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Antimicrobial Effects of Weak Acids on the Survival of *Escherichia coli* O157:H7 under Anaerobic Conditions†‡

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ABSTRACT

Outbreaks of disease due to vegetative bacterial pathogens associated with acid foods (such as apple cider) have raised concerns about acidified vegetables and related products that have a similar pH (3.2 to 4.0). *Escherichia coli* O157:H7 and related strains of enterohemorrhagic *E. coli* (EHEC) have been identified as the most acid resistant vegetative pathogens in these products. Previous research has shown that the lack of dissolved oxygen in many hermetically sealed acid or acidified food products can enhance survival of EHEC compared with their survival under aerobic conditions. We compared the antimicrobial effects of several food acids (acetic, malic, lactic, fumaric, benzoic, and sorbic acids and sulfite) on a cocktail of EHEC strains under conditions representative of non–heat-processed acidified vegetables in hermetically sealed jars, holding the pH (3.2) and ionic strength (0.342) constant under anaerobic conditions. The overall antimicrobial effectiveness of weak acids used in this study was ranked, from most effective to least effective: sulfite > benzoic acid > sorbic acid > fumaric acid > L- and D-lactic acid > acetic acid > malic acid. These rankings were based on the estimated protonated concentrations required to achieve a 5-log reduction in EHEC after 24 h of incubation at 30°C. This study provides information that can be used to formulate safer acid and acidified food products and provides insights about the mode of action of weak acids against EHEC.

Foodborne *Escherichia coli* O157:H7 infections have been a significant food safety concern in the United States because of the serious symptoms that can arise *(29, 34, 47),* the number of disease outbreaks *(31),* and the wide spectrum of food vehicles involved *(36). E. coli* O157:H7 infections may result in hemolytic uremic syndrome *(2),* which is considered to be a leading cause of kidney failure in children in the United States *(40).*

Outbreaks of *E. coli* O157:H7 infection associated with apple cider (pH 3.5 to 4.0), yogurt (pH 3.9), and mayonnaise (pH 3.6) have raised concerns about the survival of this pathogen in acidic foods *(6, 33, 45).* Prolonged survival of this pathogen under acidic conditions below pH 4 has been documented *(30, 45).* Acidified vegetable products and acid foods such as fruit juices or fermented dairy products share a similar pH range (pH 3.2 to 4.0). Breidt et al. *(10)* found that *E. coli* O157:H7 was more resistant to the effects of acid than were *Listeria monocytogenes* and *Salmonella* under conditions typical of acidified vegetables. Currently, commercial producers of acidified foods file safety processes with U.S. Food and Drug Administration (FDA) that include a 5-log reduction step for *E. coli* O157:H7 *(11).*

Acid stress is a common environmental threat to bacteria, combining the antimicrobial effects of organic acids with the stress of a low pH environment. Organic acids are weak acids naturally found in fruits, vegetables, and fermented foods and have been used for decades as food additives for preservation *(17).* Acidic pH environments can alter intracellular metabolic activities in bacteria, including protein phosphorylation (cellular signals), flagellar synthesis and rotation, and nutrient transport *(32).* The diffusion of a protonated organic acid through cell membranes followed by intracellular dissociation of the acid can result in acidification of the cytoplasm and intracellular acid anion accumulation *(8),* with eventual disruption of pH homeostasis and cellular metabolism *(4, 19).*

Comparative studies of the relative effects of acid on *E. coli* O157:H7 are confounded by the variety of food types used and inconsistent environmental factors, such as pH, oxygen content, and ionic strength. To address this issue, Bjornsdottir et al. *(7)* compared the antimicrobial action of various organic acids on *E. coli* O157:H7 under conditions

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of constant pH, ionic strength, and temperature. This research was conducted under aerobic conditions and combined the effects of acid stress and oxidative stress, which enhances acid effects *(5, 26)*. Many acidic foods such as acidified vegetables, juices, yogurt, and mayonnaise-based sauces are packaged in hermetically sealed containers, resulting in an anaerobic acid environment. Our objective was to study and compare the antimicrobial effect of various organic acids on *E. coli* O157:H7 under anaerobic conditions representative of acidified vegetable products.

