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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

ABSTRACT

Most manufacturers of acidified foods use heat pasteurization to achieve a 5-log reduction in vegetative acid-resistant pathogens and ensure product safety. For sensory reasons, mustard manufacturers may wish to avoid pasteurization. Research has validated cold-fill-hold times for a 5-log reduction of pathogens in pickle brine with pH ≤ 3.3, or with pH of 3.5 or 3.8 and formulated with specific levels of acetic and benzoic acids. We investigated survival of 5-strain single-pathogen cocktails of *Escherichia coli* O157:H7 (EC), *Salmonella* spp. (SP), and *Listeria monocytogenes* (LM) in 25 mustards over 96 h at 10°C to determine whether a cold-hold process would ensure pathogen reduction across a variety of mustard formulations. Final Δ-log CFU/g across all pathogen/mustard combinations ranged from 3.3 to > 8.0. A ≥ 5-log reduction occurred in 87, 92, and 100% of EC, SP, and LM trials, respectively. Average pH = 3.4 and a_w = 0.96 characterized all mustards, including mustards (n = 7) with ≥ 5-log reduction in all pathogens in ≤ 6 h. Slightly higher average pH (3.7) and lower a_w (0.93) characterized mustards (n = 3) in which adequate lethality was never achieved in the case of at least one pathogen. Results failed to establish a hold-time at 10°C that would ensure adequate pathogen reduction across a variety of mustard formulations.

INTRODUCTION

Acidified foods are low-acid foods to which acid(s) or acid food(s) have been added. Acidified foods have a water activity greater than 0.85 and a finished equilibrium pH of 4.6 or below *(17)*. Exempted from categorization as acidified foods are foods kept refrigerated, carbonated beverages, jams, jellies, and preserves, and acid foods (including standardized and non-standardized dressings and condiment sauces) that contain small amounts of low acid food(s) and have a resultant finished equilibrium pH that does not differ significantly from that of the predominant acid or acid food. No standard of identity for prepared mustard has been established *(14)*,

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and prepared mustards are therefore not automatically exempted from categorization as acidified foods. In September 2010, the U.S. Food and Drug Administration (FDA) issued a draft guidance for industry that attempted to describe the terms 'small amounts of low-acid foods' and 'significant shift in pH' used in defining an acidified food *(15)*. In the draft guidance, a small amount of low-acid food(s) was considered to be no more than 10% by weight in the finished product. The 2010 draft guidance also established when the pH of the finished product was considered significantly different from the pH of the predominant acid or acid food *(15)*. Given the language in the draft guidance, prepared mustards that contain more than 10% by weight of low-acid ingredients, e.g., mustard powder or eggs, or that contain a small amount of low-acid food ingredients but that vary significantly in pH from the predominant acid food, e.g., vinegar with pH 2.4, could be considered acidified foods.

The Code of Federal Regulations *(21 CFR 114; 17)* mandates that vegetative cells of microorganisms of public health significance be destroyed during the manufacture of acidified canned foods. The FDA's 2010 draft Guidance for Industry: Acidified Foods further established that "To be adequate…the scheduled process for acidified foods should be sufficient to destroy or prevent the presence of vegetative cells of…pathogenic microorganisms (such as *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* species) that are acid-tolerant"*(15)*. Destruction of pathogens of public health concern in acidified foods is usually achieved by a thermal process *(4, 5)*. However, for sensory reasons manufacturers of prepared mustards may wish to forego thermal processing. Recent research in a cucumber-brine model system has outlined cold-fill-hold conditions that 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 *(2)*, or with equilibrium pH 3.5 or 3.8 that are formulated with specific levels of acetic and benzoic acid *(3)*. Manufacturers of prepared mustard who are unable to meet these validated cold-fill-hold formulation targets would have to undertake challenge studies for each formulation to establish conditions that would ensure that the product was safe.

