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O L L S C O I L L U I M N I G H

**Anti-listerial effects of essential oils
and herbs in fresh-cut produce:
opportunities and limitations**

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**for the Degree of
Doctor of Philosophy**

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Declaration

I hereby declare that I am the sole author of this thesis and that it has not been submitted for any other academic award. References and acknowledgements have been made, where necessary, to the works of others.

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Dedication

To my wonderful daughter Katie.

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Abstract

Anti-listerial effects of essential oils and herbs in fresh-cut produce: opportunities and limitations

Johann Scollard

The potential anti-listerial benefits of essential oils and herbs in fresh-cut produce systems were investigated. Interactions with modified atmospheres and product types were examined in detail, including effects on quality. A strong anti-listerial response from rosemary herb was discovered during maceration and the chemical basis of this determined for future exploitation.

The anti-listerial properties of essential oils (thyme, oregano and rosemary), under a range of storage atmospheres (air, 5%CO₂/2%O₂/93%N₂ and 20%CO₂/1% O₂/79%N₂) and temperatures (4 and 8°C), were examined using a model vegetable system. Effectiveness was in the order thyme EO > oregano EO > rosemary EO, and greatest under 20%CO₂/1%O₂/79%N₂ and at 4°C. When headspace volatiles from the EOs were tested there was little anti-listerial effect, suggesting that the EOs needed to be in direct contact with cultures. When applied to modified atmosphere packaged fresh-cut vegetables (lettuce, cabbage, coleslaw mix and carrots), the effectiveness was in the order thyme EO > oregano EO > rosemary EO > basil EO. Applying undiluted EOs directly to the fresh produce had a detrimental effect on appearance. A product effect was seen with EOs and herbs having increased anti-listerial activity on shredded cabbage and carrot. Diluting the EOs, or using them diluted in combination, reduced adverse sensory effects, but also eliminated the anti-listerial effects.

In general, use of the fresh herb equivalents of the EOs was ineffective. However, while commercial rosemary EO was relatively ineffective, freshly macerated rosemary herb had very strong anti-listerial effects. To investigate this further, the chemical composition and anti-listerial activity of rosemary oil obtained by different extraction methods (CO₂ extraction, hydrodistillation, solvent extraction) was determined. Gas chromatography-mass spectroscopy identified the main components present as camphor, verbenone, borneol, bornyl acetate and caryophyllene. All of these individual components showed anti-listerial activity, however principal component analysis showed verbenone to be highly correlated with anti-listerial kill rate. The hydrodistillate, which had the highest antilisterial activity, contained the highest levels of verbenone. The extract of macerated rosemary herb had more than twentyfold the level of verbenone found in the unmacerated rosemary extract. When headspace analysis was carried out on uncut, freshly chopped and macerated rosemary herb, the relative levels of verbenone were 0, 6 and 118ppm. The strong anti-listerial activity of the macerated rosemary may be explained by the higher concentration of verbenone present in these extracts. Simulation of maceration by stomaching in industrial production of rosemary EOs is likely to greatly enhance their anti-listerial effectiveness.

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List of Abbreviations

atm	Atmosphere
CO ₂	Carbon dioxide
CFU	Colony forming unit
ClO ₂	Chlorine dioxide
EO	Essential oil
g	Gram
g	Relative centrifuge force
GAP	Good agricultural practice
GC-MS	Gas chromatography-mass spectroscopy
GMP	Good manufacturing practice
GRAS	Generally regarded as safe
HACCP	Hazard Analysis and Critical Control Point
HD	Hydrodistillation
H ₂ O ₂	Hydrogen peroxide
L	Litre
LSA	Listeria selective agar
MA	Modified atmosphere
MAP	Modified atmosphere packaging
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
ml	Millilitre
mm	Millimetre
N ₂	Nitrogen
NCCLS	National Committee for Clinical Laboratory Standards

NCTC	National Collection of Type Cultures
O ₂	Oxygen
P	Probability
PBS	Phosphate buffered saline
PCA	Principal component analysis
ppm	Parts per million
RTE	Ready to Eat
s.d.	Standard Deviation
SFE	Supercritical fluid extraction
TBC	Total bacterial counts
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TSB-YE	Tryptone soya broth – yeast extract
v/v	volume (of solute) per volume (of solvent)
w/v	weight (of solute) per volume (of solvent)
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	Micrometre

Publications

Scollard, J., Francis, G.A. and O'Beirne, D. (2009). Effects of Essential Oil Treatment, Gas Atmosphere, and Storage Temperature on *Listeria monocytogenes* in a model vegetable system. *Journal of Food Protection*. 72, 1209-1215. **Published.**

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Chapter 1
Literature Review

1.1 Introduction

The worldwide fresh-cut produce industry has grown rapidly in recent years, largely driven by increasing consumer demand for healthy, freshly prepared, convenient fruits and vegetables. However, fresh-cut produce harbours large and diverse populations of microorganisms, and some of this microflora may include foodborne pathogens such as *Listeria monocytogenes*.

The fresh-cut produce food system is not pasteurised and relies upon Hazard Analysis Critical Control Point (HACCP) protocols to prevent contamination, and low storage temperatures ($\leq 4^{\circ}\text{C}$) to prevent growth of pathogens should contamination occur. Products are subjected to dipping in 100 ppm of chlorine which reduces microbial populations approximately tenfold and may help prevent cross-contamination (Adams *et al.* 1989). These hurdles have been shown to be incompletely effective as demonstrated by a small number of serious food poisoning outbreaks associated with this food system over the past ten years. The addition of natural plant antimicrobials, such as plant essential oils (EOs), may represent an additional hurdle to reduce survival and/or growth of pathogens during storage.

In this thesis, the anti-listerial properties of plant EOs and fresh herbs were examined in the context of fresh-cut produce systems. A process to greatly enhance the anti-listerial properties of rosemary was discovered and studied in detail. Different extraction methods were employed to extract rosemary EOs and the chemical composition of these extracts was determined. These oils and their individual chemical components were tested for anti-listerial activity. The scientific literature relevant to these issues is reviewed.

1.2 Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, facultatively anaerobic, rod-shaped foodborne pathogen that causes a variety of diseases in humans including septicaemia, meningitis and spontaneous abortions. Currently, the genus is classified into seven species (*L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. weishimeri*, *L. seeligeri*, *L. grayi* and the newly identified *L. marthii*), however *L. monocytogenes* is the only species considered to be pathogenic to man. *L. monocytogenes* can be distinguished by its serotype (a group of closely related microorganisms distinguished by a characteristic set of antigens). The majority of human listeriosis infections are associated with serotypes 1/2a, 1/2b or 4b (Swaminathan and Gerner-Smidt 2007, Little *et al.* 2007).

L. monocytogenes is an important foodborne pathogen as it has the ability to grow at refrigeration temperatures, in acid and high salt foods and under relatively high CO₂ levels (Bourke and O' Beirne 2004, Cotter *et al.* 2001a, Mc Clure 1989, Cotter *et al.* 2001b). In many respects, *L. monocytogenes* differs from most known foodborne pathogens in that it is ubiquitous in the environment, resistant to diverse environmental conditions and is facultatively anaerobic and psychrotropic. The optimum growth range for *Listeria* is between 30 and 37 °C but numerous studies have shown *Listeria* to grow at a range of refrigeration temperatures (Junttila *et al.* 1988), at temperatures as high as 55°C (Lundén *et al.* 2008) and also at freezing temperatures of -0.5°C (Walker *et al.* 1990). *Listeria* can grow at pH levels between 5 and 9.4, however studies have shown that *Listeria* is able to adapt to acidic conditions and has been shown to multiply at pH values below 5 (Farber *et al.* 1989) and has been observed to survive pH values below 3 (Dykes and Moorhead 2000).

The various ways in which the bacterium can enter a food processing plant, its persistence in the industrial environment, its ability to grow at low temperatures and to

survive in food for prolonged periods under adverse conditions, have made this bacterium one of the hottest topics for the food industry during the last two decades (Rocourt *et al.* 2003). *L. monocytogenes* strains may persist in food processing plants for months or even years despite regular sanitation procedure (Fox *et al.* 2011), this persistence has been associated with enhanced adherence to food contact surfaces coupled with its resilient nature (Lundén *et al.* 2008).

L. monocytogenes is considered ubiquitous in the environment, with soil considered to be the primary reservoir of the organism. As a result, contamination of foods can occur at many stages from farm to fork, and the organism has been found in a wide variety of raw food materials. Foods associated with outbreaks of listeriosis include meat, poultry, seafood, dairy products and various fruits and vegetables (Lekroengsin *et al.* 2007). Due to *L. monocytogenes*' versatile nature, and its ability to cause severe illness, it is of great concern to public health authorities.

1.3 Listeriosis

Listeriosis, the general group of disorders caused by *L. monocytogenes*, is a potentially life threatening infection that affects 'high risk' sections of the population. Before 1982, *Listeria* was only thought to be associated with contaminated animal feed or silage, affecting only animals. It wasn't until 1981 that a foodborne association with *Listeria* was widely accepted, when an outbreak of listeriosis in humans was caused by ingestion of contaminated coleslaw (Schlech and Acheson 2000). The main individuals at risk from listeriosis are the immunocompromised, elderly, newborns and pregnant women. In these groups, listeriosis usually presents itself as meningitis, septicaemia, and in pregnant women it can result in spontaneous abortions or stillbirths. In severe

cases, *L. monocytogenes* can result in death and listeriosis has a mortality rate of approximately 30% in high risk groups (Carpentier and Cerf 2011).

The precise dose of *L. monocytogenes* that needs to be ingested to represent a significant risk of illness to humans is uncertain and it is thought to be highly strain and host dependant; however, the minimum infectious dose is thought to be low, possibly less than 1,000 cells (Food Safety Authority of Ireland 2008). The nature and severity of symptoms are largely dependent on age, sex and health of the host. The reported incidence of human disease appears to be low, because most normal healthy people, when infected, remain symptom-free or suffer mild flu-like symptoms of malaise, mild fever and diarrhoea.

In Ireland, there was an increase in the number of cases of listeriosis notifications in 2007 (Health Protection Surveillance Centre 2008). There were 19 cases reported in 2007, compared to 11, 12 and 7 total cases in 2004, 2005 and 2006 respectively (Figure 1.1), and Ireland is listed as one of the six European Union countries with a statistically significant increase in human listeriosis cases between the years 1999 and 2006 (O' Brien *et al.* 2009). The increase in the number of listeriosis cases appears to be primarily among pregnancy-related and neonatal cases (referred to collectively as pregnancy-associated cases).

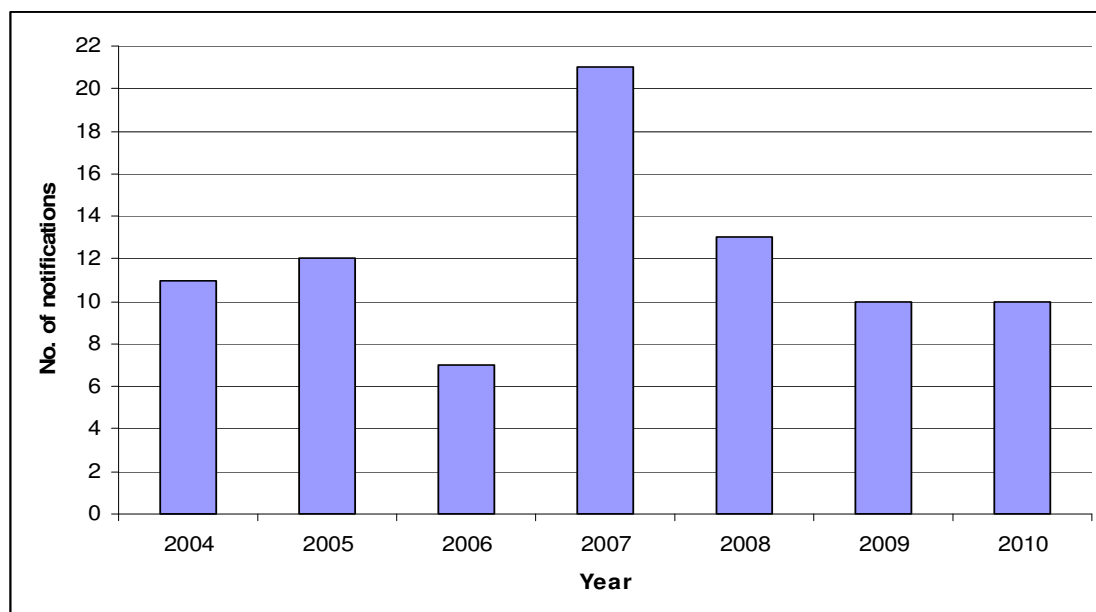


Figure 1.1: Number of listeriosis notifications, Ireland 2004-2010. Adapted from Health Protection Surveillance centre (2008).

While cases of listeriosis in Ireland have dropped since 2007, figures from the EU have shown an increase over the last number of years. Estimates from the United States place the number of listeriosis cases per annum at 1,850 with 425 deaths (Food Safety Authority of Ireland 2008). The incidence of listeriosis varies between 0.1 and 11.3/1,000,000 in different countries (Swaminathan and Gerner-Smidt 2007). In the EU, the number of listeriosis cases in humans increased by 19.1 % compared to 2008, with 1,645 confirmed cases recorded in 2009, and this resulted in 290 deaths (EFSA 2011). This increase could be due to the ever increasing consumption of ready-to-eat produce. The minimal processing used, which omits any effective microbial elimination step, results in food products that naturally would carry microorganisms, some of which may be potentially hazardous to human health

The outbreaks that have occurred over the last two decades, clearly demonstrate that control of *L. monocytogenes* in the food sector is absolutely vital and that outbreaks of listeriosis have been associated with vegetable products. The first conclusively documented foodborne outbreak of listeriosis occurred in Canada in 1981

and resulted in 18 deaths. The vehicle of transmission was coleslaw and the source of *L. monocytogenes* was stored cabbage (Schlech 1991). Ho *et al.* (1986) found that the only common foods associated with a listeriosis outbreak in Boston in 1979 were celery, tomatoes and lettuce. A gastroenteritis outbreak in northern Italy in 1997, in which 292 people were hospitalised, was found to be related to corn contaminated with *L. monocytogenes* (Aureli *et al.* 2000).

1.4 The Market for Fresh-cut vegetables

The food industry is one of the single most important industries in Ireland. In 2010, the agri-food sector in Ireland was reported to contribute €24 billion to the national economy, generate exports of €7.88 billion and provide 7.4% of national employment. The worldwide fresh-cut produce industry has grown in recent years, largely driven by increasing consumer demand for healthy, freshly prepared, convenient fruits and vegetables. Many countries have run campaigns promoting the health benefits of fresh fruit and vegetables, recommending at least five servings of fruit and vegetables to be consumed daily. Consumers demand high quality, fresh-like foods that require a minimal amount of effort and time for preparation (Manani *et al.* 2006). For this reason, the market of minimally processed fruits and vegetables has grown rapidly in recent decades as a result of changes in consumer attitudes. Figures for the consumption of fresh-cut fruits and vegetables show this to be the case (UK, 12 kg per person per year; France, 6 kg; Italy, 4 kg and Germany, Belgium and Netherlands with more than 3 kg) (Abadias *et al.* 2008). In Ireland, the retail market for fresh produce was valued at €1.203 billion in 2007 which was a 5.4% growth compared with 2006. This market is made up of sales of fruit (45.4%), salad vegetables (14.2%), vegetables (23.6%) and potatoes (16.7%). The fresh produce sector has been

growing in Ireland over the past number of years (Table 1.1). In the USA, it is one of the fastest growing sectors with annual sales of \$12 billion, with sales of packaged salads accounting for \$3 to \$4 billion of this.

Table 1.1: Retail Sales of Fresh Produce in Ireland 2007 (Safefood Ireland 2007).

Category	Share of retail sales of fresh produce in %	Change in 2007 vs 2006
Fruit	45.4	+6.6
Vegetables	23.6	+12.7
Salad Vegetables	14.2	+6.8

1.5 Fresh cut vegetables

Fresh-cut vegetables may consist of peeled, sliced, shredded, trimmed and/or washed vegetables. These products are then packaged and stored at refrigerated temperatures.

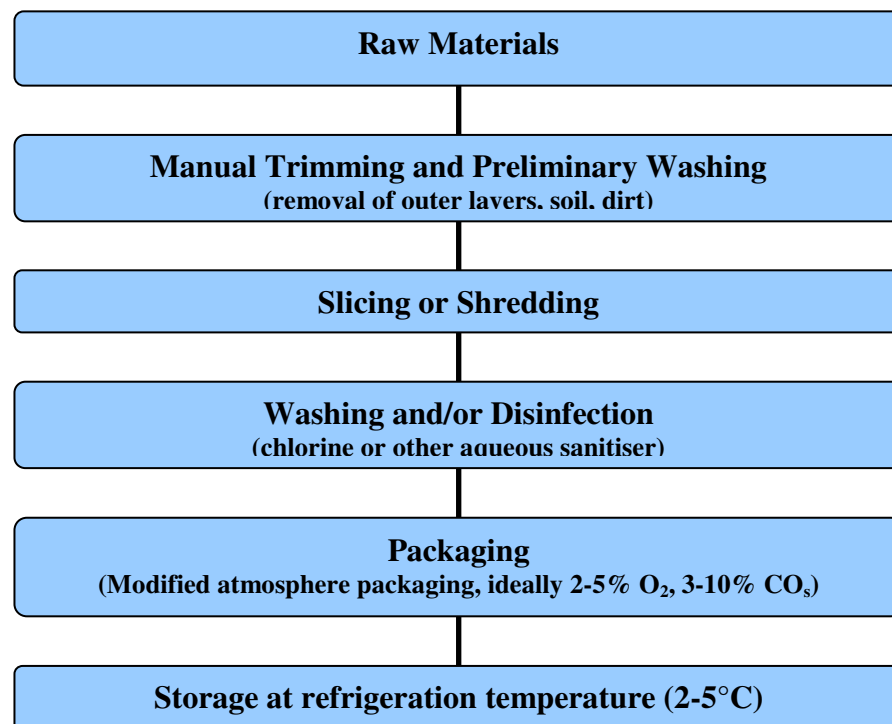


Figure 1.2: A flow diagram for the production of minimally processed vegetables. Adapted from Francis *et al.* (1999).

The initial step in the production of fresh-cut vegetables includes the removal of outer layers of the vegetables, including removal of outer leaves, core and any dirty or

damaged areas. The vegetables are then sliced, diced, shredded or grated. Minimal processing causes many physiological changes in vegetables, including increased respiration rate and physical damage such as disruption of cells; these influence product quality and shelf-life (Cliffe-Byrnes *et al.* 2003). To reduce the microbial load and to increase the shelf-life of these vegetables, they may be subjected to an antimicrobial dipping or sanitisation treatment (e.g. dipping in a chlorine solution or other aqueous sanitizer acids) and are maybe packaged in a modified atmosphere. The current sanitisation steps of minimal processing cannot be relied upon to eliminate pathogens. In order to assure the safety of fresh-cut produce, there is a need to provide complimentary/alternative antimicrobial strategies. In addition, there is increasing consumer concern about the potential harmful effects of synthetic and/or chemical antimicrobials, and this has created new market opportunities for more natural antimicrobials.

1.6 Microbiological safety of fresh-cut vegetables

The number of foodborne illnesses associated with fresh and fresh-cut fruit and vegetables has increased in the past 30 years (Warriner *et al.* 2009). Ready-to-eat (RTE) fresh cut vegetables that may be consumed without further cooking or reheating can be grouped as potentially high risk foods. Fresh-cut vegetables harbour large and diverse populations of microorganisms (Francis and O' Beirne 2001b) and can be a vehicle for the transmission of bacterial, parasitic and viral pathogens (Abadias *et al.* 2008). There is potential for contamination from seed, soil, irrigation water, organic fertilisers, harvesting, processing and packaging (Ragaert *et al.* 2007, Beuchat 1996b, Rico *et al.* 2007, Manani *et al.* 2006).

Fresh-cut vegetables retain much of their indigenous microflora after minimal processing, and counts of 10^5 - 10^7 CFU g^{-1} are frequently present (Francis *et al.* 1999). The majority of the bacteria are gram-negative rods, predominantly *Pseudomonas*, *Enterobacter* or *Erwinia* species (Froder *et al.* 2007). Pathogens may form part of this microflora leading to potential safety problems (Francis *et al.* 1999). Some human pathogens such as *Salmonella*, *Escherichia coli* and *Yersinia enterocolitica* and gram-positive bacteria such as *L. monocytogenes*, *Bacillus cereus* and *Clostridium* spp. can also be found (Froder *et al.* 2007). Growth of food poisoning microorganisms on fresh cut vegetables depends on the properties of the microorganisms, on the intrinsic properties of vegetables and on the effects of processing, packaging and storage (Francis *et al.* 1999).

Minimally processed vegetables go through a cutting or slicing step and this may help pathogens survive and grow. Cut vegetables are more susceptible to chemical and microbiological deterioration because during cutting, cells are destroyed and exudates rich in minerals, sugars, vitamins, and other compounds are released (Froder *et al.* 2007). These nutrients released at the damaged surfaces provide a nourishing environment for microbial growth.

Therefore, good agricultural and manufacturing practices are essential at every step during production and processing of these products. Slicing and shredding procedures, as well as insufficient refrigeration conditions during storage, have been associated with an increase in the number of mesophilic and psychrotrophic aerobic microorganisms (Babic *et al.* 1996). Further preservation techniques such as antimicrobial washing/dipping are used to reduce microbial counts. Pathogens may survive washing and sanitising steps however, largely due to the aqueous nature of sanitisers and their failure to reach hydrophobic regions of cut plant tissues which can

harbour significant numbers of microorganisms, including pathogens (Heaton and Jones 2008). Raw vegetables have been found to harbour potential foodborne pathogens. In the United States, contaminated fresh fruit and vegetables now account for 12% of foodborne illnesses and 6% of foodborne outbreaks (Mukherjee *et al.* 2006).

1.7 Prevalence of *Listeria* in fresh-cut vegetables

Vegetables can become contaminated with *L. monocytogenes* due to its ubiquitous nature in the environment. Since *L. monocytogenes* occurs widely in soil and in the agricultural environment generally, it is present naturally on many vegetables (Francis *et al.* 1999). Many studies have indicated that fresh-cut packaged vegetables may contain *L. monocytogenes* and therefore could represent a health risk to consumers. In recent studies, *L. monocytogenes* was found in fresh mixed salad, cabbage samples and on other fresh produce and ready-to-eat salad vegetables (Abadias *et al.* 2008, Johnston *et al.* 2006, Johannessen *et al.* 2002, Little *et al.* 2007). Three cabbage samples (1%) were positive for *L. monocytogenes* in a survey of US and Mexican fresh produce (Johnston *et al.* 2006) and the pathogen was isolated from 0.3% of fresh produce samples in Norway (Johannessen *et al.* 2002). In a microbiological study of ready-to-eat salad vegetables, Sagoo *et al.* (2003) found *L. monocytogenes* in 2.3% of samples, but only one sample contained levels of $> 10^2$ CFU/g. Similarly, studies carried out on organic mixed vegetables in Zambia detected *Listeria* in 20% of the products tested (Nguz *et al.* 2005). Arumugaswamy *et al.* (1994) isolated *L. monocytogenes* from beansprouts (85%), leafy vegetables (22%) and cucumber (80%) in Malaysia. Francis and O' Beirne (2006) found that a total of 21 *L. monocytogenes* isolates (2.9% of samples) were recovered from a range of

products, including dry coleslaw mix (80% shredded cabbage and 20% shredded carrot), bean sprouts, and leafy vegetables such as iceberg, romaine, and radicchio lettuce and mixed salad leaves (curly endive, escarole, and radicchio leaves) purchased from supermarkets in Ireland. Szabo *et al.* (2000) surveyed 120 bagged lettuce samples in Australia and reported isolation of *L. monocytogenes* in 2.5% of the samples. In Spain, 103 vegetable samples were analysed and *L. monocytogenes* was isolated from 7.8% of the samples (De Simón *et al.* 1992). A survey of 1000 samples of 10 types of fresh produce at the retail level in the USA revealed the presence of *L. monocytogenes* on cabbage, cucumbers, potatoes and radishes and, in another survey, prepared mixed salads and two individual salad ingredients were found to contain *L. monocytogenes* (Beuchat 1996a). In the latter study, the higher rate of contamination of prepared salads was attributed to cross contamination of the pathogen during chopping, mixing and packaging.

Table 1.2: Occurrence of *L. monocytogenes* on fresh-cut produce.

Vegetable	Country	Prevalence	Reference
Fresh vegetables	Spain	5/63 (7.9%)	Badosa <i>et al.</i> (2008)
Pre-packaged salad.		8/42 (19.1%)	
Mixed salads	Spain	132/236 (56%)	Abadias <i>et al.</i> (2008)
Fresh-cut lettuce		29/236 (12.3%)	
Frozen vegetable salad	Chile	88/347 (25.4%)	Cordano and Jacquet (2009)
Ready to eat salad		22/216 (10.2%)	
Fresh vegetables	Malaysia	69/306 (22.5%)	Ponniah <i>et al.</i> (2010)
Pre-packaged mixed vegetable salads	UK	129/2686 (4.8%)	Little <i>et al.</i> (2007)
Mixed vegetable salads	Italy	13/44 (29.5%)	Meloni <i>et al.</i> (2009)
Romaine lettuce	Portugal	1.32%	Santos <i>et al.</i> (2011)
Mixed salad		0.66%	

Over the years, a small number of outbreaks of listeriosis have been linked to fresh-cut produce consumption (Schlech 1991, Schlech 1996, Ho *et al.* 1986). If vegetables are contaminated in the field, the washing steps of minimal processing cannot be relied upon to eliminate *L. monocytogenes*. Studies have shown that there are harbourage sites in food industry premises and equipment where *L. monocytogenes* can persist (Carpentier and Cerf 2011). As the vegetables go through a number of slicing, shredding and cutting steps this further adds to the risk of contamination. Ells and Truelstrup Hansen (2006) found that all twenty four strains of *Listeria* tested exhibited a preference to attach to cut tissue compared to intact leaf surfaces of cabbage. The risk of listeriosis is increased as many of these products are consumed raw without any cooking or pasteurisation step before consumption. As refrigeration can sometimes be the only preservation step, strict temperature control is vital, as research has shown that *L. monocytogenes* can survive and grow at refrigeration temperatures in fresh-cut vegetables (Farber *et al.* 1998, Francis and O' Beirne 2001b). Studies by Little *et al.* (2007) however, found that prepackaged mixed salads were contaminated with *Listeria* spp. and *L. monocytogenes* more frequently when they were stored or displayed above 8°C.

The USA require absence of *L. monocytogenes* in 25 g of foods (“zero tolerance”), while the EU regulation on microbiological criteria for foodstuffs (Regulation (EC) No. 2073/2005), in force from January 2006, provides that *L. monocytogenes* should be below 100cfu/g at the point of consumption of ready-to-eat (RTE) foods, and that processing areas and equipment use in the manufacture of RTE foods must also be monitored for *L. monocytogenes* (E.C. 2005a). Therefore, in order to assure a safe food supply, the industry must employ stronger antimicrobial hurdles during storage.

1.8 Modified atmosphere packaging

Modified atmosphere packaging (MAP) of foods is increasingly being used to improve food shelf-life by inhibition of growth of spoilage microorganisms. MAP techniques are now widely used on a range of fresh or chilled foods, including raw and cooked meats and poultry, fish, fresh pasta, fruit and vegetables. Table 1.2 shows the advantages and disadvantages of modified atmosphere packaging as a food preservation technique.

Table 1.3: Potential advantages and disadvantages of MAP: Adapted from Phillips (1996)

Advantages to consumer:	Advantages to producer:
Increased shelf life High quality product Clear view of product Little or no use of chemical preservatives	Increased shelf life Centralised packaging Reduction in distribution costs
Disadvantages to consumer:	Disadvantages to producer:
Visible added cost Temperature control requirement Product safety to be established Benefits are lost once pack is opened	Visible added cost Temperature control requirement Product safety to be established Specialised training and equipment needed

The main gases used in MAP are O₂, CO₂ and N₂. These gases are used in different concentrations and combinations depending on the product and the needs of the processor and the consumer. The type of packaging material used also plays an important role in MAP. Cliffe-Byrnes *et al.* (2003) showed that using incompatible packaging material can lead to generation of unsuitable gas atmospheres within packs, which have negative effects on produce quality. If the correct material is used, generally semi-permeable packaging films for fresh-cut produce, then the correct atmosphere to suit the product will be created. Modified atmosphere packaging of fresh produce relies on the modification of atmosphere inside the package achieved by

the natural interplay between two processes: the respiration rates of the commodity and the permeability of the packaging films (Mangaraj *et al.* 2009). Vegetables continue to respire after harvest and when packaged, therefore the concentrations of CO₂ and O₂ surrounding the product will change. Ideally O₂ levels will fall from 21% to 2-5% and CO₂ levels will increase from 0.03% to 3-10% (Francis and O' Beirne 1998). In products where respiration rates are low, flushing with 100% nitrogen or low oxygen atmospheres may be used to achieve low oxygen modified atmospheres from the outset. The low O₂ levels and slightly elevated CO₂ levels, combined with refrigeration, can reduce the product's respiration rate and inhibit the activity of the ripening hormone ethylene. The gas levels also inhibit enzymatic browning at cut surfaces, chlorophyll breakdown and microbial growth, and thus extend the shelf-life. While there may be some direct effects of gas atmospheres, the main reduction of microbial growth results from improved product quality and low storage temperature.

Carbon dioxide levels above 20% have antimicrobial properties; however, this level does not generally occur in MAP fresh-cut vegetables. The CO₂ concentrations (5-10%) and low O₂ levels (2-5%) within modified atmosphere packages of fresh-cut produce may selectively inhibit a number of foodborne spoilage and pathogenic microorganisms (Daniels *et al.* 1985, Farber 1991).

The application of MAP in fresh-cut produce can be challenging and a number of factors must be considered. As the physical effects of minimal processing can induce or accelerate many physiological changes in vegetables, and most processing steps disrupt plant cells and damage membranes, increased product stability is required to prolong the shelf-life of these minimally processed products (Cliffe-Byrnes and O' Beirne 2007). However, several other factors, in addition to a suitable modified atmosphere must be considered when attempting to produce high quality minimally

processed produce. Cultivar selection has been shown to be a contributing factor and cultivars suitable for MAP should have low respiration rates and be resistant to mechanical injury and crushing. Cliffe-Byrnes and O'Beirne (2005) showed that different cultivars of cabbage samples had different respiration rates and found that gas atmospheres within packs of coleslaw varied as a result of the differences. In general, an increase in the rate of respiration leads to a decrease in shelf-life of the product. Selection of packaging film is also an important factor to consider when employing MAP in minimally processed produce. Use of unsuitable packaging materials can lead to technically unsuitable atmospheres with detrimental effects on product quality. If film permeability is low, relative to the product respiration, a rapid accumulation of CO₂ and depletion of O₂ results, leading to the formation of off-flavour compounds such as ethanol and acetaldehyde (Cliffe-Byrnes *et al.* 2003). However, if the film is too permeable then little modification of atmosphere occurs and so shelf-life extension is limited. Controlling the storage temperature is also important. Increases in storage temperature lead to increased product respiration and the atmosphere within a package can change to an unintended combination of gases, such as excessively high levels of CO₂ and low levels or absence of O₂.

1.9 MAP and *Listeria*

Listeria monocytogenes is facultatively anaerobic and is capable of survival and growth under the low O₂ and slightly elevated CO₂ concentrations found in MA packages of vegetables. *L. monocytogenes* is also a psychrotroph and is capable of surviving and growing at the low storage temperatures associated with fresh-cut MAP vegetables. In some reports, MA packaged products containing elevated carbon dioxide levels were found to inhibit the growth of *L. monocytogenes* and *Clostridium*

sporogenes (Phillips 1996). However, Francis and O' Beirne (1998) found that by comparison with air, CO₂ concentrations of 5% or 10% had no inhibitory effect on the survival and growth of *L. monocytogenes*, and these gas levels are typical of atmospheres found in MA packaged vegetables. Francis and O' Beirne (1999) reported that *L. monocytogenes* survived and grew well in MA packages of vegetables containing 2-5% O₂ and 0-15% CO₂. In the presence of 10% CO₂, growth of *L. monocytogenes* was stimulated in minimally processed carrots (Amanatidou *et al.* 1999), and *L. monocytogenes* was shown to survive in an atmosphere of 100% N₂ (Francis and O' Beirne 1998).

Although MAP can be an effective mild preservation technique, a number of studies have indicated that survival and growth of facultative anaerobic pathogens, such as *L. monocytogenes* on MAP vegetables can be enhanced. MAP increases the shelf-life of products, therefore increasing the time available for pathogens to grow. Francis and O'Beirne (1997) showed that nitrogen flushing packages of shredded lettuce resulted in significant growth of *Listeria* during storage at 8°C, compared to storage under product modified atmospheres. Storage under product modified atmospheres in turn resulted in better growth than in air at 8°C. Carlin *et al.* (1996) examined the survival of *L. monocytogenes* on chicory leaves stored at 10°C in air, or under 10%, 30% or 50% CO₂, with 10% O₂, and found that *L. monocytogenes* grew better as the concentration of CO₂ increased. Oliveira *et al.* (2010) found that *L. monocytogenes* increased about 1 log unit in MA packaged shredded lettuce stored at 5°C. Bourke and O'Beirne (2004) showed that certain strains of *Listeria* spp. (ie. ATCC 19114) had the ability to survive at 3°C in high CO₂/low O₂ environment which would be unintended but frequently encountered sub-optimal atmospheres for fresh cut vegetables.

L. monocytogenes possesses a glutamate decarboxylase (GAD) acid resistance mechanism that plays a major role in its acid tolerance and contributes to its survival in low pH foods (Cotter *et al.* 2001b). Francis *et al.* (2007) reported that the presence of GAD in *L. monocytogenes* mutants (with either the *gadA*, *gadB* or *gadC* genes absent) contributed significantly to their improved survival in MAP vegetables especially those containing elevated CO₂ atmospheres (ie. 25-35%). In addition, Francis and O'Beirne (2001a) showed that prior acid adaptation enhanced survival of *L. monocytogenes* in MA packages of vegetables with relatively high levels of CO₂ (25%).

There are a number of reasons why modified atmosphere packaging may cause food safety concern. The gas atmospheres and refrigeration temperatures used inhibit growth of many spoilage microorganisms, some of which may be natural competitors of pathogens. Reducing the growth of these competitive spoilage microorganisms may indirectly facilitate or enhance pathogen survival and growth (Francis *et al.* 1999). In addition, MAP increases the shelf-life of produce thus allowing more time for pathogens to grow. Over extending the shelf-life may allow development of significant populations of pathogens. This could pose a health risk to consumers, as the product appears of sound quality. Scifo *et al.* (2009) found that *L. innocua* became one of the dominant species in samples packaged in MA from the third day of storage as the CO₂ levels increased to 15%. Many authors have shown that CO₂ levels higher than 15% promotes the growth of *Listeria* spp. (Farber 1991, Carlin *et al.* 1996, Phillips 1996). Studies have also indicated that fresh cut vegetables packaged under MA supported the growth of *Listeria* spp. Francis and O'Beirne (1997) observed that when packages were flushed with inert atmospheres to minimise enzymatic browning, contaminating *L.*

monocytogenes survived better than in unflushed packages, when the product experienced mild temperature abuse.

The data suggest that MA packaging of fresh-cut products in general seems safe, provided they are stored at or below 4°C. However, even at mild abuse temperatures such as the 8°C commonly experienced during distribution/retail display, safety issues can arise if contamination of fresh-cut produce has occurred. As a result, the two primary objectives in ensuring safety are prevention of contamination and storage at low temperature. This can be achieved through Good Agricultural Practice (GAP), Good Manufacturing Practice (GMP), and the application of HACCP principles throughout production, processing, packaging and storage. Since there is no listericidal step included in the production/distribution of fresh-cut vegetables, incorporation of additional hurdles could improve their safety.

1.10 Washing/Sanitisation

Fresh produce can be contaminated with soil or mud and so would normally undergo a cleaning/washing step before processing. Subsequently, washing after peeling and/or cutting removes microorganisms and tissue fluid, thus reducing microbial growth and enzymatic oxidation during subsequent storage. The recommended quantity of water that should be used is 5-10 l/kg of product before peeling and/or cutting and 3 l/kg after peeling and/or cutting (Ahvenainen 1996). However, wash water can become a source of microbial contamination during processing if reused, and a chemical disinfectant is normally added to the wash water system. These procedures reduce microbiological load on the vegetables, thus reducing the rate of subsequent microbial spoilage and reducing but not eliminating populations of potential pathogens (Francis *et al.* 1999). Chlorine-based chemicals, particularly

liquid chlorine and hypochlorite, are the most widely used sanitizers for decontaminating fresh produce. Chlorine compounds are usually used at levels of 50-200 ppm free chlorine and with typical contact time of less than 5 minutes (Francis and O' Beirne 2002). A study by Ells and Truelstrup Hansen (2006) demonstrated that *L. monocytogenes* had the ability to rapidly attach to both cut and intact cabbage tissues and adhere within 5 minutes. They found that the binding strength of these leaf associated cells was strong, as greater than 70% remained attached after vigorous washing. Nguyen-the and Carlin (1994) reported that the elimination of *L. monocytogenes* from the surface of vegetables by chlorine is limited and unpredictable. Zagory (1999) found that *L. monocytogenes* appeared to be little affected by chlorine washes and may grow faster after competing epiphytic bacteria have been removed. Studies by Zhang and Farber (1996) found that a wide variety of disinfectants, including chlorine, were not very effective, with most chemicals only causing approximately a 1 log reduction in numbers of *L. monocytogenes* on fresh-cut vegetables.

In addition, there are safety concerns associated with chlorine, such as the reaction of chlorine with natural organic matter resulting in the formation of carcinogenic halogenated disinfection by-products (Zhang and Farber 1996, Ölmez and Kretschmar 2009). In some European countries including Germany, the Netherlands, Switzerland and Belgium the use of chlorine in ready-to-use (RTU) products is prohibited (Rico *et al.* 2007). As a consequence, there is a growing number of alternative water sanitizing compounds, which are used to reduce microbial populations in fresh-cut produce, including chlorine dioxide (ClO₂), hydrogen peroxide (H₂O₂), ozone and organic acids (eg lactic acid, citric acid, acetic acid, tartaric acid). Chlorine dioxide is accepted for use in washing fruits and vegetables and

many studies have demonstrated its antimicrobial activity (Rico *et al.* 2007). However, Rodgers *et al.* (2004) found that while chlorine dioxide was effective against *L. monocytogenes* on surface inoculated whole lettuce, it was ineffective on shredded or sliced lettuce. Again, while hydrogen peroxide has been shown to have antimicrobial properties, Bennik *et al.* (1996) found that *L. monocytogenes* grew better on H₂O₂ disinfected produce than on non –disinfected or water-rinsed produce. Parish *et al.* (2003) found that shredded lettuce was severely browned upon dipping in a solution of H₂O₂.

1.11 Essential oils

Concern caused by traditional food preservatives such as chemical sanitisers, reporting of occasional allergic reactions in sensitive individuals and the formation of potentially carcinogenic by-products among other problems, has increased the interest in antimicrobial compounds found in nature (Rico *et al.* 2007). Consumers are looking for more organically produced foods which they consider to be safer. In addition, there is growing concern of microbial resistance towards conventional preservatives. The use of natural plant antimicrobials, such as plant Eos, may represent an alternative/complimentary strategy to reduce contamination and growth of pathogens on fresh-cut produce.

Essential oils, also known as volatile or ethereal oils, are aromatic oily liquids obtained from plant materials. They can be obtained by expression, fermentation or extraction, but the most common method used is steam distillation (Burt 2004). Antimicrobial compounds in EOs have great potential as natural antimicrobial agents in foods. Plant oils and extracts have been used for a wide variety of purposes for thousands of years. The greatest use of EOs in the European Union is in food (as

flavourings), perfumes and pharmaceuticals. An estimated 3000 EOs are known, of which 300 are commercially important, mostly in the flavours and fragrance market (Prabuseenivasan *et al.* 2006). EOs, or some of their components, are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservatives and additives, and as natural remedies (Bakkali *et al.* 2008).

Essential oils from aromatic and medicinal plants have been known since antiquity to possess biological activity, notably antibacterial, antifungal and antioxidant properties (Baratta *et al.* 1998). More recent studies have confirmed that some EOs and their components have strong antimicrobial properties (Burt 2004). The growing interest in EOs by the food industry is due to their safe status, their wide acceptance by consumers, and their potential exploitation for multi-functional use (Sacchetti *et al.* 2005).

1.12 Extraction of EOs

Today the most common EOs are obtained by either hydro- or steam distillation. Raw plant materials (flowers, leaves, wood, bark, roots, seeds, or peel) are put into a distillation apparatus. As the water is heated, the steam or water passes through the plant material, vaporizing the volatile compounds. The vapours flow through a cooling coil, where they condense and the condensate is collected in a receiving vessel.

Another method commonly used is solvent extraction. A hydrocarbon solvent, such as ether, ethanol, methanol, hexane or alcohol, is added to the plant material to help dissolve the EO. However, solvent extraction has some disadvantages including low yield, losses of volatile compounds, long extraction times, toxic solvent residues, and thermal degradation of unsaturated compounds producing off-flavour compounds

(Khajeh *et al.* 2004). Extracts obtained by solvents can contain solvent residues that can pollute the foods to which they are subsequently added (Guan *et al.* 2007).

New separation techniques have gained increasing importance in the chemical and food industries, primarily due to recent environmental and public health regulations and the necessity for minimizing energy requirements (Fadel *et al.* 1999). In recent years, supercritical fluid extraction (SFE) has become an alternative to more conventional extraction procedures, chiefly because the dissolving power of supercritical fluids can be adjusted by regulating the pressure and temperature conditions employed (Díaz-Maroto *et al.* 2002). CO₂ is the most widely used fluid for supercritical extraction as it is non toxic, non flammable, chemically stable, and retains no solvent residue in the extract (Díaz-Maroto *et al.* 2002). This method uses CO₂, liquefied under pressure, to extract the EO from the plant material. SFE has been demonstrated to be a valuable process alternative, because it requires less solvent, has a short extraction time (30 min versus 4 h for hydrodistillation) and a capability to extract thermally labile compounds under mild conditions (Yamini *et al.* 2008). Moreover, the possibility of manipulating the composition of the oil, by changing the parameters of the extraction (pressure, temperature and dynamic extraction time), is more attainable in SFE (Khajeh *et al.* 2004). Studies by Guan *et al.* (2007) have also shown that extracts obtained by SFE can better retain the organoleptic characteristics of the starting plant material.

1.13 Antimicrobial effects of EOs

It has long been known that EOs have antimicrobial properties. The mechanism of antimicrobial action of EOs has not been studied in great detail, but a number of different mechanisms are believed to be involved. EOs and their components are hydrophobic, and it is believed that this enables them to dissolve in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures of the membranes and rendering them more permeable. Leakage of ions and other cell contents will occur, and although a certain amount of leakage can be tolerated, extensive loss of cell contents or the exit of critical molecules and ions will eventually lead to cell death (Burt 2004). Generally, the EOs possessing the strongest antibacterial properties against foodborne pathogens contain a high percentage of phenolic compounds, such as carvacrol, eugenol and thymol (Cosentino *et al.* 1999, Dorman and Deans 2000). The mechanism of action of phenolic compounds is considered to be the disturbance of the cytoplasmic membrane, disruption of the proton motive force, electron flow and active transport, and coagulation of cell contents (Burt 2004). The mode of action of carvacrol, one of the major components of oregano and thyme, has received much attention from researchers. Thymol and carvacrol are structurally very similar having the hydroxyl group at different locations in the phenolic ring. Both substances appear to act by making bacterial cell membranes more permeable (Lambert *et al.* 2001).

Other components of EOs appear to act on cell proteins in the cytoplasmic membrane (Knobloch *et al.* 1989). Two possible mechanisms have been suggested, whereby cyclic hydrocarbons act on ATPases which are located in the cytoplasmic membrane and bordered by lipid molecules. Lipophilic carbon molecules may accumulate in the lipid bilayer and distort the lipid protein interaction. Alternatively,

direct interaction of the lipophilic compounds with hydrophobic parts of proteins is possible (Burt 2004).

