Elite Herb Extracts Containing High Rosmarinic Acid and Inhibition of *Listeria monocytogenes*

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(The research that is an integral part of this report formed the basis of the MS thesis of Andy Seaberg and will be part of continuation of doctoral work by Yuan-Lin Tong)

Abstract

Food safety continues to be a major concern for the food industry in recent years. One of the industry's top priorities has been to find alternative ways to preserve their existing and newly developed foods while satisfying the increasing consumer demand to produce safe, all-natural products. In order to achieve this "clean label", much research has been devoted to the use of effective plant-based antimicrobials, such as those from herbs and spices, to replace chemical preservatives. However, due to the cross-pollination character of herbs and spices, there is a lot of genetic heterogeneity among different batches of the same plant species. This poses a problem for the routine use of plants, and their extracts, as a barrier towards microbial growth. In order to combat this, a unique tissue-culture-based selection strategy was used to isolate an elite phenolic phytochemical-producing clonal line of oregano (*Origanum vulgare*). Ethanol extracts of this elite clonal line of oregano were then used to study its inhibitory action against *Listeria monocytogenes* in both broth and meat systems. Thymol and carvacrol, two of the main phenolic constituents of oregano extracts, were also tested in both systems to evaluate their activity against that of the whole oregano extract. Initial results indicate that thymol, carvacrol, and the clonal oregano line were all effective in inhibiting the growth of *L. monocytogenes* in both systems. Approximately 150-200 ppm of pure carvacrol or thymol was needed in order to significantly inhibit the growth of *L. monocytogenes* in broth, while at least 1200 ppm (corresponding to 27.8 µg phenolics/ml) of the elite clonal oregano extract was needed to do the same. Inconclusive results were obtained when the clonal line was compared to store-brand samples of oregano. In meat systems, 800 ppm of the oregano extract was able to significantly inhibit the growth of the pathogen more so than 800 ppm of carvacrol. A possible explanation for this is that the oregano extract was able to work more effectively at the interface of the lipid and water-soluble portions of the meat due to higher content of rosmarinic acid than the carvacrol alone These results are promising for the food industry since we have now developed an approach for a highly consistent and reliable natural source of antimicrobial activity for further studies and evaluation for applications.

Introduction

The contamination of food by foodborne pathogens is still a major problem for the food industry despite the wide range of preservation techniques available (e.g., freezing, drying, preservatives). The number of outbreaks continues to increase in the United States. Part of this may be due to better detection techniques but a larger part of it is probably due to changes in the eating habits of people and to the more tolerant nature of emerging pathogens. The introduction of minimally processed foods has created many new concerns for food safety since these products have few barriers to microbial growth. Many times, refrigeration is used as the primary barrier for these types of foods. This poses a problem since one of the most dangerous foodborne pathogens present in the United States, *Listeria monocytogenes*, can grow at refrigerated temperatures. *L. monocytogenes* is a gram-positive, facultatively anaerobic, non-spore-forming rod that has been implicated in many outbreaks, especially in the last 25 years. Because of the dangerous health effects associated with *L. monocytogenes*, especially towards pregnant women, the United States government has issued a zero tolerance policy for *L. monocytogenes* in food.

The main barrier used in the food industry to prevent foodborne pathogens, such as *L. monocytogenes*, is to use chemical preservatives. But with consumer preferences towards a "clean label", the food industry has to respond accordingly and look for other methods to inhibit the growth of dangerous microorganisms in food besides using chemicals. One alternative that can work is to use antimicrobials that are plant-based. Since these substances are generally recognized as safe (GRAS), effective plant-based antimicrobials, such as those from herbs and spices, could replace chemical preservatives. Furthermore, many studies have been done to show the powerful antimicrobial activity of different types of phenolic compounds present in herbs and spices. However, due to the cross-pollinating character of herbs, there is a lot of genetic heterogeneity among different batches of the same plant species. This poses a problem for the routine use of plant-based extracts as a barrier towards microbial growth. One solution to this problem is to screen for high phenolic producing clonal lines from the original heterogeneous bulk population. This would create a reliable source for antimicrobial applications. Our research focused on the use of extracts of an elite clonal line of oregano (*Origanum vulgare*) to inhibit the growth of *L. monocytogenes* in food systems. These elite high rosmarinic acid clonal extracts were compared to other heterogeneous oregano samples that were found in supermarkets as well as to thymol and carvacrol, two of the major phenolic compounds found in the extracts of oregano that have been previously evaluated by other research groups.

Objectives

1) To evaluate the effectiveness of high rosmarinic acid-containing herb extracts to inhibit *L.monocytogenes* in laboratory media.

2) To evaluate the effectiveness of high rosmarinic acid-containing herb extracts in ready to eat meat and poultry products**.**

Literature Review

Important Challenges to Food Safety

Food safety has become a major concern in the food industry in recent years. In the United States alone, there are an estimated 6-33 million cases of foodborne illness each year (Shetty & Labbe, 1998). Also, the number of outbreaks has been increasing over the years. Some of this may be attributed to better methods to detect foodborne illnesses but a larger, more significant factor, may be due to changes in eating habits. Besides the common food vehicles responsible for foodborne illness, such as raw poultry and raw meat, other types of food are becoming are problem. One such food in demand is "fresh" minimally processed foods. These foods include melon, carrots, alfalfa sprouts, and unpasteurized apple cider. As the demand for these products has increased, so too has the incidents of foodborne illness (Shetty and Labbe, 1998).

In addition to fruits and vegetables, the food industry has also been addressing the interests of time-constrained consumers. This has led to many new trends in the food industry. One such trend is the explosive growth in sales of value-added prepared meats, poultry, and seafood (Hao et al, 1998). These refrigerated ready-to-eat entrees are becoming an important class of products in supermarket and convenience stores. Refrigeration is often the principal, and sometimes only factor relied upon to control foodborne pathogens in these types of foods. Hence, temperature abuse of such foods could lead to foodborne illness. In addition, some psychrotrophic pathogens can grow in refrigerated foods with little or no obvious change in sensory quality (Berrang et al., 1989). The result of all these measures is that we now have more serious and unexpected sources of foodborne pathogens.

Background Information on *Listeria monocytogenes*

One important pathogen that has been a major concern to food safety in recent years is *Listeria monocytogenes*. *L. monocytogenes* is a gram-positive, facultatively anaerobic, non-spore-forming rod that has been implicated in many outbreaks, especially in the last 25 years (Table 1). This psychrotrophic bacterium grows over a wide temperature range of 2.5 - 44.0 °C (Seelinger, 1961), thus refrigerated foods are a large concern, in regards to Listeria outbreaks, in the food industry. Also, this pathogen can grow over a wide pH range, although they grow best in the range of 6-8 (Jay, 1996). This is also a concern to the food industry since many foods fall within these pH ranges, especially meat products. *L. monocytogenes* can also grow at low a_w. In a study done using trypticase soy broth base at a pH of 6.8 and a 30°C incubation, the minimum a_w that permitted growth was 0.92 with sucrose as a humectant (Petran et al., 1989). In another study using brain heart infusion broth (BHI), three humectants, and 30°C incubation, the minimum a_w that permitted growth of serotypes 1, 3a, and 4b of *L. monocytogenes* revealed the following: with glycerol 0.90, 0.93 with sucrose, and 0.92 with NaCl (Farber et al., 1992). Based on these studies, *L. monocytogenes* can be viewed as high as second, only to *Staphylococcus aureus*, as foodborne pathogens being able to grow at a_w values < 0.93.

Year	Source	Cases/Deaths	Location
1953	Raw milk	2/1	Germany
1959	Fresh meat/poultry[a]	4/2	Sweden
1960–61	Various/unknown	81/?	Germany
1966	Milk/products	279/109	Germany
1979	Veggies/milk?[b]	23/3	Boston
1980	Shellfish	22/6	New Zealand
1981	Cole slaw	41/18	Canada
1983	Pasteurized milk[b]	49/14	Boston
1983–87	Vacherin Mont D'Or	122/34	Switzerland
1985	Mexican-style cheese	142/48	California
1986–87	Vegetables?[b]	36/16	Philadelphia
1987–89	Pâté	366/63	United Kingdom
1987	Soft cheese	1	United Kingdom
1988	Goats' milk cheese	1	United Kingdom
1988	Cooked-chld-chick.	1	United Kingdom
1988	Cooked-chld-chick.	2	United Kingdom
1988	Turkey franks	1	Oklahoma
1989	Pork sausage	1	Italy
1988	Alfalfa tablets	1	Canada
1989	Salted mushrooms	1	Finland
1989	Shrimp	9/1	United States (Conn.)
1989	Pork sausage	1	Italy
1990	Raw milk	1	Vermont
1990	Pork sausage	1	Italy
1990	Pâté	11/6	Australia
1991	Smoked mussels	3/0	Australia
1992	Smoked mussels	4/2	New Zealand
1992	Goat meat (from Calif.)	1	Canada
1992	Pork tongue in jelly	279/85	France
1993	Pork rillettes	39/0	France
1994	Chocolate milk	52/0	USA
1994	Pickled olives	1	Italy
1995	Brie cheese	17/0	France

[a]Suspected.
[b]Epidemiologically linked; organisms not found.

Table 1. Some of the suspected and proven foodborne Listeriosis outbreaks and cases (from Jay, 1996).

Listeria are widely distributed in nature and can be found on decaying vegetation and in soils, animal feces, sewage, silage, and water (Jay, 1996). In foods, the organism has been found in raw and improperly pasteurized milk; soft cheese; fresh and frozen meat, poultry, and seafood products; and on fruits and vegetables. It is interesting to note that in a study done by Green (1990), 2.8% of a variety of ready-to-eat meats from 4105 processing plants throughout the United States were positive for this organism. This represents a very high and dangerous number for American consumers. Also, the most common type of serovar found in outbreak cases for *L. monocytogenes*, by far, is 4b, followed by 1/2a and 1/2 b (Cossart et al., 1989).