Mayerhauser determined survival of three individual strains of *E. coli* O157:H7 in three varieties of retail mustard: Dijon, yellow, and deli-style *(8)*. *E. coli* O157:H7 was not detected in Dijon-style mustard beyond 3 h at 25°C and beyond 2 days at 5°C. Pathogen survival in yellow and deli-style mustard was not detected beyond 1 h. Tsai and Ingham inoculated acidic condiments (ketchup, prepared mustard, and sweet pickle relish) with individual strains of *E. coli* O157:H7 (n = 3) and *Salmonella* spp. (n = 3) and noted complete die-off of all pathogen strains within 1 h at either 5 or 23°C in the prepared mustard and sweet pickle relish *(12)*. Small numbers of *E. coli* O157:H7 and *Salmonella* survived in ketchup for periods ranging from several hours to several days. Mustard essential oil contains an antimicrobial compound not naturally present in pickles or ketchup, allyl isothiocyanate *(6)*, and this compound may contribute to pathogen destruction in prepared mustard. We investigated survival of *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* in 25 mustards over 96 h at 10°C, with the goal of determining whether a cold-hold process would ensure pathogen reduction across a variety of mustard formulations.

MATERIALS AND METHODS

Bacterial strains

Strains of *L. monocytogenes, E. coli* O157:H7, and *S. enterica* previously used in process validation studies with acidified foods *(4, 5)* were used in this study *(Table 1)*. One strain of *E. coli* O157:H7 linked to an outbreak of illness associated with ground beef and with known acid tolerance, 43895 (American Type Culture Collection, Manassas, VA), was used *(Table 1)*. Stock cultures of each pathogen strain were maintained in Tryptic Soy Broth (TSB; Difco, Becton, Dickinson and Company, Sparks, MD) containing 10% (vol/vol) glycerol (Fisher Scientific, Itasca, IL) and kept frozen at -20°C. Working cultures were prepared monthly by streaking for isolation from partially thawed stock cultures as follows: *L. monocytogenes* on Listeria Selective agar (LSA; Oxoid LTD, Basingstoke, Hampshire, England) with added Listeria Selective Supplement (Oxoid), and *Salmonella* and *E. coli* O157 on Levine's Eosin Methylene Blue agar (Difco) modified with added D-sorbitol (10 g/l; Fisher) and NaCl (5g/l; Fisher) (m-EMB). Working culture plates were incubated (35°C) for 24 h (*Salmonella* and *E. coli* O157:H7) or 48 h *(L. monocytogenes)* and then stored at 4°C for < 40 days. Periodically, strain identity was confirmed based on Gram reaction, cell and colony morphology, and biochemical identification (API 20E, bioMerieux, Durham, NC).

Culture preparation

Inoculation cultures were prepared by transferring a single isolated colony of each strain from a working culture plate into a separate tube containing 9 ml TSB, which was then incubated for 24 h at 35°C to obtain stationary phase cells. Following incubation, the contents of all tubes containing a stationary-phase culture of a given pathogen were combined in a sterile 50-ml centrifuge tube. Each resulting pathogen cocktail was centrifuged for 10 min at 8000 RPM to create an inoculum pellet. The supernatant was then discarded, and the pellet was suspended in 5-ml Butterfield's Phosphate Diluent (BPD; 3M, Minneapolis, MN) and vortexed, resulting in a cocktail inoculum concentration of ~10^8 CFU/ml. Pathogen levels in the cocktails were determined by serially diluting and spread plating (0.1 ml) the inocula as follows: *L. monocytogenes* on LSA with added Listeria Selective Supplement; *Salmonella* and *E. coli* O157:H7 on m-EMB agar. Serial dilutions were prepared using BPD, and

Table 1. Pathogen strains used

Strain Name	ID[a]	Food Origin
Listeria monocytogenes	SRCC 529	Pepperoni
	SRCC 1791	Yogurt
	SRCC 1506	Ice cream
	SRCC 1838	Cabbage
	SRCC 2075	Diced coleslaw
Escherichia coli O157:H7	ATCC 43895	Ground beef
	SRCC 1675	Apple cider outbreak
	SRCC 1486	Salami outbreak
	SRCC 2061	Ground beef
	SRCC 1941	Pork
Salmonella Braenderup[b]	SRCC 1093	10% salted yolk
Salmonella Cerro	SRCC 400	Cheese powder
Salmonella Enteritidis	SRCC 1434	Ice cream
Salmonella Newport	SRCC 551	Broccoli with cheese
Salmonella Typhimurium	SRCC 1846	Liquid egg

[a]SRCC strains courtesy of North Carolina State University via Mérieux NutriSciences, Chicago, IL;
ATCC, American Type Culture Collection, Manassas, VA
[b]*Salmonella enterica* strains with the serotype

plates were incubated at 35°C for 24 h (*Salmonella* and *E. coli* O157) or 48 h *(L. monocytogenes)*, after which colonies were enumerated.

