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Evaluation of NaCl, pH, and lactic acid on the growth of Shiga toxin-producing *Escherichia coli* in a liquid Cheddar cheese extract

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ABSTRACT

A Cheddar cheese model system, Cheddar cheese extract, was used to examine how different levels of known microbial hurdles (NaCl, pH, and lactic acid) in Cheddar cheese contribute to inhibition of bacterial pathogens. This knowledge is critical to evaluate the safety of Cheddar varieties with altered compositions. The range of levels used covered the lowest and highest level of these factors present in low-sodium, low-fat, and traditional Cheddar cheeses. Four pathogens were examined in this model system at 11°C for 6 wk, with the lowest levels of these inhibitory factors that would be encountered in these products. The 4 pathogens examined were *Salmonella enterica*, *Staphylococcus aureus*, *Listeria monocytogenes*, and Shiga toxin-producing *Escherichia coli* (STEC). None of these organisms were capable of growth under these conditions. The STEC exhibited the highest survival and hence was used to examine which of these inhibitory factors (NaCl, pH, and lactic acid) was primarily responsible for the observed inhibition. The STEC survival was examined in Cheddar cheese extract varying in NaCl (1.2 vs. 4.8%), lactic acid (2.7 vs. 4.3%), and pH (4.8 vs. 5.3) at 11°C for 6 wk. The microbial hurdle found to have the greatest effect on STEC survival was pH. The interactions between pH and levels of protonated lactic acid and anionic lactic acid with STEC survival was also evaluated; only the concentration of protonated lactic acid was determined to have a significant effect on STEC survival. These results indicate that, of the pathogens examined, STEC is of the greatest concern in Cheddar varieties with altered compositions and that pH is the microbial hurdle primarily responsible for controlling STEC in these products.

Key words: low-sodium cheese safety, survival of pathogen, Cheddar cheese compositional factor

INTRODUCTION

Traditional Cheddar cheese, when manufactured from pasteurized milk under good manufacturing practices, has a well-established history of safety. The safety of Cheddar cheese is due, in large part, to the large number of hurdles to microbial growth and survival that are present in Cheddar cheese. These factors include the relatively low ripening temperature, pH, water activity, nutrients for microbial growth (i.e., lactose), the presence of a competing microbiota, and the relatively high levels of inhibitory compounds, such as organic acids (primarily lactic acid) and NaCl (Johnson et al., 1990). However, there has been significant interest in the manufacture of Cheddar cheese with nontraditional compositions (e.g., low Na and low fat) and the microbial safety of these products is not well established. Low-fat Cheddar cheeses typically have significantly higher moisture content, which results in the dilution of inhibitors such as lactic acid and NaCl (Johnson et al., 1990, 2009). Salt-in-moisture is the critical process parameter. In the manufacture of the alternative Cheddar cheeses (i.e., low fat), the salt-in-moisture is lower, as addition of sufficient salt to retain the salt-in-moisture of traditional Cheddar results in a cheese with excessive saltiness (Johnson et al., 2009). Low-Na Cheddar cheeses, of course, have a significantly lower level of NaCl (Johnson et al., 2009). Additionally, these Cheddar varieties commonly use a curd wash step, which results in cheeses with higher pH and lower levels of lactic acid (Johnson et al. 1990, 2009; Grummer and Schoenfuss, 2011). These alterations in Cheddar cheese composition result in products more likely to support the growth of pathogenic microorganisms (Johnson et al., 2009; Doyle and Glass, 2010).

Bacterial pathogens can be introduced into cheese via postpasteurization contamination of the milk, curd, or finished cheese or the use of contaminated raw milk (Altekruse et al., 1998). Recalls and foodborne illness outbreaks have been associated with Cheddar cheese due to growth or long-term survival of *Salmonella en-*

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terica (Goepfert et al., 1968; Cody et al., 1999), *Listeria monocytogenes* (Ryser and Marth, 1987; Altekruse et al., 1998; CDC, 2000; Ryser, 2001), Shiga toxin-producing *Escherichia coli* (**STEC**; Reitsma and Henning, 1996), *Staphylococcus aureus* (Takahashi and Johns, 1959), and *Campylobacter jejuni* (CDC, 2000; Ryser, 2001), and less frequently with *Brucella melitensis* or *abortus* (Gilman et al., 1946; Eckman, 1975), *Yersinia enterocolitica* (Schiemann, 1978), and *Mycobacterium paratuberculosis* (Donaghy et al., 2004). The bacterial pathogens selected for evaluation in this study were *S. enterica*, *Staph. aureus*, *L. monocytogenes*, and STEC, based on their noted persistence in natural cheese, infectious dose, or the severity of disease (Table 1). Each of these pathogens has a different tolerance to salt and acidity; enteric gram-negative pathogens such as *E. coli* O157:H7 and *Salmonella* are typically very acid tolerant, whereas the gram-positive bacteria such as *L. monocytogenes* and *Staph. aureus* are salt tolerant.

