EFFICACY OF ROSEMARY AND/OR THYME OIL AS ANTILISTERIAL INGREDIENTS IN MOZZARELLA TYPE CHEESE OR IN CHEESE PACKAGES

By

Dhaval Patel

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FACULTY OF GRADUATE STUDIES

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Dhaval Patel

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

Of

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ABSTRACT

Shredded mozzarella cheese is popular and consumption is growing at a rate of 4% every year. By the year 2009, mozzarella cheese is expected to be the most popular variety of cheese in North America. Listeriosis, a food-borne disease caused by *Listeria monocytogenes* has been linked with the consumption of soft varieties of cheese. As markets change with consumers demanding more natural foods free from chemical preservatives, researchers are investigating the use of naturally occurring antimicrobials instead. The present work examined the effectiveness of rosemary and thyme oil as volatile antimicrobial agents in cheese against *L. monocytogenes*. The experiments involved inoculation of low and full fat cheese with a cocktail of different strains of *L. monocytogenes* at 3 $\log_{10}$ cfu/g. The inoculated cheese was treated with rosemary and thyme oil either separately or together and stored at 4 and 10°C for 20 or 15 days, respectively. Preliminary experiments showed that one of the five strains of *L. monocytogenes* used was resistant to rosemary and thyme oil. Therefore, further experiments were done with a cocktail of four strains.

When the antimicrobials were tested for their antilisterial activity in full fat cheese separately or in combination, they failed to eliminate *L. monocytogenes*, producing a reduction of 0.3 and 0.4 $\log_{10}$ cfu/g, respectively, at 10°C. When tested at 4°C, the numbers in controls and samples treated with rosemary or thyme oil separately or together were similar. Thus, in full fat cheese the oils were ineffective against *L. monocytogenes*. When sodium diacetate was used along with rosemary and thyme oil, its presence alone or with the oils produced a bacteriostatic effect at 4°C. Treatment of inoculated cheese with all three antimicrobials reduced *L. monocytogenes* growth by 2.6 $\log_{10}$ cfu/g at the end of day 6. However, this early inhibition was reversed by 15 d, when

the reduction was only 0.6 $\log_{10}$ cfu/g. Sodium diacetate along with rosemary and thyme oils were slightly more effective at 4°C than at 10°C.

When evaluated against *L. monocytogenes* in low fat cheese, the oils were more effective. Rosemary or thyme oil produced a significant difference of 0.5 and 1.0 $\log_{10}$ cfu/g, respectively, at the end of 20 d storage at 4°C, but the difference increased to 1.7 $\log_{10}$ cfu/g when the oils were used together. Rosemary and thyme oils were less effective at 10°C, and when used separately they did not produce a significant difference in numbers from those in control samples. However, when the oils were combined, numbers were reduced by 0.7 $\log_{10}$ cfu/g at the end of 15 d storage. When both oils were released from a sachet containing microcellular foam (MCF) starch, they were slightly more effective initially but were only able to produce a difference of 0.7 $\log_{10}$ cfu/g by the end of 15 d storage at 10°C. Neither rosemary or thyme oil was effective when used alone in the sachet. Results showed that 1% (w/w) rosemary and/or thyme oils could slightly reduce *L. monocytogenes* growth in cheese but were unable to eliminate the pathogen.

Chapter 1

INTRODUCTION

Many food products are perishable by nature. A variety of physical, chemical and microbiological changes can take place after harvest or manufacture that can lead to deterioration of their overall quality and cause reduction in shelf-life. Factors responsible for food spoilage include the presence of undesirable microorganisms, availability of nutrients, environmental conditions occurring in foods (preservatives, pH and water activity) and storage conditions (temperature and atmosphere) (Luck and Jager, 1997).

Traditional food preservation techniques involve the use of heat, refrigeration, freezing, drying and fermentation. In addition to these techniques, chemical preservatives have frequently been relied upon to delay spoilage (Branen, 1983). However, nowadays, consumers are becoming more and more health conscious and are demanding foods of high quality, with few if any preservatives and greater shelf-life. Along with these changes in consumer demands, labeling regulations and more stringent requirements regarding chemical preservative use have made it difficult for food processors to meet current needs (Brul and Coote, 1999).

Food additives normally perform one or more of five main functions when added to foods such as a contribution to desirable color or flavor, improvement of body and/or texture, and nutritive value. Antimicrobials are additives that prevent or retard biological deterioration (Branen, 1983). The effectiveness of a preservative depends on the chemical composition of treated foods, the number and type of bacteria present, the inherent chemical characteristics of the preservative and the conditions under which the target food is stored. Cost and safety are two additional considerations which influence preservative use (Wagner and Moberg, 1989).

In concert with the proper use of processing techniques, food packaging methods and materials (especially active food packaging), can play an important role in determining the overall quality and safety of packaged foods. Active, as opposed to conventional passive packaging, involves interaction between the food, the packaging material and the internal gaseous environment. In these systems the packaging material can contain a compound(s) that affect the contained environment, providing better chemical or microbiological stability (Debeaufort et al., 1998; Han, 2000).

The search to satisfy increased demands for minimally processed and preservative-free foods with extended shelf-life has led to the examination of naturally occurring inhibitory compounds such as organic acids, essential oils, bacteriocins and other products for their potential value in this application (Lemay et al., 2002).

Within recent years, cheese consumption has doubled over what it was 20 years ago. Mozzarella cheese is the second most popular cheese in North America with per capita consumption of 3.8 kg/year. It is believed that by 2009, mozzarella cheese will represent the largest fraction of cheese consumed in the US, with per capita consumption rising to 5.8 kg/person/year (Ferrari et al., 2003). In addition, shredded cheese which includes mainly Cheddar and mozzarella is becoming popular. The sale of shredded cheese has increased at steady rate of 4.4% since 1996 and is estimated to be $1.3 billion of the $8.8 billion cheese market (Elayedath and Barringer, 2002).

The objective of this research work was two fold. First, rosemary and thyme oils were used as natural antimicrobials to evaluate their effectiveness in shredded cheese against *Listeria monocytogenes*. Second, the antimicrobials were used to develop an antimicrobial packaging system for shredded cheese where the volatilized oils were tested for their effectiveness against *L. monocytogenes*.

Chapter 2

LITERATURE REVIEW

2.0 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, catalase-positive, non-sporeforming, facultatively anaerobic intracellular pathogenic organism. Young cells often appear as diplococci or cocci when viewed under microscope (Ryser and Marth, 1991). They show tumbling motility when grown at 20-25 °C. When grown in that temperature range, *L. monocytogenes* has peritrichous flagella, which are responsible for their motility. However, higher growth temperature (37 °C) inhibits production of flagella, making the cells non-motile (Farber and Peterkin, 1991).

The pathogen was first isolated from the blood of a laboratory animal by Murray and coworkers in 1926. The first human listeric infection was confirmed in 1929 (El-Shenaway and Marth, 1989). Food was not recognized as a vehicle for human infection by *Listeria* until 1982 (Hoff, 2003).

There are eight recognized *Listeria* species. These include *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. murrayi*, *L. denitrificans* and *L. grayi*. Of these species, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic in nature (Comi et al., 1990).

2.1 *Listeria monocytogenes*, its ecology and listeriosis

L. monocytogenes is widely distributed in nature. It can be isolated from agricultural environments such as soil, water, plants, animal waste, feed and silage. It is also present in food processing environments (Park et al., 2002). The organism can grow over a temperature range of -1 to 45 °C, with the optimum growth temperature being in the range of 35 – 37 °C. Its lag phase increases with decrease in the storage temperature (5 – 10 d at 4°C). However, it is more virulent when grown at low temperature (4°C) (Olarte et al., 2002; Prasad and Gupta, 1990).

In addition to its ability to grow over a wide temperature range, the organism is also known for its ability to survive and/or grow in the presence of salt up to10 % NaCl. However, salt concentrations higher than this inhibit its growth. *L. monocytogenes* was inhibited at 37 °C in Trypticase Soy Broth containing 25.5% NaCl at 4 d. However, when incubated at low temperature (4°C), it survived for more than 132 days under same conditions (Ryser and Marth, 1991). *L. monocytogenes* can also grow over a wide pH range (5 to 9.6), with the best growth occurring at neutral to slightly alkaline pH (El-Shenawy and Marth, 1989; Ryser and Marth, 1991).

Because of its ability to grow over wide temperature and pH ranges as well as at higher salt concentrations, the pathogen can be of great concern for food processors. *L. monocytogenes* has been associated with illnesses related to consumption of a wide variety of contaminated foods including meat, seafood, dairy products and vegetables (Guerra et al., 2001).

The presence of the pathogen in raw milk has been linked with poor dairy farm management and poor hygienic conditions of the animals. Since *L. monocytogenes* is heat labile, a standard pasteurization treatment will eliminate it from milk. Its presence

in pasteurized milk and milk products clearly indicates post-processing contamination (Hassan et al., 2001; Prasad and Gupta, 1990).

Several outbreaks of listeriosis have been associated with consumption of dairy products, especially soft and semi-soft cheese. Though *L. monocytogens* has been identified as human pathogen for many years, its risk to public health has only been recognized over the past two decades (Comi et al., 1990; Hassan et al., 2001).

Listeriosis occurs mainly in certain groups of people such as the young, old, pregnant and immunologically compromised. The mortality rates can sometimes approach as high as 50%. The infection in pregnant women can cause abortion or stillbirth (Ooi and Lorber, 2005; Rudolf and Scherer, 2001). The infectious dose varies. In healthy adults, the level of *L. monocytogenes* required to cause infection has varied from 9 log cfu/ml in chocolate milk to 5 log cfu/ml in cold-smoked rainbow trout. For higher risk groups, the level varied from < 2 to 4 log cfu/g in rainbow trout to 4 log cfu/g in ice cream (Maijala et al., 2001). In Canada, Farber et al. (1996) reported that doses of 7 and 9 log cfu/g in susceptible or normal individuals, respectively, were sufficient to cause illness. In addition to the level of the pathogen in food, the amount of contaminated food consumed is also an important factor.

2.2 Occurrence of *Listeria monocytogenes* in cheese

Following outbreaks of listeriosis in North America and Europe during the 1980s and 1990s, *L. monocytogenes* has received considerable interest among food processors and food microbiologists. Listeriosis is now linked with the consumption of ready-to-eat food products that are usually processed and consumed without further heat treatment.

These products when stored at refrigeration temperature have an extended shelf-life and provide a unique opportunity for *L. monocytogenes* to grow during storage (Guerra et al., 2001).

The Centre for Disease Control and Prevention (CDC) has reported that in the United States, food-borne diseases cause 76 million illnesses and 5000 deaths each year (CDC, 2000). Though the frequency of listeriosis is very low, the impact on public health is still of great concern. Among all the food-borne pathogens, *Listeria* spp. have been recognized as the second leading cause of death. Due to close surveillance and implementation of Hazard Analysis Critical Control Point (HACCP) systems at food manufacturing plants, there has been a steady decline in the frequency of listeriosis outbreaks (e.g., a 19% decrease in the incidence of infection over the period from 1996 to 1999). However, the number of people affected in each outbreak is still unchanged (Meyer-Broseta et al., 2003; Park et al., 2002).

L. monocytogenes was recognized as food-borne pathogen only after an outbreak in Nova Scotia, Canada in 1982 where the vehicle of illness was coleslaw (Hoff, 2003). The first outbreaks due to consumption of dairy products were reported in 1985 when pasteurized milk and Mexican-style soft cheese contaminated with *L. monocytogenes* were found responsible. During the late 1980s, many cases of listeriosis were reported in Switzerland. The food vehicle was again contaminated soft cheese (Comi et al., 1990).

Listeriosis outbreaks are both epidemic as well as sporadic. They became a great concern for public health after a major outbreak occurred in California during January to August 1985. A total of 142 people were diagnosed with the disease. The outbreak showed a mortality rate of 34% and involved 30 fetuses and newborn infants, as well as nonpregnant adults. Upon further investigation, it was revealed that Mexican-style soft

cheese was responsible for the spread of the disease and the cheese was recalled from the market place (Farber and Peterkin, 1991).

During 1983 and 1987, a major outbreak of listeriosis involving *L. monocytogenes* occurred in Switzerland. The outbreak involved 122 cases and there were 31 deaths. Upon an extensive epidemiological study, it was found that Vacherin Mont d'Or soft cheese was the source of contamination. Again the cheese was recalled (EL-Shenawy and Marth, 1989; Farber and Peterkin, 1991).

In November 2000, health care providers reported three cases of listeriosis over a period of two weeks in North Carolina. In conjunction with the FDA and the CDC, state officials investigated the outbreak. A total of 12 cases were identified which involved 11 women (10 who were pregnant) with an average age of 21 yr. The infection caused five stillbirths, three premature deliveries and two infected newborns. Upon further investigation, it was revealed that all patients consumed a soft Mexican-style queso fresco cheese. This variety of cheese is made from raw milk. The investigators found cheese being sold in two small local community grocery stores without being labeled unpasteurized. It was also found that a commercial dairy plant was selling raw milk to unskilled manufacturers. As a result of this outbreak, authorities prohibited the sale of raw milk. They also added listeriosis to the list of reportable diseases in the US (CDC, 2001).

A number of other listeriosis outbreaks from cheese have been reported in Europe (Table 2.1). Buyser et al. (2001) conducted a study to determine the involvement of milk and milk product-related diseases in France and other countries after 1980. Occurrence of listeriosis in France during 1995 and 1997 resulted in 50 reported cases, 4 deaths and 7 neonatal cases. The food vehicles for these outbreaks were Brie de Meaux

cheese and Pont Leveque cheese. Listeriosis outbreaks due to consumption of cheeses either made from pasteurized or unpasteurized milk have been reported in countries such as Denmark, Switzerland, Luxembourg as well as the USA.

Because of these high mortality rates and ability of *Listeria* to survive in ready-to-eat foods stored at refrigeration temperatures, efforts have been made to track down the pathogen in various foods throughout the world (Table 2.2). In Portugal, from 1998 to 2000, 429 food samples were collected from various food manufacturing plants. *L. monocytogenes* was detected in 8 cheese samples and other listeria species were detected in 15 others. It was also evident that processed foods were more frequently contaminated than raw foods. Cheese samples sold unpackaged tested negative for the pathogen, whereas those sold wrapped were positive for *L. monocytogenes*. Clearly, *Listeria* can contaminate food products even at a late stage of processing, which demonstrates the need for HACCP systems in food manufacturing environments (Guuerra et al., 2001). In another similar study conducted in Navarra, Spain, more than one *Listeria* species was detected in soft cheeses. *L. monocytogenes* was detected in one sample only. Other listeria species detected in cheese samples included *L. innocua* (6 samples) and *L. grayi* (1 sample). In total 8 samples of 99 collected were positive for *listeria* (Vitas and Garcia-Jalon, 2003).

2.4 Plant essential oils

Despite achievements in modernizing food production and processing technologies, food safety is still of great concern to the food industry. Food safety and preservation have been challenges for mankind since ancient time. In addition consumers, aware of issues regarding nutrition and safety of foods, are now demanding foods that are more 'natural', minimally processed and free of chemical preservatives. In concert with this gradual, yet quite promising, change in market demand for foods, regulatory agencies are continuously evaluating the safety of additives currently available and have restricted the use of some from food production (Brul and Coote, 1999; Burt 2004; Devlieghere et al., 2004; Wanger and Moberg, 1989).

Food additives perform five main functions. These include the addition of flavor and/or color, enhancement of body and texture and improvement of nutritional value. Antimicrobials are also additives and their main function is to prevent and/or restrict biological deterioration (Branen, 1983). They can be either present naturally in the foods, formed in the foods during processing or added as ingredients during manufacture of foods (Beuchet and Golden, 1989). Though chemically synthesized antimicrobial agents are often highly effective, their safety has been questioned by both consumers and food researchers. As a result, a search for potent natural antimicrobials has been conducted for the last couple of decades. Spices and herbs have been used for many years as flavoring agents in foods and are known for their preservative and medicinal power (Buechat and Golden, 1989; Zaika, 1988).

Plant essential oils are derived from various parts of plants including flowers, buds, roots, leaves, seeds, twigs, bark, wood and fruits. Obtained either by expression, fermentation or steam distillation, essential oils are liquid aromatic oils. Their

antimicrobial activity is attributed, quite often, to their major components (Burt, 2004; Zaika, 1988). Over the past few years there has been an increase in the use of plant oils as antimicrobial agents in foods. Lowering salt and sugar content has promoted interest in the addition of seasonings containing spices and herbs to compensate for the bland taste (Shelef, 1983).

The antimicrobial activities of plant essential oils have been examined against both pathogens and spoilage microorganisms. Among them, the most extensively studied plant essential oils include oils of cinnamon, clove, thyme, rosemary, oregano, basil, nutmeg, mint, tea tree, cilantro, dill, garlic, onion, horse radish, pimento and bay. In addition to whole plant essential oils, their major components have also been analyzed for antimicrobial activity. These include oil components like cinnamaldehyde, eugenol, thymol, carvacrol and allyl isothiocyanate (Baydar et al., 2004; Chang et al., 2001; Charai et al., 1996; Cox et al., 2000; Delaquis et al., 2002; Hao et al., 1998; Iscan et al., 2002; Muthukumarasamy et al., 2003; Singh et al., 2003; Sivropoulou et al., 1995, 1996; Tassou et al., 1995; Walsh et al., 2003; Wan et al., 1998). The antimicrobial activity of various plant essential oils and their major components against pathogenic and spoilage organisms as well as against various fungi has been recently reviewed by Holley and Patel (2005).

2.5 Rosemary oil

Rosemary oil is a naturally occurring plant essential oil extracted from the spice *Rosemarinus officinalis* L. The word *Rosemarinus* is based on the Latin word "*ros-roris*" meaning dew. During ancient time in Greece, it was also known as "antos" which means 'the flower' for excellence or 'libanotis' for its incense (Pintore et al., 2002). It grows well wild in areas of Europe, Asia and Africa. It is also found in some areas near the Mediterranean Sea (Domokos et al., 1997; Pintore et al., 2002). It contains about 1% oil and has been used in traditional medicine as an antiseptic, a coleretic, a cholagogic, antirheumatic and antidiarrheic agent. In addition, it has also been widely used in perfumes and liquors (Domokos et al., 1997; Pintore et al., 2002).

The essential oil obtained from *Rosemarinus officinalis* L. is usually colorless or pale yellow and has a characteristic camphoraceous taste. α-pinene, 1,8- cineole and camphor are the major components present in rosemary oil. In addition to these compounds, it also contains considerable amounts of camphene, limonene and borneol. The amount of oil and its composition also vary with respect to the environment and location. For example, rosemary oil obtained from plants grown in Morocco, Tunisia, Turkey, Greece, Yugoslavia, Italy and France contained more than 40% of 1,8- cineole, whereas oil from plants grown in Spain and Bulgaria contained equal amounts (20-30%) of 1,8- cineole, α-pinene and camphor (Boutekedjiret et al., 1998; Boutekedjiret et al., 1999; Rezzoug et al., 1998).

2.5.1 Antimicrobial activity of rosemary oil

Rosemary oil has been analyzed for its antimicrobial activity against both Gram-positive and Gram-negative bacteria. Usually Gram-positive organisms are more sensitive (Baratta et al., 1998; Mangena and Muyima, 1999). Domokos and coworkers (1997) evaluated antimicrobial activity of rosemary oil against Gram-positive and Gram-negative bacteria using an agar diffusion method. They reported that the presence of 0.2 ml of rosemary oil in agar wells completely inhibited Gram-positive bacteria, while the oil failed to exert a strong antibacterial activity against Gram-negative bacteria. Similar observations were reported by Smith-Palmer et al. (1998). They analyzed 21 plant essential oils against five different foodborne pathogens. A concentration of >1% rosemary oil was required to exert either bacteriostatic or bactericidal effects against *Escherichia coli*, *Salmonella* Enteritidis and *Campylobacter jejuni*. When analyzed against *L. monocytogenes* and *Staphylococcus aureus* in TSB, only 0.02 and 0.04% rosemary oil, respectively, was required to show bacteriostatic activity. A concentration of 0.1% was bactericidal against both Gram-positive pathogens (Table 2.3). Mangena and Muyima (1999) reported that when undiluted rosemary oil was evaluated against various bacteria using the agar diffusion technique, it was able to produce larger inhibition zones against Gram-positive than against Gram-negative bacteria. The inhibition zones of *L. monocytogenes*, *S. aureus* and *Streptococcus pyogenes* were 17, 12 and 12 mm, respectively, whereas *Pseudomonas aeruginosa* was the most resistant and the oil was not able to produce any zone of inhibition. Similar observations were also reported by Deans and Ritchie (1987). These authors tested 50 different plant essential oils against 25 genera of bacteria. They reported that *Bacillus subtilis*, *S. aureus* and *Enterococcus faecalis* were the most sensitive organisms while *P. aeruginosa* was

extremely resistant and the oil failed to produce any inhibition zone. Thus, cell wall type plays an important role in determining the overall activity of antimicrobial essential oils. The presence of an outer membrane containing lipopolysaccharide, phospholipid and some proteins in Gram-negative bacteria may provide protection to the cytoplasmic membrane by obstructing the uptake of essential oil or oil components, resulting in increased resistance to the natural hydrophobic antimicrobials. The complexity of the Gram-negative bacterial cell wall, which has three component layers, contributes to the generally greater resistance of these bacterial cells to the natural antimicrobials. In the case of Gram-positive bacteria, only the peptidoglycan layer covers the cytoplasmic membrane making it available for more direct contact with the oils and thereby increasing susceptibility (Chao et al., 2000).

Cox et al. (2000) reported that the presence of 0.25% and 0.5% (v/v) tea tree oil inhibited respiration of both *E. coli* and *S. aureus* cells. In addition, exposure of *E. coli* and *S. aureus* to 0.25% (v/v) tea tree oil for 30 min increased cell permeability to the nucleic acid stain, propidium iodide, to which the bacterial cell wall is usually impermeable. Also treatment of cells with tea tree oil resulted in leakage of potassium ions. In contrast with the generally greater susceptibility of Gram-positive cells to essential oils the leakage of potassium started immediately after adding oil to the suspension containing *E. coli* and after 5 min with *S. aureus*. These findings clearly indicate that the oil damages cell membrane structure in *E. coli* and *S. aureus*.

That the cell membrane is the primary site of action of these plant essential oils was also confirmed in another study conducted by Lambert et al. (2001). They evaluated the mode of action of oregano essential oil, thymol and carvacrol against *P. aeruginosa* and *S. aureus*. Addition of 0.1% antimicrobial resulted in an increased uptake of

ethidium bromide, a positively charged, fluorescent nuclear stain to which the intact cell membrane is impermeable. Addition of essential oil and oil compounds also led to leakage of potassium and phosphate in both *P. aeruginosa* and *S. aureus*. When Helander et al. (1998) evaluated the mechanism of action of essential oil components against Gram-negative bacteria, they found that trans-cinnamaldehyde strongly inhibited growth and bioluminescence of *Photobacterium leiognathi* without disintegrating the outer membrane (OM). This was taken to indicate that the oil component had passed through the porin proteins of the OM.

Studies have also been conducted to evaluate the antimicrobial activity of rosemary oil in food systems. Pandit and Shelef (1994) evaluated antilisterial activity of rosemary oil in liver sausage. The presence of 1% rosemary oil in sausage suppressed growth of the pathogen. The latter authors observed a difference of 1 to 2 log cfu/g between control and treatment at the end of 50 d storage at 5 °C. Mendoza-Yepes et al. (1997) used a commercially available preservative containing a mixture of rosemary, sage and citric compounds. At 2500 ppm, the preservative was able to inhibit the growth of *L. monocytogenes* in Spanish soft cheese stored for 10 d at 7 °C. Addition of 5% rosemary spice extract (RSE) in mechanically deboned poultry meat resulted in a 4 log cfu/g reduction of *S. aureus* at the end of 12 d storage at 5 °C. However, at lower concentrations (0.1 and 1.0%), RSE failed to control the growth of *S. aureus*, and at the end of storage the number in control as well as treated samples was similar.

2.6 Thyme oil

Thyme oil is frequently obtained from the plant *Thymus vulgaris* L. which belongs to the Labiateae family. The genus *Thymus* contains more than 300 different species. It is widely grown in Southern Europe and Asia. In Turkey it is also called 'Kekik' for its thymol or carvacrol-like odor. The plants usually contain a large amount of oil (>2%). They are well known for their medicinal power and have been used as flavoring agents for many years in variety of food products such as sauces, meat and canned foods. In addition, they are also known for their antiseptic and antimicrobial properties (Baydar et al., 2004; Bhaskara Reddy et al., 1998; Vardar-Unlu et al., 2003).

More than 44 compounds in thyme oil have been identified and the major compounds include thymol, γ- terpinene, ρ-cymene and carvacrol. Among the major components in thyme oil, thymol and carvacrol have been recognized as the most effective antimicrobial agents (Baydar et al., 2004; Bhaskara Reddy, 1998).

2.6.1 Antimicrobial activity of thyme oil

Baydar et al. (2004) evaluated antimicrobial activity of thyme oil from *Thymus spicata* L. (containing about 75 % carvacrol) against 15 different bacteria using a paper disc diffusion technique. A 1/50 solution of thyme oil in absolute alcohol was able to inhibit all test organisms. *Bacillus brevis* was the most sensitive organism, showing a 45 mm inhibition zone. The oil was also able to inhibit *L. monocytogenes* Scott A, producing a 33.5 mm inhibition zone. In another similar study, Firouzi and coworkers (1998) reported that a sterile filter paper disc containing 1:6400 diluted thyme oil was

highly effective against *L. monocytogenes*, giving a 25 mm inhibition zone after 24 h incubation at 37 °C (Table 2.3).

