PLANT OILS AS ANTIBACTERIAL AGENTS

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Submitted to the Faculty

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By

Alexander Ogilvie Gill

In Partial Fulfillment of the

Requirements for the Degree

of

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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DOCTOR OF PHILOSOPHY

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DEDICATION

This thesis is dedicated to my wife Cara. Also to my parents Carol and Colin Gill, who always said I could be whatever I want, even a garbage man.

FOREWORD

This thesis is composed of four papers prepared for journal publication with the addition of a literature review (Chapter 2), and consolidated introduction (Chapter 1), conclusion (Chapter 7) and references. The Chapters 3 to 6 are presented as originally submitted for publication, with minor changes to format for the purposes of standardizing presentation of this thesis. I, Alexander Gill, was the primary author and experimenter for all the work described in the following chapters with the exception of Sections 3.3.3., 3.3.12. and paragraphs 1 and 5 of 3.4. written by Pascal Delaquis, and Section 3.3.10. written by Peter Russo.

Chapter 3 was originally published as "Evaluation of antilisterial action of cilantro oil on vacuum packed ham" by A.O. Gill, P. Delaquis, P. Russo and R.A. Holley (2002) in the International Journal of Food Microbiology. Vol. 73 pp. 83-92.

Chapter 4 was originally published as "Mechanisms of bactericidal action of cinnamaldehyde and eugenol against *Listeria monocytogenes* and eugenol against *Lactobacillus sakei*" by A.O. Gill and R.A. Holley (2004) in Applied and Environmental Microbiology. Vol. 70 pp. 5750-5755.

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ABSTRACT

Essential oils of herbs and spices and their components, such as eugenol, carvacrol and cinnamaldehyde, are active as antimicrobials against a wide range of Gram negative and positive bacteria. These agents have potential as antimicrobials for use in food products. The mechanism of action of these agents is poorly understood. The development of effective antimicrobial treatments based on essential oils requires an understanding of their mechanism of action.

The potential of cilantro oil to control the growth of *Listeria monocytogenes* on vacuum packed ham was investigated. Cilantro oil is an essential oil preparation extracted from the plant *Coriandrum sativum*.

When tested against 5 strains of *L. monocytogenes* minimal inhibitory concentrations were found to range from 0.074 to 0.018%. Cilantro oil treatments were tested on ham disks inoculated with a cocktail of five *L. monocytogenes* strains. The treatments studied were 0.1, 0.5, and 6% cilantro oil diluted in sterile canola oil or incorporated into a gelatin gel containing lecithin. Gelatin gel treatments with 6% cilantro oil and 1.4% monolaurin were also tested to determine if an interaction between the antimicrobials could increase inhibition of *L. monocytogenes*. Treated ham was vacuum packed and stored at 10°C for up to 4 weeks.

The only treatment that inhibited growth of *L. monocytogenes* was the 6% cilantro oil gel. Samples receiving this treatment had populations of *L. monocytogenes* 1.3 log CFU/ml lower than controls at week one of storage, though there was no difference between treatments from week two onward. Immobilization of the antimicrobial in a gel appeared to enhance the effectiveness of treatments.

In broth media at 20°C, 5 mM eugenol or 30 mM cinnamaldehyde were bactericidal (>1 log CFU/ml reduction in 1 h) to *L. monocytogenes.* At 6 mM eugenol was bactericidal to *Lactobacillus sakei*, but treatment with 0.5 M cinnamaldehyde had no significant effect.

To investigate the role of interference with energy generation in the mechanism of action, the cellular and extracellular ATP of cells in HEPES buffer at 20°C were measured. Treatment of non-energised *L. monocytogenes* with 5 mM eugenol, 40 mM cinnamaldehyde or 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) for 5 min prevented an increase in cellular ATP upon addition of glucose. Treatment of energised *L. monocytogenes* with 40 mM cinnamaldehyde or 10 μM CCCP caused a rapid decline in cellular ATP levels, but 5 mM eugenol had no effect on cellular ATP. Treatment of *Lb. sakei* with 10 mM eugenol prevented ATP generation by non-energised cells, and had no effect on the cellular ATP of energised cells. CCCP at 100 μM had no significant effects on the cellular ATP of *Lb. sakei.* Due to their rapidity, effects on energy generation clearly play a major role in the activity of eugenol and cinnamaldehyde at bactericidal concentration.

The role of membrane disruption in the bactericidal activity of the plant oil aromatic compounds eugenol, carvacrol and cinnamaldehyde was investigated using confocal laser scanning microscopy, changes in ATP levels and cell viability. In 25 mM HEPES buffer pH 7 at 20°C, 10 mM eugenol or carvacrol increased uptake of propidium iodide by *Escherichia coli*, *L. monocytogenes* and *Lb. sakei* over a 10 min period. The same treatments resulted in lowered viability, rapid depletion of cellular ATP and release of ATP, with the exception of *Lb. sakei* treated with carvacrol. Eugenol or carvacrol at 5

mM to 10 mM inhibited *E. coli* and *L. monocytogenes* motility.

Lb. sakei was resistant to cinnamaldehyde. Thus, its effects were only studied on *E. coli* and *L. monocytogenes.* At 10 mM cinnamaldehyde caused a slight but statistically significant increase in propidium iodide staining of *E. coli*, but had no effect on *L. monocytogenes*. Cinnamaldehyde treatment of *E. coli* at 10 mM and *L. monocytogenes* at 40 mM resulted in decreased cellular ATP, but there was no concomitant release of ATP. Cinnamaldehyde at 5 and 10 mM inhibited *E. coli* and *L. monocytogenes* motility.

Results for eugenol and carvacrol are consistent with non-specific permeabilization of the cytoplasmic membrane. Evidence for increased membrane permeability by cinnamaldehyde is less conclusive.

The capacity of the aromatics to inhibit the membrane bound ATPase activity of *E. coli* and *L. monocytogenes* was investigated using isolated membranes. Inhibition of the ATPase activity of *E. coli* membranes was observed with 5 mM or 10 mM eugenol or carvacrol. Progressively greater inhibition by cinnamaldehyde was observed as concentration increased from 0.1 to 10 mM. *L. monocytogenes* ATPase activity was significantly inhibited by eugenol (5 or 10 mM), carvacrol (10 mM) and cinnamaldehyde (10 mM).

To determine whether the resistance of *Lb. sakei* to cinnamaldehyde was related to the relative hydrophobicity of the cell surface and hence the ability of the cell to take up the aromatics, the percentage of the three organisms partitioning in dodecane was compared. No significant difference was found between the partitioning percentage of *L. monocytogenes* (17.2%) and *Lb. sakei* (13.8%), indicating that surface hydrophobicity may not explain the differing sensitivity to cinnamaldehyde of these two organism. The

percent partitioning of *E. coli* was significantly greater than both other organisms (23.3%) and may explain the greater sensitivity of *E. coli* to all three aromatics.

The results of these experiments indicate that eugenol and carvacrol cause cell death by disrupting the cellular membrane leading to leakage of cell contents. Disruptive membrane interactions are less clearly indicated for cinnamaldehyde. The antimicrobials also inhibited membrane associated ATPase activity. ATPase inhibition may be a secondary mechanism in cell death, resulting from non-specific hydrophobic interactions of the molecules with membrane bound enzymes.

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1. Introduction

There is much research interest among food microbiologists in the development of novel antimicrobial systems for use in food products. This interest is driven by conflicting consumer demands for foods that are minimally processed, contain lower quantities of additives, while being microbiologically safe, convenient and shelf stable.

Essential oils extracted from plants, and the small hydrophobic molecules of which they are composed, have been proposed as suitable agents for use in novel antimicrobial systems. These agents are effective at inhibiting the growth or killing a wide range of bacterial species, including pathogens. These agents are commonly extracted from herbs and spices that are traditional food ingredients, which potentially makes them highly acceptable to consumers. Unfortunately, the concentrations of essential oil antimicrobials required for the control of bacterial targets in model systems and foods have significant sensory effects.

It has been proposed that the problem of sensory effects can be reduced by incorporating essential oil antimicrobials into systems composed of several reinforcing antimicrobial agents (Roller and Board, 2003; Burt, 2004; Holley and Patel, 2005). Ideally, such combinations would be synergistic, where the effect of two or more agents is greater than the additive effect of the single agents. However, the rational development of antimicrobial systems requires understanding of the mechanism of action of agents and for essential oil antimicrobials these are poorly understood.

The research program detailed in this thesis was undertaken with the objective of determining the mechanism by which the essential oil components, eugenol, cinnamaldehyde and carvacrol kill bacterial cells. Though there is now a significant body of work on the mechanism of action of carvacrol, there has been very little work done on

investigating other essential oil components. Additionally, much of the work that has been done is difficult to interpret due to the frequent failure to distinguish clearly between growth inhibition and cell death.

In the experiments detailed in this thesis, we first determined the concentrations at which the eugenol and cinnamladehyde were bactericidal to the test organisms *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei*. There are a small number of fundamental activities in cell metabolism that can lead to rapid cell death. These include inhibition of energy generation, substrate uptake, protein, lipid or cell wall synthesis. For this reason we first investigated the effects of the agents on energy generation. If energy generation is impaired cell metabolism will come to a halt as no metabolic work can be done. Having determined that generation of ATP was rapidly inhibited at bactericidal concentrations we then proceeded to investigate whether this was associated with membrane disruption and ATPase inhibition.

2. Literature Review

2.1. Introduction

As Shelef (1983) correctly recognized in the early 1980's consumer concerns about potential health affects associated with synthetic and traditional preservatives would create a demand for alternatives. Consumers expressed this demand, and continue to do so, as a request for foods perceived as "natural". Shelef (1983) suggested that this demand could be answered by the development of novel antimicrobial systems employing the oil fractions isolated from various spices and herbs.

These oil fractions are extracted by a number of distillation methods and are commonly referred to as "essential oils" (Burt, 2004; Holley and Patel, 2005). The term essential oil derived historically from the term *Quinta essentia* introduced by the 16th century Swiss physician and alchemist Paracelsus to describe medically active agents (Burt, 2004). The term essential oil for plant oil extracts seems to have been popularized by its use in such fringe medical areas as homeopathy and aromatherapy, which have long made use of essential oils.

The antimicrobial activity of certain spices, herbs and the essential oils prepared from them has long been recognized. Perhaps the earliest scientific report of such activity is in a letter dated October 9, 1676 by Antony van Leeuwenhoek. In this letter van Leeuwenhoek described the decline in the number and activity of "animalcules" in a sample of well water following the addition of pepper (Dobell, 1960). From these humble beginnings an enormous body of literature describing observations of antimicrobial activity of essential oils continues to be published. Activity has been reported against a wide range of bacteria, fungi and single celled eukaryotes (Burt, 2004; Holley and Patel,

2005). However, though there is a voluminous amount of descriptive literature on the antimicrobial activity of essential oils, comparatively little work has been published investigating the mechanisms by which organisms are inhibited or killed by these agents.

It is only comparatively recently that any attempt to understand the mechanisms by which essential oils function as antimicrobial agents has been made. Progress in resolving this issue has been initially slow for two reasons. Firstly, essential oils are composed of a complex mixture of molecules, whose composition may vary greatly with raw materials and method of preparation. Secondly, essential oils and their components are hydrophobic and the standard protocols for the evaluation of antimicrobials have been developed for hydrophilic molecules (Nychas and Skandamis, 2003).

An understanding of the mechanism of action of essential oil components is necessary for the development of effective applications for food products. Successful applications must take into account modification of agent activity by the food environment. Treatments intended to increase the safety of the product must be lethal to target pathogens due to the small numbers commonly required for infection. If the development of such treatments is to proceed beyond largely random testing of agents and products a clear understanding the mechanism of action is required. Additionally, effects on flavour and odour that occur at bactericidal concentrations limit application of essential oils or their components. Though these sensory changes may not be undesirable for specific products, it does limit potential applications. An understanding of the mechanisms of action holds out the possibility that effective treatments composed of combinations of agents may be developed that have enhanced antimicrobial and/or lowered sensory effects.

2.2. Methodological problems of essential oil studies

In evaluating the results of studies of the antimicrobial activity of essential oils and their components there are two major methodological issues that should be recognised. Firstly, many evaluations of agents on bacterial cells fail to distinguish between bacteriostatic and bactericidal effects. Secondly, challenges in experimental design are posed by the hydrophobic nature of essential oils and their components, and the need to study their activity against microorganisms in aqueous systems.

The methods available to determine the antimicrobial activity of essential oils and their components can be summarised as follows:

1. Inhibition Zone: the size of the zone of growth inhibition around a paper disk or well in agar medium is measured.
2. Optical Density and Impedance: the time required for cells to reach a specific cell density in aqueous suspensions.
3. Inhibition on Agar Media: cells are plated onto agar medium incorporating the antimicrobial.
4. Microscopic Quantification: intact cells in a liquid medium are enumerated by microscopy.
5. Fluorescence Quantification: the proportion of viable and nonviable cells is distinguished by one or more fluorescent probes.
6. Plate Count Quantification: cells are exposed to the agent in liquid medium and then plated on a non-selective agar medium.

The first three techniques can not be used to determine whether treatment with an agent is lethal to the cells tested. These techniques can only be used to determine whether inhibition of growth is occurring, as they detect whether or not cell density has reached a

critical level. Further, the ability of these techniques to distinguish between growth inhibition and slowed growth may be compromised if relatively short incubation periods are used. The continuing popularity of these techniques is due to their low cost, rapidity and ease of automation. For these reasons they are suitable for initial screening of agents for antimicrobial activity.

Perhaps most surprising is the use of optical density (OD) measurements to follow the response of a bacterial population to an agent. OD measurements can only detect cell numbers in excess of 10^6 CFU/ml. OD can not reliably determine cell death as a decline in the OD of a sample will only occur if there is complete lysis of cells.

Microscopic methods also pose difficulties in distinguishing between viable and non-viable cells and require a minimum cell density. This method is also tedious and time consuming.

Fluorescent viability probes may be used to augment microscopic methods of quantification or used in conjunction with spectrophotometry or flow cytometry. These methods are popular with those laboratories that can afford the appropriate equipment as they lend themselves to automated data collection. However, the generally accepted indicator of cell viability is the ability to reproduce. Since these methods commonly function by the differential uptake of probes based on intactness of the cell membranes or other indicators of physiological state, care must be taken in interpretation of results.

As Nychas and Skandamis (2003) observed, plate counting is "the gold standard" for quantifying cell viability. Though time consuming the results of plating a sequential time series of samples leaves little ambiguity as to whether or not a cell population is remaining static or declining.

Distinguishing between lethal and inhibitory treatments is essential if applications to protect against bacterial pathogens are to be developed. Since many bacterial pathogens have low infectious doses (10 to 1000 cells), treatments that only inhibit cell reproduction will have a minimal impact on product safety.

The methods described above all have an important failing when applied to hydrophobic essential oils and their components. These methods were developed originally for the study of soluble antibiotics and assume that the agent under investigation will diffuse evenly and rapidly through the test medium.

The problem of limited diffusion of the agent is particularly acute with inhibition zone and agar inhibition experiments where the antimicrobial is expected to diffuse through a water saturated agar matrix. An additional complication is the possible contribution to activity by agents in the gaseous phase (Inouye et al., 2000; Inouye, 2003). These issues pose serious difficulties in interpreting the results of inhibition studies of cells on solid surfaces.

Similar problems of poor diffusion of hydrophobic molecules exist when the agent is added to an aqueous liquid system such as buffer or broth media. For this reason many experimenters make use of an emulsifying agent (ethanol, tween, propylene glycol) to improve dispersal of the agent (for review see Burt, 2004). Unfortunately, the use of emulsifiers does not provide a simple solution to this problem. Many of the emulsifiers used are themselves bioactive. The antimicrobial activity of oregano, thyme, and clove essential oils has been reported to be reduced by the presence of ethanol (0.2%) or tween 80 (0.25%) (Remmal et al., 1993a,b). Remmal et al. (1993a,b) and Mann and Markham (1998) reported that adequate dispersal of essential oils could be achieved with 0.15 to

0.2% agar. A number of researchers have reported adequate results without the use of any emulsifier (for review see Burt, 2004).

It is commonly held that the maximum activity of a hydrophobic antimicrobial occurs at the solubility limit of the molecule. This view is based on the assumption that only molecules dissolved in the aqueous phase are available for interaction with cells (Sikkema et al., 1995). This is a reasonable assumption in situations where interactions between molecules and cells involve interaction of the molecules with cellular enzymes and when partitioning between phases is at equilibrium. However, essential oils and their components are regularly reported to be inhibitory or lethal to cells when present in amounts in excess of their maximum solubility (for tables of reported values see Burt, 2004; Holley and Patel, 2005).

In the presence of an insoluble bulk phase the availability of a hydrophobic molecule may exceed the level predicted by solubility and is instead governed by the dissolution rate (Stucki and Alexander, 1987). The dissolution rate may be increased by the presence of bacteria (Thomas et al., 1986), as molecules are metabolised or partition into the cell membrane from the aqueous phase. Further, the presence of undissolved droplets in suspension allows direct partitioning of molecules to the cell membrane.

2.3. Activity and application of essential oil antimicrobials

Essential oils and their components (Fig. 2.1.) have been demonstrated to have antimicrobial activity against a wide range of microorganisms. Antimicrobial activity has been reported against Gram negative and Gram positive bacteria including *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Escherichia (E.) coli*, *Listeria (L.) monocytogenes*, *Pseudomonas (Ps.)* spp., *Staphylococcus (S.) aureus*, *Shigella spp.*,

Salmonella enterica Typhimurium and *Vibrio parahaemolyticus* (Burt, 2004; Holley and Patel, 2005). Fungal species include, *Aspergillus* spp., *Fusarium moniliforme*, *Rhizopus oligosporus* and *Saccharomyces cervisiae* (Holley and Patel, 2005).

Similarly there are numerous reports of essential oils or oil components being tested in food systems. Bactericidal and bacteriostatic activity have been reported against bacteria on fresh and cooked meats, cheese, processed fruit and vegetable products (Holley and Patel, 2005). Commonly it has been found higher levels of agent are required in foods than laboratory media for successful treatment (Burt, 2004; Holley and Patel, 2005). Possible explanations for this phenomenon are increased nutrient availability, binding to proteins (Juven et al., 1994) or partitioning into lipids present in foods.

The greatest limitation to the application of essential oil antimicrobials to food products is that effective treatments require agents at concentrations that alter the sensory qualities of the product (Burt, 2004; Holley and Patel, 2005). Some authors have reported that such sensory changes may be favourable (Mejlholm and Dalgaard, 2002) or negligible after cooking (Tsigarida et al., 2000). Thus, essential oil antimicrobials may have the greatest application when incorporated into the formulation of processed foods. Holley and Patel (2005) suggest smoked foods as excellent subjects for this approach.

Many reviewers have suggested that the key to overcoming the sensory limitations of essential oils is the use of combinations of agents with amplifying effects on antimicrobial activity (Roller and Board, 2003; Burt, 2004; Holley and Patel, 2005). The ideal is to find synergistic combinations of agents, where the effect of two or more agents is greater than the additive effect of the single agents. Some positive results in this direction have been reported. Blaszyk and Holley (1998) reported enhanced effectiveness of combinations of eugenol, monolaurin and sodium citrate against *Brochothrix*

thermosphacta, *Lactobacillus(Lb.) curvatus*, *Lb. sakei* and *Leuconostoc(Lc.) mesenteroides*, *E. coli* and *L. monocytogenes.* Carvacrol has been reported to interact synergistically with *p*-cymene (Ultee et al., 2000) and nisin (Pol et al., 2002).

Ultimately, the most successful approach may be that suggested by Roller and Board (2003) where we create antimicrobial systems that mimic those found in nature. Natural antimicrobial systems found in plants and animals involve groups of molecules which interact and reinforce one another, rather than relying on a single "magic bullet". To develop such systems will require a full understanding of the mechanism of action and chemistry of the molecules involved.

2.4. Mechanism studies of essential oils and phenolics with bacterial cells

Serious attempts to understand the mechanism of action of essential oil components began with the work of Ultee et al. (1998, 1999, 2002) on the affects of carvacrol against *Bacillus* (*B.*) *cereus.* However, speculations on mechanism of action began much earlier guided by studies of phenolic compounds, which are a major component of essential oils, and other results from trials of essential oils in model systems.

The earliest use of a phenolic compound as an antimicrobial was the introduction of carbolic acid by Lister in 1867 as a surgical sanitizer. Since then a number of other phenolic compounds have been investigated for use as antimicrobials within the food industry. These include esters of *p*-hydroxybenzoic acid (parabens) and phenolic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) (Branen et al., 1980; Davidson, 1993) (Fig. 2.2.).

In an early paper, Roberts and Rahn (1946) reported changes in dehydrogenase and catalase activity, as well as oxygen uptake by *E. coli* cells in the presence of phenol. They reported that at concentrations sufficient to slow but not inhibit growth there was no effect on catalase and dehydrogenase activity but O_2 consumption was 85% of control. At growth inhibiting concentration there was no effect on dehydrogenase activity, catalase activity was 90%, and O_2 consumption was 25-50%. A lethal dose of phenol resulted in no dehydrogenase activity or O_2 consumption, while catalase activity was reduced to 60%. Roberts and Rahn (1946) suggested that the observed inhibition of enzyme activity could be a consequence of the antimicrobial mechanism, not the cause.

A series of papers were published on the interaction of phenolic compounds with membrane lipids during the 1970's. Kaye and Proudfoot (1971) reported that phenolic compounds were capable of disrupting phosphatidylethanolamine monolayers. They arranged four phenolic compounds in order of their effectiveness in disrupting phosphatidylethanolamine monolayers as follows: phenol < *o*-cresol < *p*-cresol < 2,6-xylenol. Oleuropein, a phenolic compound extracted from green olives, was reported by Juven et al. (1972) to induce leakage of L-glutamate, potassium and inorganic phosphate from *Lb. plantarum*. On the basis of the swelling of cells of *Ps. aeruginosa* suspended in water Bernheim (1972) argued that phenol and chlorophenol interact with the phospholipids of the cell membrane. Eletr et al. (1974) reported that BHT perturbed the structure of sodium dodecylsulfate micelles and phospholipid vesicles lowering the transition temperature by 10 °C.

Furr and Russell (1972) in study of four paraben compounds (methyl, ethyl, propyl, and butyl) on *Serratia marcescens* in nutrient broth at 30 °C observed that the minimum inhibitory concentration (MIC) decreased with increasing alkyl chain length.

Treatment of cells with parabens, particularly propyl and butyl esters was observed to result in significant leakage of cell contents as measured by leakage of pentoses. The addition of 0.25% (w/v) $MgSO_4$ or 0.33 M sucrose or both had no effect on the MIC but $MgSO_4$ reduced the rate and extent of pentose leakage. Since the addition of magnesium ions would stabilise the cell membrane but did not alter the MICs of the paraben compounds tested, it was evident that leakage was not provoked by chelation of outer membrane ions.

BHT interaction with phospholipid vesicles was reported by Singer and Wan (1977) to reduce the permeability to $^{22}Na^+$ and lower the temperature at which fatty acyl chain motion increased (measured by electron spin resonance spectroscopy). Singer and Wan (1977) proposed a model in which BHT partitioned into the lipid bilayer, but due to the presence of the hydroxyl group it was believed anchored at the lipid-water interface where it disrupted lipid packing resulting in increased fluidity. They proposed that the observed reduction in Na^+ permeability could have resulted from alteration in the surface dipole potential and/or organisation of interfacial water.

Eklund (1980) compared the affects of the weak organic acids benzoate, sorbate and propionate, as well as parabens (methyl, ethyl, propyl, butyl) on growth and amino acid uptake by whole cells and membrane vesicles of *B. subtilis, E. coli, Ps. aeruginosa* in minimal media at 37 °C. Eklund examined the relative concentrations required for 50% inhibition. It was observed that there did not appear to be a correlation between the inhibition of uptake or growth by the weak organic acids. By comparison, the concentrations required for inhibition of growth by the alkyl-esters of benzoic acids were closely correlated with inhibition of amino acid transport. Additionally, the concentrations of parabens required for inhibition was reduced with increasing alkyl

chain length. Eklund (1980) concluded that growth inhibition by the parabens may be a consequence of transport inhibition.

BHA was shown to introduce electrons into the electron transport chain by the reduction of a + a_3 and b type cytochromes in cell free extracts of *S. aureus* (Degré et al., 1983). BHA was also able to reduce mammalian cytochrome c. However, BHA was also observed to inhibit oxidation of NADH and succinate by whole *S. aureus* cells. Degré et al. (1983) noted these results indicate that BHA does not interfere with the functioning of the electron transport chain and that inhibition of electron transport chain substrates, NADH and succinate, is a consequence of other effects on cell physiology. Degré et al. (1983) suggested that inhibition of lipid synthesis may explain these results, based on the involvement of lipids in the respiratory process of *S. aureus* and the reported interference of BHA in the lipid synthesis of *Tetrahymena pyriformis* (Surak, 1980). However, the same results would not be inconsistent with the disruption of the proton motive force (PMF) by membrane disruption.

Clear support for the role of membrane disruption in growth inhibition of *E. coli* by phenol was reported in a study Heipieper et al. (1991). At growth inhibitory concentrations phenol was observed to induce leakage of ATP, potassium ions and UV (260 nm) absorbing cell components in a dose dependent manner.

Disruptive interactions of phenolics with cellular membranes appear to be a property shared by a wide range of cyclic hydrocarbons. In their study of the interactions between cyclic hydrocarbons and *E. coli* liposomes, Sikkemma et al. (1994), reported that accumulation of cyclic hydrocarbons resulted in the efflux of protons and a membrane impermeable probe carboxyfluorescein (molecular weight 376 g/mol). This efflux was accompanied by gross perturbations of the model membranes, swelling of liposomes,

increased membrane fluidity and the release of phospolipids labelled with fluorescent probes.

From the early 1990's onward the amount of available literature indicates a decline in interest in the antibacterial of activity of phenolics, particularly in the food science literature. This is probably a consequence of the failure of these compounds to be widely adopted in the food industry, primarily because of their marginal effectiveness as antimicrobials at the concentrations permitted by regulations (Davidson, 1993). However, there has been an increasing body of literature investigating the activity of specific essential oil components.