Gram-positive bacteria are usually more sensitive to EOs than Gram-negative bacteria (Burt 2004, Lambert *et al.* 2001, Smith-Palmer *et al.* 1998). This is to be expected, as Gram-negative bacteria possess an outer membrane surrounding the cell wall. Baratta *et al.* (1998) found that the volatile oils of marjoram, oregano, rosemary and lemon exhibited considerable inhibitory effects against twenty five different genera of bacteria including animal and plant pathogens, food poisoning and spoilage bacteria and a spoilage fungus. Studies by Gutierrez *et al.* (2008b) found that different EOs exerted different antimicrobial activity. Oregano and thyme EOs had the highest activity against all the bacteria tested, marjoram and basil EOs had selectively high activity against *B. cereus*, *E. aerogenes*, *E. coli* and *Salmonella*, while lemon balm and sage EOs displayed activity against *L. monocytogenes* and *S. aureus*. Elgayyar *et al.* (2001) showed that oregano EO was inhibitory against *L. monocytogenes*, *Staphylococcus aureus*, *E. coli* O:157:H7, *Y. enterocolitica*, *P. aeruginos* and *Lactobacillus plantarum*. Dorman and Deans (2000) found that oregano and thyme oils were effective against 25 different genera of bacteria, including animal and plant pathogens and food poisoning and spoilage bacteria. A study carried out by Viuda-Martos *et al.* (2008) showed that the EOs from oregano, thyme, rosemary, sage, cumin and clove showed antibacterial activity against some bacteria commonly found in the food industry; *L. curvatus*, *L. sakei*, *S. carnosus* and *S. xylosus* or related to food spoilage *E. gergoviae*, *E. amnigenus*. Thyme EO was found to be the most potent inhibitor and rosemary EO performed the worst.

While these studies all show antimicrobial effects of EOs, there is a general lack of scientific information concerning the antimicrobial effectiveness of EOs in the

vapour phase compared with direct contact. Nedorostova *et al.* (2008) found that certain EOs, including *Origanum vulgare*, *O.majorana*, *Thymus vulgaris* and *T. serpyllum*, were highly effective in vapour phase and could be potentially used against foodborne bacterial pathogens. López *et al.* (2005) found that cinnamon and clove oils in the vapour phase had significant antimicrobial activity against both Gram-positive bacteria (*Staphylococcus aureus*, *B. cereus*, *Enterococcus faecalis*, and *L. monocytogenes*) and Gram-negative bacteria (*E. coli*, *Y. enterocolitica*, *S. choleraesuis*, and *Aspergillus flavus*). However, most studies have found EOs are more effective when in direct contact with cultures (Lopez *et al.* 2005, Suhr 2003, Scollard *et al.* 2009).

Table 1.4 below shows further results for the antibacterial effect of numerous EOs.

Table 1.4: Antibacterial effects of EOs against foodborne pathogens. Adapted from Burt (2004)

Plant from which EO is derived	Species of bacteria	MIC	Reference(s)
Rosemary	<i>B. cereus</i>	16,500 ppm	(Gutierrez <i>et al.</i> 2008b)
	<i>E. coli</i>	0.3-0.5 %v/v	(Fu <i>et al.</i> 2007, Jiang <i>et al.</i> 2011)
		0.6-6.4 mg/ml	(Prabuseenivasan <i>et al.</i> 2006, Moreira <i>et al.</i> 2005)
	<i>S. aureus</i>	12,500 ppm	(Gutierrez <i>et al.</i> 2008b)
		0.03-0.125 %v/v	(Fu <i>et al.</i> 2007, Jiang <i>et al.</i> 2011)
	<i>S. typhimurium</i> <i>L. monocytogenes</i>	6-12.8 mg/ml	(Prabuseenivasan <i>et al.</i> 2006, Van Vuuren <i>et al.</i> 2009)
7,500 ppm		(Gutierrez <i>et al.</i> 2008b)	
12,500 ppm		(Gutierrez <i>et al.</i> 2008b)	
Oregano	<i>B. cereus</i> <i>E. coli</i>	8,750 ppm	(Gutierrez <i>et al.</i> 2008b)
		425 ppm	(Gutierrez <i>et al.</i> 2008b)
	<i>E. coli</i>	0.12-0.25 %v/v	(Oussalah <i>et al.</i> 2007, Hammer <i>et al.</i> 1999)
		0.5-6.25 mg/ml	(Burt 2003, Soković <i>et al.</i> 2007, Moreira <i>et al.</i> 2005)
	<i>S. aureus</i>	350 ppm	(Gutierrez <i>et al.</i> 2008b)
		0.12-0.13 %v/v	(Oussalah <i>et al.</i> 2007, Hammer <i>et al.</i> 1999)
	<i>S. typhimurium</i>	30 ppm	(Gutierrez <i>et al.</i> 2008b)
		0.05-0.12 %v/v	(Oussalah <i>et al.</i> 2007, Hammer <i>et al.</i> 1999)
	<i>L. monocytogenes</i>	0.5 mg/ml	(Soković <i>et al.</i> 2007)
		550 ppm	(Gutierrez <i>et al.</i> 2008b)
0.1 %v/v		(Oussalah <i>et al.</i> 2007)	
0.5 mg/ml		(Soković <i>et al.</i> 2007)	
Thyme	<i>E. coli</i>	70 ppm	(Gutierrez <i>et al.</i> 2008b)
		0.05 % v/v	(Oussalah <i>et al.</i> 2007)
		1-1.25 mg/ml	(Soković <i>et al.</i> 2007, Burt 2003)
	<i>S. aureus</i>	660 ppm	(Gutierrez <i>et al.</i> 2008b)
		0.025 %v/v	(Oussalah <i>et al.</i> 2007)
	<i>S. aureus</i>	0.5 mg/ml	(Soković <i>et al.</i> 2007, Van Vuuren <i>et al.</i> 2009)
		175 ppm	(Gutierrez <i>et al.</i> 2008b)
	<i>S. typhimurium</i>	0.05 %v/v	(Oussalah <i>et al.</i> 2007)
		1500 ppm	(Gutierrez <i>et al.</i> 2008b)
	<i>L. monocytogenes</i>	0.1 % v/v	(Oussalah <i>et al.</i> 2007)
110 ppm		(Gutierrez <i>et al.</i> 2008b)	

1.14 Anti-listerial effects of EOs

In vitro studies found that thyme oil was the most inhibitory EO against *Listeria*, whereas sage and rosemary EOs were not very effective (Singh *et al.* 2003). Oussalah *et al.* (2006) found that oils such as oregano and thyme showed strong activity against *L. monocytogenes*, and that the organism was destroyed completely by twenty EOs at concentrations $\leq 0.8\%$ (vol/vol). Friedman *et al.* (2002) and Nevas *et al.* (2004) showed that oregano, savory and thyme had antimicrobial effects on *L. monocytogenes* as well as *E. coli*, *S. aureus*, *C. perfringens* and *C. botulinum*, and that oregano, cinnamon, thyme and clove oils showed strong activity against *L. monocytogenes*, *Campylobacter jejuni*, *E. coli* O157:H7, and *S. enterica*. Smith-Palmer *et al.* (1998) also found that cinnamon and thyme EOs were active against *L. monocytogenes*. Studies by Lis-Balchin and Deans (1997) found EOs from rosemary and *Eucalyptus citriodora* to be effective against 20 strains of *Listeria* tested. Pandit and Shelef (1994) found that out of 18 spices screened only clove ($\geq 1\%$, w/v) and rosemary ($\geq 0.5\%$, w/v) EOs were listericidal. Mangena and Muyima (1999) found that rosemary EO displayed strong activity against *L. monocytogenes*. Hazzit *et al.* (2006) found that 13 strains of *L. monocytogenes* tested were not resistant to the action of EOs of either *Origanum* or *Thymus* species. In addition to some of the contradicting data reported above, there is considerable variation in the activity of the same EO against different strains of *L. monocytogenes* (Lis-Balchin and Deans 1997) with some strains exhibiting greater resistance to EOs. Nguefack *et al.* (2004) also found that there was significant differences in sensitivity between strains of *Listeria* observed when tested against five EOs.

Essential oils have also been shown to have additive or synergistic effects when used in combinations, and recently it was shown that the combination of oregano with

thyme had greater efficacy against *L. monocytogenes* than when either of the EOs were tested individually (Gutierrez *et al.* 2008a). Overall, many studies have shown that the EOs of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) are among the most active against a number of bacteria including *L. monocytogenes* (Scollard *et al.* 2009, Smith-Palmer *et al.* 1998, Dimitrijevic *et al.* 2007, Gutierrez *et al.* 2008b). It has been reported that there is a relationship between the chemical composition of the tested oil and the antimicrobial activity. Mourey and Canillac (2002) found that the constituents of EOs, such as monoterpenes, contribute to the antimicrobial effect, particularly against *L. monocytogenes*.

1.15 Chemical composition of EOs

EOs have great potential as natural antimicrobial agents in foods. It is believed that EOs can comprise more than sixty different chemical components (Russo *et al.* 1998). The major components constitute up to 85% of the EO, while the remaining components exist as trace amounts (Faleiro *et al.* 2003). The main components are terpenes and terpenoids, and the minor components are aromatic and aliphatic compounds (Figure 1.3).

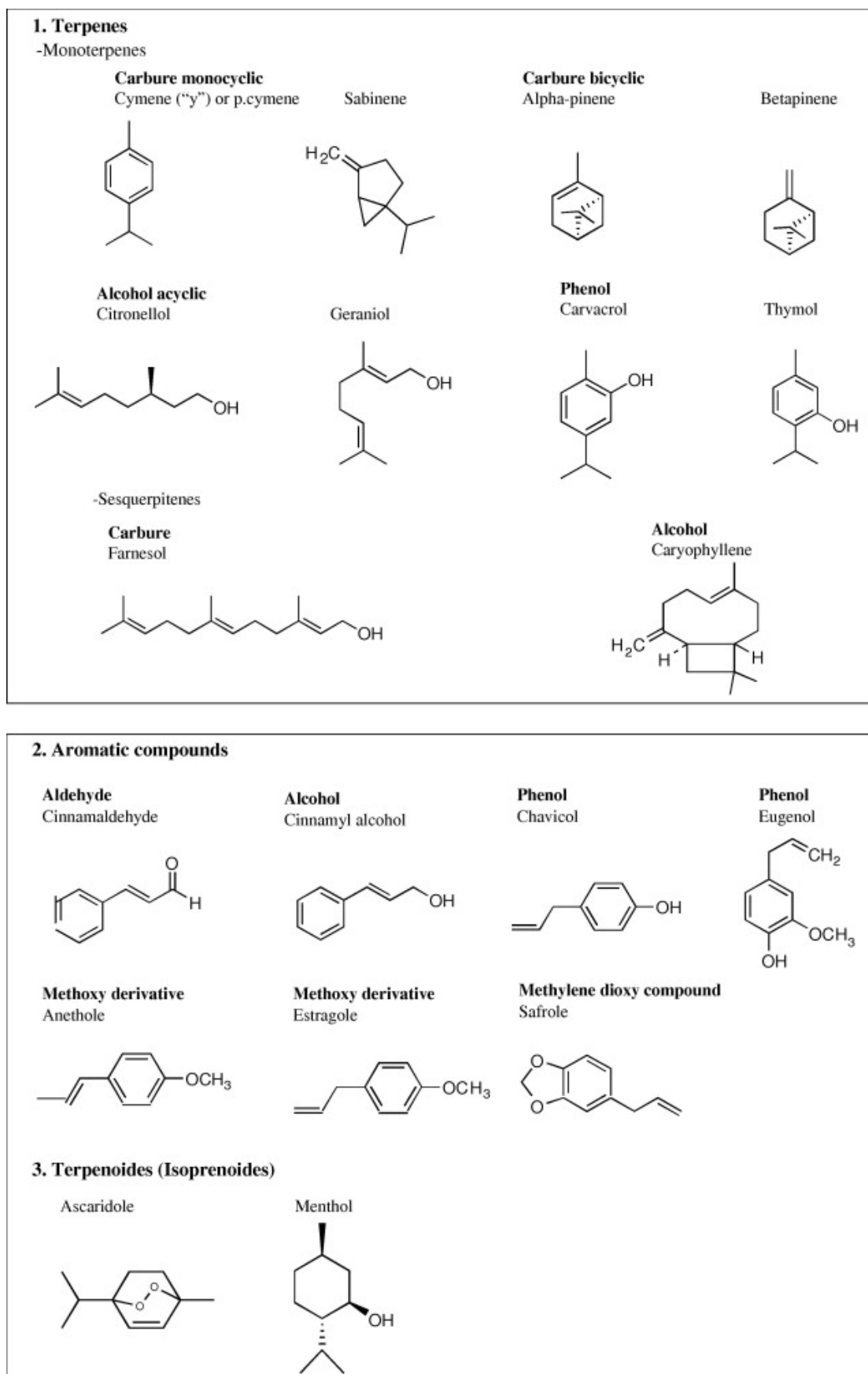


Figure 1.3: Chemical structures of selected components of EOs (Bakkali *et al.* 2008).

While it is believed that the phenolic components are chiefly responsible for their antibacterial properties (Cosentino *et al.* 1999), there is some evidence that minor components may also play a role in antibacterial activity, possibly by producing a synergistic or additive effect between components (Burt 2004). The composition of the EOs and of the extracts obtained from plants are determined by genetics (species and variety), agronomic conditions, harvest time and the type of processing followed (Guillén *et al.* 1996). Effects of soil, climate and harvest time mean that no chemical will be present in the same proportions at each extraction. Studies show that although the composition of oils obtained from different varieties of herbs differed quantitatively, it did not differ qualitatively (Khajeh *et al.* 2004). Rosemary, oregano, basil and thyme were used in the current study. Table 1.5 shows the main components reported in these EOs.

Table 1.5: Oils with antibacterial activity and their main components. Adapted from Tajkarimi *et al.* (2010)

Species	Plant part	Major components
Oregano (<i>Ocimum basilicum</i>)	Leaves/flowers	Carvacrol/Thymol
Rosemary (<i>Rosmarinum officinalis</i>)	Leaves	Camphor/1-8,-cineole/borneol/camphor
Basil (<i>Ocimum basilicum</i>)	Leaves	Linalool/methyl chavicol
Thyme (<i>Thymus vulgares</i>)	Leaves	Thymol/carvacrol

Chemical composition of EOs can also be affected by post-harvest treatments such as chopping or grinding. Grinding facilitates the release of aroma/flavour principles for mixing with food materials (Gopalkrishnan *et al.* 1991). Studies on coriander herb found that a change in oil chemistry was triggered by the chopping of the plant material, probably due to the release of an enzyme which reduces the

aldehydes to the corresponding alcohols (Smallfield *et al.* 1994). Crushing garlic, was found to transform the alliin present into the biologically active allicin molecule, and it was this allicin molecule that was responsible for its remarkable antibacterial activity (Rahman 2007). Smallfield *et al.* (2000) showed that different degrees of crushing of coriander gave differences in oil composition and yield, however this could have been due to the heavy crushing rupturing all vittae in the sample compared to the light crushing. Studies also found that oil yields obtained from ground black pepper are far above those from whole fruits (Rouatbi *et al.* 2007, Murthy *et al.* 1999). This difference is due to the increase of the area for material exchanges and to the burst of cells obtained by grinding.

EOs typically contain terpenoids, sesquiterpenes and possibly diterpenes, together with different groups of aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters of lactones (Tajkarimi *et al.* 2010). EOs with high monoterpene hydrocarbon levels were very active against bacteria. This relationship was apparent when the terpenes included pinenes, camphene, α -terpinene, γ -terpinene, myrcene or limonene (Lis-Balchin *et al.* 1998). Other components in EOs showing high effectiveness were linalool, linalyl acetate, 1,8-cineole, thymol and many monoterpene hydrocarbons. For example, the EOs obtained from oregano and thyme are well known for their antimicrobial activity and are characterized by a very high content of monoterpenes, both hydrocarbons (such as γ -terpinene and p-cymene) and oxygenated compounds (mainly thymol and carvacrol). The antimicrobial activity of the EOs may be explained by the lipophilic character of the monoterpenes which act by disrupting the microbial cytoplasmic membrane (Cristani *et al.* 2007).

The monoterpenes borneol and camphor, which are present in rosemary EO have been shown to have strong antibacterial effects. Tabanca *et al.* (2001) found that

borneol showed inhibitory effects on Gram-negative and Gram-positive pathogenic microorganisms. In addition, borneol was found to be the most active compound tested against a variety of microorganisms including *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* (Santoyo et al. 2005). Studies by Sokovic *et al.* (2007) showed that camphor exhibited inhibitory activity at 5-7 µg/ml and was bactericidal at 6-10 µg/ml against a variety of human pathogenic bacteria.

Studies have shown that greater antimicrobial potential could be ascribed to the oxygenated terpenes, especially phenolic compounds. Compounds present in EOs such as carvacrol, eugenol, linalool, and thymol inhibit a wide variety of microorganisms (Delaquis *et al.* 2002). Studies by Cristani *et al.* (2007) found that thymol was considerably more toxic of the four terpenes tested against *S. aureus* and that carvacrol was the most inhibitory against *E. coli*. Camphor and thymol were also found to be inhibitory against *S. aureus* and *P. aeruginosa* and seemed to have an additive effect when mixed (Lambert *et al.* 2001).

1.16 Effect of extraction method on effectiveness of EOs

The chemical composition of the volatile oils isolated from aromatic plants depends strongly on the extraction method used. Several authors have reported a difference in the composition of an EO due to extraction method (Yamini *et al.* 2008, Carvalho *et al.* 2005, Reverchon *et al.* 1995). Chemical transformations such as hydrolysis or isomerisation can occur during hydrodistillation (HD) or simultaneous distillation-solvent extraction (Fadel *et al.* 1999). Monoterpenes are well known to be vulnerable to chemical changes under steam distillation conditions, and even conventional solvent extraction is likely to involve losses of more volatile compounds during removal of the solvent (Díaz-Maroto *et al.* 2002). Guan *et al.* (2007) showed

that the composition of clove oil extracted by different methods was mostly similar but that the relative concentrations of its components were different. Fadel *et al.* (1999) found that the yield of monoterpene hydrocarbons in HD oil was slightly higher than in the SFE extract, while the SFE extract possessed higher concentrations of the sesquiterpenes, light oxygenated compounds and heavy oxygenated compounds. Boutekedjiret *et al.* (2003) found that the monoterpene hydrocarbon compounds are in comparatively small proportions in the hydrodistilled oil than in the steam distilled oil, presumably due to chemical conversions in the presence of water. Several authors have compared the EO obtained by steam distillation and the product obtained by SFE, and found that steam distilled oil contained relatively more terpene hydrocarbons. By contrast, the SFE oil contained a higher percentage of oxygenated compounds (Reverchon and Senatore 1992). Steam distillation is based on the evaporation of volatile compounds induced by steam, therefore compounds with low vapour pressure cannot be completely extracted by this technique (Reverchon *et al.* 1995). Supercritical fluid extraction on the other hand can provide high levels of solubilisation as the operation can be manipulated by changing the pressure or temperature (Roy *et al.* 1996).

As the chemical composition of oils differ due to extraction method, so too does the antimicrobial activity of the different oil extracts. Vági *et al.* (2005) found that extracts obtained by SFE showed significantly stronger antimicrobial properties against *E. coli*, *P. fluorescens* and *B. cereus*, in comparison with the slightly inhibitory effects of the ethanolic extract. They suggested that the stronger antimicrobial activity of the SFE extracts could be explained by the higher concentration of particular compounds in the extract. Studies by Celiktas *et al.* (2007) also found that methanol extracts of rosemary exhibited very low antimicrobial activities compared to the EOs

obtained by HD. Other studies found that the antimicrobial activity of rosemary EOs obtained by microwave hydrodiffusion and gravity (MHG) was slightly higher than that obtained with HD, and that the antimicrobial activity of MHG EO can be linked to its higher content of oxygenated compounds (Bousbia *et al.* 2009).

1.17 EOs in food

There are still relatively few studies on the effectiveness of EOs in real food systems. Although EOs perform well in antibacterial assays *in vitro*, it has been found that a greater concentration of EO is needed to achieve the same effect in foods (Burt 2004). This may be because of the greater availability of nutrients in food compared to laboratory media, which may enable bacterial cells to repair faster after injury by the EO (Gill *et al.* 2002). Intrinsic factors of foods also play a role in the antimicrobial effects. Generally, a decrease in pH increases the antimicrobial effects of EOs. In addition, high levels of fat and/or protein in foodstuffs may protect bacteria against damaging effects of EOs (Gutierrez *et al.* 2008a). Since vegetables generally have a low fat content, this may contribute to the inhibitory effects of EOs in vegetable products. In the high fat product pâté, mint oil exhibited little antibacterial effect against *L. monocytogenes* and *S. enteritidis*, whereas in cucumber the same EO was much more effective (Tassou *et al.* 1995). The study found that the antibacterial action of the mint EO depended mainly on its concentration, food pH, composition, storage temperature and the nature of the micro-organism. Studies have also shown that the antimicrobial activity of EOs benefits by a decrease in storage temperature (Skandamis and Nychas 2000). Further work on the effects of temperature on the efficacy of EOs is needed to enable them to be used effectively in food systems.

As higher concentrations of plant EOs are generally required in foods than *in vitro* (Shelef 1984), the application of EOs in foods may be limited due to changes in the sensory quality of the food or to interactions of EOs with food components (Devlieghere *et al.* 2004). To try and eliminate the negative flavour and sensory issues associated with the addition of highly concentrated EOs, lower/more dilute combination treatments need to be developed to maintain product safety and shelf-life (Gutierrez *et al.* 2008a). EOs derived from herbs such as basil, marjoram, thyme and bay with proven anti-listerial activity, have been recommended for use in foods. The addition of EOs to food products improved the sensory attributes (odour and appearance) of fish and meat (Goulas and Kontominas 2007, Fernández-López *et al.* 2005), however, clove and cinnamon EOs had unpalatable effects in meats and cheeses (Lis-Balchin and Deans 1997). Bagamboula *et al.* (2004) showed that addition of 0.1% (v/v) thyme oil (diluted in ethanol) proved detrimental to the sensory quality of lettuce leading to browning and strong odour development. The detrimental effect of thyme oil on the sensory quality of chopped bell peppers was also reported by Uyttendaele *et al.* (2004).

Undesirable organoleptic effects might possibly be limited by careful selection of EOs according to the type of food, by diluting EOs, and/or using EOs in different combinations. Studies by Gutierrez *et al.* (2008a) found that combinations of EOs could minimize application concentrations of EOs and consequently reduce any adverse sensory impact in food. Another method to possibly reduce the negative organoleptic impact of the EOs is to dilute the oils or use the main chemical components of the EOs in food products. Studies have shown that mixing some of the individual chemical components of EOs have additive antimicrobial effects and in

some cases exceeded the inhibition achieved by the individual oils (García-García *et al.* 2011, Lambert *et al.* 2001, Delaquis *et al.* 2002).

The antibacterial activity of EOs is also influenced by the degree to which oxygen is available. There are only a few studies examining the influence of gas atmosphere on the effectiveness of EOs. The activity of thyme and oregano EOs was greatly enhanced against *S. typhimurium* and *S. aureus* at low oxygen levels (Paster *et al.* 1990). The use of vacuum packaging in combination with oregano EO was shown to have a synergistic effect on the inhibition of *L. monocytogenes* and spoilage flora on beef fillets. In addition, oregano EO was found to be more effective in meat samples packed under vacuum using low-permeability films when compared to aerobically stored samples, and samples packaged under vacuum with high permeability films (Tsigarida *et al.* 2000). Skandamis *et al.* (2002) found that oregano EO delayed microbial growth and suppressed final counts of spoilage microorganisms in minced beef stored under a modified atmosphere (40% CO₂, 30% N₂ and 30% O₂), whereas no inhibition of microbial growth was seen in beef packaged in air. Studies on fresh chicken found that MAP (70% CO₂/30% N₂) in combination with oregano EO had a clear inhibition on total viable cell counts, and that the combination of the 1% oregano EO with MAP resulted in a shelf-life of more than 20 days (Chouliara *et al.* 2007). Guillén *et al.* (2007) found that the use of MAP combined with thymol, eugenol and carvacrol to table grapes, significantly improved weight loss, colour changes and softening. Very little work has been done on vegetable products, therefore, work on the combined effects of gas atmosphere and EOs against *Listeria* on fresh-cut vegetables is needed.

1.18 Other plant antimicrobials

Antimicrobial activities of fresh herbs and spices are well known. Yano et al. (2006), found that basil, clove, garlic, horseradish, marjoram, oregano, rosemary, and thyme herbs were effective against *Vibrio parahaemolyticus*, and that rosemary, clove, marjoram and oregano herbs were effective against *E. coli*. Pandit and Shelef (1994) found that rosemary herb (> 0.5% w/v) had listericidal properties. Shelef et al. (1980) found that a concentration of 0.3% of either sage or rosemary herb in growth media inhibited growth of 21 Gram positive organisms, 9 of which were enteropathogenic. While the antibacterial effects of EOs are widely studied, further work is needed on the effects of fresh herbs.

Some fruit and vegetable tissues have naturally occurring antimicrobials that provide varying levels of protection against pathogens. Beuchat and Doyle (1995) found that treatment of lettuce with carrot juice retarded the growth of *L. monocytogenes* at 12°C but did not control growth at 20°C. Other studies by Beuchat and Brackett (1990) found that very small populations or no viable *L. monocytogenes* cells were detected on shredded carrots compared to whole carrots after treatment. Most authors have attributed the anti-listerial activity to intrinsic factors of carrot tissue, suggesting that naturally occurring antimicrobials or phytoalexins in cellular and vascular fluids may be released as a result of rupturing carrot cells, and have a lethal effect. Nguyen-the and Lund (1991) demonstrated a rapid fall in viable counts of *L. monocytogenes* during the first three days of storage on ready-to-use carrot pieces, and further tests found that a contact time of 90 minutes between carrot slices and a suspension of *L. monocytogenes* reduced the number of viable cells by three log cycles at 4°C. Other studies suggested that oxygen was necessary for the anti-listerial activity of carrot, and that the anti-listerial effect was only inhibited when anaerobic conditions

were applied (Nguyen-The and Lund 1992). Some reports suggest that the indigenous microflora of carrots might exhibit antagonistic activity against *L. monocytogenes* (Liao 2007). However, Noriega *et al.* (2010) found that the anti-listerial activity had to be attributable to intrinsic physiological factors of carrots and not to the antagonistic effect of the indigenous microflora, since the experiments were carried out on sterile carrot discs.

1.19 Thesis objectives

Many studies have been carried out on the effectiveness of EOs, showing them to have good antimicrobial properties. However, many questions still remain. While modified atmosphere packaging has been utilised in the fresh-cut vegetable industry for a number of years, and EOs have been shown to have strong anti-listerial effects, little work has been done on their combined effects.

The first objective of this study was to examine the combined effects of EOs (thyme, rosemary, oregano, marjoram, basil and fennel) and atmosphere on survival and growth of different *Listeria* strains on a model vegetable medium. The anti-listerial effectiveness of the EOs was studied in combination with storage atmospheres (Air, 5% CO₂/2% O₂/93% N₂ and 20% CO₂/1% O₂/79% N₂) typical of fresh-cut systems. Storage temperatures of 4 and 8°C were investigated.

Although EOs perform well in antibacterial assays *in vitro*, there are still relatively few studies in real food systems. Some work has been carried out in meat, fish and dairy products but little work has been done on fruits and vegetables (Burt 2004).

The second objective was to examine the effectiveness of EOs, fresh herbs and plants against *L. monocytogenes* and the natural microflora of fresh-cut vegetables. Interactions with product type were studied in order to examine whether components of produce may enhance the effectiveness of these natural plant antimicrobials.

While EOs have been shown to have good anti-listerial effects, studies have shown that they have negative organoleptic effects. Work by Gutierrez *et al.* (2008a) showed that oregano combined with marjoram, thyme or basil had an additive effect against *E. coli*, and that mixtures of marjoram or thyme also displayed additive effects in combination with basil, rosemary or sage against *L. monocytogenes*.

The third objective was to examine the effects of using EOs in lowered concentrations and also in mixtures at lower concentrations in an attempt to exploit their antimicrobial effects without negative sensory effects.

A significant discovery during this project was that crushed rosemary herb had a much greater anti-listerial effect than rosemary EO. This suggests that components in the herb may be critical for the anti-listerial effect and that some of these may be lost in the preparation of EOs. While the chemical composition of rosemary EO is well documented, components vary depending on the variety of the herb, season, country of origin and method of extraction.

The fourth objective of this study was to compare methods of extraction to see whether certain extraction methods or pretreatments might be more effective and perhaps replicate the stronger anti-listerial effect observed with the crushed fresh herb. Gas chromatography-mass spectrometry was used to

determine the chemical composition of rosemary herb and rosemary EO/extracts, to see whether their components and concentrations differed, and to relate this to differences in anti-listerial effects.

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Chapter 2

Effects of essential oil treatment, gas atmosphere and storage temperature on survival of *Listeria monocytogenes* in a model vegetable system

2.1 Abstract

Natural plant antimicrobials such as plant EOs are generally recognised as safe (GRAS), and may be useful for controlling pathogenic bacteria on vegetable produce. The anti-listerial properties of EOs (thyme, oregano and rosemary), in combination with varying storage atmospheres (air, 5% CO₂/2% O₂/93%N₂ and 20% CO₂/1% O₂/79%N₂) and temperatures (4 and 8°C), were examined using a gas flow-through model vegetable system. The antimicrobial effects of the EOs varied depending on the oil, the *Listeria* strain and species, the method of application and the storage conditions tested. Using the disc diffusion assay, the anti-listerial effectiveness of the oils was in the order of: thyme EO > oregano EO > rosemary EO. Volatiles released from the EOs resulted in very small anti-listerial effects, indicating that the oils needed to be in direct contact with cultures in order to be effective. There was a strain and species variation effect observed, with *L. innocua* NCTC 11288 exhibiting the strongest resistance to the EOs, and *L. monocytogenes* NCTC 7973 being the most sensitive strain. In addition, the effectiveness of the EOs was influenced by storage atmosphere and temperature conditions. The application of the EOs in combination with a gas atmosphere of 20% CO₂/1% O₂/79%N₂ had the greatest anti-listerial effect, suggesting that high CO₂ atmospheres enhanced the anti-listerial properties of EOs. Lowering the storage temperature from 8 to 4°C improved the anti-listerial activity of thyme oil. In conclusion, the results demonstrate that thyme and oregano EOs display strong inhibitory effects against *Listeria*, and increasing CO₂ levels and reducing storage temperatures further enhance these anti-listerial effects.

2.2 Materials and Methods

2.2.1 Preparation of inocula

The *Listeria* strains used to assess the anti-listerial properties of the essential oils were as follows: two *L. monocytogenes* reference strains (i.e. NCTC 11994 and NCTC 7973), two *L. monocytogenes* strains which had been previously isolated from packaged vegetables (*L. monocytogenes* IL 323 and CW 329) and one *L. innocua* strain (i.e. NCTC 11288). *Listeria* strains were maintained at -20°C in tryptone soya broth (TSB, Oxoid CM129, Fannin Healthcare) supplemented with 20% (v/v) glycerol. Resuscitation was achieved by thawing cultures at room temperature (17 to 22°C) followed by loop-transfer in TSB (10 ml) and overnight incubation at 37°C . Cultures were then centrifuged (5000 g, 15 min), resuspended, mixed (for some experiments) and diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to the desired concentration of approximately 10^6 CFU/g.

2.2.2 Preliminary antimicrobial screening (Disc Diffusion Assay)

The EOs used in this work were thyme, oregano, rosemary, marjoram, basil and fennel (Guinness Chemicals Ltd., Ireland). The EOs were screened for antimicrobial activity against the five different *Listeria* strains using the agar disc diffusion method (NCCLS, National Committee for Clinical Laboratory Standards, 1997). In brief, cultures (100 μl) were surface spread onto Brain Heart Infusion agar plates (Oxoid, BO0961T). Sterile filter paper discs (Whatman No. 1, 6 mm in diameter) soaked in 10 μl of undiluted EO were applied to the centre of each inoculated plate, and the plates stored at 4°C for 2 hours. The plates were then incubated overnight at 37°C , and the diameters of the resulting zones of inhibition were measured in millimetres. Each assay

was carried out in triplicate and the average result and standard deviation calculated. The results indicated in Figure 2.1 represent the anti-listerial effects of the EOs. The scale of measurement was the following: ≥ 20 mm zone of inhibition indicates strong inhibition; 12- <20 mm zone of inhibition indicates moderate inhibition; and <12 mm indicates little or no inhibition.

2.2.3 Preparation of the surface model medium

A model vegetable agar medium was prepared using surface washings from shredded lettuce. To prepare the model medium, 3 x 60 g batches of shredded lettuce leaves were washed in 250 ml of sterile distilled water by gentle shaking. This wash water was filtered aseptically using sterile 0.45 μm nitrocellulose filters. For final preparation of the agar medium, the wash water was heated to 50°C, and two parts were mixed with one part 3% agar (Oxoid, Agar bacteriological, CML11). This gave a final concentration of 1% agar. Approximately 10 ml aliquots were poured into 55 mm diameter petri dishes (Sterilin, UK) and allowed to solidify. The surface model medium was then inoculated with the desired concentration of bacteria ($\sim 1 \times 10^5$ CFU/ml).

2.2.4 Effects of application method

Diluted cultures of *L. innocua* (50 μl) were surface spread onto the model medium. The anti-listerial effects of EOs and volatiles from EOs were determined during storage using the agar disc diffusion assay and a modified disc volatilisation method (Lopez *et al.*, 2005), respectively. For the agar disc diffusion assay, a sterile disc, impregnated with 10 μl of EO (rosemary, thyme and oregano EO) was placed in the centre of each 55 mm inoculated plate. The plates (in duplicate) were then placed into

2 L flasks (kilner jars) at 4°C or 8°C and microbiological analysis was performed during storage (as described below).

In order to examine the effects of volatiles from the EOs on *Listeria* a flow-through model system was used. Inoculated plates, each in duplicate, were placed into the 2 L flasks, along with an open jar containing 20 ml of EO (thyme, oregano or rosemary). Each flask was sealed with a wide silicone bung (AGB Scientific Ltd, Ireland, bottom diameter 86 mm, top diameter 96 mm, height 54 mm). The wide neck enabled the plates to be easily placed into the flasks and the gas tight seal had been tested under pressure. In addition, the anti-bacterial activity of volatiles from EOs was evaluated by the modified disc volatilisation method (Lopez *et al.*, 2005). Briefly, 10ml of model medium were poured into 55 mm diameter petri dishes (Sterilin, UK) and 3 ml into the lid and allowed to solidify. The EOs were added to 3 sterile paper discs (6 mm) and placed on the agar in the lid of each petri dish. The agar medium in the petri dish lid served as a sealing and prevented adsorption of EOs onto the plastic material of the petri dish cover.

2.2.5 Experimental design

A flow-through surface model system was used to study the effects of gas atmospheres on the growth of *L. monocytogenes*. Inoculated plates, each in duplicate were placed into the 2 L flasks. The flasks were sealed, connected up to one another and flushed continuously (60 ml min⁻¹) with the desired gas mixture. The gas atmospheres studied were: (a) air, (b) 5% CO₂, 2% O₂, 93% N₂ and (c) 20% CO₂, 1% O₂, 79% N₂. The agar plates were stored in the gas atmospheres at either 4 or 8°C for 9 days

2.2.6 Microbiological analyses

Listeria monocytogenes populations were determined by analysing samples of the model agar medium in duplicate as follows: all of the agar in the plate was aseptically transferred into stomacher bags. Samples were homogenised for 2 min, with 90 ml PBS, using a Seward laboratory stomacher (Stomacher 400, AGB Scientific, Ireland). Serial dilutions of each homogenised sample were made in 10 ml PBS and were plated on *Listeria* selective agar plates (Oxoid, CM856). Counts of *Listeria* were determined on LSA plates after incubation for 48 h at 37°C.

2.2.7 Statistical analysis

All experiments were carried out twice. Duplicate plates of each sample were serially diluted and plated in duplicate at each analysis time. Reported populations therefore represent the means of four values. Figures 2-6 show the means \pm standard deviation. Statistical differences were determined using a Two-way ANOVA with Bonferroni's post test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA).

2.3 Results

2.3.1 Preliminary antimicrobial screening

Preliminary screening of the antimicrobial activity of six EOs against five *Listeria* strains was performed using the agar disc diffusion assay. The anti-listerial properties of plant EOs varied depending on the oil (Figure 2.1); thyme, oregano and marjoram EOs showed the highest anti-listerial activities, rosemary EO displayed moderate antimicrobial properties, and basil and fennel EOs displayed little or no antimicrobial activity against the strains tested. In terms of anti-listerial effectiveness: thyme EO > oregano EO > marjoram EO > rosemary EO > basil EO > fennel EO.

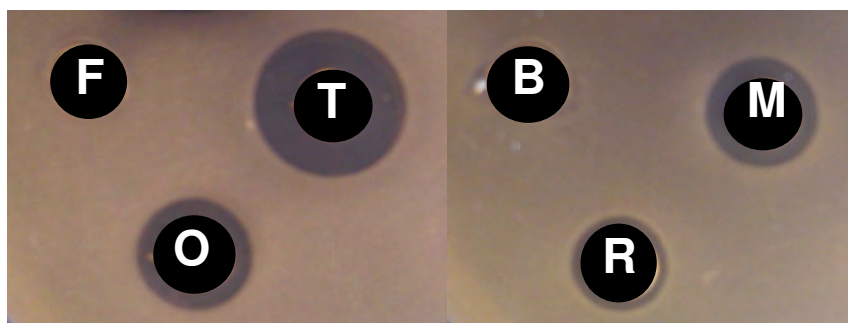


Figure 2.1: Zones of inhibition showing anti-listerial activity for a number of plant EOs.

Fennel (F), Thyme (T), Basil (B), Marjoram (M), Oregano (O) and Rosemary (R)

Based on these data, thyme, oregano and rosemary oils were the oils selected for further experiments.

2.3.2 Effect of application method

The effectiveness of EOs also depended on the method used to introduce the oil.

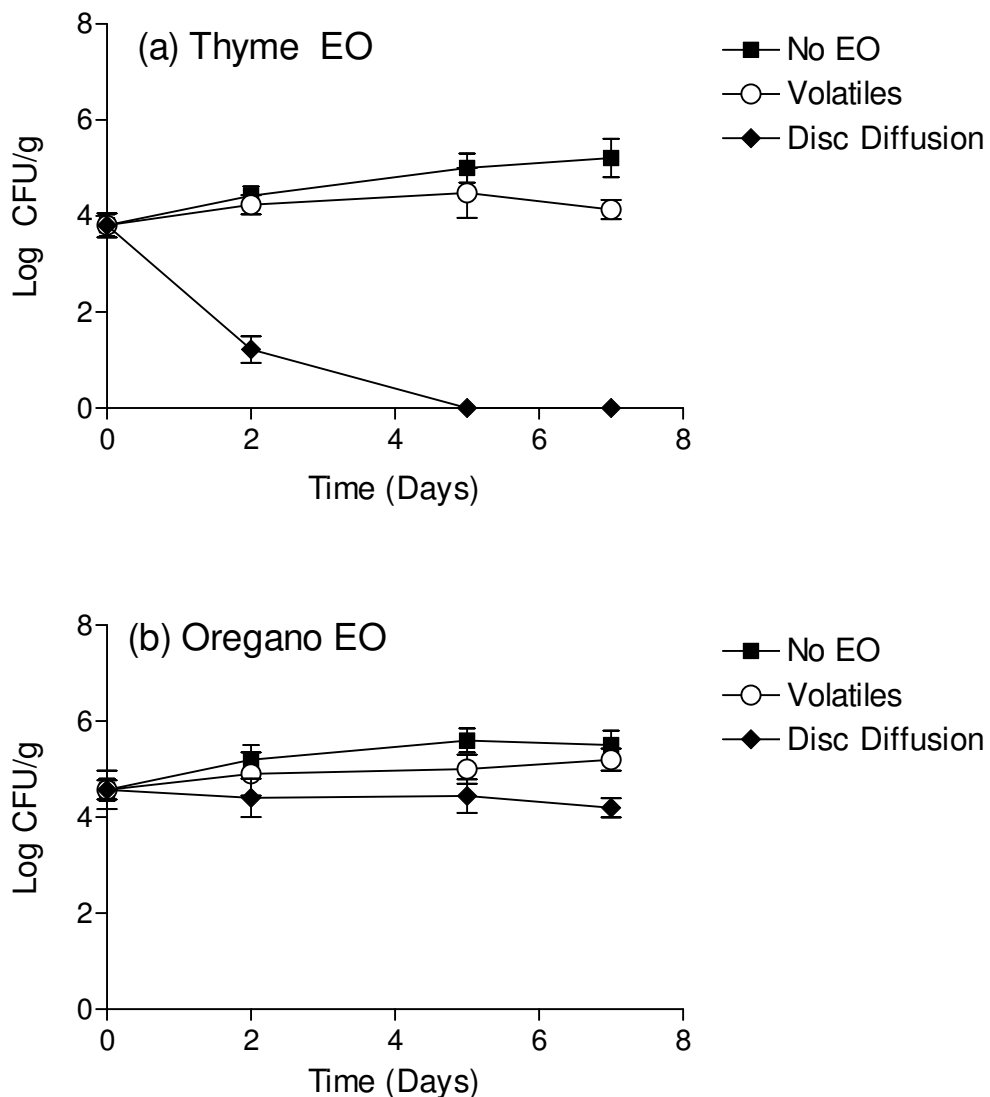


Figure 2.2: Effects of application method of (a) thyme EO and (b) oregano EO on survival of *L. monocytogenes* IL323 on a model vegetable medium during storage at 4°C. Reported populations are means of 4 values. Error bars show s.d.

Volatiles released from thyme, rosemary or oregano oils generally resulted in only small growth inhibitory effects that were not as strong as when the oils were in direct contact with *Listeria* cultures (Figure 2.2). This suggested that the EOs needed to be in direct contact with *Listeria* cultures in order to be effective.

2.3.3 Effects of storage atmosphere on *Listeria* counts

The effects of storage atmosphere and time on the survival and growth of *Listeria* IL 323 on the model vegetable medium during storage at 4°C are shown in Figure 2.3. During storage in air, *Listeria* populations reached a final population density of $\sim 1 \times 10^6$ CFU/g by the end of storage. Increasing CO₂ concentrations to 5% had no inhibitory effect on the survival and growth of *L. monocytogenes* on the model medium. An atmosphere of 20% CO₂ did not reduce growth of *Listeria* until the end of storage when final population densities were 1 log cycle lower. Similar results were found for all strains tested.

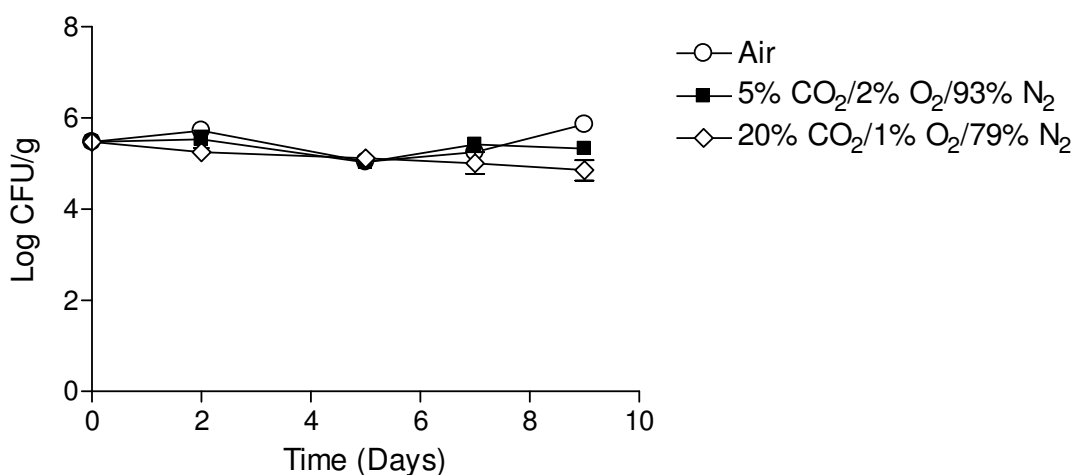


Figure 2.3: Effects of gas atmosphere on the survival and growth of *Listeria monocytogenes* IL323 on a model vegetable medium during storage at 4°C. Reported populations are means of 4 values. Error bars show s.d.

