MICROBIOLOGICAL CONSEQUENCES OF USING SODIUM DICHLOROISOCYANURATE AS A SURFACE DISINFECTANT FOR MINIMALLY PROCESSED SALAD VEGETABLES

SUBMITTED BY
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LIST OF ABBREVIATIONS

g	Gram
ml	Millilitre
L	Litre
cm	Centimetres
mm	Millimetres
lb	Pound
ppm	Parts Per Million
M	Molar
CFU/g	Colony Forming Units per Gram
pH	- Log (H^+)
°C	Degrees Celsius
n	Sample number
rpm	Revolutions Per Minute
Min	Minute
approx.	Approximately
pKa	Dissociation constant
ATCC	American Type Culture Collection
in-vitro	meaning "in glass". Biological process occurring, usually under experimental conditions.
in-vivo	Biological process occurring within a living situation.
GRAS	Generally Regarded As Safe.

MICROBIOLOGICAL CONSEQUENCES OF USING SODIUM DICHLOROISOCYANURATE AS A SURFACE DISINFECTANT FOR MINIMALLY PROCESSED SALAD VEGETABLES

ABSTRACT

The perception of Minimally processed (MP) salad vegetables as healthy and convenient has led to an increasing popularity for such foods. Concerns regarding the microbiological safety of minimally processed salad vegetables arise as their unique characteristics enhances the growth of microorganisms, including those which can grow at refrigeration temperatures. For food processors the challenge is to produce a safe product with extended shelf-life. Normal catering operations require well defined instructions for the safe handling and preparation of salad vegetables which exist for other food groups such as meat, dairy and fish products.

This research project was devised to examine the microbiological consequences of using NaDCC (sodium dichlorisocyanurate), commercially available as Aquatabs, as a surface disinfectant during the preparation of salad vegetables in catering or processing operations.

Experiments were designed to do the following:

1. To reduce the microbial load on the surface of cut butterhead lettuce during it's preparation using NaDCC as a chlorine dip.

2. To examine the antimicrobial effect of NaDCC on the different physiological groups of microorganisms present on cut lettuce and cabbage.

3. To examine the effect of NaDCC on foodborne pathogens commonly associated with vegetables *in-vivo*.

4. To compare Aquatabs to another commercially available chlorine product and two organic acid products as surface disinfectants for cut lettuce.

Results showed that treatment of cut lettuce with NaDCC (Aquatabs) significantly improved the microbiological quality. When applying the disinfectant to salad vegetables intended for refrigerated storage, the pH of the chlorine solution, drying post treatment and type of vegetable are important factors to be considered. Treatment of cut lettuce with NaDCC also significantly reduced pathogens *in-vivo* contributing to the microbiological safety of the product. The efficiency of NaDCC disinfectants was similar to the lactic acid disinfectants. The survival of microorganisms was attributed to the surface microenvironment of the salad vegetable which protected the microoganisms from the biocidal action of the disinfectants.

CHAPTER 1 : GENERAL INTRODUCTION

1.1 MINIMAL PROCESSING

Part one of this chapter outlines a general definition of minimal processing and what this term means in relation to vegetables. It points to the increased demand for these products and the safety considerations.

Part two considers the various criteria used to assess quality of minimally processed vegetables.

Part three discusses the antimicrobial activity of chlorine disinfectants.

1.1.1 MINIMAL PROCESSING ~ A DEFINITION

Minimal processing of foods has been defined by Ohlsson (1994) as covering a wide range of technologies and methods for preserving food during their transport from the agricultural producter to the consumer changing the inherent fresh-like attributes of the food as little as possible (minimally) at the same time as giving the food product sufficient shelf-life. King and Bolin (1989) defined minimal processing as encompassing any procedure short of traditional complete preservation procedures such as heat sterilization or freezing that adds value.

Several definitions have been put forward to describe minimally processed (MP) vegetables. Huxsoll and Bolin (1989) while describing MP vegetables as products that have the 'attributes of convenience and fresh-like quality', stated that 'the minimally processed product is raw and the cells of the tissues are alive, but these characteristics are not required'. King and Bolin (1989) describe MP vegetables as 'those prepared for convenient consumption and distributed to the consumer in a fresh-like state'. Cantwell (1991) called MP vegetables 'cut vegetables which are lightly processed'. In general, MP vegetables are viewed as products that contain live tissues or

those that have been only slightly modified from the fresh condition and are fresh-like in character and quality.

MP vegetable products are available in many forms as ready-to-cook or ready-to-eat vegetables. Examples of MP vegetable products are given in Table 1.1

Table 1.1: Examples of ready-to-eat and ready-to-cook minimally processed vegetable products.

Ready-to-eat	
Salad vegetables	Shredded carrots and shredded cabbage for coleslaw, diced onions, halved and chopped peppers, whole parsley, shredded lettuce, sliced tomatoes, cut endive and chicory.
Sandwich vegetables	Sliced tomatoes, shredded lettuce, diced onions.
Snack vegetables	Whole and sliced onions, celery strips, cut carrots, sliced cucumber, whole lettuce
Ready-to-cook	
Pizza topping vegetables	Strip-cut peppers, sliced mushrooms, sliced tomatoes.
Soup vegetables	Diced peppers, diced mushrooms, diced onions, strip-cut parsley , diced garlic, cross-cut leeks.
Stew vegetables	Cut green beans, sliced onions, diced mushrooms and potatoes, brussel sprouts, diced tomatoes

1.1.2 MINIMAL PROCESSING OF VEGETABLES

The major differences between MP vegetables and raw vegetables are the specific processing and preservation steps taken. Processing of MP vegetables has been described by Rolle and Chism (1987) as including all unit operations such as washing, sorting, peeling or slicing that might be used prior to blanching on a processing line. The result of processing is a highly

perishable product with a short shelf-life. Refrigeration and packaging which may be optional for raw fresh intact vegetables become mandatory for MP vegetables.

MP vegetables require special preservation methods for the purpose of extending storage life and preventing spoilage. The problems can relate to both enzyme and microbe control. Minimal processing technologies and methods discussed by Ohlsson (1994) include:

- Modified Atmosphere (MA) storage;
- post-harvest treatments ~ chemical treatment with disinfectants or preservatives;
- clean room technologies ~ elimination of microbiological contamination from humans and the environment;
- protective microbiological treatment ~ lactic acid bacteria producing bacteriocins;
- non-thermal processing ~ high pressure, irradiation, electric pulses,
- thermal processing ~ mild heating methods, sous vide;
- packaging ~ Modified atmosphere packaging, active packaging, edible coatings;
- combination method ~ more than one minimal processing method.

Commercial preparation of salad vegetables in salad manufacturing plants and catering operations like delicatessens, supermarkets and restaurants is becoming common practice. Generally, the process involves minimal processing of large volumes of salad vegetables by washing along with cutting, peeling, slicing etc... followed by refrigerated storage, so that the produce is ready-to-use. Commercial manufacturers can use modified atmosphere packaging to extend the shelf-life and satisfy the growing consumer demand for such products. In catering operations minimal processing can save time and be more cost effective.

The need for steps to ensure microbiological safety of these foods during preparation and storage is highlighted by the rise in food related illnesses in the USA. associated with the consumption of raw vegetables (Bryan 1988). Normal catering operations may not include well-defined instructions for the safe handling and preparation of salad vegetables as may exist for the other food groups such as meat, fish and dairy products. The safety issues concerning salad vegetables are often overlooked. This study deals with the commercial preparation of salad vegetables and the microbiological consequences of using chlorine as a surface disinfectant.

1.1.3 CONSUMER DEMAND AND MARKET TRENDS

Minimally processed fresh fruits and vegetables have been described as the 'new cornucopia of produce' reflecting the increase in demand for quality and freshness (Lucier and Bertelsen, 1994). Fresh produce processing is not a new business, but commercial processing of fresh produce is relatively new to the food industry. Hurst and Schuler (1992) describes fresh produce processing as a 'symbol of the times'.

Trends in the food markets are strongly influenced by the changing attitudes of consumers to foods. Ohlsson (1994) identified four major trends in the food habits during the nineties to include:

- diet and health;
- convenience and simplicity;
- uncertainty concerning food safety and the need for guarantees;
- food is part of the good-life ~ a rising interest in food and cooking with emphasis on natural ingredients and cooking from scratch, including ethnic foods.

Consumers perceive MP fresh produce as being healthy and convenient, offering a clean, ready-to-serve product having fresh-like qualities. The food service industry considers MP fresh vegetable products, such as shredded cabbage for coleslaw or sliced potatoes, as standardised products reducing waste and labour costs (Hurst and Schuler, 1992). Some industry analysts predict MP fresh produce will be a 4 to 8 billion dollar market by the year 2000.

The increase in sales of MP vegetables reflects the expanding market for vegetables. For example, the annual value of the USA lettuce crop from 1986 to 1990 was estimated to be $850 million (USDA, 1988) and partially processed lettuce increased from 3.2% to 10% of total lettuce production. A large percentage of fresh produce is marketed through fast food outlets. One fast chain alone consumes more than 5% of the total USA production of iceberg lettuce (Bradshaw, 1988).

Over the last 10 to 15 years, increases in consumption of vegetables in the US were for items traditionally found in salads (Lucier and Bertelsen, 1994). The annual increase in fresh vegetable consumption per person since 1980 included tomatoes (up 3.1 lb), all lettuce varieties (up 1.5 lb), green peppers (up 3 lb), onions (up 4.3 lb) and spinach (up over 0.5 lb). Speciality varieties of lettuce accounted for almost all of the increase in lettuce consumption in the U.S. since 1980. Fresh broccoli and cauliflower, which are also frequently used in salads were viewed as the vegetable market stars during the 1980's. Consumption more than doubled for fresh-market broccoli and was up 109% for fresh cauliflower.

In Ireland, consumers' expenditure on fresh vegetables was £151 million at retail level in 1995, a 4.5% increase on 1994 (An Bord Glas, 1995). Loose vegetables accounted for 51% of vegetable sales and prepacked vegetables accounted for 41% of sales. A market research also completed by an Bord Glas (1995) on the prepared, wet and dry salad / vegetable market

estimated this market to be worth £25 million. The value of vegetable raw materials used to manufacture the salads was £4.2 million. Coleslaw cabbage, carrots, potatoes and lettuce were the most important vegetables and further growth in the sector was predicted for the following years.

1.1.4 SAFETY CONSIDERATIONS FOR MP VEGETABLES

Minimal processing of vegetables changes the product from a microbiologically stable to unstable product. The cutting, peeling and slicing of the intact vegetables affects the microbiology in several ways. Firstly, cutting releases nutrients from the ruptured cells that can be used by the microorganisms. Cutting also provides an increased surface area which can lead to faster microbial growth. Consequently, higher populations of microbes usually develop in cut versus whole vegetables. Priepke *et al.* (1976) showed that cut salad vegetables (lettuce, carrots and celery) had a significantly higher total plate count than intact vegetables after 4 days storage at 4.4°C in sealed packages under normal atmosphere.

Such proliferation can lead to microbial spoilage and growth of pathogens which may be present. The primary safety issue with MP foods, in particular ready-to-eat foods, is the presence of pathogens especially the Listerias, Yersinias and Aeromonads which are psychrotrophic and capable of growing at refrigeration temperatures, which is widely relied upon to combat microbial growth and ensure quality retention. In addition, the high moisture content of MP vegetables, the lack of a terminal treatment step, and the potential for temperature abuse by consumers further intensifies the risk of foodborne illness. Pathogens causing foodborne illness attributed to vegetables include *Salmonella* spp. (O'Mahony *et al.*, 1990), *Shigella* spp. (Davis *et al.*, 1988), *Vibrio cholerae* (Swerdlow *et al,*. 1992), *Listeria monocytogenes* (Ho *et al.*, 1986), *Bacillus cereus* (Portnoy *et al.*, 1976),

Enteropathogenic *Escherichia coli* (Merson *et al.*, 1976) and *Clostridium botulinum* (Solomon *et al*,. 1990). Bryan (1988) reported that vegetables caused 4.9% of foodborne disease outbreaks.

Another important reason for paying close attention to food safety is that foodborne disease can cause catastrophic economic problems for the companies and individuals involved. Todd (1989) estimated that over 12 million cases of foodborne disease costing $8.4 billion occur each year in the USA. This estimate did not include the costs to food companies resulting from litigation, recall procedures and lost of sales due to adverse publicity.

1.2 QUALITY OF MINIMALLY PROCESSED VEGETABLES

1.2.1 QUALITY ~ A DEFINITION

The term "Quality" is one of the most defined terms used in the food industry. The British standard (BS 4778:1979) states that the word quality is used for several distinct purposes. Quality can be 'comparative', whereby products are ranked relative to certain criteria. Alternatively quality can be 'quantitative' as is common in food manufacturing when discussing the acceptable quality level of a batch of product, or in other words the extent of departure from the ideal. The third, and perhaps most widely used concept of quality is the 'fitness for purpose' which relates to the ability of a product to satisfy a given need. Arthey (1975) defined quality as the relative value of several characteristics of a product to the buyer and ultimately the consumer. A simpler definition, therefore, is "the totality of features and characteristics of a product that bear on its ability to satisfy a given need" (European Organisation for Quality Control, 1976).

1.2.2 PERCEPTION OF QUALITY

In a USA survey carried out by Zind (1989) on consumer attitudes towards fresh fruit and vegetables, 96% of respondents cited ripeness and freshness as important selection criteria, while an equal percentage cited 'taste'. In addition, 94% indicated the importance of appearance and condition, while 66% used nutritive value and 63% used price as a guide. The survey report indicated that 40% of produce-purchase decisions of supermarket shoppers are made in the produce department while only 20% are made in advance. This emphasises the importance of appearance in the purchase decision.

According to Zind (1989) external characteristics, those perceived by the senses of sight and touch without ingesting the product, and internal

characteristics, those perceived by the senses of taste and smell and touch (mouth feel) all combine in determining acceptability and presumably the decision to purchase the product. Other less-tangible characteristics, such as nutritional value, wholesomeness, and safety of a product require sophisticated equipment to measure and are not readily determined by most consumers, but the perceptions of these attributes by the consumer affect both differentiation and acceptability of a product (King and Bolin, 1989).

1.2.3 SENSORY QUALITY OF MP VEGETABLES

Minimally processed cut vegetables are highly perishable and subject to sensory deterioration caused by physical, biochemical and microbiological changes. All spoilage mechanisms are interdependent and contribute to disorders in MP vegetables. Physical damage of plant tissue releases cellular nutrients and moisture which increases the growth of spoilage microorganisms. The action of released cellular enzymes and spoilage microorganisms can cause further physical deterioration.

1.2.3.1 *Physical changes*

Wounding stress caused by cutting and shredding of MP vegetables results in metabolic activation. The main physiological manifestations of this phenomenon include increased respiration rate, and the responses depend on the magnitude of the stress. The O_2 consumption rate of shredded endive is 1.2 times that of intact endive (Chambroy, 1989). This ratio increases to 1.4 for broccoli (Ballantyne, 1987) and to 2 for shredded lettuce (Ballantyne, 1986). For more damaged plant tissue, respiration averages 3 to 7 times that of intact tissue, for example, 4 to 7 times for grated carrots (Carlin, 1989; MacLachlan and Stark, 1985).

1.2.3.2 *Biochemical changes*

Enzymes and substrates are normally located in different cellular compartments and their transfer activity regulated. However, when they are brought into contact as a result of cellular damage, enzymatic reactions follow which can cause sensory deterioration such as discolouration , loss of flavour and loss of firmness. Deterioration of colour is primarily due to enzymatic browning of cut surfaces. The yellowish brown to black pigments that are formed can appear very rapidly and are unappetizing. In the intact tissue the enzymes responsible, generically referred to as the 'phenolases' are separated from the substrate. However, when they are brought into contact as a result of damage, the naturally occurring phenolic compounds are enzymatically oxidised to form yellowish quinone compounds (Mayer, 1987). A sequence of polymerization reactions follows to give brown products called melanins. Changes in chlorophyll due to loss of membrane integrity (Rolle and Chism, 1987) and degrading enzymes (Watada *et al.*, 1990) can also cause colour changes in MP vegetables.

Loss of texture is related to the weakening of the polymeric structures of the plant cell wall and may be accompanied by a loss of turgor (King and Bolin, 1989) which is a result of exudation (Carlin *et al.*, 1990). Bolin *et al.* (1977) and Bolin and Huxoll (1991) found that shredded lettuce darkened during storage and that this change was accompanied by a loss of visual green pigmentation, which was greater at 10°C than at 5°C, and greater at 5°C than at 2°C. Flavour and appearance of stored cut vegetables (lettuce, carrot, endive, celery and radish) were judged on a hedonic scale of 1 to 9 by Priepke *et al.* (1976). A score of 9 was considered to be excellent, 5 just acceptable and 1 extremely poor. A high score indicated presence of typically fresh flavour. Results showed that flavour and appearance deteriorated faster in cut vegetable than in intact vegetable tissues. Williams (1990) found discolouration, odour development and drying of tissues in prepared mixed

salads to be determinants of the shelf-life, and showed that the temperature of these changes followed a square root relationship. Loss of moisture from vegetable products (as little as 5% by weight) mainly due to transpiration can lead to shrivelling and wilting and increases the rate of senescence.

1.2.3.3 *Microbiological changes*

Microflora responsible for spoilage of MP vegetables include a large number of indigenous fungal and bacterial species. The majority of the bacteria responsible for spoilage of vegetables are Gram negative. Of these, *Erwinia* is the most aggressive, causing soft rot in most vegetables. The *Erwinia* soft rots initially starts as soft, water soaked areas on the vegetable tissue. As the infection proceeds, the area of rotting expands until complete collapse of the tissue occurs (Lund, 1983). The fluorescent pseudomonads are another common and important group of spoilage organisms causing soft rot in many types of vegetables including celery, potato, chicory, lettuce, Chinese chard and cabbage (Brocklehurst and Lund, 1981). The psychrotrophic nature of these pseudomonads makes them important spoilage organisms in MP vegetables. Although many different types of fungi can be associated with the spoilage of vegetables (Bulgarelli and Brackett, 1990), only relatively few cause most spoilage problems. Fewer still are able to spoil vegetables at refrigeration temperatures. Spoilage by fungi is more likely to occur in humid conditions and when refrigeration is not maintained.

Chilled storage is a major factor in the stability of MP cut vegetables. Storage at low temperatures can retard the deteriorative changes by reducing the rate of respiration, loss of colour and growth of spoilage microorganisms that limit shelf-life. Attention to the selection of the raw vegetables in order to achieve high quality cannot be over emphasized, since subsequent processing cannot compensate for selection of poor quality raw produce, particularly for ready-to-eat vegetable salads as 'freshness' is one of the most important criteria for their purchase.

1.2.4 NUTRITIONAL QUALITY OF MP VEGETABLES

Most studies of MP vegetables have been concerned with market quality as determined objectively and subjectively by colour, flavour and texture measurements. Little is known of the nutritional consequences, which primarily relate to the losses of vitamin C (ascorbic acid), of minimally processing vegetables. Vitamin C is mainly present in vegetables as L-ascorbic and to a lesser extent as L-dehydro ascorbic acid, both compounds showing full vitamin C activity.

The stability of vitamin C depends on the internal factors of the vegetable and for leafy vegetables on the condition and time of harvest (Salunkhe *et al.*, 1987). Ascorbic acid in vegetables degrades mainly by enzymatic oxidation to 2,3 diketogulonic acid which has no vitamin C effect. Factors which influence the oxidation rate, such as temperature and storage atmosphere, affect the stability of vitamin C. Bognár (1990) investigated the vitamin status of chilled food and found that vitamin C decreased as storage temperature increased. The effects of controlled atmospheres and modified atmospheres on the vitamin C of intact vegetables varied. Kurki (1979) found that the vitamin C content of leek over a period of 4 months in controlled atmospheres (1% O_2 and 10% CO_2) and cold air storage showed little difference.

Early results on the effect of minimal processing on vegetables suggest that higher losses of vitamin C might be expected than in untreated vegetables (Bognár, 1987). Cutting of the vegetable accelerates the enzymatic degradation of ascorbic acid. Vitamin C losses, for instance, of cut parsley and white cabbage after 2 days storage at 4°C and 20°C were nearly twice as high as for untreated produce.

1.3 CHLORINE

1.3.1 THE USE OF CHLORINE IN THE FOOD INDUSTRY

Chlorine was first produced for use as a bleaching agent for the textile industry in 1785, 24 years before the discovery of chlorine as an element by Sir Humphrey Davy in 1809. It was not until the nineteenth century that the disinfecting properties of chlorine were recognised and chlorinated lime was used for water treatment and disinfection in hospitals. In 1881, the German bacteriologist Koch demonstrated that bacteria could be destroyed by hypochlorite. Five years later, the American Public Health Association issued a favourable report on the use of hypochlorites as disinfectants (Hadfield, 1957). Today, it is rare to find municipal water that is not treated by chlorination (Dychdala, 1983). Later, the use of chlorine or 'active chlorine compounds' gained widespread acceptance in other industries. The United States Milk Ordinance and Code of 1939 recommended chlorine as one of the agents available for sanitizing milk equipment after cleaning (Mercer and Somers, 1957). The canning industry also began to use chlorinated water to cool heat-sterilized cans to prevent 'leaker' damage (Wei *et al.*, 1938).

Extensive use of chlorine in food industry plants began with the development of 'break-point' or 'in-plant chlorination'. Break-point chlorination refers to chlorination beyond the water demand resulting in a residual of free available chlorine (Griffin, 1946). As a result of in-plant chlorination, bacteria and microbial slimes were practically eliminated from processing equipment and bacterial counts in the end products were significantly lower (Mercer and Somers, 1957).

By 1946, several conferences had been held concerning the use of chlorine in the food industry and conclusions reached include the following (Kirk and Mitchell, 1980):

- The use of chlorine prevents or greatly reduces the accumulation of microbial slimes on equipment that is continuously or frequently washed with chlorinated water. It also prevents the development of off-odours from fermentation and decay.
- The use of chlorine permits longer hours of operation by reducing the time for clean-up.
- The total bacterial counts on finished products are reduced when raw products are washed in chlorinated water.
- No apparent corrosion of equipment is observed when reasonable levels of chlorine are used.
- Chlorine should not be used indiscriminately.

Thus, the use of chlorine as a sanitizer in the food industry has become common-place because of its effectiveness as an antimicrobial agent. Chlorine is presently a Generally Recognised As Safe (GRAS) chemical. The use of chlorine as a direct or indirect food additive is permitted because of its GRAS status.

1.3.2 USE OF CHLORINE ON MP VEGETABLES

In the recent past, conventional wisdom suggested that refrigeration temperatures from -2.2°C to 4.4°C (Anon, 1989) would generally control the growth of pathogenic and spoilage organisms. Refrigeration provides effective protection to the consumer from risk of food poisoning and minimises the possibility of poor quality. However, the emergence of psychrotrophic pathogens (*Listeria monocytogenes* and *Yersinia enterocolytica*) in refrigerated foods, in particular MP foods which rely heavily on refrigeration as the main preservation method, has increased the need for diligence in preventing microbial contamination of MP foods. Gibson (1990) posed the question 'Microbiology of chilled foods – is refrigeration enough?' and concluded that

refrigeration alone cannot ensure food safety but is still of great importance for the safe handling of food.

Included in the minimal processing methods of foods outlined by Ohlsson (1994) is post harvest treatment of vegetables with disinfectants. One disinfectant commonly used in industry for the surface disinfection of vegetables is chlorine (Lund, 1983). Chlorine compounds are used in connection with washing MP vegetables and are sometimes the primary preservation agent. Adams *et al.* (1989) found that treatment of shredded lettuce with 100ppm free chlorine significantly reduced the microbial load. Garg *et al.* (1990) found that washing of fresh-cut vegetables with 300ppm chlorine significantly reduced the microbial populations but the positive was dependent on maintaining high levels of free chlorine.

Treatment of vegetables in chlorinated water has been shown to be effective against a number of pathogens on salad vegetables under laboratory conditions. Such experiments include significant reductions of *Salmonella montevideo* on tomatoes (Wei *et al.*, 1995; Zhuang *et al.*, 1995), *L. monocytogenes* on shredded lettuce (Zhang and Farber, 1996) and brussel sprouts (Brackett, 1987a) and *Vibrio cholerae* on lettuce (Uboldi Eiroa and Porto, 1995). In all these studies the free chlorine solutions were prepared using the commercially available active chlorine compounds: sodium/calcium hypochlorite or sodium dichloroisocyanurate.

1.3.3 MECHANISM OF CHLORINE DISINFECTION

Chlorine in an aqueous solution, even in minute amounts, exhibits fast bactericidal action in the form of Hypochlorous acid (HOCl). Hypochlorous acid was first identified in 1904 to be responsible for the destruction of microorganisms. However, the mechanism of this activity is not fully understood despite extensive research in this field (Dychdala, 1983). In

general, it is considered that the lethal action on organisms is due to chlorination of cell protein or enzyme systems by non-ionised hypochlorous acid, causing hydrolysis of peptidic chains of cellular membranes of bacteria (Green and Stumpf, 1946; Knox *et al,*. 1948).

1.3.4 FACTORS AFFECTING THE BIOCIDAL ACTIVITY OF CHLORINE

A long history and wide usage of chlorine compounds have yielded much laboratory and field evaluation, mostly concerning hypochlorites, but with application to all active chlorine compounds to some extent (Dychdala, 1983). Chlorination is a dynamic chemical process. The efficiency of the chlorination process is ever-changing and factors normally influencing the biocidal capacity include pH, concentration, temperature, organic material and hardness of water.

1.3.4.1 *pH*

pH has perhaps the greatest influence on the antimicrobial activity of chlorine in solution. The relationship between pH and the degree of dissociation of HOCl to hydrogen ions (H^+) and hypochlorite ions (OCl^-) (figure 1.1b) plays a major part in determining the antimicrobial action of chlorine. Hypochlorous acid is the desired form for chlorination, having a stronger bactericidal and sporicidal activity than the hypochlorite ion which is relatively inactive. At a pH slightly above neutral, half of the chlorine will be in the form HOCl and reducing the pH of the solution increases the concentration of HOCl and hence the biocidal capacity.

The greater biocidal capacity of sodium dichloroisocyanurate (NaDCC) compared to sodium hypochlorite (NaOCl) has been partially attributed to the lower pH of NaDCC solutions. Hence, the pH of NaDCC solution (approximately 6.0), being lower than pH of NaOCl solutions (ranging from

8.5 to 11.5), gives a much higher percentage of undissociated hypochlorous acid (Bloomfield and Miles, 1979).

1.3.4.2 *Concentration*

The concentration of chlorine in solution is normally measured in parts per million (ppm), which indicates the number of units of available chlorine by weight. It is logical to assume that an increase in concentration in available chlorine in a solution would bring a corresponding increase in antimicrobial activity. This supposition holds true as long as other factors such as pH, temperature and organic content are held constant. Weber and Levine (1944) tested hypochlorite at concentrations of 25, 100 and 500ppm of available chlorine at a constant pH of 10 and temperature of 20°C. The times required to provide a 99.9% kill of the resistant *Bacillus metiens* spores were 31 minutes for 500ppm, 63.5 mins for 100ppm and 121 mins for 25ppm available chlorine. They concluded that a 4-fold increase in concentration of free chlorine using hypochlorite will result in a 50% reduction in killing time, and a 2-fold increase resulting in a 30% reduction.

1.3.4.3 *Temperature*

The activity of chlorine increases as the temperature of the solution increases. Collins (1995) showed that a 3ppm available chlorine solution using calcium hypochlorite ($Ca(OCl_2)$) produced 99% kill in 4 mins at 21°C but, on average, in 10 mins at 4.4°C. The effects of temperature on the bactericidal action of free available chlorine are especially evident at pH levels higher than 8.5 and when the chlorine residuals are low (0.02 to 0.03ppm available chlorine) (Butterfield *et al.*, 1943).

1.3.4.4 *Organic Material*

Chlorine has a particular affinity for organic matter. The presence of organic material in chlorine solutions will cause a reduction in free available chlorine and thus lower the capacity for bactericidal action, particularly at low concentrations (Dychdala, 1983). A comparison of the two active chlorine compounds NaOCl and NaDCC, found that NaDCC solutions were less inactivated in the presence of organic matter (Coates, 1988; Bloomfield and Uso, 1985). This can be explained by the fact that 50% of the total available chlorine is 'free' with NaDCC and the rest is 'combined' in the form of mono and dichloroisocyanurates. The equilibrium remains constant until there is a chlorine demand on the solution from microorganisms, organic or nitrogenous material, which displaces hypochlorous acid. This equilibrium provides for the self regulation, progressive release of 'free chlorine' found using NaDCC solutions giving improved efficiency when compared to NaOCl solutions (Bloomfield and Miles, 1979; Bloomfield, 1973).

