Effect of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during storage of full-fat Cheddar cheese¹

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ABSTRACT

Sodium reduction in cheese can assist in reducing overall dietary Na intake, yet saltiness is an important aspect of cheese flavor. Our objective was to evaluate the effect of partial substitution of Na with K on survival of lactic acid bacteria (LAB) and nonstarter LAB (NSLAB), pH, organic acid production, and extent of proteolysis as water-soluble nitrogen (WSN) and protein profiles using urea-PAGE, in Cheddar cheese during 9 mo of storage. Seven Cheddar cheeses with molar salt contents equivalent to 1.7% salt but with different ratios of Na, K, Ca, and Mg cations were manufactured as well as a low-salt cheese with 0.7% salt. The 1.7% salt cheeses had a mean composition of 352 g of moisture/kg, 259 g of protein/kg and 50% fat-on-dry-basis, and 17.5 g of salt/kg (measured as Cl⁻). After salting, a faster initial decrease in cheese pH occurred with low salt or K substitution and it remained lower throughout storage. No difference in intact casein levels or percentage WSN levels between the various cheeses was observed, with the percentage WSN increasing from 5% at d 1 to 25% at 9 mo. A greater decrease in intact α_{s1} -casein than β -casein was detected, and the ratio of α_{s1} -casein (f121–199) to α_{s1} -casein could be used as an index of ripening. Typical changes in bacteria microflora occurred during storage, with lactococci decreasing gradually and NSLAB increasing. Lowering the Na content, even with K replacement, extended the crossover time when NSLAB became dominant. The crossover time was 4.5 mo for the control cheese and was delayed to 5.2, 6.0, 6.1, and 6.2 mo for cheeses with 10, 25, 50, and 75% K substitution. Including

10% Mg or Ca, along with 40% K, further increased crossover time, whereas the longest crossover time (7.3 mo) was for low-salt cheese. By 9 mo, NSLAB levels in all cheeses had increased from initial levels of $\leq 10^2$ to approximately 10^6 cfu/g. Lactococci remained at 10^6 cfu/g in the low-salt cheese even after 9 mo of storage. The propionic acid concentration in the cheese increased when NSLAB numbers were high. Few other trends in organic acid concentration were observed as a function of Na content.

Key words: cheese, sodium, potassium, propionic acid, nonstarter

INTRODUCTION

The promotion of lower-Na foods for better health when applied to cheese has the potential to affect the development of normal cheese flavor during aging. Challenges in reducing Na levels in cheese have been recently reviewed, emphasizing the importance of salt in cheese flavor, food safety, and general consumer acceptability (Johnson et al., 2009). Small reductions (10 to 25%) in salt content of cheese are acceptable to consumers, but some saltiness is an expected part of cheese flavor (Lawrence et al., 2009). As salt content is reduced further, consumer liking of cheese decreases for both Cheddar and Mozzarella cheeses (Ganesan et al., 2014).

Therefore, if a Na reduction of 30% or more is to be achieved, then the saltiness of the cheese has to be maintained using something other than sodium. To replace the salty taste in reduced-sodium cheese, other chloride salts such as K, Mg, and Ca (but especially KCl) can be used as cation substitutes (Johnson et al., 2009). However, these other cations do not provide the same level of saltiness as Na ions and some have inherent off-flavors, such as bitter or metallic, which limit their acceptability in cheese (Fitzgerald and Buckley, 1985; Grummer et al., 2013).

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Potassium chloride is the most chemically similar to NaCl and hence the obvious choice for NaCl replacement (Johnson et al., 2009). Fitzgerald and Buckley (1985) reported that Cheddar cheese containing a 50% replacement of Na with K did not significantly differ from 100% NaCl cheese in proteolysis, flavor, texture, or FA patterns. This was not the case when either CaCl_2 or MgCl_2 was used as a NaCl replacement, as texture and taste scores were much different. Toelstede and Hofmann (2008) also found that bitterness in Gouda cheese matured for 44 wk was induced by replacement of NaCl with either CaCl_2 or MgCl_2 . In Kefalograviera cheese, Katsiari et al. (1998) reported that replacing Na with K up to 50% had no significant effect on cheese composition or textural or sensory characteristics up to 180 d of aging. Aly (1995) also reported that a 1:1 mixture of NaCl:KCl produced a Feta-like cheese of acceptable quality. In contrast, Lindsay et al. (1982) found that consumers preferred cheese containing a 50% KCl replacement of NaCl less than cheese containing 100% NaCl.

Besides its direct influence on cheese flavor, salting cheese plays other roles important in cheese manufacture and aging. For most cheeses (except when Ca content is lowered; Paulson et al., 1998), adding salt promotes whey expulsion from the cheese and aids in surface rind formation (Guinee, and Fox, 2004) allowing the cheese to attain its expected characteristics. Salt content in cheese can also be critical from a microbiological aspect because adding salt can be essential to restrict growth of unwanted bacteria in ripening curd. The salt concentration in cheese also helps control survival and metabolism of desirable bacteria such as the starter lactic acid bacteria (**LAB**), as well as secondary organisms [nonstarter LAB (**NSLAB**)] that may grow and create flavors during extended storage.

Reducing the overall salt level in cheese, or replacing Na with other cations, alters the salt-related response of bacteria and is likely to redirect starter bacteria and NSLAB survival along with their metabolism, hence affecting flavor production during aging. The stress response of LAB to Na (Rallu et al., 1996; van de Guchte et al., 2002; Xie et al., 2004) and other metal ions, such as Cu, Ni, Ar, and Cd (Efstathiou and McKay, 1977; Boutibonnes et al., 1995; Barré et al., 2007; Turner et al., 2007), are well known. Several studies have examined the effect of partial or total replacement of NaCl with KCl on cheese microflora and found no significant differences (Aly, 1995; Reddy and Marth, 1995b; Wachowska, 2011; Ayyash et al., 2012).

Both starter LAB (usually lactococci in Cheddar cheese) and NSLAB produce organic acids during

metabolism; some are generally intermediates in metabolism, whereas other organic acids are end products retained in the cheese. Correlating organic acid production and their individual concentrations over the ripening period to changes in cheese chemistry, microbial populations, and, eventually, flavor differences may provide insight into how LAB react to changes in cations and cation concentrations. Upreti et al. (2006a) examined the influence of Ca, P, lactose, and salt-to-moisture in Cheddar cheese to organic acid production over 12 mo of ripening. For many acids (citric, uric, butyric, and propionic), concentrations increased in the later stages of ripening, whereas other organic acid concentrations either decreased (orotic) or only showed a very modest increase (pyruvic) over time. Ayyash et al. (2012) found differences in organic acid concentrations in Akawi cheese after 30 d when K was used to replace Na. Higher concentrations of citric, lactic, and acetic acids were found in 75% KCl:25% NaCl cheese compared with control cheese (100% NaCl).

Compared with just lowering salt content, using KCl should help maintain control of bacteria during aging of Cheddar cheese (Reddy and Marth, 1995a). Most research using salts with different cations has focused on flavor with limited examination of the factors that influence flavor development (Lindsay et al., 1982; Fitzgerald and Buckley, 1985). We conducted a systematic study into how substitution of NaCl with KCl affects the microbiology of Cheddar cheese and the consequent cheese flavor. Potassium substitution levels of 10, 25, 50, and 75% were compared with a control cheese and the effect of including a low level (10%) of MgCl_2 or CaCl_2 was investigated. As a negative control, a low-Na cheese without any cation substitution was made so we could separate the effect of reducing Na from that of replacing Na with K.

Our overall research focused on (1) factors influencing survival and growth of bacteria in cheese, such as type of salts and amount of water available for bacteria; (2) metabolic output from bacteria that can directly influence flavor development, such as organic acids, protein breakdown, and cheese pH; and (3) an understanding of which bacteria predominate during various stages of cheese aging and how different salts may influence their number and metabolism. Parameters tested during storage included lactococci and NSLAB numbers, and various chemical parameters [pH, water activity, organic acids, water-soluble N (**WSN**), and extent of casein proteolysis]. Here, we report on the effect of cation substitution during salting on cheese chemical composition and the cheese microbiome during 9 mo storage of full-fat Cheddar cheeses.

