2011 Annual Report

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WDC Annual Report

Published May 1, 2012 by the Western Dairy Center.

Our annual report is a technical overview of WDC funded research and other Center activities during fiscal year 2011. We prepared this report for organizations funding WDC and for fellow dairy researchers. This report describes projects in progress and interpretations of data gathered to date. It is not a peer-reviewed publication.

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Cover design by Kandice Johnson.
Edited by Donald J. McMahon and Carl Brothersen.
Mission of the Western Dairy Center

To foster innovation and technology transfer by addressing strategic needs of the dairy industry, as a

National dairy foods research center and

Regional hub for dairy science and education.
Industry Members

DSM
http://www.dsm.com/

Chr. Hansen
http://www.chr-hansen.com/

Glanbia Nutritionals
http://www.glanbianutritionals.com/

Grande Cheese Company
http://www.grande.com/

Schreiber Foods Inc.
http://www.schreiberfoods.com/

Kraft Foods Inc.
http://www.kraftfoodscompany.com/

Lin Manufacturing & Design, LLC
http://www.linmfg.com/
Principal Investigators & Students

The following principal investigators, and their students, contributed to research and program activities conducted within the Western Dairy Center during the reporting period.

David Britt, USU
Jeffery R. Broadbent, USU
   Craig Brighton, USU
   Taylor Oberg, USU
Carl Brothersen, USU
Daren Cornforth, USU
MaryAnne Drake, North Carolina State University
E.Allen Foegeding, North Carolina State University
Balasubramanian Ganesan, USU
Conly Hansen, USU
Korry Hintze, USU
Silvana Martini, USU
Donald J. McMahon, USU
   Kelly M. Brown, USU
   Nicoletta Fucà, visiting scholar from CoRFiLaC in Italy
   Ying Lu, USU
   Erik Oberg, USU
   Faith Ortakci, USU
   Jess Perry, USU
   Ranjeka Wadhwani, USU
William R. McManus, USU
Michael Qian, Oregon State University
J.A. Torres, Oregon State University
Marie Walsh, USU
Robert Ward, USU
   Mike Young, USU
   Albert Lihong Zhou, USU
Bart Weimer, University of California at Davis

DTIL Staff

Donald McMahon, Director
Carl Brothersen, Associate Director
Kimberly Rasmussen, Administrative Assistant
Kandice Johnson, Publications Assistant
David A. Irish, Dairy Processing Specialist
William McManus, Research Scientist
Balasubramanian Ganesan, Research Scientist
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Technology Transfer
PUBLICATIONS

Journal Articles & Papers


Hintze, K.J., Benninghoff, A.D. and Ward, R.E. 2012. Formulation of the Total Western Diet (TWD) as a basal diet for rodent cancer studies. Journal of Agricultural and Food Chemistry Accepted December 2011


Seminars & Presentations


Hintze, K.J., Ward, R.E., and Benninghoff, A. 2011. Formulation of the Total Western Diet (TWD) as basal diet for rodent cancer studies. American Chemical Society Annual Meeting, August 28-September 1, Denver, CO (oral presentation)

Jimenez-Flores, R., and Ward, R.E. 2011. The MFGM in whey and its physical and nutritional properties. 6th International Whey Conference, September 18-21, Chicago, IL (oral presentation)


Tansawat, R., Ward, R.E., Martini, S. and Cornforth, D. 2011. Chemical characterization of grass- and grain-fed beef related to meat quality and flavor attributes. IFT/AECT Utah Food & Candy Expo, Institute for Food Technologists Bonneville Section and American Association of Candy Technologists Rocky Mountain Section (poster presentation)


Graduate Theses & Dissertations


Additional Publications

### 2011 Workshops & ShortCourses

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<tr>
<td>Artisan Cheese Symposium</td>
<td>February 8, 2011</td>
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<td>Basic Cheesemaking Course</td>
<td>February 9-11, 2011</td>
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<td>Advanced Cheesemaking Course</td>
<td>February 22-25, 2011</td>
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<td>Western Dairy Center Annual Meeting</td>
<td>May 10-11, 2011</td>
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<td>Quality Control Workshop (GMP)</td>
<td>May 16-17, 2011</td>
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<td>HACCP Workshop</td>
<td>May 18-20, 2011</td>
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<td>Advanced Sanitation Workshop</td>
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<td>Employee Based Food Safety Workshop</td>
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### 2012 Workshops & ShortCourses

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<tr>
<td>Artisan Cheese Symposium</td>
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<td>Basic Cheesemaking Course</td>
<td>February 8-10, 2012</td>
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<tr>
<td>Advanced Cheesemaking Course</td>
<td>February 21-24, 2012</td>
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<tr>
<td>Western Dairy Center Annual Meeting</td>
<td>May 8, 2012</td>
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<td>Quality Control Workshop (GMP)</td>
<td>May 14-15, 2012</td>
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<td>HACCP Workshop</td>
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<td>Advanced Sanitation Workshop</td>
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<td>Employee Based Food Safety Workshop</td>
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<td>Food Safety Modernization Act Workshop</td>
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<td>SQF Workshop</td>
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2013 WORKSHOPS & SHORTCOURSES

Artisan Cheese Symposium  February 5, 2013
Basic Cheesemaking Course  February 6-8, 2013
Advanced Cheesemaking Course  February 19-22, 2013
Western Dairy Center Annual Meeting  May 7-8, 2013
Quality Control Workshop (GMP)  May 13-14, 2013
HACCP Workshop  May 15-16, 2013
Advanced Sanitation Workshop  May 20-21, 2013
Employee Based Food Safety Workshop  May 22-24, 2013
Statistical Process Control Workshop  May 29-30, 2013
Food Safety Modernization Act Workshop (dates are tentative)  June 4, 2013
SQF Workshop (dates are tentative)  June 5-6, 2013
PREPARATION OF CHEDDAR CHEESE EXTRACT (CCE)

Cheddar Cheese Extract (CCE) is a concentrated extract of the water soluble fraction of Cheddar cheese. The cheese used for the manufacture of CCE should be congruent with the objectives of the study in which it is being used. For example, if the study is to determine the metabolism of non-starter lactic acid bacteria, an aged cheese should be used. CCE can be made from any type of cheese.

To manufacture CCE, the cheese is first cut into cubes and comminuted to particles ~2 mm in size. Twice the weight of distilled water is added, and the mixture is stirred and gradually heated to 50°C. At about 40-45°C the cheese aggregates to form a continuous mass. The temperature is maintained at 50°C for 20 minutes with constant stirring. The solid cheese is then removed from the mixture and discarded. The liquid portion is passed through a cloth filter to remove the particulates then ultrafiltered, with the retentate recycled to the feed tank. Diafiltration water is added continuously to the retentate until the permeate is clear. The UF permeate is then concentrated using reverse osmosis and the retentate collected. The solids level in the RO permeate is determined and the product is refrigerated or frozen for later use. When used, the product can be diluted to the needed solids level.

The DTIL’s Continuing Involvement

The Process of making CCE by DTIL

- Cheese
- Heat 122°F
- Stir 20 min
- Drain Liqu.
- Dil Water
- Cheese
- Filter 10<sup>−2</sup> – 10<sup>−1</sup> μM
- 1 Pass
- Multiple passes
- Max. Conc.
- Freeze liquid
CHEESE AWARDS

Many of the graduates of the Western Dairy Center cheesemaking courses are now making award winning cheese. A list of awards and cheesemakers is given below.

Beehive Cheese Company

World Cheese Awards 2010
Full Moon. Earthy cheese made with raw milk.

Idaho Milk Producers Association 2009 Competition
Promontory, with Rosemary. This Irish style cheese is buttery, full-bodied, and lively with snappy, citrus-like, and fruity notes. Warms on your tongue and leaves a subtle sharp note in your mouth.

Idaho Milk Producers Association 2010 Competition
Promontory, with Habanero. This Irish style cheese is buttery, full-bodied, and lively with snappy, citrus-like, and fruity notes. Warms on your tongue and leaves a subtle sharp note in your mouth.

American Cheese Society 2011 Competition, World Cheese Awards 2010
Promontory. This Irish style cheese is buttery, full-bodied, and lively with snappy, citrus-like, and fruity notes. Warms on your tongue and leaves a subtle sharp note in your mouth.

Barely Buzzed. A full bodied cheese with a nutty flavor and smooth texture. The cheese is hand rubbed with a Turkish grind of Colorado Legacy Coffee Company's (The Cheesemakers brother) "Beehive Blend". The blend consists of a mix of South American, Central American, and Indonesian beans roasted to different styles. French Superior Lavendar buds are ground with the coffee, and the mixture is diluted with oil to suspend the dry ingredients in the rub. The rub imparts notes of butterscotch and caramel, which are prevalent near the rind but find their way to the center of the cheese. The cheese is aged on Utah Blue Spruce aging racks in our humidity controlled caves and moved to different temperatures during the aging process to develop texture and flavor.

American Cheese Society Competition 2011
SeaHive. Hand rubbed with Beehive wildflower honey and local Redmond RealSalt. The honey is harvested from a local farm where the bees visit wildflowers and fruit orchards. The salt is from an ancient sea bed near Redmond, Utah and contains unique flecks of color that are the result of more than 50 natural trace minerals.

World Cheese Awards 2009
American Cheese Society Competition 2008
Apple Walnut Smoked. This cheese is cold-smoked in small batches using local Utah walnut shells and slices of red apple. Apple Walnut Smoked has a subtle sweet and nutty flavor with a hint of smoke.

American Cheese Society Competition 2009, 2011
Big John’s Cajun. This spicy hand-rubbed cheese packs a heated punch as you near the rind. The spiciness of the rub is a nice compliment to the creamy texture of the cheese.
Gold Creek Farms

World Championship Cheese Competition 2010, 2012
American Cheese Society Competition 2010, 2011
Smoked White Cheddar. Lightly smoked with cherry wood chips.
Cheesemaker, Fernando Chavez

World Championship Cheese Competition 2012
American Cheese Society Competition 2011
Smoked Parmesan. Tastefully smoked with cherry wood chips.
Cheesemaker, Fernando Chavez

Lark's Meadow Farms

2011 American Cheese Society Competition
Dulcinea. A Basque style cheese with a long lasting flavor that has a subtle, tangy twist and fruity undertones.
Cheesemaker, Kendall Russell

Snowy Mountain Sheep Creamery

2011 United States Championship Cheese Contest
Timpanogos Peak Blue Cheese. A creamy cheese with a little blue flavor.
Cheesemaker, Stig Hansen

Utah State University

Idaho Milk Processors Competition 2010, 2011
Old Juniper Cheese. A cheddar-style aged cheese that has become a favorite among our cheese connoisseurs looking for a fully mature cheese. Its unique warm rich flavor will be a success on any occasion.
Cheesemaker, Doug Palmer

Idaho Milk Processors Competition 2011
Aggiano Cheese. Aged for 12 months or more to give it an old style parmesan flavor. Great for snacking or shredding on your favorite pasta dish or salad.
Cheesemaker, Doug Palmer
Research Project Reports
Effect of fat removal on cheese microenvironment and starter culture metabolism in cheddar cheese

Jeff Broadbent*

*Principal Investigator: Utah State University, Western Dairy Center, Logan, UT 84322-8700
email: jeff.broadbent@usu.edu


ABSTRACT

Flavor development in bacterial-ripened cheese (e.g., Cheddar) is primary due to the action of lactic acid bacteria (LAB) and enzymes in the ripening curd. Knowledge of the mechanisms by which LAB affect cheese flavor has facilitated industry efforts to accelerate or intensify flavor development in many traditional cheese varieties. Unfortunately, empirical efforts to extend this information into low-fat cheese systems have not proved successful, and low-fat products continue to suffer from low intensity of desirable flavors and/or from pronounced off-flavor defects.

It is the hypothesis of this project that flavor problems in low-fat products are most likely explained by a scenario wherein starter physiology (and thus overall metabolism) is altered by differences in the physico-chemical environment in ways that affect the production of flavor- and aroma-active metabolites. To this hypothesis, we are working to determine how Lactococcus lactis metabolism is affected by the changes in cheese microenvironment, and especially S/M ratio, that occur as a consequence of fat reduction. Specifically, we are evaluating gene expression and volatile metabolite profiles from L. lactis strains cultured in Cheddar cheese extract (CCE) medium adjusted to reflect the differing microenvironments of low- and full-fat cheese.

Efforts to develop effective culture systems for low-fat cheese will require more specific knowledge of how the cheese physico-chemical environment affects starter cell physiology. Knowledge generated from this study will facilitate industry efforts to develop starter strains, through mutagenesis or other methods, that can enhance flavor development in low-fat and nonfat Cheddar cheese.

BACKGROUND

Transformation of bland-flavored curd into delicious mature cheese is a complex and dynamic process whose intricacies are scripted by the milk type and composition, the cultures and enzymes present or added to the cheese milk, and the manufacturing and ripening conditions used. Many cheeses need to be stored at low temperature for months or even years before they attain characteristic flavor and body attributes. During this time, termed the curing or ripening period, microorganisms and enzymes in the cheese matrix act on milk constituents in a manner that is partly dictated by the curd microenvironment (e.g., cheese pH, a空气, salt content, Eh, temperature, etc.) and which ultimately gives the desired product (Fox et al., 1993).

Types of LAB that occur in internally bacterial-ripened cheeses such as Cheddar, Dutch, Swiss, and Italian varieties, include deliberately added strains (e.g., starters and adjunct cultures) and adventitious species (primarily nonstarter LAB or NSLAB) that enter cheese through milk or processing equipment. Modern sanitation and Good Manufacturing Practices help minimize initial numbers of NSLAB in cheese, yet these organisms invariably appear and grow to high numbers during ripening (Peterson and Marshall, 1990). In Cheddar cheese, numbers of Lactococcus lactis starter bacteria frequently exceed 10⁹ colony-forming units (cfu) per gram when ripening begins. As maturation proceeds, the harsh cheese ripening environment (little or no residual lactose, pH 5.0 to 5.3, 4-6% salt in moisture, 5-13°C) gradually takes its toll and starter viability declines. A fraction of the dying starter cells undergo autolysis, which releases intracellular enzymes and cellular components (e.g., sugars and nucleic acids) into the cheese matrix (Fryer, 1969). At the same time,
NSLAB populations (whose initial numbers are typically less than $10^2$ cfu/g in cheese made under good sanitary conditions with high quality pasteurized milk) begin to grow and eventually plateau at cell densities of $10^4-10^5$ cfu/gram after 3-9 mo of aging (Peterson and Marshall, 1990). Microbiological studies have shown NSLAB populations in bacterial-ripened cheeses may be quite diverse, but are usually dominated by facultatively heterofermentative species of lactobacilli or, far less frequently, by pediococci (Fryer, 1969; Beresford et al., 2001; Broadbent et al., 2003; Broome et al., 1990a; Crow et al., 2001; Sherwood, 1939). Depending on the species that is used (and whether or not the particular strain can grow in ripening cheese), populations of adjunct bacteria may mirror the trend for starter or NSLAB fractions.

Though a link between LAB activity and cheese flavor attributes was postulated more than 100 years ago (see Fryer, 1969), the variation and complexity that exists in cheese microbiota and enzyme content confounded early efforts to establish a causal role for these bacteria in flavor development. This limitation was overcome in the late-1950's, when sensory studies of aseptically manufactured Cheddar cheese showed that starter-free, gluconolactone-acidified cheese failed to develop Cheddar flavor, while cheese made with *Lc. lactis* starter bacteria developed characteristic, balanced flavor (Law et al., 1976; Reiter et al., 1967). The same investigations also showed NSLAB could modify basic flavor notes and accelerate flavor development. More recently, use of *Lactobacillus* spp. NSLAB isolates as adjunct cultures for Cheddar cheese manufacture has indicated these bacteria may influence flavor in at least 3 ways: they may intensify (i.e., accelerate) typical flavor development, impart atypical (but desirable) flavor notes, or promote off-flavor development (Fryer, 1969; Crow et al., 2001; Sherwood, 1939; Broome et al., 1990b; Lynch et al., 1999; McSweeney et al., 1994; Swearingen et al., 2001). In addition, NSLAB have also been associated with cheese quality defects such as open body (via gas production) and formation of calcium lactate crystals (Fryer, 1969; Johnson et al., 1990; Khalid et al., 1990).

Given the causal role of LAB in flavor development, efforts to define the biochemical basis for flavor changes in cheese have logically focused on the microbiology and physiology of species found in cheese (for recent reviews see Beresford et al., 2001; El Soda et al., 2000; Fox and Wallace, 1997; Marilley and Casey, 2004; Rattray and Fox, 1999). Those efforts have identified many of the most important biochemical and chemical processes in maturation, and have shown starter, adjunct, and NSLAB have an intimate role in most of those processes including lactose fermentation, conversion of milk proteins (primarily caseins) into peptides and free amino acids, catabolism of amino acids into volatile aroma compounds, lipase/esterase activity, and citrate catabolism.

Knowledge of the mechanisms by which LAB affect cheese flavor has facilitated industry efforts to promote flavor development in many traditional cheese varieties, but empirical efforts to extend this information into low-fat cheese systems have not proved successful. As a result, low-fat or nonfat bacterial-ripened cheeses continue to suffer from low intensity of desirable flavor or pronounced off-flavors. From a purely technological perspective, many of the flavor limitations in low-fat cheese might be overcome through the addition of dairy flavors or enzymes. The costs associated with this technology are estimated to be 1-5 cents per pound (T. Bhowmik, pers. Comm.), however, and would be incurred on top of the already premium cost to make low-fat cheese. As a result, exclusive use of flavors or enzyme technologies to solve the flavor problems in low-fat products is not an attractive option at this time. Industry concerns about product cost is supported by a recent consumer study that found there is little support for low-fat cheese product, if their cost is substantially higher than that of full fat cheese (M.A. Drake, pers. comm.).

A more cost-effective solution to the flavor problems in low-fat cheese can likely be found through combining flavors or enzymes with culture systems that deliver better flavor in these products. Compared to dairy flavors or enzyme addition, culture technology is an inexpensive means to secure flavor development, and one that should be further explored and optimized for industry to offset the price concerns associated with low-fat cheese products.

These deficiencies in low-fat cheese flavor are most likely explained by one of two scenarios: 1) starter physiology (and thus metabolic end-product profile) is the same in all cheeses, but sensory perception of those metabolites is altered by differences in the physicochemical environment (e.g., fat, moisture, or salt in moisture contents); or 2) starter physiology itself (and thus overall metabolism) is altered by differences in the physicochemical environment in ways that affect the production of flavor- and aroma-active metabolites. A third possibility, of course, is that each of these scenarios has a role in the atypical flavor profile of low- and nonfat cheese systems.

Though little information is available on the differences in flavor- or aroma-active metabolites in full versus reduced fat cheese, work by Milo and Reinuccius (1997) noted important differences in volatile components from full fat versus 40% fat reduced Cheddar cheese, and suggested that these differences might be due to the higher water content in the reduced fat cheese. More recently, Carunchia Whetstine et al. (2006) investigated sensory properties and volatile chemical profiles in full- and 50% reduced fat Cheddar manufactured by a novel fat removal process after aging is complete. Those authors discovered that the great majority of volatile aroma-active compounds remained in the aqueous-containing cheese matrix versus the removed fat fraction and, more interestingly, found
that cheeses with either fat level had nearly identical flavor profiles. Given these findings, and the central role of lactic acid bacteria in flavor development, it is our hypothesis that the second scenario provided above (i.e., starter physiology and metabolism is altered by perturbations in the cheese microenvironment) has the greatest effect on flavor development in low- or nonfat cheese. If this hypothesis is correct, efforts to develop effective culture systems for low-fat cheese will require more specific knowledge of how the cheese physico-chemical environment affects starter cell physiology. Thus, the goal of this study is to determine how Lactococcus lactis metabolism is affected by fat reduction and its concomitant changes in cheese make procedure impart on the cheese microenvironment (e.g., S/M ratio, lactate content, pH, etc.). This knowledge is expected to generate basic information industry needs to develop starter strains, through mutagenesis or other methods, which enhance flavor development in low-fat and nonfat Cheddar cheese. To attain the goals of this project, we will complete the following experiments:

RESEARCH PLAN

Objective 1.

Develop and utilize a model system to investigate the impact of different cheese microenvironments on the physiology of commercial L. lactis starter bacteria.

MATERIALS AND METHODS

Objective 1. Develop and utilize a model system to investigate the impact of different cheese microenvironments on physiology of commercial L. lactis subsp. lactis and L. lactis subsp. cremoris starter bacteria.

To ensure their relevance to cheese, experiments performed under this objective will utilize cells incubated in a Cheddar Cheese Extract (CCE) medium using laboratory-scale (1 to 2 L) bioreactors. The CCE medium for these experiments was prepared from a large batch (>500 lbs) of full and low-fat Cheddar cheeses so that we would have sufficient uniform CCE powder to meet all the needs of the project and the partner NSLAB project submitted by Dr. Steele entitled “Selecting bacterial cultures to enhance low-fat cheese flavor”. Full and low-fat Cheddar cheeses were manufactured at the USU dairy plant and aged 6 weeks (the time at which HPLC analysis confirmed residual lactose and galactose levels had fallen below quantifiable levels), then shredded and frozen in preparation for subsequent processing into CCE powder. Because the lyophilization step for CCE preparation imposed a significant time delay, we eventually worked with the Western Dairy Center staff to develop a novel, high-throughput system for generating the final CCE aqueous extract using our pilot plant UF capabilities. Once complete, samples of the CCE were collected for analysis of residual lactose and galactose, pH, NaCl, D/L lactic acid, and small peptides and amino acids using standard methods (Marshall, 1993), then aliquots were frozen at -20°C. Some samples were shipped to Dr. Steele’s lab for use in the partner NSLAB project.

To determine the impact of cheese microenvironment on starter volatiles production and transcriptional profile, we selected six different L. lactis starter bacteria that include strains recommended by industry suppliers for aged full fat or low-fat cheese production (Table 1). To avoid potential overlap in strains from different culture companies, the uniqueness of each strain was analyzed by plasmid DNA profiles. Working cultures were prepared from frozen stock cultures through two successive transfers (0.1% inoculum) in sterile skim milk at 30°C for 16-18 h without pH control.

Microbes in cheese are found in the aqueous fraction, so experiments to replicate the microenvironment of low-fat and full-fat cheese will be performed by adjusting CCE composition so reflect the aqueous fraction of either low-fat or full-fat cheese (Table 2). To accomplish this, CCE collected from low-fat cheese will be aseptically transferred into 1 L bioreactors, adjusted to obtain specific environmental conditions listed in Table 2, then incubated under temperature (10° or 30°C) and pH (5.1) control. Important note: Test conditions listed in Table 2 were established by consultation between Dr. Broadbent and Drs. Donald J. McMahon, Mark E. Johnson, and James L. Steele.

The first series of experiments will evaluate differences in the transcriptomes and in volatile compounds production by L. lactis strains during incubation in CCE designed to mimic low-fat or full-fat cheese microenvironments (Table 2) at 10°C, except that redox will not be adjusted. Those experiments will be followed by a series of independent experiments to investigate the effects of salt-in-moisture (3.7 vs 4.75%), temperature (10 vs 30°C), and redox (+340 vs -200).

For each of these experiments, CCE composition will be adjusted as desired (Table 2), then the bioreactor will be inoculated at approximately 1 x 10⁶ CFU/ml with a single strain of fresh, milk-grown working culture and the pH will be maintained at 5.1 throughout incubation by addition of 15% (v/v) NH₄OH with an agitation rate of 100 rpm. Cells will be incubated for various times (see relevant sections below) before samples are collected for transcriptome or volatiles analysis.

Production of volatile flavor compounds.

The influence of cheese environment on volatiles production by the starter will be performed under the supervision of Dr. Robert Ward using a solid phase
microextraction GC-MS approach essentially as described by Lee et al. (2007). Cells will be incubated in the CCE as described above then samples will be collected for volatiles analysis at time 0 and after 72 h. Once volatiles analyses are complete, Drs. Broadbent, Ward and Steele will individually review the volatiles data obtained from these experiments and prioritize the lactococcal RNA samples that should be included in the transcriptome component of this work. Priority for these selections will be based on pair-wise comparisons that each professor feels will provide the greatest insight to effect of microenvironment on cell physiology. It is our expectation that prioritization will allow us to reduce the number of strains that will be included in transcriptional profiling from 6 down to 3 or fewer.

**Transcriptional studies.**

The influence of cheese environment on the transcriptional profile of different starter bacteria will be performed under the supervision of Dr. Jeff Broadbent using a lactococcal full-genome Affymetrix microarray that is available through the USU Center for Integrated Biosystems. RNA samples for microarray studies will be extracted from 20-100 ml of cell culture (depending on growth phase and cell density) collected at time 0 and after 72 h as described above. The cells will be harvested by centrifugation at 4000 rpm for 4 min in a rotor prewarmed to culture growth-temperature because preliminary microarray hybridizations indicated cell exposure to a chilled rotor resulted in the induction of several cold-shock genes (Wechter and Steele, unpublished). The cell pellet will immediately be suspended in 10 ml of RNAProtect (Qiagen, Valencia, CA) and incubated at room temperature for 10 min. After RNAProtect treatment, the cells are pelleted by centrifugation, suspended in 1 ml RNase-free sterile H2O containing 20 mg/ml lysozyme, 8 U mutanolysin, and 75 uL rifampicin (25 mg/mL in methanol). The cells are incubated at 37°C for 15 minutes in a shaker incubator (240 rpm), then total RNA is isolated using the Aurum Total RNA mini Kit (Bio-Rad Laboratories, Catalog #732-6820) scaled to 10X as directed by the kit supplier. The resulting total RNA sample is frozen at -80°C until needed.

Synthesis and biotin labeling of cDNA from selected total RNA samples will be performed using a series of protocols recommended by Affymetrix (see GeneChip® Expression Analysis Technical Section 3: Prokaryotic Sample and Array Processing; www.affymetrix.com/support/technical/manual/expression_manual.aifix). Hybridization of cDNA samples to microarrays and array scanning will be performed in the Affymetrix core facility at the USU Center for Integrated Biosystems. Statistical analysis and interpretation of microarray data will be performed by personnel in Drs. Broadbent’s lab as described previously (Smeianov et al. 2007; Broadbent et al. 2010).

Measurement of microarray spots intensity levels is, hypothetically, directly correlated with the abundance of the corresponding mRNA. However, the intensity unit is arbitrary, and ratios are relative between samples being compared. As a result, there is a need for absolute quantification of mRNA (or indirectly via cDNA) levels between samples, in order to confirm the findings suggested by microarray data. Real-time RT-PCR is a method that allows confirmation and quantification of microarray results with a higher throughput and accuracy than those of Northern blots or RNA slot blots. Thus, key genes and operons identified in the microarray analysis will be confirmed by real-time RT-PCR. Genes will be chosen for analysis by real-time RT-PCR based upon the level and reproducibility of changes in expression observed in the microarray experiments. Equipment and methods for real-time RT-PCR are established in Dr. Broadbent’s laboratory (Smeianov et al. 2007; Broadbent et al. 2010).

In summary, it is our expectation that the combined results from volatiles profiling and transcriptional studies will generate critical knowledge needed to understand how starter physiology is altered by perturbations in the cheese microenvironment. The molecular information derived from this work should allow us to build a “metabolic fingerprint” for flavor production in low-fat and full-fat cheeses that may reveal new strategies to develop starter strains, through mutagenesis or other methods, that produce more intense and desirable flavor notes in low-fat Cheddar cheese.

**RESULTS AND DISCUSSION**

Our work continues to focus on starter fermentations in Cheddar cheese extract (CCE). To obtain uniform results, we prepared a large batch (>500 lbs) of CCE powder that will meet all the needs of the project. Full and low-fat Cheddar cheeses were manufactured at the USU dairy plant and aged 6 weeks (the time at which HPLC analysis confirmed residual lactose and galactose levels had fallen below quantifiable levels), then shredded and frozen in preparation for subsequent processing into CCE powder. To accelerate this process, we worked with the Western Dairy Center staff to develop a novel, high-throughput system for generating the final CCE aqueous extract using our pilot plant UF capabilities. Importantly, this process is also amenable to using frozen shredded cheese as the input, and gives an aqueous concentrate that we believe will allow us to completely eliminate the need for lyophilization in future CCE preparations. Experiments confirmed that NSLAB growth was comparable in CCE concentrate versus powder prepared from the same cheese, although compositional analysis of CCE concentrate and powder revealed minor differences in sugar and organic acids content (Table 1). Because of this finding, we opted
Effect of fat removal in cheese microenvironment and starter culture metabolism in cheddar cheese

I. Broadbent

Table 1. Composition of powder and liquid concentrate forms of CCE prepared from low-fat cheese.

<table>
<thead>
<tr>
<th></th>
<th>Powder %(g/100mL)</th>
<th>Liquid %(g/100mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>st.dev</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Formate</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>1.68</td>
<td>0.01</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>Salt</td>
<td>1.07</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Figure 1. Plasmid DNA content of lactococci selected for the study.

to rely exclusively on concentrate for this project, and use the powder for other experiments.

As part of our preliminary work on this project, we collected and characterized six strains of Lactococcus lactis for use in this study. These include: *L. lactis* M70, *L. lactis* MM100, *L. lactis* S2, *L. lactis* SCO213, *L. lactis* LL 071, and *L. lactis* LL 011. The cultures were grown in M-17 lactose broth at 30°C, streaked for single colony isolation on M-17 lactose agar, and their identity as *Lactococcus lactis* was confirmed by PCR and sequencing of the 16S rRNA gene. The plasmid DNA profile of each strain was also determined (Fig. 1), and a software program called “r” was customized to suit the lactococcal microarray that will be used to analyze gene expression profiles in CCE-grown lactococci.

Because the liquid CCE that we prepared contains more than 0.1% residual sugar (Table 1), we initially believed our lactococcal cultures would display modest growth during inoculation into this medium (as is normally the case when starters transition from milk to fresh curd). However, our first experiments in 10 ml CCE samples indicated growth was negligible. Because modest growth is desirable to our goals to mimic the cheese environment, we performed follow-up studies to establish conditions that would facilitate it. As is shown in Fig. 2, we found that adjustment of the lactose concentration to 0.2% provides for a 1.5-2 log increase in cell numbers after incubation for 2 days at 30°C. As a result of these studies, we supplement our liquid CCE with lactose to 0.2% final concentration prior when we use this medium to prepare cells for transcriptomics and volatiles studies.

We coordinated our methodology for volatiles analysis with Dr. MaryAnne Drake at North Carolina State University, so that results from this study can be directly compared with data collected from the DMI low-fat cheese platform study, and analyzed the volatiles content of CCE fermented with different starter cultures (Table 1 and Fig. 3). We also performed comparative genome hybridizations with genomic DNA from each of the six strains against an Affymetrix custom microarray for *L. lactis*. Together, these experiments confirmed the suitability of our methodology for microarray and volatiles studies of lactococci incubated in CCE, and also provided us with a more detailed picture of the genetic differences between these strains.
Table 1. Examples of aroma compounds detected in CCE after incubation with L. lactis starter cultures^1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Flavor</th>
<th>Compound</th>
<th>Flavor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Butanone, 3-hydroxy</td>
<td>sour milk</td>
<td>Nonanal</td>
<td>green, fatty</td>
</tr>
<tr>
<td>2,3-Butanediol, [S-(R^<em>,R^</em>)]</td>
<td>cheesy, caramel</td>
<td>Octanoic Acid</td>
<td>green odor</td>
</tr>
<tr>
<td>Butanoic acid</td>
<td>cheesy, rotten, sharp</td>
<td>Decanal</td>
<td>green</td>
</tr>
<tr>
<td>Butanoic acid, 3-methyl</td>
<td>tootsie roll</td>
<td>Nonanoic acid</td>
<td>goat</td>
</tr>
<tr>
<td>2-Heptanone</td>
<td>fruity, spicy, fatty</td>
<td>Pyrazine, trimethyl</td>
<td>nutty, musty, beans</td>
</tr>
<tr>
<td>Methional</td>
<td>roast potato</td>
<td>Heptanal</td>
<td>soapy</td>
</tr>
<tr>
<td>Pyrazine, 2,5-dimethyl</td>
<td>nutty, roast grain</td>
<td>Heptanoic acid</td>
<td>rancid</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>almond</td>
<td>Acetic acid</td>
<td>vinegar</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>popcorn goaty</td>
<td>Pyrazine, tetramethyl</td>
<td>raw potato, beans</td>
</tr>
<tr>
<td>Benzeneacetaldehyde</td>
<td>rossy</td>
<td>Acetophenone</td>
<td>sweet</td>
</tr>
</tbody>
</table>

^A total of 150 compounds were detected.

As is shown in Fig. 4, we also discovered that addition of milk fat globular membrane (MFGM) material promotes growth of some lactococci at 10°C. This is an important finding because it expands our understanding of the CCE model for cheese with evidence that MFGM may contribute to cytoplasmic membrane fluidity (and thus membrane function, including solute uptake and efflux) and/or some components of MFGM may serve as substrates (and thus contribute to the profile of volatile aroma compounds in cheese). Since either of these possibilities has obvious implications on flavor development in low-fat versus full fat cheese, we prepared a large amount of MFGM and include it, at an appropriate concentration, in CCE designed to mimic each type of cheese (Table 2).

Based on the outcomes described above, we performed transcriptome and volatile studies using L. lactis M70. We selected this strain because it was the culture used for the low-fat platform study, so we will be able to compare data from the CCE trials to chemical data collected in the platform study. Analysis of growth and volatiles production in CCE with high (4.8%) or low (1.2%) salt levels by the other strains shown in Fig. 1 was performed in parallel work by Dr. James Steele at the University of Wisconsin. Those data confirmed environment and strain-specific differences among L. lactis cultures with respect to volatiles production (Fig. 5).

For fermentations with L. lactis M70 incubated at 30 or 10°C in CCE adjusted to mimic low-fat or full fat cheese, without redox control (see Table 2). L. lactis M70 was added at 10^7-10^8 cfu/ml to the bioreactor, and cells are sampled at time 0, day 1, and at weekly intervals thereafter (2 wks total for 30C, 6 wks for 10C). At each sampling time, cells were enumerated and volatiles measured. A cell-free CCE control, which is also incubated at the 30 or 10°C during the fermentation and sampled for volatiles, was used to collect information on volatile compounds that most likely are not produced by the starter.

Observations from the fermentations that were performed without redox control confirm:

- Starter viability in LF and FF CCE are similar.
- Once again, there are negligible differences in the levels of some compounds between the FF and the LF samples. However, large differences have been noted in the levels of other compounds, and a few volatiles were detected at much higher levels in either FF or LF samples.
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Figure 3. Production or degradation of the aroma compounds methional (A), benzeneacetaldehyde (B), and acetoin (C) in CCE by different \textit{L. lactis} during incubation at 10° or 30°C. CCE = cell-free medium.

Figure 4. Growth of \textit{Lactococcus lactis} M70 or SC0213 in CCE with or without added MFGM.

Table 2. CCE conditions under study.

<table>
<thead>
<tr>
<th>Components</th>
<th>Low-fat Model</th>
<th>Full-fat Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/M (%)</td>
<td>3.7</td>
<td>4.75</td>
</tr>
<tr>
<td>MFGM</td>
<td>0.12%</td>
<td>0.88%</td>
</tr>
<tr>
<td>lactate</td>
<td>5500 ppm L-lactate</td>
<td>same</td>
</tr>
<tr>
<td>temperature</td>
<td>10, 30°C</td>
<td>same</td>
</tr>
<tr>
<td>redox</td>
<td>+340, -200, none</td>
<td>same</td>
</tr>
<tr>
<td>pH</td>
<td>5.1</td>
<td>same</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.21%</td>
<td>0.21%</td>
</tr>
</tbody>
</table>
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Next, we performed experiments at 30°C in CCE with 24 mmol potassium ferricyanide \([K_3Fe(CN)_6]\) or 30 mmol dithiothreitol (DTT) added to create oxidative or reducing conditions \((Eh = 340 \pm 30 \text{ mV} \text{ or } -200 \pm 30 \text{ mV}, \text{respectively})\). Trials with \(K_3Fe(CN)_6\) showed this compound could not maintain a high redox, so no further experiments were pursued. In contrast, DTT addition did keep the redox low for a full week, and even by week 2 it was still below the control. Thus, we have completed two full trials with DTT and the third and final fermentation is underway. Once again, \(L. lactis\) M70 was added at \(10^7-10^8\) cfu/ml to each bioreactor, and cells are sampled at time 0 and again at day 1, 7, and 14 for enumeration and volatiles measurements. Data analysis from the low redox trials is still underway.

One important finding was that cells incubated in CCE do not provide sufficient RNA for transcriptome analysis. To overcome this limitation, we performed RNA extractions under conditions more akin to those used by our group for the analysis of stress-responsive genes, except that the stress treatment will be incubation in CCE modified to mimic FF or LF cheese. Those experiments were successful, and data analysis for microarray hybridizations results is underway.

**CONCLUSIONS**

The differences we have observed in volatiles production by \(L. lactis\) M70 can only be attributed to CCE microenvironment. Hence, results to date clearly support our hypothesis that starter physiology (and hence metabolism) is affected by differences in the physico-chemical environment in ways that affect the production of flavor- and aroma-active metabolites.

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Designing filler particles to imitate fat in cheddar cheese

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ABSTRACT

The goal of this project is to systematically evaluate the application of filler particles for replacement of fat cheddar cheese. Based on our prior research on cheeses at different fat levels (3 to 33%), cheese firmness increases as fat decreases due to there being more protein gel network per unit volume. The difference in chewdown properties, such as adhesiveness, smoothness and cohesiveness, appears to be related to different fracture patterns in the cheese network due to the absence of fat. This leads us to hypothesize that the two critical factors required for a successful filler particle will be appropriate phase volume and a similar effect on the fracture pattern. This project will have two phases. In the first, we will study the effects of different types and amounts of interacting and non-interacting filler particles on rheological and microstructure of cheese. The goal being to screen treatments making 1 kg batches of cheese. Dr. Vardhanabhuti will be responsible for making (obtaining) the particles and all rheological and microstructural analysis, while Dr. McMahon will make the cheese. At the end of the first phase, we should be able to select various filler particles that produce the textural properties of full fat cheese. Selected filler particles will be extensively investigated in the second phase which will include the sensory analysis. At the end of the project, we should be able to identify the critical properties of filler particles that allow them to function like fat.

BACKGROUND

Introduction

The first 10 months of DMI project entitled “Investigating the filled gel model for the role of fat in cheese” has been completed and produced some unique insight. In order to determine the role of fat in cheddar cheese texture, a set of cheeses were developed that ranged in fat content from 3 to 33%. The texture of these cheeses was measured over an aging time of 12 weeks to determine the combined effects of fat and the early events in the aging process. The first replication of this experiment is completed and the second replication is underway. The results from the first replication will be discussed as a foundation for this proposed project. In the second phase of the current project, we will determine the general effect of an inert, non-active filler (sephadex carbohydrate beads). This is needed because, as will be discussed below, fat globules undergo several changes as the fat content increases.

Results from Phase 1

As seen in Figure 1, there were discernible differences in texture due to fat level and aging.

The first data point at two weeks showed that cheeses with 3 or 8% fat were much firmer than the other treatments. However, by 4 weeks, the cheeses were starting to separate based on fat content. This is showing that extremely low fat cheeses (3 and 8% fat) have different structures from the start, and that these structure do not change much with aging. In contrast, cheeses with ≥ 13% fat undergo structural rearrangement in the first 4 weeks. This is most logically due to a redistribution of water between trapped pockets of whey and the casein gel network to produce a semi-homogeneous casein gel network.

As aging time progressed, differences between almost every fat level appeared such that at 12 weeks there was a general step-wise increase in firmness as the fat content was decreased. An increase in firmness has historically been seen as a problem with low fat cheese texture (Gwartney et al., 2002), and this could be due to
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Time (Weeks)

Figure 1. Changes in sensory firmness of cheddar cheese containing different levels of fat (3 to 13%).

Figure 2. Changes in sensory adhesiveness of cheddar cheese containing different levels of fat (3 to 13%).

Figure 3. Color image of structure seen under confocal microscopy. Image on the left is of a cheese containing 3% fat and the image on the right shows a 33% fat cheese. Protein matrix is stained red and the fat is stained blue.

changes in the concentration of protein in the gel network or the amount of gel network. The water:protein ratio for the cheeses varied from 1.41 to 1.66 w/w, suggesting that there was minimal difference in the protein concentration of the gel network. This would indicate that the texture differences are due to the amount of gel network rather than the inherent strength of the gel network; and this will be further justified in the discussion of fracture properties.

Firmness is a sensory first bite term that is evaluated during the first chew. While it is important to the overall impression of texture, the texture perceived during chewing, called chewdown terms, have been shown to be a major factor differentiating low fat from normal cheeses (DMI Annual Report, Low Fat Natural Cheese Strategic Platform, 2008). The sensory term of adhesiveness serves to illustrate the general trend seen in all chewdown terms (Figure 2).

As was seen with sensory firmness, the cheeses started at a similar adhesiveness then aged into structures that showed major differences due to fat level. In this case, a high level of adhesiveness is desired to prevent a “rubber ball-like” texture seen in low fat cheeses where elastic, non-interacting particles are created during chewing. By 12 weeks, the treatments with ≥ 23% fat had a desired level of adhesiveness while the rest remain much lower.

Sensory texture is an assessment of the breakdown pattern of the cheese during chewing. With that in mind, we analyzed the microstructure of cheeses to see if the initial structure could provide some insight into how fracture processes would occur.

As can be seen in Figure 3, increasing fat content changed not only the amount of fat observed in the microstructure, but also increased size and altered the shape of fat globules. This clearly showed that the choice of particle size used to imitate fat is more complex than just adding particles in the range of milk fat globules (0.1 – 10 µm; Walstra et al, 1999). The deviation of a particle from a spherical geometry can be measured by determining the shape factor. A sphere would have a shape factor of 1 and values lower than one indicates the degree of non-spherical shape. There was a general trend of the fat particles becoming more non-spherical as the fat content increased (Figure 4). The mean fat globule area is an indication of size and, as observed in Figure 3, area increased with the amount of fat.

One goal of this project was to see if the filled gel model
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Figure 4. Shape factor and mean area of fat globules in cheddar cheese with different levels of fat.

Figure 5. Composition of cheddar cheese. Treatment indicated % target fat level.

could be used to predict how fat would function in cheddar cheese. This model states that the elastic modulus (G') of a gel filled with particles will increase or decrease due to the relative moduli of the gel network and filler particle (G'filler / G'gel network), the amount of filler particles and if the particles interact (active filler) or do not interact (inactive filler) with the gel network (Tolstoguzov and Braudo 1983; van Vliet 1988). Previous research with Gouda cheese has shown that the filled gel model can be used to explain various rheological transitions, especially those associated with fat and temperature changes (Visser, 1991).

The compositions of the cheeses were adjusted such that as fat content was increased (3 to 33%) there was a decrease in protein content (35 to 24%), while the water:protein ratio was targeted to be held constant. This approach allowed for the cheese to be viewed as a filled gel with filler particles (fat) dispersed in a protein network of constant concentration (i.e., constant water:protein ratio). The actual compositions of the cheeses were very close to the target values (Figure 5). While the water:protein ratio varied from 1.42 to 1.72, it did not show a systematic variation and the three cheeses with the highest amount of fat had the same water:protein ratio.

At 10°C, the filler particles are expected to be more solid than liquid and G'filler > G'gel network. There was an increase in G'cheese as filler particle (fat) percentage increased, and gel network concentration (protein) decreased (Figure 6). This trend shows a reinforcing of the network by the filler particle and suggests that fat is acting as an active filler at 10°C (Figures 6).

Rheological properties were much different when the temperature was increased to 25°C; the temperature of cheeses during sensory evaluation (Figure 7). At 25°C, the fat is more liquid than solid and therefore G'filler < G'gel network and little effect is seen on the gel network. Note that all G'cheese values in Figure 6 (10°C) were > 200 Pa while those in Figure 7 were around or below 200 Pa. Similar temperature-dependent trends in G'cheese were observed with Gouda cheese (Visser, 1991).

Changes in G'cheese reflect the overall stiffness of the gel network at deformations that do not cause irreversible changes in the gel structure. Like microscopy that provides visual images of the structure, G'cheese provides a mechanical description of the cheese structure. In contrast, fracture properties (fracture stress and fracture strain) tell us how much force (stress) and deformation is needed.
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Figure 6. Changes in G' (10°C; 1 Hz) with fat (left) and protein (right) content.

Figure 7. Changes in G' (25°C; 1 Hz) with fat content.

Figure 8. Changes in fracture stress with fat (left) and protein (right) content.

... changes in fracture stress with fat content and increase with protein content. This is consistent with the concept that fracture stress represents the network strands that need to be ruptured to fracture through the cheese and that an increase in protein content would increase the network strand density. Note that fracture stress increased most substantially for cheeses with 3 and 8% fat (Figure 8), that had on average higher (1.66) and lower (1.41) water:protein ratios than the other cheese (Figure 5). Therefore, it appears that the amount of gel network, instead of the concentration of protein in the gel network, is the critical factor determining fracture properties (Figure 8) and sensory firmness (Figure 1). This suggests that low fat cheese with acceptable texture can be made with filler particles that interact (or do not) with the gel network like that of fat, and have the same filler volume.

There are several key conclusions that can be drawn from our current and past investigations. First, there are two main textural differences between low fat and full fat cheeses. Low fat cheeses tend to have a greater firmness and breakdown into larger, more rubbery, less smooth particles...
Designing filler particles to imitate fat in cheddar cheese / D. J. McMahon

During chewing. The initial firmness can be explained by an increase in the relative amount of protein network as compared to filled fat particles but the differences in chewdown properties (smoothness, adhesiveness, particle size) are more complex. One can envision the chewdown properties being different due to variation in crack propagation through the cheese structure. Three possible fracture paths are: 1) proceeding through the gel network and along the fat particle surface; 2) proceeding through the gel network and through the fat particles and 3) proceeding through the gel network and leaving the fat-gel network interfaces intact. The plausibility of each path may be discussed based on findings in model systems.

Using mixed protein-poly saccharide gels as a model, van den Berg et al. (2007) showed different fracture patterns that were dependent on microstructure. Gels with protein continuous and bicontinuous networks were perceived as crumbly, whereas those with coarse stranded gels were viewed as spreadable. Moreover, these textural differences were associated with different crack propagation mechanisms observed by confocal microscopy. In protein continuous structures, those most closely resembling cheese, micro-cracks appeared during deformation (strain of 0.3) before the actual fracture point (fracture strain of 1.04 to 1.15). The main fracture path went through the gel network and the dispersed, fluid hydrocolloid solution particles. This suggests that either path 1 or 2 are logical for cheese, and would depend on the fluid/solid nature of the fat. Therefore, if we knew how to design a low fat cheese microstructure with all the essential physical/chemical elements of a full fat cheese microstructure, there should be little or no textural differences. The questions become what microstructural elements are key to full fat cheese texture and how do we create such structures without fat?

One approach is to use starch granules as fat replacers. The logic underpinning of this approach is that starch can act as an inactive filler (e.g., not interaction between starch granules and the protein matrix), thereby taking up space and, hopefully, acting like fat particles. However, in an imitation cheese, addition of starch cause a major increase in fracture stress and decrease in fracture strain (Noronha et al., 2008). These rheological transitions are indicative of a firmer, more brittle sensory texture. The increase in firmness/toughness was associated with a decrease in water mobility, suggesting that water was partitioning inside of the starch granules and thereby increasing the protein concentration of the gel network. These results show that ability of a filler particle to remove or add water to the protein gel network phase is essential to how they will function.

Besides sensory analysis, an evaluation of the chewing process helps us understand differences among cheeses. We have used three dimensional jaw tracking to measure the chewing process of a line of commercial cheddar cheeses with different fat levels. This brand was picked because it had 50% and 75% reduced fat cheeses, and the 75% reduced fat cheese contained starch. The jaw tracking data showed that as fat was reduced in the cheese, it required more extensive chewing before swallowing. In addition, with the 75% reduced fat cheese, there was at least one intermediate swallow before the final clearing swallow (Figure 9). This showed directly from mastication during chewing.
measurements that as fat is reduced the cheese becomes
tougher and requires more extensive chewing before
swallowing. While we cannot determine how the added
starch is affecting the cheese texture, it is clear that it is not
reducing the overall toughness.

Proposal

Our proposal for the next project is to build on our
current findings and start investigating a range of filler
particles. The following particles will be investigated:

Polar whey protein isolate gel particles.

Whey proteins can be heated to form polymers then
added to calcium chloride solution under shear to produce
gel particles of a range of sizes depending on shearing
conditions. The particles would be made at the same
water activity as the cheese to prevent moisture migration.
The water activity of the particles will be adjusted to
that of cheese (0.93 to 0.97) by adding sugar alcohols.
Alternatively, it may be desirable to cause moisture
migration to or from the particles and this can also be done
by adjusting particle water activity.

Non-polar whey protein isolate gel particles.

A similar approach as outlined in #3 would be used
but glucono-delta-lactone is added to the whey protein
polymer solution prior to being added to oil under shear.
The protein-polymer solutions will form emulsion droplets
in the oil and the glucono-delta-lactone will slowly lower
the pH causing gelation of the droplet. The surfaces of
these particles are expected to be hydrophobic.

Whey protein-hydrocolloid particles.

Various approaches have been used to form particles
with whey protein and hydrocolloids, such as pectin
(Bédié et al., 2008). In all cases, the particle properties
are adjusted by altering solution conditions (pH, polymer
concentration) and protein:polysaccharide ratio. We would
produce a range of particles for evaluation.

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Walstra, P., Geurts, T. J., Noomen, A., Jellema, A., and van
Milk, Properties and Processes. Marcel Dekker, Inc. New
York, NY.

RESEARCH PLAN

Objective 1.

Develop whey protein-based filler particles.

Objective 2.

Evaluate whey protein-based filler particles in a model
system.

Objective 3.

Manufacture cheeses containing whey protein-based
filler particles.

Objective 4.

Evaluate textural properties of cheeses containing
whey protein-based filler particles.

Objective 5.

Evaluate textural properties of cheeses containing
whey protein-polysaccharide particles.

Objective 6.

Cheese manufactured in 20-lb blocks with preferred
filler particles (n=9 cheeses in duplicate).

Objective 7.

Sensory texture analysis results through 24 weeks of
storage from cheeses containing particles showing optimal
rheological and microstructural results.
MATERIALS AND METHODS

Overall, the project will consist of two approaches. In the first approach, we will study the effects of different types and amounts of interacting and non-interacting filler particles on rheological and microstructure of cheese. The goal being to screen a large number of treatments making 1 kg batches of cheese. At the end of the first phase, we should be able to select various filler particles that produce the textural properties of full fat cheese. Selected filler particles will be extensively investigated in the second phase which will include the sensory analysis and mastication evaluation. At the end of the project, we should be able to identify the critical properties of filler particles that allow them to function like fat. We expect to be able to provide the knowledge needed to make low fat cheese having the texture quality of full fat cheese.

Part I. Screening of Filler Particles Based on Textural and Microstructural Properties

In the first part of our study, we will investigate different types of interacting and non-interacting filler particles at different fat replacement levels (15 and 27%) in corresponding to reduced fat (18%) and low fat cheese (6%), respectively. The initial screening of particles will be determined over a 2 month storage using rheological analysis and confocal microscopy. Particles that produce textural changes similar to what was observed in the full fat control will be further analyzed in Part II. Treatments in Part I are listed in Table M1.

<table>
<thead>
<tr>
<th>Table M1. List of treatments.</th>
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<tbody>
<tr>
<td><strong>Treatments no.</strong></td>
</tr>
<tr>
<td>1</td>
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<td>11</td>
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<tr>
<td>12</td>
</tr>
</tbody>
</table>

These particles will be made at North Carolina State University and shipped to Utah State University for cheese making. One kilogram of cheese per treatment will be made in duplicate. For each set of experiments (6 cheeses per 2 month storage), full fat cheese will be included as control. The rheological properties and confocal microscopy of cheese samples will be studied. Sampling will be done at 2, 4, 6 and 8 weeks.

Part II. Sensory Evaluation of Cheese Made with Select Particles

Filler particles that result in cheese with rheological and microstructural properties similar to full fat cheese will be further investigated (select 3). Some select rheological properties will be evaluated but the main focus will be on sensory analysis. Similar to what is outlined in Part I, each set of experiments will include full fat cheese as a control. Each treatment will be made and analyzed in duplicate (i.e. two replications). Sampling will be done at 2, 8, 12 and 24 weeks.

At the end of the project, we should be able to determine the key properties of filler particles that will provide the overall properties similar to fat in full fat cheese.

Preparation of filler particles.

**Polar whey protein isolate gel particles.**

Whey protein isolates at 4 and 10% protein (w/w) will be heated at 85°C for 30 min (pH 7) to form polymers and added to calcium chloride solution under shear to produce gel particles of different sizes depending on initial protein concentration and shearing conditions. The water activity of the particles will be adjusted to that of cheese (0.93 to 0.97) by adding sugar alcohols to prevent moisture migration. Alternatively, it may be desirable to cause moisture migration to or from the particles and this can be done by adjusting particle water activity.