The extent of pathogen survival or growth in cheese is dependent on the product's pH, total acidity, water activity, water-phase salt, competitive microflora, as well as naturally occurring or added antimicrobials. Of these factors, NaCl, lactic acid, and pH were included in this study because these factors can be controlled by the cheese manufacturer, whereas naturally occurring microflora and antimicrobials are difficult to control. Cheddar cheese is a complex product with many of the variables (i.e., pH and lactic acid concentration) being interrelated. For example, protonation of organic acids is dependent on pH change, and chemical composition change in the moisture phase might affect microflora composition. In addition, microflora composition might affect both enzymatic and physicochemical characteristics in cheese. Additionally, a relatively high natural variability occurs in cheese composition. Therefore, the development of a model system that allows for independent adjustments of variables (i.e., NaCl concentration), is devoid of confounding competitive microflora, and is highly reproducible is desirable. Our laboratory developed a model system to study cheese ripening based on Cheddar cheese extract (**CCE**), the aqueous fraction of cheese (Díaz-Muñiz et al., 2006; Budinich et al., 2011). This model was subsequently refined for use in microbial growth studies at low temperatures (8°C) via the incorporation of 1% milk fat (Tan et al., 2012). This model is founded on the assumption that microorganisms grow in the aqueous phase of cheese and, hence, a medium that replicates the conditions in this phase will replicate the conditions that microorganisms encounter in ripening cheese. In addition, CCE contains milk-derived nutrients for bacterial growth, such as simple carbohydrates (i.e., lactose and galactose), complex carbohydrates (i.e., glycomacrope-

Table 1. Bacterial strains used in this study

Organism	Strain designation	Source
Listeria monocytogenes	FSL R2-500	Cheese isolate associated with 2000 outbreak, North Carolina soft Hispanic-style cheese, serotype 4b (ILSI[1] North American *Listeria monocytogenes* Strain Collection)
	LM101	Hard salami isolate, serotype 4b
	LM310	Goat cheese isolate associated with human spontaneous abortion, serotype 4b
Salmonella enterica	Serovar Enteritidis E40	Chicken ovary isolate
	Serovar Heidelberg S13	Human isolate; Wisconsin State Laboratory of Hygiene (Madison) SHL 39902
	Serovar Typhimurium M-09-0001A-1	Peanut butter isolate associated with 2009 outbreak; Minnesota Department of Agriculture (St. Paul)
Staphylococcus aureus	FRI S6	Enterotoxin types A, B
	FRI 952	Enterotoxin type A; outbreak associated with whipped butter
	FRI 196[E]	Enterotoxin types A, D; outbreak associated with ham
Shiga toxin-producing *Escherichia coli*	O103:H2 strain 01-3002	Clinical isolate; Wisconsin State Laboratory of Hygiene
	O157:H7 strain F-90	Sausage isolate; 1994 outbreak in California associated with dry-cured salami
	O157:H7 strain F-5854	Cheese curd isolate; 1998 outbreak in Wisconsin associated with cheese curds

[1]ILSI = International Life Sciences Institute (Washington, DC).

ptide, glycoprotein, and glycolipids), citrate, FA, and phosphoserine-containing peptides (Budinich et al., 2011). In this study, we modified the CCE manufacturing process previously reported to use unsalted fresh cheese and the aqueous phase was isolated via UF and then by reverse osmosis.

The goal of this study was to examine concentration ranges of several compounds (NaCl, H^+, and lactic acid) that vary between Cheddar cheese varieties and their contribution to the safety of these products. To achieve this goal, the following objectives were pursued: (1) examine the growth and survival of *S. enterica*, *L. monocytogenes*, *Staph. aureus*, and STEC in a CCE in the most permissive Cheddar cheese condition, which mimics the internal curd environment of low-Na Cheddar cheese (low NaCl, high pH, and low lactate), and (2) examine the concentration range of NaCl, H^+, and lactic acid present in Cheddar cheese varieties on the survival of the pathogen identified in objective 1 as having the longest survival among the 4 pathogens examined.

