The effect of sodium reduction with and without potassium chloride on the survival of *Listeria monocytogenes* in Cheddar cheese

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ABSTRACT

Sodium chloride (NaCl) in cheese contributes to flavor and texture directly and by its effect on microbial and enzymatic activity. The salt-to-moisture ratio (S/M) is used to gauge if conditions for producing good-quality cheese have been met. Reductions in salt that deviate from the ideal S/M range could result in changing culture acidification profiles during cheese making. *Lactococcus lactis* ssp. *lactis* or *Lc. lactis* ssp. *cremoris* are both used as cultures in Cheddar cheese manufacture, but *Lc. lactis* ssp. *lactis* has a higher salt and pH tolerance than *Lc. lactis* ssp. *cremoris*. Both salt and pH are used to control growth and survival of *Listeria monocytogenes* and salts such as KCl are commonly used to replace the effects of NaCl in food when NaCl is reduced. The objectives of this project were to determine the effects of sodium reduction, KCl use, and the subspecies of *Lc. lactis* used on *L. monocytogenes* survival in stirred-curd Cheddar cheese. Cheese was manufactured with either *Lc. lactis* ssp. *lactis* or *Lc. lactis* ssp. *cremoris*. At the salting step, curd was divided and salted with a concentration targeted to produce a final cheese with 600 mg of sodium/100 g (control), 25% reduced sodium (450 mg of sodium/100 g; both with and without KCl), and low sodium (53% sodium reduction or 280 mg of sodium/100 g; both with and without KCl). Potassium chloride was added on a molar equivalent to the NaCl it replaced to maintain an equivalent S/M. Cheese was inoculated with a 5-strain cocktail of *L. monocytogenes* at different times during aging to simulate postprocessing contamination, and counts were monitored over 27 or 50 d, depending on incubation temperature (12 or 5°C, respectively). In cheese inoculated with 4 log₁₀ cfu of *L. monocytogenes*/g 2 wk after manufacture, viable counts declined by more than 3 log₁₀ cfu/g in all treatments over 60 d. When inoculated with 5 log₁₀ cfu/g at 3 mo of cheese age, *L. monocytogenes* counts in Cheddar cheese were also reduced during storage, but by less than 1.5 log₁₀ cfu/g after 50 d. However,

cheese with a 50% reduction in sodium without KCl had higher counts than full-sodium cheese at the end of 50 d of incubation at 4°C when inoculated at 3 mo. When inoculated at 8 mo postmanufacture, this trend was only observed in 50% reduced sodium with KCl, for cheese manufactured with both cultures. This enhanced survival for 50% reduced-sodium cheese was not seen when a higher incubation temperature (12°C) was used when cheese was inoculated at 3 mo of age and monitored for 27 d (no difference in treatments was observed at this incubation temperature). In the event of postprocessing contamination during later stages of ripening, *L. monocytogenes* was capable of survival in Cheddar cheese regardless of which culture was used, whether or not sodium had been reduced by as much as 50% from standard concentrations, or if KCl had been added to maintain the effective S/M of full-sodium Cheddar cheese.

Key words: *Listeria monocytogenes*, KCl, sodium reduction, Cheddar cheese

INTRODUCTION

Reduction of dietary sodium is a public health priority. It is estimated that 75% of women and 95% of men in the United States exceed the upper daily intake level recommended for sodium (between 1,500 and 2,300 mg per day, depending on demographics) (Cohen, et al., 2008). Excessive consumption of sodium may lead to hypertension, cardiovascular disease, and kidney failure, among other negative health consequences (Appel et al., 2012). Because it has a relatively high salt content (approximately 600 mg of sodium per 100 g), Cheddar cheese has been targeted for sodium reduction.

Sodium chloride (NaCl) is an important ingredient used in the manufacture of Cheddar and other cheeses and contributes to the flavor, texture, and microbiological stability of the cheese (Guinee and Fox, 2004). Dry salting, as is applied in Cheddar manufacture, is done to control the production of lactic acid by the activity of the starter cultures so that a targeted pH and moisture can be reached in the final cheese, thereby setting up the appropriate conditions for aging that will produce desirable flavor and texture. The amount

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of acid produced after salting also affects the amount of moisture removed from the curd during salting and pressing because of its effect on the casein proteins, thereby affecting the final moisture. This effect on the amount of moisture removed at these steps also affects the amount of salt lost with the whey (Sutherland, 1974; Nair et al., 2004).

The ratio of the percentage salt to percentage moisture (**S/M**) in the final cheese is used as a way to assess if the appropriate conditions for aging have been achieved, and each cheese has a target range to achieve this. The S/M also relates to water activity (a_w), especially during the early stages of ripening (Fox et al., 2000; Guinee, 2004; Hickey et al., 2013) and this can, therefore, be an indicator of microbial safety, as a_w is used as a hurdle to microbial growth. In a recent study of 650 commercial Cheddar samples in the US market, the average S/M was 4.3 for branded cheeses and 4.1 for private label product (Agarwal et al., 2011).