We investigated a variety of acids classified as generally recognized as safe (GRAS) for their ability to kill *E. coli* O157:H7, including acetic, lactic, malic, and fumaric acids. We also evaluated benzoic and sorbic acids and sulfite, which may be present in acid and acidified foods. Acids were compared in buffered solutions at pH 3.2, typical of non–heat-processed acidified foods. Currently, a 5-log reduction process is achieved in non–heat processed acidified foods by a holding time of 6 days at 10°C or 48 h at 25°C *(10)*. This holding time is defined only for products with acetic acid as the primary acidulent. Results from this study revealed that most GRAS acids are more effective for killing *E. coli* O157:H7 strains than acetic acid. These results may allow producers to reduce the holding times needed for a 5-log reduction of acid-resistant pathogens in acidified foods.

MATERIALS AND METHODS

Preparation of acid solutions. Various chemicals were used in the preparation of weak acid solutions: L-lactic acid, sodium D-lactate, acetic acid, L-malic acid, potassium sorbate, D-gluconic acid sodium salt, and disodium sulfite (Sigma-Aldrich Chemical, Co., St. Louis, MO), sodium fumarate (Alfa Aesar, Fair Lawn, NJ), and sodium benzoate (Fisher Scientific Co., Fair Lawn, NJ). Gluconic acid (20 mM) was used as a noninhibitory buffer in all acid solutions, as described by Breidt et al. *(9)*. The pH was adjusted to 3.2 with 3 N HCl using an AR25 Accumet pH meter (Fisher Scientific Co., Pittsburgh, PA). The concentrations of the weak acids in this study are given as protonated acid concentrations at pH 3.2, calculated using Matlab $_{pH}$Tools software (Dr. Daniel Dougherty, Michigan State University, East Lansing). The ionic strength of acid solutions was adjusted to 0.342 by adding NaCl as determined using $_{pH}$Tools.

Bacterial cultures and growth medium. Five *E. coli* O157:H7 strains were used in this study (Table 1). Stock cultures were stored at −80°C in Luria-Bertani broth (LB; BD, Franklin Lakes, NJ) supplemented with 1% glucose (Sigma) and 16% glycerol (Sigma). Each strain was grown statically at 37°C in 10 ml of LB broth supplemented with 1% glucose overnight (17 h) to induce acid resistance *(12, 18)*. Independent cultures were prepared for each of the five strains. Cells were harvested by centrifugation and washed with sterile saline solution (0.85% NaCl). The five strains were then combined and resuspended in 20 mM gluconic acid solution (pH 3.2). The five-strain suspension (0.2 ml) was then immediately inoculated into 1.8 ml of each organic acid solution prepared as described above, adjusted to a final pH of 3.2 and an ionic strength of 0.342 using NaCl. The initial inoculum was approximately 1×10^8 CFU/ml, as determined by serial dilution and spiral plating (model 4000, Spiral Biotech, Inc., Norwood, MA) of the inoculum on LB agar plates. Plates were incubated at 37°C for 24 h, and colonies were counted with an automated plate reader (Q-count, Spiral Biotech).

TABLE 1. Escherichia coli *O157:H7 strains used in this study*

Study ID no.	Other ID no.	Isolate source
B200	ATCC 43888	Human feces
B201	SRCC 1675	Apple cider outbreak
B202	SRCC 1486	Salami outbreak
B203	SRCC 206	Ground beef
B204	SRCC 1941	Pork

Acid challenge, dissolved oxygen, and redox potential measurements. Before inoculation, all the acid solutions were incubated in the anaerobic chamber (COY, Grass Lake MI) for 16 h at 23°C to ensure there was no dissolved oxygen present. A portable dissolved oxygen meter (standardized by adding 220 mV; model Oxi330i, WTW, Weilheim, Germany) and a conductivity meter (Conductivity TDS, Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan) were used to measure the oxygen level and redox potential change, respectively, in 3 ml of acid solutions held in disposable tubes (10 by 75 mm; BD). For acid killing experiments, the *E. coli* O157:H7 strain mixture (0.2 ml) was inoculated into each 1.8-ml organic acid solution and maintained at 30°C for 24 h in the anaerobic chamber. After the acid challenge, inoculated acid solutions were immediately neutralized by 1:10 dilution into 3-*N*-morpholino-propanesulfonic acid buffer (Sigma) with 0.85% NaCl at pH 7.0. Samples were then diluted and plated as described above, and LB agar plates were incubated in an anaerobic jar (Gaspak 100 system, BD) at 37°C overnight.