The most common clinical picture of listeriosis is of meningitis and/or septicemia (Eley, 1992). Usually, listeriosis is only associated with people whose cell-mediated immunity is thought to be impaired. This group of people would include: unborn and newborn infants, pregnant women, elderly, and individuals who are

immunocompromised (such as AIDS patients). Most healthy persons who are infected with Listeria are probably symptomless or suffer only mild symptoms that go unnoticed. Pregnant women who contract the disease may not present any symptoms, but when they do they are often mild and influenza-like (Jay, 1996). These symptoms often include nausea, vomiting, abdominal pain, and fever. The incubation period may be as short as one day or may take up to 3 weeks, depending on how heavily contaminated the food was. Unfortunately, the consequence of listeriosis in pregnant women is abortion, premature birth, or stillbirth. Because of these consequences and because of the increasing number of outbreaks of *L. monocytogenes*, the United States government instituted a zero-tolerance policy towards *L. monocytogenes* in 50 g samples. Any food that contains this organism can be considered adulterated and, thus, be subject to recall and/or seizure.

Current Techniques Used in the Food Industry to Inhibit Foodborne Pathogens

In order to prevent infections from Listeria or any other foodborne pathogen, cooking and pasteurizing methods can be employed. These are effective ways to inhibit the growth of foodborne pathogens; however, their use is limited. Foods such as fruits and vegetables are not appropriate towards cooking and pasteurization since their sensory and textural properties will change. Other procedures, such as irradiation, have been slow to be approved and adopted (Shetty and Labbe, 1998).

Currently, the food processing industry uses chemical preservatives and chemical antimicrobials to inhibit food spoilage by chemicals, enzymes, and microorganisms and to reduce the risk of foodborne pathogens entering into the marketplace. Common preservatives used are BHA, BHT, and TBHQ and common antimicrobials are benzoic acid, sorbic acid, and sulfites. However, consumers often do not prefer the inclusion of artificial or chemical additives (Shetty and Labbe, 1998). Such compounds do not satisfy the concept of "natural" and "healthy" food that consumers prefer and that the food industry, consequently, needs to develop. Therefore, the use of natural preservatives to replace the synthetic forms is gaining importance and studies have shown that the consumer is willing to pay higher prices for natural products (Stroh, 1993). Since plant extracts and other naturally occurring plant products are classified as GRAS (generally recognized as safe) substances, their inclusion into food products to control foodborne pathogens may be one solution to this problem.

Phenolic Compounds and Their Antimicrobial Activity

Several biological systems are known to contain many different types of antimicrobial compounds, which play an important role in the natural defense or competition systems of all types of living organisms. Plants, such as spices and herbs, are well known to inhibit the growth of bacteria, yeasts, and molds. In fact, one class of compounds present in plants that are responsible for this effect is the phenolic compounds. Many studies have been done to prove this and will be discussed below. Phenolic compounds in plant foods include a wide range of compounds and a broad spectrum of functional activities. In simple chemical terms, phenolic compounds are characterized by having an aromatic ring bearing one or more hydroxy substituents,

including functional derivatives (Fenema, 1996). These compounds have been considered important in plant foods because of their flavor and color (particularly with enzymatic browning reactions) (Fenema, 1996). However, much of the current interest with these compounds lie with fact that they have been shown to have potential health benefits (Ho et al., 1992), antioxidant activity (Pratt, 1992), anti-inflammatory properties (Lopez Arnaldos et al., 1994), and antimicrobial effects (Beuchat, 1976).

One of the many plants whose extracts have been studied extensively for antimicrobial activity is oregano (*Origanum vulgare*). Oregano belongs to the family Labiatae (*Lamiaceae*), which also includes thyme and rosemary. The major component of commercial essential oils of oregano is carvacrol, which was found as high as 60-75 % (Fleisher and Sheer, 1982). The other major component found in these samples is thymol. These two phenolic compounds, thymol and carvacrol (Figure 3), are generally reported in ratios of 1:10-1:20 (Salzer, 1977). The reported antimicrobial properties of carvacrol and thymol are 1.5 and 20.0 times that of phenol respectively (Aeschbach et al, 1994).

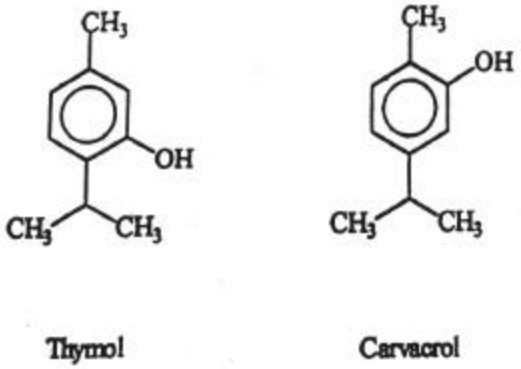

Figure 1. Chemical structures of the phenolic compounds, thymol and carvacrol.

Many studies have been done to display the antimicrobial activity of oregano and two of its major components, carvacrol and thymol. Beuchat (1976) reported that the growth of the foodborne pathogen, *Vibrio parahaemolyticus*, was delayed by the presence of 100 ppm of the essential oils of oregano ant thyme. Shetty and Labbe (1998) reported that 150 ppm of either carvacrol or thymol inhibited the growth of *E. coli 0157:H7* in tryticase soy broth at pH 6 (pH near that of meat). Also, Kim et al.(1995) reported that carvacrol was the most antibacterial phenolic compound they tested against five foodborne pathogens (*Escherichia coli*, *E. coli 0157:H7*, *Salmonella typhimurium*, *L. monocytogenes*, and *Vibrio vulnificus*). The paper disk assay was used to test each phenolic compound against the five foodborne pathogens. Carvacrol was shown to have a larger zone of inhibition than did the other phenolic compounds at the same concentrations (citral, geraniol, terpineol, perillaldehyde, eugenol, linalool, and citronellal).

Studies done with hurdle technology have also been applied to these phenolic compounds. In one particular study, nisin was used in combination with carvacrol to inhibit the growth of *Bacillus cereus* and *L. monocytogenes* (Pol and Snid, 1999). One of the results found in this study was that at 20°C, growth of *L. monocytogenes* was completely inhibited in the presence of a concentration of nisin that was 16-fold lower

(when used in combination with carvacrol) than when nisin was applied as the sole preservative. Similar results were found for *B. cereus*. Thus, using carvacrol in combination with nisin did increase the effectiveness of the antimicrobial and allowed it to be used at much lower concentrations than it was used previously. Hurdle technology was also used in a study done by Karatzas et al. (2001), which showed the combined action of carvacrol and high hydrostatic pressure on *L. monocytogenes*. The results of this study indicated that carvacrol and high hydrostatic pressure act synergistically and the antimicrobial effects of the combined treatment were greater at lower temperatures.

Terpenes and Terpenoid Biosynthesis

Plants produce thousands of compounds that contain one or more phenolic residues. Most of these compounds arise from a common biosynthetic intermediate, phenylalanine (phenylpropanoid pathway) or its close precursor shikimic acid, both produced via the shikimate pathway (Goodwin and Mercer, 1983). These compounds include: phenols, phenolic acids, hydroxycinnamic acids, coumarins, quinines, lignans, tannins, and xanthones. However, a significant number of compounds are not derived from this phenylpropanoid pathway. One class of compounds that fits this description is the terpenes. In general, the term terpene is used to denote compounds containing an integral number of 5C units, whether or not they contain other elements such as oxygen (Goodwin and Mercer, 1983). Terpenoids are compounds with varying numbers of carbon atoms clearly defined from 5C units. These compounds are found throughout a plant and contribute greatly to the essential life of the plant.

Terpenoids precursors are produced by the acetyl-CoA pathway (Goodwin and Mercer, 1983). In this pathway, 2 molecules of acetyl-CoA go through a complex set of reactions to produce ?^{3}-iso-pentenyl pyrophosphate (IPP) (Figure 2). The key precursor of IPP is the 6-C hydroxy acid, (3R)-mevalonic acid (MVA).

