

Influence of Freezing and Freezing plus Acidic Calcium Sulfate Addition on Thermal Inactivation of *Escherichia coli* O157:H7 in Ground Beef

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ABSTRACT

Undercooked ground beef is a leading vehicle for acquiring *Escherichia coli* O157:H7 infections through consumption of foods. Studies were done to determine the effect of freezing and the combined effect of freezing and addition of 20% acidic calcium sulfate and 10% lactic acid (ACS) on the thermal sensitivity of *E. coli* O157:H7 in ground beef. Five strains of *E. coli* O157:H7 were separately inoculated into ground beef and held at 5°C for up to 10 days or –20°C for up to 3 weeks, then heated at 57, 60, 62.8, 64.3 and 68.3°C to determine rates of thermal inactivation. Results revealed that D-values at equivalent temperatures of four of five *E. coli* O157:H7 strains were less in the previously frozen than refrigerated ground beef. Only one strain of *E. coli* O157:H7 was used to determine the effect of ACS in previously frozen or refrigerated ground beef on rates of thermal inactivation. The addition of ACS to ground beef at a ratio of 20 ml per kg increased the thermal sensitivity of *E. coli* O157:H7 in both previously frozen and refrigerated ground beef, with greatest rates of inactivation occurring in previously frozen ground beef containing ACS. Reducing the ACS concentration by half increased the sensitivity of *E. coli* O157:H7 in ground beef to heat treatment regardless of whether the beef was previously refrigerated or frozen. D-values at 57, 60, 62.8, and 64.3°C obtained for *E. coli* O157:H7 in frozen ground beef containing ACS were significantly ($P < 0.05$) less at equivalent heating temperatures than those obtained from previously frozen ground beef with no ACS added. Results revealed that the addition of ACS to ground beef, whether frozen or refrigerated can have a substantial effect on reducing the temperature or time required to kill *E. coli* O157:H7 during heating.

Escherichia coli O157:H7, first identified as a human pathogen in 1982, has emerged as a major cause of both sporadic cases and outbreaks of bloody diarrhea throughout much of the world

(13). Most outbreaks of *E. coli* O157:H7 infection are foodborne of which foods of bovine origin have been identified as the principal vehicle (13,19, 20). The low infectious dose and high degree of virulence of *E. coli* O157:H7 make this pathogen of particular concern (19).

The objectives of this study were: (1) to compare the rates of thermal inactivation in ground beef of several *E. coli* O157:H7 isolates obtained from ground beef at different periods of time to determine if unusual heat resistance occurs among these isolates; (2) to determine whether freezing increases the susceptibility of cells of *E. coli* O157:H7 to thermal inactivation; and (3) to evaluate the potential value of a mixture of acidic calcium sulfate and lactic acid (ACS) and freezing for increasing the thermal sensitivity of *E. coli* O157:H7 in ground beef.

MATERIALS AND METHODS

Bacteria used for evaluation. All *E. coli* O157:H7 strains were originally isolated from ground beef and included strain 401 (isolated March 1999), 419 (isolated May 1999), 431 (isolated January 2000), OH1395 (isolated May 1994) and E1993 (isolated March 1993). Two successive 24-h culture transfers were made for each isolate before the culture was used as an inoculum for each trial.

Bacteria were grown to the late stationary phase in 10 ml of brain heart infusion broth at 37°C for 18 h with agitation (100 rpm). Bacteria were sedimented by centrifugation at 4,000 x g for 20 min and washed in 0.1 M phosphate buffer, pH 7.2, with 0.85% NaCl (PBS) for three times by the same method. Sedimented bacteria were suspended in PBS and adjusted to an OD reading of 0.5 at 630 nm (ca. 10⁸ CFU/ml). Actual *E. coli* O157:H7 cell counts were confirmed by enumeration on tryptic soy agar after incubation at 37°C for 24 h.

