

Influence of Temperature, pH, and Glycerol Monolaurate on Growth and Survival of *Listeria monocytogenes*¹

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(Received for publication November 17, 1992)

ABSTRACT

The effect of temperature, pH, and monolaurin on the growth and survival of *Listeria monocytogenes* in tryptic soy broth with yeast extract (TSBYE) was investigated. TSBYE medium containing 5, 6, 7, 8, or 9 µg/ml monolaurin was adjusted to pH 5.0, 5.5, or 7.0, inoculated with *L. monocytogenes*, and incubated at 7, 15, or 35°C. The effect of sublethal concentrations of monolaurin was measured by calculating generation times. Results indicated that temperature and pH were very important factors influencing the efficacy of monolaurin against *L. monocytogenes*. Bactericidal effects were observed at pH 5.0 but not observed at pH 5.5 or pH 7.0. Further, 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. Generation time of the organism as significantly ($P < 0.05$) influenced by pH, temperature, concentration of monolaurin, or their combined interactions. Thus, the results indicate that pH and temperature interact to affect the antimicrobial potential of monolaurin against *L. monocytogenes*.

Listeria monocytogenes is a gram-positive, nonsporeforming, catalase-positive, short, rod-shaped psychrotrophic bacterium that can cause foodborne disease in humans and animals (5,11,20). The disease, listeriosis, has emerged as a serious food safety issue (3,19). The bacterium is widespread in the environment and has been isolated from soil, animals, seafood, milk, and meat products (1,8,12,24,32). Recent outbreaks of foodborne listeriosis have generated much interest in investigating the behavior of the bacterium in food systems. Since this organism is a pathogen and can grow at refrigeration temperatures, foods from refrigerated storage cannot safeguard against its growth (25). The pH of foods can have an important preservative effect against bacteria. *L. monocytogenes* has been reported to be unable to survive, or grows poorly, in low pH environments (10). However, recent work has indicated that the organism is more acid tolerant than most foodborne pathogens and can grow or survive in broth media at pH values as low as 4.3-5.0 (23,30).

The use of chemical preservatives to control microorganisms in food products is continually being debated, due to public concerns about food quality. Some chemical agents are being seriously considered for removal from the market because of their potential or perceived toxic or carcinogenic effects (31). Consequently, consumers may reject foods containing preservatives, due to questions about food safety. Fatty acids and their esters are nontoxic, naturally occurring substances in foods and may be used as antimicrobial preservatives. 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 (21 CFR GRAS 182.4205) and has been extensively investigated (2,13-16,29).

The antimicrobial effects of fatty acids against pathogenic or spoilage organisms have been reported (14,18), but little information is available on the antimicrobial effect of monolaurin against *L. monocytogenes*. We previously reported on the effect of pH on the minimum inhibitory concentration of monolaurin against this bacterium (21), but the dynamic effects of sublethal concentrations have not been investigated. Therefore, the present research was conducted to determine the effect of temperature and pH on the effectiveness of monolaurin against growth and survival of *L. monocytogenes*.

MATERIALS AND METHODS

Test organism

Strain Scott A of *L. monocytogenes* (Economic Laboratories, St. Paul, MN) was used throughout this study because previous research (20) showed little difference in sensitivity to monolaurin among four different strains. A stock culture was maintained through monthly transfers on tryptic soy agar (Difco) slants and stored at 4°C until subcultured for use. The culture was subcultured in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; Difco) for 24 h at 35°C before use. Serial dilutions were made in TSBYE medium to prepare a working culture of approximately 10⁵ CFU/ml.

Preparation of media and preservative

Rehydrated TSBYE was adjusted to pH 5.0, 5.5, and 7.0 with 1 N HCl. Since previous research (20) showed little difference in monolaurin sensitivity between pH values of 6-7, tests were not

¹Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript No. 92-21-6373.

conducted in media having pH values within this range. After autoclaving, appropriate aliquots of TSBYE were aseptically dispensed into 250-ml sterilized Erlenmeyer flasks. A 1% stock solution of monolaurin (Sigma Chemical Co., St. Louis, MO) in absolute ethanol was prepared by filter sterilization using a 0.45- μm membrane filter (Millipore Products Division, Bedford, MA).