Table 1. Target salt level, molar cation ratio, and weight of salts added to 11.9-kg portions of Cheddar cheese curd

Cheese	Target salt level ¹ (%)	Na:K:Mg:Ca	NaCl (g)	KCl (g)	MgCl ₂ ·6H ₂ O (g)	CaCl ₂ ·2H ₂ O (g)
Control	1.7	100:0:0:0	300	— ²	—	—
10% K	1.7	90:10:0:0	270	38	—	—
25% K	1.7	75:25:0:0	225	96	—	—
50% K	1.7	50:50:0:0	150	191	—	—
75% K	1.7	25:75:0:0	75	287	—	—
10% Mg	1.7	50:40:10:0	150	153	101	—
10% Ca	1.7	50:40:0:10	150	153	—	74
Low salt	0.7	100:0:0:0	85	—	—	—

¹Salt level refers to salt in cheese measured by chloride analysis when only NaCl was used or in a general manner to other cheeses in which a molar cation substitution was made.

²— = none added.

MATERIALS AND METHODS

Cheese Manufacture

Fresh cow milk was obtained from the George B. Caine Dairy Research and Teaching Center (Wellsville, UT) and transported to the Gary Haight Richardson Dairy Products Laboratory at Utah State University (Logan). The milk was standardized to a protein-to-fat ratio of 0.83, pasteurized at 73°C for 15 s, and then 700 kg was pumped into a Tetra Scherping horizontal cheese vat (Tetra Pak Cheese and Powder Systems Inc., Winsted, MN) and 270 kg into an open stainless steel vat. Both batches of milk were warmed to 31°C and 0.14 g/kg of frozen pellets containing *Lactococcus lactis* ssp. *lactis/cremoris* starter culture (DVS850; Chr. Hansen Inc., Milwaukee, WI) was added and the milk allowed to ripen for 45 min. Then, 0.073 mL/kg of a 32% (wt/wt) CaCl₂ solution (Nelson-Jameson Inc., Marshfield, WI) and 0.073 mL/kg of double-strength (~650 international milk clotting units/mL) chymosin rennet (Maxiren; DSM Food Specialties USA Inc., Eaganville, PA) were added, and the milk allowed to set undisturbed for 30 min. After cutting and healing, the curd/whey mixtures were stirred for 25 min, heated to 39°C over 35 min, and then stirred for another 15 min.

The curd and whey from both vats were then combined by transferring onto a drain table (Kusel Equipment Co., Watertown, WI) with partial whey drainage and stirred until a curd pH of 6.3 was reached (~20 min). The remaining whey was then drained and the dry curd stirred for approximately 5 min (15 passes of agitators along the drain table). The curd was allowed to mat together, cut into slabs, and cheddared for approximately 135 min until the curd pH reached 5.4. Curd was milled, separated into eight 11.9-kg portions, placed into open plastic containers, and individually salted according to Table 1. Each aliquot of salts was added and manually mixed using 3 applications with 5 min between each application. Salted curd was allowed to stand for 10 min before being placed into plastic

cheesecloth-lined stainless steel hoops and pressed overnight (140 kPa, ~18 h, ~20°C). The cheese was then de-hooped, vacuum packaged, and stored at 6°C. This produced 7 blocks of high-salt cheese consisting of a cheese salted with 100% NaCl (control), a series of cheeses with increasing K substitution for Na, designated as 10% K, 25% K, 50% K, 75% K, and 2 cheeses with a 40% K substitution plus an additional 10% substitution using either MgCl₂ (10% Mg) or CaCl₂ (10% Ca), and 1 low-salt cheese that would meet the requirement for a low-Na food (i.e., ≤140 mg/50 g of cheese; FDA, 2008) without any cation substitution. After 7 d of storage, the cheese was cut into 1- and 2-kg pieces and returned to storage.

Cheese Sampling

Cheese samples were collected before and after salting (d 0), after pressing (d 1), on d 7 and 28, and then monthly through 9 mo of aging. These samples were either immediately distributed for sample preparation for the various analyses or stored frozen at -80°C. In replicate 2, additional samples were collected of (1) curd before salting, (2) curd after salting, and (3) unsalted curd that was pressed overnight. These were tested for microbial populations to provide a baseline for starter culture numbers in curd before pressing, and to help determine the influence of salt content on starter culture die-off during overnight pressing of curd into cheese blocks.

Chemical Analysis

Proximate composition of the cheeses was determined after approximately 5 d. Moisture content was measured by weight loss using approximately 3.7 g of grated cheese in a microwave moisture analyzer (model SMART System5; CEM Corp., Matthews, NC) at 100% power with an endpoint setting of <0.5-mg weight change over 10 s. Fat content was measured by a modified Babcock method (Richardson, 1985). Protein

content was calculated from total N as measured by the Kjeldahl method, and multiplied by 6.38. Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher 400 (Seward Ltd., Worthing, UK). The slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for NaCl using a chloride analyzer (model 926; Corning Diagnostic Corp., Medfield, MA). Salt-in-moisture concentration was calculated as salt/(moisture + salt) and expressed as a percentage. For mineral analysis, cheese samples were ashed in a muffle furnace (model 550-126; Fisher Scientific, Pittsburgh, PA) at 100°C for 18 to 24 h, then 24 to 36 h at 300°C and 12 to 24 h at 550°C (until the ash was white), and then cooled to room temperature. The sample before ashing and ash were accurately weighed (± 0.05 mg) and the ash sent to Analab (Fulton, IL) for mineral analysis by inductively coupled plasma spectroscopy. The pH was measured using a glass electrode after stomaching 20 g of grated cheese with 10 g of distilled water for 1 min at 260 rpm. Water activity was measured by relative humidity using an AquaLab CX2 instrument (Decagon Devices Inc., Pullman, WA).

Organic Acids Analysis

Organic acids were extracted from cheeses using water and acetonitrile, and separated by HPLC using the methods described by Marsili et al. (1981). Calibration curves were determined for the various analytes, and then the analyte concentration in the cheese was calculated using a dilution factor based on sample weight of the cheese and weight of 0.013 *N* H₂SO₄ in which the cheese was dissolved. Acids were identified by comparison of retention times to authentic standards injected under identical conditions. Calibration curves were established for each acid based on peak area using linear regression lines ($R^2 = 0.99$ for each acid).

Proteolysis

Proteolysis in the cheese was determined by urea-PAGE and measuring percentage WSN. Water-soluble fractions of the cheeses were prepared as described by Kuchroo and Fox (1982) and analyzed for N by the Kjeldahl method. Urea-PAGE of cheeses was performed as described by Farkye (1995) with sodium caseinate used as a reference, showing bands of α_{s1} -CN and β -CN. Ten milligrams of each sample was weighed, mixed with 1 mL of 1 $\times$ sample buffer, and then heated for 5 min at 50°C and vortexed. Four microliters of each sample was loaded onto the urea-PAGE gel consisting of a 12% (wt/vol) acrylamide resolving gel with a 4% (wt/vol) acrylamide stacking gel. The gel was

run at 150 V for 60 min. Presumptive identification of hydrolysis products: α_{s1} -CN (f24–199) (also known as α_{s1-I} -CN), α_{s1} -CN (f121–199), β -CN (f106–209), β -CN (f29–209), and β -CN (f108–209) (also known as γ_1 -, γ_2 -, and γ_3 -CN, respectively) were made by comparison to those from Hinz et al. (2012). Gels were scanned by densitometry and band densities for α_{s1} -CN, β -CN, and α_{s1} -CN (f121–199) were measured and corrected based on band densities for the sodium caseinate standards.

Microbial Enumeration in Cheese

Cheese samples [11 g added to 99 mL of sterile 2% (wt/wt) sodium citrate buffer] were enumerated as described by Oberg et al. (2011) using M17-L medium incubated aerobically at 30°C for 24 h for *L. lactis* starter cells, de Man, Rogosa, and Sharpe (MRS) medium supplemented with sorbitol for total LAB, and MRS-vancomycin for NSLAB, with the latter 2 media incubated anaerobically at 37°C for 48 h. A “crossover time” as described by Oberg et al. (2011) for the time point when NSLAB numbers equaled and then surpassed lactococcal numbers was determined. To account for any between-replicate differences, the ratios of the logarithm of the number of lactococci and the logarithm of the number of NSLAB to the logarithm of the number of total LAB for each time point was determined for each replicate. These mean logarithmic ratios were plotted against storage time and a trend line fitted to each set of data (second-order polynomial trend line for starter and logarithmic trend line for NSLAB). The time (mo) when the lines intersect was considered the crossover time.

