PROCESSING AND INTRINSIC FACTORS AFFECTING THE OCCURRENCE OF CALCIUM LACTATE CRYSTALS IN CHEDDAR CHEESE

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SHANTANU AGARWAL find it satisfactory and recommend that it be accepted.

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ABSTRACT

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Calcium lactate crystals (CLC) in hard cheeses are a continual expense to the cheese industry. Appearance of a white haze on cheese is unappealing to consumers, who may refrain from buying, resulting in lost revenue to manufacturers. Improved sanitation and better cheese manufacturing practices reduced the occurrence of D(–)-lactate crystals in cheese, but in recent years there increased occurrence of L(+)-lactate crystals in Cheddar cheese. We believe that there are correlations among total lactic acid in cheese, cheese pH and concentration of calcium ions with formation of CLC.

Objective one was to determine whether gas flushing of Cheddar cheese contributes to the occurrence of calcium lactate crystals (CLC). Two different cheese milk compositions were used: Standard (lactose:protein=1.47, protein:fat=0.90, lactose=4.8%) and Ultrafiltered (UF) (lactose:protein=1.23, protein:fat=0.84, lactose=4.8%), with or without adjunct *Lb.curvatus*. After aging at 7.2^{o}C for 6 mo, cheeses were cubed and either vacuum packaged or gas flushed. High intensity of crystals were observed on surfaces of cubed cheeses that were gas flushed, but not on cheeses that were vacuum packaged. Cheeses made without *Lb. curvatus* exhibited L(+)-CLC on surfaces, while cheeses made with *Lb. curvatus* exhibited racemic mixtures of L(+)/D(-)-CLC throughout the cheese matrix. Gas flushing, milk composition and presence of nonstarter lactic acid bacteria (NSLAB) contribute to the development of CLC on cheese surfaces. These findings stress the importance of packaging to cheese quality.

Objective two was to investigate the effect of concentrating milk (using UF) or increasing solids with nonfat dry milk (NFDM), and cheese pH, upon formation of CLC. In cheeses made

from skim milk supplemented with UF (CSM2) and NFDM (CSM3), total calcium was 26% greater than in cheeses made from skim milk (SM2). As the pH of cheeses made from SM2 decreased from 5.4 to 5.1, the concentration of soluble calcium increased by 61.6%. In cheeses made from CSM2 and CSM3, the concentration of soluble calcium increased by 41.4% and 45.5%, respectively. CLC were observed in cheeses made from SM2 at and pH 5.1, while CLC were observed in cheeses from CSM2 and CSM3 at pH 5.3 and lower. Increased concentrations of soluble calcium can increase the potential for development of CLC in cheese manufactured with increased concentrations of milk solids, particularly at and less than pH 5.1.

Objective three was to determine the effect of salt and starter bacteria on the cheese pH and occurrence of CLC. A commercial starter was selected based on its sensitivity for salt-to-moisture (S/M) smaller than and greater than 4.0. Cheddar cheese was made using either whole milk (3.25% protein, 3.85% fat) or whole milk supplemented with ultrafiltered milk and cream (4.5% protein, 5.3% fat). Calculated amounts of salt were added at milling (pH 5.40 ± 0.02) to obtain low S/M whole milk cheese (LSWMC), low S/M concentrated milk cheese (LSCMC) with 3.5 S/M and high S/M whole milk cheese (HSWMC), high S/M concentrated milk cheese (HSCMC) with 4.5 S/M. The cheeses were either vacuum packaged or gas flushed with N_2 and aged at 7.2^{o}C for 15 weeks. Soluble calcium was 41 to 35% higher in LSWMC and LSCMC compared to HSWMC and HSCMC. Concentration of lactic acid in high S/M cheeses was double of smaller S/M cheeses at end of 15 weeks of aging. CLC were observed in smaller S/M cheeses but greater intensity of CLC were observed in cheeses made with milk with high protein concentration and gas flushed packaging. Cheese manufacturers must be careful about the salt tolerance of the starter culture used in their cheese plant and should make sure adequate salt is added to inhibit the metabolism of starter bacteria.

The results from the three studies confirm that occurrence of CLC is dependent on cheese milk concentration and pH of cheese, which can be influenced not only by S/M but also by cheese microflora. This research explains to cheese manufacturers why CLC occur and describes methods to prevent it.

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I. INTRODUCTION

The presence of white crystals on the surface of Cheddar cheese troubled cheese manufacturers for quite some time (Van Slyke and Publow, 1910). The first appearance of white crystals on cheese surfaces was reported by Van Slyke and Publow (1910), who thought the crystals were calcium salts of fatty acids. Later, McDowall and McDowell (1939) identified crystals as calcium lactate. Since then, the appearance of white crystals was reported by numerous authors (Brooker et al., 1975; Chou et al., 2003; Conochie et al., 1960; Dybing et al., 1988; Harper et al., 1953; Johnson et al., 1990; Pearce et al., 1973 and Severn et al 1986). The appearance of white crystals on Cheddar cheese renders cheese unappealing to consumers, who think the cheese is either infested with mold or is exhibiting serious manufacturing defects. Vendors and consumers complain to manufacturing plants about the appearance of white haze of crystals on surfaces of both vacuum packaged and gas flushed packaged cheeses. The problem remains a challenge and expense to cheese manufacturers (Agarwal et al., 2005; Chou et al., 2003; Rajbhandari and Kindstedt, 2005; Swearingen, et al., 2004), with significant amounts of Cheddar cheese manufactured in US forming calcium lactate crystals (CLC) during aging (Johnson, 2004). Cheese manufacturers suffer millions of dollars loss of sales and revenue when CLC are present (Somer et al., 2001). The financial losses that CLC cost the cheese industry warrant research into intervention to reduce occurrence of CLC on hard and semi-hard cheeses.

White haze formed within 2 to 8 mo is commonly identified as calcium lactate pentahydrate (Brooker et al., 1975; Chou et al., 2003; Conochie et al., 1960; Dybing et al., 1988; Harper et al., 1953; Johnson et al., 1990; Pearce et al., 1973; Severn et al., 1986; Somers et al., 2001; and Washam et al., 1985). CLC may be attributed to a number of variables, including differences in milk composition (Pearce et al., 1973), cheesemaking procedures (Dybing et al.,

1986), aging temperatures (Chou et al., 2003; Dybing et al., 1988; Johnson et al., 1990b; Pearce et al., 1973), and the growth of nonstarter lactic acid bacteria (NSLAB) in cheese during aging (Chou et al., 2003; Khalid and Marth, 1990; Somers et al., 2001). Certain NSLAB encourage development of D(–)-lactate, which is smaller soluble than L(+)-lactate, thus promoting CLC formation (Agarwal, et al., 2006, Chou, et al., 2003). Temperature fluctuations during cheese aging encourage CLC (Chou et al., 2003) and both L(+)- and D(–)-lactate crystal development are stimulated by gas-flush packaging (Agarwal et al., 2005). Cleaning, sanitizing, prevention of cheese milk contamination with lactate-racemizing NSLAB, consistent storage temperatures and vacuum packaging are encouraged to minimize the occurrence of CLC (Agarwal et al., 2006; Agarwal et al., 2005; Chou et al., 2003).

Although improved sanitation and good cheese manufacturing practices are reducing the occurrence of D(–)-lactate crystals in cheese, in recent years there is increased occurrence of L(+)-lactate crystals in Cheddar cheese (Agarwal et al., 2005; Johnson, 2004; Linke, 1958; Rajbhandari and Kindstedt, 2005; Swearingen et al., 2004). Previous researchers concluded that non-starter lactic acid bacteria (NSLAB) and storage temperatures can lead to CLC in cheese (Blake et al., 2005; Chou et al., 2003; Somers et al, 2001). It is still not clear why some cheeses manufactured in a facility develop crystals while other cheeses do not.

Large commercial cheese manufacturing facilities manufacture Cheddar cheese differently from small traditional Cheddar cheese manufactures. Most large commercial cheese plants concentrate cheese milk to increase through-put. Manufactures may increase the total solids of cheese milk by adding milk powder, evaporated milk, or milk concentrated using membrane filtration (reverse osmosis or ultrafiltration). But when milk powders, evaporated milk and reverse osmosis are used to increase the total solids of cheese milk, not only are milk

proteins concentrated, but also calcium both lactose and associated with proteins and lactose are concentrated, which may exacerbate development of CLC. Most commercial cheese plants also use a faster cheese making schedule than small traditional cheese plants, mill cheese at higher pH, and use salt tolerant starter bacteria to cut down the time of actual cheese production. Most commercial Cheddar cheese plants press cheese curd in block sizes ranging from 18 kg to 290 kg, prior to then packaged and cooled. Uneven salting of cheese curd after milling and slow cooling of cheese blocks, due to slow heat transfer, leads to huge variation in salt to moisture ratios, lactic acid and moisture concentrations within cheese blocks (Drake, 2007). Cheese blocks are cut and packaged into smaller sizes for retail sale, either in the manufacturing facility itself or by a distributor or retailer. The distributor may age the cheese further or repackage the cheese in either gas flushed or vacuum packaging for immediate retail sale. All these changes in manufacturing, storage, and distribution can influence occurrence of CLC.

The present research is designed to determine and understand if and when calcium solubility changes at certain pH and lactic acid concentrations favor CLC formation. The other objectives of this study were to determine whether cheese making procedures (salt, starter bacteria, nonstarter lactic acid bacteria and pH of cheese) and cheese packaging (vacuum and gas flush packaging) affect the occurrence of CLC in Cheddar cheese. Our goal was to study the complex relationships among cheese milk composition, starter lactic acid bacteria, and salt to moisture ratio and packaging on occurrence of CLC in cheese. The results of this research will enable us to guide cheese manufacturers in cheese aging and product sale schedules.

The objectives of these study were to:

1. Study the effect of packaging and different gas used for packaging of Cheddar cheese upon CLC formation.

2. Determinate changes in total and soluble calcium in cheese milk and cheeses at different pH and evaluate how soluble calcium interacts with lactate, citrate and phosphate in cheese serum to form CLC.
3. Study the effect of salt to moisture ratios (S/M) on the activity of starter bacteria and the influence on cheese pH, lactic acid, and soluble calcium upon occurrence of CLC in cheese; and
4. Investigate the effects of aging on the migration of calcium and lactate ions in cheese and occurrence of CLC.

The hypotheses of this study were:

1. Cheese flushed with CO_2 will exhibit a larger intensity of CLC compared to cheeses flushed with N_2 or N_2+CO_2; Volume of packages with CO_2 will be smaller than packages flushed N_2.
2. Increased concentrations of total and soluble calcium in cheese made from increased total solids results in increased occurrence of CLC in cheeses made from cheese milk with higher total solids;
3. Cheeses having smaller S/M will have higher acidity, lower pH and increased occurrence of CLC than cheeses with high S/M; and
4. Migration of calcium ions and lactate to the cheese surface during aging will promote growth of CLC.

REFERENCES

Agarwal, S., M. Costello, and S. Clark. 2005. Gas-Flushed packaging contributes to calcium lactate crystals in Cheddar cheese. J. Dairy Sci. 88:3773-3783.

Agarwal, S., J. R. Powers, B. G. Swanson, S. Chen, and S. Clark. 2006a. Cheese pH, protein concentration, and formation of calcium lactate crystals. J. Dairy Sci. 89:4144-4155.

Blake, A. J., J. R. Powers, L. O. Luedecke, and S. Clark. 2005. Enhanced lactose cheese milk does not guarantee calcium lactate crystals in finished Cheddar cheese. J. Dairy Sci. 88, no. 7:2302-2311.

Brooker, B. E., D. G. Hobbs, and A. Turvey. 1975. Observations on the microscopic crystalline inclusions in Cheddar cheese. J Dairy Res. 42:341-348.

Chou, Y. E., C. G. Edwards, L. O. Luedecke, M. P. Bates, and S. Clark. 2003. Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. J. Dairy Sci. 86:2516-2524.

Conochie, J., J. Czulak, A. J. Lawrence, and W. F. Cole. 1960. Tyrosine and calcium lactate crystals on rindless cheese. Aust. J. Dairy Technol. 15:120.

Drake, M. A. 2007. Defining dairy flavors. 22nd Annual Cheese Short Course. Pullman, WA.

Dybing, S. T., S. A. Brudvig, J. A. Wiegand, and E. A. Huang. 1986. A simple method for estimating the extent of surface crystal development on colored Cheddar cheese. J. Food Prot. 49:421-422.

Dybing, S. T., J. A. Wiegand, S. A. Brudvig, E. A. Huang, and R. C. Chandan. 1988. Effect of processing variables on the formation of calcium lactate crystals on Cheddar cheese. J. Dairy Sci. 71:1701-1710.

Harper, W. J., A. M. Swanson, and H. H. Sommer. 1953. Observations on the chemical composition of white particles in several lots of Cheddar cheese. J. Dairy Sci. 36:368-372.

Johnson, M. E., B. A. Riesterer, C. Chen, B. Tricomi, and N. F. Olson. 1990a. Effect of packaging and storage conditions on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:3033-3041.

Johnson, M. E., B. A. Riesterer, and N. F. Olson. 1990b. Influence on nonstarter bacteria on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:1145-1149.

Khalid, N. M. and E. H. Marth. 1990. Lactobacilli--their enzymes and role in ripening and spoilage of cheese: a review. J. Dairy Sci. 73:2669-2684.

Johnson, M. E. 2004. Calcium lactate crystals. International Cheese Technology Exposition, Madison, WI.

Linke, W. F. 1958. Solubilities of Inorganic and Metal Organic Compounds. *in* Am. Chem. Soc. 4th ed. W. F. Linke, ed, Washington, DC.

Pearce, K. N., L. K. Creamer, and J. Gilles. 1973. Calcium Lactate Deposits on Rindless Cheddar Cheese. N. Z. J. Dairy Sci. Tech. 8:3-7.

Rajbhandari, P. and P. S. Kindstedt. 2005. Compositional factors associated with calcium lactate crystallization in smoked Cheddar cheese. J. Dairy Sci. 88:3737-3744.

Severn, D. J., M. E. Johnson, and N. F. Olson. 1986a. Determination of lactic acid in Cheddar cheese and calcium lactate crystals. J. Dairy Sci. 69:2027-2030.

Somers, E. B., M. E. Johnson, and A. C. L. Wong. 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84:1926-1936.

Swearingen, P. A., D. E. Adams, and T. L. Lensmire. 2004. Factors affecting calcium lactate and liquid expulsion defects in Cheddar cheese. J. Dairy Sci. 87:574-582.

Van Slyke, L. L. and C. A. Publow. 1909. The Science and Practice of Cheese-Making. Pages 332-333. Orange Judd Pub. Co., Inc., New York.

Washam, C. J., T. J. Kerr, V. J. Hurst, and W. E. Rigsby. 1985. A scanning electron microscopy study of crystalline structures on commercial cheese. Devel. Industrial Micro. 749-761.

II. LITERATURE REVIEW

BACKGROUND OF CHEDDAR CHEESE

The word "Cheddar" is the name of the village in Somerset, England where Cheddar cheese was first made in the beginning of the 19th century (Kosikowski and Mistry, 1997). "Cheddaring" is the process of piling and re-piling of blocks of warm curd in cheese vats. During the period of about 2 h, lactic acid increases rapidly and gives the characteristic body and texture to Cheddar cheese. As the curd blocks are piled, the individual curd particles lose their identity and form a closely knit structure. The Cheddaring steps also helps in moisture control, since much of the moisture is retained in blocks of cheese curd as the blocks are piled and re-piled. The concept of Cheddar cheese manufacture was popularized in America in 1876 by Robert McCadam, leading to the evolution of the American Cheddar cheese industry. Cheddar cheese has a waxy, cohesive texture, closed body, and with pleasingly clean nutty flavor.

The U.S. is currently the largest producer of Cheddar cheese in the world, with the largest production in Wisconsin, followed closely by California, Idaho, New York, and Minnesota (IDFA, 2006). Development in Cheddar cheese manufacture has taken place with the introduction of continuous Cheddar cheese manufacturing systems in large establishments. The curd is ripened, cut and cooked in vertical or horizontal cheese vats. From cheese vats the cheese curd is pumped onto a perforated conveyor belt, which automatically Cheddars the cheese. The speed of the belt is adjusted so that at the end of the conveyor the desired acidity is achieved and the curd is milled, salted and pressed to form blocks. Cheese blocks are aged in coolers at 4^{o}C to 13^{o}C.

Regardless of automation, starter cultures are always used in the manufacture of Cheddar cheese. The primary function of starter cultures is to produce acid during the fermentation process, but starters also contribute to cheese ripening, as their enzymes are involved in proteolysis and formation of flavor compounds (Fox and Wallace, 1997). Starter bacteria can be defined as isolates that produce sufficient acid to reduce the pH of milk to < 5.3 in 6 h at 30-37^{o}C. Defined mesophilic starter cultures, *Lactococcus lactis* ssp. *lactic* and *Lactococcus lactic* ssp. *cremoris*, are used for Cheddar cheese production worldwide. These homofermentative starter cultures are added deliberately at the start of Cheddar cheese manufacture. The starter bacteria produce L(+)-lactate from lactose and they grow, typically attaining cell densities of 10^{8}cfu/g within hours of the beginning of manufacture. Production of homogeneous, high quality Cheddar cheese requires uniform lactose fermentation and proteolysis. The rate and extent of both fermentation and proteolysis depends on temperature and salt concentration (Thomas and Pearce, 1981). One of the main roles of starter bacteria is to provide a suitable environment for enzyme activity from rennet and favorable growth of secondary microflora with respect to redox potential, pH and moisture content in cheese.

Although, one of the most popular cheeses in world many defects may be associated with Cheddar cheese to affect its acceptability. Common defects in Cheddar cheese are flavor and texture defects and appearance of CLC. This thesis focuses on potential cause and prevention of CLC in Cheddar cheese.

CALCIUM LACTATE CRYSTALS

The first case of white crystals on cheese was reported by Van Slyke and Publow (1910), who thought the crystals were calcium salts of fatty acids. More recently, the crystals were

identified as crystals of calcium lactate or racemic mixture of L(+)- lactate and D(-)- lactate, calcium phosphate, tyrosine, or salts of amino acids (Harper et al., 1953; Conochie et al., 1960; Pearce et al., 1973; Brooker et al., 1975; Washam et al., 1985; Severn et al., 1986; Dybing et al., 1988; Johnson et al., 1990; Somers et al., 2001; Chou et al., 2003). While the crystals in cheese aged more than one year are mostly tyrosine, the crystals found in Cheddar cheese aged less than one year are mostly CLC (Severn et al., 1986; Dybing et al., 1988; Johnson et al., 1990a; Johnson et al., 1990b). CLC have been observed in Cheddar cheese aged for as little as a few weeks to 9 mo (Harper et al., 1953; Conochie et al., 1960; Dybing et al., 1988).

CLC on cheese are in the form of pentahydrate crystals, such that five molecules of water per molecule of calcium lactate are incorporated into a crystal lattice. The lactate can be present in D(-)/L(+)-isomeric forms. Studies conducted in the 1980s and 1990s (Thomas and Crow, 1983; Severn et al., 1986; Dybing et al., 1988; Johnson et al., 1990a) showed that racemic mixtures of D(-)/L(+)-calcium lactate were the reason for crystal formation, due to lower solubility of D(-)/L(+)-lactate in water than L(+)-lactate. Since calcium lactate is not a native component of milk, sufficient quantity of lactate and calcium ions must be present in cheese before crystals form (Pearce et al., 1973). Dybing et al. (1988) showed us how the calcium and lactate ions can react and form crystals in Cheddar cheese.

$$Ca^{2+} + 2\ \text{Lactate} \Leftrightarrow \underset{\text{(soluble)}}{\text{Ca Lactate}} \Leftrightarrow \underset{\text{(nucleated crystals)}}{\text{Ca Lactate}} \Leftrightarrow \underset{\text{(macro crystals)}}{\text{Ca Lactate}}$$

Many factors or combinations of factors are responsible for CLC in cheese, including racemization of L(+)- lactate to D(-)- lactate by non-starter lactic acid bacteria (NSLAB) (Martin and Crow, 1983; Johnson et al., 1990a; Chou et al., 2003), composition of cheese milk (Dybing et al., 1988), acidity of curd at the time of milling (Dybing et al., 1988; Johnson et al., 1990a),

storage temperature (Pearce et al., 1973; Chou et al., 2003), and packaging (Dybing et al., 1988). Although all the above concepts are not closely related, one or a combination of these factors may be responsible for the formation of CLC in Cheddar cheese. In the next several sections, these factors will be examined in more detail.

LACTIC AND NON STARTER LACTIC ACID BACTERIA IN CHEDDAR CHEESE

Starter bacteria make a significant contribution to the microbial biomass of lactic acid bacteria (LAB) in fresh curd. As the cheese ages, starter bacteria lyse and their population becomes insignificant in comparison to the microbial population of non-starter lactic acid bacteria (NSLAB). The high starter biomass provides considerable biocatalytic potential for the cheese ripening reactions, which are started by the autolysis of starter cells. Autolysis of starter cultures may be influenced by the salt concentration and associated S/M values of cheese (Bie and Sjostrom, 1976), and varying manufacturing conditions like elevated cook temperatures (Lowrie, Lawrence and Peberdy 1974). The rate of lactose fermentation by starters in Cheddar cheese depends upon S/M concentrations (Thomas and Pearce, 1981). In the work conducted by Thomas and Pearce (1981), all of the lactose was utilized within 8 d at 4% S/M, but at 6% S/M the lactose concentration remained high for several weeks after manufacture. S/M during manufacturing and temperature of cheese after pressing should be controlled to ensure that the activity of the starter is inhibited once the required pH to be reached. The growth and uninhibited metabolization of lactose to lactic acid by starter bacteria increases the amount of L(+)-lactate in cheese.

NSLAB are typically lactobacilli and pediococci that form a significant portion of the microbial flora of most cheese varieties during ripening. NSLAB are not part of normal starter flora generally do not grow well in milk, and do not contribute to acid production in the cheese vat (Cogan et al., 1997). Lactobacilli are divided into three groups on the basis of being i) obligatory homofermentative, ii) facultatively heterofermentative, or iii) obligatory heterofermentative (Kandler and Weiss 1986). The NSLAB lactobacilli found in cheese are generally facultatively heterofermentative. The common species isolated from Cheddar cheese are *Lactobacillus casei, Lb. paracasei, Lb. plantarum, Lb. rhamnosus* and *Lb. curvatus* (Jordan and Cogan 1993; Coppola et al., 1997; Fitzsimonas et al., 1999). *Pediococcus acidilactici* and *Pe. pentosaceus* are the most frequently encountered pediococci in Cheddar cheese.

Although starter bacteria make up the majority of the cheese microflora initially, NSLAB dominate the viable population in cheese for much of the ripening period (Williams et al., 2000; Khalid and Marth, 1990). Even if the initial population of NSLAB in pasteurized milk is 10 cfu/g, the population of NSLAB increases rapidly to 10^{7-8} cfu/g in the first few weeks of aging (Fox et al., 2000). The source of NSLAB in Cheddar cheese is a focus of debate, as they are found in cheese made from both raw and pasteurized milk. In Cheddar cheese made from pasteurized milk, the likely source of NSLAB is post-pasteurization contamination or failure of pasteurization to fully inactivate NSLAB (Turner, Lawrence and LeLievre, 1986; Martin and Crow 1993). A small number of NSLAB may survive pasteurization in an injured state, revive during cheese ripening, and subsequently grow in the cheese (Jordan and Cogan, 1999). This shows that low concentrations of contamination can result in NSLAB rapidly becoming a significant proportion (99%) of the total cheese flora. NSLAB can grow in Cheddar cheese with generation time of 8.5 d in cheese ripened at 6^{o}C (Jordan and Cogan, 1993). Fox, McSweeney

and Lynch, (1996) showed that the final population of NSLAB is independent of initial contamination of cheese curd, and the population of NSLAB can reaches 10^{7-8} cfu/g in 90 d. Although ripening temperature does not affect the final number of NSLAB, the growth rate is strongly affected by ripening temperature (Folkertsma et al., 1996). So, irrespective of aging temperature, the final population of NSLAB can reach 10^{7-8} cfu/g, although faster at high aging temperatures than in cheeses aged at low temperatures.

Most heterofermentative NSLAB produce a racemic mixture of D(-)/L(+)-lactate (Thomas and Turner, 1980; Johnson et al., 1990; Chou et al., 2003). Heterofermentative NSLAB also tend to produce lactic acid from other substrates, present in cheese as a result of starter metabolism, that are not utilized by starter bacteria (glactose and citrate), leading to increased concentration of lactic acid in cheeses with NSLAB. The calcium salt of D(-)-lactate is less soluble than the calcium salt of L(+)-lactate (Cao et al., 2001), leading to crystallization of D(-)-calcium lactate on cheese surfaces. Several authors (Johnson et al., 1990; Somers et al., 2001; Chou et al., 2003) demonstrated that *Lb. curvatus*, a heterofermentative NSLAB, produces racemic mixture of L(+)- and D(-)-lactic acid in cheese and tend to produce CLC on cheese surfaces.

PROCESSING FACTORS AFFECTING THE DEVELOPMENT OF CLC

Milk composition, unit operations, aging temperatures, and packaging may affect undesirable crystal formation in or on Cheddar cheese. In the following sections the ways in which each factor might affect the crystal formation will be discussed.

Milk Composition

The composition of milk, especially fat and proteins, strongly influences the quality of cheese. Considering CLC, the concentrations of calcium and lactose are especially important. Lactose is metabolized to lactic acid and can react with calcium in milk to form CLC (Pearce et al., 1973; Sutherland et al., 1981). The typical composition of milk is 12.7% total solids, 4.8% lactose, 3.7% fat, 3.4% protein, 0.7% ash (Fox et al., 2000). Calcium, lactose, and protein will be discussed in more detail because they contribute to CLC formation in Cheddar cheese.

a) Calcium

The calcium content in cow's milk is approximately 1.2 g/L (Fox et al. 2000). Approximately 34% of calcium is soluble and the rest is present as insoluble colloidal calcium bound to casein micelles and phosphate complexes (Parry et al., 1974; Dybing et al., 1988; Fox et al., 2000). Fifty five percent of the soluble calcium is present as calcium citrate, 10% is present as calcium phosphate and 35% is present as ionic calcium (Ca^{2+}) (Dybing et al., 1988; Fox et al., 2000). The calcium content in Cheddar cheese is approximately 7.6g/kg (Fox et al., 2000). The amount of calcium in cheese is dependent upon the cheese manufacturing process conditions that affect the distribution of calcium between soluble calcium and insoluble calcium. The equilibrium between soluble calcium and colloidal calcium is dependent on pH of the milk or cheese curd. As the pH declines, some colloidal calcium is converted to soluble calcium, which is lost along with whey during the course of cheese manufacture (Kosikowski and Mistry, 1997). Approximately 40% of the total milk calcium is lost in during Cheddar cheese manufacture, mostly with the drainage of whey (Lucey and Fox, 1993).

The amount of calcium available for CLC formation depends upon the initial calcium concentration in the milk and the cheese manufacturing process. Dybing et al. (1988) conclude that casein bound calcium is the major source of calcium in CLC. Dybing et al. (1988) showed that fast acid production and high milling acidities are associated with reduced CLC formation in Cheddar cheese due to reduced concentration of casein bound calcium. Seasonal changes affecting milk casein and calcium affect CLC; low casein to calcium ratios are related to increased CLC formation (Dybing et al., 1988). Low casein to calcium ratio leads to increased amount of bound calcium in milk, contributing to increased amount of calcium in cheese.

Calcium plays a very important role in cheese manufacture since it influences rennet coagulation of milk and curd structure (Fox et al., 2000; Lucey and Fox, 1993). In fact, cheese manufactures often add $CaCl_2$ to cheese milk to assist faster and firmer setting of curd. The added calcium ions neutralize negatively charged residues on casein micelles and increase the curd firmness (Fox et al., 2000; Lucey and Fox, 1993). Since the added calcium is in ionic form, and most of it is lost in whey, it does not contributes to CLC formation.

b) Lactose

Lactose, a disaccharide composed of glucose and galactose, is the principle carbohydrate found in milk. Cow's milk contains approximately 4.8% lactose (Fox et al., 2000). In Cheddar cheese manufacture, approximately 96% of the lactose is lost with whey at draining (Fox et al., 2000). Starter bacteria present in cheese convert residual lactose to lactic acid. This is an essential process in Cheddar cheese manufacture. Lactose is hydrolyzed by starter bacteria to glucose and galactose; of which glucose is metabolized rapidly by starter bacteria to produce lactic acid (Kosikowski and Mistry, 1997). Unlike glucose metabolism, galactose metabolism is slow and incomplete, as galactose is converted to glucose before being metabolized (Kosikowski

and Mistry, 1997). Glucose and galactose are metabolized though the Embden Meyerhof Parnas pathway to pyruvate, and pyruvate is converted to lactic acid by lactate dehydrogenases in lactic acid bacteria (Kosikowski and Mistry, 1997). During Cheddar cheese manufacture, most of the lactose is converted to lactic acid (Huffman et al., 1984). Starter bacteria or NSLAB metabolize the lactose remaining in cheese within the first week of aging (Dybing et al., 1988; Fox et al., 2000; Thomas and Crow, 1983). The presence of higher lactose (more than 4.8%) in cheese milk leads to increased lactose in cheese, which can be used by starter bacteria or NSLAB to produce lactate in cheese and increase calcium lactate concentrations (Dybing et al., 1988; Pearce et al., 1973; Blake et al., 2005). In experiments conducted by Pearce at al. (1973), cheese made from milk having high lactose concentration (5.4%) formed CLC. Pearce et al. (1973) suggested seasonal variation in lactose concentration in cheese milk; higher lactose in milk (more than 4.8%) can be responsible for CLC formation. In contrast, Blake (2005) showed that Cheddar cheese manufactured with milk having high lactose (5.24%) alone may not result in CLC formation; presence of NSLAB were the critical to formation of CLC, as NSALB could have fermented excessive lactose to D(-)/L(+)- lactate. So there is a definite risk of CLC in cheese made from high lactose milk since high concentrations of lactose provide additional substrate for NSLAB to grow, leading to increased concentrations of D(-)/L(+)- lactic acid in cheese.

Manufacturing Processes

a) Milling and Salting

The acidity at which the cheese curd is milled has an impact upon the quality of Cheddar cheese. As the curd pH decreases, the serum calcium increases at the expense of casein-bound calcium. The result is that more calcium is lost in whey. Fast acid development and high milling

acidity are associated with lower occurrence of CLC in cheese (Dybing et al., 1988) because much of the serum calcium is lost in the whey during pressing, so less is available to bind with lactate.

The size of the milled curd also influences the amount of serum calcium in cheese curd (Fox et al., 2000). Milling cheese curd to smaller pieces increases the cheese surface area exposed to salt. Increase in surface area of the cheese curd at the time of salting means higher concentrations of salt at the surface, leading to increased expulsion of moisture, calcium, lactose and lactate from the entire cube of cheese curd (Conochie and Sutherland, 1965).

Salt plays an important role in cheese manufacture because it controls the growth and acid production activity of microorganisms, controls various enzyme activities in cheese, and influences syneresis of the curd. O'Connor (1971) showed an inverse relationship between salt and moisture concentration, suggesting higher salt concentrations lead to more expulsion of whey. Salt increases the solubility of casein micelles though replacing calcium ions present in casein micelle with sodium ions (Lawrence et al., 2001). Replacement of calcium ions bound to casein with sodium ions increases the number of calcium ions available in serum to form calcium lactate. Therefore, salting of cheese to higher salt concentrations (6% S/M) may release large amounts of calcium ions and facilitate greater CLC formation (Dybing et al., 1988). Salting of cheese curd at lower pH (less than 5.4) allows for large amounts of solubilized calcium ions to escape with whey before cheese curd is pressed. But there is a limitation; as pH lower than 5.1 will detrimentally affects cheese texture. The texture of Cheddar cheese at 35 d can vary from curdy (pH > 5.3) to waxy (pH 5.3 to 5.1) to mealy (pH < 5.1). This change in Cheddar cheese quality is attributed to changes in structure of the casein micelles due to loss of calcium. Low pH of cheese blocks after pressing increases the concentration of serum calcium, as more

colloidal calcium becomes soluble, but reduces the solubility of calcium lactate (Cao et al., 2001) making cheese more prone to CLC.

Thomas et al. (1981) determined the S/M concentrations at the surface and the center of individual curd pieces and found that S/M equilibria is established in curd pieces within 24 to 48 h after hooping. Smaller curd size will ensures uniform distribution of salt within cheese at faster rate thus inhibiting the starter bacteria sooner . So milling of cheese curd to smaller curd size is desirable in reducing the occurrence of CLC in cheese.

