Running head: pathogen growth on cheese

Growth of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* on Cheese during Extended Storage at 25°C

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ABSTRACT

A potentially hazardous food (PHF) requires time/temperature control to maintain safety. The US Food and Drug Administration would classify most cheeses as PHF based on pH and $a_{w,}$ and a product assessment would be required to evaluate safety for >6 h storage at 21°C. We tested the ability of 67 market cheeses to support growth of *Listeria monocytogenes* (LM), *Salmonella* spp. (SALM), *Escherichia coli* O157:H7 (EC), and *Staphylococcus aureus* (SA) over 15-day storage at 25°C. Hard (Asiago and Cheddar), semi-hard (Colby and Havarti), and soft cheeses (Mozzarella and Mexican-style) were among types tested, and included some reduced-sodium and reduced-fat types. Single-pathogen cocktails were prepared and individually inoculated onto cheese slices (~10^5CFU/g). Cocktails were comprised of 10 strains of LM, six of SALM, or five of EC or SA. Inoculated slices were vacuum packaged and stored at 25˚C for ≤ 15 days, with surviving inocula enumerated every three days. Salt-in-the-moisture phase (%SMP), calculated from measured moisture (%) and salt (%), titratable acidity (%), pH, and a_w were measured. Pathogens did not grow on 53 cheeses, while 14 cheeses supported growth of SA, six of SALM, four of LM, and three of EC. Of the cheeses supporting pathogen growth, all supported growth of SA, ranging from 0.57 to 3.08 log CFU/g (avg. 1.70 log CFU/g). Growth of SALM, LM, and EC ranged from 1.01 to 2.05 log CFU/g (avg. 2.05 log CFU/g), 0.60 to 2.68 log CFU/g (avg. 1.60 CFU/g), and 0.41 to 2.90 log CFU/g (avg. 1.69 CFU/g), respectively. Cheese pH and %SMP most affected pathogen growth, with pH having a dominant effect. Pathogen growth/no-growth varied within some cheese types or lots. Except for Swiss-type cheeses, mold- or bacterial-ripened cheeses, and cheeses made with non-bovine milk where insufficient data exists, the pathogen growth/no-growth interface could be modeled and boundary conditions established for safe, extended storage (≤25°C) of cheeses based on pH and %SMP.

INTRODUCTION

Temperature-dependent storage of most cheeses has three major roles – to allow for curing/ripening of cheeses that contain added or indigenous bacteria and enzymes, to prevent quality defects, and to control pathogen growth (*3*). The 2009 US Food and Drug Administration (FDA) Food Code (*40*) defines a potentially hazardous food as a food that requires time/temperature control to limit the growth of pathogenic microorganisms or toxin formation. In this publication, potentially hazardous foods are also designated as Time/Temperature Control for Safety (TCS) foods. This latter designation has been adopted in the 2013 Food Code (*45*). In both versions of the Food Code, foods with a pH of <4.2 and any $a_{w,}$ or a_w of <0.88 and any pH are not considered potentially hazardous. Foods considered potentially hazardous, unless shown to be safe by a product assessment, fall into one of the following categories: $a_w \geq 0.88$ and pH >5.0, a_w >0.90-0.92 and pH >4.6, or a_w >0.92 and pH >4.2. The Food Code indicates that TCS foods must be maintained at ≤5°C, or, if placed outside refrigeration, can be stored for up to 6 h at a temperature no greater than 21°C, after which the product must be discarded.

The composition of many cheeses, when evaluated using the Food Code criteria, places them into the category of TCS foods, thus limiting the ability of retailers to market the cheeses under room-temperature conditions which could enhance cheese flavor and aroma (*12*). The Food Code-mandated time and temperature control may also limit industry flexibility in the transportation, handling, and storage of cheeses. It has, however, been suggested that the biochemical changes that occur during cheese ripening create an environment hostile for pathogen growth, and that time/temperature control of some cheese is primarily needed to maintain the organoleptic quality of cheese, not to maintain safety (*3*). Bishop and Smukowski conducted a thorough review of the literature available up until 2006 and recommended that cheeses meeting certain criteria, e.g. cheeses manufactured in the US with pasteurized or heat-treated milk ($\geq$63°C for $\geq$16 sec), cheeses manufactured following Good Manufacturing Practices and under the principles of HACCP (Hazard Analysis and Critical Control Points), and cheeses manufactured meeting standards of identity outlined in 21 CFR (Code of Federal Regulations) part 133 (*43*), should be exempted from refrigeration requirements during ripening, storage, shipping, and display (*3*). Bishop and Smukowski recommended that the following cheeses could meet these criteria: Asiago (medium and old), Cheddar, Colby, Feta, Monterey Jack, Muenster, Parmesan, Pasteurized process, Provolone, Romano, and Swiss/Emmentaler.

In order to establish whether a particular food, e.g. cheese, can be exempted from TCS-requirements, the Food Code allows processors or retailers to conduct a microbial challenge study in order to assess the ability of a food product to inhibit pathogenic bacterial growth or inactivate these microorganisms. The Food and Drug Administration (FDA) has outlined parameters for conducting such challenge studies (*44*).

When experts consider the major microbiological hazards across the food supply, the risk of bacterial illness from dairy products such as milk and cheese can be attributed primarily to *Listeria monocytogenes*, *Yersinia entercolitica*, *Campylobacter* spp., and non-typhoidal *Salmonella* spp. (*2*). Between 1990 and 2011, there were 105 reported foodborne illness outbreaks in the US, with over 2000 illnesses, linked to cheese/cheese products in the US (*11*). Pathogens linked to these cheese-related outbreaks included *Salmonella* spp. (37 outbreaks), *Listeria monocytogenes* (16 outbreaks), pathogenic *Escherichia coli* (6 outbreaks), *Staphylococcus aureus* (4 outbreaks), Norovirus (21 outbreaks), *Campylobacter* spp. (9 outbreaks), and *Brucella* spp. (5 outbreaks) (*11*). Among the 105 outbreaks, 17 were linked to cheeses made with pasteurized milk, 30 were linked to cheese made with raw milk, and the pasteurization status of cheeses involved in the remaining 58 outbreaks was unspecified. The pathogenic bacteria primarily responsible for foodborne illness outbreaks linked to cheese manufactured with pasteurized milk were *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7. Cheeses implicated in these outbreaks included process cheese, Mozzarella, and Mexican-style cheeses (*7, 11*). The low incidence of *S. aureus*-linked outbreaks related to cheese is presumed to be due to the low incidence of this pathogen in pasteurized milk, and the growth characteristics of this bacterium (*21*). However, *S. aureus* is commonly carried by humans and thus could contaminate cheese during post-pasteurization handling (*16*). *S. aureus* is also the bacterial pathogen considered to have the highest tolerance to reduced-moisture conditions or increased salt concentration (*22*), and therefore could be considered a target pathogen in determining the safety of cheese contaminated post-processing and stored for extended periods of time at room temperature.

The goal of this project was to evaluate survival of strains of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* on natural market cheeses during extended storage at 25°C, and to determine the effect of cheese compositional factors such as pH, a_w, and %salt on pathogen survival. Pathogen-survival data from laboratory research and data from published literature were then combined in order to model the boundary conditions for pathogen growth / no-growth during storage of cheese at room temperature.

MATERIALS AND METHODS

Cheeses. Sixty-seven cheeses were purchased from local retail establishments or obtained directly from the manufacturer and stored at 4°C. Cheeses studied were Asiago (aged, young), Brick (2 brands), Cheddar (mild, regular, sharp), Cheddar-Mozzarella, Colby, Colby-Jack, Farmer's, Feta, Gouda, Gruyere, Havarti (2 brands), Jack (goats' milk), Monterey Jack, Muenster (2 brands), Parmesan, Pepper Jack (2 brands), Provolone (mild, regular; 2 brands sharp), Provolone-Mozzarella, Queso Blanco, Queso Fresco, Queso Quesadilla, String cheese (2 brands), Swiss (Baby, 2 brands; Lacey, regular), reduced-fat cheeses (Cheddar, Colby-Jack, Provolone) and reduced-sodium cheeses (Colby Jack, Provolone). Where a type of cheese was tested more than once, tested cheeses were from different brands and/or from different production dates of the same brand. All cheeses were manufactured in the United States from pasteurized milk (Table 1, 2).

Proximate analysis. The cheeses tested in this study were characterized by % moisture, % salt, and a_w at the beginning of each trial. Changes in both % titratable acidity (%TA) and pH were anticipated over time; thus pH was measured on pathogen-inoculated cheeses at every sampling time (days 0, 3, 6, 9, 12 and 15), and %TA was measured on un-inoculated cheeses on days 0, 6 and 15. Duplicate trials were performed for each compositional analysis, and average values were reported.