Experimental Design

The experiment was conducted as a randomized block design, with cheese made on 3 separate occasions and curd divided into batches and then salted and pressed. Each block was cut into 0.5- and 1-kg pieces, individually vacuum packaged, stored at 6°C, and pieces randomly selected for testing during storage. Statistical analysis of the effect of storage time was performed as a split plot design. ANOVA was performed using PROC GLM in SAS (version 9.1; SAS Institute Inc., Cary, NC) and differences between means determined using REGW multiple range test and Tukey least squares means.

RESULTS

Proximate Composition and Mineral Content

Significant differences existed in cheese composition as a function of salting treatment for all variables

except fat and Ca, as shown in Table 2. Fat content was the same for all cheeses, whereas the variation in Ca measurement was such that the addition of 10% of the salt as CaCl_2 was not sufficient to cause statistical significance, although a trend existed for the 10% Ca cheese to have slightly higher Ca content. Moisture, salt (measured by Cl^- content) and water activity were similar for high-salt cheeses, with mean values of 349 g of moisture/kg, 17.1 g of salt/kg, and 0.956 water activity. The cheese that received the low-salt treatment was consistently 25 g/kg higher in moisture content than the other cheeses. For low-salt cheese, no curd treatment was applied before salting to lower the curd moisture content, so less whey expulsion occurred. A significant replicate effect was detected for all variables except salt content and pH. Because each replicate of cheese curd was manufactured about a month apart, some slight differences existed in milk composition and cheesemaking. For example, in replicates 1, 2, and 3, when the mean cheese moisture was 346, 346, and 354 g/kg, the mean d-7 pH was 5.13, 5.09, and 5.15; mean salt-in-moisture concentration was 4.74, 4.95, and 4.77%; and mean water activity was 0.956, 0.956, and 0.962.

Salt levels for the high-salt cheeses were within the range of 16 to 20 g/kg. This was expected, as substitution of Na^+ by K^+ was made on a molar basis. The divalent Mg^{++} and Ca^{++} salts were only added as 10% of total salt, so the extra Cl^- included in these salts was less than the normal variation that occurs during salting of curd. The low-salt cheese had a mean salt level of 6.8 g/kg and mean Na level of 2.3 mg/g, which is at the level required for the cheese to be classified as low Na (i.e., ≤ 140 mg of Na/50 g; FDA 2008).

Mean Na contents decreased from 6.5 mg/g in the control cheese (mean salt = 17.4 g/kg) to 2.1 mg/g in the 75% K cheese. A corresponding increase in K concentration was detected from 1.1 mg/g when no KCl was added (control) to 10.4 mg/g in the 75% K cheese. Mean Na content of 10% K cheese was the same as the control cheese, and 25% K cheese was only 10% less than the control in mean Na content. Based on their mean Na content, 50% K and 75% K cheeses could be classified as a 40%-reduced-Na cheese and low-Na cheese, respectively. The 10% Mg and 10% Ca cheeses could be classified as 40- and 50%-reduced-sodium cheeses, respectively. They had mean K levels of 6.1 and 5.1 mg/g, and an approximately 1 mg/g increase in Mg and Ca concentration, respectively.

Cheese pH

When NaCl was substituted with KCl, a greater decrease occurred in cheese pH during overnight pressing, such that the d-1 mean pH for the control cheese was 5.23, whereas cheese made with a 25:75 molar ratio of Na to K (75% K cheese) had a d-1 mean pH of 5.15. Cheese made using 40% K plus 10% Mg or 10% Ca substitution had a further decrease in pH during pressing at d 1 to 5.09 and 5.05, respectively. The low-salt cheese had the lowest d-1 mean pH of 4.97. Differences in pH between the cheeses were smaller after 7 d of storage (Table 2). The pH of the high-salt cheeses decreased during the first week of storage, as expected, as Cheddar cheese typically contains 0.8 to 1.0% lactose before salting and this is metabolized quickly through the continued activity of the starter culture lactococci (McSweeney and Fox, 2004).

Table 2. Composition of experimental cheeses described in Table 1 (n = 3)

Cheese ¹	Moisture (g/kg)	Fat (g/kg)	Protein (g/kg)	Salt ² (g/kg)	[Salt] ³ (%)	Na (g/kg)	K (g/kg)	Mg (g/kg)	Ca (g/kg)	d-7 pH	Pooled pH ⁴	a _w ⁵
Control	350 ^b	327 ^a	260	17.4 ^a	4.73 ^a	6.5 ^a	1.1 ^c	0.30 ^b	7.0 ^a	5.16 ^a	5.30 ^a	0.956 ^b
10% K	350 ^b	326 ^a	252	17.0 ^a	4.64 ^a	6.5 ^a	2.2 ^{de}	0.34 ^b	7.9 ^a	5.17 ^a	5.30 ^a	0.954 ^b
25% K	349 ^b	323 ^a	259	17.6 ^a	4.80 ^a	5.8 ^a	3.9 ^{cd}	0.34 ^b	8.0 ^a	5.17 ^a	5.26 ^a	0.956 ^b
50% K	349 ^b	325 ^a	255	17.8 ^a	4.84 ^a	3.9 ^b	7.3 ^b	0.34 ^b	7.9 ^a	5.15 ^a	5.25 ^{ab}	0.958 ^b
75% K	349 ^b	320 ^a	263	18.0 ^a	4.91 ^a	2.1 ^c	10.4 ^a	0.35 ^b	8.4 ^a	5.11 ^{ab}	5.19 ^{bc}	0.955 ^b
10% Mg	348 ^b	319 ^a	258	18.1 ^a	4.94 ^a	3.8 ^b	6.1 ^{bc}	1.13 ^a	8.1 ^a	5.07 ^{ab}	5.16 ^{cd}	0.957 ^b
10% Ca	345 ^b	332 ^a	269	17.7 ^a	4.87 ^a	3.0 ^{bc}	5.1 ^{bc}	0.34 ^b	9.0 ^a	5.01 ^b	5.11 ^d	0.957 ^b
Low salt	373 ^a	315 ^a	254	6.8 ^b	1.79 ^b	2.3 ^c	1.0 ^e	0.31 ^b	7.0 ^a	5.01 ^b	5.11 ^d	0.975 ^a
P-value	<0.001	0.53		<0.001	<0.001	<0.001	<0.001	<0.001	0.19	0.003	<0.001	<0.001
P _{rep} ⁶	<0.001	0.006		0.72	0.68	<0.001	0.014	0.010	0.014	0.091	0.29	0.014

^{a-e}Means within a column followed by the same superscript letter were not significantly different ($\alpha = 0.05$).

¹The K cheeses refer to cheeses in which KCl was substituted for NaCl at molar levels of 10, 25, 50, or 75%; 10% Mg = cheese with 40% K substitution and 10% Mg substitution; 10% Ca = cheese with 40% K substitution and 10% Ca substitution; low salt = 50% K substitution for Na.

²Measured by Cl^- analysis.

³Calculated as salt/(moisture + salt).

⁴Mean pH over all storage times.

⁵Water activity, as measured on d 28.

⁶Probability of no difference between replicates (*rep*).

Table 3. Pooled mean pH during 9 mo of storage of cheese salted with various cation substitutions as shown in Table 1 (n = 24)

Item	Storage time										
	1 d	7 d	28 d	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Mean pH	5.14 ^{cd}	5.11 ^d	5.19 ^{bc}	5.26 ^{ab}	5.23 ^{ab}	5.29 ^a	5.18 ^{bcd}	5.22 ^{abc}	5.24 ^{ab}	5.21 ^{abc}	5.22 ^{abc}

^{a-c}Means followed by the same superscript letter were not significantly different ($\alpha = 0.05$).

At d 7, a trend still existed for lower pH as Na substitution increased and the same trend was apparent when cheese pH was pooled over all time points (Table 2). After 2 mo of storage at 6°C, the pooled mean pH for all cheeses significantly increased approximately 0.10 units, with a trend for another approximately 0.05-unit increase through 4 mo of storage (Table 3). A trend existed for pH to decrease slightly during the remaining storage, such that the pH at 9 mo was similar to the 1-mo and 2-mo pH. This occurred even though the total organic acid content of the cheeses increased throughout storage (Figure 1B).

Water Activity

No significant difference was detected in water activity between the high-salt cheeses, with all measurements in the range of 0.95 to 0.96, with mean water activities at 28 d of storage at 0.956 ± 0.002 (Table 2). This was expected, given that salt substitution was performed on a molar basis and these cheeses all had a mean salt-in-moisture concentration of 4.6 to 4.9%. Low-salt cheese had higher water activity (about 0.02 higher) than the other cheeses, as expected, as it had a mean salt-in-moisture concentration of only 1.8%. During storage, a trend existed in the high-salt cheeses for water activity to decrease approximately 0.01 units from 1 d (pooled mean of 0.959) to 9 mo (pooled mean of 0.951) and a similar trend occurred for the low-salt cheese.