1.3.4.5 *Hardness of water*

Water hardness components such as Mg^{2+} and Ca^{2+} ions do not exhibit any slowing affect on the antibacterial action of hypochlorite solution. Shere (1948) evaluated a sodium hypochlorite solution with 5ppm available chlorine at 0 and 400ppm hardness at 20°C. He obtained a complete kill of bacterial organisms at the two examined levels of hardness, indicating that hardness in water exhibited no inhibitory action on the bacterial kill by hypochlorite solution.

1.3.5 SODIUM DICHLOROISOCYANURATE

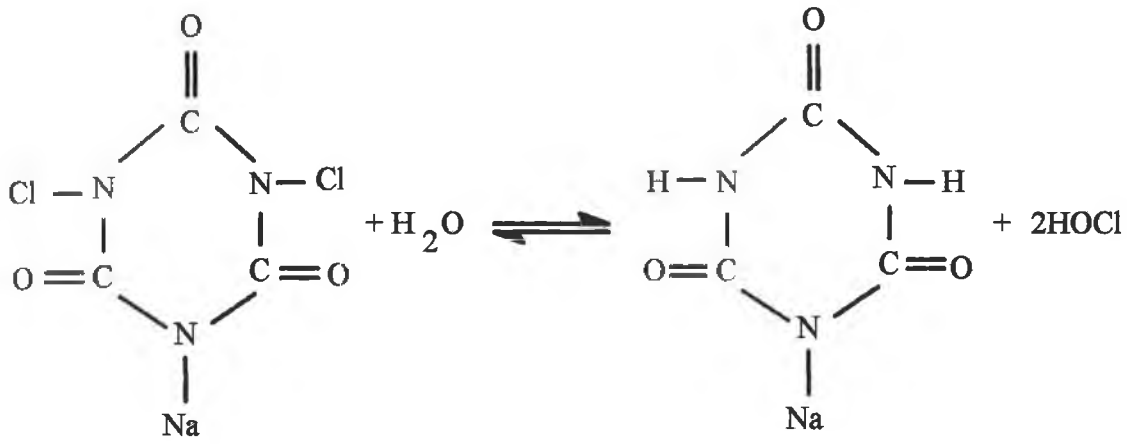
The active chlorine compound chosen for the purpose of this study was Sodium dichloroisocyanurate (NaDCC). NaDCC was chosen instead of HOCl

due to it's greater biocidal capacity than NaOCl and also because it is least inactivated by organic material.

NaDCC is the sodium salt of 1,3-dichloro-1,3,5-triazine-2,4,6 (1H,3H,5H)-trione. NaDCC is a synthetic organic, chlorine donor derived from isocyanurate. It is a white crystalline or granular powder of molecular weight 219.9 containing approximately 62% of 'available chlorine'. A description of sodium dichloroisocyanurate is in the chemical abstracts: 2893-78-9 (Reynolds, 1989).

Chloroisocyanurates have been approved by the Department of the Environment (UK) to be used in tap water applications (The Water Supply [Water Quality] Regulations 1989 as amended by the Water Supply [Water Quality][Amendment] Regulations 1991, Regulations 25 and 26). NaDCC containing up to 100ppm free chlorine was added in the USA to the list of chemical disinfectants permitted in the food-processing industry for use on equipment and utensils and on food contact surfaces in public eating places (USDA, 1976).

When NaDCC is added to water, monosodium cyanurate (a non-toxic compound) and hypochlorous acid (the active compound) are very quickly liberated by the mechanisms shown in Figure 1.1a. The hypochlorous acid molecule dissociates to produce ions of hydrogen and hypochlorite as shown in Figure 1.1b depending on the pH of the solution.



Sodium Dicloroisocyanurate **Water** **Monosodium cyanurate** **Hypochlorous acid**

(a)

$$HOCl \rightleftharpoons H^{+} + OCl^{-}$$

Hypochlorous acid **Hydrogen ion** **Hypochlorite ion**

(b)

Figure 1.1: a) Chemical reaction of sodium dichloroisocyanurtate with water, liberating monosodium cyanurate and hypochlorous acid.

b) Dissociation of hypochlorous acid.

1.4 OBJECTIVES

1.4.1 THE OBJECTIVES OF THE PRESENT STUDY

The aim of this study is to investigate the microbiological quality of ready-to-use vegetables can be improved through the application of a chlorine based surface disinfectant. The principle vegetable type examined was butterhead lettuce; this vegetable is widely consumed as a salad ingredient. In some experiments a second vegetable type was examined for comparative purposes; white cabbage was chosen as it is commonly used in dry salads and as a constituent for coleslaw preparation.

Surface disinfection has two aims:
1) to enhance the shelf-life of the product
2) to ensure a safer product for consumption.

In order to achieve these aims in the commercial preparation of vegetables a surface disinfectant should meet the following criteria:

- capable of dilution in tap water
- safe to apply in a food operation
- act to reduce the total microbial load on the vegetable surface
- act to reduce spoilage organisms, in particular the psychrotrophic organisms which can contribute to spoilage during refrigeration.
- act to reduce the growth of potential pathogens which may contaminate the produce during cultivation or processing.

The disinfectant used in this study is marketed under the trade name of *Aquatabs* and contains sodium dichloroisocyanurate as active ingredient; its antimicrobial effect was to be assessed in terms of achieving a significant reduction in microbial load on vegetables soaked in tap water containing NaDCC compared to those soaked in tap water only.

Objectives:

1. To establish an experimental method to assess the effect of applying a surface disinfectant to butterhead lettuce in conditions simulating the preparation and minimal processing of this vegetable in commercial practice;

2. Having established a reduction in total microbial load, to determine the antimicrobial effect of the disinfectant on selected microbial populations on both lettuce and white cabbage;

3. To develop a method of inoculating lettuce leaves with a pathogen and establishing growth of this organism on the vegetable surface with a view to determining the antimicrobial effect of NaDCC on a selection of pathogens;

4. To compare the antimicrobial activity of NaDCC on microbes present on lettuce with that of three other commercial disinfectants.

CHAPTER 2 : THE APPLICATION OF NADCC AS A SURFACE DISINFECTANT IN THE PREPARATION OF LETTUCE

2.1 INTRODUCTION

2.1.1 MICROBIAL LOAD AND SPOILAGE OF MP SALAD VEGETABLES

Microbial spoilage of MP vegetables has been investigated by several authors and attempts have been made to correlate spoilage with the microbial load. In many cases the level of mesophilic bacteria at the beginning of storage can be a useful indicator of storage stability. Bolin *et al.* (1977) found that the microbial load of the initial product influenced storage stability. Shredded iceberg lettuce that initially had a count of 5.8×10^3 bacteria per gram was inoculated with organisms, extracted from salad leaves, to give two higher levels of inoculation, 1.2×10^5 and 5.1×10^6 organisms per gram. Results showed that samples containing the higher levels of microbial load had a shortened shelf-life in heat sealed polyethylene bags at 5°C. Barriga *et al.* (1991) reported a linear relationship between the number of psychrotrophs on shredded iceberg lettuce and a decrease in visual score during storage at 4°C, however, a controlled atmosphere (3% O_2 and 10% CO_2) delayed spoilage development without reducing the microbial load. They concluded that a high CO_2 level controlled both microbial and plant enzyme activity without appreciably affecting microbial growth. However, other published reports have shown total bacterial counts at the end of storage to be unrelated to sample quality. For shredded carrots, total counts of mesophilic bacteria were similar in spoiled samples and in samples with good appearances (Carlin *et al.*, 1989) and despite the high numbers and types of spoilage bacteria found in retail packs of mixed vegetable salads one day after the sell-by date, they appeared to be of acceptable quality (Brocklehurst *et al.*,1987).

Studies carried out by King *et al.* (1991) plotted the visual score of shredded iceberg lettuce, the percentage of leaf pieces with discolourations, and the number of microorganisms against length of storage at 2°C over 26 days. The produce was sealed in polyethylene bags. Results showed that the

extent of microbial growth and visual score were not linear, in contrast to the increase in discoloured leaves. Examinations of pieces of lettuce showed they were not visually affected by microbial growth. Instead, the discoloured and soft spots seemed to be related to physical damage of the lettuce tissue as the discoloured areas were usually edges of lettuce pieces that had physical damage from bruising or bending. A relationship between microbial number and visual score did occur only after a period of storage in which respiration and lettuce metabolism caused the tissue to become stressed and less resistant to microbial attack. They concluded that spoilage of lettuce is complex and is caused by many factors and that microbial spoilage becomes significant during the last days of storage.

The complex relationship between microbial load and shelf-life is an important factor when processing a MP salad vegetable and extending the shelf-life of the product. In this study the potential of a surface disinfectant to reduce the initial microbial load on the surface of cut lettuce was investigated. The effectiveness of introducing a disinfectant step in the wash protocol was measured in terms of a reduction in the initial microbial load post-disinfection and the impact of refrigerated storage on this load.

2.1.2 PROCESSING OF SALAD VEGETABLES

The commercial preparation of salad vegetables falls within the disinfection of a minimal process. In general, preparation includes processing (peeling, cutting, slicing...) of salad vegetables along with more than one wash step. Washing serves a number of purposes:

- Pre-washing before processing removes soil, mud, and sand residues.
- A second wash after processing removes microbes and tissue fluid containing the released cellular nutrients and oxidation enzymes, thus

retarding microbial growth and reducing enzymatic oxidation during storage.

- Washing acts as an effective kill step in the processing of vegetables with the addition of antimicrobials to the wash water.

Many compounds have been evaluated for their ability to sanitise ready-to-use vegetables including citric acid (Shapiro and Holder, 1960), lactic acid (Diez *et al.*, 1979), antibiotics (Shapiro and Holder, 1960; Lund, 1983) and probably the most widely used, free available chlorine (Lund, 1983).

Other factors which are important in the processing of cut lettuce are cutting, rinsing and drying procedures. When lettuce leaves are cut microbial growth is accelerated due to the presence of nutritious internal fluid leaked from cut tissue. Also, the cut surface provides increased surface area on which the microorganisms can grow. Studies carried out by Priepke *et al.* (1976) showed that total aerobic microorganisms on intact lettuce and celery increased more slowly than the cut vegetables stored in permeable polyvinylidene bags at 4.4°C. They also showed an increase in respiration rates for cut vegetables and a decrease in sensory quality. Bolin *et al.* (1977) found that cell rupturing, caused by cutting lettuce, is a critical factor that influences the physiological breakdown due to the exudation of cellular fluids. Results showed that an increase in cell exudation leads to a decrease in storage-life. They recommended tearing lettuce or the use of sharp blades to minimise cell rupturing as dull blades caused greater damage to tissue, and consequently a greater loss of cellular fluids.

Herner and Krahn (1973) indicated the importance of keeping cut lettuce dry, and even advocated not rinsing at all before storage. However, later studies carried out by Bolin *et al.* (1977) found that fluids, in particular the active enzymes which coat the cut product after slicing, need to be removed by rinsing and drying. Enzymes such as polyphenol oxidase, catalase and peroxidase were all found to be present on the surface of cut lettuce and

can account for rapid deterioration of the cut product. Also, studies showed a decrease in lettuce quality during storage if the surface was left wet (Bolin *et al.*, 1977).

Hence, rinsing and drying procedures can have a significant effect on the microbial load on MP salad vegetables as they act to remove cellular nutrients and moisture, which influence microbial growth, from the vegetable surface. This study compared the use of two drying systems in the preparation of lettuce. The impact on the microbial load after applying a surface disinfectant was investigated with and without a rinse step post-disinfection. Counts were carried out immediately after treatment and after refrigerated storage of the product.

2.1.3 CHLORINE TREATMENT OF LETTUCE

Adams *et al.*, (1989) achieved a 92.4% reduction in aerobic plate counts by washing of lettuce leaves with tap water containing 0.2ppm available chlorine. A smaller reduction was observed by Shapiro and Holder (1960) who reported a 79.5 % reduction in bacterial count for mixed salads rinsed in tap water. A 98 % reduction in total coliforms was observed by Rosas *et al.* (1984) when whole lettuce leaves, grown in soil irrigated with domestic waste, were rinsed with tap water for 30 seconds. Variation in these results was most likely dependent on washing protocol and plant material (Garg *et al.*, 1990). However, increasing the total available chlorine in the wash water to 100ppm using a hypochlorite solution by Adams *et al.* (1989) found that the total count was further reduced. They also found that a three-fold increase in available chlorine to 300ppm produced a roughly proportionate decrease in the total bacterial count.

Due to the dynamic nature of the chlorination process, the antimicrobial efficiency is influenced by a number of factors including changes in the pH,

concentration and temperature of the chlorine solution, the amount of organic matter and exposure time. According to Boyette *et al.* (1993), pH of a chlorine solution has the greatest affect on the activity of chlorine water Another factor which influences the antimicrobial activity of chlorine treatment is length of exposure time. Shorter exposure times being less effective than longer exposures. However, most of the sanitising action of the chlorine can be accomplished within the first several minutes of exposure (Boyette *et al.*, 1993).

Hence, when reducing the microbial load on ready-to-use lettuce in this study the concentration, pH and treatment time of the NaDCC solution used during the process were considered to be important factors.

2.1.4 OBJECTIVES

The objectives of this section of the study were:

1. To devise an experimental method simulating the commercial preparation of cut lettuce which will allow a comparison of effect of soaking prepared produce in tap water containing NaDCC with soaking in tap water only;
2. To determine the concentration of NaDCC to use;
3. To determine the antimicrobial effect of NaDCC on the total aerobic mesophiles both after treatment with chlorine and after refrigerated storage of produce;
4. To assess the impact on antimicrobial activity of;
    a) removing the rinse step after treatment
    b) comparing an automatic drying system and a hand drying system after treatment with chlorine;

5. To examine the effect of pH and exposure time on the antimicrobial activity of NaDCC.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Lettuce

Butterhead lettuce was purchased from a retail outlet where it was stored at ambient temperature (10 to 15°C) in open polyethylene bags. All the samples were chosen at random and transported to the laboratory within one hour of purchase.

Hand Salad Dryer

A domestic hand salad dryer consisting of a rotating inner plastic basket in a plastic container was purchased from a retail outlet and used to hand dry the salad ingredients.

Automatic salad dryer

Salad Green manufactured by Crypto Peerless, Bordesley green rd., Birmingham B9 4UA, England. The Salad Green consists of a removable inner basket for convenient filling and cleaning after use. The spin speed of approximately 350 rpm is automatically controlled by a 0 to 4 minute timer.

Food bags

Polyethylene food bags, 25cm x 32 cm in dimension with folding closure produced by Albal GLAD, Dowbrands S.A., France.

Cooled Incubators

LMS model 303. LMS Ltd., The Modern Forges Riverhead, Sevenoakes, Kent.

Autoclave

Tomy, Model SS-325, Tomy Seiko Co. Ltd., Tokyo 179, Japan.

Lab blender

Stomacher (Model 400) manufacterued by Seward Medical Ltd., 131 Great Sulfolk St. London SEI IPP, UK. The lab blender is used for homogenising a food sample immersed in a diluent within a special disposable plastic bag in preparation for microbiological analysis.

Incubator

Gallenkamp, Economy Incubator with fan, Size 2. AGB Scientific Ltd.

Sanitiser

Virkon 1% solution. Active ingredient Potassium monopersulphate contains an anionic surfactant. Non tainting to food when used on food preparation surfaces. Jencons (Scientific) Ltd., Bedfordshire, England.

pH meter

Orion Model 410 A. Orion Research Inc., Boston, USA.

Sodium Dichloroisocyanurate (NaDCC)

Commercially available Aquatabs supplied by Medentech Ltd. Ireland were used to prepare the NaDCC solutions. Each 85mg effervescent tablet contained 50 mg free available chlorine.

Hydrochloric acid

0.1 Molar solution of hydrochloric acid

Ringers Solution ($\frac{1}{4}$ strength)

An osmotically controlled solution for the preparation of suspensions of food samples and for use as a diluent in dilution techniques for bacterial enumeration.

Lab M 100Z: One tablet was dissolved in 500mls deionised water. When completely dissolved the solution was dispensed into containers as required and sterilised by autoclaving at 121°C for 15 minutes.

Ringers Solution Thiosulphate

An isotonic rinse containing thiosulphate to neutralise chlorine.

100mls of Lab M thiosulpate ringer solution neutralises 7mg of chlorine.

LAB M 102: 2.8g of powder was dissolved in 1L of deionised water. The solution was then dispensed into containers as required and sterilised by autoclaving at 121°C for 15 minutes.

Nutrient Agar

A general purpose medium for the cultivation of organisms that are not demanding in their nutritional requirements.

Lab M 8: 28g of powder was dispersed in 1L of deionised water. After soaking for 10 minutes the medium was mixed by swirling and then sterilised by autoclaving for 15 minutes at 121°C. The medium was cooled to 47°C before pouring plates.

Chlorine DPD

A test for measuring free available chlorine in water using the Palintest Comparator Colour Match method (Thomas and Chamberlain 1974). The system uses diethyl-p-phenylene diamine (DPD) in tablet form which reacts with free chlorine in solution to produce a pink colouration. The intensity of the colour is proportional to the free chlorine concentration. The colour intensities are measured by comparison against colour standards using a palintest comparator and disc. The calibration of colour standards is carried out in the Palintest Laboratories by matching test solutions against master colours from the Palintest Colour Scale. Standard solutions used in the calibration of the tests are checked against standard analytical methods.

2.2.2 PREPARATION OF LETTUCE

The lettuce heads were prepared for treatment with tap water and chlorine by removing the outer leaves and inner core. The remaining leaves were soaked for 5 minutes in tap water then placed in a colander and rinsed for approximately 2 minutes.

A hand salad dryer was then used for 1 minute to dry the lettuce for the experiments investigating the effect of NaDCC concentration on the aerobic mesophiles on cut lettuce after treatment and after refrigerated storage. Drying of the lettuce in subsequent experiments was carried out using the automatic salad dryer, unless otherwise stated.

After drying, the lettuce was cut into pieces approximately 5cm × 2cm using a vegetable knife.

In some experiments the microbial load on unwashed cut lettuce was determined for comparison. This produce was trimmed, cored and cut (5cm × 2cm) without washing.

2.2.3 PREPARATION OF SODIUM DICHLOROISOCYANURATE SOLUTION

A 100ppm chlorine solution was prepared by dissolving 2 Aquatab 85mg tablets in 1L of tap water. An 80ppm solution was prepared by dissolving two 85mg Aquatabs in 1.25 litres of tap water. Tap water was used as the diluent for the chlorine solutions to mimic commercial preparation of the disinfectants. The pH of the 100ppm NaDCC solution was determined using a pH meter and gave a reading of approximately pH 6.0. The pH of this solution was adjusted to pH 5.0 using 0.1M hydrochloric acid when necessary.

2.2.4 EXPERIMENTAL METHOD

To meet the first objective a scheme for investigating the effect of the chlorine solutions on aerobic mesophiles was developed and is shown in Figure 2.1. For experiments where the number of samples tested was greater than 4 a second batch of randomly chosen butterhead lettuce was prepared and treated.

This method was adapted from the method used by Adams *et al.*(1989) who investigated the factors affecting the efficacy of washing procedures used in the production of prepared salads. Controls for each treatment contained tap water instead of chlorine disinfectant as carried out by Best *et al.* (1990) and Shapiro and Holder (1960). Adams *et al.* (1989) also used tap water as wash water for lettuce and examined the effect of chlorine on the microflora present on lettuce by adding sodium hypochlorite into the wash water.

Treatment of lettuce

Approximately 70 grams of prepared lettuce were fully immersed in either one litre of tap water (control) or chlorine solution (test) so that the cut lettuce pieces were fully immersed during treatment and left for 30 minutes.

Rinsing and Drying

After treatment the lettuce was rinsed by swirling 3 times in tap water. Lettuce used in the first set of experiment (the effect of NaDCC concentration on the total aerobic mesophiles on lettuce after treatment and after 7 days storage at 5°C) was dried using the hand salad dryer for 2-3 minutes. Drying of lettuce in subsequent experiment used the automatic salad dryer for 4 minutes.

Microbiological Analysis and Storage

10g samples of lettuce were weighed out for microbiological analysis (n=3 / n=4). The remainder (approx. 30-40g) was packed into closed polyethylene food bags and refrigerated in a cooled incubator at 5°C.

For the purpose of the investigations certain changes were made to the method outlined and are highlighted later in the text.

To meet the second and third objectives a series of experiments were set up to examine the effect of 80 and 100ppm chlorine solutions on aerobic mesophiles present on cut lettuce immediately after treatment and after 7 days storage at 5°C.

To meet the fourth objective:

1. The impact of rinsing was examined. The experimental method outlined in Figure 2.1 includes a rinse step following treatment with 100ppm NaDCC pH5.0 solution or tap water. It was decided to investigate the effect of removing this step.
2. The effect of drying procedure was examined by comparing the use of the domestic salad dryer with the automatic salad dryer when drying the lettuce for 1 minute after washing and for 4 minutes after treatment (100ppm NaDCC / Tap water).

Microbiological analysis was carried out after treatment and after 7 days storage at 5°C

To meet the fifth objective the effect of lowering the pH on the biocidal capacity of the 100ppm chlorine solution was assessed by reducing the pH of the solution from pH 6.0 to 5.0. The total aerobic mesophilic count on lettuce was determined after treatment and after 7 days storage at 5°C.

To examine the effect of treatment time on the efficiency of 100ppm NaDCC pH5.0 solution, the soaking period of 30 minutes was reduced to 20 and 10 minutes.

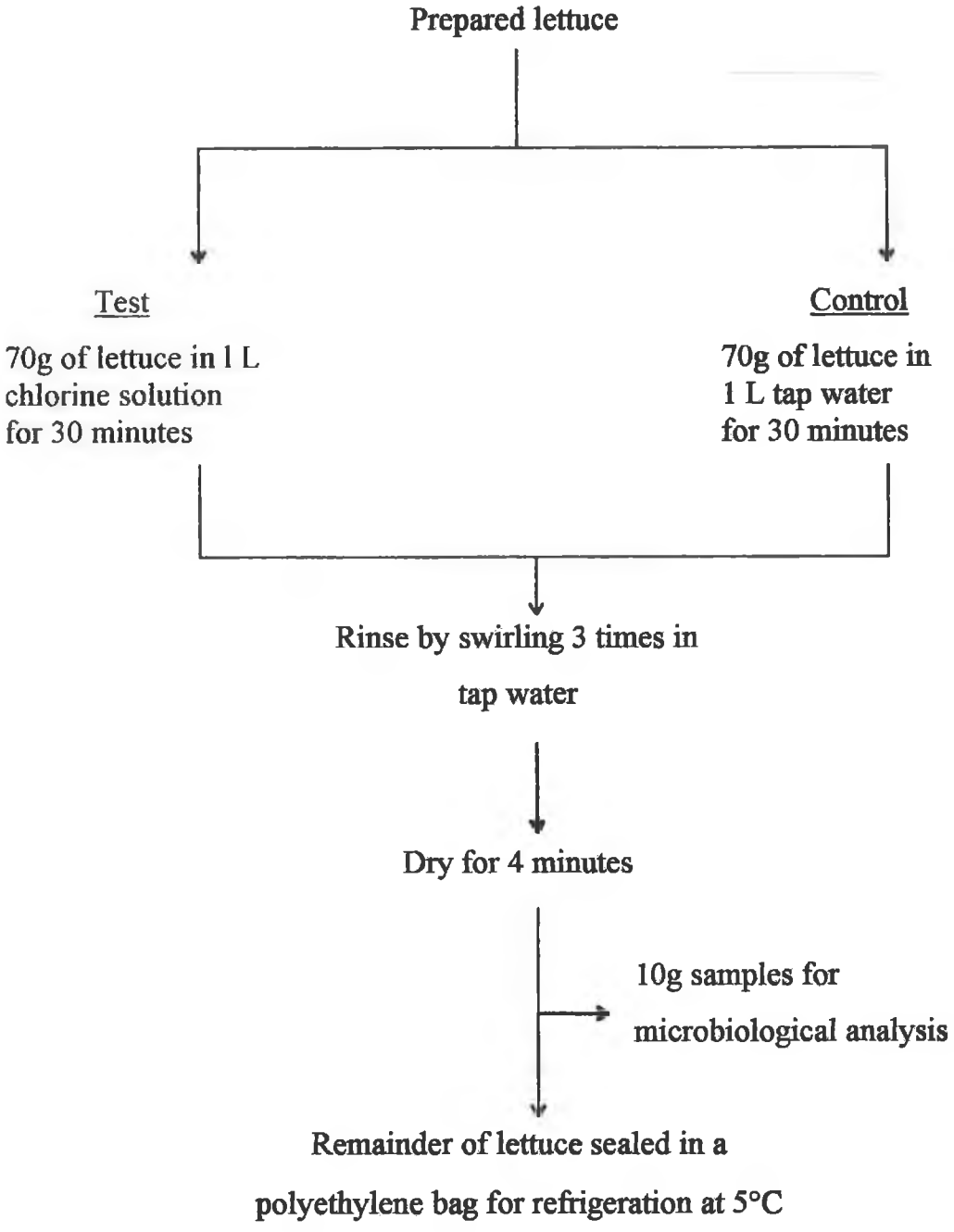


Figure 2.1: A Scheme for investigating the antimicrobial effect of chlorine solution on the aerobic mesophiles present on cut lettuce.

2.2.5 MICROBIOLOGICAL ANALYSIS

10g samples from all treatments (Test and Control) were diluted in 90 mls sterile Ringers Thiosulphate solution and blended for 1 minute in a lab blender. Further dilutions were made in sterile Ringers solution. Duplicate plates were prepared using onto appropriate media using surface streaking for single colonies.

Isolation and enumeration of mesophilic aerobes were carried out using Nutrient agar with incubation at 37°C for 48 hours. In experiments where the rinse step was removed the thiosulphate diluent was used at double strength to ensure that all the residual chlorine was inactivated.

Microbiological analysis of the tap water used in the experiments, for total coliforms, feacal coliformd and heterotrophs, were carried out at intervals by Dublin Corporation Central Laboratory and results are contained in Appendix A.

2.2.6 CHLORINE MONITORING

The colour match method using the Palintest Comparator (Chlorine DPD, Thomas and Chamberlain 1974) was used for testing the free available chlorine of the test solutions and chlorine residues on the treated lettuce. 10g samples of treated lettuce were blended with 90 ml distilled water in a laboratory blender for 2 minutes to measure the free available chlorine residues on the treated lettuce samples. Tests carried out at intervals by Dublin Corporation to detect total residual chlorine in the water supply used in the tests are given in Appendix A. The levels of chlorine detected ranged for the most part between 0.02 and 0.07mg/L. Tests carried out in the laboratory at intervals on the water supply were not detectable using the Palintest Comparator system as the minimum detection level of the system is 0.2mg/L.

2.2.7 PREPARATION OF FOOD CONTACT SURFACES AND UTENSILS

All equipment used during the preparation of the lettuce including knives, cutting board, colander, hand and automatic dryers, basins and glassware were washed with a 1% sanitising solution and rinsed thoroughly with tap water and allowed to drain before and after use.

2.2.8 STATISTICAL ANALYSIS

Statistical analysis of the results was carried out using the SPSS for Windows release 6.1. An independent t-test was used to determine significant differences between samples using a 95 % confidence interval.

