternative Decontamination Strategies

Research and commercial applications have verified that chlorine dioxide, ozone and natural antimicrobial solutions can replace traditional sanitizing agents and provide other benefits (Graham, 1997; Cherry, 1999). Consumer demand for minimally processed foods with reduced use of chemical additives have led to substantial developments in nonthermal food preservation technologies. The FDA (1998) has allowed the use of aqueous chlorine dioxide in washing fruits and vegetables. Ozone has been recently declared Generally Recognized As Safe (GRAS) by an expert panel for use in food processing (Graham, 1997). To date, no single treatment has been found which is capable of completely eliminating all pathogenic bacteria (Singh *et al.*, 2002).

1.5.2.1 Organic Acids

Organic acids are commonly used as antimicrobial acidulants to preserve foods either by direct addition or through microbiological fermentation. Since many pathogens generally cannot grow at pH values much below 4.5, acidification may act to prevent microbial proliferation. Organics acids may also possess bactericidal capabilities. The antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by the dissociation of hydrogen ions from the acid. Many types of produce, especially fruit, naturally possess significant concentrations of organic acids such as acetic, benzoic, citric, malic, sorbic, succinic and tartaric acids, which negatively affect the viability of contaminating bacteria (Parish *et al.*, 2003).

Organic acids have been used traditionally in abattoirs, in animal feedstuffs and in the food and pharmaceutical industries to control pathogens. Recent evidence has suggested that these acids or their salts may be effective in controlling the proliferation of *E. coli* O157:H7 (Jordan *et al.*, 1999). One of the most effective salts against this organism *in vitro* is lactate, and its effectiveness in combination with its wide availability, low cost, and generally "recognized-as-safe" status makes lactate a promising candidate as a control measure for *E. coli* O157:H7 in farm and slaughterhouse environments (McWilliam & Steward, 2002).

Treatment with citric acid in the form of lemon juice has been shown to reduce populations of *Salmonella enterica* serovar Typhi (Fernandez Escartin *et al.*, 1989). Treatment in 5% acetic acid for 30 minutes did not result in any recovery of aerobic bacteria and treatment of whole parsley leaves for 5 minutes at 21 °C with 7.6% acetic acid reduced populations of *Shigella sonnei* more than 7 logs per gram (Wu *et*

al., 2000). Coliforms and fecal coliforms were reduced about 2 and 1 logs per gram respectively, on mixed salad vegetables treated with 1% lactic acid (Torriani *et al.* 1997; Parish *et al.*, 2003).

Acids generally inhibit molecular reactions essential to the micro-organisms by increasing the hydrogen ion concentration, which results in a decrease in internal pH (pH_i). This fall in pH_i is a major cause of growth inhibition by weak acids. The pH of the environment and the dissociation constant (pK_a) of the weak acid determine the proportion of the hydrophobic (undissociated) form in the medium and thus the effectiveness of the weak acid (Vasseur *et al.*, 1999). The strength of an acid is defined by its dissociation constant (pK_a). Thus at a pH between 3 and 6, strong acids will be dissociated whereas weak acids will be undissociated. This latter form is membrane-permeable and thus allows the weak acid to enter the microbial cell. Once inside the cell, weak acids which encounter a higher pH due to the cell buffers, dissociate and become toxic, which ultimately inhibits cell growth due to the acidification of the cell interior. Therefore the lower the pH value, the greater the proportion of the acid in the undissociated form and thus the greater the antimicrobial effect (Beales, 2004).

An acid pH and the presence of organic acids in certain foods play important roles in both conferring flavor and preventing the growth of contaminating micro-organisms. Among the many food preservatives, weak acid preservatives have several mechanisms of action that adversely affect several systems in an organism and thus exhaust the microbial cells, limiting their ability to grow. However, in the presence of weak acid preservatives, many organisms have been shown to have stress adaptations, which aid microbial survival by inducing a specific pattern of gene expression, which appears to be required for optimal adaptation to weak acid conditions. Most of these

stress protection systems include a mechanism for sustaining cytoplasmic pH (Beales, 2004). The resistance or adaptation of micro-organisms to such conditions may result in microbial spoilage of products and affect food safety and so is clearly of significance to the food industry in relation to survival of pathogens and growth of spoilage organisms in food (Beales, 2004). The tolerance of *E. coli* O157:H7 to acidic pH is well documented (Zhao et *al.*, 1993; Conner & Kotrola, 1995), where the organism survived for 35 days when held at 5°C in mayonnaise based sauces (pH~4.0) (Weagant *et al.*, 1994; Francis *et al.*, 1999).

Lactic and acetic acids (1%) reduced numbers of *Listeria monocytogenes* on lettuce by only 0.5 and 0.2 log cycles respectively, and were therefore not very effective (Zhang & Farber, 1996; Francis *et al.*, 1999). More extensive bactericidal activity was observed when lactic acid was used in combination with hydrogen peroxide, however the sensory quality of lettuce was compromised by the inclusion of lactic acid in the treatment. The treatment of lettuce with hydrogen peroxide with or without lactic acid at elevated temperatures was suggested as a practical approach to inactivating pathogenic bacteria without compromising sensory quality (Lin *et al.*, 2002).

A wide range of organic acids have been tested for their efficiency in the disinfection of RTE vegetables, such as acetic, lactic, citric and propionic acids at 300 to 500 mg ml^{-1}. The reduction in total counts was not significantly different from that obtained with water. Shapiro & Holder (1960) obtained similar results with citric acid at concentrations up to 1500 mg l^{-1}: bacterial growth during 4 days of storage at 10°C did not differ from that observed on controls. Tartaric acid at 1500 mg l^{-1} reduced total counts tenfold and the differences with controls were maintained during 4 days of storage at 10°C. The reduction in total counts obtained with ascorbic acid, sorbate,

or a combination of both compounds on mixed salads, was always below 1 log cycle, and no differences were noted after 10 days of storage at 4.4°C. The authors concluded that such treatments were ineffective (Nguyen-the & Carlin, 1994).

Karapinar & Gonul (1992) succeeded in reducing the counts of *Yersinia enterolitica* inoculated on parsley leaves from 10^7 CFU g^{-1} to below 1 CFU g^{-1} by immersing the leaves in a solution of 2% (v/v) acetic acid or 40% (v/v) vinegar for 15 minutes. The author did not describe the appearance of the leaves after disinfection (Nguyen-the & Carlin, 1994).

Shapiro & Holder (1960) reported that dipping mixed salad vegetables in a 500 mg l^{-1} citric acid solution reduced the total bacterial numbers substantially compared to dipping in tap water, and that the optimum concentration for maximum growth restriction of representative isolates from vegetables was 1000 mg l^{-1}. The numbers of background microflora on shredded lettuce dipped in citric acid (1%) were significantly lower than those on lettuce washed in water throughout a storage period of 14 days at both 3 and 8°C (Francis & O'Beirne, 1997). By contrast, Adams *et al.*, (1989) found that the reduction in total counts on minimally processed lettuce achieved using 300 to 500 mg l^{-1} of acetic, citric, lactic or propionic acids was not significantly different from the reduction obtained using tap-water.

Calcium lactate is a natural product that gives good sensorial results, however it is expensive. Other organic acid such as citric and acetic acid are less expensive. An organoleptic issue with the use of citric acid is that it alters the taste. Acetic acid also has organoleptic limitations in that it deteriorates flavour, odour and sensorial qualities of the product.

1.5.2.2 Calcium Lactate

Shelf life extension of fresh-cut products is relevant for the industry because of its economic impact. It is important that treatments applied to fresh-cut fruits help maintain their appearance (i. e. color, integrity, absence of excessive drippings in the package), as this is the first characteristic a consumer perceives as quality of the product. However, parallel to maintaining adequate appearance and texture of the product, potential treatments should not negatively affect fresh-cut product flavour or jeopardize microbiological safety (Luna-Guzman & Barett, 2000).

Luna-Guzman & Barett (2000) found that calcium lactate treated samples of cantaloupe at any of the assayed concentrations were less bitter than untreated samples, and only 1% calcium lactate samples were firmer than just-cut samples ($P < 0.07$). Additionally, the bitterness of heat treated samples (1% calcium lactate at 60°C) was not significantly different from either 1% calcium lactate at 25°C or controls. These results indicate that 1% dips using the calcium salt maintain the firmness of fresh-cut cantaloupes. Neither bitterness nor firmness increased with increased dip temperatures.

Calcification of diced tomatoes increased the firmness in all samples. Calcium lactate had the greatest impact on firmness, increasing firmness by 48% as compared with the control. Irradiation at 1.25 kGy decreased firmness in all samples with the greatest loss in the control (35%), whereas, 1% $CaCl_2$ and 2% calcium lactate remained significantly firmer then the control following irradiation. After 8 days of storage at 4°C, diced tomatoes dipped in 1 or 2% calcium salts maintained firmness, while the 0.2% $CaCl_2$ and control samples were considerably softer (Magee *et al.*, 2002).

The essential role of Ca^{2+} in delaying plant senescence is largely associated with its stabilizing influence on cell membranes (Ferguson, 1984), and the induction of membrane lipid catabolism is a characteristic feature of wounded, quiescent plant storage organs and of fresh-cut products. Accordingly, the use of Ca^{2+} has been suggested as a way to delay senescence in fresh-cut tissues. The effect of Ca^{2+} involves deferral of senescence-related membrane lipid changes. Because of the fundamental importance of membrane function in senescing tissues and the growing interest in fresh-cut and table-ready fruits and vegetables, this area of postharvest biology is in need of greater attention (Picchioni *et al.*, 1996).

Calcium maintains the cell wall structure of vegetables by interacting with pectin to form calcium pectate and it is reported to maintain firmness by crosslinking with cells wall and middle lamella pectins (Grant *et al.*, 1973). Thus, fruit and vegetables treated with calcium are generally firmer than controls during storage (Camire *et al.*, 1994, Luna-Guzman *et al.*, 1999). Calcium lactate (0.5-2%) has been used as a firming agent for fruits such as cantaloupes, strawberry and others (Main *et al.*, 1986). It has been reported to be a good alternative to calcium chloride because it avoids the bitterness or off-flavours associated with this salt (Luna-Guzman & Barrett, 2000). The effects of calcium lactate washing solutions on natural microflora have been described for treatment of honey-dew melon and minimally processed vegetables (Saftner *et al.*, 2003; Martin-Diana *et al.*, 2004).

Calcium is an essential mineral for the body and is one of the most popular dietary supplements in the U. S. While sufficient calcium can be obtained exclusively from food (from dairy products and other foods, including leafy vegetables), many people do not get the recommended daily amount of calcium from their diet (Consumerlab, 2003).

Calcium is critical for building and maintaining strong bones and teeth, where 99% of the mineral found in the body. The rest is present in the blood, extracellular fluid, muscle and other tissues, where it plays a role in contraction and vasodilatation, muscle contraction, nerve transmission and glandular secretions (Consumerlab, 2003).

Calcium is a mineral that is found naturally in foods. It is necessary for many normal functions of the body, especially bone formation and maintenance. It can also bind to other minerals (such as phosphate) and aid in their removal from the body. Calcium lactate is used to prevent and to treat calcium deficiencies (Yale New Haven Health, 2003).

1.5.2.3 Ozone

Ozone is one of the most potent sanitisers known. Excess ozone auto-decomposes rapidly to produce oxygen, and thus it leaves no residues in food. The sanitiser is active against all forms of micro-organism at relatively low concentrations. Because of this, the technology is attracting the attention of the food industry. Khadre *et al.* (2001) reported the need by food processors for relevant and concise information about this sanitiser.

Ozone (O_3) results from the rearrangement of atoms when oxygen molecules are subjected to high-voltage electric discharge. The product is a bluish gas with pungent odour and strong oxidizing properties (Khadre *et al.*, 2001). The gas does not appreciably react with water; therefore it forms a true physical solution. Solubility of gases can be compared if their â values are known. Solubility in water is greater for ozone than for nitrogen and oxygen; â values are 0.64, 0.0235, and 0.049,

respectively. Ozone, however, is less soluble in water than carbon dioxide (β=4.54) (Khadre *et al.*, 2001).

Dissolution of ozone in water can also be expressed in a more practical term, the solubility ratio (Sr), where:

Sr =mg l^{-1} O_3 in water/mg l^{-1} O_3 in the gas phase

Solubility ratio for ozone increases as the temperature of water decreases (Bablon *et al.*, 1991). In addition to pressure and temperature, which directly affect the solubility, other parameters practically influence the dissolution of ozone in water. When a solution is prepared by bubbling ozone in water, smaller bubble sizes result in larger surface area of contact which increases the solubility, and optimum dissolution of ozone in water occurs when bubbles are 1 to 3 mm in diameter (Khadre *et al.*, 2001). Purity and pH of water greatly affect the rate of ozone solubilisation. Kim (1999) found that ozone gas dissolved faster in deionised and distilled water than in tap water. The high pH of tap water may have destabilized ozone, and thus the apparent rate of solubilisation decreased. In addition, tap water may contain organic matter that consumes ozone. Presence of minerals in water may also catalyze ozone decomposition (Hoigne & Bader, 1985). Therefore, solubility of ozone increases when purity of water increases and the half-life of ozone in distilled water at 20°C is generally considered to be 20 to 30 minutes (Khadre *et al.*, 2001). Kim (1999) reported that the stability of ozone in solution was greatest at pH 5.0. Ozone stability decreased as pH increased, and no ozone was detected in buffers with pH 9.0.

The ozone molecule acts as dipole with electrophilic and nucleophilic properties. Organic and inorganic compounds in aqueous solutions react with ozone in one of two pathways (Staehelin & Hoigne 1985):

(a) Direct reaction of organic compound (M) with molecular ozone.

$$O3 + M \longrightarrow Mox \text{ (Oxidated organic compound)}$$

(b) Decomposition of ozone in water into a radical (for example, OH) which reacts with the compound (M).

$$O3 \xrightarrow{OH-} OH \xrightarrow{M} Mox$$

Molecular ozone reactions are selective and limited to unsaturated aromatic and aliphatic compounds (Khadre *et al.*, 2001).

Bacteria studies show that 0.12-to 3.8 mg l^{-1} aqueous ozone inactivated Gram-positive bacteria by 1 to 7 logs. When Gram-positive bacteria were treated with 0.004-to 6.5 mg l^{-1} aqueous ozone, their population decreased by 0.5 to 6.5 logs (Khadre *et al.*, 2001).

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, peptidoglycan in cell envelopes, enzymes and nucleic acids in the cytoplasm, and proteins and peptidoglycan is spore coats and virus capsids. There are two theories for the anti-microbial effect of ozone; molecular ozone as the main inactivator of micro-organisms, or the antimicrobial activity of the reactive by-products of ozone decomposition such as $^{\cdot}OH$, $^{\cdot}O^{2-}$, and $HO^{\cdot 3}$ (Hunt & Marinas, 1997; Khadre *et al.*, 2001).

Restaino *et al.* (1995) investigated the antimicrobial effects of ozonated water against food related micro-organisms and determined that ozone effectively killed such Gram-positive bacteria as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis*, and such Gram-negative bateria as *Pseudomonas aeruginosa* and *Yersinia enterocolitica.* Treating fruits and vegetables with ozone has been used to increase shelf-life. Onions have been treated with ozone during storage.

Mold and bacterial counts were greatly decreased without any charge in chemical composition and sensory quality. Treatment of shredded lettuce in water bubbled with ozone gas decreased bacterial content (Kim *et al.*, 1999; Guzel-Seydim, *et al.*, 2003). Currently, ozone is the most likely alternative to chlorine and hydrogen peroxide in food applications, however further research is still needed to explore new applications for ozone and to best utilize the unique features of this sanitizer (Khadre *et al.*, 2001). The main advantages and disadvantages of ozone are shown in Table 1. 3.

Ozone must be produced on-site, and the initial cost of ozone generators may be of concern to small processors; however, long-term application may justify these costs (Khadre *et al.*, 2001).

Table 1.3 Advantages and limitations of ozone (Adapted from Parish *et al.*, 2003).