Organic Acids

As shown in Figure 1, the overall concentration of organic acids in all the cheeses increased during storage. No appreciative differences caused by adding 10% Mg or Ca along with 40% K (10% Mg and 10% Ca cheeses) compared with 50% K cheese with 50% K substitution were detected, so curves for organic acids in 10% Mg and 10% Ca cheeses are not shown to provide clarity in the figures. The effect of cation substitution will, therefore, focus on changes in the Na:K ratio, with low-salt cheese used as a negative comparison for cheese with low-Na and low-salt content.

Lactic Acid. Mean d-1 lactic acid content for the control cheese was 14.3 g/kg, which is close to the 15

g/kg for Cheddar cheese stated by McSweeney and Fox (2004) and 11 g/kg observed for a similar Cheddar cheese by Upreti et al. (2006a). Lactic acid content would be expected to increase during the first week of storage due to continuing action of the starter bacteria, as shown by the decrease in pH by d 7 (Figure 1A). For example, the mean pH of control cheese decreased from pH 5.4 before salting to pH 5.23 after pressing, to pH 5.20 after 7 d. As the percentage of K replacement

Figure 1. Change in (A) pH and (B) total organic acid concentration in a control Cheddar cheese, cheese in which KCl was substituted for NaCl at molar levels of 10, 25, 50, and 75%, and a low-salt cheese during 9 mo of storage at 6°C. Error bars = SE (n = 3).

increased in the cheeses, a corresponding increase in lactic acid concentration was detected in the cheeses. In accordance with no significant difference existing in pH or Na or K content of control and 10% K cheeses, both of these cheeses had similar lactic acid concentrations. This was the case for virtually all comparisons between these cheeses; insufficient change was made by substituting 10% K for Na during salting. Concentrations of lactic acid were also significantly lower for the control and 10% K cheeses throughout the 9 mo of storage, compared with 25% K cheese (Figure 2A) and the other cheeses. The low-salt cheese had the highest lactic acid content (Figure 2A), whereas 10% Mg and 10% Ca cheeses (data not shown) were similar to 50% K and 75% K cheeses. All cheeses exhibited an increase in lactic acid concentration over the 9 mo of ripening, with a marked increase occurring between 6 and 9 mo. This is similar to the lactic acid concentration increase observed by Upreti et al. (2006a) in Cheddar cheese aged for 48 wk, whereas St-Gelais et al. (1991) observed an increase up to 2 mo and then a leveling off up to 6 mo in a Cheddar cheese-like product.

Propionic Acid. The initial propionic acid concentration was about 6 to 16 mM (0.15 to 0.43 g/kg) in the cheeses and remained virtually constant through 3 mo of storage, which is similar to the findings of Upreti et al. (2006a). Lues and Bekker (2002) had reported that after 3 wk of maturation, propionic acid in Cheddar cheese levels increased, following an initial decrease in concentration. At 3 mo, propionic acid concentration was similar in all the high-salt cheeses at approximately 20 mM, with the low-salt cheese being lower at 11 mM, which was a slight increase from its initial level of 5 mM. A 3-fold increase in propionic acid concentration occurred after 3 mo for the control and 10% K cheeses to approximately 60 mM at 6 mo (Figure 2B). Upreti et al. (2006a) also noted a similar 10-fold increase in propionic acid in Cheddar cheese after 3 mo of storage. Cheeses with a 25% or more K substitution did not show an increase in propionic acid until after 6 mo of storage. After 9 mo of storage, no significant difference existed between the high-salt cheeses, with propionic acid concentration at approximately 70 mM. From 3 to 6 mo, cheeses containing 10% K substitution or less showed a much larger increase in propionic acid concentration. For the low-salt cheese, propionic acid concentration increased after 3 mo to approximately 30 mM at 6 mo, and then exhibited a very large increase between 6 and 9 mo to about twice the level of the high-salt cheeses.

Formic Acid. Initial formic acid concentrations in all cheeses were similar, with a mean of 26.3 mM (0.42 g/kg); Upreti et al. (2006a) reported similar levels of 0.3 g/kg. An increase in formic acid concentration oc-

Figure 2. Change in concentrations of (A) lactic acid, (B) propionic acid, (C) formic acid, and (D) acetic acid in a control Cheddar cheese, cheese in which KCl was substituted for NaCl at molar levels of 10, 25, 50, and 75%, and a low-salt cheese during 9 mo of storage at 6°C. Error bars = SE ($n = 3$).

curred in all cheeses up to 3 mo, with 75% K cheese having lower formic acid concentration than the other cheeses (Figure 2C). Only the control cheese containing no K substitution continued to show a steady increase in formic acid concentration up to 9 mo. Even 10% K cheese with only 10% K substitution, had significantly lower formic acid concentration than control cheese at 6 and 9 mo. Formic acid production in all other cheeses (except 10% Ca cheese), including the low-salt cheese, slightly increased throughout storage and reached 50 to 70 mM by 9 mo. The presence of increased K appeared to have a bigger influence on lowering formic acid concentration than did lowering Na because 10% K cheese and low-salt cheese had higher 9-mo formic acid concentrations than the 25% K, 50% K, and 75% K cheeses. The 10% Ca cheese (40% K and 10% Ca substitution) had a similar 9-mo level to 10% K cheese, with a mean formic acid concentration of 77 mM (data not shown). In comparison, Upreti et al. (2006a) reported a gradual increase in formic acid in Cheddar cheese until 32 wk storage, which was followed by a decrease during the next 20 wk of storage. Akalin et al. (2002) found an increase in formic acid concentration during the first few months of storage, followed by no substantial increase up to 12 mo, whereas Lues and Bekker (2002) found no pattern in formic acid concentration in Cheddar cheese aged to 96 d.

Acetic Acid. Very little acetic acid was present in the cheese initially: approximately 1 mM (0.03 g/kg) for the high-salt cheeses and 2 mM for the low-salt cheese. Considerable acetic acid production occurred in all cheeses during the first month of storage to 6 to 14 mM (Figure 2D). In comparison, Lues and Bekker (2002) reported that initially acetic acid concentration in Cheddar cheese rapidly decreased and then increased after 3 wk of storage. Upreti et al. (2006a) also reported a decrease in acetic acid content during the first few months of storage from an initial concentration of 0.15 to 0.06 g/kg. Although highly significant differences in acetic acid concentration existed between the cheeses, no discernable pattern was evident based on Na reduction or K substitution. For example, at 3 mo, the control cheese had the lowest mean acetic acid concentration at 5.7 mM and 75% K cheese had the highest (21.4 mM), but 50% K cheese had a mean acetic acid concentration of 7.8 mM, which was similar to the control cheese. At 3 mo, the low-salt cheese also had a high mean acetic acid concentration of 17.2 mM. All the cheeses showed some increase in acetic acid concentration over the ripening period, but several cheeses decreased in acetic acid concentration from 6 to 9 mo.

Other Organic Acids. Concentrations of butyric, citric, hippuric, orotic, and pyruvic acids in cheeses during aging are shown in Figure 3. Initial mean lev-

Figure 3. Change in concentrations of (A) pyruvic acid, (B) orotic acid, (C) butyric acid, (D) citric acid, and (E) hippuric acid in a control Cheddar cheese, cheese in which KCl was substituted for NaCl at molar levels of 10, 25, 50, and 75%, and a low-salt cheese during 9 mo of storage at 6°C. Error bars = SE ($n = 3$).

els of these acids in the higher-salt cheeses were 1.9 mM (0.058 g/kg) butyric acid, 14.3 mM (0.96 g/kg) citric acid, 0.25 mM (0.016 g/kg) hippuric acid, 0.25 mM (0.014 g/kg) orotic acid, and 0.95 mM (0.029 g/kg) pyruvic acid. Statistically significant differences existed between cheeses, but no discernable pattern or correlations existed between acid concentration and substitution of Na with K, Mg, or Ca in the cheeses. The pyruvic acid, orotic acid, and butyric acid concentrations increased during storage, whereas citric acid and hippuric acid concentrations remained flat. Upreti et al. (2006a) showed an increase in both citric and pyruvic acid during 48 wk of storage. We also noted a steady increase in pyruvic acid concentration up to 4 mM during 9 mo of storage, whereas citric acid concentration remained constant (~ 15 mM). Lues and Bekker (2002) reported an increase in pyruvic acid concentration up to 12 d of storage after an initial lag phase. Other researchers have not noted any trend for pyruvic acid concentration during cheese aging (Bouzas et al., 1993; Gonzalez De Llano et al., 1996).