Strategies to remove sodium should maintain the original product attributes, especially safety (Doyle and Glass, 2010). Salt concentration, a_w , pH and temperature are all important hurdles in controlling the growth of psychrotrophic pathogens such as *Listeria monocytogenes* (Cole et al., 1990; Dykes and Moorhead, 2000; Phan-Thanh et al., 2000; Hill et al., 2002; Ryan et al., 2008; Taormina 2010). One common strategy used in food sodium-reduction initiatives is to replace a portion of sodium chloride with small-molecular-weight compounds such as salts and sugars. Potassium chloride (KCl) is commonly used because it provides a salty taste, although it can contribute to a bitter or metallic note (Brandsma, 2006). It has been demonstrated to have similar inhibitory effects on *L. monocytogenes* when added at the same molar concentration NaCl in broth studies (Boziaris et al., 2007). In Cheddar cheese, KCl has been used to replace a portion of NaCl (Lindsay et al., 1982; Fitzgerald and Buckley, 1985; Reddy and Marth, 1995), but it is of note that the amount of KCl used to replace NaCl should be on a molar basis, equating to the same effective S/M such as in the study of Grummer et al. (2013).

Listeria monocytogenes is a very serious foodborne pathogen and is attributed to causing 1,590 infections and 255 deaths in the United States annually (19% of the deaths attributed to foodborne causes) (Scallan et al., 2011). Ready-to-eat dairy products are susceptible to postpasteurization contamination, and cheese has been implicated in *Listeria* contamination and cases of listeriosis (Linnan et al., 1988; McLauchlin et al., 1990; Rudolf and Scherer, 2001; Gianfranceschi et al., 2006; Pagotto et al., 2006; CDC, 2013). From 1998 to 2012, cheese had been implicated in 10 of 12 multi-state outbreaks involving dairy products in the United

States (CDC, 2013). None of these outbreaks involved Cheddar cheese, but there were 3 Class 1 recalls in the United States from 2011 to 2012 for *L. monocytogenes* in Cheddar cheese (FDA, 2013a). Cheddar cheese, similar to any other cheese, can be contaminated with *L. monocytogenes* after pasteurization and cheese making because the pathogen is often present in dairy plants and has been found on product contact surfaces (Walker et al., 1990; Pritchard et al., 1995; Tompkin, 2002; Gianfranceschi et al., 2006). In fact, a recall in 2012 involved multiple cheese types, including Cheddar, when *L. monocytogenes* was detected in shredded cheese through routine screening, and follow-up analysis detected *L. monocytogenes* on product contact surfaces on the slicing and conversion line used for processing this product and multiple other cheeses (FDA, 2013b). Conditions in cheese such as pH vary during aging, so understanding the implications of contamination at different stages during the ripening process (as could occur with cutting, shredding, and wrapping operations) is desirable.

Although many studies have been conducted to develop low- or reduced-sodium cheeses, focusing on quality aspects, less research has been undertaken to determine the effects of sodium reduction on their microbiological safety. Reducing sodium in Cheddar cheese has been reported to reduce the pH (Shrestha et al., 2011a) and to increase the moisture of the final cheese (Schroeder et al., 1988; Shrestha et al., 2011a). These factors could affect *Listeria* survivability in contaminated cheese (both positively and negatively). A study by Shrestha et al. (2011a) evaluated the effect of sodium reduction, pH, and storage temperature on the survival of *L. monocytogenes* in Cheddar cheese. They demonstrated that low-salt treatments led to a higher bacterial survival rate after 90 d of aging than full-salt cheese, but noted that the pathogen also survived in full-salt samples, with neither pH nor salt reduction effectively altering survivability.

The cultures used in cheese making provide hurdles to pathogen growth through their growth and metabolism, such as pH reduction and generation of low-molecular-weight compounds that contribute to reducing a_w . They also can produce inhibitory compounds, such as bacteriocins and organic acids beyond just lactic (Maisnier-Patin et al., 1992; Buyong et al., 1998; O'Sullivan et al., 2002; Galvez et al., 2008; Zacharof and Lovitt, 2012; Milillo et al., 2013; O'Shea et al., 2013;). From the 2 subspecies that are typically used as components of mixed starter cultures in Cheddar cheese manufacture in the United States, *Lactococcus lactis* ssp. *lactis* has been shown to have higher temperature, pH, and salt tolerance than *Lc. lactis* ssp. *cremoris* (Kim et al., 1999; Sanders et al., 1999). Differences have also been

reported between *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* in regard to the effect of sodium on their metabolism of lactose, which could then affect cheese pH (Turner and Thomas, 1980). In studies in milk meant to simulate cheese, Turner and Thomas (1980) found that *Lc. lactis* ssp. *lactis* was able to grow up to an S/M of 4 and ferment lactose and produce acid up to an S/M of 5. In contrast, *Lc. lactis* ssp. *cremoris* was inhibited at an S/M of 3. Reductions in sodium in cheese could, therefore, affect these cultures differently, resulting in different acidification profiles and culture survival. For a reduction to “low sodium” by US definition, the S/M would be approximately 1.9 if the cheese was 37% moisture and would, therefore, be less inhibitory to culture activity than full-sodium cheese.

Because of the effects of sodium on controlling microbial growth, we hypothesized that sodium reduction in Cheddar cheese without replacement by KCl would enhance the survival of *L. monocytogenes* at lower concentrations of NaCl. Additionally, we hypothesized that the selection of cheese starter culture subspecies would affect the survival of *L. monocytogenes* due to different acidification and culture survival profiles, because of differences in stress tolerance to NaCl. The objective of this study was to determine the effect of sodium reduction, with or without the addition of KCl, in Cheddar cheese cultured separately with different subspecies of *Lc. lactis*, on the ability of *L. monocytogenes* to survive when inoculated at different times during aging to simulate postprocessing contamination.