Prepared mustards

Because prepared mustard lacks a standard of identity, this study defined mustard as any product labeled as 'mustard' in compliance with the Code of Federal Regulations *(21 CFR 102; 18)*. Twenty-five mustards marketed under 12 brands were purchased from retailers or obtained directly from the manufacturer *(Table 2)*. Replicate samples of the same type of mustard sold under the same brand were from different production lots. Because of differences in retail availability or manufacturers' production schedules, duplicate trials for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* were conducted in 14, 16, and 14 mustards, respectively. The number of individual pathogen/mustard trials for *Salmonella, L. monocytogenes,* or *E. coli* O157:H7 was 11, 9, and 11, respectively. All 25 mustards were challenged with each pathogen at least once. Processing conditions, length of storage, and handling conditions prior to purchase or prior to samples arriving in the laboratory were unknown. Mustards were grouped by marketing/label category as follows: Dijon-style (6), yellow (4), honey (6), deli/brown (6) and horseradish/garlic (3), and it was noted whether prepared mustards were smooth- or coarse-ground. Mustards that, when spread to a thickness of ca. 2 mm, were visibly uneven because of the presence of partially ground or unground mustard seeds were classified as 'coarse-ground'; those without visible particulates were classified as smooth. Mustards were characterized by water activity (a_w; AquaLab LITE, Decagon Devices Inc., Pullman, WA), and pH (Accumet AB15; Fisher).

Challenge study

For each pathogen/mustard trial (n = 119), nine sample bags (WhirlPak; Nasco, Ft. Atkinson, WI), each containing 25 g of mustard, were prepared. Eight of the nine bags were each inoculated with 0.1 ml of pathogen cocktail and massaged gently for 10 s to produce a starting inoculum of ~10^8 log CFU/g. One bag, which was not inoculated, was analyzed to determine levels of microorganisms already in the product. Inoculated bags were held at 10°C and sampled after 0, 6, 18, 24, 30, 48, 72 and 96 h of holding.

Surviving inocula and native microflora (uninoculated sample) were enumerated as follows: sample bag was

Table 2. Survival of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* in prepared mustards at 10°C

Mustard Type[a]			*Salmonella*			*E. coli* O157:H7			*L. monocytogenes*		
	pH	a_w	n[b]	Δ log CFU/g[c]	Time (h)[d]	n[b]	Δ log CFU/g[c]	Time (h)[d]	n[b]	Δ log CFU/g[c]	Time (h)[d]
Dijon											
A[e]	3.80	0.973	1	5.8	24	1	7.1	96	1	6.0	24
B	3.36	0.950	2	5.5	24, 18	2	5.7	6, 6	2	5.0	6, 6
C	3.42	0.933	1	6.0	72	1	5.1	96	1	6.1	6
D	3.40	0.927	2	6.8	6, 6	2	6.4	6, 6	2	7.2	6, 6
E[e]	3.46	0.946	1	5.0	6	1	5.5	6	1	6.3	6
F	3.61	0.945	1	5.8	6	1	7.1	18	1	5.3	6
Yellow											
A	3.39	0.980	2	5.6	6, 6	2	6.4	6, 6	2	6.4	6, 6
B	3.31	0.975	2	6.3	6, 6	2	5.2	18, 6	2	7.2	6, 6
C	3.35	0.980	1	6.0	6	1	5.6	30	1	6.0	6
D	3.24	0.972	2	6.2	6, 6	2	6.1	6, 6	2	7.0	6, 6
Honey											
A	3.65	0.975	1	6.3	6	1	6.1	6	1	6.4	6
B	3.82	0.926	2	NA[f]	>96, >96	2	NA	>96, >96	2	6.2	72, 96
C	3.34	0.940	2	5.3	6, 6	1	6.0	6	2	6.4	6, 18
D	3.50	0.931	2	5.3	96, 96	2	NA	>96, >96	2	(6.1)[g]	48, 72
E	3.42	0.950	1	5.2	72	1	7.2	24	1	5.6	18
F	3.38	0.961	1	5.3	6	1	7.1	18	1	5.3	6
Deli/Brown											
A	3.65	0.975	1	5.7	6	1	5.1	18	1	5.1	18
B[e]	3.87	0.939	2	NA, 5.1	>96, 96	1	NA	>96	2	5.5	30, 24
C	3.36	0.967	2	7.4	6, 6	2	7.1	6, 6	2	6.4	18, 6
D[e]	3.12	0.934	2	6.1	6, 6	2	(5.2)	6, 6	2	5.8	24, 24
E	3.45	0.974	2	7.2	6, 6	2	5.8	18, 6	2	6.4	6, 6
F	3.41	0.950	2	6.2	6, 6	2	7.0	6, 6	2	6.2	6, 6
Horseradish											
A	3.32	0.968	1	6.3	6	2	5.1	6, 24	2	6.0	6, 6
B	3.48	0.973	1	6.7	6	2	5.9	6, 6	2	6.9	6, 6
C	3.63	0.945	2	5.1	30, 24	2	5.1	48, 48	2	7.3	6, 6

