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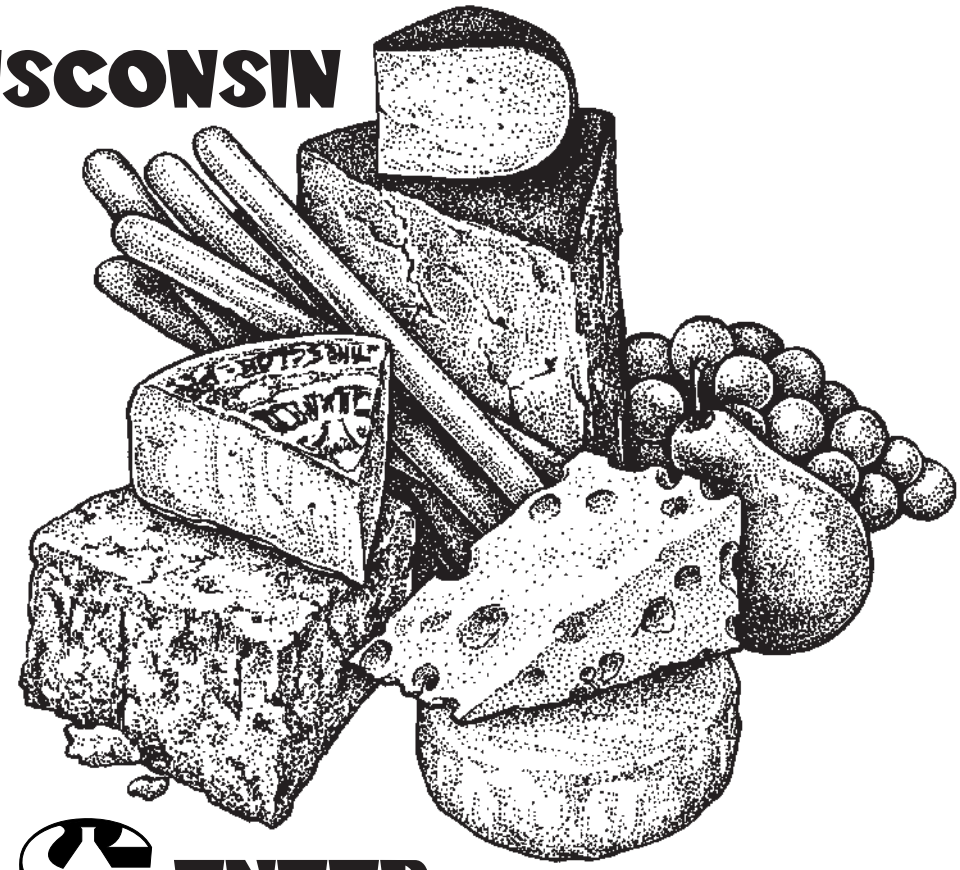

Center for Dairy Research

Annual Report 2001

Wisconsin
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CDR Annual Report

Published March 1, 2002, by the Wisconsin Center for Dairy Research.

Our annual report is a technical overview of CDR funded research and other Center activities during fiscal year 2001. This document was prepared for organizations funding CDR and for fellow dairy researchers. Although it describes projects in progress and interpretations of data gathered to date, it is not a peer-reviewed publication.

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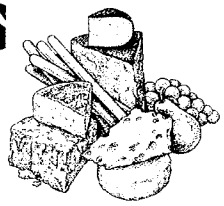
CONTENTS



Chapter One, Interim reports

Relationship between cheese melt profiles and chemical/textural/sensory properties 3

Development and application of a cheese shred/texture map delineated by cheese rheological, sensory and chemical analysis 3

Identification of physical/chemical changes in shredded cheese over time 4

Alpha-lactabumin production for clear bottled drinks and nutraceutical 4

Technical and economic development of a milk refinery 5

"Whey Refinery" for producing proteins for beverages and nutraceuticals 6

Development of a meltability test for process cheese 6

Developing pH-sensitive biodegradable smart hydrogels using whey protein concentrate 7

Large amplitude nonlinear viscoelastic behavior of mozzarella cheese during twin-screw extrusion 8

Model development for manipulation of rheological properties of cheese 9

Texurization of butter and butter spreads 9

Crystallization kinetics of calcium lactate 10

Development of Parmesan cheese flavor using selected bacteria 10

Use of whey proteins in pasteurized processed cheese products 11

Biochemistry of full and reduced fat Cheddar shred ripening 11

Mother liquor for production of lactose and a calcium-based product 12

Development of information manuals on controlling whey flavor 12

Identification and characterization of components of the proteolytic enzyme system of *Lactobacillus helveticus* which effect bioactive peptide accumulation 13

New starter systems for accelerated ripened Cheddar cheese 14

Production of intensely flavored Cheddar-type cheeses by adjunct cultures 14

Mechanisms for intensifying and modulating cheese flavor: A global approach 15

Determination of the relationship between galactose and lactic acid content of sweet whey and the effect on the spray drying process 15

Analysis of the economic impact of cheese defects 16

Control of annatto cheese colors in whey products 16

Chapter Two, Final reports

Inhibition of pathogens on process cheese slices at abuse temperature 19

Determination of caloric bioavailability and apparent lipid digestibility of liquid mikfat fractions 32

Minimizing the watering-off of unripened lower fat and no-fat mozzarella cheese 34

Investigating reasons for hardening of reduced fat Cheddar cheese during heating 39

Identification and characterization of components of the proteolytic enzyme system of *Lactobacillus helveticus* which affect bioactive peptide accumulation 51

Using neural networks to predict the pH of finished cheese 53

Improvement of Cheddar cheese quality through identification and characterization of microbial dation of bitter peptides in cheese 58

Growth of non-starter lactic acid bacteria in reduced fat Cheddar cheese 67

Succinate production by Lactobacillus casei: Pathways responsible and development of strategies to control its accumulation 72

Genomic survey sequencing of *Bifidobacterium longum* VMKB44 82

Chapter Three, CDR Applications program

Cheese Industry and Applications Program 89

CDR Communications Program 95

Safety/Quality Applications Program 100

Whey Applications Research Program 102

CDR specialty cheese applications program 105

Dairy marketing and economics program 107

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International Flavors and Fragrances (IFF)
Kraft Foods Technology Center
Land O' Lakes Inc
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Our Mission Statement

The Wisconsin Center for Dairy Research will serve as a national leader in strategic research to improve the competitive position of the dairy industry by linking Center/ University faculty, staff, students and the dairy/food industries to address key issues resulting in transfer of technology and communication of information.

Chapter One
Interim Reports

Relationship between cheese melt profiles and chemical/textural/sensory properties

Personnel

Chen, Carol; Muthukumarappan, K.

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 12/31/2002

Objecives

1. To characterize the effect of selected manufacturing protocols on cheese melt profiles.

2. To correlate cheese melt profile characteristics to chemical/textural/sensory properties.

3. To develop strategies based on correlations that enable cheesemakers to design manufacturing practices which result in specific melt/flow characteristics for food application systems.

Development and application of a cheese shred/texture map delineated by cheese rheological, sensory and chemical analysis

Personnel

Chen, Carol

Funding

Wisconsin Milk Marketing Board

Dates

7/1/2000 to 12/31/2002

Objectives

1. To develop a shred/texture map of cheese based on rheological, sensory and chemical measurements.

2. To define manufacturing protocols of Cheddar and mozzarella tailored for shredding.

Identification of physical/chemical changes in shredded cheese over time

Personnel

Chen, Carol

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 12/31/2002

Objectives

1. To characterize physical/chemical/sensory characteristics over time of shredded cheese in consumer-sized packages.

2. To determine the effect of flow agents on the physical/chemical/sensory projects of shredded cheese.

Alpha-lactabumin production for clear bottled drinks and nutraceutical

Personnel

Etzel, Mark

Funding

Dairy Management, Inc.

Dates

1/2/2001 to 12/31/2002

Objective

Produce alpha-lactalbumin of a purity and absence of denatured protein, denatured protein, suitable for use in development of clear, bottled drinks and nutraceutical beverages.

Technical and economic development of a milk refinery

Personnel

Etzel, Mark

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 12/31/2002

Objectives

1. Determine the technical capabilities of various MF systems from different suppliers for the separation of casein from milk serum proteins (i.e., how complete and clean is the separation) and the efficiency of subsequent UF concentration of the serum proteins.

2. Determine the throughput, yield, and recovery of the ion exchange chromatography step as a function of feed stream properties and target protein fractions.

3. Determine the technical properties and opportunities for use of casein concentrates (liquid or dry), and casein and milk serum protein fractions as dairy ingredients in non-cheese applications.

4. Determine the costs (capital, fixed, variable, operational, etc.) for the MF/UF and ion exchange chromatography aspects of fractionation and concentration of the milk protein streams.

5. Determine the potential market and utilization of milk refinery products (i.e., opportunities).

"Whey Refinery" for producing proteins for beverages and nutraceuticals

Personnel

Etzel, Mark

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 12/31/2002

Objectives

Purified whey proteins are uniquely suited for use in clear bottled drinks and nutraceutical foods for enhancing infant nutrition, suppressing appetite, treating diseases such as phenylketonuria, and other applications. The objective of this project is to develop a "whey refinery" technology to economically manufacture purified whey proteins suitable for new uses and novel application in beverages and nutraceutical foods. Specifically, we will:

1. develop and demonstrate an ion exchange process to produce purified alphalactalbumin, beta-lactoglobulin, lactoferrin, lactoperoxidase, and κ-casein glycomacropeptide from a single stream of whey.

2.Test the purified whey proteins for purity, activity, and/or clarity after heat treatment.

Development of a meltability test for process cheese

Personnel

Sundaram Gunasekaran

Funding

Dairy Management, Inc.

Dates

7/1/2000 to 6/30/2002

Objectives

Cheese meltability is a manifestation of combined effects of structure and phase changes in cheese during heating–i.e. both rheological and thermal effects should be considered simultaneously. By continuously measuring the cheese flow vs. time data, under lubricated squeeze flow configuration, we can evaluate the cheese meltability and related parameters objectively and reliably. By incorporating unique design elements, multiple samples can be tested simultaneously within a short time (about 5 min.).

1. To develop an automated test device and procedure to determine melt characteristics of process cheese for routine use in plant and quality assurance laboratories.

2. To validate the results from the new melt measurement device and procedure based on the information about process cheese composition and technological factors.

3. To relate process cheese melt characteristics to dynamic rheological data to ensure that we are measuring some valid physical/rheological properties.

Developing pH-sensitive biodegradable smart hydrogels using whey protein concentrate

Personnel

Sundaram Gunasekaran

Funding

Dairy Management, Inc.

Dates

7/1/2001 to 6/30/2003

Objectives

The overall objective is to develop new biodegradable smart hydrogels using whey protein concentrate (WPC).
Hypothesis: Whey protein-based hydrogels exhibit a pH-sensitive swelling behavior. Therefore, they can be used as carrier matrices for pH-sensitive controlled delivery applications.

1. To develop new pH-sensitive hydrogels using whey protein concentrate and characterize their swelling behavior as a function of swelling medium and gel preparation conditions.

2. To determine the release kinetics of some model biologically active substances from whey protein-based hydrogels in various pH media.

Large Amplitude Nonlinear Viscoelastic Behavior of Mozzarella Cheese During Twin-Screw Extrusion

Personnel

Gunasekaran, Sundaram

Funding

Dairy Management, Inc.

Dates

4/1/2000 to 12/31/2001

Objectives

1.To measure rheological parameters via dynamic, nonlinear viscoelastic experiments using a sliding-plate rheometer.

2. Test and develop constitutive equations (rheological equations of state) that describe the flow of cheese in a win-screw extruder.

3. To obtain optimal extrusion and operating conditions as a function of cheese type, composition and the desired end-use quality.

4. To evaluate the functional properties (melt, stretch, and oiling-0ff) of the extruded cheese.

Model development for manipulation of rheological properties of cheese

Personnel

Gunasekaran, Sundaram

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 6/30/2002

Objectives

1. Develop a model that defines physical and functional properties (melt, stretch, end-use properties, etc.) by rheological and other measurements at room and elevated temperatures that are related to typical industry measurements and ultimate cheese use. This model will establish a defined target for cheese makers to reach which is crucial for tailor-making of specified cheeses.

2. Validate the model(s) developed for their applicability using cheeses manufactured with specific make parameters to manipulate certain functional properties.

Texurization of butter and butter spreads

Personnel

Hartel, Richard

Funding

Dairy Management, Inc.

Dates

4/1/2000 to 6/30/2002

Objectives

The primatry objective of this project is to understand how to control lipid crystallization rates during tecturization of dariy spreads in order to influence the rheological properties. Specifically, the objecytives are:

1. To dertermine the effects of processing conditions in the Gerstenberg and Agger texturizer unit on crystallization of milk fat in dairybased spreads.

2. To correlate the rheological properties of these products to their crystalline structure, based on processing conditions, types of fats mixed together and storage conditions.

Crystallization kinetics of calcium lactate

Personnel

Hartel, Richard

Funding

Dairy Management, Inc.

Dates

7/1/2001 to 12/31/2002

Objectives

The primary objective of this project is to investigate the factors that influence crystallization kinetics of calcium lactate. We will study crystallization kinetics in model solutions, in expressed cheese serum and on the surface of cheese itself. The effects of various storage conditions (temperature, temperature fluctuations, etc.) and chemical composition (calcium and lactate content, pH, other salts, other constituents of importance in cheese, etc.) will be evaluated.

Development of Parmesan cheese flavor using selected bacteria

Personnel

Johnson, Mark; Steele, James; Lindsay,Robert

Funding

Dairy Management, Inc.

Dates

7/1/2001 to 12/31/2003

Objectives

1. Define and verify the chemistry of flavors produced in Parmesan cheese made with specifically selected adjunct lactic acid bacteria that provide flavor notes known to characterize high quality, aged Parmesan cheese. Hypothesis: By correlating chemical and sensory data from experimental Parmesan cheese, we will be able to identify and establish commercially viable starters and adjunct lactic acid bacteria and cheese manufacturing methods to produce Parmesan cheese with intensified flavors.

2. Construct derivatives of *Lactobacillus helveticus* CNRZ32 that overexpress specific esterase activity. Hypothesis:We believe that esterase activity, i.e. production of specific esters, provides specific desirable flavor notes in Parmesan cheese. The lactobacilli used as starters for Parmesan cheese lack sufficient esterase activity to adequately develop full, aged Parmesan flavor.

Use of whey proteins in pasteurized processed cheese products

Personnel

Lucey, John

Funding

Dairy Management, Inc.

Dates

7/1/2001 to 6/30/2002

Objectives

1. To determine the influence on the rheological, textural, and sensory properties of pasteurized processed cheese products.

2. To investigate the influence of denaturation and further processing treatments on whey products when they are subsequently incorporated into processed cheese products.

Biochemistry of full and reduced fat Cheddar shred ripening

Personnel

Rankin, Scott

Funding

Dairy Management, Inc.

Dates

4/15/2001 to 12/31/2001

Objectives

Characterize the effects of gas composition (CO2, N2, CO2/N2 blends) and light exposure on the biochemical, microbial, and textural ripening indicies of MAP packaged
Cheddar cheese shreds. An understanding of the degree to which MAP storage variables influence cheese ripening will enable processors to start to select conditions condusive to optimal ripening and storage quality.

Mother liquor for production of lactose and a calcium-based product

Personnel

Smith, Karen

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 12/31/2002

Objectives

1. Evaluate microfiltration (MF) system and centrifugation for ability to remove calcium from DLP.

2. Initial determination of feasibility of separating calcium from DLP.

3. Determine the composition and type of calcium product produced from DLP.

4. Compare resulting calcium from DLP product with currently available dairy calcium products.

5. Produce acceptable products containing calcium ingredients

Development of information manuals on controlling whey flavor

Personnel

Smith, Karen

Funding

Dairy Management, Inc.

Dates

7/1/2000 to 12/31/2002

Objectives

1. Develop two manuals: a) Manual for whey producers (cheese makers) on how they can affect whey flavor and minimize problems.b) Manual for whey handlers that outlines methods for handling whey which minimize flavor problems.

Identification and characterization of components of the proteolytic enzyme system of *Lactobacillus helveticus* which effect bioactive peptide accumulation

Personnel

Steele, Jim

Funding

Dairy Management Inc.

Dates

4/1/2000 to 12/31/2001

Objectives

1.To screen strains of *Lactobacillus helveticus* for the type and level of bioactive peptides/bioactive peptide precursors which accumulate as the result of the organisms growth in milk.

2. Determine which components of the proteolytic systems of the selected strains of *Lb. Helveticus* are essential for the accumulation of the bioactive peptides/bioactive peptide precursors from milk.

3. Construct strains of *Lb. Helveticus* which accumulate elevated levels of the bioactive peptides/bioactive peptide precursors of interest.

New starter systems for accelerated ripened Cheddar cheese

Personnel

Steele, Jim; Broadbent, Jeff

Funding

Dairy Management, Inc.

Dates

7/1/2000 to 12/31/2002

Objectives

1. Determine bitter taste thresholds for casein derived peptides in a cheese model system.

2. Define the contribution of *Lactobacillus helveticus* CNRZ32 peptidases to the hydrolysis of casein derived bitter peptides.

3. Construct food-grade *Lactococcus lactis* S2 derivatives with enhanced activity of peptidases demonstrated to be important in hydrolysis of bitter peptides.

4. Develop a food-grade, genetic system for proteinase gene exchange in industrial strain of *Lactococcus lactis.*

Production of intensely flavored Cheddar-type cheeses by adjunct cultures

Personnel

Steele, Jim

Funding

Dairy Management, Inc.

Dates

3/16/2000 to 12/31/2002

Objectives

1. Construct strains of *Lactobacillus casei* which produce elevated levels of diacetyl.

2. Construct strains of *Lactobacillus casei* which over-express a bacterial lipase known to enhance cheese flavor.

3. Manufacture processed cheese from Cheddar cheeses having significantly elevated levels of free fatty acids or furanones and pyrazines.

Mechanisms for intensifying and modulating cheese flavor: A global approach

Personnel

Steele, James L.

Funding

Dairy Management, Inc.

Dates

1/1/2001 to 12/31/2003

Objectives

The overall objective of this project is to assemble a comprehensive database on the metabolic potential of *Lactobacillus helveticus* CNRZ32, an important cheese flavor-enhancing bacterium, for modulating and intensifying cheese flavor development. Based on our previous experience in cheese flavor research, it is our hypothesis that genome sequence analysis of CNRZ32 is the most expedient method to identify many of this bacterium's genes encoding enzymes involved in cheese flavor development. Because of their similarities, it is also our hypothesis that this knowledge can be applied to other important dairy lactic acid bacteria.

Specific objectives:
1. Determine the nucleotide sequence of the *Lactobacillus helveticus* CNRZ32 genome

2. Assemble a comprehensive database of CNRZ32 genes likely to be involved in modulating or intensifying cheese flavor.

Determination of the relationship between galactose and lactic acid content of sweet whey and the effect on the spray drying process

Personnel

Wendorff, Bill

Funding

Dairy Management, Inc.

Dates

7/1/2001 to 6/30/2002

Objective

To determine the relationship between galactose and lactic acid content of whey and its effects on spray drying.

Analysis of the economic impact of cheese defects

Personnel

Smukowski, Marianne; Wendorff, Bill

Funding

Dairy Management, Inc.

Dates

7/1/2001 to 12/31/2002

Objectives

The purpose of this study is to collect data concerning cheese defects and to examine the severity to which manufacturers in the cheese industry are financially penalized for such defects. This study will identify the most common defects and assist the industry in correcting these defects

1. Survey cheese manufacturers for specific defects

2. Assess economics of the industry-wide impacts of the specific defects

3. Extrapolate data based on tonnage

Control of annatto cheese colors in whey products

Personnel

Wendorff, Bill; Lindsay, R. C.

Funding

Dairy Management, Inc.

Dates

1/1/2001 to 12/31/2002

Objectives

Hypothesis: The annatto-based off-colors in dry whey products are caused by the adsorption of annatto colorants onto protein or protein-lipid particles, and these off-colors can be minimized by oxidative bleaching and/or processing to disrupt and remove the adsorptive complexes.

1. Determine the quantitative binding capability of commercially-important forms (native, denatured, and delipidated) of whey proteins for annatto cheese colorants.

2. Devise commercially-applicable methods to minimize or eliminate annatto off-colors in dry whey products.

Chapter Two
Final Reports

Inhibition of pathogens on process cheese slices at abuse temperature

Personnel

Larson, Ann; Glass, Kathleen; Granberg, Dawn; McDonnell, Lindsey; and Johnson, Eric, Food Research Institute

Funding

Dairy Management Inc.

Dates

March 2000 to March 2001

Objectives

1. Evaluate the survival of *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* serotypes, *Escherichia coli* O157:H7, and *Bacillus cereus* on pasteurized process cheese (PPC) slices stored at 27°C for up to 7 days.

2. Analyze the variation in formulations of PPC slices with regards to moisture, pH, salt, and water activity.

3. Identify major factors that control pathogen growth in PPC slices, providing a basis for which predictive models evaluating safety of process cheese slices can be developed.

Summary

According to its Federal Standard of Identity (CFR, 1999) pasteurized process cheese (PPC) is heated during manufacture to >150°F for >30 seconds. Such heating is sufficient to inactivate bacterial foodborne pathogens, but not the spores of *Clostridium botulinum.* Certain combinations of moisture, salt, and pH can inhibit botulinal growth and toxin production in formulation-safe products (Kosikowski 1997, Tanaka, 1986).

The U.S. Food Code defines a "Potentially Hazardous Food" as one that can support the "growth and toxin production of *C. botulinum*" or the "rapid and progressive growth of infectious or toxigenic microorganisms" (U.S. 1999). "Rapid" is measured in hours rather than in days or weeks (U.S. FDA, 1986). This category includes foods with pH >4.6 and water activity >0.85. By virtue of its pH and water activity, PPC is classified as a potentially hazardous food and thus, according to the Food Code, must be kept refrigerated at ≤5°C unless laboratory evidence verifies its safety.

A recent study (Glass, 1998) evaluated the ability of six lots of commercial PPC slices from two manufacturers to support the growth of four foodborne pathogens during a 4 day incubation at 30°C. The formulations tested allowed survival of *S. aureus*, but not rapid growth of the pathogen (defined as growth in <12 hours). In addition, numbers of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 decreased during incubation. Results of that research supported the position that unopened packages of properly formulated pasteurized process cheese can be safely stored unrefrigerated for limit times (Nelson, 1984).

Additional work in this laboratory (K. Glass, J. McDermott, and J.H. Nelson, unpublished results) demonstrated a 1 log CFU/g increase in *S. aureus* levels on retail cheese food slices stored seven days at 27°C, but not at four days. The moisture levels of cheese food (44%) are higher than for process cheese slices (40%), yet growth of *S. aureus* was delayed in the cheese food. Results of these two studies confirm that *S. aureus* may grow slowly on process cheese and cheese food slices if contaminated and held at abuse temperatures for >4 days.

The objective of this study was to more thoroughly assess the safety of process cheese stored at abuse temperature by determining the potential for growth of five foodborne bacterial pathogens inoculated on PPC slices and stored at 27°C for up to 7 days. This study was designed to generate safety information on representative process cheese formulations in part to determine if process cheese slices can be exempt from the potentially hazardous food category, allowing limited out-of-refrigeration storage.

Cheese slices

Two lots each (different production dates) of twenty formulations of PPC slices produced by 13 different manufacturers were tested. These included 15 formulations of American, two Sharp American, two Swiss, and one combination American/Swiss formulation. Slices were obtained either directly from manufacturers or were purchased at local grocery stores, and were stored at 4°C until inoculation.

Principal ingredients in all of the process cheeses tested included American, Cheddar, and/or Swiss cheese, cream (milkfat), water, and citrate- and/or phosphate-based emulsifiers. Other ingredients in some cheeses included sorbic acid, enzyme-modified Cheddar cheese, acetic acid, color, lecithin, and (additional) salt.

Bacterial strains and media

The survival of five pathogens, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* serotypes, *E. coli* O157:H7, and *Bacillus cereus*, was evaluated separately in PPC slices stored at 27°C. Where possible, acid tolerant strains, outbreak strains, or strains used in previous challenge studies were chosen (Table 1).

Strains were grown individually in Trypticase soy broth (TSB; BBL Microbiological Systems, Cockeysville, MD), at 37°C for 18 hours, harvested by centrigation at 2,500 X g for 20 minutes and washed with PBS. Three strains of each pathogen were mixed in approximately equal concentrations and the inocula diluted in PBS to deliver approximately 10^3 CFU/g of cheese.

Population of each three-strain mixture and of individual strains were verified by plating serial dilutions on Trypticase soy agar (TSA) and on appropriate selecive media. *S. aureus* was plated on Baird-Parker Agar (Accumedia Manufacturers, Inc., Baltimore, MD) with egg yolk-tellurite enrichment, *L. monocytogenes* on modified Oxford agar (Oxoid, Unipath LTD, Ogdensberg, NY), *Salmonella* serotypes on xylose lysine desoxycholate agar (XLD;

Table 1 Bacterial strains used to challenge process cheese slices.

Strain designation	Source	Reference
Staphylococcus aureus		
FRI 196E	Ham isolate; toxin types A, D	
FRI S6	Shrimp isolate; toxin types A, B	
FRI 952	Whipped butter isolate; toxin type A	
Listeria monocytogenes		
Scott A	Human isolate	Glass 89,91
LM101	Hard salami isolate	Glass 89,91
LM310	Goat cheese isolate associated with human spontaneous abortion	
Salmonella		
S. enteritidis E40	Chicken ovary isolate	Glass (91) Leyer, 92
S. heidelberg S13	Human isolate; SHL 39902	Glass (91) Leyer(92)
S. typhimurium S9	Human isolate; SHL 40467	Glass (91)
Escherichia coli O157:H7		
C7927	Human isolate; 1991 MA apple cider outbreak	Besser
F-90	Sausage isolate; 1994 CA dry-cured salami outbreak	CDR (95)
F-5854	Cheese curd isolate; 1998 WI cheese curd outbreak	CDR (00)
Bacillus cereus		
B4AC	ham isolate associated with diarrheal outbreak	
F4491	vanilla pudding isolate associated with diarrheal outbreak	
BC101E	pasteurized liquid egg isolate	

BBL), *E. coli* O157:H7 on MacConkey agar with sorbitol (Accumedia), and *B. cereus* on mannitol-egg yolk-polymyxin agar (MYP; Difco, Beckton Dickinson Microbiological Systems, Sparks, MD).

Inoculation method

Slices were equilibrated at room temperature (21°C) for approximately 30 minutes before inoculation. Individual slices were surface-inoculated on one half of the cheese slice with 2.63 µl/g (50 µl/19 g slice) of a three-strain mixture for a given pathogen to yield approximately 10^3 CFU/g. Control slices for each lot were similarly treated with 2.63 µl/g of sterile PBS. Slices were folded so that the inoculated surface was toward the inside, placed into sterile oxygen-permeable polyethylene sampling bags (*Fisherbrand* 18 oz. capacity, Fisher Scientific, Pittsburgh, PA) and the bags closed securely. Slices were stored at 27°C for up to 7 days (168h).

Proximate analyses of slices

Proximate analyses including pH, moisture, NaCl, and water activity were performed for each lot on duplicate uninoculated slices and on control slices treated with 2.63 µl/g of 0.01 M phosphate-buffered saline (PBS, pH 7.2) per slice. The pH was determined directly with a Corning 340 pH meter (Corning Glass Works, Corning, NY) and an Orion 8104 combination pH electrode (Orion Research, Boston, MA). Moisture was measured by the AOAC 926.08 vacuum oven method (5 h, 100°C) (Richardson, 1990). Salt was analyzed as chloride by automated silver nitrate titration (Metrohm 682 titrator, Brinkman, Westbury, NY). Water activity (a_w) was determined with a CX-2 water activity meter (Decagon Devices, Pullman, WA).

Phosphate, citrate (citric acid), and sorbate (sorbic acid) levels were determined for duplicate uninoculated control slices of each lot using AOAC procedures 962.02 (Johnson, 1990), 920.126 (Richardson, 1990), and 974.10 (Fazio, 1990), respectively. Protein analysis was performed by the Soil and Plant Analysis Lab, University of Wisconsin-Extension.

Microbial analyses

Samples were assayed at time-0 and after 1, 12, 24, 36, 48. 72, 96, and 168 h storage at 27°C. At each testing interval, three slices for each lot/pathogen combination were assayed for pathogen populations. One slice for each PBS-treated control was assayed for standard plate counts as well as yeast and mold counts, and an additional control slice monitored for pH changes.

For microbial analysis, the entire sample was diluted 1:3 (w/v) in PBS and thoroughly homogenized using a LabBlender Stomacher Model 400 (Cooke Laboratory Products, Alexandria, VA). Serial dilutions were made in PBS, and 0.1 ml aliquots surface-inoculated onto duplicate plates of appropriate selective media (listed above). Plates were incubated at 37°C for 24-48 h. The appearance of typical colonies on selective agar was considered confirmatory.

Aerobic plate counts and yeast and mold counts were determined for single control samples of each cheese lot at each testing interval by diluting samples as described above, and plating on plate count agar (PCA; Difco Laboratories, Detroit, MI) and potato dextrose agar (PDA; Difco) acidified to pH 3.5 with tartaric acid. PCA plates were incubated at 37°C for 24-48 h, and PDA plates were incubated at 25°C for up to 5 days.

Proximate analysis of control samples

Proximate analyses, including % moisture, pH, % NaCl, and A_w of untreated slices (no PBS added) of both lots of each formulation of PPC tested are summarized in Table 2. The addition of PBS to control slices did not significantly alter A_w or moisture values; therefore it was not considered a variable in this study. The results of phosphate, sorbate, citrate, and protein analysis for uninoculated control samples are also summarized in Table 2.

The Federal Standard of Identity 21 CFR Part 133.169 (U.S.FDA, 1996) for pasteurized process cheese (PPC) made from a single variety of cheese allows for a moisture content not more than 1% greater than the maximum moisture content for the cheese itself, although an exception allows for maximum 44% moisture in pasteurized process Swiss cheese. The maximum moisture content for PPC made from two varieties of cheese must not be more than 1% greater than the average of the allowable maximum moisture contents for the two cheeses. Thus, maximum allowable moistures of Standard-of-Identity PPC made from Cheddar, Swiss, or a mixture of the two are 40%, 44%, and 41%, respectively.

Moisture values of American (including Sharp American) PPC slices tested in this study ranged from 37.68%-41.05%. Seventeen of 34 American lots tested had moisture values above 40%. Moisture values of all four lots of Swiss PPC slices were below 44%, ranging from 42.81-43.71%. The two lots of Swiss/American PPC had moisture values (39.27%-39.39%) below the allowed maximum. For 16 of 20 formulations, moisture values varied <1% between duplicate lots.

The pH of slices ranged from 5.48-5.99. For eight of 20 formulations, pH values varied more than 0.1 units between duplicate lots. Salt levels (% NaCl) ranged from 1.30%-2.79%. For five of 20 formulations, salt levels varied more than 0.1% between duplicate lots. Water activity values ranged from 0.908-0.950. For six of 20 formulations, A_w values varied more than 0.01 units between duplicate lots.

Phosphate levels in cheese samples ranged from 0.33% to 4.15%, and in most cases were similar between duplicate lots. Citric acid (citrate) levels varied widely, ranging from 0.393% to 4.704%. Citric acid levels often varied widely between duplicate lots. Protein levels ranged from 16.88% to 22.50%. Sorbic acid levels ranged from 0.0008% to 0.358%. Tested sorbic acid levels were >0.1% in cheeses for which sorbic acid was listed as an ingredient on the product label.

Microbial analysis of inoculated samples

Growth or survival of pathogens on inoculated PPC slices are summarized in Table 2. Numbers of *L. monocytogenes*, *Salmonella* serotypes, *E. coli* O157:H7, and *B. cereus* remained steady or decreased on both lots of all formulations during 7 days of incubation at 27°C. More specifically, in most formulations, *L. monocytogenes* decreased approximately 0.5-1.0 log CFU/g in 7 days, but was still detected by direct plating on all samples (>1.18 log CFU/g). Numbers of *Salmonella* serotypes, *E. coli* O157:H7, and *B. cereus* on most formulations decreased approximately 1-2 log CFUg over 7 days, and were not detected by direct plating on 2, 3, and 6 lots out of 40, respectively, at day 7. There were no obvious correlations between survival of these four pathogens and moisture levels, pH, % NaCl, or a_w of cheeses.

Numbers of *S. aureus* increased on five lots of PPC during 7 days of incubation at 27°C. More specifically, *S. aureus* increased approximately 1 log CFU/g in 7 days on cheeses 4A and 4B. On cheeses 6A, 13A, and 13B, numbers of *S. aureus* increased approximately 4 log CFU/g in 7 days, to levels of 6.2, 8.6, and 8.0 log CFU/g, respectively. Growth of *S.aureus* on cheese 6A was evident at 36 hours, but numbers above 6 log CFU/g were not noted until day 7. Growth of *S. aureus* on cheeses 13A and 13B was evident by 24 hours, and numbers had increased above 7 log CFU/g by 4 days. Staphylococcal enterotoxin generally is not detected in a food until numbers of *S. aureus* reach at least 6-7 log CFU/g, but this number varies widely between food substrates (Jay, 2000).