MATERIALS AND METHODS

Preparation of CCE

The CCE used in this study was produced at the Western Dairy Center (Utah State University, Logan), using a modified procedure, based upon previous methods for CCE production (Díaz-Muñiz et al., 2006; Budinich et al., 2011; Tan et al., 2012). Unsalted Cheddar cheese was manufactured using a standard making procedure and aged for 5 d at 8°C. After 5 d of ripening, the composition of Cheddar curd was 38% moisture and 30% fat, with a pH of 5.25 and the lactose was under the detection limit (<10 mg/L). Shredded Cheddar curd and distilled water were added to a jacketed vat at a ratio of 4:1 (wt/wt) and heated to 50°C; this temperature was maintained for 20 min with continuous stirring. Diafiltration using a UF membrane was used to isolate the aqueous fraction of the cheese slurry. Reverse osmosis of the UF permeate was used to concentrate CCE to 1.5-fold relative to moisture content of the original cheese (1.5× CCE). The resulting 1.5× CCE was held at −20°C until used.

Chemical Analysis of CCE

The NaCl concentration in the 1.5× CCE was determined using Chloride Analyzer 926 (Nelson-Jameson Inc., Marshfield, WI). The lactose, galactose, citrate, acetate, formate, and D/L-lactic acid concentrations in the CCE concentrate were determined by using lactose/D-galactose, citrate, acetate, formate, and D/L-lactic acid enzymatic test kits from R-Biopharm Inc. (Marshall, MI). The kits were used as directed by the supplier except that the total volume of the assay was decreased from 3 mL to 600 μL while maintaining the proportions described in the manufacturer's instructions.

The level of protonated lactic acid was calculated using the Henderson-Hasselbalch equation (Equation [1]), where the acid dissociation constant (pK_a) of lactic acid is 3.86 and the pH is 5.3 or 4.8. Additionally, in Equation [1], [*Base*] and [*Acid*] indicate conjugate base and protonated lactic acid, respectively. Percentage lactic acid protonation was calculated based on the proportion of [*Base*] and [*Acid*] using Equation [2].

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\left(\frac{[Base]}{[Acid]}\right); \quad [1]$$

$$\text{Protonation}(\%) = \left[\frac{\mathrm{A}}{(\mathrm{A}+\mathrm{B})}\right]\times 100\%, \text{ when } [Acid]:[Base] = \mathrm{A}:\mathrm{B}. \quad [2]$$

Bacterial Strains

Four pathogens: *S. enterica*, *L. monocytogenes*, *Staph. aureus,* and STEC were used for the study because of their potential persistence in natural cheese, infectious dose, the severity of disease, and availability in the bacterial culture collection at the Food Research Institute, University of Wisconsin-Madison (courtesy of K. Glass; Table 1). In addition, many nondairy strains were selected in this study because strains of dairy origin are not necessarily the most robust under high-salt and low-pH conditions. When possible, cheese isolates were used, but the other strains used (e.g., from fermented, dried meats, low-water-activity peanut butter, and high-salt ham) are robust under the conditions of the study. Clinical isolates are also representative of isolates that would be found in foods. Frozen stock cultures were maintained at −80°C in tryptic soy broth (TSB; BD Difco Laboratories Inc., Sparks, MD) supplemented with 15% (vol/vol) glycerol. Working cultures of individual strains were prepared from frozen stocks by 2 sequential transfers in 10 mL of TSB at 37°C for 18 h with aeration. Prior to inoculating CCE media, cells were harvested by centrifugation (2,500 × *g* for 20 min at room temperature, 25°C), washed twice with PBS [8.0 g of NaCl, 0.20 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter (pH 7.2)], and suspended in 4.5 mL of PBS. Inoculum for each pathogen type (3-strain mixture) was prepared by combining equivalent populations of each of the 3 strains to yield a final concentration of approximately

4.0 log cfu/mL of CCE. Populations of each strain and the mixtures were verified for purity and enumeration by plating on tryptic soy agar (**TSA**), and appropriate selective agar: modified Oxford agar for *L. monocytogenes*, Baird-Parker agar with egg yolk-tellurite enrichment for *Staph. aureus*, MacConkey sorbitol agar for STEC, and xylose lysine deoxycholate (XLD) agar for *Salmonella* (all Difco, BD Biosciences, Sparks, MD)

Bacterial Survival Experiments

Phase 1 determined which of the 4 pathogens exhibited the highest level of survival and growth in CCE with the NaCl, lactic acid, and pH levels present in low-Na Cheddar cheese (designated **CCE-P**). The CCE-P was prepared by adding sufficient NaCl and L-lactic acid to the 1.5× CCE that the final levels were 1.89% NaCl and 2.7% L-lactic acid after the CCE was diluted to 1.0×. The pH of the media was then adjusted to 5.4 (Orion Star A111 pH meter with 8220BNWP micro pH probe; Thermo Scientific, Waltham, MA) via the addition of 10.0 *M* KOH. The CCE was subsequently filter sterilized with 0.2-μm polyethersulfone (PES) membrane filter units (Nalge Nunc International Corp., Rochester, NY) and stored at 4°C before use. Phase 2 examined the influence of NaCl, lactic acid, and pH on the organism exhibiting the highest level of survival in CCE-P; 8 CCE with varying NaCl (1.2 vs. 4.8%) and lactic acid (2.7 vs. 4.3%) concentrations and pH (4.8 vs. 5.3) were used.