[a]Prepared mustards grouped by marketing/label designation. pH and a_w are average values, where appropriate

[b]n = number of replicates for a given pathogen/mustard combination

[c]Change log CFU/g from time 0 to where ≥5-log reduction first noted on sampling; minimum is noted across multiple trials, if applicable

[d]Time at which ≥5-log reduction first noted on sampling. Multiple entries reflect time for individual trials

[e]**Bolded** letter = coarse-ground mustard. Non-bolded letter = smooth mustard

[f]NA = not applicable; minimum 5-log reduction not achieved within 96 h

[g]( ) = log reduction was not maintained over time

aseptically opened and 99 ml BPD was added. Each bag was stomached (AES Smasher, AES Chemunex, Bruz, France) for 30 s at high speed. Stomached samples were serially diluted in BPD and spread plated. Mustards inoculated with *L. monocytogenes* were plated onto LSA with added Listeria Selective Supplement; mustards inoculated with *Salmonella* or *E. coli* O157 were plated onto m-EMB agar; and uninoculated mustards were plated onto Tryptic Soy Agar (TSA; Difco) (at time 0 only). Spread plates for enumeration of indigenous bacteria, *Salmonella,* and *E. coli* O15:H7 were incubated for 24 h at 35°C; those for enumeration of *L. monocytogenes* were incubated for 48 h at 35°C. Typical colonies were counted after incubation, and CFU/g and reduction in log CFU/g relative to 0 h samples (Δ-log CFU/g) were calculated for each sample at each time point. When no colonies were observed, a value of 0.5 CFU was assigned to the least dilute plate. On m-EMB agar, typical colonies of *E. coli* O157:H7 appeared colorless to pink, while colonies of *Salmonella* were dark red-black, with a metallic green sheen. On LSA, *L. monocytogenes* colonies were grey and surrounded by a black halo.

Statistical analysis

Because adequate cold-fill-hold condition(s) would be based on the survival pattern of the most tolerant pathogen across all mustards, each trial (n = 119) was treated as an individual experiment for statistical analysis. The Δ-log CFU/g, relative to time 0, was calculated for each sampling point in each trial. The time was recorded for each trial at which adequate pathogen lethality for a pathogen/mustard combination was first achieved (Δ-log CFU/g $\geq$ 5.0). To determine the effect of mustard texture (coarse vs smooth) on reduction of each pathogen, Δ-log counts at 6, 18 and 24 h were evaluated by *t*-tests (Microsoft Excel; Redmond, WA) and ANOVA (Minitab; State College, PA). A significant difference occurred when $P < 0.05$. Linear regression was used to determine the relationship between Δ-log CFU/g at 96 h against pH and against a_w for each pathogen (Excel).

RESULTS AND DISCUSSION

Twenty-five prepared mustards were challenged at least once with each of three pathogens: *Salmonella, E. coli* O157:H7, and *L. monocytogenes* *(Table 2)*. The pH of mustards ranged from 3.05 to 3.89, with an average of 3.44. The variation in pH between lots of the same mustard from the same manufacturer ranged from 0.01 to 0.11. Water activity (a_w) ranged from 0.919 to 0.981, with an average of 0.957. The variation in a_w between lots ranged from 0 to 0.01. Mustards were grouped based on their marketing/label designation as Dijon-style (n = 6), Yellow (n = 4), Honey (n = 6), 'Deli' or 'Brown' mustard (n = 6), and mustards with added horseradish or garlic (n = 3), and represented a wide array of prepared mustard available at retail. Each category included mustards with 'add-ins' such as 'Classic Yellow Mustard with Dill,' categorized for purposes of this study as 'Yellow' mustard. Of the 25 prepared mustards, four had a visibly uneven texture caused by partially ground or unground mustard seeds apparent when spread to a thickness of ca. 2-mm and were classified as 'coarse-ground;' those without visible particulates were classified as smooth *(Table 2)*.

