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Effect of Organic Acids on the Survival of *Escherichia coli* O157:H7

Deog-Hwan Oh†, Jong-Hyun Park* and Boo-Kil Park

Division of Food and Biotechnology, Kangwon National University, Chuncheon 200-701, Korea
**Department of Food and Bioengineering, Kyungwon University, Sungnam 461-701, Korea*

Abstract

The inhibitory effect of various organic acids on the growth and survival of *Escherichia coli* O157:H7 in tryptic soy broth with 0.6% yeast extract at 37°C or 4°C was determined. Minimal inhibitory pHs of acetic acid, citric acid, fumaric acid, hydrochloric acid and lactic acid were 5.0, 4.0, 4.5, 4.0 and 4.5, respectively. Acetic acid (0.012 M) showed the strongest antimicrobial activity, based on the pH values or equivalent molar concentrations, followed by lactic acid (0.006 M), fumaric acid (0.004 M) and citric acid (0.004 M), respectively. *E. coli* O157:H7 with an initial inoculum of 10^7 CFU/ml and 10^5 CFU/ml in tryptic soy broth supplemented with 0.6% yeast extract, acidified to target pH with citric, fumaric and lactic acids at 37°C, was completely inactivated after 7 d and 5 d incubation, respectively, except for the acetic acid (9 d). The bactericidal effect decreased at the same pH when the incubation temperature was reduced from 37°C to 4°C. The pH values of 0.2% acetic (pH 5.1), 0.6% citric (pH 4.2) and 0.4% lactic acid (pH 4.3) in TSBYE were almost correspondent to the minimal inhibitory pH values on *E. coli* O157:H7 of acetic (pH 5.0), citric (pH 4.0) and lactic acids (pH 4.5).

Key words: *Escherichia coli* O157:H7, organic acids, growth and survival

INTRODUCTION

Enterohaemorrhagic *Eschrichia coli* (EHEC) has been reported as the causative agent of hemorrhagic diarrhoca and hemolytic uremic syndrome (HUS) since its recognition as a foodborne pathogen in 1982. The pathogenicity of EHEC appears to be associated with several cytotoxins referred to as shiga-like toxin (SLT) or verotoxins (1).

Several foodborne-outbreaks of *E. coli* O157:H7 infection have been epidemiologically linked to the consumption of undercooked ground beef and raw milk, suggesting that dairy cattle may be a reservoir for this organism. *E. coli* O157:H7 has been isolated from retail ground beef, poultry, pork, lamb and from fecal samples of herds associated with cases of HUS (2-6). Additionally raw milk, cold sandwiches, vegetables and water have been implicated as sources of some outbreaks (2-6). Two recent outbreaks were unusual in that they were both linked to consumption of low pH foods, which have traditionally been considered safe. An outbreak in Massachusetts was associated with the drinking of apple cider, which indicates that resistance to acidic pH is an additional characteristic that distinguishes the O157:H7 serotype from other *E. coli* (7,8). The other outbreak was associated with ingestion of a mayonnaise-containing food from an Oregon restaurant chain (9). In both cases, laboratory experiments showed that *E. coli* O157:H7 survived for weeks at refrigeration temperature. These outbreaks suggest that *E. coli* O157:H7 may be tolerant to acidic conditions, particularly at lower temperatures. Zhao et al. (10) reported that the pathogen grew and persisted in apple cider (pH 3.6 to 4.0). Furthermore, Brackett et al. (11) 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 (12). Acidification is extensively used in food processing to control the growth and survival of spoilage-causing and pathogenic microorganisms. The ability of food-borne pathogens, such as *E. coli, Listeria monocytogenes* and *Salmonella typhimurium,* to adapt to acidic conditions is a concern in food safety (13). Recently, Leyer and Johnson (14) reported that acid-adapted *S. typhimurium* survived better than non-adapted cells during milk fermentation and curing of various cheeses.

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 food-associated organic acids against *E. coli* O157:H7. To better assess the role of *E. coli* O157:H7 in food-borne disease as well as to develop potential means of controlling this bacterium in foods, it is important to identify and quantify the effects of various environmental factors on the behavior of *E. coli* O157:H7. Therefore, this study was undertaken to investigate the fate of several strains of *E. coli* O157:H7 in the presence of selected organic acids and hydrochloric acid in a laboratory medium.