For both phases, separate portions of CCE were inoculated with the target pathogen to yield approximately 4.0 log cfu/mL of CCE. The inoculated CCE was aliquoted (1 mL) into sterile 2-mL crimp-top vials and incubated at 11°C for up to 6 wk. A 6-wk incubation time was used, based upon results obtained in a preliminary trial in which significant differences in pathogen survival were observed by 6 wk. Triplicate samples (technical replicates) were taken from individual vials at each time point to ensure that the conditions in the vials would not be influenced by sampling. Cells were enumerated at d 2 and then weekly thereafter by plating serial dilutions on TSA plates via the drop plate method (Mallmann and Broitman, 1956). Drop plating was conducted in triplicate using 20 μL per drop, and plates were incubated aerobically at 37°C for 20 h before enumeration. Each study was replicated twice (biological replicates).

Statistical Analysis

To identify significant treatment effect on the logarithmic reduction of pathogens in CCE, ANOVA and the Tukey honestly significant difference for multiple comparisons were used. The mean value and standard error of viable cell numbers were calculated based on the average number of colonies on TSA plates in each biological replicate. The effect of CCE composition and pH on the growth and survival of pathogens were also examined by principal component analysis (XLSTAT 7.5.2; Addinsoft SARL, New York, NY). Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Determination of the pH Ranges and Composition of Low-Na Cheddar Cheese

Cheddar cheese composition has a significant effect on the ability of microorganisms to grow and survive. Microorganisms grow in the aqueous phase of Cheddar cheese and, hence, it is the composition of this phase that is of primary interest relative to microbial growth. Therefore, production of Cheddar cheese varieties with altered aqueous compositions may represent a food safety concern. Estimates of the levels of moisture, NaCl, lactic acid, and pH in traditional, low-fat, Lite-Na [cheese salted with Lite Salt (NaCl:KCl = 1:1; Morton Salt Inc., Chicago, IL], and low-Na Cheddar cheeses made either with or without a curd wash step are presented in Table 2 (Johnson et al., 2009; M. E. Johnson, unpublished survey from the Center for Dairy Research, University of Wisconsin-Madison). These values were used to calculate the NaCl in the moisture (salt-in-moisture) and lactic acid in the moisture levels and were used throughout this study to select compositions to represent the conditions present in nontraditional Cheddar varieties.

The levels of NaCl, lactose, galactose, citrate, acetate, formate, D-lactic acid, and L-lactic acid in the base 1.0× CCE used in this study were 3.5 m*M*, 0.13 m*M*, below quantifiable limit (**BQL**; <38.86 μ*M*), 1.43 m*M*, BQL (<2.4 μ*M*), BQL (<50 μ*M*), BQL (<3.34 μ*M*), and 9.38 m*M*, respectively.

Phase 1: Survival and Growth of 4 Select Bacterial Pathogens in CCE-P

To determine which of the 4 selected bacterial pathogens is best able to survive and grow in low-Na Cheddar cheese, 3 strain cocktails of *L. monocytogenes*, *S. enterica*, *Staph. aureus*, and STEC were inoculated (~4.0 log cfu/mL) into CCE-P, a medium that mimics the aqueous phase of low-Na Cheddar cheese, and incubated at 11°C for 6 wk. No significant changes in pH (±0.05) were observed between initial and 6-wk time point in any of samples. This was the expected result, as CCE has high buffering capacity and the CCE used

Table 2. Physicochemical composition of Cheddar cheese varieties with an emphasis on factors likely to influence microbial growth and survival[1]

Cheese	Curd wash	Moisture (%)	NaCl (%)	Lactic acid (%)	NaCl/M^2 (%)	Lactic acid/M^2 (%)	pH
Traditional	Yes	37[3,4]	1.7[3,4]	1.3[4]	4.60[4]	3.50[4]	5.2[4]
	No	37[3,4]	1.7[3,4]	1.6[4]	4.60[4]	4.32[4]	5.0[4]
Low fat	Yes	55[3,4]	1.9[4]	1.6[4]	3.45[4]	2.90[4]	5.4[3,4]
	No	55[3,4]	2.0[3,4]	2.0[4]	3.60[4]	3.63[4]	5.0[4]
Lite Na[5]	Yes	37[3,4]	1.35[4]	1.1[4]	3.64[4]	2.97[4]	5.4[3,4]
	No[6]	37[3,4]	1.35[4]	Unknown	3.64[4]	Unknown	4.8[4]
Low Na	Yes	37[3,4]	0.7[3,4]	1.0[4]	1.89[4]	2.70[4]	5.4[4]
	No[6]	37[3,4]	0.7[3,4]	Unknown	1.89[4]	Unknown	4.8[4]

[1]These estimated values are based on the studies cited by Johnson et al. (2009) and an unpublished survey conducted by the Center for Dairy Research (M. E. Johnson, University of Wisconsin-Madison).