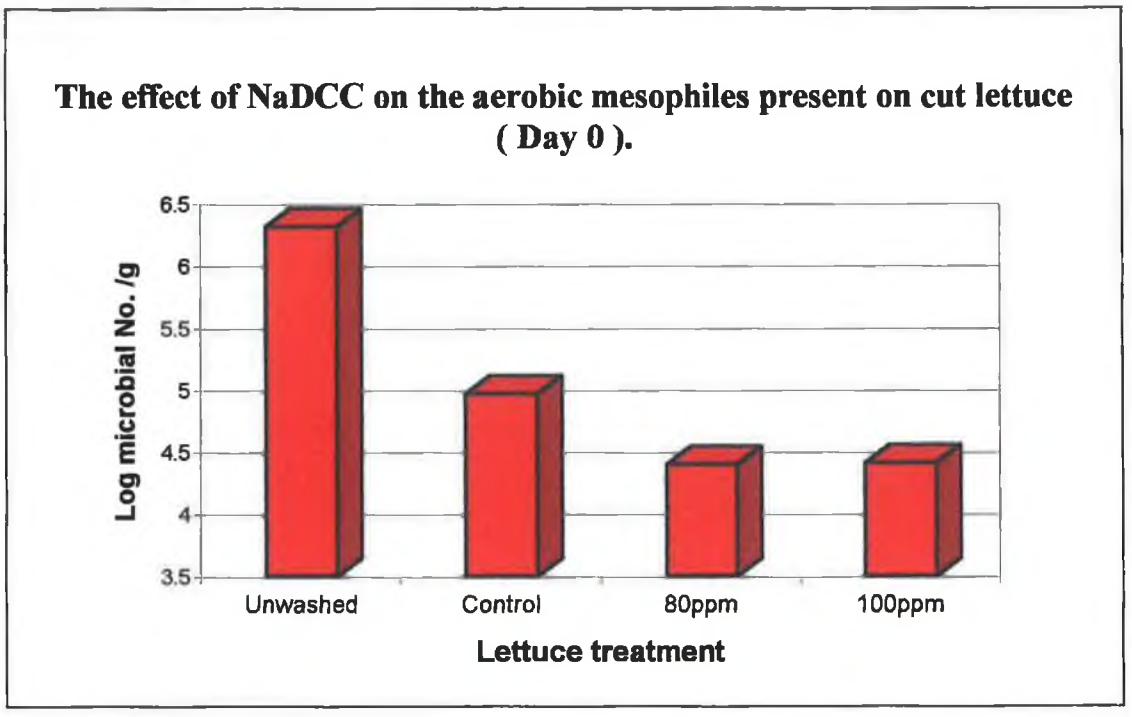
2.3 RESULTS

2.3.1 THE EFFECT OF TREATING CUT LETTUCE WITH NADCC CHLORINE SOLUTION

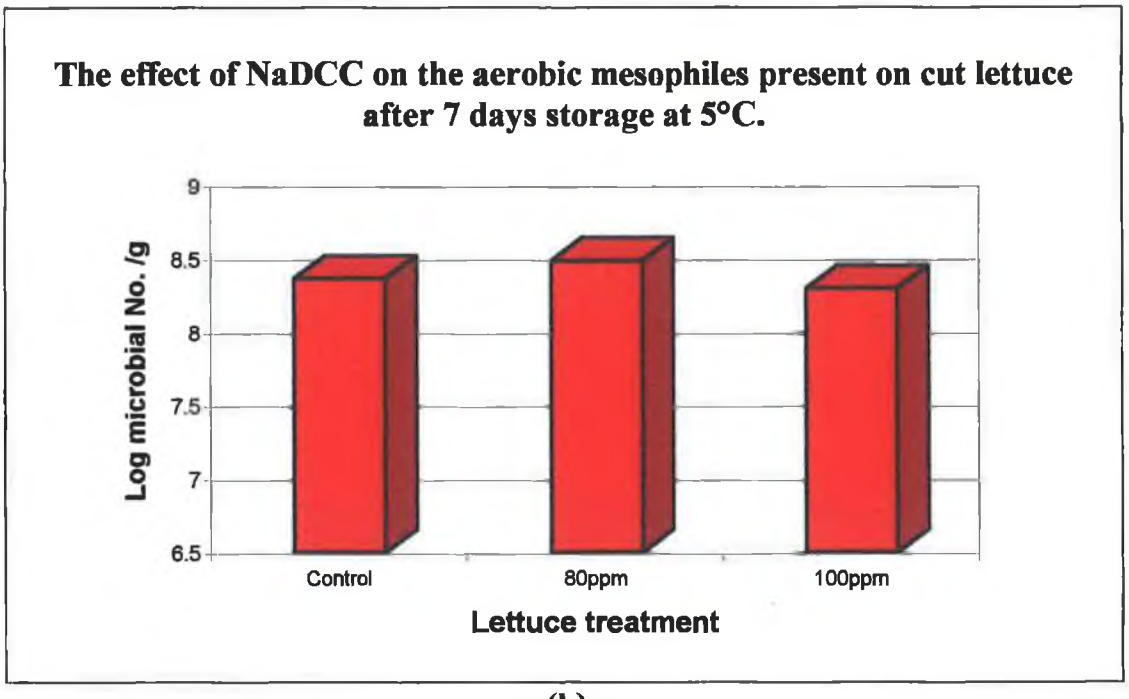
Counts per gram of unwashed / untreated lettuce were found to be approximately 10^6 CFU/g. After treatment of the lettuce with tap water results showed a significant reduction (1.3 log cycle) in aerobic mesophiles (Figure 2.2a, $n = 8$). A further 0.6 log cycle reduction was achieved when the samples were treated with 80 and 100ppm NaDCC solutions. No significant differences were observed between the antimicrobial effect of the 80 and 100ppm NaDCC solutions. After 7 days storage there was no significant difference in total aerobic mesophiles on samples treated with water and those treated with chlorine (Figure 2.2 b, $n = 8$).

2.3.2 THE IMPACT OF RINSING AND DRYING POST-TREATMENT ON THE ANTIMICROBIAL ACTIVITY OF CHLORINE ON CUT LETTUCE

Results showed that rinsing the lettuce post treatment had no significant effect on the microbial load (Figure 2.3 a, $n = 4$). The level of chlorine residues detected on the surface of the lettuce samples is given in Table 2.1. Levels of chlorine were detected on unwashed cut lettuce between 0.6 and 0.8ppm per gram of lettuce. An increase in chlorine residues to 1.4ppm was detected only on samples that were not rinsed after treatment with chlorine. After 7 days storage there was no significant difference in the microbial load present on the samples treated with tap water and chlorine, with or without rinsing post treatment (Figure 2.3 b, $n = 4$). Thus, the presence of a 1.4ppm chlorine residue on the surface of lettuce did not alter significantly the antimicrobial effect for long-term storage.



(a)



(b)

Figure 2.2: The total aerobic mesophiles present on unwashed cut lettuce and after treatment with tap water and 80ppm and 100ppm NaDCC pH6.0 (a) for same-day use (n = 8) and (b) after 7 days storage at 5°C (n = 8).

Table 2.1: The level of free chlorine detected on lettuce samples before washing and after treatment of the lettuce with either tap water or chlorine with respect to rinsing post treatment.

Lettuce treatment (n=2)	Free available chlorine/g (ppm)
Unwashed	0.6 - 0.8
Tap water :	
without rinsing	0.6
with rinsing	0.8
Chlorine (100ppm):	
without rinsing	1.4
with rinsing	0.8

The impact of drying on the antimicrobial activity of chlorine on the aerobic mesphlies examined two drying methods, hand drying in a salad dryer (uncontrolled drying) and automatic drying in a salad green dryer (consistent and controlled drying). For same-day use, results showed no significant difference in microbial load on the lettuce samples treated with tap water and chlorine with respect to drying procedure (Figure 2.4 a, $n = 7$). After 7 days storage the control samples again showed no significant difference in microbial load with respect to drying procedure. However, the microbial load on the lettuce samples treated with chlorine and hand dried was significantly greater than the chlorine treated samples dried in the automatic salad dryer (Figure 2.4 b, $n = 7$).

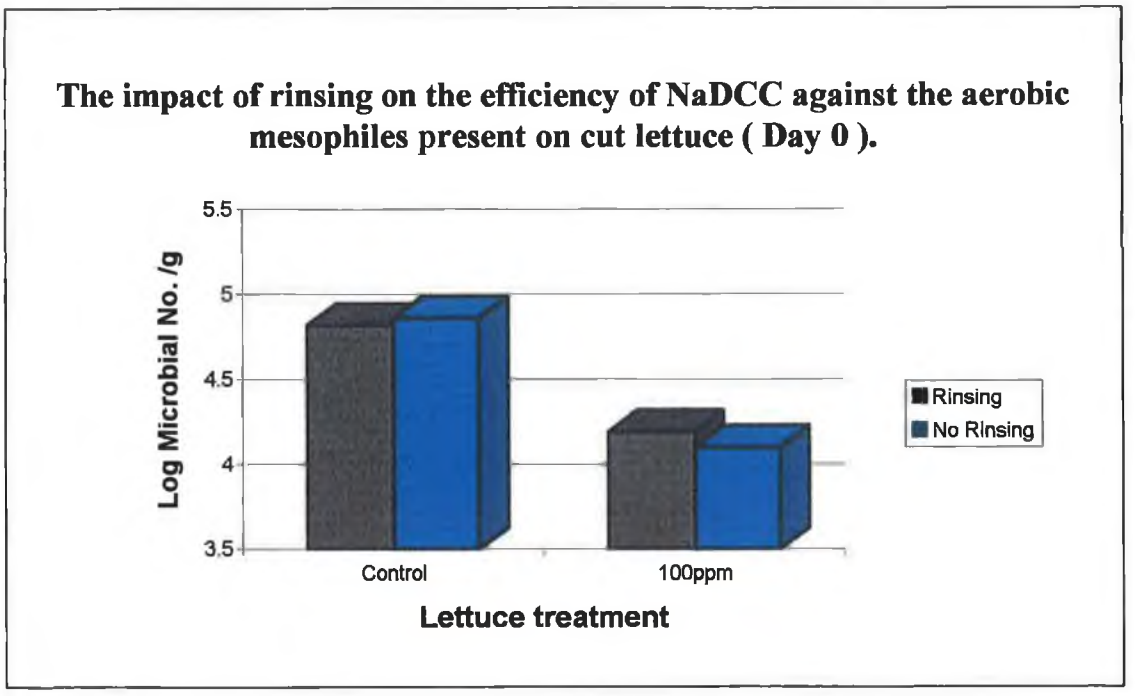
2.3.3 THE EFFECT OF LOWERING THE PH ON THE ANTIMICROBIAL ACTIVITY OF THE NADCC SOLUTION

When the pH of the 100ppm NaDCC solution was decreased from 6.0 to 5.0 there was a further significant reduction of 0.4 log cycles in the microbial load present on the cut lettuce (Figure 2.5 a, $n = 8$). However, after 7 days storage at 5°C no significant difference was observed between samples

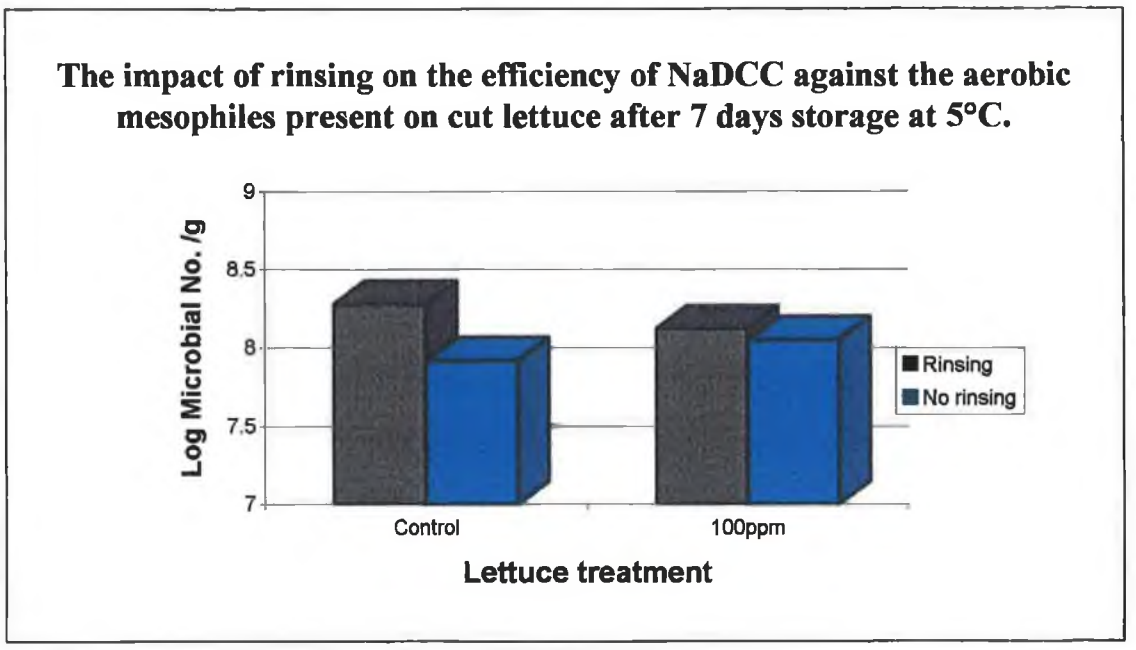
treated with 100ppm NaDCC pH 6.0 and 100ppm NaDCC pH 5.0 (Figure 2.5 b, $n = 8$).

2.3.4 THE ANTIMICROBIAL ACTIVITY OF NaDCC ON CUT LETTUCE AND TREATMENT TIME

In this study a 30 minute treatment time was chosen to mimic normal commercial preparation of cut lettuce and to facilitate easy application of disinfectant. The antimicrobial effects of reduced treatment times of 20 mins and 10 mins were determined. Results showed that reducing the treatment time from 30 mins to 10 mins did not significantly alter the antimicrobial effect of the disinfectant on the microbial load present on the cut lettuce (Figure 2.6, $n = 3$).

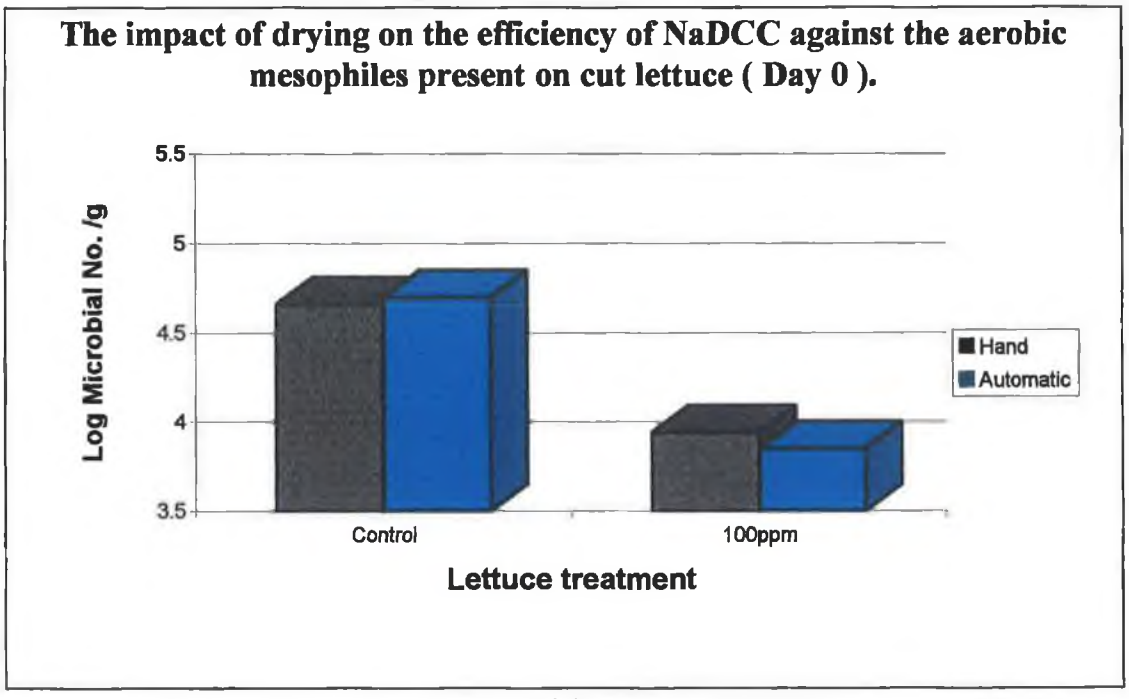


(a)

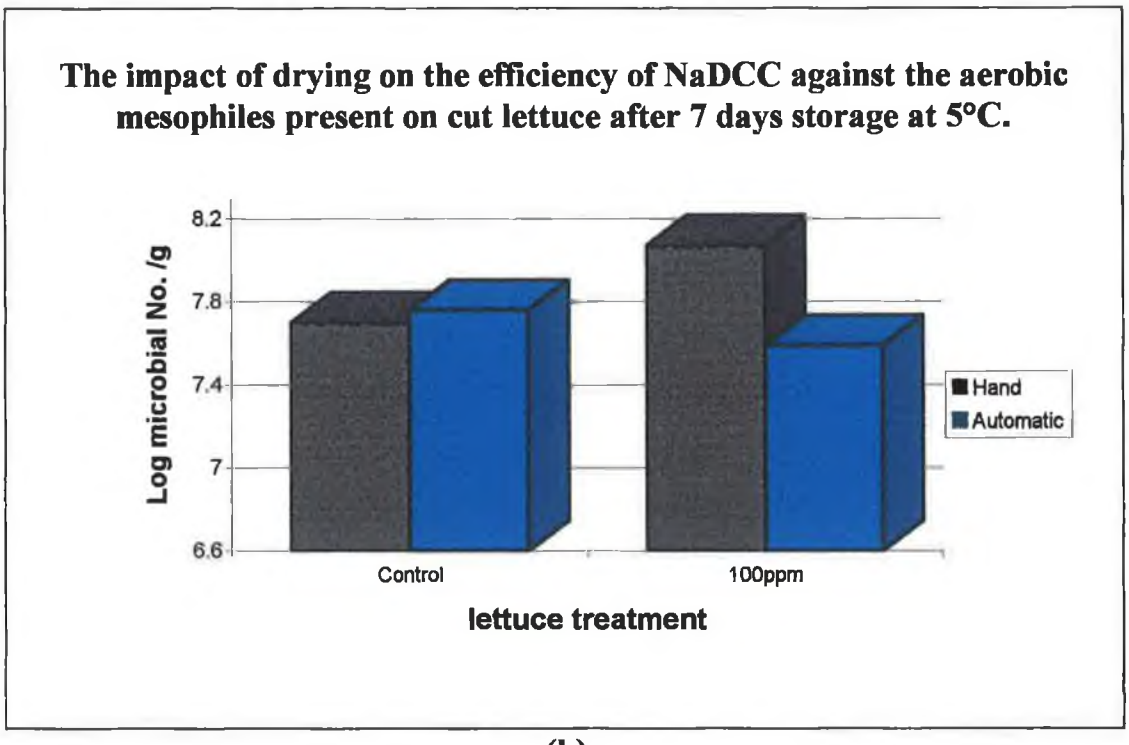


(b)

Figure 2.3: The impact of rinsing on the total aerobic mesophiles present on cut lettuce after treatment with tap water and 100ppm NaDCC pH 6.0 (a) for same-day use (n = 4)and (b) after 7 days storage at 5°C (n = 4).

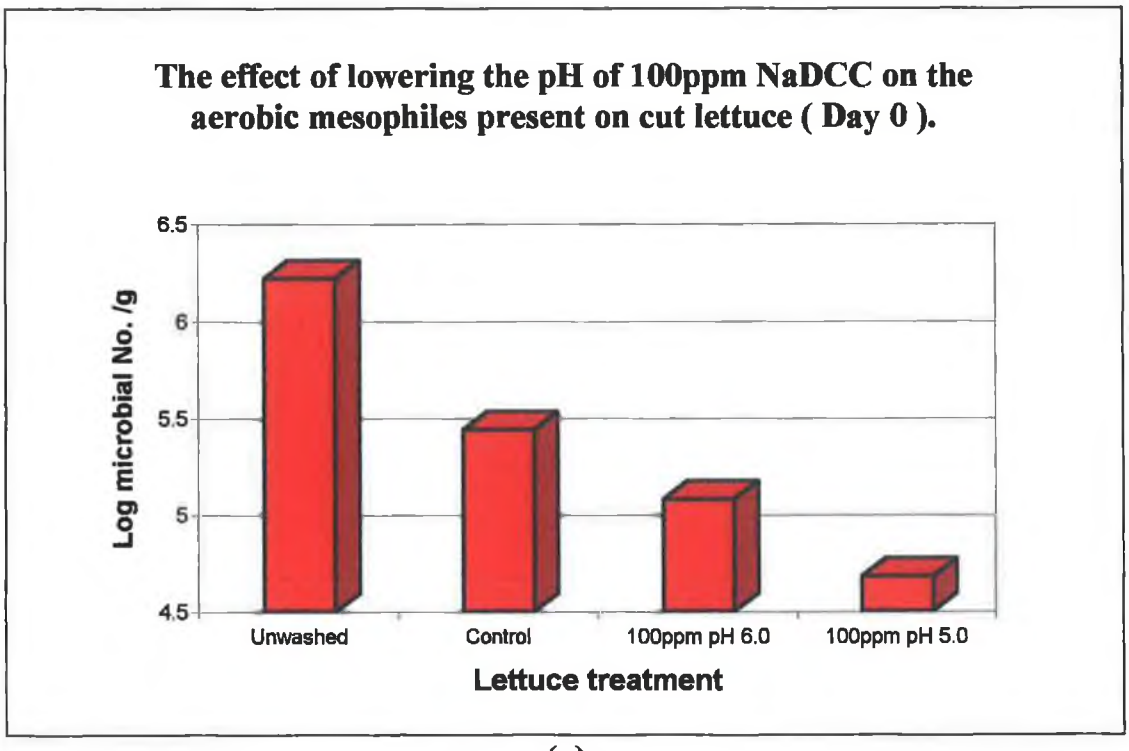


(a)

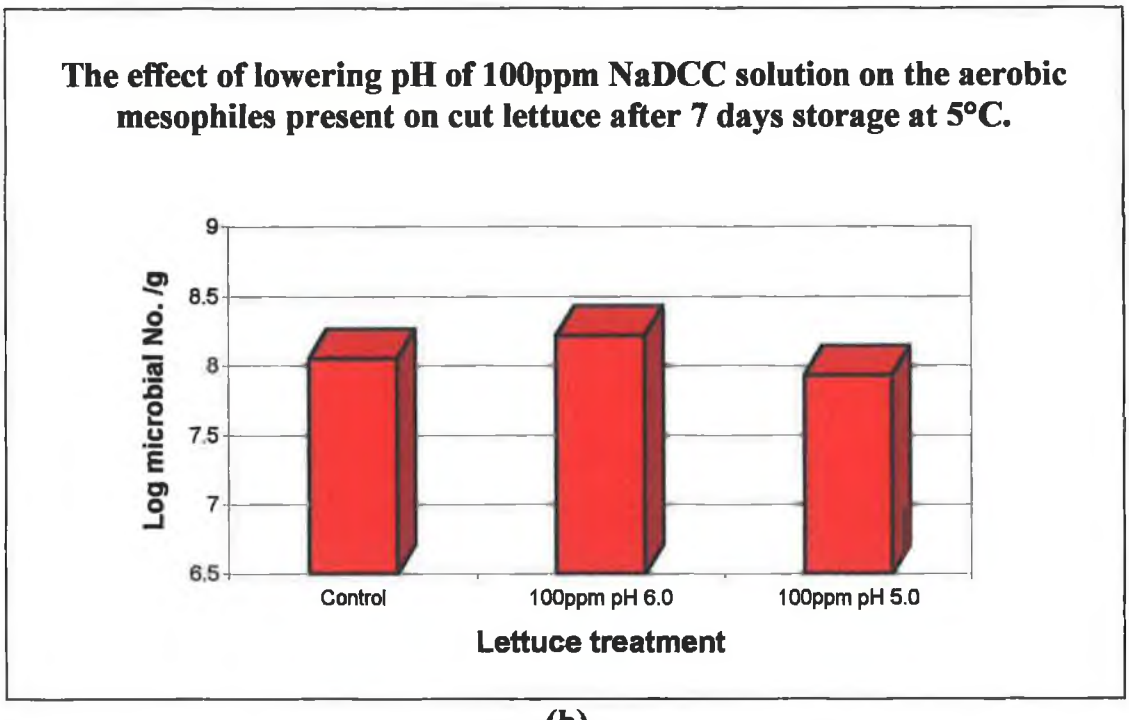


(b)

Figure 2.4: The impact of drying on the total aerobic mesophiles present on cut lettuce after treatment with tap water and 100ppm NaDCC pH 6.0 (a) for same-day use (n = 7) and (b) after 7 days storage at 5°C (n = 7).



(a)



(b)

Figure 2.5: The antimicrobial effect of lowering the pH of 100ppm NaDCC from pH 6.0 to 5.0 on the aerobic mesophiles present on ready-to-use lettuce (a) for same-day use (n =8) and (b) after storage at 5°C for 7 days (n = 8).

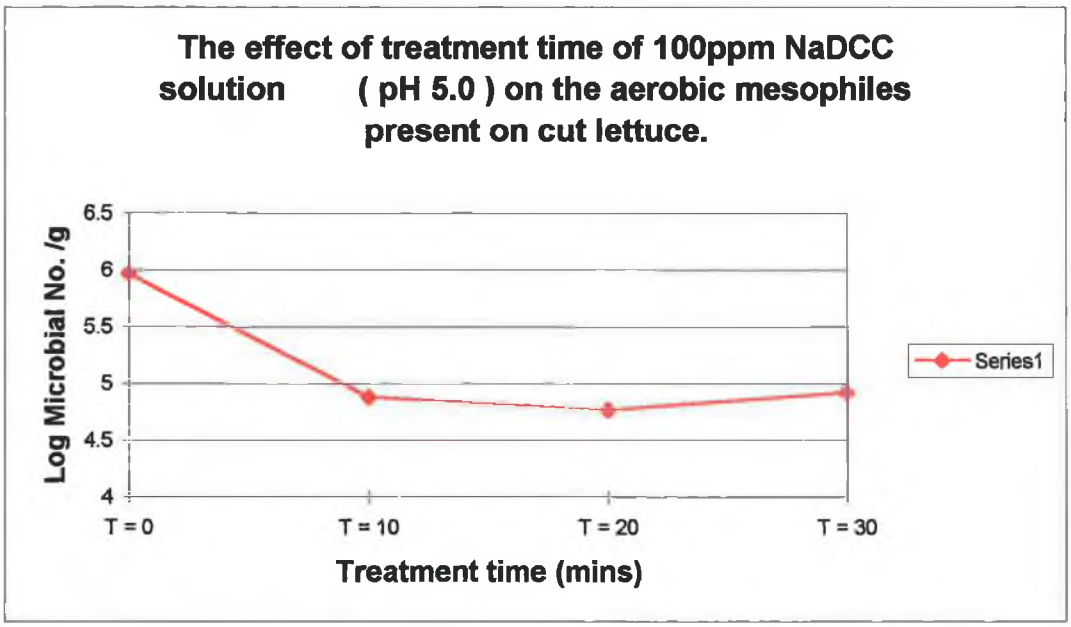


Figure 2.6: The effect of treatment time on the antimicrobial activity of 100ppm NaDCC pH 5.0 on the aerobic mesophiles present on cut lettuce ($n = 3$).

2.4 DISCUSSION

2.4.1 THE ANTIMICROBIAL EFFECT OF CHLORINE AS A SURFACE DISINFECTANT ON READY-TO-USE LETTUCE

The experimental method devised to assess the antimicrobial activity of NaDCC showed consistent significant differences between cut lettuce treated with tap water only and tap water / chlorine solutions. The total aerobic mesophiles present on unwashed lettuce was approx. 10^6 CFU/g. This number was reduced by 95.5% (1.35 log cycles) when the lettuce was prepared and treated with tap water for 30 minutes. When unwashed lettuce was prepared and treated with 80 and 100ppm NaDCC (pH 6) solutions for 30 minutes the total aerobic mesophiles was reduced by 98.8% (1.92 log cycles; Figure 2.2 a). These observations are similar to studies carried out by Adams *et al.* (1989) who found that washing lettuce produced a 92.4 ± 5% reduction in aerobic plate count and when a 100ppm NaOCl solution pH 8.8 was used the total count was reduced by 96.3 - 98% of the total unwashed count. No significant differences in anitmicrobial activities of the 80 and 100ppm solutions were observed. These results show that the concentrations of the NaDCC disinfectant used during treatment was not a limiting factor.

Studies comparing the disinfection capacity of NaDCC and NaOCl solutions by Bloomfield (1973) showed a significantly higher activity for NaDCC when 125ppm solutions of both disinfectants were tested against bacterial species. Therefore, a lower count on cut lettuce samples treated with NaDCC is expected in this study compared to counts on lettuce treated with 100ppm NaOCl carried out by Adams *et al.* (1989). Although the percentage reductions were approx. 98% in both studies the results of this count did show a lower count of aerobic mesophiles ($10^{4.4}$ CFU/g) on cut lettuce after treatment with 100ppm NaDCC pH6.0. Counts on cut lettuce treated with 100ppm NaOCl pH8.8 by Adams *et al.* (1989) were not reduced below $10^{5.8}$

CFU/g, even after the pH was reduced down to pH 4 using acetic acid and H_2SO_4.

However, the different results may only reflect a natural limit to the achievable reduction in microbial numbers and not a difference in disinfection capacity of NaOCl and NaDCC. Scanning electron micrographs showed that adhering microorganisms were present in grooves or hollows in hydrophobic protective pockets due to the waxy surface cuticle (Adams *et al.*, 1989) and in biofilms (Gras *et al.*, 1994). Hence, contact between disinfectants and microorganisms on the surface of lettuce may be limited, which would also explain the lack of difference between the 80 and 100ppm NaDCC solutions.

2.4.2 THE IMPACT OF RINSING AND DRYING PROCEDURES ON THE MICROBIAL LOAD PRESENT ON CUT LETTUCE AFTER PROCESSING WITH CHLORINE

Removal of cellular fluids containing active enzymes which cause rapid deterioration of cut lettuce by rinsing and drying procedures is a critical factor in the preparation and storage quality of ready-to-use lettuce. Studies carried out by Bolin and Huxoll (1991) concluded that both rinsing to remove cellular fluid from cut pieces of iceberg lettuce and centrifuging at 2000 rpm for an increasing length of time, to the point of slight desiccation, resulted in an increased storage life. An increased storage life was also reported by Bolin *et al.* (1977) for shredded iceberg lettuce when cellular fluids and surface water was removed by rinsing and centrifuging. However, they concluded that centrifuging time was not important as long as it was just adequate to remove the surface water.