Statistical methods. Linear or quadratic regression models for killing curves were formulated by inspection of plots generated using SigmaPlot software (version 10, Systat Software, Inc., Chicago, IL). The concentration needed for a 5-log reduction in *E. coli* O157:H7 populations was estimated as a nonlinear function of the regression coefficients for each of these models as appropriate. Approximate standard errors for the estimated concentrations were obtained using asymptotic theory for nonlinear regression models *(39)* and the NLIN procedure of the SAS statistical software package (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Dissolved oxygen levels and redox potentials were determined for the solutions incubated in the anaerobic chamber (Fig. 1). There was no significant difference in the dissolved oxygen level among the control, fumaric, and sulfite solutions, although the redox potential for the sulfite solution was significantly higher than that of the gluconic acid control and fumaric acid solutions. After 16 h inside the chamber, the dissolved oxygen level reached approximately 0.15 mg/liter (near the limit of detection for the meter), and the redox potential reached the minimum values of −70.5 and −150 mV for sulfite and the other acid (gluconic acid control and fumaric acid) solutions, respectively. Both dissolved oxygen content and redox potential remained the same for up to 3 days (data not shown).

The log reduction in the *E. coli* O157:H7 populations was positively correlated with the protonated acid concentration for the organic acid treatments, with the exception of citric acid (Fig. 2). Citric acid was not included in Table 2

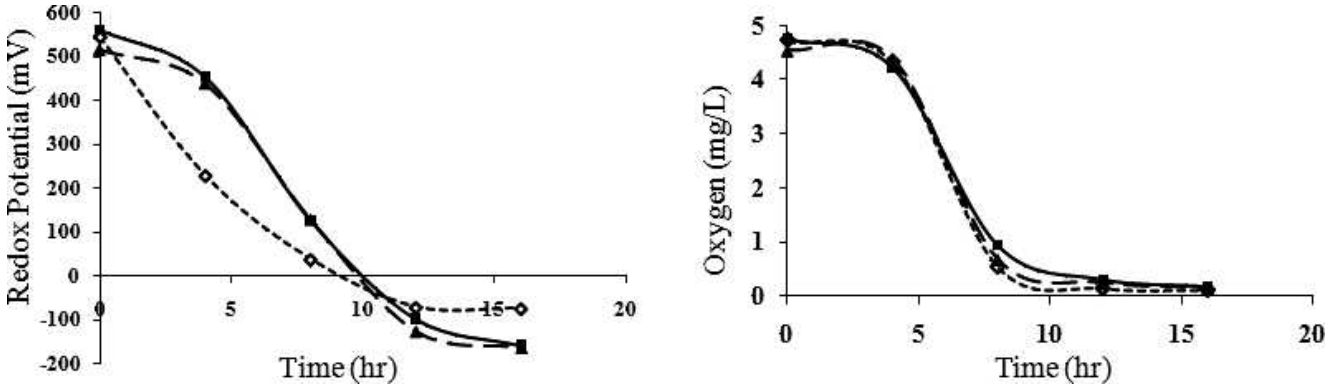

FIGURE 1. *Curves for redox potential (Eh, mV) (left) and dissolved oxygen change (right) of 3 ml of acid solution during the first 16 h inside the anaerobic chamber. Solutions included control (containing no organic acid; solid lines), 40 mM fumaric acid (long dash lines), and 3 mM sulfite (short dash lines) solutions. The pH and ionic strength were consistently maintained at 3.2 and 0.342, respectively, for all the acid solutions. Fumaric acid represented other organic acid solutions, which are significantly different from sulfite solution, based on the results of a pilot experiment. Each data point represents the means of three replicates.*

