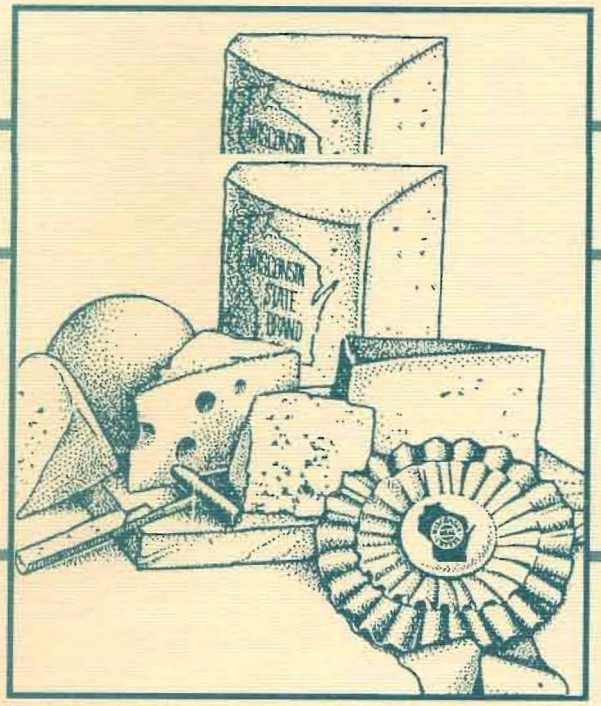
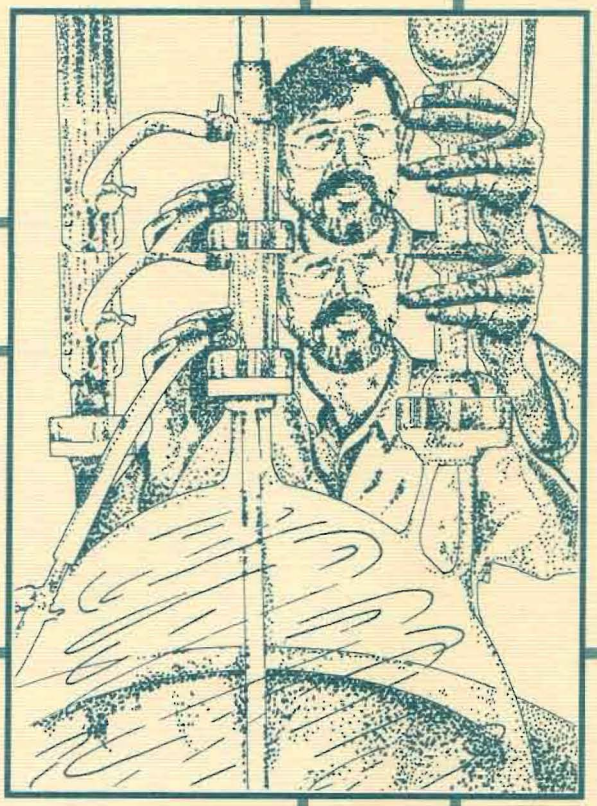
CDR

Annual Report 1989-1990





Center for Dairy Research

University of Wisconsin-Madison • College of Agricultural and Life Sciences

Direct your inquiries about CDR to :

Center for Dairy Research
University of Wisconsin - Madison
1605 Linden Dr., Rm. 241
Madison, Wisconsin 53706
Phone: 608/262-2217

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From the Director

As you will see from this report, in the past year the Center for Dairy Research attained several substantial goals in both our research program and our information and technology transfer activities. Our desire for interdisciplinary research has become a reality with cooperative and complementary projects between scientists within departments, across departmental and college boundaries, and with other U.S. universities and international research organizations.

The research reports herein reflect the efforts of researchers in the Center for Dairy Research, Walter V. Price Cheese Research Institute, and the Departments of Food Science, Food Microbiology and Toxicology, Chemical Engineering, Dairy Science, Nutritional Sciences, Agricultural Economics, and Meat and Animal Science, and of researchers at Purdue University, and the University of Nebraska, plus visiting scientists at CDR.

Milk component utilization, cheese technology, product and process development, and dairy food safety and quality continue to be areas of emphasis in the Center. Milkfat research encompasses control of milk composition, the fractionation and modification of milkfat, basic research to facilitate use of milkfat as a food ingredient, plus demand analysis to project milkfat usage in the future.

Fractionation procedures, unique methods of milkfat modification by interesterification, and characterization of milkfat fractions show promise for enhancing milkfat utilization. Research on whey is more limited in scope, but studies on production of polysaccharides from lactose and isolation of minor whey proteins have prompted interest by industry. Major thrusts of our cheese research program include expanding the market for cheese and improving the flavor and quality of cheese. Basic research to improve the characteristics of low-fat and low-sodium cheeses continues our efforts to increase the range of high-quality cheeses available to the consumer. A multi-project program on enhancing and controlling cheese flavor includes research on characterization of enzymes of potential flavor-producing bacteria, technologies such as spray-drying to provide functional cultures to industry, and evaluation of these cultures in cheese.

The Center's program includes an emphasis on maintaining the safety of dairy products. Projects underway investigate methods for the detection of pathogens in dairy products and the effects of environmental conditions on survival of those pathogens. Methods for detecting only virulent strains of *Listeria monocytogenes* and a rapid assay for a pathogenic *Escherichia coli* strain are being developed. Impacts of processing technologies on pathogen survival should be directly applicable to the cheese industry. Subpasteurization heat treatments greatly reduced the survival of several pathogens in Cheddar cheese, and the curd-cooking process had similar effects in Parmesan cheese.

The Worldwide Information and Technology Exchange Program is a catalyst to our research program and a conduit for information and technology transfer. Our scientist exchange program has contributed directly to research output but, equally as important, has facilitated longer-term collaboration with domestic and international laboratories. A mentor program was established to provide input to CDR from experienced dairy researchers. It will be expanded because of its positive impact on our overall research program and on individual researchers.

Dairy technology is always evolving which presents challenges and opportunities that CDR is addressing. Our success results from the combined and cooperative efforts of the researchers, the CDR Administrative Program, and WITEP.

Sincerely,

Norman F. Olson

Chapter 1

An Overview of CDR

CDR Staff

Director

Norman F. Olson

Administrative Officer

Thomas Szalkucki

Administrative Program

Melinda Adams, administrative program coordinator

Shannon Daggett, word processing operator

Shawna McGee, word processing operator

Al Muelling, program assistant

Mary Ann Murray, fiscal clerk

Brenda Williams, fiscal clerk

Research

Tarun Bhowmik, MS, research associate

Dave Bogenrief, assistant researcher

Carol Chen, cheese technologist, associate researcher

J.P. Chen, PhD, chemical engineer, assistant scientist

David Everett, graduate student

Jeremy Foltz, graduate student

Brian Gould, PhD, dairy economist, associate scientist

Mark Johnson, PhD, cheese microbiologist, senior scientist

Eric Kettner, graduate student

Dale McGill, research specialist

research, continued

Hong Pai, graduate student

Laura Paluch, graduate student

Lisa Pannell, graduate student

Carlo-Fererico Perali, graduate student

Brian Riesterer, associate researcher

Michael Thomsen, graduate student

Bill Tricomi, MS, laboratory manager, assistant researcher

Cheng-Hsin Wang, graduate student

Baokang Yang, graduate student

Worldwide Information and Technology Exchange Program (WITEP)

Sarah Quinones, program coordinator

Charlie Behnke, associate information processing consultant

Beth Carney, program assistant

David Gaeuman, technical writer/editor

Alice Winter Oakey, student assistant writer/editor

Visiting Scientists

José Coca, University of Oveido, Oveido, Spain

Morsi El Soda, Alexandria University, Alexandria, Egypt

Moustafa El-Shenawy, National Research Center, Cairo, Egypt

Geraldine Farrell, St. Angela's College of Education, Lough Gill, Sligo, Ireland

W. James Harper, Ohio State University, Galena, OH

H.J. Lee, Seoul National University, Seoul, Korea

S.K. Lee, DooSan Research Institute, Seoul, Korea

M.A. van Boekel, Wageningen Agricultural University, Wageningen, The Netherlands

Advisory Committees

Administrative Advisory Committee

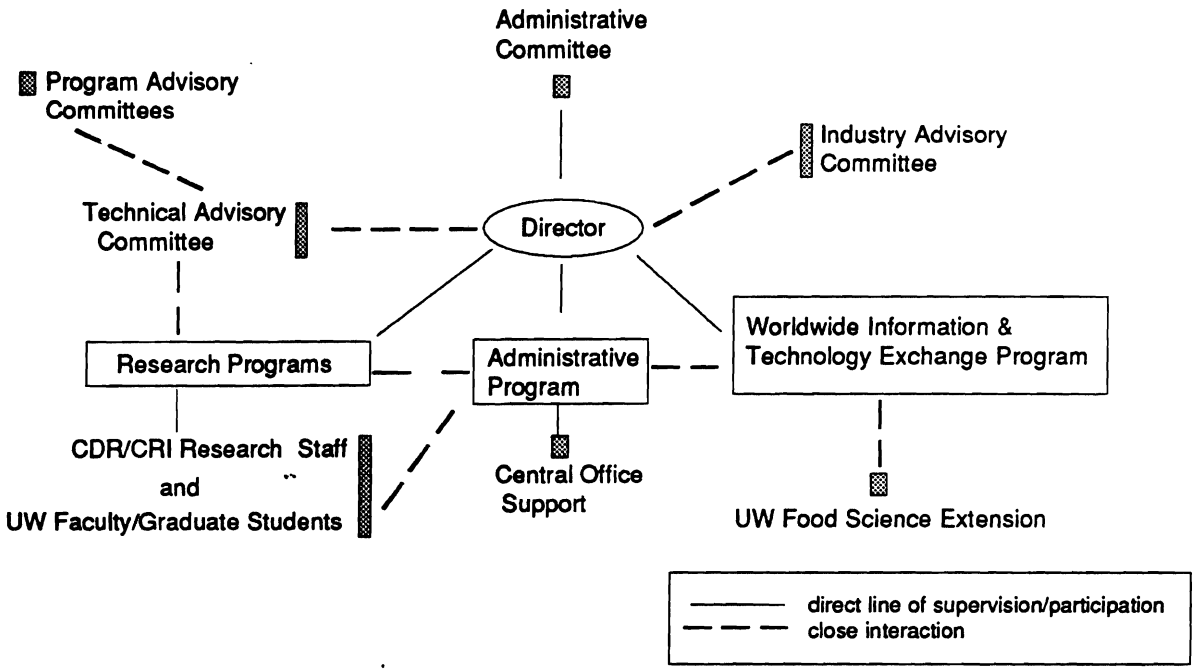
This committee establishes the overall policies and program goals of the Center.

Norman Olson, Director, Center for Dairy Research
Dean Smith, Associate Dean of Graduate School
Harvey Grosskopf, Board Member, Wisconsin Milk Marketing Board
Leo Walsh, Dean, College of Agricultural and Life Sciences
David Ward, Board Member, Wisconsin Milk Marketing Board
Janet Williams, Director of Research, National Dairy Promotion and Research Board
Neal Jorgenson (ex officio), Associate Dean, College of Agricultural and Life Sciences
Leslie Lamb (ex officio), Director of Research and Education,Wisconsin Milk Marketing Board

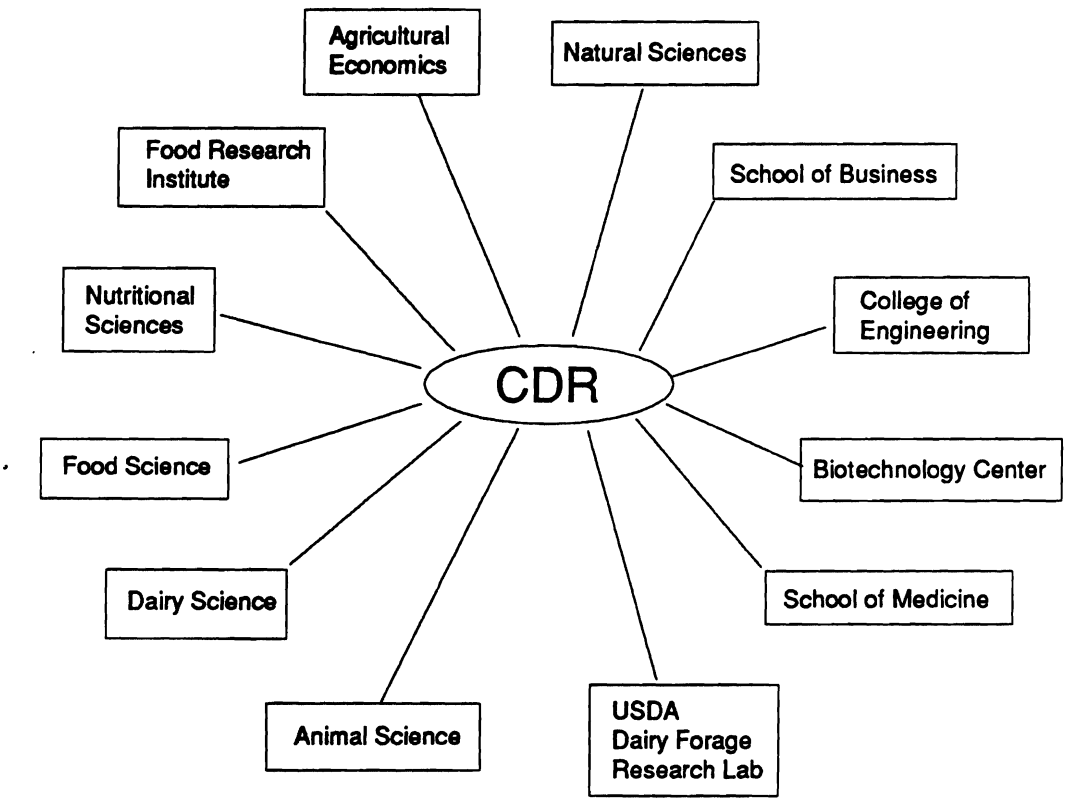
Technical Advisory Committee

This committee assists in planning the research program of the Center.

Robert Bremel, Dept. of Dairy Science
Michael Doyle, Dept. of Food Microbiology and Toxicology (Food Research Institute)
Janet Greger, Dept. of Nutritional Sciences
Charles Hill, Dept. of Chemical Engineering
Edward Jesse, Dept. of Agricultural Economics
Eric Johnson, Dept. of Food Microbiology and Toxicology (Food Research Institute)
Ken Lee, Dept. of Food Science
Robert Lindsay, Dept. of Food Science
Norman Olson, Director, Center for Dairy Research, Dept. of Food Science
Thomas Szalkucki, Administrative Officer, Center for Dairy Research
Mark Johnson (ex officio), Center for Dairy Research
Neal Jorgenson (ex officio), Associate Dean, College of Agricultural and Life Sciences
Janet Williams(ex officio), Director of Research, National Dairy Promotion and Research Board



Organizational Components of CDR



CDR Interactions within the University

Chapter 2

CDR Research Project Reports

Research Report List

Safety and Quality of Dairy Products

Safety

Nutrition

Cheese Research

Technology

Ripening

Milk Components and Their Uses

Milkfat Modification

Milk Composition Control

Whey Utilization

New Process and Product Development

Economics

Project Title:

Differentiating Virulent from Avirulent *Listeria monocytogenes*

Personnel:

Michael Doyle, professor, Food Research Institute (PI); Anna Lammerding, graduate student, Food Research Institute; Kathleen Glass, assistant researcher, Food Research Institute; Patrick Ford, laboratory assistant, Food Research Institute; Annette Gendron-Fitzpatrick, assistant director for education and co-director, Pathology Research Animal Resource Center Diagnostic Lab

Funding:

National Dairy Promotion and Research Board

Funding Code:

88-8

Dates:

July 1, 1988 - June 30, 1990

Objectives:

1. To determine if a difference in virulence of different isolates of *L. monocytogenes* could be identified with a pregnant mouse model.

2. To identify and characterize pathogenic factors of isolates of *L. monocytogenes* causing infection of mice fetal tissue.

Summary of Findings:

The work on this project has continued with testing different isolates of *Listeria monocytogenes* to determine if they are capable of invading internal tissues, including the placenta and fetus, when fed orally to pregnant mice.

- One isolate (#19113), a typical hemolytic strain of *L. monocytogenes*, did not survive in the gastrointestinal tract, nor did it infect any of the maternal or fetal tissues. This demonstrated that not all hemolytic *L. monocytogenes* can be regarded as virulent.

- Two additional strains isolated from foods were tested in the pregnant mouse model. These isolates behaved like the virulent Scott A strain, i.e., they were invasive and could infect placental and fetal tissues.

From the above results, screening of additional strains isolated from dairy products is warranted to determine what percent behave as the 19113 strain, i.e., avirulent.

- The pregnant mouse model, to determine the invasive properties of *L. monocytogenes*, was developed using germ-free reared (gnotobiotic) mice, i.e., animals which had been raised in a sterile environment and had not been exposed to any bacteria before inoculation. The virulence of *L. monocytogenes* Scott A was also tested in pregnant mice raised under normal conditions, with a normal immunity to bacteria living in the gastrointestinal tract. In such 'conventional' mice, the organism was also invasive, but the infection was not as extensive as in gnotobiotic mice. This indicated that:

1. The gnotobiotic mouse model is a very sensitive assay to test virulence of *L. monocytogenes* strains.

2. The immunity of the host may limit the infection caused by virulent strains.

A component of the cell wall of *L. monocytogenes* strains isolated from foodborne outbreaks appears to be unique to these particular strains. Work is focusing on this component to determine if it may be a marker for, or somehow involved in, virulence of the epidemic strains.

Seminars/Posters Presented on the Project:

'A Pregnant Animal Model to Assess the Virulence of Different *Listeria monocytogenes* Strains after Oral Ingestion,' at the 40th Conference of Canadian Laboratory Workers in Animal Diseases. Ottawa, Canada. May, 1990.

'Virulence of *Listeria monocytogenes* in a Pregnant Animal Model,' to be presented at the Annual Meeting of the International Association of Milk, Food and Environmental Sanitarians (IAMFES). Chicago, Illinois. August, 1990.

Project Title:

Procedure for Detection of *Escherichia coli* 0157:H7 in Dairy Products

Personnel:

Michael Doyle, professor, Food Research Institute (PI); Zhao Tong, research associate, Food Research Institute; Rick Kittell, research specialist, Food Research Institute; Mi Sun Kim, research associate, Food Research Institute; Nisha Padhye, graduate student, Food Research Institute

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-57

Dates:

July 1, 1988 - July 14, 1989

Objectives:

1) To produce and characterize monoclonal antibodies specific for *E. coli* 0157:H7 and verotoxins.

2) Develop immunoassays for detection of *E. coli* 0157:H7 and verotoxins.

3) Verify efficacy of immunoassays for detecting *E. coli* 0157:H7 in dairy products.

Summary of Findings:

1) Produced monoclonal antibody highly specific to *E. coli* 0157:H7 and a related enterohemorrhagic *E. coli* 026:H11.

2) Developed two immunoassays, an ELISA procedure and a dipstick assay, based on the above monoclonal antibody that can detect *E. coli* 0157:H7 in enrichment cultures within 2 hours.

3) Produced polyclonal and monoclonal antibodies to verotoxin-1 and verotoxins-2 of *E. coli* 0157:H7.

4) Developed immunoassays; i.e., ELISA procedures, using antibodies to verotoxins, that can detect verotoxin in milk.

Significance to Dairy Industry:

This project has resulted in the development of assays for detecting a severe dairy cattle-borne pathogen (*E. coli* 0157:H7) and its toxins in milk and dairy products.

Seminars/Posters Presented on the Project:

1) Finnish Veterinary Association, Annual Meeting, Helsinki, Finland. November 17, 1989. "*Escherichia coli* 0157:H7 — a newly recognized cause of hemorrhagic colitis and hemolytic uremic syndrome."

2) American Society for Microbiology, Annual Meeting, Anaheim, CA. May 16, 1990. "Production and characterization of monoclonal antibody to *Escherichia coli* 0157:H7."

3) Food Research Institute Annual Meeting, University of Wisconsin-Madison, WI. May 23, 1990. "Production of a monoclonal antibody highly specific for *Escherichia coli* 0157:H7."

4) University of Guelph, *E. coli* 0157:H7 Round Table, Guelph, Ontario, Canada. June 26, 1990. "Detection of *Escherichia coli* 0157:H7 in foods."

5) Society for Industrial Microbiology, 1990 Annual Meeting, Orlando, Florida. July 30, 1990. "*Escherichia coli* 0157:H7—A foodborne pathogen with severe consequences."

6) Food Micro 90, 14th International Symposium of the International Committee on Food Microbiology and Hygiene, Bolkesjo, Tele-

mark, Norway. August 18, 1990. "*Escherichia coli* 0157:H7 and its significance in foods."

Publications:

1) Padhye, V. V., T. Zhao, and M. P. Doyle. 1989. Production and characterization of monoclonal antibodies to verotoxins-1 and -2 from *Escherichia coli* of serotype 0157:H7. *J. Med. Microbiol.* 30:219-226.

2) Padhye, N. V. and M. P. Doyle. Production and characterization of a monoclonal antibody specific for enterohemorrhagic *Escherichia coli* of serotypes 0157:H7 and 026:H11 (submitted).

3) Kittell, F. B., V. V. Padhye, and M. P. Doyle. Characterization and inactivation of verotoxin-1 produced by *Escherichia coli* 0157:H7 (submitted).

Project title:

Prevention of Survival and Growth of Pathogens in Milk and Cheese by Enhancement of the Activity of Lactoferrin/Lysozyme

Personnel:

Eric A. Johnson, assistant professor, Food Research Institute; Ariane Nara, research specialist, Food Research Institute

Funding:

National Dairy Promotion and Research Board

Funding Code:

'88-7

Dates:

July 1, 1988 - June 30, 1990

Objective:

Determine the antimicrobial activity of lysozyme and lactoferrin against pathogens in milk and cheese.

Summary of Findings:

Listeria monocytogenes

Listeria monocytogenes strain Scott A was not inhibited in culture media (Brain Heart Infusion) at 37°C by lactoferrin (0.5 or 1.0 mg/ml; lactoferrin was determined to be 17% saturated with iron). Bicarbonate (1 mg/ml) also did not enhance the activity of lactoferrin against *L. monocytogenes*. However, when lysozyme (100 ppm) was added to lactoferrin + bicarbonate, then substantial inhibition was observed with several *L. monocytogenes* strains (Figure 1). *L. monocytogenes* in milk at 18°C or 37°C was resistant to lactoferrin/bicarbonate ± lysozyme. *L. monocytogenes* also grew equally well in Cheddar cheese prepared with lactoferrin (data not shown). These data suggest that lactoferrin would not be a useful adjunct in cheese to prevent the growth of *L. monocytogenes*.

Our laboratory has observed that several factors influence the susceptibility to *L. monocytogenes* to lysozyme. In particular, divalent metal chelators (especially EDTA) potentiated the activity of lysozyme in milk. *L. monocytogenes* was stubbornly resistant to lysis in whole milk (Figure 2), although the same cells were susceptible in phosphate buffer. Also *Micrococcus luteus* was susceptible in milk indicating that lysozyme was not inactivated (data not shown). These results indicate that milk protects *L. monocytogenes* from lysis or that the cells undergo physiological changes when suspended in milk. A series of experiments were carried out to sensitize *L. monocytogenes* in milk. We found that susceptibility to lysozyme was not increased if proteins were coagulated by addition of acid to milk, and lysis was attempted in the whey by addition of lysozyme. However, if the whey fraction was demineralized by passage through a metal affinity column, lysis was observed. This result suggested that metals may protect *L. monocytogenes* in milk. When metals were depleted from whole pasteurized milk by dialysis against 20 mM sodium citrate followed by passage through a metal-affinity column then *L. monocytogenes* became sensitized (Figure 2). Furthermore, if *L. monocytogenes* was sublethally heat-treated (60°C for 15s), then lysis was dramatically improved in the demineralized milk (Figure 2). These results indicate that divalent metals protect *L. monocytogenes* against lysis in milk. This was confirmed when we replaced various metals in the demineralized milk: replacement of calcium or magnesium reinstated the resistance (Figure 3).

In conclusion, the limiting factor for the activity of lysozyme against *L. monocytogenes* appears to be an outer surface structure, which prevents penetration of lysozyme. EDTA or heat treatment disrupts this layer and enables lysozyme penetration. Preliminary results indicate that sublethal heat treatments sensitize *L. monocytogenes* in Cheddar cheese. Further trials are presently being carried out.

Other pathogenic bacteria

Lactoferrin alone did not effectively inhibit germination and growth of the spores of two proteolytic strains of *Clostridium botulinum* in minimal media (Figure 4). However, lysozyme together with lactoferrin was inhibitory (Figure 4). In milk, the combination was not effective. Similar results were found with *Bacillus cereus* and *Yersinia enterocolitica*. We have not investigated methods to sensitize these pathogens in milk.

Significance to the Dairy Industry

Our results indicate that *L. monocytogenes* can be sensitized to inactivation by lysozyme using sublethal heat-treatment and metal limitation. This observation should help to develop a preservation system in milk to help rid *L. monocytogenes* from dairy products.

Seminars:

Food Research Institute Annual Meeting, May 1990, University of Wisconsin-Madison. "Effectiveness of antibacterial substances against pathogens. Presented by E. A. Johnson."

Invited speaker- 84th Annual Meeting of the American Dairy Association, Lexington, Kentucky, August 2, 1989. "The potential application of antimicrobial proteins in food preservation."

Invited speaker- Tenth Annual Food Microbiology Symposium, University of Wisconsin-River Falls. "Novel approaches for controlling *Listeria monocytogenes* and *Clostridium botulinum* in foods."

Invited seminar- General Foods Corporation, White Plains, New York, November 28, 1989. "Natural antimicrobial systems for control of pathogens in foods."

Figure 1. *Inhibition of various* L. monocytogenes *strains in brain heart infusion broth by lysozyme (100 ppm), lactoferrin (0.5 mg/ml) and sodium bicarbonate (1 mg/ml).*

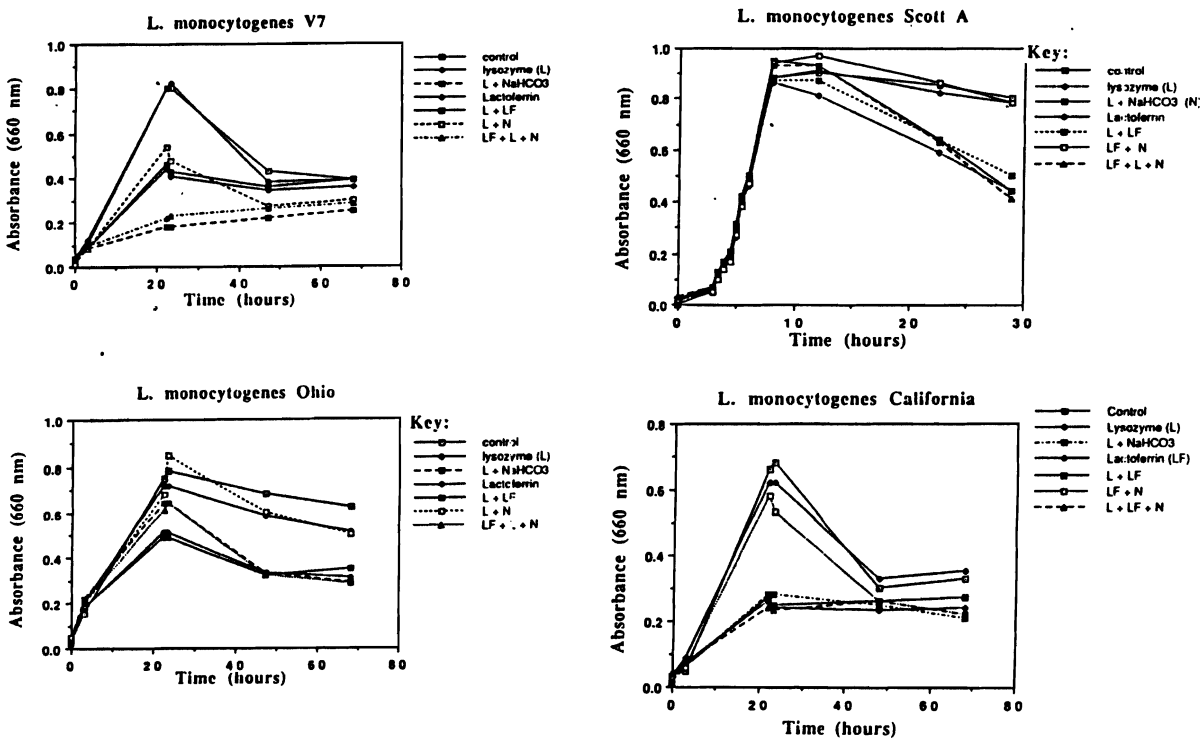


Figure 2. Lysis of L. monocytogenes Scott A in whole milk and milk treated to remove metals (see text for experimental details). In certain incubations, L. monocytogenes was sublethally heated in the milk (60°C for 15 s) prior to addition of lysozyme.

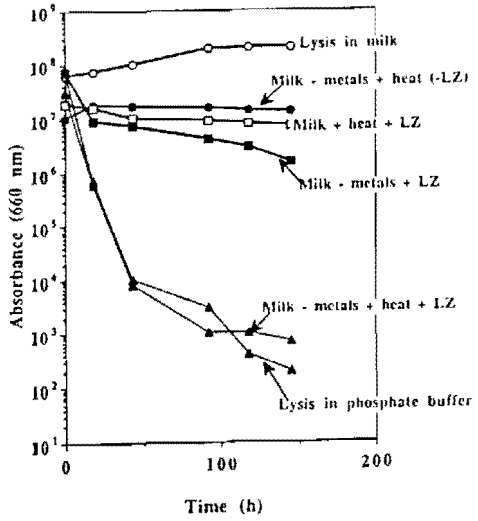


Figure 3. Protection against lysis of L. monocytogenes in demineralized milk by replacement of various metals [Ca, 666 ppm; Mg, 83 ppm; Zn, 3.0 ppm]. Metals were replaced at about the concentrations that are found in milk (determined by inductively coupled plasma (ICP) emission spectroscopy).

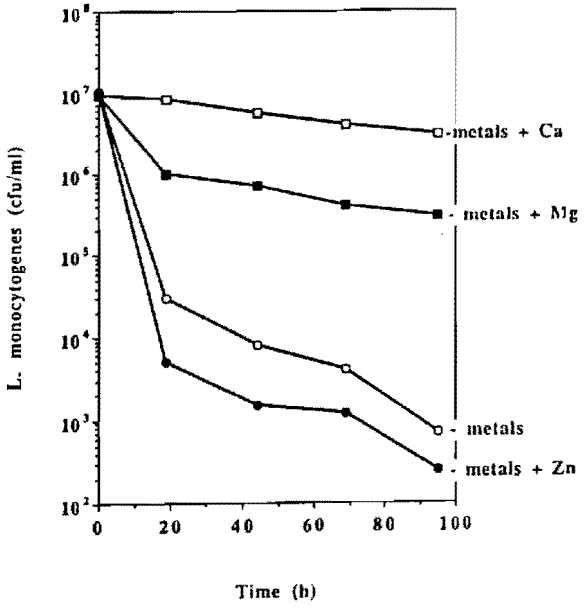
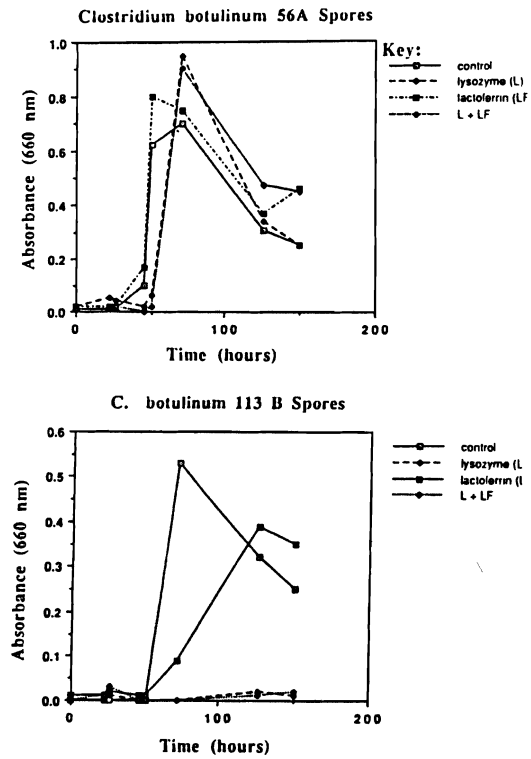


Figure 4. Inhibition of growth of C. botulinum spores by lysozyme and lactoferrin in minimal medium.



Project Title:

Behavior of *Listeria monocytogenes* during the Preparation of Lactic Starter Cultures and in Raw Milk Inoculated with Lactic Acid Bacteria

Personnel:

Elmer H. Marth, professor, Dept. of Food Science (PI); Jane Wenzel, graduate student, Dept. of Food Science

Funding:

Wisconsin Milk Marketing Board

Funding Code:

IV-53

Dates:

September 1, 1987 - August 31, 1989

Objectives:

1. Determine the behavior of *Listeria monocytogenes* when in the presence of starter cultures during their preparation by external or internal control of pH of the culture medium.

2. Determine the behavior of *L. monocytogenes* in raw and pasteurized milk when lactic acid bacteria are added to such milks.

3. Determine the effectiveness of the activated lactoperoxidase/thiocyanate system in raw milk for the control of *L. monocytogenes*.

Summary of Findings:

The overall objective of this project was to develop additional information which could be useful to insure the safety of milk products, particularly cheese and other cultured dairy foods. In this work we sought to answer the question, "How would *L. monocytogenes* behave if present when a bulk starter is prepared using a medium with internal pH control?" The results of this project have practical as well as scientific significance relating to our knowledge of *Listeria monocytogenes*.

Of practical significance is the fact that internal pH control medium supported growth of each of three strains of *L. monocytogenes* in the absence and presence of lactic acid bacteria. Populations of listeria did decrease in the presence of each of the lactic cultures but the actual decline usually began at pH 5.5; the pH at which the medium is said to be ready for use. No complete inhibition of listeria was seen at this pH. In the past, fermented products were considered to be free of pathogens because of their acid content. When medium with internal pH control is used to prepare bulk starter, this assumption can no longer be made. We found that this medium could contain listeria populations of up to 10^5 cfu/ml at the time the medium is said to be ready for use when the original population was 10^3 cfu/ml.

We also saw the ineffectiveness of a raw milk inoculant in the control of *Listeria monocytogenes* in raw milk. This product may be of use in the control of other psychrotrophs, but it does not control growth of listeria.

In contrast, we also saw the effects of activation of the lactoperoxidase system on growth of listeria in several media including raw milk. This system was found to have a bactericidal effect on listeria depending on strain and number of the pathogen and culture medium. The lactoperoxidase system may be an economical and safe way of controlling *L. monocytogenes*.