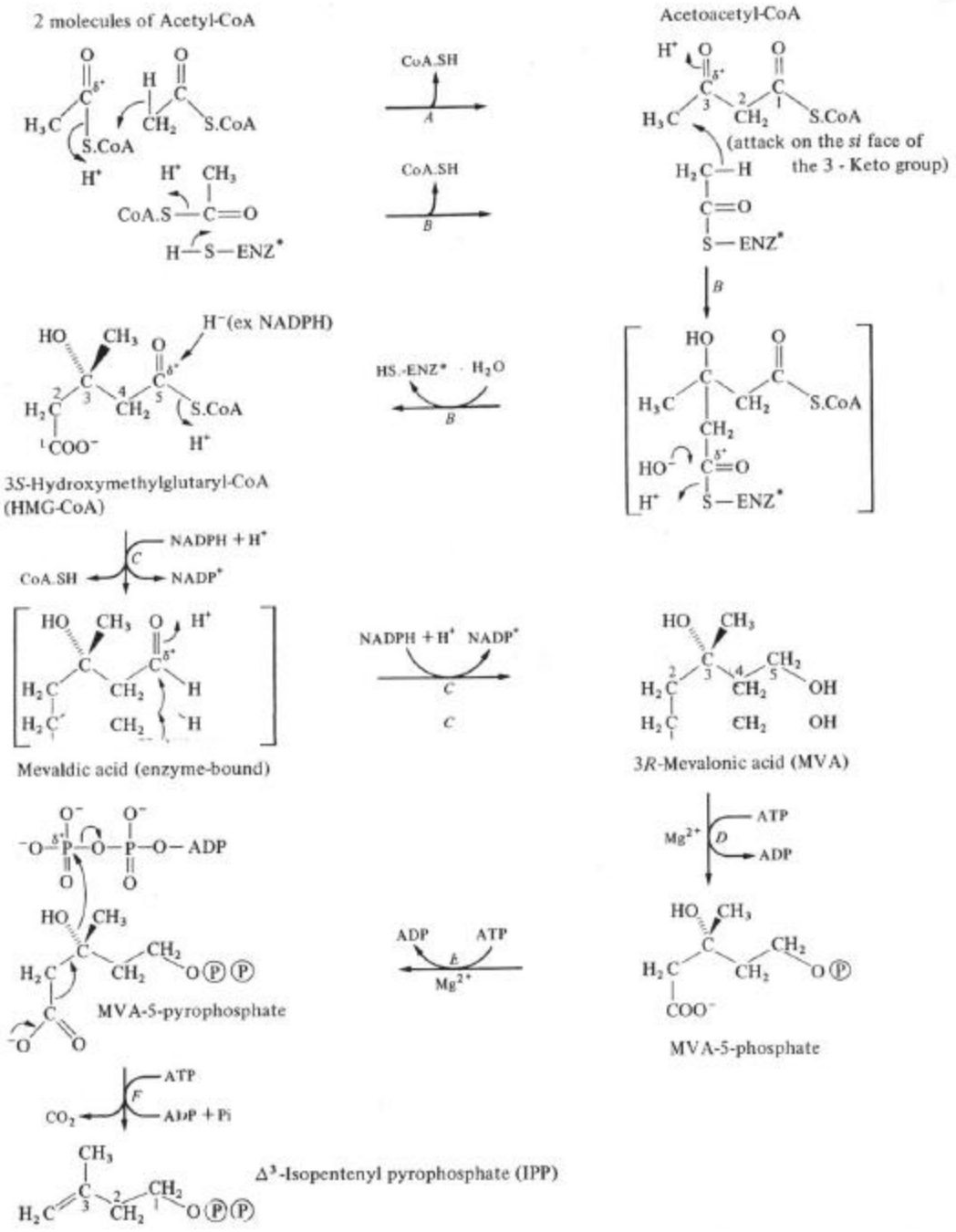

Figure 2. The formation of ?^{3}-iso-pentenyl pyrophosphate (IPP) from acetyl CoA. (A = Acetyl-CoA: acetyl-CoA C-transferase; B and ENZ* = hydroxymetylglutaryl-CoA synthase; C = hydroxymethylglutaryl-CoA reductase (NADPH); D = mevalonate kinase; E = phosphomevalonate kinase; F = pyrophosphomevalonate decarboxylase.) (from Goodwin and Mercer, 1983).

IPP is then converted into all the different terpenes and terpenoids found in nature according to the scheme shown in Figure 3. Much of the research done in food safety with terpenes has been done with thymol and carvacrol, which both have aromatic rings. Both of these compounds are cyclic monoterpenes that are phenolic in nature and will be discussed below. Monoterpenes are produced by a series of steps. First, IPP is isomerized to dimethylallyl pyrophosphate (DMAPP). DMAPP then condenses with a molecule of IPP to form the compound geranyl pyrophosphate (GPP). GPP can then be channeled into monoterpene biosynthesis to produce carvacrol or thymol. Like other phenolic pathways, this pathway is probably induced by stress response in order to drive the pathway to overproduce phenolic compounds as a defense mechanism for the plant.

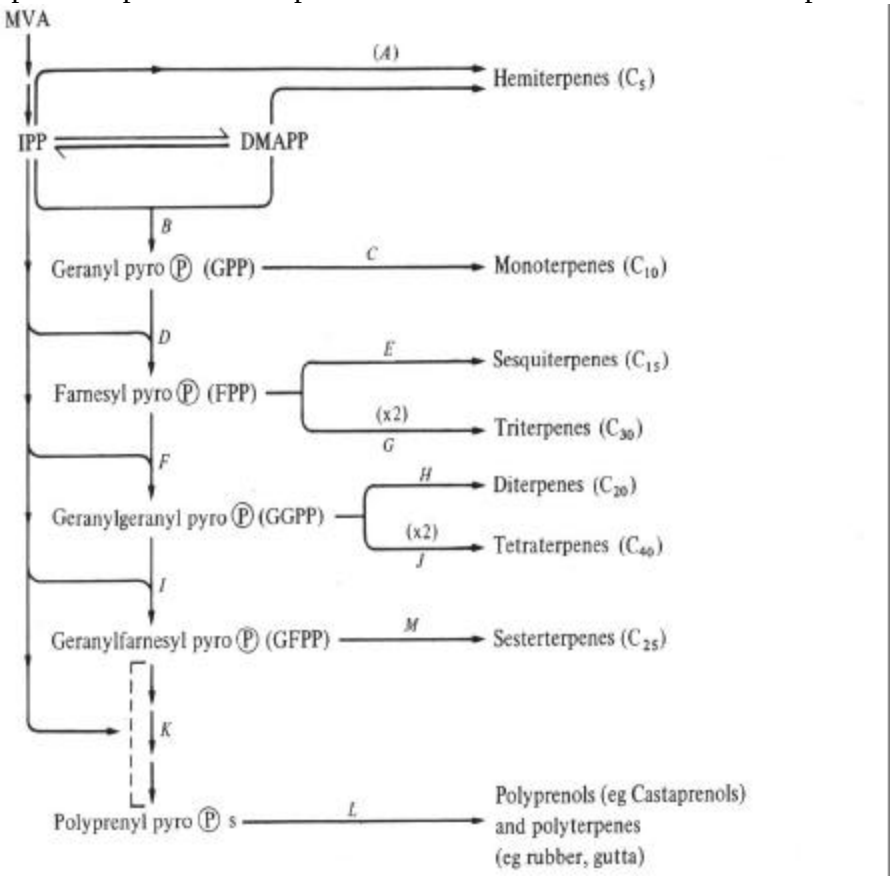

Figure 3. General scheme of terpenoid biosynthesis (MVA = (3R)-mevalonic acid; IPP = ?^{3}-isopentyl pyrophosphate; DMAPP = dimethylallyl pyrophosphate; X2 = tail-to-tail dimerization.) (from Goodwin and Mercer, 1983).

Advances in Biotechnology for Phenolic Research

As mentioned earlier, the use of herbs and spices as a potential preservative for the food industry does have excellent prospects for improving hurdle technology for controlling foodborne pathogens. However, due to their natural cross-pollination nature, these herbs and spices are genetically heterogeneous. This heterogeneity results in a high

degree of variability in the levels of phenolics in these plants (Fleisher and Sneer, 1982). Furthermore, problems associated with climate and diseases can lead to even more quality variation in the plants. This produces many serious problems for the routine use of these plants as a hurdle to inhibit the growth of foodborne pathogens in products developed by the food industry.

Biotechnology can play an important role in plant improvement. Many advantages can arise by using clonal plant propagation, also known as tissue culture, to harvest plants. Tissue culture under *in vitro* conditions in a controlled environment ensures freedom from changes in climate, temperature, and they are also free from many types of contamination (Deans et. al, 1993). Also, this technique ensures that plants will maintain their genetic integrity from generation to generation. Furthermore, tissue culture allows these plants to grow rapidly and thus creates a large production of these plants in a relatively small space (Chu and Kurtz, 1990). Clonal propagation also allows us to obtain more detailed analysis of biosynthetic pathways of primary and secondary metabolites from uniform genetic material. This also allows us to study the effects of manipulating the pathways, such as adding specific precursors of a pathway or manipulating or deleting key enzymes (Shetty and Labbe, 1998).

In order to screen for high phenolic stimulated clonal lines, microbial elicitors, such as *Pseudomonas sp*., are often employed (Al-Amier et al., 1999; Yang et al., 1997; Eguchi et al., 1996; and Shetty et al., 1996). The rationale for this screening approach is that phenolic metabolites are known to be stimulated in response to microbial elicitors (Dixon and Paiva, 1995) and, therefore, any clonal line tolerant to *Pseudomonas* must be a phenolic overexpressing phenotype (Shetty et al., 1996). By using these techniques, it is now possible to obtain a reliable and a genetically uniform plant source with excellent functionality for antioxidant and antimicrobial applications that can be used in the food industry. Using this method we have isolated clonal lines of oregano high in diphenyl. Rosmarnic acid and also also contain the terpenoid pathway derived phenols, carvacrol and thymol. Such a profile we hypothesized would be ideal for application in meat systems to control *Listeria monocytogenes*.

Materials and Methods

Tissue Culture and Clonal Propagation of Oregano
(Method was adapted from Eguchi et al, 1996)

The shoot based clonal line of oregano (extracts from experimental field grown oregano O-1 clonal line grown in a greenhouse in Massachusetts) was generated following germination of a heterogeneous seed population. This clonal line originated from a single heterozygous seed. Prior to germination, the seeds were disinfected based on earlier methods (Shetty et al., 1995). After 30 d of germination, individual shoot apices arising from multiple areas of seedling were excised aseptically and transferred to petri plates containing Murashige and Skoog (MS) medium (Sigma Chemical Co.) supplemented with 1 mg/L benzylaminopurine (BAP) (Sigma Shemical Co.) and 3 % sucrose. The initial medium pH was 5.8. Each petri plate had 8 apices with each apex having two lateral leaves below it. The petri plates containing shoot apex explants were incubated at 24°C, 16 h light cycle with a light intensity of 40 $\mu mol.\mu m^{-2}.s^{-1}$. After 30 d the shoot-apex explants had regenerated several more shoot apices through axillary shoot proliferation. Transferring the shoot apices of the clonal line to half-strength MS medium without hormones resulted in their rooting. The rooted shoots, after careful conditioning under 90 % humidity and 24°C temperature, developed into normal plants for transfer to outdoors or in a greenhouse.