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 $\mu\text{g}/\text{ml}$ of monolaurin, respectively, in 50 ml total volume. 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. This was incubated at given temperatures for designated time periods. TSBYE medium containing 0.1% ethanol without monolaurin was used as a control.

L. monocytogenes Scott A was determined by surface plating 0.1-ml amounts of undiluted or serially diluted (0.1% peptone water) samples on duplicate tryptic soy agar plates. Sampling was done at appropriate time intervals to determine the 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 entire experiments. Numbers were converted to log CFU/ml and generation times (GT) were calculated from logarithmic phase data, as previously described (6).

Statistical analysis

Analysis of variance procedures using the Statistical Analysis Systems program (SAS, 26) were used to determine generation time differences among pH, temperature, or concentrations of monolaurin. Also, Duncan's test was used to determine interactions when analysis of variance indicated significant differences.

RESULTS

Effect of monolaurin at 7°C

The GT of *L. monocytogenes* at different temperatures, pH values, and monolaurin concentrations are shown in Table 1. *L. monocytogenes* initiated growth in the control medium at 7°C after 1 d with a GT of 5.3 h at pH 7.0 (Fig. 1). Lag phases were extended in the presence of 5, 6, 7, or 8 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$ monolaurin compared with the control and other treatments. At pH 5.5, the bacterium

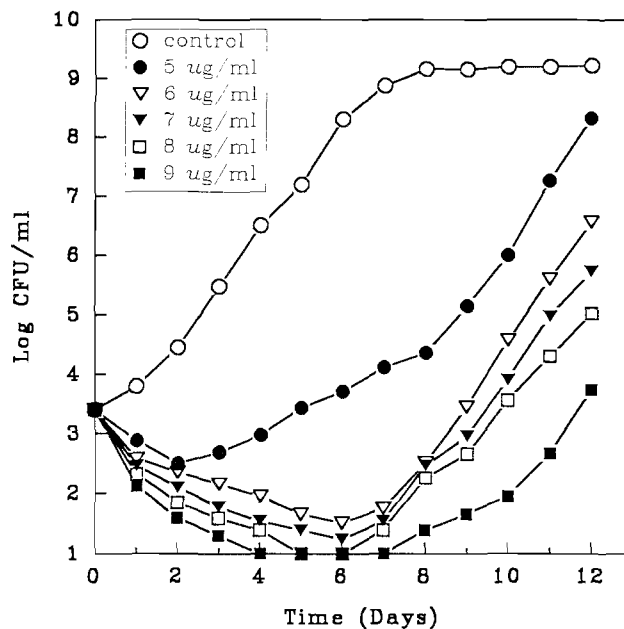


Figure 1. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 7°C and pH 7.0.

started to grow in the control after 2 d with a GT of 10.0 h (Fig. 2). Addition of 5, 6, 7, or 8 $\mu\text{g}/\text{ml}$ monolaurin to the medium extended lag phase of *L. monocytogenes* until 12 d of incubation prior to growth. GT at these concentrations were not significantly different ($P > 0.05$) from the control. In the presence of 9 $\mu\text{g}/\text{ml}$ monolaurin, the lag phase was lengthened to 16 d before slow growth was initiated. When the pH was reduced to 5.0, the pathogen started to grow after 3 d with a GT of approximately 12.9 h in the control medium (Fig. 3). No growth was observed at any concentration of monolaurin. Addition of 8 or 9 $\mu\text{g}/\text{ml}$ monolaurin to the medium reduced counts to undetectable levels after 22 or 20 d, respectively. GT of all monolaurin treatments were significantly different ($P < 0.05$) from the control.

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). Addition of 5 or 6 $\mu\text{g}/\text{ml}$ monolaurin extended the lag phase to 12 h, while not significantly affecting ($P > 0.05$) GT. Addition of 7 or 8 $\mu\text{g}/\text{ml}$ monolaurin extended the lag phase to 18 or 24

TABLE 1. Generation time of *L. monocytogenes* at different temperatures, pH, and concentrations of monolaurin.