MATERIALS AND METHODS

Cheese Manufacturing

Stirred-curd Cheddar cheese was manufactured, in duplicate, in the Joseph J. Warthesen Food Processing facility located at the University of Minnesota (Saint Paul). Each cheese batch was produced according to

the stirred-curd Cheddar cheese-making procedure described in Grummer et al. (2012). Pasteurized milk was divided into 2 open stainless steel vats with automatic agitation (Kusel Equipment Co., Watertown, WI) at 31.1°C, and separately inoculated with either *Lc. lactis* ssp. *lactis* (M58, direct vat set; Danisco USA Inc., New Century, KS), or *Lc. lactis* ssp. *cremoris* (D71, bulk set; Danisco USA Inc.) at a level of 0.01% or 1% (wt/wt) of the cheese milk, respectively. The bulk-set D71 culture was manufactured by inoculating 1% of frozen culture into pasteurized whole milk and incubating at 22.2°C for 16 h the day before cheese making.

When the final titratable acidity target was achieved for salting (0.21% expressed as lactic acid), 13.6 kg of curd was portioned into 5 separate salting bins where the appropriate concentrations of NaCl and KCl (if used) were applied to create the salting treatments (amounts were applied in 3 equal additions, with 5 min of stirring between additions; Table 1). The amount of NaCl (Top-Flo Evaporated Salt; Cargill Inc., Minneapolis, MN), and KCl (Premier Potassium Chloride 8799; Cargill Inc.) used in each treatment was calculated based on the amount of NaCl used in our standard stirred-curd Cheddar cheese and the amount of KCl required to maintain the same a_w as in a full-sodium stirred-curd Cheddar cheese (molar replacement), as indicated by previous research (Grummer and Schoenfuss, 2011; Grummer et al., 2012, 2013). When salting was complete, the curds were packed into 9.1-kg Wilson-style hoops, and pressed overnight at 276 kPa. Pressed blocks were removed from the hoops, vacuum-sealed (Multivac Inc., Kansas City, MO), and stored at 4 to 5°C.

Preparation of Cheese for Chemical and Microbiological Testing

After cheese was manufactured and stored for 1 wk to allow for curd knitting and salt equilibration, each

Table 1. Concentration of NaCl and KCl added to curd during the salting step (before pressing) in the manufacture of stirred-curd Cheddar cheese

Treatment	NaCl ¹ added (g/100 g of curd)	KCl ² added (g/100 g of curd)
Full-sodium ³	2.90	0.00
Reduced-sodium ⁴	2.18	0.00
Reduced-sodium with KCl	2.18	0.93
Low-sodium ⁵	1.31	0.00
Low-sodium with KCl	1.31	2.04

¹Top-Flo Evaporated Salt (Cargill Inc., MN).
²Premier Potassium Chloride 8799 (Cargill Inc., MN).
³Full-sodium final cheese target: 600 mg of sodium/100 g of cheese.
⁴Reduced-sodium final cheese target: 450 mg of sodium/100 g of cheese.
⁵Low-sodium final cheese target: 280 mg of sodium/100 g of cheese.

block was cut into 8 equal subsections and separately vacuum packaged. Two samples were randomly chosen as replicates for chemical and microbiological testing. These blocks were shredded separately in a food processor (Cuisinart DFP-14BCN; Cuisinart, East Windsor, NJ), stored separately, and used for chemical and microbiological testing.

Cheese Compositional Analysis

Titrate acidity was measured during cheese manufacture to monitor the acidity level of the cheese as per the standard methods for milk and whey (Marshall, 1992). The following measurements were made in duplicate for each replicate from each treatment. Moisture was determined by vacuum oven as specified in Wehr and Frank (2004). Moisture was determined after initial manufacture and after 1 and 2 mo of storage at 4°C. The pH of the cheese was measured (uninoculated samples only), initially after manufacture, and periodically during refrigerated storage during 9 mo of aging. The measurement was performed according to standard methods (Marshall, 1992) using a pH meter with an Ag/AgCl combination electrode (Oakton pH meter 510 series; Oakton Instruments, Vernon Hills, IL). Protein content was determined by measuring percentage nitrogen on a Leco TruSpec N analyzer (Leco Corp., St. Joseph, MI) using the Dumas combustion method (AOAC International, 2005; method 993.13) and multiplying by a conversion factor of 6.38. Fat content was measured by the Babcock method according to the standard method for cheese (Marshall, 1992). Ash content was determined by a combustion method as specified in Richardson (1985).

Sodium and potassium content were determined by atomic absorption of the dry ashed cheese samples (ISO/IDF, 2007; method 119:2007). Samples were analyzed by atomic absorption using a PerkinElmer AAnalyst spectrometer 100 (PerkinElmer Inc., Waltham, MA) with compressed air as the oxidant, and acetylene gas as the fuel. Samples were analyzed separately for Na and K using a Na-K cathode lamp after calibration of the instrument with each mineral at the appropriate wavelength.

Experimental Design

Stirred-curd Cheddar cheese was manufactured in duplicate batches separately for each starter culture (4 total) and used throughout the duration of this project. Cheese was aged at 4°C and samples were removed from storage and inoculated with *L. monocytogenes* at different times during cheese aging (2, 15, 22, or 33 wk) to simulate episodes of postpasteurization contamination.