Inoculation of ground beef. Treated ground beef was prepared for Trial 1 by adding 4.5 kg of ground beef containing 24% fat, 90.8 ml of 10% lactic acid, 20% acidic calcium sulfate (ACS,

Safe₂₀TM Brand Ground Beef Additive, Mionix Co., Roseville, CA) and for Trial 2 by reducing the concentration of ACS to 5% lactic acid and 10% acid calcium sulfate (half dosage), while mixing in a Hobart ribbon mixer (Hobart Corporation, Troy, Ohio) for 3.5 min at 5°C. Untreated ground beef was prepared under similar conditions but sterilized PBS was used instead of ACS. ACS was added during the final one minute of mixing. The ground beef was then ground through a 15-mm (5/8 inch) plate for three times for uniform distribution of the additive. Washed cells of *E. coli* O157:H7 at a ratio of 1 ml of 10⁸ CFU per 100 g of ground beef was added into ground beef and mixed by massaging the meat with gloved hands for 2 min under a laminar flow hood. After mixing, ca. 30-g portions of the inoculated ground beef preparation were added to 12 to 14 Whirl-Pak (120-g) bags and held at either 5°C or -20°C prior to use in thermal inactivation trials. ACS-treated and untreated ground beef held at 5°C were used within 10 days following inoculation, whereas ACS-treated and untreated ground beef held at -20°C were used within 3 weeks.

Thermal inactivation. One-gram portions of refrigerated treated or untreated (control) ground beef samples were weighed and lightly packed in a laminar hood into each of 24 Pyrex test tubes (10 x 75 mm) capped with rubber stoppers. Frozen ground beef samples were thawed at 21°C in a laminar flow hood for 20 to 30 min, then 1-g portions were lightly packed into test tubes as described above. Temperature was monitored by thermocouples placed in the center of several meat samples. All tubes were submerged in water in a circulating water bath (Model 1265PC, VWR Scientific, Cornelius, OR) pre-adjusted to the appropriate temperature (2°C greater than the desired temperature of the study). Once the ground beef reached the desired temperature (57, 60, 62.8, 64.3 or 68.3°C), two tubes were immediately removed and cooled in iced water (3°C). *E. coli* O157:H7 counts in these samples were the initial number reported at zero-time. Duplicate samples were taken at appropriate time intervals and enumerated for *E. coli* O157:H7. Duplicate tests were done for each

temperature treatment. Sampling times for refrigerated ground beef included: 0, 5, 10, 15 and 20 min at 57°C; 0, 2, 5, 10, and 15 min at 60°C; 0, 1, 3, 5, 7, and 9 min at 62.8°C; 0, 0.5, 0.75, 1, and 1.25 min at 64.3°C; and 0, 0.67, 1, 2, and 5 min at 68.3°C. Sampling times for frozen ground beef included: 0, 1, 3, 5, 10 and 15 min at 57°C; 0, 0.5, 1, 2, 5 and 10 min at 60°C; 0, 0.17, 0.33, 0.5, 1 and 1.5 min at 62.8°C; 0, 1.7, 0.33, 0.5, 0.67 and 1 min at 64.3°C; 0, 0.17, 0.33, 0.5, 0.67, 0.83 and 1 min at 68.3°C.

Enumeration of *E. coli* O157:H7. Following heat treatment and cooling, the ground beef was transferred to 9 ml of 0.1% peptone water. Using a sterilized inoculation loop, the sample was macerated and 1 ml was serially (1:10) diluted in 0.1% peptone to 10^{-3} . A 0.1-ml portion from each dilution was plated in duplicate on tryptic soy agar (Becton Dickinson, Sparks, MD) and Rainbow agar O157 plates (Biolog Inc., Hayward, CA). The plates were incubated at 37°C for 24 h and colonies were enumerated. Five colonies from TSA plates with the highest dilution having colonies were randomly selected and confirmed as O157 by *E. coli* O157 latex agglutination assay (Unipath, Oxoid Division, Ogdensburg, N.Y.). *E. coli* O157:H7 counts were determined based on the portion of O157-confirmed colonies from TSA plates at the appropriate dilution. Black colonies typical of *E. coli* O157:H7 on Rainbow agar plates with the highest dilution were randomly selected and confirmed as O157 by the *E. coli* O157 latex agglutination assay. Without exception, all black colonies on Rainbow agar were *E. coli* O157:H7; hence all black colonies on the medium were counted as *E. coli* O157:H7.

Statistical analysis. Least square (LS) means of D-values of *E. coli* O157 in ground beef patties at the different temperatures with different treatments were determined using the general linear model of the Statistical Analysis System Procedure (SAS Institute, Cary, NC).

