Synergistic Effect of High Temperature and High pH on the Destruction of Salmonella enteritidis and Escherichia coli O157:H7

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ABSTRACT

This study was undertaken to determine if high temperature and high pH interact synergistically to enhance the rate of destruction of two important gram-negative foodborne pathogens, Escherichia coli O157:H7 and Salmonella enteritidis. The rates of destruction in NaHCO$_3$-NaOH buffers at pH 7.0, 10.0, and 11.0 were determined at 35, 40, 45, 50, 55, 60, and 65°C. Use of an improved heating protocol eliminated a "tailing effect" at longer exposure times. The present study demonstrated that the combination of high pH and high temperature resulted in a highly significant synergistic interaction ($P > F = 0.0001$), which caused rapid death of both E. coli O157:H7 and S. enteritidis. This "alka-therm" technology might be used commercially to destroy gram-negative foodborne pathogens on various raw agricultural commodities.

Key words: Salmonella enteritidis, E. coli O157:H7 high pH, high temperature, synergistic, pathogens

There are an estimated 33 million cases of foodborne illness in the United States each year (5), at an approximate cost of $8.4 billion annually (32). The majority of these cases result from the undercooking of raw animal products in food-service operations and in homes, which allows pathogenic bacteria to survive and cause illness when ingested. Compared to gram-positive pathogens, gram-negative intestinal pathogens, such as Salmonella spp., Campylobacter spp., pathogenic E. coli, Shigella spp., Vibrio spp., and Yersinia spp., are more frequently implicated in outbreaks of foodborne illness and cause an estimated 69% of all cases (2).

With the exception of food irradiation, which has a high initial capital expense, few if any processing technologies currently exist that significantly reduce or eliminate gram-negative pathogens on the surface of raw animal products. Lactic acid (1%) and hydrogen peroxide (0.5%) have yielded 4-log unit reductions of Salmonella typhimurium from pure cultures (29) and with inoculated broiler carcasses (29); however, such treatments were later shown to discolor the treated meat (21). Hot water immersion of whole turkey carcasses has also been studied; however, the appearance of the turkey carcasses was negatively affected by hot water treatments above 77°C (31). A similar study by Cox et al. (14) demonstrated that broiler carcasses subjected to a 60°C water treatment exhibited a partially cooked appearance.

Several studies have examined the effectiveness of high pH treatments on the destruction of gram-negative foodborne pathogens. Salmonella typhimurium in culture broth was shown to be eliminated in less than 1 min at pH 11.0 and 50°C (25). Humphrey et al. (20) found that S. typhimurium attached to chicken skin could be killed more rapidly in water at pH > 9.0 than pH 6.0. NaOH and KOH treatments (10%) have been found to be effective in lowering S. typhimurium by as much as 4 log units on lean and fat beef tissue (15). Catalano and Knabel (6) demonstrated that Salmonella spp. were rapidly destroyed in egg washwater maintained at a pH of 11.0 or higher, with D values determined to be less than 0.14 minutes when the temperature was increased to 43.3°C. Another study by Catalano and Knabel (7) determined that no cross-contamination or penetration of Salmonella enteritidis occurred when eggs were washed in pH 11.0 washwater at 37.7°C. Trisodium phosphate dip solutions (8 to 12%) have been used to reduce Salmonella spp., E. coli O157:H7, and Listeria monocytogenes on the surface of beef tissue (16, 23, 34). Coppen (12) indicated that these solutions were effective due to the removal of a layer of fat from the animal skin and their intrinsic high pH.

Elimination of gram-negative foodborne pathogens, including Salmonella spp., Campylobacter spp., and E. coli O157:H7 at the production level is not currently feasible. Therefore, an intervention step to substantially reduce or eliminate them during processing is desperately needed to ensure the safety of raw animal products. Mendonca et al. (27) recently demonstrated that destruction of gram-negative foodborne pathogens by high pH is due to the disruption of their cytoplasmic membranes. The rate of destruction increased dramatically with increasing pH and...
temperature (up to 45°C); however, this research did not
determine whether a combination of these two parameters
resulted in significant synergistic interaction that enhanced
the rate of destruction. The USDA’s Food Safety and
Inspection Service recently approved the use of hot water
(“... heated to any temperature provided sufficient safe-
guards exist”) as an acceptable antimicrobial treatment
during final washing of carcasses (36). Therefore, the
purpose of the present study was to determine if high
temperature interacts synergistically with high pH to rapidly
destroy gram-negative foodborne pathogens.