Proteolysis

No significant difference in percentage WSN existed between the cheeses as a consequence of cation substitution. Typical trends of increasing percentage WSN during storage of the cheeses were observed (Figure 4). When breakdown of β -CN and α_{s1} -CN was monitored using urea-PAGE, also no difference was observed in breakdown patterns between cheeses, with a typical pattern showing more hydrolysis of α_{s1} -CN and β -CN and formation of α_{s1} -CN (f24–199) and α_{s1} -CN (f121–199) and various β -CN peptides, as shown in Figure 5A. As shown by Hinz et al. (2012), only a faint band was detected for α_{s1} -CN (f24–199) compared with the α_{s1} -CN (f121–199) band, indicating that even after cleavage at peptide bond 23–24, further hydrolysis at peptide bond 120–121 occurs so that little α_{s1} -CN (f24–199) accumulates. During storage, the pattern of proteolysis was typical for Cheddar cheese (Upadhyay et al., 2004). The expected trend of more hydrolysis of α_{s1} -CN than β -CN (Fenelon and Guinee, 2000) was observed, with a declining ratio of intact α_{s1} -CN to β -CN throughout the 9 mo of storage (Figure 5B). This occurs when the milk is coagulated using chymosin (primarily active against α_{s1} -CN), whereas β -CN is principally hydrolyzed by residual native milk proteinases such as plasmin (Hinz et al., 2012). As the cheeses aged, the ratio of α_{s1} -CN (f121–199) to intact α_{s1} -CN increased, as shown in Figure 5C. When all the data was combined, this ratio served as an index of cheese ripening, with a linear correlation of 0.79 in which cheese age (mo) = $0.140 \times [\alpha_{s1}\text{-CN (f121–199)} / \alpha_{s1}\text{-CN}] + 0.635$.

Figure 4. Changes in percentage water-soluble N (WSN) in a control Cheddar cheese, cheese in which KCl was substituted for NaCl at molar levels of 10, 25, 50, and 75%, and a low-salt cheese during 9 mo of storage at 6°C. Error bars = SE (n = 3).

At d 1, proteolytic products from both α_{s1} -CN and β -CN were present in the cheeses (Figure 6), as shown previously by Fenelon and Guinee (2000). Although α_{s1} -CN (f24–199) and α_{s1} -CN (f121–199) are the first hydrolysis products from enzymatic cleavage of α_{s1} -CN by the residual coagulant enzymes (Upadhyay et al., 2004), they are further hydrolyzed to smaller peptides. Therefore, although the ratio of α_{s1} -CN (f121–199) to α_{s1} -CN increased as the cheese aged (Figure 5C), the quantities of both α_{s1} -CN and α_{s1} -CN (f121–199) decreased during aging, as shown in Figure 6. No differences existed between any of the cheeses in their protein-peptide profile throughout storage and after 6 mo of storage, the density of the various peptide bands that had been visualized on the urea-PAGE gels had also diminished, suggesting that these had been further hydrolyzed into smaller peptides that were no longer apparent on the gels.

Fitzgerald and Buckley (1985) reported that substitution of KCl for NaCl (up to 50%) did not influence proteolysis rates in Cheddar cheese, whereas a 100% substitution did enhance proteolysis. Reddy and Marth (1993) also found no significant differences in proteolysis in cheeses made with NaCl or KCl used alone or in mixtures. In Kefalograviera cheese, partial substitution of NaCl by KCl did not significantly change either the characteristics or extent of proteolysis during 6 mo of aging (Katsiari et al., 2001). Rulikowska et al. (2013) reported that using KCl decreases bacterial peptidase activity in lower-salt cheeses, resulting in slower flavor formation and higher bitterness, although urea-PAGE shows primarily hydrolysis of caseins by coagulant and native milk proteinases.

Figure 5. Extent of proteolysis based urea-PAGE electrophoretograms of Cheddar cheeses in which KCl was substituted for NaCl at molar levels of 10, 25, 50, and 75%, as well as a control and low-salt cheese during 9 mo of storage at 6°C, showing (A) pooled mean levels of intact β -CN, α_{s1} -CN, and α_{s1} -CN (f121-199) ($n = 24$); (B) ratio of intact α_{s1} -CN to β -CN ($n = 3$); and (C) pooled cheese ripening index based on the ratio of α_{s1} -CN (f121-199) with a linear trend line ($n = 24$). Error bars = SE.

Microbial Numbers

Initial Microbial Numbers. Before salting, cheese curd contained about 6×10^8 cfu of lactococci/g and the same levels continued overnight when unsalted cheese was pressed. In contrast, the lactococci in high-salt cheeses had decreased to 8×10^7 to 2×10^8 cfu/g after overnight pressing, representing a 65 to 85% immediate (i.e., within 1 d) decline in starter viability after salting. The low-salt cheese had less starter decline than the other cheeses and was at 4×10^8 cfu/g on d 1. It was also observed that the counts on M17-L agar for d-1 and -7 cheeses were sometimes higher than the counts on MRS plus 10 g of sorbitol/L (MRS+S) agar, which had been found as a suitable medium to enumerate total LAB in cheese (Oberg et al., 2011). Because the cheeses had very low NSLAB numbers initially ($<10^3$ and often $<10^2$ cfu/g), it would predominantly be the lactococcal starter culture growing on MRS+S agar, so the higher numbers observed on M17-L agar were probably a result of the differences in incubation conditions between these media (i.e., anaerobic for 48 h at 37°C for MRS+S versus aerobic for 18 to 24 h at 30°C for M17-L). For some of the cheeses (e.g., replicate 2 at d 7), the lactococcal numbers were higher than the total LAB numbers, as small pinpoint colonies on the MRS+S media were not included in the total LAB count. On further investigation, these were determined to be gram-positive cocci and are assumed to be colonies from metabolically injured starter culture bacteria. These plates were recounted to obtain population numbers for total LAB with and without such metabolically injured cells.

Within each replicate, levels and patterns of growth of NSLAB were similar, which was expected, as the curd from each replicate was divided into separate portions for salting, so all cheeses would be expected to have the same starting microbial background population. Between batches of milk, some variations were expected in initial levels of NSLAB because of intrinsic and extrinsic factors, such as bacterial content of the incoming milk and presence of NSLAB in biofilms on equipment and pipe surfaces. In the past, we have observed initial levels of NSLAB in Cheddar cheese ranging from 10^2 to 10^6 cfu/g (data not shown). In the current experiment, the lowest dilution used for bacterial enumeration was 10^{-2} . Thus, for many cheeses, initial NSLAB counts were reported as $<10^2$ cfu/g, as no colonies were observed on the lowest-dilution plates. For calculating mean numbers and when making plots of microbial numbers, a value of 5×10^1 cfu/g was used for samples with counts $<10^2$ cfu/g. The actual number of bacteria present was probably lower but we

Figure 6. Urea-PAGE electrophoretograms of Cheddar cheeses during 9 mo of storage at 6°C in which designation of α_{s1} -CN and β -CN was based on sodium caseinate and designation of hydrolysis products was by comparison with those from Hinz et al. (2012).

made a conservative estimate rather than minimizing the estimated number.

As enumeration data for each replicate was accumulated, it became apparent that differences existed in the rate at which NSLAB numbers in each replicate increased throughout storage. In both replicates 1 and 2, the control cheeses had d 1 counts of NSLAB of $\leq 10^2$ cfu/g and starter culture counts at d 1 were approximately 10^8 cfu/g (Figure 7A and 7B). In contrast, in replicate 3, the d-1 counts of NSLAB were higher (10^3 cfu/g) and starter culture counts were lower ($\sim 10^7$ cfu/g; Figure 7C). Differences also existed in microbial patterns during storage, and for the NSLAB counts to be consistently $\geq 10^5$ cfu/g took 6, 1, and 7 mo for replicates 1, 2, and 3, respectively. Nevertheless, as shown in Figures 2 and 3, considerable consistency existed in organic acid levels between replicates, even with this difference in microbial numbers.

