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Growth and Survival of *Escherichia coli* O157:H7 under Acidic Conditions†

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The effect of pH reduction with acetic (pH 5.2), citric (pH 4.0), lactic (pH 4.7), malic (pH 4.0), mandelic (pH 5.0), or tartaric (pH 4.1) acid on growth and survival of *Escherichia coli* O157:H7 in tryptic soy broth with 0.6% yeast extract held at 25, 10, or 4°C for 56 days was determined. Triplicate flasks were prepared for each acid treatment at each temperature. At 25°C, populations increased 2 to 4 $\log_{10}$ CFU/ml in all treatments except that with mandelic acid, whereas no growth occurred at 10 or 4°C in any treatments except the control. However, at all sampling times, higher ($P < 0.05$) populations were recovered from treatments held at 4°C than from those held at 10°C. At 10°C, *E. coli* O157:H7 was inactivated at higher rates in citric, malic, and mandelic acid treatments than in the other treatments. At the pH values tested, the presence of the organic acids enhanced survival of the pathogen at 4°C compared with the unacidified control. *E. coli* O157:H7 has the ability to survive in acidic conditions (pH, ≥4.0) for up to 56 days, but survival is affected by type of acidulant and temperature.

Since its recognition as a food-borne pathogen in 1982 (12, 13), there have been at least 16 documented outbreaks of food-borne infection due to *Escherichia coli* O157:H7 (11), most of which have been attributed to contaminated ground beef or raw milk. However, in a 1991 outbreak of *E. coli* O157:H7 infection (hemorrhagic colitis) in Massachusetts, apple cider was the vehicle of transmission (17). In a 1980 outbreak in Canada involving apple cider, *E. coli* O157:H7 was suspected as the etiological agent on the basis of the symptoms of the victims (16). These outbreaks suggest that *E. coli* O157:H7 may be tolerant to acidic conditions. Work by Zhao et al. (17) showed that the pathogen grew and persisted in apple cider (pH 3.6 to 4.0). Furthermore, Brackett et al. (5) indicated that hot (55°C) sprays of acetic, citric, and lactic acids did not affect survival of *E. coli* O157:H7 on raw beef, and relatedly, Cutter and Siragusa (8) reported that organic acid carcass washes did not completely inactivate the pathogen on beef tissues. Growth of *E. coli* O157:H7 can also occur in fermented dairy products (2), further indicating that the pathogen is resistant to organic acids.

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. The goal of this research, therefore, was to determine the growth, survival, and death characteristics of the pathogen as affected by type of acid, pH, and temperature. The specific objectives were to determine the pH required to inhibit the growth of *E. coli* O157:H7 at different temperatures in a liquid medium acidified with acetic, citric, lactic, malic, mandelic, or tartaric acid and to quantify population changes in acidified media.

Three isolates of *E. coli* O157:H7 from M. P. Doyle, University of Georgia, were used: 301C, isolated from retail chicken; 240P, isolated from retail pork; and 505B, isolated from retail beef. Cultures were grown in brain heart infusion (Difco, Detroit, Mich.) and maintained in brain heart infusion-glycerol (50:50, vol/vol) at −80°C. To prepare inocula for the test media, cultures were activated (revived) by two successive transfers in tryptic soy broth with 0.6% yeast extract (TSBYE; Difco) at 37°C for 24 h.

Stock solutions of known concentrations of reagent-grade acetic, citric, lactic, malic, mandelic, and tartaric acids (Sigma Chemical Co., St. Louis, Mo.) were prepared, filter sterilized, and used to aseptically acidify batches of TSBYE to pH 4.0, 4.5, 5.0, and 5.5. 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-Hasselbalch equation (4), using only pK_{a1}.

Preliminary screening was done in 96-well tissue culture plates (365 μl). For each of the six acids tested, 10 wells, each containing 200 μl of appropriately acidified TSBYE, were inoculated (10 μl) to provide an initial population of 1,000 CFU/ml, using a cell suspension containing equal numbers of the three isolates of *E. coli* O157:H7, and then the plates were held statically at 4, 10, 25, or 37°C for 21 days. Wells were examined daily for turbidity.