RESULTS

Escherichia coli O157:H7 counts obtained on TSA were very similar to results obtained from Rainbow agar, with counts on TSA being slightly higher. Hence, *E. coli* O157:H7 counts obtained on TSA were used to determine D-values. D-values of the five ground beef strains of *E. coli* O157:H7 that were isolated from products of different ground beef processors at different periods of time (between 1993 and 2000) were approximately the same when determined in previously frozen patties, and were similar for 4 of the 5 strains when determined in previously refrigerated ground beef (Table 1). The exception was *E. coli* O157:H7 strain 401 that had considerably higher D-values at equivalent heating temperatures in previously refrigerated, but not previously frozen, ground beef.

Comparing D-values of *E. coli* O157:H7 in previously frozen ground beef to those of the pathogens in previously refrigerated ground beef revealed that freezing injures *E. coli* O157:H7 cells sensitizing them to subsequent thermal inactivation. However, statistical analysis did not reveal a significant difference ($P>0.05$). Four of 5 strains were consistently more sensitive to heat following freezing in beef, whereas one strain, 419, was equally sensitive or slightly more resistant to thermal inactivation when previously frozen in beef than in previously refrigerated meat.

The addition of ACS (10% lactic acid and 20% acidic calcium sulfate) reduced the pH of the ground beef from 6.0 to 5.0, whereas the addition of only 10% lactic acid did not noticeably change the pH of ground beef. Results of D-values of *E. coli* O157:H7 in ground beef with or without ACS and previously held at refrigeration temperature revealed that the addition of ACS consistently reduced the time required for thermal inactivation of the pathogen (Table 2). Similarly, the D-values of *E. coli* O157:H7 in ACS-treated frozen ground beef were considerably less than those determined for the control frozen ground beef not treated with ACS. Statistical analysis revealed inactivation of *E. coli* O157:H7 was significantly ($P<0.05$) greater in frozen ground beef treated with ACS than in

control (no ACS) frozen ground beef when heated at 57°C for 5 min, 60°C for 2 min and 62.8°C for 1 min.

When the ACS treatment of ground beef was reduced by one half, results revealed that *E. coli* O157:H7 was more rapidly inactivated at equivalent temperatures than occurred in the control ground beef (without treatment); however, not as rapidly as occurred when the higher concentration of ACS was used (Table 2). The D-values for *E. coli* O157:H7 in the one-half ACS concentration-treated ground beef were not statistically different ($P>0.05$) than those when the ground beef was not treated with ACS.

DISCUSSION

Depending on conditions, freezing ground beef may reduce *E. coli* O157:H7 cell numbers by 1 log₁₀ CFU/g or not substantially at all (2, 9, 17). Rapidly freezing *E. coli* O157:H7-inoculated ground beef patties at -20°C and holding them at -20°C for up to 12 months resulted in an approximate reduction of 1.0 log₁₀ CFU/g (2). Survival of individual *E. coli* O157:H7 strains did not differ significantly from each other or from an *E. coli* control strain (2).

Although results from some previous studies are conflicting, several studies have revealed that organic acids such as acetic, citric and lactic acid do not substantially reduce *E. coli* O157:H7 cell numbers in beef (1, 6), which may be explained by the exceptional acid tolerance of many strains of *E. coli* O157:H7 (5, 10, 12, 15). It has been determined that *E. coli* O157:H7 can tolerate acidic conditions in a variety of fermented and acidified meats such as during the processing of dry fermented sausage (10), in processed salami (7) and in acidified ground, roasted beef (1).

Bolton et al. (5) determined the potential value of individual and combined applications of some GRAS (generally recognized as safe) additives with freezing and pulsed-electric field (PEF)

treatments on reducing *E. coli* O157:H7 cell numbers in beef patties. Sequential application of 2% (v/v) lactic acid and freezing at -20°C for 2 h resulted in a decrease of approximately $6 \log_{10}$ CFU cm^{-1} in *E. coli* O157:H7, but only on filter paper. Their results indicated that currently available methods for controlling *E. coli* O157:H7 in beef burgers during production are ineffective and that freezing does not provide a significant intervention strategy to reduce the risks of human infection with *E. coli* O157:H7 transmitted in contaminated beef burgers.