Observations made in this study were of scientific significance. We noted differences in overall inhibition of listeria depending on temperature, percentage, and type of lactic culture and strain of listeria. As with most scientific research, more questions were raised than were answered. Some of the questions resulting from this work that deserve further study include:

1. Why was *Listeria monocytogenes* strain *California* more sensitive to the effects of certain lactic acid bacteria?

2. How can we explain differences in inhibition of listeria seen when different percentages of lactic culture caused a similar pH?

3. What is causing the inhibition of listeria? We found pH plays a role but is not solely responsible for the inhibition.

4. Why was *Streptococcus cremoris* much more inhibitory to listeria in IPCM-1 (internal pH control medium-1) after the 30-hour incubation than *Streptococcus lactis*?

Significance to the Dairy Industry:

Fermented dairy products can no longer be assumed to be free of pathogens because of their acid content. The responsibility for production of a safe product lies with the manufacturer. *Listeria monocytogenes* is everywhere in the environment. It has been isolated from soil and poor quality silage; and it is difficult to imagine how listeria could not enter the dairy environment. This pathogen has been isolated from food processing plants, including dairies. Production processes must be examined step-by-step to prevent contamination with pathogens, like listeria. Implementation of hazard control programs, such as HACCP, should be mandatory for every product made. Dairy processors should be very concerned about listeria because, unlike many pathogens, it survives at refrigerator temperatures.

Publications:

El-Shenawy, M.A, H.S. Garcia, and E.H. Marth. 1990. Inhibition and inactivation of *Listeria monocytogenes* by the lactoperoxidase system in raw milk, buffer, or a synthetic medium. *Milchwissenschaft* (in press).

Wenzel, J.M. and E.H. Marth. 1990. Behavior of *Escherichia coli* 0157:H7 or *Yersinia enterocolitica* in the Presence of *Streptococcus cremoris* in a Medium with Internal pH Control. *Lebensmittel-Wissenschaft & Technologie* (accepted).

Wenzel, J.M. and E.H. Marth. 1990. Behavior of *Listeria monocytogenes* in the presence of Lactic Acid Bacteria in an Agitated Medium with Internal pH Control. *Journal of Food Science* (accepted).

Wenzel, J.M. and E.H. Marth. 1990. Behavior of *Listeria monocytogenes* in the presence of *Streptococcus lactis* in a Medium with Internal pH Control. *Journal of Food Protection* (accepted).

Wenzel, J.M. and E.H. Marth. 1990. Behavior of *Listeria monocytogenes* at 4 and 7°C in Raw Milk with a Commercial Culture of Lactic Acid Bacteria. *Milchwissenschaft* (accepted).

Wenzel, J.M. and E.H. Marth. 1990. Changes in Populations of *Listeria monocytogenes* in a Medium with Internal pH Control Containing *Streptococcus cremoris*. *Journal of Dairy Science* (accepted).

Wenzel, J.M, and E.H. Marth. 1988. Behavior of *Listeria monocytogenes* in the presence of *Streptococcus lactis* in a medium with internal pH control. *J. Dairy Sci.* 71. (suppl. 1):86 (abstr.).

Wenzel, J.M. and E.H. Marth. 1988. Behavior of *Listeria monocytogenes* in the presence of *Streptococcus lactis* in a medium with internal pH control. Abstr. Annu. Meeting North Central Branch. Amer. Soc. Microbiol., Madison, WI. Oct. 7- 8, 1988, p. 3 (abstr.)

Wenzel, J.M. and E.H. Marth. 1988. Behavior of *Listeria monocytogenes* in the presence of *Streptococcus cremoris* in Media with internal pH control. *J. Dairy Sci.* 72(suppl. 1): 137 (abstr.).

Project Title:

Behavior of *Listeria monocytogenes* During Manufacture and Ripening of Cheese

Personnel:

Elmer H. Marth, professor, Dept. of Food Science (PI); A.E. Yousef, research associate, Dept. of Food Science; M.A. El-Shenawy, visiting scientist, Center for Dairy Research

Funding:

National Dairy Promotion and Research Board

Funding Code: Dept. of Food Science

Project Dates:

February 1989 - February 1990

Objectives:

1. Determine the behavior of *L. monocytogenes* during the manufacture of Parmesan, Swiss, and Mozzarella cheeses.

2. Determine the fate of *L. monocytogenes* during the ripening/storage of Parmesan, Swiss, and Mozzarella cheeses.

3. Determine the presence of *L. monocytogenes* in whey when different varieties of cheese are made.

4. The hypothesis being tested: *L. monocytogenes* will not grow during cheesemaking, most cells of *L. monocytogenes* in milk will be trapped in curd rather than be lost in whey, and *L. monocytogenes* will remain in cheese for an extended time during ripening/storage.

Summary of Findings:

I. Survival of *L. monocytogenes* during the manufacture and ripening of Parmesan cheese

Parmesan cheese was made from a mixture of pasteurized whole and skim milk that was inoculated to contain 10^4 - 10^5 cells of *L. monocytogenes*/ml of milk. Cooking was done at 51°C for ca. 45 min. Numbers of *L. monocytogenes*, during cheesemaking, increased 0.6 - 1.0 orders of magnitude (0.6-1.0 log). During the cooking, the count did not decrease at the rate expected with such a heat-treatment. Numbers of *L. monocytogenes* decreased appreciably during pressing (Figures 1 and 2). These results support our hypothesis about entrapment of cells of *L. monocytogenes* in the curd.

During cheese ripening, numbers of *L. monocytogenes* decreased, almost logarithmically, (Figures 1 and 2) at a faster rate than what was reported for other hard cheeses (See Table 1 for information on hard cheeses). *L. monocytogenes* strain *California* died at a faster rate than strain V7. Average D-values for inactivation of *L. monocytogenes* during ripening of Parmesan cheese were 29 and 17 d for strains V7 and California, respectively. We were unable to detect *L. monocytogenes* in Parmesan cheese (by direct plating) after 3 - 16 weeks of ripening. The period of survival depended on the strain of the pathogen, initial inoculum in the milk, and the lot of cheese. Contrary to our hypothesis, *L. monocytogenes* did not survive during ripening of Parmesan cheese for an extended period. It is likely that a large proportion of the population of *L. monocytogenes* in the curd was injured during cooking. It is also likely that these injured cells did not survive as well in cheese during ripening as did normal healthy cells.

Whey produced during the manufacture of Parmesan cheese contained fewer cells of *L. monocytogenes* than cheese curd. Numbers of *L. monocytogenes* in whey remained relatively constant during cheesemaking before the cooking step. Numbers of *L. monocytogenes* in whey decreased during cooking because cells were inactivated by heat. These results support our hypothesis, and they also show that most of *L. monocytogenes* in whey result from the cutting step. Development of a semi-permeable film around curd particles may prevent further release of cells to the whey.

Figure 1. Listeria monocytogenes strain V7 in Parmesan cheese.

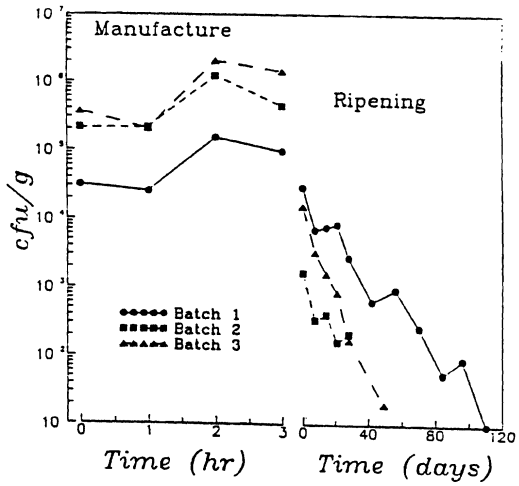


Figure 2. Listeria monocytogenes strain CA in Parmesan cheese.

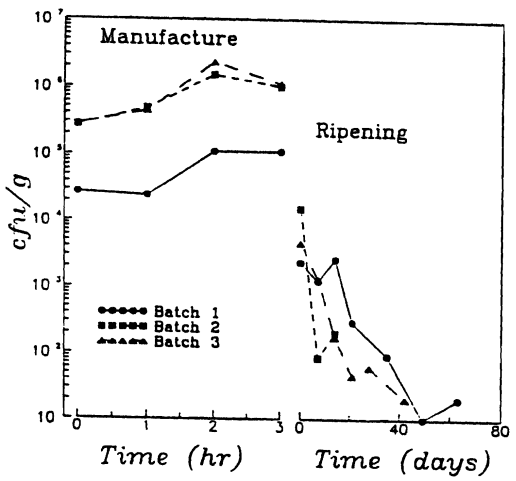


Table 1. Average D-values for inactivation of L. monocytogenes during ripening of Parmesan and Colby[a] cheeses.

Cheese	Strain	D-value (days)
Parmesan	V7	29
Parmesan	CA	17
Colby	V7	59
Colby	CA	57
High-moisture Colby[b]	V7	124

[a]Results from Yousef and Marth (jj).
[b]Average frmm two lots of Colby cheese that contained 42.5% moisture.

Significance to the Dairy Industry:

Parmesan cheese made in this study was not a favorable medium for survival of *L. monocytogenes*. FDA regulations state that Parmesan cheese should be ripened for at least 10 months. According to data from our study, this long ripening period should be sufficient to result in listeria-free Parmesan cheese. Some manufacturers, however, cook Parmesan cheese curd at lower temperatures than we did in this study. Such a practice may produce a more favorable cheese for survival of *L. monocytogenes*.

Presentations:

1989 Conference of the Center for Dairy Research, Madison, Wisconsin.

1989 American Dairy Science Association Annual Meeting, Lexington, Kentucky.

Abstracts:

Yousef, A. E. and E. H. Marth. 1989. Behavior of *L. monocytogenes* in Parmesan cheese. *J. Dairy Sci.* 72 (suppl. 1), 138.

II. Thermal Inactivation and Injury of *Listeria monocytogenes* in Milk

Objectives:

Some results from our earlier study contradict our hypothesis and current literature about the general behavior of *L. monocytogenes* in cheeses. Numbers of *L. monocytogenes* decreased rapidly during pressing, salting, and ripening of these curds. Additionally, numbers of *L. monocytogenes* did not decline during cooking of Parmesan cheese curd at the rate expected for such a heat treatment. Therefore, we did this study to investigate the following topics:

1. Determine the thermal inactivation of *L. monocytogenes* at temperatures similar to those used to heat-treat cheesemilk and cook cheese curd.

2. Determine the degree of injury of cells of *L. monocytogenes* caused by heat treatments similar to those used in processing cheesemilk, and in cooking the curd.

Summary of Findings:

Cells of *L. monocytogenes* strain *V7* suspended in reconstituted (10%) nonfat dry milk were placed in capillary tubes and heated at 50°, 55°, 60°, 65°, 70°, and 75° C. Heat-treated cells were counted on a permissive medium (tryptose agar) so that both healthy and heat-injured cells could form colonies on the agar medium. The pathogen had D-values of 1900, 270, 57, 6.0, 1.4, and 0.4 s. at those temperatures, respectively, when heated cells were plated on tryptose agar. Estimated D-values for inactivation of *L. monocytogenes* at temperatures normally used for pasteurization of milk (62.8° and 71.7° C) are 20 and 0.94 s., respectively. Therefore, High-Temperature-Short-Time pasteurization of milk is a 16-D process. The D-value at temperatures that may be used to cook the curd of Parmesan cheese is 18.5 min. Heat-treatments similar to those used to cook the curd of Parmesan cheese (51° for 45 min) should decrease the population of *L. monocytogenes* by ca. 2.4 orders of magnitude. Results of the study on Parmesan cheese show that cooking the curd inactivated fewer cells of *L. monocytogenes* than expected from this study. It is likely that poor transfer of heat in curd particles, compared with that in milk, may account for this discrepancy.

Heat-treated milk was plated on a restrictive medium (tryptose agar containing 6% salt) to permit only healthy cells to form colonies on the agar medium. The D'-value (decimal reduction time as determined with the salt-containing medium) were 1600, 170, 36, 4.7, 1.2, and 0.46 s. at 50°, 55°, 60°, 65°, 70°, and 75° C, respectively. Injury was somewhat more pronounced when cells were heated at the lower temperatures, e.g., 50° - 65° C. Cooking Parmesan cheese curd may have injured cells of *L. monocytogenes* appreciably since both heat and acidity are involved in the process. As suggested earlier, injured cells may be inactivated in cheese during ripening at a faster rate than normal, healthy cells.

Significance to the Dairy Industry:

1. Our results confirm that pasteurization of milk as defined by FDA should inactivate *L. monocytogenes*. Cheesemilk should receive a heat-treatment similar to that of pasteurization to insure freedom from *L. monocytogenes*.

2. Cells of *L. monocytogenes* may be injured appreciably during processing of dairy products. Therefore, methods to detect and isolate the pathogen from these products should include a resuscitation procedure to allow for recovery of injured cells.

Publications:

El-Shenawy, M.A., A.E. Yousef, and E.H. Marth. 1990. Fate of *L. monocytogenes* during the Manufacture and Ripening of Parmesan Cheese. *Journal of Dairy Science* (accepted).

El-Shenawy, M.A., A.E. Yousef, and E.H. Marth. 1989. Thermal inactivation and injury of *L. monocytogenes* in reconstituted nonfat dry milk. *Milchwissenschaft*, 44:741-745.

Project Title:

Behavior of *Listeria monocytogenes* in Ultrafiltered Milk with and without Added Lactic Acid Bacteria.

Personnel:

Elmer H. Marth, professor, Dept. of Food Science (PI); Fathy E. El-Gazzar, visiting scientist, Dept. of Food Science

Funding:

National Dairy Promotion and Research Board

Funding Code:

MAR89-2

Dates:

January 1, 1990 to December 31, 1990

Objectives:

1. Determine the growth/survival behavior of *L. monocytogenes* in ultrafiltered milk (concentrated 2 to 5X) during storage at refrigeration temperature (e.g., 4°C) and at temperatures used in cheesemaking (e.g., 32° - 40°C).

2. Determine the growth/survival behavior of *L. monocytogenes* in ultrafiltered milk (concentrated 2 to 5X) inoculated with lactic acid bacteria and held at temperatures used in cheesemaking (e.g., 32° - 40°C).

3. Determine the heat resistance of *L. monocytogenes* in ultrafiltered milk of various concentrations.

Summary of Findings:

Ultrafiltration (UF) can be used to concentrate milk and the resulting product can be made into cheese. Thus, UF has been used to produce concentrated milk for subsequent manufacture of Mozzarella, Cheddar, cottage, Havarti, brick and Colby cheeses. The principal advantage of using UF milk is the 16% - 20% increase in cheese yield; reduction in costs of energy, equipment, and labor; improved consistency of cheese flavor; and possible production of new by-products.

Listeria monocytogenes, the cause of at least four outbreaks of illness associated with contaminated dairy products, could enter ultrafiltered milk either from the ultrafiltration device or from the environment of the dairy factory. Presently, information on behavior of foodborne pathogens in UF milk and permeate is limited. Hence, work reported in this communication was done to answer the first question we have, Does *L. monocytogenes* grow or survive in UF milk and permeate during storage at 4°C and at temperatures used in cheesemaking (e.g., 32° - 40°C)? If so, what is the rate of growth or how long does the pathogen survive?

Pasteurized skim milk and retentate (skim milk concentrated 5X or 2X by volume) and permeate from ultrafiltered skim milk were inoculated with *L. monocytogenes* strains *California (CA)* or *V7* and incubated at 4°C. The growth rates of both strains were greater in retentate at 4°C than in skim milk. Maximum populations were Ca. 1 to 2 orders of magnitude greater in retentate (5X or 2X) than in skim milk when incubation was at 4°C and attained maximum populations of Ca. 10^6 to 10^7/ml.

Tyndallized samples were inoculated with the same strains of *L. monocytogenes* and incubated at 32° or 40°C. At these temperatures, populations achieved by the pathogen Ca. 10^7 or 10^8/ml, were similar in skim milk, retentate, and permeate.

Significance to the Dairy Industry:

Delivery of safe food to the consumer must be the first priority of the dairy industry. To accomplish that, it is necessary to know how the pathogens behave during manufacture and storage of dairy foods. Our results contribute to the knowledge that is needed (e.g. potential growth of *L. monocytogenes* in the products tested was established). With this knowledge, appropriate action can be taken by industry, and other organizations, to

minimize risk, and insure using UF milk free of *Listeria* for cheesemaking or other foods.

Publications:

El-Gazzar, F.E., H.F. Bohner, and E.H. Marth. 1990. Growth of *Listeria monocytogenes* at 4°, 32° and 40°C in skim milk and in retentate and permeate from ultrafiltered skim milk. *J. Food Prot.* (submitted).

Project title:

Effects of Heat Treatment and Cheesemaking Variables on Pathogen Survival and Growth

Personnel:

Eric A. Johnson, assistant professor, Food Research Institute (co-PI); John H. Nelson, senior research manager, Food Research Institute (co-PI); David J. Kihm, research specialist, Food Research Institute; and Brian Radtke, student assistant, Food Research Institute

Funding:

National Dairy Promotion and Research Board

Funding Code:

88-5

Dates:

July 1, 1988 -June 30-1991

Objectives:

1. Determine effect of subpasteurization heat treatments [60°C - 65°C (140°F - 150°F) for 15 - 20 seconds] on survival of pathogens in milk and in cheeses.

2. Quantitatively determine heat resistances of pathogens of concern in cheeses including strains of *Listeria monocytogenes, Salmonella,* and enteropathogenic *Escherichia coli.*

Summary of Findings:

Effect of heat treatment on the survival of *Salmonella heidelberg, Listeria monocytogenes* Scott A, and *Escherichia coli* O157:H7 during preparation and ripening of laboratory prepared Cheddar cheese:

Experimental Methods:

Salmonella heidelberg, Listeria monocytogenes Scott A, and *Escherichia coli* O157:H7 were used in this study.

Heat treatments and cheese preparation pathogens were cultured, harvested, and suspended in buffer. They were added (10^6/ml) to whole, non-homogenized pasteurized milk preheated to 60°C, 62.5°C, or 65°C and held for 15 seconds. Controls were also added to heated and cooled milk. This milk was used for cheese preparation.

Cheese was prepared as follows: Starter culture (freeze-dried) was first grown in skim milk at 23°C for 12-16 hours until a pH of 5.0 was reached. Milk for cheesemaking (containing pathogen) was tempered to 31.5°C, 1% starter was added, the milk was incubated for 45 minutes, rennet was added, and the milk was allowed to coagulate for 30 minutes at 31.5°C. The curd was cut, and whey expelled gently for 15 minutes, and the cheese was cooked by raising the temperature from 31.5° to 38.9°C over 20 minutes. The cheese was held at 38.9°C for 45 minutes and the whey removed by compressing in cheesecloth. The cheese was pressed in a beaker, and salted to a final concentration of 1.8% (w/w). The cheese was divided into four equal samples (~25 g), and packaged in Whirl-Pak bags. These were incubated at 5°C for up to 8 weeks.

Determination of Pathogen Growth and Survival:

Samples were taken at various times in the cheesemaking process. Pathogens were determined by enrichment and plating on selective media.

Results:

Pathogen survival was significantly affected by the heat-treatment employed. Compared to the controls, cells heated at 60°C or 62.5°C survived poorly in the cheese, and no cells were recovered at 65°C. *E. coli* was less affected than the other two pathogens. See Figures 1-3 for results.

We have also found that sublethal heat treatments sensitize *L. monocytogenes* to lysis by lysozyme in phosphate buffer and in milk that has been treated to remove divalent

cations. In whole milk, *L. monocytogenes* was completely resistant to lysozyme. When the pathogen was treated at 60°C for 15 seconds, a mild sensitization to lysozyme was observed (viable counts dropped by ~1 log over 4 hours). In demineralized milk, viable counts of heat-treated cells dropped by 3-4 logs when treated with lysozyme. These preliminary results indicate that *L. monocytogenes* can be sensitized for inactivation in milk by heat treatment and that cells are protected by divalent metals. Further work is being carried out to understand the mechanism of inactivation.

Significance to the Dairy Industry:

L. monocytogenes is widespread in dairy plants and has been found in several dairy products. The FDA and USDA have enforced a zero tolerance level for *L. monocytogenes*. Hence, effective methods must be developed to keep it out of dairy products. Our research has provided important advances in this direction.

Seminars:

Food Research Institute Annual Meeting, May 1990, University of Wisconsin-Madison. "Effectiveness of antibacterial substances against pathogens." Presented by E. A. Johnson.

Poster presentation. Food Research Institute Annual Meeting, May 1990, University of Wisconsin-Madison. David J. Kihm and Eric A. Johnson. "Influence of heat-treatments on survival of pathogens in cheese."

Publications:

Johnson, E. A., J. H. Nelson and M. Johnson. 1990. Microbiological safety of cheese made from heat-treated milk. I. Introduction and historical review. *J. Food Prot.* 53: 441-452.

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

Johnson, E. A., J. H. Nelson and M. Johnson. 1990. Microbiological safety of cheese made from heat-treated milk. III. Effects on processing. *J. Food Prot.* (in press, July).

Figure 1. Effect of heat treatments on survival of pathogens in Cheddar cheese. Pathogen survival is presented as a function of curing time: Escherichia coli 0157:H7 at 60°C (left) and at 62.5°C (right).

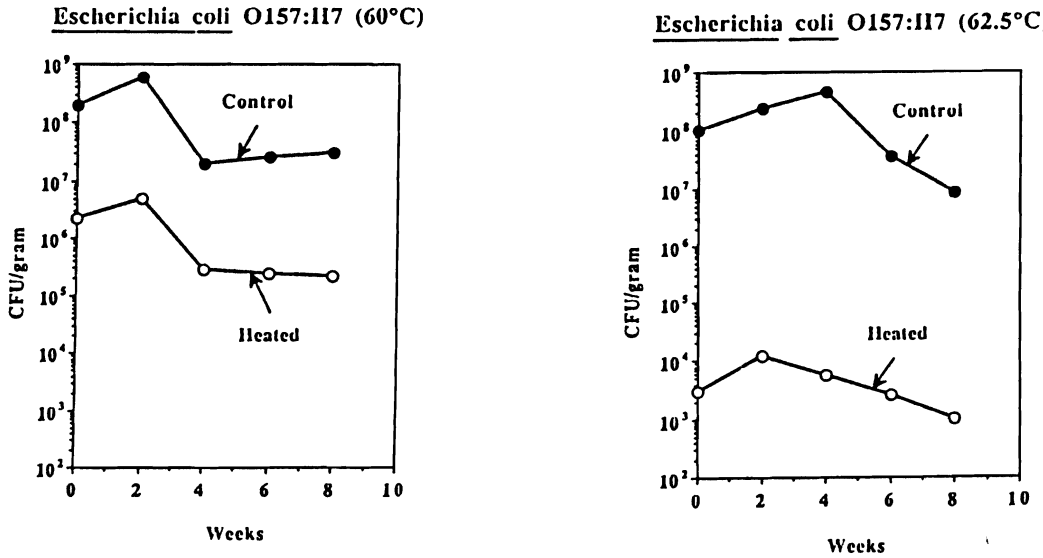


Figure 2. Effect of heat treatments on survival of pathogens in Cheddar cheese. Pathogen survival is presented as a function of curing time: Salmonella heidelberg at 60°C (left) and at 62.5°C (right).

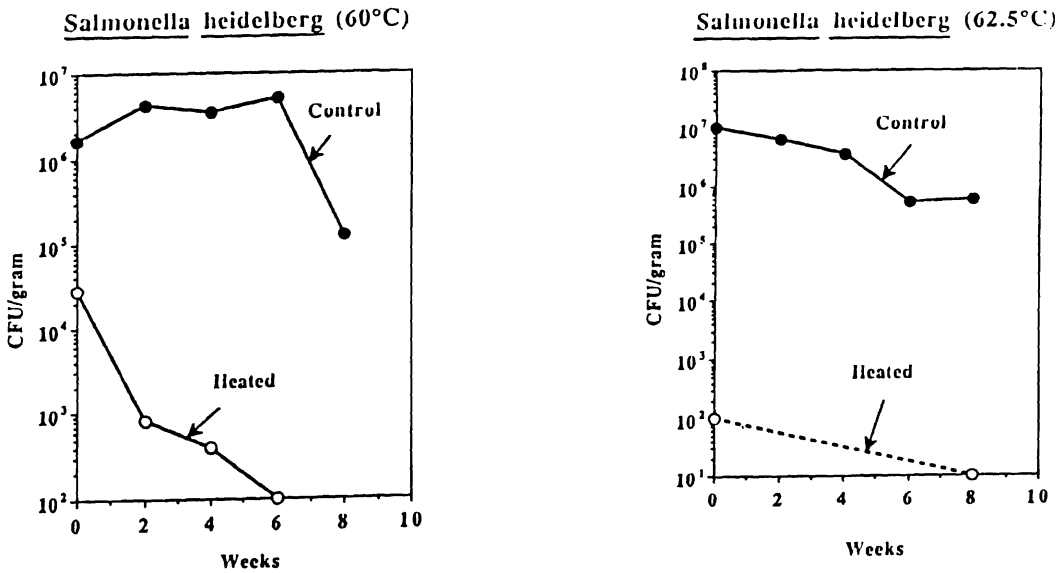
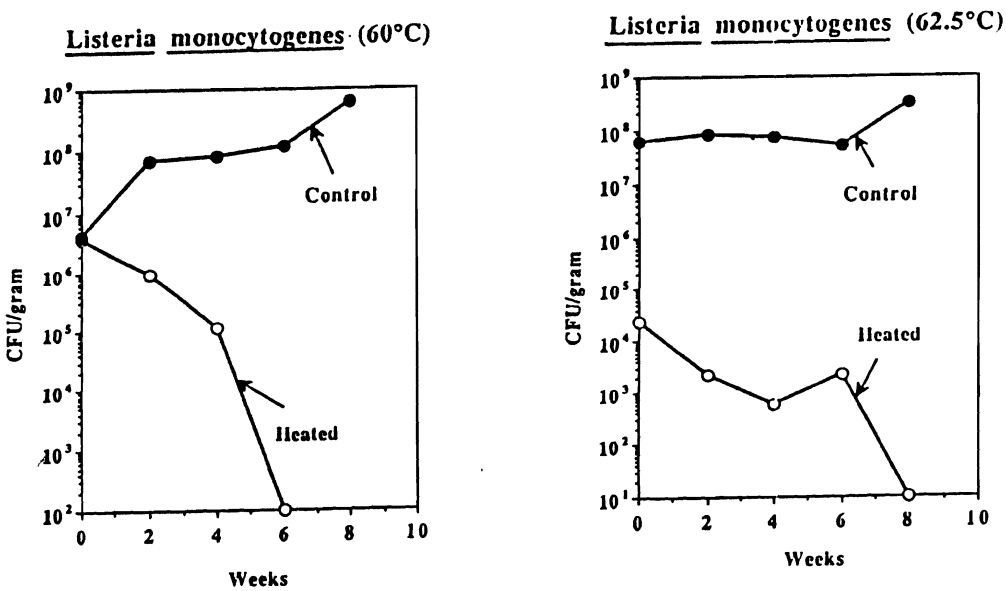


Figure 3. Effect of heat treatments on survival of pathogens in Cheddar cheese. Pathogen survival is presented as a function of curing time: Listeria monocytogenes at 60°C (left) and at 62.5°C (right)



Project Title:

Identification of Environmental Sources of *Listeria monocytogenes* in Dairy Product Manufacturing Plants and Development of HACCP Programs Designed to Prevent *Listeria* Contamination of Dairy Products

Personnel:

Eric A. Johnson, assistant professor, Food Research Institute (PI); John H. Nelson, senior research manager, Food Research Institute

Funding:

Wisconsin Milk Marketing Board

Funding Code:

IV - 65

Dates:

July 1, 1989 - June 30, 1991

Objectives:

1. Determine the status of New York state dairy plants with respect to *Listeria* contamination of dairy products and identify field conditions that promote contamination.

2. Identify other factors in the typical dairy plant environment outside the direct processing areas that may be reservoirs of *Listeria.*

3. Develop generic Hazard Analysis Critical Control Point (HACCP) methodologies to minimize product contamination with pathogenic bacteria.

4. Communicate useful information to the dairy industry on approaches for reducing the risk of dairy product contamination with pathogens.

5. Compare physiological and biochemical properties of *Listeria* isolates from dairy plants in order to determine bacteriological properties that contribute to the organism's ability to colonize dairy plants. Properties examined will include resistance to antibiotics and sanitizers, sensitivity to lysozyme, etc.

Summary of Findings:

Data has been compiled and analyzed for *Listeria* testing done on over 880 environmental samples from 62 dairy plants during 1987-88. Plant sampling was not statistically based, but was biased toward *Listeria* detection. Locations giving positive results were frequently resampled. The objective of these samplings carried out by industry was to determine the location of *Listeria* in the plant environment.

Listeria samplings for six different types of dairy plants were analyzed. The incidence of *Listeria* was 10% in fluid milk plants and 12% in frozen product plants. The incidence was lower in butter (5%), processed cheese (4%), natural cheese (3%), and dry product (1%) plants. Positive tests in which *Listeria* species were confirmed indicated that 439 of 566 isolates were *Listeria monocytogenes.*

The results show that the overall incidence of *Listeria* was 10% in the six types of dairy plants. Fluid milk and frozen product plants had a higher incidence of *Listeria* positives than the other four types of processing plants. *Listeria* was detected frequently in wet locations, including conveyors, floors, and drains. These results suggest that moist, difficultly-maintained locations are likely sites of *Listeria* contamination in dairy plants.

Significance to the Dairy Industry:

Wisconsin dairy plants will be provided with identification of Critical Control Points and HAACP procedures to help eliminate listeria contamination from Wisconsin dairy products.

Publications:

Nelson, J. H. 1990. Where are *Listeria* likely to be found in dairy plants? *Dairy and Food Sanitation* (accepted for publication).

Project title:

Generation and Roles of Proline in Providing Flavor and Pathogen Protection in Cheese

Personnel:

Eric A. Johnson, assistant professor, Food Research Institute; Greg Leyer, graduate student, Food Research Institute

Funding:

National Dairy Promotion and Research Board

Funding Code:

88-6

Dates:

July 1, 1989 - June 30, 1991

Objectives:

1. Determine the role of proline in protecting against osmotic stress in cheese in *Salmonella typhimurium*.

2. Determine the role of acid adaptation in providing pathogen protection in cheese.

3. Assay certain certain lactic acid bacteria for the presence of arginine deiminase enzymes.

Summary of Findings:

Role of proline in protecting against osmotic stress

It is well-known that in *Salmonella*, accumulation of proline can alleviate growth inhibition imposed by osmotic stress. Bacteria can accumulate proline to high intracellular concentrations by increased net synthesis or by enhanced uptake from the medium.

To understand the importance of proline in protecting against osmotic stress, we obtained a proline overproducing mutant of *Salmonella typhimurium* (TL 978) from Dr. Laszlo Csonka, Purdue University. To determine if the proline overproducing mutant has a competitive edge over wild-type *S. typhimurium* TL1, we prepared a synthetic medium (Medium 63) with various salt and proline concentrations. Cells were incubated at 37°C and growth was monitored by following absorbance at 660 nm. We observed that in most cases, strain TL 978 initiated growth more rapidly than the wild-type, but within a day or two, the wild-type reached a similar optical density (data not shown).

To better understand the role of proline overproduction against osmotic stress in a food system, Cheddar cheese was prepared with salmonella-inoculated milk. Salmonella was added at an initial concentration of 10^4 CFU/ml. After cheese preparation, the cheese was salted to a final concentration of 1.3% NaCl. The cheese had a high moisture content of 49%. The cheese was then incubated at an abusive temperature of 30°C, and samples were taken over a five-week period. We found that at the 1.3% NaCl and high moisture level, the wild-type and the proline overproducer grew equally well (Table 1).

We repeated this experiment with brined cheese to increase the salt content to 4.6%. Under these conditions, the cells died off over time at a fairly equal rate (Table 2). These results suggest that in cheese, overcoming salt concentration by proline overproduction is not the only factor necessary to allow pathogen growth and this area needs further study. In particular, we are finding evidence that adaptation to acid is important for pathogen survival in cheese.

Role of acid adaptation in protecting against acid stress

The ability of a pathogen to overcome acid stress in fermented milk products undoubtedly aids in its ability to survive and grow. We have examined the ability of *S. typhimurium* to become adapted at lower pHs, and have determined how this affects survival at lethal pHs. *S. typhimurium* was grown in a synthetic medium, Medium E (pH 7.6), to a cell number of approximately 1 x 10^8 CFU/ml. The pH was then adjusted to 5.8 with hydro-

Table 1. Growth of Salmonella in Cheese (1.3% NaCl)

Day	Strain	CFU/gm
0	TL1	5.4×10^5
	TL 978	9.2×10^4
3	TL1	5.9×10^6
	TL978	1.3×10^8
7	TL1	4.1×10^7
	TL978	6.2×10^7
21	TL1	7.0×10^7
	TL978	8.9×10^7
35	TL1	2.5×10^8
	TL978	8.3×10^7

Table 2. Death of Salmonella in NaCl Cheese (4.6% NaCl)

Day	Strain	CFU/gm
0	TL1	5.7×10^5
	TL978	2.2×10^5
4	TL1	7.2×10^3
	TL978	7.3×10^3
9	TL1	3.1×10^3
	TL978	1.3×10^3
16	TL1	2.65×10^2
	TL978	1.78×10^2
24	TL1	9.2×10^1
	TL978	6.4×10^2
38	TL1	<10
	TL978	<10

chloric acid, and the cells were grown for one doubling. An identical flask was prepared and cells grown to ~2×10^8 CFU/ml, and the pH was lowered to 3.45 (when challenged with HCl) or to 3.8 (when challenged with lactic, acetic, or propionic acid). Viable cells were enumerated after various time points by plating onto LB agar. The differences in survival between adapted and non-adapted cultures were dramatic (Figure 1). Future experiments using adapted cells in fermentation studies will enable us to determine how acid tolerance aids in pathogen survival.