MATERIALS AND METHODS

Bacterial cultures

E. coli strains O157:H7 932, 933 and 1009 strains were acquired from the Department of Food Science, University of Georgia. Cultures were grown in tryptic soy broth (Difco

†Corresponding author. E-mail: dcoghwa@cc.kangwon.ac kr
Phone. 82-33-250-6457, Fax: 82-33-250-6457

Laboratories, Detroit, MI) and maintained in tryptic soy broth (TSB) - glycerol (50:50, vol/vol) at -20°C. To prepare inocula for the test media, the bacteria were grown in tryptic soy broth supplemented with 0.6% 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 organic acids

Ten percent stock solution of reagent-grade HCl, acetic, citric and lactic acids (Mallinkrodt Inc., Paris, KY) in distilled water and 5% stock solution of fumaric acid in absolute ethanol were prepared. These were prepared fresh before each experiment. All organic acids were individually filter-sterilized using 0.22 μm membrane filters (Millipore Products Division, Bedford, MA).

Experimental procedure

Appropriate amounts of organic acid stock solutions were aseptically added to 250-ml Erlenmeyer flasks containing 50 ml of sterile TSBYE to adjust pH 4.0, 4.5, 5.0, 5.5, and 6.0. Then, the minimal inhibitory pH (target pH) 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 the flask containing appropriate amounts of each organic acid to give a final inoculum of 10^5 CFU/ml. The flasks were incubated at 37°C for 24 h.

From the minimal inhibitory pH data, the effect of the target pH on the survival of *E. coli* O157:H7 cells was determined for each acid. Triplicate flasks of TSBYE (100 ml) acidified to the target pH values (acetic acid, pH 5.0; citric acid, pH 5.0; fumaric acid, pH 4.5; hydrochloric acid, pH 4.0; lactic acid, pH 4.5) were prepared. The volume of acid added to achieve the desired pH was noted and used to calculate the concentration of each acid in TSBYE at each test pH. Concentrations of undissociated forms of organic acids at each pH were calculated by the Henderson-Hasselbach equation (15). Also, the inhibitory effect of the organic acids on *E. coli* O157:H7 was determined. Triplicate flasks of TSBYE containing 0.2%, 0.4% and 0.6% of acetic, citric and lactic acids were prepared and the pH values determined for the given concentration of each acid. From the working culture, 0.1 ml aliquots were aseptically transferred into each flask to give a final inoculum of 10^3 CFU/ml. TSBYE without organic acid (pH 7.1) served as the control. For the fumaric acid, the same amount of ethanol was used as a control. All samples were held at 4°C and 37°C for designated periods. Samples were removed from each flask and viable counts of *E. coli* O157:H7 were enumerated by the plate count agar method.

Statistical analyses

All the experiments were performed in triplicate to provide mean viable count data that were subjected to analysis of variance, using the SAS general linear model procedure (16) with a significance at $p<0.05$ to determine the inhibitory effects of organic acids.

RESULTS AND DISCUSSION

The growth of *E. coli* O157:H7 in TSBYE acidified to different pHs with organic acids at 37°C is shown in Table 1. The minimal inhibitory pHs of acetic acid, citric acid, fumaric acid, hydrochloric acid and lactic acid were 5.0, 4.0, 4.5, 4.0 and 4.5, respectively. These results indicate that the minimum pH at which *E. coli* O157:H7 did not grow in acidified TSBYE was dependent on the acid tested. Based only on pH, acetic acid (pH 5.0) showed the strongest antimicrobial activ-

Table 1. Screen test for survival of *E. coli* O157:H7 in TSBYE acidified to different pH with various organic acids at 37°C

pH	Incubation time (d)	Growth[1] in TSBYE acidified with given acid[2]									
		HCl		Acetic acid		Citric acid		Lactic acid		Fumaric acid	
4.0	1	-[3]	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
4.5	1	+	+	-	-	+	+	-	-	-	-
	2	+	+	-	-	+	+	-	-	+	+
	3	+	+	-	-	+	+	-	-	+	+
5.0	1	+	+	-	-	+	+	+	+	+	+
	2	+	+	-	-	+	+	+	+	+	+
	3	+	+	-	-	+	+	+	+	+	+
5.5	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+
6.0	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+

[1]Initial inoculum of 10^5 CFU/ml was used for each treatment.
[2]1 M acetic, citric and lactic acids, and 0.25 M fumaric acid was used for this experiment.
[3]- : no growth, + : growth

ity on *E. coli* O157:H7, followed by lactic and fumaric acids (pH 4.5), citric and hydrochloric acids (pH 4.0). This data is similar to the inhibitory pH values reported by Conner and Kotrola (17) who also reported that, on an equivalent pH basis, acetic acid was more inhibitory than lactic and citric acids. These results are also consistent with other reports that factors other than pH, such as type of acid, temperature, concentration, and extent of dissociation, could affect the antibacterial effect of various organic acids on *E. coli* O157:H7 (18-21).

