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Combined effects of heat, acetic acid, and salt for inactivating *Escherichia coli* O157:H7 in laboratory media

Sun-Young Lee [c], Dong-Hyun Kang [a,b,*]

[a] Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99164-6376, USA
[b] Department of Food and Animal Biotechnology, Seoul National University, San 56-1, Sillim-dong, Gwanak-gu, Seoul 151-742, Korea
[c] Department of Food and Nutrition, Chung-Ang University, 72-1 Nae-ri, Daedeok-myeon, Anseong-si, Gyeonggi-do 456-756, South Korea

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ABSTRACT

Heat, acid, and salt have been commonly used to ensure the microbial safety of foods and are often used in combinations in many food products. When combined, they can produce different results, such as additive, synergistic, and antagonistic effects. However, there has been little investigation into the effect of these combination treatments. Therefore, in this study, the effect of combined treatment of heat, acid, and salt was investigated in laboratory media. All possible paired combinations among three factors, heat (55 °C), acid (0.25% acetic acid, [v/v]) and salt (3%, [w/v]) were tested and compared with individual treatments for killing *E. coli* O157:H7 in laboratory media. When salt was combined with heat, there was no significant difference in reduction of *E. coli* O157:H7 (additive effect). However, when acid was combined with heat, there was a higher reduction of *E. coli* O157:H7 (synergistic effect). When salt was combined with acid treatment, salt gave protection against acid treatment (antagonistic effect), thus, there was lower reduction of *E. coli* O157:H7 in the combined treatment than in the single acid treatment. Depending on the combination of preserving factors, results were different.

1. Introduction

Escherichia coli O157:H7 is a member of the enterohemorrhagic group of pathogenic *E. coli* that has emerged as a foodborne and waterborne pathogen of major public health concern. A wide variety of foods have been implicated as vehicles of *E. coli* O157:H7 infection, including meat, milk, fruit juices, and vegetables (Buchanan & Doyle, 1997). Unlike most foodborne pathogens, *E. coli* O157:H7 is tolerant of acidic environments. Survival in apple cider (pH 3.6–4.0) and mayonnaise (pH 3.6–3.9) has been reported and *E. coli* O157:H7 survived fermentation of buttermilk (pH 4.4) and drying and storage of fermented sausage (pH 4.5) (Buchanan & Doyle, 1997). These organisms cause a spectrum of disease increasing in severity from a mild diarrheal illness to hemorrhagic colitis, hemolytic uremic syndrome, and, in some cases, death (Bolton & Aird, 1998).

Several years ago, hurdle technology was developed as a new concept for the realization of safe, stable, nutritious, tasty, and economical foods. This approach uses a combination of suboptimal growth factors, e.g. heating, chilling, drying, salting, conserving, acidification, oxygen-removal, fermenting, adding various preservatives, to establish growth inhibition of microorganisms in foods (McMeekin et al., 2000). The essence of this approach is that foods can remain stable and safe even without refrigeration, and are acceptable organoleptically and nutritionally due to the mild processes applied (Leistner, 1978). Consumers demand fresher and more natural products. This prompts food manufacturers to use milder preservation techniques and could be stimulating the current trend to hurdle technology. The mode of action of combined hurdles may be additive or even synergistic with the latter deserving particular attention as a means to select constraints that best achieve microbial stability and safety (Leistner, 1992). That synergism is anticipated derives from the effect of hurdles on separate targets within the cell which disturb homeostasis by different mechanisms (Leistner, 2000).

Hurdle technology is employed because we expect that a combination of two or more factors is more inhibitory effect than any one agent alone. However, recently, some studies showed that combination treatments were less effective at reducing levels of microorganism than were single treatments alone (Casey & Condon, 2002) therefore application of the hurdle concept for preservation of food may inhibit outgrowth but induce prolonged survival of *E. coli* O157:H7 in foods (Uyttendaele, Taverniers, & Debevere, 2001). The various responses of microorganisms under stress might hamper food preservation and could turn out to be problematic for the application of hurdle technology. Heating, acidification, and adding salt is widely used in the food industry, and are commonly used in combination for food preservation. For an example, acidified pickled vegetables are made by

* Corresponding author. Address: Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99164-6376, USA. Tel.: +1 509 335 3937; fax: +1 509 335 4815.
E-mail address: dhkang@wau.edu (D.-H. Kang).

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immersing raw vegetables in brine containing vinegar (acetic acid) and salt, and then heat-treated. However, the relationships of these hurdles on the survival of pathogens are less clear. Therefore, in this study, the effect of combined treatment of heat, acid, and salt, which are major inhibitory factors used in many types of foods including pickled vegetables, was investigated in laboratory media.

2. Materials and methods

2.1. Bacterial cultures

Five strains of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890, ATCC 43894, and ATCC 43895) were obtained from the Food Science and Human Nutrition Culture Collection at Washington State University (Pullman, WA, USA). All cultures were maintained on tryptic soy agar (TSA; Difco laboratories, Detroit, MI, USA) slants and subcultured monthly.

2.2. Cell suspension

Each strain of *E. coli* O157:H7 was cultured separately in Tryptic soy broth (TSB; Difco) at 37 °C for 22 h, harvested by centrifugation at 9000*g* for 5 min at 4 °C, and washed twice with buffered peptone water (Difco). The final pellet was resuspended in buffered peptone water to a concentration calculated to yield approximately 10^{10} CFU/ml. Then, each of the five strains was used as an inoculum.