Dorman and Deans (2000) reported the antimicrobial activity of thyme oil against 25 different bacteria. The oil showed antimicrobial activity against all bacteria tested. Again thyme oil was more effective against Gram-positive than against Gram-negative bacteria.

When tested in cheese, Smith-Palmer et al. (2001) found thyme oil bactericidal, resulting in a 4 log cfu/g reduction of *L. monocytogenes* in low fat (16%) cheese over a period of 14 d (Table 2.4). Singh et al. (2003) showed that dipping of inoculated fat-free hot dogs in a thyme oil solution (10 ml/L) for 10 to 15 min resulted in a 0.86 to 1.33 log cfu/g reduction of *L. monocytogenes.* However, there was a decrease in the antilisterial activity of thyme oil when evaluated in full fat (26%) hot dogs. The oil was able to reduce the pathogen by only 0.53 log cfu/g when treated under same conditions.

When tested against the same pathogen in chicken breast, the presence of 2% thyme oil was not able to produce any significant difference between treated and untreated control samples. At the end of 14 d storage at 5 °C, the number in treated samples was 5.10 log cfu/g compared to 4.8 log cfu/g in untreated samples (Hao et al., 1998).

Cutter (2000) used a commercially available herbal mixture against *L. monocytogenes*, *S.* Typhimurium and *E. coil* O157:H7 in beef. Treatment of ground beef with 2.5% liquid Protecta one (a proprietary herbal mixture dispersed in sodium citrate) resulted in reduction of *L. monocytogenes, S.* Typhimurium and *E. coli* on days 0 and 1 by 0.3, 0.8 and 0.3 log cfu/g. However, the herbal mixture was not able to maintain this

initial reduction and at the end of 14 d storage at 4 °C there was no significant difference between treatment and untreated control samples.

2.7 Antimicrobial packaging

With increased consumer demand for chemical (preservative) free and minimally processed foods, and globalization of food trade, the safety and shelf-life of perishable food products have created challenges for food processors and food microbiologists (Appendini and Hotchkiss, 2002; Suppakul et al., 2003). The quality of foods is determined by organoleptic properties, nutritional profile and hygienic status. These aspects sometimes depend on mass transfer between foods and the immediate surrounding environment (Debeaufort et al., 1998). Packaging can play important roles in preventing contamination and in controlling the exchange of gases between the environment and food products (Debeaufort et al., 1998).

Apart from providing barrier functions, the functionality of food packaging materials can also be improved by adding various active substances such as oxygen scavenging materials, ethanol generating and ethylene absorbing substances. Such packaging is called 'active' packaging (Han, 2000). Active packaging systems involve interactions between the packaging material, the internal environment and the packaged food. The main goal of active packaging systems is to extend the shelf-life of packaged food products (Cutter, 2002).

Antimicrobial packaging is a form of active packaging that involves incorporation of antimicrobial substances to control and/or eliminate undesirable microorganisms (Devileghere et al., 2004).

The various antimicrobial packaging systems that have been developed (Table 2.5) can be classified as migrating, adsorptive, regenerating or non-migrating (Han, 2003). They can be prepared by incorporating an antimicrobial agent directly into polymers, by coating the antimicrobial onto the inside surface of the packaging material, by immobilizing the active antimicrobial onto the surface of the food or by using pads or sachets containing antimicrobial agents. In addition to these different antimicrobial packaging systems, inherently antimicrobial polymers can also be used as primary packaging materials (Appendini and Hotchkiss, 2002).

2.7.1 Design of an antimicrobial packaging system

Several factors determine the overall effectiveness of an antimicrobial packaging system. These factors include characteristics of the antimicrobial substance, the methods by which it is incorporated into the packaging system, its activity against microorganisms, characteristics of the target microorganisms, casting methods, safety of antimicrobial agents, and relevant regulations (Han, 2003).

The chemical stability of an antimicrobial agent is of prime importance if the antimicrobial agent is incorporated into packaging film during the casting process. For example, heat labile antimicrobial agents may be destroyed while making a packaging film because of high temperature and pressure conditions employed during the extrusion process (Han and Floros, 1999).

Another factor of importance in the design of antimicrobial packaging systems is the type of food to be packaged. Foods with different composition will have different chemical and biological characteristics, with the potential to interact favourably or

otherwise with microorganisms present or with the antimicrobials used. For example, the pH of foods can influence growth and proliferation of microorganisms and can change the ionization of chemical preservatives, thereby affecting their antimicrobial activity (Quintavalla and Vicini, 2002). In addition to pH, the water activity of food is also an important factor which influences the growth rate of microorganisms. The presence of oxygen in the package headspace can promote the growth of aerobic organisms. Thus pH, water activity of foods and oxygen permeability of packaging material are important factors to be considered in the design of antimicrobial packaging systems (Han, 2000).

The solubility of the antimicrobial agents is also an important factor. If the antimicrobial is hydrophobic, during the casting process it may damage the integrity of hydrophobic packaging films, leading to development of holes, crystallization of antimicrobials and even loss of film transparency. The solubility of the antimicrobial in food is also important. Higher solubility in food will lead to free diffusion, whereas lower solubility will lead to accumulation of the antimicrobial on the food surface. Under the latter conditions the actual concentration of antimicrobial beneath the food surface will be negligible. Thus, solubility and migration capability of the antimicrobial agent in the food matrix are also very important parameters (Han, 2003).

2.7.2 Natural preservative use in antimicrobial packaging systems

Bacteriocins are cationic peptide antimicrobial compounds produced by bacteria. They are effective against closely related microorganisms. Among various bacteriocins available, those that are produced by LAB have been extensively studied and evaluated for their effectiveness in food preservation (Cleveland et al., 2001).

The bacteriocins produced by lactic acid bacteria include diplococcin, lactostrepcins, nisin, lacticin 481, lactococcin and many more. Nisin is the most extensively studied bacteriocin. It is produced by *Lactococcus lactis* subsp. *lactis*. It has been given Generally Regarded As Safe (GRAS) status in the US and is permitted in the manufacture of a variety foods. It has shown antimicrobial activity against wide range of Gram-positive, food-borne pathogenic bacteria including *Staphylococccus aureus*, *Listeria monocytogenes* and sporeformers such as *Clostridium botulinum* (Nettles and Barefoot, 1993; Teerakaran et al., 2002). Antimicrobial packaging films containing nisin have been evaluated for their effectiveness in both laboratory media as well as in food systems.

2.7.3 Effectiveness of antimicrobial films on bacterial growth media

Hoffman et al. (2001) evaluated activity of corn zein films containing nisin against *L. monocytogenes* and *Salmonella* Enteritidis (Table 5). The required amount of nisin was mixed with corn zein solution (6.75 g in 40.6 ml 95% ethanol) just before casting. Nisin at 0.188 mg/film was able to reduce *L. monocytogenes* by 5.5 log after 48 h at 23 °C. When combined with lactic acid, nisin reduced *Listeria* numbers to <1 log cfu/g at the end of 12 h. In another study, edible films based on hydroxypropylmethylcellulose (HPMC) containing nisin were evaluated against *Micrococcus luteus*. At 10^5 IU/ml of nisin in film, a 14 mm inhibition zone was observed. Addition of stearic acid reduced the effectiveness of nisin. The interaction between positively charged nisin and the negatively charged fatty acid may have prevented the release of nisin from the film into agar media, resulting in reduced antimicrobial activity

(Coma et al., 2001). In another similar study, nisin at 5×10^3 IU ml^{-1} concentration in HPMC film reduced *L. innouca* by 0.4 log cfu/plate within 48 h at 37 °C (Sebti and Coma, 2002).

2.7.4 Effectiveness of antimicrobial packaging films in food systems

Janes et al. (2002) developed a corn zein film containing nisin and evaluated its activity against *L. monocytogenes* on the surface of refrigerated, ready-to-eat chicken. It was observed that zein films containing 1000 IU/g nisin were able to reduce *L. monocytogenes* by 1 to 3 log within 8 d when stored at 4°C. When zein films that combined either ethanol or propylene glycol with nisin were evaluated, the films were able to suppress the growth of the organism. A reduction of 4.6 log was observed when zein propylene glycol film containing nisin was used and the organism was inhibited below the detection limit when zein ethanol film containing nisin was used. Thus a synergistic effect of nisin and the solvent used for making zein films was observed.

The most common method for making packaging films is extrusion. Siragusa et al. (1999) made low density polyethylene (LDPE) film containing nisin by extrusion. The film was tested for its effectiveness *in vitro* and on vacuum packaged refrigerated meat. The film was extruded at low temperature (120 °C). Nisin was incorporated along with milk solids to yield final nisin concentration of 0.1% by weight. Nisin-containing films showed antimicrobial activity against *Brochothrix thermosphacta* and *Lactobacillus helveticus* in suitable agar media. When analyzed on a 5 cm^2 sample of refrigerated beef carcass surface tissue wrapped with a nisin-containing LDPE film, it restricted *B. thermosphacta* to less than 6.09 log/cm^2 by the end of 20 d storage at 4 °C.

Numbers on the untreated control samples were >7 log cfu/cm^2 at the end of storage. The conditions used for manufacture of LDPE films containing nisin did not have any adverse effect on the activity of nisin. In another study, a nisin formulation containing EDTA was coated onto commercially available plastic films (linear low density polyethylene, LDPE, polyvinyl chloride, PVC, or nylon). The coated films were then applied directly onto broiler skins inoculated with *S.* Typhimurium (5.0 log CFU/cm^2 skin). After 24 h, the coated skin was vortexed for 2 min in 20 ml 0.1% peptone water. The numbers were enumerated by plating an appropriately diluted sample on BHI plates. The level of bacterial survivors recovered from skin coated with PVC, LLDPE and Nylon film containing nisin was 4.3, 4.0 and 4.2 log cfu/ml of rinse, respectively. The presence of EDTA enhanced the antimicrobial activity of nisin-containing films. This may have been due to the fact that EDTA, a chelating agent, binds magnesium and other cations. The loss of divalent cations from the outer membrane of Gram-negative bacteria may increase cell permeability, destroy the lipopolysaccharide layer, affect cytoplasmic membrane integrity and lead to loss of viability (Natrajan and Sheldon, 2000a).

Natrajan and Sheldon (2000b) reported effectiveness of protein and polysaccharide-based edible films containing nisin against *S.* Typhimurium on poultry skin. In calcium alginate film containing 500 μg/ml nisin a 3.01 log reduction was observed after 72 h at 4°C. Five hundred μg/ml of nisin in polysaccharide film (agar, 0.75%) resulted in a 1.9 and 4.4 log reduction of test organisms at the end of 72 and 96 h, respectively, as compared to control untreated samples. With an increase in agar concentration to 1.25%, there was a decrease in bacterial lethality. Greater cross- linking of agar at higher concentrations within the film may have limited the migration of nisin onto the drumstick skin. The agar structure was thought to be open and elastic when

0.75% agar was used, allowing greater release of nisin compared to films with 1.25% agar.

Scannell et al. (2000) evaluated a bioactive cellulose-based package insert containing nisaplin (a proprietary form of nisin) for its antilisterial activity when vacuum packaged with cheese. A 5 cm^2 piece of paper was covered with 20 ml of the bacteriocin solution. The paper insert and bacteriocin solution were allowed to stay in contact for 8 h at 4°C to facilitate adsorption. The concentration of Nisaplin adsorbed onto the paper insert was approximately 2560 AU (Activity Unit) per cm^2. Nisin treated paper was placed on the surface of inoculated cheese slices. The samples were then placed into Polystyrene/ Ethylene-vinyl alcohol/ PE bags and sealed under modified atmosphere containing 60% nitrogen and 40% carbon dioxide gas. The bioactive cellular insert was able to reduce the number of *L. innocua* in cheese by 3 log cfu/g during one week of refrigerated storage. With up to 24 d further storage, the number of *L. innocua* dropped one more log cfu/g. *S. aureus* was also inhibited showing a 3 log reduction after the 24 d storage.

In another study, pediocin was used in developing an antimicrobial packaging system. Pediocin powder (7.75 mg/cm^2) was evenly distributed on the inside surface of packaging bags. Turkey breasts, ham and beef inoculated with *L. monocytogenes* (1000-5000 CFU/item) were placed in the bags, a vacuum was drawn and sealed bags were stored at 4°C for 12 weeks. Pediocin restricted the growth of *L. monocytogenes* during storage. At the end of the storage period, *L. monocytogenes* numbers had dropped by 1 and 0.5 log in beef and turkey breasts, respectively, while the bacteriocin showed bacteriostatic activity against *L. monocytogenes* in ham (Ming et al., 1997).

2.7.5 Use of plant essential oils in antimicrobial packaging systems

For many years spices and herbs have been used to extend shelf-life or improve flavor of foods. Their ability to enhance shelf-life results from the presence of ingredients which are antimicrobial in nature. Some of these substances are also known to contribute to the self-defense of plants against infectious organisms (Deans and Ritchie, 1987; Kim et al., 2001). In addition they also possess limited antimicrobial activity. The known antiseptic power of spices and herbs is often attributed to a major component present in the oil derived from them (Alzoreky and Nakahara, 2002; Beuchat and Golden, 1989; Zaika, 1988).

Hong et al. (2000) developed antimicrobial packaging films containing propolis extract, chitosan polymer and oligomer, or clove extract as natural compounds. LDPE resins and each natural antimicrobial compound was mixed and extruded at 115 °C to yield master batch pellets. The concentration of the natural compounds in the pellets was 20% w/w. These pellets were again mixed with LDPE resins at a ratio of 1:3. The mixture was extruded using a single screw extruder. The melting and compression zones were maintained at 120-125 °C. The antimicrobials were present at 5% (w/w) in the final film. The film containing clove extract was brown in color while the other films were white to turbid in appearance. The films were effective against *Lactobacillus plantarum* and *Fusarium oxysporum*, but not against *Escherichia coli* and *Saccharomyces cerevisiae*. Films containing chitosan or clove extract were able to reduce *L. plantarum* counts by about 2-2.5 log after 16 h at 30 °C. Films containing propolis extract showed weak antimicrobial activity against *S. cerevisiae*, but were highly effective against *F. oxysporum*.

Allyl isothiocyanate (AIT), a major component in mustard oil, has been shown to have antimicrobial activity against bacteria, yeasts and molds. One µl of AIT prevented growth of *Aspergillus flavus, Penicillium commune, P. roqueforti and P. solitum* (Neilsen and Rios, 2000). Nadarajah et al. (2002) evaluated the antimicrobial activity of AIT in ground beef against *E. coli* O157:H7. Filter paper saturated with AIT was inserted in oxygen impermeable packaging bags containing ground beef inoculated with about 3 or 6 log cfu *E. coli* O157:H7 per g meat. The bags were flushed with nitrogen and then heat-sealed. Vaporized AIT at 474 ppm was able to reduce number of *E. coli* from an initial level of 6.2 log to 2.5 log cfu/g and to below the detection limit from an initial level of 3.6 log by 21 d storage at 4 °C. It was also observed that cooking the ground beef evaporated residual AIT and there was no adverse effect on the sensory properties of ground beef after cooking.

Grapefruit seed extract (GFSE) has been used in the development of multilayer antimicrobial packaging film by the solution coating method (Ha et al., 2001). GFSE was mixed with a 40% (w/v) polyamide solution and the final concentration was adjusted to 0.5% to 1% (w/v). The solution was then spread on LDPE film and dried at room temperature for 24 h. The film was antimicrobial against *E. coli*, *S. aureus*, *Bacillus cereus*, *Bacillus subtilis*, *S. cerevisiae* and *Micrococcus flavus* when evaluated using an agar diffusion method. When tested in ground beef, the antimicrobial film suppressed the growth of aerobic and coliform bacteria. It extended the period for total aerobic numbers of bacteria to reach the same level as the control (7 log CFU/ml) by 5 d at 3 °C. However there was no significant difference between the two concentrations of GFSE. The change in pH of the beef was less when packaged with films containing GFSE, compared to the control samples.

In another study involving GFSE, Cha et al. (2002) developed sodium-alginate and κ-carrageenan based edible antimicrobial packaging films. Na-alginate film containing GFSE (0.1% w/w) showed strong antimicrobial activity against *Micrococcus luteus*, giving an inhibition zone of 2-5 mm when evaluated by an agar diffusion method. κ-carrageenan film containing the same amount of GFSE was highly effective against *M. luteus*, *L. innocua*, *E. coli* and *S. aureus*.

2.7.6 Use of other natural antimicrobial agents in antimicrobial films

Chitosan is a linear polymer prepared by deacetylation of chitin. Chitin is one of the most abundant polymers present in living organisms such as crustaceans, insects, and fungi (Coma et al., 2002). Chitosan is inexpensive, inert, hydrophilic, biocompatible, can be used for immobilization of enzymes and is often used in medical and biomedical applications (Cetinus and Oztop, 2003; Chen et al., 2003). It has some potential for use in many food applications such as a gelling agent depending upon its degree of deacetylation (Chen et al., 2003). Although hydrophilic, chitosan shows poor solubility in water and the need for use of organic solvents has restricted its widespread application in the food industry. However its solubility characteristics can be improved by carboxymethylation. This process yields carboxymethyl chitosan which is soluble in water (Chen et al., 2003).

Kittur at al. (2003) reported that low molecular weight chitosans with an average molecular weight of 5000-20000 Da had superior biological activities. Fractions with molecular weight of 5000-10000 Da have shown antimicrobial activity against *E. coli*, *E. coli* O157:H7, *S.* Typhi, *Pseudomonas aeruginosa*, *M. luteus*, *S. aureus*, *Bacillus*

subtilis, and lactic acid bacteria including *Lactobacillus bulgaricus*, *L. casei* and *Streptococcus faecalis* (Jeon et al., 2001). Both Gram-positive and Gram-negative bacteria were inhibited by antimicrobial LDPE film with incorporated chitosan at >1.43% (Park et al., 2002). When 8% (v/v) chitosan polymer solution was incorporated directly into agar inoculated with *L. monocytogenes*, it resulted in a complete inhibition of the test organism for 8 d. This indicates that the use of chitosan as a food additive could significantly reduce *L. monocytogenes* development. When the films were evaluated for their effectiveness in cheese against *L. innocua*, the organisms were inhibited below the detection limit at the end of 5 d at 37 °C. Thus, chitosan may be used as an effective natural antimicrobial agent in foods (Coma et al., 2002).

Another important natural antimicrobial agent is lysozyme. It is present in tears, milk, saliva, and eggs. Appendini and Hotchkiss (1997) immobilized egg white lysozyme on polyvinyl alcohol, nylon and cellulose triacetate (CTA). The most effective film was CTA with incorporated lysozyme. With an increase in the amount of lysozyme added, the activity of the resultant film also increased. The highest activity was obtained when lysozyme was added at a level of 150-250 mg/g of polymer. In addition to the amount of lysozyme added, film thickness also had an influence on the activity of the film. With an increase in the film thickness from 20 to 60 μm, there was an increase in the activity from 0.6 to 1.4 units/cm^2 film. CTA films containing lysozyme reduced the viable numbers of *M. lysodeikticus* from 8 to 1 log cfu/ml after 12 h incubation at 30 °C when tested in Tryptic Soy Broth at a film surface area to TSB volume ratio of 1:100.

Chapter 3

MATERIALS AND METHODS

3.1 Manufacture of direct acidified low and high fat mozzarella cheese

Direct acidified full fat and low fat mozzarella cheese was made by following a modified procedure based on a method outlined by Kosikowski and Mistri (1997). Full fat cheese was made from fresh pasteurized whole milk (3.25% fat). Low fat cheese was made from fresh pasteurized skimmed milk with a fat content standardized to 1.6%. Fresh cream with 40% fat was used for standardization of skimmed milk. Milk and cream were purchased from the local market and were received at 4 °C. Once the fat content was standardized, the milk was repasteurized using an HTST pasteurization system (APV plate pasteurizer, Toronto, ON) with a time-temperature combination of 72 °C for 16 sec. After pasteurization, milk was cooled to 4°C and was transferred to a 200 L cheese vat that had been cleaned and sanitized using a 200 ppm solution of sodium hypochlorite sanitizer (Ecolab XY-12, Ecolab Ltd., Mississauga, ON). At a time 120 L of pasteurized milk was taken for cheese making. The pasteurized and cooled milk was slowly warmed up to 25 °C using hot water circulated in the jacket of the cheese vat. Once the desired temperature had been reached, the pH of milk was adjusted from its initial pH of 6.6 to a final pH of 5.8 with 5% (w/w) citric acid (Fisher Scientific, Fair Lawn, NJ). After adjusting the pH of milk, 12 ml of double strength rennet (Rhodia Inc., Dairy Business, Madison, WI) was added. The rennet was diluted with 80 parts of pasteurized water before addition to the milk. After mixing rennet and milk for about 30 sec, the milk was allowed to stand undisturbed for 30 min to facilitate curd formation. After 30 min, the strength of the curd was determined by inserting a dairy knife and

lifting the cut surface. Once the desired strength of curd was obtained, it was cut using cheese knives. The curd cubes (1 cm^3) were allowed to stand in whey for about 5 min.

The curd was then cooked at 40 °C for 30 min. After cooking was completed, the whey was drained and the curd was packed in the cheese vat to facilitate further removal of whey. Upon removal of whey, salt at 2% w/w was added to the curd. After thorough mixing of salt, the curd was filled into one 40 lb cheese hoop. The filled hoop was covered with a lid and pressed in a horizontal cheese press (Model- 18 FT, DeLaval Co. Ltd., Peterborough, Canada) at 35 psi for about 5 h. After pressing, the cheese was cut into 500 g blocks and vacuum packed in Deli*1 bags (Winpak, Winnipeg, MB) using a vacuum packaging machine (Model GM-2000, Bizerba Canada Inc., Mississauga, ON).

3.1.1 Determination of moisture content in cheese

An AOAC (1990) method was used for determination of the moisture content of cheese. About 1 to 2 g of shredded cheese prepared with a power operated food processor (Moulinex Jeanette, Concord, ON) was weighed into previously cleaned and dried flat bottom aluminum moisture dishes. They were loosely covered with a lid and placed in a vacuum oven at 100 °C. The samples were allowed to remain inside the oven until a constant weight was obtained. After 4 h, the dishes were tightly covered, cooled to room temperature and weighed.

3.1.2 Determination of fat content of the cheese

Fat content of both low and high fat cheese was determined by following the Babcock method (Case et al., 1985). Exactly 9 g of shredded cheese was placed in a babcock bottle. To this cheese, 10 ml of hot water (60 °C) was added and the contents mixed to create a suspension of cheese in water. This was followed by addition of 15 ml of concentrated sulfuric acid (Sp. Gr. 1.82-1.83, Fisher Scientific) in 3 additions of 5 ml. The addition of acid was completed in 20 sec. The contents were thoroughly mixed to digest the cheese particles by placing the bottles on a mechanical shaker for about 5 min. Following shaking, the bottles were counterbalanced in a centrifuge (The Jalco Motor Co., Union City, IN) and were centrifuged for 5 min. To these bottles a sufficient amount of hot water (60 °C) was added such that the final level of the content reached the neck of the bottle. The bottles were again centrifuged for 2 min. After centrifugation, the bottles were transferred into a water bath and held at 55 °C for 5 min. Immediately following the fat column was measured and the fat content was expressed as % fat of cheese.

3.1.3 Determination of cheese pH

The pH of cheese was determined as per the method outlined by Case et al. (1985). Fifty g thawed cheese was shredded using the food processor. The shredded cheese was tightly packed in a 100 ml capacity glass beaker and maintained at 25 °C.

The pH meter (Accumet, Model 910, Fisher Sci., USA) was standardized using buffers at pH 4 and pH 7. After standardization, the pH meter probe was inserted into

closely packed cheese and was allowed to stay in contact with the cheese for 45 sec until the pH meter gave a stable reading.

3.2 Standardization of Gas Chromatograph

One hundred or 200 ppm rosemary or thyme oil were weighed separately into a 180 ml glass jar which was closed with a metal lid. A 2 mm diameter hole was drilled through the metal lid. The hole was covered with a Teflon-fluorocarbon-resin/silicone septum (Fisher Scientific, Whitby, ON) and sealed with silicon glue to provide an air tight seal. The jars were allowed to stand at 37 °C for 2 h. Once the liquid content of the jar was converted into the gaseous phase, a 0.3 ml sample was withdrawn from jars by inserting the needle of a gas tight syringe (Series A-2, Valco Precision Sampling Syringe, Sigma-Aldrich, Oakville, ON) through the septum and the syringe contents were immediately inserted into the Gas Chromatograph (GC). The area under curve obtained from the chromatograph was plotted against the essential oil concentration to prepare standard curves for each oil.

3.2.1 Analysis of rosemary and thyme oil in the headspace

At intervals a gaseous sample of 0.3 cm^3 was withdrawn from jars prepared in the previous section by inserting the needle of a gas tight syringe through the septum and was immediately inserted into the GC.

The headspace concentration of gaseous rosemary and thyme oil was determined using a Model 560 GC (Tracor Inc, Tracor Lane Austin, TX). The GC was equipped

with a flame ionization detector and a column measuring 15 m with 0.25 mm internal diameter and a wall thickness of 0.25 μm (J&W DB5MS, Mandel Scientific Co. Ltd., Guelph, ON).

The injector port and flame ionization detector were maintained at 240 °C. Following sample injection the column temperature was increased from the initial 45 °C to 220 °C at a rate of 2 °C/min. Helium was used as a carrier gas and hydrogen was used to facilitate functioning of the flame ionization detector.