[2]M designates the moisture level.

[3]Johnson et al. (2009).

[4]Survey conducted by Center for Dairy Research.

[5]Lite-Na Cheddar cheese salted with Lite Salt (NaCl:KCl = 1:1; Morton Salt Inc., Chicago, IL).

[6]It is unknown if these Cheddar cheeses are made commercially.

in this study contained only 0.13 m*M* lactose. The results from this experiment are presented in Figure 1. The logarithmic reductions of colony-forming units per milliliter of STEC, *S. enterica*, *Staph. aureus*, and *L. monocytogenes* were 0.36 ± 0.04, 1.08 ± 0.34, 1.10 ± 0.60, and 1.87 ± 0.29, respectively. The results indicate that STEC survived at a significantly ($P < 0.05$) higher level than the other 3 bacterial pathogens examined. Based on this observation, STEC are expected to survive for a long ripening time if the contamination occurs during processing of raw milk and, hence, it was selected for phase 2 of this study. Considerable variability was observed in the survival of *L. monocytogenes*, *Staph. aureus*, and *S. enterica* between biological replicates; these results are likely the result of either differences in medium composition [i.e., redox, level of oxygen, and (or) level of nutrients] or physiological differences in the inocula (i.e., initial cell number or growth phase; Johnston et al., 2000). This variability also has affected studies done in natural cheeses (Shrestha et al., 2011a,b; Hystead et al., 2013).

In general, pathogen numbers decrease during Cheddar cheese ripening, with more rapid inactivation observed in products at the lower range of pH and at higher ripening temperatures (Takahashi and Johns, 1959; Goepfert et al., 1968; Ryser and Marth, 1987). Populations of *Salmonella*, *L. monocytogenes*, and STEC decreased 1 to 2 log during ripening of Cheddar with an initial pH of 5.1 to 5.2 during 8 wk of storage at 2 to 13°C (Goepfert et al., 1968; Ryser and Marth, 1987; Reitsma and Henning, 1996; D'Amico et al., 2010). Similar logarithmic reduction of *Salmonella* and *L. monocytogenes* in between low-Na CCE and full-Na Cheddar conditions suggests that logarithmic reduction of select pathogenic bacteria in Cheddar cheese were less likely affected by low Na levels. However, these cheeses differed from each other in composition, including pH, salt, organic acid concentrations, and competing microbiota, making comparisons between these studies as well as with our results in CCE difficult. In addition, survival of *Salmonella* and *L. monocytogenes*, when inoculated on the surface of reduced-salt Cheddar cheese, has been evaluated (Shrestha et al., 2011a,b; Hystead et al., 2013). The results of those investigations demonstrated that a significant difference in pathogen survival existed between surface contamination and cells entrapped in the moisture phase in ripening cheese and suggested that *Listeria* was better able to survive after surface contamination during later periods of aging, such as cutting, shredding, and wrapping. Those results indicate that our results should not be extrapolated to

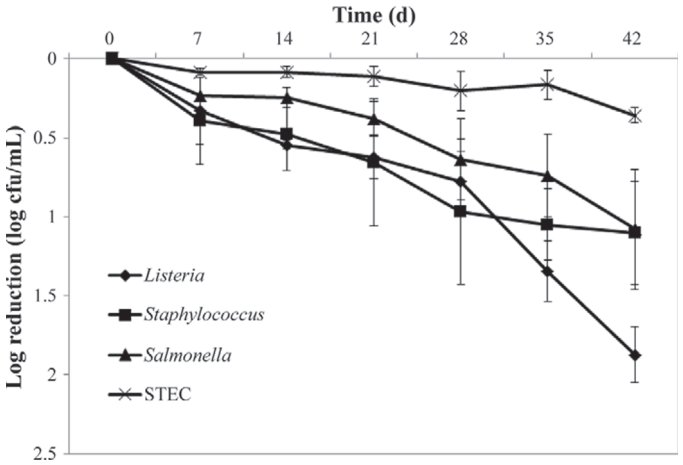

Figure 1. Survival of *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and Shiga toxin-producing *Escherichia coli* (STEC) in Cheddar cheese extract (CCE) with 1.89% NaCl and 2.7% L-lactic acid (CCE-P). Each pathogen was inoculated in CCE-P (pH 5.4) at 11°C for 6 wk. Initial inoculum (~4 log cfu/mL) was normalized to 0 at d 0. Error bars indicate SD of data from 2 biological replicates conducted with 3 technical replicates.

