PEER-REVIEWED ARTICLE

Food Protection Trends, Vol 38, No. 5, p. 322–328

6200 Aurora Ave., Suite 200W, Des Moines, IA 50322-2864

Fred Breidt, Jr.,[1] Elizabeth L. Andress[2] and Barbara Ingham[3*]

[1]USDA/ARS, 322 Schaub Hall, Box 7624, North Carolina State University, Raleigh, NC 27695, USA
[2]208 Hoke Smith Annex, 300 Carlton St., University of Georgia, Athens, GA 30602, USA
[3*]1605 Linden Drive, 113B Babcock Hall, University of Wisconsin-Madison, Madison, WI 53706, USA

Recommendations for Designing and Conducting Cold-fill Hold Challenge Studies for Acidified Food Products

ABSTRACT

A scheduled process for an acidified food must be validated with existing data or a product-specific challenge study. We outline recommendations for designing, conducting and interpreting cold-fill-hold challenge studies for acidified foods and discuss information that should be included in reporting challenge study results to the U.S. Food and Drug Administration. Cold-fill challenge studies for acidified foods, designed and evaluated by expert food microbiologists, must take into account variability in ingredients, packaging, preservatives, and native microbiota. The studies should clearly document a 5-log pathogen reduction over four or more sampling times from at least two independent replicate trials, with pH measured at each sampling point. Inoculation is generally done in bulk and should account for no more than 1% of the product volume. The inoculum is most often a cocktail of at least three strains grown statically in broth containing 1% glucose to induce acid resistance. The pH challenged becomes the maximum pH for the scheduled process; the temperature becomes the lower limit for the hold time. Data analysis should consider the expected non-linearity of survival curves. These recommendations will help ensure that design, implementation, and interpretation of challenge studies for cold-fill-hold acidified foods meet scientific standards and adequately support product safety.

INTRODUCTION

Several factors must be considered when conducting microbial challenge studies for acid and acidified shelf-stable foods in hermetically sealed containers. According to the United States Food and Drug Administration (U.S. FDA), acid foods are those with a natural or normal pH below 4.6. Acidified foods are low-acid foods to which acid(s) or acid food(s) are added and which have a final water activity greater than 0.85 and a finished equilibrium pH of 4.6 or below *(21)*. Destruction of pathogens of public health concern in acidified foods is usually achieved by a thermal process *(5, 6)*. However, for sensory reasons, manufacturers of some shelf-stable canned foods may wish to forego thermal processing. Research in a cucumber-brine model system has outlined cold-fill-hold conditions that

*Author for correspondence: Phone: +1 608.263.7383, Fax: +1 608.262.6872; E-mail: bhingham@wisc.edu

would ensure a target 5-log pathogen reduction in acidified foods with either an equilibrium pH of 3.3 or below and acetic acid as the primary acidulant *(3)*, or equilibrium pH 3.5 or 3.8 that are formulated with specific levels of acetic and benzoic acid *(4)*. Manufacturers of acidified foods may use these validated minimum cold-fill-hold targets for appropriately formulated products. In the absence of additional published studies, manufacturers who cannot meet these validated formulation requirements must undertake product-specific challenge studies to ensure product safety and establish shelf stability.

A product-specific challenge study would evaluate the ability of a particular formulation, a manufacturing practice, or their combination to achieve at least a 5-log reduction of target pathogens *(18)*. These studies must also account for food storage and packaging conditions that may affect pathogen survival. Challenge studies must be designed and evaluated by an expert food microbiologist *(18)* and are often carried out under the direction of these experts. University faculty, licensed food testing laboratories, or qualified independent consultants may have the expertise needed to design and evaluate challenge studies. The goal of this document is to provide recommendations for designing, conducting and interpreting cold-fill-hold challenge studies for acidified food products and to discuss information that should be included in reporting challenge study results to the U.S. FDA. These recommendations were developed specifically for acidified food manufacturers based on the National Advisory Committee on Microbial Criteria for Food's report *Parameters for Determining Inoculated Pack/ Challenge Study Protocols (18)* and informed by current science and industry and regulatory practice. A cold-fill-hold challenge study for an acidified food that is designed, carried out and interpreted following these recommendations should meet regulatory standards for supporting process documentation. The recommendations outlined herein may also be useful in developing studies to support processing of other foods, e.g., acid foods.