Although freezing or organic acid treatments of ground beef by themselves may not eliminate *E. coli* O157:H7 from beef patties, prior exposure to these conditions could sensitize *E. coli* O157:H7 to subsequent bactericidal treatments such as heating (3, 4, 8, 11, 14, 17, 18). Our results revealed that freezing *E. coli* O157:H7 in ground beef substantially increased the rate at which most strains of the pathogen are killed during heating. Furthermore, the addition of ACS (10% lactic acid and 20% acidic calcium sulfate) to ground beef significantly increases the rate of thermal inactivation of *E. coli* O157:H7, with greater rates of inactivation occurring when the ground beef is previously frozen than refrigerated. Reducing the ACS concentration in ground beef by one-half reduced the sensitivity of *E. coli* O157:H7 to thermal inactivation irrespective of whether the ground beef was previously frozen or refrigerated; however, rates of thermal inactivation were still greater than those in control (no ACS) ground beef. The combined effect of freezing and ACS addition to ground beef provides an increased margin of safety to cooking hamburgers.

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Table 1: D and z values of *E. coli* O157:H7 strains in refrigerated or frozen ground beef

Strain	Refrigerated or frozen	D-value (min) ^a at:				z value (°C)
		60°C	62.8°C	64.3°C	68.3°C	
401	Refrigerated	11.3±0.8	6.3±0.1	2.5±0.0	NS ^b	10±0.3
401	Frozen	4.5±0.4	1.4±0.1	NS	NS	IS ^c
419	Refrigerated	3.6±0.2	1.8±0.2	0.4±0.0	NS	6.2±0.5
419	Frozen	3.6±0.2	3.0±0.4	1.5±0.1	NS	11.4±0.4
431	Refrigerated	4.3±0.3	3.6±0.5	IS	NS	IS
431	Frozen	4.5±0.5	1.3±0.0	NS	NS	IS
E1993 ^b	Refrigerated	4.5±0.1	1.5±0.0	IS	NS	IS
E1993 ^b	Frozen	2.5±0.1	0.5±0.2	IS	NS	IS
OH1395	Refrigerated	3.0±0.3	1.1±0.3	0.3±0.0	NS	7.3±0.3
OH1395	Frozen	2.1±0.1	0.3±0.1	0.2±0.1	NS	8.3±0.5

^a Average±S.D.

^b NS, no survivors; no detectable cells at zero time (initial cell counts were ca. 10⁷ CFU/g before heating), ca. 10⁵ *E. coli* O157:H7/g were inactivated during the come-up time.

^c IS, insufficient number of data points to calculate D-value or z-value.

Table 2: D and z values of *E. coli* O157:H7 strain OH1395 in previously refrigerated or frozen ground beef with and without acid calcium sulfate (ACS)

Refrigerated or frozen	D-value (min) ^a at:					z value (°C)
	57°C	60°C	62.8°C	64.3°C	68.3°C	
Refrigerated, no ACS	7.7±0.1	3.0±0.3	1.1±0.3	0.3±0.0	NS ^b	2.7±0.3
Refrigerated, with ACS	5.3±0.4	1.0±0.4	IS ^c	NS	NS	IS
Frozen, no ACS	5.7±0.3	2.1±0.1	0.3±0.1	0.2±0.1	NS	3.1±0.4
Frozen, with ACS	2.7±0.3	0.5±0.0	0.1±0.0	IS	NS	0.4±0.4
Refrigerated, no ACS	10±0.2	2.4±0.0	1.1±0.0	0.3±0.0		3.0±0.8
Refrigerated, with ACS (1/2)	5.1±0.4	2.1±0.2	IS	IS		IS
Frozen, no ACS	6.3±0.5	2.0±0.0	0.3±0.0	IS		1.0±0.1
Frozen, with ACS (1/2)	3.8±0.1	1.6±0.1	IS	NS		IS

^a Average±S.D.

^b NS, no survivors; no detectable cells at zero time (initial cell counts were ca. 10⁷ CFU/g before heating), ca. 10⁵ *E. coli* O157:H7/g were inactivated during the come-up time.

^c IS, insufficient number of data points to calculate D-value or z-value.