In all other lots of cheese, numbers of *S. aureus* remained steady or decreased <1 log CFU/g during 7 days of incubation. Additionally, numbers of *S. aureus* had increased 1-3 log CFU/g in sporadic samples of cheeses 3A, 3B, 10A, 10B, 12A, 12B, 15A, 18A, 19A, and 19B tested after 14 and 28-30 days of incubation at 27°C (data not shown).

Proximate and microbial analysis of control samples treated with PBS

The pH of control samples of all formulations treated with PBS remained relatively constant during 7 days of incubation at 27°C (Table 2; Figures 1A-20B). Initial (time-0) yeast and mold counts ranged from below detectable levels (<1.18 log CFU/g) to 1.88 log CFU/g, and increased to 3-4 log CFU/g in sporadic samples of cheeses 6A, 6B, 12A, 12A, and 17B during incubation (data not shown).

Initial (time-0) aerobic plate counts ranged from 1.48-2.99 log CFU/g and remained relatively stable in most formulations during 7 days at 27°C (data not shown). Interestingly, aerobic plate counts increased >2 log CFU/g within 7 days only in cheeses 6A, 13A, and 13B, which also supported significant growth of *S. aureus.*

Pasteurized process cheese (PPC) and related products have an excellent safety record in the United States (M. Johnson, 1990). During the past 50 years, very few foodborne outbreaks have

Table 2 Proximate analysis for pasteurized process cheese slices

Product	Manuf.	Type	lot	% moisture	ph	% NaCl	water activity	% phosphate	% citric acid	% sorbic acid	% protein
1	C	American	A	37.68	5.63	2.08	0.908	0.92	0.645	0.247	18.79
			B	38.60	5.74	2.01	0.936	0.72	2.132	0.249	19.03
2	D	American	A	39.21	5.66	1.74	0.935	0.77	1.554	0.302	18.18
			B	39.38	5.67	1.73	0.936	0.49	1.952	0.240	18.57
3	D	American/Swiss	A	39.39	5.74	1.77	0.939	0.33	1.674	0.181	18.11
			B	39.27	5.61	1.72	0.928	0.89	1.124	0.235	18.47
4	E	American	A	39.05	5.61	1.30	0.943	2.30	0.963	0.138	18.29
			B	40.63	5.48	1.61	0.931	3.35	0.604	0.155	18.03
5	F	American	A	39.48	5.67	1.90	0.950	1.10	1.505	.190	18.43
			B	38.60	5.62	1.98	0.940	1.16	1.508	0.177	17.19
6	G	American	A	38.17	5.66	1.71	0.916	1.88	0.393	0.0011	20.16
			B	40.03	5.97	1.68	0.927	2.18	0.945	.0008	18.58
7	H	American	A	40.47	5.65	1.69	0.937	0.91	0.840	0.242	17.53
			B	40.31	5.68	1.61	0.919	0.89	2.040	0.256	17.84
8	I	American	A	40.21	5.53	2.02	0.929	1.57	0.87	0.0011	20.94
			B	40.26	5.51	1.83	0.931	1.41	0.94	0.0034	19.47
9	I	Sharp American	A	41.05	5.57	1.46	0.932	1.38	0.48	0.0107	18.00
			B	40.47	5.63	1.65	0.933	1.81	0.68	0.0017	19.38
10	I	Swiss	A	43.38	5.66	1.50	0.922	1.93	0.48	0.0023	21.60
			B	43.71	5.60	1.44	0.921	1.70	0.40	0.0027	19.90
11	J	American	A	40.34	5.77	1.69	0.946	0.90	1.330	0.193	17.02
			B	39.71	5.73	1.66	0.944	1.12	1.558	0.251	16.88
12	K	American	A	40.05	5.72	2.05	0.937	1.06	0.945	0.191	19.45
			B	38.72	5.63	1.98	0.933	1.00	0.895	0.178	19.18
13	L	American	A	39.90	5.99	2.39	0.935	1.62	0.534	0.0022	18.47
			B	40.10	5.93	2.43	0.934	1.86	1.044	0.0017	18.78
14	L	American	A	39.22	5.86	2.54	0.920	1.34	1.025	0.181	18.77
			B	39.93	5.67	2.65	0.917	1.70	0.874	0.192	18.75
15	L	American	A	39.63	5.76	2.36	0.927	0.93	1.390	0.182	18.43
			B	39.64	5.62	2.33	0.935	1.02	1.153	0.171	17.61
16	L	Swiss	A	42.81	5.65	2.38	0.920	1.96	1.265	0.191	22.05
			B	43.45	5.79	2.46	0.931	2.02	0.405	0.162	22.50
17	M	American	A	40.02	5.61	2.51	0.932	1.21	0.63	0.218	18.00
			B	38.98	5.62	2.30	0.927	1.17	4.704	0.262	17.86
18	N	American	A	40.30	5.70	2.52	0.918	0.92	0.944	0.236	17.79
			B	40.04	5.65	2.61	0.926	4.15	0.703	0.183	18.15
19	N	Sharp American	A	39.82	5.75	2.79	0.915	1.07	1.043	0.184	17.88
			B	40.41	5.79	2.70	0.923	0.92	0.813	0.167	17.43
20	O	American	A	40.15	5.70	1.62	0.919	1.16	2.816	0.358	17.82
			B	40.29	5.54	1.64	0.925	1.01	1.837	0.266	17.47

been attributed to contaminated process cheese products. One exception is a botulism outbreak in 1993 associated with the consumption of contaminated cheese sauce served in a delicatessen (Townes 1996). The investigation revealed that the canned cheese sauce had been properly processed to inactivate spores, but botulinal toxin was produced in the sauce after a can was opened, contaminated with *Clostridium botulinum*, and left unrefrigerated. Because previous work in this laboratory has shown that botulinal toxin was not produced in formulations of PPC similar to those tested in this study (K. Glass, unpublished data), toxin formation by *C. botulinum* was not evaluated in this study.

Although most standardized process cheese products produced in the United States are formulated to inhibit botulinal growth and are heated to inactivate vegetative pathogens, post-processing contamination at home or in a food service environment by vegetative pathogens is considered a potential risk factor for these products. In the Netherlands, a survey of household kitchens found that 20% were contaminated with *Listeria* spp. (Cox, 1989). Spores of *Bacillus cereus* are heat resistant, can usually survive pasteurization conditions, and may be common postpasteurization contaminants in dairy products (Meer, 1991). *B. cereus* is widely distributed in nature, and is frequently isolated from dairy products (Wong, 1988).

The levels of salt found in cheese products don't always inhibit growth of bacterial foodborne pathogens, especially *S. aureus* and *L. monocytogenes*, which are salt tolerant. Seven of 11 imitation cheeses supported extensive growth and toxin production by *S. aureus* after a minimum of 4 days at 26°C, but growth and toxin production could not be correlated with pH, a_w, or product formulation (Bennett, 1983). Numbers of *L. monocytogenes* increased >4 log CFU/g in 3-4 days in whey or skim milk containing 6% NaCl during incubation at 22°C (Papageorgiou, 1989).

Even if bacterial pathogens don't grow in cheese or cheese products, many can survive over extended periods of time in such products. Survival of even a few organisms can be a concern with pathogens such as *Salmonella* and *E. coli* O157:H7, both of which have a low infectious dose. In two cheese-associated outbreaks of *Salmonella* infections, fewer than 10 *Salmonella* per 100 g of cheese were detected (Alterkruse, 1998). Three serotypes of *Salmonella* were capable of extended survival in Cheddar cheese (White 1976). *E. coli* O157:H7 is capable of extended survival in Cheddar cheese during manufacture and curing at 2.75 to 3.76% salt in the moisture phase (Reitsma 1996). *L. monocytogenes* is capable of extended survival in Cheddar cheese during ripening (Ryser 1987), and survived in Swiss cheese for up to 66-80 days during ripening (Buazzi, 1992).

Microbial stability

Microbial stability of PPC slices is formulation dependent. Moisture, pH, and salt may act in combination to inhibit growth of many vegetative bacterial pathogens, similar to the mechanism of inhibition for *C. botulinum* (Tanaka, 1986; ter Steeg, 1995). The pH values of all formulations tested in this study (range from 5.4-

5.9) were above the accepted minimum pH for growth of all five pathogens tested. (ICMSF).

Although the a_w of formulations tested in this study ranged from 0.908-0.950, the a_w of most of the cheeses tested (0.92-0.93) was at or below the minimum required for growth of most foodborne pathogens (ICMSF, 1996). The minimum a_w for growth of *L. monocytogenes* was 0.92 in media containing NaCl (Miller, 1992). Minimum a_w for growth of *E. coli* O157:H7 is 0.95 (ICMSF, 1996). *Salmonella* can grow in certain foods with a_w as low as 0.93 (Christian, 1953). *E. coli* O157:H7 and *Salmonella* are able to survive at aw much lower than the minimum required for growth (Christian, 1953; ICMSF, 1996). *B. cereus* will not grow at a_w 0.92-0.93 in media with NaCl as the humectant. (ICMSF, 1996). Although *S. aureus* can grow aerobically at a_w as low as 0.86, the rate of growth is reduced substantially when the water activity is less than 0.94 (Scott, 1953.)

It is well established that *S. aureus*, can grow at significantly lower a_w than other bacterial foodborne pathogens. However, other factors may affect growth of the organism. Levels of 500 or 100 ppm potassium sorbate were shown to inhibit growth of *S. aureus* in broth media (Stewart, 2001). The addition of sorbate has been found to delay growth of *S. aureus* in process cheese food products (Krieisman, 1978) and in an intermediate moisture food (Boylan, 1975). *S. aureus* grew and produced enterotoxin A in Cheddar cheese slurries at 32°C containing 45 and 60% moisture (2-log CFU/g increase in 1 day), but growth was inhibited by 0.2-0.3% sorbic acid (Gandhi, 1973).

Sorbate and sorbic acid have also been shown to affect growth or survival of other bacterial foodborne pathogens. Sorbic acid exhibited a listericidal effect in cold-pack cheese food during storage at 4°C (Ryser 1988). However, although sorbates were effective as preservatives, they were not listericidal in cottage cheese (Piccinin, 1995). Sodium sorbate decreased survival of *E. coli* O157:H7 in apple cider (Zhao, 1993). A level of 0.3% potassium sorbate significantly delayed or prevented growth of *Salmonella* serotypes in a soft Hispanic type cheese (Kasrazadeh,1994). Growth of *Bacillus cereus* is inhibited by 0.26% sorbic acid at pH 5.5 and by 0.39% potassium sorbate at pH 6.6. (ICMSF, 1996).

The greatest increase in *S. aureus* populations in this study occurred in both lots (13A and 13B) of one formulation of PPC with high pH (5.99 and 5.93). However, there was no obvious correlation between growth or survival of *S. aureus* and initial moisture, % NaCl, a_w , % protein, % citric acid, % phosphate, or type (American or Swiss) of cheeses. Four formulations of PPC tested (6, 8, 9, and 13) did not list sorbic acid as an ingredient on the product labels. The pH of formulations 8 and 9 (5.51-5.63) were among the lowest of all formulations tested. Thus it is possible that the combination of high pH and lack of sorbate may have contributed to the growth of *S. aureus* in formulation 13.

In this study, *S. aureus* grew to high numbers in only 3 lots tested within 7 days at abuse temperature. None of the formulations tested supported ("rapid") pathogen growth within 12 hours. In addition, populations of *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*, and *B. cereus* decreased or remained constant during the testing interval.

While sorbate and water activity may have inhibited pathogen growth on most formulations of PPC tested, moisture levels, pH, and NaCl probably contributed to the safety of these products. Results of this study confirm the importance of preventing post-processing contamination of pasteurized process cheese slices to maintain the safety of these products.

References

Altekruse, S.F., B.B. Timbo, J.C. Mowbray, N.H. Bean, and M.E. Potter. 1998. Cheese-associated outbreaks of human illness in the United States, 1973 to 1992: sanitary manufacturing practices protect customers. J. Food Prot. 61:1405-1407.

Bennett, R.W. and W.T. Amos. 1983. *Staphylococcus aureus* growth and toxin production in imitation cheeses. J. Food Sci. 48:1670-1673.

Boylan, S.L., K.M. Acott, and T.P. Labuza. 1976. *Staphylococcus aureus* challenge study in an intermediate moisture food. J. Food Sci. 41:918-921.

Buazzi, M.M., M.E. Johnson, E.H. Marth. 1992. Survival of *Listeria monocytogenes* during the manufacture and ripening of Swiss cheese. J. Dairy Sci. 75:380-386.

Centers for Disease Control and Prevention. 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami-Washington and California, 1994. Morbid. Mortal. Weekly Rep. 44:157-160.

Christian, J.H.B. and W.J. Scott. 1953. Water relations of salmonellae at 30°C. Aust. J. Biol. Sci. 6:564-573.

Cox, L.J., T. Kleiss, J.L. Cordier, C. Cordellana, P. Konkel, C. Pedrazzini, R. Beumer, and A. Siebenga. 1989. *Listeria* spp. in food processing, non-food and domestic environments. Food Microbiol. 6:49-61.

Fazio, T. 1990. Food Additives: Direct. p. 1137-1175. *In* K. Herlich (ed.), Official methods of analysis, 15th ed. Association of Official Analytical Chemists, Arlington, VA.

Gandhi, N.R. and G.H. Richardson. 1973. Staphylococcal enterotoxin A development in Cheddar cheese slurries. J. Dairy Sci. 56:1004-1010.

Glass, K.A. and M.P. Doyle. 1989. Fate and thermal inactivation of *Listeria monocytogenes* in beaker sausage and pepperoni. J. Food Prot. 52:226-231.

Glass, K.A. and M.P. Doyle. 1991. Fate of *Salmonella* and *Listeria monocytogenes* in commercial, reduced-calorie mayonnaise. J. Food Prot. 54:691-695.

Glass, K.A., K.M. Kaufman, and E.A. Johnson. 1998. Survival of bacterial pathogens in pasteurized process cheese slices stored at 30°C. J. Food Prot. 61:290-294.

Hargrove, R.E., F.E. McDonough, and W.A. Mattingly. Factors affecting survival of *Salmonella* in Cheddar and Colby cheese.

Hudson, L.M., J. Chen, A.R. Hill, and M.W. Griffiths. 1997. Bioluminescence: a rapid indicator of *Escherichia coli* O157:H7 in selected yogurt and cheese varieties. J. Food Prot. 60:891-897.

International Commission on Microbiological Specifications for Foods. 1996. *In* T.A. Roberts, A.C. Baird-Parker, and R.B. Tompkin (ed.), Microorganisms in foods 5: microbiological specifications of food pathogens, p. 20-35, 126-140, 141-182, 217-264, 299-332. Blackie Academic and Professional, London.

Johnson, F.J. 1990. Fertilizers. p. 9-39. *In* K. Herlich (ed.), Official methods of analysis, 15th ed. Association of Official Analytical Chemists, Arlington, VA.

Johnson, E.A. , J.H. Nelson, and M. Johnson. 1990. Microbiological safety of cheese made from heat-treated milk, part II. Microbiology. J. Food Prot. 53:519-540.

Kasrazadeh, M. and C. Genigeorgis. 1994. Potential growth and control of *Salmonella* in Hispanic type soft cheese. Int. J. Food Microbiol. 22:127-140.

Kosikowski, F.V. and V.V. Mistry. 1997. Cheese and Fermented Milk Products, Vol. 1: Origins and Principles, 3rd ed. F.V. Kosikowski, L.L.C., Wesport, CN. p. 328-352.

Kreisman, L.N. and T.P. Labuza. 1978. Storage stability of intermediate moisture food process cheese food products. J. Food Sci. 43:341-344.

Leyer, G.J. and E.A. Johnson. 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. 58:2075-2080.

Meer, R.R., J. Baker, F.W. Bodyfelt, and M.W. Griffiths. 1991. Psychrotrophic *Bacillus* spp. in fluid milk products: a review. J. Food Prot. 54:969-979.

Miller, A.J. 1992. Combined water activity and solute effects on growth and survival of *Listeria monocytogenes* Scott A. J. Food Prot. 55:414-418.

Nelson, J.H. 1984. Some regulatory compliance concerns of the cheese industry. J. Dairy Sci. 67:2108-2119.

Papageorgiou, D.K. and E.H. Marth. 1989. Behavior of *Listeria monocytogenes* at 4 and 22°C in whey and skim milk containing 6 or 12% sodium chloride. J. Food Prot. 52:625-630.

Park, H.S., E.H. Marth, J.M. Goepfert, and N.F. Olson. The fate of *Salmonella typhimurium* in the manufacture and ripening of low-acid Cheddar cheese.

Piccinin, D.M. and L.A. Shelef. 1995. Survival of *Listeria monocytogenes* in cottage cheese. J. Food Prot. 58:128-131.

Rietsma, C.J. and D.R. Henning. 1996. Survival of Enterohemorrhagic *Escherichia coli* O157:H7 during the manufacture and curing of Cheddar cheese. J. Food Prot. 59:460-464.

Richardson, G.H. 1990. Dairy products, p. 802-852. *In* K. Herlich (ed.), Official methods of analysis, 15th ed. Association of Official Analytical Chemists, Arlington, VA.

Ryser, E.T. and E.H. Marth. 1987. Behavior of *Listeria monocytogenes* during the manufacture and ripening of Cheddar cheese. J. Food Prot. 50:7-13.

Ryser, E.T. and E.H. Marth. 1988. Survival of *Listeria monocytogenes* in cold-pack cheese food during refrigerated storage. J. Food Prot. 51:615-621.

Scott, W.J. 1953. Water relations of *Staphylococcus aureus* at 30°C. Aust. J. Biol. 6:549-564.

Stewart, C.M., M.B. Cole, J.D. Legan, L. Slade, M.H. Vandeven, and D.W. Schaffner. 2001. Modeling the growth boundary of *Staphylococcus aureus* for risk assessment purposes. J. Food Prot. 64:51-57.

Tanaka, N., E. Traisman, P. Plantinga, L. Finn, W. Flom, L. Meske, and J. Guggisberg. 1986. Evaluation of factors involved in antibotulinal properties of pasteurized process cheese spreads. J. Food Prot. 49:526-531.

ter Steeg, P.F., and H.G.A.M. Cuppers. 1995. Growth of proteolytic *Clostridium botulinum* in process cheese products: II. Predictive modeling. J. Food Prot. 58:1108-1110.

Townes, J.M., P.R. Cieslak, C.L. Hatheway, H.M. Solomon, J.T. Holloway, M.P. Baker, C.F. Keller, L.M. McCroskey, and P.M. Griffin. 1996. An outbreak of type A botulism associated with a commercial cheese sauce. Ann. Intern. Med. 125:558-563.

U.S. Food and Drug Administration. 1986. Retail Food Protection Program Information Manual. Part 6-Inspection, Ch. 01-Code Interpretations, #1-102. Definitions-Potentially Hazardous Food. Center for Food Safety and Applied Nutrition, Retail Food Protection Branch. Wahsington, DC. 5/9/86.

U.S. Food and Drug Administration. 1998. Cheese and related cheese products. Code of Federal Regulations, Title 21, Part 133. Office of the Federal Register, U.S. Government Printing Office, Washington, D.C.

U.S. Food and Drug Administration. 1999. Food Code. National

Technical Information Service. Springfield, VA.

White, C.H. and E.W. Custer. 1976. Survival of *Salmonella* in Cheddar cheese. J. Milk Food Technol. 39:328-331.

Wong, H.-C., M.-H. Chang, and J.-Y. Fan. 1988. Incidence and characterization of *Bacillus cereus* isolates contaminating dairy products. Appl. Environ. Microbiol. 54:699-702.

Zhao, T., M.P. Doyle, and R.E. Besser. 1993. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in apple cider with and withotg preservatives. Appl. Environ. Microbiol. 59:2526-2530.

Determination of caloric bioavailability and apparent lipid digestibility of liquid mikfat fractions

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Funding

Wisconsin Milk Marketing Board

Dates

July 1996 to December 2000

Objectives

1. To determine growth and apparent lipid digestibility of weanling rats fed diets containing liquid milkfat fractions, intact milkfat or corn oil.

2. To determine the caloric bioavailability of liquid milkfat and intact milkfat with a bioassay method based on the growth of weanling rats fed a basal diet supplemented with corn oil. This objective was not completed due to the delay in the UW-Madison CDR providing the needed liquid milkfat fractions. The fractions were not available until August 1998 although the grant started July 1996 and we spent considerable time testing fractions from Grasslands and the CDR in an attempt to obtain a liquid milkfat fraction obtained by thermal fractionation with a suitable fatty acid profile.

Summary

The Center for Dairy Research provided 5 kg of a very low melting milkfat fraction (dropping unit $< 10^{o}C$) and intact anhydrous milkfat in August 1998. The liquid milkfat fraction contains a decreased proportion of 16:0 and 18:0 saturated fatty acids and an increased proportion of 18:1 monosaturated fatty acid compared to the intact milkfat (see Table 1). Both fractions contain approximately 10% of fatty acids with less than or equal to 10 carbon atoms.

We obtained a profile of the triacylglycerol species present in the milkfat fractions using high temperature capillary gas chromatography in consultation with Dr. Eric Lien at Wyeth Nutritionals International. The liquid milkfat fraction contains higher levels of triacylglycerols with unsaturated fatty acids, especially 18:1 and the intact milkfat contains higher levels of triacylglycerols with trisaturates including: tripalmitate, myristate-myristate-palmitate, myristate-palmitate-palmitate and stearate-stearate-myristate.

We speculated that the lower levels of trisaturated triacylglycerols in liquid milkfat compared to intact milkfat would improve the lipid digestibility of the liquid milkfat. An animal feeding study comparing the apparent lipid digestibility of diets containing corn

Table 1.
Fatty acid profiles of intact milkfat and a liquid milkfat fraction, expressed as % butyl esters

Fatty Acid	Intact Milkfat	Liquid Milkfat
4:0	4.8	5.4
6:0	2.3	2.7
8:0	1.3	1.6
10:0	2.9	3.5
12:0	3.2	3.8
12:1	ND	ND
14:0	11.1	10.5
14:1	ND	ND
16:0	32.0	24.1
16:1	1.6	2.3
18:0	12.7	8.0
18:1	24.4	33.0
18:2	3.2	4.3
18:3	0.4	0.5
Fatty Acids ≤	0.4	0.5

oil, liquid milkfat, intact milkfat and medium chain triacylglycerols was conducted to test this concept. Liquid milkfat showed improved digestibility associated with the lower levels of trisaturated triacylglycerols such that the apparent lipid digestibility of liquid milkfat was not significantly different from corn oil (96%) and significantly improved compared to intact milkfat (90%). These data demonstrate that temperature fractionation of intact milkfat to reduce the proportion of trisaturated triacylglycerols significantly improved the lipid digestibility of milkfat.

The goal of this research was to assess the apparent lipid digestibility of liquid milkfat fractions, which we hypothesize will be improved compared to intact milkfat in association with decreased levels of trisaturated triacylglycerols. Our hypothesis was supported by our experimental data. We have shown that thermal fractionation of milkfat to produce a liquid milkfat fraction improved its apparent lipid digestibility such that the liquid milkfat fraction was not different from corn oil, 96% absorbed.

This observation is significant because milkfat is rarely used as an ingredient for human nutritional products in the United States because of cost and concern that milkfat is not digested as well as unsaturated vegetable fats. Nutritional products may include infant formulas, supplements for healthy individuals such as the elderly or athletes, and medical foods for individuals with specific conditions. Markets for these nutritional products are expanding with an aging population and opportunities for use of liquid milkfat as a food ingredient will increase once the barrier of "poor digestibility" is overcome. This research helps to establish that liquid milkfat is as well digested as corn oil.

Publications/ Presentations

Lai, HC and Ney, DM. Gastric digestion modifies absorption of butterfat into lymph chylomicrone in rats. J Nutr 128:2403-2410, 1998.

Lai, HC. Postprandial lipid metabolism with ingestion of defined butterfat fractions in the rat. Ph.D. thesis. University of Wisconsin-Madison, 275 pp, 1994.

I have made presentations on the "Nutritional Properties of Milkfat" at the UW-Madison Center for Dairy Research Short courses on October 1996 and March 1998.

Minimizing the watering-off of unripened lower fat and no-fat mozzarella cheese

Personnel

Chen, Carol M.; Johnson, Mark E.

Funding

Dairy Management Inc.

Dates

January 1996 to June 2001

Objectives

To evaluate how manipulations to cheese milk, different manufacturing protocol, or the addition of specific casein hydrolyzing enzymes can minimize the watering off of unripened high moisture lower and no fat mozzarella cheese. Specifically, manipulations will focus on protein-to-water interactions to maximize the water absorption capability of the cheeses.

Summary

A newly manufactured mozzarella melts to a tough, granular consistency, has low free oil release and readily losses water. During the first days of ripening cheese texture becomes softer, melts more readily, increases in free oil release and no longer exhibits free moisture. Typically, mozzarella cheese requires anywhere from 1 to 14 days to equilibrate depending on pH and levels of colloidal calcium phosphate and casein to moisture ratios.

Typical moisture contents for low moisture part skim (LMPS), lower fat and skim milk mozzarella cheeses are 48%, 54% and 62%, respectively. Moisture levels are increased as fat content is decreased in part to maintain a similar percentage of moisture to non-fat solids (MNFS, indicator of cheese casein to moisture ratio). While higher moisture contents improve the body and meltability of lower and no fat mozzarella cheese, it may magnify the functional defect of watering off. This can lead to problems because these high moisture cheeses may be shredded and frozen for use as an ingredient on pizza pies or other prepared entrees within 1 to 4 days after manufacturing. When baked, these cheeses exhibit a severe watering off defect. This is observed in conventional ovens, but may be beneficial in forced air ovens.

This research explored different manufacturing manipulations and their effect on the water holding capacity of lower and no-fat mozzarella cheese: addition of NaCl, higher than normal milk pasteurization temperatures, addition of trypsin, cheese storage temperature, addition of Methocel K® (Table 1). Water holding capacity of unripened cheeses was measured by the percentage of expressible serum (wt expressible serum/wt cheese). The manufacturing manipulations focused on altering the protein/water associations, in an attempt to increase the rate at which free

Table. 1

Manufacturing Manipulation	Rational and Results
NaCl addition, Salt balance	Addition of NaCl changes the protein-salt balance. Sodium replaces calcium in the casein micelle. This loosens the protein structure allowing it to associate with free water. NaCl is also capable of binding water. At the start of the cooking of the curd during cheese-making, 0.5% (wt salt to wt milk) NaCl was added to the curd / whey slurry. The addition of salt increased the moisture content of the cheese from 55 to 56% for non-fat Mozzarella. The pH values were similar for control and experimental cheeses. No differences were noted in levels of expressible serum.
Trypsin addition, Selective hydrolysis of β casein	Trypsin can selectively hydrolyze β casein. The presence of β casein in the casein network, due to strong hydrophobic interactions, may prevent water absorption by the casein network. Hydrolysis of β casein would remove it from the network allowing the network to absorb more serum. Trypsin (1 g/ 1000 lb milk) was added prior to coagulation. No differences were noted in levels of expressible serum.
Higher pasteurization temperatures, Denatured whey proteins	Heating milk causes denaturation of whey proteins. Denatured β lactoglobulin forms a complex with β casein, which associates with more water and slows syneresis from the curd. Milk for cheese-making was pasteurized at 164 (normal) and 184^{0}F / 16 s. Higher pasteurization temperatures increased cheese moisture by 1.8%, and doubled the level of expressible serum. However, cheese moisture (not able to be expressed) to protein ratio remained the same.
Cheese ripening temperatures, swelling of casein.	As temperature is reduced, hydrophobic interactions are reduced, but electrostatic repulsion between casein molecule increase. The net result is that the casein network absorbs free serum. However, this is highly pH dependent. Lower fat and non fat Mozzarella cheese lots were split and ripened at three temperatures to evaluate how ripening temperatures of 35^{0}F, 45^{0}F, and 55^{0}F affected the levels of expressible serum during early ripening. No differences were noted in levels of expressible serum
Addition of Methocel K®, chemical absorption of water.	Methocel K® (0.05, 0.10 and 0.20%) was added to the cheese milk after pasteurization but before coagulant addition. Methocel ® absorbs water upon cooling after a heat treatment (mixer 135^{0}F). The addition of Methocel K® completely eliminated expressible serum in both lowers fat and no fat mozzarella cheese. See Table 2 for more details.

Table 2.

	Skim milk mozzarella cheese			
Percentage Methocel®	None	0.05%	0.10%	0.20%
Cheese Composition				
% Moisture	62.2	65.4	64.1	62.8
% Fat	1.3	1.1	1.2	1.1
% Protein	30.7	27.4	29.1	29.8
% Salt	1.3	1.4	1.6	1.5
% FDM	3.4	3.1	3.4	3.0
% MNFS	63.0	66.1	64.9	63.4
% Expressible Serum (wt exp serum/wt cheese)				
1 day	13.9	9.3	0.0	0.0
3-4 days	7.7	6.8	0.0	0.0
7 days	5.2	4.7	0.0	0.0
14 days	3.9	6.4	0.0	0.0
Ratio Moisture (not able to be expressed) to Total Protein[1]				
1 day	1.57	2.06	2.20	2.11
3-4 days	1.77	2.15	2.20	2.11
7 days	1.85	2.23	2.20	2.11
14 days	1.95	2.16	2.20	2.11
pH				
1-day	5.26	5.19	5.12	5.35
7 days	5.25	5.22	4.97	5.38
14 days	5.14	5.06	4.94	5.13

water associates with casein. With the exception of Methocel K® addition, manufacturing alterations used in this research study did not significantly alter the expressible serum of lower and no fat mozzarella over time.

Methocel K®, made by the Dow Chemical Company, is a water soluble gum made from the cellulose esters, methylcellulose and hydroxypropyl methylcellulose. Methocel gum solutions gel when heated, then return to their original viscosity when cooled. These polymers dissolve in water by first swelling, then hydrating. At warmer temperatures the gums are insoluble and readily disperse into solution. As the solution cools, the Methocel K® solublizes. In our cheese manufacturing application, the Methocel K® was dispersed in warm water (39°C), then added to the milk prior to coagulation. Throughout cheesemaking and mixing of the mozzarella curd, Methocel K ® was dispersed, but not

hydrated. The curd temperatures in the mixer averaged 65°C, below the critical gelation temperature of 70°C. The Methocel K® entrapped within the mozzarella cheese hydrated upon the cooling of the mozzarella block in the chill tank. As Methocel ® hydrated, it absorbed free water in the cheese, resulting in no expressible serum in the unripened mozzarella cheese.

In theory, all of the manufacturing manipulations tested in this project should decrease the levels of expressible serum in young mozzarella cheese. However, we worked within a pH range (5.00 to 5.30) where differences were minimal and difficult to significantly quantify. A higher or lower pH range in the cheese would have likely produced a greater difference in the levels of expressible serum.

Increases in water holding capacity or decreases in levels of expressible serum require a change in the confirmation of the cheese proteins. In order for the casein to absorb water, there must be a loss of calcium. With the loss of calcium, there will be a negative charge on the casein molecule. This is partially neutralized by sodium or hydrogen ions, both monovalent positive charged ions, in the cheese. However, these ions are associated with water. This is important since the water in cheese is absorbed, or becomes closely associated with the casein, through these ions. In essence, water shares the ions with the casein molecules. At higher cheese pH ranges, the casein conformation is closer to its native state (higher levels of calcium associated with the casein) and smaller changes in pH, salt concentration, or temperature are more likely to affect the calcium/protein balance. At lower cheese pH ranges, the isoelectric point of casein is approached, and hydrophobic interactions play a significant roll.

Table 3 summarizes the composition, pH and levels of expressible serum for control lots of mozzarella cheese at three different fat levels. Initial (1 to 4 days) levels of expressible serum are similar for LMPS, lower and no fat mozzarella cheeses. However, the protein in the LMPS mozzarella appeared to have a greater ability to associate with water during later stages of testing (10 to 14 days), as shown by the increase in the ratio of moisture (not able to be expressed) to total protein. This difference is likely due milk preparations. In the manufacture of lower and no fat mozzarella cheeses, milk is preacidified to pH 6.30 and coagulated at pH 6.05 - 6.10 (lower fat) or pH 5.95 - 6.00 (no fat), resulting in an early loss of calcium. All mozzarella curds were stretched in the mixer at pH 5.30. Lower and no fat mozzarella curd have a smaller drop in pH during manufacture, which is enough to affect the calcium/protein balance or the water holding capacity of the cheese over time.