The inhibitory effect of the essential oils of oregano and thyme against *S. aureus* (Paster et al., 1990) and of oil components of thymol and carvacrol against *Salmonella* Typhimurium (Juven et al., 1994) was reported to be enhanced when these organisms were incubated under anaerobic conditions. Two explanations for this phenomenon were suggested by Paster et al. (1990). These were that oxidative changes occur in the oil which reduce its activity aerobically, or reduced energy from anaerobic metabolism makes the bacteria more vulnerable to the antimicrobial effects of the oils. The latter explanation implies that the inhibitory mechanism can be repaired or that it reduces the yields from energy metabolism. Juven et al. (1994) also reported protective effects by complex nutrient media compared to minimal media and reproduced the protective effect by the addition of bovine serum albumin. Juven et al. (1994) suggested that the protective effect could be due to complexing of the phenolic agent with peptides.

While searching for chemicals that could reduce the formation of biogenic amines in foods, Wedakoon and Sakaguchi (1995) demonstrated that cinnamaldehyde and eugenol were capable of enzyme inhibition. Ethanol extracts of cloves, cinnamon, sage,

nutmeg and allspice were observed to inhibit the enzymatic activities of hystidine, lysine and ornithine decarboxylase in a crude extract of *Enterobacter aerogenes*. Pure eugenol and cinnamaldehyde were also shown to inhibit hystidine decarboxylase. Wedakoon and Sakaguchi (1995) did not suggest a mechanism to explain the observed inhibition, nor did they address the issue of the role of decarboxylase inhibition in the observed antimicrobial properties of eugenol and cinnamaldehyde.

Combinations of eugenol, monolaurin and sodium citrate have been shown to have a greater antimicrobial effect against a number of food spoilage (*Brochrothrix thermosphacta*, *Lb. curvatus*, *Lb. sakei* and *Lc. mesenteroides*) and pathogenic bacteria (*E. coli, L. monocytogenes*) than alone (Blaszyk and Holley, 1998). The bacteria were grown in broth media at pH 6.5 for 4 days and relative growth determined. Eugenol at 500 ppm completely inhibited *E. coli* at 7 °C. At 18 °C 500 ppm inhibited *E. coli* growth to 35% of control and 1000 pm caused complete inhibition. A concentration of 1000 ppm eugenol inhibited *L. monocytogenes* completely at 7 and 18 °C. Eugenol was able to inhibit *Lb. sakei* to 60% of control at 1000 ppm, but had no effect on *Lb. curvatus* at 7 °C. Eugenol (1000 ppm) inhibited the growth of *Lc. mesenteroides* at 7 and 18 °C, but had a greater effect at 7 °C. A wide number of combinations of the agents were observed to be more effective at restricting growth of all the organisms than treatment with the individual agents (Blaszyk and Holley, 1998). The authors suggested that the increased activity of the combinations was a consequence of all three agents disrupting the cell membrane structure (Blaszyk and Holley, 1998). However, similar results could be expected if one or more agent had effects on the cytoplasmic contents and the other agents increased its access to the cytoplasm by membrane disruption.

Helander et al. (1998) studied the effects of essential oil components, carvacrol, (+)carvone, thymol and trans-cinnamaldehyde on *E. coli* O157:H7 and *Salmonella* Typhimurium. Uptake of the hydrophobic fluorescent dye 1-N-phenylnapthylamine (NPN) by the cell membrane, at growth inhibitory concentrations of the antimicrobials, was used as an indicator of disruption of lipopolysaccharide (LPS) which presents a hydrophilic barrier to NPN. Carvacrol and thymol (0.5 to 2 mM) were demonstrated to disrupt the cell membranes of *E. coli* O157:H7 and *Salmonella* Typhimurium by observation of increased NPN uptake. NPN uptake was not affected by the addition of 2 mM $MgCl_2$ indicating that carvacrol and thymol did not function as chelators (Helander et al., 1998). Carvacrol and thymol treatment also resulted in a significant decrease in cellular ATP pools and a significant increase in external ATP. In contrast, carvone (10 mM) and cinnamaldehyde (2 mM) had no effect on NPN uptake or cellular or external ATP (Helander et al., 1998). The authors interpreted these results as evidence that cinnamaldehyde and carvone were able to gain access to the periplasm or cytoplasm of the cell and that their activity was dependent upon interaction with cell components other than the cell membrane.

Scanning electron microscopy of *B. cereus* cells treated with 0.3 ml/l of cinnamaldehyde revealed cells that had developed a filamentous morphology, with incomplete septa (Kwon et al., 2003). However, the same treatment had no visible effect on *S. aureus*. Kwon et al. (2003) also claimed that cinnamaldehyde caused protein leakage from *B. cereus* and *S. aureus*, as measured by the protein content of the supernatant extracted after centrifugation of cell suspensions. Since cinnamaldehyde is poorly soluble and heavier than water it is conceivable that the observed protein leakage

was a consequence of pelleted cells being exposed temporarily to an extremely high concentration of the hydrophobic molecule.

Evidence for membrane disruption by eugenol and thymol was provided by Walsh et al. (2003) who reported potassium leakage from *E. coli* cells (at 0.05% v/v eugenol) and *S. aureus* (at 0.1% v/v eugenol) taken from agar colonies and suspended unbuffered in water. Unfortunately, since the cells were not buffered against osmotic shock the relevance of these results to cells that were not osmotically stressed is questionable.

Vanillin has been reported to have membrane disruptive effects on *E. coli*, *Lb. plantarum* and *L. innocua* (Fitzgerald et al., 2004). Vanillin at 50-70 mM increased propidium iodide uptake and potassium leakage. However, no effect on cellular ATP pools was observed (Fitzgerald et al., 2004).

The most extensive investigation of the antimicrobial mechanism of a substituted aromatic has been conducted on the thyme oil constituent carvacrol and its effects on *Bacillus cereus*. In the first of a series of papers, Ultee et al. (1998) showed that carvacrol at concentrations in excess of 0.75 mM was bactericidal to both vegetative cells and spores of *B. cereus*, though spores had a greater resistance. The effectiveness of carvacrol was found to increase at pH above and below 7 (Ultee et al., 1998). The apparent increased effectiveness above or below pH 7 was probably due to pH stress on the cells as there was little change in the dissociation constant of carvacrol at pH values lower than 9 (Ultee et al., 1998). When exposed to carvacrol at 30 °C vegetative cells of *B. cereus* grown at 8 °C had a higher death rate than cells grown at 30 °C. The authors proposed that cells grown at 8 °C were more vulnerable due to the increased fluidity of the cell membrane allowing easier incorporation of carvacrol molecules.

In their second paper, Ultee et al. (1999) demonstrated that the mechanism of carvacrol action involved inhibition of energy generation. Addition of 2 mM carvacrol to glucose-energised *B. cereus* caused a rapid decline in cellular ATP and a small, non-proportional increase in external ATP. Further experiments indicated that inhibition involved dissipation of the PMF. Carvacrol was demonstrated to dissipate the membrane potential ($\Delta\Psi$) as measured by the fluorescent dye 3,3-dipropylthiacarbocyanine, the membrane pH and potassium ion gradients (Ultee et al., 1999). Further evidence of the involvement of membrane interaction in the action carvacrol was the observation that *B. cereus* altered the phospholipid composition of its cellular membrane in response to sublethal levels of carvacrol (Ultee et al., 2000). Additionally, Pol and Smid (1999) demonstrated that addition of carvacrol increased the rate that nisin dissipated the PMF.

The phenolic hydroxyl group of carvacrol was demonstrated to play an important role in the mechanism of action of carvacrol against *B. cereus* (Ultee et al., 2002). Comparison was made of the antimicrobial effectiveness of carvacrol and several structurally related molecules (thymol, menthol, carvacrol methylester and cymene). Complete growth inhibition of *B. cereus* was observed with 0.75 mM thymol or carvacrol which differ only in the relative position of the hydroxyl group. Menthol differs from thymol in being composed of a hexane ring and was far less inhibitory with 10 mM being required for inhibition. Carvacrol methylester or cymene which lacks any hydroxyl group had no effect on *B. cereus* growth at 10 mM (Ultee et al., 2002). The importance of the hydroxyl group in carvacrol activity was reinforced by experiments to determine the effect of cymene on the membrane potential, intracellular pH and ATP pools of *B. cereus*. Like carvacrol, treatment with cymene dissipated the membrane potential of *B. cereus*,

though higher concentrations of cymene were required. In contrast to carvacrol, 2.4 mM cymene had no significant effect on cellular or extracellular ATP pools and 2 mM cymene had no significant effect on intracellular pH (Ultee et al., 2002). Observations of carvacrol and cymene interaction with phosphatidylethanolamine (PE) liposomes indicated that though both molecules inserted into the membrane causing its expansion, the degree of expansion caused by cymene was 2.7 times greater and maximum expansion was reached at 0.5 μM carvacrol/mg PE compared to 2 μM cymene/mg PE (Ultee et. al., 2002). Based on these observations Ultee et al., (2002) proposed a model of carvacrol action in which a molecule like cymene inserts into the membrane disrupting its structure, but the presence of an hydroxyl group also allows carvacrol to act "as a transmembrane carrier of monovalent cations by exchanging its hydroxyl proton for another ion such as a potassium ion".

Evidence of membrane disruption by carvacrol was also reported by Lambert et al. (2001). *Ps. aeruginosa* and *S. aureus* cells in trypto soy broth treated with 0.1% carvacrol or thymol for 10 min showed increased ethidium bromide uptake. Carvacrol and thymol also caused dissipation of cellular pH gradients and leakage of potassium and phosphate ions (Lambert et al., 2001).

Other non-aromatic molecules may contribute to the ability of essential oils to disrupt cellular membranes. The essential oil of the Australian tea tree, *Melaleuca alternifolia*, is composed primarily of monoterpenes. A series of studies on tea tree oil reported that membrane disruption of *E. coli* was indicated by electron microscope observations (Gustafson et al., 1998) and potassium leakage (Cox et al., 1998).

Interpretation of these results is complicated by the use of a complex essential oil preparation rather than individual components.

2.5. Studies of essential oil components in non-bacterial systems

The possibility that essential oils may interfere with the cellular machinery of microorganisms was raised by Conner and Beuchat (1984a) in a study of the recovery of eight strains of yeast from heat stress. In general the number of colonies recovered from non-heat stressed yeast cells on agar media containing up to 200 ppm of the essential oil extracts of the spices, allspice, cinnamon, clove, garlic, onion, oregano, savory and thyme was not reduced. The number of colonies recovered from heat stressed cells was reduced in a concentration dependent manner by the addition of essential oils (Conner and Beuchat, 1984a). The authors suggested that the observed effects could be the result of an increase in the interaction of essential oil components with cellular machinery following disruption of the cell membrane by heat stress. In a further study of the effects of spice essential oil extracts on food spoilage yeast, Conner and Beuchat (1984b) observed that essential oil of cinnamon stimulated pseudomycelium production by the yeasts *Saccharomyces cerevisiae* and *Lodderomyces elongisporous* indicating interference with cell wall synthesis.

Inhibition of yeast cell wall synthesis was also investigated by Bang et al.(2000) who reported that trans-cinnamaldehyde inhibited several cell wall synthesis enzymes in *S. cerevisiae*. Experiments with isolated enzymes demonstrated non-competitive inhibition of β-(1,3)-glucan synthase and mixed inhibition of chitin synthases 1,2 and 3. Thymol and eugenol were also reported to interact with the cell wall and membrane of *S.*

cerevisiae. An electron microscopy study found these compounds caused damage to cell surfaces and released metabolites that absorbed at 260 nm (Bennis et al., 2004).

Eugenol and cinnamaldehyde have been reported to inhibit the ATPase activity of rat mitochondria (Usta et al., 2003). Inhibition of the P-type Na^+/K^+ ATPase (eugenol at 1.1 mM and cinnamaldehyde at 4.7 mM) and CPx-type Cu^{2+} ATPase (eugenol at 0.65 mM and cinnamaldehyde at 0.94 mM) were reported. In contrast, Usta et al. (2003) also reported that eugenol (0.3 to 30 mM) and cinnamaldehyde (0.37 to 37 mM) stimulated the mitochondrial F_1F_0-ATPase. However, this conclusion may not be fully accurate as the activity of the F_1F_0-ATPase was determined by mitochondrial potassium production from ATP rather than by membrane preparations or purified enzymes. Thus the apparent stimulation of the F_1F_0-ATPase could be due to uncoupling of respiration by membrane disruption.

Thymol and carvacrol have been observed to bind to purified DNA by hydrogen bond interactions in a 40% ethanol solution (Nafisi et al., 2004). The significance of this to any interactions with cells is unclear.

2.6. Conclusions

Though the available literature clearly indicates the involvement of membrane interactions in the antimicrobial activity of essential oil components, the precise nature of these interactions and their significance in inhibition or cell death remains controversial. Individual results can be interpreted to indicate three potential mechanisms: (1) energy uncoupling through proton transport; (2) membrane disruption leading to energy uncoupling and leakage of cell contents; (3) inhibition of essential enzymes. Due to the

diverse chemical structures of essential oil components it may be inappropriate to generalise about the mechanism of action.

Much of the uncertainty surrounding this issue arises from the failure of experimenters to distinguish clearly between bactericidal and bacteriostatic effects and the frequent use of non-purified antimicrobial agents in experiments.

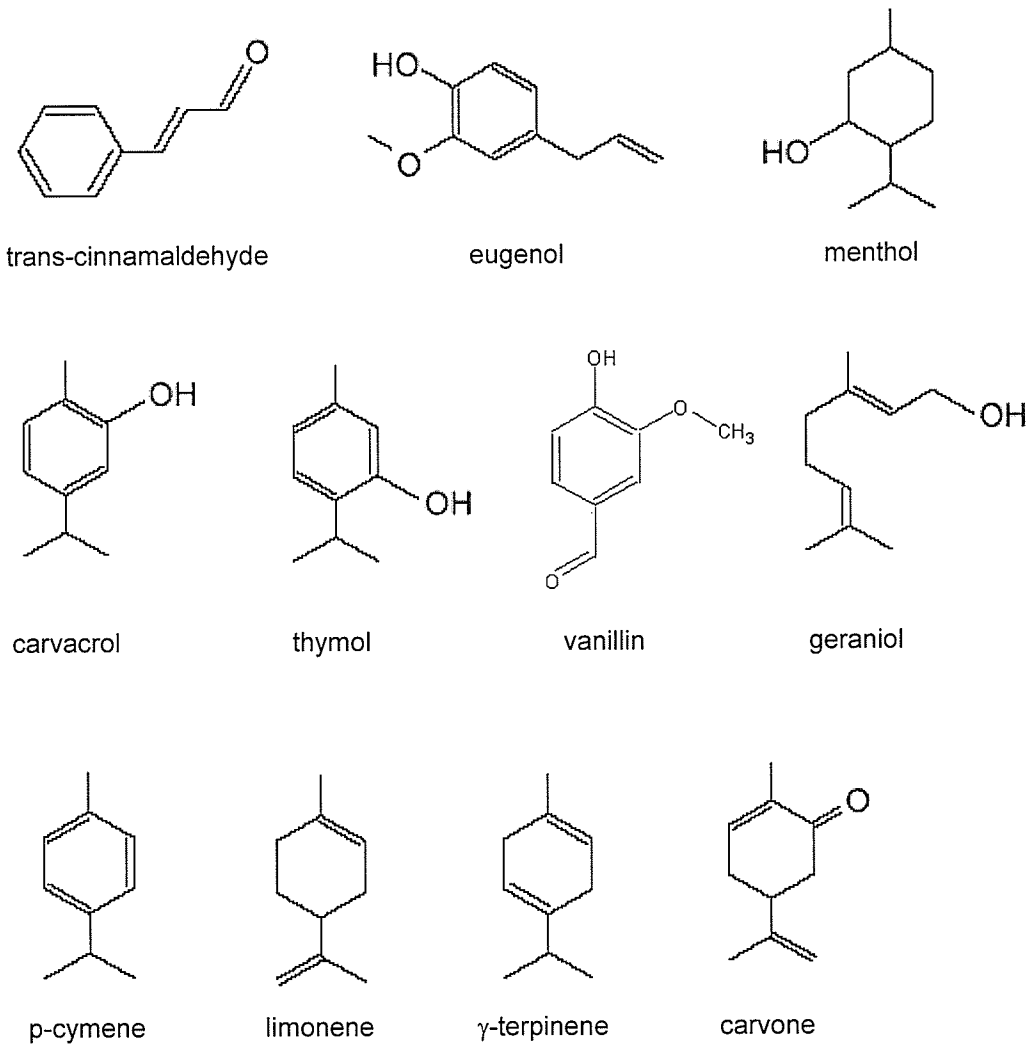
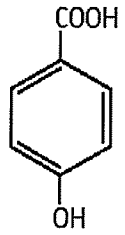


Fig. 2.1. Molecular structures of selected essential oil components



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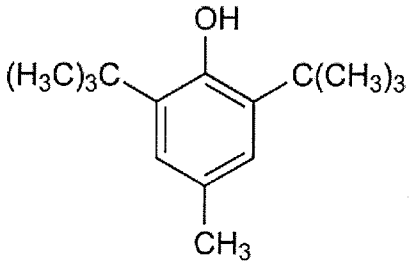
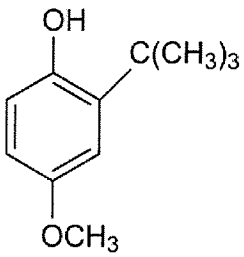


butylated hydroxytoluene



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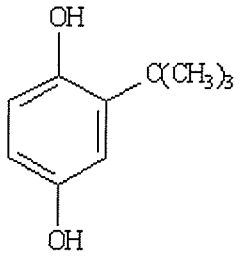


tertiary butylhydroquinone

Fig. 2.2. Molecular structures of selected phenolic compounds

Table 2.1. Properties of Essential Oil Components

	Carvacrol 5-isopropyl-2-methylphenol	**Cinnamaldehyde** 3-phenyl-2-propenal	**Eugenol** 2-methoxy-4-(2-propenyl)phenol
Mol. Wt.[a]	150.22	132.16	164.204
Density g/ml[a]	0.976[c]	$1.050^{25}{}_{25}$	$1.0664^{20}{}_{4}$
Refractive Index[a]	1.523[c]	1.6219^{20}	1.5408^{20}
Melting Point[a]	3.5°C	-7.5°C	-9.2°C
Boiling Point[a]	237-238°C	246°C	255°C
pKa[b]	10.62 (20°C)[d]	NA	10.19 (25°C)
LD_{50} Rat (oral)[b]	810 mg/kg	2220 mg/kg	1930 mg/kg
Solubility in 100 parts water[a]	0.1[d]	0.014 in 100 parts	NA

a. Data obtained from Dean, (1999).
b. Data obtained from Combined Chemical Dictionary, CRC Press, http://www.chemnetbase.com.
c. Data from Sigma-Aldrich Material Safety Data Sheet.
d. Values given for carvacrol isomer thymol. NA – not available

3. Evaluation of antilisterial action of cilantro oil on vacuum packed ham.

3.1. Abstract

Cilantro oil is an essential oil preparation extracted from the plant *Coriandrum sativum*. A series of experiments were conducted to evaluate the ability of cilantro oil to control the growth of *Listeria monocytogenes* on vacuum packed ham.

The *in vitro* minimum inhibitory concentration for 5 strains of *L. monocytogenes* was found to vary from 0.018 to 0.074% depending on strain. Cilantro oil treatments were then tested on ham disks inoculated with a cocktail of the five *L. monocytogenes* strains. The treatments studied were 0.1, 0.5, and 6% cilantro oil diluted in sterile canola oil or incorporated into a gelatin gel in which lecithin was used to enhance incorporation of the cilantro oil. Gelatin gel treatments were also conducted with 1.4% monolaurin with or without 6% cilantro oil to determine if an interaction between the antimicrobials could increase inhibition of *L. monocytogenes*. Treated ham was then vacuum packed and stored at 10°C for up to 4 weeks.

The only cilantro oil treatment which inhibited growth of *L. monocytogenes* on the ham samples was the 6% cilantro oil gel. Samples receiving this treatment had populations of *L. monocytogenes* 1.3 log CFU/ml lower than controls at week one of storage, though there was no difference between treatments from week two onward. It appears that immobilization of the antimicrobial in a gel enhanced the effect of treatments.

Cilantro oil does not appear to be suitable agent for the control of *L. monocytogenes* on ham. Possible reasons for reduced effectiveness of cilantro oil against *L. monocytogenes* on ham are discussed.

3.2. Introduction

Spices have been used as food preservatives throughout history and the antimicrobial activity of specific spices was documented as early as the late 19th century (Boyle, 1955). However, these compounds have received surprisingly little study as antimicrobials until comparatively recently (Shelef, 1983). In the late 1970's there was a renewal of interest in the antimicrobial properties of spices as a consequence of changes in consumer attitudes to the use of agents such as nitrates and NaCl in foods (Shelef, 1983).

Cilantro oil is an essential oil preparation extracted from cilantro or coriander, *Coriandrum sativum.* Cilantro is used in food preparation in the Middle East and South East Asia and is ascribed medicinal properties in China (Brown, 1995). Cilantro oil contains a complex mixture of organic molecules, with C_{10} to C_{12} aldehydes predominating (Potter, 1996). Since it has been has reported that significant quantitative differences exist between samples of cilantro at different growth stages (Potter, 1996), the composition of the cilantro oil used in these experiments was determined by gas chromatography/mass spectroscopy.

The ability of spices to inhibit the growth of microorganisms is well established. The essential oils of spices have been reported to inhibit the growth of a wide range of pathogenic or food spoilage bacterial species (Aureli et al., 1992; Sivropoulou et al., 1995; Ouattara et al., 1997). Specific essential oil components have also been demonstrated to inhibit a wide range of bacterial species (Moleyar and Narasimham, 1992; Jay and Rivers, 1984).

The antimicrobial mechanism of spices remains unclear. Most reviews of the topic ascribe the effect to disruption of the cell membranes, leading to depletion of cellular

energy by lipophilic molecules (Shelef, 1983; Conner, 1993). Recent studies suggest that the mechanism of action of spice components may be more complex. While searching for chemicals that could reduce the formation of biogenic amines in foods, Wendakoon and Sakaguchi (1995) found that spice oils can inhibit enzyme activity. Ethanol extracts of cloves, cinnamon, sage, nutmeg and allspice were observed to inhibit the enzymatic activities of histidine, lysine and ornithine decarboxylases in a crude extract of *Enterobacter aerogenes*. Eugenol and cinnamaldehyde, major components of the spice oils, were demonstrated to inhibit histidine decarboxylase (Wedakoon and Sakaguchi, 1995). Helander et al., 1998, studied the effects of spice essential oil components, carvacrol, carvone, thymol and cinnamaldehyde on *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Photobacterium leiognathi*. Analysis of intracellular and extracellular ATP pools by luciferase assay and release of cytoplasmic proteins indicated that carvacrol and thymol disrupt the cell's membranes. However, membrane disruption did not appear to be involved in growth inhibition by cinnamaldehyde and carvone.

Blaszyk and Holley (1998) previously reported that monolaurin glycerol may interact with the spice component eugenol to increase inhibition of meat spoilage and pathogenic bacteria, including *Listeria monocytogenes* at 250 ppm, in broth media. The addition of monolaurin glycerol to agar plates has been reported to reduce the tolerance of *L. monocytogenes* to salt and low pH (Bal'a and Marshall, 1996). Inhibition of *L. monocytogenes* growth by monolaurin glycerol has been reported in beef roasts (Stillmunkes et al., 1993) and beef and turkey frank slurries (Wang and Johnson, 1997).

In this paper we describe studies evaluating the use of cilantro oil to control the growth of *L. monocytogenes* on ham. Since microbial growth occurs on the surface of processed meat products such as ham, we developed treatments that can be applied to the

meat surface. The application of surface treatments to ham has been previously shown to enhance the effectiveness of lysozyme and nisin-based treatments (Gill and Holley, 2000). Due to the hydrophobic nature of the cilantro oil, the surface treatments were applied by using either a vegetable oil or a gelatin gel containing lecithin as an amphillic binding molecule. Lecithin was supplied in the form of powdered egg yolk or 40% soya bean lecithin. In one set of experiments, monolaurin glycerol was added to a gel containing cilantro oil to determine whether interactions with cilantro oil components could increase the antimicrobial effect.

3.3. Materials and Methods

3.3.1. Cultures and incubation conditions

Experiments were conducted using five *L. monocytogenes* strains. These strains are meat plant isolates provided by Dr. M.W. Griffiths, University of Guelph, Guelph, ON. The Canadian Research Institute for Food Safety culture collection numbers for the strains are C716, C717, C718, C719 and C720. All the strains were identified as somatic serotype 1. C718, C719 and C720 have identical genetic fingerprints as determined by pulsed field electrophoresis using Apa1 and Sma1 restriction enzymes. C716 and C717 are unrelated to each other and the other three (Griffiths, 2001). Stock *L. monocytogenes* cultures were frozen at –85°C in glycerol. For experimental use each strain was streak-plated on BHI agar. Cultures were prepared by inoculation of a single colony into BHI broth and incubated overnight at 30°C aerobically. *L. monocytogenes* was enumerated by spread plating on BHI agar or selectively by spread plating on modified oxford (MOX); agar plates were then incubated for 48 h at 24°C.