Microbial Numbers During Storage. Mean populations of starter culture decreased during storage and NSLAB numbers increased (Figure 8), as was previously observed for Cheddar cheese (Oberg et al., 2011). The best fit of the data was most often obtained using power trend lines, especially during the first few months of aging when the greatest growth of the NSLAB and greatest decrease in starter culture counts occurred. Also, as expected, the NSLAB population eventually exceeded that of the starter culture, and it was determined during the current study that the predominant NSLAB in

all cheeses made during this experiment was *Lactobacillus curvatus* (Porcellato et al., 2014), which agrees with previous findings (Broadbent et al., 2013). Reddy and Marth (1995b) also found no detectable differences in types of LAB (including starter cultures and NSLAB), that developed in ripening Cheddar cheese containing up to 100% replacement of NaCl with KCl over 36 wk. Also, no observable differences existed for aerobic sporeformers, coliforms, and yeasts and molds (Reddy and Marth, 1995a). Wachowska (2011) reported similar results for the microflora of Edam-like cheese brined in mixtures of NaCl/KCl, whereas Aly (1995) found lower total counts in Feta-like cheeses salted with mixtures of NaCl/KCl compared with control cheese salted with only NaCl. In Akawi cheese aged to 30 d, no pattern was found in the growth of microbial populations based on KCl substitution for NaCl (Ayyash et al., 2012).

Because adjunct cultures were not used during cheesemaking for this experiment, the total LAB counts always coincided with the M17-L counts (lactococci) or the MRS-vancomycin counts (NSLAB), depending on which was higher. No apparent differences existed in total LAB numbers observed in the cheeses as a function of cation substitution. The only major difference was how long during storage it took before the crossover point between starter and NSLAB was reached. Decreases in starter population (both rate of decrease and extent of decrease over 9 mo) correlated to both salt and Na concentrations in the cheese. For

Figure 7. Change in mean number of total lactic acid bacteria (LAB), lactococci, and nonstarter LAB (NSLAB) in (A) replicate 1, (B) replicate 2, and (C) replicate 3 based on pooled data from control, 10% K, 25% K, and 50% K cheeses (representing increasing percentages of K substitution for Na) during 9 mo of storage at 6°C, including trend lines for starter (solid line) and NSLAB (dashed line). Error bars = SE ($n = 4$).

the control cheese, mean lactococci numbers decreased from approximately 10^8 cfu/g at d 1 to approximately 10^3 cfu/g after 9 mo of storage. When salt-in-moisture concentration was retained at approximately 4.7% but Na was reduced sufficiently low by substituting 75% of it with K (cheese 75% K), or when Mg or Ca were added (cheeses 10% Mg and 10% Ca, respectively) the

mean lactococcal counts at 9 mo were approximately 10^4 cfu/g. Starter populations in the low-salt cheese with a salt-in-moisture concentration of only 18 g/kg remained high and were still at 10^6 cfu/g after 9 mo.

Mean crossover times for the cheeses are shown in Table 4. In the control cheese, the crossover occurred more quickly at 4.5 mo of storage and as storage continued, the NSLAB became the predominant bacteria in cheese, whereas the starter-culture colony-forming units continued to decrease (as the starter bacteria either died or became nonculturable). Even a 10% substitution of K for Na (10% K cheese) caused the crossover time to be extended slightly (5.2 mo) and it eventually reached 6.2 mo with a 75% substitution (75% K cheese). Interestingly, when Na was reduced to 50%, and K was 40%, the cheeses with 10% Mg or 10% Ca had further extended crossover times of 6.4 and 7.0 mo, respectively. In the low-salt cheese when no substitution was made for Na, the crossover did not occur until 7.3 mo of storage. In the low-salt cheese, even though NSLAB increased to approximately 10^6 cfu/g within 3 mo, starter lactococci remained above 10^6 cfu/g for 6 mo and stayed at approximately 10^6 cfu/g throughout the 9 mo of storage.

Sodium content had the greatest effect on crossover time, with a linear correlation of 0.70 in which crossover time (mo) = $-0.4202[\text{Na}] + 7.87$. This effect of Na on cheese microflora agrees with Upreti et al. (2006a) in that cheeses with low salt concentrations support higher numbers of starter bacteria for an extended period and exhibit a more rapid increase in the NSLAB population during ripening, although they reported higher NSLAB counts in their lower-salt cheese than was observed in our study (all cheese contained 10^6 to 10^7 NSLAB cfu/g at 9 mo). Similar retention of higher numbers of viable LAB in cheese with lower salt concentrations for longer times was shown by Mistry and Kasperson (1998) when comparing cheeses with salt-in-moisture concentrations of 2.7, 3.7, or 4.5%.

DISCUSSION

Cheese pH

The pH of the cheese tended to decrease during the first 7 d of storage, with the larger decreases occurring in cheeses containing the greater levels of Na substitution, or no Na substitution (low-salt cheese). Presumably, this reflects continued conversion of lactose to lactic acid by starter culture as was bioinformatically predicted by Ganesan and Brown (2014). Apparently, the starters in cheeses with K addition use most of the lactose during the first day and so the pH decrease during the following 6 d is minimal. Intracellular K^+ cat-

Figure 8. Mean bacterial counts of starter lactococci, total lactic acid bacteria (LAB), and nonstarter LAB (NSLAB) for (A) control Cheddar cheese; cheese in which (B) 10%, (C) 25%, (D) 50%, and (E) 75% of Na was replaced with K; cheese with 40% K substitution and 10% (F) Mg and (G) Ca substitution; and (H) a low-salt cheese during 9 mo of storage at 6°C, including trend lines for lactococci (solid line) and NSLAB (dashed line). Error bars = SE ($n = 3$).

Table 4. Estimated crossover time for nonstarter lactic acid bacteria (NSLAB) to become equal to and then exceed starter lactococci numbers during 9 mo of storage of cheese salted with various cation substitutions as shown in Table 1

Item	Cheese ¹							
	Control	10% K	25% K	50% K	75% K	10% Mg	10% Ca	Low salt
Crossover time (mo)	4.5	5.2	6.0	6.1	6.2	6.4	7.0	7.3

¹The K cheeses refer to cheeses in which KCl was substituted for NaCl at molar levels of 10, 25, 50, or 75%; 10% Mg = cheese with 40% K substitution and 10% Mg substitution; 10% Ca = cheese with 40% K substitution and 10% Ca substitution..

ions provide a counterion for fermentation acid anions, which accumulate inside bacteria as the pH decreases and can impede viability (Russell and Diez-Gonzalez, 1998). This may help explain differences seen in lactic acid concentration and hence, the pH (Figure 1), as LAB in cheese with increased K produced more lactic acid than was seen from bacteria in cheese containing the highest levels of Na. In addition, cheese containing more K generally had a lower pH over the storage period, although not as low as the low-salt cheese.

The increase in pH during the first 3 mo of storage can be attributed to the slow rebalancing of the calcium phosphate equilibrium in cheese that occurs upon cooling the cheese after pressing and continued storage at 6°C. Hassan et al. (2004) showed that during this 3-mo period approximately 20% of the insoluble Ca in cheese becomes soluble, and a corresponding amount of phosphate ions would also be released from the protein. At the pH of cheese, the phosphate ions will be mainly H_2PO_4^- , whereas it is generally unprotonated when present as insoluble calcium phosphate. Thus, during the first 3 mo of storage, the pH of cheese increases as the concentration of H^+ ions decreases as protons are absorbed by the phosphate ions as they dissolve into the cheese serum.

Salt Concentration

Lactococcus lactis starter cultures, along with many other LAB, are sensitive to the salt concentration in cheese. When high levels of salt are added to curd during cheese manufacture, the ability of starter bacteria to metabolize lactose to lactic acid is impaired, which would also contribute to stabilizing the cheese pH (Olson and Johnson, 1990) and the salt concentration can continue to affect the growth of bacteria during cheese storage. Although the typical salt-in-moisture concentration in Cheddar cheese of 4.0 to 5.5% is not sufficient to prevent all microbial growth, in combination with a low pH and refrigerated ripening temperature, it prevents growth of pathogens and influences LAB populations. If the salt-in-moisture concentration

is low (<4.5%), starter bacteria numbers can remain at a higher concentration in the aging cheese (Lane et al., 1997), whereas increasing salt-in-moisture concentration in cheese above 4.5% results in a more rapid decrease in starter lactococci during initial ripening (Upreti et al., 2006b). A high salt concentration also decreases lactose metabolism by starter lactococci, leaving more residual lactose available to be used by NSLAB during ripening (Turner and Thomas, 1980). Thus, salt concentration affects the population dynamics of NSLAB by determining how quickly NSLAB will grow in the ripening cheese as well as the final number of NSLAB.