Destroying vegetative cells of public health significance is essential to processing safe acidified canned foods (21 CFR 114) *(17)*. The FDA recommends a 5-log reduction in pathogenic microorganisms, specifically those that are acid tolerant *(15)*. While post-packaging pasteurization of mustards can ensure that a 5-log reduction is met, manufacturers may wish to avoid heating. For this reason, the lethality of a cold-fill-hold process for a wide range of prepared mustards was examined. Mustards were inoculated and stored at 10°C for up to 4 days, with periodic sampling. Average survival pattern for each pathogen across all mustards is shown in *Fig. 1*. Survival of pathogens in acidic or acidified foods is greater at lower than at higher temperatures in a model cucumber juice medium *(2)* as well as in acidic condiments *(8, 12)*, so a holding temperature of 10°C was used for this study. A temperature of 10°C could, therefore, be seen as representing a 'best case' for pathogen survival and, if validated hold conditions were to ensue from this study at 10°C, holding a product at room temperature would ensure a margin of safety.

Each prepared mustard was challenged with three pathogens: *Salmonella, E. coli* O157:H7, and *L. monocytogenes.* When a minimum 5-log reduction in a given pathogen was first noted in a given pathogen/mustard trial, the time was recorded, along with Δ-log CFU/g (from time 0 h). Where duplicate trials were conducted, the lowest Δ-log CFU/g that was greater than the 5.0 threshold was recorded across both trials, and the time when a $\geq$ 5-log reduction was first observed was noted for each trial *(Table 2)*. *E. coli* O157:H7 out-survived both *Salmonella* and *L. monocytogenes* in 14 of 25 mustards (56%), with the minimum required lethality in *E. coli* O157:H7 eventually noted. *Salmonella* out-survived both *E. coli* O157:H7 and *L. monocytogenes* in trials of 2 mustards: Dijon-type (sample B; 2 trials) and Honey mustard (sample E); *L. monocytogenes* exhibited extended survival in two mustards, both in the Deli/Brown category: sample B, D *(Table 2)*. The number of surviving *E. coli* O157:H7 fluctuated in Deli/Brown sample D over the entire 96 h, and minimum required lethality in this mustard could not be assured. In one other Deli/Brown mustard (sample A), both *E. coli* O157:H7 and *L. monocytogenes* out-survived *Salmonella*, with minimum required lethality for *E. coli* O157:H7 and *L. monocytogenes* ($\geq$ 5-log reduction) observed by 18 h. For three mustards (Honey mustard (sample B and D), and Deli/Brown mustard sample B), minimum required lethality across all pathogens was never observed. Minimum required lethality ($\geq$ 5-log reduction) in *Salmonella* and *E. coli* O157:H7 was never achieved in Honey mustard sample B and in one of two runs with Deli/Brown mustard B. Minimum lethality

