Antimicrobial Properties of Glycerol Monolaurate Either Alone or Combined With Selected Organic Acids Against Listeria Monocytogenes.

Deog-hwan Oh
Louisiana State University and Agricultural & Mechanical College

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Antimicrobial properties of glycerol monolaurate either alone or combined with selected organic acids against *Listeria monocytogenes*

Oh, Deog-Hwan, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1993
ANTIMICROBIAL PROPERTIES OF GLYCEROL MONOLAURATE EITHER ALONE OR COMBINED WITH SELECTED ORGANIC ACIDS AGAINST LISTERIA MONOCYTOGENES

A Dissertation
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Food Science

by
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LIST OF ABBREVIATIONS AND SYMBOLS

AA: acetic acid
BA: benzoic acid
°C: degrees Celsius
CA: citric acid
CFU: colony forming units
cm: centimeter
CO₂: carbon dioxide
d: day (s)
DT: detection time
g: gram (s)
GML: glycerol monolaurate
GT: generation time
h: hour (s)
H⁺: hydrogen ion
IDT: impedance detection time
L: liter
LA: lactic acid
log: logarithm
MAP: modified atmosphere packaging
MIC: minimal inhibitory concentration
min: minute (s)
ml: milliliter (s)
M.W.: molecular weight
N: normal or normality
N₂: nitrogen
O₂: oxygen
P: probability
PBS: phosphate-buffered saline
pH: negative logarithm of the hydrogen ion concentration
pKₘ: negative logarithm of the equilibrium constant
s: second
sp.: species
sq.: square
SS: stainless steel
TSA: tryptic soy agar
TSB: tryptic soy broth
TSBYE: tryptic soy broth supplemented with yeast extract
μg: microgram (s)
%: percent
ABSTRACT

The objective of this study was to evaluate the antimicrobial properties of glycerol monolaurate (monolaurin) either alone or in combination with organic acids against Listeria monocytogenes in model broth or food. The minimal inhibitory concentration (MIC) of monolaurin was reduced by decreasing the pH value of the medium. The contribution of temperature to monolaurin effectiveness showed that lethal effects of monolaurin increased at higher temperatures and lower pH values, whereas, bacteriostatic effects on growth increased as temperature and pH decreased. The inhibitory effect of ethanol on the growth of L. monocytogenes was slight up to 2.5% ethanol, but was significant in the presence of 5% ethanol. When monolaurin was combined with ethanol, MIC value of the combination was not changed compared to that of the most active single compound alone. MIC value was lower when monolaurin was combined with lactic acid. Synergistic effects were observed when monolaurin was combined with acetic, benzoic, or lactic acid, whereas, there was little interaction when monolaurin was combined with citric acid or ethanol. Planktonic cells exposed to 50 or 100 \( \mu g/ml \) monolaurin were much more sensitive than were adherent cells, while 1-d adherent cells on stainless steel exposed to 50 or 100 \( \mu g/ml \) monolaurin were more sensitive than 7-d adherent cells in tryptic soy broth or diluted tryptic soy broth. However, the
inactivation effect on those cells significantly increased (P<0.05) when 50 or 100 μg/ml monolaurin was combined with 1% acetic acid. Heat effects on planktonic cells, 1-d, or 7-d adherent cells at 55°C were lower, but inactivation was more enhanced at 65°C. However, planktonic cells, 1-d, or 7-d adherent cells exposed to 50 or 100 μg/ml monolaurin at 65°C were completely inactivated. Air-, vacuum-, or modified atmosphere-package effects combined with 200 μg/ml monolaurin or 0.5% lactic acid on crawfish tail meat inoculated with 10³ cells/g of *L. monocytogenes* at 4°C were not different (P>0.05) compared with controls. However, each package containing crawfish tail meat treated with 200 μg/g monolaurin combined with 0.5% lactic acid significantly enhanced (P<0.05) the inhibitory effect. Results indicate that 200 μg/ml monolaurin, 0.5% lactic acid, and MAP had the greatest potential to inhibit growth of the bacterium.
CHAPTER 1
INTRODUCTION

*Listeria monocytogenes* is widespread in the environment and has been isolated from soil, animals, seafoods, dairy products, poultry, and meats (Bailey et al., 1989; Fuchs and Surendran, 1989; Johnson et al., 1988; Pini and Gilbert, 1988; Watkins and Sleath, 1981). The organism has emerged as one of the most serious pathogen due to its ability to cause food-borne disease (Pearson and Marth, 1990b). Recent outbreaks of listeriosis by *L. monocytogenes*, have generated much interest in investigating the behavior of the bacterium in foods. Human listeriosis has a high mortality, but the disease is largely confined to pregnant women, fetuses, newly born infants, and the immunosuppressed (Nieman and Lorber, 1980). The organism can cause meningitis, septicemia, and abortion among susceptible individuals.

Growth conditions for this bacterium depend on incubation temperature, pH level, medium composition, water activity, and type of microbial flora (El-Shenawy and Marth, 1988; Rosenow and Marth, 1987; Sorrels et al., 1989). Juntila et al. (1989) reported a mean minimum growth temperature of 1.1°C for *L. monocytogenes*. Indeed, at refrigeration temperatures, survival of *Listeria* may be prolonged. In general, *Listeria* is very resistant to freezing, though cell injury does occur. Golden et al. (1988) determined that up to 82% of *L.*
monocytogenes are injured but not killed during the freezing process.

The pH of foods can have an important preservative effect against bacteria. L. monocytogenes has been reported to be unable to survive or to grow poorly in low pH environments (George et al., 1988). However, other work has indicated that the bacterium is more acid tolerant than most foodborne pathogens and can grow or survive in broth media as low as pH 4.3 as well as cabbage juice adjusted to pH 5.0 (Conner et al., 1986; Rosenow and Marth, 1987; Parish and Higgins, 1989). However, the effect of acidic conditions on this organism varies with type of acid used (Conner et al., 1990; Polla and Hutkins, 1991).

Contamination of food products by foodborne disease organisms has caused great concern in the food industry. One of the most important reasons for contamination may be due to increased resistance of L. monocytogenes to sanitizers or other antimicrobial agents. The ability of microorganisms to develop increase resistance to sanitizers and other antimicrobial agents once they become attached to a surface has been documented (Le-Chevallier et al., 1988; Anwar and Costerton, 1990; Frank and Koffi, 1990; Mafu et al., 1990; Krysinski et al., 1992). L. monocytogenes has been reported to attach to a variety of surfaces; especially important is attachment of cells on the surface of stainless steel, which results in increased resistance to conventional chemical
sanitizers such as quaternary ammonium compounds or acid anionic sanitizer (Frank and Koffi, 1990; Krysinski et al., 1992; Mustapha and Liewen, 1989).

The fact that the pathogen can adhere to stainless steel surfaces and form biofilms, which is a microcolony of bacteria closely associated with an inert surface attached to it by a matrix composed of complex polysaccharide material, may indicate possible attachment of *L. monocytogenes* to non-food contact surfaces such as walls or inside floor-drains as well as food contact surfaces (Spurlock and Zottola, 1991). These biofilm formations also may possibly serve as an important reservoir of the bacterium in food processing plants. The presence of *L. monocytogenes* in food processing plants as well as in foods represents a health hazard and makes cleaning and sanitizing of the processing plant and equipment important for controlling the pathogen. Therefore, it is necessary to evaluate the increased resistance of the bacterium to various food industry sanitizers.

Recent trends toward commercialization of a variety of cooked, modified atmosphere-packaged (MAP), refrigerated, ready-to-eat food products could have the potential to seriously compromise an excellent food safety record because some psychrotrophic bacteria, such as nonproteolytic *clostridia*, *L. monocytogenes*, *Yersinia enterocolitica*, or *Aeromonas hydrophila*, can grow at refrigeration temperature in certain MAP products. Widespread occurrence of *L.*
monocytogenes in fresh or frozen seafood products has been documented (Jemmi, 1990; Lovett et al., 1987; Weagant et al., 1988). Cross-contamination of ready-to-eat seafood products with *L. monocytogenes* from food handlers or food contact surfaces during slicing and packaging operations may be likely. Postprocessing contamination with *L. monocytogenes* has been identified as a major source of contamination of many food products (Farber and Peterkin, 1991). Ready-to-eat foods can be expected to be occasionally consumed without reheating. Also, shelf life of refrigerated seafood products at the retail level is related to the microbiological condition of the seafood as received at retail, retail handling, and the temperature of storage.

Generally, CO₂-modified atmosphere packaging (MAP) provides for improved stability of fresh fish, with higher initial CO₂ levels resulting in increased shelf-life extensions (Farber, 1991). However, storage under controlled or modified atmospheres will not improve the quality of the product, but will only delay the rate of spoilage (Farber, 1991). Also, some pathogenic organisms, such as *L. monocytogenes, Y. enterocolitica*, or *A. hydrophila*, appear capable of growing at refrigerated temperatures in certain MAP foods. Therefore, MAP products with extended refrigerated shelf lives could be a problem.

The use of chemical preservatives to control microorganisms in food products has been debated due to public
concerns about food quality. Even though many chemical agents used as food preservatives are known to have good bactericidal activity, some are being considered for removal from the market because of their perceived toxic or carcinogenic potential (Tanaka et al., 1977). Consequently, consumers may reject foods containing preservatives due to questions about their safety. The use of non-toxic, naturally occurring substances as components of food has become important. Many workers have investigated alternatives to synthetic chemicals. For example, fatty acids and their esters have been used as antimicrobial preservatives. In particular, glycerol monolaurate (monolaurin) has been extensively investigated (Baker et al., 1982; Kabara et al., 1972a; Kabara, 1979b; Kabara, 1980; Kabara, 1981; Smith and Palumbo, 1980).

Monolaurin, a food-grade glycerol monoester of lauric acid, is approved in the U.S. as a food emulsifier. In addition to its emulsification properties, monolaurin also possesses broad spectrum antimicrobial activity in culture media against Gram-positive bacteria, yeasts, and molds, but is less effective against Gram-negative bacteria (Kabara et al., 1977). However, the range of use of monolaurin as a food preservative may be limited because its activity is antagonized by many food components such as starch, protein, and monopalmitin (Kato and Shibasaki, 1975).
This limitation of monolaurin when used alone might be overcome using a combined system.

Conner et al. (1990) reported that organic acids such as acetic, citric, lactic, and propionic acid inhibited *L. monocytogenes* at very low concentration of undissociated acid, but their effects were primarily bacteriostatic. Furthermore, the observation that the antilisterial activity of the acids was greater at 30°C than at 10°C indicates that environmental factors and/or treatments can influence antibacterial activity of acids. One approach to overcome limited antimicrobial activity of food preservatives when used alone is to adopt a combined system. El-Shenawy and Marth (1989) reported that sodium benzoate combined with organic acids decreased growth of *L. monocytogenes* more than sodium benzoate alone. Unda et al. (1991) reported that the greatest destruction of *L. monocytogenes* in beef roasts occurred with brines containing a phosphate blend and sodium lactate or monolaurin in combination with one, and particularly two, cookings.

The overall objective of the present study was to evaluate the antimicrobial properties of monolaurin, either alone or in combination with organic acids, against *L. monocytogenes* in a model broth system. The aim was to determine if monolaurin has potential as a new food preservative. In addition, the potential sanitizer effects of monolaurin, either alone or in combination with acetic acid or
heat, on cells of *L. monocytogenes* attached to stainless steel were determined. Specific objectives were:

1. To determine the effect of pH on the minimal inhibitory concentration (MIC) of monolaurin on *L. monocytogenes*.
2. To determine combined inhibitory effects of pH, temperature, and monolaurin on *L. monocytogenes*.
3. To investigate the minimal inhibitory concentration (MIC) and antimicrobial activity of monolaurin combined with selected organic acids (acetic, benzoic, citric, and lactic acid) or ethanol against *L. monocytogenes*.
4. To determine the effectiveness of monolaurin as a sanitizer against *L. monocytogenes* planktonic cells or adherent cells attached to stainless steel.
5. To evaluate the combined effect of monolaurin and acetic acid as sanitizers against *L. monocytogenes* planktonic cells or adherent cells attached to stainless steel.
6. To determine the effect of air, vacuum, or modified atmosphere packaging with or without monolaurin or lactic acid on precooked crawfish tail meat inoculated with *L. monocytogenes* and stored at refrigerated temperature.
CHAPTER 2
REVIEW OF LITERATURE

2.1 Characteristics of *Listeria monocytogenes*

*Listeria monocytogenes* is a Gram-positive, nonspore-forming, facultatively anaerobic, short, rod-shaped bacterium that can grow at refrigeration temperatures (Wilkins, 1972). Cells are 0.4-0.5 μm in diameter and 0.5-2.0 μm in length. It is catalase-positive, oxidase-negative and expresses a β-hemolysin. The bacterium possess peritrichous flagella, which give it a characteristic tumbling motility, occurring only in a narrow temperature range. When the organism is grown between 20 and 25°C, flagellin is both produced and assembled at the cell surface, but at 37°C flagellin production is markedly reduced (Peel et al., 1988). Colonies grown for 24 h at 37°C are small, round, translucent with a watery appearance and appear blue-grey when observed in obliquely transmitted light (Henry, 1933). While optimum growth temperature range of the organism is 30-37°C, *L. monocytogenes* is capable of growth over a temperature range of 1-45°C, making the bacterium of potential food safety concern in refrigerated foods. The pH range for growth is pH 5-9 (Conner et al., 1986; George et al, 1988; Parish and Higgins, 1989). Table 2.1 illustrates the biochemical characteristics of the genus *Listeria*. Table 2.2 demonstrates the biochemical tests used to differentiate seven different *Listeria* species.
Table 2.1. Biochemical characteristics differentiating species of the genus *Listeria*.

<table>
<thead>
<tr>
<th>Biochemical characteristic</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen requirement</td>
<td>Facultative</td>
</tr>
<tr>
<td>Growth at 35°C</td>
<td>+</td>
</tr>
<tr>
<td>Motility at 20-25°C</td>
<td>+</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>+</td>
</tr>
<tr>
<td>Hydrogen sulfide production</td>
<td>-</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red reaction</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer reaction</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
</tr>
<tr>
<td>Urease activity</td>
<td>-</td>
</tr>
</tbody>
</table>

Source: Adapted from Parrisus et al. (1986)
Table 2.2. Biochemical tests for differentiation of seven *Listeria* species.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Species⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LM</td>
</tr>
<tr>
<td>β-Haemolytic</td>
<td>+</td>
</tr>
<tr>
<td>Acid from:</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
</tr>
<tr>
<td>Hydrolysis of aesculin</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
</tr>
<tr>
<td>CAMP test⁴</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Rhodococcus equi</em></td>
<td>-</td>
</tr>
</tbody>
</table>


b Slightly haemolytic

c Variable

d CAMP test. A positive (+) reaction means enhanced haemolysis near either *S. aureus* or *R. equi* streak lines

Source: Adapted from Prentice and Neaves (1988)
2.2 Human Infections and Significance as a Pathogen

*L. monocytogenes* is now recognized as a food-borne pathogen, capable of causing epidemic and sporadic illness. Although humans have become infected after direct contact with diseased animals (Twedt, 1988), and soil may often be the origin of the organism, *L. monocytogenes* was considered an environmental contaminant whose primary means of transmission to humans is through food contaminated during production and processing (Anonymous, 1988d).

There are many possible routes for the transmission of *L. monocytogenes* to humans other than food, and several have been documented. Transmission of *L. monocytogenes* from diseased animals to humans has been described (Seeliger, 1961). Listeriosis has been observed to occur naturally in sheep, cattle, goats, swine, horses, geese, ducks, pigeons, turkeys, chickens, dogs, cats, and deer (Seeliger, 1961). *L. monocytogenes* also has been found in fish and crustaceans (Armstrong, 1985). Infected animals, and sometimes healthy animals, often shed *L. monocytogenes* in their feces. Consequently, humans can become infected by direct contact with diseased or healthy animals. Furthermore, it is possible that *L. monocytogenes* can be transmitted from rodents to domestic animals or to humans.
\( L. \) monocytogenes has been recognized as ubiquitous throughout the environment and as a causal agent of disease in humans. Listeriosis, a potential foodborne disease caused by \( L. \) monocytogenes, has emerged as a serious food safety issue. \( L. \) monocytogenes is a pathogenic bacterium that can cause meningitis, septicemia, abortion, central nervous system infections, immunosuppression, and diabetes mellitus (Nieman and Lorber, 1980). Those at high risk are pregnant women and their newly born infants as well as older individuals (Gray and Killinger, 1966). There are, however, instances in which apparently normal healthy individuals have become ill with listeriosis in foodborne epidemics (Schlech et al., 1983) and sporadic cases (Azadian et al., 1989). Susceptibility to acquiring listeriosis varies within the population, the overall risk being quite low. It is estimated that the rate of infection is approximately 3.7 persons per 1,000,000. A high mortality rate of approximately 30% is associated with the disease (Schlech, 1988). The most common manifestation of listeriosis is meningitis. The clinical course develops and progresses suddenly, and the fatality rate of those with meningitis can be as high as 70%. All forms of listeriosis are more likely to accompany immunocompromised states, whether natural or induced as a result of medical treatment. Therefore, populations at greatest risk for listeriosis are the unborn, the newborn, and the immunocompromised.
2.3 Foodborne Listeriosis

The fact that food can carry *L. monocytogenes* which, when ingested, can cause listeriosis in humans was widely demonstrated in the past few years by outbreaks associated with coleslaw (Schlech et al., 1983), pasteurized milk (Fleming et al., 1985), Mexican-style cheese (CDC, 1985), and raw vegetables (Ho et al., 1986). That the organism can be harbored in food processing facilities and then contaminate processed food products also has been observed repeatedly. Furthermore, the persistence of *L. monocytogenes* in contaminated foods (Doyle et al., 1985; Ryser et al., 1985; Ryser and Marth, 1987a) and the ability of the organism to grow in foods during refrigerated storage (Rosenow and Marth, 1987b) magnifies the problem of foodborne listeriosis.

An outbreak of listeriosis associated with *L. monocytogenes* serotype 4b occurred among adult patients in eight Boston hospitals in 1979 (20 cases), although it was not reported until several years later (Ho et al., 1986). Case-control studies tentatively identified three foods as being preferred by cases compared to controls: tuna fish, chicken salad, and cheese. Three patients died of listeriosis (15% mortality rate). Ten of the patients were immunosuppressed as a result of chemotherapy or steroid treatment. During a Canadian outbreak of human listeriosis, raw cabbage and coleslaw were suspected as the source of the organism (Schlech et al., 1983). Of 34 perinatal cases, there were 23 live
births of ill infants with a subsequent 27% mortality rate. There was also a 28.6% adult mortality rate. Coleslaw obtained from the refrigerator of one of the patients was shown to contain \textit{L. monocytogenes} type 4b, the epidemic strain. Another outbreak which raised listeriosis to a higher level of concern among food manufacturers and regulatory agencies, occurred in California from January to August 1985 (Linnan et al., 1988). Of 142 cases, 93 were perinatal and 49 were adult, with a total of 48 deaths (34% mortality rate) involving 30 fetuses and newborn infants, and 18 nonpregnant adults. Among the 49 adult cases, 48 were immunosuppressed, elderly, or had a severe chronic illness. Case-control studies implicated Mexican-style soft cheese of a certain brand. The presence of \textit{L. monocytogenes} type 4b in this cheese was confirmed.