2.3.4 Effects of EOs in combination with varying gas atmospheres

The effectiveness of the EOs was influenced by gas atmosphere conditions. Figure 2.4 shows the effects of rosemary, oregano, thyme EO on *L. monocytogenes* in air, 5% CO₂ and 20% CO₂.

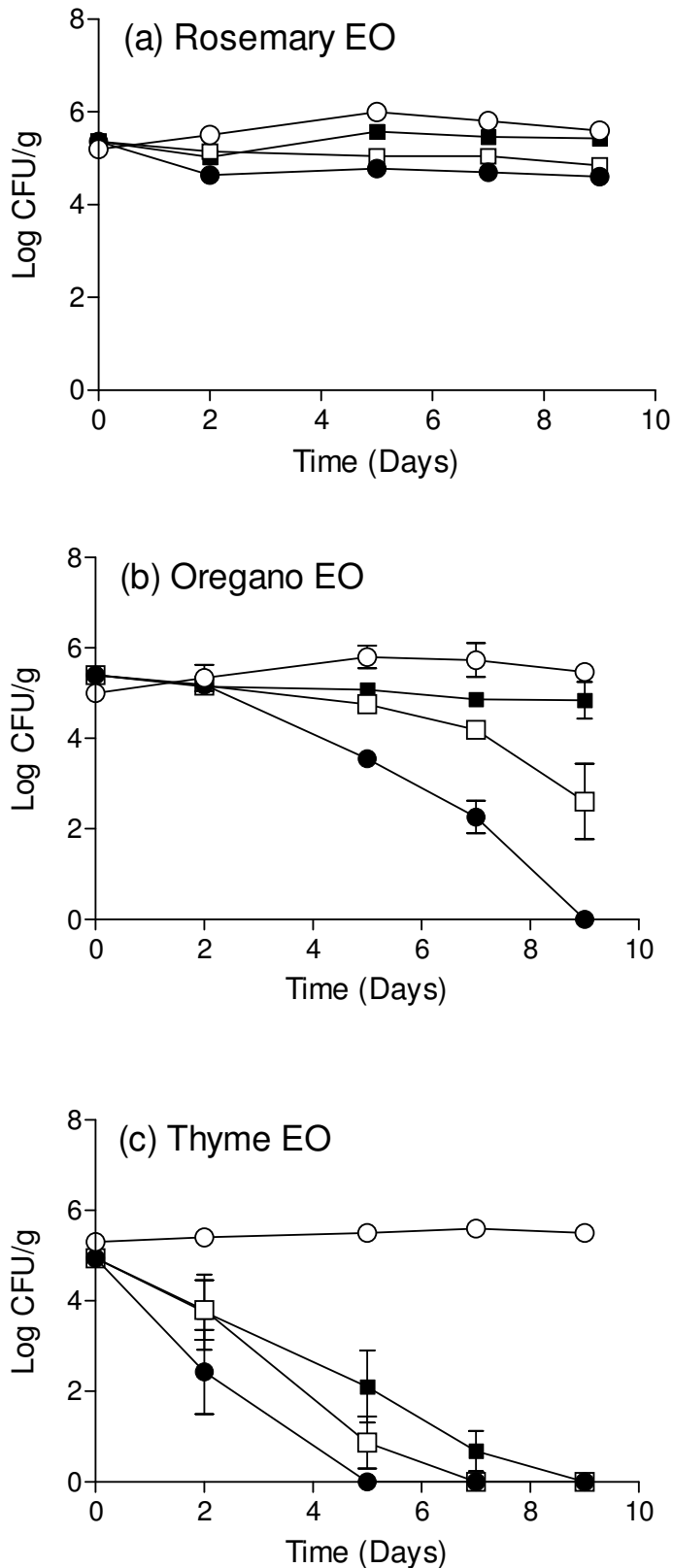


Figure 2.4: Effects of (a) rosemary EO, (b) oregano EO and (c) thyme EO on survival of *L. monocytogenes* on a model vegetable medium during storage under different gas atmospheres at 4°C. Reported populations are means of 4 values. Error bars show s.d. (○ No EO; ■ air; □ 5% CO₂/2% O₂/93% N₂; ● 20% CO₂/1% O₂/79% N₂)

The anti-listerial effects of all the EOs were increased at reduced levels of O₂ and increased levels of CO₂. This is clearly evident in Figures 2.4(b) and 2.4(c) where increasing the CO₂ level from 5 to 20%, enhanced the anti-listerial effectiveness of oregano and thyme oils. Thyme oil had a particularly strong anti-listerial effect, and in air, there were no viable *Listeria* cells detected by Day 9 of storage. In 5% CO₂ and 20% CO₂, no viable cells were detected by Day 7 and by Day 5 of storage, respectively, showing the synergy between thyme oil and gas atmosphere. The application of all EOs in combination with a gas atmosphere of 20% CO₂/1% O₂/79% N₂ had the greatest anti-listerial effect, suggesting that increasing CO₂ levels enhanced the anti-listerial properties of the EOs.

2.3.5 Effects of gas atmosphere and oregano oil on growth of *Listeria*

Figure 2.5 shows the effect of oregano oil and gas atmosphere on survival and growth of different *Listeria* strains. Again, increasing the CO₂ levels greatly increased the effectiveness of oregano oil for all strains, with gas levels of 20% CO₂ having the greatest antimicrobial effect. There were major differences between the *Listeria* strains tested, with some strains exhibiting stronger resistance to the EO. *L. innocua* NCTC 11288 generally appeared to be the most resistant strain, while strains NCTC 7973 and NCTC 11994 were more susceptible to oregano oil (Figure 2.5). Similar strain variation results were observed when rosemary and thyme oil were tested.

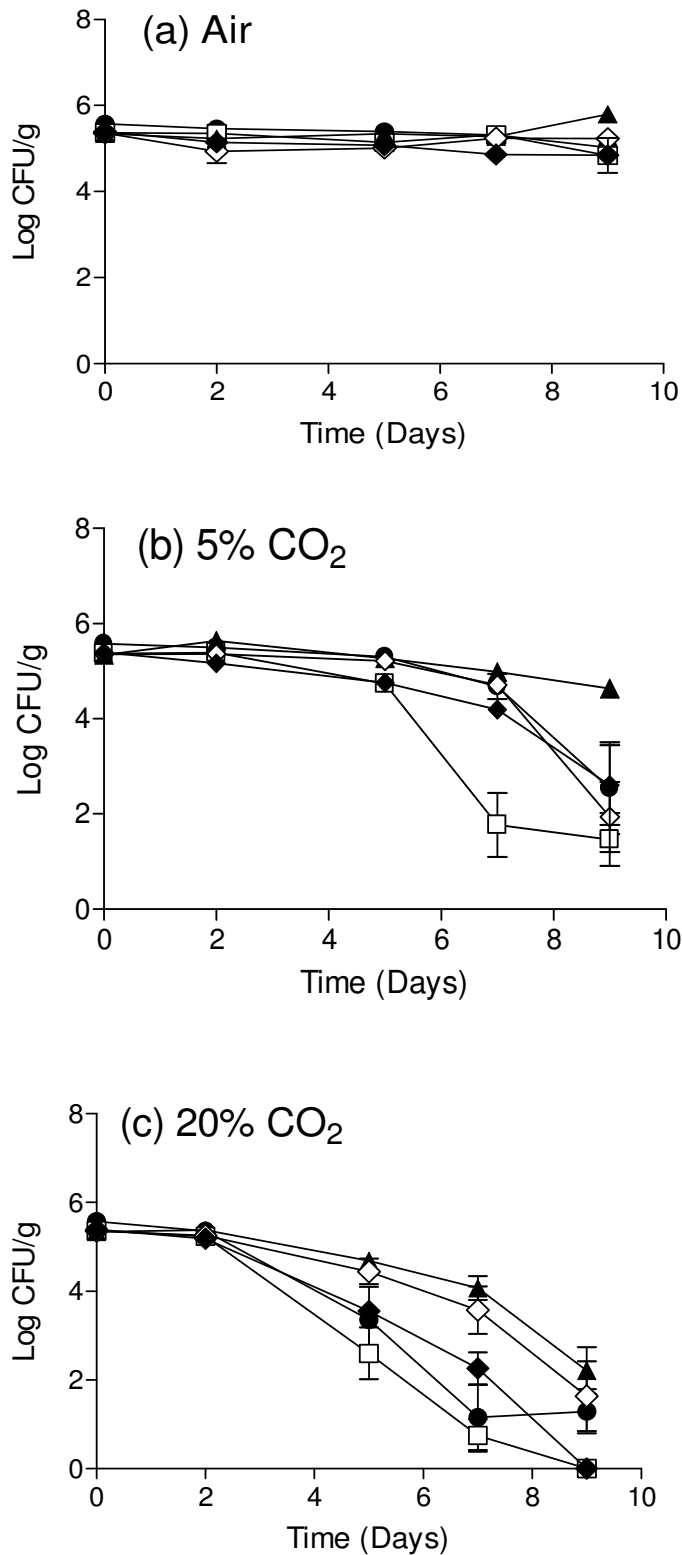


Figure 2.5: Effects of oregano EO on growth of different *Listeria* strains on a model vegetable medium during storage at 4°C in: (a) Air, (b) 5% CO₂/2% O₂/93% N₂, (c) 20% CO₂/1% O₂/79% N₂. Reported populations are means of 4 values. Error bars show s.d. (▲ *L. innocua* NCTC 11288; ● *L. monocytogenes* strains NCTC 11994, □ NCTC 7973, ◇ IL 323, ◆ CW 329)

2.3.6 Effects of storage temperature on effectiveness of EOs against *Listeria*

Figure 2.6 shows the effects of storage temperature (4°C and 8°C) on the effectiveness of thyme oil on survival and growth of two different *Listeria* strains during storage.

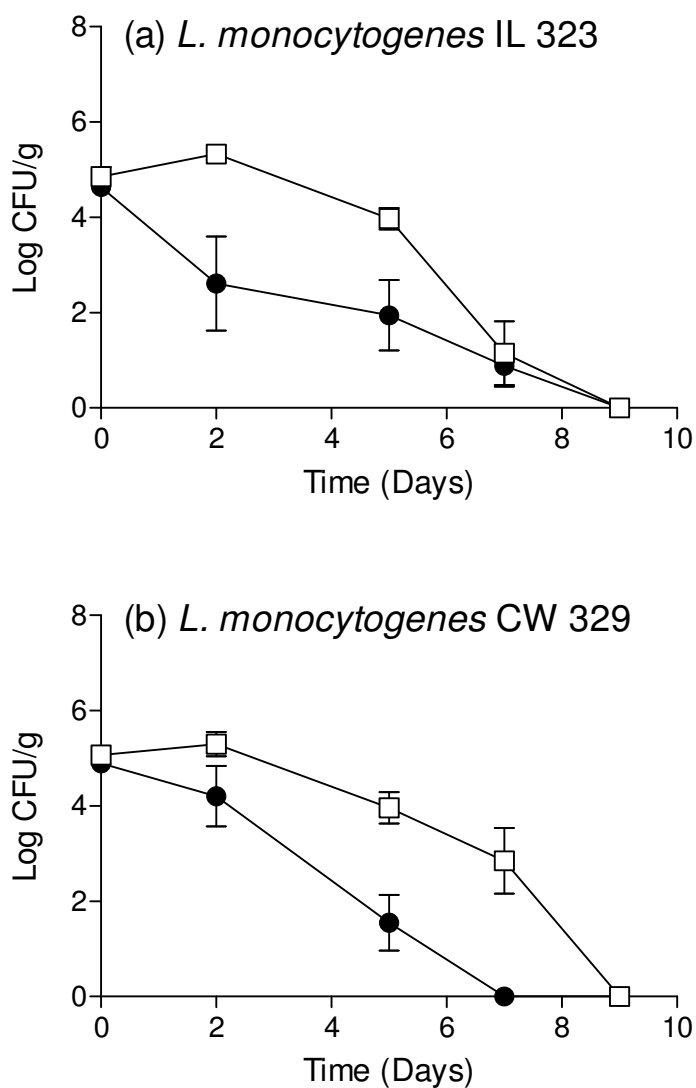


Figure 2.6: Effects of storage temperature (4°C ●, 8°C □) on the effectiveness of thyme EO against *Listeria monocytogenes* strains (a) IL 323 and (b) CW 329 during storage on a model vegetable medium. Reported populations are means of 4 values. Error bars show s.d.

For all strains examined, reducing the storage temperature from 8° to 4°C significantly increased ($P<0.05$) the anti-listerial effect of thyme oil between Day 0 and 5. Therefore, the lower the storage temperature (4°C) the greater the anti-listerial effect of thyme oil.

2.4 Discussion

The work reported here demonstrates that antimicrobial effects of EOs varied depending on the oil, the *Listeria* strain and species, the method of application and the storage conditions tested. In this study, the addition of rosemary EO had little or no inhibitory effect on *Listeria* survival and growth. Similar results were found by Singh *et al* (2003), who showed that rosemary was not very effective against *L. monocytogenes*, and that levels as high as 62.5-125.0 ml/L were needed to produce a clear zone of inhibition compared to other EOs. However, other studies demonstrated that *L. monocytogenes* was sensitive to rosemary EO, with a bacteriocidal concentration of only 0.1 µl/ml needed to inhibit growth (Smith-Palmer *et al.* 1998). Lis-Balchin and Deans (1997) found that rosemary EO was effective against 16 out of 20 different strains of *Listeria monocytogenes*, illustrating a strain variation effect. Rosemary oil in broth cultures produced listeristatic effects after 2 hours of incubation at concentrations of 10 µl/ml, however, these results were only temporary and cell numbers increased after 48 h of incubation (Pandit and Shelef 1994).

The antimicrobial activity of essential oils is assigned to a small number of terpenoid and phenolic compounds and chemical analysis of these oils have shown that the principal active compounds are carvacrol, thymol, citral, eugenol, 1-8 cineole, limonene, pinene, linalool and their precursors (Viuda-Martos *et al.* 2008). In this study, oregano and thyme EOs were found to have much greater anti-listerial effects than rosemary oil. This is in accordance with Burt (2004) who noted that in general, EOs possessing the strongest anti-bacterial properties contain a high percentage of carvacrol and/or thymol, such as oregano or thyme. These active compounds appear to act on the cell membrane, rendering it more permeable (Lambert *et al.* 2001). Dorman and Deans (2000) demonstrated that the oil with the widest spectrum of activity was

found to be thyme EO followed by oregano, and that the component with the widest spectrum of activity was thymol followed by carvacrol. Previous studies have shown thyme oil is a very effective antimicrobial; Smith Palmer *et al.* (1998) showed that thyme EO had a bacteriostatic effect against *Listeria* at a concentration of 0.02%. In addition, thyme oil was found to be the most inhibitory EO against all strains of *L. monocytogenes* in a study carried out by Singh *et al.* (2003).

Some studies have been carried out on the effects of application method on the effectiveness of EOs. Suhr (2003) found that thyme oil had the best anti-fungal effects when applied directly to a medium than when applied as a volatile. Lopez *et al.* (2005) found that basil and rosemary oil had good anti-bacterial properties when in direct contact with bacteria, however rosemary and basil EOs showed no inhibitory activity towards any of the microorganisms in the vapour phase test. In another study, Lopez *et al.* (2007) also found that thyme oil was ineffective against *L. monocytogenes* in its vapour phase. This corresponds with results found in this study where only a slight reduction in *Listeria* counts was seen when oregano and thyme volatiles were used in comparison with the control.

Many studies have shown that the effectiveness of EOs also varies depending on the strain of *Listeria* used. Lis-Balchin and Deans (1997) found that thyme oil was effective against 15 strains of *L. monocytogenes* and rosemary was effective against 16 strains out of 20 tested, showing that some strains are more resistant to EOs than others. In another study, bacterial strains were found to respond differently to various EOs (Singh *et al.* 2003). In this study, there was a strain and species variation effect observed, with *L. innocua* NCTC 11288 exhibiting the strongest resistance to the EOs and *L. monocytogenes* NCTC 7973 being the most sensitive strain. However, further

studies are needed to determine the molecular basis for the strain variation effects observed.

The data presented show that increasing the CO₂ levels to either 5% or 20% had little or no inhibitory effect on *Listeria* survival and growth, in comparison with air. Similar results were found by Francis and O'Beirne (1998), who observed that at CO₂ levels of 20% the final population densities were not different from samples stored in air. It is well known that modified atmosphere systems can be an effective means to inhibit microbial growth and improve the shelf-life of a food product, however, other antimicrobial methods in combination with modified atmosphere packaging (MAP) are needed to reduce or eliminate foodborne pathogens. In this study, the anti-listerial effectiveness of EOs was influenced by gas atmosphere and the use of high CO₂ gas atmospheres increased the anti-listerial effects of the oils. In air, thyme oil was shown to have a strong anti-listerial effect, however, this effect was enhanced when CO₂ levels were increased to 5% or 20%. Similarly for oregano, increasing CO₂ levels to 5% resulted in a significant reduction in growth of all *Listeria* strains, and increasing to 20% CO₂ resulted in a rapid decline in *Listeria* populations from Day 2 onwards with no viable cells detected by the end of storage. These results are in accordance with Tsigarida *et al.* (2000) who showed that oregano oil eliminated *L. monocytogenes* under vacuum packaging and in a modified atmosphere of 40% CO₂/30% O₂/30% N₂, while no major anti-listerial effects were seen in air. Furthermore, EOs in combination with a gas atmosphere of 40% CO₂/30% O₂/30% N₂ were found to be effective against *S. typhimurium* (Skandamis *et al.* 2002). In addition, clove and cinnamon oils were found to be most effective in inhibiting the growth of microorganisms when used in combination with low oxygen levels (<0.05%) and high CO₂ concentrations (40%) (Matan *et al.* 2006).

Listeria monocytogenes is one of the few foodborne pathogens capable of growing at refrigeration temperatures (Carlin *et al.* 1995). Many studies have shown that even mild temperature abuse can increase growth of *Listeria* species (Francis and O' Beirne 1997, Harrison *et al.* 2000), where it was found that increasing the temperature from 4°C to 8°C significantly increased the growth of *L. monocytogenes*. In this work, the effectiveness of thyme EO was greater at 4°C than at 8°C with a difference of between 2-4 log cycles seen for some strains, showing the importance of maintaining low temperature control. This is in accordance with Smith-Palmer *et al.* (1998) who found that thyme oil retained a lower bacteriostatic and bacteriocidal concentration at 4°C compared to at 35°C.

In summary, thyme and oregano oils displayed powerful antimicrobial effects against *Listeria* survival and growth, while rosemary oil showed small anti-listerial effects. Increasing CO₂ levels to 20% and decreasing the storage temperature to 4°C further enhanced the antimicrobial effectiveness of EOs. The use of EOs in combination with MAP and refrigeration temperatures may be useful to improve the microbiological safety of fresh-cut packaged produce.

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Chapter 3

**The effects of natural plant antimicrobials on *Listeria*
survival and growth on fresh-cut lettuce**

3.1 Abstract

The effectiveness of a number of commercial EO preparations and of fresh shredded herbs were examined against *Listeria innocua* on modified atmosphere packaged (MAP) fresh-cut lettuce. In terms of anti-listerial effectiveness: thyme EO > oregano EO > rosemary EO > basil EO. Thyme EO had a major anti-listerial effect; however, no anti-listerial effects were seen when fresh thyme herb or thyme EO volatiles were examined. Similar results were found for oregano EO and oregano herb, whereas basil EO and herb showed little or no anti-listerial effects. While rosemary EO showed little anti-listerial effects, adding fresh shredded rosemary herb significantly reduced *Listeria* populations ($P < 0.05$) on lettuce during stomaching. Applying undiluted EOs directly to the lettuce had a detrimental effect on the appearance of the lettuce. However, the addition of fresh thyme improved sensory appearance scores. The results demonstrate that thyme EO, rosemary herb and oregano EO displayed strong antimicrobial effects against *Listeria* on MAP packaged lettuce. By contrast, basil and rosemary EO and thyme, oregano and basil fresh herbs displayed no anti-listerial effects.

3.2 Materials and Methods

3.2.1 Preparation of fresh-cut MAP lettuce

Heads of Iceberg lettuce were purchased from a local fruit and vegetable supplier (Richardson's Foods, Limerick) on the day of testing. The outer and damaged leaves as well as the core of the heads were removed and discarded. Inner leaves were sliced manually to approximately 10mm strips using a sharp stainless steel knife.

Lettuce strips (25 g) were transferred into bags (18 cm x 10 cm), composed of 35 µm oriented polypropylene packaging film (Cannings Packaging Ltd., Dublin, Ireland), which were later heat-sealed. According to the manufacturer, this film had a permeability to O₂ of 1,200 ml/m²/day/atm and to CO₂ of 4,000 ml/m²/day/atm.

3.2.2 Preparation of inocula

For all EO, herb and EO volatile treatments *Listeria innocua* NCTC 11288 was used. *L. innocua* has been used successfully as a model organism for *L. monocytogenes* in previous studies (Omary *et al.* 1993, Francis and O' Beirne 1998). The organisms have similar growth and biochemical characteristics (Omary *et al.* 1993) and work carried out by Francis and O' Beirne (1997) has shown that *L. innocua* strain 11288 is a valid model for *L. monocytogenes* behaviour on minimally processed lettuce. The cultures were maintained at -20°C in tryptone soya broth (TSB) supplemented with 20% (v/v) glycerol, and were grown in tryptone soya broth (10 ml TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) at 35°C for 24 h. Cultures were then centrifuged (5000 g, 15 min), resuspended in PBS, mixed and diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow for contamination of vegetables at initial levels of approximately 10⁵-10⁶ CFU/g of vegetable.

3.2.3 Inoculation of vegetables

After appropriate dilutions, 100 µl aliquots of the cell suspension were distributed uniformly over the vegetables contained within each of the packages. Following inoculation, the antimicrobial treatments were applied to the vegetables and the bags were heat-sealed using an impulse bench-top heat sealer (Relco, UK Ltd., England). After sealing, packs were gently shaken to assist distribution of the inoculum.

3.2.4 Effects of antimicrobial treatments and storage

The effectiveness of the plant antimicrobial treatments on the survival and growth of *Listeria* were examined as described below:

1. Essential Oils

Thyme, oregano, basil and rosemary EOs (CO₂ supercritical fluid extracts) were obtained from Guinness Chemicals Ltd. (Portlaoise, Ireland). The oils (0.5 g) were sprayed over the vegetable products (25 g) in a fine mist using a spray bottle.

2. Fresh herbs

Fresh thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*), basil (*Ocimum basilicum*) and rosemary (*Rosmarinus officinalis*) herbs were obtained from a local supplier. The fresh herbs were cut into approx 10mm pieces using a sterile sharp knife and 5 g quantities were added to samples of 25 g portions of lettuce and mixed.

3. Aroma volatiles

Essential oils were placed in a separate compartment within the sealed vegetable packs. A 20 mm gap was open at the top of the compartment to allow the volatiles to surround the lettuce in the packs (Figure 3.1). As volatiles were found to be ineffective, this experiment was not carried out for all the EOs tested.

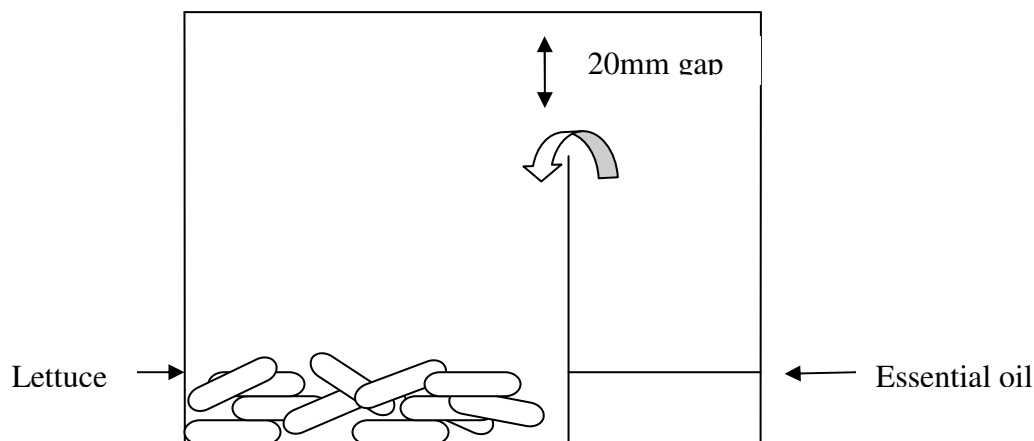


Figure 3.1: Vegetable packs (oriented polypropylene) used to measure the antimicrobial effectiveness of essential oil volatiles

3.2.5 Microbiological analyses

Microbiological analyses were carried out on Day 0 (Day of inoculation) and at regular intervals throughout the storage period (Days 2, 6, 9). At each sampling, duplicate packs each of two experiments were analysed for *Listeria* populations and total viable cell counts. The 25 g lettuce sample of each package was transferred into a stomacher bag and samples were homogenised for 2 min at high speed with 225 ml of phosphate buffer saline (PBS, Oxoid BR0014) using a Seward laboratory stomacher (Stomacher Model 400, AGB Scientific, Ireland). Serial dilutions of each homogenised sample were made in PBS and were surface spread (100 μ l/ plate) in duplicate on to appropriate media.

Populations of *L. innocua* were determined on *Listeria* selective agar (LSA, Oxoid CM856) containing a modified *Listeria* selective supplement (Oxoid SR0206) after 48 h at 35°C. A total count of aerobic mesophilic microflora was made on tryptone soya agar (TSA Oxoid CM131) after incubation at 35°C for 48 h. Following incubation, colonies were counted and results were expressed as colony forming units per gram of sample.

3.2.6 Analyses of the gaseous atmospheres inside the packages

On each of the sampling dates (Days 0, 2, 6, 9), gases within two packages for each treatment were analysed using an O₂ and CO₂ gas analyser (PBI-Dansensor, PBI Development, Denmark, Model TIA-III LV). An airtight needle attached to a flexible probe loop was inserted into packages and the readings of O₂ and CO₂ levels were recorded.

3.2.7 Evaluation of sensory appearance

Evaluation of appearance was performed on lettuce packages during storage (Days 0, 2, 6 and 9) by a 5 member panel (members of our laboratory with experience in sensory evaluation of fresh-cut vegetables). The panellists were asked to score the appearance of samples, on an 11 point scale ranging from 10 to 0, where 10 represented excellent appearance and 0 represented very poor appearance. The samples were coded (three random digits) and randomly offered to panellists. The evaluations were carried out under typical daylight conditions and at a temperature of 18 to 20°C.

3.2.8 Statistical analyses

All experiments were carried out independently in duplicate and replicated twice. Duplicate packs were serially diluted and plated in duplicate at each analysis time. Populations were reported as the means of four values \pm standard deviations. Means were compared using analysis of variance (ANOVA) followed by least significance difference testing at $P < 0.05$ level. Significant changes in sensory appearance were examined using paired-sample t-tests ($P < 0.05$).

3.3 Results

Fresh-cut MAP lettuce was inoculated with *Listeria innocua* and the effectiveness of plant antimicrobial treatments against *Listeria* was examined during storage.

3.3.1 Effects of thyme EO and fresh herb on *L. innocua* in MAP lettuce

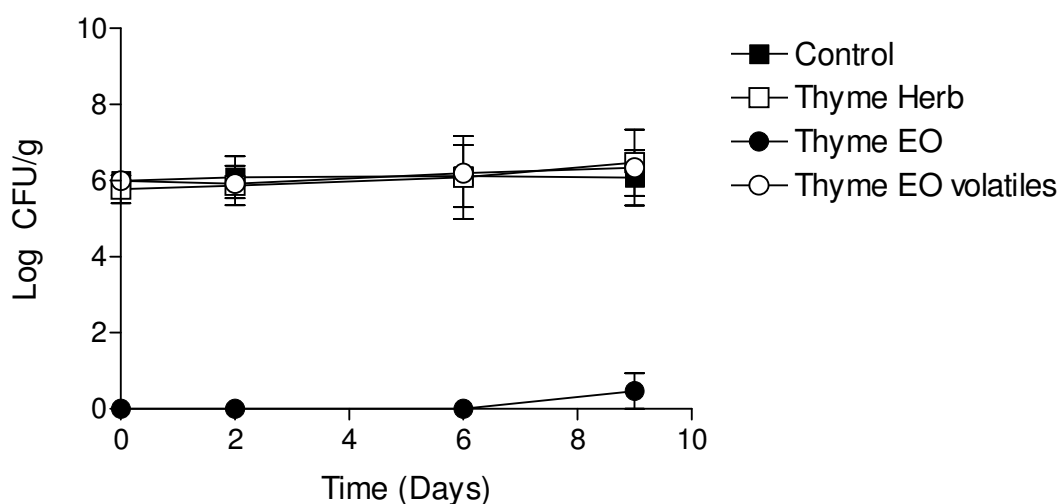


Figure 3.2: Effects of thyme (EO, EO volatiles and shredded fresh herb) on survival and growth of *Listeria innocua* on MAP fresh-cut lettuce stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 3.2 shows the effects of thyme EO, thyme EO volatiles and thyme herb on *Listeria* survival and growth during storage on MAP fresh-cut lettuce at 8°C. Thyme EO significantly reduced *Listeria* populations ($P < 0.001$) by 6 log counts with no viable cells being detected from Day 0 to Day 6 of storage. For the untreated lettuce, populations of *Listeria* remained at levels of $\sim 1 \times 10^6$ CFU/g throughout the storage period. Thyme herb and thyme EO volatiles had no inhibitory effects on *Listeria* survival and growth. Similar trends were found at 4°C (data not shown).

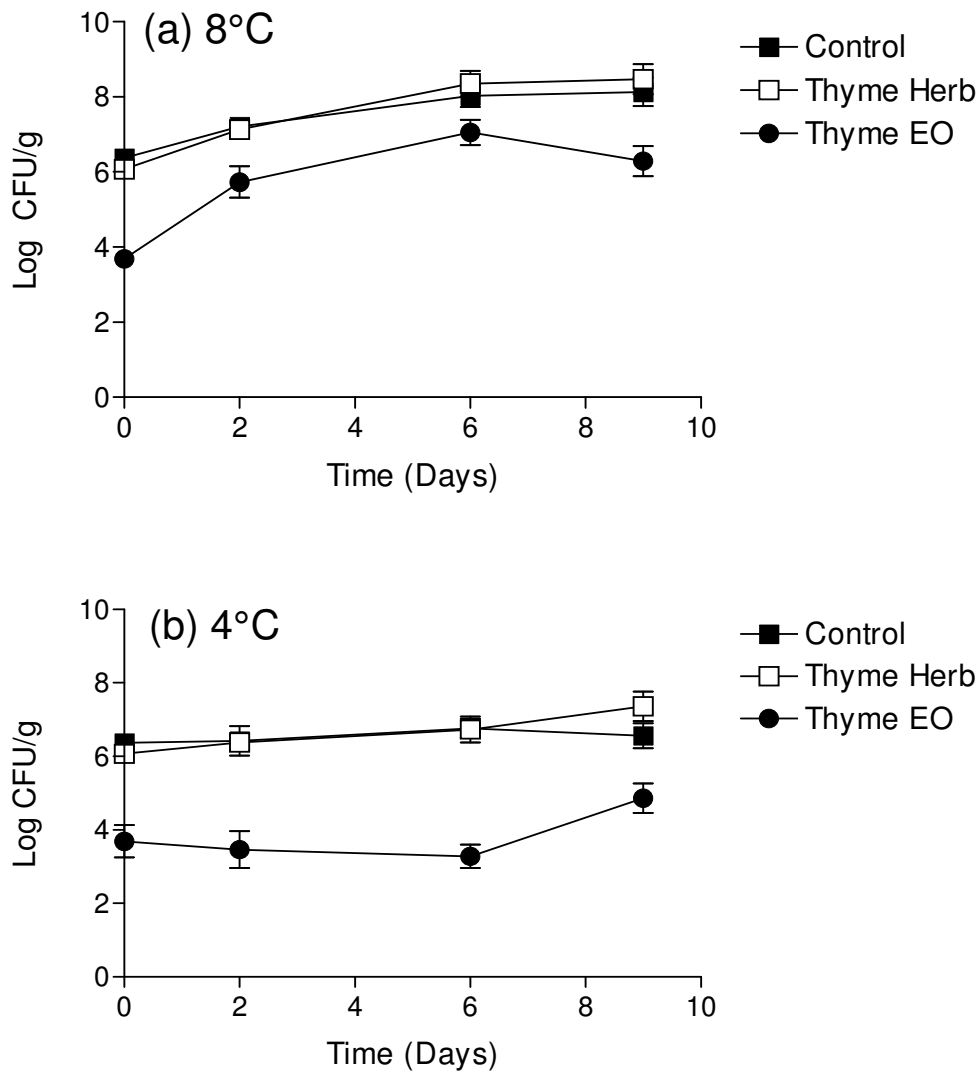


Figure 3.3: Effects of thyme (EO and shredded fresh herb) on total bacterial counts (TBCs) on MAP fresh-cut lettuce stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

In Figure 3.3, the effects of thyme on total bacterial counts on MAP fresh-cut lettuce stored at both 4 and 8°C are shown. At 8°C, addition of thyme EO reduced growth of TBCs and initial counts were 2 log cycles lower than the control. This effect remained throughout storage. Lowering the storage temperature to 4°C increased the antibacterial effect of the thyme oil on Days 2 and 6. Adding thyme herb to lettuce had no effect on TBCs at either 4 or 8°C storage.

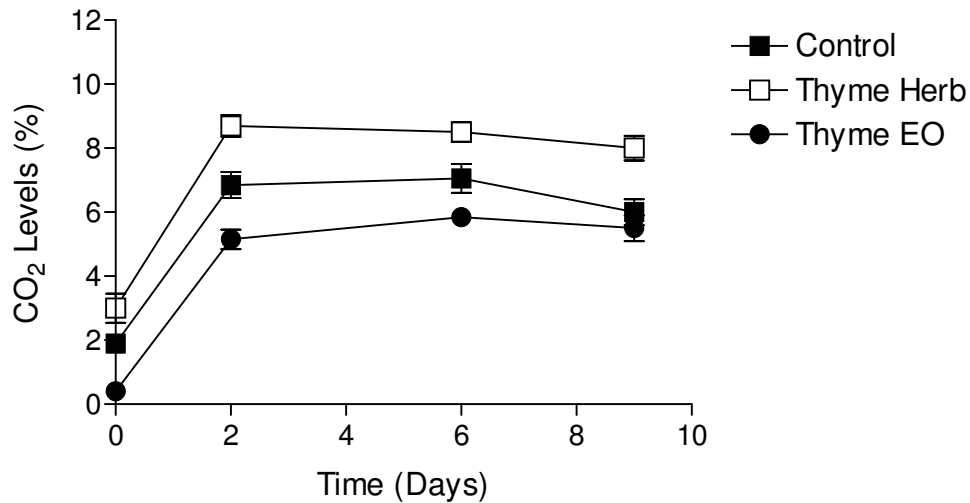


Figure 3.4: Effects of thyme (EO and shredded fresh herb) on gas atmospheres (CO₂ levels) in MA packages of fresh-cut lettuce stored at 8°C. Data are means of 4 values \pm s.d.

Figure 3.4 presents the effects of thyme EO and herb on levels of CO₂ in MAP fresh-cut lettuce at 8°C. For untreated lettuce, CO₂ levels increased to 7% on Day 2 of storage and remained at that level throughout storage. Addition of thyme herb increased levels of CO₂ to ~9% on Day 2 and stayed at this level up to Day 9. Carbon dioxide levels were lower (~5%) when the thyme EO was applied to the lettuce. At 4°C, similar trends were observed, where herb increased CO₂ levels and thyme EO reduced CO₂ levels (data not shown).

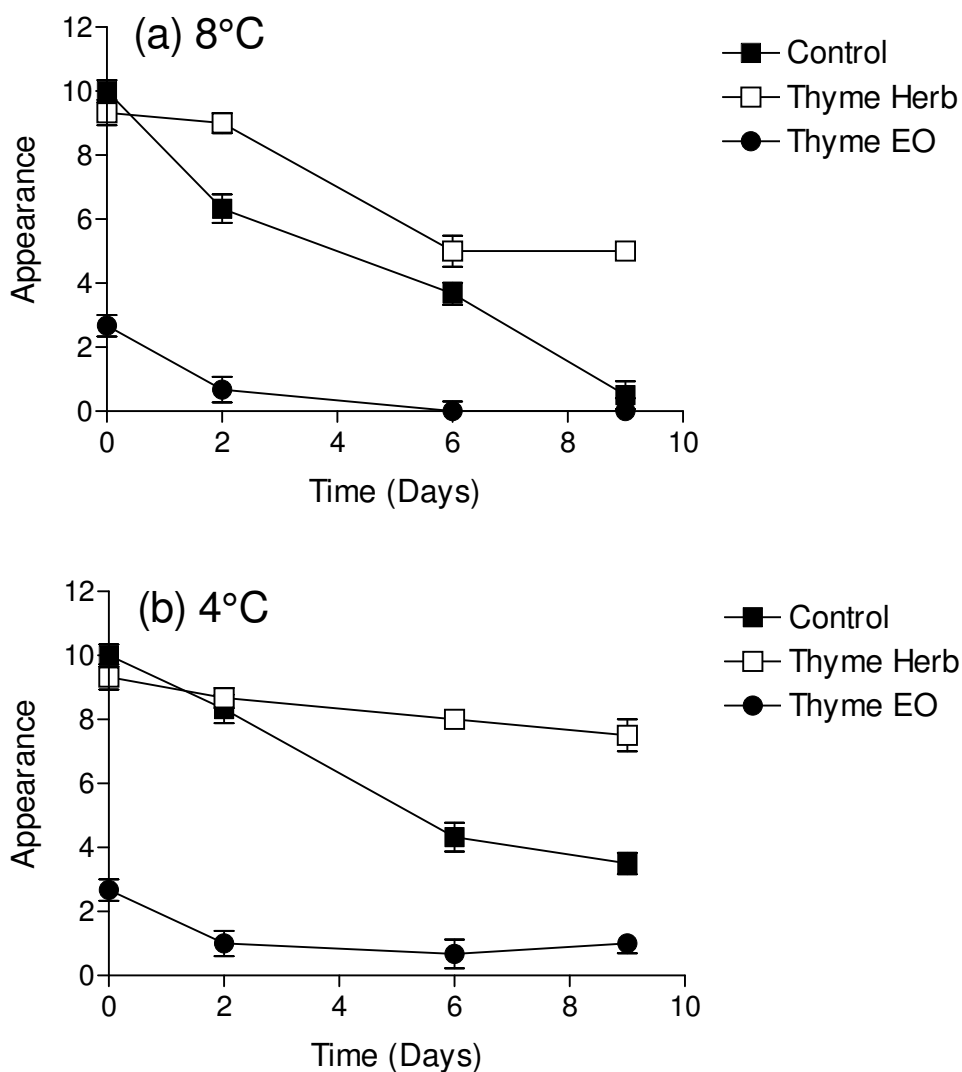


Figure 3.5: Effects of thyme (EO and shredded fresh herb) on appearance scores for sensory appearance of MAP fresh-cut lettuce stored at (a) 8°C and (b) 4°C. Reported scores are means of 5 values. Error bars show s.d.

At 8°C, the addition of thyme EO reduced mean appearance score to 2.5 (versus 10 for control) on Day 0 (Figure 3.5). This fell further to 0 by Day 6 of storage. Appearance scores for the untreated lettuce also fell during storage, to 6 by Day 2 and then to 1 by Day 9. By contrast, thyme herb improved the appearance scores for lettuce throughout storage in comparison to the untreated lettuce; on Day 9 the appearance score was 6 versus 1 for the controls. Thyme herb also increased appearance scores at 4°C; on Day 9 of storage, appearance score was 4 points above that of the control ($P < 0.05$).

3.3.2 Effects of oregano EO and fresh herb on *L. innocua* in MAP lettuce

Figure 3.6 shows the effects of oregano EO and fresh herb on *Listeria* survival and growth during storage on MAP fresh-cut lettuce at 8°C. For the untreated lettuce, populations of *Listeria* remained at levels of $\sim 1 \times 10^6$ CFU/g until Day 9 when they increased slightly. The addition of oregano EO resulted in about a 2 log inactivation by Day 2 ($P < 0.001$). However, this effect was lost by the end of the storage period. Oregano herb had no inhibitory effect on *Listeria* survival.

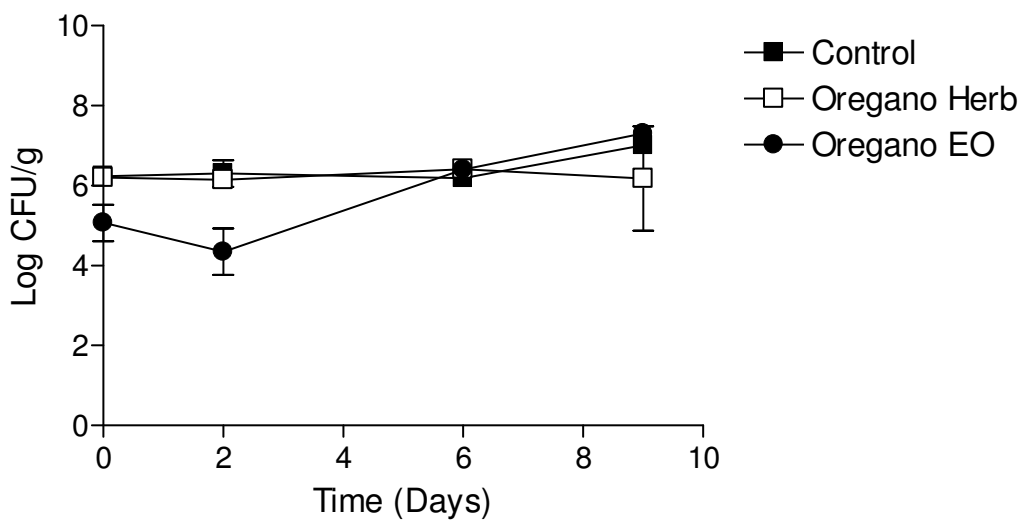


Figure 3.6: Effects of oregano (EO and shredded fresh herb) on survival and growth of *Listeria innocua* on MAP fresh-cut lettuce stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

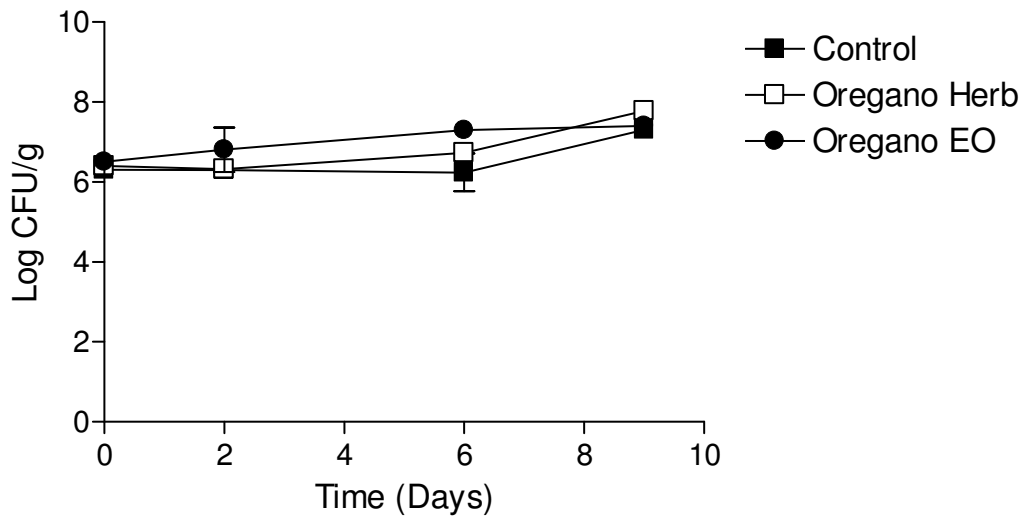


Figure 3.7: Effects of oregano (EO and shredded fresh herb) on total bacterial counts (TBCs) on MAP fresh-cut lettuce stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 3.7 demonstrates that there were no effects of the addition of either oregano EO or oregano herb on total bacterial counts on MAP fresh-cut lettuce.

3.3.3 Effects of basil EO and fresh herb on *L. innocua* in MAP lettuce

Figure 3.8 shows the effects of basil EO and fresh herb on *Listeria* survival and growth during storage on MAP fresh-cut lettuce at 8°C. Basil EO or herb had no significant effect ($P > 0.05$) on *Listeria* survival and growth at 8°C, with populations remaining at $\sim 1 \times 10^6$ CFU/g throughout the storage period.