3.3.2. Materials

Commercially prepared chopped ham mix (12.5% meat protein, pork, water, salt, sugar, dextrose, sodium phosphate, carrageenan, sodium erythorbate and sodium nitrite) was kindly provided by Maple Leaf Meats (Winnipeg, MB). Pasteurized dried egg yolk was provided by Canadian Inovatech Inc. (Winnipeg, MB). Cilantro oil was prepared by hydrodistillation of dried cilantro leaves and stems in a custom built, proprietary column. Lecithin (L-α-phosphatidyl-choline) and monolaurin glycerol (1-monolauroyl-rac-glycerol) were obtained from Sigma Chemical Co. (St Louis, MO). Sausage casings (hog intestine, 30 mm diameter, Canada Compound Western, Winnipeg, MB) were stuffed with meat batter using a 9 litre capacity mechanical sausage stuffer (F. Dick Gmbh, Germany). Knox Gelatin (Tomas J. Lipton Ltd., Toronto, ON), and Canola Harvest, 100 % canola oil, (Canbra Foods Ltd, Lethbridge, AB), were used where required. Low oxygen permeable (2.3 $cm^3.m^{-2}.day^{-1}.atm^{-1}$ 23°C, thickness 75μM) polyethylene-nylon laminated plastic bags (Deli*1) and (0.7 $cm^3.m^{-2}.day^{-1}.atm^{-1}$ 23°C thickness 62μM) metal foil laminate bags (MESE 1250 R) were kindly provided by Winpak (Winnipeg, MB). Standard methods agar (SMA), BHI broth, granulated agar, proteose peptone No. 3, oxford medium base and modified oxford antimicrobic supplement (colistin sulfate 10 mg/l, moxalactam 20 mg/l) for MOX, were all obtained from Difco, Becton-Dickinson (Franklin Lakes, NJ). An Autoplater 4000 equipped with a CASBA-4 automated counting system was used for surface plating on pre-poured agar media and enumeration (Spiral Biotech, Inc., Bethesda, MD). The anaerobic incubator (model 3640-6) was from the National Appliance Co (Portland, OR). Anaerobic conditions were created by flushing twice with 30% CO_2/ 70 % N_2. A model GM 2002 vacuum packaging machine (Bizerba, Mississauga, ON), was used to vacuum package and seal pouches. A Tekmar LSC 2000

purge and trap unit supplied by Tekmar Corp., (Cincinnati, OH) was used to sample headspace gas composition for essential oil volatiles.

3.3.3. Essential Oil Analysis

Diluted oil aliquots (1 µl) were injected into a gas chromatograph (Model HP 5890, Model HP 3396A integrator) equipped with an autosampler (model 7673A, Hewlett Packard, Avondale, PA). Components were separated on a Supelcowax 10 fused silica capillary column (60 m length x 0.25 mm i.d. x 0.25 µm film thickness) with helium and nitrogen as carrier and make-up gases, respectively. Column headpressure was maintained at 207 kPa (30 psi) and the injector split ratio was adjusted to 20:1. The injector and detector (flame ionization) temperatures were both set at 250°C; the oven was programmed to increase from 35°C to 200°C at a rate of 3 °C/min, followed by a final hold time of 10 min.

Mass spectra were recorded with a HP 5890-5970 GC-MSD system. The mass spectrometer was operated with an ion source at 250°C, ionizing energy of 70eV, scan range of 25-250 amu, threshold at 400, and a frequency of 2.6 scans/sec. Column and temperature programming for separation on the gas chromatograph was similar to that described above. Eluted compounds were identified using HP G1034C MS ChemStation software containing a HP G1035A Wiley (138.1) PBM library. Confirmation of several compounds was accomplished by comparing retention times with reference standards (Aldrich Chem. Co., Milwaukee, WI).

3.3.4. Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of cilantro oil was determined for individual *L. monocytogenes* strains by the resazurin indicator method of Mann and Markham (1998). The MIC is the lowest concentration of agent that prevents detectable growth within the period of incubation. The protocol of Mann and Markham was modified by conducting experiments in BHI broth with 0.15% agar at 24°C, with an initial inoculum of 5.3 log CFU/cm^2. Eight replicates were prepared simultaneously in 96 well microtitre plate wells.

3.3.5. Preparation of Ham Sausage

A single batch of raw meat batter obtained from the manufacturer was stored at –20°C until needed. The batter was thawed and casings were soaked in distilled water to remove salt for 24 h at 4°C prior to processing. Ham sausages were prepared by filling batter into the casings with the stuffer. The sausages (approximately 30 cm) were clipped with metal rings and cooked to an internal temperature of 69°C in a jacketed steam kettle for three 20 min intervals at 53, 64, and 75°C. The cooked sausages were then vacuum-packed in heat-sealed O_2 barrier plastic bags and frozen at –20°C.

Ham sausage was thawed overnight at 4°C and cut into 14 mm thick disks on a sterile board using a flame-sterilized knife. This produced discs of meat 29-31 mm in diameter and 14 mm thick, with a surface area (maximum) of 28.73 cm^2, weighing 10 g ± 1 g. Three disks were prepared for each treatment group and for each sampling time point.

3.3.6. Preparation of Inoculum for Ham Experiments

A single colony of each of the five strains of *L. monocytogenes* was transferred to 9 ml of BHI broth, which was incubated aerobically overnight at 30°C. A dipping bath for inoculation of the samples with *L. monocytogenes* was prepared by adding 0.3 ml of each of the 5 cultures to 1.5 litre of 0.1% peptone. This produced a 5 strain cocktail of *L. monocytogenes* which yielded an initial population of approximately 4 log CFU/cm^2 on the inoculated ham.

3.3.7. Sampling for Ham Experiments

Three disks were removed from the packages at each sampling time, were placed separately in a stomacher bag with 90 ml of 0.1% peptone and blended for 2 min (Stomacher 400, A.J. Seward, Canlab, Toronto, ON). The mixed samples were then serially diluted to produce dilutions ranging from 10^{-3} to 10^{-5}. Each dilution was plated in duplicate on modified oxford media (MOX) and SMA using the spiral plater.

The MOX plates were incubated at 24°C aerobically for 48 h to provide selective enumeration of *L. monocytogenes*. The SMA plates were incubated anaerobically for 48 h at 24°C to provide an estimate of the total anaerobic number of bacteria (TAN). The number of CFU/ml per sample was converted to CFU/cm^2 of sample surface.

3.3.8. Treatment of Ham Sausage with Cilantro oil in Canola Oil

The meat disks were inoculated by a 30 sec immersion in the bacterial cocktail using a 21 cm diameter stainless steel mesh basket with 2 mm holes. The meat disks were then allowed to dry on sterile filter paper in a laminar flow hood for 15 min prior to treatment with cilantro oil.

Canola oil, sterilized by autoclaving, was used for dilution of the cilantro oil. The meat disks were apportioned to receive the following treatments: no further treatment (control), dipping in sterile canola oil, and dipping in 0.1%, 0.5% or 6.0% (v/v) cilantro oil. To apply the oil coating, the inoculated meat pieces were dipped with flame-sterilized tweezers into the oil for 5 sec. Excess oil was allowed to drain for 15 sec and the sample was immediately placed into packaging bags.

Three disks from each sample group were retained as time zero samples. These samples were stored at 4°C and analyses were started within 4 h. The remaining samples were divided into groups of three for each time point and vacuum packed in heat-sealed, polyethylene-nylon plastic pouches. The samples were incubated in their sealed pouches at 10°C for up to four weeks after inoculation. Packaging of the 6% (v/v) cilantro oil-treated samples differed. Vacuum packages were placed in gasket-sealed 1 litre glass Mason jars to limit changes in headspace volatile composition that could result from gas penetration in or diffusion through the packaging film.

3.3.9. Treatment of Ham Sausage with Cilantro oil in Gelatin Gel

The ham disks received the following treatments prior to inoculation: no further treatment (control), coating with 7% (w/v) gelatin gel containing egg yolk, and coating with 7% (w/v) gelatin gel containing egg yolk with 0.1% or 0.5% (v/v) cilantro oil.

The gelatin gel was prepared as follows: an emulsion containing 1 g pasteurized dried egg yolk with 3 ml sterile distilled water was prepared by mixing for 5 min in a sterile beaker with a sterile magnetic mixing bar. Cilantro oil (0.1 ml or 0.5 ml) was added to the egg yolk emulsion as appropriate and mixed for a further 10 min. The egg yolk emulsion was then added to a 7% gelatin solution in boiling sterile water. The ham

disks where then treated by dipping into the gelatin solution. The gel was set by placing the coated disks on a sterile grill at 4°C for 5 min, before a second dipping in the gel followed by setting for a further 10 minutes at 4°C.

The treated ham disks were inoculated as described above. Time zero samples were put aside for immediate sampling and the remaining samples were vacuum packed in heat-sealed, polyethylene-nylon barrier bags. The samples were incubated in their pouches at 10°C for up to four weeks after inoculation.

3.3.10. Treatment of Ham Sausage with Cilantro oil and Monolaurin in Gelatin Gel

In a refinement of the preparation of the gelatin gel, pure lecithin was substituted for powdered egg yolk in these experiments. The gelatin gel was prepared with 7% (w/v) gelatin and 2 g lecithin. Four treatment gels were prepared, gel without antimicrobials (control), gel with 6% (v/v) cilantro oil, or 1.40% (w/v) monolaurin glycerol (dissolved in ethanol, 0.3 g/ml) or gel with both 6% cilantro oil and 1.40% monolaurin glycerol. The 6% (v/v) cilantro oil, or 1.40% (w/v) monolaurin glycerol were equivalent to approximately 1000 ppm and 250 ppm, respectively, per ham disk with an average 0.2 g coating of gelatin per 10 g ham sample.

The treated ham disks were inoculated as described above. Time zero samples were put aside for immediate analysis and the remaining samples were vacuum packed in heat-sealed, foil laminate (MESE 1250 R) plastic pouches. The samples were incubated in their bags at 10°C for up to four weeks after inoculation.

3.3.11. Data Treatment

The data generated from this experiment was analyzed to determine if the presence of cilantro oil in the oil or gel-coated samples resulted in significantly different bacterial populations of the test microorganism and TAN compared to control and treated samples.

The data were first screened to determine if there were practical differences between bacterial populations in control and treated samples at each time point during storage. A practical difference in populations was defined as a difference between the means for the control and treatment ≥ 1 log CFU/cm^2. A value of one log was chosen for practical significance as differences of one order of magnitude are generally regarded as being of microbial significance (Gill and Baker, 1998; Jarvis, 1989). If the difference of the two means was of practical significance, a two way t-test with an $\alpha = 0.05$ was conducted to determine whether the difference between the means was statistically significant. For results to be considered significant, the difference between means of control and treatment were required to meet the conditions of both practical and statistical significance.

3.3.12. Analysis of the Permeability of Plastic Bags to Cilantro Oil

Filter paper disks (Whatman #1) were placed in glass petri dishes (50 mm internal diameter) without lids. Except for two control dishes, 0.5 ml of cilantro oil was added to the petri dishes. The open petri dishes were vacuum packed in 15 x15 cm bags composed of either polyethylene-nylon or foil laminates. Care was taken to avoid contact between the film and oil, and the films were tightly stretched across the open face of the dish. The

packaged petri dishes were then placed in 1 litre glass Mason jars and sealed with canning jar lids modified to accommodate a septum. The jars were stored at 12°C.

The headspace volatiles were recovered from each jar after 24 hours by a needle introduced through the septum and connected to a Tekmar LSC 2000 purge and trap unit. Using purified nitrogen at a purging rate of 100 ml/min, volatiles were collected for 5 min on the glass trap. Separation and identification of volatile compounds by gas chromatography and mass spectrometry was done as previously described (Section 3.3.3.).

3.4. Results

The results of the cilantro oil analysis are presented in Table 3.1. The two single largest components were linalool at 25.86% and (E)-2-decenal at 20.22%.

Minimum inhibitory concentrations of cilantro oil determined by *in vitro* tests were found to depend on the isolate of *L. monocytogenes* tested, with values ranging between 0.074% and 0.018% obtained in BHI broth at 24°C (Table 3.2.). Since the highest value for MIC was 0.074%, 0.1% cilantro oil was used as the lowest value for ham surface treatment experiments.

No practical or statistical difference in *L. monocytogenes* or total anaerobic populations were observed between untreated controls and ham samples receiving coating with oil or gel without antimicrobial (results not reported). Cilantro oil applied to ham at concentrations of 0.1, 0.5 and 6.0% in canola oil had no effect on the growth of *L. monocytogenes* or total anaerobic populations. Treatment of ham with cilantro oil incorporated into a gelatin gel at 0.1 or 0.5% also had no significant effect on *L. monocytogenes* growth or total anaerobic populations (results not reported).

Coating with a gelatin gel containing 6% cilantro oil emulsified with lecithin was the only treatment found to have any effect on the fate of *L.* monocytogenes on ham (Fig. 3.1.). There was no significant difference between the number of colonies recovered on aerobic MOX or anaerobic SMA media from the samples. Samples treated with 6% cilantro alone or with 1.4% monolaurin glycerol had numbers of *L. monocytogenes* 1.3 log CFU/cm^2 lower than the other treatments at week 1. No significant differences were observed in *L. monocytogenes* populations between control samples and samples treated with 1.4% monolaurin glycerol. There was also no significant difference between samples treated with cilantro oil or both cilantro oil and monolaurin glycerol together. Thus it appears that treatment with monolaurin glycerol at 1.4% had no effect on *L. monocytogenes*. The experiments were discontinued after week 3 as the numbers of *L. monocytogenes* were greater than 8 log CFU/cm^2 on both control and treated samples.

To determine if the low antimicrobial activity of cilantro oil in the packaged ham experiments was due to diffusion of cilantro oil, the permeability of the polyethylene-nylon and foil laminate films was examined. The polyethylene-nylon film used in these experiments was observed to be permeable to several cilantro oil components (Fig. 3.2A. and 3.2B.) which were detected by gas chromatography. The release of volatiles from the foil laminate film makes it difficult to determine whether the permeability to cilantro oil components influenced the outcome (Fig. 3.3A. and 3.3B.). The detection of peaks for nonane and 1-propoxy-2-propanal suggests that the foil laminate was permeable to some cilantro oil components, although this was limited compared to the polyethylene-nylon film.

3.5. Discussion

The composition of the cilantro oil used in these experiments was considerably different from the composition of the two samples as described by Potter (1996). However, the values for the two largest components (E)-2-decenal (20.22%) and linalool (25.86%) fall within the range of reported values (Potter, 1996; Potter and Fagerson, 1990). The composition of cilantro oil is highly dependent upon the part of the plant used and the stage of growth at harvest (Potter, 1996).

(E)-2-decenal has been previously reported to have antimicrobial activity against *Escherichia coli, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae and Streptococcus pyogenes* at concentrations of 500 to 15.6 μg/ml as measured by disc diffusion assay (Bisignano et al., 2001).

Linalool is a major component (greater than 10%) of several essential oils with known antimicrobial action, including cinnamon, pimento, black pepper, oregano and thyme (Ouattara et al., 1997). Moleyar and Narasimham (1992), reported that 1000 μg /ml of linalool slowed the growth of unspecified *Staphylococcus*, *Micrococcus* and *Bacillus* species on solid agar medium. Carvone, a minor component of cilantro oil (1.2%) has also been demonstrated to have an antilisterial effect at a concentration of 5 mM at 45°C, but not at 8°C (Karatzas et al., 1999). Interestingly, Bisignano et al., (2001) reported that a combination of four olive aldehydes was significantly more effective at inhibiting bacterial growth than any of the aldehydes singly. This suggests that spice oil extracts may be more effective antimicrobials than their purified components. The variation in cilantro oil composition may explain why Aureli et al., (1992) previously

reported that cilantro oil had a limited inhibitory effect on the growth of 5 strains of *L. monocytogenes*, as measured by disc diffusion assay.

The MIC determinations conducted in these experiments indicated that cilantro oil inhibited the growth of *L. monocytogenes* in broth media. The effectiveness of cilantro oil against *L. monocytogenes* was found to vary among strains, with values ranging from 0.074 and 0.018%. Since the difference in MIC between the closely related strains C719, C720 and C718 was a two-fold dilution, it is likely that this difference reflects the sensitivity of the technique used. Nevertheless, this plant extract was found to exert a potent *in vitro* antimicrobial effect against these species at relatively low concentrations.

Variation in the effectiveness of spices against different serotypes of *L. monocytogenes* has been reported by Hefnaway et al. (1992). The spices sage, allspice, cumin, garlic powder, paprika, and red pepper were all effective against *L. monocytogenes* Scott A (serotype 4b), but only sage was observed to have any activity against *L. monocytogenes* V7 (serotype 1)(Hefnaway et al., 1992). Since all five of the strains used in our experiments had the same somatic serotype, our results show that non-serotype strain variation can result in a variable response to spice oil extract.

The surface treatments examined in these experiments were found to be ineffective in controlling the growth of the five *L. monocytogenes* strains on ham. Significantly fewer viable cells of *L. monocytogenes* were recovered after one week on samples treated with 6% cilantro (with or without monolaurin) immobilized in a surface gelatin gel. It appears that the immobilization of cilantro oil in a surface gel increases the effectiveness of the treatment, an observation in agreement with previous work conducted with other antimicrobials (Gill and Holley, 2000). It is possible that the slight increase in effect could be due to the use of the foil laminate film, which has a lower permeability to

cilantro oil components or the presence of bioactive volatile compounds released from the film. The absence of any immediate antibacterial effect (time zero) by cilantro oil or significant effect by 6% cilantro oil in canola oil when packaged in impermeable glass jars suggests that diffusion through the packaging film is not the primary reason for the ineffectiveness of the cilantro treatments.

The ineffectiveness of the cilantro oil treatments when applied to ham may be due to a number of factors. It is possible that the antibacterial cilantro oil components remained partitioned in hydrophobic environments created by lecithin, canola oil or fat in the ham. Thus, it is possible that there was little opportunity for cilantro oil come into contact with bacterial cells growing on more hydrophilic ham surfaces (Holley, 1997).

A correlation between fat content and the effectiveness of spices is supported by the observation of Ceylan et al. (1998) that the effectiveness of spices against *E. coil* O157:H7 was considerably greater in agar than ground beef or salami with 20% fat content. Partitioning of spice components into meat lipids was also suggested by Cutter (2000) as an explanation for the ineffectiveness of spice treatments in ground beef against *E. coli*, *L. monocytogenes* and *S.* Typhimurium.

Another possibility is that cells attached to the meat surface were in a different physiological state than those in 0.15% agar, which could have rendered them less susceptible to the antimicrobial properties of cilantro oil. The physiological state of bacterial cells has been shown to affect the activity of spice components. In a study of the response of *L. monocytogenes* to combined carvone and mild thermal treatment (45°C), Karatzas et al. (1999) found that the treatment had a bactericidal effect resulting in a reduction of 2 log CFU/ml on exponential phase cells grown at 8°C. The same treatment

had no effect on stationary phase cells grown at 8°C or on cells from either phase grown at 35°C (Karatzas et al., 1999).

A number of different of physiological changes may be proposed to explain the increased resistance of bacterial cells localized at the surface of meats. One possibility is that membrane composition is altered by changes in temperature and growth rates resulting in altered interaction with the antimicrobial. Ming and Daeschel (1995) observed that nisin resistant strains of *L. monocytogenes* Scott A had significantly lower levels of the phospholipids phosphatidylglycerol, diphosphatdylglycerol and bis-phosphatidylglyceryl phosphate, compared to sensitive strains. Another possibility is that the cells growing in a complex, nutrient rich environment such as meat, will reach their maximum replication rate and still have excess nutrients available for the repair or increased turnover of cellular components. Such cells can be expected to have increased resistance to many different stresses. To our knowledge this possibility has yet to be investigated. Alternatively, cells growing on the meat surface may form a microbial biofilm community. Cells in such communities have been observed to possess higher resistance than planktonic cells to a wide range of antimicrobials (Kumar and Anand, 1998; Bower and Daeschel, 1999).

In conclusion, it appears that cilantro oil is not a suitable agent for applications to control *L. monocytogenes* on meat products. The phenomenon of a significant reduction in the effectiveness of antimicrobial essential oil treatments when they are moved from model media to food applications is well established. Determining whether this effect is due to interactions of the agent with environmental components or physiological differences exhibited by target organisms in these different environments may allow for better assessment of novel antimicrobial agents for food use.

Table 3.1. Major components of cilantro oil separated by gas chromatography and identified by mass spectroscopy.

Compound	% (v/v)
Nonane	2.54
α-Pinene	2.74
p-Cymene	3.51
Linalool oxide	1.52
Decanol	8.42
Camphor	1.85
Linalool	25.86
Terpinene	1.09
(E)-2-Decenal	20.22
Carvone	1.20
1-Decanol	3.88
(E)-2-Decen-1-ol	7.90
(E)-2-Dodecenal	3.72
Octanoic	1.69
Minor components	13.86

Table 3.2. Minimum inhibitory concentrations of cilantro oil as determined for *Listeria monocytogenes* strains in BHI broth at 24 °C.

Strain	MIC % (v/v)[1]	Proportion[2]
C716	0.018	3
C717	0.074	4
C718	0.074	6
C719	0.037	7
C720	0.037	3

1. Minimum inhibitory concentration, the lowest concentration at which no growth observed in any replicates.
2. Proportion of replicates, out of eight, with growth at the serial dilution below the MIC.

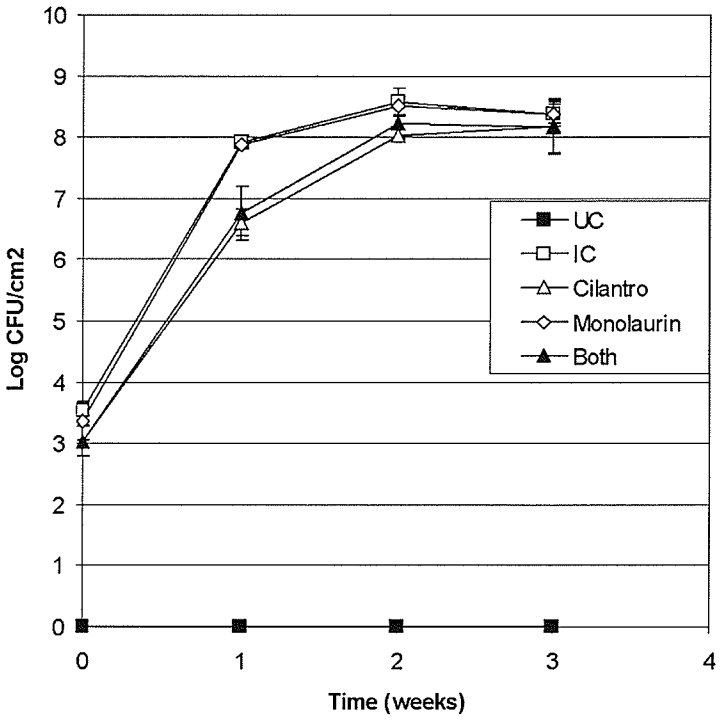


Figure 3.1. Growth of *Listeria monocytogenes* on vacuum packaged ham at 10°C, recovered on aerobic modified oxford media. UC –uninoculated control; IC – inoculated control. Cilantro – samples coated with gelatin gel containing 6% cilantro oil. Monolaurin - samples coated with gelatin gel containing 1.4% monolaurin glycerol. Both - samples coated with gelatin gel containing 6% cilantro oil and 1.4% monlaurin. Average of three replicates. Error bars indicate 95% confidence interval.

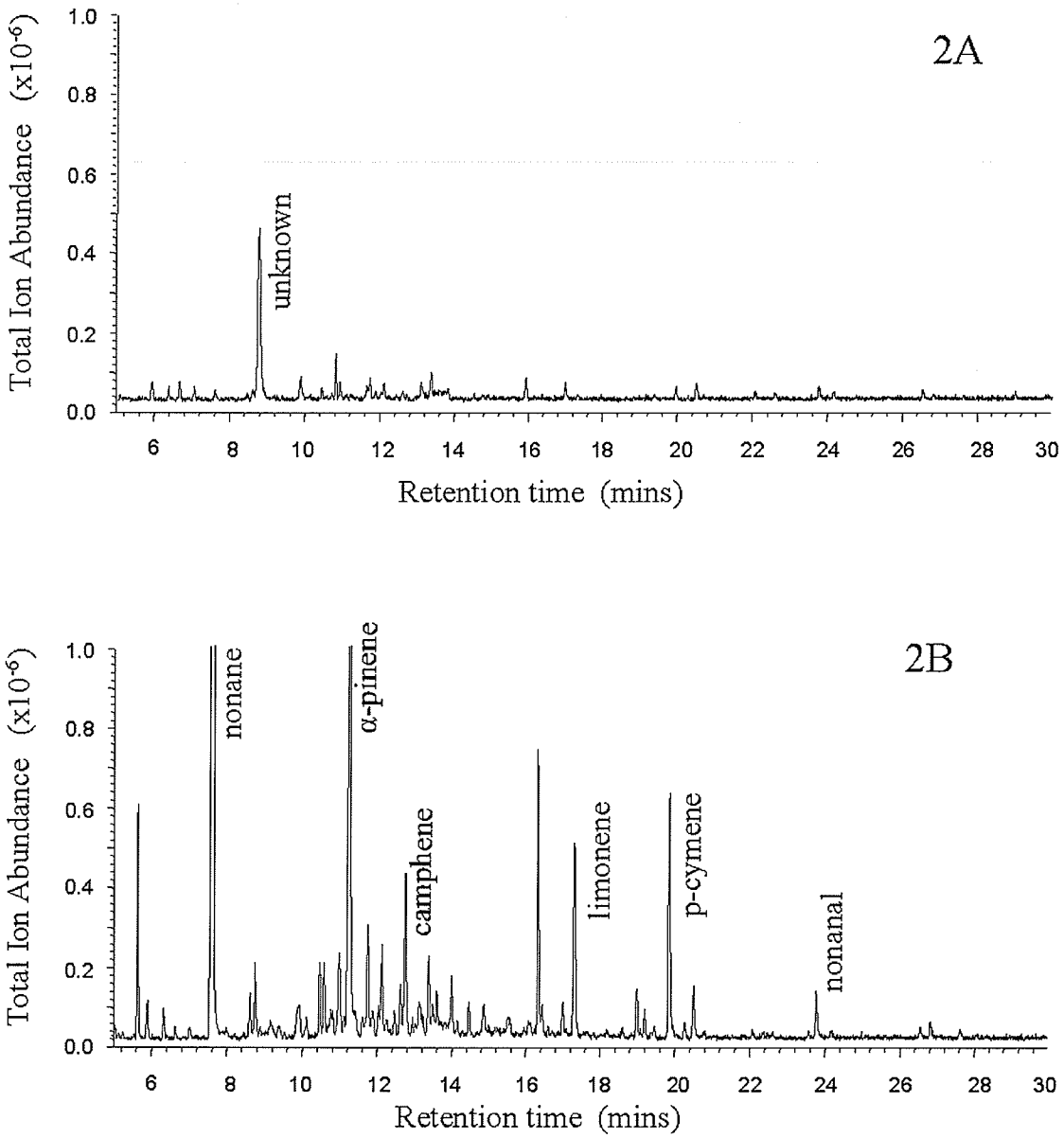


Figure 3.2. Gas chromatograms of samples withdrawn from the glass jar headspace above cilantro oil packed in a polyethylene-nylon pouch. A- without cilantro oil. B- with cilantro oil. Analysis of headspace after one day in a glass container at 12 °C.