2.4 Incidence and Survival in Foods

A. Dairy Products

\textit{L. monocytogenes} has been found in a wide variety of dairy products (Table 2.3). Among these products, cheese has been the most intensively examined because of its known association with foodborne listeriosis. Levels of \textit{L. monocytogenes} as high as $10^7$ CFU/g have been found in some naturally contaminated cheeses (van Renterghem et al., 1990). The organism has the ability to survive in many different
Table 2.3. Incidence of *L. monocytogenes* in a Variety of Foods

<table>
<thead>
<tr>
<th>Food Products</th>
<th>Number of Samples</th>
<th>Number of Samples Positive (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dairy and Milk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soft cheese</td>
<td>338</td>
<td>6 (1.8)</td>
<td>Venables, 1989</td>
</tr>
<tr>
<td>Semihard cheese</td>
<td>205</td>
<td>4 (2.0)</td>
<td>Breer and Schopfer, 1989</td>
</tr>
<tr>
<td>Hard cheese</td>
<td>66</td>
<td>1 (1.5)</td>
<td>Greenwood et al., 1991</td>
</tr>
<tr>
<td>Red-smear cheese</td>
<td>343</td>
<td>23 (10.0)</td>
<td>Breer and Schopfer, 1989</td>
</tr>
<tr>
<td>Raw milk</td>
<td>100</td>
<td>14 (7.0)</td>
<td>Lovett et al., 1987</td>
</tr>
<tr>
<td>Raw milk</td>
<td>200</td>
<td>8 (4.0)</td>
<td>Liewen and Plautz, 1988</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>180</td>
<td>4 (2.2)</td>
<td>Greenwood et al., 1991</td>
</tr>
<tr>
<td>Ice cream</td>
<td>394</td>
<td>1 (0.3)</td>
<td>Farber et al., 1989</td>
</tr>
<tr>
<td><strong>Meat and Poultry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>25</td>
<td>23 (92.0)</td>
<td>Lowry and Tiong, 1988</td>
</tr>
<tr>
<td>Raw beef</td>
<td>658</td>
<td>41 (6.2)</td>
<td>Carosella, 1990</td>
</tr>
<tr>
<td>Frozen ground beef</td>
<td>41</td>
<td>20 (49.0)</td>
<td>McClain and Lee, 1988</td>
</tr>
<tr>
<td>Minced beef</td>
<td>67</td>
<td>19 (28.0)</td>
<td>Skovgaard and Norrung, 1988</td>
</tr>
<tr>
<td>Beef steaks</td>
<td>25</td>
<td>6 (24.0)</td>
<td>Wong et al., 1990</td>
</tr>
<tr>
<td>Cooked beef</td>
<td>844</td>
<td>23 (2.7)</td>
<td>Anonymous, 1988</td>
</tr>
<tr>
<td>Pork</td>
<td>34</td>
<td>20 (58.5)</td>
<td>Won et al., 1990</td>
</tr>
<tr>
<td>Pork</td>
<td>25</td>
<td>17 (78.0)</td>
<td>Lowry and Tiong, 1988</td>
</tr>
<tr>
<td>Chilled meats</td>
<td>74</td>
<td>13 (18.0)</td>
<td>Gilbert et al., 1989</td>
</tr>
<tr>
<td>Chicken legs</td>
<td>16</td>
<td>9 (56.3)</td>
<td>Farber et al., 1989</td>
</tr>
<tr>
<td>Precooked chicken</td>
<td>102</td>
<td>27 (26.5)</td>
<td>Kerr et al., 1990</td>
</tr>
<tr>
<td><strong>Vegetables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetables</td>
<td>49</td>
<td>6 (12.2)</td>
<td>Wong et al., 1990</td>
</tr>
<tr>
<td>Potatoes</td>
<td>132</td>
<td>34 (25.8)</td>
<td>Heisick et al., 1989</td>
</tr>
<tr>
<td>Cabbage</td>
<td>92</td>
<td>2 (2.2)</td>
<td>Heisick et al., 1989</td>
</tr>
<tr>
<td>Cucumbers</td>
<td>92</td>
<td>10 (10.9)</td>
<td>Heisick et al., 1989</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>92</td>
<td>11 (12.0)</td>
<td>Heisick et al., 1989</td>
</tr>
<tr>
<td>Lettuce</td>
<td>92</td>
<td>1 (1.1)</td>
<td>Heisick et al., 1989</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>20</td>
<td>0 (0)</td>
<td>Farber et al., 1989</td>
</tr>
<tr>
<td>Radishes</td>
<td>10</td>
<td>0 (0)</td>
<td>Farber et al., 1989</td>
</tr>
<tr>
<td><strong>Seafood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seafoods</td>
<td>57</td>
<td>6 (10.5)</td>
<td>Wong et al., 1990</td>
</tr>
<tr>
<td>Frozen cooked crab</td>
<td>24</td>
<td>7 (29.0)</td>
<td>Weagant et al., 1988</td>
</tr>
<tr>
<td>Frozen raw shrimp</td>
<td>7</td>
<td>2 (29.0)</td>
<td>Weagant et al., 1988</td>
</tr>
<tr>
<td>Smoked fish</td>
<td>377</td>
<td>47 (12.5)</td>
<td>Jemmi, 1990</td>
</tr>
</tbody>
</table>
dairy products such as butter, camembert cheese, cultured buttermilk, and yoghurt (Choi et al., 1988; Olsen et al., 1990; Ryser and Marth, 1987b; Siragusa and Johnson, 1988).

*L. monocytogenes* grows well in both naturally and artificially contaminated fluid dairy products at temperatures ranging from 4 to 35°C (Ferguson and Shelef, 1990; Marshall and Schmidt, 1988; Pearson and Marth, 1990a; Rosenow and Marth, 1987). Marshall and Schmidt (1988) reported that the presence of pseudomonads in milk may enhance the growth of *L. monocytogenes*. They demonstrated that *L. monocytogenes* can grow in the presence of other common psychrotrophic microorganisms in milk.

B. Meat and Poultry

A wide variety of meats can be contaminated with *L. monocytogenes* (Table 3), with the incidence of contamination varying greatly. This variation is partly due to differences in methods of detection including the method used, the sample size, and the source of analyzed samples. Most meat contamination is on the surface; however, Johnson et al. (1990) found *L. monocytogenes* in the interior muscle cores of 5 of 110 total samples of beef, pork, and lamb roasts. Chicken also seems to be heavily contaminated with *L. monocytogenes* as surveys show contamination rates ranging from 27 to 56% (Table 3).
Although studies (Johnson et al., 1988; Shelef, 1989) have shown that *L. monocytogenes* may be unable to grow on meat stored at 4 or 25°C, other researchers have indicated that the organism is definitely capable of growing on meat (Chung et al., 1989; Dickson, 1990; Glass and Doyle, 1989; Marshall et al., 1991). However, growth appears to be highly dependent on temperature, pH, type of tissue, and the amount of competing microflora present. Glass and Doyle (1989) found that growth of *L. monocytogenes* on meat was highly dependent on product type and pH. The organism tended to grow well on meat products with a pH value near or above 6.0, whereas, it grew poorly or not at all on meats near or below pH 5.0. *L. monocytogenes* also grew better at 0°C on vacuum-packaged beef at pH 6.0 than on at pH 5.6 (Grau and Vanderlinde, 1988). They found that regardless of storage temperature or pH, *L. monocytogenes* grew to higher levels on fat than on lean meat, probably as a result of a much shorter lag phase. In contrast, similar growth patterns of *L. monocytogenes* on both lean and fat beef tissue were observed, with slightly longer lag periods occurring on fatty tissue (Dickson, 1990).

C. Vegetables

Although many different types of vegetables have been analyzed for the presence of *L. monocytogenes* (Farber et al., 1989b, Heisick et al., 1989), only potatoes and cucumbers appear to be regularly contaminated (Heisick et al., 1989).
L. monocytogenes appeared to grow quite well in lettuce juice stored at 5°C (Steinbreugge et al., 1988), but it grew in heat-sterilized cabbage juice only when stored at 30°C and containing less than 2.0% NaCl. At 4°C the organism did not grow in the cabbage juice, but was able to survive for long periods (Conner et al., 1986). Steinbreugge et al. (1988) found that L. monocytogenes grew on shredded lettuce stored at 5, 12, and 25°C, although the increase was only about 1 log after 14 d at 5 and 25°C, and about 3 logs after 14 d at 12°C. In addition, in several trials, the organism either did not grow or died after 14 d. L. monocytogenes does not appear to be able to grow well on carrots unless they are cooked (Beuchat and Brackett, 1990). Indeed, an anti-listeria effect was observed in raw carrots stored at 5 or 15°C that spoiled before L. monocytogenes could develop. Also, it was found that a broth culture medium containing as little as 1% raw carrot juice substantially inhibited growth of organism.

D. Seafood

Seafood products have received less study than have other foods. Weagant et al. (1988) reported that 15% of 57 samples of frozen seafood products, including shrimp, crabmeat, lobster tail, fin fish, and surimi-based seafood, were positive for L. monocytogenes. Jemmi (1990) tested 377 samples of smoked and marinated fish and found 47 to be positive for L. monocytogenes. Of 100 smoked samples, 24%
were positive for the organism. Very little work has been done to examine the growth of *L. monocytogenes* in seafoods. Lovett et al. (1987) examined the growth of *L. monocytogenes* in shrimp, crabmeat, surimi, and white fish stored at 7°C. The bacterium inoculated into samples of these products, which had been sterilized prior to inoculation, increased in number by about 5 logs within 14 d. Many fish products including frozen cooked shrimp, smoked salmon, frozen canned lobster, and surimi products have been found to be contaminated with *L. monocytogenes*, and have been recalled from the market (Anonymous, 1987, 1988a, 1988b, 1988c).

2.5 METHODS FOR DETECTION IN FOODS

A. Conventional Methods

1). Direct Plating

Many isolation media have been developed to recover *Listeria* spp. from foods. A selective medium developed by McBride and Girard (1960), which was the first solid medium for recovering *L. monocytogenes* from mixed cultures, was modified by Lovett (1988), and called modified McBride's agar (MMA). An improved selective medium was developed by Lee and McClain (1986) for isolation of *L. monocytogenes* from meats. This medium is better than MMA for recovery of the organism from mixed cultures. Other useful formulations include a
modification of Vogel Johnson agar (MVJ), Oxford agar, and modified Oxford agar (Buchanan et al., 1988; Van Netten et al., 1988; Curtis et al., 1989; McClain and Lee, 1988). There are several reports comparing different selective media for isolation and enumeration of *Listeria* spp. in foods (Buchanan et al., 1988; Cassiday et al., 1989; Jatisatienr and Busse, 1989). These studies indicated that lithium chloride and phenylethanol (LPM) agar was the best overall medium for enumerating *L. monocytogenes* in foods, since it only inhibited the growth of other species while supporting the growth of all *L. monocytogenes* strains. MVJ and Oxford agars had an advantage over LPM agar in that *Listeria* spp. could be visually differentiated from other bacteria. Accordingly, no single medium has emerged as superior, although LPM and Oxford media are the most widely used in North America.

### 2). Selective Enrichment

Among the earliest methods used to recover *L. monocytogenes* from food and environmental samples used cold enrichment (Gray and Killinger, 1966). More recently, the incorporation of specific selective agents in enrichment media has shortened the time required to isolate the bacterium. Nalidixic acid and acriflavine have been used in the U.S. Food and Drug Administration (FDA) enrichment broth (EB), which also contains cycloheximide as an antifungal agent (Pini and Gilbert, 1988b; Lovett et al., 1987). Several investigators
have compared enrichment procedures for the isolation of *Listeria* spp. from dairy products and meats. Doyle and Schoeni (1987) compared the detection of *L. monocytogenes* in cheese using cold enrichment, FDA enrichment, and selective enrichment procedures (Doyle and Schoeni, 1986). *L. monocytogenes* was isolated from 41 of 90 (46%) samples of soft, surface-ripened, cheese. In most cases, the organism was isolated from a cheese sample by only one of the three procedures. Pini and Gilbert (1988b) compared detection of *L. monocytogenes* in chicken or soft cheese by cold enrichment in tryptose phosphate broth at 4°C over a period of 12 weeks and by the FDA enrichment procedure. *L. monocytogenes* was isolated from 70 of 160 (44%) chicken samples and from 23 of 222 (10%) cheese samples. Neither method alone yielded all isolates from the two food types.

B. Alternative Rapid Methods

Conventional methods are tedious and are variable in their results. To monitor the incidence of *L. monocytogenes* in foods, reliable methods must be developed for the rapid detection of the organism. Suggested techniques have included fluorescent-antibody assay, enzyme immunoassay (EIA), flow cytometry (FCM), and DNA hybridization (Klinger, 1988). The production of monoclonal antibodies (MAbs) for use in identification of *Listeria* spp. in EIA methods was first reported by Farber and Speirs (1987). MAbs were developed
which reacted in the presence of either H antigen A, B, or C. Detection of L. monocytogenes by colony hybridization was first reported by Datta et al. (1987) using a radio-labeled DNA probe. The method was tested against 52 pure cultures of Listeria spp., and homology was detected only with β-hemolytic strains. The possibility of developing specific probes based on unique regions of rRNA was exploited in the development of a commercial hybridization assay (Gene-Track) for Listeria spp. in foods (Klinger and Johnson, 1988; King et al., 1989). Detection of L. monocytogenes by means of polymerase chain reaction amplification, followed by either agarose gel electrophoresis or dot blot analysis with a $^{32}$P-labeled internal probe, has been reported (Bessesen et al., 1990; Border et al., 1990).

### 2.6 Biofilm Formation and Its Resistance

Microorganisms can attach firmly to solid surfaces such as stainless steel, glass, and polypropylene, as well as clinical devices (Anwar et al., 1989a; Frank and Koffi, 1990; Herald and Zottola, 1988; Lee and Frank, 1991; Mafu et al., 1990). Attached microorganisms might cause potential contamination problems for the food industry because such cells are resistant to a wide range of sanitizers. If attached microorganisms are not completely removed, they could contaminate processing systems, resulting in undesirable food products and economic loss.
Many workers have reported on the structural and functional nature of biofilms, which are defined as microcolony formation of bacteria associated with an inert surface with attachment by matrix formation (Charscklis and Cooksey, 1983; Costerton et al., 1987; Hamilton and Characklis, 1989). Biofilms are ubiquitous in nature and equally widespread in manmade and industrial environments. Marshall et al. (1971) suggested that adhesion occurs in two phases, reversible and irreversible. During the initial reversible phase, bacteria are held to the surface by weak attractions. Firmer binding occurs when physical and chemical forces combine to hold the bacterial cells irreversibly to the surface. Some of these forces include the production of exopolysaccharides termed a glycocalyx (Costerton et al., 1981a). The glycocalyx matrix modifies the environment of the biofilm by concentrating nutrients and protecting the cells from surfactants (Frank and Koffi, 1990; Petrocii, 1983), biocides (Mosley et al., 1976), phagocytic cells (Costerton et al., 1981b), and antibiotics (Nickel et al., 1985).

Herald and Zottola (1988) reported that *L. monocytogenes* attached to inert stainless steel surfaces in nutrient medium at various pH values and temperatures. Cell numbers of *L. monocytogenes* on stainless steel surfaces increased at lower temperatures and increased with increasing incubation time. They postulated that *L. monocytogenes* attachment to stainless
steel may be mediated through a combination of motility and any polymer surrounding the cells, although flagella and/or fibrils were not observed in all preparations.

2.7 General Background of Glycerol Monolaurate

Many investigators have reported on the structure-function relationship of fatty acids (Kabara et al., 1972a; Kabara et al., 1972b; Kabara, 1979a; Kabara, 1984a) and polyol esters (Conley and Kabara, 1973). These studies showed that optimum activity for even numbered carbon aliphatic fatty acids was saturated \( C_{12} \) (lauric acid) and mono- and diunsaturated \( C_{18} \) (oleic and linoleic acid). An unsaturated derivatives of a long-chain fatty acid are more active than the corresponding saturated fatty acid, but ester production from unsaturated fatty acid is fraught with a number of problems because unsaturated fatty acids produce toxic peroxides and can change from cis- (active) to trans- (inactive) and can become growth factors for microorganisms at low concentration (Altenbern, 1977; Nieman, 1954). Kabara et al. (1972a) tested the effects of esterification on lauric acid biocidal activity. They found that esterification with polyhydroxy compounds might be active, whereas, ester formation with monohydroxy compounds were less active or inactive. When one of the more common polyhydroxy alcohols, glycerol, was esterified with fatty acids, it was found to be active (Conley and Kabara, 1973; Kabara et al., 1972a; Kabara
et al., 1977). They found that the monoester of glycerin gave the most active effects, while di- and triesters were generally inactive. In most instances, the monoglyceride was more active than the free fatty acid. Among fatty acids or their derivatives, the monoester of glycerol with lauric acid was found to have the greatest antimicrobial activity (Kato and Shibasaki, 1975).

Glycerol monolaurate (monolaurin), a food grade glycerol monoester of lauric acid, is approved as an emulsifier in foods by the U.S. Food and Drug Administration (FDA) regulation and is recognized as a generally regarded as safe (GRAS) material (21 CFR GRAS 182.4205). Even though GRAS food ingredients are approved only for those uses that have been specified, monolaurin can be used in food as a potentiator of preservative action, thus lowering the need for other classic preservatives without fear of toxicity (Kabara, 1981). The antimicrobial aspects of the compound were originally identified by screening 600 lipid derivatives (Conley and Kabara, 1973; Kabara et al., 1972b; Kabara, et al., 1977).

2.8 Combined Effects of Monolaurin with Antimicrobial Agents

Monolaurin has the greatest overall antimicrobial activity among all fatty acids and their esters and would appear to have the highest potential for use in foods and cosmetics (Shibasaki and Kato, 1979). However, the range of use of monolaurin as a food preservative may be limited
because its activity is antagonized by many food components such as starch, lipid, and protein (Kato and Shibasaki, 1975). Monolaurin has antimicrobial activity against Gram-positive bacteria, yeasts, and molds, but is less effective against Gram-negative bacteria. Lack of effectiveness against Gram-negative bacteria can be overcome by combining monolaurin with chelating agents (Kabara, 1980). He found that the monoglyceride with ethylenediamine-tetraacetic acid (EDTA) was very effective, particularly when used to inhibit or kill Pseudomonas aeruginosa. However, the presence of a chelating agent is not always necessary for Gram-negative activity, since Beuchat (1980) found that 5 µg/ml monolaurin was more effective than 70 µg/ml sorbic acid or 300 µg/ml sodium benzoate against Vibrio parahaemolyticus.