Table 3. Selected composition of experimental Cheddar cheese extracts (CCE)[1]

Item	CCE1	CCE2	CCE3	CCE4	CCE5	CCE6	CCE7	CCE8
NaCl (%)	Low (1.2)	Low (1.2)	Low (1.2)	Low (1.2)	High (4.8)	High (4.8)	High (4.8)	High (4.8)
Lactic acid[2] (%)	Low (2.7)	Low (2.7)	High (4.3)	High (4.3)	Low (2.7)	Low (2.7)	High (4.3)	High (4.3)
pH	Low (4.8)	High (5.3)	Low (4.8)	High (5.3)	Low (4.8)	High (5.3)	Low (4.8)	High (5.3)
Total lactic acid[2] (m*M*)	299.7	299.7	477.3	477.3	299.7	299.7	477.3	477.3
Protonated lactic acid[3] (m*M*)	93.0	29.4	236.5	74.8	93.0	29.4	236.5	74.8
Anionic lactic acid[4] (m*M*)	206.7	270.3	240.8	402.5	206.7	270.3	240.8	402.5

[1]Three-strain cocktails of *Escherichia coli* serotype O103, O157:H7 (strain F-90), and O157:H7 (strain F-5894) were inoculated in gas chromatography vials at a level of 1.0×10^4 cfu/mL and incubated at 11°C for 6 wk.

[2]Total L-lactic acid in each CCE.

[3]Protonated lactic acid was calculated based on the Henderson-Hasselbalch equation: $\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{[Base]}{[Acid]}\right)$, where the acid dissociation constant ($\mathrm{p}K_a$) of lactic acid is 3.86 and the pH is 5.3 or 4.8; [*Base*] and [*Acid*] indicate conjugate base and protonated lactic acid, respectively.

[4]Anionic lactic acid = total lactic acid − protonated lactic acid.

the survival of pathogens on the surface of Cheddar cheese.

Phase 2: Survival of STEC Strains in CCE Varying in NaCl and Lactic Acid Concentrations and pH

Based on the relative survival rates in CCE-P, STEC was chosen for further study as the pathogen type with the greatest likelihood of survival in low-Na CCE. To examine the influence of NaCl, pH, and lactic acid on STEC survival with the nutrients and temperatures present in ripening Cheddar cheese, a 3-strain cocktail of STEC was inoculated (~4.0 log cfu/mL) into 8 CCE varying in NaCl (1.2 vs. 4.8%) and lactic acid (2.7 vs. 4.3%) concentrations and pH (4.8 vs. 5.3) and incubated at 11°C for 6 wk (Table 3). The results from this experiment are presented in Table 4. Considerable variability on the survival of STEC strains between biological replicates (Table 4) was observed, as discussed previously; this was likely due to physiological differences in the inocula. After the 6-wk incubation, the 4 CCE (CCE1, CCE3, CCE5, and CCE7) at the lower pH (pH 4.8) exhibited significantly ($P < 0.05$) greater reductions in viability than 2 of the CCE (CCE4 and CCE6) having the higher pH (pH 5.3), whereas no significant effect was observed with percentage NaCl or percentage lactic acid, suggesting that pH is the primary determinant of STEC cell death under the conditions examined. Principal component analysis was also used to examine the relative contribution of NaCl, pH, and lactic acid to reductions in STEC viability, and the results of this analysis are presented in Figure 2. This analysis supports the conclusion that pH is the primary determinant of STEC survival under these conditions and that the percentages of lactic acid and NaCl alone did not affect STEC survival. It is important to note that the conditions examined represent the extremes of percentage lactic acid, percentage NaCl, and pH present in Cheddar varieties; therefore, it is difficult to extrapolate to conditions present in typical Cheddar cheese.