Advantages	**Disavantages**
Effective at low concentrations	Physiological injury of produce possible
Effective at short contact time	Corrosive to equipment
Broad spectrum	Deterioration of produce flavor and color
Good penetration ability	Unstable; very highly reactive
Effectiveness against protozoa	Possible human toxic effects in processing facilities
Decomposes to nontoxic products	
Low cost	
Good for the environment	

1.6 Microbiological Safety Issues

Vegetables and fruits have been associated with outbreaks of foodborne disease in many countries. Organisms involved include bacteria, viruses and parasites (De Rover, 1998) These outbreaks vary in size from a few persons affected to many thousands. However, they represent only a small proportion of the total number of cases reported. For example, in the U. S. between 1993 and 1997 fruits and vegetables were associated with only 1.4% to 3% of outbreaks. However, according to the Center for Disease Control and Prevention, the number of produce-associated outbreaks per year in US doubled between the periods 1973-1987 and 1988-1992 (Olsen *et al.*, 2000; European Commission, 2002)

The world's largest reported vegetable borne outbreak to date occurred in Japan in 1996 and of the over 11,000 people affected, about 6,000 were culture confirmed. The outbreak involved the death of three school children and was caused by *E. coli* O157:H7 (European Commission, 2002). Although the incidence of foodborne illness linked to fresh produce is still low, there is evidence that it is increasing. Fresh produce is of special concern because it is likely to be consumed raw, without any type of microbiologically lethal processing. Verocytotoxin producing *Escherichia coli* (VTEC), of which *E. coli* O157:H7 is the most common member, has emerged over the last decade as a serious global public health concern. Since 1982 there have been notable outbreaks in Japan and the Unites States.

1.6.1 Causes of Contamination and Outbreaks

Fruits and vegetables can become contaminated whilst growing in the fields, or during harvest, handling, processing, distribution and use (Beuchat, 1995). At the

retail stage any microbial contamination present is likely to reflect the environment through which the product has passed. The extent of microbial contamination of vegetables and the impact of this contamination on consumer health need further examination (European Commission, 2002). Outbreaks may be related to cross-contamination between animal effluents (such as runoff from manure piles) and the growing fields, even if manure was not directly applied to the crop (European Commission, 2002).

Table 1.4 shows the characteristics of some pathogens with regard to RTE Vegetables. Fruit juices, and minimally processed fruits and vegetables have also been involved in foodborne outbreaks (European Commission, 2002). Soil is a reservoir of foodborne pathogens, such as *Bacillus cereus, Clostridium botulinum*, and *Clostridium perfringens. Listeria monocytogenes* has been isolated from non-cultivated soil (Welshimer & Donker-Voet, 1971). Pathogenic organisms from the human/ animal reservoir can be found in soil due to irrigation and fertilization with manure and sludge or due to droppings of animals in the farming area. Water is mainly used for irrigation of plants and its quality will vary depending on whether it is surface water or potable water. Water may be a source of contaminating micro-organisms. Surface water from streams and lakes may be contaminated with pathogenic protozoa, bacteria and viruses. The occurrence of *Listeria monocytogenes, Salmonella* and viruses has been reported in surface waters (Nguyen-the & Carlin, 1994).

The transfer of foodborne pathogenic micro-organisms from irrigation water to fruits and vegetables will depend on the irrigation technique and on the nature of the produce (NACMCF, 1999). Spray irrigation would be expected to increase the risk of contamination in comparison to drip irrigation or flooding. Leafy vegetables provide

large surfaces for contact with water and for the attachment of micro-organisms (European Commission, 2002).

Table 1.4 Characteristics of some microbial pathogens that have been linked to outbreaks of produce-associated illness (adapted from FDA, 2001)

Micro-organisms	Typical Incubation Period	Infectious Dose (Number of eells)	Source	Symptoms
BACTERIA				
Clostridium Botulinum	12 to 36 h	intoxication growth and toxin production in food	soil, lakes, streams, decaying Vegetation, reptiles.	Nausea, vomiting, fatigue, dizziness, Dyness of mouth and throat, muscle Paralysis, difficulty swallowing, double or blurred vision, drooping eyelids, and breathing difficulties
Escherichia coli O157:H7	2 to 5 d	10 to 1000	Animal feces, especially Cattle, deer and human; cross contamination from raw meat.	Bloody diarrhea, abdominal pain. Can lead to hemolytie uremic Syndrome and kidney failure Especially in children and the elderly
Salmonella spp.	18 to 72 h	10 to 100,000	Animal and human feces; Cross contamination from raw meat, poultry, or eggs	Abdominal pain, diarrhea, chills, Fever, nausea, vomiting
Shigella spp	1 to 3 d	~10	Human feces	Abdominal pain, diarrhea, fever, vomiting
Listeria monocytogenes	1 d to 5 or more wk	unknown dependent upon health of individual	soil, food processing environments	Febrile gastroenteritis in healthy adults; may lead to spontaneous abortion or stillbirth in pregnant women; severe septicaemia and meningitis in neonates and immunocompromised adults, mortality may be 20 to 40%
PARASITES				
Cryptoporidium Spp.	1 to 12 d	~30	Animal and human feces	Profuse watery diarrhea, abdominal pain, anorexia, vomiting
Cyclospora spp.	1 to 11 d	unknown, probably low	others? Specific environmental sources unknown at this time	Watery diarrhea, nausea, Anorexia, abdominal cramps (duration 7 to 40 d)
VIRUSES				
Hepatitis A	25 to 30 d	10 to 50	human feces and urine	Fever, malaise, anorexia, nausea, abdominal pain, jaundice, dark urine
Norwalk/ Norwalk-like Virus	12 to 48 h	unknown, probably low	human feces, vomitus	Vomiting diarrhea, malaise, fever, Nausea, abdominal cramps

An increasing number of VTEC outbreaks have recently been associated with the consumption of fresh fruit and vegetables. These have raised concerns regarding the safety of such foods. While there have been no reported cases of VTEC associated with fruit and vegetables in Ireland, results of investigations of outbreaks elsewhere suggest that these are attributed to changes in production practices, food preparation and consumption patterns. The principal sources of VTEC contamination are most likely to be animal manure, used as a crop fertilizer, or contaminated water used for growing or washing produce. Other sources may include the entry of livestock or wildlife into fields of fruit or vegetable crops near harvest time (FSAI, 1999). VTEC can survive and multiply on common salad vegetables at room temperature without causing any adverse change in visual appearance, but gradually decline in numbers if the salad vegetables are kept at 5°C. Research has also shown that VTEC strains can penetrate damaged lettuce leaves at cut edges. It is thought that once embedded in tissue, the organisms are protected from sanitizing chemicals (e. g. chlorine) that have little penetration power. The safety, therefore, of lettuce and, perhaps, other leafy produce may be enhanced by the packer discarding damaged leaves and exposing undamaged leaves to effective levels of sanitizer before cutting (FSAI, 1999)

Refrigerated temperatures cannot be relied upon to prevent growth of pathogens micro-organisms on produce. Populations of *Listeria monocytogenes* remained constant or grew on a variety of whole and cut produce stored at refrigerated temperatures (Farber *et al.*, 1998). Under certain chilled storage conditions, spoilage of the product by the native microflora might not occur until after pathogen populations reach levels capable of causing disease. Austin *et al.* (1998) reported toxin production by *Clostridium botulinum* on unspoiled onions and butternut squash stored under modified atmosphere at 15°C. Piagentini *et al.* (1997) reported that

Salmonella enterica serovar Hadar could survive and proliferate on chilled shredded cabbage prior to detection of spoilage. While growth of some pathogens may be inhibited by chilled temperatures, survival can be enhanced under certain conditions. For example, *Salmonella* spp. and *E. coli* O157:H7 survive for a longer time period in fruit juices under refrigeration than at room temperature (Parish *et al.*, 1997; Zhao *et al.*, 1993; FDA, 2001).

The susceptibility of vegetable processing and storage procedures to microbial contamination and temperature abuse is well recognised (Francis *et al.*, 1999). The possibility of contamination with psychrotrophic foodborne pathogens (e. g. *Listeria monocytogenes* & *Aeromonas caviae*) is a recognized concern (Jacxsens *et al.*, 1999; Szabo *et al.*, 2000). Given enough time, psychrotrophic pathogens can grow to high populations in packaged produce, even if proper temperatures are maintained (Jacxsens, 2002).

1.6.2 Prevention

The safety of food must be assured by a preventative approach based on the application of a food safety management system, i.e. Hazard Analysis Critical Control Points (HACCP) at all stages in the food supply system.

Good Hygienic Practice (GHP) as defined in the Codex document on "General Principles of Food Hygiene" in combination with HACCP is the basis for safe food production (Codex Alimentarius, 1997). The application of HACCP for safety of fruits and vegetables is considered to be problematical, particularly the identification of robust Critical Control Points (CCP) for the destruction of pathogens and record keeping for measures taken at the CCP. Effective HACCP systems involve a

systematic approach to identification, evaluation and control of food safety hazards in a food operation (European Commission, 2002).

The microbiological safety of fresh, raw, fruits and vegetables is determined by a variety of factors and conditions as already outlined. Plant protection products, washing, irradiation and decontaminants can be applied for improvement of hygiene. Risk management legislation within the EU or its Members States governing the use of such products or processes fall within different areas like environmental protection, food additives and food hygiene (European Commission, 2002).

1.6.3 Increased Consumer Risk

Apart from pre-weaning infants, no group of the population is excluded from eating raw vegetables, sprouted seeds and fruits, etc. The young, the old, the pregnant and immunocompromised consumers potentially pose higher risks. A particular concern relates to infection of young children with *E. coli* O157:H7 and the potential for these infections to progress to Haemolytic Uraemic Syndrome (HUS) (Parry and Palmer, 2000). The very young, the very old and the immuno-suppressed are more susceptible to VTEC infection than are other groups. Furthermore, the symptoms resulting from the infection are more severe amongst these groups. Haemorrhagic colitis, leading to bloody diarrhea, can be a serious and life threatening illness in elderly patients. In children, there is a higher risk of HUS, which can lead to kidney failure (FSAI, 1999)

1.7 Modeling of microbial growth and survival

Modeling the growth and survival of food poisoning or spoilage organisms is a basic tool for the prediction of food safety and/or microbial deterioration of food products during processing and storage (McMeekin *et al.*, 1993; McClure *et al.*, 1994). Accurate predictions provide valuable data on the rate of deterioration of foods, enabling manufacturers to reduce the amount of experimental work that is required to ensure product safety and assign adequate shelf life. In addition, more realistic shelf lives can be applied to produce with higher profits for the manufacturer, less wastage and, in turn, cheaper food for the consumer (Bovill *et al.*, 2000).

Temperature is a major factor responsible for microbial spoilage. Both the growth rate and lag phase are highly temperature dependent. Thus, the effect of temperature on microbial stability has been widely studied (Zwietering *et al.*, 1994; McMeekin *et al.,* 1992, Buchanan 1993; Almonacid-Merino and Torres, 1993).

Both primary and secondary models have been used in predictive microbiology. Primary models describe the changes in microbial population as a function of time such as the sigmoid Gompertz function, which is considered to be the most popular to fit sigmoid bacterial growth curves. Secondary models describe the responses by parameters of primary models to changes in environmental conditions such as temperature, pH and water activity (Whiting, 1995). Examples of such models include Arrhenius and the square-root model which are considered to be the major classes of secondary models that have been proposed to describe the effect of temperature on microbial growth.

Tertiary models express secondary model predictions in a primary model using spreadsheet and computer software. In the 90's, two major datasets were generated on bacterial responses to food environments: one was the basis of Food MicroModel

(FMM), a commercial package supported by MAFF (Ministry of Agriculture Fisheries and Food) and subsequently FSA (Food Standards Agency) in the UK; the other was behind the Pathogen Modeling Program (PMP) of the Eastern Regional Research Center (ERRC), Agricultural Research Service (ARS), US Department of Agriculture (USDA).

The FMM and PMP datasets have been supplemented with additional information submitted by members of the ERRC, ARS, USDA Center of Excellence in Microbial Modeling and Informatics (CEMMI) and by that compiled from the scientific literature by the Institute of Food Research (IFR), Norwich, UK, through the support of the UK Food Standards Agency. Ultimately, all these data sets have now been unified in a common database known as ComBase, following a structure developed by the IFR.

1.8 Guidelines for the Microbiological Quality of RTE Food

A microbiological guideline is a criterion, which relates to the microbiological condition of the food sample that is applied at any stage of food processing and retailing. It aids in identifying situations requiring attention for food safety or quality reasons. Guidelines arise from many sources; the food industry, enforcement agencies and national and international committees, and are especially applied to indicator organisms. While guidelines may be written in law they are not legally enforceable, but serve to provide assistance to enforcement agencies in interpreting whether producers are complying with the general policy in relation to standards (Codex Alimentarius, 1997).

Tables 1.5 and 1.6 show the number of bacteria allowed in RTE vegetable products (Guidelines for the interpretation of results of microbiological analysis of some Ready-to-eat foods sampled at point of sale, Food Safety Authority Ireland, 2001).

Table 1.5 Guidelines for the interpretation of results of microbiological analysis of some relevant Ready-to-Eat food samples at the point of sale (Adapted from FSAI, 2001b)

Food category (see Table 1. 6)	Criterion	Microbiological quality (CFU per gram unless stated)			
		Satisfactory	Acceptable	Unsatisfactory	Unacceptable/ potentially hazardous
	Aerobic colony count 30°C/48 h				
3		$<10^5$	$10^5<10^6$	$\geq10^6$	N/A
4		$<10^6$	$10^6<10^7$	$\geq10^7$	N/A
5		N/A	N/A	N/A	N/A
	Indicator organisms		100<10^4	$\geq10^4$	N/A
3-5	Enterobacteriaceae	<100	20<100	≥100	N/A
3-5	*E. coli* (total)	<20	20<100	≥100	N/A
3-5	*Listeria* spp. (total)	<20			not detected in 25 g
	Pathogens				not detected in 25 g
3-5	*Salmonella* spp.	not deteeted in 25 g			not detected in 25 g
3-5	*Campylobacter* spp	not detected in 25 g	20<100		not detected in 25 g
3-5	*E. coli* O157 & other VTEC	not detected in 25 g	20<100		$\geq10^3$
3-5	*V. cholerae*	not detected in 25 g	20<100	$100<10^3$	≥100
3-5	*V. parahaemolyticus*	<20	20<100	N/A	$\geq10^4$
3-5	*L. monocytogenes*	<20**		$100<10^4$	$\geq10^4$
3-5	*S. aureus*	<20	$10^3<10^4$	$100<10^4$	
3-5	*C. perfingens*	<20			$\geq10^5$
3-5	*B. cereus* and other pathogenic			$10^4<10^5$	
	Bacillus spp.	$<10^3$			

Footnote: Foods contained in each of these categories are listed in Table 1.6.

Table 1.6 Colony count categories for relevant types of Ready-to-Eat foods (Adapted from FSAI, 2001b)

Food group	Product	Category
Vegetable	coleslaw	3
	fruit and vegetables (fresh)	5
	prepared mixed salad and crudités	4

1.9 Research Needs

The primary method to eliminate, or significantly reduce, pathogens on produce is strict adherence to Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), HACCP, and other relevant strategies that prevent contamination from occurring. Although the frequency of produce contamination by pathogens is thought to be very small, there are no known mitigation strategies that will completely remove pathogens after contamination has occurred while maintaining produce freshness. A variety of mitigation regimens and sanitisers are available to reduce microbial populations depending upon the type of several factors, including characteristics of the produce surface, water quality, cleaner/sanitiser used, contact time, and presence and type of scrubbing action. Based on reported data, it is likely that different sanitation strategies are needed for different produce items (Parish *et al.*, 2003).

Several gaps exist in our understanding of the effects of minimal processing on the microbiological safety of vegetables. The work that follows tries to address these gaps, through investigating the effects of a range of alternative anti-microbial dipping treatments and some temperature abuse conditions on shredded lettuce.

1.10 Aims and Objectives of this Study

The aim of this study was to examine the effects of a number of decontamination treatments as alternatives to chlorine principally against the natural microflora of RTE Iceberg lettuce.

Specifically the objectives of the study were as follows:

- To compare ozone and chlorine as decontamination treatments on the natural microflora of RTE Iceberg lettuce;
- To examine the effects of a combination method of ozone plus calcium lactate as a decontaminant of RTE Iceberg lettuce in comparison with chlorine;
- To determine, in comparison with a standard chlorine treatment, the effects of different concentrations of calcium lactate used at various wash temperatures on the natural microflora of RTE Iceberg lettuce;
- To determine the effects of organic acids as decontaminants for RTE Iceberg lettuce.
- To investigate and compare the effects of calcium lactate and chlorine on the lag phase of challenge populations of *Escherichia coli* in RTE Iceberg lettuce stored at different temperatures (4°C and 10°C) over a 10-day storage period.

CHAPTER 2: Materials and Methods

2.1 Materials and Equipment

Absolute alcohol.

Acetic acid (glacial), 100%, 17.5 M.

Anaerobic Gas Jar and GasPak System: BBL

Anaerobic indicator Anaerotest: Merck

Anhydrous citric acid: SIGMA

Autoclave: Tomy SS-325.

Autoclave bags: Greiner-Labortechnik.

Autovortex mixer: Stuart Scientific.

Balance: Sartorius, GMBH.

Bromocresol purple, pH=5.2-6.8, yellow to violet: BDH indicators.

Buchner conical flask: Millipore 1000 ml.