2.3. Heat treatment with or without combination of acid or salt

In this study, individual heat treatment (55 °C) was compared with heat treatment combined with 3% (w/v) sodium chloride or 0.25% (v/v) acetic acid. Luria–Bertani (LB) broth containing 1% (w/v) tryptone (Difco) and 0.5% (w/v) yeast extract (Difco) without salt and with 3% salt or with 0.25% acetic acid was prepared. Each cell suspension was 100-fold diluted into 2 ml of the three different media and subjected to heat treatment. For heat treatment, inoculated tubes were completely immersed in a water (55 °C) using water bath. At selected time intervals (0, 5, 10, 20, and 30 min), tubes were removed, cooled in ice water and used for the enumeration of survival bacteria as describe below.

2.4. Acid treatment with or without combination of salt

Two milliliters of LB broth without salt and with 3% salt was prepared. Acetic acid (0.25%) was incorporated into both LB and LB containing 3% salt using 25% acetic acid solution. Each cell suspension was added to the media as described previously and stored at room temperature (22 °C). At selected time intervals (0, 1, 5, and 7 days), samples were enumerated as described below.

2.5. Bacterial enumeration

Treated samples were serially 10-fold diluted with 9 ml sterile buffered peptone water. Diluted samples were spread-plated onto TSA as a non-selective agar and Sorbitol MacConkey agar (SMAC; Difco) as a selective agar for *E. coli* O157:H7 and incubated at 37 °C for 24 h before counting. The difference of numbers between TSA and SMAC were used to calculate the level of sublethally injured *E. coli* O157:H7.

2.6. Statistical analysis

All experiments were performed in duplicate and repeated three times. Before analysis, the average of duplicate plate counts from three replications was converted to $\log_{10}$ CFU/ml for analysis of variance. To determine the slope of the regression line and corresponding standard error, the LINEST function from Excel (Microsoft Office XP, Microsoft, Redmond, WA, USA) was used. In simple linear regression, the least squares and maximum likelihood estimate of the slope are the same. To determine the upper 95% confidence limit for *D*-values (decimal reduction time) at 55 °C or at pH 4.2 (0.25% acetic acid), the negative inverse of confidence limits for the slope was used. Statistical inferences concerning the similarity of *D*-values were determined with the general linear models (GLM) procedure of SAS (Version 8.1, SAS Institute, Cary, NC) for a completely randomized design. When the effect is significant ($P < 0.05$), mean separation was accomplished with the probability option (PDIFF, a pairwise *t* test).

3. Results and discussion

In this study, the combined effect of each of two factors of heat, acid, and salt for reducing *E. coli* O157:H7 were investigated in laboratory media. LB broth containing 1% tryptone and 0.5% yeast extract was used as a control (none of treatment), and for acid and salt treatment, 0.25% acetic acid and 3% salt were added into LB broth. When 0.25% acetic acid was added into LB, pH was 4.2. Adding 3% salt decreased pH from 7.0 and 4.2 to 6.8 and 3.9 for LB and LB containing 0.25% acetic acid, respectively (data not shown). Further decrease of pH when 3% salt was added could be due to a change of ionic strength of solution. From the Debye–Hückel limiting law, lower pH values were calculated in acetic acid solution when the ionic strength of solution was increased by adding potassium chloride compared to solution without potassium chloride (Blackburn, 1969).

Each paired combination among three factors (heat, acid, and salt) was compared with heat treatment alone or acid treatment alone. For combination of heat and salt or heat and acid, it was observed that 0.25% acetic acid and 3% salt did not affect the levels of *E. coli* O157:H7 during heat treatment (up to 30 min) at 55 °C; heating was the major factor that reduced levels of *E. coli* O157:H7 inoculated into broth. Therefore, the result of these two combinations ('combination treatment') was compared with that of heat treatment alone ('single treatment'). For the same reason, since 3% salt did not affect levels of *E. coli* O157 during acid treatment time, the combination of acid and salt was compared with acid treatment alone.

Fig. 1 shows the effect of combined heat and salt combination and heat alone on killing five different strains of *E. coli* O157:H7 in laboratory media. Initial levels of all five strains of *E. coli* O157:H7 in laboratory broth before treatments were approximately 10^8 CFU/ml. There were significant decreases in the levels of *E. coli* O157:H7 with each increase in treatment time when they were treated with both heat alone and heat combined with salt and enumerated on both TSA and SMAC ($P < 0.05$). However, for some strains, somewhat greater reductions were observed when they were treated with combination of heat and salt and enumerated on SMAC than when they treated with heat alone. D-values at 55 °C were in the range of 7.4–9.6 min and 4.8–6.5 min when five strains of *E. coli* O157:H7 were treated with heat alone and enumerated on TSA and SMAC, respectively, and *D*-values were in the range of 6.7–11.1 min and 3.7–5.5 min when they were treated with a combination of heat and salt. There was no significant difference in the results among the five different strains ($P > 0.05$). The method of enumerating survivors of heat inactivation experiments can affect the percent recovery of heat-injured cells and hence has a bearing on the calculated heat resistance of the organism. Enumeration on TSA led to *D*-values up to twice as great as