Activity of ADI enzymes in *Lactobacillus* and *Pediococcus*

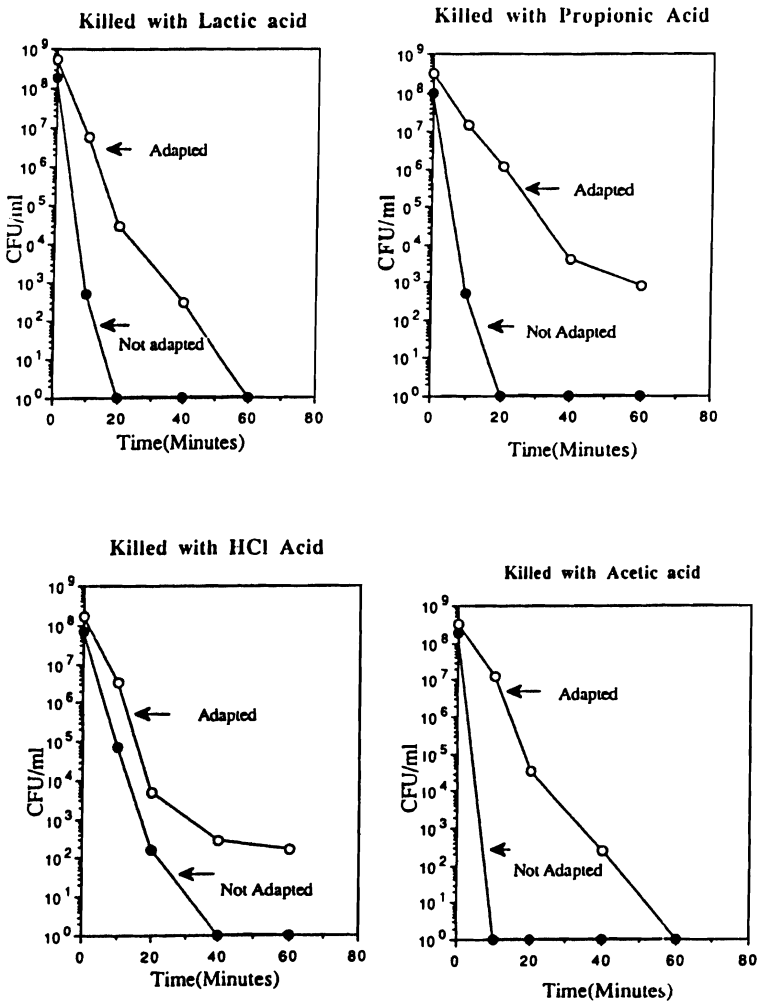
Proline can be synthesized from arginine through a series of reactions. Preliminary experiments were performed to determine if *Lactobacillus helveticus* CDR 101 and *Pediococcus pentosaceus* NCDO 559 contained arginine deiminase enzymes. The *Pediococcus* strain expressed arginine deiminase and ornithine transcarbamylase activity whereas the *Lactobacillus* strain did not. Further studies are necessary to determine how much proline is being produced and what impact it has on cheese flavor and safety.

Significance to the Dairy Industry:

The completion of this project will provide us with a better understanding of the factors that provide pathogen protection in cheese. Knowledge of proline and acid production in cheese could provide a predictive method for the safety of the cheese, and could eventually lead to improved safety.

See next page for Figure 1.

Figure 1. Survival of acid-adapted and non-adapted cultures of Salmonella typhimurium to various acids.



Project Title:

Milk, a Point of Entry into the Human Diet for Mevalionate-Suppressive Plant Secondary Metabolites

Personnel:

Charles E. Elson, professor, Dept. of Nutritional Sciences (PI); Naji Abuirmeileh, PhD; Wendy Altmann, graduate student, Dept. of Poultry Science; Suzanne Shoff, graduate student, Dept. of Nutritional Sciences

Funding:

Wisconsin Milk Marketing Board

Funding Code:

IV-61

Dates:

January 1, 1989 - December 31, 1990

Objectives:

1. Isolate and identify constituents of alfalfa which suppress the synthesis of mevalonic acid (MVA).

2. Confirm that the MVA-suppressive constituents are passed into milk.

Summary of Findings:

Use of Alfalfa to Lower Blood Cholesterol

The addition of alfalfa (50,000 ppm) to the cholesterol-free diet of chicks effected a 15% lowering of blood cholesterol and a 60% suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity. HMGR catalyzes the synthesis of MVA, the rate-limiting step in the synthesis of cholesterol and in the synthesis of products essential for the growth of tumor cells.

Alfalfa Fractionation Results

The constituents of alfalfa have been fractionated according to their solubilities in organic solvents. The fractionation consists of the sequential extraction of alfalfa with petroleum ether (PE solubles) and methyl alcohol (ME solubles). The ME solubles were fractionated by chromatography on silica gel G plates with a mobile phase consisting of diethyl ether: petroleum ether: acetic acid (50:50:1). Band 5, the leading band, contained MVA-suppressive components. Constituents of this band were isolated by high performance liquid chromatography on a C-18 column with methyl alcohol as the mobile phase. Fraction 5 collected from the column contained the MVA-suppressive component.

Mass Spectral Analysis

Mass spectral analysis indicates a constituent with a molecular ion peak at m/e 246+. The PE solubles were fractionated into seven bands by thin layer chromatography on silica gel G with a mobile phase of petroleum ether: diethyl ether: acetic acid (95:5:0.5). MVA-suppressive activity was limited to band 1. The constituents of band 1 were subfractionated by high performance liquid chromatography on a C-18 column with acetonitrile as the mobile phase. Eight fractions were isolated; the major MVA-suppressive activity was associated with HPLC fraction 2.

Mass spectral analysis of the HPLC fraction 2 constituents showed one with a molecular ion peak at m/e 246+ (C-17, H-26, O-1), tentatively 2,4,6,6-tetramethyl-1-cyclohexen-1-yl-3,5-dipenten-4,6-dimethyl-2-one, a second with a molecular ion peak at m/e 232+ (C-16, H-24, O-1), tentatively 2,6,6,-trimethyl-1-cyclohexen-1-yl-3,5-dipenten-4,6-dimethyl-2-one and a third with a molecular ion peak at m/e 192+ (C-13, H-20, O-1), tentatively 2,6,6-trimethyl-1-cyclohexen-1-yl-3-buten-2-one (beta-ionone).

Use of B-Ionone and Geranoil to Lower Total Cholesterol

Commercial B-ionone co-elutes with HPLC fraction 2. Commercial B-ionone elicited a concentration dependent suppression of MVA biosynthesis. The in vivo impact of dietary B-ionone on MVA and cholesterol metabolism has been assessed in two studies. In the first, four-week-old pullets were fed a diet containing 250 ppm b-ionone or 250 ppm geraniol, a second isoprenoid constituent of alfalfa, for four weeks (Table 1).

B-ionone elicited a 33% decrease in total cholesterol and a 20% decrease in HDL cholesterol, changes consistent with the 3-fold lower HMGR activity. Similar changes were noted when geraniol was fed. A second trial provided evidence of the mechanism through which b-ionone exerts its cholesterol-lowering action. In this trial, B-ionone and geraniol were fed (100 ppm) for four weeks to two-week-old cockerels.

Addition of Pentobarbital

In parallel groups, pentobarbital (20 ppm) was fed to induce a microsomal P450 monooxygenase that degrades geraniol and B-ionone.

The cockerels were fasted for two days and refed for three days prior to sacrifice and tissue collection in order to maximize HMGR activity. During the refeeding phase, mevinolin (500 ppm) was added to the diets of B-ionone and control subsets of birds. Mevinolin, a competitive inhibitor of HMGR, induces the synthesis of the enzyme. The results of these trials are shown on Table 2.

Consistent with the results of the first test, birds fed 100 ppm B-ionone or geraniol had lower total cholesterol values. The data suggest a dose-dependent action since at the level of 250 ppm, both B-ionone and geraniol depressed HDL cholesterol, as well as total cholesterol. The data suggest a dose-dependent action since at the level of 250 ppm, both ionone and geraniol depressed HDL cholesterol, as well as total cholesterol. Both substances blocked the induction of HMGR activity. Pentobarbital had no impact on the sterol metabolism of control birds.

Induction of the P450 monooxygenase totally voided the effect of the two isoprenoids. The final findings of substance were that mevinolin-induced HMGR activity to a level 50% greater that effected by fasting/refeeding

Table 1. Impact of 250 ppm B-Ionone and Geraniol on Lipid Metabolism of 8-week-old Pullets.

Treatment	Cholesterol total	HDL	HMGR pmol/min/mg protein
Control	120+/-7	70+/-3	468+/-41
B-ionone	80+/-2	56+/12	110+/-19
Geraniol	70+/-4·	54+/-3	252+/-50

and to a level 300% greater than that recorded for birds not subjected to fasting/refeeding. B-ionone attenuated this adaptive response. Our interpretation of these findings is that B-ionone and geraniol bring into play the regulatory action that suppresses HMGR activity at the level of translation.

Significance to the Dairy Industry:

These results demonstrate that isoprenoid constituents of alfalfa suppress cholesterol synthesis. The cholesterol-suppressive constituents identified in the PE solubles of alfalfa are related to the carotenoids. We anticipate that during the final six months of this project we will be able to identify MVA-suppressive plant metabolites in butterfat.

Presentations:

Abuirmeileh, N. and C. E. Elson, Jordon University of Science and Technology, Irbid and the Department of Food Sciences, Madison, WI. "The Potentiation of the Mevalonate-Reversible Isoprenoid-Mediated Suppression of H. Halobium Growth by Pentobarbital." Federation of American Societies for Experimental Biology. Washington, DC. April 1-5, 1990 Poster 3835 (FASEB J 4:A927, 1990).

Table 2. Impact of 100 ppm B-Ionone and Geraniol of Sterol Metabolism of 6-week-old Cockerels.

Treatment	Cholesterol total	HDL	HMGR pmol/min/mg protein
Control	133+/-4	72+/-9	1064+/-130
B-ionone	113+/-3	80+/-9	562+/-150
Geraniol	125+/-3	75+/-6	838+/-90
Pentobarbital	133+/-7	73+/-9	1029+/-120
B-ionone + Pentobarbital	130+/-8	77+/-5	912+/-230
Geraniol + Pentobarbital	135+/-3	72+/-3	1016+/-250
Control + Mevinolin	138+/-3	68+/-3	1519+/-130
B-ionone + Mevinolin	122+/-0	77+/-6	1100+/-60

Project Title:

Relationship between Butterfat Short-Chain Fatty Acids and Lipoprotein Metabolism in the Rat

Personnel:

Denise M. Ney, assistant professor, Dept. of Nutritional Sciences (PI); John B. Lasekan (PhD), research associate, Dept. of Nutritional Sciences; Hui-Chuan Lai, graduate student, Dept. of Nutritional Sciences

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-39

Dates:

May 1, 1988 - June 30, 1990

Hypothesis and Objectives:

Hypothesis:

We hypothesize that butterfat differs from other dietary saturated fats, such as beef tallow, coconut oil, and palm oil, in its effects on VLDL composition and subsequent HDL metabolism. Differences in lipoprotein metabolism with butterfat ingestion may be due to the fact that 10% of the fatty acids in butterfat comprise short- and medium-chain length fatty acids (less than 12 carbon atoms) which differ in metabolism from longer chain fatty acids.

Objectives:

1. To compare the response of rats to the ingestion of diets with 16% fat and 0.035% cholesterol (w/w). Five dietary fats (butterfat, beef tallow, coconut, palm, or corn oil) will be compared to determine:

a. the length of feeding period necessary to observe differences in plasma cholesterol, triglyceride, and apolipoprotein levels;

b. if the experimental variation for laboratory measurements differs among the dietary groups;

c. if differences in food intake exist.

2. To determine if feeding rats butterfat, beef tallow, coconut oil, palm oil, or corn oil based diets has different effects on:

a. hepatic and plasma cholesterol and triglyceride content;

b. lipid and protein composition of VLDL, LDL and HDL;

c. HDL apolipoprotein distribution;

d. lipid digestibility.

3. To determine if alterations in the quantity and composition of VLDL with butterfat feeding (objective #1) are associated with differences in:

a. in vivo plasma triglyceride secretion or turnover using 2-^{3}H glycerol;

b. the chemical composition and relative size of VLDL;

c. post-heparin plasma lipoprotein lipase activity.

Summary of Findings:

Our objective was to compare the lipogenic effects of milkfat (MF) vs. other dietary saturated fats. Male, Sprague Dawley rats were fed diets containing 2% corn oil (CO) + 14% MF, beef tallow (BT), palm oil (PO), or coconut oil (CN) vs. a 16% CO control diet for 5 or 6 weeks. Food intakes and weight gains were similar; apparent lipid digestibility was lower ($p<0.05$) with PO and BT (87 ± 0.4%) vs. CO, MF, and CN (94 ± 0.3%). Total plasma cholesterol level was lower with MF than PO feeding, $p<0.05$; plasma cholesterol levels were not significantly different among the MF, CN, BT, and CO groups.

Non-denaturing gradient gel electrophoresis immunoblot analysis indicated that HDL apo A-I and apo E resided on particles with significantly smaller model diameters for saturated fats vs. CO. Fasting triglyceride (TG) levels in plasma, VLDL, and HDL were significantly higher with BT, PO, and CN than CO; TG levels were not different with MF vs CO. In vivo hepatic TG secretion was determined by compartmental analysis of the changes in plasma TG specific radioactivity (dpm/mg TG) after injection of 2-^{3}H-glycerol. The rate of hepatic TG secretion (mg/min/100 g body wt) was not different in rats fed MF or CN vs. CO; whereas BT was associated with a higher rate of hepatic TG secretion than CO feeding, $p<0.05$. These data suggest that ingestion of saturated dietary fats altered HDL and TG metabolism, relative to CO; but, MF and BT demonstrated unique effects on VLDL composition. Results also suggest that higher plasma triglyceride levels in response to feeding saturated fats versus corn oil can be explained, in part, by an increased flux of hepatic triglyceride secretion.

Significance to the Dairy Industry:

This research is important to the dairy industry because it will provide needed information regarding the effects of milkfat/butterfat ingestion on lipid metabolism. This information will help in the marketing of dairy products as consumers continue to request information regarding the health implications of specific foods. These data will also provide insight regarding which portion of butterfat has a negative impact on blood cholesterol levels. This information could then be used as a basis for altering the fatty acid composition of butterfat for individuals with elevated plasma cholesterol levels.

Publications and Theses:

Lai, Hui-Chuan (1990). Plasma Lipoprotein Composition and Hepatic Triglyceride Secretion in Response to Ingestion of Dietary Saturated Fats in the Rat. M.S. Thesis. UW-Madison.

Abstracts:

Lai, H.-C., D.M. Ney, J.B. Lasekan, and M. Lefevre, Effects of different dietary saturated fats on lipoprotein composition in rats. *FASEB J.* 3:4230, 1989.

Lai, H.-C., D.M. Ney, J.B. Lasekan, and M.K. Clayton, In vivo determination of triglyceride secretion in rats fed different dietary saturated fats using [2-^{3}H-glycerol]. *FASEB J.* 4:2297, 1990.

Ney, D.M., H.-C. Lai, M. Lefevre, and J.B. Lasekan, Relative effects of milkfat on lipoprotein composition and in vivo hepatic triglyceride secretion in rats. *INFORM* 1:222-223, 1990.

Ney, D. M. Potential for enhancing the nutritional properties of milkfat. *J. Dairy Science* 73 (suppl) :98, 1990.

Manuscripts based on the above abstracts are in preparation.

Presentations:

Title/Function	Location	Date
Served on Expert Panel "Cholesterol Reduction: Insights into Emerging Technologies"	Wisconsin Milk Marketing Board Industry Forum; Madison, WI	10-25-89
Presented a lecture entitled "Effects of Milkfat on Human Lipid Metabolism"	Wisconsin Dairy Technology Society; Milwaukee, WI	1-16-90

continued on next page

Title/Function	Location	Date
Presented a lecture entitled "Potential for Enhancing the Nutritional Properties of Milkfat" as part of a symposium.	American Dairy Science Association Annual Meeting; Raleigh, NC	6-26-90
Presented a lecture entitled "Milkfat - Nutritional Issues of the 1990s"	Wisconsin Dairy Products Assoc. Annual Meeting; Madison, WI	8-21-90
Participated in a symposium on milk lipids by presenting a poster entitled "Relative Effects of Milkfat on Lipoprotein Composition and In Vivo Hepatic Triglyceride Secretion in Rats"	American Oil Chemists' Society Annual Meeting; Baltimore, MD	4-24-90
Presented a lecture entitled "Effects of Milkfat on Human Lipid Metabolism."	Dean Foods Annual Research Meeting, Eagle Ridge, IL	9-20-90

Project Title:

Texture Development in Cheese Made from Ultrafiltered Milk

Personnel:

Damodaran Srinivasan, associate professor, Dept. of Food Science; Peggy Clark, graduate student, Dept. of Food Science

Funding:

Wisconsin Milk Marketing Board

Funding Code:

III-12

Dates:

September 1, 1986 - August 31, 1989

Objectives:

1. To elucidate the fundamental causes for poor texture development in cheese made from ultrafiltered milk.

2. To develop practical methods to improve texture development of hard cheeses made from ultrafiltered milk.

Summary of Findings:

The main objective of this study was to elucidate the fundamental reasons for poor texture development in cheeses made from ultrafiltered (UF) milk. It was hypothesized that compositional changes in UF milk, higher retention of whey proteins in the cheese curd of UF milk, and insufficient proteolysis of k-casein might be responsible for the poor textural properties of hard cheeses made from UF milk. It was found that a significantly higher amount of ß-lactoglobulin was retained in UF curd than in regular milk curd.

Variations in the concentrations of citrate and calcium affected the rate of coagulation and curd formation of UF milk. However, neither the changes in the mineral composition nor the rate of setting of the curd affected the final structure of the UF curd as judged from the scanning electron microscopic analysis. It was concluded that the texture defects in UF cheese were most probably related to the inability of the para-casein micelles to fuse together to form a network structure. This may be due either to the presence of excess amount of whey proteins or the insufficient proteolysis of k-caseins by rennin.

1. Effect of Ultrafiltration on the State of Equilibrium of Milk.

Ultrafiltration of milk at 50°C for an extended period of time might cause alterations in the state of equilibrium of various components between the serum and micellar phases. Such changes may affect the aggregation properties of the casein micelles. To elucidate whether there is a change in the equilibrium state of casein micelles during ultrafiltration and what effect such changes have on their aggregation properties, the following studies were conducted.

Pasteurized skimmilk was ultrafiltered to 5-fold concentration using E-500 membrane (Desalination Systems, Inc., CA) in a laboratory scale ultrafiltration unit (Tri-Clover, Kenosha, WI). Ultrafiltration was done at 25°, 40°, 50° and 55°C. After ultrafiltration the 5-fold concentrated sample was diluted to 50-fold (i.e., one-tenth of single strength) with the permeate. The temperature of the sample was immediately lowered to 30°C and incubated in a water bath at that temperature. Aliquots of the sample were taken at regular time intervals and the kinetics of rennet-induced aggregation of the casein micelles was studied using an aggregometer. The changes in the rate of aggregation as a function of incubation time were determined.

In a control experiment, pasteurized milk was stored at 4°C. After 24 hours, the milk was diluted 10-fold (one-tenth of single strength milk) using the permeate from the ultrafiltration experiment. The temperature was immediately raised to 30°C and incubated at that temperature. Aliquots were withdrawn at

regular time intervals and the rennet-induced aggregation of the casein micelles were studied. As before, the changes in the rate of aggregation as a function of incubation time were determined. The rationale for these studies is as follows: If ultrafiltration of milk caused unusual changes in the state of equilibrium of the milk components, one could observe these changes from the time-dependent relaxation to the equilibrium state upon reconstitution of the milk to the original conditions.

Results of these studies are shown in Figure 1. In the control (i.e, the sample whose temperature was increased from 4° to 30°C and incubated at that temperature), the rate of rennet-induced aggregation of casein micelles increased with incubation time. This indicated that upon changing the temperature from 4° to 30°C, the milk system undergoes a slow change in the distribution of components between the serum and micellar phases, and reaches a new equilibrium state after about 3 to 4 hours. During this slow equilibration process, the changes that occur in the physico-chemical properties of casein micelles promote their enzymatic aggregation/coagulation properties. It is known that pre-cooling of milk before renneting increases the coagulation time. However, incubation or aging of pre-cooled milk at renneting temperature (i.e, 30°C) has been shown to restore its coagulation properties to that of unchilled milk. The results shown here, in fact, support those observations and provide kinetic evidence for those observations.

The time dependent aggregation behavior of casein micelles in ultrafiltered milks is also shown in Figure 1. In contrast to the behavior of the control, the rate of aggregation of the micelles of ultrafiltered milk decreased with incubation time at renneting temperature. This indicates that the casein micelles of the ultrafiltered milk also undergo a slow equilibration process. However, this equilibration process is different from that of the control. In other words, ultrafiltration of milk at higher temperatures alters the chemical equilibrium of the milk system, but the changes in the distribution of components during ultrafiltration are different from those at low temperatures, i.e., in opposite direction. When aged at the renneting temperature, the casein micelles of ultrafiltered milk undergo a re-equilibration process which decreases their renneting properties; after 3 hours, the renneting properties of the ultrafiltered milk seem to approach that of the regular milk.

Since the rate of aggregation and coagulation of casein micelles has a pronounced effect on curd structure and subsequent texture development, an understanding of the factors affecting the coagulation rate of casein micelles in ultrafiltered milk could provide ways to manipulate cheese texture.

2. Effects of Changes in Milk Composition on its Coagulation Properties.

Several studies have indicated that substantial changes in the composition of milk occur during ultrafiltration. These include changes in the levels of calcium, phosphate, and citrate. To examine whether adding citrate or calcium affects the rate of aggregation /coagulation of micelles, the effects of these ions on the rennet-induced coagulation of k-casein were studied in a model system. The essence of these studies is shown in Figure 2. At lower concentrations, citrate decreased the clotting time; however, above 7mM (ionic strength = 0.035), the rate of rennet-induced coagulation of k-casein decreased and the clotting time increased dramatically. When 10mM calcium chloride was included in the reaction medium, the rate of coagulation decreased and the clotting time increased with citrate concentration. However, in both cases, addition of 40mM sodium citrate increased the clotting time by about 2 (in the absence of $CaCl_2$) to 10-fold (in the presence of 10mM $CaCl_2$). Although these results were obtained with pure k-casein, the data suggest that it is possible to manipulate the aggregation/coagulation properties of casein micelles of ultrafiltered milk by adding sodium citrate.

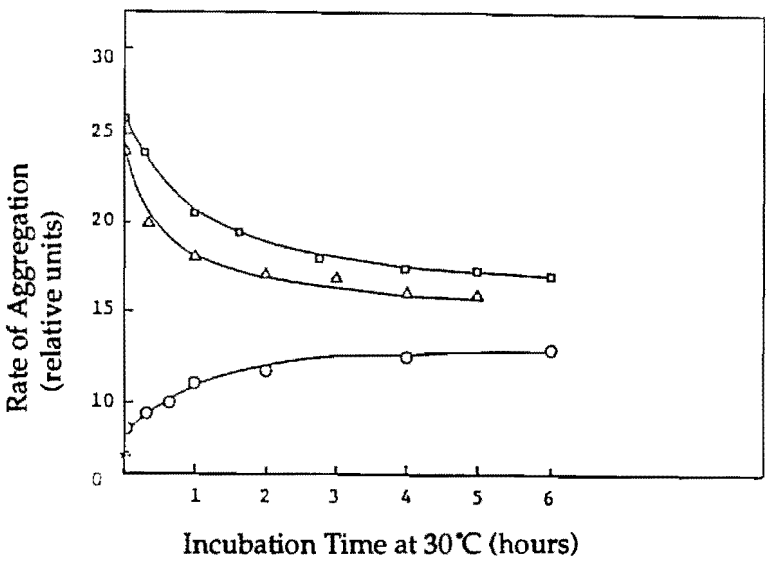
A reliable, nondestructive methodology was developed to study the effects of various milk constituents on the kinetics of coagulation of UF milk. The method involves the use of an

Instron Universal Testing Instrument with a 500g tension load cell. The probe was made of 1.5mm diameter stainless steel rod, about 15cm long with a 6cm crosspiece welded horizontally at the bottom of the vertical rod. To measure the kinetics of coagulation, a probe is lowered to the bottom of the sample; after adding rennet to the milk, the probe is pulled upward at a speed of 0.02 inch/min. The typical force versus time curve obtained by this method is shown in Figure 3. The initial slope of the curve in Figure 3 was used as a parameter to compare the effects of various experimental parameters on the rennet induced coagulation of ultrafiltered milk.

The above methodology was used to study the effects of changes in the mineral composition on the coagulation properties of UF milk. The important observations of these studies are summarized in Figures 4 and 5. Figure 4 shows the effects of calcium chloride, sodium chloride, and sodium citrate on the initial rate of coagulation of IX milk which had been heated at 50°C for one hour and cooled to 30°C. The initial rate of coagulation increased with calcium chloride concentration up to about 10mM and remained unaffected above 10mM. On the other hand, addition of sodium chloride caused little change in the initial rate of coagulation up to about 15mM while causing a slight decrease above 15mM. However, addition of sodium citrate caused a dramatic decrease in the rate of coagulation of heated IX milk; no coagulation of the IX milk was observed above 7mM sodium citrate. These results indicate that at low concentrations, i.e., at 0 to 15mM, calcium chloride, sodium citrate, and sodium chloride have positive, negative, and indifferent effects, respectively, on the coagulation of IX milk. These effects are mainly due to ion specific effects, rather than ionic strength effects, of these salts on the properties of casein micelles.

In contrast to the behavior of IX milk, the effects of the above three salts on the coagulation behavior of 4X milk are shown in Figure 5. Addition of calcium chloride apparently had no effect on the initial rate of coagulation of 4X milk, although a slight increase at 10mM and a decreasing trend above 10mM was observed. On the other hand, sodium chloride slightly increased the rate of coagulation up to about 40mM and leveled off above 40mM. In contrast to these two salts, sodium citrate exhibited a dramatic negative affect on the rate of coagulation; above 50mM, sodium citrate totally inhibited the coagulation of 4X milk. It should be noted that in regular IX milk this

Figure 1. Effect of aging at 30° C on the aggregation properties of casein micelles of ultrafiltered milk. Key: O—O, Sample was stored at 4° C and then incubated at 30° C (control). , Milk was ultrafiltered at 50° C and then incubated at 30° C. Δ—Δ, Milk was ultrafiltered at 55° C and then incubated at 30° C.



point of no coagulation was reached at 7mM sodium citrate (Figure 4).

The higher citrate concentration needed to inhibit coagulation of 4X milk might be related to the higher protein concentration, as well as higher calcium concentration. The apparent lack of influence of calcium on the rate of coagulation of 4X milk could be due to the fact that the level of free calcium in the 4X milk might be above the saturation level; under these conditions, addition of more calcium might not cause further change in the rate of coagulation. However, the rate of coagulation of 4X milk is highly dependent on the citrate concentration. This observation has some potential in terms of practical applications. It has been hypothesized by many investigators that the coarse structure of cheese curd made from concentrated milks might be due to the higher rate of coagulation, which traps the casein micelles in larger agglomerates and prevents formation of a uniform gel network in the curd. This implies that by controlling the rate of coagulation, one should be able to modify the curd structure and thus the textural properties of the cheese curd. This can be accomplished by adding sodium citrate to the concentrated milk in the concentration range 0 to 50mM.

The effect of rennet concentration on the rate of coagulation of 4X milk in the presence of 0, 10, and 30mM sodium citrate was studied. At any given rennet concentration, an increase in citrate concentration increased the lag time and decreased the rate of coagulation (Figure 6). These results indicated that the rate of setting of the 4X milk curd could be manipulated by varying both rennet and citrate concentrations.

The amount of ß-lactoglobulin entrapped in the curd of 4X milk was determined by SDS-PAGE. It was found that a significantly higher amount of ß-lactoglobulin was entrapped in the 4X milk curd than in the 1X milk curd.

3. Scanning Electron Microscopy of 4X Milk Curds.

In order to characterize the structure of milk curds obtained under various conditions of coagulation, scanning electron microscopy of this section of curds was performed. The milk curds were prepared under the following conditions:

1. 1X milk coagulated with 0.04% rennet,
2. 4X milk coagulated with 0.04% rennet, and
3. 4X milk + 30mM sodium citrate coagulated with 0.04% rennet.

Figure 2. Effect of citrate on the rennet induced clotting of k-casein in the presence and absence of 10 mM $CaCl_2$. O—O, in the absence of $CaCl_2$; Δ—Δ, in the presence of $CaCl_2$.

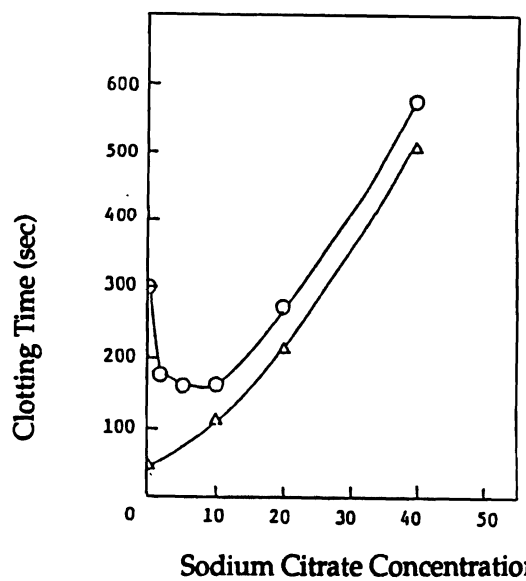
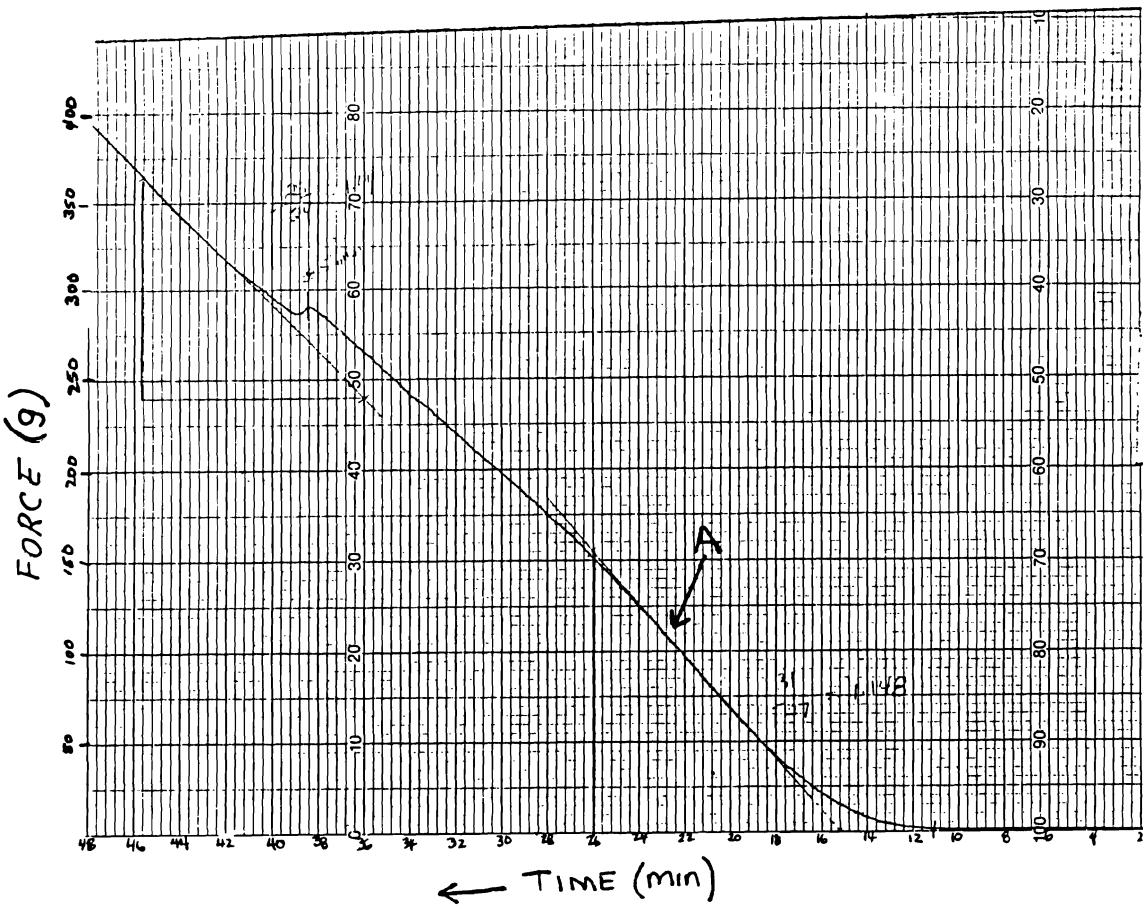


Figure 3. A typical Force-Time curve obtained during rennet-induced coagulation of 4X milk using the Instron Universal Testing Instrument. "A" is the initial slope.