Moisture (%) was determined using a standard method (*4*) by drying a representative 3-g sample at 100°C for 5 h in a vacuum oven maintained at -98kPa throughout the drying process (M.D.O. Vacuum Oven, Model 3623, Lab-Line Instrument Inc., Melrose Park, IL). Salt (%) was determined by titration of chloride using the silver titration standard method (*4*). For each trial, a representative 5-g sample was diluted with distilled water 1:20 (w/v) and % chloride was determined according to the standard method using a Model M926 Chloride Analyzer (Nelson Jameson, Marshfield, WI). The % chloride content was automatically calculated by the analyzer and expressed as mg%/liter of sodium chloride, which was converted to % salt by multiplying the appropriate dilution and conversion factors. Salt (%) and moisture (%) of an individual cheese sample were used to calculate % salt-in-moisture-phase (%SMP) using Equation 1:

$$\%SMP = (\%\ salt \times 100) / (\%\ salt + \%\ moisture) \quad (1)$$

Water activity (a_w) was determined for each cheese at the beginning of each trial using an AquaLab LITE water activity meter (Decagon Devices Inc., Pullman, WA) according to a standard method (*1*). Titratable acidity (%) was monitored during storage (days 0, 6, and 15) according to a standard method (*4*). Briefly, for each cheese/trial, one sample (10.0 $\pm$ 0.5 g) that had been manually crumbled was automatically blended with 50-ml distilled water and titrated using a Model DL22 Automatic Titrator (Mettler Toledo, Schwerzenbach, Switzerland), which was set to calculate % titratable acidity (%TA) using the molecular weight of lactic acid. To determine the impact, if any, of the presence of inoculum bacteria or growth of indigenous bacteria on cheese pH, the surface pH was measured for individual inoculated cheese slices at each sampling time (days 0, 3, 6, 9, 12, and 15) using an Accumet AB15 pH meter equipped with a flat surface combination electrode (Fisher Scientific, Itasca, IL).

Inoculum preparation. Ten strains of *L. monocytogenes,* six strains of *Salmonella* spp., five strains of *E. coli* O157:H7, and five strains of *S. aureus*, representing a wide variety of sources and serotypes, were used in this study (Table 3). Stock cultures were maintained at −20ºC in brain heart infusion broth (BHIB; Difco, Becton Dickinson, Sparks, MD) with 10%

(wt/vol) added glycerol (Fisher). Fresh working cultures were prepared monthly by thawing stock cultures and streaking for isolation as follows: *L. monocytogenes* on Listeria Selective agar (LSA; Oxoid, Ogdensburg, NY) with added Listeria Selective Supplement (Oxford formulation, Oxoid), *Salmonella* and *E. coli* O157:H7 on modified Levine's Eosin Methylene Blue agar (m-LEMB), prepared from lactose-free LEMB agar (Difco) with the addition of 10 g/liter D-sorbitol (Fisher) and 5 g/liter NaCl (Fisher); and *S. aureus* on Baird-Parker agar (BP; Difco) with added egg yolk Tellurite enrichment (Difco). Working culture plates were incubated for 24 h at 35°C for *Salmonella* spp. and *E. coli* O157:H7, and 48 h at 35°C for *L. monocytogenes* and *S. aureus*, whereupon all cultures were observed for consistent colony morphology and stored at 4°C for <40 days. Inoculation cultures were prepared for individual strains by transferring a single colony of each strain into a separate tube containing 9 ml of Nutrient broth (NB; Difco) for *L. monocytogenes*, or BHIB for *Salmonella* spp., *E. coli* O157:H7 and *S. aureus*. Preliminary cheese challenge studies showed better survival of *L. monocytogenes* over 15 days at 25°C on Cheddar and Swiss cheeses when inocula had been grown in NB, while the other three pathogens survived better on cheeses when inocula had been grown in BHIB (n=2, data not shown). Following incubation for 20 to 24 h at 35ºC, 1 ml of stationary-phase culture of each strain for a designated pathogen (10^8 CFU/ml for *L. monocytogenes*, and 10^9 CFU/ml for *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*) was transferred to a sterile 9-ml tube to produce a single-pathogen, multi-strain cocktail. Each pathogen cocktail was mixed by vortexing and diluted, as necessary, to produce a starting inoculum cocktail of 10^7 CFU/ml. Pathogen levels in the cocktails were estimated by plating the inocula on brain heart infusion agar (BHIA; Difco) and incubating at 35°C for 24 h.

Sample inoculation. The working surface of a biosafety cabinet was sterilized with 70% (v/v) ethanol and covered with aluminum foil prior to cheese inoculation. Cheese slices (approx. 25-30 g, approx 70-80cm^2) were placed on the aluminum foil aseptically, six cheese slices per trial. An aliquot (0.1ml) of a single-pathogen cocktail (10^7 CFU/ml) was pipetted onto each of the six cheese slices. An L-shaped spreader was used to evenly distribute the inoculum over the surface of the six slices, then samples were left to air-dry under the hood for 15 min to allow bacterial attachment and evaporation of excess liquid. The a_w values of control and air-dried inoculated samples were not significantly different (n=3; p>0.05; data not shown). Inoculated cheese slices were folded into half, with the inoculated cheese surfaces facing inward. Folded cheese samples were weighed, then individually vacuum-packaged in standard retail barrier bags (B-2175; Cryovac Food Packaging and Food Solutions, Duncan, SC) and stored at 25°C for up to 15 days. Oxygen transmission rate for the bags was 3-6 cm^3/m^2 at 40˚F in 24 h. The initial inoculum level on each cheese slice was ~10^5 CFU/g.

Sampling and enumeration. Packaged cheese samples were analyzed following inoculation (time 0) and throughout storage for up to 15 days. Every three days, one cheese slice per pathogen was removed from incubation, the storage/barrier bag was aseptically opened, and Butterfield's phosphate diluent (BPD; Nelson Jameson, Marshfield, WI) was added to create a 1:10 (w/w) dilution. The cheese/diluent mixture was stomached in the bag (AES Smasher, AES Chemunex, Bruz, France) for 2 min at high speed. Stomached samples were serially diluted in BPD, and 0.1-ml portions were spread-plated onto LSA, m-LEMB, m-LEMB, and BP for cheeses inoculated with *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*, respectively. A preliminary trial confirmed better recovery of *Salmonella* spp. by plating on m-LEMB rather than on Xylose Lysine Desoxycholate agar (XLD; Difco), and better recovery of *E.*

coli O157:H7 by plating on m-LEMB rather than on Sorbitol MacConkey agar (SMAC; Difco). Inoculated samples were also spread-plated on deMan-Rogosa-Sharpe agar (MRS; Difco) at 0, 6, and 15 days to monitor changes in lactic acid bacteria (LAB) populations during storage, and to thereby investigate the impact, if any, of indigenous, starter, or adjunct bacterial growth on inoculum survival. The m-LEMB spread-plates were incubated 24 h at 35ºC, LSA and BP plates 48 h at 35ºC, and MRS plates 72 h at 35ºC, after which time counts were recorded for each plate, with countable plate counts converted to log CFU/g. On m-LEMB, typical colonies of *E. coli* O157:H7 appear colorless to pink, while colonies of *Salmonella* spp. are dark red-black with metallic green sheen. Colonies of *S. aureus* are typically shiny black and surrounded with clear zone on BP agar. *L. monocytogenes* colonies are normally grey in color surrounded by black halo on LSA. Data were used to calculate Δlog CFU/g, relative to time 0, over the 15-day storage period for each pathogen/cheese combination.

Literature data search and selection. To provide additional data to augment our product assessment, data from published literature were combined with data from this study. In searching for relevant published studies, keywords including, but not limited to, “pathogen, survival, cheeses, temperature, pH, salt” were entered into online scientific databases. Reference lists of publications were also screened for relevant studies with appropriate data. Published challenge studies that met the following criteria were selected: (i) the inoculated cheeses were made with pasteurized cow’s milk, (ii) the cheeses were inoculated with at least one of the pathogens: *L. monocytogenes, Salmonella, E. coli* O157:H7, or *S. aureus,* (iii) the pathogen(s) was inoculated on the finished cheese, and (iv) inoculated cheeses were stored at 20-30˚C. Studies with surface-ripened, mold-ripened, Swiss, or processed cheeses, or cheese made with non-bovine milk were excluded. Of 155 studies published between 1959 and 2012 and which investigated pathogen behavior in or on cheeses, six published studies met the criteria (*14*, *24*, *25*, *33*, *34*, *39*). From each publication, the following information was extracted: type of cheese, temperature and length of storage, type and number of pathogen strains, composition (all available information for pH, a_w, % moisture, %SMP, % TA) of cheeses and behavior (growth vs. no-growth) of pathogen(s) (Table 4).

Evaluating compositional characteristics affecting pathogen growth. The relationship between compositional factors and behavior of pathogens on cheeses was explored. Compositional factors of cheese: moisture (%), initial pH, %SMP, a_w, and initial TA (%), were paired, i.e. one compositional factor as "x" and one as "y", and a growth vs. no-growth outcome was plotted for each cheese as a function of the x and y values to analyze the influences of the paired compositional factors on pathogen growth. Values of compositional factors were normalized to a 100-point scale before plotting as follows: for each compositional factor, the minimum value of the data set was subtracted from the observed value and the total was divided by the range of the values and multiplied by 100 to obtain the normalized value, as shown in Equation 2.

$$\text{Normalized value} = [(\text{value} - \text{minimum value})/\text{range}] \times 100 \qquad (2)$$

In this analysis, a "growth" result was indicated for a cheese when the Δ-log CFU/g for any cheese/pathogen combination over the 15-day storage period was a positive value that exceeded the pathogen-specific plating variability: 0.39, 0.41, 0.27, and 0.25 log CFU/g for *L. monocytogenes*, *Salmonella* spp. *E. coli* O157:H7 and *S. aureus*, respectively. The growth / no-growth outcome plot from each pair of compositional factors was inspected and compared with predictions from a logistic regression equation (SAS 9.2, SAS Institute, Cary, NC). A model at

P=0.05 based on the variables pH and %SMP was generated according to the method of McMeekin et al. (*29*) (Figure 1).