MATERIALS AND METHODS

Bacterial cultures

Escherichia coli O157:H7 was obtained from the E. coli
Reference Center (The Pennsylvania State University, University
Park, PA). Salmonella enteritidis ATCC 13076 was obtained from
the American Type Culture Collection (Rockville, MD). Overnight
cultures were grown on Trypticase soy agar supplemented with
0.6% yeast extract (TSAYE) and in Trypticase soy broth supple-
mented with 0.6% yeast extract (TSBYE) (BBL Microbiology
Systems, Cockeysville, MD, and Difco Laboratories, Detroit, MI).
The cultures were then incubated at 35°C for 18 h. Stock cultures
were maintained in TSBYE with 40% (vol/vol) glycerol at −40°C.

One milliliter of the overnight culture of either E. coli
O157:H7 or S. enteritidis ATCC 13076 was transferred into 100 ml
of fresh TSBYE and incubated for 18 h at 35°C. Cells were then
harvested by centrifugation in a Beckman J2-21 centrifuge (Beck-
man Instruments Inc., Palo Alto, CA) operating at 9,000 × g for 10
min at 4°C. The cell pellets were washed twice with 0.6% saline
solution and resuspended in 7.5 ml of 0.6% saline, resulting in a
bacterial cell suspension of approximately 1.8 × 10^10 CFU/ml.

Chemicals

Sodium chloride (NaCl), sodium bicarbonate (NaHCO3),
sodium hydroxide (NaOH), and hydrochloric acid (HCl) were
obtained from Sigma Chemical Company, St. Louis, MO.

Preparation of buffers

Stock solutions of 0.05 M (0.41%) NaHCO3 were prepared
in 0.6% saline and titrated with 10 N NaOH to buffer pHs of 10.0
and 11.0 at 25°C. A stock solution of 0.05 M NaH2CO3 was also
back-titrated with 12 N HCl to pH 7.0 at 25°C. All buffer solutions
were freshly prepared on the day of use and filter-sterilized (1-liter,
0.2-μm pore size Nalgene Filter Units; Nalge Co., Rochester, NY).
Each buffer (100 ml) was aseptically transferred into a sterile
250-ml Erlenmeyer flask which contained a magnetic stir bar.

Heating protocol

A flask containing the appropriate NaHCO3-NaOH buffer (pH
7.0, 10.0, or 11.0) was tempered in a water bath to the desired
temperature (35, 40, 45, 50, 55, 60, or 65°C) for 20 min prior to
inoculation. In order to monitor the temperature of the heating
menstruum, a mercury thermometer was inserted into an identical
uninoculated flask, which was placed into the water bath at the
same time as the treatment flask. To ensure complete and uniform
heating, the water level in the water bath was maintained at 5.0 cm
above the heating menstruum. When the menstruum reached the
desired temperature, 1 ml of the appropriate bacterial suspension
was added to provide a final cell concentration at time 0 of
approximately 1.8 × 10^10 CFU/ml. Cells were uniformly distributed
in the flask through the use of a magnetic stir bar, which was rotated
by a Nuova II stirrer set at position 5 (Thermolyne Corporation,
Dubuque, IA) placed beneath the water bath.

In initial experiments, the level of water in the water bath was
only 2.0 cm above the surface of the heating menstruum and hand
swirling of flasks was used to mix the cells. This initial heating
protocol resulted in a tailing effect, described in the results and
discussion sections.