3.3 *Listeria monocytogenes* stock culture preservation

The five strains of *L. monocytogenes* serotype 1 used in this experiment were C716, C717, C718, C719 and C720. The strains were kindly donated by Dr M.W. Griffiths, University of Guelph, Guelph, ON. A loopful of each strain was streaked on solidified Trypticase Soy Agar (TSA, BBL, Becton Dickinson, Sparks, MD) in Petri dishes to check purity of the cultures. A characteristic *L. monocytogenes* colony was then transferred to sterilized Trypticase Soy Broth (TSB, BBL, Becton Dickinson) and incubated at 35 °C for 24 h. After incubation, 30 ml of each culture was centrifuged at 9,000 rpm for 10 min at 10°C (Sorvall RC-5 refrigerated centrifuge, Du Pont, Newtown, CT). After centrifugation, the pellets were separated from the supernatant. The pellets were washed with 30 ml of 0.1% peptone water (Sigma, St. Louis, MO) and re-centrifuged at 9,000 rpm for 10 min at 10°C. The supernatant was discarded and the pellets were held at 4 °C and re-suspended in 1.5 ml of sterilized TSB. Following the addition of TSB, 0.75 ml of filter-sterilized glycerol (50% v/v) was added. The content was treated by vortex mixing for 1 min. After thorough mixing, about 0.3 ml of the cell

suspension was placed in 1.2 ml cryogenic vials (Corning Inc., NY). The vials were maintained at -80 °C until used.

3.4 *L. monocytogenes* strain preparation

Strains preserved at -80 °C were activated by two sequential transfers in 10 ml portions of TSB followed by incubation at 35 °C for 24 h each. Thirty ml of each strain was transferred to separate centrifuge bottles and centrifuged at 9,000 rpm for 10 min at 10°C. The supernatants were removed and the pellets were re-suspended in 30 ml of 0.1% peptone water and again centrifuged at 9,000 rpm for 10 min at 10°C. After washing and centrifugation, the cultures were again re-suspended in peptone water, diluted such that a final bacterial cell density of 8 log cfu/ml was obtained by adjusting optical density (OD) at 600 nm to 0.4 in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech., Baie d'Urfe, QC).

3.5 *L. monocytogenes* strain standard curve preparation

About 30 ml of an overnight culture of each strain was transferred to a separate centrifuge bottle and centrifuged at 9000 rpm for 10 min at 10 °C. After centrifugation the supernatant was removed and the pellets were re-suspended in 30 ml of 0.1% peptone water. The contents were centrifuged again using the same conditions. After removing the supernatant the pellets were allowed to stand briefly at 4 °C and were mixed with 0.1% peptone water by vortexing. The OD of the resultant mixture was adjusted to 0.2, 0.4, 0.6, 0.8 at 600 nm and the corresponding solution was further

serially diluted using 0.1% peptone water. Appropriately diluted sample was then surface plated on prepoured solidified TSA plates. The plates were incubated at 35 °C for 48 h. Colonies were then counted and multiplied by the dilution factor to obtain numbers of cells/ml. The resultant bacterial numbers for a particular OD was plotted. The standard curve obtained was used for further experiments to adjust the bacterial cell density.

3.6 Antilisterial activity of gaseous rosemary and thyme oil

Separate overnight cultures of the five strains of *L. monocytogenes* were centrifuged to yield pellets. The pellets were washed with 0.1% peptone water and then again centrifuged at 9000 rpm for 10 min at 10 °C. The bacterial cell density was adjusted to 8 log cfu/ml at 0.4 OD at 600 nm. A cocktail of five strains was made by combining 1 ml of each strain. The cocktail was serially diluted and 0.1 ml of appropriately diluted sample was plated on the surface of 15 ml prepoured solidified TSA on the bottom of each glass jar such that the final bacterial level on the agar surface became 3 log cfu/cm^2. After 5 min, rosemary oil at 0.1, 0.2, 0.4, 0.5 % (v/v) or thyme oil (0.05, 0.075, 0.1%, v/v) (Aldrich Chemical, Milwaukee, WI) were separately placed in a disposable polystyrene beaker (Fisher Scientific, Whitby, ON) which was then placed on the inside of the jar lid. The glass jar containing inoculated agar was then closed in an inverted position. The jar assembly was then transferred to 10 °C. At 24 h intervals the headspace concentration of oils was measured after sampling by syringe as previously described and by immediate insertion of the sample into the GC. To check the lethality

of gaseous antimicrobials the lids containing the antimicrobials were removed from the jars. The jars were covered with aluminum foil and were incubated at 35 °C for 4 d.

3.7 Inoculation of cheese

A cocktail of five *L. monocytogenes* strains was made by mixing 1 ml of the cell suspension of each strain prepared as described above. After thorough mixing, 1 ml of the cocktail was serially diluted by its addition to 9 ml of 0.1% sterile peptone water and the final bacterial level was adjusted to 5 log cfu/ml by serial dilution using a standard curve.

Previously vacuum packed and frozen cheese was thawed overnight at 4°C. Exactly 1 kg cheese was weighed into a sterile aluminum tray (44×34 cm^2). The cheese was shredded using the food processor. Shredded cheese (2×0.5 cm^2) was then inoculated with 10 ml of the 5 strain cocktail of *L. monocytogenes*. The cocktail was evenly dripped on the surface of the shredded cheese and mixed by hand for 5 min. This gave an initial population of *L. monocytogenes* in the shredded cheese of approximately 3 log cfu/g. The inoculated cheese was then again shredded to facilitate even mixing and distribution of *L. monocytogenes*.

3.8 Treatment of cheese with rosemary and thyme oil

For each treatment, 1 kg shredded and inoculated cheese was placed in a sterilized aluminum tray. Then undiluted rosemary or thyme oil was sprinkled using a one ml capacity pipetter separately as well as together on the surface of shredded cheese

such that their final concentration in cheese became 1% (w/w). Oil and cheese were mixed together by hand for 5 min. For microbial analysis, 11 g of inoculated cheese was weighed in Deli*1 bags. These bags were flushed with 100% nitrogen and then heat sealed. For measurement of pH, 25 g of cheese was weighed into Del*1 bags, flushed with 100% nitrogen and then heat sealed. All bags were stored at 4 or 10°C for up to 20 or 15 days, respectively.

3.9 Microbial analysis of inoculated cheese

Triplicate bags were taken every 3 d at 10 °C and at 4 d intervals for 4°C stored cheese samples. To each bag containing 11 g cheese, 99 ml 0.1% sterile peptone water was added. The mixture was then homogenized in the stomacher (Model 400, Seward, London) for 30 sec. Samples were then serially diluted using 0.1% peptone water and 0.1 ml of the appropriately diluted sample was plated using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD) on TSA for enumeration of total aerobic bacteria (TAB). Lactic acid bacteria (LAB) were determined by plating samples on MRS lactobacilli agar (BBL, Becton Dickinson).

Listeria selective agar medium (Oxford formula, Oxoid Ltd., Basingstoke, Hants, England) supplemented with Listeria selective supplement (Oxford formula, Oxoid Ltd) was used for determination of viable *L. monocytogenes*. Preliminary experiments done in our lab showed that the commercially available Listeria selective agar with selective supplement supported the growth of the natural cheese flora along with *L. monocytogenes*. In order to make the medium strictly selective for *L. monocytogenes*, acriflavin (Sigma-Aldrich Co., St. Louis, MO) was added before sterilization to yield a

final concentration of 10 mg/L. *L. monocytogenes* numbers were determined by plating 0.1 ml of sample on Listeria selective agar containing acriflavin. This concentration of acriflavin did not affect recovery of the test strains of *L. monocytogenes*. The Listera selective agar plates were incubated anaerobically using Gas Pak Jars (BBL, Becton-Dickinson) with the Gas Pak Plus anaerobic system containing a palladium catalyst at 35 °C for 36 h. The TSA plates were incubated at 37 °C for 24 h. The MRS plates were incubated anaerobically in an anaerobic incubator (model 3640-6, National Appliance, Portland, OR). The anaerobic condition was generated by flushing the incubator with 30% CO_2/70% N_2. The plates were incubated for 3 d at 22 °C. All colonies were counted on a Quebec colony counter. On each sampling day, 3 samples were taken from each treatment and were plated in duplicate.

3.10 Evaluation of bacterial resistance towards antimicrobial plant oils in cheese

Resistance of all five strains of *L. monocytogenes* towards the antimicrobial plant oils was determined in this set of experiments. Each of five strains was grown separately overnight, centrifuged to harvest the bacterial cells and their density was adjusted as mentioned before to yield 8 log cfu/ml. About 100 g of thawed cheese was weighed in a sterile aluminum tray and shredded using the food processor. The cheese was inoculated with each strain separately by sprinkling 1 ml of the OD adjusted bacterial inoculum. This was followed by mixing the cheese and *L. monocytogenes* by hand for 5 min. The cheese was allowed to stand for another five min to facilitate adsorption of bacteria on to the surface of cheese. The inoculated cheese was again shredded to facilitate even distribution and mixing of *L. monocytogenes* in the cheese. Rosemary and thyme oil

(each at 1% w/w) were added together and the content was again mixed thoroughly by hand. After mixing, 11 g of inoculated cheese containing both plant oils was weighed into each Deli*1 bag. The bags were flushed with nitrogen and heat sealed. The samples were stored at 4 or 10 °C for 5 d. Samples were taken every 2 d and analyzed for *L. monocytogenes* level by plating 0.1 ml of appropriately diluted sample on Listeria selective agar containing acriflavin. The plates were incubated anaerobically at 35 °C for 36 h as mentioned before.

3.11 Antiliserial activity of sodium diacetate along with rosemary and thyme oils

Sodium diacetate (Aldrich Chemical Co.) was used as an antilisterial agent along with rosemary and thyme oil in this set of experiments. One kg of shredded cheese was weighed in a sterile aluminum tray. The cheese was inoculated with a cocktail of only four strains of *L. monocytogenes* (C716, C718, C719, C720) using methods previously described. A 20% (w/v) sodium diacetate solution was prepared by dissolving 2 g sodium diacetate in 10 ml of sterile distilled water. Then 1 ml of this solution was evenly dripped on the surface of the shredded cheese. Following hand mixing for 5 min, rosemary and thyme oils were added to the inoculated cheese to achieve final concentrations of 1% (w/w). Following the addition of rosemary and thyme oil, the cheese was thoroughly mixed by hand. Eleven g of inoculated cheese containing the antimicrobials was weighed into Deli*1 bags. The bags were flushed with nitrogen, heat sealed and stored at 4 and 10 °C for 20 and 15 d, respectively. Samples were taken every 3 and 4 d for cheese stored at 10 and 4 °C, respectively, and were analyzed for *L. monocytogenes*, LAB and TAB as previously described.

3.12 Use of sachets as an antimicrobial reservoir

3.12.1 Preparation of microcellular foam (MCF) starch

Microcellular foams (MCF) were made following a modified procedure that was based on a method outlined by Buttery et al. (1999). For each test, 250 ml of 5% (w/w) pea starch solution (Parrheim Foods Co., Portage-La-Prairie, MB) was prepared in distilled water. The solution was heated to its boiling point with continuous stirring and held at this temperature for 15 min. After boiling, the solution was cooled to 35 °C. The gelatinized pea starch solution was then added to about 500 ml absolute ethanol with continuous stirring to facilitate bead formation. The mixture was allowed to equilibrate at room temperature for an hour. After equilibration, the starch beads were separated from ethanol by centrifugation at 4000 rpm for 10 min at 10 °C. The separated starch beads were then spread on an aluminum tray and dried in an oven at 80 °C.

3.12.2 Delayed release of rosemary and thyme oil from MCF

Undiluted rosemary or thyme oil was mixed at 1% and 10% (w/w) with 500 mg MCF. The MCF and oil mixture was then placed in a 180 ml lidded glass jar. The lids were prepared to facilitate headspace sampling as previously described by drilling a 2 mm hole which was then covered with a Teflon-fluorocarbon-resin/silicon septum and sealed with silicon glue. Along with the mixture of MCF and rosemary or thyme oil, 2 ml distilled water was also added. The whole assembly was allowed to stand at room temperature (25 °C) for 0.5 h. Then 0.3 ml of headspace sample was drawn from the jar by inserting a syringe needle and the sample was immediately injected into the GC. The

jars containing undiluted rosemary or thyme oil mixed with MCF at 1 or 10% (w/w) served as a control.

3.12.3 Preparation of sachets containing antimicrobials

A 4 cm^2 sachet base was made using a square of Whatman # 4 filter paper (Springfield Mill, Maidstone, Kent, England). While making the sachet, two sides of the filter paper were folded towards each other followed by folding of a third side. These folds were sealed with masking tape. The last side of the sachet was kept open. The MCF along with 10% (w/w) rosemary and thyme oil separately or together was added through the open side. After adding the MCF and rosemary or thyme oil mixture, the sachet was immediately sealed with masking tape.

3.12.4 Treatment of inoculated cheese with antimicrobials contained in sachets

The MCF sachets were inserted in Deli*1 bags containing 11 g cheese inoculated with 3 log cfu/g *L. monocytogenes*. The bags were flushed with 100 % nitrogen and then heat sealed. The sealed bags were stored at 10 °C for 15 d. Samples were taken at 3 d intervals and analyzed for TAB, LAB and *L. monocytogenes* numbers. In addition, the headspace concentration of rosemary and thyme oil was also measured by GC as previously described.

3.13 Statistical analysis

All microbiological analysis were based on six replicates. Data were analyzed using Statistical Analysis System software program, version 8.1 (SAS Institute, Inc., NC). Microbiological data were analyzed using general linear model (GLM) procedure and Tukey's t-test for examination of significant differences ($p<0.05$) at each storage interval for individual treatments.

Chapter 4

RESULTS AND DISCUSSION

4.1 Preparation of direct acidified low and full-fat mozzarella cheese

Direct acidified low and full-fat mozzarella cheese was made as per the procedure outlined in section 1.1 and the average composition of the cheeses is given in Table 3.1. Values of fat and moisture for full- and low-fat cheeses manufactured in the pilot plant were similar to those of commercial products. Commercially available mozzarella cheese usually has about 5.3 pH, 52% moisture with a fat level of 25% (Bergamo et al., 2003). The initial fat content of milk used for making cheese as well as the losses that might occur during processing can play an important role in determining the overall fat content of cheese.

Table 4.1. Average composition of direct acidified low fat and full fat mozzarella cheese

Component of cheese	Full fat cheese	Low fat cheese
Fat (%)	23	15
Moisture (%)	53	50
pH	5.8	5.8

4.2 Antimicrobial activity of gaseous rosemary and thyme oil

The antimicrobial activity of rosemary and thyme oil was examined separately against a cocktail of five different strains of *L. monocytogenes* at 10°C. Rosemary oil was analyzed at 0.1, 0.2, 0.4, 0.5% (v/v). Rosemary oil at 0.5% v/v (1.3 mg/L in vapor) was bactericidal after 48 h storage at 10°C resulting in elimination of inoculated bacteria (Table 4.2). When thyme oil was analyzed at 0.05, 0.075 and 0.1% v/v levels, 0.1% (1.5 mg/L in vapor) proved to be bactericidal under the same conditions.

Considerable effort has been made to evaluate these two oils in bacterial media against *L. monocytogenes* as well as against other bacteria and yeast and molds. Smith-Palmer et al. (1998) determined antilisterial activity of thyme oil at 4 and 35 °C. A concentration of 0.03 % v/v was bactericidal at both temperatures. Rosemary oil at 0.1% (v/v) was required to exert a bactericidal effect. Inouye et al. (2001) evaluated the antimicrobial activity of 14 essential oils in their vapor phase against five pathogens. Thyme oil was required at 3.1 to 12.5 mg/L air in order to inhibit the growth of all five pathogens. Rosemary oil at $\geq$ 50 mg/L air was required in order to exert the same effect.

In another similar study, Inouye et al. (2003) compared antimicrobial activity of 4 different essential oils in their vapor form and by solution contact. There was a decrease in the concentration required to inhibit the test organisms when the oils were analyzed in the gaseous form. Thyme oil inhibited the growth of *S. aureus* and *E. coli* at 12.5 μg/cm^3 in air, whereas concentrations of 200 and 400 μg/ml against the same pathogens were required, respectively, to achieve the same effect when analyzed by agar dilution. They concluded that the antimicrobial activity of the essential oils was improved by direct contact between vapor and bacterial cells. Also the oil vapor adsorbed onto the agar surface can contribute to overall antimicrobial activity. The latter

effect was described in a study conducted by Inouye et al. (2001). The authors analyzed 7 essential oils for their antifungal activity against *Aspergillus fumigatus*. Thyme oil at 6.3 µg/ml air retarded the elongation of hypha and elongation was completely inhibited in the presence of 63 µg thyme oil/ml air.

Table 4.2. Concentraion dependent lethality[1] of Rosemary and Thyme oils against a 5 strain cocktail of *L. monocytogenes*.

Essential oil	Concentration (% v/v)	Effect[2]
Rosemary	0.1	-
	0.2	-
	0.4	-
	0.5	+
Thyme	0.05	-
	0.075	-
	0.1	+

[1] Lethality measured by absence of colony development on inoculated agar exposed to spice oil vapors for 48 h at 10°C.
[2] - No effect, + Bactericidal

4.3 Antimicrobial activity of rosemary and thyme oil against a five-strain cocktail of *L. monocytogenes*, TAC and LAB in full fat mozzarella cheese at 4 and 10°C.

4.3.1 *L. monocytogenes*

The antilisterial activity of rosemary and thyme oils against *L. monocytogenes* in full fat (23%) mozzarella cheese at 4 and 10°C is shown in Figure 4.1. The initial inoculation level of *L. monocytogenes* in the cheese was about 3.7 $\log_{10}$ cfu/g. Growth was observed in both inoculated untreated as well as inoculated treated cheese containing rosemary and thyme oil added either separately or together. At the end of 15 d storage at both 4°C and 10°C, *L. monocytogenes* numbers in the control untreated cheese increased to 7.6 $\log_{10}$ cfu/g. Rosemary oil at 1% (w/w) was not effective against *L. monocytogenes*, although *L. monocytogenes* numbers in treated cheese stored at 4°C were significantly ($p<0.05$) lower at days 4 and 12. The level of *L. monocytogenes* in cheese samples treated with rosemary oil was the same as in untreated cheese at 15 d. The presence of 1% (w/w) thyme oil in the cheese significantly ($p<0.05$) reduced numbers of *L. monocytogenes* from day 3 onwards and this difference was maintained during subsequent storage. A difference of 0.8 $\log_{10}$ cfu/g between control and treated samples was observed by the end of storage. However, when both oils were added together, the difference between treated and untreated samples towards the end of storage was 1.5 $\log_{10}$ cfu/g. Similar results were obtained during storage at 10°C where the reduction in *L. monocytogenes* numbers was 1.4 $\log_{10}$ cfu/g at the end of storage. Thus, at both temperatures the combination of rosemary and thyme oil resulted in an additive or possibly synergistic effect against *L. monocytogenes*. At the higher storage

temperature *L. monocytogenes* initially grew faster than at 4°C but at both temperatures, regardless of treatment, *L. monocytogenes* did not grow beyond 7.7 $\log_{10}$ cfu/g.

The average fat content of cheese used in these experiments was 23%. Smith-Palmer et al. (2001) evaluated effect of thyme oil against *L. monocytogenes* in Kraft Philadelphia full fat (30%) soft cheese. The authors evaluated thyme oil at 3 different concentrations (0.1, 0.5, 1% v/v). The presence of 0.1% thyme oil did not have any significant effect on *L. monocytogenes* and the numbers were similar to that of the control sample at the end of 14 d storage. Treatment of cheese with 0.5% (v/v) thyme oil resulted in a bacteriostatic effect. When thyme oil was tested at 1% (v/v), it resulted in a 1 $\log_{10}$ cfu/ml reduction of *L. monocytogenes* by the end of 14 d storage. However, when they tested thyme oil against *S.* Enteritidis under same conditions, the oil at 1% v/v failed to exert any antimicrobial activity throughout storage. The antimicrobial activity of the oil was examined in cheese diluted 1 in 10 with phosphate buffered saline. So the actual antimicrobial activity of oil in undiluted cheeses might be different.

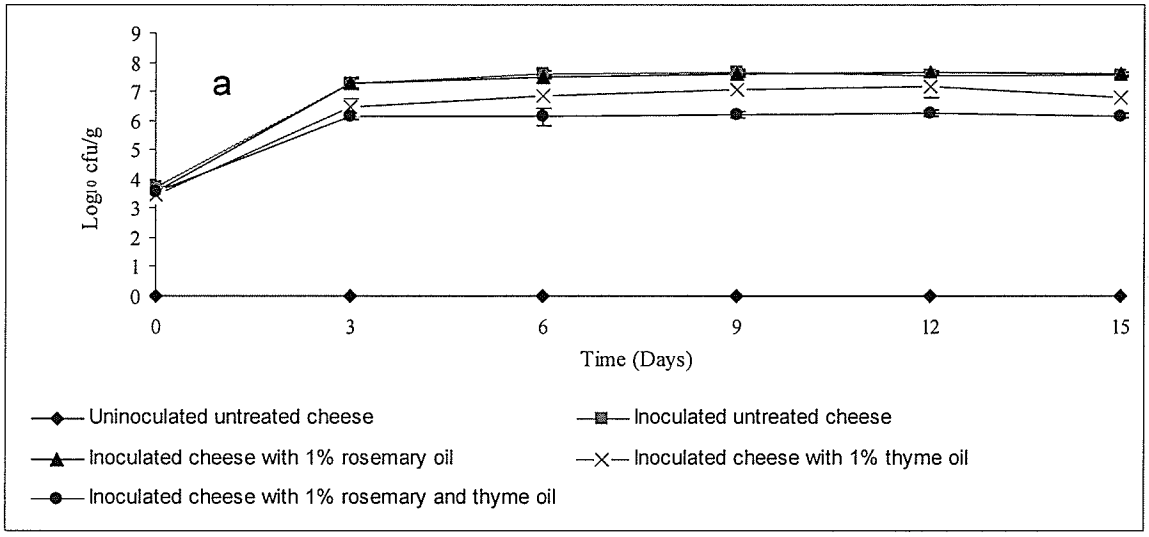
In another similar study reported by Hao et al. (1998), thyme oil at 2% (v/w) failed to produce antilisterial activity in chicken breast at 5°C. By the end of 14 d storage, the numbers of *L. monocytogenes* in control and treated samples were not significantly different at 4.8 and 5.0 $\log_{10}$ cfu/g. When they evaluated the antilisterial activity of thyme oil (2%) at 15°C, again, no significant difference was observed. *L. monocytogenes* numbers increased to 7.8 and 7.7 $\log_{10}$ cfu/g from initial numbers of 4.9 and 4.7 $\log_{10}$ cfu/g in control and treated samples, respectively, by the end of 14 d.

Pandit and Shelef (1994) evaluated the antilisterial activity of rosemary spice and oil in a commercially obtained pork liver sausage. The presence of rosemary spice at 0.5% (w/w) retarded the growth of *L. monocytogenes* over a period of 28 d at 5°C,

producing a difference of 1 $\log_{10}$ cfu/g. The numbers of *L. monocytogenes* were >9 $\log_{10}$ cfu/g in control samples by the end of storage. The presence of 1% rosemary oil suppressed the growth of *L. monocytogenes* and a difference of 1-2 $\log_{10}$ cfu/g was observed between control and treated samples. The authors also used 5% encapsulated rosemary oil with 18% oil content. Use of encapsulated rosemary oil retarded the growth of *L. monocytogenes* and the numbers increased from an initial level of 3.5 $\log_{10}$ cfu/g to about 4.2 $\log_{10}$ cfu/g, producing a difference of 4.8 $\log_{10}$ cfu/g compared to the control by the end of 28 d at 5°C. The authors also reported the antilisterial activity of rosemary spice and oil when tested in agar media. Use of rosemary spice at 0.5% (w/v) in BHI agar was listericidal and the organism was eliminated after 48 h at 35°C. When rosemary oil was tested at 10 µl/100 ml in BHI broth, the oil produced a listeriostatic effect after 24 h at 35°C. However, the inhibition was temporary and cells were able to recover after 48 h of incubation. They concluded that there was a need for higher concentrations of antimicrobials when tested in food systems. They also observed a decrease in the antilisterial activity of rosemary oil and spice during storage. They observed that the stability of spice and oil is an important factor in determining the overall antimicrobial activity.

The ineffectiveness of rosemary oil in the present study may be due to its interaction with the fat phase of cheese. The inverse relationship between fat content of product and the antimicrobial activity of spices and their oils is well documented (Cutter 2000; Gill et al., 2002; Singh et al., 2003; Smith-Palmer et al., 2001). Farbood et al. (1976) suggested that the lower water activity together with higher lipid and solid content of food systems than in laboratory media might result in greater absorption of the spice extract in the fat, reducing its concentration in the aqueous phase and

decreasing its effectiveness in foods. Also coating of bacterial cells with lipid can reduce the antimicrobial activity (Gill et al., 2002). Apart from lipid content of foods, interaction between food proteins and antimicrobial oils can also lead to loss of antimicrobial activity. This was evident in a study reported by Juven et al. (1994). The authors reported loss of thymol antimicrobial activity, a major component of thyme oil, in the presence of bovine serum albumin.