**Non-polar whey protein isolate gel particles.**

Whey protein polymers will be made similar to polar whey protein isolate gel particles. Different polymer sizes can be achieved by altering initial protein concentration. Instead of adding calcium chloride solution to form gel particles, glucono-delta-lactone will be added to the whey protein polymer solution prior to being added to oil under shear. The protein-polymer solutions will form a water-in-oil emulsion and the whey protein solution droplets will be formed into a gel by glucono-delta-lactone slowly lower the pH causing gelation of the droplets. The surfaces of these particles are expected to be hydrophobic.

**Whey protein-hydrocolloid particles.**

Particles formed by complex formation between whey
protein and pectin will be made. Preliminary studies will be conducted to determine protein concentration, polysaccharide concentration, and pH to determine the appropriate conditions which will produce three different sizes of particles.

Cheese making and chemical analysis.

Cheese will be made in small stainless steel vats using 40 lb of milk. Stirred curd make procedures will be used based on the full fat, reduced fat and low fat cheeses made for the systematic platform project. The cheese will be pressed into a 3 to 4-lb block overnight, and then vacuum packaged and stored at 6°C. Four vats of cheese will be made simultaneously on each day of manufacture. Duplicate sets of cheese will be made.

After 1 wk of storage the cheese will be cut into four pieces. One piece will be used for proximate analysis of moisture (by vacuum oven), fat (by babcock), protein (by N), pH (by glass electrode), salt (by chloride), and calcium (by ICP spectroscopy). The remaining three cheese pieces will then be vacuum packaged, and stored at 6°C. Cheese will be shipped to NCSU at 2 and 7 wk for rheological screening at 3 and 8 wk and one piece of cheese retained for additional testing/storage if needed.

Rheological analysis.

Two general types of rheological analysis will be used. Small-strain testing will be done to determine the overall properties of the network. Our ongoing cheese texture research has shown that critical stress, critical strain and maximum compliance ($J_{\text{max}}$) are all good indicators of network properties that are associated with textural properties. The second type of testing is large-strain torsional testing, where the samples are twisted to fracture. This determines fracture stress and strain, which characterize the breakdown of cheese structure and closely linked to sensory properties.

I. Small strain testing.

Determination of the linear viscoelastic region and critical stress and strain. Stress sweeps of cheeses will be completed using a Stress Tech controlled stress rheometer (ATS Rheosystems, Bordentown, NJ) with 20mm smooth parallel plate geometry. Temperature will be controlled using an integrated, induction heating device set at 25°C. Cheese samples will be cut 4mm thick, and trimmed to the size of the plate. Samples will be glued to both plates prevent slipping using cyanoacrylate glue (Loctite 401: Loctite Corp. Rocky Hill, CT). Once the plates are glued, the exposed edges of the samples will have a thin layer of synthetic lubricant (SuperLube: Synco Chemical Bohemia, NY) applied to prevent dehydration.

Stress sweep tests will be conducted at 10 Hz with a stress range from 1 to 100 Pa. The critical stress and strain will be determined when $G^*$ values begin to regularly decrease; the stress and strain on the sample at this point will be used as the critical stress and critical strain.

Creep/Recovery Analysis. Creep/Recovery tests will be conducted using a Stress Tech controlled stress rheometer (ATS Rheosystems, Bordentown, NJ) as outlined above. Tests will be conducted at a constant stress within or near the linear viscoelastic region. Forces will be applied to the sample for 200 seconds then removed and the recovery of the sample will be measured for an additional 200 seconds. Tests at each force (100 and 150 Pa) will be conducted twice for each replication. The values of maximum compliance ($J_{\text{max}}$), instantaneous compliance ($J_1$), retardation time ($\lambda$), and percent recovery ($\text{crp}$) will be determined. Maximum compliance is the maximum compliance reached before the force is removed. Instantaneous compliance is the initial compliance as time approaches zero. Retardation time is calculated as the time it takes for the strain to reach 63.2% of its final value. The percent recovery is calculated using the following equation:

$$\frac{J_{\text{rec}}} {J_{\text{max}}} = \frac{(J_{\text{max}}) - (J_1)} {J_{\text{max}}}$$

Where $J_{\text{max}}$ is the maximum compliance and $J_1$ is the compliance after the sample has been allowed to fully recovered (Brown et al., 2003). Mechanical Spectra. The storage modulus ($G'$) and loss modulus ($G''$) will be measured during a frequency sweep varying from 0.01 to 10 Hz at a constant stress (150 Pa), which is within the linear viscoelastic region of the materials, at 10 and 25°C (based on previous research with cheddar cheese). These moduli represent the amount of energy elastically stored and recovered per cycle (elastic component, $G'$) and the amount of energy lost per cycle by viscous dissipation ($G''$). The change of these moduli with frequency (1/time) provides a “mechanical spectra” that represents the viscoelastic characteristics of the cheese.

II. Large-strain torsional analysis.

Torsional methods will be used to determine the nonlinear and fracture properties of the cheeses. Cylinders of cheese will be formed using a 19-mm internal diameter cork borer. The cylinders were cut to a length of 28.7-mm, and plastic disks (Gel Consultants, Raleigh, NC) were glued to the ends of the cylinder using cyanoacrylate glue (Loctite 100- Loctite Corporation, Rocky Hill, CT) to enable the samples to be mounted to the grinding and twisting apparatuses. The cylinders will be shaped into a capstan shape having a minimum diameter of 10-mm using a precision grinding machine (Gel Consultants, Raleigh,
NC). Measurements of the total cylinder length \(L_{tg}\), length of curved section \(L_{curv}\), upper cylinder radius \(r_{total}\), and minimum radius \(r_{min}\) will be taken in order to calculate the geometry of the curved section. Samples will be twisted using a Haake 550 viscometer (Gebruder Haake GmbH, Karlsruhe, Germany) fitted with a fabricated apparatus that enabled torsional measurement. The capstans will be twisted at 0.045, 0.45, and 4.5 rpm, and three replications at each speed of each treatment replication will be made. These speeds correspond to strain rates of 0.0047, 0.047, and 0.47 s\(^{-1}\) allowing for comparison with mechanical spectra data. True shear stress \(\sigma\) and true shear strain \(\gamma_{true}\) will be calculated at each point from time zero to time at fracture according to Nadai (1937), Diehl et al. (1979), and Hamann (1983).

Confocal microscopy.

Cheese samples will be imaged using confocal scanning laser microscopy (CSLM). The method for imaging samples will be similar to one used by Auty and others (2001). Cheese samples will be kept at 10°C until sliced into sections approximately 5 mm x 5 mm x 1 mm thick using a razor blade. Fluorescent dyes will be pipetted on the cut surface of the cheese: 15 µL of Nile Red solution and 15 µL of Rhodamine B solution. Samples stand for at least 10 min to absorb the dyes and return to room temperature. Cheese samples will then be turned over onto a single-welled slide with a #1.5 coverslip attached to the bottom with silicone grease, which allows the dye side to be in contact with the coverslip. Samples will then be imaged on an inverted Leica CSLM using a 40x (oil immersion) objective. A 488nm laser (to excite Nile Red in the fat phase) and a 561nm laser (to excite Rhodamine B in the protein phase) will be used to image the samples. For each cheese treatment, 2 samples will be prepared and 5 images will be taken per sample, resulting in 10 images per treatment.

Part II. Sensory Evaluation of Cheese Made with Select Particles

Filler particles that produce 1 Kg cheeses with rheological and microstructural properties similar to full fat cheeses will be used to make 10 Kg batches of cheese. A few key rheological properties will be measured but the primary focus will be on sensory analysis and mastication evaluation.

Cheese making and analysis.

Cheese will be made in stainless steel vats using 300 lb of milk. Stirred curd make procedures will be used based on the full fat, reduced fat and low fat cheeses made for the systematic platform project. The cheese will be pressed into a 20-lb block overnight and then vacuum packaged and stored at 6°C. Duplicate sets of cheese will be made four weeks apart.

After 1 wk of storage the cheese will be cut into 2-lb and 1-lb blocks. One of the 1-lb blocks of cheese will be sampled for proximate analysis of moisture (by vacuum oven), fat (by babcock), protein (by N), salt (by chloride), ash (by furnace), pH (by glass electrode), calcium (by ICP spectroscopy), soluble calcium and protein hydrolysis (by pH 4.6 soluble N). The other blocks will be vacuum packaged, two 2-lb blocks will be shipped to NCSU for the 2 wk sensory analysis, and the others will be returned for storage at 6°C.

At 8 wk of storage, a 1-lb block of cheese will be tested for soluble calcium (by water extraction and ICP spectroscopy), and protein hydrolysis (pH 4.6 soluble N), pH (by glass electrode).

At 7, 11 and 23 wk, two 2-lb block of cheese will be sent to NCSU for sensory analysis.

Sensory analysis.

Descriptive sensory analysis will be conducted using the methods laid out by Brown et al (2003) and Yates and Drake (2007). Analysis will be conducted using an experienced texture panel consisting of seven panelists with approximately 100 hours of experience in descriptive texture analysis utilizing the Spectrum method. Samples will be characterized on a 15 point scale anchored on the right by the term “very” and on the left by term “not” using terms from the texture lexicon laid out by Brown et al (2003). Panelists will be given 8 cubes of each cheese, measuring 1.27cm³, in lidded 4oz. plastic cups labeled with three digit codes. Panelists will be given deionized water to clean their palettes between each sample, and reference cheeses will be made available for each session. The basic terms described in Table M2 will serve as core terms and new ones may be added. Samples will be evaluated in triplicate for each replication. Data will be analyzed by univariate and multivariate statistical analyses using SAS (V 9.1, Cary, NC). Specifically, analysis of variance with means separation will be used to identify differences between treatments and attributes. Principal components analysis will be used to identify gross relationships between multiple treatments and attributes.

A superscript letter indicated that the term was evaluated in the investigations of Brown et al, Gwartney et al, or Barrangou et al. *Definition for terms used in Gwartney et al.*

References

Auty MAE, Twomey M, Guinee TP, Mulvihill DM. 2001. Development and application of confocal scanning laser microscopy methods for studying the distribution of fat and
Table M2. Sensory terms used for descriptive analysis of gels.

<table>
<thead>
<tr>
<th>Evaluation Phase</th>
<th>Term</th>
<th>Definition*</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-fracture</td>
<td>Smoothness&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Degree to which sample was perceived as smooth when evaluated with tongue</td>
<td>Move gel in mouth without chewing</td>
</tr>
<tr>
<td></td>
<td>Small-strain&lt;sup&gt;c&lt;/sup&gt; force</td>
<td>Force required to cause ~10% deformation</td>
<td>Compress to ~10 using molar's</td>
</tr>
<tr>
<td></td>
<td>Firmness&lt;sup&gt;b&lt;/sup&gt; (Fracture force)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Force required to fracture sample with molar's</td>
<td>Completely bite through sample using molar's</td>
</tr>
<tr>
<td></td>
<td>Moisture release&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Extent of moisture released</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Crumbliness/ fractionability&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Degree to which the sample fractures into pieces</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Deformability&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Degree of deformation prior to fracture</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Degree of Breakdown&lt;sup&gt;ab&lt;/sup&gt; (Particle breakdown)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Amount of breakdown</td>
<td>Chew the sample for 3 times,&lt;sup&gt;c&lt;/sup&gt; 5 times,&lt;sup&gt;a&lt;/sup&gt; or 8 to 10 times&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Particle size&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Size after chews (small to large)</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Particle size distribution&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Degree of homogeneity in distribution</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Particle shape&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Degree of irregular particle shape (irregular means distinct edges)</td>
<td>Same as above</td>
</tr>
<tr>
<td>Chewdown</td>
<td>Cohesiveness&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Degree to which the chewed mass holds together</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Adhesiveness&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Degree to which the chewed mass sticks to mouth surfaces</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Smoothness&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Degree to which the chewed mass surface is smooth</td>
<td>Same as above</td>
</tr>
<tr>
<td></td>
<td>Number of&lt;sup&gt;b&lt;/sup&gt; chews (Chewiness)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Number of chews required for swallowing</td>
<td>Complete chewing</td>
</tr>
<tr>
<td></td>
<td>Smoothness of mouth coating&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Smoothness felt after expectoration</td>
<td>Evaluate after expectorating</td>
</tr>
</tbody>
</table>


RESULTS AND DISCUSSION

Objective 1.

Develop whey protein-based filler particles.

We previously developed a method for particle formation based on cold gelation of individual droplets. While effective in producing functional particles, there were limitations to scale up, and so a method was developed whereby particles were generated by mechanical grind. The method affords greater manipulation of particle composition, as a heat set whey protein gel is formed and subsequently milled to micro-scale particles using
Designing filler particles to imitate fat in cheddar cheese / D. J. McMahon

a high speed mixer. This allows for control of particle microstructure during heating (i.e. stranded or particulate) as well as generation of a composite gel particle type.

**Objective 3.**

*Manufacture cheeses containing whey protein-based filler particles.*

<table>
<thead>
<tr>
<th>Table 1. Composition of whey protein gels.</th>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>Protein % (w/w)</td>
</tr>
<tr>
<td>NaCl (mM)</td>
</tr>
<tr>
<td>Fat % (w/w)</td>
</tr>
</tbody>
</table>

Using the mechanical grind method, three particle types were manufactured and shipped to Utah State for incorporation into cheese (Table 1). The treatments for this batch included a particulate (Treatment A) and stranded (Treatment B) whey protein gel microstructure type, as well as a composite gel particle in which the 6% fat allowed in a low fat cheese was concentrated into the particles rather than the continuous casein matrix (Treatment C). The sampling period was based on previous work that found rheological values of aging particle filled cheese (after curd is fully knit) to be more characteristic of finished product structure than a younger cheese.

A compositional analysis of the cheeses (Table 2) revealed similar fat levels in all the treatment cheeses. As was expected, moisture values for the treatment cheeses were higher than the full fat and homogenized controls, yet were in alignment with the low fat control.

Rheological analysis of particle filled cheeses is depicted in table 3. Recoverable energy reflects changes in structure associated with damage or flow during testing. Fracture stress and strain are the respective strength and deformability of cheese. While treatments A and B still most closely resemble the low fat control, treatment C exhibited fracture stress and recoverable energy values similar to the full fat control cheese (Table 3).

As compositional differences between treatment cheeses were minimal, we suspect that the greater efficacy of the composite type particle is due to a localization effect, concentrating fat at the fracture interface as it propagates through or around the particles.

The homogenized control, which contained smaller particles surrounded by an interfacial film of disrupted fat globule membrane and casein, was used to act more

<table>
<thead>
<tr>
<th>Table 2. Composition of particle filled cheese.</th>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Particle A-1</td>
</tr>
<tr>
<td>Particles A-2</td>
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<tr>
<td>Particles B-1</td>
</tr>
<tr>
<td>Particles B-2</td>
</tr>
<tr>
<td>Particles C-1</td>
</tr>
<tr>
<td>Particles C-2</td>
</tr>
<tr>
<td>Low fat control</td>
</tr>
<tr>
<td>Homogenized full fat</td>
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<tr>
<td>Full fat control</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3. Rheological properties of particle filled cheeses (34 weeks aging).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Low Fat Control</td>
</tr>
<tr>
<td>Full Fat Control</td>
</tr>
<tr>
<td>Homogenized Control</td>
</tr>
<tr>
<td>Particles A-1</td>
</tr>
<tr>
<td>Particles A-2</td>
</tr>
<tr>
<td>Particles B-1</td>
</tr>
<tr>
<td>Particles B-2</td>
</tr>
<tr>
<td>Particles C-1</td>
</tr>
<tr>
<td>Particles C-2</td>
</tr>
</tbody>
</table>
as an active filler and possibly mimic the effect of low fat content. However, rather than increasing rheological values, fracture stress and recoverable energy were lower than the full fat control. A notable challenge in interpreting these results hinges on the difficulty of quantifying particle retention in the cheese. Particles can be visually recognized using confocal microscopy, but transforming the particle area to a filler volume is difficult. The importance of filler volume in a composite gel system is well established and thus inconsistent or sub-target particle retention is apt to blur differentiation among treatment types. Based on the highlighted results in Table 2b, it was predicted that, among the particle treatments, treatment C would be the most similar in texture to the full fat control.

**Objective 4.**

Evaluate textural properties of cheeses containing whey protein-based filler particles.

Despite the rheological similarities between the composite gel particle treatment (treatment C) and the full fat control, descriptive texture panel results revealed a larger gap in the sensory properties (Table 3). Treatment C impacted the fracture hardness and springiness of the cheese at fracture (Table 4a), but had little effect on any of the breakdown terms (Table 4b). These adhesive/cohesive and smoothness properties, which are not directly assessed with a rheological evaluation, comprise an essential element of post fracture properties and bolus formation. Additionally, fat globule structure seemingly plays an important role in force-deformation texture terms, as the homogenized control imparted far lower firmness and springiness values than would be surmised from its rheological values (Table 3). Note that all terms evaluated after chewing were the same between full fat control and homogenized full fat (Table 4b).

As the composite gel type (treatment C) demonstrated the most favorable rheological properties, our future work seeks to determine why this is the case. Cheeses have been made in 2012 with all of the fat incorporated in the whey protein particles, allowing tracking of particle retention and unhindered assessment of the particle’s ability to comprehensively mimic the functionality of fat. Particles will be made containing both homogenized and non homogenized fat to explore structural effects (previously treatment C contained only half of the possible fat allowed in the particle structure), and localization at the fracture interface will be assessed using a particle treatment with no added fat.

**CONCLUSIONS**

The incorporation of fat into the whey particles and forming cheese with particles and no-fat milk appears to be the best approach. This will allow for a clear analysis of particle incorporation into the cheese matrix. This approach will be comprehensively analyzed in 2012.

**Table 4a. Texture of particle filled cheese: force-deformation terms (34 weeks aging).**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>hand firmness</th>
<th>hand springiness</th>
<th>hand rate of recovery</th>
<th>firmness</th>
<th>fracturability</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>14.2 ± 0.4</td>
<td>14.0 ± 1.0</td>
<td>13.1 ± 1.5</td>
<td>11.0 ± 1.0</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>B1</td>
<td>14.1 ± 0.5</td>
<td>14.0 ± 0.8</td>
<td>13.8 ± 0.8</td>
<td>10.8 ± 1.0</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td>C1</td>
<td>13.2 ± 0.6</td>
<td>13.5 ± 1.1</td>
<td>12.7 ± 1.7</td>
<td>10.5 ± 1.1</td>
<td>6.3 ± 1.6</td>
</tr>
<tr>
<td>LFC</td>
<td>14.1 ± 0.7</td>
<td>14.1 ± 0.8</td>
<td>13.7 ± 1.0</td>
<td>10.6 ± 0.7</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>FFC</td>
<td>6.6 ± 0.6</td>
<td>10.5 ± 0.8</td>
<td>8.9 ± 1.9</td>
<td>4.2 ± 0.8</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>HC</td>
<td>4.5 ± 0.7</td>
<td>4.5 ± 2.2</td>
<td>4.6 ± 2.3</td>
<td>3.6 ± 0.4</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>A2</td>
<td>13.5 ± 0.2</td>
<td>14.6 ± 0.5</td>
<td>14.3 ± 0.6</td>
<td>10.6 ± 0.4</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td>B2</td>
<td>12.7 ± 0.5</td>
<td>14.1 ± 0.8</td>
<td>14.0 ± 0.7</td>
<td>9.7 ± 0.4</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>C2</td>
<td>13.9 ± 0.4</td>
<td>14.6 ± 0.3</td>
<td>14.5 ± 0.2</td>
<td>10.5 ± 0.5</td>
<td>6.3 ± 0.6</td>
</tr>
</tbody>
</table>

**Table 4b. Texture of particle filled cheese: particle breakdown & bolus formation terms (34 weeks aging).**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>degree of breakdown</th>
<th>cohesion</th>
<th>adhesion</th>
<th>smoothness of mass</th>
<th>smoothness of mct</th>
<th>fracturability</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>4.0 ± 0.8</td>
<td>4.9 ± 1.0</td>
<td>4.5 ± 0.9</td>
<td>6.3 ± 1.3</td>
<td>7.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>5.2 ± 1.3</td>
<td>5.9 ± 1.0</td>
<td>5.0 ± 0.9</td>
<td>6.2 ± 0.7</td>
<td>7.0 ± 0.7</td>
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<td>6.0 ± 0.8</td>
<td>5.2 ± 0.5</td>
<td>6.6 ± 0.7</td>
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<td>6.4 ± 0.7</td>
<td>5.2 ± 0.4</td>
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<td>11.4 ± 0.6</td>
<td>10.1 ± 0.6</td>
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<td>HC</td>
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<td>11.4 ± 0.4</td>
<td>10.4 ± 0.4</td>
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<td>10.9 ± 0.7</td>
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<tr>
<td>A2</td>
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<td>7.4 ± 0.4</td>
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</tr>
<tr>
<td>B2</td>
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<tr>
<td>C2</td>
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<td>6.5 ± 0.6</td>
<td>5.9 ± 0.6</td>
<td>7.4 ± 0.4</td>
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Increasing stringiness of low fat Mozzarella cheese using polysaccharides

Erik N. Oberg and Donald McMahon*

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Funded by: Dairy Management Inc., as administered by the Dairy Research Institute

ABSTRACT

We examined the ability of polysaccharides to function as fat mimetics in low fat mozzarella string cheese to improve functionality by acting to form protein fibers during cheese extrusion. Low fat (LF) mozzarella cheese curd made from 273 kg of 0.7-fat milk was salted at a rate of 10 g/kg then divided into 3.6-kg batches that were hand-stretched in 5% brine at 80 degrees C and formed into a homogeneous mass. The hot cheese was hand mixed with a hot 80° polysaccharide slurry and placed into a small piston-driven extruder and cheese forced through a 16-mm die to form the string cheese and cut manually into about 15-cm lengths. The data from preliminary trials using starches (waxy corn, waxy rice, and instant tapioca starch), xanthan and guar gum, and polydextrose we determined that low fat string cheese made using xanthan gum most resembled commercial string cheese. Cheese was then made using 10% xanthan gum slurry added at 0.25%, 0.5%, 1.0%, 1.5%, and 2.0% levels as well as a control with no added gum. Cheeses were analyzed for fat, salt, pH, and moisture. After 2 wk of storage, the cheese was analyzed for extent of stringiness by pulling apart the cheese longitudinally, visually observing and photographing size, length and appearance of individual strings of cheese. A consumer liking test was conducted at 2 and 8 weeks storage time. Hardness was tested using a penetrometer shear test. At 2 weeks storage time, using a hedonic scale (1 to 9) for overall liking the LF with 1% added xanthan slurry (score=6.76) was liked more (P<0.05) than a retail comparison string cheese (score=6.18) and the LF cheese with no added gum scored lower (5.86). When considered on a JAR scale, 71% of panelists scored the LF cheese with added xanthan as having the right texture, while only 49% did so for the retail cheese. The no added gum LF cheese was considered too firm. At 8 weeks storage time, using a hedonic scale (1 to 9) for overall liking the LF with 1% added xanthan slurry (score=6.13) was liked more (P<0.05) than a retail comparison string cheese (score=6.43) and the LF cheese with no added gum scored lower (5.72). When considered on a JAR scale, most of the panelists scored the LF cheese with added xanthan as having the right texture, while only some did so for the retail cheese. The no added gum LF cheese was considered too firm. By visual comparison, adding the xanthan gum slurry produced greater fiber formation with the longest and best string separation. After 8 wk storage, the LF cheeses had softened extensively with fracture stress for LF cheese decreasing from 12-20 kg at 2 wk to 1.5-3 kg at 8 wk. Extent of stringiness also decreased considerably during storage.

BACKGROUND

Low moisture part skim mozzarella string cheese is considered a snack cheese. String cheese continues to grow, at least in terms or dollars, while unit sales of string have flattened (Dairy Foods). It is a popular snack among children and a low fat alternative may improve sales. In low moisture part skim mozzarella string cheese the fat globules form channels between the proteins and allow fibers to form and separate giving the cheese its stringiness. Removing fat from mozzarella cheese decreases fiber formation because protein strands fuse together during stretching and extruding. Given that stringiness is a favorable characteristic of mozzarella string cheese, it must be preserved when removing the fat from the cheese. Many studies have been conducted on low fat cheeses with added fat replacers, including polysaccharides, but there has not been studies done on polysaccharides as fat replacers in snack cheese. This study examined the ability of polysaccharides to act as fat mimetics and aid in fiber
formation in manufacture of string cheese. Polysaccharide materials that do not bind to the proteins have the potential to mimic the action of fat globules in cheese by physically blocking protein fusion and allowing protein strands to form. Polysaccharides could be a good alternative for the fat in string cheese and the removed fat could be used for other products such as ice cream.

MATERIALS AND METHODS

Cheese Making

The low-fat mozzarella string cheese was made from 273 kg of 0.7%-fat milk obtained from USU Caine dairy. We followed a standard mozzarella make procedure with a few changes that were based on prior research. The changes in the make procedure have been used in other low fat cheese makes and are aimed at reducing the firmness of the curd. Fat in cheese has a light scattering effect and makes the cheese appear white; because of this the milk was colored with TiO2 and annatto to preserve the whiteness of the cheese. The cheese was pre-acidified with 5 % acetic acid to a pH of 6.3. The starter culture used was Streptococcus thermophilus TS-20D from DSM, Logan, Utah. The cooking and stirring temperature was lowered from 102°F to 94°F; this was done to slow down the acid production of the starter culture and raise the moisture of the curd. The curd was salted at a rate of 10 g/kg then divided into 3.6-kg batches that were hand-stretched in 5% (wt/wt) hot brine (80°C) and formed into a homogeneous mass. The hot cheese was hand mixed with a hot (80°C) polysaccharide slurry and then placed into a small piston-driven extruder and cheese forced through a 16-mm die to form the string cheese and cut manually into about 15-cm lengths. The xanthan gum powder (Grindsted® Xanthan 80, Danisco) was first made into a 10% wt/wt slurry. The xanthan gum slurry was then added to the cheese at different concentrations: 0% control, 0.25%, 0.5%, 1.0%, 1.5% and 2.0% (wt/wt). After one week of storage the cheeses from all three trials were analyzed for fat, salt, pH, and moisture in the Western Dairy Center Analytical laboratory.

Stringiness

Stringiness was measured objectively by comparing the different low fat cheeses to each other and to commercial low-moisture-part-skim mozzarella string cheese. After 2 and 8 weeks of storage at 4°C the extent of stringiness was measured by pulling apart the cheese longitudinally by hand, visually observing and photographing size and appearance of individual strings of cheese. Cheeses that produced strings with smaller diameters and greater lengths were considered stringier.

TPA

Many people consume string cheese by just biting it instead of pulling off strings. This TPA test was done to simulate a person biting into the cheese and measured the force of resistance to the bite and shows the firmness of the cheese. TPA testing was also done on the string cheese using a shear test with a Warner-Batzler Blade with a round edge mounted on a Stable Micro Systems TA XT Plus Texture Analyzer and samples were cut perpendicular to the strings and compared to commercial string cheese. The TPA protocol was modified from a tofu shear test. The results were interpreted by force and penetration into the cheese.

Confocal Microscopy

Five different cheeses were also analyzed using confocal laser scanning microscopy. Cheeses from trial 2 containing polydextrose and instant tapioca starch and 2 cheese from trial 4 on with added xanthan gum one with no added polysaccharide. A commercial string cheese was also analyzed. The purpose of the confocal microscopy is to visualize the polysaccharides within the cheese protein matrix. The cheeses were frozen and thin sections cut perpendicular to the protein fibers using a cryostat sample preparation. The proteins were fixed by immersing them in osmium tetroxide vapor for 18 h. The polysaccharides were treated with periodic acid to produce aldehyde groups. The polysaccharides were then stained with 0.1 % Acriflavin and the proteins stained with 0.1 % Rhodamine B.

Consumer Panels

After 2 and 8 weeks of storage time, consumer panels were conducted at the Utah State University Food Sensory Kitchen using volunteers from the community. The low-fat cheeses were compared to a LMPS commercial string cheese. Each panelist was given a 15 mm section of each cheese and the order of the cheeses was randomized. The tests were conducted using a 9-point Hedonic liking scale and a “just right” texture scale.

RESULTS AND DISCUSSION

Rep. 1

The moisture, fat, pH, and NaCl analyses from Trial 3 are found in Table 3. The cheeses with added xanthan gum produced the longest strings with the smallest diameter. While the cheese with no added polysaccharide had little to no string formation. After 2 weeks of storage the string formation was relatively similar with the different concentrations of the xanthan gum in the cheeses (figure 5). The cheeses with concentrations of 0.5% to 1.0% had
Increasing stringiness of low fat Mozzarella cheese using polysaccharides / D. J. McMahon

slightly better string formation producing string with smaller diameters. After 8 weeks of storage the string formation decreased and differed in the cheeses (Figure 6). The cheeses with 0.5% and 1.0% added xanthan gum slurry produced the longest strings with the smallest diameter. The cheese with no added xanthan gum had little to no string formation. The cheese with 2.0% added xanthan had little to no string formation after 8 weeks of storage. As with Trial 2 string formation decreased with storage time as seen when comparing figures 3 and 4.

Analysis with TPA of commercial string cheeses compared to low-fat string cheeses with added xanthan gum indicated that the commercial string cheeses deformed under the force of the blade and fractured at around 8 to 11 mm penetration. The low fat cheeses were analyzed after 2 weeks of storage and exhibited a more brittle fracture at 9 to 12 mm that was manifest by a sharp peak in force followed by a sharp drop off in force as shown in Chart 4. The commercial cheese deformed and compressed more than it fractured as manifest by the lack of a sharp spike in the force measured. After 8 weeks of storage time the low-fat string cheeses had a decrease in firmness and fracture intensity as seen in chart 4. The low fat cheeses became softer as seen by the decrease in force. The addition of xanthan gum produced cheeses with more similar TPA curves to those of commercial string cheese, but the cheese still fractures.

The consumer panel conducted after 2 weeks of storage showed that the cheese with 1.0% xanthan gum was the most preferred by the panelists of the low-fat cheeses with a score of 6.44 (table 7). The low-fat control was the least preferred by consumers in both taste and texture tending to be too firm with a score of 5.89 and JAR of 4.01. The commercial cheese was most preferred of all the cheeses, both in taste and texture with a score of 7.27 and a JAR of 2.96. The consumer panel conducted after 8 weeks of storage had slightly different results (table 8). The commercial cheese was preferred most in taste and texture among all cheeses with a score of 6.43 and a JAR of 3.01. The 1.0% xanthan gum cheese was most preferred in taste among the low fat cheeses with a score of 6.13. The 1.5% xanthan gum cheese was most preferred for texture among the low-fat cheeses with a JAR of 2.95. The least preferred for taste and texture was the 0.25% xanthan gum cheese and some described it as having an oxidized taste.

CONCLUSIONS

The addition of polysaccharides to low-fat string cheese increased fiber formation so that the cheeses were more similar to low moisture part skim commercial string cheese in stringiness. The string formation in the low-fat cheeses did decrease over time and could be problematic for length of shelf life. There could be several reasons why this happened: 1-The moisture in the channels between the proteins that carried the polysaccharides was absorbed into the protein matrix and the channels were lost. 2- It could have been a function of vacuum packaging used for storage of experimental cheeses the vacuum may have been too strong and some of the moisture from the channels was lost to syneresis. 3- Fusion occurring between the polysaccharides and protein matrix. Further research is needed to understand the interactions between the proteins and the polysaccharides. A solution for the packaging would be to use gas flushing similar to what is done commercially and the cheese would be packaged with no extra pressure exerted from the packaging.

The TPA helped to show the difference between the low-fat string cheese and the commercial string cheese. The low fat cheeses tended to fracture and this would indicate that when pulling strings from the cheese the strings will break. The low fat cheese also had a higher force exerted on them which indicates that they are firmer than the commercial cheeses which is an unfavorable characteristic, but the results are promising in that the cheeses with added polysaccharides preformed more like commercial cheeses.

The confocal laser scanning microscopy method used gave promising results in being able to image the
polysaccharides distributed throughout the cheese and visualize the polysaccharides in the protein matrix.

The results from the consumer panels showed that the low fat cheeses with added xanthan gum were preferred over the low fat cheeses with no added polysaccharides and in one consumer taste test the low fat cheese with added xanthan gum was preferred over all other cheeses including the LMPS commercial string cheese. The liking scores for the low fat cheeses did decrease over time, but the cheeses with added xanthan gum were still preferred over the low fat cheeses with no added xanthan. The consumer panel at 8 weeks was a little different in that the cheese with no added xanthan was preferred over the cheese with 0.25% xanthan gum, and explanation for this could have been that the cheese had oxidized as indicated by some of the panelist's comments.

From the data it can be concluded that adding polysaccharides to the low fat string cheese improved the stringiness of the cheese as well as the functionality.


<table>
<thead>
<tr>
<th>Xanthan Conc.</th>
<th>Moisture %</th>
<th>Fat %</th>
<th>pH</th>
<th>Salt %</th>
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<table>
<thead>
<tr>
<th>Xanthan Conc.</th>
<th>Moisture %</th>
<th>Fat %</th>
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<td>6.0</td>
<td>5.49</td>
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<tr>
<td>1.5%</td>
<td>60.32</td>
<td>6.0</td>
<td>5.46</td>
<td>1.86</td>
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Figure 3. Photographs of string formation at 2 weeks storage time, Trial 3, Rep. 1.
Figure 4. Photographs of string formation at 8 weeks storage time, Trial 3, Rep. 1.

Figure 6. Photograph of the piston-driven extruder.
Increasing stringiness of low fat Mozzarella cheese using polysaccharides / D. J. McMahon

Figure 12. TPA graph of Shear test for commercial LMPS mozzarella string cheeses.

Figure 14. TPA graphs of low fat string cheeses at 2 weeks storage time Trial 3.

Figure 15. TPA graphs of low fat string cheeses at 8 weeks storage time Trial 3.
Table 5. Hedonic liking scale averages for string cheeses at 2 weeks storage time Trial 3, Rep 1.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>0% Control</th>
<th>0.25% Xanthan</th>
<th>0.5% Xanthan</th>
<th>1.0% Xanthan</th>
<th>1.5% Xanthan</th>
<th>Commercial Cheese</th>
<th>P-Value</th>
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<tr>
<td>Liking</td>
<td>c</td>
<td>bc</td>
<td>bc</td>
<td>a</td>
<td>ab</td>
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Table 6. JAR scale averages for string cheeses at 2 weeks storage time Trial 3, Rep. 1.

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<th>1.5% Xanthan</th>
<th>Commercial Cheese</th>
<th>P-Value</th>
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<tr>
<td>Frequency in %</td>
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<td>23%</td>
<td>19%</td>
<td>19%</td>
<td>19%</td>
<td>19%</td>
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<tr>
<td>&lt; Center</td>
<td>23%</td>
<td>76%</td>
<td>39%</td>
<td>39%</td>
<td>39%</td>
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Table 7. Hedonic liking scale averages and JAR scores for string cheeses at 2 weeks storage time Trial 3, Rep. 2.

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<th>1.0% Xanthan</th>
<th>1.5% Xanthan</th>
<th>Commercial Cheese</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>Liking</td>
<td>c</td>
<td>bc</td>
<td>bc</td>
<td>b</td>
<td>b</td>
<td>a</td>
<td>0.0001</td>
</tr>
<tr>
<td>JAR - Texture</td>
<td>4.01</td>
<td>3.52</td>
<td>3.86</td>
<td>3.77</td>
<td>3.66</td>
<td>2.96</td>
<td>0.0001</td>
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Table 8. Hedonic liking scale averages and JAR scores for string cheeses at 8 weeks storage time Trial 3, Rep. 2.

<table>
<thead>
<tr>
<th>Attribute</th>
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<th>0.5% Xanthan</th>
<th>1.0% Xanthan</th>
<th>1.5% Xanthan</th>
<th>Commercial Cheese</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liking</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>a</td>
<td>0.0001</td>
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<tr>
<td>JAR - Texture</td>
<td>3.2</td>
<td>3.64</td>
<td>3.35</td>
<td>3.38</td>
<td>2.95</td>
<td>3.01</td>
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Isolation of an oligotrophic *Lactobacillus* species that may be associated with late gas production and splits in cheese

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Funded by: Dairy Management Inc., as administered by the Dairy Research Institute

**ABSTRACT**

A wide variety of facultative and obligate heterofermentative bacteria, including a number of lactobacilli, have been associated with late gas production in aged cheese. Such cheese can suffer from splits and slit defects during cheese storage, especially when storage temperature is increased to accelerate flavor development. The objective of this study was to identify bacteria in aged Cheddar cheese that causes late gas production. We isolated a novel heterofermentative *Lactobacillus* species (WDC04) following incubation on MRS agar at 6°C for 35 d. BLAST analysis against the 16S rRNA gene database of Genbank revealed WDC04 had 97% sequence identity with *Lactobacillus suebicus* strain CECT5917 (AJ575744), *Lactobacillus vaccinostercus* (AB218793), and an uncultured compost bacterial sequence (FN667177). Cellular morphology and colony morphology were consistent with related species. API CH50 fermentation panels showed a preference for utilization of ribose and galactose over other carbohydrate sources, and WDC04 is difficult to grow expect on MRS broth supplemented with galactose and ribose. As a nonstarter lactic acid bacteria (NSLAB), WDC04 ecologically falls into the category of being an oligotroph that undergoes slow growth in conditions of low nutrient availability. In contrast, lactococcal starter bacteria have copiotroph attributes and exhibit high growth rates when resources are abundant such as occurs in milk. In the harsh environment of ripening cheese (no residual lactose, low pH, low temperature and high salt concentration), viability of starter bacteria usually declines. At the same time, oligotrophic NSLABs utilize amino acids and bacterial debris to supply their energy needs, and will slowly increase in numbers until they are the predominant organism(s) of aged cheese microflora. Gas production by WDC04 was observed at 5 d in MRS broth incubated at 25°C with significant gas production by 9 d. Gas production was also observed after incubation for 28 d in MRS at 8°C. It was concluded that WDC04 is a potential cause of gas production during storage of cheddar cheese.
A systematic study of cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese

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Funded by: Dairy Management Inc., as administered by the Dairy Research Institute

ABSTRACT

Use of 50% potassium chloride, and small amounts of magnesium and calcium chlorides has been suggested to maintain salty flavor while minimizing sodium in cheese. Addition of these salts (especially potassium chloride) helps maintain control of bacteria during aging of cheddar cheese. Some research using different salts has been reported on flavor but with no examination of what is taking place in the cheese during aging that is responsible for flavor development. We are proposing a systematic examination of flavor of reduced sodium cheeses and various parameters responsible for generation of cheese flavor. This includes (i) factors influencing survival and growth of bacteria in cheese such as type of salts and amount of water available for bacteria, (ii) metabolic output from bacteria that can directly influence flavor development such as organic acids, volatile flavor compounds, protein breakdown and cheese pH, (iii) an understanding of which bacteria predominant during various stages of cheese aging and how different salts influence their metabolism, (iv) all of which is related to flavor of cheeses at different aging levels (mild, medium, sharp), and (v) whether consumers who are wanting to restrict their sodium intake are willing to accept any flavor changes that may occur as a result of lowering the sodium level in cheese while using other salt (especially potassium chloride) to impart a salty taste to the cheese. Because of known difference in flavor development and perception when fat content is varied, this study will be performed on both regular and reduced fat cheeses.

BACKGROUND

Function of Salt in Cheese.

One of the final steps in the cheese making process is the addition of salt. Salt addition is the major source of sodium in natural cheese. If Cheddar-type cheese were made without added salt, the sodium content would be about 12 mg per 50 g of cheese. This is in comparison to the typical sodium content of 310 mg sodium per 50 g. In dry salted cheeses like Cheddar, the salt is added directly to the curds just before hooping and pressing. Salt serves at least 6 purposes in the manufacture and aging of good-quality cheese:

- to promote syneresis and control final moisture of the cheese,
- to control the metabolism and survival of the starter bacteria,
- to influence the types of secondary organisms that may grow and create flavors during the ripening period,
- to control enzyme activity in the final cheese,
- to control texture of the final cheese as the salt influences the solubility of the proteins in cheese,
- to be a component of the expected taste of the cheese.
The best Cheddar cheeses are manufactured when the salt content of the cheeses is about 1.7% to 2.1% corresponding to about 340 to 420 mg sodium per 50 g cheese. Since moisture content of Cheddar cheese can also vary between about 34% and 39%, the controlling factor is the salt content expressed as a percentage of salt in the water phase of the cheese, which for high quality Cheddar cheese should be in the range of 4.7% to 5.7% salt-in-water concentration (Guinee and O’Kennedy, 2007).

Salt (sodium chloride) addition is a common way to suppress bacterial proliferation in food products (Weimer et al., 1997). In hard cheeses like Cheddar, salt is added prior to pressing the cheese into blocks to allow better whey expulsion and allowing the cheese to knit in the press. Whey release increases salt concentration in the moisture phase of cheese and provides an additional stress barrier to slow down bacterial growth and metabolism and ultimately limit microbial spoilage (Rallu et al., 1996). Salt also lends to better tasting cheese by masking many unwanted flavors and potentiating umami flavor (Drake et al., 2001). The advent of low sodium foods for better health alters manufacturing and ageing conditions that need to be commensurate with cheese flavor development.

Salt and Flavor

A slight salty flavor is an expected aspect of the flavor of cheese (Phan et al., 2008) and challenges reducing sodium levels in cheese have been recently reviewed (Johnson et al., 2009). There has been little published research on the effects of sodium reduction in cheese, with most of the literature being from 10 to 20 years ago. When salt levels in cheese are reduced, the cheese was perceived as having less cheese intensity and more unpleasant aftertastes (Schroeder et al., 1988) and can be more bitter (Banks et al., 1993; Mistry and Kasperson, 1998). Although what often occurs in such studies is there is no correction made during cheese manufacture for decreased whey expulsion when less salt is applied so lower salt cheeses are usually higher moisture and softer than the higher salt cheeses. This exacerbates the cheese defects as it further decreases the salt-in-water content of the lower salt cheeses. In such studies, consumers can recognize and prefer cheddar cheese with normal salt levels (1.7% to 1.8%) compared to cheeses with 1.5% salt or less (Lindsay et al., 1982). Although whether this is a direct effect of salt on flavor perception or a result of changes in flavor development during aging was not determined.

Salt Cations

To replace the salty taste that is absent when salt (sodium chloride) use in cheese is restricted when making a reduced sodium cheese, other metal salt of chloride such as potassium, magnesium, and calcium (but especially potassium chloride) can be used as a cation substitute. However, these other metal ions do not have the same level of saltiness as sodium ions and have inherent off-flavors (e.g., bitter, metallic flavors (Fitzgerald and Buckley, 1985)) that limit their use. Potassium chloride is the most chemically similar to sodium chloride and is the most obvious choice for sodium chloride replacement (Johnson et al., 2009). However, consumers prefer cheese containing only sodium chloride to cheese containing a partial substitution (50:50) with potassium chloride (Lindsay et al., 1982). Replacement of sodium with cations such as potassium, calcium, and magnesium have also recently been investigated (Toelstede and Hofmann, 2008).

Cheese Microbiology

Since cheddar cheese flavor development is known to require lactic acid bacteria (LAB), cheese microbiology has long been a focal point for research to accelerate or enhance flavor reactions. Interestingly, microbial examination of ripening curd has shown that in addition to deliberately added LAB, bacterial-ripened cheeses also contain large populations of adventitious ("contaminant") bacteria that gain access to cheese through milk or the milk-processing environment (Peterson and Marshall, 1990). In cheese made from pasteurized milk, these contaminants are always non-pathogenic species of LAB, termed nonstarter LAB or “NSLAB” that pose no threat to human health. Modern sanitation and Good Manufacturing Practices help keep NSLAB to very low levels in young cheese, but these bacteria inevitably begin to grow and will reach high numbers within a few months of ripening. In Cheddar cheese, for example, numbers of Lactococcus lactis starter bacteria commonly exceed 10^9 colony-forming units (cfu) per gram when ripening begins. As maturation proceeds, the harsh cheese ripening environment (no residual lactose, acidic pH, presence of added salt, and low ripening temperature) gradually takes its toll and starter cells begin to lose their viability. At the same time, NSLAB populations (whose initial numbers are typically well below 10^2 cfu/g in cheese made under good sanitary conditions with high quality milk) begin to multiply and will eventually plateau at cell densities of 10^9-10^10 cfu/gram after 3-9 mo of aging.

Microbiological characterization of NSLAB populations in bacterial-ripened cheeses has shown they may be quite diverse, but are usually dominated by facultatively heterofermentative species of lactobacilli or, far less frequently, by Pediococcus pentosaceus (Beresford et al., 2001; Broadbent et al., 2003; Broome et al., 1990a; Crow et al., 2001; Fryer, 1969; Sherwood. 1939). Use of Lactobacillus spp. NSLAB isolates as adjunct cultures for Cheddar cheese manufacture has indicated these bacteria may influence flavor in at least 3 ways: they may intensify (i.e., accelerate) typical flavor development, impart atypical (but desirable) flavor notes, or promote off-
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon

flavor development (Broome et al., 1990b; Cogan et al., 2007; Crow et al., 2001; Fryer, 1969; Lynch et al., 1999; McSweeney et al., 1994; Sherwood, 1939; Swearingen et al., 2001). In addition, NSLAB have also been associated with cheese quality defects such as open body (via gas production) and formation of calcium lactate crystals (Fryer, 1969; Johnson et al., 1990; Khalid et al., 1990).

Given the causal role of LAB in flavor development, efforts to define the biochemical basis for flavor changes in cheese have logically focused on the microbiology and physiology of species found in cheese (for recent reviews see Beresford et al., 2001; El Soda et al., 2000; Fox and Wallace, 1997; Marilley and Casey, 2004; Rattray and Fox, 1999). Those efforts have identified many of the most important biochemical and chemical processes in maturation, and have shown starter, adjunct, and NSLAB have an intimate role in most of those processes including lactose fermentation, conversion of milk proteins (primarily caseins) into peptides and free amino acids, catabolism of amino acids into volatile aroma compounds, lipase/esterase activity, and citrate catabolism.

Knowledge of the mechanisms by which LAB affect cheese flavor has facilitated industry efforts to promote flavor development in many traditional cheese varieties, but empirical efforts to extend this information into low-sodium and low-fat Cheddar has not proved as successful. It is our hypothesis that lack of proper flavor development in low sodium Cheddar cheese is most likely explained by one of two scenarios: 1) sodium reduction alters the composition of NSLAB populations in cheese, which results in a changed flavor profile; or 2) sodium reduction alters LAB physiology itself (and thus culture metabolism) in ways that fail to give rise to production of flavor- and aroma-active metabolites that are more typical of Cheddar.

Salt and Bacteria

Starter and nonstarter survive for longer in cheese when the salt is lowered to 1.25% compared to cheese with 1.8% salt (Rulikowski et al., 2008). This is related to the stress placed on bacteria by the combined influence of salt, acid, lowered water activity, and cold storage of cheese. Enzyme activity is also affected by salt level because of its relationship to water activity. There is scant information on how the bacterial metabolism and enzyme actions in cheese during aging are affected by lowering sodium levels or adding other metal cations. Rulikowski et al. (2008) reported decreases in bacterial peptidase activity in lower salt cheeses resulting in slower flavor formation and higher bitterness. Fitzgerald and Buckley (1985) reported increased amounts of proteolysis and lipolysis when Ca, K or Mg levels where used to replace part of the sodium. Reddy and Marth (1993) found no significant differences in proteolysis in cheeses made with NaCl, KCl or mixtures of the two salts. Starter and non-starter bacteria have very limited proteolytic abilities towards whole caseins in cheese, although proteinase-positive strains of lactococci have cell envelop associated serine proteinases that hydrolyze caseins.

Reducing the salt level in cheese, or changing cations, alters the components of salt-related stress for bacteria and is likely to unpredictably redirect the starter and nonstarter survival and metabolism for flavor production during aging. The stress response of bacteria to sodium (Rallu et al., 1996; van de Gucht et al., 2002; Xie et al., 2004) and transition metal ions such as copper, nickel, tellurite, arsenate, and cadmium (Barre et al., 2007; Boutibonnes et al., 1995; Efstathiou and McKay, 1977; Liu et al., 2002; Turner et al., 2007) are well known. However, how lactic acid bacteria respond to substitution of sodium with potassium, calcium, and magnesium are not well defined. And in general, survival and stress response to these salt combinations in foods is uncharacterized.

Bacteria Identification

Species fingerprinting by DGGE is a powerful method for characterizing the structure of complex bacterial communities (Fromin et al., 2002). Briefly, DGGE involves discrete separation of small PCR-derived products from individual bacterial species as a function of G + C content and their relative distribution in the sample. As a result, this method is well suited for monitoring and characterization of complex microbial communities, and provides data on the occurrence and relative abundance of microbes in that community.

In DGGE analysis, the DNA band pattern that is obtained from a sample provides a snapshot of the bacterial community in that sample, with each discrete band referring to a unique species. It should be recognized, however, that only the main populations present in a sample (species representing at least 0.1-1% of the total population) are actually displayed in the profile (Fromin et al., 2002). Thus, the gel pattern obtained by DGGE fingerprinting is a better reflection of community structure (i.e., the relative abundance of the main bacterial populations) than of its total richness. Still, the method is one of the most discriminating tools available to dissect complex communities, and it provides a far greater level of sensitivity than is available through traditional culture-based methods of identification.

Automated ribosomal intergenic spacer analysis (ARISA) was first developed to examine microbial diversity and community composition in freshwater (Fisher and Triplett, 1999). However, in recent years, it has been applied to a wide variety of samples including agricultural soil, silage, milk, brine, and cow feces (Cardinale et al., 2004; Welkje et al., 2009). ARISA’s ability to detect unculturable microorganisms is a significant advantage of this technique, as is its ability to quickly identify the composition of microbiota of various environments at a
Figure 1. Internal transcribed spacer of prokaryotes vary from 143-1529 bp in length but primers 1406f and 23Sr (T) could add more 125-140 bp into the PCR products (Fisher and Triplett, 1999). Fragments amplified by ITSF and ITSReub (C) were 197 bp shorter (Jones et al., 2007).

relatively low cost. The technique is based on variations in length and sequence that exist in the intergenic spacer region between 16S and 23S rRNA genes of microorganisms. By using a fluorescent primer to amplify 16S-23S rRNA spacer region, various sizes of PCR products can be detected in the automated capillary electrophoresis systems utilized for DNA sequencing. The use of these capillary electrophoresis systems results in a highly sensitive and reproducible method that is capable of differentiating DNA fragments that differ in length by a single nucleotide.

The position of two primer pairs utilized in ARISA is presented in Figure 1. Historically, primers designated A1406f and 23Sr (T in Fig. 1) were utilized. Subsequently, Cardinale et al. (2004) suggested that the primer pair designated ITSF and ITSReub would have greater utility in differentiating a wider set of microorganisms. However, Jones et al. (2007) argued that the overall pattern and the change of ecological composition over time or across space were identical with either primer pair.

**Bacterial Stress**

Previous studies have shown that sodium stress response in lactococci consists of expression alterations in a few regulatory genes (Rallu et al., 1996). Mainly, sodium alters expression of many metabolic genes such as those related to fatty acid biosynthesis and nucleotide metabolism (Xie et al., 2004). In this aspect sodium stress response of LAB differs from acid and temperature stresses that led to DNA damage response and protein repair (Xie et al., 2004). These studies highlight the importance of cation stress in determining the outcome of LAB metabolism or beneficial flavor compounds in Cheddar cheese. Consequently, changing the component cations in added salts is likely to provide a novel flavor outcome due to salt stress modulation.

Quantitative PCR analysis (qPCR) measures the levels of a gene of a particular organism estimated from DNA extracted from cheese from which can be estimated the number of cells present (Jany and Barbier, 2008). Cation stress response of starter bacteria can be determined by measuring the expression changes of genes related to sodium stress (Xie et al., 2004), which will allow us to understand its role in subsequent cheese flavor development.

**Salt and Proteolysis**

Proteolysis of the major milk proteins, \( \alpha_\text{S1} \), \( \alpha_\text{S2} \), and \( \beta \)-caseins, generally starts enzyme action from residual coagulant (usually chymosin) that is active against \( \alpha_\text{S1} \)-casein, and by plasmin, an indigenous milk proteinase, hydrolyzes all the caseins except \( \kappa \)-casein. In general, the lowest level of proteolysis occurs in cheese made with porcine pepsin while the most extensive proteolysis occurs in cheese made with microbial rennets (Farky, 1995).