RESULTS AND DISCUSSION

In this study, 67 cheese samples, representing a variety of national brands, were tested for their ability to support pathogen growth during extended storage at 25°C (Table 1, 2). Cheeses were manufactured using pasteurized milk in facilities meeting applicable federal and state food safety regulatory requirements. Cheeses met a standard of identity, where applicable. Among the 67 cheese samples tested, 52 were duplicate samples of cheeses from different lots/production dates of the same brand. The majority of cheeses that were tested in this study would be labeled as ‘hard’ or ‘semi-hard’ cheeses according to FDA classification (*43*), and were expected to be safe for extended room-temperature storage due to reduced moisture level and low pH. Trials on ‘soft’ cheeses with higher moisture were also included in this study in order to clarify compositional differences affecting pathogen growth/no-growth outcomes. Inoculated cheeses were vacuum packaged to prevent mold growth and moisture loss which could inhibit pathogen growth.

The FDA, in its guide to microbial challenge testing, notes that it can be important to evaluate a range of intrinsic factors which can influence the safety of a food during its intended shelf life (*44*). Compositional factors in cheese that could influence pathogen behavior were analyzed: surface pH (Day 0, 3, 6, 9, 12, 15), % moisture, % salt, and a_w (Day 0); and % TA (Day 0, 6, 15). Change in lactic acid bacteria (LAB) count was determined on Day 0, 6, and 15. Across all cheese samples, moisture content ranged from a low of 32.07% to a high of 57.64%, for one lot of Gruyere and Feta cheese, respectively. Salt content ranged from 0.33% for one lot of Lacey Swiss to 3.30% for Queso Blanco. Salt-in-moisture phase (%SMP) was calculated from % moisture and % salt (Equation 1) with values ranging from 0.73% for one lot of Lacey Swiss to 7.21% for one lot of Parmesan. Water activity (a_w) varied little across the cheese samples tested, ranging from 0.96 to 0.99, except for Parmesan (average a_w=0.93) (Table 1).

Cheese pH measured at the surface, ranged from 4.33 to 6.49 for Feta (average of two lots) and Queso Fresco, respectively (Table 1, 2) on Day 0. Over the 15-day storage period, change in pH ranged from -1.44 to +0.53 pH units, for Queso Fresco and Baby Swiss (average of 4 lot), respectively, with most cheeses exhibiting only slight change in pH. To quantify the amount of organic acid present in each cheese at the beginning of storage and to determine the effect, if any, of storage on changes in organic acid level, % TA was measured (Table 1, 2). The %TA across the cheeses tested ranged from 0.26% to 2.83% for Queso Blanco and Feta (average of 2 lots), respectively, at the beginning of storage. Change in %TA over storage was not clearly linked with change in pH and bacterial survival (data not shown). Change in LAB count in cheese samples was estimated during extended storage at 25°C storage (Table 1, 2). LAB count on Day 0 across the cheeses ranged from 2.00 to 8.08 log CFU/g for one lot of Pepper Jack and Monterey Jack, respectively. Initial LAB counts on similar cheese samples from different brands, or different lots of the same brand, could vary widely. The Day 0 count for LAB on different lots of Provolone (reduced-fat) (Brand 3) varied by 3.25 log CFU/g between purchase dates. Similarly, one sample of Provolone (Brand 3) had one of the lowest Day 0 LAB counts, 2.70 log CFU/g, while another sample of a different brand of Provolone (Brand 4) had one of the highest initial LAB counts, 7.70 log CFU/g. The Day 0 LAB counts for the two samples of Brand 3 Provolone were 2.70 log CFU/g and 3.78 log CFU/g, and these rose to 5.40 log CFU/g and 7.19 log CFU/g, respectively; equivalent to a Δ-log of 2.70 and 3.41 log CFU/g, respectively. The

LAB count for the one lot of Brand 4 Provolone increased by one order of magnitude, from 7.70 log CFU/g (Day 0) to 8.70 log CFU/g (Day 15). Throughout the storage period and across all cheese samples tested, changes in LAB count ranged from -2.92 CFU/g for one lot of Parmesan to +5.66 log CFU/g for one lot of Pepper Jack (Brand 4). Of the 67 cheese samples tested, LAB population increased on storage in 47 cheese samples tested. LAB count was relatively constant ($0 < \Delta \log \leq 0.3$ log CFU/g) in 7 cheese samples tested, and declined ($\Delta \log \geq -0.3$ log CFU/g) in 13 other cheese samples during storage.

Cheeses were tested for their ability to support growth of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* (Table 1, 2). Pathogens did not grow on 53 cheese samples over the 15 days (Table 1), while 14 cheese samples supported growth of *S. aureus*, six of *Salmonella*, four of *L. monocytogenes*, and three of *E. coli* O157:H7 (Table 2). The pattern of pathogen survival for each cheese lot was consistent over storage except for Queso Quesadilla (Table 2). We observed growth of *S. aureus* (+0.57 log CFU/g) at Day 6 on Queso Quesadilla however by Day 15 we noted a decrease in pathogen population (overall Δ log =-0.40 log CFU/g). Of the cheese samples which did support pathogen growth, all supported growth of *S. aureus*, ranging from 0.57 to 3.08 log CFU/g (avg. 1.62 log CFU/g across all 14 cheeses). Growth of *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7, ranged from 0.60 to 2.68 log CFU/g (avg. 1.60 log CFU/g), 1.01 to 3.02 log CFU/g (avg. 2.05 log CFU/g), and 0.41 to 2.90 log CFU/g (avg. 1.69 CFU/g), respectively. Cheese samples which supported growth of *S. aureus* included Farmer's, Gruyere (2 lots), Jack (goat's milk), Muenster (Brand 6), Provolone (Brand 3; 2 lots), reduced-sodium Provolone (2 lots), Queso Blanco, Queso Fresco, and 2 brands of String cheese. The six cheeses that supported growth of *Salmonella* spp. included: Gruyere (2 lots), Jack (goats' milk), Muenster (Brand 6), Queso Fresco, and one brand of String cheese

(Brand 14). The four cheeses that supported growth of *L. monocytogenes* included: Gruyere (one lot), Queso Blanco, Queso Fresco, one brand of String cheese (Brand 14), and the three cheeses that supported growth of *E. coli* O157:H7 included: Muenster (Brand 6), Queso Fresco, and String (Brand 14).

Among the cheeses which supported pathogen growth at some point during the 15-day storage period, seven supported only the growth of *S. aureus*: Farmer's, Provolone (Brand 3; 2 trials), reduced-sodium Provolone (Brand 6; 2 trials), String cheese (Brand 6) and Queso Quesadilla (at Day 6 sampling point only) (Table 2). Three cheeses supported the growth of *S. aureus* and one other pathogen: one lot of Gruyere and Jack (goats' milk) cheese each supported the growth of *S. aureus* and *Salmonella*, while Queso Blanco supported the growth of *S. aureus* and *L. monocytogenes*. Two cheeses supported the growth of three pathogens: one lot of Gruyere supported the growth of *L. monocytogenes*, *Salmonella* spp, and *S. aureus*, and one lot of Muenster (Brand 6) supported the growth of *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* (Table 2). There were two cheeses which supported growth of all four pathogens, Queso Fresco and one brand of String cheese (Brand 14).

Salmonella spp., *L. monocytogenes* and *E. coli* O157:H7 have, in recent years, been implicated in foodborne illness outbreaks linked to cheeses made with pasteurized milk (*7, 11*). *S. aureus* has not often been associated with foodborne illness outbreaks linked to cheese, even though this pathogen is generally linked to foods, like cheese, which are often hand-manipulated during processing and packaging (*8, 42*). We included *S. aureus* in the study design not only because of its link to poor sanitation and post-processing contamination but also because it is the pathogen most likely to grow in or on foods with reduced moisture and/or low a_w (*21*). For ready-to-eat food products, the FDA has established a zero-tolerance policy for *L.*

monocytogenes, *Salmonella* spp., and *E. coli* O157:H7, due to the potentially low infectious dose of *E. coli* O157:H7 and *Salmonella* spp, and the high mortality rate (15-30%) associated with *L. monocytogenes* infections (*41*). Although none of these pathogens should be present in finished cheeses made from pasteurized or heat-treated milk, the composition of a cheese supporting growth of any of these bacteria during extended room-temperature storage presents an unacceptable risk. A zero-tolerance policy is not in place for *S. aureus* in ready-to-eat foods because staphylococcal food poisoning occurs as a result of ingestion of a preformed enterotoxin which is only produced in amounts sufficient to cause illness as a result of extended temperature abuse and growth of the pathogen to a high concentration (~10^5 CFU) (*30*). Thus a cheese with compositional characteristics allowing growth of *S. aureus* during storage is also an unacceptable risk. For these reasons, growth of four target pathogens: *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*, as post-processing contaminants on cheeses was investigated.

Pathogen strains used in this study represented a variety of sources and serotypes (Table 3). The strains of *L. monocytogenes* and *Salmonella* spp. had been screened in previous research in our laboratory to confirm tolerance to salt and pH conditions typical of cheese (*13*). Strains of *E.* coli O157:H7, *Salmonella* spp., and *S. aureus* were exposed to acid during inoculum preparation in BHIB, as a pH drop of ~ 1 unit was observed during overnight incubation. *L. monocytogenes* was grown in NB, with no acid production or pH drop during inoculum preparation. Where it occurred, the slight exposure to acid during inoculum preparation was unlikely to have led to acid adaptation of strains. Therefore, the key characteristic of strains selected for use in this study was their human or animal/animal-product origin, making these strains perhaps representative of organisms to be found in a food processing or handling environment.