Rinsing and drying procedures also play a role in the removal of microbes present on the surface of cut salad vegetables. This study showed that removing the rinse step, after treatment with tap water and chlorine, had no significant effect on the number of aerobic mesophiles present on ready-to-

use lettuce for same-day use and after 7 days storage at 5°C (Figure 2.3 a&b). The impact of drying procedures after treatment also showed no significant differences between samples treated with both chlorine and tap water for same-day use (Figure 2.4 a). However, a significantly higher count on lettuce treated chlorine and hand dried after 7 days storage at 5°C (Figure 2.4 b) was observed compared to chlorine treated lettuce dried with the automatic salad dryer. Results suggest that rinsing post treatment and drying procedures are not the critical factors for the removal of microbes when salad vegetables are treated with chlorine for same-day use. However, when samples were treated with chlorine and hand dried the microbial load was significantly greater than chlorine treated samples dried automatically after 7 days storage at 5°C. This result may reflect an increased growth rate during storage of mesophiles which were not inactivated by chlorine treatment due to the increase in surface moisture on the lettuce. Thus, when salad vegetables are treated with a chlorine disinfectant, effective drying can improve the microbiological quality of the stored produce.

2.4.3 THE EFFECT OF TREATMENT TIME AND PH OF THE NADCC SOLUTION ON THE MICROBIAL LOAD ON CUT LETTUCE

Investigations by Bloomfield (1973) using capacity test methods indicated significantly higher activity for NaDCC formulations as compared with NaOCl. Studies were carried out by Bloomfield *et al.* (1979) to determine whether the observed differences in activity of NaDCC pH 6.0 and NaOCl pH 9.0/9.6 were due entirely to pH or if there were differences in the fundamental properties of the two systems, which may affect their properties as disinfectants. The authors concluded the latter to be true. However, the present study showed that the pH of the NaDCC solution significantly influences the biocidal capacity against the total aerobic mesophiles present on ready-to-use lettuce. By lowering the pH of the NaDCC solution from pH 6.0

to pH 5.0 the antimicrobial effect against the aerobic mesophiles was increased by a factor of two as compared to the reduction by treatment with tap water (Figure 2.5 a). Adams *et al.* (1989) found that a decrease in pH of 100ppm NaOCl solution pH 8.0 to pH 5.0 increased the antimicrobial activity by a factor of 1.5.

The experiment investigating the impact of treatment time on the antimicrobial effect of chlorine showed that the effect achieved using 100ppm NaDCC pH5.0 was maintained when the treatment time was reduced from 30 to 10 mins (Figure 2.6). Adams *et al.* (1989) also found that extending the washing period from 5 to 30 minutes of lettuce with NaOCl showed no significant differences in log reductions. This could reflect a decrease in available chlorine due to the particular affinity of free chlorine to organic matter. Kotula *et al.* (1997) studied the reduction of aqueous chlorine by organic material and found that available chlorine reduction is dependent on exposure time, chlorine concentration and the amount of organic material. Thus, after 10 minutes treatment of lettuce the active chlorine may have been "used up". In this study, a 30 minute treatment time was used to facilitate easy application of disinfectant to large volumes of salad vegetables in a catering operation.

2.4.4 CONCLUSIONS

Surface disinfection of cut lettuce with 80 and 100ppm NaDCC can significantly reduce the microbial load. Reducing the pH of the formulation from pH 6.0 to 5.0 significantly improved the biocidal activity. For use in catering or processing operations a treatment time of 10 minutes is adequate to achieve this biocidal effect. The reduction in microbial load was not maintained over the storage period of seven days, however, improved drying

for 4 minutes in an automatic salad dryer showed benefits for long term storage compared to drying using a hand salad dryer.

From the results obtained in this section, it was decided to alter the scheme for investigating the antimicrobial efficiency of NaDCC (Figure 2.1) in all subsequent experiments as follows:

1. A 100ppm NaDCC solution pH5.0 would be used as the chlorine treatment for the salad vegetables;

2. Drying of lettuce would be carried out in an automatic salad dryer during preparation of the lettuce for 1 minute and after treatment of the lettuce with disinfectant for 4 minutes.

CHAPTER 3 : COMPARING THE ACTIVITY OF NaDCC ON TWO TYPES OF SALAD VEGETABLES

3.1 INTRODUCTION

Results from experiments in chapter two showed that the aerobic mesophiles on cut lettuce are significantly reduced after treatment with chlorine (100ppm NaDCC pH5.0). However, there was no significant difference between levels on chlorine treated lettuce and controls after 7 days storage at 5°C. Therefore, further investigations were carried out to determine the growth dynamics of aerobic mesophiles on treated and untreated samples over a 7 day refrigerated period. Also, a second salad vegetable was introduced to the experiments to compare the antimicrobial activity of NaDCC on different salad vegetables.

To investigate the behaviour of the microorganisms on each salad vegetable the different physiological groups of microorganisms were examined. The antimicrobial effect of chlorine on the different microbial populations was then compared on each salad vegetable after treatment and during refrigerated storage.

3.1.1 MICROBIAL CONTAMINATION OF READY TO USE SALAD VEGETABLES

The microbial species that prevail on MP vegetables are also usually found on plants in the field or after harvest and probably originate from the epiphytic microflora of the raw material (Nguyen-The and Carlin, 1994). Surveys of vegetables indicate that bacteria are the predominant flora although lesser numbers of moulds and yeasts are also present. Most of the bacteria will reside on the surface of vegetables, although internal tissue can also harbour microorganisms (Samish *et al.*, 1961, 1963).

In this study, the physiological groups of microorganims commonly associated with salad vegetables were examined and include the mesophilic

aerobes, coliform bacteria, lactic acid bacteria, psychrotrophic aerobes and the yeasts and moulds.

3.1.1.1 *Aerobic mesophiles*

Microbial evaluations of MP vegetable products have shown the mesophilic aerobes as the most numerous group of bacteria present on vegetables giving highly variable counts ranging from 10^3 to 10^9 CFU/g (Nguyen-The and Carlin, 1994). Microbial analysis of vegetable ingredients in salad bars including lettuce, broccoli, tomatoes and cauliflower showed the total aerobic mesophiles ranging from 10^5 to 10^7 CFU/g (Albrecht *et al.*, 1995). Bacterial counts on products analysed soon after processing were lower and ranged from 10^3 to 10^6 CFU/g on grated carrots (Carlin *et al.*, 1989), fresh cut vegetables (Garg *et al.*, 1990) and partially processed iceberg lettuce (King *et al.*, 1991).

Most of the bacteria (80-90%) counted on media for mesophilic bacteria were Gram negative rods; *Pseudomonas* spp., *Enterobacter* spp. or *Erwinia* spp. Pseudomonads usually prevailed over the other genera and were the only bacteria isolated from all samples of MP salads by Marchetti *et al.* (1992). King *et al.* (1991) estimated the frequency of pseudomonads as 56.7% of the bacterial population. Most of the pseudomonads identified were *Ps. fluorescens* and *Enterobacter agglomerans. Erwinia herbicola and Rahnella aquatilis* were the most frequent Enterobacteriacae found in MP fresh vegetables. Other mesophilic species identified were *Flavobacterium spp., Xanthomonas spp., Serratia spp., Alcaligenies spp., Bacillus spp.,* and *Chryseomonas spp.* (King *et al.*,1991; Magnuson *et al.*, 1990; Marchetti *et al.*, 1992).

3.1.1.2 *Lactic acid bacteria*

Second to the mesophilic bacteria in numerical importance are the lactic acid bacteria. Brocklehurst *et al.* (1987) recorded counts reaching 10^9 CFU/g in retail packs of mixed salad vegetables. However, counts for a given sample are usually lower than the mesophilic bacteria. In general shredded carrots were more contaminated by lactic acid bacteria than lettuce or chicory salads (Nguyen-The and Carlin, 1994). The lactic acid bacteria isolated from MP fresh vegetables have been identified as *Leuconostoc* spp., and more particularly *L. mesenteroides* (Carlin *et al.*, 1989).

3.1.1.3 *Coliform bacteria*

The numbers of coliforms present on salad vegetables are normally found to comprise of a smaller proportion of total bacterial levels (Fowler and Foster, 1976; Nguyen-The and Carlin, 1994). However this is not always the case. A survey of vegetable ingredients in salad bars by Albrecht *et al.* (1995) showed that high counts of aerobic mesophiles from $10^{5.25}$ to $10^{6.63}$ CFU/g were accompanied by high numbers of coliforms ranging from $10^{4.89}$ to $10^{6.39}$ CFU/g. It was found that all vegetables were held at temperatures higher than the recommended 4°C and concluded that the ambient temperature of the vegetables along with possible excessive handling were contributing factors to the high number of microorganisms. Brocklehurst et *al.* (1987) also found high numbers of coliforms approximately 10^5 to 10^6 CFU/g, in retail packs of mixed salad vegetables at the end of shelf-life. Despite these high levels of coliforms the level was below that of the total aerobic mesophiles and they did not detect *E. coli* type 1, in general, higher than the tolerable range; a count of more than 10 *E. coli* type 1/g specified by the International Commission on Microbiological Specifications for Foods (ICMSF, 1974).

A bacteriological profile of salad vegetables in Bangladesh identified the coliforms present as *E. coli, Klebsiella* spp., *Enterobacter* spp. and

Serratia spp. (Khan *et al.*, 1992). In Italy an assessment of fresh marketed lettuce and fennel identified total coliforms and faecal coliforms to include the genera *Citrobacter, Enterobacter, Esherichia* and *Klebsiella* (Ercolani, 1976).

3.1.1.4 *Aerobic psychrotrophs*

The cold tolerant psychrotrophic bacteria are widespread in foods that undergo thermal fluctuations. The increased use of frozen and especially chilled foods in recent years and the increasingly long periods of time between their production and consumption have greatly enlarged the importance of psychrotrophic bacteria in the food industry (Gounot, 1991).

Levels of psychrotrophic bacteria present on ready-to-use salad vegetables were found to be similar to the level of mesophilic aerobes on shredded iceberg lettuce, tested immediately after harvest (Barriga *et al.*, 1991) and on ready-to-use vegetable salads, purchased one day after packaging from retail outlets (Marchetti *et al.*, 1992). The composition of the psychrotrophic microflora isolated from the ready-to-use vegetable salads included *Pseudomonas* spp. (*Ps. fluorescens* was the most frequently isolated species), *Xanthomonas* spp., *Chromobacterium violaceum*, *Chryseomonas luteola*, *Enterobacter cloacae* and *Rahnella aquatilis*.

3.1.1.5 *Yeasts and Moulds*

The number of yeasts and moulds present on salad vegetables reported in literature have been highly variable, ranging from 10^2 CFU/g for cut lettuce to 10^7 CFU/g for mixed salad vegetables (Nguyen-The and Carlin, 1994). However, in general, numbers of yeasts and moulds are significantly lower than the bacterial load on salad vegetables. Magnusson *et al,* (1990) found bacterial counts of MP iceberg lettuce ranging from 10^5 to 10^7 CFU/g while the yeast populations were lower in the range 10^3 to 10^6 CFU/g and moulds were noted infrequently. Similar results were observed by Barriga *et al.* (1991) who found that yeasts were always present on shredded iceberg lettuce

but at low levels (10^1 to 10^2 CFU/g) and that moulds were also present infrequently. In contrast, the level of yeasts and moulds were reported to be higher than the total aerobic mesophiles present in retail packs of mixed vegetable salads (Albrecht *et al.*, 1995). Many different yeast species have been identified in MP vegetables including *Candida* spp., *Cryptococcus* spp., *Rodotorula* spp., *Trichosporon* spp., *Pichia* spp., and *Torulaspora* spp. The most frequently isolated moulds have been identified as *Sclerotina, Mucor, Aspergillus, Phoma, Cladosporium, Rhizopus* and *Penicillium* (King *et al.*, 1991).

3.1.2 MICROORGANISMS AS A CAUSE OF VEGETABLE SPOILAGE

Most decay of leafy vegetables in the fields are caused by the bacteria, *Pseudomonas chichori, Pseudomonas marginaliis and Erwinia carotovora* (Lund, 1983) or by fungi, *Sclerotinia spp. and Botrytis cinerea* (Dennis, 1983). *E. carotovora* plays a major role in storage rot of many vegetable crops (Perombelon and Kelman, 1980), whereas pectinolytic pseudomonads, mainly strains of *Ps. marginalis* which are also regarded as biotypes of *Ps. fluorescens* (Doudoroff and Palleroni, 1974), seem to cause most post harvest decay of leafy vegetables including celery (Harrison and Barlow, 1904), chicory (Friedman, 1951), lettuce (Ceponis, 1970), chinese chard (Burton, 1971) and cabbage (Bobbin and Geeson, 1977).

Brocklehurst and Lund (1981) reported that strains of pectinolytic fluorescent Pseudomonads caused soft rot of wounded, but not of unwounded petioles of celery and cabbage stored at 20°C and 10°C to 20°C respectively. As wounding was required before the pseudomonads cause rot they stated that damage caused during harvesting and handling vegetables can be a major factor leading to microbial spoilage. The ability of the strains to grow well at 1°C indicated their importance in spoilage of vegetables at low temperatures.

Nguyen-The and Prunier (1989) investigated the involvement of pseudomonads in the deterioration of ready-to-use chicory salads packed in polypropylene bags and stored at 10°C. Of the soft rot bacteria known to cause rot in vegetables *Ps. marginalis* was the only one found in the salads analysed. Because *Ps. marginalis* was also found in undamaged crude salads they concluded that it did not induce chicory deterioration in the field or during storage under normal conditions. However, they concluded that *Ps. marginalis* plays an important role in the deterioration of ready-to-use salads for the following reasons:

a. It is able to cause chicory soft rot when inoculated into leaves and

b. In industrial products, the higher the proportion of *Ps. marginalis* among strains of pseudomonads isolated, the higher the level of deterioration.

The theory that bacteria cause of spoilage of salad vegetables was further supported by studies carried out by Robbs *et al.* (1996), on the causes of decay of fresh cut celery, who found that the predominant bacteria, identified as *Ps. marginalis* and *Ps. fluorescens* caused water soaking, soft rot and discoloration in freshly inoculated celery tissues stored at 5°C or 25°C in sealed film bags. *Leuconostoc mesenteroides* was also isolated and thought to have been responsible for slime production. In addition, lactic acid bacteria, in particular *L. mesenteroides,* were identified as a major causes of spoilage of grated carrots (Carlin *et al.*, 1989). The main type ofspoilage was a loss of firmness and a development of off flavours, closely related to the high counts of lactic acid bacteria, and to the production of compounds of fermentative metabolism of microorganisms (lactic and acetic acid, and ethanol). It was concluded that the spoilage of grated carrots was typically a lactic acid fermentation and that aerobic mesophilic bacteria and pectinolytic pseudomonads isolated from grated carrots had negligible effects on the spoilage.

Due to the pH values within vegetables (4.5 to 7.0) spoilage of vegetables by fungi is uncommon compared to bacterial spoilage. Occasional opportunistic moulds can ultimately spoil a vegetable by infecting a lesion created by spoilage bacteria (Brackett, 1987b). However, due to emerging MP vegetable products and new technologies, anticipating and preventing spoilage is proving to be a bigger challenge and unusual spoilage organisms have been isolated from MP salads and identified as *Pichia membranaefaciens, Saccharomyces exiguus, Candida sake, C. lambica, C. lypolytica* and *S. dairensis* (Brocklehurst and Lund, 1984).

3.1.3 OBJECTIVES

Disinfectant application to MP salad vegetables can be evaluated in terms of the impact the disinfectant has on the microbial load and how effective the treatment is over refrigerated storage. It is essential when examining the microbial spoilage and safety of minimally processed salad vegetables to consider the sensitivity of the different physiological groups of microorganisms present to chlorine treatment and their growth dynamics during refrigerated storage.

In this section the objectives were:

1) To compare the antimicrobial effect of 100ppm NaDCC pH5.0 solution on the microbial load on cut lettuce and white cabbage after treatment and during refrigerated storage;
2) To enumerate the physiological groups of microorganism which include aerobic mesophiles and psychrotrophs, total coliforms, lactic acid bacteria and yeasts and moulds present on lettuce and white cabbage;
3) To compare the antimicrobial effect of the chlorine disinfectant on the different microbial groups on each salad vegetable after treatment and during refrigerated storage.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

de Man, Rogosa and Sharpe (M.R..S.) Agar

LAB M 93: A medium for the cultivation and enumeration of *Lactobacillus* spp. and most lactic acid bacteria.

70g of powder was added to 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix. The media was then autoclaved at 121°C for 15 minutes.

Malt Extract Agar

LAB M 37: An acidic medium which will support the growth of most yeasts and moulds whilst inhibiting most bacteria.

50g of powder was dispersed in 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix. The mixture was then autoclaved at 115°C for 10 minutes. After cooling to 47°C 4 x 5ml vials and Lactic acid supplement (LAB M: XO37) was added to lower the pH of the medium to pH3.5 - 4.0.

Violet Red Bile Glucose Agar (V.R.B.G.A.)

LAB M 88: A medium for the enumeration of coliform organisms in food and dairy products. The selectivity of this medium is due to the presence of bile salts and crystal violet.

38.5g of powder was dispersed in 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix. The medium was then brought to the boil for sterilisation.

3.2.2 PREPARATION OF LETTUCE AND WHITE CABBAGE

The Butterhead lettuce was prepared according to the preparation of lettuce in section 2.2.2 (drying for 1 minute using the automatic salad dryer). Heads of white cabbage were purchased from a retail outlet and transported to the laboratory within 1 hour of purchase. This produce was unwashed, wrapped in cling film, and stored at 5°C. To prepare the white cabbage for treatment the inner core and outer leaves were removed and the remainder was cut, into pieces approximately 0.5 × 5.0 cm, using a vegetable knife.

3.2.3 PREPARATION OF SODIUM DICHLOROISOCYANURATE SOLUTION

A 100ppm NaDCC pH 5.0 solution was used for the chlorine treatment of salad vegetables. The NaDCC solution was prepared as previously in section 2.2.3.

3.2.4 EXPERIMENTAL METHOD

The antimicrobial effect of 100ppm NaDCC pH5.0 solution on the microbial load on cut lettuce and white cabbage was determined using the schemes for investigations in Figures 3.1 and 3.2. respectively.

To meet the first objective the total aerobic mesophiles were determined on the cut vegetables after treatment with either chlorine or tap water (control) and during storage. Counts were carried out on a daily basis for a 7 day refrigeration period.

To meet the second objective:

1) the numbers of aerobic mesophiles and psychrotrophs, coliform bacteria, lactic acid bacteria and yeasts and moulds were enumerated on unwashed cut lettuce and cabbage.

2) The effect of chlorine on each group of microorganisms present on cut lettuce and cabbage was then determined after treatment and after 3 and 5 days storage in polyethylene food bags at 5°C.

3.2.5 MICROBIOLOGICAL ANALYSIS

10g samples were used for isolation and enumeration of the microbial populations. Preparation of samples were carried out according to microbiological analysis in section 2.2.5. The culture media, methods and incubation conditions employed are listed in Table 3.1.

Table 3.1: Table of culture media, methods and incubation conditions for the isolation and enumeration of microbial populations present on salad ingredients.

Test	Method	Culture media	Incubation	
			Temp. °C	Time (days)
Total aerobic mesophiles	Surface plate	Nutrient agar	37	2
Total aerobic psychrotrophs	Surface plate	Nutrient agar	4	10
Lactic acid bacteria	Pour plate with overlay	MRS[1]	25	5
Yeasts and Moulds	Surface plate	MEA[2] with Lactic acid supplement	25	5
Total coliform bacteria	Pour plate with overlay	VRBGA[3]	37	1

1. de Man, Rogosa and Sharpe agar.
2. Malt Extract agar.
3. Violet Red Bile Glucose Agar.

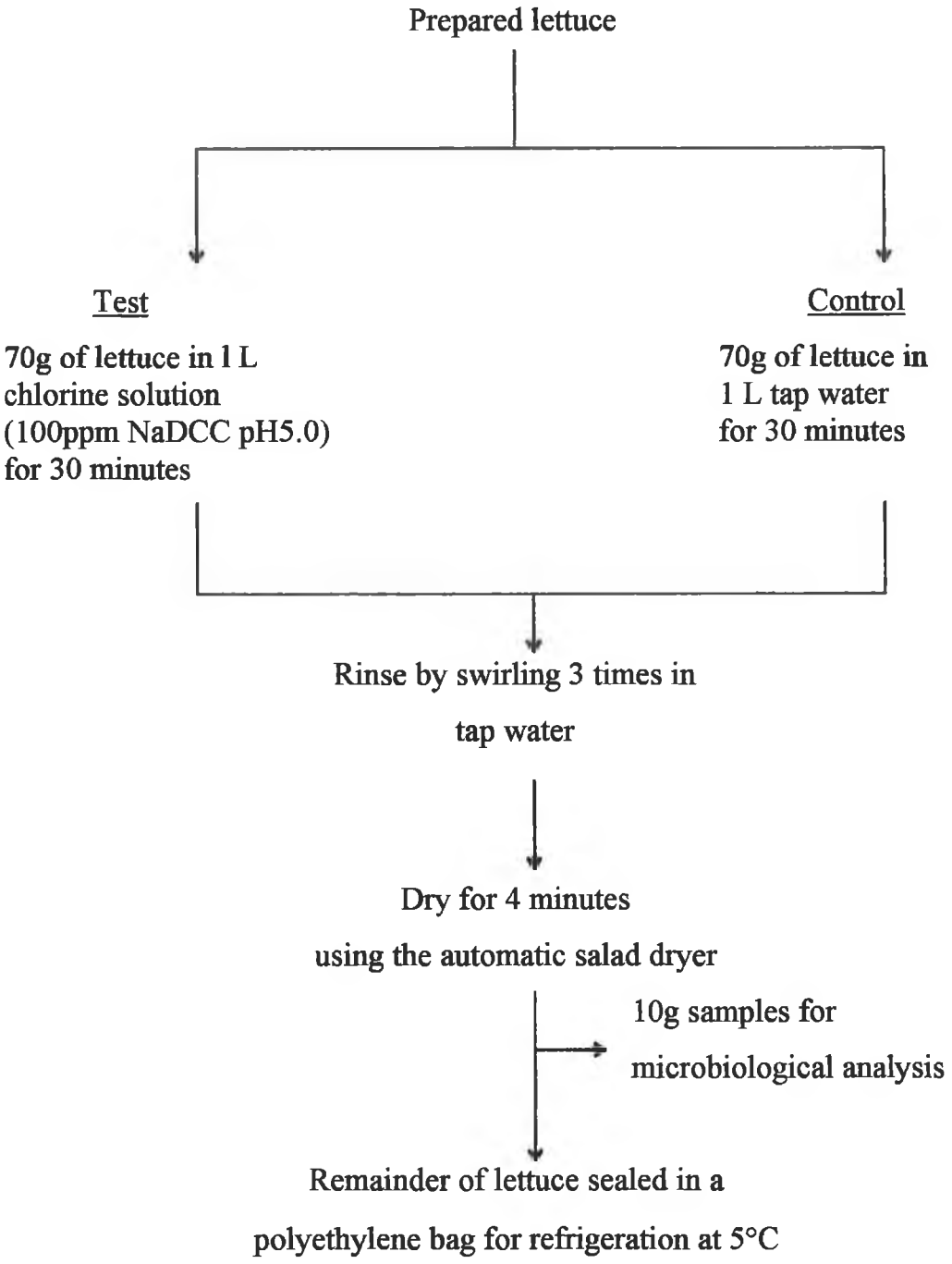


Figure 3.1: A Scheme for investigating the antimicrobial effect of chlorine solution on the microbial load present on cut lettuce.

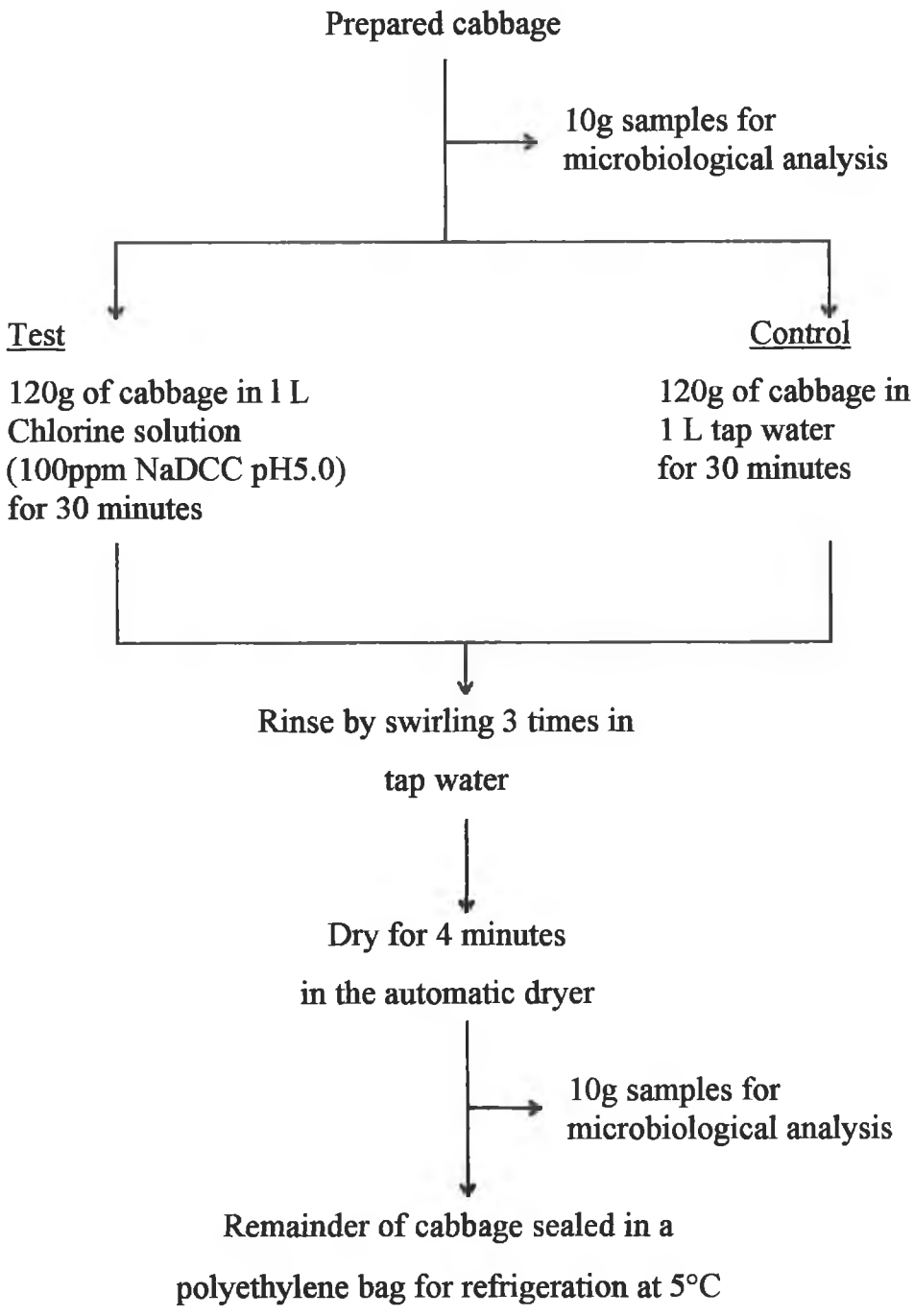


Figure 3.2: A scheme for investigating the antimicrobial effect of chlorine solution on the microbial load present on cut cabbage.

3.3 RESULTS

3.3.1 THE EFFECT OF CHLORINE TREATMENT ON THE MICROBIAL LOAD PRESENT ON CUT LETTUCE AND CABBAGE DURING REFRIGERATED STORAGE

The microbial load (total aerobic mesophiles) present on both cut lettuce and cabbage was determined after treatment with tap water and chlorine. Results given in Figure 3.3 show the microbial load present on cut lettuce and cabbage after treatment with 100ppm NaDCC solution pH 5.0 was significantly lower (approximately 1.2 and 0.5 log cycles respectively) than lettuce and cabbage treated with tap water. After 3 days storage no significant differences were observed between the microbial loads on treated and control samples. The microbial loads on the lettuce continued to increase, regardless of treatment, to approximately 10^8 CFU/g over the 7 days refrigerated storage. The cut cabbage treated with chlorine also showed no significant difference in microbial load compared to the cabbage treated with tap water after 3 days storage. However, the chlorine treated cabbage showed a significantly greater increase in microbial load than samples treated with tap water after 5 days storage. After 7 days storage this difference increased to approximately 1 log cycle.

3.3.2 ENUMERATION OF THE PHYSIOLOGICAL GROUPS OF MICROORGANISMS ON LETTUCE AND CABBAGE

The number of aerobic mesophiles and psychrotrophs present on the unwashed cut lettuce were approximately equal and gave the highest count at 10^6 CFU/g (Figure 3.4a). Second to these were the numbers of lactic acid bacteria and yeasts and moulds which also gave an approximate equal count of 10^5 CFU/g. The number of coliforms gave the lowest count at 10^4 CFU/g.