A challenge study should address each of the following factors:

1. Food product preparation

Manufacturing. The product should be prepared and held under conditions most conducive to microbial growth or survival, i.e., represent a 'worst case scenario.' Product should be prepared using realistic manufacturing standards, with variability in formulation accounted for, and with the presence of native microbiota taken into consideration. If the risk to human health is assumed to be survival of pathogens in a formulated product, inoculation should occur prior to equilibration and in regions of the product that are considered the most permissive to growth, provided these are areas reasonably likely to be contaminated. If, however, the risk is assumed to be recontamination, inoculation would occur after equilibration *(18)*.

Knowledge of product variability is needed to determine the appropriate test parameters for a challenge study *(18)*. It is important to understand the degree of variability expected within and among production lots so as to determine the appropriate number of samples to be evaluated or the number of experimental trials to be conducted.

Food product used in a challenge should be obtained from a commercial production facility or manufactured in a pilot-scale production facility *(18)*. Production steps such as cooking time and temperature (prior to filling), homogenization, and product slicing or dicing must be replicated in the test batches. Food that is being challenged must be representative of normal production with the exception of necessary adjustments to factors such as pH or temperature, to yield conditions most conducive to pathogen growth or survival at each formulation control limit *(18)*.

Formulation. According to the National Advisory Committee on Microbial Criteria for Food's report *Parameters for Determining Inoculated Pack/Challenge Study Protocols,* "When pH is one of the controlling factors, the food should be prepared with the lowest amount of acid allowed in the formulation so that the pH is at the upper range and adjustment in the laboratory is not necessary" *(18)*. The pH that is evaluated during a challenge study on an acidified food, therefore, becomes the upper limit when the product is subsequently manufactured. If pH adjustment is necessary, the titratable acidity or organic acid concentration should be measured before and after adjustment to assure the acid concentration remains within the range typical of the product.

All formulations must be prepared using Good Manufacturing Practices *(22)*, which includes listing ingredients by prepared weight. The type of acidulant and concentration must be clearly defined. Acidulants exert different degrees of antimicrobial activity at the same pH, and antimicrobial activity is primarily attributed to the protonated form of the acid *(19)*. Lu et al. *(17)* noted that the overall antimicrobial effectiveness of weak acids against a cocktail of *Escherichia coli* O157:H7 at pH 3.2 was sulfite > benzoic acid > sorbic acid > fumaric acid > L- and D- lactic acid > acetic acid > malic acid at 30°C, based on protonated acid effects. Citric acid solutions were found to have little or no acid-specific antimicrobial effect, only pH effects. Ahamad and Marth found that, based on total acid concentration and holding at 7–35°C, acetic acid was most detrimental to *Listeria monocytogenes*, followed in order by lactic and citric acids *(1)*. Target limits would establish both the maximum pH and the primary acidulant for pH control.

The presence of any legally allowed preservatives such as benzoate, sorbate, or sulfite should be carefully controlled and accounted for. Preservatives may have significant effects on pathogen survival. Benzoate has been shown to decrease by ten-fold the hold times needed to achieve a 5 log reduction of *Escherichia coli* O157:H7 in acetic acid solutions

(4). The process authority must establish the efficacy of any preservatives in the formulation. Any product imported into the United States must comply with all U.S. laws, including those regulations related to food additives *(23)*.

The presence of natural antimicrobial compounds such as sulfurous compounds in allium species should be considered only when these chemicals are consistently present and their levels can be documented in the initial and final product. The type and concentration of other components such as salt and sucrose should be standardized in the formulation. In a study of the time needed to achieve a 3-log reduction of *E. coli* O157 in 81 acidic formulations, Chapman et al. *(9)* found that in formulations containing 10% sucrose, O157 was relatively protected by 3% (wt/wt) NaCl, compared with formulations containing 1% NaCl. Subsequent research with additional *E. coli* O157 and *Salmonella enterica* strains showed that at pH 4.0 (acetic acid), up to 4% NaCl was protective, while at pH 3.8 up to 2% NaCl delayed the onset of inactivation *(8)*. Bae and Lee *(2)* found that the addition of 3% salt to an acid brine increased the resistance of *E. coli* O157:H7 and *Salmonella* to acid treatments using acetic, propionic and lactic acid, but not to treatments using malic, tartaric, citric, or phosphoric acid.