Acidulants such as citric, malic, lactic, or polyphosphoric acids increased the antimicrobial spectrum and activity of monolaurin against Gram-negative bacteria (Kato and Shibasaki, 1975). The mechanism of a combination of monolaurin with citric acid, polyphosphoric acid, or EDTA was discribed by Kato and Shibasaki (1976). They reported that cells pretreated with citric acid were more susceptible to monolaurin and exhibited a marked reduction in the population after incubation with monolaurin. Also, they found that monolaurin or citric acid alone did not inhibit uptake of amino acids by Escherichia coli, but the two combined inhibited amino acid uptake by about 90%.
In general, a phenolic antioxidant such as butylated hydroxy-anisole (BHA) exhibits wide spectrum activity, but it is less active toward Gram-negative bacteria compared with other organisms (Kabara, 1979; Kabara, 1980). However, Gram-negative bacteria were inhibited when BHA was combined with monolaurin (Kabara, 1979).

2.9 Application of Monolaurin in Food Products

Monolaurin inactivated *Staphylococcus aureus* in laboratory media at low levels (Kabara et al., 1972a), but was less effective in a model sausage system (Smith and Palumbo, 1981). They reported that anaerobic growth of *S. aureus* was inhibited by 2500 ppm potassium sorbate or 2500 ppm monolaurin, whereas twice as much of sorbate or monolaurin was required to inhibit aerobic growth. However, the combination of sorbic acid and monolaurin gave a greater inhibitory effect than either of the substances alone (Kabara, 1984b).

The utility of monolaurin in meat products was underscored by Notermans and Dufrenne (1981). Monolaurin, when used in the proportion of 5 g/kg of meat slurry (pH 6.0-6.2), completely inhibited toxin production by *Clostridium botulinum* types A, B, and E. The inhibitory effect of monolaurin was not improved by addition of lactic acid to the meat slurry until the pH was less than 5.2. Under these same conditions potassium sorbate inhibited toxin production of type B at 32 g/kg (3.2%). Since monolaurin was six times more
active than potassium sorbate, they recommended it as a possible alternative to nitrite for preservation of meat products. Baker et al. (1982) examined antimicrobial properties of monolaurin in deboned chicken meat, minced fish, and chicken sausage. The addition of 0.2% citric acid, 0.2% ascorbic acid, or 250 ppm monolaurin alone extended shelf-life of the products by 2 d. Shelf-life of the deboned chicken meat increased by 7 d, that of minced fish by 5 d, and that of chicken sausage by 8 d when monolaurin was combined with citric or ascorbic acid.

Robach et al. (1981) compared antimicrobial effects of monolaurin and sorbic acid in broth media, cottage cheese, and a pork homogenate. Two hundred fifty to 1000 ppm sorbic acid was more effective than 250 to 1000 ppm monolaurin in inhibiting growth of Salmonella enteritidis 13311 in broth. In cottage cheese, 800 ppm monolaurin was ineffective in controlling growth of any of the microorganisms tested, whereas 800 ppm sorbic acid effectively inhibited the growth of coliforms, yeasts, and molds, but only slightly inhibited growth of psychrotrophs. A concentration of 3000 ppm monolaurin in a pork homogenate had no effect on outgrowth and gas formation by Clostridium sporogenes PA3679, whereas, 3000 ppm sorbic acid delayed both.

Chipley et al. (1981) tested the antifungal and anti-aflatoxigenic effects of monolaurin. While sorbic acid levels of 1000 ppm were required for inhibition of aflatoxin
formation, monolaurin was active at 750 ppm. Lisker and Poster (1982) carried out extensive studies with monolaurin and related food-grade agents as potential antifungal agents for citrus products. Laubric (monolaurin plus sorbic acid) proved to be more active than either compound alone. The most effective combination was a mixture of monolaurin, tert-butyl hydroxyanisole, and ethylenediaminetetraacetic acid (EDTA).

2.10 Effect of Monolaurin against *L. monocytogenes*

A wide application of monolaurin in food products against Gram-positive bacteria, Gram-negative bacteria, and fungi has been reported (Chipley et al., 1981; Lisker and Poster, 1982; Notermans and Dufrenne, 1981; Baker et al, 1982). However, little information exists on the application of this compound in food products or in vitro on its potential to inhibit growth of *L. monocytogenes*.

Unda et al. (1991) examined survival and inhibitory effect of microwave-ready beef roasts containing selected antimicrobials against *L. monocytogenes*. They found that growth of *L. monocytogenes* was reduced by sodium lactate or monolaurin in recooked surface-inoculated roast beef.

Wang and Johnson (1992) demonstrated the growth inhibiting effect of fatty acids and monoglycerides in brain heart infusion (BHI) and in milk against *L. monocytogenes*. They found that monolaurin had strongest antimicrobial activity in BHI broth and was bactericidal at 10 μg/ml,
whereas, lauric acid, stearic acid, and potassium-conjugated linoleic acid (K-CLA) were bactericidal at 20, 100, and 200 μg/ml, respectively. Bactericidal activity in BHI broth was higher at pH 5 than at pH 6. *L. monocytogenes* in skim milk at 4°C did not grow in the presence of monolaurin, but the inhibitory effect of monolaurin was less at 23°C.

### 2.11 Effect of Organic Acids against *L. monocytogenes*

#### A. Acetic Acid

Acetic acid (60.05 M.W., 4.76 pKₐ) is water soluble and generally regarded as safe (GRAS) for miscellaneous and general-purpose usage under FDA regulations (FDA, 1990). Acetic acid inhibits many species of bacteria, yeasts, and molds. The inhibitory effect of acetic acid on microorganisms is greater than that due to pH alone and is greater than either citric or lactic acid at the same pH (Conner et al., 1990; Sorrells et al., 1989). Undissociated acetic acid can penetrate the microbial cell and exert its toxic effect (Brock, 1979). Antimicrobial activity is greater at pH values approaching the pKₐ, at which the amount of undissociated acid is greater. Several studies have reported on the effects of low pH on growth and viability of *L. monocytogenes* (Ahamad and Marth, 1989; Conner et al., 1990; Ita and Hutkins, 1991). These studies indicate that the effect of low pH on *L. monocytogenes* varies with the acid used. Acetic acid was
more detrimental to L. monocytogenes than either lactic, citric, or hydrochloric acid. Acetic acid has the highest pKₐ value of the four acids studied, thus, this acid would be expected to accumulate in cells in higher concentrations than the other acids studied (Ahamad and Marth, 1989; Kashket, 1987). Ita and Hutkins (1991) demonstrated that a greater than 4-log reduction in cell numbers occurred when L. monocytogenes was held in acetic acid-treated broth for 24 h and at pH 3.5, whereas only one or less log reduction in lactic, citric, or hydrochloric acid-treated broth was seen at the same pH. Based on equal pH values, the antimicrobial activity against L. monocytogenes was acetic acid > lactic acid > citric acid > hydrochloric acid (Sorrells et al., 1989).

B. Benzoic Acid

Benzoic acid (122.2 M.W., 4.2 pKₐ) long has been used as an antimicrobial additive in the food industry. Benzoic acid is used primarily in foods and beverages with pH values at or below 4.0 to 4.5. According to FDA regulations (21CFR 184.1021), benzoic acid is an additive generally regarded as safe (GRAS), and its use in food is permitted up to a maximum concentration of 0.1%. This preservative causes no deleterious effects in humans when consumed in small amounts and it occurs naturally in cinnamon, cloves, cranberries, plums, and prunes (Lewis, 1989). Benzoic acid is usually used in the form of
sodium benzoate because of low aqueous solubility of the free acid. El-Shenawy and Marth (1988a) reported that the antimicrobial effect of sodium benzoate against *L. monocytogenes* in a tryptose soy broth increased as temperature and pH decreased. The fact that a low temperature enhanced the effect of sodium benzoate is important because *L. monocytogenes* is a psychrotrophic bacterium that can grow at refrigerator temperatures. Furthermore, the antimicrobial activity of sodium benzoate was enhanced when the pH of the medium was adjusted with organic acids such as acetic, citric, lactic, or tartaric (EL-Shenawy and Marth, 1989). Acetic and tartaric acids were most effective followed by lactic and citric acids. Ryser and Marth (1988b) observed that survival of *L. monocytogenes* in cold-pack cheese food decreases when acetic, rather than lactic acid, was used to acidify the product. Yousef et al. (1989) demonstrated that inactivation and injury of *L. monocytogenes* in a minimal medium was affected by benzoic acid and incubation temperature. They found that cells of *L. monocytogenes* were inactivated at a rate dependent on temperature of incubation, while the presence of benzoic acid had relatively little effect on the rate of death of the organism.

C. Citric Acid

Citric acid (192.12 M.W., 3.13 pKₐ) is an acidifying agent with unique flavor characteristics and is generally
regarded as safe (GRAS) for use in foods under FDA regulations (FDA, 1990). As a weak acid, citric acid causes leakage of hydrogen ions across the cell membrane, acidifying the cell interior, and inhibiting nutrient transport. Since many microorganisms can metabolize citrate, this anion rarely has antimicrobial activity. Therefore, citric acid is considered a less effective antimicrobial than other acids.

Ahamad and Marth (1989) studied the behavior of \textit{L. monocytogenes} in tryptose broth acidified with acetic, citric, or lactic acid. They found that 0.2% citric acid prevented growth, while 0.5% was lethal to the bacterium. Their results indicate that increased acid concentration and higher temperature of incubation enhanced the lethal effect of citric acid on \textit{L. monocytogenes}. Because citric and lactic acids have higher dissociation constants, they exert weaker bactericidal or inhibitory action than acetic acid (Ahamad and Marth, 1989; El-Shenawy and Marth, 1989; Ita and Hutkins, 1991; Sorrells et al., 1989). Ahamad and Marth (1990) evaluated acid-injury of \textit{L. monocytogenes}. Results showed that acetic acid caused greatest inactivation, but citric acid caused the greatest degree of injury followed by lactic and acetic acid. They suggested that injured cells frequently lose some of their cellular material through leakage into the surrounding medium. Ita and Hutkins (1991) determined the intracellular pH of \textit{L. monocytogenes} during growth in a pH controlled fermentation vessel treated with organic acids.
They found that even though citric and lactic acids were effective in lowering the internal pH values, acetic acid had the greatest effect on cell survival.

D. Lactic Acid

Lactic acid (90.08 M.W., 3.79 pKₐ) has been used as a flavor enhancer, antimicrobial agent, and pH control agent and is listed as generally regarded as safe (GRAS) for use in food products under FDA regulations because of the lack of acute and/or chronic toxicity in humans (FDA, 1990; Lewis, 1989). This acknowledged absence of toxicity has led to the widespread choice of lactic acid as a decontaminating agent in the food industry (Ockerman et al., 1974; Woolthuis and Smulders, 1985; Woolthuis et al., 1984; Dezeure-Wallays and Hoof, 1980).

Ryser and Marth (1988b) determined the survival of *L. monocytogenes* in cold-pack cheese during refrigerated storage. They found that when cheese food contained sodium propionate, lactic acid, acetic acid or lactic acid plus acetic acid, average survival times were significantly shorter than when sodium propionate was used without acid. When cheese food contained sorbic acid, numbers of *L. monocytogenes* failed to decrease significantly when acidified with lactic acid. However, significantly shorter survival times were observed in cheese food adjusted with either acetic acid or lactic acid plus acetic acid compared to the non-acidified control.
Ahmad and Marth (1989) observed that the degree of inhibition or inactivation of *L. monocytogenes* by lactic acid was greatly affected by temperature of incubation and concentration of acid. No growth of *L. monocytogenes* occurred when the concentration of lactic acid was 0.2%, but at 0.3 and 0.5% lactic acid had a lethal effect on the organism.

Little information on the antimicrobial effect of lactic acid salts or lactates on *L. monocytogenes* have been published. Shelef and Yang (1991) determined the growth effect of sodium lactate on *L. monocytogenes* in broth, chicken, and beef. They found that growth supression of *L. monocytogenes* occurred in the presence of more than 5% sodium lactate, whereas, growth supression of the bacterium in sterile comminuted chicken and beef occurred with 4% sodium lactate. The bacterium was consisantly more sensitive to lactate in beef than in chicken, followed by broth medium. These results are in contrast to the more pronounced inhibitory effects normally observed in tests conducted in broth media. Addition of sodium or potassium lactate to foods, unlike that of lactic acid, did not influence the pH of the food.
2.12 Mechanism of Action of Antimicrobials

A. Fatty acids and Their Monoglycerides

Long-chain fatty acids can cause lysis of protoplasts and inhibit both oxygen uptake and amino acid uptake (Galbraith and Miller, 1973b). Thus, the presence of fatty acids might change cell membrane permeability leading to inhibition of synthesis of macromolecules or inhibition of oxygen uptake (Galbraith and Miller, 1973c). Furthermore, lowering pH of the suspending medium has been shown to increase uptake of fatty acids by Bacillus megaterium and reduce cell-medium interfacial tension (Galbraith and Miller, 1973a).

Tsuchido et al. (1985) reported that short- and medium-chain fatty acids lyse actively growing cells of Bacillus subtilis by induction of autolysins. Lysis of B. subtilis cells induced with glycerol dodecanoate and sucrose hexadecanoate may be due to the action of autolytic enzymes and not to the direct action of solubilization of the bacterial membrane. Ved et al. (1984) demonstrated that dodecylglycerol, which is an ether bond between glycerol and dodecanoic acid, had a stronger lytic action (7 μg/ml) on Streptococcus faecium than did glycerol dodecanoate (18 μg/ml) resulting from autolytic enzymes.

Lower concentrations of long-chain fatty acids stimulated oxygen uptake by Bacillus megaterium and Micrococcus lysodeikticus at pH 7.4, whereas, higher concentrations of the
fatty acids produced complete inhibition of oxygen uptake (Galbraith et al., 1971; Galbraith and Miller, 1973a; Galbraith and Miller, 1973b). Storek and Wachsman (1957) stated that cell membranes are sites of action for the fatty acids, since the enzymes involved in oxygen uptake are membrane bound.

Sheu et al. (1972) showed that short-chain fatty acids noncompetitively inhibit the uptake of amino acids in both whole cells and isolated membrane vesicles, which is energized by the cytochrome-linked electron transport system (Konings and Freese, 1971). In contrast, uptake of \( \alpha \)-methyl glucoside or fructose, which is affected by the phosphoenol pyruvate transferase system (Kundig and Roseman, 1971), was only slightly inhibited and the reduced nicotinamide adenine dinucleotide (NADH) oxidation system of membrane vesicles was not inhibited by fatty acids (Sheu et al., 1972).

It was evident that the mode of action and type of inhibition produced by fatty acids depend on the concentration used. It was apparent that at high concentrations, the effects were irreversible and bactericidal. Under these conditions (at pH 7.4), fatty acids in general, induced lysis of protoplasts and stimulated uptake of oxygen. In this respect, their activity resembled that found with mitochondria. Fatty acids could exert their bactericidal activity by producing irreversible bonding distortions as a result of surfactant effects on the bacterial cell membrane,
and hence dislocate the components of the mitochondrial energy system and inhibit synthesis of ATP (Galbraith and Miller, 1973c; Gale and Llewellen, 1971).

The activity of antibacterial compounds against amino acid transport in bacterial cells has been reported (Hugo and Bloomfield, 1971). They indicated that inhibition was due to interference of coupling of energy with transport. They further observed that these antibacterial agents at bacteriostatic concentrations produced both stimulation of oxygen uptake and inhibition of energy-dependent amino acid transport.

Another mechanism of action can be proposed from the fact that fatty acids are known to decrease glycolysis and stimulate gluconeogenesis (Sheu et al., 1975). Growth inhibition of \textit{B. subtilis} by fatty acids was reduced by glycolytic compounds, especially glucose and fructose, but only slightly, or not at all, by compounds in the citric acid cycle (Coutelle and Schewe, 1970).

Inhibition of amino acid uptake by bacterial cells has been suggested as one mode of action of fatty acids against various bacteria, but little has been reported on the mode of action of monoglycerides. Tsuchido et al. (1987) found that autolysis of \textit{Bacillus subtilis} cells was induced by glycerol and sucrose esters of fatty acids. They demonstrated that the esters caused morphological changes in cells, suggesting that inhibition of some processes of synthesis or regulation of the

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cell envelop, which is possibly related to the induction of autolysis.

Coutelle and Schewe (1970) demonstrated the action of fatty acids and monoglycerides on a NADH₂ oxidase system. The activity of the system was depressed by 50% in the presence of fatty acids or monoglycerides. Also, results on the inhibitory effect of monoglycerides on various enzyme systems showed that monoglycerides act only on the oxygen side of flavin in NADH₂ dehydrogenase, while fatty acids are less specific inhibitors, acting on several sites.

B. Lipophilic Acids

Most generally used antimicrobial food preservatives are lipophilic acids. Lipophilic acids contain either a carboxyl or a phenolic group as the essential acidic moiety. These compounds either reversibly inhibit the growth of cells or kills cells depending on the concentration used. Freese and Levin (1978) demonstrated that the inhibitory effect of lipophilic acids was due to the conduction of protons through membranes, effectively destroying the proton motive force that is needed for substrate transport. At significantly higher concentrations than those needed for reversible growth inhibition, the cell-killing effect results from puncturing the cell membrane, causing leakage of cytoplasm into aqueous surroundings (Sheu et al., 1975; Freese and Levin, 1978).

Growth inhibition by lipophilic acids also depended on
the pH of the culture (Freese et al., 1973; Leo et al., 1972). Freese (1978) proposed that only the uncharged lipophilic acid molecules partition into the membrane, since negatively charged ions usually cannot enter a lipid phase to any significant degree. He demonstrated that negatively charged lipophilic acid ions play a more important role in the inhibitory process than previously assumed. Another mechanism of inhibition by lipophilic acids proposed by Mitchell (1976) may result from the destruction of the proton motive force that is the energy source for oxidative phosphorylation and the transport of many substrates. This mechanism was further described by Freese and Levin (1978).

Many lipophilic acids have been shown to inhibit growth by inhibition of transport of amino acids into cells and membrane vesicles. Furthermore, growth inhibition in an amino acid medium as a carbon source can be counteracted by addition of glucose as another carbon source, whose active transport does not depend on the proton motive force (Freese et al., 1973; Sheu et al., 1972). Freese (1978) emphasized the mutual interdependence of the proton motive force and amino acid transport. When lipophilic acids move protons into cells, reducing the proton motive force, they reduce the uptake of amino acids and thus reduce available energy needed for the function of the electron transport system. Thus, lipophilic acids decrease the extrusion of protons that are needed to maintain the proton motive force.
C. Organic Acids

Inhibitory effects of organic acids can be correlated with their dissociation constants or pKₐ values. In general, weak acids with higher pKₐ values are thought to be more inhibitory to *L. monocytogenes* at a given pH than strong acids at the same pH (Ita and Hutkins, 1991). The amount of molecule in the undissociated form is determined by pH. As a result, this, along with solubility properties, determines the foods in which these acids are most effective. For example, the antimicrobial effectiveness of potassium sorbate increases as the pH value approaches its pKₐ of 4.74, the upper pH limit for its activity being 6.0-6.5.