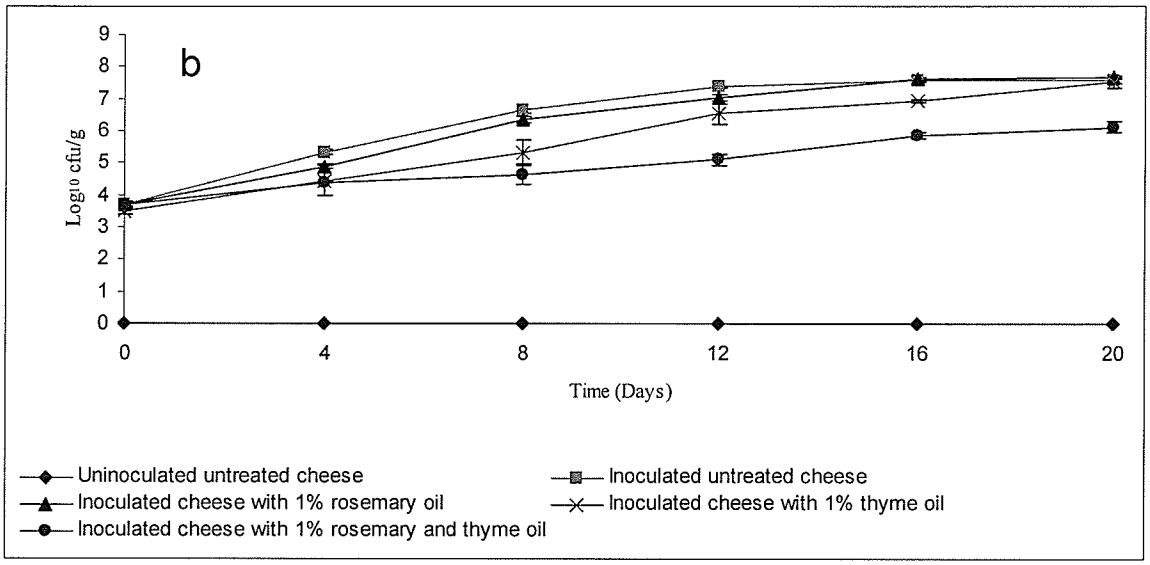


Figure 4.1. Antimicrobial activity of rosemary and thyme oil (1% w/w each) either separately or in combination against a cocktail of 5 strains of *L. monocytogenes* in shredded full-fat cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.

4.3.2 TAC and LAB

The total numbers of bacteria (total aerobic count, TAC) and lactic acid bacteria (LAB) increased in all cheese samples stored at 4°C (Figures 4.2, 4.3). TAC in uninoculated untreated control cheese increased from 6.3 to 7.1 $\log_{10}$ cfu/g by the end of 20 d storage, whereas numbers increased from 6.6 to 7.5 $\log_{10}$ cfu/g in inoculated untreated cheese samples. The presence of rosemary or thyme oil did not have any inhibitory effect on the TAC. The numbers increased from 6.2 and 5.7 $\log_{10}$ cfu/g to 7.7 and 7.8 $\log_{10}$ cfu/g in the presence of rosemary or thyme oil, respectively. However, when oils were added together, they were able to restrict the growth of TAC by about 1 $\log_{10}$ cfu/g and the numbers were fairly stable during 20 d storage. At 20 d storage, TAC at 6.4 $\log_{10}$ cfu/g was significantly lower than the control sample. The LAB increased from 5.9 to 7.6 $\log_{10}$ cfu/g in the control samples. The presence of rosemary and thyme oil also allowed growth of LAB. Their numbers increased from 5.3 and 5.0 to 7.5 and 7.4 $\log_{10}$ cfu/g in the presence of rosemary and thyme oil, respectively. Treatment of cheese with rosemary and thyme oil separately did not produce any significant difference in cell numbers. However, LAB growth was restricted by about 1 $\log_{10}$ cfu/g when both oils were added together. The numbers increased from 6.0 $\log_{10}$ cfu/g on day 0 to 6.3 $\log_{10}$ cfu/g on day 20 and were different ($p<0.05$) from that of untreated inoculated cheese samples.

Larger numbers of both TAC and LAB were found in cheese stored at 10°C. LAB and TAC numbers increased from 6.1 and 6.6 $\log_{10}$ cfu/g to 8.7 and 9.2 $\log_{10}$ cfu/g, respectively, in control untreated cheese samples by 15 d storage. LAB and TAC increased from 5.8 and 6.3 $\log_{10}$ cfu/g to 7.6 and 8.6 $\log_{10}$ cfu/g, respectively, in the presence of rosemary oil and were significantly different from control samples by the

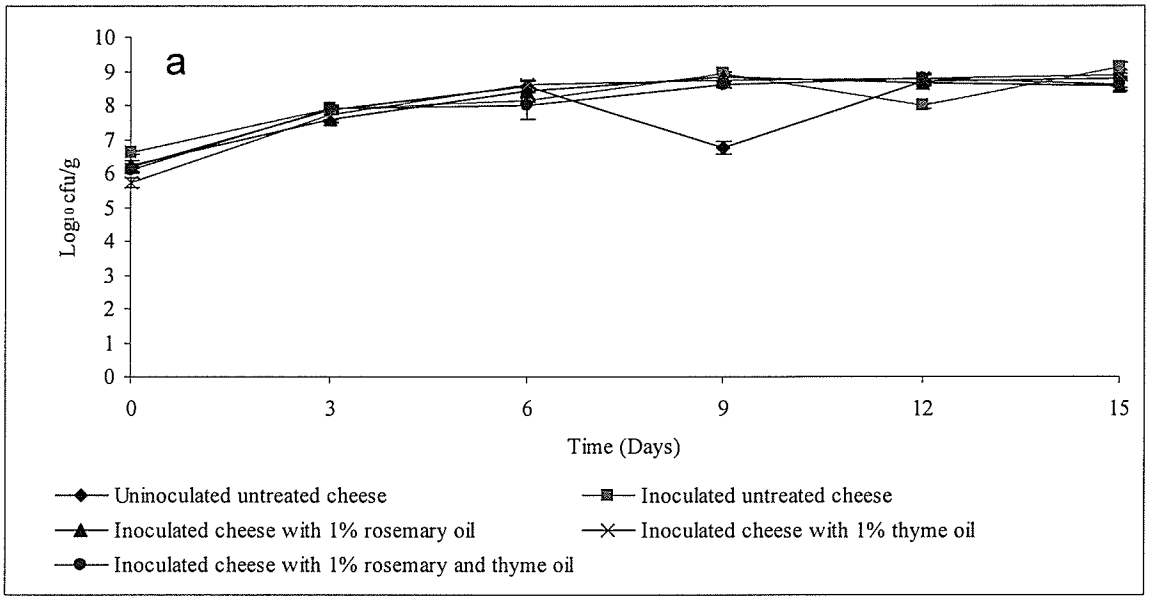
end of storage. Rosemary alone and rosemary plus thyme inhibited TAC by about 0.5 $\log_{10}$ cfu/g, whereas rosemary alone (not thyme or the combined oils) inhibited LAB by 1.0 $\log_{10}$ cfu/g at 15 d. Growth was also observed in the presence of thyme oil and the numbers of LAB and TAC increased from 5.2 and 5.7 $\log_{10}$ cfu/g to 8.8 and 8.9 $\log_{10}$ cfu/g, respectively, by 15 d storage.

The initial pH of cheese was 5.8 and decreased to 5.6 by d 15 at 10°C in controls. Neither rosemary nor thyme oil alone or together affected the pH of treated samples. The average moisture content of the cheese was 52%. This moisture level and pH will allow the growth of LAB and TAC. Similar values were found in samples stored at 4°C.

The greater extents of growth by total bacteria and LAB at 10°C than at 4°C was not unexpected. Sabia et al. (2003) used *Enterococcus casseliflavus* as a natural antagonist in Italian sausage for controlling *L. monocytogenes*. They observed a fall in pH of sausage containing *E. casseliflavus* and *L. monocytogenes* from 5.8 to 5.1 during ripening for 10 d which increased to 5.4 during further storage for 10 d at 4°C. Lactobacilli increased by about 4 $\log_{10}$ cfu/g during first 3 d of drying and then remained at the same level during ripening and storage. Olarte et al. (2002) observed a 0.6-0.8 unit decrease in the pH of fresh goat cheese stored under modified atmosphere at 4°C. The mesophilic count increased quickly to >7 $\log_{10}$ cfu/g after 14 d storage at 4°C in non-inoculated cheese, whereas it reached the same level on day 7 and remained slightly higher during subsequent storage in inoculated cheese samples. This was also observed in the present study. The numbers of TAC in noninoculated cheese samples stored at 4 and 10°C were 7.1 and 8.8 $\log_{10}$ cfu/g, respectively. Whereas the numbers were 7.6 and 9.1 $\log_{10}$ cfu/g, respectively, in inoculated untreated samples stored at 4 and 10°C. The increased bacterial numbers in inoculated cheese samples was probably due to the

presence of *L. monocytogenes*. Also in the present study, the fall in pH was slightly less than that observed in other studies (Olarte et al., 2002; Sabia et al., 2003). Manufacture of direct acidified mozzarella cheese from milk that was pasteurized twice and the use of 5% citric acid to lower the pH of milk from 6.6 to 5.8 might have affected subsequent decreases in the pH of cheese during further storage. Nonetheless, the pH values of 5.5 observed here were not unusual.

Rosemary oil and thyme oil together at 4°C and rosemary oil alone at 10°C reduced the maximum numbers of LAB and TAC by 1 $\log_{10}$ cfu/g in the present study. Campo et al. (2000) reported some antimicrobial activity by rosemary spice extract. A 1% (v/v) rosemary extract did not inhibit *Lactobacillus plantarum* and the minimum lethal concentration required was two to fourfold higher than for *L. monocytogenes*. Holley and Patel (2005) reported that among Gram-positive bacteria, LAB are usually quite resistant to plant essential oils. Shelef (1983) also reported that some spices have a stimulatory effect on the LAB resulting in an increase in their growth. This was believed due to the presence of Mn^{+} in the spice materials added. Nes and Skjelkvale (1982) reported that the growth of *L. plantarum* was enhanced in the presence of natural spices than in the presence of their oleoresins. The TAC in the present study included those bacteria that are naturally present in cheese along with *L. monocytogenes* (where inoculated). Some of the bacteria naturally present may have adapted to growth in the cheese environment and be more resistant to challenge by rosemary or thyme oil.



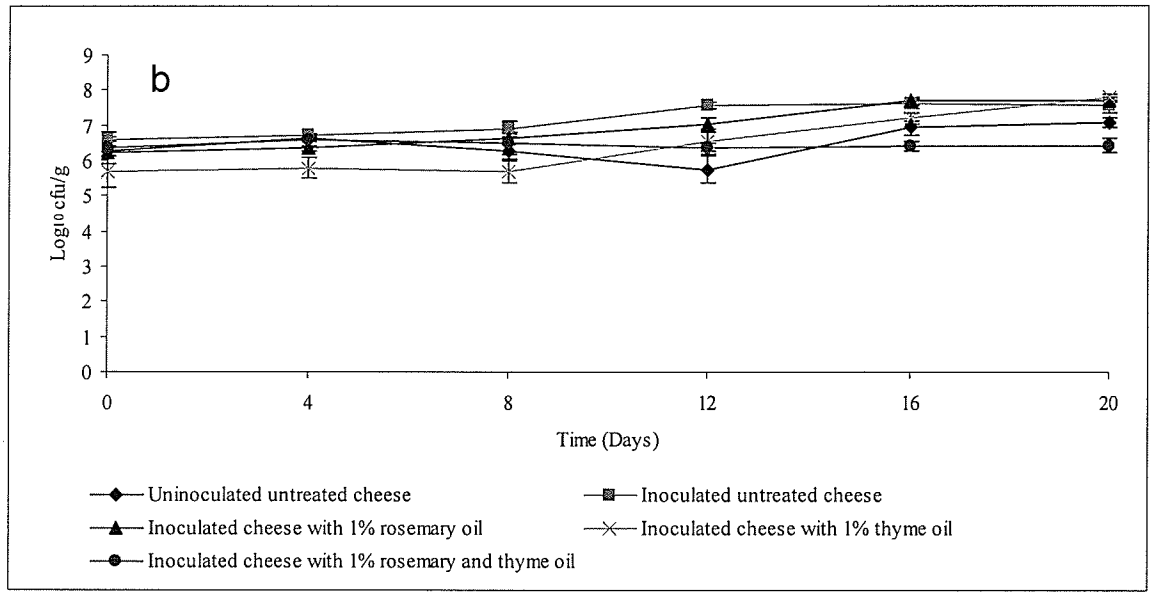
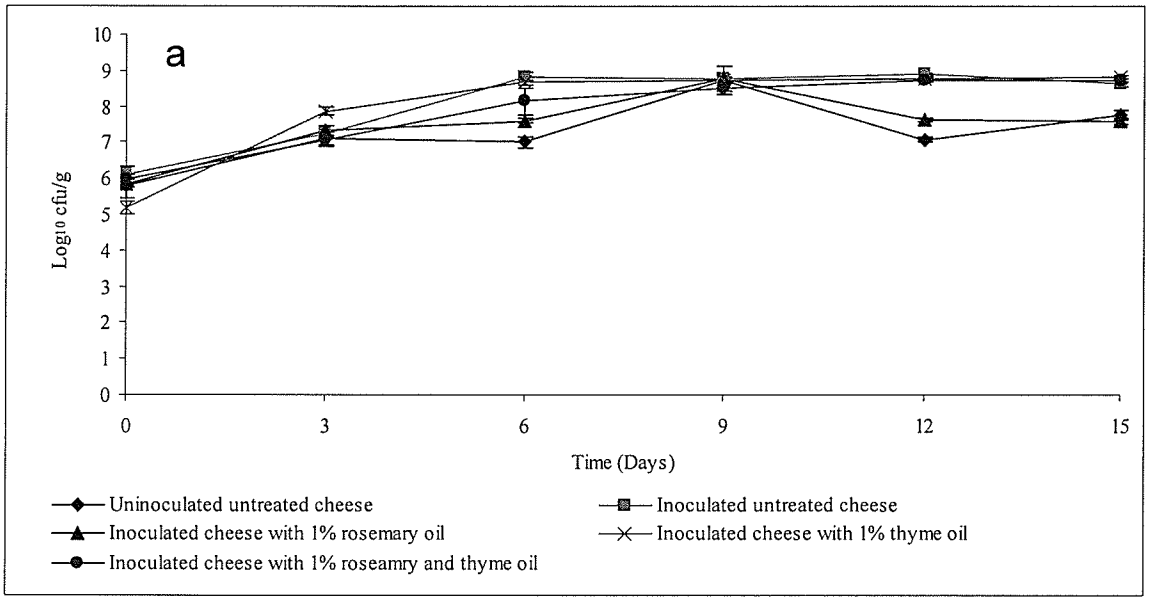


Figure 4.2. Antimicrobial activity of rosemary and thyme oil (1% w/w each) either separately or in combination against total aerobic count (TAC) in shredded full-fat mozzarella cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.



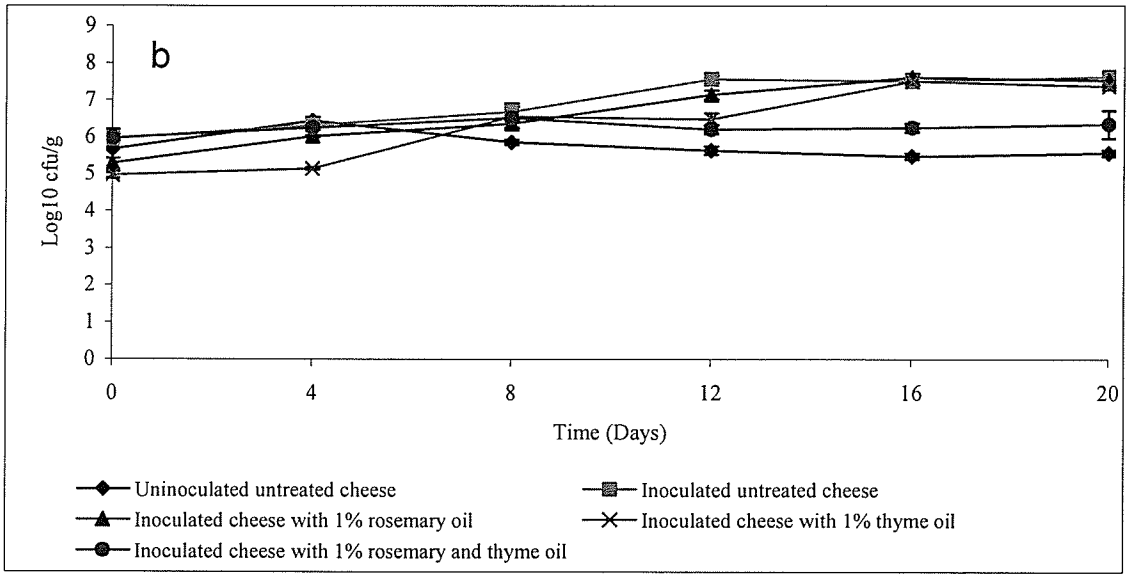


Figure 4.3. Antimicrobial activity of rosemary and thyme oil (1% w/w each) either separately or in combination against lactic acid bacteria (LAB) in shredded full-fat mozzarella cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.

4.4 Identification of an *L. monocytogenes* strain resistant to rosemary and thyme oil

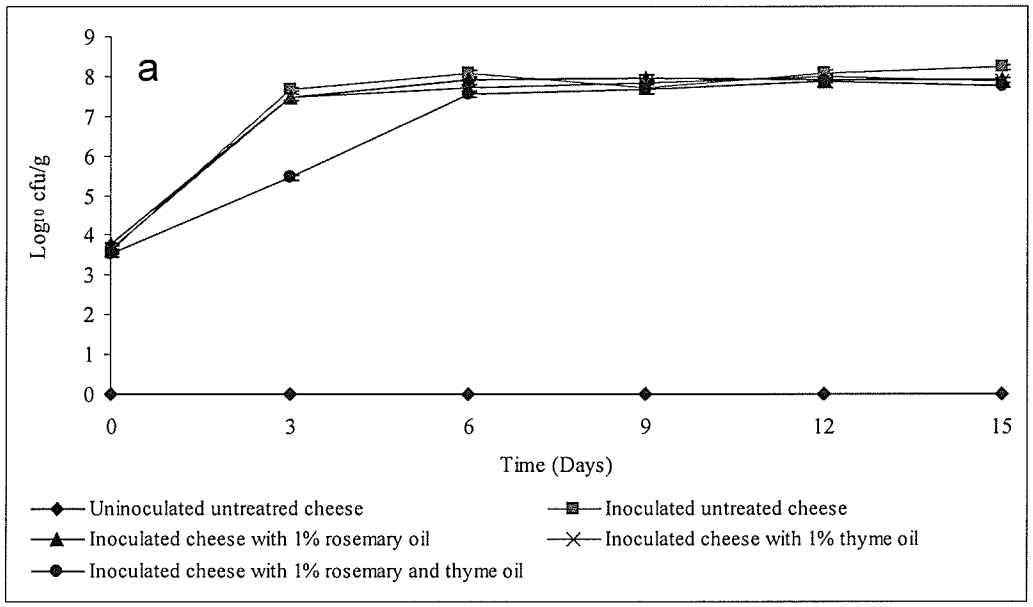
When the antilisterial activity of rosemary and thyme oil at 1% (w/w) was tested separately or in combination against a cocktail of five strains of *L. monocytogenes* in cheese, both oils showed very weak and unsatisfactory activity. Since the oils were effective against the mixed strains of *L. monocytogenes* when tested in agar media but not in cheese, the response of individual strains to the oils was examined in cheese using the previously described system. In untreated control samples at 4°C and 10°C *L. monocytogenes* strain C717 grew from 3.8 to 5.0 $\log_{10}$ cfu/g cheese in 5 d (Tables 4.3, 4.4). In the presence of rosemary and thyme oil, *L. monocytogenes* C717 increased from 3.5 to 4.3 $\log_{10}$ cfu/g at both 4 and 10°C. Growth during 5 d storage was not observed in treated cheese inoculated separately with strains C716, C718, C719 and C720. Gill et al. (2002) evaluated the antilisterial activity of cilantro oil against these strains in BHI broth. They found the concentration required to inhibit strain C717 was 2 to 4 fold higher than that required to inhibit the other strains. There are several factors that can lead to development of resistance towards antimicrobials. If the bacteria are exposed to foods that have low pH then it may lead to acid adaptation. Acid-adapted bacteria can show resistance towards natural antimicrobials (Davidson and Harrison, 2002). This was shown in a study reported by van Schaik et al. (1999) who investigated the antilisterial activity of nisin. They found that acid adaptation at pH 5.5 increased the resistance of *L. monocytogenes* towards nisin and lacticin 3147.

4.5 Antimicrobial activity of rosemary and thyme oil against a four-strain cocktail of *L. monocytogenes* in full-fat mozzarella cheese at 4 and 10°C

4.5.1 *L. monocytogenes*

Since one of five strains used in the previous experiments was found resistant to rosemary and thyme oil, its use was terminated and a cocktail of only four strains was used for further experiments. When the previous experiments reported in Figures 4.1, 4.2 and 4.3 were repeated, *L. monocytogenes* in untreated cheese increased from 3.7 $\log_{10}$ cfu/g to 8.2 $\log_{10}$ cfu/g by the end of 15 d storage at 10°C (Figure 4.4). Rosemary or thyme oil separately produced a slight inhibitory effect on growth and the numbers increased to 7.9 $\log_{10}$ cfu/g at day 15. This resulted in a 0.3 $\log_{10}$ cfu/g reduction which was significantly different from the control. When both oils were evaluated together, they produced a greater inhibitory effect on the growth rate of *L. monocytogenes* and the numbers increased from 3.6 to 7.8 $\log_{10}$ cfu/g by the end of 15 d storage. This resulted in a difference of 0.4 $\log_{10}$ cfu/g which was significant compared to controls. When the individual and combined antilisterial activity of rosemary and thyme oil against the five strain cocktail of *L. monocytogenes* was compared with the results from the four strain cocktail, the oils appeared less effective against the four-strain cocktail. It is suspected that a comparison of a larger number of analysis where the four strain cocktail was used would have yielded no difference in the results with the two cocktails. Unfortunately, elimination of the apparently resistant strain did not result in greater inhibition of growth of *L. monocytogenes* at 10 or 4°C. As discussed earlier, the presence of fat and protein in foods can interfere with antimicrobial action. The availability of nutrients in foods can help bacteria to repair injury and facilitate their growth.

When the antimicrobials were tested for their antilisterial activity in cheese at 4°C, they failed to exert significant activity. The numbers in samples treated with rosemary and thyme oil separately or together were similar (7.8, 7.6 and 7.7 $\log_{10}$ cfu/g) to that of the control (7.4 $\log_{10}$ cfu/g). The pH of cheese treated with rosemary and thyme oil separately or together was not different from the pH of control cheese (~ pH 5.5) Thyme oil alone restricted the growth of *L. monocytogenes* in cheese and yielded significantly lower numbers on days 4, 8 and 12. Similar observations were recorded for samples containing rosemary and thyme oil together. However, on day 16 and 20, no significant difference was observed between test and controls.



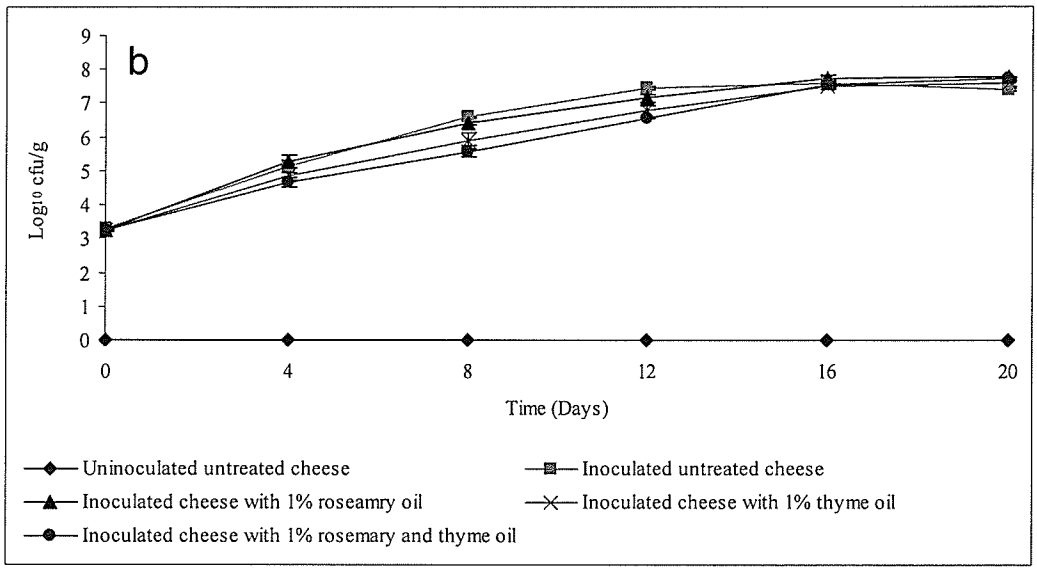


Figure 4.4. Antimicrobial activity of rosemary and thyme oil (1% w/w each) either separately or in combination against a cocktail of 4 strains of *L. monocytogenes* in shredded full-fat mozzarella cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.

4.5.2 TAC and LAB

TAC were able to grow in all samples (Figure 4.5) and increased from 4.7 to 8.5 $\log_{10}$ cfu/g in control samples at 15 d storage at 10°C. Also they were able to grow in the presence of rosemary and thyme oil either separately or in combination and the numbers were almost similar to that of control samples by the end of storage. As observed in the previous work with the five strain cocktail of *L. monocytogenes*, the growth of TAC was observed throughout storage. Similar observations were also recorded for LAB (Figure 4.6). The presence of rosemary and thyme oil separately and together restricted growth and the numbers increased from 4.3, 4.2 and 5.5 $\log_{10}$ cfu/g to 8.0, 7.6 and 7.2 $\log_{10}$ cfu/g, respectively. This resulted in a significant difference between control samples and those containing rosemary and thyme oil. As observed before, rosemary and thyme oil were not particularly effective against TAC and LAB.

When the antimicrobial activity of rosemary and thyme oil at 4°C was analyzed, the TAC increased from 6.3 $\log_{10}$ cfu/g in samples containing rosemary oil, thyme oil and rosemary and thyme oil together to 7.6, 7.2 and 7.3 $\log_{10}$ cfu/g, respectively, at 20 d storage. Treatment of samples with rosemary oil alone yielded results that were not significantly different from controls. However, addition of thyme oil restricted growth and a significant difference of 0.4 $\log_{10}$ cfu/g was observed. Combination of rosemary and thyme oil also produced a significant difference of 0.3 $\log_{10}$ cfu/g. LAB increased from 6.8 to 8.0 $\log_{10}$ cfu/g in control samples. Although there were some differences that were statistically significant, those <1 $\log_{10}$ cfu/g are not of microbiological significance (Jarvis, 1989).