because with as citric acid concentrations increased up to 1 M there was no apparent trend of decreasing cell survival (data not shown). All the parameters for each mathematical model were significantly different from 0 ($P \leq 0.05$). The minimum protonated concentration (Table 2) of each acid required to achieve a 5-log reduction of *E. coli* O157:H7 was estimated by the mathematical models shown in Figure 2, with the corresponding total acid concentrations and standard errors. Although a 5-log reduction was not achieved with L-malic acid, higher malic acid concentrations were not used because the ionic strength would exceed 0.342. An ionic strength of 0.342 was used for our experiments because it is equivalent to 2% NaCl, which is typical of acidified vegetable products. With the exception of malic and citric acids, acetic acid was the least effective of the GRAS acids used in the study for achieving a 5-log reduction of *E. coli* O157:H7 (Fig. 3). These results indicate that supplementing or replacing acetic acid in acidified foods with other acids (such as fumaric acid) may accelerate acid killing of vegetative pathogens in acidified foods. Accelerated acid killing would be useful, allowing producers to reduce the holding times required for FDA-approved processes *(10)*. Further studies will be needed to determine the effects of combined acids.

Previous studies have been conducted on the effectiveness of GRAS organic acids for killing *E. coli* O157:H7. Hardin et al. *(22)* and Bjornsdottir et al. *(7)* found that lactic acid was more effective than acetic acid for killing *E. coli* O157:H7. The antimicrobial effect of fumaric acid on *E. coli* O157:H7 has been demonstrated in several studies *(14, 16, 24)*. A correlation between protonated fumaric acid and the log reduction of *E. coli* O157:H7 was observed in apple cider treated for 4 h at 25°C *(16)*. Synergistic effects of cinnamon and sorbate or benzoate in apple cider against *E. coli* O157:H7 also have been reported at 8 and 25°C *(13)*. A 5-log reduction treatment for *E. coli* O157:H7 in unpasteurized apple cider was achieved by combining benzoic and fumaric acids *(16)* or dimethyl dicarbonate and sulfite *(3)*.

The antimicrobial effects of the two lactic acid isomers (D-lactic acid and L-lactic acid) on *E. coli* have differed in previous studies. Bjornsdottir et al. *(7)* and Leitch and Stewart *(27)* found that L-lactic acid was significantly more effective than D-lactic acid for killing *E. coli* O157:H7. These studies were conducted with acid solutions that were prepared in an aerobic environment. In our study under anaerobic conditions, there was no significant difference in bactericidal effect between the L and D isomers of lactic acid

TABLE 2. *Estimated 5-log reduction concentrations of weak acids with corresponding Log P values*[a]

Acid	Protonated acid concn, mM (SE)	Total acid concn, mM (SE)	Log P[b]
Malic	547.00 (128.00)	1158.22 (271.03)	−1.26
Acetic	377.00 (21.00)	393.63 (21.93)	−0.017
D-Lactic	140.00 (7.00)	192.18 (9.61)	−0.062
L-Lactic	124.00 (3.00)	170.21 (4.12)	−0.62
Fumaric	24.11 (3.28)	94.51 (12.86)	0.274
Sorbic	6.59 (0.54)	6.89 (0.56)	1.41
Benzoic	6.47 (0.38)	7.57 (0.44)	1.87
Sulfite	1.27 (0.12)	54.34 (5.14)	

[a] Total acid concentration is the protonated acid plus acid anion concentrations. Estimated concentration for malic acid would have exceeded the solubility of the acid under the conditions used.

[b] Log P (the logarithm of the partition coefficient) values were determined as described by Leo et al. *(28)* and Wolkowski et al. *(46)*, with the exception of the values for fumaric acid and sorbic acid, which were determined by the methods of Hansch *(21)* and Stratford et al. *(42)*, respectively.