Chymosin hydrolyzes the Phe\(_{23}\)-Phe\(_{24}\) (and Phe\(_{24}\)-Val\(_{25}\)) bond of \( \alpha_\text{S1} \)-casein to produce \( \alpha_\text{S1} \)-I [also known as \( \alpha_\text{S1} \)-CN(f24/25-199)] peptide. The hydrolysis of this bond is probably the most important reaction responsible for the initial softening of cheese. Subsequent degradation of the \( \alpha_\text{S1} \)-I casein by rennet occurs during ripening and the peptide, \( \alpha_\text{S1} \)-CN(f1-24/25), is rapidly hydrolyzed by starter cell wall-associated proteinases. Proteolysis of \( \beta \)-casein is less extensive than that of \( \alpha_\text{S1} \)-casein in cheeses made with chymosin, bovine pepsin or porcine pepsin but more extensive breakdown of \( \beta \)-casein occurs in cheeses made with proteases from \( M. \text{mihceli} \), \( M. \text{puisslus} \), and \( C. \text{parastica} \). Plasmin, an indigenous milk proteinase, hydrolyzes all the caseins except \( \kappa \)-casein. Specifically, plasmin hydrolyzes \( \beta \)-casein to \( \gamma \)-caseins [\( \beta \text{-CN}(f29-209, 106-209 \text{ and } 108-209) \)] and protease peptidases. The activity of plasmin is high in cheeses like Romano and Emmental for which high cooking temperatures are used during manufacture, and in which the coagulant is denatured.

Reddy and Marth (1993) found no significant differences in proteolysis in cheeses made with NaCl, KCl or mixtures of the two salts at identical ripening times. However, based on studies on unsalted cheddar cheese (Thakur et al., 1974) there can be a retardation of proteolysis as salt content of cheese is reduced. Salt influences the rate of proteolysis in cheese in 3 different ways: (a) it changes the aggregation status of protein substrate molecules (Mulvihill & Fox, 1978), (b) it influences growth of starter and non-starter bacteria (Turner & Thomas, 1980), and (c) it directly affects the enzyme activities (Kelly et al., 1996). Fitzgerald and Buckley (1985) reported that not partial but
total substitution of KCl for NaCl enhanced proteolysis rates in Cheddar cheese.

References


**RESEARCH PLAN**

**Objective 1.**

Manufacture (1A) full and (1B) reduced fat cheddar cheeses that have the same salt chloride content but different ratios of sodium, potassium, calcium and magnesium. **Donald McMahon, USU**

**Objective 2.**

2A. Perform descriptive sensory flavor analysis and determine changes in cheese flavor profile based on salt cation content. **MaryAnne Drake, NCSU**

2B. Perform consumer testing of the cheeses with a sodium-avoidance population. **MaryAnne Drake, NCSU**

**Objective 3.**

Measure changes in (3A) organic acids and (3B) volatile flavor compounds during aging of cheese made with varying salt cations.

**Objective 4.**

Determine if changes in salt cations influences populations numbers of lactococci and lactobacilli in cheese during aging. **Donald McMahon, USU**

**Objective 5.**

Measure water activity and pH of the cheeses and any changes that occur during aging. **Jeff Broadbent, USU**

**Objective 6.**

Identify the predominant species of lactic acid bacteria present in the cheese during aging.

**Objective 7.**

Investigate whether salt cations other than sodium impart the same stress on starter culture lactococci during cheese aging. **Bala Ganesan, USU**

**Objective 8.**

Measure water activity and pH of the cheeses and any changes that occur during aging. **Donald McMahon, USU**

**Objective 9.**

Make correlations between chemical, microbial and sensory data to produce a knowledge base for use of salt cation substitution in cheddar cheeses. **Donald McMahon**
MATERIALS AND METHODS

Objective 1.

Full fat cheese manufacture.

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

The curd and whey from both vats were then combined by transferring onto a drain table (Kusel Equipment Co., Watertown, WI) with partial whey drainage and stirred until a curd pH of 6.3 was reached (~20 min). The whey was then drained and the dry curd stirred for ~5 min (15 passes of agitators along drain table). Then the curd was allowed to mat together, cut into slabs and cheddared for ~135 min until curd pH reached 5.4. The curd was milled and then separated into eight 11.92-kg portions and placed into open plastic containers and individually salted according to Table 1.0.

Each aliquot of salts was added and manually mixed using 3 applications with 5 min between applications and then allowed to stand for 10 min before filling into plastic cheesecloth-lined stainless steel hoops and pressed overnight (140 kPa, ~18 h, ~20°C). The cheese was then de-hooped, vacuum packaged and stored at 6°C. After 7 d of storage the cheese was cut into 1- and 2-kg pieces and returned to storage.

Cheese sampling.

Samples of cheese were collected before and after salting (d 0), after pressing (d 1), on d 7 and d 28, and then monthly through 9 mo of aging as shown in Table 1.2. These samples were then either immediately distributed for sample preparation for the various analyses or stored frozen at ~80°C. In Rep 2, additional samples were collected of (i) curd prior to salting, (ii) curd after salting, and (ii) unsalted curd that was pressed overnight. These were tested for microbial populations to provide a baseline for starter culture numbers in curd prior to pressing, and the influence of salt content on starter culture die-off during overnight pressing of curd into cheese blocks.

Chemical analysis.

Proximate composition of the cheeses was determined after ~5 d as described by McMahon et al. (2009). Moisture content was measured by weight loss using ~3.7 g of grated cheese in a microwave moisture analyzer (Model SMART System 5; CEM Corporation, Matthews, NC) using program CHEESELF. Fat content was measured by a modified Babcock method (Richardson, 1985). Protein was calculated from total N as measured by Kjeldahl, and multiplied by 6.38. Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher 400 (Seward, England). The slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (model 926, Coming, Medfield, MA), and pH by glass electrode. The pH was measured using a glass electrode after stomaching 20 g of grated cheese with 10 g of distilled water for 1 min at 260 rpm.

For mineral analysis, cheese samples were ashed in a muffle furnace (Model 550-126; Fisher Scientific, Pittsburgh, PA) at 100°C for 18 to 24 h, then 24 to 36 h at 300°C and 12 to 24 h at 550°C (until the ash is white), then cooled to room temperature. The sample before ashing, and ash were accurately weighed (~±0.05 mg) and the ash sent to Analab (Fulton, IL) for mineral analysis by inductively-coupled plasma spectroscopy.

Objective 2.

Flavor properties of cheeses.

Cheeses were received at North Carolina State University at each time point by overnight shipment on cold packs. Upon receipt, cheeses were carefully examined for damage and then placed at 3°C in the dark until sensory and instrumental analyses. Cheese blocks were removed from vacuum-sealed packaging and 1 cm was trimmed from all sides of each block prior to sensory or instrumental analyses. Sensory properties and headspace volatiles of cheeses were evaluated within 1 week of receipt. Additional samples were immediately frozen at -80°C for solvent extraction and volatile compound analysis (within 6 mo of receipt).

Sensory testing was conducted in compliance with the NCSU Institutional Review Board for Human Subjects approval as described in Drake et al. (2010). Cheddar cheeses were cut into 3.5-cm cubes for descriptive sensory analysis. The cheeses were placed into lidded 58-mL
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese

D. J. McMahon

Table 1.0. Weight of sodium chloride, potassium chloride, magnesium chloride and calcium chloride used to salt 11.9 kg portions of cheese curd to produce cheese.

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Target Salt Level Na:K:Mg:Ca (^{1})</th>
<th>NaCl (g)</th>
<th>KCl (g)</th>
<th>MgCl(_2).6H(_2)O (g)</th>
<th>CaCl(_2).2H(_2)O (g)</th>
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<tbody>
<tr>
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<td>300</td>
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<tr>
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<td>90:10:0:0</td>
<td>270</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>1.7</td>
<td>75:25:0:0</td>
<td>225</td>
<td>96</td>
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</tr>
<tr>
<td>D</td>
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<td>150</td>
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<tr>
<td>E</td>
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<td>153</td>
<td>101</td>
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<tr>
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<td>74</td>
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<tr>
<td>H</td>
<td>0.7</td>
<td>100:0:0:0</td>
<td>85</td>
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\(^{1}\)molar ratios
\(^{2}\)none added

Table 1.2. Sample collection protocol with amount of cheese (g) provided to each investigator.

<table>
<thead>
<tr>
<th>Wk</th>
<th>pH/Aw Desc (^{3})</th>
<th>Cons (^{2})</th>
<th>Org (^{3})</th>
<th>Micro</th>
<th>Sol N (^{4})</th>
<th>PAGE (^{5})</th>
<th>Comm (^{6})</th>
<th>ARISA</th>
<th>Stress (^{8})</th>
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</table>

\(^{3}\)Descriptive sensory analysis
\(^{2}\)Consumer sensory analysis
\(^{3}\)Organic acids and volatiles
\(^{4}\)Soluble N
\(^{5}\)Urea-PAGE
\(^{6}\)Microbial community by DGGE
\(^{7}\)Lactococcal stress genes.
\(^{8}\)Investigator receiving samples: DJM=McMahon, MAD=Drake, CJO=Oberg, NF=Farkye, JRB=Broadbent, JS=Steele, BG=Ganesan

souffle' cups with randomly generated 3-digit codes. The cheeses were tempered at 12°C for 1 h and were served at this temperature with room temperature deionized water and unsalted crackers for palate cleansing. Descriptive analysis was conducted using a 0 to 15-point universal intensity scale with the Spectrum\(^{TM}\) method (Meilgaard and others 1999; Drake and Civille 2003) and an established cheese flavor sensory language (Drake and others 2001; 2008a, 2008b, 2009; Drake 2007). A trained descriptive sensory panel (n = 10, 9 females, 1 male, ages 23-47 y), with over 150 h of experience each with descriptive analysis of cheese flavor, evaluated the cheeses. Consistent with Spectrum\(^{TM}\) descriptive analysis training, panelists were presented with reference solutions of sweet, sour, salty, and bitter tastes to learn to use the universal intensity scale (Meilgaard and others 1999; Drake and Civille 2003) followed by training with Cheddar cheese flavor references (Drake and others 2001) and discussion and evaluation of Cheddar cheeses to clarify descriptor concepts and to consistently score product attributes. Analysis of variance of data collected from the last part of training indicated that the panelists could consistently use the attributes to differentiate the products.

Cheeses were evaluated by panelists monadically in a randomized balanced design. Each cheese was evaluated in triplicate by each panelist. Evaluations were conducted individually using Compusense Five v4.6 (Compusense, Guelph, Ontario, Canada) or paper ballots
in an enclosed room with positive air pressure dedicated to sensory analysis and free from external aromas, noise, and distractions. Panelists were instructed to expectorate samples after evaluation.

**Consumer acceptance testing.**

Sensory testing will be conducted in compliance with the NCSU Institutional Review Board for Human Subjects approval. Cheeses will be dispensed into lidded 58 mL souffle' cups numbered with 3-digit codes. Cheeses will be served at 10°C. Consumers of cheeses (50% on sodium restricted diet, 50% no dietary restrictions) will be recruited from the consumer database maintained by the sensory service center at North Carolina State University. The database has more than 3000 members. Consumers (n = 150 for each test, 75 each demographic category) are provided with consent forms consistent with NCSU Human Subjects approval, a demographic screener, and a scoring ballot. Cheeses are presented individually in a randomized balanced order. The scoring ballot will be designed to evaluate consumer evaluations of cheese for: overall acceptance, flavor and texture liking, and salty taste liking using a 9-point hedonic scale where 1 = dislike extremely or low intensity and 9 = like extremely or high intensity. Consumers may also rate salty taste using a just-about-right (JAR) scale for further product development guidance. Consumers were provided with deionized water and unsalted crackers for palate cleansing. Responses are collected using Compusense Five v4.6 (Compusense, Guelph, Ontario, Canada). Consumers are provided with gift certificates for their participation.

**Objective 3.**

**Volatile compounds.**

Volatile flavor-related compounds were measured as described by Drake et al. (2010).

**SolidPhaseMicro-ExtractionGasChromatographyMassSpectrometry (SPME GC MS).**

Headspace volatiles of cheeses were evaluated by SPME GC MS. Samples of each cheese (5 g) were loaded in triplicate into 20 mL autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Sawanee, FL). Internal standard solution (2-methyl-3-heptanone in methanol, Sigma-Aldrich, Milwaukee, WI) was added to each vial to control for analysis variability.

Samples were injected using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890N gas chromatograph (GC) with 5973 inert MS detection (Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 10°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1 cm DVB/CAR/PDMS fiber at 31 mm with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm. The GC method used an initial temperature of 40°C for 5 min with a ramp rate of 8°C/min to 250°C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250°C. An RTx-5ms column (30 m length x 0.25 mm inner diameter x 0.25 um film thickness) was used for all analyses at a constant helium flow rate of 1 ml/min. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the Quad at 150°C and Source at 250°C. Samples were maintained at 10°C prior to fiber exposure.

**Organic acids.**

Organic acids were extracted from cheeses using water and acetonitrile and separated by HPLC using the methods described by Marsili et al. (1981). Calibration curves were determined for the various analytes, and then analyte concentration in the cheese calculated using a dilution factor (DF) based on sample weight of the cheese and weight of 0.013 N H₂SO₄ in which the cheese was dissolved. Acids were identified by comparison of retention times to authentic standards injected under identical conditions.

**Objective 4.**

**Microbial analysis.**

**Enumeration by Selective Media.** Cheese was analyzed for lactococci, total lactic acid bacteria (LAB) and nonstarter LAB (NSLAB) using selective media as described by Oberg et al. (2011). In brief, 11 g of cheese was added to 99 mL of sterile 2% (w/w) sodium citrate buffer and stomached for 4 min at 260 rpm then serially diluted using Butterfield Phosphate buffer dilution blanks (Hardy Diagnostics, Santa Maria, CA). The pour plate method was used with 3 selective media. Lactococci were enumerated on M17 lactose (M17-L) agar (Difco, Becton, Dickinson and Co., Sparks, MD) after 18 to 24 h aerobic incubation at 30°C. Total LAB were enumerated on de Man, Rogosa and Sharp (MRS) agar (Difco, Becton, Dickinson and Co.) supplemented with 10 g/L sorbitol (MRS+S), after 48 h anaerobic incubation at 37°C. Nonstarter LAB were enumerated on MRS agar containing 1.0 mg/L vancomycin (Sigma-Aldrich Inc., St. Louis, MO) (MRS-V) after 48 h anaerobic incubation at 37°C.

Samples of cheese were transported to Weber State University (Ogden, UT) for microbial analysis at d1, wk 1 and 4, and then monthly. In replicates 2, samples of unsalted and salted cheese curd (refrigerated overnight at 4°C) and pressed unsalted cheeses were also sent for microbial analysis with d1 samples.
Objective 5.

Proteolysis.

Proteolysis in cheese was determined by urea-PAGE and measuring % water-soluble N. Urea-PAGE of cheeses was performed as described by Farkye et al. (1995). Ten milligram of each sample was weighed and mixed with 1mL of 1x sample buffer then heated for 5 min at 50°C and vortexed. Then 4uL of each sample was loaded onto the urea-PAGE gel consisting of 12% (wt/vol) acrylamide resolving gel with a 4% (wt/vol) acrylamide stacking gel. The gel was run at 150 V for 60 min.

Water-soluble fractions of the cheeses were prepared as described by Kuchroo & Fox (1982) and analyzed for N by Kjeldahl.

Objective 6.

Denaturing gradient gel electrophoresis.

As cheese samples were collected for microbiological sampling, additional samples were plated in duplicate on MRS agar with 0.5% sorbitol (MRS+S) to propagate cells for analysis by denaturing gradient gel electrophoresis (DGGE). Cells for DGGE were prepared by spread plating 0.1-mL samples of the 10^4 dilution on MRS+S agar with aerobic or anaerobic incubation at 30 or 45°C for 48 h, or 10°C for 1 wk. After incubation, 500 uL DNase/RNase-free water (VWR International, West Chester, PA) was added to each plate, the cells were scraped from the agar and pooled using a glass plate spreader, then transferred into a 1.5-mL microcentrifuge tube. The cells were pelleted by centrifugation at 14,000 rpm for 1 min, then the supernatant was discarded and the pellet-containing tubes placed at -80°C.

Total genomic DNA was isolated from the cell pellets and directly from thawed cheese samples (250 mg) using the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) as directed by the kit supplier. The variable V3 region of the bacterial 16S rRNA gene was amplified from template DNA isolated from cells or cheese by PCR with forward (5'-GBBCCGC CGCGCGC GGC GCCGC GGGC GCCGC GACGGGGGCTACGGAGGC AGCAG-3') and reverse (5'-ATTACCG CGGCTGC TGG-3') primers designed to bind highly conserved regions of the gene (the resulting amplicon corresponds to nt positions 341 to 534 in the 16S rRNA gene of E. coli). The forward primer contained a GC rich sequence (GC clamp; underlined) to help stabilize melting characteristics (Muyzer, et al., 1993).

To create a positive control for PCR and DGGE, template DNA was extracted from pure cultures of L. lactis ssp. lactis starter 850, Lactobacillus casei ATCC 334, Lactobacillus curvatus WSU1, Lactobacillus plantarum ATCC 4008, Lactobacillus helveticus CNRZ32, Streptococcus thermophilus ATCC 19987, and Escherichia coli ATCC 4700, which were obtained from our laboratory collection.

The PCR was performed with a DNA Thermal Cycler (PerkinElmer model 480, Wellesley, MA) in a final volume of 50 uL that contained 1x TaqMaster PCR Enhancer (5 Prime Inc., Gaithersburg, MD), 1x Buffer B (Fisher Scientific, Pittsburgh, PA), 2.5 mM MgCl2, 25 uM of each dNTP, 2.5 IU Taq DNA polymerase (Fisher Scientific, Pittsburgh, PA), 1 uM of the primers, and 1 uL template DNA solution. The PCR reaction was overlaid with 50 uL mineral oil (PerkinElmer, Waltham, MA). To minimize spurious priming, touchdown PCR was used (Don et al. 1991), wherein the annealing temperature of the first PCR cycle was 10°C higher (65°C) that the optimum annealing temperature, and every second cycle the anneal temperature was dropped 1°C until it reached the optimum annealing temperature, whereupon 15 additional cycles were completed. The presence of amplicon was verified by electrophoresis in 1% (wt/vol) agarose gels and ethidium bromide staining.

Denaturing gradient gel electrophoresis was performed with the CBS Scientific Company (Solam: Beach, CA) DGGE system. Gels were made with 8% (wt/vol) acrylamide (acylamide-N,N-methylene-isisacrylamide, 27:1; VWR) in 1x TAE buffer (40 mM Trs acetate [pH 7.4], 20 mM sodium acetate, 1 mM Na2-EDTA) with a gradient of 25%-60% denaturant (100% denaturant is 7M urea and 40% [wt/vol] deionized formamide). Ten microliters of PCR product was combined with 5 uL 6x loading buffer (0.25% bromophenol blue, 12.5% xylene cyanol, and 40% [wt/vol] sucrose in ddH2O) and loaded on the polyacrylamide gel. The DGGE was performed at 60°C and 150 V for 30 min, then for an additional 5 hr at 200 V at the same temperature. Gels were stained by spreading 15 mL of SYBR Gold solution (Molecular Probes, Inc., Carlsbad, CA) diluted 1/10,000 over the surface of the gel. The gel was incubated in the dark for 1 min, washed repeatedly with ddH2O, then photographed using a UV transilluminator (UVP, Upland, CA). Bands that were prominent, unique to the sample, or changed in intensity (i.e., appeared or disappeared as a function of sampling date), were excised with a clean razor blade and placed in 1.5-mL microcentrifuge tubes for further analysis.

Cloning and sequencing of 16S rDNA fragments.

DNA was eluted from denaturing gradient gels using the procedure developed by Koo and Jaykus (2000) and amplified once more by PCR with the same primers and reaction conditions used to prepare the original amplicons. After PCR, newly formed amplicons were purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA), then cloned into E. coli 10G using the pSNaRT GC HK.
cloning Kit (Lucigen, Middleton, WI). Five kanamycin-resistant cfu from each cloning reaction were streaked for purity, then single colony isolates were inoculated into 10 mL Luria-Bertani (LB) broth and grown overnight at 37°C with mild shaking. Plasmid DNA was isolated from these cells by the alkaline lysis method, and the presence of the plasmid was confirmed by electrophoresis in 1% (wt/vol) agarose gels with ethidium bromide staining (Sambrook et al., 1989).

Plasmid DNA was then purified using the QIAquick PCR purification kit (Qiagen) then sent to the Center for Integrated BioSystems at Utah State University for bidirectional sequencing by fluorescent dye terminator chemistry. Nucleotide sequence similarity searches were performed using BLAST tools available through the National Institutes of Health Center for Biotechnology Information (www.ncbi.nlm.nih.gov) to determine the operational taxonomic unit (OTU) bacterial species from which each amplicon was most likely derived.

Objective 7.

**Water activity and pH.**

The pH was measured using a glass electrode after stomaching 20 g of grated cheese with 10 g of distilled water for 1 min at 260 rpm. Water activity was measured by relative humidity using an Aqualab CX2 instrument and pH was measured by glass electrode.

Objective 8.

**Extraction of genomic DNA and total RNA from cheese.**

Total genomic DNA and total RNA will be extracted from cheese after cell lysis using the TRIzolLS reagent (Invitrogen, Carlsbad, CA) with minor modifications to procedures described earlier (Ganesan et al., 2007; Xie et al., 2004). Grated cheese (0.25 g) will be treated with bacterial lytic enzymes (lysozyme+mutanolysin at 37°C/1 hr, followed by proteinase K at 50°C/1 hr) and then suspended in TRIzolLS (500 μL) to lyse bacteria, and shaken with chloroform (200 μL) for 30 s to allow phase separation. The top aqueous phase will be removed after centrifugation (12,000 x g for 15 min at room temperature) and used for RNA extraction by isopropanol precipitation. The RNA pellet will be washed with 75% ethanol and air-dried prior to resuspension in nuclease-free water. Similarly, DNA will be precipitated from the organic phase with 100% ethanol for 3 min. The DNA will be collected by centrifugation (2,000 x g for 5 min at 4°C), washed twice with 0.1M sodium citrate in 10% ethanol (30 min at room temperature), once with 75% ethanol (15 min at room temperature), pelleted, air-dried, and resuspended in autoclaved ddH₂O.

RNA reverse transcription.

RNA will be reverse transcribed using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) as per manufacturer’s instructions. Briefly, the total RNA (1 μg) will be incubated with dNTPs, random hexamers, and reverse transcriptase enzyme for 1h at 50°C, followed by treatment with 0.1 M NaOH at 65°C for 30 min to degrade RNA. The cDNA will be isolated from this mixture using the QIAQuick PCR purification kit (Qiagen, Valencia, CA) as described by the manufacturer.
Quantitative PCR.

Bacterial levels in cheese using DNA and gene expression analysis from RNA will be determined by quantitative PCR (qPCR). For gene expression assays, RNA will be reverse-transcribed to make cDNA (described above) from which 1 μL will be used for qPCR. Starter bacterial primers will be selected from previous studies that designed genus- and species-specific primers for the organisms. Primers for gene expression qPCR will be designed in this study for the organisms of interest using the web interface for the Primer3 software (Rozen and Skaltsky, 2000). Briefly, the qPCR reaction will be set up as follows: 1 μL of the total DNA extracted from cheese will be used in a 25 μL reaction that includes qPCR master Mix (HotStart-IT® SYBR® Green, USB Corp., Cleveland, OH) and 10 pmol of genus- and species-specific primers targeting the 16s ribosomal gene. The qPCR will be performed on a DNA Engine OPTICON2 (Bio-Rad Labs Inc., Hercules, CA) with initial enzyme activation at 95°C for 5 min, followed by 40 cycles of: denaturation at 95°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min.

Gene expression analysis.

Upon completion of stress alteration analysis in full fat and reduced fat Cheddar cheese, based on the results, gene expression analysis will be conducted on a limited subset of salt treatments in full fat cheese during the 3rd year using methods described by Xie et al. (2004) and Ganesan et al. (2007). Three salt levels will be compared at 3 selected times during ageing to determine how different salt stresses alter the flavor-forming pathways of starter bacteria. RNA previously extracted for qPCR analysis will be stored at -70°C until gene expression analysis. The cDNA will be fragmented into 50-200 bp using DNase I (Promega, Madison, WI) and then labeled with biotin as described by Affymetrix (Santa Clara, CA). The labeled cDNA will be hybridized overnight on a microarray designed by Dr. Weimer’s group for L. lactis, washed, stained, and raw gene expression data acquired by a Genechip scanner (Affymetrix).

Statistical analysis.

Cheese manufacture and bacterial addition will be done in triplicate. For bacterial survival, cycle times from qPCR analysis will be matched against standard curves created with logarithmic phase cells’ DNA to estimate the bacterial numbers in cheese. Changes in gene expression over cheese age for different bacteria and the effect of salts on physiological stress will be analyzed using a repeated measures model in JMP7 to attribute statistical significance (α=0.05) for the expression changes. The data will be analyzed as a repeated measures model using PROC GLM, with salt reduction as the treatment.

Microarray gene expression data will be normalized by the robust multichip average method and analyzed for statistically significant differences using a repeated measures model in R using the nlme package as described earlier (Ganesan et al., 2007). The false discovery rate for significant changes in gene expression will be set at 10%.

RESULTS AND DISCUSSION

Proximate Composition and Mineral Content (Objectives 1 and 5)

Composition of the cheese is shown in Table 1.2. Moisture contents of the cheeses A through G were in the range 342 to 356 g/kg and were consistent within each replicate (less than ± 4 g/kg). Cheese H that received the low salt treatment was consistently 25 g/kg higher than the other cheeses. No curd treatment was applied prior to salting to lower curd moisture content and less whey expulsion occurs at this salt level. There were no significant differences in fat content.

Salt levels when measured by chloride ion content were similar among cheeses A through G and in the range 16 to 20 g/kg. This was expected as substitution of Na+ by K+ was made on a molar basis, and the divalent Mg++ and Ca++ salts were only added as a 10% of total salt and so the extra Cl- included in these salts less than the variation that occurs during salting of curd. Cheese H had mean salt levels of 6.8 g/kg and mean sodium level of 2.7 mg/g that is at the level required to be classified as a low-sodium cheese (i.e., ≤ 140 mg/50 g of sodium (FDA 2008)).

Mean sodium contents decreased from 6.5 mg/g in the control cheese (Cheese A, mean salt = 17.4 g/kg to 2.1 g/kg in cheese E, that had a 75% molar substitution of KCl. There was a corresponding increase of potassium from 1.1 mg/g when no KCl was added (cheese A) to 10.4 mg/g in cheese E. The mean sodium content of cheese B (10% molar substitution) was the same as the control cheese, and for cheese C (25% molar substitution) was only 10% less than the control. Based on their mean sodium content, cheeses D and E could be classified as a 40%-reduced sodium cheese and low sodium cheese, respectively. Cheeses F and G could be classified as 40%- and 50%-reduced sodium cheeses, respectively, had mean potassium levels of 6.1 and 5.1 mg/g, and ~1 mg/g increase in magnesium and calcium, respectively.

Sensory Properties (Objective 2)

As might be expected for young cheeses, few sensory differences were noted after 3 mo storage of the cheeses (Table 2.1). Differences were detected in basic tastes, most notably salty and bitter tastes. Cheeses with sodium
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon

Table 1.2. Composition of cheeses (n=3).

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Target</th>
<th>Na:K:Ca:Mg</th>
<th>Moisture</th>
<th>Fat</th>
<th>Protein</th>
<th>Salt</th>
<th>Ash</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
<th>Phos</th>
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<td>0.70</td>
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<td>32.63</td>
<td>25.25</td>
<td>1.70</td>
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<td>0.03</td>
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<td>31.97</td>
<td>26.29</td>
<td>1.80</td>
<td>5.55</td>
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<td>0.84</td>
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<td>0.61</td>
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STDEV

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<th>Cheese</th>
<th>Target</th>
<th>Na:K:Ca:Mg</th>
<th>Moisture</th>
<th>Fat</th>
<th>Protein</th>
<th>Salt</th>
<th>Ash</th>
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<td>0.01</td>
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<td>0.04</td>
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1 Measured by Cl analysis.
2 Calculated as PO4 from measurement of P.

Table 2.1. Sensory flavor attribute means of cheeses1 (Rep 1 and Rep 2) after 3 mo of storage at 6°C.

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Cooked</th>
<th>Whey</th>
<th>Milkfat</th>
<th>Sulfur</th>
<th>Brothy</th>
<th>Sour</th>
<th>Bitter</th>
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<td>3.2abc</td>
<td>2.2b</td>
<td>2.6b</td>
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<tr>
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<td>3.1a</td>
<td>1.0a</td>
<td>1.6a</td>
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<td>H</td>
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<td>1.6a</td>
<td>3.2a</td>
<td>1.5a</td>
<td>1.5a</td>
<td>3.2a</td>
<td>ND3</td>
<td>2.3d</td>
<td>1.9b</td>
<td>2.4b</td>
</tr>
</tbody>
</table>

1 Cheeses salted as described in Table 1.0.
2 Attributes were scored using a 0- to 15-point universal intensity scale (Meilgaard et al., 1999) using an established cheese flavor language (Drake et al., 2001; Drake, 2007). Most cheese flavor attributes fall between 0 and 5 on this scale (Drake et al., 2008a,b, 2009).
3 ND = not detected.

Figure 2.2. Sensory profile scores for selected attributes in selected cheeses that were significantly different (p<0.05) at 3 mo of aging of all 3 reps. Cheeses were made using the normal amount of salt (control, cheese A), salted using a 75/25, 50/50 or 25/75 ratio of sodium chloride and potassium chloride (cheeses C, D and E, respectively) or salted at the low sodium level (low salt, cheese H).

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cation substitution had low but detectable levels of bitterness that increased as amount of sodium in the cheese was decreased (cheeses A through E). No bitterness was detected in the low salt cheese (cheese H), implying the increasing bitterness in cheeses A through E resulted from the increased potassium substitution for sodium of 10%, 25%, 50% and 75% on a molar basis for cheeses B, C, D, and E, respectively.

**Consumer Acceptance (Objective 2B.)**

Consumer sensory evaluation of the cheeses was performed on Reps 1 and 2 after 6 mo of aging with panelists who reported as consuming either a regular diet or a sodium-restricted diet.

**Organic Acids (Objective 3)**

Calibration curves were established for various organic acids as shown in Table 3.1. The values calculated using the equations were multiplied by the dilution factor (DF) to get the concentration of analyte in the cheese.

Organic acids changed with storage time (new figures added) but it is currently too soon to make any concrete observations on changes.

**Instrumental Analysis (Objective 3.)**

Volatile flavor compound analysis has been performed on Reps 1 and 2 through 6 mo of aging as seen in Table 3.2

---

**Figure 2.3.** Mean liking scores (using a 9-point hedonic scale) by consumers on a regular or salt restricted diet, for cheeses made using the normal amount of salt (control, cheese A), salted using a 75/25, 50/50 or 25/75 ratio of sodium chloride and potassium chloride (cheeses C, D and E, respectively) or salted at the low sodium level (low salt, cheese H).

**Table 3.1.** Retention time (RT) for different organic acids measured using HPLC along with calibration equations and $R^2$ for the calibration curves.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>RT (min)</th>
<th>Calibration equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td>5.86</td>
<td>(UV 210nm area - 26387) + 2013.5</td>
<td>0.99</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>6.12</td>
<td>(UV 210nm area - 16346) + 74094</td>
<td>0.99</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>7.08</td>
<td>(UV 210nm area +20843) + 11421</td>
<td>0.99</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>8.39</td>
<td>(UV 210nm area+22811) + 121.87</td>
<td>0.99</td>
</tr>
<tr>
<td>Formic acid</td>
<td>10.2</td>
<td>(UV 210nm area-7958.6) + 1412.7</td>
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<tr>
<td>Acetic acid</td>
<td>11.1</td>
<td>(UV 210nm area+3037.3) + 841.44</td>
<td>0.99</td>
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<tr>
<td>Propionic acid</td>
<td>13.0</td>
<td>(UV 210nm area-10695) + 834.42</td>
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</tr>
<tr>
<td>Butyric acid</td>
<td>15.9</td>
<td>(UV 210nm area+2497.5) + 820.02</td>
<td>0.99</td>
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<tr>
<td>Hippuric acid</td>
<td>26.8</td>
<td>(UV 210nm area-8970.9) + 55216</td>
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</table>
Table 3.2. Mean relative abundance (µg/kg) of selected aroma-active compounds as determined using solid phase microextraction gas chromatography of Rep 1 and Rep 2 cheeses¹ (as described in Table 1.0) after 6 mo of storage at 6°C.

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<th>Compounds</th>
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<td>DMS</td>
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<tr>
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<td>13</td>
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<td>2-methylbutanal</td>
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<td></td>
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<tr>
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<tr>
<td>δ-dodecalactone</td>
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</tbody>
</table>

¹Cheeses salted as described in Table 1.0.
²ND = not detected.
³Values within a column followed with the same letter were not significantly different (α = 0.05).
Table 3.2. Continued.

<table>
<thead>
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<th>Compounds</th>
<th>week</th>
<th>A</th>
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<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
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<td>1.74a</td>
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<td></td>
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<td>1.02ab</td>
<td>0.906ab</td>
<td>0.676ab</td>
<td>0.559b</td>
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<td>0.888ab</td>
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</tr>
<tr>
<td>1-octen-3-one</td>
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<td>15.3ab</td>
<td>15.7ab</td>
<td>10.6b</td>
<td>16.2a</td>
<td>13.9ab</td>
<td>15.8ab</td>
</tr>
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<td>10.4ab</td>
<td>3.51c</td>
<td>7.31bc</td>
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<td>74.3b</td>
<td>81.9b</td>
<td>85.5b</td>
<td>113ab</td>
</tr>
</tbody>
</table>

1 Cheeses salted as described in Table 1.0.
2 ND = not detected.
3 Values within a column followed with the same letter were not significantly different (α = 0.05).

Figure 3.1. Citric acid content of cheeses (as described in Table 1.0) at 0, 1, 3, 6 and 9 mo storage at 6°C.

Figure 3.2. Orotic acid content of cheeses (as described in Table 1.0) at 0, 1, 3, 6 and 9 mo storage at 6°C.

Figure 3.3. Pyruvic acid content of cheeses (as described in Table 1.0) at 0, 1, 3, 6 and 9 mo storage at 6°C.

Figure 3.4. Lactic acid content of cheeses (as described in Table 1.0) at 0, 1, 3, 6 and 9 mo storage at 6°C.
Microbial Numbers (Objective 4.)

Microbial populations in the Rep 1 cheeses during storage are shown in Figures 4.1 and 4.2. The Rep 1 cheeses all had levels of NSLAB of ≤10^2 cfu/g. Initial starter culture lactococcal levels in Rep 1 cheeses were 2.4 ×10^6 cfu/g as evidenced by the low salt cheese (H) (0.64% salt, 1.70% S/M) that would have the least inhibitory effect of salt on the starter culture. In comparison, the starter culture in the cheese that received the standard level of salt addition (A) (1.7% salt, 4.64% S/M) had already suffered a 95% die-off and dropped to 2.1 ×10^7 cfu/g.

Microbial populations: mean log numbers.

In Rep 1 cheeses (except for cheeses G and H) the NSLAB numbers did not increase during 5 mo of storage. In contrast, the NSLAB numbers in Rep 2 cheeses had increased to 10^6 cfu/g by 2 mo of storage. Such variation in microflora of cheeses made the same way (and with similar chemical composition) but at different times is common in cheesemaking and may reflect changes in strain of NSLAB that is growing.

For some of the cheeses (Rep 2 at d 7), the lactococcal numbers were higher than the total LAB numbers as small pinpoint colonies on the MRS+S media were not included in the total LAB count. On further investigation, these were determined to be cocci and are assumed to be colonies from metabolically-injured starter culture bacteria. These plates will be re-counted to obtain population numbers for total LAB with and without such metabolically-injured cells.

The samples of cheese curd and pressed unsalted cheese (d 1) from Rep 2 were both observed to have 6 ×10^8 cfu/g lactococci, compared to 8 ×10^7 to 2 ×10^8 cfu/g for the salted cheeses (A through G). Thus, there is about a 0.5 log die-off of starter culture occurring during overnight pressing of the cheese. The low-salt cheese (H) has less die-off than the other cheeses and was at 4 ×10^8 cfu/g lactococci at d 1. It was also observed that the counts on M17-L agar for d 1 and 7 cheeses were sometimes higher than the counts on MRS+S agar. Since the cheeses had very low NSLAB numbers at these times (<10^3 and often <10^2) it would only be the lactococcal starter culture that is growing on MRS+S agar and the higher numbers on M17-L agar is probably a result of the differences in incubation conditions between these media (i.e., anaerobic for 48 h at 37°C and aerobic for 18 to 24 h at 30°C).

To account for differences in total LAB populations...
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon

Figure 4.1. Mean microbial populations in control cheeses made using 100% NaCl with standard salt (A) and low salt (H) levels during 7 months of storage. Bars = SEM.

Figure 4.2. Mean microbial populations in cheeses made using 1.7% salt consisting of NaCl/KCl blends in molar ratios of 90:10 (B), 75:25 (C), 50:50 (D) and 25:75 (E) during 7 months of storage. Bars = SEM.

Figure 4.3. Mean microbial populations in cheeses made using 1.7% salt consisting of a 50:40:10 molar ratio blend of NaCl/KCl/MgCl₂ (F) and NaCl/KCl/CaCl₂ (G) during 7 months of storage. Bars = SEM.
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Figure 4.4. Mean relative log microbial populations (with trend lines) for control cheeses made using 100% NaCl with standard salt (A) and low salt (H) levels during 7 months of storage. Bars = SEM.

Figure 4.5. Mean relative log microbial populations (with polynomial trend lines) in cheeses made using 1.7% salt consisting of NaCl/KCl blends in molar ratios of 90:10 (B), 75:25 (C), 50:50 (D) and 25:75 (E) during 7 months of storage. Bars = SEM.

Figure 4.6. Mean relative log microbial populations (with polynomial trend lines) in cheeses made using 1.7% salt consisting of a 50:40:10 molar ratio blend of NaCl/KCl/MgCl₂ (F) and NaCl/KCl/CaCl₂ (G) during 7 months of storage. Bars = SEM.
between the cheeses, the relative log proportion of starter and NSLAB bacteria was calculated by dividing the log number of starter and NSLAB by the log number of total LAB. This provides a representation of whether the starter bacteria or NSLAB dominate the microbial population as shown in Figures 4.4, 4.5 and 4.6. At the normal salting level (cheese A) the concomitant die-off of the lactococci and growth of NSLAB is such that by 3 mo the cheese contains equal numbers of lactococci and NSLAB, and after that time the NSLABs predominate (Figure 4.4). In contrast, when only 0.7% salt is added, the starter cultures remain the dominant microbe throughout at least 7 months storage.

Adding KCl does not appear to change this trend (Figure 4.4). While adding CaCl2 does appear to also support longer survival of the lactococci (Figure 4.5).

Proteolysis (Objective 5)

%WSN (Objective 5)

Mean changes in water-soluble N (%WSN) levels are shown in Figures 5.1 and 5.2 Typical trends of increasing levels of %WSN during storage of the cheeses were observed. No difference in production of %WSN was observed based upon cation content of the cheeses.

Urea-PAGE (Objective 5)

Electrophoresis has been performed on month 6 from the rep 1 and month 3, 4, and 5 from the rep 2. Densitometry has been performed on all gels analyzed to date. Urea-PAGE gel electrophoretogram of the different cheeses at 1 d, 7 d and 4 wk after manufacture are shown in Figure 5.3.

Levels of $\alpha_\text{S-1}$-casein, beta-casein, and $\alpha_\text{S-2}$-casein-1 (i.e., $\alpha_\text{S-1}$-casein, (f24-199)) throughout 6 mo of aging for Rep 1 are shown in Figure 5.4.

Preparation of an ARISA database for dairy lactic acid bacteria

In silico analysis of ARISA phylotypes.

Sequences of rRNA operons were obtained from the complete genome sequences of predominant lactic acid bacteria in cheese, which are available on the National Center for Biotechnology Information (NCBI) website. These included Lactococcus lactis subsp. lactis CV56 and KF147 as well as subsp. cremoris A76, MG1363, NZ9000, and SK11, Streptococcus thermophilus JIM 8232, LMD-9, and ND03, Lactobacillus casei ATCC 334 and Zhang, Lb. rhamnosus ATCC 8530, ATCC 53103 or GG, Lc 705, and Lcr35, Lb. fermentum CECT 5716 and IFO 3956, Lb.
Figure 5.3. Urea-PAGE of Rep 1 cheeses A through H at d 1, d 7 and wk 4 after manufacture compared to sodium caseinate (Std).
Figure 5.4. Band intensities for \( \alpha_1 \)-casein, \( \beta \)-casein, and \( \alpha_{1\prime} \)-casein-1 (i.e., \( \alpha_{1\prime} \)-casein, (f24-199)) throughout 6 mo of aging for Rep 1.
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon

Table 6.1. Species of bacteria identified in samples collected from cheese.

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest relative</th>
<th>% Ident</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
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<td>Lactobacillus graminis</td>
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<td>GU470987.1</td>
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<td>Lactobacillus sakei</td>
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<td>2</td>
<td>Lactococcus lactis lactis</td>
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<td>Enterococcus faecium</td>
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<tr>
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<td>E. durans</td>
<td>99</td>
<td>JN560933.1</td>
</tr>
</tbody>
</table>

Figure 6.1. DGGE of Rep 1 cheeses plated on MRS+sorbitol and incubated at 10°C unless noted.
Lanes: 1 – Cheese A at 1 day; 2 – Cheese A at 3 months; 3 and 8 – marker: A – Lactobacillus plantarum ATCC 4008; B – Lactobacillus helveticus CNRZ32; C – Starter 850; D – E. coli ATCC 47009; 4 - Cheese D at 1 day; 5 - Cheese D at 3 months; 6 – Cheese H at 1 day; 7 – Cheese H at 3 months; 9 – Cheese A (45°C) at 1 day; 10 – Cheese D (45°C) at 1 day; 11 – Cheese H (45°C) at 1 day.
Table 6.2: Calculated actual sizes of ARISA phylotypes for various cheese-related bacteria compared to anticipated sizes with and without tRNA.

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<th>Strains</th>
<th>Anticipated sizes of ARISA phylotypes (bp)</th>
<th>Actual sizes of ARISA phylotypes (bp)</th>
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<tbody>
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<td>With tRNA</td>
<td>Without tRNA</td>
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<td>Lactobacillus curvatus WSU-1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Lactobacillus fermentum ATCC 14931</td>
<td>673</td>
<td>478</td>
</tr>
<tr>
<td>Lactobacillus helveticus ATCC 15009</td>
<td>733</td>
<td>483</td>
</tr>
<tr>
<td>Lactobacillus plantarum ATCC 14917</td>
<td>715</td>
<td>486</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus GG</td>
<td>716</td>
<td>503</td>
</tr>
<tr>
<td>Lactococcus lactis IL 1403</td>
<td>-</td>
<td>584</td>
</tr>
<tr>
<td>Streptococcus thermophilus MTC 330</td>
<td>-</td>
<td>554</td>
</tr>
</tbody>
</table>

helveticus DPC 4571 and H10, and Lb. plantarum JDM1, ST-III and WCFS1. The sequences were aligned with the primer sequences 1406f (5’ TGYACACACC GGCCGT 3’) and 23Sr (5’ GGGTBCCCCCATTCRG 3’) to predict the sizes of ARISA phylotypes amplified by PCR using MEGA 5 (Tamura et al., 2007).

Amplification of ARISA phylotypes.

Genomic DNA from the pure cultures of Lactococcus lactis IL 1403, Streptococcus thermophilus MTC 330, Lactobacillus casei ATCC 334, Lb. curvatus WSU-1, Lb. rhamnosus GG, Lb. fermentum ATCC 14931, Lb. helveticus ATCC 15009, and Lb. plantarum ATCC 14917 was isolated using the DNeasy Blood & Tissue Kit (Qiagen). ARISAPCR was conducted using 5-FAM labeled primer 1406f and primer 23Sr (Fisher and Triplett, 1999). Reaction mixtures contained 1X Pfx50™ PCR mix, 300µM each dNTP, 400µM each primer, 5U Pfx50™ DNA Polymerase (Invitrogen), and genomic DNA in a final volume of 50 µl. PCR conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min and a final extension at 68°C for 5 min. PCR reactions were performed in an iCycler thermal cycler (Biorad). 1 µl of PCR products, 0.4 µl of custom 100- to 2000-bp standard labeled with Rhodamine X (Bioventures), and 10 µl of highly deionized formamide (Applied Biosystems) were submitted to the University of Wisconsin-Madison Biotech Center for denaturing capillary electrophoresis in an ABI 3700 Genetic Analyzer (Jones et al., 2007).

The actual sizes of ARISA phylotypes were calculated by comparing the resulting data (Table 6.2) to the internal size standard using PeakScanner (Applied Biosystems).

Lactococcal Stress Genes (Objective 7)

RNA processing is progressing with a small delay. Sequences of 6 genes, 3 induced by salt stress and 3 repressed, were obtained from the genomes of L. lactis IL1403 and L. cremoris SK11 and compared and a common primer pair for qPCR for each gene has been designed amidst some minor constraints. Additional primer sets were designed to preclude any time lag during optimization. Primers were designed for each gene on the Primer-BLAST tool and all primers were automatically compared against the nr database at NCBI to avoid any cross reactivity with non-lactococcal species.

There has been a delay this quarter in running qPCR gene expression assays due to a significant health setback with one of the collaborators.

RNA extraction is progressing as per schedule. Sequences of 6 genes, 3 induced by salt stress and 3 repressed, were obtained from the genomes of L. lactis IL1403 and L. cremoris SK11 and compared to design a common primer pair for qPCR for each gene.

pH (Objective 8)

The pH and water activity of the cheese were measured throughout storage as shown in Tables 8.1 and 8.2. When NaCl was substituted with KCl there was a greater decrease in cheese pH during pressing such that mean pH for Cheese A (100% NaCl) was 5.23 (SD= 0.08) while cheese made with a 25:75 molar ratio of sodium to potassium (Cheese E) had a day 1 pH of 5.15 (SD=0.04). The cheese made using 50% NaCl, 40% KCl and 10% MgCl2 or CaCl2 had a further drop in pH during pressing to mean pH at d 1 or 5.09 and 5.05, respectively. The low salt cheese (Cheese H) had the lowest pH of 4.97 (SD=0.01). This corresponds
### Table 8.1. Mean pH (and SD) of cheese after overnight pressing (1 d) and during 9 mo storage at 6°C.

<table>
<thead>
<tr>
<th>Cheese</th>
<th>d 1</th>
<th>d 7</th>
<th>d 28</th>
<th>mo 2</th>
<th>mo 3</th>
<th>mo 4</th>
<th>mo 5</th>
<th>mo 6</th>
<th>mo 7</th>
<th>8 mo</th>
<th>9 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>5.23</td>
<td>5.16</td>
<td>5.26</td>
<td>5.40</td>
<td>5.33</td>
<td>5.43</td>
<td>5.36</td>
<td>5.41</td>
<td>5.23</td>
<td>5.16</td>
<td>5.26</td>
</tr>
<tr>
<td>A</td>
<td>5.20</td>
<td>5.17</td>
<td>5.28</td>
<td>5.35</td>
<td>5.30</td>
<td>5.43</td>
<td>5.26</td>
<td>5.37</td>
<td>5.20</td>
<td>5.17</td>
<td>5.28</td>
</tr>
<tr>
<td>B</td>
<td>5.22</td>
<td>5.17</td>
<td>5.24</td>
<td>5.29</td>
<td>5.25</td>
<td>5.36</td>
<td>5.20</td>
<td>5.22</td>
<td>5.22</td>
<td>5.17</td>
<td>5.24</td>
</tr>
<tr>
<td>C</td>
<td>5.21</td>
<td>5.15</td>
<td>5.16</td>
<td>5.25</td>
<td>5.23</td>
<td>5.36</td>
<td>5.20</td>
<td>5.21</td>
<td>5.21</td>
<td>5.15</td>
<td>5.16</td>
</tr>
<tr>
<td>D</td>
<td>5.15</td>
<td>5.11</td>
<td>5.19</td>
<td>5.24</td>
<td>5.21</td>
<td>5.29</td>
<td>5.19</td>
<td>5.25</td>
<td>5.15</td>
<td>5.11</td>
<td>5.19</td>
</tr>
<tr>
<td>E</td>
<td>5.09</td>
<td>5.07</td>
<td>5.15</td>
<td>5.25</td>
<td>5.24</td>
<td>5.25</td>
<td>5.15</td>
<td>5.19</td>
<td>5.09</td>
<td>5.07</td>
<td>5.15</td>
</tr>
<tr>
<td>F</td>
<td>5.05</td>
<td>5.01</td>
<td>5.13</td>
<td>5.16</td>
<td>5.15</td>
<td>5.23</td>
<td>5.09</td>
<td>5.13</td>
<td>5.05</td>
<td>5.01</td>
<td>5.13</td>
</tr>
<tr>
<td>G</td>
<td>4.97</td>
<td>5.01</td>
<td>5.11</td>
<td>5.15</td>
<td>5.14</td>
<td>5.19</td>
<td>5.10</td>
<td>5.14</td>
<td>4.97</td>
<td>5.01</td>
<td>5.11</td>
</tr>
<tr>
<td>H</td>
<td>0.01</td>
<td>0.02</td>
<td>0.07</td>
<td>0.06</td>
<td>0.19</td>
<td>0.08</td>
<td>0.16</td>
<td>0.19</td>
<td>0.08</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

### Table 8.2. Mean (and SD) water activity of cheese after overnight pressing (1 d) and during 9 mo storage at 6°C.

<table>
<thead>
<tr>
<th>Cheese</th>
<th>1 d</th>
<th>7 d</th>
<th>28 d</th>
<th>2 mo</th>
<th>3 mo</th>
<th>4 mo</th>
<th>5 mo</th>
<th>6 mo</th>
<th>7 mo</th>
<th>8 mo</th>
<th>9 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.960</td>
<td>0.956</td>
<td>0.956</td>
<td>0.954</td>
<td>0.953</td>
<td>0.955</td>
<td>0.957</td>
<td>0.963</td>
<td>0.948</td>
<td>0.953</td>
<td>0.954</td>
</tr>
<tr>
<td>A</td>
<td>0.959</td>
<td>0.956</td>
<td>0.954</td>
<td>0.957</td>
<td>0.956</td>
<td>0.964</td>
<td>0.954</td>
<td>0.953</td>
<td>0.950</td>
<td>0.954</td>
<td>0.949</td>
</tr>
<tr>
<td>B</td>
<td>0.957</td>
<td>0.955</td>
<td>0.956</td>
<td>0.959</td>
<td>0.955</td>
<td>0.953</td>
<td>0.955</td>
<td>0.952</td>
<td>0.947</td>
<td>0.949</td>
<td>0.952</td>
</tr>
<tr>
<td>C</td>
<td>0.957</td>
<td>0.959</td>
<td>0.958</td>
<td>0.957</td>
<td>0.959</td>
<td>0.957</td>
<td>0.956</td>
<td>0.957</td>
<td>0.954</td>
<td>0.964</td>
<td>0.978</td>
</tr>
<tr>
<td>D</td>
<td>0.961</td>
<td>0.960</td>
<td>0.955</td>
<td>0.955</td>
<td>0.954</td>
<td>0.953</td>
<td>0.953</td>
<td>0.954</td>
<td>0.959</td>
<td>0.950</td>
<td>0.957</td>
</tr>
<tr>
<td>E</td>
<td>0.962</td>
<td>0.960</td>
<td>0.957</td>
<td>0.957</td>
<td>0.958</td>
<td>0.961</td>
<td>0.967</td>
<td>0.956</td>
<td>0.951</td>
<td>0.957</td>
<td>0.956</td>
</tr>
<tr>
<td>F</td>
<td>0.959</td>
<td>0.961</td>
<td>0.957</td>
<td>0.960</td>
<td>0.964</td>
<td>0.966</td>
<td>0.955</td>
<td>0.957</td>
<td>0.954</td>
<td>0.953</td>
<td>0.955</td>
</tr>
<tr>
<td>G</td>
<td>0.971</td>
<td>0.979</td>
<td>0.975</td>
<td>0.975</td>
<td>0.970</td>
<td>0.966</td>
<td>0.964</td>
<td>0.963</td>
<td>0.964</td>
<td>0.952</td>
<td>0.954</td>
</tr>
<tr>
<td>H</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
</tbody>
</table>

| Mean   | -----| -----| -----| ------| ------| ------| ------| ------| ------| ------| ------|
| A      | 0.009| 0.007| 0.002| 0.003| 0.004| 0.013| 0.009| 0.007| ---- | ---- | ---- |
| B      | 0.006| 0.009| 0.002| 0.017| 0.001| 0.003| 0.006| 0.009| ---- | ---- | ---- |
| C      | 0.009| 0.012| 0.003| 0.006| 0.002| 0.003| 0.001| 0.009| 0.012| ---- | ---- |
| D      | 0.008| 0.006| 0.003| 0.004| 0.000| 0.001| 0.007| 0.008| 0.006| ---- | ---- |
| E      | 0.007| 0.006| 0.004| 0.004| 0.002| 0.001| 0.018| 0.007| 0.006| ---- | ---- |
| F      | 0.006| 0.005| 0.002| 0.004| 0.016| 0.006| 0.006| 0.005| ---- | ---- | ---- |
| G      | 0.002| 0.005| 0.009| 0.003| 0.003| 0.002| 0.002| 0.005| ---- | ---- | ---- |
| H      | 0.005| 0.007| 0.014| 0.016| 0.018| 0.017| 0.013| 0.005| 0.007| ---- | ---- |

*Cheeses salted as described in Table 1.*
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese

D. J. McMahon

with the trend in d-1 lactic acid content of the cheeses as shown Figure 8.1.