The structures of these curds at two different magnifications are shown in Figure 7. While the curds prepared from 1X milk had a fibrous network of large, fused casein micelle agglomerates, the curd from 4X milk (both in the presence and absence of citrate) contained small globular-shaped particles uniformly distributed in the network. It appears that the casein micelles in the 4X curd were left intact in the monomeric state, probably because of insufficient cleavage of k-casein by rennet, although the rennet concentration used in these experiments was higher than that used in commercial operations. On the other hand, the fusion of para-casein micelles into agglomerates in the case of 1X milk curd could have been due to sufficient cleavage of the k-casein on these micelles. It is also evident from these results that decreasing the rate of coagulation of 4X milk by the addition of 30mM sodium

Figure 4. Effects of salts on the coagulation rate of 1X milk at pH 6.0 in terms of mM concentration. ■—■ *, NaCl;* ◆—◆ *, $CaCl_2$;* ⊡—⊡ *, Na Citrate.*

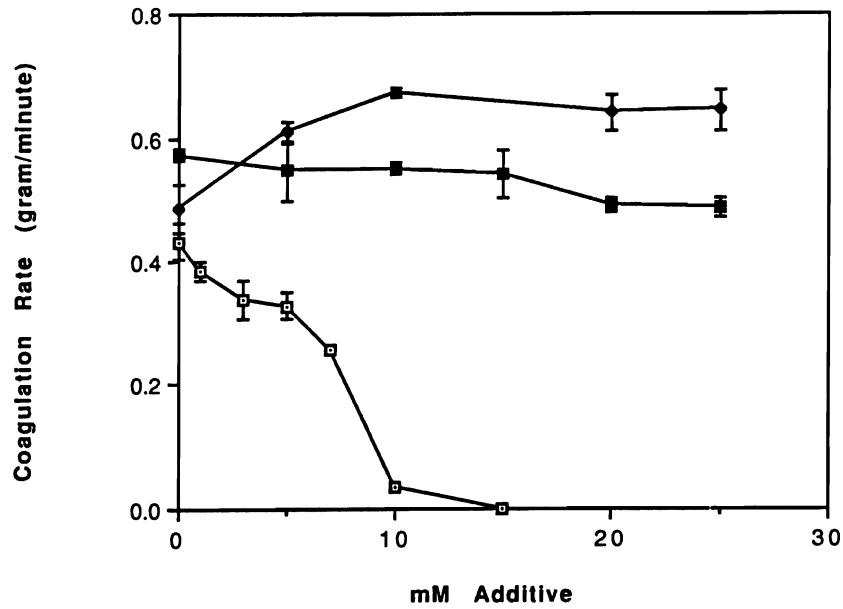


Figure 5. Effect of salts on the coagulation rate of 4X milk at pH 6.0 in terms of mM concentration. ◆—◆ *, $CaCl_2$;* ⊡—⊡ *, Na Citrate;* ■—■ *, NaCl.*

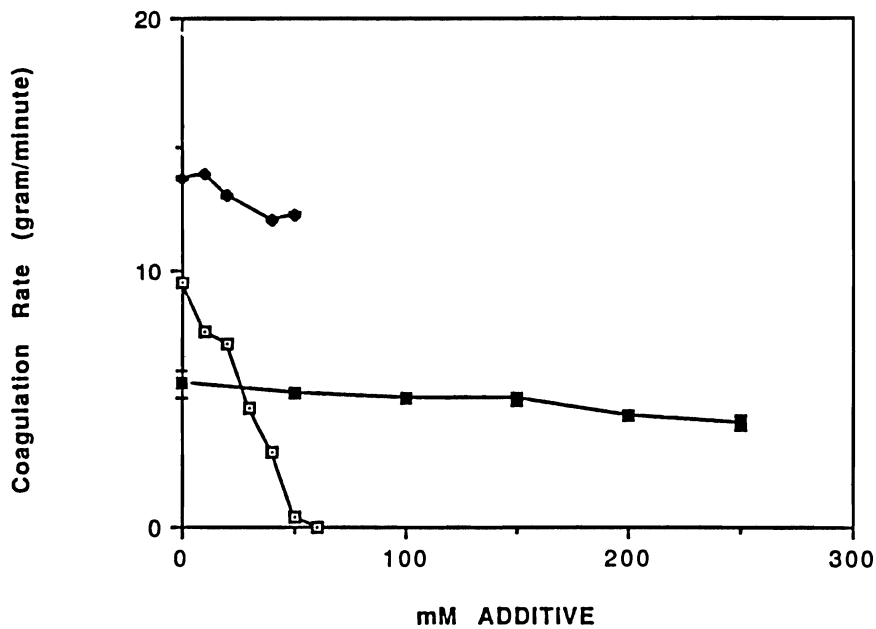
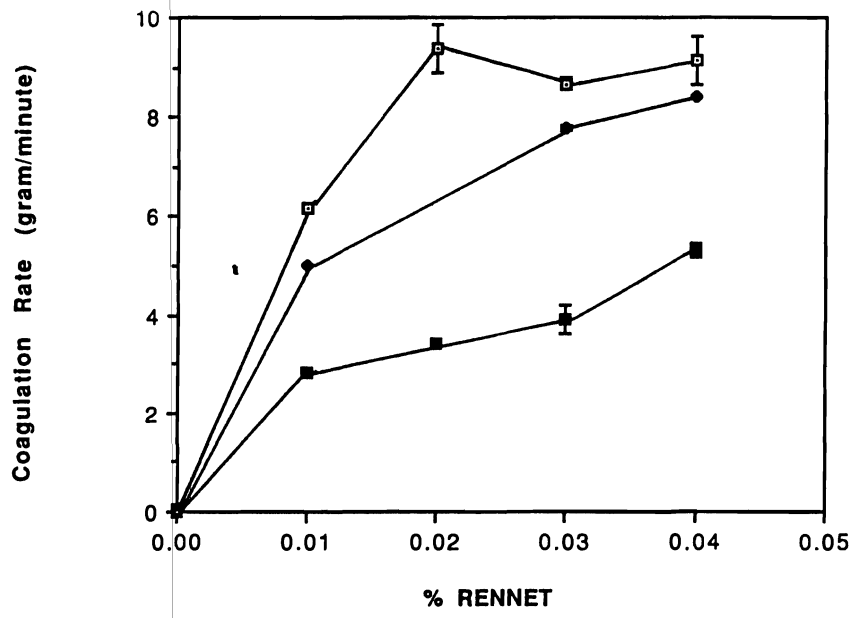
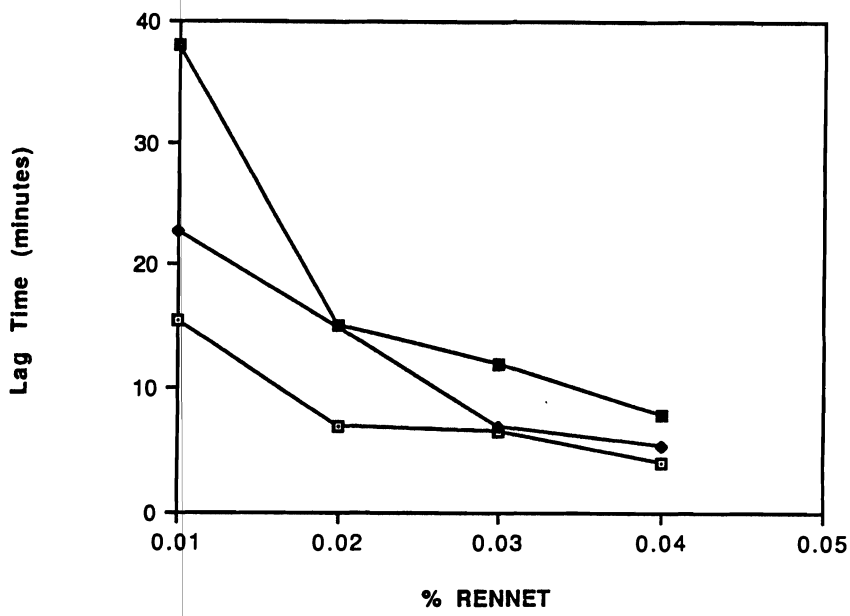


Figure 6. The effect of rennet concentration on the rate of coagulation (A) and lag time (B) of 4X milk in the presence of sodium citrate. ⊡—⊡, 0mM citrate; ◆—◆, 10mM citrate; ■—■, 30 mM citrate.

A: COAGULATION RATE: 10mM/30mM Citrate vs. % Rennet



B: LAG TIME: 10mM/30mM Citrate vs. % Rennet



citrate does not seem to improve the structure of the curd. This could tentatively be interpreted to mean that the rate of setting of curd may not be a critical factor in the development of network structures in the curd.

To determine whether partial destabilization of casein micelles by heat treatment of the 4X milk at 80°C prior to rennet addition would improve the structure formation, the 4X milk was heated at 80°C for 5 minutes in the presence or absence of 40mM sodium citrate, then cooled to 30°C and rennetted. The structure of the curd made from 4X milk heated at 80°C in the absence of citrate was very similar to the one with no heat treatment, indicating that the heat treatment was ineffective in improving the structure formation in 4X curd. On the other hand, the SEM structure of 4X milk heated in the presence of 40mM citrate did show some fusion of the casein micelle. However, when Cheddar cheese samples were prepared under all these conditions, all the cheese samples were crumbly and brittle.

Significance to the Dairy Industry:

For cheesemakers contemplating the use of ultrafiltered milk, this study provides information on the potential effects of independent variables.

Publications:

Clark, P.S. and Damodaran Srinivasan. (1989) Effects of salts on coagulation and the structure of curd made from ultrafiltered milk. American Dairy Science Association Annual Meeting, *J. Dairy Sci.* 72, Supplement 1, p. 136, Abstract # 334G.

A Masters thesis is under preparation.

Figure 7A. 1X milk, 0.04% rennet, 0 mM Na Citrate, no heat treatment.

Figure 7B. 4X milk, 0.04% rennet, 0 mM Na Citrate, no heat treatment.

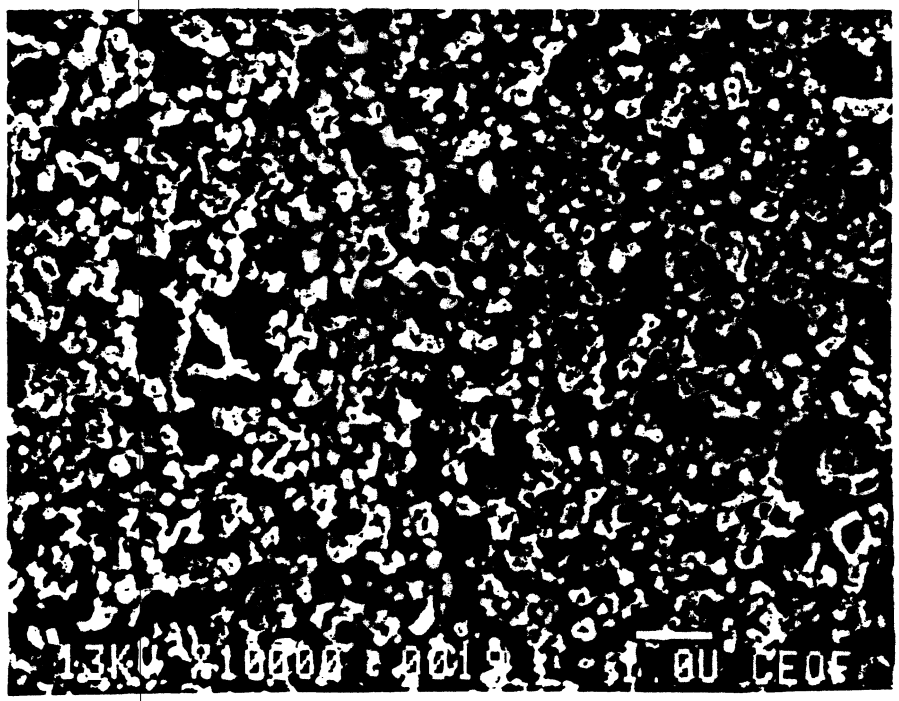
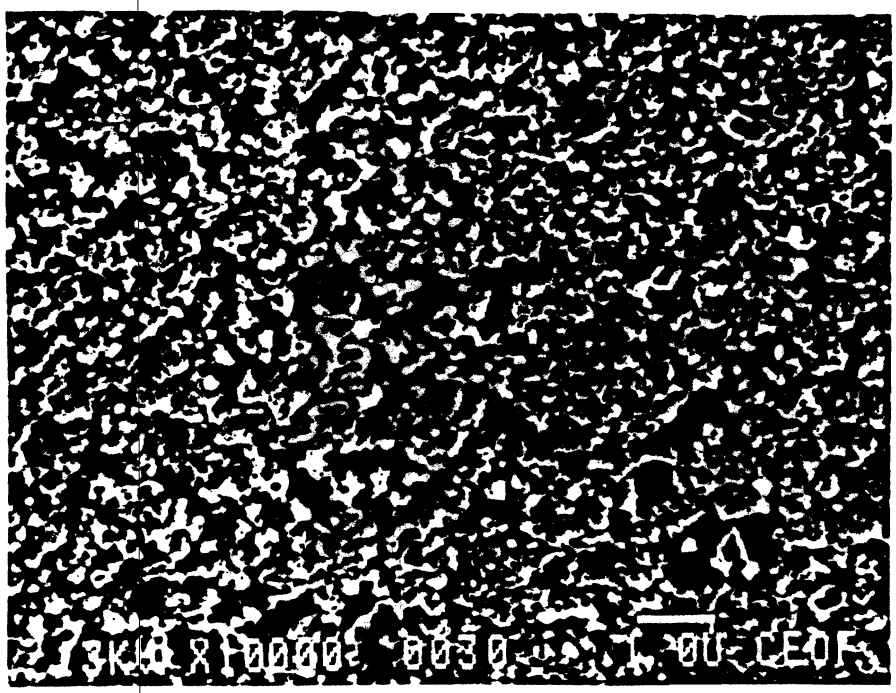


Figure 7C. 4X milk, 0.04% rennet, 30 mM Na Citrate, no heat treatment.



Project Title:

Development of Basic Technology for Improving the Flavor and Consumer Acceptability of Reduced-Sodium Cheddar Cheese

Personnel:

Robert C. Lindsay, professor, Dept. of Food Science (co-PI); E.A. Johnson, assistant professor, Food Research Institute (co-PI); Stephen L. Taylor, University of Nebraska (co-PI); Brian Guthrie, graduate student, Dept. of Food Science; Shane McDonald, graduate student, Dept. of Food Science

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-41

Dates:

July 1, 1988 - June 30, 1991

Objectives:

1. To investigate the flavor chemistry and microbiology of the production of unclean, Strecker-type flavor compounds in low-salt Cheddar cheese made from conventional, UF- and RO-retentate supplemented milk.

2. To develop methods to analytically measure the ability of starter and cheese ripening lactic acid bacteria to produce unclean, Strecker-type flavor compounds, and to screen various lactic culture and wild isolates for this metabolic activity.

3. To investigate the potential for growth of food pathogens that are capable of causing histamine or botulinal toxigenesis in low-salt Cheddar cheese, and characterize the behavior of these organisms in low-salt Cheddar cheese.

Summary of Findings:

Producing Unclean Flavors via Media and Model Systems

Under objectives 1 and 2, several media and model systems were prepared and evaluated for use in screening bacteria for the ability to produce unclean flavors. The media combined elements of methods employed for dairy starter culture propagation, accelerated cheese slurry ripening, and minimal nutrient media compositions.

Minimal media employed 0.5 mM succinic acid adjusted to pH 5.5 with 0.5 N KOH (autoclave sterilized) combined with either filter sterilized tyrosine or phenylalanine. Several type-cultures (lactobacilli) and wild isolates (rods) from unclean cheeses were inoculated and held at 37° C for up to 2 days before odor assessment and headspace gas chromatographic analysis for phenylacetaldehyde and phenethanol. The cultures failed to thrive, and corresponding unclean compounds were not isolated and detected by analytical or sensory assessments. The cultures may require an initial limiting amount of glucose to initiate cell growth before the organisms can produce unclean compounds.

Skimmilk Media

Skimmilk media was prepared with 25% solids (NFDMS) and 4mM of tyrosine and 4mM of phenylalanine, added before adjusting the pH to either 5.2 or 7.2. This nutritionally complex medium was tested with *Lactobacillus casei* (ATCC 393), and after incubation at either 32° or 40° C for 3, 5, or 10 days, it was analyzed by headspace gas chromatography and odor assessments. Although the *L. casei* grew well, it produced excessive levels of acid which probably killed the cells before they were able to convert from carbohydrate metabolism to the added amino acids. As a result, only minimal odors were observed and these were supported by detection of very small concentrations by gas chromatography. Since the milk medium became too caramelized when

autoclaving was attempted, the base medium was steamed before use. Use of control samples showed that this seemed to provide adequate destruction of potentially competitive microorganisms. The experiments indicate that the *L.casei* does not produce unclean compounds from amino acids under non-stressed conditions or perhaps the strain did not possess the biochemical mechanisms for the conversions.

Sweet Cream Buttermilk Medium

Based on previous experience with unclean aromas in dairy-related products, a high solids, sweet cream buttermilk medium was devised to screen more complex initial mixed-culture samples from cheeses. This medium has shown good ability to yield unclean aromas with corresponding flavor compounds when various mixed and single strain cultures are grown in it. A range of solids concentrations ranging from 30% down to 2% buttermilk (sweet cream) have been used, but the more rapid aroma development occurs with from 2% to 5% solids levels. The media is also buffered with either an insoluble buffer (calcium carbonate) or it is maintained at near pH 6.2 with ammonium hydroxide or sodium hydroxide to prevent acid-related lethality of the organisms. Steaming of the medium before inoculation appeared to provide suitable reduction of extraneous microbes to allow the inoculated microorganisms to dominate in the cultures.

Unclean Development

The research showed that some micrococcus organisms produced indole-like unclean aromas and compounds, while other wild isolates synthesized unclean aromas and compounds from phenylalanine and tyrosine (i.e., p-cresol, phenethanol, and phenylacetaldehyde). Unclean aromas were apparent with some cultures in 2 to 3 days, but some require longer incubation times in this medium.

While the screening method needs refinement to accelerate the rate of unclean aroma development, it was used to select one particularly potent unclean-producing isolate from a no-salt experimental Cheddar cheese for detailed characterization in the next project segment. This organism, and others, will be used later to investigate the intermediary metabolism of the unclean compound production, thus serving as a contrasting organism for cultures that lack the ability to produce the unclean flavors.

Investigation of Factors Influencing Production of Unclean Compounds in Cheddar Cheese

In addition to trials conducted with model media and isolates from various unclean low-salt cheeses, factors that may influence production of unclean compounds in Cheddar cheese manufactured in the University of Wisconsin Dairy Plant were investigated.

Effects of Differing Sodium Levels

One trial compared the flavor and properties of Cheddar cheeses manufactured with three levels of sodium chloride (fully-salted, one-half normal, and no-added salt), and ripened at 5° and 10° C. The salt-in-moisture (S/M) ratio is an important parameter in relation to producing desirable Cheddar cheese flavor. A ratio of less than 4.0 usually leads to excessive microbial metabolism, excessive proteolysis, and undesirable flavors. The S/M of the cheeses averaged 4.8 for the full-salt samples, 2.6 for the half-salt samples, and 0.2 for the no-salt samples. Evaluation of the cheeses routinely during ripening showed that unclean and bitter flavors developed at both 5° and 10° C in the reduced sodium samples, and were accompanied by bland/astringent flavors. The production of unclean compounds was measured by gas chromatography and showed that phenethanol was present in these particular cheeses made with traditional commercial starter cultures.

Effects of Differing Sodium Chloride Concentrations

Cheddar cheese was manufactured also with various concentrations of sodium chloride and 50 gm each of L-tyrosine, L-methionine, and L-phenylalanine added at the time of milling and

pressing. These cheeses were analyzed and evaluated for the production of corresponding unclean, Strecker-type flavor compounds during ripening. It was found that initially the cheeses lacked noticeable flavors, except for slightly brothy notes. However, pronounced unclean flavors, that could be attributed to conversion of amino acids to unclean aldehydes and corresponding alcohols and phenols becames apparent. There was some indication that possible peptides were required for transport of the amino acid precursors into the cells for conversion, but these experiments indicate that the amino acids can be utilized directly.

Effects of Added *Lactobacillus helveticus* and Amino Acids

Cheeses have also been manufactured with added *Lactobacillus helveticus*, along with added amino acids. The intensity and rapidity of unclean flavor development seems to be consistent with the involvement of metabolites from this organism in some of the unclean flavor development. Micrococci have been inoculated into cheeses to determine their possible role in the flavor development. These cheeses will be analyzed for unclean, Strecker-type flavors.

Investigation of Histamine Production

Two independent cheesemaking trials were completed. Histamine production was investigated in both trials, in which three vats of milk were inoculated post-pasteurization with either 10^2, 10^3, or 10^4 *Lactobacillus buchneri* per ml of milk. This culture was chosen because it produces histamine readily. A fourth vat was uninoculated and served as the control. Samples of milk, whey, and curd were taken during cheesemaking and during subsequent aging at 7°- 8° C for analysis. Enumeration of total microorganisms and presumptive *L. buchneri* were preformed by standard plate count procedures on Plate Count Agar and MRS-Melezitose, respectively. Presumptive *L. buchneri* were picked and their identity confirmed by biochemical tests. For all experimental treatments, numbers of total microorganisms and *L. buchneri* in cheese had increased by approximately 10-fold by the fourteenth day. Ten-fold increases were also observed for both after 28 and 56 days. Total counts in the control cheese remained low. Histamine analysis via the AOAC method indicated that the milk, prior to cheesemaking, was essentially histamine-free. Histamine analysis of cheese is currently in progress.

Botulinum and Role of Proline as Osmoprotectants

Using a minimal defined medium developed for *C. botulinum* groups I and II, compatible solutes have been identified that provide for salt tolerance in *C. botulinum* types A, B, and E. In both groups of *C. botulinum*, glycine-betaine and proline worked effectively as osmoprotectants. Proline facilitated the fastest growth rate of many potential osmoprotectants tested. This finding is of importance since milk proteins contain considerable proline in their amino acid composition. Proline could be produced in cheese by three means:

1. proteolytic degradation of milk proteins,
2. synthesis by normal bacterial flora, and
3. catabolism of certain amino acids, especially arginine.

The deiminase pathway that converts arginine to proline is present in many starter lactobacilli and lactococci and also in secondary ripening flora, including lactobacilli and pediococci. Thus, there is considerable potential for generation of proline in milk and cheese by proteolysis and catabolism of arginine. Proline can contribute to flavor and to osmoprotection of pathogenic and normal flora of cheese.

Identifying Metabolic Changes in *C. botulinum* Due to Salt Stress

Work has begun on the identification of metabolic changes that occur in *C. botulinum* when the pathogen is exposed to salt stress. These metabolic changes may be important for understanding survival of pathogens in cheese. In proteolytic serotypes of *C. botulinum*, exposure to salt leads to a severe de-

crease in expression of glutamate dehydrogenase (15.9 units/mg, no salt versus 1.9 units/mg protein of NAD-glutamate dehydrogenase for sample with 3% NaCl). Its decrease in expression would prevent catabolism of arginine to glutamate and cause an increase of proline in the cell. This enzyme is present only at very low levels in nonproteolytic *C. botulinum* type E, and therefore metabolic response to salt probably involves a different response. The identification of metabolic enzymes whose expression and activity is influenced by exposure to salt is a focal point of continuing research.

Research Plan for Next Year:

Flavor Chemistry and Microbiology Objectives (Under Overall Project Objectives 1 & 2):

a) To investigate the role of direct microbial Strecker degradation of amino acids to unclean flavor compounds through decarboxylation and transamination compared to indirect microbial/chemical Strecker degradation through reaction of microbial alpha-dicarbonyls and amino acids.

b) To investigate the role of individual selected microbial isolates and cultures in the development of unclean flavors in reduced-sodium Cheddar cheeses using a model cheesemaking system and media for the enhanced production of unclean flavor compounds.

c To study taxonomic properties of isolates from cheeses which produce unclean flavor compounds, and develop biochemical mapping of enzymic systems involved in the production of flavor compounds.

Microbial Safety - *Clostridium botulinum* Objectives (Under Overall Project Objective 3):

a) To define the nutritional conditions in Cheddar cheese and in milk that enable *C. botulinum* to grow and produce toxin.

b) To determine cheesemaking procedures and curing conditions that contribute salt inhibition of *C. botulinum* in Cheddar cheese.

Microbial Safety - Histamine Production Objectives (Under Overall Project Objective 3):

a) To determine the concentrations of histamine in experimentally-inoculated aging Cheddar cheese, and to determine the degree of proteolysis in the cheeses at selected intervals.

b) To isolate organisms from reduced-sodium cheese that are capable of producing histamine.

Significance to the Dairy Industry:

Information on a relatively unknown group of detrimental flavor compounds found in low-salt cheeses will be gained and identification of the proper lactic acid bacteria for producing clean cheese flavors in low-salt products will be determined. This work will help processors create quality, reduced-sodium cheeses that are free of the flavor defects sometimes found in low-salt cheeses today.

Publications:

McDonald, S.T. and R.C. Lindsay. 1989. Improvement of the Quality of Low-Sodium Cheddar Cheese (Abstract). Proceeding of the Cheese Research and Technology Conference, Center for Dairy Research, University of Wisconsin-Madison. March 29 - 30. p. 116 (Presented at Poster Session)

Leyer, Greg J. 1989. Nutritional regulation of toxin formation in *Clostridium botulinum* type E. M.S. Thesis, University of Wisconsin-Madison.

Presentations:

Lindsay, R. C. 1989. "Sensory Properties of Low-Fat and Low-Sodium Cheeses." Presented to the WMMB Technical Seminar, Dairy Products in Transition - - The Next Generation. Madison, June 13, to Food Writers from around Wisconsin and Chicago area.

Project Title:

Development of Process Technology and Flavor Enhancement of Reduced-Fat Cheese

Personnel:

Mark Johnson, senior scientist, Center for Dairy Research; Carol Chen, associate researcher, Center for Dairy Research; R. C. Lindsay, professor, Dept. of Food Science; James L. Steele, assistant professor, Dept. of Food Science; Tarun Bhowmik, research associate, Dept. of Food Science

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-54

Dates:

January 1, 1989 - December 31, 1990

Objectives:

1. To investigate the flavor chemistry of undesirable meaty-brothy off-flavors in reduced-fat Colby- and Cheddar-type cheeses, and to investigate methods to eliminate this defect and extend shelf-life;

2. To define the technology necessary to manufacture high quality reduced-fat cheese;

3. To enhance and introduce unique flavor characteristics into reduced-fat cheese;

4. To assess the consumer acceptability of the reduced-fat cheeses.

Summary of Findings for Objective #1: (Dr. R. C. Lindsay)

Reduced-fat cheeses from retail and experimental lots were obtained for investigations. These cheeses were at least 30% reduced in fat content compared to full-fat cheeses. Peptide fractions were prepared according to the method of Harwalker and Elliot, and were designed to be the same as collected by Rank when he observed the brothy flavors in certain fractions. The fractions were obtained from a Sephadex G-25 size-exclusion type separation, and UV absorption at 280 nm was used to visualize the peptide fractions. After collection of each of seven fractions, UV absorption curves were determined for each of the fractions. Most of the fractions showed absorption maxima at 280 which reflected the contribution of peptides (those with aromatic rings-tyrosine, phenylalanine, tryptophan). However, a fraction designated E was found to exhibit an absorption maximum at 255 nm.

Individual fractions were assessed for flavors, and it was found that fraction E had a very brothy flavor. Other fractions also had varying degrees of brothy-meaty flavors. Then, each of the fractions were extracted and analyzed by gas chromatography for volatile flavor compounds. In each of the fractions, many potential volatile flavor compounds were found. Thus, in order to focus on the most important compounds first, individual peaks were assessed for aromas as they eluted from gas chromatographic separations. Peaks possessed specific aromas of sweet, nutty, brothy, burnt caramel-like, burnt rubber, sweet candy-like, popcorn-like, and pyrazine-like. Notably, there were significant differences between the sizes of peaks (concentrations) between the low-fat cheeses and the full-fat cheeses. However, the larger peaks were found in the reduced-fat cheese rather than the expected full-fat cheeses.

Lactones

The unexpected finding relative to the lactones has been the focus of much effort in an attempt to rationalize the finding. Lactones are derived from the fat content of cheeses, and it would be expected that the full-fat cheese would contain the larger amount. The lactones are sweet, peachy, and coconuty compounds that could contribute to the brothy flavor. It is the current working hypothesis that the lactones are more water soluble in the

low-fat environment, and thus are recovered more extensively than from full-fat cheese. When total lactones are analyzed, it is found that the full-fat cheese contains the greatest quantity. The importance of this observation is under current further study.

The unusual burnt caramel-like substance in Sephadex fraction E has been the focus of considerable study to ascertain the structure of the substance. Data (mass spectra, GC and odor) support the identification as a furanone. The maximum UV absorption of the sugar furanone and the maple lactone both reside near the 255 nm maximum observed, and the data are thus in agreement with this interpretation. Trials have been carried out on the formation of sugar furanone using model systems of reducing sugars, and it is hypothesized that the formation of the compound in the reduced-fat cheese may be accentuated because of the high water content, lower fat level, and possibly higher levels of either galactose or glucose. The carbonyl groups of the reducing sugars would react with the free amino acids formed in the reduced-fat cheese, and lead to the slow formation of the furanone. This would be more rapid in the reduced-fat cheese because of the aqueous nature of the medium.

Developing Quantitative Measuring

The popcorny-nutty, brothy peaks are also of significance in the brothy-meaty flavor of reduced-fat cheeses. Efforts are currently underway to develop a quantitative method to measure the pyrazines which are responsible for these flavor notes. The most abundant compound in this category is 2,5-dimethylpyrazine, and it is viewed as a candidate for indexing the degree of brothy-meaty flavor in reduced-fat cheeses.

Emphasis is currently placed on methods to quantitatively measure the important compounds in the brothy flavor defect. It is also a current area of emphasis to relate the occurrence of the compounds to the conditions existing in reduced-fat cheeses. The role of alpha-dicarbonyls produced by certain lactobacillus adventitious organisms is a possible key to situations where the flavor defect develops to an extraordinary degree. This is often the case observed for commercially available reduced-fat cheeses.

Work will continue on the objectives of the project which focus on the flavor system, and means to enhance flavor via alternate culturing strategies will be investigated.

Summary of Findings for Objectives #2 and #3: (Dr. Mark Johnson)

Several reduced-fat Cheddar cheese trials were run in the past year. The sensory analysis has now been completed. In trial 1, cheeses were manufactured to compare the effects of 3 experimental variables on the body and flavor of the cheese. The variables tested were starter (2% vs. .5%), drain pH (6.5 vs. 6.0) and mill pH (5.8 vs. 5.4). The manufacturing process was identical to milled curd Cheddar cheese with the time between steps varying to obtain the desired pH. The moisture content of the cheeses was lower than anticipated so the fat content of the cheeses was slightly above (20.6% - 22.9%) the maximum allowed (20.43%).

The lactose content of the cheeses was high at 1 week (.20% - .57%) and remained high in some cheeses even after 3 months of aging. The cheeses with the highest residual lactose were made with .5% starter. There was also higher residual lactose in cheeses made with the higher drain pH. Mill pH did not appear to affect lactose retention. The high lactose contents of the cheeses may have ramifications for off- flavor development in light of Lindsay's comments. Residual lactose may cause problems (although none were noted in our cheeses) if heterofermentative microorganisms metabolize it. Gas (slits-openness) and fruity-fermented flavors may be produced. The lactic acid content of the cheeses was also high (1.52% - 1.86% at 3 months) and as the cheese aged, some cheese exhibited calcium lactate crystals. All the cheeses had some mechanical openings.

All cheeses were graded at 3 months, and only selected cheeses at 7 months, using hedonic

scales. Some cheeses were so poor in quality at 3 months that further evaluation was not done. Although the cheese graders consisted of experienced and novice cheese tasters, it is felt that the results are suitable for guiding subsequent cheese manufacturing trials. The manufacturing procedures which produced the smoothest-bodied cheese were high drain pH and high mill pH. These cheeses were highest in moisture (42.7% - 44.0%). However, at 7 months the highest moisture cheese (44.0%) became too soft and weak while the 42.7% moisture cheese did not. The later cheese was made with 2% starter, the former with .5% starter. The lowest moisture (39.6%) cheeses were curdy and crumbly.

Cheeses made with 2% starter were graded as too acid at 3 months but were not considered acid at 7 months. Cheeses made with .5% starter were not considered acid at 3 or 7 months. Starter levels did not significantly affect body or moisture, i.e. better body or higher moisture cannot be attributed to a particular starter level.

Flavor Intensity

There was not a clear difference in Cheddar flavor intensity among the cheeses at 3 months. However, cheeses made with high drain and mill pH were consistently ranked highest in flavor intensity, quality and preference. The flavor of these cheeses was described as mild at 3 months. At 7 months, cheese made with 2% starter (high drain and mill pH) was ranked highest in flavor intensity, as well as preference, but because of the wide range of scores among the various graders, any differences between cheeses were not statistically significant.

However, while one manufacturing schedule using 2% starter produced the best cheese, the other schedules with 2% starter were not graded very high compared to cheeses made with .5% starter. These other cheeses were downgraded due to unclean flavors and very slight bitterness (not objectionable). Only one cheese produced with .5% starter developed bitterness. Meaty-brothy type flavors tended to be a characteristic of cheeses manufactured at low mill pH (5.4).

The highest and lowest ranked cheeses (at 7 months) were compared to retail reduced-fat cheeses. In a blind taste test, our 2 cheeses ranked considerably higher than the retail cheeses in Cheddar flavor quality, intensity of flavor, body and preference. The ages of the retail cheeses were unknown so it may be an unfair comparison. There was considerable variation in all attributes among the retail samples except in flavor intensity (all low). One of the retail samples had the lowest off-flavor intensity score and one had the highest off-flavor score.

High Moisture Impact

In trial 2, high moisture (46.5% - 48.7%) reduced-fat Cheddar was made. The fat contents ranged from 19.7% to 20.6%. These cheeses were high in lactic acid (1.9% - 2.0%) but low in lactose (negligible) at 1 month. At 3 months, the cheeses were graded as extremely acid, bitter, and not acceptable. The body, however, was soft. Although not graded again it would be expected that these cheeses would become too soft and weak with age.

Milk Pasteurization Impact

In trial 3, the effects of milk pasteurization (164° vs. 180°/16 sec) and a 25% whey dilution (wash) were evaluated. The cheeses were made with .5% starter, pH 6.5 at drain and pH 5.8 at mill. With the addition of one ounce of $CaCl_2$, the curd cutting time of the milks was 30-35 minutes. At 3 months there were no statistically significant differences between the cheeses in body, flavor quality, acid flavor intensity, off-flavor intensity or preference. The whey dilution reduced lactic acid levels by about .2% and the washed cheeses were higher in pH than non-washed cheeses. However, the cheese made from milk pasteurized at 180°F and no wash was scored highest in flavor intensity and had the least off-flavor. All cheeses were graded as acceptable. These cheeses will be graded at 6 months of age.

Starter Adjunct

In trial 4, a non-lactose fermenting, non-proteolytic derivative of *Lactococcus lactis* ssp. *cremoris E8* was used as a starter adjunct. This strain was developed by Tarun Bhowmik working with Dr. Jim Steele. (A summary of Dr. Steele's findings is located later in this report.) We noticed more flavor in reduced-fat cheese using 2% starter, but as a 3-month-old cheese, the cheese was too acid. To circumvent this problem we tested the mutant strain. Cheese was made with .5% starter and 2% added mutant cells. Compared to control vats at 3 months, these cheeses exhibited a slight increase in flavor, the flavor quality was higher, the body was preferred, and the cheeses were not acid. All cheeses were considered acceptable. Microbial analysis at one month indicated that the non-starter population in cheeses made with the added mutant were significantly lower than in cheeses without the added cells. Curing the cheeses at 50°F vs. 45°F increased flavor development without adversely affecting the Cheddar flavor quality. At 6 months similar trends continued. However, at 6 months, some panelists objected to the more intense flavors when the cheeses were ripened at 50°F The most preferred cheese was made with the mutant cells and ripened at 45°F. Bitterness and off-flavors were apparent at 50°F, bitterness was reduced with the added cells. Bitterness was not evident at 45°F. The main off-flavors at 50°F were bitterness; sulphury; and slight rosy, fruity-fermented. Curiously, to some panelists, the cheeses ripened at 50°F were more preferred.

It has become apparent during the cheese grading sessions that there is a wide range of what people prefer, with regard to flavor. Mild cheeses free of meaty-brothy or bitter flavors are most acceptable. However, stronger tasting cheeses free of bitterness or meaty-brothy flavors score lower because of sulphury flavors (H_2S). A few judges prefer these cheeses and refer to them as having "New York" Cheddar flavor.