The population of total aerobic mesophiles found on the unwashed cut cabbage gave the highest count at approximately 10^5 CFU/g. This was followed by the aerobic psychrotrophs ($10^{4.5}$ CFU/g), coliforms (10^4 CFU/g) and yeasts and moulds ($10^{3.7}$ CFU/g). The lactic acid bacteria gave the lowest count at $10^{3.1}$ CFU/g (Figure 3.4b).

3.3.3 THE EFFECT OF NADCC ON THE MICROBIAL POPULATIONS PRESENT ON CUT LETTUCE

When the lettuce samples were treated with tap water (control) there was a significant reduction in each population of approximately 1 log cycle, with the exception of the coliforms which was reduced by 1.4 log cycles (Figure 3.4a). When the lettuce samples were treated with chlorine (100ppm NaDCC pH 5.0) there was a further significant reduction for each population. The population of lactic acid bacteria was reduced by 1.5 log cycles and the numbers of aerobic mesophiles and psychrotrophs, coliforms and yeasts and moulds were reduced by approximately 1.9-2.3 log cycles with respect to the unwashed samples.

After three days storage at 5°C there was a significant increase in numbers of aerobic mesophiles and psychrotrophs to approximately 10^7 CFU/g while the numbers of yeasts and moulds, lactic acid bacteria and coliforms remained lower between 10^3 and $10^{4.5}$ CFU/g (figure3.5a).

After 5 days storage a further increase in the total aerobic mesophiles and psychrotrophs to approximately $10^{7.5}$ CFU/g was observed (Figure 3.6a). The numbers of lactic acid bacteria, yeasts and moulds and coliforms remained between 10^3 and $10^{4.5}$ CFU/g. There were no significant differences in the numbers of each population present on the lettuce samples treated with tap water and those treated with chlorine at the end of the storage period.

3.3.4 THE EFFECT OF NaDCC ON THE MICROBIAL POPULATIONS PRESENT ON CUT WHITE CABBAGE

After treatment of the cabbage with tap water there was a significant reduction in the total aerobic mesophiles, coliforms and yeasts and moulds of approximately 1 log cycle (Figure 3.4a). A greater reduction of these populations was observed after treatment of the samples with chlorine of approximately 1.5 to 1.8 log cycles. No significant reductions of lactic acid bacteria and the aerobic psychrotrophs were observed after treatment with tap water, however, when the cabbage was treated with chlorine the populations were significantly reduced by approximately 0.6 and 0.9 log cycles respectively.

After 3 days storage at 5°C the numbers of aerobic mesophiles and psychrotrophs had significantly increased and the total aerobic psychrotrophs was significantly greater than the total aerobic mesophiles by approximately 1 log cycle. For both populations, the levels of microbes on the chlorine treated cabbage was not significantly different to the levels found on the cabbage treated with tap water (Figure 3.5b). The number of coliforms present on the cabbage treated with tap water also significantly increased by approximately 1 log cycle over the storage period. However, the levels of coliforms present on the chlorine treated cabbage remained significantly lower by approximately 1.5 log cycles. There was no significant change in the yeast and mould levels found on the cabbage treated with tap water and chlorine over the 3 day storage period. The number of lactic acid bacteria on the cabbage treated with tap water also showed no significant change over the storage period, but the level of lactic acid bacteria on the cabbage treated with chlorine showed a significant decrease.

Results after 5 days storage showed a further increase in the total aerobic psychrotrophs, with the numbers present on the cabbage treated with chlorine significantly greater (approximately 0.8 log cycle) than the numbers present on the cabbage treated with tap water (Figure 3.6b). A significantly higher count of mesophiles was also found on the chlorine treated cabbage after 5 days storage. However, the number of aerobic mesophiles remained lower than the number of psychrotrophs. After the 5 days storage the level of coliforms present on the cabbage treated with tap water ($10^{4.2}$ CFU/g) showed no significant increase. For the chlorine treated cabbage, a significant increase in coliforms was observed to approximately 0.5 log cycle below levels found on the cabbage treated with tap water. This difference was not significant at the 95% confidence interval but was significant at the 90% confidence interval. For both the yeasts and moulds and the lactic acid bacteria, no significant differences were observed between the levels present on the cabbage treated with tap water or chlorine after the 5 day storage period and both microbial populations remained below 10^3 CFU/g. In addition, it was noted that the appearance of the cut cabbage treated with chlorine after 5 days had a noticeable brown discolouration, whereas the samples treated with tap water had no discolouration and a good fresh-like appearance.

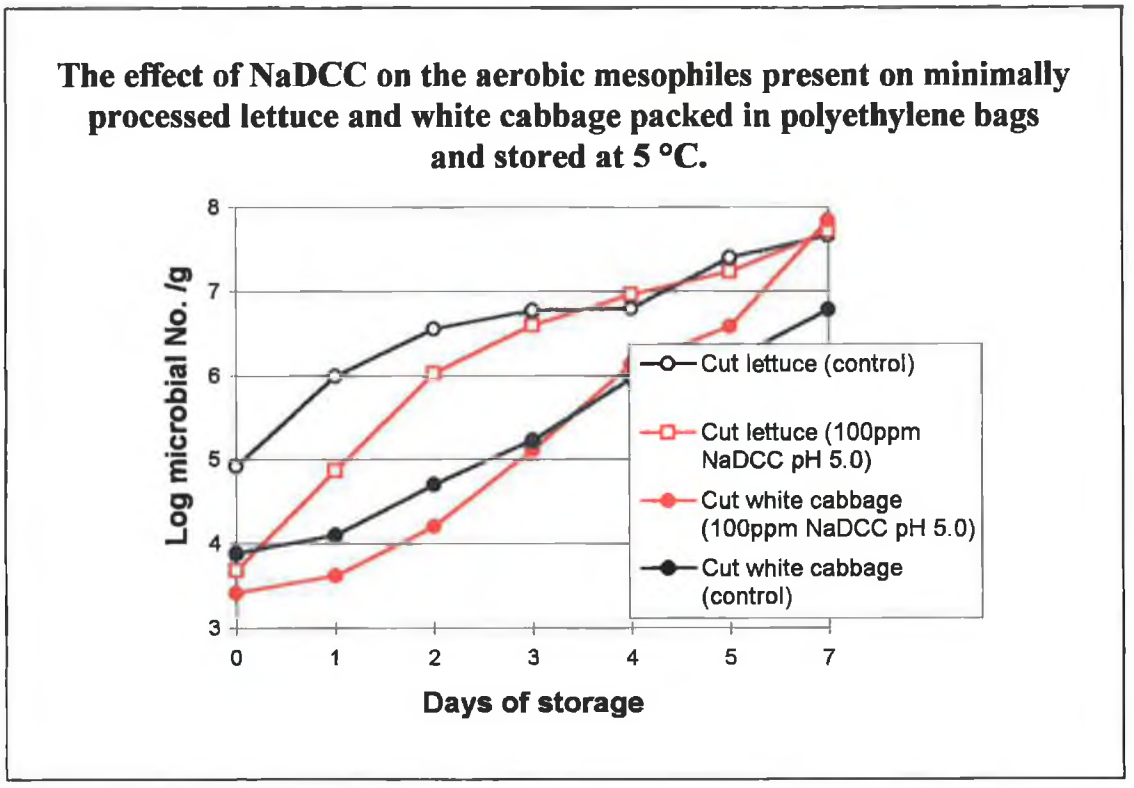
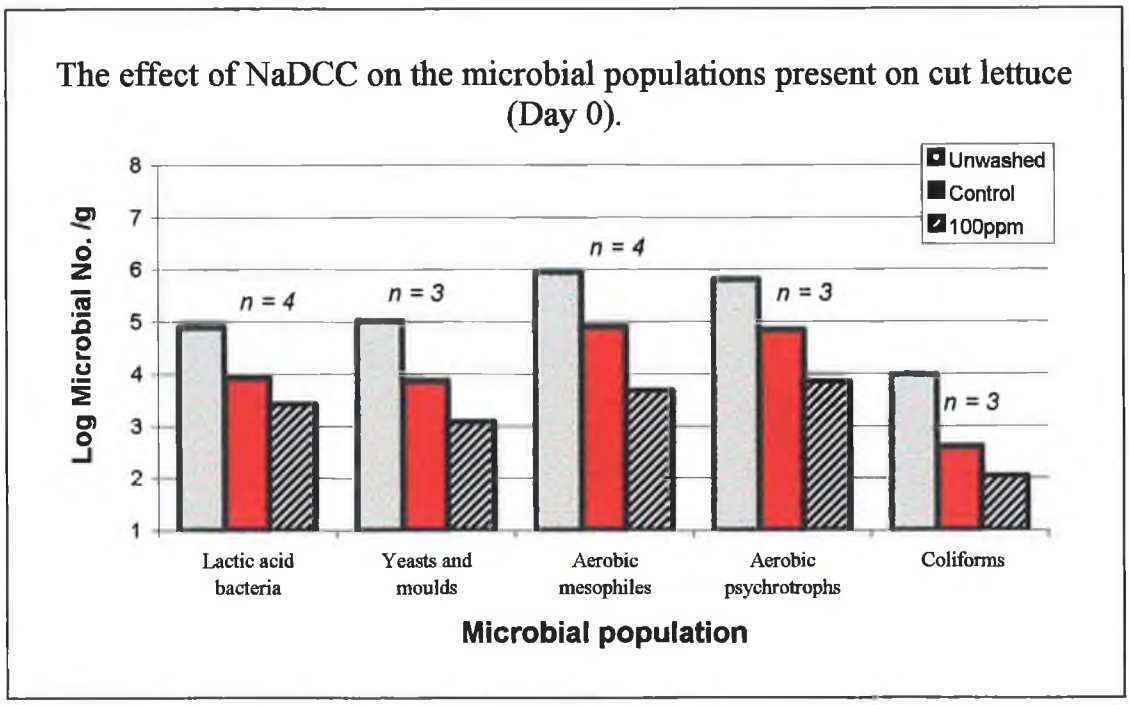
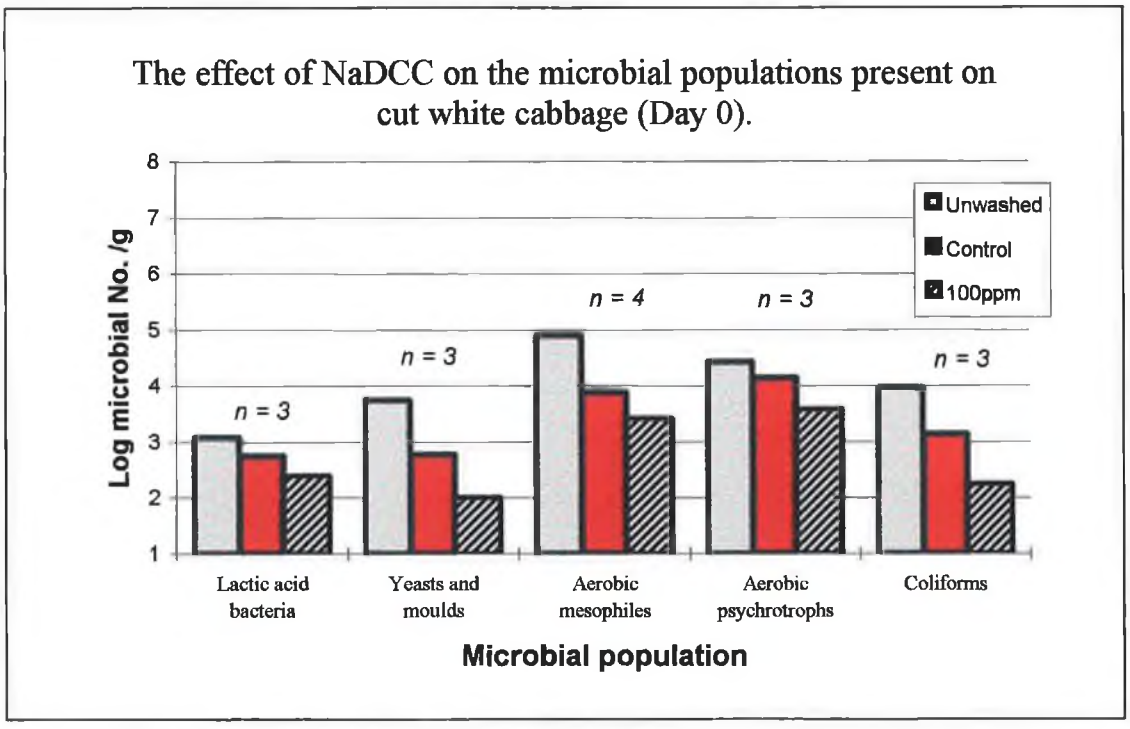


Figure 3.3: The effect of chlorine treatment (100ppm NaDCC pH 5.0) on the total aerobic mesophiles present on cut lettuce and cabbage over a period of 7 days storage at 5°C (n=5).

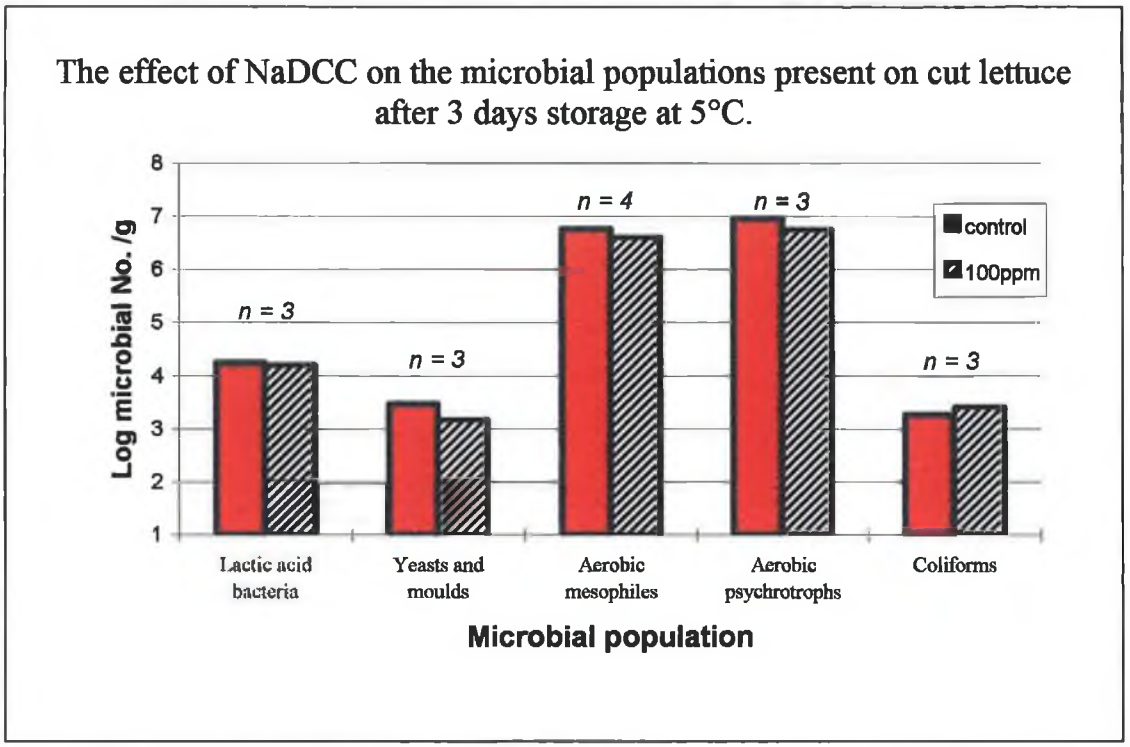


(a)

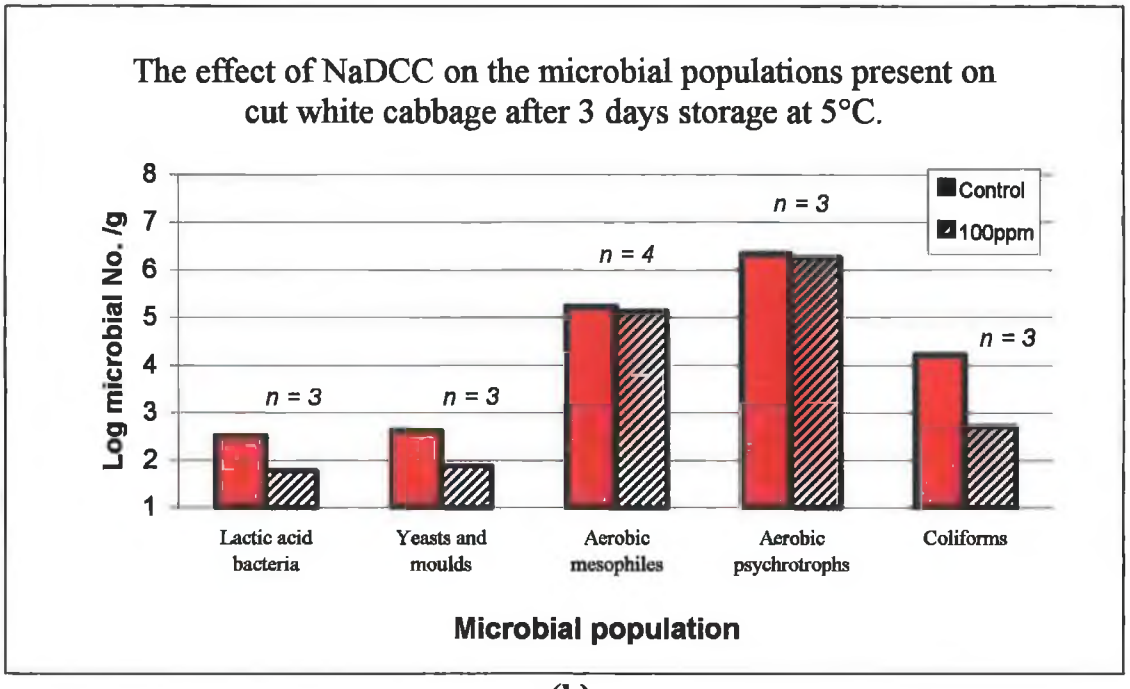


(b)

Figure 3.4: The effect of chlorine treatment (100ppm NaDCC pH 5.0) on the initial populations present on a) cut lettuce and b) cut cabbage.

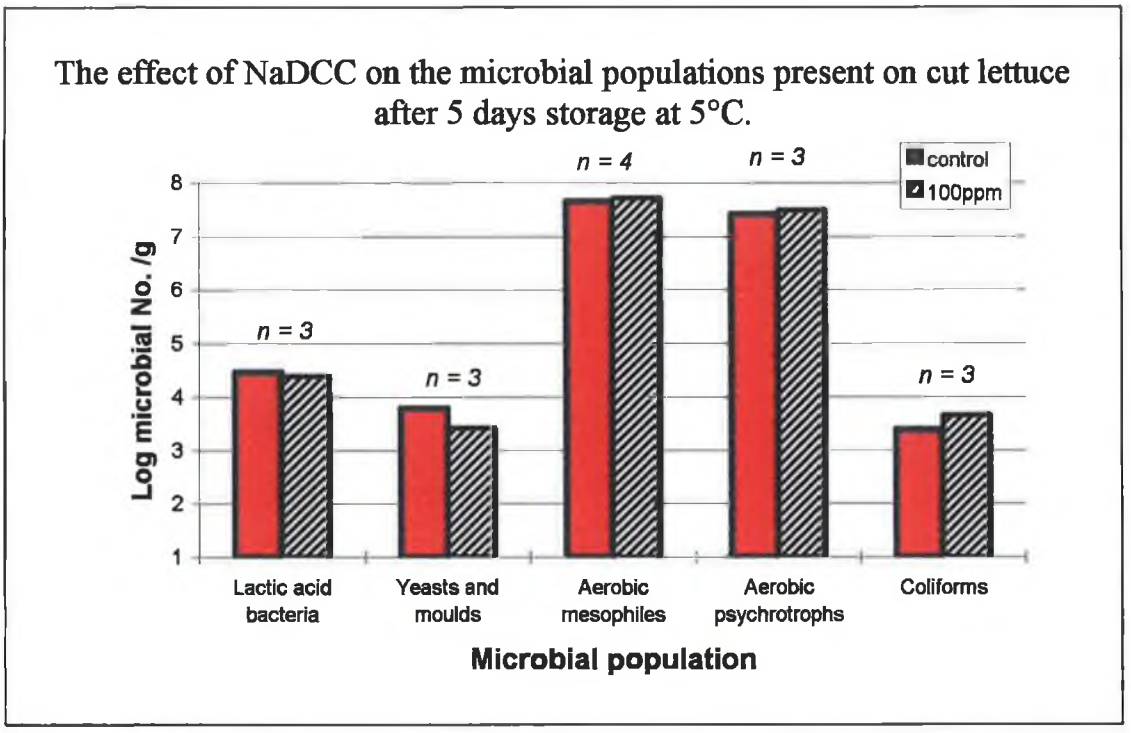


(a)

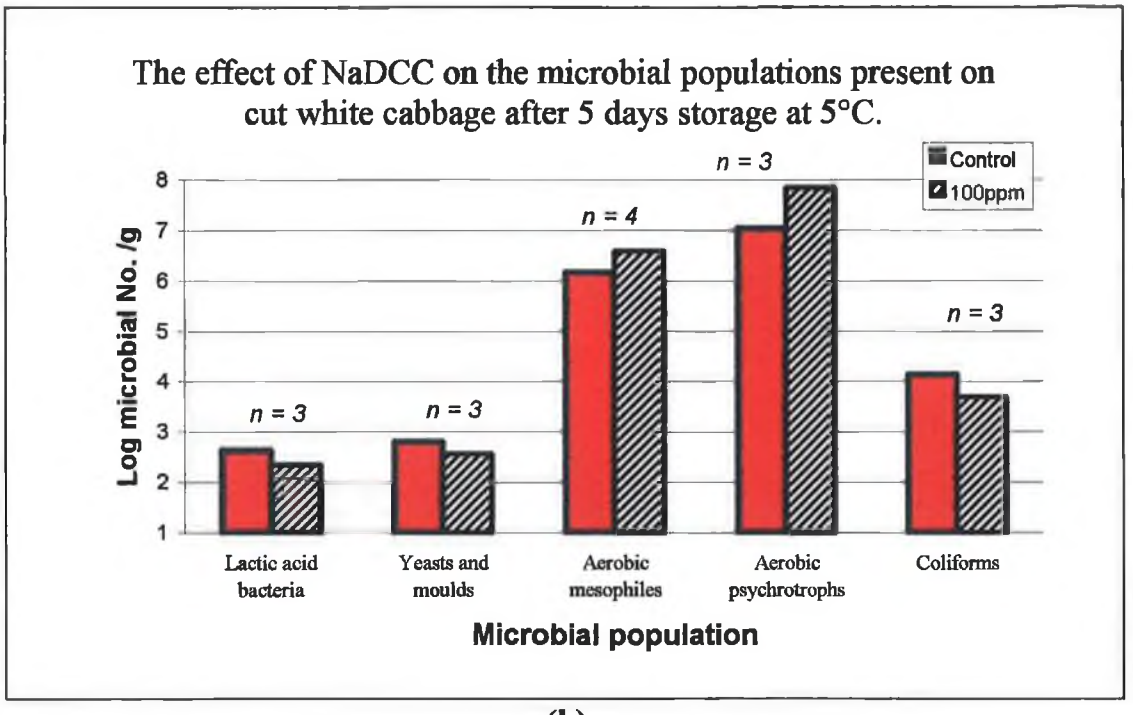


(b)

Figure 3.5: The effect of chlorine treatment (100ppm NaDCC pH 5.0) on the microbial populations present on a) cut lettuce and b) cut cabbage after 3 days storage at 5°C.



(a)



(b)

Figure 3.6: The effect of chlorine treatment (100ppm NaDCC pH 5.0) on the microbial populations present on a) cut lettuce and b) cut cabbage after 5 days storage at 5°C.

3.4 DISCUSSION

3.4.1 ANTIMICROBIAL EFFECT OF CHLORINE ON SALAD VEGETABLES DURING REFRIGERATED STORAGE

The addition of a second vegetable type allowed a comparison of the efficiency of a chlorine disinfectant on different vegetable types. Results showed that the antimicrobial activity of chlorine was less effective against the aerobic mesophiles present on cabbage than those present on lettuce. Cabbage treated with chlorine showed an increase in the aerobic mesophiles after 4 days refrigerated storage (Figure 3.3). This effect was not observed on the chlorine treated lettuce. The findings highlight changes in population dynamics that can occur when salad vegetables are treated with a chlorine disinfectant. This prompted further investigation into the growth of different physiological groups of microorganisms during refrigerated storage on lettuce and cabbage treated with chlorine.

3.4.2 MICROBIAL CONTAMINATION OF SALAD INGREDIENTS

Aerobic mesophiles and Psychrotrophs

Microbial analysis of the unwashed cut lettuce and white cabbage showed the level of microbial contamination on the cabbage was lower than on the lettuce (Figure 3.4a&b). On both salad vegetables the number of aerobic mesophiles and psychrotrophs constituted the greater proportion of microbial contamination. The number of aerobic mesophiles and psychrotrophs ranged from 10^5 to 10^6 CFU/g on lettuce and on white cabbage from 10^4 to 10^5 CFU/g. Similar results were observed by Marchetti *et al.* (1992) who examined ready-to-use salad ingredients including red and green chicory, carrots and mixed salads and found approximately equal counts of mesophiles and psychrotrophs. Studies on fresh cut vegetables carried out by Garg *et al.*

(1990) also found numbers of psychrotrophic bacteria comparable to the mesophilic bacteria. The high levels of psychrotrophs isolated from cut vegetables may indicate that the process of selecting for psychrotrophs began soon after the vegetables were harvested.

Yeasts and moulds

The yeasts and moulds present were approximately 1 log cycle lower than the aerobic mesophiles found on the unwashed vegetables (Figure 3.4a&b). Yeasts were predominant and the presence of moulds were noted infrequently. Similar counts of yeasts and moulds (1 log cycle lower than the mesophiles) was observed on unprocessed iceberg lettuce (King *et al.*, 1991), and for total yeasts on unprocessed white cabbage (Geeson, 1979). A review of the microbiology of MP vegetables by Nguyen-The and Carlin (1994) showed that the number of yeasts and moulds are generally lower than the mesophiles, however, levels have been reported higher than the mesophiles by Albrecht *et al.* (1995) on salad bar ingredients.

Lactic acid bacteria

The lactic acid bacteria present on the unwashed lettuce yielded significantly higher counts than levels found on unwashed cabbage. Numbers of lactic acid bacteria present on other MP vegetables were also found to be variable ranging from 10 for cauliflower florets (Garg *et al.*, 1991) to 10^9 CFU/g for mixed salad vegetables (Nguyen-The and Carlin, 1994). It has been observed by Manvell and Ackland (1986) that the lactic acid bacteria may predominate in vegetable salads when held at an abuse temperature of 30°C.

Coliform bacteria

Equal levels of coliform bacteria, 10^4 CFU/g, were obtained from the unwashed lettuce and cabbage. Ercolani (1976) also found no significant differences between total coliform counts, of about 10^5 CFU/g, on fresh market lettuce and fennel sampled over a 2 year period and concluded that the level of contamination of lettuce and fennel was mostly under the control of

their rate of exposure to pollution. Nichols *et al.* (1971) evaluated lettuce one day after irrigation with effluent containing human sewage and found levels of coliform bacteria of approximately 10^4 CFU/g and levels of *E. coli* 1 of about 10^3 CFU/g. Results also showed that the most reliable indication of faecal contamination were tests for *E. coli* 1 and coliform bacteria at 37°C. Such high levels of coliform bacteria are not uncommon on salad vegetables. High counts have also been reported on on ready-to-eat salad vegetables from hotels and restaurant in Bangladesh of $10^{2.3}$ to $10^{4.8}$ CFU/g (Khan *et al.*, 1994) and on ready-to-use vegetable salads between $10^{3.8}$ and $10^{6.5}$ CFU/g (Marchetti *et al.*, 1992). Hall et al. (1967) suggested that a limit of 10 coliform per gram should be a standard for raw food. However, a study carried out by Fowler and Foster (1976) to determine the feasibility of developing control guidelines for the quality of fresh green salads and coleslaw without dressing found levels of coliforms ranging from 10^1 to $10^{5.2}$/g. The authors concluded that it would be difficult to do so due to the extreme variability of analysis within a given product. Neither the total plate counts nor the total coliform counts exhibited enough uniformity to recommend microbiological guidelines. They did suggest that if special applications, such as hospital feeding or for special patient feeding are intended, then special procedures of analysis for all pathogens could be carried out.