The pH of the cheese tended to drop during the first 7 d of storage with the larger drops occurring in the cheeses containing the greater ratios of NaCl. Presumably this reflects continued conversion of lactose into lactic acid by the residual starter culture. Apparently the cheeses containing the KCl use most of the lactose during the first day and so the pH drop during the following 6 da is minimal.

During further storage there was a overall trend for all cheeses to increase in pH during the 9 mo storage, with up to a 0.3 pH increase in some cheeses with less change in most of the cheese containing KCI. This occurs even though the level of lactic acid increases during storage for all the cheeses (see Figure 8.1)

Water Activity (Objective 8)

Water activity of the cheeses was measured throughout storage as shown in Table 8.2. Water activities of the cheeses A through G were in the range 0.95 to 0.97 after pressing and remained constant throughout 9 mo storage. The low salt cheese H had higher water activity (about 0.01 higher) than the other cheeses as expected for having a lower salt-in-moisture content. However, during storage the water activity of cheese H gradually decreased from its initial value of 0.975 to 0.963 at 6 mo and then became comparable to the other cheeses by 8 mo with water activity of 0.952.

CONCLUSIONS

Sampling of the cheeses continued, although Rep 3 1-day samples intended for volatile and organic acid analysis were lost at Utah State University. Otherwise sampling and testing is progressing according to plan. Storage of Rep 1 and Rep 2 cheeses have been completed through 9 mo, and samples sent for testing are being analyzed according to individual PIs schedules.

The expected variations in mineral composition based upon salting were observed, although substitution of NaCl with 10% KCl did not cause a significant change sodium content of the cheese, although an increase in potassium content was observed. This is probably because of variations in salt (chloride) content in cheese typically observed in cheese manufacture and inherent variation occurring during analysis.

Less suppression of lactic acid generation by starter culture during overnight pressing occurs when KCl is substituted for NaCl resulting in a lower pH at day 1.

No difference in production of % WSN was observed based upon cation content of the cheeses.

Early analyses indicate that there are volatile compound and organic acid differences among samples but there is currently not enough data collected to make any conclusions on these differences.

Consumer sensory testing shows a trend of the cheese with 50% substitution with KCl being as well liked as the control. Consumers on a sodium restricted diet tended to score all cheeses higher, especially the low salt cheese in which the cheese still had a mean liking score >5.0
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon

compared to <5.0 from regular consumers.

Increasing bitterness in cheese occurs with KCl usage. There was no bitterness detected in the low salt cheese.

Preliminary observations using DGGE to determine predominant species in cheeses A, D and H, appear to correspond to microbial numbers observed by plate counting.

Most of the predominant lactic acid bacteria in cheese, except Lactococcus lactis and Streptococcus thermophilus, contain two sizes of ARISA phylotypes since there are tRNA genes within the 16S-23S spacer.

We are able to discriminate the predominant lactic acid bacteria in cheese using ARISA.

REFERENCES
Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon


Cheese microbiology and flavor based on salt cation substitution in lower sodium cheddar cheese / D. J. McMahon


Whey syneresis upon salting of Cheddar cheese curd

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Email: donald.mcmahon@usu.edu

Funded by: Dairy Management Inc., as administered by the Dairy Research Institute

ABSTRACT

The rate and extent of syneresis (whey expulsion) strongly affects cheese composition and quality. During salting, curd syneresis is influenced by the combined effect of both osmotic pressure and protein hydration. Our objective is to examine how cheese composition and whey expulsion is influenced salting of Cheddar cheese curd with different intervals, salt levels, rates and potassium chloride (KCI) substitution. Unsalted fresh Cheddar curd s were obtained and salted with different methods, with 3 replicates of each set on separate days. Set A was salted with 30 g/kg NaCl over 3 applications either 5 or 10 min apart. Set B was salted with 30, 25 and 20 g/kg NaCl over 3 applications 5 min apart. Whey was collected every 5 or 10 min until 30 or 40 min after the start of salting. Salted curds were pressed for 3 h. Using 10-min intervals delayed whey syneresis but after pressing there was no significant influence on final cheese composition. Lowering salting levels significantly reduced the amount of whey expelled prior to pressing and resulted in cheeses with higher moisture and slightly lower pH. Adding salt over different applications did not significantly affect cheese composition. Partial substitution with KCl did not affect amount of whey expelled or cheese moisture composition. Set C to be salted with 20 g/kg NaCl using 1, 2 or 3 applications and Set D to receive salt consisting of a 2:1 molar ratio of NaCl and KCl over 3 applications 5 min apart, will be completed during 2012.

BACKGROUND

Role of Salt in Cheese Manufacture

Salt is added to cheese for several purposes: controlling microbial growth and enzyme activity, promoting curd syneresis (whey expulsion), modifying flavor, texture and other physical properties (Guinee, 2004; Johnson et al., 2009). In general, bacterial and enzyme activity decreases as salt concentration increases. Salt to moisture (S/M) level is significantly correlated with cheese quality (Guinee, 2004). For Cheddar cheese, S/M levels of 4.5% to 5.5% have been reported to produce the best quality cheese. The high ratio of S/M reduces the bacterial growth and enzyme activity. Therefore it can decrease the rate of pH dropping and proteolysis during cheese ripening, and slow flavor development (Guinee, 2004; Lawrence et al., 2004). At low percent S/M, there is a high rate of microbial growth, proteolysis, incidence of bitterness and off-flavors (Guinee, 2004; Lawrence et al., 2004; Upreti and Metzger, 2007).

Reduced Sodium Cheese

Schroeder et al. (1988) assessed Cheddar cheeses with sodium levels from 275 to 5,667 mg/kg. These cheeses were salted at different rates, aged and evaluated over 7 mo. Cheeses containing sodium of more than 4,408 mg/kg received significantly higher scores of flavoring than cheeses with less sodium. However, this was not attributable to salt content alone as the cheeses with lower sodium also had higher acidity and moisture because of the impact of salt on whey syneresis during pressing. In general, there was a lower Cheddar intensity, more pronouncing bitterness and more disagreeable aftertaste associated with the low sodium cheeses.

In a more recent study of consumer perception regarding salt reduction (Drake et al., 2011), a salt reduction of no more than 20% was noticeable and correctly identifiable by customers, although a small 2% to 5% salt decrease was unnoticeable. Customers are used to the saltiness of cheese, so large reductions of sodium may lose the customers’ loyalty. In addition, simply reducing sodium in cheese...
causes defects such as higher rates of proteolysis, water activity, acidity, bitterness as well as decreasing firmness and saltiness (Lindsay et al., 1982; Fitzgerald and Buckley, 1985; Ayyash and Shah, 2010; Ayyash et al., 2011; Drake et al., 2011; Gomes et al., 2011).

Instead of adding less salt, sodium content in cheese can also be reduced by substituting salt (i.e., NaCl) with KCl or other minerals (Johnson et al., 2009). The most researched sodium replacer is KCl, which has the most similar chemical structure to sodium chloride (Johnson et al., 2009). Potassium is considered as one of the concern nutrients for Americans, especially for African Americans and individuals with hypertension (USDA/USDAHHS, 2010). However, typical American diets usually reach only 56% of the potassium adequate intake (AI) for adults (4700 mg per day) (USDA, 2008). Randomized trials have shown that increasing potassium intake in the diet is linked with lower cardiovascular mortality (He and MacGregor, 2008; He et al., 2010), lower blood pressure (He et al., 2005), and is likely to prevent or at least slow down the progression of renal disease (He and MacGregor, 2008). In addition, while potassium reduces calcium absorption in the intestinal tract, it decreases calcium loss in urine (Lemann et al., 1993; Zhu et al., 2009). So overall, potassium contributes to greater bone mineral density, especially for elderly people (Tucker et al., 1999; Whiting et al., 2002).

Partial replacement of NaCl with KCl appears feasible for producing a cheese without significant changing its quality. Katsiari et al. (1998) made Kefalograviera cheese with 50% replacement of NaCl by KCl. This cheese was not significantly different from the normal salted cheese in composition (moisture, fat and protein), chemistry (pH and water activity) and texture. Similar observations has also been observed by Fitzgerald and Buckley (1985) in Cheddar cheese, Katsiari et al. (2000a, b) in feta cheese, Karagözli et al. (2008) in white pickled cheese, and Dorosti et al. (2010) in Iranian white cheese. However, cheeses containing KCl have been reported as having less saltiness, more bitter and metallic flavor, softer body and more lipase activity (Lindsay et al., 1982; Fitzgerald and Buckley, 1985; Karagözli et al., 2008; Johnson et al., 2009).

**Cheese Syneresis**

In cheese manufacture, the process of whey being expelled out of cheese curds is called syneresis (Pearse and Mackinlay, 1989). The rate and extent of syneresis strongly affects the cheese processing method, the loss of fat and protein in whey, cheese moisture, acidification, and proteolysis, and therefore strongly influences the cheese composition and quality (Daviau et al., 2000; Dejmek and Walstra, 2004; Everard et al., 2008). There are several factors that influence cheese syneresis rate including physical (mechanical pressure and homogenization) and chemical factors (temperature-induced changes, pH and ionic strength).

Usually, mechanical pressure (such as cutting, stirring and cheddaring) promotes syneresis (Lodait et al., 2000; Dejmek and Walstra, 2004; Geng et al., 2011). Small curd sizes usually result in a high syneresis rate (Grundelius et al., 2000; Dejmek and Walstra, 2004) although it has been reported that the initial syneresis rate increases proportionally to gel (curd) thickness (Lodait et al., 2000).

Increasing temperature within a certain range can promote syneresis (Pearse and Mackinlay, 1989; Van Vliet et al., 1991; Geng et al., 2011). It was observed that increasing heating temperature at a range 20 to 35°C promoted one-dimensional gel shrinkage (Van Vliet et al., 1991). In a more recent study, temperature rise from 32°C to 40°C strongly stimulated curds shrinkage (Geng et al., 2011).

Reducing pH can increase syneresis rate (Pearse and Mackinlay, 1989; Grundelius et al., 2000; Lodait et al., 2000; Dejmek and Walstra, 2004; Thomann et al., 2008). This occurs because at a low pH, the net micelle charges and electrostatic repulsion are diminished, and thus, more whey is expelled as stronger attractive forces will cause the gel network to shrink (Pearse and Mackinlay, 1989; Dejmek and Walstra, 2004). It should also be noted that at the casein isoelectric pH (~pH 5), electrostatic bonds are quite strong and cause casein molecules to aggregate (Dejmek and Walstra, 2004).

Generally, salting of Cheddar cheese curd (i.e., increasing the ionic strength) promotes syneresis and results in a decreased moisture level (Kindstedt et al., 1992; Pastorino et al., 2003a; Agarwal et al., 2008). Although in some cases (such as cheese with low calcium levels), it was observed that adding salt does not lead to moisture loss of cheese (Cervantes et al., 1983; Paulson et al., 1998).

When dry salt is applied to milled cheese curds, salt dissolves slowly in the moisture on curd surfaces and forms a thin highly saturated salt solution. The osmotic pressure difference between the saturated solution on curd surfaces and the serum inside curd particles is considered as the driving forth of the salt diffusion. Sodium chloride ions and water molecules respond to different osmotic pressure by traveling through the serum portion inside protein matrix of curds. The salt diffusion creates a salt concentration gradient inside the curds with the highest level on surfaces and the lowest at the center of curds (Guinee, 2004; Guinee and Fox, 2004).

However, syneresis may also be influenced by the protein matrix response to different salt levels. Pastorino et al. (2003a) injected a 20% (wt/wt) NaCl solution into Muenster cheese and found after 5 injections, the moisture level of cheese decreased from 41% to 38%. Scanning electron micrographs showed that 84% of the un.injected cheese matrix was occupied by protein and the rest (16%) was occupied by fat/serum pockets. After 5 injections, the salt-injected cheese had 4% increase in protein matrix.
(88% of cheese matrix) with 12% being occupied by fat/serum. This indicates that the protein hydration ability was increased by NaCl solution injection, although there was also a moisture loss during 40-d storage of cheese that had been injected 5 times with the NaCl solution.

The effect of CaCl₂ addition is still under controversy. Cheeseman (1962) reported a reduction in curds syneresis rate with 10, 50 and 100 mM CaCl₂ addition to fresh or reconstituted milk prior to cheese manufacture. Fagan et al. (2007) also observed that at the beginning of adding up to 18 mM CaCl₂ to milk, curds syneresis rate slightly decreased due to the increasing curd rigidity. Pastorino et al. (2003b) injected 40% (w/w) CaCl₂ solution under high pressure to Mozzarella cheese causing a drop in pH and moisture level, as well as a more compact protein matrix with larger fat/serum void space. Cheese with 5 injections reached a Ca level of 1.4% and lost about 12% moisture. This indicates that the Ca addition reduces casein hydration and promotes protein-protein interactions.

But it was also reported that in synthetic milk solution without phosphate, the syneresis was slightly enhanced by increasing calcium addition amount from 5.4 to 11.25 mM, but diminished significantly with further calcium addition (Aiyar and Wallace, 1970). Marshall (1982) observed that curds syneresis was stimulated by 2 mM addition of CaCl₂ and also 4 mM CaCl₂ if cutting times were reduced. In fact, the variable effect on syneresis may depend on the amount of CaCl₂ added (Pearse and Mackinlay, 1989), and the time after CaCl₂ addition at which parameters are measured (Dejmek and Walstra, 2004).

MATERIALS AND METHODS

Cheese Manufacture

Full fat Cheddar cheese curds were manufactured following the method of Rogers et al. (2010). Cold milk was transported to the Gary Haight Richardson Dairy Products Laboratory at Utah State University (Logan) and 700-kg batches were standardized to ~0.83 protein to fat ratios. The milk was pasteurized (73°C for 16 s) and pumped into a Tetra Scherping horizontal cheese vat (Tetra Pak Cheese & Powder Systems Inc., Winsted, MN) and heated to 31°C. After 10 min, starter culture was added at 21g/100 kg of milk and the milk was allowed to ripen for 10 min. CaCl₂ (32% wt/wt), annatto (single strength) and chymosin (double strength) were added at 12, 7 and 7 mL/100 kg, respectively. Followed by 2-min stirring, then milk was allowed to coagulate without stirring until a firm set was reached (~30 min). The curd was cut at 10, 11 and 12 rpm for 1 min each, reversed for 30 sec, and then cut at 14 rpm for 1 min. Allowed to rest for 5 min, the curd was stirred starting at 8, 9, 10, 11, and 12 rpm of 1 min each, cut at 14 rpm for 1 min, stirred at 10 rpm for 9 min, cut at 14 rpm for 1 min again, and stirred at 12 rpm for 14 min. Sixty-five min after renneting, the curd and whey was heated to 39°C over 30 min. After stirring for 35 min, the curd and whey were pumped to a drain table (Kusel Equipment Co., Watertown, WI) and stirring continued until the curd pH reached 6.3, and whey drained. After draining the whey, the curd was formed in pack of 3 to 4 inches deep and cut into slabs about 6 inches wide after 5 min. The curd slabs were flipped over and stacked, and curd temperature had dropped to ~34°C. Cheeses were milled at pH 5.4. Then unsalted curd was collected or the curd was salted at a rate of 30 g/kg over 3 applications with 5 min apart.

Cheese pH, Moisture and NaCl Measurement

Cheese pH was measured using a glass electrode after stomaching 20 g of grated cheese and 10 g of deionized water at 260 rpm for 1 minute in a Stomacher 400 (Seward, London, UK). Moisture was analyzed by weight loss in triplicate using a microwave oven (CEM Corp., Indian Trial, NC) (McMahon et al., 2009). In Experiment 1, total NaCl content was determined by the mineral analysis of ash from 50 g of grated cheese at 500°C in a muffle furnace (model 550-126, Fisher Scientific, Hanover Park, IL). In Experiment 2 and 3, total NaCl content was measured by stomaching 5 g of grated cheese with 98.2 g of deionized water, filtering the slurry through Whatman #1 filter paper, and analyzing the filtrate using a chloride analyzer (model 926; Corning Scientific, Medfield, MA). All measurements were conducted in triplicates.

Dry Salting of Cheddar Cheese Curd

Salting interval.

Separate 12-kg portions of unsalted milled Cheddar cheese curd were salted at a rate of 30 g/kg using NaCl over 3 equal amount applications, spaced either 5 or 10 min apart, and whey was collected every 5 or 10 min until 40 or 30 min after salting, respectively. Curds were weighed before and after salting. Salted curds were placed in a cloth-lined stainless steel hoop, a plastic bag to collect whey placed around the hoop, then the hoops were placed in a vertical press and pressed at 60 psi for 3 h at room temperature (~20°C). The weight, pH, salt and moisture of pressed cheese were measured, as well as the weight of expressed whey.

Salting level.

Cheddar curd was salted with 20, 25 or 30 g/kg of NaCl using 3 equal applications 5 min apart. Whey was collected and weighed every 5 min until 30 min after salting. Salted curds were then pressed into a block and whey collected as described above.
Whey syneresis upon salting of Cheddar cheese curd / D. J. McMahon

Figure 1. Whey expulsion over time from the first salt application of milled Cheddar curd with 30 g/kg salt added over 3 applications using either 5-min (○) or 10-min (●) intervals. The percentage of total whey expulsion is also shown. Bars = SEM.

Table 1. Composition of cheese after salting with different levels of salt (20, 25 and 30 g/kg) over 3 applications with 5 min apart and pressed for 3h at room temperature.

<table>
<thead>
<tr>
<th>Salting levels</th>
<th>Moisture</th>
<th>Salt</th>
<th>S/M¹</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>370a</td>
<td>15.4a</td>
<td>40.0a</td>
<td>5.2a</td>
</tr>
<tr>
<td>25</td>
<td>359b</td>
<td>17.6b</td>
<td>46.7b</td>
<td>5.3b</td>
</tr>
<tr>
<td>30</td>
<td>356c</td>
<td>18.9c</td>
<td>50.4c</td>
<td>5.3c</td>
</tr>
</tbody>
</table>

¹S/M: salt-in-moisture
a,b,c Means within a column with the same superscript letter were not significantly different

Figure 2. Whey expulsion over time from the first salt application of milled Cheddar curd with 30 g/kg (●), 25 g/kg (○), or 20 g/kg salt (▲) added over 3 applications using 5-min intervals. The percentage of total whey expulsion is also shown. Bars = SEM.
Statistical Analysis

A randomized block design was used to investigate the effect on cheese curd syneresis of salting time intervals, salting levels, and 33% KCl molar substitution of NaCl. All data were analyzed for statistical significance at the 95% level using the proc glm or proc mixed function in Statistical Analysis Software (SAS) version 9.3 (SAS Institute, Inc. Cary, NC). The data of experiments in which cheese were dry salting was square-root transformed to meet the normality and homoscedasticity assumptions before significance testing. Post-hoc means comparisons were made based on p-values ($\alpha = 0.05$) using the Tukey-Kramer adjustment to obtain differences of least means squares.

RESULTS AND DISCUSSION

Salting Interval

Cheese curd had typical moisture content of 420 g/kg. After salting with 5-min or 10-min intervals followed by 6-h pressing, there was no significant difference ($P > 0.4$) between cheese composition with mean ($\pm$SD) values of moisture $362 \pm 6.98$ g/kg, salt $18.6 \pm 1.31$g/kg, S/M $48.9 \pm 3.68$ g/kg, and pH $5.2 \pm 0.06$.

When the typical level of salt was added to Cheddar cheese curd (i.e. 30 g/kg), there was very little whey expelled (~2%) prior to the third salt addition in both 5-min and 10-min intervals (Figure 1). Applying the salt at 5-min intervals caused faster syneresis than using 10-min intervals, with major syneresis occurring at 15 and 25 min after the start of salting, respectively. Even when considered as the time after the third salting (e.g., 20 min after the third salting), there was still a trend ($P = 0.054$) for slightly more whey expulsion when using the 5-min intervals than the 10-min intervals.

Curd syneresis is strongly influenced by protein matrix hydration state (Paulson et al., 1998; Pastorino et al., 2003a; Guinee, 2004, Guinee and Fox, 2004; McMahon et al., 2005, 2009). When salt is applied to curd, a portion of the salt is dissolved in the moisture located on and slightly within the surface of the curd. As the salt dissolves, potentially reaching a saturated solution at the curd surface, there is movement of water based on osmotic forces, to the surface causing a dehydration of surface protein matrix (Geurts et al., 1974; Guinee, 2004, Guinee and Fox, 2004). Water inside the curd pieces continues to drive outwards in response to osmotic pressure as the sodium and chloride ions from the dissolved salt diffuse inwards. This forms a salt gradient with extremely low levels at the center of the curd pieces and gradually increasing as towards to curd surfaces.

By 20 min after the third salt application (i.e., 30 or 40 min after commencement of salting for 5 and 10-min salting intervals, respectively), the amount of whey being expelled reaches a plateau. This suggests that ratio of salt in the center of the block compared to the surface has reached a constant level that would depend upon the salting levels used. The combined driving forces of osmotic movement of water molecules in response to the presence of a salt gradient and contraction/expansion of the curd protein network in response to salt concentration would then be in equilibrium and whey expulsion ceases.

Salting Level

Unsalted curds were obtained with moisture of 41% and pH of 5.4. Applying salt with increasing salting levels (i.e. 20, 25 and 30 g/kg) significantly influenced cheese composition by decreasing moisture ($P < 0.001$), increasing pH ($P < 0.006$), salt level ($P < 0.001$) and S/M ($P < 0.001$) (Table 1). When salting with levels of 20, 25 and 30 g/kg, there was little whey expelled (less than 5%) before the third salting (Figure 2). The major syneresis occurred at 15 min after the start of salting, and reached 84.3%, 90.8% and 90.5% within 10 min, respectively.

Moisture, salt, S/M and pH was influenced by the salting levels in agreement with Guinee (2004). Cheese with less whey expulsion tends to be higher in moisture since more water is held in the protein matrix (Guinee and Fox, 2004). Salt absorption of cheese increases with the salting level (Guinee, 2004), and therefore salt and S/M increased significantly as higher salting levels. The inhibition of Lactococcus starter culture growth in cheese rises as increasing S/M level ($> 50$ g/kg especially). Therefore, pH of cheese was lower (i.e. 5.2) at low salting level (i.e. 20 g/kg) than pH (i.e. 5.3) at salting levels of 25 or 30 g/kg.

When curds were salted at increasing levels of 20, 25 and 30 g/kg, the salt diffusion and water movement are both promoted by the increasing osmotic pressure and cause a higher rate of whey expulsion in cheese. Therefore, whey expulsion was significantly decreased at lower salting levels (Figure 2).

CONCLUSIONS

Using 10-min salting intervals delayed whey syneresis but did not affect the final cheese composition after pressing. Lowering salting levels significantly reduced whey expulsion prior to pressing and resulted in cheeses with higher moisture and slightly lower pH.
REFERENCES


Whey syneresis upon salting of Cheddar cheese curd / D. J. McMahon


Effect of dairy product consumption on cognitive performance among elderly participants of the Cache County Study on Memory, Health, and Aging

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ABSTRACT

The focus of this project is to investigate the relationship between dairy product consumption and cognitive decline in participants in the Cache County Study on Memory Health and Aging. The work involves both recall of dairy product intake via Food Frequency Questionnaires as well as measurement of C15:0 and C17:0 fatty acids in erythrocytes collected in the third wave of the study. These fatty acids are primarily acquired through milk fat, and have previously been positively correlated with milk fat intake.

The erythrocyte fatty acids were previously prepared for analysis in a prior study on PUFA and cognition, but the C15:0 and C17:0 fatty acids were not able to be measured due to coelution of contaminants from the derivitization process. Thus, prior to reanalysis they require a clean up procedure with solid phase extraction. Approximately 2/3 of the samples (out of ~2,200) have been cleaned up and 1/3 have been reanalyzed at this point. Thus, no correlations have been conducted at this point.

However, a preliminary database analysis of the food intake data and cognitive decline has been conducted. Among the 3,364 subjects evaluated at baseline, a total of eleven categories of dairy products were estimated. These included skim milk, 2% milk and higher fat, chocolate milk, cream, sour cream, ice cream, yogurt, cottage cheese, cream cheese, other cheese, and butter. The intakes of these products were estimated from the Food Frequency Questionnaires, yet at this point all analyses have been restricted to total intake and thus has not been adjusted to energy. The mean baseline intake of dairy in this population is 2.7 cups per day with a standard deviation of 1.9. A very low percentage (~9%) of the respondents report to abstain from dairy. Sixty percent report consuming over 0.5 units per day.

The baseline 3MS scores were compared between groups separated by daily intake of dairy products and found to be highly significant. It would appear from a preliminary analysis that intake of 2.5 units of dairy is associated with the highest 3MS scores at baseline. This association is also noted at Wave 2, although there was not a FFQ given at this evaluation. The association remains at wave 3 when there was a FFQ administered and it is from this time point that the RBCs were collected. In moving forward, we will look at confounding factors that may explain the results including specific dairy product interactions.

BACKGROUND

Review of Literature

Background on diet and cognitive decline.

Alzheimer’s Disease (AD) is the major form of dementia among the elderly and has emerged as a major public health threat. AD is now the third most costly medical condition in the U.S. (Alzheimer’s research and prevention foundation, About Alzheimer’s Disease. 2009). There are no known cures for AD. Diagnosis of AD and other dementias are preceded by a progressive decline in cognitive function (Bennett, Wilson et al. 2002). Strategies aimed at preventing or slowing age-related cognitive decline may have substantial importance for preventing
or delaying the onset of dementia and thus curbing the enormous public health burden associated with this disease. Delaying the onset of AD by five years is projected to reduce the prevalence by 50 percent after 50 years (Brookmeyer, Gray et al. 1998). Hence, lifestyle changes with modest effects could translate into large reductions in disease burden in the population.

The etiology of AD is not yet fully understood, but is likely influenced by diet. Similar to other chronic conditions, AD is likely a result of multiple genetic and environmental risk factors. A growing body of biologically plausible evidence from animal and human studies supports the role of diet in the pathogenesis of AD (Krumen, Kumaravel et al. 2002; Seshadri and Wolf 2003). Diet-related mechanisms that may increase the risk of AD include increased inflammation, oxidative stress, vascular dysfunction, altered cerebral glucose metabolism, insulin resistance, decreased neurogenesis and repair, and altered transport and degradation of amyloid-beta protein. Several large observational studies have found positive associations between cardiovascular risk factors, including those that compromise the metabolic syndrome (impaired glucose tolerance, abdominal obesity, hypertension, hypertriglyceridemia, and dyslipidemia), and increased risk of age-related cognitive decline and Alzheimer’s Disease (Frisardi, Solfrizzi et al. 2010).

**Biomarkers of milk fat consumption and metabolic health.**

In the last decade, several studies have assessed the correlation between intakes of dietary fatty acids and their deposition in various tissues, as well as the correlation of tissue levels with various health outcomes. This technique is possible as humans are unable to synthesize several fatty acids that are common in the diet. For example, those with double bonds in the omega-6 and omega-3 position (the essential fatty acids), and those with double bonds in the trans conformation which either come from industrially hydrogenated fats, or from ruminants. Of special interest in this context are foods that contain unique fatty acids, and thus which leave a specific fatty acid signature in the tissues of those consuming them.

Assessing the impact of dietary patterns on health outcomes is limited by the accuracy and precision of dietary assessment. Although methods based on subjective recall are the most common, there are several shortcomings of this technique that limit its effectiveness as reviewed by Hodson, et al. (2008). First, it is common for subjects to under report consumption, and this appears to be more common in certain population subsets. Second, subjects may consciously or unconsciously alter their consumption behavior during the period being recorded and thus the dietary record may not accurately reflect true consumption patterns. Third, databases of food items are often not complete and/or do not necessarily match up well with the items in individual’s diet. Despite these limitations such methods are both necessary and useful for studies focused on linking specific dietary components to health outcomes. Therefore, it is of great interest that in recent years several groups have shown that fatty acid analysis of tissues does reflect dietary intake. In fact, in studies in which intakes of fat have been well controlled, the results indicate the potential for this technique as a quantitative tool (Hodson, Skea et al. 2008).

Milk fat has a unique fatty acid composition compared to other dietary fats (MacGibbon and Taylor 2006). For example, approximately 20% of the fatty acids in milk contain 14 carbons or fewer. In addition, milk fat contains many fatty acids that are byproducts of the microbes in the rumen, such as those with odd numbers of carbons, and those with double bonds in the trans configuration. Even though these fatty acids are only minor components of milk fat, they are only found in milk (and to some extent meat) and can be used as an index of consumption of these products.

Below, relevant studies from the literature in which biomarkers of milk fat intake were compared to health outcomes in human populations are reviewed and summarized.

1999


This manuscript describes a study conducted with 62 70-year-old Swedish men in which 7d diet records were collected and plasma cholesterol ester (CE) and phospholipids (PL) were profiled for fatty acid composition. Their results indicated that the pentadecanoic acid (C15:0) content of both CE and PL reflected milk fat consumption. In addition, there was an inverse relationship between milk fat consumption and body mass index (BMI), waist circumference, the LDL-HDL ratio, HDL triglycerides and fasting glucose. In addition, there was a positive relationship between serum apolipoprotein A-I and HDL.

This group also looked at the levels of C17:0 in both the CE and PL fractions of their samples, but found the levels were not detectable in many subjects. Consequently, they did not find any significant effect for dairy fat consumption and C17:0 levels.

2001

In this study, the proportions of C14:0, C15:0 and C17:0 in adipose tissue and serum cholesterol esters were investigated as long-term markers of dairy consumption. The fatty acid composition of subcutaneous adipose and serum cholesterol esters was compared to 2 1-wk weighed food records made 6 months apart and 14 24-h dietary recall interviews which took part over one year. According to the authors, there were significant correlations with dairy fat intake and the milk fat biomarkers measured in both tissues.

2004


This was a prospective case-control study that investigated the relationship between milk fat consumption, as measured by serum ester C15:0 and C17:0 and a first-ever acute myocardial infarction (AMI) in a population in Sweden. The proportions of C15:0 and C17:0 in serum esters were negatively correlated with serum concentrations of plasminogen activator inhibitor-1, tissue type plasminogen activator, triglycerides, insulin, and leptin all of which the authors indicate suggest a negative relationship to insulin-resistance and CHD risk. Although there did appear to be a negative association between milk fat consumption and a first-ever AMI, this was removed by adjustment for clinical risk factors.

2005


The focus of this work was to evaluate the intake of milk fat on the adipose and serum concentration of C15:0 and C17:0 in healthy men ages 21-55. The men provided adipose and blood samples and completed both 14 day weighed records (WR) and a 180-item food frequency questionnaire. The results indicated that the strongest correlation was between milk fat consumption as estimated by the WR and the C15:0 content of the adipose tissue. Interestingly, they also found an inverse correlation between milk fat intake and serum C17:0. The main conclusion from this work is that the content of C15:0 in adipose and serum is a useful biomarker of total dairy fat, whereas the FFQ may provide a better estimate of the intake of fat from milk.


In this study, C14:0, C15:0 and C17:0 were measured in adipose and serum and correlated with dietary intake as assessed by a pre-coded 7 day food record. The results indicated that all three fatty acids were positively correlated with milk fat intake and inversely correlated with alcohol intake.

2007


In this study biomarkers of dairy fat intake in plasma and RBCs were assessed in relation to ischemic heart disease (IHD) in US women. In this case-control study, which was conducted with data from the Nurses’ Health Study, 166 cases of IHD were matched with 327 controls for age, smoking, fasting status and the date of the blood draw. The results indicated that C15:0 and C16:1n7t can be used as biomarkers of dairy fat intake and that a higher intake of dairy fat are associated with a greater risk of IHD.

2008


In this study, red blood cell fatty acids were measured in 350 non-diabetic Swedish individuals at baseline, and then development of the disease was monitored for approximately 5 years. Lower proportions of C15:0 and C17:0 in RBC FAME were associated with higher risk for the disease. After adjustment for other risk factors, the only significant protective factors were C15:0 and C17:0 while C22:4n6 in RBC fame correlated positively with risk.

2009

Effect of dairy product consumption on cognitive performance among elderly participants / R. Ward


In this prospective case-control study, the total plasma phospholipid fatty acid composition was compared in stroke cases (129) and matched controls (257). C15:0 and C15:0 + C17:0 were significantly higher in female controls compared to the cases, but not in men.


The goal of this study was to compare the two methods of diet analysis, an 84 food item food frequency questionnaire (FFQ) and a 24 hour diet recall, in their ability to measure dietary fat consumption as reflected in the red blood cell fatty acids. The correlation coefficients for common dietary fatty acids for the FFQ and 24H were all significant and ranged from 0.29 to 0.60. However, significant correlations between FFQ estimated fat intake and RBC fatty acids were only noted for C14:0, C15:0 and C17:0 (a marker of milk fat consumption) and C20:5n3 and C22:6n3 (a marker of fatty fish consumption).

2010


This manuscript describes a prospective case-control study in a Swedish cohort of 1000 individuals in which the association of serum milk fat biomarkers (plasma phospholipids) was correlated with the risk of a first myocardial infarction (MI). The results indicated that in women the proportions of milk fat biomarkers were significantly higher in controls than cases and were weakly negatively correlated with risk factors for metabolic syndrome. After adjustment for multiple risk factors, the inverse association of dietary milk fat biomarkers and risk of MI remained, but was only significant for women. In addition, quartiles of reported cheese intake and fermented milk products were inversely related to a first MI.


This study addressed the question as to whether or not circulating trans-palmitoleate (C16:1n7) is related to risk for Type 2 diabetes. The prospective cohort study included 3,736 subjects, of whom 305 developed the disease over the 14-year period. The results indicated that whole fat dairy consumption was most strongly associated with plasma phospholipid transpalmitoleate, which in turn, was associated with lower adiposity, higher HDL levels, lower triglycerides, a lower total cholesterol-HDL ration and lower insulin resistance.

Summary of studies with biomarkers of milk fat.

In summarizing the information reviewed above, several tissues have been used to investigate the correlation of milk fat intake with tissue fatty acid biomarkers. In general, the results seem to indicate that blood can be used instead of adipose, which requires more invasive sampling procedures. In blood, plasma phospholipids, cholesterol esters and RBCs have all been used and their C15:0, C16:1n7t and/or C17:0 percentages found to correlate with milk fat intake. Although some studies did not find that C17:0 was a reliable indicator of milk fat intake and some did not report C16:1n7, the correlation of C15:0 with milk fat intake appears to be consistent across studies and the class of lipid analyzed. While most of the studies found positive associations of milk fat biomarkers and cardiovascular and other metabolic indices (Smedman, Gustafsson et al. 1999) (Waren~jo, Jansson et al. 2004) (Krachler, Norberg et al. 2008) (Waren~jo, Smedman et al. 2009) (Waren~jo, Jansson et al. 2010) (Mozaffarian, Cao et al. 2010), at least one was negative (Sun, Ma et al. 2007). These results would appear to support a role for at least some full fat dairy products in a healthy diet.

Dairy products and cognitive decline.

To date, few studies have investigated the role of dairy products in cognitive health, and it appears none have linked biomarkers of fat intake. In one recently reported study, Crichton et al. (2010) tested the hypothesis that dairy consumption may benefit cognition via positive effects on cardiometabolic health. The study was a retrospective, cross-sectional analysis of data from food frequency questionnaires and self-reported health of 432 men and 751 women aged 39-65. Data collected included cardiometabolic health indicators, cognitive and memory functioning, mental health, anxiety, stress, depression and self-esteem, which were assessed by standard questionnaires. After adjusting for total energy intake and other health confounders, regression analyses indicated that consumption of low fat yogurt was associated with increased quality of memory recall and greater social functioning in men, which low fat cheese consumption was associated with greater social functioning and decreased stress in women. Conversely, consumption of full fat dairy

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products including ice cream and cream were associated with increased depression, anxiety, stress and poorer memory functioning and general health.

These same authors recently conducted a review of the literature with respect to dairy products in the diet and cognitive performance (Crichton, Bryan et al. 2010). In their analysis they identified 3 cross-sectional and 5 prospective studies of suitable design for meta analysis. In the three cross-sectional studies described, the first found that lower dairy product consumption was associated with greater risk for poor cognitive function in women, and no effect was noted for men. A second study found no effects between dairy products and cognitive function, but those consuming low levels of dairy products did experience more depressive symptoms. The third cross-sectional-study reported found that cheese consumption was inversely associated with cognitive impairment.

In the first of the five prospective studies described, consumption of full cream milk was associated with impaired cognitive function. In the second, intake of milk and sour milks was not associated with dementia, but saturated fat from food spreads, presumably from butter, was. In the third study, almost daily intake of milk was associated with a lower risk for vascular dementia compared to those consuming less milk. Results from the fourth prospective study described suggested that high saturated fat intake from dairy products was associated with increased risk from myocardial infarction and poorer cognitive function. Last, a fifth study found that there was no association between milk, cheese and yogurt consumption with cognitive decline, but there was for dairy desserts such as ice cream. Summarizing their overall interpretation of the results from these studies were that poorer cognitive functioning and vascular dementia were associated with lower levels of dairy consumption. However, they add that high fat dairy products may be associated with cognitive decline in the elderly.

Preliminary Findings or Research Efforts by the Principal Investigators

Drs. Ward, Wengreen and Munger represent a food scientist with a background in lipid analytics, and two nutritional epidemiologists, respectively. Dr. Ward’s lab is equipped with two gas chromatographs dedicated to fatty acid methyl esters analysis, one with an FID detector, and one with a mass spectrometer. Dr. Ward has several publications using gas chromatography for analysis of lipids and other food constituents (Ataire, Ward et al. 2003; Snow, Jimenez-Flores et al. 2010) (Ward, Ninonuevo et al. 2007).

Dr. Wengreen has significant experience in the area of diet and late life cognition and has three publications in the area. The first examined associations between antioxidant intake, from both food and supplement sources, and late-life cognition (Wengreen, Munger et al. 2007). Greater vitamin C intake from food, but not supplements, or other antioxidants, was associated with higher cognitive scores at the baseline interview that were maintained across three years of follow-up. The magnitude of difference in cognitive scores at the baseline interview was 0.68 points, the same magnitude of difference observed for those with diabetes, compared to those without diabetes. Diabetes is a known important risk factor for AD (Craft 2007). In a subsequent analysis, participants who took both NSAIDS, a non-steroidal anti-inflammatory agent with known cognitive benefits, and a supplement containing both vitamins E and C declined by an average 0.33 points less per year, on a test of global cognitive function, than did nonusers.

In addition, Dr. Wengreen conducted another analysis that examined associations between nutrients (folate, omega-3 fatty acids), foods (fruits, vegetables, ready-to-eat cereal, fish) and the Recommended Food Score (RFS) as an index of diet variety and quality. Of the dietary factors explored, the RFS was most striking (Wengreen, Neilson et al. 2009). Among Cache Memory Study participants, those with the most diverse diets had the healthiest dietary profiles including diets that were higher in all micronutrients and fiber and lower in total fat, saturated fat, and cholesterol as compared to those with the lowest diet diversity scores. In addition, participants with highest RFS scored 1.8 points higher on a baseline test of cognitive function than did those with the least varied diet; a difference that was strengthened after 11 years of follow-up (Figure A). The magnitude of this effect was greater than that observed for antioxidants and similar in magnitude to the effect of having one copy of the APOE e-4 allele, an important genetic risk factor of AD.

Figure A. Mean change in 3MS scores by increasing quartile of the recommended food score (RFS) across 11 years of follow-up (reference 0) = quartile 1, time 0) using a multivariable mixed effects linear regression model.
Table 1. Characteristics (mean (standard deviation) or percent) of participants across increasing quintile (Q) of omega-3 index; Cache County Study on Memory, Health, and Aging.

<table>
<thead>
<tr>
<th>Range</th>
<th>Q1 (n=356)</th>
<th>Q2-3 (n=1066)</th>
<th>Q5 (n=355)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>(0.1 – 3.35)</td>
<td>(3.4 – 5.17)</td>
<td>(5.18 – 11.6)</td>
<td>0.780</td>
</tr>
<tr>
<td>Female (%)</td>
<td>72 (5)</td>
<td>72 (5)</td>
<td>72 (5)</td>
<td>0.008</td>
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<tr>
<td>Education (%)</td>
<td>25.7 (4.0)</td>
<td>26.6 (4.3)</td>
<td>26.0 (4.3)</td>
<td>0.46</td>
</tr>
<tr>
<td>E4 allele (%)</td>
<td>55</td>
<td>59</td>
<td>60</td>
<td>0.130</td>
</tr>
<tr>
<td>Fatty fish intake (sv/d)</td>
<td>0.10 (0.13)</td>
<td>0.16 (0.16)</td>
<td>0.21 (0.20)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total Fat (% of energy)</td>
<td>34.1 (6)</td>
<td>33.8 (6)</td>
<td>31.8 (6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total kcalories (kcal/d)</td>
<td>1906 (718)</td>
<td>1943 (656)</td>
<td>1862 (588)</td>
<td>0.130</td>
</tr>
<tr>
<td>Baseline 3MS score</td>
<td>92.8 (4.7)</td>
<td>93.4 (4.6)</td>
<td>93.7 (4.3)</td>
<td>0.072</td>
</tr>
<tr>
<td>V3 3MS score</td>
<td>90.4 (7.7)</td>
<td>91.7 (7.2)</td>
<td>92.6 (6.3)</td>
<td>0.004</td>
</tr>
<tr>
<td>Dementia (%)</td>
<td>16</td>
<td>14</td>
<td>12</td>
<td>0.481</td>
</tr>
</tbody>
</table>

**DASH diet, Mediterranean diet, and cognitive decline.**

Healthy dietary patterns may protect against age-related neurodegeneration because nutrients act synergistically to improve multiple metabolic factors. Associations between level of adherence to a DASH (Dietary Approaches to Stop Hypertension) and Mediterranean dietary patterns and agerelated cognitive function were assessed using methods previously described. Low-fat dairy consumption represented one of 9 components of the DASH diet. Higher DASH and Mediterranean scores were each similarly associated with higher 3MS scores at baseline and over time. Whole grains, low-fat dairy, vegetables, and nuts are food groups common to both the DASH and Mediterranean diets. A diet score based on these four food groups revealed stronger associations than the full DASH or Mediterranean scores: those in the highest four foodgroup quintile scored 1.72 (0.38) points higher at baseline and 3.73 (0.26) points higher after 11 years than did those in the lowest quintile (p-values < 0.001). Consumption of foods common to both the DASH and Mediterranean dietary patterns including whole grains, low-fat dairy, vegetables, and nuts was associated with higher baseline 3MS scores and less decline over 11 years. These foods may provide a means for dietary approaches for cognitive benefits that span diverse cultures and regions.

**Red blood cell profiling of wave 3.**

In 2009 Drs Ward and Wengreen received funding from the General Mills Bell Institute of Nutrition to profile the fatty acid composition of red blood cells (RBC) collected in Wave 3 of the study. Our objective was to examine associations between dietary intakes of long-chain n3 (LCn3) and n6 polyunsaturated fatty acids (PUFAs), red blood cell (RBC) fatty acids (FAs) including eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids, and C-reactive protein (CRP), a marker of inflammation, among elderly men and women. RBCs were subjected to direct transesterification with acetylchloide in methanol and analyzed via gas chromatography with flame ionization detection. In total, 16 fatty acids were quantified from the RBCs of 1,795 individuals.

Individuals were categorized into quintiles of fatty acid indexes according to the distribution of fatty acid index in the population. PASW was used to run the statistical analysis. Associations between quintiles (Q1, Q2-4, Q5) of fatty acid indexes and continuous variables were examined using ANOVA. Associations between quintiles of fatty acid indexes and categorical variables were examined using Pearson chi-squared statistics. The results indicate the Omega-3 index is associated with fatty fish consumption and with improved 3MS scores (Table 1). There was no effect of age or sex, but was for BMI. The most interesting significant effects were the association of the Omega-3 index with fatty fish consumption and with Wave 3 3MS scores, a measure of cognition. According to the results, the RBC Omega-3 index is positively and significantly associated with improved cognition. While the effect was not significant for Dementia, the trend was for protection. One other notable result was the lack of association with the ApoE4 allele, as this gene product is associated with lipid metabolism and with cognitive outcomes.

These results are consistent with the work of Wennberg (2009) who found that RBC PUFA levels correlate well with reported intakes of fatty fish. Interestingly, this group reported that intakes of dairy fat also correlated with the
RESEARCH PLAN

Hypotheses

Among elderly participants of the Cache County Study on Memory, Health and Aging:

A) dietary intakes of milk or dairy products or both will be correlated with concentrations of the fatty acids C15:0, C16:1n7t and C17:0 FA in red blood cells (RBC);

B) greater intakes of full or reduced fat milk or dairy products or both will be associated with better cognitive performance and decreased risk of dementias;

C) higher levels of C15:0, C16:1n7t and C17:0 FA in RBC will be more strongly associated with better cognitive performance and decreased risk of dementia than is estimated dietary intakes of milk and dairy.

Objective 1.

Conduct red blood cell fatty acid profiling to determine concentrations of fatty acids in RBC membranes; blood was collected from 2200 participants in 2002-2004.

Objective 2.

Quantify usual intake of full and reduced fat milk and dairy products among elderly participants of the Cache County Study on Memory, Health and Aging. Semi quantitative food frequency questionnaires that included multiple questions about milk and dairy foods were collected from 3700 participants in 1995-1997, and 2300 participants in 2002-2004.

Objective 3.

Examine correlations between full and reduced fat milk and dairy products and measured concentrations of FA in RBC.

Objective 4.

Examine prospective associations between dietary intake of full and reduced fat milk and dairy products and cognitive outcomes including trajectories of change in cognitive function and incidence of Alzheimer's disease and other dementias over a 11-year period. Confounding and effect modification of these associations by age, sex, education, APOE genotype, CRP (a marker of inflammation), weight status, history of physical activity, history of comorbidities, and as well as other dietary factors will be examined.

MATERIALS AND METHODS

Cache County Study

The Cache County Study is a prospective, population-based study of exceptionally long-lived individuals living in a mountain valley of Northern Utah. A major strength of the study design is the careful clinical assessment of incident cognitive-related endpoints over an 11-year period (1995 – 2006) in relation to participant characteristics and exposures ascertained early in the study.

The Cache County Study on Memory, Health and Aging was established in 1994 as a collaborative research project between Utah State University and Duke University with funding from NIA (R01-AG11380). The original specific aims included the investigation of the age-specific prevalence and incidence of AD, the role of the APOE e-4 allele in the age-specific expression of AD, and the role of anti-inflammatory medications in the risk of AD. Table 2 shows the relevant data collection periods of the project.

The Cache County Study Population

The participants of the Cache County study include both men and women who were over 65 years of age or older on January 1, 1995 and the remaining cohort is now between the ages of 75 and 105. The Cache Cohort is predominantly Caucasian, of LDS faith, and exceptionally long-lived. Cache County Utah was the top-ranked county in the United States for male life expectancy in 1990 (77.5 years) and is well-suited for population studies of aging and health. The predominant faith discourages the use of alcohol and tobacco and Cache participants report low rates of both smoking and drinking. The LDS lifestyle most

Table 2. Data collection schedule for Cache County Study.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Wave 1</td>
<td>Wave 2</td>
<td>Wave 3</td>
<td>Wave 4</td>
</tr>
<tr>
<td>3MS screen = 5.092</td>
<td>3MS screen = 3.411</td>
<td>3MS screen = 2.344</td>
<td>3MS screen = 1.481</td>
</tr>
<tr>
<td>FFQ = 3.831</td>
<td>FFQ = 2.186</td>
<td>FFQ = 2.086</td>
<td>FFQ = 2.186</td>
</tr>
<tr>
<td>357 prevalent AD</td>
<td>128 incident AD</td>
<td>175 incident AD</td>
<td>85 incident AD</td>
</tr>
</tbody>
</table>
likely contributes to the increased longevity and low rates of cardiovascular disease and cancer observed in Utah. In addition, >80% of Cache study participants have at least a high school education and many have been affiliated with Utah State University during their lifetime. These factors most likely contribute to exceptionally high participation rates that range from 89.9% in 1995 at the outset to 79% at the last wave of follow-up.

Overview of Dietary Assessment

Participants’ usual dietary intake was estimated using a food frequency questionnaire (FFQ) at the baseline interview in 1995 and again at the third follow-up assessment (Wave 3) in 2002-2004. Food frequency questionnaires are the most commonly used method of dietary assessment in large population-based studies. The FFQ used in the Cache Studies is based on the format and food list from the Harvard Nurses’ Health Study. The Nurses’ Health Study questionnaire has been validated for use in a wide range of populations including elderly women. The food frequency questionnaire foods included 10 questions on milk and dairy products. Both the baseline and wave 3 assessment differentiates between low fat and full fat milk, and the wave 3 assessment differentiates between low fat and full fat yogurt, cheese, and ice cream. Interestingly, the Cache population is a high dairy consuming cohort. Dairy consumption was reported by 96% of the cohort and the average number of servings of dairy consumed was 2.14 per day (range: 0 – 13). Sixty percent of dairy consumption was milk.

Reanalysis of Red Blood Cell Fatty Acids

We recently analyzed the fatty acid from RBCs obtained from Wave 3 of the Cache County Study using an extremely rapid GC protocol for a project focused on omega-3 fatty acids. However, in our analysis, we were unable to quantify the contribution of C15:0 and C17:0 as they each co eluted with plasmalogen species under the rapid assay conditions we were employing. To determine if these fatty acids were measurable in these samples, we recently reanalyzed a subset of samples using a longer GC column. The results indicate that both fatty acids can be quantified in our RBC samples, and are within the range of values commonly reported for these fatty acids. Thus, to produce this data, we are requesting funds to reanalyze the samples using different GC conditions. In the interim, the prepared GC samples have been stored at -80°C and each contains the antioxidant BHT.

Power Analysis

Statistical power calculations provided below were calculated with a sample size of 3600, the number of participants who completed the baseline survey and cognitive screening test and provided complete and plausible dietary data. All power calculations consider the dietary variable to be dichotomous. For continuous-type and multinomial variables, as is more appropriate for the characterization of dietary patterns, this is a more conservative approach to power estimation given that statistical power is reduced as a result of the reduction in precision inherent in recoding a continuous variable to a discrete variable.

The following power calculations demonstrate our ability to detect relative differences in the risk of AD over the study period. As discussed previously, such analyses will require methods for categorical data analysis, such as logistic regression. Our calculations follow the formulas derived in Rosner. Computations are based on a significance level of 0.05 for a two-sided test, and are summarized in the following table. Rows of the table contain differing levels of the proportion of the population exposed to a given risk or protective factor, and columns of the table contain relative risks comparing exposed to unexposed. Cells of the table contain the estimated power for the corresponding level of exposure and relative risk.

Power for Comparing Rates of Cognitive Change

The following power calculations demonstrate our ability to detect differences in the rate of cognitive decline over time, as measured by the 3MS-R. As mentioned previously, such analyses will require methods for continuous longitudinal data, such as linear mixed effects models. To facilitate power analysis we make the simplifying assumption that change scores are used in the final analysis. A change score is defined for any given subject as the last measure of their cognitive ability minus their first measure. Our calculations thus follow the method shown in Rosner with respect to computing power for a t-test of two independent samples. Computations are based on a significance level of 0.05 for a two-sided test. These simplifying assumptions make our computations fairly conservative. First, naïve use of change scores alone would force one to eliminate subjects who have died between waves of data collection, thus sacrificing useful information. Second, the use of change scores further ignores data collected at intermediate time points, which also results in loss of information. Nevertheless, these results at least provide some approximate lower bound on our ability to detect differing cognitive trajectories.

In preliminary analyses, we computed an average decline over 11 years of 2.4 among those with the highest diet quality versus an average decline of 5.1 points for those with the lowest diet quality. While our currently available characterization of diet quality provides only a rough illustration of the potential impact of total diet, this suggests an average decline in cognition that is about 2 times greater
among those with the lowest quality diet compared to those with the highest quality diet. Power computations for making this kind of two-group comparison are summarized in the following table. Rows of the table contain differing levels of proportion exposed to a given risk factor, and the columns of the table respectively correspond to differences in rate of 3MS decline, compared to a baseline rate of decline equal to 2.4 points over 11 years.

### Main Study

Mixed effects models will be used to examine associations between dietary intake of milk and dairy products, RBC concentration of select FA and trajectories of change in cognitive function. Trajectories of cognitive change will be modeled using repeated measures of the 3MS which was administered to each non-demented participant at each wave of data collection. For nondemented participants who continue to participate in the study this includes four 3MS assessments spanning approximately 11-years (1995-2006). Mixed effects modeling will allow us to detect baseline differences in 3MS score as well as differing rates of change (3MS decline/trajectories of change) between subgroups of interest. Other variables to be included in mixed effects models include age, gender, years of education, body mass index, history of cardiovascular disease and other medical history variables, as well as history of smoking and alcohol use. We will test for effect modification by smoking status, APOE status, gender, and age by testing interactions between these variables and antioxidant intake. Analyses will be stratified if necessary based on results of the tests for interactions. We have previously successfully used mixed modeling approaches to examine relationships between other dietrelated variables and trajectories of 3MS decline including our work on antioxidants and cognitive function.