Starter bacteria play a major role in the utilization of remaining lactose in cheese curd. Using salt tolerant starter bacteria or lesser S/M concentration enables starter bacteria to utilize residual lactose, which otherwise would be used by NSLAB. Some NSLAB are able to grow in cheese with 6% S/M or higher. At 4% S/M, starter bacteria likely utilize all the lactose in 8 d, while at 6% S/M the concentration of lactose may remain high even after several weeks of aging. Although NSLAB use other sources of carbon for metabolism, the presence of a high concentration of lactose leads to rapid increase in the population of NSLAB. NSLAB are more salt tolerant than the SLAB and there is little effect on their growth and racemizing abilities by variation in salt from 4% to 6% S/M (Turner and Thomas, 1980).

b) Packaging

With the large scale production of rindless cheese, cheese packaging plays an important role in maintaining the quality of cheese. Various flexible and barrier films are used to package cheese for aging. The two main consumer packaging methods used by cheese manufactures are vacuum packaging and gas flushed packaging. In vacuum packaging, the packaging material clings closely to the cheese surface, as the air is removed from the package before heat sealing.

Gas flushed packaging methods are used to package cheese shreds and cubed cheeses to prevent aggregation and loss of individual identity of cheese particles. The gasses generally used to flush out air are CO_2, N_2 or a combination of the two.

Johnson et al. (1990a) observed faster and greater crystal formation on cheeses that were gas flushed than on cheeses that were vacuum packaged. Only a small number of crystals were seen on vacuum packed cheese after five months of aging compared to a high number of crystals in gas flushed packaged cheese. According to Dybing et al. (1988), free ionic calcium may combine with lactate though a mechanism involving carbonic acid, as shown below:

$$CO_2 + H_2O \Leftrightarrow H_2CO_3$$

$$H_2CO_3 \Leftrightarrow 2H^+ + CO_3^{2-}$$

$$CO_3^{2-} + Ca^{2+} \Leftrightarrow CaCO_3$$

$$CaCO_3 + \text{Lactate} \Leftrightarrow \text{Calcium Lactate} + CO_3^{2-}$$

It has been speculated (Dybing et al., 1988) that packaged cheese flushed with CO_2 tends to absorb CO_2. As the CO_2 is absorbed on the surface of the cheese, it tends to lower the pH of the serum phase of cheese. Lower pH leads to decreased solubility of calcium lactate and increase in serum calcium concentration, leading to faster CLC formation.

CLC have also been observed on vacuum packaged cheeses where the package has lost integrity (Johnson et al., 1990a). Since cheese serum tends to migrate to the surface of the cheese or to cracks or crevices inside the cheese during aging, the concentration of lactic acid at those locations tends to increase. In loosely packaged cheese, the surface of the cheese tends to dry due to surface evaporation, forming nucleation sites that cause faster crystal formation. Since CLC develop faster in both gas flushed or vacuum packaged cheeses that have lost

tightness or integrity, packaging plays an important role in the appearance of CLC on cheese surfaces.

c) Aging Temperature

To attain desired sharpness Cheddar cheese is generally aged at a temperature ranging from 4°C to 13°C for three months to as long as 18. Storage temperature of Cheddar cheese affects CLC formation in two ways. First, low temperatures are associated with reduced solubility of calcium lactate (Cao et al., 2001). The solubility of calcium lactate in 100 g of water is 3.1 g at 0°C, 5.4 g at 15°C and 7.9 g at 30°C (Linke, 1958). Second, increased growth rate of heterofermentative NSLAB at elevated aging temperatures leads to increased chances of production of D(-)-lactate, and racemization of L(+)-lactate to D(-)/L(+)-isomer (Turner and Thomas, 1980). In agreement with Turner and Thomas (1980), Johnson et al. (1990a; 1990b) reported that CLC developed more quickly and to a greater extent at aging temperatures of 3.3°C and 4.3°C than at 7.2°C. Chou (2003) observed CLC developed faster in cheese aged at 4°C than in cheeses aged at 13°C.

Maintaining consistent aging temperature is important, as high aging temperatures may lead to increased growth of NSLAB, while low aging temperatures reduce the solubility of calcium lactate. Research by Chou et al. (2003) showed that fluctuation in aging temperature of cheeses should be avoided during transportation and on retail shelves of grocery stores. Cheeses aged at 13°C initially and then at 4°C developed crystals more quickly than cheeses maintained consistently at either 13°C or 4°C (Chou et al., 2003).

SUMMARY

CLC in Cheddar cheese are due to many factors: NSLAB; concentration of calcium, lactose, D(-)- and L(+)-lactic acid and S/M; pH of cheese; storage temperature; and cheese packaging. Mostly, CLC are seen in commercial cheeses because of a combination of these factors. Previous research suggested different approaches to minimize development of CLC. Residual lactose should be minimized because high concentration of residual lactose in cheese will lead to higher concentration of L(+)- or D(-)-lactate, which can readily combine with calcium ions in cheese serum. High milling acidity and fast acid development are desirable because they enable expulsion of calcium ions and lactate in the whey at the time of pressing prior to packaging. High S/M, combined with quick cooling of cheese blocks after pressing, may limit primary and secondary fermentation of lactose and lowering of pH of cheese block post packaging and reduce the occurrence of CLC. At low temperatures, the solubility of calcium lactate decreases. At high temperatures, the growth of NSLAB increases. Thus, to prevent CLC, handlers must avoid temperature abuse during storage, transportation and retail marketing. Manufacturers and distributors should avoid loose packaging or gas flushed packaging.

Many critical steps must be monitored during cheese manufacture, including plant hygiene, cheese milk composition, starter bacteria, S/M, and cheese packaging, cooling of cheese blocks and pH of cheese during aging to prevent occurrence of CLC. Current research was designed to specifically focus on how the above-mentioned steps influence the occurrence of CLC.

Objective one was to determine whether gas flushing of Cheddar cheese or if different gasses CO_2, N_2, or combination of both on increased occurrence of CLC. Our hypothesis was that Cheddar cheeses that are gas flushed with CO_2 will have a higher intensity of CLC compared to cheeses flushed with N_2 or N_2+CO_2 and volume of cheeses packages that are gas flushed with CO_2 will be lower than packages gas flushed with N_2. It was believed that CO_2 would readily absorb in the moist cheese surfaces, lowering the surface pH of cheese and inducing CLC.

Objective two was to investigate the effect of concentrating milk (using UF) or increasing solids with nonfat dry milk (NFDM), and cheese pH, upon formation of CLC. We hypothesized that increasing the protein concentration of cheese milk leads to increased concentration of total and soluble calcium in cheeses, resulting in increased occurrence of CLC in cheeses made from cheese milk with higher total solids

Objective three was to determine the effect of salt and starter bacteria on cheese pH, lactic acid, soluble calcium and occurrence of CLC in cheese. We hypothesized that cheeses having lower S/M have increased concentration of lactic acid, lower pH and increased occurrence of CLC than cheeses with high S/M.

Objective four was to study if migration of calcium and lactate ions in cheese takes place and to determine the influence of migration on occurrence of CLC. We hypothesized that migration of calcium and lactate ions to the cheese surface during aging promotes growth of CLC

REFERENCES

Bie, R. and G. Sjostrom. 1975a. Autolytic Properties of Some Lactic Acid Bacteria Used in Cheese Production. 1. Material and Methods. Milchwissenschaft. 30:653-657.

Bie, R. and G. Sjostrom. 1975b. Autolytic properties of some lactic acid bacteria used in cheese production. II. experiments with fluid substrates and cheese. Milchwissenschaft. 30:739-747.

Blake, A. J., J. R. Powers, L. O. Luedecke, and S. Clark. 2005. Enhanced lactose cheese milk does not guarantee calcium lactate crystals in finished Cheddar cheese. J. Dairy Sci. 88, no. 7:2302-2311.

Brooker, B. E., D. G. Hobbs, and A. Turvey. 1975. Observations on the microscopic crystalline inclusions in Cheddar cheese. J Dairy Res. 42:341-348.

Cao, X., H. J. Lee, H. S. Yun, and Y. M. Koo. 2001. Solubilities of calcium and zinc lactate in water and water-ethanol mixture. Korean J. Chem. Eng. 18:133-135.

Carsberg, H. 2000. Why bacteria survive your sanitizers. Sanitation Techno. pp 56-58.

Chou, Y. E., C. G. Edwards, L. O. Luedecke, M. P. Bates, and S. Clark. 2003. Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. J. Dairy Sci. 86:2516-2524.

Cogan, T. M. and C. Hill. 1999. Cheese Starter Cultures. Pages 193-256 *in* Cheese: Chemistry, Physics and Microbiology. Vol. 1. P. F. Fox, ed. An Aspen Publication, Gaithersburg, Maryland.

Conochie, J., J. Czulak, A. J. Lawrence, and W. F. Cole. 1960. Tyrosine and calcium lactate crystals on rindless cheese. Aust. J. Dairy Technol. 15:120.

Conochie, J. and B. J. Sutherland. 1965. The nature and cause of seaminess in Cheddar cheese. J. Dairy Res. 32:35.

Coppola, R., M. Nanni, M. Iorizzo, A. Sorrento, E. Sorrentino, and L. Grazia. 1997. Survey of lactic acid bacteria isolated during the advanced stages of the ripening of Parmigiano Reggiano cheese. J. Dairy Res. 64:305-310.

Drake, M. A. 2007. Defining dairy flavors. 22nd Annual Cheese Short Course. Pullman, WA.

Dybing, S. T., S. A. Brudvig, J. A. Wiegand, and E. A. Huang. 1986. A simple method for estimating the extent of surface crystal development on colored Cheddar cheese. J. Food Prot. 49:421-422.

Dybing, S. T., J. A. Wiegand, S. A. Brudvig, E. A. Huang, and R. C. Chandan. 1988. Effect of processing variables on the formation of calcium lactate crystals on Cheddar cheese. J. Dairy Sci. 71:1701-1710.

Diggin, M. B., Waldron, D.S., McGolrick, M. A., Cogan, T. M. and Fox, P. F. (1990). Growth substrate for mesophillic nonstarter lactic acid bacteria in Cheddar cheese. Irish J. Agri. Food Res. 38, 183.

Fenelon, M., and Guinee, T. P. (1999). The effect of milk fat on Cheddar cheese yield and its prediction, using modification of the Van Slyke cheese yield formula. J. Dairy Sci. 82, 2287-2299.

Fenelon, M., O'Connor, P., and Guineee, T.P. (2000). The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. J. Dairy Sci. 10, 2173-2183.

Fitzsimons, N. A., Cogan, T. M., Condon, S., and Beresford, T. (2001). Spatial and temporal distribution of nonstarter lactic acid bacteria in Cheddar cheese. J. App. Micro. 90, 600-608.

Folertsma, B., Fox, P.F., and McSweeney, P. L. H. (1996). Accelerated ripening of Cheddar cheese at elevated temperature. Int. Dairy J. 6, 1117-1134.

Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2000. Fundamentals of Cheese Science. Aspen Publishers, Inc., Gaithersburg.

Fox, P. F., P. L. H. McSweeney, and C. M. Lynch. 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust. J. Dairy Technol. 53:83-89.

Frank, J. F., R. A. N. Gillett, and G. O. Ware. 1990. Association of *Listeria spp*. contamination in the dairy processing plant environment with the presence of Staphylococci. J. Food Pro. 53:928-932.

Harper, W. J., A. M. Swanson, and H. H. Sommer. 1953. Observations on the chemical composition of white particles in several lots of Cheddar cheese. J. Dairy Sci. 36:368-372.

Huffman, L. M. and T. Kristoffersen. 1984. Does lactose concentration affect Cheddar quality? Ohio report on research and development in agriculture, home economics, and natural resources - Ohio Agricultural Research and Development Center. 69:69-70.

IDFA, 2006. Dairy Facts. International Dairy Foods Association. Washington, DC.

Johnson, M. E., B. A. Riesterer, C. Chen, B. Tricomi, and N. F. Olson. 1990a. Effect of packaging and storage conditions on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:3033-3041 ill.

Johnson, M. E., B. A. Riesterer, and N. F. Olson. 1990b. Influence on nonstarter bacteria on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:1145-1149.

Jordan, K. N. and T. M. Cogan. 1993. Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. Irish J. Agri. Food Res. 32:47-55.

Jordan, K. N. and T. M. Cogan. 1999. Heat resistance of *Lactobacillus spp*. isolated from Cheddar cheese. Letters in App. Micro. 29:136-140.

Kandler, O., and Weiss, N. (1986). Regular, nonsporing , Gram positive rods. In P. H.A. Sneath, N.S. Mair, M.E. Sharpe, and J. G. Holt (Eds.), *Bergeys manual of systematic bacteriology,* Vol. 2 (pp. 1208-1234). Baltimore: Williams and Wilkins Co.

Khalid, N. M. and E. H. Marth. 1990. Lactobacilli--their enzymes and role in ripening and spoilage of cheese: a review. J. Dairy Sci. 73:2669-2684.

Kosikowski, F. and V. V. Mistry. 1997. Cheese and Fermented Milk Foods. Vol. 2. 3rd ed. Westport, CT, Westport, Conn.

Lane, C. N., P. F. Fox, D. E. Johnston, and P. L. H. McSweeney. 1997. Contribution of coagulant to proteolysis and textural changes in Cheddar cheese during ripening. Int. Dairy J. 7:455-464.

Lawrence, R. C. and J. Gilles. 1980. The assessment of the potential quality of young Cheddar cheese. N. Z. J. Dairy Sci. Tech. 15:1-12 ill.

Lawrence et al. 2001, Cheese Chemistry and Microbiology, Fox (eds.), Chap. Cheddar cheese volume 2 page 22-24.

Linke, W. F. 1958. Solubilities of Inorganic and Metal Organic Compounds. *in* Am. Chem. Soc. 4th ed. W. F. Linke, ed, Washington, DC.

Lowrie, R. J., R. C. Lawrence, and M. F. Peberdy. 1974. Cheddar cheese flavour. V. Influence of bacteriophage and cooking temperature on cheese made under controlled bacteriological conditions. N. Z. J. Dairy Sci. Tech. 9:116-121.

Lucey, J. A. and P. F. Fox. 1993. Importance of calcium and phosphate in cheese manufacture: a review. J. Dairy Sci. 76:1714-1724.

Lynch, C. M., P. L. H. McSweeney, P. F. Fox, T. M. Cogan, and F. D. Drinan. 1996. Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. Int. Dairy J. 6:851-867.

Mattila, T., M. Manninen, and A. L. Kylasiurola. 1990. Effect of cleaning-in-place disinfectants on wild bacterial strains isolated from a milking line. J. Dairy Res. 57:33-39.

O'Connor, C. B. 1974. The Quality and Composition of Cheddar Cheese: Effect of Various Rates of Salt Addition. III. Irish Agric. Cream. 27:11-13.

Pearce, K. N., L. K. Creamer, and J. Gilles. 1973. Calcium Lactate Deposits on Rindless Cheddar Cheese. N. Z. J. Dairy Sci. Tech. 8:3-7.

Ruegg, M. and B. Blanc. 1981. Influence of water activity on the manufacture and aging of cheese.791-811.

Severn, D. J., M. E. Johnson, and N. F. Olson. 1986a. Determination of lactic acid in Cheddar cheese and calcium lactate crystals. J. Dairy Sci. 69:2027-2030.

Somers, E. B., M. E. Johnson, and A. C. L. Wong. 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84:1926-1936.

Sutherland, B. J. and G. W. Jameson. 1981. Composition of hard cheese manufactured by ultrafiltration. Aust. J. Dairy Technol. 36:136-143.

Thomas, T. D. and R. D. Batt. 1969. Degradation of cell constituents by starved *Streptococcus Lactis* in relation to survival. J Gen Microbiol. 58:347-362.

Thomas, T. D. and V. L. Crow. 1983. Mechanism of D(-)-Lactic acid formation in Cheddar cheese. N. Z. J. Dairy Sci. Tech. 18:131-141.

Thomas, T. D. and K. N. Pearce. 1981. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. N. Z. J. Dairy Sci. Tech. 16:253-259.

Thomas, T. D. (1987). Cannibalism among bacteria found in cheese. N. Z. J. Dairy Sci. Tech. 22, 215-219.

Turner, K. W., R. C. Lawrence, and J. Lelievre. 1986. A microbiological specification for milk for aseptic cheesemaking. N. Z. J. Dairy Sci. Tech. 21:249-254.

Turner, K. W. and T. D. Thomas. 1980. Lactose fermentation in Cheddar cheese and the effect of salt. N. Z. J. Dairy Sci. Tech. 15:265-276.

Van Slyke, L. L. and C. A. Publow. 1909. The Science and Practice of Cheese-Making. Pages 332-333. Orange Judd Pub. Co., Inc., New York.

Wallace, J. M. and P. F. Fox. 1997. Effect of adding free amino acids to Cheddar cheese curd on proteolysis, flavour and texture development. Int. Dairy J. 7:157-167.

Washam, C. J., T. J. Kerr, V. J. Hurst, and W. E. Rigsby. 1985. A scanning electron microscopy study of crystalline structures on commercial cheese. Devel. Industrial Micro. 749-761.

Williams, A. G. and J. M. Banks. 1997. Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. Int. Dairy J. 7:763-774.

Williams, A. G., S. E. Withers, and J. M. Banks. 2000. Energy sources of non-starter lactic acid bacteria isolated from Cheddar cheese. Int. Dairy J. 10:17-23.

III. GAS FLUSHED PACKAGING CONTRIBUTES TO CALCIUM LACTATE CRYSTALS IN CHEDDAR CHEESE.

Agarwal, S., M. Costello, and S. Clark†. 2005. Gas-Flushed Packaging Contributes to Calcium Lactate Crystals in Cheddar Cheese. J. Dairy Sci. 88:3773-3783.

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ABSTRACT

Gas flushed packaging is commonly used for cheese shreds and cubes to prevent aggregation and loss of individual identity. Appearance of a white haze on cubed cheese is unappealing to consumers, who may refrain from buying, resulting in lost revenue to manufacturers. The objective of this study was to determine whether gas flushing of Cheddar cheese contributes to the occurrence of CLC. Cheddar cheese was manufactured using standard methods, with addition of starter culture, annatto and chymosin. Two different cheese milk compositions were used: Standard (lactose:protein=1.47, protein:fat=0.90, lactose=4.8%) and Ultrafiltered (UF) (lactose:protein=1.23, protein:fat=0.84, lactose=4.8%), with or without adjunct *Lb.curvatus*. Curds were milled when whey reached 0.45% titratable acidity, and pressed for 16h. After aging at 7.2^{o}C for 6 mo, cheeses were cubed (1cm*1cm*4cm) and either vacuum packaged or gas flushed with carbon dioxide, nitrogen, or a 50:50 mixture of carbon dioxide and nitrogen, then aged three additional months. Heavy crystals were observed on surfaces of cubed cheeses that were gas flushed, but not on cheeses that were vacuum packaged.

Cheeses without *Lb. curvatus* exhibited L(+)-CLC on surfaces, while cheeses with *Lb. curvatus* exhibited racemic mixtures of L(+)/D(-)-CLC throughout the cheese matrix. The results show that gas flushing, regardless of gas composition, milk composition and presence of nonstarter lactic acid bacteria (NSLAB), can contribute to the development of CLC on cheese surfaces. These findings stress the importance of packaging to cheese quality.
(**KEY WORDS:** Gas flushing, calcium lactate crystals, Cheddar cheese, *Lb. curvatus*, Packaging.

ABBREVIATION KEY: CLC = calcium lactate crystals, NSLAB = nonstarter lactic acid bacteria, UF = Ultrafiltered.

INTRODUCTION

Quality and appearance defects in Cheddar cheeses discourage repeat purchases by consumers, so white crystals on the surface of Cheddar cheese detrimentally affect sales. Unattractive crystals on Cheddar cheese have been documented since the 1930's (McDowall and McDowell, 1939), and yet the problem remains a challenge and expense to cheese manufacturers (Chou et al., 2003). A high number of Cheddar cheeses manufactured in US have the problem of calcium lactate crystals (CLC) (Johnson, 2004). According to Johnson (2004), almost all cheese plants experience CLC problems in mild and medium Cheddar cheese, which previously was a common problem found only in aged cheeses.

With the large-scale production of rindless cheese, packaging plays an important role in maintaining the quality of cheese. Various flexible and barrier films are used to package cheese for aging. The two main consumer packaging methods used by cheese manufactures are vacuum packaging and gas flushed packaging. Gas flushing and heat sealing are used to package cheese

shreds and cubed cheeses to prevent aggregation and loss of individual identity of cheese particles. The gases generally used to flush out air are carbon dioxide (CO_2), nitrogen (N_2) or a combination of the two. Johnson et al. (1990a) observed faster and greater crystal formation on cheeses that were gas flushed using CO_2 than on cheeses that were vacuum packaged. A small number of crystals were seen on vacuum packed cheese after five months of aging compared to a high number of crystals in gas flushed packaged cheese. Dybing et al. (1988) hypothesized that free ionic calcium combines with lactate though a mechanism involving carbonic acid. According to Dybing, et al. (1988), packaged cheese flushed with CO_2 absorbs CO_2. As CO_2 is absorbed, the pH of the serum phase is reduced. Low pH in cheese is hypothesized to shift colloidal calcium to soluble calcium (Hassan et al., 2004), and increased serum calcium concentration facilitates CLC formation.

CLC have also been observed on vacuum packaged cheeses where the package has lost integrity (Johnson, et al., 1990a). Since cheese serum tends to move to the surface of the cheese or to cracks or crevices inside the cheese during aging, lactic acid concentration in those spaces increases. In loosely packaged cheese, the surface of the cheese dries due to evaporation, forming nucleation sites that accelerate crystal formation.

Residual calcium and lactose, after pressing, also contribute to CLC. Lactose is metabolized to lactic acid and can react with calcium in cheese to form CLC (Pearce, et al., 1973, Sutherland and Jameson, 1981). The calcium content in Cheddar cheese is approximately 7.6 g/kg (Fox, et al., 2000) but cheese processing conditions affect the distribution of calcium between soluble and insoluble compartments. During Cheddar cheese manufacture and within the first few weeks of aging, most of the lactose is converted to lactic acid (Huffman and Kristoffersen, 1984) by starter bacteria or nonstarter lactic acid bacteria (NSLAB) (Dybing, et

al., 1988, Fox, et al., 2000, Thomas and Crow, 1983). Elevated lactose (more than 4.8%) in cheese milk yields increased lactose in cheese, which can be used by starter bacteria or NSLAB to produce lactate in cheese and potentially increase calcium lactate concentrations (Dybing, et al., 1988, Pearce, et al., 1973). However, Blake et al. (2005) showed that high lactose in cheese milk does not guarantee CLC.

Another major contributor to CLC in Cheddar cheese is NSLAB. Most facultative heterofermentative NSLAB produce a racemic mixture of D(-)/L(+)- lactate (Chou, et al., 2003, Johnson, et al., 1990b, Turner and Thomas, 1980). Facultative heterofermentative NSLAB also tend to produce lactic acid from other substrates present in cheese such as galactose and citrate, contributing to increased concentration of lactic acid in cheeses with NSLAB (Sharma, 2003). Galactose and citrate are starter metabolites not utilized by starter bacteria. Also, the presence of facultative heterofermentative NSLAB like *Lb. curvatus*, which produces a racemic mixture of L(+)- and D(-)-lactic acid in cheese, contribute to CLC (Chou, et al., 2003, Johnson, et al., 1990b, Somers, et al., 2001). The calcium salt of D(-)-lactate is less soluble than the calcium salt of L(+)-lactate (Cao, et al., 2001; Kubantseva, et al., 2004), so the presence of D(-)-lactate often results in crystallization of D(-)-calcium lactate on cheese surfaces.

Visible CLC, especially on cubed or shredded cheese surfaces, have troubled the cheese industry for some time. Recently, CLC have been observed on cubed and shredded cheeses with predominantly L(+)-lactic acid and no or less D(-)-lactic acid. A desire to understand the formation of L(+)-lactate crystals on cheese surfaces led us to investigate the effect of gas flushing on the formation of CLC. The objective of this study was to determine effects of flushing CO_2, N_2 and CO_2:N_2 (50:50) on formation of CLC in Cheddar cheese made from two different milk compositions, both in the absence and presence of the NSLAB *Lb. curvatus*.

MATERIALS AND METHODS

Cheese Manufacture and Packaging

Two different batches of milk were standardized to make cheeses, based on selected cheese-milk formulations used in industry. Cheeses were manufactured in duplicate, with each replicate made from 90.8 kg of milk for a total of 8 cheeses. **Standard milk** (lactose : protein =1.47, protein : fat = 0.90, lactose = 4.8%): Standard milk plus starter culture (batch 1), and Standard milk plus starter culture plus *Lb. curvatus* adjunct culture (batch 2). Two duplicate batches of cheese were made from **Ultrafiltered (UF) milk** (lactose : protein = 1.23, protein: fat = 0.84, lactose = 4.8%): UF milk plus starter culture (batch 3), and UF milk plus starter culture plus *Lb. curvatus* adjunct culture (batch 4). Cheeses were made using standard procedures followed at the Washington State University Creamery. The standardized and pasteurized cheese milk was added to a hot water jacketed cheese vat. Starter culture *Lc. lactis* ssp. *cremoris* #98 (Chr. Hansen, Milwaukee, WI), was grown to a cell density of 10^8 cfu/ml in sterilized internal pH controlled buffer media (Vivolac, Indianapolis, IN) and inoculated into standardized milk at a rate of 1% (w/w) at 32°C. *Lb. curvatus* were grown to a cell density of 10^8 cfu/ml in Lactobacillus MRS broth (Becton Dickinson and Co., Sparks, MD) and added to milk to achieve initial populations of 500-700 cfu/ml in the cheese milk to mimic the low initial NSLAB counts typically observed in pasteurized cheese milk (Johnson, et al., 1990b). Double-strength coagulator (Chy-Max, Chr. Hansen Laboratories, Milwaukee, WI), diluted 1:40 with tap water, was used to assist coagulation of the milk. At the time of cutting with 6-mm cutter grid cheese knives, titratable acidity (TA) (as % lactic acid) of cheese whey was 0.12%. Curds were cooked by raising the temperature from 31 to 38°C at the rate of 1°C every 5 min over a 30-min period.

Curds and whey were stirred at 38°C for 45 min before draining, at whey TA of 0.15%, and Cheddaring.

When the TA of the whey reached 0.45 to 0.47%, the loaves were milled and curds were salted (0.3% w/w of milk). After overnight pressing at 2.8 X 10^5 psi, cheeses were cut into wedges (7.5 cm X 5.5 cm X 4 cm) approximately 150 g and vacuum packaged (Model X180, Koch Supplies Inc., Kansas City, MO) in 15 X 20 cm, 3 mil high barrier Nylon/EVOH/PE vacuum pouches (Koch Supplies Inc., Kansas City, MO). Finished cheeses were then aged at 7.2^{o}C for a period of 6 mo.

After 6 mo of aging, the cheeses were cut and repackaged as in a cut and wrap facility. The cheeses from individual treatments were cut into 1 cm x 1 cm x 4 cm pieces with a French fry cutter (Shaver Specialty Co., Los Angles, CA). Randomly, 10 pieces of cut cheese were either vacuum packaged or gas flushed packaged (Model X180, Koch Supplies Inc., Kansas City, MO) in 15 X 20 cm, 3 mil high barrier Nylon/EVOH/PE vacuum pouches (Koch Supplies Inc., Kansas City, MO) with 1) carbon dioxide, 2) nitrogen or 3) 50:50 mixture of both gases (CO_2:N_2).

Analyses

Proximate analysis of cheese was conducted using standard procedures (Table 3; Sharma, 2003). The volume of gas inside each gas flushed package was determined by measuring the volume of water (21^{o}C) displaced by submerging the cheese packages in a graduated cylinder. The volume of gas in each flushed package was measured after weeks 1, 2, 4, 8, 12 of gas flushing. The pH of the cheeses was measured after 1, 2, 4, 8, 12 weeks of packaging using a pH electrode (Orion 91-5500, Beverly, MA) calibrated with pH buffer: 4 and 7 (Fisher Scientific, Fair Lawn, NJ) before each use. Total counts and Lactobacilli were measured in cheeses at the

end of the 12th week of aging after gas flushing. Cheese microflora were enumerated by emulsifying 11 g of aseptically obtained cheese samples in 2% (w/v) trisodium citrate buffer at 45°C (pH 8.75), and serially diluted with 0.2% (w/v) peptone and plated on four sets of Petri dishes. M17 agar (Difco, Detroit, MI) containing 0.5% (w/v) lactose, LM17 agar plates were selected to determine total counts (starter culture and NSLAB). Rogosa SL agar (Difco, Detroit, MI) plates, selective for enumeration of lactobacillus species, were used to enumerate *Lb. curvatus* contaminants, and incubated at 32°C for 5 days under anaerobic conditions.

Crystal Development

The cheeses were observed for the development of crystals after weeks 1, 2, 4, 8 and 12. All pieces of cheese were thoroughly examined for occurrence of crystals and graded based on a 1 to 10 scale (Figure 1). The location, size and intensity of crystals were recorded, along with the pH, and headspace volume of cheese packages after 1, 2, 4, 8, and 12 weeks. The observed crystals, at the end of 12 weeks of gas flushed storage, were assayed for the presence of L(+) and D(-) lactic acid using D-lactic acid/L-lactic acid enzyme test kits, according to detailed inserts (Boehringer Mannheim, Indianapolis, IN). In the presence of the D-lactate dehydrogenase (D-LDH), D-lactic acid is oxidized to pyruvate by nicotinamide-adenine dinucleotide (NAD). The oxidation of L-lactic acid requires the presence of enzyme L-lactate dehydrogenase ((L-LDH). The amount of NADH formed in the reaction is stoichiometric to the amount of L(+) and D(-) lactic acid, respectively. The increase in NADH was determined by measuring its absorbance at 340 nm using an Ultraspec 4000 spectrophotometer (Pharmacia Biotech Inc., San Francisco, CA). Data obtained were analyzed with LSD using SAS Proc GLM (SAS Institute, 1989).

RESULTS AND DISCUSSION

Gas flushing and effect on volume

In Standard cheeses, the control (no NSLAB) cheeses flushed with CO_2 and CO_2:N_2 (50:50), had 6.7% and 3.8% reductions in volume, respectively during 2 weeks of storage (Figure 2A). Similarly, for Standard cheeses inoculated with NSLAB, in packages flushed with CO_2 and CO_2:N_2, an 8.0% and 5.6% reduction in volume was observed during 2 weeks of storage, respectively (Figure 2A). The percentage reduction in headspace volume remained stable throughout the 12-week storage period. Little change was observed in the headspace volume of packages flushed with N_2 throughout the 12 weeks of storage in both Standard cheeses without and with NSLAB adjunct, respectively (Figure 2A). In UF milk cheeses, control (no NSLAB adjunct) cheeses flushed with CO_2 and CO_2:N_2 had 4.7% and 3.6% reduction in volume during two weeks of storage, respectively (Figure 2B). The UF cheeses inoculated with NSLAB and flushed with CO_2 and CO_2:N_2, had 8.3% and 7.6% reduction in volume during two weeks of storage, respectively (Figure 2B). As with the Standard cheeses, no appreciable change was observed in the volume of packages flushed with N_2 gas.

One apparent reason for the larger percentage change in headspace volume in packages of both Standard and UF cheeses inoculated with *Lb. curvatus*, compared to control cheeses could be pH. Lower pH were observed in Standard and UF cheeses (4.90 to 4.94) inoculated with *Lb. curvatus* compared to pH of Standard and UF control cheeses (5.05 to 5.12) at the end of 6 mo of aging. At low pH, the water holding capacity of casein reduces (Fox, et al., 2000) and leads to increased expulsion of serum onto the cheese surface (Walstra, 1999). Increased serum

on the cheese surface leads to increased absorption of CO_2 (Fava and Piergiovanni, 1992, Sivertsvik, et al., 2004).

Gas flushing and effect on pH

Lactic acid produced by cheese micoflora (starter and NSLAB) is the major source of H^+ ions in cheese (Cogan and Hill, 1999), while another source of H^+ ions can be carbonic acid formed when CO_2 is absorbed by cheese serum (Fava and Piergiovanni, 1992). The pH of a cheese is not affected by NSLAB alone, but also by the CO_2 used to flush the package. It was observed that pH of the Standard cheeses in packages flushed with CO_2 (5.01) or CO_2:N_2 (5.02) were significantly different ($p < 0.05$) from cheeses that were either vacuum packaged or only N_2 flushed (5.08-5.09), after 12 weeks of storage (Table 1). No significant differences were observed in the pH of Standard control cheeses that were either vacuum packaged (5.08) or N_2 gas flushed (5.09), after 12 weeks of storage (Table 1). Similarly, there were no significant differences in cheeses that were flushed with either CO_2 (5.01) or CO_2:N_2 (5.02), after 12 weeks of storage (Table 1). However, in UF cheeses, no significant differences in pH were observed in cheeses vacuum packaged or gas flushed with N_2, CO_2, or CO_2:N_2, after 12 weeks of storage (Table 1). One possible reason for lack of pH differences in UF cheeses can be the increased quantity of calcium para-caseinate in the pH range of 4.8 to 5.2 (Fox, et al., 2000). Higher level of calcium para-caseinate provides increased buffering capacity, minimizing differences in pH of cheese. The pH of all Standard and UF cheeses were in pH range of 4.90 to 5.20 (Tables 1 and 2).