Summary of Findings by Dr. J. L. Steele:

A lactose-negative (Lac^-), proteinase-negative (Prt^-) derivative of *Lactococcus lactis* ssp. *cremoris E8*, designated E8-4, was isolated via novobiocin induced plasmid curing. Electroporation of *Lactococcus lactis* ssp. *lactis LM0230* with the E8 plasmid pool resulted in Lac^+ Prt^+ transformants. Examination of the plasmid pools of E8, E8-4, and the Lac^+ Prt^+ transformants indicated that a 33 megadalton plasmid, designated pSB33, encodes the Lac^+ Prt^+ phenotypes. Intracellular proteinase, aminopeptidase, dipeptidase, and dipeptidyl aminopeptidase activity were examined and compared in both E8 and E8-4; no significant differences were observed. After polyacrylamide gel electrophoresis, one active aminopeptidase, dipeptidase, and dipeptidyl aminopeptidase were detected in both strains. These results indicate that pSB33 does not effect peptidase activity. The effect of E8-4 on accelerated ripening of low-fat Cheddar cheese is under investigation.

Future work will include attempts to construct a Lac^- derivative of *Lactobacillus helveticus* CDR101. This strain could then be used as a starter adjunct in the manufacture of low-fat Cheddar cheese without concerns related to acid development.

Remainder of project:

High moisture, reduced-fat cheese is more profitable than low moisture, reduced-fat cheese. However, the intense acid flavor of the high moisture cheeses necessitates the reduction of lactose/lactic acid in the curd. We believe that this will result in less flavor development and a higher incidence of off-flavors. These cheeses will never develop intense Cheddar cheese flavor and may become too soft-pasty with age. We can now make a low moisture reduced-fat Cheddar with good flavor and body, but because of the high lactic acid content, calcium lactate crystals can develop. We also notice more intense flavors, particularly sulphury flavors, more typical of Cheddar cheese made in New York and Vermont. These flavors are objectionable to some and desired by others. Work will

continue with the application of starter adjuncts to decrease off-flavors and increase desirable flavor characteristics. If high moisture reduced-fat cheeses are to be more fully-developed, more attention will have to be given to the starter culture. The selection of non-bitter starters is necessary. Earlier work in our laboratory has shown that starter cultures producing non-bitter, high-fat cheeses may produce bitter, reduced fat cheeses. We plan to screen starters for characteristics leading to bitter cheese, i.e. high growth rate, and/or high proteinase activity at low cook temperatures (94°F-97°F). Starter adjuncts that may reduce or eliminate bitterness will also be tested in cheesemaking trials. Larger consumer acceptability taste panels are also planned.

Significance to the Dairy Industry:

A challenge to the cheese industry is to provide consumers with a variety of reduced-fat cheese that meet their dietary needs, without sacrificing quality. Identification of the conditions contributing to the excess formation of brothy flavor compounds and selection of manufacturing conditions and cultures that greatly suppress production of these compounds will result in good-flavored, textured lowfat cheese varieties.

Project Title:

Manufacture of Cheese from Milk Pasteurized at High Temperatures

Personnel:

Mark Johnson, senior scientist, Center for Dairy Research; Carol Chen, associate scientist, Center for Dairy Research; Laura Paluch, graduate student, Center for Dairy Research; and Brian Riesterer, associate researcher, Center for Dairy Research

Funding:

Wisconsin Milk Marketing Board

Funding Code:

IV-55

Project Dates:

September 1, 1988 - August 31, 1990

Objectives:

1. Identify changes (and corrective measures) in cheese manufacture necessary if pasteurization temperature/time standards are raised.

2. The project will provide information to extension and other resource personnel (serve as a database) about anticipated changes, or modifications, required during cheese manufacture using high temperature pasteurized milk. This research may also provide information that could be used to improve shelf-life of low-flavor, high-moisture (i.e., low-fat) cheeses.

Summary of Findings:

Cheddar cheese was manufactured from milk pasteurized at 73.3°C, 75.6°C, or 77.8°C for 16 seconds. Rennet clotting time was increased by approximately 12 minutes in milk heated to 77.8°C compared to milks heated to either 73.3°C or 75.6°C. No difference in rennet clotting time was observed between the two lower temperatures. Addition of .02% calcium chloride to milk heated at 77.8°C reduced the rennet clotting time by approximately 16 minutes. The heat treatments of 73.3°C, 75.6°C, and 77.8°C resulted in 4%, 6%, and 10% denaturation of ß-lactoglobulin and 1%, 6%, and 10% denaturation of α-lactalbumin, respectively.

A descriptive sensory panel (N=27) evaluation of 6-month-old cheese found no statistical difference as to overall preference, cooked flavor, or off-flavor intensity. However, cheeses made from milk pasteurized at higher temperatures were perceived as firmer and dispersed less readily in the mouth during chewing.

Cheeses made from milk heated to 73.3°C were less bitter and less acid than cheeses made from milk heated to 77.8°C. Based on these results, a quality cheese can be made from milk pasteurized as high as 77.8°C. However, since bitter flavors develop in aged cheese, use of high pasteurization temperatures is not recommended for long-hold cheese.

Significance to the Dairy Industry:

Use of high-heat-treated milk for cheesemaking, coupled with strict sanitary manufacturing conditions could lower the incidence of microbially-produced defects and help ensure production of quality cheese.

Presentations:

"Cheddar Cheese Manufactured from Milk HTST Pasteurized at 73.3° C, 75.6° C, and 77.8° C," L.J. Paluch, M.E. Johnson, B.Riesterer and N.F. Olson, 85th Annual ADSA Meeting, Raleigh, N.C., 1990

Project Title:

Construction of a Gene Bank of *Lactobacillus helveticus* CNRZ 32: Cloning and Characterization of the Aminopeptidase and Threonine Aldolase genes.

Personnel:

James L. Steele, assistant professor, Dept. of Food Science; Tarun Bhowmik, research associate, Dept. of Food Sciences; Christine Gutkowski, graduate student, Dept. of Food Science

Funding:

National Dairy Promotion and Research Board

Funding Code:

89-4

Dates:

July 1, 1989 - June 30, 1992

Objectives:

1. Construction of a gene bank of *Lactobacillus helveticus* CNRZ 32 in *Escherichia coli.*
2. Screening the gene bank for peptidase genes and identifying isolates which encode an aminopeptidase.
3. Screening the gene bank for the threonine aldolase gene.
4. Constructing strains of starter cultures with an altered ability to produce acetaldehyde and degrade peptides.

Summary of Findings:

The construction of the gene bank of *L. helveticus* CNRZ 32 in *E. coli* is now complete. The gene bank was constructed by ligating *L. helveticus* CNRZ 32 chromosomal DNA fragments ranging in size from 7 to 12 kilobases (kb), generated by partial digestion of total genomic DNA with Sau3A, into pJDC9 and transforming *E. coli* DH5a. A total of 2,728 white colonies (indicates a transformant containing an insert) were obtained from the transformation of eight different ligation mixtures. When plasmid DNA obtained from these white colonies was examined for inserts, approximately 77% of the plasmids contained inserts, with the average insert size being 7.8 kb. Assuming a genome size of 3,500 kb, the probability of the bank containing a specific gene is greater than 99%.

Histochemical staining was used to determine the number and specificity of the peptidases present in L. helveticus CNRZ 32. An aminopeptidase, prolyl-dipeptidyl aminopeptidase, tripeptidase, and dipeptidase were detected. Their ability to release leucine from a variety of peptides has been determined. These results will allow us to select the substrates to be used in screening the gene bank for the presence of the lactobacilli peptidases.

The ability of *L. helveticus* CNRZ 32 to produce acetaldehyde during growth in milk was examined using a colorimetric assay. *L. helveticus* CNRZ 32 was found to produce 1.14 ppm acetaldehyde.

Next, the gene bank will be screened for the peptidase genes and threonine aldolase gene by procedures developed with the information above.

Significance to the Dairy Industry:

This study will be a starting point for the use of genetic engineering to produce dairy products with enhanced or unique flavors. The gene bank will be used for further analysis of enzymes involved in cheese flavor development. By constructing a derivative of *L. helveticus* CNRZ 32 which lacks an aminopeptidase, the role of that enzyme in flavor development can be unequivocally demonstrated. The transfer of a lactobacilli aminopeptidase gene to a thermolytic strain of lactococci should enhance that strain's ability to produce non-bitter Cheddar cheese, and may accelerate Cheddar flavor development. The construction of a threonine aldolase negative derivative of *L. helveticus* CNRZ 32 will result in a starter adjunct with enhanced utility; this derivative could be used as a starter adjunct for cheese varieties in which acetaldehyde is not acceptable. As our understanding of the enzymes involved in cheese flavor increases, so will our ability to control and enhance cheese flavor.

Project Title:

D-(-) Lactic Acid Formation by a *Lactobacillus* Species

Personnel:

Eric A. Johnson, assistant professor, Food Research Institute (PI) and Sirirat Rengpipat, research assistant, Food Research Institute

Please note: Si Rengpipat graduated and left for Thailand in November. Therefore, this report is from July 1989 - October 1989.

Funding:

Cheese Research Institute

Funding Code:

2-89

Dates:

July 1, 1989 - June 30, 1990

Objectives:

To determine the biochemical and physiological factors that control D-(-) lactate formation in *Lactobacillus* (ATCC #49178) [UW 1].

Summary of Findings:

D-lactate formation is involved in the white crystal defect in cheese. In this study, we characterized an unusual strain of *Lactobacillus* that produces high concentrations of D-(-)-lactate. During the completion of this project, we concentrated on the physiology of lactic acid production by *Lactobacillus* #49178.

Presently, there is controversy concerning the mechanism of formation and function of L-(+)- and D-(-)-lactic acid isomers. Lactate racemases were once thought to be abundant in lactobacilli, but studies by Kandler (1983) have, in fact, shown that these racemases are rare in the lactobacilli and other lactic acid bacteria. Only two species have been shown to definitely possess lactate racemases (*L. sake* and *L. plantarum*). What, then, is the mechanism of D-(-)-lactate formation?

We have found evidence that the production of D-(-)-lactate enables *Lactobacillus* #49178 to continue to grow when the concentration of L-(+)-lactate accumulates in the growth medium. L-(+)-lactate is produced during active growth and D-(-)-lactate is mostly produced in late log phase. When cells were inoculated to media (basic medium + glucose) containing various concentrations of L-(+)-lactate, D-(-)-lactate production increased with increasing initial concentrations of the L isomer (Figure 1). Furthermore, D-(-)-lactate production increased when the initial pH was lowered from 8.0 to 4.5 (Figure 2). Formation of D-(-)-lactate was maximum at pH 5.5, and production fell rapidly above this pH. These results indicate that external pH is important in directing the syntheses of the different lactate isomers. The data suggest that the proton gradient across the cell membrane is important in growth and survival, and that the cell's ability to change export of the different isomers may be a mechanism for coping with pH stress.

If racemization of lactate did occur via a specific racemase in *Lactobacillus* #49178, then the transport of L-(+)-lactate into the cell would be required. Lactate transport into cells was determined by adding ^{14}C—L-(+)-lactate to a suspension of exponentially growing cells. No transport of labeled lactate was observed in the lactobacillus, whereas significant transport was found with *E. coli* used as positive control in the experiment (Figure 3).

During cheese manufacture, high quantities of of L-(+)-lactate are produced during starter activity, and the L-(+)-lactate persists in the cheese during storage at low temperatures. Based on the results of our study, it is conceivable that certain lactobacilli and pediococci have evolved the ability to survive in the presence of high concentrations of the L-isomer by adjusting their metabolic pathways for energy generation. The mechanism may involve a metabolic switch that results in production of D-(-)-lactate instead of the L-isomer. This provides for continued metabolism in the conditions found in cheese

Figure 1. Maximumgrowth (□—□) and maximum specific growth rate [μ (h^{-1})] (■—■) of UW 1 grown in BSMG with various concentrations of L(+)-lactate (mM) at three incubation temperatures: (a) 15°C, (b) 25°C, and (c) 37°C.

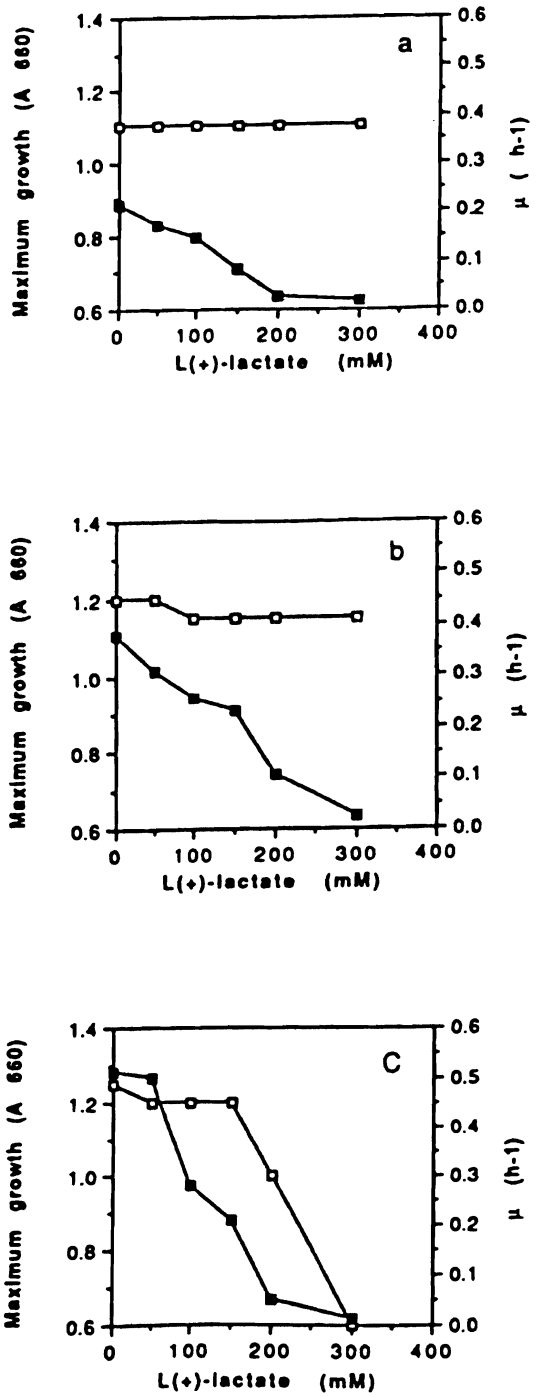


Figure 2. Production fo L(+)-lactate (□) and D(-)-lactate (■) by UW 1 grown at 25°C in BSMG at various pHs with (a) 0 mM and (b) 100 mM L(+)-lactate.

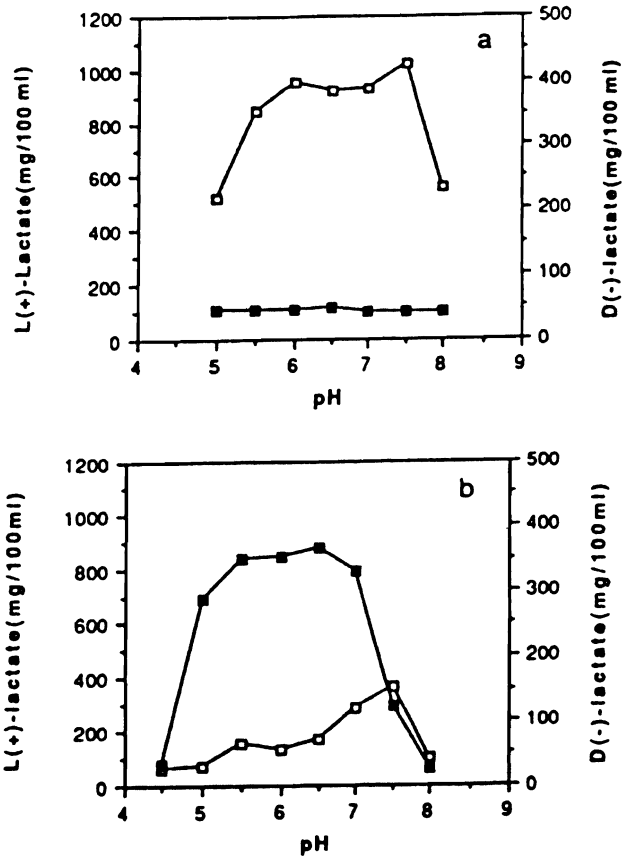
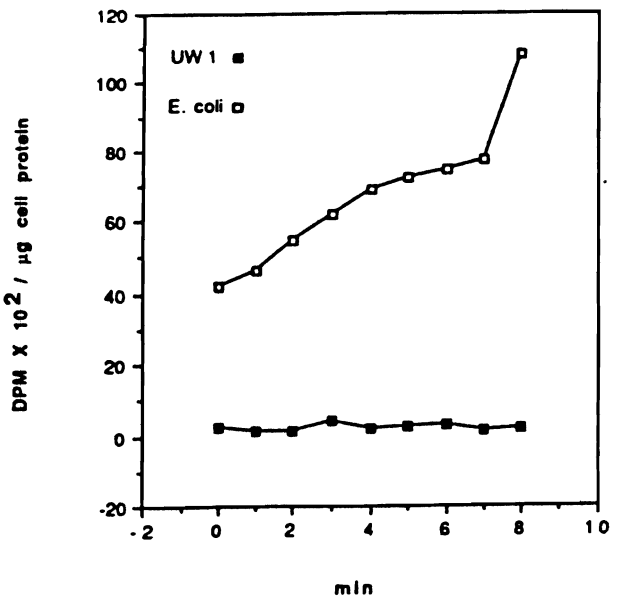


Figure 3. ^{14}C-L(+)-lactate transport in UW 1 culture and E. coli.



of high concentrations of the L-isomer and high external proton concentration (low pH).

Publications:

Rengpipat, S. and E. A. Johnson. 1989. Characterization of a Lactobacillus strain producing white crystals on Cheddar cheese. *Applied and Environmental Microbiology* 55: 2579-2582.

S. Rengipipat, M. Johnson, and E. A. Johnson. 1988. D-lactate formation by a *Lactobacillus* sp. and its role in white haze defect on Cheddar cheese. *Abstr. Amer. Dairy Sci. Assoc.*, D-148, p. 111.

Project Title:

Effect of Post-processing on Cell Viability, Cell Permeability, and Enzyme Activity of *L. helveticus* Cheese Starter Culture Adjunct

Personnel:

Mark R. Etzel, assistant professor, Dept. of Food Science; Julie Johnson, graduate student, Dept. of Food Science

Funding:

National Dairy Promotion and Research Board

Funding Code:

ETZ89-6

Dates:

October 1, 1989 - September 30, 1992

Objectives:

We are developing new technology to stimulate the flavor development and reduce the bitterness of cheese. This is especially important for low-fat cheeses which frequently have a bitter taste and a weak or nonexistent aroma. Our procedure is based on the addition of inactivated dried *L. helveticus* cells during cheesemaking. These cells are inactivated during drying but still contain active enzymes that are important for the development of desirable flavors. During cheese ripening the active enzymes are thought to leak out of the now permeable cell wall and accelerate the development of desirable cheese flavor. Addition of heat-treated and freeze-treated *L. helveticus*, during cheese making has already been shown to accelerate and improve desirable flavor development during cheese ripening. We seek to produce dry and stable *L. helveticus* cells which can be used on a large scale to enhance flavor development in cheese. We are emphasizing spray drying and freeze drying for this purpose.

Summary of Findings:

We have established the techniques and equipment needed to grow *L. helveticus* for our research. We determined the effect of freezing on cell viability and we have spray dried *L. helveticus* to determine the operating conditions required for inactivation.

There is little fundamental knowledge in the area of spray drying microorganisms. Metwally et. al. (1989) experimentally found up to 47% retention in viability after spray drying starter cultures. These authors studied the effects of spray drying on strains of *Streptococcus lactis, Streptococcus thermophilus,* and *Lactobacillus bulgaricus.* They found that spray dryer parameters that affect the survival of microorganisms are the inlet and outlet temperatures from the dryer, type of atomization, and direction of air flow in the dryer. They found that the culture pH, growth stage during harvest, and the addition of dextrin all affected inactivation during spray drying. It was found that the highest survival was 47% for *S. thermophilus* which was obtained at an outlet air temperature of 70°C using a two-fluid atomizer and counter-current air flow.

Daemen et al. (1981, 1982, 1983, 1984) investigated the effects of spray drying on three enzymes: alkaline phosphatase, rennin, and a-amylase; and two bacteria: *Serratia marcescens* and *Staphylococcus* C131. They found that changes in the outlet air temperature had a large effect on cell inactivation whereas the effect of the inlet temperature was very small. The destruction of bacteria was attributed to both a thermal effect and a non-thermal drying effect. An increase in total solids content corresponded to a decrease in the percentage of surviving bacteria. This last finding was unexpected because previous studies demonstrated that thermoresistance increased with increasing total solids content. A higher thermoresistance should result in higher cell viability. Daemen attributed the decrease in cell survival to a slower drying rate (higher resistance), and thus a higher moisture content at the center of the drying particle with the greater solids content. A higher moisture content decreases thermoresistance.

Considering the work that has been done thus far, studies on *L. helveticus* have been initiated. As a first step, batches of *L. helveticus* cells were cultivated according to Chen (1989), reconstituted with 12% (vol./vol.) sterile

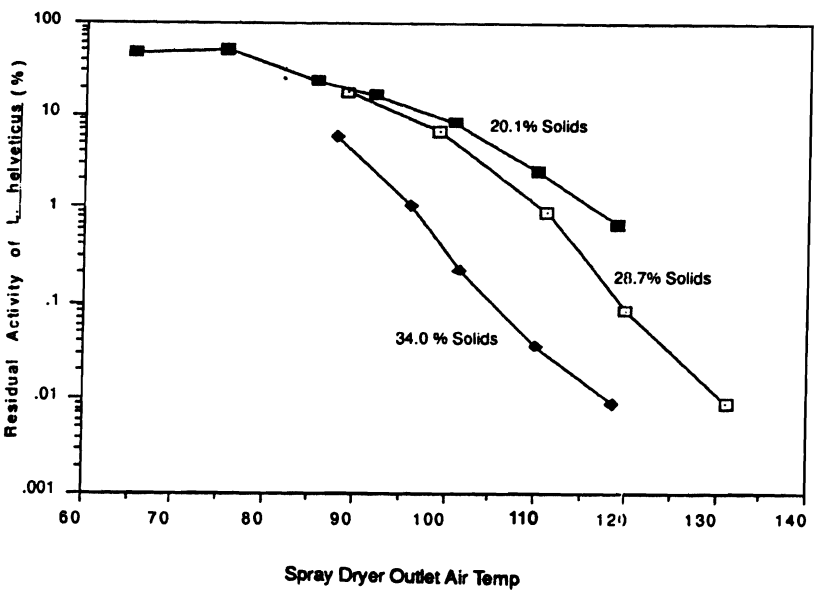
nonfat dried milk, distributed to 2 ml plastic screw-cap ampoules, and frozen in a mechanical freezer at -88°C. Upon thawing, the Standard Plate Count Method was employed in determining cell viability. Glycerol was added to some of the samples as a cryoprotective agent; however, as indicated in Table I, it did not appear to have any effect on preserving cell survival. In addition, some of the samples were refrigerated at a temperature of 4°C for 30 minutes prior to being placed in the freezer. This was done in order to compare rapid freezing with controlled-rate freezing. Again, this technique had no noticeable effect on the number of colonies that formed after freezing.

Spray drying experiments on *L. helveticus* cultures were begun using cells suspended in different concentrations of condensed skim milk. Frozen cultures were thawed and 1 ml quantities were dispersed in 500 to 1000 ml of condensed skim milk. The total solids content of the condensed skim milk varied, ranging from 20% to 34%. Plate counts were conducted on samples before spray drying and then on samples collected at various outlet air temperatures during the course of each spray drying trial. Outlet air temperatures were obtained by adjusting the rate at which the feed solution was pumped through the atomizer. The inlet air temperature was maintained constant at 220°C.

Table I.
L. helveticus viable cell population counts in Colony Forming Units (CFU) per ml of cell suspension.

Frozen w/o glycerol, No refrigeration	Frozen w/ glycerol, No refrigeration	Frozen w/o glycerol, Refrigeration	Frozen w/ glycerol, Refrigeration
1.7e10	1.6e10	1.5e10	1.7e10
1.5e10	1.6e10		

Figure 1. L. helvetica *was dispersed in different concentrations of condensed skim milk. The solutions were spray dried and samples were collected at several outlet air temperatures. The residual activity was determined by N/No x 100 where No is the initial activity and N is the activity after spray drying. Population counts were obtained using the Standard Plate Count Method.*



As shown in Figure 1, semi-logarithmic plots of % survival vs. temperature follow a linear relationship. This corresponds well with data collected by Daemen et. al. (1981, 1982, 1983, 1984). In addition, higher % survival was achieved with a decrease in the initial solids content. Again, these results are supported by Daemen.

We have achieved residual *L. helveticus* viabilities ranging from 0.03% to 49% after spray drying. This is significant for at least two reasons. First, viabilities of 0.03% should be low enough to eliminate significant repopulation of *L. helveticus* during cheesemaking. This is necessary to eliminate undesirable flavor development which may occur from the metabolism of active *L. helveticus*. Second, viabilities of 49% are high enough to suggest using spray drying for the large-scale distribution of active starter cultures. Starter cultures are now produced by diluting the cell paste in 12.5% non-fat milk and freezing to -40° C. These frozen blocks are shipped to the cheese plant and thawed just before addition to the cheese. Spray dried starter cultures may eliminate the need to ship dilute frozen suspensions and therefore may reduce the weight and volume of culture material that must be transported and stored.

Work in progress includes continuing to measure *L. helveticus* cell viability as a function of the processing conditions. We will also measure the cell permeability and enzyme activity of these cultures.

Significance to the Dairy Industry:

The demand for low-fat cheese is increasing as consumers become more health conscious. New technology needs to be developed to produce low-fat cheeses with an aroma and flavor as attractive as the normal fat varieties. This research is aimed at developing this technology and especially at a technology suitable for use on a large scale. The method we are investigating is based on adding inactive starter cultures during the cheesemaking process. These cultures contain active enzymes which are thought to leak out during cheese ripening to increase the rate of formation and intensity of desirable flavor.

We have established techniques and equipment needed to culture, assay and dry *L. helveticus*, the starter culture used in our studies. Our research indicates that spray drying can be used to produce starter cultures with various residual viabilities. Low viability cultures may be suitable for accelerated flavor development in cheese.

In addition to low viability cultures, spray dried cultures with promisingly high viabilities were also obtained in our investigation. This is significant because starter cultures play an important role in the dairy industry, however, preparing large quantities of cultures is difficult and time consuming. Cultures must be frozen or freeze-dried in order to preserve their viability during transportation and storage. Although freezing and freeze-drying yield high survival rates, these techniques are time consuming and costly. For these reasons, other methods for culture preservation are actively being sought.

Our research indicates that spray drying is one such alternative method. We measured as high as 49% viability after spray drying active *L. helveticus* cultures. Higher retentions are probable after further optimization of the culturing and processing conditions. The continuous nature of the spray drying operation, the high throughput and the high thermal efficiency label it as an economically attractive technique for culture preservation and distribution. Also, because the product is dry, it may not need to be stored in a freezer, reducing storage and transportation costs as well.

Presentations:

"Freeze-drying and Spray-drying of Microorganisms," M.R. Etzel. Invited speaker in the Biotechnology and Industry Symposium, Pewaukee, WI, March 29 (1990).

"Freeze-drying and Spray-drying of Microorganisms," M.R. Etzel. Invited speaker in the Industrial and Environmental Biotechnology Seminar Series, Madison, WI, April 16 (1990).

"Inactivation of Lactobacilli During Spray Drying," J.A. Clemons and M.R. Etzel. A.I.Ch.E. Annual Meeting, Chicago, IL, November 16 (1990).

References:

M. M. Metwally, I. A. ABD El-Gawad, S. A. El-Nockrashy, and K. E. Ahmed (1989) *Egyptian J. Dairy Sci.*, 17, pp. 35-43 and 273-281.

C. Chen (1989) "Cell Cultivation of *Lactobacillus helveticus*", Center for Dairy Research internal memorandum, University of Wisconsin-Madison.

A. L. Daemen (1981) *Neth. Milk Dairy J.*, 35, pp. 133-144.

A. L. Daemen and H. J. van der Stege (1982) *Neth. Milk Dairy J.*, 36, pp. 211-229.

A. L. Daemen, A. Kruk, and H. J. van der Stege (1983) *Neth. Milk Dairy J.*, 37, pp. 213-228.

A. L. Daemen (1984) *Neth. Milk Dairy J.*, 38, pp.

Project Title:

Improving the Flavor of Enzyme-Modified Cheeses by Control of Lipase Action in Supercritical CO_2

Personnel:

Richard W. Hartel, assistant professor, Dept. of Food Engineering; Janice M. Johnson, graduate student

Funding:

Cheese Research Institute

Project Dates:

July 1, 1989 - June 30, 1992

Objectives:

1. To assess the feasibility of using supercritical (SC) CO_2 as a means to control lipolytic action on butterfat.

2. To determine the specificity of lipase action on butterfat in SC CO_2 as influenced by the type of lipase used, the level of water as an entrainer and the reactor operating temperature and pressure.

3. To develop a fundamental understanding of how lipase action on butterfat in SC CO_2 may be controlled to improve the flavor balance and fatty acid profile of enzyme modified butterfat.

Summary of Findings:

The original objectives of this project are to determine the lipase action on butterfat in SC CO_2 under various processing conditions. Prior to studying the enzyme reaction on butterfat, a model system will be studied using pure triglycerides to determine the lipase activity in SC CO_2. In preparation for these experiments, preliminary studies are underway to determine the solubility of important triglycerides, tripalmitin and tristearin, in SC CO_2.

A standard flow-through extraction apparatus was used to measure triglyceride solubilities. SC CO_2 was passed through an extraction column where solubilization occurred. The extract phase was then passed through an expansion valve and the precipitated extract collected in the sample tube. The masses of extract and CO_2 were measured to yield solubility values. Standard operating conditions of 40°C and approximately 4400 psi (303.2 bar) were chosen.

Two methods of packing the extraction columns with triglycerides were tested. In the first, tripalmitin, which is a solid at 40°C, was melted at 70°C and thoroughly mixed with glass beads to form a thin layer of triglyceride on the beads. This method provides an increased surface contact area between the SC CO_2 and the triglyceride and should reduce any mass transfer limitations during the extraction. In the second packing method, tristearin was packed into the column in alternating beds of glass wool, triglyceride and large glass beads. In all, there were five of these alternating beds stacked in the column. To prevent any carryover of triglyceride not solubilized in SC CO_2, a thick bed of glass wool (approximately 10 cm) was packed on top of the last bed followed by a layer of glass beads. After preparing the column, the system was pressurized and the temperature increased to the operating conditions. A low flow rate of SC CO_2 was passed through the column (approximately 2500 ml/min) and collected in a sampling tube. After reducing the pressure, the solubilized triglyceride precipitated onto the a glass collection tube of known weight and the increase in weight of the tube determined. The CO_2 was vented to a mass flow meter and the volume of expanded CO_2 determined.

Solubility data for two triglycerides, tripalmitin and tristearin, have been obtained at 40°C and approximately 4400 psi (303 bar). The solubility of tripalmitin was determined to have a mole fraction $4.56(10^{-5})$ in SC CO_2 at 40°C and 4387 psi (302.3 bar). By comparison, previous research has determined that tripalmitin has a solubility of mole fraction $2.71(10^{-5})$ at 40°C and 296 bar. This difference may be attributable to experimental variability. Further experiments

are underway to determine the extent of experimental variability with this technique.

The mole fraction of tristearin in SC CO_2 was determined to be $5.86(10^{-6})$ at 40°C and an average pressure of 4419 psi (304.5 bar). No previous data on tristearin solubility in SC CO_2 is available for comparison. However, extrapolation of previous data on other triglycerides suggests a value of $2.29(10^{-6})$ based on an approximate 10-fold decrease in mole fraction for every 2 carbon increase in the carbon chain length of the fatty acid in the pure triglyceride. These results are in reasonable agreement although further experiments are underway to more accurately determine these solubilities.

Significance to the Dairy Industry:

These results on triglyceride solubility in SC CO_2 are important for the future understanding of enzyme reactions in SC CO_2. Extraction of triglyceride components of butterfat using SC CO_2 and their subsequent modification by enzyme reaction will potentially lead to the development of tailor-made fats for specific food ingredient applications.

Project Title:

Acceleration of Low Fat Cheese Ripening Using the Crude Cell Free Extract of Some Cheese Related Microorganisms- Detection and Characterization of the Aminopeptidases and Dipeplidylpeptidases

Personnel:

M. El Soda, visiting scientist, Center for Dairy Research; A. Macedo, technical assistant, Center for Dairy Research; N.F. Olson, professor, Center for Dairy Research

Funding:

WITEP Visiting Scientist

Dates:

June 1, 1989 - September 30, 1989

Objectives:

During the last 15 years a great deal of attention has been directed to the peptide hydrolase system of the *Lactococci* and, to some extent, the *Lactobacilli*. On the other hand, information concerning other cheese related microorganisms is still limited despite the fact that bacteria like *Propionibacterium, Brevibacterium,* and *Leuconostoc* are used as starters for the manufacture of a wide range of cheeses, including Swiss, surface-ripened cheeses, Gouda, and Edam. Also, organisms like *Pediococci* and *Lactobacillus casei* are isolated in fairly large numbers from most ripened cheeses. A better knowledge of the enzyme systems of these microorganisms is essential for an understanding of their role during ripening, and their possible use as accelerated ripening agents.

The present work investigated the aminopeptidase and dipeplidyl aminopeptidase systems of several cheese related microorganisms. An attempt to purify and characterize the enzymes was also undertaken.

Summary:

The first part of the work was devoted to the detection of the aminopeptidase and dipeptidyl aminopeptidase systems of *Brevibacterium linens, Leuconostoc mesenteroides, Lactobacillus casei, Propionibacterium,* and *Pediococcus* species. This was followed by a partial purification and characterization of the different enzyme systems.

Aminopeptidase (AP) activities were detected in the different microorganisms tested (Table 1a). It was, however, of interest to notice the difference in the specificities of their enzyme systems. *Brevibacterium linens* was significantly more active than the other strains on Glycine P - nitroanilide, while it did not hydrolyze the Leu, Lys, Pro and Arg derivatives. *Propionibacterium shermanii* was distinguished from the other organisms tested by its relatively high activity on Pro P - nitroanilide, while very little hydrolysis of the other substrates was observed. *Leuconostoc mesenteroides* showed rather low aminopeptidase activity on Leu, and Ala P - nitroanilides and did not hydrolyze the Lys, Pro, Gly and Arg derivatives. *L. casei* and *Pediococci* showed similarities in their specifity, but *Pediococci* was distinguished by its inability to hydrolyze Pro and Gly P - nitroanilides. The best substrate for the *L. casei* AP was Leucyl P - nitroanilide, while Lysine P - nitroanilides was best for the *Pediococci.*

As far as dipeptidyl aminopeptidase (DAP) activity is concerned, (Table 1b) *Leuconostoc* and *Brevibacterium* were unable to hydrolyze any of the DAP substrates tested. As a general rule Arg-Pro, and Gly-Pro were hydrolyzed faster than Gly-Phe P - nitroanilides. The enzyme could therefore be considered an x Prolyl dipeptidyl peptidase. *Pediococci* seems to possess the most active DAP system, while *Propionibacterium* showed the lowest levels of activity.