Many weak acids have the ability to penetrate the cell membrane and accumulate within the cell cytoplasm (Ita and Hutkins, 1991). If the interior of the cell is more alkaline than the pKₐ of the acid, more of the acid will dissociate, releasing a proton and acidifying the cytoplasm of the cell (Booth, 1985). These events are thought to result in a variety of detrimental effects (Kashket, 1987). As a defense, many bacteria possess proton pumps or proton/cation exchange systems to deal with the influx of protons and to maintain the cytoplasm near neutral. However, if these pH regulatory systems are unable to function sufficiently, then the pH gradient will collapse. Intracellular acidification will then result in the loss of cell viability or cell destruction (Booth, 1985).
The toxicity of organic acids is not primarily caused by hydrogen ions but by the action of the undissociated molecule. Toxicity of organic acids against microorganisms has been related to interference with permeability of the microbial cell membrane, causing uncoupling of both substrate transport and oxidative phosphorylation from the electron transport system (Freese et al., 1973). Anions of the organic acids are then metabolized by the cell wall with the release of H⁺ which leads to acidification of cell contents (Freese et al., 1973). This probably is the main cause of inhibition and death of microorganisms by organic acids.
CHAPTER 3
MATERIALS AND METHODS

3.1 Effect of pH on the Minimal Inhibitory Concentration of Monolaurin against L. monocytogenes

A. Bacterial Strains

Four strains of L. monocytogenes: Scott A (clinical isolate, serotype 4b; provided by Economic Laboratories, St. Paul, MN), VA (Veterans Administration Hospital, Gainesville, FL), F5027 and F5067 (C. Donnelly, University of Vermont, Burlington, VT) were used in this study. Stock cultures were maintained through monthly transfers on tryptic soy agar (TSA; Difco, Detroit, MI) slants and stored at 4°C. Cultures were grown in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; Difco, Detroit, MI) for 24 h at 35°C before use. Serial dilutions were made from these subcultures in TSBYE medium to prepare working cultures of approximately 10^5 CFU/ml.

B. Media and Monolaurin Preparation

Rehydrated TSBYE was adjusted to pH 5.0, 5.5, 6.0, and 7.0 with 1 N NaOH or 1 N HCl before autoclaving using an Orion pH Meter SA 520 (Orion, Boston, MA). Ten-ml aliquots of TSBYE were dispensed into sterile test tubes after sterilization. A 1% stock solution of monolaurin (Sigma
Chemical Co., St. Louis, MO) dissolved in absolute ethanol was prepared fresh before each experiment. The stock solution was filter-sterilized using a 0.45-μm membrane filter (Millipore Products Division, Bedford, MA).

C. Determination of Minimal Inhibitory Concentration

Appropriate amounts of monolaurin stock solution were added to 10 ml of pH-adjusted TSBYE to give final concentrations of 0, 1, 3, 5, 7, 9, 10, and 12 μg/ml. From the 10⁵ CFU/ml working culture of each strain, 0.1 ml aliquots were aseptically transferred into each test tube to obtain a final inoculum of approximately 10³ CFU/ml. Positive controls consisted of inoculated TSBYE containing 0.1% ethanol without monolaurin. Negative controls used for sterility tests were uninoculated TSBYE containing 0.1% ethanol without monolaurin.

The MIC values were determined using an impedance method (Firstenberg-Eden and Eden, 1984). One ml aliquots of inoculated broth containing appropriate monolaurin concentrations were aseptically transferred into wells of a Bactometer module (bioMérieux Vitek Systems Inc., Hazelwood, MO). The modules were incubated in the Bactometer at 35°C for 24 h. As microorganisms grow and metabolize substrates, a threshold impedance value is reached, which can be detected by the Bactometer. This point of impedance change is called the detection time (DT). The lowest concentration of monolaurin that shows no impedance detection time was designated as the...
3.2 Growth and Survival of *L. monocytogenes*

A. Test Organism

Method used was as described previously in section 3.1-A. *L. monocytogenes* strain Scott A was used for this study.

B. Preparation of Media and Preservative

Rehydrated TSBYE was adjusted to pH 5.0, 5.5, and 7.0 with 1 N HCl. Because the previous study showed little difference between pH values of 6-7 on monolaurin effectiveness, tests were not conducted in media having pH values within this range. After autoclaving, appropriate aliquots of TSB-YE were aseptically dispensed into 250 ml sterilized Erlenmeyer flasks. Preparation of monolaurin was conducted as described previously in section 3.1-B.

C. Experimental Procedure

Quantities of 25, 30, 35, 40, and 45 µl of 1% monolaurin were added to 49.975, 49.970, 49.965, 49.960, and 49.955 ml of TSBYE broth to give final concentrations of 5, 6, 7, 8, and 9 µg/ml of monolaurin, respectively. From the 10^5 CFU/ml working culture, 0.5 ml aliquots were aseptically transferred into each flask containing different concentrations of monolaurin to obtain a final inoculum of approximately 10^3.
CFU/ml. These were incubated at given temperatures for designated time periods. TSBYE medium containing 0.1% ethanol without monolaurin was used as a control.

D. Enumeration Method

*L. monocytogenes* populations were determined by surface-plating 0.1 ml amounts of undiluted or serially diluted (0.1% peptone water) samples on duplicate TSA agar plates. Sampling was done at appropriate time intervals to determine growth inhibitory effects from all tested conditions. Plates were incubated at 35°C for 48 h, and colonies were counted. Two replicates were tested during the entire experiment. Numbers were converted to log CFU/ml and generation times (GT) were calculated from logarithmic phase data (EL-Shenawy and Marth. 1988b).

E. Statistical Analysis

Analysis of variance (ANOVA) procedures using the Statistical Analysis Systems program (SAS, 1985) were used to determine generation time (GT) differences among pH, temperature, or concentration of monolaurin. Also, Duncan's test was used to determine different between means when ANOVA indicated significant differences.
3.3 Antimicrobial Effect of Ethanol, Glycerol Monolaurate, or Lactic Acid

A. Preparation of Inoculum

Method used was as described previously in section 3.2-A, except that subculture serial dilutions were made in TSBYE to prepare working cultures of approximately 10^7 CFU/ml.

B. Preparation of Antimicrobial Agents

A 10% lactic acid (Mallinkrodt Inc., Paris, KY) stock solution was prepared in sterile distilled water and filter-sterilized using a 0.45-μm membrane filter (Millipore Products Division, Bedford, MA). Preparation of monolaurin was as described previously in section 3.1-B.

C. Experimental Procedure

Appropriate amounts of antimicrobial stock solutions were added to 250-ml flasks containing 50 ml of sterile TSBYE to give final ethanol concentrations of 0, 0.625, 1.25, 2.5, 5.0, and 10%, lactic acid concentrations of 0, 0.0625, 0.125, 0.25, and 0.5%, and monolaurin concentrations of 0, 0.625, 1.25, 2.5, 10, and 20 μg/ml. Combined effects were analyzed by adding antimicrobial agents in dual combinations at various concentrations. Concentrations used were 0, 1.25, 2.5, 5.0, 10, 20, and 40 μg/ml monolaurin combined with 0, 1.25, 2.5, 5.0, 10, and 20% ethanol. Comparable concentrations of
ethanol were combined with 0, 0.125, 0.25, 0.5, and 1% lactic acid. From the 10⁷ CFU/ml working culture of the bacterium, 0.5-ml aliquots were aseptically transferred into each flask containing appropriate concentrations of antimicrobial agent alone or in combination to obtain a final initial inoculum of approximately 10⁵ CFU/ml. Positive controls consisted of inoculated TSBYE containing 0.1% ethanol and no antimicrobial agents. Negative controls used for sterility tests were uninoculated TSBYE with 0.1% ethanol without antimicrobial agents present.

D. Determination of MIC and Growth Effects

Determination of MIC values was conducted as described previously in 3.1-C. Combined effects of the agents on *L. monocytogenes* were determined using the standard plate count method (APHA, 1985). The method of Krogstad and Moellering (1986) was used to detect additive, synergistic, or antagonistic interactions. The inoculated 250-ml flasks containing 50 ml of pH 7.0-adjusted TSBYE with appropriate concentrations of each antimicrobial agent, either alone or in combination, were incubated at 35°C for 24 h. Samples were removed periodically, and *L. monocytogenes* counts were determined by surface plating on TSA and incubating the plates at 35°C for 48 h.
3.4 Antimicrobial Activity of Glycerol Monolaurate Combined with Selected Organic Acids

A. Preparation of Inoculum

Method used was as described previously in section 3.2-A.

B. Preparation of Antimicrobial Agents

Ten percent stock solutions of citric acid (EM Industries Inc., Gibbstown, NJ), lactic acid (Mallinckrodt Inc., Paris, KY), and acetic acid (Mallinckrodt) were prepared in sterile distilled water. Preparation of monolaurin was as described previously in section 3.3-B.

C. Experimental Procedure

Appropriate amounts of diluted antimicrobial stock solutions were added to 250-ml flasks containing 50 ml of sterile TSBYE, with final concentrations of 1250 µg/ml acetic acid, 1250 µg/ml benzoic acid, 2500 µg/ml citric acid, 2500 µg/ml lactic acid, and 5 µg/ml monolaurin. Combined effects were analyzed by combining each organic acid individually with monolaurin at the same concentrations. The pH of each flask with antimicrobial agents was measured using a SA 520 pH meter (Orion, Boston, MA) and is shown in Table 9. From the 10⁷ CFU/ml working culture of the organism, 0.5-ml aliquots were aseptically transferred into each flask containing appropriate concentrations of each antimicrobial agent, either alone or
combined, to obtain a final initial inoculum of approximately $10^5$ CFU/ml. Positive controls consisted of inoculated TSBYE with 0.1% ethanol without antimicrobial agents. Negative controls (pH 7.4) used for sterility tests were uninoculated TSBYE containing 0.1% ethanol without antimicrobial agents.

D. Determination of MIC and Growth Effects

Methods used were as described previously in section 3.3-D.

3.5 Biofilm Inactivation

A. Bacteria Culture

Method used was as described previously in section 3.2-A. Rich nutrient (TSB) and depleted nutrient medium (1:10 dilution of TSB) was used for preparation of adherent cells.

B. Preparation of Sanitizer Solutions

Preparation of monolaurin was as described previously in section 3.2-A. Solutions containing 50 or 100 $\mu$g/ml of monolaurin (final concentrations of ethanol were 0.5 or 1%) were made from stock solution using 0.01M phosphate-buffered saline (PBS, pH 7.2) as diluent. Tween 80 at 0.1% was used as neutralizing agent for monolaurin.
C. Preparation of Stainless Steel Chips

Stainless steel (SS) chips (type 304, No. 2B finish) were cut into 1 by 1 cm squares and cleaned by ultrasonic cleaning in 2% Micro laboratory cleaning solution (International Products Corp., Trenton, NJ) for 15 min, rinsed in distilled water, and sterilized at 121°C for 15 min prior to use.

D. Preparation of Planktonic (Broth Media Grown) Cells

Planktonic cells were obtained using TSB and 1:10 diluted TSB. One ml of a 24 h TSB subculture was inoculated into 100 ml sterile TSB or diluted TSB medium in a 250 ml flasks and incubated at 25°C for 24 h. Cells were harvested from the medium by centrifugation at 17,000 x g for 5 min (RC5C, Sorvall Instrument, Norwalk, CT) and resuspended in PBS. This suspension, used for inhibition studies of monolaurin as a sanitizer, was $10^9$ CFU/ml in media grown from TSB and $10^8$ CFU/ml in diluted TSB.

E. Preparation of Adherent Cells

Sterile stainless chips were aseptically placed in 250 ml flasks containing sterile 100 ml TSB or diluted TSB. The media were inoculated with 1 ml of a 24 h TSB subculture and incubated at 25°C for 1-d or 7-d. After incubation, each chip was aseptically removed from each flask with sterile forceps and rinsed 6 times with 100 ml PBS using a 250 ml sterile teflon squeeze washing bottle to remove unattached cells. For
7-d adherent cells, old media were discarded and fresh media were transferred every 2 d.

F. Exposure of Planktonic Cells to Monolaurin

One ml of planktonic cell suspension in TSB and 10 ml of planktonic cell suspension in diluted TSB were transferred into 99 ml and 90 ml of sterile PSB to give final cell concentrations of 10^7 CFU/ml without sanitizer as a control. Appropriate concentrations of monolaurin (50 or 100 μg/ml) were added to the final planktonic cell suspension in TSB or diluted TSB, and allowed to remain at room temperature for exposure times of 0, 20, 40, 60, 80, 100, or 120 min. Plating was performed in duplicate, and plates were incubated at 35°C for 48 h and counted using a colony counter. The entire experiment was repeated three times.

G. Exposure of 1-d or 7-d Adherent Cells to Monolaurin

Washed SS chips containing 1-d or 7-d adherent cells were exposed in a 250 ml flask containing 100 ml of monolaurin at 50 or 100 μg/ml for 0, 20, 40, 60, 80, 100, or 120 min without stirring. After exposure, the SS chips were removed and placed in 0.1% tween 80 neutralizing solution for 2 min and rinsed 6 times in a sterile 250 ml flask containing 100 ml PBS. Each washed SS chip was placed in a sterile test tube containing 10 ml of PBS and 0.5 g of sterile microscopic glass beads (100 μm diameter, BioSpec Products, Inc., Bartlesville, OK) and
vortexed for 2 min to remove adherent surface cells. Plating was performed in duplicate with incubation at 35°C for 48 h and colonies counted using a colony counter. The entire experiment was repeated three times.

H. Exposure of Planktonic Cells to Monolaurin Combined with Acetic Acid

One ml of planktonic cell suspension in TSB was transferred into 99 ml to give a final cell concentration of 10^7 CFU/ml without sanitizer as a control. Appropriate concentrations of monolaurin (50 or 100 μg/ml) combined with acetic acid (0.5% or 1%) were added to the final planktonic cell suspension in TSB, and allowed to remain at room temperature for exposure times of 0, 5, 10, 15, 20, or 25 min. After exposure, appropriate dilutions were enumerated by the pour plate procedure using TSA. Plating was performed in duplicate and plates were incubated at 35°C for 48 h and colonies counted using a colony counter. The entire experiment was repeated three times.

I. Exposure of 1-d or 7-d Adherent Cells to Monolaurin Combined with Acetic Acid

Washed SS chips containing 1-d or 7-d adherent cells were exposed to appropriate concentrations of monolaurin (50 or 100 μg/ml) combined with acetic acid (0.5% or 1%), and allowed to remain at room temperature for specific exposure times of 0,
5, 10, 15, 20, and 25 min without stirring. After exposure, the SS chips were removed and placed in 0.1% tween 80 neutralizing solution for 2 min and rinsed 6 times in a sterile 250 ml flask containing 100 ml PBS. Each washed SS chip was individually placed in a sterile test tube with 10 ml of PBS and 0.5 g of sterile microscopic glass beads (100 μm diameter, BioSpec Products, Inc., Bartlesville, OK) and vortexed for 2 min to remove adherent cells on the surface of the SS. Appropriate dilutions were pour plated with TSA, incubated at 35°C for 24 h, and colonies enumerated.

J. Effect of Heat on Planktonic Cells, 1-d, and 7-d Adherent Cells

The 10^7 CFU/ml of planktonic cells and washed SS chips with adherent cells were submerged in test tubes containing PBS tempered at either 55°C or 65°C in a water bath for 0, 1, 3, or 5 min. After heating, test tubes containing planktonic cells or SS chips with adherent cells were immediately placed on ice. Enumeration was as described in section 3.5-I. All tests were run in duplicate and repeated five times.

K. Combined Effect of Monolaurin and Heat Treatment

The 10^7 CFU/ml of planktonic cells and washed SS chips containing adherent cells were exposed in test tubes containing 50 or 100 μg/ml of monolaurin in PBS at either 55°C or 65°C in a water bath for 5 min. After heating, test tubes
containing planktonic or SS chips with adherent cells were immediately placed on ice and then, the SS chips were removed and placed in 0.1% tween 80 neutralizing solution for 2 min and rinsed 6 times using sterile teflon squeeze washing bottle. Enumeration was as described previous in section 3.5-I. All tests were run in duplicate and repeated five times.

L. Statistical Analyses

All data were subjected to analysis of variance (SAS, 1985). Statistical analyses were performed using mean log CFU/ml of L. monocytogenes to determine differences between TSB and diluted TSB on the effect of monolaurin, either alone or combined with acetic acid or heat, against planktonic cells, 1-d or 7-d adherent cells. Fisher's Protected Least Significant Difference Test was used to determine significant (P<0.05) differences between treatment means (SAS,1985).

3.6 Effect of Air, Vacuum, or Modified Atmosphere Packaging with or without Antimicrobials in crawfish tailmeat

A. Preparation of Cell Suspension

Preparation of L. monocytogenes Scott A subculture was as described previously in section 3.2-A. From subculture, 1 ml was transferred into 100 ml sterile TSB medium in a 250 ml flasks and incubated at 35°C for 24 h. Cells were harvested from the medium by centrifugation at 17,000 x g for 5 min and
resuspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2). This suspension was diluted and approximately $10^5$ CFU/ml of the suspension was used for inoculation of crawfish tail meat.

B. Preparation of Antimicrobial Agents

Stock solutions of lactic acid or monolaurin were prepared as described previously in section 3.3-B. Solutions containing 200 or 400 $\mu$g/ml of monolaurin (final concentrations of ethanol were 2 or 4%) or 0.5, 1, 2 or 3% lactic acid were made from these stock solutions.

C. Sample Preparation and Inoculation

Crawfish (*Procambarus clarkii*) tail meat used for this study was purchased from local suppliers and stored at -20°C prior to use. After thawing, 200 g of crawfish tail meat was placed in cheese cloth gauze and sterilized by autoclaving for 15 min. The product was removed and immediately placed on ice for cooling after sterilization. The product was then added to a sterile container containing a $10^5$ CFU/ml suspension of *L. monocytogenes* in 2 L attachment medium (Notermans and Kampelmacher, 1974). The attachment experiments were conducted by dipping the product for 10 min in attachment medium (8.7 g NaCl, 1.66 g Na$_2$HPO$_4$, 0.33 g NaH$_2$PO$_4$, 0.37 g EDTA, 1 L distilled water). Preliminary work was done to determine the effect of dipping time from 5 to 60 min on attachment of the organism to crawfish tail meat. Dipping time was not
found to affect attachment (data not shown). After dipping, the product was immediately placed on sterile paper towels to dry, then placed in a Waring blender jar (Waring Products Division, New Hartford, CT) and monolaurin and/or lactic acid were added to give final concentrations of 200 or 400 µg/g monolaurin or 0.5, 1, 2, or 3% lactic acid, either alone or in combination. Each sample was thoroughly mixed for 30 s.