Gas flushing and CLC

After storing gas flushed Standard control cheese packages for 4 weeks, small light patches of CLC (crystal intensity 7) were observed in cheeses flushed with CO_2 and N_2 (Figure

1), while no crystals were observed in vacuum packaged cheeses or cheeses flushed with CO_2:N_2. After eight weeks of storage, Standard control cheeses gas flushed with CO_2 developed a medium haze all over the cheese surfaces, while only a light haze was observed in cheeses flushed with N_2. Standard control cheeses flushed with CO_2:N_2 developed small light patches (crystal intensity 7) at the end of eight weeks storage after gas flushing (Figure 1). After 12 weeks of storage, Standard control cheeses flushed with either N_2 or CO_2 developed heavy crystals (crystal intensity 9), while medium intensity crystals (crystal intensity 8) developed on Standard control cheeses gas flushed with CO_2:N_2. No crystals were observed on vacuum packaged cheeses after 12 weeks of storage (Figure 4A).

In Standard cheese inoculated with *Lb. curvatus*, needlepoint size CLC were observed throughout the cheese matrix (inside the cheese and on the surface), even before gas flushing. The crystal intensity in Standard cheeses inoculated with *Lb. curvatus* (Figure 4B) and gas flushed with N_2, CO_2, or CO_2:N_2, increased from the size of tiny needle points to the size of pin heads and the crystal intensity increased from 2 to 6 (Figure 1) during 12 weeks of storage after gas flushing. No differences in the size and intensity of crystals were observed in Standard cheeses inoculated with *Lb. curvatus* that were flushed with different gases N_2, CO_2, or CO_2:N_2.

The size and intensity of crystals in Standard control cheeses (without NSLAB) and Standard cheeses with *Lb. curvatus* were different throughout aging. The CLC observed on Standard control cheeses were more of a surface phenomena, with crystals tending to develop on edges and in depressions of cheese surfaces. A CLC intensity of eight was observed in Standard control cheeses after 12 weeks of storage after gas flushing. In the case of Standard cheese with *Lb. curvatus*, CLC were observed both on surfaces and inside the cheeses and the CLC observed were pin head size, with a CLC intensity of four after 12 weeks of storage after gas flushing,

explaining the lighter intensity of CLC observed on the surface. Therefore, after 12 weeks of storage fewer crystals were observed on the surfaces of Standard cheeses with *Lb. curvatus* compared to control cheeses that were gas flushed.

After storing gas flushed UF control cheese packages for 4 weeks, small light patches of CLC (crystal intensity 7) were observed in cheeses flushed with N_2, CO_2, and CO_2:N_2 while no crystals were observed in vacuum packaged cheeses. The CLC intensity in UF control cheeses increased from small light patches to medium haze (crystal intensity 8) in cheese flushed with CO_2, N_2 and CO_2:N_2 at the end of eight weeks. At the end of 12 weeks of storage, heavy crystals (crystal intensity 10) were observed on all gas flushed cheeses (CO_2, N_2 or CO_2:N_2). No crystals were observed on vacuum packaged cheeses after 12 weeks of storage (Figure 5A). The CLC observed in UF control cheeses gas flushed with N_2, CO_2, or CO_2:N_2 were surface phenomena and crystals were not observed inside the cheese matrix.

In UF cheeses inoculated with *Lb. curvatus*, small needle point size (crystal intensity 2) CLC were observed throughout the cheese matrix, before gas flushing. The crystal intensity in UF cheeses, inoculated with *Lb. curvatus* and gas flushed increased from size of light needle point (crystal intensity 2) to size of large pin head (crystal intensity 6) during 12 weeks of storage (Figure 5B). No differences in the size and intensity of crystals were observed in cheeses that were flushed with CO_2, N_2 or CO_2:N_2. The lighter surface intensity of CLC in UF cheeses inoculated with *Lb. curvatus* can be explained by the fact that CLC were distributed both inside and on the surfaces of cheeses.

The size and intensity of CLC in vacuum packaged Standard cheese with *Lb. curvatus* remained the same (crystal intensity 2) throughout 12 weeks of storage. UF control cheeses and UF cheeses inoculated with *Lb. curvatus* that were gas flushed had higher crystal intensity than

to Standard control cheeses and Standard cheeses inoculated with *Lb. curvatus*. A CLC intensity of ten was observed in UF control cheese compared to CLC intensity of nine in Standard control cheese (Figure 4A, 5A), while CLC intensity of six was observed in UF cheeses with *Lb. curvatus* compared to CLC intensity of four in Standard cheese with *Lb. curvatus* (Figure 4B, 5B). The increased occurrence of CLC in UF cheeses, when compared to Standard cheeses, was consistent throughout all packages that were either flushed with CO_2, N_2 or CO_2:N_2, suggesting concentrated cheese milk will lead to increased intensity of crystal formation compared to Standard cheese milk. In UF milk, even though lactose was maintained at 4.8%, increased intensity of CLC was seen, substantiating the important role of calcium in formation of CLC. Concentration of milk concentrates protein and results in higher bound calcium in cheese that may contribute to greater intensity of CLC formation.

Cheese microbiology and CLC

High NSLAB counts were observed in Standard cheese (5.98 log CFU/g) and UF cheese (5.38 log CFU/g) inoculated with *Lb. curvatus* compared to control Standard (1.97 log CFU/g) and UF (1.86 log CFU/g) control cheeses after nine mo of storage (Figure 3). The CLC present in Standard and UF cheeses inoculated with *Lb. curvatus* were identified as 50:50 racemic mixtures of D(-)/L(+)-lactate crystals. High NSLAB counts (Figure 3) accounted for racemization of L(+) lactic acid to D(-) lactic acid. Crystals observed on control Standard and UF cheeses were identified as L(+)-lactate crystals, irrespective of CO_2, N_2 or CO_2:N_2 gas used to flush the cheese packages. NSLAB population such as *Lactobacillus* and *Pediococci* spp. are capable of racemizing L(+)-lactate to D(-)-lactate in cheese during aging (Thomas and Crow, 1983). NSLAB population as high as 4.0 log CFU/g are necessary in cheese to induce any appreciable increase in D(-)-lactate (Chou et al., 2003; Johnson et al., 1990b; Somers et al.,

2001). Again, the calcium salt of D(-)-lactic acid has decreased solubility than L(+)-lactic acid (Cao, et al., 2001, Kubantseva, et al., 2004). However, the presence of L(+)-lactate crystals in control Standard and UF cheeses that were gas flushed with either CO_2, N_2 or CO_2:N_2, coupled with low NSLAB counts in control Standard and UF cheeses, suggest NSLAB are not always involved in CLC formation on cheeses. This result consolidates the fact that appearance of CLC on control Standard and UF cheeses was more due to a concentration of calcium and lactate ions on the cheese surface than the presence of NSLAB.

A summary of the results and the implications of this research are compiled in Table 2. There are three possible reasons why CLC developed in gas flushed packages of Standard and UF cheeses. First, increased surface area may have led to a loss of moisture to the surrounding environment. Second, movement of free moisture from inside of cheese to surfaces may have led to increased concentration of calcium and lactate ions and third, concentration of calcium lactate on the surface of cheese due to loss of moisture from surface, may have initiated crystallization of calcium lactate.

In this research, CLC were observed in gas-flushed cheeses after one mo of storage. Thus, cheese manufacturers should plan to sell the cubed and shredded cheese well within this period instead of stocking shelves with cubed cheeses. Alternatively cheeses destined for gas flushing should not have high total solids (calcium) or low pH, as they contribute to CLC formation. Research is currently underway to study migration dynamics of calcium and lactate ions in cubed cheeses at different storage conditions. Flushing cubed cheeses with gases high in relative humidity, or reducing headspace volume are practices that should be researched further.

CONCLUSIONS

Calcium lactate crystals were observed in control cheeses that were gas flushed but not control cheeses that were vacuum packaged. L(+)-lactate crystals were observed in both Standard and UF cheeses (control), and a 50:50 racemic mixture of D(-)/ L(+)-lactate was observed in cheeses with NSLAB, indicating that NSLAB are not always necessary for CLC, but influence the form and severity of CLC. Increased CLC intensity was observed in UF cheeses compared to Standard cheeses, demonstrating the importance of available calcium to CLC formation. Gas flushing of cheese packages with different gases exhibited a significant effect on pH in Standard cheeses but not on UF cheeses. Loss of headspace gas volume was observed in both Standard and UF cheeses flushed with either CO_2 or CO_2:N_2 (50:50) when compared to cheese packages that were only flushed with N_2 gas, indicating that some of the CO_2 in the package dissolved readily in cheese serum. The lower pH observed in cheese packages flushed with either CO_2 or CO_2:N_2 compared to cheese packages that were either flushed with N_2 gas or vacuum packaged is consistent with the formation of carbonic acid.

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REFERENCES

Blake, A. J., J. R. Powers, L. O. Luedecke and S. Clark. 2005. Enhanced lactose cheese-milk does not guarantee calcium lactate crystals in finished Cheddar cheese. J. Dairy Sci. (In press 88: 4746 take H515)

Cao, X., H. J. Lee, H. S. Yun, and Y. M. Koo. 2001. Solubilities of calcium and zinc lactate in water and water-ethanol mixture. Korean J. Chem. Eng. 18:133-135.

Chou, Y. E., C. G. Edwards, L. O. Luedecke, M. P. Bates, and S. Clark. 2003. Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. J. Dairy Sci. 86:2516-2524.

Cogan, T. M. and C. Hill. 1999. Cheese Starter Cultures. Pages 193-256 *in* Cheese: Chemistry,Physics and Microbiology. Vol. 1. P. F. Fox, ed. An Aspen Publication, Gaithersburg, Maryland.

Dybing, S. T., J. A. Wiegand, S. A. Brudvig, E. A. Huang, and R. C. Chandan. 1988. Effect of processing variables on the formation of calcium lactate crystals on Cheddar cheese. J. Dairy Sci. 71(7):1701-1710.

Fava, P. and L. Piergiovanni. 1992. Carbon dioxide Solubility in foods packaged with modified atmosphere II: correlation with some chemical–physical characteristics and composition. Industrie Alimentari. 31(5):424-430.

Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2000. Fundamentals of Cheese Science. Aspen Publishers, Inc., Gaithersburg.

Hassan, A., M.E. Johonson, and J. A. Lucey. 2004. Changes in the proportion of soluble and insoluble calcium during the ripening of Cheddar cheese. J. Dairy Sci. 87:854-862

Huffman, L. M. and T. Kristoffersen. 1984. Role of lactose in Cheddar cheese manufacturing and ripening. N Z J Dairy Sci & Technol. 19(2):151-162 ill.

Johnson, M. E., B. A. Riesterer, C. Chen, B. Tricomi, and N. F. Olson. 1990a. Effect of packaging and storage conditions on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73(11):3033-3041 ill.

Johnson, M. E., B. A. Riesterer, and N. F. Olson. 1990b. Influence on nonstarter bacteria on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73(5):1145-1149.

Kubantseva, N., R. W. Hartel, and P. A. Swearingen. 2004. Factors affecting solubility of calcium lactate in aqueous solutions. J. Dairy Sci. 87:863-867.

McDowall, F. H. and A. K. R. McDowell. 1939. The white particles in mature Cheddar cheese. J. Dairy Res. 10:118-119.

Pearce, K. N., L. K. Creamer, and J. Gilles. 1973. Calcium Lactate Deposits on Rindless Cheddar Cheese. N. Z. J. Dairy Sci & Technol. 8(1):3-7.

Sharma, K. 2003. Cheese milk composition, non-starter lactic acid bacteria and aging temperature affect calcium lactate crystals in Cheddar cheese. Thesis (M.S.), Washington State Univ., 2003.

Sivertsvik, M., J. T. Rosnes, and W. K. Jeksrud. 2004. Solubility and absorption rate of carbon dioxide into non-respiring foods. Part 2: Raw fish fillets. Journal of Food Engineering. 63(4):451-458.

Somers, E. B., M. E. Johnson, and A. C. L. Wong. 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84(9):1926-1936.

Sutherland, B. J. and G. W. Jameson. 1981. Composition of hard cheese manufactured by ultrafiltration. Aust. J. Dairy Technol. 36(4):136-143 ill.

Thomas, T. D. and V. L. Crow. 1983. Mechanism of D(-)-Lactic acid formation in Cheddar cheese. N. Z. J. Dairy Sci. Tech. 18(2):131-141.

Turner, K. W. and T. D. Thomas. 1980. Lactose fermentation in Cheddar cheese and the effect of salt. N. Z. J. Dairy Sci. Tech. 15(3):265-176.

Walstra, P. 1999. The Syneresis of Curd. Pages 141-192 *in* Cheese: Chemistry,Physics and Microbiology. Vol. 1. P. F. Fox, ed. An Aspen Publication, Gaithersburg, Maryland.

Table 1. The pH[1] of Standard and UF control cheeses and Standard and UF cheeses with added *Lb. curvatus* throughout 12 weeks of storage after gas flushing at 7.2°C.

	Standard Control Cheese				**Standard Cheese with *Lb. curvatus***				**UF Control Cheese**				**UF Cheese with *Lb. curvatus***			
	Vacuum	N_2	CO_2	CO_2+N_2	Vacuum	N_2	CO_2	CO_2+N_2	Vacuum	N_2	CO_2	CO_2+N_2	Vacuum	N_2	CO_2	CO_2+N_2
Initial[2]	5.12 [a]	5.12 [a]	5.12 [a]	5.12 [a]	5.06[c]	5.06 [c]	5.06 [c]	5.06 [c]	5.06 [a]	5.06 [a]	5.06 [a]	5.06 [a]	5.03 [b]	5.03[b]	5.03[b]	5.03 [b]
Week 1	5.10 [a]	5.14 [a]	5.05 [b]	5.08 [b]	5.05 [c]	5.06 [c]	5.03 [c]	5.04 [c]	5.04 [a]	5.05 [a]	5.04 [a]	5.02 [a]	5.01 [b]	5.00[b]	4.99[b]	5.00 [b]
Week 2	5.08 [a]	5.10 [a]	5.01 [b]	5.03 [b]	5.04 [c]	5.05 [c]	5.01 [c]	5.02 [c]	5.02 [a]	5.01 [a]	5.00 [a]	4.99 [a]	4.98 [b]	4.97[b]	4.96[b]	4.98 [b]
Week 4	5.07 [a]	5.08 [a]	5.00 [b]	5.03 [b]	5.04 [c]	5.04 [c]	4.99 [d]	5.02 [c]	4.99 [a]	4.98 [a]	4.99 [a]	4.97 [a]	4.96 [b]	4.96[b]	4.95[b]	4.96 [b]
Week 8	5.07 [a]	5.08 [a]	5.02 [b]	5.02 [b]	5.03 [c]	5.04 [c]	4.98 [d]	5.01 [c]	4.97 [a]	4.99 [a]	4.97 [a]	4.97 [a]	4.94 [b]	4.97[b]	4.96[b]	4.95 [b]
Week 12	5.08 [a]	5.09 [a]	5.01 [b]	5.02 [b]	5.04 [c]	5.04 [c]	4.98 [d]	5.01 [c]	4.97 [a]	4.99 [a]	4.97 [a]	4.98 [a]	4.95 [b]	4.97[b]	4.95[b]	4.96 [b]

[1] pH expressed as the mean of three reading from vacuum packaged or gas flushed cheese in each category.

[2] Mean of three measurements after cheese was aged for 6 mo at 7.2°C.

[a, b, c, d] Means within the same category (Standard, or UF control cheeses or Standard or UF cheeses with *Lb curvatus*) not sharing common superscripts are different ($P < 0.05$).

Table 2. Summary of results and implications of calcium lactate crystals research.

		Vacuum	N_2	CO_2	CO_2:N_2
Standard Cheese	**Control**	No crystals observed	Heavy crystal cover found all over cheese. **Crystal intensity 9**	Heavy crystal cover found all over cheese. **Crystal intensity 9**	Medium Crystal cover found on the edges of cheese. **Crystal intensity 8**
	Lb. curvatus	Small needle point crystals barely visible appearing all over cheese. **Crystal intensity 2**	Pin head size crystals visible all over cheese. **Crystal intensity 4**	Pin head size crystals visible all over cheese. **Crystal intensity 4**	Pin head size crystals visible all over cheese. **Crystal intensity 3**
Implications		Avoid contamination of cheeses with NSLAB and gas flushing of cheeses having low pH (pH $\leq$ 5.1). Use vacuum for packaging cheese with pH less than 5.1. Avoid gas flushing of cheeses having NSLAB.			
UF Milk	**Control**	No crystals observed	Heavy crystal cover found all over cheese. **Crystal intensity 10**	Heavy crystal cover found all over cheese. **Crystal intensity 10**	Heavy crystal cover found all over cheese. **Crystal intensity 10**
	Lb. curvatus	Small needle point crystals barely visible appearing all over cheese. **Crystal intensity 2**	Large pin head size crystals visible all over cheese. **Crystal intensity 6**	Large pin head size crystals visible all over cheese. **Crystal intensity 6**	Large pin head size crystals visible all over cheese. **Crystal intensity 6**
Implications		Avoid contamination of cheeses with NSLAB. Use vacuum to package cheeses made from concentrated milk, and having low pH (pH $\leq$ 5.1). Avoid gas flushing of cheeses having NSLAB.			

Table 3. Proximate analysis of experimental Cheddar cheeses at day 2 compared with typical Cheddar cheese (Sharma, 2003)

Treatments	*Fat*[e] *(% wwb*[d]*)*	*Protein*[e] *(% wwb*[d]*)*	*Moisture*[e] *(% wwb*[d]*)*	*Salt*[e] *(% wwb*[d]*)*	*S/M* [e,f] *(% wwb*[d]*)*	*Lactose*[g] *(% g/100 g cheese)*
Standard cheese + 0 adjunct	34.5[a]	24.0[a]	35.1[a]	1.66[a]	4.73[a]	0.44[a]
Standard cheese + 1 adjunct (*Lb. curvatus*)	33.8[a]	24.6[a]	36.2[ab]	1.22[b]	3.36[b]	0.36[ab]
Ultrafiltered cheese + 0 adjunct	34.0[a]	25.5[b]	36.4[ab]	1.61[a]	4.44[a]	0.41[a]
Ultrafiltered cheese + 1 adjunct (*Lb. curvatus*)	34.5[a]	24.7[ab]	36.9[b]	1.16[b]	3.15[b]	0.47[a]
Typical Cheddar cheese[c]	32.0	25.0	37.0	1.50	4.05	N/A

[a, b] Means within the same category (Fat, Protein, Moisture, Salt, S/M, pH and lactose) with different superscripts significantly differ ($P < 0.05$).

[c] Kosikowski and Mistry (1997)

[d] Wet weight basis

[e] Analyzed second day after cheese manufacture

[f] Salt to moisture

[g] Analyzed first day after cheese manufacture

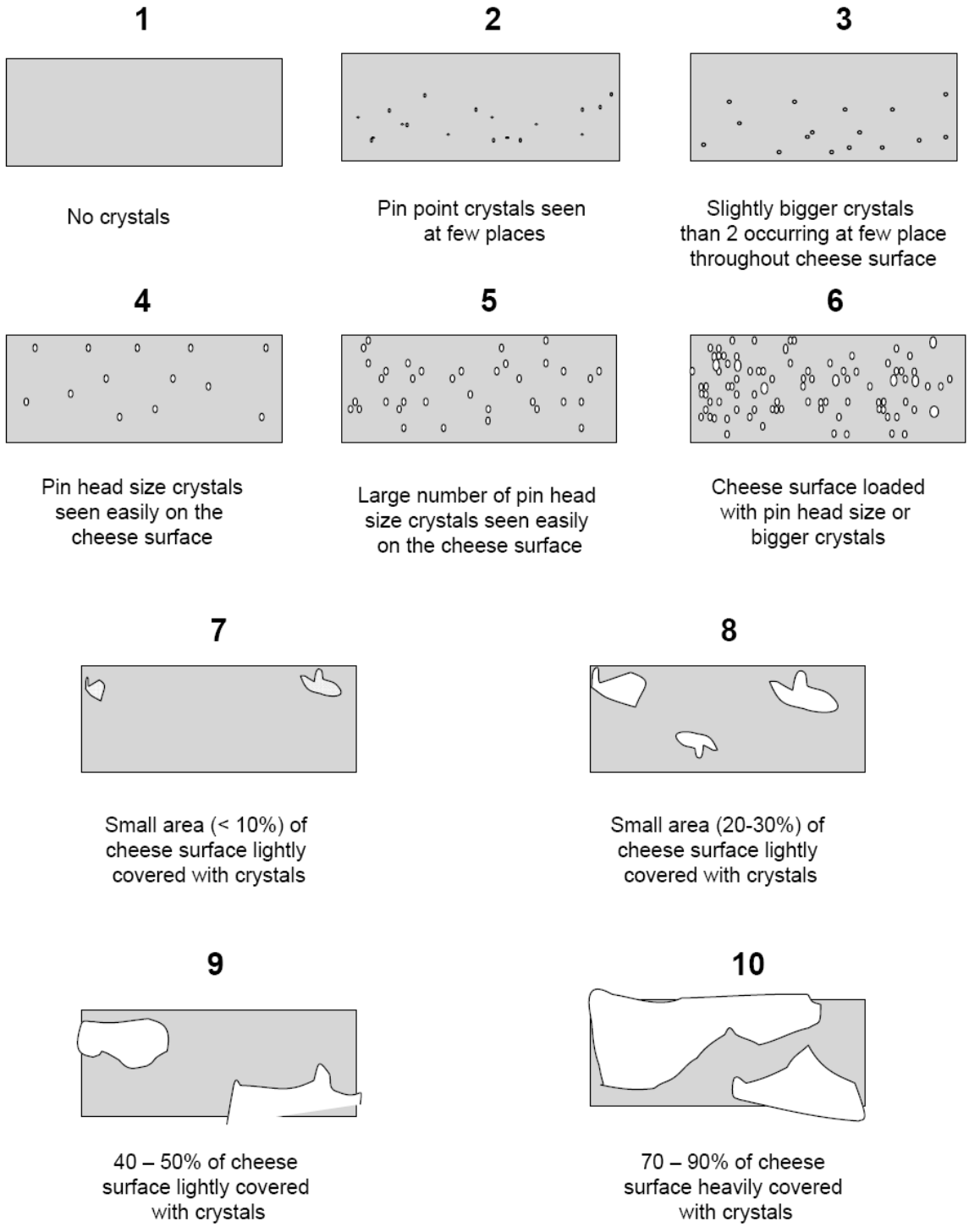


Figure 1. Chart developed for grading CLC intensity in cheeses during storage

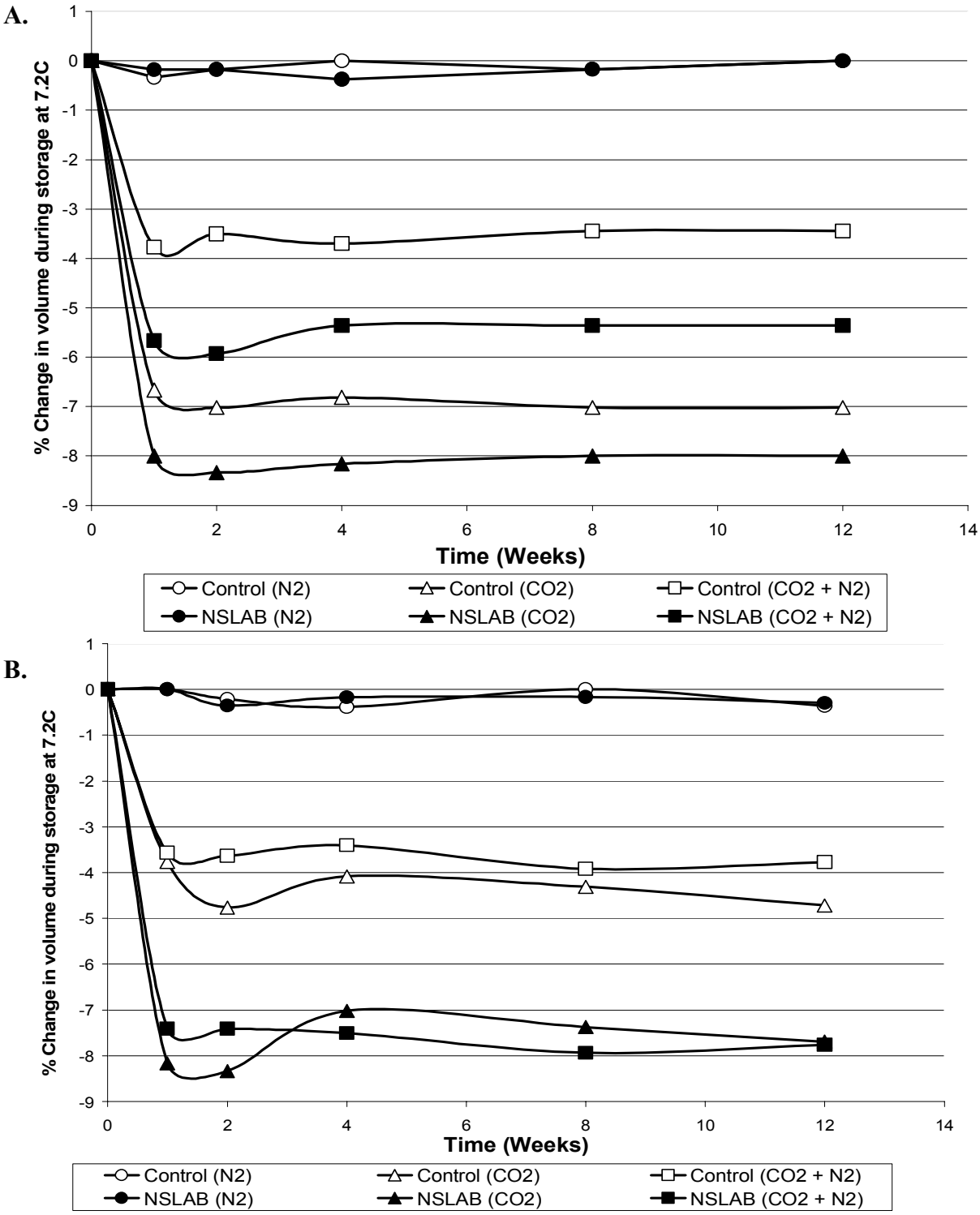
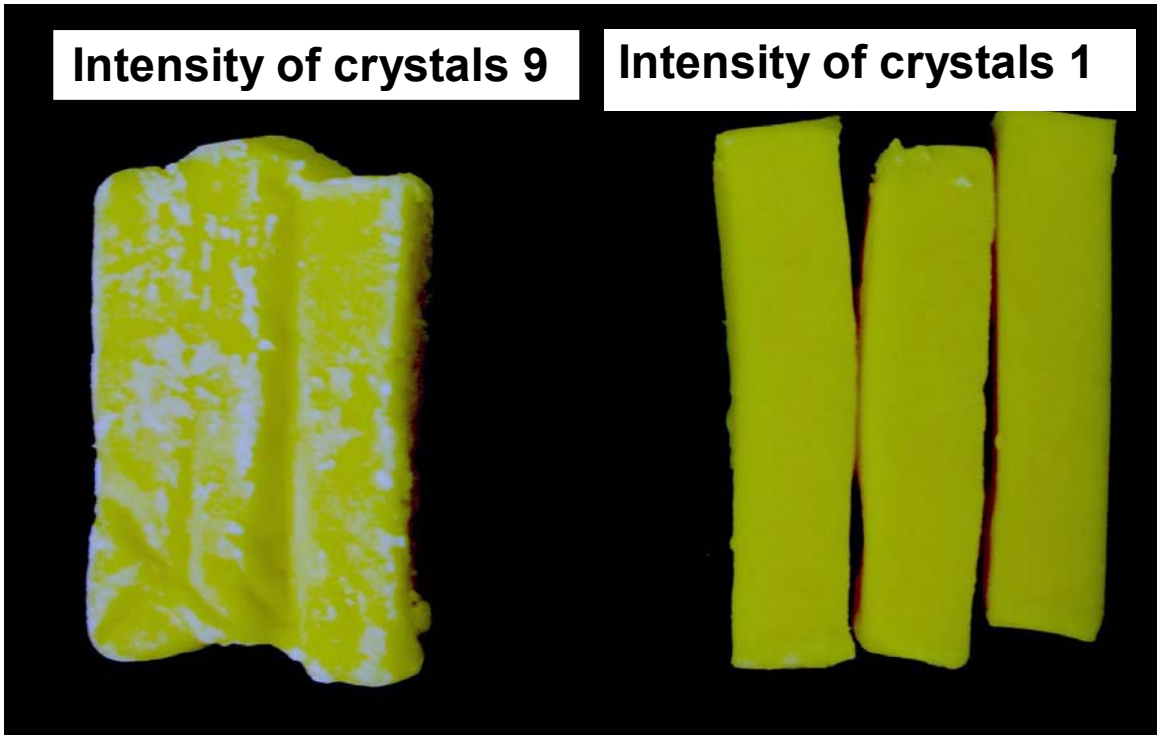


Figure 2. Percentage change in initial gas volume of packages after gas flushing of **A)** Standard cheese, **B)** UF cheese (control and cheese inoculated with *Lb. curvatus*), during 12 weeks storage at 7.2^{o}C.

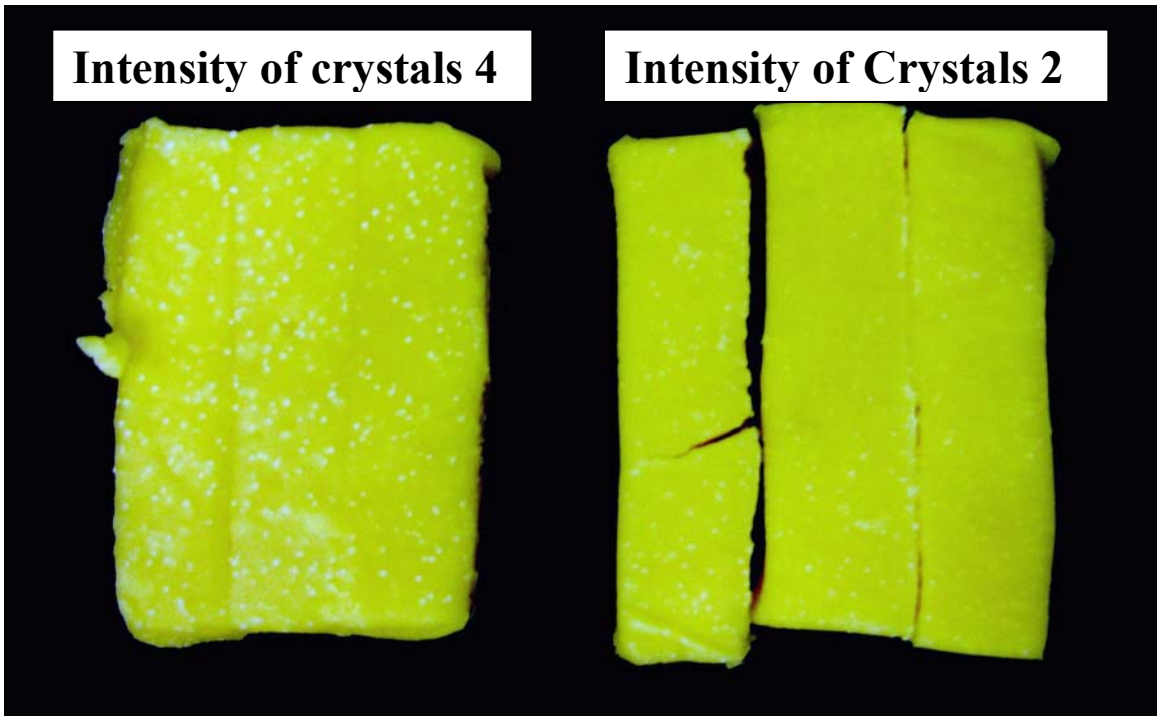
Figure 3. Microbial counts observed in Standard and UF cheeses flushed with CO_2 and stored for 12 weeks at 7.2^{o}C.

A.



B.