Methocel K® at levels above 0.10% (wt Methocel/wt milk) resulted in cheeses with no expressible serum. Other mozzarella manufacturing alterations used in these experiments (NaCl addition, trypsin addition, higher than normal pasteurization temperatures and modified cheese ripening temperatures) were not effective at increasing the rate of expressible serum absorption in unripened lower and no fat mozzarella cheeses. The established theory that the absorption of free serum in cheese is due to the combined effect of charge on the casein molecules (as determined by pH and the loss of colloidal calcium phosphate) and temperature appears to be valid. However,

Table 3.

	Mozzarella Cheese Type		
	LMPS (n=6)	Lower fat (n=16)	Skim Milk (n=13)
Composition			
% Moisture	43.6	54.3	59.0
% Fat	23.0	9.03	1.6
% Protein	26.0	31.3	33.0
% Salt	1.4	1.3	1.6
% FDM	42.9	19.7	3.8
% MNFS	60.2	59.7	59.9
Ratio Moisture (not able to be expressed) to Total Proten)			
1 day	1.50	1.51	1.52
3-4 days	1.54	1.55	1.56
7 days	1.65	1.63	1.61
10-11 days	1.73	1.67	1.67
14 days	1.77	1.70	1.66
pH			
milk (initial)	6.60	6.60	6.60
milk and preacidification	------	6.30	6.30
milk during coagulaiton	6.40-6.45	6.05-6.10	5.95-6.00
curd in mixer	5.30	5.30	5.30
cheese at 1 day	5.15	5.16	5.19
cheese at 7 days	5.15	5.16	5.17

the pH of cheeses in this experiment was 5.0 to 5.3, a range in which differences were minimal and difficult to significantly quantify.

Investigating reasons for hardening of reduced fat Cheddar cheese during heating

Personnel
Gunasekaran, Sundaram

Funding
Dairy Management Inc.

Dates
September 1997 to August 2000

Objectives

1. Evaluate the effects of heat treatments on nature and extent of different protein interactions: hydrophobic interactions, hydrogen bonding, and ionic bonding

2. Evaluate the usefulness of certain emulsifying agents and a surfactant in reducing hardening of the lower-fat cheeses

3. Assess the possibility that the experimental approaches proposed in this project will help in alleviating the skin formation which occurs when very low-fat cheeses are heated

4. Evaluate the possibility of independently controlling the meltability and firmness of the cheese by combined use of chymosin and the enzyme from *C. parasitica.*

Summary

Independent control of cheese meltability and hardness
Proteolysis is considered the main cause of changes in functional properties of Cheddar cheeses, and the degree of proteolysis over time is affected by the coagulant type. Therefore, four Cheddar cheeses were made with different *C. parasitica* proteinase to chymosin ratios. The enzyme ratios and composition of the cheeses made are listed in Table 1. The composition of all cheeses was statistically

Table 1. Composition of cheeses made with different enzyme ratios at day one after manufacturing.

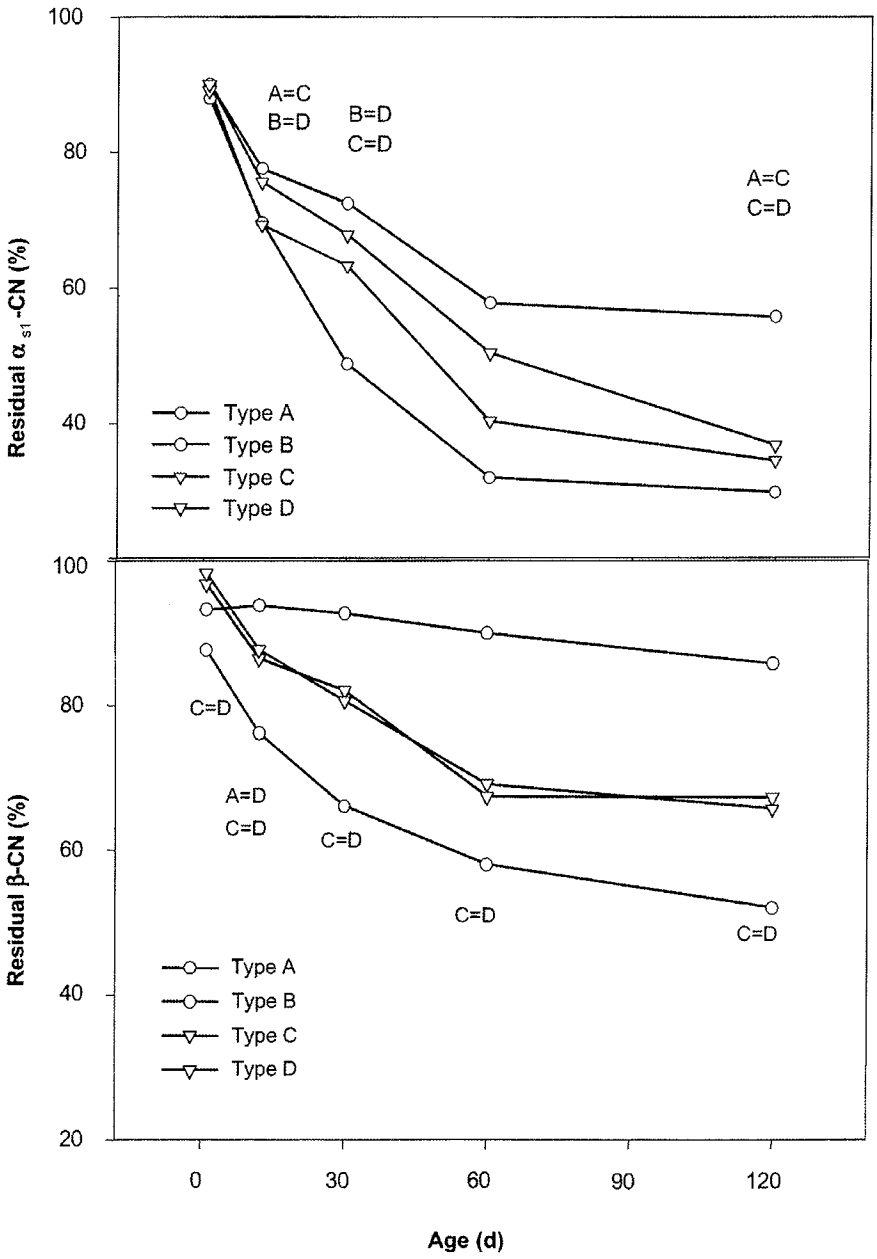
Cheese	Enzyme Ratio* (quantities used, mL/kg of milk)	Moisture %	MNFP %	FDM %	Salt %	S/M %	Initial pH	Total Protein %
A	1:0 (75:0)	37.82	55.53	51.07	1.38	3.66	5.15	39.29
B	0:1 (0:49)	37.78	55.56	51.43	1.38	3.66	5.13	38.83
C	2:1 (50:16)	37.71	55.12	50.03	1.40	3.72	5.16	39.46
D	1:2 (24:32)	37.59	54.94	50.34	1.39	3.71	5.12	38.92

**Ratio of enzymes* chymosin (Sure-curd®) to *C. parasitica* proteinase (Maxiren®).

(p>0.05) similar. Cheese samples were evaluated for relative hydrolysis of α_{s1}- and β-caseins, and bitter and off flavor intensities at 0, 10, 30, 60, and 120 d.

As shown in Figure 1, residual α_{s1}-CN decreased in all samples over time, but the rate of decrease was especially pronounced during the first 60 d. The rate of α_{s1}-CN reduction differed between samples; cheese B (100% *C. parasitica*) contained a much higher residual α_{s1}-CN than cheese A (100% chymosin) at all ages. The degrees of hydrolysis of β-CNin all cheeses were less than the corresponding hydrolysis of α_{s1}-CN. The amount of b-CN in cheese A, made with 100% chymosin, decreased only slightly over 120 days. The different levels and rates of hydrolysis of α_{s1}-CNand β-CN caseins by the two coagulants are due to the specificity and accessibility of the peptide bonds to the enzymes.

Figure 1.
Hydrolysis of α_{s1}CN (top) and β-CN (bottom) of Cheddar cheeses made with different ratios of coagulants as a function of cheese age.



The composite data for all cheeses at all ages (Figures 2 and 3) show relationships between hydrolysis of caseins, meltability (measured using the tube test of Olson & Price, 1958), and hardness (by uniaxial compression test) during maturation. The hardness and hydrolysis of α_{s1}-CN, and meltability and hydrolysis of β-CN were closely related (R^2=-0.792 and 0.714, respectively, Figure 2A,B). However meltability and hydrolysis α_{s1}-CN and hardness and hydrolysis of β-CN were poorly related (R^2=-0.443 and 0.328, respectively, Figure 2C,D). Therefore, independently control of hydrolysis of α_{s1}- and β-CN can be attained, as shown by the low correlation (R^2 = 0.108) between hydrolysis of b-CN & α_{s1}-CN (Figure 3A). A negative relation between hardness and meltability (R^2 = -0.641, Figure 3) was expected.

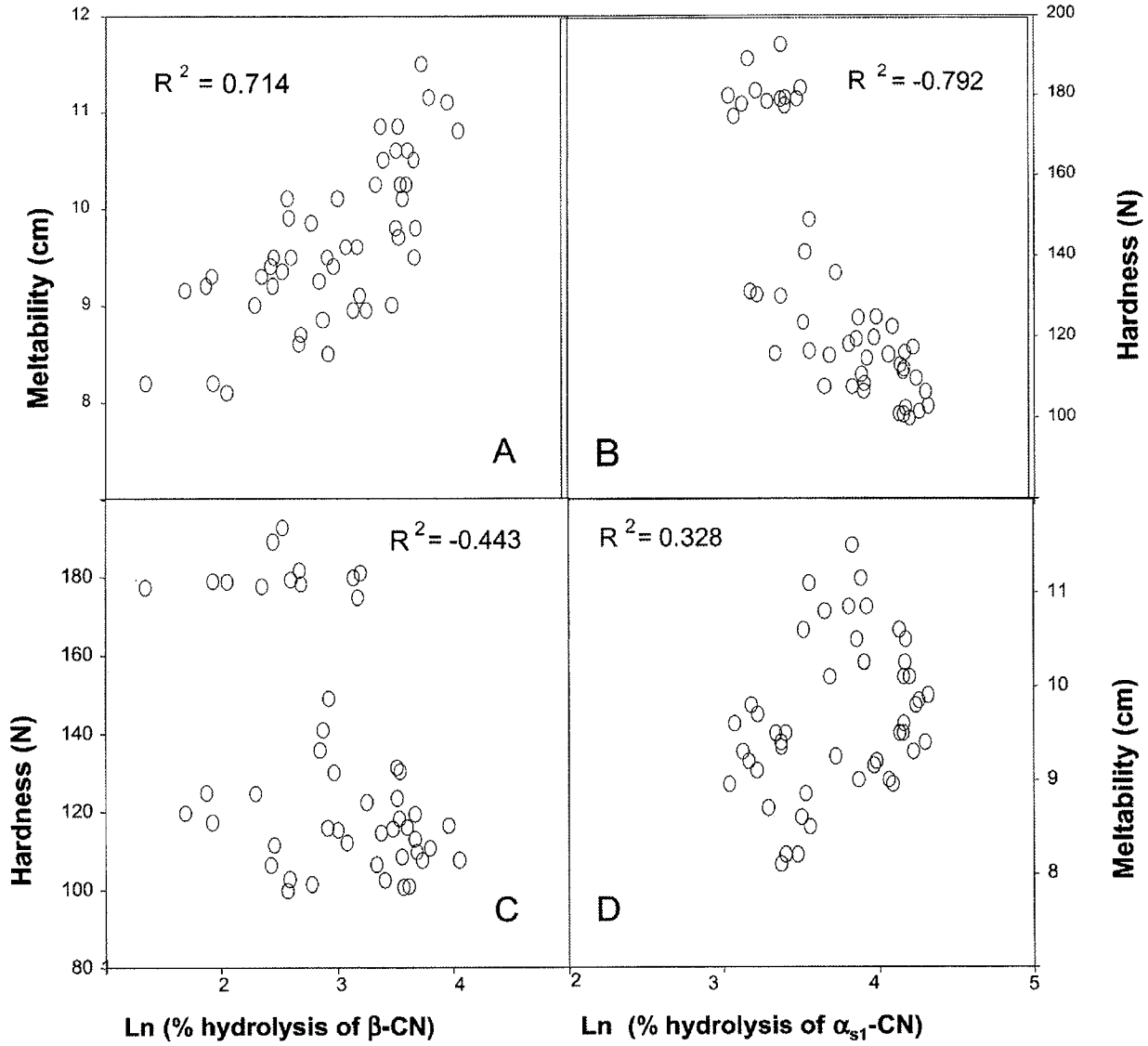


Figure 2.
Correlation between hydrolyses of caseins (α_{s1}-CN (right), β-CN (left)) and cheese meltability, and hardness during maturation, at 10, 30, 60, and 120 d for all cheeses.

Meltability of cheese B was significantly greater than that of cheese A at all ages, and this difference increased with age (Figure 4). The trend of higher meltability with higher *C. parasitica* enzyme is in agreement with previous studies, which reported that hydrolysis of b-casein was greatly enhanced in Cheddar cheese by using the clotting enzyme from *C. parasitica,* and meltability was closely correlated with extent of b-casein hydrolysis in cheese but not with hydrolysis of α_{s1}-casein.

Fracturability (uniaxial compression test) of cheeses A, B, C, and D at day 1 was as follows: 101.41, 122.90, 156.90, and 158.58 N, respectively. These results support the published findings, that is, close relationship between the disappearance of α_{s1}-CN & the magnitude of softening of a soft Dutch cheese, Meshanger, and; good correlation between decrease in percent compression force at fracture point & the hydrolysis of α_{s1}-CN of Cheddar cheese, but not related to that of β-CN, respectively.

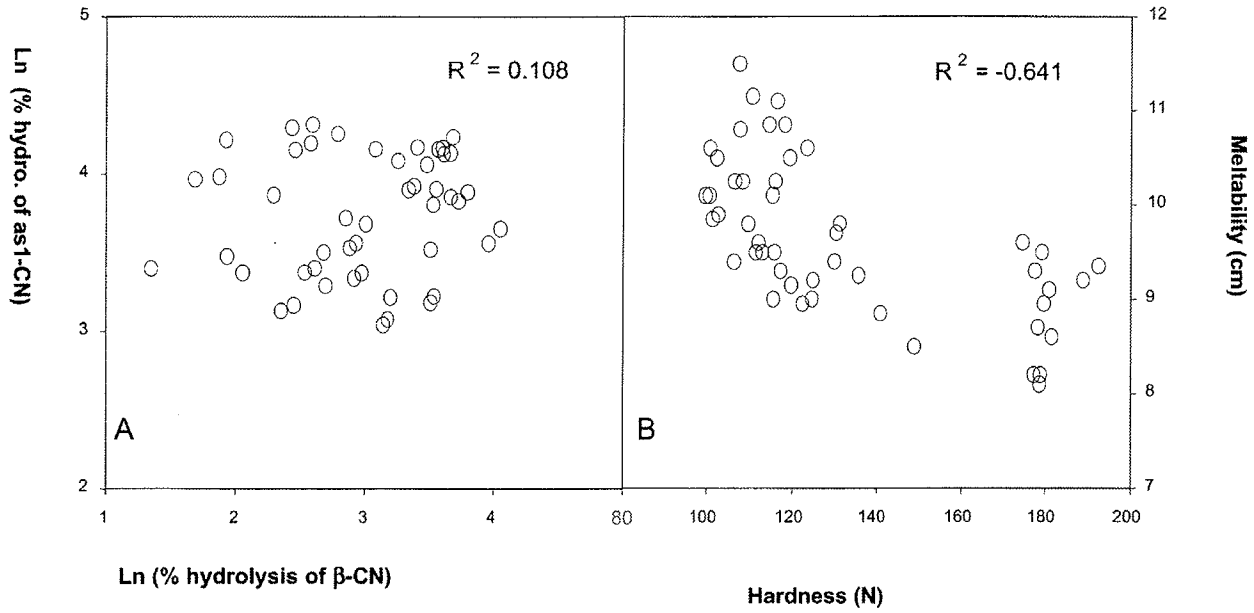


Figure 3.
Correlation between hydrolyses α_{s1}-CN and β-CN (A) and between cheese hardness and meltability (B) during maturation, at 10, 30, 60, and 120 d for all cheeses.

As shown in Figure 5, the degree of hardness for all cheeses decreased at similar rates after 10 d, but the cheeses B and C showed higher degrees of hardness from 60 d on. The hardness of cheeses A and D (made with 100 and 66% chymosin, respectively) were significantly lower than for cheeses B and D (0 and 33% chymosin, respectively) at 60 and 120 d.

Sensory results also confirmed that hardness of cheese A was lower than that of cheeses B, C, and D. However cheeses made with large amounts of *C. parasitica* protease, cheeses B and D (100% and 67% *C. parasitica* protease) had higher levels of bitter flavor intensity than cheeses A and C cheeses (0% and 33% *C. parasitica* protease).

Therefore, a ratio of the two coagulants in between those used for cheeses B and C is recommended for manufacturing Cheddar cheese in order to increase the meltability and hardness simultaneously, while reducing bitterness.

Heat-induced changes in cheese meltability

One of the reasons for changes in the viscosity of cheese during heating is thought to be the protein aggregation by hydrophobic interactions among caseins, because of the high hydrophobic residues. The caseins undergo strong temperature-dependent association. Consequently, the aggregation among hydrophobic molecules would decrease protein solubility, which then would force other components, such as fat and water, to easily separate from the protein matrix in the cheese structure. Therefore, the hydrophobic interactions in cheese may lead to a collective change in protein-water-fat network of cheese.

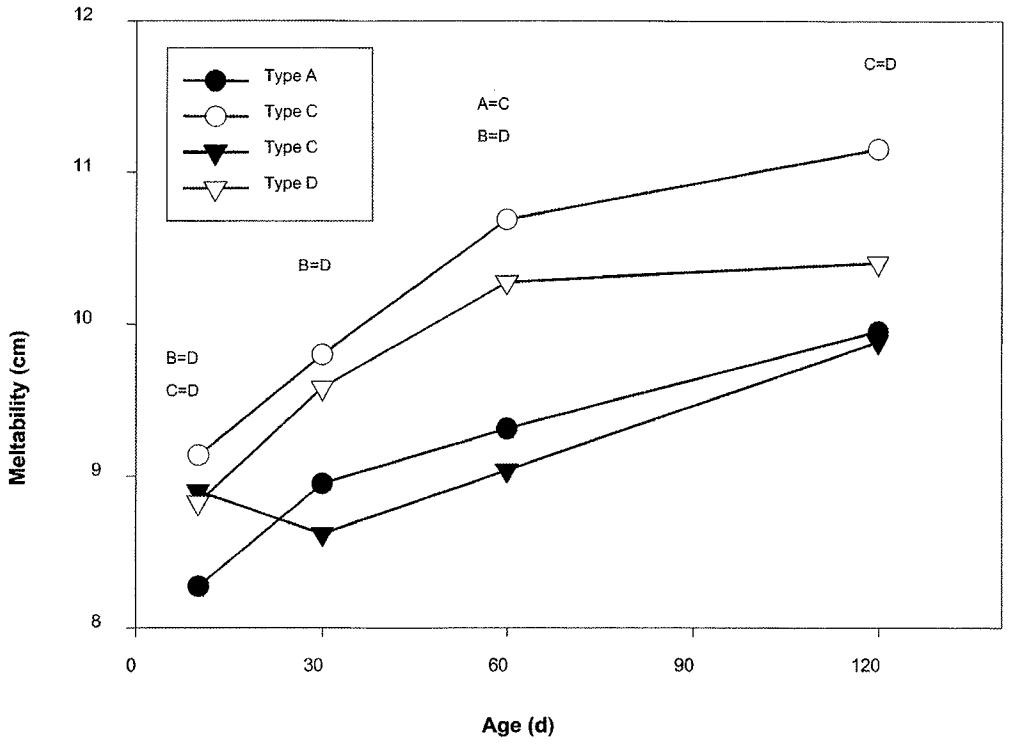


Figure 4.
Meltability (measured by the tube method) of Cheddar cheeses made with various ratios of coagulants

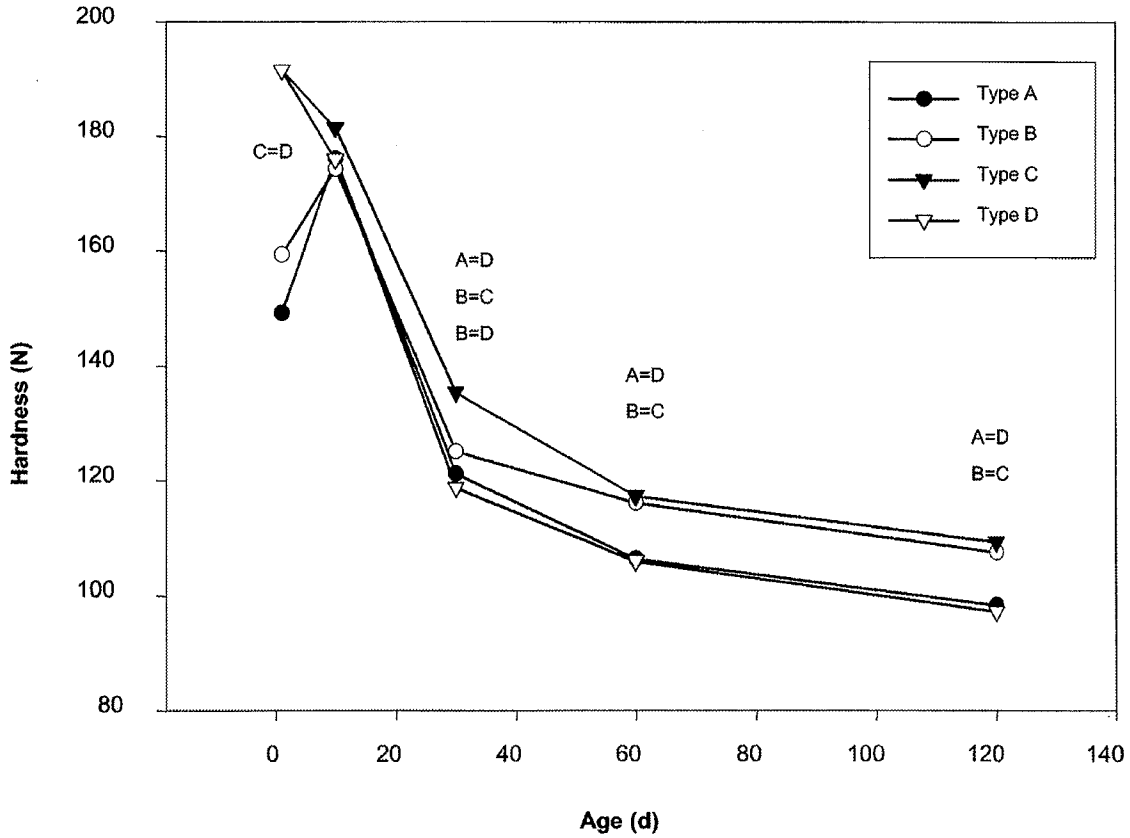


Figure 5.
Hardness (measured by uniaxial compression test) of four Cheddar cheeses made with various ratios of coagulants.

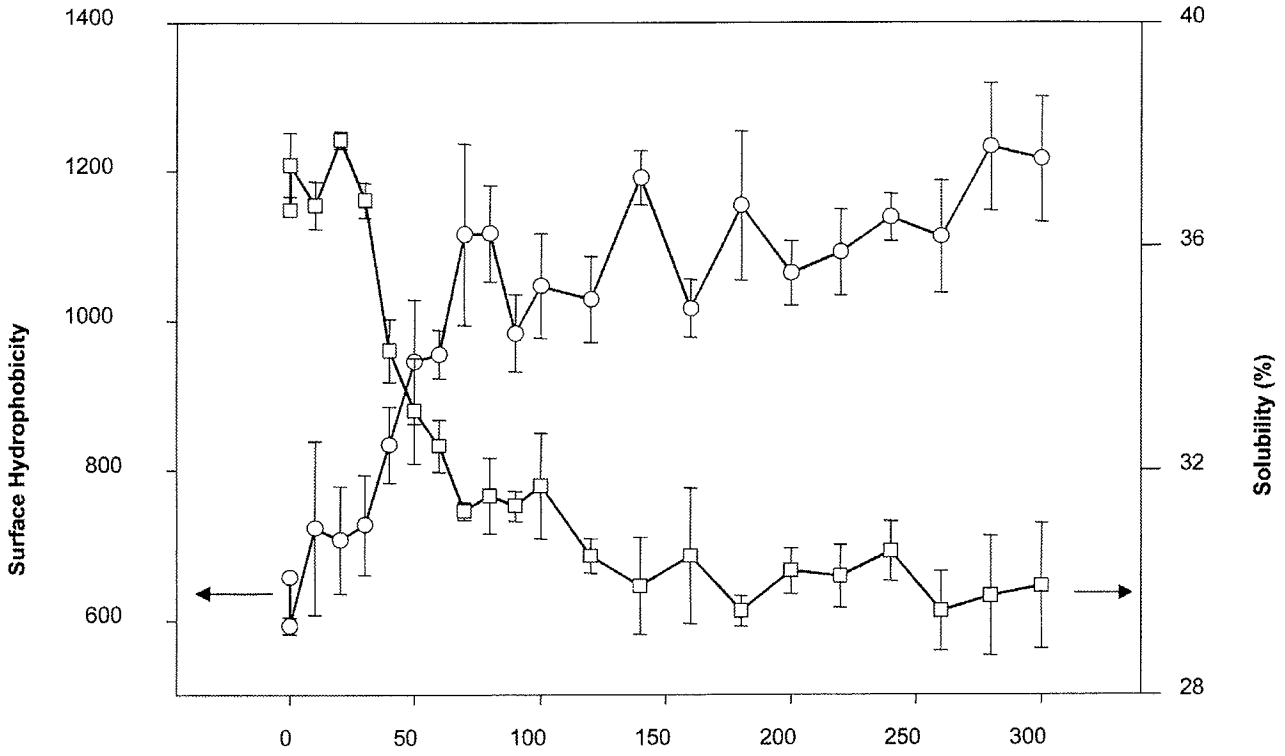


Figure 6.
Change in surface hydrophobicity and solubility of caseins in Cheddar cheese as a function of time the melted cheese held at 60°C

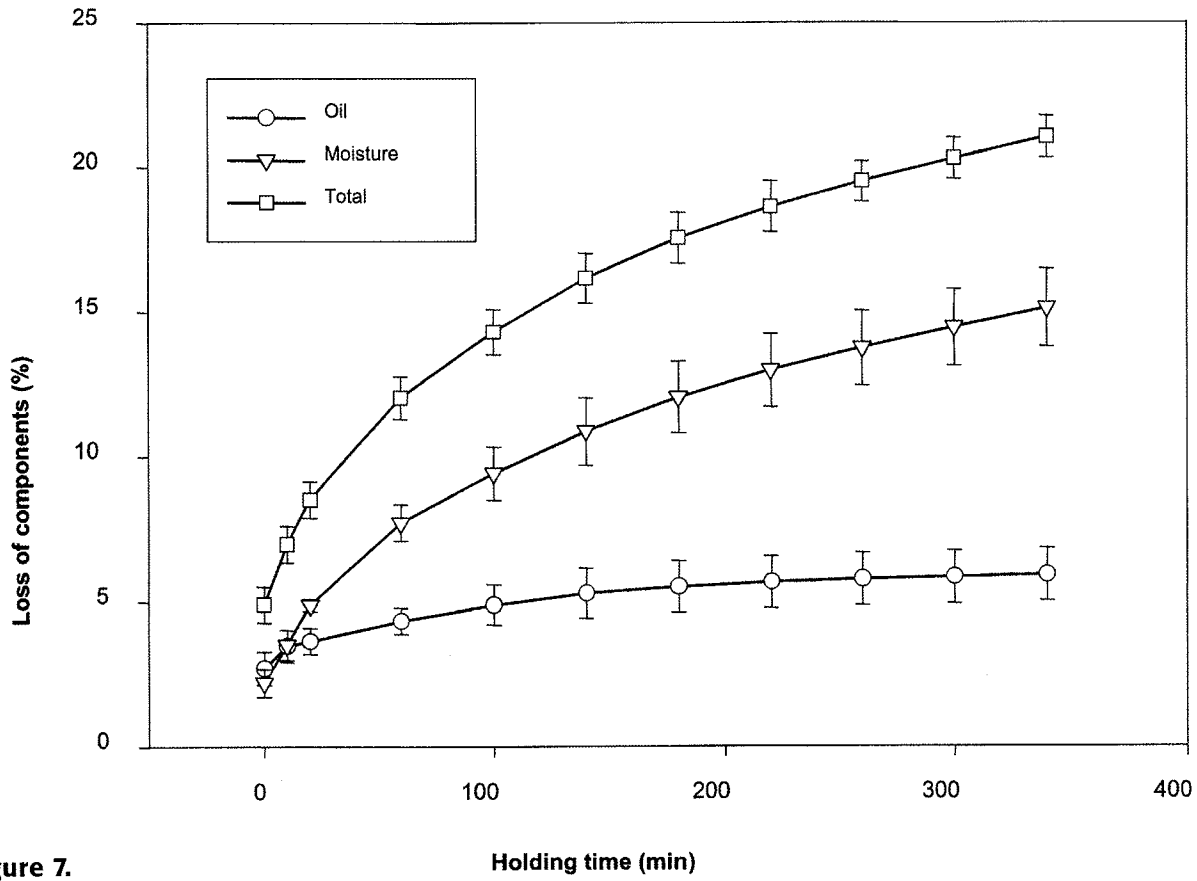


Figure 7.
Moisture and fat loss in Cheddar cheese as a function of time the melted cheese held at 60 °C.

We observed that the surface of Cheddar cheese at the age of 12 wk became rough and wrinkled after heating, compared to intact cheese, because of interactions between components. Protein interactions caused by heating may lead to the stimulated loss of fat and water, and this assumption is the basis of this study. The Cheddar cheese (moisture: 38.8%; fat-in-the-dry-matter: 53.9%; total protein: 39.3%; salt: 1.1%) for this set of experiments was manufactured at the UW Dairy Plant.

As shown in Figure 6, the change in solubility (measured by the spectrophotometric Biuret method) during heating appear to be negatively correlated to surface hydrophobicity (measured by the cis-parinaric acid fluororescence method) Figure 7 shows the fat and moisture loss profile, which change at differing rates with the greatest loss occurring during the first 100 min of holding time. Holding time is the period over which heated cheese is held at that temperature (60°C) before being allowed to flow. Most of the melted fat separated from the cheese body at the beginning of heating, and then exuded more, but the moisture loss occurred steadily. Even though the hydrophobic interactions start, as shown in Figure 6, the melting fat is considered to help the body of cheese move easier when the heated cheese is under an applied force at the beginning of heating. However, the accumulated hydrophobic aggregation of caseins causes the extended loss of fat and water from the cheese body, and the accumulated hydrophobic aggregation of caseins led to an eventual decrease in meltability. Surface hydrophobicity showed good correlation with water and fat loss (R^2=0.932 and 0.846, respectively).

A regression equation was obtained to predict the meltability of Cheddar cheese at 60°C based on fat loss, solubility, and emulsifying activity index (EAI, measured by the turbidometric method) data:

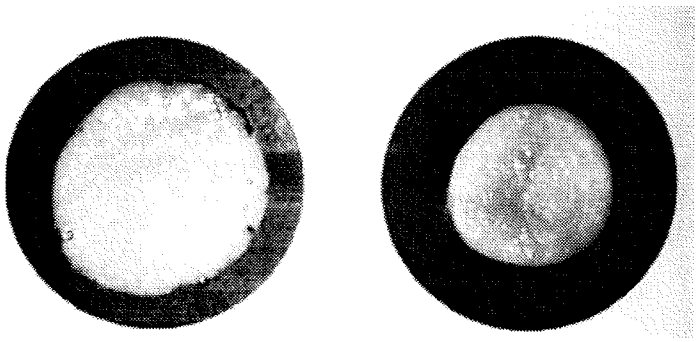
$$Y = 17.6 + 1.52\ X_1 - 4.10\ X_2 + 24.7\ X_3,$$

where Y; thermal meltability, X_1; fat loss, X_2; EAI, X_3; natural logarithm of solubility. In addition, because of close correlation ($R^2 = -0.965$) between solubility and hydrophobicity, it is possible to replace the X_3 with natural logarithm of hydrophobicity.

Investigation of skin formation during heating of reduced fat cheeses

Reduced fat and nonfat cheeses usually develop a defect called 'skin formation', i.e. formation of a dry and hard film on the cheese surface, after heating and cooling. Since there is a lack of free oil, the surface of cheese tends to be more damaged than sub-surface regions with heating time, i.e. the moisture will dry and a hard skin will form. However, there is enough free oil exuding from the full-fat cheese when heated, which covers the surface of the full-fat cheese and prevents it from drying. This allows the cheese to melt more evenly without forming a hard skin on its surface. Figure 8 shows the appearance of full-fat and reduced-fat cheeses after heating and cooling.