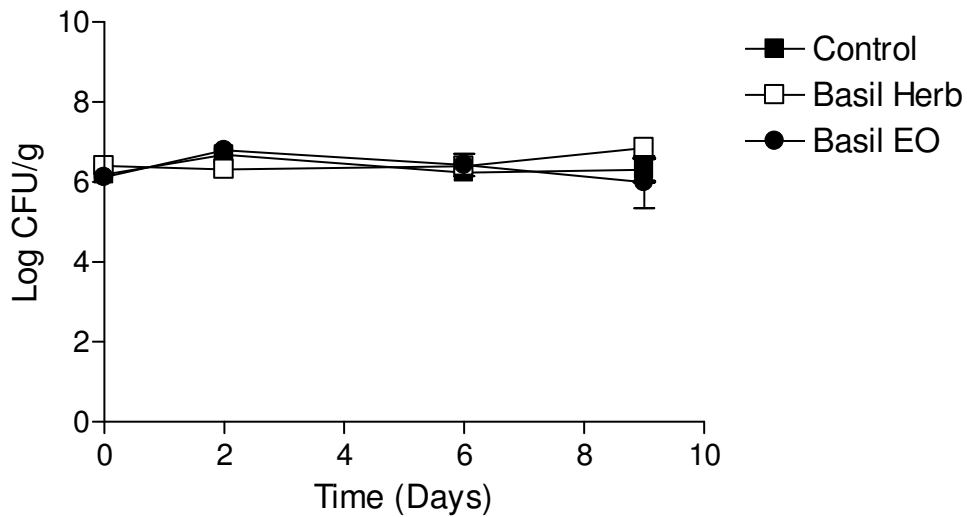


Figure 3.8: Effects of basil (EO and shredded fresh herb) on survival and growth of *Listeria innocua* on MAP fresh-cut lettuce stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

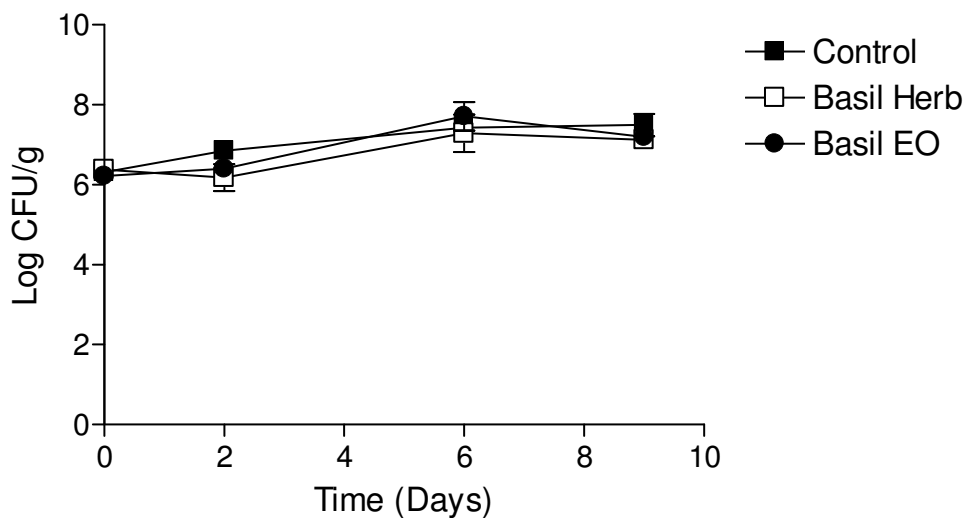


Figure 3.9: Effects of basil (EO and shredded fresh herb) on total bacterial counts (TBCs) on MAP fresh-cut lettuce stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

The effects of basil herb and EO on total bacterial counts on fresh-cut lettuce were also examined (Figure 3.9). Again basil EO and herb had no significant effect on TBCs with populations remaining at $\sim 1 \times 10^6$ CFU/g throughout storage.

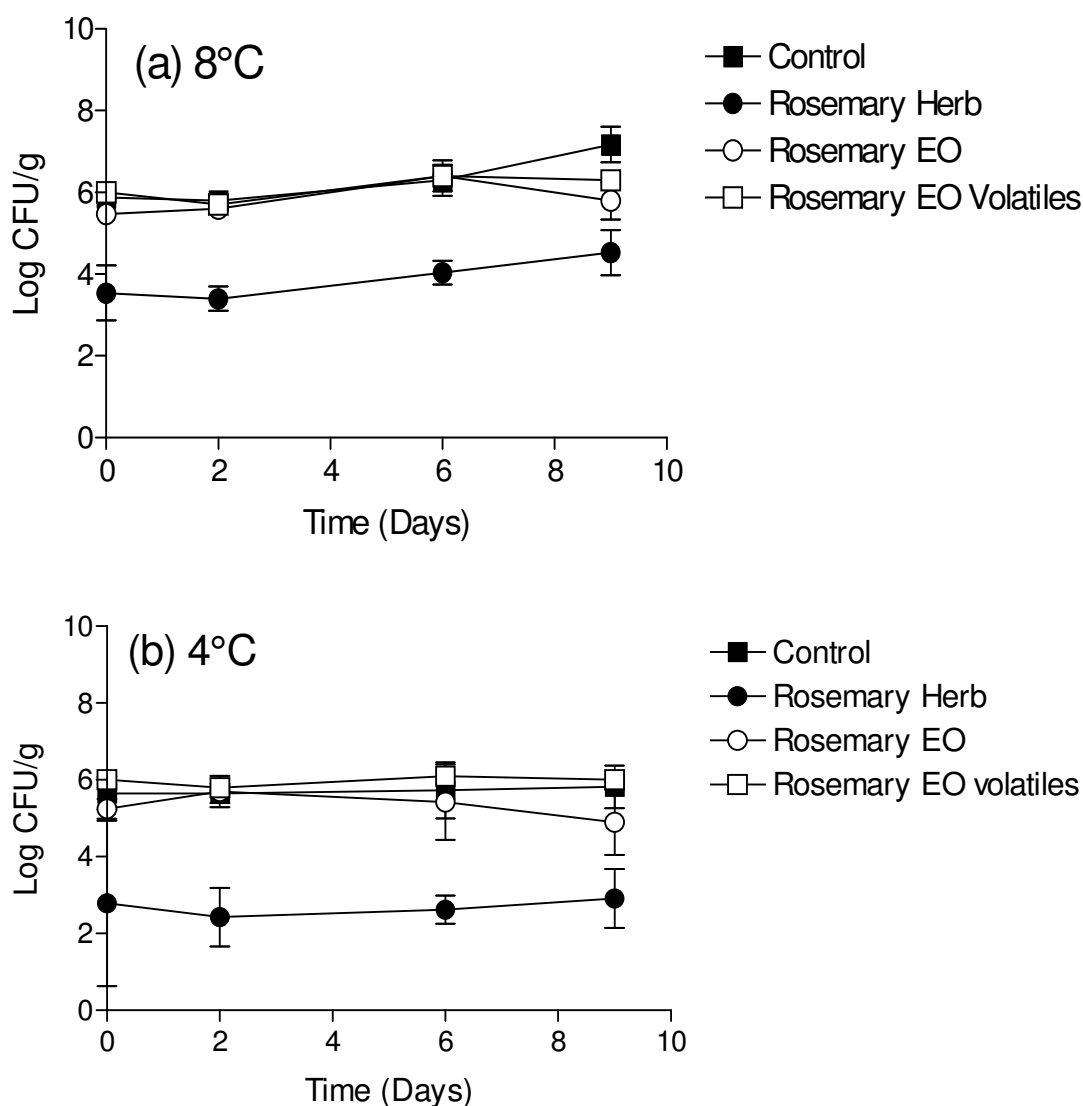
3.3.4 Effects of rosemary EO and fresh herb on *L. innocua* in MAP lettuce

Figure 3.10: Effects of rosemary (EO, EO volatiles and shredded fresh herb) on survival and growth of *Listeria* on fresh-cut MAP lettuce stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 3.10 shows the effects of fresh rosemary herb, rosemary EO and rosemary EO volatiles on *Listeria* survival and growth on lettuce stored at 4 and 8°C. At both storage temperatures, no anti-listerial effect was seen for either the rosemary EO or EO volatiles with only a slight reduction in *Listeria* counts seen for the rosemary EO at the end of storage. By contrast, adding fresh rosemary herb significantly ($P < 0.001$)

reduced *Listeria* counts by 3 log cycles on lettuce from Day 0, and reductions of this order persisted up Day 9. Counts on herb treated lettuce increased marginally at 8°C during storage but populations still remained significantly ($P < 0.001$) lower than those on untreated samples. At 4°C, a 3 log reduction in *Listeria* populations was maintained throughout storage (Figure 3.10b).

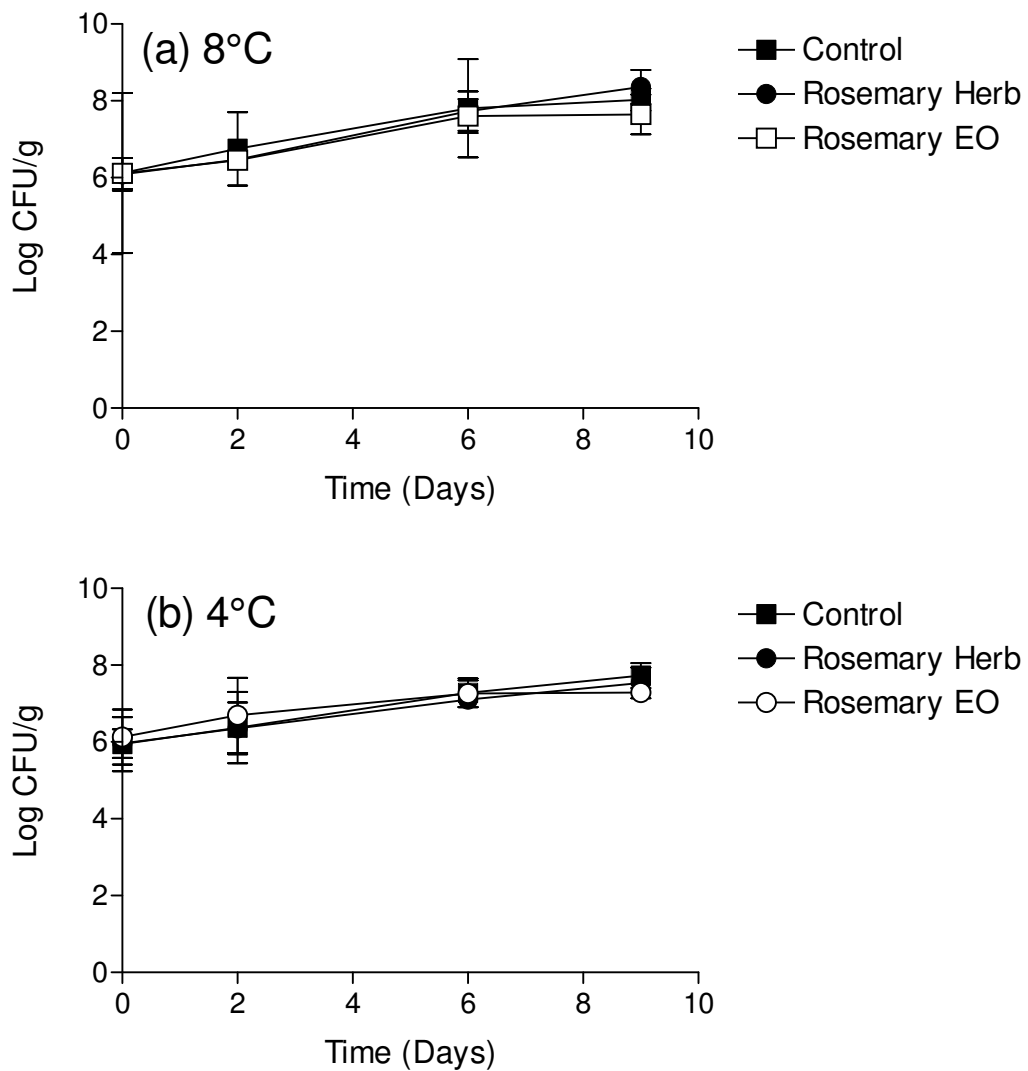


Figure 3.11: Effects of rosemary (EO and shredded fresh herb) on total bacterial counts (TBCs) on MAP fresh-cut lettuce stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

Rosemary herb or rosemary EO treatments did not inhibit growth on lettuce in comparison to the untreated lettuce during storage at either storage temperature (Figure 3.11)

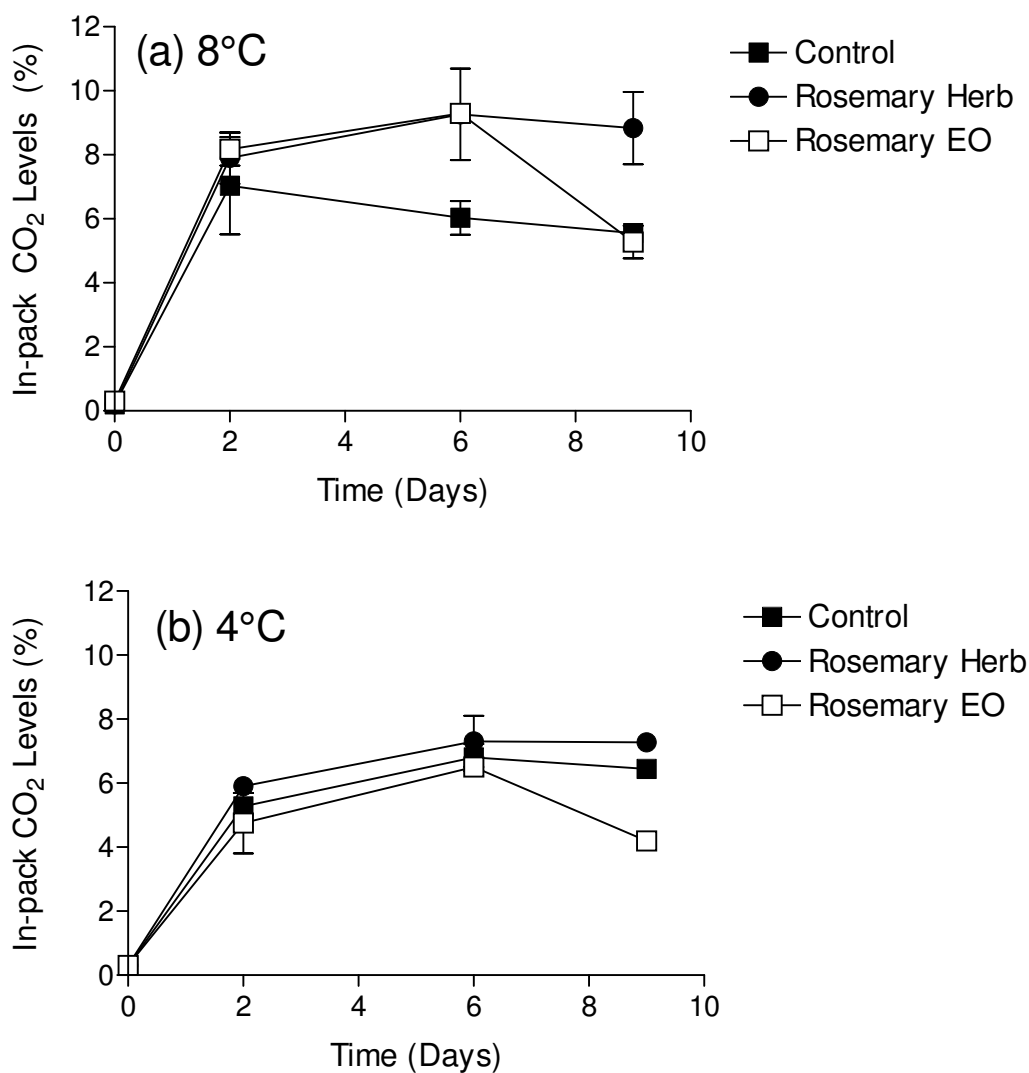


Figure 3.12: Effects of rosemary (fresh herb and EO) on gas atmospheres (CO₂ levels) in packages of MAP lettuce stored at (a) 8°C and (b) 4°C. CO₂ levels are means of 4 values. Error bars show s.d.

Gas levels in packages were affected by the herb treatments and storage temperatures. Carbon dioxide levels were slightly higher in packs when the rosemary herb was used as a treatment and at 8°C the difference in CO₂ levels was almost 4% higher than the

control, presumably due to the higher respiration rate. Use of rosemary EO was found to lower the CO₂ levels in the packs at 4°C.

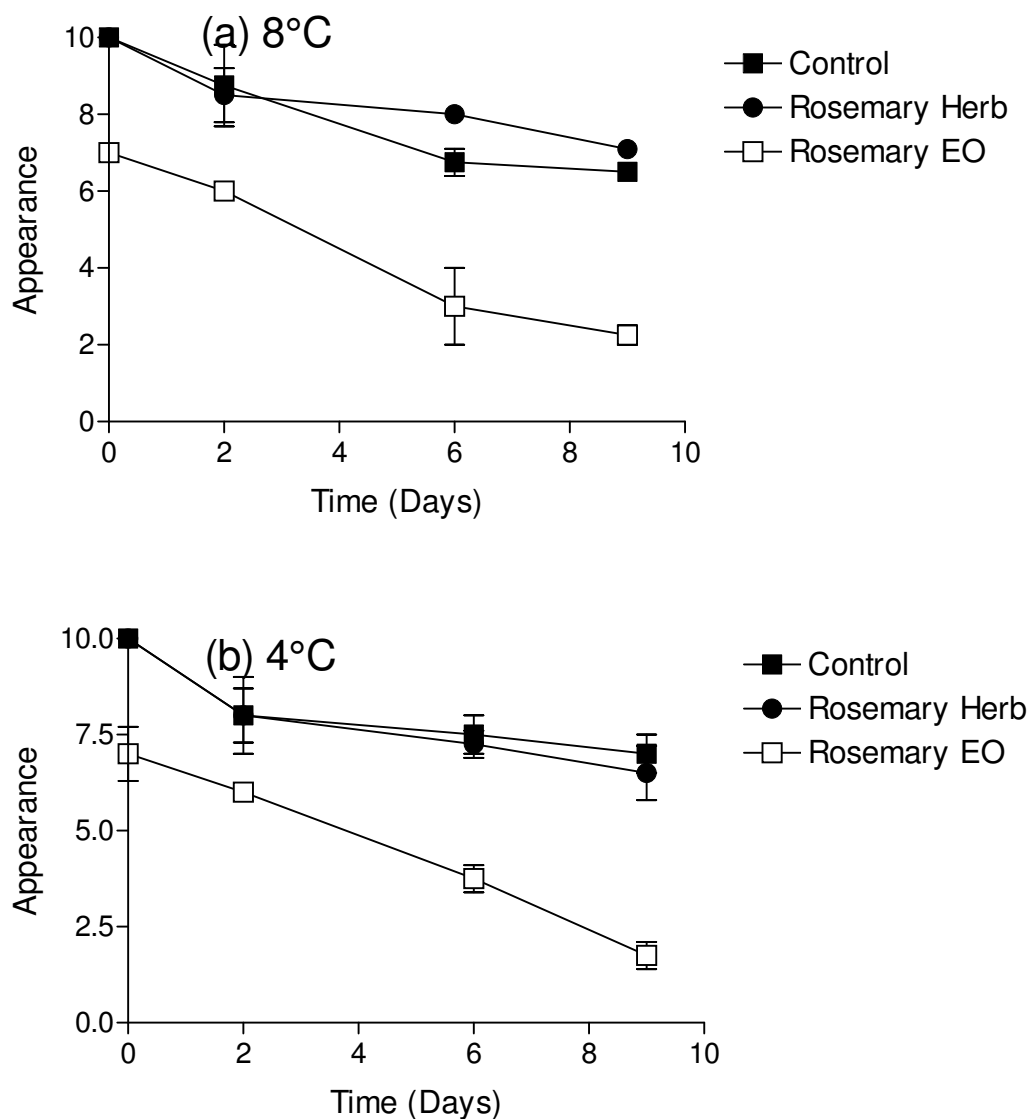


Figure 3.13: Effects of rosemary (fresh shredded herb and EO) on sensory appearance of MAP shredded lettuce stored at (a) 8°C and (b) 4°C. Reported populations are means of 5 values. Error bars show s.d.

At both 4 and 8°C, addition of rosemary EO reduced appearance score to 7 (versus 10 for control) on Day 0 (Figure 3.13). This fell further to 2 by Day 9 of storage. Appearance scores for the untreated lettuce also fell during storage to 7 by Day 9. Addition of rosemary herb had no effect on appearance scores.

3.4 Discussion

Fresh-cut lettuce was chosen for this evaluation because *Listeria* grows well on it and the phytotoxic effects of the essential oils can be easily seen. *L. innocua* NCTC 11288 was used for all experiments as *L. innocua* has been used successfully as a model organism for *L. monocytogenes* in previous studies (Omary *et al.* 1993, Francis and O' Beirne 1998). Of the EOs tested, thyme EO had the highest anti-listerial activity followed by rosemary herb. Oregano EO had moderate activity; basil and rosemary EOs, and thyme, basil and oregano fresh herbs had little or no anti-listerial effects.

Thyme EO was very effective, resulting in a 6 log reduction in counts. It also had a general antimicrobial effectiveness as TBCs were also reduced. Thyme EO has been shown previously to be effective against *L. monocytogenes*, *E. coli*, *S. Typhimurium*, and *Staph. aureus* (Burt 2004). Nguetack *et al.* (2004) found that thyme EO showed strong inhibitory activity against the four strains of *Listeria* tested. Lis-Balchin and Deans (1997) found thyme EO to be effective against 15 different strains of *L. monocytogenes*. In the current study, oregano EO also had anti-listerial effects but these were not as strong as those of thyme EO. This is supported by the data of Gutierrez *et al.* (2008) who reported that thyme and oregano EO had the highest antimicrobial activity against *Listeria* spp., *S. aureus*, *Lactobacillus* spp., *B. cereus*, *Salmonella*, *Enterobacter* spp., *E. coli*, and *Pseudomonas* spp. Dorman and Deans (2000) found that the oil with the widest spectrum of antimicrobial activity was thyme EO, followed by oregano EO. Oregano EO has also been shown to be effective against a number of food borne pathogens, including *E. coli*, *L. monocytogenes*, *S. aureus*, *S. Typhimurium*, *C. botulinum* (Oussalah *et al.* 2006, Friedman *et al.* 2002).

Oregano and thyme EOs contain high levels of thymol and carvacrol (Burt 2004, Cosentino *et al.* 1999, Lambert *et al.* 2001) and these compounds are known for their strong antibacterial activity (Dorman and Deans 2000, Lambert *et al.* 2001). Generally, the EOs with highest levels of antibacterial activity contain the highest levels of carvacrol and/or thymol (Gutierrez *et al.* 2008). Basil EO was shown to have no anti-listerial effect in this study. This may be due to the absence of phenols in the EO as suggested by Gutierrez *et al.* (2008)

Rosemary EO was found to be ineffective in reducing listerial growth on lettuce, with counts remaining similar to the untreated lettuce throughout storage. This is supported by the data of Singh *et al.* (2003) who found that rosemary EO was not effective against *L. monocytogenes* in peptone water. Rosemary was also found to be the least inhibitory EO when used as an antimicrobial against *Staphylococcus*, *Lactobacillus* and *Enterobacter* (Viuda-Martos *et al.* 2008). The major components in rosemary EO are monoterpenes, notably borneol, α -pinene, camphor, verbenone and 1,8-cineole (Tajkarimi *et al.* 2010, Pintore *et al.* 2002, Baratta *et al.* 1998), and contrary to above, many studies over the past number of years have shown these components to have significant antimicrobial activity (Soković *et al.* 2007, Tabanca *et al.* 2001, Mourey and Canillac 2002, Santoyo *et al.* 2005).

By contrast, fresh rosemary herb had major anti-listerial effects producing a 2-3 log reduction in *Listeria* populations on stomached lettuce. Pandit and Shelef (1994) found that rosemary herb (>0.5% w/v) was listericidal and that the addition of 0.5% finely ground rosemary herb to ready-to-eat pork sausage prior to cooking delayed listerial growth during refrigerated storage, whereas 1% rosemary EO was ineffective. This suggests that an important chemical component, which may have anti-listerial properties, could be lost during the EO extraction process. There is a major difference

in the anti-listerial effect between rosemary herb and rosemary EO and further investigation is required to explain this difference. These data contrast with equivalent data for thyme and oregano where both oils had large anti-listerial effects but the fresh shredded herb had no anti-listerial effects.

The volatile components of thyme and rosemary oil had no anti-listerial effect in this study. This contrasts with the data of Nedorostova *et al.* (2008) who found that oregano and thyme were highly effective in vapour phase and could be potentially used to control bacterial foodborne pathogens. One explanation for this could be that the temperature at which the current study was carried out (8°C) may have been too low to volatilise sufficient antimicrobial compounds. However, most studies have found that EOs are more effective when in direct contact with cells (Suhr 2003, Lopez *et al.* 2005). Lopez *et al.* (2005) reported that some oils (cinnamon, clove) have antimicrobial effects when in the vapour phase; however, rosemary EO was shown to be effective only when in direct contact with the cultures which included *L. monocytogenes*.

Since higher concentrations of plant EOs or undiluted EOs are generally required when added to food, their application in food may be limited due to changes in organoleptic and textural quality (Devlieghere *et al.* 2004). In this study, the application of EOs in an undiluted form to lettuce led to unacceptable damage to appearance. This negative effect on appearance is probably due to the dark colour of the oils and phytotoxic effects. Similar results were seen by De Azeredo *et al.* (2011) who found that the EOs of rosemary and oregano caused undesirable effects on the sensory quality of minimally processed vegetables. Uyttendaele *et al.* (2004) found that the addition of thyme EO to chopped bell peppers led to softening of the tissue and moisture loss, suggesting thyme EO probably has a cytotoxic effect to fresh produce

which are still respiring. Zunino and Zygodlo (2004) suggested that monoterpenes can cause oxidative stress in roots, a property which could explain some physiological and lipid composition changes caused by those compounds in intact tissues. In addition, some panellists commented on the unattractive odour associated with the oil. By contrast, the vegetable samples packaged with shredded fresh herbs displayed less enzymatic browning compared to untreated samples. This may have been because the combined respiration of the vegetables and herbs inside the packs increased the CO₂ levels and reduced the O₂ levels inside the packs more rapidly, which reduced the rate of enzymatic browning and deterioration. Diluted EOs alone or in mixtures can be used to avoid phytotoxicity but lack antimicrobial effectiveness.

In summary, in terms of EOs, thyme EO displayed the strongest antimicrobial effects against *Listeria*. Oregano EO had a moderate anti-listerial effect while basil and rosemary EO showed no anti-listerial properties. In contrast to rosemary EO, fresh chopped rosemary herb had high antimicrobial activity against *Listeria* whereas chopped fresh thyme, basil and oregano herbs showed no anti-listerial effects. While thyme EO demonstrated the best anti-listerial effect, direct application of the EO damaged appearance. However, there is potential for fresh rosemary herb to be used as it had good anti-listerial properties and also helped to maintain the fresh appearance of the lettuce. In the next chapter, opportunities to exploit the use of rosemary herb as an antimicrobial are explored further.

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Chapter 4

The effects of natural plant antimicrobials on *Listeria* survival and growth in a range of fresh-cut vegetables

4.1 Abstract

The anti-listerial effectiveness of selected EOs and shredded fresh herbs (thyme, oregano and rosemary) was examined on a range of MAP fresh-cut vegetables (carrot discs, cabbage and dry coleslaw mix). Anti-listerial effects of the antimicrobials were in the order: thyme EO > oregano EO > rosemary herb > rosemary EO. Shredded fresh rosemary herb again had a major anti-listerial effect during maceration in the stomacher, however, shredded fresh thyme and oregano herbs had no anti-listerial effects. The effectiveness of these antimicrobials varied depending on the product type. Adding shredded carrot to the vegetable packages enhanced the apparent anti-listerial effects, suggesting a synergistic effect between carrot and rosemary. Diluting thyme, oregano or rosemary EOs or using them in combination with diluted basil, oregano, thyme or rosemary EO reduced adverse sensory effects on produce, however, they eliminated the anti-listerial effects.

4.2 Materials and Methods

4.2.1 Preparation of the vegetable products

1. Carrot Discs

Locally grown Irish carrots were purchased at Richardson's Foods, Limerick and used for the production of carrot discs. Carrots were topped and tailed using a sharp knife and the end slices were discarded. Carrots were then peeled using a hand-held peeler and were sliced into 6 mm thick slices, using a sharp knife.

2. Dry Coleslaw Mix

The dry coleslaw mix (80% shredded white cabbage, 20% shredded carrot) was prepared by the local processor (Richardson's Foods, Limerick).

3. Cabbage

Heads of white cabbage were purchased from a local fruit and vegetable supplier (Richardson's Foods, Limerick). On the day of testing, the outer and damaged leaves as well as the core of the heads were removed and discarded. Inner leaves were sliced manually to approximately 10 mm strips using a sharp stainless steel knife.

4. Packaging of vegetable samples

Vegetable portions (25 g) were transferred into bags (18 cm x 10 cm), composed of 35 μm oriented polypropylene packaging film (Cannings Packaging Ltd., Dublin, Ireland), which were later heat-sealed. According to the manufacturer, this film had a permeability to O_2 of 1,200 $\text{ml/m}^2/\text{day}/\text{atm}$ and to CO_2 of 4,000 $\text{ml/m}^2/\text{day}/\text{atm}$.

4.2.2 *Listeria* strains

For all EO, herb and volatiles individual treatments on carrot discs the *Listeria innocua* strain was used as *L. innocua* very much resembles its other family member, the pathogenic *L. monocytogenes* (Francis and O' Beirne 1997). For the work carried out on the coleslaw mix, *L. monocytogenes* NCTC 11994 was used. For the combination treatments a mixture of *Listeria* strains were used, where all five strains mentioned below were mixed together equally and then used to inoculate the vegetables.

The *Listeria* strains used were as follows: two *L. monocytogenes* reference strains (i.e. NCTC 11994 and NCTC 7973), two *L. monocytogenes* strains which had been previously isolated from packaged vegetables (*L. monocytogenes* IL323 and CW 329) and one *L. innocua* strain (i.e. NCTC 11288). All cultures were maintained at -20°C in Tryptone soya broth (TSB) supplemented with 20% (v/v) glycerol, and were grown in tryptone soya broth (10 ml TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) at 35°C for 24 h. Cultures were then centrifuged (5000 g, 15 min), resuspended in PBS, mixed and diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow for contamination of vegetables at initial levels of approximately 10^5 - 10^6 CFU/g of vegetable.

4.2.3 Inoculation of vegetables

After appropriate dilutions, 100 µl aliquots of the cell suspension were distributed over the vegetables contained within each of the packages. Following inoculation, the antimicrobial treatments were applied to the vegetables and the bags were heat-sealed using an impulse bench-top heat sealer (Relco, UK Ltd., England). After sealing, packs were gently shaken to assist distribution of the inoculum.

4.2.4 Effects of antimicrobial treatments and storage

The effectiveness of the plant antimicrobial treatments on the survival and growth of *Listeria* were examined as described below:

1. *Essential Oils*

CO₂ supercritical fluid extracts of thyme, oregano and rosemary EOs were obtained from Guinness Chemicals Ltd. Portlaoise Ireland. These oils were chosen as they were shown to have the highest antimicrobial effects (Chapter 3). The oil (0.5 g) was sprayed over the vegetable products in a fine mist using a spray bottle.

2. *Fresh herbs*

Fresh thyme, oregano and rosemary herbs were obtained from a local supplier. The fresh herbs were cut into approx 30 mm portions using a sterile sharp knife and 5 g quantities were added to samples of packaged vegetables and mixed.

3. *Aroma volatiles*

Rosemary essential oil was placed in an individual compartment in the sealed vegetable packs. A 20 mm gap was left unsealed at the top of the compartment to allow the volatiles to surround the vegetables in the packs (Figure 3.1).

4. *Fresh shredded carrot*

As testing was carried out it became evident that carrot had an anti-listerial effect, therefore, fresh carrot was incorporated into the antimicrobial treatments. Carrots were topped and tailed using a sharp knife and the end slices were discarded. Carrots were then peeled using a hand-held peeler and were shredded using a grater and 5 g of shreds (10 mm) were added to samples of packaged vegetables and mixed.

5. Combination treatments

The effects of the EOs, at different dilutions and in different combinations, were also examined. Essential oil was diluted in distilled water to different concentrations and sprayed on the vegetables in different combinations, immediately after vigorously mixing. Combinations were selected based on synergistic effects seen *in vitro* in a previous study (Gutierrez *et al.* 2008a).

For rosemary EO, the concentrations and combinations used were:

	Treatments	Concentration of EO (ppm)
1)	No EO	
2)	Rosemary EO	Neat
3)	Rosemary EO	10,000
4)	Rosemary EO + Basil EO	5,000 10,000
5)	Rosemary EO + Oregano EO	5,000 100
6)	Rosemary EO + Thyme EO	5,000 100

Table 4.1: Rosemary EO concentrations and combinations for antimicrobial testing

For thyme, the concentrations and combinations used were:

	Treatments	Concentration of EO (ppm)
1)	No EO	
2)	Thyme EO	Neat
3)	Thyme EO	200
4)	Thyme EO + Rosemary EO	100 5,000
5)	Thyme EO + Oregano EO	100 100
6)	Thyme EO + Basil EO	100 10,000

Table 4.2: Thyme EO concentrations and combinations for antimicrobial testing

For oregano the concentrations and combinations were:

	Treatments	Concentration of EO (ppm)
1)	No EO	
2)	Oregano EO	Neat
3)	Oregano EO	200
4)	Oregano EO + Basil EO	100 10,000
5)	Oregano EO + Rosemary EO	100 5,000
6)	Oregano EO + Thyme EO	100 100

Table 4.3: Oregano EO concentrations and combinations for antimicrobial testing

Immediately after application of antimicrobial treatments and sealing of packages, vegetable samples were transferred to temperatures of 4°C and 8°C in order to test the effects of storage temperature on the anti-listerial effectiveness of treatments.

4.2.5 Microbiological analyses

Microbiological analyses were carried out on Day 0 (Day of inoculation) and at regular intervals throughout the storage period (Days 2, 6, 9). At each sampling duplicate packs from each of two experiment were analysed for *Listeria* populations and total viable cell counts. The 25 g sample of each package was transferred into a stomacher bag and samples were homogenised for 2 minutes at high speed with 225 ml of phosphate buffer saline (PBS, Oxoid BR0014) using a Seward laboratory stomacher (Stomacher Model 400, AGB Scientific, Ireland). Serial dilutions of each homogenised sample were made in PBS and were surface spread (100 µl/ plate) in duplicate on to appropriate media.

Numbers of *Listeria* spp. were determined on *Listeria* selective agar (LSA, Oxoid CM 856) containing a modified *Listeria* selective supplement (Oxoid SR0206) after 48 h at 35°C. A total count of aerobic mesophilic microflora was made on tryptone soya agar (TSA, Oxoid CM131) after incubation at 35°C for 48 h. Following incubation, colonies were counted and results were expressed as colony forming units per gram of sample.

4.2.6 Analyses of the gaseous atmospheres inside the packages

On each of the sampling dates, gases within three of each package type were analysed using an O₂ and CO₂ gas analyser (PBI-Dansensor, PBI Development, Denmark, Model TIA-III LV). An airtight needle that was attached to a flexible probe loop was inserted into packages and the readings of O₂ and CO₂ levels were recorded.

4.2.7 Evaluation of sensory appearance

Sensory evaluation was performed on sampling days on vegetable products during storage by a 5 member panel (members of our laboratory with experience in evaluation of fresh-cut vegetables). The panellists were asked to rate the appearance of samples, based on a rating scale for 'appearance score' ranging from 10 to 0, where 10 represents excellent appearance and 0 represents very poor quality. The samples were three digit coded and randomly offered to panellists. Analyses were carried out under typical daylight conditions and at a temperature of 18 to 20°C.

4.2.8 Statistical analyses

All experiments were carried out independently in duplicate and replicated twice. Duplicate packs were serially diluted and plated in duplicate at each analysis time. Reported populations therefore represent the means of four values \pm standard deviation. Means were compared by analysis of variance (ANOVA) followed by least difference testing at $P < 0.05$. Changes in appearance score were followed with paired-sample t tests ($P < 0.05$).

4.3 Results

4.3.1 Carrot Discs

4.3.1.1 Effects of thyme EO and fresh herb on *L. innocua* in MAP carrot discs

Figure 4.1 shows the effects of thyme EO and shredded herb on *L. innocua* survival and growth on carrot discs stored at 8°C. Thyme EO had a major anti-listerial effect, with no viable cells being detected from Day 0 of storage onwards. By contrast, thyme herb had little or no effect on *L. innocua* survival and growth on carrot discs and populations were slightly higher than the control by the end of storage. Similar results were found at 4°C (data not shown).

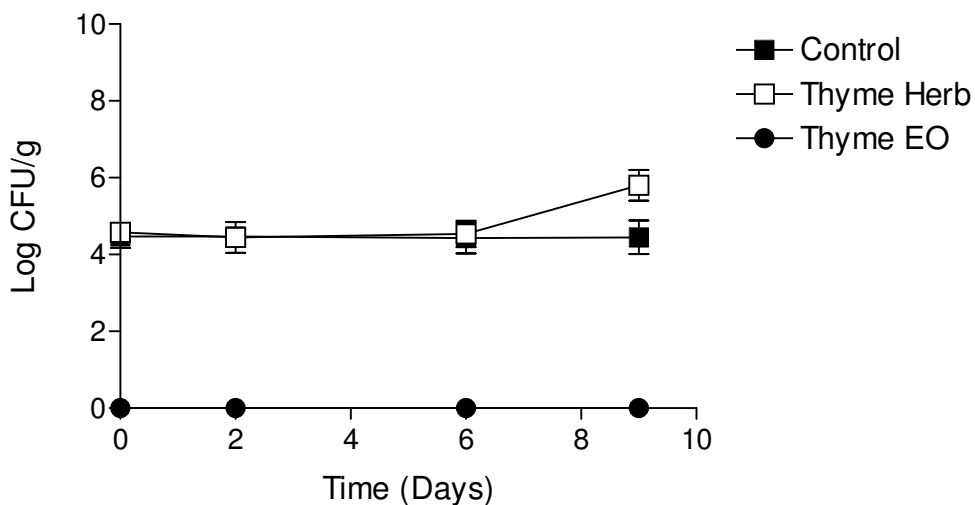


Figure 4.1: Effects of thyme (EO and shredded fresh herb) on survival and growth of *Listeria innocua* on MAP carrot discs stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

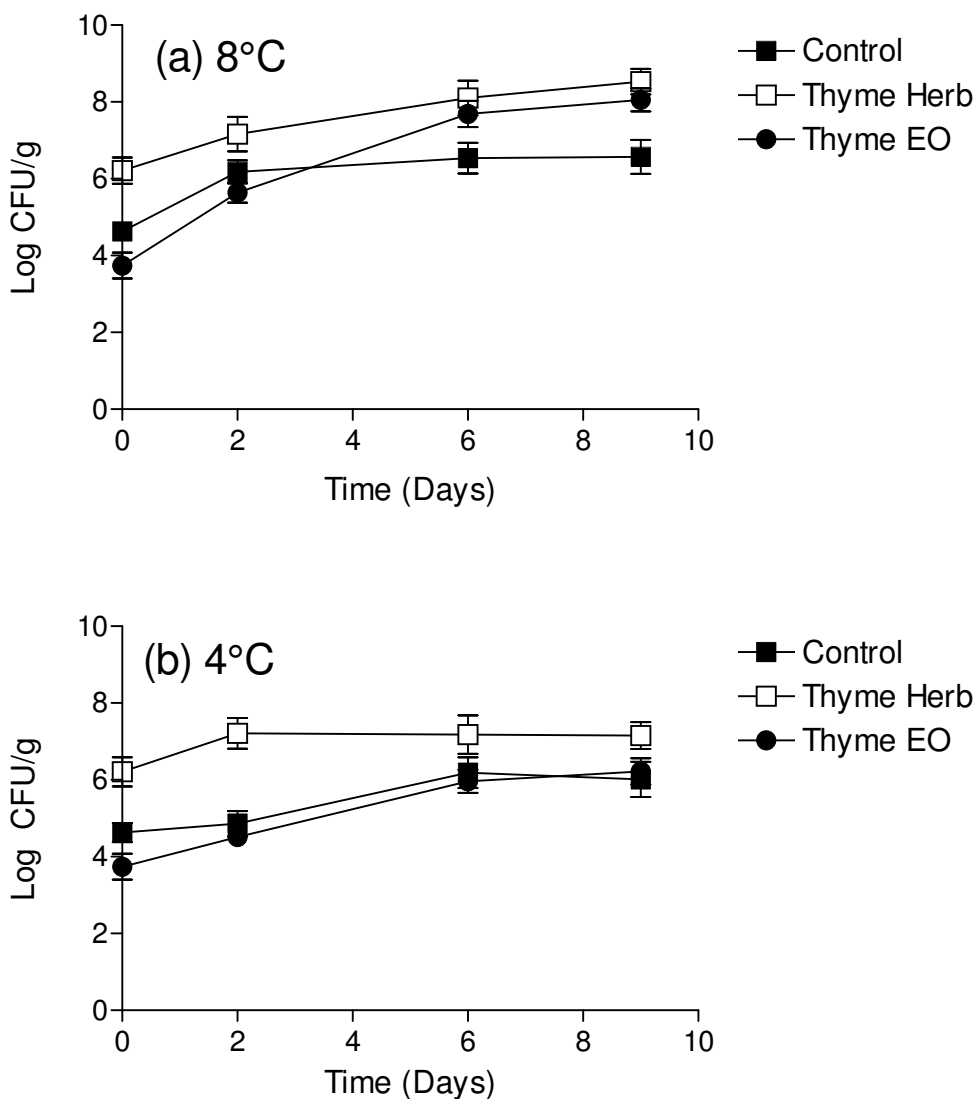


Figure 4.2: Effects of thyme (EO and shredded fresh herb) on total bacterial counts (TBCs) on MAP carrot discs stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.2 presents the results for the effect of thyme EO and herb on total bacterial counts on MAP carrot discs stored at 4 and 8°C. Applying thyme EO resulted in a 0.5-1 log reduction in initial bacterial counts, however this beneficial effect was lost by Day 2 of storage at both 4 and 8°C. Thyme herb had no effect on total bacterial counts; in fact populations were higher than the control throughout storage.

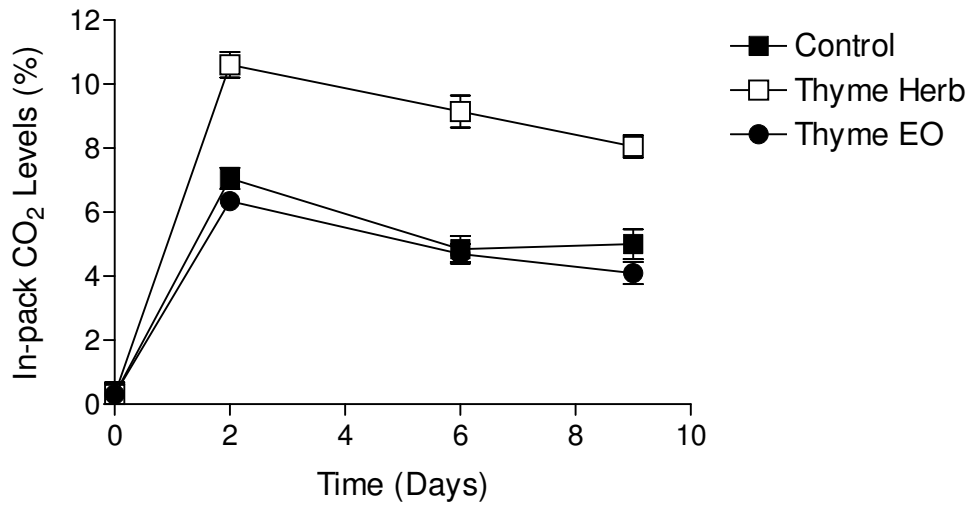


Figure 4.3: Effects of thyme (EO and shredded fresh herb) on gas atmospheres (CO₂ levels) in MA packages of carrot discs stored at 8°C. Reported levels are means of 4 values. Error bars show s.d.