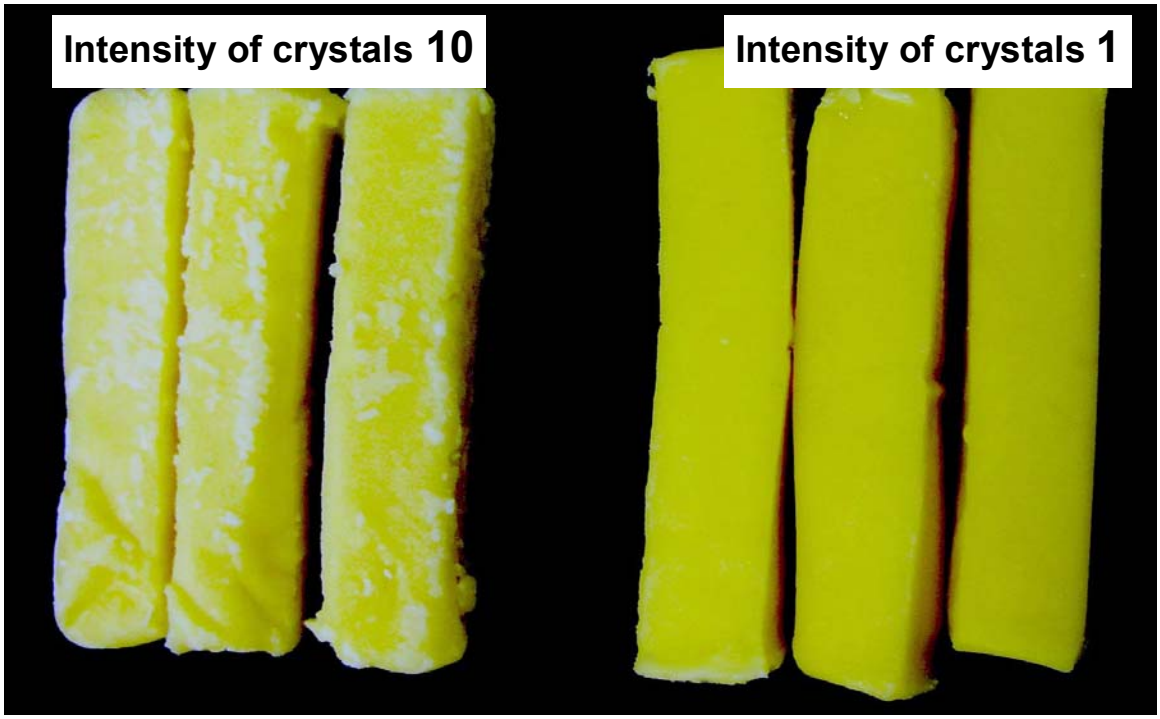
Gas Flushed Vacuum Packaged



Gas Flushed Vacuum Packaged

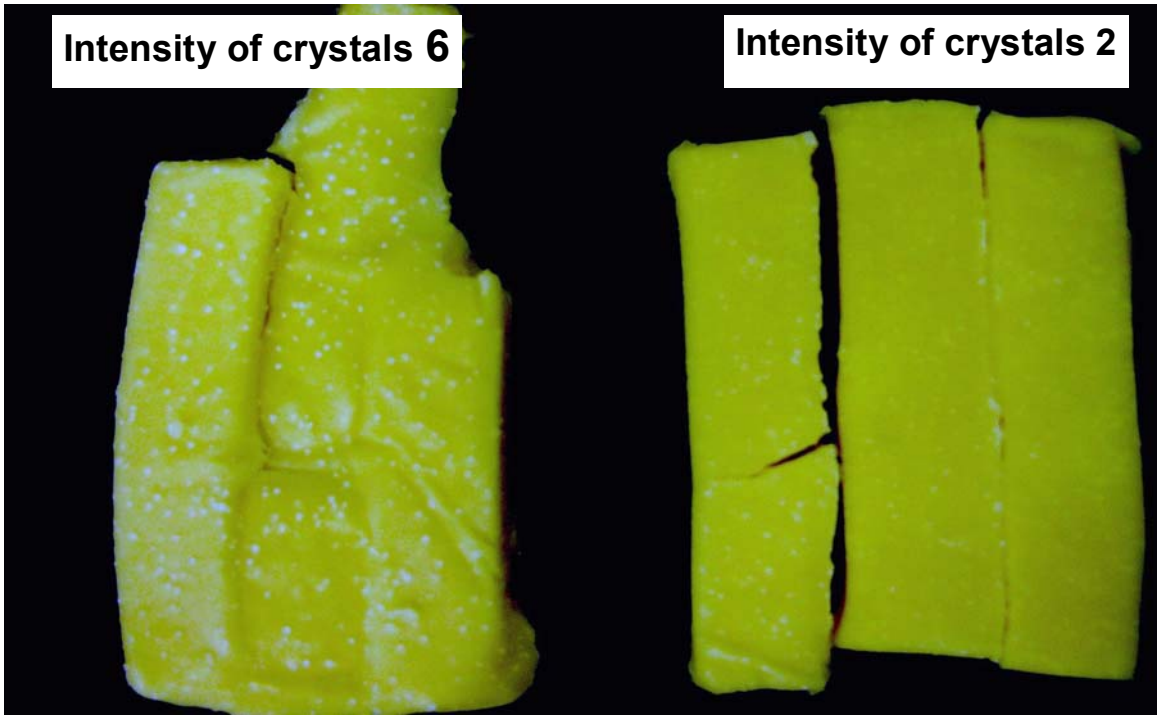
Figure 4. Comparison of CLC observed in Standard cheese that was gas flushed or vacuum packaged after 12 weeks of aging at 7.2°C **A)** Control, **B)** Cheese inoculated with *Lb. curvatus*.

A.



Gas Flushed Vacuum Packaged

B.



Gas Flushed Vacuum Packaged

Figure 5. Comparison of CLC observed in UF cheese that was gas flushed or vacuum packaged after 12 weeks of aging at 7.2°C **A)** Control, **B)** Cheese inoculated with *Lb. curvatus*.

IV. CHEESE PH, PROTEIN CONCENTRATION AND FORMATION OF CALCIUM LACTATE CRYSTALS

Agarwal, S[1]., J. R. Powers[1], B. G. Swanson[1], S. Chen[2], and S. Clark[1†]. 2006. Cheese pH, Protein Concentration, and Formation of Calcium Lactate Crystals. J. Dairy Sci. 89:4144-4155. Online. http://jds.fass.org/cgi/content/abstract/89/11/4144.

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ABSTRACT

Occurrence of calcium lactate crystals (CLC) in hard cheeses is a continual expense to the cheese industry. This research investigates the effects of protein concentration of cheese milk and pH of cheese on the occurrence of CLC. Atomic absorption spectroscopy was used to determine total and soluble calcium concentrations in skim milk (SM1, 8.7% TS), and skim milk supplemented with NFDM (CSM1, 13.5% TS). Calcium, phosphorus, lactic acid and citrate were determined in cheeses made with skim milk (SM2, 3.14% protein), skim milk supplemented with UF (CSM2, 6.80% protein) and NFDM (CSM3, 6.80% protein). Supplementation with NFDM increased the initial total calcium in CSM1 (210 mg/100 g milk) by 52%, compared to total calcium in SM1 (138 mg/100 g milk). At pH 5.4, soluble calcium concentrations in CSM1 were 68% greater than soluble calcium in SM1. In cheeses made from CSM2 and CSM3, total calcium was 26% greater than in cheeses made from SM2. As the pH of cheeses made from SM2 decreased from 5.4 to 5.1, the concentration of soluble calcium increased by

61.6%. In cheeses made from CSM2 and CSM3, the concentration of soluble calcium increased by 41.4% and 45.5%, respectively. CLC were observed in cheeses made from SM2 at and below pH 5.1, while CLC were observed in cheeses from CSM2 and CSM3 at and below pH 5.3. Increased presence of soluble calcium can be a potential for occurrence of CLC in cheese manufactured with increased concentration of milk solids particularly at and below pH 5.1.

INTRODUCTION

Quality defects in Cheddar cheeses discourage repeat purchases by consumers. White crystals on the surface of Cheddar cheese have exerted a detrimental effect on sales since the 1930s (McDowall and McDowell, 1939). The problem remains a challenge and expense to cheese manufacturers (Agarwal et al., 2005; Chou et al., 2003; Rajbhandari and Kindstedt, 2005; Swearingen, et al., 2004), with significant amounts of Cheddar cheese manufactured in US forming calcium lactate crystals (**CLC**) during aging (Johnson, 2004). The financial losses that CLC cost the cheese industry warrant research into intervention to reduce occurrence of CLC on hard and semi-hard cheeses.

CLC may be attributed to a number of variables, including differences in milk composition (Pearce et al., 1973), cheesemaking procedure (Dybing et al., 1986), aging temperature (Chou et al., 2003; Dybing et al., 1988; Johnson et al., 1990b; Pearce et al., 1973), and the growth of nonstarter lactic acid bacteria (NSLAB) in cheese during aging (Chou et al., 2003; Khalid and Marth, 1990; Somers et al., 2001). Certain NSLAB encourage development of D(–)-lactate, which is less soluble than L(+)-lactate, thus promoting CLC formation (Agarwal, et al., 2006, Chou, et al., 2003). Temperature

fluctuations during cheese aging encourage CLC (Chou et al., 2003) and both L(+)- and D(–)-lactate crystal development are stimulated by gas-flush packaging (Agarwal et al., 2005). Cleaning, sanitizing, prevention of cheese milk contamination with lactate-racemizing NSLAB, consistent storage temperatures and vacuum packaging are encouraged to minimize the occurrence of CLC (Agarwal et al., 2006; Agarwal et al., 2005; Chou et al., 2003).

Although improved sanitation and good cheese manufacturing practices are reducing the occurrence of D(–)-lactate crystals in cheese, in recent years there is increased occurrence of L(+)-lactate crystals in Cheddar cheese (Agarwal et al., 2005; Johnson, 2004; Linke, 1958; Rajbhandari and Kindstedt, 2005; Swearingen et al., 2004). Calcium from cheese milk and lactate from lactose fermentation are the principle components of CLC or more specifically calcium lactate pentahydrate ($Ca(CH_3CHOHCOO)_2.5H_2O$) (Dybing et al., 1988; Kubantseva et al., 2004; McDowall and McDowell, 1939). Increases in occurrence of CLC are attributed to changes in cheese manufacturing techniques such as use of ultrafiltration (UF), reverse osmosis, evaporated or non-fat dry milk (NFDM) to increase the total solids of cheese milk (Johnson, 2004). Calcium in milk and cheese is present in two forms, soluble (dissociated from casein) and colloidal or insoluble (associated with casein). The soluble form of calcium can readily combine with lactate to form calcium lactate. As calcium lactate concentration exceeds saturation, micro-crystals of calcium lactate are formed. Micro-crystals can accrue in size to form macro-crystals that are visible to the human eye (Dybing et al., 1988). Large concentrations of protein in cheese milk are accompanied by high colloidal calcium, and the potential for increased soluble calcium (**SC**)

concentrations in cheese. Little previous research directly relates protein concentration in cheese milk, SC, and final pH of cheese with CLC. The objective of this study is to identify the relationship between protein concentrations in cheese milk and cheese pH in formation of CLC. We hypothesize that cheeses made from cheese milk with increased protein concentrations, and having low pH, will develop more CLC than standard cheeses. The results of this research will enable us to guide cheese manufacturers in cheese making techniques and sales schedules.

METHODS AND MATERIALS

Skim milk was chosen to study the effect of milk protein concentration on the total calcium (**TC**) (sum of colloidal calcium and SC), SC and formation of CLC, to remove the variable of milk fat from the study.

Preparation of Milk and Cheese

Milk: Two different 1.0 kg batches of milk were prepared in replicate; skim milk (**SM1**) (Medow Gold Dairies, Dallas, TX) with total solids 8.7% and skim milk fortified with NFDM (**CSM1**) (Westfarm Foods, Seattle, WA) with total solids 13.5%. SM1 and CSM1 were incubated in a covered stainless steel vessel (Leedal Image Making Equipment, Chicago, IL) in a water bath (Isotemp 120, Fisher Scientific, Fair Lawn, NJ) at 30 ± 0.5^{o}C. Starter culture *Lc. lactis* ssp. *cremoris* #98 (Chr. Hansen, Milwaukee, WI) was grown to a cell density of 10^{8} cfu/ml in sterilized internal pH controlled buffer media (Vivolac, Indianapolis, IN) and inoculated into SM1 and CSM1 at a rate of 1% (w/w) at 30 ± 0.5^{o}C. SM1 and CSM1 were analyzed for TC and SC using atomic absorption

spectrometry (model SpectrAA 220, Varian Inc., Palo Alto, CA), initially and at pH 6.3, 5.8, 5.6, 5.4, 5.3, 5.2, 5.1 and 5.0.

***Cheese*:** Three different batches of milk were standardized to make skim milk cheeses, based on milk protein concentration and source of milk proteins. Skim milk cheeses were manufactured in duplicate, with each replicate made from 10.0 kg of milk, for a total of 6 cheeses. All batches of cheese were randomly distributed, with one batch of cheese made each day. The entire cheese making process lasted over one and-a-half weeks and utilized the same base lot of skim milk. **Skim milk (SM2)** (9.15% TS, and 3.14% protein), **skim milk supplemented with UF milk (CSM2)** (12.70% TS and 6.8% protein) and **skim milk supplemented with NFDM (CSM3)** (15.8% TS and 6.8% protein) were made prior to cheese making. Cheeses corresponding to SM2 (**SMC2**), CSM2 (**CSMC2**) and CSM3 (**CSMC3**) were made using standard procedures followed at the Washington State University Creamery, with slight modifications. The standardized and pasteurized cheese milk was added to a stainless steel vessel (Leedal Image Making Equipment, Chicago, IL), which was placed inside a water bath (Isotemp 120, Fisher Scientific, Fair Lawn, NJ). UF milk for standardization of CSM2 was prepared by ultrafiltration of skim milk (Medow Gold Dairies, Dallas, TX) as described by Agarwal et al. (2005). CSM3 cheese milk was prepared by slowly adding a calculated amount of NFDM (Westfarm Foods, Seattle, WA) to skim milk (Medow Gold Dairies, Dallas, TX) at room temperature in a glass beaker with continuous stirring using a magnetic stirrer. Care was taken to ensure that all the NFDM was incorporated before storing CSM3 cheese milk overnight under refrigeration. Holding milk overnight allowed hydration of NFDM and reduced any foam produced during incorporation of NFDM. Mesophillic

starter culture, *Lc. lactis* ssp. *cremoris* #98 (Chr. Hansen, Milwaukee, WI), was grown to a cell density of 10^8 cfu/ml in sterilized internal pH controlled buffer media (Vivolac, Indianapolis, IN) and inoculated into standardized milk at a rate of 1% (w/w) along with thermophillic starter culture *Streptococcus thermophilus* # STM4 (Chr. Hansen, Milwaukee, WI) at the rate of 0.1% (w/w) at 32°C. *S. thermophilus* was added to achieve the low pH during cheese making needed for this experiment. *S. thermophilus* is used by cheese manufacturers to reduce the manufacturing time of Cheddar cheese (Feagan, 1956). Double-strength coagulator (Chy-Max, Chr. Hansen Laboratories, Milwaukee, WI), diluted 1:40 with tap water, was used to assist coagulation of the milk. At the time of cutting with 6-mm cutter grid cheese knives, pH of cheese whey was 6.60±0.02 Curds were cooked by raising the temperature from 31 to 38°C at the rate of 1°C every 5 min over a 30 min period. Curds and whey were stirred at 38°C for 45 min before draining, at whey pH 6.20. The cheese was Cheddared and 60 g of cheese curd were removed from the vat at pH 5.40 ± 0.02, 5.30 ± 0.02, 5.20 ± 0.02, 5.10 ± 0.02, 5.00 ± 0.02, 4.90 ± 0.02 and 4.80 ± 0.02. At each point, the cheese curd was shredded in a food processor (Cuisinart® Model DLC -2011, Cuisinart, East Windsor, NJ) and mixed with salt (2.5% w/w of cheese curd) and erythromycin (5 mg/gm of cheese) (BP920, Fisher Scientific, Fair Lawn, NJ) to stop growth of microorganisms. Curd shreds were then pressed in a laboratory press (Carver Laboratory Press, Fred S. Carver Inc., Summit NJ) at 6000 psi for 30 min. The pH of the cheese was checked once again after pressing and adjusted with lactic acid (85% solution, J.T. Baker, Phillipsburg, NJ) to pH 5.40 ± 0.02, 5.30 ± 0.02, 5.20 ± 0.02, 5.10 ± 0.02, 5.00 ± 0.02, 4.90 ± 0.02 and 4.80 ± 0.02, respectively, as the pH of cheese increased by a factor of 0.03 to 0.06 after pressing (Table 2). The

cheese was re-shredded to enable homogenous mixture of lactic acid for uniform pH of the cheese. The cheeses were vacuum packaged (model X180, koch Supplies Inc., Kansas City, MO) in 4 x 6 cm, 3-mil high barrier Nylon/ethyl vinyl alcohol/ polyethylene vacuum pouches (Koch Supplies Inc.). The cheeses were then stored at 10^{o}C for one week before analysis for total and soluble calcium, total and soluble phosphorus and total citrate. After analysis, the cheeses were stored at -16^{o}F for later analysis of protein, salt and moisture.

Compositional Analyses

All compositional analyses were conducted in duplicate. Milk was analyzed for total solids (Marshall, 1992) and total and soluble calcium (Metzger, et al., 2001). Cheeses were analyzed for moisture (Marshall, 1992), protein was analyzed using protein analyzer (UDY Corporation, Fort. Collins CO), salt by Corning Salt Analyzer (Marshall, 1992), and pH by pH meter (Accumet® AP61, Fisher Scientific, Fair Lawn, NJ) using an Accumet electrode (serial # 208804, Fisher Scientific, Fair Lawn, NJ) calibrated with pH buffer 4 and 7 (Fisher Scientific, Fair Lawn, NJ) before each use.

Calcium Analysis: Total calcium was determined by atomic absorption spectroscopy as adapted from Metzger et al. (2000). Respectively, 0.75 and 1.5 g of milk and cheese were used. Milk and cheese were mixed with 29.25 and 45 g of 12% (wt/vol) trichloroacetic acid (J.T. Baker, Phillipsburg, NJ), respectively, and homogenized for 60 sec (Ultra-Turrax® TP-18/10S1, Cincinnati, OH). After 10 min rest, homogenates were filtered using Whatman filter paper # 4 (Whatman® international Ltd., Maidstone, England). Water soluble calcium in milk and cheese were assayed using the same technique of Metzger et al. (2001), with slight modifications in determination of SC in

cheese. To extract SC, 5 g of milk was mixed with 50 g of water (60°C) for 60 s with a homogenizer (Ultra-Turrax® TP-18/10S1, Cincinnati, OH). To extract SC, 5 g of cheese was blended with 50 g of phosphate buffer (60°C) for 60 sec. The phosphate buffer had equivalent pH as that of the cheese to prevent any changes in pH that might influence modification of the calcium equilibrium between insoluble and soluble phases (Hassan, et al., 2004). Cheeses were not analyzed using the cheese juice method (Lucey and Fox, 1993) because of the large sample size required (approximately 800 g for one analysis) (Hassan, et al., 2004). The milk and cheese slurry was then filtered using Whatman filter paper # 4 (Whatman® international Ltd., Maidstone, England). For milk, 1 g of filtrate was mixed with 9.6 g of a 3% (wt/vol) nitric acid solution (J.T. Baker, Phillipsburg, NJ) and 0.4 g of a 5% (wt/vol) lanthanum oxide solution (Sigma Aldrich, St. Louis, MO). For cheese, 1 g of filtrate was mixed with 9.6 g of a 3% (wt/vol) nitric acid solution (J.T. Baker, Phillipsburg, NJ) and 0.4 g of a 5% (wt/vol) lanthanum oxide solution (Sigma Aldrich, St. Louis, MO) and milk and cheese filtrate were further diluted 10 and 20 times, respectively, with 3% nitric acid (J.T. Baker, Phillipsburg, NJ). Diluted milk and cheese filtrate were aspirated into the atomic absorption spectrophotometer (model # SpectrAA 220, Varian Inc., Palo Alto, CA) fitted with a Varian Ca/Mg lamp (Serial # 5610107100, Varian Inc., Palo Alto, CA) for calcium determination at wavelength of 422.7 nm, in accordance with manufacturers instructions. The atomic absorption spectrophotometer was calibrated with reference standards 0, 1, 2, 4 and 5 ppm, prepared from calcium reference solution 1000±1% (SC191-100, Fisher Scientific, Fair Lawn, NJ). Reference standards contained 3% nitric acid and 0.02% wt/vol lanthanum oxide (Sigma Aldrich, St. Louis, MO).

***Phosphorus*:** Total and soluble phosphorus were determined using colorimetry (Pollman, 1991) following modifications in determination of total and soluble phosphorus in cheese. To determine non protein linked phosphorus, 1.5 g of cheese was mixed with 45 g of trichloroacetic acid (J.T. Baker, Phillipsburg, NJ) and blended for 60 sec with a homogenizer (Ultra-Turrax® TP-18/10S1, Cincinnati, OH). To extract soluble phosphate, 5 g of cheese was blended with 50 g of water for 60 sec with a homogenizer (Ultra-Turrax® TP-18/10S1, Cincinnati, OH). The cheese slurry was filtered using Whatman filter paper # 4 (Whatman® international Ltd., Maidstone, England) and 1 g of filtrate was mixed with 3 g concentrated nitric acid (J.T. Baker, Phillipsburg, NJ), to convert any organic phosphate into inorganic phosphate. Preparation of standard curve and determination of phosphorus in sample was adapted from Pollman (1991). Total and soluble phosphorus samples were analysed using a Ultrospec 4000 UV/visible spectrophotometer (Pharmacia Biotech Co., Cambridge, England).

Citrate, lactate and lactose: Citrate, lactate and lactose was measured using R-Biopharm Enzymatic Bioanalysis test kits (Boehringer Mannheim Enzymatic Bioanalysis/ Food Analysis Test Kits, Darmstadt, Germany) following the instructions provided by the manufacturer. Intensity of CLC was measured using the scale used by Agarwal et al. (2005). White crystals on the surface of cheeses were analyzed for lactic acid using R-Biopharm Enzymatic Bioanalysis test kits (Boehringer Mannheim Enzymatic Bioanalysis/ Food Analysis Test Kits, Darmstadt, Germany).

Statistical Analysis

The statistical design (ANOVA) was based on a split plot experimental design (Kuehl, 2000). A 3 x 7 factorial design with 2 replications was used for statistical

analysis. The same batch of pasteurized skim milk was used for making all cheeses. Data were analyzed with LSD using SAS PROC GLM and involved total calcium, soluble calcium, non protein linked phosphorus, soluble phosphorus, lactic acid and salt as class variables. To determine correlations among different class variables (total calcium, soluble calcium, non protein linked phosphorus, soluble phosphorus, lactic acid, salt and CLC) SAS PROC CORR was used (SAS Institute, 2005). To determine correlation between pH and occurrence of CLC in cheese, the presence of crystals on cheese surfaces was recorded as one and absence of crystals on cheese surfaces was taken as zero and SAS PROC CORR Kendall Tau-b was used (SAS Institute, 2005).

RESULTS

Milk

In trial 1, the initial pH of the CSM1 (pH = 6.52) was lower than the pH of the SM1 (pH = 6.74) because of the increased total solids (Table 1). Increase in TS generally decreases pH of milk because of increased concentration of casein micelles that are negatively charged, which lead to decreases in the pH and increases in the buffering capacity of milk (Fox, 2003). The pH of the SM1 and CSM1 were not buffered to an equivalent initial pH to ensure a process similar to when a cheese maker concentrates cheese milk with milk solids. A significantly larger concentration of calcium ($P < 0.05$) was observed in CSM1 compared to SM1 (Figure 1), similar to results recorded by Lee et al. (2005). As the pH of the SM1 was lowered from initial pH 6.74 to pH 5.00, the amount of soluble calcium increased from 51.5 to 116.5 mg/100 g of milk, an increase of 126%. Similarly, as the pH of CSM1 was lowered from pH 6.52 to pH 5.00 the amount

of soluble calcium increased from 65.5 to 157.4 mg/100 g of milk, an increase of 140%. Larger TC concentrations in CSM1 (average 210 mg/100 g) compared to SM1 (average 145 mg/100 g) shows that increasing TS in milk increases the TC concentration. Increased cheese TC leads to increased SC in final cheese unless some of the SC is removed during cheese making by including steps like washing of curd. During manufacture of Cheddar cheeses, cheese curds are milled and salted between pH 5.5 and 5.4, and final pH of the cheese after manufacture can range from 5.3 to 4.9 (Fox, 1993). Increasing the total solids of SM1 from 8.7% to 13.5% by addition of NFDM increased the amount of SC in SM1 at pH 5.2 from 98.8 to 136.5 mg/100 gm of milk, an increase of 38% (Figure 1). Increased SC in serum phase of cheese can easily combine with lactate to form CLC if the concentration of SC and lactate increase above saturation (Dybing, et al., 1988). This also explains why cheeses with CLC have low pH compared to cheeses with no CLC, as lower pH in cheeses can lead to increased concentration of SC and lactate (Rajbhandari and Kindstedt, 2005).

Cheese

Significantly ($P < 0.05$) higher protein concentrations were observed in CSMC2 (48.43%) and CSMC3 (48.02%) cheeses compared to SMC (46.28%) (Table 3). Significantly higher ($P < 0.05$) lactose concentration was observed in CSMC3 compared to SMC2 and CSMC2 cheeses (Table 3). The moisture concentration in cheeses ranged from (42 to 45%), which was on the high side compared to commercial full fat cheese; but well below the legal limit of not more than 50% for skim milk cheese (FDA, 2005). Mean salt-to-moisture ratio in SMC2, CSMC2 and CSMC3 cheeses ranged from 3.5% to 4.6% (Table 3); close to 4.0% range is the aim of most cheese manufactures. Total lactic

acid (sum of L(+) and D(-) lactic acid) percentage in cheeses increased as the pH of the cheeses decreased from 5.4 to 4.8, as expected. Over 90% of the lactic acid present in cheeses was in the L(+) form. In SMC2, total lactic acid increased from 1.25 g/100g at pH 5.4 to 2.87 g/100g at pH 4.8, an increase of 130%. Similarly, in CSMC2 and CSMC3 total lactic acid increased from 1.44 and 1.47 g/100g to 3.06 and 3.15 g/100g of lactic acid; an increase of 112 and 114% respectively, as the pH of the cheese decreased from 5.4 to 4.8. Increased concentration of lactic acid (> 1.4%) in Cheddar cheese is implicated in occurrence of CLC (Johnson, 2004). In the current study, increased lactic acid concentrations (> 1.4%) were related to CLC in cheeses made only from concentrated cheese milk with increased protein concentration. CLC were observed in CSMC2 and CSMC3 at pH 5.3, with lactic acid concentration of 1.68 and 1.78%, while no CLC were observed in SMC2 at pH 5.2 even though lactic acid concentration was 1.89% (Table 3).

Total and Soluble Calcium

No significant differences were observed between mean TC in CSMC2 (1367 mg/100g) and CSMC3 (1375 mg/100g) cheeses, but both were significantly ($P < 0.05$) larger than SMC2 (1066 mg/100g) (Figure 2). Larger TC concentration in CSMC2 and CSMC3 compared to SMC2 confirms that calcium is associated with proteins in cheese milk, as the protein concentration of CSMC2 and CSMC3 were equivalent, 6.80% (Table 3). Lee et al. (2005) also showed that cheeses made from reverse osmosis treated milk contained increased protein and calcium compared to cheeses made from regular milk. The values of TC in SCM2, CSMC2 and CSMC3 were all larger than those observed by other researchers in whole milk cheeses (680 to 833 mg/100 g) (Hassan et al., 2004;

Swearingen et al., 2004). The difference can be explained by the presence of 28 to 35% fat in whole milk cheese (Fox, 1993), which reduces the TC observed in whole milk cheese by equal percentages compared to skim milk cheeses. A significant ($P < 0.05$) negative correlation (-0.60) was observed between TC and salt in all the cheeses, demonstrating that as the concentration of salt increases, TC decreases. Increased salt concentration tends to expel more moisture from cheese, leading to decreased concentration of TC and other minerals in cheese.

The SC levels in skim milk cheeses were similar to the SC values observed by Swearingen et al. (2004) (about 450 mg/100 g). No significant differences were observed in amount of SC present in CSMC2 and CSMC3 cheeses throughout the pH range of 5.4 to 4.8 (Figure 2). Significantly ($P < 0.05$) higher SC was observed throughout pH range 5.4 to 4.8 in CSMC2 and CSMC3 cheeses when compared to SMC2 cheeses (Figure 2). A significant increase in SC was observed as the pH of the cheeses decreased by 0.1 unit throughout the pH range of 5.4 to 4.8. Increase in SC with decrease in pH of cheese is relevant because pH of finished Cheddar cheese typically ranges from 5.2 to 5.0 (Swearingen, et al., 2004). In SMC2, as the pH of the cheeses decreased from pH 5.4 to 4.8, the amount of SC increased significantly ($P < 0.05$). As the pH of the cheese declined from pH 5.4 to pH 5.1, the amount of SC increased by 61.6% in SMC2, 41.4% in CSMC2 and 45.5% in CSMC3. As the pH of the experimental cheeses declined from pH 5.4 to 5.1 or lower, the amount of SC increased (Figure 2) along with increases in concentrations of lactic acid (Table 3).

Non Protein Linked and Soluble Phosphorus

Concentrations of non protein linked phosphorus in SMC2, CSMC2 and CSMC3 were lower than those reported by Lucey and Fox (1993) and Upreti et al. (2006). One possible reason for low values of total phosphorus can be the form in which phosphorus exists. Bound phosphorus can exist in two forms: organic phosphate (covalently linked to phosphoserine residues of milk protein) and bound (associated with milk protein as calcium phosphate) (Upreti and Metzger, 2006). Use of trichloroacetic acid only extracted bound calcium and not the organic phosphate, as it is difficult to solubilize organic phosphate because it is covalently linked to phosphoserine at low pH. No significant differences were observed in mean non protein linked phosphorus in CSMC2 (241 mg/100g) and CSMC3 (247 mg/100g) cheeses (Figure 3), confirming that phosphorus is associated with proteins in cheese milk, as the protein concentrations of CSMC2 and CSMC3 were equal, 6.80% (Table 1). Significantly ($P < 0.05$) higher mean non protein linked phosphorus was observed throughout the pH range from 5.4 to 4.8 in CSMC2 (241 mg/100g) and CSMC3 (247 mg/100g) compared to SMC2 (134 mg/100g) cheeses (Figure 3). Significant ($P < 0.05$) negative correlations (-0.63) were observed between non protein linked phosphorus and salt in the cheeses, showing that as the concentrations of salt increase, the concentrations of non protein linked phosphorus decrease. Increased salt concentration tends to expel more moisture from cheese leading to decreased concentration of non protein linked phosphorus in cheese.

No significant differences were observed in soluble phosphorus in CSMC2 and CSMC3 cheeses (Figure 3). No significant differences were observed in concentrations of soluble phosphorus present in CSMC2 and CSMC3 cheeses throughout pH 5.4 to 4.8.

Significantly ($P < 0.05$) greater soluble phosphorus concentrations were observed throughout pH ranging from 5.4 to 4.8 in CSMC2 and CSMC3 cheeses when compared to SMC2 cheeses (Figure 3).

In SMC2, as the pH of the cheeses decreased from pH 5.4 to 4.8, the concentration of soluble phosphorus increased significantly ($P < 0.05$). As the pH of the cheese reduced from pH 5.4 to 5.1 the concentration of soluble phosphorus increased by 18.2% in SMC2, by 10.8% in CSMC2 and by 15.4% in CSMC3. In between pH 5.0 and 4.8, no significant changes in concentrations of soluble phosphorus were observed (Figure 3). Lucey and Fox (1993) reported that virtually all the bound phosphate in milk is soluble at pH 5.0, supporting the results.

Citrate

Significantly ($P < 0.05$) greater total citrate concentrations were observed throughout pH ranging from 5.4 to 4.8 in CSMC3 compared to CSMC2 and SMC2 cheeses (Figure 4). A probable reason for smaller total citrate concentrations in CSMC2 compared to CSMC3 is the loss of citrate in permeate during ultrafiltration, while no loss of citrate takes place while manufacturing NFDM. Significant ($P < 0.05$) differences were observed in total citrate between CSMC2 and SMC2 cheeses (Figure 4), due to concentrations of a portion citrate that might be adsorbed to casein during ultrafiltration.

pH and CLC

Analyses of white crystals showed total lactic acid ranged from 48.2 to 54.6% of the weight of crystals, confirming the crystals observed were CLC. As the pH of the cheeses was reduced from 5.4 to 4.8, increased occurrence of CLC was observed in skim

milk cheeses (Figure 5). The increased occurrence of CLC with the reduction in pH shows a positive relationship between increased SC and lactic acid in skim milk cheese. In SMC2, CLC were not observed in cheeses at pH 5.4, 5.3, and 5.2, but CLC were observed in cheeses at and below pH 5.1 (Figure 5). The intensity of CLC increased in cheeses with decrease in pH to an extent that cheese surfaces were covered with CLC at or below pH 5.1. Similarly, in CSMC2 and CSMC3, no CLC were observed in cheeses at pH 5.4 but CLC were observed in cheeses at or below pH 5.3 (Figure 5). Again, the intensity of CLC increased in cheeses with decrease in pH, to an extent that entire cheese surfaces were covered with CLC at and below pH 5.3. As the pH of the cheese continues to decrease, more and more colloidal calcium becomes soluble and can combine with lactic acid to form CLC. Occurrence of CLC at low pH corroborates the results obtained by Swearingen et al. (2004) and Rajbhandari and Kindstedt (2005), which concluded that cheeses with lowest pH and highest lactate concentration within the first month of aging, developed CLC. The pH of cheese is critical in formation of CLC, because pH directly influences the amount of SC and lactic acid in cheese serum.