This phenomenon of skin formation in 50%-reduced-fat Cheddar cheeses was investigated in terms of protein interactions measured before and after heating by dispersing the cheese in different dissociating agents (sodium dodecyl sulfate, SDS; urea; EDTA; and



Full fat 50% reduced fat

Figure 8.
Surface appearance of full fat and 50% reduced fat cheeses after heating.

mercaptoethanol), homogenization, and ultracentrifugation.

Duplicates (2 vats) of 50%-reduced-fat and full-fat Cheddar cheeses were manufactured. Cheese block was vacuum sealed in barrier bags and stored at 8°C storage room. Cheeses at the age of 12 wk were used. The composition of these cheeses is given in Table 2.

Cheese samples (45 g of ground cheese) was heated in a Petri dish at 180°C for 25 min. To make the initial protein content the same, 18 g of 50% reduced-fat Cheddar and 21.8 g of full-fat Cheddar cheese were dispersed in 90 mL of sodium phosphate buffer, including dissociating agents at pH 5.1 and homogenized for 30 s with a homogenizer (PowerGen Model 125, Fisher Scientific, Pittsburgh, PA) at 9000 rev/min. The dispersed cheese slurry was used for ultracentrifugation with a Beckman centrifuge (25-50B, 50.2 Ti rotor, Beckman Instruments France SA, 93220 Gagny). The pellet weight was expressed as percentage of precipitate vs. original cheese weight. Pellet weight resulting from ultracentrifugation indicates cheese components still interacting in the presence of a dissociating agent.

Figure 9 shows the degree of protein interactions when different denaturants (dissociating agents) were used to dissolve proteins of full-fat and 50% reduced-fat Cheddar cheeses after heating. The 'protein interaction index' was expressed as reciprocal of the response, the pellet weight obtained after ultracentrifugation, multiplied by 100. As shown in the figure, only interactions expressed by SDS and urea appeared to contribute to a protein gel network in both cheeses, with protein interactions expressed by urea contributing the most, followed by that by SDS. There was no difference in protein interaction index between in water, mercaptoethanol, and EDTA, when they are used separately ($p>0.05$). We did not observe any significant difference in protein interactions expressed by SDS between two cheeses, but by urea ($p < 0.05$). This suggests that breaking of both ionic bonds formed with calcium and disulphide bonds by EDTA and mercaptoethanol, respectively, on the surface of protein aggregate was not strong enough to increase the solubility of heated protein in the solvent.

Table 2. Composition of full-fat and 50% reduced-fat Cheddar cheeses at 12 wks

Cheese	MNFP %	FDM %	Salt %	S/M %	Moisture %	Fat %	Protein %	pH
Full fat	56.31	45.63	1.34	3.47	38.67	31.33	38.67	4.94
50% reduced fat	53.43	14.94	1.65	3.55	46.48	13.00	46.38	4.87

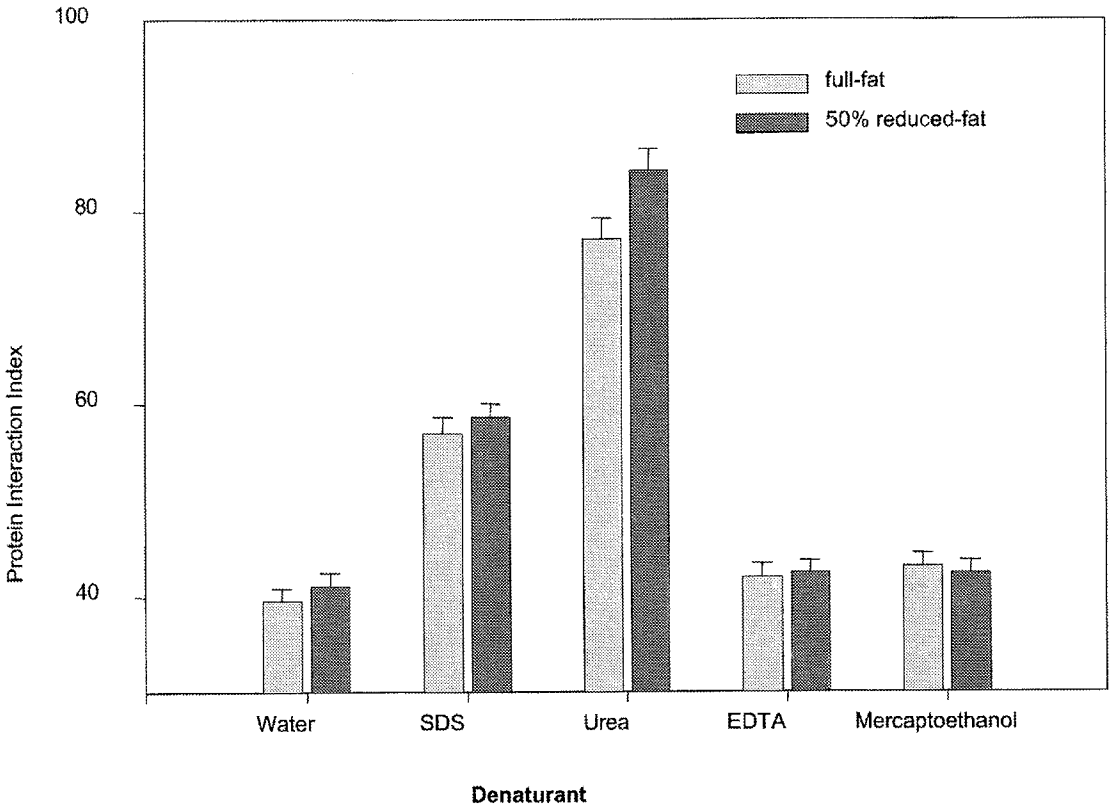


Figure 9.
Protein interaction index measured with different denaturants.

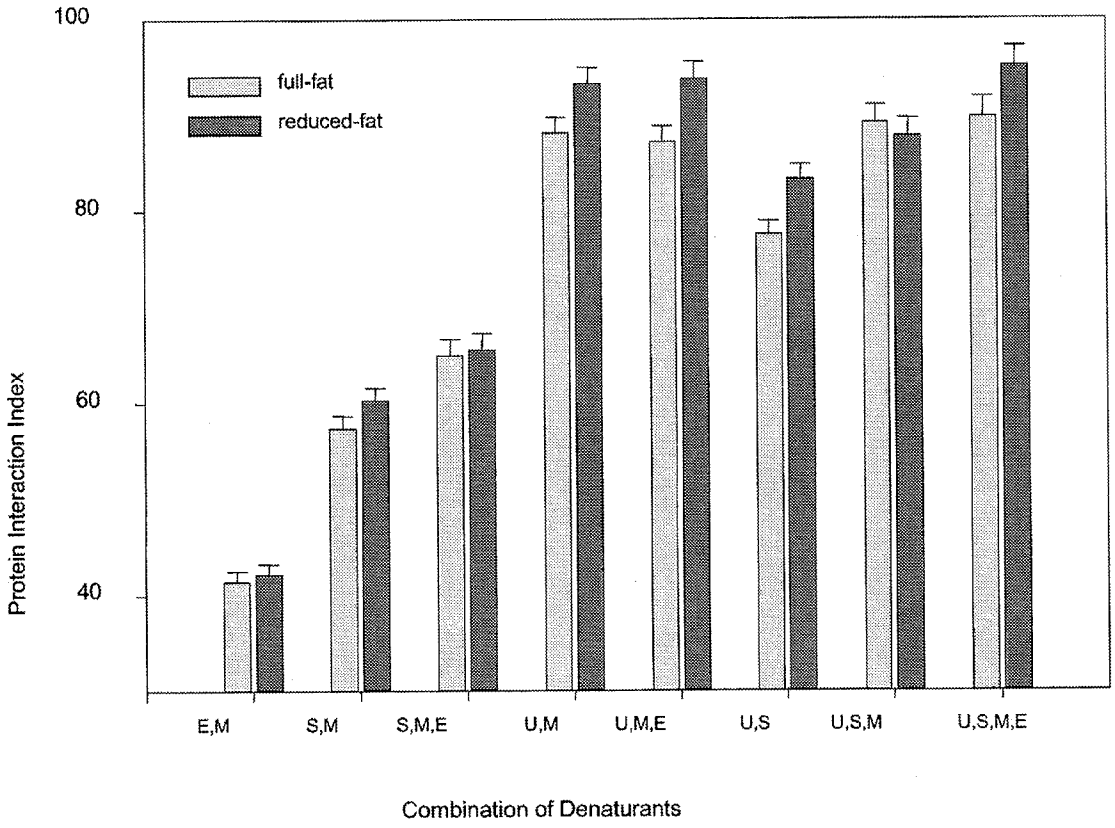


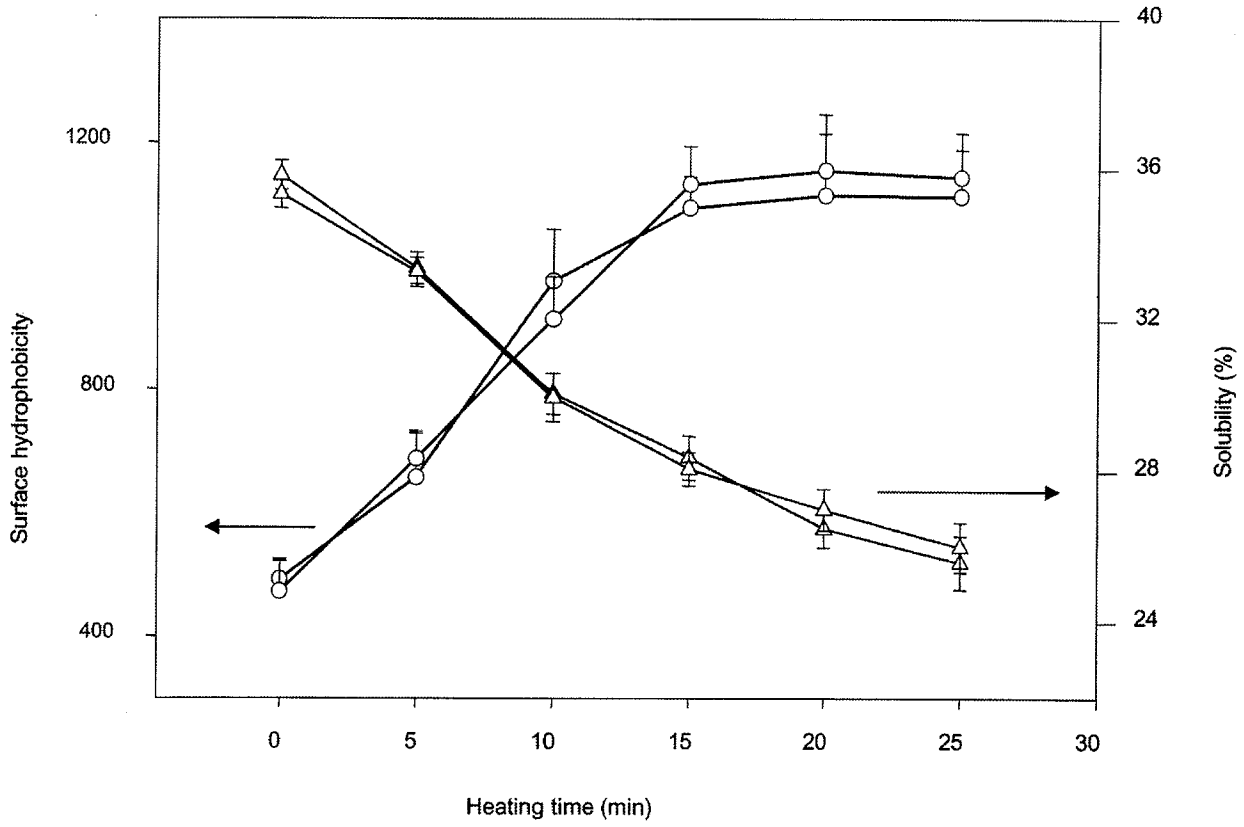
Figure 10.
Protein interaction index measured with different combination of denaturants (E – EDTA; S – SDS; U – Urea; M – Mercaptoethanol).

However, as shown in Figure 10, EDTA and mercaptoethanol accelerated the solubility of heated proteins ($p < 0.05$), when used together with SDS and/or urea. Once breakage of hydrogen bonds and hydrophobic interactions occur by urea and SDS, which were thought to be primary forces in heated protein gels, and the aggregates open, the ionic bonds with calcium and disulphide bonds seem to have more chances to be attacked by EDTA and mercaptoethanol. In other words, hydrogen bonds, ionic bonds with calcium salt, and disulphide bonds, as well as hydrophobic interactions play an important role in aggregation protein gel after heating. These results show that 50% reduced-fat Cheddar cheese was more aggregated under the same heating condition (180 °C for 25 min) than the full-fat cheese. These results can be substantiated by several similar studies

Figure 11 shows the changes in surface hydrophobicity and solubility of full fat and 50% reduced-fat Cheddar cheeses during heating at 180°C. It seems that there is no significant difference ($p>0.05$) in terms of these characteristics between these cheeses.

Figure 11.

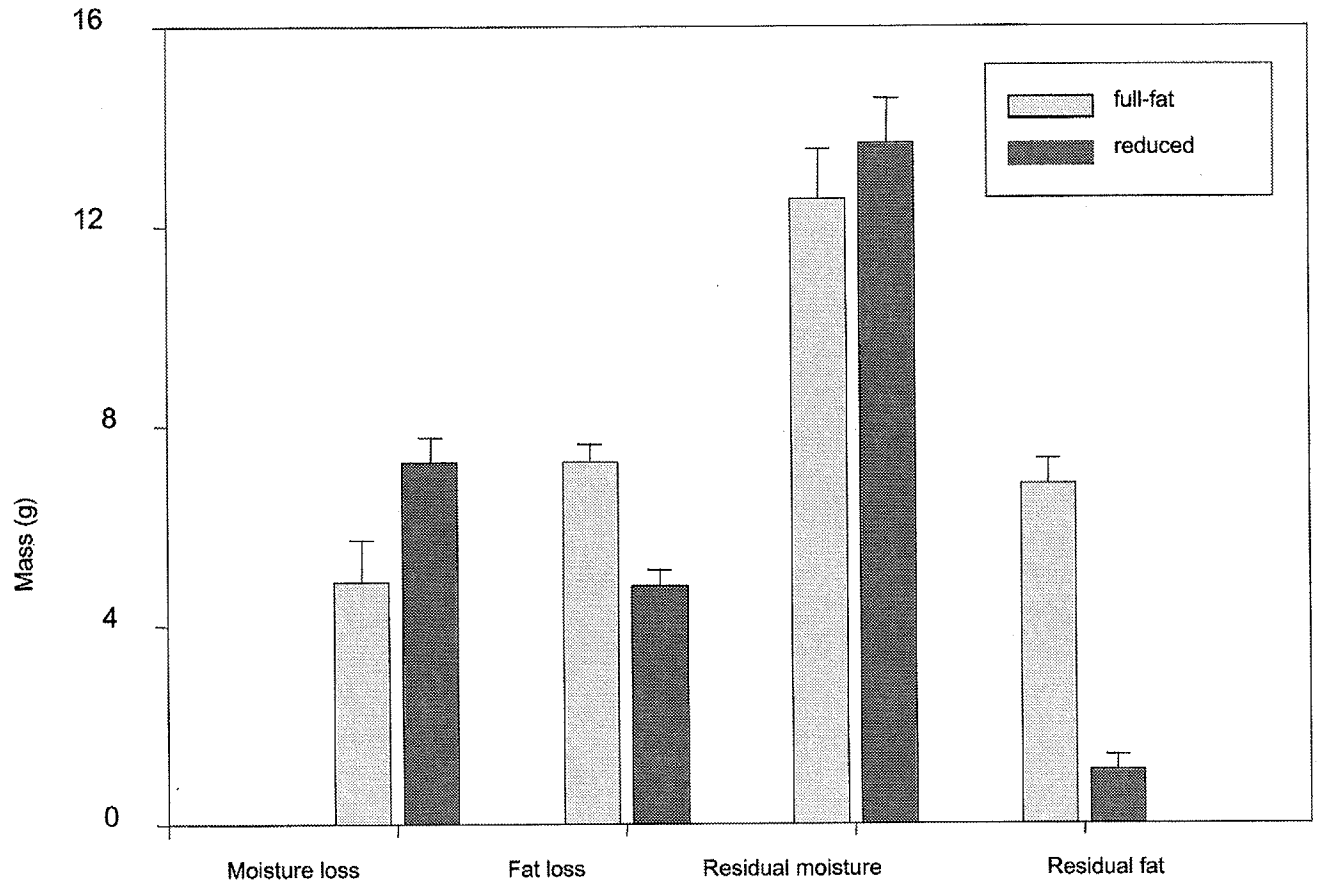
Changes in surface hydrophobicity and solubility of full fat and 50% reduced fat Cheddar cheeses during heating at 180 °C.



The initial moisture contents of 45 g full-fat and reduced-fat cheeses were 17.4 g and 20.9 g, and the initial fat contents 14.1 g and 5.9 g, respectively. As shown in Figure 12, the moisture evaporation rate is much greater for reduced-fat cheese, while the fat separation rate is greater in full-fat cheese, i.e. the rate of component loss appeared to be proportional to the initial amount. The resulting residual amount of moisture is nearly the same in both cheeses, but the amount of residual fat of reduced-fat cheese is less. The resulting protein contents of cheeses increased from 17.4% to 30.0% and 20.8% to 40.6% for full fat and reduced fat, respectively. In other words, higher protein-to-fat & protein-to-moisture ratios in reduced-fat Cheddar cheese after heating, the capacity of fat and moisture to cover and prevent the surface of cheese seemed to be short from heat damage. Not enough fat exuding from the cheese body during heating may accelerate the evaporation of moisture in reduced-fat cheese.

Reduced fat Cheddar cheese seemed to aggregate more under the same heating condition at 180°C for 25 min, compared to full fat cheese. Higher protein to fat and protein to moisture ratio of cheese is considered one of cause for skin formation during heating, which is unique in reduced-fat cheese, resulting from higher degree of protein interactions.

Figure 12.
Fat and moisture separation from 45 g of Cheddar cheese after heating at 180 °C for 25 min and the residual amount of components.



Presentations

Kim, SY and S Gunasekaran. 1997. Changes in physicochemical properties of protein and fat in Cheddar cheese upon heating. Abstract No. 69-8. IFT Annual Meeting, Book of Abstract. Orlando, FL, June 14-18.

Kim SY, HJ Kim, NF Olson, and S Gunasekaran. 1998. Comparison of protein interactions in low fat and full fat Cheddar cheeses during heating. Abstract No. 69-8. IFT Annual Meeting Program and Abstracts, Atlanta, GA, June 20-24.

Kim SY, S Gunasekaran, and NF Olson. 1999. Independent control of melting and softening of Cheddar cheese. Abstract No. 79A-41, IFT Annual Meeting Program and Abstracts, Chicago, IL, July 24-28.

Identification and characterization of components of the proteolytic enzyme system of *Lactobacillus helveticus* which affect bioactive peptide accumulation

Personnel

Steele, James L., University of Wisconsin-Madison, Broadbent, Jeffery R., Utah State University; Weimer, Bart, Utah State University

Funding

Dairy Management Inc.

Dates

June 1997 to June 2000

Objectives

1. To screen strains of *Lactobacillus helveticus* for the type and level of bioactive peptides/bioactive peptide precursors which accumulate as the result of the organisms growth in milk.

2. Determine which components of the proteolytic systems of the selected strains of *Lb. helveticus* are essential for the accumulation of the bioactive peptides/bioactive peptide precursors from milk.

3. Construct strains of *Lb. helveticus* which accumulate elevated levels of the bioactive peptides/bioactive peptide precursors of interest.

Summary

A number of bioactive casein-derived peptides, which may have beneficial affects on human health, have been described. The physiological effects being investigated include immunostimulation, antihypertensive activity, anticarcinogenic activity, mineral absorption, anti-diarrheal action, and prolonged gastrointestinal transit time. In general, significantly greater beneficial effect has been observed with fermented milk products then with unfermented products. These observations support the hypothesis that the action of lactic acid bacteria proteolytic enzymes on casein prior to human consumption alters the peptides that are produced during digestion. This research attempted to determine in greater detail the specific enzymes from *Lb. helveticus* were involved in the formation of bioactive peptides.

The University of Wisconsin-Madison component of this research was to continue our efforts to define and characterize the proteolytic enzyme system of *Lb. helveticus* CNRZ32. Genes that encode for ten peptidases have now been characterized from this organism making it one of the best studied proteolytic enzyme systems among lactic acid bacteria. This study focused on a cell envelop-associated proteinase from CNRZ32 as this class of enzymes would initiate the formation of bioactive peptides in fermented milk products. A cell envelop-associated proteinase gene, designated *prt*H, was identified in CNRZ32 via inverse PCR and characterized. The *prt*H gene encodes

a protein of 1,849 amino acids with a predicted molecular mass of 204 kDa. The deduced amino acid sequence of the *prt*H product has significant identity with lactococcal cell envelop-associated proteinases. Southern blot analysis indicates that this gene is not widely distributed within *Lb. helveticus* suggesting that the presence or absence of this enzyme may have a key role in the strain to strain variation observed in bioactive peptide formation. A prtH deletion mutant of CNRZ32 was constructed to determine if other cell envelop-associated proteinases were also present in *Lb. helveticus* CNRZ32. Cell surface proteinase activity and specificity were determined by monitoring the hydrolysis of the ϑ_{s1}casein fragment 1-23. The results clearly demonstrated that CNRZ32 has at least two cell envelop-associated proteinases with distinct specificities. The contribution of *prt*H, and all other component of the proteolytic system, to the formation of bioactive peptides awaits the development of a rapid method for the determination of bioactivity. Those results will then be coupled with peptide identification by standard techniques to clearly identify targets for the manipulation of proteolytic systems for the overproduction of bioactive peptides in fermented milks.

Publications/Presentations

Pederson, J.A., G.J. Mileski, B.C. Weimer and J.L. Steele. 1999. Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. J. Bacteriol. 181:4592-4597.

Christensen, J.E., E.G. Dudley, and J.R. Pederson, J.L. Steele. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76:217-246.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the Sixth Symposium on Lactic Acid Bacteria. September 1999.

Pederson, J.R. and J.L. Steele. (1999). Characterization and physiological role of a cell surface proteinase from *Lactobacillus helveticus* CNRZ32. Poster presentation at the Sixth Symposium on Lactic Acid Bacteria. September 1999.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the symposium on Dairy Flavors and Biotechnology. IFT Annual Meeting, July 1999.

Christensen, J.E. and J.L. Steele. 1998. Hydrolysis of casein-derived peptides by peptidase-deficient *Lactobacillus helveticus* CNRZ32 derivatives. Oral presentation at the 1998 annual meeting of the American Dairy Science Association. July 1998.

Using neural networks to predict the pH of finished cheese

Personnel
Norback, John P.

Funding
Wisconsin Milk Marketing Board

Dates
May 1999 to June 2001

Objectives
1. To improve the performance of neural networks to estimate the pH of finished cheese during the cook step of cheese making.

2. To identify the pattern of pH change of the whey during the cook step in cheese manufacture and to identify the connection of this pattern to pH of finished cheese.

3. To suggest a system to automatically control the temperature during cook step for the purpose of hitting specified and pH targets in the finished cheese.

Summary
A data acquisition system was developed. This system is able to collect the temperature and pH data during the cook step of Monterey cheese manufacturing. The system was developed as a separate unit from the main cheese vat because the pH electrode was surrounded by glass and could not be mounted in the vat. Such a system is not a fully automated because it needs manual operation to start and shut down data collecting program. This system has been used to collect all required data from real cheese manufacturing without any problems during the entire operation. Newer electrode technology fit may allow us to mount the pH sensor in the vat and provide a way to automate data collection. This will allow tighter integration of pH data with other data collected during vat operation.

The variations occurring from the training artificial neural network (ANN) with the backpropagation algorithm were investigated. The best combination of parameters and network archetecture was determined and used to enhance the network prediction. It is important to facilitate the training process because ANNs establish the relationship between input and corresponding output through the examples of provided training data. These relationships provide the ability of a trained network to recognize or predict the solution. The variations included: the number of hidden neurons, the learning rate, the smoothing factor or momentum term, and the number of training iteration. Different ANNs have different optimal-training conditions, determined by empirical methods. The best results are determined by measuring and minimizing the value of Root Mean Square Error (RMSE).

ANNs were developed and trained to predict the pH of finished cheese within the first 15 minutes of the cook step of cheese production. Two ANNs were trained with (0/1) binary graphs of whey pH and temperature change during the cook stage of cheese production. One ANN used 30-minute graphs and the other used 15-minute graphs–the first half of the previous graph. After obtaining the optimal-training condition, both trained networks were evaluated for their predictive ability with the new set of data. Both ANNs were able to predict finished cheese pH. ANNs were also trained with numerical (not graphic) inputs. These ANNs were also evaluated for their training performance and predictive capacity. The results showed that the numerical ANN was able to predict for the pH of finished cheese by the first 15 minutes of cook process. Both kinds of ANN were able to give 100% correct prediction within actual pH.

ANNs were trained with different format of input vector, one with binary graphic or pictorial type and the other with the numeric time series data input. After training, both networks were tuned to their empirically optimal condition, they were tested for the ability of prediction for the pH of finished cheese. Although, ANN trained with numeric input required a longer training time, these networks gave a better prediction with in actual pH,± 0.05. However at actual pH ±0.10, both ANNs have the same ability of prediction.

The hybrid system between neural network and fuzzy logic is proposed to control the temperature during cook stage. The system concept is using developed ANN that predicts the pH of finished cheese within the first 15 minutes of cook process as a tool to design and initiate the fuzzy system. The fuzzy rule-based system then designs the temperature and time control for the rest of cook process. The ultimate objective of this control system is to make the pH of finished cheese hit the target value.

Whey pH and temperature monitoring system

The following system was set up for gathering the whey pH and temperature values during the cook step of Monterey cheese production. Because it is a hazard to introduce any glass objects into the cheese vat, it was not advisable to place the pH sensor directly in the vat. Thus, it was necessary to develop a whey monitoring system as an additional station, separated from the main vat. During the cook stage, whey solution was sampled from the main production vat to monitor the pH and temperature values. Figure 1 shows the developed station, operated as a separate unit from the main cheese vat. All required components were placed on a cart (16 x 24 x 30 inches) allowing movement from vat to vat during the data collecting process.

There are four main components in the pH and temperature monitoring station. Each unit serves either for data acquisition or data storage. The first component is a whey-sampling screen custom made for installation at an opening edge part inside of the main vat. The slim pocket-shaped screen with a small tube inside was immersed in the whey solution after the cut stage of cheese production was completed. The second unit, a peristaltic pump

Figure 1.
The whey pH and temperature monitoring system, used for data collection.

drive (Masterflex model 07554-80 with its compatible L/S Standard Pump Head model 07013-21 and Silicone Pump Tubing 96400-14 from Cole-Parmer Instrument Co.) was used to control the whey sample flow rate from the vat. Sampled whey solution was pumped out of a main vat through the flow cell of pH electrode and thermometer probe (MicroFlow Thru pH System model FTpH2 from Lazar Research Laboratories, Inc.). After pH and temperature measurement, sampled whey solution was discarded (It was not allowed to reenter the vat). For one instance of data collection, the amount of whey used was used about 130-150 ml. At the flow cell of pH and thermometer probe, both whey pH and temperature were monitored by pH / temperature meter (Microcomputer based bench pH meter model JENCO 6171 from Jenco Instruments, Inc.) and recorded to a connected computer unit as a text file, together with their corresponding vat identification number, date and time of recording. All information from pH meter was communicated to a PC via RS-232 cable.

Determining optimal training condition for neural networks

Three useful ANN models were trained and tested on many variations of ANN assumptions. These included the variation of network structure, the training algorithm and the ability to produce a satisfactory prediction. The best combinations among such variations were identified by using empirical training method and then evaluating the training performance. We did this by applying periodically testing validation procedure to search for the model with the lowest testing error. Since an ANN provides a way that establishes a relation between input and output by leaning the association from the provided data, the empirical method is a common way to search for the best combination of all neural network variations (Huang et al., 1998, Edwards and Cobb, 1997, Peters et al., 1996).

The following are three developed ANN models, based on variations of their training process. The statistical values of Root Mean Square Error (RMSE) from training and testing process were used as criteria. The first developed ANN model (fullPic) was trained with 30-minute graphs of whey pH and temperature change during the cook process as input. This network was developed to compare with the second model (Pic-71) that trained with only 15-minutes graphs, half of the cook graph input from the previous network. The last ANN model (Num-64) was developed and trained with numeric whey pH and temperature change time series and this network was compared with the pictorial ANN. Table1. presents the optimal training condition for a particular network.

Table 1.

Summary of the best training variables for three well-trained neural network

ANN Model [a]	Learning Rate	Smoothing Factor	Training Iteration [b]	Trained RMSE	Tested RMSE
fullPic-249	0.25	0.9	97	0.1215	0.2956
Pic-71	0.15	0.9	161	0.1519	0.2428
Num-64	0.01	0.8	1000	0.1432	0.1240

[a] a number after each developed model indicate the best fit number of hidden neurons corresponding to each neural network

[b] the stop training iteration when the corresponding RMSE of testing process reaches the lowest value.

Evaluation of developed ANN models

Three well-trained ANN models were evaluated for their predictive performance. The results from this evaluation represented a predictive ability index for each ANN. Table 2 compiles the predictive results of three ANN models, presented together.

The prediction of pH of finished cheese by (fullPic-247) ANN model was compared with the result from (Pic-71) model to investigate if an ANN can be trained to predict the pH of finished cheese before ending the cook stage. According to the above table, both (fullPic-247) and (Pic-71) gave the equal value of Mean Absolute Error at 0.04. However the distributions of their absolute errors were different. (Pic-71) gave 75% correct prediction of pH of finished cheese within (0.05 from the actual pH while (fullPic-247) had 60% correct prediction. At a predictive pH, within ± 0.10 from actual pH, both pictorial models were able to give 100% correct prediction. Due to the predictive error distribution, a (Pic-71) model indicated a better prediction than (fullPic-249). The prediction results suggest that an ANN is able to be

Table 2
Comparison of three well trained ANN models for prediction of pH of finished cheese.

Absolute Error [a]	(fullPic-249)	(Pic-71)	(Num-64)
0.00 - 0.05	60%	75%	80%
0.06 - 0.10	40%	25%	20%
Mean Absolute Error [b]	0.04	0.04	0.03
Standard Deviation of Error	0.0274	0.0200	0.0297

[a] is an absolute error between predicted and actual pH of finished cheese.

[b] equals (1/20) x

trained to predict the pH of finished cheese before ending of cook stage by using whey pH and temperature graph obtained from the first 15 minutes during cook phase of cheese making process as an input vector.

The other comparison was conducted between (Pic-71) and (Num-64) to determined the effect of pictorial and numeric input vector on the generalizability of ANN. Num64 had lower Mean Absolute Error (0.03) as well as a better distribution of model predictive error. Within the actual pH ±0.05, (Num-64) was able to predict up to 80% correction while (Pic-71) was able to give 75% correct prediction. However within actual pH ±0.10, both models were able to predict the pH of finished cheese correctly. The predictive results in this research indicated that an ANN trained with numeric input vector showed a better predictive performance than an ANN that trained with binary pictorial inputs. However, an ANN trained with a numeric input required a longer time for the training process.

Publications/presentations

Using Neural Network for Pattern Recognition of Monterey Cheese (Institute of Food Technologists Annual Meeting, June 2001)

Improvement of Cheddar cheese quality through identification and characterization of microbial enzymes responsible for the production or degradation of bitter peptides in cheese

Personnel

Steele, James L., University of Wisconsin-Madison; Johnson, Mark E., Center for Dairy Research; Broadbent, Jeffery R., Utah State University; Brennand, Charlotte, Utah State University

Funding

Dairy Management Inc.