More than one batch of product must be challenged, and three independent batches is preferable. Where variation in ingredients is anticipated, e.g., variety and maturity of tomatoes or variation due to seasonality or geographic location, the independent replicates must be prepared from independent lots of ingredients. Independent replication is particularly important if ingredients have natural antimicrobial compounds. The number of independent batches that must be challenged increases as anticipated variation increases.

*Native microbiota***.** Microbiota naturally present on raw materials can affect the outcome of a cold-fill-hold challenge study in which a non-commercially sterile product is inoculated. Research has shown that the presence of native microbiota can enhance survival of pathogens on raw agricultural products *(10, 15)*. Care should be taken not to introduce atypical spoilage microorganisms into the product during the inoculation step. The inoculated product should contain typical levels of native microbiota *(18)*. Any cold-fill hold-challenge study must include an un-inoculated control that is sampled on the same schedule as the inoculated product to evaluate the potential for growth/survival of indigenous microflora.

*Packaging***.** The packaging and atmosphere within the container should reflect manufacturing and handling practice. Many acid and acidified foods are sold in hermetically sealed containers with oxygen limiting conditions. Kreske and others demonstrated that *E. coli* O157:H7 survived better in acid solutions under oxygen-limiting conditions, compared to acid solutions containing dissolved oxygen *(Fig. 1)* *(16)*. Care should be taken to ensure that headspace volume and gas composition of the challenge study samples mimics the commercial food products as closely as possible *(18)*.

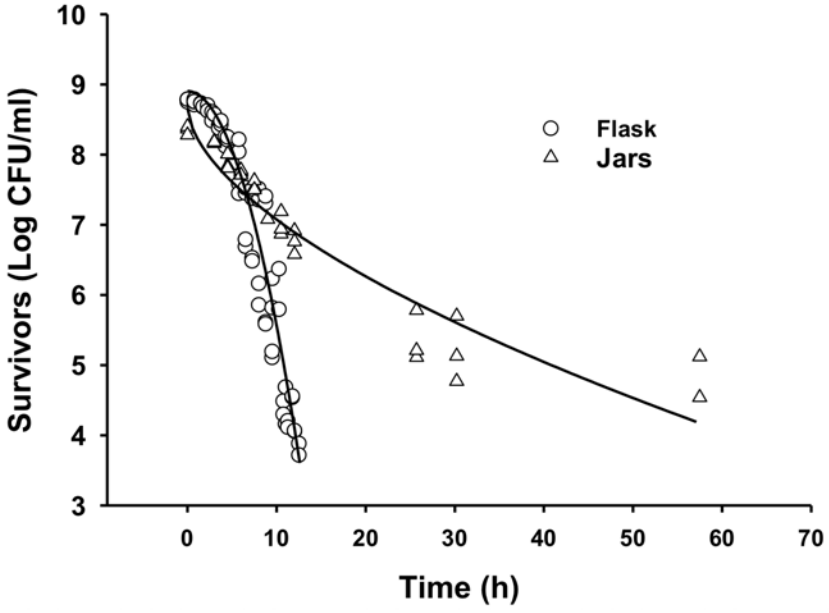

FIGURE 1. Survival of E. coli *O157:H7 in acidified pickle brines. Bacteria cell counts were obtained from sealed jars through rubber septa (triangles) or in brine removed from jars and exposed to air (circles). The lines represent the Weibull models of each data point with three replications (16). Reprinted with permission of the authors.*

2. Target organisms

An expert food microbiologist should determine the appropriate organisms for challenge testing. Contrary to prior suggestions that *Salmonella* species and *Staphylococcus aureus* are target pathogens for salad dressings and sauces stored at ambient temperature *(13)*, recent published research points to *E. coli* O157:H7 as the target pathogen for acidified foods *(3–6)*. Researchers did find that *S. aureus* survived longer than *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* in containers of pickled eggs, but only when the product was held refrigerated prior to achieving pH < 4.6 *(20)*. Once the product was removed from refrigeration and placed at ambient temperature, there was rapid die-off of *S. aureus*. Likewise, while survival of pathogens in 25 prepared mustards at 10°C varied by product, results could be broadly categorized as *E. coli* O157:H7 > *Salmonella* > *L. monocytogenes (12)*. Under certain conditions, e.g., higher pH or refrigerated hold, the process authority may need to conduct preliminary experiments to firmly establish the target pathogen for a given food product. A suitable non-pathogenic surrogate organism has not yet been established for cold-filled acidified foods.