Inoculation of Oregano Shoot Clones with Mucoid *Pseudomonas spp.*
(Method adapted from Eguchi et al., 1996)

To obtain an oregano plant with high amounts of total phenolics, the following procedure was used. After clonal multiplication, individual shoots of the clonal line were inoculated with mucoid *Pseudomonas sp*; strain F. This bacterium was originally isolated from oregano and it mediates a reduction of hyperhydricity which is characterized by enhanced phenolics in many clonal lines (Shetty et al, 1995). This bacterial strain was grown on yeast extract-mannitol medium (Difco Co.) until inoculum density reached 10^9 CFU/ml. The bacterial suspension was diluted to 10^{-6} in sterile distilled water and then dispersed into petri plates. Individual shoots of the clonal line of oregano were then inoculated by dipping into diluted bacterial suspension and transferred to half-strength hormone-free MS medium. After 30 d, the shoots were evaluated for total phenolics.

Total Phenolics Assay

Total phenolics were determined based on an assay originally developed by Chandler and Dodds (1983) and modified by Shetty et al. (1995). Approximately 50 mg of the oregano plant (wet basis) was placed in 2.5 ml of 95 % ethanol and kept at –4°C for 48-72 h. Each sample was then homogenized using a Tissue Tearor^{TM} (Biospec Products) and centrifuged at 13,000 rpm for 8-10 minutes. From each centrifuged sample, 1 ml of the supernatant was transferred to a 16 x 100-mm test tube and mixed

with 1 ml of 95 % ethanol and 5 ml distilled water. To each tube, 0.5 ml of 50% Folin-Ciocalteu phenol reagent (Sigma Chemical Co.) was added, vortexed, and allowed to incubate for 5 min at room temperature. Next, 1 ml of 5 % Na_2CO_3 was added to each sample and all samples were then vortexed. The samples were then covered with aluminum foil and placed in the dark for 1 h. After 1 hour elapsed, the samples were vortexed again and absorbance was measured at a wavelength of 725 nm using a UV spectrophotometer (Spectronic Genesys 5, Milton Roy Co.).

Standard curves were established for each assay using concentrations of 25, 50, 75, 100, 150, and 200 µg/ml of gallic acid in 95 % ethanol. Absorbance values were converted to mg of phenolics per gram dry weight of tissue.

Other Plant Materials

Retail samples of oregano were bought from stores in the Amherst area. This included samples of Mediterranean McCormick oregano leaves as well as CVS oregano leaves. These samples were used for comparison purposes to the clonal line O-1 oregano sample to see if the clonal line contained higher antimicrobial activity than the retail samples. All samples were stored at room temperature for all experiments.

Preparation of the Oregano Extract

Ethanol extracts were made for each oregano sample. This was done by adding 25 ml of 95 % ethanol to 1.0 g of crushed oregano leaves in a 100 ml beaker. Oregano leaves were crushed using a mortar and pestle. The sample was placed in the 2-5°C incubator for 48-72 hours with a stirbar to ensure proper mixing of the oregano leaves with the ethanol. After incubation, the sample was vacuum filtered using Whatman #1 filter paper. Washing of the beaker with additional 95 % ethanol was also done. All the ethanol oregano extract was collected into an evaporating dish and the filtered sample was placed into the 55°C incubator for at least 4 h to evaporate off the ethanol. The remaining extract was diluted to a total known volume of 25 ml into a large capped sterile test tube and vortexed for 1 minute. This sample was centrifuged at 5000 rpm for 10 minutes and the supernatant was used as the stock solution.

Growth Studies in Broth

Listeria monocytogenes 4b (Department of Food Science, University of Massachusetts, Amherst) was used for all studies. The stock culture was kept at –80°C in 15% (v/v) glycerol. The stock cultures were transferred to 9 ml sterile Tryptose Soy Broth (Difco Laboratories, Detroit, MI) with 1.5% yeast extract (Difco Laboratories). This broth solution was incubated overnight for 18-24 h to allow the *L. monocytogenes* to grow. An initial inoculum size of 1.0% (v/v) of the overnight culture was added to the 5 ml tryptose soy broth with yeast extract (TSBYE) for all the broth studies. Serial dilutions of this inoculated broth were made (when necessary) in order to adjust the initial CFU/mL of the inoculum that was applied to the samples. The initial pH of the broth varied from 5.5 –7.0 to see the combined effect of pH and phenolic compounds on the

growth of the *L. monocytogenes*. Both lactic acid and acetic acid was used to change the pH of the broth.

In order to test the pure compounds of thymol and carvacrol and their ability to inhibit the growth of *Listeria monocytogenes*, stock solutions of 1.0 % thymol and 1.0 % carvacrol were made. The solutions were kept in sterile 100 ml bottles with 50:50 mixture of distilled water and 95 % ethanol. This ratio of ethanol: water produced the best solubility for each terpene. Samples of thymol and carvacrol were added to 5 ml TSBYE broth at concentrations from 0 – 250 ppm. The broth samples with the inoculated *L. monocytogenes* and terpene was incubated in a shaking incubator (200 rev min^{-1}) at 37°C. A growth curve was made of each sample by taking spectrophotometric measurements at 600 nm on a UV spectrophotometer (Spectronic Genesys 5, Milton Roy Co.) every 2 h for 10-12 h or until a stationary phase was reached. The number of colony forming units per ml (CFU/ml) was determined for the zero time measurement (in order to compare to the CFU/ml at the end of the experiment). A description of how this is done is outlined below.

Oregano extract was tested using the same methods except the amount of extract tested was standardized and calculated on a phenolic basis (µg phenolics/ml) in addition to a total volume basis (ppm phenolics per sample). The µg phenolics/ml was tested at varying concentrations from 0.0-37.1 µg phenolics/ml. The volume studies were tested from 0-2000 ppm of oregano extract.

At the completion of the some of the growth studies, serial decimal dilutions (through 10^{-7} for high absorbance samples) from the broth were prepared. 0.1 ml of each dilution was plated on pre-poured tryptose soy agar + yeast extract (TSAYE) plates. Plates were incubated upside-down for 18-24 hrs at 37°C. The number of colony forming units per ml (CFU/ml) was determined.

Growth Studies in Meat

Listeria monocytogenes 4b was stored and grown overnight in the same manner as described in the previous section. After the *L. monocyt*ogenes cells were grown overnight, 1000 µL of the culture was pipetted into a sterile microcentrifuge tube and then centrifuged for 5 minutes at 13,000 rpm. After discarding the supernatant, 1000 µL of 0.1 % sterile phosphate buffer solution {NaCl = 7.650 g, Na_2HPO_4 (anhydrous) = 0.724 g, KH_2PO4 = 0.210 g, dH_2O = 1L, pH = 7.4} was added to the precipitate of cells left in the tube, and vortexed. After discarding the supernatant, the above procedure was repeated 2 more times to maximize the purity of the pellet of cells. After re-suspending the pellet of cells in the 1000 µL of the phosphate buffer solution, serial dilutions of the solution was made in order to decrease the amount of the initial inoculum that was applied to the exterior of each meat sample.

The meat tested in this experiment was thin-sliced USDA choice beef obtained from the Stop & Shop supermarket in Hadley, MA. All samples had 93% fat content. This set of experiments was run to mimic conditions seen in ready-to-eat foods that have already been pre-cooked. 10 g samples of the meat were autoclaved to eliminate the background microflora. The *L. monocytogenes* was applied to the cooled meat. This was done by pipetting 100 µL of the cells (in the peptone water) to the surface of the meat. The cells were spread over the surface of the entire meat sample by using a sterile glass

rod. The oregano samples, as well as the pure carvacrol and thymol samples, were treated onto the surface of the meat. These samples were adjusted to a pH of 5.5. The pH of the entire meat sample was then confirmed to be 5.5 as well by using a stomacher to crush a sample and then measuring the pH of that sample. The concentration and pH of the phenolic sample to be applied to the meat was determined based upon the results obtained from the broth studies. The phenolic sample was spread onto the surface of the meat using a sterile glass rod. Two control samples were prepared from the autoclaved beef; one that had only the test phenolic/oregano extract applied to the surface of the beef and one that had only *L. monocytogenes* spread onto the meat.

After all samples were adequately prepared, the meat was incubated for 48 h at room temperature in dark, sealed containers, which represented a temperature abuse situation. After the incubation, each sample was crushed in 90 ml of sterile 0.1% peptone water using a stomacher. Serial dilutions (up to 10^{-6}) of each sample were made using sterile 0.1 % peptone water. Spread plates were made of each sample (in duplicate) using trypticase soy agar with yeast extract and all plates were placed in an incubator set to 37°C for 12 hours. Colonies were counted and the results were reported as CFU/g for each sample. This test gave a good indication of the effect of phenolic compounds and their ability to inhibit the growth of pathogens in cooked beef samples that had been cross-contaminated with *L. monocytogenes*.

Results and Discussion

Total Phenolics of Oregano Samples

The total phenolics assay was performed on three different oregano samples over the course of three weeks. The samples included 2 commercial samples of oregano, Mediterranean McCormick oregano leaves and CVS oregano leaves, as well as a dried sample of oregano leaves from our University of Massachusetts (UMass) 0-1 clonal line. After analyzing the samples for total phenolic content, results indicated that the UMass clonal line had the highest amount total phenolics per gram of dry weight oregano (Figure 4). The general trend observed showed that as the sample was extracted in ethanol over a longer period of time, the total phenolic levels increased over time. The length of time observed in this study to obtain an adequate extraction of the phenolics suggests that the protocol developed by Chandler and Dodds (1983) and modified by Shetty (1996), should be modified further when dealing with samples on a dry weight basis (current protocol applies to samples on a wet basis only). The modification made should be to allow the sample to sit in the ethanol for at least 8 days when dealing with samples on a dry weight basis. This is required in order to achieve a better migration of phenolic compounds into the solvent. The current procedure calls for the sample to sit in ethanol for 48-72 hrs. When the original protocol was followed, the clonal line was analyzed to have a lower phenolic content (data not shown). In addition, care should be taken to make sure that all samples are stored in containers that prohibit oxidation by light. Samples of the UMass clonal oregano decreased in phenolic content over time (from 23 mg phenolics/g to 14 mg phenolics/g) when exposed to light over the course of one

month. Newer, fresher samples of the clonal oregano were used before testing of the clonal oregano began. The newer samples were kept at room temperature, but they were placed in dark, tightly sealed containers to prevent oxidation of the phenolic compounds. After the commercial samples were purchased, they too were kept under the same conditions as the clonal oregano. Periodic testing of all oregano samples were done to assure that there was minimal change in phenolic content of all samples over the course of the experiments.