To examine for interaction between the 3 factors being studied, the 2-way interactions (pH × total lactic acid, pH × NaCl, and total lactic acid × NaCl) were examined for their effect on STEC survival. The result of this analysis was that none of these interactions were found to be significant ($P < 0.05$). However, due to uncertainty over the mechanism of lactic acid inhibition in mildly acidic foods (Carpenter and Broadbent, 2009), we decided to also examine the 2-way interactions of pH × protonated lactic acid and pH × anionic lactic acid for their effect on STEC survival. The results of this analysis was that pH × protonated lactic acid had a significant ($P < 0.05$) effect on the survival of STEC when the highest protonated lactic acid levels (236.5 m*M* in CCE3 and CCE7) were compared with the lowest protonated lactic acid levels (29.4 m*M* in CCE2 and CCE6). The results from the pH × anionic lactic acid effect on STEC survival analysis suggested that significantly ($P < 0.05$) higher survival of STEC occurred in CCE (402.5 m*M* in CCE4 and CCE8) with higher levels of anionic lactic acid than the CCE (206.7 m*M* in CCE1 and CCE5) with lower levels of anionic lactic acid.

Microbial inhibition by organic acids in mildly acidic foods has been used in fermented products to extend shelf life and enhance safety since their development approximately 10,000 yr ago. However, the mechanisms responsible for the inhibition of microorganisms by weak acids in these products are not fully understood. It is believed that protonated acids diffuse across the cytosolic membrane and then dissociate, due to the higher intracellular pH, releasing protons and anions in the cell. The released protons result in acidification of the cytoplasm and when the reduction in internal pH results in inhibition of an essential enzyme, es-

Table 4. Logarithmic reduction of Shiga toxin-producing *Escherichia coli* (STEC) in Cheddar cheese extracts (CCE) varying in percentage NaCl, percentage lactic acid, and pH[1,2]

	Log cfu/mL (SE)							
Day	CCE1	CCE2	CCE3	CCE4	CCE5	CCE6	CCE7	CCE8
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.04^{a} (0.04)	0.07^{ab} (0.06)	0.03^{b} (0.03)	0.09^{ab} (0.09)	0.11^{ab} (0.10)	0.07^{ab} (0.07)	0.15^{ab} (0.06)	0.17^{a} (0.12)
7	0.15^{de} (0.09)	0.58^{a} (0.19)	0.32^{bcd} (0.04)	0.09^{e} (0.07)	0.39^{abc} (0.07)	0.17^{de} (0.13)	0.45^{ab} (0.14)	0.24^{cde} (0.10)
14	0.60^{b} (0.17)	1.34^{a} (0.79)	0.68^{b} (0.08)	0.35^{b} (0.14)	0.63^{b} (0.40)	0.18^{b} (0.09)	0.54^{b} (0.10)	0.65^{b} (0.31)
21	1.63^{ab} (0.27)	2.11^{a} (1.56)	1.35^{abc} (0.56)	0.62^{bc} (0.27)	1.02^{abc} (0.62)	0.18^{c} (0.12)	0.90^{bc} (0.33)	0.42^{c} (0.06)
28	3.09^{a} (0.31)	2.53^{ab} (1.67)	2.59^{ab} (0.85)	0.53^{c} (0.15)	1.63^{bc} (1.03)	0.42^{c} (0.18)	1.29^{bc} (0.23)	0.49^{c} (0.09)
35	**3.33^{a} (0.53)**	2.50^{ab} (1.26)	**3.48^{a} (0.57)**	0.75^{bc} (0.12)	**2.73^{a} (1.40)**	0.48^{c} (0.25)	**3.03^{a} (1.06)**	1.66^{abc} (1.82)
42	**3.37^{a} (0.53)**	1.71^{ab} (1.26)	**3.48^{a} (0.57)**	1.07^{b} (0.33)	**2.94^{a} (1.17)**	0.58^{b} (0.23)	**3.42^{a} (0.64)**	2.33^{ab} (1.83)

$^{a-e}$Different superscript letters indicate that values differ significantly ($P < 0.05$) within a row as determined by the Tukey multiple range test.

[1]A cocktail of 3 strains was inoculated in CCE and was incubated at 11°C for 6 wk. Composition of Cheddar cheese extracts (CCE) prepared with an initial inoculum of 4.0 log cfu/mL: CCE1 = low NaCl, low lactic acid, and low pH; CCE2 = low NaCl, low lactic acid, and high pH; CCE3 = low NaCl, high lactic acid, and low pH; CCE4 = low NaCl, high lactic acid, and high pH; CCE5 = high NaCl, low lactic acid, and low pH; CCE6 = high NaCl, low lactic acid, and high pH; CCE7 = high NaCl, high lactic acid, and low pH; and CCE8 = high NaCl, high lactic acid, and high pH.