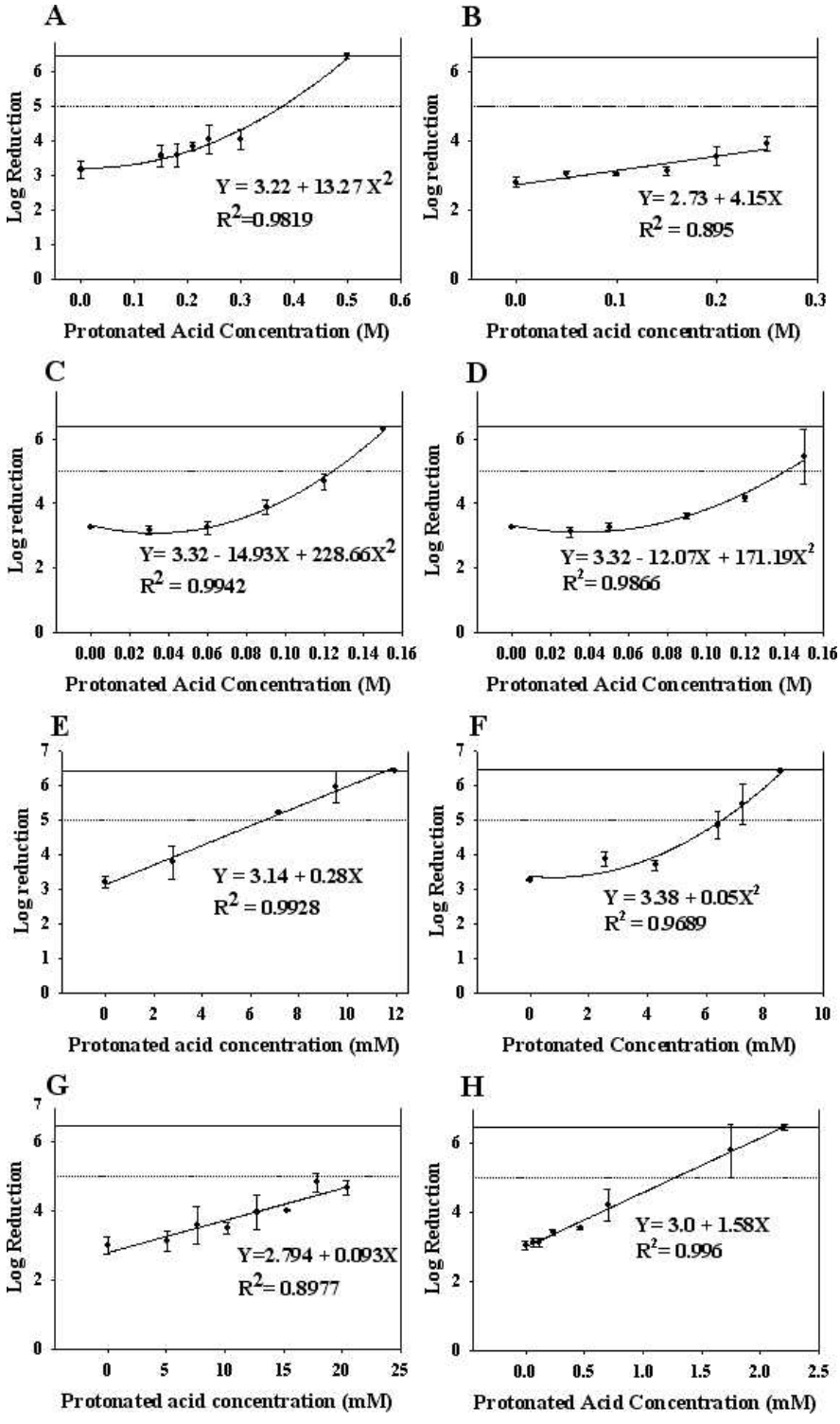

FIGURE 2. *Mathematical models for the effects of organic acids against* Escherichia coli *O157:H7. Log reductions in* E. coli *O157:H7 populations are shown after 24 h of treatment with acetic acid (A),* L*-malic acid (B),* L*-lactic acid (C),* D*-lactic acid (D), sorbic acid (E), benzoic acid (F), fumaric acid (G), and sulfite (H). Experiments were conducted at 30°C under anaerobic conditions. All of the solutions were consistently maintained at pH 3.2, with an ionic strength of 0.342 (equivalent to 2% salt). Each data point represents the means of three independent replicates, and error bars denote one standard deviation. The solid lines refer to a detection limit of 6.4 log units; the dotted lines indicate a 5-log reduction.*

($P = 0.2$). These results support the conclusions of Kreske et al. *(26)* that there is a synergistic effect of oxygen and lactic acid, leading to a differential response of *E. coli* to L- and D-lactic acid.