Viable cell counts

Aliquots (1 ml) of treated cells were aseptically removed for
viable cell counts after the first 30 s (time 0, the fastest practical
sampling time) and after 1, 2, 3, 4, 5, 10, 15, and 20 min of stirring.
One milliliter of the treated cells was immediately placed into 9.0
ml of buffered peptone water (BPW) (Difco Laboratories, Detroit,
MI) at pH 7.0 and 25°C to neutralize the sample and thus prevent
further destruction due to high pH. One milliliter of this 10^-1
dilution was spread-plated onto the surfaces of 3 TSAYE plates;
therefore, the lowest limit of detection was 10 CFU per ml. Serial
dilutions were also performed in BPW (pH 7.0) and 0.1-ml aliquots
were spread-plated onto TSAYE. All plates were incubated at 37°C
and counted after 24 h of incubation. The above procedure was
performed twice for each pH-temperature combination for both S.
enteritidis and E. coli O157:H7.

Determination of lethal rates

The D value (minutes per log-unit reduction) was determined
from the best-fit line of the survivor curve (35). The lethal rate
(log-unit reductions per minute) is the reciprocal of the D value.

Statistical analysis

All experimental results were analyzed using the Statistical
Analysis System’s Procedures for the General Linear Model (SAS
Temperature and pH level were treated as factors (pHs at 7.0, 10.0,
and 11.0; temperatures at 35, 40, 45, 50, 55, and 60°C) and time as
a quantitative variable. The general linear model terms included in
the analysis were the main effects of pH and temperature; the
interactions between pH and temperature; the effects of time or
(time)^2 with the main effects of pH and temperature, and also with
the interactions of these two terms. This model was used to analyze
survival data for both E. coli O157:H7 and S. enteritidis.

RESULTS

Results from initial experiments showed a “tailing
effect” of surviving cells at later pull times (Fig. 1). After
various changes were made (see heating protocol in Materi-
als and Methods), the “tailing effect” was no longer
observed (Fig. 2 to 7).

There was little or no destruction of E. coli O157:H7 at
pH 7.0 at temperatures at or less than 45°C within the 20 min
of heating. However, there was a dramatic decrease in total
cell counts after 5 min of exposure to pH 7.0 at temperatures
of 55°C or higher. At 55°C and pH 7.0, the original cell
count of E. coli O157:H7 was reduced by 2.75 log units after
5 min of exposure, after which time viable cells could not be
detected (dotted lines) (Fig. 2). At pH 10.0, a 4.35-log-unit
decrease of E. coli O157:H7 was observed after 5 min of
exposure at 45°C (Fig. 3). At pH 11.0, there was a
2.75-log unit reduction of E. coli O157:H7 within 3 min at
35°C (Fig. 4).

As with E. coli O157:H7, there was no destruction of S.
FIGURE 1. Effect of low water bath level and hand swirling on tailing effect during exposure of E. coli O157:H7 to pH 10.0 at various temperatures. Symbols for all seven heating temperatures are superimposed at time 0.

FIGURE 2. Effect of temperature on the rate of destruction of E. coli O157:H7 at pH 7.0. Symbols for all seven heating temperatures are superimposed at time 0. The linear portion of each survivor curve was extended by a dotted line (-----) since no survivors were detected at the next sampling time on 10^-1 plates.
FIGURE 3. Effect of temperature on the rate of destruction of E. coli 0157:H7 at pH 10.0. Symbols for all seven heating temperatures are superimposed at time 0. The linear portion of each survivor curve was extended by a dotted line (-----) since no survivors were detected at the next sampling time on $10^{-1}$ plates.

FIGURE 4. Effect of temperature on the rate of destruction of E. coli 0157:H7 at pH 11.0. Symbols for all seven heating temperatures are superimposed at time 0. The linear portion of each survivor curve was extended by a dotted line (-----) since no survivors were detected at the next sampling time on $10^{-1}$ plates.
FIGURE 5. Effect of temperature on the rate of destruction of S. enteritidis at pH 7.0. Symbols for all seven heating temperatures are superimposed at time 0. The linear portion of each survivor curve was extended by a dotted line (-----) since no survivors were detected at the next sampling time on 10⁻¹ plates.