The obtained electrophorectic zymograms of the various species showed the presence of a single aminopeptidase activity in all the species tested. The enzyme showed different electrophoretic mobility in each species. For the dipeptidyl aminopeptidases, the activity bands were detected in the stained gel of *L. casei, Pediococcus* and *Propionibacterium.* The other species did not exhibit dipeptidyl amino peptidase activity. The RF value was 0.27 for *L. casei,* while it was 0.32 for the *Pediococci.*

Table 1. Peptide hydrolase activities of several cheese related microorganisms. Results are expressed as specific activity, which is defined as the number of units per mg protein. A unit of aminopeptidase or dipeptidylpeptidase activity is defined as the increase of 0.01 absorbence units at 410 nm during 1 min of assay.

1a. Aminopeptidase

Species	Substrates					
	Leu*	Lys.*	Pro.*	Gly.*	Ala.*	Arg.*
L. casei	13.5	6.0	0.5	0	4.0	30.4
Pediococcus sp.	7.5	1.0	0	0	0.9	15.2
L. mesenteroides	0.4	0	0	0	0.9	0
B. linens	0	0	0	63.8	0.6	0
P. shermanii	0.7	0.7	6.0	0.5	0.9	1.1

*All the substrates used are the P. nitroanilide derivatives

1b. Dipeptidyl peptidase

	Substrates		
Species	Arg.Pro*	Gly. Pro*	Gly. Phe*
L. casei	9.0	4.0	1.0
Pediococcus sp.	35	22	0.12
L. mesenteroides	0	0	0
B. linens	0	0	0
P. shermanii	5.0	0.4	0.1

*The substrates used are P. nitroanilide derivatives

Partial Purification and Characterization of the Aminopeptidase and Dipeptidyl Aminopeptidase Activity

Gel filtration on Sephacryl S 300 (Pharmacia LKB) led to separation of the aminopeptidase from the dipeptidyl aminopeptidase activities of *L. casei, Proprionibacterium,* and *Pediococcus* species. Partial purification of the aminopeptidase from *Leuconostoc mesenteroides* and *Brevibacterium linens* was also accomplished on the same gel (results not shown). No further purification steps were undertaken. The separated fractions from each species were then pooled for the characterization of the enzymes.

Table 2 indicates that the aminopeptidases from the five cheese related species showed an optimum pH of 7.5. The optimum temperature was 30°C for *Brevibacterium linens,* while it was 40°C for the four other species. The isolated aminopeptidases are strongly inhibited in the presence of the metal chelator 1,10 phenanthroline at a concentration of $1x10^{-3}$M. Enzyme inhibition was also noticed in the case of *L. casei, Propionibacterium, Pedicoccus,* and *B. linens* in the presence of $1x10^{-3}$M parahydroxymercuribenzoate. With an optimum pH of 7.5 (Table 3), the dipeptidyl aminopeptidase activities are comparable to the previously described aminopeptidases. On the other hand, the 50°C optimum temperature of the *Pediococci* and *Propionibacterium* is higher than the value reported for the aminopeptidases. The dipeptidyl aminopeptidases were also distinguished by their inactivation in the presence of phenylmethylsulfonyl fluoride at a concentration of $1X10^{-3}$M, which might indicate the involvement of a serine residue in the active site of the enzymes.

Significance to the Dairy Industry:

The aminopeptidases and the more recently studied dietpeptidyl aminopeptidases seem to have an important role in the ripening of cheeses. Bartels, et al. (1987) showed that addition of freeze shocked *L. helveticus* CNRZ 32 to Gouda cheese milk led to an acceleration of cheese ripening. The resultant cheese showed better organoleptic properties than cheeses made with other *L. helveticus* or *L. bulgaricus* strains. The major difference between *L. helveticus* CNRZ 32 and the other lactobacilli was the higher number and higher activity of aminopeptidase in *L. helveticus* CNRZ 32. Similar conclusions were reached by El Abboudi, et al. (1990) when they noticed that the only difference in the peptide hydrolase system of bitter and non-bitter strains of *L. casei* was the significantly higher levels of aminopeptidase activity in the non-bitter strains.

The differences in the specificity of the aminopeptidases observed in this work also show that these enzymes can be an important tool in liberating amino acids that favor the development of desirable flavors in cheeses or other food products.

References:

Bartels, M.J., M.E. Johnson, and N.F. Olson. (1987) *Milchwissenschaft,* 42:139-143

El Abboudi, M., M. El Soda, S. Pandian, M. Drapeau, and R. Simard. Brief Communication, Int. Dairy Congress, Montreal, October 1990.

Publications:

El Soda, M., A. Macedo, and N. Olson. Aminopeptidase and Dipeplidyl Aminopeptidase Activities of Several Cheese-Related Microorganisms. *Milchwissenschaft* (to be submitted).

Table 2. Characterization of the Aminopeptidases from several cheese related microorganisms.

Species	Opt. temp.	Opt. pH	Inhibitors	Activators
L. casei	40°C	7.5	OPhe, PHHB	Mg++Co++DTT
L. mesenteroides	30-40°C	7.5	OPhe, PMSF	Mg++Co++
Pediococcus sp.	40°C	7.5	OPhe, PHMB	Mg++Co++DTT
P. shermanii	40°C	7.5	OPhe, PHMB	Mg++Co++DTT
B. linens	30°C	7.5	OPhe, PHMB	Mg++Co++DTT

Table 3. Characterization of the Dipeptidyl peptidases from several cheese related microorganisms.

Species	Opt. temp.	Opt. pH	Inhibitors
L. casei	40	7.5	PMSF
Pediococcus sp.	50	7.5	PMSF
P. shermanii	50	7.5	PMSF

Project Title:

Acceleration of Low Fat Cheese Ripening Using the Crude Cell Free Extract of Some Cheese Related Microorganisms- Influence of Lyophilized Extracts and Frozen Cells on the Rate of Protein Breakdown

Personnel:

M. El Soda, visiting scientist, Center for Dairy Research; Carol Chen, associate researcher, Center for Dairy Research; Brian Riesterer, associate researcher, Center for Dairy Research; N.F. Olson, professor, Center for Dairy Research

Funding:

WITEP Visiting Scientist

Dates:

June 1, 1989 - September 30, 1989

Objectives:

Enzymes and attenuated cells from cheese-related bacteria were used to reduce curing time of cheese. In this project the use of the lyophilized extracts of *Brevibacterium linens, Leuconostoc mesenteroides, Lactobacillus casei, Pediococci,* and *Propionibacterium* for the acceleration of reduced fat cheese is described. The addition of freeze shocked cells of some of the microorganisms is also considered.

Summary:

Reduced fat Cheddar cheese was manufactured using the conventional procedure. The lyophilized extracts were mixed with salt and added to the curd before milling, while the frozen cells were mixed with the milk prior to renneting. The cheese was then analyzed monthly during a ripening period of 6 months.

Influence of Lyophilized Extract on the Rate of Flavor Development in Cheddar Cheese

The addition of the crude cell free extract of *Lactobacillus casei, Propionibacterium, Pediococcus sp., Leuconostoc mesenteroides,* and *Brevibacterium linens* to a reduced fat Cheddar cheese did not affect the composition of the cheese. The values obtained for moisture, fat, and pH were rather similar in the control and enzyme treated cheese.

Figure 1 illustrating the evolution of 12% TCA soluble nitrogen indicates a slight increase in the rate of ripening of enzyme treated cheeses when compared to the control. Flavor and texture evaluation of the cheeses revealed no significant differences in the texture of the control and enzyme treated cheese. No major flavor defects were noticed in the enzyme treated cheeses. A sulfur-type flavor note was noticed in the cheese treated with *Lactobacillus casei* and *Brevibacterium linens,* which may be attributed to methionine aminopeptidase and demethiolase activity in these microorganisms. Bitter flavor evaluation showed that the control was always more bitter. Among the enzyme treated cheeses, the lowest levels of bitterness were detected in *L. casei* and *Propionibacterium* treated cheese, which would indicate a debittering activity in the extract of these organisms. As far as the influence of the freeze shocked cells of *L. casei* and *Pediococci* on the rate of flavor development is concerned (Figure 2), a significant increase in TCA soluble nitrogen in the freeze shocked cells treated cheese was noticed. A 52% increase in proteolysis was found in the 6 month old cheese made with *L. casei* frozen cells. The increase was 33% when frozen *Pediococci* cells were used.

When the crude cell free extract obtained from an equivalent amount of *Lactobacillus casei* cells was added to the curd, no significant increase in proteolysis could be measured. This confirms the results shown in Figure 1. Flavor evaluation of the cheese revealed higher bitter intensity scores in the control cheese compared to the treated cheese. The lowest levels of bitterness were detected in *L. casei* cheese followed by the *Pediococcus sp.* treated cheese. Both *L. casei* and *Pediococci* treated cheeses showed a pronounced acid flavor, and calcium lactate crystals were detected in the interior of *Pediococci* treated cheese after the second month of ripening. This study also revealed that no significant increase in the gross proteolysis of cheese is obtained if the crude cell free

Figure 1. Effect of the lyophilized extract of: (◇—◇) =control; (□—□) = Pediococcus sp.; (■—■) = Propionibacterium shermanii; (○—○) = Brevibacterium linens; (●—●) = Lactobacillus casei; and (◆—◆) = Leuconostoc mesenteroides on proteolysis in Cheddar cheese as measured by TCA soluble nitrogen.

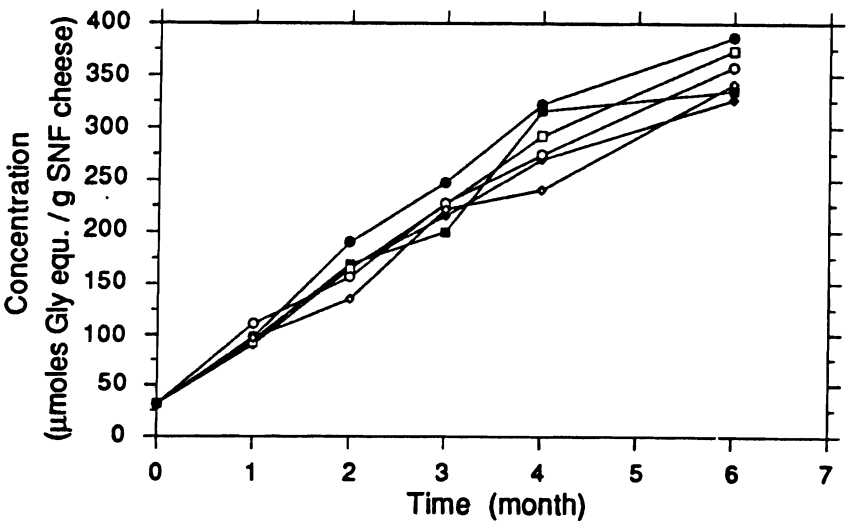
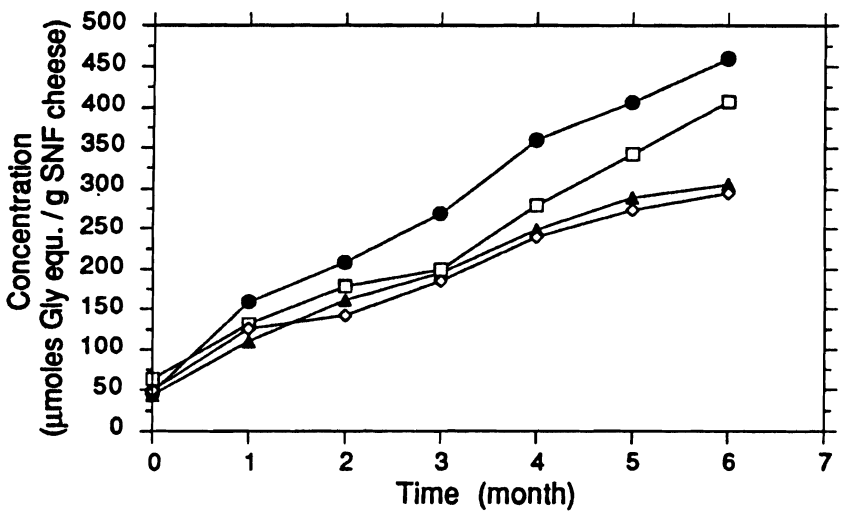


Figure 2. Effect of freeze shocked Lactobacillus casei and Pediococcus sp. and the crude cell free extract on proteolysis in Cheddar cheese. (◇—◇) = control; (□—□) = freeze-shocked Pediococcus sp. cells; (●—●) = freeze-shocked Lactobacillus casei cells; and (▲—▲) = cell-free extract of L. casei.



extract of cheese related microorganisms is added to the curd. However, some influence on flavor was noticeable and is probably due to the low proteolytic activity and fairly high peptidase activity of the cheese related microorganisms. The low levels of overall activity of these extracts in cheese could also be attributed to the inhibition of the free enzymes in the early weeks of ripening due to the low pH and probably to the salt concentration of the cheese. A greater loss of enzyme of the cell free extract may have occurred compared to loss of the attenuated cells.

The significantly higher rates of proteolysis observed in the frozen cells treated cheese are probably due to the action of the cell-wall proteinases of *L. casei* and the *Pediococci* that may have been inactivated. The cell-wall associated proteinases probably are retained in their original cellular position, which could increase their effectiveness in the successive conversion of polypeptides to short-chain peptides.

Significance to the Dairy Industry:

Attenuated cells seem to be a more appropriate tool for accelerated cheese ripening. They however lead to excess acid development or to crystal formation. These problems could be overcome by using Lac - variants or by entrapping the desired enzyme mixtures in microcapsules or liposomes (El Soda, et al. 1989).

References:

El Soda, M., Pannell, L., and Olson, N.F. (1989) *Journal of Microencapsulation*, 3:319-326

Publications:

Chen, C., M. El Soda, B. Riesterer, and N. Olson. Acceleration of Reduced-Fat Cheese Ripening Using Lyophilized Extracts or Freeze Shocked Cells of Some Cheese-Related Microorganisms. *Milchwissenschaft* (to be submitted).

Project Title:

Enzymic Modification of Butterfat in Supercritical Carbon Dioxide

Personnel:

Richard W. Hartel, assistant professor, Dept. of Food Engineering (co-PI); Kirk L. Parkin, associate professor, Dept. of Food Science (co-PI); Theresa White, graduate student; Jerome Elliott, graduate student; Jungro Yoon, Ph.D., post-doctoral researcher.

Funding:

Wisconsin Milk Marketing Board, National Dairy Promotion and Research Board

Funding Code:

88-40, 88-1

Dates:

July 1, 1988 - June 30, 1991

Objectives:

1. Assess the feasibility of using supercritical (SC) fluids as a means to control lipase action on butterfat.

2. Determine the specificity of lipase action on butterfat in SC CO_2 as influenced by the reactor operating temperature and pressure, as well as the level of an aqueous entrainer.

3. Develop a fundamental understanding of how lipase action on butterfat in SC CO_2 may be controlled by determining the reaction kinetics and specificities under various processing conditions.

Summary of Findings:

Research has continued in several areas, including:

- enzymic modification of butteroil in nonaqueous media,
-investigating the solubilities of free fatty acids in SC CO_2,
-determining the extraction capabilities of SC CO_2 for butterfat.

Enzymic Modification of Butteroil in Nonaqueous Solvents

The environmental parameters governing lipase-mediated acyl-exchange reactions with butteroil in nonaqueous media were established at atmospheric pressure. Routinely, reaction mixtures contained 10 ml solvent/ butteroil, 1 g desiccated crude porcine pancreatic lipase powder, and 50 mM free fatty acid (FFA: usually undecanoic acid, C11:0). The pH of the enzyme powder was 6.0, the reaction temperature was 60°C (unless otherwise stated), and the mixtures were magnetically stirred at 950 RPM. Reaction rates were determined as initial velocities by measuring the disappearance of the FFA or the esterification of the FFA to butteroil acylglycerides (AGs).

Hexane Content

Initial studies evaluated the effect of varying hexane content on the rate of FFA esterification to butteroil AGs (Figure 1).

Butteroil Content

Increasing the butteroil content to 5% accelerated the rate of C11:0 esterification to butteroil AGs. Above 5% butteroil, the reaction rate was fairly constant except for a slight decline at 100% butteroil. This decline is probably due to acyl-exchange reactions becoming more favored between butteroil AGs than between free C11:0 and butteroil AGs. An important observation was that no hexane was required for the reaction to take place. Thus, butteroil could act as the dispersing medium for the reaction as well as the substrate. This eliminated the need to use a solvent of questionable toxicity. The remainder of these studies used 100% butteroil in the absence of hexane as the continuous phase.

Enzyme pH

Figure 2 shows the effect of enzyme pH on the reaction rate. The optimum pH was 6.5 to 7.0, with activity declining as the pH was raised to

Figure 1. The effect of varying hexane content on the rate of FFA esterification to butteroil AGs.

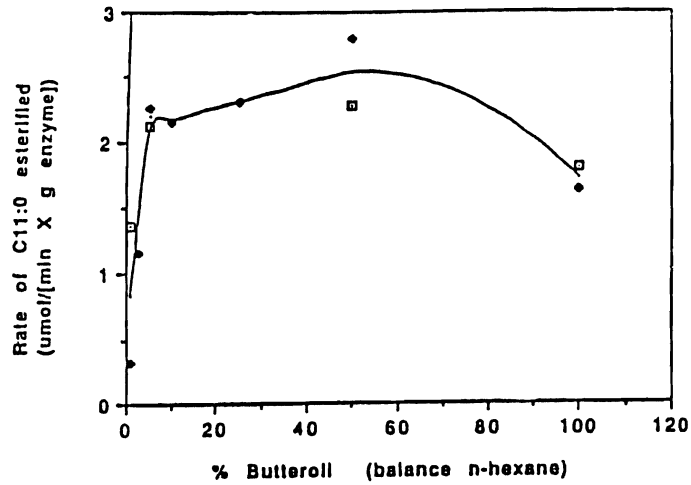


Figure 2. The effect of enzyme pH on the reaction rate.

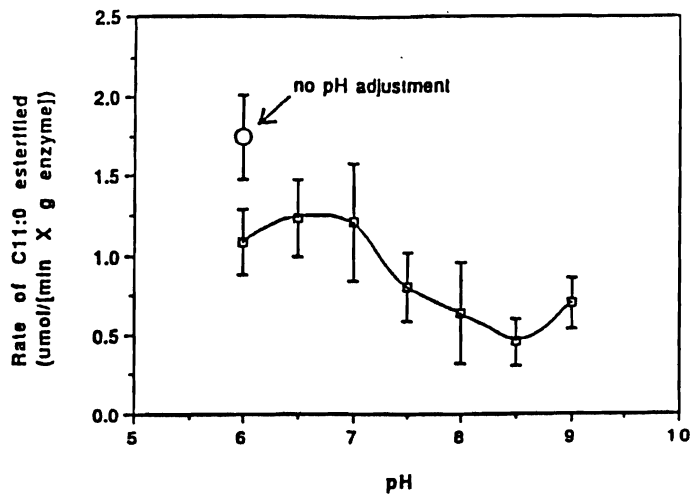


Figure 3. The effect of added water content on the reaction rate.

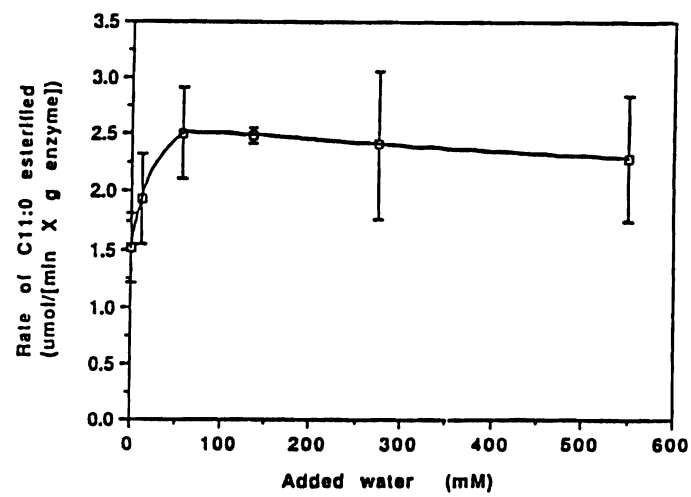


Figure 4. The effect of added water content on the level of FFA accumulation in reaction mixture.

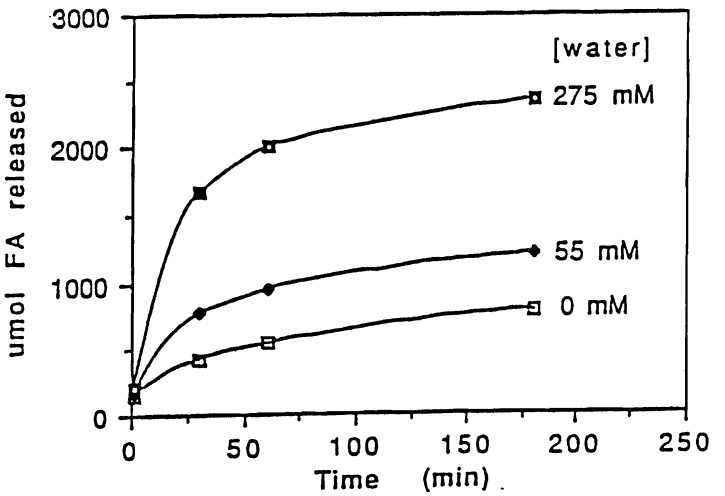


Figure 5. The effect of temperature on reaction rate.

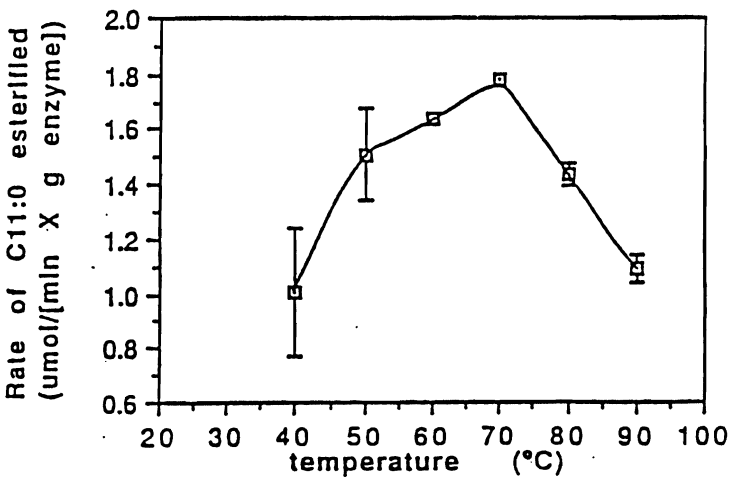
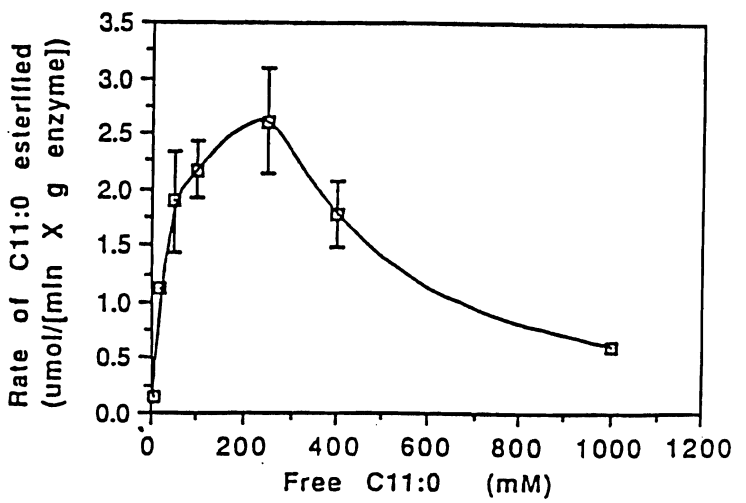


Figure 6. The effect of FFA concentration on reaction rate.



8.5. The steps required to adjust pH (titration and lyophilization) resulted in a loss of enzyme activity of 40%. Therefore, it was judged to be of little value to adjust pH of the enzyme, and it was routinely used without modification (pH of 6.0).

Water Content

Routinely, no water was added to the reaction mixtures. The water content of the desiccated enzyme was 0.93%. As added water content was increased to 50 mM, an acceleration of the reaction was observed (Figure 3), likely due to an acceleration of the initial step in acyl-exchange (hydrolysis). Above 50 mM added water, little effect on initial reaction rate was observed. However, progress curves of the reactions for 0 to 250 mM added water revealed that as added water content was increased, so was the level of FFA accumulating in the reaction mixture (Figure 4). Therefore, the absence of added water in these reactive mixtures was most favorable to acyl-exchange activity.

Temperature Effect on Reaction Rates

The effect of temperature on reaction rate is shown in Figure 5. Optimum temperature for the reaction was 70°C. This was an increase of 20°C over the optimum temperature for lipase hydrolysis of a 2% tributyrin suspension (data not shown). Thus, the enzyme is more thermostable in nonaqueous media than in aqueous media.

The effect of FFA concentration on reaction rate is shown in Figure 6. Optimum rates were observed at 250 mM free C11:0, and substrate inhibition was apparent above these levels. The reaction yield after 18 h of incubation was 81% at 5 mM, 90% at 20 and 50 mM, 86% at 100 mM, 61% at 250 mM, 36% at 400 mM 7.5% at 1000 mM. Recent results indicate that no substrate inhibition is apparent for acyl-donors (methyl and glycerol esters).

These results are essential to the selection of environmental parameters that will favor lipase-mediated acyl-exchange reactions involving butteroil in SC CO_2. This is the next stage of this project.

Enzymic Modification of Triglycerides in SC CO_2

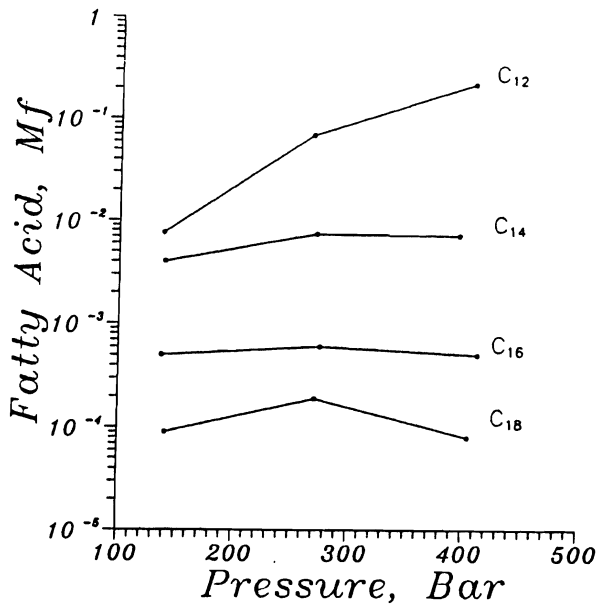
Experiments are underway to investigate the enzymic reactions on model triglyceride systems in SC CO_2. Preliminary experiments are being conducted to determine the solubilities of important triglycerides in SC CO_2 at various operating pressures and temperatures. Solubilities of triglycerides, tripalmitin and tristearin, are being quantified using the same experimental apparatus and technique as described previously for measuring free fatty acid solubilities. Results to date indicate that the solubilities of these triglycerides are much lower than the solubilities of the corresponding free fatty acids. Again, the solubility of the lower molecular weight triglyceride, tripalmitin, is higher than that of the tristearin. Studies on the lipase activity on these triglycerides is currently underway.

Solubilities of Free Fatty Acids

Investigations continue on determining the solubilities of free fatty acids and triglycerides in SC CO_2 at various temperatures and pressures. This information is necessary for optimizing enzymic reactions on butterfat in SC CO_2 and possibilities for subsequent fractionation of reaction products. Several experimental methodologies were investigated for determining fatty acid solubilities. Initial results showed that different experimental techniques resulted in different solubility values under certain conditions. The final methodology selected, based on results for solubility of naphthalene in SC CO_2 as well as on previous results on solubilities of free fatty acids, involved a flow through column for extracting fatty acids followed by gravimetric analysis of the extracted phase. The extracted fatty acid was collected and weighed after expansion of the CO_2 to atmospheric pressure. The CO_2 flow was totalized to determine the solubility values.

Results on free fatty acid solubilities in SC CO_2 as a function of pressures at 35°C are shown in Figure 7. It can be seen that the solubility was generally constant with increasing pressure over the range studied with the exception of lauric acid. At higher temperatures (45 and 55°C), solubilities were seen to increase

Figure 7. Solubilities (in mole fractions) of free fatty acids (C12 to C18) in supercritical CO_2.



slightly with increasing pressure. Increases in temperature resulted in significant increases in solubility. For example, the mole fraction of stearic acid (C18) at 35°C was about 10^{-4} whereas, at 55°C, the solubility mole fraction ranged between $1(10^{-3})$ to $5(10^{-2})$. It was also seen that the solubility of various free fatty acids increased as the carbon chain length of the fatty acid decreased. That is, the shorter the chain length of the fatty acid, the higher the solubility. This is due primarily to the molecular weight dependence of solubility.

It was also found that a supercritical end point was reached for capric acid at all pressures and temperatures studied and for lauric acid at the higher temperatures and pressures. This phenomenon was demonstrated by the different values of extraction concentration found when different experimental techniques were used. At these conditions, the fatty acid becomes essentially miscible with the SC CO_2 and the concentrations observed depend on how the CO_2 and fatty acid were mixed rather than on an equilibrium condition. This phenomenon has been suspected for free fatty acids based on previous research. The exact limits of this supercritical endpoint could not be determined with the existing equipment.

Extraction of Butterfat in SC CO_2

Experiments continue on the extraction of butterfat by SC CO_2 using a flow through extraction apparatus. Details concerning the extraction parameters are important in future development of enzyme processing systems. The rate of extraction at various conditions is being studied in addition to the physical and chemical characterization of the extracted components. In these experiments, butterfat is loaded onto glass beads which are then packed into an extraction column. SC CO_2 is allowed to flow through the extraction vessel at a slow rate of flow such that equilibrium conditions are attained. Fractions (up to 12 fractions per run) of extracted material are collected in a sample tube following the expansion of the SC CO_2 through a pressure reduction valve. The amount of CO_2 collected with each fraction is monitored using a mass totalizer. These experiments allow determination of the extractable components of butterfat in SC CO_2 at various operating conditions. The rate of extraction of these components can also be quantified. Physical and chemical properties of these fractions are then determined using such techniques as DSC for melting curves, NMR for solid fat content, and GC for chemical analysis.

Figure 8. Butterfat vs. SC CO_2 at 40°C and 300 bar.

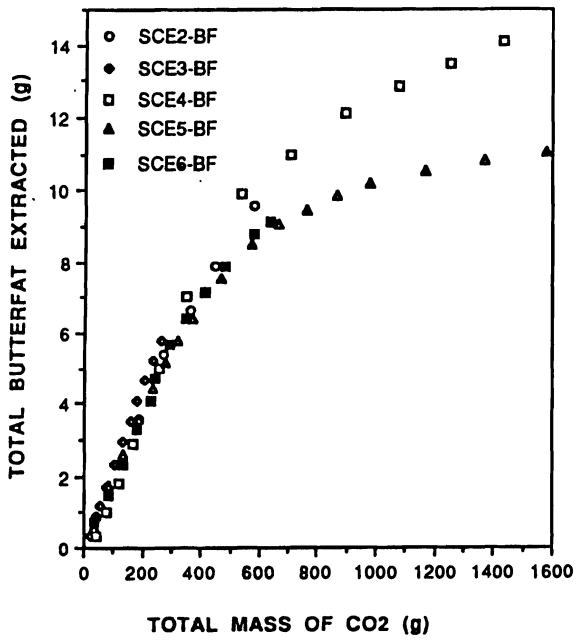


Figure 9. Change in melting properties of the fractions (as seen on the DSC) with change in order of extraction.

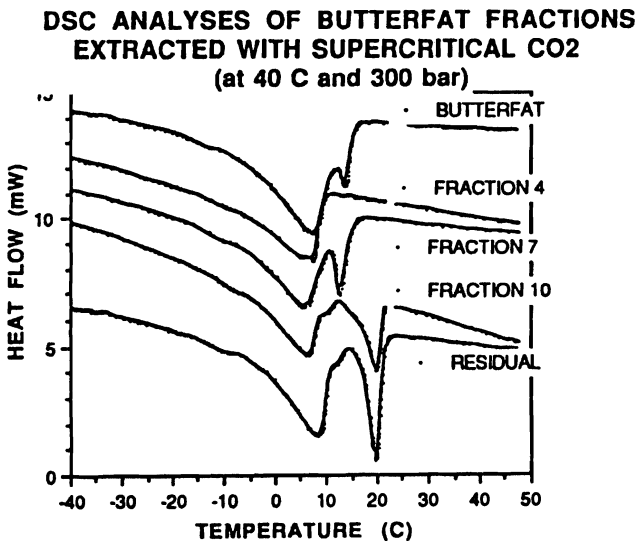


Figure 8 shows the extraction process at 40°C and 300 bar. The rate of extraction of butterfat is constant for an initial period followed by a decreasing rate of extraction. This decreased rate is due to the depletion of the more volatile components in the original butterfat. Figure 9 shows how the melting properties (as seen on the DSC) of the fractions change with the order of extraction. It can be seen that the butterfat components that exhibit the lower melting behavior are preferentially extracted in the initial stages while the latter fractions contain a higher degree of the components that exhibit higher melting point behavior. GC analyses of these fractions are underway to help in understanding which components of the butterfat are responsible for this behavior.

Future work will focus on the effects of operating pressure and temperature on the extraction rates as well as on the properties of the fractions. These fractions will then be evaluated for use as substrate for enzymic reactions in SC CO_2.

Significance to the Dairy Industry:

The results of the enzyme reaction experiments demonstrate that lipase-mediated acyl-exchange reactions with butteroil in nonaqueous environments are possible. No organic solvent is required and butteroil can act as the dispersing medium. Thus, the use of solvents of questionable toxicity and the need to remove solvent after butteroil modification can be avoided. The temperatures at which the reaction takes place are conducive to pasteurizing the product. These results will be of value in directing specific modifications of butteroil to obtain value-added products.

Presentations:

1. Elliott, J.M., M. Mani, S-J. Kuo and K.L. Parkin. 1990. "Lipase-mediated transesterifications of butteroil in nonaqueous media," presented at the IFT Annual Meeting, Anaheim, CA, June 16-20, 1990.

Publications/Theses:

1. Elliott, J.M. 1990. Modification of butteroil by lipase-catalyzed acyl-exchange reactions in anhydrous media, MS Thesis, UW-Madison.