Buffer solutions pH 4.0 and 7.0: Fisher Scientific.

Calcium lactate: SIGMA (C-8356).

Catalase test reagent: Biomerieux.

Centrifuge: Sigma 2K15

Cooled incubator: LMS

Cover slips and microscope slides: Mendez-Glazer.

Crystal violet.

Escherichia coli ATCC 25922: Oxoid Culti-loop.

Gas analyzer: Hictech Instruments, MAPTEST 4000.

Gas generating kit, Anaerocult A: Merck Microbiology.

Gram's iodine.

Hypodermic needle: SRP-25, 0.45μm.

Iceberg lettuce: purchased from Dunnes Stores Ltd. on day of first analysis.

Incubators: Gallenkamp.

Lactose broth: Oxoid Code CM137.

Laminar air flow cabinet: Faster BHA 48.

Microbiological media: Table 2.1.

Micropippettes: Gilson (200, 1000 and 5000 μliter).

Oak Ridge centrifuge tubes. PPCO: Nalgene.

Olympus microscope: CHK-G

Oxidase test: Oxoid.

Ozone generator: Ozone Water System, Model HV-103, Analytical Technology, Inc., connected to a dissolved ozone monitor (Model C15-64, Analytical Technology, Inc.).

Packaging film: Micro-perforated orientated polypropylene (OPP) film 35PA Plain 420 nm.

Packaging film sealer: Packer Impulse Sealer.

pH meter: Orion Model 420A.

Pipette tips: Diamond D1000, D2000 and D5000, Tipack: Gilson

Plastic cuvettes (1 ml)

Safranin.

Salad shaker: Dynamic. Model EM98.

Sodium hypochlorite (14% chlorine): Aldrich.

Spectrophotometer: Thermo Spectronic, Genesys 20.

Spiral platter: Autoplate 4000, Spiral Biotech.

Sterile peptone water, International Diagnostic Group.

Stirrer hot plate: Heidolph Intruments MR 300 IK.

Stomacher Filter Bags: Seward Lab System. Model 400

Stomacher laboratory blender: Seward Stomacher Model 400.

Tryptone Soya Broth: International Diagnostic Group.

Vacuum pump: Millipore.

Water activity analyzer: Aqualab CX3. Decagon devices.

Water bath: Grant W22.

Water purification system: Millipore, Mill-u10

Yeast Extract: International Diagnostic Group.

2.1.2 Microbiological Media

The isolation and/or plating media used are shown in Table 2.1.

Table 2.1: Isolation and/or plating media used for target microbial populations

Target population	**Medium**
Total Viable Count (TVC) Mesophiles Psychrophiles	Plate Count Agar (PCA) International Diagnostic Group.
Lactic Acid Bacteria (LAB)	de Man, Rogosa and Sharpe Agar (MRS), pH adjusted to pH 5.8 using lactic acid International Diagnostic Group.
Pseudomonads	Pseudomonas Agar base + C F C Supplement OXOID
Coliforms	Violet Red Bile Agar (VRBA) International Diagnostic Group.
Yeast and Moulds	Malt Extract Agar (MEA) International Diagnostic Group.
E. coli	Sorbitol McConkey Agar International Diagnostic Group.

2. 2 Methods

2.2.1 Lettuce Samples

For these experiments samples of Iceberg lettuce were obtained from a reputable supplier (Dunnes Stores Ltd., Talbot Street, Dublin 1) and were immediately subjected to anti-microbial dip treatment.

2.2.2 Anti-microbial Dip Treatments

Six anti-microbial dipping treatments were prepared:

- Chlorine solution (100 mg l^{-1});
- Calcium lactate solutions (0.5, 1.5, 2.5 and 3%, w/v);
- Ozone solution (1 mg l^{-1});
- Ozone/calcium lactate combination (1 mg l^{-1} + 2.5% , w/v, calcium lactate);
- Citric acid solution (2%, w/v);
- Acetic acid solution (1%, v/v).

The following control samples were included in each experiment:

- water washed lettuce samples;
- unwashed lettuce samples.

2.2.2.1 Preparation of Anti-microbial Dipping Solutions

Chlorine dip

A 100 mg l^{-1} chlorine solution was prepared by adding 1 ml of sodium hypochlorite (14% chlorine, Aldrich) to 1 litre of sterile deionised water.

Calcium lactate dip

Calcium lactate solutions of 0.5%, 1.5%, 2.5% and 3% were prepared by adding 5, 15, 25 and 30 grams respectively of calcium lactate to 1 litre of sterile deionised water.

Ozone dip

A 1 mg l^{-1} ozone solution was prepared using an ozone generator that in approximately one hour after switching on, reaches a level of concentration of 1 mg l^{-1} The generator was connected to a dissolved ozone monitor which allowed the ozone concentration to be monitored.

Ozone/calcium lactate combination dip

A 1 mg l^{-1} ozone solution was prepared as above and a 2.5% calcium lactate solution was prepared by adding 25 gram of calcium lactate per littre of deionised water.

Acetic acid dip

A 1% solution of acetic acid was prepared by adding 10 ml glacial acetic acid to 1 litre of sterile deionised water.

Citric acid dip

A 2% solution of citric acid was prepared by adding 20 grams citric acid to 1 litre of sterile deionised water.

2.2.2.2 Dipping Procedure

One hundred grams of whole cored heads of lettuce were placed in a dipping basket and immersed in 1 litre of dip solution for a period of 1 minute with agitation at room temperature. The water was removed after the treatment by shaking using the salad shaker for 5 minutes.

The combination dip was carried out by placing the sample in the ozone solution for 1 minute, the water was removed by shaking using the salad shaker for 5 minutes, followed by immersion in the calcium lactate solution for 1 minute. The water was removed after the procedure.

2.2.3 Packaging and Storage of Lettuce Samples

Following treatment the lettuce samples were aseptically packaged in 25 gram portions in micro-perforated orientated polypropylene (OPP) film and heat sealed to form sealed bags. The bags were stored at 4°C in a cooled incubator for up to 10 days. Bags were removed for microbiological analysis of indigenous microflora on Days 1, 3, 7 and 10. Experiments were replicated three times.

2.2.4 Microbiological Analysis

2.2.4.1 Homogenization and Dilution of Samples

All of a twenty five gram bagged sample of treated or untreated lettuce was aseptically transferred to a Stomacher bag. Sterile peptone water (225ml) was added and the sample was homogenized for 2 minutes using the Stomacher. Further decimal dilutions of the homogenate (10^{-1} dilution) sample were prepared using 9 ml volumes of sterile peptone water.

2.2.4.2 Plating Methods and Incubation

Aliquots of appropriate dilutions were plated using either the spiral platter (2 replicates of 3 dilutions) or pour plate method (3 replicates of 3 dilutions) onto appropriate media and incubated as shown in Table 2.2. The overlay plate method was used for the coliforms counts, i. e. the 1 ml of the food suspension was plated followed by molten agar. When this layer had set, an additional thin layer of VRBA was added to prevent spreading of surface colonies, thus facilitating colony counting. Anaerobic conditions for the LAB were provided using an anaerobic gas jar with an anaerobic GasPak generator; the anaerobic conditions were checked using an anaerobic indicator.

Table 2.2: Plating method, volumes plated and incubation conditions for media used to quantify the target microbial populations

Target Population	**Media**	**Plating Method and volumes plated**	**Incubation Time/Temperature**
TVC Mesophiles TVC Psychrophiles	PCA	Spiral plate method: 0.05 ml	30°C for 24 h 4°C for 7 days
Lactic Acid Bacteria (LAB)	MRS Agar	Spiral plate method: 0.05 ml	30°C for 48 h
Pseudomonads	*Pseudomonas* Agar	Spiral plate method: 0.05 ml	30°C for 24 h
Coliforms	VRBA	Pour overlay plate: 1 ml	37°C for 24 h
Yeast and Moulds	MEA	Spiral plate method: 0.05 ml	30°C for 24 h

2.2.4.3 Enumeration of Colonies on Spiral Plates

The spiral plater involves a microprocessor-controlled dispenser that deposits a liquid sample in a spiral pattern on the surface of a rotating agar plate. The dispensing stylus deposits precisely controlled amounts of sample as it moves outward from the near-center of the plate. Because of this unique plating action, microbial suspensions (from approximately 80 to 4.0 x 10^5 CFU ml^{-1} on 100mm plates) that might normally require several dilutions prior to plating can be plated directly.

Automated cleaning assures reliable disinfection of the stylus and syringe. Disinfectant and water, held at recommended levels in large reservoirs, assure the required depth of stylus immersion during programmed cycles of alternative liquid/air intake.

Colonies in the outer region of the plate where the colonies were well separated were counted and the count was divided by the volume of sample deposited in the counted regions in order to calculate CFU g^{-1} (Spiral Biotech Inc., Autoplate manual).

2.2.4.4 Enumeration of Colonies on Pour Plates

Plates chosen for counting ideally had between 30 and 300 colonies. The CFU g^{-1} was calculated by multiplying the average of the 3 replicates plate counts by the inverse of the dilution factor.

2.2.5 Gas Analysis of Package Atmospheres

The atmospheric composition of the sealed packages was analysed using a Gas-space gas analyzer (MAPTEST 4000), which utilizes a zirconium oven and an infrared detector to quantify O_2 and CO_2 levels respectively. Each package was pierced with a hypodermic needle. Gas was extracted via the airtight syringe through the flexible probe loop from the OPP bags into the analyzer.

2.2.6 Measurement Lettuce Samples pH

The pH of uninoculated packaged lettuce samples was measured by blending 25 grams with 50 ml of distilled water followed by the immersion of the pH electrode (Orion. Model 420A). The pH meter was calibrated prior to use on each day of analysis using buffers of pH 4.0 and 7.0.

2.2.7 Measurement of Packaged Samples Water Activity (a_w)

The a_w of the packaged samples was analysed in duplicate using the AquaLab CX3. In order to provide the most accurate readings, the AquaLab was ideally allowed a warm-up period of at least 15 minutes after turning on. This allows the air inside the AquaLab to equilibrate to the temperature of its surroundings. A lettuce verification standard at ambient temperature was chosen. The lettuces samples (a small amount of lettuce leaves), were placed into a sample cup and placed in the AquaLab's sample drawer. The drawer was closed carefully and the drawer knob was turned to the READ position to take a reading (AquaLab manual, Decagon Devices, 2003).

2.2.8 Statistical Analysis of Results

All experiments which investigated the effects of anti-microbial dipping were carried out in triplicate.

Statistical differences were determined using ANOVA followed by LSD tesing at $p < 0.05$ level (SPSS Version 11).

2.2.9 Estimation of Growth Rate and Survival of *Escherichia coli*

The effect of 100 mg l^{-1} chlorine and 2.5% calcium lactate on the survival and growth of *Escherichia coli* was examined.

Each treated sample was compared with an undipped lettuce control and with a sterile water dipped sample. Samples were dipped and shaken as described in 2.2.2.2.

2.2.9.4 Sample Storage of Inoculated Lettuce Samples

The packaged lettuce samples were stored at 4°C and 10°C for 10 days. Sample packages from both storage temperatures were removed for analysis at Days 0, 1, 2. 3, 5, 6, 7 and 10.

2.2.9.5 Homogenization and Dilution of Inoculated Lettuce Samples

Samples were homogenized as described in 2.2.4.1 and aliquots of appropriate dilutions were plated using the spread method (0.1 ml per plate) onto Sorbitol McConkey Agar.

2.2.9.6 Incubation of Plates

All plates were incubated at 37°C for 24h.

2.2.9.7 Confirmation and Counting Procedure

E. coli colonies were confirmed using Gram stain, catalase (Biomerieux) test and oxidase (Oxoid) test. Colonies of *E. coli* were counted on appropriate plates.

2.2.9.8 Statistical Analysis

Experiments on anti-microbial dipping were carried out in duplicate.

Statistical differences were determined using ANOVA followed by LSD tesing at $p < 0.05$ level (SPSS Version 11)

2.2.9.9 Modeling of Shelf-Life

The Gompertz function has become the most widely used primary model for describing microbial growth. The modified Gompertz equation was applied in this study to bacterial counts obtained upon the inoculation of shredded lettuce with *E-coli* ATCC 25922. Three washing treatments were studied: water, chlorine and calcium lactate at ambient temperature. Also, two storage temperatures were compared: 4°C and 10°C. The modified Gompertz model could be described as:

$\text{Log } N_{(t)} = A + C \exp\{-\exp[-B(t - M)]\}$ where,

t = time

$\text{Log } N_{(t)}$ = population density at time t

A = regression constant, log of initial level {Log CFU g^{-1}}

C = Regression constant, difference in log counts between inoculum and stationary phase {Log CFU g^{-1}}

B = Regression constant, relative growth rate at M{Log CFU g^{-1}}

M = Regression constant, time at which the absolute growth is maximal {h}

Fitting of data was performed using nonlinear regression analysis using SigmaPlot 2000 with the Marquardt-Levenberg algorithm; this is a search method for minimizing the sum of squares of the differences between the fitted and the experimental data. The algorithm calculates a set of parameters: regression coefficient

(R^2), residual sum of squares, and the value for the parameters of A, C, B and M. The curve fitter SigmaPlot worked by varying A, C, B and M of the Gompertz equation and found the parameters which caused the Gompertz equation to most closely fit the experimental data.

In this study the obtained Gompertz parameters were used to calculate the lag phase duration as:

Lag phase = M – (1/B)

The values for the lag phase of *E-coli* were compared for the washing treatments and storage temperatures studied.

CHAPTER 3: Effects of calcium lactate, ozone and combination treatments for decontamination of the indigenous microflora of ready-to-eat lettuce

The effects of the novel decontamination treatments: calcium lactate, ozone and a combination of calcium lactate plus ozone were compared with chlorine and water for effects on the indigenous microflora of RTE lettuce. Anti-microbial dipping had significant effects on the population densities of mesophiles, psychrophiles, pseudomonads, yeasts and coliforms.

3.1 Results

The most effective decontamination treatment for reducing the number of mesophiles in prepared lettuce was 100 mg l^{-1} chlorine (Fig. 3.1–a). Initial total aerobic mesophile counts on unwashed lettuce were 2.05 x 10^6 CFU g^{-1}. Following treatment with chlorine, numbers were significantly reduced to 8.13 x 10^4 CFU g^{-1} ($p < 0.05$). This effect persisted until Day 3 of storage at 4°C where populations were still over 1 log cycle lower on chlorine treated lettuce than on unwashed lettuce ($p < 0.05$). Treatment with ozone alone or in combination with calcium lactate resulted in a similar reduction to chlorine for total aerobic mesophiles. At Day 3, counts on unwashed lettuce were higher by one log cycle ($p < 0.05$), than those observed on lettuce treated with ozone alone or in combination with calcium lactate. Treatment with water or calcium lactate alone reduced mesophile populations compared with unwashed lettuce, but not significantly. By Day 7 there was no difference in the

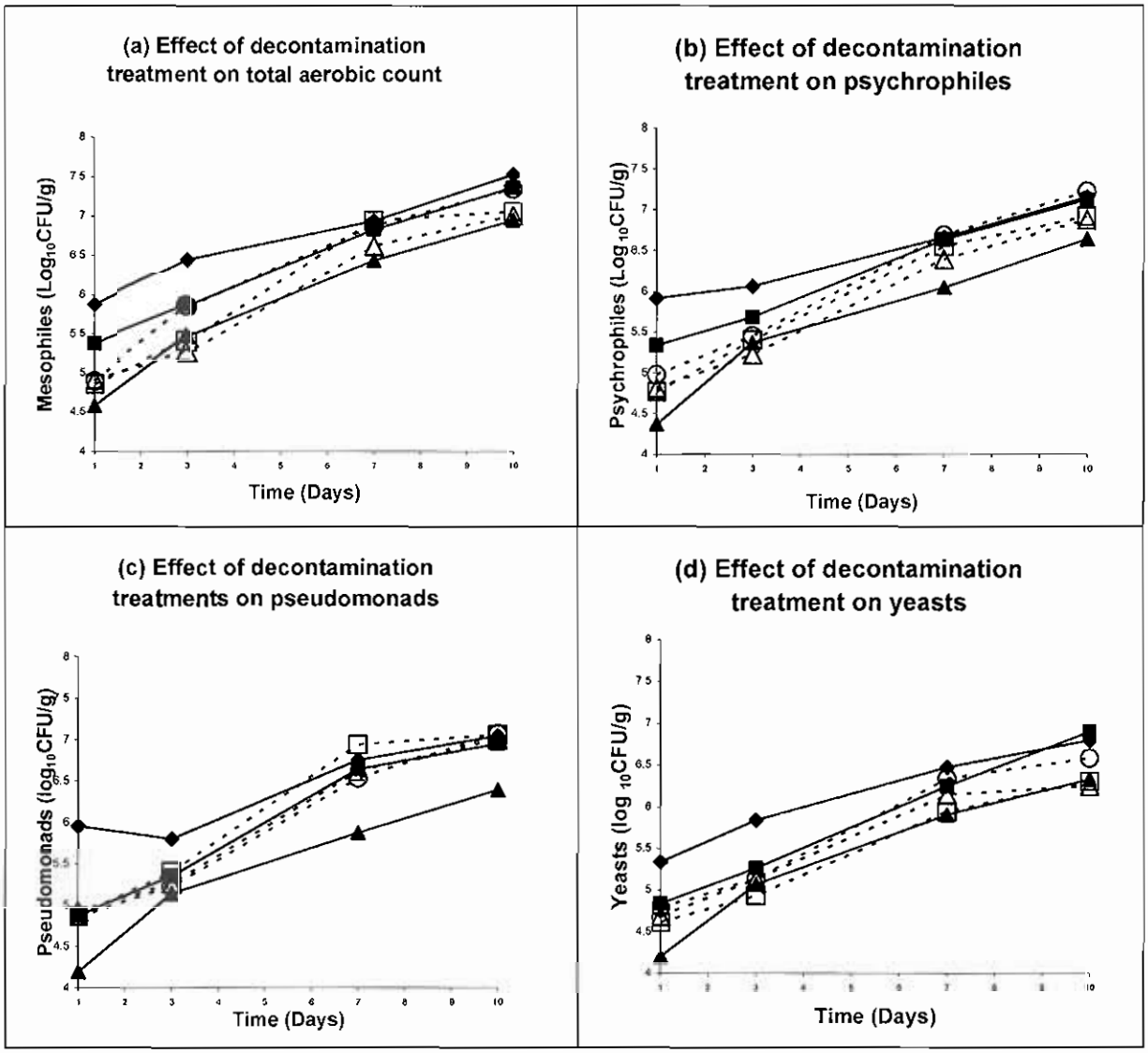

Figure 3. 1: Effects of anti-microbial dipping treatments on the growth characteristics of total aerobic mesophiles (a), psychrophiles (b), pseudomonads (c) and yeasts (d) in ready-to-eat lettuce. Data represent the means of 3 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (□): Ozone and (Δ): Ozone plus Calcium lactate.

effect of decontamination treatments; total aerobic mesophile counts had recovered to levels similar to those observed on unwashed lettuce ($p > 0.05$) (Fig. 3.1–a). Generally, total aerobic mesophile populations increased over the 10 day storage period.