The effect of thyme EO and herb on gas atmospheres in MAP carrot discs stored at 8°C are shown in Figure 4.3. Higher CO₂ levels were measured in packages containing shredded thyme herb for both storage temperatures (4°C data not shown). CO₂ levels increased to ~10% for packs with herb, while packages without herb reached levels of ~6%.

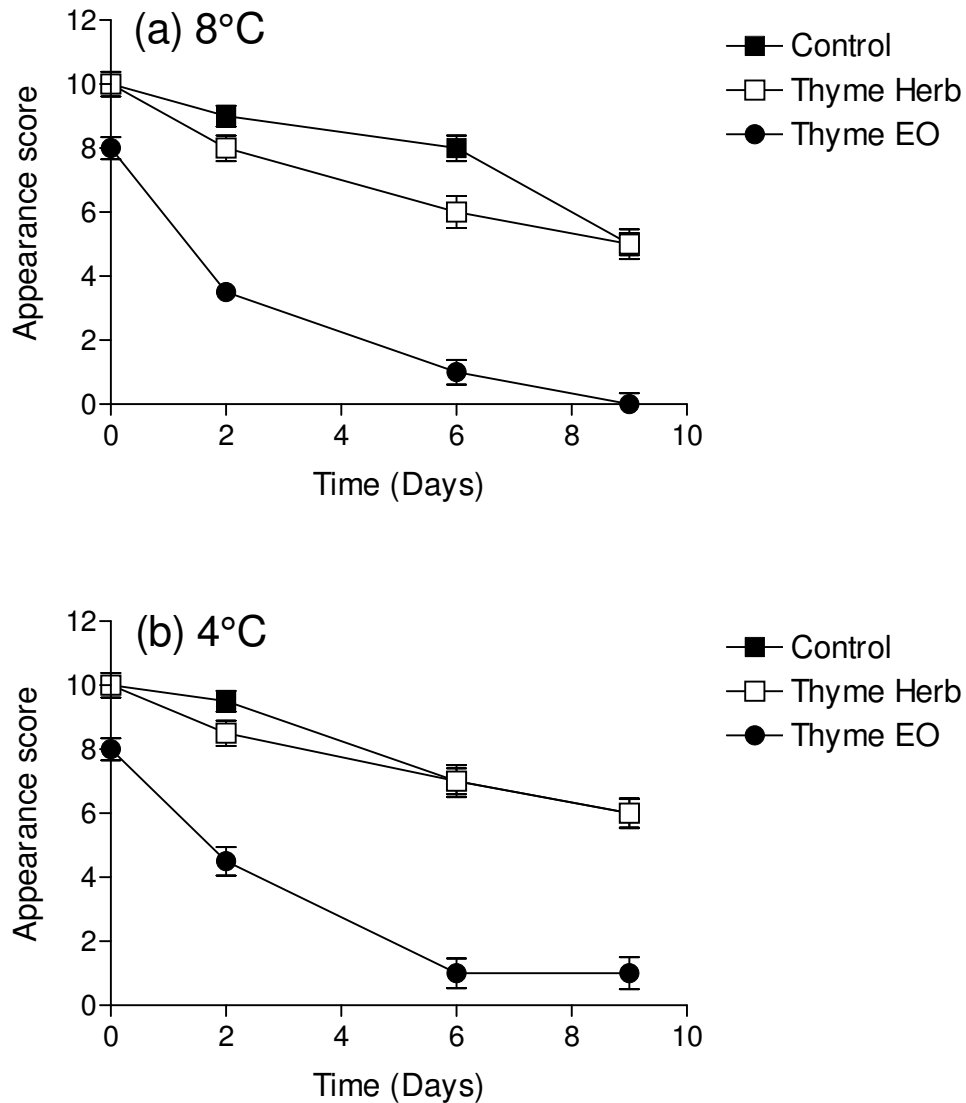


Figure 4.4: Effects thyme (EO and shredded fresh herb) on appearance score of MAP carrot discs stored at (a) 8°C and (b) 4°C. Reported scores are means of 4 values. Error bars show s.d.

Thyme EO had a detrimental effect on the appearance score of the carrot discs at both storage temperatures (Figure 4.4). The addition of thyme EO resulted in a drop in appearance score of 2 points on Day 0 and up to 9 points by Day 6 of storage. Little or no difference was seen between the sample with and without thyme herb by Day 9 at both 4 and 8°C.

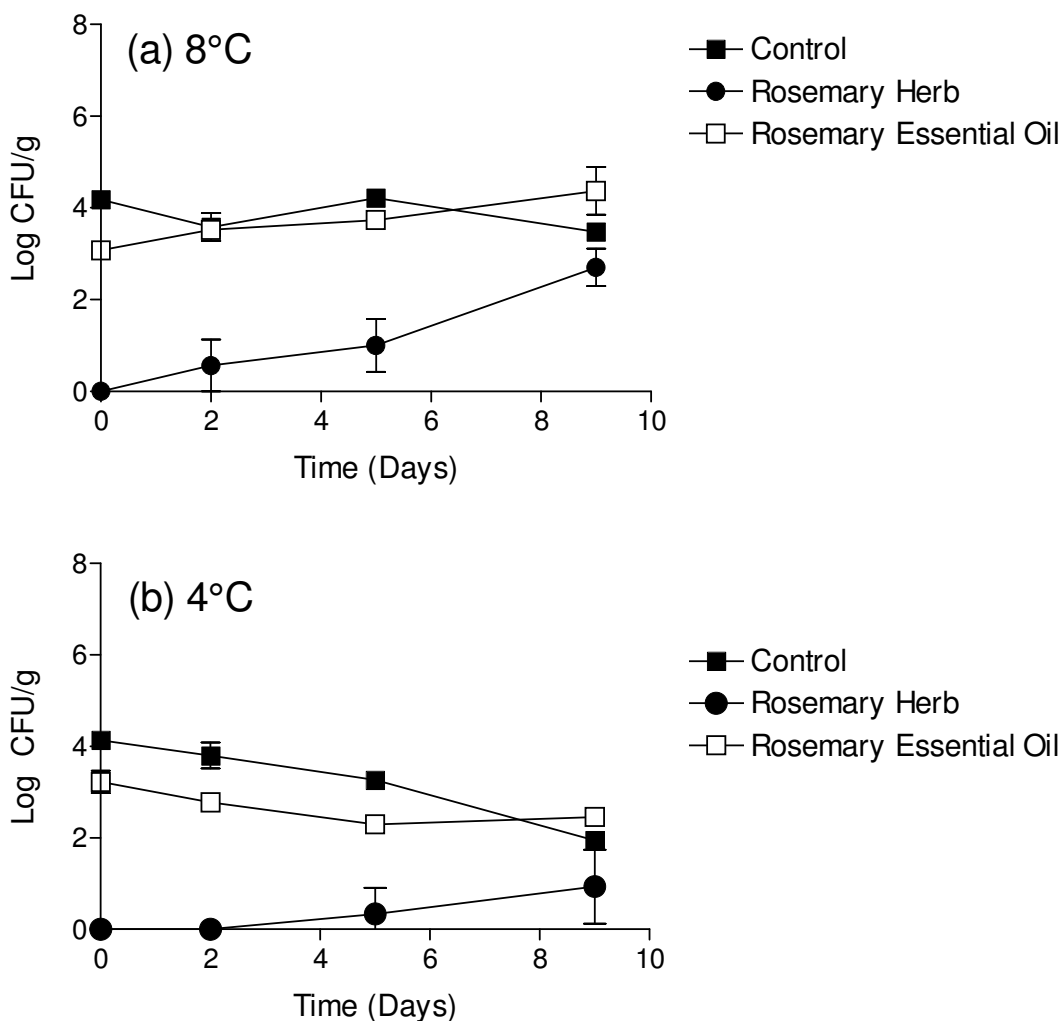
4.3.1.2 Effects of rosemary EO and fresh herbs on *L. innocua* in MAP carrot discs

Figure 4.5: Effects of rosemary (fresh shredded herb and EO) on *Listeria innocua* counts on MAP carrot discs stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.5 shows the effects of rosemary EO and shredded herb on *L. innocua* counts on MAP carrot discs stored at both 4 and 8°C. At 8°C, the rosemary EO had an anti-listerial effect on Day 0 with a 1 log reduction in populations evident but this effect was lost by Day 2, and counts were higher than the control by the end of storage. Rosemary herb had a good antimicrobial effect and significantly reduced *L. innocua* populations with no viable cells being detected on Day 0 at 8°C ($P > 0.001$). However, *L. innocua* populations increased during storage and by Day 9, populations were only 0.5 log cycles lower than the untreated carrot discs ($P < 0.05$).

At 4°C, rosemary EO had a small effect on *L. innocua* survival and growth at the beginning of storage and up until Day 6, but this effect was lost by Day 9 of storage. For rosemary herb, a major anti-listerial effect was seen with populations 4log cycles lower than the control (P<0.001), with no viable cells detected on Day 0 or Day 2. By the end of storage populations had increased, however they were still significantly lower than the control (P<0.05).

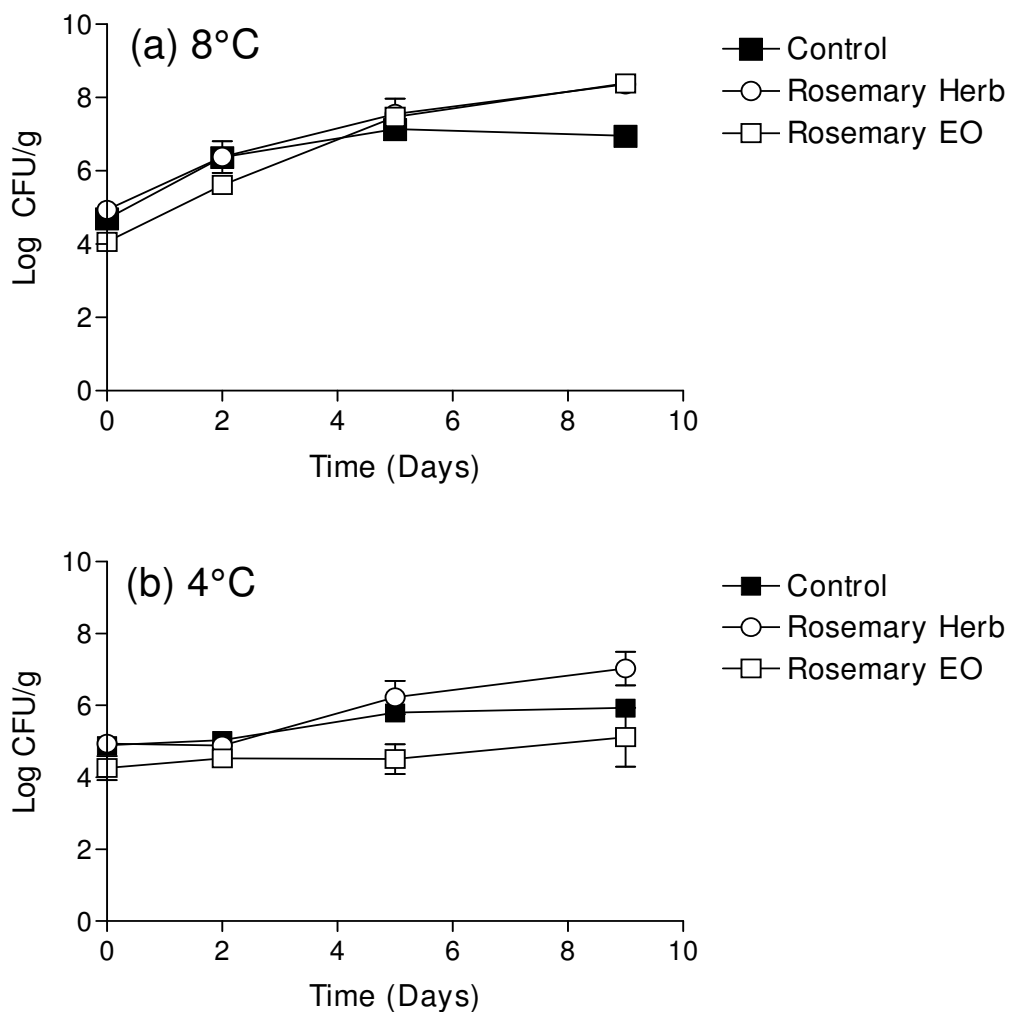


Figure 4.6: Effects of rosemary (fresh shredded herb and EO) on total bacterial counts (TBCs) on MAP carrot discs stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.6 shows the effects of rosemary EO and shredded herb on total bacterial counts on MAP carrot discs stored at both 4 and 8°C. For the untreated carrot discs at

8°C, final populations were $\sim 1 \times 10^6$. The addition of either rosemary EO or herb had little or no effect on total counts from Day 0 to Day 6, however, on Day 9 of storage populations were higher than the control. As the storage temperature was lowered to 4°C, the rosemary EO had a slight effect at reducing microbial load ($P < 0.05$) and this persisted throughout storage.

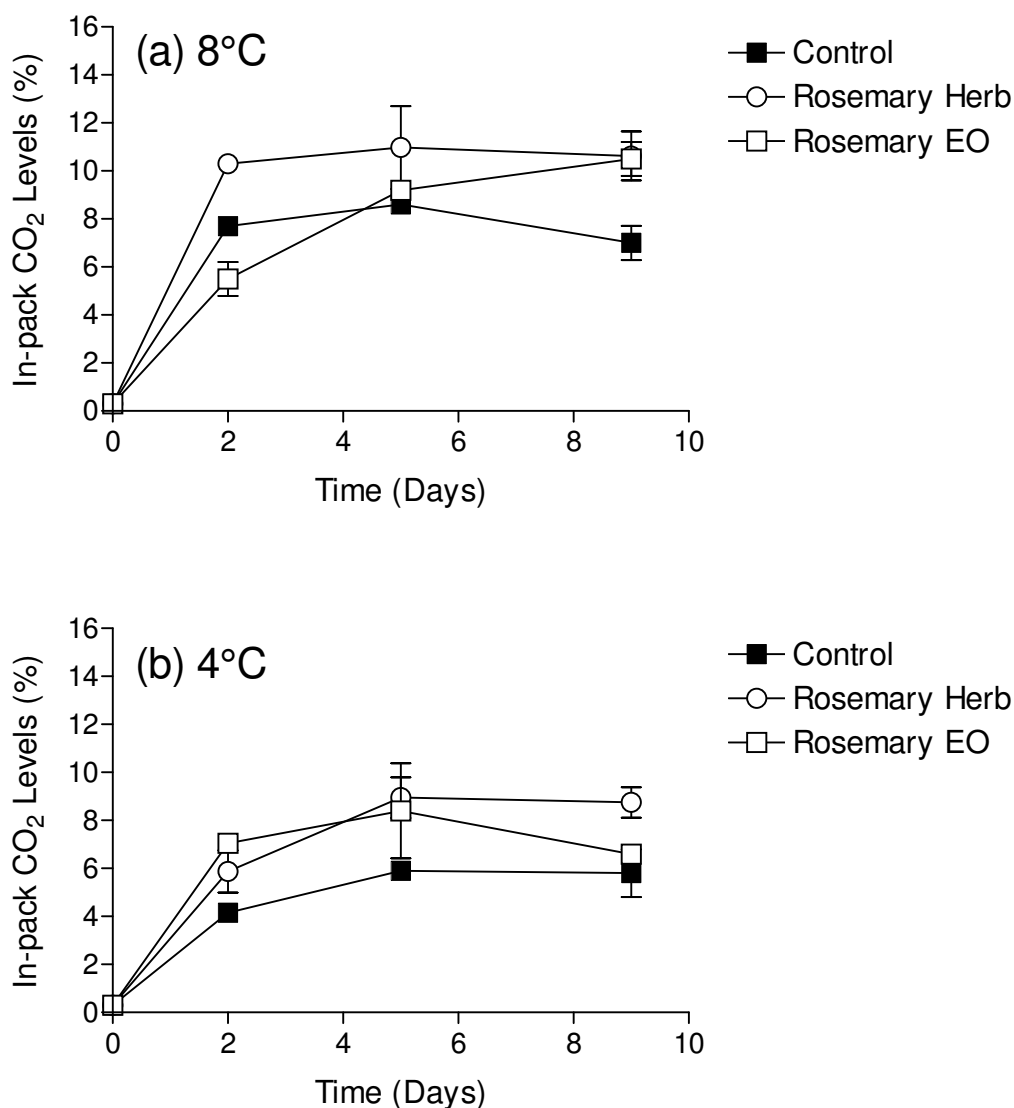


Figure 4.7: Effects of rosemary (fresh herb and EO) on gas atmospheres (CO_2 levels) in packages of MAP carrot discs stored at (a) 8°C and (b) 4°C. Reported levels are means of 4 values. Error bars show s.d.

At both storage temperatures the gas levels increased in packs of carrot discs for both the rosemary herb and the rosemary EO samples at 8°C (Figure 4.7). Addition of rosemary herb quickly increased the CO₂ levels to 11% and this level was maintained throughout storage. Adding rosemary EO also increased the CO₂ levels, with levels higher than the control seen by Day 6 and a final gas level of 11% present at the end of storage. Again, gas levels were reduced at the lower storage temperature of 4°C.

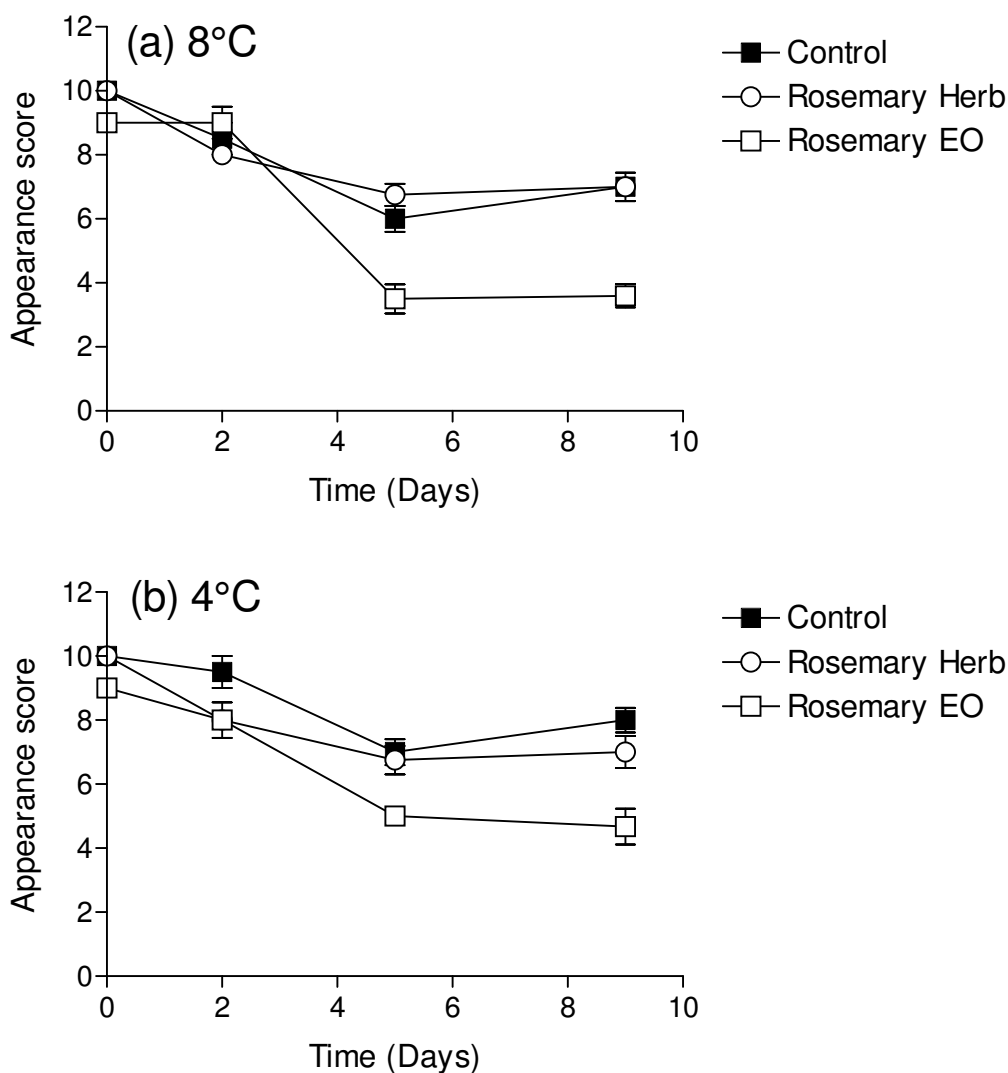


Figure 4.8: Effects of rosemary (fresh herb and EO) on appearance score of MAP carrot discs stored at (a) 8°C and (b) 4°C. Reported scores are means of 4 values. Error bars show s.d.

Figure 4.8 presents the effects of rosemary EO and shredded herb on the appearance score of MAP carrots at different storage temperatures. At 4°C the appearance of the control was slightly better than that of the rosemary herb sample, but overall little difference was seen in the sensory quality of either sample. The rosemary EO had a negative effect on the appearance of the carrot discs with a drop of 5 points in the appearance score seen by the end of storage. At 8°C, similar trends were seen.

4.3.2 Dry Coleslaw mix

4.3.2.1 Effects of thyme EO and fresh herb on *L. monocytogenes* on MAP coleslaw mix

Figure 4.9 shows the effects of thyme EO, thyme shredded herb and thyme EO volatiles on *Listeria* survival and growth in MAP dry coleslaw mix during storage at 8°C. The thyme herb and the thyme EO volatiles had no anti-listerial effect, with listerial populations behaving similarly to those on untreated coleslaw. By contrast, thyme EO resulted in no viable cells being detected from Day 0 and throughout storage ($P < 0.001$). Similar results were found at 4°C (data not shown).

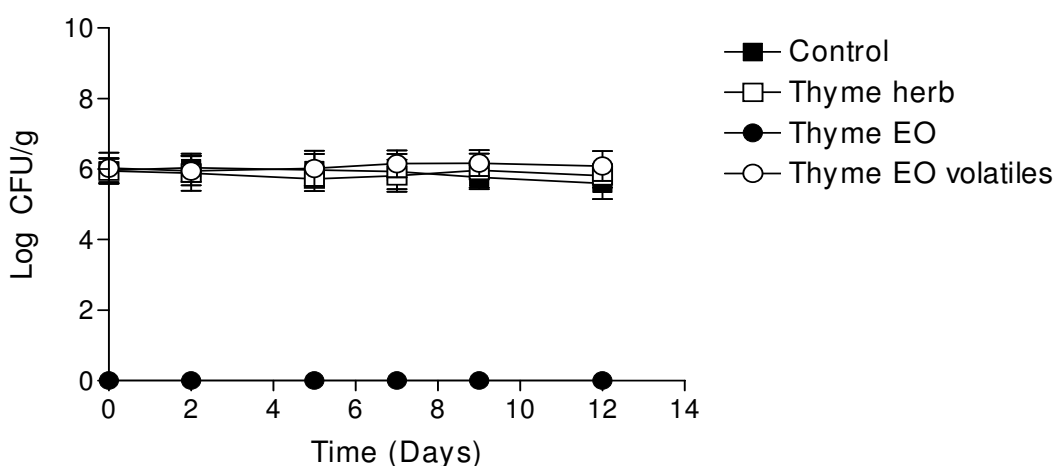


Figure 4.9: Effects of thyme (EO, EO volatiles and shredded herb) on survival and growth of *Listeria monocytogenes* (NCTC 11994) on MAP coleslaw mix stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

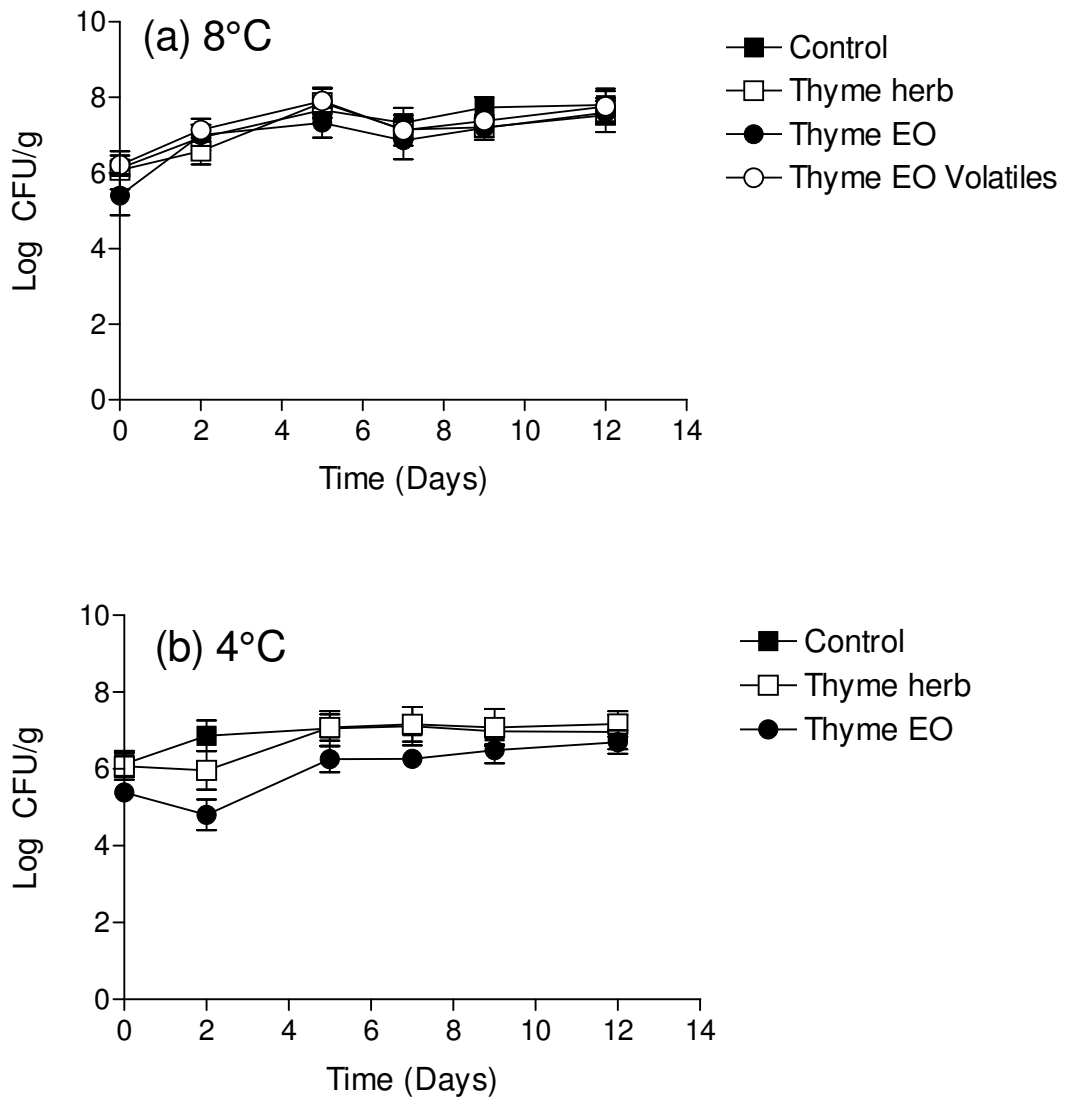


Figure 4.10: Effects of thyme (EO, EO volatiles and shredded fresh herb) on total bacterial counts (TBCs) on MAP coleslaw mix stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

The effects of thyme EO and herb on total bacterial counts on MAP coleslaw mix are presented in Figure 4.10. The thyme treatments did not affect the total bacterial counts on coleslaw stored at 8°C. At 4°C, thyme EO reduced initial TBCs by 1-2 log cycles but this effect was lost by the end of storage.

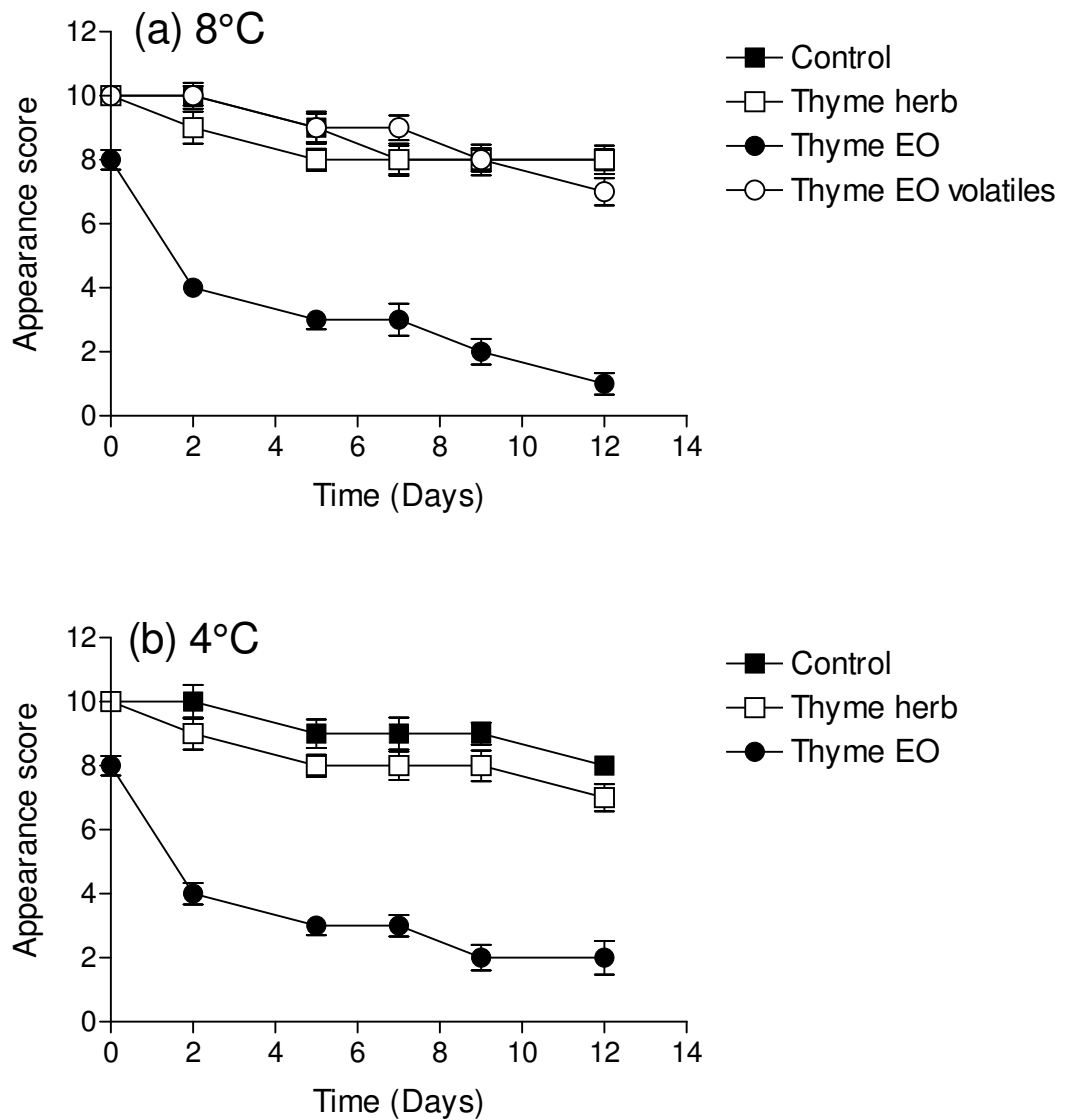


Figure 4.11: Effects of thyme (EO, EO volatiles and shredded fresh herb) on appearance score of MAP coleslaw mix stored at (a) 8°C and (b) 4°C. Reported scores are means of 4 values. Error bars show s.d.

Adding thyme EO to coleslaw mix reduced its appearance score from 10 to 8 (Day 0), and scores were significantly reduced from Day 2 onwards ($P < 0.05$) with a final appearance score of 2 on Day 9 (Figure 4.11). The addition of shredded thyme herb resulted in a drop of 1 point in the appearance score compared to the control.

4.3.2.2 Effects of oregano EO and fresh herb on *L. monocytogenes* in MAP coleslaw mix

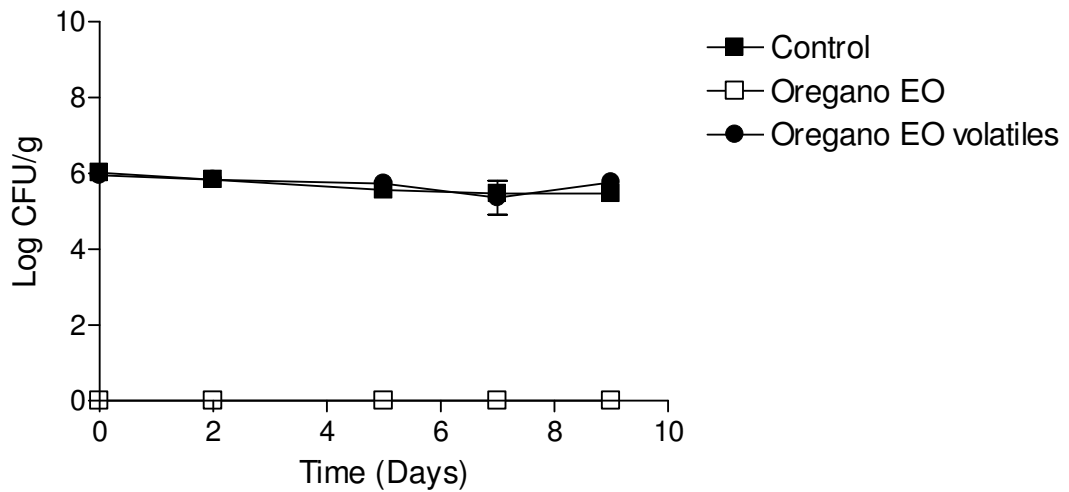


Figure 4.12: Effects of oregano (EO and EO volatiles) on survival and growth of *Listeria monocytogenes* (NCTC 11994) on MAP fresh-cut coleslaw mix stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

The effect of oregano EO and EO volatiles on *Listeria* survival and growth was also examined on MAP coleslaw mix (Figure 4.12). When oregano EO was applied directly to the coleslaw mix a major anti-listerial effect was evident with a 6 log reduction in *Listeria* populations ($P < 0.001$), and this persisted with no growth seen throughout the storage period. Volatiles released from the oregano EO had little or no effect on *Listeria* survival and growth with populations remaining similar to that of the control.

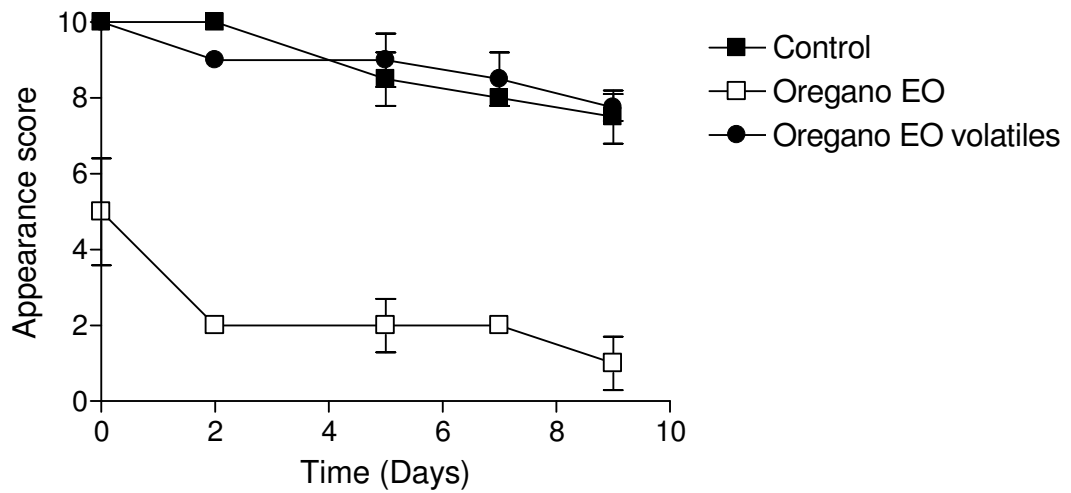


Figure 4.13: Effects of oregano (EO and EO volatiles) on appearance score of MAP coleslaw mix stored at 8°C. Reported scores are means of 4 values Error bars show s.d.

An analysis of the coleslaw mix was also carried out during storage (Figure 4.13).

Oregano EO reduced appearance scores by 5 points immediately and by 9 points at the end of storage. Oregano EO volatiles had no effect on the appearance score of the coleslaw mix throughout storage.

4.3.2.3 Effects of rosemary EO and fresh herbs on *L. monocytogenes* in MAP coleslaw mix

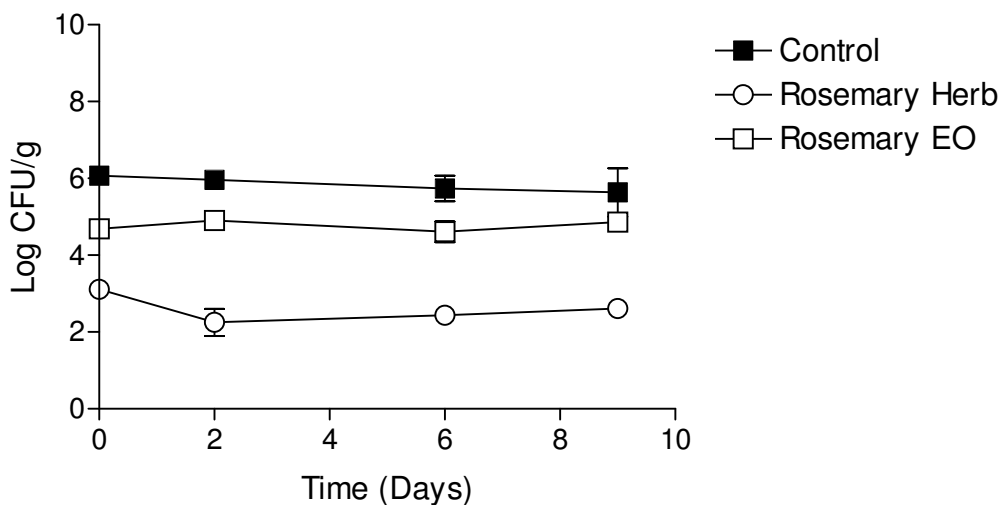


Figure 4.14: Effects of rosemary (fresh shredded herb and EO) on *Listeria monocytogenes* (NCTC 11994) counts on MAP coleslaw mix stored at 4°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.14 shows the effects of rosemary EO and fresh shredded herb on *L. monocytogenes* counts on MAP coleslaw mix during storage at 4°C. Rosemary EO had an anti-microbial effect on *Listeria* counts with a 1 log reduction in counts, in comparison to the untreated coleslaw mix, evident throughout storage ($P < 0.05$). Rosemary herb, however, had a greater effect on *Listeria* survival, producing a 3 log reduction in initial counts, and a 4 log reduction in counts by the end of storage ($P < 0.001$). Similar results were recorded at 8°C (data not shown).

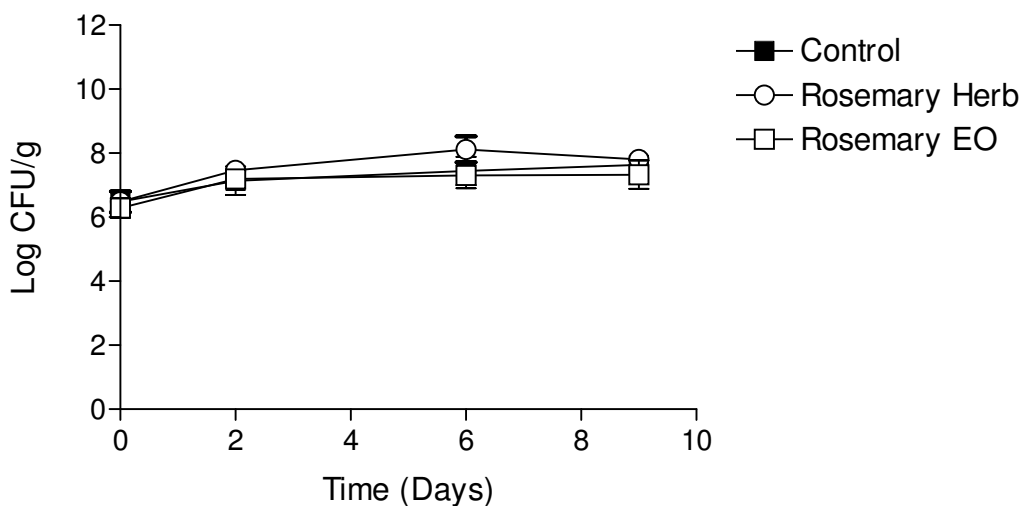


Figure 4.15: Effects of rosemary (fresh shredded herb and EO) on total bacterial counts (TBCs) on MAP coleslaw mix stored at 4°C. Reported populations are means of 4 values. Error bars show s.d.

The addition of rosemary EO or herb did not reduce total bacterial counts on coleslaw mix during storage at 4°C (Figure 4.15). Similar results were found at 8°C (data not shown).

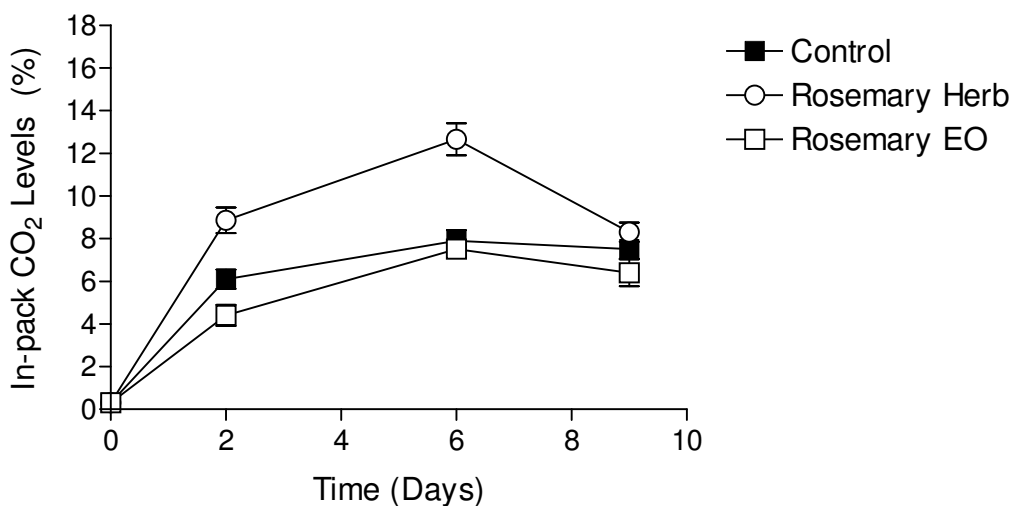


Figure 4.16: Effects of rosemary (fresh shredded herb and EO) on gas atmospheres (CO₂ levels) in packages of MAP coleslaw mix stored at 4°C. Reported levels are means of 4 values. Error bars show s.d.

Figure 4.16 presents results for the effects of rosemary EO and herb on gas atmospheres in packages of MAP coleslaw at 4°C. The addition of rosemary EO reduced the CO₂ levels found in packs. The addition of the rosemary herb to packs increased CO₂ levels to 12% CO₂ at 4°C (P<0.001) compared to the control. Similar trends were seen at 8°C (data not shown).