Because it is the undissociated acid molecule that accounts for the antibacterial activity of acids, the concentrations of the undissociated form of each acid at the test pH were determined. Acetic acid (0.012 M) had the highest concentration, followed by lactic acid (0.006 M), fumaric acid (0.004 M) and citric acid (0.004 M), respectively. However, Podolak et al. (21) reported that 1.0% fumaric acid exerted greater inhibitory effect on *E. coli* O157:H7 than 1% acetic and lactic acids. These results could have been affected by the decomposition of fumaric acid to carbon oxide, which is initiated by catalysis of transition-metal oxides (22). Also, differences of inhibitory effect among organic acids has been reported to be due to the extent of inhibition of glycolysis in intact cells, decrease of internal pH, or the destruction of a specific enzyme system (23).

From Table 2, a survival study was performed on whether recovery of *E. coli* O157:H7 could be affected by the type of acid, temperature and initial cell number. Fig. 1 shows the survival of *E. coli* O157:H7 with an initial inoculum of 10^7 CFU/ml in TSBYE acidified to a target pH at 37°C for 9 d. *E. coli* O157:H7 was completely inactivated after 7 d in the presence of all organic acids tested except for the acetic acid (9 d). As initial cell number was decreased to 10^5 CFU/ml, similar results were observed with Fig. 1, but the inactivation rate was faster than that of higher initial cell number (Fig. 2). However, the bactericidal effect decreased at the same pH when the incubation temperature was reduced from 37°C to 4°C (Fig. 3). The viable *E. coli* O157:H7 cells were recovered over a much longer period of storage at 4°C compared to 37°C. *E. coli* O157:H7 was completely inactivated within 24 to 39 d in the presence of all organic acids tested except for the acetic acid, which showed no growth but survival. In TSBYE without acid, *E. coli* O157:H7 did not grow up to 42 d of the incubation period.

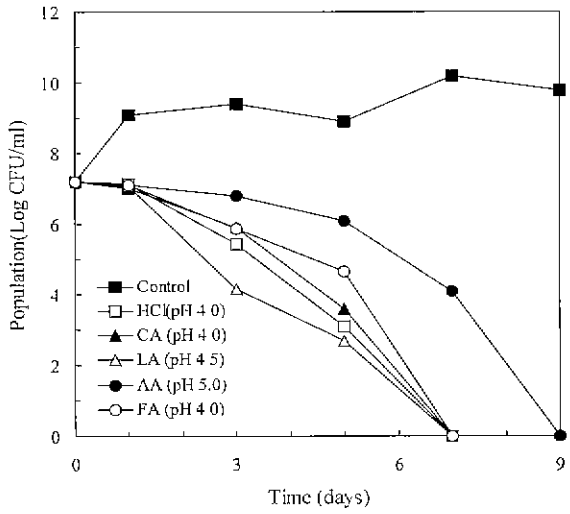

Fig. 1. Growth and survival of *E. coli* O157:H7 with initial inoculumn of 10^7 CFU/ml in the tryptic soy broth supplemented with yeast extract acidified with different organic acids at 37°C HCl: hydrochloric acid, CA: citric acid, LA. lactic acid, AA: acetic acid, FA: fumaric acid.

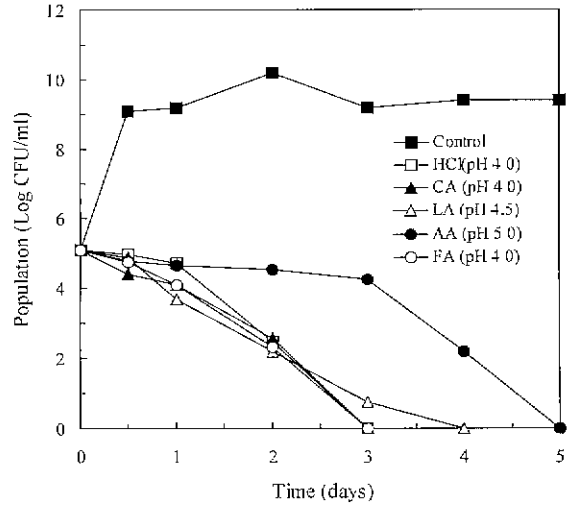

Fig. 2. Growth and survival of *E. coli* O157.H7 with initial inoculumn of 10^5 CFU/ml in the tryptic soy broth supplemented with yeast extract acidified with different organic acids at 37°C. HCl: hydrochloric acid, CA: citric acid, LA: lactic acid, AA: acetic acid, FA: fumaric acid.