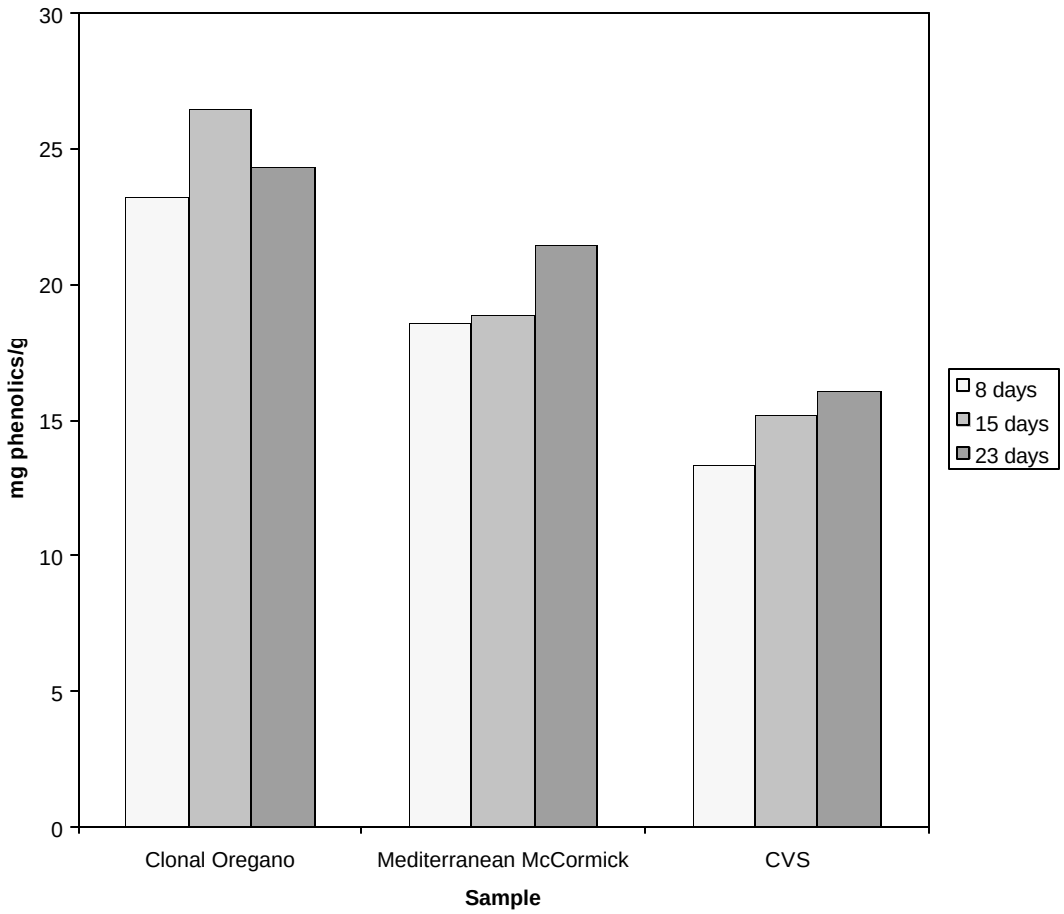

Figure 4: Comparison of total phenolic levels in various oregano samples after varying times of extractions.

The clonal line was expected to have more total phenolics than the commercial samples. As seen in Figure 4, the clonal line had considerably more total phenolics than both the Mediterranean McCormick and CVS samples. However, since these commercial samples are from heterogenetic sources, their phenolic levels should vary greatly between samples of the same company. Previous data (not shown) has shown that this would not be a problem in the clonal line.

Thymol Studies

Thymol, one of the major phenolic constituents of oregano extracts, was tested to see its efficacy in inhibiting the growth of *L. monocytogenes*. These results were compared to the results using the entire clonal oregano extract. Tests were performed, in broth, at varying pH levels in order to see any combined effect of pH and thymol inhibition. To alter the pH of the broth, both acetic acid and lactic acid were used and compared to see if any additional inhibition resulted from the type of acid used to control pH. In addition, Tween 20, an emulsifier, was added to some of the samples with the goal of observing any change in the amount of *L. monocytogenes* inhibition. Tween 20 was used since it is often utilized in the food industry as an effective emulsifier and was thus believed to bring about more interaction of the thymol solution with the aqueous broth used to cultivate the bacteria. Also, since all oregano extracts used in this study were diluted in ethanol, this study would help us to observe if the some of the major phenolic constituents of the oregano extract, such as the phenolic terpenoids, would be enhanced or hampered in their activity by incorporating the Tween 20.

As shown in Table 2 and Table 3, approximately 100 ppm to 150 ppm of thymol was required to inhibit the growth of *L. monocytogenes* in broth that had been adjusted to an initial pH of 5.5 or 6.0. Although there was less growth in the samples that were incubated in broth that was adjusted to pH = 5.5, the percent inhibition when compared to a control (samples containing no thymol) did not appear to be significantly different when the pH was raised to 6.0. These results are similar to those obtained by Shetty and Labbe (1998). In this work, thymol (or carvacrol) at concentrations of 150 ppm was also able to inhibit the growth of *E. coli 0157:H7* in trypticase soy broth at pH=6.5 (not shown). Also, in a study done by Juven et al. (1994), 175 $\mu g\ ml^{-1}$ of thymol significantly inhibited the growth of *S. typhimurium* in nutrient agar supplemented with 125 $\mu l\ l^{-1}$ of Tween 80. Less than 1 log cfu ml^{-1} was present in these samples that had this amount of thymol incorporated into the media. Thymol was also shown to be a key component in inhibiting the growth of *L. monocytogenes* in thyme essential oils (Lis-Balchin, 1997). Thyme essential oils that did not have thymol as its major component (such as Thyme sweet) did not produce the same extent of antibacterial action towards the pathogen as the thyme essential oil samples that did have thymol as one of its major components. These studies support the findings in our study that show that thymol does have powerful antimicrobial action in laboratory media.

ppm thymol	% Inhibition of Absorbance Compared to Control	
	Acetic Acid	Lactic Acid
50	0.0	16.9
100	66.2	90.5
150	98.9	98.5
200	99.3	97.6
250	99.2	99.1

Table 2: Inhibition of *L. monocytogenes* by thymol. Samples were evaluated after 30 hrs of growth in broth that was adjusted to pH 5.5 using either acetic acid or lactic acid.

ppm thymol	% Inhibition of Absorbance Compared to Control	
	Acetic Acid	Lactic Acid
50	20.4	2.0
100	89.1	99.5
150	91.8	99.9
200	97.6	99.4
250	98.9	98.6

Table 3: Inhibition of *L. monocytogenes* by thymol. Samples were evaluated after 30 hrs of growth in broth that was adjusted to pH 6.0 using either acetic acid or lactic acid.

In addition to testing the effect of pH on the growth of Listeria, two different types of acids were tested for their ability to not only adjust pH but also in their ability to inhibit the growth of microorganisms. For most of the samples, the difference in the % inhibition was either minimal or favored the lactic acid. In only one case, 50 ppm thymol at pH=6.0, did the acetic acid have a more significant inhibition of the Listeria than the lactic acid. Further testing should be conducted to verify the results seen in the experiment for the differences seen between acetic and lactic acid.

In addition to the absorbance readings, CFU/ml readings were taken after 30 hrs for 0, 50, and 100 ppm samples at pH 5.5. The pH in these samples was adjusted using acetic acid. The CFU/ml was taken in order to verify that the results obtained from the absorbance readings corresponded well with the actual count of live bacteria found in each sample.

According to the results in Table 4, there was approximately one log decrease in growth of Listeria in the presence of 50 ppm of thymol, representing a 91.8 % inhibition in growth. This data is different than the absorbance data, which reflected an inhibition of 66.2% when compared to the control. However, the data closely coincides with the lactic acid absorbance reading at the same point (90.5% inhibition). At 100 ppm, the CFU/ml was actually less than the amount that was originally inoculated at zero time. Therefore, after 30 hrs, we can infer that the sample was in its death phase. Even though there was not a complete inhibition of cells (a CFU/ml = 0), the sample was more bacteriostatic than any of the other samples. Since no log phase was detected at 100 ppm, we can conclude that there was a complete inhibition of growth of *L. monocytogenes* at this thymol concentration.

Sample (time, thymol)	CFU/ml	% Inhibition at 30 hours
0 hrs, 0 ppm	2.95×10^7	Not applicable
30 hrs, 0 ppm	2.03×10^9	0.0
30 hrs, 50 ppm	1.67×10^8	91.8
30 hrs, 100 ppm	3.37×10^6	99.8

Table 4: Growth of *L. monocytogenes* in the presence of thymol at pH 5.5. The initial pH was adjusted by acetic acid.

In separate experiments, Tween 20 was incorporated into the media to see its combined effect with thymol on the inhibition of *L. monocytogenes*. The results on Figures 5 and 6 indicate that at higher concentrations of thymol, there was an initial quenching effect of the Tween 20 after the first couple of hours of the growth study before the thymol was able to inhibit the growth of the Listeria. These results are similar to observations of Juven et al. (1994) in their study done with Tween 80. In that study, higher concentrations of Tween 80 in a medium that included thymol were not as effective as lower concentrations of Tween 80 in the same medium in inhibiting the growth of *S. typhimurium*. They concluded that Tween 80 would cause both the thymol and the membrane proteins to become less hydrophobic and thus reduce the degree of binding between them. This may have occurred in this work as well, resulting in a decreased interaction between the thymol and the Tween 20. Based on these findings, it was not recommended to use the popular Tween 20 as an emulsifier when conducting studies with plant phenolics. It would be better advised to explore other types of emulsifiers or to find an improved system for their use in order to inhibit the growth of pathogenic bacteria in hydrophilic media.