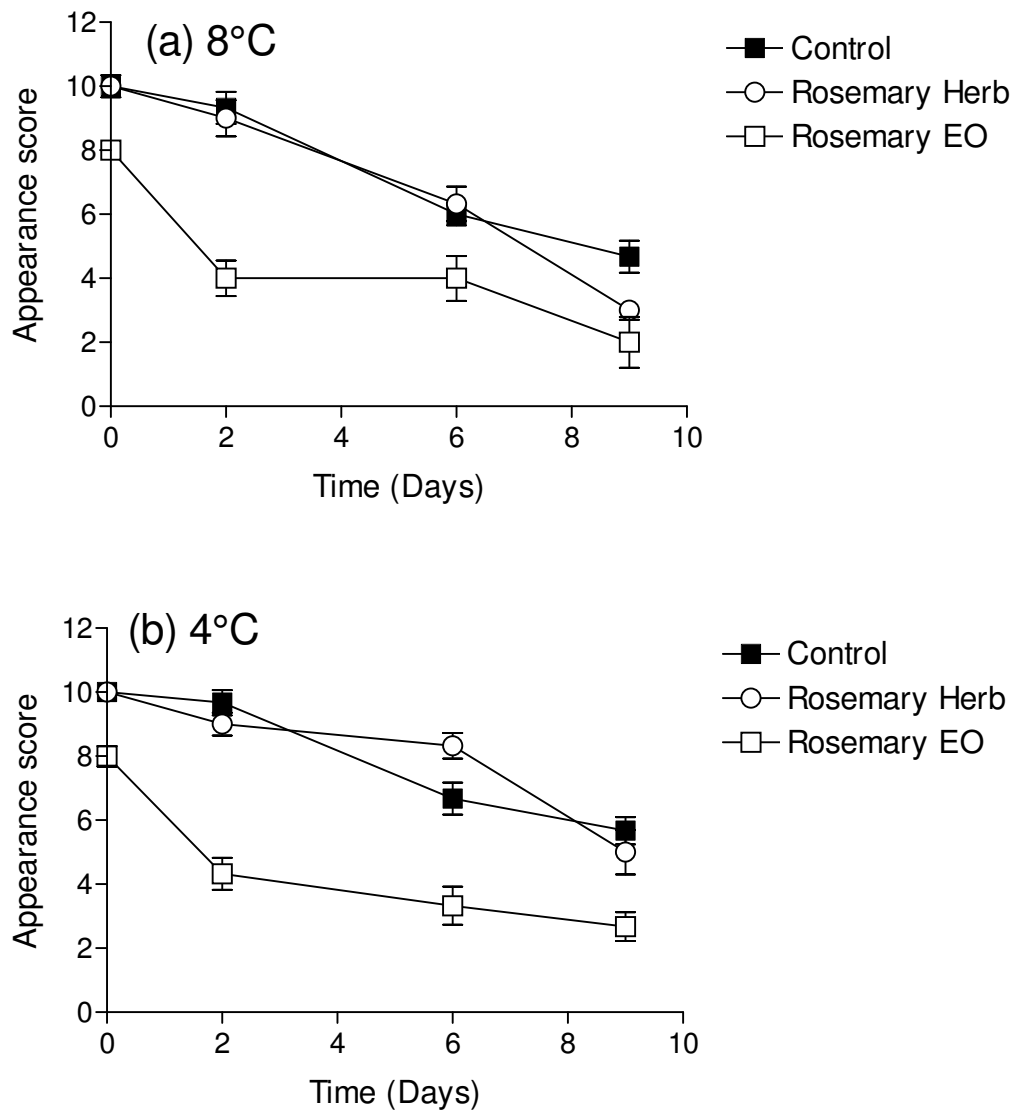


Figure 4.17: Effects of rosemary (fresh shredded herb and EO) on appearance score of MAP coleslaw mix stored at (a) 8°C and (b) 4°C. Reported scores are means of 4 values. Error bars show s.d.

The addition of fresh rosemary herb had no effect on the appearance score of the coleslaw, however, the appearance of both products had deteriorated significantly by Day 9 of storage. The rosemary EO reduced the appearance score of the coleslaw mix from Day 0 onwards. Similar trends were seen at both storage temperatures (Figure 4.17).

4.3.3 Fresh shredded cabbage

4.3.4.1 Effects of rosemary and carrot combination treatments on *Listeria* (mixed strain) in MAP shredded cabbage

As discussed previously (4.2.4) carrots were introduced as an additional antimicrobial treatment. The effects of different rosemary and carrot combination treatments on *Listeria* survival and growth on shredded cabbage during storage at 4 and 8°C are presented in Figure 4.18. At 8°C, the addition of carrot significantly ($P<0.05$) reduced *Listeria* counts. On Day 0, the addition of rosemary EO resulted in a 2 log reduction and increased to a 3 log reduction by Day 9 of storage ($P<0.001$). When the rosemary EO was combined with carrot this increased the anti-listerial effects slightly. Rosemary herb had a major anti-listerial effect with a 5 log reduction in counts seen on Day 0, however, this effect decreased during storage with a 3 log reduction in counts evident at the end of storage ($P<0.001$). When carrot was added to rosemary herb an additive effect was seen, where *Listeria* populations were reduced by a further 2 log cycle than with rosemary herb alone, and the carrot-herb combination resulted in significantly lower growth, that was 3 log cycles lower than the control by the end of storage ($P<0.001$).

At 4°C, similar effects were seen; however, in the case of the carrot-rosemary herb combination the anti-listerial effects were enhanced with no viable cells detected from

Day 0 to Day 6 ($P < 0.001$). The beneficial effects of adding carrot were lost by the end of storage, however, with similar results seen for rosemary herb and rosemary herb with carrot at Day 9 of storage.

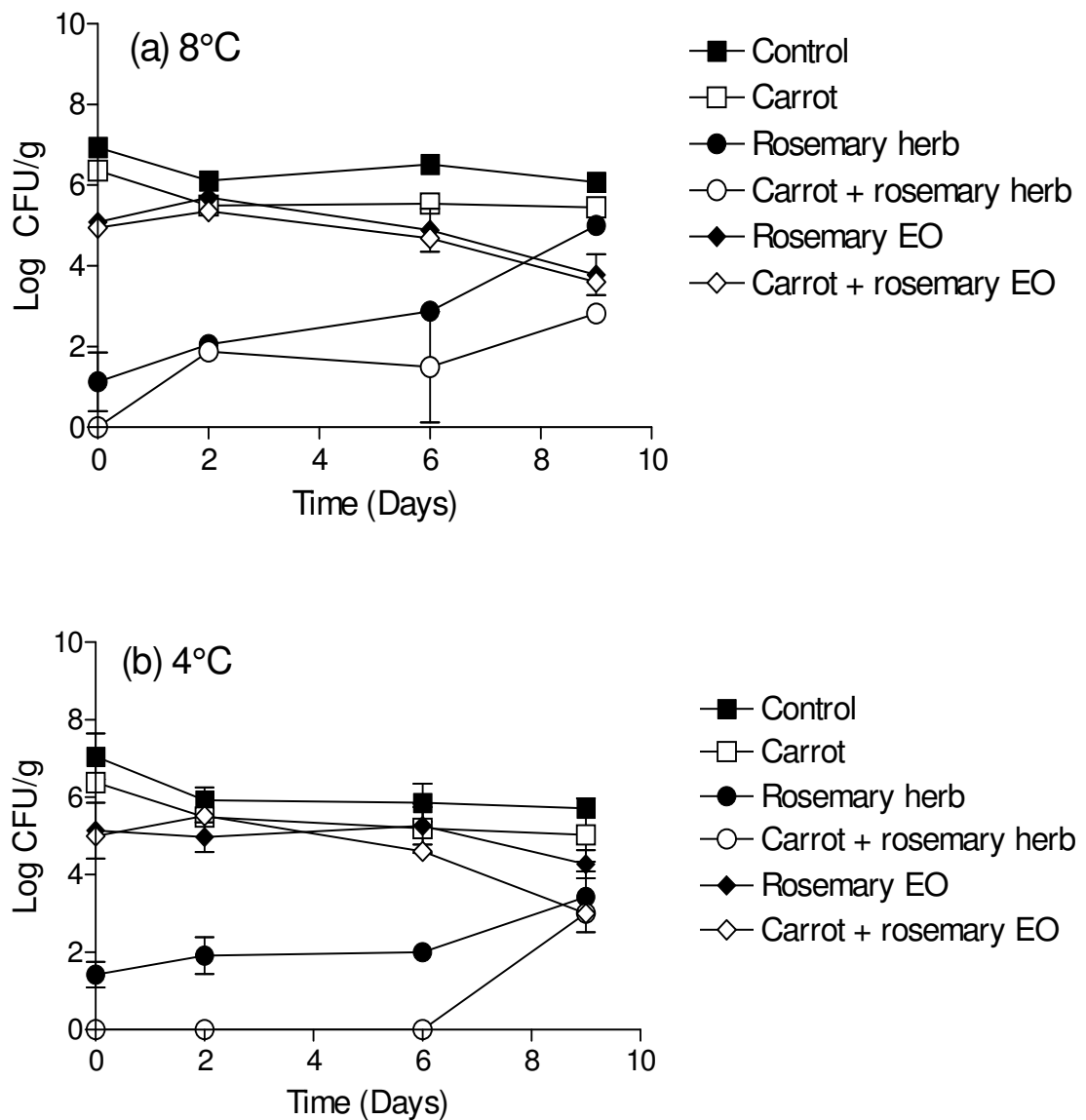


Figure 4.18: Effects of rosemary (EO and shredded fresh herb), carrot and rosemary and carrot combinations on survival and growth of *Listeria monocytogenes* (mixed strain) on fresh-cut MAP cabbage stored at (a) 8°C and (b) 4°C. Reported populations are means of 4 values. Error bars show s.d.

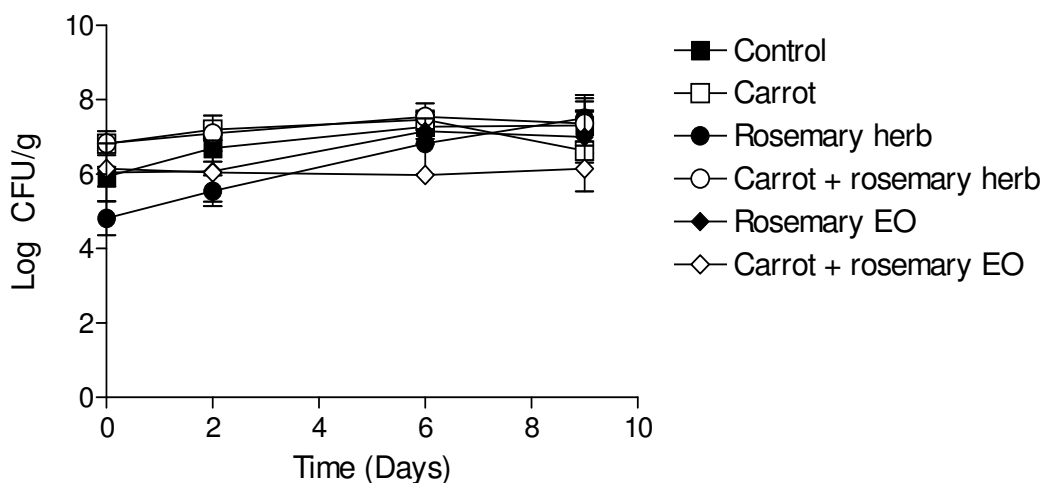


Figure 4.19: Effects of rosemary (EO and shredded fresh herb), carrot and rosemary and carrot combinations on total bacterial counts (TBCs) on fresh-cut MAP cabbage stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.19 shows the effects of rosemary and carrot combination treatments on total bacterial counts in MAP cabbage during storage at 8°C. There was no significant effect of carrot, rosemary herb or carrot and rosemary herb on the total bacterial counts in MAP cabbage in comparison to the control. Rosemary herb resulted in a slight decrease in TBCs on Day 0, but this effect was lost by Day 6 of storage. The addition of rosemary EO and carrot led to a 1 log reduction in total bacterial counts and this persisted throughout storage ($P < 0.05$). Similar trends were seen at 4°C (data not shown)

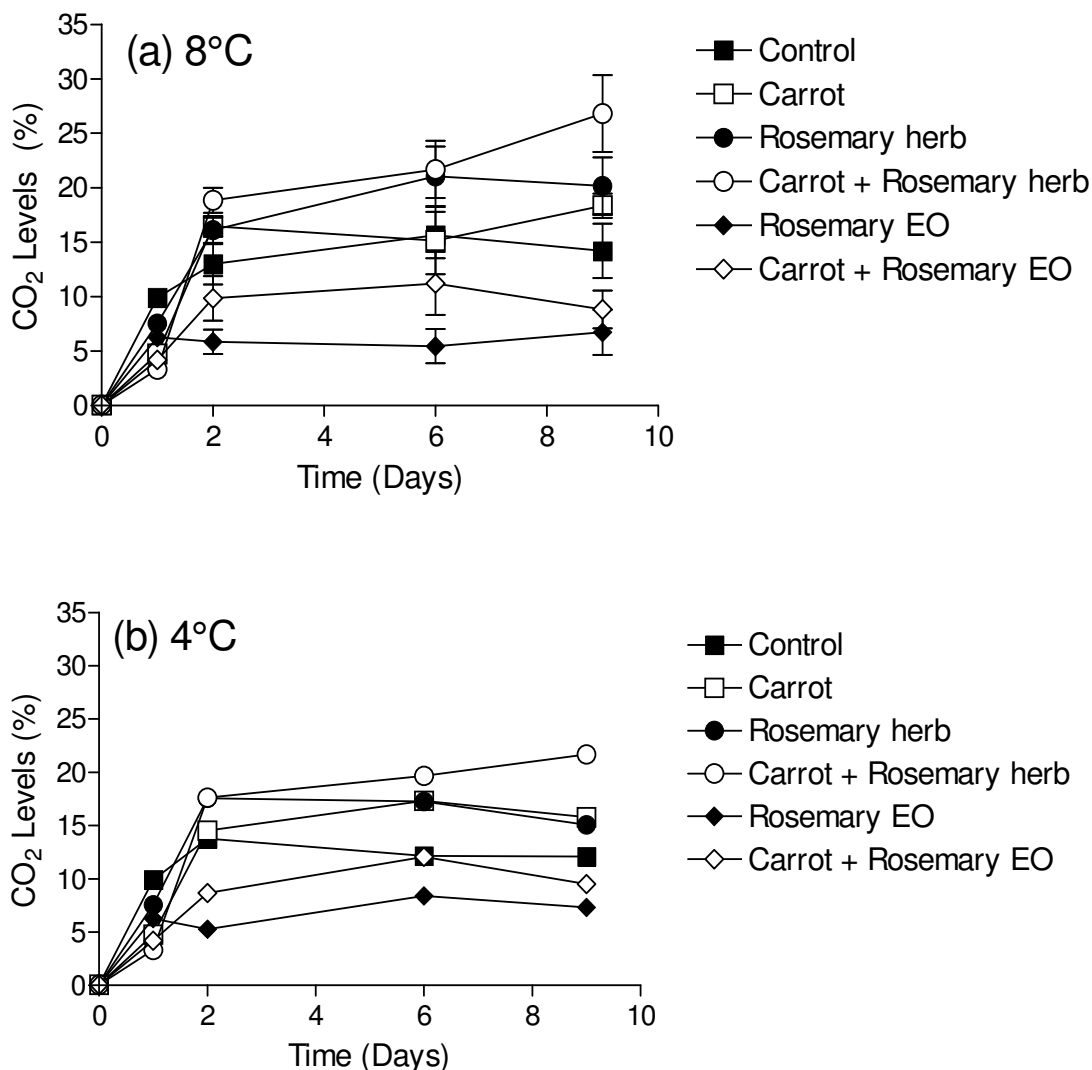


Figure 4.20: Effects of rosemary (EO and shredded fresh herb), carrot and rosemary and carrot combinations on gas atmospheres (CO_2 levels) in packages of MAP cabbage stored at (a) 8°C and (b) 4°C . Reported levels are means of 4 values. Error bars show s.d.

Figure 4.20 presents the results for the effects of rosemary and carrot treatments on gas atmospheres in packages of MAP cabbage at different storage temperatures. The addition of rosemary herb and carrot-rosemary herb combinations increased the CO_2 levels found in packs, particularly at 8°C , where gas levels rose to as high as 27% CO_2 . The addition of rosemary EO reduced the gas levels to ~ 6% CO_2 at both storage temperatures. However, the addition of carrot to the EO increased the levels slightly to

levels of 10% CO₂. Similar trends were seen at 4°C, however, these effects were reduced, presumably due to a reduction in respiration due to the lower temperature.

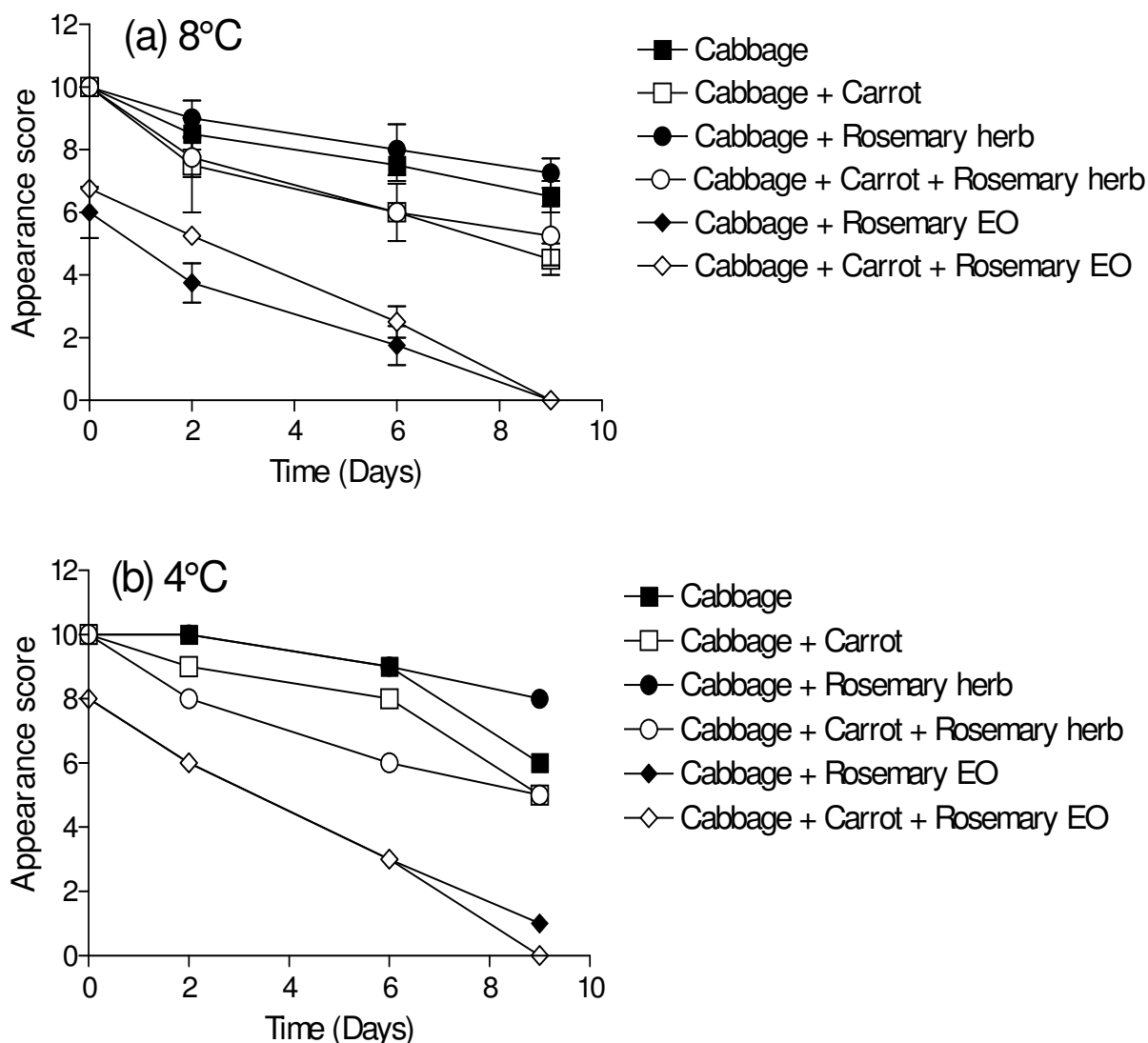


Figure 4.21: Effects of rosemary (EO and shredded fresh herb), carrot and rosemary and carrot combinations on appearance scores of MAP cabbage stored at (a) 8°C and (b) 4°C. Reported scores are means of 4 values. Error bars show s.d.

Addition of carrot lowered the appearance score of the cabbage by Day 2 of storage with levels dropping to a score of 5 for both carrot and carrot-rosemary herb combination treatments by Day 9 of storage at 8°C. The addition of rosemary EO rapidly reduced the appearance of the cabbage throughout storage resulting in an

appearance score of 1 by Day 9. The addition of rosemary herb maintained the appearance of the cabbage in comparison to the control at 8°C.

4.4.3 Effects of removing the herb prior to maceration in stomacher on *L. innocua* survival and growth

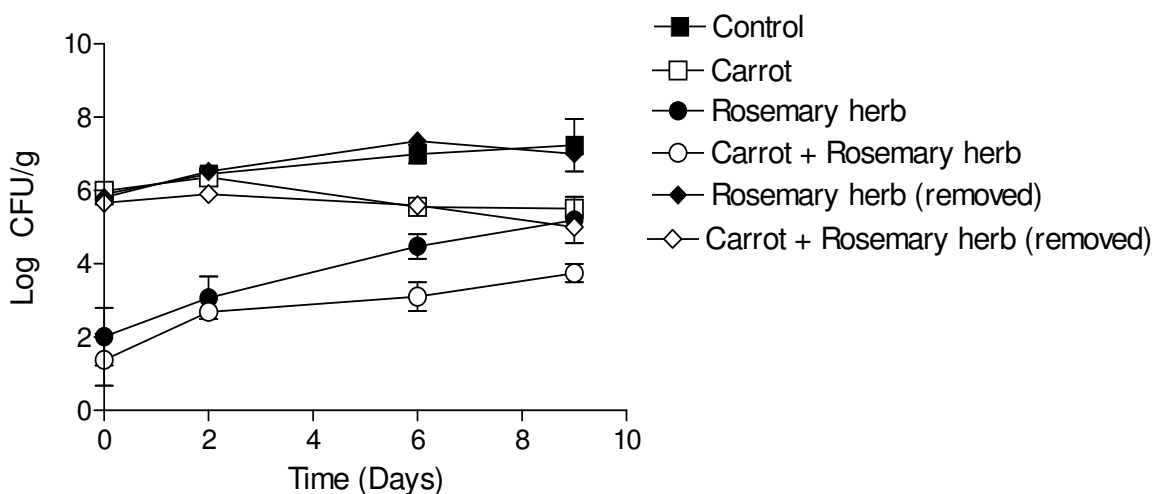


Figure 4.22: Effects of removing rosemary herb before maceration on survival and growth of *Listeria innocua* on fresh-cut MAP cabbage stored at 8°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.22 shows the effects of removing the rosemary herb prior to maceration in the stomacher on *Listeria* populations on MAP cabbage at 8°C. The addition of carrot had a slight anti-listerial effect. The addition of rosemary herb had a good anti-listerial effect with a 4 log reduction seen on Day 0 of storage ($P < 0.001$). Again, the combination of carrot and rosemary herb had an enhanced effect and a 5 log reduction was seen on Day 0 of storage, and while *Listeria* populations increased during storage, numbers were still significantly lower ($P > 0.001$) than the untreated cabbage by the end of storage. However, when rosemary herb was removed prior to maceration in the stomacher, the anti-listerial effects were not seen ($P > 0.05$), suggesting that the rosemary herb may also need to be stomached in order to release its anti-listerial effects.

4.3.5 The effect of different combinations and concentrations of EOs on *Listeria* on fresh shredded cabbage

Different concentrations and combinations of various EOs were examined to determine whether synergistic effects which would be likely to avoid damage to appearance could be achieved at lower concentrations.

4.3.5.1 Effects of different concentrations and combinations of thyme EO on *Listeria* (mixed strain)

In Figure 4.23, the effects of different concentrations and combinations of EOs against *Listeria* (mixed strain) on MAP cabbage are presented. Applying undiluted thyme EO to the shredded cabbage resulted in no viable cells being detected during storage ($P < 0.001$). By contrast, when the thyme EO was used diluted (200 ppm) no anti-listerial effects were seen. Similarly, when diluted thyme EO was used in combination with diluted rosemary (5,000 ppm), basil (10,000 ppm) or oregano EO (100 ppm), there were no anti-listerial effects. Adding thyme herb resulted in no reduction in *Listeria* counts.

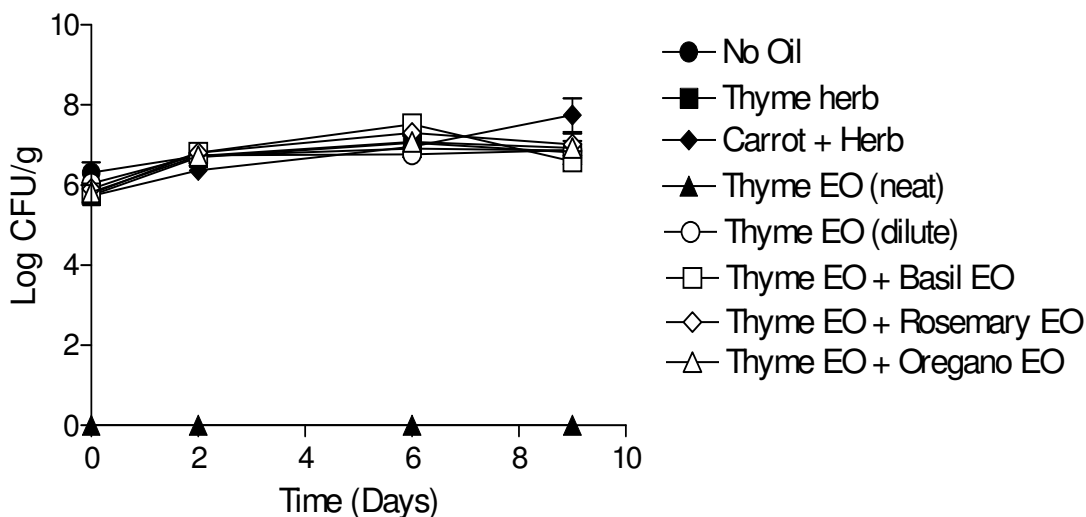


Figure 4.23: Effects of different concentrations and combinations of thyme (EO and fresh shredded herb) on survival and growth of *Listeria monocytogenes* (mixed strain) on MAP shredded cabbage during storage at 8°C. Reported populations are means of 4 values. Error bars show s.d.

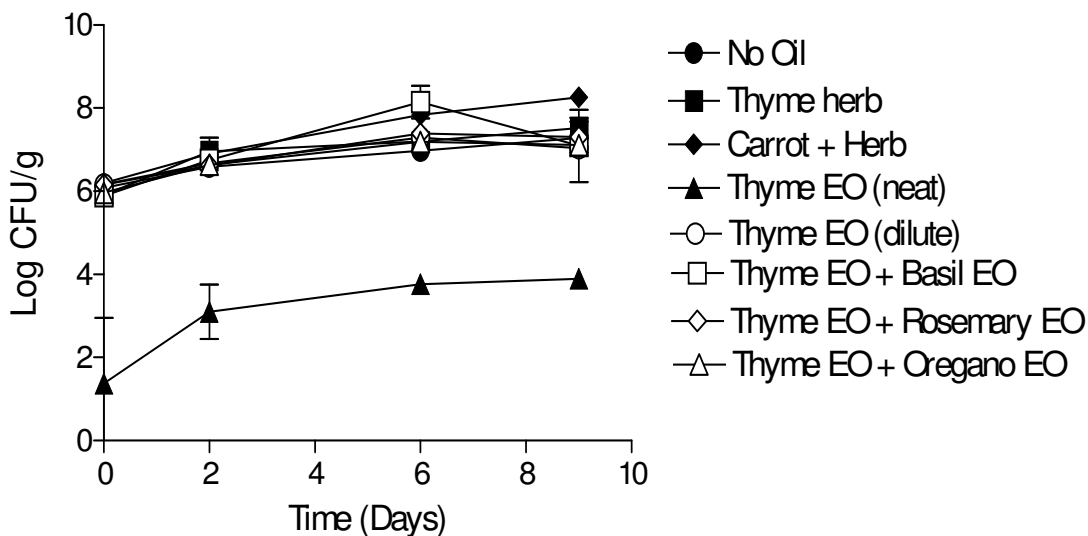


Figure 4.24: Effects of different concentrations and combinations of thyme (EO and fresh shredded herb) on total bacterial counts (TBCs) on MAP shredded cabbage during storage at 8°C. Reported populations are means of 4 values. Error bars show s.d.

The effects of different concentrations and combinations of thyme EO and fresh herb on total bacterial counts on MAP shredded cabbage during storage at 8°C are shown in

Figure 4.24. Undiluted thyme EO reduced initial populations by 5 log and produced a 3 log reduction in counts by Day 9 of storage. However, no reduction in total bacterial counts were seen for any of the combination treatments or for the diluted thyme EO.

The effects of the different combination treatments on the appearance score were also examined. While undiluted thyme had detrimental effects on the appearance of the coleslaw mix, diluting the thyme EO, and using dilute thyme EO in combination with rosemary, basil and oregano EO had no significant effect on the appearance scores (data not shown).

4.3.5.2 Effects of different concentrations and combinations of oregano EO on *Listeria* (mixed strain)

In Figure 4.25, the effects of different concentrations and combinations of oregano EO with basil, thyme and rosemary against *Listeria* on MAP cabbage are presented. Applying undiluted oregano EO to the shredded cabbage had a major anti-listerial effect with a 4 log inactivation on Day 0 and no viable cells being detected by the end of storage ($P < 0.001$). When the oregano EO was used diluted (200 ppm) no anti-listerial effects were seen. Similarly, when diluted oregano EO was used in combination with diluted rosemary (5,000 ppm), basil (10,000 ppm) or thyme EO (100 ppm), no anti-listerial effects were seen.

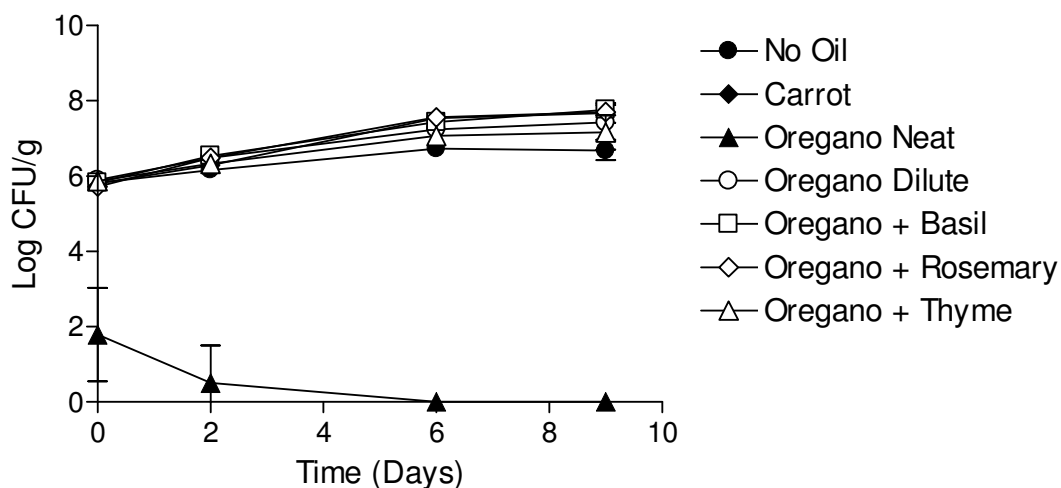


Figure 4.25: Effects of different concentrations and combinations of oregano (EO and fresh shredded herb) on *Listeria monocytogenes* (mixed strain) survival and growth on MAP shredded cabbage during storage at 8°C. Reported populations are means of 4 values. Error bars show s.d.

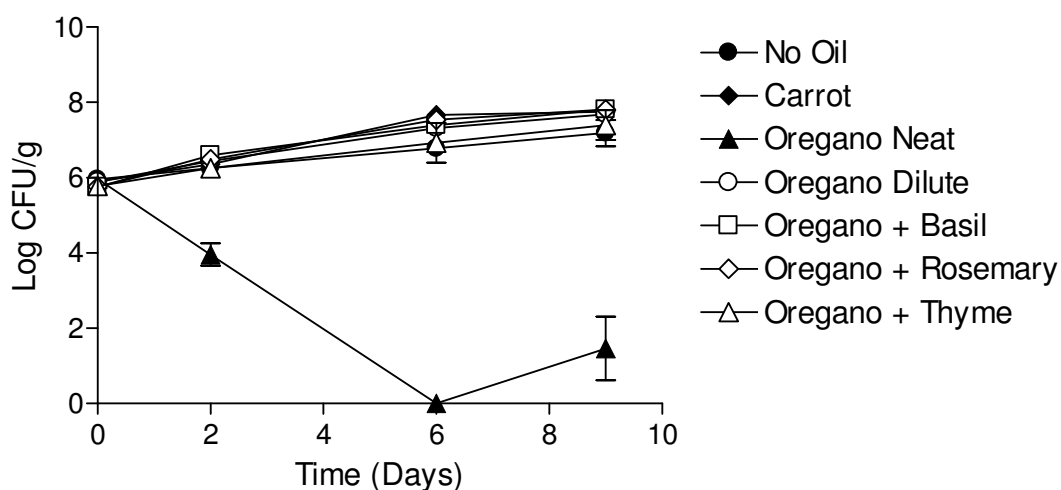


Figure 4.26: Effects of different concentrations and combinations of oregano (EO and fresh shredded herb) on total bacterial counts (TBCs) on MAP shredded cabbage during storage at 8°C. Reported populations are means of 4 values. Error bars show s.d.

Figure 4.26 shows the effects of different concentrations and combinations of oregano EO and herb on total bacterial counts on MAP shredded cabbage during storage at 8°C. Again when the EO was used neat a reduction in growth was seen with no viable cells being detected on Day 6 of storage and a 5 log inactivation was seen at the end of

storage. When the oregano EO was diluted or used in combination with basil, rosemary and thyme EOs no reduction in growth was seen, with populations remaining similar to the control.

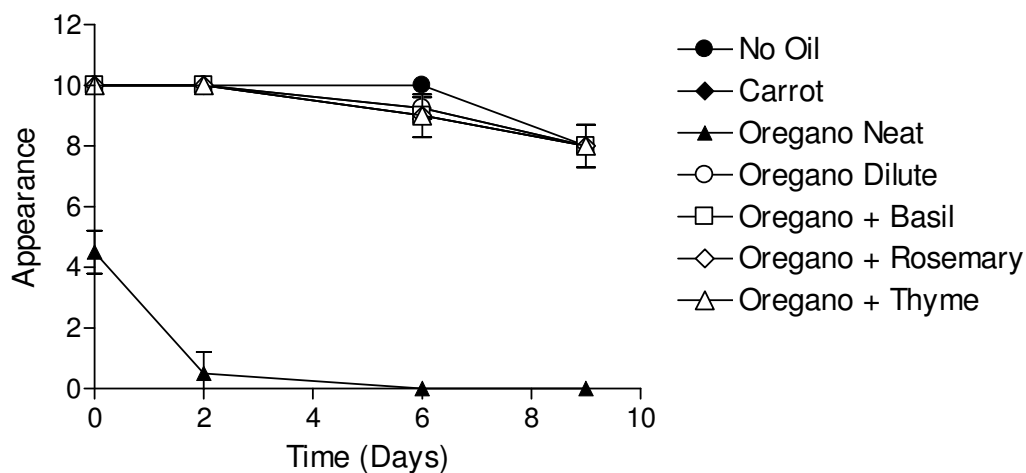


Figure 4.27: Effects of different concentrations and combinations of oregano (EO and fresh shredded herb) on the sensory quality of MAP shredded cabbage during storage at 8°C. Error bars show s.d.

The appearance of the shredded cabbage was also examined during storage (Figure 4.27). The untreated cabbage remained in good condition with a high appearance rating seen throughout the storage period. The addition of oregano EO undiluted had a detrimental effect with a rating score of 0 seen from Day 2 onwards. Diluting the EO and using it in combination with basil, rosemary and thyme EO had no affect on the appearance of the product.

4.3.5.3 Effects of different concentrations and combinations of rosemary EO on *Listeria* (mixed strain)

Figure 4.28 shows the effects of different concentrations and combinations of rosemary EO on *Listeria* counts on MAP cabbage during storage at 8°C. When the rosemary EO was used neat on the shredded cabbage, a 2 log reduction in *Listeria* populations were seen and this increased during storage to a 6 log reduction in counts on Day 9 (P<0.001). Rosemary herb resulted in a significant 5 log reduction in *Listeria* populations on Day 2 of storage (P<0.001) but populations increased during storage and this effect was lost by Day 9. When the rosemary EO was used diluted, no anti-listerial effects were seen. Similarly, when rosemary EO was used in combination with basil, oregano or thyme, counts were seen to be higher than those of the control.

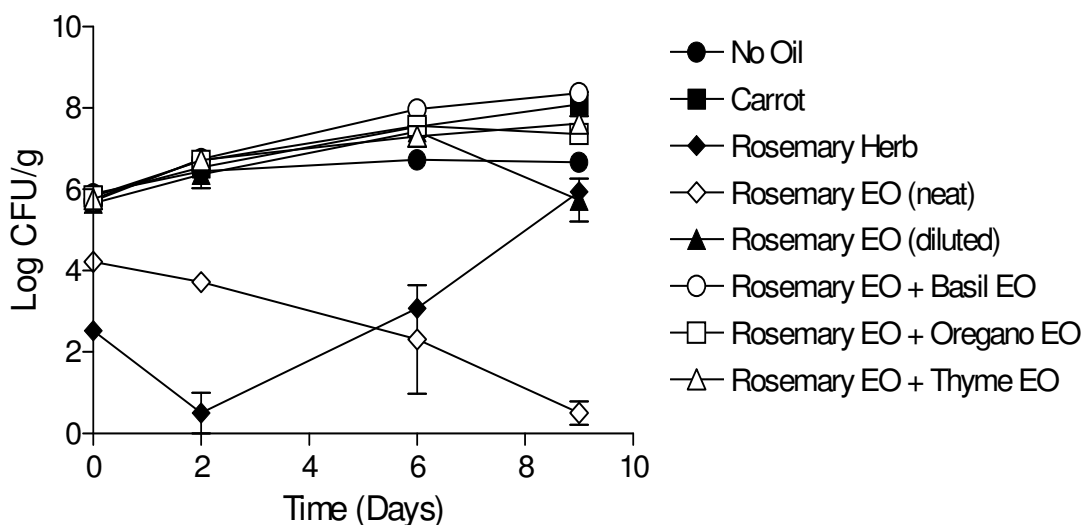


Figure 4.28: Effects of different concentrations and combinations of rosemary (EO and fresh shredded herb) on survival and growth of *Listeria monocytogenes* (mixed strain) on MAP shredded cabbage during storage at 8°C. Reported populations are means of 4 values. Error bars show s.d

Similar results were found for the total bacterial counts where no reductions in total counts occurred for any of the combination treatments or dilutions of the rosemary EO.

However, rosemary EO used neat and fresh rosemary herb produced approximately a 2 log reduction in initial total counts, but this effect was lost by the end of storage.

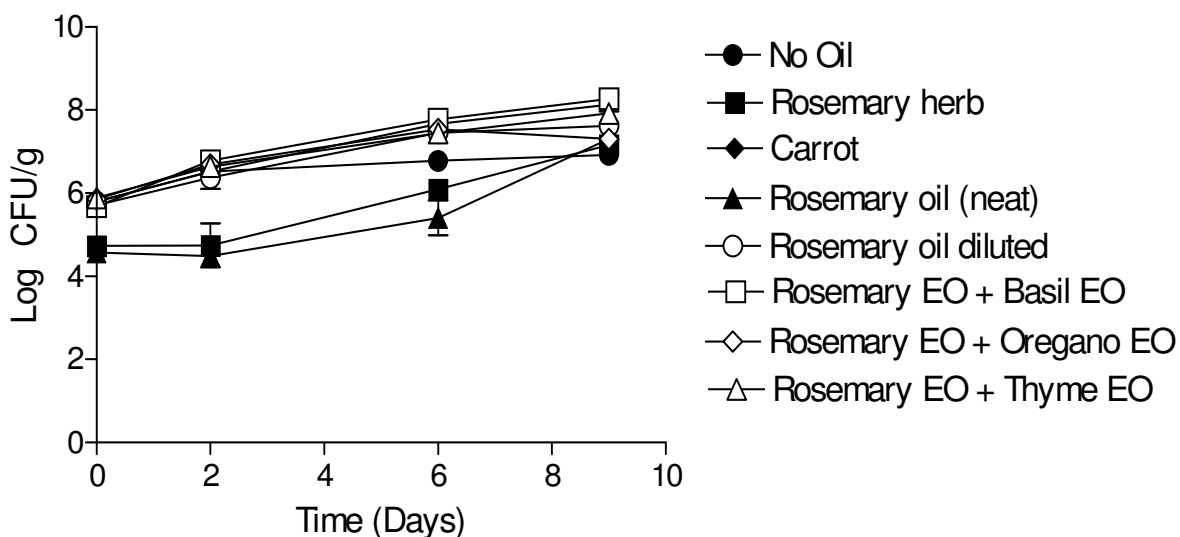


Figure 4.29: Effects of different concentrations and combinations of rosemary (EO and fresh shredded herb) on total bacterial counts (TBCs) on MAP shredded cabbage during storage at 8°C. Reported populations are means of 4 values. Error bars show s.d.

The effects of different concentrations and combinations of rosemary EO and herb on the appearance score of MAP shredded cabbage during storage at 8°C are shown in Figure 4.30. Adding carrot or rosemary herb to the shredded cabbage had no effect on the appearance of the cabbage throughout storage with the final appearance rating being the same as the control. As before the use of rosemary EO in its neat form had a negative effect on the appearance of the cabbage with a drop of 8 points seen in the appearance rating by Day 9 of storage. However, diluting the rosemary EO to 10,000 ppm and using dilute rosemary EO in combination with basil, oregano and thyme did not affect the appearance score of the cabbage until the end of storage.

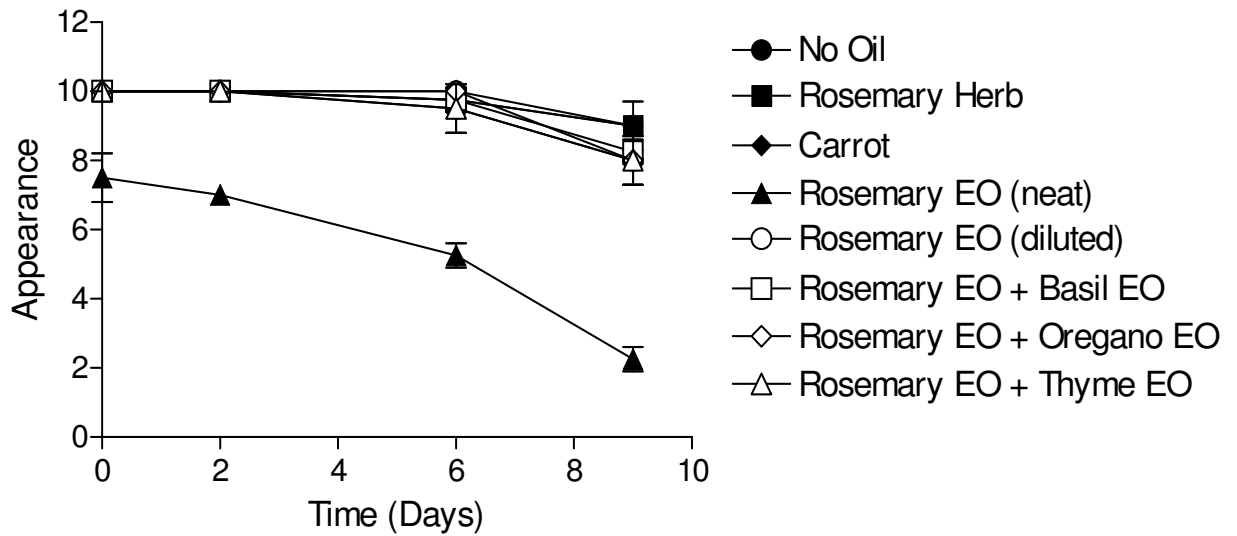


Figure 4.30: Effects of different concentrations and combinations of rosemary (EO and fresh shredded herb) on appearance score of MAP shredded cabbage during storage at 8°C. Reported populations are means of 8 values. Error bars show s.d.

A summary of the anti-listerial effects of thyme, rosemary and oregano EOs and fresh herbs on each different vegetable type is shown in Table 4.4.

	Reduction in counts Log CFU/g	Thyme		Rosemary		Oregano	
		EO	Herb	EO	Herb	EO	Herb
Lettuce *	<i>Listeria</i> counts	6	0	0	3	2	0
	TBCs	2	0	0	0	0	0
Carrot Discs	<i>Listeria</i> counts	6	0	3	6	ND	ND
	TBCs	0.5	0	0	0	ND	ND
Coleslaw Mix	<i>Listeria</i> counts	6	0	1	4	6	0
	TBCs	0.5	0	0	0	ND	ND
Shredded Cabbage	<i>Listeria</i> counts	6	0	2	6	6	ND
	TBCs	5	0	0	0	5	ND

Table 4.4 Summary of the overall reduction in counts (Day 0) for all the antimicrobial treatments tested on lettuce, carrot discs, coleslaw mix and shredded cabbage at 8°C. (* Results in chapter 3). Independent values (n = 4) were within 10% of each other. (ND = not determined).