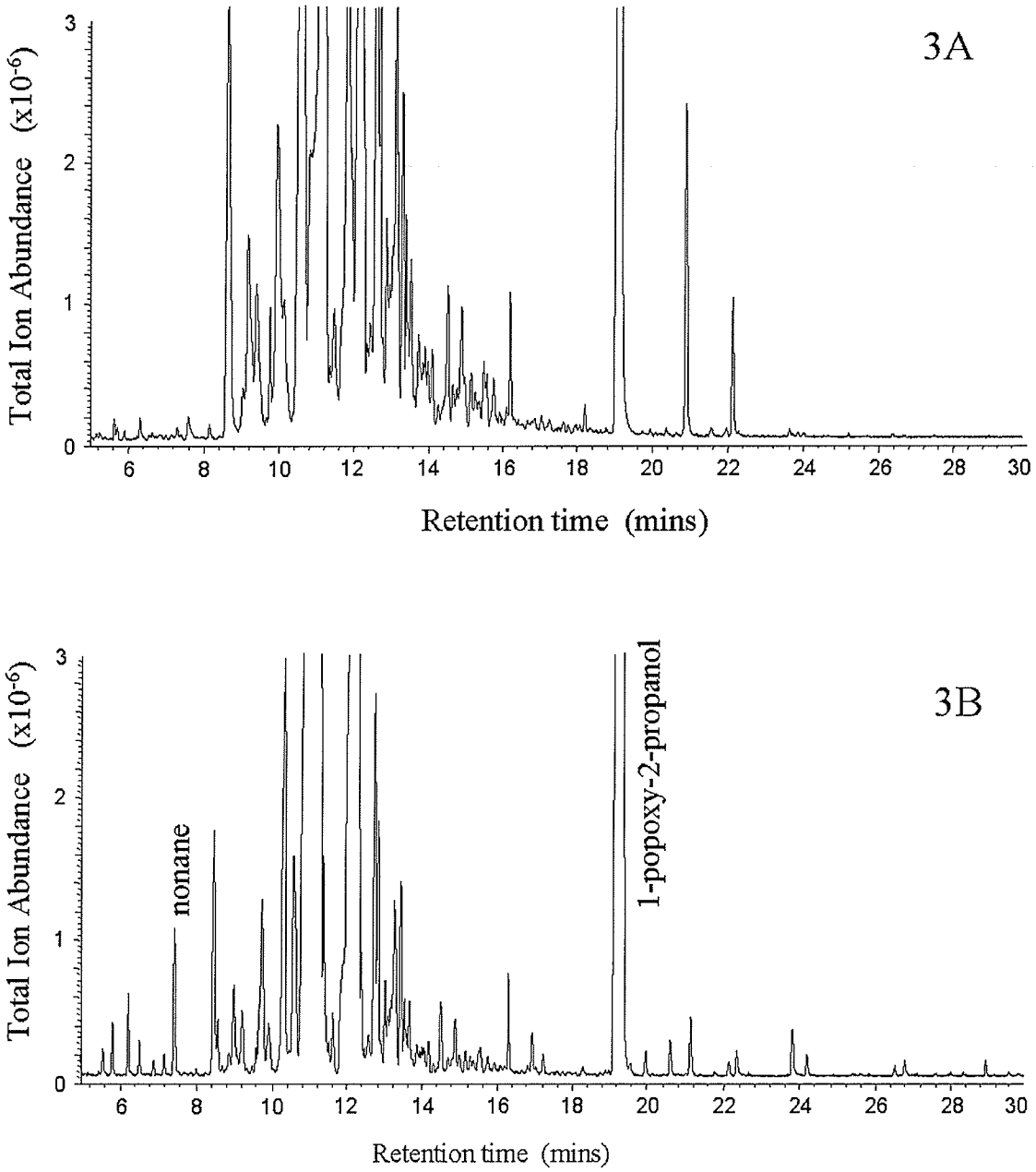


Figure 3.3. Gas chromatograms of samples withdrawn from the glass jar headspace above cilantro oil packed in a foil laminate pouch. A- without cilantro oil. B- with cilantro oil. Analysis of head space after one day in a glass container at 12 °C.

4. Mechanisms of bactericidal action of cinnamaldehyde and eugenol against *Listeria monocytogenes* and eugenol against *Lactobacillus sakei.*

4.1. Abstract

The spice oil components eugenol and cinnamaldehyde possess activity against both Gram positive and negative bacteria, but the mechanisms of action remain obscure. In broth media at 20°C, 5 mM eugenol or 30 mM cinnamaldehyde were bactericidal (>1 log CFU/ml reduction in 1 h) to *Listeria monocytogenes.* At 6 mM eugenol was bactericidal to *Lactobacillus sakei*, but treatment with 0.5 M cinnamaldehyde had no significant effect.

To investigate the role of interference with energy generation in the mechanism of action, the cellular and extracellular ATP of cells in HEPES buffer at 20°C were measured. Treatment of non-energised *L. monocytogenes* with 5 mM eugenol, 40 mM cinnamaldehyde or 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) for 5 min prevented an increase in cellular ATP upon addition of glucose. Treatment of energised *L. monocytogenes* with 40 mM cinnamaldehyde or 10 μM CCCP caused a rapid decline in cellular ATP levels, but 5 mM eugenol had no effect on cellular ATP. Treatment of *Lb. sakei* with 10 mM eugenol prevented ATP generation by non-energised cells, and had no effect on the cellular ATP of energised cells. CCCP at 100 μM had no significant effects on the cellular ATP of *Lb. sakei*. No significant changes in extracellular ATP were observed.

Due to their rapidity, effects on energy generation clearly play a major role in the activity of eugenol and cinnamaldehyde at bactericidal concentration. The possible

mechanisms of inhibition of energy generation are inhibition of glucose uptake or its utilisation and effects on membrane permeability.

4.2. Introduction

Spices have traditionally been used to preserve foods, as well as to enhance flavour and odour. The earliest description of antimicrobial effects by a spice was made by Antony van Leeuwenhoek. In a letter dated October 9 1676, Leeuwenhoek described the decline in the number and activity of "animalcules" in a sample of well water following the addition of pepper (Dobell, 1960). Current interest in the use of compounds derived from spices as antimicrobials was sparked in the 1980s by changes in consumer attitudes to the use of preservative agents such as nitrates and NaCl in foods (Shelef, 1983). However, progress in the application of spice derived compounds as antimicrobials, in food products has been slow. The major problems include accurate identification of active components and the apparent requirement of concentrations that alter the sensory qualities of the food (Nychas and Skandamis, 2003; Roller and Board, 2003).

The antimicrobial activity of spice oils has been attributed to a number of substituted aromatic molecules, such as eugenol, cinnamaldehyde and carvacrol (Fig. 4.1.)(Jay and Rivers, 1984; Juven et al., 1994; Moleyar and Narasimham, 1992). Both eugenol and cinnamaldehyde are of interest for development as food antimicrobials due to their demonstrated activity against both Gram positive and Gram negative bacteria, including organisms of safety concern. Eugenol has been reported to inhibit the growth of *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Blaszyk and Holley, 1998). Cinnamaldehyde has been reported to inhibit the growth of *Clostridium botulinum*

(Bowles and Miller 1993), *Staphylococcus aureus* (Bowles et al., 1995), *E. coli* O157:H7 and *Salmonella* Typhimurium (Helander et al., 1998).

To resolve the problem of high concentrations it has been proposed that spice derived compounds should be utilised in a system of antimicrobial agents, in a form of hurdle technology (Adams and Smid, 2003; Blaszyk and Holley 1998; Nychas and Scandamis, 2003; Roller and Board, 2003). The development of multicomponent antimicrobial systems for food products requires a greater understanding of the mechanism action of specific agents so that attention can be focused on potentially effective combinations.

Though it is common for reviewers of the topic of spice oils to ascribe the antimicrobial effects to interactions with the cell membrane, the precise mechanisms of bactericidal or inhibitory action remains unclear (Brul and Coote, 1999; Roller and Board, 2003). This problem is due to some extent to the use of methodologies that fail to adequately distinguish between inhibitory or lethal effects (Nychas and Scandamis, 2003). The available experimental evidence for eugenol and cinnamaldehyde is contradictory with existing evidence supporting membrane interaction and inhibition of specific cellular processes or enzymes (Helander et al., 1998;Kwon et al., 2003; Walsh et al., 2003; Wendakoon and Sakaguchi, 1995).

The aim of the experiments described in this paper was to investigate the role played by inhibition of ATP generation at bactericidal concentrations of eugenol and cinnamaldehyde. Bactericidal concentrations being defined by the prevention of reproduction by treated cells following transfer to a non-inhibitory medium. More than one mechanism may be involved in the activity of eugenol and cinnamaldehyde. However, the relevance of alternate mechanisms can be discounted if rapid inhibition of

energy generation occurs. This is because cells that are unable to generate energy are unable to reproduce or alter metabolism to adapt to antimicrobial challenge.

Experiments were conducted on the Gram positive organisms *Lactobacillus sakei* and *Listeria monocytogenes*. These organisms were selected due to their relevancy to a wide range of food products; *L. monocytogenes* is an important food borne pathogen and lactic acid bacteria such *Lactobacillus sakei* are of importance in spoilage and fermentation of many foods. Additionally, though both bacteria are not greatly dissimilar physiologically, their energy metabolisms are significantly different. *L. monocytogenes* possesses an electron transport chain and generates energy by respiration, whereas *Lb. sakei* generates energy solely by fermentative metabolism (Kandler and Weiss, 1986; Seeliger and Jones, 1986).

4.3. Materials and Methods

4.3.1. Materials

All Purpose Tween (APT) broth, M17 broth, Brain Heart Infusion (BHI) broth, Dextrose (glucose) and Proteose Peptone #3 were supplied by Difco, Becton-Dickinson, Sparks, MD. Agar Granulated and Trypticase Soy broth (TSB) were supplied by BBL, Becton-Dickinson, Sparks, MD. Yeast Extract (YE), eugenol (2-methoxy-4-[2-propenyl]phenol), carbonyl cyanide m-chlorophenylhydrazone (CCCP), diethylaminoethyl-dextran chloride avg M.W. 500,000 (DEAE-dextran), ATP assay mix (FL-AAM), ATP standard (FL-AAS), ATP assay mix dilution buffer (FL-AAB) and Protein Assay Kit P5656 were supplied by Sigma Chemicals, St Louis, MO. *trans*-Cinnamaldehyde was supplied by Aldrich Chemical Co, Milwaukee, WI. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was supplied by FisherBiotech,

Fairlawn, NJ. An Isotemp refrigerated circulator 1016S and a Micro-12 microcentrifuge were from Fisher Scientific, Fairlawn, NJ. LB 9509 Berthold Junior luminometer was from Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), EDTA disodium salt and trichloroacetic acid (TCA) were supplied by FisherChemicals, Fisher Scientific. ISO-Grid membranes were supplied by Neogen Co, Lansing, MI. Ultrafree-MC 0.22 μm filter unit with Durapore membrane, were supplied by Millipore Co, Bedford, MA. The spiral plater used was an Autoplate 4000, which was supplied by Spiral Biotech, Norwood, MA.

4.3.2. Bacteria and culture conditions

The *Listeria monocytogenes* strain used was a somatic serotype 1 meat plant isolate, with the Canadian Research Institute for Food Safety culture collection number of C717 (M. W. Griffiths, Univ. of Guelph, ON, Canada, personal communication). The *Lactobacillus sakei* strain used was isolated from spoiled cured meats (Holley et al., 1996). Stock cultures were frozen at –75°C in glycerol. For experimental use *L. monocytogenes* was streak-plated on BHI agar and *Lb. sakei* on APT agar.

4.3.3. Preparation of antimicrobials

Suspensions of the antimicrobials to be tested were prepared immediately prior to use by the addition of eugenol or cinnamaldehyde to sterile TSB+YE pH 7.0 or 25 mM HEPES buffer pH 7.0 and vortex mixed to form a suspension.

4.3.4. Determination of bactericidal concentration of antimicrobials.

A single colony of the bacterium to be tested was used to inoculate 10 ml of TSB+YE pH 7.0 at 20 °C and grown to log phase (OD_{650} between 0.1 and 0.3). The culture was then adjusted by dilution with TSB+YE pH 7.0 to an OD_{650} of 0.1 ± 0.04, then diluted 1/1000 into fresh TSB+YE pH 7.0. A 0.5 ml aliquot of the bacterial cell suspension was then added to a 1.5 ml microcentrifuge tube containing 1 ml of the antimicrobial to be tested, suspended in TSB+YE pH 7.0. Controls composed of cells in TSB+YE pH 7.0 untreated with antimicrobial were run simultaneously. The microcentrifuge tubes were incubated for 60 min in a water bath at 20°C. At the experiment start and every 15 min for 60 min the tubes were mixed by vortexing and duplicate samples spread-plated by spiral plater on BHI (*L. monocytogenes*) or APT (*Lb. sakei*) agar plates. Plates were incubated at 25°C for 48 h and enumerated.

To confirm that the bactericidal effects of the antimicrobial were not due to inhibition of growth by residual antimicrobial, experiments at bactericidal concentration were repeated with duplicate 100 µl samples recovered on ISO-Grid membranes. Samples were washed twice with 20 ml portions of 0.1% peptone before filter incubation on BHI or APT agars.

When ATP measurements were conducted it was found necessary to use a concentration of cells 30 times greater than was used in the experiments used to determine bactericidal concentration. Necessarily, the lethality of the concentration used against cells suspended in 25 mM HEPES buffer was confirmed by spread plating.

4.3.5. Measurement of extracellular and intracellular ATP. The bacterium to be tested was grown to log phase cells in TSB+YE pH 7.0 as described above. The culture was

then harvested by centrifugation at 10,000 x g and washed twice with 25 mM HEPES buffer pH 7.0 before being resuspended in HEPES buffer to an OD_{650} of 0.1 ± 0.04. The resuspended cells were diluted 0.3 ml into 9.7 ml of HEPES buffer and the suspension stored for 2 h at 20°C to deplete cellular ATP from the energised level. Depletion of cellular ATP under these conditions was confirmed during preliminary experiments.

The effect on ATP levels of the antimicrobial was determined for cells that were first energised with glucose or treated with antimicrobial before addition of glucose.

In energised cell experiments 0.5 ml of cells in buffer was added to a 1.5 ml microcentrifuge tube containing 1.0 ml of buffer with 0.375% glucose. The microcentrifuge tubes were incubated for 5 min at 20°C to energise the cells and a 200 µl sample was taken. Then 200 µl of the antimicrobial at 7.5 x final concentration in 25 mM HEPES buffer with 0.25% glucose was immediately added to the tube and the incubation continued for 15 min with 200 µl samples taken every 5 min.

In experiments in which cells were first exposed to antimicrobial, 0.5 ml of cells in buffer was added to a 1.5 ml microcentrifuge tube containing 1.0 ml of the antimicrobial in buffer at 1.5 x the final concentration. The microcentrifuge tubes were incubated for 5 min at 20°C and a 200 µl sample was taken. Then 200 µl of the antimicrobial at the final concentration in buffer with 1.88% glucose was added to the tube and the incubation continued for 15 min with samples taken every 5 min.

At each time point 200 µl samples were placed in ultrafree-MC centrifuge filtration units (0.22 µM). The cells in the samples were separated from the surrounding buffer by centrifuging for 30 s at 8000 rpm in the Micro-12 microcentrifuge. A 100 µl aliquot of 2.5% trichloracetic acid (TCA) with 2 mM EDTA was added to the filter unit.

After a 10 min incubation, the TCA solution was mixed by pipettor and a 20 µl aliquot removed and diluted in 380 µl of FL-AAB and retained as the cellular ATP sample. The filtrate was retained as the external ATP sample. Samples were stored at -75°C until analysed.

4.3.6. ATP analysis

The ATP content of cellular ATP and external ATP samples was assayed by a continuous light output luciferase reaction (Lundin, 2000) with the light output amplified with DEAE-dextran (Ishida et al., 2002). Each 75 µl sample was assayed with 50 µl FL-AAM luceriferase assay mix with 25 µl of 1% DEAE-dextran in 5 mM HEPES pH 7.8. Light output was quantified as relative light units (RLU) with the Junior LB 9509 luminometer. Then 5 µl ATP standard was added and measured as an internal standard. The ATP content of the sample was calculated from the ratio of the RLU of the sample to the RLU of the standard.

4.3.7. Measurement of protein content

The protein content of cell suspensions was determined by the Modified Lowry Method using a Protein Assay Kit according to the manufacturer's instructions.

4.3.8. Statistical Analysis

To determine whether significant differences existed between cells recovered by direct plating and ISO-Grid recovery, as well as between ATP measurements following

different treatments, Student's t-tests were used. The value for α for each analysis is indicated in the appropriate section of the results.

4.4. Results

4.4.1. Response of L. monocytogenes *and* Lb. sakei *to cinnamaldehyde and eugenol*

Experiments were conducted to determine the concentration of eugenol and cinnamaldehyde required for a bactericidal effect on log phase cells of *L. monocytogenes* and *Lb. sakei*. A bactericidal effect was defined as a > 1 log reduction in colony forming units recovered compared to untreated controls within 1 h.

The minimum concentrations of antimicrobial required for a bactericidal effect on *L. monocytogenes* in TSB+YE pH 7.0 was found to be 5 mM of eugenol (Fig. 4.2A.) and 30 mM cinnamaldehyde (Fig. 4.2B.). Use of 6 mM of eugenol was required for a bactericidal effect on *Lb. sakei* in TSB+YE pH 7.0 (Fig. 4.3.), but treatment with up to 500 mM cinnamaldehyde had no effect over a 1 h incubation (results not shown). When experiments were repeated with cells recovered by ISO-Grid filter after washing with 0.1% peptone there was no significant difference (t-test $\alpha = 0.05$) in numbers, with unwashed cells recovered following direct plating on agar (Appendix 2).

The lethality of the antimicrobial treatments towards bacteria suspended in 25 mM HEPES pH 7.0 with 0.25% glucose was verified (Fig. 4.4A. and 4.4B.).

4.4.2. Inhibitory effects on ATP generation by L. monocytogenes

When 0.25% glucose was supplied to *L. monocytogenes* cells in 25 mM HEPES pH 7.0 following 5 min incubation at 20°C, the cellular ATP levels significantly increased

compared to controls without glucose (Fig. 4.5A). Incubation of *L. monocytogenes* with 5 mM eugenol, 40 mM cinnamaldehyde or 10 μM CCCP for 5 min prior to addition of glucose prevented a significant increase in cellular ATP (Fig. 4.5A.).

When *L. monocytogenes* cells were energised by 5 min incubation with 0.25% glucose before addition of the antimicrobial, it was observed that 40 mM cinnamaldehyde and 10 μM CCCP provoked a significant reduction in cellular ATP. However, 5 mM eugenol was observed to have no significant effect on the cellular ATP levels when energised cells were challenged (Fig. 4.5B.).

No significant difference was observed in extracellular ATP levels between controls and treatments for *L. monocytogenes* supplied first with either glucose or antimicrobial (Appendix 3) (t-test α=0.05).

4.4.3. Inhibitory effects on ATP generation by Lb. sakei

Lb. sakei cells treated with 10 mM eugenol had significantly lower amounts of cellular ATP following addition of 0.25% glucose compared to cells in buffer (Fig. 4.6A.). The cellular ATP levels of *Lb. sakei* cells treated with 100 μM CCCP were not significantly different than glucose only controls.

Treatment of energised *Lb. sakei* with 10 mM eugenol or 100 μM CCCP resulted in values for ATP that were not significantly different from either energised or unenergised controls (Fig. 4.6B.).

Again, no significant difference was observed in extracellular ATP levels between controls and treatments for *Lb. sakei* supplied first with either glucose or antimicrobial (Appendix 3) (t-test α=0.10).

4.5. Discussion

4.5.1. Response of L. monocytogenes *and* Lb. sakei *to cinnamaldehyde and eugenol*

The experiments described here demonstrate that above a minimum threshold concentration eugenol has a dose dependent bactericidal effect on growing log phase cells of both *L. monocytogenes* and *Lb. sakei* within 15 min of exposure. Cinnamaldehyde was a less effective bactericidal agent than eugenol, requiring a concentration six times greater for a bactericidal effect on *L. monocytogenes*. Additionally, no effect on *Lb. sakei* was observed with up to 0.5 M cinnamaldehyde. The failure of cinnamaldehyde to have any appreciable effect on *Lb. sakei* suggests three possibilities: i. *Lb. sakei* possesses a cell envelope that is less permeable to cinnamaldehyde than *L. monocytogenes*. ii. *Lb. sakei* possesses a resistance mechanism, which allows it to export or inactivate cinnamaldehyde. iii. *Lb. sakei* does not possess the target for cinnamaldehyde activity found in *L. monocytogenes*. If the third scenario is true then it would seem that the mechanism of cinnamaldehyde action is different from that of eugenol, which affects both organisms at similar concentrations.

4.5.2. Effects of eugenol on ATP generation by L. monocytogenes *and* Lb. sakei

When supplied to *L. monocytogenes* cells prior to glucose, eugenol was observed to prevent the rise in cellular ATP levels that occurred in untreated controls. However, eugenol was not observed to cause ATP depletion from cells previously energised with glucose. In contrast the protonophore CCCP prevented ATP generation from glucose by non-energised cells and caused rapid ATP depletion of energised *L. monocytogenes* cells.

These results appear to contradict the ion transport model for activity proposed by Ultee et al., (2002) for carvacrol, which like eugenol is a substituted phenolic. Carvacrol

is bactericidal to *B. cereus* at 1.5-2 mM in HEPES buffer at 8 °C (Ultee et al., 1998). Carvacrol (2 mM) was observed to cause a rapid decline in the cellular ATP pools and a slight increase in the extracellular ATP of glucose energised *B. cereus* (Ultee et al., 1999). Addition of 0.15 mM carvacrol was shown to dissipate the membrane potential, as measured by the fluorescent dye 3,3-dipropylthiacarbocyanine and 1 mM was sufficient to dissipate the membrane gradients of pH and potassium (Ultee et al., 1999). By comparison of the effect of carvacrol with structurally related molecules (thymol, menthol, carvacrol methylester and cymene) on the growth rate of *B. cereus*, it was found that lower concentrations were required for growth inhibition by molecules possessing a hydroxyl group (Ultee et al., 2002). Based on this observation and the observed effects on membrane ion gradients without apparent ATP leakage, Ultee et al. (2002) proposed an ion transporter model of carvacrol action. In this model the molecule inserts itself into the membrane disrupting its structure, but the hydroxyl group allows carvacrol to act "as a transmembrane carrier of monovalent cations by exchanging its hydroxyl proton for another ion such as a potassium ion." But, it is difficult to distinguish between membrane disruption and ion transport and 6.7 mM carvacrol has been reported to increase eithidium bromide staining of *Ps. aeruginosa* and *S. aureus,* a clear indicator of membrane disruption (Lambert et al., 2001).

If eugenol acted as an ion transporter it would be expected to cause ATP depletion from energised cells as was observed with CCCP, a known ion transporter. CCCP and other protonophores can be predicted to cause depletion of ATP pools since to maintain its normal intracellular pH of 8, *L. monocytogenes* uses electron transport and ATPase to export H^+ (Shabala et al., 2002). Since ATP is not depleted following addition of eugenol to energised *L. monocytogenes*, either the proton gradient of the membrane has not been

dissipated or the activity of the F_1F_0-ATPase has been inhibited. ATPase inhibition is a very real possibility as Rico-Munoz et al. (1987) reported that the phenolic compounds tertiary butylhydroquinine and propyl gallate can reduce the activity of *S. aureus* ATPase in isolated membranes. However, in the same paper a significant increase in ATPase activity was noted in samples treated with butylated hydroxyanisole.

The absence of any observed increase in extracellular ATP from energised cells does not support disruption of the cell membrane as an explanation for lethality. The results observed would be consistent with inhibition of glucose import or utilisation by *L. monocytogenes*. If this is the case, inhibition of glucose utilisation rather than inhibition of uptake seems more probable. *L. monocytogenes* is known to possess two glucose import systems, a high-affinity phosphoenolpyruvate-dependent phosphotransferase system (PTS) (K_m = 0.11 mM) and a low-affinity proton motive force dependent system (K_m = 2.9 mM) (Parker and Hutkins, 1997). It seems unlikely that eugenol would inactivate both transport mechanisms by interaction with the enzymes involved. If inhibition of glucose utilisation occurs then it most probably involves inhibition of an enzyme involved in glycolysis, as inhibition of the TCA cycle or respiration would allow the cell to continue generating ATP by fermentation.

Membrane effects can not be discounted as Walsh et al. (2003) have reported potassium leakage from *E. coli* (3.03 mM) and *S. aureus* (6.06 mM) treated with eugenol. If eugenol is acting as an ion transporter or causes membrane leakage it is conceivable that glucose uptake could continue by PTS, and ATP pools could be maintained by fermentative metabolism. Cells in this position would still be severely injured and could be expected to expend most energy generated on futile attempts to re-establish membrane gradients.

Similar conclusions can be drawn from the results of eugenol treatment on cellular ATP levels of *Lb. sakei*. Like *L. monocytogenes*, *Lb. sakei* imports glucose by PTS and a poorly characterised non-PTS system (Lauret et al., 1996). Further, since *Lb. sakei* generation of ATP is purely fermentative if inhibition of glucose utilisation is occurring it must involve a key step in glycolysis. This conclusion is supported by the failure of 100 μM CCCP to alter ATP generation by *Lb. sakei*, which is presumably able to continue ATP generation by substrate level phosphorylation.

Though Wendakoon and Sakaguchi (1995) found that eugenol at >6 mM is inhibitory to the histidine decarboxylase of *Enterobacter aerogenes,* the role of inhibition of biosynthetic enzymes can be discounted, as the rapidity of effects on energy metabolism at bactericidal concentration make any such activity irrelevant.