FIGURE 6. Effect of temperature on the rate of destruction of S. enteritidis at pH 10.0. Symbols for all seven heating temperatures are superimposed at time 0. The linear portion of each survivor curve was extended by a dotted line (-----) since no survivors were detected at the next sampling time on 10⁻¹ plates.
enteritidis at pH 7.0 and temperatures at or below 45°C. At pH 7.0 and 55°C, the level of S. enteritidis was reduced by 7 log units after 5 min of exposure time (Fig. 5). At pH 10.0, a 3.7-log-unit decrease of S. enteritidis was observed after 5 min of heating at 45°C (Fig. 6). At pH 11.0, a 4.5-log-unit reduction of S. enteritidis occurred after 3 min of exposure at 35°C (Fig. 7). In the case of both E. coli O157:H7 and S. enteritidis, at lower temperatures and/or lower pHs a "shoulder effect" was observed at early times of exposure (Fig. 1 to 7).

The lethal rates (1/D value) for both E. coli O157:H7 and S. enteritidis were calculated and plotted as three-dimensional bar-charts (Fig. 8 and 9). No destructive effects were observed at pH 7.0 at or below 50°C; however, the lethal rates for both organisms increased exponentially above this temperature. At pH 10.0, the lethal rates for both organisms began to increase exponentially above 35°C. At pH 11.0, the lethal rates for both organisms appeared to increase exponentially below 35°C. The lethal rates of >16 log units per min (bars with arrows) were based on total destruction (>8-log-unit decrease) at the fastest practical sampling time of 30 s (time 0).

FIGURE 7. Effect of temperature on the rate of destruction of S. enteritidis at pH 11.0. Symbols for all seven heating temperatures are superimposed at time 0. The linear portion of each survivor curve was extended by a dotted line (-----) since no survivors were detected at the next sampling time on 10⁻¹¹ plates.

FIGURE 8. Effect of pH and temperature on the rate of destruction of E. coli O157:H7. The lethal rates (log-unit reductions per min) were calculated from Fig. 2 to 4.

FIGURE 9. Effect of pH and temperature on the rate of destruction of S. enteritidis. The lethal rates (log-unit reductions per min) were calculated from Fig. 5 to 7.
The combination of high pH and high temperature resulted in a highly significant synergistic interaction \( (P > F = 0.0001) \), which caused rapid death of both \( E.\ coli \) O157:H7 and \( S.\ enteritidis \) (Fig. 2 to 9). Regression equations of the survival of \( E.\ coli \) O157:H7 and \( S.\ enteritidis \) were dependent on temperature and pH and were quadratic in time. As a result, separate regressions were run with log number of survivors as the dependent variable and time and \( (\text{time})^2 \) as independent variables for each combination of temperature and pH. Regression analysis revealed that all interaction terms were statistically significant for survival of \( E.\ coli \) O157:H7 \( (\alpha = 0.0001) \), while all interaction terms except \( (\text{time})^2 \) by pH were statistically significant for the survival of \( S.\ enteritidis \) \( (\alpha = 0.0001) \).

**DISCUSSION**

Little or no research has been conducted on the combined effect of high pH and high temperature on the destruction of gram-negative foodborne pathogens. Research by Mendonca et al. (27) indicated a possible interaction between high pH and moderate temperatures (up to 45°C). Since then, the USDA has approved the use of antimicrobial agents, including elevated temperatures, to decontaminate carcasses (36). The present study demonstrated that the combination of high pH and high temperature resulted in a highly significant synergistic interaction \( (P > F = 0.0001) \), which caused rapid cell death (Fig. 2 to 9).

The use of nonsubmerged vessels instead of sealed, completely submerged thermal-death-time tubes was necessary in the present study to permit rapid removal and pH neutralization of samples. The initial use of a nonsubmerged, low water level-handling system likely caused the "tailing effect" observed in Figure 1. This tailing effect was likely due to cells being splashed above the water level in the water bath, resulting in a less severe heat treatment for those cells. Donnelly et al. (17) reported similar results when heating vessels were not completely submerged. Our results (Fig. 2 to 7) demonstrate that the "tailing effect" can be removed by a more stringent nonsubmerged heating protocol, which ensures that all cells receive the same heat treatment. The "shoulder effect" observed in this study may be due to an initial requirement for cells to sustain sufficient injury before first-order logarithmic death kinetics occur (35).