Monolaurin $\mu\text{g}/\text{ml}$	Generation time (h)								
	pH 7.0			pH 5.5			pH 5.0		
	35°C	15°C	7°C	35°C	15°C	7°C	35°C	15°C	7°C
0	0.5 ^a	1.6 ^a	5.3 ^a	0.6 ^a	2.5 ^a	10.0 ^a	1.1 ^a	4.7 ^a	12.9 ^a
5	0.5 ^a	1.7 ^a	5.4 ^a	0.7 ^{ab}	3.0 ^a	11.4 ^a	1.6 ^b	4.9 ^a	NG ^b
6	0.5 ^a	1.7 ^a	5.7 ^a	0.7 ^{ab}	3.0 ^a	12.6 ^a	1.7 ^b	5.0 ^a	NG ^b
7	0.5 ^a	2.0 ^b	6.3 ^{ab}	0.8 ^{ab}	3.1 ^a	13.5 ^a	1.9 ^b	5.3 ^a	NG ^b
8	0.5 ^a	2.3 ^{bc}	7.4 ^{ab}	0.9 ^b	3.2 ^a	16.9 ^a	NG ^c	9.2 ^b	ND ^b
9	0.9 ^b	2.6 ^c	10.1 ^b	1.3 ^c	4.5 ^a	28.2 ^b	ND ^c	ND ^c	ND ^b

NG: No growth occurred.

ND: No detectable generation time.

Means within a column with different superscripts are significantly different ($P < 0.05$).

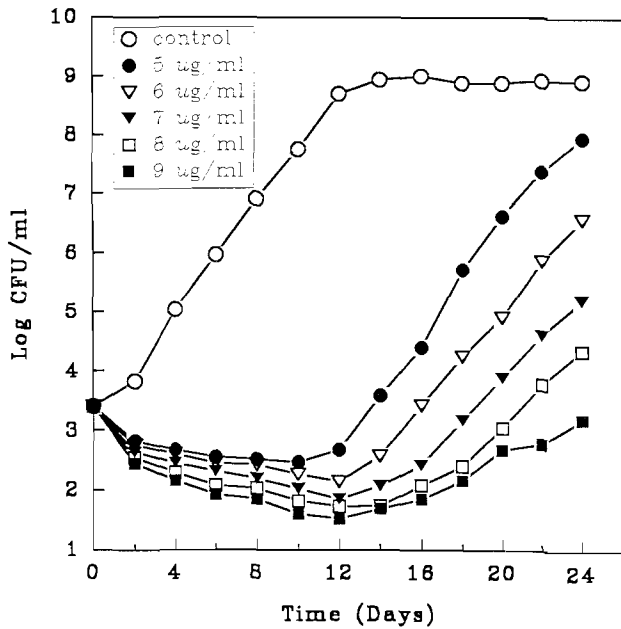


Figure 2. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 7°C and pH 5.5.

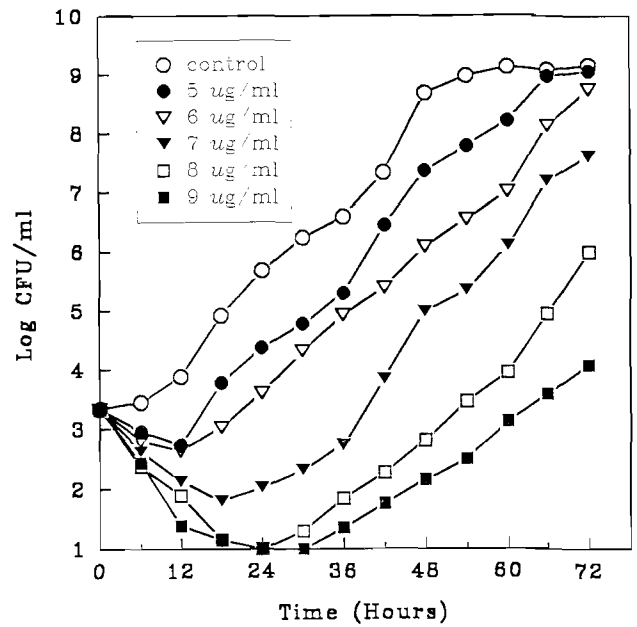


Figure 4. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 15°C and pH 7.0.