D. Packaging of Inoculated Crawfish Tail Meat Containing Antimicrobial Agents

From each treated sample, 11-g subsamples were aseptically placed in sterile 7" x 8" plastic barrier bags (Koch Model 014609, Kansas City, MO). These barrier bags have O₂ transmission rates of 9 cc/m² in 24 h. Bags containing air storage samples were heat-sealed without evacuation or gas flush. Bags containing vacuum storage samples were evacuated to a negative pressure of 980 mbar and heat-sealed using a Multivac A300122 packing machine (Kansas City, MO). Bags with samples for modified atmosphere storage were evacuated to -980 mbar, backflushed with a commercial gas mix containing 74.8% CO₂:15.2% N₂:10% O₂ (Aligal 18, A-L Compressed Gases, Inc., Baton Rouge, LA) and heat-sealed. Concentration of ethanol in the 200 or 400 µg/g monolaurin treated samples were 2 or 4%, respectively. Thus, 2 or 4% ethanol were used as controls to determine the effects of 200 or 400 µg/g monolaurin. Negative controls used for sterility tests were uninoculated heat...
treated crawfish tail meat without antimicrobial agents. Positive controls consisted of inoculated TSBYE without antimicrobial agents. Triplicate packed samples were prepared and stored at 4°C for 21 d.

E. Enumeration of Bacteria

Three samples containing monolaurin or lactic acid, either alone or in combination during air storage at 4°C, were tested for the number of *L. monocytogenes* at 0, 3, 7, 10, 14, 17, and 21 d. Also, three samples per packaging treatment were analyzed for the population of the organism after 14 d at 4°C. Enumeration was as described in section 3.2-D.

F. Determination of pH

An Orion pH Meter SA 520 (Orion, Boston, MA) was used to measure pH changes of the crawfish tail meat treated with or without monolaurin or lactic acid, either alone or in combination, during storage at 4°C.

G. Statistical Analyses

Statistical analyses were performed using mean log CFU/g of *L. monocytogenes* to determine differences among samples treated with or without antimicrobials during air, vacuum, or modified atmosphere storage. Also, generation times were calculated from log phases growth curves. Statistical analyses were as described in section 3.5-L.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Effect of pH on the Minimal Inhibitory Concentration of Monolaurin Against *L. monocytogenes*

The effect of pH on antibacterial activity of monolaurin against four strains of *L. monocytogenes* is shown in Table 4.1. There was little difference in susceptibility to monolaurin among the strains. When the pH of the medium was reduced from 7.0 to 5.0, the MIC of monolaurin reduced from 10 \( \mu g/ml \) to 3 \( \mu g/ml \) for three of four strains. There was, however, little difference in MIC values in broth with pH 6.0 or pH 7.0. Wang and Johnson (1992) demonstrated the growth effect of fatty acids and monoglycerides in brain heart infusion (BHI) and in milk against *L. monocytogenes*. They found that monolaurin had the strongest antimicrobial activity in BHI broth and was bactericidal at 10 \( \mu g/ml \), whereas lauric acid (C\(_{12}\)), stearic acid (C\(_{18}\)), and potassium-conjugated linoleic acid (K-CLA) were bactericidal at 20, 100, and 200 \( \mu g/ml \), respectively. Bactericidal activity in BHI broth was higher at pH 5 than at pH 6. *L. monocytogenes* in skim milk at 4°C did not grow in the presence of monolaurin, but the inhibitory effect of monolaurin was less at 23°C.

Other antimicrobial agents that have been tested against *L. monocytogenes* were less effective than monolaurin. The most effective phenolic compounds reported were tertiary...
Table 4.1. Effect of pH on the minimal inhibitory concentration (MIC) of monolaurin against four strains of *L. monocytogenes*

<table>
<thead>
<tr>
<th>pH</th>
<th>Scott A</th>
<th>F-5027</th>
<th>F-5069</th>
<th>VA</th>
</tr>
</thead>
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<tr>
<td>5.0</td>
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<td>3</td>
<td>3</td>
<td>3</td>
</tr>
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<td>5.5</td>
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<td>6.0</td>
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</tr>
<tr>
<td>7.0</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

*The MIC represents the lowest concentration of monolaurin that completely inhibited growth of *L. monocytogenes.*
butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and propyl paraben with MIC values of 64, 128 and 512 μg/ml, respectively (Kim et al., 1989). Lowering the pH of the medium has been shown previously to increase the inhibitory activity of antimicrobial agents. For example, El-Shenawy and Marth (1988b) reported that *L. monocytogenes* was capable of slight growth in the presence of 3,000 μg/ml potassium sorbate at pH 5.6 but only 1,500 μg/ml was needed for complete growth inhibition at pH 5.0.

The present results show that the antimicrobial activity of monolaurin was highly dependent upon the pH of the suspending medium. Consequently, the properties of monolaurin in the suspending solution must be considered. Long-chain fatty acids can: 1) cause lysis of protoplasts, 2) cause leakage of 260 nm-absorbing material and protein from both bacteria and protoplasts, and 3) inhibit oxygen uptake and amino acid uptake (Galbraith and Miller, 1973b). Thus, the presence of fatty acids might change cell membrane permeability leading to inhibition of synthesis of macromolecules or inhibition of oxygen uptake (Galbraith and Miller, 1973c). Indeed, monolaurin has been shown to cause extensive leakage of 260 nm-absorbing intracellular proteins from *Staphylococcus aureus* (Vadehra et al., 1985). Furthermore, lowering the pH of the suspending medium has been shown to increase the uptake of fatty acids by *Bacillus megaterium* and reduce cell-medium interfacial tension.
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(Galbraith and Miller, 1973a). The increased inhibition of *L. monocytogenes* by monolaurin with decreasing pH values may result from decreased interfacial tension at the bacterial lipid membrane-aqueous medium interface or increased uptake of monolaurin.

Results from the present study indicate that the four strains of *L. monocytogenes* were sensitive to monolaurin, with sensitivity increasing with decreasing pH. Since the effect of monolaurin against *L. monocytogenes* was greater than that reported for other commonly used food antimicrobials, these data can serve as a basis for evaluating the potential of monolaurin for control of *L. monocytogenes* in foods.

4.2 Influence of Temperature, pH, and Monolaurin on Growth and Survival of *L. monocytogenes*

A. Effect of Monolaurin at 7°C

The generation time (GT) of *L. monocytogenes* at different temperatures, pH values, and monolaurin concentrations are shown in Table 4.2. *L. monocytogenes* growth in the control medium at 7°C was detected at 1 d with a GT of 5.3 h at pH 7.0 (Fig. 4.1). Lag phases were extended in the presence of 5, 6, 7, or 8 μg/ml monolaurin, but GT of the pathogen at those concentrations did not significantly differ (P>0.05) from the control. However, GT was significantly different (P<0.05) in the presence of 9 μg/ml monolaurin compared with the control.
Table 4.2. Generation time of *L. monocytogenes* at different temperatures, pH, and concentrations of monolaurin.

<table>
<thead>
<tr>
<th>Monolaurin (μg/ml)</th>
<th>pH 7.0</th>
<th>pH 5.5</th>
<th>pH 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>15°C</td>
<td>7°C</td>
</tr>
<tr>
<td></td>
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<td>15°C</td>
<td>7°C</td>
</tr>
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<td>5.7ª</td>
<td>12.6ª</td>
</tr>
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</tr>
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<td>2.3ª</td>
<td>7.4ª</td>
<td>16.9ª</td>
</tr>
<tr>
<td>9</td>
<td>2.6ª</td>
<td>10.1ª</td>
<td>4.5ª</td>
</tr>
</tbody>
</table>

NG: No growth occurred.
ND: No detectable generation time.

Means within a column with different superscripts are significantly different (p<0.05).
Fig. 4.1. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 7°C and pH 7.0.
and other treatments. At pH 5.5, the bacterium started to grow in the control after 2 d with a GT of 10.0 h (Fig. 4.2). Addition of 5, 6, 7, or 8 μg/ml monolaurin to the medium extended lag phase of L. monocytogenes until 12 d of incubation prior to growth. GT at these concentrations was not significantly different (P>0.05) from the control. In the presence of 9 μg/ml monolaurin the lag phase was lengthened to 16 d before slow growth was initiated. When the pH was reduced to pH 5.0, the pathogen started to grow after 3 d with a GT of approximately 12.9 h in the control medium (Fig. 4.3). No growth was observed at any concentration of monolaurin at this pH. Addition of 7, 8, or 9 μg/ml monolaurin to the medium reduced counts to undetectable levels after 24, 22, or 20 d, respectively. GT of all monolaurin treatments were significantly different (P<0.05) from the control.

B. Effect of Monolaurin at 15°C

The bacterium grew in the control medium at 15°C after 6 h with a GT of 1.6 h at pH 7.0 (Fig. 4.4). Addition of 5 or 6 μg/ml monolaurin extended the lag phase to 12 h, while not significantly affecting (P>0.05) GT. Addition of 7 or 8 μg/ml monolaurin extended the lag phase to 18 or 24 h, respectively. GT at these levels was significantly different (P<0.05) from previous treatments. Nine μg/ml monolaurin extended the lag phase to 30 h and resulted in a significantly longer (P<0.05) GT than seen at other concentrations. At pH 5.5, the pathogen
Fig. 4.2. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 7°C and pH 5.5.

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Fig. 4.3. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 7°C and pH 5.0.
Fig. 4.4. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 15°C and pH 7.0.
initiated growth in the control medium after 12 h with a GT of 2.5 h (Fig. 4.5). Addition of 5 μg/ml monolaurin to the medium extended the lag phase to 24 h prior to growth. Addition of 6, 7, 8, or 9 μg/ml monolaurin to the medium extended the lag phase to 36 h. None of the GT of monolaurin treated samples were significantly different (P<0.05) from the control. When the pH was decreased to 5.0, the bacterium started to grow after 1 d, with a GT of 4.7 h for the control (Fig. 4.6). Addition of 5, 6, or 7 μg/ml monolaurin to the medium extended the lag phase to 48, 72, or 72 h, respectively, while not significantly affecting (P>0.05) GT. Addition of 8 μg/ml monolaurin extended the lag phase to 120 h and significantly increased (P<0.05) the GT compared with the other treatments. Counts of the bacterium were reduced to undetectable levels after 120 h in the presence of 9 μg/ml monolaurin.

C. Effect of Monolaurin at 35°C

*L. monocytogenes* began to grow during incubation at 35°C after 2 h with a GT of 0.5 h in the control at pH 7.0 (Fig. 4.7). The bacterium grew after lag phases of 4, 6, 8, 10, or 12 h in the presence of 5, 6, 7, 8, or 9 μg/ml monolaurin, respectively. GT at 5, 6, 7, or 8 μg/ml monolaurin was not significantly different (P>0.05) from the control, while the GT with 9 μg/ml monolaurin was different (P<0.05) from the other treatments. At pH 5.5, growth of *L. monocytogenes*
Fig. 4.5. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 15°C and pH 5.5.
Fig. 4.6. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 15°C and pH 5.0.
Fig. 4.7. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 35°C and pH 7.0.
initiated in the control broth after 3 h with a GT of 0.6 h (Fig. 4.8). Addition of 5, 6, or 7 μg/ml monolaurin to the medium extended the lag phase to 3, 6, or 15 h, but GT did not significantly differ (P>0.05) from the control. Addition of 8 μg/ml monolaurin extended the lag phase to 15 h and gave a GT that was different (P<0.05) from the control, but not different from the previous treatments. Increasing monolaurin to 9 μg/ml extended the lag phase to 21 h. GT of this treatment was greater (P<0.05) than the other treatments. When the pH was reduced to 5.0, L. monocytogenes began to grow after 6 h with a GT of 1.1 h (Fig. 4.9). No growth occurred in the presence of 5, 6, 7, or 8 μg/ml monolaurin until 42 h. Counts were reduced to below detectable levels with 9 μg/ml monolaurin within 6 h. GT of all treatments were significantly different (P<0.05) from the control.

These results demonstrated that differences in the antimicrobial efficacy of monolaurin depend on the interaction between pH and temperature. It was previously reported that L. monocytogenes was notably more sensitive to monolaurin as pH decreases (Table 4.1). This finding was confirmed in the present study where L. monocytogenes was inactivated by lower concentrations of monolaurin with a decrease in the pH. The contribution of temperature to monolaurin effectiveness showed that inactivation occurs more rapidly at higher temperatures. Thus, the most rapid inactivation of the pathogen by monolaurin occurs with the highest temperature and lowest pH.
Fig. 4.8. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 35°C and pH 5.5.
Fig. 4.9. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 35°C and pH 5.0.
Combination. Bacteriostatic effects of sublethal monolaurin concentrations increased as temperature and pH decreased. Thus, interactions between temperature, pH, and monolaurin depend on the levels of each factor. Similar lethal and sublethal effects have been found in investigations of the combination of temperature and pH on sodium benzoate activity against *L. monocytogenes* (EL-Shenawy and Marth, 1988a).

Lowering medium pH has been shown to increase uptake of fatty acids by *Bacillus megaterium* and to reduce cell-medium interfacial tension (Galbraith and Miller, 1973a). The increased inhibition of *L. monocytogenes* by monolaurin with decreasing pH values may result from decreased interfacial tension at the bacterial lipid membrane-aqueous medium interface. It also may be due to increased uptake of monolaurin. In addition to pH and temperature, other environmental factors, i.e., initial inoculum levels, competing organisms, type of food components, or water activity, may influence the effectiveness of monolaurin against *L. monocytogenes*.

The present study also showed that populations of *L. monocytogenes* decreased during the lag phase in the presence of sublethal concentrations of monolaurin, followed by an increase to initial inoculum levels and continued growth. This pattern was termed the "Phoenix phenomenon" by Collee et al. (1961). They observed an apparent decrease in cell numbers of *Clostridium perfringens* within the first few hours.
of incubation at 50°C, followed by an increase to a maximum population within 6 h. This phenomenon was explained by Shoemaker and Pierson (1976), reporting on the growth of C. perfringens at sublethal temperatures. They showed a decrease in cell numbers (phase I), followed by an increase in count to the initial level (phase II) and a further increase above the initial level of cell numbers (phase III). The researchers investigated the possibility that the "Phoenix phenomenon" involved an injury-recovery process using experiments with nalidixic acid that eliminated the possibility of cell division. They concluded that viable cells became sensitive to assay conditions (phase I), but after a period of time these cells apparently recovered their tolerance to assay conditions (phase II) and finally grew (III) as a result of the initial temperature shock. The same phenomenon was observed in the present data with the presence of sublethal concentrations of monolaurin against L. monocytogenes instead of at sublethal temperatures. Sublethal concentrations may injure some cells in the culture, but remaining active cells can use more substrate for growth than injured cells. Effectiveness of sublethal concentrations of monolaurin may depend on whether the cells are metabolically active or resting. Yousef et al. (1991) assumed that metabolically active cells may detoxify antimicrobial agents up to a certain concentration, or may be poisoned by it. If this detoxication can occur, then growth would be delayed until the cells
reduced the concentration of the particular agent to a safe level. If the microorganism is metabolically active, but cannot detoxify sublethal concentrations, then generation time and maximum growth would be unfavorably affected, resulting in inhibition of growth. These assumptions were illustrated by Yousef and Marth (1981) who reported that sublethal concentrations of sorbic acid delayed growth of *Aspergillus parasiticus*, but apparently did not affect other growth parameters. This result may be demonstrated by the ability of the mold to detoxify sorbic acid at the concentrations used.

It has been reported that monolaurin has the greatest overall antimicrobial activity among fatty acids and their esters and would appear to have the greatest potential for use in foods and cosmetics (Shibasaki and Kato, 1979). However, the range of use of monolaurin as a food preservatives may be limited since its activity is antagonized by many food components such as starch, protein, lipid, and monopalmitin (Kato and Shibasaki, 1975). Furthermore, monolaurin has been found to be active against Gram-positive bacteria, yeasts, and molds, but less active against Gram-negative bacteria. More research is needed on the combined effects of monolaurin with other agents to maximize their effectiveness when used directly in foods as a preservative. Kato and Shibasaki (1976) examined the combined effects of fatty acids and monoglycerides on the thermal destruction of Gram-negative bacteria. They found that lauric acid and monolaurin were
highly effective at low concentrations in combination with heating.

An important observation made in this study is that *L. monocytogenes* was highly sensitive to monolaurin. Since monolaurin had a greater inhibitory effect at low temperature, it might be considered as a preservative to control psychrotrophic organisms like *L. monocytogenes* that can grow at refrigerator temperatures. Also, the possible enhanced antimicrobial effectiveness of monolaurin in combination with heating, or with other chemical agents, may warrant its use as a new alternative food preservative in direct food applications.

4.3 Combined Antimicrobial Effect of Ethanol with Monolaurin or with Lactic Acid

A. Minimal Inhibitory Concentration Effect

Minimal inhibitory concentration (MIC) values of ethanol and monolaurin, either alone or in combination against *L. monocytogenes*, are shown in Table 4.3. MIC values of monolaurin and ethanol alone were 10 μg/ml (0.001%) and 50,000 μg/ml (5%), respectively. However, MIC values increased when monolaurin was combined with ethanol. For example, when 5% ethanol was combined with sublethal concentrations of monolaurin, the combination allowed growth of the organism, whereas, 5% ethanol used alone prevented growth. This result suggests antagonism when the two agents are combined.
Table 4.3. Minimal Inhibitory Concentration (MIC)* of combinations of monolaurin and ethanol against *L. monocytogenes.*

<table>
<thead>
<tr>
<th>Ethanol (%)</th>
<th>0</th>
<th>0.625</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+(^b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-(^c)</td>
<td>-</td>
</tr>
<tr>
<td>0.625</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1.25</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.50</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5.00</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The MIC represents the lowest concentration of antimicrobials that shows no impedance detection (IDT).

\(^b\): Growth-the concentrations of antimicrobials that show IDT.

\(^c\): No growth-the concentrations of antimicrobials that show no IDT.
Table 4.4 shows MIC values of ethanol and lactic acid either alone or in combination. MIC value of lactic acid alone was 5,000 μg/ml (0.5%), but was lower when 1.25% ethanol was combined with 0.25% lactic acid. This indicates additive or synergistic effects when the agents are combined. Thus, sublethal concentrations of combinations of monolaurin and ethanol or ethanol and lactic acid were chosen to further evaluate potential additive, synergistic, or antagonistic effects on L. monocytogenes (Krogstad and Moellering, 1986).

B. Inhibitory Effect

Monolaurin is generally insoluble in water, but soluble in organic solvents such as methanol, ethanol, or glycerol. Thus, to incorporate monolaurin into aqueous TSBYE, the compound was dissolved in ethanol before addition to the medium. Therefore, this study first investigated the effect of ethanol on the growth of L. monocytogenes. There was no inhibition of the organism in media containing 1.25% ethanol, while in 2.5% ethanol slight growth inhibition was observed (Fig. 4.10). Growth of L. monocytogenes was almost completely inhibited in the presence of 5% ethanol. The concentration of ethanol in the TSBYE medium used as control was at a subinhibitory level of 0.1%.