The initial reduction in populations and subsequent growth patterns of psychrophiles on control or decontaminated lettuce followed similar trends to those observed for

total aerobic mesophiles. There was a general increase in psychrophile counts over the 10 day storage period (Fig. 3.1-b). Psychrophile counts on prepared lettuce were reduced by over 1 log cycle when treated with chlorine (100 mg l^{-1}), ozone or ozone in combination with calcium lactate ($p < 0.05$). From Day 3 of storage at 4°C, there was no significant effect of decontamination treatment in comparison with unwashed lettuce where similar counts of psychrophiles were recorded ($p > 0.05$) (Fig. 3.1-b). Pseudomonad counts were reduced from 1.21 x 10^6 CFU g^{-1} on unwashed lettuce to 8.88 x 10^4 CFU g^{-1} on lettuce treated with 100 mg l^{-1} of chlorine ($p < 0.05$). There seemed to be a residual effect of chlorine as pseudomonad populations were consistently lower on chlorine treated lettuce than for any other treatment during the storage period. All other decontamination treatments resulted in an initial reduction in pseudomonad numbers but on subsequent storage at 4°C, there were no further effects of treatment and counts increased gradually throughout storage at (Fig. 3.1–c). Similar trends were recorded for the initial effect of the decontamination treatments on yeast counts in prepared lettuce to those observed for the other microflora analysed (Fig 3.1). Again, treatment with chlorine was the most effective where counts of 7.16 x 10^5 CFU g^{-1} on unwashed lettuce were reduced to 9.22 x 10^4 CFU g^{-1} on the lettuce treated with chlorine (Fig 3.1-d). By Day 3, yeast counts observed on lettuce treated with ozone or chlorine were still lower by comparison with unwashed lettuce ($p < 0.05$). Calcium lactate treatments did not effect the initial or subsequent levels of yeasts on lettuce by comparison with unwashed lettuce (Fig 3.1-d). Yeast counts increased over storage and were similar for all treatments from Day 7 of storage.

The combination treatment of ozone plus calcium lactate was the most effective for initial reduction of coliform populations (in excess of 1 log cycle) on prepared lettuce (Fig 3.2). However, on subsequent storage at 4°C, there seemed to be a residual

effect of treatment with chlorine. Coliform numbers were consistently lower on chlorine treated lettuce in comparison with other decontamination methods from Day 3 ($p < 0.05$). Coliform counts generally increased over the 10 day storage period, with more growth observed for coliforms on unwashed lettuce than on combination, ozone, calcium lactate, water or chlorine treated samples in that order.

No LAB or moulds were found in any sample.

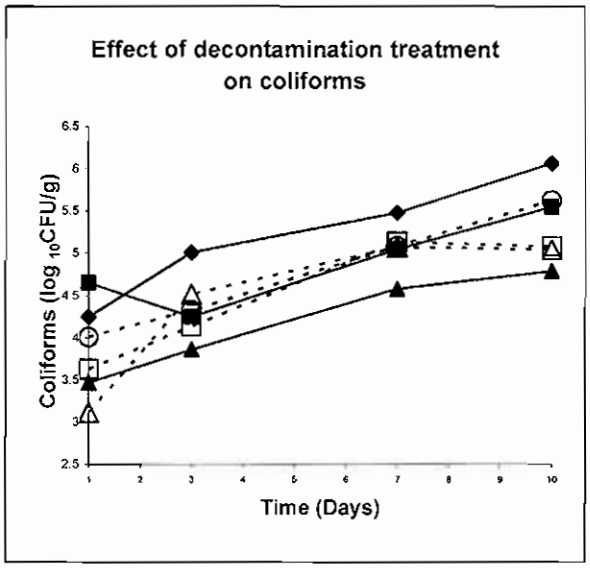

Figure 3.2: Effects of anti-microbial dipping treatments on the growth characteristics of total Coliforms in ready-to-eat lettuce. Data represents the means of 3 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (□): Ozone and (Δ): Ozone plus Calcium lactate.

3.2 Discussion

Washing and/or disinfection procedures form part of the minimal processing technology used to produce fresh RTE vegetables. The objectives are to reduce the rate of microbial spoilage and to reduce the concentration of naturally occurring pathogens leading to an extension of shelf life and enhancement of safety of RTE vegetables. Chlorine is the most widely used disinfectant, however, in some EU

countries chlorine is not permitted as a wash water additive due to concerns regarding toxic by product formation (Seymour & Appleton, 2001).

The main objective of this work was to investigate alternatives to chlorine for decontamination treatment of RTE lettuce. Calcium lactate was proposed as an alternative decontamination method as it has been reported to maintain firmness and texture of vegetables, and contribute nutritional benefits. Calcium is an essential mineral for the body and it is critical to many body functions (Consumer Lab, 2003). A second alternative strategy investigated was decontamination with ozone, which has characteristics that make it suitable for use as a sanitizer in food processing; it has a strong oxidising power and does not remain in water for a very long period of time (Guzel-Seydim *et al.*, 2003). The combination method of both ozone and calcium lactate was proposed to combine the strong oxidizing power of ozone with the firmness maintenance of calcium lactate on vegetables. Typical storage life for RTE vegetables is approximately one week and the following discussion will focus on results obtained up to Day 7.

The results showed similar trends for effects of treatments against initial populations of total aerobic mesophiles, psychrophiles and yeasts. The use of chlorine or ozone alone, and ozone in combination with calcium lactate reduced initial populations by approximately 1.5 or 1 log cycle respectively, compared with untreated samples, and this effect persisted until Day 3 of storage. Baur *et al.* (2004) also reported a 1.5 log cycle reduction in aerobic mesophiles on shredded lettuce treated with chlorine. These authors also reported that treatment with ozone was more effective than treatment with tap water alone for reduction of aerobic mesophiles, but that chlorine was still the most effective treatment. This is similar to the results presented here, where the alternative treatments were less effective than chlorine for reduction of aerobic mesophiles, psychrophiles and yeasts. After 1 week at 4°C, populations were

slightly lower on chlorine treated lettuce but there was no significant effect of decontamination treatment. Beuchat & Brackett (1990) observed a 2 log cycle reduction of mesophiles after treatment with 100 mg l^{-1} chlorine, but by Day 4 of the study, no significant differences in populations of aerobic mesophiles were noted on lettuce, regardless of treatment. It was reported by Beltran *et al.* (2005) that populations of mesophiles, psychrophiles and yeasts were not initially significantly reduced after treatment with 20 mg l^{-1} ozone on fresh-cut potato strips, and by Day 5 of their experiment mesophiles and yeast populations were higher on the ozone treated sample. This contrasts with the results obtained in this work on shredded lettuce, where a significant reduction for mesophiles, psychrophiles and yeasts compared with untreated lettuce was initially observed using ozone treatments. However by Day 3 of storage there was no significant effect of decontamination with ozone compared to unwashed lettuce.

Martin Diana *et al.* (2005) treated shredded lettuce with calcium lactate and reported that lettuce treated with calcium lactate was generally firmer than untreated lettuce. Gorny *et al.* (1999) reported that a 2% ascorbic acid plus 1% calcium lactate post cutting dip resulted in reduction of cut surface browning on peaches and nectarines. However Gorny *et al.* (2002) reported that using calcium lactate alone at 20°C did not prevent cut surface darkening in pear slices. Treatment with 2.5% calcium lactate did reduce initial populations of mesophiles, psychrophiles and yeasts in the current work, and was slightly more effective than treatment with water but not significantly so. Luna-Guzman & Barrett (2000) found that treatment with 2.5% calcium lactate maintained melon firmness throughout storage, however, they also found that the effects of calcium lactate were similar to water treatment for initial reduction of total plate counts and yeasts with no effect of treatment compared to water control throughout storage.

Pseudomonads are major components of the indigenous microflora of RTE vegetables (Nguyen-the & Carlin, 1994). In this work all decontamination treatments reduced initial pseudomonad populations on shredded lettuce, however chlorine was the most effective and a residual effect was observed over storage period. The greater initial reduction of pseudomonads was reflected in lower counts throughout storage. Baur *et al.* (2004) also reported greater reductions in pseudomonad populations subjected to chlorine treatment compared with ozone or water treatments. A similar residual effect of chlorine was recorded by Cliffe-Byrnes & O'Beirne (2005) on fresh cut coleslaw where pseudomonad counts were lower by comparison with water washed coleslaw throughout storage at 4 or 8°C.

Escherichia coli O157:H7 has been involved in outbreaks of foodborne diseases associated with the consumption of fresh produce (Francis *et al.*, 1999), therefore coliform counts were performed to assess the efficacy of the decontamination procedures with regard to food hygiene. The best initial decontamination treatment for reduction of coliforms was the combination treatment: ozone plus calcium lactate, this was the only population for which a synergistic decontaminant effect of the combination treatment was recorded. However, after 2 days storage, numbers had increased above those recorded for chlorine. There was a residual effect of chlorine on coliforms similar to that observed for pseudomonads.

In the current work, treatment of lettuce with chlorine was more effective than any other treatment for initial decontamination and counts throughout storage of aerobic mesophiles, psychrophiles, yeasts and pseudomonads. The alternative strategies using calcium lactate or ozone alone or in combination resulted in a decontamination efficacy comparable to or better than washing in water.

CHAPTER 4: Effects of organic acid anti-microbial dipping treatments on the indigenous microflora of ready-to-eat lettuce

Acetic and citric acid dipping solutions (1% and 2% respectively) were compared with calcium lactate, chlorine and water dipping treatments for effects on the indigenous microflora of ready-to-eat lettuce. Treatment with organic acids had significant effects on the population densities of aerobic mesophiles, psychrophiles, pseudomonads, yeasts and coliforms.

4.1 Results

All decontamination treatments reduced the initial mesophile populations, but counts increased over the storage period in all lettuce samples except those dipped in acetic acid solution (Fig. 4.1-a). Acetic acid and citric acid were the most effective decontamination treatments, reducing the number of mesophiles by over 2 log cycles compared with undipped lettuce (Fig. 4.1-a). This trend continued throughout the storage period, where mesophile counts were consistently lower on acetic or citric acid dipped lettuce by comparison with the other treatments. The 2% citric acid treatment in particular was very effective in maintaining low levels of mesophiles. At the end of storage, the mesophile counts of 2.08×10^4 CFU g^{-1} recorded on acetic acid dipped lettuce were significantly lower compared with counts of 4.95×10^7 CFU g^{-1} recorded for undipped samples ($p < 0.05$). Chlorine or calcium lactate treatments reduced initial mesophile counts by comparison with the untreated lettuce ($p<0.05$).

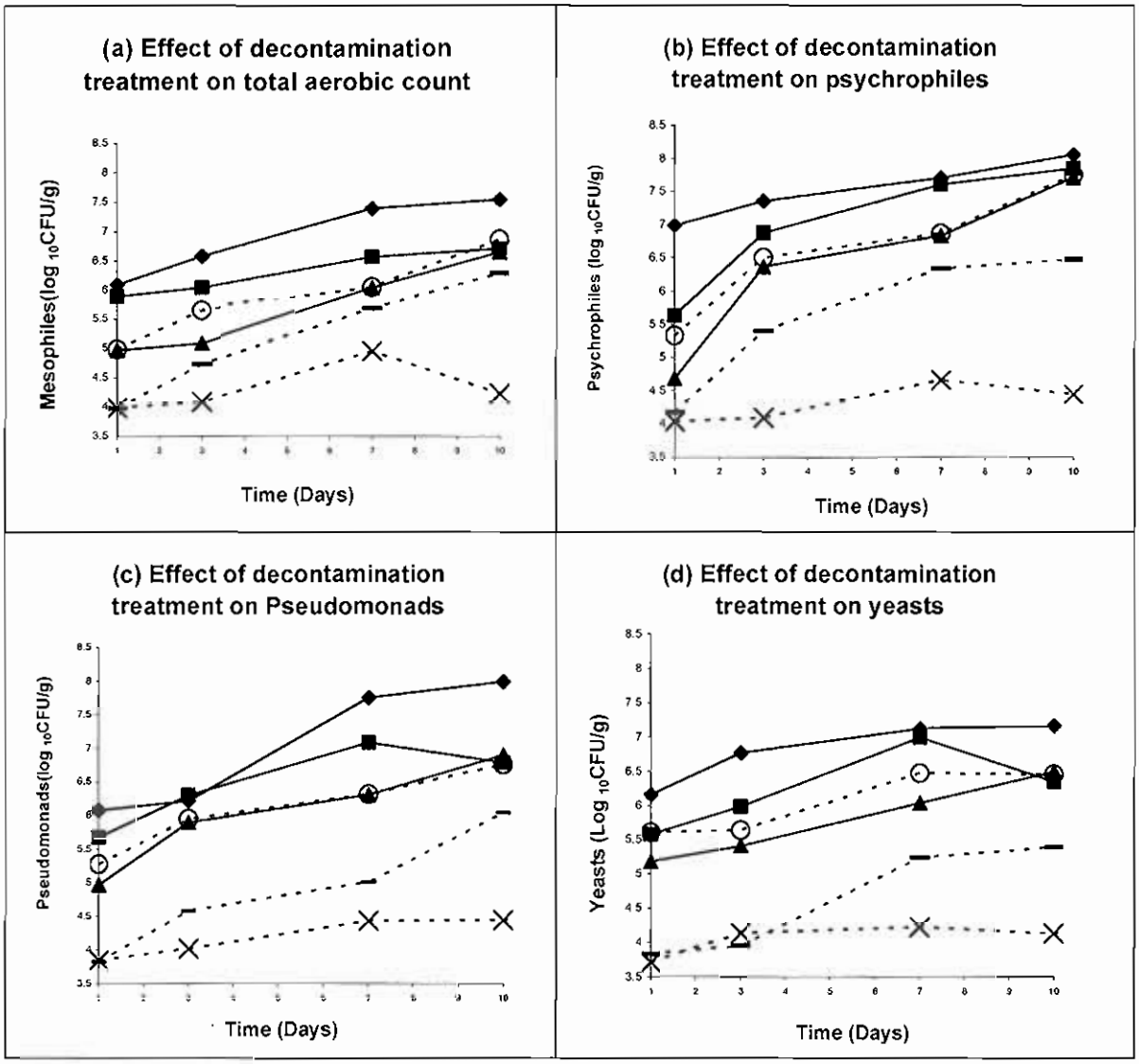

Figure 4.1: Effects of anti-microbial dipping treatments on the growth characteristics of (a) total aerobic mesophiles, (b) psychrophiles, (c) pseudomonads and (d) yeasts in ready-to-eat lettuce. Data represent the means of 3 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (×): Acetic acid (1%) and (—): Citric acid (2%).