Growth of *L. monocytogenes* was observed on four cheese samples: Gruyere, Queso Blanco, Queso Fresco, and one brand of String cheese (Brand 14) (Table 2), ranging from 0.60 to 2.68 log CFU/g. Growth of *L. monocytogenes* on Muenster (Brand 6, 0.17 log CFU/g) and String (Brand 6, 0.22 log CFU/g) did not exceed the plating variability for the pathogen (0.39 log CFU/g) and 'growth' was not declared. Genigeorgis et al. studied the survival of *L. monocytogenes* on 11 different types of market cheeses stored at 30°C (*14*). Pathogen growth was observed only on Hispanic-style cheeses: Queso Fresco, Queso Ranchero, and Queso Panela, and ranged from 0.38 to 3.18 log CFU/g (*14*). Uhlich et al. observed an increase of more than 5 log CFU/g of *L. monocytogenes* on Queso Blanco stored at 25°C for up to 6.25 days (*39*). In the present study, we observed growth of *L. monocytogenes* on one brand of String cheese (Brand 14) that slightly exceeded the plating variability, i.e. the observed growth of 0.60 log CFU/g exceeded the plating variability of 0.39 log CFU/g. Genigeorgis et al. (*14*) did not observe growth of *L. monocytogenes* on String cheese, instead noting a drop in *L. monocytogenes* population of 2.36 log CFU/g over 9 days at 30°C. The String cheese that Genigeorgis et al. tested had similar pH and %SMP values to the cheese sample that we evaluated, but an unknown level of LAB. The String cheese sample in our study allowing some growth of pathogen simultaneously supported a dramatic increase in LAB population, from 4.87 log CFU/g at Day 0 to 8.86 log CFU/g by Day 15 (Table 2).

Growth of *L.* monocytogenes was not observed on 63 samples of cheese tested (Table 1, 2), many of the cheeses which did not support pathogen growth would be classified as 'hard' or 'semi-hard' cheeses based on FDA classification (*43*) and may be suitable for extended room temperature storage. Shrestha et al. (*33*) did not observe growth of *L. monocytogenes* on a range of Cheddar-type cheeses stored at 21°C for 30 days, with counts of *L. monocytogenes* dropping by ≤ 1.1 log CFU/g during storage. We also observed a slight decrease in the population of *L. monocytogenes* on mild, reduced-fat, and sharp Cheddar cheeses during storage at 25° (Table 1). Pathogen populations decreased from 0.00 to 0.76 log CFU/g across samples and Cheddar cheese-type tested. Genigeorgis et al. also reported a decrease of *L. monocytogenes* population on mild Cheddar cheeses during storage (*14*). Similarly, Genigeorgis et al. evaluated the growth of *L. monocytogenes* on Monterey Jack, Colby, Provolone, Muenster, and Feta cheeses during storage, and observed a decrease in pathogen population of >1-2 log CFU/g in all cases. In our study, we noted an average decrease in pathogen population of 0.2 log CFU/g for Colby, 4.74 log CFU/g for Feta, 1.83 log CFU/g for Monterey Jack, 0.25 log CFU/g for Muenster (Brand 3), and 0.99 log CFU/g for several different types of Provolone (regular, mild, sharp) (Table 1, 2). Two lots of Provolone (Brand 3) which supported growth of *S. aureus* did not support the growth of *L. monocytogenes* (Table 2). One brand of Muenster (Brand 6) appeared to support a slight growth of *L. monocytogenes* during storage (0.17 log CFU/g), but this was found not to exceed the plating variability associated with this pathogen (0.39 log CFU/g), and thus ‘no growth’ was declared.

Growth of *Salmonella* was observed on six cheeses: Gruyere, Jack (goat’s milk), Muenster (Brand 6), Queso Fresco, and String (Brand 14), ranging from 1.01 to 3.02 log CFU/g over 15 days. Slight growth of *Salmonella* was also observed for Brand 6 of String cheese (0.39 log CFu/g) but this was below the plating variability for this pathogen (0.41 log CFU/g), and therefore counted as ‘no growth.’ Kasrazadeh and Genigeorgis (*25*) studied the growth of *Salmonella* inoculated onto sliced Queso Fresco stored at 20°C. They noted rapid growth, a lag time of 2.5-3.5 h and a generation time of 1.65-2.17 h, for *Salmonella* on Queso Fresco. We observed an increase in *Salmonella* concentration of 3.02 log CFU/g on Queso Fresco stored at

25°C over 15 days. This was the highest level of *Salmonella* growth observed over all 67 cheese samples tested.

There were 61 cheeses which did not support the growth of *Salmonella* in this study. Shrestha et al. (*34*) examined the survival of *Salmonella* on a range of Cheddar-type cheeses stored for up to 30 days at 21°C. Cheddar cheese manufactured to standards of pH and salt was comminuted, inoculated with *Salmonella* spp., and stored at 21°C for up to 30 days. *Salmonella* spp. counts decreased significantly at 21°C for all cheese-types. We evaluated the survival of *Salmonella* spp. on mild, reduced-fat, and sharp Cheddar cheeses and observed average decreases of 0.3, 1.12, and 1.26 log CFU/g, respectively, for the brands tested. Growth of *E. coli* O157:H7 was observed on three cheeses: Muenster (Brand 6), Queso Fresco, and String (Brand 14), ranging from 0.41 log CFU/g (Muenster) to 2.90 log CFU/g (Queso Fresco) over 15 days. Kasrazadeh and Genigeorgis (*24*) also observed rapid growth of *E. coli* O157:H7 on Queso Fresco stored at 20°C. There were 64 cheese samples in this study which did not support the growth of this pathogen.

The survival pattern for pathogens on cheeses was consistent during storage, with the exception of the survival of *S. aureus* on Queso Quesadilla which increased by 0.57 log CFU/g on Day 6 of storage, but decreased by 0.40 log CFU/g relative to the time-zero level by Day 15. In all other cases, pathogen growth/no-growth was consistent, displaying an increase or decrease over the 15-day storage period. LAB count increased in 47 of 67 cheeses tested in this study. With one exception, cheeses which supported pathogen growth also supported LAB growth. LAB count decreased in Jack (goat's milk) cheese which supported growth of *Salmonella* (+2.50 log CFU/g) and *S. aureus* (+1.62 log CFU/g); otherwise LAB count increased from 1.54 to 4.47 log CFU/g in cheeses which supported pathogen growth. The level of inoculum on each cheese slice at time 0 averaged 4.7 log CFU/g (n=268). This level allowed for accurate enumeration of growth or death without reaching the limits of research methodology. This inoculum level could have placed pathogens at a level to effectively compete with active indigeneous organisms. LAB count on Day 0 averaged 5.03 log CFU/g for cheeses which supported pathogen growth (n=14, Table 2). While previous studies have shown that initial inoculum level does not affect the survivability or growth kinetics of pathogens (*6*, *26*, *46*), a higher proportion of *S. aureus* compared to LAB may aid in the survival of this particular pathogen (*17*). Although growth of *S. aureus* is reported to be weak when a high load of competitive bacteria, e. g. lactic acid bacteria (LAB) is present, increasing the proportion of *S. aureus* to LAB has been shown to aid in survival of this pathogen (*17*, *23*).

The change in pH on storage among cheeses that supported pathogen growth showed no clear trend, remaining the same (Δ pH $\leq$ 0.3 units) in 7 samples, and increasing in 6 samples (Table 2). Cheese samples that supported pathogen growth had %TA which ranged from 0.26 % to 1.67 % at the beginning of storage (Table 2); while cheese samples which did not support pathogen growth had %TA ranging from 0.66% to 2.86% at the beginning of storage (Table 1). Change in %TA over storage (data not shown) had no apparent relationship with the change of pH and LAB count. Among cheeses that supported pathogen growth, LAB count increased in all but one sample (Jack (goats' milk cheese)), with an increase ranging from 1.54 log CFU/g for one lot of reduced sodium Provolone to 4.43 log CFU/g for Farmer's cheese. Correlation between changes in pH and LAB count in cheeses was weak (r^2=0.25).

A total of 53 cheeses did not support the growth of any pathogen tested. These cheeses were most notably characterized by lower pH; there was little difference in % moisture and %SMP between these cheeses and those that supported pathogen growth. When cheese samples were separated into roughly equal groups by initial pH value: 4.29 - 5.20 (29 cheeses), 5.21 – 5.40 (18 cheeses), and 5.41 – 6.50 (20 cheeses), it was readily apparent that pathogen growth was better supported on higher pH cheeses. With the exception of Provolone (Brand 3; pH 5.15) and reduced-sodium Provolone (pH 5.15), cheeses with Day 0 pH ranging from 4.8-5.2 did not support growth of any pathogens (Table 1). Feta was the most acidic cheese tested (average pH 4.33, n=2 lots), and pathogen viability on this cheese type decreased over time more than for any other cheese with average reductions of 4.74 log CFU/g for *L. monocytogenes*, 4.82 log CFU/g for *Salmonella* spp., 4.34 log CFU/g for *E. coli* O157:H7, and 3.84 log CFU/g for *S. aureus.* As pH increased to 5.21 - 5.40, four of 18 cheeses supported growth: Provolone (Brand 3; 1 lot), reduced-sodium Provolone (1 lot), String cheese (Brand 6) and Queso Quesadilla; all supporting the growth of *S. aureus* (average 1.14 log CFU/g across all 3 cheeses), but no other pathogen (Table 2). In the pH range 5.41 - 6.50, eight cheeses supported pathogen growth: Jack (goats' milk) (pH 5.41), String (Brand 14, pH 5.44), Farmer's (pH 5.46), Muenster (pH 5.48), Gruyere (2 lots; pH 5.68; 6.28), Queso Blanco (pH 6.37), and Queso Fresco (pH 6.49). Pathogen growth on Queso Fresco was the greatest across all cheeses - tested; this was also the cheese with the highest initial pH. Cheeses with an initial pH $\geq$5.46 supported growth of at least one pathogen, with the exception of Swiss-style cheeses (Baby Swiss, Swiss, Lacey Swiss – pH range 5.50 – 6.02), and one lot of Havarti (pH 5.49) which did not support growth. Optimal pH for growth of *S. aureus* is between pH 6.0 and 7.0, with pH 4.0 as the reported minimum for growth (*20*). Minimum pH values for growth that have been reported for *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 are 4.39, 4.20, and 4.40, respectively (*20*). Only Feta cheese (pH 4.29, 4.38) was below the reported minimum pH for growth of any of the pathogens tested.