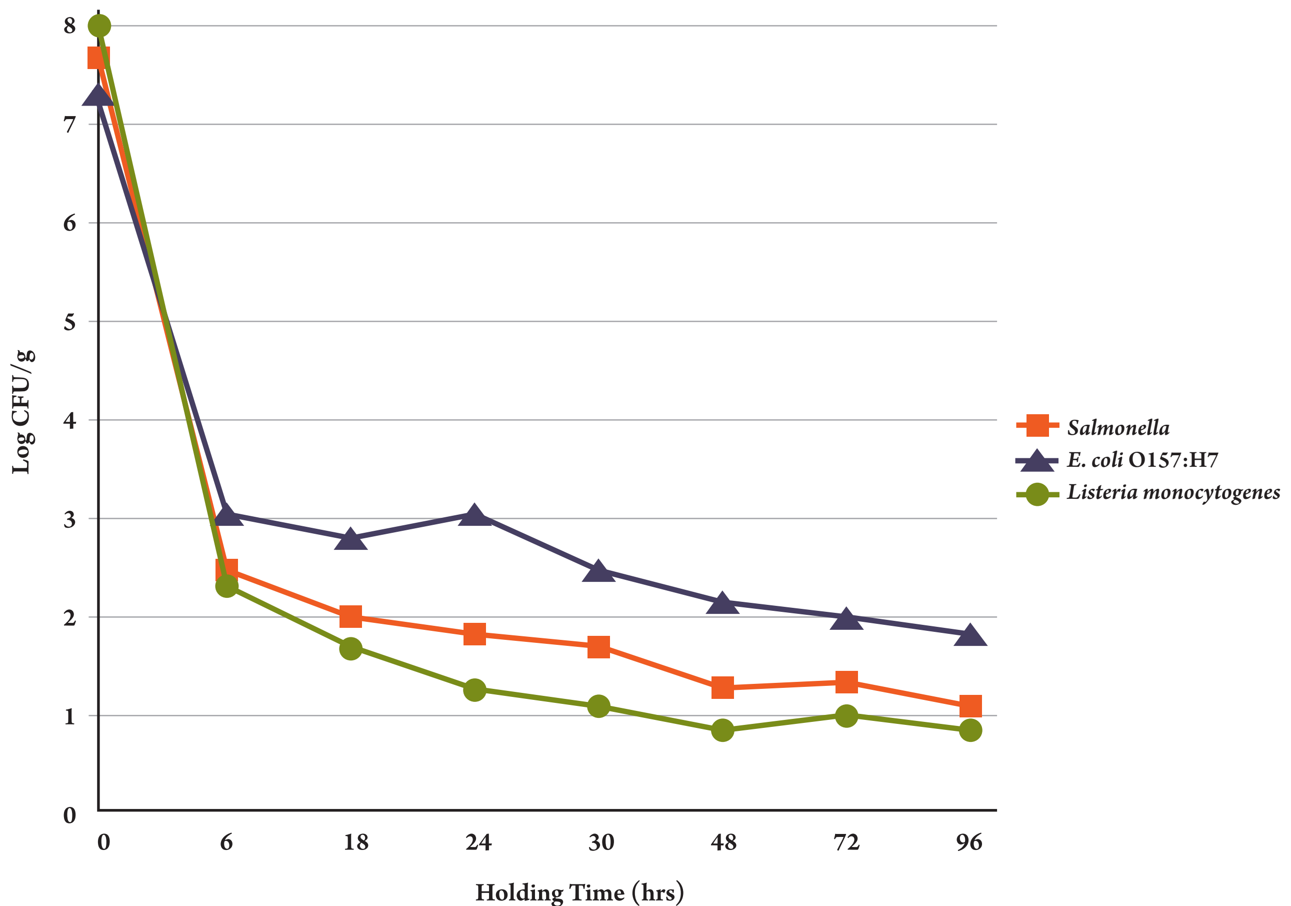

Figure 1. Average survival of E. coli *O157,* Salmonella *spp., and* L. monocytogenes *in prepared mustard (n = 25) held for 96 h at 10°C*

for *Salmonella* in Honey mustard sample D was achieved by 96 h, and appeared to be achieved for *L. monocytogenes* by 72 h; however, the number of surviving *L. monocytogenes* increased from 72 to 96 h. Whether this increase was due to experimental error is not known. Minimum required lethality for *E. coli* O157:H7 was never achieved in Honey mustard sample D. Where minimum lethality across all pathogens was never achieved, these mustards provided conditions slightly more favorable for survival – higher pH (3.73) and lower a_w (0.932) – although these conditions were not statistically different from those of the average sample. In seven mustards, a $\geq$ 5-log reduction was noted in all pathogens at the first 6-h sampling point. Rapid pathogen die-off was distributed across mustard categories, with at least one mustard per category (Dijon D, E; Yellow A, D; Honey A; Deli/Brown F; and Horseradish B). The pH and a_w of the seven mustards in which pathogens rapidly died off were representative of the collection as a whole, with average pH and a_w of 3.43 and 0.960, respectively.