Shapiro et al. (1978) found that the effectiveness of ethanol against Staphylococcus aureus was a function of a_w and the test medium, but was greater than could be attributed to
Table 4.4. Minimal Inhibitory Concentration (MIC)\(^a\) of combinations of lactic acid and ethanol against *L. monocytogenes*.

<table>
<thead>
<tr>
<th>Lactic Acid (%)</th>
<th>Ethanol (%)</th>
<th>0</th>
<th>0.625</th>
<th>1.25</th>
<th>2.5</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+(^b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-(^c)</td>
</tr>
<tr>
<td>0.0625</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.125</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) The MIC represents the lowest concentration of antimicrobials that shows no impedance detection (IDT).

\(^b\): Growth-the concentrations of antimicrobials that show IDT.

\(^c\): No growth-the concentrations of antimicrobials that show no IDT.
Fig. 4.10. Effect of ethanol (EtOH) alone on *L. monocytogenes* in tryptic soy broth supplemented with 0.6% yeast extract at 35°C.
a_\text{w} \text{ lowering ability of ethanol. They suggested that ethanol has potential as an antimicrobial agent in low-a_\text{w} \text{ foods. Other alcohols possess desirable properties of disinfection, but only a few such as ethanol and iso-propyl alcohol have found any real practical applications as such. Inhibitory effects on the growth of many bacteria, yeasts, and molds require concentrations of 4-8\% ethanol in ordinary laboratory media (Shibasaki, 1982). Further, the shelf life of foods can be extended by the addition of ethanol (Shibasaki, 1982). Although much information on the antimicrobial activity of simple or complex alcohols has been reported, only ethanol has been used as a food disinfectant or preservative.}

Monolaurin had the greatest overall antimicrobial activity among all fatty acids and their esters and would appear to have the highest potential for use in foods and cosmetics (Shibasaki and Kato, 1979). However, use of monolaurin as a food preservative may have limitations since its activity is antagonized by many food components (Kato and Shibasaki, 1975). The combination effect of monolaurin and ethanol was of considerable interest because ethanol is frequently used as a dissolving agent for monolaurin. The combined effect of monolaurin with ethanol is shown in Fig. 4.11. The inhibitory effect of the combination was similar to the most active compound alone after 24 h incubation. This indicates little interaction between monolaurin and ethanol against \textit{L. monocytogenes}, and is judged to be an additive
Fig. 4.11. Effect of ethanol (EtOH) and monolaurin (GML), either alone or combined, against *L. monocytogenes* in tryptic soy broth supplemented with 0.6% yeast extract at 35°C.
effect. Fig. 4.12 shows the combination effect of 2.5% ethanol and 0.25% lactic acid. Likewise, the combination did not increase the inhibitory effect of the most active single compound alone. This result also suggests an additive interaction between ethanol and lactic acid. Thus, MIC results correlated poorly with growth curves when studying combined effects.

MIC tests for combined antimicrobial compounds in vitro represent an important tool in detecting their interactions (Parish and Carroll, 1988). The present study has shown that the nature of combined antimicrobial response against *L. monocytogenes* is a complex one, and it is unlikely that any one laboratory test can fully evaluate the response. Nevertheless, this information would be useful for determining interactions that could compromise effectiveness in food systems.

4.4 Enhanced Antimicrobial Activity of Monolaurin Combined with Selected Organic Acids

A. Minimal Inhibitory Concentration Effect

Ethanol concentrations up to 1.25% had little effect on the growth of *L. monocytogenes*, but the organism was almost completely inhibited in the presence of 5% ethanol. It was demonstrated that additive effects occurred when monolaurin was combined with high levels of ethanol. An additive effect
Fig. 4.12. Effect of ethanol (EtOH) and lactic acid (LA), either alone or combined, against *L. monocytogenes* in tryptic soy broth supplemented with 0.6% yeast extract at 35°C.
indicates that one compound neither enhanced nor interfered with the antimicrobial activity of the other. Thus, use of ethanol in low amounts as a solvent would have no effect on the growth and survival of *L. monocytogenes* in the present experiments. MIC results of organic acids and monolaurin alone against *L. monocytogenes* are shown in Table 4.5. The MIC of monolaurin was 10 μg/ml, acetic and benzoic acid were both 2500 μg/ml, and citric and lactic acid were both 5000 μg/ml. MIC values were lower when monolaurin was combined with lactic acid (Table 4.6). The combined MIC values were 1.25-2500 μg/ml or 2.5-2500 μg/ml with the monolaurin-lactic acid combination. Based upon these results and similar MIC results for monolaurin combined with the other acids (data not shown), sublethal concentrations of combinations of monolaurin with the organic acids were chosen to further study the population dynamics of *L. monocytogenes* and to confirm interaction effects.

**B. Inhibitory Effect**

The combined effect of monolaurin with lactic acid is shown in Fig. 4.13. When the two compounds were combined, inhibition of growth of *L. monocytogenes* was greater than when each antimicrobial agent was added alone. Growth was slightly inhibited in the presence of 2500 μg/ml lactic acid, but when 5 μg/ml monolaurin was combined with 2500 μg/ml lactic acid, an extended lag period was observed. These results agree with
Table 4.5. Minimal Inhibitory Concentration (MIC) of organic acids against *L. monocytogenes* at 35°C.

<table>
<thead>
<tr>
<th>Organic Acids</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolaurin</td>
<td>10 (w/v)</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>2500 (v/v)</td>
</tr>
<tr>
<td>Benzoic Acid</td>
<td>2500 (w/v)</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>5000 (w/v)</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>5000 (v/v)</td>
</tr>
</tbody>
</table>
Table 4.6. Minimal Inhibitory Concentration (MIC)* of combinations of monolaurin and lactic acid against *L. monocytogenes*

<table>
<thead>
<tr>
<th>Lactic Acid (%)</th>
<th>0</th>
<th>0.625</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+[^b]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-[^c]</td>
<td>-</td>
</tr>
<tr>
<td>0.0625</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.125</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The MIC represents the lowest concentration of antimicrobials that shows no impedance detection time (IDT).
[^b]: Growth—the concentrations of antimicrobials that show IDT.
[^c]: No growth—the concentrations of antimicrobials that show no IDT.

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Fig. 4.13. Effect of lactic acid (LA) and monolaurin (GML), either alone or combined, against *L. monocytogenes* at 35°C.
that of MIC values (Table 4.6), where synergistic interactions were observed. The inhibitory effect of monolaurin combined with citric acid was slightly greater than the most active compound alone during the early stages of incubation, while little difference was noted during the latter stages (Fig. 4.14). This indicates an additive interaction between monolaurin and citric acid against *L. monocytogenes*. The combination of 5 μg/ml monolaurin with 1250 μg/ml acetic acid synergistically inhibited growth of the pathogen in the broth system (Fig. 4.15). Similar results are illustrated in Fig. 4.16, where 5 μg/ml monolaurin combined with 1250 μg/ml benzoic acid acted synergistically. Monolaurin reportedly has the greatest overall antimicrobial activity among all fatty acids and their esters and would appear to have the highest potential for use in foods and cosmetics (Shibasaki and Kato, 1979). However, the range of use of monolaurin as a food preservative has been limited because its activity is antagonized by other food components (Kato and Shibasaki, 1975). Furthermore, monolaurin is active against Gram-positive bacteria, yeasts, and molds but to a lesser degree against Gram-negative bacteria. On the other hand, several organic acids have been used as food preservatives and as decontaminating agents because of their broad antimicrobial spectrum. Therefore, the combined effects of monolaurin and organic acids might prove to be effective in foods by removing the limitation of using monolaurin alone as a preservative.
Fig. 4.14. Effect of citric acid (CA) and monolaurin (GML), either alone or combined, against *L. monocytogenes* at 35°C.
Fig. 4.15. Effect of acetic acid (AA) and monolaurin (GML), either alone or combined, against *L. monocytogenes* at 35°C.
Fig. 4.16. Effect of benzoic acid (BA) and monolaurin (GML), either alone or combined, against *L. monocytogenes* at 35°C.
Kato and Shibasaki (1976) described a potential mechanism of inhibition of bacterial cells using a combination of monolaurin with citric acid, polyphosphoric acid, or ethylenediaminetetraacetic acid (EDTA). They reported that *Escherichia coli* cells pretreated with citric acid became more susceptible to monolaurin, resulting in a marked reduction in the population. However, most of the cells pretreated with monolaurin survived subsequent incubation with citric acid. It was suggested that *E. coli* received sublethal damage during pretreatment with citric acid and became more susceptible to monolaurin. The inhibition of amino acid uptake by bacterial cells was considered a mode of action of fatty acids against microorganisms (Freese et al., 1973; Galbraith and Miller, 1973a; Sheu et al., 1972). Kato and Shibasaki (1976) investigated the effects of monoglycerides, citric acid, or polyphosphoric acid on amino acid uptake by *E. coli*. They found that monolaurin or citric acid alone did not inhibit uptake, but the combination of monolaurin and citric acid inhibited uptake by approximately 90%.

Many weak acids can penetrate the cell membrane and accumulate within the cell cytoplasm in their undissociated form, resulting in detrimental effects (Booth, 1985). Kashket (1987) demonstrated that undissociated weak lipophilic acids, such as acetic acid and sorbic acid, are more permeable through the cell membrane than those such as lactic acid or
citric acid. Therefore, more undissociated acetic or sorbic acid would be expected to accumulate in the cytoplasm to higher concentrations than lactic or citric acid. Polla and Hutkins (1991) investigated the effect of organic acids on *L. monocytogenes* by measuring intracellular pH in cells exposed to organic acids. They found that inhibition of the pathogen by organic acids was not caused by decreasing intracellular pH, but rather by a specific effect of the undissociated acids on metabolic activities. Researchers have reported that *L. monocytogenes* was most inhibited by acetic acid followed by lactic acid, citric acid, and HCl, in descending order (Ahamad and Marth, 1989; Polla and Hutkins, 1991). The results obtained here are consistent with other reports indicating that *L. monocytogenes* was most inhibited by acetic acid, followed by benzoic, lactic, and citric acids.

Results showed that temperature and pH were very important factors influencing the efficacy of monolaurin against *L. monocytogenes*. Lethal effects of monolaurin increased as temperature increased at constant pH. Conversely, bacteriostatic effects on growth increased as temperature decreased at constant pH. At constant temperature, the bactericidal and bacteriostatic effects of monolaurin increased as the pH of the medium decreased. The effects of organic acids on growth of *L. monocytogenes* are influenced by type of acid as well as by other factors, such as salt content, pH, and temperature (Conner et al., 1986;
Parish and Higgins, 1989; Polla and Hutkins, 1991). Since the previous work used HCl to adjust the pH of the growth medium, it was of interest to determine whether other acids were active against *L. monocytogenes* over and above that of simple pH effect on monolaurin activity. El-Shenwy and Marth (1989) showed that inhibition of *L. monocytogenes* increased when sodium benzoate was combined with organic acids. Table 4.7 shows the pH values of the growth medium with sublethal or lethal concentrations of organic acids. The pH values at the MIC for each organic acid were relatively low, except for benzoic acid, whereas, pH values of sublethal concentration of each organic acid were higher. The pH values of sublethal concentrations of each organic acid were not changed when 5 μg/ml monolaurin was added. However, the antimicrobial effect of monolaurin combined with organic acids increased compared with single organic acids used alone.

It was shown earlier that at 35°C *L. monocytogenes* grew well in the presence of 5 μg/ml monolaurin at pH 5.5 in a medium adjusted with HCl. However, when the pH was further reduced to 5.0, the bacterium failed to grow. In the present study, *L. monocytogenes* did not grow at 35°C in the presence of 5 μg/ml monolaurin at pH 5.5, 6.0, or 6.9 in media adjusted with 2500 μg/ml lactic acid, 1250 μg/ml acetic acid, or 1250 μg/ml benzoic acid, respectively. In contrast, the bacterium grew in the presence of 5 μg/ml monolaurin in the medium adjusted to pH 5.4 with 2500 μg/ml citric acid. These results
Table 4.7. Medium pH values with added antimicrobial agents either alone or combined.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Concentration (µg/ml)</th>
<th>pH values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid (AA)</td>
<td>1250</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>5.0</td>
</tr>
<tr>
<td>Benzoic acid (BA)</td>
<td>1250</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>6.3</td>
</tr>
<tr>
<td>Citric acid (CA)</td>
<td>2500</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>4.5</td>
</tr>
<tr>
<td>Lactic acid (LA)</td>
<td>2500</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>4.3</td>
</tr>
<tr>
<td>Monolaurin (GML)</td>
<td>5</td>
<td>7.4</td>
</tr>
<tr>
<td>AA-GML</td>
<td>1250-5</td>
<td>6.0</td>
</tr>
<tr>
<td>BA-GML</td>
<td>1250-5</td>
<td>6.9</td>
</tr>
<tr>
<td>CA-GML</td>
<td>2500-5</td>
<td>5.4</td>
</tr>
<tr>
<td>LA-GML</td>
<td>2500-5</td>
<td>5.5</td>
</tr>
</tbody>
</table>
indicate that the enhanced inhibitory effect of combinations of monolaurin with organic acids was influenced by both pH and type of organic acid used. Since monolaurin shows relatively high anti-listerial activity alone and significantly enhanced activity with organic acids, combinations might be considered for use in food systems to create environments hostile to \textit{L. monocytogenes}. Therefore, further research will be needed to determine the efficacy of these combinations in actual food products.

4.5 Sanitizer Effect of Monolaurin either Alone or Combined with Acetic Acid or Heat on Biofilms of \textit{L. monocytogenes}

A. Inactivation Effect of Monolaurin

Fig. 4.17 shows the rate of adherent cell formation on the surface of stainless steel when \textit{L. monocytogenes} was cultivated in rich nutrient (TSB) or depleted nutrient conditions (diluted TSB). The number of adherent cells attached on stainless steel slowly increased from 1 d to 7 d until reaching a maximum of $2.3 \times 10^6$ CFU/sq. cm in TSB or $1.9 \times 10^6$ CFU/sq. cm in diluted TSB was reached, whereas, the population of planktonic cells grown in TSB or diluted TSB reached $1.47 \times 10^9$/ml or $2.1 \times 10^8$/ml after 1 d, respectively (data not shown).

The effects of monolaurin on planktonic cells in TSB or diluted TSB are shown in Fig. 4.18 and Fig. 4.19,
Fig. 4.17. Kinetics of biofilm formation of *L. monocytogenes* grown on stainless steel in tryptic soy broth or diluted tryptic soy broth at 25°C.
Fig. 4.18. Effect of monolaurin (GML) on inactivation of planktonic cells of *L. monocytogenes* in tryptic soy broth at 25°C.
Fig. 4.19. Effect of monolaurin (GML) on inactivation of planktonic cells of *L. monocytogenes* in diluted tryptic soy broth at 25°C.
respectively. Planktonic cells were rapidly inactivated by 50 or 100 μg/ml monolaurin and exhibited a 5 log decrease in number after 120 min, while planktonic cells grown in diluted TSB were completely inactivated in the presence of 100 μg/ml monolaurin by 100 min of incubation and showed more than a 5 log reduction in the presence of 50 μg/ml by 120 min. Effects of 50 or 100 μg/ml monolaurin on planktonic cells in TSB were significantly different (P<0.05) from those in diluted TSB.

Because of an interest was in the susceptibility of adherent cells to monolaurin, experiments on cells attached to SS. Fig. 4.20 shows that 1-d adherent cells exposed to 50 or 100 μg/ml monolaurin in TSB exhibited an approximate 2 log decrease in number after 120 min with no significant difference (P>0.05) noted between the two concentrations. Also, 1-d adherent cells exposed to monolaurin in diluted TSB showed no significant change (P>0.05) compared with that of TSB after 120 min in the presence of 50 or 100 μg/ml monolaurin with no significant (P>0.05) difference between both concentrations (Fig. 4.21). Seven-d adherent cells exposed to monolaurin in TSB, however, were more resistant than 1-d adherent cells. Approximately 1 log decrease in number occurred in the presence of 50 or 100 μg/ml monolaurin in either TSB (Fig. 4.22) or diluted TSB (Fig. 4.23). Effects of 50 or 100 μg/ml monolaurin on 7-d adherent in TSB were not significantly different (P>0.05) from those in diluted TSB.
Fig. 4.20. Effect of monolaurin (GML) on inactivation of 1 d adherent cells of *L. monocytogenes* in tryptic soy broth at 25°C.

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Fig. 4.21. Effect of monolaurin (GML) on inactivation of 1 d adherent cells of *L. monocytogenes* in diluted tryptic soy broth at 25°C.
Fig. 4.22. Effect of monolaurin (GML) on inactivation of 7 d adherent cells of *L. monocytogenes* in tryptic soy broth at 25°C.
Fig. 4.23. Effect of monolaurin (GML) on inactivation of 7 d adherent cells of *L. monocytogenes* in diluted tryptic soy broth at 25°C.
The susceptibility of adherent cells of *L. monocytogenes* to 50 or 100 μg/ml monolaurin was similar in diluted TSB and TSB, while planktonic cells grown in diluted TSB were more sensitive to monolaurin than those in TSB. Planktonic cells were much more sensitive to monolaurin than were adherent cells, while 1-d adherent cells were more sensitive than 7-d adherent cells in TSB. These results confirm observations by other workers (Anwar et al., 1989a; Anwar and Costerton, 1990) who showed that that planktonic cells of both mucoid and nonmucoid *Pseudomonas aeruginosa* were more susceptible to tobramycin than were adherent cells. Their results indicated that young 2-d biofilms (sessile bacteria) of both mucoid and nonmucoid *P. aeruginosa* were equally susceptible to tobramycin. However, old 7-d biofilms were extremely resistant to this antibiotic (Anwar et al., 1989a; Anwar et al., 1989b). These studies indicate that eradication of cells in biofilms should be as early as possible.

Many studies have been reported the surface attachment and adherent microcolony formation potential of *L. monocytogenes*. Bacteria growing in biofilms are more resistant to antimicrobial agents than are those grown in culture (Frank and Koffi, 1990; Lee and Frank, 1991; Mustapha and Liewen, 1989; Nickel et al., 1985). Frank and Koffi (1990) demonstrated that formation of microcolonies of *L. monocytogenes* on the surface of glass strips exhibit substantially increased resistance to a quaternary ammonium
compound and an acid anionic sanitizer. They suggested that the increased resistance of microcolonies might be from a surface-bound lipopolysaccharide-like substance produced by *L. monocytogenes*, which could increase cell envelope lipophilicity (Wexler and Oppenheim, 1979). In addition, Gram-positive bacteria can produce extracellular lipoteichoic acids that may protect against sanitizer penetration (Hammond et al., 1984).