Dates

June 1997 to December 2000

Objectives

1. Define the contribution of starter CEP specificity on peptide pools and bitterness in Cheddar cheese

2. Develop a cheese-based test for bitterness in Cheddar cheese and establish factors that influence sensory perception of bitterness in Cheddar cheese

3. Determine bitter taste thresholds for β-CN (f193-209) and α_{S1}-CN (f1-9).

4. Define the contribution of *Lactobacillus helveticus* CNRZ32 peptidases to degradation of β-CN (f193-209) and α_{S1}-CN (f1-9)

5. Construct *L. lactis* SK11 derivatives with enhanced activity of peptidases demonstrated to be important in hydrolysis of β-CN (f193-209) & α_{S1}-CN (f1-9)

Summary

The first three objectives were primarily conducted at Utah State University under the direction of Drs. Jeff Broadbent and Charlotte Brennand. Previous research by our group has shown that the lactococcal cell envelope proteinase (CEP) is a primary determinant in whether or not a starter culture will produce bitter peptides. One of the limitations in that preliminary study was that peptide accumulation data were confounded by variability in the degree of autolysis and intracellular peptidase activity among strains of *Lactococcus lactis.* To overcome this limitation, Dr. Broadbent's laboratory constructed a series of isogenic strains which differ only in proteinase specificity and which lack the gene for the major lactococcal autolysin, AcmA. The proteinase that were evaluated included the *L. lactis* Wg2 group e proteinase, CEP, the *L. lactis* SK11 group a proteinase, and the group h

proteinase from the bitter starter *L. lactis* S3. The proteinase specificity of each isogenic construct was confirmed by in vitro incubation of whole cells with α_{S1}-CN (f1-23) at pH 5.2 in 4% NaCl and, for the S3 clone, by DNA sequence analysis of the substrate binding regions. Permission to perform sensory analysis on cheeses manufactured with these bacteria was then obtained from the Utah State Biosafety Committee and the USU Institutional Review Board (for experiments with human subjects). 50% reduced-fat Cheddar cheese was manufactured at UW-Madison and HPLC analysis has confirmed that peptide accumulation in the experimental cheeses occurred as predicted by the CEP specificity of each starter. Trained sensory analysis of the experimental cheeses after 2, 4, and 6 mo of ripening has established a clear role for CEP specificity in bitterness. As expected, strains carrying the group a, e, or h proteinase had low, intermediate, or high propensities for bitterness, respectively. These results confirm our previous findings that starter culture proteinase specificity is a key determinate of whether or not a cheese will develop bitterness.

A number of casein-derived peptides with bitter flavor notes have been characterized, but the actual peptides that are most frequently responsible for bitterness in cheese have not yet been identified. In the past, researchers seeking to determine the contribution of specific peptides to bitterness in cheese have relied on sensory evaluation of peptides in aqueous solutions to measure bitterness. However, sensory studies have clearly established that taste thresholds for a compound increase when viscosity increases or when competing tastes are present. For this reason, the quantity of any peptide necessary to evoke a bitter response will always be much higher in cheese than in water, so water dispersion data cannot be reliably applied to cheese. Dr. Brennand's work has demonstrated that dispersal of bitter compounds in a cheese model system is a representative and effective means to study bitterness in cheese. To our knowledge, we are the first group to study the contribution of individual peptides to bitterness in model cheese system, and our work on bitter taste thresholds for β-CN (f193-209) and α_{S1}-CN (f1-9) has provided valuable new insight into the role of specific peptides in bitterness. In the case of both peptides the bitter taste threshold was approximately 10-fold higher in the model cheese system than in water. When the bitter taste threshold of these peptides in the model cheese system were compare to the levels of these peptides observed in a bitter cheese, it was concluded that the (f1-9) was primarily responsible for bitterness in this cheese. While the β-CN (f193-209) peptide likely had a complementary function, rather than a dominant role, in the perception of bitterness in this cheese.

The ability of lactic acid bacteria peptidases to hydrolyze bitter peptides to non-bitter peptides and amino acids is well established, but the relative contribution of individual enzymes to this process is largely unknown. The peptidase system of *Lactobacillus helveticus* CNRZ32, an adjunct that reduces bitterness in cheese, has been investigated in detail by Dr. Steele's laboratory. Genes for ten peptidases have been cloned and sequenced from this organism. Of these enzymes, the contribution of 2 general aminopeptidases (PepC and PepN), a proline-specific aminopeptidase (PepX), and two endopeptidases (PepO and PepE) to the hydrolysis of the known bitter peptides β-CN (f193-209) and α_{S1}-CN (f1-9) has been evaluated. As a preliminary study, the ability of CNRZ32 and isogenic derivatives lacking

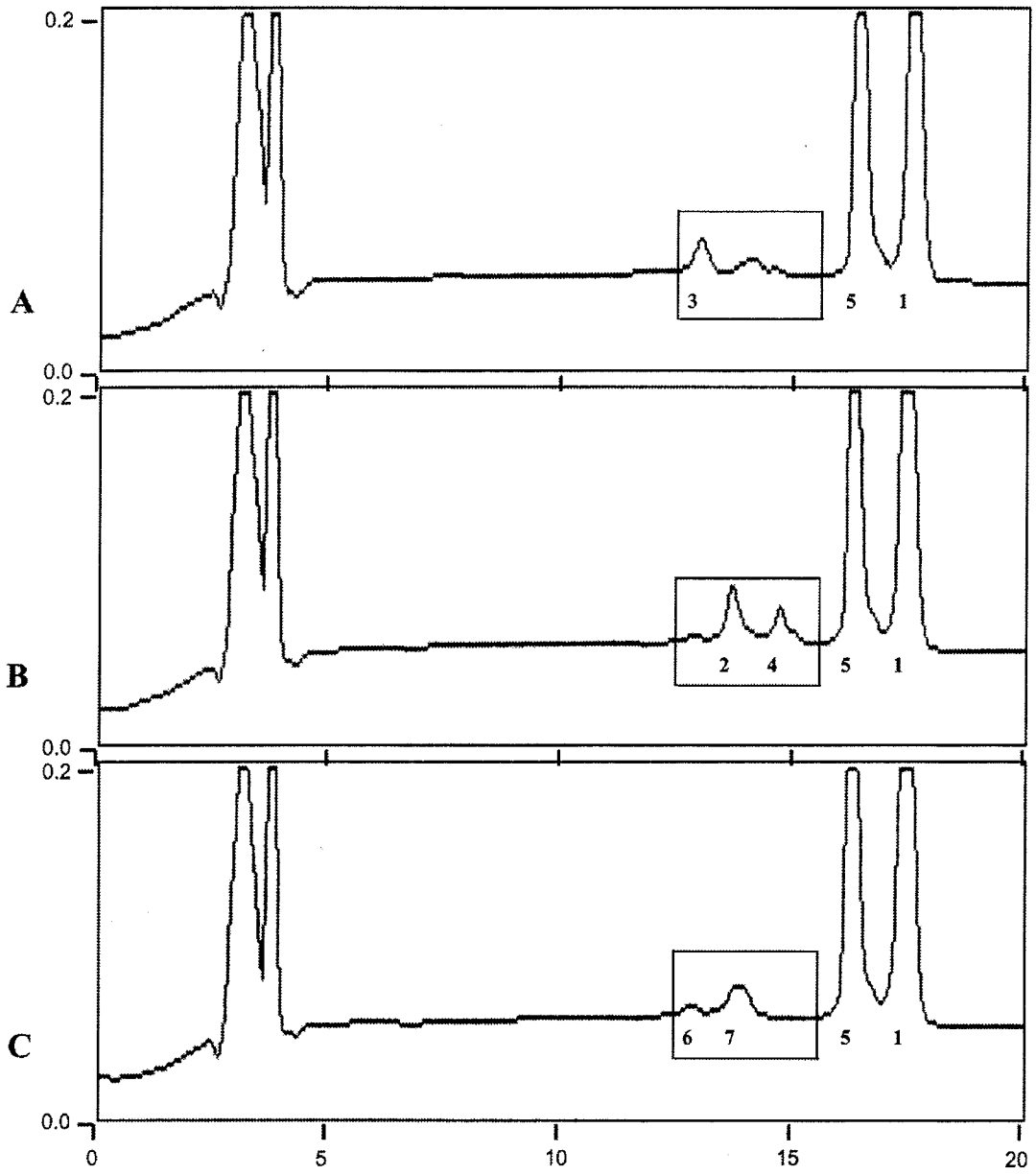


Figure. 2.

Chromatograms of peptide separations from hydrolysis of α_S1-CN(f1-9). The chromatogram shows an A220 cross section of the DAD data from 200-300 nm wavelength. Chromatogram A is from a reaction with CFE from wild type, but is representative of chromatograms from the reactions with CFE from ΔpepC, ΔpepO, and ΔpepE strains. Chromatogram B and C are from reaction with CFE from ΔpepN and ΔpepX strains, respectively. The boxed region of the chromatograms (RT 12.5-15.5 min) show peak profiles which correspond to the differences in identified peptides between strains. Peaks that were identifiable by mass spectrometry are numbered and correspond to the order presented in Table 2: 1, α_S1-CN(f1-9); 2, α_S1-CN(f3-9); 3, α_S1-CN(f4-9); 4, α_S1-CN(f5-9); 5, α_S1-CN(f1-7); 6, α_S1-CN(f4-7); 7,α_S1-CN(f1-5).

PepC (JLS241), PepN (JLS242), PepX (JLS243), PepE (JLS233) or PepO (JLS232) to obtain essential amino acids for growth from α_{S1}-CN (f1-9) and β-CN (f193-209) and was evaluated. The peptides sequences and essential amino acids for growth of *Lb. helveticus* CNRZ32 are shown in figure 1.

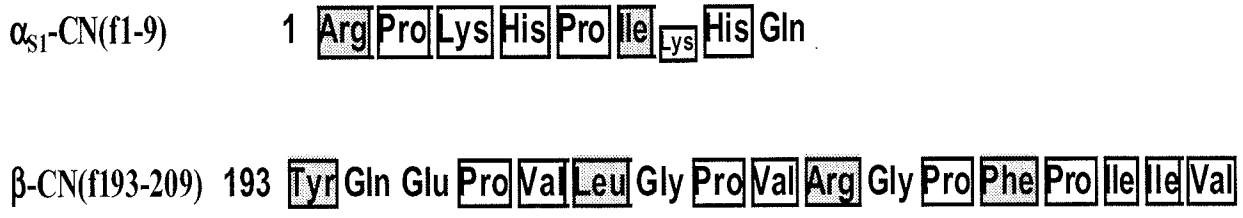
No deficiencies were observed for the growth of *Lb. helveticus* wild type or any of the peptidase deletion mutant strains requiring hydrolysis to obtain essential AA from of either α_{S1}-CN(f1-9) or β-CN(f193-209). The lack of growth deficiencies of single peptidase deletion mutants (Δ*pepC*, Δ*pepE*, Δ*pepN*, Δ*pepO*, or Δ*pepX*) in defined peptide media may be explained several ways. First, the mutated peptidases may not be involved in the hydrolysis of either α_{S1}-CN(f1-9) or β-CN(f193-209). Second, the absence of a single peptidase may be compensated for by another peptidase with similar specificity. Third, the essential AA may be liberated via a different pathway of hydrolysis in the absence of a given peptidase.

Reactions of CFE of *Lb. helveticus* wild type and the peptidase deletion mutants with α_{S1}-CN(f1-9) or β-CN(f193-209) were investigated to assess differences in the hydrolysis patterns in the absence of a given peptidase. The results for the hydrolysis of α_{S1}-CN (f1-9) are shown in figure 2 and the transitional peptides derived from these reactions are summarized in table 1. Similarly, the results of the hydrolysis of β-CN(f193-209) are shown in figure 3.

Differences in hydrolysis were detected from reactions with JLS242 (ΔpepN) and/or JLS243 (ΔpepX). Several unique peptides accumulated in the absence of PepN activity. For the hydrolysis reactions of α_{S1}-CN (f1-9), these peptides corresponded to a decreased ability to liberate Lys3 from α_{S1}-CN (f3-9) (Table 2). For the hydrolysis of b-CN(f193-209), these peptides corresponded to a decreased ability to liberate Tyr193, Val197, and Leu198 from derived peptides (Table 3). The results for liberation of Lys and Leu residues are consistent with the reported AA specificities of PepN for AA-rNA substrates. The activities measured for liberation of Tyr and Val from dipeptide substrates is routinely reported to be relatively low. However, an evaluation of activity of purified lactococcal PepN for a tryptic digest of β-CN also indicated the ability of this peptidase to liberate Tyr from β-CN(f193-202) and Val from β-CN(f170-176), as well as peptides containing Glu at the N-terminus. The absence of PepX activity from CFE resulted in the accumulation of α_{S1}-CN(f1-5) and α_{S1}-

Figure. 1.

Peptide sequences of αS1-CN(f1-9) and β-CN(f193-209) derived from Bos taurus casein. The residues that are boxed are essential AA for *Lb. helveticus*. The residues that are shaded are essential AA that only occur once in the peptide and must therefore be liberated for growth of *Lb. helveticus*.



	Arg 1	Pro 2	Lys 3	His 4	Pro 5	Ile 6	Lys 7	His 8	Gln 9	Strain
f1-9	R	P	K	H	P	I	K	H	Q	WT, JLS242, JLS243
f3-9			K	H	P	I	K	H	Q	JLS242
f4-9				H	P	I	K	H	Q	WT
f5-9					P	I	K	H	Q	WT & JLS242
f1-7	R	P	K	H	P	I	K			WT, JLS242, JLS243
f4-7				H	P	I	K			JLS243
f1-5	R	P	K	H	P					JLS243

Table 1.

Transitional peptides derived from hydrolysis of α_S1-CN(f1-9) by CFE from *Lb. helveticus* wild type, JLS242 (_pepN), and JLS243 (_pepX). The peptides correspond to unique values obtained for RP-HPLC fractions from mass spectrometry data. The strain column indicates from which reactions with CFE a given peptide was identified.

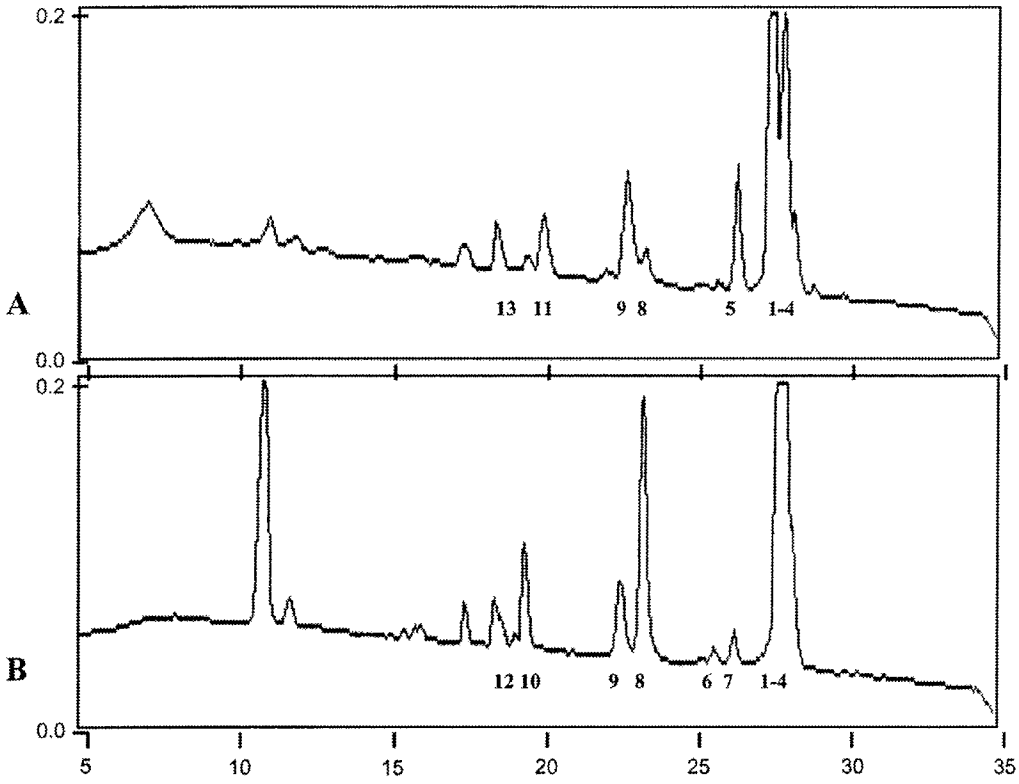


Figure 3.

Chromatograms of peptide separations from hydrolysis of β-CN(f193-209). The chromatogram shows an A220 cross section of the DAD data from 200-300 nm wavelength. Chromatogram A is from a reaction with CFE from wild type, but is representative of chromatograms from the reactions with CFE from ΔpepC, ΔpepO, ΔpepE, and ΔpepX strains. Chromatogram B is from reaction with CFE from the ΔpepN strain. Peaks that were identifiable by mass spectrometry are numbered and correspond to the order presented in Table 3: 1, β-CN(f193-209); 2, β-CN(f194-209); 3, β-CN(f195-209); 4, β-CN(f197-209); 5, β-CN(f199-209); 6, β-CN(f201-209); 7, β-CN(f193-208); 8, β-CN(f193-206); 9, β-CN(f194-206); 10, β-CN(f197-206); 11, β-CN(f199-206); 12, β-CN(f193-204); 13, β-CN(f194-204).

	Tyr	Gln	Glu	Pro	Val	Leu	Gly	Pro	Val	Arg	Gly	Pro	Phe	Pro	Ile	Ile	Val	
	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	Strain
f193-209	Y	Q	E	P	V	L	G	P	V	R	G	P	F	P	I	I	V	WT & JLS242
f194-209		Q	E	P	V	L	G	P	V	R	G	P	F	P	I	I	V	WT & JLS242
f195-209			E	P	V	L	G	P	V	R	G	P	F	P	I	I	V	WT
f197-209					V	L	G	P	V	R	G	P	F	P	I	I	V	WT & JLS242
f199-209							G	P	V	R	G	P	F	P	I	I	V	WT
f201-209									V	R	G	P	F	P	I	I	V	JLS242
f193-208	Y	Q	E	P	V	L	G	P	V	R	G	P	F	P	I	I		JLS242
f193-206	Y	Q	E	P	V	L	G	P	V	R	G	P	F	P				WT & JLS242
f194-206		Q	E	P	V	L	G	P	V	R	G	P	F	P				WT & JLS242
f197-206					V	L	G	P	V	R	G	P	F	P				JLS242
f199-206							G	P	V	R	G	P	F	P				WT
f193-204	Y	Q	E	P	V	L	G	P	V	R	G	P						JLS242
f194-204		Q	E	P	V	L	G	P	V	R	G	P						WT

Table 2.

Transitional peptides derived from hydrolysis of β-CN(f193-209) by CFE from *Lb. helveticus* wild type and JLS242 (_pepN). The peptides correspond to unique values obtained for RP-HPLC fractions from mass spectrometry data. The strain column indicates from which reactions with CFE a given peptide was identified.

CN(f4-7), both peptides having an Xaa-Pro N-terminus (Table 2). This is consistent with the known substrate specificity of PepX.

The chromatograms for hydrolysis reactions by all six strains were virtually indistinguishable in terms of the rate in reduction ofα_{S1}-CN(f1-9) and the accumulation of the primary product, α_{S1}-CN(f1-7). Also, the peptides identified from hydrolysis reactions by all six strains contained several β-CN(f193-209) derived fragments with Pro204 or Pro206 at the C-terminus. Since the deletion of PepE and PepO did not affect the rate of formation of these peptides, these results suggest the hydrolysis was due to an unidentified post-proline endopeptidase. Confirmation of a post-proline endopeptidase in CNRZ32 was obtained by the ability of CNRZ32 CFEs to hydrolyze C- and N-blocked β-CN (f203-209). The identification of a post-proline endopeptidase in CNRZ32 is significant, as this enzymes substrate specificity suggests it may contribute to the hydrolysis of numerous bitter peptides. Therefore, Objective 5 was delayed to identify endopeptidase essential for the hydrolysis of β-CN (f193-209).

A genomic library of *Lb. helveticus* CNRZ32 in *E. coli* DH5a was screened for endopeptidase activities with Ac-β-CN(f203-209)-rNA. Two isolates of the 1880 screened had activity in a coupled reaction with PepN. Restriction endonuclease profiles of the two isolates were

visually indistinguishable. One plasmid, designated pSUW99, was selected for further analysis. The endopeptidase activity was subcloned on a 3.0 kb SstI fragment. The complete nucleotide sequence of the 3.0 kb SstI fragment was determined, and an open reading frame (ORF) of 1947 bp identified. This ORF could encode a polypeptide of 649 amino acids with deduced mass of 71.4 kDa. Protein sequence homology searches using current BLAST databases revealed high amino acid sequence similarity between the deduced amino acid sequence and other LAB PepO endopeptidases. This protein has 56% identity and 70% similarity to Lb. helveticus CNRZ32 endopeptidase PepO; therefore, this gene was designated pepO2. Northern hybridization using total RNA from an exponential culture of *Lb. helveticus* CNRZ32 resulted in the detection of a transcript with a size of 2.1 kb. This size corresponds to the size of the pepO2 ORF, and indicates that pepO2 is monocistronic.

Reactions of CFEs prepared from E. coli DH5a containing only the cloning vector (pJDC9) and containing pSUW99 with α_{S1}-CN(f1-9) or β-CN(f193-209) were investigated to assess PepO2's hydrolysis specificities with these substrates. No significant hydrolysis of either substrate was detected with CFEs from E. coli DH5a (pJDC9). However, significant hydrolysis of both β-CN(f193-209) andα_{S1}-CN(f1-9) was detected with CFEs from E. coli DH5a(pSUW99). The predominant peptide fractions were collected and analyzed. PepO2 was determined to hydrolyze b-CN(f193-209) at bonds Pro196-Val197, Pro200-Val201, and Pro206-Ile207. Hydrolysis of α_{S1}-CN(f1-9) was observed at Pro5-Ile6 bond. All of the bonds hydrolyzed in these peptides by PepO2 were either Pro-Val or Pro-Ile, indicating that PepO2 is a post-proline endopeptidase. Hydrolysis of the β-CN(f193-209) Pro204-Phe205 and α_{S1}-CN(f1-9) Pro2-Lys3 bonds was not observed, suggesting that PepO2 may have a preference for small uncharged amino acids on the carboxy side of the scissile bond. The hydrolysis of peptide bonds involving Pro is likely to be important in the hydrolysis of CN-derived peptides as Pro constitutes 16.7% of b-CN and 8.5% of α_{S1}-CN amino acid residues. Additionally, CN-derived bitter peptides have been observed to contain relatively large amounts of Pro and it has been proposed that the spatial structure resulting from the presence of Pro in a peptide directly relates to bitterness. Therefore, the specificity of PepO2 for bonds containing Pro suggest that this enzyme is likely to have a central role in the demonstrated ability of Lb. helveticus CNRZ32 to reduce bitterness in cheese.

Subsequent studies will examine if strains over-expressing PepO2 can reduce bitterness and increase flavor development in a bacterial ripened cheeses (i.e. Cheddar and Gouda). Additionally, the possible interaction between PepO2 and other components of the Lb. helveticus CNRZ32 proteolytic system, such as PepN, will be assessed through the use of combinations of strains over-expressing different enzymes.

Publications/Presentations

Christensen, J.E. Broadbent, J.R. and J.L. Steele. 2002. Hydrolysis of casein derived peptides α_S1-CN(f1-9) and β-CN(f193-209) by *Lactobacillus helveticus* peptidase deletion mutants. In final preparation.

Chen, Y.S., J.E. Christensen, M. Strickland, Broadbent, J.R., and J.L. Steele. 2002. Cloning and characterization of a post-prolyl endopeptidase from *Lactobacillus helveticus* CNRZ32. In final preparation.

Broadbent, J.R., M. Barnes, C. Brennand, M. Strickland, K. Houck, M.E. Johnson and J.L. Steele. 2002. Contribution of Lactococcus lactis cell envelope proteinase specificity to peptide accumulation and bitterness in Cheddar cheese. J. Dairy Sci. 85: in press.

Christensen, J.E., E.G. Dudley, and J.R. Pederson, J.L. Steele. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76:217-246.

Steele, J.L., M.E. Johnson, J.R. Broadbent, and B.C. Weimer. 1998. Starter culture attributes which affect cheese flavor development, pp. 157-170. In, Proc. LACTIC '97 conference, Which strains? For which products?

Johnson, M.E., J.L. Steele, J. Broadbent, and B.C. Weimer. 1998. Manufacture of Gouda and flavor development in reduced-fat Cheddar cheese. Aust. J. Dairy Technol. 53:67-69.

Broadbent, J.R., M. Strickland, B. Weimer, M.E. Johnson, and J.L. Steele. 1998. Peptide accumulation and bitterness in Cheddar cheese made using single-strain *Lactococcus lactis* starters with distinct proteinase specificities. J. Dairy Sci. 81:327-337.

Christensen, J.E. 2000. Peptidases of *Lactobacillus helveticus*: role in physiology and caein hydrolysis. Ph.D. Dissertation, University of Wisconsin-Madison

Chen, Y.S. 2001. Endopeptidases of *Lactobacillus helveticus* CNRZ32: Identification and Characterization. Ph.D. Dissertation, University of Wisconsin-Madison

Chen, Y.-S., J.E. Christensen, M, Strickland and J.L. Steele. 2001. Identification and characterization of endopeptidase O2 from *Lactobacillus helveticus* CNRZ32, an enzyme involved in the hydrolysis of a β-casein derived bitter peptides. Oral presentation at the 2001 annual meeting of the Institute of Food Technologists.

Broadbent, J.R. 2000. Role of lactic acid bacteria in cheese flavor development –Part I. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

Steele, J.L. 2000. Role of lactic acid bacteria in cheese flavor development –Part II. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese.

Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

Chen, Y.-S., J.E. Christensen and J.L. Steele. 2000. Identification and characterization of PepO2 from *Lactobacillus helveticus* CNRZ32, an enzyme involved in the hydrolysis of α_s1-CN-casein derived bitter peptides. Oral presentation at the 2000 annual meeting of the American Dairy Science Association.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the symposium on Dairy Flavors and Biotechnology. IFT Annual Meeting, July.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the Sixth Symposium on Lactic Acid Bacteria. September, The Netherlands.

Broadbent, J.R. 1999. Cheese curing and flavor development. Invited oral presentation for the 15th Cheese Making Short Course. February 9-11, Utah State University, Logan.

Broadbent, J.R. 1998. How starter bacteria direct cheese flavor development. Invited oral presentation for the 13th Biennial Cheese Conference. Aug. 10-12, Utah State University.

Broadbent, J.R. 1998. Influence of *Lactococcus lactis* starter bacteria on peptide accumulation and bitterness in Cheddar cheese. Invited oral presentation for Texel International. April 30, Dange, France.

Broadbent, J.R. 1998. Cheese curing and flavor development. Invited oral presentation for the 14th Cheese Making Short Course. March 26-28, Utah State University, Logan.

Christensen, J.E. and J.L. Steele. 1998. Hydrolysis of casein-derived peptides by peptidase-deficient *Lactobacillus helveticus* CNRZ32 derivatives. Oral presentation at the 1998 annual meeting of the American Dairy Science Association.

Broadbent, J.R. 1997. Influence of starter bacteria on peptide accumulation and bitterness in Cheddar cheese. Invited oral presentation for the National Cheese Technology Forum sponsored by Dairy Management, Inc., Dec. 9-10. Chicago.

Growth of non-starter lactic acid bacteria in reduced fat Cheddar cheese

Personnel

Steele, James, University of Wisconsin-Madison; Broadbent, Jeff, Utah State University; Johnson, Mark, Wisconsin Center Dairy for Research

Funding

Dairy Management Inc.

Dates

July 1997 to December 2000

Objectives

1. To establish the population dynamics between starter, non-starter, and adjunct bacteria during ripening of 50% reduced fat Cheddar cheese.

2. To establish the population dynamics between starter, non-starter, and adjunct bacteria during ripening of 50% reduced fat Colby cheese.

3. To construct derivatives of the adjunct *Lactobacillus casei* subsp. *pseudoplantarum* that are unable to co-metabolize citrate and lactate and to test the influence of the loss of this metabolism on the ability of the adjunct to grow in cheese.

4. To establish the impact on the sensory attributes of reduced fat Cheddar cheese to which adjunct bacteria have been added by monitoring the relationship between growth of starter, adjunct and non-starter bacteria and flavor attributes during aging of the cheese.

Summary

Work at Utah State University was focused on Objectives 1 and 2, while objectives 3 and 4 were the focus of research at the University of Wisconsin-Madison. The population dynamics between starter, non-starter, and adjunct bacteria during cheese ripening has been examined by random amplified polymorphic DNA (RAPD) fingerprinting of cheese isolates. Initial studies confirmed the utility of this technique for differentiation among individual strains of *Lactococcus lactis*, *Lactobacillus casei*, and *Lactobacillus helveticus*. For the cheese studies, 8 vats of 50% reduced-fat Cheddar and Colby cheese was manufactured at UW-Madison in November of 1998. Template DNA for RAPD was isolated from 80 individual colonies (10 per vat) collected on Rogosa or Ellikers agar after day 1, 2 weeks, 1 mo, 2 mo, 3 mo, 4 mo and 6 mo of ripening. RAPD fingerprints have now been collected from day 1, 2wk, 2 mo, 4 mo, and 6 mo cheese isolates. Isolates which displayed a unique RAPD fingerprint were identified by nucleotide sequence analysis of their 16S rRNA gene. By combining strain fingerprint and species data, we have been able to monitor the diversity of NSLAB in each of the cheeses over time and have been able to evaluate the ability of an adjunct *Lactobacillus casei* strain

to dominate the NSLAB biota. As part of this work, we also isolated DNA from our 6 mo-old experimental cheese and used it as a template for the amplification of bacterial 16S rRNA genes. It was our hope that this approach would allow us to speciate nonculturable bacteria that might dominate the NSLAB population. Unfortunately, the only species identified to date by DNA sequence analysis of 10 different 16S rDNA clones is the starter, *Lc. lactis*. The significant conclusions from this part of the study were: (i) RAPD is an effective method for the analysis of NSLAB population dynamics in ripening cheese; (ii) the NSLAB biota in both cheeses changed over time, but NSLAB populations in Colby cheese retained a greater degree of heterogeneity than those of Cheddar, (iii) the *Lb. casei* Lila adjunct did not dominate the NSLAB populations beyond 3 mo, but it's use did limit the heterogeneity of the NSLAB population in Colby and in young Cheddar; and (iv) all cheese finished with a NSLAB population dominated by more than 1 strain of *Lb. casei*; the dominant strains of *Lb. casei* in each cheese appeared to be most affected by adjunct treatment and not cheese variety. Sensory analysis of these cheeses indicated panelists preferred the cheeses that lacked adjunct, and that the adjunct used, *Lb. casei* Lila, either directly or indirectly resulted in off-flavors.

The lactic acid bacteria (LAB) family encompasses a group of gram-positive bacteria that ferment carbohydrates primarily to lactic acid, generating ATP by substrate-level phosphorylation. It is believed that the fermentation of carbohydrates is required for the growth of LAB. However, carbohydrates are depleted within the first weeks of cheese ripening and while NSLAB increase from low levels (approximately 10^3 CFU/gr. of cheese) to relatively high levels (10^8 CFU/gr. of cheese). It has been postulated that NSLAB generate sufficient energy from the fermentation of organic acids (such as citrate) and catabolism of amino acids to reach high cell density in cheese. Due to relatively high concentration of citrate in ripening cheese and the ability of NSLAB to catabolized citrate, it is commonly thought that citrate catabolism has a role in supporting the growth of NSLAB to high cell density in ripening cheese. Additionally, citrate is a potential precursor to flavor compounds found in fermented products. We have used ^{13}C NMR to identify the metabolites and metabolic intermediates produced from citrate by NSLAB (*Lb. zeae, Lb. casei, and Lb. rhamnosus*). These organisms have been shown to produce acetate, lactate, and ethanol from citrate. These studies are covered in significantly greater detail in the report on succinate production in this volume. Additionally, we have demonstrated that citrate enhances the growth yield of non-starter lactobacilli under conditions that simulate those found in ripening cheese. Therefore, our laboratory has attempted to examine the mechanism by which NSLAB metabolize citrate.

To facilitate efforts to understand the contribution of citrate catabolism to the ability of organisms from the "*Lb. casei*" cluster (*Lb. zeae, Lb. casei, and Lb. rhamnosus*), we choose to clone the citrate cluster from *Lb. zeae* ATCC 393. The computer program PRODOM 99 and amino acid sequence alignments of OadA homologs reported in GenBank, were used to identify regions of

Table 1.

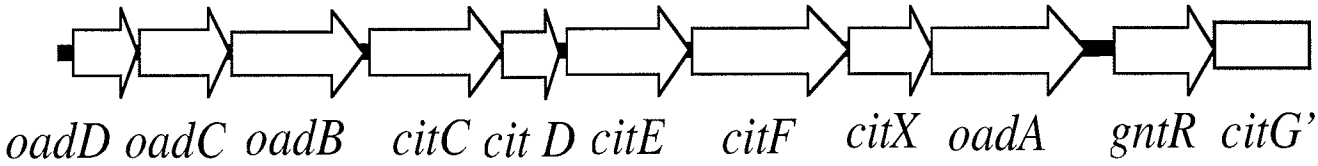
Open reading frames identified in the 8858-bp DNA sequenced from *Lactobacillus zeae* ATCC 393.