4.5.3. Effects of cinnamaldehyde on ATP generation by L. monocytogenes

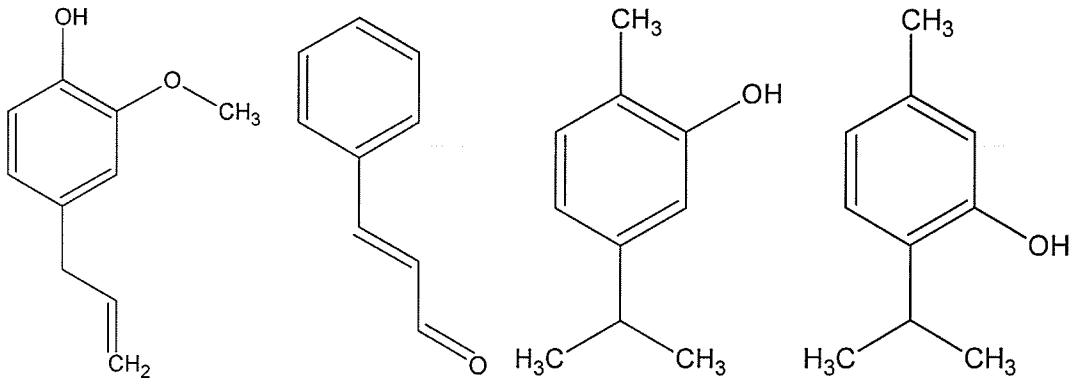
As with eugenol, the possibility that the mechanism of cinnamaldehyde activity involves inhibition of cell wall synthesis (2.36 mM, *B. cereus*, Kwon et al., 2003) or inhibition of biosynthetic enzymes (>7.5 mM, histidine decarboxylase, Wendakoon and Sakaguchi 1995) is unlikely because of the rapidity of ATP inhibition or depletion.

Treatment with 40 mM cinnamaldehyde was observed to have an effect identical to 10 μM CCCP in preventing a rise in cellular ATP following addition of glucose to cells pre-exposed to inhibitor, and also in causing a rapid depletion in the cellular ATP of energised cells. The hypothesis that cinnamaldehyde functions as a ion transporter like CCCP can be dismissed as it lacks the presence of any chemical group, such as a hydroxyl, which would allow it to function in such a manner. The results seen would be

consistent with a mechanism for cinnamaldehyde action where interaction with the cell membrane is causing disruption sufficient to disperse the PMF by the leakage of small ions without leakage of larger cell components such as ATP. The observed results would also be consistent with either inhibition of glucose import or inhibition of glycolysis.

4.6. Conclusions.

The results of these experiments clearly indicate rapid inhibition of the energy metabolism of *L. monocytogenes* when exposed to bactericidal concentrations of eugenol and cinnamaldehyde, and similar effects were shown by eugenol against *Lb. sakei*. Further experiments are necessary to determine whether the mode of inhibition involves glucose utilisation or membrane interactions. Additionally, the possibility of ATPase inhibition by eugenol should be investigated.



Eugenol trans-Cinnamaldhyde Carvacrol Thymol

Fig. 4.1. Structures of the substituted aromatic compounds, eugenol, *trans*-cinnamaldehdye, carvacrol and thymol.

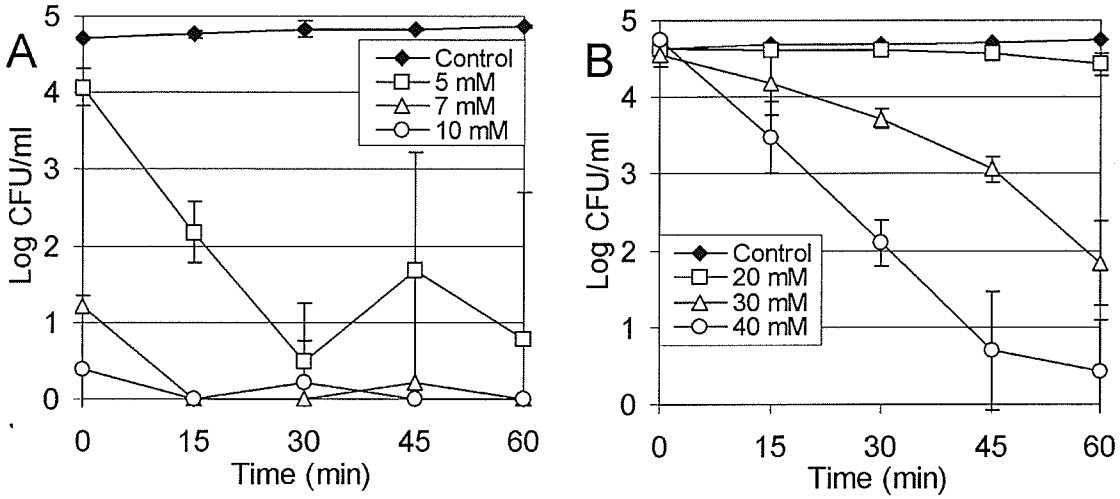


Fig. 4.2. Effect of **A.** eugenol and **B.** cinnamaldehyde on *L. monocytogenes* in TSB+YE at 20°C and pH 7.0. Average of three experiments. Error bars indicate one standard deviation.

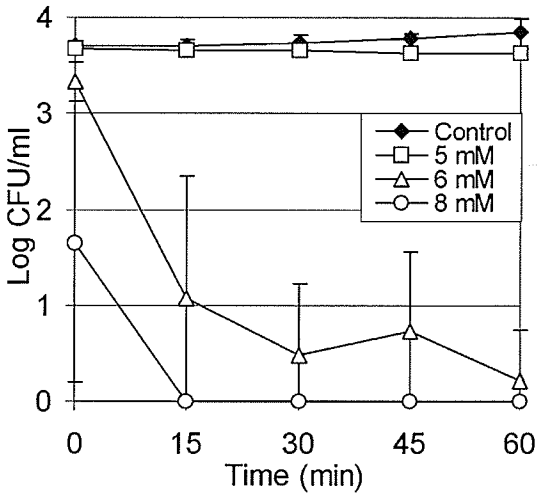


Fig. 4.3. Effect of eugenol on *Lb. sakei* in TSB+YE at 20°C and pH 7.0. Average of three experiments. Error bars indicate one standard deviation.

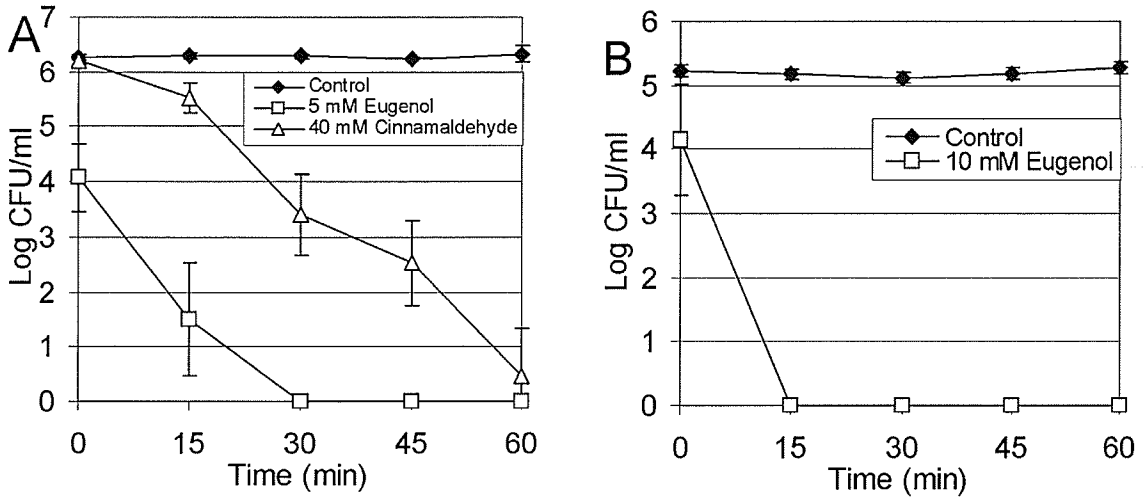


Fig. 4.4. Effect of antimicrobials on **A.** *L. monocytogenes* and **B.** *Lb. sakei* in 25 mM HEPES pH 7.0 at 20°C with 0.25% glucose. Average of two experiments. Error bars represent 1 standard deviation.

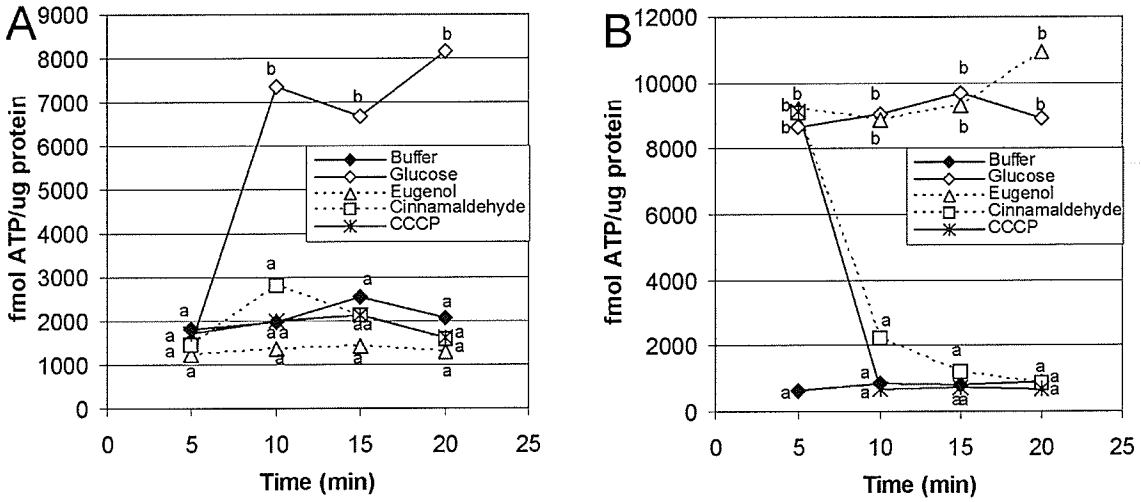


Fig. 4.5. Cellular ATP fmol/μg of protein from *L. monocytogenes* in 25 mM HEPES buffer pH 7.0 at 20°C. **A.** Cells were exposed to antimicrobials at 0 min and all treatments except Buffer were energised with 0.25% glucose at 5 min. **B.** All treatments except Buffer were energised with 0.25% glucose at 0 min and antimicrobials were added at 5 min. Treatments: Buffer, Glucose, Eugenol (5 mM), Cinnamaldehyde (40 mM), CCCP (10 μM). Average of four experiments, values that are significantly different by t-test ($\alpha = 0.05$) are denoted by different letters.

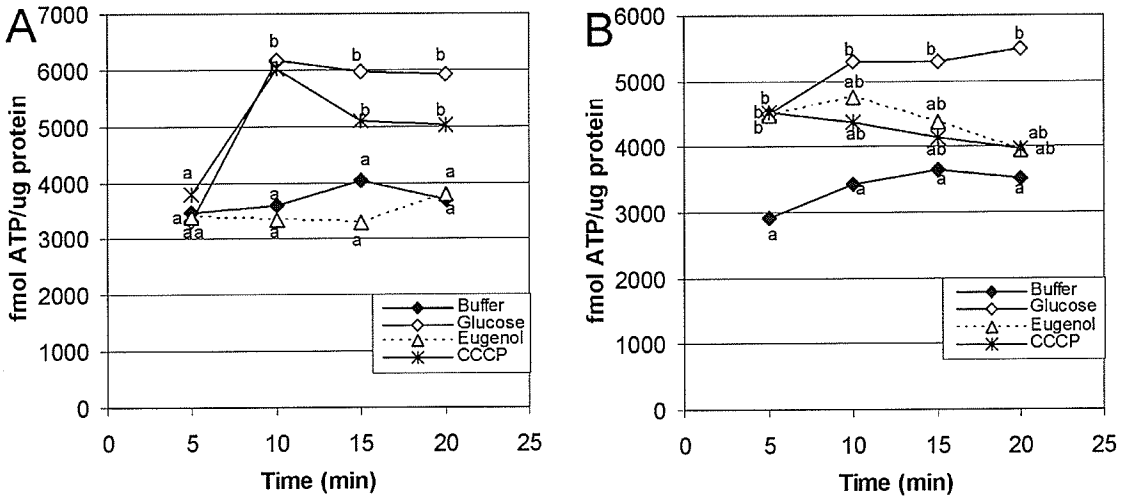


Fig. 4.6. Cellular ATP fmol/μg of protein from *Lb. sakei* in 25 mM HEPES buffer pH 7.0 at 20°C. **A.** Cells were exposed to antimicrobials at 0 min and all treatments except Buffer were energised with 0.25% glucose at 5 min. **B.** All treatments except Buffer were energised with 0.25% glucose at 0 min and antimicrobials were added at 5 min. Treatments: Buffer, Glucose, Eugenol (10 mM), CCCP (100 μM). Average of four experiments, values that are significantly different by t-test ($\alpha = 0.10$) are denoted by different letters.

5. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics.

5.1. Abstract

The role of membrane disruption in the bactericidal activity of the plant oil aromatic compounds eugenol, carvacrol and cinnamaldehyde was investigated using confocal laser scanning microscopy, changes in ATP levels and cell viability. In 25 mM HEPES buffer pH 7 at 20°C, 10 mM eugenol or carvacrol increased uptake of propidium iodide by *Escherichia (E.) coli*, *Listeria (L.) monocytogenes* and *Lactobacillus (Lb.) sakei* over a 10 min period. The same treatments resulted in lowered viability, rapid depletion of cellular ATP and release of ATP, with the exception of *Lb. sakei* treated with carvacrol. Eugenol or carvacrol at 5 mM to 10 mM inhibited *E. coli* and *L. monocytogenes* motility.

Lb. sakei was resistant to cinnamaldehyde. Thus, its effects were only studied on *E. coli* and *L. monocytogenes.* At 10 mM cinnamaldehyde caused a slight but statistically significant increase in propidium iodide staining of *E. coli*, but had no effect on *L. monocytogenes*. Cinnamaldehyde treatment of *E. coli* at 10 mM and *L. monocytogenes* at 40 mM resulted in decreased cellular ATP, but there was no concomitant release of ATP. Cinnamaldehyde at 5 and 10 mM inhibited *E. coli* and *L. monocytogenes* motility.

Results for eugenol and carvacrol are consistent with non-specific permeabilization of the cytoplasmic membrane. Evidence for increased membrane permeability by cinnamaldehyde is less conclusive. The release of ATP from eugenol and carvacrol-treated cells and absence of release from cinnamaldehyde-treated cells could indicate that eugenol and carvacrol possess ATPase inhibiting activity. Secondary effects would also be consistent with membrane disruption.

5.2. Introduction

An increasing body of research is being compiled on the antimicrobial activity of various plant oil extracts and their specific components for possible application in fields ranging from the food industry to dentistry (Burt, 2004; Holley and Patel, 2005). A number of the active components of these oil extracts have been identified, primarily substituted aromatic molecules including eugenol, cinnamaldehyde and carvacrol. These specific agents have been demonstrated to have activity against a number of gram-positive and gram-negative bacteria. Eugenol is active against the bacterial pathogens *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Blaszyk and Holley, 1998). Activity of cinnamaldehyde has been reported against *Clostridium botulinum* (Bowles and Miller, 1993), *Staphylococcus aureus* (Bowles et al., 1995), *E. coli* O157:H7 and *Salmonella* Typhimurium (Helander et al., 1998). Carvacrol is active against *Bacillus cereus* (Ultee et al., 1998), *L. monocytogenes, S.* Typhimurium and *E. coli* O157:H7 (Cosentino et al., 1999; Kim et al., 1995).

With the exception of the effects of carvacrol on *Bacillus cereus* (Ultee et al., 1998, 1999, 2002), *Pseudomonas aeruginosa* and *S. aureus* (Lambert et al., 2001) little research has been done on the mechanism of antibacterial activity of spice oil components. Previous studies of eugenol and cinnamaldehyde have presented evidence indicating possible roles for membrane interactions and the inhibition of specific cellular processes or enzymes (Helander et al., 1998; Kwon et al., 2003; Walsh et al., 2003; Wendakoon and Sakaguchi 1995).

In previously published experiments treatment with bactericidal concentrations of eugenol inhibited the generation of ATP from glucose by unenergised *L. monocytogenes* (5 mM) and *Lb. sakei* (10 mM) (Chapter 4). Treatment with 40 mM cinnamaldehyde

inhibited ATP generation by unenergised *L. monocytogenes* (Chapter 4). Treatment with cinnamaldehyde resulted in a rapid depletion in cellular ATP from *L. monocytogenes* previously energised by incubation with glucose. However, no depletion of ATP was observed in energised *L. monocytogenes* or *Lb. sakei* cells treated with eugenol (Chapter 4). These results were considered consistent with inhibition of glucose utilisation or membrane interactions.

The aim of the experiments described in this paper was to determine if disruption of the cellular membrane occurs when *L. monocytogenes, Lb. sakei* or *E. coli* O157:H7 are exposed to bactericidal concentrations of eugenol, cinnamaldehyde and carvacrol.

Due to the low infectious dose of many food-borne pathogens the application of spice oil components to improve food safety requires the development of bactericidal treatments. An understanding of the mechanism of action of these agents would allow prediction of organisms that can be expected to demonstrate sensitivity and food products to which these agents may be effectively applied. Further, knowledge of mechanism of action would allow the rational development of treatments using combinations of antimicrobial agents, that target other cell functions in a complimentary manner

5.3. Materials and Methods

5.3.1. Materials

All Purpose Tween (APT) broth, M17 broth, Brain Heart Infusion (BHI) broth, Dextrose (glucose) and Proteose Peptone #3 were supplied by Difco, Becton-Dickinson, Sparks, MD. Agar Granulated and Trypticase Soy broth were supplied by BBL, Becton-Dickinson, Sparks, MD. Yeast Extract, eugenol (2-methoxy-4-[2-propenyl]phenol), diethylaminoethyl-dextran chloride avg M.W. 500,000 (DEAE-dextran), ATP assay mix

(FL-AAM), ATP standard (FL-AAS), ATP assay mix dilution buffer (FL-AAB), propidium iodide, fluorescein sodium salt and Protein Assay Kit P5656 were supplied by Sigma Chemicals, St Louis, MO. *trans*-cinnamaldehyde and carvacrol were supplied by Aldrich Chemical Co, Milwaukee, WI. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was supplied by FisherBiotech, Fairlawn, NJ. An Isotemp refrigerated circulator 1016S and a Micro-12 microcentrifuge were from Fisher Scientific, Fairlawn, NJ. The LB 9509 Junior luminometer was from Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany. EDTA disodium salt and trichloroacetic acid (TCA) were supplied by FisherChemicals, Fisher Scientific. Ultrafree-MC 0.22 μm filter units with Durapore membrane, were supplied by Millipore Co, Bedford, MA. The spiral plater used was an Autoplate 4000, which was supplied by Spiral Biotech, Bethesda, MD. Lab-Tek 8 well 0.4 ml chambered coverglass slides of #1 borosilicate glass were supplied by Nalge Nunc Int., Naperville, IL. Confocal laser scanning microscopy (CLSM) was performed with an Olympus IX70 Scanning Confocal Microscope system using Fluoview imaging software and an argon laser (488 nm) as an emission source, supplied by the Carsen Group Inc. Markham, ON, Canada. Phase contrast microscopy was done with a Zeiss Photomicroscope, Zeiss, Göttingen, Germany.

5.3.2. Bacteria and culture conditions

The *Escherichia coli* O157:H7 strain used was a human isolate supplied by the Laboratory Centre for Disease Control, Ottawa, ON, Canada (Strain #7283). The *Listeria monocytogenes* strain used was a somatic serotype 1 meat plant isolate, with the Canadian Research Institute for Food Safety culture collection number of C717 (M. W. Griffiths,

Univ. of Guelph, ON, Canada). The *Lactobacillus sakei* strain used was isolated from spoiled cured meats (Holley et al., 1996). Stock cultures were frozen at -75°C in glycerol. For experimental use *E. coli* and *L. monocytogenes* were streaked for isolation on BHI agar and *Lb. sakei* on APT agar.

5.3.3. Preparation of antimicrobials

Suspensions of the antimicrobials to be tested were prepared immediately prior to use by the addition of eugenol, cinnamaldehyde or carvacrol to sterile 25 mM HEPES buffer pH 7.0 to form suspensions with antimicrobial contents ranging from 7.5 mM to 320 mM. Suspensions were made and vortex mixed immediately prior to use to form uniform suspensions that upon addition to cell suspensions would give the desired final antimicrobial content. The use of emulsifiers such as ethanol or tween was avoided as these agents may reduce the antimicrobial effect of plant oil components and possess membrane disrupting activity (Remmal et al., 1993a).

5.3.4. Preparation of bacterial cells

A single colony of the bacterium to be tested was used to inoculate 10 ml of TSB+YE pH 7.0 at 20°C and grown to log phase (OD_{650} between 0.1 and 0.3). The culture was then harvested by centrifugation at 10,000 x g for 15 min and washed twice with 25 mM HEPES buffer pH 7.0 before being resuspended in HEPES buffer to an OD_{650} of 0.1 ± 0.04.

5.3.5. Observation of propidium iodide uptake by confocal laser scanning microscopy

A 1 ml aliquot of bacterial cells in buffer was mixed with 3 µl of 5 mM propidium iodide (PI) and 10 µl of 0.25% (w/v) fluorescein. Aliquots of the cells with stain, 350 µl, were then transferred to the wells of chambered coverglass slides and then incubated in the dark for 10 min.

The CLSM was then focused on the layer of cells that formed on the bottom of the well. The experiments were begun by adding 50 µl of the antimicrobial suspension to be tested or HEPES buffer as a control. CLSM was then immediately begun. The field of view was scanned at 15 sec intervals with 3.3 sec scans for 10 min at 800x. Duplicate scans were made of the same field of view in two separate channels. The first channel was used to visualise fluorescein (excitation 490 nm, emission 515 nm) which is cell impermeable and which acts as a negative stain of cells (Caldwell et al., 1992). The second channel was used to visualise the nucleic acid stain PI (excitation 490 nm, emission 635 nm). PI is also cell impermeable but increases its fluorescence by an order of magnitude when complexed with nucleic acids, thus acting as a selective stain for cells with damaged membranes (Arndt-Jovin and Jovin., 1989).

The images collected were analysed with ImagePro Plus software (Media Cybernetics, Silver Spring, MD) to produce cell counts from both channels. The proportion of cells stained by PI in the field of view was calculated from the ratio of cells visible in channel 2 at each time point with the number of cells visible in channel 1 at the first time point. Only the first time point for channel 1 was used, since quenching of fluorescein occurred, making it difficult to get consistent cell counts in channel 1 slides after the first time point. Data were calculated from slide sets showing a minimum of 100 cells in channel 1.

5.3.6. Enumeration of Bacterial Cells

Aliquots of 710 µl of cells in buffer were transferred to micro-centrifuge tubes. A 10 µl sample was taken from the tube, diluted in a 10 ml buffer blank and immediately surface plated with the spiral plater. The experiment was begun by adding 100 µl of antimicrobial or buffer to the cells in buffer in the micro-centrifuge tube. Samples were then taken and plated by spiral plater at 2 min intervals over 10 min.

5.3.7. Measurement of extracellular and intracellular ATP

A suspension of the bacterium to be studied in 25 mM HEPES buffer pH 7.0 was prepared as described above. The cellular and extracellular levels of ATP from cells at 20°C energised with 0.25% glucose were determined as described previously (Chapter 4). To improve the recovery of extracellular ATP the protocol was modified by filtering 200 µl samples by centrifugation for 30 s at 4600 xg through ultrafree-MC centrifuge filtration units (0.22 µM) containing 100 µl of 7.5% trichloracetic acid (TCA) with 6 mM EDTA in the lower compartment. An aliquot of TCA 2.5% with 2 mM EDTA 100 µl was then added to the upper compartment of the filter unit. After a 10 min incubation, 20 µl aliquots of the TCA solutions in each compartment were removed and diluted in 380 µl of FL-AAB. The sample from the upper compartment comprised the cellular ATP and the filtrate from the lower compartment contained the external ATP sample. ATP samples were stored at -75°C until analysed.

The ATP content of the samples was assayed by a continuous light output luciferase reaction (Lundin, 2000) with the light output amplified with DEAE-dextran

(Ishida et al., 2002) with quantification by the addition of an internal sample as previously described (Chapter 4).

5.3.8. Measurement of protein content

The protein content of cell suspensions was determined by the Modified Lowry Method using the Protein Assay Kit according to the manufacturer's instructions.

5.3.9. Changes in motility

Motility experiments were performed with *E. coli* and *L. monocytogenes* only as the *Lb. sakei* strain used is non-motile. Cells in buffer were prepared as described above, with the exception of being resuspended to an OD_{650} of 0.2 ± 0.04. A 350 µl aliquot of the cells in buffer was then placed in micro-centrifuge tubes with 350 µl of TSB+YE at 2x concentration. Following 5 min incubation at 20°C, 100 µl of antimicrobial or buffer was added. A 10 µl sample was immediately placed on a glass slide with coverslip and the sample examined by phase contrast microscope at a magnification of 1000x for 10 min. Samples in which the majority of cells could be observed to be both actively running and tumbling were scored as motile positive. Samples in which only a minority of cells could be observed to display either running or tumbling behaviour were scored as reduced motility. Samples in which only stationary vibrational movement occurred were scored as nonmotile.

5.3.10. Statistical Analysis

To determine whether significant differences existed between controls and treatments observed by CLSM, as well as for plating and ATP extraction experiments two tailed Student's t-tests were used (Johnson and Bhattacharyya, 1992). The value of α used to determine statistical significance was 0.10 except for viable cell recoveries (Figs. 4-6) where α=0.05 was used.

5.4. Results

5.4.1. Propidium Iodide uptake by E. coli

Following treatment with eugenol, cinnamaldehyde or carvacrol at 10 mM the proportion of *E. coli* cells in the field of view stained with PI was significantly greater than control cells (Fig. 5.1.). The period of incubation required for significantly greater staining was 1 min 30 sec for 10 mM eugenol, 6 min 30 sec for 10 mM cinnamaldehyde and 5 min 30 sec for 10 mM carvacrol. Treatment with either 10 mM eugenol or carvacrol resulted in 100% staining of cells by 10 min, whereas 10 mM cinnamaldehyde resulted in only 10% staining by 10 min.