Protein Concentration and CLC

Heavier occurrences of CLC were observed in cheeses made from CSM2 and CSM3 compared to cheeses made from SM2. CLC were observed in SMC2 in pH range of 5.1 to 4.8, while CLC were observed at pH 5.3 to 4.8 in CSMC2 and CSMC3. At equivalent pH, CLC were observed at increased intensity in cheeses manufactured with increased protein compared to cheeses made with smaller concentrations of protein. Total calcium in cheese is positively related to protein in cheese because calcium is structurally linked to caseins (Lee et al., 2005; Lucey and Fox, 1993). With the increase

in protein concentration, there was an increase in calcium, phosphate and citrate concentrations in cheese. Using techniques like ultrafiltration, reverse osmosis or addition of NFDM to cheese milk to increase protein concentration of cheese milk can lead to increased occurrence of CLC in cheeses (Agarwal et al., 2005) even at pH ranges of 5.3 to 5.0. Calcium concentration in cheese is critical to CLC formation because as the pH of the cheese continues to decrease during aging, calcium becomes soluble and can combine with lactic acid to form CLC.

DISCUSSION

The objective of the research was to study the effect of protein concentration (3.14 and 6.80%) and pH (5.4 to 4.8) on the occurrence of CLC in Cheddar cheese while keeping other factors constant. CLC were visible within a week in cheeses made from skim milk and concentrated milk having low pH. A high correlation (0.67) was observed between pH of the cheese and occurrence of CLC. A positive correlation (0.82) was observed between lactic acid and SC. As the pH of cheese decreased, increased concentrations of SC were observed. The saturation limit of L(+) lactate, as observed by Kubantseva, et al., (2004), is 4.18 g of anhydrous calcium lactate at 10^{o}C in 100 g of water at pH 5.00. The amount of SC necessary to exceed the critical limit is 766.97 mg in 100 g of water. In skim milk cheeses having 42 to 45% moisture, only 322 to 345 mg of calcium is required to reach saturation, while in full fat cheese having 36 to 38% moisture only 276 to 291 mg is required to reach saturation. The concentrations of SC in the experimental cheeses at all pH exceeded the theoretical saturation concentration of SC to form CLC, yet we did not see CLC in some of the cheeses. One possible reason for absence of crystals in some of the cheeses is the reaction of part of the SC with

soluble phosphates and/or citrates and a subsequent reduction in the concentration of SC available to combine with lactate to form CLC. Most of the soluble phosphorus in milk and cheese exist in several forms of phosphates (Gaucheron, 2005; Upreti, and Metzger, 2006). In SMC at pH 5.1, CLC were observed (Figure 5). The cheese contained about 480 mg of SC and about 65 mg of soluble phosphorus per 100 g of cheese (Table 3). It is assumed that much of the calcium combined with phosphorus (present in the form of phosphate, PO_4^{3-} equivalent to ~2.1 mmole), to form calcium mono-hydrogen phosphate ($CaHPO_4$). Since 2.1 mmole of phosphate will combine with 2.1 mmole of calcium, equivalent to 83.9 mg/100 g of cheese, 396.1 mg of SC are available, which is higher than the theoretical saturation concentration of calcium (322 to 345 mg/100 gm cheese at 42 to 45% moisture) to form CLC (Figure 2). In CSMC2 at pH 5.4, no CLC were observed (Figure 5). The cheese contained about 432 mg of SC and about 92.5 mg of soluble phosphorus per 100 g of cheese (Table 3). It is assumed that much of the calcium combined with phosphorus (as described above), to form calcium mono-hydrogen phosphate. Since 3 mmole of phosphate will combine with 3 mmole of ionic calcium, equivalent to 120 mg/100 g of cheese, 312 mg of SC remain to combine with lactate, which is less than the theoretical saturation concentration of calcium (322 to 345 mg/100 gm cheese at 42 to 45% moisture) to form CLC.

It is rare to identify a cheese manufacturer concentrating the cheese milk to 6.8% protein; most cheese manufacturers concentrate cheese milk to 3.8% to 5.0% protein to increase the throughput of their cheese plant. This research shows that increasing the protein concentration increases the risk of occurrence of CLC at low pH; follow-up research needs to be done with larger batches of cheeses using whole milk.

Crystal formation is influenced by lactate and calcium ions in the cheese serum. Additionally, the presence of nucleation sites inside and/or on the cheese, such as dead cells of microorganisms and crevices, promote growth of crystals. Nucleation sites enable a smaller energy requirement for development of crystals (Hartel, 2001). Also, CLC formation in cheese is influenced by other factors such as lactose concentration in cheese milk, starter bacteria, NSLAB, salt to moisture, storage temperature and packaging (Agarwal et al., 2006; Agarwal et al., 2005; Blake et al., 2005; Chou et al., 2003; Johnson et al., 1990a; Johnson et al., 1990b; Swearingen et al., 2004). The above-mentioned factors affect the concentration of SC and lactate in cheese. For instance, increased starter or NSLAB activity lead to low pH, leading to increased concentrations of SC and lactate. Less salt-to-moisture ratio in cheese promotes growth of starter and NSLAB bacteria. Solubility of calcium lactate is positively related to storage temperature (Chou et al., 2003; Kubantseva et al., 2004). Warmer storage temperatures increase solubility of calcium lactate, while solubility of calcium lactate decreases at colder temperatures (Kubantseva et al., 2004). Warmer storage temperatures promote increased starter and NSLAB activity (Agarwal et al., 2006), again leading to lower pH. Loose packaging or packaging with poor barrier properties will promote surface drying of cheese, increasing the concentrations of lactate and SC, leading to formation of CLC (Agarwal, et al., 2005). Factors such as lactose concentration in cheese milk, starter bacteria, NSLAB, salt-to-moisture ratio, storage temperature and packaging promote the underlying cause of occurrence of CLC, that is large concentration of SC and lactate in cheese serum. Concentrating the cheese milk or making cheeses with low pH favor the occurrence of CLC in cheese even when few NSLAB are present in cheese.

CONCLUSIONS

Protein concentrations in cheese milk and pH of cheese positively impacted the appearance of CLC on cheese. Concentrations of lactate and SC increased as the pH of cheese decreased. CLC were observed in skim milk cheeses at or below pH 5.1 and in cheeses made from concentrated skim milks at and below pH 5.3. The occurrence of calcium L(+) lactate crystals in Cheddar cheese is enhanced by large protein concentrations in cheese milk and low post-manufacture pH of cheese, which lead to greater concentrations of calcium and lactate ions in cheese serum. Cheese manufactures can reduce the occurrence of CLC in cheeses by focusing on cheese making techniques that reduce the concentration of TC and SC in final cheese, which can be done by setting milk at low pH and introducing a curd washing step.

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REFERENCES

Agarwal, S., B. G. Swanson, G. U. Yuksel, and S. Clark. 2006. Non-starter lactic acid bacteria biofilms and calcium lactate crystals in Cheddar cheese. J. Dairy Sci. 89:1452-1466.

Agarwal, S., M. Costello, and S. Clark. 2005. Gas-flushed packaging contributes to calcium lactate crystals in Cheddar cheese. J. Dairy Sci. 88:3773-3783.

Blake, A. J., J. R. Powers, L. O. Luedecke, and S. Clark. 2005. Enhanced lactose cheese-milk does not guarantee calcium lactate crystals in finished Cheddar cheese. J. Dairy Sci. 88:2302-2311.

FDA, Code of Federal Regulations. Title 21, Section 133.189. 2005. Skim Milk Cheese for Manufacturing. Page 335. U.S. Government Printing Office, Pittsburgh , PA.

Chou, Y. E., C. G. Edwards, L. O. Luedecke, M. P. Bates, and S. Clark. 2003. Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. J. Dairy Sci. 86:2516-2524.

Dybing, S. T., S. A. Brudvig, J. A. Wiegand, and E. A. Huang. 1986. A simple method for estimating the extent of surface crystal development on colored Cheddar cheese. J. Food Prot. 49:421-422.

Dybing, S. T., J. A. Wiegand, S. A. Brudvig, E. A. Huang, and R. C. Chandan. 1988. Effect of processing variables on the formation of calcium lactate crystals on Cheddar cheese. J. Dairy Sci. 71:1701-1710.

Feagan, J. T. 1956. A modification to the short method for Cheddar cheese manufacture. Aust J Dairy Technol. October-December: 149-153.

Fox, P. F. 1993. Cheese : chemistry, physics and microbiology. 2nd ed. Chapman & Hall, London ; New York.

Gaucheron, F. 2005. The minerals of milk. Reprod. Nutr. Dev. 45:473-483.

Hartel, R. W. 2001. Crystallization in foods. Aspen Publishers, Gaithersburg, Md.

Hassan, A., M. E. Johnson, and J. A. Lucey. 2004. Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. J. Dairy Sci. 87:854-862.

Johnson, M. E. 2004. Calcium lactate crystals. International Cheese Technology Exposition, Madison, WI.

Johnson, M. E., B. A. Riesterer, C. Chen, B. Tricomi, and N. F. Olson. 1990a. Effect of packaging and storage conditions on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:3033-3041 ill.

Johnson, M. E., B. A. Riesterer, and N. F. Olson. 1990b. Influence on nonstarter bacteria on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:1145-1149.

Khalid, N. M. and E. H. Marth. 1990. Lactobacilli--their enzymes and role in ripening and spoilage of cheese: a review. J. Dairy Sci. 73:2669-2684.

Kubantseva, N., R. W. Hartel, and P. A. Swearingen. 2004. Factors affecting solubility of calcium lactate in aqueous solutions. J. Dairy Sci. 87:863-867.

Kuehl, R. O. 2000. Design of Experiments: Statistical Principles of Research Design and Analysis. 2nd ed. Duxbury Press, Pacific Grove, CA

Lee, M. R., M. E. Johnson, and J. A. Lucey. 2005. Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. J. Dairy Sci., 88:3798-3809.

Linke, W. F. 1958. Solubilities of Inorganic and Metal Organic Compounds. *in* Am. Chem. Soc. 4th ed. W. F. Linke, ed, Washington, DC.

Lucey, J. A. and P. F. Fox. 1993. Importance of calcium and phosphate in cheese manufacture: a review. J. Dairy Sci. 76:1714-1724.

Marshall, R. T. 1992. Standard methods for the examination of dairy products. 16th ed. American Public Health Association, Washington, DC.

McDowall, F. H. and A. K. R. McDowell. 1939. The white particles in mature Cheddar cheese. J. Dairy Res. 10:118-119.

Metzger, L. E., D. M. Barbano, and P. S. Kindstedt. 2001. Effect of milk preacidification on low fat Mozzarella cheese. III. Post-melt chewiness and whiteness. J. Dairy Sci. 84:1357-1366.

Metzger, L. E., D. M. Barbano, M. A. Rudan, and P. S. Kindstedt. 2000. Effect of milk preacidification on low fat Mozzarella cheese. I. Composition and yield. J. Dairy Sci. 83:648-658.

Pearce, K. N., L. K. Creamer, and J. Gilles. 1973. Calcium Lactate Deposits on Rindless Cheddar Cheese. N. Z. J. Dairy Sci. Technol. 8:3-7.

Pollman, R. M. 1991. Atomic absorption spectrophotometric determination of calcium and magnesium and colorimetric determination of phosphorus in cheese: collaborative study. J. AOAC. 74:27-31.

Rajbhandari, P. and P. S. Kindstedt. 2005. Compositional factors associated with calcium lactate crystallization in smoked Cheddar cheese. J. Dairy Sci. 88:3737-3744.

SAS Institute. 2005. SAS User's Guide. Version 9.1. SAS Inst., Inc., Cary, NC.

Smit, L. E., H. C. Schonfeldt, W. H. J. De Beer, and M. F. Smith. 2001. The influence of factory and region on the composition of South African Cheddar and Gouda cheese. J. Food Comp. Anal. 14:177-198.

Somers, E. B., M. E. Johnson, and A. C. L. Wong. 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84:1926-1936.

Swearingen, P. A., D. E. Adams, and T. L. Lensmire. 2004. Factors affecting calcium lactate and liquid expulsion defects in Cheddar cheese. J. Dairy Sci. 87:574-582.

Upreti, P. and L. E. Metzger. 2006. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: manufacture and composition. J. Dairy Sci. 89:420-428.

Table 1. Composition of skim milk (SM1), skim milk supplemented with NFDM (CSM1), milk used for making cheeses, skim milk (SM2) and skim milk supplemented with UF milk (CSM2) and skim milk supplemented with NFDM (CSM3) (means of replicate ± SD).

Milk Type	Total solids %	Protein %	Lactose %
SM1	8.7 ± 0.16	2.98 ± 0.06	4.68 ± 0.12
CSM1	13.5 ± 0.18	5.78 ± 0.14	6.84 ± 0.18
SM2	9.1 ± 0.12	3.14 ± 0.08	4.72 ± 0.14
CSM2	12.7 ± 0.08	6.80 ± 0.16	4.95 ± 0.28
CSM3	15.8 ± 0.07	6.84 ± 0.03	7.84 ± 0.16

Table 2. pH of cheese after pressing, pH of cheese after addition of lactic acid and approximate volume of lactic acid added to attain the target pH.

Cheese	Desired pH	pH after Pressing	Final pH	Lactic acid added (85% wt/vol) (ml)
CSM2 Batch 1	5.4	5.46	5.39	0.38
	5.3	5.33	5.29	0.26
	5.2	5.24	5.20	0.24
	5.1	5.12	5.10	0.07
	5.0	5.05	5.01	0.24
	4.9	4.95	4.90	0.25
	4.8	4.85	4.80	0.22
CSM2 Batch 2	5.4	5.42	5.40	0.10
	5.3	5.35	5.30	0.32
	5.2	5.23	5.19	0.24
	5.1	5.16	5.11	0.19
	5.0	5.05	4.99	0.36
	4.9	4.94	4.91	0.15
	4.8	4.86	4.79	0.30
CSMC2 Batch 1	5.4	5.46	5.38	0.48
	5.3	5.32	5.31	0.05
	5.2	5.25	5.18	0.15
	5.1	5.13	5.10	0.21
	5.0	5.06	5.10	0.30
	4.9	4.95	4.90	0.40
	4.8	4.87	4.81	0.22
CSMC2 Batch 2	5.4	5.46	5.39	0.46
	5.3	5.35	5.30	0.24
	5.2	5.26	5.21	0.11
	5.1	5.13	5.11	0.14
	5.0	5.04	5.00	0.24
	4.9	4.90	4.90	0.00
	4.8	4.84	4.78	0.22
CSMC3 Batch 3	5.4	5.43	5.41	0.12
	5.3	5.34	5.28	0.31
	5.2	5.25	5.20	0.18
	5.1	5.15	5.09	0.29
	5.0	5.03	5.01	0.16
	4.9	4.93	4.91	0.12
	4.8	4.81	4.81	0.00
CSMC3 Batch 3	5.4	5.44	5.40	0.24
	5.3	5.33	5.31	0.10
	5.2	5.21	5.21	0.00
	5.1	5.11	5.11	0.00
	5.0	5.04	4.99	0.39
	4.9	4.93	4.89	0.24
	4.8	4.86	4.80	0.34

Table 3. Composition of cheeses made from skim milk (SM2) and skim milk supplemented with UF milk (CSMC2) and NFDM (CSMC3) (means of replicates ± SD).

SMC2							
Protein %	46.28 ± 0.75						
pH	**5.4**	**5.3**	**5.2**	**5.1**	**5.0**	**4.9**	**4.8**
Salt %	1.83 ± 0.00^{a}	1.88 ± 0.01^{a}	1.65 ± 0.05^{a}	1.75 ± 0.08^{a}	2.00 ± 0.12^{a}	1.70 ± 0.06^{a}	1.80 ± 0.15^{a}
Moisture %	42.03 ± 0.75^{a}	41.68 ± 0.84^{a}	42.26 ± 1.18^{a}	41.58 ± 1.27^{a}	41.78 ± 1.36^{a}	42.14 ± 1.58^{a}	42.22 ± 1.54^{a}
Salt to moisture ratio	4.36 ± 0.55^{a}	4.48 ± 0.64^{a}	3.92 ± 0.22^{a}	4.17 ± 0.15^{a}	4.77 ± 0.24^{a}	4.06 ± 0.07^{a}	4.28 ± 0.26^{a}
Lactose %	0.65 ± 0.23^{a}	0.56 ± 0.19^{ab}	0.47 ± 0.17^{b}	0.42 ± 0.08^{b}	0.34 ± 0.13^{bc}	0.27 ± 0.03^{c}	0.20 ± 0.11^{c}
Lactic acid %	1.25 ± 0.06^{a}	1.58 ± 0.02^{b}	1.89± 0.16^{c}	2.08 ± 0.23^{d}	2.39 ± 0.03^{e}	2.65 ± 0.43^{e}	2.87 ± 0.39^{f}
Total calcium (mg/100 g)	1064.5±31.5^{a}	1072.3±84.7^{a}	1081.6±27.4^{a}	1059.8±94.9^{a}	1042.6±66.4^{a}	1071.5±64.2^{a}	1071.1±86.5^{a}
Soluble calcium (mg/100g)	297.2 ± 12.2^{a}	405.0 ± 29.3^{b}	441.7 ± 18.5^{c}	480.2 ± 36.0^{d}	497.9 ± 36.4^{e}	508.5 ± 24.7^{f}	565.6 ± 28.2^{g}
Non protein linked phosphorus (mg/100g)	141.3 ± 1.1^{a}	134.6 ± 2.2^{a}	135.1 ± 9.0^{a}	135.2 ± 3.9^{a}	132.8 ± 9.8^{a}	129.8 ± 11.1^{a}	129.0 ± 6.3^{a}
Soluble phosphorus (mg/100g)	55.0 ± 0.4^{a}	57.8 ± 0.3^{b}	61.9 ± 1.3^{c}	65.2 ± 2.5^{d}	66.1 ± 4.8^{e}	68.7 ± 6.6^{e}	66.6 ± 4.8^{e}
CSMC2							
Protein %	48.43 ± 0.44						
pH	**5.4**	**5.3**	**5.2**	**5.1**	**5.0**	**4.9**	**4.8**
Salt %	1.67 ± 0.03^{a}	1.54 ± 0.01^{a}	1.75 ± 0.08^{a}	1.76 ± 0.02^{a}	1.72 ± 0.04^{a}	1.82 ± 0.11^{a}	1.62 ± 0.04^{a}
Moisture %	44.53 ± 1.38	43.11 ± 1.52	43.41 ± 1.65	45.00 ± 1.45	42.77 ± 1.27	43.96 ± 1.73	43.73 ± 1.86
Salt to moisture ratio	3.74 ± 0.40^{a}	3.58 ± 0.02^{a}	4.03 ± 0.08^{a}	3.91 ± 0.04^{a}	4.02 ± 0.07^{a}	4.14 ± 0.43^{a}	3.70 ± 0.09^{a}
Lactose %	0.78 ± 0.10^{a}	0.70 ± 0.04^{a}	0.67 ± 0.16^{ab}	0.55 ± 0.06^{b}	0.45 ± 0.11^{bc}	0.32 ± 0.08^{c}	0.25 ± 0.09^{c}
Lactic acid %	1.44 ± 0.21^{a}	1.68 ± 0.04^{b}	1.79 ± 0.09^{c}	2.15 ± 0.07^{d}	2.46 ± 0.01^{e}	2.87 ± 0.01^{f}	3.06 ± 0.07^{g}
Total calcium (mg/100 g)	1351.8±29.3^{a}	1375.7±42.4^{a}	1376.1±26.6^{a}	1367.3±28.2^{a}	1370.3±66.3^{a}	1356.7±22.8^{a}	1371.3±52.8^{a}
Soluble calcium (mg/100g)	432.5 ± 12.2^{a}	499.0 ± 5.8^{b}	550.9 ± 18.2^{c}	610.7 ± 6.3^{d}	647.7 ± 22.2^{e}	705.4 ± 38,3^{f}	728.9 ± 11.2^{g}
Non protein linked phosphorus (mg/100g)	245.2 ± 9.4^{a}	240.4 ± 14.3^{a}	251.2 ± 16.0^{a}	243.4 ± 10.6^{a}	251.0 ± 9.8^{a}	247.6 ± 12.4^{a}	250.5 ± 11.6^{a}
Soluble phosphorus (mg/100g)	92.5 ± 2.0^{a}	92.0 ± 3.3^{a}	98.1 ± 1.7^{b}	102.3 ± 1.8^{c}	109.2 ± 6.2^{d}	110.0 ± 2.9^{d}	107.2 ± 3.4^{d}
CSMC3							
Protein %	48.02 ± 0.20						
pH	**5.4**	**5.3**	**5.2**	**5.1**	**5.0**	**4.9**	**4.8**
Salt %	1.84 ± 0.01^{a}	1.93 ± 0.06^{a}	2.01 ± 0.11^{a}	1.85 ± 0.05^{a}	1.95 ± 0.10^{a}	1.67 ± 0.04^{a}	1.93 ± 0.09^{a}
Moisture %	44.79 ± 1.03^{a}	45.99 ± 1.26^{a}	43.81 ± 1.81^{a}	43.67 ± 1.65^{a}	43.35 ± 1.94^{a}	43.59 ± 1.79^{a}	42.47 ± 0.98^{a}
Salt to moisture ratio	4.11 ± 0.52^{a}	4.19 ± 0.58^{a}	4.58 ± 0.35^{a}	4.24 ± 0.67^{a}	4.49 ± 0.62^{a}	3.84 ± 0.57^{a}	4.54 ± 0.32^{a}
Lactose %	1.68 ± 0.26^{a}	1.59 ± 0.11^{a}	1.53 ± 0.17^{ab}	1.45 ± 0.05^{b}	1.31 ± 0.07^{c}	1.21 ± 0.13^{c}	1.11 ± 0.06^{c}
Lactic acid %	1.47 ± 0.23^{a}	1.73 ± 0.08^{b}	1.91 ± 0.25^{c}	2.16 ± 0.38^{d}	2.56 ± 0.22^{e}	2.86 ± 0.52^{e}	3.15 ± 0.25^{f}
Total calcium (mg/100g)	1352.9±15.3^{a}	1374.2±63.6^{a}	1402.8±53.5^{a}	1391.0±65.6^{a}	1377.5±78.3^{a}	1373.8±103.3^{a}	1359.4±82.4^{a}
Soluble calcium (mg/100g)	420.6 ± 21.9^{a}	491.5 ± 30.7^{b}	558.5 ± 38.9^{c}	610.9 ± 35.7^{d}	629.7 ± 15.2^{e}	677.0 ± 14.0^{f}	728.5 ± 35.6^{g}
Non protein linked phosphorus (mg/100g)	241.7 ± 10.6^{a}	239.7 ± 11.6^{a}	239.8 ± 11.1^{a}	246.3 ± 5.6^{a}	250.4 ± 13.3^{a}	231.0 ± 7.7^{a}	238.4 ± 15.7^{a}
Soluble phosphorus (mg/100g)	91.2 ± 1.5^{a}	93.1 ± 1.4^{b}	94.0 ± 4.0^{b}	104.8 ± 4.5^{c}	108.1 ± 4.8^{d}	111.0 ± 3.7^{e}	111.6 ± 3.0^{e}

$^{a, b, c, d, e, f, g}$ Means within the same category (Salt, salt-in moisture, lactic acid, total calcium, soluble calcium, non protein linked phosphorus, and soluble phosphorus) sharing common superscripts are similar ($P > 0.05$).

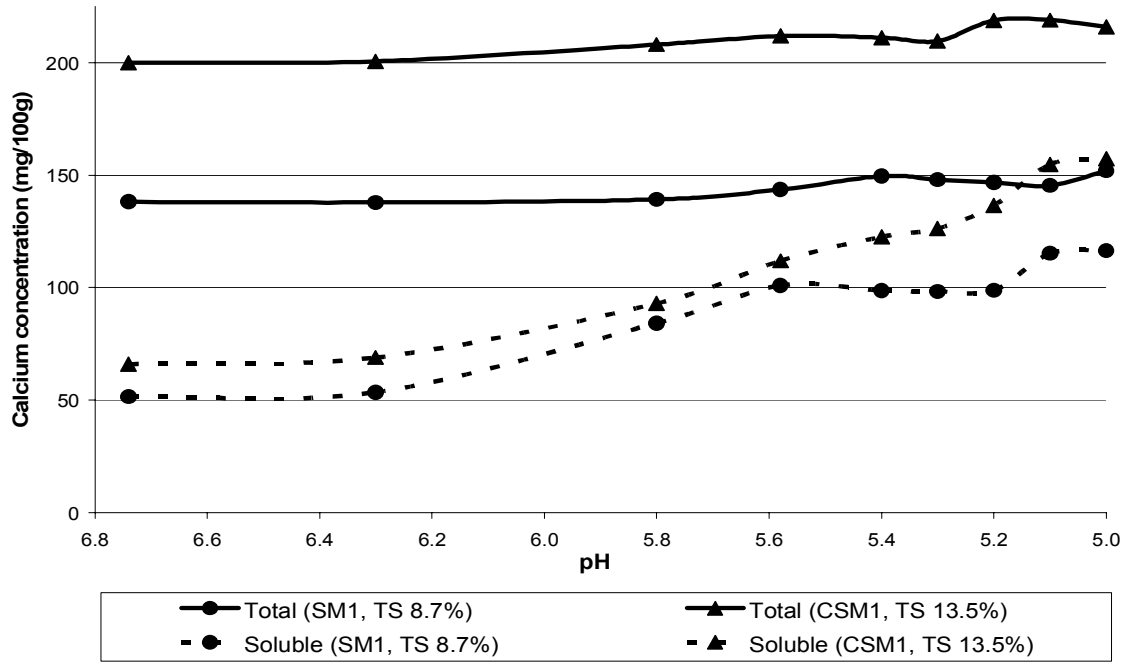

Figure 1. Total and soluble calcium in skim milk (SM1) and concentrated skim milk (CSM1) at pH 6.8 to 5.0.

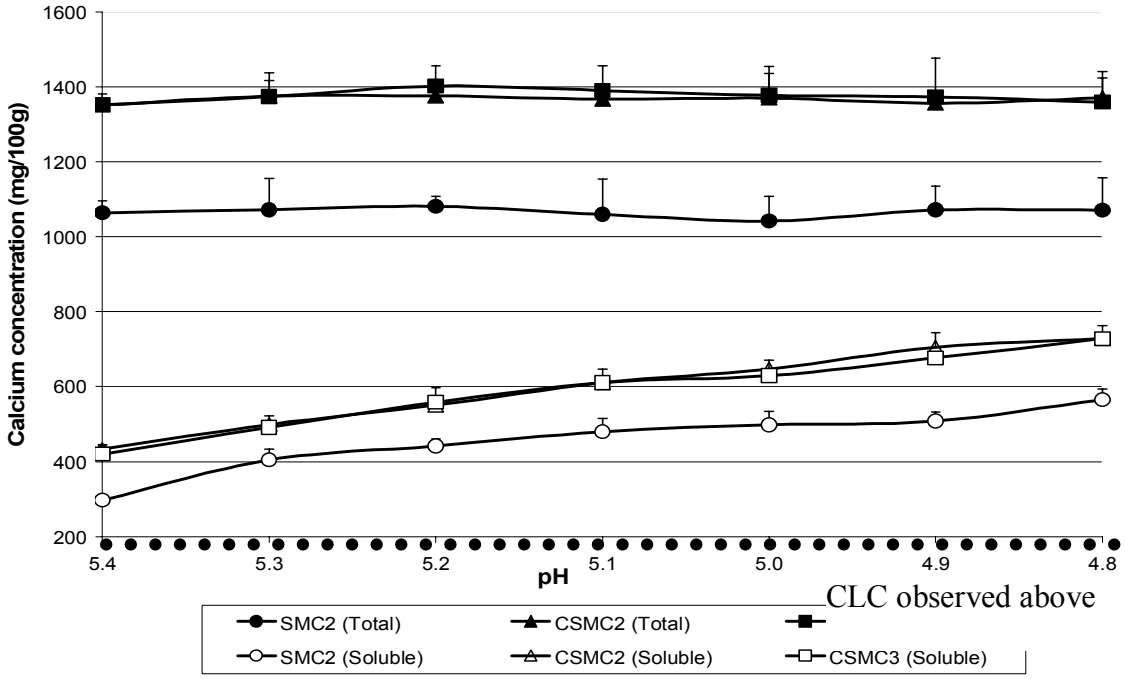

Figure 2. Total and soluble calcium in cheeses made from skim milk (SCM2), skim milk supplemented with ultrafiltered milk (CSMC2) and skim milk supplemented with NFDM (CSMC3) at pH 5.4 to 4.8.

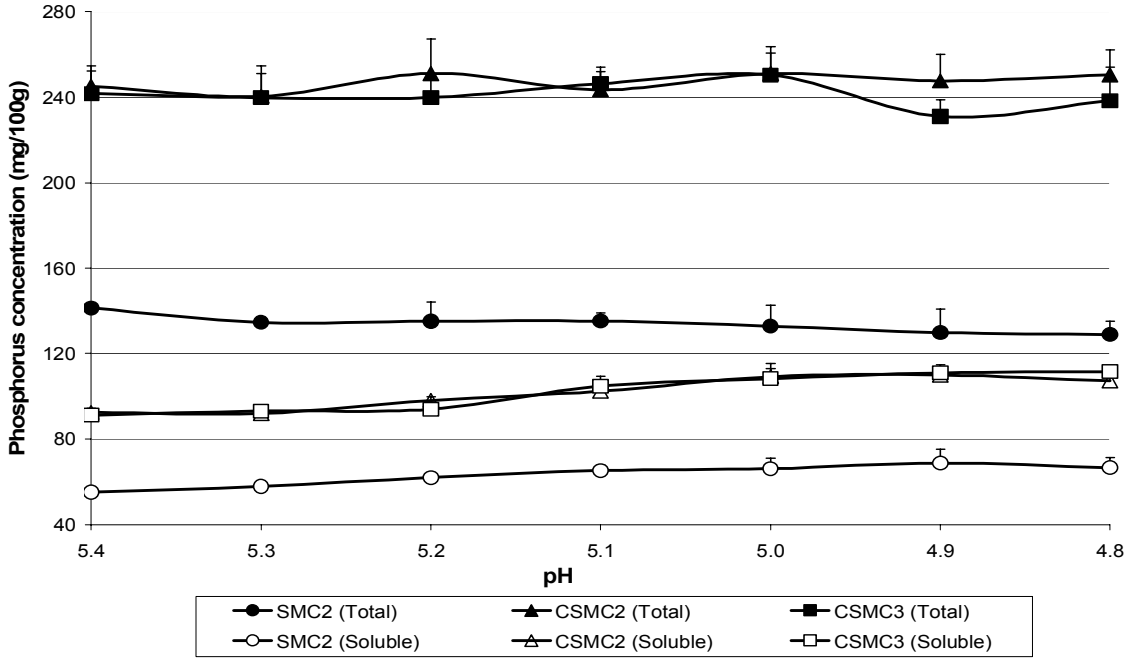

Figure 3. Non protein linked and soluble phosphorus in cheeses made from skim milk (SCM2), skim milk supplemented with ultrafiltered milk (CSMC2) and skim milk supplemented with NFDM (CSMC3) at pH 5.4 to 4.8.

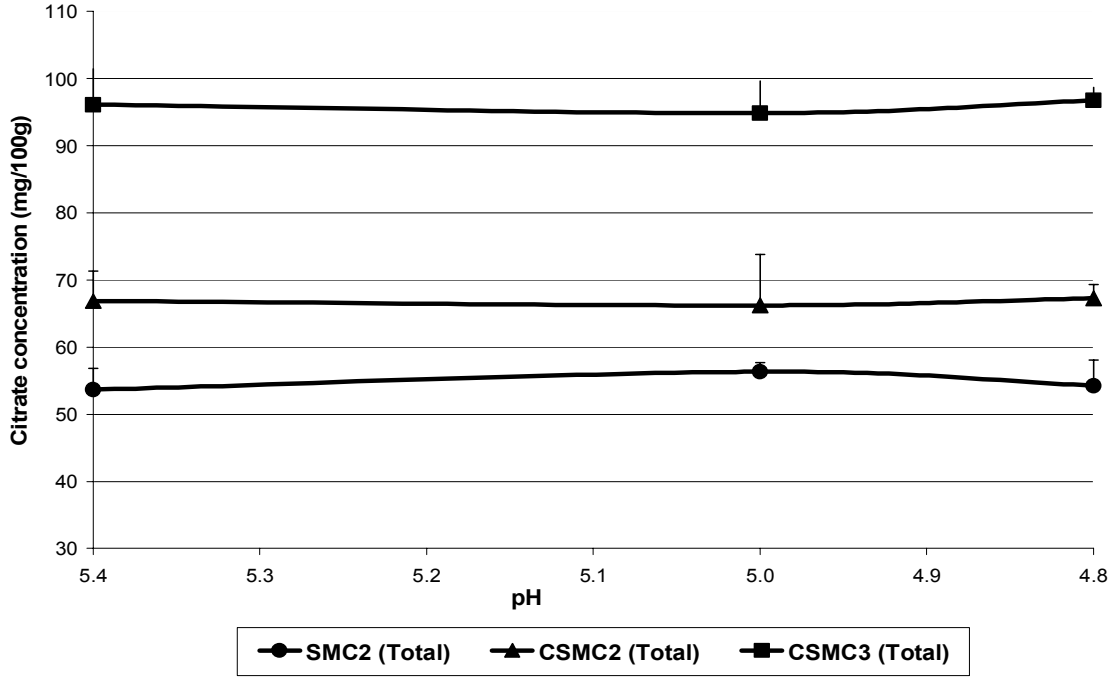

Figure 4. Citrate concentration in cheeses made from skim milk (SCM2), skim milk supplemented with ultrafiltered milk (CSMC2) and skim milk supplemented with NFDM (CSMC3) at pH 5.4 to 4.8.

