J Food Sci. Nutr
Vol. 6, No. 1, p. 6~9 (2001)

Antimicrobial Activity of Glycerol Monolaurate and Organic Acids on the Survival of *Escherichia coli* O157:H7

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Abstract

Minimal inhibitory concentrations (MIC) and antimicrobial effects of glycerol monolaurate (monolaurin) and organic acids, either alone or in combination, against *Escherichia coli* O157:H7 in tryptic soy broth were determined. MIC values of monolaurin (ML), acetic (AA), citric (CA), lactic (LA) and hydrochloric acid (HCl) were 300 μg/mL (0.03%), 1250 μg/mL (0.125%), 5000 μg/mL (0.5%), 2500 μg/mL (0.25%) and 2500 μg/mL (0.25%), respectively. When 150 μg/mL of ML was combined with 50 μg/mL AA, 250 μg/mL HCl and 125 μg/mL LA, the combined agents did not increase the inhibitory effect of the most active single compound alone. This result indicates that there was little interaction between ML and AA, HCl and LA. However, the combination of 150 μg/mL ML and 250 μg/ mL CA synergistically inhibited growth of *E. coli* O157:H7. The present study showed that the nature of combined antimicrobial response against *E. coli* O157:H7 was complex, but this information would be useful for determinig interaction that could compromise effectiveness in food systems.

Key words: glycerol monolaurate, organic acids, *Escherichia coli* O157:H7, MIC, growth inhibition

INTRODUCTION

The first food related outbreaks of *E. coli* O157:H7 infection occurred in USA in 1982 and the outbreaks were associated with eating ground beef sandwiches (1). Since then, several additional outbreaks have been reported from raw ground beef, raw milk, turkey sandwiches, potatoes, roast beef, apple cider, and dry-cured salami (2-8). The pathogen is typical of most *E. coli*, but with some notable exceptions. Differences in biochemical characteristics include sorbitol fermentation and β-glucuronidase activity. Most *E. coli* of human origin can ferment sorbitol within 24 h and produce B-glucronidase, but *E. coli* O157:H7 typically does not (9).

Outbreaks involving acidic foods, such as mayonnaise (10) and apple cider (11), have underscored the unusual acid tolerance of this organism. Acidic foods are generally considered to be at low risk for transmission of pathogenic bacteria, but *E. coli* O157:H7 can survive at pHs as low as 2.0 and can persist for up to several weeks when inoculated into apple cider or mayonnaise. This acid tolerance may have also contributed to a 1994 outbreak involving dry-cured salami (8). This product, considered as ready to eat, is not cooked during processing because the pH of the product is decreased during fermentation to kill pathogenic organisms. Zhao et al. (12) reported that the pathogen grew and persisted in apple cider (pH 3.6 to 4.0). Furthermore, Brakett et al. (13) indicated that hot spray (55°C) of acetic, citric, and lactic acids did not affect survival of *E. coli* O157:H7 on raw beef. Also, the pathogen grew in fermented dairy products (14).

Given the recent emergence of *E. coli* O157:H7 as a recognized food-borne pathogen and its apparent ability to survive under acidic conditions, there is a need to quantify the antibacterial activity of organic acids against *E. coli* O157:H7. Monolaurin, a food-grade glycerol monoester of lauric acid, is approved in the USA as a food emulsifier. In addition, monolaurin also possesses a broad spectrum of antimicrobial activity in culture media against Gram-positive bacteria, yeast and mold, but generally not against gram-negative bacteria (15). An approach to overcome limitations of antimicrobial activity of food preservatives used alone, is to use them in combination. Oh and Marshall (16) reported that minimal inhibitory concentrations (MIC) values on *Listeria monocytogenes* were lower when monolaurin was combined with selected organic acids, such as acetic acid, benzoic acid and lactic acid in dual combination. Thus, the present study was conducted to determine MIC and antimicrobial effects for interaction between selected organic acids and monolaurin against *E. coli* O157: H7 in a model broth system.