At the beginning of each inoculation event, replicate samples of cheese from each cheese-making batch were prepared and inoculated as described under the microbiological analysis method. Inoculated samples were stored at 4 or 12°C, depending on the testing period.

Microbiological Analysis

Bacterial Strains and Inoculation of Cheese Samples. Five strains of *L. monocytogenes* [strains J1-119 (lineage I, human, epidemic, Los Angeles, cheese, 1985), N1-227 (lineage I, food, epidemic, US, ready to eat meat, 1998–1999), N3-031 (lineage II, sporadic, US, hot dogs), R2-500 (lineage I, food, epidemic, North Carolina, cheese, 2000), and R2-502 (lineage I, food, epidemic, Illinois, chocolate milk, 1994)] were procured from the outbreak set of the International Life Sciences Institute collection of Martin Wiedmann (Cornell University, Ithaca, NY) and are described in Fugett et al., (2006). Frozen cultures of *L. monocytogenes* strains were kept at –80°C in 20% glycerol. Working cultures were prepared by transferring frozen cultures (individually) into tryptic soy broth (TSB; Neogen Corp., Lansing, MI) and incubated at 37°C for 24 h. The individual strain TSB cultures were mixed with the other strains (2 mL per strain) to prepare the inoculation cocktail. The mixed strain pool cultures of *L. monocytogenes* were serially diluted and spread plated at the start of the study to confirm viability and that counts were greater than 8 log₁₀ cfu/mL.

Cheese was inoculated at 3 different times during cheese aging with approximately 4 to 5 log₁₀ cfu/g. After the results showing the decline in numbers when inoculated at 4 log₁₀ cfu/g at 2 wk of ripening, the inoculation rate was increased to 5 log₁₀ cfu/g. To inoculate cheese samples, 1 mL volumes of *L. monocytogenes* cocktail were added to small plastic bags containing 99 g of the shredded treatment cheese. The bags were held closed and the contents were manually agitated for 90 s to ensure adequate mixing and homogeneous distribution of the inoculum. The inoculated cheese samples were transferred in 10-g portions into separate bags, depending on the number of samples to be taken over the time course of testing. Additionally, a corresponding number of uninoculated control samples were prepared for each treatment based on the number of samples taken over the duration of the study. The bags were vacuum sealed and stored at 4 or 12°C, depending on the objective.

Enumeration. On each sampling occasion, 2 sample bags per treatment were removed from storage and homogenized at approximately 230 rpm (Stomacher Lab Blender 400; Seward Laboratory Systems Inc., Port Saint Lucie, FL) with 90 mL of buffered peptone water

(Neogen Corp.) for 2 min. After homogenization, serial dilutions (2 dilutions per sample) were performed and the samples were spread plated (2 plates per dilution, 0.1 mL spread plated) on PALCAM agar (Neogen Corp.). When the counts of *L. monocytogenes* were below the limit of detection (15 cfu/g), cheese samples were enriched with UVM modified *Listeria* enrichment broth (Neogen Corp.) for 24 h at 37°C. After enrichment, samples were plated on PALCAM agar (0.5 mL per plate, 4 plates enumerated). Typical colonies with dark precipitate were enumerated as *L. monocytogenes* (the appearance was the same as pure cultures streak plated on that agar).

Starting at 153 d of ripening, background microflora were enumerated. After homogenization and serial dilutions to achieve plates capable of enumerating, uninoculated controls were spread plated on plate count agar (PCA; Neogen Corp.) to obtain a total aerobic plate count, and on de Man, Rogosa, and Sharpe (MRS) agar (Neogen Corp.) to enumerate lactic acid bacteria. All plates were incubated at 37°C for 24 h, and if no growth occurred, held for 48 h before enumeration. All plating was performed in duplicate for each treatment for each cheese-making replicate.

Statistical Analysis

Data were analyzed using PROC MIXED in SAS computer software (version 9.2; SAS Institute Inc., Cary, NC). Fisher's least significant difference was used as a summary statistic. All error bars in displayed figures represent 1 standard deviation above and below the mean.

RESULTS AND DISCUSSION

Compositional Analysis

No differences were observed in the gross composition (moisture, fat, and protein) of cheeses based on the starter culture used to manufacture the cheese, or the salt treatment applied to the cheese (Table 2). The mean ash, sodium, and potassium contents varied depending on salt treatment, as expected, although a lower-than-expected sodium concentration was measured in the 4 cheese batches in the full-sodium control (488 to 501 mg/100 g). This equated to an average S/M of 3.35 and was due to excessive NaCl loss during the salting and pressing stage (approximately 57% loss). This was a higher salt loss than what we typically see (approximately 30–50%), although Nair et al. (2004) reported 60% salt losses in their control cheese in a study evaluating the effects of Cheddar formula on salt losses. The average sodium concentration for full-

sodium Cheddar in the United States is 615 mg/100 g, although a broad range exists in full-sodium products in retail distribution (450 to 850 mg/100 g; Agarwal, et al., 2011). The S/M is used as an indicator of potential cheese quality and it relates to a_w (Guinee, 2004; Hickey et al., 2013). Sodium reduction in Cheddar cheese has been reported to increase cheese moisture because lower sodium leads to reduced curd syneresis (Guinee and Fox, 2004). We expected that the reduced- and low-sodium treatments without KCl would contain more moisture than with KCl, but this was not the case. The studies of Schroeder et al. (1988) and Shrestha et al. (2011a) with milled-curd Cheddar cheese reported an increase in moisture in reduced-sodium cheese without KCl. Reddy and Marth (1993) reported no difference in moisture in reduced-sodium cheese when KCl was used, and Grummer et al. (2012, 2013) reported increased moisture or no difference, depending on KCl source in stirred-curd Cheddar cheese.