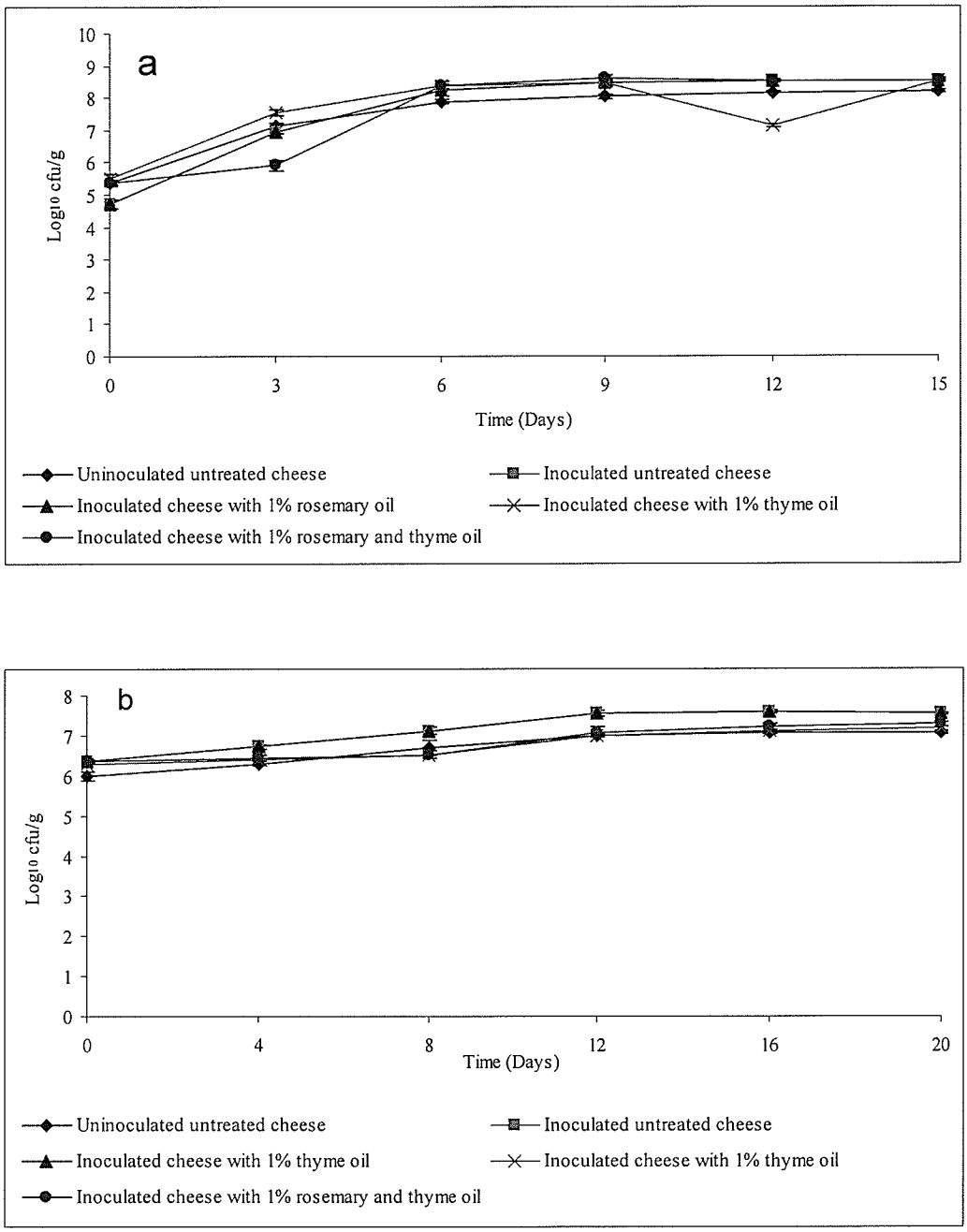
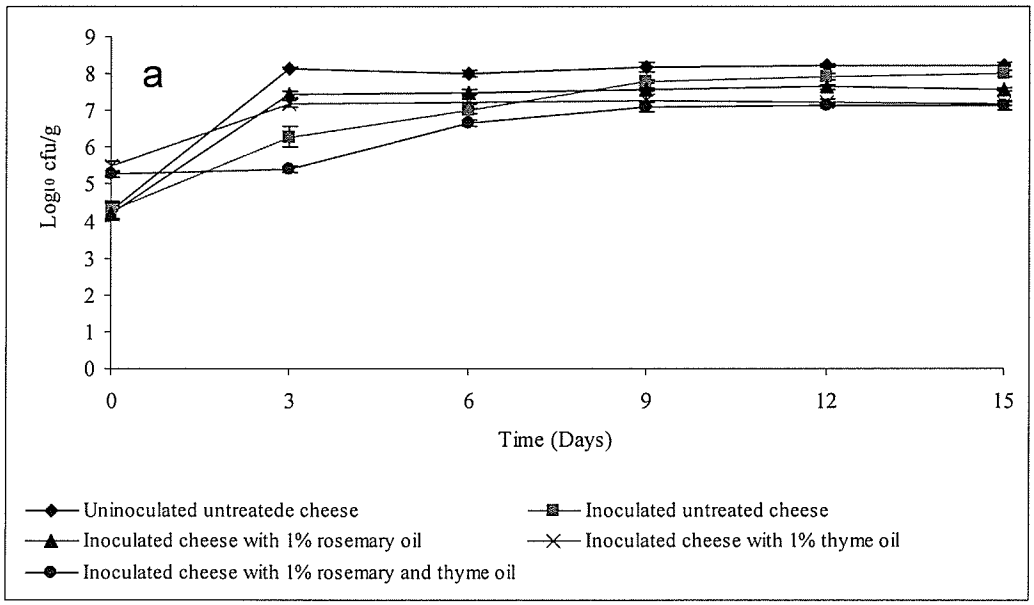


Figure 4.5. Antimicrobial activity of rosemary and thyme oil (1% w/w each) either separately or in combination against total aerobic count (TAC) in shredded full-fat mozzarella cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.



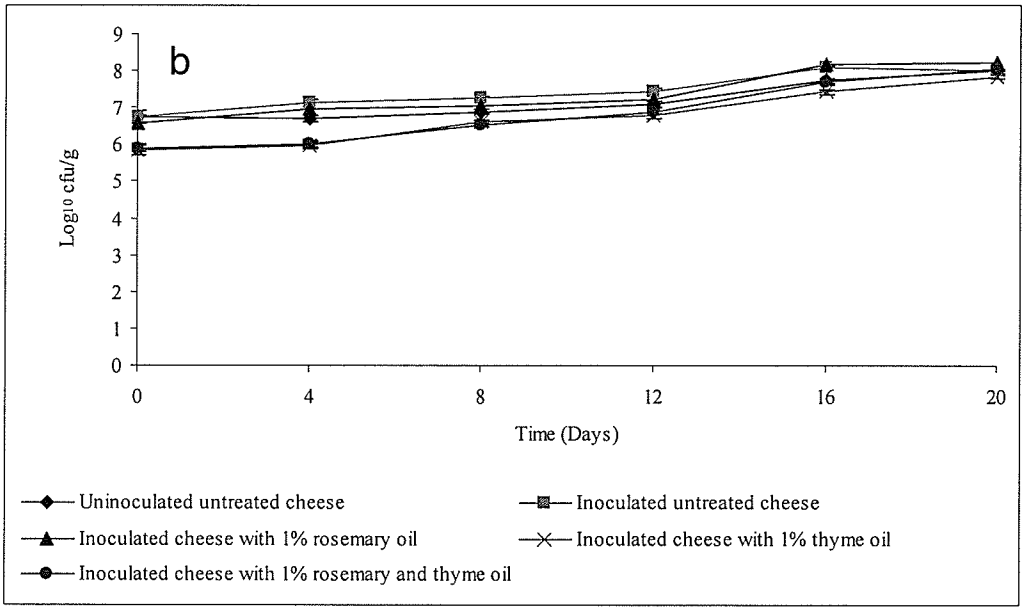


Figure 4.6. Antimicrobial activity of rosemary and thyme oil (1% w/w each) either separately or in combination against lactic acid bacteria (LAB) in shredded full-fat mozzarella cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.

4.6 Antimicrobial activity of sodium diacetate plus rosemary and thyme oil against a four-strain cocktail of *L. monocytogenes*, LAB and TAC in full fat cheese at 4 and 10°C

4.6.1 *L. monocytogenes*

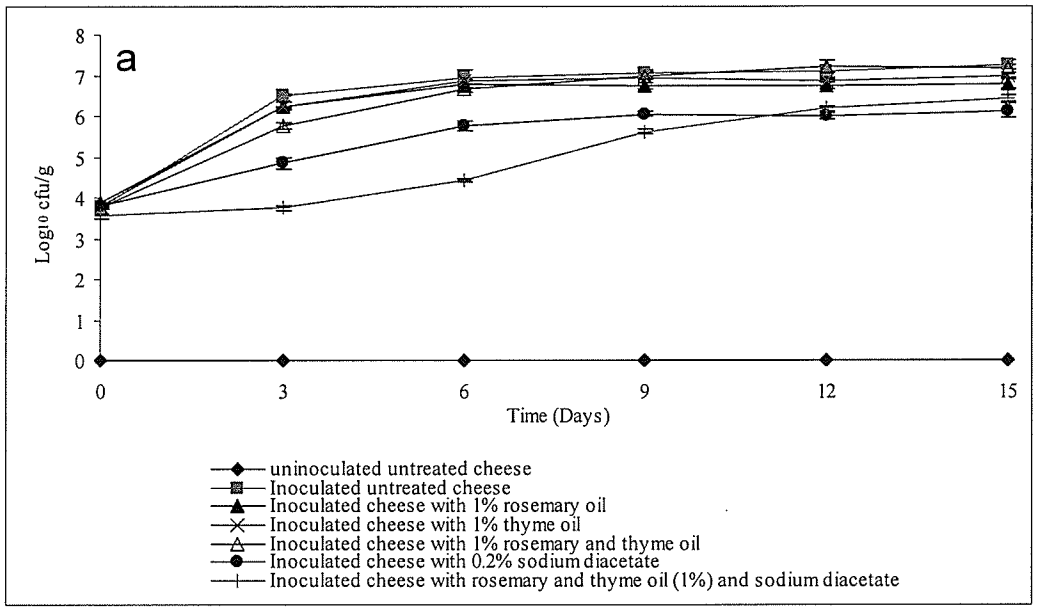
Figure 4.7 shows the antimicrobial activity of rosemary and thyme oil separately at 4 and 10°C against a cocktail of four strains of *L. monocytogenes*. The numbers of *L. monocytogenes* in control samples increased from 3.8 $\log_{10}$ cfu/g to 7.2 $\log_{10}$ cfu/g by the end of the experiment at 10°C. The presence of rosemary oil at 1% (w/w) significantly suppressed the growth of *L. monocytogenes* on days 3, 9, 12 and 15. The numbers increased from 3.9 to 6.8 $\log_{10}$ cfu/g producing a difference between the treatment and the control of 0.5 $\log_{10}$ cfu/g at 15 d. Thyme oil at same level also showed retardation in the growth of *L. monocytogenes* and the numbers in treated cheese samples increased from 3.8 to 7.0 $\log_{10}$ cfu/g. This resulted in a significant difference of 0.3 $\log_{10}$ cfu/g with the control. However, when both oils were combined, they failed to produce a significant difference. The previous experiment done with the five-strain cocktail of *L. monocytogenes* showed greater inhibition of growth of *L. monocytogenes* in the presence of both oils than when the oils were analyzed separately. However, in this set of experiments, the oils were more effective when tested separately than when used together. The presence of sodium diacetate at 0.2% (w/v) resulted in a greater retardation of the growth and the numbers increased from 3.8 to 6.1 $\log_{10}$ cfu/g by the end of experiment. This produced a significant difference of 1.1 $\log_{10}$ cfu/g between the treatment and control. When sodium diacetate was combined with rosemary and thyme oil, it produced greater inhibition of the growth of *L. monocytogenes* during the first 6 d.

The numbers in control samples reached 7.0 $\log_{10}$ cfu/g by the end of 6 d, whereas the combined treatment contained 4.4 $\log_{10}$ cfu/g which was significantly lower than the control. However, by 15 d continued growth by *L. monocytogenes* in the combined treatment yielded 6.4 $\log_{10}$ cfu/g which was still significantly lower than the control. Weak organic acids such as acetate, lactate and sorbate are the most commonly used preservatives in the food industry. They are known to act against bacteria, fungi as well as spores (Sofos and Busta, 1981). These acids show optimum inhibitory activity in the undissociated state. Upon entering the bacterial cell through the cell membrane in an undissociated form, they dissociate. This changes the intracellular pH. The movement of these weak acid molecules across the bacterial cell membrane continues until an equilibrium is reached with respect to the pH gradient across the membrane. This leads to accumulation of ions resulting in stress on pH homeostasis (Burl and Coote, 1999).

Rosemary oil, thyme oil and sodium diacetate showed more potent antilisterial activity when tested at 4°C. *Listeria* numbers in control samples increased from 3.7 $\log_{10}$ cfu/g to 8.0 $\log_{10}$ cfu/g by 20 d storage. The presence of 0.2% (w/v) sodium diacetate along with rosemary and thyme oil (1% w/w each) restricted the growth of *L. monocytogenes* and numbers increased from 3.6 to 3.8 $\log_{10}$ cfu/g by 20 d storage. This produced a difference of 4.3 $\log_{10}$ cfu/g between the treatment and control. Treatment of cheese samples with sodium diactetate alone also produced strong antilisterial activity and by the end of 20 d storage numbers increased from 3.7 to 4.1 $\log_{10}$ cfu/g which were significantly lower than in control samples. Rosemary oil also slowed the growth rate and the numbers increased from 3.8 to 7.7 $\log_{10}$ cfu/g and were significantly lower than the control samples. Similar observations were recorded for samples treated with thyme

oil and rosemary plus thyme, but differences between the treatments and control were ≤ 0.6 $\log_{10}$ cfu/g.

The antimicrobials used in this study are believed to have different modes of action. Rosemary and thyme oil are natural plant essential oils rich in phenolics. On the other hand, sodium diacetate is a sodium salt of acetic acid, which is a weak organic acid. The well recognized mode of action of phenolic antimicrobials results in disruption of the cell membrane, whereas weak organic acids cause changes in the pH equilibrium across the bacterial cell membrane and can change intracellular pH. Bacterial resistance to internal pH change has an energy cost and this may translate into reduced bacterial growth. The inhibition was greater at 4°C than at 10°C. It is also well known that the lag phase increases with decrease in temperature. This increase in the lag phase together with addition of antimicrobials with different modes of action might have produced greater inhibition at the lower temperature. In a study conducted by Samelis et al. (2005), it was observed that dipping bologna slices inoculated with a cocktail of 10 strains of *L. monocytogenes* in a solution containing nisin (5000 IU/ml) and sodium diacetate (3% w/v) for 1 min inhibited growth for about 90 d when stored at 4°C after vacuum packaging. The inhibition was extended to 120 d when the concentration of sodium diacetate was increased to 5% (w/v).



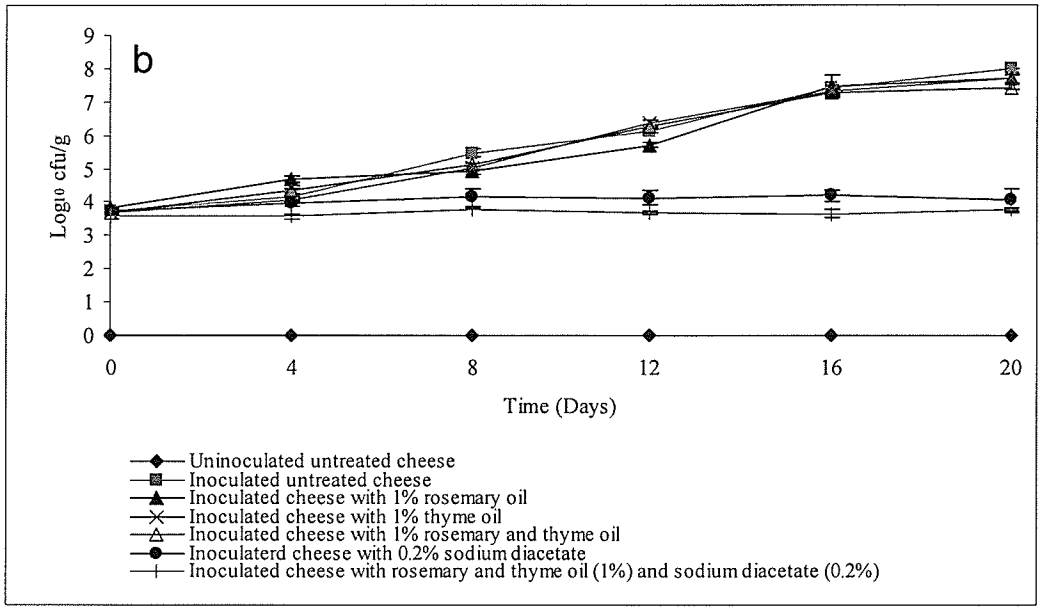


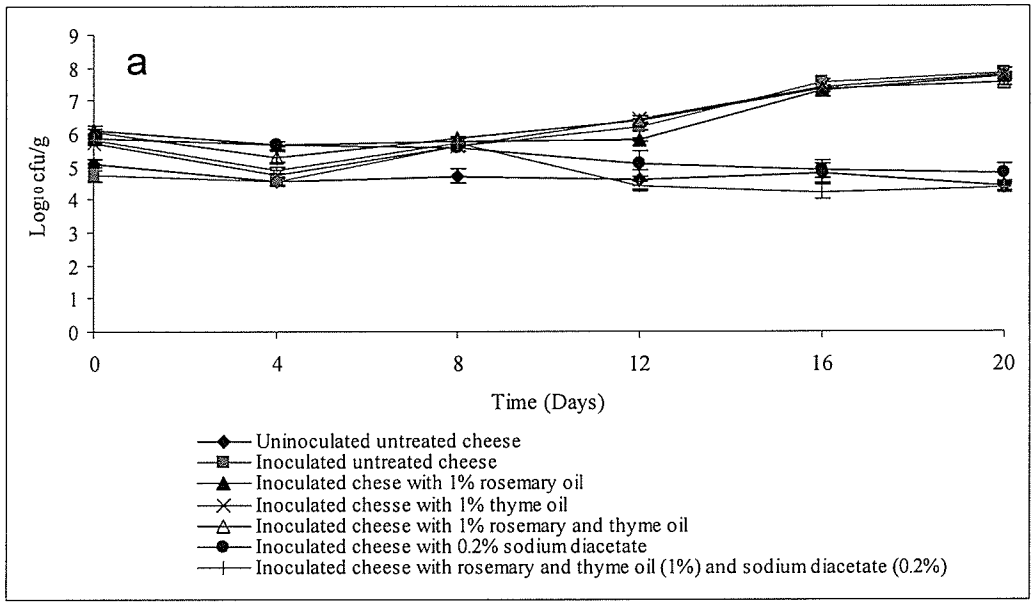
Figure 4.7. Antimicrobial activity of rosemary oil, thyme oil and sodium diacetate either separately or in combination against a cocktail of four strains of *L. monocytogenes* in shredded full-fat mozzarella cheese at 10°C (a) and 4°C (b). Six replicates were used to generate the standard deviation bars.

4.6.2 TAC and LAB

Sodium diacetate also controlled proliferation of LAB and TAC (Figures 4.8, 4.9). At 4°C, the numbers of LAB in samples treated with rosemary oil, thyme oil and sodium diacetate changed from 5.8 to 4.4 $\log_{10}$ cfu/g by of 20 d storage. The numbers increased from 4.6 to 7.8 $\log_{10}$ cfu/g in controls. Sodium diacetate alone also reduced the level of LAB from 5.8 to 4.8 $\log_{10}$ cfu/g by the end of storage. However, rosemary and thyme oil separately as well as together did not have strong antimicrobial activity against LAB and the numbers were 7.7, 7.9 and 7.5 $\log_{10}$ cfu/g at the study end, respectively. Inhibition was not as strong at 10°C. With respect to TAC at 4°C (Figure 4.9), numbers increased from 5.2 to 7.7 $\log_{10}$ cfu/g by the end of 20 d in controls. Thyme and rosemary oil separately and together failed to exert an antimicrobial effect and the numbers were not different (7.8, 7.7 and 7.5 $\log_{10}$ cfu/g, respectively). When sodium diacetate was used alone it restricted the growth of TAC and at 20 d, the number was 5.3 $\log_{10}$ cfu/g. This resulted in a significant difference of 2.4 $\log_{10}$ cfu/g compared to the control. Growth was also restricted in the presence of all three antimicrobials. The TAC decreased from 6.2 to 5.6 $\log_{10}$ cfu/g at the end of storage at 4°C.

When the antimicrobials were tested at 10°C, the LAB increased from 5.2 to 8.4 $\log_{10}$ cfu/g in control samples (Figure 4.8). Treatment of cheese with rosemary and thyme oil separately or together did not have any antimicrobial activity and the numbers reached 8.9, 8.7 and 8.9 $\log_{10}$ cfu/g, respectively, by 15 d storage. Addition of sodium diacetate alone or in combination with rosemary plus thyme oil was less effective than at 4°C. The LAB increased from 5.6 and 5.7 to 7.8 and 7.7 $\log_{10}$ cfu/g at the end of experimental storage in samples containing sodium diacetate alone and with rosemary and thyme oil, respectively. This resulted in a difference of 0.6 and 0.7 $\log_{10}$ cfu/g in

samples treated with sodium diacetate alone and sodium diacetate together with rosemary and thyme oil, respectively. The numbers in treated samples were significantly different from the control. Similar observations were recorded for TAC at 10°C (Figure 4.9). The level of TAC in samples treated with rosemary and thyme oil separately and together was higher (9.0, 8.7 and 8.9 $\log_{10}$ cfu/g, respectively) than in control samples (8.5 $\log_{10}$ cfu/g) by 15 d storage. The numbers in samples treated with sodium diacetate alone increased from 6.2 to 8.5 $\log_{10}$ cfu/g by 15 d storage and was not significantly different from controls. Treatment of cheese with all three antimicrobials allowed the TAC to increase from 6.2 to 8.1 $\log_{10}$ cfu/g by 15 d. This resulted in a significant difference of 0.4 $\log_{10}$ cfu/g compared with the control. Barmpalia et al. (2005) also reported similar findings. The total bacterial population on the surface of bologna treated with 0.125% sodium diacetate increased from 3 $\log_{10}$ cfu/g to about 8 $\log_{10}$ cfu/g after 28 d storage at 10°C. When they evaluated the antimicrobial activity of 0.125% sodium diacetate at 4°C, the population of total bacteria increased from 3 to 8 $\log_{10}$ cfu/g at the end of 90 d and was not significantly different from that of control samples. However an increase in the concentration of sodium diacetate (0.25%) and its combination with 1.8% sodium lactate resulted in stronger antimicrobial activity and the total bacterial numbers on the surface of bologna at 4°C remained unchanged, whereas it increased from 3 $\log_{10}$ cfu/g to about 5 $\log_{10}$ cfu/g by the end of 28 d at 10°C. The greater antimicrobial activity of a combination of two antimicrobials can result from different modes of action. Sodium lactate was able to reduce the a_w of meat. Also, the increased concentration of sodium diacetate contributed to reduced a_w. The reduction in the a_w of meat along with the ability of sodium diacetate to change the intracellular pH might have brought about the stronger antimicrobial activity.



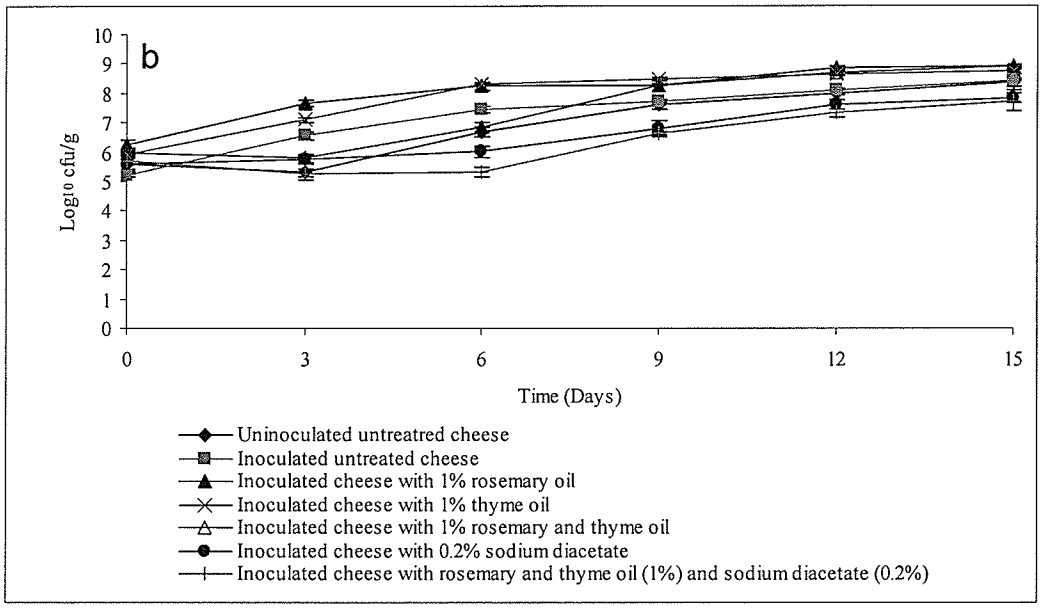


Figure 4.8. Antimicrobial activity of rosemary oil, thyme oil and sodium diacetate either separately or in combination against lactic acid bacteria (LAB) in shredded full-fat mozzarella cheese at 4°C (a) and 10°C (b). Six replicates were used to generate the standard deviation bars.