[2]Highest logarithmic reduction levels are highlighted in bold.

sential cellular functions cease, and cell death occurs (Booth, 1985; Davidson and Taylor, 2007; Carpenter and Broadbent, 2009). However, evidence also exists to support the hypothesis that the intracellular released anion is the actual inhibitory molecule (Carpenter and Broadbent, 2009). The mechanism of inhibition in this case is related to cellular osmolarity, indirect acidification of the cytoplasm, and feedback inhibition of enzymes in essential metabolic pathways (Padan et al., 1976; Russell, 1992; Conner and Kotrola, 1995; Russell and Diez-Gonzalez, 1997; Roe et al., 1998, 2002; Davidson and Taylor, 2007; Carpenter and Broadbent, 2009). Carpenter and Broadbent (2009) hypothesized that intracellular accumulation of anions is driven by 2 factors: external anion concentration and the internal-external pH gradient, a gradient where the pH outside the cell is lower than the pH within the cell, which would be the case in mildly acidic foods such as Cheddar cheese. However, the results described in the current study did not show that an increase in external anion concentration resulted in an increase in cell death. Rather, an increase in STEC death was observed with an increased concentration of protonated lactic acid. These results suggest that, in the case of STEC, in this environment, the primary cause of cell death is intracellular acidification.

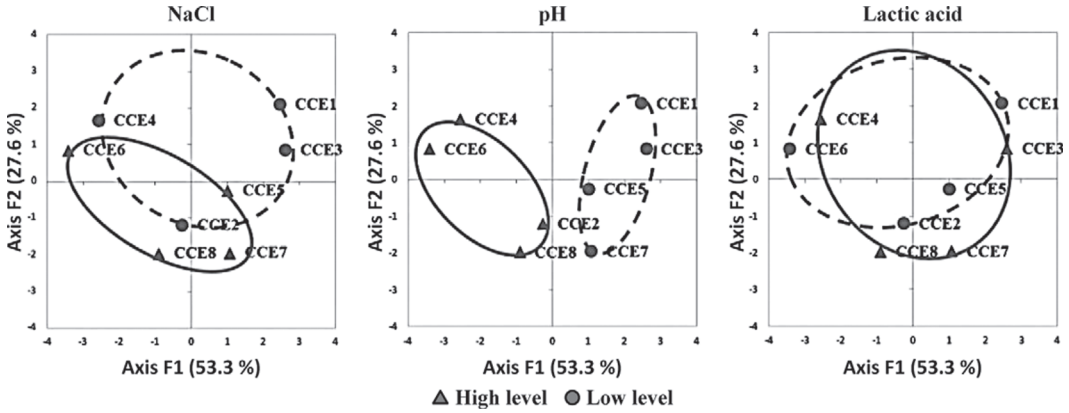

Figure 2. Principal component analysis of the Shiga toxin-producing *Escherichia coli* (STEC) logarithmic reduction. The different shapes of dots (circles and triangles) plotted in 2-dimensional space represent the effect of pH, NaCl, and lactic acid levels on the survival of STEC strains, and are clustered in 2 circles in each chart (high level in closed circle and low level in dotted circle). Axis F1 (x-axis) and axis F2 (y-axis) indicate principal component 1 and 2, respectively. Composition of Cheddar cheese extracts (CCE): CCE1 = low NaCl, low lactic acid, and low pH; CCE2 = low NaCl, low lactic acid, and high pH; CCE3 = low NaCl, high lactic acid, and low pH; CCE4 = low NaCl, high lactic acid, and high pH; CCE5 = high NaCl, low lactic acid, and low pH; CCE6 = high NaCl, low lactic acid, and high pH; CCE7 = high NaCl, high lactic acid, and low pH; and CCE8 = high NaCl, high lactic acid, and high pH.

CONCLUSIONS

The safety of Cheddar cheeses with nontraditional compositions (e.g., low-Na Cheddar cheese) is not well established. Of particular concern is that these varieties have lower concentration of known microbial hurdles, such as NaCl and lactic acid, and higher pH (Johnson et al., 2009). Therefore a Cheddar cheese model system was used to examine how different concentrations of these known microbial hurdles influence the growth and survival of *L. monocytogenes*, *S. enterica*, *Staph. aureus*, and STEC. None of these organisms were capable of growth under these conditions. However, the logarithmic reductions after 6 wk at 11°C of STEC, *S. enterica*, *Staph. aureus*, and *L. monocytogenes* were only 0.36 ± 0.04, 1.08 ± 0.34, 1.10 ± 0.60, and 1.87 ± 0.29, respectively. Shiga toxin-producing *E. coli* was used to examine which of NaCl, pH, and lactic acid was primarily responsible for the observed inhibition. Our results indicate that of the pathogens examined, STEC are of greatest concern in Cheddar varieties with altered compositions and that pH among the hurdles investigated is the microbial hurdle primarily responsible for controlling this pathogen in these products.

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