From results of the screening study, the test pH, which represented a "growth-no growth" threshold, was determined for each acid. Triplicate flasks of TSBYE (100 ml) acidified to these target pH values (acetic acid, pH 5.0; citric acid, pH 4.0; lactic acid, pH 4.5; malic acid, pH 4.0; mandelic acid, pH 5.0; tartaric acid, pH 4.0) were prepared, allowed to reach the appropriate temperature, and inoculated with a composite suspension containing equal numbers of cells of each of the three *E. coli* O157:H7 isolates to give an initial population of 10^5 CFU/ml in each flask. Measured pHs of each batch of acidified TSBYE are given in Table 1. TSBYE with no added acid (pH 7.0) served as the control. Sets of acidified TSBYE were held statically at 4, 10, or 25°C. Immediately after inoculation, and weekly thereafter for 8 consecutive weeks, samples (5.0 ml) from each flask were withdrawn and viable *E. coli* O157:H7 organisms were enumerated. Experimental samples and inoculum suspensions were enumerated by the spiral plate method (Spiral Systems, Inc., Bethesda, Md.) with TSBYE as the plat-

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TABLE 1. Acids in TSBYE used for survival study

Acid	Target pH	Measured pH	Total acid concn (M)	Concn of undissociated acid (M)[a]
Acetic	5.0	5.2	0.027	0.007
Citric	4.0	4.0	0.031	0.004
Lactic	4.5	4.7	0.033	0.001
Malic	4.0	4.0	0.039	0.008
Mandelic	5.0	5.0	0.025	0.001
Tartaric	4.0	4.1	0.027	0.002

[a] Calculated at measured pH with the Henderson-Hasselbalch equation.

ing medium; plates were incubated at 37°C for 24 h before colonies were counted. Recovered populations from each flask were converted to $\log_{10}$ CFU per milliliter and subjected to analysis of variance ($3 \times 6 \times 9$ factorial), using the SAS general linear model procedure (14) with significance set at $P \leq 0.05$.

In earlier work in this laboratory, *E. coli* O157:H7 growth occurred at 4°C in tryptic soy broth with 3 and 4% sodium lactate (6). However, in the present screening study, no growth was evident at 4°C with any of the treatments (Table 2). At 10°C, *E. coli* O157:H7 grew at pH 5.5, but not at pH 5.0, 4.5, or 4.0 in TSBYE acidified with all acids except acetic acid, with which no growth was observed at any tested pH. At 25 and 37°C, the acids were less inhibitory, as evidenced by growth at lower pH values compared with the 10°C storage temperature. Acetic and mandelic acids were most inhibitory, while tartaric acid was least inhibitory. Similar patterns of growth were observed at 25 and 37°C.

In the survival study, in which TSBYE was acidified to the no growth-growth threshold found in the screening study (Table 2), recovery of *E. coli* O157:H7 was also affected ($P \leq 0.01$) by type of acid, temperature, and storage time, and there were significant ($P \leq 0.01$) interactions of the factors. Therefore, data are presented (Fig. 1) and discussed in regard to the interaction of these factors.

At 25°C, populations increased 2 to 4 $\log_{10}$ CFU/ml in all treatments except that with mandelic acid, in which *E. coli* O157:H7 was inactivated. In all acidified TSBYE, no growth occurred at 4 and 10°C. *E. coli* O157:H7 did grow in unacidified TSBYE at 10°C. Survival patterns at 4 and 10°C were similar in all acid treatments; however, at all sampling times, populations from treatments held at 4°C were equal to or higher than those from corresponding treatments held at 10°C. At 10°C, *E. coli* O157:H7 was inactivated at slightly higher rates by citric, malic, and mandelic acids than by acetic and lactic acids. Interestingly, higher populations survived at 4°C in all of the acid treatments versus the control.