The average moisture content for cheese samples which supported growth (43.11%) varied little from moisture content for cheese samples which did not support growth (40.38%) (Table 1 ,2). An even narrower difference in -average SMP was observed between cheeses which supported growth (3.76%) and cheeses which did not support growth (3.52%) (Table 1, 2), however the range of values in each category (growth/no-growth) was much wider, ranging from 0.73 to 7.21 %SMP for cheese samples which did not support growth, and from 2.26 to 6.56 %SMP for cheese samples that did. The greater growth potential that we observed for *S. aureus* on cheeses could be attributed, in part, to the high salt-tolerance of this pathogen. Nunheimer and Fabian reported that some strains of *S. aureus* are able to tolerate up to 20% NaCl (*31*). Sutherland et al. (*36*) reported growth of *S. aureus* in BHIB with pH 4.48 and 8.5% NaCl at 25˚C. Ingham et al. reported greater tolerance of *S. aureus* than of *L. monocytogenes* to high salt- and low a_w in meat products stored at 21°C (*19*).

Where applicable, we tested cheeses from different brands, or from different lots within the same brand, to allow us to determine lot-to-lot or brand-to-brand variation for a similar type of cheese. For example, Muenster cheese from two manufacturers was tested; cheese from one brand (Brand 6, Table 2) supported growth of three pathogens, *S. aureus*, *Salmonella* spp., and *E. coli* O157:H7 (+0.41 to +1.77 log CFU/g;), while Muenster cheese from a different brand (Brand 3, Table 1) did not support growth of any pathogen (-0.00 to -0.75 log CFU/g). Among ten Provolone cheeses tested (mild, sharp, regular (3 lots from 2 brands), reduced-fat (2 lots), reduced-sodium (2 lots), and a Provolone-Mozzarella blend), six cheeses (2 lots of reduced-fat, regular, sharp, mild, and Provolone-Mozzarella blend) did not support growth of any pathogen (Table 1). The contribution of pH, %SMP, and other inhibitory compounds present in cheese, such as metabolites of LAB and the presence of free fatty acids may have varied from lot-to-lot, brand-to-brand, and between cheese types, resulting in differences in pathogen growth during non-refrigerated storage. The effect of these factors on microbial survival has been shown to be highly dependent on the concentration of inhibitory compound and the species and strain of both LAB and pathogen (*10, 15, 17, 35*). The apparent inconsistencies in pathogen growth patterns observed for cheeses of a similar type supports the assertion that it may be compositional characteristics, more than cheese type, that determine the likelihood of pathogen growth on a sample of cheese.

The compositional factors of pH, %SMP, a_w, and %TA were paired in all combinations and a pathogen growth / no-growth outcome for each cheese was plotted as a function of each pair of factors. Plotting growth / no-growth outcome as a function of pH and %SMP, combined with logistic regression, created a growth / no-growth interface that could be used to clearly differentiate cheeses which inhibited pathogen growth from those that allowed pathogen growth (Figure 1). A similar approach using other pairs of compositional factors was not successful in generating a clear growth / no-growth interface (data not shown). These results are consistent with those of Oh et al. who evaluated the effect of compositional factors of low-sodium Cheddar cheeses on the growth of strains of *Salmonella* spp., *L. monocytogenes*, *S. aureus*, and Shiga toxin-producing *E. coli* (STEC). In a model low-sodium Cheddar-cheese extract, STEC survived significantly better than the other three pathogens. Principal component analysis indicated that STEC survival was primarily determined by pH, and not by % salt or % lactate (*32*).

The eight Swiss-style cheese samples tested did not fit the pattern established by data from the other cheeses tested. These Swiss-style cheeses had the lowest %SMP (0.73-1.87%) of all cheeses tested, a relatively high pH (5.36-6.02), and a high a_w (0.98-0.99). Despite compositional factors which seem to be permissive for growth, none of the Swiss-style cheeses supported pathogen growth. Leyer and Johnson reported poorer survival of *Salmonella* spp. on Swiss cheeses than on Cheddar and Mozzarella (*27*). Swiss-style cheeses are unique among the types of cheeses that we tested due to the addition of propionic acid bacteria added as an adjunct culture in cheese manufacture. The added propionic acid bacteria can produce metabolites with antimicrobial properties, such as propionic acid, acetic acid, and diacetyl (*9*). Studies have shown greater antimicrobial properties linked to propionic acid (pK_a=4.87) as compared to lactic acid (pK_a=3.86) (*37*). The results of our study would suggest that target pathogens will not grow on Swiss-style cheeses during extended storage at 25°C, but the safety of such cheeses should be evaluated independently from cheeses which are fermented using only lactic acid-producing bacteria. Similarly, research suggests that the ability of pathogens to grow on bacterial surface-ripened or mold-ripened should be evaluated independently from cheeses manufactured without these ripening adjuncts. Bacterial surface-ripened and mold-ripened cheeses have added cultures that are capable of growing and altering the environment for pathogen growth. Growth of added bacterial and/or mold cultures can result in the production of antimicrobial compounds (e.g. bacteriocins) which could hinder pathogen growth, but can also lead to lactate metabolism which can subsequently increase cheese pH and enhance pathogen growth (*5*). Genigeorgis et al. found a significant reduction of *L. monocytogenes* (> -2.36 log CFU/g) when inoculated onto Limburger, a bacterial surface-ripened cheese (*14*). While the high pH of Limburger (pH 7.2) would suggest that this cheese could support pathogen growth, the growth of smear bacteria results in extensive lipolysis which produces a high concentration of free fatty acids, which are compounds known to have antimicrobial activity (*35*). Goats' milk cheese may also contain high levels of free fatty acids. Woo et al. evaluated the free fatty acid content in a variety of cheeses and concluded that Blue, Swiss, Limburger, and goats' milk cheeses contained high concentrations of free fatty acids (*47*). Thus, we conclude that surface-ripened cheeses, mold-ripened cheeses, and non-cow's milk cheeses, along with Swiss-style cheeses should be evaluated separately, perhaps by group, and more data gathered in order to assess their suitability for extended non-refrigerated storage.

Water activity (a_w) and pH are the two criteria used in the FDA Food Code to determine the shelf stability of food products (*45*). However, %SMP can be seen as a more appropriate factor than a_w in assessing the likelihood of pathogen survival on cheese. In addition to salt, other solutes in cheese such as non-protein nitrogen-containing compounds and products released during proteolysis, could contribute to the reduction of a_w, yet these compounds may not play a role in inhibiting pathogen growth (*28*). Tapia et al. (*38*) suggested that the usefulness of measured a_w as an indicator of microbial safety or stability is diminished by the 'specific solute effect'; that is that the solute in the food matrix dramatically alters the minimum a_w for microbial growth. Hilderbrand (*18*) supported %SMP as a more reliable factor than a_w in determining bacterial growth in smoked fish. In addition, %SMP is routinely determined and has historically been used in the cheese industry as a measure of product quality. Our search of published literature indicated that other researchers investigating survival of pathogens as post-processing contaminants on cheese routinely monitored %SMP (*14, 24, 25, 33, 34, 39*), while only a few studies investigating pathogen survival on cheese considered the impact of product a_w (*33, 34, 39*). Furthermore we identified that pH and %SMP were the two compositional factors which could be used to clearly differentiate cheeses which supported pathogen growth from those that inhibited growth (Figure 1), while the compositional factors of pH and a_w were not similarly effective.

Of the 67 market cheeses studied, 53 did not support the growth of *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7, or *S. aureus* and could safely be kept at ≤ 25°C for an extended period of time. The risk of pathogen growth for those cheeses which supported growth can be characterized as follows: *S. aureus* (growth on 14 of 14 cheeses supporting pathogen growth) >> *Salmonella* (growth on 6 of 14) > *L. monocytogenes* (growth on 4 of 14) > *E. coli* O157:H7 (growth on 3 of 14). Of several intrinsic compositional factors associated with cheese, i.e. pH, $a_{w,}$ %SMP, and %TA, cheese pH has the clearest effect on pathogen growth. Laboratory data was combined with relevant published research in order to expand our product assessment. Pathogen growth/no-growth outcomes for 82 cheeses, 56 cheeses tested in our laboratory and 26 cheeses for which published results were available in the literature, were plotted on a graph with axes of pH and %SMP. Logistic regression analysis generated a *P*=0.05 boundary line, which indicated a clear differentiation between cheese compositions (in terms of pH and %SMP) which supported pathogen growth and those which did not. Data from Swiss-type cheeses, mold-or bacterial-ripened cheeses, or cheeses made with non-bovine milk were excluded from this analysis due to insufficient data or lack-of-fit. The growth/no-growth interface established by the logistic regression line clearly shows that many common cheese types, if made from pasteurized cows' milk in compliance with US regulatory standards, can safely be considered non-TCS foods. Non-TCS cheeses should be described in terms of pH and %SMP rather than cheese-type or brand, and would include cheeses with pH/%SMP values more restrictive than any of the following combinations drawn from Figure 1 (in order of increasing pH): ≤4.60/≥0.24; 4.61-4.70/0.25-0.91; 4.71-4.80/0.92-1.58; 4.81-4.90/1.59-2.24; 4.91-5.00/2.25-2.91; 5.01-5.10/2.92-3.58; 5.11-5.20/3.59-4.25; 5.21-5.30/4.26-4.92; 5.31-5.40/4.93-5.59; 5.41-5.50/5.60-6.26; and 5.51-5.60/6.27-6.93. More research would be necessary to develop boundary conditions for safe, extended room-temperature storage of cheeses not covered in this model, including Swiss-type cheeses, bacterial surface-ripened or mold-ripened cheeses, cheeses made from non-bovine milk, or cheeses made from unpasteurized milk.