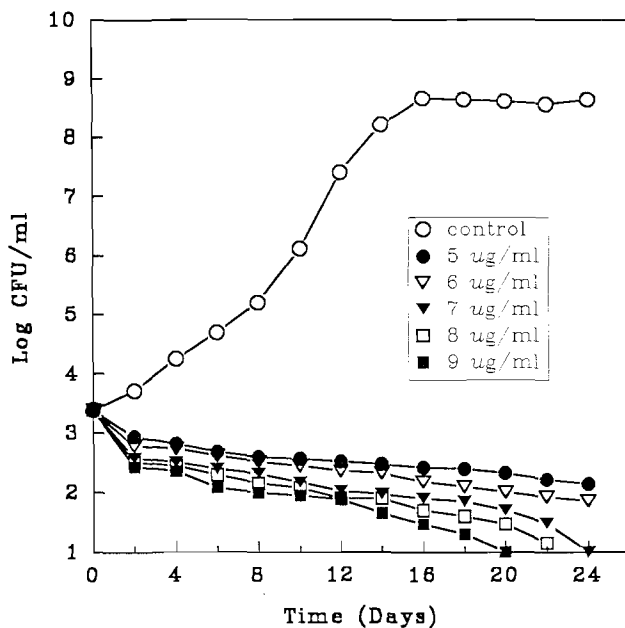


Figure 3. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 7°C and pH 5.0.

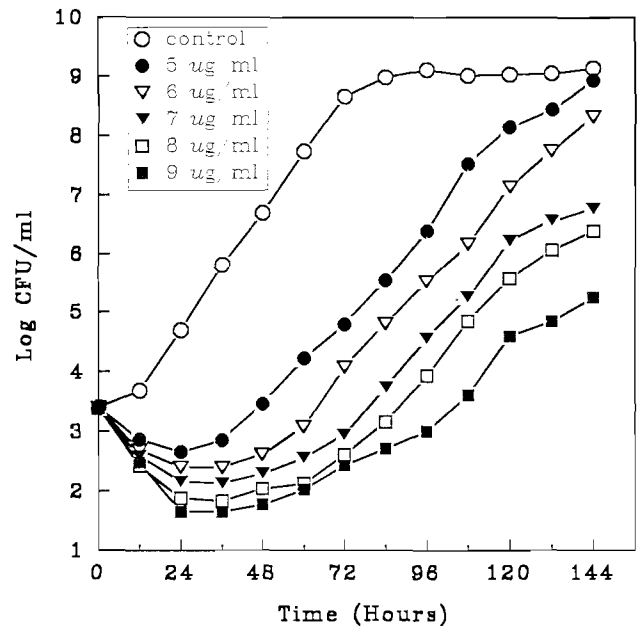


Figure 5. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 15°C and pH 5.5.

h, respectively. GT at these levels were significantly different ($P < 0.05$) from previous treatments. Nine $\mu\text{g/ml}$ 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 initiated growth in the control medium after 12 h with a GT of 2.5 h (Fig. 5). Addition of 5 $\mu\text{g/ml}$ monolaurin to the medium extended the lag phase to 24 h prior to growth. Addition of 6, 7, 8, or 9 $\mu\text{g/ml}$ 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. 6). Addition of 5, 6, or 7 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ monolaurin extended the lag phase to 120 h and significantly increased ($P < 0.05$) the GT compared to the other treatments. Counts of the bacterium were reduced to undetectable levels after 144 h in the presence of 9 $\mu\text{g/ml}$ monolaurin.