The test pH at which the pathogen could not grow in acidified TSBYE was different depending on temperature and type of acid tested. At 10°C, the inhibitory pH for all of the acids in TSBYE was ≤5.5; however, the inhibitory pH values were considerably lower at the higher incubation temperatures. At the higher temperatures and solely on the basis of pH, mandelic and acetic acids, which inhibited bacterial growth at pH 4.5, were the most inhibitory. The effects of acetic acid were similar to those seen in acidified beef slurry (1). In the present study, malic, citric, and lactic acids were inhibitory at pH 4.0, whereas tartaric acid was not inhibitory at pH 4.0. Zhao et al. (17) reported that *E. coli* O157:H7 grew slightly (ca. 1 $\log_{10}$ CFU/ml) in apple cider at pH 3.8 to 4.0, indicating more acid tolerance than observed here. Siragusa and Dickson (15) reported that *E. coli* O157:H7 on beef tissue was more resistant to acetic and lactic acids contained in alginate gels than was *Salmonella typhimurium* or *Listeria monocytogenes*. In contrast, however, Glass et al. (10) reported a higher inhibitory pH (5.0) for lactic acid in tryptic soy broth than was observed here.

TABLE 2. Preliminary results of screening for *E. coli* growth[a]

Temp (°C)	pH	Growth[b] in TSBYE with given acid						
		Control	Malic	Tartaric	Citric	Lactic	Acetic	Mandelic
4°C	4.0		−	−	−	−	−	−
	4.5		−	−	−	−	−	−
	5.0		−	−	−	−	−	−
	5.5		−	−	−	−	−	−
	7.0	−						
10°C	4.0		−	−	−	−	−	−
	4.5		−	−	−	−	−	−
	5.0		−	−	−	−	−	−
	5.5		+++	+++	+++	+++	−	+++
	7.0	+++						
25°C	4.0		−	+	−	−	−	−
	4.5		+++	+++	−	+	−	−
	5.0		+++	+++	+++	+++	+	+
	5.5		+++	+++	+++	+++	+++	+++
	7.0	+++						
37°C	4.0		−	++	−	−	−	−
	4.5		+++	+++	+++	+	−	−
	5.0		+++	+++	+++	+++	+	−
	5.5		+++	+++	+++	+++	+++	+++
	7.0	+++						

[a] Growth of *E. coli* O157:H7 in TSBYE adjusted to different pHs with six different acids and incubated at 4, 10, 25, or 37°C for 21 days.

[b] Ten wells (200 μl) per acid-pH combination inoculated (initial population, 10^3 CFU/ml). −, no growth; +, growth in 1 to 5 wells; ++, growth in 6 to 9 wells; +++, growth in 10 wells.