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Table 1. Composition of natural cheeses that did not support growth of *L. monocytogenes, Salmonella* spp., *E. coli* O157:H7, and *S. aureus* and pathogen survival (Δlog CFU/g) during storage for 15-days at 25°C.

Cheese[a]	Brand	% Moisture[b]	% Salt[c]	%SMP[d]	a_w[e]	pH[f] 0 d	pH[f] 15 d	%TA[g] 0 d	LAB count[h] 0 d	LAB count[h] 15 d	Pathogen survival (Δlog CFU/g)[i] LM	SALM	EC	SA
Asiago (Young)	1	36.00	1.71	4.53	0.96	5.36	5.21	2.16	7.36	6.87	-2.05	-3.74	-2.12	-1.13
Asiago (Young)	1	38.63	1.83	4.52	0.96	5.12	5.01	2.82	7.79	7.40	-2.26	-2.12	-0.68	-1.07
Asiago (Aged)	10	38.84	0.96	2.41	0.97	5.15	4.98	1.78	6.22	7.16	-2.92	-2.79	-3.70	-3.53
Asiago (Aged)	10	43.30	1.94	4.29	0.97	5.09	5.06	2.02	6.02	6.94	-3.84	-3.63	-1.59	-2.67
Baby Swiss	5	38.36	0.61	1.57	0.98	5.77	6.28	-[j]	7.07	7.50	-0.71	-1.38	-1.98	-0.62
Baby Swiss	5	36.25	0.69	1.87	0.98	5.79	6.32	-	6.94	8.05	-0.67	-0.76	-0.75	-1.15
Baby Swiss	9	37.21	0.62	1.64	0.99	5.55	6.04	-	7.19	7.72	-1.00	-2.43	-1.27	-0.79
Baby Swiss	9	35.58	0.65	1.79	0.99	5.71	6.27	-	7.35	7.72	-0.39	-1.45	-0.61	-1.02
Brick	11	40.39	1.52	3.63	0.96	5.43	4.90	1.29	7.23	7.66	-0.40	-0.71	-0.38	-0.74
Brick	11	41.21	1.95	4.52	0.97	5.30	4.98	0.90	7.19	7.82	-0.32	-0.70	-0.40	-0.98
Brick	2	38.28	1.52	3.82	-	5.25	5.37	1.07	6.33	8.08	-0.09	-0.22	-0.42	-0.79
Cheddar (Mild)	3	37.34	1.57	4.04	0.96	5.09	5.00	1.89	7.41	6.78	-0.70	-0.88	-0.30	-0.43
Cheddar (Mild)	3	36.59	1.77	4.61	0.97	5.09	5.06	1.44	7.39	6.81	-0.76	-1.00	-0.80	-0.17
Cheddar (Reduced-Fat)	6	40.26	1.60	3.82	0.97	5.19	5.11	1.15	5.35	6.21	-0.13	-0.65	-0.43	-1.28
Cheddar (Reduced-Fat)	6	44.00	1.66	3.64	0.98	4.99	5.27	0.90	5.52	5.79	-0.69	-0.57	-0.55	-0.97
Cheddar (Sharp)	3	36.34	1.78	4.67	0.96	5.27	5.27	1.69	4.30	6.39	-0.35	-0.75	-0.96	-1.19
Cheddar (Sharp)	3	36.57	1.32	3.48	0.97	5.19	5.28	1.71	4.63	5.84	0.00	-1.03	-0.59	-1.34
Cheddar-Mozzarella	6	40.09	1.62	3.88	-	5.19	5.33	1.42	6.99	6.24	-0.09	-0.27	-0.31	-0.48
Colby	4	35.96	1.61	4.28	0.96	5.45	5.61	1.09	5.76	7.39	-0.39	-0.50	-0.21	-0.57
Colby	4	40.14	1.60	3.83	0.97	5.30	5.47	1.78	5.91	6.38	-0.11	-0.63	-0.24	-0.39
Colby Jack	5	36.13	1.42	3.78	0.96	5.17	5.10	1.26	7.19	7.19	-0.20	-0.97	-0.80	-0.46
Colby Jack	5	36.85	1.35	3.53	0.98	5.01	5.40	1.37	7.70	7.38	-0.44	-0.59	-0.08	-0.46
Colby Jack (Reduced-Fat)	6	43.96	1.64	3.60	0.97	5.29	5.00	1.09	5.79	7.68	0.02	-0.90	-0.76	-1.12
Colby Jack (Reduced-Fat)	6	46.00	1.76	3.69	0.97	5.08	5.11	1.39	4.52	6.91	-0.56	-0.74	-0.73	-1.05
Colby Jack (Reduced-Na)	6	36.30	1.26	3.35	0.97	5.11	5.03	1.48	4.52	6.91	-0.17	-0.46	-1.03	-1.09
Colby Jack (Reduced-Na)	6	36.45	1.13	3.01	0.98	5.09	5.17	0.89	4.12	5.40	-0.69	-0.96	-0.39	-0.64
Feta	3	57.10	2.35	3.95	0.99	4.29	4.60	2.80	4.80	6.57	-4.58	-4.71	-4.60	-2.93
Feta	3	57.64	1.72	2.90	0.98	4.38	4.53	2.86	3.30	3.40	-4.89	-4.94	-4.07	-4.74
Gouda	6	41.15	1.62	3.79	0.97	5.28	5.25	0.88	7.29	7.38	-0.51	-0.32	-0.23	-0.83

Gouda	6	41.08	1.39	3.27	0.97	5.30	5.28	1.24	7.40	7.48	-0.44	-0.46	-0.34	-0.79
Havarti	3	37.79	1.33	3.40	0.97	5.49	5.52	1.08	6.88	7.26	-0.25	-0.61	-0.21	-0.73
Havarti	3	38.17	1.20	3.05	0.98	5.34	5.59	0.66	6.88	7.20	-0.51	+0.21[k]	-0.29	+0.01[k]
Havarti	6	41.32	1.27	2.98	-	5.11	5.26	1.40	8.28	7.75	-0.16	-0.61	-0.37	-0.70
Monterey Jack	5	45.10	1.87	3.98	0.98	5.15	5.20	2.41	8.08	8.16	-1.03	-0.91	-0.33	-0.37
Monterey Jack	5	35.45	1.64	4.42	0.97	5.08	5.11	2.28	8.06	7.98	-2.63	-1.17	-0.91	-0.66
Muenster	3	42.20	1.63	3.72	0.97	5.20	5.28	1.27	6.90	7.80	-0.49	-0.25	-0.24	0.00
Muenster	3	41.94	1.75	4.01	0.98	5.29	5.12	0.74	7.11	6.26	-0.10	-0.75	-0.45	-0.46
Parmesan	8	32.44	2.52	7.21	0.93	5.41	5.36	1.40	6.92	4.00	-0.88	-1.45	-1.25	-0.59
Parmesan	8	32.70	2.35	6.70	0.92	5.45	5.40	1.48	5.31	7.23	-1.51	-1.66	-1.86	-1.80
Pepper Jack	4	36.13	1.58	4.19	0.98	5.11	4.76	2.12	2.00	7.66	-0.85	-3.87	-0.81	-1.09
Pepper Jack	4	38.69	1.60	3.97	0.97	4.93	5.12	1.94	4.69	7.14	-2.86	-3.40	-3.25	-3.58
Pepper Jack	3	40.42	1.64	3.90	0.97	5.14	5.12	1.53	4.65	7.30	-2.39	-2.32	-2.10	-0.72
Pepper Jack	3	38.27	1.54	3.87	0.97	5.21	5.15	1.45	8.25	7.39	-0.62	-0.73	-0.35	-0.30
Provolone	4	42.15	1.38	3.17	-	5.24	4.97	1.81	7.70	8.70	-1.34	-0.97	-0.16	-0.72
Provolone (Mild)	8	43.05	2.08	4.61	-	5.18	5.22	1.80	5.53	6.70	-0.50	-1.84	-0.57	-0.71
Provolone (Sharp)	10	40.02	1.72	4.12	-	5.09	5.17	2.20	6.43	7.45	-1.59	-2.83	-1.27	-1.73
Provolone (Reduced-Fat)	3	48.98	1.43	2.84	0.97	4.97	4.67	1.83	6.95	7.98	-2.80	-2.23	-0.62	-1.55
Provolone (Reduced-Fat)	3	52.71	1.35	2.50	0.98	4.98	4.94	-	3.70	7.94	-0.56	-0.95	-0.24	-0.97
Provolone-Mozzarella	6	42.26	1.68	3.82	-	5.38	5.33	1.61	7.67	7.28	-0.25	-0.19	-0.17	-0.68
Swiss	6	38.57	0.52	1.33	0.98	5.36	5.50	-	5.95	6.59	-1.20	-1.11	-0.73	-2.32
Swiss	6	36.91	0.64	1.70	0.99	5.50	5.80	-	5.28	6.19	-0.93	-1.30	-0.36	-1.20
Swiss (Lacey)	5	45.17	0.33	0.73	0.99	6.02	5.87	-	7.00	8.18	-0.43	-1.19	-0.46	-1.02
Swiss (Lacey)	5	45.92	0.37	0.80	0.99	5.65	5.94	-	7.92	5.70	-1.83	-1.21	-0.31	-1.06

[a] Cheeses were national brands obtained from local retail outlets or directly from manufacturers. Qualifying descriptive information, e.g. 'mild,' 'sharp' is reproduced where provided on the package.