Logistic regression and discrete-time hazard models will be used to evaluate the risk of incident AD by milk and dairy exposures. Discrete-time hazard models divides the observation period into discrete time intervals (years) and incorporates covariates to predict the hazard of AD; that is, it estimates the probability of developing AD in a given year for previously non-demented subjects. This method has been used by other Cache investigators to assess how AD incidence relates to other characteristics or exposures, such as age.

### RESULTS AND DISCUSSION

A method for cleaning up the samples with solid phase extraction was developed and validated. According to the results from this work, there appears to be no degradation of the samples during the 2 years they were stored at -80 degrees (Figure 1). This was not unexpected as the sample all contained an antioxidant (butylated hydroxotoluene). While there are some differences, this is likely explained by the different analytical methods used to generate the data.

The solid phase extraction technique was implemented on a subset of the samples in October and shown to be effective at removing the contaminants which resulted from the initial sample preparation.

Among the 3,364 subjects evaluated at baseline, a total of eleven categories of dairy products were estimated. These included skim milk, 2% milk and higher fat, chocolate milk, cream, sour cream, ice cream, yogurt, cottage cheese, cream cheese, other cheese, and butter. The intakes of these products were estimated from the Food Frequency Questionnaires, yet at this point all analyses have been restricted to total intake and thus has not been adjusted to energy. The mean baseline intake of dairy in this population is 2.7 cups per day with a standard deviation of 1.9. The cumulative daily frequency of dairy product intake is shown in Figure 2. According to

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### Table 3. Statistical power for comparing two binomial proportions.

<table>
<thead>
<tr>
<th>Prevalence of Risk Factor</th>
<th>Odds Ratio (n=3,600)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>0.025</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>0.05</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>0.10</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>0.25</td>
<td>&gt; 0.99</td>
</tr>
</tbody>
</table>

### Table 4. Statistical power for comparing rates of cognitive change.

<table>
<thead>
<tr>
<th>Prevalence of Risk Factor</th>
<th>Relative Rate of 3MS Decline (n=3,600)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>0.025</td>
<td>0.35</td>
</tr>
<tr>
<td>0.05</td>
<td>0.59</td>
</tr>
<tr>
<td>0.10</td>
<td>0.86</td>
</tr>
<tr>
<td>0.25</td>
<td>&gt; 0.99</td>
</tr>
</tbody>
</table>
Effect of dairy product consumption on cognitive performance among elderly participants

Figure 1. Comparison of average fatty acid concentrations in red blood cells of participants from the cognition study.

Figure 2. Plot of dairy intake in units vs. cumulative percent of participants at baseline.

the figure, a very low percentage (~9%) of the respondents report to abstain from dairy. Sixty percent report consuming over 0.5 units per day.

The baseline 3MS scores were compared between groups separated by daily intake of dairy products and found to be highly significant. A plot of baseline, wave 2 and wave 3 3MS scores vs. intake of daily dairy intake is shown in Figure 3. It would appear from a preliminary analysis that intake of 2.5 units of dairy is associated with the highest 3MS scores at baseline. This association is also noted at Wave 2, although there was not a FFQ given at this evaluation. The association remains at wave 3 when there was a FFQ administered and it is from this time point that the RBCs were collected. In moving forward, we will look at confounding factors that may explain the results including specific dairy product interactions.

REFERENCES


Crichton, G. E., K. J. Murphy, et al. (2010). “Dairy intake and cognitive health in middle-aged South Australians.” Asia Pac
Effect of dairy product consumption on cognitive performance among elderly participants / R. Ward


Effect of milk fat globule membrane (MFGM) on gut barrier protection in runners

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Funded by: United Dairymen of Idaho, January 2011 - December 2011

BACKGROUND

Review of Literature

Sports nutrition basics.

It is well established that dietary macronutrients (carbohydrates, proteins and fats) can enhance the physiological response to exercise and facilitate recovery. In a recent position paper on nutrient timing and exercise, Kerkvink et al. reviewed the accumulated evidence for beneficial effects of dietary interventions before, during and after both resistance and endurance training (Kerkvink, Harvey et al. 2008). Prior to listing their specific recommendations, it is important to establish a few general points with respect to exercise and recovery. First, intense or prolonged exercise is associated with substantial changes in endogenous energy stores, imbalances in nutrients (sodium, amino acids, lipids), loss of fluid (dehydration), as well as effects on hormonal signaling and the immune system (Dempsey 2003). Second, the level of physical output achievable by an individual is correlated with energy stores, and the inability to maintain a given output, or fatigue, results when energy reserves are low. Third, foods and drinks provide energy substrates, biochemical building blocks and nutrients, which facilitate restoration of energy stores and repair and replacement of damaged tissues. Importantly, the composition of the dietary intervention in terms of macronutrients (carbohydrates, proteins and fats) as well as the timing of the food intake (pre-exercise, during exercise and post-exercise) may significantly affect an individual’s ability to restore muscle energy stores and repair and rebuild tissues.

Nutrition strategies for optimum performance are organized according to the time of ingestion, pre-exercise, during exercise, post-exercise. These points will be of interest when the experimental design for this study is described in the following section.

Nutrition pre-exercise.

- Muscle and liver glycogen storage is limited and levels depend on nutritional status and training level (Coyle, Coggan et al. 1985).
- During medium to high intensity exercise (65-85% VO2max) glycogen stores provide adequate energy for approximately 90 min to 3 h of exercise (Tarnopolsky, Gibala et al. 2005).
- The intensity of exercise (pace, work output) is reduced as glycogen is progressively depleted (Coyle, Coggan et al. 1985).
- Muscle tissue breakdown and immune suppression are correlated with glycogen depletion (2000; Gleeson, Nieman et al. 2004).
- Pre-exercise glycogen levels are highest with a high-glycemic, high-CHO diet (Bussau, Fairchild et al. 2002; Tarnopolsky, Gibala et al. 2005).
- For a pre-event meal, the optimal carbohydrate and protein content is influenced by factors such as fitness level and duration. However, in general it is suggested athletes consume 1-2 g CHO/kg and 0.15-0.25 g PRO/kg 3-4 hours before competition (Tarnopolsky, Gibala et al. 2005).
- Ingestion of amino acids or PRO before exercise increases protein synthesis and PRO + CHO produces greater levels of muscle accretion (Tipton, Rasmussen et al. 2001; Tipton, Elliott et al. 2004)
- Consumption of different PRO sources along with CHO leads to greater increases in strength and improved body composition compared to CHO alone (Coburn, Housh et al. 2006; Cribb and Hayes 2006; Willoughby, Stout et al. 2007).
Nutrition during exercise.

- During exercise, the availability of CHO and muscle glycogen levels significantly affect exercise performance, and supplementation of CHO during exercise is most important when pre-exercise glycogen levels are low (Widrick, Costill et al. 1993; McConell, Snow et al. 1999).
- When the length of the exercise session lasts longer than 60 min, dietary sources of CHO gain importance for maintenance of blood glucose and muscle glycogen (Jeukendrup, Jentjens et al. 2005).
- Muscle CHO oxidation can be increased when different sources (monosaccharides, dextrins) are combined, and increased CHO oxidation associated with improved performance. Combinations can include glucose, fructose, sucrose and maltodextrins, but large quantities of fructose are associated with gastrointestinal discomfort (Currell and Jeukendrup 2008).
- Adding PRO to CHO at a ratio of 3:4:1 can improve endurance performance in both acute and endurance events (Ivy, Res et al. 2003; Saunders, Kane et al. 2004).
- In resistance training, CHO or CHO + PRO consumption are associated with increased muscle glycogen storage, and less muscle damage (Haff, Koch et al. 2000; Baty, Hwang et al. 2007).

Nutrition post exercise.

- To maximally stimulate glycogen synthesis following exercise, 8-10 g CHO/kg/day should be consumed within 30 minutes of completion (Jentjens and Jeukendrup 2003; Tamopolsky, Gibala et al. 2005).
- When PRO is added to CHO post exercise at 0.2-0.5/kg/day at a ratio of ~3:1, glycogen re-synthesis is enhanced (Ivy, Goforth et al. 2002).
- After exercise, ingestion of amino acids (primarily those essential) is associated with a measurable increase in muscle hypertrophy (Rasmussen, Tipton et al. 2000; Tipton, Elliott et al. 2004). The addition of CHO to post exercise amino acids appears to enhance the effect, while pre-exercise supplementation results in the best response (Tipton, Rasmussen et al. 2001).

Summary of nutrition strategies for optimal performance.

Intense and or prolonged exercise depletes energy stores, damages tissues, leads to nutrient imbalances, fluid loss and aberrations in hormonal signaling and immunity. While the focus of recovery from exercise stress should be the restoration in all of these systems, most work to date has focused on repletion of energy stores. Based on our preliminary data (presented below) we believe MFGM has the potential to significantly improve the barrier properties of the gut against exercise stress. As the role of milk in biology is to effectively deliver macronutrients as well as provide protection from stressors, there is precedent for such activity. Furthermore, demonstration of this activity will likely open up new markets to MFGM as an ingredient.

Sports nutrition and dairy products.

Dairy derived ingredients are well represented in foods formulated for and marketed to athletes, primarily due to their composition and the functional benefits they provide. For example, whey proteins are high in essential as well as branched-chain amino acids. The former are necessary substrates for muscle building and the latter are thought to facilitate anabolic processes (Ha and Zemel 2003). To date, the majority of studies that have investigated the role of dairy derived ingredients in sports nutrition have focused on basic endpoints such as recovery and rehydration. However, as the biological role of milk is to promote health and provide protection against stress, it stands to reason that constituents of milk might provide other benefits and protections to athletes beyond provision of macronutrients, and that these activities would differentiate dairy ingredients from those isolated from other sources. For example, it has been suggested that milk and ingredients derived from milk may be valuable in influencing nutrient partitioning, in promoting gut health, in supporting the immune system and in reducing oxidative stress (Ha and Zemel 2003). Below the recent evidence from recovery studies conducted with chocolate milk is summarized. Following this, other aspects of exercise and stress are discussed and potential for dairy ingredients to affect these processes will be presented.

Dairy products and recovery.

In recent years, several studies have investigated the effects using chocolate milk as a recovery drink for improving athletic performance. Karp et al. (2006) compared the ability of three beverages to facilitate glycogen resynthesis, as measured by an endurance trial to exhaustion at 70% VO2max, after a preliminary interval workout designed to deplete muscle glycogen (Karp, Johnston et al. 2006). The three beverages were Gatorade,
Effect of milk fat globule membrane (MFGM) on gut barrier protection in runners / R. Ward

a fluid replacement (FR) drink, Endurox, a commercially available carbohydrate replacement (CR) drink and low-fat chocolate milk (CM). Nine, male, trained cyclists performed the trials in a counterbalanced, single-blind, randomized design. The carbohydrate content of the CR and CM were the same, while that of the FR drink was lower. Variables measured in the second trial, average heart rate, rating of perceived exertion, and total work performed (WT). According to their results, both the TTE and the WT were significantly greater for the FR and CM beverages compared to the CR drink.

Prior to this study, the authors hypothesized that both the CM and the CR beverages would lead to a greater TTE versus the FR drink due to their higher carbohydrate contents and the inclusion of protein. In fact, their results were in contrast to Williams et al. (2003), who found that the same CR drink led to 55% improvement in exercise time in cyclists over the same FR drink in a trial conducted at 85% VO2max (Williams, Raven et al. 2003). Karp et al. suggested that the difference in exercise intensity might have accounted for the disparity, as there may be a greater usage of muscle glycogen at the higher intensity. Furthermore, they suggested that the short recovery time might have prevented complete digestion of the complex carbohydrates in the CR beverage slowing the rate of glucose entry into circulation. One other notable difference in the CM and the CR beverages is the presence of fat. At the lower exercise intensity, they speculated, there may have been a greater reliance on circulating free fatty acids for energy, which would delay glycogen depletion. However, they also pointed out that the fat in the chocolate milk may have increased gastric emptying and hypothesized that non-fat chocolate milk may have increased TTE even more.

In a study with a similar design, Thomas et al (2009) investigated the same three beverages for the ability to facilitate recovery from an initial exercise stress, a fluid replacement drink (FR; Gatorade), a carbohydrate replacement drink (CR; Endurox) and chocolate milk (CM) (Thomas, Morris et al. 2009). This study used nine cyclists in a randomized counterbalanced order and employed the same protocol for glycogen-depletion and the same conditions for the endurance capacity trial (time to exhaustion at 70% VO2max). However, instead of providing the same carbohydrate content, their treatment normalized the CR and CM beverages to total calories. Thus, the CR beverage was provided in slightly greater volume and contained more protein and carbohydrate and less fat to match the caloric content. In contrast to the results of Karp, they found that the CM led to a 51% longer TTE than the CR beverage and 43% longer than the FR beverage.

While it is unclear from the results of this study what parameters of the CM led to the increased cycling times, the authors speculated that it might have been due to the presence of sucrose in the CM which is not present in the CR beverage. Additionally, they pointed out that the higher fat content of the CM may have increased circulating plasma free fatty acids and fat oxidation, which have been previously correlated with improvement in endurance capacity at similar exercise intensities (i.e.~70% VO2max (Pitsiladis, Smith et al. 1999; Stevenson, Williams et al. 2005). Lastly, they also referenced the study by Williams et al. (Williams, Raven et al. 2003) wherein the CR beverage was shown to improve post exercise recovery over the same FR drink at a higher submaximal intensity level (85% VO2max), and suggested that the higher exertion rate may have favored carbohydrate oxidation.

In a third study comparing chocolate milk (CM) and a carbohydrate replacement beverage (CR) but not the FR drink, a different experimental design was used and no significant differences were found between the two drinks. Pritchett et al. used a different protocol for the first exercise test designed to deplete muscle glycogen, and then had the cyclists perform a TTE trial at 85% of VO2max 15-18 h later (Pritchett, Bishop et al. 2009). After the first event, the cyclists were provided the beverages at t=0 and again at 2 h targeting 1.0 kg CHO/kg/h. Similar to the study of Thomas, the beverages were isocaloric. During the 4 h recovery the athletes were not permitted any other food or drink, but water was provided ad libitum. In the 15-18 h separating the two trials, the subjects were instructed to replicate their dietary intake. While the TTE was not significantly different, the research team did find that the change in creatine kinase levels were significantly higher in the group consuming the CR beverage. However, there were no significant differences in muscle soreness as determined with a visual analog scale. Interestingly, all of the participants preferred the taste and consistency of the CM over the CR beverage.

In a recent study conducted with 13 NCAA Division I male soccer players, chocolate milk was compared to a common carbohydrate replacement gel (Clif Shots) for the ability to facilitate recovery and prevent indices of muscle damage (Gilson, Saunders et al. 2010). Unlike the previous studies, the physical parameters tested in this study were of alternating intensity and contained more resistance type training. In a repeated, crossover design, the athletes completed one week of normal baseline training followed by four days of increased training duration (ITD). The baseline training was similar to normal off-season training, and the ITD trials were designed to increase total training duration by >25% over four consecutive days. The parameters measured included serum creatine kinase (CK), myoglobin, muscle soreness, fatigue rating, and isometric quadriceps force (IQF). In addition, two performance tests, the T-drill and vertical jump, were performed in each session. The results from this study indicated that no treatment effects were measured for serum myoglobin, muscle soreness,
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IQF or for the performance tests. However, similar to the results of Pritchett, there was a significant reduction in the serum CK levels when the athletes consumed chocolate milk as the recovery beverage. Interestingly, the authors did note that increases in IQF scores were higher in those consuming chocolate milk, and hypothesized this effect may reach significance in future studies utilizing greater training volumes and longer periods of time.

Summary of evidence from chocolate milk studies and resulting questions.

Taken together, the studies reviewed above indicate that chocolate milk, when used as a recovery beverage after an initial trial to deplete muscle glycogen, has equal or perhaps superior properties to products formulated for and marketed to athletes. However, the effects may be dependent on the specifics of the experimental design and/or the intensity at which the endurance trial is conducted. Furthermore, two of the research groups that conducted these studies have noted that low-fat chocolate milk contains more fat than either of the other beverages used, and speculated that this may mediate recovery via the provision of circulating free fatty acids. In addition, the results of these studies suggest that in comparison to the other beverages tested, chocolate milk appears to reduce circulating levels of creatine kinase, an index of sarcolemma disruption. Although these measures have failed to correlate with either subjective measures of muscle pain or power output, there may be a beneficial effect of chocolate milk consumption on physiological stress.

Physiological effects of exercise beyond depletion of energy stores.

Besides effects on energy substrates and fluid and nutrient loss, intensive exercise has been shown to stress many areas of an athlete’s physiology. For example, it is well established that heavy training loads lead to damaged muscle tissue (Cockburn, Hayes et al. 2008), intestinal permeability (Pals, Chang et al. 1997), increased levels of oxidative stress (Ha and Zemel 2003), and depression of the immune system (Gleeson, Nieman et al. 2004). To date, few studies have addressed the ability of foods and/or ingredients to either prevent or attenuate these processes. However, there are some indications, from our preliminary data as well as from other studies, that dairy-based recovery beverages may effectively attenuate some of these physiological insults.

Of the four stresses mentioned, there appears to be the best evidence that specific nutritional strategies can affect parameters of the response of skeletal muscles to exercise. For example, creatine kinase (CK) is an intercellular skeletal muscle enzyme that can be found in plasma after exhaustive exercise (Brancaccio, Maffulli et al. 2007). Levels of serum CK are variable across individuals according to lean muscle mass, race, sex, physical activity and even climate. Within individuals, serum CK levels are a good measure of intensity of exercise, and are exacerbated by activities which cause sarcomeric damage, such as ultramarathon running, running downhill and weight-bearing exercise (Brancaccio, Maffulli et al. 2007). Interestingly, changes in CK do not correlate with recovery time or with impairment of muscular performance. However, as there does clearly appear to be an effect mediated by chocolate milk on plasma CK (and possibly muscle damage) we propose to measure this variable in our study.

A well-known consequence of endurance exercise is compromised gut barrier integrity. Endurance exercise increases body core temperature and reduces splanchnic blood flow causing localized intestinal hypoxia and increased intestinal membrane permeability (Lambert 2008). In the Comrades Ultra marathon, 81% of the 89 athletes admitted to the medical tent following the race had clinical endotoxemia and 2% had levels considered in

Figure 1. (A) Effect of rest (R) and running for 60 minutes at 40%, 60% and 80% of VO2max on the urinary Lactulose/Rhamnose ratio. (B) Correlation between urinary lactulose/rhamnose ratio and final rectal temperature (Pals, Chang et al. 1997).
the fatal range (Brock-Utne, Gaffin et al. 1988). Increased intestinal permeability in athletes is thought to be the mechanistic link between endotoxiaemia, increased plasma inflammatory cytokines and gastrointestinal complaints associated with exercise (Lambert 2008) (Ng, Lee et al. 2008) (Jeukendrup, Vet-Joop et al. 2000) (Camus, Poortmans et al. 1997). According to the data reproduced in Figure 1A (left), intestinal permeability doubles in subjects running at 80% VO2max for an hour (Pals, Chang et al. 1997). In this study, the urinary lactulose/rhamnose ratio was significantly and positively correlated to core temperature (Figure 1B, right). This suggests that increased gut permeability and associated morbidities can affect athletes competing in many types of sporting events and is not exclusive to ultra endurance type events. Work conducted in our labs (see below) and by others has indicated that components of milk, specifically the MFGM, may strengthen the gut against stress-induced leakiness. For example, Dial and Lichtenberger (1984) showed the milk phospholipids can prevent stomach ulcers induced by ethanol and strong acid (Dial and Lichtenberger 1987). Additionally, they have shown more recently that orally administered phosphatidylcholine prevents LPS induced gut leakiness when administered 1 before the LPS injection (Dial, Zayat et al. 2008). Furthermore, Park et al. (1998) have shown that gangliosides isolated from milk provide the same protection against LPS mediated gut leakiness when added to a mouse diet (Park, Suh et al. 2007). We believe that these data suggest that MFGM may effectively prevent and/or treat leaky guts induced by exercise stress.

Intense exercise is also associated with systemic inflammation. For example, Ng et al., (Ng, Lee et al. 2008) demonstrated increased plasma levels of IL-10 and IL-6 (50% and 65.2% increase from baseline respectively) in runners immediately following a half marathon. Similarly, plasma levels of IL-6 and TNFα have been demonstrated to be increased from baseline immediately after a marathon (Camus, Poortmans et al. 1997). Dairy products, on the other hand, have been associated with reductions in inflammation either caused by exercise (Ha and Zemel 2003) or via obesity mediated metabolic stress (Zemel, Sun et al.). To date, the studies conducted with chocolate milk as a recovery beverage have not addressed whether or not there is an effect on inflammation, yet data available from other fields suggest there may be an effect.

Based on our preliminary data, we believe that incorporation of MFGM into a sports drink may abrogate exercise induced intestinal permeability with the net result of less endotoxiaemia, systemic inflammation and associated gastrointestinal complaints. Exercise induced gut permeability is a major issue in athletics that is not addressed by sports drinks now in the marketplace making our study novel with high commercial potential for the dairy industry.

**Effect of milk fat globule membrane (MFGM) on gut barrier protection in runners / R. Ward**

**Preliminary findings or research efforts by the principal investigator.**

PIs Ward and Hintze have collaborated on several studies over the last few years investigating bioactive effects of milk fat globule membrane (MFGM). To date we have utilized both in vitro approaches with Caco-2 cells and in vivo approaches with both rats and mice. In a study published this year we reported on the protective effects of MFGM supplemented diets against chemically induced colon preneoplastic lesions (Snow, Jimenez-Flores et al. 2010).

Caco-2 cells are an intestinal cell line that establishes barrier properties when grown on permeable supports between two chambers of media. Various stresses may be applied to the cells to investigate effects on the barrier properties. In a preliminary set of studies we applied MFGM to the apical side of Caco-2 cells for 24 h at 1mg/ml which we approximated to be a normal physiological concentration. Subsequently, the media was replaced with media containing 2mg/ml EGTA for 2 h, which stresses cellular tight junctions. This media was then replaced with one that contained phenol red on the apical side. After 24 h the concentration of phenol red was determined on the basolateral media side. Our treatments included a) a blank control, b) MFGM, c) MFGM treated with pancreatic enzymes, and d) MFGM treated with both gastric and pancreatic enzymes. According to our results (Figure 1A) stressing cells with EGTA leads to greater phenol red on the basolateral side, and untreated cells were the leakiest. Both the untreated MFGM and the MFGM treated with both the pancreatic and gastric enzymes significantly prevented this effect.

In a follow up study we utilized the same mode to compare the protective effects of MFGM to an emulsion formulated with similar macronutrient proportions of whey protein, lecithin and anhydrous milk fat (AMF) (50:25:25). In this study we measured the barrier properties of the cells using transepithelial electrical resistance (TEER). According to our results (Figure 1B), only the treated (or digested) MFGM reduced the EGTA induced leakiness. Based on this preliminary data, we tested the effects of supplementing mouse diets with a mixture of MFGM and AMF on gut protection and inflammation compared to a control diet formulated with corn oil. The mice were fed the diets for 5 weeks and then gut stress was induced via an intraperitoneal injection of lipopolysaccharide (LPS). Immediately after the LPS injection the mice were gavaged with an indigestible high molecular weight fluorescent polymer. Mice (n=6 gp) were sacrificed after 24 h and 48 h and plasma fluorescence and 17 cytokines were measured. The fluorescence data are shown in Figure 2 on the left side and four cytokines are shown on the right side.

According to the Figure, at 24 hours plasma fluorescence was significantly higher in the mice fed the control diet.
Figure 2. Effect of MFGM treatment on barrier properties of Caco-2 cells grown on permeable supports in bicameral chambers. Left (A): Cells were treated with control media, or media that had been supplemented with 1mg/ml MFGM. The MFGM was subjected to an in vitro digestion using pancreatic enzymes at pH 7.0 for 1 h, or treated with gastric enzymes at pH 3.0 for 30 min then treated with pancreatic enzymes. To induce barrier stress, cells were treated with EGTA for 2 h. After stress, the media was changed and the media in the top chamber was spiked with phenol red. Leakiness was assessed via phenol red detection in the lower chamber. Right (B): Change in trans epithelial electrical resistance (TEER) of Caco-2 cells treated with either a whey:lecithin:anhydrous milk fat (50:25:25) emulsion or MFGM each untreated and treated (digested) as in the first study. Only the digested MFGM (marked with an asterisk) led to a significant reduction in the ΔTEER.

Figure 3. Effect of dietary milk fat globule membrane (MFGM) on gut permeability (left) and serum cytokines (right). Animals (6/gp) were fed the AIN-76A diet (control) or the control diet formulated to contain ~1% polar lipids by weight supplied by MFGM for 28 days. Animals were injected i.p. with LPS (10mg/kg), gavaged with dextran-FITC, and sacrificed 24 hours later. Dietary MFGM significantly prevented gut leakiness (left) and led to lower levels of 6 of sixteen measured cytokines (Shown are A: IL-6, B: IL-10, C:IL-17, and D:MCP-1). White bar (control), grey bar (MFGM 24h), and dark grey bar (MFGM 48h). Other significantly affected cytokines were INF-γ and TNF-α. All animals fed the control diet died at 48h.
than in those fed the MFGM-AMF mix, indicating gut leakiness. In addition, levels of IL-6, IL-10, IL-17, MCP-1, IFN-γ, TNF-α and IL-3 were all lower in the MFGM-AMF fed animals at 24 h compared to controls. Interestingly, at 48 h all of the animals fed the control diet had died, whereas only one animal fed the MFGM-AMF did not survive. We interpreted this data to indicate that the initial injection of the LPS intraperitoneally induced inflammation in the gut, which subsequently led to decreased barrier properties and increased fluorescence. Translocation of LPS and other gut contents further induced an inflammatory response in the control animals resulting in septic shock. The most interesting result of this study was that the MFGM-AMF diet appeared to prevent the leakiness of the gut.

In sum, our preliminary cell culture work and mouse data strongly suggest that dietary MFGM enhances the barrier properties of the gut against stressors. However, to date there is no information on this effect in humans. Interestingly, recent studies indicate that high fat diets and obesity negatively affect the barrier properties of the gut, and lead to increases in the blood of the gram negative bacterial cell wall molecule, lipopolysaccharide (LPS), which can influence insulin signaling (Cani, Possemiers et al. 2009). According to a rapidly developing hypothesis, the low concentration of LPS in the blood may be responsible for the chronic low-grade inflammation consistently measured in obese individuals, and may precipitate the subsequent metabolic dysregulation (Cani and Delzenne 2009). While it is unclear if the same mechanisms are responsible for these effects in our preliminary studies and in the studies cited above, it would appear that dietary strategies to improve gut barrier properties are of current interest. We feel that the use of trained endurance athletes, a phenotype with documented occurrence of gut dysfunction, make an excellent human model in which to test the hypothesis that MFGM can protect the gut against hypoxia induced leakiness. In addition, based on the recent findings that implicate leakiness of the gut in compromised health, positive results from this study would indicate an exciting new and valuable nutritional bioactivity of an underutilized milk fraction.

Validation of the exercise-induced gut leakiness model.

To determine whether our proposed exercise model would induce changes in gut permeability and appearance of LPS in the systemic circulation; two of the investigators completed an exercise challenge. One hour prior to the exercise challenge, blood was drawn to determine baseline LPS levels and core temperature was measured rectally. Both investigators exercised for 1 hour at 80% maximal heart rate, one on a treadmill the other on an elliptical machine. The exercise was conducted at room temperature without ventilation. Thirty minutes into the challenge, the investigators consumed the lactulose/rhamnose sugar probe. Immediately after exercise core temperature was measured and blood was collected 1 hour post-exercise. All urine was collected for 4.5 hrs post-exercise. Three days after the exercise challenge, the investigators again ingested the sugar probe and collected urine for 4.5 hrs to determine baseline gut permeability.

Plasma LPS levels were determined by spectrophotometry using a commercial kit (Charles River
Labs, Wilmington, MA). Urinary lactulose and rhamnose were quantified by GC mass spectrometry according to a published method (Abazia, Ferrara et al. 2003).

The exercise challenge raised core temperature from 37.1°C ± 0.2 to 39.8°C ± 0.4. Core temperatures between 39 and 40°C have been demonstrated to increase gut permeability in previous studies (Figure 1A and (Pals, Chang et al. 1997; Lambert 2008)) suggesting that our proposed exercise challenge is at an appropriate intensity. Plasma LPS increased ~2-fold from baseline to 1 hr post-exercise (Figure 4A). Gut permeability as assessed by the ratio of urinary lactulose to rhamnose increased more than 2-fold in response to the exercise challenge (Figure 4B). In a similar study using the lactulose/rhamnose probe, Gisolfi et al., observed a similar increase in gut permeability when subjects ran for 1 hr at 80% VO2 max and that gut permeability is highly correlated to core temperature (Figure 1A) (Pals, Chang et al. 1997).

Although our preliminary data is limited by a small sample size, we believe our proposed model induces changes in both gut permeability and plasma LPS concentrations. Moreover, we now have the analytical protocols in place to measure our primary endpoints which we believe greatly enhances chances for success if the project is funded.

**RESEARCH PLAN**

**Project Objectives and Hypothesis**

Milk fat globule membrane (MFGM) added to a fluid replacement (FR) drink will reduce gut leakiness, lipopolysaccharide (LPS) absorption, systemic inflammation, and parameters of muscle damage compared to the FR drink alone.

**MATERIALS AND METHODS**

**Project Design**

*Overview of experimental design.*

To test the hypothesis milk fat globule membrane (MFGM) will prevent gut leakiness induced by intense exercise, we will conduct a randomized, double-blind crossover exercise trial with 10 runners on Utah State University campus. This population of 10 runners will be selected from a larger population of 20 that will be recruited to complete two initial trials. In two preliminary visits we will determine the parameters necessary for conducting the experimental trial and will select athletes for a second trial. During the first visit, we will determine a baseline value for gut permeability as well as aerobic capacity of runners. In the second visit we will ensure the capacity to complete the rigorous at 80% VO2max and will determine the effect of the exercise stress on gut permeability, plasma LPS and core temperature. From the data collected in this trial we will select 10 athletes to participate in the second trial, and will pick athletes that are the greatest responders to the exercise stress in terms of gut leakiness and heat generation.

Once a test population is selected, we will proceed to the randomized, double-blind, crossover trial in which MFGM will be tested for the ability to prevent the exercise induced gut permeability. Each trial will be conducted in precisely the same way, and the only difference will be in whether or not the MFGM in included into the fluid replacement beverage.

During each testing session we will monitor heart rate (HR), work output and core temperature (CT). HR and CT will be monitored by a CorTemp™ temperature pill, which will be ingested by the athletes prior to the trial (http://www.hqinc.net/pages/pill_page.html). In addition, blood will be drawn at baseline (before depletion trial), immediately after the time trial, and again 2 h after. Blood will be analyzed for indices of training intensity (lactate), muscle damage (creatine kinase, cytokines), and gut barrier dysfunction (lipopolysaccharide, cytokines). In addition, half way through the trial athletes will consume a beverage with two carbohydrates (lactulose, and rhamnose). During the following six hours urine will be collected and these sugars will be measured via HPLC to determine the permeability of the gut.

**Subject recruitment.**

**First visit.**

Twenty, male runners (ages 18-50) will be initially recruited via campus flyers, advertisements in the local paper and weekly sports guide. Participants who have medical histories including: heart disease, uncontrolled hypertension, diabetes, Crohn’s Disease, irritable bowel syndrome, colitis, celiac disease, inflammatory or autoimmune diseases or lactose intolerance will be excluded from the study. In addition, all subjects will be instructed to avoid all anti-inflammatory medications for at least 24 h prior to any testing activity. On the morning of the first visit, approved subjects will consume a standardized 400 kcal breakfast and have body composition measured by BodPod (Life Measurement, Inc., Concord, CA). Participants will be asked to refrain from any heavy exercise for 48 hrs before the first visit. Subjects will be given a 50 ml tube containing the 5 g lactulose and 2 g rhamnose sugar probe and instructed to drink the probe 4hrs after they have finished breakfast to determine baseline gut permeability.

Subjects will be given a urine collection container and instructed to collect all urine for 6 hrs after ingestion of the probe. Subjects will also be given a standardized 400 kcal lunch that will be consumed 1.5 hrs after drinking the sugar probe. To reduce variability related food intake and gut fill,
Subjects will only be able to consume the standardized lunch during the urine collection period and allowed only to drink water. After 5 hrs of urine collection, subjects will return to the test site and perform a running VO2 max test.

**Second visit (approximately one week after first visit).**

Subjects will be provided with the standardized breakfast the night before the second visit and instructed to only consume the provided breakfast and water. Participants will be asked to refrain from any heavy exercise for 24 hrs before the second visit. Approximately 3 hrs after completion of breakfast, subjects will report to the test site to have blood drawn (to determine baseline plasma LPS), weight measured and to ingest a temperature probe. Participants will start the exercise challenge (60 minute run at 80% VO2 max) approximately 4 hrs after completion of breakfast. The exercise challenge will be conducted in a climate-controlled room at 22 °C without a fan. Subjects will be monitored to ensure they are running at 80% VO2 max and not allowed to consume water during the challenge. Thirty minutes into the challenge, participants will consume the sugar probe. Immediately after the exercise challenge weight will be measured, blood drawn (post-exercise plasma LPS) and subjects will instructed to collect their urine for the next six hrs. Subjects will be given a standardized lunch which will be the only food allowed during the urine collection period. To facilitate rehydration and urine production, subjects will be instructed to replace 150% of their fluid losses (pre to post exercise weight) by water intake.

**Final participant selection.**

Ten participants will be selected to take part in the study. The investigators will select participants based on the following criteria ranked in order:

- Ability to complete the exercise challenge.
- Largest difference between baseline and post-exercise gut permeability.
- Largest difference between baseline and post-exercise plasma LPS.
- Largest increase between baseline and post-exercise core temperature.
- Availability of the subject to complete the study.

**Third and fourth visits.**

The third and fourth visits will be the crossover portion of the experiment comparing MFGM to the vehicle using the ten subjects chosen from the 1st and 2nd visits. The 3rd and 4th visits will be identical to the 2nd visit except participants will drink either the fluid replacement vehicle or fluid replacement + MFGM 1 hr before the exercise challenge and again immediately following the exercise. Moreover, additional blood draws will be taken at 1 and 5hrs following the exercise challenge. Participants and personnel administering experimental protocols will be blinded to treatments by use of coded, non-transparent drink containers. Subjects will complete the 4th visit one week later and consume the vehicle beverage or the vehicle + MFGM. For a schematic see Figure 5.

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**Figure 5.** Experimental design.
Treatments.

For the crossover portion of the study, athletes will be provided with a fluid replacement beverage (FR) or the same beverage formulated with MFGM both before and after the exercise trial. The beverages will be provided one hour prior to the exercise trial as well as immediately after.

Fluid replacement (FR) beverage.

For the fluid replacement we will use Accelerade from Pacific Health Laboratories. Subjects will be asked to consume a 32 oz. bottle in which 3 scoops of powder will have been added. At this level, the drink will contain 360 calories (60g sugars, 15g protein and 3g fat).

Fluid replacement beverage + MFGM.

We will formulate this beverage by supplementing the Gatorade with milk fat globule membrane. Arla Foods markets a high MFGM product (Lacprodan PL-20) which contains roughly 20% phospholipids and 60% protein, and we will use this ingredient or one similar from another manufacturer. In studies with mice, Dial et al have noted effects on gut protection in 1 h when animals are provided with phospholipids at 100mg/kg body weight (Dial, Zayat et al. 2008). Accordingly, we will select an appropriate amount of MFGM to add to the Gatorade according to the athlete’s weight. For a 70kg male this would mean adding 35g of MFGM. A 32 oz bottle of Gatorade® contains 56 g of carbohydrates and 200 calories. The supplemented MFGM would add 21g of protein, and 7 g lipids, or another 180 calories.

Endpoints measured.

Gut permeability.

To determine resting, baseline intestinal permeability the method of Pals will be used (Pals, Chang et al. 1997). This method determines intestinal permeability by supplying the non-digestable sugars lactulose, and rhamnose and estimates intestinal and gastric permeability by recovery of these sugar probes in the urine. In healthy gut epithelia, lactulose has very limited transport across the gut barrier. Rhamnose crosses the small intestinal epithelia via a transcellular route and serves as internal control to account for gastric emptying rate, intestinal transit time and renal function. The larger lactulose molecule can only cross gut epithelia via a paracellular route through tight junctions. Therefore a higher ratio of lactulose to rhamnose recovered in the urine indicates compromised intestinal barrier function. Exercise stress has been demonstrated to significantly increase the urinary lactulose/rhamnose ratio (Pals, Chang et al. 1997) (Davis, Willard et al. 2005) (Lambert 2008) (Smetanka, Lambert et al. 1999). Similar to our proposed exercise stress model, this method has been used to demonstrate that running at 80% VO2max for an hour doubles gut permeability compared to baseline (Pals, Chang et al. 1997).

Plasma LPS.

To assess endotoxemia, plasma LPS concentrations will be measured via a kinetic activity as described by Nieman (Nieman, Henson et al. 2006). Plasma LPS will be measured from blood taken before exercise (baseline), immediately following, and at 1 and 5 hours after the exercise trial. Plasma LPS has been demonstrated to increase after endurance exercise bouts and is hypothesized to be the result of aberrant bacterial translocation of gut bacteria through compromised gut barrier. Elevated plasma LPS from baseline immediately after exercise has been demonstrated previously after a half-marathon (Ng, Lee et al. 2008), full marathon (Camus, Poortmans et al. 1997) and a long distance triathlon (13). We anticipate plasma LPS levels will increase after exercise stress and will be correlated to core body temperature and the lactulose/rhamnose index of gut barrier integrity.

Inflammatory cytokines.

Plasma levels of IL-6, TNFα, IL-10, IL-17, INFγ, IL-3, MCP-1, IL-15 and GMCSF will be measured in the baseline and in the 1 h post exercise samples. Cytokine concentrations will be determined by multiplex ELISA by a commercial vendor (Quansys Biosystems, Logan, UT). These cytokines were selected based on a previous study investigating gut leakiness and MFGM consumption in an LPS-stimulated gut leakiness trial with mice. All cytokines were significantly upregulated in animals with leaky guts except GMCSF, and were significantly lowered in MFGM-fed animals compared to controls. Moreover, Ng et al., (Ng, Lee et al. 2008) demonstrated increased plasma levels of IL-10 and IL-6 (50% and 65.2% increase from baseline respectively) in runners immediately following a half marathon. Similarly, plasma levels of IL-6 and TNFα have been demonstrated to be increased from baseline immediately after a marathon (Camus, Poortmans et al. 1997). Based on our preliminary data and the aforementioned studies, we anticipate increased levels of inflammatory cytokines after the exercise challenge and hypothesize MFGM will abrogate this effect compared to the other recovery beverages.

Plasma Creatine kinase.

Creatine kinase will be measured in samples taken before, immediately after and 1 hours after the exercise challenge. Creatine kinase will be measured by using
a commercial colorimetric kit (Sigma) in a 96-well UV/vis plate reader (Molecular Devices). Increased plasma creatine kinase is a well-established index of exercise induced muscle damage. In a similar study, cyclists riding at 85% VO2 max until exhaustion after glycogen depletion the previous day had less plasma creatine kinase when they consumed chocolate milk as a recovery drink compared to a carbohydrate replacement beverage (Pritchett, Bishop et al. 2009).

Plasma lactate.

To assess anaerobic metabolism, plasma lactate will be measured at baseline, immediately after and 2 hours subsequent the exercise stress. Lactate will be measured using an enzymatic test kit (r-Biopharm, Marshall, MI).

Data analysis and interpretation.

The primary endpoints measured in this study will be the lactulose/rhamnose ratios in urine and plasma LPS. The data will be analyzed by single factor ANOVA and significant differences will be explored with post-hoc testing. Core body temperature will be measured in real time as it is correlated with gut hypoxia and with gut leakiness. Thus, any effects in gut leakiness will be compared to core temperature data. If our hypothesis concerning MFGM is correct, we envision we will see leaky guts in runners consuming the control but not the MFGM supplemented beverage.

All components measured in the blood (lactate, LPS, cytokines, and CK) will be analyzed by a two factor ANOVA with time and treatment as variables. Lactate is included as it is generally considered to be an index of anaerobic activity. One other parameter we are interested in is creatine kinase. As mentioned in the introduction, this enzyme is detected in the blood after intense exercise and is thought to correlate with damage to muscles. Furthermore, at least two studies with chocolate milk have noted that plasma CK is lower in athletes drinking chocolate milk vs. other recovery drinks. Thus, if we do determine that plasma CK is lower in the runners drinking the chocolate milk, then we will investigate whether or not this is correlated with any of the other measurements.

After the ANOVAs are performed, we plan to explore the relationships of the measured parameters using both principle component analysis (PCA) as well as linear regression. Specifically, we will investigate whether or not gut leakiness tends to correlate with increased inflammation, and if so, with which specific cytokines.

Management plan.

All three PIs will be actively involved in the planning and execution of this study, as well as in the sample processing and the analysis. Dr. Ward will be responsible for formulation of the MFGM-FR beverage. Depending on the data available for the Lacprodan PL-20 (Arla Foods), Dr. Ward may conduct analyses on this ingredient to insure proper beverage formulation. In addition, Dr. Ward will be responsible for the HPLC analysis of the urine. In addition, Dr. Ward will supervise a graduate student supported by this project.

Dr. Bressel will be tasked with recruiting the athletes and with organizing the tests. In addition, Dr. Bressel will supervise all of the performance trials and the collection of the exercise data.

Dr. Hintze will be in charge of the blood and urine samples. The student (and perhaps Dr. Hintze) will conduct the LPS the lactate and the CK assays. In addition, Dr. Hintze will be responsible for proper sample handing of the blood and for the specimen delivery to Quansys Biosystems for the cytokine analysis.

All three PIs will collaborate on the data analysis and on manuscript preparation.

Facilities and equipment.

All testing will take place in the human performance laboratory at Utah State University. The facility includes state of the art biomechanics and exercise physiology testing equipment. The laboratory space is approximately 4,000 square feet and is adjacent to a sports medicine clinic that employs team doctors, physical therapists, and athletic trainers.

Measurements and equipment.

- Volume of oxygen consumed (VO2). Oxygen consumption during the VO2max test and the submax 60 min time trial will be recorded using a computerized on-line metabolic measurement system (Parvomedics True One 2400, Sandy UT). Calculations of VO2 (ml • kg-l • min-l) will be made from expired air samples taken from participants breathing through a two-way valve mouthpiece (Hans Rudolph 700 series, Kansas City MO).
- Body fat percentage. Body weight and percent body fat will be assessed using the Bod Pod air displacement plethysmography system (Life Measurement, Inc., Concord, CA, USA). The system provides a calibrated measurement of body weight, and pilot testing in our laboratory indicated that our system was reliable at estimating percent body fat (ICC = 0.99; F1,10 = 230).
- Power output during running. Athletes will complete all tests on one of the treadmills housed in the lab. The treadmill will provide
real time power and pace to control for exercise intensity.
- Heart rate and core body temperature. Heart rate and core temperature will be monitored in real time using the CorTemp ingestible pill and data recorder. The temperature probes are disposable pills and there is a data logger available in the department.

Several analyses in this study will be conducted in the Department of Nutrition, Dietetics and Food Sciences at USU. Both Dr. Ward and Dr. Hintze maintain laboratories in the department, which together are equipped with the necessary equipment for the following analyses.

- The LPS, creatine kinase and lactate measurements will all be conducted using a microplate reader in the lab of Dr. Hintze.
- There is an HPLC in the NDFS department that is available for use in measuring the urinary sugars for the gut permeability analysis. The instrument is equipped with an evaporating light scattering detector (ELSD) that is helpful for carbohydrate detection.

REFERENCES

Effect of milk fat globule membrane (MFGM) on gut barrier protection in runners / R. Ward


Effects of dietary milk polar lipids on gut permeability and systemic inflammation during the development of diet-induced obesity in C57BL/6J wild type mice

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ABSTRACT

This project explored the effects of dietary milk polar lipids on gut permeability and systemic inflammation during the development of diet-induced obesity. When C57BL/6J mice were fed high fat diet with 35% fat as energy, the animals developed died-induced obesity after 70 days. During the development of died-induced obesity, gut permeability to bigger molecule (FITC-dextran, 4000kD) decreased over time while gut permeability to smaller molecules (sugar probes and endotoxin, 0.3-100kD) increased. There was no significant increase of major inflammatory cytokines (IL-6, TNF-α, MCP-1 and tPAI-1) levels even though plasma endotoxin level increased significantly. These data revealed that a diet with 35% fat as energy and/or diet-induced obesity itself may not pose strong inflammatory stress for C57BL/6J mice. The data indicated that dietary phospholipids may have beneficial effect before the establishment of diet-induced obesity and could have negative effect once obesity is established while dietary gangliosides may have beneficial effect after establishment of obesity.

BACKGROUND

A link between high fat diet and endotoxemia has been suggested. A chronic high fat diet could increase endotoxin absorption during the digestion of dietary lipids. The resultant metabolic endotoxemia leads to low-grade metabolic inflammation [1]. Current evidence suggests that high fat diets may decrease gut barrier integrity and/or increase endotoxin absorption, which may lead to systemic inflammation independent of obesity. Obesity may also decrease intestinal barrier integrity and increase systemic inflammation. The systemic inflammatory state in obesity is associated with endotoxemia resulting from increased gut barrier permeability [2].

In general, current evidence suggests that during the development of high-fat diet-induced obesity (DIO), increased gut barrier permeability, endotoxemia, systemic inflammation and obesity may occur in sequential steps. It is also possible that in the context of high-fat DIO, obesity develops before any other aforementioned conditions. Once obesity is developed, the adipose tissue may release proinflammatory cytokines that may initiate systemic inflammation. The inflammatory state may increase gut barrier permeability which could lead to endotoxemia and worsen the condition of obesity. Therefore, increased gut barrier permeability, endotoxemia, systemic inflammation and DIO are complexly interrelated events. Milk polar lipids have been shown to have beneficial effects on intestinal barrier integrity [3] and reduce inflammatory response [4-7]. The effects of milk polar lipids on intestinal barrier integrity could have an influence on endotoxemia and systemic inflammation. Dietary supplementation of milk polar lipids during the development of DIO may facilitate the understanding of the interrelationships among intestinal barrier integrity, systemic inflammation and obesity.

RESEARCH PLAN

Objective 1.

To study the dynamic changes of gut permeability and
systemic inflammation during the development of diet-induced obesity (DIO) in C57BL/6J wild type mice fed a diet with 35% fat by energy.

**Objective 2.**

To study the effects of dietary milk polar lipids on gut permeability and systemic inflammation during the development of diet-induced obesity (DIO) in C57BL/6J wild type mice fed a diet with 35% fat by energy.

**MATERIALS AND METHODS**

5-week old C57BL/6J mice were fed rodent chow diet for 2 weeks during which the animals were acclimatized and baseline data were collected. Then the mice were randomly assigned to three groups: control (n=6), G600 (n=6) and PC700 (n=6). The mice were fed experimental diets (Table 1) for 14 weeks. Food intake and body weight were measured every other day. Body compositions were determined by EchoMRI-900 every two weeks. Fasting blood samples were collected at baseline, 5 and 14 week 5 h after oral gavage of FITC-dextran. Plasma FITC level was measured by spectrophotometry. Plasma inflammatory cytokines levels were measured by immunoassay. 24 h urine samples were collected at baseline, 2, 4, 6, 10 and 12 week after oral gavage of sugar probes solution containing mannitol, sucrose, lactulose and sucralose. Urinary sugars levels were determined by gas chromatography.

After the mice were sacrificed, mucosa from jejunum, ileum and colon were collected immediately. Expression of tight junction proteins zonula occludens-1 (ZO-1) and occludin in intestinal mucosa were assessed by Western Blot.

**RESULTS AND DISCUSSION**

Dietary treatment did not affect food intake and weight gain (Figure 1). Body fat composition data (by MRI) indicated that the mice developed obesity after 70 days of experimental feeding and dietary polar lipids (esp. phospholipids) supplementation facilitated fat accumulation after establishment of obesity (Figure 2). Dietary phospholipids decreased gut permeability to FITC-dextran before DIO and increased it after DIO was achieved (Figure 3). Differential sugar-absorption test (DST) indicated that gut permeability decreased over time, which could be due to maturation of the gut during development. DST indicated that dietary phospholipids increased permeability of small intestine and decreased permeability of colon. Gangliosides decreased gut permeability after DIO as demonstrated by DST. Western Blot revealed that dietary polar lipids (esp. phospholipids) suppressed occludin expression in jejunum mucosa (Figure 4). In general, dietary phospholipids increased obesity and permeability of small intestine while dietary gangliosides increased adiposity slightly and reduced gut permeability after DIO.

Plasma endotoxin level increased significantly in all groups once DIO was established and plasma endotoxin level was not affected by dietary treatments. There were no significant dietary treatment effect on major plasma inflammatory cytokines and adipokines, including IL-6, TNF-α, t-PAI-1, MCP-1, resistin and leptin. Plasma resistin and leptin increased over time. Dietary phospholipids increased plasma insulin level after DIO while dietary gangliosides did affect plasma insulin level (Figure 5).

**CONCLUSIONS**

During development of DIO in C57BL/6J mice fed high fat diets, gut permeability to FITC-dextran decreased and to LPS increased. High dietary fat increased plasma leptin and resistin levels. Plasma inflammatory cytokines were not significantly affected by high fat feeding or dietary treatments. A diet with 35% fat as energy and/or diet-induced obesity itself may not pose strong inflammatory stress for C57BL/6J mice. Milk gangliosides had little effect on inflammation and some beneficial effect on gut permeability. Milk phospholipids increased gut permeability, decreased intestinal occludin level and increased plasma insulin level. Milk phospholipids did not affect plasma inflammatory cytokines. Milk polar lipids were incorporated into the diets as concentrates. In order to pinpoint which specific compound is responsible for the observed effect, further studies are needed to investigate the effects of purified individual milk polar lipid class.

**REFERENCES**


Effects of dietary milk polar lipids on gut permeability and systemic inflammation / R. Ward

Neither invention disclosure nor patent application was filed.

Table 1. Macronutrient composition of dietary treatments.

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>Diets</th>
<th>Control</th>
<th>G600</th>
<th>PC700</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/kg diet)</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g/kg diet)</td>
<td>548</td>
<td>548</td>
<td>548</td>
<td></td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Lard</td>
<td>95.3</td>
<td>91.93</td>
<td>83.2</td>
<td></td>
</tr>
<tr>
<td>Milk Gangliosides+other</td>
<td>-</td>
<td>0.2+3.17</td>
<td>-</td>
<td></td>
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<tr>
<td>Milk Phospholipids+other</td>
<td>-</td>
<td>-</td>
<td>10+2.1</td>
<td></td>
</tr>
</tbody>
</table>

Dietary treatments did not affect food intake & weight gain

Interaction Plot for DIET*TIME

Figure 1. There were no significant differences in food consumption and weight gain.

Figure 2. PC700 increased body fat percentage.

Effects of dietary milk polar lipids on gut permeability and systemic inflammation / R. Ward

**Figure 3.** PC700 decreased plasma FITC (4000 kD) level and increased it upon DIO.

**Figure 4.** PC700 decreased tight junction protein occludin in jejunum mucosa.

**Figure 5.** PC700 increased plasma insulin level upon DIO.

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Microbial quality and safety of Juustoleipa cheese

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Funded by: Dairy Management Inc., as administered by the Dairy Research Institute, October 2011 - March 2012

ABSTRACT

Juustoleipa cheese is manufactured by direct acidification of milk and stored either refrigerated or frozen. The cheese has a shelf life ranging from 2 weeks to over several months depending on mode of storage. The cheese is cooked (either toasted or mildly sautéed) prior to use in foods, which is a post manufacture step that helps destroy unwanted bacteria. However, even such heat treatments do not exclusively limit bacterial load. To understand microbial quality, spoilage-, and safety-related issues arising from Juustoleipa cheese-making, we hypothesized that survival of unwanted bacteria in Juustoleipa cheese is reduced by frozen storage, thereby altering its shelf life, quality and safety. The objective of the study was to understand how storage conditions and pre-storage baking of Juustoleipa cheese impact its microbial quality. Juustoleipa cheese was made in three replicates. Escherichia coli and Pseudomonas fluorescens were added to separate cheese portions at an initial level of 10^4 CFU/gm cheese along with a control (bacteria not added). Bacterial addition was performed 30 min after salting and cheeses were further pressed for 1.5 h and stored refrigerated or frozen with or without pre-storage cooking in a pizza oven (internal temperature of 82°C). We compared the survival of these bacteria by plate counts on selective media in refrigerated and frozen storage conditions that were sampled at a) 0, 14, and 28 d for refrigerated cheese and b) 0, 30, and 90 d for frozen cheese. Bacterial survival was reduced (p<0.05) by 1,000-10,000-fold when samples were stored frozen, justifying our hypothesis. Pre-storage cooking of cheese reduced E. coli and Pseudomonas below detection by plate counts, but aerobic thermotolerant bacteria that were incident during manufacture survived cooking at 82°C. As expected, microbial counts in cheeses that were not cooked were higher (p<0.05) by 10-1000-fold than in cooked samples. Added Pseudomonas numbers were reduced significantly (p<0.05) by 100-fold due to cooking, but storage at refrigeration or frozen conditions did not limit Pseudomonas growth. Characterization of the thermotolerant bacteria is underway to comprehend their survival mechanisms. We concluded that pre-storage cooking and frozen storage reduce survival of but do not fully eliminate thermotolerant bacteria and do not limit Pseudomonas survival in Juustoleipa cheese, which might impact its shelf life and microbial quality.