Gene	nucleo-tides	putative product M.W.	Best match*	PROSITE
oadD	327	11832	none	none
oadC	405	13492	*Streptococcus mutans* biotin carboxy carrier protein (P29337; 52% over 143 aa)	PS00188
oadB	1122	39802	*Vibrio cholerae* oxaloacetate decarboxylase beta subunit (AAF93719; 61% over 373 aa)	none
citC	1002	36843	*Weissella paramesenteroides* putative citrate lyase ligase (CAB60040; 54% over 318 aa)	PS00136
citD	303	10774	*Escherichia coli* citrate lyase acyl carrier protein (P77618; 53% over 96 aa)	none
citE	876	31440	*Leuconostoc mesenteroides* citrate lyase beta chain (O53078; 73% over 287 aa)	none
citF	1530	54700	*Leuconostoc mesenteroides* citrate lyase alpha chain (Y10621; 72% over 510 aa)	none
citX	558	20501	*Vibrio cholera* CitX protein (AE004165; 46% over 164 aa)	none
oadA	1401	51880	*Methanococcus jannaschii* oxaloacetate decarboxylase alpha subunit (U67563; 75% over 441 aa)	PS00165
gntR	768	30010	*Bacillus subtilus* gluconate operon repressor (BAA19694; 45% over 214 aa)	PS00043
citG (partial)	261		*Vibrio cholerae* CitG (AE004165; 65% over 84 aa)	none

* Indicates the sequence in GenBank with the highest sequence identity to the deduced amino acid sequence from Lb. zeae, the GenBank accession number of this sequence, and the percent identity shared by the two sequences.
† PROSITE (Hofmann et al., 1999) pattern identified within the deduced amino acid sequence. PS00188, Biotin-requiring enzymes attachment site; PS00136, Serine proteases, subtilase family, aspartic acid active site; PS00165, Serine/threonine dehydratases pyridoxal-phosphate attachment site; PS00043, Bacterial regulatory proteins, gntR family signature.

Figure 1.

Schematic representation of the open-reading frames deduced from the 8.9 kb sequenced region of Lb. zeae ATCC 393 DNA. Each open-reading frame is designated by an arrow, and the arrowhead points in the direction of transcription deduced from the DNA sequence. The direction of transcription for the incomplete citG sequence (designated citG') is in the same direction as the other genes represented here, as determined from the deduced amino acid sequence.



sequence similarity between bacterial OadA sequences. Based upon these alignments, two degenerate primers with sequences were designed, which could hybridize to nucleotides 935-960 and 1715-1740 of the *Salmonella typhimurium oadA* sequence, generating a 806-bp product. The sequences of these two primers were selected to bias the codon frequency used by *Lb. casei* and *Lb. plantarum*, using a *Lactobacillus* codon frequency table (Dr. Peter Pouwels, TNO, The Netherlands, personal communication). Degenerate PCR was performed using *Lb. zeae* ATCC 393 genomic DNA as the template and successfully amplified a 0.8-kb product. BLAST searches indicated that this fragment could encode a protein with 62% sequence similarity (135 of 213 amino acid residues) to the *S. typhimurium* oxaloacetate decarboxylase alpha-subunit. Inverse PCR was used to isolate *Lb. zeae* ATCC 393 DNA fragments that flank the product obtained by degenerate PCR. The PCR products obtained used directly for nucleotide sequencing. The complete nucleotide sequence of an 8.9 kb region of the *Lb. zeae* ATCC 393 genome was determined utilizing this approach. A summary of the open reading frames (ORFs) identified within the sequenced region is shown in Table 1 and a schematic representation of the ORFs within this segment is shown in Fig.1.

Within the citrate lyase cluster, the structural subunits CitD, CitE, and CitF were identified based upon BLAST searches, which comprise the a, b, and g subunits of citrate lyase in other bacteria. Also, the gene that putatively encodes citrate lyase-ligase (CitC), which activates CitDEF through acetylation of the CitF prosthetic group, was identified 5' of *citDEF*. An ORF identified as CitX, and a partial ORF similar to CitG were identified. CitG is a protein of unknown function that is found associated with all citrate lyase operons sequenced to date. CitX is another protein of unknown function, which is associated with putative citrate lyase genes of *E. coli*, and *Vibrio cholerae.*

Oxaloacetate decarboxylase in *K. pneumoniae* is a three-subunit protein designated OadGAB. In *Lb. zeae*, products of ORFs corresponding to OadA and OadB were identified. Unlike the corresponding enzymes from *K. pneumoniae* and *S. typhimurium*, an amino acid sequence conforming to the biotin-binding pattern of PROSITE was not identified in OadA. Upstream of *oadB* in the *Lb. zeae* sequence is an ORF, designated *oadC*. The deduced amino acid sequence of *oadC* has identity to the *Streptococcus mutans* biotin-carrier protein as well as the biotin-binding proteins of other bacterial enzyme complexes. Located 5' of *oadC* is an ORF, designated *oadD*, which does not have significant identity to any other sequences in GenBank. Lastly, the deduced amino acid sequence of an identified ORF has high identity to transcriptional regulators of the GntR family, and contains an amino acid sequence that conforms to the PROSITE signature sequence for proteins of the GntR family. Therefore, this ORF was designated *gntR*.

The *Lb. zeae* genes encoding oxaloacetate subunits are of particular interest. The deduced amino acid sequence of the a-subunit suggests that the *Lb. zeae* OadA is a 467-aa protein, which is

significantly (>120 aa) shorter than the characterized OadA homologs from *K. pneumoniae*, *S. typhimurium*, or *Legionella pneumophila*. Sequence comparisons suggest the *Lb. zeae* enzyme is a C-terminal truncated form of the other enzymes. This truncation is expected to eliminate the biotin-binding site. Towards the 5'-end of *citD*, genes designated *oadB* and *oadC* were identified encoding products with similarity to membrane-spanning carboxybiotin-carrier protein decarboxylases and biotin-binding proteins of other organisms, respectively. The presence of these subunits suggests that the structure of the *Lb. zeae* oxaloacetate decarboxylase may be similar to the decarboxylase involved in the malonate utilization pathway of *Malonomonas rubra*, the methylmalonyl-CoA decarboxylase of *Propionigenium modestum* and *Veillonella parvula*, and the glutaconyl-CoA decarboxylase of *Acidaminococcus fermentans*. These systems are comprised of a substrate decarboxylase, a biotin-carrier protein, and a membrane-spanning carboxybiotin-carrier protein decarboxylase. Substrate decarboxylation by these enzyme systems is a two-step process. First, the substrate is decarboxylated and the liberated CO_2 is covalently attached to the prosthetic group of the biotin-carrier protein. Subsequently the biotin-carrier protein is decarboxylated by the membrane-spanning carboxybiotin-carrier protein decarboxylase, and the energy liberated through this reaction [–17 to –25 kJ/mol] is used to pump a sodium ion from the intracellular to the extracellular compartment. In this manner, the energy of decarboxylation is conserved in the form of a chemical gradient. It can be hypothesized that the oxaloacetate decarboxylase of *Lb. zeae* functions in a similar manner. Experiments are ongoing to evaluate whether the sequenced region encodes functional protein products, and whether the reactions catalyzed by these proteins enhance the growth rate of *Lb. zeae* in the presence of citrate

Publications/Presentations

Broadbent, J.R., B. McKernan, K. Houck, and M. E. Johnson. 2001. Influence of adjunct use and cheese microenvironment on nonstarter lactic acid bacteria in Cheddar-type cheese. In final preparation.

Díaz-Muñiz, I, E.G. Dudley, J.A. Pederson, and J.L. Steele. 2001. Citrate catabolism by *Lactobacillus zeae* and its influence on growth in ripening cheese. American Society for Microbiol. Abstr., 2001, . (manuscript also in preparation)

Broadbent, J.R. 2000. Role of lactic acid bacteria in cheese flavor development –Part I. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

Steele, J.L. 2000. Role of lactic acid bacteria in cheese flavor development –Part II. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

Succinate production by *Lactobacillus casei*: Pathways responsible and development of strategies to control its accumulation

Personnel

Steele, James L., University of Wisconsin-Madison

Funding

Dairy Management Inc.

Dates

July 1997 to December 2000

Objectives

1. Screening strains of *Lactobacillus casei* for the ability to metabolize citrate and produce succinate.

2. Construction and characterization of *Lb. casei* mutants defective in lactate dehydrogenase and oxaloacetate decarboxylase.

3. Evaluation of the effect of the lactate dehydrogenase and oxaloacetate decarboxylase mutations on the ability of *Lb. casei* to produce succinate in a model cheese ripening system.

Summary

Succinate is an organic acid that affects the flavor of fermented dairy products, however little is known concerning how succinate is produced in aged cheese such as Cheddar. Therefore, this research was initiated to better understand the mechanisms of succinate production by *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus zeae*, and *Lactobacillus rhamnosus*, which are organisms that can be isolated from Cheddar during the ripening process. The identified potential pathways for the formation of succinate are presented in Figure 1.

As strains of *Lb. plantarum* have been shown to produce succinate and occur in ripening cheese, we choose to initiate this study by screening stationary phase *Lb. plantarum* strains ATCC 14917, ATCC 14431 and RL3 for the ability to produce succinate from citrate, L-lactate, L-Asp, and isocitrate. *Lb. plantarum* ATCC 14917 was previously been shown to produce succinate during growth in a citrate- and mannitol-containing medium. *Lb. plantarum* RL3 is a natural cheese isolate that has been suggested to produce succinate when present in cheese during the ripening process (R. C. Lindsay, personal communication).

All three strains of *Lb. plantarum* produced detectable levels of succinate when incubated in the presence of citrate (Table 1), however the concentration varied depending upon whether L-lactate was initially present or not. *Lb. plantarum* ATCC 14917, ATCC 14431, and RL3 accumulated approximately 2.3-, 1.3-, and 2.4-fold more succinate when citrate and L-lactate were both added to whole-cell suspensions, compared to incubations per-

formed without added L-lactate. No succinate accumulation was detected from 10 mM L-lactate (Table 1), 10 mM L-Asp (data not shown), or 10 mM isocitrate (data not shown).

All three strains catabolized citrate in the presence or absence of L-lactate. However, strains ATCC 14917 and RL3 catabolized <13% of the citrate initially present under either condition, while strain ATCC 14431 catabolized approximately 41% and 48% of the citrate in the absence and presence of L-lactate, respectively. Whole cells of *Lb. plantarum* ATCC 14431 accumulated more succinate under these conditions than did ATCC 14917 or RL3, however the stoichiometry of succinate produced per citrate molecule catabolized for the latter two strains was greater than that for ATCC 14431. The strains ATCC 14917 and RL3 synthesized approximately 1 mole of succinate per 2 moles of citrate utilized, while ATCC 14431 accumulated approximately 1 mole of succinate per 5 moles of citrate utilized.

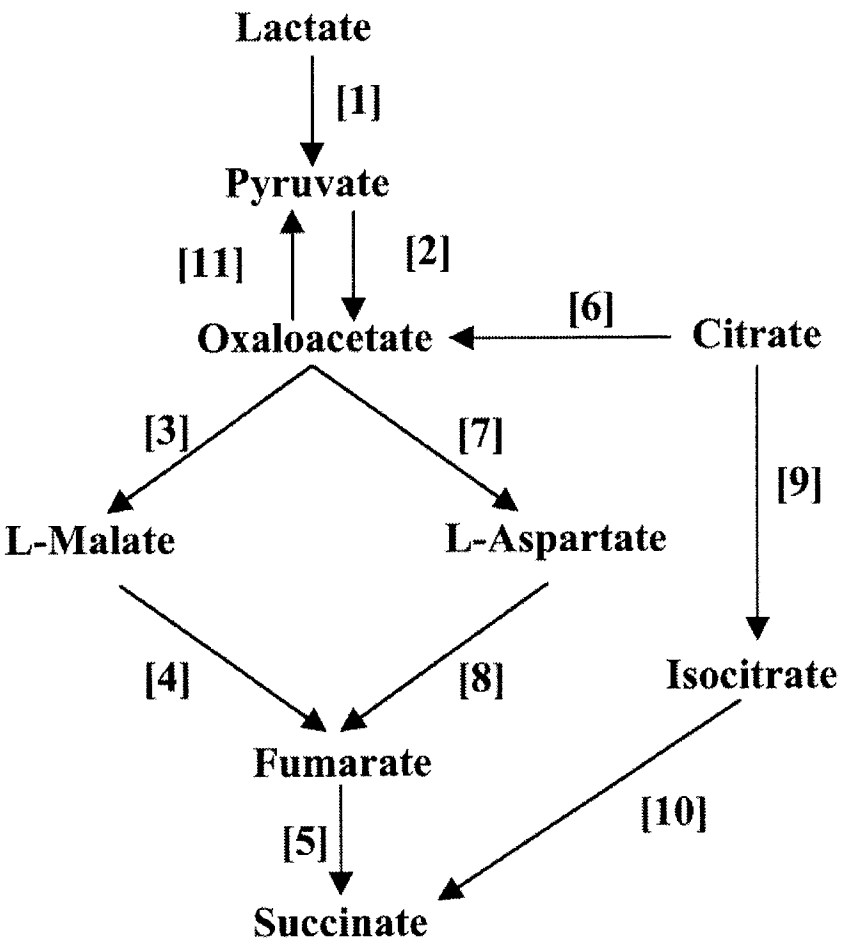


Figure 1.

Succinate producing pathways screened for in strains of Lactobacillus. The enzymes indicated are: [1] lactate dehydrogenase; [2] pyruvate carboxylase; [3] malate dehydrogenase; [4] fumarase; [5] fumarate reductase; [6] citrate lyase; [7] aspartate aminotransferase; [8] aspartase; [9] aconitase; [10] isocitrate lyase; [11] oxaloacetate decarboxylase.

Table 1.
Succinate production, L-lactate utilization, and citrate utilization by whole cells of Lb. plantarum*

Strain	Substrate	mM succinate produced	mM L-lactate remaining	mM citrate remaining
Lb. plantarum ATCC 14917	10 mM citrate	0.19 ± 0.03	N. D.	9.81 ± 0.16
Lb. plantarum ATCC 14431	10 mM citrate	0.76 ± 0.09	N. D.	6.02 ± 0.27
Lb. plantarum RL3	10 mM citrate	0.28 ± 0.03	N. D.	9.64 ± 0.10
Blank	10 mM citrate	Bql	N. D.	10.24 ± 0.11
Lb. plantarum ATCC 14917	10 mM citrate 10 mM L-lactate	0.43 ± 0.03	6.93 ± 0.11	9.11 ± 0.16
Lb. plantarum ATCC 14431	10 mM citrate 10 mM L-lactate	0.97 ± 0.09	7.81 ± 0.08	5.19 ± 0.25
Lb. plantarum RL3	10 mM citrate 10 mM L-lactate	0.67 ± 0.03	6.99 ± 0.11	8.73 ± 0.06
Blank	10 mM citrate 10 mM L-lactate	Bql	10.03 ± 0.14	9.98 ± 0.17
Lb. plantarum ATCC 14917	10 mM L-lactate	Bql	8.94 ± 0.10	N. D.
Lb. plantarum ATCC 14431	10 mM L-lactate	Bql	9.59 ± 0.21	N. D.
Lb. plantarum RL3	10 mM L-lactate	Bql	9.47 ± 0.29	N. D.
Blank	10 mM L-lactate	Bql	10.07 ± 0.15	N. D.

* Whole cells were incubated in the presence of the substrate(s) indicated, and assayed for succinate production after 24 h incubation at 37 oC. All values are reported as the average of duplicate assays from two independent experiments ± standard deviation. N. D.; not determined. Bql; below the quantifiable limit of 0.10 mM succinate.

All three *Lb. plantarum* strains catabolized L-lactate, whether citrate was initially present or not. The strains ATCC 14917, ATCC 14431, and RL3 catabolized approximately 11%, 5%, and 6%, respectively, of the L-lactate initially present when incubated in the absence of citrate, and approximately 31%, 22%, and 30%, respectively, of the L-lactate when incubated in the presence of citrate.

To identify the products of citrate catabolism by *Lb. plantarum* as well as catabolic intermediates, ^{13}C-NMR spectroscopy was utilized as a noninvasive *in vivo* method. Data from Table 2 suggested that strain ATCC 14431 catabolizes citrate in a manner different than strains ATCC 14917 and RL3, which appear to be similar. Strains ATCC 14431 and RL3 were thus chosen for subsequent NMR experiments. When whole cells of strain ATCC 14431 were analyzed, peaks corresponding to Asp, succinate, acetate, and lactate were observed 15 h after the addition of labeled citrate (Fig. 2). This is well past the time at which the substrate was fully utilized. Additionally in spectra analyzed approximately 0.25 h after citrate addition, the metabolic intermediates oxaloacetate and malate were identified. When strain RL3 was analyzed, the same four products of citrate catabolism were observed (Fig. 3), and the intermediates oxaloacetate and malate were also identified in spectra collected approximately 0.25 h after the addition of citrate.

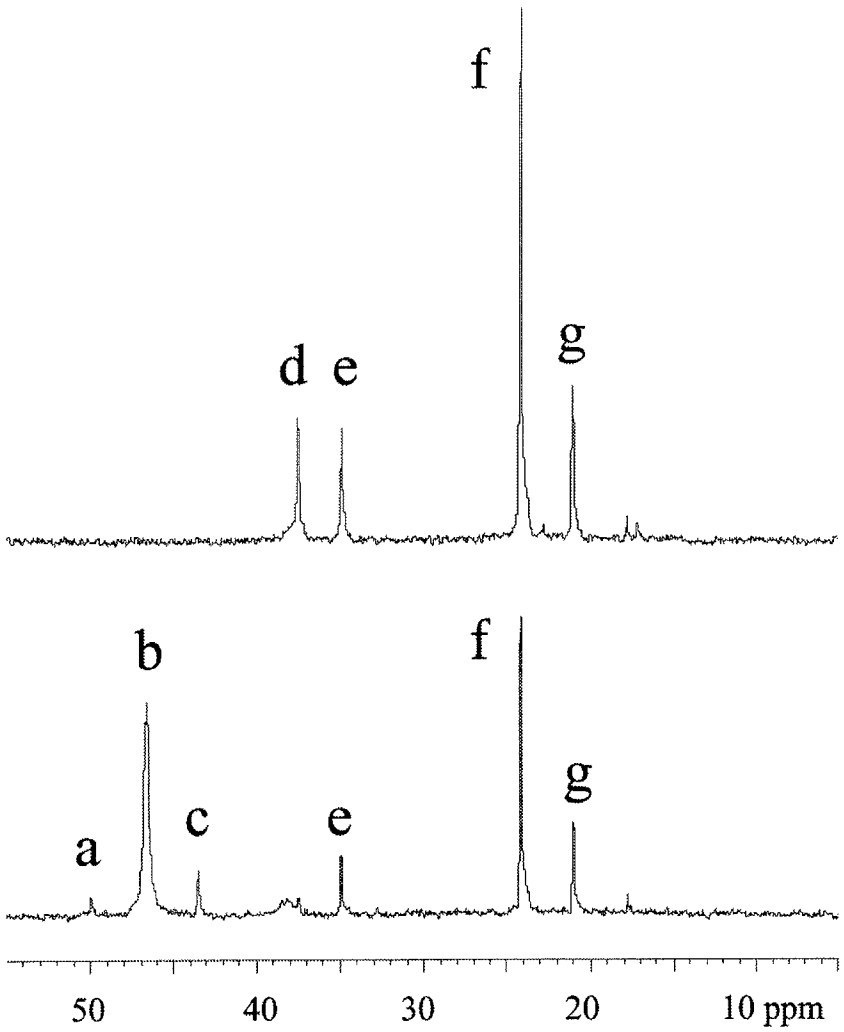


Figure 2.
13C-2,4 citrate catabolism by whole cells of *Lactobacillus plantarum* ATCC 14431 under anoxic conditions at pH 7.0 and 37°C. The bottom and top panels represent data collected approximately 0.25 h and 15 h after the addition of labeled citrate, respectively. Peak assignments are: a, oxaloacetate; b, citrate; c, malate; d, Asp; e, succinate; f, acetate; and g, lactate. The small peak that appears around 18 ppm in both spectra was also present in the spectra collected prior to the addition of citrate, and was not identified.

When these experiments were repeated using $^{13}C_3$-L-lactate instead of ^{13}C-2,4 citrate, two sets of doublet peaks were observed in the whole-cell sample of ATCC 14431 incubated at 37 °C (Fig. 4). Based upon the chemical shifts, the doublets around 182.1 ppm and 24.1 ppm were assigned to the carboxy and methyl carbons of acetate, respectively. No putative intermediates of L-lactate catabolism to acetate were identified in any spectra collected.

The data collected from NMR studies suggested that *Lb. plantarum* strains produced succinate from citrate via the reductive TCA pathway, so the enzymes from this pathway were assayed in CFEs. All three strains of *Lb. plantarum* produced detectable levels of citrate lyase, malate dehydrogenase, and fumarase (Table 2). In general, the activities of these three enzymes were similar in all three strains; the activities of citrate lyase, malate dehydrogenase, and fumarase varied by approximately 4.5-, 3.1-, and 1.6-fold, respectively, among strains. Fumarate reductase assays following the oxidation of methylviologen were inconclusive due to high blank background. Therefore, this enzyme was detected by screening whole-cell suspensions for the ability to produce succinate from fumarate. The three *Lb. plantarum* strains produced between 1.90 and 5.11 mM succinate within 24 h when resuspended in buffer at pH 7 containing 10 mM fumarate. It is

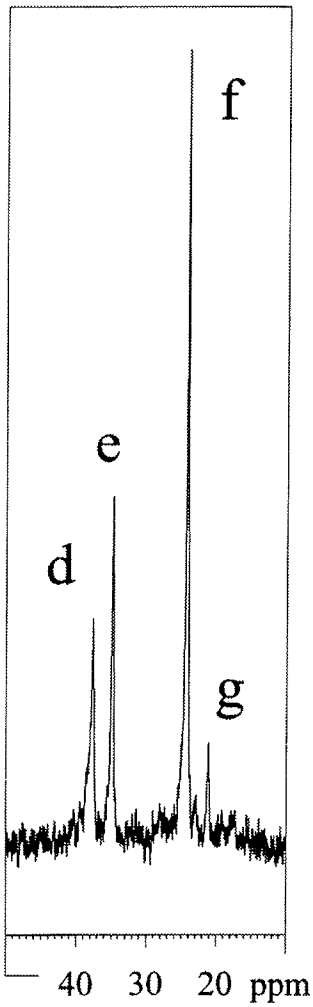


Figure 3.
Catabolism of 13C-2,4 citrate by *Lactobacillus plantarum* RL3 under anoxic conditions at pH 7.0 and 37°C. The above spectrum represents data collected approximately 14 h after the addition of labeled citrate. Peak assignments are: d, Asp; e, succinate; f, acetate; and g, lactate.

not known, however, how much fumarate was utilized in each of these experiments. None of the CFEs contained detectable aspartase or isocitrate lyase activities. The limits of detection for aspartase and isocitrate lyase assays were approximately 0.0076 units and 0.039 units, respectively.

As shown previously, *Lb. plantarum* strains produce Asp, succinate, acetate and lactate from citrate when the reactions are followed at pH 7.0 and 37 °C. During cheese ripening, conditions typically include pH 5.1, 4% NaCl, and a temperature of 13 °C. Therefore, we were interested in repeating the NMR and succinate assay studies under conditions more representative of the environment *Lb. plantarum* would encounter during ripening cheese. Comparison of spectra collected from the catabolism of labeled citrate by strain ATCC 14431 under these two conditions suggested that citrate was fully catabolized during both experiments, and significantly less succinate was produced at pH 5.1, 4% NaCl, and 13 °C compared to pH 7.0 and 37 °C (Fig. 5). Quantification of succinate production revealed that at pH 5.1, 4% NaCl and 13 °C, ATCC 14917, ATCC 14431, and RL3 accumulated approximately 3-, 3-, and 2-fold less succinate, respectively, than at pH 7.0 and 37°C.

The conclusions of this component of the study were that *Lb. plantarum* strains produce succinate from citrate via the reductive TCA pathway. Future experiments to identify the steps limiting the production of succinate by *Lb. plantarum* during cheese ripening are required.

The next component of this study was to determine if *Lb. casei* and related species could produce succinate from L-lactate, L-Asp, or isocitrate. We chose to study *Lb. casei* ATCC 334, *Lb. zeae* ATCC 393, and *Lb. rhamnosus* ATCC 7469, which are the type strains of their respective species. Whole cells were screened for the ability to produce succinate from citrate, L-lactate, L-Asp, and isocitrate. After a 24 h incubation at 37 °C, none of the supernatants from any whole-cell suspension contained detectable levels of succinate. The lack of succinate production from citrate was not due to the inability to catabolize citrate. Under these conditions, ATCC 334, ATCC 393, and ATCC 7469 catabolized approximately 91-94%, 42% and 97% of the citrate initially present whether L-lactate was included in the reaction or not. These strains also produced L-lactate from citrate when incubated in 10 mM citrate + 10 mM L-lactate, as L-lactate concentrations found in supernatants were higher than the blank. Whole cells of ATCC 334 catabolized approximately 3% of the L-lactate initially present when incubated in the presence of L- lactate without additional citrate. No significant catabolism of L-lactate was seen for ATCC 7469 or ATCC 393.

^{13}C-NMR spectroscopy was used to identify the citrate catabolic pathway(s) of the three *Lactobacillus* strains. All three strains accumulated acetate, lactate, and ethanol (Fig. 6) when catabolism of ^{13}C-2,4 citrate was followed at 37 °C. The spectra were indistinguishable whether only labeled citrate was added, or whether labeled citrate was added together with 10 mM of

Figure 4.

13C3-L-lactate catabolism by whole cells of *Lactobacillus plantarum* ATCC 14431. The spectrum represents data collected 12.5 h after the addition of labeled L-lactate to a final concentration of 10 mM. Whole cells were incubated at 37°C. The letter assignments are: a, carboxy- and methyl carbons for L-lactate; b, carboxy- and methyl carbons for acetate. The peak around 18 ppm was also present in a spectrum collected prior to the addition of labeled L-lactate, and was not identified.

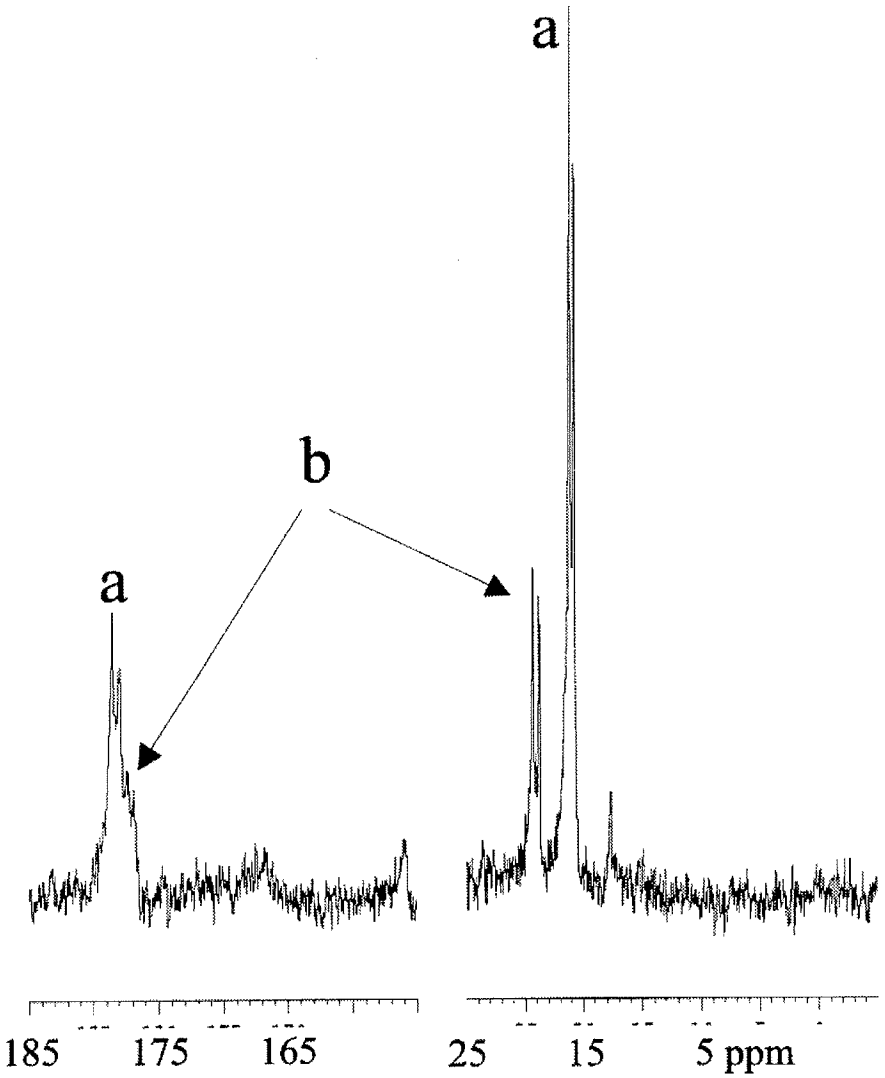


Table 2.

Detection of citrate lyase, malate dehydrogenase, fumarase, and fumarate reductase activities in cell-free extracts or whole cells of Lactobacillus plantarum strains*

Strain	Citrate lyase (U)†	Malate dehydrogenase (U)†	Fumarase (U)‡	Fumarate reductase (mM) §
ATCC 14917	0.39 ± 0.01	1.00 ± 0.05	0.38 ± 0.01	3.00 ± 0.44
ATCC 14431	0.86 ± 0.10	3.05 ± 0.94	0.17 ± 0.05	5.11 ± 0.76
RL3	0.19 ± 0.00	0.87 ± 0.04	0.62 ± 0.11	1.90 ± 0.07

* All values are reported as the average of duplicate assays on two independent experiments ± standard deviation.
† Citrate lyase and malate dehydrogenase activities are reported as mmol NADH oxidized min-1 (mg protein)-1.
‡ Fumarase activities are reported as mmol fumarate formed min-1 (mg protein)-1.
§ Fumarate reductase activities are reported as mM succinate produced from whole-cell suspensions in 100 mM potassium phosphate pH 7.0 and 10 mM fumarate at 37°C after 24 h.

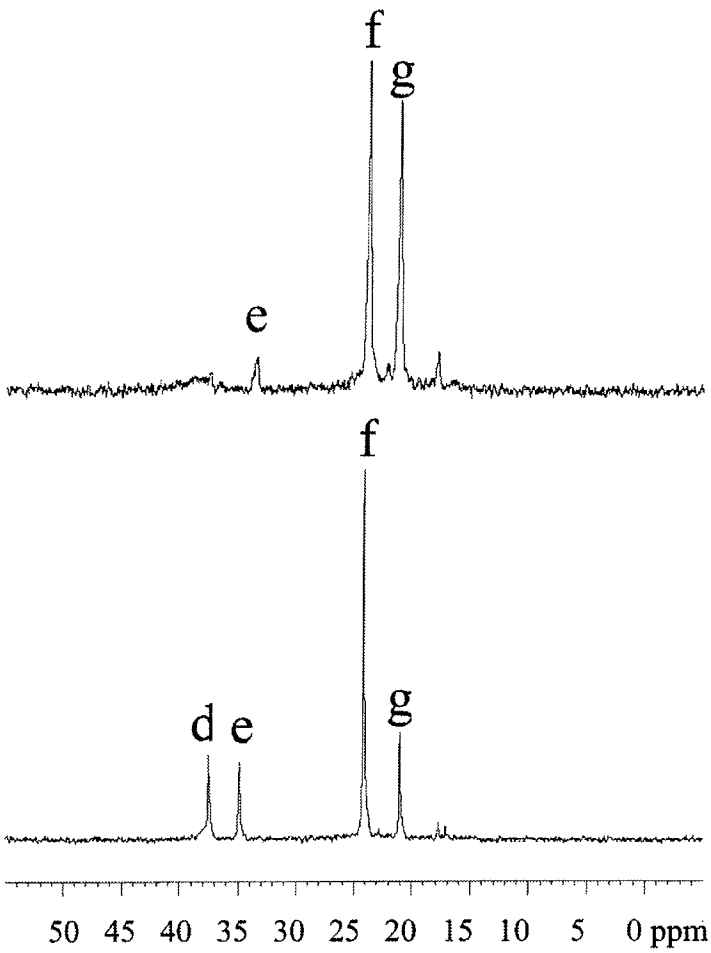


Figure 5.
Catabolism of ^{13}C-2,4 citrate at pH 7.0, 37°C (bottom panel) and pH 5.1, 4% NaCl, and 13°C (top panel). The spectra represent data collected approximately 14-15 h after the addition of labeled citrate. The peak assignments are: d, Asp; e, succinate; f, acetate; and g, lactate

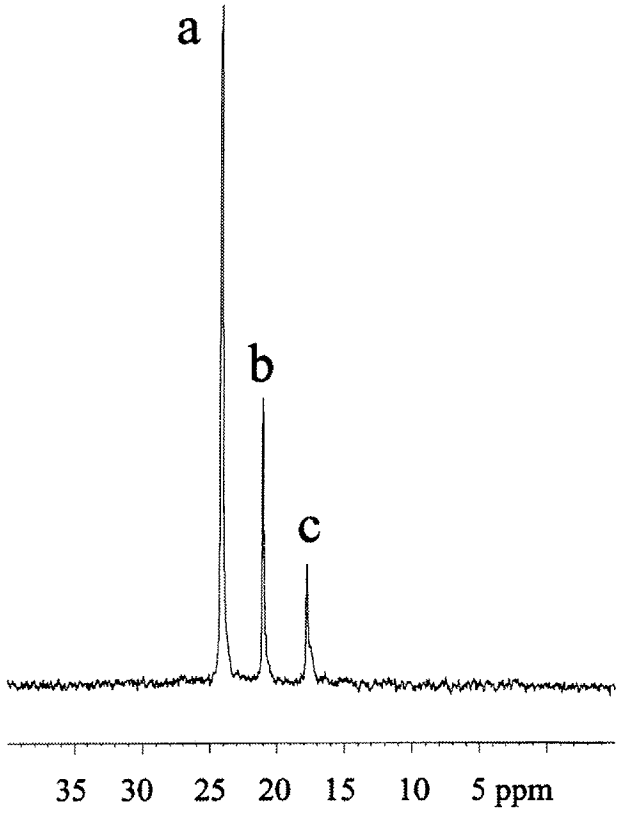


Figure 6.
Catabolism of ^{13}C-2,4 citrate by *Lb. rhamnosus* ATCC 7469 recorded approximately 15 min after the addition of labeled citrate. The incubation was performed at 37°C in 100 mM potassium phosphate pH 7.0 under anoxic conditions. This spectrum is also representative of the results seen for *Lb. casei* ATCC 334 and *Lb. zeae* ATCC 393. Peak assignments are: a, acetate; b, lactate; and c, ethanol.

unlabelled L-lactate. For all strains, the peak corresponding to citrate was absent in all spectra collected 26 min after the addition of citrate. No intermediates of citrate catabolism were identified in any spectra collected during experiments performed at 37 °C. The *Lactobacillus* stains produced acetate, lactate, and ethanol from citrate when ^{13}C-2,4 citrate catabolism was followed in the presence of L-lactate. The same products were also seen when whole cells were incubated anoxically at pH 5.1 and 13 °C in the presence of 4% NaCl.