The present study provides the means to investigate the interaction of adherent cells with monolaurin under variable conditions mimicking those found in a food processing plant, i.e., ranging from planktonic growth in rich media to slow growth in nutrient depleted media. The data show that *L. monocytogenes* grown on SS surfaces over time will significantly increase its resistance to monolaurin in rich or depleted nutrient conditions, but there was little difference in monolaurin effectiveness. Results further indicate that effectiveness of monolaurin as a sanitizer for adherent cells was similar to that reported for other commonly used sanitizers (Frank and Koffi, 1990; Krysinski et al., 1992; Lee and Frank, 1991). However, it was very effective against planktonic cells.
B. Effect of Monolaurin Combined with Acetic Acid

1). Inactivation Effect on Planktonic Cells

Fig. 4.24 shows the inactivation effect of monolaurin combined with acetic acid on planktonic cells. The 0.5% acetic acid or 50 μg/ml monolaurin treatments reduced counts more than 1 log cycle, but exposure of the cells to 50 μg/ml monolaurin combined with 0.5% acetic acid significantly reduced (P<0.05) the population by approximately 5 logs after 25 min exposure. Exposure of planktonic cells to 100 μg/ml monolaurin resulted in a 2 log reduction, but more than 5 log reduction occurred when 100 μg/ml monolaurin was combined with 0.5% acetic acid (Fig. 4.25). Inactivation effectiveness increased (P<0.05) when 50 or 100 μg/ml monolaurin was combined with 0.5% acetic acid. When 1% acetic acid was combined with 50 μg/ml monolaurin, complete inactivation occurred, while 1% acetic acid alone only caused a 2 log reduction (Fig. 4.26). However, the inactivation effectiveness increased (P<0.05) when 100 μg/ml monolaurin was combined with 1% acetic acid. Complete inactivation occurred with the combined treatment after 20 min (Fig. 4.27). These results indicate that planktonic cells of L. monocytogenes can be easily inactivated by the combination of monolaurin with acetic acid. Enhanced activity was clearly observed with the combined treatment compared to each single compound alone.
Fig. 4.24. Inactivation of planktonic cells treated with 50 μg/ml monolaurin (GML) combined with 0.5% acetic acid (AA) in tryptic soy broth at 25°C.
Fig. 4.25. Inactivation of planktonic cells treated with 100 µg/ml monolaurin (GML) combined with 0.5% acetic acid (AA) in tryptic soy broth at 25°C.
Fig. 4.26. Inactivation of planktonic cells treated with 50 μg/ml monolaurin (GML) combined with 1% acetic acid (AA) in tryptic soy broth at 25°C.
Fig. 4.27. Inactivation of planktonic cells treated with 100 $\mu$g/ml monolaurin (GML) combined with 1% acetic acid (AA) in tryptic soy broth at 25°C.
2). Inactivation Effect on 1-d Adherent Cells

Fig. 4.28 shows the inactivation effect of monolaurin combined with acetic acid on 1-d adherent cells. Exposure of cells to 0.5% acetic acid caused less than a 1 log reduction, while 50 µg/ml monolaurin reduced counts by approximately 1 log. Inactivation effects of 50 µg/ml monolaurin combined with 0.5% acetic acid were higher (P<0.05) than the most active single compound alone after 25 min. Exposure of the cells to 100 µg/ml monolaurin caused a 1 log reduction, but a approximately a 2 log reduction occurred when 100 µg/ml monolaurin was combined with 0.5% acetic acid (Fig. 4.29). When 1% acetic acid was combined with 50 µg/ml monolaurin, more than a 3 log reduction occurred, while 1% acetic acid alone caused an approximately 1.5 log reduction (Fig. 4.30). There were significantly different (P<0.05) inactivation effects when 50 or 100 µg/ml monolaurin were combined with 0.5% acetic acid. Inactivation was more enhanced (P<0.05) with 100 µg/ml monolaurin combined with 1% acetic acid compared to that of 0.5% acetic acid. No cells were detected in this combined concentration after 25 min (Fig. 4.31). This indicates that 1-d adherent cells of L. monocytogenes could be inactivated with the combination of monolaurin with acetic acid, but were more resistant than planktonic cells.
Fig. 4.28. Inactivation of 1 d adherent cells treated with 50 μg/ml monolaurin (GML) combined with 0.5% acetic acid (AA) in tryptic soy broth at 25°C.
Fig. 4.29. Inactivation of 1 d adherent cells treated with 100 μg/ml monolaurin (GML) combined with 0.5% acetic acid (AA) in tryptic soy broth at 25°C.
Fig. 4.30. Inactivation of 1 d adherent cells treated with 50 μg/ml monolaurin (GML) combined with 1% acetic acid (AA) in tryptic soy broth at 25°C.
Fig. 4.31. Inactivation of 1 d adherent cells treated with 100 μg/ml monolaurin (GML) combined with 1% acetic acid (AA) in tryptic soy broth at 25°C.

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3). Inactivation Effect on 7-d Adherent cells

The inactivation effect of monolaurin combined with acetic acid on 7-d adherent cells. Exposure of cells to 0.5% acetic acid or 50 μg/ml monolaurin caused less than 1 log reduction (Fig. 4.32). Inactivation effects of 50 μg/ml monolaurin combined with 0.5% acetic acid were significantly different (P<0.05) from each single compound alone after 25 min. Exposure of the cells to 100 μg/ml monolaurin caused less than 1 log reduction, but more than 1 log reduction occurred (P<0.05) when 100 μg/ml monolaurin was combined with 0.5% acetic acid (Fig. 4.33). When 1% acetic acid was combined with 50 μg/ml monolaurin, approximately a 1.5 log reduction occurred, while 1% acetic acid caused approximately a 1 log reduction after 25 min (Fig. 4.34). However, the inactivation effect was enhanced (P<0.05) when 100 μg/ml monolaurin was combined with 1% acetic acid. Approximately a 2 log reduction occurred with the combined concentrations after 25 min (Fig. 4.35). There were no significant differences (P>0.05) on inactivation effects between 50 or 100 μg/ml monolaurin combined with 0.5% acetic acid. However, significant differences (P>0.05) occurred between 50 or 100 μg/ml monolaurin combined with 1% acetic acid. These results indicate that 7-d adherent cells of L. monocytogenes were more resistant than 1-d adherent cells and could not be easily inactivated with the combination of monolaurin with acetic acid.
Fig. 4.32. Inactivation of 7 d adherent cells treated with 50 μg/ml monolaurin (GML) combined with 0.5% acetic acid (AA) in tryptic soy broth at 25°C.

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Fig. 4.33. Inactivation of 7 d adherent cells treated with 100 μg/ml monolaurin (GML) combined with 0.5% acetic acid (AA) in tryptic soy broth at 25°C.

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Fig. 4.34. Inactivation of 7 d adherent cells treated with 50 µg/ml monolaurin (GML) combined with 1% acetic acid (AA) in tryptic soy broth at 25°C.

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Fig. 4.35. Inactivation of 7 d adherent cells treated with 100 μg/ml monolaurin (GML) combined with 1% acetic acid (AA) in tryptic soy broth at 25°C.
There is a direct relationship between the number of cells attached to stainless steel and contact time (Herald and Zottola, 1988). They found that single cells of *L. monocytogenes* attached to stainless steel chips after 4 or 8 h in TSB at 21°C, whereas pairs and small groups of cells attached after 12 or 18 h. The 12 or 18 h observation corresponds to the early or late log phases of *L. monocytogenes*, respectively. Stanley (1983) reported that the greatest attachment of *P. aeruginosa* cells occurred in the early to late log phase as opposed to the stationary phase. They suggested that dead cells attached to surfaces are not important in situations where growth of bacteria is the problem. However, adherence of dead cells could provide a primary biofilm and might affect systems that are sensitive to biofilm thickness.

Stone and Zottola (1985) explained that culture age, cell concentration, and time have an effect on the number of cells attached and the amount of attachment produced by a cell. They suggested that attachment of a microbe to stainless steel occurs shortly after the organism is introduced into the new environment. As growth continues, large microcolonies of the organism appear, and these are attached both to the surface and to other cells through the production of flexible polar and rigid fibrils. Therefore, 7-d adherent cells may be more resistant to monolaurin than 1-d adherent cells due to increased thickness of microcolonies.
Biofilms may not be eradicated by conventional cleaners or sanitizers commonly used in food processing plants. As environmental factors on food contact surfaces or equipment are highly variable and unknown, the effectiveness of cleaners or sanitizers to remove adherent microcolonies will be incomplete, resulting in potential contamination of food. Therefore, the introduction of Hazard Analysis Critical Control Point concepts might be helpful to establish cleanliness standards of food contact surfaces, resulting in biofilm prevention. The results indicate that effectiveness of monolaurin itself as sanitizer for adherent cells of *L. monocytogenes* was not as efficient as many other sanitizers, such as chlorine or quaternary ammonium compounds commonly used in the food industry (Frank and Koffi, 1990; Krysinski et al., 1992; Mustapha and Liewen, 1989). However, when monolaurin was combined with acetic acid or heat to control *L. monocytogenes* biofilms on stainless steel, effectiveness was significantly enhanced. Thus, use of combination systems with chemical or physical treatments may be effective to control biofilm problems in the food industry as well as clinical environments.

C. Heat Inactivation of Planktonic Cells or Surface Adherent Cells with or without Monolaurin

Table 4.8 shows the effects of heat on planktonic cells, 1-d, and 7-d adherent cells of *L. monocytogenes* in TSB.
Table 4.8. Heat inactivation of planktonic cells (A), 1-d adherent (B), and 7-d adherent cells (C) of *L. monocytogenes* Scott A in tryptic soy broth.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Cell type</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0°</td>
</tr>
<tr>
<td>A</td>
<td>7.27±0.08</td>
<td>7.18±0.16</td>
</tr>
<tr>
<td>55</td>
<td>B</td>
<td>5.21±0.17</td>
</tr>
<tr>
<td>C</td>
<td>6.25±0.12</td>
<td>6.33±0.27</td>
</tr>
<tr>
<td>A</td>
<td>7.27±0.08</td>
<td>7.09±0.04</td>
</tr>
<tr>
<td>65</td>
<td>B</td>
<td>5.21±0.17</td>
</tr>
<tr>
<td>C</td>
<td>6.25±0.12</td>
<td>6.20±0.04</td>
</tr>
</tbody>
</table>

*Initial cell numbers before heating: A=mean log CFL/ml, B and C=mean log CFU/sq.cm ± standard deviation, N=5.*

<sup>b</sup>Not detectable levels.
When exposed to 55°C for 5 min, planktonic cells decreased approximately 0.6 log, while 1-d or 7-d adherent cells decreased approximately 0.3 or 0.25 log, respectively by this temperature. Similar results are observed in diluted TSB (Table 4.9). Planktonic cells decreased approximately 0.8 log, while 1-d or 7-d adherent cells decreased approximately 0.3 log or 0.2 log, respectively, after 5 min. There were no differences (P>0.05) in planktonic cells, 1-d, or 7-d adherent cells between TSB and diluted TSB at 55°C. When temperature was increased to 65°C, inactivation effects on the cells were significantly enhanced (P<0.05) (Table 4.8). Planktonic cells grown in TSB exhibited approximately 3.6 log reduction after a 5 min treatment, while 1-d adherent cells were completely inactivated, and 7-d adherent cells were reduced by approximately 1.4 log (Table 4.8). Similar results were observed in diluted TSB at 65°C (Table 4.9). Counts of planktonic cells were rapidly reduced by approximately 4.9 log CFU/ml after 5 min, whereas 1-d adherent cells were completely inactivated and 7-d adherent cells were reduced by approximately 1.6 log CFU/sq. cm. Effects of heat at 65°C on planktonic cells or 7-d adherent cells were different (P<0.05) between TSB and diluted TSB, while 1-d adherent cells showed no difference (P>0.05) between media.

The lethal effect of heat combined with monolaurin on planktonic and adherent cells in TSB is shown in Table 4.10. When planktonic cells were exposed to 50 or 100 μg/ml
Table 4.9. Heat inactivation of planktonic cells (A), 1-d adherent (B), and 7-d adherent cells (C) of *L. monocytogenes* Scott A in diluted tryptic soy broth.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Cell type</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7.30±0.04</td>
<td>7.33±0.06</td>
</tr>
<tr>
<td></td>
<td>5.17±0.21</td>
<td>5.14±0.05</td>
</tr>
<tr>
<td></td>
<td>6.18±0.22</td>
<td>6.23±0.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>Cell type</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7.30±0.04</td>
<td>6.71±0.06</td>
</tr>
<tr>
<td></td>
<td>5.17±0.21</td>
<td>5.07±0.04</td>
</tr>
<tr>
<td></td>
<td>6.18±0.22</td>
<td>6.13±0.06</td>
</tr>
</tbody>
</table>

<sup>a</sup>Initial cell numbers before heating: A=mean log CFL/ml, B and C=mean log CFU/sq.cm ± standard deviation, N=5.

<sup>b</sup>Not detectable levels.
Table 4.10. Combined inactivation of planktonic cells, 1-d adherent, and 7-d adherent cells of *L. monocytogenes* Scott A by monolaurin and heating for 5 min in tryptic soy broth.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>50</th>
<th>100</th>
<th>50</th>
<th>100</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolaurin (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>ND</td>
<td>ND</td>
<td>4.6±0.11</td>
<td>3.6±0.21</td>
<td>6.0±0.13</td>
<td>5.1±0.05</td>
</tr>
<tr>
<td>65</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Cell numbers before treatment were planktonic cells=7.27±0.08 log CFU/ml, 1-d adherent cells=5.21±0.17, and 7-d adherent cells=6.25±0.12 log CFU/sq.cm.*

*bHeat treatment was done after monolaurin treatment at the given concentrations.*

*cNot detectable levels.*

*dMean ± standard deviation, N=5.*
monolaurin with heat, cells were very sensitive and completely inactivated at 55 or 65°C, while 1-d or 7-d adherent cells were more resistant to combined treatments at 55°C. One-d adherent cells exposed to 50 or 100 μg/ml monolaurin at 55°C decreased 0.6 or 1.7 log CFU/sq. cm, respectively, whereas, 7-d adherent cells decreased 0.2 or 1.2 log CFU/sq. cm, respectively. However, planktonic cells and both adherent cells exposed to monolaurin at 65°C in TSB were completely inactivated (Table 4.10). Similar results were observed in diluted TSB (Table 4.11). When planktonic cells were exposed to 50 or 100 μg/ml monolaurin at 55°C for 5 min, cells were completely inactivated. One d adherent cells exposed to 50 or 100 μg/ml monolaurin at 55°C for 5 min decreased 1.4 or 2.3 log CFU/sq. cm, respectively, while 7-d adherent cells decreased 0.4 or 1.3 log CFU/sq. cm, respectively. When planktonic cells, 1-d, or 7-d adherent cells were exposed to 50 or 100 μg/ml monolaurin at 65°C, all cells were completely inactivated in diluted TSB. There were no difference (P>0.05) in planktonic cells between TSB and diluted TSB treated with monolaurin at 55°C, while 1-d or 7-d adherent cells showed significant differences (P<0.05) between both media. These results are consistent with those of Frank and Koffi (1990) who observed that planktonic cells in number slowly decreased at 55°C, whereas, 14-d adherent microcolonies on the surface of glass slides declined only slightly. Furthermore, the adherent cells treated with benzalkonium chloride were reduced by only 2 log counts at 55°C for 5 min.
Table 4.11. Combined inactivation of planktonic cells, 1-d adherent, and 7-d adherent cells of *L. monocytogenes* Scott A by monolaurin and heating for 5 min in diluted tryptic soy broth.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>50</th>
<th>100</th>
<th>50</th>
<th>100</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Planktonic</td>
<td>1-d Adherent</td>
<td>7-d Adherent</td>
<td>Monolaurin (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>55b</td>
<td>ND</td>
<td>ND</td>
<td>3.8±0.48</td>
<td>2.9±0.23</td>
<td>6.6±0.15</td>
<td>4.9±0.17d</td>
</tr>
<tr>
<td>65</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Cell numbers before treatment were planktonic cells=7.27±0.08 log CFU/ml, 1-d adherent cells=5.21±0.17, and 7-d adherent cells=6.25±0.12 log CFU/sq.cm.*

*b Heat treatment was done after monolaurin treatment at the given concentrations.

*Not detectable levels.

d Mean ± standard deviation, N=5.
The fact that bacteria growing in biofilms are more resistant to antimicrobial agents than those grown in planktonic culture has been reported (Anwar et al., 1989a; Anwar et al., 1989b; Nickel et al., 1985). In the present work it was found that planktonic cells of L. monocytogenes were much more sensitive to monolaurin than adherent cells, with 1-d adherent cells being more sensitive than 7-d adherent cells in TSB or diluted TSB. These results are consistent with observations of other workers (Anwar et al., 1989a; Anwar and Costerton, 1990) who showed that young 2-d biofilms (sessile bacteria) of both mucoid and nonmucoid P. aeruginosa were equally susceptible to tobramycin, while old 7-d biofilms were extremely resistant to this antibiotic compound.

Lee and Frank (1991) demonstrated inactivation of L. monocytogenes adherent cells on stainless steel by hypochlorite combined with heat. They reported that 4-h or 8-d adherent cells were reduced by approximately 3.8 log in number at 65°C for 30 s, but both adherent cells were completely inactivated after 5 min. These results were different from the present data showing that 1-d adherent cells were completely inactivated at 65°C after 5 min, but 7-d adherent cells decreased only 1.6 log CFU/sq. cm. These different findings may be due to different recovery methods, medium compositions, incubation times, or incubation temperatures. On the other hand, Frank and Koffi (1990) found
that 14-d adherent microcolonies on glass slides had a 5 log decrease and were still detectable at 70°C for 5 min. Lee and Frank (1991) recovered approximately 5 log CFU/sq. cm from 8-d adherent cells on stainless steel using a scraping method to remove the adherent cells. Presently it was found that more than 6 log CFU/sq. cm of 7-d adherent cells were obtained by vortexing with microscopic beads, even though smaller stainless steel chips (1 x 1 cm) were used than their stainless steel slides (2 x 4 cm). Krysinski et al. (1992) demonstrated that using microscopic beads to remove attached cells produced highly reproducible results despite a 20% bacteria cell damage. When both methods were compared for recovery efficiency in the preliminary research, microscopic beads method gave greater and more reproducible recovery, especially for removing old adherent cells, than the scraping technique to determine viable cell numbers (data not shown).

The sanitizer effects of monolaurin on planktonic or adherent cells were significantly enhanced at 65°C. Planktonic and adherent cells treated with monolaurin were completely inactivated at 65°C. This study indicates that old adherent cells are significantly more resistant to heat or monolaurin. The behavior of L. monocytogenes in biofilms needs to be further investigated to prevent the proliferation of the organism within a food processing plant environment or to determine proper formulation of detergents and sanitizers for use in the food processing industry.
4.6 Extended Shelf-Life of Precooked Crawfish Tail Meat using Packaging Combined with Antimicrobials

A. Effect of Monolaurin or Lactic Acid on Generation Time of L. monocytogenes

The generation time (GT) of L. monocytogenes on minced crawfish tail meat treated with different antimicrobial agents during 4°C storage is shown in Fig. 4.36. Effects of monolaurin on growth and survival of L. monocytogenes in minced crawfish tail meat at 4°C storage are examined in Fig. 4.37. Two or 4% ethanol were used as controls for 200 or 400 μg/g monolaurin, respectively. The organism initiated growth rapidly in the control crawfish tail meat with a GT of 11.3 h, while addition of 200 μg/ml monolaurin to crawfish tail meat significantly reduced (P<0.05) the growth rate of the organism with a GT of 15.8 h until 17 d. Addition of 2% ethanol to crawfish tail meat was not significantly different (P>0.05) compared with the control. However, growth of the organism in the presence of 4% ethanol was significantly reduced (P<0.05) with a GT of 28 h, whereas, addition of 400 μg/g monolaurin to crawfish tail meat significantly reduced (P<0.05) the growth of the pathogen with a GT of 31.4 h.