Levels of native microflora in retail mustards ranged from 0.60 to 3.86 log CFU/g, with an average of ~2-log CFU/g. The level of indigenous organisms tended to be lower in coarse-ground mustards than in smooth mustards (data not shown). There was no clear trend for the relationship of level of indigenous bacteria to mustard type (data not shown). The level of native microflora was not sampled after time 0, so it is not clear whether there was growth or survival of indigenous organisms during storage. However, none of the prepared mustards were sampled directly after bottling, and all were either purchased at retail or obtained from a manufacturer's warehouse, so we may assume that the level of native organisms may have stabilized in the product prior to use in our trials.

Tsai and Ingham compared the survival of three strains of both *E. coli* O157:H7 and *Salmonella* in a prepared mustard and found that all strains died within 1 h at either 5 or 23°C *(12)*. The pH of the mustard that Tsai and Ingham studied was 3.1, lower than the pH of 24 of the 25 prepared mustards used in this study; only Deli/Brown sample D had a similar

pH (3.12). More recently, Mayerhauser investigated survival of three test strains of *E. coli* O157:H7 individually inoculated into three lots of each of three varieties of retail mustard – Dijon, yellow and deli – at 5°C or 25°C, where the pH of the mustards ranged from 3.17 to 3.63 *(8)*. Mayerhauser did not detect *E. coli* O157:H7 in yellow or deli-style mustard beyond 1 h at either 5 or 25°C. Survival in Dijon-style mustard was not detected beyond 3 h at 25°C or 2 days at 5°C. The fact that, in some cases, we observed extended survival of *E. coli* O157:H7 in retail mustards compared with findings of other researchers is likely due to differences in strain selection, variation in formulation of mustards challenged with the pathogen, and differences in study design, e.g., holding temperature.

The overall survival trend for *Salmonella, E. coli* O157, and *L. monocytogenes* in mustard may be useful for the process authority seeking to identify critical control points in a scheduled process, as required under the Food Safety Modernization Act *(16)*. Over 96 h at 10°C, pathogen survival in prepared mustards can broadly be categorized as: *E. coli* O157:H7 > *Salmonella* > *L. monocytogenes* *(Fig. 1)*. Importantly, *E. coli* O157:H7 survived beyond the hold period at 10°C in three mustards: Honey samples B and D and Deli-style sample B; survival of *Salmonella* was also extended in the same samples. Samples that allowed for extended pathogen survival were characterized by higher-than-average pH (3.73 vs 3.44) and lower-than-average a_w (0.932 vs 0.957). Linear regression of Δ-log CFU/g at 96 h against pH showed a slight negative correlation ($r^2 = 0.23$ for *Salmonella*, 0.03 for *E. coli* O157:H7, and 0.01 for *L. monocytogenes*). A similar regression of Δ-log CFU/g at 96 h against a_w showed a slight positive correlation ($r^2 = 0.25$ for *Salmonella*, 0.24 for *E. coli* O157:H7, and 0.04 for *L. monocytogenes*). In 36 of 39 trials across all three pathogens, when mustard pH ≤ 3.45 and $a_w \geq 0.960$, a 5-log reduction in all pathogens occurred within 6 h. Evidence suggests, however, that boundaries for pH and a_w alone would not be sufficient to establish critical factors that would validate a cold-fill-hold time and ensure sufficient pathogen lethality across all mustards held at 10°C.

Smittle, in a review of the microbiological safety of mayonnaise, salad dressings and sauces produced in the United States *(10)*, noted that these products, when commercially produced, have a long record of food safety. Smittle attributed the lethality of such products against *Salmonella, E. coli* O157:H7, and *L. monocytogenes* to the toxic effect of acetic and, to a lesser extent, lactic and citric acids. Smittle analyzed published data and found that the most important and significant factor in destroying pathogens in salad dressings is pH as adjusted with acetic acid, followed by the concentration of acetic acid in the water phase. Smittle noted that the pK_a of acetic acid is 4.75, ensuring that prepared mustards, with acetic acid as the primary acidulant, are inherently bactericidal to some extent. Likewise, Beuchat et al. *(1)* determined the death rates of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* in 10 shelf-stable, dairy-based pourable salad dressings with initial product pH ranging from 2.83 to 3.87 and varying by manufacturer. Dressings were inoculated with test pathogens and stored at 25°C for up to 15 days. The type of dressing did not affect pathogen survival. All pathogens died rapidly in all salad dressings, with *Salmonella* undetectable within 1 day and both *E. coli* O157:H7 and *L. monocytogenes* undetectable within 8 days of inoculation.