As shown in Figure 1, the pH of the cheese increased after 3 mo of age (from pH 4.8 to 5.4). No difference was observed in pH due to the starter culture used in manufacture, indicating that the change in sodium levels did not affect the 2 cultures differently with regard to lactose fermentation. The increase in pH observed is typical during cheese aging and due to the production of alkaline compounds (such as ammonia) during proteolysis (McSweeney, 2004). Surprisingly, no difference in pH was observed due to the salt treatment, although, as mentioned earlier, our control S/M was lower than expected and could have contributed to this lack of difference. We expected to observe lower pH values with the reduced-sodium treatments that did not contain KCl. Fitzgerald and Buckley (1985) reported the same pH between full-sodium Cheddar cheese, cheese made with KCl alone (replaced on an ionic strength basis), or a 1:1 mixture of NaCl and KCl. The S/M, or effective S/M in the case of the KCl-containing samples, were all above the minimum ratio for high-quality Cheddar cheese (8.7, 4.5, and 5.0, respectively) in the Fitzgerald and Buckley (1985) study. Shrestha et al. (2011a) observed differences in pH between 50%-reduced-sodium-chloride Cheddar cheese (no KCl added) and control that were evident in cheese as early as 1 wk after manufacture. The reduced-sodium cheeses produced in the Shrestha et al. (2011a) study had S/M of 1.74 and 1.78 and their controls had S/M of 5.2 and 4.99, respectively. This would contribute to the lower pH observed by allowing increased culture metabolism and acidification, but would not have occurred in the Fitzgerald and Buckley (1985) study because of their higher effective S/M with the use of KCl. However, we did not see a difference in pH, and the S/M of the reduced- and low-sodium cheeses cultured with *Lc. lactis* ssp. *lactis*, and with

Table 2. Composition (means; n = 8) of full-sodium, reduced-sodium, and low-sodium stirred-curd Cheddar cheese

Salt treatment	Fat (%)	Moisture (%)	Protein (%)	Ash (%)	Sodium (mg/100 g)	Potassium (mg/100 g)	S/M ¹ (effective S/M ²)
Cheddar made with <i>Lactococcus lactis</i> ssp. <i>lactis</i>							
Full-sodium ³	32.5 ^a	38.45 ^a	23.87 ^a	3.31 ^a	501 ^a	36 ^a	3.31
Reduced-sodium ⁴	33.0 ^a	37.20 ^a	24.38 ^a	2.87 ^b	393 ^b	33 ^a	2.68
Reduced-sodium with KCl	33.0 ^a	37.11 ^a	24.72 ^a	3.48 ^c	441 ^b	292 ^b	(4.01)
Low-sodium ⁵	33.0 ^a	37.01 ^a	24.08 ^a	2.39 ^d	201 ^c	29 ^a	1.26
Low-sodium with KCl	33.0 ^a	37.40 ^a	24.91 ^a	3.57 ^e	251 ^c	576 ^c	(3.78)
Cheese made with <i>L. lactis</i> ssp. <i>cremoris</i>							
Full-sodium	32.5 ^a	38.92 ^a	24.05 ^a	3.34 ^a	488 ^a	31 ^a	3.18
Reduced-sodium	32.5 ^a	39.02 ^a	24.39 ^a	3.07 ^b	432 ^b	32 ^a	2.95
Reduced-sodium with KCl	32.0 ^a	38.57 ^a	24.36 ^a	3.45 ^c	415 ^b	305 ^b	(3.88)
Low-sodium	33.0 ^a	37.86 ^a	24.02 ^a	2.49 ^d	264 ^c	36 ^a	1.65
Low-sodium with KCl	32.0 ^a	38.45 ^a	24.76 ^a	3.68 ^e	273 ^c	644 ^c	(4.17)

^{a-e}Mean values within a column (both cultures combined) with different superscripts differ ($P < 0.05$).

¹S/M = percent salt divided by percent moisture.

²Effective S/M = salt-to-moisture ratio calculated by multiplying Na and K concentrations by 2.54 and 2.18, respectively, to obtain the NaCl and KCl concentrations. Naturally occurring K (present in control cheese) was subtracted before calculating KCl. The KCl concentration was divided by 1.27 to determine the equivalent amount of NaCl in terms of molar concentration. This value was added to the actual NaCl, and divided by the moisture in the cheese to determine the effective S/M.

³Full-sodium final cheese target: 600 mg of sodium/100 g of cheese.

⁴Reduced-sodium final cheese target: 450 mg of sodium/100 g of cheese.

⁵Low-sodium final cheese target: 280 mg of sodium/100 g of cheese.

L. lactis ssp. *cremoris* without KCl were much lower than control and treatments with KCl (Table 2). Other studies also observed a lower cheese pH when NaCl was reduced and KCl was absent (Kosikowski, 1983), or even when KCl was used together with NaCl (Koenig and Marth, 1982; Reddy and Marth, 1995; Grummer et al., 2012).