At lower concentrations the agents yielded smaller changes in cell permeability to PI. Treatment with 5 mM eugenol gave an apparent but non-statistically significant increase in PI staining. No increase in PI staining was observed for cells treated with 5 mM cinnamaldehyde or carvacrol (Fig. 5.1.). There was also no significant difference between controls and samples treated with 1mM eugenol, cinnamaldehyde or carvacrol (results not shown).

5.4.2. Propidium Iodide uptake by L. monocytogenes

Treatment of *L. monocytogenes* with 10 mM eugenol resulted in significantly greater PI staining after 1 min incubation (Fig. 5.2.). Carvacrol at 10 mM was similarly effective in increasing PI staining and the effect became significantly greater after 1 min and 15 sec incubation. *L. monocytogenes* cells treated with 5 mM eugenol had an apparent but non-significant increase in staining. Treatment with 5 mM carvacrol resulted in significantly greater PI staining than the control at 2 min 30 sec and thereafter intermittently at 17 of 30 time points during the duration of the test to 10 min.

Treatment with 10 mM, 5 mM, or 1 mM cinnamaldehyde, 1 mM eugenol or 1 mM carvacrol had no significant effect on PI staining (results not shown).

5.4.3. Propidium Iodide uptake by Lb. sakei

PI staining of *Lb. sakei* was significantly greater than the control following 30 sec exposure to 10 mM eugenol, 45 sec exposure to 10 mM carvacrol or 2 min exposure to 5 mM carvacrol (Fig. 5.3.). Significantly greater PI staining following treatment was observed with 5 mM eugenol at 3 min 30 sec to 4 min 15 sec, 5 min, 5 min 15 sec and 6 min 45 sec (Fig. 5.3.). Neither 1 mM eugenol nor carvacrol resulted in significantly greater PI staining than the controls (results not shown).

The effect of cinnamaldehyde on *Lb. sakei* was not investigated as previous experiments found that suspensions of up to 0.4 M had no effect on growth or survival of this organism (Chapter 4).

5.4.4. Effect of antimicrobials on viable cell recovery

To determine whether the observed increase in PI staining coincided with cell death, cells were recovered by spread plating during exposure to the antimicrobials under conditions mimicking the CLSM experiments. *E. coli* (Fig. 5.4.) and *L. monocytogenes* (Fig. 5.5.) cells were exposed to 10 mM, 5 mM and 1 mM eugenol, cinnamaldehyde, carvacrol or buffer control. *Lb.sakei* was exposed to the antimicrobials eugenol and carvacrol only (Fig. 5.6.).

Treatment of *E. coli* with 10 mM eugenol or carvacrol resulted in significantly lower numbers of recovered cells compared to controls following 2 min exposure as did 5mM carvacrol after 4 min of exposure. Significantly lower numbers of *L. monocytogenes* were recovered following 4 min exposure to 10 mM eugenol or 2 min exposure to 10 mM carvacrol. Treatment of *Lb. sakei* with 10 mM eugenol or carvacrol resulted in significantly lower numbers of recovered cells following 8 min and 2 min exposure, respectively.

All other treatments had no significant effect on the cell numbers recovered thoughout the 10 min incubation.

5.4.5. Effect of antimicrobials on the cellular ATP of E. coli

Cells of *E. coli* (Fig. 5.7A), *L. monocytogenes* (Fig. 5.8A.) and *Lb. sakei* (Fig. 5.9A.) energised with dextrose had significantly greater cellular ATP levels than cells given buffer only. There was no significant difference between the cellular ATP levels of dextrose energised treatments and all energised treatments were significantly greater than the buffer control, prior to addition of the antimicrobials at 5 min (Fig. 5.7A., 5.8A., 5.9A.).

Addition of 10 mM eugenol to *E. coli* cells resulted in significantly lower levels of ATP compared to both dextrose and buffer-treated cells by 10 min (Fig. 5.7A.). The cellular ATP of cells treated with 10 mM carvacrol declined to a level significantly lower than dextrose and was not different from buffer at 10 min. The cellular ATP level of cells treated with 10 mM cinnamaldehyde similarly declined but took 20 min before it became significantly different from dextrose but was not different from the buffer only treatment.

5.4.6. Effect of antimicrobials on the extracellular ATP of E. coli

The ATP levels in extracellular samples treated with dextrose or buffer were not significantly different (Fig. 5.7B.). Treatment with 10 mM eugenol or carvacrol resulted in an increase in extracellular ATP significantly greater than buffer or dextrose treated cells at 10 min onward. The ATP in extracellular samples from cinnamaldehyde treated cells was not significantly different from dextrose or buffer controls except at 20 min when it was significantly lower.

5.4.7. Effect of antimicrobials on the cellular ATP of L. monocytogenes

Addition of 10 mM eugenol to *L. monocytogenes* cells resulted in cellular ATP levels that were significantly lower than dextrose-treated cells and not significantly different from buffer-treated cells at 10, 15 and 20 min (Fig 5.8A.). Addition of 10 mM eugenol resulted in cellular ATP levels that were significantly lower than dextrose-treated cells and not significantly different from buffer-treated cells at 10, 15 and 20 min. Treatment with 10 mM cinnamaldehyde resulted in a decline in cellular ATP to levels non-significantly different than buffer control at 20 min. At 10 and 15 min the cellular ATP levels of cinnamaldehyde-treated cells was significantly lower than dextrose control

and significantly greater than buffer control. The cellular ATP of carvacrol-treated cells was significantly lower than dextrose-treated cells at 15 and 20 min and was not significantly different from the buffer at 10, 15 and 20 min.

5.4.8. Effect of antimicrobials on the extracellular ATP of L. monocytogenes

The ATP levels in extracellular samples treated with dextrose or buffer were not significantly different (Fig. 5.8B.). Treatment with 10 mM eugenol or carvacrol resulted in extracellular ATP levels significantly greater than buffer or dextrose-treated cells at 10 min and onward. The ATP in extracellular samples from cinnamaldehyde-treated cells was not significantly different from either dextrose or buffer-treated cells.

5.4.9. Effect of antimicrobials on the cellular ATP of Lb. sakei

Eugenol-treated cells of *Lb. sakei* had significantly higher levels of ATP than buffer treated cells except at 20 min (Fig 5.9A.). The cellular ATP of carvacrol-treated cells was not significantly different from dextrose-treated cells at any time and was consistently greater than buffer-treated cells.

5.4.10. Effect of antimicrobials on the extracellular ATP of Lb. sakei

The ATP levels in extracellular samples treated with dextrose or buffer were not significantly different (Fig. 5.9B.). Treatment with 10 mM eugenol resulted in extracellular ATP levels significantly greater than buffer or dextrose-treated cells at 10 min and onward. Treatment with carvacrol had no significant effect on extracellular ATP.

5.4.11. Effects of antimicrobials on motility

The antimicrobials were observed to inhibit the motility of both *E. coli* and *L. monocytogenes* in TSB+YE with 12.5 mM HEPES pH 7.0. Treatment of either *E. coli* or *L. monocytogenes* with 10 mM eugenol or carvacrol resulted in an immediate cessation of motile behaviour. Treatment with 5 mM of eugenol or carvacrol resulted in an immediate reduction in motile behaviour; tumbling and limited running was observed, where the speed and length of running was clearly reduced. The inhibition of motility persisted for the 10 min of observation.

Treatment with 5 mM or 10 mM cinnamaldehyde resulted in reduced motility for both organisms, which appeared to increase in severity over the 10 min period of observation. However, tumbling and running of reduced speed and duration could be observed over the full observation period for both organisms at both concentrations.

Treatment with 1 mM of eugenol, cinnamaldehyde or carvacrol had no noticeable effect on motility of either organism. Experiments were repeated twice.

5.5. Discussion

5.5.1. Permeabilization of the cellular membrane by eugenol and carvacrol

The results of the PI uptake and companion agar plating experiments clearly demonstrate that eugenol and carvacrol disrupt the cellular membranes of *E. coli*, *L. monocytogenes* and *Lb. sakei* when supplied at 10 mM, a concentration sufficient to have an immediate bactericidal effect. Since PI is a hydrophilic molecule of molecular weight 414 g/mol, the increased membrane permeability is sufficient to allow free diffusion of any small ions and consequent dissipation of the proton motive force. Treatment with 5 mM eugenol caused an observable but statistically non-significant increase in PI staining of *E. coli*, *L. monocytogenes* and *Lb. sakei*. This concentration, though not bactericidal

over 10 min of exposure, was found to be bactericidal in previous experiments in which cells were incubated with eugenol for up to 1 h (Chapter 4; Appendix).

In previously reported experiments we observed that eugenol at bactericidal concentrations inhibited ATP generation from glucose by *L. monocytogenes* and *Lb. sakei,* though no significant difference in extracellular ATP was observed (Chapter 4). The results of the PI experiments described presently indicate that treatment with 10 mM eugenol or carvacrol should result in the release of ATP (molecular weight, 505 g/mol). Thus the effect of the antimicrobials on the cellular and extracellular ATP levels of energised cells was reexamined with a modified protocol, designed to improve recovery of ATP by denaturing any free enzymes present in the extracellular sample capable of destroying ATP. Consequently, membrane disruption was confirmed by increases in extracellular ATP and decreases in cellular ATP from cells treated with 10 mM eugenol or carvacrol. The exception was the response of *Lb. sakei* to carvacrol where no statistically significant change in extracellular ATP was observed (Fig. 5.9B.).

The observed effects of eugenol and carvacrol on the motility of *E. coli* and *L. monocytogenes* provide further evidence to support membrane disruption as the major lethal event. The motility of planktonic cells of *E. coli* and *L. monocytogenes* is dependent on flagella which are structurally integrated into the membrane and which are supplied with energy by the membrane proton gradient rather than by phosphorylated intermediates such as ATP (Silverman, 1980). The speed of the flagellar motor of *E. coli* has been demonstrated to be linearly related to the proton motive force (Gabel and Berg, 2003). The inhibition of motility observed in the presence of 5 mM and 10 mM eugenol or carvacrol most probably results from direct inhibition of the flagella motor by

dissipation of membrane proton gradient. However, loss of motility due to structural changes such as loss of flagella can not be discounted.

Previous reports by other authors also contain evidence consistent with disruption of bacterial cellular membranes by substituted aromatic compounds. In their study of the interactions between cyclic hydrocarbons and *E. coli* liposomes, Sikkemma et al. (1994), reported that accumulation of cyclic hydrocarbons resulted in the efflux of protons and a membrane impermeable probe, carboxyfluorescein (molecular weight 376 g/mol). This efflux was accompanied by gross perturbations of the model membranes, swelling of liposomes, increased membrane fluidity and the release of phospolipids labelled with fluorescent probes.

Carvacrol at 1mM has been reported to dissipate the membrane gradients of hydrogen ions and potassium in *B. cereus*, as well as causing a small leakage of ATP at 2 mM (Ultee et al., 1999). Dissipation of pH gradients, potassium and phosphate leakage from *Ps. aeruginosa* and *S. aureus* was reported by Lambert et al. (2001) following treatment with carvacrol-containing oregano essential oil. Membrane damage by oregano essential oil and pure carvacrol (6.7 mM) were indicated by increased ethidium bromide staining following 10 min incubation. Increased uptake of PI and potassium leakage from *E. coli*, *Lb. plantarum* and *Listeria innocua* occurred following treatment with 50-70 mM vanillin (Fitzgerald et al., 2004). Eugenol has been reported to cause potassium leakage from *E. coli* (3.03 mM) and *S. aureus* (6.06 mM) (Walsh et al., 2003).

From experiments that compared the minimum inhibitory concentrations of carvacrol and a number of structurally related compounds and that demonstrated the importance of the carvacrol hydroxyl group, Ultee et al. (2002) proposed that carvacrol acts as a protonophore. Though this possibility can not be discounted at sublethal

concentrations, it seems clear that non-selective permeabilization of the membrane is responsible for the bactericidal effect of carvacrol and eugenol. Sikkemma et al. (1995) noted that the ability of cyclic hydrocarbons to interact with cell membranes may be limited by their solubility. The presence of the hydroxyl group on eugenol and carvacrol may increase the solubility of these molecules in aqueous suspensions and improve their ability to pass through the hydrophilic portions of the cell envelope (Sikkemma et al., 1995).

A role for secondary effects of carvacrol and eugenol on bacterial cells can not be discounted, particularly at sub-lethal concentration. The interaction of hydrophobic molecules with cell membranes is known to affect the activity of membrane-bound or embedded enzymes (Sikkemma et al., 1995). This may explain the reports of enzyme inhibition and altered growth reported by other authors (Helander et al., 1998; Kwon et al., 2003; Walsh et al., 2003; Wendakoon and Sakaguchi, 1995).

5.5.2. Permeabilization of the cellular membrane by cinnamaldehyde

The case for permeabilization of the cellular membrane by cinnamaldehyde at bactericidal concentrations is not as definitive as for eugenol and carvacrol. A significant increase in PI staining of *E. coli* occurred when treated with 10 mM cinnamaldehyde, a concentration that was not bactericidal over 10 min but was previously found to be bactericidal over a 1 h period (Appendix). No increase in PI staining of *L. monocytogenes* was observed following treatment with 10 mM cinnamaldehyde, a concentration below the 30 - 40 mM that was required for a bactericidal effect (Chapter 4). Unfortunately, we found that at concentrations above 10 mM the presence of large numbers of

cinnamaldehyde globules in the suspension interfered with the acquisition of CLSM images.

Treatment of *E. coli* with cinnamaldehyde (10 mM) and *L. monocytogenes* (40 mM) resulted in significant decreases in cellular ATP but no increase of extracellular ATP. This result is inconsistent with membrane permeabilization. However, membrane permeabilization is consistent with the inhibition of *E. coli* and *L. monocytogenes* motility following treatment with 10 mM cinnamaldehyde. The loss of motility would be expected to be due to a non-specific increase in permeability, as cinnamaldehyde is structurally unsuitable to serve as a protonophore.

Our previous finding that 5 mM eugenol inhibited ATP generation from glucose by starved *L. monocytogenes*, but did not provoke a decrease in cellular ATP from energised cells (Chapter 4), suggests a hypothesis which would reconcile these observations. Since *L. monocytogenes* will rapidly deplete ATP to maintain the intracellular pH (Shabala et al., 2002), we proposed that the results would be consistent with inhibition of the F_1F_0-ATPase. If eugenol and carvacrol inhibit the F_1F_0-ATPase and cinnamaldehyde does not, then the difference in extracellular ATP levels could be explained by the active ATPase consuming ATP in cinnamaldehyde-treated cells.

5.5.3. Hydrophobic Nature of Plant Oil Aromatics

Bactericidal response of the tested bacteria to eugenol, cinnamaldehyde and carvacrol often requires that these sparingly soluble molecules be present in amounts approaching or exceeding their maximum solubility. The literature solubility value for cinnamaldehyde is 1.1 mM and for the carvacrol isomer thymol is 6.4 mM (Dean, 1999). Aqueous solubility may be estimated by calculation from AQUAFAC group values

(Mackay, 2000) which gives solubility values for eugenol of 9.7 mM and 3.7 mM for carvacrol. However, these estimates should be treated cautiously since AQUAFAC calculation gives a value of 10.6 mM for cinnamaldehyde.

Consideration of the application of hydrophobic molecules as antimicrobials is often governed by the assumption that only molecules dissolved in the aqueous phase are available for interaction with cells (Sikkema et al., 1995). This is a reasonable assumption in situations where interactions between molecules and cells involve interaction of the molecules with cellular enzymes and where partitioning between phases is at equilibrium. Under such conditions it can be expected that the effect of an antimicrobial would be maximal at the maximum solubility of the respective molecules. However, the results presented here and elsewhere indicate that this is not the case for eugenol, cinnamaldehyde and carvacrol (Chapter 4).

In the presence of an insoluble bulk phase the availability of a hydrophobic molecule may exceed the level predicted by solubility and is instead governed by the dissolution rate (Stucki and Alexander, 1987). The dissolution rate may be increased by the presence of bacteria (Thomas et al., 1986), as molecules are metabolised or partition into the cell membrane from the aqueous phase. Further, the presence of undissolved droplets in suspension allows direct partitioning of molecules to the cell membranes. During our CLSM experiments we observed rapid PI staining of cells in close proximity to droplets of undissolved antimicrobial.

5.6. Conclusions

The results of these experiments clearly indicate that at bactericidal concentrations the primary mechanism of action of carvacrol and eugenol is disruption of the

cytoplasmic membrane, which increases its non-specific permeability. Other secondary effects at sub-lethal concentrations can not be discounted, and can be expected as a consequence of membrane interactions.

The evidence for membrane disruption and increase in permeability caused by cinnamaldehyde is less conclusive. The absence of an increase in extracellular ATP from cinnamaldehyde-treated cells could suggest that eugenol and carvacrol possess ATPase inhibiting activity which cinnamaldehyde lacks.

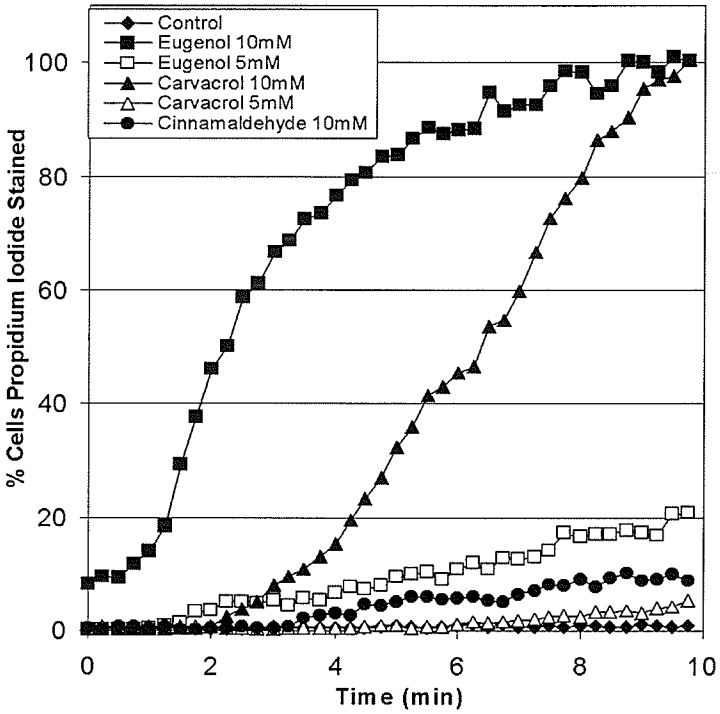


Fig 5.1. Percentage of *Escherichia coli* cells in field of view fluorescing with propidium iodide uptake following treatment with eugenol, carvacrol or cinnamaldehyde or buffer control. Average of triplicate experiments.

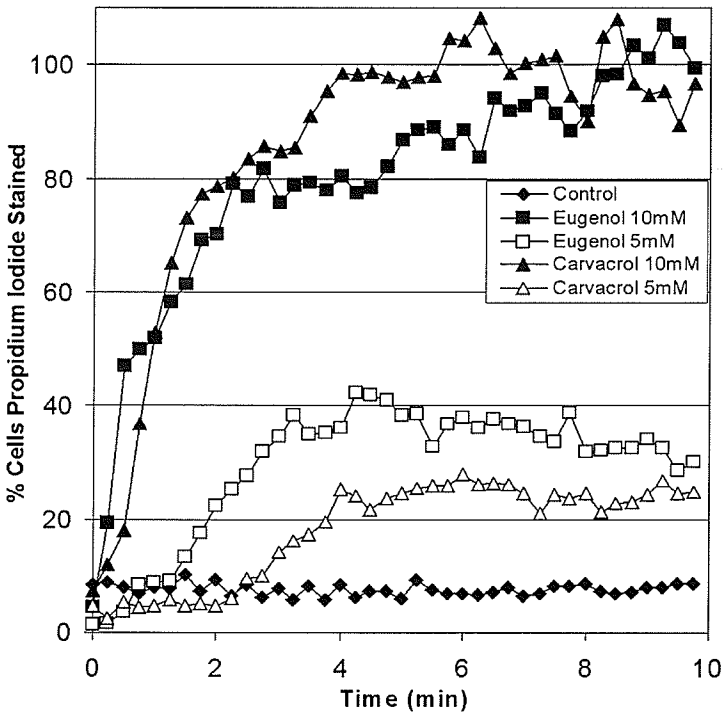


Fig 5.2. Percentage of *Listeria monocytogenes* cells in field of view fluorescing with propidium iodide uptake following treatment with eugenol, carvacrol or buffer control. Average of triplicate experiments.

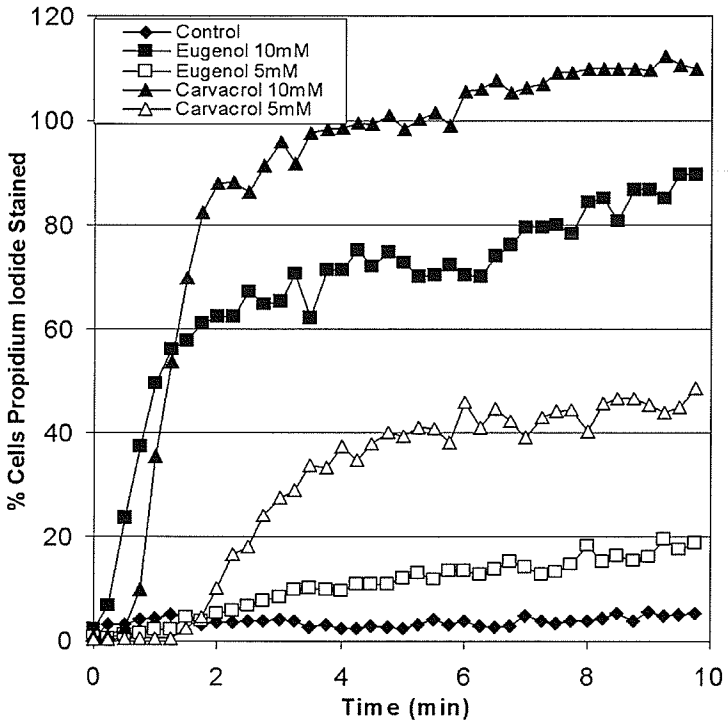


Fig 5.3. Percentage of *Lactobacillus sakei* cells in field of view fluorescing with propidium iodide uptake following treatment with eugenol, carvacrol or buffer control. Average of triplicate experiments.

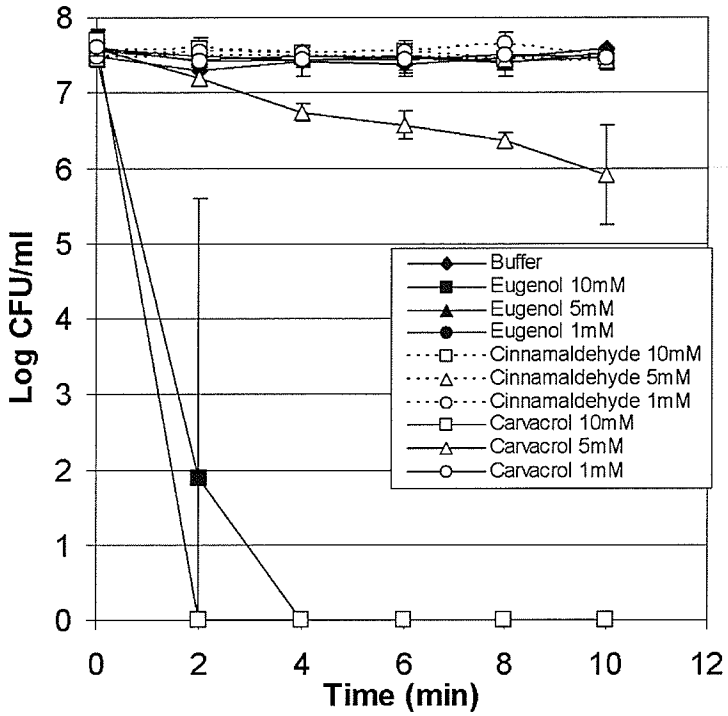


Fig 5.4. Effect of antimicrobials on *E. coli* viability in 25 mM HEPES buffer at 20°C and pH 7.0. Average of three experiments. Error bars indicate 95% confidence limits.

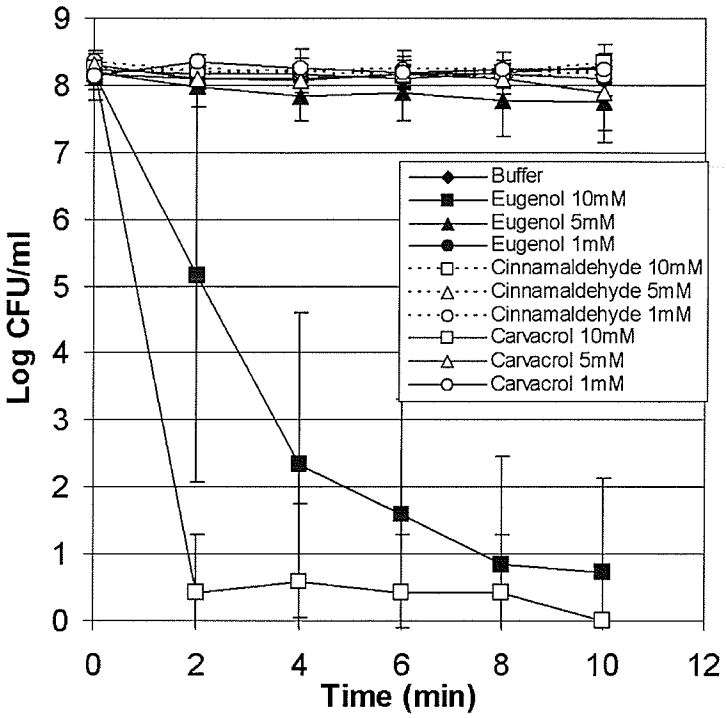


Fig 5.5. Effect of antimicrobials on *L. monocytogenes* viability in 25 mM HEPES buffer at 20°C and pH 7.0. Average of three experiments. Error bars indicate 95% confidence limits.