Because moisture content of Cheddar cheese can vary between about 340 and 390 g/kg, a controlling factor for microbial growth is the salt concentration in the water phase of the cheese, rather than just total salt content. For high-quality Cheddar cheese, the salt-in-moisture concentration should be in the range of 4.7 to 5.7% (Guinee and O’Kennedy, 2007). Starter bacteria and NSLAB survive longer in cheese when salt content is decreased to 12.5 g/kg compared with cheese with 18 g/kg salt (Rulikowska et al., 2013). This is related to the stress placed on bacteria by the combined influence of salt, acid, lowered water activity, and cold storage of cheese. Enzyme activity is also affected by salt level because of its relationship with water activity. Adding salt to Cheddar cheese plays a further role by promoting whey expulsion after salting and during pressing, hence producing cheese with lower moisture content. This was evident in that the low-salt cheese had approximately 20 g/kg higher moisture than all the high-salt cheeses. So overall, a high salt-in-moisture concentration provides a hurdle that slows down bacterial growth and metabolism, and ultimately limits microbial spoilage (Rallu et al., 1996).

This function of adding salt to suppress metabolic function of starter lactococci and slow their ability to convert lactose to lactic acid was traditionally considered to occur with additions of >10 g of salt/kg to milled Cheddar curd (Van Slyke and Price, 1979). However, at that time, starter cultures were primarily made

using traditional bulk sets and the culture fermented the milk-based medium to pH 4.5 or less. When pH-controlled starter culture systems were developed, not only were lower inoculums needed (e.g., 0.5% compared with 2%), the bacteria were no longer acid damaged. Now, starter lactococci (used either as direct vat set cultures or as bulk starter) are grown with pH control so they retain greater salt tolerance (personnel communication, R. Thunell, DSM Food Specialties USA Inc., Logan, Utah, 2014). For such cultures, more than the normal 30 g/kg of salt would need to be added to curd to inhibit their further conversion of lactose to lactic acid. For *L. lactis* ssp. *cremoris* strains, a salt-in-moisture concentration of 5.25% in cheese is needed to prevent further acid development, whereas inhibition of *L. lactis* ssp. *lactis* strains require even higher salt concentrations. Upreti et al. (2006a) found that cheeses with salt concentrations of approximately 6.6% produced less lactic acid than cheeses with salt-in-moisture concentrations of approximately 4.7%. Cheese curd made with the starter culture used in this experiment can be salted as soon as the curd reaches pH 5.9 and the same final pH will be reached as salting at pH 5.4 (D. J. McMahon, unpublished data). Also, when using mixed-strain cultures, differences exist in salt tolerance between strains so that after salting, some strains can die off, whereas more salt-tolerant strains remain.

Organic Acids

Lactic Acid. Increased lactic acid production in cheeses with 25% or greater K substitution was probably due to a slower die-off or inactivation of the starter bacteria, as K is less inhibitory than Na. Ayyash et al. (2012) also found significantly higher levels of lactic acid in Akawi cheese with 75% K substitution after 1 mo of storage. The low-salt cheese also showed a similar pattern of higher lactic acid concentrations and higher lactococcal numbers during aging, as found in the K replacement cheeses. Lawrence et al. (1984) noted that cheese with a salt-in-moisture concentration <4.0% has insufficient salt to control bacterial growth and flavor development during aging. The increase in lactic acid from 6 to 9 mo may have been due to NSLAB metabolic activity converting a variety of substrates (amino acids and ribose, among others) to lactic acid. Williams et al. (2000) found that lactate was not a common substrate for most NSLAB they examined, concluding that lactate may be important as a substrate only to a small portion of NSLAB and so lactate would continue to accumulate in the cheese.

Propionic Acid. Increases in propionic acid concentration during ripening of cheese seem to follow crossover times between starter lactococci and NSLAB. The

control and 10% K cheeses (both with typical Cheddar cheese Na levels) had a more rapid decline in starter populations, which allowed NSLAB to become dominant earlier during storage. Nonstarter LAB activity has been reported to increase propionic acid concentration in cheese during storage (St-Gelais et al., 1991; Bouzas et al., 1993). The control and 10% K cheeses had elevated propionic acid concentrations at 6 mo and both had crossover times <6 mo (Table 4). All other cheeses had crossover times from 6.0 to 7.3 mo and an increase in propionic acid concentration was only observed when the cheeses were sampled at 9 mo.

At least one common NSLAB in these cheeses, *Lactobacillus curvatus*, appears to have the metabolic capability to produce propionic acid from lactate (J. R. Broadbent and C. J. Oberg, unpublished data). However, no decrease in lactic acid concentration (Figure 2A) corresponding to the 40 mM increase in propionic acid (Figure 2B) was observed between 3 and 6 mo for the control and 10% K cheeses or between 6 and 9 mo for the other high-salt cheeses. The low-salt cheese had the greatest increase in propionic acid concentration between 6 and 9 mo, and lactic acid concentration was the same at 6 and 9 mo, whereas most of the other cheeses had a further increase in lactic acid concentration over that time period (Figure 2A).

Alternatively, proteolysis of amino acid side chains and nonspecific esterase activities by NSLAB have also been suggested as sources of propionic acid (González de Llano et al., 1996). Propionic acid can be produced from branched-chain amino acids that are used as an energy source by LAB under sugar starvation conditions prevalent in aged cheese (Ganesan et al., 2004). In Cheddar cheese augmented with α -ketoglutarate (a transaminase acceptor that enhances amino acid catabolism), propionic acid concentration was higher at 24 wk of aging than in untreated cheeses (Banks et al., 2001). In our study, at 9 mo, the NSLAB populations were similar in all cheeses and by then propionic acid concentrations in all the high-salt cheeses were also the same. In the low-salt cheese, having a lower salt-in-moisture concentration perhaps allowed increased metabolic turnover of substrates, resulting in the highest propionic acid concentration at 9 mo.

Formic Acid. From the differences in formic acid concentration between the control and all other cheeses, it appears that both a decrease in Na^+ ion concentration in the cheese and an increase in K^+ ion concentration slows down accumulation of formic acid after 3 mo of storage. Either a lack of production of formic acid occurs or an equilibrium because of conversion of formic acid to other end products occurs. In LAB, formate is chiefly generated by metabolism of pyruvate to acetate and formate via the enzyme pyruvate-formate lyase,

which is typically active only under strict anaerobic conditions. As changes in formic acid concentration (Figure 2C) do not trend with changes in acetic acid concentration (Figure 2D), an alternative pathway is likely functional, or the acetyl-CoA could have been converted to other end products such as ethanol (also a potential end product from pyruvate-formate lyase). Using a database prediction on LAB metabolic pathways (Ganesan and Brown, 2014), a pathway exists involving oxidation of aldehydes to formate that is inhibited by K, which may lead to lower formate levels. However, as lower Na levels in cheese also resulted in lower formic acid concentration, this suggests multiple shifts in metabolic control of formate synthesis occurring in LAB in cheese.

Acetic Acid. Other than an increase during the first month of storage for all cheeses, no trend or pattern was observed between cheese based on Na and K concentrations in the cheese. However, it should be noted the organic acid levels in cheeses were very similar between replicates (SE bars in Figures 2B to D and in Figure 3 are smaller than the symbols), so although no trend based on Na and K concentrations was observed, very significant statistical differences existed in this metabolite concentration in the cheese. The levels of acetic acid during 9 mo were only 7 to 25 mM, whereas increases in lactic acid levels during the same period ranged from 60 to 150 mM.

Upreti et al. (2006a) also found that levels of acetic acid fluctuated during 12 mo of ripening, independent of differences among Cheddar cheese types and speculated this was related to acetate's role as an intermediate in biochemical pathways. In a Cheddar cheese-like product, a decrease in acetic acid concentration between 2 and 4 mo was followed by a gradual increase to 6 mo (St-Gelais et al., 1991), a pattern similar to the one observed in cheeses made in the current experiment (Figure 2D). Ong et al. (2007) also reported an increase in acetic acid concentration in Cheddar cheese during 6 mo of ripening. Cheeses with high levels of *Lactobacillus casei* and *Lactobacillus paracasei* can produce higher concentrations of acetic acid, perhaps from lactate, citrate, or amino acids (Fox and McSweeney, 1996).