MATERIALS AND METHODS

Bacterial cultures

E. coli strains O157:H7 932 and 933 strains were obtained from the Department of Food Science at the University of Georgia. Cultures were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) and maintained in TSB-glycerol (50: 50, vol/vol) at -20°C. To prepare inocula for the test media, the bacteria were grown in TSB supplemented with 0.6%

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yeast extract (TSBYE; Difco) at 37°C for 24 h. Each working culture was combined in equal volumes to serve as test organisms.

Preparation of antimicrobial agents

Ten% (v/v) stock solutions of known concentrations of reagent-grade acetic, citric, hydrochloric and lactic acid (Sigma Chemical Co., St. Louis, Mo) were prepared in sterile distilled water. A 1% (w/v) stock solution of monolaurin (Sigma Chemical Co., St. Louis, Mo) dissolved in absolute ethanol was prepared fresh before each experiment. The antimicrobial solutions were filter-sterilized using a 0.22 μm membrane filter (Milipore Products Division, Bedford, MA). These were prepared fresh before each experiment.

Experimental procedure

Appropriate amounts of antimicrobial stock solutions were added to 300-mL flasks containing 50 mL of sterile TSBYE to give 625, 1,250, 2,500, and 5,000 μg/mL of each organic acid and 25, 50, 100, 250, and 500 μg/mL monolaurin. Then, MIC was determined with visual growth-no growth threshold, and it was confirmed using the pour plate count agar method. Briefly, from the 10^7 CFU/mL working culture of the organism, 0.5 mL aliquots were aseptically transferred into each flask above containing appropriate amounts of each organic acid to give a final inoculum of 10^5 CFU/mL and the flasks were incubated at 37°C for 24 h.

From MIC data, the inhibitory effect of antimicrobial agents on *E. coli* O157:H7 was determined using the standard plate count method. 0.5 mL aliquots were aseptically transferred into the 300-mL flasks containing 50 mL of pH 7.0-adjusted TSBYE with appropriate concentrations of each antimicrobial agent, either alone or in combination, and were incubated at 35°C for 24 h. Positive controls consisted of inoculated TSBYE containing 0.1% ethanol and no antimicrobial agents. Negative controls used for sterility tests were uninoculated TSBYE containing 0.1% ethanol as dissolving agent and no antimicrobial agents. All samples were held at 35°C for designated periods and removed periodically, serially diluted in PBS, and spread on TSA plates to enumerate *E. coli* O157:H7. Plates were incubated at 35°C for 24 h before colonies were counted and triplicate experiments were performed.

Statistical analyses

Experiments were performed in triplicate to provide mean microbial count data that were subjected to analysis of variance, using the SAS general linear model procedure (17) with significance at $p<0.05$ to determine inhibitory effects of antimicrobial agents.

RESULTS AND DISCUSSION

Effect of ethanol on the growth of *E. coli* O157:H7 was first investigated because ethanol was used as a solvent for monolaurin. There was no inhibition of the organism in media containing 5% ethanol, but complete inactivation was observed in the presence of 10% ethanol (data not shown). The concentration of ethanol in the TSBYE medium used in this study as a control was at a subinhibitory level of 0.1%. Thus, use of low levels of ethanol (0.1%) as a solvent should have no effect on growth and survival of *E. coli* O157:H7 in our *in vitro* experiments. Oh and Marshall (18) reported that ethanol concentrations up to 2,500 μg/mL (0.25%) had little effect on the growth of *L. monocytogenes*, but the bacterium was almost completely inhibited in the presence of 5,000 μg/mL ethanol.

Table 1 shows MIC values of organic acid and monolaurin against *Escherichia coli* O157:H7 at 35°C. The MIC values of monolaurin (ML), acetic (AA), citric (CA), lactic (LA) and hydrochloric acid (HCl) alone were 300 μg/mL (0.03%), 1250 μg/mL (0.125%), 5000 μg/mL (0.5%), 2500 μg/mL (0.25%) and 2500 μg/mL (0.25%), respectively. 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 (19). However, the use of monolaurin as a food preservative may have limitations because its activity is antagonized by many food components (20). Furthermore, monolaurin is active against Gram-positive bacteria, yeasts, and molds but to a lesser degree against gram-negative bacteria. Thus, the combined effect of monolaurin and organic acid was of much interest because Oh and Marshall (16) previously reported that the MIC values were lower when monolaurin was combined with lactic acid against *L. monocytogenes*. MIC values of citric acid and monolaurin, either alone or in combination against *E. coli* O157:H7, are shown in Table 2. The MIC value of citric acid alone was 500 μg/mL, but the MIC value was lower when 200 μg/mL was combined with 125 μg/mL citric acid. This result implies synergistic effect when the agents are combined. However, additive MIC effect was observed when lactic acid was combined with monolaurin