Similar trends were observed for psychrophile counts (Fig. 4.1-b) on treated lettuce as those recorded for mesophiles. All decontamination treatments were effective for reducing initial numbers by comparison with those present on undipped lettuce ($p < 0.05$). Acetic acid was the best decontamination treatment and no increase in counts was observed over the storage period. Initial population reductions for citric acid were the same as those observed with acetic acid. On subsequent storage at 4°C,

psychrophile counts increased for all treatments except acetic acid. However, populations recorded on citric acid dipped lettuce were consistently lower throughout storage than those on undipped lettuce samples ($p < 0.05$). Dipping lettuce in chlorine was a more effective treatment than calcium lactate where counts of 5.54×10^4 CFU g^{-1} (chlorine) were recorded on Day 1 compared with counts of 3.06×10^5 CFU g^{-1} (calcium lactate).

Similar reductions in numbers of pseudomonads were achieved on lettuce treated with acetic acid and citric acid on Day 1 (Fig. 4.1-c). Counts were reduced from 1.30×10^6 CFU g^{-1} on undipped lettuce to 7.29×10^3 CFU g^{-1} and 6.79×10^3 CFU g^{-1}on acetic acid and citric acid dipped lettuce samples respectively. Pseudomonad populations followed similar trends to those observed for mesophiles, where the best disinfectant treatment was acetic acid ($p<0.05$), followed by citric acid treatment ($p<0.05$). These effects were observed throughout the storage time. There was a general increase in pseudomonads counts over the 10 day storage period. Chlorine or calcium lactate treatments had similar effects, showing a significant reduction by comparison with the undipped lettuce ($p<0.05$), (Fig. 4.1. -c), but treatment with water was ineffective for control of pseudomonads.

Citric and acetic acid were the most successful treatments for reduction of yeast populations; a difference in excess of 2 log cycles compared with numbers on undipped lettuce was recorded ($p < 0.05$). This effect persisted over the 10 days storage (Fig. 4.1-d). Treatment with chlorine reduced populations from 1.97×10^6 CFU g^{-1} (untreated lettuce) to 2.23×10^5 CFU g^{-1} and these remained lower throughout storage at 4°C. Effects of calcium lactate treatment were similar to those recorded for dipping in water alone. There was an increase in yeast populations over the 10 day storage on all lettuce samples excepting those treated with acetic acid.

Reductions in coliform numbers of 2 log cycles on lettuce treated with acetic or citric acid and a reduction of 1 log cycle on lettuce treated with chlorine were observed after initial treatment compared with untreated lettuce (Fig. 4.2). Generally, coliform counts increased over the 10 days storage period ($p < 0.05$), following initial trends until Day 3. As observed with other microflora populations examined, coliforms in acetic acid dipped lettuce were lower throughout storage than those on all other lettuce samples. Coliform counts declined on acetic acid dipped lettuce during storage, indicating a possible residual effect of the treatment.

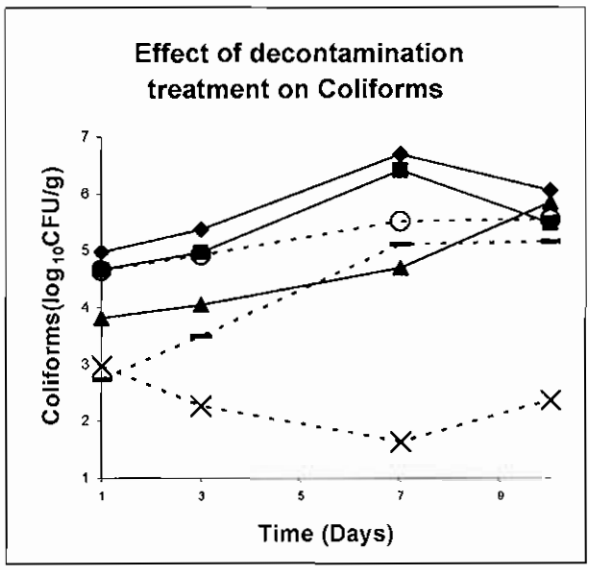

Figure 4.2: Effects of anti-microbial dipping treatments on the growth characteristics of total Coliforms in ready-to-eat lettuce. Data represent the means of 3 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (×): Acetic acid (1%) and (—): Citric acid (2%).

The concentration of oxygen in lettuce packages decreased from atmospheric levels to 16-18% by Day 2 of storage at 4°C (Fig 4.3-a). This was accompanied by an increase in carbon dioxide levels from 0.03% to 1.5 to 2.5 % (Fig 4.3-b). There was an effect of storage temperature observed where the gas levels were modified by product respiration more rapidly at 10°C than at 4°C. By Day 1, oxygen levels were

in the range of 14-18% (Fig. 4.4-a) and carbon dioxide levels reached 1.5 – 4% (Fig. 4.4-b). There was no consistent difference in the effect of the treatments on atmosphere modification over the storage period.

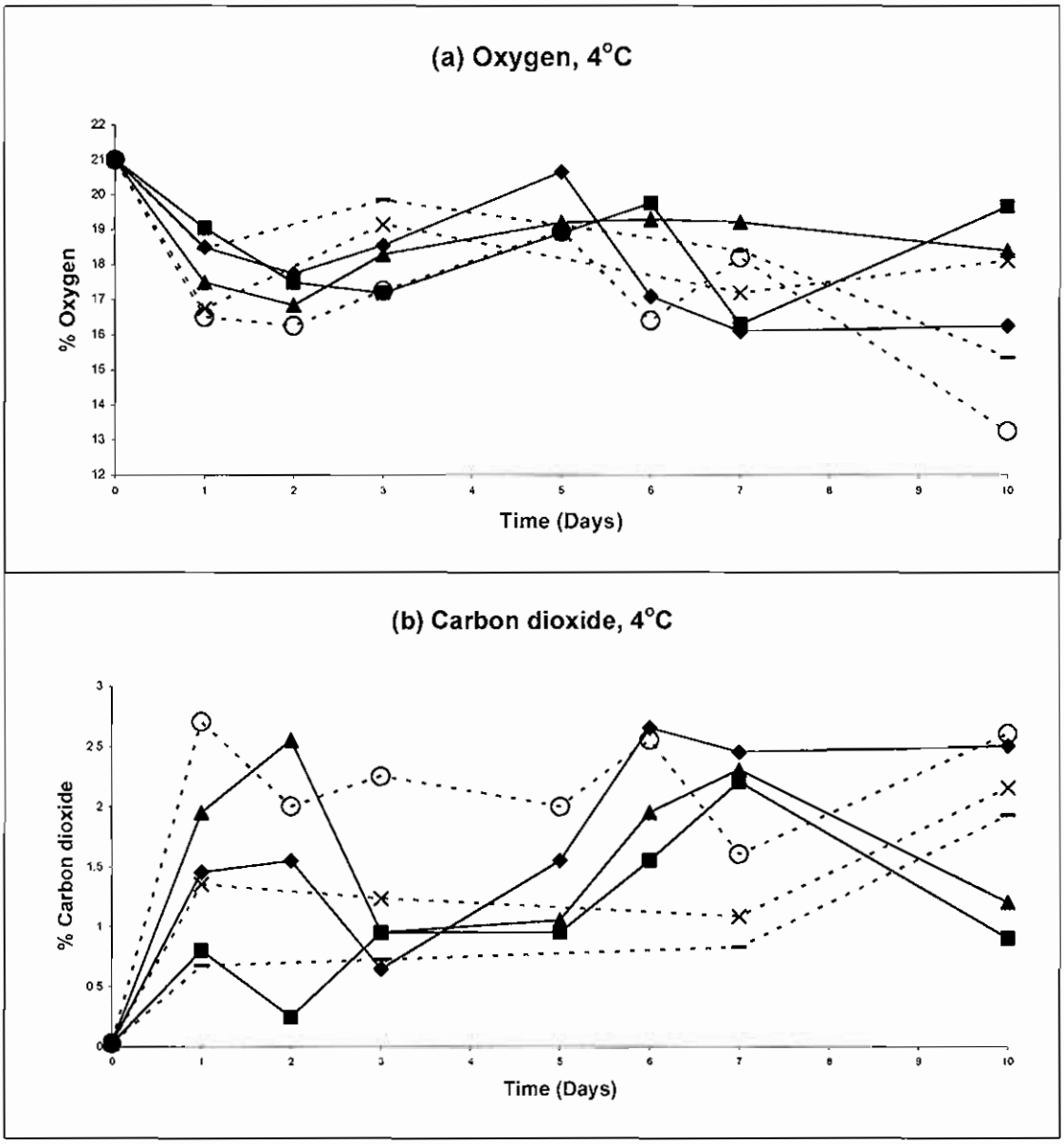

Figure 4.3: Gaseous changes within lettuce packages at 4°C a) Oxygen levels, b) Carbon dioxide levels. Data represent the means of 2 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (×): Acetic acid (1%) and (—): Citric acid (2%).

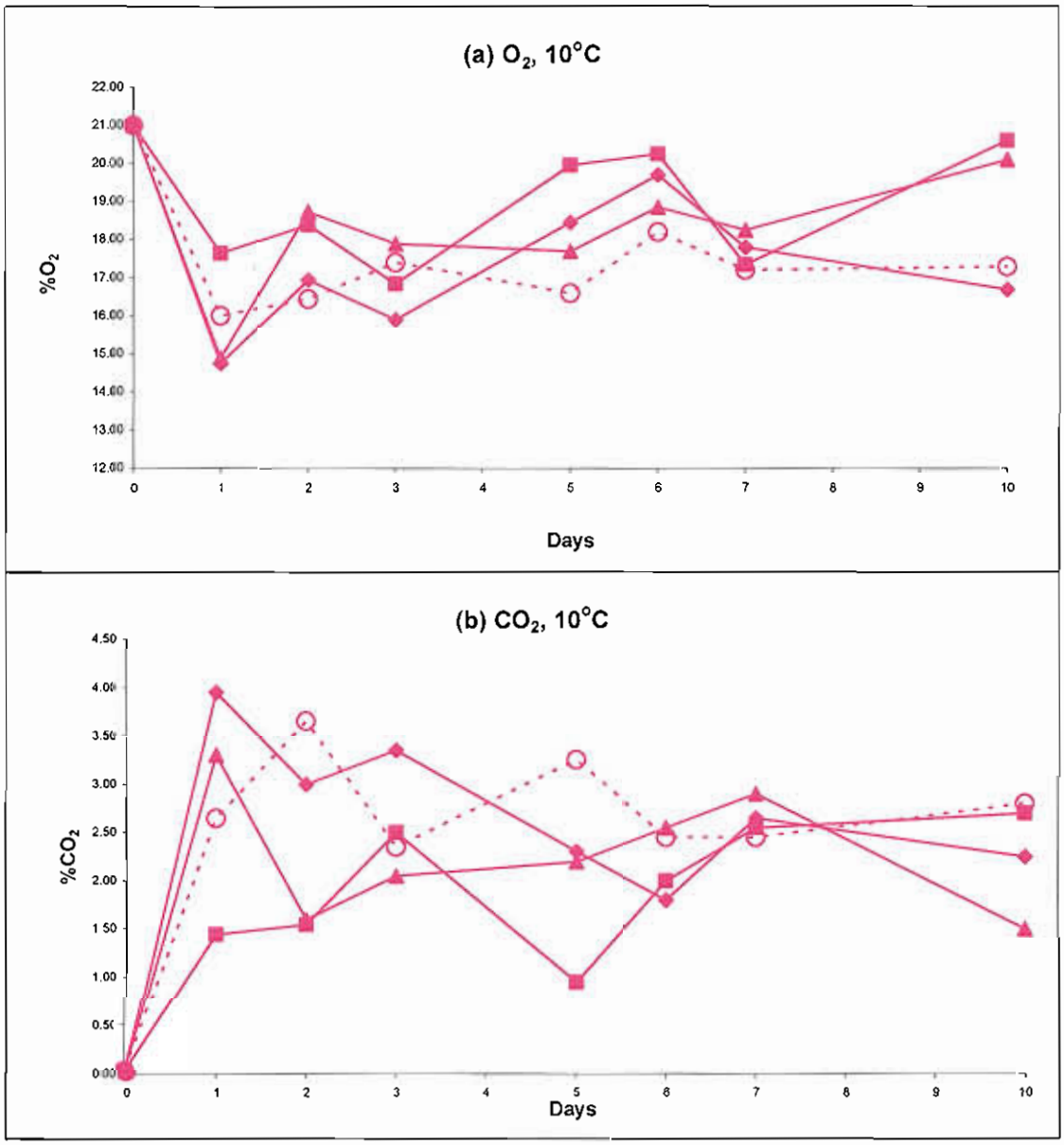

Figure 4.4: Gaseous changes within lettuce packages at 10°C a) Oxygen levels, b) Carbon dioxide levels. Data represent the means of 2 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (×): Acetic acid (1%) and (—): Citric acid (2%).

Dipping lettuce in a 1% acetic or a 2% citric acid solution resulted in lower pH levels than those observed for lettuce treated with calcium lactate, chlorine or water. This was apparent at Day 1, where lettuce treated with acetic or citric acids had lower pH levels, (of 5.6 and 5.75 respectively), compared with all other treatments (Fig. 4.5). This trend continued throughout storage where pH levels were consistently lower for citric acid or acetic acid treated lettuce by comparison with other treatments.

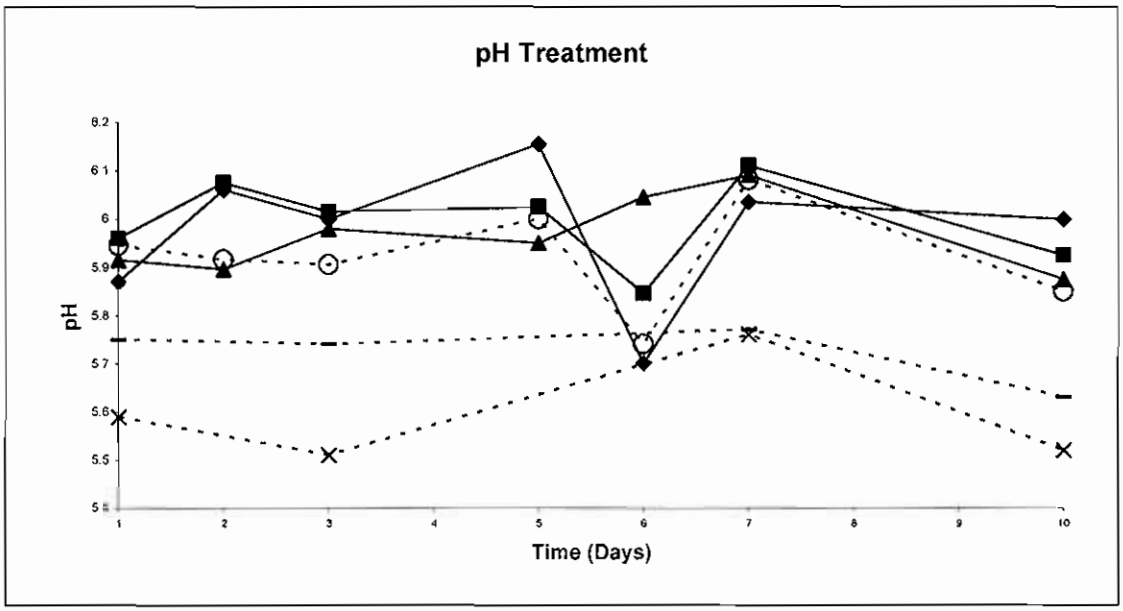

Figure 4.5: Effect of anti-microbial dipping treatments on pH at 4°C. Data represent the means of 2 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (×): Acetic acid (1%) and (—): Citric acid (2%).