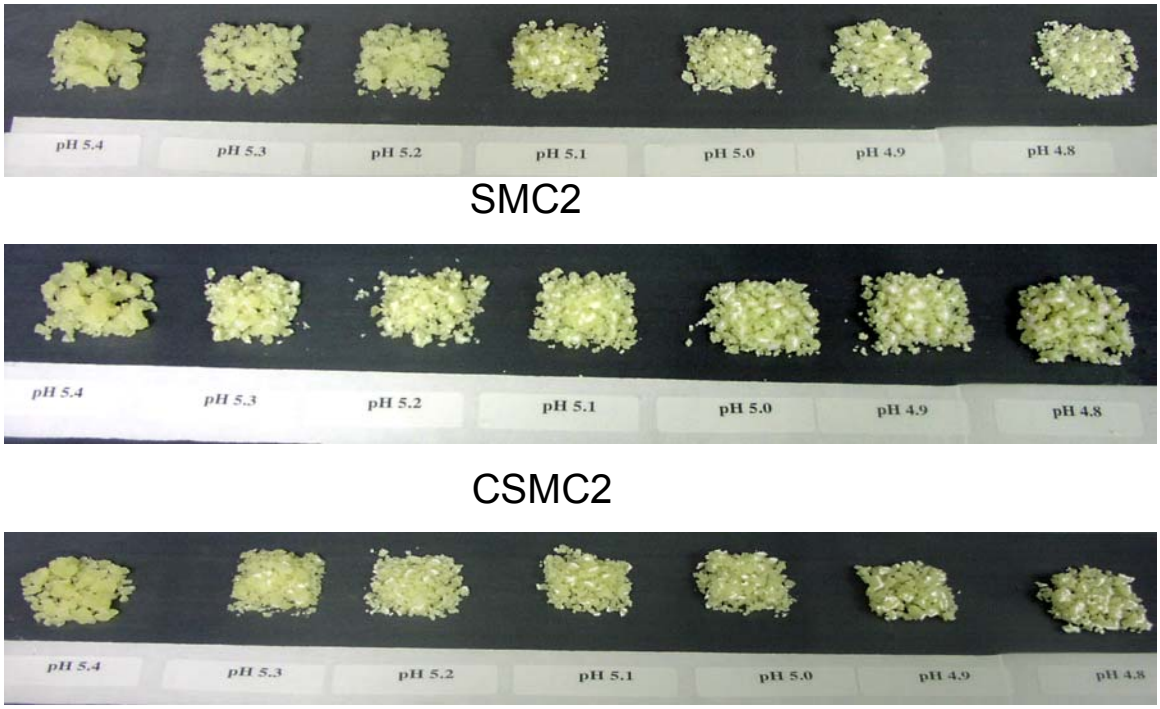

Figure 5. Calcium lactate crystals (white bright areas on the surface of cheese) in cheeses made from skim milk (SCM), skim milk supplemented with ultrafiltered milk (CSMC2) and skim milk supplemented with NFDM (CSMC3) at pH 5.4 to 4.8.

V. INFLUENCE OF SALT TO MOISTURE RATIO ON STARTER BACTERIA AND CALCIUM LACTATE CRYSTAL FORMATION

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ABSTRACT

Occurrence of L(+)-lactate crystals in hard cheeses continues to be an expense to the cheese industry. Salt tolerance of starter bacteria and salt to moisture ratio (S/M) in cheese dictate final pH of cheese, which influences calcium lactate crystals (CLC) formation. The research investigates these interactions on incidences of CLC.

A commercial starter was selected based on its sensitivity to salt less than and greater than 4.0 S/M. Cheddar cheese was made using either whole milk (3.25% protein, 3.85% fat) or whole milk supplemented with cream and ultrafiltered milk (4.5% protein, 5.3% fat). Calculated amounts of salt were added at milling (pH 5.40 ± 0.02) to obtain cheeses with smaller (3.5) and greater (4.5) S/M. Total and soluble calcium, lactic acid and pH were measured and development of CLC were observed in cheeses. All cheeses were both vacuum packaged and gas flushed with N_2 and aged at 7.2^{o}C for 15 weeks.

Concentration of lactic acid in increased S/M cheeses ranged from 0.73 to 0.80 g/100 g of cheese while in smaller S/M cheeses ranged from 1.86 to 1.97 g/100 g at the end of 15 weeks of aging due to starter bacteria salt sensitivity. Smaller and increased

S/M concentrated milk cheeses (LSCMC and HSCMC), exhibited 30 to 28% increased total calcium (1242 and 1239 mg/100g cheese, respectively), than smaller and increased S/M whole milk cheeses (LSWMC and HSWMC; 954 and 967 mg/100g of cheese, respectively) throughout aging. Soluble calcium was 41 to 35% greater in smaller S/M cheeses (LSWMC and LSCMC; 496 and 524 mg/100g cheese, respectively) compared to increased S/M cheeses (HSWMC and HSCMC; 351 mg/100g and 387 mg/100g cheese, respectively). Due to the lower pH of the smaller S/M cheeses CLC were observed in smaller S/M cheeses. However, the greatest intensity of CLC was observed in gas flushed cheeses made with milk of increased protein concentration due to the increased content of calcium available for CLC formation.

These results confirm that occurrence of CLC is dependent on cheese milk concentration and pH of cheese, which can be influenced by S/M and cheese microflora.

(**KEY WORDS**: Salt to moisture ratio, calcium lactate crystals, Cheddar cheese, starter culture, packaging).

INTRODUCTION

Quality defects in Cheddar cheeses discourage repeat purchases by consumers. White crystals on surfaces of Cheddar cheeses exert detrimental effects on sales (McDowall and McDowell, 1939). The problem remains a challenge and expense to cheese manufacturers (Agarwal, et al., 2005; Agarwal, et al., 2006a; Agarwal, et al., 2006b; Chou, et al., 2003; Swearingen, et al., 2004). The financial losses that CLC cost the cheese industry warrant research into intervention to reduce occurrence of CLC on hard and semi-hard cheeses.

CLC may be attributed to a number of variables, including differences in milk composition (Pearce, et al., 1973), cheese making procedure (Dybing, et al., 1986), aging temperature (Agarwal, et al., 2006b; Chou, et al., 2003; Johnson, et al., 1990b; Pearce, et al., 1973), growth of nonstarter lactic acid bacteria (NSLAB) in cheese during aging (Chou et al., 2003; Khalid and Marth, 1990; Somers et al., 2001) and cheese pH (Agarwal, et al., 2006a). Although improved sanitation and good cheese manufacturing practices are leading to reduced NSLAB counts and reducing the occurrence of D(–)-lactate crystals in cheese, in recent years there has been documented increased occurrence of L(+)-lactate crystals in Cheddar cheese (Agarwal, et al., 2006a; Johnson, 2004; Swearingen, et al., 2004). Calcium from cheese milk and lactate from lactose fermentation are the principle components of CLC, or more specifically calcium lactate pentahydrate ($Ca(CH_3CHOHCOO)_2.5H_2O$) (Dybing, et al., 1988; Kubantseva, et al., 2004). Increases in occurrence of CLC are attributed to changes in cheese manufacturing techniques such as use of ultrafiltration (UF) or reverse osmosis, and the use of evaporated or non-fat dry milk (NFDM) to increase the total solids of cheese milk (Johnson, 2004; Agarwal et al., 2006a). Large concentrations of protein in cheese milk are accompanied by increased colloidal calcium (Agarwal, et al., 2006a; Upreti and Metzger, 2007). Calcium in milk and cheese is present in two forms: soluble (dissociated from casein) and colloidal or insoluble (associated with casein). Increase in colloidal (casein-bound) calcium in cheese milk leads to increased soluble calcium concentrations in cheeses because at low pH, increased quantity of colloidal calcium associated with proteins becomes soluble (Agarwal, et al., 2006a). The soluble form of calcium can readily combine with lactate to form calcium lactate. As calcium lactate concentration

exceeds saturation, micro-crystals of calcium lactate are formed. Micro-crystals can accrue in size to form macro-crystals that are visible to the human eye (Dybing et al., 1988).

Various factors like cheese milk quality, cheese making environment, starter bacteria used, cheese make procedures and cheese storage conditions influence the pH of cheese, which leads to various textural and appearance attributes in Cheddar cheese. The final pH of cheese is influenced by many factors, including fermentable sugars in cheese serum, S/M, starter lactic acid bacteria (SLAB) and NSLAB, and storage conditions (Fox, et al., 2004; Thomas and Pearce, 1981; Turner and Thomas, 1980).

S/M is very important for controlling the growth of SLAB and NSLAB. Dybing et al. (1988) showed that cheeses having increased S/M content had lesser occurrence of CLC compared to cheeses with smaller S/M content. Smaller S/M enables salt tolerant SLAB to ferment lactose and produce an elevated concentration of lactic acid, which can lead to occurrence of CLC (Turner and Thomas, 1980). Salt tolerance of SLAB varies, and salt tolerant SLAB can significantly lower the pH of the cheese, especially in regions of smaller S/M (Turner and Thomas, 1980). Turner and Thomas (1980) reported differences in growth and utilization of lactose at different S/M concentrations. Research conducted by Pearce et al. (1973) concluded that cheeses with low pH (< 5.0) stored at 4.5^{o}C developed CLC, while cheeses with higher pH (> 5.1) did not develop CLC. Swearingen et al. (2004) also observed that SLAB used in making cheese exert a significant influence on the occurrence of CLC in Cheddar cheese. Little previous research directly relates SLAB or S/M to occurrence of CLC. The objective of this study was to identify relationships among protein concentrations in cheese milk, S/M, and

SLAB that affect development of CLC. We hypothesize that cheeses made from cheese milk with increased protein concentrations and smaller S/M ratio will exhibit low pH and will develop more CLC than standard cheeses and cheeses with greater S/M. The results of this research will enable us to guide cheese manufacturers in cheese starter culture selection and cheese making techniques. It is also expected that differences in concentrations of calcium and lactic acid will be observed at the surface compared to the core of cheese, indicating migration of calcium and lactate ions cheese surfaces and formation of CLC.

MATERIALS AND METHODS

Selection of Starter Culture

Three commercial direct vat set cultures DVS 850, R603, R608 (Chr. Hansen, Milwaukee, WI) were tested for salt sensitivity at different salt concentration levels in whole milk. Salt was added to 100 ml pasteurized whole milk (Wilcox Farms, Roy, WA) at the rate 3.0, 3.5, 4.0, 4.5, and 5.0%. The milk samples were incubated in a water bath (Isotemp 120, Fisher Scientific, Fair Lawn, NJ) at 35$\pm$0.5^{o}C and inoculated with 0.1% of DVS 850, R603, R608 culture. The pH of each milk sample was measured after 0, 30, 45, 60, 90 and 120 and 960 min. This experiment was repeated three times on three different days. Enumeration of starter bacteria was not done as the aim of the trial was to find a commercial starter that has variable sensitivity to S/M and can exploit the variation in S/M in cheeses leading to different cheese pH even in same batch of cheese manufactured.

Cheese Manufacture

Four different Cheddar cheese batches were manufactured using both two different concentrations of milk protein and two different S/M. The four different treatments included increased and smaller salt whole milk cheeses made from (3.25% protein, 3.85% fat) and increased and smaller salt cheeses made from whole milk supplemented with ultrafiltered milk and cream (4.5% protein, 5.3% fat). Cheeses were manufactured in duplicate, with whole milk cheeses made from 114 kg of whole milk (WSU Creamery) and concentrated milk cheeses made from 68 kg milk (to account for greater yield), for a total of 8 batches of Cheddar cheese. Concentrated milk used in preparing concentrated milk cheeses was standardized by using UF skim milk (Agarwal, 2005), whole milk (WSU Creamery) and heavy cream (Darigold, Seattle, WA). Processing of batches of cheese were randomly distributed across days, with two batches of cheese made each day. The entire cheese making process lasted for four days. Two **High Salt Whole Milk Cheeses** (**HSWM**) (4.5 S/M), two **Low Salt Whole Milk Cheeses (LSWM)** (3.5 S/M), **High Salt Concentrated Milk Cheeses** (**HSCM**) (4.5 S/M), and **Low Salt Concentrated Milk Cheeses** (**LSCM**) (3.5 S/M) composed the 8 treatments. Cheeses were made using standard procedures followed at the WSU Creamery, with slight modifications. Mesophillic starter culture, *DVS 603* (Chr. Hansen, Milwaukee, WI), at the rate of 0.1% (w/w) was added at 31°C to milk. Double-strength coagulator (Chy-Max, Chr. Hansen Laboratories, Milwaukee, WI), diluted 1:40 with tap water, was used to assist coagulation of the milk. At the time of cutting with 6-mm grid cheese knives, pH of cheese whey was 6.60±0.02. Curds were cooked by raising the temperature from 31 to 38°C at the rate of 1°C every 5 min over a 30 min period. Curds and whey were

stirred at 38°C for 45 min before draining, at whey pH 6.20. The cheese was cheddared until a pH of 5.45 was reached and then milled to a size of 1cm x 2cm and mixed with salt (2.95% w/w of cheese curd for increased S/M cheeses and 2.29% w/w for smaller S/M cheeses). A increased percentage of salt was used than in traditional cheese making because of increased loss of salt during salting in smaller batches. The cheeses were pressed in a pneumatic cheese press at 55 psi for 16 h. The cheeses were cut in 850 g cylinders and were vacuum packaged (model X180, Koch Supplies Inc., Kansas City, MO) in 25.4 x 33.0 cm, 3-mil high barrier Nylon/ethyl vinyl alcohol/ polyethylene vacuum pouches (Koch Supplies Inc.). The cheeses were then stored at 7.2^{o}C for one week. Later half of the cheese blocks were randomly selected, opened, and gas flushed with nitrogen gas then aged at 7.2^{o}C for 15 weeks.

Samples from cheese cylinders (diameter 16 cm) were cut in 4 concentric circles at fixed distances from the center of the cheese cylinder (Figure 1) at week 1, 10 and 15. First, a sample ring of 0.5 cm radius was cut from the center of the cheese, which was followed by another concentric ring of 2 cm thickness that was discarded. This procedure was repeated until 4 sample rings resulted, including the outer-most ring of 0.5 cm diameter (Figure 1). These concentric sample rings were shredded and packaged in Ziploc® Freezer Bags (S C Johnson, Racine, WI) and stored at -26^{o}C prior to analysis.

Compositional Analyses

All compositional analyses were conducted in duplicate on weeks 1, 10 and 15. Milk was analyzed for total solids (Marshall, 1992), protein was analyzed using protein analyzer (UDY Corporation, Fort. Collins CO), fat (Marshall, 1992). Cheeses were analyzed for moisture (Marshall, 1992), salt by Corning Salt Analyzer (Marshall, 1992),

and pH by pH meter (Accumet® AP61, Fisher Scientific, Fair Lawn, NJ) using an Accumet electrode (serial # 208804, Fisher Scientific, Fair Lawn, NJ) calibrated with pH 4 and 7 buffers (Fisher Scientific, Fair Lawn, NJ). L(+)-lactic acid concentration in cheese was determined by using enzyme assay (Severn, et al., 1986). Total and soluble calcium were determined by atomic absorption spectroscopy (Agarwal et al., 2006a). Lactose was measured using R-Biopharm Enzymatic Bioanalysis test kits (Boehringer Mannheim Enzymatic Bioanalysis/ Food Analysis Test Kits, Darmstadt, Germany) following the instructions provided by the manufacturer. Intensity of occurrence of CLC was measured using the scale used by Agarwal et al. (2005). White crystals on the surface of cheeses were analyzed for lactic acid using R-Biopharm Enzymatic Bioanalysis test kits (Boehringer Mannheim Enzymatic Bioanalysis/ Food Analysis Test Kits, Darmstadt, Germany).

Statistical Analysis

Effects of milk type, S/M, packaging and distance from the edge of a cheese cylinder on total calcium, soluble calcium, lactic acid, and salt content of the cheeses was studied. The design was a split plot design with repeated measures. The whole plot design structure was completely randomized as the cheeses batches were replicated with a one-way treatment structure (milk type). The subplot structure was a randomized complete block design with a three-way treatment structure (salt, packaging and time). The repeated measure was represented by the position sampled on the cheese cylinder. Least squares means for mean comparison was used to determine significant differences at $P = 0.05$.

RESULTS AND DISCUSSION

Starter Culture Selection

As expected, acid production rate of starter bacteria was influenced by salt, which influenced the final pH of the milk. Starter culture DVS 850 was the least salt tolerant of the three starter cultures tested. The lowest pH attained was about pH 5.2 at 3.0% salt concentration, while pH ranged from 5.8 to 5.9 at 5.0% salt concentration (Figure 2). Starter culture DVS 608 was the most salt tolerant of the three starter cultures tested. DVS 608 starter culture attained a pH of 5.0 at 3.0% salt and about pH 5.4 at 5.0% salt concentration (Figure 3). The average pH of milk incubated with starter culture DVS 603 at 3.0 and 3.5% salt concentration was 4.8 to 4.9 but at 4.5 and 5.0% average pH of milk reached only pH 5.6 at the end of 960 minutes of incubation (Figure 4). Starter culture DVS 603 was selected for conducting cheese trials to observe the effect of salt on cultures, final pH of the cheese after pressing, and subsequent impact upon CLC occurrence.

Effects of Cheese Milk and Salt on Cheese Composition

The effects of cheese milk composition and S/M on final cheese composition was significant. As expected, greater S/M cheeses (HSWMC and HSCMC) had significantly increased S/M compared to smaller S/M cheeses (LSWMC and LSCMC) (Table 1). Average S/M in increased salt cheeses ranged from 4.48 to 4.55 while in decreased salt cheeses S/M ranged from 3.27 to 3.52. Average moisture content in HSWMC (33.92%), LSWMC (36.12%), HSCMC (36.69%), and LSCMC (37.28%) after 1 week of storage revealed increased moisture content in cheeses with smaller S/M compared to

corresponding cheeses with increased S/M. The decreased moisture content in increased S/M cheeses compared to smaller S/M cheeses can be explained by the fact that syneresis is enhanced by S/M; more serum was lost during pressing of cheeses with increased salt compared to cheeses with smaller S/M content.

Protein concentration was significantly ($P < 0.05$) higher in increased S/M cheeses than smaller S/M cheeses. Increased protein concentration in increased S/M cheeses is explained by the decreased moisture content compared to the smaller S/M cheeses. As expected, use of ultrafiltered milk had a significant ($P < 0.05$) effect on protein concentration as well. HSCMC (30.6%) and LSCMC (29.6%) had significantly increased protein concentration compared to HSWMC (28.9%) and LSWMC (25.6%) (Table 1). Similar results were obtained by Agarwal (2006a), Acharya (2004) and Jensen (1987), where authors saw an increase in protein concentration of cheeses manufactured with cheese milk supplemented with UF milk.

Effect of Cheese Milk, Salt and Aging on pH

Concurrently, the pH of cheese was significantly ($P < 0.05$) influenced by salting rate and was closely related to amount of lactic acid developed. The average pH of increased S/M cheeses (HSWMC and HSCMC) at the end of 1 week of aging decreased to only 5.35 and 5.37 compared to smaller S/M cheeses (LSWMC and LSCMC; 5.16 and 5.25) (Table 1). Higher pH of LSCMC compared to LSWMC at the end of 1 week of aging can be explained by the increased concentration of proteins and calcium in concentrated milk, which increased buffering capacity of the concentrated milk cheeses, though LSWMC and LSCMC had comparable concentrations of lactic acid. Slower decrease of pH in cheeses having increased concentration of calcium and phosphorus was

also observed by Upreti and Metzger, (2007). The pH of all the cheeses changed throughout the aging process. In the case of smaller S/M cheeses (LSWMC and LSCMC) the pH decreased for the first 10 weeks and then started increasing slowly, while in the case of HSWMC and HSCMC, an increase in pH was observed throughout the aging process (Figure 5). Greater pH (5.3 to 5.5) were observed in cheeses having increased S/M compared to smaller pH (4.9 to 5.0) in cheeses having smaller S/M after 15 weeks of aging. Similar observations of decreased and increased pH during aging of Cheddar cheese are well established (Swearingen, et al., 2004; Upreti and Metzger, 2007). In the present study, the difference in pH of cheeses was due to inhibition of the starter bacteria (DVS 603) at increased S/M. Elevated lactose concentration in increased S/M cheeses (HSWMC and HSCMC) after 15 weeks of aging confirmed inhibition of starter bacteria. In contrast, absence of lactose in smaller S/M cheeses (LSWMC and LSCMC) showed that starter bacteria were active and metabolized available lactose (Figure 6).

A high correlation ($r = 0.76$) was observed between S/M and pH during 15 weeks of aging (Figure 7). Several authors have documented the influence of salt on pH of Cheddar cheese. A strong correlation ($r = 0.94$) between S/M and cheese pH was shown by Thomas and Pearce (1981) during the first 2 weeks of aging. The authors also suggested that at smaller S/M (< 4.0) all the lactose present in the cheese will be utilized by cheese microflora, resulting in the lowest possible pH. Upreti and Metzger (2007) showed that cheeses having increased S/M (6.4) had higher pH compared to cheeses with smaller S/M (4.8) during 48 weeks of aging. Similar results were shown by O'Connor (1974) when 8 week old Cheddar cheese with smaller S/M of 1.4 to 4.9 had low pH

(ranging from 5.08 to 5.12), while cheeses with greater S/M (6.3 to 8.3) resulted in cheeses with high pH (ranging from 5.41 to 5.49). The fact that different starter bacteria have different salt tolerances and different SLAB are used in research explains why lower pH can be seen in cheeses having S/M of 4.8 than cheeses having S/M of 6.3 to 8.3 (O'Connor, 1974; Upreti and Metzger, 2007).

Effect of Cheese Milk, Salt and Aging on Lactose and Lactic Acid

Lactose and lactic acid content in cheese significantly ($P < 0.05$) differed among cheeses with different S/M. As expected, increased lactose content was observed in increased S/M cheeses (HSWMC and HSCMC; 1.40 to 1.55 g/100g of cheese) compared to negligible amount of lactose in smaller S/M cheeses (LSWMC and LSCMC; smaller than 0.01 g/100g of cheese) (Figure 6). The average lactic acid concentration of increased S/M cheeses (HSWMC and HSCMC; 0.73 and 0.80 g/100g of cheese) at the end of 1 week of pressing was lower than smaller S/M cheeses (LSWMC and LSCMC; 1.08 and 1.01 g/100g of cheese) due to inhibition of SLAB metabolism (Figure 8). A strong correlation ($r = 0.91$) was observed between S/M and amount of lactic acid present in cheeses after 15 weeks of aging (Figure 9). The amount of lactic acid in smaller S/M cheeses (LSWMC and LSCMC; 1.86 to 1.97%) was more than twice the amount of lactic acid present in increased S/M cheeses (HSWMC and HSCMC; 0.71 and 0.85%) after 15 weeks of aging (Figure 8). Similar results were observed by Upreti et al. (2006), where they observed that cheeses with increased S/M had significantly lower lactic acid concentration than cheeses with smaller S/M. Both SLAB and NSLAB have the ability to convert lactose to lactic acid in cheese during aging (Agarwal, et al., 2006b; Shakeel Ur, et al., 2000; Thomas and Crow, 1983), but presence of increased lactose does not

always guarantee increased amount of lactic acid (Blake, et al., 2005). Salt influences the water activity (a_w), thus influencing the growth of SLAB and NSLAB (Thomas and Pearce, 1981). Knowing the salt tolerance level of SLAB used in cheese making can enable manufacturers to salt appropriately to control residual lactose and subsequent CLC formation.

Effect of Cheese Milk, Salt and Aging on Total and Soluble Calcium

Milk concentration had a significant ($P < 0.05$) effect on the total calcium concentration of cheeses. Average total calcium content in standard milk cheeses (HSWMC and LSWMC) was (953.84 and 966.61 mg/100g of cheese), than in concentrated milk cheeses (HSCMC and LSCMC; 1239.44 and 1242.20 mg/100g of cheese) showing that concentration of cheese milk leads to increased concentration of total calcium in cheeses (Figure 10). Lee et al. (2005) also showed that cheeses made from reverse osmosis treated milk contained increased protein and total calcium compared to cheeses made from standard milk. Similar results were obtained by Agarwal et al. (2006a), where cheeses manufactured from milk concentrated with either non fat dry milk or UF milk had increased protein and total calcium concentrations. But S/M had no significant effect on total calcium of either whole milk or concentrated milk cheeses. The value of total calcium of whole milk cheeses (930 mg/100g) was larger than those observed by other researchers in whole milk cheeses (680 to 833 mg/100 g) (Hassan et al., 2004; Swearingen et al., 2004) and can be explained due to seasonal variation in casein to calcium in cheese milk (Dybing et al., 1988).

The concentration of soluble calcium in cheeses was influenced more by pH of cheeses than protein concentration of milk used for manufacturing. Average soluble

calcium content in increased S/M cheeses (HSWMC and HSCMC; 351.20 and 387.30 mg/100g of cheese) was lower than in smaller S/M cheeses (LSWMC and LSCMC; 496.33 and 524.13 mg/100g of cheese) (Figure 11). Since increased S/M concentration inhibited culture metabolism, pH remained high and soluble calcium remained smaller in cheeses even though are cheeses were salted at pH 5.40. Low pH and concentration of cheese milk leads to increased concentration of soluble calcium in cheeses. The soluble calcium levels in experimental cheeses were comparable to the soluble calcium values in the commercial cheeses (about 450 mg/100 g of cheese) studied by Swearingen et al. (2004). Salt had a significant effect on the amount of soluble calcium, as S/M directly affected the activity of SLAB thus the pH and lactic acid concentration in cheese. A strong correlation ($r = 0.81$) was observed between amount of lactic acid in cheese and amount of soluble calcium at the end of 15 weeks of aging (Figure 12). Also, a correlation ($r = 0.83$) was observed between pH of cheese and soluble calcium at end of 15 weeks of aging (Figure 13). Concentration of soluble calcium increased significantly ($P < 0.05$) during the first 15 weeks of aging in smaller S/M cheeses (LSWMC and LSCMC), while no significant changes observed in increased S/M cheeses (HSWMC and HSCMC) (Table 1). Changes in lactic acid, pH and soluble calcium are correlated such that cheeses with smaller S/M had increased concentrations of lactic acid, lower pH and increased concentrations of soluble calcium compared to cheeses with increased S/M. Similar results were obtained by Agarwal et al. (2006a) who showed an increased concentration of soluble calcium and lactic acid with a decrease in cheese pH (5.4 to 4.8). Effect of S/M on lactic acid, pH and soluble calcium were also discussed in detail by Upreti and Metzger (2007), where the authors found cheeses with increased S/M had

decreased concentration of soluble calcium and inorganic acids compared to cheeses with smaller S/M. Upreti and Metzger (2007) also found a high correlation (r = 0.78) between pH of cheese and soluble calcium. Cheese manufacturers can introduce washing step before milling of cheese curd, thus washing off excess of lactose, lactic acid and soluble calcium from the cheese and significantly lowering the risk of having excessive lactic acid and calcium for CLC to occur.

Effect of Cheese Milk Composition, Salt and Packaging on Occurrence of CLC

CLC were seen earlier in cheeses that had low pH (< 5.1) than cheeses with higher pH (> 5.1) regardless of milk type or S/M. CLC started appearing at a light intensity on smaller S/M cheeses, in particular LSWMC that were gas flushed, as early as 5 weeks (2 on a scale of 1 to 10). At the end of 10 weeks of aging, pH of all smaller S/M cheeses ranged from 4.9 to 5.1 and a increased intensity of CLC were observed in all these cheeses. Generally, increased intensity of CLC were observed in LSCMC compared to LSWMC. Additionally, low pH in cheeses led to a defect called sweating, which occurs due to loss in moisture holding capacity of cheese protein at low pH (Swearingen et al., 2004). Cheese packaging had no significant effect on the amount of total calcium, soluble calcium, lactic acid, salt concentration or pH of the cheeses which was also seen by (Agarwal, 2006b). CLC were observed earlier and to a greater extent in cheeses that were gas flushed (crystal intensity 4 to 6) compared to cheeses that were vacuum packaged (crystal intensity 2 to 3) probably due to loss of moisture to surrounding air, which led to concentration of calcium ions and lactate on the cheese surfaces. CLC were also observed to a increased intensity in cheeses that were made from concentrated milk compared (crystal intensity 6) to cheeses that were made from whole milk cheese (crystal

intensity 4) due to increased concentration of soluble calcium and lactic acid concentration (Table 1). CLC were observed in cheese that had smaller S/M but not in cheeses that had increased S/M, showing the important relationship between salting rate and starter bacteria selection. Similar conclusions were also drawn by Rajbhandari and Kindstedt (2005), who blamed within-vat variation in salting efficacy on formation of CLC. L(+)-lactate was the predominant form of lactate and no D(-)-lactate was determined from the analysis of CLC in the present study.

Migration of Total and Soluble Calcium during Aging

Significant ($P < 0.05$) differences were not only observed in composition of cheeses having smaller and increased S/M but also in the way calcium and lactate ions migrated to surfaces, leading to growth of CLC. Changes in total calcium, soluble calcium, and lactic acid were studied at different concentric locations over 15 weeks of aging. Significantly ($P < 0.05$) Increased concentration of total calcium was observed on the surfaces (position D) of LSWMC and LSCMC than at the core (position A, B and C) in the same cheeses after 15 weeks of aging (Figure 14). Also, significantly ($P < 0.05$) increased concentrations of total calcium were observed at weeks 10 and 15 between positions D and C. This shows that most of the calcium migration to the surface migrated from surrounding areas within cheese (position C). Significantly ($P < 0.05$) increased concentrations of soluble calcium and lactic acid were observed within smaller S/M cheeses (position A, B and C) than on their surfaces (position D) after 10 and 15 weeks of aging (Figure 16 & 18). Increased concentrations of soluble calcium and lactic acid within the cheeses (position A, B and C) compared to surfaces (position D) suggests that gradients may encourage migration of calcium ions to surfaces. The gradient theory is

supported by the fact that no differences in increased S/M cheeses were noted as no gradient existed. Occurrence of CLC on the surface of smaller S/M cheeses (LSWMC and LSCMC) can explain increased concentration of total calcium compared to other parts of the cheeses.

No significant differences in concentration of total calcium, soluble calcium and lactic acid were observed between the core and surface of increased S/M cheeses (HSWMC and HSCMC) after 10 and 15 weeks of aging (Figure 15, 17 & 18). This lack of gradient in increased S/M cheeses (HSWMC and HSCMC) can be explained by lack of fermentation of lactose by starter bacteria leading to not enough soluble calcium and lactate in cheese serum to form CLC.

CONCLUSIONS

Results of this study suggest that cheese manufactures must carefully select starter bacteria and carefully monitor the salting of cheese during manufacture. Use of salt tolerant SLAB enables post-press fermentation of residual lactose, solubilization of calcium and CLC formation, particularly at smaller S/M and increased protein concentrations and gas flushed packaging conditions. Cheese manufactures can reduce the occurrence of CLC in cheeses by selecting starter cultures that are salt sensitive, carefully monitoring the salting process for uniform distribution of salt, and carefully documenting post-manufacture pH of cheese during storage. Cheese manufactures facing problems of CLC can consider reducing the concentrations of total calcium and soluble calcium in final cheese, which can be accomplished by introducing a curd washing step.

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REFERENCES

Acharya, M. R. and V. V. Mistry. 2004. Comparison of effect of vacuum-condensed and ultrafiltered milk on Cheddar cheese. J. Dairy Sci. 87, no. 12:4004-4012.

Agarwal, S., M. Costello, and S. Clark. 2005. Gas-Flushed packaging contributes to calcium lactate crystals in Cheddar cheese. J. Dairy Sci. 88:3773-3783.

Agarwal, S., J. R. Powers, B. G. Swanson, S. Chen, and S. Clark. 2006a. Cheese pH, protein concentration, and formation of calcium lactate crystals. J. Dairy Sci. 89:4144-4155.