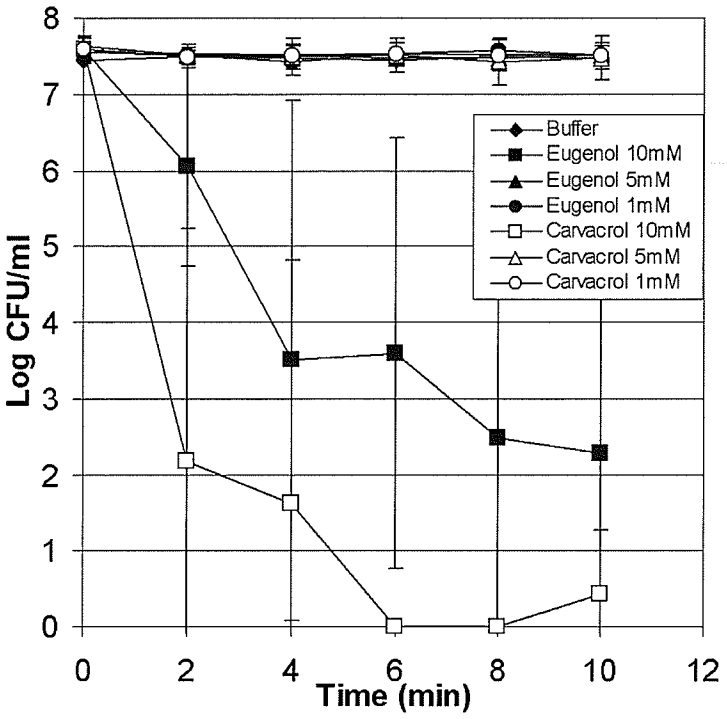


Fig 5.6. Effect of antimicrobials on *Lb. sakei* viability in 25 mM HEPES buffer at 20°C and pH 7.0. Average of three experiments. Error bars indicate 95% confidence limits.

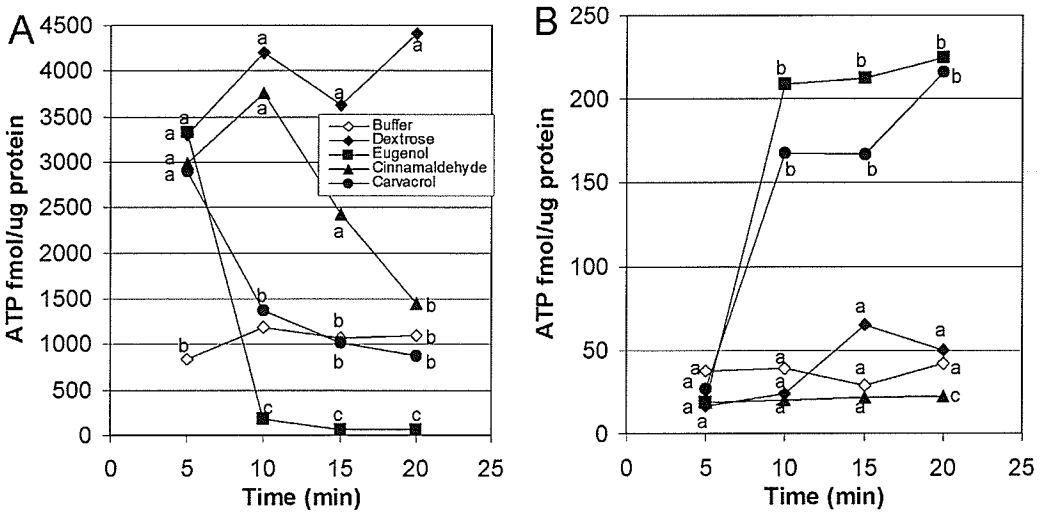


Fig 5.7. ATP fmol/μg of protein from *E. coli* in 25 mM HEPES buffer pH 7.0 at 20°C in cellular (A) and extracellular (B) samples. All treatments except Buffer were energised with 0.25% glucose at 0 min and antimicrobials were added at 5 min. Treatments: Buffer, Glucose, Eugenol (10 mM), Cinnamaldehyde (10 mM), Carvacrol (10 mM). Average of three experiments, values that are significantly different from buffer or dextrose controls by t test $\alpha = 0.1$ are indicated by different letters. Symbols in (A) and (B) are used for the same treatments.

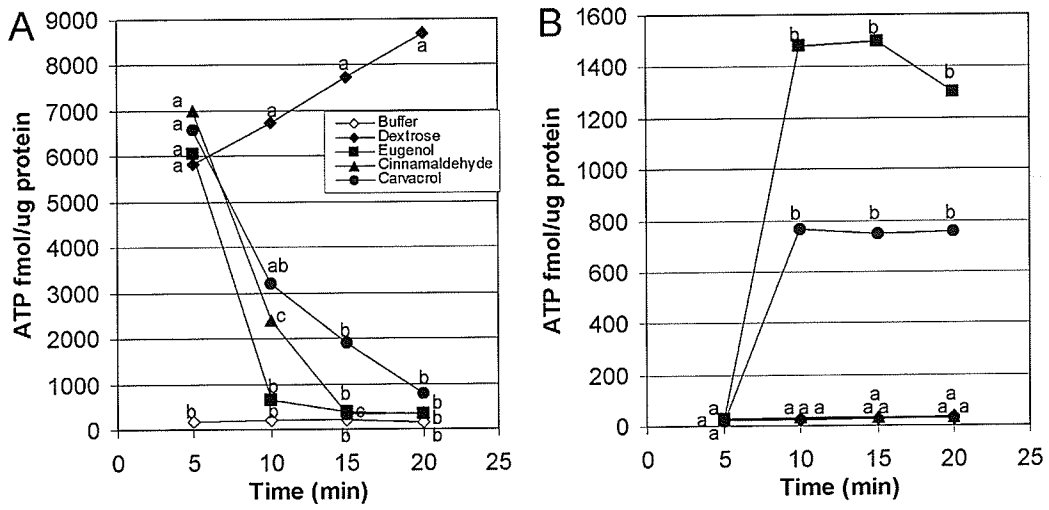


Fig 5.8. ATP fmol/μg of protein from *L. monocytogenes* in 25 mM HEPES buffer pH 7.0 at 20°C in cellular (A) and extracellular (B) samples. All treatments except Buffer were energised with 0.25% glucose at 0 min and antimicrobials were added at 5 min. Treatments: Buffer, Glucose, Eugenol (10 mM), Cinnamaldehyde (40 mM), Carvacrol (10 mM). Average of three experiments, values that are significantly different from buffer or dextrose controls by t test $\alpha = 0.1$ are indicated by different letters. In (B), symbols for Buffer, Glucose and Cinnamaldehyde are superimposed. Symbols in (A) and (B) are used for the same treatments.

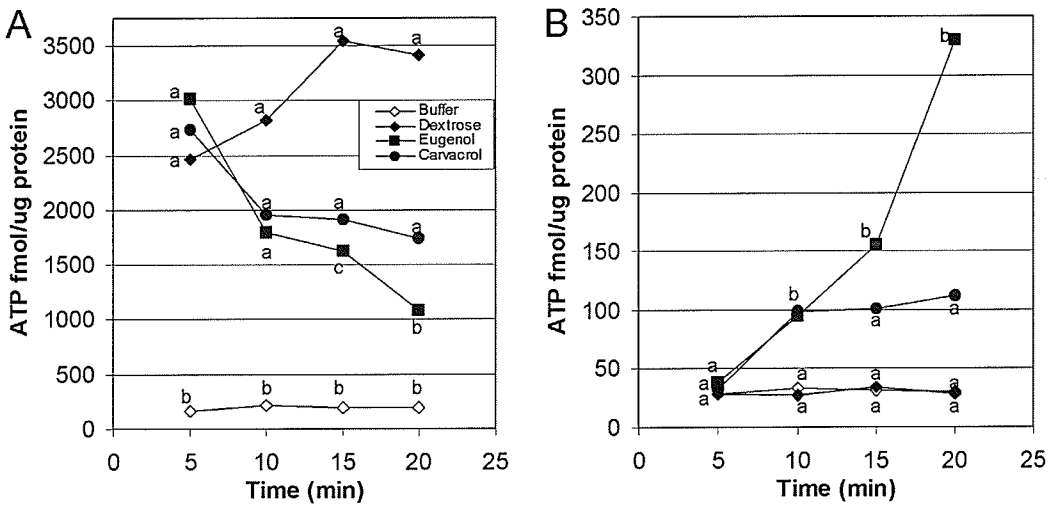


Fig 5.9. ATP fmol/μg of protein from *Lb. sakei* in 25 mM HEPES buffer pH 7.0 at 20°C in cellular (A) and extracellular (B) samples. All treatments except Buffer were energised with 0.25% glucose at 0 min and antimicrobials were added at 5 min. Treatments: Buffer, Glucose, Eugenol (10 mM), Carvacrol (10 mM). Average of three experiments, values that are significantly different from buffer or dextrose controls by t test $\alpha = 0.1$ are indicated by different letters. Symbols in (A) and (B) are used for the same treatments.

6. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics.

6.1. Abstract

Previous studies have reported that the mechanism of bactericidal action of the plant oil aromatics, eugenol, carvacrol and cinnamaldehyde involves inhibition of adenosine triphosphate generation and membrane disruption.

In this study the capacity of the aromatics to inhibit the membrane bound ATPase activity of *Escherichia coli* and *Listeria monocytogenes* was investigated by experiments on isolated membranes. Inhibition of the ATPase activity of *E. coli* membranes was observed with 5 mM or 10 mM eugenol or carvacrol. Progressively greater inhibition by cinnamaldehyde was observed as the concentration increased from 0.1 to 10 mM. *L. monocytogenes* ATPase activity was significantly inhibited by eugenol (5 or 10 mM), carvacrol (10 mM) and cinnamaldehyde (10 mM).

Lactobacillus sakei is highly resistant to cinnamaldehyde compared to *E. coli* and *L. monocytogenes*. To determine whether this resistance was related to the relative hydrophobicity of the cell surface and hence the ability of the cell to take up the aromatics, the percentage of the three organisms partitioning in dodecane was compared. No significant difference was found between the partitioning percentage of *L. monocytogenes* (17.2%) and *Lb. sakei* (13.8%), indicating that surface hydrophobicity does not explain the differing sensitivity to cinnamaldehyde of these two organism. The percent partitioning of *E. coli* was significantly greater than both other organisms (23.3%) and may explain its greater sensitivity to all three aromatics.

6.2. Introduction

In previous experiments investigating the mechanism of bactericidal action of the plant oil aromatics, eugenol, cinnamaldehyde and carvacrol it was observed that treatment of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cells with these agents inhibited generation of adenosine triphosphate from dextrose and disrupted the cell membrane (Chapters 4 and 5).

Analysis of the cellular and extracellular ATP levels of cells treated with eugenol, cinnamaldehyde and carvacrol suggested that these compounds might inhibit the ATPase activity of bacterial cells. Inhibition of rat liver ATPases by eugenol and cinnamaldehyde has been reported by Usta et al. (2003). Other phenolic compounds, tertiary butylhydroquinine and propyl gallate, have been reported to inhibit the ATPase activity of isolated *Staphylococcus aureus* membranes (Rico-Munoz et al., 1987). To investigate this possibility membrane fractions from *E. coli* and *L. monocytogenes* were isolated and the effect of the aromatics on ATPase activity was examined.

It was also observed that although similar concentrations of eugenol or carvacrol (5 to 10 mM) had a bactericidal effect on *E. coli*, *L. monocytogenes* and *Lb. sakei*, there was a greater difference in response among the three organisms to cinnamaldehyde (Chapter 4 and 5). Cinnamaldehyde was bactericidal to *E. coli* and *L. monocytogenes* at 10 mM and 30 mM respectively. However *Lb. sakei* was observed to be unaffected by cinnamaldehyde at 0.5 M (Chapter 4). A possible explanation for the difference in response to cinnamaldehyde of the three bacterial species is that there may be differences in the ability of this small hydrophobic molecule to interact with the outer surface of the cells and thus gain access to the cell membrane. To investigate this possibility the relative surface hydrophobicity of the three bacteria was measured.

6.3. Materials and Methods

6.3.1. Materials

All Purpose Tween (APT) and Brain Heart Infusion (BHI) broth were supplied by Difco, Becton-Dickinson, Sparks, MD. Agar Granulated and Trypticase Soy Broth (TSB) were supplied by BBL, Becton-Dickinson, Sparks, MD. Yeast Extract (YE), eugenol (2-methoxy-4-[2-propenyl]phenol), ATP assay mix (FL-AAM), ATP standard (FL-AAS), ATP assay mix dilution buffer (FL-AAB), dithiothreitol, deoxyribonuclease I, dodecane, magnesium sulfate, tris(hydroxymethyl) aminomethane HCL (TRIZMA preset crystals pH 7.8) and Protein Assay Kit P5656 were supplied by Sigma Chemicals, St Louis, MO. *trans*-cinnamaldehyde and carvacrol were supplied by Aldrich Chemical Co, Milwaukee, WI. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was supplied by FisherBiotech, Fairlawn, NJ. An Isotemp refrigerated circulator 1016S and a Micro-12 microcentrifuge were from Fisher Scientific, Fairlawn, NJ. The LB 9509 Junior luminometer was from Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany. EDTA disodium salt and trichloroacetic acid (TCA) were supplied by FisherChemicals, Fisher Scientific. Ultrafree-MC 0.22 μm filter units with Durapore membrane, were supplied by Millipore Co, Bedford, MA. Cells were sonicated with a Sonic Dismembrator Model 150, supplied by Artek Systems Corp., Farmingdale, NY. The Beckman L8-80 ultracentrifuge was by Beckman Coulter Canada, Inc., Mississauga, ON. Polypropylene micro-titre plates (96 well) were supplied by Corning-Costar, Corning Inc., Acton, MA.

6.3.2. Bacteria and culture conditions

The *E. coli* O157:H7 strain used was a human isolate supplied by the Laboratory Centre for Disease Control, Ottawa, ON, Canada (Strain #7283). The *L. monocytogenes* strain used was a somatic serotype 1 meat plant isolate, with the Canadian Research Institute for Food Safety culture collection number of C717 (M. W. Griffiths, Univ. of Guelph, ON, Canada). The *Lb. sakei* strain used was isolated from spoiled cured meats (Holley et al., 1996). Stock cultures were frozen at -75°C in glycerol. For experimental use *E. coli* and *L. monocytogenes* were streaked for isolation on BHI agar and *Lb. sakei* on APT agar.

6.3.3. Preparation of bacterial membranes

Isolated cell membrane preparations expressing ATPase activity were prepared from *E. coli* and *L. moncytogenes* cells using the following protocol adapted from Fillingame (1979).

Single colonies of *E. coli* or *L. monocytogenes* were inoculated into four 500 ml flasks containing 125 ml of TSB + YE (5g/L), pH 7.0. The flasks were then incubated for 16 hours with shaking at room temperature.

Cell cultures were pooled, harvested by centrifugation at 5000xg for 15 and resuspend in 50 mM tris(hydroxymethyl) aminomethane HCl (TrisHCl), pH 7.8 with 10 mM $MgSO_4$ (Tris Buffer).

The *L. monocytogenes* cell suspension was centrifuged (5000xg, 15 min) and resuspend in Tris Buffer with 500 mg/l of lysozyme. *L. monocytogenes* cells were then incubated for 90 min at 37°C. *E. coli* cells were not subjected to a lysozyme digestion step.

The cells were then centrifuged (5000xg, 15 min) and resuspended in 10-15 ml of Tris Buffer with 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mg/ml deoxyribonuclease I. The cell suspensions were then frozen at –20 °C and thawed to room temperature. This procedure was repeated three times.

Following the freeze/thaw cycles, the cell membranes were disrupted by sonication. The cell suspensions were treated with the Sonic Dismembrator at 90% power in 10-15 sec bursts interspersed with 15 sec cool down periods for 1 hour, while suspended in an ice bath.

Whole cells were removed from the supernatant by two sequential centrifugations (5000xg for 15 min). Isolated membranes were recovered from the supernatants by centrifugation at 141,000xg (max) for 75 min in a Beckman L8-80 ultracentrifuge. The membrane pellets were resuspended in Tris Buffer, then centrifuged (141,000xg max, 75 min) and resuspended in Tris Buffer with 1 mM dithiothreitol. The membrane preparations were divided into 1.5 ml aliquots and stored in microcentrifuge tubes at –80 °C.

6.3.4. Measurement of protein content

The protein content of membrane preparations was determined by triplicate analysis of samples by the Modified Lowry Method using the Protein Assay Kit according to the manufacturer's instructions.

6.3.5. Estimation of specific ATPase activity of membrane preparations

The membrane preparations were diluted in Tris Buffer to a protein content of 0.25 mg/ml for *E. coli* or 1.0 mg/ml for *L. monocytogenes*. Membrane aliquots of 200 µl

were placed in microtitreplate wells. The plates were equilibrated to 30°C in a water bath for 5 min. The reaction was initiated by the addition of 50 µl of ATP. At 30 sec intervals over 2 min, 50 µl samples were removed and immediately quenched in 50 µl of 10% TCA. The 10 µl aliquots of the quenched samples were then diluted in 390 µl of FL-AAB buffer. Duplicate experiments were performed over a range of ATP concentrations from 5 to 60 µM.

6.3.6. Inhibition of membrane ATPase activity

The membrane preparations were diluted in Tris Buffer to a protein content of 0.4 mg/ml for *E. coli* or 1.6 mg/ml for *L. monocytogenes*.

Reaction volumes of 75 µl were prepared in the wells of micro-titre plates. The following treatments were prepared: positive control (50 µl membrane suspension, 25 µl Tris Buffer); negative control (75 µl Tris Buffer); buffer blank (100 µl Tris Buffer); aromatic blank (50 µl Tris Buffer, 25 µl plant oil aromatic); aromatic treatment (50 µl membrane suspension, 25 µl plant oil aromatic). The microtitre plates were then equilibrated to 30°C in a water bath for 5 min. Samples were treated with the plant oil aromatics eugenol, cinnamaldehyde or carvacrol prepared in Tris Buffer at 0.1, 0.5, 1, 5 and 10 mM.

The reaction was initiated by the addition of 25 µl of 40 µM ATP to all treatments except the buffer blank. The reaction in each well was terminated after 10 min by the addition of 100 µl of 10% TCA. Under these conditions positive controls would consume approximately 70% of the ATP present during the 10 min incubation.

Samples were prepared for ATP analysis by diluting 10 µl aliquots from each well in 390 µl of FL-AAB buffer. Diluted samples were either sampled immediately or stored overnight at –80°C. Four replicates of each treatment were conducted.

6.3.7. ATP Analysis

The ATP content of the FL-AAB diluted samples was assayed by a continuous light output luciferase reaction (Lundin, 2000). Each 100 µl aliquot of sample was assayed with 100 µl luciferase assay mix (FL-AAM diluted 1/10 in FL-AAB buffer). Light output was quantified as relative light units (RLU) with the Junior LB 9509 luminometer. Then 5 µl ATP standard was added and measured as an internal standard. The ATP content of the sample was calculated from the ratio of the RLU of the sample to the RLU of the standard.

6.3.8. Cell surface hydrophobicity

The relative hydrophobicity of the bacterial cells was assayed by percent partitioning of the bacterial cells in dodecane (Pembrey et al., 1999). An overnight culture of the bacterium to be tested was grown in TSB +YE pH 7.0 at 20°C. The cells were harvested by centrifugation at 10,000xg for 10 min. The cells were washed twice and resuspended in 25 mM HEPES buffer pH 7.0 to an optical density at 650 nm (OD_{650}) of 0.200 ± 0.004. Three ml aliquots of the cells were transferred to test tubes containing 1 ml of dodecane. The tubes were vortexed at full power for 2 min. Phase partitioning was then allowed to take place for 20 min before removal of 1 ml aliquots of the aqueous phase for OD_{650} measurement. Four replicates of each experiment were conducted, including buffer

blanks prepared simultaneously. The percentage of cells partitioning in dodecane was calculated as [(OD_{650} Initial- OD_{650} Final)/OD_{650} Initial]x100.

6.3.9. Statistical Analysis

To determine whether significant differences existed between controls and treatments observed in the ATPase inhibition and dodecane partitioning experiments two tailed Student's t-tests were used (Johnson and Bhattacharyya, 1992). The value of α used to determine statistical significance was 0.05.

6.4. Results

6.4.1. Specific activity of membrane bound ATPase

The protein content of the membrane preparations was determined to be 2.62 mg/ml for *E. coli* and 2.08 mg/ml for *L. monocytogenes*.

The initial velocity of the reactions over a range of substrate concentrations was estimated from the slope of four time points on an ATP concentration versus time graph. To estimate the Michaelis constant (K_m) and maximum velocity (V_{max}) Eadie-Hofstee plots of four points at four concentrations of ATP were prepared (Copeland, 1996).

The ATPase activity of the *E. coli* membrane had an apparent K_m of 2095 μM and a V_{max} of 5.28 μM/s per mg protein at pH 7.8 and 30°C. The ATPase activity of the *L. monocytogenes* membrane had an apparent K_m of 141 μM and a V_{max} of 0.282 μM/s per mg protein at pH 7.8 and 30°C.

6.4.2. Inhibition of membrane ATPase activity

There was no significant difference between the apparent ATP concentration of negative control samples and aromatic blanks (results not shown).

All three plant oil aromatics tested demonstrated inhibition of *E. coli* membrane ATPase activity (Fig. 6.1.). Treatment with 5 mM or 10 mM of eugenol or carvacrol resulted in significant inhibition of ATPase activity. Treatment with cinnamaldehyde resulted in significant inhibition of ATPase activity for all concentrations tested. All aromatic treatments resulted in final ATP concentrations significantly lower than the negative control without membrane, with the exception of 10 mM cinnamaldehyde.

Inhibition of membrane bound ATPase activity of the *L. monocytogenes* membrane preparations by the plant oil aromatics was also observed (Fig. 6.2.). Significant ATPase inhibition occurred in samples treated with 5 mM or 10 mM eugenol, 10 mM carvacrol and 10 mM cinnamaldehyde. All treatments of *L. monocytogenes* membrane resulted in final ATP concentrations significantly lower than the negative control.

6.4.3. Relative hydrophobicity

The relative hydrophobicity of the of the three organisms as determined by percent partitioning into dodecane were in order: *E. coli* 23.3%, *Lb. sakei* 17.2%, and *L. monocytogenes* 13.8%. The percent partitioning of *E. coli* was significantly greater than both *Lb. sakei* and *L. monocytogenes*. There was no significant difference between the percent partitioning of *Lb. sakei* and *L. monocytogenes*.

6.5. Discussion

The results presented here indicate that eugenol, carvacrol and cinnamaldehyde are capable of inhibiting the membrane bound ATPase activity of *E. coli* and *L. monocytogenes.* Bacterial membranes contain a number of differing enzymes with ATPase activity including ATP dependent transport proteins and the F_1F_0-ATPase which is involved in ATP generation and cellular pH regulation (Shabala et al., 2002). Though inhibition of these functions would impair cell survival, that significant ATPase inhibition occurs at concentrations within the same range required for membrane disruption (5 to 10 mM) suggests that this is a secondary rather than a primary cause of cell death (Chapter 5). However, enzyme inhibition may play a significant role in reducing growth rate at sublethal concentrations.

Non-specific inhibition of membrane bound or embedded enzymes can be caused by small hydrophobic molecules, presumably due to changes in protein conformation as a consequence of hydrophobic interactions (Sikkemma et al., 1995). Such a mechanism may cause the observed inhibition of ATPase activity and also the reported inhibition of other enzymes and altered bacterial growth by carvacrol and eugenol (Helander et al., 1998; Kwon et al., 2003; Walsh et al., 2003; Wendakoon and Sakaguchi, 1995).

To determine whether or not the observed inhibition of the ATPase activity is due to a specific or non-specific interaction would require a study of the affect of the aromatics upon the kinetics of purified enzymes. It would be informative to compare the results of a study of the primary membrane bound ATPase, the F_1F_0 complex, with other membrane-associated enzymes with different substrates.

The only previously published report of the effects of eugenol and cinnamaldehyde on ATPase activity was a study on eukaryotic rat mitochondria (Usta et al., 2003). Inhibition of the P-type Na^+/K^+ ATPase (eugenol 1.1 mM and cinnamaldehyde

4.7 mM) and CPx-type Cu^{2+} ATPase (eugenol 0.65 mM and cinnamaldehyde 0.94 mM) was reported. In contrast, Usta et al. (2003) reported that eugenol (0.3 to 30 mM) and cinnamaldehyde (0.37 to 37 mM) stimulated the mitochondrial F_1F_0-ATPase. However, this conclusion may not be fully accurate as the activity of the F_1F_0-ATPase was determined by potassium production from ATP by mitochondria rather than membrane preparations or purified enzymes. Thus the apparent stimulation of the F_1F_0-ATPase could be due to uncoupling of respiration by membrane disruption.

Comparison of the relative surface hydrophobicity, as measured by dodecane partitioning, of the three organisms does not explain the resistance of *Lb. sakei* to cinnamaldehyde. Though the bacterium with the most hydrophobic surface, *E. coli*, was most sensitive to the aromatics, there was no significant difference between the hydrophobicity of *Lb. sakei* and *L. monocytogenes*. The nature of *Lb sakei* resistance to cinnamaldehyde remains obscure. However, the greater hydrophobicity of the *E. coli* surface may explain its relative sensitivity to the aromatics compared to the two Gram positive bacteria, as greater hydrophobicity would facilitate interaction of the aromatic with the membrane.

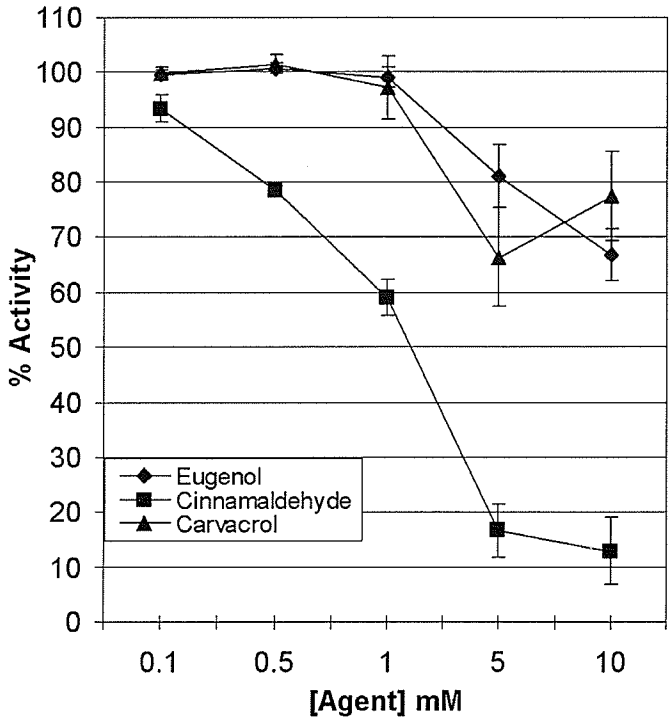


Fig. 6.1. Percentage ATPase activity of *E. coli* membrane preparations treated with aromatic agents and incubated for 10 min at 30°C. Average of four replicates. Error bars indicate 95% confidence intervals.