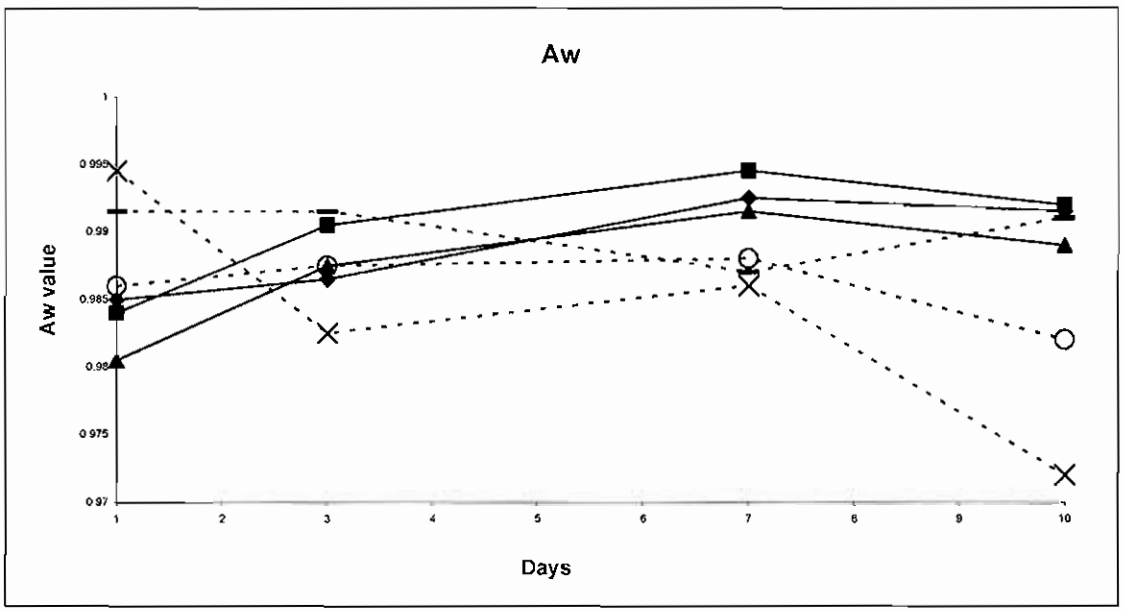

Figure 4.6: The effect of anti-microbial dipping treatments on Aw. Data represent the means of 2 determinations. (♦): Undipped, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate (2.5%), (×): Acetic acid (1%) and (—): Citric acid (2%).

The water activity of the chlorine treated, water dipped or undipped lettuce samples was initially lower than that recorded for calcium lactate, acetic or citric acid treated

lettuce (Fig. 4.6). However, during storage, the water activity of lettuce treated with the organic acids declined , whereas the water activity increased up to Day 7 for chlorine dipping, water dipping or undipped lettuce.

4.2 Discussion

Organic acids can occur naturally in many fruits and vegetables and retard the growth of micro-organisms. Due to recent changes in legislation, washes and sprays containing organic acids are becoming more popular for the processing of organically grown fruits and vegetables (Seymour & Appleton, 2001). The most commercially important preservatives are still the organic acids which although naturally occurring, the bulk of these substances used in foods are synthetically produced. The group of organic acids containing acetic, citric, tartaric, malic and lactic acid show an anti-microbial activity owing primarily to their pH-reducing effect. They work either directly by lowering the pH of the food and thus adding stress to the micro-organisms, or in the undissociated form by migrating though the cell membrane into the cytoplasm of the micro-organisms where they dissociate and lower the internal pH of the cell (Meyer *et al.*, 2002). However, several reports have shown that the undissociated form of acetic acid also has anti-microbial action (Rusul *et al.*, 1987).

Similar trends were observed in the effects of the decontamination treatments against pseudomonad and total aerobic mesophile populations in this study. Decontamination with acetic or citric acid were the best treatments for reduction of initial populations, with a persisting effect throughout the storage period. The greater efficacy of the organic acid treatments was probably associated with a pH lowering effect; lower pHs were recorded for citric acid or acetic acid dipped lettuce. A 2 log cycle reduction was

achieved using 1% acetic or 2% citric acid solutions. Although counts did increase on citric acid dipped lettuce, levels were maintained at initial levels on lettuce treated with acetic acid. Escudero *et al.* (1999) used 0.5% acetic acid to decontaminate lettuce spiked with challenge populations of *Yersinia enterocolitica* and achieved a reduction of 2.73 log cycles. Jiang *et al.* (2004) recorded that treatment with 0.1 M citric acid markedly extended the shelf life of fresh cut Chinese water chestnuts. Vijayakumar & Wolf-hall (2002) reported a 1.2 log reduction in total mesophile counts on lettuce following treatment with apple cider vinegar (0.3% acetic acid). However in the current work, the sensory properties of acetic acid dipped lettuce were unacceptable in appearance and odour, with the vinegar odour remaining throughout storage. The lower water activity recorded for acetic acid dipped lettuce in combination with the lower pH levels may have had an additive anti-microbial effect.

Although acetic or citric acid dipping was more effective than chlorine or calcium lactate treatments, a 1 log reduction in initial populations of aerobic mesophiles and pseudomonads was achieved using either chlorine or calcium lactate in comparison with water or untreated lettuce. This is in contrast to results recorded by Luna-Guzman (2000) where the efficacy of a 2.5% calcium lactate treatment was comparable to that of water alone. Populations recorded on calcium lactate or chlorine dipped lettuce remained at lower levels than those found on untreated lettuce but were similar to those recorded on water dipped lettuce after 1 week. The anti-microbial effects achieved with chlorine or calcium lactate decontaminating treatments were not as prolonged as those achieved with acetic or citric acid.

Acetic and citric acid solutions were again the best treatments for initial reduction of psychrophile, yeast and coliform populations, achieving at least a 2 log cycle reduction in comparison with untreated lettuce. Eggenberger-Solorzano *et al.* (2002) recorded a 1 log cycle reduction on coliforms after treatment with 1.8% acetic acid. In the current

work, acetic acid was also the most effective treatment for maintenance of microflora populations at initial levels throughout the storage period. However, the efficacy of citric acid dipping was better than water or chlorine treatments throughout storage, particularly for yeast and psychrophile populations. Psychrophile and yeast counts on chlorine or calcium lactate treated lettuce were similar during storage, but for coliforms, chlorine was more effective.

The numbers of background microflora on shredded lettuce dipped in citric acid (1%) were significantly lower than those on lettuce washed in water throughout a storage period of 14 days at both 3 and 8°C in a study conducted by Francis & O'Beirne (1997). By contrast, Adams *et al.* (1989) found that the reduction in total counts on minimally processed lettuce achieved using 300 to 500 mg l^{-1} of acetic, citric, lactic or propionic acids was not significantly different from the reduction obtained using tap-water. In the present study, anti-microbial dipping with the organic acids had significant effects on background microflora populations and a lesser effect was achieved with chlorine or calcium lactate treatments. Although the initial microflora populations analysed were reduced by the decontamination treatments, their subsequent recovery may be attributed to the survival of sufficient numbers in the natural grooves of the vegetable tissue or those located in biofilms. Gras *et al.* (1994) attributed the limited efficiency of decontamination procedures on salad leaves to the location of most of the bacterial microflora in biofilms. Beuchat & Golden (1989) suggested that the mode of action of organic acids was related to direct pH reduction of the substrate as well as some reduction in internal pH of the cell or disruption of transport mechanisms through the cell membrane. The lack of a rinsing step could have contributed to the maintenance of the more acidic conditions imposed particularly with citric acid or acetic acid dipping.

storage period, but this was slower in lettuce treated with calcium lactate at 50°C up to Day 3.

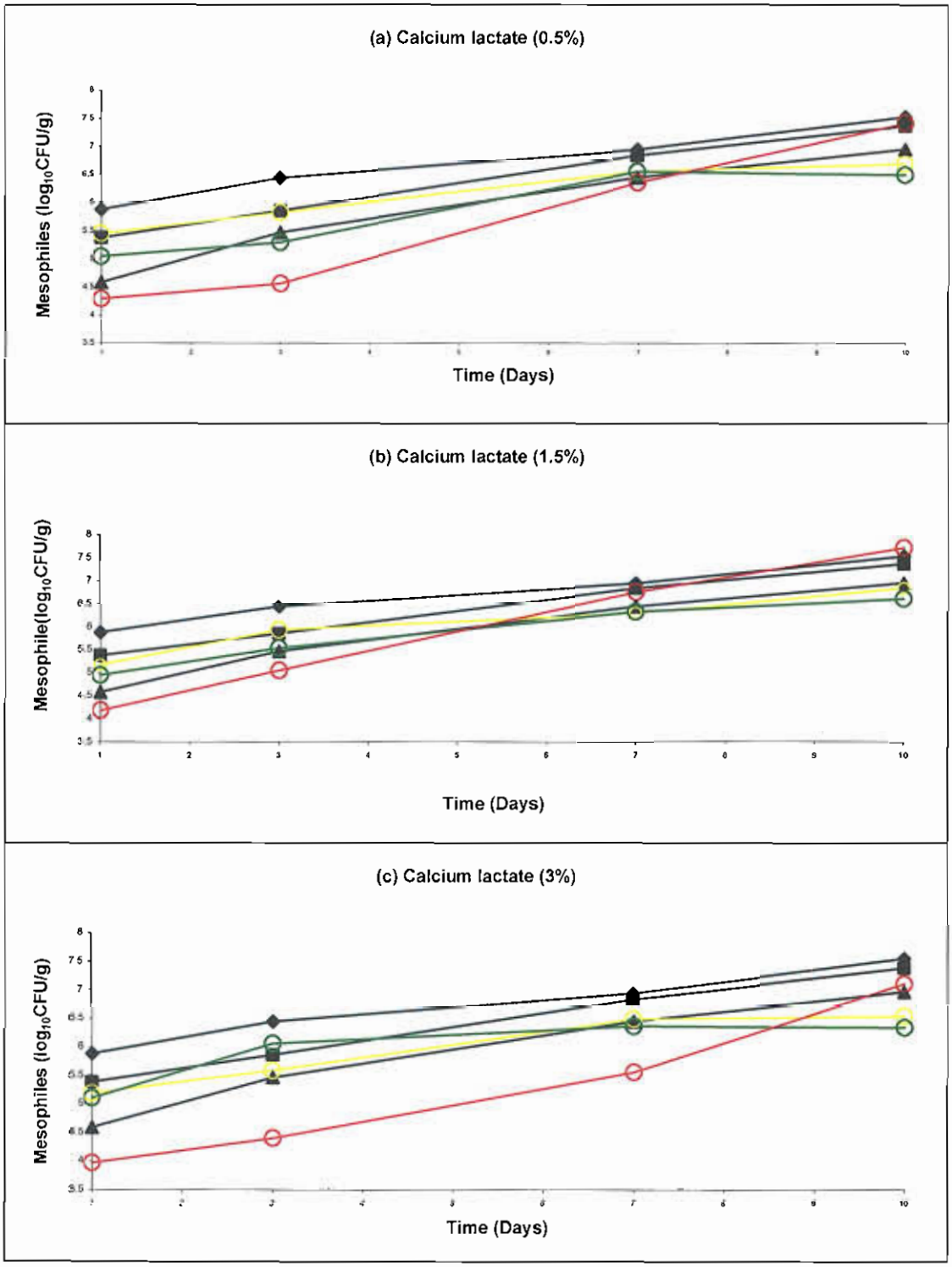

Figure 5.1: Effects of calcium lactate concentration and temperature on aerobic mesophiles in ready-to-eat lettuce. Data represents the mean of 3 determinations. (♦): Unwashed, (■): Water, (▲): Chlorine (100 mg l^{-1}), ( ): Calcium lactate 4°C, (○): Calcium lactate 25°C, (○): Calcium lactate 50°C.

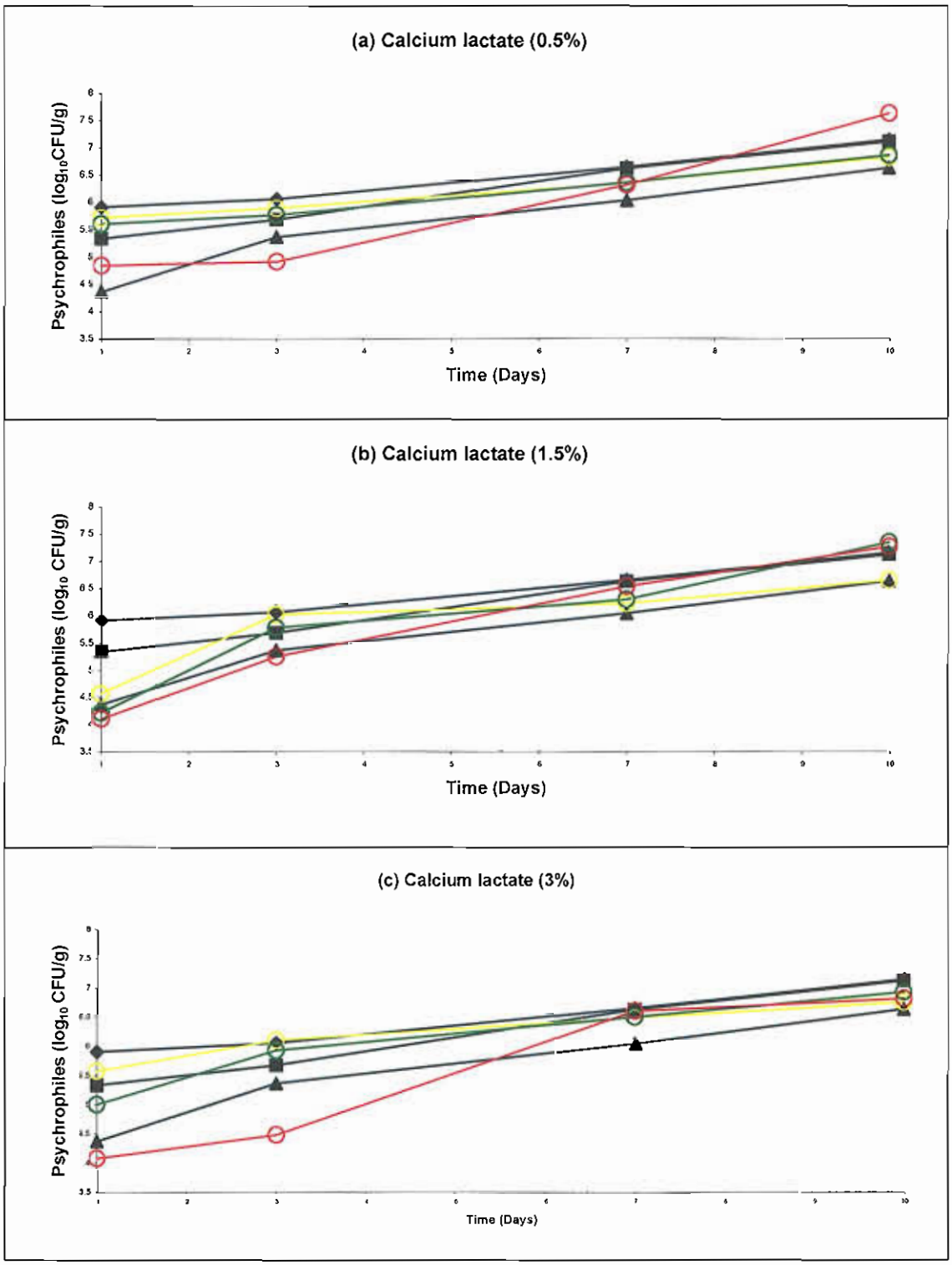

Figure 5.2: Effects of calcium lactate concentration and temperature on psychrophiles in ready-to-eat lettuce. Data represents the mean of 3 determinations. (♦): Unwashed, (■): Water, (▲): Chlorine (100 mg l^{-1}), ( ): Calcium lactate 4°C, (○): Calcium lactate 25°C, (○): Calcium lactate 50°C.

All decontamination treatments reduced the initial number of psychrophiles by comparison with counts on unwashed lettuce. Similar trends were observed for initial decontamination and subsequent growth characteristics of psychrophiles as mesophiles. Calcium lactate treatment at 50°C, regardless of concentration was comparable to or more effective than chlorine for reduction of initial psychrophile counts. This trend was also apparent on Day 3 of storage, but thereafter, the lowest counts were observed on chlorine treated lettuce (Fig. 5.2). There was a general increase in the number of psychrophiles over storage period, but this was again slower in lettuce treated with calcium lactate at 50°C up to Day 3.

All decontamination treatments reduced initial populations of pseudomonads by comparison with counts on unwashed lettuce ($p < 0.05$). Regardless of concentration, treating lettuce with calcium lactate at 50°C was as effective or more effective than chlorine for reduction of initial pseudomonad numbers (Fig. 5.3). Chlorine or calcium lactate at 50°C were the most effective treatments ($p < 0.05$), leading to an average 2 log cycle reduction in initial numbers. This effect persisted until Day 3 of storage at 4°C where lower counts were recorded compared to all other treatments. Treatment of lettuce with 3% calcium lactate at 50°C noticeably delayed growth of pseudomonads until after Day 3 of storage (Fig. 5.3-c) and led to significantly lower numbers of pseudomonads than for all other treatments ($p < 0.05$). There was a general increase in the pseudomonad counts over the 10 day storage period, particularly after Day 3. By Day 7 pseudomonads had grown to similar levels ranging from 5.5 to 6.5 $\log_{10}$ CFU g^{-1} on all lettuce samples; with the lowest counts again generally obtained on chlorine or calcium lactate (3% at 50°C) treated lettuce.