Table 2. Minimal inhibitory pH in TSBYE at which *E. coli* O157:H7 did not grow

Acid	Measured pH	Total acid concentration (M)	Concentration of undissociated acid (M)
Hydrochloric acid	4.0	0.034	-[1]
Acetic acid	5.0	0.038	0.012
Citric acid	4.0	0.035	0.004
Lactic acid	4.5	0.034	0.006
Fumaric acid	4.5	0.016	0.004

[1]Hydrochloric acid completely dissociated.

We were interested in the survival differences between minimal inhibitory pH and inhibitory concentrations for each acid. Thus, 0.2, 0.4, and 0.6% of acetic, citric and lactic acids in TSBYE were tested for their pH (Table 3). The pH values were 5.1, 4.4 and 4.2 in the TSBYE containing 0.2, 0.4 and 0.6% acetic acid, respectively. As the concentration of acetic acid increased, the corresponding pH value decreased. The pH value in TSBYE containing 0.2% acetic acid was almost the same as the minimal inhibitory pH value of the acetic acid

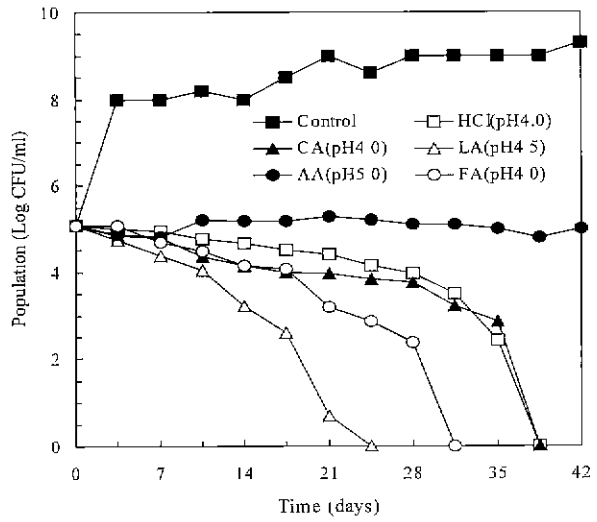

Fig. 3. Growth and survival of *E. coli* O157:H7 with initial inoculumn of 10^5 CFU/ml in the tryptic soy broth supplemented with yeast extract acidified with different organic acids at 4°C. HCl: hydrochloric acid, CA: citric acid, LA: lactic acid. AA: acetic acid.

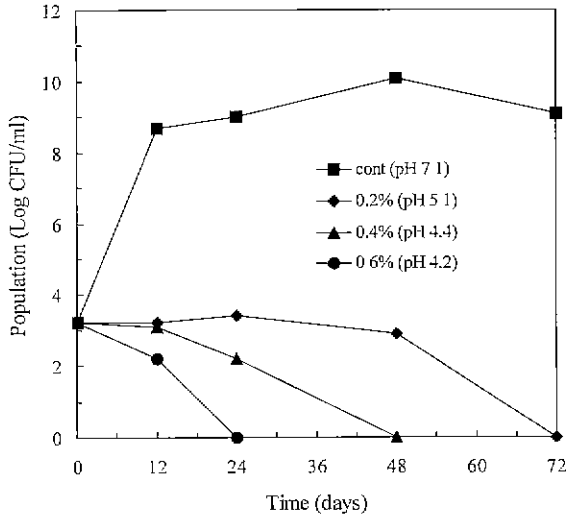

Fig. 4. Growth and survival of *E. coli* O157:H7 in tryptic soy broth supplemented with yeast extract containing acetic acid at 37°C.

Table 3. pH values of different kinds of acids

Solutions	Measured pH	Molarity (M)	Conc. of undissociated acid (M)
0.2% Acetic acid	5.1	0.033	0 010
0.4% Acetic acid	4.4	0.067	0.045
0 6% Acetic acid	4.2	0.100	0.078
0.2% Citric acid	5.5	0.013	0 0001
0.4% Citric acid	4.7	0.021	0.001
0.6% Citric acid	4.2	0.031	0.003
0.2% Lactic acid	5.4	0.022	0.001
0.4% Lactic acid	4.3	0.044	0.012
0.6% Lactic acid	4.0	0.066	0.027