BACKGROUND

Juustoleipa cheese is typically manufactured by direct acidification of milk and stored either refrigerated or frozen. The cheese has a shelf life ranging from 2 weeks to over several months depending on mode of storage. The cheese is cooked (either toasted or mildly sautéed) prior to use in foods, which is a post manufacture step that helps destroy unwanted bacteria. However, even such heat treatments do not exclusively limit bacterial load. We seek to understand microbial quality, spoilage-, and safety-related issues arising from the Juustoleipa cheese-making process.

We hypothesized that survival of unwanted bacteria in Juustoleipa cheese is reduced by frozen storage, thereby improving its shelf life, quality and safety.

RESEARCH PLAN

Overall Project Experimental Design

Juustoleipa cheese was made and stored refrigerated and frozen with and without pre-storage cooking in a pizza oven (internal temperature of 180°F). To the cheese curd 2 different bacteria were added separately along with a control (no added bacteria). This gave us 3 cheeses with 3 reps and 3 bacterial conditions (no addition, E. coli,
and Pseudomonas) to be sampled at a) 0, 14, and 28 d for refrigerated samples and b) 0, 30, and 90 d for frozen samples, to examine bacterial survival.

**Objective 1.**

Evaluate the microbial quality of Juustoleipa cheese manufactured at the DTIL with and without cooking and stored refrigerated and frozen.

**Objective 2.**

Understand incident bacterial survival by adding *E. coli* and *Pseudomonas* in Juustoleipa cheese and enumerating their numbers during storage.

**MATERIALS AND METHODS**

**Cheese Manufacture**

Juustoleipa cheese was manufactured using current processes developed at the DTIL. Briefly, cow’s milk (pH 6.6-6.7) was obtained from Utah State University’s Caine Dairy Research and Teaching Center (Wellsville, UT), and HTST-pasteurized at 164 °F for 16 Seconds. Milk in cheese vats was heated to 37°C, and set using Maxiren double-strength rennet. After 30 min, the curd was cut with 1.9-cm knives, allowed to heal for 5 min, and stirred constantly for 20 min. The whey was drained and the curd salted using 12-15 grams of salt per gallon of milk (2-2.5% of curd weight). After salting the curd was allowed to sit for 30 minutes to allow salt absorption.

After salting, the curd was divided into nine seven-pound portions, placed in tubs, and randomly assigned culture treatment as follows.

<table>
<thead>
<tr>
<th>Portion</th>
<th>Culture</th>
<th>Rep</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td><em>E. coli</em></td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
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</tr>
<tr>
<td>5</td>
<td><em>Pseudomonas</em></td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td><em>E. coli</em></td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td><em>Pseudomonas</em></td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td><em>Pseudomonas</em></td>
<td>3</td>
</tr>
</tbody>
</table>

The previously prepared cultures were diluted in 100 mL of 4% brine to give approximately 1,000 CFU/g curd. The diluted culture was added to the curd and stirred for 5 minutes. The curd was then be placed in round hoops and pressed for 90 minutes. The pressed cheese was cut into slices ~0.75 inches thick, quartered and each quarter vacuum packaged separately. One half of the packages were stored at ~45°F and one half at ~20°F.

At 1, 14 and 28 days of age, three refrigerated samples were selected for microbial enumeration (see procedure below), one of which was baked in an Impinger oven to an internal temperature of 180°F before enumeration. Two frozen samples were selected for microbial enumeration at 1, 30 and 90 days of storage. Both samples were thawed and one sample then baked in an Impinger oven to an internal temperature of 180°F before enumeration.

**Proximate Analysis**

Proximate analysis was conducted on all cheeses at 5 d of storage. Moisture content was determined by weight loss using a microwave oven (CEM Corp., Indian trail, NC) at 70% power with an endpoint setting of <0.4 mg weight change over 2 s. Fat content was determined using a modified Babcock method (Richardson, 1985). Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher 400 (Seward, England). The slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (model 926, Corning, Medfield, MA). Cheese pH was measured using a glass electrode after stomaching 20 g grated cheese with 10 g distilled water for 1 min at 260 rpm.

**Microbe Addition to Juustoleipa Cheese**

Juustoleipa cheese made at the DTIL was separated into three portions, a) no microbe added, b) added *E. coli* at an initial level of 10^3-10^4 CFU/ml, and c) added *Pseudomonas* at an initial level of 10^3-10^4 CFU/ml. Bacteria to be added was grown overnight at least twice at 30°C for 48 h in respective optimal liquid broth media and moved to refrigeration at least 1 h prior to inoculation onto cheese.

**Aerobic Bacteria, *E. Coli*, and *Pseudomonas* Enumeration**

Juustoleipa cheese was grated prior to stomaching in a blender (200 rpm for 2 min; Seward, England) along with 0.85% sterile unbuffered saline. The stomached suspension was further diluted and cheese without added bacteria was plated on aerobic count plate petrifilm (3M, St. Paul, MN), cheese with added *E. coli* plated on E. coli/coliform count plate petrifilm (3M, St. Paul, MN) and cheese with added *Pseudomonas* was plated on cetrimide agar (BD, Franklin Lakes, NJ). Inoculated petrifilms and agar plates were incubated at 30°C for at least 48 h prior to bacterial enumeration.
RESULTS AND DISCUSSION

Objective 1. Microbial Quality of Juustoleipa Cheese.

Figure 1. Aerobic plate counts of Juustoleipa cheeses during storage in refrigeration.

Figure 2. Aerobic plate counts of Juustoleipa cheeses during frozen storage.

Objective 2. Survival of *E. Coli* and *Pseudomonas* in Juustoleipa cheese.

Figure 3. *E. coli* plate counts of Juustoleipa cheeses during storage in refrigeration.
Figure 4. *E. coli* plate counts of Juustoleipa cheeses during frozen storage.

Figure 5. *Pseudomonas* plate counts of Juustoleipa cheeses during storage in refrigeration.

Figure 6. *Pseudomonas* plate counts of Juustoleipa cheeses during frozen storage.
CONCLUSIONS

Juu stoleipa cheese was successfully made without and with pre-storage cooking at 82°C for microbial evaluation. *E. coli* and *Pseudomonas* were added at a targeted initial level of $10^4$ CFU/gm cheese. Microbial analyses showed that even in cheese without added bacteria, aerobic bacteria were reduced by 100-fold but not fully eliminated under refrigerated storage. Also, bacteria grew by 10- to 100-fold over storage in the raw and cooked cheeses, suggesting that incident aerobic bacteria were not limited by cooking. However, frozen storage conditions did cause a reduction in aerobic bacteria by 10-fold in the raw cheese by 28 d, but no change was observed in the cooked cheese during the same period, potentially due to the low initial microbial levels.

*E. coli* added to Juustoleipa cheese did not increase in numbers over 4-wk refrigerated storage and was not detected in the cooked cheeses, suggesting that at 82°C, incident coliforms are likely to be destroyed. Frozen storage also reduced *E. coli* levels by 100-fold over 28 days as opposed to no reduction in refrigeration, highlighting the importance of storage conditions in effective reduction of coliform-type bacteria.

Added *Pseudomonas* ranged initially at $10^5$ CFU/gm, but population levels remained closer to initial levels during refrigerated storage of raw cheese up to 28 days. Cooking appeared to limit initial numbers of *Pseudomonas*, but their numbers increased 1,000-fold over 28 days to reach initial levels, suggesting that cooking is insufficient to remove it post incidence. Frozen storage also had minimal impact on *Pseudomonas*, wherein no reduction was observed after 28 d storage of raw cheese and numbers increased by 100-fold in cooked cheese. Potentially, *Pseudomonas* was attenuated but not destroyed by the cooking process, which may have led to the observed initial low numbers. *Pseudomonas* is an important cold storage spoilage organism in other hard and semi-hard cheeses and preventing its incidence may be the optimal solution to avoid *Pseudomonas*-related lipolytic or proteolytic spoilage.

In summary, Juustoleipa cheese appears to support aerobic and coliform-type bacterial growth under refrigerated storage but not under frozen storage, which may help reduce its deterioration. However, incidence of *Pseudomonas* and other related bacteria must be reduced during cheese processing and handling in order to improve its shelf life.

NEXT STEPS

A manuscript is being prepared for publication in a peer-reviewed journal.

REFERENCES

Manuscripts and Abstracts

Assessing the sliceability of cheese

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Funded by: Dairy Management Inc., as administered by the Dairy Research Institute

BACKGROUND

Sliced cheese is a valuable and desirable product. Pre-sliced packages have become increasingly popular in grocery stores, and deli counters require cheese that is easy to slice and handle. Sliced cheese should appear appetizing and eye-appealing for both the delis and cheese sections of grocery stores, because it is the first attribute consumers measure quality and identity (Brown et al., 2003; Hort et al., 2001; Jack et al., 1993). Cheese that is easy to slice doesn’t crumble, tear, or turn to mush as the slicing blade moves through it (Chen, 2000). Currently, slice evaluation methods are only visual. Emphasis on sliced cheese is placed on the integrity of the slices, so it is essential to ensure that the slices retain desirable characteristics during handling, distribution and storage (Ni et al., 2004; Childs et al., 2007).

Sliceability is a term that embodies slicing qualities, and is defined as a cheese’s ability to cut cleanly into thin slices, resist breakage or fracture at slices edges, and undergo a high level of bending before breaking (Guinee et al., 2004). Shreddability is a comparable property to sliceability because they both involve the rheological and adhesive properties of the cheese. Childs et al. (2007) found that both composition and age influences cheese rheology and shreddability.

Shreddability is defined as a cheese’s ability to cut cleanly into long thin uniform strips, have low susceptibility to form curd fines, and resist sticking, matting or clumping when loosely packed (Guinee et al., 2004). Shreddability is influenced by cheese age and moisture. Masi et al. (1986) and Childs et al. (2007) correlated increases in moisture contents of Mozzarella cheese with decreases in elasticity, which indicates poor shredding properties. In addition to moisture, Kinstedt (1995) and Childs et al. (2007) both found that a cheese’s age at the time of shredding regulates quality, in that young Mozzarella cheese does not shred properly compared to a three month old Monterey Jack. If a cheese is too brittle, i.e. too old or low in moisture, it will end up with fractured shreds that are broken and inconsistent. On the other hand, if a cheese is too sticky or too soft, i.e. too young or high in moisture, the shreds will be inconsistent in shape, bent or curled, and vary in length (Chen, 2007).

Rheological properties, such as firmness, and tack energy, also affect shreddability. For example, it is difficult to cleanly shred a hard cheese, such as a Parmesan because it has have a relatively low fracture strain and it is also challenging to evenly cut an over acid Cheddar cheese because it fractures in a jagged fashion and breaks at edges (Guinee, 2002). Childs et al. (2007) evaluated the adhesive properties of cheese by measuring tack energy; the energy required to separate two materials that are not bound permanently. From the results, Childs et al. (2007) concluded that an increase in tack energy was associated with an increase in cheese adherence to the blade. The tack energy for Monterey Jack and process cheese was greater than the tack energy for Mozzarella cheese, meaning that Monterey Jack and process cheese have greater adhesion to the blade than the high moisture Mozzarella. In this study, the rheological properties were the best indicators of shredding defects, in that adherence to the blade was associated cheese viscosity and the production of fines was associated with increases in firmness. It is assumed that the rheological and adhesive properties that affect shreddability will also affect sliceability.

Cheese shreddability is influenced by age, moisture and rheology, and so it can be predicted that these properties also have an effect on sliceability. Since trends were found with moisture and age, it is important to focus on those factors with regards to sliceability.

Effect of Moisture Content on Sliceability

Sliceability incorporates cheese textural and rheological properties, which are influenced by moisture content. If the moisture content of a cheese is increased, then the ratio of moisture to protein is increased, and the protein matrix
weakens because the protein volume fraction concentration decreases (Lucey et al., 2003; Guinee et al., 2004). A high ratio of moisture to casein will give a softer cheese texture, which may lead to greater adhesion. Carunchia-Westine et al. (2007) noted in her research that increased moisture content contributed to softer texture and lower moisture content contributed to more brittle cheeses. Watkinson et al. (2002) found that an increase from 40 to 48% moisture of model Cheddar-like cheeses resulted in a large decrease in elasticity and large increase in adhesiveness. It can therefore be seen that increases in moisture content causes greater blade adhesion, and potentially poor sliceability.

Variations in the ratio of moisture to protein also attribute to differences in rheological properties and ultimately sliceability. Creamer and Olson (1982) reported a linear decrease in the strength of the protein matrix as the moisture of Cheddar increased from 34.0 to 39.7%. Tunick et al. (1993) and Masi et al. (1986) also found that a variation in the ratio of water to protein led to differences in textural quality, and the elastic and storage moduli decreased with increasing moisture. These findings are supported by research performed by both Luyten et al. (1991ab) and Venugopal et al. (2003). Luyten et al. (1991ab), increasing the moisture content of 7.5 month old Gouda cheese from 32% to 46% resulted in decreases in elastic modulus and fracture stress, and Venugopal et al. (2003) found that the elastic modulus of high moisture model Cheddar-like cheese (62.4%) was significantly less than the lower moisture content (58.1%), implying that higher moisture content made the cheese softer and less elastic compared to the normal moisture.

Effect of Age on Sliceability

Cheese texture changes markedly during maturation due to chemical changes within the casein matrix, and thus, sliceability may change over time as well. Lawrence et al. (1987) identified two distinct phases in the development of cheese texture. The first phase occurs within the first 14 days, and the second during the remainder of the ripening period. Understanding if there are any differences between these two phases with regard to sliceability will assist in obtaining high quality slices.

In two studies, Hort et al. (1996, 2001) reported that the textural properties of Cheddar cheese changed as maturation progressed. Hort et al. found that young Cheddar within the first texture phase was very springy, then the springiness of the cheese changed as it moved into the second phase. Once the cheese entered the second texture development phase, there was a large decrease in springiness and increase in crumbliness and creaminess. Everard et al. (2006), as well, found that cheeses decreased in instrumental springiness with maturation. Springiness relates to sliceability, and as the cheese moves through the slicing machine, if it recovers quickly, cohesive slices will be produced (O’Callaghan and Guinee, 2004). Chevanen et al. (2006) found an increase in adhesiveness during ripening, and the cheese became stickier with age. A cheese that is springy and low in adhesiveness will have good sliceability.

Slicing has been long established in the industry; however, there is no specific scientific research about how compositional, textural and rheological properties influence slice quality. Intuitively, sliceability depends on the microstructural and rheological properties of the casein network. A cheese that is easy to slice doesn’t crumble, tear, or turn to mush as the slicing blade moves through it. It is known that moisture control and maturation are important factors that influence functionality of cheese, and so it is accepted that they affect sliceability as well. Research must be performed in order to make further logical and scientific conclusions.

MATERIALS AND METHODS

Slice Quality Evaluation

A 12 kg block of Cheddar was purchased the day after manufacture from Gary H. Richardson Dairy Products Laboratory (Utah State University, Logan) for preliminary slice quality evaluation. The block of cheese was separated into six sections, and each section was evaluated for slice quality every 7 d over 5 wk of storage. Each section was sliced multiple times at four different thicknesses (1.1, 0.9, 0.6, and 0.3 mm) in order to determine the grading scale. The 1.1-mm slice represented a typical thickness found in most commercial cheeses. The three other thicknesses were chosen in order to determine quality differentiation between slices.

Cheeses

Commercial cheese.

Cheddar cheese (3 mo) was purchased as one 0.9 kg block and in 3 different typical thicknesses (1.20 mm, 0.90 mm, and 0.60 mm) that are normally used for sliced cheese, from Gossner’s Food, Inc. (Logan, UT) on 3 separate occasions, and are referred to as replicates. Cheese composition, cheese grading, tack, flexibility and rheology were determined in as described below within a 2 day time period of cheese purchase, and were replicated in triplicate over the 3 different lots of cheeses.

Experimental cheddar cheeses.

Three different groups of moisture contents (33-34%, 36-38%, and 40-41%) were obtained through 2 different cook temperatures (35°C, 39°F), and 2 different mill pH values (5.60, 5.40).
Raw milk was obtained from the Gary H. Richardson Dairy Products Laboratory (Utah State University, Logan) to produce cheddar cheese. Milk (227 kg) for the low moisture cheeses was prepared in an enclosed vat using the same method as Rogers et al. (2009). Milk (454g) for the high and medium moisture cheeses was prepared in open vats using the same method as Oberg et al. (2011). Milk was pasteurized at 74°C for 16 s, then warmed to 31°C and inoculated with 96 g of S. lactis (DVS 850, Danisco Cultures Plant, Madison, WI) starter culture for 45 min. After the ripening period, 60 mL double-strength chymosin (Maxiren, DSM Food Specialties USA, Logan, UT) at a 1:20 dilution was added. After the coagulation time of 30 to 45 min, the high and medium moisture content gels were cut with 1/2" knives, and the low moisture content gels were cut with 1/4" knives, and allowed to heal for 10 min. The low moisture curd was gradually heated to 39°C for 40 minutes and held at that temperature for another 30 min, the middle moisture curd 37°C, and the high moisture curd 35°C in open vats. For all cheeses, the whey was drained when the pH of the curd reached 6.30. Only the lower moisture content cheese was dry stirred after whey drainage for 5 min. The curd was allowed to mat together to form a pack for 10 min, then the pack was cut into slabs (15.24 cm in width) and flipped every 5 min for 40 min in order to keep the curd warm. Acid development was monitored throughout the curding period and at pH 5.85, the slabs were cut in half and stacked 2 high. Low moisture curd slabs were cut milled using a kitchen knife at pH 5.40 and the middle and high moisture curd slabs were cut at pH 5.60. Curd was salted (632 g) in three applications, with 5 min for each application, and put into a cheese hoops (12 kg each), and then placed in a horizontal cheese press (413.7 kPa) for 12 to 15 h. The cheese was then removed from the hoops. Each cheese block was cut into 6 separate cubes and the cubes were individually vacuumed sealed. The 6 corresponding cubes were then placed into one box for ripening, and were allowed to ripen at 6°C.

Out of a total of 24 cheeses made, 9 were selected for analyzing based on similar moisture contents. Cheeses within the moisture range of 33-34% will be referred to as LM, 36-38% as MM, and 40-41% as HM. Cheese composition was determined after one wk of aging as described below. Cheese grading, tack, and flexibility were determined as described below every 14 d, for 84 d. Rheological analysis was performed twice; at ages of one month and 3 month.

Cheese Composition

Moisture, salt, fat, mineral and pH were analyzed for composition on d 14 for experimental cheeses and day or purchase for commercial cheeses. Cheese pH was measured using the gold electrode/quinhydrone method (Marshall, 1992). Moisture was analyzed using a microwave oven (CEM Corp., Indian Trail, NC), and moisture was determined as weight loss (AOAC, 1990). Fat content was determined using a modified Babcock method (Marshall, 1992; method 15.8.A). Total NaCl content was measured using a chloride analyzer (Model 926; Corning Scientific, Medfield, MA) (Paulson et al., 1998). Mineral content was determined through dry ashing, in which the ash was sent to Analab (Fulton, IL) for further mineral analysis.

Cheese Grading

Each cheese was cut into a block (11 cm x 11 cm x 11 cm), and then the block was cut in half into a triangle. The triangular block was then sliced at three different on a Berkel Manual meat and cheese deli slicer (Berkel, Inc., Troy, OH) equipped with a 31 cm stainless-steel slicing blade at 3 levels (10, 8, and 6) for 3 different thicknesses (1.20 mm, 0.90 mm, and 0.60 mm). The cheese was sliced at each thickness 5 times, and cheese slices were visually analyzed using the grading scale developed during slice quality evaluation experiment.

Texture Analysis

Cheese adhesiveness was measured using a TAX-T2 texture analyzer (Texture Technologies, Scarsdale, NY) with a flat, 25.4 mm diameter stainless-steel probe (TA-11s). The cheeses were cut into 4-cm squares and sliced to a thickness of 6.35 mm with a modified wired cheese slicer. The cheese was placed on a platform below the probe arm, and the probe was brought to the surface of the cheese at a speed of 1 mm/s. Upon reaching the cheese surface, a force of 2.0 N was applied, held for 5 s, and removed at a speed of 0.1 mm/s. Tack force was determined as the maximum force recorded during separation. Tack energy was determined as the area under the force distance curve.

Cheese flexibility was measured using a TAX-T2 texture analyzer (Texture Technologies, Scarsdale, NY) with a three-point adjustable breaker fixture and an aluminum plate platform (TA-92). The three different slice thicknesses for both commercial and experimental cheeses were prepared in triplicate from the original cheese samples. A single cheese slice was placed horizontally on the adjustable breaker fixture (5.08 cm in width) and a compression test was performed, bending the cheese slice a distance of 25 mm at a speed of 2 mm/s. Flexibility was determined as the maximum bent force recorded before the slice broke in half (Guinee, 2004).

Rheology

Dynamic oscillation tests will be performed on cheese samples to evaluate the linear viscoelastic region. The elastic modulus (G'), viscous modulus (G''), and complex modulus (G*) was determined with an AR-G2
Experimental Design

A randomized block design with fixed measures was used for the commercial cheeses and a randomized complete split-plot design with fixed measures was used for the experimental cheeses, with an alpha level of 0.05. For the experimental cheese, the different sub-plot treatments of the whole-plot unit were the days of testing (14, 28, 42, 56, 70, and 84 d).

The general linearized model (PROC GLM) in the SAS statistical software package (Version 9.3, SAS Institute, Inc., Cary, NC) allowed statistically significant differences between whole-plot and sub-plot treatments in the experimental cheeses to be distinguished. A correlation analysis (PROC CORR) using the SAS statistical software package allowed for determination of relationships among cheese properties.

RESULTS AND DISCUSSION

Slice Quality Testing

A slice quality grading scale was developed based on visual observation of defects in cheese slices as shown in Table 1. This takes into account that slices can be defective if they have splits and cracks or if the corners are broken and not intact. Such breaking of corners is especially problematic if slices are in a triangle shape as 2 of the corners form an acute angle of only 45°. Triangular cheese slices are commonly sold in the food service market for use in sandwich quick service restaurants, while square or round slices are more commonly sold in the retail market. This grading scale makes it possible to compare slice quality between different cheeses even though they may have different defects. Examples of cheese slices with the various defects and grading scores are shown in Figs 1 to 5.

The more cracks and breaks a slice had, the higher the score and the worse its slicing quality. A grading score of one represents a highly sliceable cheese while a grading score of 5 indicates the cheese has very poor slicing attributes.

Composition of cheese used for the initial experiment was 36.4% moisture, 1.31% salt and 34% fat, with a pH of 5.09. Mean slicing grade for this cheese when sliced at 7, 14, 21, 28 and 35 d after manufacture is shown in Table 2. The 1.1-mm cheese slices represented a typical commercial cheese slice thickness, and thinner slices were used to investigate their potential for differentiating slice quality between cheeses by exaggerating the slices defects. At 7 d of age, the cheeses had poor slice quality with numerous defects (no cheeses were free of defects, i.e., all scores >1) with mean quality scores of 2.0 to 4.4 depending on slice thickness.

Slicing the cheese at 14 d compared to 7 d caused an improvement in slice quality in the 1.1- and 0.9-slices with grading scores of 1.6 and 1.4, respectively, being obtained. With the 0.6-mm slices it required 21 d of aging to obtain any slices that were free of defects, (mean score = 1.2). Using a very thin slice (0.3 mm) provided no benefit to differentiating between cheese slicing quality as no cheese slices could be produced that were free of defects with most cheeses receiving scores of 3 or higher. Such slices were never a complete piece and were broken in half or thirds. A thickness of 1.1 mm provided many examples of good slice quality, in that the cheese remained in a whole shape and did not crumble, break, or split during slicing. Aging the cheese for 35 d compared to 14 d before slicing did not produce any further increase in quality when it was sliced at 0.9 or 1.1 mm of thickness. However, the best slice quality for the 0.6-mm slices was not reached until the cheese was 35 d old when none of the slices contained any defects.

Commercial Cheeses

For the commercial cheeses the mean fat content was 35%, salt content 1.68%, moisture content 37.5%, and the cheeses had a mean pH 5.10. Mineral analysis was performed and the mean calcium and phosphate contents were determined to be 0.87% and 1.42%, respectively.

Experimental Cheese

The mean values for proximate compositions of the experimental cheese groups are found in Table 3. The high moisture group had a mean moisture content value of 40.6%, the medium 37.03% and the low 33.95%. The higher moisture group had the lowest pH at 5.05, while the low moisture group had the highest pH at 5.28. Fat varied from 30.67 to 34%, salt varied from 1.27 to 1.83%, calcium from 17.73 to 19.88%, and phosphate from 37.01 to 42.74% for all cheeses.

Slicing Analysis

The commercial cheese mean slice defect scores for 1.25, 1.15, and 1.05-mm thicknesses were 1.33, 1.46, and 1.88, respectively. All three commercial cheeses expressed relatively good slice quality. The commercial cheeses provided a reference for the development of the slice quality grades in that the three thicknesses covered the
Table 1. Grading scale for qualitative analysis of slice slices.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slice does not have any cracks or breaks within interior of slice.</td>
</tr>
<tr>
<td></td>
<td>Slice contains all corners on the edges of slice, none are broken off.</td>
</tr>
<tr>
<td>2</td>
<td>Slice contains one crack within interior and has all corners on edge.</td>
</tr>
<tr>
<td></td>
<td>Slice contains two cracks within interior and has all corners on edge.</td>
</tr>
<tr>
<td>3</td>
<td>Slice contains no cracks within interior and has one corner broken off.</td>
</tr>
<tr>
<td></td>
<td>Slice contains three cracks within interior and has all corners on edge.</td>
</tr>
<tr>
<td>4</td>
<td>Slice contains one crack within interior and has one corner broken off.</td>
</tr>
<tr>
<td></td>
<td>Slice contains three cracks within interior and has two corners broken off.</td>
</tr>
<tr>
<td>5</td>
<td>Slice contains more than three cracks within interior and has two corners broken off.</td>
</tr>
</tbody>
</table>

Figure 1. Examples of cheeses with a slice defect score = 1.
Figure 2. Examples of cheeses with a slice defect score = 2.
Figure 3. Examples of cheeses with a slice defect score = 3.
Figure 4. Examples of cheeses with a slice defect score = 4.
Figure 5. Examples of cheeses with a slice defect score = 5.
Table 2: Mean (±SD) slice defect scores for Cheddar cheese cut at a thickness of 1.1, 0.9, 0.6 and 0.3 mm at 7, 14, 21, 28 and 35 d after manufacture (n=5).

<table>
<thead>
<tr>
<th>Slice thickness</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
<th>28 d</th>
<th>35 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mm</td>
<td>4.4 (0.55)</td>
<td>3.4 (0.55)</td>
<td>3.6 (0.89)</td>
<td>2.6 (0.55)</td>
<td>3.2 (1.0)</td>
</tr>
<tr>
<td>0.6 mm</td>
<td>2.0 (0)</td>
<td>2.2 (0.45)</td>
<td>1.2 (0.45)</td>
<td>1.8 (0.45)</td>
<td>1.0 (0)</td>
</tr>
<tr>
<td>0.9 mm</td>
<td>3.0 (0)</td>
<td>1.4 (0.55)</td>
<td>2.2 (0.45)</td>
<td>1.2 (0.45)</td>
<td>1.4 (0.55)</td>
</tr>
<tr>
<td>1.1 mm</td>
<td>2.6 (0.55)</td>
<td>1.6 (0.55)</td>
<td>1.6 (0.55)</td>
<td>1.4 (0.55)</td>
<td>1.8 (0.45)</td>
</tr>
</tbody>
</table>

Table 3: Mean values of moisture, pH, salt, fat for experimental cheese groups.

<table>
<thead>
<tr>
<th>Cheese Group</th>
<th>pH</th>
<th>Moisture</th>
<th>Salt</th>
<th>Fat</th>
<th>Ca</th>
<th>PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM</td>
<td>5.05α</td>
<td>40.6α</td>
<td>1.27α</td>
<td>31.0α</td>
<td>0.67α</td>
<td>1.40α</td>
</tr>
<tr>
<td>MM</td>
<td>5.17β</td>
<td>37.0β</td>
<td>1.44β</td>
<td>30.0β</td>
<td>0.71β</td>
<td>1.49β</td>
</tr>
<tr>
<td>LM</td>
<td>5.28γ</td>
<td>33.9γ</td>
<td>1.83γ</td>
<td>34.0γ</td>
<td>0.64γ</td>
<td>1.38γ</td>
</tr>
</tbody>
</table>

α,β,γ Means with the same letter superscripts within the same column were not significantly different.

The defect scores for slice thicknesses 1.1-mm, 0.9-mm, and 0.6-mm are shown in Figure 6. The slice scores for 1.1-mm thickness of the experimental cheeses expressed the most difference between moisture groups than the other slice thicknesses. The high moisture cheeses had significantly higher defect scores than the medium and low moisture cheeses, and all three moisture groups' scores improved over time. At slice both 0.9-mm and 0.6-mm thicknesses, the high moisture slice defect scores were significantly higher than the medium and low moisture slice scores, especially at ages 14 d and 84 d.

At all three thicknesses, the HM slice defect score results were higher than the MM and LM groups, with the lowest scores coming from the LM group. Overall, it was visually observed that the higher moisture cheeses adhered and caked onto the slicing blade more than the lower moisture cheeses, which created more fines and uneven slices. When the cheese sticks to the blade, slices do not result in whole pieces and many cracks in the slice result. Childs et al. (2007) also found that high moisture Mozzarella adhered to the blade, as well as produced more fines during shredding. The HM group also had the lowest pH value, and low pH values lead to a more crumbly cheese. The combination stickiness, due to high moisture content, and crumbliness, due to a low pH, creates an overall bad combination for cheese sliceability.

As a result of blade adherence being a dominate observation, as opposed to crumbliness, it can be assumed that the moisture content was more involved with slice quality than pH, though pH did correlate with slice scores. At 28 d moisture content and pH were significantly correlated with slice defect scores at 1.1-mm (R=0.60721, R=-0.46252) and at 0.6-mm (R=0.72289, R=-0.55102). Similar correlations were found at 84 d with slice defect scores at 1.1-mm (R=0.46609, R=-0.47202), at 0.9-mm range at which commercial cheese slices are sold.
Assessing the sliceability of cheese / D. J. McMahon

(R=0.782, R=-0.61650), and at 0.6-mm (R=0.69326, R=-
0.71284). Higher moisture contents and lower pH values
led to higher defect scores at all three thicknesses. As the
cheese aged, no matter what thickness cheese slice quality
improved.

Rheology

Commercial cheese rheological properties are
presented in Table 4. Within the linear viscoelastic region
of the commercial cheeses, the elastic modulus ranged
from 5.03 to 4.49 log Pa, the viscous modulus from 4.49
to 4.34 log Pa, and the complex modulus from 5.05 to 4.66
log Pa, with increasing oscillatory stress frequency.

Table 4: Mean values for \( G' \), \( G'' \), and \( G^* \) of commercial
cheeses.

<table>
<thead>
<tr>
<th>Oscillatory Stress (Pa)</th>
<th>( G' ) (log Pa)</th>
<th>( G'' ) (log Pa)</th>
<th>( G^* ) (log Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>5.03</td>
<td>4.49</td>
<td>5.05</td>
</tr>
<tr>
<td>150</td>
<td>5.02</td>
<td>4.48</td>
<td>5.04</td>
</tr>
<tr>
<td>1000</td>
<td>4.93</td>
<td>4.45</td>
<td>4.95</td>
</tr>
<tr>
<td>4000</td>
<td>4.49</td>
<td>4.34</td>
<td>4.66</td>
</tr>
</tbody>
</table>

Pairwise comparison of the three moisture groups
resulted in a significant difference between the high and the
low moisture groups for \( G' \) and \( G^* \), and between all three
moisture groups for the \( G'' \) at all stresses. For all moduli,
however, pairwise comparison expressed no significant
differences between cheese age, and differences were
determined from the interaction between moisture and
storage time for \( G' \), \( G'' \), and \( G^* \).

The elastic modulus for the HM group at all stresses
was significantly lower than the LM groups at age 84 d
(Figure 7). The elastic modulus at 28 d for the oscillatory
stresses 1000 and 4000 Pa also expressed significant
decreases with increasing in cheese moisture. The viscous
modulus of the high moisture group at all oscillatory
stresses was significantly lower than both medium and low
moisture groups at 28 d and 84 d. The complex modulus
of the high moisture group at an oscillatory stress of 20
Pa was significantly lower than the low moisture groups at
both ages.

Cheese is considered viscoelastic because during and
after deformation part of the mechanical energy supplied
to the cheese is stored in the material (elastic part) and part
is dissipated (viscous part) (Lucey et al., 2003). Masi and
Addeo (1986) found that increases in moisture contents of
Mozzarella cheese were accompanied by a decrease in the
modulus of elasticity, which caused difficulty in shredding.
Venugopal et al. (2003) and Tunick et al. (1992) also found
that the \( G' \) of high moisture cheese was significantly lesser
than normal moisture cheese because elevated moisture

Figure 7: Pooled mean values for \( G' \) (A), \( G'' \) (B), and \( G^* \) (C) at 28 d and \( G' \) (D), \( G'' \)
(E), and \( G^* \) (F) of high moisture (●), medium moisture (●) and low moisture (▲)
experimental cheeses during 84 d storage at 6°C. Error = SE
content makes cheese softer due to greater hydration of
the casein network. Results from the current study support
the mentioned findings, and there was a definite distinction
between high moisture and low moisture cheeses.

The HM cheeses had low elastic modulus value, which
may be a possible indicator of poor slicing, and higher
elastic modulus values seen in the LM cheeses an indicator
of good slicing. It was found that the viscous modulus at 20
Pa (R = -0.324), 150 (R= -0.32302), 1000 Pa (R= -0.30836)
and 4000 Pa (R= -0.26646) had significant correlations
with the slice score at 1.1-mm thickness. Lower values for
the viscous modulus, or fluid behavior, of a cheese were
correlated with the improvement of slice quality.

**Tack Force**

Mean results for tack force for the commercial cheeses
are shown in Table 5, and the mean tack force was 0.37 N.
The tack force results for all the experimental cheeses are
shown in Figure 8. The HM group showed an increasing
linear trend in tack force (R² = 0.7117, y=0.005x + 0.21) as
storage time increased. The MM and LM did not express
any increasing or decreasing trends, and both seemed to
remain constant as storage time increased.

Pairwise comparison expressed a significant difference
between high, medium and low moisture groups, and
between the older 70/84 d cheeses and the younger 14/28
d cheeses. Investigating the interaction between moisture
and age, all three moisture groups were not significantly
different at 14 d, but there was a difference between high
and low moisture groups at age 84 d. Within each moisture
group individually, the young cheeses at 14 d of the HM
group were significantly different than their older 70/84 d
counterparts. The low moisture group did not express any
differences for all ages.

There were correlations between moisture content, pH
and tack force. At 14 d, pH had a significant correlation
with tack force (R= -0.3358), and at 28 d the moisture
content and pH were significantly correlated with tack force
(R= -0.65453), as well as at 84 d (R= -0.38837). As moisture content increased, tack force
increased due to greater adhesion to the tack probe, and
as pH increased, tack force decreased, due to the cheese
becoming more crumbly.

**Tack Energy**

Mean results for tack energy for the commercial cheeses
are shown in Table 7, and the mean tack energy was 75.1 mJ/m². The tack energies for all the experimental
cheeses are shown in Figure 9. The HM group showed an
increasing linear trend in tack energy (R² = 0.7246, y=0.93x + 50.3) as storage time increased. The MM and LM did
not express any increasing or decreasing trends, and both
seemed to remain constant as storage time increased.

There was a significant difference between high,
medium and low moisture groups for tack energy, and between all of the older (84/70d) and younger cheeses (14d). There was no significant difference between moisture groups at 14 d, but at age 84 d, the medium moisture group was significantly larger than the low moisture group. Within the high moisture group, tack energy significantly increased with age, however, the medium and low moisture groups did not express any differences between ages.

There were significant correlations between tack energy and slice quality score. It was found that tack energy (R = 0.1798) was significantly correlated with the slice score at 1.1-mm thickness, and higher tack energy values were correlated with higher defect slice scores. It was also determined at 14 d that moisture content and pH were significantly correlated with tack energy (R=0.47375, R=-0.39310), as well as at 28 d (R=0.70981, R=-0.71469), and at 84 d (R=0.59688, R=0.44254). As moisture content increased, tack energy increased, and as pH increased, tack energy decreased.

**Flexibility Force**

Results for flexibility forces at three different slice thicknesses (1.25, 1.15, and 1.05 mm) for the commercial cheeses are shown in Table 5. At a thickness of 1.25-mm, the commercial cheeses had a mean flexibility force of 1.84 N, at 1.15-mm the cheeses had a flexibility force of 1.19 N and at 1.05-mm 2.5, 0.83 N. Overall, as slice thickness decreased with the commercial cheeses, the flexibility force decreased as well.

The flexibility force at all three slice thicknesses for all the experimental cheeses are shown in Figure 10. Overall, there was a significant difference between the HM group and MM and LM groups, as well as between the young 28 d cheeses and the older 70/84 d cheeses, for flexibility force at slice thickness 1.1-mm, and 0.9-mm. Hort et al. (2001, 1997) determined that young, green cheddar was harder than older cheddar and Tunick et al. (2007) determined that increased storage resulted in decreases in TPA hardness. Current results support Hort et al and Tunick et al’s research in that the younger cheeses resisted breakage at bending more than the older cheeses, the moisture groups. There was, however, a significant difference between the 28 d cheeses and the 70/84 d cheeses, as previously seen at the other two thicknesses.

Moisture content and pH were correlated with flexibility force. It was determined at 14 d that moisture content and pH were significantly correlated with flexibility forces at 1.1-mm (R=-0.76896, R=0.58415), at 0.9-mm (R=-0.73889, R=0.55106), and at 0.6-mm (R=-0.58113, R=0.58156). At 28 d the moisture content was significantly correlated with flexibility force at 1.1-mm (R=-0.46624), and pH was significantly correlated with flexibility forces at 0.9-mm (R=-0.39223) and at 0.6-mm (R=-0.44827). Lastly, at 84 d the moisture content and pH were significantly correlated with flexibility forces at 1.1-mm (R=-0.80888, R=0.65693) and at 0.9-mm (R=-0.77063, R=0.69217). Increasing moisture content led to decreases in flexibility force, and increasing pH leads to increases in flexibility force, as a result of the cheese becoming less crumby.

The P-values from the textural tests and slice scores are shown in Table 7. Moisture content and age, as well as a combination of the two, had a significant effect on all of the test parameters and slice scores, except the flexibility force at 0.9-mm slice thickness. Correlation tests, however, did not express a strong connection between moisture and age and cheese slice quality, overall. Therefore, conclusions regarding cheese slice quality cannot be determined from the textural and rheological tests alone. A combination of the slice quality evaluation and textural parameter tests will allow for determination of cheese sliceability.

![Figure 10: Mean flex force of 1.2-mm (A), 0.9-mm (B), and 0.6-mm (C) cheese slices with high moisture (●), medium moisture (■) and low moisture (▲) during 84d storage at 6°C. Error = SE.](image-url)
Table 7: P-values for tack force, tack energy and flex force of 1.1-mm (1), 0.9-mm (2), and 0.6-mm (3) experimental cheeses.

<table>
<thead>
<tr>
<th>dv</th>
<th>Tack Force</th>
<th>Tack Energy</th>
<th>Flexibility Force 1 (N)</th>
<th>Flexibility Force 2 (N)</th>
<th>Flexibility Force 2 (N)</th>
<th>Slice Score 1</th>
<th>Slice Score 2</th>
<th>Slice Score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental Moisture Group</td>
<td>2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.92</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Experimental Storage Time</td>
<td>5</td>
<td>&lt;0.01</td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Experimental Moisture*storage</td>
<td>10</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.07</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

CONCLUSIONS

A slice quality evaluation method was created based on a combination of defects that can be visually observed in sliced cheese. In general, a combination of tack energy and slice evaluation can be used to predict cheese sliceability. Higher moisture cheeses leads to poor slice quality. Future research should examine composition specifically, and whether or not cheese slice quality is a function of the fusion of curd particles.
REFERENCES


Effect of brine composition and brining temperature on cheese physical properties in Ragusano cheese

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ABSTRACT

Composition and physical properties of cheeses are influenced by temperature, salt and calcium concentration of brine. This work aimed to examine conditions of brine under which the cheese matrix contracts or expands in absence of restrictions imposed by surface rind development during overnight block formation. Three experimental 4-kg blocks of Ragusano cheese were produced at three different stretching temperatures (70, 80, 90 °C) and cut into pieces weighing ~40 to 50 g. One piece from each was chemically analyzed at time 0. All other pieces were measured for weight and volume and placed in plastic bags containing 300 ml of different brine solutions (2% NaCl with 0.1% Ca, 10% NaCl with 0%, 0.1%, 0.2% or 0.4% Ca, 18% NaCl with 0.1% Ca, and 26% NaCl with 0.1% Ca) at 3 different temperatures (4, 12, and 20°C). After 24 h brining, the cheeses were analyzed for weight, volume, chemical, and microstructural changes. Salt concentration in brine significantly (P < 0.01) influenced composition, weight and volume of the cheeses after brining. Salt concentration was inversely related with cheese volume and weight. Changes in weight caused by altering the brining temperature were not sufficient to reach statistical significance (P = 0.16), whereas statistically significant (P = 0.01) volume changes were induced by brining temperature and its interaction with salt content (P = 0.04). The highest volume increase (30%) occurred in the cheese stored in the 2% NaCl brine at the coldest temperature, whereas the greatest decrease was recorded in cheeses brined in the 26%NaCl brine. Composition was not affected by brining temperature. Calcium concentration did influence (P = 0.01) weight, volume and composition, except for fat-on-dry-basis (P = 0.58). When cheeses were brined without added calcium there was an increase in cheese volume and weight at all temperatures. At high calcium levels (0.4%), syneresis occurred and there was a decrease in volume, especially at 20°C (-16.5%). Microstructural investigation with porosity measurement confirmed weight and volume changes.

BACKGROUND

Ragusano cheese is a traditional Sicilian cheese made from cows raw milk in the province of Ragusa (Licitra et al., 1998). It is a pasta filata cheese produced using traditional wooden tools in which microorganisms from a biofilm in a wooden vat acidify the milk and coagulation occurs by adding of rennet paste (from kid and lamb) (Licitra et al., 2007). Curd is heated, drained, re-heated in whey or water, drained, then allowed to acidify overnight at ambient temperature. The fermented curd is stretched in hot (~85°C) water into a coherent mass to produce a smooth surface, then pressed into a rectangular block. Typically, 16-kg blocks are immersed in saturated salt brine at ambient temperature for 3 wk, after which they are aged up to 12 mo without vacuum packaging at ambient temperature. Two blocks of cheese are tied together with rope and hung over rafters to facilitate even moisture loss from the blocks (Licitra et al., 1998).

Changes in cheese composition during brining are typically a balance between weight gain resulting from salt penetration into the cheese and weight loss as moisture
Exits the cheese (Geurts et al., 1974b). There can also be a swelling or shrinkage of the cheese that is dependent on both the salt and calcium content of the brine (Geurts et al., 1972). For Gouda cheese brined at 12°C the crossover salt concentration at which there was no change in cheese volume occurred at ~15% (wt/wt) when the brine contained 0.1% (wt/vol) calcium. When calcium content was increased to 0.5% (wt/vol), cheese samples contracted when the brine salt concentration was >8%. At lower salt concentrations the cheese expanded. For some cheeses, such as feta, temperature of the brine solution and pH of the cheese can also influence changes in weight and volume of cheese during brining (McMahon et al., 2009). At cold temperature (e.g., ≤6°C) there is an increase in cheese volume when feta is immersed in brine with salt concentrations of 6.5% to 9.5% (wt/wt) and calcium content of 0.1% (wt/wt), whereas at higher temperature (22°C) shrinkage occurs. When feta cheese is brined at 10°C, whether the cheese gains or loses volume is dependent on brine salt and calcium concentration, brining temperature, and initial pH of the cheese (McMahon et al., 2009).

Penetration of salt into cheese during brining has been described as an impeded diffusion process (Guinee and Fox, 2004). When a block of cheese is placed into brine there is a net movement of Na⁺ and Cl⁻ ions from the brine into the cheese. There is also movement of any ions, such as Ca²⁺ and H₂PO₄⁻ into the brine if their concentration is higher in the cheese than in the brine. If the osmotic pressure difference between the cheese and the brine is sufficiently high, then water molecules will also diffuse out of the cheese. Geurts et al. (1974b) determined that in general, water moves out of the cheese about twice as fast as salt moves into the cheese as a consequence of the diffusion process. They proposed that such water displacement is not a result of independent shrinking of the matrix but rather the reduction in volume follows from the net transport of water and salt. However, the observation by McMahon et al. (2009) that cheese can shrink or expand when cheese in a brine solution is moved from cold to warm, or warm to cold, temperature implies that the inherent properties of cheese are just as important as osmotic effects. Changes in cheese volume can thus occur in the absence of any salt concentration gradients between cheese and surrounding brine.

Traditionally, Ragusano cheese has been brined in a saturated salt solution at ambient temperature, and loss of moisture from the cheese is greater than absorption of salt resulting in an 11% decrease in cheese weight (Licita and Bottazzi, 2001). Subsequent storage for 12 mo produces a further 6% moisture loss. The greatest changes in salt and moisture occur at the surface of the cheese block and a surface barrier layer is formed. It has been calculated that the porosity of the exterior 1-mm portion of Ragusano cheese blocks decreases by ~50% after immersion in saturated brine for 12 d (Melilli et al., 2005).

Having rapid salt penetration into the cheese is important because if salt content in the interior of the block of Ragusano cheese remains low, growth of undesirable gas-forming bacteria (such as coliforms) is favoured and early blowing of the cheese can occur (Melilli et al., 2004). Reducing brine temperature from 18°C to 12°C can reduce the incidence of early gas formation (Melilli et al., 2004). Also, the rate of salt uptake in cheese is influenced by brine concentration, and salt diffuses faster into cheese when a non-saturated brine is used (Resmini et al., 1974). For Ragusano cheese, using an 18% brine provides faster salt uptake than using a saturated brine (Melilli et al., 2003a, 2005). This apparently is caused by the smaller outward migration of water that occurs when using lower concentration brines (Guinee and Fox, 2004). Having less moisture loss in lower concentration brines results in an increase in cheese yield with an 18% brine for at least the first 8 d of brining compared to using saturated brine (Melilli et al., 2003, 2006). Brining of a 4-kg block of Ragusano cheese in saturated brine for 24 d results in shrinkage of the cheese with an overall 10% loss of weight (Melilli et al., 2003b). This weight loss is the net result of ~12.5% moisture loss occurring concomitantly with ~2.5% gain in weight from salt diffusion into the cheese.

Geurts et al. (1974b) estimated pore size of the protein matrix in cheese to be about 2.5 nm. This exerts a sieving effect on both the inwardly diffusing Na⁺ and Cl⁻ ions and the outward-moving H₂O molecules (Guinee and Fox, 2004). During pressing of Ragusano cheese (that occurs in wooden tables with the cheese exposed to air, and some whey drainage occurs), there is partial dehydration of the surface of the cheese block. This combined with surface dehydration occurring during brining (Geurts et al., 1972) and subsequent storage, eventually produces a 4-mm thick rind on Ragusano cheese (Melilli et al., 2003a). Development of such a compressed protein matrix either resulting from pressing or during brining would further impede the penetration of salt into Ragusano cheese.

Calcium is also an important factor in brining of cheeses and the initial surface dehydration during brining occurs faster when the cheese lacks calcium (Geurts et al., 1972). This rapid loss of moisture (within 2 h for Gouda cheese) is followed by a slower loss of calcium from the cheese surface layer and a consequent increase in surface moisture content above its original level. Thus, swelling or shrinkage of cheese during brining can be dependent on both brine salt and calcium concentrations (Geurts et al., 1972) and temperature (McMahon et al., 2009) and interactions between these factors. For example, softening of the cheese surface when using a weak brine can be avoided by increasing the calcium content of the brine (Geurts et al., 1972).

Such changes in moisture content of cheese only occur in those regions in which some salt has penetrated (Geurts et al., 1972). So while the outer portions of the cheese
are responding to high salt levels, the interior portions of the cheese block are responding to progressively lower salt concentrations. This implies that different portions of the cheese block will have a tendency to become more hydrated or dehydrated—for the protein matrix to expand or contract—based upon the chemical environment being experienced. Our goal was to study the conditions under which the cheese matrix contracts or expands in response to salt and calcium concentration, and temperature in the absence of restrictions imposed by surface rind development during overnight block formation.

MATERIALS AND METHODS

Cheese Making and Sampling

Ragusano curd, made according to the traditional procedure, was bought from a farm in the Hyblean area of Sicily and transported to the CoRFiLaC pilot plant. It was cut into strips and 4-kg batches were manually-stretched at 3 different temperatures (70, 80, 90°C). The hot cheese was formed into a coherent block and then shaped into cubes using a traditional wooden tool (mastroredda) and allowed to cool overnight at room temperature (~23°C). The following day, the outer 6-mm was cut from the block surface so as to eliminate any interference from the rind that had formed through surface dehydration. The remainder of the cheese was cut into 3 x 3 x 6 mm pieces weighing ~ 40 to 50 g.

Brining Cheese

Brine solutions (adjusted to pH 5.2) of the following concentrations (wt/wt) were prepared: 2% NaCl with 0.1% Ca (added as CaCl₂), 10% NaCl with 0%, 0.1%, 0.2% or 0.4% Ca, 18% NaCl with 0.1% Ca, and 26% NaCl with 0.1% Ca. A portion of each brine solution was tempered to 4, 12, or 20°C. Each piece of cheese was accurately weighed and its volume measured by displacement and then placed in a plastic bag. Three hundred millilitres of brine was added, then the bag was sealed and stored at either 4, 12 or 20°C. After 6 h, the cheese was removed from the bag, weighed and then placed in a plastic bag with a fresh 300-ml aliquot of the appropriate brine. The cheeses were stored for an additional 18 h and then analysed for weight, volume, chemical, and microstructural changes.

Cheese Composition Analysis

Moisture content was determined by drying a 3-g sample in a forced-air oven at 100°C for 24 h (APHA, 2004). Total nitrogen was measuring by Kjeldahl using a 1-g sample size (ISO/TS 17837:2008/IDF/RM 25) with conversion to protein content using a factor of 6.38. Calcium content was measured using a complexometric method (Kindstedt and Kosikowski, 1985). Ash was measured using AOAC, 2000 (method 932.42) and salt was calculated as ash minus 3 x calcium. Fat content of cheese was determined by Van Gulik method (ISO 3433:2008). All testing was performed in duplicate.

Microstructural Analysis

Small slices (13 x 3 x 1 mm) were taken from the central portion of each piece of cheese and they were prepared for scanning electron microscopy by the cryofracturing technique according to McManus et al. (1993). Samples were critical point dried in a Polaron CPD 7501 (Polaron, Watford, United Kingdom) with CO₂. Dried samples were mounted on aluminium stubs, using a carbon adhesive (SPI Supplies Structure probe, West Chester, Pennsylvania, USA), and gold–palladium coated for 15 s in a Polaron SC7620 mini sputter coater (Polaron, Watford, United Kingdom). Samples were examined by JSM–5900 LV scanning electron microscope (JEOL, Tokyo, Japan) operating at an accelerating voltage of 5 KV, a working distance of 10 - 17 mm, and a magnification of 400X. To avoid observer bias in selection of fields to compare, 5 fields were used for digital imaging as shown in figure 1. For each sample 5 SEM images were recorded and quantitatively analysed using Image J software equipped with the structure analysis plugin.

Figure 1. Scheme showing the fields selected for imaging in samples of cheese prepared for examination using scanning electron microscopy.
Effect of brine composition and brining temperature on cheese physical properties in Ragusano cheese / D. J. McMahon

with a “Pore Analysis” plug-in (Impoco et al., 2006). Porosity was measured as the percentage of pore area with respect to the total sampled area.