In most cases, therefore, *E. coli* O157:H7 would be the target of the challenge study and, regardless of pathogen, a cocktail of strains should be used. Strains used should exhibit heightened, but not atypical, resistance to acid/low pH, and this information is generally obtained from preliminary screening studies. Comparative studies of the acid resistance of pathogenic *E. coli* serotypes (including O157:H7 and others) have been conducted *(14)*. Challenge studies should generally be conducted with three to five bacterial strains, either individually or in combination. Where there is considerable variability among strains, as many as 10 strains may be needed *(18)*. If modified strains are used, e.g., those carrying antibiotic resistance, it is important to establish that the modified strains possess the same characteristics as the parent strain without the marker, with respect to factors critical to the challenge study *(18)*.

Strains used in the challenge study should be appropriate for the food product being challenged *(18)*. Generally, but not always, for acid or acidified foods this would mean selecting pathogen strains with known acid resistance isolated from foods and, often most appropriately, from produce or produce-related outbreaks. Periodically, biochemical characteristics, serology and other strain characteristics should be confirmed.

3. Challenge study

Inoculum. Strains used in challenge studies must be maintained according to accepted laboratory procedures *(18)*. Cells should be exposed to acid during inoculum preparation by growing strains statically in broth containing added 1% glucose *(7, 11)*. Static growth is important; *E. coli* cells that are shaken during growth may exhibit an oxidative metabolism, which may not lower pH sufficiently to induce acid resistance. A recommended medium is Luria broth or tryptic soy broth containing 1% glucose *(3, 4)*. Stationary phase cells should be harvested within 20 hours of inoculation (to prevent pH increase due to subsequent amino acid metabolism) by centrifugation and washed in buffer or carrier medium to remove nutrients or metabolites in the spent medium that could affect growth in the test product *(18)*. The pH of the spent medium should be recorded, following filtration to remove residual cells, to assure acidic conditions occurred. Typically, a pH between pH 5 and pH 6 will be recorded. Cell pellets should be suspended in an isotonic medium (0.85% NaCl is commonly used) prior to inoculation of the food.

The multiple strains comprising the inoculum should be grown separately, then combined for harvesting and resuspension, with the multiple strains combined in approximately equal numbers. One set of cultures for the strain cocktail should be prepared for each independent (biological) replication. It is important to verify the numbers of viable organisms in the inoculum used *(18)*. In addition to enumerating the inoculum suspension, it is important to obtain a time-zero count in the inoculated food. The time-zero count should be taken from a sample taken as soon after inoculation as possible. The presence of bactericidal ingredients in the product formulation, which could lead to rapid and significant reductions in the inoculum population and need to be considered when designing experiments.

At least two independent biological replicates must be tested for each formulation and for each study variable that is challenged, such as storage temperature or product packaging.

Method of inoculation. High numbers of cells at time-zero, e.g., 7–9 log CFU per ml or per g, are necessary to quantify at least a 5-log pathogen reduction in the product during the incubation period. It is important that the lower limit of detection (CFU per ml) for the plating method is clearly defined for the study. To help ensure that critical parameters of the product formulation undergoing challenge are not changed, inoculum volume should generally be limited to 1% of the volume of the challenge sample *(13)*. Ideally, the product should be bulk-inoculated, thoroughly mixed, and then dispensed into the packaging material. Dispersal of the inoculum within the food matrix should place the inoculum on or within the product in a manner that realistically simulates potential contamination that might occur during manufacture *(18)*. Inoculum must be rapidly and evenly dispersed so that time-zero sampling is not delayed. Inoculation must not introduce additional contamination into the food matrix. Co-inoculation of a sample with multiple pathogen cocktails is generally not acceptable unless preliminary experiments present clear evidence to the contrary.

Sufficient food volume must be challenged so the formulation and packaging represent a 'real world' scenario. Packaging should be under an appropriate atmosphere that duplicates the packaging system to be used during commercial production.

Sampling and enumeration. Sample preparation for enumeration should be based on acceptable microbiological methods *(18)*. Wherever possible, non-selective plating media should be used for enumeration; if selective media must be used, then samples should be plated on non-selective media, with a subsequent selective agar overlay applied after several hours of incubation. The time prior to applying the overlay containing the selective ingredients may allow for recovery of injured cells. In addition to inoculum cells, native microbiota should be enumerated using the same plating method at each sampling point from control samples that were not inoculated. Testing for native microbiota cannot substitute for pathogen testing. In addition, the presence or absence of spoilage bacteria cannot be used as an indicator of safety *(18)*. The product must not support the growth of vegetative spoilage organisms if the product is to be declared 'shelf stable.'