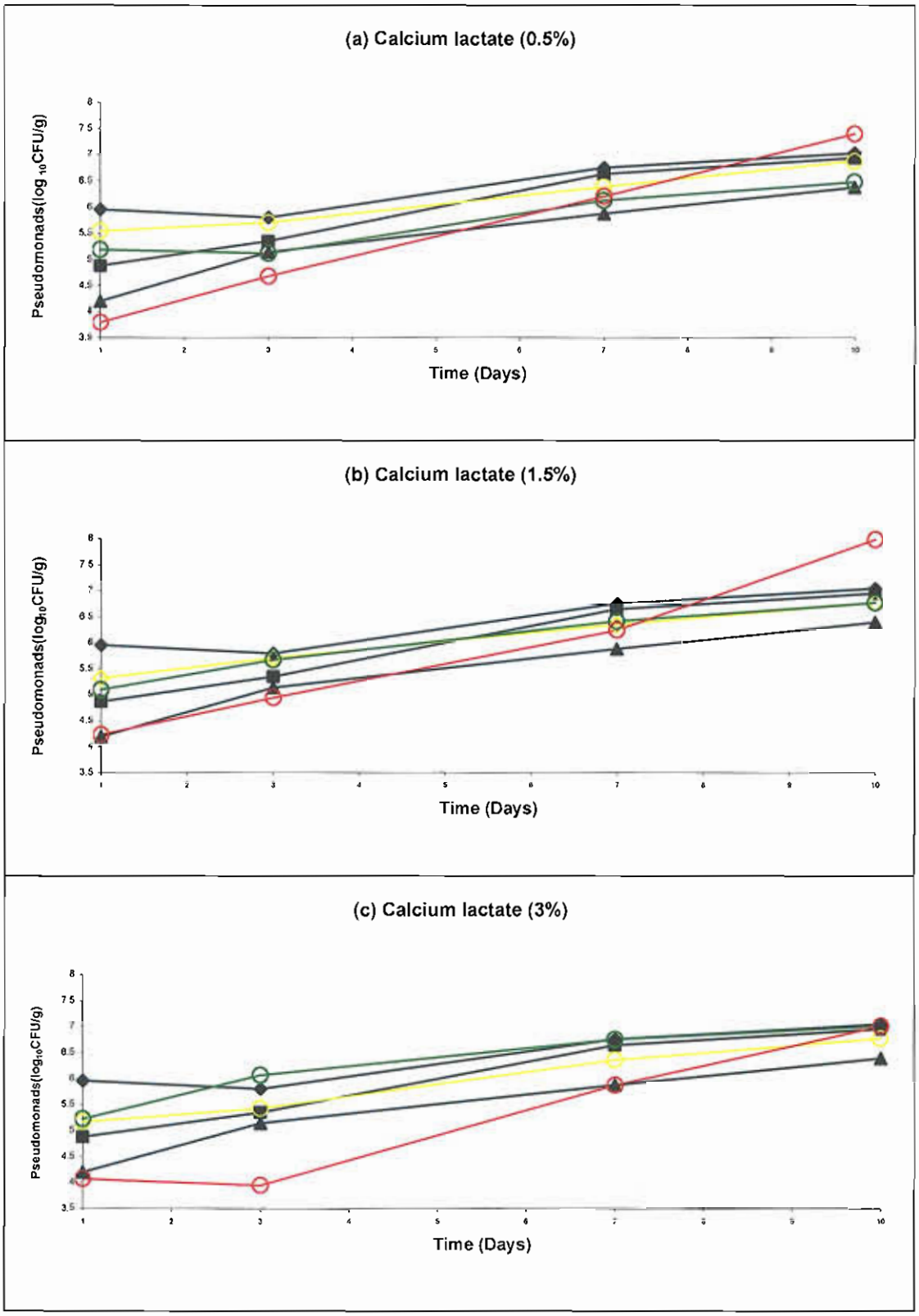

Figure 5.3: Effects of calcium lactate concentration and temperature on pseudomonads in ready-to-eat lettuce. Data represents the mean of 3 determinations. (♦): Unwashed, (■): Water, (▲): Chlorine (100 mg l^{-1}), ( ): Calcium lactate 4°C, (○): Calcium lactate 25°C, ( ): Calcium lactate 50°C.

There was an effect of treatment temperature on yeast populations on shredded lettuce. Decontamination with calcium lactate at 50°C regardless of concentration was more effective for reducing yeast numbers compared with unwashed lettuce ($p < 0.05$); a reduction of approximately 2 log cycles was achieved (Fig. 5.4). Dipping in calcium lactate at 4°C or at room temperature yielded similar results to water alone; no reduction or a reduction less than 0.5 log cycles was recorded compared to numbers on unwashed lettuce (Fig. 5.4). As with other microflora populations, the most effective decontamination method for reducing initial yeast counts was calcium lactate 3% at 50°C and this trend persisted until Day 3 of storage, where counts were maintained at initial levels. Counts were also lower than those on chlorine treated lettuce at Day 7 ($p < 0.05$). Generally, yeast counts increased over 10 day storage. Although lower levels of yeasts were recorded on lettuce washed in 3% calcium lactate up to Day 7, by the end of the storage time, counts were similar to or higher than those found recorded for all other treatments.

Similar trends to those for other microflora populations were observed for initial effects of treatments against coliforms. The biggest reduction was after treatment with calcium lactate at 50°C regardless of concentration, with the greatest reduction of 1.5 log cycles achieved using a 0.5% solution (Fig 5.5). However, by Day 3 of storage at 4°C, although there was no significant difference found between the treatments ($p > 0.05$), coliform counts were consistently lower on chlorine or water treated lettuce (Fig. 5.5). By Day 10 the number of coliforms was higher on the calcium lactate (50°C) treated samples ($p < 0.05$). Generally, there was an increase in coliform counts over the storage time, but levels on chlorine treated lettuce were lower throughout storage.

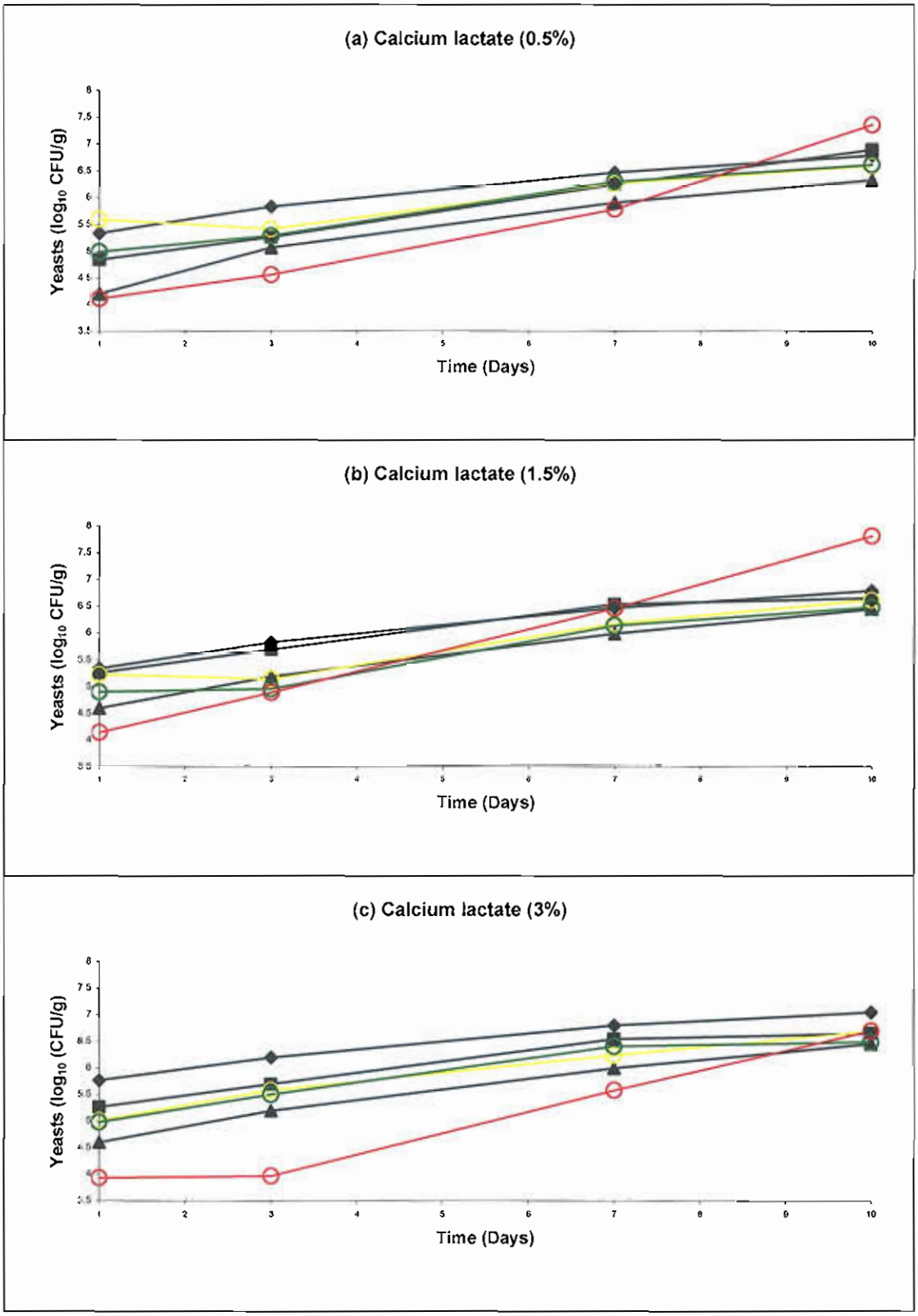

Figure 5.4: Effects of calcium lactate concentration and temperature on yeasts in ready-to-eat lettuce. Data represents the mean of 3 determinations. (♦): Unwashed, (■): Water, (▲): Chlorine (100 mg l^{-1}), (○): Calcium lactate 4°C, (○): Calcium lactate 25°C, (○): Calcium lactate 50°C.

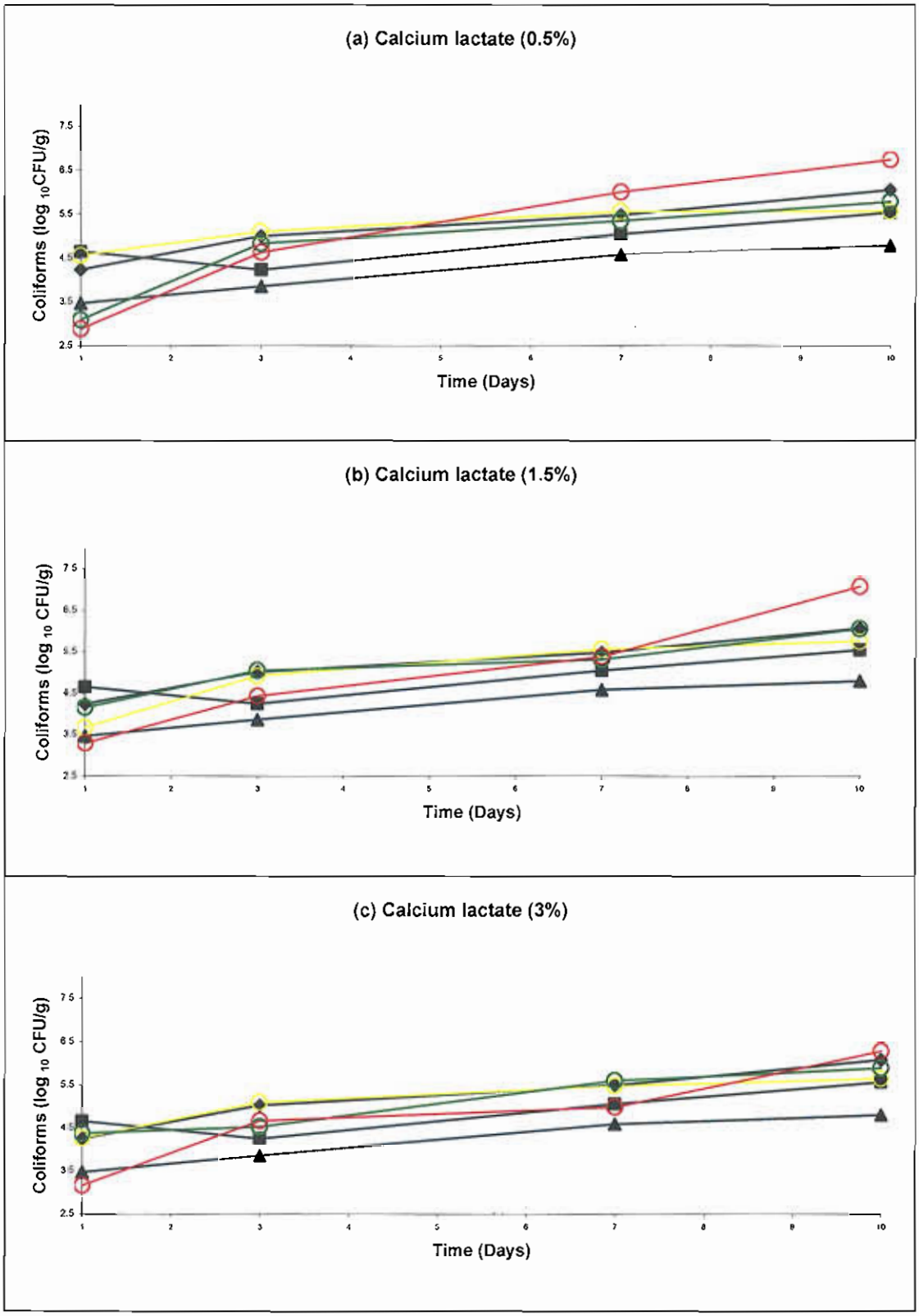

Figure 5.5: Effects of calcium lactate concentration and temperature on coliforms in ready-to-eat lettuce. Data represents the mean of 3 determinations. (♦): Unwashed, (■): Water, (▲): Chlorine (100 mg l^{-1}), (□): Calcium lactate 4°C, (○): Calcium lactate 25°C, (◌): Calcium lactate 50°C.

Table 5.1: Number of LAB found in Ready-to-Eat lettuce (log_{10} CFU g^{-1})

Days		1	3	7	10
Treatment					
Unwashed		4.38	2.60	2.75	----------
Water		----------	2.29	2.29	----------
Chlorine		----------	----------	2.60	----------
Calcium lactate Concentration	Temperature				
0.50%	4°C	----------	----------	----------	3.07
0.50%	4°C	3.29	----------	----------	2.90
0.50%	4°C	2.90	----------	----------	3.52
1.50%	25°C	3.60	3.14	3.20	----------
1.50%	25°C	----------	2.29	----------	----------
1.50%	25°C	----------	2.29	----------	3.05
3%	50°C	----------	3.94	----------	----------
3%	50°C	----------	----------	----------	2.29
3%	50°C	3.57	3.37	3.06	2.90

Lactic acid bacteria if detected were at very low levels. The numbers declined slowly over the storage period (Table 5.1). No effects of treatment could be discerned.

5.2 Discussion

Minimally processed Iceberg lettuce has all the attributes to be considered a potential cause of a public health hazard with a pH greater than 4.6 and a water activity in excess of 0.5; it also does not receive a thermal process and it has no barrier by either intrinsic factor (e. g. presence of nitrites, salt content, the presence of competitive flora, etc) or extrinsic factor (e. g. a heat treatment to control pathogens) to the growth of spoilage or pathogenic micro-organisms (Wiley, 1994). The application of a mild heat treatment as a hurdle has the potential to reduce micro-organisms and inhibit enzyme activity (Wiley, 1994). Previous chapters have focused on the application of ozone or organic acids as novel decontamination strategies. This chapter focussed on

optimising the temperature and concentration conditions for application of calcium lactate as a decontamination step, as it has been previously reported to have sensory and nutritional benefits for minimally processed fruit and vegetables (Gorny *et al.*, 1999; Martin-Diana *et al.*, 2004, 2005).