Banks et al. (2001) also found an irregular pattern of acetic acid concentration over 24 wk of ripening in Cheddar cheese. Their results indicated that in cheese supplemented with α -ketoglutarate, an increase occurred in the catabolism of alanine or serine to acetic acid. As acetic acid is also a product of fat and carbohydrate metabolism, this obfuscated their conclusions. Both lactic acid and acetic acid can be generated from pyruvic acid, so while lactic acid is still accumulating, increases in acetic acid suggest heterofermentative

metabolism of sugars. During further storage, the continuing increases in pyruvic acid (though only at low levels of 2 to 4 mM, as it is rapidly utilized through various metabolic pathways) also suggest continuing heterofermentative metabolism, either from scavenged carbohydrates of lysed bacteria or a shift from sugars to amino acids for energy. A heterofermentative NSLAB has recently been isolated from cheeses made in the same facility as used for the current experiment (Oberget et al., 2013). Bouzas et al. (1993) suggested that acetic acid concentration can be affected by the degree of heterofermentative metabolism (or pentose utilization, such as deoxyribose from DNA) because it can be produced from citric acid, lactose, or amino acids, thus making its concentration heavily influenced by type of NSLAB and metabolic activity of remaining starter lactococci in the cheese.

Other Organic Acids. The lack of a consistent trend in pyruvic acid concentration during cheese aging comes about because of its role as a carbon source for, and intermediate in the metabolic pathways of NSLAB (Bouzas et al., 1993; González de Llano et al., 1996). Various reports on citric acid levels exist that differ from ours. Lues and Bekker (2002) observed an increase after 40 d, whereas McGregor and White (1990) showed a steady increase in citric acid concentration during storage. Williams et al. (2000) demonstrated that citric acid did not appear to be a significant energy source for NSLAB.

It may be necessary to take into consideration (1) the role of citrate fermentation where LAB convert citrate to pyruvate, acetate, and CO₂; (2) that large differences in metabolic ability can exist between the various strains of *L. lactis* that are used in making Cheddar cheese; and (3) the differences in NSLAB populations between cheese-manufacturing sites. In a comparison of flavor in Cheddar cheese made at various locations, the controlling factors that produced differences in flavor were intrinsic to each facility rather than regional or whether the cheese was made using stirred curd or milled curd methods of manufacture (Drake et al., 2008). Selection of starter culture and NSLAB strains present in the cheesemaking microbiome would have a large effect on metabolic processes taking place in cheeses made in those facilities.

Increases in butyric acid concentration during Cheddar cheese ripening have been attributed to lipase activity in the cheese (St-Gelais et al., 1991; Lues and Bekker, 2002). Catabolism of various amino acids after sugar starvation occurs is another possibility (Ganesan et al., 2004). The low-salt cheese had the greatest increase in butyric acid (between 3 and 6 mo) and perhaps the lack of inhibitory effect by salt allows other bacteria in the cheese to be metabolically active.

Although orotic acid concentration was very low (≤ 0.5 mM), the most significant increase occurred between 1 and 3 mo. Cheeses with normal Na content (control and 10% K cheeses) had the highest orotic acid concentration, whereas it took 9 mo for low-salt cheese to reach the same orotic acid concentration as the control cheese did in 3 mo. This suggests that orotic acid concentration is a function of the starter lactococci, as NSLAB levels were only about 10^2 at the beginning of storage. Orotic acid is an intermediate in nucleotide biosynthesis and is a product of carbon fixation that would happen under carbon starvation when lactococci need to scavenge excess carbon from non-sugar sources for their continued metabolism. Lactococci have all the necessary enzymes involved in orotic acid synthesis and the increase at 3 mo would coincide with expected lactose depletion in the cheese. Another possible source of orotic acid is from arginine catabolism that occurs upon sugar starvation (Chou et al., 2001) and this generates CO_2 and carbamoyl phosphate, both of which are precursors to orotic acid.

Microbial Populations

The longer starter culture survival with lower Na content and higher K content of cheeses, and consequently longer crossover times, was not unexpected. Potassium ions are the most prevalent cations in bacterial cytoplasm and interfere less with intracellular metabolic activity. Also, K^+ is considered to be one of a few compatible solutes that bacterial cells can take up and concentrate in the cell interior to balance an increase in external osmolarity and restore cell turgor (Csonka, 1989). For many bacteria, the intracellular K^+ ion concentration has been found to be almost proportional to the osmolarity of the surrounding medium as a consequence of loss of turgor or possibly the reduction in cytoplasmic volume (Csonka, 1989). Therefore, adding K to cheese should exert less stress on LAB present than Na^+ ions. Increased intracellular K^+ ion concentration allows cells to tolerate higher external Na^+ ion concentrations and fermentation anions (Russell and Diez-Gonzalez, 1998). In studies on NaCl-induced osmotic stress, Glaasker et al. (1998) observed that *Lactobacillus plantarum* cells could not adequately respond by accumulating sufficient K^+ ions. In the presence of 0.8 M solutions of either NaCl or KCl (which corresponds to a salt-in-moisture concentration of approximately 4.7%), intracellular levels of Na and K increased to approximately 2,000 nmol/mg of protein, respectively, when stressed with the corresponding salt.

MacLeod and Snell (1948) found that whereas Na^+ inhibits LAB growth, increasing the K^+ ion concentration in the growth medium counteracts the inhibitory

action of Na^+ . This inhibitory action of Na^+ may be due to its competitive interference with normal K^+ utilization by cells as intracellular Na^+ ion concentration increases, and further diminished by addition of K^+ to the medium and consequent increase in intracellular K^+ ion concentration. Thus, K competitively interfered with the inhibitory action of Na on LAB as its concentration increased, and this effect was noted at even low levels of Na replacement.

Knowledge of the mechanisms by which LAB metabolism affects cheese flavor has facilitated industry efforts to promote flavor development in many traditional cheese varieties, but empirical efforts to extend this information into low-Na Cheddar cheese has not proved as successful. During the first 4 mo of aging, the predominant culturable bacteria in cheese are the starter lactococci and in the first replicate of this study, the NSLAB population remained approximately 10^3 cfu/g for approximately 5 mo, whereas they reached 10^6 cfu/g within 2 mo in the second replicate (Figure 7). The differences in organic acid metabolites based upon cation content and level during this 4 to 5 mo can, therefore, be attributed primarily to activity of the lactococci because no differences existed in organic acid levels between the replicates even though NSLAB levels differed considerably.

CONCLUSIONS

Cheeses with a standard (1.7%) salt level were made with the target variations in cation content (Na, K, Mg, and Ca) and they had similar moisture and fat composition and water activity. The low-salt cheese was about 20 g/kg higher in moisture content and had a consequent higher water activity and lower pH. Substitution of Na with 10% K did not cause a detectable change in cheese Na content, although an increase in K was observed. Presence of 1.7% salt in the cheese produced an approximately 1 log cfu/g decrease in starter lactococci during overnight pressing, whereas this initial decrease was smaller when Na was lower, even when K was increased proportionally so as to maintain the same overall salt concentration. This decrease in starter lactococci number was minimal in the low-salt cheese containing only 0.7% salt. The initial decrease in pH was also inversely proportional to cheese Na content and although changes in pH during storage were similar for all cheeses, cheese with higher Na had a higher pH throughout storage. We observed no difference in production of percentage WSN or protein hydrolysis profiles throughout storage based upon cheese cation content. Because chymosin was used as coagulant, more breakdown of α_{s1} -CN than β -CN occurred, and during storage the ratio of α_{s1} -CN (f121–199) to intact α_{s1} -CN

increased in proportion to time and could be useful as an index for cheese ripening. Decreasing Na in cheese led to higher lactic acid concentration in the cheese and some changes in other organic acids. Including 10% Mg or 10% Ca, along with 40% K in the salting mixtures did not have any apparent major effect on changes in proteolysis or organic acids during 9 mo of storage. Within the 3 replicates of cheese that were made, differences existed in the rate at which NSLAB levels increased during aging but no differences existed in organic acid content between the replicates. This suggests that differences during the first 4 to 6 mo of storage could be attributed to different metabolic activities of the starter lactococci. Propionic acid synthesis in the cheese occurred after 3 or 6 mo, depending on Na content of the cheese. A longer time for propionic acid concentration to increase occurred when Na content of cheese was reduced by 25% or more. This was attributed to synthesis of propionic acid by NSLAB and the NSLAB becoming dominant later during the aging process of such lower-Na cheese. The time when NSLAB numbers became equal to and then exceeded starter lactococci progressively increased as Na content was decreased. Crossover times ranged from 4.3 mo in the control cheese to 7.3 mo in the low-salt cheese, with cheeses containing K substitution having crossovers at intermediate times.

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