Total Aerobic Counts and Lactic Acid Bacteria in Control, Reduced-, and Low-Sodium Cheddar Cheese

No difference was observed in total plate count (TPC) based on salt treatment in cheese stored at 4°C monitored from 5 through 8 mo of age (data not shown). The average TPC were between 5 and 6 log₁₀ cfu/g. Reddy and Marth (1995) also observed no difference in TPC in milled-curd Cheddar cheese made with varying levels of sodium reduction and KCl replacement during 36 wk of aging. The total population of lactic acid bacteria as enumerated in our study on MRS agar at 5 mo of age were approximately 8.5 log₁₀ cfu/g in cheese stored at 4°C and no difference was observed based on treatment (data not shown). This result is consistent with studies of lactic acid bacteria in stirred-curd and milled-curd Cheddar cheese (Turner and Thomas, 1980; Hou et al., 2012), although Schroeder et al. (1988) reported significantly higher lactic acid bacterial growth during the first 4 mo of aging when the S/M was less than 2.0 (compared with cheese with S/M of 4.12 and 3.13).

Listeria monocytogenes Survival in Control and Reduced-Sodium Cheddar Cheese

Listeria monocytogenes was inoculated into cheese and enumerated at 4 different cheese ages to simulate post pasteurization contamination events in dairy plants, as has been demonstrated to occur in cheese (McLauchlin et al., 1990). Cheeses were initially inoculated at a level of 4 log₁₀ cfu/g after 2 wk of storage but later inoculations were done at 5 log₁₀ cfu/g to allow the monitoring of survival without the use of recovery methods. After inoculation at 2 wk postmanufacture, *L. monocytogenes* populations declined steadily. No difference was observed in survival based on starter culture or salt treatment (Figures 2a and 2b). Data from time points 28 and 63 d were collected using enrichment methods for cheese from both cultures, as the counts had dropped below the limit of detection.

The *L. lactis* ssp. *cremoris*-cultured treatments were inoculated at 15 wk of age. When stored at 4°C, the *L. monocytogenes* populations declined from 5.5 to 4.5 log₁₀ cfu/g in all treatments except in the 55%-reduced-sodium-without-KCl samples (Figure 3). The counts enumerated for this treatment were higher than full-sodium, reduced-sodium (with and without KCl), and low-sodium-with-KCl treatments from d 42 through 49.

Lactococcus lactis ssp. *cremoris*-cultured treatments were inoculated at 22 wk of age and stored at 12°C to simulate temperature abuse. The populations of *L. monocytogenes* remained closer to the 5 log₁₀ cfu/g inoculation level throughout the experiment, with no

Figure 1. The effect of sodium reduction and KCl addition on the pH of Cheddar cheese manufactured with *Lactococcus lactis* ssp. *cremoris*. Error bars represent sample SD; each data point represents the mean pH of uninoculated cheese manufactured separately ($n = 2$) and stored at 4°C. Full-sodium = final cheese target of 600 mg of sodium/100 g of cheese; reduced-sodium = final cheese target of 450 mg of sodium/100 g of cheese; low-sodium = final cheese target of 280 mg of sodium/100 g of cheese.

observed difference in survival based on salt treatment (Figure 4).

After a final inoculation with *L. monocytogenes* at 33 wk of age into cheeses manufactured with *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*, the populations of *L. monocytogenes* in control sodium cheese, reduced-, and low-sodium Cheddar cheese declined gradually from 5 to 3.5 log₁₀ cfu/g over a period of 49 d of enumeration in all treatments except the low-sodium treatment with KCl, in which *L. monocytogenes* survived at a level of 4 log₁₀ cfu/g (Figures 5a and 5b). No difference was observed in survival of *L. monocytogenes* between the 2 starter cultures.

With the exception of later periods of incubation in low-sodium treatments when *L. monocytogenes* was inoculated into aged cheeses (Figures 3, 5a, and 5b), differences in survival were not observed between treatments. This is surprising, as NaCl, along with pH, is used to control the growth and survival of *L. monocytogenes*. The rapid decline in *L. monocytogenes* populations we observed during the early stages of inoculation is interesting, however. This may have been the result of the active growth of the starter cultures, the presence of inhibitory substances produced by them, or, most likely, the effect of the lower initial pH. Or, conversely, the alteration in starter culture metabolism,

compounds responsible for the increased pH later in ripening (i.e., free amino acids), or the effect of the higher pH (i.e., change in organic acid effectiveness) could have contributed to the increased survival of *L. monocytogenes* compared with our earlier inoculation events. *Listeria monocytogenes* has been classified as an auxotroph and free amino acids, as a result of proteolysis, could have acted as an additional source of nutrients and increased the survival of *L. monocytogenes* later during aging (Verheul et al., 1998).