Samples must be prepared under conditions that will lead to detectable colonies on direct plating and allow a measurement of surviving inoculum *(18)*. For acid challenge studies, an initial 1 to 10 dilution in isotonic medium buffered at or near neutral pH is recommended. This will serve to help neutralize the acid prior to plating. A diluent containing a biological buffer such as 50 mM MOPS (morpholinepropanesulfonic acid) in 0.85% NaCl, with the pH adjusted to 7.0, is ideal. Sample analysis must be done using methods that permit the accurate and reproducible recovery of microorganisms *(18)*, including standardization of buffer or diluent used and control of temperature and holding time.

At each sampling point, pH must be measured on a representative sample. The process authority may decide that other product characteristics such as gaseous atmosphere also need to be documented at each sampling point.

Destructive sampling must be weighed against sampling from bulk. In most cases, a sealed, inoculated package/container should be sampled at each interval. When sampling is from bulk, care must be taken to avoid changes to the microbial environment (e.g., headspace, gas environment, chemical composition) and to prevent contamination.

Storage temperature should be constant, documented, and less than or equal to the lowest temperature at which the food will be stored or held prior to release into commerce. Acid resistance of most bacterial pathogens, including *E. coli* O157:H7, increases as temperature is reduced. For non-refrigerated shelf stable acidified vegetables, the challenge study should be conducted at or below the lowest temperature of storage of the product during the shelf life, 50°F is recommended.

To define the killing curve, the test microorganisms should be enumerated a minimum of four times during the course of a challenge study. Sampling intervals must allow enumeration of surviving inoculum until at least a 5-log reduction is observed; the inability to count survivors is not equated with a 5-log reduction. For spread plating methods, colony counts between 20 and 200 CFU/ml are typically considered to be statistically accurate. If the detection limit is reached before a 5-log reduction is achieved, the study should be repeated and the initial inoculum level increased. It may be necessary to increase the number of samples taken at a given time-point in order to ensure accurate enumeration *(18)*.

4. Data handling and interpretation

Interpreting the results of microbiological inactivation studies requires evaluation by expert microbiologists who will consider all relevant factors *(13, 18)*. Graphical representation of the data to examine trends may be useful, and often necessary, to accurately assess survival *(18)*. Survivor curves of CFU per ml or g versus time often exhibit nonlinear decay *(3)*, and data handling must account for nonlinear microbial inactivation *(Fig. 2)*. A regression line with reported R^2 or root mean square error term is appropriate for fitting linear decay. Non-linear decay is best fit using a method such as the Weibull model as described by van Boekel *(24)*:

$$\log_{10} \text{survivors} = N_0 - [1/\text{Ln}(10)](t/\alpha)^{1/\beta}$$

Parameters of the model include the initial cell numbers (N_0) and two shape parameters (α and β). The predicted survivor curve is plotted as log survivors versus time (t) *(3)*.

Breidt and colleagues have modified the Weibull model to allow for prediction of a 5-log reduction time with normalized error distribution as follows *(3–6)*.

$$\text{Estimated 5-log reduction time} = \alpha\,[-\text{Ln}(10^{-5})]^{\beta}$$

Variation in the experimental data used for generating linear or non-linear models, and therefore 5-log reduction times, must be accounted for when analyzing the data. Calculated time-temperature data from laboratory experiments generally represent minimum processing parameters. When determining recommended manufacturing parameters, the process authority must consider goodness-of-fit of laboratory data, expected product variability, and other factors. For either linear or Weibull models, the standard error for estimating the 5-log reduction times is generally reported *(4, 6)* as a measure of goodness-of-fit and incorporated when determining recommended processing times.

Any changes to formulation, including acidulant or the presence of intrinsic factors, or changes to container or closure require review by a process authority; new challenge studies may be needed.