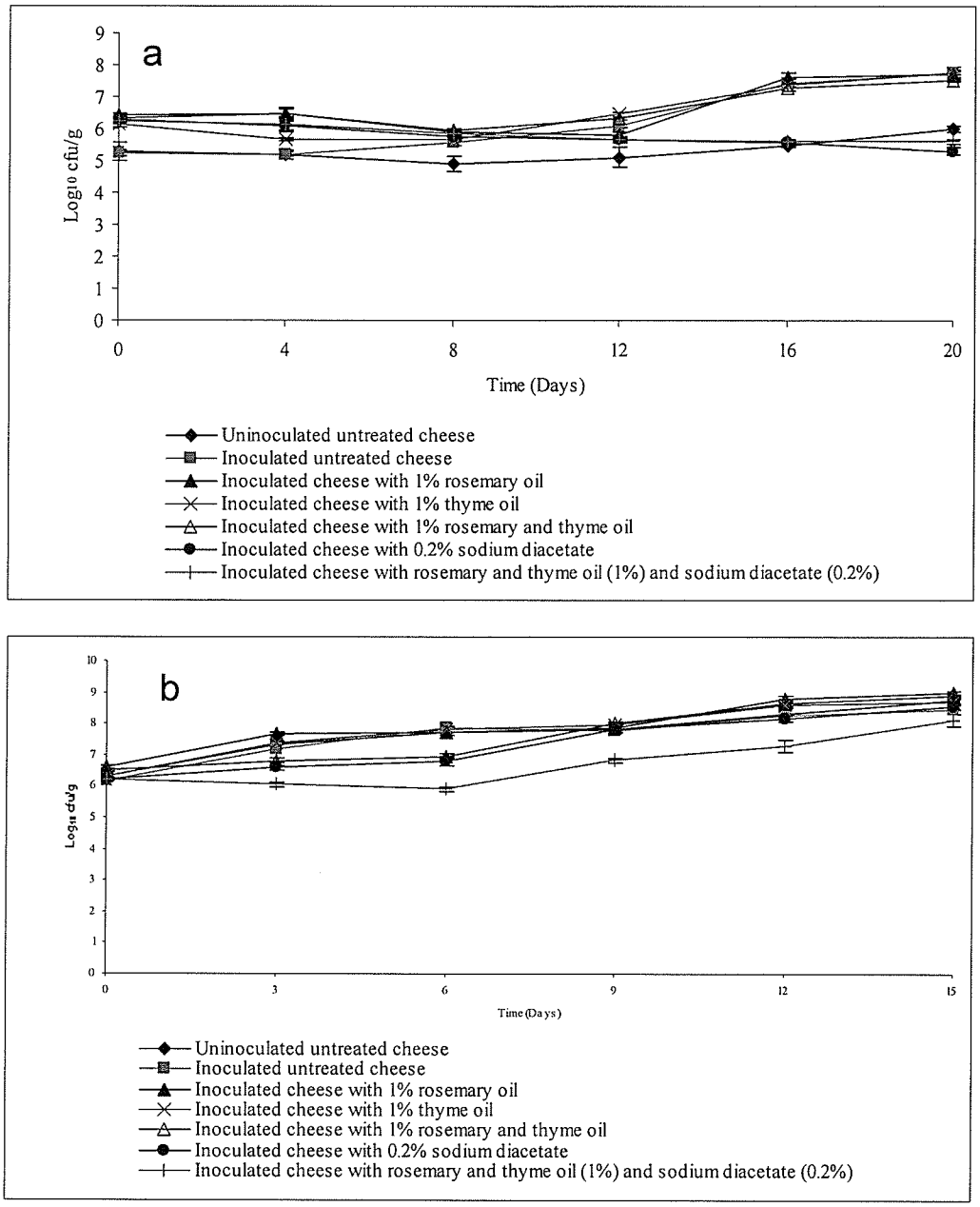


Figure 4.9. Antimicrobial activity of rosemary oil, thyme oil and sodium diacetate either separately or in combination against total aerobic count (TAC) in shredded full-fat mozzarella cheese at 4°C (a) and 10°C (b). Six replicates were used to generate the standard deviation bars.

4.7 Effect of rosemary and thyme oil alone and together in low fat mozzarella cheese

4.7.1 *L. monocytogenes*

In this set of experiments, the antimicrobial activity of rosemary and thyme oil (1% w/w each) was studied separately and together (1:1 of 1% (w/w) rosemary and thyme oil) in shredded low-fat mozzarella cheese at 4 and 10°C. Rosemary and thyme oil separately as well as together were inhibitory to the growth of *L. monocytogenes* (Figure 4.10). The numbers increased from 3.5 to 6 $\log_{10}$ cfu/g by 20 d at 4°C in samples treated with rosemary oil, which were significantly lower than that in control samples (6.5 $\log_{10}$ cfu/g by the end of storage). Thyme oil also showed inhibition of growth when added to cheese. By the end of storage for 20 d, the number in treated samples was 5.5 $\log_{10}$ cfu/g. This resulted in a significant difference of 1.0 $\log_{10}$ cfu/g. The antilisterial activity of both oils was even stronger when they were combined. Rosemary and thyme oil in cheese produced a significant difference of 1.7 $\log_{10}$ cfu/g at 20 d. Thus, addition of rosemary and thyme oil produced an additive inhibitory effect against the growth of *L. monocytogenes*.

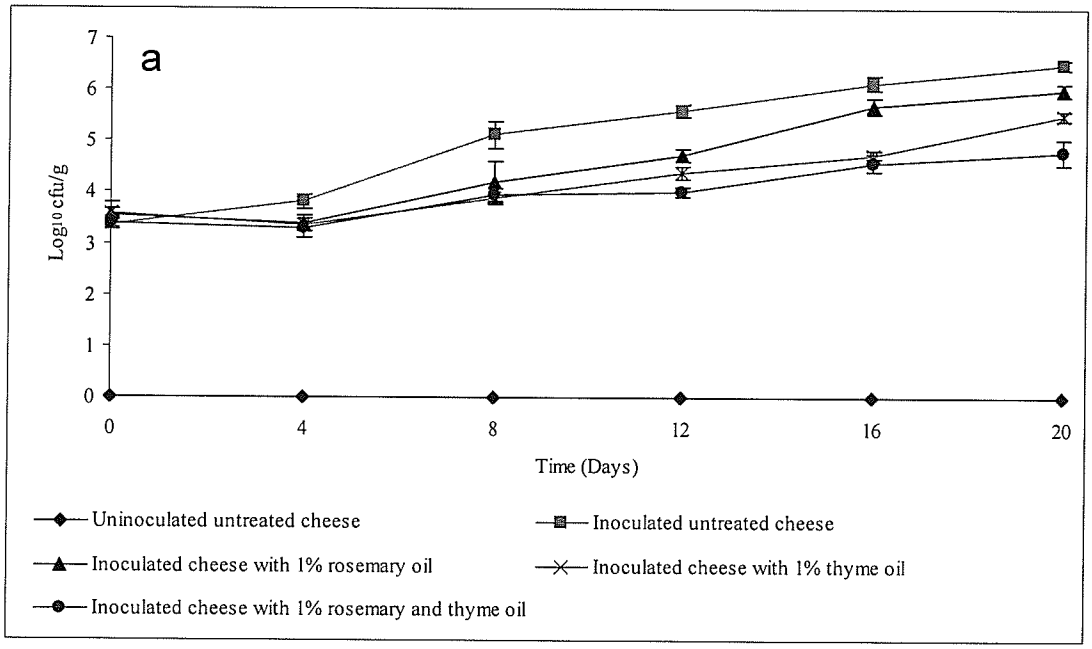
However, when rosemary and thyme oil were tested for their activity in cheese at 10°C, they produced weaker inhibition of *L. monocytogenes*. Rosemary and thyme oil separately did not produce any significant difference. *Listeria* numbers in control samples and samples treated with rosemary or thyme oil increased from 3.6, 3.8 and 3.7 $\log_{10}$ cfu/g, respectively, to 6.7, 6.6 and 6.6 $\log_{10}$ cfu/g, respectively, by 15 d. However, treatment of samples with rosemary and thyme oil together produced a significant

difference of 0.7 $\log_{10}$ cfu/g and restricted the growth of *L. monocytogenes* to 6.0 $\log_{10}$ cfu/g by 15 d.

The oils were more effective against *L. monocytogenes* in low fat cheese than in full fat cheese as mentioned. The numbers in full fat cheese increased from 3.2 $\log_{10}$ cfu/g to 7.7 $\log_{10}$ cfu/g by day 20 at 4°C. No significant difference was observed between control samples and samples treated with rosemary and thyme oil together. Treatment of full fat cheese samples with rosemary or thyme oil separately was also not effective and the numbers of *L. monocytogenes* were similar to that of control samples. Thus, the effect of the level of fat was quite obvious and seemed to play an important role in determining the overall antimicrobial activity of rosemary and thyme oil. Similar observations have been reported in the literature. Smith-Palmer et al. (2001) used commercially available Kraft Philadelphia low fat (16%) and full fat (30%) cheese. They found 1% (v/v) of thyme oil was inhibitory to *L. monocytogenes* in low fat cheese. Thyme oil at that concentration reduced *Listeria* numbers from 5 $\log_{10}$ cfu/g to below the detection limit after 10 d at 4°C. However, when they evaluated the antilisterial activity in full fat cheese, thyme oil was able to reduce the numbers by 1 $\log_{10}$ cfu/g by day 14. Unfortunately, they reported the antimicrobial activity in cheese that was diluted 1 in 10 with phosphate buffered saline. It is possible that the antilisterial activity in undiluted cheese might be different. They also evaluated antilisterial activity against just one strain of *L. monocytogenes*, whereas in the present study a cocktail of four strains of *L. monocytogenes* was used. It might be possible that the only strain used in work done by Smith-Palmer et al. (2001) was sensitive to the antimicrobial tested. Mbandi and Shelef (2002) compared the antilisterial activity of sodium lactate (2.5%) and sodium diacetate (0.2%) together in bologna at 5 °C. They evaluated the antilisterial activity

against *L. monocytogenes* Scott A alone as well as against a cocktail of six different strains of *L. monocytogenes* including *L. monocytogenes* Scott A. Sodium diacetate and sodium lactate in bologna inoculated with *L. monocytogenes* Scott A resulted in listericidal activity and the pathogen was inhibited below the detection limit by the end of 45 d storage. When the salts were analyzed against the cocktail of strains under the same conditions they appeared less inhibitory, but still produced a listeriostatic effect. Gill et al. (2002) evaluated antilisterial activity of cilantro oil in vacuum packed ham. They also evaluated the antilisterial activity of cilantro oil against five different strains in BHI broth at 24°C. They found variation in the sensitivity of strains towards cilantro oil. Thus, in a view of the differences in the sensitivity of different strains of *L. monocytogenes*, antimicrobial potency should be determined against a mixture of different strains rather than against one single strain.

The effect of fat on antilisterial activity was also confirmed in a study reported by Singh et al. (2003). They analyzed the antilisterial activity of thyme oil solution against a mixture of three different strains of *L. monocytogenes* in beef hot dogs with three different fat levels. Dipping of fat-free (0%) hot dogs for 15 min in a solution containing 10 ml/L thyme oil reduced the population by 0.9-1.3 $\log_{10}$ cfu/g. The antimicrobial activity of thyme oil solution was reduced when tested in low-fat (9%) and full-fat (26%) hot dogs resulting in 0.61-0.66 and 0.4-0.6 $\log_{10}$ cfu/g reduction of *L. monocytogenes*, respectively. Thus, it is evident that the composition of foods plays an important role in determination of antimicrobial activity.



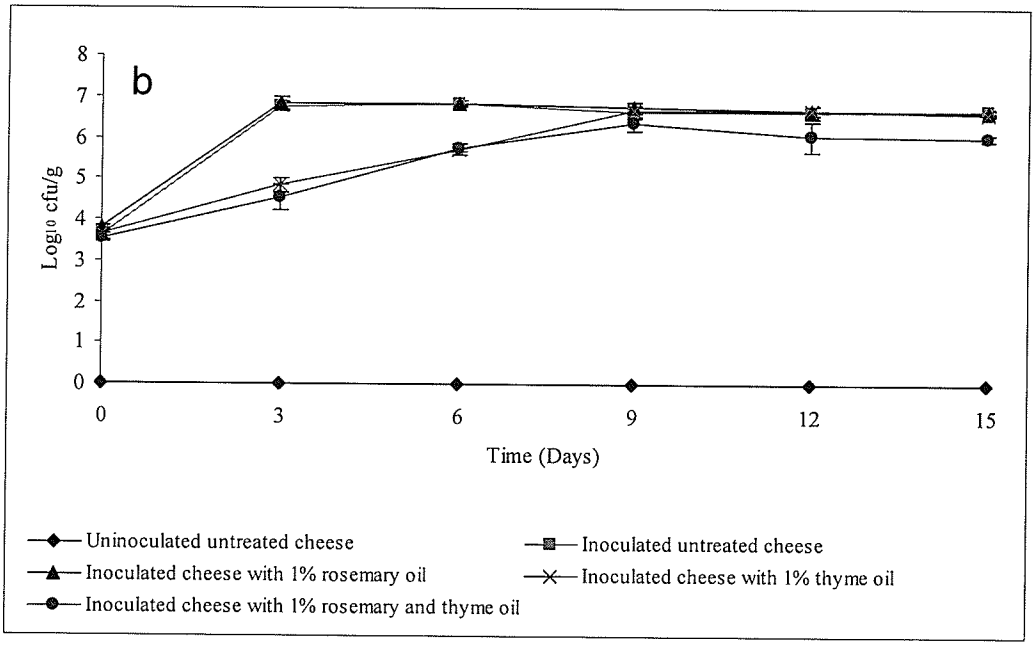
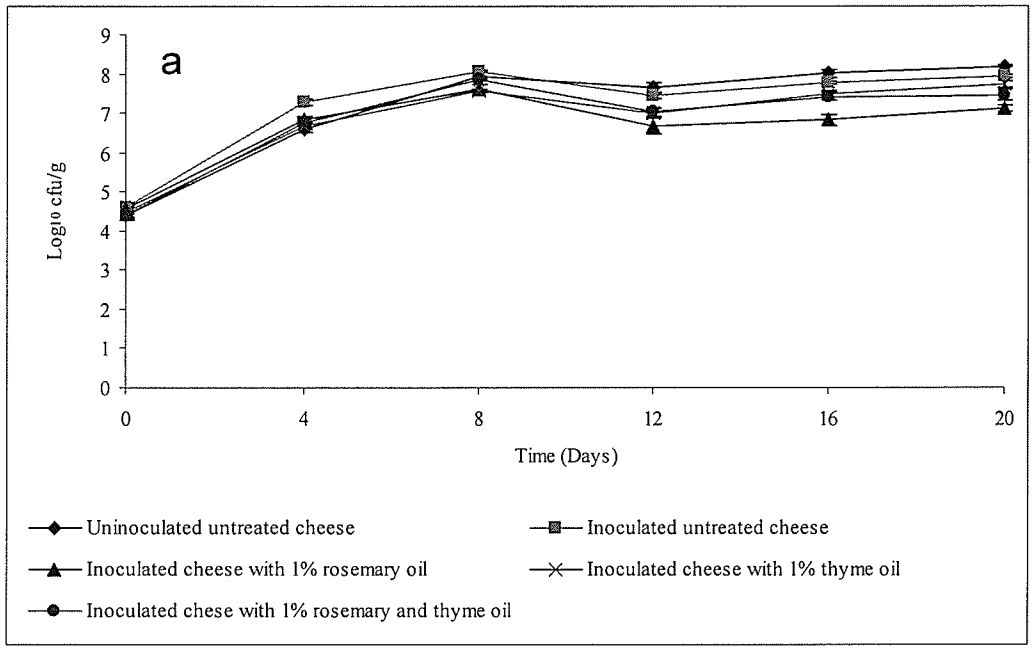


Figure 4.10. Antimicrobial activity of rosemary oil and thyme oil (1% w/w each) either separately or in combination against a cocktail of four strains of *L. monocytogenes* in shredded low-fat mozzarella cheese at 4°C (a) and 10°C (b). Six replicates were used to generate the standard deviation bars.

4.7.2 LAB and TAC

Bacterial numbers in control samples (both LAB and TAC) were higher in the cheese stored at 10°C than at 4°C. LAB were able to grow in low fat cheese, as they did in high fat cheese (Figure 4.11). However, the final numbers in control samples and samples treated with rosemary and thyme oil either separately or in combination were lower than in high fat cheese. The numbers of LAB increased to 7.9 $\log_{10}$ cfu/g by 20 d storage at 4°C in control samples. Rosemary and thyme oil also allowed growth and the numbers were 7.1 and 7.7 $\log_{10}$ cfu/g at 20 d. LAB were also able to grow when rosemary and thyme oil were added together. Their numbers increased from 4.6 $\log_{10}$ cfu/g to 7.5 $\log_{10}$ cfu/g. Similar observations were observed for TAC (Figure 4.12). Their numbers in control samples and samples treated with rosemary and thyme oil increased from 4.6, 5.7 and 5.8 $\log_{10}$ cfu/g, respectively, to 8.2, 8.0 and 8.0 $\log_{10}$ cfu/g, respectively, by the end of the storage study. The numbers increased from 5.6 to 7.9 $\log_{10}$ cfu/g in samples containing both oils together.



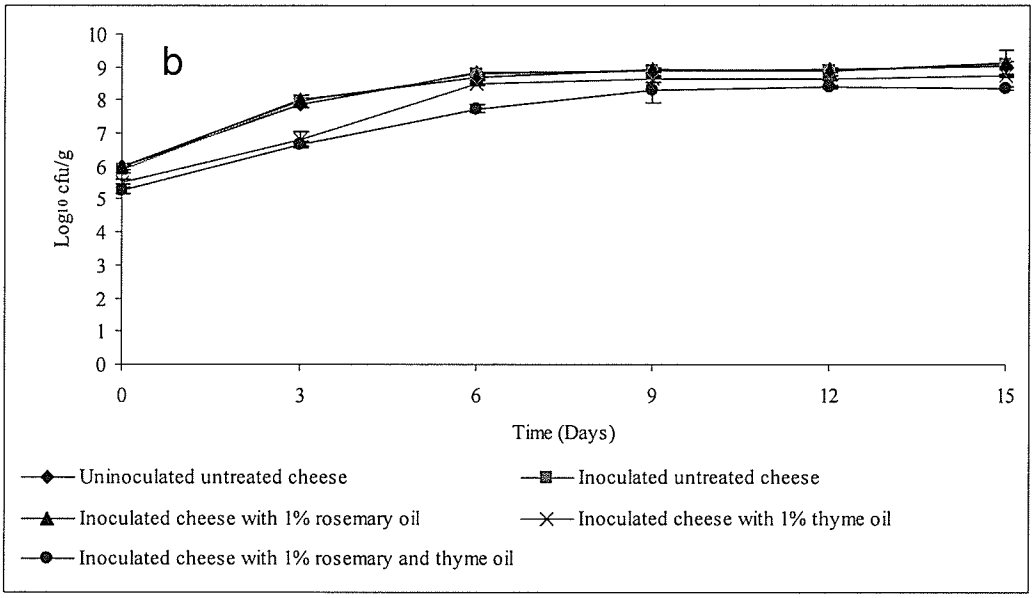
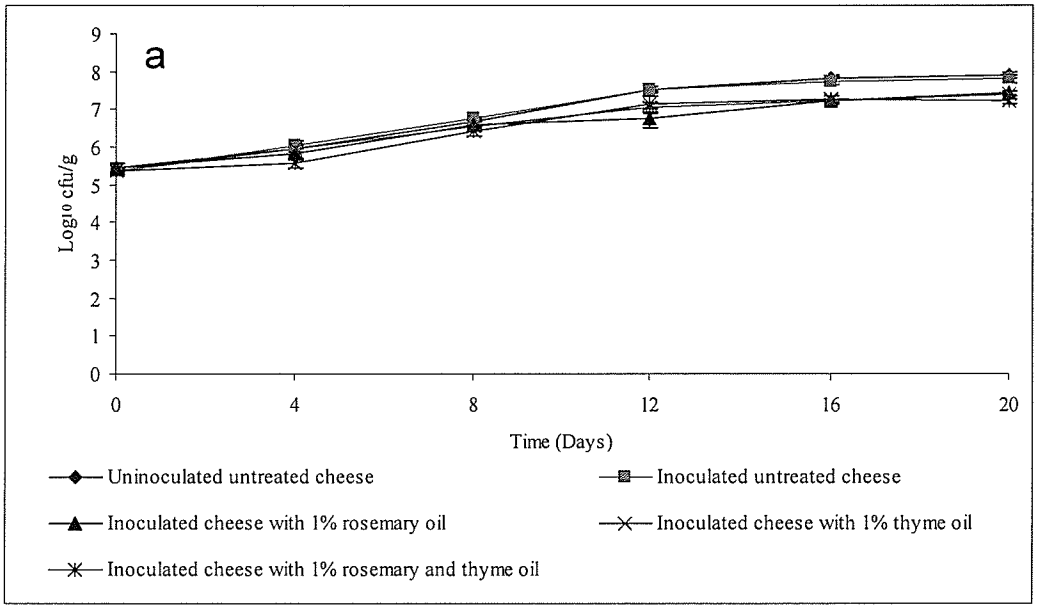


Figure 4.11. Antimicrobial activity of rosemary oil and thyme oil (1% w/w each) either separately or in combination against lactic acid bacteria (LAB) in shredded low-fat mozzarella cheese at 4°C (a) and 10°C (b). Six replicates were used to generate the standard deviation bars.



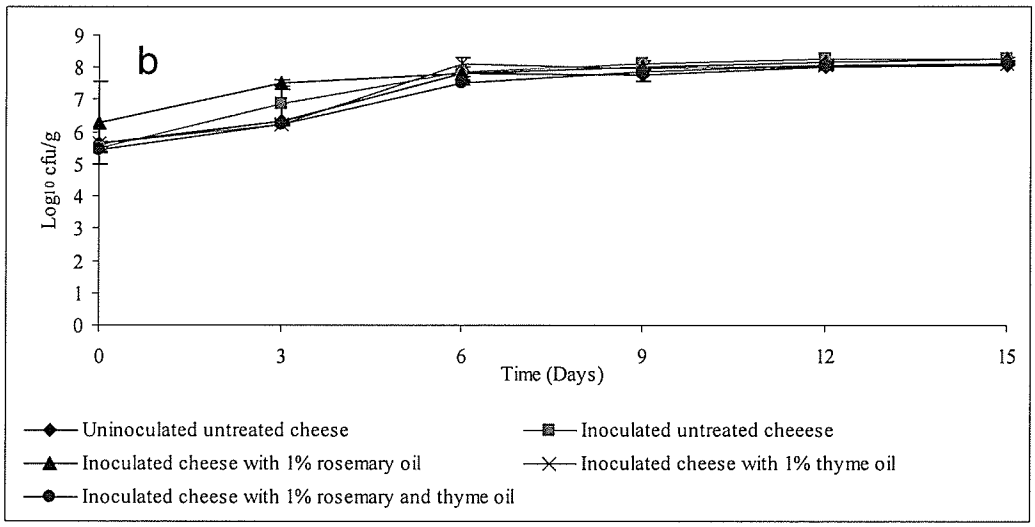


Figure 4.12. Antimicrobial activity of rosemary oil and thyme oil (1% w/w each) either separately or in combination against total aerobic count (TAC) in shredded low-fat mozzarella cheese at 4°C (a) and 10°C (b). Six replicates were used to generate the standard deviation bars.

4.8 Use of rosemary and thyme oil as antimicrobial agents delivered from a paper sachet

4.8.1 *L. monocytogenes*

In this experiment, a sachet was used containing microcellular foam (MCF) starch mixed with rosemary and thyme oil separately or combined. The initial bacterial level in control untreated samples was 3.5 $\log_{10}$ cfu/g. By 15 d storage at 10°C, numbers increased to 7.2 $\log_{10}$ cfu/g (Figure 4.13). The sachet containing rosemary or thyme oil separately mixed with MCF starch did not exert antilisterial activity and, surprisingly, *Listeria* numbers were higher (7.6 $\log_{10}$ cfu/g,) than in control samples by 15 d. However, numbers in samples containing a sachet with a mixture of rosemary and thyme oil together were lower than the control. Rosemary and thyme oil together retarded *Listeria* growth and produced a significant difference of 0.7 $\log_{10}$ cfu/g by day 15. The gaseous concentrations of rosemary and thyme oil volatile compounds were 9.0 and 17.8 ppm, respectively at day 15.

Release of rosemary and thyme oil from MCF occurred at high relative humidity in the cheese packages. In a separate experiment it was observed that the rosemary and thyme oil (1% and 10% w/w), once mixed into MCF were trapped, as no compound could be detected by the GC. This indicated that preparation of MCF produced a structure(s) sufficient to completely encapsulate the rosemary and thyme oil. However, in the presence of moisture the MCF released the oil compounds and when gaseous samples were analyzed by GC, two or four components from thyme or rosemary oil, respectively, were detected, and the GC profiles were identical to those of the pure oils. The total of each oil released from the MCF starch is shown in Table 4.5.