3.4.3 THE ANTIMICROBIAL EFFECT OF CHLORINE ON THE MICROBIAL POPULATIONS ON SALAD INGREDIENTS

Treatment of the salad ingredients with 100ppm NaDCC pH 5.0 significantly reduced the initial microbial load which included the aerobic mesophiles and psychrotrophs, coliforms, lactic acid bacteria and yeasts and moulds (Figure 3.4a&b). Chlorine treatment of shredded iceberg lettuce with 200ppm free chlorine by Beuchat and Brackett (1990) also found a significant reduction in initial populations of aerobic mesophiles and psychrotrophs but

not of the yeasts and mould. However, the chlorine treatment was not as effective in reducing the populations of psychrotrophs as it was in reducing the mesophiles. Studies carried out by Barriga *et al.* (1991) found the initial levels of aerobic mesophiles and psychrotrophs present on iceberg lettuce were reduced to approximately 10^4 CFU/g after treatment with 100ppm hypochlorite solution for 5 minutes at 15°C, which is comparable to levels achieved in this study after chlorination of the cut lettuce and cabbage. Low levels of lactic acid bacteria and coliforms were isolated by Barriga *et al.* (1991) from the iceberg lettuce after chlorine treatment and varied between 10^0 and 10^2 CFU/g.

No antimicrobial effect of chlorine on the microbial populations was observed on cut lettuce after 3 and 5 days storage and the levels of microbes on the lettuce treated with tap water were equal to the levels found on the chlorine treated lettuce. In contrast, the lactic acid bacteria, coliforms and yeasts and moulds remained significantly lower after 3 days storage on the cabbage treated with chlorine. No significant difference was observed after 5 days storage. Moreover, the populations of aerobic mesophiles and psychrotrophs present on cabbage treated with chlorine, which were significantly reduced on day 0, were significantly higher on samples treated with chlorine than on samples treated with tap water after 5 days storage. These results show a fundamental difference between microbial population dynamics on the two vegetables.

One explanation for the increase in aerobic mesophiles and psychrotrophs on chlorine treated cabbage may be that chlorine is rarely effective in preventing spoilage by bacteria closely associated with the plant tissue (Eckert, 1977). This is probably due to (a) the free chlorine is being reduced by constituents of the host tissue before it reaches the spoilage bacteria and (b) that most bacteria found on the surface of vegetables have been found in hydrophobic protective pockets (Adams *et al.*, 1989) and in

biofilms (Gras *et al.*, 1994). Spoilage bacteria normally associated with plant tissue include the pectinolytic *Pseudomonas* spp. (aerobic psychrotrophs) and *Erwinia* spp. (aerobic mesophiles) which are known to cause soft rot in most vegetables. More importantly the pectinolytic fluorescent pseudomonads, frequently found on vegetables, are able to grow and macerate tissue at 6°C or below (Brocklehurst and Lund, 1981; Liao and Wells, 1987). The fact that psychrotrophs present on cut cabbage were not reduced by tap water (Figure 3.4) would suggest their close association with the plant tissue surface. It is therefore possible to hypothesise that aerobic psychrotrophs and mesophiles on cabbage are closely associated with surface tissue, which protect them from the biocidal activity of chlorine. During refrigerated storage it is possible that the aerobic mesophiles and psychrotrophs surviving chlorine can grow more rapidly due to fewer bacterial competitors for nutrients. The rapid growth of pectinolytic psychrotrophs would also account for the brown discoloration observed only on the cut cabbage treated with chlorine.

Another contributing factor that may explain the different microbial behaviour on the two salad vegetables was the lower levels of lactic acid bacteria found on the unwashed cabbage (approximately $10^{2.5}$) compared to the levels isolated from the unwashed lettuce (approximately $10^{5.0}$). After treatment of the cut cabbage with chlorine, the level of lactic acid bacteria was significantly lower than the level found on the cabbage treated with tap water. This reduction was maintained over 3 days storage and after 5 days storage the levels of lactic acid bacteria on cabbage treated with chlorine and tap water, although not significantly different, remained below 10^3 CFU/g. In comparison, treatment of the cut lettuce with chlorine also reduced the levels of lactic acid bacteria, but the reduction was not maintained after 3 days storage and the levels increased to approximately $10^{4.5}$ CFU/g after 5 days storage.

It is well recognised that, in addition to acids, some lactic acid bacteria produce inhibitory compounds such as bacteriocins, antibiotic or bacteriocin-like substance (Kim, 1993). A study investigating the inhibitory effect of selected lactic acid bacteria on microflora associated with ready-to-use vegetables found that the use of lactic acid bacteria as inoculant, in particular *Lactobacillus casei,* caused a significant reduction in total aerobic mesophiles during refrigerated storage (Vescovo *et al.*, 1995). Thus, the higher levels of lactic acid bacteria on the cut lettuce, even after treatment with chlorine, could have inhibited to a greater extent the growth of the aerobic mesophiles and psychrotrophs during storage compared to the levels present on the cabbage. The lower levels of lactic acid bacteria on the cut cabbage during storage also reduces the level of competition with the aerobic mesophiles and psychrotrophs for nutrients.

Other studies on the antimicrobial effect of chlorine treatment on the different microbial population present on salad vegetables during storage have, in general, been carried out under modified atmospheres. Populations of 10^8 CFU/g aerobic mesophiles on the shredded iceberg lettuce treated with 200ppm free chlorine were detected after 15 days at 5°C, under modified air containing 3% O_2 and 97% N_2 in sealed bags and populations of psychrotrophs generally exceeded those of mesophilic aerobic microorganisms (Beuchat and Brackett, 1990). The numbers of mesophilic and psychrotrophic aerobes on iceberg lettuce treated with 100ppm hypochlorite solution (Barriga *et al.* 1991) after 12 days storage at 4°C in air and in a modified atmosphere (2.5% O_2 + 10.5% CO_2 and 3% O_2) increased to approximately 10^7 CFU/g regardless of storage conditions. Total coliforms were present in low numbers and increased to 10^3 CFU/g throughout storage. Controlled atmosphere did not influence their development and the levels of coliforms were considered acceptable.

3.4.4 CONCLUSIONS

Treatment of the lettuce and cabbage with chlorine effectively reduces the microbial load. All the microbial populations were reduced by the chlorine disinfectant. The biocidal effect of NaDCC varied when applied to the dufferent vegetable types. More notably, the numbers of lactic acid bacteria and aerobic psychrotrophs were reduced on the cut cabbage only after chlorine treatment and not treatment with tap water. In general, the antimicrobial effect did not last more than three days.

Chlorine treatment of the vegetables significantly reduced the levels of coliforms on these two salad vegetables. Although the levels increased during refrigerated storage, they did not exceed the initial levels found on the unwashed vegetables. In addition, the levels found on the chlorine treated cabbage remained lower than the cabbage treated with water. Hence the application of chlorine as a disinfectant for vegetables would successfully reduce levels of coliform bacteria particularly when the microbiological quality is a concern. For example, vegetables that are consumed in Mexico city come mainly from agricultural areas which are irrigated with municipal wastewater (Rosas *et al.*, 1984) and pose a significant threat to public health. Treatment of these vegetables with chlorine to reduce the levels of coliform bacteria could therefore be recommended.

CHAPTER 4 : THE IMPACT OF NaDCC ON MICROBIAL PATHOGENS PRESENT ON SALAD VEGETABLES

4.1 INTRODUCTION

Chapter 3 compared the effect of NaDCC on the different microbial populations present on lettuce and cabbage and included the groups of microorganisms commonly associated with bacterial spoilage of vegetables. This chapter investigates the effect of chlorine on pathogens commonly associated with salad vegetables and considers the application of chlorine as a method to improve the microbiological safety of raw salad vegetables.

4.1.1 SAFETY ASPECTS OF SALAD VEGETABLES

For the most part, fresh vegetables are viewed as the safest of foods. Pathogens are unlikely to grow in vegetables because of competition from spoilage microorganisms. However vegetables, and in particular MP vegetables, are not without risk. The high moisture content of fresh cut vegetables, along with the lack of a heat processing step and the potential for temperature abuse increases the risk of foodborne illness. Some foodborne infections linked to the consumption of raw vegetables are listed in Table 4.1.

A survey on foodborne disease outbreaks in the United States from 1973 to 1987 (Bean and Griffin, 1990) found that the highest incidents of food poisoning outbreaks associated with fruit and vegetables were caused by *Clostridium botulinum* (99 cases) followed by *Salmonella* spp. (9 cases), *Staphylococcus aureus* (4 cases), *Shigella* spp. (3 cases), *Bacillus cereus* (3 cases), *Yersinia enterocolitica* (2 cases) and 1 case was reported each of *Escherichia coli, Clostridium perfringens* and *Streptococcus* spp. Bacteria not previously recognised as important foodborne pathogens that emerged during the study period included *Campylobacter jejuni*, *Escherichia coli* 0157:H7 and *Listeria monocytogenes*. Bacterial pathogens accounted for 90% of deaths

recorded during the study period, with *L. monocytogenes* having the highest death to case ratio (317/1000) followed by *Clostridium botulinum* (197/1000).

Table 4.1: Foodborne infections linked to the consumption of raw vegetables.

Pathogen	Vegetable
Salmonella	Beansprouts[a]
Vibrio cholerae	Cabbage[b]
Shigella sonnei	Shredded lettuce[c]
Bacillus cereus	Vegetable sprouts[d]
Listeria monocytogenes	Raw vegetables in salads[e] Shredded cabbage in coleslaw[f]
Escherichia coli (Enterotoxic)	Salads of raw vegetables[g]
Clostridium botulinum	Shredded cabbage in coleslaw[h]

a) O'Mahony *et al.*, 1990; b) Swerdlow *et al.*, 1992; c) Davis *et al.*, 1988; d) Portnoy *et al.*, 1976; e) Ho *et al.*, 1986; f) Schlech *et al.*, 1983; g) Merson *et al.*, 1976; h) Solomon *et al.*, 1990.

4.1.2 SOURCES OF CONTAMINATION OF MP VEGETABLES WITH PATHOGENIC MICROORGANISMS

The potential for MP vegetables to become contaminated with pathogenic microbes is high because of their exposure to a wide variety of conditions during growth, harvesting and distribution. According to Madden (1992) the sources of contamination on vegetables and conditions that allow them to become potentially dangerous include:

i) contaminated irrigation water;

ii) human handling;

iii) animal waste fertiliser;

iv) wild and domestic animals;

v) post harvest washing;

vi) improper storage;

vii) new packaging technologies;

viii) improper packaging;

ix) contamination from other food in the preparation area.

Typically, diseases associated with vegetables are infections resulting from contamination of the food with Gram negative enteric pathogens. These bacteria can gain entry to foods when animal fertilisers or contaminated irrigation water are used during production (Roberts, 1984). *Vibrio cholerae* infections in South America were associated with consumption of raw vegetables contaminated with polluted water (Swerdlow *et al.*, 1992). The 1981 outbreak of *Listeria monocytogenes* in Canada was linked to the consumption of coleslaw made with cabbage that was probably contaminated with sheep manure. Poor sanitary conditions during cultivation or harvest were also incriminated in contamination of vegetables by *Campylobacter* spp. (Park and Sanders, 1992). Vegetables may also be contaminated with faecal indicators by food handlers during harvest and transport, as suspected for a range of vegetables (Geldreich and Bordner, 1971) and for parsley (Käferstein, 1976).

The risk of contamination of vegetables during harvesting and processing is also a function of the ability of pathogens to survive in the field. *L. monocytogenes* can survive for several months in samples of soil, in the environment (Watkins and Sleath, 1981) and in the laboratory (Welshimer, 1960). Bryan (1977) reported cases of *Salmonella* surviving in soils for more than 200 days although in most experiments the bacteria disappeared after a few days. Studies carried out by Nichols *et al.* (1971) on lettuce irrigated with sewage effluent found that *E. coli* survived more than 21 days on lettuce in the field irrigated with sewage.

4.1.3 BACTERIAL PATHOGENS ASSOCIATED WITH SALAD VEGETABLES

For the purpose of this study seven bacterial pathogens associated with salad vegetables were chosen and include: *Listeria monocytogenes Salmonella enteritidis, Shigella sonnei, Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*. All of the chosen pathogens have been implicated in foodborne disease outbreaks caused by consumption of contaminated vegetables and are of increasing concern in MP vegetable products.

4.1.3.1 *Listeria monocytogenes*

Listeria spp. are widely distributed in nature; they are found in soil, plants and animals. Up to 30% of healthy asymptomatic individuals carry these bacteria in their stools. Consequently, endogenous as well as exogenous sources exist. Contaminated food is therefore, only one of several possible origins of infections although there is general agreement that it might be the most frequent one (Hof *et al.*, 1994). *Listeria* can be detected fairly often in various food items at high frequency, including raw milk (1-5%), soft cheese (10-20%), raw meat (≤ 5%), cooked meat (1-3%), poultry (≤60%), seafood (≤20), salad vegetables (10-30%). Fortunately, most of these bacteria belong to the non pathogenic species; only *L. monocytogenes* is a potential health risk for humans.

L. monocytogenes is capable of causing serious and life threatening illnesses including septic abortion, meningoencephalitis and septicaemia in immunocompromised individuals (Farber and Peterkin, 1991). Outbreaks have been traced to foods such as soft cheese and pâté (Gilbert, 1992; Roberts, 1994). *L. monocytogenes* is of special importance as it can grow at refrigerated temperature (temperature growth range of -0.4°C to 44°C), persists as an environmental contaminant and has the potential to cause mortality associated with outbreaks.

L. monocytogenes has been isolated from MP vegetables at frequencies that vary from 0 to 19% of samples of pre-packed salads (Sizmur and Walker, 1988) and of mixed salads and salad ingredients (Velani and Roberts, 1991). In a study on the occurrence of *Listeria* spp. found on fresh market produce, Heisick *et al.* (1989) isolated *L. monocytogenes* from cabbage, cucumber, potato and radish. More significant amounts of contamination were found on potatoes and radishes than on other positive produce. Previous work has also shown that *L. monocytogenes* survives and grows on many raw and processed vegetables, such as iceberg lettuce (Steinbruegge *et al.*, 1988), shredded cabbage (Kallender *et al.*, 1991) asparagus, broccoli and cauliflower (Berrang *et al.*, 1989). Ryser and Marth (1991) also considered raw vegetables as possible sources of Listeriosis.

In 1981, a large outbreak of both adult (7 cases) and perinatal (34 cases) infection due to *L. monocytogenes* in Canada was attributed to consumption of coleslaw made from contaminated shredded cabbage (Schlech *et al.*, 1983). A previous outbreak, in 1979, of *L. monocytogenes* infection involving patients from 8 Boston hospitals was suspected to have been caused by consumption of raw vegetables, in particular lettuce and celery (Ho *et al.*, 1986). The importance of *L. monocytogenes* in MP vegetable salads held at refrigeration temperature must be considered when extending the shelf-life.

4.1.3.2 *Salmonella*

Salmonella spp. are the normal inhabitants of the intestinal tracts of humans and other invertebrates. Salmonellosis continues to be one of the most serious forms of human enteric infection. A marked increase in human salmonellosis has been reported in many countries including the UK. (Maguire *et al.*, 1993) and USA (Tauxe, 1991). Various foods have been implicated as vehicles of *Salmonella* infection. Food of animal origin such as poultry and other meat products, eggs and dairy products are the most commonly implicated sources of salmonellosis outbreaks (Tauxe, 1991).

Fresh fruit and vegetables are implicated less frequently in outbreaks of salmonellosis, however, between 1983 and 1987 2.2% of salmonella food poisoning was caused by the consumption of fruits and vegetables (Tauxe, 1991). An outbreak of infection associated with beansprouts contaminated with *Salmonella saint-paul* was reported by O'Mahony *et al.* (1990). Also four large multi-state outbreaks of salmonellosis have been attributed to fresh produce since 1990 (Hedberg *et al.*, 1993). Two outbreaks involved the consumption of tomatoes contaminated with *Salmonella javiana* and *Salmonella montevideo*. Despite such few outbreaks caused by *Salmonella* on MP vegetables, *Salmonella* spp. have been frequently isolated from several types of salad vegetables, including fresh marketed lettuce and fennel (Ercolani, 1976), leafy vegetables and beansprouts (Arumugaswany *et al.*, 1995), fresh vegetables (Garcia-Villanova Ruiz *et al.*, 1987) and in a range of MP vegetables (Denis and Picoche, 1986). The presence of *Salmonella* spp. in raw produce and consequently in MP vegetable salads is of concern due to the increasing antimicrobial resistance of *Salmonella* to antibiotics and the increased severity of salmonellosis in the infant, elderly and the immunocompromised patient.

4.1.3.3 *Shigella sonnei*

The vast majority of cases of shigellosis are spread by person to person transmission, but many foodborne and waterborne disease outbreaks occur each year. Food almost always becomes contaminated by human faeces, usually from the hand of an asymptomatic person with mild, unrecognised disease (Doyle, 1989). Because the infective dose is small, low numbers of *Shigella* in foods may be infective. Conditions leading to outbreaks occur when infected persons handle foods and the risk of poisoning is increased in foods that require extensive handling. Between 1973 and 1987 the number of outbreaks caused by *Shigella* spp. was 5.6% of the bacterial foodborne

outbreaks reported and poor personal hygiene was the factor most frequently reported (91%) cause of the *Shigella* outbreaks.

Two large outbreaks of gastroenteritis caused by *S. sonnei* was attributed to the consumption of contaminated lettuce. In one outbreak contamination of the product may have occurred in the field or in a warehouse (Martin *et al.*, 1986). A food handler was suspected to have been responsible for the other *S. sonnei* outbreak associated with shredded lettuce. The worker who fed the lettuce into the shredding machine had symptoms comparable with mild shigellosis. Although a subsequent stool specimen did not grow *Shigella*, the specimen was obtained 13 to 14 days after the worker's illness and therefore did not exclude shigellosis. The lettuce shredding process may have allowed cross contamination of the lettuce to occur. None of the surfaces that came into contact with the lettuce were washed until the day's shredding was completed. As a result, any lettuce that became contaminated could have contaminated the shredding equipment and thereby subsequent lettuce. After shredding the lettuce was held at elevated temperatures for up 6 hours. Thus, improper storage temperature was a contributing factor to the outbreak of *S. sonnei.*

Subsequent experiments carried out by Davies *et al.* (1988) found that the strain responsible for the outbreak grew well on shredded and intact lettuce at 22°C and survived at refrigeration temperatures for at least one week. Satchel *et al.* (1990) studied the survival of *S. sonnei* on shredded cabbage packaged under vacuum and modified atmospheres as well as aerobic conditions and also found that it grew well at room temperature (24±2°C) and survived well at refrigeration temperatures (0° to 6°C). Thus, the presence of *Shigella* spp. on MP vegetables poses a potential hazard to the customer as the infective dose is low and time-temperature abuse may increase the safety risk.

In 1994, an increase in the number of cases of *Shigella sonnei* infection occurred in several European countries, including Norway, Sweden and the

UK. In all three countries epidemiological evidence incriminated iceberg lettuce of Spanish origin as a vehicle of transmission (Kapperud *et al.*, 1995).

4.1.3.4 *Escherichia coli*

E. coli encompasses a great variety of strains that include purely commensal organisms as well as those possessing combinations of virulence determinants that enable them to act as specific pathogens of the gut and of extra intestinal sites, especially the urinary tract, viz enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli* and enterohemorragic *E. coli* (Gross, 1992). The presence of *E. coli* type 1 is usually considered to indicate recent excretal pollution. Food may be contaminated by infective food handlers who practice poor personal hygiene or by contact with water contaminated by human sewage (Doyle, 1989). Hall and Hauser (1966) found that 6.4% of healthy workers to be carriers of enteropathogenic *E. coli*. Geldreich and Bordner (1971) found that the number of faecal coliforms present on many vegetables was likely to be relatively low provided that vegetables were not subject to pollution by humans, animals or irrigation waters. Microbiological limits applicable immediately after preparation for sale, for fresh salad vegetables to be consumed raw are specified by the International Commission on Microbiological Specifications for Foods (ICMSF, 1974); a count of 10 *E. coli* type 1/g would be regarded as undesirable and more than 1000/g would be unacceptable.

Contamination with *E. coli* was reported on salad vegetables offered for retail sale by Brocklehurst *et al.* (1987). A study on traveller's diarrhoea in Mexico City revealed that enterotoxigenic *E. coli* accounted for 45% of the cases and that the illness was associated with the consumption of salads containing raw vegetables (Merson *et al.*, 1976). As salad vegetables are consumed raw, exposure of salad crops to faecal contamination including *E. coli* is a cause of concern in the processing of MP salad vegetables.

4.1.3.5 *Staphylococcus aureus*

Staphylococcal food poisoning is a common foodborne illness around the world. Poisoning results from the ingestion of enterotoxins produced in foods by *S. aureus* (Doyle, 1989). The main reservoirs of *S. aureus* are the human nasal cavity, skin and hair. Thus, foods that require handling can easily become contaminated. Staphylococcal food poisonings are usually caused by unsanitary handling of food or holding food at the wrong temperature and therefore the risk of staphylococcal contamination and potential production of enterotoxins is high on ready-to-eat salads, in particular, those purchased from salad bars.

Studies carried out by Maxcy (1978) showed that *S. aureus* inoculated onto lettuce and stored at 23° to 25°C showed some growth after a lag period of 2 hours. Gourama *et al.* (1991) who examined the growth and enterotoxin production on salad bar ingredients and clam chowder, found that *S. aureus* survived on salad ingredients including lettuce for more than 48 hours. However, no enterotoxins were produced by the *S. aureus* on the salad ingredients. Daoud and Debevere (1984) also found that despite good growth of *S. aureus* in heated vegetables the production of enterotoxin was low. Gourama *et al.* (1991) concluded that even though enterotoxin production was not demonstrated on salad bar ingredients, there is a risk of contamination of other items found in salad bars, such as the clam chowder which was found to support enterotoxin production. Thus, cross contamination of *S. aureus* from salad vegetables onto ingredients that can support entertoxin production could lead to staphylococcal food poisoning and is more likely to occur if temperature is not properly controlled.

4.1.3.6 *Bacillus cereus*

Aerobic, endosporeforming bacteria have been suspected or implicated as agents of food poisoning since the early days of microbiology.

Nevertheless, a certain amount of scepticism has surrounded their association with gastroenteritis, and it is only in relatively recent times that *Bacillus* spp. have been given increasingly more attention, often under the guise of "new" or "Emerging" foodborne enteropathogens. The European literature from the beginning of this century contains many accounts of food poisoning outbreaks by *B. cereus* or *B. cereus*-like organisms. One of the earliest episodes of a *B. cereus*-like food poisoning outbreak was reported in 1908, involving 300 patients and staff in a hospital who had eaten contaminated meatballs. In more recent times, food vehicles responsible are almost invariably farinaceous, containing either cereal (mainly rice) or cereal derived ingredients such as flour (Doyle, 1989).

B. cereus caused 3.1% of reported food poisoning outbreaks between 1973 and 1987 (Bean and Griffin, 1990) in the USA. The foods involved included Chinese food (24 outbreaks), Mexican food (5 outbreaks), beef (3 outbreaks) and fruit and vegetable (3 outbreaks). Improper storage or holding temperature was the factor most frequently reported (94%) as a cause of the outbreaks of *B. cereus*. In 1973 four cases of *B. cereus* food poisoning were caused by contamination of raw vegetable sprouts that had been grown in a home seed sprouting kit purchased from a local health food store.

B. cereus is found widely in the environment, including soils and clays (particularly those associated with the rhizosphere), dust, natural waters and vegetation (Doyle, 1989). Hence the occurrence of *B. cereus* in nature, the temperature at which the vegetative cells can grow and multiply (between 10° and 50°C) and the temperature range at which spores can germinate (between 5° and 50°C) increases concern for the presence of *B. cereus* in MP vegetables, in particular, if time - temperature abuse occurs.

4.1.4 OBJECTIVES

Chlorine is an effective disinfectant agent against bacterial pathogens. Nevertheless, there are few reports in literature about the use of disinfectants against pathogens in foods, especially on fresh cut vegetables. Hence, the objectives of this part of the study are as follows:

1. To inoculate pathogens commonly associated with salad vegetables onto cut lettuce and allow them to become established on the surface;

2. To examine the antimicrobial activity of a chlorine solution (100ppm NaDCC pH 5.0) on pathogens present on the surface of cut lettuce.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Salmonella enteritidis (ATCC 3076)

Salmonella enteritidis (ATCC 3076)

Shigella sonnei (ATCC 25931)

Staphylococcus aureus (ATCC 29213)

Bacillus cereus (ATCC 11778)

Escherichia coli (ATCC 110497)

Tryptone soya broth

LAB M 4: A general purpose nutritious broth capable of growing a wide range of bacteria and fungi.

30g of powder was dispersed in 1 litre of deionised water and swirled to mix. The media was dispensed into Duran bottles and then autoclaved at 121°C for 15 minutes.

Brilliant Green Agar Modified

LAB M 34: A selective medium for the isolation of Salmonellae (except *S. typhi*).

52g of powder was dispersed in 1 litre of deionised water and allowed to dissolve for 10 minutes. The mixture was brought to the boil with frequent swirling to dissolve the solids and cooled to 47°C before pouring into sterile petri dishes.

Eosin Methylene Blue Agar

LAB M 61: A selective medium for the enumeration of *E. coli,* which produce blue black colonies with a distinctive metallic sheen on this medium.

37.5g of powder was dispersed in 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix. The mixture was then sterilised by autoclaving at 121°C for 15 minutes, the medium was cooled to 50° and

agitated gently to ensure uniform distribution of the flocculant precipitate before pouring into sterile petri dishes.

Bacillus Cereus Medium

LAB M 73: A medium for the enumeration of *B. cereus* in foods.

48g of powder was dispersed in 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix. The mixture was then sterilised by autoclaving at 121°C for 15 minutes. After cooling to 47°C 100 mls of XO73 egg yolk emulsion and 2 vials of XO74 polymixin was aseptically added before pouring into sterile petri dishes.

Baird-Parker Medium

LAB M 85: A complex medium for the enumeration of *S. aureus* in foods.

65.5g of powder was dispersed in 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix before autoclaving for 15 minutes at 121°C. After cooling to 47°C, 50mls of XO85 sterile egg yolk tellurite emulsion was aseptically added before pouring into sterile petri dishes.

Xylose Lysine Decarboxylase

LAB M 32: A selective medium for the recovery and recognition of *Shigella* spp.

53.5g of powder was dispersed in 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix. The mixture was rapidly brought to the boil with frequent stirring and allowed to cool to 47°C before pouring into sterile petri dishes.

Listeria Isolation Medium

LAB M 122: A selective identification medium for the isolation of *Listeria monocytogenes* from food.

57.5g of powder was added into 1 litre of deionised water, allowed to soak for 10 minutes and swirled to mix before autoclaving at 121°C for 15 minutes.

After cooling to 47°C 2 vials of selective supplement X122 was aseptically added and mixed well before pouring into sterile petri dishes

Biological Safety Cabinet

Class II cabinet, series "SE", Make :MDH. Model: M51424/2. AGB Scientific LTD., Ireland

4.2.2 PREPARATION OF LETTUCE LEAVES

The inner core and outer leaves were removed from the butterhead lettuce heads. The remainder of leaves were soaked in tap water for 5 minutes and then drained. Leaves of similar morphology were cut into approximately 5cm × 5cm for inoculation.

4.2.3 PREPARATION OF INOCULANT

All work with the pathogens were carried out in the Biological safety cabinet. Inoculation dips of *Salmonella enteritidis, Shigella sonnei, Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* were prepared as follows: A 24 hour (37°C) culture maintained on nutrient agar was used to prepare an 18 hour (37°C) culture in Tryptone Soya Broth. 5 mls of the 18 hour culture was centrifuged for 5 minutes and then resuspended in 450 mls ringers solution to obtain approximately 10^4 to 10^6 CFU/ml which was checked using the appropriate media.

For *L. monocytogenes* the method of Carlin and Nguyen-The (1994) was used. A 24 hour culture was maintained on nutrient agar (30°C). The bacteria was then cultured for 4 days at 10°C in Tryptone Soya Broth and 5 mls from this was centrifuged for 5 minutes and resuspended in 450 mls ringers solution to obtain an inoculant dip of approximately 10^6 CFU/ml.

4.2.4 INOCULATION OF LETTUCE LEAVES

Inoculation of the lettuce leaves was adapted from the method used by Carlin and Nguyen-The (1994). The cut lettuce leaves were completely submerged into the bacterial suspensions for 15 minutes. Inoculated leaves were then drained twice on absorbent paper and each placed in the polyethylene food bags closed using the fold-closure mechanism.

For all strains tested, except *L. monocytogenes,* the inoculated leaves were incubated in the food bags for 24 hours at 30°C in order for the bacteria to become established on the lettuce. A greater than 1 log increase after incubation was confirmation of this.

Due to the concern about the growth of *Listeria monocytogenes* at refrigeration temperatures the inoculated lettuce leaves were incubated for 2 days at 10°C. Under these conditions *Listeria monocytogenes* on butterhead lettuce was shown by Carlin and Nguyen-The (1994) to increase by more than 1 log cycle.