The effects of different lactic acid concentrations on growth and survival of L. monocytogenes are shown in Fig. 4.38. The bacterium initiated growth rapidly in the presence of 0.5% lactic acid with a GT of 12.2 h, without a lag phase.
Fig. 4.36. Generation time of *L. monocytogenes* on inoculated crawfish tail meat treated with or without antimicrobials after 21 d storage at 4°C.

a-e: Means having different letters are significantly different (P < 0.05).
Fig. 4.37. Effect of monolaurin (GML) or ethanol (EtOH) on growth of *L. monocytogenes* Scott A in crawfish tail meat during incubation at 4°C.
Fig. 4.38. Effect of lactic acid (LA) alone on growth of *L. monocytogenes* Scott A in crawfish tail meat during incubation at 4°C.
and with a growth rate not significantly different (P>0.05) from the control. Addition of 1% lactic acid to crawfish tail meat did not extend the lag phase, but significantly reduced (P<0.05) the growth rate of the organism with a GT of 16 h resulting a 1 log reduction in numbers after 21 d of storage (P<0.05). Similar results were reported for *L. monocytogenes* on crawfish tail meat held at 6°C in which a rapid increase in numbers were seen without a lag phase (Dorsa et al., 1992). No growth occurred in the presence of 2% lactic acid during the entire storage time and the organism was not detected in the presence of 3% lactic acid after 10 d of storage. Several organic acids have been used as food preservatives and as decontaminating agents because of their broad antimicrobial spectrum. Monolaurin has the highest potential for use in foods and cosmetics (Shibasaki and Kato, 1979), but the its range of use as a food preservative has been limited because its activity is antagonized by other food components (Kato and Shibasaki, 1975). Synergistic effects when monolaurin was combined with organic acids has been previously shown in section 4.4. Therefore, combination effects of monolaurin with organic acids might prove to be effective in foods by removing the limitation of monolaurin alone as a preservative.
B. Effect of Monolaurin Combined with Lactic Acid during Air Storage

The effects of monolaurin and lactic acid, either alone or in combination, on crawfish tail meat were examined at 4°C (Fig. 4.39). Addition of 200 μg/g monolaurin to crawfish samples significantly inhibited (P<0.05) growth until 14 d, but the inhibitory effect was not significantly different (P>0.05) from the control at 21 d of storage. Addition of 0.5% lactic acid did not inhibit (P>0.05) the bacterium during the 21 d incubation period. However, inhibition of growth was enhanced (P<0.05) when 200 μg/g monolaurin was combined with 0.5% lactic acid, but the combination did not extend the lag phase. Combining 200 μg/g monolaurin with 1% lactic acid significantly increased (P<0.05) inhibitory effects and extended lag phase until 10 d with a GT of 24.6 h (Fig. 4.40). No viable cells were detected when 200 μg/g monolaurin was combined with 2% lactic acid after 10 d (Fig. 4.41).

Many workers have reported that the effect of organic acids on growth of L. monocytogenes is influenced by type of acid used in addition to other factors such as salt content, pH, and temperature (Conner et al., 1986; Parish and Higgins, 1989; Sorrells et al., 1989). It was previously found that L. monocytogenes did not grow at 35°C in the presence of 5 μg/ml monolaurin at pH 5.5, 6.0, or 6.9 in media adjusted with 0.25% lactic acid, 0.125% acetic acid, or 0.125% benzoic acid, respectively, whereas the bacterium grew in the presence of...
Fig. 4.39. Effect of 200 µg/ml monolaurin (GML) and 0.5% lactic acid (LA), either alone or combined, on growth of *L. monocytogenes* Scott A in crawfish tail meat during storage at 4°C.
Fig. 4.40. Effect of 200 μg/ml monolaurin (GML) and 1% lactic acid (LA), either alone or combined on growth of *L. monocytogenes* Scott A in crawfish tail meat during storage at 4°C.
Fig. 4.41. Effect of 200 μg/ml monolaurin (GML) and 2% lactic acid (LA), either alone or combined on growth of *L. monocytogenes* Scott A in crawfish tail meat during storage at 4°C.
5 μg/ml monolaurin in the medium adjusted to pH 5.4 with 0.25% citric acid (see section 4.4). Thus, results demonstrated that the enhanced inhibitory effects of monolaurin combined with organic acids were influenced by both pH and type of organic acid used.

The pH changes of crawfish tail meat inoculated with *L. monocytogenes* before and after 21 d storage are presented in Table 4.12. The pH values in the control, 200 μg/g monolaurin, or 200 μg/g monolaurin combined with each lactic acid were significantly lowered (P<0.05), whereas, the pH values in the crawfish sample treated with different lactic acid concentrations or 400 μg/g monolaurin were not significantly changed (P>0.05). *L. monocytogenes* grew well in the pH 6.7 crawfish sample adjusted with 0.5% lactic acid. The bacterium was completely inactivated in the pH 4.4 samples adjusted with 3% lactic acid. On the other hand, *L. monocytogenes* grew well in pH 6.1 samples adjusted with 1% lactic acid. The organism was significantly inhibited (P<0.05) in pH 6.3 samples adjusted with lactic acid combined with monolaurin. Therefore, the results indicate that enhanced inhibitory effects were influenced by pH as well as by the interaction of both compounds.

*L. monocytogenes* was highly sensitive to monolaurin in a broth system and the MIC of monolaurin was reduced when the pH decreased from pH 7.0 (10 μg/ml) to pH 5.0 (3 μg/ml). However, inhibitory effect of monolaurin against *L.
Table 4.12. Change in pH of crawfish tail meat treated with monolaurin or lactic acid either alone or combined after 21 d storage at 4°C.

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Concentration (%)</th>
<th>pH values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 d</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>7.8a</td>
</tr>
<tr>
<td>Monolaurin (GML)</td>
<td>0.02</td>
<td>7.8a</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>7.8a</td>
</tr>
<tr>
<td>Lactic acid (LA)</td>
<td>0.5</td>
<td>6.7a</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.1a</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>5.3a</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>4.6a</td>
</tr>
<tr>
<td>LA-GML</td>
<td>0.5-0.02</td>
<td>6.9a</td>
</tr>
<tr>
<td></td>
<td>1.0-0.02</td>
<td>6.3a</td>
</tr>
<tr>
<td></td>
<td>2.0-0.02</td>
<td>5.4a</td>
</tr>
</tbody>
</table>

*a,b Means within a row with different subscripts are significantly different (P<0.05).

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monocytogenes was significantly reduced in crawfish tail meat.

The composition of a food can affect the inhibitory activity of monolaurin. Kato and Shibasaki (1975) demonstrated that monolaurin activity was antagonized by many components such as lipophilic proteins, Tween 20, starch, or fat globules, and others. Crawfish tail meat consists of 75% water, 24% protein, 1.4% lipid, and 2.5% ash (USDA, 1987). Thus, reduced inhibitory effects of monolaurin in crawfish may result from an antagonizing effect due to the high protein content. Wang and Johnson (1992) reported that 10 to 20 μg/ml monolaurin in brain heart infusion broth at 35°C was bactericidal to L. monocytogenes. In skim milk, 200 μg/ml monolaurin inhibited the bacterium, but in whole milk, because of the higher fat content, this concentration was not effective.

C. Effect of Modified Atmosphere Packaging with Monolaurin or Lactic Acid on Growth of L. monocytogenes

Effects of modified atmosphere packaging, vacuum packaging, or air packaging, either alone or combined with antimicrobials, on crawfish tail meat inoculated with 10³ cells/g of L. monocytogenes and held at 4°C for 14 d are shown in Fig. 4.42. The bacterium in air storage rapidly grew and reached 9.25 log CFU/g after 14 d, while the pathogen in vacuum-packaged sample also rapidly reached 8.7 log CFU/g. Both package environments were not significantly different
Fig. 4.42. Effect of air, vacuum, or modified atmosphere packaging with or without antimicrobials on growth of *L. monocytogenes* Scott A in crawfish tail meat after 14 d storage at 4°C. Means having different letters are significantly different (*P* < 0.05).
However, the pathogen in modified atmosphere storage reached only 6.8 log CFU/ml after 14 d and growth was significantly inhibited (P<0.05). Wang and Brown (1983) showed that total aerobic counts were significantly reduced in CO₂ stored crawfish samples, while counts in air-stored crawfish rapidly exceeded $10^7$ cells/g after 14 d storage at 4°C. When crawfish tail meat samples in air, vacuum, or MA packages were stored at 11°C, *L. monocytogenes* rapidly reached 9.3, 9.2, or 8.6 log cells/g, respectively, after 6 d of storage (data not shown). These values were not significantly different (P>0.05). Marshall et al. (1991) demonstrated that the effectiveness of modified atmospheres against *L. monocytogenes* on precooked chicken nuggets was reduced with increased storage temperature. Result indicates that storage temperature of MA packaged crawfish samples should be kept as low as possible since the solubility of CO₂ decreases rapidly with increasing temperature (Daniels et al., 1985; Finne, 1982; Gill and Tan, 1980).

Addition of 0.5% lactic acid to crawfish samples in air, vacuum, or MA package did not enhance (P>0.05) previously described inhibitory effects after 14 d storage at 4°C. Likewise, addition of 200 µg/g monolaurin to crawfish samples in air or MA packaged samples did not increase (P>0.05) inhibitory effects compared with the control. Conversely, 200 µg/g monolaurin in vacuum-packaged samples significantly reduced (P<0.05) the growth of *L. monocytogenes*. When 200
μg/g monolaurin was combined with 0.5% lactic acid, significant inhibitory effects were observed (P<0.05) for each packaging treatment, indicating that crawfish tail meat treated with 200 μg/g GML-0.5% LA, and MAP had the greatest inhibiting effect on the organism.

Fey and Regenstein (1982) investigated the possibility of combining MAP with potassium sorbate in the form of ice at 1°C to extend shelf-life of fresh fish. They demonstrated that this treatment significantly improved flavor and overall acceptability as well as inhibiting microorganisms. Generally seafoods are very susceptible to both microbiological and chemical deterioration. MAP can provide consistently improved stability of fresh fish, with higher initial CO₂ levels resulting in increased shelf-life. However, this fact can not be assured unless chilled products are hygienically handled from time of harvesting and held at low temperatures.

Most pathogens would not be able to survive and/or grow any better in a MA package compared with air storage, but some clostridia or Campylobacter species may be able to survive better in MA compared with an air atmosphere (Farber, 1991). Nonproteolytic clostridia, L. monocytogenes, or Y. enterocolitica could grow at refrigerated temperature in certain MAP environments. This concern has been raised about the safety of MAP fish held at refrigeration temperatures. Therefore, MAP products with extended refrigerated shelf lives could be a problem. Based upon this, introduction of combined
multibarrier concepts, such as acidity, water activity, temperature, a competitive flora, or preservatives with MAP systems may be necessary to prevent development of potential health hazards (Leistner, 1978). These hurdles could interact either directly or synergistically to secure microbial stability of a particular food product.
CHAPTER 5

SUMMARY AND CONCLUSIONS

*Listeria monocytogenes* has emerged as a serious foodborne pathogen because of its ability to grow in foods during refrigerated storage. Thus, it is necessary to have useful measures to control the bacterium in foods. Use of chemical preservatives for this purpose in food products has been debated due to public concerns about food quality because of perceived toxic and carcinogenic potential (Tanaka et al., 1977). Therefore, use of non-toxic substances as components of food has become essential. Monolaurin, a naturally occurring substance, has the greatest overall antimicrobial activity among fatty acids and their esters, and may have greatest potential for use in foods. Thus, the present study was conducted to evaluate the antimicrobial properties of monolaurin, either alone or combined with organic acids, against *L. monocytogenes*.

Medium pH played an important role in reducing MIC of monolaurin against *L. monocytogenes*. Increased inhibition of the bacterium by monolaurin with decreased pH values may result from decreased interfacial tension at the bacterial lipid membrane-aqueous medium interface or increased uptake of monolaurin. The contribution of temperature to monolaurin effectiveness showed that inactivation occurred more rapidly at higher temperatures. Thus, the most rapid inactivation of
the pathogen by monolaurin occurred with the highest temperature and lowest pH combination. Bacteriostatic effects of sublethal monolaurin concentrations increased as temperature and pH decreased. The results demonstrate that pH and temperature interact to affect antimicrobial potential of monolaurin against *L. monocytogenes*. Other environmental factors such as initial inoculum levels, competing organisms, type of food components, or water activity may additionally influence the effectiveness of monolaurin against *L. monocytogenes*.

Because ethanol was used as dissolving agent for monolaurin, it was necessary to determine the effect of ethanol, either alone or combined with monolaurin, against the growth of *L. monocytogenes*. Results showed that the bacterium was inhibited only slightly up to 2.5% ethanol, but was significantly inhibited in the presence of 5% ethanol. When monolaurin was combined with ethanol to determine interaction effects, MIC values, or inhibitory effects of the combination, were not different compared to the most active single compound alone. These data indicate little interaction between ethanol and monolaurin against *L. monocytogenes*.

Since medium pH adjusted with HCl played an important role in reducing MIC of monolaurin against *L. monocytogenes*, it was of interest to determine whether other acids were active against *L. monocytogenes* over and above a simple pH effect on monolaurin activity. Synergistic effects were
observed when monolaurin was combined with acetic, benzoic, or
lactic acid, whereas, there was little interaction when
monolaurin was combined with citric acid. Results presented
indicate that the enhanced inhibitory effect of combinations
of monolaurin with organic acids was influenced by both pH and
type of organic acid used.

*L. monocytogenes* has been reported to attach to a variety
of surfaces. Adherent cells on the surface of stainless steel
showed increased resistance to conventional chemical
sanitizers such as a quaternary ammonium compound and an acid
anionic sanitizer (Frank and Koffi, 1990; Petrocii, 1983).
Planktonic cells were much more sensitive to monolaurin than
were adherent cells, with 1-d adherent cells being more
sensitive than 7-d adherent cells in tryptic soy broth or
diluted tryptic soy broth. Results indicate that *L.*
*monocytogenes* grown on SS surfaces for several days are
significantly more resistant to monolaurin in rich or depleted
nutrient conditions. However, inactivation effects of
monolaurin combined with acetic acid on planktonic cells, 1-d,
or 7-d adherent cells greater than monolaurin or acetic acid
used alone. These results indicate that *L. monocytogenes*
planktonic and 1-d adherent cells can be readily inactivated
with the combination of monolaurin and acetic acid, but 7 d
adherent cells were not easily inactivated. Heat effects on
planktonic cells, 1-d or 7-d adherent cells at 55°C were low,
but inactivation effects were significantly enhanced at 65°C.
Planktonic and adherent cells treated with monolaurin at 65°C were completely inactivated. Thus, results demonstrate that the use of combination systems with chemical or physical treatments may be effective to control *L. monocytogenes* biofilm problems in the food industry.

When monolaurin or lactic acid were used on crawfish tail meat inoculated with *L. monocytogenes* at 4°C, their inhibitory ability was significantly reduced compared with the broth control system. However, this problem could be overcome using monolaurin combined with lactic acid in air-packaged or modified atmosphere-packaged crawfish tail meat. The bacterium was significantly reduced by 3 log CFU/g when 200 μg monolaurin was combined with 1% lactic acid. Furthermore, this combination extended the lag phase until 10 d of the 21-d storage period. No *L. monocytogenes* was detected when 200 μg/ml monolaurin was combined with 2% lactic acid. These results suggest that enhanced inhibitory effects were influenced by pH as well as by interaction of both compounds used. Modified atmosphere packaging significantly enhanced the inhibitory effect of the combination more than air or vacuum packaging. *L. monocytogenes* in air or vacuum storage at 4°C reached 9.3 or 8.7 log CFU/g after 14 d storage, respectively, while the pathogen in the modified atmosphere package reached only 6.8 log CFU/g. Crawfish tail meat treated with 200 μg/g monolaurin, 0.5% lactic acid, and MAP had the greatest potential to inhibit growth of the bacterium.
In conclusion, an important observation made in this dissertation is that \textit{L. monocytogenes} was highly sensitive to monolaurin. Since monolaurin had excellent inhibitory activity at low temperature, it might be considered as a preservative to control psychrotrophic organisms like \textit{L. monocytogenes} that can grow at refrigerator temperatures. Also, the inhibitory effect of monolaurin against \textit{L. monocytogenes} was greater than that widely reported for other commonly used food antimicrobials, such as tertiary butylhydroquinone, butylated hydroxyanisole, propyl paraben, sorbic acid, etc. However, this inhibitory effect of monolaurin against \textit{L. monocytogenes} was reduced in crawfish tail meat, showing that the composition of foods can affect the inhibitory activity of monolaurin. On the other hand, synergistic effects against \textit{L. monocytogenes} were observed when monolaurin was combined with selected organic acids, such as acetic, benzoic, or lactic acid in laboratory media or crawfish. Therefore, the enhanced antimicrobial effectiveness of monolaurin combined with heating or with organic acids may warrant its use as an alternative food preservative in foods or in food contact surface applications.

Most pathogens would not be able to survive and/or grow any better in a MA package compared with air storage, but psychrotrophic pathogens, such as \textit{L. monocytogenes} or \textit{Y. enterocolitica} could grow at chill temperature in certain MAP conditions. Thus, MA products with extended refrigerated
shelf lives could be a problem. Combining MAP with monolaurin and lactic acid might solve this concern with *L. monocytogenes*.

Since monolaurin activity was antagonized by food components, additional research is needed to determine factors that cause reduced monolaurin activity and the mechanisms by which synergistic interactions occur in combined systems. Also, introduction of combined multibarrier concepts, such as acidity, water activity, temperature, competitive flora, or preservatives with MAP systems will be necessary to prevent the development of potential health hazards. Therefore, further research will be needed to determine the efficacy of these multiple barriers with monolaurin to control *L. monocytogenes* in actual food products.
REFERENCES


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While he was studying for the Ph.D., he received the Honorable Mention Award for the J.C. Ayers Competition, Food Microbiology Division, Institute of Food Technologists in 1991.

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Major Field: Food Science

Title of Dissertation: Antimicrobial Properties of Glycerol Monolaurate Either Alone or Combined with Selected Organic Acids Against Listeria monocytogenes

Approved:

[Signatures]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:
March 19, 1993