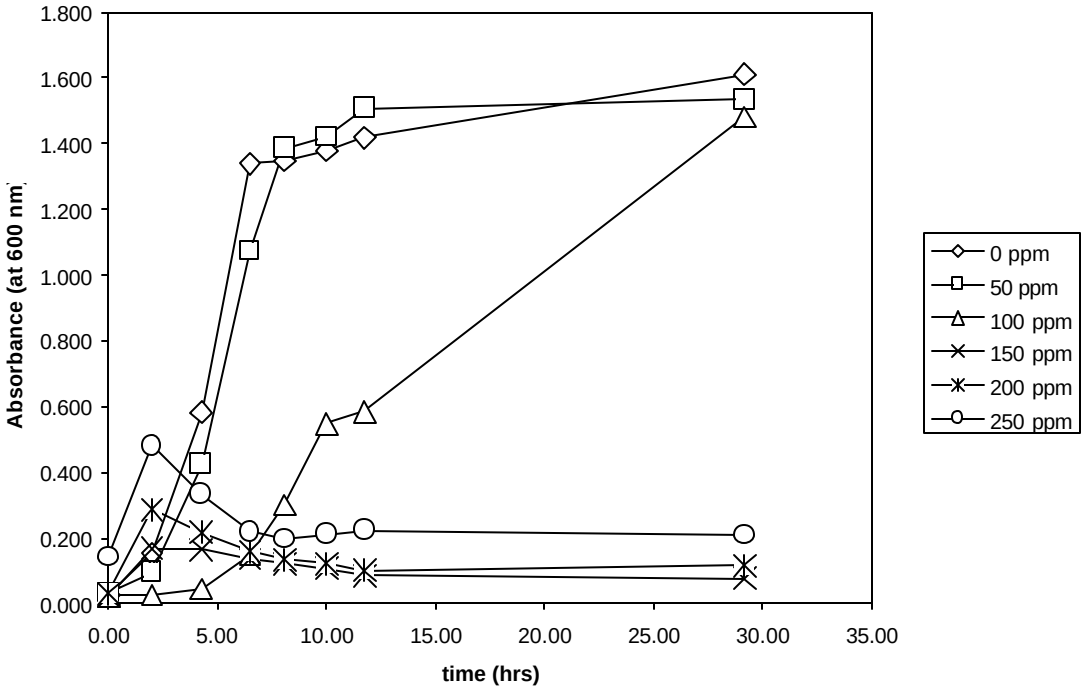

Figure 5: Effect of thymol and Tween 20 (0.1 % v/v) on the growth of *L. monocytogenes* at pH 6.0.

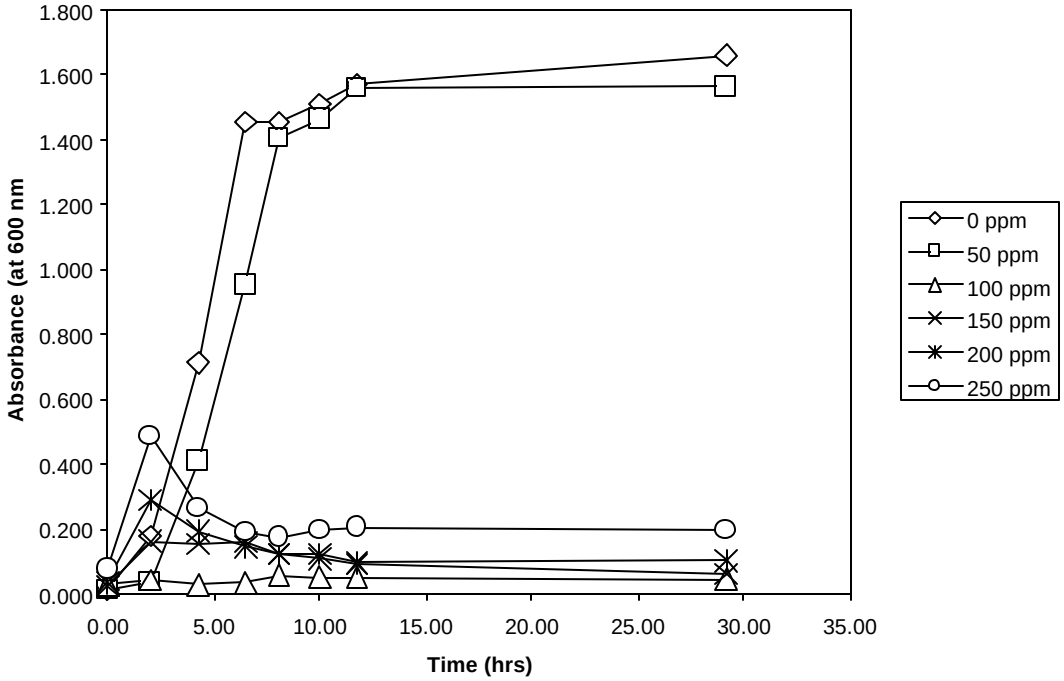

Figure 6: Effect of thymol and Tween 20 (0.1 % v/v) on the growth of *L. monocytogenes* at pH 7.0.

Carvacrol Studies

Carvacrol, another one of the major phenolic constituents of oregano extracts, was also tested to determine its efficacy in inhibiting the growth of *L. monocytogenes*. These tests mimicked those of the thymol experiments in order to directly compare the results of the tests. No Tween studies were done with the carvacrol studies. However, the effect of carvacrol when different initial CFU/ml of *L. monocytogenes* was inoculated was tested. This was done to observe if a decrease in the initial CFU/ml would alter the amount of carvacrol needed to observe an inhibition against the pathogen. Although it is useful to determine if carvacrol can inhibit the growth of a high concentration *of L. monocytogenes* (as seen in the previous experiments), it is also worthwhile to determine the extent of inhibition at concentrations that are more typical of those in contaminated food.

According to Table 5, approximately 100-150 ppm of carvacrol is required to inhibit the growth of *L. monocytogenes* in broth adjusted to pH=5.5. However, a greater concentration of carvacrol, between 150-200 ppm was required to inhibit the same amount of the pathogen at pH=6.0 (Table 6). This is in contrast to the data seen in the thymol studies where the same amount of thymol was required to inhibit *L. monocytogenes* at both pH values of 5.5 and 6.0. This is more typical of the result that is expected since an increase in pH creates a less harsh environment for the bacteria to grow, thus more carvacrol is needed to inhibit the same amount of bacteria. A possible explanation of why thymol was more inhibitory at a higher pH than carvacrol is because its slight difference in structure from carvacrol allows a greater percentage of the

terpenoid to penetrate into the cell membrane. This would allow the thymol to further destabilize the microenvironment around the cell until the bacteria cannot survive due to energy depletion and eventually die. More studies should be done involving aspects of this study as well as the possible mechanisms that occur in the bacteria in response to phenolics in order to better understand the circumstances leading to the death of the pathogen. The results discussed above are similar to those that were conducted by Shetty and Labbe (1998) and mentioned earlier in the thymol discussion. Also, Kim at al (1995) stated that, according to their studies, carvacrol showed strong bactericidal activity against all tester strains, which included *Escherichia coli, E. coli 0157:H7, Salmonella typhimurium, Vibrio vulnificus,* and *Listeria monocytogenes* using disk assay-based zones of inhibition. These studies further support the results in our experiments in which we observed the powerful inhibitory action of carvacrol towards foodborne pathogens.

ppm carvacrol	% Inhibition of Absorbance Compared to Control	
	Acetic Acid	Lactic Acid
50	-2.9	13.8
100	82.1	53.6
150	93.3	88.9
200	98.9	98.0
250	99.0	99.5

Table 5: Inhibition of *L. monocytogenes* by carvacrol. Samples were evaluated after 30 hrs of growth in broth that was adjusted to pH 5.5 using either acetic acid or lactic acid.

ppm carvacrol	% Inhibition of Absorbance Compared to Control	
	Acetic Acid	Lactic Acid
50	-1.7	11.2
100	18.2	50.4
150	74.3	75.3
200	94.6	94.1
250	96.8	98.3

Table 6: Inhibition of *L. monocytogenes* by carvacrol. Samples were evaluated after 30 hrs of growth in broth that was adjusted to pH 6.0 using either acetic acid or lactic acid.

Similar to the thymol studies, experiments were also conducted to see the added inhibitory effects of lactic and acetic acid on the growth of *L. monocytogenes*. According to Table 5, acetic acid had a greater inhibitory action against *L. monocytogenes* than lactic acid at pH 5.5. This was more profoundly seen at a carvacrol concentration of 100 ppm at pH=5.5. However, at pH=6.0, lactic acid had a slightly better inhibition against the pathogen at lower concentrations of carvacrol. At higher concentrations, minimal differences were observed between the two types of acids. Since there was no distinct, consistent difference observed between the two types of acids tested, it is concluded that either acid can be used together with the phenolic terpenoids to control pH and inhibit the growth of pathogens. However, for the remainder of the studies, acetic acid was used to control the pH of the system used. Acetic acid was already shown to be effective in

inhibiting the growth of *L. monocytogenes* in systems that had carvacrol in them. Since carvacrol is found in a higher concentration in oregano extracts than thymol, acetic acid was used for all future studies in order to contribute more to the total antimicrobial functionality in the systems tested. This decision to use acetic acid instead of lactic acid is supported by Jay (1996) who showed that at the same pH, the antimicrobial activity of acetic acid was greater than lactic acid.