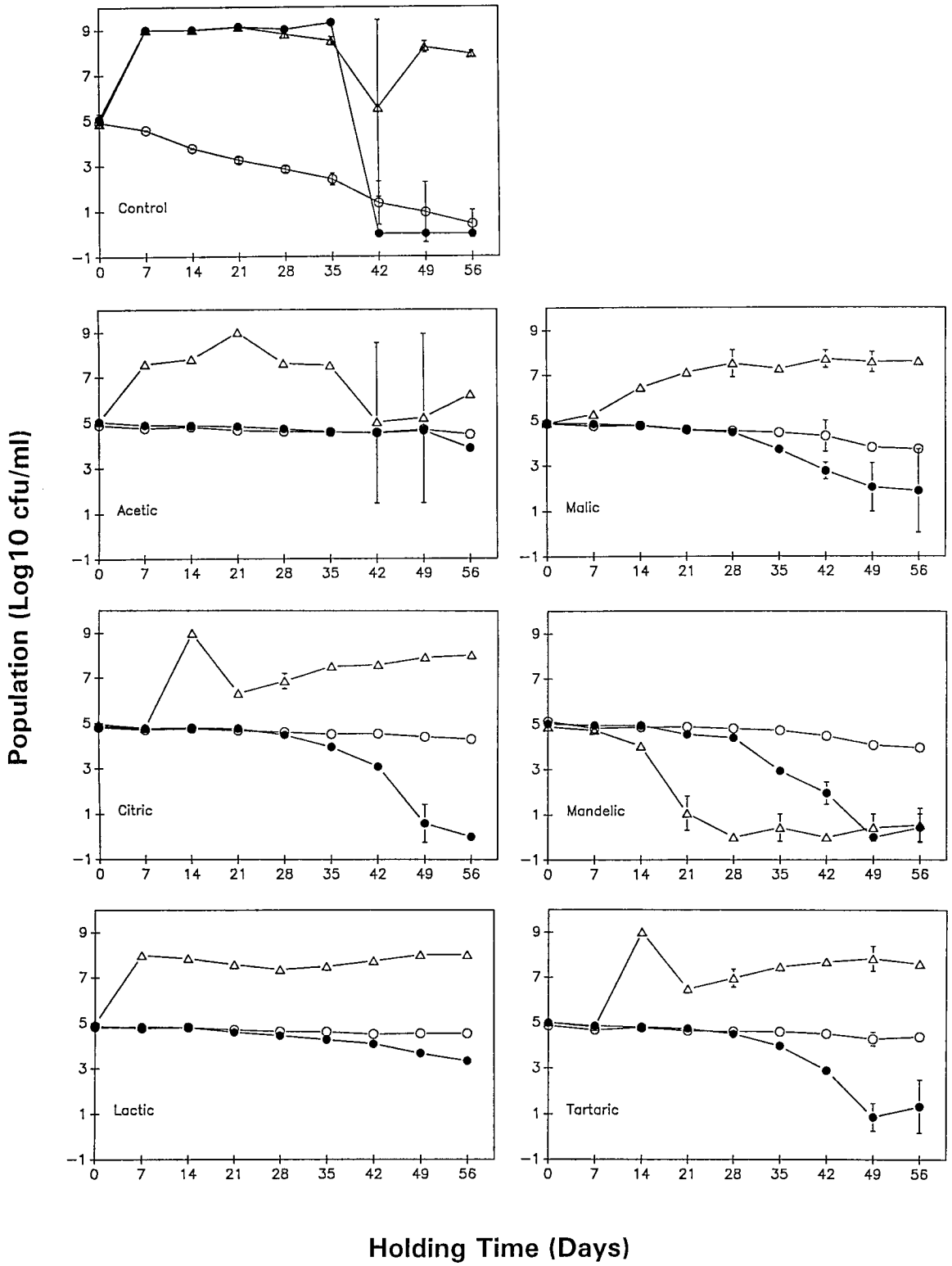

FIG. 1. Recovery of *E. coli* O157:H7 from TSBYE acidified with different organic acids and held at 4°C (○), 10°C (●), or 25°C (△). See Table 1 for pH values. Error bars = ±1 standard deviation; $n = 3$.

Because of differences in dissociation properties of the test acids, different amounts of each acid were required to achieve the tested pH values, which were not the same for each acid tested. Thus, the concentrations at the tested pH values were calculated and found to differ among the tested acids (Table 1). On this basis, tested concentrations of mandelic (0.025 M), acetic (0.027 M), and tartaric (0.027 M) acids were the lowest observed, while the concentration of malic acid (0.039 M) was

the highest. Because it is the undissociated acid molecule that accounts for an acid's antibacterial activity (3, 9), the concentration of the undissociated form of each test acid at the test pH (survival study) was determined. Lactic (0.001 M) and mandelic (0.001 M) acids had the lowest concentrations, while malic (0.008 M) and acetic (0.007 M) acids had the highest. Thus, these factors must be taken into account when antibacterial activities, against this bacterium or other food-borne pathogens, are compared (7).

Interestingly, the 25°C screening results did not completely correspond to results of the 25°C survival study. In the screening study, acidification of TSBYE to pH 4.0 with malic and citric acids prevented growth; however, an increase in population occurred at these pH values in the survival study. A higher inoculum level may have accounted for the difference. For the lactic acid treatment, the measured pH was 0.2 U higher than the target of 4.5, which did not allow growth of the pathogen. This difference permitted growth at 25°C.

Overall, mandelic acid appeared to have the greatest activity against *E. coli* O157:H7; unfortunately, this acid is not currently generally regarded as safe. Acetic and lactic acids, which have been tested as treatments for eliminating *E. coli* O157:H7 from beef surfaces (1, 5, 8, 15), showed little activity, although acetic acid was inhibitory at pH 4.5 to 5.0, but at a relatively high concentration. One of the more interesting observations was that the presence of organic acids in TSBYE stored at 4°C enhanced survival compared with the control. This apparent protective effect, as well as the previously observed stimulatory effect (6) of organic acids and salts, warrants more inquiry.

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