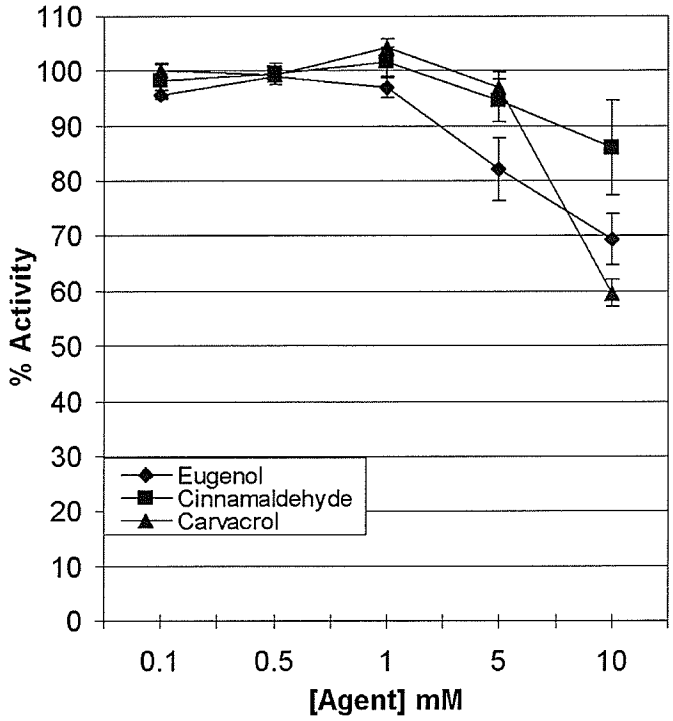


Fig. 6.2. Percentage ATPase activity of *L. monocytogenes* membrane preparations treated with aromatic agents and incubated for 10 min at 30°C. Average of four replicates. Error bars indicate 95% confidence intervals.

7. Conclusions

Chapter 3 of this thesis provides a demonstration of the current limitations in developing essential oils as antimicrobials for food products. Though cilantro oil (>0.08%) was found to be effective at inhibiting *Listeria monocytogenes* growth in broth media with 0.15% agar it was far less effective when applied to ham. Incorporation of the antimicrobial into a gel film led to a slight increase in effectiveness. Application of essential oil antimicrobials to foods composed of a solid matrix may be most effective when incorporated in packaging systems that bring the antimicrobial into contact with the target organisms where they grow better, on the surface.

From the results of the experiments presented in Chapters 4, 5 and 6 of this thesis I believe that it is possible to construct a much clearer model of the antimicrobial mechanism of action of eugenol, cinnamaldehyde and carvacrol. Further, I believe that this model can be generalised to other essential oil components.

The experiments described in Chapter 4 indicated eugenol and cinnamaldehyde inhibit ATP generation in sensitive organisms. At bactericidal concentration eugenol (5 mM) and cinnamaldhyde (40 mM) inhibited ATP generation from glucose by *L. monocytogenes*. When supplied to energised cells cinnamaldehyde caused depletion of cellular ATP, while the cellular ATP levels of eugenol treated energised cells remained high. Eugenol (10 mM) also inhibited ATP production from glucose by *Lactobacillus sakei* and caused a slight but statistically non-significant decline in cellular ATP in energised cells.

It had been proposed by Ultee et al., (2002) that the carvacrol phenol group increases its ability to inhibit growth of *B. cereus* by allowing the molecule to function as a protonophore. For this reason our experiments in Chapter 3 included carbonyl cyanide

m-chlorophenylhydrazone (CCCP) as a known protonophore. CCCP at 10 μM inhibited ATP generation by *L. monocytogenes*, and caused rapid ATP depletion of energised cells. CCCP at 100 μM had no effect on the ability of *Lb. sakei* to generate ATP. *L. monocytogenes* generates ATP by respiration and hydrolyzes ATP with the F_1F_0-ATPase to maintain cellular pH (Shabala et al., 2002). In contrast *Lb. sakei* generates ATP by substrate level phosphorylation and is less sensitive to changes in cellular pH. Thus, the response of these organisms to CCCP is what would be expected on exposure to a potent uncoupler of the proton motive force. Since, *L. monocytogenes* did not respond to eugenol as it did to CCCP, it was clear that eugenol could not function solely as a protonophore. The possibility that cinnamaldehyde functions as a protonophore was discounted on the basis of its molecular structure.

The results of Chapter 4 experiments established the primacy of energy metabolism inhibition in the bactericidal mechanism of eugenol and cinnamaldehyde. Though other effects could not be discounted, the cells were effectively and rapidly crippled by inhibition of energy generation. Cells that can not generate energy can neither reproduce nor repair themselves. The mechanism of this inhibition still needs to be resolved and a number of possibilities exist.

Disruption of the cellular membrane would be consistent with the results of Chapter 4 experiments, and many studies of other essential oil antimicrobials have generated reports of the leakage of cell contents. Thus, in the next series of experiments described in Chapter 5, we proceeded to investigate the possibility of membrane disruption. The experiments had to answer the following questions: Does non-specific

permeability, indicating membrane disruption, increase in the presence of the agents? If it does occur, does membrane disruption occur in the same time frame as cell death?

To answer these questions cells of *E. coli*, *L. monocytogenes* and *Lb. sakei* were exposed to eugenol, cinnamaldehyde and carvacrol and uptake of propidium iodide (PI) was observed by examining a sequence of scanning laser microscopy images (SLCM) over a 10 min period. Eugenol and carvacrol were observed to increase PI staining of all three organisms rapidly and in a dose dependent manner. Plating of cells exposed to the agents under the same conditions confirmed that increased membrane permeability to PI correlated with cell death. Treatment of energised cells of all three organisms with eugenol (10 mM) or carvacrol (10 mM) resulted in rapid depletion of cellular ATP and significant ATP leakage, with the exception of *Lb. sakei* treated with carvacrol. Finally, the agents were also observed to inhibit the flagellar motility of *E. coli and L. monocytogenes*, which is coupled to the membrane pH gradient, indicating its dispersal. These observations support the hypothesis that the immediate cause of cell death on exposure to euegnol or carvacrol is the loss of cellular membrane integrity.

The results for cinnamaldehyde were less conclusive. Cinnamaldehyde at 10 mM was observed to cause a small but significant increase in PI staining of *E. coli* and to inhibit *E. coli* and *L. monocytogenes* motility. However, though treatment with cinnamaldehyde depleted the cellular ATP of *E. coli* (10 mM) and *L. monocytogenes* (40 mM), it did not cause an increase in extracellular ATP.

The possibility that the eugenol in particular inhibited cellular ATPase activity had been considered initially during the interpretation of Chapter 4 results. This was because cellular ATP of energised *L. monocytogenes* cells treated with 5 mM eugenol was not depleted as would be expected. In contrast rapid depletion coupled with ATP

release was observed in the Chapter 5 experiments where 10 mM eugenol was used. However, these results might not be inconsistent. If 5 mM eugenol results in increased permeability to small ions but leaves the membrane relatively intact, then ATPase inhibition may prevent ATP consumption while diffusion from the cell is low. The PI uptake experiments indicated that when exposed to 10 mM eugenol the membrane ceased to prevent diffusion of molecules of the size of ATP within minutes, which when coupled with ATPase inhibition, would result in rapid depletion of cellular ATP and ATP leakage, as was observed.

In Chapter 6 experiments it was found that all three agents, eugenol, cinnamaldehyde and carvacrol were capable of inhibiting the ATPase activity of isolated *E. coli* and *L. monocytogenes* membranes. It remains to be determined whether this inhibition is specific for the F_1F_0-ATPase or is non-specific for a wide range of enzymes. Previous reports of enzyme inhibition (Wendakoon and Sakaguchi, 1995) and deformed cellular morphology (Kwon et al., 2003) support the hypothesis that the inhibition is non-specific.

To summarise, the following model is proposed to explain the antimicrobial action of eugenol, carvacrol and cinnamaldehyde, and by extension for other essential oil components. These molecules would appear to function as antimicrobials by engaging in hydrophobic interactions with the cell membrane. These interactions are disruptive of the membrane structure and increase membrane permeability to an extent which is dependent upon their availability for uptake into the membrane. The cell membrane is a complex structure including many embedded proteins, which perform essential roles in the transport and metabolism of substrates. When dissolved in the membrane small hydrophobic molecules alter the interaction of these proteins with the lipids of the

membrane resulting in conformational changes and consequent impairment of function. Cell death is an immediate consequence of increased membrane permeability coupled to inhibition of enzymes. Bacteriostatic effects would be observed when cell leakage and minor enzyme inhibition is sufficient to impair growth but not prevent recovery of the cells. In essence essential oil components are toxic to bacterial cells in a manner similar to that proposed for cyclic hydrocarbons (Sikkema et. al., 1995).

The capacity of eugenol, cinnamaldehyde and carvacrol to disrupt the cell membrane is well established. Testing the model proposed above would require investigation of the potential of these molecules to inhibit membrane bound enzyme activity. Specifically, what is the nature of the ATPase inhibition observed in Chapter 6 experiments? What other enzymes may be inhibited in the presence of these molecules? If the physical chemistry of the interaction of these molecules with membranes and membrane components is adequately understood it will be possible to predict which essential oil components have the greatest potential as antimicrobials. Further, it may be possible to construct analogous molecules with minimal sensory characteristics.

If this model is correct the following inferences can be drawn regarding potential applications of these agents. Since these agents increase the permeability of cell they could be used to increase the uptake of hydrophilic antimicrobials that target cytoplasmic contents. It is predicted that the susceptibility of target organisms is determined by the nature of their cell envelope and the consequent ability of the essential oil antimicrobials to dissolve in the membrane. Organisms that are more tolerant of increased membrane permeability will be less responsive to the antimicrobials.

Appendix 1: Response of *Eschericia coli* to Eugenol and Cinnamaldehyde

A1. 1. Antimicrobial concentration bactericidal to Escherichia coli *O157:H7*

The concentration of eugenol or cinnamaldehyde that is bactericidal to log phase cells of *Escherichia coli* O157:H7 (Laboratory Centre for Disease Control, Ottawa, ON, Canada. Strain #7283) in broth media was determined. A bactericidal effect was defined as a >1 log reduction in colony forming units recovered compared to untreated controls within 1 h. All materials used are described in Section 4.3.1.

A single colony of *E. coli* O157:H7 was inoculated to 10 ml of TSB+YE pH 7.0 at 20 °C and grown to log phase (OD_{650} between 0.1 and 0.3). The culture was then adjusted by dilution with TSB+YE pH 7.0 to an OD_{650} of 0.1 ± 0.04, then diluted 1/1000 into fresh TSB+YE pH 7.0. A 0.5 ml aliquot of the bacterial cell suspension was then added to a 1.5 ml microcentrifuge tube containing 1 ml of the antimicrobial to be tested, suspended in TSB+YE pH 7.0. Controls composed of cells in TSB+YE pH 7.0 untreated with antimicrobial were run simultaneously. The microcentrifuge tubes were incubated for 60 min in a water bath at 20°C. At the experiment start and every 15 min for 60 min the tubes were mixed by vortexing and duplicate samples spread-plated by spiral plater on Brain Heart Infusion (BHI) agar plates. Plates were incubated at 25°C for 48 h and enumerated.

To confirm that the bactericidal effects of the antimicrobial were not due to inhibition of growth by residual antimicrobial, experiments at bactericidal concentration were repeated with duplicate 100 µl samples recovered on ISO-Grid membranes. Samples were washed twice with 20 ml portions of 0.1% peptone before filter incubation on BHI agar.

A1.2. Results

The minimum concentrations of antimicrobial required for a bactericidal effect on *E. coli* in TSB+YE pH 7.0 was found to be 4 mM of eugenol (Fig. A1.1.) and 7 mM cinnamaldehyde (Fig. A1.2.). When experiments were repeated with cells recovered by ISO-Grid filtration after washing with 0.1% peptone there was no significant difference (t-test $\alpha = 0.05$) in numbers, with unwashed cells recovered following direct plating on agar (Table A1.1.).

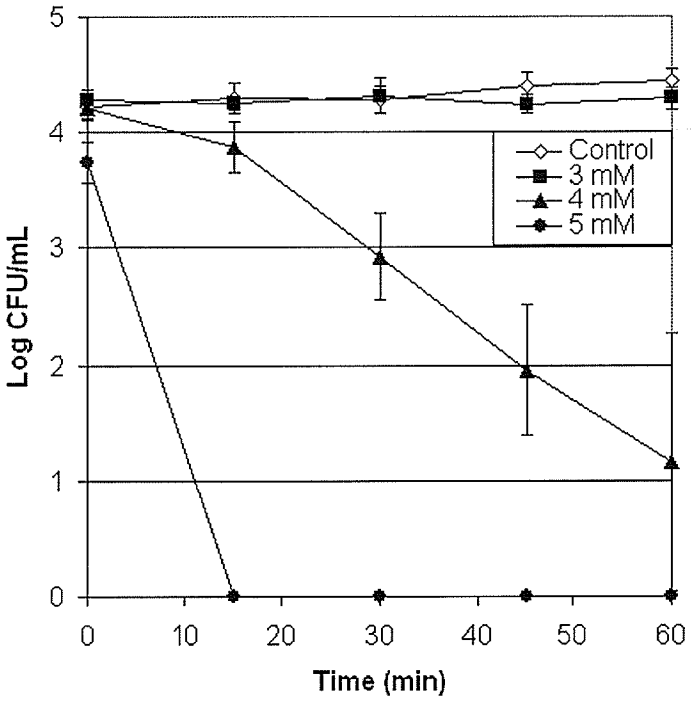


Fig. A1.1. Effect of eugenol on *E. coli* in TSB+YE at 20°C and pH 7.0. Average of three experiments. Error bars indicate 95% confidence intervals.

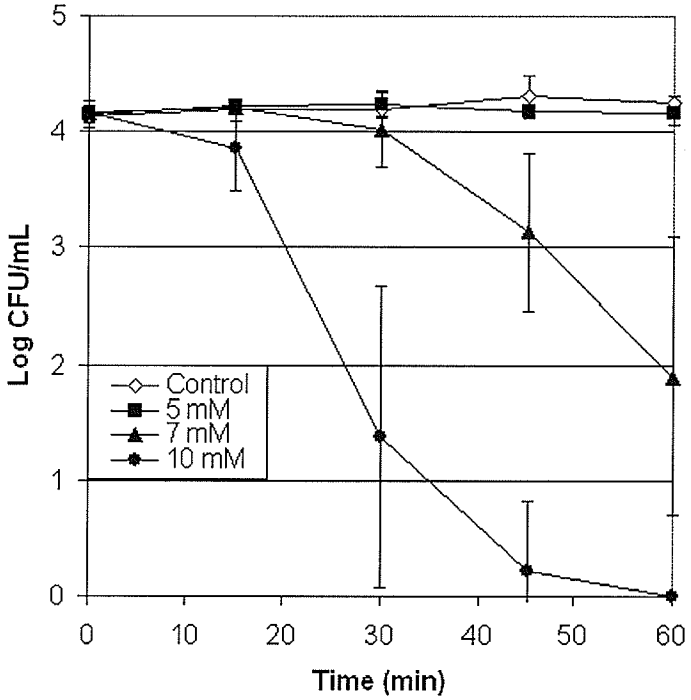


Fig. A1.2. Effect of cinnamaldehyde on *E. coli* in TSB+YE at 20°C and pH 7.0. Average of three experiments. Error bars indicate 95% confidence intervals.

Table A1.1. Recovery of *Escherichia coli* cells in trypo soy broth with yeast extract treated with eugenol or cinnamaldehdye by ISO-Grid membrane. Average of duplicate samples, 95% confidence intervals.

	Control		**5mM Eugenol**		**10mM Cinnamaldehyde**	
Time	Log CFU/ml	95% Con	Log CFU/ml	95% Con	Log CFU/ml	95% Con
0	4.12	0.085	0	0	3.70	0.195
1 hr.	4.22	0.116	0	0	0	0

Appendix 2: Recovery of *Lactobacillus sakei* and *Listeria monocytogenes* by ISO-Grid.

Table A2.1. Recovery of *Lactobacillus sakei* cells in trypo soy broth with yeast extract treated with eugenol by ISO-Grid membrane

	Control		**8mM Eugenol**	
Time	Log CFU/ml	95% Con	Log CFU/ml	95% Con
0	3.78	0.026	0.33	0.800
1 hr.	3.90	0.094	0	0

Average of duplicate samples, 95% confidence intervals.

Table A2.2. Recovery of *Listeria monocytogenes* cells in in trypo soy broth with yeast extract treated with eugenol or cinnamaldehdye by ISO-Grid membrane.

	Control		**5mM Eugenol**		**40mM Cinnamaldehyde**	
Time	Log CFU/ml	95% Con	Log CFU/ml	95% Con	Log CFU/ml	95% Con
0	4.71	0.080	4.75	0.026	4.63	0.187
1 hr.	4.80	0.068	1.52	0.668	2.08	0.859

Average of duplicate samples, 95% confidence intervals.

Appendix 3: External ATP Results for *Lactobacillus sakei* and *Listeria monocytogenes.*

Table. A3.1a. External ATP from *Listeria monocytogenes* samples exposed to glucose then treated with 40 mM cinnamaldehyde, 5 mM eugenol and 10 μ CCCP.

	5min		10min		15min		20min	
	Avg	**Std**	**Avg**	**Std**	**Avg**	**Std**	**Avg**	**Std**
Buffer	13.3	5.25	12.8	1.26	12.8	1.71	13.7	2.06
Glucose	19.3	5.38	27.3	9.21	43.8	29.1	39.3	11.3
Eugenol	18.5	6.86	666	538	751	610	723	647
Cinnamaldehyde	32.0	27.6	40.0	11.3	36.3	6.65	47.3	3.86
CCCP	17.5	16.0	28.8	7.09	26.8	6.40	27.0	8.91

Avg – average of four experiments.
Std – standard deviation.

Table. A3.1b. Results of students t-test on Table A3.1a. data.

	5min	**10min**	**15min**	**20min**
Buffer/Glucose	0.2505	0.0500	0.1279	0.0189
Buffer/Eugenol	0.4376	0.0931	0.0937	0.0977
Buffer/Cinnamaldehyde	0.3006	0.0161	0.0069	0.0002
Buffer/CCCP	0.6004	0.0142	0.0098	0.0549
Glucose/Eugenol	0.7913	0.0980	0.1015	0.1055
Glucose/Cinnamaldehyde	0.4029	0.0071	0.5915	0.2010
Glucose/CCCP	0.8205	0.8346	0.3709	0.2464

Table. A3.2a. External ATP from *Listeria monocytogenes* samples treated with 40mM cinnamaldehyde, 5 mM eugenol and 10μ CCCP, then exposed to glucose.

	5min		10min		15min		20min	
	Avg	**Std**	**Avg**	**Std**	**Avg**	**Std**	**Avg**	**Std**
Buffer	19.2	13.9	19.0	6.15	21.7	8.26	58.7	81.0
Glucose	17.8	5.57	21.3	4.95	24.5	3.55	25.5	4.47
Eugenol	84.7	69.4	79.6	51.9	103	89.7	85.8	68.5
Cinnamaldehyde	33.8	23.0	78.5	62.1	38.4	33.0	41.8	28.3
CCCP	33.4	25.8	39.7	26.4	41.4	34.0	37.8	29.5

Avg – average of four experiments.
Std – standard deviation.

Table. A3.2b. Results of students t-test on Table A3.2a.

	5min	**10min**	**15min**	**20min**
Buffer/Glucose	0.7993	0.3278	0.6329	0.4801
Buffer/Eugenol	0.1322	0.0946	0.1840	0.1836
Buffer/Cinnamaldehyde	0.1075	0.0490	0.1669	0.1180
Buffer/CCCP	0.3831	0.3020	0.3783	0.3723
Glucose/Eugenol	0.5948	0.2373	0.3264	0.4910
Glucose/Cinnamaldehyde	0.3403	0.2163	0.2006	0.6367
Glucose/CCCP	0.2124	0.1188	0.1476	0.6974

Table. A3.3a. External ATP from *Lactobacillus sakei* samples exposed to glucose then treated with 10 mM eugenol and 100 μM CCCP.

	5min		10min		15min		20min	
	Avg	**Std**	**Avg**	**Std**	**Avg**	**Std**	**Avg**	**Std**
Buffer	14.0	13.7	20.8	16.3	15.5	4.51	17.0	4.55
Glucose	23.5	9.26	27.3	22.7	56	40.5	24.5	15.0
Eugenol	14.5	8.54	53.8	62.1	65.5	82.8	76.5	83.9
CCCP	13.8	4.99	15.3	3.86	18.3	4.03	17.8	5.44

Avg – average of four experiments.
Std – standard deviation.

Table. A3.3b. Results of students t-test on Table A3.3a.

	5min	**10min**	**15min**	**20min**
Buffer/Glucose	0.3891	0.7077	0.1418	0.2691
Buffer/Eugenol	0.9633	0.2465	0.2945	0.2360
Buffer/CCCP	0.9765	0.4917	0.1514	0.7529
Glucose/Eugenol	0.2960	0.5207	0.8137	0.2344
Glucose/CCCP	0.0490	0.4138	0.1463	0.2840

Table. A3.4a. External ATP from *Lactobacillus sakei* samples treated with 40mM cinnamaldehyde, 5 mM eugenol and 10μ CCCP, then exposed to glucose.

	5min		10min		15min		20min	
	Avg	**Std**	**Avg**	**Std**	**Avg**	**Std**	**Avg**	**Std**
Buffer	43.9	30.2	56.0	28.5	52.2	27.0	44.7	25.6
Glucose	45.2	25.6	47.7	28.9	45.2	23.0	185	231
Eugenol	47.6	9.41	50.9	14.3	75.0	25.1	55.6	10.0
CCCP	38.1	10.7	40.5	13.2	42.7	16.5	39.0	14.2

Avg – average of four experiments.
Std – standard deviation.

Table. A3.4b. Results of students t-test on Table A3.4a.

	5min	**10min**	**15min**	**20min**
Buffer/Glucose	0.7905	0.4985	0.3375	0.3263
Buffer/Eugenol	0.7882	0.6019	0.1039	0.4329
Buffer/CCCP	0.4790	0.1622	0.2140	0.5079
Glucose/Eugenol	0.8135	0.7963	0.1216	0.3309
Glucose/CCCP	0.4737	0.5025	0.6147	0.2872

Appendix 4: Calculation of V_{max} and K_m of Membrane

The maximum velocity (V_{max}) and Michaelis constant (K_m) for the ATPase activity of the estimated by Eadie-Hofstee plots. The plots were constructed from initial velocity measurements made at four different substrate concentrations.

From an Eadie-Hofstee plot of velocity versus velocity divided by substrate concentration K_m (μM) is indicated by the negative of the slope and V_{max} (μM/s) by the Y axis intercept. The slope and intercept were calculated from the data set by linear regression.

The activity assays contained 0.05 mg of *Escherichia coli* membrane protein or 0.2 mg of *Listeria monocytogenes* membrane protein. Thus the specific activity of the membrane preparation per mg of protein for *E. coli* was K_m = 2096 μM, V_{max} = 5.28 μM/s and *L. monocytogenes* K_m = of 141 μM, V_{max} = 0.282 μM/s at pH 7.8 and 30°C.

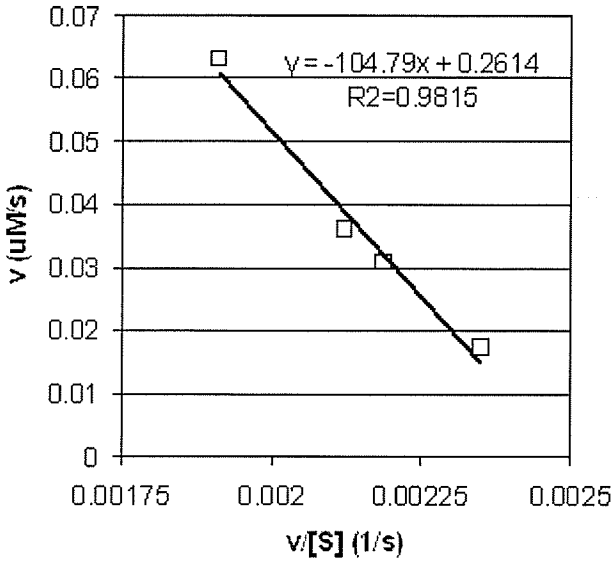


Fig. A4.1. Eadie-Hofstee plot of ATPase activity of *Escherichia coli* membrane preparations.

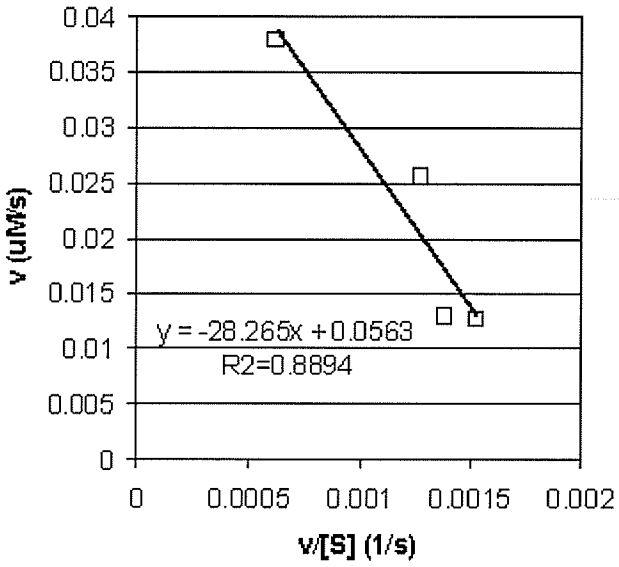


Fig. A4.2. Eadie-Hofstee plot of ATPase activity of *Listeria monocytogenes* membrane preparations.

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