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. 7). The bacterium grew after lag phases of 4, 6, 8, 10,

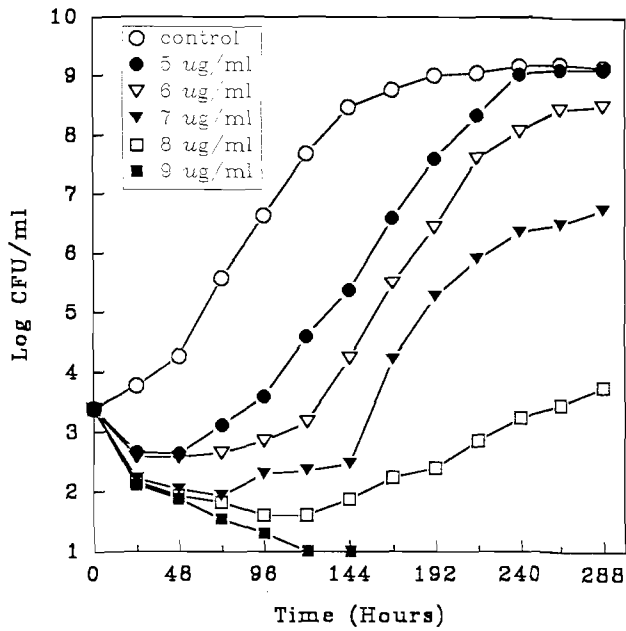


Figure 6. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 15°C and pH 5.0.

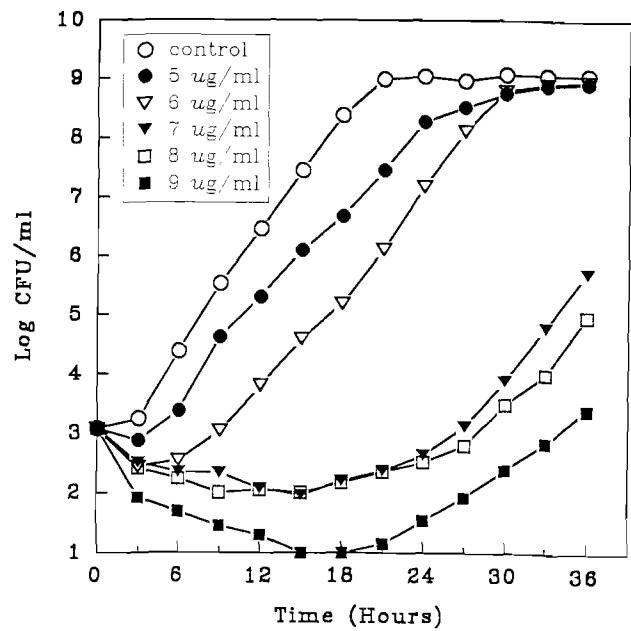


Figure 8. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 35°C and pH 5.5.

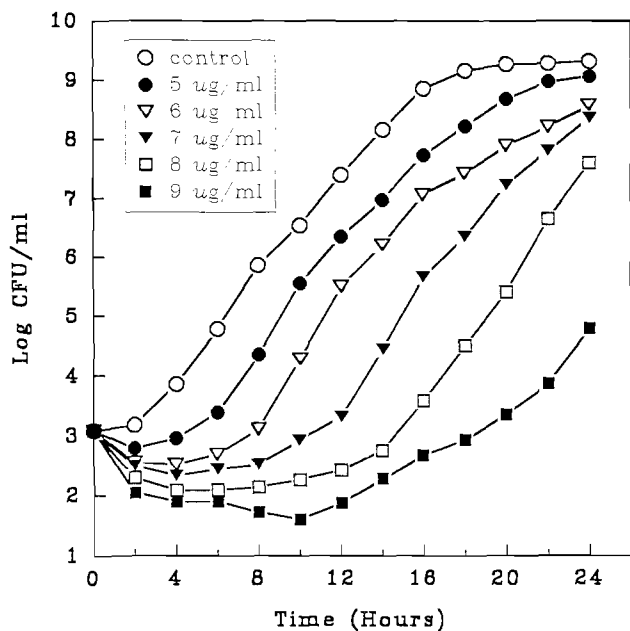


Figure 7. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 35°C and pH 7.0.