In addition to these studies, an experiment was performed to determine the effect of carvacrol when the amount of the initial inoculum of *L. monocytogenes* was decreased (Table 7). The results indicate that at a lower initial CFU/ml of *L. monocytogenes*, the same amount of carvacrol had more antimicrobial activity than at larger initial CFU/ml values. The most noticeable difference occurred in the presence of 100 ppm of carvacrol, pH=6.0. At this concentration, the percentage inhibition increased dramatically, from 25.8% up to 71.6% when the initial CFU/ml of the *L. monocytogenes* was decreased from 5.8×10^7 to 7.1×10^3. This effect was even more drastic at pH=5.5, when all *L. monocytogenes* samples below an initial innoculum of 5.8×10^7, did not grow at all (data not shown). These results suggest that the size of the inoculum plays an important role in the amount of inhibition observed. This suggests that lower amounts of carvacrol could be used to inhibit the growth *L. monocytogenes* in situations that could arise in contaminated food systems.

ppm carvacrol	% Inhibition of Absorbance Compared to Control		
	5.8×10^7/ml initial	6.0×10^5/ml initial	7.1×10^3/ml initial
50	17.5	21.8	36.7
100	25.8	63.3	71.6

Table 7. Inhibition of *L. monocytogenes* by carvacrol at lower inoculum levels. Samples were evaluated after 30 hrs of growth in broth that was adjusted to pH 6.0 using acetic acid. Initial CFU/ml of *L. monocytogenes* inoculated was varied from 5.8×10^7/ml to 7.1×10^3/ml.

Oregano Studies

In the next series of studies, ethanol extracts of different oregano samples were prepared and then tested in broth systems that were inoculated with *L. monocytogenes*. All tests were done at pH of 6.0, the pH that most closely resembles that of beef-based upon the data collected from experiments conducted in the laboratory. Different initial concentrations of Listeria were tested in order to observe the amount of oregano extract needed to achieve an inhibition of the pathogen.

According to the results seen on Table 8, the UMass clonal oregano sample had slightly more inhibition of *L. monocytogenes* at 1200 ppm and at 1600 ppm than the McCormick or CVS samples did. These results were expected since the clonal line had more total phenolics than the other two extracts (Figure 4). The Elite clonal line was also confirmed to have the highest percentage of rosmarinic acid (up to 1.4% of dry weight). The most noticeable difference was at the 1200 ppm concentration where the clonal oregano was 13% more effective in inhibiting the growth of the Listeria than the CVS sample at the same oregano concentration. When the initial CFU/ml of the *L.*

monocytogenes was decreased from 6.8 x 10^5 to 6.5 x 10^3, similar results were seen (Table 9). Since fewer Listeria were present, less oregano was needed to see an inhibition of the pathogen over the course of the experiment. The increased inhibitory effect of the clonal oregano sample, when compared to the other two samples, was more visibly seen at the 400 ppm and 800 ppm concentrations (Table 9). In fact, approximately the same difference in percentage inhibition was seen between the clonal sample and the CVS sample at the 800 ppm oregano concentration level (Table 9) and the 1200 ppm concentration (Table 8). Among the samples tested, the clonal oregano had the greatest concentration of total phenolics and the CVS sample had the least amount (Figure 4).

ppm Oregano extract	% Inhibition of Absorbance Compared to Control		
	Clonal	Mediterranean McCormick	CVS
400	-5.4	-5.8	-0.6
800	5.6	5.8	2.3
1200	55.7	52.9	42.2
1600	97.7	93.1	94.3

Table 8. Inhibition of *L. monocytogenes* by oregano extracts. Samples were evaluated after 25 hrs of growth in broth that was adjusted to pH 6.0 using acetic acid. Initial CFU/mL of *L. monocytogenes* inoculated was 6.8 x 10^5/ml.

ppm Oregano extract	% Inhibition of Absorbance Compared to Control		
	Clonal	Mediterranean McCormick	CVS
400	12.1	7.4	0.1
800	55.2	50.9	42.0
1200	96.1	95.2	97.2
1600	100.0	97.7	100.0

Table 9. Inhibition of *L. monocytogenes* by oregano extracts. Samples were evaluated after 25 hrs of growth in broth that was adjusted to pH 6.0 using acetic acid. Initial CFU/mL of *L. monocytogenes* inoculated was 6.5 x 10^3/ml.

When the Listeria were exposed to oregano extracts based on equivalent phenolic concentrations, the general trend observed was that the CVS was most effective in inhibiting the growth of the pathogen (Table 10 and Table 11). These results indicated that the amount of inhibition is more closely related to the volume of oregano introduced into the system. Since the CVS sample had the least amount of phenolics of the three samples tested, it needed to have the most amount of oregano extract placed into the broth in order to equalize the amount of total phenolics tested. Therefore, in each case, the CVS oregano had the most amount of inhibition seen in the experiments summarized on Table 10 and 11.

µg phenolics/mL	% Inhibition of Absorbance Compared to Control		
	Clonal	Mediterranean McCormick	CVS
9.3	-0.1	-1.2	10.1
18.5	6.7	26.7	55.3
27.8	69.6	85.9	98.8
37.1	97.4	100.0	100.0

Table 10. Inhibition of *L. monocytogenes* by oregano extracts. Samples were evaluated after 25 hrs of growth in broth that was adjusted to pH 6.0 using acetic acid. Initial CFU/mL of *L. monocytogenes* inoculated was 6.1 x 10^5/ml.

µg phenolics/mL	% Inhibition of Absorbance Compared to Control		
	Clonal	Mediterranean McCormick	CVS
9.3	10.0	18.3	41.2
18.5	54.3	79.5	98.3
27.8	97.0	98.4	100.0
37.1	98.9	100.0	100.0

Table 11. Inhibition of *L. monocytogenes* by oregano extracts. Samples were evaluated after 25 hrs of growth in broth that was adjusted to pH 6.0 using acetic acid. Initial CFU/mL of *L. monocytogenes* inoculated was 5.4 x 10^3/ml.

It is important to emphasize that the UMass oregano extract was developed from a clonal system. Therefore, use of clonal oregano can provide consistent data between experiments and reliable antimicrobial activity. Also, if the same line of oregano is used, experiments conducted should be highly repeatable if data is represented on a phenolic basis. However, comparing different samples on the same phenolic basis cannot ensure that the same amount of antimicrobial activity will be present. Different phenolic profiles with variable types of individual phenolic compounds and different amount of phenolic interaction can prevent this from occurring. This could be a reason why the CVS sample displayed the most amount of inhibition when all samples were standardized to have the same level of phenolics inoculated in the broth system.

Also, these studies were done using ethanol extractable phenolics to test for antimicrobial activity. Many studies that have already been conducted have focused on the use of essential oils of oregano for testing. These essential oils are more lipid-compatible. An example of one such study is the work of Paster et al (1990). These experiments showed that approximately 250 µg/ml of essential oils of oregano were required in order to significantly inhibit the growth of *S. typhimurium*, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Clostridium sporogenes*. Much like the experiments conducted in our research, very low levels of the oregano extract were needed to produce an inhibitory effect on the foodborne pathogen. Many of the other studies that have been done using essential oils have been done using the essential oils of thyme. These studies

are also highly relevant to the studies conducted above since thymol is one of the major components of thyme (as well as oregano). One such study was done by Hammer et al (1999) in which thyme oil was shown to be the most effective in inhibiting the growth of *E. coli* among the samples tested. This result is significant since *E. coli* is a Gram-negative bacteria and thus has a more rigid cell wall and is usually less susceptible to attack than a Gram-positive bacteria (such as *L monocytogenes*). As can be observed by these studies, essential oils do have powerful antimicrobial activity. However, we focused on ethanol soluble extracts of oregano since they are more relevant to food use. These types of extracts can provide antimicrobial activity at an interface of both lipid and water-soluble components in a food matrix.

Use of clonal oregano systems would offer many advantages for food safety as well as the food industry that are not currently available using the techniques and practices that have become standard today. Instead of using extracts that come from heterogeneous sources whose antimicrobial activity can vary between samples, clonal samples from a single genetically uniform source can be used each time. In addition, after the effective phenolic level or volume level of the clonal extract is established to inhibit the growth of a pathogen, that same level can be used with confidence each time in the same application. This constant, reliable antimicrobial source can provide the food industry with a natural preservative and thus increase the efforts of the food industry to provide a "clean label" for its consumer

Meat Studies

A significant decrease in the CFU/g was observed when the control sample of *L. monocytogenes* was compared to the remaining samples that had either carvacrol or oregano (Table 12). Excluding the control sample of carvacrol (which was used to test the efficacy of the meat sterilization techniques), the clonal oregano at 800 ppm displayed the most amount of inhibition when compared to the control. Repetition of this experiment verified the results seen in Table 12 (results not shown). It appears that 250 ppm of either the carvacrol or the oregano was not a substantial enough of a concentration to significantly inhibit the growth of the pathogen. At 800 ppm, carvacrol alone did not have as significant of an impact as either oregano sample (clonal oregano and CVS oregano).

	CFU/g	
Sample	250 ppm	800 ppm
Carvacrol Control	0.00	0.00
L. monocytogenes + carvacrol	1.73×10^6	6.77×10^5
L monocytogenes + clonal oregano	2.66×10^7	2.30×10^3
L. monocytogenes + CVS oregano	9.10×10^6	1.17×10^4

Table 12: Growth of *L. monocytogenes* in meat samples inoculated with oregano and carvacrol. CFU of *L. monocytogenes* applied to beef = 6.30×10^2/gm beef; final CFU/g: 2.01 x 10 pH of beef = 5.5 ; Temperature of Incubation = Room Temperature (approximately 72°F);Length of incubation = 48 hrs

A possible explanation for the results seen in Table 12 is that the oregano samples were working more effectively at the interface of the lipid and water-soluble portions of the meat than the carvacrol. Most likely, the carvacrol was only working in the lipid portions of the meat. Thus, the Listeria that were growing in the other portions of the meat were not exposed to the inhibitory action of the phenolic. In the meat studies, it is likely that the effective antimicrobial activity could have been from water and ethanol soluble phenolics such as rosmarinic acid found in the oregano (Kikuzaki and Nakatani, 1989). These ethanol and water-soluble oregano phenolics, such as rosmarinic acid, simple monophenols, and flavonoids, are probably more effective at the interface of membranes in meat systems. This could increase hyperacidification more. This study shows the difference between broth and meat studies very significantly since the concentrated phenolic, carvacrol, appeared to be more effective in the broth studies than in the meat study. Since broth studies are aqueous systems and the small test tubes were constantly shaking for the entire duration of the experiment, more of the carvacrol was able to distribute itself in the broth. In the meat studies, the carvacrol selectively interacts with the most lipid-compatible components only, thus it is probably only inhibiting the pathogen in the lipid portions of the meat.