4.2.5 CHLORINE TREATMENT OF LETTUCE LEAVES

A 100ppm NaDCC pH 5.0 solution was prepared using Aquatabs as already described in section 2.2.3. The lettuce leaves were submerged in 1 litre chlorine solution (test) and 1 litre tap water solution (control) for 30 minutes. After treatment the leaves were removed and dried twice on absorbent paper.

4.2.6 EXPERIMENTAL METHOD

To meet the first objective each pathogen was inoculated onto the lettuce surface and allowed to become established on the surface. The level of

each pathogen on the lettuce was determined before and after incubation to confirm a minimum 1 log cycle increase.

To meet the second objective the antimicrobial effect of chlorine against pathogens established on the surface of cut lettuce was examined. The inoculated lettuce leaves were treated with 100ppm NaDCC pH 5.0. Inoculated leaves treated with tap water for 30 minutes were used as a control.

4.2.7 ISOLATION AND ENUMERATION OF PATHOGENS FROM LETTUCE

10g samples were used for isolation and enumeration of the microbial pathogens. Preparation of samples were carried out according to microbiological analysis in section 2.2.5. The culture media, methods and incubation conditions employed are listed in Table 4.2. All plates were incubated aerobically with surface spreading.

Table 4.2: Table of culture media and incubation conditions for the isolation and enumeration of the microbial pathogens present on the cut lettuce pieces.

TEST	CULTURE MEDIA	SUPPLEMENT	TEMPERATURE (°C)	TIME (DAYS)
Salm. enteritidis	BGA modified[1]	none	37	1
E. coli	EMB agar[2]	none	37	1
B. cereus	BCM[3]	Egg yolk emulsion Polymixin	37	1
Staph. aureus	Baird-Parker	Egg yolk tellurite emulsion	37	1
Shig. sonnei	XLD agar[4]	none	37	1
L. monocytogenes	Listeria isolation medium	Listeria selective supplement	30	1

1. Brilliant Green Agar
2. Eosin Methylene Blue
3. Bacillus Cereus Medium
4. Xylose Lysine Decarboxylase

4.3 RESULTS

4.3.1 INCUBATION OF LETTUCE LEAVES WITH PATHOGENS

To ensure each pathogen became established on the vegetables surface levels were determined before and after incubation. Approximate values for the levels of pathogens are given in Table 4.3. Approximate values for the levels of pathogens on the lettuce before incubation ranged between 10^3 to 10^6 CFU/g and after incubation between 10^6 to 10^9 CFU/g.

Table 4.3: Levels of pathogens present on lettuce before and after incubation in polyethylene food bags.

Pathogen	Before incubation (CFU/g) *n*=1	After Incubation (CFU/g)*n*=1
S. enteritidis	~10^5	~10^8
E. coli	~10^5	~10^9
B. cereus	~10^3	~10^6
S. aureus	~10^4	~10^6
S. sonnei	~10^3	~10^8
L. monocytogenes	~10^5	~10^7

(~ Approximate value)

4.3.2 THE ANTIMICROBIAL EFFECT OF NaDCC AGAINST PATHOGENS PRESENT ON THE SURFACE OF CUT LETTUCE

The antimicrobial activity of chlorine on pathogens inoculated onto the surface of cut lettuce is given in Table 4.4. The effect of treatment on the pathogens present on the lettuce with tap water and chlorine solution is illustrated in Figure 4.1.

Table 4.4: The antimicrobial effect of tap water (control) and 100ppm NaDCC pH5.0 on bacterial pathogens inoculated onto cut lettuce

Pathogen	Tap water (log CFU/g) *n = 3*	100ppm NaDCC pH5.0 (log CFU/g) *n = 3*	Log reduction (CFU/g)
S. enteritidis	7.61	6.91	0.70
E. coli	8.30	7.65	0.65
B. cereus	4.85	3.08	1.77
S. aureus	5.61	4.10	1.51
S. sonnei	6.86	6.10	0.76
L. monocytogenes	6.30	5.20	1.10

With reference to soaking in tap water, disinfection of the lettuce leaves in NaDCC solution showed a significant (95% confidence interval) reduction in pathogen levels for all strains tested. The log difference between treatment of the cut lettuce with tap water and chlorine was greatest for *B. cereus* (1.77 $\log_{10}$ CFU/g) and *Staph. aureus* (1.51 $\log_{10}$ CFU/g), followed by *L. monocytogenes* (1.10 $\log_{10}$ CFU/g). The least difference was shown for *Salm. enteritidis* (0.70 $\log_{10}$ CFU/g), *Shig. sonnei* (0.76 $\log_{10}$ CFU/g) and *E. coli* (0.65 $\log_{10}$ CFU/g). In addition, the pathogen levels least effected by chlorine treatment were those that were present at higher counts before treatment (*S. enteritidis*, *S. sonnei* and *E. coli*) (Table 4.3).

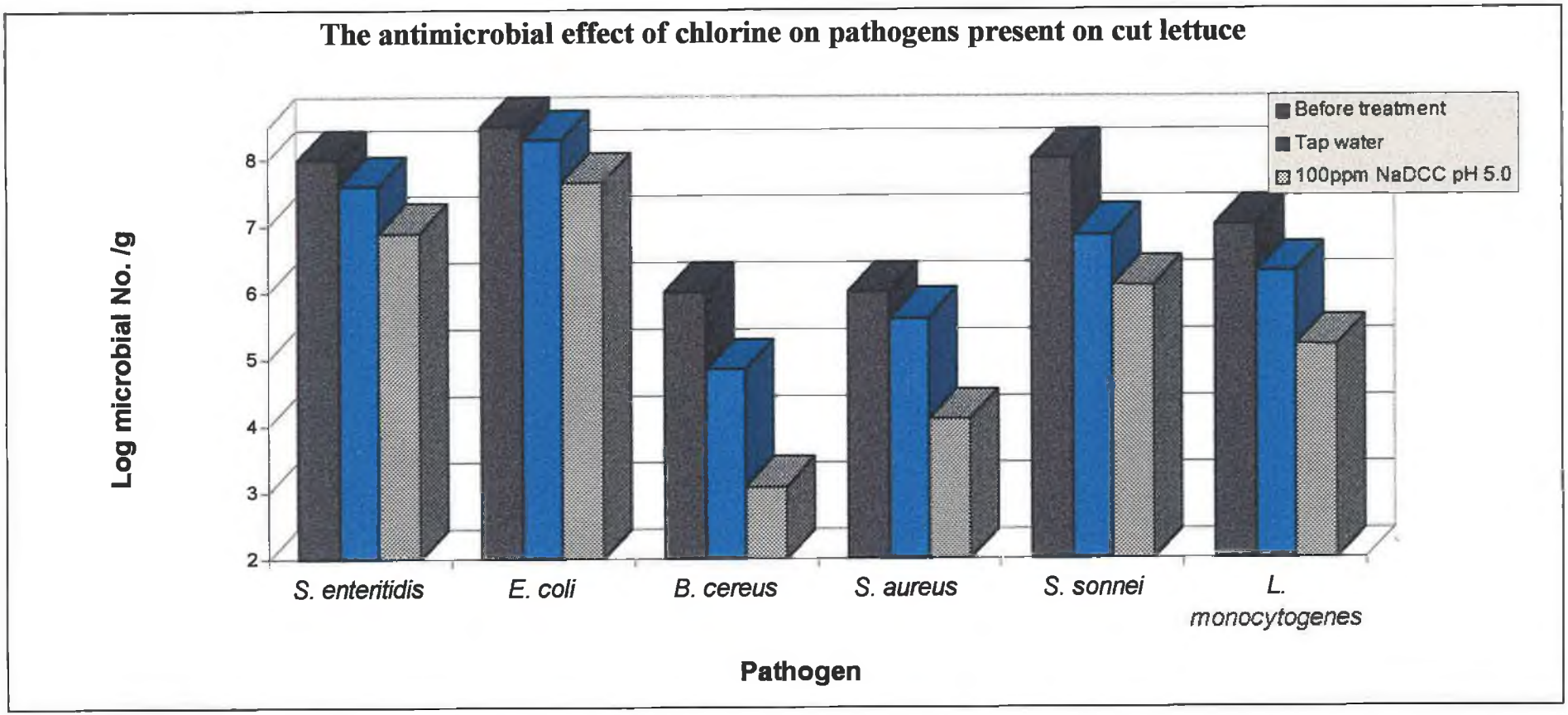


Figure 4.1: The effect of treating pathogens present on the surface of cut lettuce with tap water and chlorine solution (100ppm NaDCC pH 5.0).

4.4 DISCUSSION

4.4.1 THE EFFECT OF CHLORINE ON PATHOGENS PRESENT ON THE SURFACE OF VEGETABLES

The effect of chlorine on pathogens has been widely tested *in-vitro* and shown to be very effective. Chlorine acts rapidly against pathogens even when it is used in small amounts. For example, *E. coli* is more resistant to chlorine than some other vegetative bacteria, yet it is destroyed by 0.055ppm available chlorine in only one minute (Dychdala, 1983). All of the pathogens chosen for this study have no inherent resistance to chlorine. However, this study showed that even though use of 100ppm NaDCC pH5.0 significantly reduced all the pathogens inoculated onto the lettuce surface, the treatment of lettuce with chlorine did not reduce the pathogens by more than 3 log cycles over unwashed samples.

Reduction of pathogens by treatment with chlorine with respect to tap water (control) ranged between 0.65 to 1.71 log CFU/g (Table 4.3). Other studies investigating the antimicrobial effect of chlorine against pathogens on vegetables show similar results. Zhang and Farber (1996) studied the effects of various disinfectants against *L. monocytogenes* on fresh cut vegetables and found that levels were reduced by 1.2 log cycles after treatment with 100ppm free chlorine as compared to the corresponding tap water control.

Brackett (1987a), found that levels of *L. monocytogenes* inoculated onto the surface of brussel sprouts were decreased by about 2 log cycles when they were dipped in 200ppm hypochlorite solution and when dipped in sterile chlorine free water the number of viable cells was reduced by about 1 log cycle. This was much less than destruction found *in-vitro*. The observation that water alone reduced the population by one order of magnitude made the hypochlorite solution appear even less effective. Brackett (1987a)

hypothesised that the the addition of brussel sprouts to the wash solution could have reduced the free available chlorine in the wash solution and consequently reduced the effectiveness of the sanitizer. However, this was rejected as the addition of the brussel sprouts or culture to a wash did not appreciably reduce the free residual chlorine. Brackett concluded that it was possible that surviving cells on the brussel sprouts were protected by the surface microenvironment and hence reduced the activity of the hypochlorite solution.

Studies carried out by Zhuang *et al.* (1995) found that populations of *Salmonella montevideo* on the surfaces and in core tissues of tomatoes were significantly reduced by dipping for 2 minutes in a solution containing 60 or 100ppm chlorine and treatment in a solution containing 320ppm chlorine did not result in complete inactivation. Similar studies on the effect of chlorine on *S. montevideo* on the surface of tomatoes by Wei *et al.* (1995) also found that treatment with 100ppm of aqueous chlorine for up to 2 minutes failed to kill *S. montevideo* on unbroken surfaces, in puncture wounds and in stem scars. The authors concluded that organic material reducing the free available chlorine may have provided a protective function for *Salmonella*. Moreover, the stem scars and growth cracks provided a better protective environment for *S. montevideo* on the surface of tomatoes against washing with tap water or aqueous chlorine. They also found that effectiveness of such protection is dependent on the shape and degree of damage of stem scars and growth cracks.

An evaluation of different commercially available chlorine based disinfectants against *Vibrio cholerae* present on lettuce by Uboldi Eiroa and Porto (1995) showed levels of reduction ranging from 1.45 log MPN/g (Most Probable Number) for 100ppm sodium hypochlorite Milton (Merrel Lepetit Farmacêutica Ltda.) to 4.32 log MPN/g for 100ppm sodium dichloroisocyanurate Divosan K-06 (Diversey Brazil, Barueri, SP, Brazil). The authors concluded that the total removal of *V. cholerae* was not possible and

that the survival was possibly due to the microenvironment on the surface of the lettuce.

Treatment of the cut lettuce with 100ppm NaDCC pH5.0 in this study showed a varied effect on the pathogens present (figure 4.1). The three Gram negative *Enterobacteriacae* (*E. coli, Salm. enteritidis* and *Shig. sonnei*) exhibited a greater resistance to chlorine treatment. Larger reductions of the Gram positive pathogens (*B. cereus, Staph. aureus* and *L. monocytogenes*) after chlorine treatment were linked to lower levels of organisms present before treatment.

During the incubation time the Gram positive pathogens did not increase on the lettuce as much as the Gram negative pathogens. This observation may explain why the Gram negative were more resistant to chlorine than others, as the purpose of incubation time is to allow the bacteria to become attached and established on the lettuce surface. Frank and Koffi (1990) stated that attachment of cells to surfaces can provide protection against chemical sanitizers and when attachment is followed by growth, additional resistance develops. Therefore, the Gram negative pathogens that increased the most during incubation with the lettuce would exhibit a greater resistance to chlorine treatment. In addition, the ability of *Enterobacteriacae* to attach to surfaces using surface adhesions plays an essential role in their pathogenicity and hence can explain their greater increase on the lettuce during incubation and hence their greater resistance to chlorine.

The behaviour of microorganisms on the surface of lettuce may also explain the large difference observed between the high levels of reductions of *V. cholerae* (4.32 log cycles) on lettuce leaves treated with 100ppm NaDCC (Divosan K-06) achieved by Uboldi Eiroa and Porto (1995) and the significantly lower reductions achieved in this study, using the same concentration of NaDCC (Aquatab). Uboldi Eiroa and Porto (1995) used overnight refrigeration as the method to allow the population of *V. cholerae* on

the lettuce surface to become established. While this method may promote surface adherence to the lettuce surface, the refrigeration temperature does not promote growth. The method used in this study was designed to promote surface adherence of the pathogens and growth of at least 1 log cycle over a 24 hour period. Therefore the pathogens would be more likely to exhibit a greater resistance to chlorine. The greater bactericidal power achieved using NaDCC (Divosan K-06) may also be attributed to the presence of other associated compounds such as an ingredient, like surfactants, whose identity is known only by the manufacturer.

4.4.2 CONCLUSIONS

Treatment of lettuce with chlorine significantly reduced the levels of all bacterial pathogens present. However, the application of this disinfectant on vegetables cannot be relied upon to eliminate pathogens. The establishment of the bacteria on the surface of the lettuce, although time consuming, was an important step in the experimental method to mimic the presence of the pathogens on salad vegetables in the environment. The determination of the effect of chlorine gave reproducible results. The standard deviation of 10g samples tested ranged between 0.06 to 0.4 Log CFU/g. The straight forward method can also be adapted to examine the growth of pathogens on salad vegetables at various temperatures or under modified atmospheres.

The use of chlorine during food processing, in particular during cleaning and washing operations, is an important step in the processing of safe MP ready-to-eat vegetables. The quality of water is one of the key elements in the safety of MP vegetables as washing if done properly can reduce microbial populations on vegetables (Brackett, 1992). Inadequate control of washing can spread contaminants over the produce, increasing the potential for microbial spoilage and the risk of foodborne illness. Addition of chlorine to the wash

waters can improve the microbiological quality of the produce and may help prevent microbial contamination of pathogens during processing.

CHAPTER 5 : COMPARISON OF FOUR COMMERCIALLY AVAILABLE ANTIMICROBIALS AS SURFACE DISINFECTANTS

5.1 INTRODUCTION

Chapters 2,3 and 4 investigated the antimicrobial effect of one disinfectant, NaDCC from Aquatabs, when applied as a surface disinfectant on salad vegetables. In this chapter, the antimicrobial effect of NaDCC (Aquatabs) is compared to other commercially available disinfectants for use as surface disinfectants for salad vegetables.

5.1.1 ANTIMICROBIALS AS SURFACE DISINFECTANTS FOR SALAD VEGETABLES

Microbial quality of vegetables is a primary safety issue in the processing of fresh cut vegetables. Processors recognise that pre-cutting and packaging of ready-to-eat cut vegetables does not have a kill step such as blanching in traditional vegetable processing. They depend on maintaining proper temperature control and good plant and employee sanitation for produce quality and safety. Thus, there is growing interest in the application of surface disinfectants as a kill step to control the microbial load on vegetables. Such disinfectants include chlorine compounds (Lund, 1983); organic acids ~ acetic and lactic acid (Adams and Hall, 1988), sorbic acid (Liewen and Marth, 1985), propionic acid (Dziezak, 1986), benzoic acid (Jay, 1986); and antibiotics (Shapiro and Holder, 1960; Jay, 1986).

In this study, the efficiencies of four commercial disinfectant preparations were evaluated as surface disinfectants for cut lettuce. Two of the formulations used, Milton and Aquatab, were chlorine based and two were organic acids, Purac Fresh S and Purac FCC 80.

5.1.2 CHLORINE

Dychdala (1983) stated that chlorination of food, when carefully controlled, checks the microbial population and makes the food safer for consumption, without adversely affecting the original nutritional value and palatability. Chlorination of food is an established practice and specific uses of chlorine in the food industry have been identified at the following concentrations (Kirk and Mitchel, 1980);

1) 50 - 200 ppm ~ hydrocooling raw products
2) 5 - 10ppm ~ fish thawing
3) 200 - 500ppm ~ thawing of frogs legs
4) 1 - 5ppm ~ rinse water.

Chlorine dips and sprays using sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) can be used to reduce populations of microorganisms on the surface of raw vegetables. Somers (1963) reported that a 5ppm of residual chlorine reduced populations on several vegetables and the use of 60-80ppm hypochlorite in wash water has been recommended for inactivation of enteric pathogens which may be present on fruits and vegetables (Hobbs, 1978). Reductions of the natural microflora present on the surface of lettuce have been reported using 100- 300ppm chlorine solutions (Adams *et al.*, 1989; Beuchat and Brackett, 1990; Garg *et al.*, 1990). Significant reductions of microflora on cut lettuce was achieved in this study using 100ppm NaDCC as a chlorine dip. The mechanism of chlorine disinfection is described in 1.3.4.

5.1.3 ORGANIC ACIDS

Acetic and lactic acids, generated microbiologically *in situ* or exogenously, are traditional means of improving food safety and shelf-life.

General recognition of their low toxicity to human has led to new applications, such as reducing the microbial load on carcass meat. Their antimicrobial action appears to be the result of the ability of lipophilic, undissociated acid molecules to penetrate the bacterial plasma membrane. In the higher pH environment of the cytoplasm, the acid dissociates to release protons and conjugate bases, which disrupts the membrane proton motive force, thus disabling the energy yielding and transport process dependent upon it (ICMSF, 1980). The antimicrobial effect of an organic acid is therefore dependent upon its pK_a value and the pH of the external medium.

5.1.3.1 *Acetic acid*

Acetic acid (CH_3COOH) is water soluble; it has pK_a of 4.76 and low toxicity. The main application of acetic acid is in the form of vinegar (a concentration of 4% or greater in vinegar). Acetic acid inhibits many species of bacteria, yeasts and moulds. Both vinegar and acetic acid are listed as GRAS in the US. Vinegar or acetic acid is used as a preservative in many foods including prepared mustard, mayonnaise, salad dressing, pickles and marinades (Busta and Foegeding, 1983). Acetic acid from mayonnaise or salad dressing is the principal agent in prepared salads, where it may exert both microstatic and microbiocidal effects. Uboldi Eiroa and Porto (1995) compared the antimicrobial effect of chlorine based disinfectants and vinegar against *Vibrio cholerae* present on lettuce and found that vinegar, made from red wines, gave significantly higher log reductions in bacterial numbers.

5.1.3.1 *Lactic acid*

Lactic acid ($CH_3CHOHCOOH$) has a pK_a of 3.79 and is highly water soluble. As a natural constituent of some foods, lactic acid is one of the oldest preservatives used. It is a GRAS substance of low toxicity and is non mutagenic (Lueck, 1980). However, the antimicrobial action of lactic acid is only moderate and concentrations above 0.5% are needed for a preservative

effect. The antimicrobial action of lactic acid is directed primarily against anaerobic bacteria such as putrefying anaerobes and butyric acid bacteria (Woolford, 1975). Many moulds and yeasts are capable of growing at acidic pH values and can metabolise lactic acid. Consequently, lactic acid is frequently combined with another antimicrobial agent.

Smulders *et al.*, (1986) recommended the use of lactic acid as a surface decontaminant for fresh meats, slaughter by-products and poultry. The microbiological consequences of treating meat with lactic acid was examined with special reference to enteropathogenic *Enterobacteriaceae* and *Camplylobacter* spp. The authors found that treatment of approx. 1% lactic acid did not discolour meat surfaces and up to 2% did not cause off-flavours in meat. Treatment also resulted in a significant reduction of the bacterial flora, not only by a means of a pH drop but also by a specific action of the acid in an undissociated form.

5.1.4 OBJECTIVES

The objective of this study is to compare four commercially available disinfectants for use on salad vegetables. The disinfectants include two NaDCC-based chlorine disinfectants (Milton and Aquatabs) and two organic acid disinfectants (Purac FCC 80 ~ 80 % lactic acid and Purac fresh S ~ mixture of lactic and acetic acid)

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

Purac Fresh S

L(+) Lactic acid and acetic acid (mixture of lactic acid and acetic acid - proportion of acids not given by the manufacture) aqueous solution manufactured by PURAC biochem, Birmingham ,UK.

Purac FCC 80

L(+) Lactic acid (80%) aqueous solution manufactured by PURAC biochem, Birmingham, UK.

Milton sterilising tablets

Effervescent Sodium dichloroisocyanurate tablets manufactured by Proctor and Gamble (Beauty and Healthcare) Ltd., Egham, Surrey, UK.

5.2.2 PREPARATION OF THE DISINFECTANTS

1 litre solutions of the disinfectants were prepared as follows; a 100ppm NaDCC pH 5.0 using Aquatabs was prepared as in the preparation of sodium dichloroisocyanurate described in section 2.2.3. A 100ppm NaDCC solution using Milton tablets was prepared according to the manufacturers' instructions using tap water. To check that the solution contained 100ppm free chlorine the Palintest DPD comparator was used. The pH of the 100ppm NaDCC (Milton) was recorded as 6.45. A 2% w/v solution of Purac FCC 80 (80% lactic acid) and a 2% w/v solution of Purac Fresh S were prepared using tap water.

5.2.3 EXPERIMENTAL METHOD

A scheme for investigating the effect of the disinfectant solutions is shown in figure 5.1. Approximately 70g samples of prepared lettuce (see preparation of lettuce in section 2.2.2) was treated for 30 minutes in solutions of tap water (control), 100ppm NaDCC pH 5.0 (Aquatab) and 100ppm NaDCC pH 6.45 (Milton). Samples of 70g were also dipped for 1.5 minutes in solutions of Purac FCC 80 and Purac fresh S as recommended by the manufacturer for the surface disinfection of salad vegetables. After treatment, the lettuce was rinsed by swirling 3 times in tap water and dried for four minutes in the automatic dryer. 10g samples were taken for microbiological analysis and the remainder was packed in closed polyethylene bags and stored for 5 days at 5°C in a cooled incubator.

5.2.4 MICROBIOLOGICAL ANALYSIS

10g samples were used for isolation and enumeration of total aerobic mesophiles after treatment for same-day-use and after 5 days refrigerated storage. Preparation of samples were carried out according to microbiological analysis in section 2.2.5.

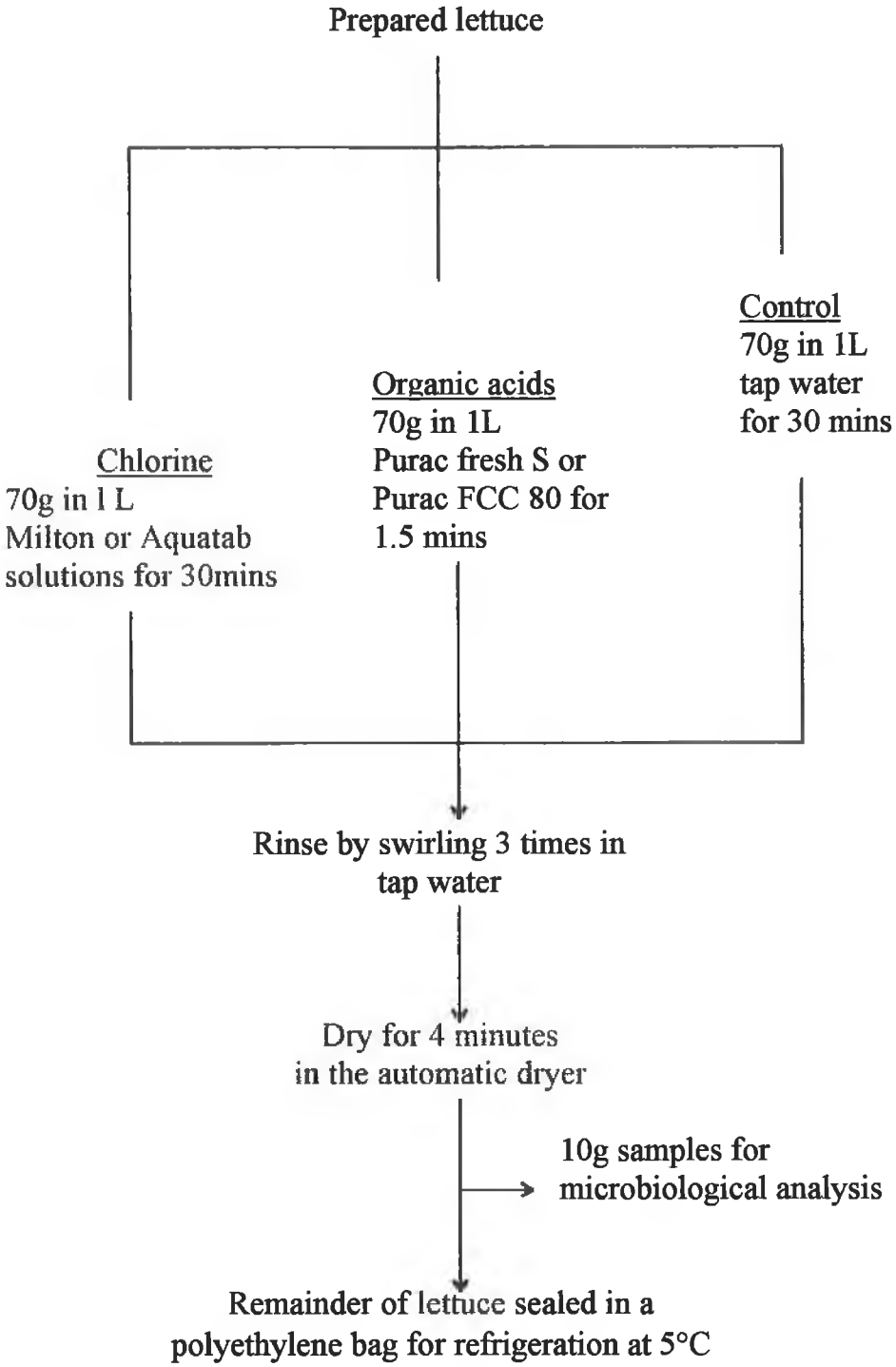


Figure 5.1: Schematic representation of test procedure used to examine the antimicrobial effect of disinfectant solutions on the microbial load present on cut lettuce.

5.3 RESULTS

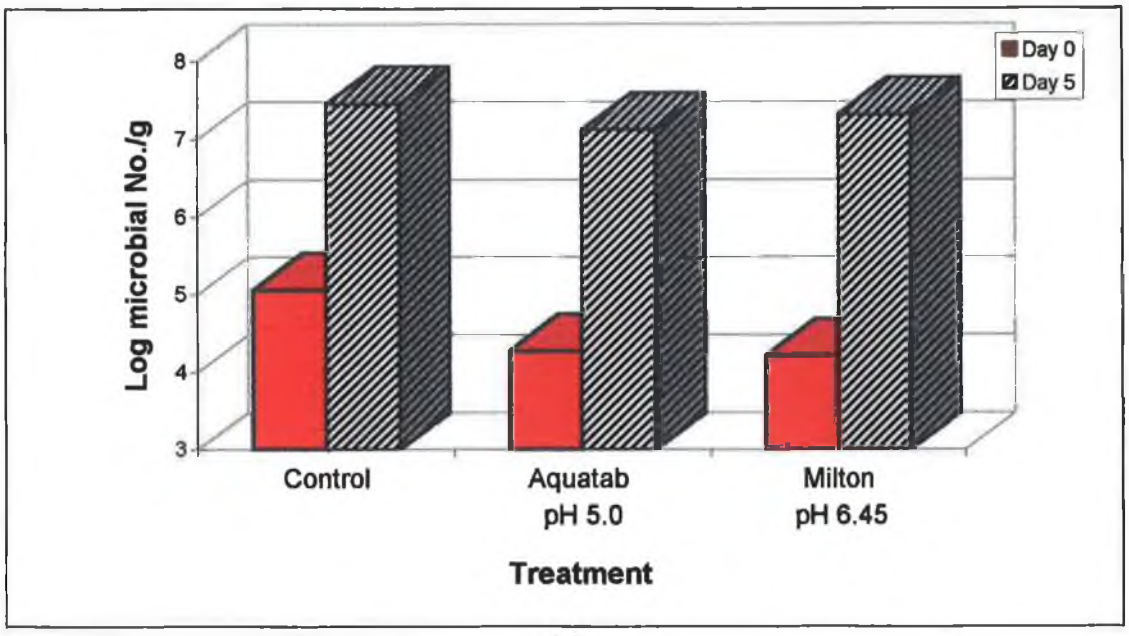
5.3.1 THE EFFECT OF NADCC (AQUATAB) AND NADCC (MILTON) AS SURFACE DISINFECTANTS FOR SALAD VEGETABLES

The two commercially available chlorine disinfectants studied are both NaDCC-based products and sold in an effervescent tablet form. The pH of the solutions containing 100ppm free chlorine was recorded as pH 6.45 for the Milton solution and at pH 5.0 (adjusted) for the Aquatab solution. Results showed that treatment of the lettuce with the disinfectants for 30 minutes reduced the microbial load by 0.8 log cycles as compared to treatment with tap water (Figure 5.2 a). No significant differences were observed between the two disinfectants despite the differences in pH. After 5 days storage at 5°C, there was no significant difference in microbial load on lettuce after treatment with Aquatab and Milton solutions.