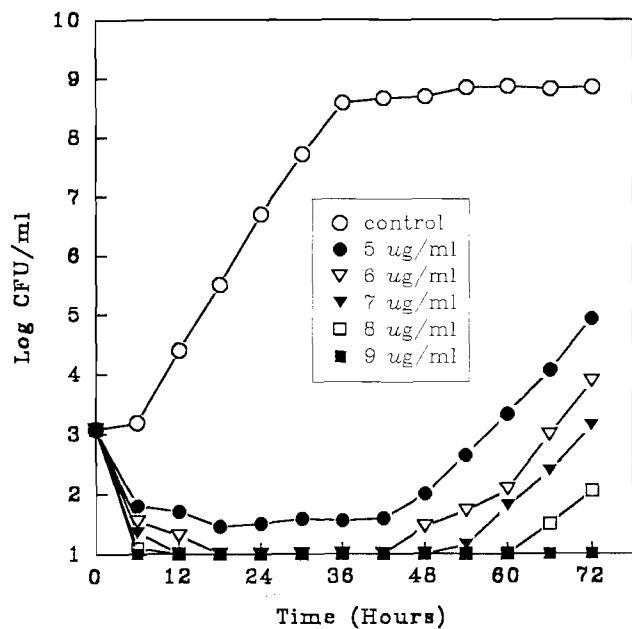


Figure 9. Growth and survival of *L. monocytogenes* as affected by various concentrations of monolaurin at 35°C and pH 5.0.

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 were not significantly different ($P > 0.05$) from the control, while the GT with 9 µg/ml monolaurin was significantly different ($P < 0.05$) from the other treatments. At pH 5.5, growth of *L. monocytogenes* initiated in the control broth after 3 h with a GT of 0.6 h (Fig. 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 significantly different ($P < 0.05$) from the control but not the previous treatments. Increasing monolaurin to 9 µg/ml extended the

lag phase to 21 h. GT of this treatment was significantly longer ($P < 0.05$) than the other treatments. When the pH was reduced to 5.0, *L. monocytogenes* began to grow after 6 h with a GT of approximately 1.1 h (Fig. 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 12 h. GT of all treatments were significantly different ($P < 0.05$) from the control.

DISCUSSION

Our results demonstrated that differences in the antimicrobial efficacy of monolaurin depend on the interaction

between pH and temperature. We previously reported that *L. monocytogenes* was notably more sensitive to monolaurin as pH decreases (20). This finding is confirmed in the present data where *L. monocytogenes* was inactivated by lower concentrations of monolaurin as the pH was reduced. The contribution of temperature on 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 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 when studying the combination of temperature and pH on sodium benzoate activity against *L. monocytogenes* (6).

Lowering medium pH has been shown to increase uptake of fatty acids by *Bacillus megaterium* and to reduce cell-medium interfacial tension (9). 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 may also be due to increased uptake of monolaurin (21). In addition to pH and temperature, other environmental factors such as 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. (4) and further explained by Shoemaker and Pierson (28) who reported on the growth of *C. perfringens* at sublethal temperatures. Viable cells apparently became sensitive to assay conditions initially, but after a period of time recovered their tolerance to assay conditions and finally grew as a result of the initial temperature shock. A similar phenomenon was observed in our study using sublethal concentrations of monolaurin instead of sublethal temperatures.

Effectiveness of sublethal concentrations of monolaurin may depend on whether the cells were metabolically active or resting. Yousef et al. (33) assumed that metabolically active cells may detoxify the antimicrobial agent 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 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.

It has been reported that, among fatty acids and their esters, monolaurin has the greatest overall antimicrobial activity and would appear to have the greatest potential for use in foods and cosmetics (27). Monolaurin has been found to be active against gram-positive bacteria, yeasts, and molds but less active against gram-negative bacteria. More research must be conducted on the combined effects of monolaurin with other agents to maximize its effective-

ness when used directly in foods as a preservative. Kato and Shibasaki (17) 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. Oh and Marshall (22) found synergistic effects against growth of *L. monocytogenes* when monolaurin was combined with organic acids, i.e., acetic, benzoic, and lactic.

An important observation made in this study is that *L. monocytogenes* was highly sensitive to monolaurin. Since monolaurin had a good 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 combined with heating or with another chemical agent may warrant its use as a new alternative food preservative in direct food systems applications.

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