4.4 Discussion

Complex anti-listerial and anti-bacterial effects in EOs and shredded herb equivalents were seen in this study. The work demonstrates that the antimicrobial effects of EOs varied depending on which EO was used, the method of application, the concentration of the EO and the vegetable products to which these were applied. Both anti-listerial and anti-bacterial effects were seen for thyme and oregano EOs. Thyme EO was found to be the most effective treatment against *L. monocytogenes* on all the vegetable products tested. This agrees with the results of Lis-Balchin and Deans (1997) who found thyme EO to be effective against 15 different strains of *L. monocytogenes*. Smith-Palmer *et al.* (1998) found that thyme EO in tryptone soy broth exerted a strong inhibitory effect against *L. monocytogenes*. Dorman and Deans (2000) showed thyme EO to be the oil with the widest spectrum of antimicrobial activity. Viuda-Martos *et al.* (2008) also found thyme EO to be a very potent inhibitor of six common food spoilage bacteria.

In this study, oregano EO was also found to have strong anti-listerial effects, but not as strong as those of thyme EO. Burt (2004) reported oregano EO to be effective against bacteria such as *Listeria*, *S. aureus* and *E. coli*. Gutierrez *et al.* (2008b) found that oregano and thyme EOs had the highest activity against all the tested bacteria including *Listeria* spp. Govaris *et al.* (2010) found that both oregano and thyme EO exhibited equal bacterial activity against *L. monocytogenes*. Chemical analysis of EOs, have found carvacrol and thymol to be present in high quantities in both oregano and thyme EOs. Carvacrol and thymol possess antimicrobial activity *in vitro* against a broad spectrum of Gram-positive bacteria including *L. monocytogenes* (Lis-Balchin and Deans 1997, Lambert *et al.* 2001). Thymol and carvacrol showed

inhibitory effects against *L. monocytogenes* at 24 °C in a microbiological medium at 0.5–0.7 w/v and were bacteriostatic at 0.5–1.0% (Davidson 2000).

Rosemary herb (but not rosemary EO) had a strong anti-listerial effect on all the vegetable products with a 4 log reduction seen on carrot and cabbage products. Rosemary herb has been shown to exhibit inhibitory effects against a number of bacteria. Yano *et al.* (2006) found that rosemary herb was effective against *E. coli* at both 5 and 30°C. Shelef *et al.* (1980) found that a concentration of 0.3% rosemary in the media inhibited growth of 21 Gram positive organisms. However, of the fresh herbs tested only rosemary had these effects, as no reductions in *Listeria* populations were evident with oregano or thyme herb. Pandit and Shelef (1994) also found that thyme herb did not exhibit any anti-listerial effects. The herb results are interesting and anomalous as there were no anti-listerial effects of the commercial EOs used.

However, during the course of this study a limitation on the effectiveness of the rosemary herb was discovered. Removing the rosemary herb prior to stomaching resulted in the loss of the anti-listerial effects; it appears that the herb is only effective when it is completely macerated with the vegetable sample in the stomacher. This effect may be due to the stomaching releasing particular antimicrobial components from the herb which are effective against *Listeria*. Studies by Smallfield *et al.* (2000) found that coriander subjected to heavy crushing had an increased oil yield due to all vittae being ruptured. Crushing garlic, was found to transform the alliin present into the biologically active allicin molecule, and it was this allicin molecule that was responsible for its remarkable antibacterial activity (Ankri and Mirelman 1999). In some cases, rosemary herb was found to lose its anti-listerial effectiveness by the end of storage. This is presumably due to a loss of the volatile components (which are

released upon maceration) due to evaporation/time. As antimicrobial levels are weaker, this may allow *Listeria* to adapt to these sub-lethal levels and survive and grow.

There appeared to be significant interactions between some antimicrobial effects of EOs/herbs and the presence of other vegetable components, notably carrot and cabbage. In the previous chapter, rosemary EO was found to have little or no anti-listerial effects on lettuce. However, rosemary EO showed stronger anti-listerial effects on cabbage and coleslaw. Thyme EO, rosemary EO and rosemary herb effects were enhanced on shredded cabbage. Rosemary EO reduced initial *Listeria* populations by 1 log cycle on coleslaw mix. The anti-listerial effects of rosemary EO were even greater on cabbage, with a 3 log reduction in *Listeria* populations by the end of storage. The effectiveness of rosemary herb was increased from 3 log cycles on lettuce to 6 log cycles when applied to shredded cabbage. Cabbage has been shown to generate methyl methanethiosulfonate (MMTSO), as a result of enzyme reaction when the tissue of the vegetable is disrupted (Kyung and Lee 2001), and MMTSO has been found to demonstrate antimicrobial activity (Kyung and Fleming 1994). Kyung and Fleming (1997) found that MMTSO was inhibitory against *Listeria monocytogenes*. The combination of thyme EO, rosemary EO and rosemary herb with cabbage appeared to enhance the effectiveness of the antimicrobials, and this effect was seen in both the coleslaw mix and in the shredded cabbage.

This effect of product was again seen in the carrot discs and was particularly evident when rosemary herb is added to carrot discs. Addition of rosemary herb to lettuce resulted in a 3 log reduction in *Listeria* populations on Day 0 (Figure. 3.9), whereas the addition of rosemary herb to carrot discs resulted in a 4 log reduction in initial listerial populations. This heightened anti-listerial effect seen on the carrot discs led to the inclusion of carrot as an antimicrobial treatment in experiments on other

products. To our knowledge, no studies have been carried out on the combination effect of carrot and EOs. However, carrot itself has long since been recognised as having strong anti-listerial effects. The lethal effect of shredded carrot on *Listeria monocytogenes* has been previously reported (Beuchat and Brackett 1990). Studies showed that treatment with 20 or 50% carrot juice resulted in significantly lower populations of viable *L. monocytogenes* (Beuchat and Doyle 1995). Noriega *et al.* (2010) showed that the anti-listerial effect was maximum on sliced carrots at 10 and 4°C. While the anti-listerial effect of carrot is known, its synergy with rosemary observed here has not been previously reported. On cabbage at 4°C, rosemary herb alone resulted in a 5 log reduction in *Listeria*. However, the combination of rosemary herb and shredded carrot resulted in a 6 log reduction with no viable cells detected from Day 0 to Day 6 of storage. The effect seemed to be additive.

While EOs have been shown to have antimicrobial effects *in vitro*, many studies suggest that greater concentrations are needed to achieve the same effects in foods (Shelef 1984). Accordingly, a challenge for practical application of EOs is to develop optimised low dose combinations to maintain product safety and shelf-life, thereby minimising the undesirable flavour and sensory changes associated with the addition of high concentrations of EOs. Gutierrez *et al.* (2008a) found that oregano in combination with thyme had a greater efficacy than when the EOs were tested separately against *E. coli* and *L. monocytogenes*, and combinations of marjoram or thyme mixed with basil, rosemary or sage showed additive effects against *L. monocytogenes*. Shelef *et al.* (1980) showed that a combination of sage and rosemary had enhanced antibacterial effects against 22 Gram negative and 24 Gram positive bacteria.

In this work, combinations of selected EOs (thyme, rosemary, oregano and basil) were tested against *L. monocytogenes* in shredded cabbage. Results showed that when thyme EO was diluted or used in combination with diluted rosemary, basil or oregano EO, no anti-listerial effects were seen, whereas when thyme EO was used undiluted it was very effective and no viable *Listeria* cells were detected throughout storage. Similar results were found for oregano EO combinations. Gutierrez et al (2008a) showed some EO combinations to have an additive effect *in vitro*, however, this effect was not seen when combinations were applied to the shredded cabbage samples for either thyme or oregano, and in fact the combination of these oils appeared to reduce the anti-listerial effects. For rosemary, when used diluted or in combinations, populations were higher than the untreated cabbage by the end of storage, showing the combinations to be enhancing listerial growth rather than reducing it. Similar results were found by Lis-Balchin and Deans (1998) who showed that there was an apparent 'dilution' of activity of the most active antibacterial essential oil by the other oils used in the combinations and that, in most cases, the antibacterial activity of the mixture was found to be less than that of the most active individual essential oil.

In this study, the application of all EOs (thyme, rosemary and oregano) in their undiluted form resulted in unacceptable appearance scores. Application of the EOs directly to the vegetable products led to discolouration and a loss of texture which the panellists found unappealing. Undesirable organoleptic effects might be limited by careful selection of EOs according to the type of food, by diluting EOs, by using combinations of EOs, or by using EOs in combination with plant material such as carrot or cabbage shreds. In this work, the combinations of the diluted EOs had no negative effects on the appearance of the vegetable samples, but had no anti-listerial effects. Surprisingly, Gutierrez *et al.* (2008b) reported that on a carrot model product,

basil, lemon balm, marjoram, oregano and thyme EOs were deemed organoleptically acceptable; and that only oregano and marjoram EOs were deemed acceptable for lettuce. Although the use of EOs at lower concentrations did not eliminate *Listeria*, they might be useful for reducing bacterial growth in combination with other preservation methods, thus reducing sensory impact of the EOs.

In summary, in terms of anti-listerial properties: thyme EO > oregano EO > rosemary fresh herb > rosemary EO. Thyme and oregano fresh herb had no anti-listerial effects. However, while rosemary herb appeared to have good anti-listerial effects, it was only effective when stomached. Product type also had a major impact on the effectiveness of the plant antimicrobials with greater reductions on carrot discs, coleslaw and cabbage than on lettuce. Furthermore, combinations of rosemary herb and shredded carrot as antimicrobials had an additive effect.

Of all these effects, the apparent emergence of strong anti-listerial activity in rosemary herb during stomaching, in contrast to little or no anti-listerial activity in commercial rosemary EO, was considered the most novel finding and this will be explored further in the next chapter. The effect seemed uniquely anti-listerial with little or no effect on other bacteria. This contrasts with the observations of both antibacterial and anti-listerial effects in thyme and oregano EOs. The suggestion that components in the herb could be synthesised in response to the stresses of the stomaching process, and may be critical for the anti-listerial effect, will be explored. GC-MS will be used to evaluate the effect of extraction methods and stomaching on the chemistry of rosemary EO, in order to discover whether critical anti-listerial chemicals are lost/formed during these processes.

4.5 References

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Chapter 5

Effects of extraction methods on anti-listerial effectiveness of rosemary essential oils

5.1 Abstract

In order to investigate the anti-listerial effect of macerated (in stomacher) rosemary herb, the anti-listerial activity and chemical composition of rosemary oil obtained by different extraction methods was initially determined. In terms of anti-listerial effectiveness (1% v/v): hydrodistillate > CO₂ extracted EO > hexane/acetone extracted EO > methanol extracted EO. The chemicals present were similar in all of the rosemary extracts tested but the percentage composition varied considerably. Gas chromatography-mass spectroscopy identified the main components as camphor, verbenone, borneol, bornyl acetate and caryophyllene. Each of these individual components showed good anti-listerial activity, with borneol and verbenone the most effective, however, principal component analysis showed that levels of verbenone made the biggest contribution to anti-listerial activity. The hydrodistillate, which had the highest anti-listerial activity, contained the highest levels verbenone (5,126 ppm). The extract of macerated rosemary herb had more than twentyfold the level of verbenone found in the un-macerated rosemary extract. When headspace analysis was carried out on uncut, freshly chopped and macerated rosemary herb, the relative levels of verbenone were 0, 6 and 118 ppm. The strong anti-listerial activity of rosemary herb during stomaching may be explained by this very high concentration of verbenone.

5.2 Materials and Methods

5.2.1 *Listeria monocytogenes*

For all antimicrobial testing carried out the *Listeria innocua* strain (NCTC 11288) was used as a model for pathogenic *Listeria monocytogenes*. Cultures were maintained at -20°C in Tryptone soya broth (TSB) supplemented with 20% (v/v) glycerol, and were grown in tryptone soya broth (10 ml TSB, Oxoid CM129, Fannin Healthcare, Cork, Ireland) at 35°C for 24 h. Cultures were then centrifuged (5000 g, 15 min) and diluted in phosphate buffered saline (PBS, Oxoid BR014, Fannin Healthcare) to allow for contamination of agar plates/broth at initial levels of approximately 10⁵-10⁶ CFU/g.

5.2.2 Essential oil extracts

The following essential oil extracts of rosemary were compared:

1. Supercritical CO₂ extract

Commercial rosemary essential oil (CO₂ supercritical fluid extract) was obtained from Guinness Chemicals Ltd. Portlaoise, Ireland.

2. Hydrodistillate

Fresh rosemary herb was purchased from Richardson's Foods, Limerick. The herb (50 g) was chopped and EO extracted in the distillate with 450 ml of water for 90 min until no more EO was obtained (Figure 5.1). The essential oil was collected and stored at 4°C in the dark until used.

3. Hexane:Acetone extract

Rosemary herb (5 g) was ground using a blender in 45 ml of solvent. The solvent used was a mixture of hexane (Sigma-Aldrich 34859, Germany) and Acetone (00570 Fluka Switzerland) in a 70:30 ratio. Extracts were stored at 4°C until used.

4. Methanol extract

Rosemary herb (5 g) was ground using a blender in 45 ml of methanol (Sigma-Aldrich 34859, Germany). Extracts were stored at 4°C until used.

4. Stomached extract

Rosemary herb (5 g) was homogenised for 2 min, with 45 ml of hexane/acetone (Sigma-Aldrich 34859, Germany) in a 70:30 ratio, using a Seward laboratory stomacher (Stomacher 400, AGB Scientific, Ireland).

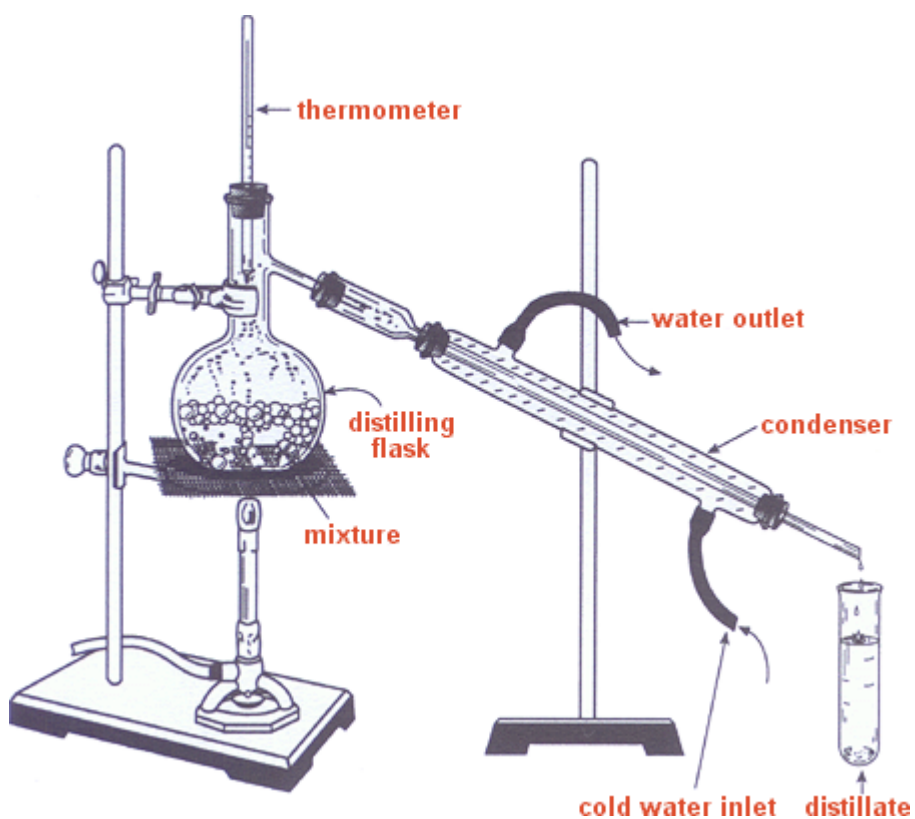


Figure 5.1: Hydrodistillation apparatus

5.2.3 Anti-listerial tests

5.2.3.1 Disc Diffusion

Rosemary EO extracts and their major chemical components (borneol, bornyl acetate, camphor, caryophyllene, verbenone) were screened for antimicrobial activity against *L. innocua* using the agar disk diffusion method of the 1997 NCCLS approved standard M2-A6. In brief, the *Listeria* culture (100 µl) was surface spread onto LSA in different concentrations (10^3 to 10^6 CFU/g). Sterile filter paper disks (Whatman no. 1, 6 mm in diameter) with 10 µl of the EO extracts or chemical components applied at different concentrations (0.01% to 1% v/v) were applied to the centre of each inoculated plate. For the component mixtures, 5 µl of each component (combined 10 µl) was added to the disc. The plates were incubated at 35°C overnight, and the diameters of the resulting zones of inhibition were measured in millimetres.

5.2.3.2 Broth dilution

A broth dilution method was used to determine the anti-listerial effectiveness of the rosemary EO and extracts. Overnight broth cultures of *L. innocua* were prepared in TSB-YE. The supercritical CO₂ extract, hydrodistillate, hexane:acetone extract and methanol extract were added to the broths in concentrations ranging from 0.01% to 1% v/v. Broths were incubated at 35°C for up to 24 h. Microbiological analyses were carried out at Time 0 and at intervals throughout the storage period (Time 3, 6 and 24 h).

At time of testing, serial dilutions of each broth sample were made in PBS and were surface spread (100 µl/ plate) in duplicate on media. Numbers of *L. monocytogenes* were determined on *Listeria* selective agar (LSA, Oxoid CM 856) containing a modified *Listeria* selective supplement (Oxoid SR0206) after 48 h at

35°C. Following incubation, colonies were counted and results were expressed as colony forming units per gram of sample. Tests were performed in duplicate.

5.2.4 Gas Chromatography – Mass Spectrometry

The chemical composition of the extracts was determined using gas chromatography-mass spectroscopy (Varian model 431 gas chromatograph coupled to a Varian model 210 mass detector). A CP-Wax 52 CB fused-silica capillary column (30 m x 25 mm i.d., $df = 0.25 \mu\text{m}$) was used. The oven temperature was programmed from 60°C to 230°C at 2°C/min and then held isothermally at 230°C for 35 min.

Injector and detector temperatures were maintained at 280°C. Samples were injected in the splitless mode, using helium as the carrier gas (1ml/min); injection volume was 0.1 μl of sample.

Standards were injected into the GC-MS at concentrations of 100-10,000ppm. A calibration curve was constructed and used to quantify the individual chemical components.

For analysis of volatiles, the following packs were compared:

1) Fresh rosemary herb

Fresh rosemary herb (5 g) was placed in a pack and sealed before testing.

2) Fresh chopped rosemary herb

Fresh rosemary herb (5 g) was chopped, placed immediately into packs and sealed prior to testing.

3) *Stomached rosemary herb*

Fresh rosemary herb (5 g) was placed in packs with 50 ml of distilled water and sealed. Packs were then macerated in a stomacher on high speed for 2 min.

For the measurement of the volatiles the following protocol was used: Following sample preparation, 1 ml of volatiles was removed from the headspace of sealed packs using a gastight syringe. Samples were then injected into GC-MS and analysed under the same conditions as above.

5.2.5 Statistical analysis

All experiments were carried out twice and in duplicate. Reported populations therefore represent the means of two values. Figures show the means \pm standard deviation. Statistical differences were determined using a Two-way ANOVA with Bonferroni's post test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA).

In order to elucidate which chemical in the mixture was most important in inducing the kill of *Listeria*, a principal components analysis (PCA) was carried out using MVSP (multivariate statistics package, version 3.1, Kovach Computing Services, 2004; www.kovcomp.com/mvsp).

5.3 Results

5.3.1 Effects of extraction method on effectiveness of rosemary EO against *L. innocua*

Preliminary testing was carried out using the agar disc diffusion method.

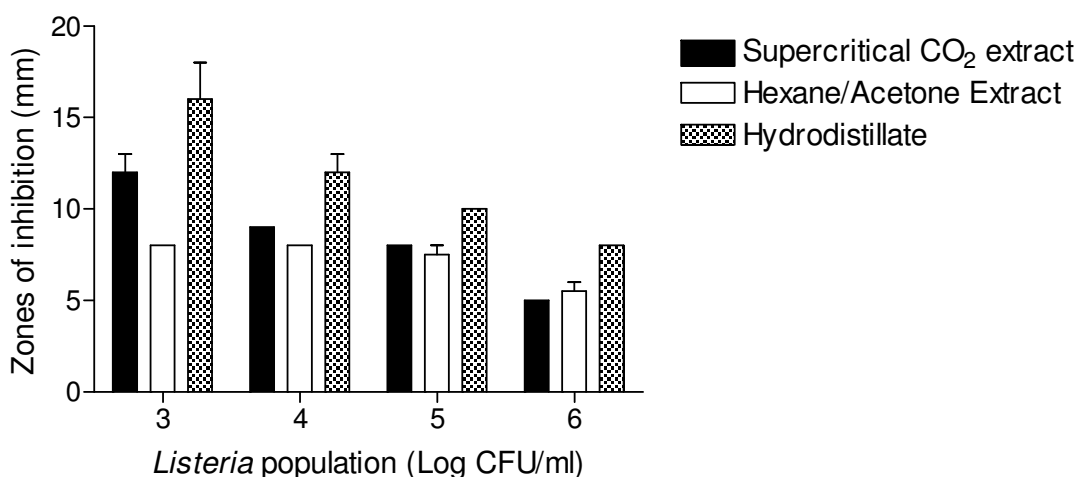


Figure 5.2: Effects of extraction method on the effectiveness of EO against *Listeria innocua* using the agar disc diffusion method. Reported results are means of 4 values. Error bars show s.d.

Figure 5.2 shows the effects of different extraction methods on *L. innocua* survival; at levels of 3 log CFU/ml of *Listeria innocua*, the hydrodistillate had the strongest anti-listerial effect with a 16 mm zone of inhibition. This was followed by supercritical CO₂ extract (12 mm), and the hexane/acetone extract (8 mm). This was true for all other *Listeria* counts, where again the hydrodistillate was more effective than the other two extraction methods, and proved to be a good anti-listerial agent up to 6 log CFU/g.

Studies have shown that the agar disc diffusion assay is not suited to natural antimicrobial compounds that are scarcely soluble or insoluble in water and thus their hydrophobic nature prevents uniform diffusion through the agar media (Klancnik *et al.* 2010). As a result, further antimicrobial testing using the broth dilution method was carried out.

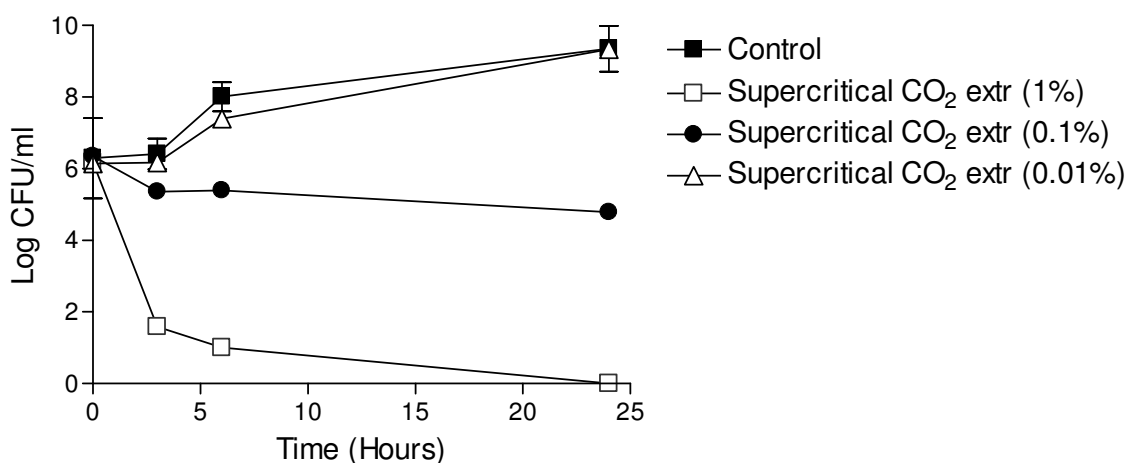


Figure 5.3: Effects of supercritical CO₂ extract of rosemary on survival and growth of *Listeria innocua* in TSB over 24 hours. Reported populations are means of 4 values. Error bars show s.d. where present.

Addition of supercritical CO₂ extract was effective in reducing populations of *L. innocua* at both 0.1% and 1% v/v at all sampling times ($P < 0.05$, Figure 5.3). The 0.1% v/v level inactivated growth by 3 hours and by 4 log cycles after 24 hours ($P < 0.001$). A reduction in the *Listeria* population was evident at levels of 1% v/v, where a 5 log reduction in growth was seen after 3 hours and no viable cells were detected by 24 hours ($P < 0.001$) compared to the control. The addition of extract at levels of 0.01% v/v had little or no anti-listerial effects.

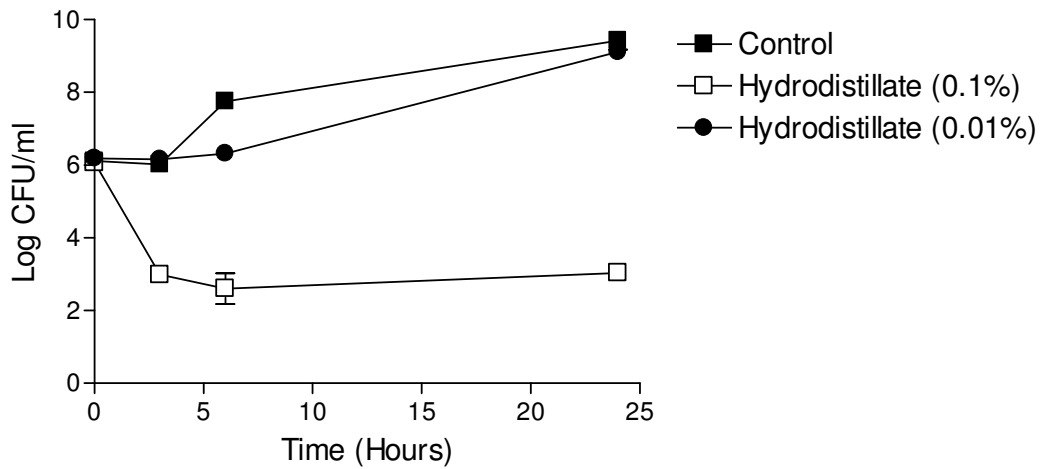


Figure 5.4: Effects of hydrodistillate of rosemary on survival and growth of *Listeria innocua* in TSB over 24 hours. Reported populations are means of 4 values. Error bars show s.d. where present.

Figure 5.4 shows the effects of the hydrodistillate on *Listeria* survival and growth. Addition of 0.01% v/v resulted in a slight anti-listerial effect at 6 hours ($P < 0.001$), however this effect was lost by the end of the 24 hours period. At 0.1% v/v, *Listeria* populations were reduced by 2 log cycles after 3 hours and remained at this level throughout storage ($P < 0.001$).

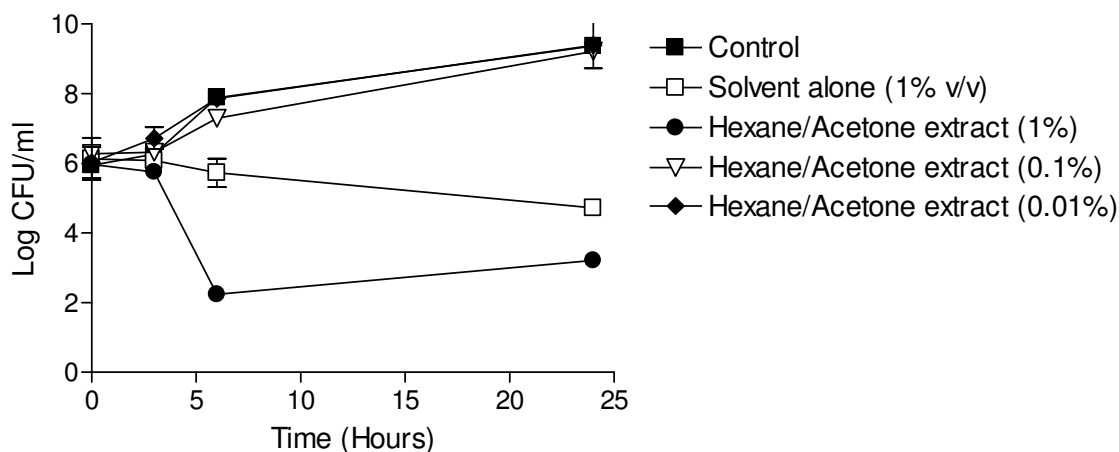


Figure 5.5: Effects of hexane/acetone extract of rosemary on survival and growth of *Listeria innocua* in TSB over 24 hours. Reported populations are means of 4 values. Error bars show s.d.

Figure 5.5 shows the effects of the hexane/acetone extract on *Listeria* survival and growth. Levels of both 0.01% and 0.1% v/v showed little or no anti-listerial effects. Addition of 1% v/v extract resulted in a 4 log reduction in growth after 6 hours ($P < 0.001$). While growth increased after this period, growth was still 5 log cycles lower than that of the control at 24 hours ($P < 0.001$). However, the solvent itself also had an anti-listerial effect, reducing *Listeria* populations by 2 log cycles after 6 hours and 4 log cycles by 24 hours ($P < 0.001$).

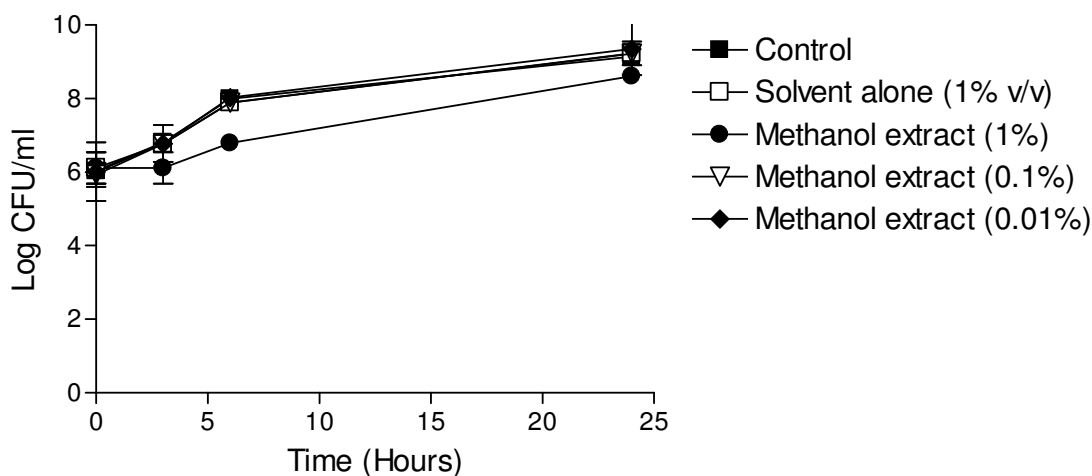


Figure 5.6: Effects of methanol extract of rosemary on survival and growth of *Listeria innocua* in TSB over 24 hours. Reported populations are means of 4 values. Error bars show s.d.

Figure 5.6 shows the effects of the methanol extract on *Listeria* survival and growth. There was no significant difference ($P > 0.05$) in *Listeria* survival and growth when 1% v/v methanol was added to the broth. Extracts at levels of 0.1% and 0.01% v/v had no anti-listerial effects throughout storage. Extract levels of 1% reduced counts by 1 log cycle from 6 hours to 24 hours ($P < 0.001$).

5.3.2 Effects of extraction method on the chemical composition of rosemary extracts

In the essential oil obtained by CO₂ extraction, borneol, bornyl acetate, camphor, caryophyllene and verbenone were identified (Figure 5.7). Camphor was the most abundant component in the essential oil (2,560 ppm), followed by verbenone (2373 ppm). The relative content of the main components was: camphor > verbenone > caryophyllene > borneol > bornyl acetate (Table 5.1).

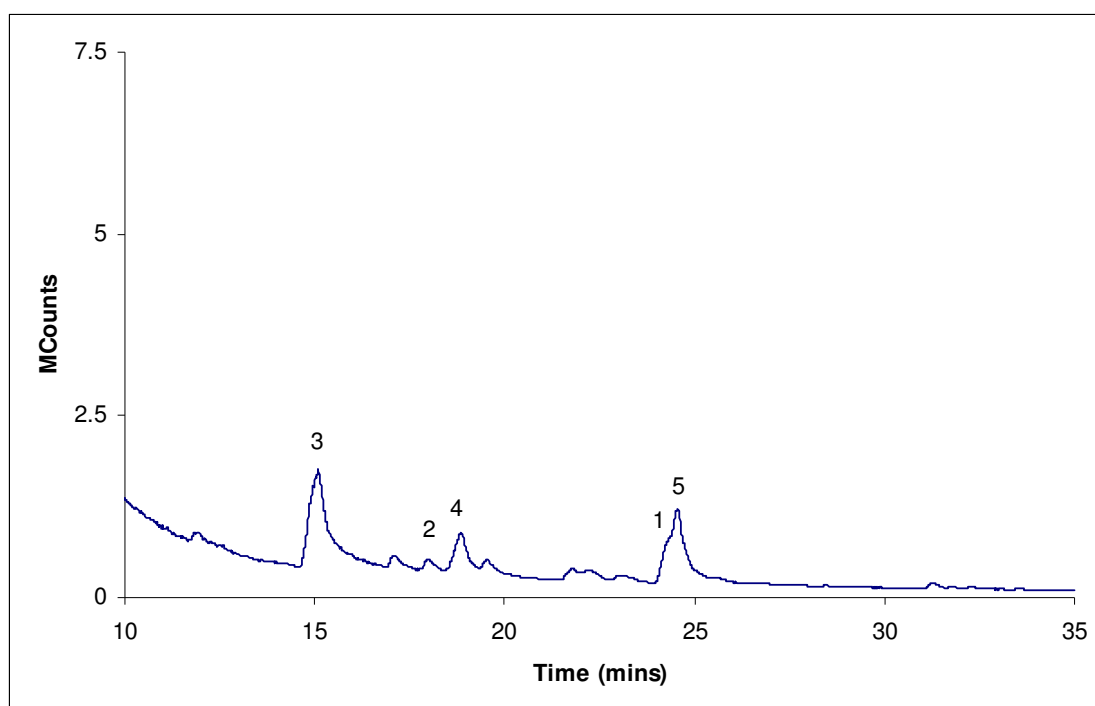


Figure 5.7: Chromatograph of supercritical CO₂ extract of rosemary (1% v/v)

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.290	95	5.30E+06	443
2	Bornyl Acetate	18.000	95	475740	1089
3	Camphor	15.056	95	1.09E+07	2,560
4	Caryophyllene	18.792	132.9	1.41E+06	943
5	Verbenone	24.540	106.9	1.85E+06	2,373

Table 5.1: Levels of main components in supercritical CO₂ extract of rosemary. Data are means of two determinants. Independent values were within 10% of each other. Five components were identified by GC-MS in the hydrodistillate extract (Figure 5.8).

Again the most abundant compound detected was camphor (10,339 ppm). Verbenone, borneol and bornyl acetate were also present in large amounts. The relative abundance of the main components was: camphor > verbenone > borneol > bornyl acetate > caryophyllene (Table 5.2).

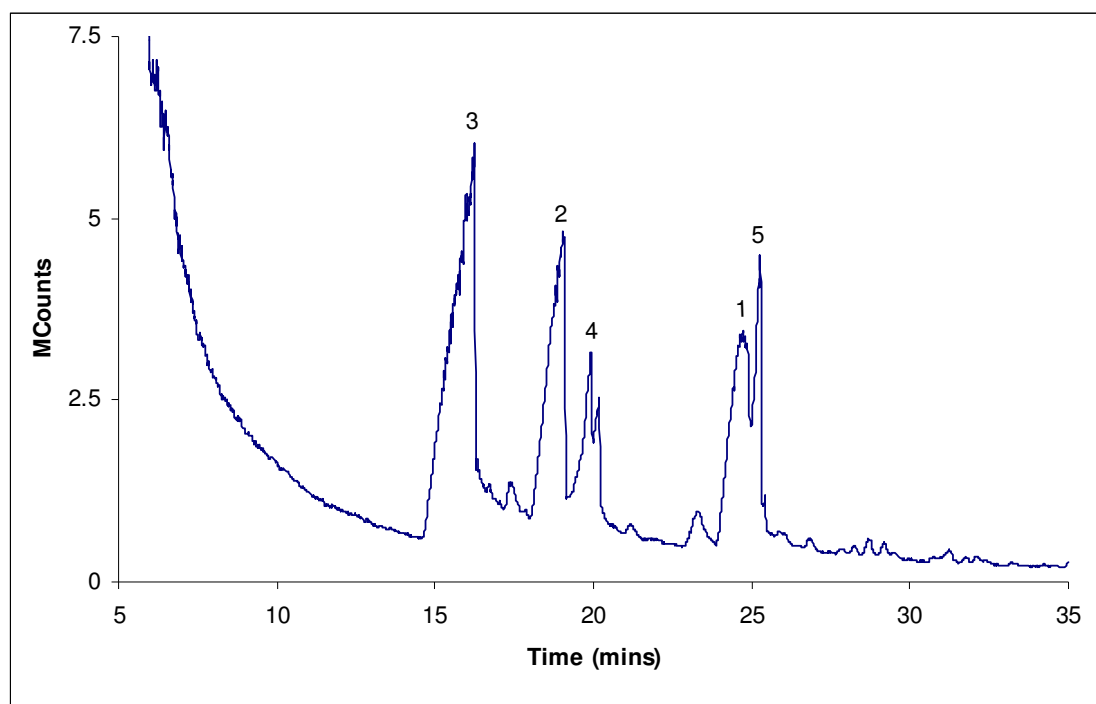


Figure 5.8: Chromatograph of hydrodistillate of rosemary (1% v/v)

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.496	95	4.01E+07	3,792
2	Bornyl Acetate	18.897	95	1.38E+07	1,319
3	Camphor	15.932	95	4.39E+07	10,339
4	Caryophyllene	18.769	132.9	240287	161
5	Verbenone	25.299	106.9	4.02E+06	5,126

Table 5.2: Levels of main components in hydrodistillate of rosemary. Data are means of two determinants. Independent values were within 10% of each other.

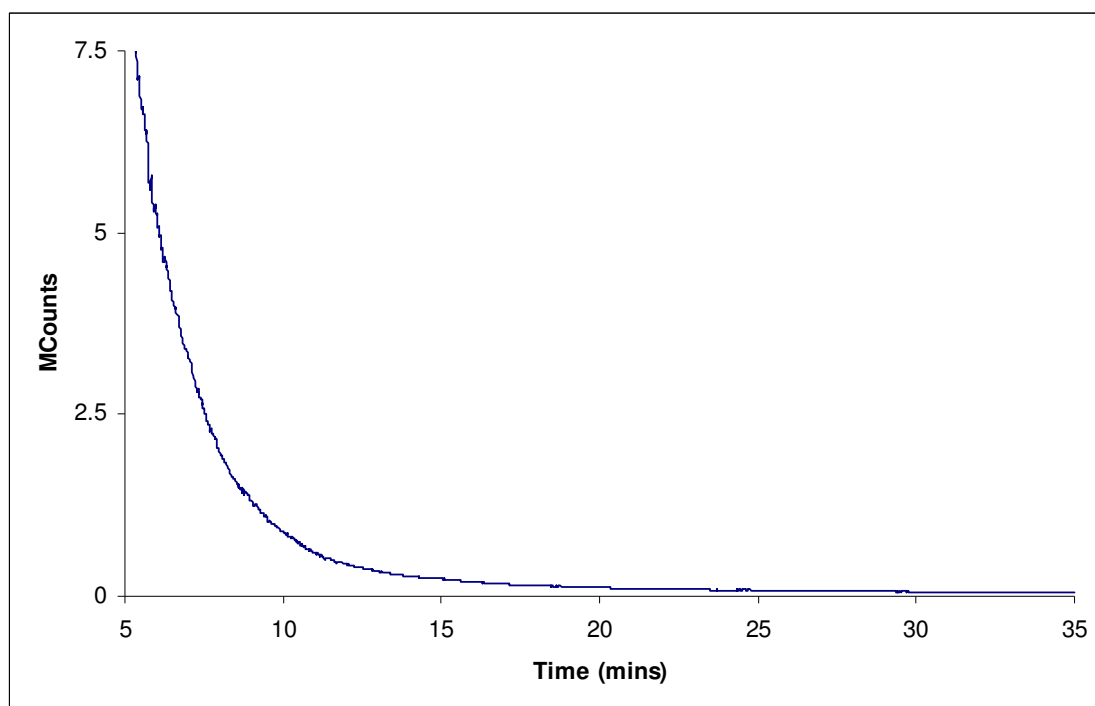


Figure 5.9: Chromatogram of hexane/acetone extract of rosemary (1% v/v)

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.395	95	56983	Trace
2	Bornyl Acetate	18.107	95	7634	1
3	Camphor	15.016	95	84464	20
4	Caryophyllene	18.858	132.9	9130	6
5	Verbenone	24.784	106.9	16178	49

Table 5.3: Levels of main components in hexane/acetone extract of rosemary. Data are means of two determinants. Independent values were within 10% of each other.

Five chemical components were also identified in the hexane/acetone extract but levels were much lower than in the other extracts. Verbenone was the most abundant compound (49 ppm), followed by camphor (20 ppm). Smaller amounts/traces of caryophyllene, bornyl acetate and borneol were also recorded. The relative amounts were: verbenone > camphor > caryophyllene > bornyl acetate > borneol (Table 5.3).

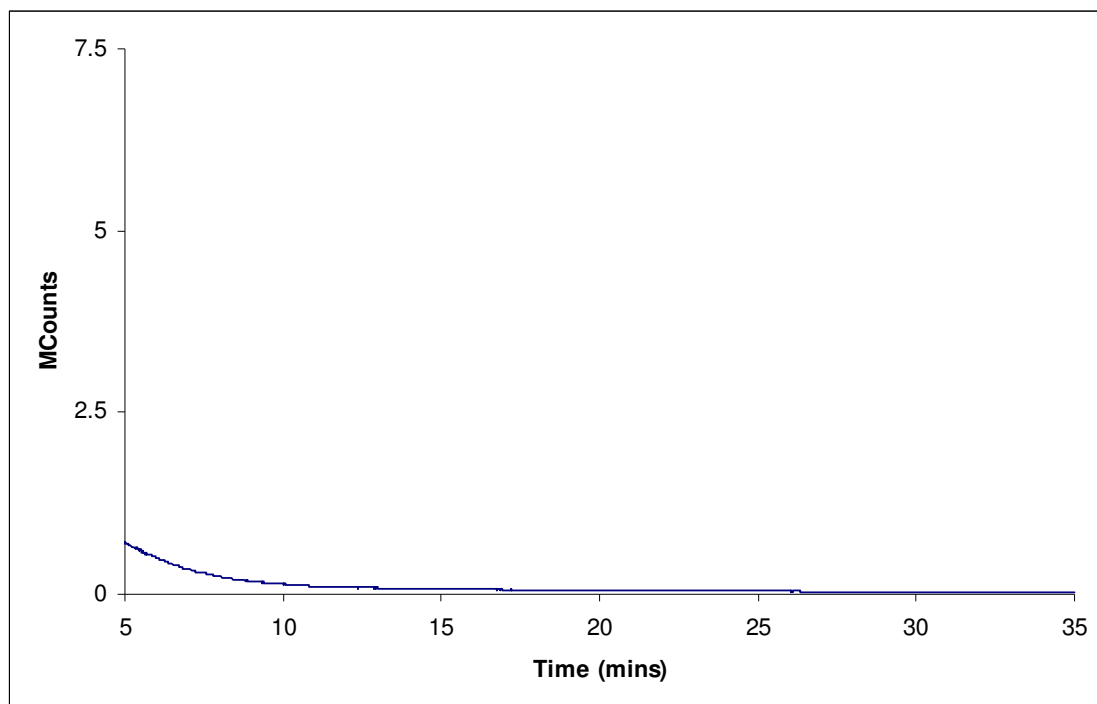


Figure 5.10: Chromatogram of methanol extract of rosemary (1% v/v)

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.287	95	81406	Trace
2	Bornyl Acetate	18.131	95	7874	1
3	Camphor	15.142	95	29678	7
4	Caryophyllene	18.615	132.9	0	0
5	Verbenone	24.907	106.9	21243	55

Table 5.4: Component yields of rosemary EO (Solvent extraction with methanol). Data are means of two determinants. Independent values were within 10% of each other.