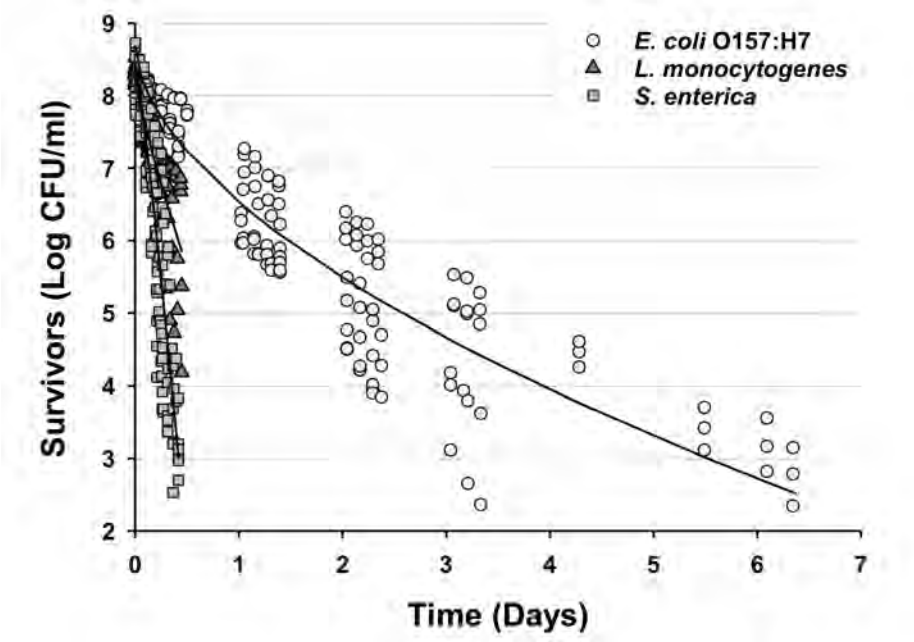

FIGURE 2. The survival of E. coli *O157:H7,* S. enterica*, and* L. monocytogenes *strains in acidified pickle jars at 10°C. The data for* E. coli *O157:H7 (circles),* S. enterica *(triangles), and* L. monocytogenes *(squares) show the log of the viable cell count from seven or more replicate experiments, each with a cocktail of five strains of a given species. The solid lines represent the predicted survival curves from the Weibull model (3). Reprinted with permission of the authors.*

5. Report

The challenge study report must provide adequate and appropriate information to allow for regulatory review. Generally, the report includes: (a) introduction that includes the purpose of the study and reviews the data supporting the experimental design; (b) information characterizing the product and manufacturing steps (a flow diagram may be included); (c) materials and methods used in the challenge study (in the format of a scientific publication); (d) raw data, clearly presented; (e) summarized data and discussion of the results, including any limitations to study design; and (f) study conclusions which include recommendations to the processor and which identify critical factors for the processing operation. The statistical design and analysis of results should be thoroughly described *(18)*.

The process authority authors the challenge study report, clearly articulating the formulation, container closure and processing steps, and critical factors in manufacture of the product. All information, such as minimum fill temperature and F- and z-values, must be collected to allow the processor to adequately file form 2541e with the U.S. FDA. It is the continuing responsibility of food canning establishments to ensure that their food products comply with all applicable statutory and regulatory requirements.

These recommendations are intended to focus existing industry guidelines *(18)* on the manufacture of acidified canned foods. The factors to consider in study design, implementation, and interpretation are based on national standards and informed by laboratory research, as well as industry and regulatory practice. This document will aid in regulatory review of cold-fill hold challenge studies of acidified foods and will help ensure scientific integrity and product safety.

REFERENCES

1. Ahamad, N., and E. H. Marth. 1989. Behavior of *Listeria monocytogenes* at 7, 13, 21, and 35°C in tryptose broth acidified with acetic, citric, or lactic acid. *J. Food Prot.* 52:688–695.
2. Bae, Y.-M., and S. –Y. Lee. 2015. Combined effects of organic acids and salt depending on type of acids and pathogens in laboratory media and acidified pickle. *J. Appl. Microbiol.* 119:455–464.
3. Breidt, F., Jr., J. Hayes, and R. F. McFeeters. 2007. Determination of 5-log reduction times for food pathogens in acidified cucumbers during storage at 10 and 25°C. *J. Food Prot.* 70:2638–2641.
4. Breidt, F., Jr., K. Kay, J. Cook, J. Osborne, B. Ingham, and F. Arritt. 2013. Determination of 5-log reduction times for *Escherichia coli* O157:H7, *Salmonella enterica*, or *Listeria monocytogenes* in acidified foods with pH 3.5 or 3.8. *J. Food Prot.* 76:1245–1249.