CFEs of *Lb. casei* ATCC 334, *Lb. zeae* ATCC 393, and *Lb. rhamnosus* ATCC 7469 were examined for enzymes required for the production of succinate from citrate via the reductive TCA pathway (Table 3). Citrate lyase and malate dehydrogenase activities were detected in CFEs from all three strains. Malate dehydrogenase activities were just above the limit of detection for the assay used. Fumarase activity was not detected in any of the CFEs. Fumarate reductase assays were conducted by screening whole-cell suspensions for the ability to accumulate succinate when incubated in the presence of fumarate. Succinate was only detected in supernatants from *Lb. casei* ATCC 334

Table 3.

Screen of *Lactobacillus casei* ATCC 334, *Lactobacillus zeae* ATCC 393, and *Lactobacillus rhamnosus* ATCC 7469 cell-free extracts for enzyme activities involved in the reductive TCA pathway.

Strain	Citrate lyase (U)*	Malate dehydrogenase (U)*	Fumarase (U)†	Fumarate reductase (mM)‡
ATCC 334	0.20 ± 0.04§	0.01 ± 0.00	< 0.02	2.28 ± 0.19
ATCC 393	0.93 ± 0.07	0.03 ± 0.01	< 0.02	< 0.10
ATCC 7469	1.48 ± 0.08	0.03 ± 0.01	< 0.01	< 0.10

Citrate lyase and malate dehydrogenase activities are reported as mmol NADH oxidized min-1 (mg protein)-1.
† Fumarase activities are reported as mmol fumarate formed min-1 (mg protein)-1.
‡ Fumarate reductase activities are reported as mM succinate produced from whole cell suspensions in 100 mM potassium phosphate pH 7.0 and 10 mM fumarate at 37 oC after 24 h.
§ All values are reported as the average of duplicate assays from two independent experiments ± standard deviation.

whole cell suspensions. None of the CFEs from these strains contained detectable aspartase or isocitrate lyase activities. These assays indicated that these three lactobacilli express citrate lyase and malate dehydrogenase activities, however the activities for the latter enzyme were just above the limit of detection for the assay used. Succinate-producing *Lb. plantarum* strains studied previously had malate dehydrogenase activities approximately 29-305 fold higher than these strains. These data, taken together with the lack of detected fumarase activities in all three strains and lack of fumarate reductase activities in ATCC 393 and ATCC 7469, suggest these strains do not produce succinate due to deficiencies in reductive TCA pathway enzymes. In conclusion, it appears *Lb. casei* ATCC 334, *Lb. zeae* ATCC 393, and *Lb. rhamnosus* ATCC 7469 do not produce succinate from citrate, L-lactate, L-Asp, or isocitrate.

The results obtained in project have provided sufficient knowledge related to citrate catabolism that lucid strategies for the construction of strains of Lb. casei capable of producing significantly elevated levels of succinate in ripening cheese is now possible.

Publications/Presentations

Christensen, J.E., E.G. Dudley, and J.R. Pederson, J.L. Steele. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek **76:**217-246.

Fenster, K.M., K.L. Parkin and J.L. Steele. 2000. Characterization of an aryl esterase from *Lactobacillus helveticus* CNRZ32. J. Appl. Microbiol. **88:**572-583.

Dudley, E.G. and J.L. Steele. 2001. *Lactococcus lactis* LM0230 contains a single aminotransferase involved in aspartate biosynthesis, which is essential for growth in milk. Microbiol. **147:**215-224.

Dudley, E.G. and J.L. Steele. 2002. *Lactobacillus plantarum* ATCC14917, ATCC14431, and RL3 catabolize citrate to succinate by the reductive TCA pathway. Manuscript in preparation

Dudley, E.G. and J.L. Steele. 2002. *Lactobacillus casei* ATCC334, *Lactobacillus zeae* ATCC393, and *Lactobacillus rhamnosus* ATCC7469, do not produce succinate because of deficiencies in the reductive TCA pathway. Manuscript in preparation

Steele, J.L. 2000. Role of lactic acid bacteria in cheese flavor development –Part II. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 2000.

Dudley, E.G. and J.L. Steele. 2000. Citrate catabolism and succinate production by Cheddar cheese nonstarter lactobacilli. Oral presentation at the 2000 annual meeting of the American Dairy Science Association. July 2000. Received 1st place in the graduate student paper competition.

Dudley, E.G., M.W. Atiles, and J.L. Steele. 1999. Characterization and physiological role of the branched-chain and aspartate aminotransferase genes of *Lactococcus lactis.* Poster presentation at the FEMS Sixth Symposium on Lactic Acid Bacteria. September 1999.

Dudley, E.G. and J.L. Steele. 1999. Production of succinate by *Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus rhamnous.* Poster presentation at the FEMS Sixth Symposium on Lactic Acid Bacteria Abstracts. September 1999.

Dudley, E.G. and J.L. Steele. 1999. Production of succinate by *Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus rhamnous.* Poster presentation at the 1999 American Society for Microbiology annual meeting. May 1999.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the symposium on Dairy Flavors and Biotechnology. IFT Annual Meeting, July 1999.

Steele, J.L.. 1998. Cultures and cheese flavor development. Invited presentation to the Dairy Ingredients Division of Rhône-Poulenc. April 1998.

Dudley, E.G., M. Atiles, and J.L. Steele. 1998. Cloning and characterization of two aminotransferases from *Lactococcus lactis* LM0230. Poster presentation at the ASM Conference on Streptococcal Genetics. April 1998.

Genomic survey sequencing of *Bifidobacterium longum* VMKB44

Personnel

Steele, James L.

Funding

Dairy Management Inc.

Dates

July 1999 to December 2000

Objective

To obtain and characterize a small genomic survey of *Bifidobacterium longum* VMKB44

Summary

Bifidobacteria are Gram-positive, non-sporeforming, catalase-negative anaerobic rods which constitute one of the major components of human indigenous intestinal microflora. The health-promoting potential of bifidobacteria has generated interest in their therapeutical use as probiotics (non-pathogenic, host-derived microorganisms which beneficially affect the host by improving the microbial balance of the target niche). In order to exert its influence, probiotic bacteria should be able to colonize, at least transiently, the intestinal tract and efficiently compete with potential pathogens. However, there is very limited understanding of the molecular mechanisms responsible for probiotic properties of bifidobacteria. This is connected, in part, with a fact that genetics of bifidobacteria has not gained a sufficient attention of researchers until very recently. As a results, only limited number of bifidobacterial genes have been described.

Potential of probiotic indigenous microorganism such as bifidobacteria to colonize intestinal tract may depend on variety of factors. This includes (i) ability of the bacteria to survive physico-chemical conditions (e.g., pH changes) during the transit of the upper gastrointestinal (GI) tract and successfully adapt to the environment of the lower intestinal tract, (ii) adhere to the intestinal epithelial surfaces, and (iii) efficiently utilize specific energy sources available in the intestine such as complex food and mucus carbohydrates. The latter two characteristics may be considered as a basis for such mechanisms of interference of the probiotic with other microorganisms, potential pathogens included, as competition for nutrients and adhesion sites. Among the other competition mechanisms, production of antimicrobial compounds (organic acids, bacteriocin-like substances, antibiotics) and stimulation of host immunity are believed to be important for probiotic activity.

Genomic survey (sample) sequence (GSS) analysis represents an efficient and low cost tool for rapid gene discovery in a previously uncharacterized bacterial genome and may be also considered as a first step toward initiation of a full genome sequencing project.

Gene function	Organism	Accession	BLASTX E-value	Clone
UDP-glycosyl transferase	*Streptococcus pneumoniae*	AAC44962	5e-31	000429
Hexosyltransferase (EC 2.4.1.-)	*Sinorhizobium meliloti*	CAB01932	7e-05	000911
Glycogen branching enzyme (Amylo-(1,4 to 1,6)transglucosidase)	*Streptomyces coelicolor*	CAB92878	2e-07	000981
Beta-1,4-glucosyltransferase	*Salmonella enterica*	AAC98402	8e-07	000181
Undecaprenyl-phosphate alpha-N-acetylglucosaminyltransferase	*Streptomyces coelicolor*	CAB94536	4e-10	000113
Capsular polysaccharide repeating unit transporter	*Streptococcus agalactiae*	AAK43614	5e-06	000269
Fine tangled pili subunit (24 kD surface protein)	*Haemophilus ducreyi*	Q47953	1e-18	000026
Avicel (bacterial microcrystalline cellulose)-binding protein, cell-wall anchored	*Streptomyces reticuli*	CAB09806	2e-07	000241
Putative fimbria-associated protein	*Actinomyces naeslundii*	AAC13546	1e-17	001050

Table 1.
Putative genes identified encoding proteins potentially involved in the utilization of complex carbohydrates.

Gene function	Organism	Accession	BLASTX E-value	Clone
Oligopeptide permease	*Bacillus subtilis*	AF027868	6e-07	000021
Glutamate uptake ATP-binding protein	*Streptomyces coelicolor*	T35147	5e-42	000178
PepC (aminopeptidase C)	*Lactobacillus helveticus*	L26223	5e-19	000245
PepX (Xaa-prolyldipeptidyl aminopeptidase)	*Caldicellulosiruptor saccharolyticus*	AAB06263	1e-09	000901
Probable proteinase (collagenase)	*Campylobacter jejuni*	H81340	6e-08	000989
Hypothetical metallopeptidase	*Streptomyces coelicolor*	CAB56374	4e-14	000517

Table 2.
Putative genes identified encoding proteins potentially involved in assimilation of proteins, peptides and amino acids.

Gene function	Organism	Accession	BLASTX E-value	Clone
Cold shock protein B	*Streptomyces coelicolor*	CAB93399	3e-13	000104
Heat-shock serine protease HtrA	*Streptococcus pyogenes*	AAK34840	2e-26	000655
DNA-damage inducible protein P	*Staphylococcus aureus*	BAB42981	6e-20	000272
LexA repressor/SOS response	*Streptomyces clavuligerus*	O86847	2e-09	000467
Probable two-component response regulator	*Streptomyces coelicolor*	CAB70633	5e-04	000540
Probable (chemotaxis) response regulator	*Streptomyces coelicolor*	T35758	2e-39	000743
Fatty-acid synthase	*Brevibacterium ammoniagenes*	S55505	3e-68	000571
DNA modification methyltransferase	*Deinococcus radiodurans*	F75294	1e-12	000023
*Eco*VIII modification methylase	*Escherichia coli*	AAA91204	1e-06	000142
*Mun*I-like methyltransferase	*Enterococcus faecalis*	AAF72345	1e-11	000264
AbiEi,abortive infection bacteriophage resistance	*Lactococcus lactis*	AAB52382	4e-05	000681
Multidrug transporter	*Lactococcus lactis*	CAA63510	4e-23	000161
Putative transmembrane efflux (chrloramphenicol) protein	*Streptomyces coelicolor*	CAB66188	2e-30	001117
Similar to tellurite resistance protein	*Staphylococcus aureus*	BAB42498	2e-14	001049

Table 3.

Putative genes identified encoding proteins related to stress-response elements, bacteriophage defense systems, antibiotic resistance, and resistance to heavy metal ions.

The aim of the present study was, therefore, to identify genes that encode putative proteins which may have a role in the ability of *Bifidobacterium longum* VMKB44, a human faecal isolate, to colonize the intestine and compete with potential pathogens.
A genomic library *B.longum* VMKB44 was constructed in *Escherichia coli* using an M13Janus vector. Random sequencing reads were conducted on 1,150 of the clones from this genomic library. Sequences were processed (vector removed and high-quality trim) using Seqman II (DNASTAR). The trimmed sequence reads were searched against the DNA and protein sequences deposited in Genbank by using BLASTN and BLASTX. Matches with an E-value $<1.0x10^{-5}$ as determined using the BSOLUM62 matrix were retained and analyzed.

The sequencing results revealed several classes of genes that may be involved in colonization and probiotic activity of this organism. These include genes which putative products may be required for utilization of specific nutrients available in the intestine (complex carbohydrates, peptides, glycoproteins and glycolipids), adhesion (e.g., glycosyltransferases; pili- and fimbria-associated proteins) and adaptation and survival (e.g., resistance to antibiotics, toxic cations and phage; shock and starvation responses). Another group of genes may encode proteins potentially responsible for the probiotic effects of bifidobacteria such as host immunomodulation (autolysins) as well as for interference with pathogens (e.g., polyketide and fatty acid synthases; iron assimilation system). Representative results from this screen are presented in Tables 1 through 3.

Further analysis of these genes and this genome will assist us in understanding how *B. longum* VMKB44 survives gastric passage and elicits its probiotic effect. This type of research is required to establish a scientific basis for health claims for probiotics. Such claims would assist in the marketing of a variety of dairy products.

Publications/Presentations

Smeianov, V.V., Y. Chen, V.M. Korschunov, B.A. Efimov, V. Burland, F.R. Blattner, J.L. Steele. 2001. Genomic survey sequencing of human intestinal indigenous organism *Bifidobacterium longum* VMKB44: insights into colonization factors and mechanisms of probiotic activity. Abstract (P166) and poster presentation at the Functional Genomics of Gram-Positive Microorganisms conference in June of 2001.

Chapter Three
Applications

Cheese Industry and Applications Program

Personnel

John Jaeggi,coordinator; Amy Bostley, research specialist; Carol Chen, researcher; Rani Govindasamy-Lucey, associate scientist; Bill Hoesly, research cheese maker; Kristen Houck, research specialist; Mark Johnson, senior scientist; Cindy Martinelli, research specialist; Alice Ping, sensory coordinator; Juan Romero, analytical coordinator; William Tricomi, assistant researcher; Matt Zimbric, research specialist

Funding

Wisconsin Milk Marketing Board

Dates

January 2001 to December 2001

Objectives

1. Provide direct technical support for the use of commodity and specialty cheese, processed cheese, cold pack/club cheese and cream cheese in food application systems through consultations, pilot plant trials, application lab evaluations and plant visits.

2. Conduct industry directed cheese applications research - modifying manufacturing processes or ingredients during cheese making to produce a cheese with specific functional characteristics.

3. Direct contact with industry, DMI, WMMB, IFT, ADSA or other cheese industry related outlets to meet informational needs.

4. Provide technical support on internal cheese trials and projects, funded by WMMB, DMI, CDR, CDR Cheese Industry Team, and/or other UW departments through consultations, pilot plant trials, and application lab evaluations.

5. Provide technical support to other CDR Application program areas and to University of Wisconsin Food Science Department through consultations/lectures, pilot plant trials/ lab sessions, and application lab evaluations/demonstrations.

Summary

The 2001 Cheese Industry and Applications program Annual Report includes Wisconsin cheese industry activities, national cheese industry activities, international cheese industry activities, and internal CDR and various UW interdepartmental cheese activities. In 2001, approximately 50% of the work conducted by the Cheese Industry and Applications program was for Wisconsin-based companies. About 30% of the work involved internal projects, short courses, and CDR Cheese Industry Team projects. The remaining 20% was split equally between national and international cheese industry clients.

Table 1 summarizes the clientele that the Cheese Industry and Applications program have had contact with in 2001. The results found in Table 1 only reflect the number of companies the program has interactions with. It does not count the multiple contacts we have from different

Table 1. Year 2001 Cheese Industry and Applications Program Clients

Client	Wisconsin	National/International
Cheese Manufacturer	38	25
End User	10	15
Equipment Manufacturer	5	5
Ingredient Supplier	6	11
Consultant	4	4
Educational Institutions	1	8
Organization	2	2
Broker	2	2
Milk Producers	4	2
Communications	1	3
Contract Lab	3	2
Regulatory/Government	1	2
Farmstead Cheese	4	2
Total	77	81

individuals from the same companies on a wide range of topics. In 2001, we worked with 77 Wisconsin and 81 national and international cheese industry clients. For Wisconsin, 50% of those clients are cheese manufacturers, which is lower than previous year figures. On a national/international level, 31% of our interactions involve cheese manufacturers. The large and growing number of interactions with everyone from milk production through end users and all facets in between demonstrates the continued commitment between the Wisconsin Center for Dairy Research and the cheese industry.

A summary of technical transfer activities (cheese making, laboratory work, visits, consultations) is shown in Table 2. As in past years, we worked directly with cheese manufacturers to develop manufacturing protocols for cheeses, which target specific flavor profiles, texture and/or functional characteristics. A typical example would involve outlining the manufacturing protocols, demonstrating cheese making parameters in the CDR pilot plant, and then assisting in the commercial scale-up of several specialty ethnic varieties or commodity types of cheese.

As in the past year, we have worked in situations where the milk producer is marketing cheese made from his milk by a particular cheese manufacturer. In 2001, we have also continued work with milk producers who are planning on building on-site manufacturing facilities.

We continue to work directly with cheese manufacturers and end users to tailor manufacture Cheddar and Mozzarella cheeses for appetizer and pizza applications. For these projects it was critical to clearly understand the desired melt characteristics so as to obtain cheeses with desired cheese functionality.

Table 2. Year 2001 Cheese Applications Program Technical Support

Industrial Cheese making trials in the CDR pilot plant Wisconsin Worked with 14 companies. Forty-one cheese making dates: 44% manufacturers, 56% ingredient suppliers & others. Manufactured approximately 27 varieties of cheese. National/International Worked with 2 companies. Twelve cheese making dates: 100% ingredient suppliers & others. Evaluated new ingredient flavor, functionality, and effect on cheese yields.
Internal Projects and Short Courses Cheese making in the CDR pilot plant Wisconsin Worked with 5 UW departments (including CDR). Twenty cheese making dates: 75% other departments, 25% short courses. Manufactured a wide variety of cheeses: Brick, Cheddar, Colby, Cold Pack/Club/Food/ Spread, Edam, Gouda, Leiden, Manchego, Masdaam, LMPS Mozzarella, Parmesan, Processed Cheese/ Food/Spread/Sauce, Swiss. National/International Worked with CDR Cheese Industry Team. Four cheese making dates: Manufactured the following cheeses: Cheddar, Colby, Parmesan, and Swiss. Evaluated effect of using UF milk to standardize regular milk stream and its effect on cheese manufacturing and cheese properties and yield. Evaluated calcium lactate crystal formation.
Analytical, applications or sensory work Wisconsin Worked with 21 companies and departments. Seventy sets of analysis at multiple time points: 90% of cheese manufactured in CDR pilot plant, 10% of cheeses commercially manufactured. Types of analysis: Composition, chemical, sensory, microbiological, physical properties, cheese functionality in end application. National/International Worked with 4 companies. Eighteen sets of analysis: 90% of the cheese manufactured in the CDR pilot plant, 10% of the cheeses commercially manufactured. Types of analysis: Composition, chemical, sensory, microbiological, physical properties, cheese functionality in end application.
CDR or Onsite visits Wisconsin Met with 24 companies. Forty-four visits: 70% visits to the CDR, 30% onsite visits. CDR visits included discussion on current research, cheese applications, general cheese technology and methods of evaluating physical properties of cheese. Onsite visit included assisting in the scale-up of specific cheese varieties, discussions of milk standardization and cheese yield and general cheese technology questions. National/International Met with 11 companies. Fifteen visits: 55% visits to the CDR, 45% onsite visits. CDR visits included discussion on current research, cheese application program, general cheese technology. Onsite visit to assist in the scale up of CDR stirred curd, direct salted mozzarella cheese.
Consultations Wisconsin Worked with 77 companies. Discussed general cheese technology issues, milk standardization, cheese yield, manufacturing equipment, controlling the meltability of cheese, cheese microbiology, cheese defects, cheese process control, cold pack formulations, processed cheese formulations, UF and RO technology, labeling/ nutrient claims issues, flavor and texture development, analytical protocol, regulatory issues, HACCP, safety. National/International Worked with 79 companies. Discussed general cheese technology issues, milk standardization, cheese yield, manufacturing equipment, controlling the meltability of cheese, cheese microbiology, cheese defects, cheese process control, cold pack formulations, processed cheese formulations, UF and RO technology, labeling/ nutrient claims issues, flavor and texture development, analytical protocol, regulatory issues, HACCP, safety.

We continue to work on cheese related projects funded by various outside or internal sources, many reported in detail elsewhere in this Annual Report. The CDR Cheese Industry Team (CIT) currently funds two on-going projects. The first is a project in which we continue to work in the area of milk standardization as it involves the use of different ultra-filtered (UF) milks to manufacture different cheese varieties. We then evaluate the effects of using these UF-standardized milks on manufacturing, whey composition, cheese properties (chemical, physical, and sensory), and cheese yield. The second CIT project involves the study of re-creation of calcium lactate crystal formation in Cheddar and Colby cheese so as to evaluate the type of crystals formed and how they differ from past years, and altering milk standardization and manufacturing protocols in attempt to minimize or eliminate this defect.

The Cheese Industry and Applications Program also continues to work with other University of Wisconsin Departments or CDR personnel on various cheese projects. One example includes the manufacture of pasta filata and stirred curd mozzarella for analysis by the Biological Systems Engineering Department. Another is a joint project between the CDR, Animal Science Department, and the Food Science Department which involved the manufacture of Manchego cheese using ovine milk sourced from different weaning systems to access manufacturing, compositional, yield and chemical differences in the cheese. The CDR Cheese Industry and Applications program is currently assisting CDR and Food Science personnel on such projects as studying calcium solubility in Cheddar cheeses of different pH's and studying the effect of specific cultures and culture adjuncts used to manufacture Gouda and Parmesan cheeses. The Cheese Industry and Applications program is also actively participating with on-going CDR projects which include working with UF and diafiltered milks in pasta filata mozzarella manufacture and how the use of these milks affect the functional characteristics of these cheeses, redox potential in Cheddar cheeses made using stirred curd, milled curd, and washed curd manufacturing methods, the shred map project, and the melt profile project.

We continue to work with a large number of ingredient suppliers to evaluate starter cultures, starter adjunct systems, enzymes, coagulants, and other additives. We then evaluate the effects of using these ingredients on flavor, texture, chemical, physical, and sensory properties, and/or cheese yield in many different cheese varieties, including processed and cold pack cheeses.

In 2001, we noted a huge increase in consultations for companies and individuals. A few of the topics included cheese making parameters, milk standardization, cheese defects (off flavor formation, gas formation, calcium lactate crystals, pinking, browning, etc.) cheese technology, cheese equipment, ingredients in cheese, cheese/starter microbiology, cheese functionality, cheese flavor, formulations for different processed and cold pack cheeses, sanitation, regulatory issues, analytical protocols and safety. These questions are generated from all facets of the

cheese industry, from the largest corporations in Wisconsin, the United States, and the world to the smallest farmstead operations. Numerous contacts are made with many different individuals, sometimes from within the same companies.

The Cheese Industry and Applications program also continues to assist the Specialty Cheese/Master Cheesemaker program area in regards to coordinating grading and sampling of Master Cheesemaker and Master Mark candidates' cheeses, grading final exams, and attending all Master Cheesemaker member and board meetings. This past year, program personnel also assisted the Specialty Cheese/Master Cheesemaker program and/or the Food Science Extension Program in providing assistance with lecturing and setting up lab sessions for the two Wisconsin Cheese Technology short courses, the two Wisconsin Processed Cheese short courses, the Dutch Artisan course, the Membrane Processing Technology short course, to name a few.

The Cheese Industry and Applications group conducted chemical, microbiological, physical property, application, and sensory testing on various cheese samples that are related to applications research projects. Approximately 80% of laboratory work conducted is in conjunction with CDR pilot plant cheese making. This again shows the cheese industry is placing an emphasis on understanding how the cheese composition/age affects the sensory and physical properties, and thus the functionality in the end application.

We hosted several industry groups to discuss the Cheese Industry and Application program and current cheese research topics. Cheese Industry and Applications group personnel continued to travel to cheese plants not only in Wisconsin, but also nationally and internationally to provide one-on-one technical transfers of cheese making protocols, milk standardization, identifying problems potentially causing cheese defects, and other cheese technology issues.

Publications and Presentations

Members of the Cheese Applications program team provided technical information at several national and regional meetings or conferences. The staff plays an important role in the Wisconsin Cheese Technology Short Course (March, October), the Wisconsin Process Cheese Seminar (February, October), the Dutch Artisan Cheese Making Seminar (September), the Membrane Processing Technology Seminar (October) sponsored by the UW Food Science Department and/or the Specialty Cheese/Master Cheesemaker program.

Throughout the year, the CDR provided tours for various journalists, councils, academia and industry groups.

Participated in two DMI-sponsored Ideation session (M. Lubbers), which concentrated on creating new menu items with cheese for a fast food restaurant and new product development, October & November 2001, in Chicago, IL.

The presentations and publications made by the Cheese Applications Program team members are listed below:

"Evaluation of sensory and chemical properties of Manchego cheese manufactured from ovine milk with different somatic cell levels." by J.J. Jaeggi*, Y.M. Berger, M.E. Johnson, S. Govindasamy-Lucey, B.C. McKusick, D.L. Thomas, and W.L. Wendorff, at the 7th Annual Great Lakes Dairy Sheep Symposium, November 2001, in Eau Claire, Wisconsin.

"Controlling the coagulation properties of high solids cheesemilks that are standardized with cold ultrafiltration retentates" by S. Govindasamy-Lucey*, J.J. Jaeggi, M.E. Johnson, and J. Lucey, at the American Dairy Science Association Annual Meeting, July 2001, in Indianapolis, IN.

"Relationship between chemical, physical, and sensory properties for pasta filata and stirred curd LMPS Mozzarella cheeses" by C.M. Chen*, A.L. Dikkeboom, M.E. Johnson, and M. L. Zimbric at the American Dairy Science Association Annual Meeting, July 2001, in Indianapolis, IN.

"Standardization of cheesemilks using cold ultrafiltration retentates for the manufacture of Parmesan cheese" by J.J. Jaeggi*, S. Govindasamey-Lucey, M.E. Johnson, and J. Lucey, at the American Dairy Science Association Annual Meeting, July 2001, in Indianapolis, IN.

"Effect of rennet coagulation time on composition, yield, and quality of reduced-fat Cheddar cheese" by C.M. Chen, J.J. Jaeggi, and M.E. Johnson, J. Dairy Sci. 2001 May; 84(5): 1027-33.

"Overview and technical considerations: Standardization of milk for cheesemaking" by Mark Johnson, at the WCMA/CDR joint Wisconsin Cheese Technology Conference, April 2001, in Green Bay, Wisconsin.

CDR Communications Program

Personnel

Mary Thompson, coordinator; Joanne Gauthier, communication specialist; Tim Hogensen, graphic designer; and Karen Paulus, editor

Funding

Wisconsin Milk Marketing Board

Dates

January 2001 to December 2001

Objective

Support CDR Applications and Applied Technology Development programs to provide Wisconsin's dairy industry the information necessary to maintain or enhance their competitive advantage. This is accomplished through industry outreach including conferences, workshops, industry training programs, publications, technology documents, the website and maintenance of an information technology system

Summary

Industry outreach events, chronological (*CDR sponsored events)

Producer Value Showcases
Sponsored by Wisconsin Milk Marketing Board
February 6, 8, 20, and 22, 2001
WMMB/Producer Value Showcases were held in Mineral Point, Loyal, Green Bay and Ripon, Wisconsin. The CDR exhibit featured cheeses from the Class of 2001 Wisconsin Master Cheesemakers and a whey display showing the flow of milk to highly refined whey components.
Attendance - 900+

Quality Milk Seminar
February 6-7, 2001
Sponsored by Wisconsin Association of Dairy Field Representatives, University of Wisconsin-Depts. of Food and Dairy Science and CDR.
Attendance - 200

DFA's Young Cooperators Conference
February 24, 2001
Madison, WI
"About CDR" was presented.
Attendance- 45+

Legislative Brunch
Senate Chamber Parlor
State Capitol, Madison, WI
Sponsored by Wisconsin Women in Agriculture
Exhibit and display on programs at CDR
Attendance - 150+

Fancy Food and Confection Show
February 24 – 27, 2001
Chicago, IL
Exhibit on Wisconsin Master Cheesemakers

DMI/Dry Dairy Ingredient Applications
April 10, 2001

Cheese Industry Team*
April 17 and November 14, 2001
Green Bay and Madison, Wisconsin
Representatives from 19 companies attended the cheese industry meetings. Members participated in discussions focused on new technologies and industry concerns. CDR program coordinators presented updates on their program areas.

Wisconsin Cheese Industry Conference*
Sponsored by Wisconsin Cheese Makers Association and CDR
April 18-19, 2001
Green Bay, Wisconsin
Conference theme:
A Critical Analysis: The National Drug Residue Milk Monitoring Program - Panel Moderator: Rusty Bishop, Wisconsin Center for Dairy Research
"M-a-86, How Did We Get Here?" – Robert Childers, Food and Drug Administration
"*Producer View*" – Ted Hickerson, Dairy Farmers of America
"*Manufacturer View*" - Dan Rackley, Dean Foods
"C*onsiderations of Risk Assessment*" - Rusty Bishop

Wisconsin Master Cheesemaker Recognition Ceremony*
April 19, 2001
Madison, Wisconsin
Four Wisconsin cheesemakers were certified as Wisconsin Master Cheesemakers. New Masters and their certified cheeses include Dave Lindgren – Mozzarella and Provolone, David Metzig – Cheddar, Duane Peterson – Gouda and Edam, Carrie Wagner – Asadero and Havarti. Two past Masters, Scott Erickson and Joe Widmer received certifications in additional cheese varieties.

United States Dairy Export Council Trade Mission*
USDEC/Japan Trade Mission – foodservice and restaurant personnel
Topic - cheese physical and functional properties
Whey Applications group on the United States Dairy Export Council mission from Latin America.

Farm Progress Days
Janesville, WI
September 18 – 20, 2001
CDR exhibit featured the applications programs with a focus on specialty cheese. Samples of the American Cheese Society - Best of Show cheese made by Mike Gingrich, Pleasant Ridge Reserve was sampled.
Attendance – 60,000+

Wisconsin Master Cheesemakers - Historical Cheesemaking Days
October 14 & 15
New Glarus, Wisconsin
CDR coordinated participation of eight Wisconsin Master Cheesemakers demonstrating the historic way to make Swiss cheese.

World Dairy Exposition
October 4 – 8, 2000
CDR worked in press room with Wisconsin Milk Marketing Board staff.

Fond du Lac County Education Day for Farm Women
November 6, 2001
"Wisconsin Center for Dairy Research boosts specialty cheese industry", by Mary Thompson
Made arrangements for a press conference with Dane County Executive, Kathleen Falk. The conference was held at CDR and recipient of Dane County Ag grants were announced.