[b] Moisture content (%) of cheese sample on Day 0, n=2.

[c] % salt of cheese sample on Day 0, n=2.

[d] % salt-in-moisture phase (%SMP) of cheese sample on Day 0. Calculated from % moisture and % salt of the same cheese.

[e] Water activity (a_w) of cheese sample on Day 0.

[f] pH of cheese slice surface on Day 0 and Day 15, n=2.

[g] % titratable acidity (%TA) of cheese sample on Day 0 and Day 15, n=2.

[h] DeMan-Rogosa-Sharpe (MRS) agar count for lactic acid bacteria (LAB) on Day 0 and Day 15 (log CFU/g), n=2.

i Survival of pathogen LM=*L. monocytogenes*, SALM=*Salmonella* spp., EC=*E. coli* O157:H7, and SA=*S. aureus.* (+) indicates growth, (-) indicates no-growth.

[j] not determined.

[k] Growth of pathogen did not exceed plating variability: 0.39, 0.41, 0.27, 0.25 log CFU/g for *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7and S. *aureus*, respectively.

Table 2. Composition of natural cheeses that supported growth of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and/or *S. aureus* and pathogen survival (Δ log CFU/g) during storage for 15-days at 25°C.

Cheese[a]	Brand	% Moisture[b]	% Salt[c]	% SMP[d]	a_w[e]	pH[f]		% TA[g]	LAB count[h]		Pathogen survival (Δlog CFU/g)[i]			
						0 d	**15 d**	**0 d**	**0 d**	**15 d**	**LM**	**SALM**	**EC**	**SA**
Farmer's	12	39.85	1.71	4.11	-[j]	5.46	4.99	1.14	4.63	9.10	-0.41	-0.10	-0.39	**+1.48**[l]
Gruyere	7	34.25	1.01	2.86	0.97	5.68	5.74	1.04	5.70	7.40	**+1.01**	**+1.01**	-0.40	**+3.08**
Gruyere	7	32.07	1.41	4.21	0.98	6.28	5.78	1.55	5.04	6.70	-0.54	**+2.13**	-0.67	**+2.19**
Jack (goats' milk)	13	45.20	2.33	4.90	-	5.41	5.24	1.44	7.74	6.88	-0.40	**+2.50**	-0.62	**+1.62**
Muenster	6	41.58	1.21	2.83	-	5.48	5.53	0.66	4.85	7.67	+0.17[k]	**+1.65**	**+0.41**	**+1.77**
Provolone	3	43.17	1.03	2.33	0.97	5.29	4.78	1.36	2.70	5.40	-1.10	-0.40	-0.88	**+0.80**
Provolone	3	44.08	1.58	3.46	0.98	5.15	5.19	1.55	3.78	7.19	-0.40	-0.80	-0.52	**+0.81**
Provolone (Reduced-Na)	6	42.93	1.05	2.39	0.98	5.15	4.95	1.24	6.25	7.79	-1.20	-0.31	-0.30	**+0.62**
Provolone (Reduced-Na)	6	44.09	1.02	2.26	0.98	5.28	5.12	1.62	5.73	7.39	-0.29	-0.27	-0.63	**+1.59**
Queso Blanco	7	47.02	3.30	6.56	0.96	6.37	6.11	0.26	4.38	6.78	**+2.68**	-1.07	-2.11	**+2.57**
Queso Fresco	7	51.19	1.85	3.49	0.98	6.49	5.05	0.31	4.86	8.68	**+2.09**	**+3.02**	**+2.90**	**+1.55**
String	14	47.91	1.98	3.97	-	5.44	4.96	1.59	4.87	8.86	**+0.60**	**+2.00**	**+1.75**	**+2.39**
String	6	47.07	2.18	4.43	-	5.33	5.02	1.67	4.85	8.65	+0.22[k]	+0.39[k]	-0.38	**+1.58**
Queso Quesadilla	7	43.10	2.18	4.81	0.97	5.35	5.39	1.21	4.57	6.29	-0.01	-0.57	-0.48	**-0.40**[m]

[a] Cheeses were national brands obtained from local retail outlets or directly from manufacturers. Qualifying descriptive information, e.g. 'mild,' 'sharp' is reproduced where provided on the package.

[b] Moisture content (%) of cheese sample on Day 0, n=2.

[c] % salt of cheese sample on Day 0, n=2.

[d] % salt-in-moisture phase (%SMP) of cheese sample on Day 0. Calculated from % moisture and % salt of the same cheese.

[e] Water activity (a_w) of cheese sample on Day 0.

[f] pH of cheese slice surface on Day 0 and Day 15, n=2.

[g] % titratable acidity (%TA) of cheese sample on Day 0 and Day 15, n=2.

[h] DeMan-Rogosa-Sharpe (MRS) agar count for lactic acid bacteria (LAB) on Day 0 and Day 15 (log CFU/g), n=2.

i Survival of pathogen LM=*L. monocytogenes*, SALM=*Salmonella* spp., EC=*E. coli* O157:H7, and SA=*S. aureus*. (+) indicates growth, (-) indicates no-growth.

[j] not determined.

[k] Growth of pathogen did not exceed plating variability: 0.39, 0.41, 0.27, 0.25 log CFU/g for *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 and S. *aureus*, respectively

[l] Bolded numbers indicate growth beyond the pathogen-plating variability.

[m] Growth (+ 0.57 log CFU/g) at Day 6 sampling; no net growth over 15-day storage period

Table 3. Pathogen strains used in laboratory cheese challenge studies.

Inoculum	**Serotype**	**Strain**[a]	**Collection**[b]	**Source**[c]
Listeria monocytogenes	4b	LM 101	FRI	Hard salami
	4b	LM 310	FRI	Goat cheese
	4b	ATCC 43256	ATCC	Mexican-style cheese, Calif. (1985 outbreak strain)
	4b	ATCC 43257	ATCC	Mexican-style cheese, Calif. (1985 outbreak strain)
	4b	ATCC 51414	ATCC	Raw milk, Massachusetts
	4b	ATCC 51776	ATCC	Cheese, Belgium
	4b	ATCC 51777	ATCC	Cheese, Belgium
	4b	ATCC 51778	ATCC	Cheese, Belgium
	4b	Scott A	FRI	Clinical
	1/2a	V7	FRI	Raw milk
Salmonella spp.	Cerro	FSL R8-370	FSL	Bovine
	Typhimurium	FSL S5-433	FSL	Bovine
	Newport	FSL S5-436	FSL	Bovine
	Agona	FSL S5-517	FSL	Human
	Typhimurium	FSL S5-536	FSL	Human
	Newport	FSL S5-639	FSL	Human
Escherichia coli O157:H7	O157:H7	FRIK 22	FRI	Unknown
	O157:H7	FRIK 2000	FRI	Bovine
	O157:H7	F5854	FRI	Cheese curds (1998 outbreak strain)
	O157:H7	039732	NMDH	Gouda cheese (2010 outbreak strain)
	O157:H7	CWD EC1	VT	Farmstead goat cheese
Staphylococcus aureus		I	FPL	Raw milk
		J	FPL	Raw milk
		FRI 100	FRI	Cake
		FRI 1007	FRI	Genoa sausage
		ATCC 25923	ATCC	Clinical

[a]Strain designation provided by Collection.

[b]Collection: FRI = Food Research Institute, University of Wisconsin-Madison, Madison, Wisc.; ATCC = American Type Culture Collection, Manassas, Va.; FSL = Food Safety Laboratory, Dr. Katherine Boor, Cornell University, Ithaca, N.Y.; NMDH = New Mexico Department of Health, Santa Fe, N.M.; VT = Vermont Institute for Artisan Cheese, Dr. D.J. D'Amico, University of Vermont, Burlington, Vt.; FPL= Food Pathogen Laboratory, Dr. Barbara Ingham, University of Wisconsin-Madison, Madison, Wisc.

[c] Source provided by Collection.