Agarwal, S., K. Sharma, B. G. Swanson, G. Ü. Yüksel, and S. Clark. 2006b. Nonstarter lactic acid bacteria biofilms and calcium lactate crystals in Cheddar cheese. J. Dairy Sci. 89:1452-1466.

Blake, A. J., J. R. Powers, L. O. Luedecke, and S. Clark. 2005. Enhanced lactose cheese milk does not guarantee calcium lactate crystals in finished Cheddar cheese. J. Dairy Sci. 88, no. 7:2302-2311.

Chou, Y. E., C. G. Edwards, L. O. Luedecke, M. P. Bates, and S. Clark. 2003. Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. J. Dairy Sci. 86:2516-2524.

Dybing, S. T., S. A. Brudvig, J. A. Wiegand, and E. A. Huang. 1986. A simple method for estimating the extent of surface crystal development on colored Cheddar cheese. J. Food Prot. 49:421-422.

Dybing, S. T., J. A. Wiegand, S. A. Brudvig, E. A. Huang, and R. C. Chandan. 1988. Effect of processing variables on the formation of calcium lactate crystals on Cheddar cheese. J. Dairy Sci. 71:1701-1710.

Fox, P. F., L. H. McSweeney, M. T. Cogan, and P. Guinee. 2004. Cheese: Chemistry, Physics and Microbiology. Vol. 2. 3rd ed. Elsevier Academic Press, London; San Diego.

Jensen, L. A., M. E. Johnson, and N. F. Olson. 1987. Composition and properties of cheeses from milk concentrated by ultrafiltration and reverse osmosis--a review of literature. Cultured Dairy Prod. J. 22:6-10, 12-14.

Johnson, M. E. 2004. Calcium lactate crystals. International Cheese Technology Exposition, Madison, WI.

Johnson, M. E., B. A. Riesterer, and N. F. Olson. 1990b. Influence on nonstarter bacteria on calcium lactate crystallization on the surface of Cheddar cheese. J. Dairy Sci. 73:1145-1149.

Kubantseva, N., R. W. Hartel, and P. A. Swearingen. 2004. Factors affecting solubility of calcium lactate in aqueous solutions. J. Dairy Sci. 87:863-867.

Lee, M. R., M. E. Johnson, and J. A. Lucey. 2005. Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. J. Dairy Sci. 88:3798-3809.

Linke, W. F. 1958. Solubilities of Inorganic and Metal Organic Compounds. Am. Chem. Soc. 4th ed. W. F. Linke, ed, Washington, DC.

McDowall, F. H. and A. K. R. McDowell. 1939. The white particles in mature Cheddar cheese. J. Dairy Res. 10:118-119.

O'Connor, C. B. 1974. The Quality and Composition of Cheddar Cheese: Effect of various rates of salt addition. III. Ir Agric Cream Rev. 27:11-13.

Pearce, K. N., L. K. Creamer, and J. Gilles. 1973. Calcium lactate deposits on rindless Cheddar cheese. N. Z. J. Dairy Sci. Technol. 8:3-7.

Rajbhandari, P. and P. S. Kindstedt. 2005. Compositional factors associated with calcium lactate crystallization in smoked Cheddar cheese. J. Dairy Sci. 88:3737-3744.

Severn, D. J., M. E. Johnson, and N. F. Olson. 1986. Determination of lactic acid in Cheddar cheese and calcium lactate crystals. J. Dairy Sci. 69:2027-2030.

Shakeel Ur, R., J. M. Banks, P. L. H. McSweeney, and P. F. Fox. 2000. Effect of ripening temperature on the growth and significance of non-starter lactic acid bacteria in Cheddar cheese made from raw of pasteurized milk. Int. Dairy J. 10:45-53.

Swearingen, P. A., D. E. Adams, and T. L. Lensmire. 2004. Factors affecting calcium lactate and liquid expulsion defects in Cheddar cheese. J. Dairy Sci. 87:574-582.

Thomas, T. D. and V. L. Crow. 1983. Mechanism of D(-)-Lactic acid formation in Cheddar cheese. N. Z. J. Dairy Sci. Tech. 18:131-141.

Thomas, T. D. and K. N. Pearce. 1981. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. N. Z. J. Dairy Sci. Technol. 16:253-259.

Turner, K. W. and T. D. Thomas. 1980. Lactose fermentation in Cheddar cheese and the effect of salt. N. Z. J. Dairy Sci. Technol. 15:265-276.

Upreti, P., L. L. McKay, and L. E. Metzger. 2006. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: changes in residual sugars and water-soluble organic acids during ripening. J. Dairy Sci. 89:429-443.

Upreti, P. and L. E. Metzger. 2007. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: pH changes during ripening. J. Dairy Sci. 90:1-12.

Table 1. Composition of smaller and increased S/M cheeses made from whole milk and UF milk at week 1, 10 and 15. All values are average of vacuum and gas flushed cheeses because significant difference not found (means of replicates ± SD).

		High salt whole milk cheese	Low salt whole milk cheese	High salt concentrated milk cheese	Low salt concentrated milk cheese
Week 1	**pH***	5.36 ± 0.01[a]	5.17 ± 0.05[b]	5.36 ± 0.01[a]	5.25 ± 0.07[b]
	Moisture (%)	33.92 ± 0.34[a]	36.11 ± 0.45[b]	36.70 ± 0.22[b]	37.29 ± 0.12[c]
	Protein (%)	28.89 ± 0.45[a]	25.63 ± 0.53[b]	30.63 ± 0.40[c]	28.32 ± 0.34[d]
	Fat (%)	33.63 ± 0.18[a]	35.50 ± 0.71[b]	29.63 ± 0.28[c]	30.13 ± 0.30[c]
	Salt (%)	1.52 ± 0.10[a]	1.23 ± 0.18[b]	1.68 ± 0.11[c]	1.25 ± 0.08[b]
	S/M ratio	4.51 ± 0.05[a]	3.27 ± 0.24[b]	4.49 ± 0.08[a]	3.51 ± 0.07[b]
	Lactose (g/100g)	1.45 ± 0.06[a]	0.32 ± 0.09[b]	1.58 ± 0.10[a]	0.38 ± 0.02[b]
	Lactic acid (g/100g)	0.73 ± 0.03[a]	1.08 ± 0.07[b]	0.80 ± 0.06[a]	1.01 ± 0.08[b]
	Total calcium (mg/100g)	916.64 ± 6.35[a]	929.36 ± 10.27[a]	1247.49 ± 10.10[b]	1219.70 ± 30.50[b]
	Soluble calcium (mg/100g)	338.93 ± 18.05[a]	438.13 ± 18.57[b]	364.83 ± 13.33[c]	454.37 ± 20.15[d]
Week 10	**pH**	5.46 ± 0.07[a]	5.03 ± 0.04[b]	5.37 ± 0.03[a]	5.02 ± 0.01[b]
	Salt (%)	1.48 ± 0.04[a]	1.17 ± 0.03[b]	1.47 ± 0.04[a]	1.26 ± 0.06[b]
	S/M ratio	4.37 ± 0.12[a]	3.26 ± 0.03[b]	4.34 ± 0.13[a]	3.44 ± 0.17[b]
	Lactose (g/100g)	1.42 ± 0.02[a]	0.06 ± 0.08[b]	1.62 ± 0.27[a]	0.04 ± 0.07[b]
	Lactic acid (g/100g)	0.75 ± 0.07[a]	1.86 ± 0.14[b]	0.83 ± 0.08[a]	1.99 ± 0.24[b]
	Total calcium (mg/100g)	988.59 ± 32.62[a]	995.12 ± 46.00[a]	1221.86 ± 20.79[b]	1228.40 ± 32.47[b]
	Soluble calcium (mg/100g)	365.24 ± 14.40[a]	489.44 ± 16.70[b]	400.73 ± 13.69[c]	537.56 ± 13.90[d]
Week 15	**pH**	5.51 ± 0.02[a]	5.09 ± 0.04[b]	5.40 ± 0.02[c]	5.11 ± 0.07[b]
	Salt (%)	1.47 ±0.03[a]	1.25 ± 0.06[b]	1.51 ± 0.05[a]	1.25 ± 0.04[b]
	S/M ratio	4.34 ± 0.08[a]	3.48 ± 0.04[b]	4.45 ± 0.05[a]	3.43 ± 0.11[b]
	Lactose (g/100g)	1.40 ± 0.14[a]	0.01 ± 0.02[b]	1.55 ± 0.17[a]	Not detected
	Lactic acid (g/100g)	0.71 ± 0.08[a]	1.86 ± 0.06[b]	0.85 ± 0.10[a]	1.97 ± 0.12[b]
	Total calcium (mg/100g)	956.29 ± 28.60[a]	975.36 ± 43.91[a]	1240.08 ± 11.08[b]	1278.50 ± 25.93[b]
	Soluble calcium (mg/100g)	349.43 ± 3.85[a]	516.29 ± 18.67[b]	403.83 ± 8.91[c]	549.63 ± 12.92[d]

[a, b, c, d,] Means within the same category (pH, moisture, protein, fat, salt, salt to moisture, lactose, lactic acid, total calcium, soluble calcium) sharing common superscripts are similar (P > 0.05).

* observations taken one day after manufacture of cheese.

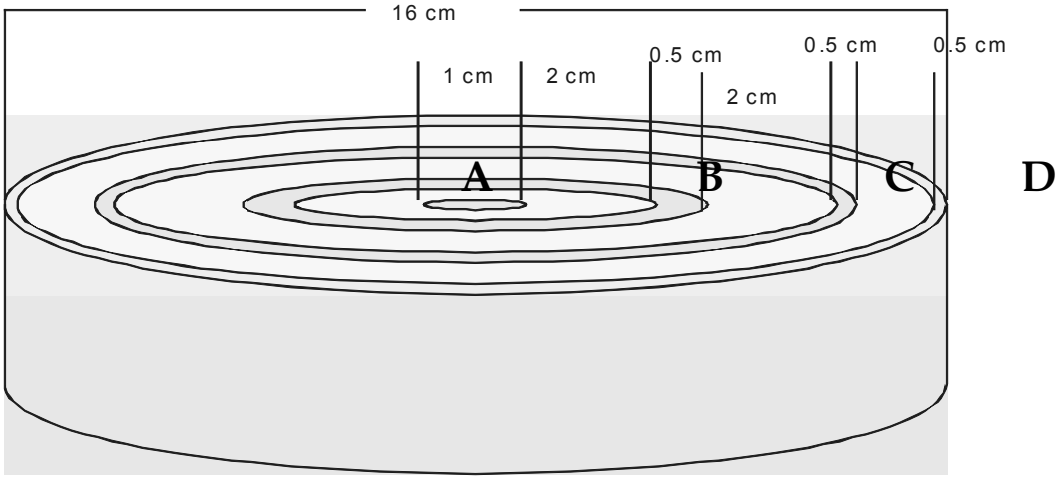

Figure 1. Schematic diagram of cheese cylinder; shaded area show sampling locations

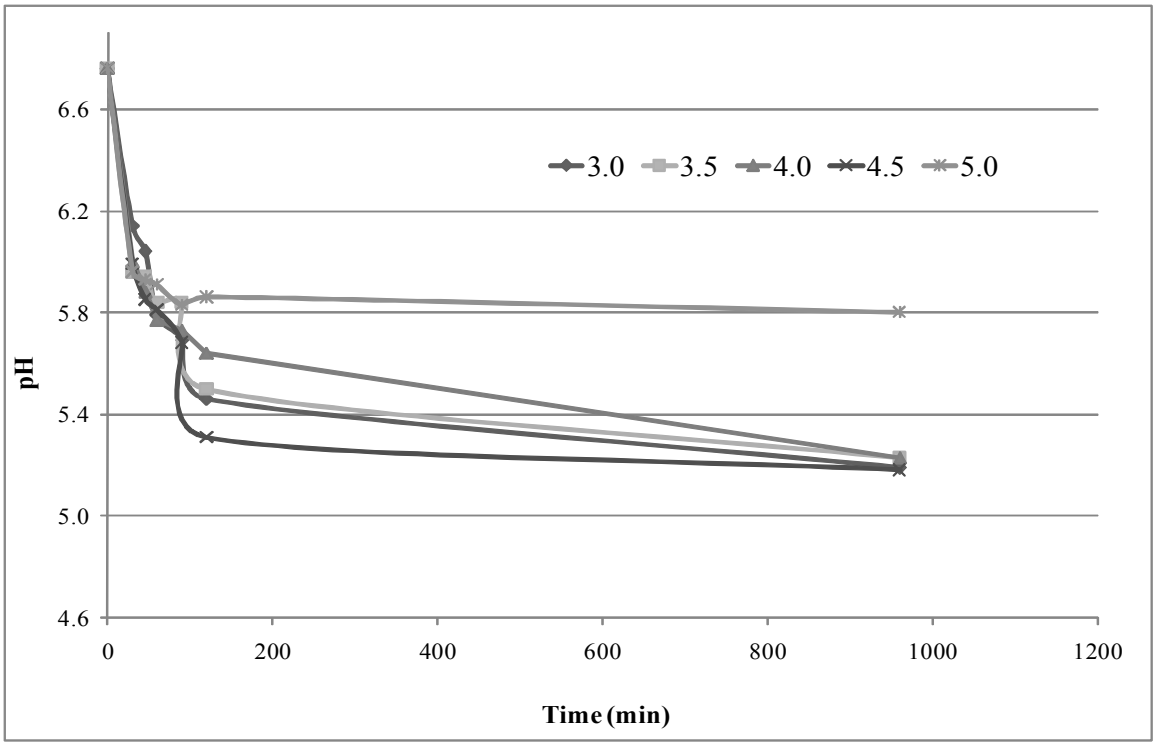

Figure 2. Effect of salt concentration (3.0, 3.5, 4.0, 4.5, and 5.0) on pH of milk inoculated with 0.1% DVS 850 and incubated for 960 min at 35±0.5°C.

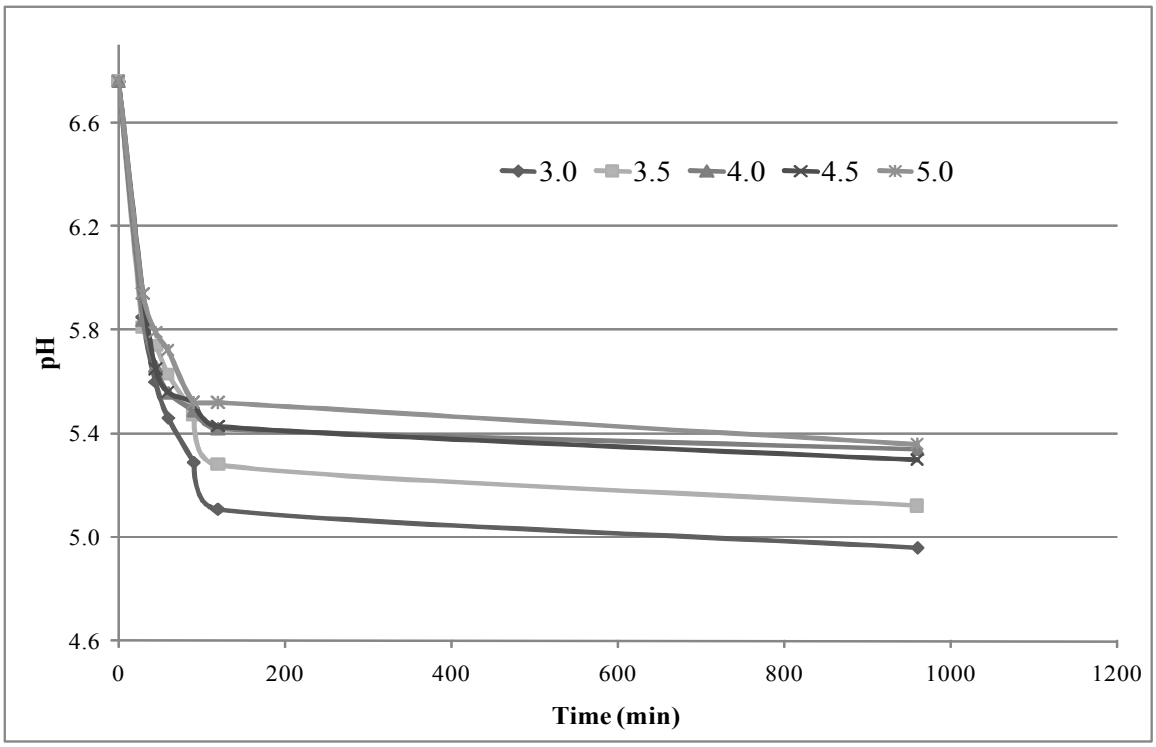

Figure 3. Effect of salt concentration (3.0, 3.5, 4.0, 4.5, and 5.0) on pH of milk inoculated with 0.1% DVS 608 and incubated for 960 min at 35±0.5°C.

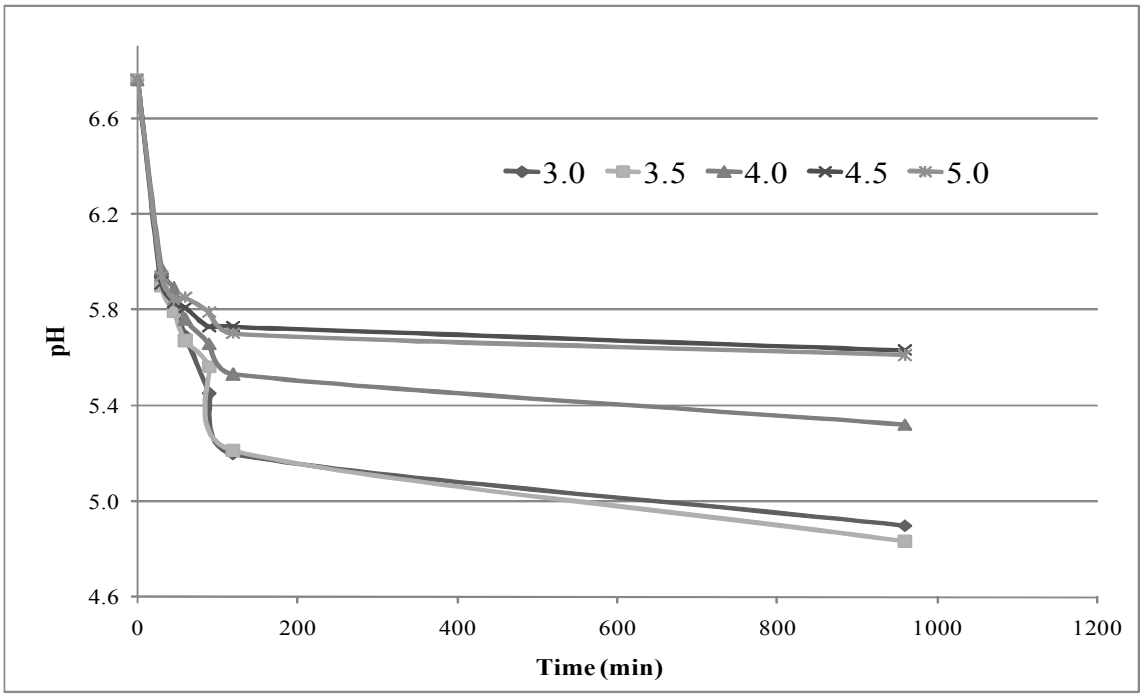

Figure 4. Effect of salt concentration (3.0, 3.5, 4.0, 4.5, and 5.0) on pH of milk inoculated with 0.1% DVS 603 and incubated for 960 min at 35±0.5°C.

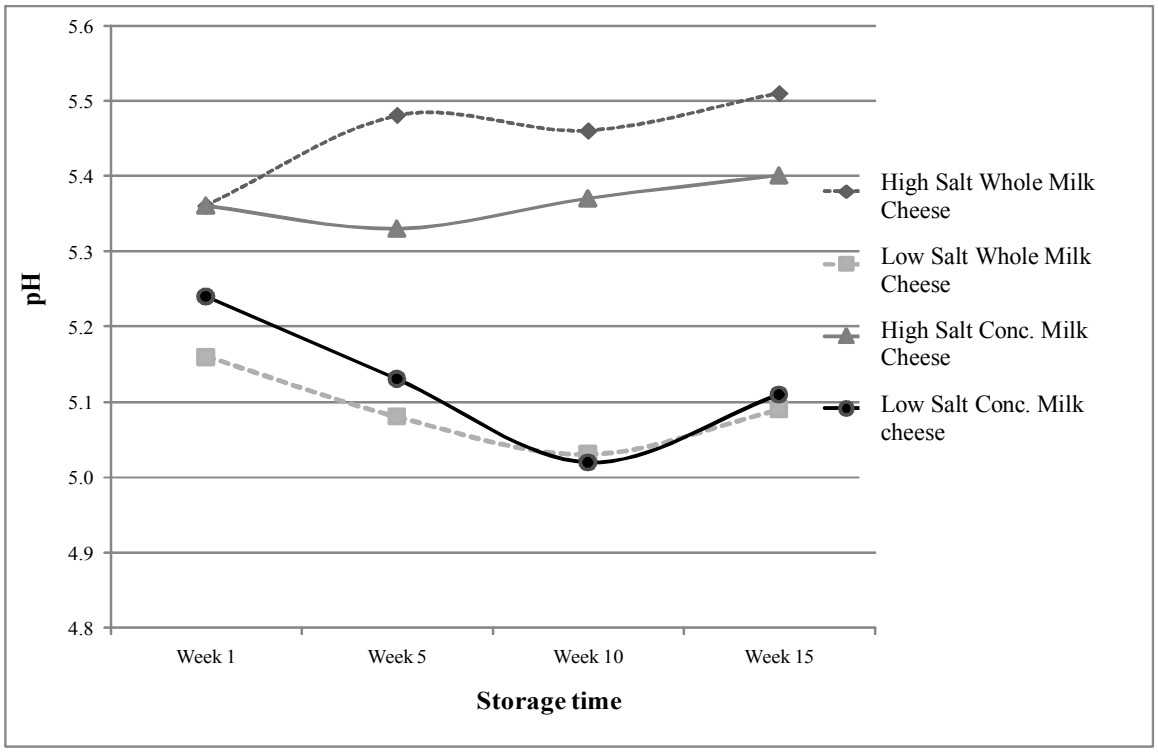

Figure 5. Changes in pH of increased and smaller S/M cheeses stored at 7.2°C for 15 weeks (average of vacuum and gas flushed packaged cheese, mean of duplicates).

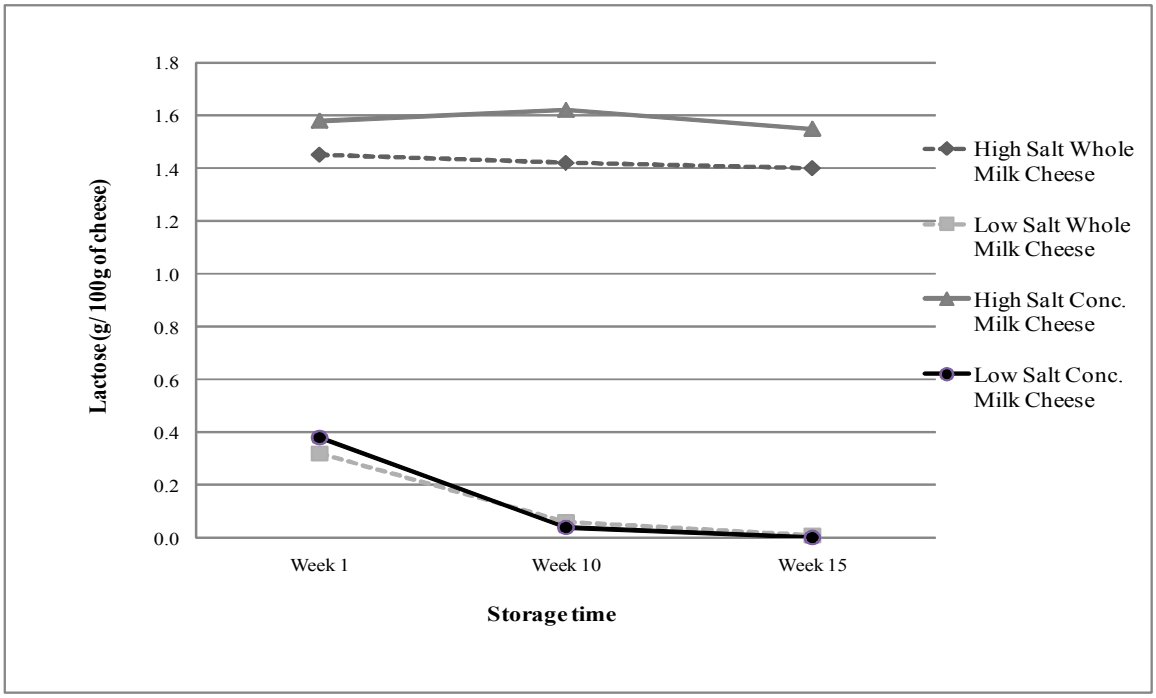

Figure 6. Changes in lactose concentration of increased and smaller S/M cheeses stored at 7.2°C for 15 weeks (average of vacuum and gas flushed packaged cheese, mean of duplicates).

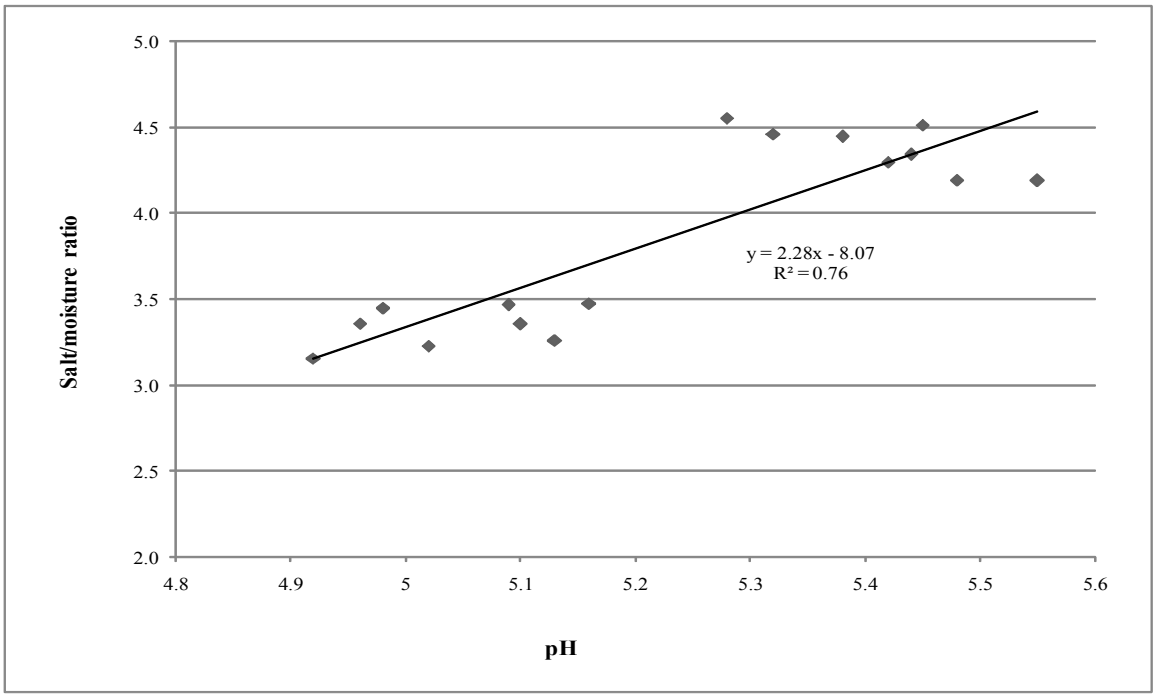

Figure 7. Scatter plot of cheese pH vs. S/M in cheese after 15 weeks of aging.

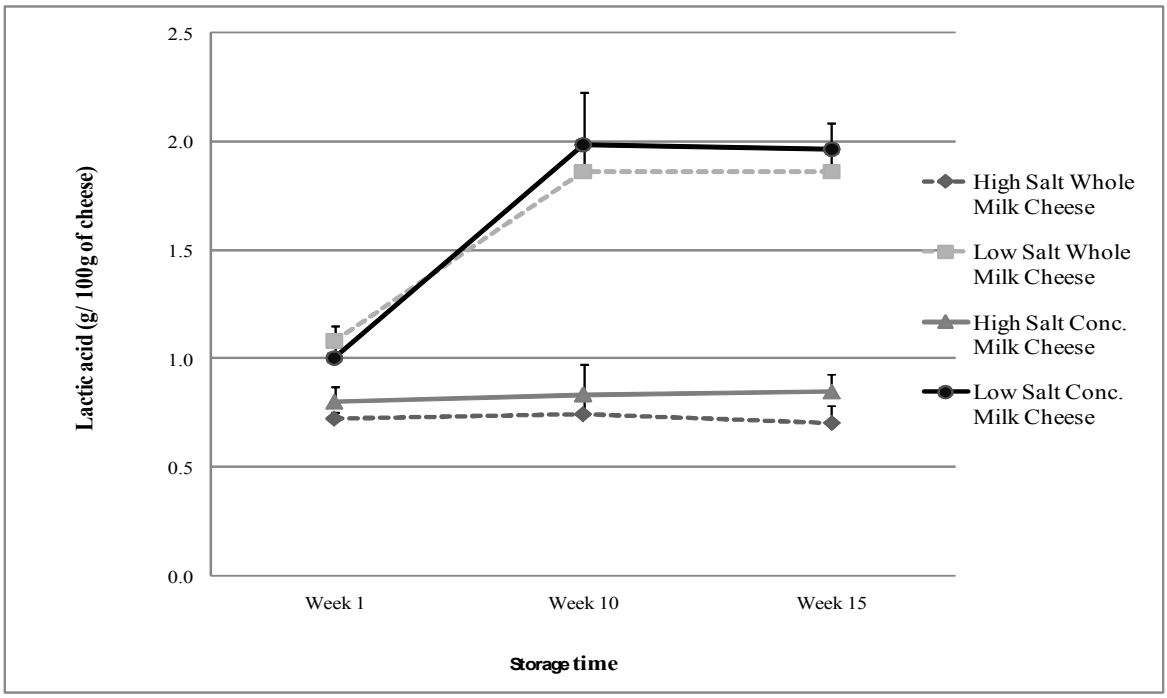

Figure 8. Changes in average lactic acid concentration of increased and smaller S/M cheeses stored at 7.2°C for 15 weeks (average of vacuum and gas flushed packaged cheese, mean of duplicates).

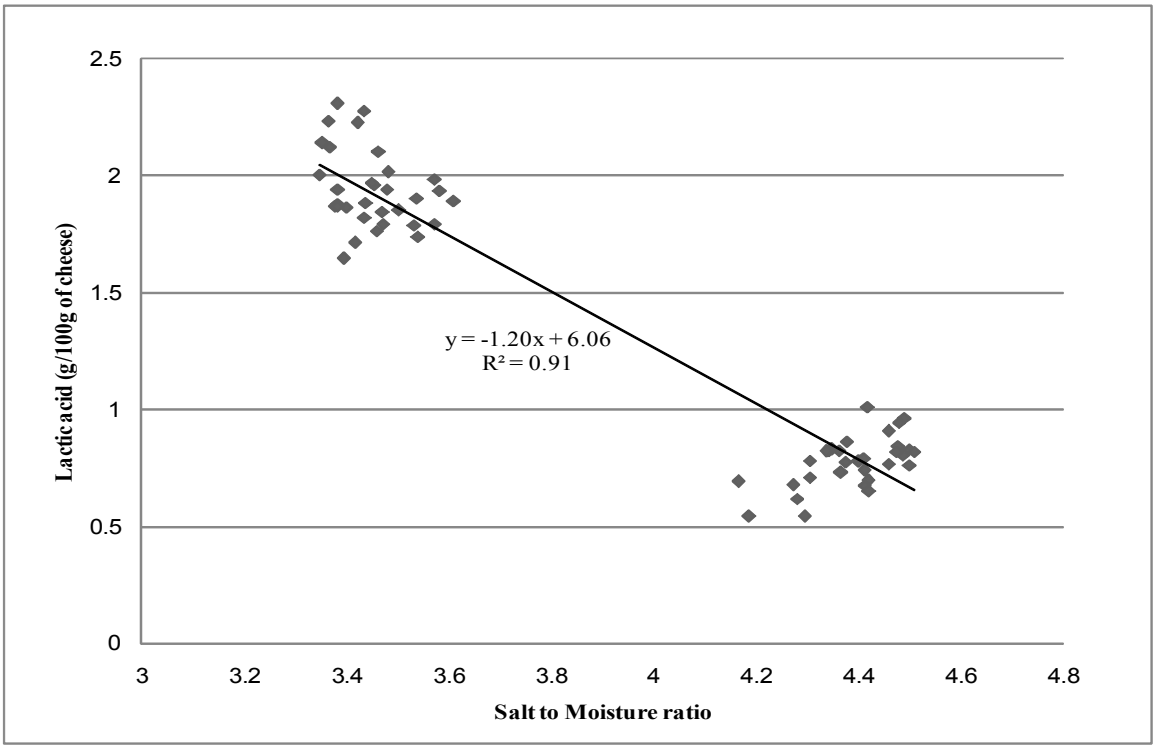

Figure 9. Scatter plot of lactic acid concentration vs. S/M in cheese after 15 weeks of aging.