Mustard essential oil contains allyl isothiocyanate (AIT), a strong antimicrobial compound produced from glucosinolates by a reaction catalyzed by the endogenous enzyme myrosinase *(7)*. The antimicrobial efficacy of mustard essential oil and purified AIT was demonstrated in studies using *E. coli* O157:H7 *(6, 13)*, *L. monocytogenes* *(6)*, and *Salmonella* Typhi *(13)*, with mustard essential oil proven more lethal than purified AIT against *E. coli* O157:H7 *(13)*. The AIT from mustard seeds displays multi-targeted mechanisms of disrupting metabolic pathways, membrane integrity, and cellular structure, ultimately resulting in cellular breakdown *(11, 13)*. In this study, statistical analysis by *t*-tests and ANOVA showed greater pathogen reduction in smooth- than coarse-ground mustards within the first day after inoculation (data not shown). Reduction in *L. monocytogenes* at 6 h and 18 h was significantly greater ($P < 0.05$) in smooth- than coarse-ground mustards, and the same difference in *E. coli* O157 survival was observed at 18 and 24 h. No significant difference ($P > 0.05$) in *Salmonella* survival between coarse- and smooth-ground mustards was seen within 24 h. These differences could result from greater concentrations of mustard essential oils in smooth-ground mustards, in which the mustard seeds have been crushed and the essential oils released. However, this study included only four coarse-ground mustards, too few to draw any firm conclusions. Because we did not have compositional data available to us from the various manufacturers, we were unable to draw any conclusions regarding the contribution that any one ingredient might have made to our results. The handling and storage history for the retail mustards that we tested or for those samples that we obtained directly from the manufacturer was unknown, information which may also have impacted our results.

The combined antimicrobial effect of mustard flour and acetic acid against *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* has been investigated *(9)*. Researchers noted that mustard flour alone, commonly used by manufacturers to prepare mustards, was effective at reducing pathogens to undetectable levels within 5 days at 5°C or 12 h at 22°C. Pathogen destruction was at least as rapid with added 1% acetic acid, product pH 3.61 to 3.81.

While this study did not validate a cold-fill-hold process that would ensure safety of prepared mustards across a range of product formulations, several guidelines for safe production of prepared mustard can be proposed. Manufacturers may consider meeting these guidelines to

improve the likelihood of producing mustard in which a 5-log pathogen-reduction target would be met during a cold-fill-hold process.

- Smooth-ground mustards may have more antimicrobial activity than coarse-ground mustards. In this study, smooth-ground mustards were more lethal to *L. monocytogenes* and *E. coli* O157:H7 than coarse-ground mustards, although the relatively small number of coarse-ground samples tested does not allow us to establish firm guidelines in this area.
- Prepared mustards with lower pH and higher a_w may have greater antimicrobial activity, with low-pH being slightly more strongly correlated with minimum pathogen lethality. In this study, *Salmonella* and *L. monocytogenes* rapidly died off within 6 h in a majority of trials when pH $\leq$ 3.45 and $a_w \geq 0.960$; corresponding values for *E. coli* O157:H7 were 3.41 and 0.948 for pH and a_w, respectively.
- The pathogen with the greatest likelihood of survival in prepared mustard is *E. coli* O157:H7. Across the samples tested, *E. coli* O157:H7 had the highest survival rate of any pathogen and *L. monocytogenes* the lowest *(Fig. 1)*. *E. coli* O157:H7 is, therefore, the pathogen of most concern in mustards. Manufacturing processes should be developed to minimize risk of *E. coli* O157:H7 contamination and survival.
- A product-specific challenge study would be required to establish validated cold-fill-hold conditions for prepared mustard. Manufacturers believing that a 5-log reduction in vegetative cells of public health significance can be achieved in their mustard within some holding time should conduct a challenge study to prove that processing their product in this way would pose no threat to public health.
- A holding period longer than 96 h at 10°C, or a holding temperature higher than 10°C, may effectively reduce pathogens in mustard and lead to the validated time-temperature hold conditions needed to ensure safety.

This study confirmed the difficulty of trying to establish a validated process for a product such as prepared mustard that lacks a standard for formulation. In the absence of such a standard, process validation is complex and, to be broadly applicable, must encompass the variability in the product that exists in the marketplace.

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