807 Table 4. Data from published research selected to augment laboratory product assessment.

Reference	Pathogen	No. of strains	Cheese	Storage (days)	Temp (˚C)	pH[a]	%SMP[b]	a_w	Growth/Death[c]
31	*Salmonella*	9	Queso Fresco	-[d]	20	6.60	1.64	-	LT[e]:2.5 - 3.5h GT: 1.65 - 2.17 h
30	*E. coli* O157:H7	2	Queso Fresco	-	20	6.60	1.61	-	LT: 3 - 3.45 h GT: 2.33 - 2.56 h
42	*L. monocytogenes*	5	Cheddar	30	21	5.06	1.70	0.98	-1.11
						5.30	1.80	0.97	-0.48
						5.66	5.00	0.95	-0.14
						5.28	4.80	0.95	-0.96
43	*Salmonella* spp.	5	Cheddar	30	2 1	5.06	1.70	0.98	-3.2
						5.30	1.80	0.97	-3.9
						5.66	5.00	0.95	-3.8
						5.28	4.80	0.95	-3.5
48	*L. monocytogenes*	5	Queso Blanco	6.25	25	6.80	4.53	0.97	> 5.00
17	*L. monocytogenes*	5	Queso Fresco	3	30	6.60	6.60	-	+0.39
			Queso Fresco	6	30	6.60	4.50	-	+0.95
			Queso Fresco	3	30	6.50	6.15	-	+0.74
			Queso Ranchero	1	30	6.20	4.10	-	+2.60
			Queso Panela	3	30	6.20	2.50	-	+1.81
			Queso Panela	1	30	6.70	3.95	-	+3.18
			Queso Panela	3	30	6.60	3.48	-	+0.79
			Cotija	8	30	5.60	9.60	-	> -2.00
			Cotija	6	30	5.50	12.50	-	> -2.00
			Monterey Jack	4	30	5.00	3.00	-	> -1.40
			Monterey Jack	13	30	5.20	2.72	-	> - 2.09
			Mild Cheddar	4	30	4.90	2.60	-	> -1.26
			Mild Cheddar	7	30	5.20	4.49	-	> -2.09
			Colby	9	30	5.50	4.93	-	> -2.36
			String Cheese	9	30	5.50	4.24	-	> -2.36
			Provolone	9	30	5.60	4.62	-	> -2.36
			Muenster	9	30	5.50	3.80	-	> -2.36
			Domestic Feta	4	30	4.30	7.50	-	> -2.04
			Domestic Feta	4	30	4.30	2.20	-	> -2.04

808 [a] pH values of cheeses at initial sampling point of experiment

[b] Certain publications stated %SMP as % brine, which was calculated using the same equation as in this study (Equation 1). For publications that included both %moisture and % salt, % SMP was calculated using Equation 1.

[c] Behavior of pathogen over storage, expressed as Δlog CFU/g or LT/GT.

[d] Not specified.

[e] LT: Lag time (h); GT: generation time (h).

Figure legend.

Figure 1. Growth (Δ) or No-Growth (x) of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* on cheeses stored at 20-30˚C based on cheese pH (Day 0) and %SMP (salt-in-moisture-phase). Data from published research (n=26; Table 4) and this study (n=55). Solid line represents the growth/no-growth interface (*P*=0.05).

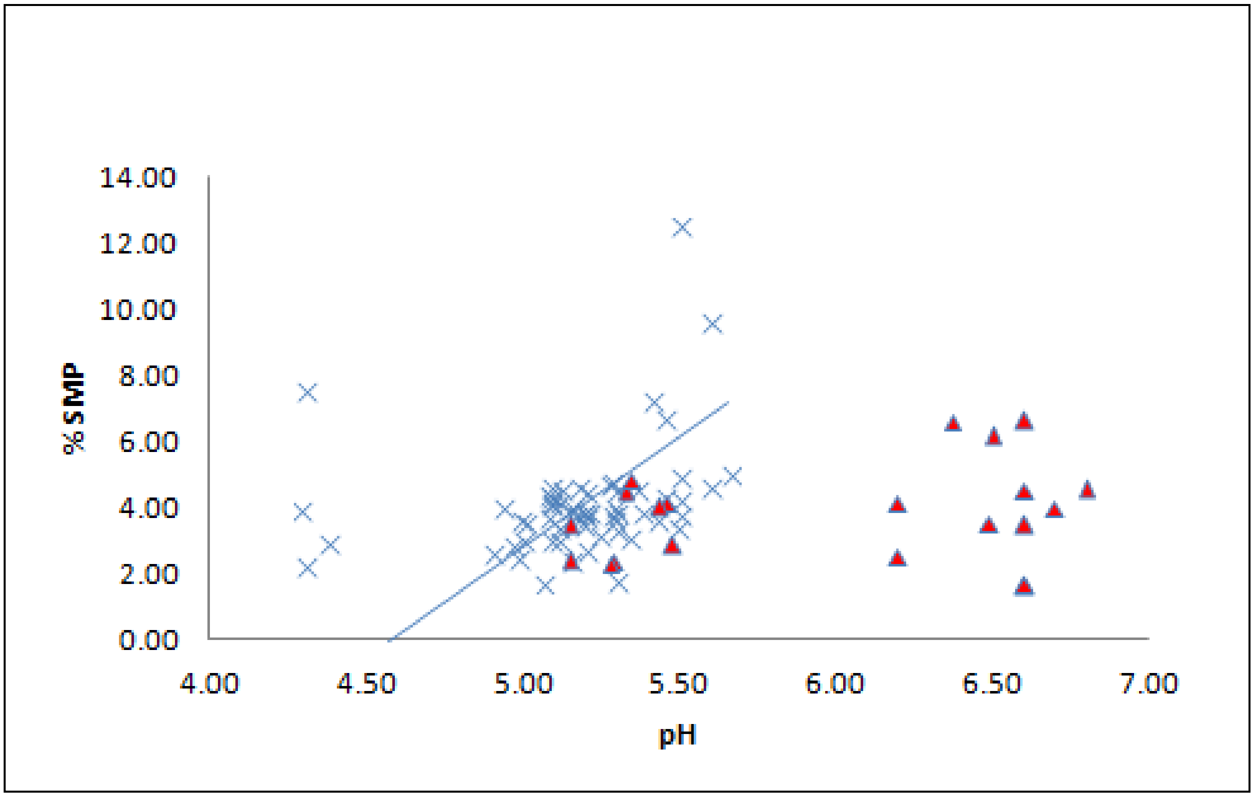
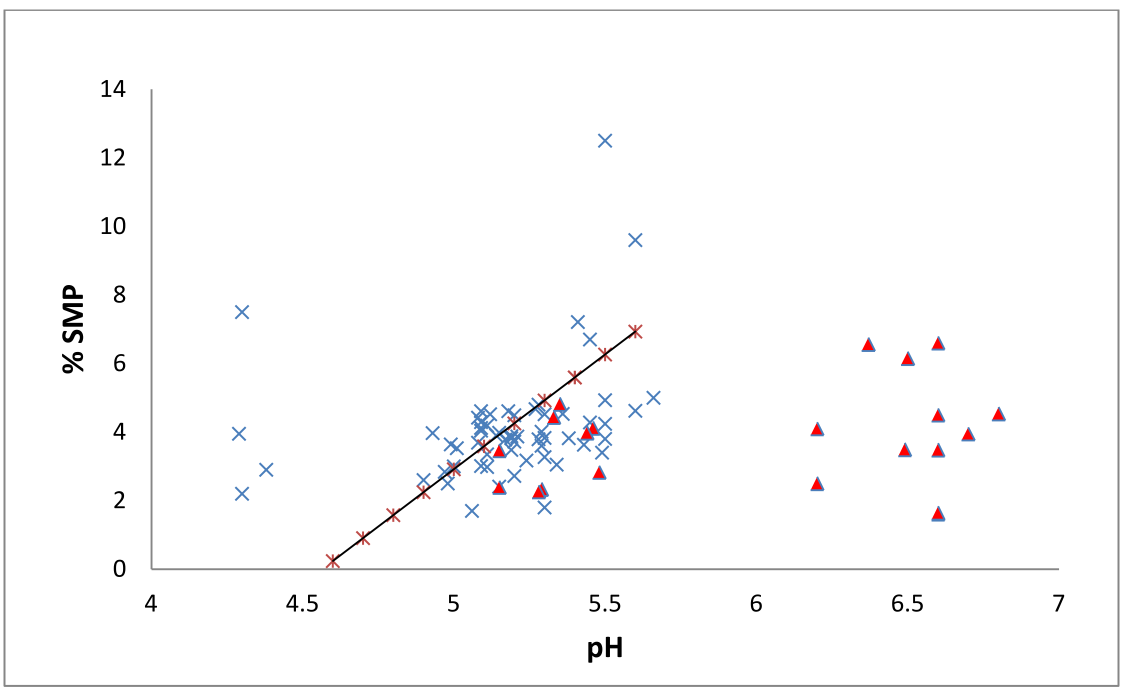

14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
% SMP
4.00
4.50
5.00
5.50
6.00
6.50
7.00
pH

Figure 1. Growth (Δ) or No-Growth (x) of at least one of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7 or *Staphylococcus aureus* on cheeses stored at 20-30˚C, with each point location indicating the pH (x-axis) and salt-in-moisture-phase (%SMP; y-axis) of the cheese at day 0. Growth/No-Growth outcomes are from previously published research (n=26 trials) and the University of Wisconsin-Madison study (n=55 trials) described in Non-TCS Cheeses Supporting Document #1. Results from four categories of cheese tested in the latter study (bacterial surface-ripened, mold-ripened, goats' milk and Swiss-style cheeses) are not shown. The sloped line depicts the proposed boundary between non-TCS cheeses and TCS cheeses, which was developed using logistic regression analysis. Cheeses with pH and % SMP values falling to the left of the TCS boundary line do not support pathogen growth and would be considered non-TCS foods, while cheeses with pH and %SMP values falling to the right of line would be considered TCS foods unless a product assessment determined otherwise. Points on the boundary line (⋇) represent the boundary pH and %SMP combinations listed below.



*Combinations of pH and %SMP to the left of the TCS boundary line include:

pH not greater than 4.60 and % SMP not less than 0.24

pH not greater than 4.70 and % SMP not less than 0.91

pH not greater than 4.80 and % SMP not less than 1.58

pH not greater than 4.90 and % SMP not less than 2.24

pH not greater than 5.00 and % SMP not less than 2.91

pH not greater than 5.10 and % SMP not less than 3.58

pH not greater than 5.20 and % SMP not less than 4.25

pH not greater than 5.30 and % SMP not less than 4.92

pH not greater than 5.40 and % SMP not less than 5.59

pH not greater than 5.50 and % SMP not less than 6.26

pH not greater than 5.60 and % SMP not less than 6.93