The classic weak acid theory attributes the antimicrobial effects of organic acids to the undissociated form, which is assumed to freely diffuse across the cell membrane causing cytoplasm acidification *(8)*. Therefore, the pK_a of the organic acid and the environmental pH determine the effectiveness of a weak acid. Our study was carried out anaerobically, eliminating the antimicrobial effects of dissolved oxygen. The amount of protonated acetic acid required to achieve a 5-log reduction was 370 mM higher than that of sorbic acid (Table 2), despite the similar pK_a of these two acids (approximately 4.75). Sorbic acid was previously identified as a more potent inhibitor of microbial growth than acetic acid *(38, 42)*. These observations suggest that the model of cytoplasmic acidification is not sufficient to explain the mode of action of weak acids. Although acid-specific effects are evident from previous studies, the LogP values, which are related to hydrophobicity and membrane permeability for organic acids *(21, 28, 42, 46)*, are

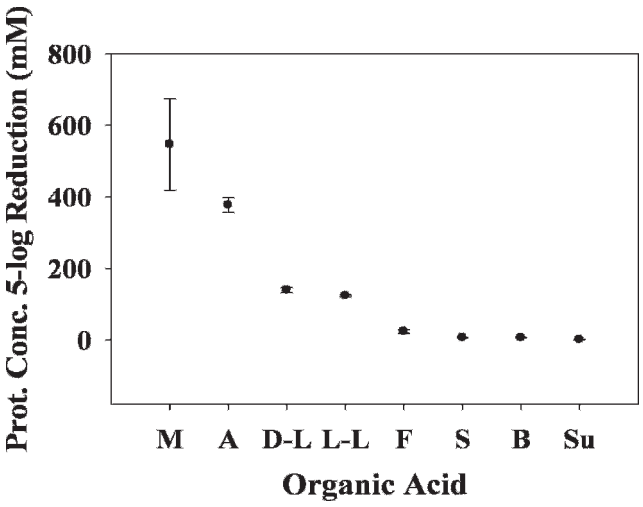

FIGURE 3. *Minimum concentrations of organic acids needed to achieve a 5-log reduction in* E. coli *O157:H7 populations. The error bars indicate the corresponding standard errors from the SAS program based on the mathematical model of each organic acid. M, malic acid; A, acetic acid; D-L, D-lactic acid; L-L, L-lactic acid; F, fumaric acid; S, sorbic acid; B, benzoic acid; Su, sulfite.*

negatively correlated with lethal effects for the acids shown in Table 2 ($r = -0.73$). Additional research will be needed to determine the significance of the relationship between molecular characteristics such as LogP and the killing kinetics for various organic acids.

To fully understand the antimicrobial performance of various weak acids, factors in addition to membrane permeability and cytoplasm acidification should be taken into consideration. A weak acid may have multiple targets in the cell, including the integrity of the cell membrane (outer and inner for gram-negative bacteria), biosynthesis of proteins and nucleic acids, and activity of critical enzymes and osmotic homeostasis *(4, 8, 19, 43)*. Gram-negative bacteria are typically less susceptible to weak acids because of their outer membrane, which serves as a barrier *(37)*. The ability of lactic acid to permeabilize the outer membrane of *E. coli* O157:H7 *(1)* may explain the greater effectiveness of lactic acid compared with acetic acid. Intracellular accumulation of acid anions has been implicated as a cause of stress in bacteria *(37, 43)*. Sulfite-mediated breakage of disulfide bonds can result in conformation changes in enzymes and metabolic disorder *(35)*. Sulfhydryl enzymes are very sensitive to sorbate *(41)*. Benzoate was suggested to attenuate anaerobic glycolysis, resulting in ATP depletion *(15, 25, 44)*. Intracellular effects of fumarate remain unknown. However, considering the role of fumarate as a critical metabolite in the tricarboxylic acid cycle and as an electron acceptor during anaerobic respiration in *E. coli (20, 23),* intracellular fumarate accumulation may alter cell metabolism. The capacity of cells to metabolize, detoxify, or export a specific chemical also may affect the resistance of cells to fumarate. Future work will include investigation of specific acid effects leading to the differences (two orders of magnitude) observed in the concentration of protonated acid needed for a 5-log reduction in *E. coli* O157:H7 populations under the conditions of this study.

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