Similar observations were reported by Buttery and coworkers (1999). They analyzed the adsorption of volatile flavor compounds by MCF and their release in the presence of water. Addition of water immediately released the flavoring compounds adsorbed by MCF. Also the release of relatively polar compounds was lower than that of apolar compounds. In the present study *Listeria monocytogenes* growth was retarded as noted above in the presence of rosemary plus thyme oil vapors showing that when these compounds were present in the package headspace together they exerted antimicrobial activity. Inhibition of growth was not observed in the presence of gaseous rosemary or thyme oil when added separately, and the numbers of *L. monocytogenes* were higher than in control samples by 15 d storage. There may have been additive antimicrobial interactions between the oils when present together. Further research is needed to study the possible mechanism by which the MCF encapsulates the plant oils and the kinetics of their release at high relative humidity.

4.8.2 LAB and TAC

When the effect of gaseous rosemary and thyme oil on the growth of LAB and TAC was evaluated (Figures 4.14, 4.15), organisms were able to grow in both control and treated samples. LAB increased from 4.7 $\log_{10}$ cfu/g to 8.1 $\log_{10}$ cfu/g, whereas they increased from 5.3 $\log_{10}$ cfu/g in samples containing rosemary or thyme and rosemary and thyme oil together to 6.2, 6.8 and 6.9 $\log_{10}$ cfu/g, respectively, by 15 d storage. The oils separately or together did have a negative effect on the extent of LAB growth. With respect to the growth of TAC, the numbers increased from 5.3 $\log_{10}$ to 7.5 $\log_{10}$ cfu/g in control samples by the end of storage. Rosemary and thyme oil separately restricted growth and the numbers increased from 5.2 $\log_{10}$ cfu/g, respectively, to 6.0 and 6.1 $\log_{10}$ cfu/g, respectively, at 15 d. The latter numbers were 1.6 and 1.7 $\log_{10}$ cfu/g less than in control samples and differences were significant. When both oils were tested together, they also resulted in growth restriction and numbers increased from 5.2 to 6.1 $\log_{10}$ cfu/g (producing a significant difference of 1.4 $\log_{10}$ cfu/g). As observed in other experiments conducted, rosemary and thyme oil were not able to prevent growth of LAB and TAC. However, in this set of experiments the oils were able to restrict their growth more effectively, and results should be further explored for possible use of the oils to extend product shelf-life.

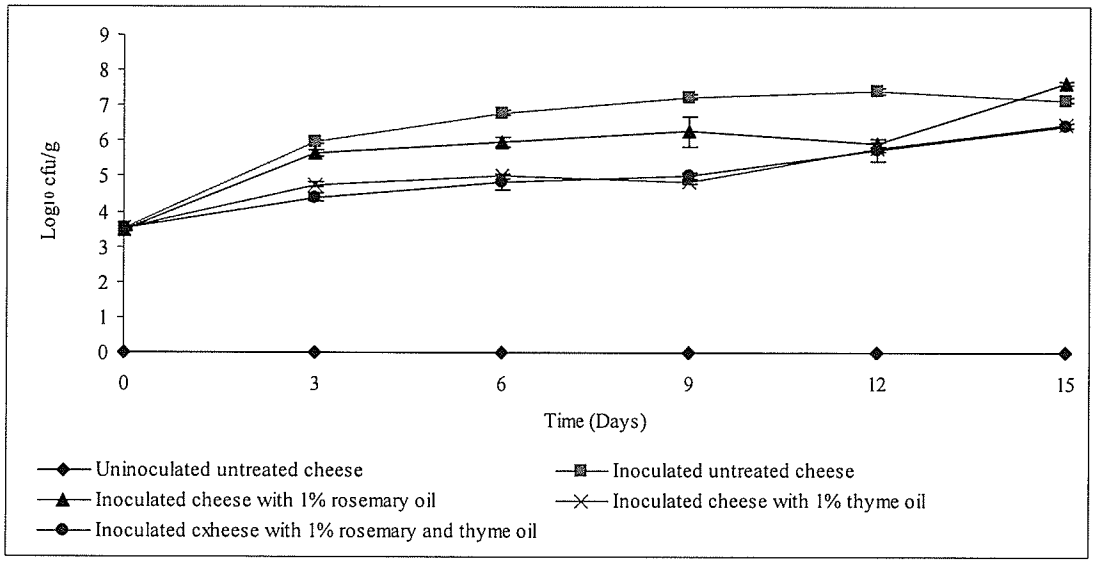


Figure 4.13. Antimicrobial activity of rosemary oil and thyme oil contained (1% w/w each) either separately or in combination in a MCF starch sachet against a cocktail of four strains of *L. monocytogenes* in shredded low-fat mozzarella cheese at 10°C. Six replicates were used to generate the standard deviation bars.

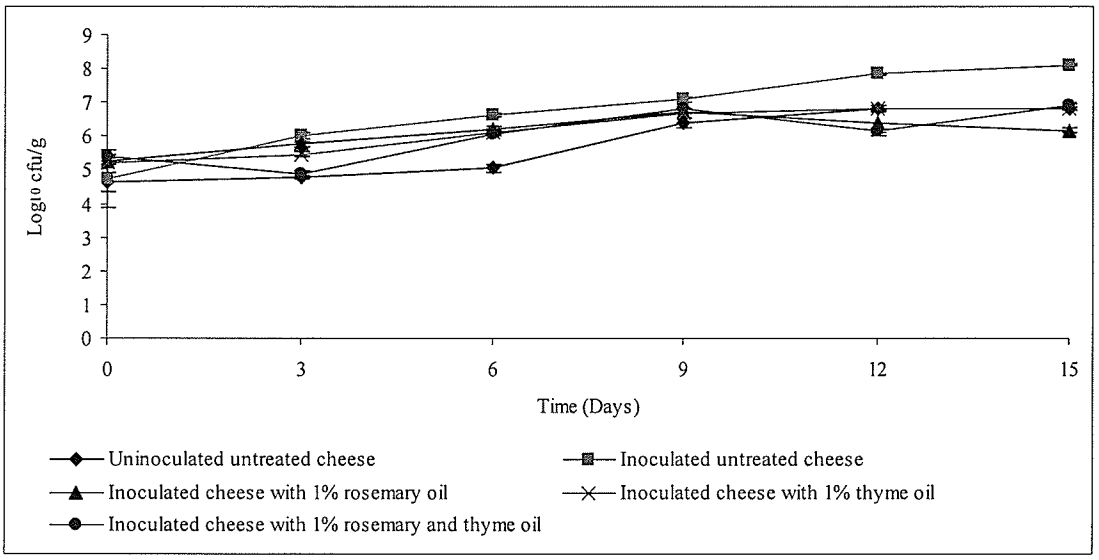


Figure 4.14. Antimicrobial activity of rosemary oil and thyme oil (1% w/w each) contained either separately or in combination in a MCF starch sachet against lactic acid bacteria (LAB) in shredded low-fat mozzarella cheese at 10°C. Six replicates were used to generate the standard deviation bars.

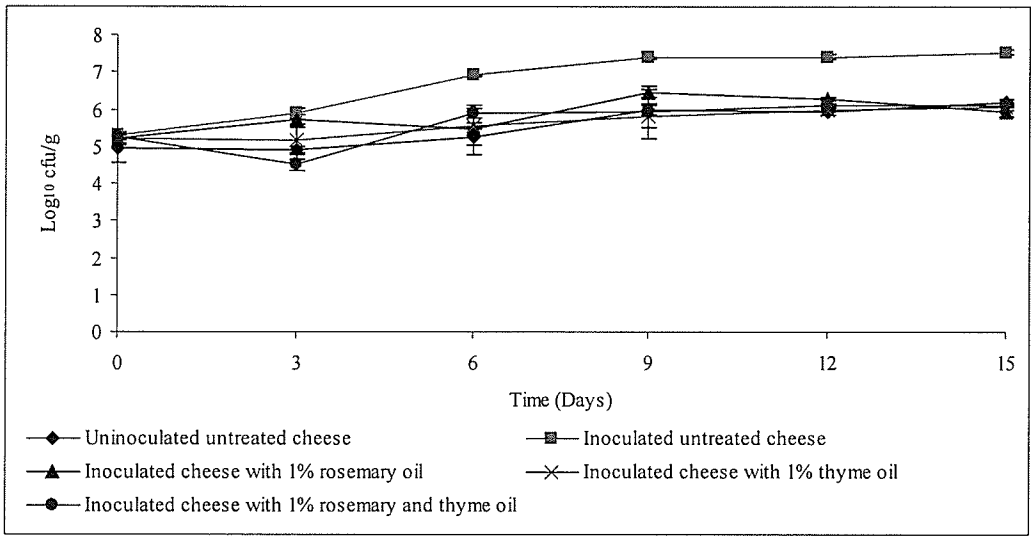


Figure 4.15. Antimicrobial activity of rosemary oil and thyme oil (1% w/w each) contained either separately or in combination in a MCF starch sachet against total aerobic count (TAC) in shredded low-fat mozzarella cheese at 10°C. Six replicates were used to generate the standard deviation bars.

Table 4.3: Identification of an essential oil resistant *L. monocytogenes* strain in cheese at 4°C.

		L. monocytogenes per g cheese[1]	
Strain	Day	T1[2]	T2[3]
C716	0	3.63±0.06	3.36±0.06
	2	3.87±0.03	3.22±0.05
	5	4.14±0.11	3.29±0.26
C717	0	3.86±0.04	3.48±0.47
	2	4.30±0.04	3.81±0.04
	5	4.98±0.07	4.19±0.05
C718	0	3.62±0.06	3.47±0.09
	2	3.99±0.05	3.32±0.04
	5	4.36±0.10	3.39±0.13
C719	0	3.67±0.08	3.45±0.10
	2	3.68±0.05	3.30±0.04
	5	3.93±0.10	3.38±0.12
C720	0	3.54±0.04	3.37±0.03
	2	3.94±0.05	3.38±0.05
	5	4.82±0.07	3.47±0.29

[1] Log_{10} cfu/g
[2] Inoculated untreated cheese
[3] Inoculated cheese with 1% rosemary and thyme oil

Table 4.4: Identification of an essential oil resistant *L. monocytogenes* strain in cheese at 10°C.

		L. monocytogenes per g cheese[1]	
Strain	Day	T1[2]	T2[3]
C716	0	3.59±0.11	3.35±0.06
	2	3.87±0.03	3.22±0.05
	5	4.46±0.13	3.38±0.09
C717	0	3.84±0.06	3.47±0.46
	2	4.30±0.04	3.81±0.04
	5	5.09±0.15	4.26±0.05
C718	0	3.62±0.06	3.47±0.09
	2	3.99±0.05	3.32±0.04
	5	4.69±0.15	4.08±0.03
C719	0	3.67±0.08	3.45±0.10
	2	3.68±0.05	3.30±0.04
	5	4.18±0.06	3.38±0.12
C720	0	3.54±0.04	3.37±0.03
	2	3.94±0.05	3.38±0.05
	5	4.82±0.07	3.47±0.29

[1] Log_{10} cfu/g
[2] Inoculated untreated cheese
[3] Inoculated cheese with 1% rosemary and thyme oil

Table 4.5: Headspace concentration of rosemary and thyme oil as released from MCF in the presence of water

Oil	Concentration (%w/w) in MCF	Concentration (ppm) in headspace
Rosemary	1	1.25
	10	1.26
Thyme	1	1.17
	10	1.55

Chapter 5

CONCLUSIONS

In this research, we examined the use of rosemary and thyme oil as natural antimicrobial agents as well as the weak organic acid sodium diacetate as against *Listeria monocytogenes* in cheese. Based on the results obtained, the conclusions drawn were as follows:

1. The volatile rosemary and thyme oils were highly effective against a cocktail of *L. monocytogenes* in agar medium. They both proved to be lethal against *L. monocytogenes* when analyzed at 10°C.

2. One of the five strains used in this research was shown to be more resistant to the volatile oils than the other strains during individual strain challenge tests.

3. Rosemary and thyme oils were not effective against *L. monocytogenes* when used in full fat cheese at 4 or 10°C. Rosemary and thyme oil either separately or together in inoculated cheese produced a statistically significant reduction in the extent of *L. monocytogenes* growth, however, this difference was not microbiologically significant.

4. The fat content of cheese seemed to play an important role in influencing the antimicrobial activity of rosemary and thyme oil. Combination of the oils in low fat cheese was more effective in controlling the growth of the pathogen.

5. Addition of sodium diacetate alone as well as together with rosemary and thyme oil showed listeriostatic activity at 4°C. *L. monocytogenes* numbers remained unchanged throughout storage for 20 d. Treatment of cheese with sodium diacetate alone or together with rosemary and thyme oil was also effective in controlling the growth of *L. monocytogenes* at 10°C.

6. When the oils were released from a microcellular foam starch matrix contained in a sachet inserted into packages to control the growth of *L. monocytogenes* in cheese, they suppressed the growth of the pathogen. However, growth was observed throughout storage for 15 d at 10°C. The oils failed to eliminate *L. monocytogenes* from cheese when the pathogen was inoculated at 3 $\log_{10}$ cfu/g.

Chapter 6

RECOMMENDATIONS FOR FUTURE RESEARCH

It may be useful to define the upper limit of rosemary and thyme oil use in cheese by organoleptic evaluation to determine whether higher concentrations of the oils would be more effective in the cheese against *L. monocytogenes*.

Determine the effectiveness of rosemary and thyme oil in combination with other plant essential oils (eg. oregano) against *L. monocytogenes* in cheese. Partitioning of the oils in the fat fraction of exposed foods should be verified analytically.

Study the antimicrobial activity of rosemary and thyme oils together with bacteriocins and other enzymes (eg. lysozyme) against *L. monocytogenes* in cheese and other food products.

The mechanism by which the MCF starch binds and releases plant essential oils should be studied in order to allow effective use of higher concentrations of these oils in packaged foods. The kinetics of oil release and volatilization should be documented.

Investigate the possible ways by which the release of volatile antimicrobials by MCF starch can be altered (change their vapor pressure) to meet the need for designing effective antimicrobial packaging systems for different food products.

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Table 2.1: Human listeriosis outbreaks related to cheese

Country	Year	Product	Level (cells/g)	Number of cases (Number of deaths)	Reference
Switzerland	1983-1987	Raw milk cheese	10^4-10^6	122 (31)	Farber and Peterkin (1991)
CA, USA	1985	Jalisco cheese	10^2-10^4	142 (48)	Farber and Peterkin (1991)
UK	1986	Soft cheese	-	1	Prasad and Gupta (1990)
UK	1989	Aneri cheese from goat milk	-	1	Prasad and Gupta (1990)
Luxembourg	1989	Camembert cheese	-	2	De Buyser et al. (2001)
Denmark	1989-1990	Hard and Blue cheese	-	26 (6)	De Buyser et al. (2001)
France	1995	Brie de Meaux cheese from raw milk	-	36 (4)	De Buyser et al. (2001)
France	1997	Pont-Leveque cheese from raw milk	-	14	De Buyser et al. (2001)
NC, USA	2000	Mexiican-style soft cheese	-	12	CDC (2001)
Canada	2002	Soft and semi-hard cheese	-	17	CCDR (2003)

Table 2.2: Incidence of *Listeria monocytogenes* in cheese

Country	Type of cheese	Samples analyzed (Positive samples)	Reference
Canada	Soft and semi soft cheese	182 (4)	Farber et al. (1987)
Italy	Soft cheese	1284 (65)	Comi et al. (1990)
Portugal	Semi hard cheese from raw sheep milk	9 (4)	Guerra et al. (2001)
	Hard cheese from pasteurized sheep and goat milk	12 (9)	
Chile	Soft cheese	256 (2)	Cordano and Rocourt (2001)
Austria	Red smear cheese	10 (1)	Rudolf and Scherer (2001)
France	Red smear cheese	150 (5)	Rudolf and Scherer (2001)
Germany	Red Smear cheese	120 (11)	Rudolf and Scherer (2001)
Italy	Red Smear cheese	23 (4)	Rudolf and Scherer (2001)
Spain	Soft cheese	99(1)	Vitas and Garcia-Jalon (2003)
Japan	Natural cheese (imported)	1387 (33)	Okutani et al. (2004)

Table 2.3: Antilisterial activity of rosemary and thyme oil in laboratory media

Antimicrobial Agent	System	MIC	Inhibition zone[a] (concentration)	Reference
Rosemary spice	Plate count agar	0.9% w/v	-	Ting and Deibel, 1992
Rosemary oil	Nutrient agar	-	15 (10 µL)	Deans and Ritchie (1987)
Rosemary oil	TSA	-	7.1 (25 µL)	Smith-Palmer et al. (1998)
Rosemary oil	TSB	0.1% v/v	-	Smith-Palmer et al. (1998)
Rosemary spice extract	TSB	0.5% v/v	-	Campo et al. (2000)
Thyme oil	TSA		10 (25 µL)	Smith-Palmer et al. (1998)
Thyme oil	TSB	0.03% v/v	-	Smith-Palmer et al. (1998)
Black thyme oil	Nutrient agar	-	33.5 (50 µL)	Baydar et al. (2004)
Thyme oil	Agar	-	25 (3 µL)	Firouzi et al. (1998)

[a] Inhibition zone diameter is expressed in terms of mm

Table 2.4: Activity of rosemary and thyme oil against *L. monocytogenes* in food systems

Antimicrobial Agent	Food	Concentration	Effect[1]	Reference
Rosemary oil	Fresh pork sausage	0.5% ground rosemary or 1% rosemary oil	b	Pandit and Shelef (1994)
Rosemary oil	Queso Fresco cheese	2500 ppm	a	Mendoza-Yepes et al. (1997)
Thyme oil	Chicken breast	2%	c	Hao et al. (1998)
Thyme oil	Low fat cheese	1%	a	Smith-Palmer et al. (2001)
	Full fat cheese	1%	a	
Thyme oil	Hot dog	10 ml/L	c	Singh et al. (2003)

[1] a= bactericidal effect
b= bacteriostatic effect
c= little or no effect

Table 2.5: Use of natural antimicrobials in development of antimicrobial packaging systems

Antimicrobial	Packaging material	Antimicrobial concentration	Test system	Target organism	Reference
Pediocin	Packaging bag	7.75 mg/cm^2	Turkey breasts Ham Beef	*L. monocytogenes*	Ming et al. (1997)
Nisin	LDPE[1]	0.1% w/w	Beef carcass	*B. thermospacta*	Siragusa et al. (1999)
Nisin	LLDPE[2] Nylon PVC[3]		Broiler skin	*S.* Typhimurium	Natrajan and Sheldon (2000a)
Nisin	Ca-alginate	500 µg/ml	Poultry skin	*S.* Typhimurium	Natrajan and Sheldon (2000b)
Nisaplin	Cellulose paper PE: polyamide	2560 AU[10]/cm^2	Cheese	*L. innocua* *S. aureus*	Scannell et al. (2000)
Nisin	Corn zein	0.188 mg/film		*L. monocytogenes*	Hoffman et al. (2001)
Nisin	HPMC[4]	1000 IU[9]/ml	Tryptose broth agar medium	*Micrococcus luteus*	Coma et al. (2001)
Nisin	HPMC	5×10^3 IU/ml	Nutritive agar	*L. innocua*	
Nisin	Corn Zein	1000 IU/ml	Chicken	*L. monocytogenes*	Janes et al. (2002)
Clove extract	Chitosan	5% w/w	0.1 M potassium citrate buffer	*L. plantarum*	Hong et al. (2000)
AIT	OPP[5]/EVOH[6]/PELD[7]/PELLD[8]	1 µL	Bread	*Aspergillus flavus*	Neilsen and Rios (2000)

Grape fruit seed extract	LDPE	0.5-1%	Ground beef	coliform	Ha et al. (2001)
Grape fruit	Na-alginate	0.1% w/w	BHI agar	*M. luteus*	Cha et al. (2002)
Seed extract	κ-carrageenan	0.1% w/w	BHI agar	*L. innocua* *S. aureus*	
Oregano	Chitosan	2% w/w	Bologna	L. monocytogenes E. coliO157:H7	Zivanovic et al. (2005)

[1] Low density polyethylene
[2] Linear low density polyethylene
[3] Polyvinyl chloride
[4] Hydroxy propyl methyl cellulose
[5] Oriented poly propylene
[6] Ethylene-vinyl alcohol
[7] Polyethylene (Low density)
[8] Polyethylene (Linear low density)
[9] International Unit

Appendix 1: Antimicrobial activity of rosemary and thyme oil against a five strain cocktail of *L. monocytogenes* in full fat (23%) mozzarella cheese stored at 4°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	3.70±0.10^{a}	5.26±0.08^{a}	6.62±0.08^{a}	7.36±0.02^{a}	7.48±0.04^{b}	7.59±0.08^{a}
Inoculated cheese with 1% rosemary oil	3.71±0.07^{a}	4.89±0.07^{b}	6.34±0.09^{a}	7.02±0.10^{b}	7.61±0.07^{a}	7.67±0.04^{a}
Inoculated cheese with 1% thyme oil	3.48±0.07^{b}	4.43±0.47^{c}	5.82±0.36^{b}	6.54±0.32^{c}	6.93±0.06^{c}	7.51±0.19^{a}
Inoculated cheese with 1% rosemary and thyme oil	3.71±0.06^{a}	4.40±0.04^{c}	4.61±0.29^{c}	5.11±0.17^{d}	5.85±0.11^{d}	6.12±0.18^{b}
Uninoculated untreated cheese	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 2: Antimicrobial activity of rosemary and thyme oil against total aerobic count (TAC) in full fat (23%) mozzarella cheese stored at 4°C

	TAC per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	6.60±0.06^{a}	6.73±0.03^{a}	6.89±0.24^{a}	7.55±0.12^{a}	7.61±0.12^{a}	7.56±0.22^{a}
Inoculated cheese with 1% rosemary oil	6.24±0.10^{a}	6.21±0.08^{b}	6.62±0.14^{a,b}	7.05±0.17^{b}	7.69±0.11^{a}	7.72±0.06^{a}
Inoculated cheese with 1% thyme oil	5.68±0.09^{b}	5.78±0.29^{c}	5.70±0.33^{c}	6.54±0.25^{c}	7.19±0.14^{b}	7.79±0.09^{a}
Inoculated cheese with 1% rosemary and thyme oil	6.35±0.44a	6.58±0.03^{a}	6.48±0.31^{a,b}	6.35±0.15^{c}	6.40±0.14^{d}	6.41±0.20^{c}
Uninoculated untreated cheese	6.26±0.14^{a}	6.63±0.05^{a}	6.25±0.23^{b}	5.75±0.37^{d}	6.93±0.21^{c}	7.07±0.15^{b}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 3: Antimicrobial activity of rosemary and thyme oil against lactic acid bacteria (LAB) in full fat (23%) mozzarella cheese stored at 4°C

	LAB per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	5.94±0.26^{a}	6.31±0.13^{a,b}	6.67±0.23^{a}	7.56±0.10^{a}	7.50±0.09^{a}	7.62±0.02^{a}
Inoculated cheese with 1% rosemary oil	5.28±0.41^{b,c}	6.01±0.03^{b}	6.35±0.12^{a}	7.13±0.12^{b}	7.60±0.10^{a}	7.52±0.10^{a}
Inoculated cheese with 1% thyme oil	4.97±0.24^{c}	5.14±0.41^{c}	6.54±0.29^{a}	6.47±0.16^{c}	7.50±0.11^{a}	7.36±0.08^{a}
Inoculated cheese with 1% rosemary and thyme oil	5.96±0.09^{a}	6.24±0.03^{a,b}	6.55±0.29^{a}	6.20±0.12^{d}	6.24±0.12^{b}	6.33±0.38^{b}
Uninoculated untreated cheese	5.66±0.24^{a,b}	6.43±0.10^{a}	5.85±0.07^{b}	5.63±0.11^{e}	5.47±0.08^{c}	5.56±0.08^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 4: Antimicrobial activity of rosemary and thyme oil against a cocktail of five strains of *L. monocytogenes* in full fat (23%) mozzarella cheese stored at 10°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	3.72±0.08^{a}	7.30±0.20^{a}	7.60±0.08^{a}	7.66±0.09^{a}	7.53±0.12^{a}	7.55±0.08^{a}
Inoculated cheese with 1% rosemary oil	3.57±0.10^{a,b}	7.26±0.20^{a}	7.51±0.05^{a}	7.60±0.13^{a}	7.65±0.04^{a}	7.59±0.07^{a}
Inoculated cheese with 1% thyme oil	3.45±0.11^{b}	6.47±0.26^{b}	6.82±0.10^{b}	7.07±0.09^{b}	7.15±0.38^{b}	6.80±0.08^{b}
Inoculated cheese with 1% rosemary and thyme oil	3.58±0.14^{a,b}	6.12±0.09^{c}	6.12±0.30^{c}	6.19±0.09^{c}	6.25±0.11^{c}	6.17±0.07^{c}
Uninoculated untreated cheese	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 5: Antimicrobial activity of rosemary and thyme oil against total aerobic count (TAC) in full fat (23%) mozzarella cheese stored at 10°C

	TAC per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	6.62 ± 0.05^{a}	7.89 ± 0.03^{a}	$8.12\pm0.02^{b,c}$	8.93 ± 0.06^{a}	7.98 ± 0.06^{b}	9.13 ± 0.11^{a}
Inoculated cheese with 1% rosemary oil	6.25 ± 0.05^{b}	7.59 ± 0.12^{c}	$8.43\pm0.26^{a,b}$	6.85 ± 0.15^{c}	8.65 ± 0.11^{a}	8.57 ± 0.17^{c}
Inoculated cheese with 1% thyme oil	5.70 ± 0.14^{c}	$7.70\pm0.10^{b,c}$	8.62 ± 0.13^{a}	$8.73\pm0.12^{a,b}$	8.78 ± 0.12^{a}	8.89 ± 0.06^{b}
Inoculated cheese with 1% rosemary and thyme oil	6.08 ± 0.05^{b}	7.89 ± 0.12^{a}	8.00 ± 0.43^{c}	8.61 ± 0.08^{b}	8.78 ± 0.14^{a}	8.59 ± 0.09^{c}
Uninoculated untreated cheese	6.19 ± 0.19^{b}	$7.86\pm0.09^{a,b}$	8.56 ± 0.18^{a}	6.75 ± 0.17^{c}	8.68 ± 0.06^{a}	8.80 ± 0.02^{b}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 6: Antimicrobial activity of rosemary and thyme oil against lactic acid bacteria (LAB) in full fat (23%) mozzarella cheese stored at 10°C