In the methanol extract, only four chemical components were identified. Verbenone was again the main compound identified (55 ppm) and no caryophyllene was found. The relative amounts were: verbenone > camphor > bornyl acetate > borneol (Table 5.4).

5.3.3 Anti-listerial effects of chemical components of rosemary extracts

Some of the main chemical components present in rosemary extracts were tested for anti-listerial activity using the agar disc diffusion method (Figure 5.11).

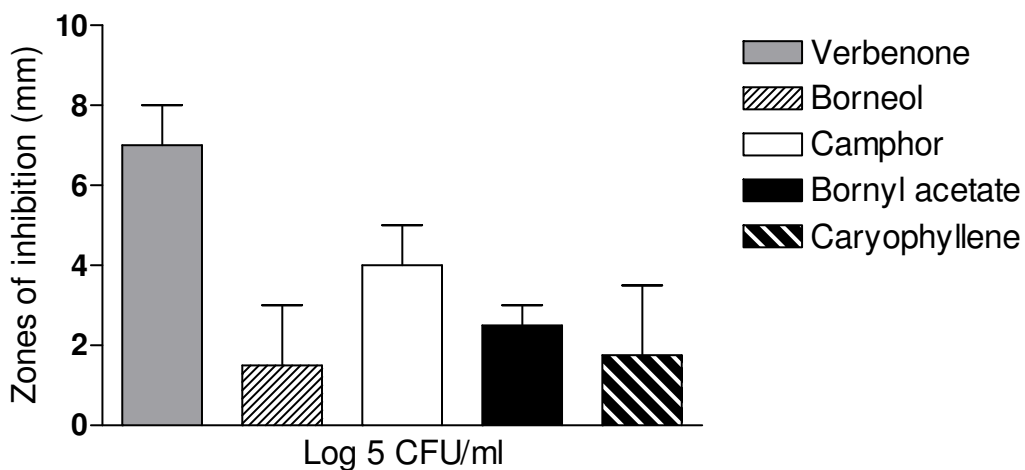


Figure 5.11: Zones of inhibition showing anti-listerial activity for a number of chemical components (1% v/v) present in rosemary. Reported results are means of 4 values. Error bars show s.d.

In terms of anti-listerial effectiveness, verbenone > camphor > bornyl acetate > caryophyllene > borneol. Verbenone was the most effective, displaying a zone of inhibition of 7 mm. Camphor and bornyl acetate showed moderate inhibition, and caryophyllene and borneol displayed lower inhibitory effects (1-2 mm).

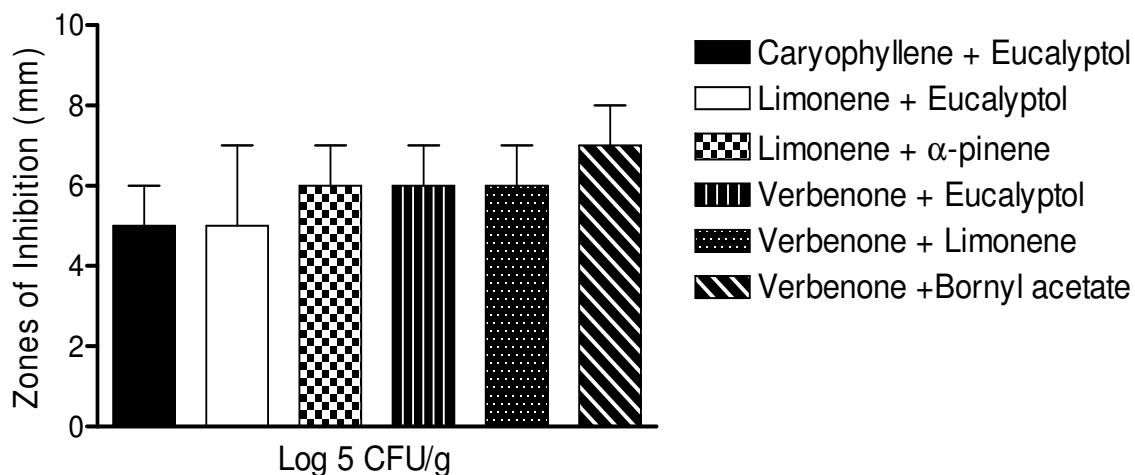


Figure 5.12: Zones of inhibition showing anti-listerial activity for a number of chemical components (1% v/v) in combination. Reported populations are means of 4 values. Error bars show s.d.

Figure 5.12 shows the anti-listerial effectiveness of different combinations (50/50 ratio) of the chemical components present in the rosemary extracts. Of the combinations tested, that of verbenone and bornyl acetate exhibited the strongest inhibition where a zone of 7 mm was recorded. The other combinations tested produced inhibition zones in the 5-6 mm range. No additive effects of the combined oils were seen in comparison to Figure 5.11.

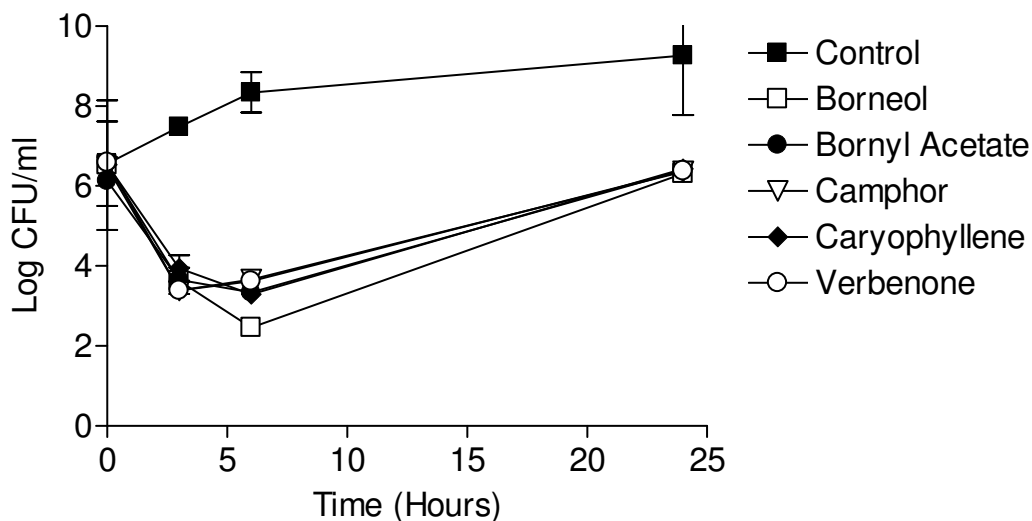


Figure 5.13: Effects of chemical components of rosemary extracts (1% v/v) on survival and growth of *Listeria innocua* in TSB over 24 hours. Reported populations are means of 4 values. Error bars show s.d. where present.

The major chemical components of rosemary all had good anti-listerial effects in broth dilution tests with counts dropping by 5-6 log cycles in the first 6 hours. Borneol had the largest effect with *Listeria* counts 1 log cycle lower than the other components after 6 hours of testing ($P < 0.001$). However these anti-listerial effects were reduced by 24 hours to 2 log cycles.

5.3.4 Effect of maceration on volatile components released from rosemary herb

In the previous chapter, rosemary herb was shown to only have an anti-listerial effect when it was macerated in the stomacher (Figure 4.22). As a result, the effects of maceration on the components present/released from fresh rosemary herb were examined using GC-MS.

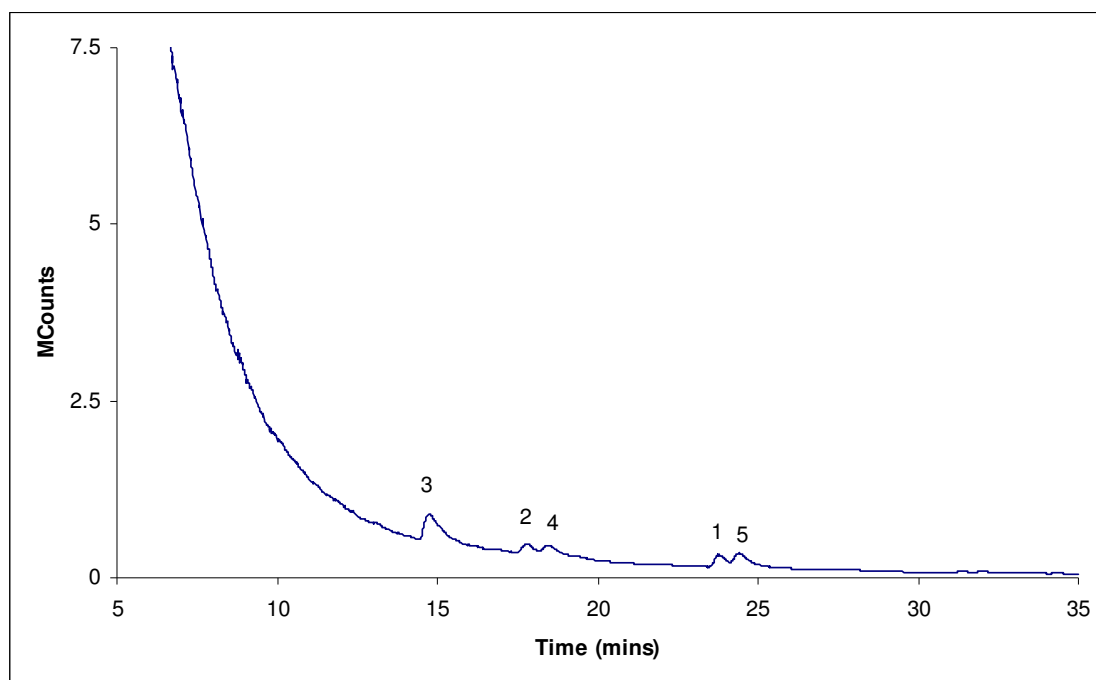


Figure 5.14: Chromatograph of macerated rosemary herb (1% v/v)

Peaks	Peak Name	RT	Quan Ions	Area	ppm
1	Borneol	23.36	95	2.62E+06	285.37
2	Bornyl acetate	18.38	95	1.20E+06	Trace
3	Camphor	13.19	95	3.40E+06	653.895
4	Caryophyllene	18.95	132.9	381784	Trace
5	Verbenone	24.32	106.9	554060	1295.03

Table 5.5: Component yields of macerated rosemary herb. Data are means of two determinants. Independent values were within 10% of each other.

When the rosemary herb was macerated in hexane/acetone (Fig 5.14), five chemical components were identified. Verbenone was present in the highest amount (1295.03

ppm), followed by camphor (695.895 ppm) and then borneol (285.37 ppm). Bornyl acetate and caryophyllene were only present in trace amounts.

The effect of maceration on the headspace components released during this process, compared to those released upon chopping and in fresh herb was also examined.

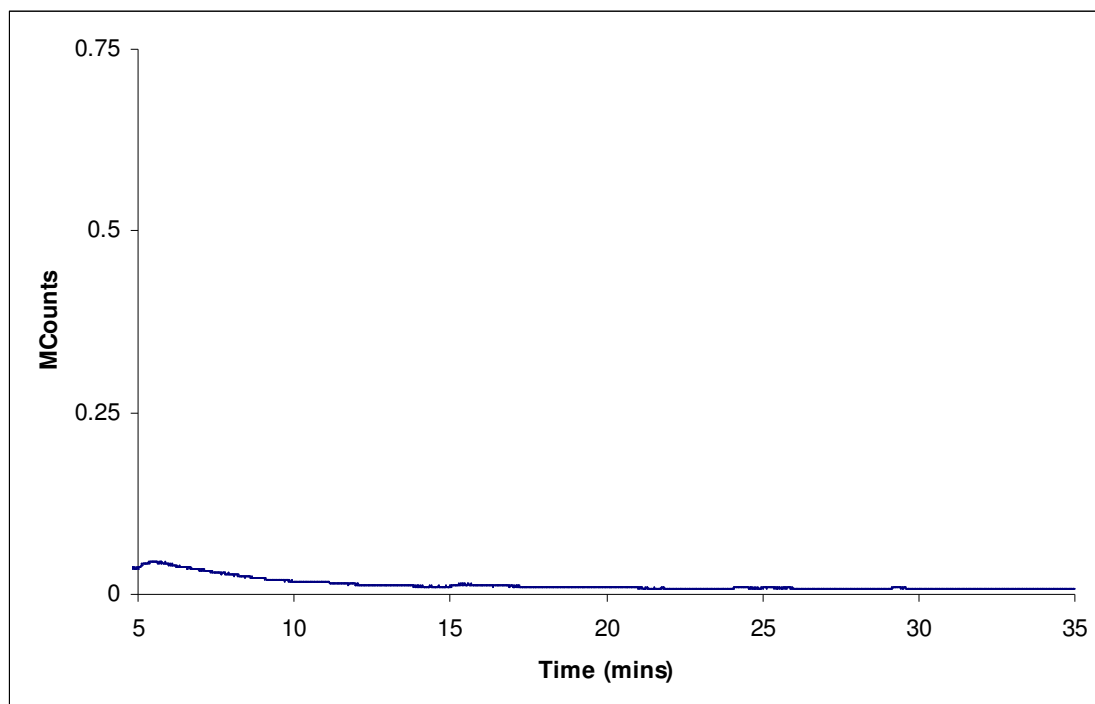


Figure 5.15: Chromatograph of volatiles released by fresh unchopped rosemary herb.

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.85	95	0	Trace
2	Bornyl Acetate	18.95	95	0	0
3	Camphor	15.18	95	0	Trace
4	Caryophyllene	19.284	132.9	0	0
5	Verbenone	24.569	106.9	0	0

Table 5.6: Component yields of volatiles released from fresh unchopped rosemary herb. Data are means of two determinants. Independent values were within 10% of each other.

When the volatiles released from fresh unchopped rosemary herb were analysed only two of the main components (borneol and camphor) were detected. These components

were present in trace amounts. Bornyl acetate, caryophyllene and verbenone were not found.

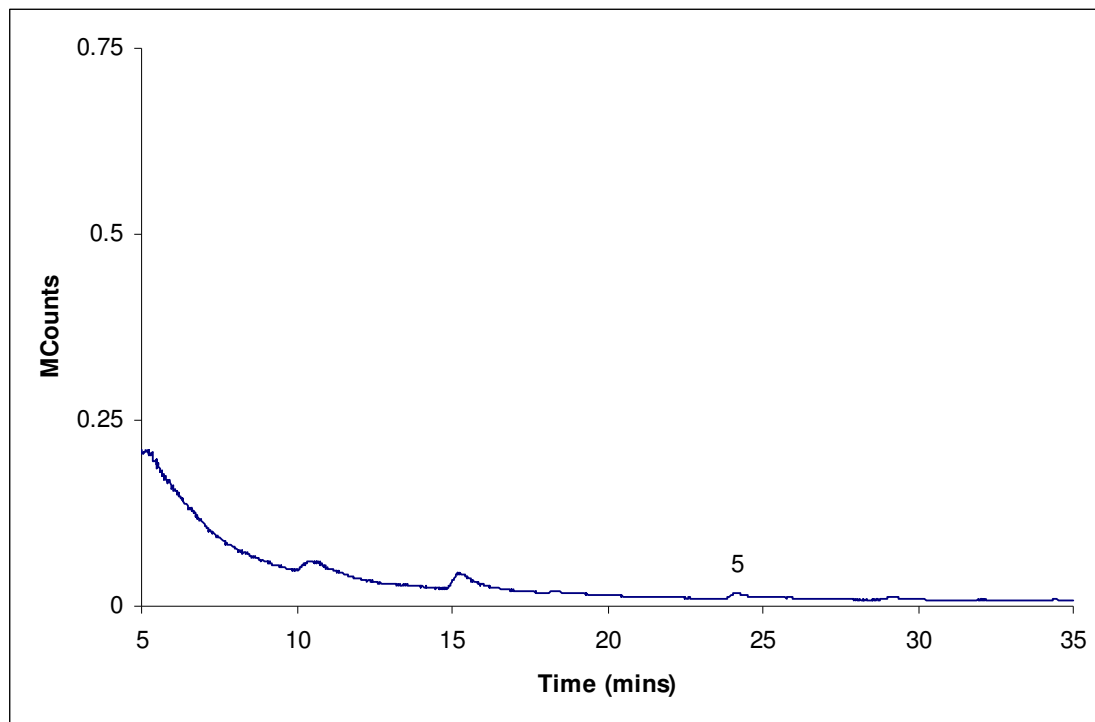


Figure 5.16: Chromatograph of volatiles released by freshly chopped rosemary herb.

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.85	95	0	Trace
2	Bornyl Acetate	18.95	95	0	0
3	Camphor	15.18	95	0	Trace
4	Caryophyllene	19.284	132.9	0	0
5	Verbenone	24.569	106.9	0	6.155

Table 5.7: Component yields of volatiles released from freshly chopped rosemary herb. Data are means of two determinants. Independent values were within 10% of each other.

Three chemical components were detected when the rosemary herb was chopped prior to headspace analysis, but levels were quite low. Small amounts of verbenone (6.155 ppm) were present, followed by trace amounts of borneol and camphor.

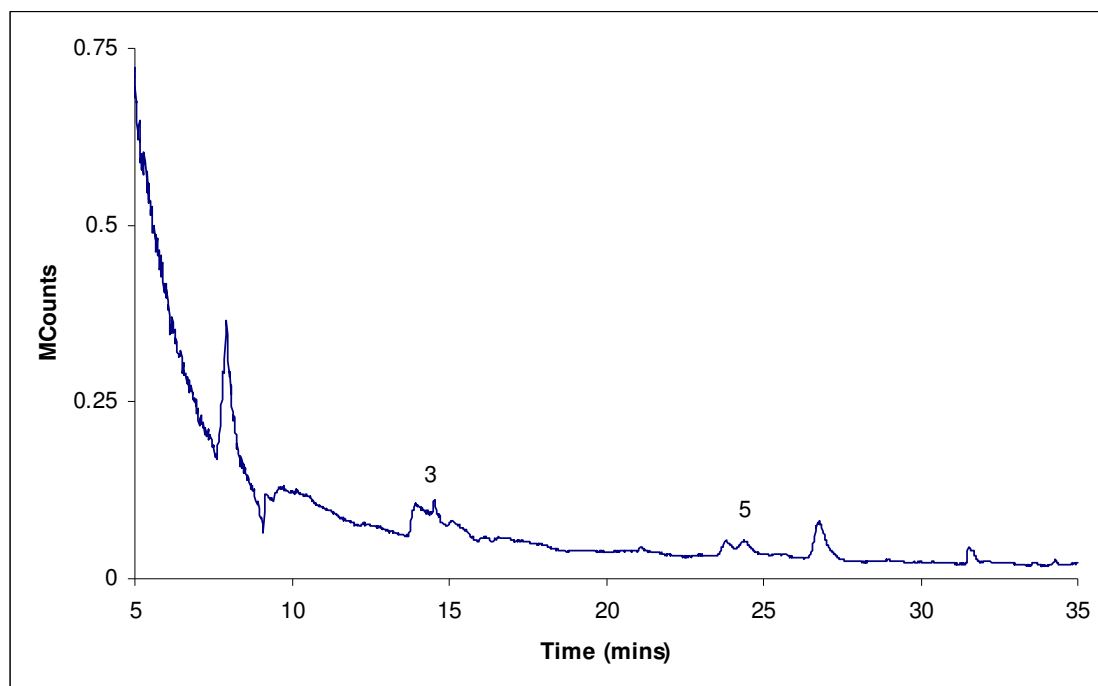


Figure 5.17: Chromatograph of volatiles released by macerated rosemary herb

Peaks	Peak Name	RT	Quan Ions	Area	Amount (ppm)
1	Borneol	24.85	95	0	0.663
2	Bornyl Acetate	18.95	95	0	0
3	Camphor	15.18	95	0	22.32
4	Caryophyllene	19.284	132.9	0	0
5	Verbenone	24.569	106.9	0	118.3

Table 5.8: Component yields of volatiles released from macerated rosemary herb. Data are means of two determinants. Independent values were within 10% of each other.

When the rosemary herb was macerated in the stomacher prior to GC-MS analysis, 3 volatile components were again identified. Verbenone was the most abundant compound (118.3 ppm), followed by camphor (22.32 ppm). Smaller amounts of borneol were also detected (0.663 ppm).

5.3.5 Statistical analysis of chemical component vs listerial kill using PCA

In an effort to elucidate which chemical in the different extracts was most important in inducing the kill of *Listeria*, a principal components analysis (PCA) was carried out.

Extract method	Kill (logs)	Sample Dilution (ppm)	Bor (ppm)	Bact (ppm)	Camph (ppm)	Cary (ppm)	Verb (ppm)
HD	7.756	10,000	3791.5	1318.84	10338.47	160.604	5126.408
CO ₂	7.009	10,000	431.83	45.646	2559.97	942.81	2372.543
H/A	5.731	10,000	Trace	0.733	19.882	6.102	48.603
Met	1.0935	10,000	0.1	0.755	6.986	0	55.026
HD	5.155	1000	257.33	348.425	895.21	30.72	598.63
CO ₂	2.6115	1000	27.739	0.1	12.625	18.038	138.943
H/A	0	1,000	14.745	0	1.333	0	29.142
Met	0	1,000	14.147	0	1.428	0	30.164

Table 5.9: Anti-listerial kill versus level of chemical components present in extracts. (Abbreviations are as follows: HD = Hydrodistillate, CO₂ = CO₂ extract, H/A = Hexane/Acetone extract, Met = Methanol extract, Bor = Borneol, Bacet = Bornyl acetate, Camph = Camphor, Cary = Caryophyllene, Verb = Verbenone).

The PCA scores are presented in Table 5.10, and they are plotted below (Figure 5.18).

	Axis 1	Axis 2	Axis 3
Bor	0.064	0.281	0.874
Bacet	-1.141	0.682	-0.186
Camph	0.479	0.230	-0.662
Cary	-0.937	-1.003	-0.036
Verb	1.535	-0.189	0.010

Table 5.10: The PCA scores of Axis 1 to Axis 3. The chemicals are abbreviated as follows: Bor = Borneol, Bacet = Bornyl acetate, Camph = Camphor, Cary = Caryophyllene, Verb = Verbenone.

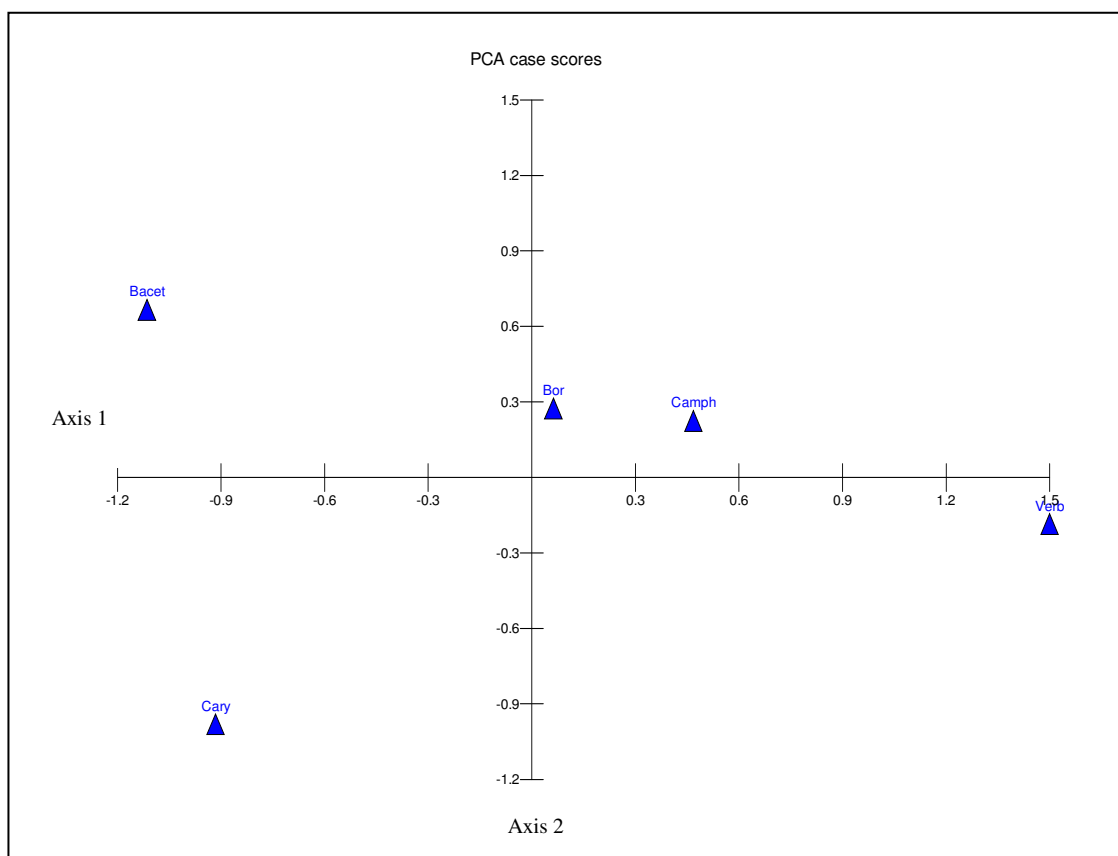


Figure 5.18: Plot of PCA Axis 1 versus PCA Axis 2. The chemicals are labelled as in table 5.10.

Figure 5.18 shows that Axis 1 had bornyl acetate and caryophyllene at the negative end and verbenone at the positive end. The Pearson correlation coefficients were calculated between kill values and each of the Axes (1 to 3). The only significant result was kill versus PCA Axis 1 ($r = -0.791$; $p = 0.019$). This shows that PCA Axis 1 was negatively correlated with values of kill. Hence, a plot of kill versus the reciprocal of PCA Axis 1 ($r = 0.74$; $p = 0.036$) is shown as this is easier to interpret:

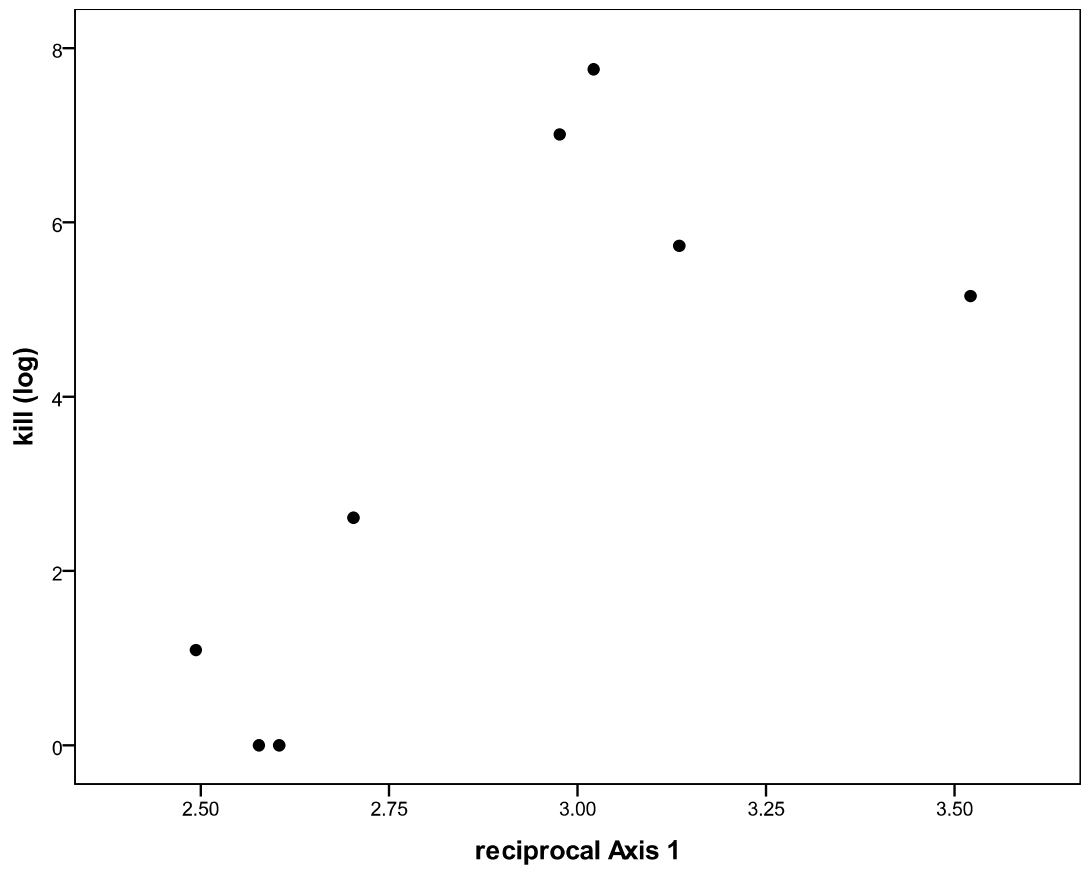


Figure 5.19: Plot of the kill (log values) versus the reciprocal of PCA Axis 1.

5.4 Discussion

This Chapter investigated the basis of the strong anti-listerial effects discovered in rosemary herb during stomaching in Chapters 3 and 4. Rosemary herb was extracted using different techniques, and the chemical composition and anti-listerial effects of whole extracts and of their individual components determined. The main components found in all of the extracts were camphor, verbenone, borneol. Reports by other investigators also found these components to make up the main composition of rosemary EO (Angioni *et al.* 2004, Okoh *et al.* 2010, Pintore *et al.* 2002). They were present in all the EOs tested here, but the relative quantity of each varied greatly with extraction method. The nature and composition of EOs are known to vary depending on the methods of extraction used (Boutekedjiret *et al.* 2003, Burt 2004, Guan *et al.* 2007). Studies show that during extraction there can be losses of some volatile compounds due to prolonged extraction times and degradation of unsaturated or ester compounds through thermal effects (Okoh *et al.* 2010, Lucchesi *et al.* 2004). Reverchon *et al.* (1995) showed that steam distillation and solvent extraction can cause the degradation of thermolabile compounds, hydrolysis of water sensitive compounds and also lead to solvent contamination.

Oils obtained by hydrodistillation contained the highest levels of verbenone (5,126 ppm), camphor (10,339 ppm), borneol (3,792 ppm) and bornyl acetate (1,319 ppm). Many studies have shown that although the compositions of oils obtained by supercritical fluid extraction and hydrodistillation were not qualitatively different, they do differ quantitatively (Vági *et al.* 2005, Reverchon and Senatore 1992).

In experiments using broth dilution, all the major components were found to have good anti-listerial effects, with a 4 log inactivation of growth seen after 6 hours. Borneol was the strongest anti-listerial agent; however, camphor, bornyl acetate,

verbenone and caryophyllene also had good anti-listerial activity. In experiments using the disc diffusion method, verbenone was found to be the strongest anti-listerial agent (Figure 5.11). Similar results were found by Santoyo *et al.* (2005) who showed that α -Pinene, 1,8-cineole, camphor, verbenone, and borneol showed antimicrobial activity against both Gram positive and Gram-negative bacteria, with borneol being the most effective followed by camphor and verbenone. The anti-listerial effects of these components was lost by the end of storage, presumably due to their highly volatile nature at 37°C, where their antimicrobial components may evaporate readily.

When the chemical components and quantitative anti-listerial effects of a series of extracts were investigated using PCA, verbenone was shown to make by far the biggest contribution. The data presented in this study shows that both the hydrodistillate and the CO₂ extract contain high concentrations of the monoterpene verbenone with levels being higher in the hydrodistillate (5,126 ppm). Fadel *et al.* (1999) also found that the yield of monoterpene hydrocarbons was slightly higher in hydrodistillate than that in the supercritical fluid extract. Previous studies have shown that components such as verbenone, borneol and camphor have displayed strong antimicrobial activity (Soković *et al.* 2007, Tabanca *et al.* 2001, Santoyo *et al.* 2005). Monoterpenes have been shown to possess strong antibacterial and antimicrobial activities and are said to exert their antimicrobial activity on microorganisms through the disruption of bacterial membrane integrity (Okoh *et al.* 2010). The most active EO in this study (obtained by hydrodistillation) contained the highest quantity of verbenone, and these high levels were correlated to the anti-listerial effects observed (Table 5.2).

Studies have shown a difference in the composition of oils obtained by solvent extraction compared to those obtained by distillation or supercritical fluid extraction,

and this may influence antimicrobial properties. Vagi *et al.* (2005) found that extracts obtained by supercritical fluid extraction showed significantly stronger antimicrobial properties in comparison to the slightly inhibitory effects of ethanolic extracts against *E. coli*, *P. fluorescens* and *B. cereus*. They concluded that the stronger antimicrobial activity of SFE extracts could be explained by the higher concentration of volatile compounds in the extract compared with those in the ethanolic extracts. In this study, very low levels of verbenone were detected in the solvent extracts; and these low levels could explain the lack of antimicrobial effects seen in the solvent extracted EOs.

The extract of macerated rosemary herb had more than twentyfold the level of verbenone found in the unstomached rosemary extract. While few studies have been carried out on the subject, Smallfield *et al.* (1994) found that a change in the oil chemistry of coriander was triggered by the chopping of the plant material. They suggested that this was probably due to the release of an enzyme, stored separately from the oil glands, which reduces the aldehydes to the corresponding alcohols. In garlic, studies have shown that once it is processed by cutting or crushing, compounds in the intact garlic are converted into organosulfur compounds, and allinase is activated to produce allicin, the active component in garlic (Rahman 2007, Amagase *et al.* 2001). This may also be the case in the current study, where the active chemicals from the herb may be released by maceration during the stomaching process. In the previous chapter (Figure 4.22), it was shown that rosemary herb was only effective when it is completely macerated with the vegetable sample in the stomacher. This could be due to the maceration of the herb creating conditions for the synthesis and/or release of particular antimicrobial components, such as verbenone, from the herb which are effective against *Listeria*.

In an experiment where the rosemary herb was macerated in the stomacher in a hexane/acetone mix, greater levels of verbenone, camphor and borneol were extracted than when the solvent extraction was carried out using hexane/acetone without maceration. Verbenone levels increased about twenty six-fold after maceration from 50 ppm to 1295 ppm. Gachkar *et al.* (2007) found that rosemary exhibited a high antimicrobial activity against *L. monocytogenes*, *E. coli* and *S. aureus* (MBC varied from 2 to 4 µl/ml) due mainly to the dominance of verbenone, borneol and camphor. A second experiment, in our study, compared the headspace composition over fresh rosemary that had been either un-chopped, chopped or macerated. The respective levels of verbenone were 0, 6 and 118 ppm. As both experiments point to the release of verbenone upon maceration of the herb in the stomacher, and PCA shows verbenone to be highly correlated with anti-listerial activity, it appears that verbenone is responsible for the strong anti-listerial effects of rosemary herb observed during maceration.

The data indicate that verbenone was the main anti-listerial component in rosemary extracts, and that modifying the industrial process used to extract essential oils from rosemary in ways that simulate the effects of maceration by stomaching, will increase the level of this key component. In the context of the damaging effects of EOs on product acceptability, identifying, enriching and possibly separating out key powerful anti-listerial components may be useful. In particular, such an approach may facilitate their direct use in ways which separate the anti-listerial and phytotoxic effects observed here, and enable this powerful natural anti-microbial agent to be exploited commercially.

5.5 References

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Chapter 6
General Discussion

6.1 General Discussion and Conclusions

The worldwide fresh-cut produce industry has grown in recent years, largely driven by increasing consumer demand for healthy, freshly prepared, convenient fruits and vegetables. However, fresh-cut produce harbours large and diverse populations of microorganisms, and some of this microflora may include foodborne pathogens such as *Listeria monocytogenes*. The fresh-cut produce food system is not pasteurised and relies upon HACCP protocols to eliminate contamination and low storage temperatures ($\leq 4^{\circ}\text{C}$) to prevent growth of pathogens should contamination occur. Products are subjected to dipping in 100 ppm chlorine which reduces microbial populations tenfold and may help prevent cross-contamination. These hurdles have been shown to be incompletely effective as demonstrated by a small number of serious food poisoning outbreaks associated with this food system over the past ten years. The addition of natural plant antimicrobials, such as plant EOs, may represent an additional hurdle to reduce contamination and growth of pathogens during storage. In this work, the anti-listerial properties of plant EOs and fresh herbs were examined to assess the opportunities and limitations of their use in modified atmosphere packaged fresh-cut vegetable systems.

To determine their performance under modified atmospheres, the anti-listerial properties of EOs (thyme, oregano and rosemary), in combination with varying storage atmospheres (air, 5% CO_2 /2% O_2 /93% N_2 and 20% CO_2 /1% O_2 /79% N_2) and temperatures (4 and 8°C), were examined using a flow-through model vegetable system (Chapter 2). The data indicated that EOs with anti-listerial effects in air at 4°C (thyme, oregano) had their effectiveness enhanced at atmospheres used in the fresh-cut sector (5% CO_2 /2% O_2 /93% N_2) including unintended atmospheres with more elevated

CO₂ levels which can develop due to poor product-package compatibility or temperature abuse. Volatiles released from the EOs showed no anti-listerial effects, indicating that the oils need to be in direct contact with the cultures in order to be effective, and that the introduction of EOs in gas form into MA packages was unlikely to be effective. However, it is likely that some EOs could have strong anti-listerial effects under MA conditions, provided that they were in direct contact with the pathogen.

Although EOs perform well in antibacterial assays *in vitro*, these effects may be lost when incorporated into a food product. This could be due to a number of intrinsic factors such as pH, fat or protein content of the food (Gutierrez *et al.* 2008). Studies have shown EOs to be effective in numerous meat and dairy products; however, few studies have been carried out on the antibacterial effects of EOs in fresh-cut vegetables. In Chapter 3, the effectiveness of a number of EOs and of fresh shredded herbs were examined against *Listeria innocua* on modified atmosphere packaged (MAP) fresh-cut lettuce as a model fresh-cut vegetable product. As before, thyme EO had a major anti-listerial effect; there were no anti-listerial effects with fresh thyme herb or thyme EO volatiles. Similar results were found for oregano EO and herb. However, applying undiluted EOs directly to the lettuce had such a detrimental effect on appearance as to make the lettuce unacceptable. Diluting thyme, oregano or rosemary EOs or using them in combination with diluted basil, oregano, thyme or rosemary EO reduced these damaging effects but also eliminated the anti-listerial effects. Thus, direct application of EOs may be impractical as a commercial solution.

By contrast, the addition of fresh herbs had more complex effects than expected. Most herbs improved appearance scores. While rosemary EO had shown

little anti-listerial effects, fresh shredded rosemary herb significantly reduced *Listeria* populations ($P < 0.05$) during maceration of lettuce in the stomacher compared to the control, suggesting some latent anti-listerial ability of rosemary associated with maceration of the fresh state.

In Chapter 4, the study of EO and herb effects was extended to other fresh-cut vegetables (carrot disc, cabbage and dry coleslaw mix). These vegetables were selected as they are among the most commonly purchased vegetables/fresh-cut produce in Ireland (Safe Food Ireland 2007). The same patterns of anti-listerial effects of EOs and rosemary herb emerged. However, in addition, some major interactions with plant materials were observed (Table 4.4). The 3 log anti-listerial effect of rosemary herb observed in lettuce was increased to 6 logs in carrot, to 4 logs in coleslaw and 6 logs in shredded cabbage. While there were no anti-listerial effects of rosemary EO on lettuce, it reduced counts by 1 log on coleslaw and 2 logs on shredded cabbage. The anti-listerial effects of oregano EO were increased from 2 logs in lettuce to 6 logs in coleslaw and shredded cabbage. Compared with lettuce, greater general anti-bacterial effects (greater reductions in TBCs) were also observed on shredded cabbage with both thyme EO and oregano EO. Previous studies have also shown carrot (Beuchat and Brackett 1990, Noriega *et al.* 2010, Beuchat *et al.* 1994) and cabbage (Kyung and Fleming 1997) to have a listericidal effect. Some experiments were carried out in which shredded carrot was introduced as an added antibacterial agent. The addition of the shredded carrot in combination with the rosemary herb and EO led to a greater inactivation of *Listeria*, suggesting a synergistic effect between the carrot and rosemary. The reason for the enhanced anti-listerial effects of rosemary herb in combination with shredded carrots is unclear, but needs to be studied further. This data

suggests that the anti-listerial effects of rosemary herb may be due to the maceration releasing particular antimicrobial components from the herb which are effective against *Listeria*.

The data in Chapters 3 and 4 have shown that rosemary herb was effective in reducing *Listeria* counts during maceration in the stomacher while rosemary EO was not. This suggests that an important chemical component, which may have anti-listerial properties, could be lost during the EO extraction process or that it may be synthesised only during specific extraction conditions. As a result, the chemical composition and latent anti-listerial power of rosemary herb was investigated further in Chapter 5. Initial antimicrobial tests, carried out using the disc diffusion and broth dilution methods, showed all extraction methods (CO₂ extraction, hydrodistillation, solvent extraction) to have an anti-listerial effect, with the hydrodistillate showing the highest anti-listerial activity. Analysis of the chemical components present in the extracts found that while the main components present were similar, percentage composition varied greatly, and the hydrodistillate was found to have the highest levels of camphor (10,339 ppm), verbenone (5,126 ppm) and borneol (3,792 ppm). Previous studies have shown that components such as borneol, camphor and verbenone have displayed strong antimicrobial activity (Soković *et al.* 2007, Tabanca *et al.* 2001, Santoyo *et al.* 2005). All of the individual chemicals showed good anti-listerial activity, however PCA showed that levels of verbenone made the biggest contribution. As seen in Chapter 4, rosemary herb was only effective when it was macerated with the vegetable product during maceration in the stomacher. An intriguing possibility emerged that the maceration might result in synthesis/release of particular antimicrobial components from the herb which are effective against *Listeria*. GC-MS

analysis found that the extract of macerated rosemary had more than twenty-five fold the level of verbenone than found in the unmacerated extract. Headspace analysis carried out on uncut, freshly chopped and stomached rosemary found the levels of verbenone were 0, 6 and 118 ppm respectively. The data indicated that verbenone was the main anti-listerial component in rosemary extracts. In the context of the damaging effects of EOs on product acceptability, identifying, enriching and possibly separating out key powerful anti-listerial components may be useful. In particular, such an approach may facilitate their direct use in ways which separate the anti-listerial and phytotoxic effects observed here, and enable this powerful natural anti-microbial agent to be exploited commercially.

The main conclusions drawn from this study were as follows:

1. Essential oils such as thyme, oregano and rosemary exhibit good anti-listerial effects *in vitro*. The data indicated that the anti-listerial effectiveness of EOs was influenced by gas atmosphere and the use of high CO₂ gas atmospheres increased the anti-listerial effects of the oils.
2. EOs were found to be effective when applied directly to a medium and not effective when used in volatile form. However, direct application of undiluted EOs to fresh-cut produce had a detrimental effect on appearance making the products unacceptable. By contrast, addition of fresh herb generally improved appearance.
3. There appeared to be significant interactions between some antimicrobial effects of EOs/herbs and the presence of other vegetable components. On shredded carrot and shredded cabbage, the anti-listerial effect of the EOs/herbs was enhanced.

4. While rosemary EO had only slight anti-listerial effects in fresh-cut produce, rosemary herb was a very effective anti-listerial agent during maceration in the stomacher.
5. Verbenone was identified as the most active anti-listerial agent in rosemary. Higher levels of verbenone were detected when rosemary herb was stomached, showing that it is possible to enhance the anti-listerial power of rosemary extracts by modifying pure extraction conditions.

Essential oils and herbs are powerful anti-listerial/anti-microbial agents with great potential for use in food systems, including fresh-cut systems. However, when used directly on fresh-cut products they cause phytotoxic effects. In order to exploit their potential, far more sophisticated approaches are needed to separate phytotoxic from anti-listerial effects. The current thesis provides important insights which point the way to possible solutions. In particular, it points to the likely benefits of further work exploiting synergistic interactions between herbs and other plant materials/product types such as carrot and cabbage, leading to opportunities to develop specific product combinations. In addition, it has shown that herb extraction technology may benefit from optimisation to increase the level of powerful anti-listerial chemicals such as verbenone. This strong antimicrobial could be used as a processing aid, maybe replacing chemical sanitisers as a wash treatment. Enhancement and/or extraction of powerful specific chemicals may reduce phytotoxic effects by, for example, enabling the chemical or EO preparation to be used in contact with products for shorter periods or in new contexts.

Because of the complexity of the challenges, successful use of natural antimicrobials in fresh-cut systems will be an enormous achievement with great benefits to the sector. This thesis provides valuable advances towards achieving that objective.

6.2 References:

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