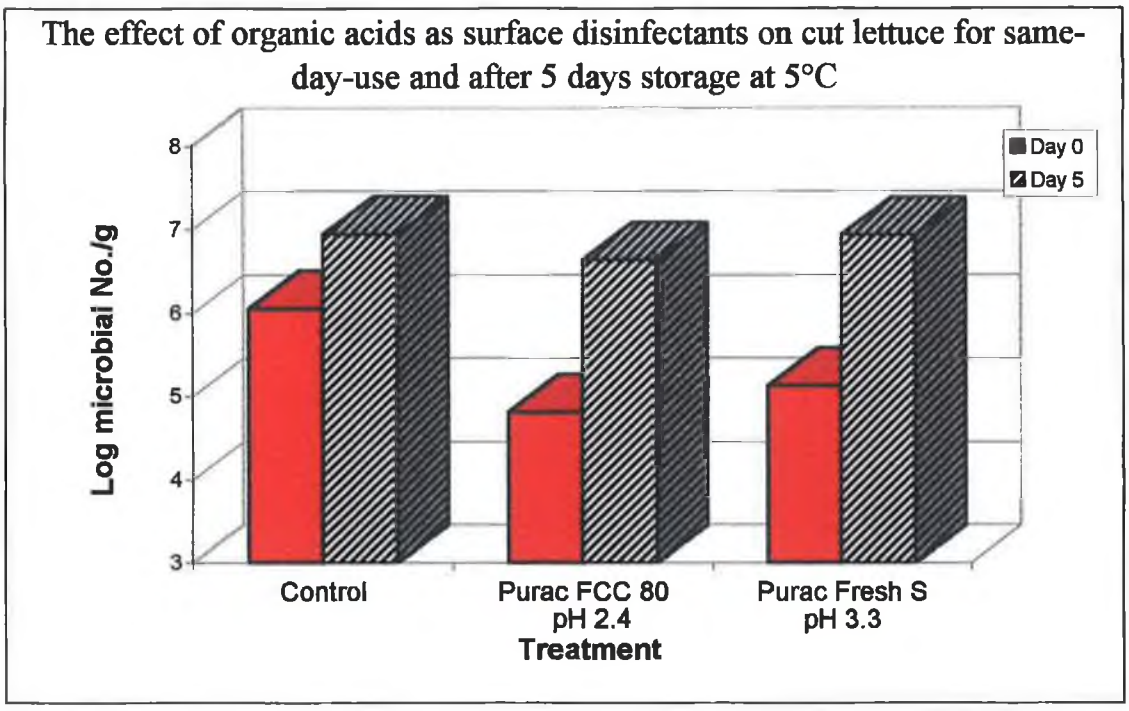
5.3.2 THE EFFECT OF ORGANIC ACIDS AS SURFACE DISINFECTANTS FOR SALAD VEGETABLES

The two organic acids Purac FCC 80 containing 80% lactic and Purac Fresh S containing a mixture of acetic acid and lactic acid was applied to cut lettuce for 1.5 minutes. The control samples were treated with tap water for 30 minutes so that a comparison could be made with the lettuce samples treated with chlorine disinfectants. The pH of the disinfectants was recorded as pH 2.4 for Purac FCC 80 and pH 3.3 for Purac Fresh S. Results showed that the Purac FCC 80 and Purac Fresh disinfectants significantly reduced the microbial load on the cut lettuce by 1.24 and 0.92 log cycles respectively as compared to treatment with tap water (Figure 5.2 b). After 5 days storage at 5°C no significant difference in the microbial load was observed between the treated samples, which increased to approx. 10^7CFU/g. The effect of chlorine

based disinfectants as surface disinfectants on cut lettuce for same-day-use and after 5 days storage at 5°C.



(a)



(b)

Figure 5.2: The effect of a) two chlorine based disinfectants ($n = 6$) and b) two organic acids ($n = 3$) on the total aerobic mesophiles present on cut lettuce for same-day-use and after 5 days storage at 5°C in closed polyethylene bags.

5.4 DISCUSSION

5.4.1 SURFACE DISINFECTION OF SALAD VEGETABLES USING CHLORINE AND ORGANIC ACIDS

Comparison of the two chlorine based disinfectants showed no significant difference in antimicrobial activity despite the pH of the Milton (pH 6.45) solution being higher than the pH of the Aquatab (pH 5.0 adjusted) solution. Previous experiments in this study have shown that by reducing the pH of the NaDCC Aquatab solution from pH 6.0 to 5.0, a significant increase in antimicrobial activity was achieved (Figure 2.5 a). Therefore, the Aquatab NaDCC solution (pH 5.0) would be expected to give a greater log reduction in microbial load on lettuce than the NaDCC Milton solution (pH 6.45). As this was not the case, it may be concluded that the bactericidal power of Milton was due to the influence of other associated compounds whose identity is known only by the manufacturer. An evaluation carried out by Uboldi Eiroa and Porto (1995) of different chlorine based disinfectants against *Vibrio cholerae* on lettuce also showed differences in antimicrobial activity of three commercially available NaDCC-based products. The authors concluded that these differences were most likely due to the influence of other unknown ingredients, such as surfactants, that influenced the activity of the disinfectants.

The disinfection capacity of the organic acid disinfectants, Purac Fresh S containing a mixture of lactic and acetic acid and Purac FCC 80 containing 80% lactic acid, were examined. As the pK_a value of lactic acid (pK_a 3.86) is lower than that of acetic acid (pK_a 4.75), the antimicrobial effect of acetate is expected to be more potent in well buffered foods with a moderately low pH (4-6). Studies by Adams and Hall (1988) clearly showed that acetic acid was more inhibitory than lactic acid against *Escherichia coli* and *Salmonella enteritidis in vitro* at pH 5 and 6. The results showed that the greater

inhibition by acetic acid reflected its higher pK_a value, and with both acids the inhibition was greater at lower pH. Busta and Feogeding (1983) also stated that the antimicrobial effect of lactic acid is only moderate and therefore is usually combined with another antimicrobial compound. Thus, a higher antimicrobial activity would be expected by the Purac Fresh S (lactic acid and acetic acid). However, results showed that the antimicrobial activity of Purac FCC 80 (80% lactic acid) was significantly greater on the microbial load present on the cut lettuce. Similar results on the treatment of foods with lactic acid and acetic acid have been reported. Greer and Dilts (1992) found that treatment of beef with lactic acid gave a significantly greater reduction in foodborne pathogens than acetic acid. Zhang and Farber (1996) also showed that the antimicrobial effect of 1% lactic acid (pH 2.1) was significantly greater than 1% acetic acid (pH 2.6). Thus, the efficiency of the two organic acids against microorganisms on food is reversed and may be attributed, in part, to the lower pH of the lactic acid disinfecting solution.

A comparison of the chlorine and organic acid products as surface disinfectants for vegetables showed that the microbial load was reduced by 0.8 log cycles by the two chlorine based disinfectants, 0.92 log cycles by Purac fresh S and by 1.24 log cycles for Purac FCC 80 as compared to treatment with tap water. Uboldi Eiroa and Porto (1995) also reported higher reductions of *V. cholerae* on lettuce treated with 6% (v/v) vinegar for 15 minutes than 100ppm NaDCC disinfectants. In contrast, Zhang and Farber (1996) found that the use of chlorine disinfectants to reduce *L. monocytogenes* on shredded lettuce achieved a greater reduction (approx. 1 log cycle) than 1% Lactic acid and 1% Acetic acid which reduced populations by 0.5 and 0.2 log cycles respectively as compared to tap water.

5.4.2 CONCLUSIONS

Results from this study suggest that the organic acids exhibits a greater antimicrobial effect than the chlorine disinfectants on the lettuce. However, previous experiments using 100ppm NaDCC have also achieved >1 log reduction in aerobic mesophiles (Fig. 3.3) similar to the reductions achieved when Purac FCC 80 was used. Therefore the results may reflect a variation in the microflora present on the different batches of lettuce treated and not a greater antimicrobial activity.

This furthers the belief that there is a limit to the achievable reduction of microflora on the surface of lettuce regardless of the type of disinfectant used. Although the surface of the vegetables appears wet the possibility that the surfaces are not thoroughly wetted to aid the disinfection cannot be discounted. Hence, surfactants are being added to disinfectant solutions to increase surface wetting. Adams *et al.* (1989) reported that addition of a surfactant, Tween 80, to hypochlorite solutions increased the antimicrobial action of the disinfectant. However, Tween 80 is not used in food production because it leads to unacceptable sensory attributes. Zhang and Farber (1996) found that the addition of Orenco peel and Tergitol, two surfactants currently allowed to be used in the USA for washing vegetables and fruits, into a 100ppm hypochlorite solution did not improve the efficacy of the disinfectant. In fact, they lessened the antimicrobial effect. Nevertheless, further studies using surfactants to increase surface wetting without altering the sensory quality of the vegetables may help to enhance the effectiveness of disinfectants.

CHAPTER 6 : GENERAL DISCUSSION

6.1 DISCUSSION AND CONCLUSIONS

6.1.1 MICROBIOLOGICAL CONSEQUENCES OF USING NaDCC AS A SURFACE DISINFECTANT FOR MP SALAD VEGETABLES

Chambers (1956) specified that a sanitizer should reduce bacterial populations by 99.999% (i.e. 5 log $_{10}$) in a 30 second exposure to be considered effective. Chlorine compounds are very effective at killing a wide variety of microorganisms and, in general, 0.15 to 0.25 ppm available chlorine is sufficient to destroy the vegetative cells within 30 seconds. The sporeforming organisms are usually more resistant to chlorine than vegetative forms (Dychdala 1983). However, application of chlorine in practical situations uses more than the minimum concentrations of chlorine despite the sensitivity of microorganisms to low concentrations. One reason for this is that free available chlorine is affected by pH, the presence of organic matter (in particular when hypochlorite solutions are used) and temperature.

Best *et al.* (1988) evaluated NaDCC as a disinfectant for use on contaminated surfaces. *Mycobacterium smegmatis* was treated with 60ppm free chlorine for 1 minute in suspension and on a carrier surface. Results showed a 6 log cycle reduction of *M. smegmatis* in suspension and between 4 and 5 log cycle reductions in carrier tests on stainless steel surfaces. *M. smegmatis* was chosen as the test organism as mycobacteria are generally more resistant to chemical disinfection than other vegetative bacteria, and the selection of 1 minute contact time gave a reproducible time interval and a realistic picture of the usual practices of routine surface disinfection.

Although the NaDCC (60ppm free available chlorine) solution performed well on the stainless steel surfaces in the work of Best *et al.* (1988), the same degree of disinfection was not found for the NaDCC, tested in this study, on the surface of cut lettuce. When the unwashed cut lettuce was rinsed

and treated for 30 minutes in 80ppm and 100ppm NaDCC (pH 6.0) the microbial load was reduced by 1.92 log cycles (98.8%). The observation that treatment for 30 minutes using tap water (0ppm) reduced the microbial load by 1.35 log cycles (95.5%) made the NaDCC solution appear less effective. The maximum reduction in microbial load of 2.3 log cycles was achieved when the pH of the 100ppm NaDCC solution was lowered to pH 5.0. The effects of rinsing and drying post-treatment, and treatment time (10, 20 and 30 minutes) of chlorine were also examined but showed no significant effect on the microbial load for same-day-use. After 7 days storage no antimicrobial effect of the chlorine treatment was observed. However, it was found that when the cut lettuce was treated with chlorine, improved drying can enhance the microbiological quality of the lettuce for long term storage.

Other studies investigating the effect of chlorine on the surface of vegetables found similar reductions of microorganisms. Adams *et al.* (1989) achieved a 97.8% reduction in lettuce leaf microflora after treatment with 100 ppm NaOCl (pH 9.0). Brackett (1987a) investigated the antimicrobial effect of chlorine on *L. monocytogenes* and found that dipping brussel sprouts contaminated with *L. monocytogenes* in 200ppm NaOCl solution for 10 seconds reduced the populations of viable cells by about 2 log cycles (approx. 99%). This was much less than the complete destruction of *L. monocytogenes* found for the same hypochlorite concentration in buffer. Other studies showed similar reductions of *L. monocytogenes* on cut lettuce of 1.7 log cycles (98%) when treated with 200ppm NaOCl solution (Zhang and Farber 1996), and on the surface of tomatoes of 1.2 log cycles (approx. 95.6%) after treatment with 110ppm NaOCl solution compared to treatment with water (0ppm) (Zhuang *et al.* 1995). Uboldi Eiroa and Porto (1995) evaluated different chlorine based disinfectants against *V. cholerae* present on lettuce and found that total removal of the bacteria from the surface was not possible, however, reductions of between 1.45 log cycles (96.5%) using hypochlorite and 4.32 (99.995%) using NaDCC were achieved.

Hence, the use of chlorine as a surface disinfectant for vegetables, in general, does not fulfil the criteria for an effective sanitizer specified by Chambers (1956). The disinfection capacity of chlorine against microorganisms present on the surface of vegetables can be described as having a limited efficiency when compared to the disinfection capacity against microorganisms in suspension. This so-called limited efficiency has been attributed to the survival of the bacteria in hydrophobic pockets or creases in the leaf surface, as made evident by Adams *et al.* (1989) and in biofilms as shown by Gras *et al.* (1994). This limited value of chlorine is not restricted to vegetables alone. Treatment of beef (Marshall *et al.*, 1977), chicken (Morrison and Fleet, 1985) and lamb (Kelly *et al.*, 1981) with at least 200ppm free chlorine all resulted in less than 2 orders of magnitude reduction in microbial populations.

Despite the limited efficiency of chlorine on food its value as a disinfectant during processing cannot be disregarded. Chlorine was shown in this study to enhance the microbiological quality of lettuce by reducing levels of aerobic mesophiles, psychrotrophs, coliforms, lactic acid bacteria and yeasts and moulds when applied as a surface disinfectant. Chlorine was also effective at reducing levels of pathogens which are commonly associated with salad vegetables. Thus, treatment of ready-to-eat salad vegetables with chlorine would be recommended when the microbial quality of the raw produce represents a health risk, in particular, for produce from developing countries where environmental sanitary conditions and agricultural practices are poor. In addition, failure to maintain adequate washing can lead to the spread and increase of microbial populations over the produce. Therefore, the processing of all salad vegetables would benefit from the addition of an antimicrobial such as chlorine to the washwater to control the spread microorganisms is beneficial.

6.1.2 TYPES OF SALAD VEGETABLES AND THE EFFICIENCY OF NaDCC

It is important when examining microorganisms as a cause of spoilage and safety problems to consider why bacteria behave the way they do. This ecological view is fundamental to the study of food microbiology. Food can be viewed as "a complex dynamic ecological niche teeming with life" (Wiley 1994) and within food there are many microenvironments. For example, the surface of food exposed to air might constitute one microenvironment and the interior of food constitutes another. Thus, the processing and handling of foods affect these microenvironments and hence microbes will likewise be affected. The changes in microflora will differ depending on the microorganisms present.

The source and type of raw produce have an important effect on the microflora. Vegetable products such as lettuce and cabbage will harbour the microorganisms from the soil in which they were grown by direct contact. Handling of these products post harvest also effects the microflora and is particularly important as more can be done to control the environment and treatment of the vegetables. Temperature, water activity, handling procedures, cutting and slicing, application of antimicrobials and types of packaging are all factors affecting the microflora of MP vegetables.

This study found higher levels of contamination on the unwashed cut lettuce than unwashed cut cabbage and differences in levels of microbial populations. Although the predominant microbes on both salad vegetables were the total aerobic mesophiles and psychrotrophs, the numbers and proportions of lactic acid bacteria, coliforms and yeasts and moulds varied on each vegetable. When the cut vegetables were treated with chlorine the groups of microbes were affected differently which became more apparent during refrigerated storage. The use of chlorine to reduce the initial levels of microorganisms on the cut cabbage changed the population dynamics of the microbes increasing the levels of aerobic mesophiles and psychrotrophs during

the refrigerated storage period. The same effect was not observed on the cut lettuce. Differences in the intrinsic characters of the two salad vegetables may explain the contrasting behaviour of the microorganisms. Firstly, a close association of psychrotrophic pectinolytic microbes with the plant tissue of the cabbage would serve to protect during chlorine treatment (Eckert, 1977). During refrigerated storage the surviving microbes could then grow with fewer competitors. Secondly, lower levels of lactic acid bacteria on the cut cabbage may have been a contributing factor to the rapid growth of aerobic mesophiles and psychrotrophs during storage. Higher levels like that found on cut lettuce would inhibit to a greater extent other microorganisms by producing bacteriocins and competing for nutrients.

Studies by Marchetti *et al.* (1992) have also highlighted differences in microbial population dynamics on a range of salad vegetables and found that these differences supported different spoilage patterns. Zhang and Farber (1995) studied the effect of *L. monocytogenes* on cut lettuce and cabbage and found that the type of vegetables can affect the antimicrobial activity of these compounds. Consequently, the types and numbers of microbes and the type of vegetable are important factors to consider when applying chlorine as a surface disinfectant to salad vegetables intended for refrigerated storage. From this study, the use of 100ppm NaDCC as a surface disinfectant on cut lettuce to reduce the microbial load can be recommended. However, for use on cabbage, additional barriers to prevent rapid growth of microorganisms during refrigerated storage is needed.

6.1.3 MICROBIAL SAFETY OF MP SALAD VEGETABLES USING NADCC

The unique characteristics of MP salad vegetables enhances the growth of microbes on the product including possible pathogenic microorganisms. Ready-to-eat salad vegetables fall within the UK Institute of Food Science and

Technology (IFST, 1990) definition of chilled foods which states: chilled foods are perishable foods which, to extend the time which they remain wholesome, are kept within the specified ranges of temperatures above -1°C and below 8°C. Therefore, MP salad vegetables are subject to the legislative constraints governing food composition, additive usage, residues, contaminants, hygiene, labelling and packaging.

Foods may be considered unsafe owing to the presence of pathogenic microorganisms. As sensory changes cannot be relied upon as an indicator of microbial safety, the growth of these microorganisms in food may not necessarily result in spoilage. It is therefore essential that an effective program is used to ensure the safety of foods from production, through processing and storage and distribution until consumption.

The storage of MP salad vegetables at refrigeration temperature cannot prevent all microbial growth, but can prevent some types and retard the rate of growth in others. Pathogenic microorganisms capable of growth at chilled temperatures include *Listeria monocytogenes* (-0.4°C), *Yersinia enterocolytica* (0°C), *Aeromonas hydrophilia* and *Escherichia coli* (7-10°C), various *Salmonella* spp. (6-7°C), *Vibrio parahaemolyticus* (3°C), *Staphylococcus aureus* (6.6°C), *Clostridium botulinum* (3.3°C), *Bacillus cereus* (5-7°C) (Brimelow and Vadehra 1991) and are therefore of increasing concern in MP salad vegetables. Provisional microbiological guidelines for ready-to-eat prepared mixed salads at the point of sale was drawn up by a subgroup of the Public Health Laboratory Service food surveillance group and is contained in table 6.1.

The potential for pathogenic organisms to survive and grow on MP vegetables has led to the possible use of chlorine as an added barrier or hurdle to enhance microbiological safety. Chlorine has been shown to be very effective at killing microbes *in-vitro*. In this study, *Listeria monocytogenes,*

Salmonella enteritidis, Staphylococcus aureus, Bacillus cereus, Escherichia coli and *Shigella sonnei* on the surface cut lettuce were all significantly reduced after treatment with chlorine. The extent of the disinfection was linked to how established the pathogen had become on the surface of the vegetable.

Table 6.1: Provisional guidelines for microbiological acceptability of ready-to-eat mixed salads at the point of sale.

Microorganisms	**Satisfactory**	**Fairly Satisfactory**	**Unsatisfactory**	**Unacceptable ~ Potentially hazardous**
Aerobic plate count (prepared mixed salads)	10^5	10^5 - 10^8	$>10^8$	–
Salmonella	Not detected in 25g			Present in 25g
V. parahaemolyticus	Not detected in 25g			Present in 25g
Campylobacter	Not detected in 25g			Present in 25g
L. monocytogenes	Not detected in 25g	Present in 25g - 10^2	10^2 - 10^3	$> 10^3$
E. coli	< 20	20 - 10^2	10^2 - 10^4	$> 10^4$
S. aureus	< 20	20 - 10^2	10^2 - 10^4	$> 10^4$
Cl. perfringens	< 200	200 - 10^3	10^3 - 10^4	$> 10^4$
B. cereus and other *Bacillus spp.*	< 200	200 - 10^4	10^4 - 10^5	$> 10^5$

(From Gilbert *et al.*, 1992; PHLS)

Modified Atmosphere Packaging (MAP) as a barrier to the growth of foodborne pathogens has been extensively studied. The use of polymeric packaging material to establish a modified atmosphere around MP vegetables can achieve a degree of product "preservation". MAP can be used to retard senescence and oxidative discolouration of cut vegetables to extend the shelf-life (Myers, 1989). The most common way to do this is to reduce the O_2 concentration while increasing the CO_2 concentration. CO_2 has antimicrobial properties that kill or inhibit various microorganisms. Most work has been done on meat and meat products, and these results show that a 10% level of

CO_2 usually gives about 50% inhibition on the basis of total counts after a given incubation time (Wagner and Moberg, 1989).

Reports on the effects of MAP to control the microbes on cut vegetables have been varied. Berrang *et al.* (1990) found that storing broccoli at 1°C in an atmosphere containing 10% CO_2 and 11% O_2 inhibited growth of total aerobic microorganisms by several logs. Other reports have shown no obvious effects on microorganisms. Priepke *et al.* (1976) found that populations of total aerobic microorganisms in salad vegetables stored refrigerated in 10.5% CO_2 and 2.25% O_2 differed by only one tenth of a log cycle. Barriga *et al.* (1991) found that the visual quality and shelf-life of shredded lettuce can be significantly extended by controlled atmospheres (10% CO_2 and 3% O_2) without appreciable reducing microbial counts. They concluded that microbial development in shredded lettuce must be controlled by maintaining low temperatures and other means.

As MAP can allow the growth of microorganisms including pathogens to go relatively unnoticed, it cannot be assumed that a given food will be totally safe. The use of chlorine as a surface disinfectant combined with temperature control and MAP could be used to provide hurdles to the growth of pathogens. However, it is important that such combinations are tested for the specific food product system before application.

6.1.4 NADCC VERSUS OTHER COMMERCIAL PRODUCTS FOR USE AS SURFACE DISINFECTANTS FOR MP SALAD VEGETABLES

Studies investigating the use of surface disinfectants for MP vegetables have included, for the most part, chlorine and chlorine-based products, and organic acids. In addition to their antimicrobial activity, these products must be cheap, commercially available and easy to apply products for widespread

use in the food industry. According to Lund (1983) chlorine is probably the most widely used as a surface disinfectant for vegetables.

A comparison of NaDCC to the two organic acid disinfectants in this study showed similar reductions in the microflora on the surface of cut lettuce. The effervescent NaDCC tablets which produced an accurate concentration of free chlorine when added to water were found to be convenient and easy to apply. The pH of the Aquatab solution did require adjustment, however, this could be accommodated with the addition of a buffer agent as an ingredient by the manufacturer. The potential of chlorine to react with organic molecules to form carcinogenic chlororganics compared to the low toxicity of orgainc acids would favour the use of organic acids as a surface disinfectant for salad vegetables. However, both the Purac FCC 80 and the Purac fresh S were supplied in a concentrated solution, which can cause irritation to the skin and eyes, and required careful dilution before being used.

Therefore, the use of chlorine products would have an advantage over the organic acid products, especially in small scale operations where a safe and easy application of a disinfectant is required. Furthermore, Zhang and Farber (1996) who investigated the effect of various disinfectants on fresh-cut vegetables, reported that organic acid residues could reduce the sensory quality of the lettuce. Thus, the effect of extending the recommended treatment time of 1.5 minutes used for the Purac organic acid disinfectants on the sensory quality of lettuce would have to be examined before recommending its use as a surface disinfectant during processing of salad vegetables.

APPENDIX

APPENDIX A

Results of the microbiological and chemical analysis carried out on the water supply to the laboratory by Dublin Corporation for 1995 and 1996.

Received Sample Date	Total Residual Chlorine mg/L	Total Coliforms CFU/100ml	Feacal Coliforms CFU/100ml	Heterotrophic Plate Count @ 22°C CFU/ml
9/1/95	0.030	0	0	151
16/1/95	0.010	0	0	32
23/1/95	0.030	0	0	11
6/2/95	0.030	0	0	52
13/2/95	0.030			19
20/2/95	0.030	0	0	26
27/2/95	0.010	0	0	21
6/3/95	0.030	0	0	30
13/3/95	0.020	0	0	30
20/3/95		0	0	25
27/3/95	0.030	0	0	17
3/4/95	0.010	0	0	41
10/4/95	0.030	0	0	29
24/4/95	0.030	0	0	9
8/5/95	0.010	0	0	6
15/5/95	0.010	0	0	7
22/5/95	0.010	0	0	1
29/5/95	0.010	0	0	10
12/6/95	0.010	1	0	22
19/6/95	0.020	0	0	4
5/7/95	0.020	0	0	
12/7/95	0.030	1	0	
19/7/95	0.010	0	0	
26/7/95	0.030	0	0	
2/8/95	0.030	0	0	
9/8/95	0.030	2	0	
16/8/95	0.010	1	0	
23/8/95	0.020	0	0	
30/8/95	0.030	4	0	
6/9/95	0.010	0	0	
13/9/95	0.020	0	0	
20/9/95	0.020	0	0	
29/9/95	0.020	0	0	
4/10/95	0.020	0	0	
11/10/95	0.030	0	0	
18/10/95	0.020	0	0	
25/10/95	0.030	0	0	
1/11/95	0.020	0	0	
8/11/95	0.020	0	0	
15/11/95	0.020	0	0	

Received Sample Date	TResCl mg/L	Total Coliforms CFU/100ml	Feacal Coliforms CFU/100ml	Heterotrophic Plate Count @ 22°C CFU/ml
22/11/95	0.020	0	0	
29/11/95	0.020	0	0	
6/12/95	0.020	0	0	
13/12/95	0.020	0	0	
20/12/95	0.030	0	0	
5/2/96	0.040	0	0	
12/2/96	0.050	0	0	21
19/2/96	0.050	0	0	4
26/2/96	0.120	0	0	0
4/3/96	0.060	0	0	15
11/3/96	0.070	0	0	1
25/3/96	0.060	0	0	5
1/4/96	0.060	0	0	4
15/4/96	0.050	0	0	14
22/4/96	0.050	0	0	20
29/4/96	0.040	0	0	7
13/5/96	0.070	0	0	12
20/5/96	0.040	0	0	18
27/5/96	0.030	0	0	3
10/6/96	0.050	0	0	70
17/6/96	0.030	0	0	2
3/7/96	0.020	0	0	
8/7/96	0.030	0	0	10
17/7/96	0.030	0	0	
24/7/96	0.030	0	0	
31/7/96	0.030	0	0	
7/8/96	0.030	0	0	
14/8/96	0.020	0	0	
21/8/96	0.030	0	0	
28/8/96	0.020	0	0	
4/9/96	0.030	0	0	
11/9/96	0.030	0	0	
17/9/96	0.030	0	0	2
25/9/96	0.020	0	0	
2/10/96	0.030	0	0	
9/10/96	0.020	0	0	
16/10/96	0.020	0	0	
23/10/96	0.030	0	0	
30/10/96	0.030	0	0	
6/11/96	0.030	0	0	
13/11/96	0.070	0	0	
20/11/96	0.070	0	0	
27/11/96	0.700	0	0	
4/12/96	0.070	0	0	
10/12/96	0.070	0	0	36
18/12/96	0.050	0	0	

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