Many studies have been done testing the effectiveness of plant extracts in food products. In the work of Hao et al. (1998), ethanol extracts of thyme (and many other plant extracts as well) were studied in refrigerated ready–to-eat-foods. Results of these studies did show that the thyme extracts did not inhibit the growth of *L. monocytogenes* when compared to the controls. Their results differ from ours for a number of possible reasons. Hao et al (1998) stated that large porous surfaces in the cooked beef used in the study could have provided a refuge for pathogens and thus reduce the amount of interaction with the plant extract. In addition, the extracts could have been solubilized into the lipid fraction of the beef. Other reasons as to why the results documented by Hao et al would differ from ours is that their original extracts were acquired from commercial chemical and flavor vendors and then diluted in ethanol. However, without knowing how that commercial extract was prepared, it would be difficult to understand how the components of the extract would interact with the meat sample. Also, it is possible that the supplier had batch-to-batch variation in their samples and thus the antimicrobial activity was not high relative to other samples. In our experimental set-up, we controlled for these variables by producing a high-phenolic clonal line of the oregano and then controlling the extraction process of that clonal line. Also, we used 93% fat-free choice beef for all of our samples, as opposed to whole sirloin tips, which could have had a higher percentage of fat in the sample. As mentioned before, this higher amount of fat can cause the extract to solubilize into this lipid portion and the antimicrobial activity for the entire sample would decrease. It is also important to realize that the pH of the food that is being tested can also affect the antimicrobial activity of a given extract. This was noted by Tassou et al (1995) who showed that as the pH of a food sample increased, the antimicrobial activity of the essential oil of mint (which usually contains large amounts of menthol, another terpene) decreased. Factors such as fat content and pH can cause variable results in the results of a given study. In addition NaCl, surface active agents, and some proteins can also decrease the antibacterial activity of a given sample (Del Campo et al, 2000). These parameters should also be controlled as much as possible in order to replicate results from one study to the next.

Further investigation (from the study we conducted) would include chromatographic analysis of the individual components of the oregano extracts in order to investigate other compounds, besides carvacrol, that could be attributing to the inhibition of the *L. monocytogenes*. In addition, testing the oregano extracts on different types of food, as well as different types of preparation of the food, would be useful. We have completed these studies in the last 2 weeks and have confirmed again the presence of higher content of rosmarinic acid in our elite clonal line of oregano.

Future Studies

The experiments discussed in this paper could be used as a foundation for many other studies related to the use of phytochemicals as antimicrobials. One of the most prominent questions that this research has revealed is exactly how the phenolic components in the extract are capable of destroying the pathogen of concern. The hypothesis put forward in this research is that the phenolic increases the hyperacidification in the microenvironment around the cell. The hyperacidification can disrupt the function of the ATP synthase in the cell membrane, which is a key component in the process of proton motive force. By overloading the ATP synthase with H+ ions, the enzyme complex will not be able to regulate the amount of protons on either side of the cellular membrane and thus the cell will begin to acidify excessively and deteriorate. In addition, disrupting the proton motive force impairs the ability of the cell to synthesize ATP and also contributes to the death of the cell. It is also possible that specific steps in the electron transport chain or important transport proteins could also be disrupted along with channels for regulating proton gradients. However, more research into the mechanism of the action of the phenolic on the pathogen is needed in order to validate the hypothesis presented here. The research should be targeted towards the enzymatic action of the ATP synthase complex in order to better understand if the phenolic disrupts the activity of the entire complex and the mechanism behind the disruption.

Other studies that can supplement the work done in this paper would be to find a better system for the incorporation of the oregano extract into a hydrophilic system, such as a broth system. In these experiments the extract was distributed in the system by a constant agitation of the broth. Studies with Tween 20 have shown that the emulsifier has a deleterious effect when incorporated with a phenolic in a broth system. Other studies that look into using different types of emulsifiers or a different type of system for distributing the phenolic components would be recommended for future testing. Finding a way to fully incorporate extracts that have some hydrophobic components would greatly benefit future experiments as well as the food industry.

In addition, HPLC could be used to complement future research in this field. By analyzing the phenolic profile of various oregano extracts, this would give us the ability to potentially screen various clonal lines for their antimicrobial activity based upon the levels of a variety of phenolic components, as opposed to just a few (such as rosmarinic acid, thymol, and carvacrol). As seen with the oregano studies described earlier, it is important to realize that standardizing different samples for the same amount of total phenolics can only give a rough estimation of each samples antimicrobial activity. In order to get a better idea of the total amount of antimicrobial activity in a sample, it is

better to know the phenolic profile of each sample. This would give us an idea of the quality of the phenolic components in the sample, as opposed to just the quantity of phenolic components in the sample.

Two of the major phenolic components in oregano, thymol and carvacrol, were used in this work. Even though rosmarinic acid is a primary phenolic constituent in oregano extracts, it was not used. This is because preliminary results in our lab indicate that pure rosmarinic acid on its own is effective at minimum levels of 250 µg of phenolics /disk. However, clonal oregano is effective at a lower concentration, approximately 100 µg of phenolics/disk (Lin and Shetty, In progress). Therefore, the focus was on oregano extracts, as well as thymol and carvacrol. At the same time, it is also clear that some high level of rosmarinic acid in oregano, in synergy with total oregano phenolics, is more effective than rosmarinic acid by itself. Therefore, any future studies with rosmarinic acid should be done with the idea of enhancing antimicrobial activity by testing the synergy between rosmarinic acid and other phenolic components in the oregano extract, as opposed to just focusing only on rosmarinic acid.

Additional studies can also be incorporated into the meat studies. The studies described earlier gave us a good indication of the extent of antimicrobial activity of the clonal oregano on samples of meat that were cross-contaminated with a pathogen after cooking. Similar studies that included the testing of cooked portions of meat that were previously inoculated with both the oregano extract and/or phenolic as well as the pathogen of concern before cooking are warranted. This would give us an indication of the amount of inhibitory action the phenolic would have, in addition to the cooking process. This would be important information for the food industry since many foodborne diseases result from undercooked meat. Phenolic components may help to eradicate any pathogens in the sample that survived a cooking process, and thus can help to decrease the chance of a foodborne outbreak in the food industry. In order to supplement research of this nature, once a level of a certain clonal extract of oregano is determined to be effective in inhibiting the growth of a pathogen on a meat sample, sensory analysis should be preformed on that meat sample. This should be done to ensure that the clonal oregano extract does not impart a noticeable flavor to the sample when compared to a control. Testing different types of meat, as well as other food systems, such as baked goods, dairy goods, and vegetarian dishes would help to give a better understanding of the extent, and effectiveness, of using clonal oregano extracts in food systems.

Other additional studies could focus on testing clonal lines of different types of herbs, such as rosemary and thyme, as well as testing the effectiveness of these clonal lines against other types of pathogens. The next type of pathogen tested should be of the Gram negative type, such as *Escherichia coli 0157:H7*, in order to compare the effectiveness of the clonal plant extract against a pathogen that has a different type of cellular wall than the Gram positive *L. monocytogenes*.

Conclusions and Implications

Initial results indicate that the clonal oregano line is effective in inhibiting the growth of *L. monocytogenes*. The high-phenolic producing UMass clonal line of oregano was able to significantly inhibit the growth of the pathogen that was inoculated into the

broth system at varying levels. Two of the main constituents of the oregano, thymol and carvacrol, also displayed a very powerful inhibition of the pathogen. When the extract was applied to a meat system that was contaminated with *L. monocytogenes*, results showed that the oregano extract is very effective at concentrations of at least 800 ppm on the meat. In addition, the meat studies also indicate that the entire oregano extract is more effective at inhibiting the growth of the pathogen than just the phenolic by itself. As stated earlier, a possible explanation for this is that the oregano samples were working more effectively at the interface of the lipid and water-soluble portions of the meat than the carvacrol.

Studies done comparing the effectiveness of the UMass clonal oregano against the store brands provided inconclusive results. Although the UMass clonal line was slightly better than the store brands when equivalent amounts of the extracts were tested, the UMass clonal line did not perform better than the store brands when compared on an equal phenolic basis. As stated earlier, this may be due to the different phenolic profiles present amongst the samples.

It is important to re-emphasize that the UMass oregano extract is made from a clonal line. This can provide the user of this extract with a consistent and reliable antimicrobial source for each sample prepared from this clonal line. The same cannot be said for oregano sources that are currently used in the food industry or used in food research. These sources are usually heterogeneous in nature and thus replicating the amount of antimicrobial activity from batch to batch would prove to be quite difficult. This is of an utmost importance for food safety, especially for its use if the food industry. The fact the studies discussed in this paper came from ethanol extracts of oregano is important to note as well. This extract provided high amounts of antimicrobial activity and can be very well utilized in a food matrix. This is because the phenolics in this extract can work at an interface of both lipid and water-soluble components in the food.

Based upon the initial studies discussed in this paper, it is highly recommended that future research in this field be directed towards the use of ethanol extracts of clonal lines of oregano (or other herbs). This would, in return, provide the food industry with a consistent and reliable source of antimicrobial activity. More importantly, it will provide the industry and the consumer with a reliable natural preservative and a “clean label” for the product’s ingredient statement.

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