Table 1. Minimal inhibitory concentration (MIC) of organic acid and monolaurin against *Escherichia coli* O157:H7 at 35°C

Organic acid	MIC (μg/mL)
Acetic acid	1250
Citric acid	5000
Lactic acid	2500
Hydrochloric acid	2500
Monolaurin	300

Table 2. Minimal inhibory concentration (MIC)[1] of monolaurin and citric acid against *Escherchia coli* O157:H7 at 35°C

Citric acid (μg/mL)	Monolaurin (μg/mL)				
	0	50	100	200	300
0	+[2]	+	+	+	−[3]
125	+	+	+	−	−
250	+	+	+	−	−
500	−	−	−	−	−

[1]The MIC represents the lowest concentration of antimicrobials that showed no growth after 24 h incubation.
[2]+, the concentrations of antimicrobials that show growth.
[3]−, the concentrations of antimicrobials that show no growth.

(Table 3). Also, similar results were observed in the combination of acetic acid or HCl and monolaurin (data not shown). Based upon these MIC results, sublethal concentrations of combinations of monolaurin with organic acids were chosen to further evaluate whether MIC data determined by "growth-no growth by spectrophotomer" correlates with growth curve data using standard plate count methodology (21).

The combined effect of monolaurin with acetic acid is shown in Fig. 1-A. The inhibitory effect at the sublethal concentration of the combination was little changed compared to the the most active compound alone after 24 h incubation. However, growth curve data confirmed that MIC value of acetic acid is showing complete inhibition in the presence of 125 μg/mL acetic acid. Similar patterns were observed in the combined effect of monolaurin with lactic acid or hydrochloric acid (Fig. 1-C and 1-D). On the other hand, enhanced inhibitory effect was observed when 150 μg/mL monolaurin was combined with 250 μg/mL citric acid and growth of *E. coli* O157:H7 was completely inhibited in the presence of 500 μg/mL citric acid (Fig. 1-B). The results implies that MIC results correlated well with growth curves through studying for combined effects.

Table 3. Minimal inhibory concentration (MIC)[1)] of monolaurin and lactic acid against *Escherchia coli* O157:H7 at 35°C

Lactic acid (μg/mL)	Monolaurin (μg/mL)				
	0	50	100	200	300
0	+[2)]	+	+	+	−[3)]
62.5	+	+	+	−	−
125	+	+	−	−	−
250	−	−	−	−	−

[1)]The MIC represents the lowest concentration of antimicrobials that showed no growth after 24 h incubation.
[2)]+, the concentrations of antimicrobials that show growth.
[3)]−, the concentrations of antimicrobials that show no growth.

The antibacterial effects of organic acid may be due to the undissociated, rather than hydrogen ions. Many weak acids can penetrate the cell membrane and accumulate within the cell cytoplasm in undissociated form, resulting in detrimental effects (22) Ita and Hutkins (23) 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 was not caused by decreasing intracellular pH, but rather by a specific effect of undissociated acid on metabolic activities. Oh and Marshall (16) reported that enhanced inhibitory effect of combinations of monolaurin with organic acids was likely influenced by both pH and type of acid.