In 2 low-sodium treatments at the end of incubation where we observed differences (Figures 3, 5a, and 5b), it is likely that some of the *L. monocytogenes* strains used for inoculation adapted to the conditions in the cheese (acid in particular) better in the lower-sodium treatments. *Listeria* has been shown to adapt to acid and osmotic stress and have increased survival after adaptation (Gahan et al., 1996; Hill et al., 2002; Cataldo et al., 2007; Ryan et al., 2008). We cannot explain why we saw increased survival in low-sodium cheese without KCl at one time period (Figure 3), and then observed the opposite (low-sodium with KCl had increased survival) during the later inoculation event (Figures 5a and 5b). This is especially surprising in the case of low-sodium cheese with KCl, as KCl has been reported to be as effective in inhibiting *L. monocytogenes* growth

Figure 2. The effect of sodium reduction on the survival of *Listeria monocytogenes* in Cheddar cheese manufactured with *Lactococcus lactis* ssp. *lactis* (a) and *Lc. lactis* ssp. *cremoris* (b) when stored at 4°C during the first 2 mo of cheese aging. Time 0 denotes inoculation with *L. monocytogenes* 13 d after cheese manufacture. Error bars represent sample SD; each data point represents the mean count of 8 plates. Full-sodium = final cheese target of 600 mg of sodium/100 g of cheese; reduced-sodium = final cheese target of 450 mg of sodium/100 g of cheese; low-sodium = final cheese target of 280 mg of sodium/100 g of cheese.

Figure 3. The effect of sodium reduction on the survival of *Listeria monocytogenes* inoculated into 5-mo-old Cheddar cheese manufactured with *Lactococcus lactis* ssp. *cremoris* stored at 4°C after inoculation. Time 0 denotes inoculation with *L. monocytogenes* at 104 d of cheese ripening. Error bars represent sample SD; each data point represents the mean count of 8 plates. Full-sodium = final cheese target of 600 mg of sodium/100 g of cheese; reduced-sodium = final cheese target of 450 mg of sodium/100 g of cheese; low-sodium = final cheese target of 280 mg of sodium/100 g of cheese.

in broth (Boziaris et al., 2007). Potassium chloride has been reported to have a less detrimental effect on other bacteria, however (Bautista-Gallego et al., 2008). The observed results are interesting and warrant further evaluation of why this occurred.

In the broth studies of Boziaris et al. (2007) to examine the effect of pH, NaCl, KCl, and bacteriocin concentration on *L. monocytogenes* growth and death, the researchers concluded that equimolar concentrations of KCl were shown to be as effective at inhibiting *L. monocytogenes* Scott A growth, lag phase duration until growth, and death rate, as NaCl. In regard to the effect of pH on death, at pH 4.5 and 1 M salt concentrations (4.8% NaCl; much higher than would be present in most Cheddar cheeses), *L. monocytogenes* was inactivated within 40 h for both salts. Genigeorgis et al. (1991), in a study in which 1.5×5 -cm cubes of multiple cheese types were surface inoculated with a 5-strain pool of *L. monocytogenes* containing approximately $4 \log_{10}$ cells, reported a significant correlation with *L. monocytogenes* growth and pH values greater than 5.5, and gradual death in cheeses that ranged from pH 4.3 to 5.6. Organic acids present in cheese

such as lactic, acetic, formic, and citric, have the ability to cross the cell membrane of microorganisms, reducing their viability (Phan-Thanh et al., 2000; Ricke, 2003; Carpenter and Broadbent, 2009; Park et al., 2011). The concentration of lactic acid in Cheddar cheese has been reported to remain relatively constant after cheese manufacture and during aging (Marsili, 1985; Bouzas et al., 1993; Upreti et al., 2006; Murtaza et al., 2012), whereas other acids such as propionic and acetic increase during aging (Marsili, 1985; Bouzas et al., 1993). The change in pH from approximately 4.8 to 5.5 over the course of cheese aging in our experiment could have resulted in different amounts of organic acid being able to enter the cells and dissociate, as described in Carpenter and Broadbent (2009), and contributed to the lower survival we saw initially, and the improved survival during later inoculations.

A complication in comparing results between various studies performed with *L. monocytogenes* in cheese is the use of different strains for inoculation. Different strains vary in their acid, salt, temperature, and bacteriocin tolerance (Dykes and Moorhead, 2000; Ennahar et al., 2000; Fugett et al., 2006). This is the reason why

Figure 4. The effect of sodium reduction on the survival of *Listeria monocytogenes* inoculated into 5-mo-old Cheddar cheese manufactured with *Lactococcus lactis* ssp. *cremoris* and stored at 12°C after inoculation. Time 0 denotes inoculation with *L. monocytogenes* 153 d after production. Error bars represent sample SD; each data point represents the mean count of 8 plates. Full-sodium = final cheese target of 600 mg of sodium/100 g of cheese; reduced-sodium = final cheese target of 450 mg of sodium/100 g of cheese; low-sodium = final cheese target of 280 mg of sodium/100 g of cheese.

we chose to use a cocktail of 5 strains from 2 different lineages that have been implicated in foodborne outbreaks. The rapid reduction in *L. monocytogenes* counts during the early stage of cheese ripening we observed is similar to what was reported by Ryser and Marth (1987). They inoculated 113-kg lots of cheese milk with 50 mL of milk containing approximately 3 log₁₀ cfu/mL of each of 3 different strains of *L. monocytogenes* [Scott A (FSL-J1-225), V7, and CA], and manufactured stirred-curd Cheddar cheese. They enumerated *L. monocytogenes* throughout cheese making and aging. In their study, after an increase in counts during cheese making and during the first 2 wk of ripening at 6°C, counts declined fairly rapidly from approximately 3.5 log₁₀ cfu/g to less than 1 log₁₀ cfu/g in as early as 75 d, depending on the strain and cheese make. One strain, however, exceeded 1 log₁₀ cfu/g for over 100 d, showing the variability that can be introduced due to strain selection. Their study had a similar S/M (4.3) ratio (or effective S/M) to our control cheese and reduced- and low-sodium cheese with KCl. A difference between this study and ours is that the *L. monocytogenes* was added before cheese manufacturing and a better distribution

of the organism would have been achieved. Also, the organism had to adapt to changing conditions throughout the cheese-making and storage processes, so it did not simulate postprocess contamination as in our study.