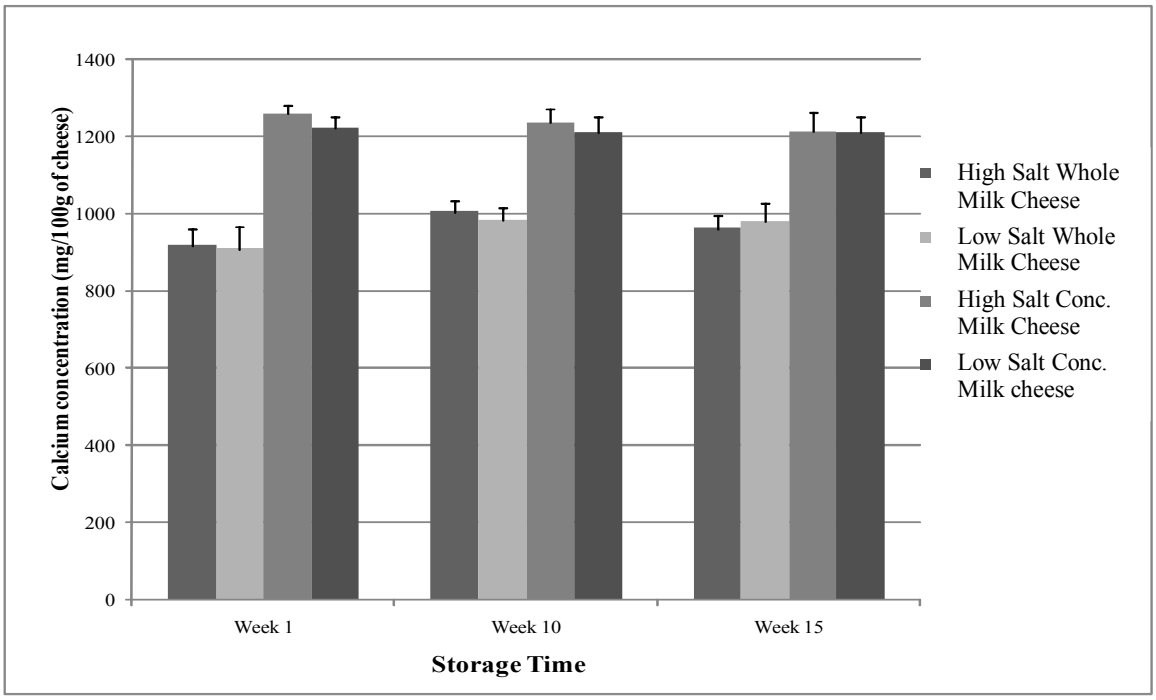

Figure 10. Changes in total calcium concentration of increased and smaller S/M cheeses stored at 7.2°C for 15 weeks (average of vacuum and gas flushed packaged cheese, mean of duplicates).

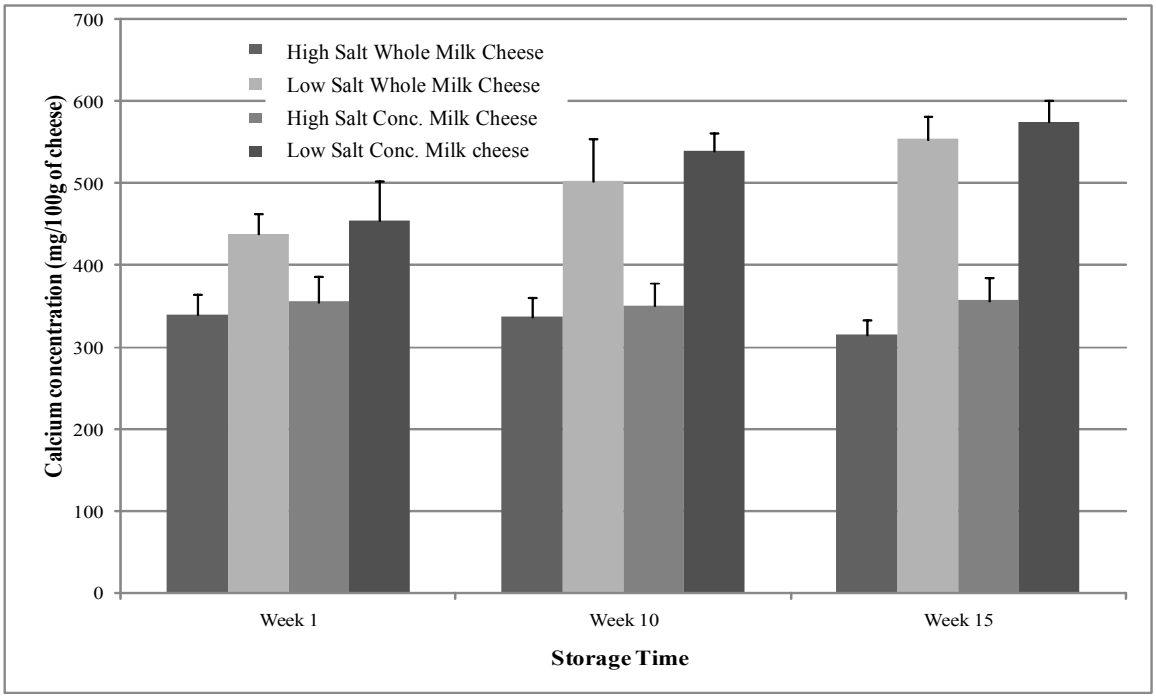

Figure 11. Changes in soluble calcium concentration of increased and smaller S/M cheeses stored at 7.2°C for 15 weeks (average of vacuum and gas flushed packaged cheese, mean of duplicates).

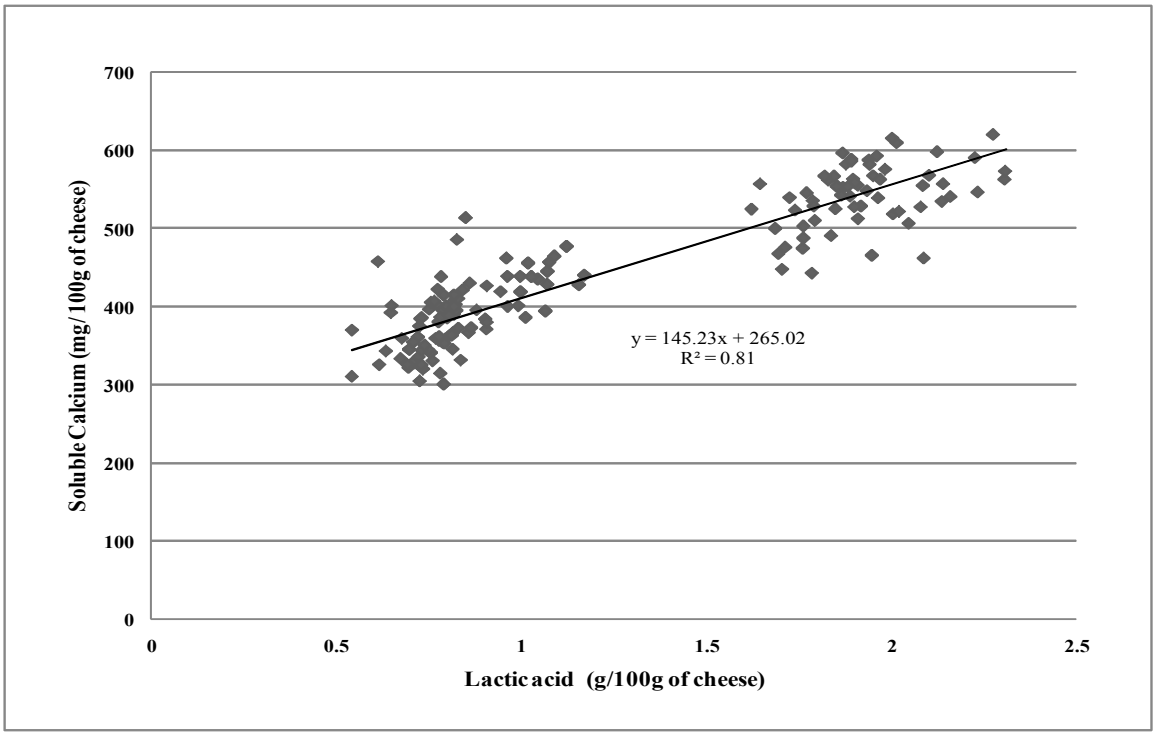

Figure 12. Scatter plot of lactic acid concentration vs. soluble calcium in cheese throughout aging.

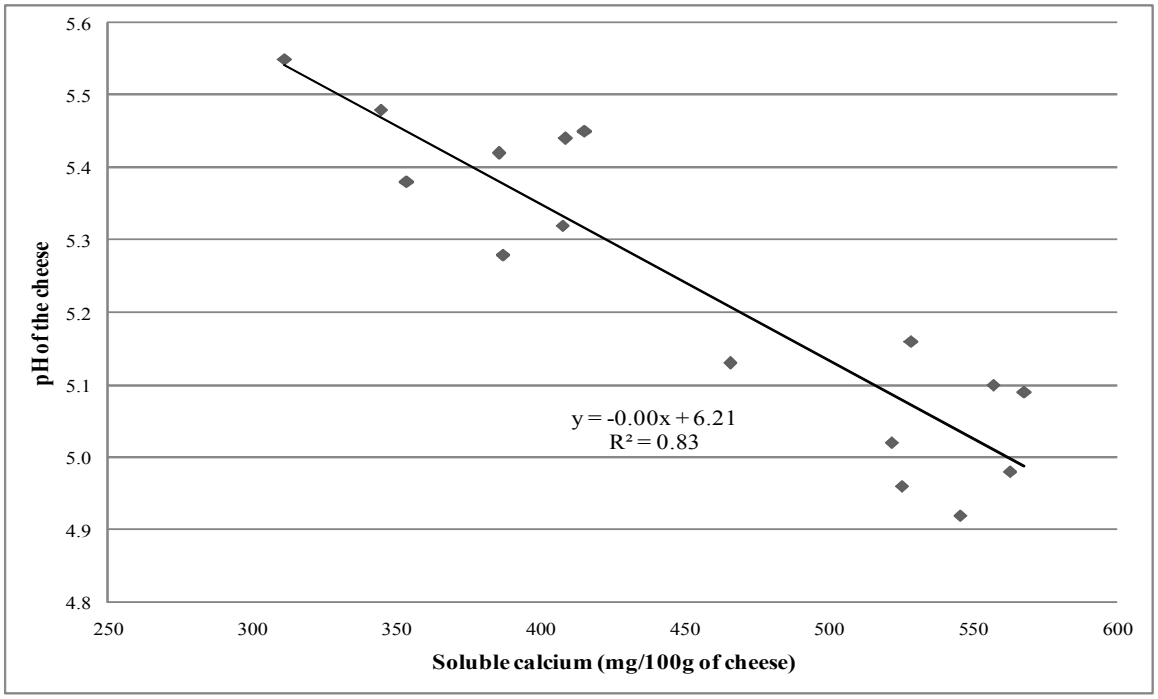

Figure 13. Scatter plot of pH vs. soluble calcium in cheese after 15 weeks of aging.

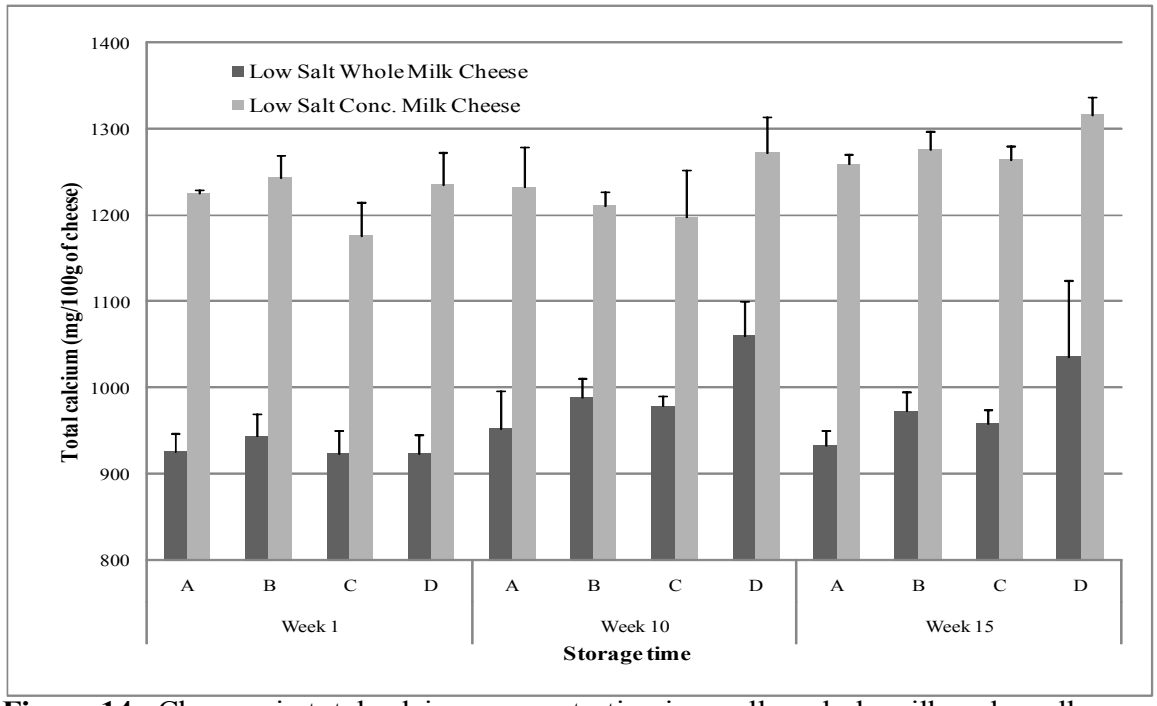

Figure 14. Changes in total calcium concentration in smaller whole milk and smaller S/M concentrated milk cheeses at different positions in cheese cylinder (with A being innermost sample and D being the surface) throughout 15 weeks of storage (average of vacuum and gas flushed packaged cheese, mean of duplicates).

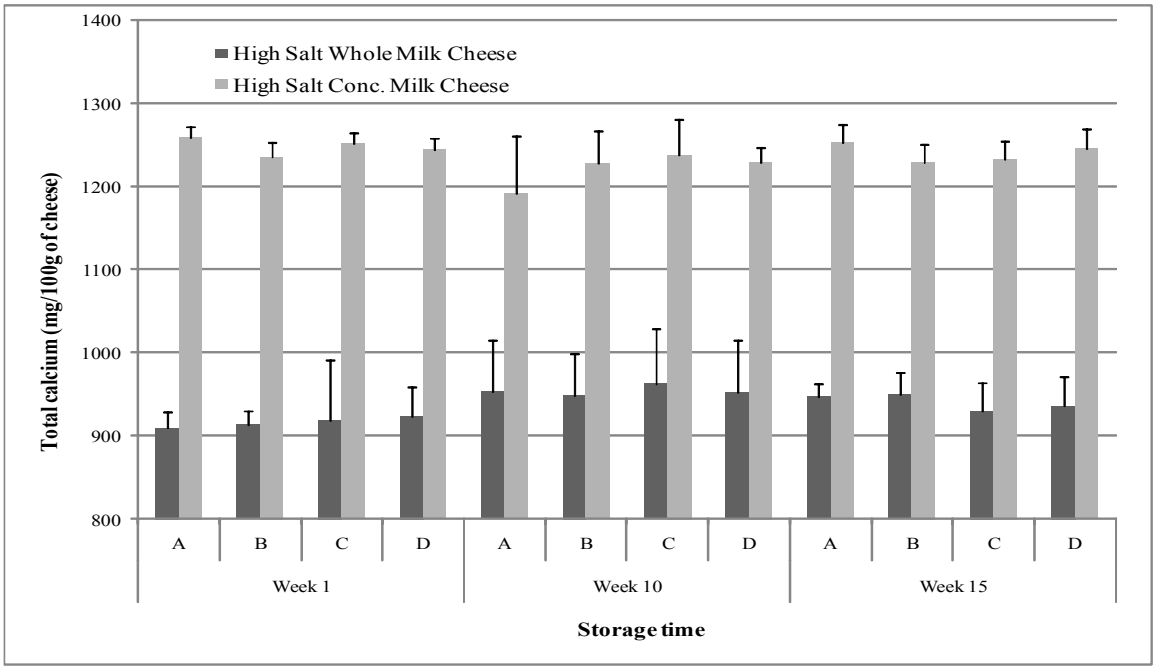

Figure 15. Changes in total calcium concentration in increased S/M whole milk and increased S/M concentrated milk cheeses at different positions in cheese cylinder (with A being innermost sample and D being the surface) throughout 15 weeks of storage. (average of vacuum and gas flushed packaged cheese, mean of duplicates).

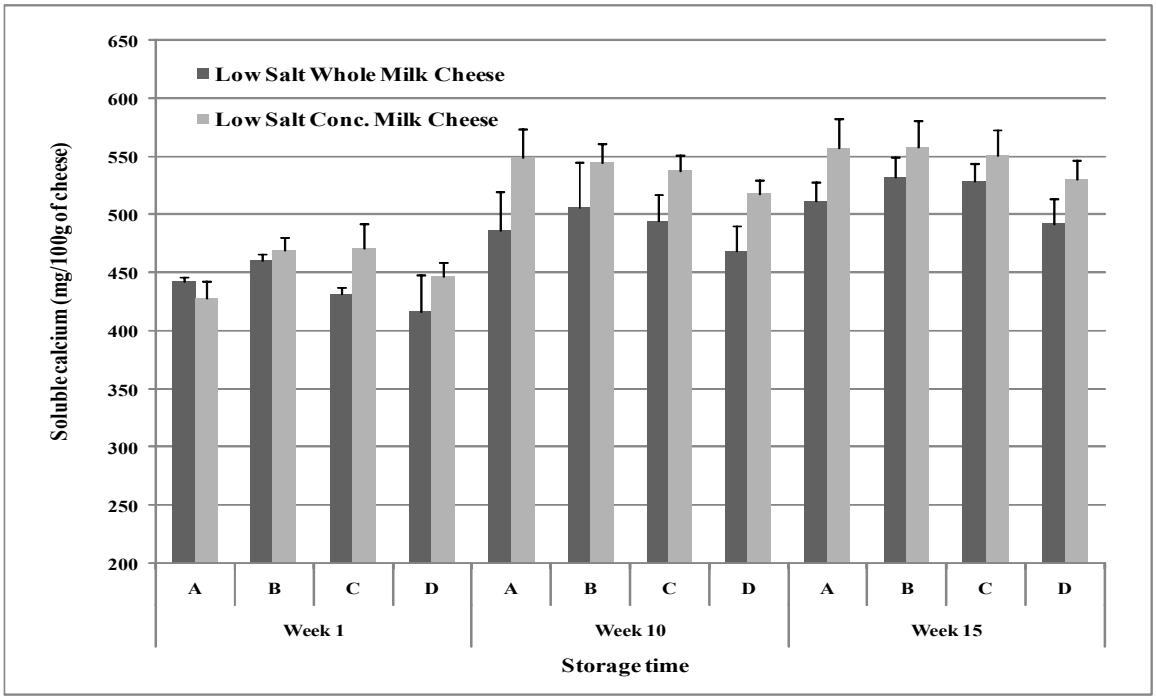

Figure 16. Changes in soluble calcium concentration in smaller S/M whole milk and smaller S/M concentrated milk cheeses at different positions in cheese cylinder (with A being innermost sample and D being the surface) throughout 15 weeks of storage (average of vacuum and gas flushed packaged cheese, mean of duplicates).

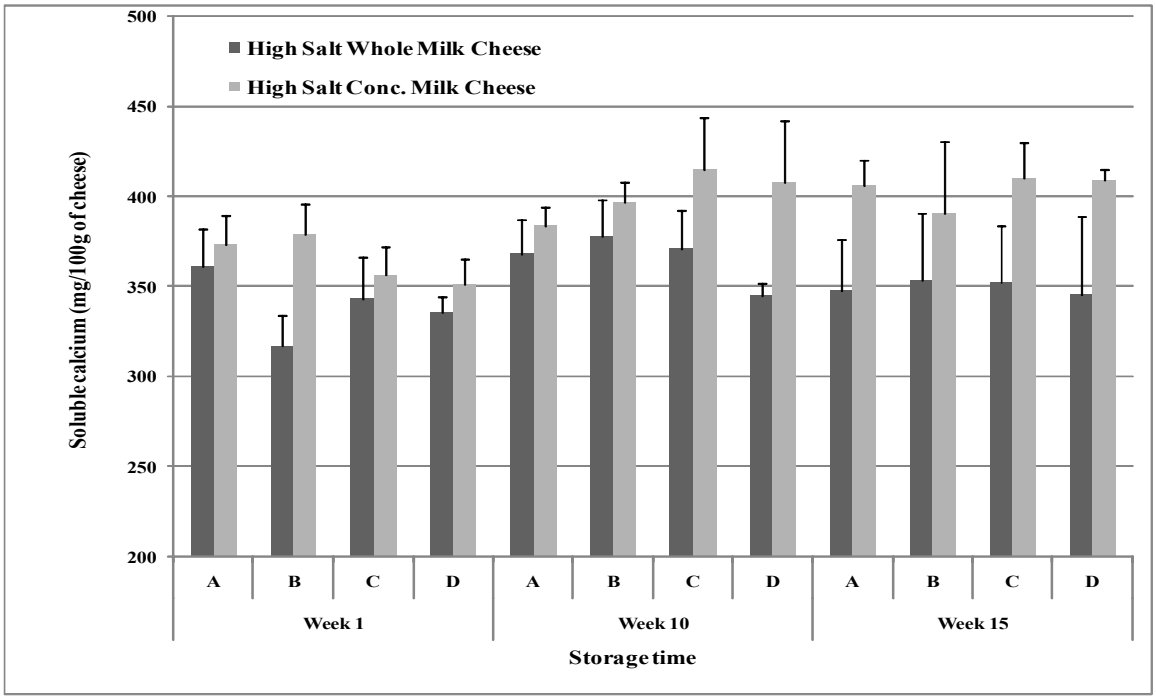

Figure 17. Changes in soluble calcium concentration in increased S/M whole milk and increased S/M concentrated milk cheeses at different positions in cheese cylinder (with A being innermost sample and D being the surface) throughout 15 weeks of storage (average of vacuum and gas flushed packaged cheese, mean of duplicates).

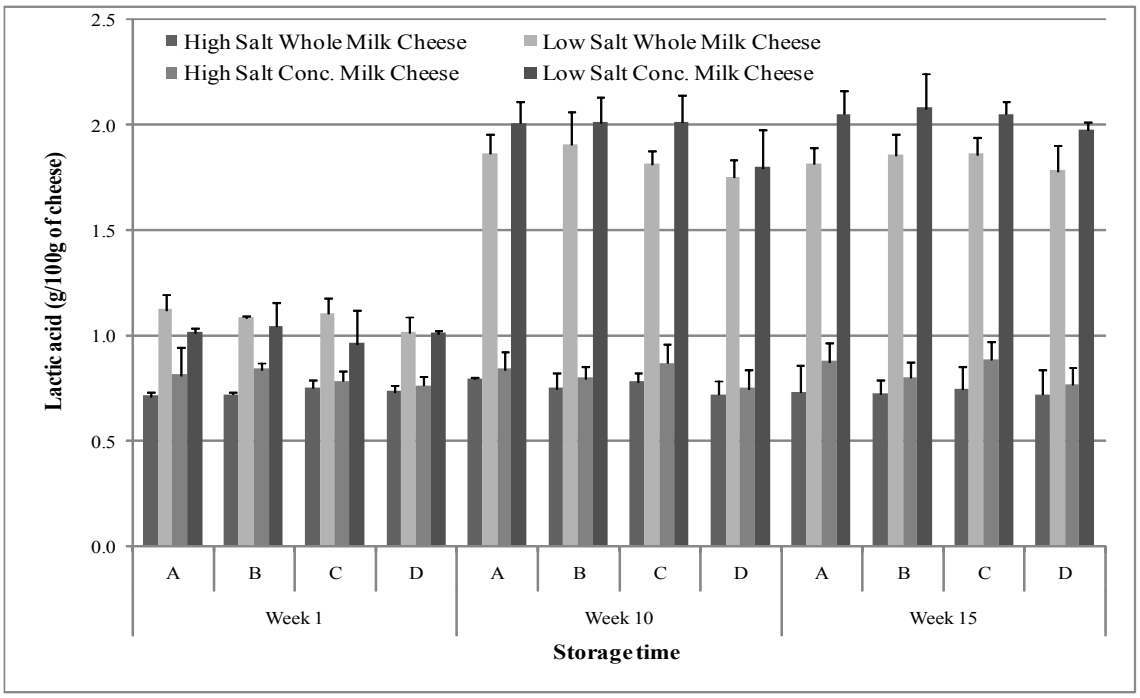

Figure 18. Changes in lactic acid concentration in both increased and smaller S/M whole milk and concentrated milk cheeses at different positions in cheese cylinder (with A being innermost sample and D being the surface) throughout 15 weeks of storage (average of vacuum and gas flushed packaged cheese, mean of duplicates).

VI. CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS

Occurrence of calcium L(+)-lactate crystals is a serious frustration for the cheese industry as L(+)-lactate crystals occur in cheese despite increased sanitation in cheese plants and smaller NSLAB counts in cheese. L(+)-lactate crystals occur in cheese when the concentration of calcium ions and L(+)-lactate increase above saturation, leading to formation of microscopic crystals. Over time, L(+)-lactate crystals can grow in size and become visible. The present research has confirmed that concentration of calcium ions and lactate ions in cheese serum are influenced by protein and lactose concentration in cheese milk, activity of starter bacteria, salt to moisture ratio (S/M), and moisture in cheeses. Factors that influence the saturation point of calcium and lactate ions are storage temperature, pH of cheese and cheese packaging. Previous research showed that storage temperature plays an important role in occurrence of CLC. The solubility of calcium lactate increases at higher temperatures but decreases significantly at low temperatures and any temperature fluctuation during storage will promote nucleation and growth of CLC. The present work contributes data to show that cheeses with reduced moisture can develop CLC earlier compared to cheeses having equivalent pH but increased moisture content. Calcium and lactate ions will reach saturation limit at a smaller concentration in cheeses with low moisture. Complicating matters is the fact that all the above mentioned factors are interactive (Table 1).

The findings stress the fact that, cheese manufactures should continue to be careful regarding sanitation, as contamination of cheese with NSLAB can lead to occurrence of

D(-)-lactate crystals. Most NSLAB can endure increased S/M, grow at low storage temperatures, and metabolize various other sources of carbohydrates to produce lactic acid, which can lead to occurrence of CLC.

This research concludes that the pH of cheese significantly influences the amount of soluble calcium in cheese serum. Lower pH solubilizes colloidal calcium that is bound to proteins, making calcium available to interact with lactate ions (Table 1). Increasing protein concentration, which is commonly used to increase the throughput of a cheese plant, also leads to increased concentration of bound calcium in cheeses. If reverse osmosis, evaporated milk or non fat dry milk is used to increase the protein concentration, then cheese manufacturers also increase the lactose concentration in cheese milk. Increased lactose concentration provides additional substrate for starter bacteria to grow and produce more lactic acid and lower the pH of the cheeses. Additionally, we concluded that the final pH of the cheese is significantly influenced by the starter bacteria and S/M in cheese. Cheeses with smaller S/M or having salt tolerant starter bacteria can end up having low pH leading to increased concentration of lactic acid and soluble calcium, which contribute to occurrence of CLC. Also, the occurrence of CLC on the surface of cheeses with smaller S/M creates a gradient that causes migration of calcium and lactate ions from inside the cheese to the surface, contributing to growth of CLC.

The present research shows that cheese packaging plays an important role in development of CLC. Gas flushed packaging tends to promote CLC sooner and to increased intensity on cheeses than vacuum packaging. Slight loss of moisture from the surface of cheese to the surrounding atmosphere increases the concentration of calcium

lactate, which leads to formation of micro-crystals on cheese surfaces. Micro-crystals grow rapidly in size to become macro-crystals, when the concentration of calcium and lactate ions is above saturation limit.

Another practice commonly employed by cheese plants to speed throughput is milling of cheese at pH 5.5 to 5.8 and use of salt tolerant starter bacteria to reduce the pH of cheese during storage. As the primary function of starter bacteria is to produce lactic acid, uninhibited starter bacteria continue to ferment available lactose to lactic acid and lower pH of the cheese post-packaging. Low pH of the cheeses leads to increased concentrations of calcium and lactate ions trapped in cheese serum, which otherwise are lost during pressing. Salt plays an important role in inhibition of starter bacteria, thus stopping the further production of lactic acid and reduction of pH of cheese post-packaging. Cheeses milled at high pH, but allowed to drop to pH below 5.1, tend to have calcium and lactate higher than the saturation limit of calcium lactate and tend to develop CLC (Table 1).

The findings of the present study point out several recommendations. Cheese manufactures having problems with CLC should consider modifying the cheese making procedure in order to reduce the amount of lactose, lactic acid and soluble calcium in their cheeses. Cheese manufacturers can do so successfully by increasing the ripening time in the cheese vat before addition of rennet, milling the cheese at lower pH, and using enough salt to ensure inhibition of starter bacteria. Introducing a curd washing step before addition of salt can also remove excess calcium and lactic acid from the cheese curd. Also, making sure that cheese blocks are cooled rapidly to storage temperature after pressing is also important to prevent NSLAB from growing during storage. Cheese

manufactures must keep complete records of the pH of each vat of cheese during manufacturing and during 10 weeks of aging in order to track and predict CLC formation. Cheeses having pH below 5.1 should be considered high risk cheeses and should not be sold as cubed or shredded cheese in retail, as CLC are likely to develop below pH 5.1.

FUTURE RESEARCH

More research needs to be done to determine the mechanism and growth rate of CLC in cheese. To expand on work done by Dr. Metzger's research group (2006),which examined the buffering properties of Cheddar cheese, and developing models to predict the availability of calcium to interact with various ion in cheese under various conditions, will be very beneficial in understanding occurrence of CLC. Research should take into consideration the interaction of calcium and lactate ions with other ions in cheese serum. Solubility curves of calcium lactate at different pH, temperature, and ionic concentrations should be developed. This research will help to develop accurate models to predict the growth rate of CLC, enabling plants to schedule sales carefully to prevent unappealing products from getting to consumers.

Another very interesting field of research in prevention of occurrence of CLC is in the direction of limiting the availability of calcium ions to interact with lactate ions. Possibilities of using starter cultures that produce equivalent amounts of citrate and lactate should also be explored, as citrate will compete with lactate to bind with calcium ions and reduce the availability of calcium to bind with lactate. Considerations should also be given to use of additives that can be blended with salt and added to cheese during salting. Additives that are likely to succeed would have the property of preferentially

binding calcium; calcium salts of additives should be highly soluble and should be already approved for use as food additive under Code of Federal Regulations.

Table 1. Table summarizing the effect of various factors on occurrence of CLC in Cheddar cheese.

pH of cheese	Measurable variable	Measured value range in cheese milk	Predicted outcome in cheese	Occurrence of CLC
pH 5.3 and above	Protein concentration	3.0 to 3.3%	Soluble calcium limited in cheese (280 to 350 mg/100 g)	no
		3.4 to 3.9%	Intermediate	no
		4.0 to 4.5%	Soluble calcium limited in cheese (300 to 380 mg/100 g of cheese)	no
	Lactose concentration	4.7 to 4.9%	Residual lactose 0.3 to 1.5 g/100 g of cheese	no
			Lactic acid limited in cheese (0.6 to 0.9 g/100 g)	
		5.0 to 5.3%	Residual lactose 0.8 to 1.8 g/100 g of cheese	no
			Lactic acid limited in cheese (0.6 to 0.9 g/100 g)	
pH 5.1 and below	Protein concentration	3.0 to 3.3%	Soluble calcium not limiting in cheese (380 to 450 mg/100 g)	yes
		3.4 to 3.9%	Intermediate	yes
		4.0 to 4.5%	Soluble calcium not limiting in cheese (400 to 480 mg/100 g)	yes
	Lactose concentration	4.7 to 4.9%	Residual lactose 0.0 to 0.2 g/100 g of cheese	yes
			Lactic acid not limiting in cheese (1.6 to 1.9 g/100 g)	
		5.0 to 5.3%	Residual lactose 0.0 to 0.3 g/100 g of cheese	yes
			Lactic acid not limiting in cheese (1.6 to 2.2 g/100 g)	
	Salt to moisture ratio	3.0 to 4.0	Lactic acid production not inhibited, all lactose fermented to lactic acid, leads to pH below 5.1	yes
		4.1 to 5.0	Lactic acid production inhibited, pH remains above 5.1	no
	Storage temperature	2 to 5°C	Decreases solubility of calcium lactate (not limiting)	likely
		7 to 10°C	Recommended	maybe
		11 to 15°C	Increased lactic acid production (not limiting)	likely