2. White, T.M. 1990. Solubilities of free fatty acids in supercritical CO_2, MS Thesis, UW-Madison.

Project Title:

Use of Immobilized Enzymes in the Treatment of Milkfat

Personnel:

C. G. Hill, Jr., professor, Dept. of Chemical Engineering; H. R. Reyes and F. X. Malcata, graduate students, Dept. of Chemical Engineering; H. S. Garcia, graduate student, Dept. of Food Science.

Funding:

National Dairy Promotion Research Board, CONACYT (Mexico), INVOTAN (Portugal), IFT, AT&T.

Funding Code:

89-3

Dates:

July 1, 1989 - June 30, 1992

Objectives:

The objective of this study is to perform a preliminary assessment of the technical feasibility of utilizing immobilized enzyme technology to bring about hydrolysis and interesterification reactions so as to change the composition of milkfat. Specific tasks to be accomplished include:

-Development of appropriate experimental procedures for the immobilization of selected lipases,

-accumulation of kinetic data for model systems,

-development of rate expressions to fit the data and,

-simulation of system performance.

Summary of Findings:

Our experimental effort to characterize the enzymatic modification of milkfat has involved three components. H.S. Garcia focused on the use of emulsified systems to effect the hydrolysis of milkfat. F.X. Malcata used a membrane reactor in which a membrane containing an immobilized enzyme is used to bring about the hydrolysis reaction. H. R. Reyes has been studying interesterification reactions in a model system. Results obtained in each of these three components of our program are described below.

Enzymatic hydrolysis in an emulsified system (Garcia):

First, a known analytical technique for quantification of free fatty acids was adapted and modified so as to permit us to determine all major fatty acid constituents of the process streams of interest. This analytical protocol uses a three-component mobile phase, where the nonpolar character is provided by methanol instead of acetonitrile.

Characterization of the enzyme indicated that its optimum temperature lies close to 35° C. The activation energy for the thermal inactivation was found to be 49.4 kJ/mole, a value which is comparable to that obtained by our group for the immobilized enzyme. A study of the effect of different salts on enzyme activity showed that the presence of monovalent cations, such as Na+ and K+, decreases the original activity by 10%-20%, whereas the presence of some divalent cations, such as Mg^{+2}, Ca^{+2}, Ba^{+2}, and Fe^{+2}, promoted an increase in activity ranging from 200% to 335% of the activity of the enzyme in the absence of other ionic species. Mercuric ions did not significantly affect the performance of the enzyme. On the other hand, the enzyme was fully inhibited by Fe+3 and Cu+2 ions when these ions were present at a concentration of 0.025 M.

The specific surface area (SSA) of the oil/water emulsion was measured by laser scattering. The effect of increased homogenization pressure on the size distribution of the fat globule was determined. Enzyme activity was highly correlated with the SSA. Using SSA as a measure of substrate concentration, we calculated values of Km and Vm on the basis of the Michaelis-Menten model for each of the

major fatty acids in milkfat. Values for Vm varied considerably because of their dependence on the concentrations of the individual fatty acids. Values for Km fell within a narrow range (0.16 to 0.34 m^2/ml).

Based on HPLC analyses, it was found that at intermediate values of pH, the enzyme showed differences in the selectivity for hydrolysis to specific fatty acids. Short chain fatty acids (C4-C8) exhibited a different pH optimum (~ 5) than that for the overall hydrolysis (6.0-7.0 for total fatty acids released), see Figure 1 for butyric acid and total fatty acids. Protonation of one or more amino acids residues associated with the active site, or possibly protonation of product species to facilitate release from the active site could be responsible for this enhanced selectivity toward the short chain (more polar) fatty acids. This hypothesis, along with the high stability of the enzyme observed at mildly acidic conditions, could help to establish criteria for selection of the operating conditions (pH) employed in industrial utilization of the enzyme.

Hydrolysis catalyzed by an immobilized lipase in a membrane reactor (Malcata):

The major thrust of this component of the research involves technical and economic assessments of the feasibility of a continuous process for the production of lipolyzed butterfat using an immobilized lipase. Virtually all processes dealing with accelerated enzymatic hydrolysis of butterfat use batch addition of crude lipases to an emulsified substrate followed by incubation and thermal treatment. Immobilized lipases can be employed to accelerate the lipolytic process. We believe that such technology is preferable to the more conventional batch addition process.

This research is of interest because there is a world surplus of butter, coupled with a trend towards consumption of increasing amounts and varieties of cheese-flavored products. In addition, more stringent specifications as to the characteristics and quality of dairy products are being demanded by the consumer.

To date, a complete literature search has been conducted relevant to the utilization of immobilized lipase technology for hydrolysis reactions and the use of lipases for flavor generation. A lipase produced by a strain of *Aspergillus niger* (APF-12™ from Amano International Enzyme Co.) has been found to be particularly suitable for lipolysis of butterfat because it selectively catalyzes the liberation of medium chain fatty acids from triglycerides and it possesses GRAS status. Two three-phase reactor configurations have been studied: a reactor similar to a plate-type blood dialyzer, and a hollow-fiber reactor. The distinctive aspect of these reactors is the utilization of two microporous hydrophobic

Figure 1. Effect of pH on the initial rate of release (Vo) of butyric acid (—Δ—) and total fatty acids (—O—). Hydrolysis as a 20 % butteroil emulsion in the presence of lipase APF-12 at 40°C.

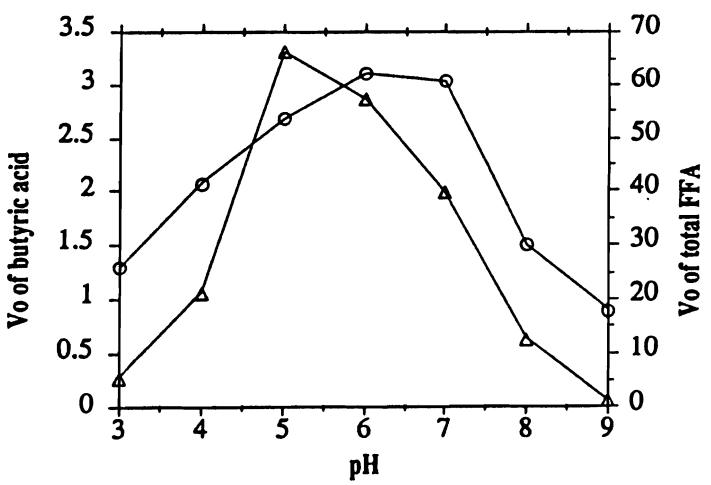


Figure 2. Lipase activity removed from solution plotted as a function of the activity of the supernatant solution.

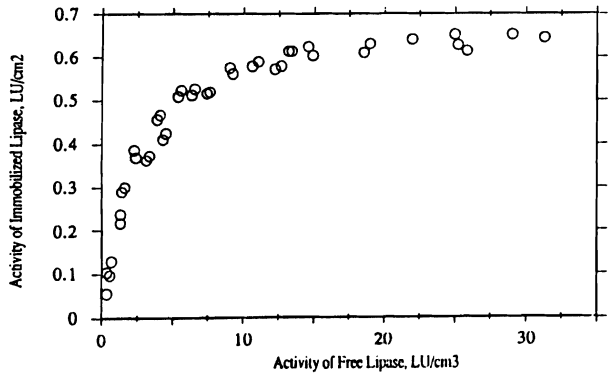
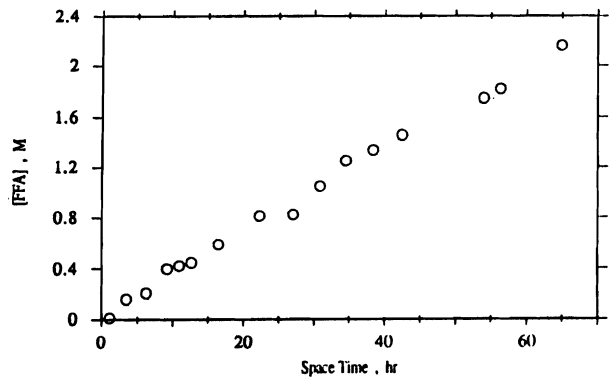


Figure 3. Release of free fatty acids versus the reactor space time at pH 7.0 and 35°C.



polypropylene flat-sheet membranes (Celgard 2500™) or hollow fibers (Celgard X10™, both from Hoechst Celanese) located at the oil/water interface. The lipase is immobilized on these membranes by physical adsorption. The adsorption follows a Langmuir-type isotherm as shown in Figure 2.

Experiments conducted with the flat-sheet membrane reactor at 35°C have demonstrated that up to 75% release of fatty acids from the sn-1,3 positions of triglycerides can be accurately modeled by simple zero order kinetics (Figure 3). Leakage of lipase is negligible in time frames of the order of the half life of the adsorbed lipase. The support can be easily reloaded with lipase after it has been thoroughly washed with ethanol. The rate of release of free fatty acids as a function of the pH of the buffer follows a diprotic Dixon-Webb model. Multi-response analysis of the experimental data on the product composition indicates that the rate of release of each free fatty acid residue has a bell shaped dependence on the number of carbon atoms in said residues. The curve is centered at C8:0.

The reactor designs selected are particularly suitable because:

-the need for addition of emulsifiers coupled with vigorous stirring no longer exists,

-natural feedstocks of butterfat with low levels of particulate material can be processed,

-there is no contamination of the product via residual lipase,

-multiple use of the lipase is possible,

-there is no requirement for thermal treatment of the effluent stream,

- opportunities for better control of both the process and product quality are enhanced, and

- auxiliary equipment requirements include only pumps and reactor jackets employing a low temperature heat transfer fluid.

Since there are no diffusional limitations in the reactor design chosen, the kinetic parameters determined using the laboratory apparatus can be readily used in the design and simulation of commercial scale systems.

Planned experimental work includes the study of the performance of the hollow-fiber reactor over a range of pH values and temperatures. Once this modeling stage is completed, an economic assessment of the technology will be carried out.

Enzymatic interesterification of triglycerides (Reyes):

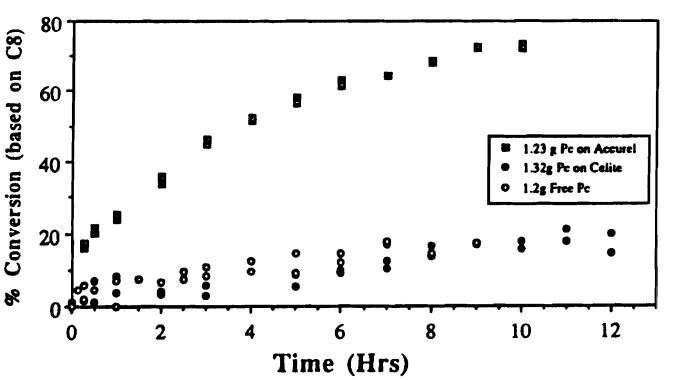
To facilitate our analyses and for economic reasons, we have opted to work with a model triglyceride system (olive oil) which contains many of the same triglycerides as milkfat, particularly those which are most abundant. We have selected octanoic acid as the fatty acid reactant for the interesterification process. The reliability of kinetic data for the interesterification reaction of olive oil and octanoic acid has been improved appreciably. A quick and efficient method for determining the concentration of free fatty acids released by reaction was developed via modification of a procedure developed by Prof. K. Parkin and his students in the Food Science Department.

The catalytic effectiveness of a new enzyme and a new support were studied. Lipase from the bacterial species *Pseudomonas cepacia* was obtained from Amano International. A generous sample of a microporous polypropylene support (Accurel powder) was donated by Akzo.

The interesterification activities of free lipase, and lipases immobilized on both Celite (diatomaceous earth, the traditional support), and Accurel were tested in well-stirred reaction vessels. Figure 4 contains plots of conversion versus time data for these systems. One can see that the Accurel support enhances the activity by a factor of 5 to 10.

Kinetic data for the *Pseudomonas cepacia*-polypropylene (Pc-PP) system were obtained under a variety of conditions. Data have been collected at the several temperatures (40, 50,

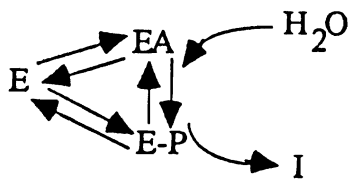
Figure 4. Interesterification reactions of octanoic acid/olive oil (1:2 molar) as catalyzed by free and immobilized lipases from Pseudomonas cepacia (Pc) at 50°C. No water added to the enzyme.



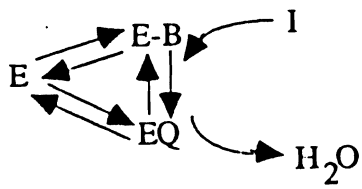
60, and 70°C) and molar ratios of octanoic acid to olive oil (0.5, 1, 2, 3). Another variable was the initial content of water in the mixture of Pc-PP and olive oil/octanoic acid. It ranged from 50 µL added to the Pc-PP and mixed with 65 ml of the molecular sieve-dried organic mixture to 350 µL added to the Pc-PP and mixed with 65 ml of the water saturated organic mixture. Karl-Fischer data for the solubility of water in olive oil/octanoic acid mixtures have been obtained. To date the results have been encouraging. Conversions as high as 75 percent have been observed in reaction times of 14 hours or less.

We will attempt to model these data using the following kinetic mechanism as a basis :

Hydrolysis Step



Reesterification Step



where E = enzyme
A = native glyceride
P = fatty acid released
B = incorporated fatty acid
Q = new glyceride
I = lower glyceride intermediate

For this purpose we are in the process of implementing a nonlinear regression algorithm on the VAX computer of the Chemical Engineering Department.

The next experimental steps of the project will involve determinations of the thermal stability of the lipase and assessments of the activity the Pc-PP system in mixtures of butter oil and octanoic acid or linolenic acid.

Publications:

García, H.S., H. R. Reyes, F. X. Malcata, C. G. Hill, Jr., and C. H. Amundson. Determination of the Major Free Fatty Acids in Milkfat Using a Three-Component Mobile Phase for HPLC Analysis, accepted for publication in *Milchwissenschaft*.

Malcata, F.X., H.R. Reyes, H.S. García, C.G. Hill, Jr., and C.H. Amundson. Determination of the Major Free Fatty Acids in Milkfat Using a Three-Component Mobile Phase for HPLC Analysis, accepted for publication in *Milchwissenschaft*.

Project Title:

Thermodynamic Modeling of Lipids in Supercritical Carbon Dioxide

Personnel:

P. D. McMahon, assistant professor, Dept. of Chemical Engineering; and B. Y. Hwang, graduate student, Dept. of Chemical Engineering

Funding:

National Dairy Promotion and Research Board

Funding Code:

88-2

Dates:

July 1, 1988 - June 30, 1990

Objectives:

Develop a model to predict fatty acid and triglyceride mixture solubilities in supercritical carbon dioxide, and to predict the extent of fractionation at various operating conditions using an equation of state model to describe the pressure-density-temperature effects and a semi-continuous mixture formulation to describe the compositional dependence.

Summary of Findings:

Correlation of the Solubilities in Supercritical Carbon Dioxide of Milkfat Fatty Acids and Triglycerides

The solubilities in supercritical carbon dioxide of milkfat fatty acids and triglycerides have been correlated using the Soave-Redlich-Kwong (SRK) cubic equation of state. Equation of state interaction parameters have been determined, and the optimum lipid phase models identified. The formalism of continuous thermodynamics was used to extend the models to predict the solubilities of continuous distributions of milkfat lipids, and several example calculations were carried out.

The SRK equation represents supercritical solubility behavior of fatty acids and triglycerides reasonably well. For fatty acids, we found that a liquid model for the solute phase best fits the binary solubility data. For triglycerides, a solid model for the solute phase fits the binary solubility data very well. For supercritical fluid-solid equilibria in multicomponent systems, the lipid-lipid binary interaction parameter in the equation of state is usually not needed. For the system of trilaurin and trimyristin, a solid solution model is more appropriate.

Description of Lipid Composition Using a Schultz Distribution

We found that the continuous thermodynamic method of moments using a Schultz distribution to describe the lipid composition is easily applied to scf-lipid solubility calculations and qualitatively predicts the expected behavior. In practice, the lipid composition of milkfat cannot be represented by a simple Schultz distribution. However, in such cases one can usually describe the lipid compositions as a combination of distributions, each having its own characteristic parameters. As more data becomes available, this extension could be applied to milkfat where one might, for example, want to distinguish between saturated and unsaturated triglycerides.

Lack of Data

Finally, this research has shown that although computational tools for modeling complex milkfat mixtures are now available, there still is a critical shortage of accurate experimental data. More solubility data is needed for individual triglycerides to determine the carbon number dependence of the CO_2-lipid interaction parameters. Data is also needed at several different temperatures to determine the temperature dependence, if any, of the equation of state parameters. And more information is needed about the physical state of lipid mixtures at high pressures. This would allow a choice between the mixture of pure solids model, the ideal solid solution model, or a more complex model in which some lipids—such as low molecular weight triglycerides—

are present in solid solution while others are not. Finally, the range of compounds studied must be increased to include mixed triglycerides, both saturated and unsaturated.

Significance to the Dairy Industry:

Separation processes based on the properties of supercritical fluids has the advantage for the food industry of effecting separations at relatively gentle temperatures, thereby avoiding thermal decomposition or oxidation of food products and the loss of natural flavors. They have the further advantage that both temperature and pressure can be used to manipulate solubilities, whereas most other separation methods are insensitive to pressure. Recently it has been suggested that supercritical processing may find an important new application in the dairy industry where there is considerable interest in the enzymatic modification of milkfats. By altering the lipid distribution of milkfats it may be possible to achieve more desirable physical properties; carrying out the process in a supercritical solvent allows for additional reaction control and provides a ready separation technique. A barrier to these new applications is that little work has been done on the properties of milkfat in supercritical solvents. This work has developed the thermodynamic models needed for the design of supercritical extraction systems for milkfats that use carbon dioxide as the supercritical solvent: CO_2 is particularly favored for food applications because it has a low critical temperature (31.1° C) and is non-toxic, nonflammable, relatively inert, and inexpensive.

Publications and Theses:

B. Y. Hwang and P. D. McMahon, 1990. Modeling Milkfat Lipid Solubilities in Supercritical Carbon Dioxide. Submitted to *J. Food Proc. Engr.*

B. Y. Hwang, 1989. Thermodynamic Modeling of Fatty Acids and Triglycerides in Supercritical Carbon Dioxide. M.S. Thesis, University of Wisconsin.

Project Title:

Development of Improved Processes for Enhanced Melt Properties and Flavor Stability of Cold-Spreadable Butter and Other Dairy-Based Spreads

Personnel:

Robert C. Lindsay, professor, Dept. of Food Science (co-PI); and Richard W. Hartel assistant professor, Dept. of Food Science (co-PI), Kerry Kaylegian, graduate student, Dept. of Food Science, Dan Grall, graduate student, Dept. of Food Science

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-56

Dates:

July 1, 1988 - June 30, 1991

Objectives:

1. To devise processes using controlled crystallization and/or supercritical carbon dioxide extraction to obtain a range of fractions from milkfat ranging from very low-melting to very high-melting.

2. To investigate and characterize the fundamental molecular structure of the milkfat fractions, and characterize thc crystallization habits of fractions that are suitable for incorporation into cold-spreadable butter.

3. To investigate means for efficiently stabilizing the flavors of milkfat fractions to be used in cold-spreadable butter.

4. To devise processes to yield improved cold-spreadable butter that exhibits suitable cold-spreadability and flavor stability.

Modification of Objectives:

The scope of the project was broadened to include the characterization of a variety of milkfat fractions obtained through existing milkfat fractionation procedures. Analysis of chemical, physical, and functional properties of the fractions, plus evaluations of selected applications in the food products industry, will be carried out. One application would be improved spreadability of butter.

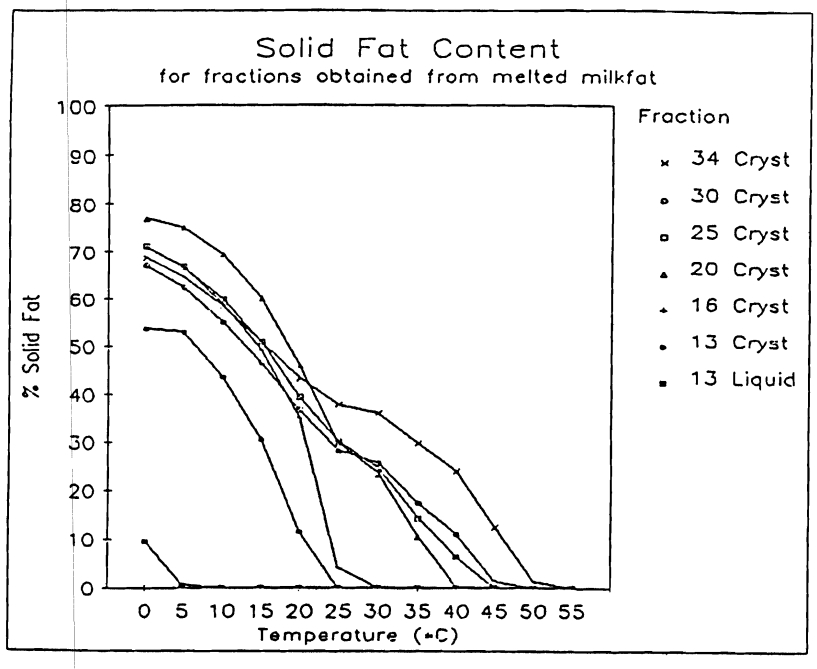
Summary of Findings:

The research has focused on Objectives 1 and 2 which are related to the development of controlled crystallization and extraction methods for separation of milkfat fractions, and the characterization of the fundamental physical and chemical properties of the resulting fractions.

The approaches for fractionation of milkfat have included crystallizations of fractions from molten milkfat and from solutions of milkfat in acetone solvent, and through supercritical carbon dioxide extractions. The first series of fractions were obtained by stepwise fractionation from the melt, creating a series of fractions that exclude the triglycerides which crystallize in any fraction of crystals formed at a higher temperature. This gives fractions having greater homogeneity than those obtained from a single temperature condition-type of crystallization. For example, a sequentially obtained 20°C fraction will not have those higher melting glycerides, which have been removed at 25° and 30°C crystallization. Conversely, fractions obtained from a single condition crystallization at 20°C will have the higher-melting glycerides contained in the crystals formed which change their compositional and functional properties compared to the sequential approach. Fractions from both of these approaches, to obtain crystals from the melt, were obtained for the range 13°- 34°C.

To obtain more homogeneous fractions, crystallization from a solvent is greatly preferred to crystallization from the melt. Considerable carry-over of liquid fat is inherent in the melt crystallization method because of the physical trapping of liquid fat in solid crystals. Even with efficient vacuum filtration, the liquid fat is carried into the solid fraction at each temperature. Crystallizations from

Figure 1. Solid fat contents of milkfat fractions.



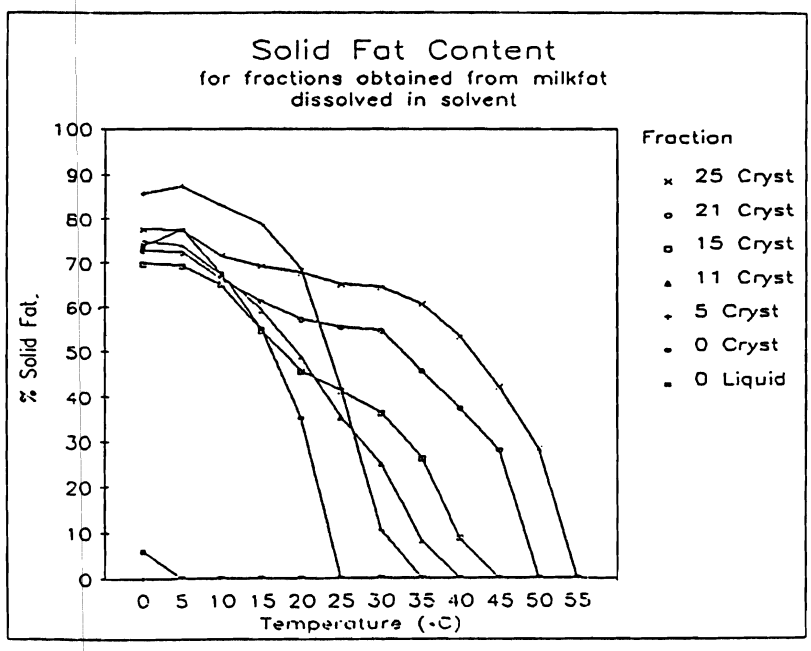
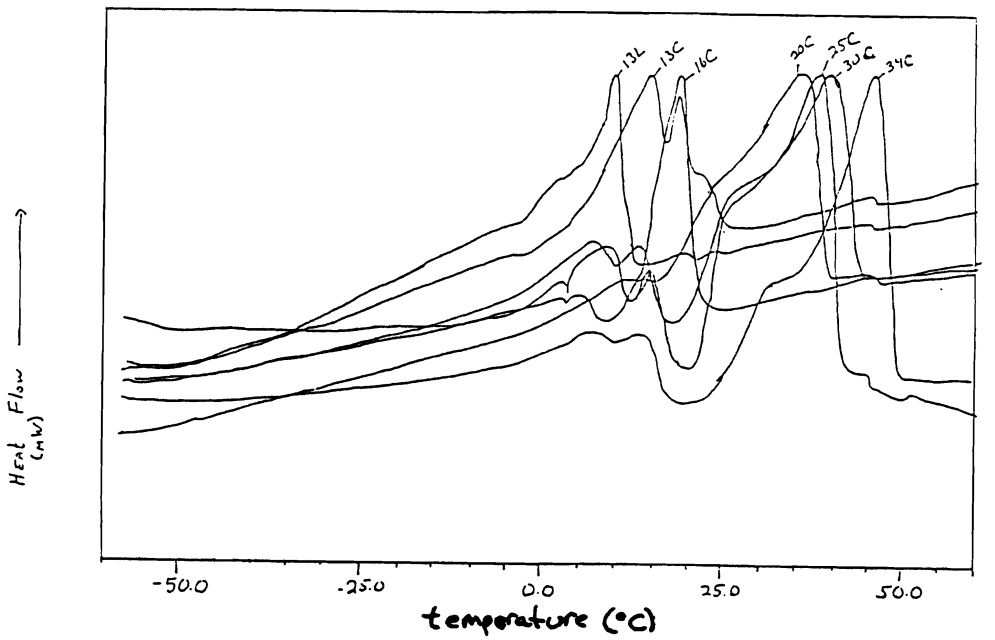


Figure 2. DSC (Differential Scanning Calorimetry) thermal profiles for fractions obtained by stepwises fractionation of molten milkfat.



acetone solutions were carried out using the sequential procedure, and this gives more homogeneous fractions from a melting point standpoint. Single condition separations will be carried out later, but they will be more hetereogeneous than the sequential crystals because of the above mentioned inclusion of all higher melting components in a given fraction.

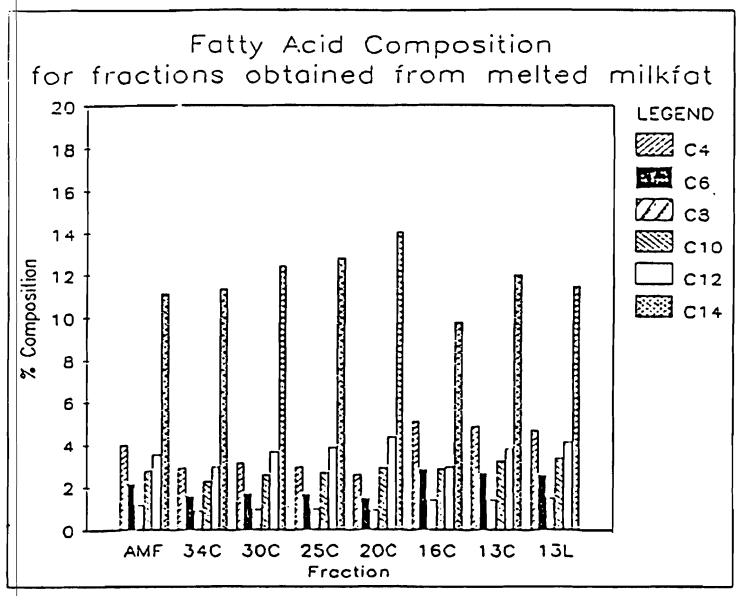
Milkfat was fractionated by supercritical carbon dioxide extraction using pressures of either 350, 300, 250, 200, or 150 bar and temperatures of 33°, 40°, 50°, or 60°C. Residual and extract samples were obtained from the conditions included in the experiments.

The analysis of the fractions creates extensive data, and illustrations of each are provided to demonstrate the ranges of physical and chemical properties that are characterized. The solid fat index of fractions is an extremely useful measurement to assess the functionalities of fractions during processing, handling, and consumption. Initially, traditional solid fat index measurements were attempted, but these were too laborious, inaccurate, and did not give the true solid fat content. Procedures were adapted for the measurement of true solid fat content of fat fractions using Pulsed Nuclear Magnetic Resonance (PNMR). Data in Figures 1A and 1B show the rather dramatically different profiles of percent solid fat in the total that is obtained for fractions from the melt (1A) and those from acetone solution (1B).

Milkfat fractions will also be characterized by Differential Scanning Calorimetry (DSC) whereby the phase transitions or crystal melting are detected during programmed heating of the samples. Data in Figure 2 illustrates milkfat fractions obtained from a sequential fractionation show different temperatures of maximum heat uptakes and different shapes of curves which reflect fundamental alterations in the physical properties of the fractions. It is noteworthy that the melting points of these fractions, determined by traditional methods, show considerably higher values than the temperatures at which the individual samples were correspondingly fractionated.

Fatty acid concentrations in the milkfat fractions provide information about the composition of the triglycerides; data in Figure 3 show the fatty acids obtained in the fractions ob-

Figure 3. Fatty acid compositions of milkfat fractions obtained from sequential crystallizations of milkfat.



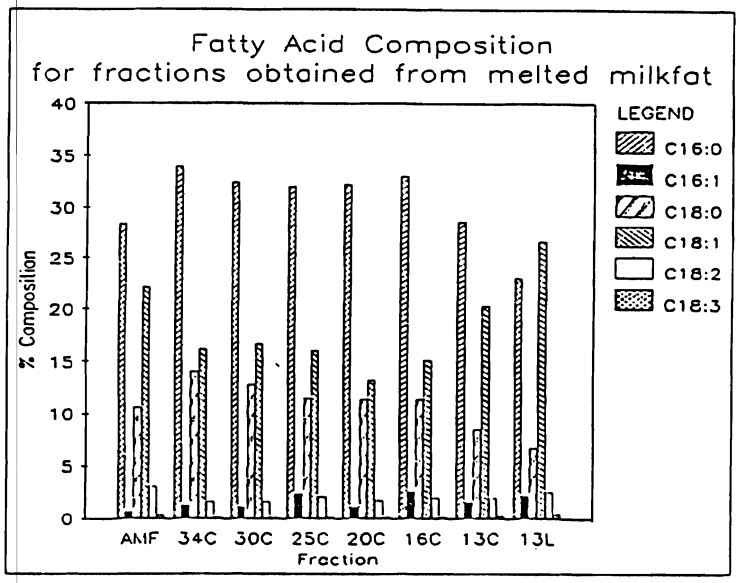
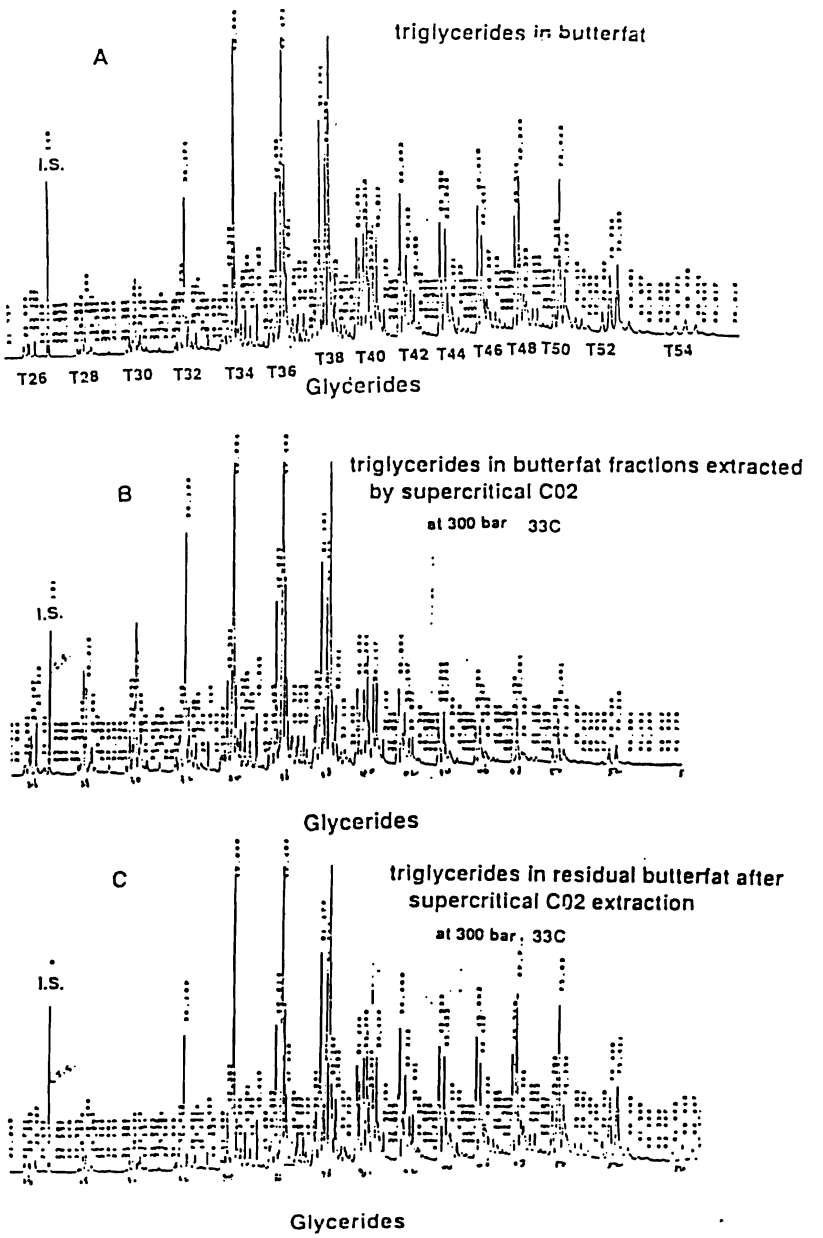


Figure 4. Triglyceride profiles of milkfat fractions obtained by supercritical carbon dioxide extraction of milkfat.



tained from sequential milkfat crystallization from melted milkfat. Such information can provide direction for milkfat fractions containing elevated levels of volatile fatty acids that can be released to provide enhanced flavors. Similarly, fractions with higher concentrations of saturated fats can be directed towards their most valuable uses. While the fatty acid distributions yield very useful data, they do not provide information about the composition of individual triglycerides in the fractions obtained from the separation methods. Capillary gas chromatography can separate individual triglycerides by the sum of carbon atoms in each of the member triglycerides. Data in Figure 4 show the type of information obtained from these analyses for fractions obtained from supercritical carbon dioxide extraction under 300 bar pressure and 30°C operating condition. Comparing Figures 4B and 4C, it can be seen that the carbon number of the extracted fraction in the lower ranges (T28-T34) are greater than for the residual fraction. Conversely, the higher carbon number fractions contain greater amounts of glycerides in the residue than in the extracted fraction.