By contrast, Mehta and Tatini (1994) also added *L. monocytogenes* during cheese making and observed only a 1 log₁₀ cfu/g reduction after cheese ripening commenced over 20 wk of storage in reduced-fat stirred-curd Cheddar cheese that was manufactured with 2 strains of *L. monocytogenes* (Scott A and V7). Their study had a higher S/M (5.2) than ours. Higher S/M in Cheddar cheese results in reduced lactic acid production by starter cultures (Upreti et al., 2006) and could have contributed to differences between the studies.

In an investigation of regular and reduced-sodium milled-curd Cheddar cheese, Shrestha et al. (2011a) inoculated cheese with a 5-strain cocktail of *L. monocytogenes*. They manufactured cheese with 2 pH targets with and with full sodium and an approximately 60% sodium reduction, which resulted in S/M of 5.2, 5.0, 1.7, and 1.8. They inoculated at 10 wk of age to simulate postprocessing contamination. In inoculated samples held at 4°C and monitored for 90 d, they

Figure 5. The effect of sodium reduction on the survival of *Listeria monocytogenes* inoculated into 7-mo-old Cheddar cheese manufactured with the starter cultures *Lactococcus lactis* ssp. *lactis* (a) and *Lc. lactis* ssp. *cremoris* (b) and stored at 4°C. Time 0 denotes inoculation with *L. monocytogenes* 231 d after cheese manufacture. Error bars represent sample SD; each data point represents the mean count of 8 plates. Full-sodium = final cheese target of 600 mg of sodium/100 g of cheese; reduced-sodium = final cheese target of 450 mg of sodium/100 g of cheese; low-sodium = final cheese target of 280 mg of sodium/100 g of cheese.

observed an approximate $1 \log_{10}$ cfu/g decline in the population of *L. monocytogenes*, and similar to what we observed, they did not see an overall effect of salt treatment on this decline. However, the exception noted was that at 90 d of enumeration, their control salt level, low-pH treatment (5.25 at the enumeration time) had lower $\log_{10}$ colony-forming units per gram than all other treatments. Their study had higher S/M in their control NaCl cheeses than ours, and their pH was also generally higher than ours. In contrast to our findings, Shrestha et al. (2011a) observed a moderate reduction in *L. monocytogenes* counts when incubated at 10 and 21°C, whereas we observed no decrease in survival in cheese inoculated at 5 mo of age and incubated at 12°C (Figure 4). The higher incubation temperature seemed to improve the survival of *L. monocytogenes* compared with cheese we inoculated at 4 mo and incubated at 4°C (Figure 3). Our results are similar to the those in the study of Ryser and Marth (1987), in which they noted that an elevated ripening temperature (13°C) did not adversely affect *L. monocytogenes* survival compared with 6°C, except in the case of strain V7 (it survived almost twice as long at 6 than 13°C). This is in contrast with what is typically observed with other pathogens such as *Salmonella* (Goepfert et al., 1968; Shrestha et al., 2011b), but has been reported as a difference between *L. monocytogenes* from different lineages, so it could also be a reason for differences observed between our studies (Bergholz et al., 2010).

CONCLUSIONS

Due to the effects of sodium on controlling microbial growth, we investigated whether the reduction in sodium in Cheddar cheese without maintaining the effective S/M by the use of KCl would enhance the survival of *L. monocytogenes*. The selection of cheese starter culture subspecies could also affect the survival of *L. monocytogenes* due to different acidification and survival profiles because of differences in stress tolerance to NaCl and pH. No difference existed in the chemical composition of the cheese related to salting treatment, or in *L. monocytogenes* survival related to the single-strain starters used. Therefore, any differences in the acid or NaCl stress tolerance between *Lc. lactis* ssp. *cremoris* and *Lc. lactis* ssp. *lactis* did not result in pH differences and did not have an effect on *L. monocytogenes* survival. After inoculating with a 5-strain cocktail of *Listeria monocytogenes* at different times to simulate postprocessing contamination, differences were only observed in survival at later periods of enumeration in low-sodium cheese. *Listeria monocytogenes* did not survive well in any of the salting treatments when inoculated 2 wk after cheese manufacturing when the pH was lower

than at later inoculation events. After the cheese had aged for a few months, however, *L. monocytogenes* was able to survive at 2 different incubation temperatures. Sodium reductions of up to 50% with S/M as low as 1.26, and the substitution of NaCl with the same molar concentration of KCl, did not alter the survival of *L. monocytogenes*. This study demonstrated, however, that *L. monocytogenes* was better able to survive in cheese when it was inoculated during later periods of aging, as could happen during cutting, shredding, and wrapping operations. This emphasizes the need for adherence to good manufacturing practices at all stages of Cheddar manufacture and handling to avoid the introduction of this pathogen.

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