	LAB per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	6.08±0.22^{a}	7.22±0.05^{b,c}	8.83±0.10^{a}	8.77±0.34^{a}	8.90±0.02^{a}	8.65±0.11^{b}
Inoculated cheese with 1% rosemary oil	5.84±0.11^{a}	7.33±0.12^{b}	7.60±0.07^{c}	8.78±0.34^{a}	7.63±0.06^{c}	7.65±0.06^{d}
Inoculated cheese with 1% thyme oil	5.17±0.17^{b}	7.86±0.11^{a}	8.70±0.11^{a}	8.72±0.09^{a}	8.79±0.06^{a,b}	8.83±0.04^{a}
Inoculated cheese with 1% rosemary and thyme oil	5.96±0.07^{a}	7.06±0.12^{c}	8.15±0.38^{b}	8.49±0.14^{a}	8.75±0.08^{b}	8.72±0.04^{a,b}
Uninoculated untreated cheese	5.78±0.32^{a}	7.11±0.20^{b,c}	7.01±0.16^{d}	8.75±0.06^{a}	7.08±0.08^{d}	7.78±0.10^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 7: Antimicrobial activity of rosemary and thyme oil against a four strain cocktail of *L. monocytogenes* in full fat (23%) mozzarella cheese stored at 10°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	3.62±0.10^{b}	7.67±0.04^{a}	8.07±0.07^{a}	7.70±0.16b^{,c}	8.08±0.07^{a}	8.21±0.04^{a}
Inoculated cheese with 1% rosemary oil	3.79±0.02^{a}	7.47±0.07^{b}	7.92±0.02^{b}	7.95±0.08^{a}	7.90±0.06^{b}	7.90±0.05^{b}
noculated cheese with 1% thyme oil	3.66±0.08^{b}	7.47±0.07^{b}	7.70±0.10^{c}	7.85±0.06^{a,b}	7.97±0.10^{a,b}	7.88±0.05^{b}
Inoculated cheese with 1% rosemary and thyme oil	3.55±0.09^{b}	5.44±0.07^{c}	7.54±0.07^{d}	7.66±0.12^{c}	7.86±0.15^{b}	7.77±0.05^{c}
Uninoculated untreated cheese	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 8: Antimicrobial activity of rosemary and thyme oil against lactic acid bacteria (LAB) in full fat (23%) mozzarella cheese stored at 10°C

	LAB per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	4.29±0.23^{b}	8.14±0.01^{d}	8.01±0.07^{d}	8.16±0.12^{b}	8.21±0.04^{b}	8.20±0.09^{b}
Inoculated cheese with 1% rosemary oil	4.27±0.20^{b}	6.28±0.28^{b}	6.99±0.05^{b}	7.78±0.05^{c}	7.93±0.08^{c}	7.99±0.07^{c}
noculated cheese with 1% thyme oil	4.21±0.18^{a}	7.45±0.08^{c}	7.50±0.04^{c}	7.55±0.04^{d}	7.65±0.06^{d}	7.57±0.05^{d}
Inoculated cheese with 1% rosemary and thyme oil	5.48±0.12^{a}	7.19±0.10^{e}	7.22±0.02^{e}	7.27±0.16^{d}	7.24±0.06^{d}	7.18±0.05^{d}
Uninoculated untreated cheese	5.26±0.07^{b}	5.40±0.10^{a}	6.67±0.08^{a}	7.09±0.11^{a}	7.16±0.05^{a}	7.14±0.12^{a}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 9: Antimicrobial activity of rosemary and thyme oil against total aerobic count (TAC) in full fat (23%) mozzarella cheese stored at 10°C

	TAC per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	4.73±0.14^{c}	6.94±0.22^{b}	8.23±0.06^{a}	8.47±0.06^{b}	8.54±0.03^{a}	8.50±0.02^{a}
Inoculated cheese with 1% rosemary oil	5.69±0.14^{a}	7.68±0.04^{a}	8.23±0.18^{a}	8.26±0.13^{b}	8.25±0.03^{a}	8.28±0.02^{b}
noculated cheese with 1% thyme oil	5.51±0.11^{a,b}	7.53±0.09^{a}	8.40±0.09^{a}	8.47±0.07^{b}	8.46±0.32^{a}	8.51±0.03^{a}
Inoculated cheese with 1% rosemary and thyme oil	5.37±0.06^{b}	5.90±0.16^{c}	8.39±0.10^{a}	8.63±0.07^{a}	8.49±0.03^{a}	8.53±0.05^{a}
Uninoculated untreated cheese	5.37±0.09^{b}	7.11±0.11^{b}	7.88±0.08^{b}	8.03±0.08^{d}	8.17±0.02^{a}	8.19±0.03^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 10: Antilisterial activity of rosemary and thyme oil against a cocktail of four strains of *L. monocytogenes* in full fat (23%) mozzarella cheese stored at 4°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	3.29±0.06^{a}	5.14±0.18^{a}	6.58±0.02^{a}	7.44±0.04^{a}	7.57±0.10a,^{b}	7.40±0.05^{c}
Inoculated cheese with 1% rosemary oil	3.24±0.05^{a}	5.26±0.18^{a}	6.40±0.04^{a}	7.15±0.15^{b}	7.71±0.12^{a}	7.77±0.02^{a}
noculated cheese with 1% thyme oil	3.24±0.06^{a}	4.83±0.12^{b}	5.90±0.23^{b}	6.76±0.16^{c}	7.49±0.08^{b}	7.56±0.07^{b}
Inoculated cheese with 1% rosemary and thyme oil	3.23±0.03^{a}	4.66±0.14^{b}	5.58±0.16^{c}	6.56±0.02^{d}	7.54±0.07^{b}	7.73±0.05^{a}
Uninoculated untreated cheese	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{d}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 11: Antimicrobial activity of rosemary and thyme oil against total aerobic count (TAC) in full fat (23%) mozzarella cheese stored at 4°C

	TAC per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	6.34±0.13^{a}	6.71±0.13^{a}	7.11±0.05^{a}	7.55±0.03^{a}	7.59±0.06^{a}	7.53±0.07^{a}
Inoculated cheese with 1% rosemary oil	6.42±0.07^{a}	6.79±0.10^{a}	6.99±0.08^{a}	7.46±0.09^{a}	7.59±0.06^{a}	7.59±0.07^{a}
noculated cheese with 1% thyme oil	6.29±0.06^{a}	6.39±0.07^{b}	6.50±0.09^{c}	6.98±0.06^{b}	7.10±0.02^{c}	7.17±0.02^{b}
Inoculated cheese with 1% rosemary and thyme oil	6.37±0.04^{a}	6.44±0.06^{b}	6.52±0.09^{b,c}	7.08±0.12^{b}	7.22±0.06^{b}	7.28±0.05^{c}
Uninoculated untreated cheese	5.98±0.11^{b}	6.29±0.04^{b}	6.69±0.19^{b}	7.00±0.02^{b}	7.07±0.04^{c}	7.08±0.03^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 12: Antimicrobial activity of rosemary and thyme oil against lactic acid bacteria (LAB) in full fat (23%) mozzarella cheese stored at 4°C

	LAB per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	6.75±0.04^{a}	7.14±0.08^{a}	7.25±0.07^{a}	7.45±0.06^{a}	8.07±0.04^{b}	7.98±0.04^{b}
Inoculated cheese with 1% rosemary oil	6.57±0.02^{a}	6.94±0.08^{b}	7.04±0.06^{b}	7.21±0.06^{b}	8.18±0.04^{a}	8.23±0.01^{a}
noculated cheese with 1% thyme oil	5.86±0.16^{b}	5.96±0.07^{d}	6.62±0.06^{d}	6.79±0.06^{c}	7.42±0.07^{c}	7.82±0.04^{c}
Inoculated cheese with 1% rosemary and thyme oil	5.89±0.10^{b}	6.00±0.08^{d}	6.52±0.04^{d}	6.85±0.16^{c}	7.69±0.07^{c}	8.04±0.01^{b}
Uninoculated untreated cheese	6.75±0.16^{a}	6.71±0.07^{c}	6.88±0.06^{c}	7.10±0.09^{b}	7.72±0.05^{c}	7.98±0.05^{b}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 13: Antimicrobial activity of sodium diacetate (0.2% w/w) alone as well as in combination with rosemary and thyme oil against a four strain cocktail of *L. monocytogenes* in full fat (23%) mozzarella cheese stored at 4°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	3.73±0.04^{b}	4.18±0.20^{b,c}	5.46±0.11^{a}	6.13±0.03^{b}	7.43±0.04^{a}	7.99±0.02^{a}
Inoculated cheese with 1% rosemary oil	3.82±0.02^{a}	4.70±0.09^{a}	4.94±0.11^{b}	5.71±0.06^{c}	7.49±0.29^{a}	7.68±0.11^{b}
Inoculated cheese with 1% thyme oil	3.69±0.03^{b}	4.08±0.07^{c,d}	5.02±0.06^{b}	6.38±0.05^{a}	7.33±0.07^{a}	7.69±0.15^{b}
Inoculated cheese with 1% rosemary and thyme oil	3.67±0.05^{b}	4.36±0.11^{b}	5.13±0.05^{b}	6.27±0.05^{a,b}	7.26±0.10^{a}	7.43±0.06^{b}
Inoculated cheese with 0.2% sodium diacetate	3.74±0.03^{b}	3.96±0.10^{d}	4.15±0.25^{c}	4.13±0.22^{d}	4.19±0.15^{b}	4.04±0.36^{c}
Inoculated cheese with all three antimicrobials together	3.57±0.08^{c}	3.57±0.08^{e}	3.80±0.03^{d}	3.67±0.05^{e}	3.65±0.11^{c}	3.77±0.04^{c}
Uninoculated untreated cheese	0.00±0.00^{d}	0.00±0.00^{f}	0.00±0.00^{e}	0.00±0.00^{f}	0.00±0.00^{d}	0.00±0.00^{d}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 14: Antimicrobial activity of sodium diacetate (0.2% w/w) alone as well as in combination with rosemary and thyme oil against lactic acid bacteria (LAB) in full fat (23%) mozzarella cheese stored at 4°C

	LAB per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	4.73±0.16^{d}	4.55±0.15^{d}	5.63±0.03^{a,b}	6.20±0.10^{a}	7.54±0.12^{a}	7.82±0.17^{a}
Inoculated cheese with 1% rosemary oil	6.11±0.12^{a}	5.64±0.13^{a}	5.77±0.07^{a,b}	5.79±0.05^{b}	7.29±0.15^{a}	7.73±0.04^{a,b}
Inoculated cheese with 1% thyme oil	5.70±0.06^{b}	4.73±0.04^{c,d}	5.60±0.13^{b}	6.41±0.12^{a}	7.39±0.03^{a}	7.79±0.05^{a,b}
Inoculated cheese with 1% rosemary and thyme oil	6.06±0.08^{a}	5.29±0.16^{b}	5.84±0.07^{a}	6.39±0.04^{a}	7.35±0.09^{a}	7.52±0.07^{b}
Inoculated cheese with 0.2% sodium diacetate	5.84±0.07^{b}	5.67±0.07^{a}	5.58±0.10^{b}	5.06±0.38^{c}	4.90±0.25^{b}	4.79±0.30^{c}
Inoculated cheese with all three antimicrobials together	5.82±0.11^{b}	4.88±0.11^{c}	5.70±0.07^{a,b}	4.42±0.14^{d}	4.22±0.22^{c}	4.35±0.12^{d}
Uninoculated untreated cheese	5.09±0.12^{c}	4.55±0.09^{d}	4.70±0.22^{c}	4.59±0.27^{d}	4.78±0.30^{b}	4.40±0.15^{d}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 15: Antimicrobial activity of sodium diacetate (0.2% w/w) alone as well as in combination with rosemary and thyme oil against the total aerobic count (TAC) in full fat (23%) mozzarella cheese stored at 4°C

	TAC per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	5.22±0.09c	5.17±0.14d	5.56±0.10c	6.09±0.05b	7.44±0.11b	7.73±0.10a,b
Inoculated cheese with 1% rosemary oil	6.35±0.04a,b	6.49±0.16a	5.90±0.04a	5.79±0.12c	7.62±0.12a	7.71±0.12a,b
Inoculated cheese with 1% thyme oil	6.14±0.07b	5.68±0.05c	5.66±0.04b,c	6.45±0.06a	7.39±0.07b,c	7.76±0.03a
Inoculated cheese with 1% rosemary and thyme oil	6.43±0.04a	6.47±0.17a	5.93±0.03a	6.34±0.04a	7.27±0.07c	7.53±0.08b
Inoculated cheese with 0.2% sodium diacetate	6.29±0.03a,b	6.10±0.14b	5.78±0.12a,b,c	5.65±0.06c	5.57±0.08d	5.30±0.12e
Inoculated cheese with all three antimicrobials together	6.24±0.05a,b	6.15±0.24b	5.88±0.08a,b	5.69±0.07c	5.62±0.05d	5.60±0.06d
Uninoculated untreated cheese	5.29±0.28c	5.19±0.07d	4.91±0.24d	5.11±0.29d	5.48±0.06d	6.00±0.10c

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 16: Antimicrobial activity of sodium diacetate (0.2% w/w) alone as well as in combination with rosemary and thyme oil against a cocktail of 4 strains of *L. monocytogenes* in full fat (23%) mozzarella cheese stored at 10°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	3.75±0.04^{b}	6.52±0.02^{a}	6.95±0.18^{a}	7.04±0.10^{a}	7.08±0.13^{a}	7.24±0.11^{a}
Inoculated cheese with 1% rosemary oil	3.87±0.02^{a}	6.23±0.02^{b}	6.78±0.07^{a,b}	6.74±0.08^{b}	6.74±0.07^{b}	6.78±0.10^{c}
Inoculated cheese with 1% thyme oil	3.82±0.04^{a,b}	6.25±0.09^{b}	6.85±0.05^{a}	6.94±0.10^{b}	6.87±0.03^{b}	6.99±0.07^{b}
Inoculated cheese with 1% rosemary and thyme oil	3.76±0.04^{b}	5.76±0.06^{c}	6.67±0.10^{b}	6.96±0.07^{a}	7.20±0.15^{a}	7.18±0.06^{a}
Inoculated cheese with 0.2% sodium diacetate	3.79±0.02^{b}	4.85±0.14^{d}	5.76±0.10^{c}	6.04±0.05^{c}	6.00±0.08^{c}	6.12±0.17^{e}
Inoculated cheese with all three antimicrobials together	3.55±0.05^{c}	3.79±0.06^{e}	4.42±0.03^{d}	5.62±0.06^{c}	6.17±0.07^{c}	6.42±0.07^{d}
Uninoculated untreated cheese	0.00±0.00^{d}	0.00±0.00^{f}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{f}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 17: Antimicrobial activity of sodium diacetate (0.2% w/w) alone as well as in combination with rosemary and thyme oil against lactic acid bacteria (LAB) in full fat (23%) mozzarella cheese stored at 10°C

	LAB per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	5.21±0.06^{d}	6.55±0.11^{c}	7.43±0.11^{b}	7.73±0.05^{b}	8.08±0.09^{c}	8.44±0.04^{b,c}
Inoculated cheese with 1% rosemary oil	6.25±0.15^{a}	7.65±0.09^{a}	8.24±0.12^{a}	8.27±0.01^{a}	8.87±0.05^{a}	8.88±0.06^{a}
Inoculated cheese with 1% thyme oil	5.93±0.15^{b}	7.11±0.08^{b}	8.32±0.02^{a}	8.46±0.04^{a}	8.66±0.09^{b}	8.72±0.09^{a,b}
Inoculated cheese with 1% rosemary and thyme oil	5.98±0.13^{a,b}	5.80±0.13^{d}	6.85±0.16^{c}	8.27±0.02^{a}	8.69±0.08^{a,b}	8.89±0.03^{a}
Inoculated cheese with 0.2% sodium diacetate	5.58±0.21^{c}	5.75±0.12^{d}	6.01±0.17^{d}	6.79±0.27^{c}	7.60±0.16^{d}	7.82±0.17^{d}
Inoculated cheese with all three antimicrobials together	5.68±0.23^{b,c}	5.28±0.13^{e}	5.33±0.14^{e}	6.16±0.05^{c}	7.32±0.14^{e}	7.73±0.35^{d}
Uninoculated untreated cheese	5.57±0.22^{c}	5.32±0.26^{e}	6.70±0.16^{c}	7.61±0.17^{b}	8.00±0.08^{c}	8.36±0.14^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 18: Antimicrobial activity of sodium diacetate (0.2% w/w) alone as well as in combination with rosemary and thyme oil against total aerobic count (TAC) in full fat (23%) mozzarella cheese stored at 10°C

	TAC per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	6.15±0.12^{c}	7.19±0.04^{c}	7.86±0.13^{a}	7.75±0.07^{d}	8.27±0.06^{b}	8.46±0.08^{c}
Inoculated cheese with 1% rosemary oil	6.59±0.04^{a}	7.66±0.04^{a}	7.72±0.09^{a}	7.88±0.05^{b,c}	8.79±0.07^{a}	8.95±0.06^{a}
Inoculated cheese with 1% thyme oil	6.29±0.05^{b}	7.36±0.05^{b}	7.83±0.08^{a}	7.95±0.02^{a,b}	8.58±0.10^{a}	8.70±0.05^{b}
Inoculated cheese with 1% rosemary and thyme oil	6.51±0.05^{a}	6.77±0.11^{d}	6.96±0.07^{b}	8.02±0.03^{a}	8.65±0.09^{a}	8.89±0.04^{a,b}
Inoculated cheese with 0.2% sodium diacetate	6.22±0.05^{b,c}	6.62±0.13^{e}	6.81±0.15^{b}	7.80±0.11^{c,d}	8.15±0.10^{b}	8.52±0.09^{c}
Inoculated cheese with all three antimicrobials together	6.21±0.06^{b,c}	6.04±0.05^{f}	5.90±0.06^{c}	6.82±0.05^{e}	7.29±0.19^{c}	8.11±0.17^{d}
Uninoculated untreated cheese	6.30±0.06^{b}	7.32±0.05^{b,c}	7.74±0.14^{a}	7.80±0.05^{c,d}	8.32±0.12^{b}	8.72±0.14^{b}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 19: Antilisterial activity of rosemary and thyme oil against a cocktail of four strains of *L. monocytogenes* in low fat (15%) mozzarella cheese stored at 4°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	3.35±0.09[a]	3.82±0.13[a]	5.13±0.26[a]	5.60±0.12[a]	6.14±0.15[a]	6.50±0.09[a]
Inoculated cheese with 1% rosemary oil	3.54±0.26[a]	3.40±0.15[b]	4.19±0.41[b]	4.72±0.13[b]	5.70±0.15[b]	6.00±0.12[b]
noculated cheese with 1% thyme oil	3.58±0.09[a]	3.35±0.12[b]	3.89±0.06[b]	4.37±0.12[c]	4.74±0.07[c]	5.51±0.09[c]
Inoculated cheese with 1% rosemary and thyme oil	3.40±0.11[a]	3.30±0.18[b]	3.93±0.13[b]	4.00±0.09[d]	4.58±0.16[c]	4.80±0.25[d]
Uninoculated untreated cheese	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[e]	0.00±0.00[d]	0.00±0.00[e]

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 20: Antimicrobial activity of rosemary and thyme oil against lactic acid bacteria (LAB) in low fat (15%) mozzarella cheese stored at 4°C

	LAB per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	4.58±0.07^{a}	7.28±0.09^{a}	8.07±0.02^{a}	7.45±0.06^{a}	7.78±0.04^{b}	7.94±0.04^{b}
Inoculated cheese with 1% rosemary oil	4.54±0.18^{a}	6.84±0.08^{b}	7.63±0.10^{c}	6.67±0.20^{c}	6.83±0.14^{d}	7.12±0.07^{e}
noculated cheese with 1% thyme oil	4.47±0.12^{a}	6.67±0.08^{b,c}	7.59±0.04^{c}	7.01±0.09^{b}	7.48±0.06^{c}	7.73±0.09^{c}
Inoculated cheese with 1% rosemary and thyme oil	4.39±0.12^{a}	6.74±0.14^{b,c}	7.85±0.01^{b}	7.02±0.11^{b}	7.43±0.11^{c}	7.46±0.13^{d}
Uninoculated untreated cheese	4.38±0.06^{a}	6.61±0.10^{c}	7.93±0.02^{b}	7.65±0.12^{a}	8.00±0.08^{a}	8.19±0.01^{a}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 21: Antimicrobial activity of rosemary and thyme oil against total aerobic count (TAC) in low fat (15%) mozzarella cheese stored at 4°C

	TAC per g cheese[1, 2]					
Days	0	4	8	12	16	20
Treatment						
Inoculated untreated cheese	5.35±0.10^{a}	6.04±0.10^{a}	6.74±0.07^{a}	7.52±0.06^{a}	7.75±0.10^{a}	7.80±0.10^{a}
Inoculated cheese with 1% rosemary oil	5.45±0.11^{a}	5.81±0.15^{b}	6.59±0.06^{a,b,c}	6.74±0.25^{c}	7.20±0.04^{b}	7.42±0.07^{b}
noculated cheese with 1% thyme oil	5.35±0.06^{a}	5.96±0.08^{a,b}	6.54±0.13^{b,c}	7.04±0.14^{b}	7.21±0.04^{b}	7.40±0.06^{b}
Inoculated cheese with 1% rosemary and thyme oil	5.36±0.08^{a}	5.57±0.12^{c}	6.42±0.12^{c}	7.14±0.22^{b}	7.26±0.09^{b}	7.20±0.06^{c}
Uninoculated untreated cheese	5.46±0.10^{a}	5.97±0.10^{a}	6.66±0.09^{a,b}	7.51±0.07^{a}	7.83±0.08^{a}	7.90±0.06^{a}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 22: Antimicrobial activity of rosemary and thyme oil against a four strain cocktail of *L. monocytogenes* in low fat (15%) mozzarella cheese stored at 10°C

	L. monocytogenes per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	3.61±0.11^{b}	6.76±0.11^{a}	6.83±0.06^{a}	6.62±0.13^{a}	6.62±0.15^{a}	6.67±0.05^{a}
Inoculated cheese with 1% rosemary oil	3.79±0.03^{a}	6.83±0.14^{a}	6.84±0.04^{a}	6.74±0.14^{a}	6.67±0.12^{a}	6.64±0.05^{a}
noculated cheese with 1% thyme oil	3.66±0.08^{b}	4.85±0.17^{b}	5.69±0.04^{b}	6.69±0.11^{a}	6.68±0.12^{a}	6.60±0.04^{a}
Inoculated cheese with 1% rosemary and thyme oil	3.55±0.09^{b}	4.54±0.29^{c}	5.72±0.14^{b}	6.36±0.17^{b}	6.04±0.32^{b}	6.00±0.07^{b}
Uninoculated untreated cheese	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 23: Antimicrobial activity of rosemary and thyme oil against lactic acid bacteria (LAB) in low fat (15%) mozzarella cheese stored at 10°C

	LAB per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	5.91±0.10^{a}	7.97±0.18^{a}	8.78±0.02^{a}	8.87±0.02^{a}	8.90±0.06^{a}	9.07±0.08^{a}
Inoculated cheese with 1% rosemary oil	5.99±0.08^{a}	8.04±0.02^{a}	8.67±0.05^{a,b}	8.93±0.03^{a}	8.91±0.05^{a}	9.14±0.35^{a}
noculated cheese with 1% thyme oil	5.52±0.07^{b}	6.82±0.21^{b}	8.51±0.08^{c}	8.64±0.07^{a}	8.65±0.07^{b}	8.72±0.04^{b}
Inoculated cheese with 1% rosemary and thyme oil	5.25±0.10^{c}	6.68±0.09^{b}	7.73±0.12^{d}	8.31±0.40^{b}	8.40±0.05^{c}	8.36±0.05^{c}
Uninoculated untreated cheese	5.96±0.19^{a}	7.89±0.09^{a}	8.85±0.03^{a}	8.89±0.01^{s}	8.92±0.09^{a}	9.02±0.09^{a}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)

Appendix 24: Antimicrobial activity of rosemary and thyme oil against total aerobic count (TAC) in low fat (15%) mozzarella cheese stored at 10°C

	TAC per g cheese[1, 2]					
Days	0	3	6	9	12	15
Treatment						
Inoculated untreated cheese	5.50±0.11^{a}	6.85±0.49^{b}	7.86±0.16^{a}	8.10±0.08^{a}	8.24±0.03^{a}	8.26±0.05^{a}
Inoculated cheese with 1% rosemary oil	5.79±0.07^{a}	7.51±0.11^{a}	7.80±0.22^{b}	8.02±0.08^{a,b}	8.15±0.04a,^{b}	8.24±0.06^{a}
noculated cheese with 1% thyme oil	5.66±0.02^{a}	6.12±0.03^{c}	8.10±0.22^{a}	7.95±0.01^{a,b}	8.04±0.10^{b}	8.09±0.04^{b}
Inoculated cheese with 1% rosemary and thyme oil	5.44±0.10^{a}	6.22±0.05^{c}	7.52±0.07^{b}	7.84±0.27^{b,c}	8.05±0.12^{b}	8.11±0.03^{b}
Uninoculated untreated cheese	5.61±0.11^{a}	6.34±0.41^{c}	7.82±0.18^{a,b}	7.74±0.14^{c}	8.02±0.05^{b}	8.08±0.02^{b}

[1] Log_{10} cfu/g
[2] Numbers in columns with the same superscript letters are not significantly different ($p>0.05$)