In the current work, total aerobic mesophile, psychrophile, pseudomonad and yeast counts were reduced significantly with calcium lactate treatment at 50°C at all concentrations. The 3% calcium lactate solution was the best decontamination treatment with almost a 2 log cycle initial reduction in aerobic mesophiles. These results are comparable to those reported by Delaquis *et al.* (1999) where a 3 minutes wash in chlorinated water at 47°C reduced total aerobic populations by 2.6 log cycles. They attributed the lethality of the treatment to a synergy between the application of chlorine and heat. It has been suggested that the application of a heat stress can prevent or reduce undesirable biochemical reactions associated with wounding (Delaquis *et al.*, 1999), and the same authors showed that the 3 minutes dip in chlorinated water at 47°C provided optimum retention of appearance in stored, packaged Iceberg lettuce. Further studies by Delaquis *et al.* (2002) recorded reductions in total aerobic mesophiles of 1 log cycle at 4°C and 2 log cycles at 47°C. This is similar to the results obtained in the current study, where initial reductions achieved using calcium lactate at 50°C were in the order of 1 log cycle greater than those achieved using calcium lactate at 4°C. Delaquis *et al.* (2004), also suggested that 1 minute at 50°C and < 1 minute at 51°C could be suitable alternatives to a 3 minutes chlorine wash at 47°C for commercial application. No significant differences were observed between calcium lactate treatments at 4°C, 25°C and the water treatment. Martin Diana *et al.* (2005) proposed calcium lactate as a good alternative to chlorine washing for salad cut lettuce. In their studies with polyphenol

oxidase (PPO); an enzyme implicated in the enzymatic browning process, there were significant effects of storage time, calcium lactate concentration and temperature on PPO activity. Lettuce washed in calcium lactate at 50°C had a lower PPO activity than samples treated at 25 or 4°C. Magee *et al.* (2002) found that calcification of diced tomatoes increased the firmness and that calcium lactate had the greatest impact, increasing firmness by 48% compared with control tomato samples. Bolin & Huxsoll (1989) suggested that calcium could enhance tissue resistance to fungal or bacterial attack, by stabilising or strengthening cell walls.

The best decontamination treatments for reduction of initial psychrophile numbers were 1.5% or 3% calcium lactate solutions with 1.5 log cycle reductions recorded. However by Day 7, lower levels were recorded for chlorine treated lettuce. Delaquis *et al.* (1999) found the number of psychrotrophic bacteria on lettuce washed at 4°C and unwashed controls were not significantly different after 5 days storage at 1°C. In contrast, they found the development of psychrotrophic bacteria to be delayed in lettuce washed at 47°C.

The microflora of iceberg lettuce is dominated by Gram-negative bacteria, particularly species of pseudomonads (56.7%) *Serratia* (8.1%) and *Erwinia* (8.1); micro-organisms with little resistance to heat (King *et al.*, 1991). Exposure to a temperature of 50°C is probably sufficient to inactivate some of the more heat sensitive species on shredded lettuce. Pseudomonad levels were optimally decreased with calcium lactate treatments at 50°C at any concentration, and numbers remained at lower levels than for other treatments until Day 7 of storage.

The best decontamination treatment for reducing initial yeast populations was 3% calcium lactate at 50°C and this treatment maintained the lowest yeast numbers until Day 7. Delaquis *et al.* (1999) also found that yeast did not grow well on lettuce

treated with chlorine at 47°C. Luna-Guzman & Barrett, (2000) found delayed growth of yeast populations on cantaloupe melon treated with 2.5% calcium lactate at 60°C. Initially treatment with calcium lactate at 50°C was as effective as chlorine for reducing coliform counts. However, on subsequent storage, the lowest populations were recorded on chlorine treated lettuce. Delaquis *et al.* (2002) found that washing lettuce in chlorinated water at 4°C reduced initial populations of *E. coli* by 1 log cycle and that the effect was enhanced at 47°C. However, they also found that treatment with the higher temperature solution resulted in a 2 log cycle increase in numbers over a 14 day storage period compared with a decline in *E. coli* viability observed following treatment at 4°C. This is similar to the results recorded in the current study; the highest coliform populations were recorded for lettuce treated with calcium lactate at 50°C by Day 10 and the lowest were found on chlorine treated lettuce.

In the current study, a 1 minute treatment with calcium lactate at 50°C offered the best alternative to chlorine. The effect of treatment temperature at 50°C exceeded that of calcium lactate solution concentration. Based on results presented here, it appears that the application of a treatment at 50°C improves the microbiological decontamination properties and that the use of calcium lactate would maintain sensory qualities as well as contributing to the delay of microbial outgrowth due to the enhancement of tissue firmness. However, some populations seemed to achieve higher levels at the end of the 10 day storage time following treatment at 50°C. The use of a calcium lactate solution at 50°C as a washing step for minimally processed shredded lettuce would need to be accompanied with good hygiene and strict temperature control to prevent outgrowth of any spoilage or pathogenic microflora.

CHAPTER 6: Effects of chlorine and calcium lactate (2.5%) on Shelf-life extension of lettuce inoculated with *E. coli.*

In the previous chapters the effect of novel methods on reducing indigenous micro-flora of ready-to-eat lettuce were discussed and proved to be viable. An essential outcome of applying decontamination treatments is to extend the shelf life while also preserving important sensorial elements such as colour and texture. In this chapter, the efficacy of calcium lactate at room temperature as a novel decontamination treatment contributing to an extended shelf life was compared with industry standards, chlorine and water against a challenge population of non pathogenic *E. coli* ATCC 25922. This was numerically estimated by data fitting into the Gompertz model where parameters to calculate the lag phase of *E. coli* are derived. Outbreaks of *E .coli* O157:H7 infection have been linked to different lettuce varieties. In this study non-pathogenic *E-coli* was used in lieu of the toxigenic *E. coli* O157:H7. There have been no reports of significant differences in their survival and growth characteristics to date (McClure & Hall, 2000; Yokoigawa *et al.*, 1999).

6.1 Results

Following decontamination treatments, the lettuce samples were stored at 4°C to represent recommended refrigeration conditions and also at 10°C to represent mild temperature abuse conditions typically encountered in retail outlets. The experiment was conducted over a 10 day storage period. There were significant effects of storage temperature and decontamination treatment on the survival and growth of *E. coli.*

Growth rate curves were obtained by entering the data generated over the 10 days storage period into the Gompertz Model using Sigma Plot 2000. The bacterial growth

curve was measured and modelled under controlled temperature conditions. Generally good agreement between experimental data and fitted values was obtained: a minimum of 7 points were determined for each curve and each point was the mean of two determinations. Typical growth curves obtained are represented in Fig 6.1 where the effect of calcium lactate at 4 and 10°C is presented. As expected, it can be deduced from these plots that the lag phase duration was longer at 4°C compared to 10°C.

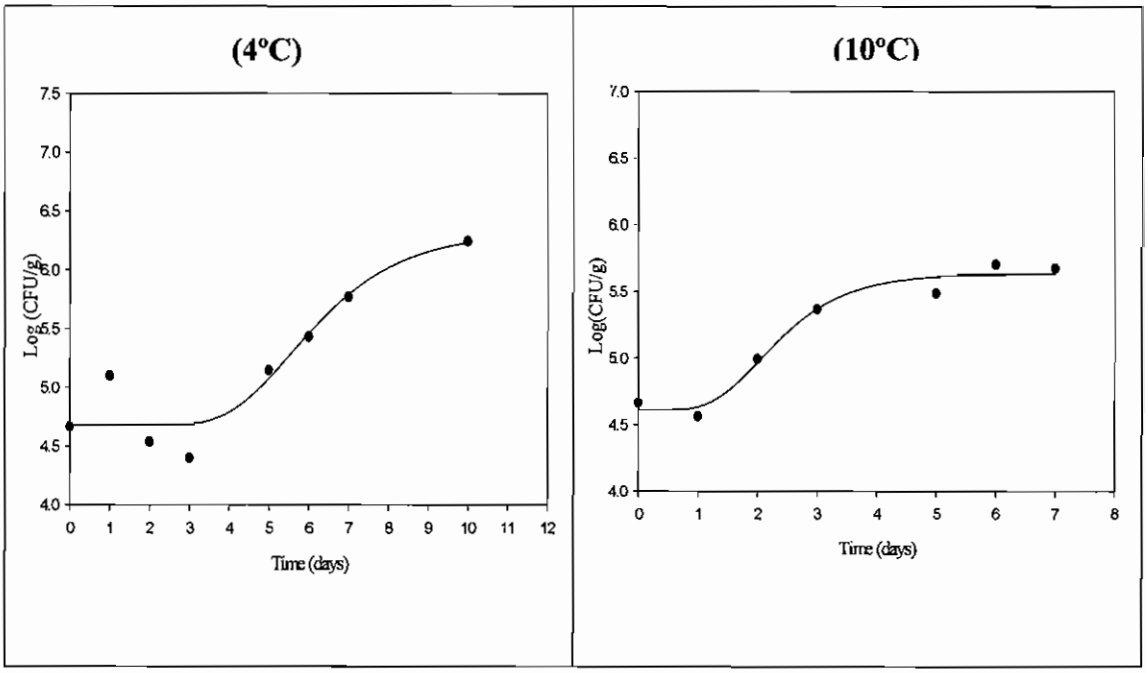

Figure 6.1: Growth rates generated from fitting the Gompertz model for lettuce samples decontaminated with calcium lactate and stored at 4 and 10°C.

The Gompertz model offers a number of parameters relevant to the growth rate curve such as the generation time, specific growth rate and the lag phase. Of particular interest to this study is calculation of the lag phase duration, which is an indirect estimation of the shelf life. Using the modified Gompertz model of:

$$\text{Log N (t)} = A+C \exp\{-\exp[-B(t-M)]\}$$

Where the lag phase duration is calculated as: M-(1/B)

Fitting the data obtained at the two storage temperatures studied, the following lag phase durations were calculated for the different treatments as shown below in table 6.1.

Table 6.1: Lag phase duration in days for *E. coli* populations at 4 and 10°C.

Treatment	Water	Chlorine	Calcium lactate
4°C	5.52	8	5.54
10°C	zero	1	0.7

Washing with water yielded a shelf life estimation of approximately 5.5 days at 4°C and was similar to washing with calcium lactate. There was a much longer estimation of shelf life for shredded lettuce using chlorine treatment.

At 10°C, the preservative effect of all treatments were negated by the elevated storage temperature and the lettuce shelf lives were estimated between 0 and 1 days (R^2 0.90—0.97). This represents a 12.5 times reduction in storage life with temperature abuse. The lag phase duration estimation was significantly different ($p< 0.05$) between the two storage temperatures studied.

6.2 Discussion

Extending the shelf-life of any RTE vegetable is of commercial interest as there are both microbiological and quality concerns to be addressed. The application of chlorine is under review in some EU countries; therefore novel washing strategies are being investigated to address both safety and quality aspects.

The results presented on the lag phase duration of *E. coli* are comparable to those reported by Conner & Kotrola, (1995) where *E. coli* populations on lettuce did not increase during 7 days storage at 4°C. Gunes & Hotchkiss, (2002) found that the

growth of *E. coli* O157:H7 populations on apple slices increased after 3 days storage in air at 15°C. Duffy *et al.* (1999) reported that the lag phase (LPD) of *E. coli* O157:H7 was approximately 0.3 days in brain heart infusion broth at 15°C. Li *et al.* (2005) in their studies on minced bison, found that at 5°C the number of *E. coli* O157:H7 remained constant after 8 day of storage. A lag phase at 5°C could not be predicted on raw sterile ground beef (Tamplin *et al.*, 2005), but at 10°C the lag phase duration was estimated to be zero.

Treatment with either calcium lactate at room temperature or water had comparable lag phases of around 5 days when lettuce was stored at 4°C. At 10°C, the lag phase duration of *E. coli* was significantly reduced below one day for all treatments, and it seems that the preservative effect of chlorine is linked to strict control of storage temperature.

Lactate and chlorine at low storage temperatures extended the shelf-life of the product, and as a typical commercial RTE lettuce product would be stored for up to 4-5 days at temperatures below 8°C, it seems relevant or possible that calcium lactate could be proposed as a substitute for chlorine.

The similarity of calcium lactate treatment to water alone for effects on background microflora populations has been discussed previously (Chapters 3, 4 & 5). Although the effects were similar, the sensory and nutritional advantages imposed with applying calcium lactate would be superior to using water alone.

Beside storage temperature, there are a number of environmental factors which influence the growth rate characteristics of micro-organisms on RTE lettuce, such as pH, water activity and the atmospheric composition. In this chapter the main focus was on storage temperature and its interactions with the three different washing treatments applied. For a perishable product like RTE lettuce, temperature abuse, as a

typical logistic issue at commercial retail outlets, would have a significant effect on shelf-life reduction. Also the effectiveness of the washing treatment was found to increase with decreasing storage temperatures.

CHAPTER 7: Conclusions

7.1 Efficacy of anti-microbial dipping treatments on the indigenous microflora of RTE Iceberg lettuce

- While all counts increased over the storage period, both traditional and innovative anti-microbial dipping treatments had significant effects on the indigenous populations of mesophiles, psychrophiles, pseudomonads, yeasts and coliforms in comparison with untreated lettuce samples.

7.2 Efficacy of calcium lactate, ozone and combination treatments in comparison with chlorine in decontamination of RTE lettuce

- The most effective decontamination treatment for reducing initial populations of total aerobic mesophiles, psychrophiles, pseudomonads and yeasts was found to be chlorine (100 mg l^{-1}) when compared with calcium lactate or ozone alone or an ozone-calcium lactate combination. This effect was observed to last throughout the 10 day storage period generally.

- The use of chlorine, ozone alone and ozone in combination with calcium lactate reduced initial populations by approximately 1.5 or 1 log cycle respectively, compared with untreated samples and this effect persisted until at least Day 3 of storage.

- The combination treatment of ozone plus calcium lactate was the most effective treatment for initial reduction of coliforms. This was the only population for which a synergistic decontaminant effect of the combination treatment was observed.

- The use of calcium lactate or ozone alone or in combination resulted in a decontamination efficacy comparable to or better than washing in water.

- While similar effects were observed using calcium lactate treatment and water alone on indigenous microflora, the sensory and nutritional advantages attributed to calcium lactate should allow it to be considered superior to using water alone.

- Lactate and chlorine at low temperatures extended the shelf-life of the product, and as a typical commercial RTE lettuce product would be stored for up to 4-5 days maximum, it seems possible to propose calcium lactate as a satisfactory alternative to chlorine.

7.3 Efficacy of organic acid anti-microbial dipping treatments on RTE lettuce

- Citric acid treatment was found to be a reasonably successful alternative to chlorine for microbiological decontamination of RTE lettuce.

- While acetic acid was also found to be significantly successful in reducing microbial populations its use would not be recommended as it was found to have a negative sensory impact.

- The persisting effects of citric and acetic acid treatments may have contributed to atmosphere modification which in turn contributed to reduced microbial populations.

7.4 Effects of treatment temperature and concentration on the efficacy of calcium lactate

- Since calcium lactate is reported to improve the sensorial properties of RTE lettuce, the effects of treatment temperature and concentration were investigated in this study. The microflora of Iceberg lettuce is dominated by Gram-negative bacteria which show little resistance to heat, particularly species of *Pseudomonas*, *Serratia* and *Erwinia*; exposure to a temperature of 50°C successfully reduced the indigenous microflora.

- Treatment with calcium lactate at 50°C for 1 minute offered the best alternative to chlorine. Initially treatment with calcium lactate at 50°C was as effective as chlorine, however, on subsequent storage, the lower populations were recorded on chlorine treated lettuce. The effect of treatment temperature at 50°C exceeded that of calcium lactate solution at lower temperatures.

- The best concentration of calcium lactate was found to be 3% for most microbial populations, with 1.5 – 2 log reductions observed.

- Based on results presented here, it appears that the application of a treatment at 50°C improves the microbiological decontamination properties and helps to maintain sensory qualities as well as contributing to the delay of microbial outgrowth due to the enhancement of tissue firmness.

- However, some populations were observed to achieve higher levels by the end of storage following treatment at 50°C. The use of a calcium lactate solution at 50°C as a washing step for minimally processed shredded lettuce would need to be accompanied with good hygiene and strict temperature control to prevent outgrowth of any spoilage or pathogenic micro-organisms.

- The application of a mild heat stress to avoid undesirable reactions associated with wounding may be an attractive method for quality retention in minimally processed lettuce.

- Lactic acid bacteria if detected were at very low levels. The numbers declined slowly over the storage period. No effects of treatment could be discerned.

7.5 Effects of chlorine and calcium Lactate (2.5%) for shelf life extension of lettuce inoculated with *E. coli*

- Treatment with either calcium lactate at room temperature or water had comparable lag phases when calculated using the Gompertz model of around 5 days when lettuce was stored at 4°C. At 10°C, the lag phase duration of *E. coli* was significantly reduced below one day for all treatments, and it seems

that the preservative effect of chlorine is linked to strict control of storage temperature. The results obtained provide preliminary indications to the potential replacement of chlorine with a novel decontaminant such as calcium lactate.

- A typical commercial RTE lettuce product would be stored for up to 4-5 days, it seems possible that calcium lactate could be utilised as a substitute for chlorine.

- A suitable combination of growth limiting factors might be the most successful approach to shelf life extension of minimally processed RTE vegetables; this could involve several sub-inhibitory treatments which when used in tandem exert a significant preservative effect.

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