5. Breidt, F., Jr., K. Kay, J. Osborne, B. Ingham, and F. Arritt. 2014. Thermal processing of acidified foods with pH 4.1 to 4.6. *Food Prot. Trends* 34:132–138.
6. Breidt, F., Jr., K. P. Sandeep, and F. M. Arritt. 2010. Use of linear models for thermal processing of acidified foods. *Food Prot. Trends* 30:268–272.
7. Buchanan, R. L., and S. G. Edelson. 1996. Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. *Appl. Environ. Microbiol.* 62:4009–4013.
8. Chapman, B., and T. Ross. 2009. *Escherichia coli* and *Salmonella enterica* are protected by acetic acid, but not by hydrochloric acid, by hypertonicity. *Appl. Environ. Microbiol.* 75:3605–3610.
9. Chapman, B., N. Jensen, T. Ross, and M. Cole. 2006. Salt, alone or in combination with sucrose, can improve the survival of *Escherichia coli* O157 (SERL 2) in model acidic sauce. *Appl. Environ. Microbiol.* 72:5165–5172.
10. Cooley, M.B., D. Chao, and R. E. Mandrell. 2006. *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *J. Food Prot.* 69:2329–2335.
11. Hill, C., B. O'Driscoll, and I. Booth. 1995. Acid adaptation and food poisoning microorganisms. *Int. J. Food Microbiol.* 28:245–254.
12. Ingham, G. A., M. Pan, F. Ranelli, and B. H. Ingham. 2017. Efficacy of a hold-time at 10°C for achieving a 5-log reduction of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in prepared mustard. *Food Prot. Trends* 37:8–15.
13. Institute of Food Technologists. 2003. Evaluation and definition of potentially hazardous foods. Chapter VI. Microbial challenge testing. *Comp. Rev. Rood Sci. Food Saf.* 2(Suppl):1–109.
14. Kim, G., F. Breidt, P. M. Fratamico, and D. Oh. 2015. Acid resistance and molecular characterization of *Escherichia coli* O157:H7 and different non-O157 Shiga toxin-producing *E. coli* serogroups. *J. Food Sci.* 80(10):M2257–M2264.
15. Koerfer, B., J. Sogin, and B. Ingham. 20xx. Comparative survival of *Listeria monocytogenes, Salmonella enterica,* or Shiga toxin-producing *Escherichia coli* and native microbiota in a produce model system and on fresh-cut retail produce. *J. Food Prot.* (submitted)
16. Kreske, A. C., K. Bjornsdottir, F. Breidt, Jr., and H. Hassan. 2008. Effects of pH, dissolved oxygen, and ionic strength on the survival of *Escherichia coli* O157:H7 in organic acid solutions. *J. Food Prot.* 71:2404–2409.
17. Lu, H. J., F. Breidt, Jr., I. M. Perez-Diaz, and J. A. Osborne. 2011. Antimicrobial effects of weak acids on the survival of *Escherichia coli* under anaerobic conditions. *J. Food Prot.* 74:893–898.
18. National Advisory Committee on Microbiological Criteria for Foods. 2010. Parameters for determining inoculated pack/challenge study protocols. *J. Food Prot.* 73:140–202.
19. Russel, J. B. 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J. Appl. Bacteriol.* 73:363–370.
20. Sullivan, E. K., D. C. Manns, J. J. Churey, R. W. Worobo, and O. I. Padilla-Zakour. 2013. Pickled egg production: Inactivation rate of *Salmonella, Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Staphylococcus aureus* during acidification step. *J. Food Prot.* 76:1846–1853.
21. U.S. Food and Drug Administration. 2012. Food for human consumption. Acidified foods. 21 CFR 114. U.S. Food and Drug Administration, Washington, D.C.
22. U.S. Food and Drug Administration. 2016. Current good manufacturing practice, hazard analysis, and risk-based preventive controls for human food. 21 CFR 117. U.S. Food and Drug Administration, Washington, D.C.
23. U.S. Food and Drug Administration. 2017. Code of Federal Regulations (CFR) citations for color additives, food ingredients and packaging. Available online: https://www.fda.gov/Food/Guidance Regulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm082463.htm Accessed 10 October 2017.
24. Van Boekel, M. A. J. S. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int. J. Food Microbiol.* 74:139–159.