Experimental Design and Statistical Analysis

The 3 blocks of cheese (from the 3 stretching temperatures) were used as replicates for statistical analysis of chemical and physical changes in the cheeses brought by the different brining treatments. Data were analyzed using the Generalized Linear Models (GLM) procedure of SAS (Version 9.1, 2002-2003, SAS Institute, Cary, NC) in a three-way factorial design of brine composition (salt and calcium) and brining temperature, and REGWQ test was used to detect differences between means of volumes and weight after 24 h for different amount of salt and calcium in the brine, with a significance value of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Before Brining

Composition of the curd prior to hot water stretching was 479 g/kg moisture, 238 g/kg Ca, 44.9% fat-on-dry-basis (FDB) with pH 5.13 indicating adequate acid development in the curd during overnight fermentation before it was cut into pieces and stretched. The traditional hot water temperature for stretching Ragusano curd is ~85°C (Licitra et al., 1998) although water temperatures as low as 73°C have also been used (Melilli et al., 2003). In this experiment, the 3 batches of cheese were stretched using water at 70, 80 and 90°C to cover the range of potential stretching conditions and provide a generalized set of cheese for the brining experiment. Composition of the 3 cheeses after stretching is shown in Table 1 and was within the expected range for Ragusano cheese (Licitra et al., 1998; Melilli et al., 2003). The 3 stretched cheeses were all similar in composition, especially those stretched at 70 and 80°C. There was an observed trend of the cheese increasing in fat-on-dry basis content and pH. This suggests there was more loss of soluble components (lactose, lactate, salt and hydrogen ions) into the water at the higher temperatures. Moisture content of the cheese stretched at 90°C was also higher than in those stretched at 70 and 80°C.

<p>| Table 1. Proximate composition of cheese before and after stretching in 70, 80, or 90°C water. |
|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Stretch Water Temperature</th>
<th>Moisture (g/kg)</th>
<th>Protein (g/kg)</th>
<th>FDB (g/kg)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>470</td>
<td>238</td>
<td>449</td>
<td>5.13</td>
</tr>
<tr>
<td>70</td>
<td>473</td>
<td>247</td>
<td>469</td>
<td>5.15</td>
</tr>
<tr>
<td>80</td>
<td>468</td>
<td>251</td>
<td>473</td>
<td>5.21</td>
</tr>
<tr>
<td>90</td>
<td>491</td>
<td>246</td>
<td>483</td>
<td>5.22</td>
</tr>
</tbody>
</table>

Composition After Brining

Significance of factors influencing cheese composition is shown in Table 2. Brine salt concentration influenced (P < 0.01) moisture, protein, FDB, salt, S/M and calcium content of the cheese. Calcium levels in the brine also influenced all these parameters except for FDB (P = 0.58).

<p>| Table 2. Mean Square (and P-value) for effect of NaCl levels (n=4), Ca levels (n=4), and temperature (n=3) of brine on chemical composition, weight and change in volume of Ragusano cheese after 24 h brining. |
|---|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Source of Variation</th>
<th>NaCl</th>
<th>Ca</th>
<th>Temperature</th>
<th>NaCl x Temperature</th>
<th>Ca x Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>1170 (&lt;0.01)</td>
<td>0.570 (0.87)</td>
<td>0.468 (3.99)</td>
<td>0.790 (0.98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>142 (&lt;0.01)</td>
<td>1.192 (0.25)</td>
<td>0.262 (0.93)</td>
<td>0.408 (0.82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDB</td>
<td>0.033 (&lt;0.01)</td>
<td>0.0002 (0.93)</td>
<td>0.0008 (0.96)</td>
<td>0.0005 (0.98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>77.7 (&lt;0.01)</td>
<td>2.90 (&lt;0.01)</td>
<td>0.082 (0.47)</td>
<td>0.078 (3.63)</td>
<td>0.044 (0.87)</td>
<td></td>
</tr>
<tr>
<td>S/M</td>
<td>609 (&lt;0.01)</td>
<td>1.07 (0.05)</td>
<td>0.290 (0.47)</td>
<td>0.039 (0.99)</td>
<td>0.138 (0.90)</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>0.158 (&lt;0.01)</td>
<td>0.232 (&lt;0.01)</td>
<td>0.0013 (0.75)</td>
<td>0.0019 (0.87)</td>
<td>0.0015 (0.92)</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>0.194 (&lt;0.01)</td>
<td>0.106 (&lt;0.01)</td>
<td>0.001 (0.69)</td>
<td>0.001 (0.94)</td>
<td>0.001 (0.87)</td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>489 (&lt;0.01)</td>
<td>6.11 (0.16)</td>
<td>5.31 (3.15)</td>
<td>3.03 (0.47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔVolume</td>
<td>502 (&lt;0.01)</td>
<td>43.3 (0.01)</td>
<td>18.8 (3.04)</td>
<td>7.71 (0.42)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Effect of brine composition and brining temperature on cheese physical properties in Ragusano cheese / D. J. McMahon

Brine temperature did not influence cheese composition either as an independent variable ($P = 0.25$ to $0.93$) or as an interaction with NaCl ($P = 0.63$ to $0.99$) or Ca ($P = 0.82$ to $0.98$) content of the brine. This is the opposite of what had been observed during the brining of feta cheese where lower moisture contents were observed as the brine temperature was increased from $3^\circ C$ to $22^\circ C$ (McMahon et al., 2009).

Pooled composition of the cheese after 24 h brining in the various salt and calcium combinations is shown in Table 3. Moisture contents ranged from 565 g/kg in cheese brined at $4^\circ C$ in the lowest salt concentration (2% NaCl) down to 280 g/kg in cheese brined at $20^\circ C$ in the highest salt concentration (26% NaCl). Such lowering of moisture content with increasing salt concentration in the brine is well known (O'Connor, 1971; Sutherland, 1974; Jordan and Cogan, 1993; Kelly et al., 1996).

There was some tendency for slightly lower moisture content (~8 to 18 g/kg reduction) in the cheese as the temperature of brining increased from $4^\circ C$ to $20^\circ C$. This was observed in all the cheeses except for the cheese brined in 10% NaCl with no added calcium, which exhibited the opposite effect. The differences in moisture were greatest at high salt levels in the brine and the least difference was observed when calcium levels were increased to 0.2% or 0.4%. Increasing the calcium content in the brine also produced cheese with lower moisture. Moisture contents of 512 g/kg were obtained when cheese was brined in 10% salt with no added calcium, compared to 416 g/kg in brine containing 0.4% calcium (Table 3).

It is commonly observed (O'Connor 1971; Thakur, 1973) that higher salt contents in cheese coincided with higher fat and protein contents. This relates to greater losses of moisture that occur as a function of whey syneresis induced by salting of Cheddar cheese curd and by immersion of cheese into high salt concentration brines. In this study, fat and protein contents of the cheese were inversely related to moisture content. This was observed for the effects of salt and calcium content of the brine as well as brining temperature.

The uptake of salt by the cheeses during brining has a diluting effect on the amount of protein per kg of cheese, but this is not sufficient to counteract the effect of moisture. For cheese brined in 2% NaCl, mean protein content expressed on a dry basis was 47.1% whereas it was only 42.5% when brined in 26% NaCl. The influence of moisture uptake or loss during brining is more apparent when the cheeses brined in 10% NaCl with the different calcium levels were compared. With no added calcium the cheese had mean composition of 512 g/kg moisture with 43.4% protein on a dry basis. With 0.4% Ca in the brine, the cheese moisture was only 416 g/kg (a 19% difference) yet protein expressed on a dry basis had only a slight increase to 44.8%.

Fat content of the brined cheeses, when expressed on a dry basis, was similar for all cheeses except that brined in 2% NaCl. Cheeses brined in 10% to 26% NaCl all had mean FDB in the range of 44% to 48%, whereas cheese from the 2% NaCl brine had 55% FDB. Cheeses with lower salt content would be expected to have higher FDB, however the reason for the cheeses from the 10% and 26% NaCl brines having the same FDB was unexpected and the reason for this was unknown.

Salt content of the cheese increased as expected with increased salt concentration in the brine, and ranged from 108 g/kg in the cheese brined in 2% NaCl, to 807 g/kg in cheese brined in 26% NaCl. In this experiment, the cheese to brine ratio was about 1:6 and a fresh portion of brine was

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**Table 3.** Mean composition for cheeses brined for 24 h in 2%, 10%, 18% or 26% NaCl solutions (all containing 0.1% Ca), or in 10% NaCl solutions containing 0%, 0.1%, 0.2%, or 0.4% Ca with data being pooled from all brining temperatures.

<table>
<thead>
<tr>
<th>Brine Parameters</th>
<th>Moisture (g/kg)</th>
<th>Salt (%)</th>
<th>FDB (g/kg)</th>
<th>Protein (g/kg)</th>
<th>Ca/Protein</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (0.1% Ca)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>561 a</td>
<td>10.8 a</td>
<td>57.2 a</td>
<td>207 a</td>
<td>26.7 a</td>
<td>5.25 a</td>
</tr>
<tr>
<td>10%</td>
<td>458 b</td>
<td>47.3 b</td>
<td>44.8 b</td>
<td>239 b</td>
<td>27.5 b</td>
<td>5.10 b</td>
</tr>
<tr>
<td>18%</td>
<td>376 c</td>
<td>60.4 c</td>
<td>47.3 b</td>
<td>270 c</td>
<td>25.6 c</td>
<td>4.99 c</td>
</tr>
<tr>
<td>26%</td>
<td>293 d</td>
<td>80.7 d</td>
<td>44.2 b</td>
<td>300 d</td>
<td>28.9 d</td>
<td>4.91 d</td>
</tr>
<tr>
<td>LSD</td>
<td>20</td>
<td>2.3</td>
<td>6.5</td>
<td>11</td>
<td>4.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Ca (10% NaCl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>512 a</td>
<td>52.3 a</td>
<td>48.3 a</td>
<td>211 a</td>
<td>23.3 a</td>
<td>5.20 a</td>
</tr>
<tr>
<td>0.1%</td>
<td>458 b</td>
<td>47.5 b</td>
<td>44.8 b</td>
<td>239 b</td>
<td>27.5 a</td>
<td>5.10 b</td>
</tr>
<tr>
<td>0.2%</td>
<td>439 b</td>
<td>42.6 c</td>
<td>46.3 a</td>
<td>249 c</td>
<td>30.0 b</td>
<td>5.06 b</td>
</tr>
<tr>
<td>0.4%</td>
<td>416 c</td>
<td>39.4 c</td>
<td>47.2 a</td>
<td>262 d</td>
<td>32.2 c</td>
<td>4.94 c</td>
</tr>
<tr>
<td>LSD</td>
<td>23</td>
<td>3.6</td>
<td>3.9</td>
<td>8</td>
<td>4.4</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Fat-on-dry-basis

Means within columns for each brine parameter with the same letter were not significantly different ($a = 0.05$).
added after 6 h to help compensate for the dilution effect as water diffuses from the cheese into the brine. The final salt-in-moisture contents of the cheeses were 1.9%, 9.4%, 13.5% and 21.6% after a total of 24 h immersion in 2%, 10%, 18% and 26% NaCl brines, respectively. The failure for the cheeses immersed in higher concentration brines (i.e., 18% and 26% NaCl) suggests that flow of sodium and chloride ions into the cheeses is more impeded and they would require longer to reach equilibrium between the cheese and brine.

Calcium content of the cheeses decreased from 8.6 g/kg to 5.5 g/kg as the NaCl content of the brine was lowered from 26% to 2% respectively. This however is a function of increased moisture content of the lower salt cheeses, and when calculated on a protein basis the cheeses all had calcium contents of 26 to 29 g/kg protein (Table 3). This implies that there is no displacement of Ca from the proteins by Na ions as the salt diffuses into the cheese from the brine. This agrees with Pastorino et al. (2003c) who also observed no increase in soluble Ca when NaCl solutions were injected into cheese. Varying the level of Ca in the brine did alter the ratio of Ca to protein in the cheese from 22 g/kg protein when no Ca was added to the brine, up to 33 g/kg protein when 0.4% Ca was included in a 10% NaCl brine solution. The levels of insoluble calcium in the cheeses were not measured but it could be assumed that some of the soluble Ca from the brine would bind to the proteins and become part of the colloidal calcium phosphate that forms crosslinks between the phosphoserine side chains of the caseins.

The pH of the cheeses after brining was dependent on both NaCl and Ca content of the brine, with lower pH occurring in cheeses brined with either higher NaCl or higher Ca levels. Each of the brine solutions had initially been adjusted to pH 5.2 to match the pH of the cheese prior to brining. When the brine contained either 2% NaCl with 0.1% Ca or 10% NaCl with 0% Ca, the pH of the cheese after 24 h brining remained at about pH 5.2 (see Table 3). Increasing the NaCl in the brine to 26% produced a cheese with pH 4.9. The same effect was observed when the Ca level in the 10% NaCl brine was increased to 0.4%. Pastorino et al. (2003a) observed a similar decrease in pH when a Ca solution was injected into cheese. Such a lowering of pH requires the release of H⁺ ions into the cheese serum.

The most likely source of H⁺ ions in cheese would be from H₂PO₄⁻ ions being precipitated as calcium phosphate in the form of Ca₅(PO₄)₂ which is the most likely form of the colloidal calcium phosphate in cheese (Lucey and Horne, 2009). It is easily understandable how increasing the calcium content of the brine would shift the position of the calcium phosphate equilibrium to an increase in insoluble calcium phosphate but it is less clear why this occurs with an increase in NaCl.

### Salt Concentration

#### Cheese Weight.

Each cheese sample was weighed before brining and after 6 and 24 h (with fresh brine solution added at 6 h). Use of different salt concentrations in brine significantly (P < 0.01) influenced the weight of the cheese after brining (Table 2). As the brine salt concentration decreased there was a linear increase in cheese weight. When the cheese was brined in a saturated 26% salt brine, there was a 10% to 20% loss of weight (Fig. 2A). Conversely, when a 2% salt brine was used the cheeses gained up to 25% of their original weight during the 24-h brining process. There were significant differences in cheese weight after brining at each salt concentration (2%, 10%, 18% and 26%).

Changes in weight induced by altering the brining temperature were not sufficient to reach statistical significance (P = 0.16). Except for the observed difference in amount of weight lost after 24 h in the 18% brine, cheeses brined at 4°C and 12°C were very similar. When the temperature was increased to 20°C, the cheeses had lower weights (Fig. 2A) with less weight gain occurring in the 2% brine (i.e., a weight gain of 12% compared to ~25%), and more weight loss in the saturated brine (i.e., a weight loss of 19% compared to ~13%).

#### Cheese Volume.

If change in weight was only a function of absorption of salt, then weight gain should be in proportion to salt concentration of the brine (Fig. 2B). Generally, when cheese is brined there is a flow of water from the cheese into the brine (Geurts et al., 1974b) brought about by osmotic effects induced by the high salt concentration (e.g., 20%) of the brine. At low salt concentrations in the brine, this osmotic effect is much smaller and the flow of moisture is in the opposite direction (i.e., into the cheese) and the cheese expands (P < 0.01). Brine temperature did influence volume change of the cheese (P = 0.01) and there was also a significant interaction of temperature with salt concentration (P = 0.04). The greatest volume increase (30%) occurred in the cheese stored in the 2% brine at the coldest temperature (Fig. 2C). Brining the cheese at 20°C counteracted the tendency of the cheese to expand and the volume increase was only ~4%. This is similar to that observed for feta cheese (McMahon et al., 2009). Mean volume of cheeses after 24 h in 2%, 10% and 18% brines were significantly different but there was no significant difference between cheeses in the 18% and 26% brines.

Based on the crossover from cheese expansion in 10% salt brine (when containing 0.1% Ca) to cheese shrinkage in 18% salt brine (Fig. 2A), it was estimated that cheese in ~13% salt brine (at 12 or 20 °C) would exhibit no weight change during brining. Implying, there would be
Effect of brine composition and brining temperature on cheese physical properties in Ragusano cheese / D. J. McMahon

A slight decrease in cheese volume that compensates for the increased density of the cheese as salt is absorbed. If the only factor influencing moisture diffusion was the salt-in-moisture concentration gradient, then it would still be expected for the cheese to lose moisture and shrink. We suggest that osmotic pressure alone does not control diffusion of water in the cheese but it is also influenced by the balance of protein-protein interactions within the protein matrix of the cheese and protein-water interactions.

At 2% salt concentrations, the osmotic effect promoting diffusion of water out of the cheese is very small while protein-protein interactions are weakened as the proteins increase in solubility (Paulson et al., 1998) and the protein matrix expands. Inhibition of protein matrix expansion occurs at higher temperature (20°C) because hydrophobic interactions between proteins become more relevant to the free energy of the system. Then as salt concentration is increased, the concentration gradient between the cheese and the brine increases such that osmotic pressure increases in magnitude and the balance of forces now results in shrinkage of the cheese.

Calcium

Varying calcium content in 10% salt brines significantly (P<0.01) (Table 2) influenced weight change of the cheese as shown in Fig. 3. At 0.4% Ca, virtually no change occurred at any temperature, there was a decrease in weight at 0.2% Ca at 20 °C whereas weight increase resulted for all the other combinations of Ca and temperature. The highest weight yield was at 4°C with 0% Ca in the brine. Temperature was not a significant source of variation of weight changes in cheese (P = 0.16) but it did influence volume (P < 0.01). (Table 2).

Volume of the cheese after brining was inversely related to calcium contents in the brine (Fig. 3). Changes in volume indicate that protein matrix structure is either expanding or contracting. An absence of calcium caused a volume increase at all temperatures, with cold temperature (4°C) causing the largest increase. While a combination of high temperature (20°C) and high calcium percentage (0.4%) resulted in a cheese volume decrease.

Cheese Microstructure

Microstructure of the Ragusano cheeses coincided with observed changes in chemical composition and volume of the cheese. At 20°C, brining in the lowest salt level of 2% NaCl produced only a 4% increase in volume (Fig. 2) and the protein matrix of this cheese (Fig. 4) appeared more expanded than cheese brined at 10% NaCl (Fig. 5) that had a 3% decrease in volume. When the porosity of the cheese in these images was quantified, the cheese brined in 10% NaCl had a porosity mean value of 12.6%, whereas the porosity of the cheese from the 2% brine was 14.4%. The higher salt cheese had smaller (area mean value 13.6512
nm against 21.8592 nm) and more numerous (1475 pores against 1046 pores) pores confirming that shrinkage occurred in cheeses brined at 10% NaCl brine.

Having a higher salt concentration in the brine causes a contraction of the cheese protein matrix that would expel whey as well as a moisture by osmosis so that there is a net whey migration from the void spaces and the protein matrix. Thus, per unit area of cheese samples viewed by electron microscopy the pores became smaller and more numerous. The protein matrix in higher salt cheese also occupied a greater area of the SEM field of view, 78% compared to 75%.

Calcium concentration in brine (and consequent calcium content in the cheese) also influenced cheese microstructure. Higher Ca in cheese produced pores that were smaller compared to cheeses brined with lower Ca. Cheese brined in 10% NaCl with 0.4% Ca (Fig. 6) also had fewer (1350 pores against 1849 pores) and smaller (area mean value 14.638 nm against 17.988 nm) pores cheese from the 0.2% Ca brine (Fig. 7). This corresponded to porosity mean values of 13.5% and 18%, respectively. Thus, the loss of moisture from the pores caused by a high calcium level in the brine appears greater than that caused by high salt. McMahon et al. (2005) also showed that increasing calcium concentration in cheese induces stronger protein-protein interactions and less whey remains entrapped within the protein matrix. Similar observations were found by Guinee et al., (2002) and Joshi et al., (2004d), in which reducing calcium content of Mozzarella cheese led to a more hydrated and expanded protein matrix. Pastorino et al. (2003a) also reported that adding calcium to cheese causes protein fiber contraction and more whey loss, inducing shrinkage of the whole cheese structure.

**Brining Cheese**

The implication of this to Ragusano cheese as it is being brined is that various portions of the cheese block will be subject to different forces depending on the salt and calcium concentrations to which the protein matrix is exposed. At the same time, as the cheese for this experiment was being manufactured, duplicate blocks of Ragusano cheese were made, and these were then brined in the normal manner. After brining these cheeses were sampled at different depths and going from the exterior layer to the central core the cheese had increasing moisture levels of 40.3%, 43.4%, 45.1% and 46.4% with corresponding S/M levels of 9.7%, 6.7%, 3.9% and 1.6%. Consequently, while the protein matrix at the surface of the cheese surface is contracting and expelling moisture, the cheese at the centre of the cheese resists shrinkage and if moisture were available it would be expected to expand (Fig. 2).

Cheese is criss-crossed by capillaries and several factors can affect permeability and the diffusion of salt into the cheese. Fat globules can block the cheese microstructure, and salt penetration will take longer in a cheese with a higher fat. The pH of the cheese can also influence the rate of salt absorption with more salt being absorbed at low pH than at higher pH. Decreased pH value, induce calcium solubility and its content decrease in the cheese (Pastorino et al., 2003b). As shown by Melilli et al. (2006), salt penetration varies with temperature with higher temperature promoting salt penetration, moisture loss and weight loss during brining.
CONCLUSIONS

Ragusano cheese has been salted in brines having different composition and temperatures. Salt and calcium concentrations of the brine had the greatest influence on cheese composition, cheese weight and volume. Higher moisture loss resulted in cheese immersed in 26% NaCl brine and in that brined in 0.4% Ca. Since high levels of salt and calcium promoted water expulsion, decreases of weight and volume were recorded. In detail, higher salt concentration in the brine enhanced expulsion of whey and moisture loss by osmosis process, causing a contraction of the cheese protein matrix. High calcium levels in the brine tightened protein-protein hydrophobic interactions, favouring whey expulsion from the cheese. Results of cheese volume changes were confirmed from the porosity measurement. Decreases in volume corresponded to a lower cheese porosity. Brining temperature and its interaction with salt and calcium had a significant influence just on volume changes.
REFERENCES


ABSTRACT

Milk gels were made by renneting and acidifying skim milk containing 5 different starches, and then compressed by centrifugation to express whey and simulate curd syneresis during the manufacture of low-fat cheese. A series of 17 starches were examined with five starches being selected for in-depth analysis: a modified waxy cornstarch (WC), a waxy rice starch (WR), an instant tapioca starch (IT), a modified tapioca starch (MT) and dextrin (DX). Milks containing WC, WR, and DX were given a 72°C heat treatment, while those containing IT and MT had a 66°C for 30 min treatment that matched their optimum gelatinization treatments. Curd yields were calculated by weight, and estimated starch content in whey was measured gravimetrically by alcohol precipitation, and starch retention in curd calculated. Curds yields were 13.1% for a control milk (no added starch) and 18.4%, 20.7%, 21.5%, 23.5%, and 13.2% for the gels containing starches WC, WR, IT, MT and DX, respectively. Estimated starch retentions in the curd were respectively 71%, 90%, 90%, 21%, and 1%. Laser scanning confocal microscopy was used to determine location of the starches in the curd and their interaction with the protein matrix. Waxy corn, WR, and IT starches have potential to improve texture of low-fat cheese because they had high retention in the curd and they generate interruptions in the protein matrix network that may help limit extensive protein-protein interactions. Modified tapioca starch interfered with formation of the protein structure of the curd and produced a soft non-cohesive gel even though most (79%) of MT starch was lost in the whey. There were few distinct starch particles in the MT curd network. Dextrin was not retained in the curd, nor did it disrupt the protein network, making it unsuitable for use in low-fat cheese.

BACKGROUND

Development of lower fat foods is often difficult because removing fat from many food systems results in foods with poor sensorial and functional qualities (Johnson et al., 2009). Low-fat natural cheese is one such food where fat reduction gives the cheese a hard, rubbery texture (Drake and Swanson, 1995). Fat has the important role in cheese of imparting discontinuity to the protein matrix (Rogers et al., 2010), so when fat is removed the cheese is composed of a homogenous, dense protein network (Paulson et al., 1998). Starch and other hydrocolloids have been suggested as possible remedies for improving the texture of low-fat cheese, and have been used in some commercial lower fat cheeses.

Starches embody a diverse array of functional attributes that through modifications such as cross-linking, acid hydrolysis, or substitution, can be manipulated to serve almost any function within a food matrix (BeMiller and Whistler, 1996). Use of starch is common in dairy products, such as ice cream and yogurt, and has also been used in mozzarella and process cheeses (Cody et al., 2007; Merrill and Anderson, 2007; Trivedi et al., 2008). Starch inclusion in ice cream provides enhanced creaminess, improves mouthfeel, and improves freeze-thaw stability (Stanley et al., 1996; Cody et al., 2007). In yogurt, it enhances mouthfeel and helps prevent syneresis (Sandoval-Castilla et al., 2004). In cheeses, starch has been used for its water binding properties and to modify baking properties.

Besides starch, other polysaccharides such as gums, as well as protein-based fat mimetics, surfactants and emulsions have been investigated in lower fat cheeses for improving texture (Mistry, 2001). This includes: β-glucan (Konuklar et al., 2004; Vithanage et al., 2008),
lecithin (Drake et al., 1996; Sipahigil et al., 1999), gum tragacanth (Rahimi et al., 2007), carageenan (Kavas et al., 2004; Totosau and Guemes-Vera, 2008), pectin (Liu et al., 2008), protein and carbohydrate microparticles (McManus et al., 1996), emulsions (Lobato-Calleros et al., 2008) and exopolysaccharide-producing bacteria (Perry et al., 1997). Under US regulations for lower fat foods (21 CFR 130.10), such ingredients may be added provided they have a functional property, even though they are not permitted in the standardized full fat cheese.

Sipahigil et al. (1999) used a modified tapioca starch and lecithin to increase moisture content and soften reduced-fat feta. Adding a modified potato starch has been used for making low-fat white pickled cheese (Kavas et al., 2004) with no difference in moisture content resulting in a cheese that was harder and chewier than low-fat cheese made without added starch. Starch will compete with the protein in cheese for water so this may have increased the cheese hardness, however, Kavas et al. (2004) did not determine losses of the starch into the whey (which can be quite high) or starch content of the cheese.

Bhasharacharya and Shah (2001) looked at inclusion of two types of maltodextrins and a modified (particulated) potato starch in low-fat mozzarella. They observed that potato starch increased hardness of the cheese and decreased moisture content. Potato starch particles were distributed in serum pockets as well as in the protein matrix of the cheese. These particles apparently swelled during storage and removed moisture from the surrounding proteins. The maltodextrin-based fat replacers had amorphous gel-like structures that improved texture characteristics of the cheese and increased openness. Bhasharacharya and Shah (2001) attributed the differing properties of the cheeses made with the different starches to the starches' sizes, degrees of microparticulation, and to their interactions with casein. In Cheddar cheese made using a blend of modified cornstarch and microparticulated microcrystalline cellulose, Aryana and Haque (2001) observed an 11% increase in cheese moisture and a softening of the cheese. However, there was no observed increase of discontinuities within the protein matrix of the cheese. McMahon et al. (1996) reported a 4% increase in moisture of low-fat mozzarella cheese when a similar polysaccharide fat replacer was used, and observed increased openness and formation of large serum channels in the cheese microstructure.

The manufacture of rennet-set cheeses involves separating the curd from whey, with the whey being converted into other products so absence of starch in the whey (i.e., starch retention in the curd) is an important factor regarding its potential for use in making low-fat cheese. Our goal was to compare the effect of starches with a diverse range of hydration properties on curd syneresis. We used a model system of rennet-induced partially acidified skim milk gels that were then centrifuged to accelerate whey expulsion, quantified partitioning of starch between curd and whey, and the impact of starch on milk gel microstructure using laser scanning confocal microscopy (LSCM). In doing so a new method for visualizing starch in the presence of proteins in a food system was developed (McManus et al., 2009).

**MATERIALS AND METHODS**

**Materials**

Pasteurized (73°C for 15 s) skim milk was obtained from the Gary H. Richardson Dairy Products Laboratory (Utah State University, Logan, UT) and stored at 4°C. Starches (Table 1) were donated by National Starch (notebook 1180346; Bridgewater, NJ). Glucono-δ-lactone was from PURAC America (Blair, NE), chymosin rennet (Maxiren DS. ~650 International clotting units (ICU/ml) was from DSM Food Specialties (Parsippany, NJ). Celite, periodic acid, acriflavine HCl and Rhodamine B were from Sigma Aldrich, (St. Louis, MO). Osmium tetroxide was from Ted Pella Inc., (Redding, CA) All other chemicals were reagent grade.

**Starch Swelling**

Swelling properties of the 17 starches (Table 1) were measured in duplicate based upon Konik-Rose et al. (2001) to determine the amount of water each starch holds when subjected to different heat treatments. In 50-mL conical centrifuge tubes, 0.40 ± 0.03 g of starch was added to 40 mL distilled deionized water (~22°C) and heated with agitation to 60, 66 or 72°C. Solutions heated to 60 or 66°C were held for 30 min at their respective temperatures, while those heated to 72°C were cooled immediately. Heat treatments examined were based on manufacturer's recommendation for ideal starch gelatinization. The solutions were cooled to room temperature and centrifuged at 2,000 g for 20 min. Following centrifugation, the pellet and supernatant were weighed and water-holding capacity calculated. Moisture content of each starch powder was determined by vacuum oven at 66°C for 18 h.

**Milk Gels**

Preliminary study.

The 17 starches (Table 1) were dispersed in cold milk at 5 g/L and 10 g/L. Six 200-mL batches of each starch-in-milk solution (plus a milk only control) were prepared in 250-mL polycarbonate Nalgene centrifuge bottles (Thermo Fischer Scientific, Rochester, NY) and then given 3 different heat treatments (in duplicate) based upon observations on optimum starch swelling: heated to 63°C then immediately cooled, heated to 72°C then immediately cooled, or heating to 63°C held for 30 min then cooled.
Four grams of glucono-δ-lactone and 400 μL of 1:10 dilute hydrochloric acid (about 130 IU/kg milk) were added to each mixture after cooling to 35°C and then incubated for 30 min to allow coagulum formation. The resultant gel was then cut with a spatula vertically with 3 cuts in each of 2 perpendicular directions. Bottles containing the curd and whey were centrifuged at 25°C for 30 min, by this time the pH of the mixture dropped to ~ pH 5.1. The whey was separated immediately after centrifugation and again after 30 min, and its volume measured using a graduated cylinder. Curd yield was calculated on a volume basis and expressed as a percentage.

### Table 1. Starch description and properties.

<table>
<thead>
<tr>
<th>Code</th>
<th>Base</th>
<th>Description</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>waxy corn</td>
<td>amyllopectin, phosphate, 2-hydroxypropyl ether, 71% StOH retention.</td>
<td>base viscosifier, CU starch with high inhibition and stabilization, high process tolerance,</td>
</tr>
<tr>
<td>2</td>
<td>waxy corn</td>
<td>amyllopectin, phosphate, 2-hydroxypropyl ether</td>
<td>base viscosifier, CU starch medium inhibition and high stabilization, medium process tolerance</td>
</tr>
<tr>
<td>3</td>
<td>waxy corn</td>
<td>amyllopectin, phosphate, 2-hydroxypropyl ether</td>
<td>base viscosifier, CU starch low inhibition and high stabilization, low process tolerance</td>
</tr>
<tr>
<td>4</td>
<td>waxy corn</td>
<td>amyllopectin, phosphate, 2-hydroxypropyl ether</td>
<td>base viscosifier, agglomerated CWS starch with high inhibition and stabilization, high process tolerance</td>
</tr>
<tr>
<td>5</td>
<td>waxy corn</td>
<td>amyllopectin, phosphate, 2-hydroxypropyl ether</td>
<td>base viscosifier, agglomerated CWS starch with low inhibition and high stabilization, medium process tolerance</td>
</tr>
<tr>
<td>6</td>
<td>potato starch</td>
<td>starch</td>
<td>base viscosifier, CU starch with medium inhibition, no chemical treatment, large granules with naturally occurring phosphate groups</td>
</tr>
<tr>
<td>7</td>
<td>waxy rice</td>
<td>Amylopectin, 90% StOH retention.</td>
<td>base viscosifier, CU starch with medium inhibition, no chemical treatment</td>
</tr>
<tr>
<td>8</td>
<td>regular corn</td>
<td>starch, phosphate, 2-hydroxypropyl ether</td>
<td>base viscosifier, CU starch with medium inhibition and high stabilization</td>
</tr>
<tr>
<td>9</td>
<td>tapioca starch</td>
<td>amyllopectin, 2-hydroxypropyl ether, 90% StOH retention</td>
<td>base viscosifier, agglomerated CWS starch with medium inhibition and medium stabilization, medium process tolerance,</td>
</tr>
<tr>
<td>10</td>
<td>tapioca starch</td>
<td>amyllopectin, 2-hydroxypropyl ether, 21% StOH retention</td>
<td>co-texturizer, CU starch, high stabilization,</td>
</tr>
<tr>
<td>11</td>
<td>tapioca starch</td>
<td>maltodextrin</td>
<td>co-texturizer, CWS starch with specialty modifications</td>
</tr>
<tr>
<td>12</td>
<td>tapioca starch</td>
<td>dextrin, 1% StOH retention</td>
<td>dextrin with specialty modifications</td>
</tr>
<tr>
<td>13</td>
<td>tapioca starch</td>
<td>starch</td>
<td>gelling starch, acid converted</td>
</tr>
<tr>
<td>14</td>
<td>regular corn</td>
<td>starch, acid hydrolyzed</td>
<td>base viscosifier, CU starch with low inhibition, no chemical treatment, large granules with naturally occurring phosphate groups</td>
</tr>
<tr>
<td>15</td>
<td>potato starch</td>
<td>starch</td>
<td>Co-texturizer, Agglomerated CWS StOH with high stabilization</td>
</tr>
<tr>
<td>16</td>
<td>tapioca starch</td>
<td>starch, 2-hydroxypropyl ether</td>
<td>Base viscosifier. Agglomerated CWS StOH with medium inhibition no chemical treatment</td>
</tr>
<tr>
<td>17</td>
<td>tapioca starch</td>
<td>amyllopectin starch</td>
<td></td>
</tr>
</tbody>
</table>

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Second study.

Five starches from Table 1 were selected that were representative of the various performance attributes of starch when incorporated into a milk gel: modified waxy corn starch 1 (WC), waxy rice starch 7 (WR), instant tapioca starch 9 (IT), modified tapioca starch 10 (MT), and dextrin starch 12 (DX). Milk-starch mixtures (with starch concentration of 5 g/L) were prepared and those containing WC, WR or DX were heated to 72°C then immediately cooled. Milk-starch mixtures containing IT and MT were heated to 66°C, and held for 30 min before cooling. Control samples without starch were heated to both temperatures.
The milk solutions were converted into gels as described above with slight modification in that centrifugation was at 500 g for 15 min followed by 1,000 g for 15 min. After centrifuging, gels were evaluated visually for curd appearance, settling of starch, clean separation of whey from curd, slimness, viscosity, and similarity of whey released from milk gels containing starch to that from controls. Whey was decanted immediately after centrifugation, and whey and curd weighed. Curd and whey yields were calculated and expressed as a percentage of original weight of milk-starch mixture. A portion of whey was used to measure moisture content (in triplicate), while another portion was frozen and retained for starch analysis. Curd was retained for microstructural analysis.

Starch Loss in Whey

Starch lost in whey was quantified using a modified version of the method for measuring total dietary fiber (AOAC, 1990). Four frozen whey samples from each starch-containing curd (and from the controls) were thawed. Approximately 12 g of each whey (in duplicate) was accurately measured into beakers and 50 mL of 0.08 M pH 6.0 phosphate buffer was added to each sample, followed by pH adjustment to between 4.0 and 4.6 with 0.275 M HCl. Then 280 mL of 95% ethanol, warmed to 60°C, was added to each sample and the precipitate allowed to form for 60 min. Coarse fritted, glass 50-mL crucibles (Cole Parmer, Vernon Hills, IL) containing 0.5 g of acid washed celite were dried over night and weighed before use; prior to filtration the bed of celite was evenly redistributed using 78% ethanol. After 60 min, each sample was filtered through the crucibles, followed by washing with three 20-mL portions of 78% ethanol, two 10-mL portions of 95% ethanol, and two 10-mL portions of acetone. Following filtration, crucibles containing residue were dried overnight in a vacuum oven at 66°C then weighed.

Analyzing starch in water solutions of known concentration was used to determine recovery of starch after ethanol precipitation. Solutions of each starch were made and given heat treatments identical to the milk used to make the model cheeses. A portion of each solution containing approximately 0.04 g of starch accurately measured on an analytical balance was analyzed as described above. Weights of residues were compared with the known amount of starch in each solution.

Microstructure

Curd samples were prepared for microstructural analysis using LSCM as described by McManus et al. (2009). Curd was cut to 1 x 1 x 1-cm cubes and protein was fixed with 1% (wt/vol) osmium tetroxide prepared in whey solution obtained from centrifuging the milk gel (to avoid changes in ionic environment during fixation). Samples in fixative were microwaved (Model 3470, Ted Pella Inc., Redding, CA) at high power under vacuum for 4 cycles of 2 min on then 2 min off, while the sample was maintained at 31°C, then stored overnight at 4°C. Prior to imaging, samples were cut into 1-mm thick slices, and placed for 20 min in 0.5% (wt/vol) aqueous periodic acid solution to oxidize the starch, and washed with several exchanges of deionized distilled water. Next, samples were immersed in 1% (wt/vol) aqueous acriflavine HCl to attach the fluorophore to aldehyde groups formed by oxidation of starch, using the same microwave procedure. Samples were washed till little fluorophore was visible in the wash water, and were next immersed in 0.01% (wt/vol) aqueous Rhodamine B to stain protein and again microwaved. Samples were again washed, then mounted on a glass slide with glycerol/gelatin (Horobin and Kieman, 2002) and covered with a coverslip.

To confirm imaging of starch was occurring, 50-mL solutions of 5-g/L starch in water were gelatinized then treated (at 22°C) with 0.1 g periodic acid and allowed to oxidize for 20 min. An additional 20 mL of water was added to each solution, then tubes were centrifuged 5 min at 3,000 g. Supernatant was decanted and more water added to bring the volumes back to 40 mL. Tubes were shaken to wash the pelleted material, then centrifuged and decanted again. Five milliliters of 0.3% (wt/vol) aqueous acriflavine was added to each tube. Solutions were allowed to stain 10 min, followed by extensive washing following the same protocol for washing out the periodic acid. Once no residual dye was apparent in supernatants, the gelatinized starch pellets were smeared on glass slides. A drop of water was placed on top of each smear, followed by a glass coverslip as described above.

Samples were imaged on a confocal microscope (MRC 20; Biorad, Hercules, CA) with a Kr/Ar laser exciting the acriflavine at 488 nm and the Rhodamine B at 568 nm. Emissions were from 488 to 650 nm and 550 to 750 nm, respectively, and exclusion filters of 512 to 532 nm and above 585 nm were used to capture the fluorescent signals of acriflavine bound to oxidized starch, and Rhodamine B bound to protein, respectively. Images were false colored with protein as red-orange and starch as yellow-green.

Statistical Analysis

Moisture contents were measured in triplicate. Curd and whey yields were calculated based on 4 samples per curd type. Alcohol precipitation was done in duplicate for 4 whey samples from each type of curd. Differences in alcohol-insoluble residue for all curd types were determined with SAS 9.0 (SAS Institute, Inc., Cary, NC) using PROC GLM with a one-way factorial design, Tukey-Kramer adjustment for mean comparison and significance declared at α = 0.05. Recovery factors were determined using 5 samples per starch. Confocal imaging was done
on at least 2 subsamples of 3 curd samples for each type of curd with images from multiple fields of each sample recorded.

RESULTS

Starch Swelling

Extent of starch swelling and the influence of heat treatment used for gelatinization are shown in Figure 1. Only starches 15 and 17 exhibited any difference in swelling based on type of heat treatment. The starches were then grouped according to water holding capacity by pooling over all heat treatments. Starches that held <1.0 g/g of water were classified as non-swelling (starches 11 and 12), 1.0 to 5.0 g/g as low swelling (starch 14), 5.1 to 15.0 g/g as medium swelling (starches 1, 2, 7, 8, 9, 13, and 17), 15.1 to 24.0 as high swelling (starches 3, 4, 5, 6, and 10), and >=24.0 g/g as very high swelling starch 15). Starches 10 and 16 separated poorly during centrifugation and so water retention values (see Fig. 1) for these starches could only be estimated or was not obtained.

Preliminary Curd Studies

Curd yield from the control milks ranged from 11.8% to 14.3% with the higher yield occurring in milk heated to 72°C and was indicative of the presence of denatured \( b \)-lactoglobulin being retained in the milk gel, hindering contraction of the casein network, and producing a higher moisture gel. The yield from all the control milks was higher than the 9% to 10% yield observed for Cheddar cheese but less curd compression occurs in the model system than in normal cheesemaking. The model system leaves out process steps applied to dry curd such as stirring, salting and pressing, that further concentrate the protein matrix and promote whey drainage from the curd. Adding starches to milk produced curd yields ranging from 11% to 28% when 5 g/L starch was added (Figure 2) and from 11% to 50% when 10 g/L starch was added (data not shown), depending on type of starch and heat treatment. Starches 11 and 12 (maltodextrin and dextrin starches) did not significantly increase curd yield. Starches 13 and 14 (tapioca starch and the traditional gelling, acid converted starch) only increased curd yield when milk had been heated to 72°C. At the 5 g/L level this increase could be explained by the anincrease in whey protein denaturation at the higher temperature (as occurs with the control milks) while at the 10 g/L level, the yields for starches 13 and 14 increased to 20% compared to 11% to 12% when heated at the lower temperatures. Starches 1 to 10, 15, 16 and 17 significantly increased curd yield, with the effect being more pronounced at higher concentration and heat treatment.

Higher heat treatment generally increases swelling of starch granules and incorporation of hydrated starch into the para-casein network that forms as the renneted milk coagulates, physically increases curd volume. In addition, presence of starch particles in the milk gel can physically block contraction of the protein network as well as reduce attractions between proteins if the starch chemically interacts with the para-casein protein matrix. This would increase curd volume (and curd yield) and produce a gel with higher moisture content.

Severity of heat treatment influenced curd yields (P < 0.001) for some of the starches (Figure 2). Undercooking

![Figure 1. Water retention.](image1)

![Figure 2. Curd yield.](image2)
Effects of starch addition on low-fat cheese curd and partitioning between curd and whey / D. J. McMahon

and overcooking can result in less than maximum swelling of the starch granules and there was some starch sedimentation in milk. For starches 11, 12, and 13 it was apparent that heating was insufficient for proper starch gelatinization. Starch settling during gel formation and centrifugation was considerable for the potato starches (6 and 15), the instant waxy corn starches (4 and 5), maltodextrin (11), dextrin (12) with the lower heat treatment, and the instant rice starch (17). There was little (if any) starch sedimentation when using the waxy corn starches (1, 2 and 3) or the waxy rice starch (7) (when heated to 72°C). There was less tendency for starch settling at 5 g/L than 10 g/L, and better curd properties and separation of whey, so 5 g/L was used in the second trial.

Physical appearance of the centrifuged control curd for all heat treatments were similar in that the curd was very firm and rubbery with good curd and whey separation. At a starch addition level of 5 g/L, there was a good whey separation obtained with starches 1, 2, 3, 7, 8, and 9. Poor separation occurred with starches 10 and 16. The whey released from gels containing starches appeared more viscous and slimy than the control gels indicating less entrapment of the starches 4, 5, 10, 16 and 17 in the curd and their release into the whey. There was an apparent demixing and settling of the gelatinized starch from the proteins in the milk gels when using tapioca starch 13 and the traditional gelling acid-converted starch 14. This produced curd after centrifugation that was softer at the bottom. The modified tapioca starches 10 and 16 had the most dramatic visible effect on curd properties. Both prevented the curd from settling during centrifugation, produced curds that lacked cohesion, and caused poor curd and whey separation. The whey from these samples was very slimy.

Curd Yield

In the second trial using WC, WR, IT, MT and DX starches, there was no difference in curd yields (or any other parameters measured) of the control milk gel after either the 72°C or 66°C for 30 min heat treatments (Table 2). A curd yield of 13.1 g/100 g milk was obtained. Curd yield was significantly (P < 0.05) increased to 18.4, 20.7, 21.5, and 23.5 g/100 g milk when WC, WR, IT and MT starches were respectively added to the milk. Adding DX starch did not significantly increase curd yield compared to the control.

Starch Retention

The 5 starches (WC, WR, IT, MT and DX) when tested alone were equally recovered using the alcohol precipitation method with recoveries calculated at 96% to 114%. When supernatant whey from the centrifuged control milk was subjected to alcohol precipitation there was a small amount of residue, 1.79 mg/g, that most likely consisted of proteins. The residues recovered from whey expelled from the WC, WR, IT, and DX starch-containing milk gels were 3.92, 3.24, 2.38 and 7.09 mg/g, respectively. Based on the amount of whey expressed, retention of these starches in the milk gels was 71%, 90%, 90%, and 1%, respectively. There was very poor separation of whey from curd when using MT starch that made it difficult to calculate total starch content because of interference from milk proteins, and its retention in the curd was estimated at 20%.

Starch Fluorescence

Starches WC, WR and IT when derivatized and stained using the periodic acid-acriflavine method fluoresced as shown for WR starch in Figure 3. The strongest fluorescence was observed around the residual starch granules, presumably because of the greater presence of oxidized glycol units that can bind the acriflavine. Both the MT and DX starches should have no granular structure, and similar particles seen using LSCM (data not shown) were probably unhydrolyzed material present in these materials. During preparation of these MT and DX starches there was almost no starch pellet following staining, washing, and centrifugation.

Curd Microstructure

Control.

Gels made with no starch had a microstructure that would be expected in a low-fat or non-fat system. The protein matrix dominated the curd structure, with few interruptions (Figure 4A) and the curd was visibly more compact than curd containing starch.

Waxy Corn Starch.

Gels containing WC starch (Figure 4B) had more openness compared to the control that was expected based on increased volume (yield) of curd obtained when WC starch was added. The protein network appeared less compact and swollen starch particles up to about 50 μm in size were distributed throughout the matrix. The swollen starch particles were relatively large, and irregularly shaped, and the granules appear to still remain somewhat intact. The starch particles appeared to be interacting with the surface of the protein network rather than being suspended in the moisture rich areas. An increased amount of dark space in the micrograph indicates more moisture was incorporated into the curd. Moisture rich areas also likely contain soluble starch, but at a level where it cannot be distinguished from background staining in the images.
Effects of starch addition on low-fat cheese curd and partitioning between curd and whey / D. J. McMahon

Table 2. Curd and whey yields after centrifugation of 200 mL of acidified renneted milk containing no starch (control) or 5 g/L waxy corn (WC), waxy rice (WR), instant tapioca (IT), modified tapioca (MT) starches or dextrin (DX), along with alcohol-insoluble residue in the whey and calculated starch content of the whey and retention in the curd.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Control</th>
<th>WC</th>
<th>WR</th>
<th>IT</th>
<th>MT</th>
<th>DX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curd yield, g/100 g milk</td>
<td>13.1a</td>
<td>18.4b</td>
<td>20.7c</td>
<td>21.5c</td>
<td>23.5d</td>
<td>13.2a</td>
</tr>
<tr>
<td>Alcohol insoluble residue, mg/g whey</td>
<td>1.79a</td>
<td>3.92abc</td>
<td>3.24ab</td>
<td>2.38a</td>
<td>6.30bc</td>
<td>7.09c</td>
</tr>
<tr>
<td>Total starch in whey, g</td>
<td>n/a</td>
<td>0.39ab</td>
<td>0.26a</td>
<td>0.10a</td>
<td>0.79bc</td>
<td>0.98c</td>
</tr>
<tr>
<td>Starch Retention in Curd, %</td>
<td>n/a</td>
<td>71ab</td>
<td>90a</td>
<td>90a</td>
<td>21bc</td>
<td>1bc</td>
</tr>
</tbody>
</table>

1calculated as residue weight less residue weight from control whey as a function of their respective volumes
2not applicable
3calculated as amount added to milk less amount lost in whey
4means within a row with the same letter were not different, α = 0.05

Figure 3. Starch granules.

Waxy Rice Starch.

The milk gel made with WR starch also had a more open structure consisting of a loose protein network extensively coated in small, round, discrete starch moieties (Figure 4C). Starch was located primarily at the protein-void interfaces, but some particles were visibly trapped within the protein network as well. The swollen starch granules were small and typically < 10 μm. From the curd structure it is clear that a high amount of starch was retained with the curd. There were large areas of continuous protein network, whereas curd with WC starch (Figure 4B) had a more interrupted protein network with smaller intact protein units. Waxy rice starch particles were smaller than WC, and there was a considerable amount of WR starch in the serum voids, unlike in the WC curd where WC appears more discrete in nature.

Instant Tapioca Starch.

Inclusion of IT starch in the skim milk gels also produced a more disrupted curd structure (Figure 4D). Unlike the WC and WR starches, IT starch did not appear to be preferentially located at the protein-void interfaces. Rather, it was dispersed throughout the protein matrix and filled the spaces it created in the protein, much as fat globules in full fat cheese do. The swollen starch particles were irregularly shaped and evenly distributed through the protein matrix.

Modified Tapioca Starch.

The milk gel made with MT produced a curd structure with the most interruptions (Figure 4E). The protein network appeared very loose, with numerous voids, although there were few distinct starch structures. The estimated MT starch retention in the curd was only 20%, and based on the lack of distinct starch regions in the curd structure this estimate seems correct. In spite of low starch retention, MT starch very effectively disrupted the protein network creating large channels and the highest curd yield (23.5 g/100 g milk). This is perhaps indicative of a starch gel being formed with a phase separation occurring between the protein and the starch polymers, with both gels being compressed during centrifugation. In solution with just water, the modified food starch has a high swelling power, and the slimy, mucous-like gel that was formed did not sediment when centrifuged at 2,000 g for 20 min. Likely
the starch interacted highly with the protein, inhibiting some of the protein-protein interactions that occur when milk is renneted and acidified, but due to the highly soluble nature of the starch, a large portion was lost with the whey and only the portion that was physically entrapped or interacting with the protein remained in the curd.

Dextrin.

Addition of DX did not increase openness of the curd (Figure 4F) and was similar to the control (Figure 4A) with its structure dominated by the protein network with few disruptions. There are some small, distinct starch particles imbedded in the protein that are likely some residual granules from preparation of the dextrin powder and are there only because they were physically entrapped in the curd as the curd formed and was compressed. Due to the low retention of DX in the curd and its inability to change the curd structure, it is likely it does not interact with casein.

DISCUSSION

Starch Imaging

Periodic acid oxidizes glucose residues of amylose and amylpectin chains at carbons 2 and 3, splitting the bond between the two carbons and converting the hydroxyl groups on each to aldehydes (Lehninger, 1975; McManus et al., 2009). The presence of the dialdehyde then facilitates binding to the starch by the pseudo-Schiff base acriflavine. The strongest fluorescence was observed around the residual starch granules, presumably because of the greater presence of oxidized glycol units that can bind the acriflavine. Amylose leached from granules would also bind acriflavine, but because of extensive rinsing steps during sample preparation soluble amylose molecules would be removed.

There was also a general background staining in all starch gels imaged because it is difficult to remove all excess acriflavine from the solutions. However, the amount of fluorescence was greater in areas where the fluorophore was concentrated and since acriflavine binds covalently to the derivatized starch there is more fluorescence in areas where there are more derivatized-starch aldehyde groups per unit volume. Both MT and DX starches are known to be smaller and more soluble than WC, WR, and IT starches, and the several washing steps required to properly gelatinize, oxidize and stain the starches may have removed much of the starch from the solutions. When staining starch within skim milk gels, there are fewer washing steps involved and the starch remains entrapped in the protein network, so washing away soluble starch materials present in the protein gel is not as much of a concern.

The curd samples used for LSCM imaging were initially stored in an osmium tetroxide in whey solution so as to fix protein, make the sample firmer and therefore easier to prepare for LSCM imaging. Previous attempts to analyze unfixed curd were unsuccessful as the curd was too soft to be thinly sliced and fell apart during washing and staining. Osmium was selected as the fixative of choice because of its ability to crosslink proteins without changing the microstructure of the material (Bourne and Danielli, 1967). Also, unlike glutaraldehyde, it did not add free aldehyde groups to the sample, which was important because of the starch-staining technique. Controlled microwaving of the samples during fixing, staining, and washing steps was done to hasten penetration of the fixative, stain, etc. (Horobin and Flemming, 1990).

Since acriflavine fluoresces without being bound to starch, a general background fluorescence from acriflavine

Figures 4A-4F. Starch milk gel.
Effects of starch addition on low-fat cheese curd and partitioning between curd and whey / D. J. McMahon

was expected, however, most excess acriflavine can be rinsed from the milk gels. With concentration of the fluorophore in starch-rich areas, the difference in level of fluorescence allowed for starch identification in the curd. Since skim milk was used to make the gel, the amount of fat present in the curd was minimal and was not imaged as there was not a fat stain that fluoresced at different wavelengths than acriflavine and rhodamine B.

Starch in Curd

The 5 starches used in the second trial were selected to represent the different performances in renneted milk observed in the initial study. Waxy corn starch 1 was selected to represent starches 1, 2 and 3 that all had similar properties. Dextrin (starch 12) was selected to represent starches with little apparent impact on curd formation and syneresis. Modified tapioca starch 10 was selected as it greatly increased the curd volume and changed the curd appearance. Instant tapioca (IT) starch 9 was selected as an example of a starch that had undergone gelatinization and drying steps during its manufacture. Waxy rice starch 7 was selected because it had a different botanical source from the other starches and has a much smaller granule size.