Short Courses, Workshops and presentations (*CDR sponsored)

Process Cheese Short Course
February 27 – 28, 2001
October 23 – 24, 2001

Clean in Place Workshop (CIP)
March 14, 2000

Dairy HACCP Workshop*
May 23, 2001

Cheese Technology Short Course
March 26 – 30, 2001
October 15 – 19, 2001.
Presentations:
Cheese Yields & Economics – Jim Path
Milk Standardization – Mark E. Johnson
Cheese Chemistry – Mark E. Johnson
Mechanization of Cheesemaking – Jim Path
Specialty Cheeses – Jim Path
Curing and Flavor Development – Carol Chen
Cheese Technology/Applications – Mark E. Johnson
Textural and Flavor Evaluation of Cheese – Amy Dikkeboom and Carol Chen
World of Surface and Mold Ripened Cheeses – John Jaeggi

Master Cheesemaker Culture Seminar*
April 11, 2000
Presentations:
Importance of Starters – Mark E. Johnson
Basics of Cheese Making as Related to Starters – Mark E. Johnson
The Role of Starters in Other Cheeses – Mark E. Johnson

Wisconsin Whey and Whey Utilization Short Course
May 2 – 3, 2000
Presentations:
Lactose Production – Karen Smith
Process Parameters Impacting Whey Functionality – Karen Smith

Whey Ingredient Functionality – KJ Burrington
Dairy and Food Applications – KJ Burrington

Cheese Grading Short Course
June 6 – 7, 2000
November 7 – 8, 2000

Managing Dairy Food Safety Workshop
Wednesday, September 5, 2001

Dutch Cheese Artisan Seminar *
September 25 – 26, 2001

Membrane Processing of Dairy Products*
October 30 – 31, 2001

Technical presentations, poster sessions, and public information

International Dairy Federation

American Dairy Science Association presentations
Standardization of cheesemilk using cold ultrafiltration retentates for the manufacture of Parmesan cheese – John Jaeggi
Controlling the coagulation properties of high solids cheesemilks that are standardized with cold ultrafiltration retentates. S. Govindasamy-Lucey (color correction) ADSA
Relationship between chemical, physical and sensory properties for pasta filata and stirred curd LMPS mozzarella cheese. C. M. Chen, ADSA

American Dairy Science Association poster session

Institute of Food Technologists Annual Meeting:

Dairy Management Inc. - Cheese Forum:
Worked with J. Jaeggi on salt infusion article for DMI publication.

United States Dairy Export Council
Coordinating CDR's part in Agricultural Products Quality training program for 4 Cochran Fellows from Latin America. Request was processed through the International Agri-Business Center/ WDATCP. The presentations was Wednesday, October 17, 2001, 10:00 – 12:00 Noon.

Coordinated and participated in CDR's involvement in the Agri-Business Legislative tour. Specialty cheese was the focus of the presentation. 50+ people attended and 10 of those were legislators.

Wisconsin Dairy Products Association Butter & Cheese Clinic
Developed and published newsletter for Wisconsin Master Cheesemakers

Work with WARD TV – Washington, D.C. coordinating details for CDR's role in a National Public Television series – "Secrets of the Sequence". Set up interview with T. Szalkucki. Worked with production staff to get footage of the cheesemaking process.

Publications

2000 Annual Report, Karen Paulus, CDR editor

Return on Investment, revised
Documentation of CDR's return on Wisconsin producer investment

Dairy Pipeline Newletter, published 4 times a year by Karen Paulus, CDR editor
Pipeline - Winter, 2000

Pipeline - Spring, 2000

Pipeline- Summer 2000

Pipeline -Fall 2000

Technical fact sheets, technology documents/reviews:
Cheese tech reviews for distribution. Currently there are 4 tech reviews.
The Melt and Stretch of Cheese
Casein – How it colors cheese
The question: Consistent Quality The answer: Cheese Yield Equations
Dairy Ingredients in cheesemaking – possibilities and problems

Agri-View producer newspaper, column once every 2 months written by Joanne Gauthier:
Wisconsin Master Cheesemakers Class of 2001

CDR Web page
The web page was redesigned for ease in navigation and finding information.

Web page calendar lists meetings, events, seminars, workshops, and short courses. Informational brochures are placed on the web as pdf's and can be downloaded for viewer use.

Current and back issues of the Dairy Pipeline newsletter are on the web and can be downloaded.

Safety/Quality Applications Program

Personnel

Marianne Smukowski, coordinator

Funding

Wisconsin Milk Marketing Board

Dates

January 2001 to December 2001

Objectives

1. Provide technical assistance to Wisconsin companies in the areas of safety/quality audits, preparation for regulatory audits, sanitation program reviews and overall GMP reviews.

2. Assist in development of HACCP plans and programs.

3. Provide technical support for safety/quality problem solving.

4. Assist in executing the Wisconsin Cheese Food Safety Initiative Program

Summary

The Safety/Quality Applications program assists WI cheese manufacturers in the following areas: safety/quality audits, third party audits, recall issues, GMP reviews, developing HACCP plans, assisting the WI Master Cheesemaker program and providing technical support in regulatory matters.

I continue to be a member of the NCIMS laboratory committee, which addresses the use of drug residue kits, and laboratory practices.

Gave presentations throughout the state as part of a series on "Overview of Recall Programs", which was sponsored by WMMB and WCMA. My topic covered "How to Prepare for a Food Safety Audit".

Numerous plant visits were made to assist plants in third party audits and HACCP implementation. I also assisted a large food company with GMP training of their employees.

Reviewed several United States Grade Standards, Specifications, and Commercial Item Descriptions for various dairy products for USDA-Dairy Division. Cheddar cheese was one of these standards, which has not been revised since 1956. The proposed revisions for Cheddar cheese will have a major impact on the Wisconsin cheese industry. Reviewed several of Wisconsin Agriculture, Trade, And Consumer Protection documents. Two documents reviewed were ATCP 60 (Dairy Farms) and ATCP 80 (Dairy Plant). Again, proposed revisions to these two documents will have an impact on the Wisconsin dairy industry.

Lastly, I am involved with the Wisconsin Cheese Food Safety Initiative program. This program was started last summer. There are four objectives for the program. The main objective is to develop a standard food safety plant audit acceptable to all dairy manufacturers. I also am involved with the HACCP subcommittee. This group is trying to establish uniform GMPs for HACCP implementation. This is a huge project and we anticipate it will take an enormous investment of our time, in numerous meetings, to meet our goals. We expect that it will take several years to develop the program.

Publications and presentations

WI cheese grading short course, Italian cheese evaluation (twice a year)

Collegiate Dairy Products Evaluation Contest (Lead Butter Judge)

WI CIP Workshop, Plant Sanitation Audits

WI Dairy Products Assoc. Cheese and Butter Evaluation Clinic, Overview of butter grading

Dairy HACCP Workshop, Program coordinator

WI Process Cheese Seminar, HACCP for Process Cheese

Presented at the American Cheese Society with the topic of HACCP Model for Small Cheese Plants

Managing Dairy Food Safety Workshop, Program Coordinator

WI Cheese Technology Short Course, Cheese Handling-Plant to Retail (Twice a year)

Contributing author for the American Society for Quality, Food, Drug, and Cosmetic Division for their Quality Auditor's HACCP Handbook

Whey Applications Research Program

Personnel

Kimberlee J. Burrington, coordinator; Karen Smith, PhD, Researcher

Funding

Wisconsin Milk Marketing Board; Dairy Management, Inc.

Dates

January 2001 to December 2001

Objectives

Enhance the value of whey-derived ingredients by providing technical support to the whey processing industry. Provide processing and applications support for whey, permeate, lactose, whey protein concentrate, whey protein isolate, and whey protein fractions.

Conduct industry directed whey applications projects, which evaluate the functional attributes of specific whey ingredients in finished food systems. Areas of food applications for whey ingredients are dairy and bakery products, beverages, soups, sauces, meats, nutraceuticals, and infant formula.

Initiate development of a pilot plant facility which provides the ability to conduct whey processing projects with industry, for the evaluation of existing and new processing conditions. The pilot plant should be able to process whey from the cheese vat to the spray dried ingredient.

Summary

This year completed the fourth year of the Whey Applications program. In 2001, the Whey Applications program was in contact with 30 Wisconsin-based companies and 67 national companies, consisting of whey processors, ingredient suppliers, and end-users. Activities were increased both in applications and processing support.

Whey applications were developed and presented at the following events, seminars, and companies: the USDEC Latin American/CDR journalist mission, Dairy Farmers of America board member visit, WMMB board members visit, Leprino visit, Glanbia visit and IFT. Applications development focused on energy bars, meal replacement drinks, sports drinks, cookies, tortillas, batters and breadings, and pizza crust. Evaluation of the DMI Whey Application Guide formulas were completed. Functionality evaluations were added to the list of services provided this year. Methods have been established to evaluate foamability, emulsification, gelation, solubility, heat stability and viscosity for existing and new whey ingredients. General whey processing, functionality, and applications information were presented 22 times over the course of the year.

Processing support has involved further development of the whey processing pilot plant, with the installation of a spray dryer, ion exchange system, and a pilot scale evaporator. Many of the needs of the whey processors and end-users have been informational needs. Typical requests are for standard methods for chemical and functional analysis, specifications, whey ingredient sources, literature searches, formulations for specific applications, and processing trouble-shooting questions. Membrane processing support has also been provided to the CDR cheese group on all projects requiring ultrafiltered milk.

Presentations and publications

Karen Smith, Ph.D.
Processing Options for Whey, for Cedar Grove Dairy, January 31.

Processing of Whey into Value-Added Ingredients, for Foremost Farms, USA, February 19.

Manufacturing Considerations During Processing of Whey into Functional Dairy Ingredients, Calpoly Concentrated and Dried Milk and Whey Products Symposium, February 28.

Spray Dried Dairy Products, UW Food Science-Food Engineering class, May 1.

U.S. Whey Protein and Lactose Products, USDEC Mission trip to SE Asia, May 22.

Processing Whey into Value Added Ingredients-Parts I and II, for USDEC Latin American Journalists Mission to CDR, June 20.

Future of Dairy Products, IFT 2001, June 27.

Whey Ingredients Hidden Treasures, World Wide Food Expo, October 19.

Converting Whey Into Value Added products/SE Asia Perceptions/US Whey Products, World Wide Food Expo, October 19.

Principles of Membrane Filtration, Feed Materials, Evaporation and Drying of Whey Products, UF/MF in the Dairy Plant, UW Membrane Processing Short Course, October 30.

Background on Milk Protein Products, for WMMB, August 2001

K.J. Burrington
Whey Applications, for Foremost Farms, USA, March 5.

Whey Ingredient Functionality, for Minute Maid, March 9.

Whey Applications Program, WMMB CEO visit, March 15.

Whey Applications Program, Cheese Industry Team meeting, April 17.

Whey Applications in Reduced Fat Bakery Products, USDEC Mission to Mexico City-Baking Industry, May 5. (sent presentation)

Whey Functionality and Applications, USDEC Latin American Journalists Mission to CDR, June 20.

Dried Dairy Ingredients-A User's Perspective- Domestic, for IFT 2001, June 27.

Processing Impacts on Whey Functionality, UW Membrane Processing Short Course, October 30.

Whey Applications Program, Cheese Industry Team Meeting, November 14.

Simply Nuts, Food Product Design, March 2001

Keeping the Crunch in Breakfast Cereals, Food Product Design, June 2001.

High Protein Drinks, Food Product Design, October 2001.

CDR specialty cheese applications program

Personnel

Jim Path, outreach specialist

Funding

Wisconsin Milk Marketing Board

Dates

January 2000 to December 2001

Objectives

1. Continue developing the artisan workshops, a module of the Wisconsin Master Cheesemaker® program.

2. Provide technical support to cheesemakers, including workshops, consulting, and on site manufacturing trials.

3. Manage the Wisconsin Master Cheesemaker® program.

Summary

Jim Path and John Jaeggi traveled to Leeuwarden, Holland to meet with persons at Tebel Equipment Company and instructors at the Van Hall Institute to finalize plans for Dutch Cheese Seminar held at the Center for Dairy Research in Madison, Wisconsin on September 25, 2001. Several cheese factories were visited and some samples brought back to the Center for Dairy Research for analysis.

The Wisconsin Process Cheese seminar was held on February 27 and 28, 2001. Registration for the class was filled with over 30 persons. Because of demand (over 30 persons on the waiting list) a one-time additional class was held on October 2001. Additional equipment, which was donated by Nutra-Sweet, will allow us to increase the class size for the next regularly scheduled class in February of 2002.

The Master Cheesemaker Honors event was held in Green Bay on April 19, 2001. Four new Wisconsin Master Cheesemakers® were honored at an evening Recognition Ceremony. The honor of Wisconsin Master Cheesemakers® is specific to a cheese type (e.g. a master in Gouda) and Masters are only allowed to have two cheese types per three year apprenticeship. For the first time two previous Masters returned into the program to gain recognition for additional cheeses types.

Jim Path visited Tillamook Cheese in Tillamook, Oregon and Washington State University at Pullman, Washington to observe cheese making. Washington State University was particularly interesting as they manufacture Cougar Gold cheese. It is one of a very few cheeses which are manufactured and then sealed in a can. We would like to thank the plant manager for allowing Jim to gather information, videotape and take pictures for future seminars and technology development.

The Wisconsin Master Cheesemakers® board met on July 12, 2001 and 4 new applicants were accepted for the apprentice phase of the program. One existing Master was accepted for an additional cheese.

Jim Path traveled to northern Wisconsin to research Juustoleipä (squeaky) farmstead cheese. Since the trip, the Center for Dairy Research has made two trial runs of Juustoleipä farmstead cheese. The cheese was brought to America from Finland by immigrants. The information that we have is that the cheese was originally produced from reindeer milk, but is now a commercial product produced from cow's milk. We have obtained some product from Finland.

Dutch Cheese Seminar held on September 25, 2001. Eyed cheeses from Holland were discussed. A great deal of information regarding Dutch cheese was obtained. About 22 persons attended.

A Master Cheesemaker dinner was held at New Glarus on Oct. 6, 2001. Received input from Masters regarding program direction.

Wisconsin Master Cheesemaker board met on December 14, 2001 and 6 new applicants and 3 masters applying for 2nd round were approved. A Masters Honors Ceremony will be held on April 25, 2002 in Madison

Jim Path traveled to Germany November 24 though December 6, 2001. The purpose of the trip was the following:
Travel to Ladenburg, Germany to produce research cheese at BK Giulini, a company which produces salts for process cheese. Work done to develop natural flavored cheeses.

Travel to Sangerhausen Germany to observe production of process cheese at Käsewerk Sangerhausen GMBH

Travel to Guben, Germany to cross (walking) boarder to Gubin, Poland and purchase samples of cheeses at the request of a Wisconsin cheese factory interested in producing Polish cheese.

Travel to Munich to observe research at the Institute for Food Process Engineering and Dairy Technology in Weihenstephan, Germany. Visit Dr. Rademacher regarding process to manufacture cheese with out culture and rennet under high pressure. Also visit major food market in Munich and various cheese manufacturing factories.

Dairy marketing and economics program

Personnel

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Funding

Wisconsin Milk Marketing Board

Dates

January 2001 to December 2001

Objectives

1. To understand the factors that influence cheese yield and to quantify the economic impacts for both the cheese manufacturer and the producer.

Summary

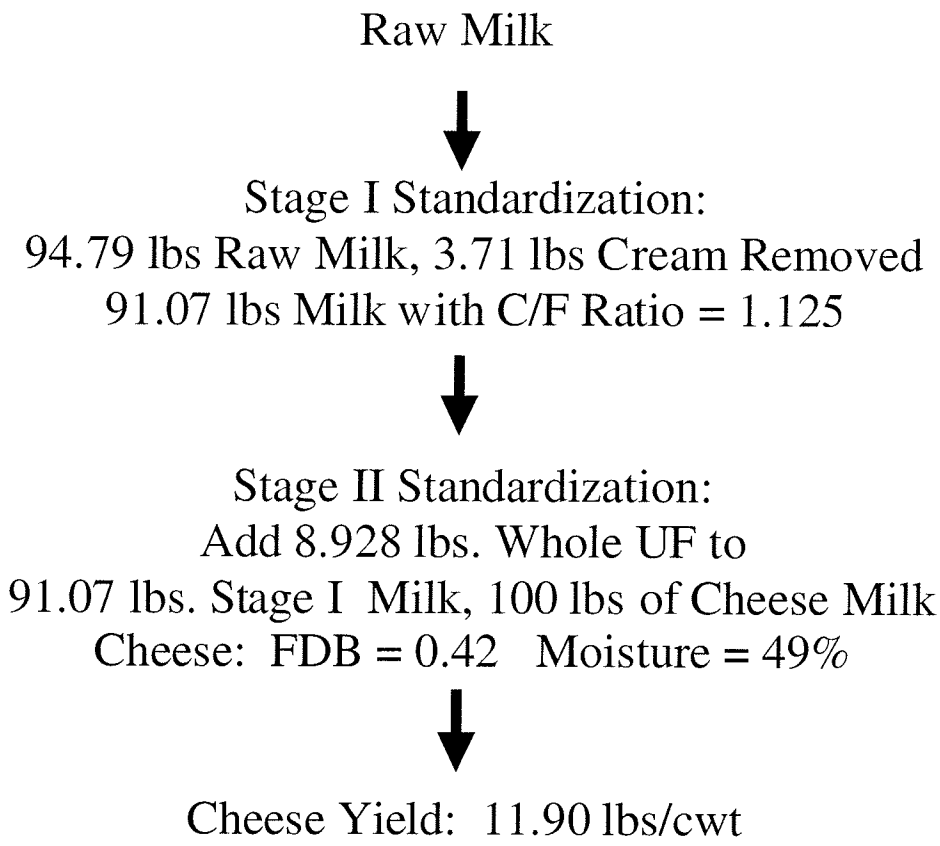
The Dairy Marketing and Economics program had four major subject areas of research during the 2001 calendar year. These included: Economic Analysis of Cheese Yield, Use of Dairy Futures Markets by the Dairy Industry for Risk Management, Economic Analysis of Alternative Multiple Component Pricing Systems, Analysis of Consumer Demand for Dairy Products.
Below we discuss the activities undertaken in these areas. If you would like more information concerning these activities contact program coordinator, Dr. Brian W. Gould at the email address: gould@cdr.wisc.edu .

Economic analysis of cheese yield

The objective of this area of research is to better understand the factors that impact cheese yield and to quantify the economic impacts for both the cheese manufacturer and ultimately the dairy farm operator. There were two main areas of research: completion of the *Economic Analysis of Cheese Yield* (EACY) software program and analysis of the use of alternative membrane and ultra-filtered milk products on cheese yield.

The EACY software development was initiated two years ago. EACY is a stand-alone Windows software system that allows the user to examine the impacts of milk quality, standardization techniques, and whey by-product utilization on the total gross returns of 100 lbs of raw (cheese) milk as well as the vat level impacts of such characteristics. This software is available electronically by contacting the author, Dr. Brian W. Gould at the email address, gould@cdr.wisc.edu. A brochure describing the software package can be obtained by accessing the following PDF file: www.cdr.wisc.edu/pdf/eacy_brochure.pdf. Using the EACY software system as a resource, we presented an analysis of the economics of the use of membrane and ultra-filtered (UF) milk products for the standardization of milk for cheese making, *Economics of Membrane Processing: Examples of Product Use and Manufacture in Cheese Making.* This presentation was part of the October short course sponsored by CDR, *Use of Membrane*

Figure 1. Using whole UF (3X) to standardize mozzarella cheese milk



and Ultra-Filtered Dairy Products. In this presentation we examined the use of whole UF milk, skim UF, non-fat dry milk and the removal of cream for the standardization of cheese milk in the manufacture of mozzarella.

Figures 1 through 4 show the resulting standardization procedures that were used to obtain a cheese milk FDB of 0.42 and a cheese moisture content of 49%. A copy of the full presentation can be obtained by accessing the following PowerPoint Presentation file: www.aae.wisc.edu/future/publications/cdr_short_course_gould_4.pdf . This file contains background information concerning the UF products used in the above analysis and the economic impacts of their use.

Use of dairy futures markets by the dairy industry for risk management

A considerable amount of effort was devoted to the maintenance and enhancement of the University of Wisconsin Dairy Market web site (www.aae.wisc.edu/future). This web site is now a major dissemination point for the University of Wisconsin Extension Dairy Risk Management teams initiative to improve the Wisconsin dairy industry's price risk awareness. As part of this effort, a number of alternative risk management curriculum have been developed and are available for download. The UW-Extension

risk management curriculum can be accessed via the following web site address: www.aae.wisc.edu/future/risk_team/risk_team_1.htm. In addition to this curriculum, other risk management educational materials can be reached via the following: www.aae.wisc.edu/future/front_tutorials.htm. Also, contained in the publications section of the University of Wisconsin Dairy Marketing web site (www.aae.wisc.edu/future/front_publications.htm) are materials useful for understanding various risk management tools available to both dairy farm operators and processors.

One major achievement this year was the development of an automated data retrieval system for daily dairy futures and options settle prices. The data is retrieved from the Chicago Mercantile Exchange (CME) where dairy futures and options are currently traded. This system automatically accesses the end-of-the-day records for all dairy futures and options and adds them to the University of Wisconsin Dairy Marketing web site. The futures data can be accessed via the following URL: www.aae.wisc.edu/future/front_futures.htm. Within 30 minutes of the market closing, our automated system has this data recorded on our web site. The options data can be accessed via the following: www.aae.wisc.edu/future/front_options.htm. Due to the schedule of the CME, the options data are updated at 11:30 p.m. the day of trading. In addition to adding this data to our web site, our automated system also automatically graphs all currently traded Class III, Class IV and Butter futures settle prices from the first day of trading to last. The daily Class III graphs can be accessed via the URL: www.aae.wisc.edu/future/daily_settle_graph.htm.

Figure 2. Using NFDM to standardize mozzarella cheese milk

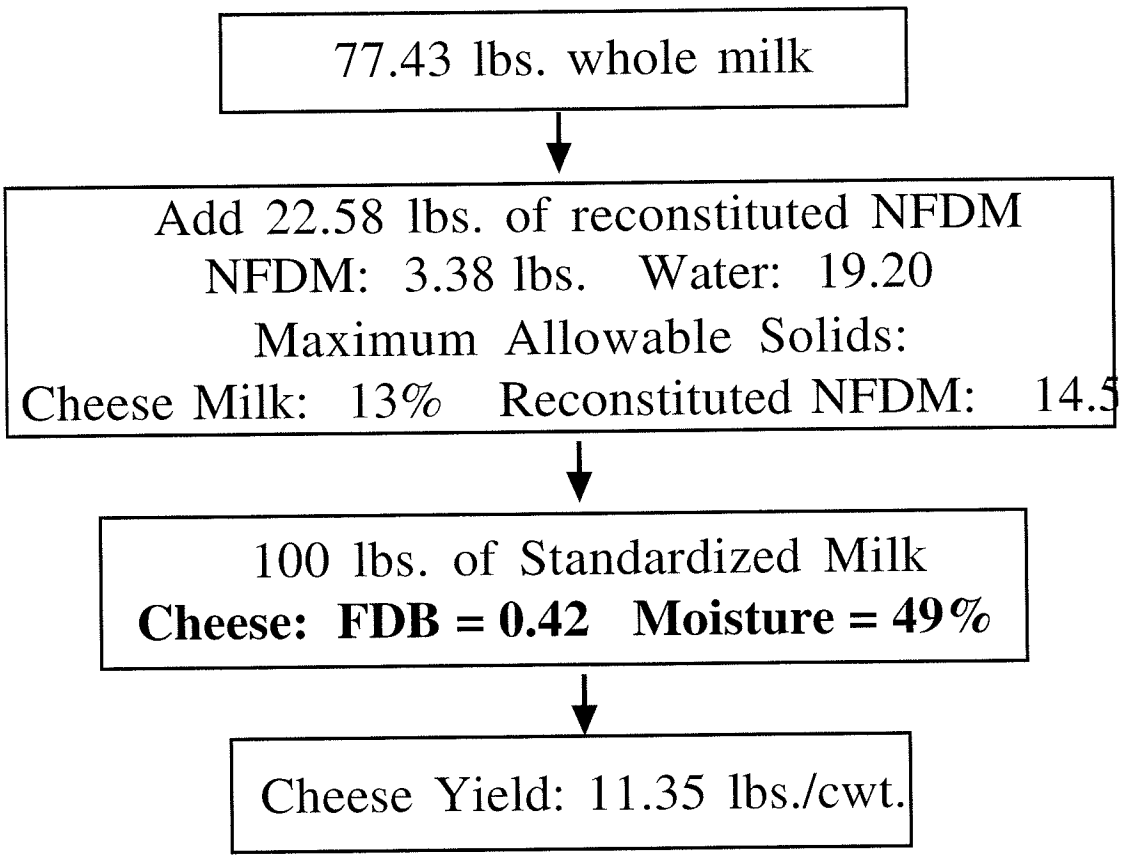
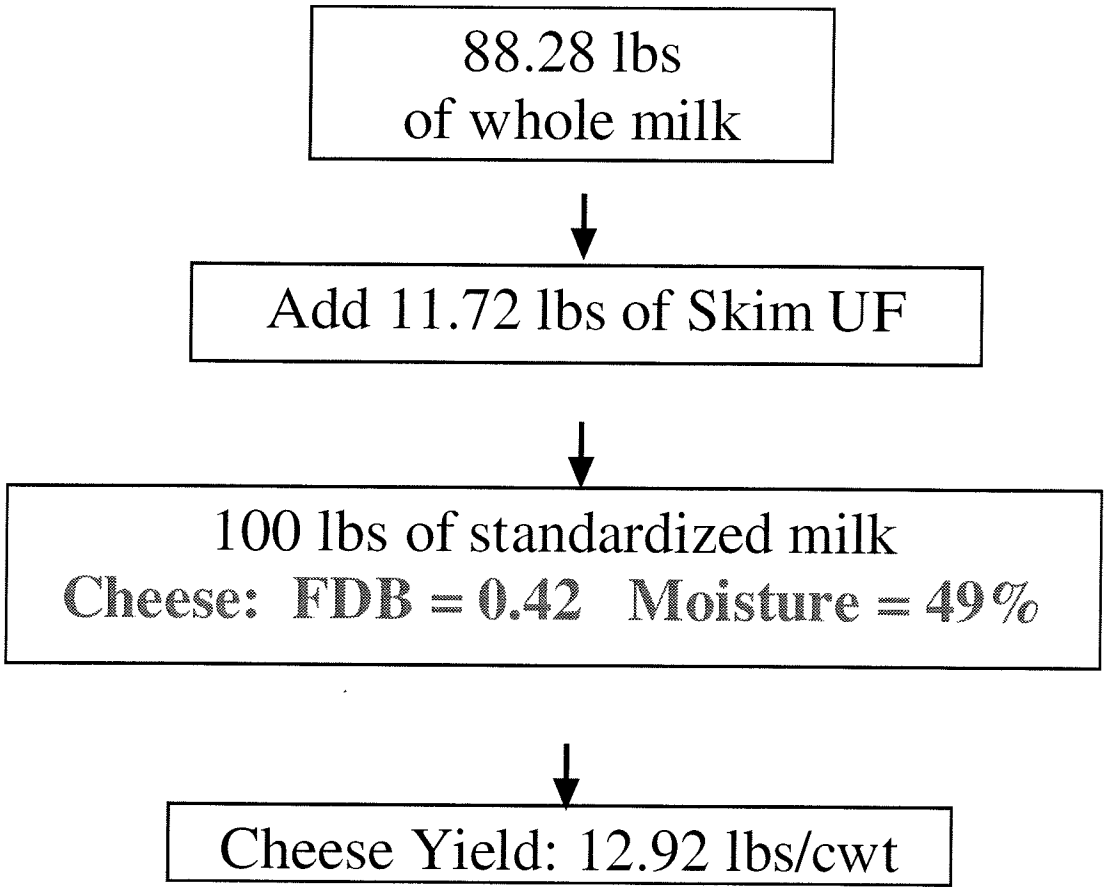


Figure 3: Use of skim UF to standardize mozzarella cheese milk



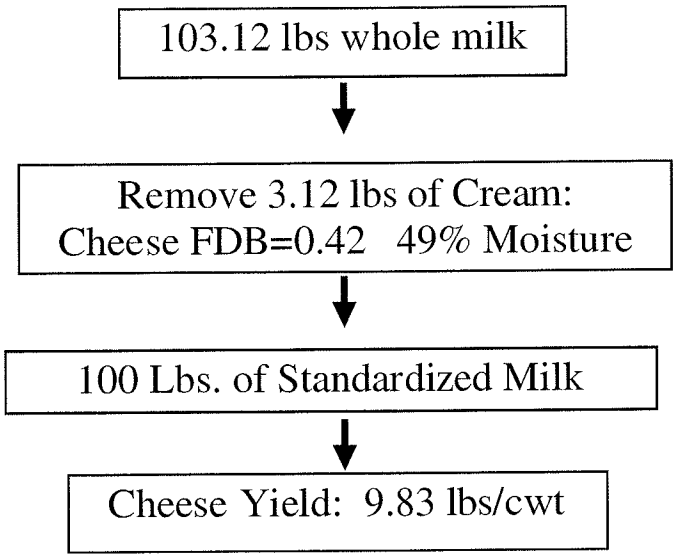
The daily Class IV graphs can be accessed via the URL: www.aae.wisc.edu/future/daily_settle_graph_cl4.htm. The daily butter futures settle price graphs can be accessed via: www.aae.wisc.edu/future/daily_settle_graph_butter.htm. The automation of these tasks reduced the time required from 1.5 hours/day to approximately 3 minutes/day.

The University of Wisconsin Dairy Marketing web site is accessed by approximately 3-4,000 unique users (i.e., IP addresses) per month. The number of these "hits" is continually increasing from month-to-month. There are approximately 6,000 files contained within this site, occupying approximately 450 MB of data, charts, publications, software, etc.

Economic analysis of alternative multiple component pricing systems

Under this sub-project we assisted a number of cheese plants investigating the economic impact of implementing farm operator pay plans based on alternative systems for the valuation of milk components. Most of this work was undertaken on a confi-

Figure 4: Removing cream to standardize mozzarella cheese milk



dential basis so we cannot provide details here concerning the specific systems investigated. Our contribution included developing spreadsheet models that accounted for the total mass balance of farm milk. The model began with the transformation of raw milk to the finished cheese product and incorporated using whey in the manufacture of whey-based products. The overall philosophy of these systems is to provide market signals to a plant's patrons regarding the appropriate composition of raw farm milk that maximizes the revenue stream to the cheese plant. As a result, returns increase for both the plant and the farmer patrons. The "optimal" composition of this farm milk will vary, depending on the products produced by the plant.

Analysis of consumer demand for dairy products
During 2001 we continued to develop a number of alternative econometric methodologies applicable to the analysis of household food (dairy product) expenditures. The first area of research focused on the analysis of food (dairy product) expenditure patterns in Mexico, Argentina, Brazil and China. Single-equation, along with demand system approaches, were developed. Within these models we incorporate a number of alternative methods for characterizing the age/gender impacts of household composition on food expenditures. We used the concept of equivalence scales to quantify impacts. With the estimation of these equivalence scales we can compare, from a household welfare perspective, expenditure patterns of households of differing sizes and composition.

A second area of research that continues is in the area of developing methodologies for estimating food demand parameters under a censored regression framework. We estimate a censored demand system in which we incorporate the above endogenously determined adult equivalent scales.

A third area of research, undertaken over the previous year, is in the examination of the impact of generic promotion efforts on the household demand for dairy products in the U.S. This research resulted in a manuscript which was submitted for publication in the *Canadian Journal of Agricultural Economics.* In this analysis we follow the biweekly purchases of this panel of consumers during 1997-99 resulting in a total of 78 bi-weekly purchase periods and 84,864 total observations. There are some unique aspects to our approach: we account for the panel nature of the data, the potential for serial correlation of equation error terms and censoring the dairy product purchases. Our econometric model is unique in that it not only allows for the use of simulated probability techniques to solve high-order integrals, but also partitions the data into smaller components to allow for analysis of longer time periods, increased accuracy, and reduced computing time. We incorporate generic advertising within the econometric model via a standard a polynomial distributed lag (PDL) specification, with end-point restrictions equal to zero. Biweekly, generic advertising expenditure data from 1996 were used to provide three full years (1997-1999) of data for estimation. An application to estimate producer marginal net returns to at-home cheese purchases revealed that the advertising program may be somewhat beyond optimal spending levels, as evidenced by small, but modest negative marginal net returns. The empirical results of this study are useful in providing additional insight regarding generic cheese advertising influence on various households' cheese purchase behavior and how this in turn influences the underlying returns to producers.

Publications/presentations

Submitted

T. Schmit, B.W. Gould, D. Dong, H. Kaiser and C. Chung, 2001. The Impact of Generic Advertising on Household Cheese Purchases: A Censored Autocorrelated Regression Approach, *Canadian Journal of Agricultural Economics.*

C. Chung, D. Dong, T. Schmit, H. Kaiser and B.W. Gould, 2001. Estimation of Price Elasticities from Cross-Section Data, *American Journal of Agricultural Economics.*

T. Schmit, C. Chung, D. Dong, H. Kaiser and B.W. Gould, 2001. Identifying the Effects of Generic Advertising on the Household Demand for Fluid Milk and Cheese: A Two-Step Panel Data Approach, *Journal of Agricultural and Resource Economics.*

Accepted

B.W. Gould, 2001, Household Composition and Food Expenditures in China, *Agribusiness.*

B.W. Gould, and H.Villarreal, 2001, Adult Equivalence Scales and Food Expenditures: An Application to Mexican Beef and Pork Purchases, forthcoming *Applied Economics.*

R. Sabates, B.W. Gould and H. Villarreal, 2001, Household Composition and Food Expenditures: A Cross-Country Comparison, forthcoming, *Food Policy.*