Our results confirm and extend those of Anellis et al. (1), Cotterill (13), Kinner and Moats (25), and Mendonca et al. (27), who found that the destruction of gram-negative pathogens increased rapidly as pH increased. At pH 7.0 (Fig. 2 and 5), cell death is not due to a pH effect, but is the consequence of exposure to higher temperatures (greater than or equal to 55°C). The synergistic effect of high pH and elevated temperatures on the destruction of both organisms is clearly shown with pH 10.0 and 40°C (Fig. 3 and 6). When the pH was increased from 7.0 to 10.0, the temperature required for a 4.5-log-unit reduction of \( E.\ coli \) O157:H7 and a 6.0-log-unit reduction of \( S.\ enteritidis \) decreased from 55 to 40°C, respectively.

Gram-negative bacteria possess a very thin peptidoglycan layer (30), which may offer little protection against internal turgor pressure when the cytoplasmic membrane is weakened by high pH and/or high temperatures (27). The fluidity of the cytoplasmic membrane is largely a reflection of membrane lipid composition (3). Katsui et al. (22) suggested that the change in the fluidity of membrane lipids may be linked with the heat resistance of bacteria. The membrane would tend to be more fluid and possibly more susceptible to higher pH at higher temperatures. The weakening of the cytoplasmic membrane by high pH may be due to the solubilization of membrane proteins (18, 26) and/or saponification of membrane lipids (37). Bowler et al. (4) suggested that the central cause of cellular heat injury is the disruption of membrane lipoprotein complexes or enzymes associated with the integrity of the cell membrane. The synergistic effect of high temperature and high pH may be due to both of these conditions directly affecting the stability of the cytoplasmic membrane, which subsequently results in cell lysis and death (37).

A NaOH-NaHCO₃ buffer was utilized in this study because NaOH is an excellent alkalinizing and saponifying agent. These characteristics may enhance the disruption of bacterial membranes and the destruction of gram-negative organisms. Furthermore, both NaOH and NaHCO₃ are listed as GRAS (generally recognized as safe) food additives in the Code of Federal Regulations (8, 9). Also, unlike trisodium phosphate (12, 16, 19, 23, 34), these chemicals do not contain phosphates, which are potential environmental pollutants.

Commercial application of this "alka-therm" technology may help prevent cross-contamination by pathogenic microorganisms during scalding and defeathering of poultry carcasses and washing of beef carcasses. Many studies have shown that \( Salmonella \) spp. and \( Campylobacter \) spp. attached to poultry carcasses can survive after immersion in 52 to 60°C scald water (24, 28, 33). These pathogens can subsequently spread to noncontaminated carcasses during defeathering and chilling (10, 11). Our results, summarized in Figures 8 and 9, demonstrate that high pH causes a dramatic exponential increase in the rate of destruction of \( S.\ enteritidis \) and \( E.\ coli \) O157:H7 at lower temperatures. High pH may facilitate the use of lower temperature treatments to achieve rapid rates of destruction that were previously only attained using much higher temperatures at pH 7.0. For example, a pH 11.0 and 50°C treatment (Fig. 7) resulted in a rate of destruction of \( S.\ enteritidis \) similar to a pH 7.0 and 65°C treatment (Fig. 5). This observation is of practical importance since rinsing carcasses at temperatures higher than 60°C has been shown to cause undesirable changes in their appearance (14). High pH alone (pH 11.0) also did not cause total destruction of \( S.\ enteritidis \) and \( E.\ coli \) O157:H7 at or below 45°C (Fig. 8 and 9). However, in a study by Hollender et al. (19), there was no significant change in appearance of whole broilers after immersion in a 12% solution of TSP (pH 12.0 to 12.5). By utilizing a combination of high pH and high temperature it may be possible to significantly reduce or eliminate gram-negative pathogens.
on carcasses without causing negative organoleptic side effects.

Pathogens attached to carcasses are much more resistant to either high pH (16) or high temperature (14). The use of a combination of high pH and high temperature may permit reduction or elimination of pathogens attached to carcasses. Research is currently being conducted in our laboratory to determine which pH-temperature combinations will rapidly destroy gram-negative pathogens on beef and poultry carcasses without affecting product quality. This “alka-therm” technology may eventually be applied to the destruction of gram-negative pathogens and spoilage organisms on all raw agricultural commodities.

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