Several organic acids have been used as food preservatives

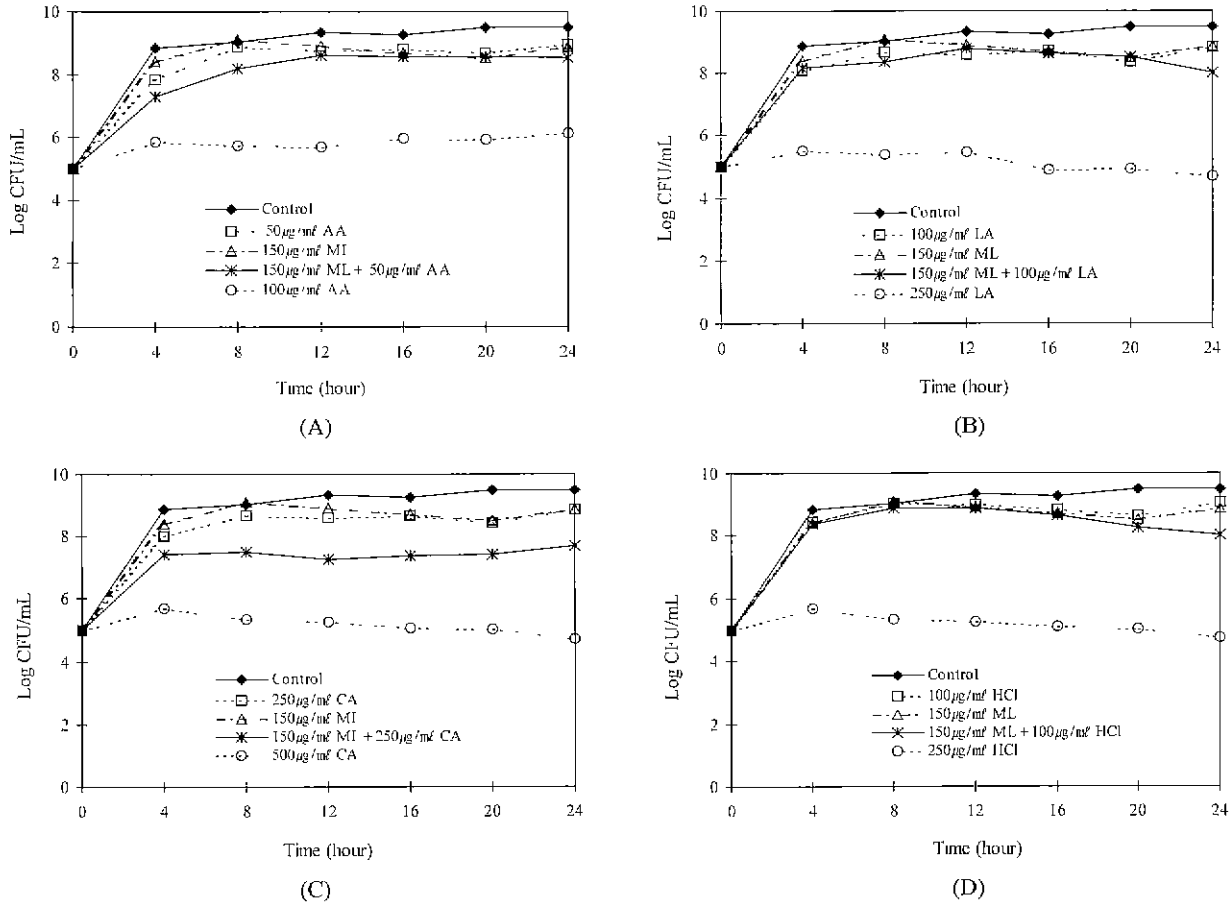

Fig. 1. Effect of monolaurin and organic acid either alone or in combination against *E. coli* O157:H7 in TSBYE at 35°C.

and as decontaminating agents because of their broad antimicrobial spectrum and it has been reported that the inhibitory effects of organic acids on food-borne pathogens in the broth systems are strong, but weak on foods or animal tissues. Organic acid treatments on red meat did not completely inactivate *E. coli* O157:H7 (13). Also, the pathogen can survive fermentation, drying and storage of fermented sausage regardless of the use of strater cultures (24).

Monolaurin showed significantly enhanced antimicrobial activity against Gram-positive bacteria, such as *L. monocytogenes, vibrio parahaemolyticus, staphylococcus aureus,* but the pathogen had significant limitation against Gram-negative bacteria (18). Thus, the combined effect of monolaurin and organic acid is of much necessity and should prove to be effective in foods by removing the limitation of using monolaurin alone as a preservative. MIC tests for combined antimicrobial compounds *in vitro* represent an important tool in detecting their interactions (5). Thus, it should be addressed to evaluate the MIC data in food systems to determine the interactions that could compromise their effectiveness.

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(Received October 11, 2000)