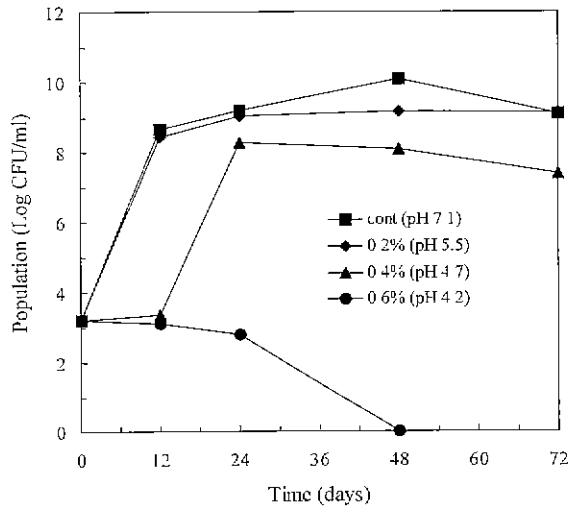

Fig. 5. Growth and survival of *E. coli* O157:H7 in tryptic soy broth supplemented with yeast extract containing citric acid at 37°C.

on *E. coli* O157:H7. These results were confirmed with dynamic kinetics in Fig. 4. The viable counts of *E. coli* O157:H7 in the control rapidly increased to approximately 10^9 CFU/ml after 12 h and then showed the same level up to 72 h of the incubation period. However, *E. coli* O157:H7 was completely inactivated within 24 to 72 h in the presence of 0.2 to 0.6% acetic acid. Similar patterns were observed in Fig. 5 and 6. The pH values were 5.5, 4.7 and 4.2 at 0.2, 0.4 and 0.6% citric acid, but 5.4, 4.3 and 4.0 in the case of lactic acid. The pH values of 0.6% citric acid and 0.4% lactic acid in TSBYE were almost the same as the minimal inhibitory pH value of each acid. These results indicate that acetic acid, on an equivalent concentration basis, is more inhibitory against *E. coli* O157:H7 than citric acid or lactic acid.

The antimicrobial effect of organic acids has been attributed to undissociated acid molecules that interfere with cellular metabolism or a decrease in biological activity as a result of a pH change in the cell's environment (24). In general, inhibition by organic acids is thought to occur by at least two mechanisms; 1) a generalized intracellular acidification and 2) the specific effect of the undissociated acid on metabolic activities. Salmond et al. (13) pointed out the latter mechanism to be more potent in inhibiting growth of *E. coli*.

Generally, it has been reported that the inhibitory effects of organic acids on food-borne pathogens in the broth systems are strong, but weak in foods or animal tissues. Organic acid treatments on red meat did not completely inactivate *E. coli* O157:H7 (25) and warm (20°C) or hot (55°C) acetic, citric and lactic acid treatments did not effectively work on the reduction of *E. coli* O157:H7 on raw beef (11). Also, the pathogen can survive fermentation, drying, and storage of fermented sausage regardless of the use of starter cultures (19). Along with its survival in high acid foods, resistance of *E. coli*

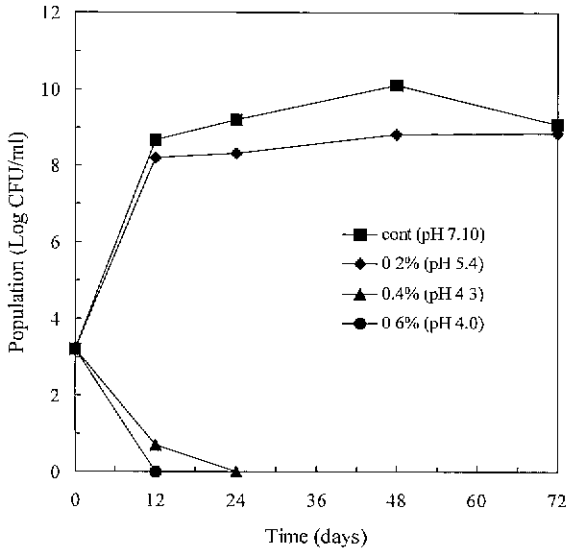

Fig. 6. Growth and survival of *E. coli* O157:H7 in tryptic soy broth supplemented with yeast extract containing lactic acid at 37°C.

O157:H7 strain to acid conditions has important implications in the use of new washing processes to remove or eliminate pathogens from animal carcasses. Organic acids have been used in an attempt to control *E. coli* O157:H7 on beef carcasses, but have been unsuccessful in the reduction of pathogen levels. Therefore, such treatments may not be sufficient as the only means to improve the overall microbiological safety of beef carcasses, but they might be beneficial as a part of an overall HACCP approach that can be implemented to enhance microbiological safety and extend the shelf-life of post-rigor beef.

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