Waxy corn starch has a high amylopectin content, and because it is crosslinked and modified it is more resistant to degradation than its native counterpart. At 72°C, WC had a high swelling power relative to lower temperatures, but likely swells more if given a more severe heat treatment. Because it is more resistant, some granules are likely still intact within the curd matrix. In imitation low-fat cheese made with resistant corn starch, the starch was shown to be granular still and dispersed homogenously throughout the matrix, rather than forming swollen clusters (Noronha et al., 2007). Curd yield was close to 19%, evidence of WC's high affinity for water combined with prevention of curd shrinkage during centrifugation

Waxy rice starch was retained well in the curd and also increased yield more than other starches with the 72°C heat treatment. This starch has very small granules, high water binding capacity, and high amylopectin content. Because the unswollen granules only range from 3 to 8 mm, they can impart a creamy mouthfeel to products in which they are used. Rice starches have often been used as a fat mimetic. In the skim milk gel with WR starch there was good retention in the curd, and also a high yield. Curd was soft and voluminous, similar to the curd made with the WC starch. In a study of acidified skim milk containing rice starch (Zuo et al., 2007), it was found that rice starch acts as an inactive filler and absorbs water from the continuous phase thereby concentrating the protein, a trait likely more desirable in acidified milk products like yogurt where whey expulsion is undesirable, but in products like low-fat cheese further protein concentration is unwanted. If the heat treatment given to the milk and starch prior to cheese making is sufficient to cause maximal granule swelling prior to renneting and acidification of the milk, it may be possible to inhibit further water uptake by the starch from the protein.

Instant tapioca starch had very good retention in the curd. This starch was soft but cohesive, and had good curd and whey separation. Low- and reduced-fat feta cheese with tapioca starch has been studied (Sipahioglu et al., 1999), and it increased moisture and hardness of cheese, but did not significantly improve texture, flavor or overall acceptability over low-fat controls. However, in that study, milk did not receive an appreciable heat treatment once starch was added, suggesting the starch was more likely to absorb water from the protein during storage, and it may have given a grainy feel to the cheese. In our study instant tapioca starch was used, which requires very little heat for dispersion and swelling to occur.

Modified tapioca starches are typically used to change mouthfeel in dairy products. In the skim milk gels, it made the curd very soft, and not as cohesive as curds made with the other starches. Boundaries where the curd was cut didn’t meld back together well, as they did in other curds. The whey decanted from this curd was thicker and slimier than control whey, indicating starch loss in the whey. This was confirmed by the whey analysis test which showed only 21% retention in the curd. Interestingly, this curd also had the highest yield, perhaps indicative of the high hygroscopicity of this starch. The MT starch apparently disrupts protein interactions that normally occur in renneted acidified milk, thus restricting curd shrinkage and syneresis. This trait was even more apparent in curds made with 10 g/L starch in milk (data not shown), where separation of curd and whey was nearly impossible. However, at 5 g/L, this starch could likely impart the desired discontinuity to low-fat cheese by interfering with hydrophobic interactions that foster contraction of the curd protein matrix.

All curds made with starch had higher yields than controls, except for the DX starch-containing curd. This was expected because in tests measuring starch swelling under the heat treatments used, DX had very low swelling power (data not shown). It also had the lowest retention in curd, probably because it is made up of smaller molecules that are more soluble and less likely to get trapped within the curd. The curd made with DX starch had an appearance and feel similar to the control gel, which was firm and rubbery.

CONCLUSIONS

Low-fat cheese curd normally has a dense protein network. Addition of WC starch produced a loose protein network with irregularly shaped starch granules evenly distributed around and throughout that matrix; retention of WC in the curd was 71%. The WR starch was 90% retained and produced a curd that was also dense, with small
granules of starch coating the protein strands rather than being distributed throughout the curd. A 90% retention in curd was also obtained with IT starch and it similarly coated protein strands but was not in granular form. The MT starch interacted highly with the protein, as the curd network was very loose. The starch seems to be in solution around, as well as incorporated into the protein, but was only 12% retained in the curd. There was virtually no retention of DX in the curd. All starches except DX increased the curd yield with the effect being MT > IT = WR > WC > DX = Control. The WC, WR, and IT starches have potential for improving texture of low-fat cheese because they are retained well in the protein network during coagulation and produce interruptions in the curd network that may help limit extensive protein-protein interactions.

REFERENCES


Effects of starch addition on low-fat cheese curd and partitioning between curd and whey / D. J. McMahon


Texture and yield of low-fat cheddar cheese incorporated with \( W_1/O/W_2 \) emulsion with inulin as primary aqueous phase (\( W_1 \))

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ABSTRACT

The objective of this study was to enrich low-fat Cheddar cheese (6% fat) with inulin in the form of a water-in-oil-in-water (\( W_1/O/W_2 \)) emulsion and simultaneously improve the texture. Four batches of 16 kg skim milk were used for cheesemaking with 4 different treatments namely, low-fat cheese with cream (LFC1), low-fat cheese with 3.5% oil-in-water emulsion (LFC2), low-fat cheese with 5.8% \( W_1/O/W_2 \) emulsion (LFE1), and low-fat cheese with 3.5% \( W_1/O/W_2 \) emulsion (LFE2). The cheeses were stored at 4°C until analyzed for composition, texture, and optical microscopy. Mean moisture content of LFE2 was higher (\( P < 0.05 \)) than the control (LFC1), resulting in higher yield (\( P < 0.05 \)), lower hardness, gumminess, and chewiness. Other three cheeses, LFE2, LFC1, and LFC2 were same for yield. The net inulin content in LFE1 was calculated as 1.66% (wt/wt) which provided about 0.5 g of fiber per serving size of 28 g cheese. However, this amount of fiber does not fulfill the label declaration for low-fat cheese and therefore, further work to increase fiber level in cheese is recommended.

BACKGROUND

With the trend of developing low-fat foods and high fiber foods alternatives, the food processors still need to make high quality low-fat foods that match consumer taste expectation. Dairy products, especially cheeses, are criticized for their high fat content (Tunick et al., 1993). Consequently, their consumption by a health conscious populace is limiting. However, these people may not want to neglect the other benefits of dairy products such as protein, calcium, phosphate, bioactive peptides, vitamins, and other minerals (Renner, 1983). Several epidemiological and clinical studies have shown that a dietary pattern that is insufficient in dairy product content is associated with higher arterial pressure in the population and/or an increased prevalence of high blood pressure or hypertension (Miller et al., 2000; Wang et al., 2008). On the other hand, consumption of dietary fiber requires attention in western countries fighting against increasing obesity (Slavin, 2005). There is a large fiber gap to fill between usual intake of dietary fiber and recommended intake. According to 2010 dietary guidelines for Americans, an adequate intake for total fiber in foods is 38 g and 25 g/d for young men and women, respectively. This target without a major change in eating patterns requires enriching more and more foods with dietary fibers.

Incorporation of inulin as a source of dietary fiber in imitation cheeses was investigated by Hennelly and group (2006). They suggested that inulin was successfully incorporated into the imitation cheese matrix at a level of 3.44 g/100 g cheese replacing 63% of the total fat in the formulation without any significant effect on the melting characteristics. Inulin has also been used to improve the sensory properties and texture of low-fat yogurts (Kip et al., 2006).

This study is an effort to incorporate inulin in cheese without compromising the cheese texture and avoiding high losses of inulin into the whey. Incorporating inulin into a double emulsion (\( W_1/O/W_2 \)) and using for the manufacture of low-fat cheese should add fiber to low-fat cheese and also improve its texture.
MATERIALS AND METHODS

Emulsion Preparation

Double (W/O/W) emulsions were prepared using two-stage emulsification with some modification from Surh et al. (2007). First, a 40% (wt/wt) inulin solution was prepared by adding inulin powder (FructaFit IQ, Sensus, Rosendaal, The Netherlands) in a 5% NaCl solution at boiling temperature, gradually and with constant stirring using magnetic stirrer until clear solution was obtained. This was designated as aqueous phase W₁. Next, 8% (wt/wt) of polyglycerol polyricinoleate (PGPR) obtained from Palsgaard Industri de Mexico (St. Louis, MO) was mixed with canola oil (Western Family Foods Inc., Tigard, OR) at 25°C and the 40% inulin solution added at 40:60 ratio (W₁ to oil emulsion blend) using magnetic stirrer. Canola oil was chosen for the formulation because of its increased efficacy in maintaining double emulsion as compared to melted butter (preliminary study described in chapter 6). The inulin solution was slowly added to the oil phase while it was hot since the solution tended to crystallize and become viscous. This primary (W/O) emulsion was then mixed with 2% (wt/wt) of whey protein isolate (WPI) (Provon 292, Southwest cheese Co., Clovis, NM) slurry using magnetic stirrer in 20:80 proportion (W₁/O to WPI) to produce a secondary emulsion (W₂/O/W₁). The emulsion mix was then homogenized using a high speed blender (Omni General Laboratory Homogenizer, Omni International, Kennesaw, GA) operated at 5000 rpm for 1 min.

An O/W₁ emulsion was also prepared by adding canola oil to 2% WPI solution in 20:80 proportions, homogenized with high speed blender for 1 minute as described previously. The emulsions were formulated, covered with foil, and kept at room temperature (~22°C) in dark overnight before adding to cheese milk the next morning. During storage, the W₁/O/W₂ emulsion mixture became thick and viscous due to gelling of inulin as the emulsion temperature decreased to room temperature.

Cheesemaking

Skim milk (Western Family Foods Inc., Portland, OR) was obtained from a local supermarket. Prior to warming, L-lactic acid (Nelson and Jameson, Marshfield, WI) was diluted (1:16) and added to the milk, to reach a pH of 6.2. Milk was then stirred well, heated to 35°C. Emulsion volume to be added to the milk was then calculated using initial fat content of skim milk of 0.17%. Four batches of low-fat cheese were designed as LFC: low-fat control (total volume of 15.9 kg skim milk plus cream); LFE1: made from skim milk plus 5.8% W₁/O/W₂ emulsion mixture; LFE2: made from skim milk plus 3.5% W₁/O/W₂ emulsion mixture; and LFC2: made from skim milk plus 3.5% O/W₂ emulsion mixture. The milk was mixed with the above mentioned ingredients and homogenized using hand held blender (Model MP550, Robot Coupe USA, Inc., Ridgeland, MS) for 1 minute.

Milk was then inoculated with 0.02% lyophilized L. lactis culture (DVS 850, Chr. Hansen Inc., Milwaukee, WI) with continuous stirring. After 20 min, annatto (7.34 g/100 kg) color (DSM Foods Specialty Inc., Parsippany, NJ) was added and the mixture was stirred thoroughly. Double-strength chymosin (ChyMax, Chr. Hansen Inc., Milwaukee, WI) was diluted 20-fold with chloride-free cold water, added to milk, stirred for 2 min and let stand for 20 min. Curd was cut when firm with 1.6-cm wire knives, heated for 5 min, and gently stirred to avoid fusion of freshly cut curd cubes and to facilitate whey. The curd particles were cooked with constant stirring for 40 min and then half of the whey was drained. Curd particles were then stirred and allowed for wet acid development to reach pH 5.95. The remaining whey was drained and dry curd particles were continued to produce more acid and reach to pH 5.20. The curd was then washed with cold water (4°C) at a rate of ~0.5 kg/kg curd to lower curd temperature to 22°C. The curd was then weighed and salted at a rate of 2.0% of curd weight and applied in 3 applications, 5 min apart. The curd particles were hooped, and pressed at 60 kPa in vertical press for 18 hrs. The cheese blocks so formed were vacuum sealed and stored at 3°C for 2 weeks.

Composition

Moisture content was determined in triplicate by weight loss using a microwave oven (CEM Corp., Indian trail, NC) at 100% power with an endpoint setting of <0.4 mg weight change over 2 s. Fat content was determined in duplicate using a modified Babcock method (Richardson, 1985). Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher 400 (Seward, England). The slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (model 926, Corning, Medfield, MA). The pH was measured using a glass electrode after stomaching 20 g of grated cheese with 10 g of distilled water for 1 min at 260 rpm.

Texture Analysis

Texture profile analysis (TPA) of the cheese was performed (in triplicate) using a Texture Analyzer TTA.XT plus (Stable Micro Systems, Godalming, Surrey, UK) equipped with 2-kg load cell. The cheese textural parameters evaluated were hardness, adhesiveness, cohesiveness, gumminess, springiness and were calculated as described by Bourne (1968). The cheese samples were cut into cylindrical specimens (10 mm diameter x 20 mm) using a cork borer. The samples were tempered for 1 h at room
temperature (22°C) before analysis. A 2-bite compression test was conducted with 60% compression.

**Optical Microscopy**

The primary (inulin-in-canola oil) and secondary (inulin-in-canola oil dispersed in WPI solution) emulsions were imaged using optical microscopy to ensure that the inulin gel was enclosed in oil droplets. The emulsions were taken in a glass tube, gently stirred to normalize the mix. Then a drop was placed on a microscope slide and covered with a cover slip. Instantly, the slide was observed under the conventional optical microscope (Nikon microscope eclipse E400, Nikon Corp., Japan) equipped with a CCD camera (CCD-300-RC, DAGE-MTI, Michigan City, IN). More than 3 images were captured for each sample and a representative image for each trial is presented.

**Statistical Analysis**

Three replicate cheesemaking trials were undertaken over 3 d; all 4 cheeses with different emulsion formulation and type were produced each day. A randomized block design (4 treatments, 3 blocks) was used for analysis of the response variables relating to the composition, yield, and texture. Analysis of variance (ANOVA) was carried out using SAS 9.1.3 (SAS Institute, 1999) where the effect of treatment and replicates were estimated for all response variables. A split plot design was used to monitor the effects of treatment, ripening time and their interaction on the textural properties (hardness, springiness, gumminess, cohesiveness, chewiness, and adhesiveness) measured at regular intervals (15 and 30-d) during ripening. Analysis of variance for the split plot design was carried out using a generalized linear model (GLM) procedure of SAS (1999). Statistically significant differences (P < 0.05) between different treatment levels were determined by Fisher’s least significant difference.

**RESULTS AND DISCUSSION**

**Optical Microscopy**

Representative optical micrographs of inulin-incorporated emulsions are presented in Figures 7.1 and 7.2. The dispersion of inulin gel within the oil phase is shown in Figure 7.1. It is evident from the micrograph that inulin is uniformly distributed in oil as a W/O emulsion. Further, the suspension of W/O emulsion (primary) to WPI solution produces a W/O/W emulsion (secondary) as presented in Figure 7.2. Again, it is confirmed that the inulin gel is located within oil droplets. The high speed homogenization and microfluidization was avoided while preparing the emulsions to prevent the breaking of the double emulsions as occurred during preliminary trials, even at lower homogenizing pressures (500 psi) (see Appendix C Figure C1).

As shown in Figure 7.2, some of the primary water phase (W1) droplets were visible bigger in size than the other. This is because of the avoidance of high speed homogenization. In general, this type of emulsion formulation depicts bimodal or even trimodal distributions of particle size (Surh et al., 2007). The use of high pressure homogenization and membrane emulsifications are recommended to avoid gravity separation (Benichou et al., 2001). However, in our study, the emulsions were used as an ingredient mixture in cheese that eliminated the challenge but fairly immediate incorporation of emulsions to milk for cheesemaking is highly recommended.

**Cheese Composition**

Mean moisture content, fat, salt, pH, fiber, and yield of four treatments (control, 5.8% W1/O/W2, 3.5% W1/O/W2, and 3.5% O/W2 emulsion blends) are summarized in Table 7.1. The cheeses were made in three replicates and composition was reproducible for all replicates with consistent results throughout replications. As shown in Table 7.1, mean moisture content of low-fat cheese with 5.8% W1/O/W2 emulsion (LFE1) which was the targeted treatment among all four treatments, was the highest (P < 0.05) and significantly different from the control (LFC1). This had a straight impact on the final yield of the cheese resulting in an increase in the yield. Our goal of retaining inulin in cheese was fulfilled by obtaining 1.6% inulin in cheese.

According to the CFR Food labeling requirements, fiber content at this level is insufficient to be declared on the label because it is providing only 0.5 g of fiber per serving size (28 g). The fat content of all cheeses were in the range of 6.0 - 6.5% and achieved as target fat percent. Emulsion blends added to skim milk were calculated based on the initial fat content of 0.17% in skim milk and to produce low-fat cheese with about 6% fat. Protein content was in the range from 30.4% - 33.7%, ash from 2.8% - 4.4%, and salt content were in the range of 1.4% to 1.8%. These cheeses were salted at pH 5.5 and interestingly, the pH recorded after 3 days was 5.1 for all the treatments (Table 7.1).

**Texture Profile Analysis**

Texture profile of all treated cheeses was conducted at 15 and 30 d of storage. These time points were chosen because most of the textural changes in cheese curd are achieved in first 15 days (Lawrence et al., 1987) and then accomplished at 30 d followed by minimal changes in texture after 30 d. Among textural properties, hardness, adhesiveness, springiness, cohesiveness, gumminess, and chewiness were recorded in triplicate and for all 3
replicates. The mean values ± SEM are presented in Table 7.2. As shown in Table 7.2, increasing moisture content of LFE1 resulted in reduced hardness as compared to LFC1 (P < 0.05). For low-fat cheese texture, hardness is one critical point to be controlled (Mistry, 2001) and this parameter needs to be reduced for such cheeses.

The incorporation of inulin helped to achieve low hardness values for low-fat cheese which is very challenging. Interestingly, it was observed that the hardness was significantly higher for control (LFC1) when aged from 15 d to 30 d, but remained unchanged for emulsion cheeses (Figure 7.3). Other textural properties also significantly changed with the addition of inulin such as adhesiveness, cohesiveness, gumminess, and chewiness (Table 7.2). Comparing all 4 treatments, LFE1 which was made with inulin emulsion was observed as less hard, less gummy and less chewy than the control cheese LFC1. Cohesiveness values for LFE1 were indifferent at 15 day but then it was significantly lowered than the control cheese LFC1. This reduction in cohesiveness could be explained due to the open structure of protein matrix facilitated by emulsified fat droplets with gelled inulin in LFE1 (Sepulveda-Ahumada et al., 2000).

At equivalent moisture level, low-fat cheese control (LFC1) and low-fat cheese with O/W2 emulsion (LFC2) demonstrated significantly different hardness values irrespective of storage time (see Table 7.1 and 7.2). This trend is difficult to explain and we speculate it as a result from a complex interaction of a number of variables such as presence of cream (LFC1) versus canola oil (LFC2) as an emulsion blend with WPI solution. Accordingly, previous study has shown that the effect of fat replacement and the

**Table 7.1.** Mean composition and yield of cream and emulsion (W1/O/W2 or O/W2) incorporated low-fat cheeses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LFC1</th>
<th>LFE1</th>
<th>LFE2</th>
<th>LFC2</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Moisture</td>
<td>55.8a</td>
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<td>57.7a</td>
<td>56.3ab</td>
<td>0.001</td>
</tr>
<tr>
<td>Fat</td>
<td>6.2</td>
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<td>6.0</td>
<td>6.5</td>
<td>0.064</td>
</tr>
<tr>
<td>Ash</td>
<td>4.4a</td>
<td>3.5b</td>
<td>2.8c</td>
<td>4.0a</td>
<td>0.015</td>
</tr>
<tr>
<td>Protein</td>
<td>33.6a</td>
<td>30.4b</td>
<td>32.3a</td>
<td>33.0a</td>
<td>0.004</td>
</tr>
<tr>
<td>Fiber²</td>
<td>0.0c</td>
<td>1.6a</td>
<td>1.2b</td>
<td>0.2c</td>
<td>0.004</td>
</tr>
<tr>
<td>pH</td>
<td>5.1</td>
<td>5.1</td>
<td>5.1</td>
<td>5.1</td>
<td>0.098</td>
</tr>
<tr>
<td>Salt</td>
<td>1.8a</td>
<td>1.6ab</td>
<td>1.4b</td>
<td>1.7a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yield³</td>
<td>7.2b</td>
<td>7.7a</td>
<td>7.3b</td>
<td>7.2b</td>
<td>0.029</td>
</tr>
</tbody>
</table>

¹LFC1 = low-fat cheese made from skim milk plus cream; LFE1 = low-fat cheese made from skim milk plus 5.8% W1/O/W2 emulsion; LFE2 = low-fat cheese made from skim milk plus 3.5% W1/O/W2 emulsion; and LFC2 = low-fat cheese made from skim milk plus 3.5% O/W2 emulsion
²Fiber% = 100% - (moisture + ash + protein)
³Yield = [Total weight of pressed cheese/initial weight of milk] x 100%

¹Mean with different superscript within same row is different
Texture and yield of low-fat cheddar cheese incorporated with W₁/O/W₂ emulsion / D. J. McMahon

In contrast to the previous study (Hennelly et al. 2006) our study suggests that the presence of inulin as a part of double emulsion (W₁/O/W₂) significantly reduced the hardness of low-fat cheese comparing LFE1 and LFC2. However, level of inulin also played a vital role in reducing the hardness because LFE2 was no different than LFC2 even though LFE2 also contained inulin incorporated as double emulsion.

**CONCLUSIONS**

Inulin was effectively incorporated as W₁/O/W₂ emulsion in low-fat cheese. The addition of inulin improved the texture of low-fat cheese by reducing the hardness, gumminess, and chewiness which has been challenging and a universal complaint about low-fat cheeses. It is evident from the present study that the addition of gelled inulin increased the yield of low-fat cheese. However, the level of inulin in low-fat cheese is very low and entirely dependent on the use of oil in emulsion formulation as well as the ultimate fat level in cheese. Further, the addition of inulin as W₁/O/W₂ emulsion can be increased in reduced and regular fat cheeses.

**Table 7.2. Textural properties**¹ (hardness, adhesiveness, cohesiveness, gumminess, and chewiness) of low-fat cheese treatments² (LFC1, LFE1, LFE2, LFC2) evaluated at 15- and 30 day of storage using Universal testing machine.

<table>
<thead>
<tr>
<th>Textural parameter¹</th>
<th>Time (d)</th>
<th>LFC1</th>
<th>LFE1</th>
<th>LFE2</th>
<th>LFC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness</td>
<td>15</td>
<td>37.26±1.85A²</td>
<td>18.03±1.33B²</td>
<td>26.39±2.56B⁵</td>
<td>28.70±0.83B⁵</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>59.38±1.31A²</td>
<td>24.17±2.65A²</td>
<td>35.56±0.95A³</td>
<td>43.47±1.91A²</td>
</tr>
<tr>
<td>Adhesiveness</td>
<td>15</td>
<td>12.87±3.44CA²</td>
<td>7.21±1.26CA²</td>
<td>3.04±0.83aA²</td>
<td>4.21±0.84aA²</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>16.17±1.20aA²</td>
<td>18.82±1.39abA²</td>
<td>19.80±0.61abB²</td>
<td>20.82±0.43AB²</td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>15</td>
<td>0.82±0.01aA²</td>
<td>0.82±0.02aA²</td>
<td>0.84±0.01aA²</td>
<td>0.83±0.01aA²</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.71±0.02aA²</td>
<td>0.54±0.03aB²</td>
<td>0.62±0.01abB²</td>
<td>0.56±0.05bB²</td>
</tr>
<tr>
<td>Gumminess</td>
<td>15</td>
<td>30.40±1.22abA²</td>
<td>14.66±1.09aCA²</td>
<td>22.08±1.89aB²</td>
<td>23.83±0.48abB²</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>42.22±1.96aA²</td>
<td>13.63±1.91aA²</td>
<td>22.30±0.80aA²</td>
<td>23.84±1.06aB²</td>
</tr>
<tr>
<td>Chewiness</td>
<td>15</td>
<td>27.06±1.13aA²</td>
<td>13.00±0.97aA²</td>
<td>20.07±1.73aA²</td>
<td>21.91±0.44aB²</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>37.36±1.91aA²</td>
<td>11.89±1.76aA²</td>
<td>19.33±0.80aA²</td>
<td>20.69±1.09abA²</td>
</tr>
</tbody>
</table>

¹Units of measurement: hardness, gumminess and chewiness in Newton; adhesiveness (absolute values) in mm², and cohesiveness is dimensionless.
²LFC1 = low-fat cheese made from skim milk plus cream; LFE1 = low-fat cheese made from skim milk plus 5.8% W₁/O/W₂ emulsion; LFE2 = low-fat cheese made from skim milk plus 3.5% W₁/O/W₂ emulsion; and LFC2 = low-fat cheese made from skim milk plus 3.5% O/W₂ emulsion.

Means with different superscript in a row denote significant differences (P < 0.05) between the different trials of low-fat cheese for the same texture parameter.

Means with different superscript in a column denote significant differences (P < 0.05) between the different trials of low-fat cheese for the same texture parameter.

*Figure 7.3. Comparison of hardness of cheese at 15- and 30 d of storage. (abc: Different letter indicates significant difference at P < 0.05)*)
REFERENCES


Survival of microencapsulated probiotic *Lactobacillus paracasei* LBC-1e during manufacture of mozzarella cheese and simulated gastric digestion

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ABSTRACT

An erythromycin resistant strain of probiotic *Lactobacillus paracasei* subsp. *paracasei* LBC-1 (LBC-1e) was added to part skim Mozzarella cheese in alginate microencapsulated or free form at a level of 10⁶ and 10⁷ cfu/g respectively. Survival of LBC-1e and total lactic acid bacteria was investigated through the *pasta filata* process of cheese making where the cheese curd was heated to 55°C and stretched in 70°C-hot brine, followed by storage at 4°C for 6 wk and subjected to simulated gastric and intestinal digestion. This included incubation in 0.1 M and 0.01 M hydrochloric acid, 0.9 M phosphoric acid and a simulated intestinal juice consisting of pancreatin and bile salts in a pH 7.4 phosphate buffer. There were some reductions in both free and encapsulated LBC-1e during heating and stretching with encapsulated LBC-1e surviving slightly better. Changes in total lactic acid bacteria losses during heating and stretching did not reach statistical significance. During storage there was a decrease in total lactic acid bacteria but no statistically significant decrease in LBC-1e. Survival during gastric digestion in HCl was dependent on extent of neutralization of HCl by the cheese with more survival in the weaker acid in which pH increased to 4.4 after cheese addition. The alginate microcapsules did not provide any protection against the HCl. Interestingly, there was greater survival of the encapsulated LBC-1e during incubation in H₂PO₄. Proper selection of simulated gastric digestion media is important for predicting delivery of probiotic bacteria into the human intestinal tract. Neither free nor encapsulated LBC-1e was affected by incubation in pancreatin/bile solution. Based on survival during simulated gastric digestion, the level of probiotic bacteria that would need to be added to cheese to provide a beneficial health benefit is lower than is generally assumed for other fermented dairy foods or when consumed as supplements.

BACKGROUND

Probiotics are live microorganisms which when administered in adequate amounts (10⁶ to 10⁷ cfu/g) confer a health benefit on the host (FAO/WHO, 2002). Probiotics may be consumed either as a food component or as a non-food preparation. Foods containing such bacteria fall within the functional foods category and these are described as foods claimed to have a positive effect on health. However, for a probiotic bacteria to provide a benefit to human health it must fulfill several criteria. It must have good technological properties so that it can be manufactured and incorporated into food products without losing viability and functionality, it must maintain that viability through storage to end of shelf life of the food, and should not create unpleasant flavors or textures. It must also survive passage through the upper gastrointestinal (GI) tract and arrive alive at its site of action, and it must be able to function in the gut environment (Mattila-Sandholm, 2002).

Yogurts and other fermented milks have been used as delivery system for probiotic cultures although cheese has been suggested as a better carrier because of better survival of the probiotic culture during storage and through the GI tract (Ong et al., 2006; Sharp et al., 2008). Various cheeses have been investigated including: cheddar (Stanton et al., 1998; Phillips et al., 2006), low fat cheddar (Sharp et al.,
The pH and reduced oxygen levels of cheese make it suitable for long-term survival of probiotic bacteria (Boylston et al., 2004; Phillips et al., 2006). In contrast, lower pH and higher oxygen levels in a liquid matrix such as fermented milks and yogurt, may not maintain sufficient numbers (i.e., >10^7 viable cell/g) of the probiotic bacteria (such as some strains of Bifidobacterium spp.) through to the end of shelf life (Gardiner et al., 1999; Shah, 2000). The high buffering capacity and lipid content of cheese has also been suggested as providing protection to probiotic bacteria in the GI tract (Phillips et al., 2006). Thus, cheese could deliver viable probiotics in sufficient numbers to provide therapeutic effects through the entire shelf life (Burns et al., 2008).

To exert a beneficial health benefit via action in the distal ileum and colon, probiotic bacteria must survive passage through the esophagus, highly acidic stomach, and alkaline small intestine (Naidu et al., 1999). Losses of up to 6 to 9 log cfu/g of probiotic bacteria in simulated gastric digestion has been reported (Sabikh et al., 2010; Ortakci, 2010) depending on bacterial strain and testing conditions. Immobilization of bacteria within an encapsulating matrix has been investigated as a means to reduce cell injury or loss and improve their survival in foods (Muthukumarasamy and Holley, 2006; Kailasapathy, 2006; Ozer et al., 2009; Ortakci, 2010; Brinques et al., 2011) and during gastric digestion (Chandromouli et al., 2004; Picot and Lacroix, 2004; Muthukumarasamy et al., 2006; Pimentel-Gonzalez et al., 2009; Ding and Shah, 2009; Ortakci, 2010; Brinques et al., 2011).

To be effective, capsules should maintain integrity in the foodstuff and during digestion and passage through the GI tract, after which they should break down and release their contents. Different types of encapsulating materials have been used to trap probiotic bacteria. The most common is alginate because it has the benefits of being nontoxic, easy to form into a gel and readily available (Ding and Shah, 2009). Capsules containing bacteria can be extruded through a small orifice (such as a syringe needle) to produce droplets that then free fall into a hardening solution (Muthukumarasamy et al., 2006). This produces capsules that are about 2 to 3 mm in diameter. Another approach is to add an aqueous polymer suspension containing the bacteria into vegetable oil under gently stirring (Sheu and Marshall 1993) to create a water-in-oil emulsion. The polymer suspension as the discontinuous phase is then cross linked to form a gel and the beads can then be separated by chemically breaking the emulsion. The size of such beads depends on shear rate used to form the emulsion and varies from about 0.025 to 2 mm (Krazeekoopt et al., 2003).

A further complication to the measurement of survival of probiotic bacteria in fermented foods is how to enumerate them when the food contains high numbers of other bacteria that may have similar growth characteristics. In cheese, nonstarter lactic acid bacteria frequently can grow on media that is assumed selective for probiotic bacteria (Oberg et al., 2011). Accurate measurement can be achieved if the probiotic bacteria used for testing has a biomarker inserted that can be used to select for its growth (Sharp et al., 2008). We measured survival of an erythromycin-resistant strain of Lactobacillus paracasei to test its survival when encapsulated in alginate during the manufacture of low moisture part skim mozzarella cheese, during storage, and after incubation in simulated gastric and intestinal juices.

**MATERIALS AND METHODS**

### Materials

*Lactobacillus paracasei* subsp. *paracasei* LBC-1e, an erythromycin-resistant derivate (Broadbent et al., 2004) of the probiotic strain LBC-1 (Cargill, Waukesha, WI, USA), was obtained from the culture collection of Dr. Jeffrey Broadbent (Utah State University, Logan, UT). Streptococcus thermophilus starter culture TS-10C was donated by DSM Food Specialties USA Inc. (Eagleville, PA). Sodium alginate, Tween-80, erythromycin, bile salts were purchased from Sigma-Aldrich Co. (St. Louis, MO). Eliker’s Agar, de Man, Rogosa, and Sharpe (MRS) broth and agar were from Becton Dickinson (Franklin Lakes, NJ), peptone from EMD Chemicals Inc. (Gibbstown, NJ), pepsin from Mallinckrodt Baker Inc. (Phillipsburg, NJ), pancreatin from Spectrum Chemical Mfg Co. (New Brunswick, NJ), corn oil (Western Family Foods Inc., Portland, OR) was purchased from a local supermarket, CaCl_2, H_2O, HCl, NaCl, NaH_2PO_4 and Na_2HPO_4 were analytical reagent grade. Double strength chymosin rennet (Maxiren) was from DSM Food Specialties USA Inc. Fluorescein isothiocyanate (FITC) was from (Sigma-Aldrich, Inc., Saint Louis, MO), SYTO® 9 was a component of a LIVE/DEAD® BacLight bacterial viability kit (Invitrogen, Carlsbad, CA), microscope slides were from Mercedes Medical (Sarasota, FL) and Taylor Lube petroleum gel was from Haynes Manufacturing Co. (Westlake, OH).

### Bacterial Growth Conditions

Working cultures of LBC-1e were prepared from frozen stocks stored at -70°C by sequential transfer twice
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into MRS broth containing 3 μg/mL erythromycin in which the cultures were incubated anaerobically at 37°C for 18 h. After incubation overnight, the media containing cells were centrifuged at 4,250 × g for 10 min at 4°C, following which the supernatant was removed and the cells further washed twice (4,250 × g for 10 min at 4°C) with sterile 0.1% (wt/vol) peptone water. The washed cells were suspended to ~10^8 cfu/mL in saline/peptone-water by comparing optical density to a previously prepared standard curve (R^2 ≥ 0.9; data not shown).

Microencapsulation

Bacterial cells were microencapsulated in alginate by the modified emulsion method of Sheu and Marshall (1993). Briefly, 200 mL of cell suspension (~10^9 cfu/mL) was mixed with 800 g of a 25 g/kg sodium alginate solution with continuous stirring at 400 rpm for 10 min to entrap bacteria. The alginate-culture mixture was then added dropwise through a 50-mL sterile syringe fitted with a needle into 5 kg of corn oil containing 0.2% (wt/vol) Tween-80 with stirring (~500 rpm). When the oil-alginate mixture formed an emulsion, as evident from increasing turbidity, sterile 0.2 M CaCl_2 solution was added immediately to polymerize the alginate and break the emulsion. After 30 min, the Ca-alginate microcapsules formed were collected by vacuum filtration through Whatman #4 filter paper, washed twice in 0.2 M CaCl_2 then stored in peptone-saline solution at 4°C until use.

Mozzarella Cheese Manufacture

Fresh bovine milk was obtained from Utah State University's George B. Caine Dairy Research and Teaching Center (Wellsville, UT) and standardized to protein:fat ratio of 1.2 and pasteurized at 73°C for 15 s in the Gary Haight Richardson Dairy Products Laboratory. Two 16-kg portions of milk were warmed to 35°C in small stainless steel vats, 1 g of starter culture added to each, and the milk ripened for 60 min. Then the milk was renneted by adding 4 mL of chymosin, the milk stirred for ~2 min, and the adjunct probiotic culture quickly added as either 10 g of broth containing free LBC-1e cells (~1×10^10 cfu/g) or 360 g of encapsulated LBC-1e (~5×10^8 cfu/g), the milk was stirred and then allowed to coagulate. After 15 min, the curd was cut using wire knives (16-mm spacing), heated for 10 min, then stirred and heated to 41°C. With continued stirring, one-third portions of whey were removed after 15 and 35 min, then whey was drained down to curd level after a further 10 min. The curd was stirred for 10 min and washed with 13°C-water to cool the curd to 22°C, then drained and the curd salted at 10 g/kg.

Each batch of curd (containing either free or encapsulated LBC-1e) was divided into three 550-g portions and a sample collected for bacterial enumeration. Each replicate portion of curd was stretched in an excess amount of hot 70°C-brine (containing 50 g/kg NaCl) for about 1 min to heat the curd to 55 ± 1°C and held for 2 min. The hot cheese was then placed in a stainless steel mold and immersed in iced brine (containing 50 g/kg NaCl and 3 g/kg CaCl_2) for 2 h. The temperature of the cheese blocks decreased to ~24°C in 30 min, ~12°C after 1 h and was at 6°C by 2 h. Each block of cold cheese was sampled for bacterial enumeration, then cut into 4 pieces, vacuum packaged and stored at 4°C.

Bacterial Enumeration

Bacteria were enumerated from mozzarella cheese containing free LBC-1e after mixing 25 g of cheese with 225 mL 0.1% peptone water at 230 rpm for 10 min (stomacher Model 400; Seward, London, UK). For cheese containing encapsulated LBC-1e, 0.2 M phosphate buffer (pH 7) was used in place of peptone-water to disrupt the alginate gel and release the encapsulated bacteria. Total lactic acid bacteria (LAB) were enumerated on MRS agar with pour-plate method and was incubated anaerobically at 37°C for 48 h. LBC-1e was selectively enumerated on MRS agar containing 3 μg/mL erythromycin.

Simulated Gastric Digestive

To investigate the influence of pH on survival of probiotic bacteria, sterile filtered simulated gastric juice (SGJ) based on Mainville et al. (2005) containing 2.0 g/kg NaCl and 0.3 g/kg pepsin was prepared using HCl. To provide a pH of the SGJ-cheese mixture similar to physiological pH of the human stomach (~pH 1.4), SGJ-1 was made using 0.1 M HCl with a cheese/SGJ ratio of 1:6. For SGJ-2, the same acid concentration was used but the cheese/SGJ ratio was changed to 1:4, and then to simulate physiological conditions in which further acid is secreted into the stomach after food ingestion, additional HCl was added during incubation of the SGJ-cheese mixture to maintain pH < 3. Then to further test the effect of pH on bacterial survival and allow a greater rise in pH after adding the cheese, SGJ-3 was made using 11 mM HCl. An additional acid digestion test (SGJ-4) was performed using 87 mM phosphoric acid (pH 2.0) instead of HCl, as a comparison to Sharp et al. (2008). Prior to adding cheese, the SGJs were tempered to 37°C, then the mixture stomached for 10 min at 230 rpm and held at 37°C for up to 2 h with periodic shaking.

Simulated Intestinal Digestive

After treatment of cheese for 60 min in 0.1 M HCl (SGJ-1) the mixture was converted to simulated intestinal juice (SJ) (Huang and Adams 2004; Annan et al., 2008)

1 Sharp et al. (2008) incorrectly reported the strength of phosphoric acid they used as 8.7 mM.
by adding to 36 mL of the mixture, 1 mg/mL pancreatic and 4.5 g/mL bile salts suspended in phosphate buffer, and adjusting to pH 7.4 with 0.1 M NaOH. The cheese-SJJ mixture was then incubated for 4 h at 37°C with periodic shaking.

**Microstructure**

Thin slices (~10 x 10 x 3 mm) of mozzarella cheese mounted on microscope slides were treated with 5 g/L FITC in acetone-water (1:1) solution to stain for protein. The sample was rinsed twice with water and then treated with 3.34 mM of the SYTO®9 fluorophore. Stained samples were mounted on standard microscope slides with glycerin jelly and then examined using an inverted laser scanning confocal microscope (Biorad, Hercules, CA) with an Ar/Kr laser that provided excitation of both FITC and SYTO 9 at a wavelength of 488 nm. Emissions from the fluorophores were captured sequentially using filters of wavelength 512 to 532 nm for SYTO 9 and ≥ 585 nm for FITC.

**Statistical Analysis**

Log reductions in bacterial numbers as a consequence of hot water stretching and chemical analysis results of both cheese samples was analyzed by one way ANOVA and Tukey Multiple comparison test (SAS® PROC GLIMMIX) with three replicates. Log reductions in bacterial numbers during storage and as a consequence of SGJ or SJJ incubation were analyzed using repeated measurement and Tukey Multiple comparison test (SAS® PROC GLIMMIX) as a 2-way factorial with encapsulation as the treatment effect with 3 replicates. Significance was declared at P ≤ 0.05.

**RESULTS**

**Cheese Composition**

The cheeses had similar moisture of 55.5% and 55.6% and pH of 5.39 and 5.41, respectively, for cheese containing free or encapsulated bacteria. While a slightly lower fat content (12.5% versus 15.8%) and higher salt content (1.88% versus 1.70%), was observed (P < 0.05) for the cheese with the encapsulated bacteria. These differences were not expected to influence bacterial survival and were assumed to relate to the presence of the alginate microcapsules during milk coagulation and curd manufacture.

As shown in Figure 1, the microcapsules containing the LBC-1e bacteria (A) were located in the serum/fat pockets that form within the curd structure as the protein matrix forms and contracts as a consequence of renneting, acid development and curd agitation and cooking. Then during the hot-water stretching process the fat droplets are oriented into channels concomitantly with the protein matrix forming into fibrous strands (Oberg et al., 1993).

**Cheese Manufacture and Storage**

Initial numbers of total LAB in cheese curd were 8.3 x 10^6 and 2.2 x 10^6 cfu/g for curds that contained free and encapsulated bacteria, respectively (Table 1). Included in this enumeration are the *St. thermophilus* starter culture and the LBC-1e probiotic culture. Addition of LBC-1e to milk had been planned to provide about 1 x 10^8 bacteria per 28-g serving of cheese, assuming there would be a 10-fold concentration of bacteria during conversion of milk into curd with an allowance for potential loss of microcapsules by sedimentation prior to coagulation. The number of LBC-1e added to the milk had been estimated at ~6 x 10^6 cfu/g and ~1 x 10^7 cfu/g of free and encapsulated bacteria, respectively, and the amount measured in the cheese curd was 6.6 x 10^7 and 5.4 x 10^6 cfu/g, respectively. Thus, there was less loss of encapsulated bacteria during the coagulation process than anticipated and the 2 sets of cheese curd contained above the target of 3.6 x 10^6 cfu/g.

When the curd was immersed and stretched in hot water, there was a slight decrease in bacterial numbers (Table 1). Total LAB had a mean log reduction of 0.20 and 0.40 for control and encapsulated treatment, respectively. The extent of decrease for total LAB was not significantly different (P= 0.095) in the control cheese but was in the cheese containing the encapsulated bacteria. The survival of encapsulated LBC-1e bacteria was slightly higher (P = 0.012) than in cheese containing free bacteria, with log reduction of 0.25 and 0.45, respectively.

During 42-d storage there was no decrease (P=0.8387) in number of either free or encapsulated LBC-1e (Table 1) in the mozzarella cheese as has been shown for Cheddar cheese storage (Stanton et al., 1998; Gardiner et al., 1999; Sharp et al., 2008). However, the total LAB numbers decreased significantly (P<0.0001) during the storage period, indicating there was a slow die-off residual starter *St. thermophilus* bacteria occurring after first week of refrigerated storage.

**Simulated Gastric Digestion**

Extent of die-off of bacteria when the cheese was incubated in HCl-based SGJ was a function of both acid concentration and extent of neutralization of acid upon cheese addition. When cheese was incubated in SGJ-1 (0.1M HCl, pH 0.65,1:6 cheese/SGJ ratio) there was a 4.2 log decrease in LBC-1e numbers after 60 min incubation (Table 2). Initial mean bacteria counts were 5.6 x 10^7 and 3.5 x 10^8 cfu/g for free and encapsulated LBC-1e, and after 1 h they had fallen to 4.7 x 10^7 and 2.7 x 10^6 cfu/g respectively (P<0.0001). After adding the cheese, the pH of the mixture had increased to pH 1.4 which is similar to the
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Table 1. Mean microbiological counts for total lactic acid bacteria (LAB) and added *L. paracasei* LBC-1e (LBC-1e) in mozzarella curd (before hot stretching) and cheese during refrigerated storage for cheese in which LBC-1e was added to milk prior to renneting as free (control) or alginate-encapsulated bacteria. The total LAB includes the *Streptococcus thermophilus* starter culture, free or encapsulated LBC-1e, respectively, and any nonstarter lactic acid bacteria present in the cheese.

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Total LAB</th>
<th>LBC-1e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Encapsulated</td>
</tr>
<tr>
<td>0 (curd)</td>
<td>$8.19 \times 10^8$</td>
<td>$1.78 \times 10^9$</td>
</tr>
<tr>
<td>2 (cheese)</td>
<td>$5.00 \times 10^8$</td>
<td>$6.62 \times 10^8$</td>
</tr>
<tr>
<td>7</td>
<td>$6.61 \times 10^8$</td>
<td>$1.82 \times 10^8$</td>
</tr>
<tr>
<td>21</td>
<td>$2.24 \times 10^8$</td>
<td>$7.38 \times 10^8$</td>
</tr>
<tr>
<td>42</td>
<td>$2.23 \times 10^8$</td>
<td>$7.13 \times 10^8$</td>
</tr>
</tbody>
</table>

*Before hot stretching process of curd*

*After hot stretching process of curd*

Table 2. Mean loss (n=3) of free (control) and encapsulated *Lactobacillus paracasei* LBC-1e in mozzarella cheese after incubation in simulated gastric juice (SGJ) and simulated intestinal juice (SIJ).

<table>
<thead>
<tr>
<th>Juice</th>
<th>Composition</th>
<th>Ratio$^1$</th>
<th>Time (min)</th>
<th>pH</th>
<th>Loss During Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Control (log$_{10}$ cfu/g)</td>
</tr>
<tr>
<td>SGJ-1</td>
<td>0.1M HCl</td>
<td>1:6</td>
<td>60</td>
<td>0.65</td>
<td>1.4</td>
</tr>
<tr>
<td>SGJ-2</td>
<td>0.1M HCl$^2$</td>
<td>1:4</td>
<td>120</td>
<td>0.65</td>
<td>2.3</td>
</tr>
<tr>
<td>SGJ-3</td>
<td>0.01M HCl</td>
<td>1:6</td>
<td>120</td>
<td>1.5</td>
<td>4.4</td>
</tr>
<tr>
<td>SGJ-4</td>
<td>0.9M H$_3$PO$_4$</td>
<td>1:9</td>
<td>120</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>SIJ$^3$</td>
<td>Bile/pancreatin</td>
<td>1:6</td>
<td>240</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

$^1$Ratio of cheese added to simulated gastric solutions.

$^2$Additional 0.1M HCl was added to SGJ-2 during incubation to maintain pH < 2.5.

$^3$Survival in SIJ was determined by adding 1 mg/mL pancreatin and 4.5 g/mL bile salts suspended in phosphate buffer after incubating cheese in SGJ-1 for 60 min, then adjusting pH to 7.4 with 0.1 M NaOH.

$^{ax}$ and $^{cx}$ Means within a same column with same letters are not significantly different (α=0.05).

$^{by}$ Means within a same row with same letters are not significantly different (α=0.05).

ph of the human stomach before ingestion of food. In such a harsh environment, there was no protection provided by encapsulation of the bacteria in calcium-alginate.

When a greater proportion of cheese is added (such as in SGJ-2), the pH of the cheese-SGJ mixture increases above that maintained in the stomach, primarily because of the buffering capacity of the protein and phosphate in the cheese. By periodically adding 0.1M HCl during incubation this buffering effect was countered and the final pH of SGJ-2 was 2.3. Under these conditions, there was greater survival of bacteria compared to SG-1 and numbers of free and encapsulated LBC-1e in SGJ-2 decreased from initial mean values of $1.5 \times 10^7$ and $2.6 \times 10^8$ cfu/g to $1.5 \times 10^6$ and $1.1 \times 10^7$ cfu/g, respectively after 2 h incubation ($P<0.0001$). Again there was no observed protection on bacterial survival in SGJ-2 provided by encapsulation.

Loss of bacteria after 2 h incubation in 11 mM HCl (SGJ-3) was minimal with 90% of LBC-1e surviving (i.e. only $\sim 1$ log reduction) for both free and encapsulated bacteria. Initial mean counts were $3.29 \times 10^7$ and $2.55 \times 10^8$ cfu/g for free and encapsulated bacteria, respectively, and these had dropped to $3.14 \times 10^6$ and $1.08 \times 10^7$ cfu/g after incubation ($P<0.0001$). Such survival can be attributed to buffering of SGJ-3 by the cheese, and it was observed that pH of this gastric solution increased to pH ≥ 4 within 2 min. This explains why Gardiner et al. (1999) found no reduction in probiotic bacteria in cheddar cheese after 2-h incubation in porcine gastric juice which had an initial pH of 2.0 and a final pH of 4.74 and did not really simulate physiological conditions encountered by probiotic bacteria.

When acid tolerance of LBC-1e was tested using phosphoric acid (SGJ-4) so that buffering affects of the cheese would be negated, there was again very poor survival ($P<0.0001$) of bacteria in the control mozzarella cheese containing free LBC-1e. The pH of SGJ-4 remained $\sim 2$ during incubation and mean numbers of free LBC-1e fell from an initial level of $3.3 \times 10^7$ cfu/g to $2.0 \times 10^7$ cfu/g after incubation ($P<0.0001$) (Table 2). This agrees with
Sharp et al. (2008) who reported a 3.8 log reduction after 2-h incubation in SGJ-4 of a low fat cheddar cheese containing *Lb. paracasei* 334e. Interestingly, greater survival was observed when the bacteria were present in cheese in encapsulated form (Table 2). Mean numbers of LBC-1e were 2.6 x 10⁸ cfu/g prior to incubation and remained at 2.3 x 10⁷ cfu/g after the 2-h incubation (P<0.0001).

When the cheese-SGJ-1 mixture was neutralized and bile and pancreatic added to form SJ1, there was a slight increase in bacterial numbers to 4.7 x 10⁷ and 2.7 x 10⁸ cfu/g for the free and encapsulated bacteria respectively. Probably because of resuscitation of some cells that were sub-lethally injured during the 1-h incubation of SGJ-1. Incubation of the cheese-SJ mixture for 4 h at 37°C did not significantly affect survival of either free or encapsulated LBC-1e (Table 2) indicating a natural bile resistance of this probiotic strain.

**DISCUSSION**

There have been conflicting reports on whether alginate encapsulation of probiotic bacteria increases their survival in SGJ. Hansen et al. (2002) found no improvement in survival of acid-sensitive bifidobacteria incubated in SGJ at pH 2, 4, or 6 using alginate microspheres with diameters <100 µm. Krasaekoopt et al. (2004) also reported no increased survival of *Bifidobacterium bifidum* in alginate capsule in HCl-SGJ at pH 1.55. In contrast, Muthukamarasamy et al. (2006), who encapsulated 5 different strain of *Lactobacillus reuteri* in 2% alginate beads (using both the emulsion (~40 µm microcapsules) and extrusion (~2 mm capsules) methods) reported a protective effect in SGJ at pH 1.5 (0.08 M HCl, 0.2%NaCl). Using the same SGJ (0.08 M HCl, 0.2% NaCl, pH 1.5), Ortakci (2010) observed a similar effect using ~2-mm alginate capsules containing *Lactobacillus acidophilus* ATCC 4356 with a ~3 log reduction in encapsulated bacteria and no survival (~9 log reduction) of free *Lb. acidophilus* ATCC 4356 under the same conditions. This supports the notion that the matrix in which the bacteria are present can influence their survival as shown by Sharp et al. (2008), and consuming bacteria as a liquid culture is not as effective as when it is present in a semi-solid matrix.

We have shown that the buffering effect of a food that is delivering probiotic bacteria can influence bacterial survival when tested in vitro using an acid medium to simulate gastric contents of the human stomach. However, such buffering provides an artifactual result because in vivo in a healthy individual, additional acid is secreted into the stomach to maintain acidity at or below pH 2. Under such conditions, a 4 log reduction in bacterial numbers can be expected in foods with greater reductions occurring when bacteria are ingested in a non-protected form.

With ingestion of 10⁷ cfu probiotic bacteria as an isolated culture, no survival following passage through the stomach would be expected, and perhaps only 10² in a liquid or soft gel food such as yogurt (Sharp et al., 2008). If the probiotic culture is delivered in a hard gel such as cheese, the expected survival after gastric passage would be 10⁵. Thus, by having 10⁶ cfu/servery of a probiotic food in cheese, 100- to 1,000-fold more living cells of this bacteria would survive into the intestinal tract where it needs to be present to have any beneficial health impact. If the aim is to deliver 10⁴ bacteria/g to the intestines, this could be achieved starting with as little as 10⁶ cfu/g in cheese since only a 2 log reduction during simulated gastric digestion occurs when pH was maintained similar to physiological conditions. In comparison, a serving of 10⁶ bacteria is now considered the minimal amount needed a semi-solid food but in liquid media the starting level is 10ⁱ⁰ bacteria/g. This would have a dramatic effect on reducing the cost of producing probiotic foods.

The *in vitro* test of gastric survival using phosphoric acid (SGJ-4) was useful to provide a buffering effect when the bacteria were present in a free form, but was not a good test for acid tolerance of the bacteria in the alginate microcapsules. There was possibly some interaction between the phosphoric acid and Ca-alginate capsules that may have prevented penetration of acid into capsules. As shown in SGJ-2 in which HCl was replenished during incubation so as to maintain acid conditions of the cheese-SGJ mixture, encapsulation was unable to provide any significant protective effect for the bacteria.

**CONCLUSIONS**

Hot stretching during Mozzarella cheese manufacturing caused a slight log reduction of 0.4 and 0.2 in the numbers of free and encapsulated *Lb. paracasei* LBC-1e respectively. Refrigerated storage did not make any difference in the numbers of both free and gel encapsulated LBC-1e, however, during storage, the total LAB numbers in both cheeses (predominantly the starter *St. thermophilus* bacteria, decreased significantly. Encapsulation did not increase the survival in SGJ containing HCl. Rather, survival was dependent on pH of the SGJ-cheese mixture with mean reduction of 4.0, 1.5 and 1.1 with final pH of 1.4, 2.3 and 4.4 respectively, showing the importance of selecting proper conditions for simulating gastric digestion. Interestingly, alginate encapsulation increased survival of LBC-1e in phosphoric acid buffer.
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