Significance to the Dairy Industry:

These fundamental data of milkfat fractions provide information that allows selection of fractions that are functional in food formulations. Further, the profiling data on these fractions will allow matching and selection of fractions that simulate the valuable melting and textural characteristics of high-value fats.

A milkfat fraction data bank will be developed and will describe the practical, functional properties of the various fractions for potential food industry uses. The use of milkfat could be expanded through increased incorporation of specialty milkfats into modern foods, such as microwave foods. The project will also provide technology and information on improved methods for the fractionation of milkfat so that the dairy industry can provide specific fractions to other segments of the food industry.

Publications:

Lindsay, R. C. 1989. Milkfat as a source of concentrated dairy flavors. In: Proceedings of the Conference on Milkfat: Trends and Utilization, Dairy Research Conference, Madison, WI, Center for Dairy Research, University of Wisconsin-Madison, Madison, p 31-39.

Kaylegian, K. and R. C. Lindsay. 1989. Milkfat fractionation (Abstract). Institute of Food Technologists, Midwest Food Processing Conference, LaCrosse, WI .

Kaylegian, K. and R. C Lindsay. 1989. Milkfat fractionation: Investigation of cold-spreadable butter (Abstract). Proceedings of the Cheese Research Technology Conference, Center for Dairy Research, University of Wisconsin-Madison, March 29, p.116.

Project Title:

Enzymatic Modification of Butterfat in Reversed Micelle Systems with a Phospholipid as the Surfactant

Personnel:

J.P. Chen, assistant scientist, Center for Dairy Research (PI); H. Pai, graduate student, Dept. of Food Science

Funding:

National Dairy Promotion and Research Board
Wisconsin Milk Marketing Board: Basic Research

Funding Codes:

89-2 (NDPRB)
89-29 1B (WMMB)

Dates:

July 1, 1989 to June 30, 1990

Objectives:

1) To characterize the properties of *Candida cylindracea* lipase in reversed micelles of lecithin for hydrolysis of milkfat.

2) To investigate the influences of system parameters on enzymatic activity of lipase in both solvent-free and isooctane reversed-micelle systems.

Summary of Findings:

New System for Hydrolyzing Milkfat Using Reversed Micelles

Controlled hydrolysis of milkfat by lipase is applied in the dairy industry to produce lipolyzed milkfat with butter-like or cheese-like flavor. A new system for hydrolyzing milkfat was developed in this study by using a micro-emulsion system containing reversed micelles. Reversed micelles are thermodynamically stable nanometer-scale aggregates of amphiphilic molecules solubilizing aqueous drops in a continuous hydrophobic medium (usually an organic solvent). The hydrophilic "head" groups of the surfactant molecules surround the aqueous droplet, while the hydrophobic surfactant "tails" protrude into the hydrophobic medium enveloping the micelles. Enzymes can be solubilized within the reversed micelles with the retention of their catalytic activities.

Previous attempts to modify oils or fats by enzymatic hydrolysis or interesterification in reversed-micelle systems have been almost exclusively conducted in an AOT/isooctane system where an anionic synthetic surfactant, sodium bis(2-ethyl)sulfosuccinate (AOT), was used with isooctane as the hydrophobic medium. We have tried to investigate a different reversed-micelle system for milkfat hydrolysis in this study. A natural surfactant (soybean lecithin) was chosen instead of the commonly used AOT due to the possible toxicity effects of synthetic surfactants and the intended food uses for the end products. Also, we found that it was possible to carry out lipolysis reactions in a solvent-free reversed-micelle system by replacing the organic solvent with the substrate butteroil as the continuous hydrophobic oil phase. A microbial lipase from *Candida cylindracea* was used as the enzyme in this study.

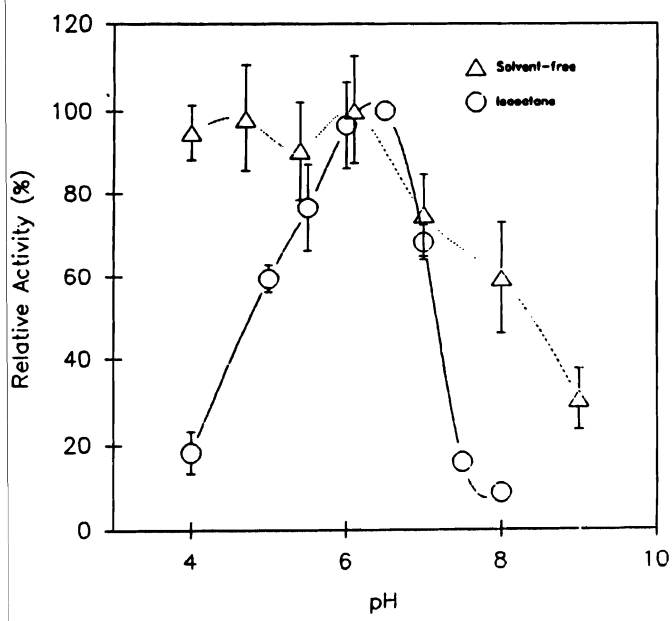
Reversed micelles containing entrapped lipase were prepared by injecting a predetermined amount of concentration enzyme solution (in buffer) into clear butteroil (or clear isooctane solution with up to 30% butteroil) containing 50 mM soybean lecithin. The mixture was either vortex-mixed or sonicated briefly to get a clear micro-emulsion. Lipase activity was measured by determining the free fatty acid contents in the system with a colorimetric method.

The water-in-oil micro-emulsion was found to be stable during the reaction and the solution stayed clear with no phase separation observed. Lipolysis reaction progressed smoothly in both solvent-free and isooctane systems, where concentration of fatty acids formed is proportional to reaction time, up to 30 minutes. Kinetic analysis was carried out

Table 1. Comparison of optimum pH and temperature and activation energy for lipolysis in different systems.

SYSTEM	OPTIMUM pH	OPTIMUM TEMP.	ACTIVATION ENERGY	REFERENCE
Reversed-micelle				
no solvent	4 to 6	55 °C	15.4 kcal/mole	This study
isooctane	6.5	55	7.5	This study
Isooctane/AOT	7.1	35	8.0	Han and Rhee, 1985
Emulsion	7.0	35	5.3 to 24.1	Park and Lee, 1985
Immobilized Enzyme	7.5	35	N.A.	Tahoun, 1986

Figure 1. Effect of pH on lipolysis rate in a reversed-micelle system.



based on initial reaction velocity calculated from the formation rate of fatty acids within this range of linear response.

Lipase Activity -pH

The dependence of lipase activity on pH in reversed-micelle solutions is shown in Figure 1. The pH values are those of the buffers from which micellar solutions were prepared. For solvent-free system, there is no significant difference in lipase activity for pH between 4.0 and 6.0 but the activity decreases rapidly thereafter. On the other hand, a pH optimum at 6.5 was observed for reversed-micelle systems using isooctane as the continuous phase. Compared to the pH optimum reported for this lipase in various systems as shown in Table 1, the pH optimum for the isooctane system is slightly shifted from around 7.0 to 6.5. The plateau of maximum activity in Figure 1 for the solvent-free system, however, represents a rather unusual and unexpected property. Changes in surfactant and oil phase seem to totally alter the pH profile of this lipase. Such a behavior will have the advantage of alleviating precise pH control when employing the solvent-free system for milkfat hydrolysis as lipase activity is less sensitive to pH changes under acidic conditions.

Lipase Activity - Temperature

The effect of temperature on lipase activity is shown in Figure 2. The optimum temperature was observed to be around 55° C for both systems; above that the enzymatic activity decreased rapidly possibly due to thermal denaturation. The optimum reaction temperature for lecithin reversed-micelle systems are about 20°C higher than that of most reaction systems reported in the literature, which include AOT/isooctane reversed-micelle, emulsion, and immobilized enzyme systems (see Table 1). Protection of protein from thermal denaturation by lipid and changes in water properties in reversed micelles of lecithin may account for the differences. This enhanced thermal stability will allow reactions to be carried out at higher temperatures in lecithin reversed-micelle systems to give faster

Figure 2. Effect of temperature on lipolysis rate in a reversed-micelle system.

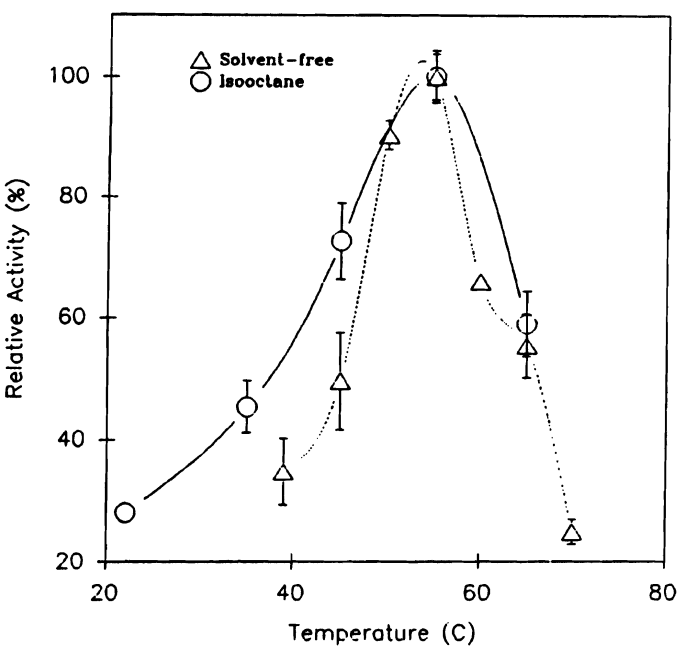


Figure 3. Effect of water content on lipolysis rate in a reversed-micelle system. R is the molar ratio of water to surfactant concentration.

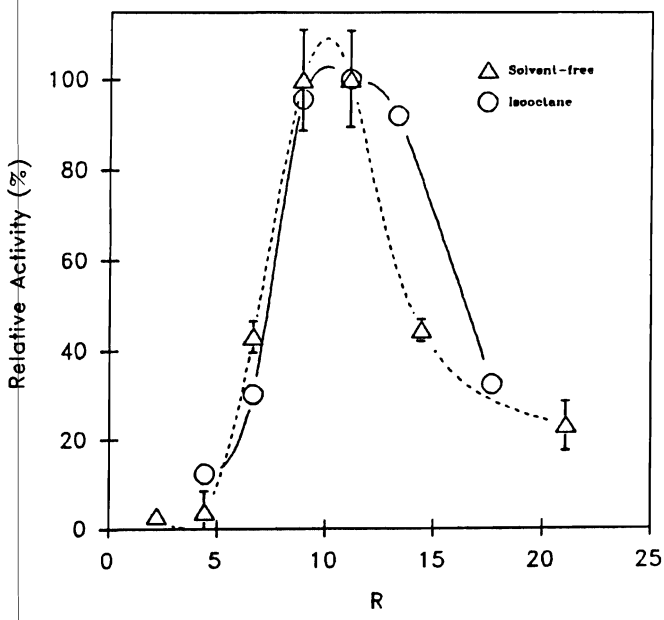
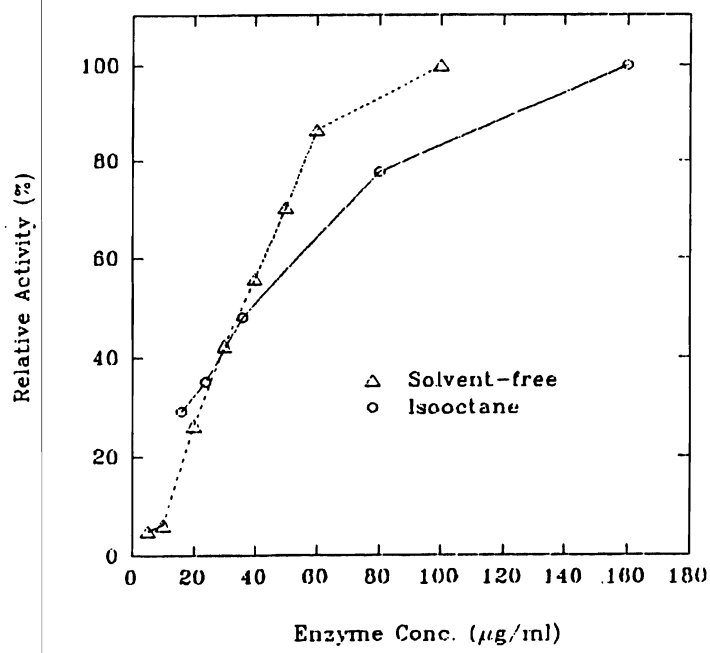


Figure 4. Effect of enzyme concentration in lipolysis rate in a reversed-micelle system.



reaction kinetics. The activation energy calculated from the slopes of Arrhenius plots are 15.4 and 7.5 kcal/mole for the solvent-free and isooctane system, respectively, which are consistent with the values reported for other systems (Table 1).

Water Content

The influence of water content on reaction rate is portrayed in Figure 3, where the value of R (the molar ratio of water to surfactant concentration) is based on 50 mM surfactant concentration. The corresponding volume fractions of water in the system are between 0.45 and 2.25% (v/v) for R values between 5 and 25. Bell-shaped curves were observed for activity dependence on water content with an optimum R value at around 10 for both systems. This behavior is consistent with previous enzymatic studies in reversed-micelle systems as a result of concerted actions of numerous factors which either compensate or supplement each other.

The effects of enzyme and surfactant concentration on initial reaction velocity have also been investigated as shown in Figures 4 and 5, respectively. In the solvent-free system, enzyme activity increased with increasing enzyme concentration up to 80 μg/ml, and eventually saturation occurred. Similar behavior was observed for the isooctane system as well. Since lipase is active almost exclusively at phase interfaces, saturation of initial velocity is expected when the catalytic reaction rate is limited by the available interfacial surface area for enzyme adsorption rather than the enzyme concentration itself. Oil-in-water emulsion systems are expected to start showing rate saturation at lower enzyme concentrations because of the limited surface area that can be created. One study in those systems reported a small increase in reaction rate from 46 to 54 units/mg when enzyme concentration was increased from 25 to 170 μg/ml. In comparison, the reversed-micelle systems in Figure 4 give a 3-fold increase in reaction rate within the same enzyme concentration range. Enzyme activity also seemed to depend on the surfactant concentration in forming reversed micelles in solvent-free systems, where a 6-fold increases in initial velocity was observed when lecithin concentration was increased from 10 to 50 mM at a constant R value. In contrast, in the isooctane system the velocity was almost independent of lecithin concentration within the same concentration range.

Significance to the Dairy Industry:

We have demonstrated the feasibility of carrying out lipolysis in lecithin reversed-micelle systems and investigated the effects of various system parameters on the reaction rate in this study. Lipase entrapped in reversed micelles seemed to possess some unusual properties, such as a higher optimum reaction temperature and a broader optimum pH range, which were obvious advantages over conventional reaction systems. It may also have fatty acid specificities different from conventional emulsion systems. A solvent-free reversed-micelle system developed in this study for fat hydrolysis looked promising in terms of eliminating the use of organic solvent.

Seminars:

H. Pai and J.P. Chen, "Enzymatic Hydrolysis of Milkfat with Lipase in Reversed Micelles of Lecithin," paper presented at 85th ADSA annual meeting, Raleigh, North Carolina, 1990.

Publications:

H. Pai and J.P. Chen, Hydrolysis of Milk Fat with Lipase in Reversed Micelles, *Journal of Food Science* (accepted for publication).

Project Title:

Incorporation of Butterfat Fractions into Chocolate and Confectionery

Personnel:

Richard W. Hartel, assistant professor, Dept. of Food Science; Chris Bunting, graduate student, Dept. of Food Science

Funding:

National Dairy Promotion and Research Board

(Dept. of Food Science)

Dates:

July 1, 1989 - June 30, 1992

Objectives:

1. To investigate the fat crystallization behavior of mixed fat systems of interest to the chocolate industry to determine the feasibility of using butterfat fractions as cocoa butter replacers or extenders.

2. To enhance the utilization of butterfat by optimizing the use of butterfat fractions in chocolate products.

3. To investigate the use of butterfat fractions in confections to potentially replace the vegetable fats in current use.

Objective 1:

Research in this area has focused on the development of techniques for comparing mixtures of butterfat fractions and cocoa butter. In particular, the use of NMR spectroscopy to evaluate solid fat content of various mixtures has been applied. In addition, the DSC has been used to quantify the changes in melting profiles of the various cocoa butter-milkfat fraction products. This information is useful in understanding the physical chemistry of these mixtures. From these results, a clearer picture of the effects of butterfat components on cocoa butter crystallization will arise.

Experimental:

Comparison of Cocoa Butter and Butterfat Fraction Mixtures

The physical properties of cocoa butter obtained from Guittard Chocolates were compared to various mixtures of cocoa butter and butterfat fractions using NMR and DSC. The characteristics of anhydrous milkfat (AMF) and each of the butterfat fractions, as prepared by melt crystallization, were also evaluated. The butterfat fractions included a high-melting fraction (crystals above 30°C), an intermediate-melting fraction (crystals between 20° and 30°C), and a low-melting fraction (liquid below 20°C). Blends of these fractions and cocoa butter were studied at ratios of 80/20, 60/40, and 40/60 of cocoa butter to butterfat fraction.

Butterfat fractions were manufactured in a lab-scale melt crystallization apparatus. The butterfat is crystallized by holding melted butterfat (anhydrous) at a cool temperature until a crystal equilibrium has been attained. The crystals are then separated from the liquid fraction by filtration. The liquid fraction is then cooled further to allow another separation at a lower temperature. In this way, high-melting, intermediate-melting, and low-melting fractions of butterfat have been obtained. Thus far, the high-melting fraction is the solid crystallized at 30°C and the liquid below 20°C is the low-melting fraction. The intermediate fraction is the solid crystal phase from the second crystallization.

Tempering Profiles of Blends

Samples of mixtures were prepared for NMR and DSC analysis by proper tempering. Several tempering profiles have been recommended for use with these techniques, however, the optimal tempering profile for any blend is probably quite different from the standard tempering profiles recommended. Two options exist for comparison of these blends. The first is to compare the melting profiles under identical tempering conditions. In this comparison, the optimal temper is not

considered. Alternatively, the melting profiles can be compared for optimal tempering conditions for each blend. This involves determining the optimal temper for each unique blend and then generating melting profiles at that condition. Thus, the tempering conditions will be different for each blend. The former method is best for scientific comparisons. However, the latter method is more appropriate for commercial comparisons since the chocolates would be tempered at their ideal conditions. In this study, we hope to quantify the differences that tempering profiles have on the melting properties of these blends and to recommend, based on these results, the most appropriate comparison conditions. At this point, however, we have been using one typical tempering profile for comparisons. This profile includes 60°C for 30 minutes, 27°C for 15 minutes, 0°C for 15 minutes, 27°C for 30 minutes and 0°C for 30 minutes. This tempering profile is based on a combination of published techniques and has been used, initially, due to the relatively short time required for tempering.

Determination of Solid Fat Content

Solid fat content was determined using the National NMR facility at the UW-Madison. Solid fat contents were determined at 0°, 5°, 10°, 15°, 20°, 25°, 30°, 35°, 40°, 45°, 50°, 55°, and 60°C for each product or blend. Olive oil was used as the reference fat for these calculations. From the solid fat profiles obtained in this way, isosolid diagrams were drawn for the blends of cocoa butter and butterfat fractions.

Determination of Melting Profiles

The melting profiles of these samples were also investigated using the Perkin Elmer DSC 7 purchased specifically for this project. Fat samples were tempered directly in the DSC pan and cooled to -40°C prior to melting. The samples were then heated at a rate of 10°C/ minute up to 80°C, and the resulting heat output due to phase changes was recorded.

Results:

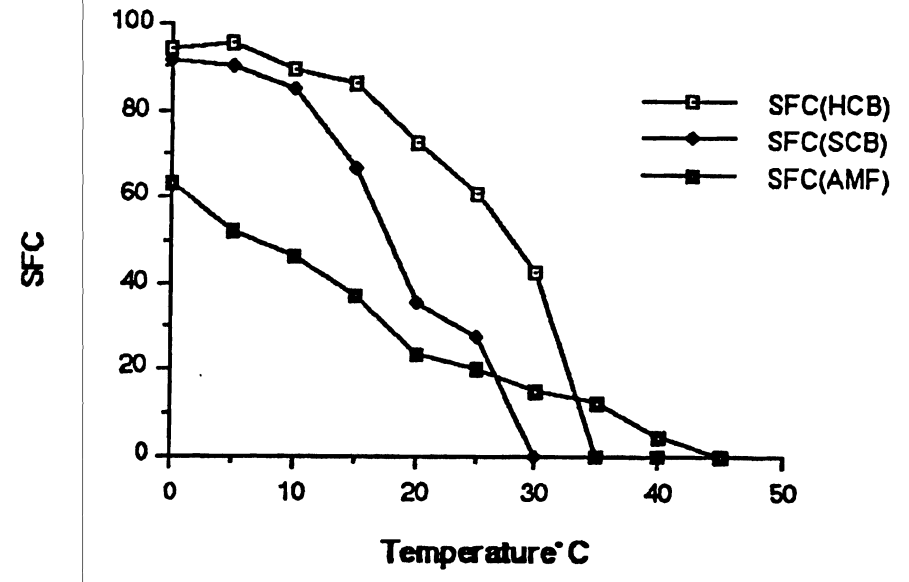
Effect of Adding Butterfat Fractions to Cocoa Butter

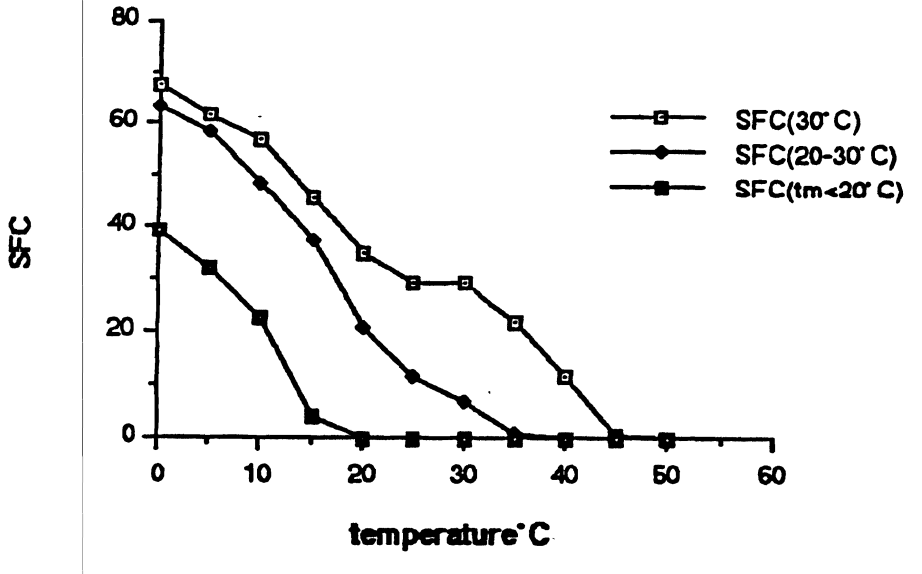
Solid fat contents of the various components used in this study were determined initially. Figure 1 shows the SFC curves for two cocoa butters (hard and soft) and the initial AMF used in fractionation, as well as for each of the fractions generated by melt crystallization. Significant differences in solid fat content can be seen between the different butterfat fractions. The effects of adding the butterfat fractions to cocoa butter can be seen in Figure 2. As expected, the lowest melting fraction of butterfat softens the blend more than the high melting fraction, although addition of each of the blends results in some softening of the pure cocoa butter. Greater softening occurs at a ratio of 40:60 butterfat fraction to cocoa butter. That is, the more butterfat fraction added, the greater the degree of softening.

Isosolid Diagrams

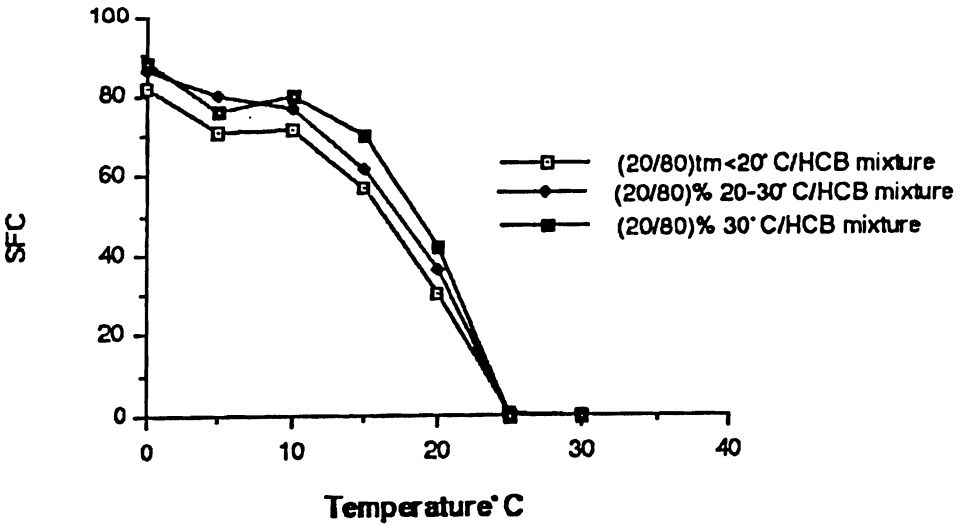
NMR curves such as these can be analyzed in terms of an isosolid diagram. In these plots, lines of constant solid fat content are plotted as a function of temperature for various compositions of a blend. Figure 3 shows these isosolid diagrams, based on data obtained to date, for the blends of cocoa butter and each of the three butterfat fractions. Parallel lines would indicate a complete mixing of the two components such that no changes in crystallization behavior were realized. Maxima and minima in these curves represent significant effects of one component on the crystallization behavior of the other. A minimum in the curves indicates that a eutectic formation is taking place resulting in a softening of the blend below what would be expected from a dilution effect. It can be seen that minima appear in the curves for the high and low melting fraction blends. These curves will aid in the identification of appropriate blends of butterfat fractions and cocoa butter that result in satisfactory chocolates. Further work on quantifying the crystalline structure for each of these blends will be required to provide a full understanding of the physical chemistry of these mixtures.

.Figure 1. Solid fat content SCB, HCB, and AMF (top figure), Milkfat fractions (lower figure)





.Figure 2. Solid fat content on milkfat fractions and hard cocoa butter. Top figure: 20/80 (fractions/HCB) Bottom figure: 40/60 (fractions/HCB)



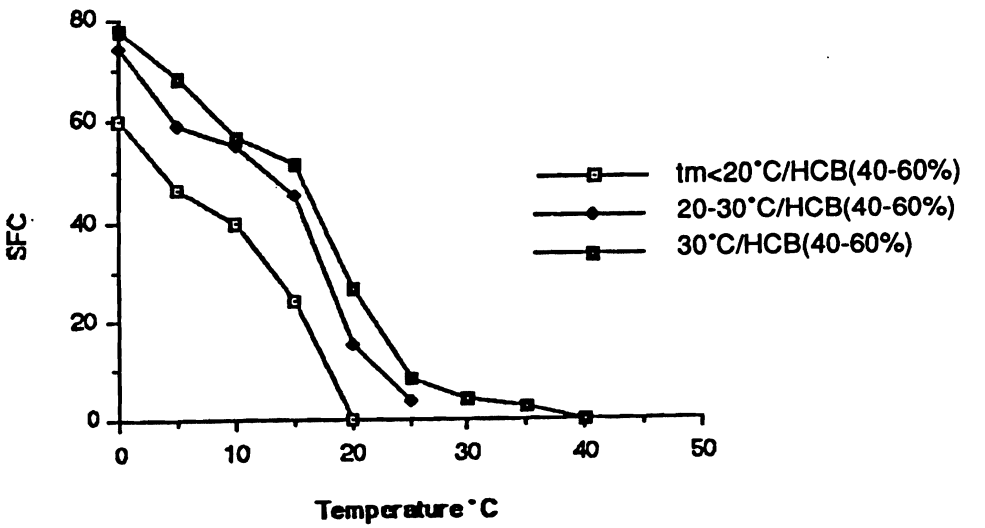


Figure 3. Isosolid diagrams of mixtures of cocoa butter with milkfat fractions. A. Low melting fraction/cocoa butter, B. Middle melting fraction/cocoa butter, C. High melting fraction/cocoa butter.

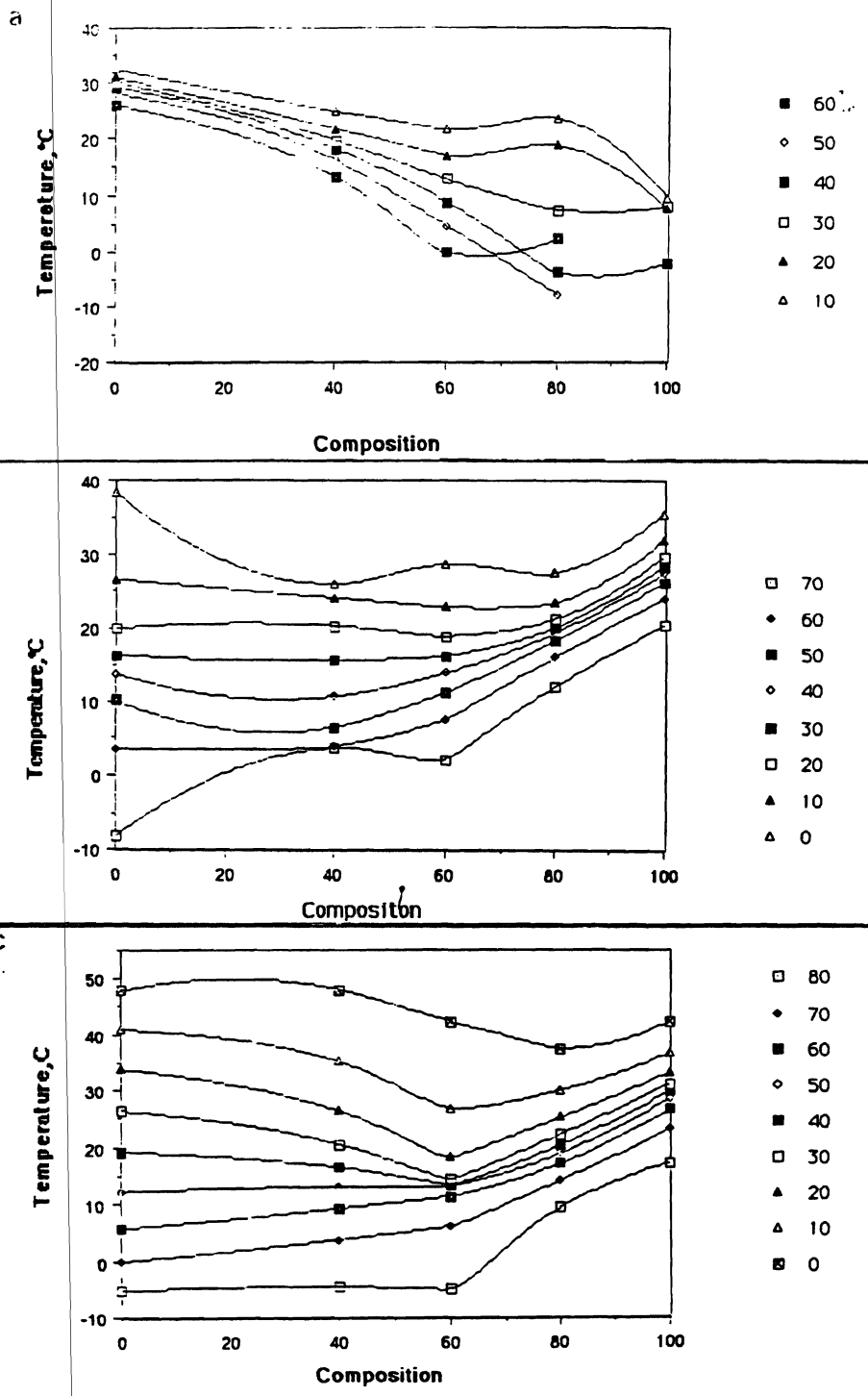


Figure 4. DSC freezing and melting curves of anhydrous milkfat.

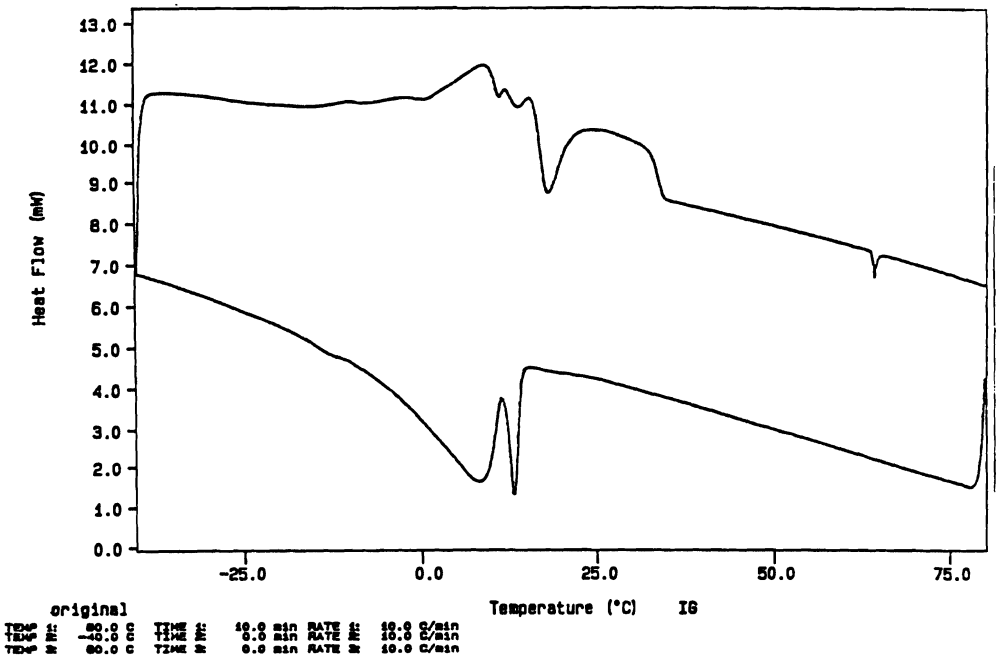


Figure 5. DSC freezing and melting curves of low melting fraction (tm <20°C).

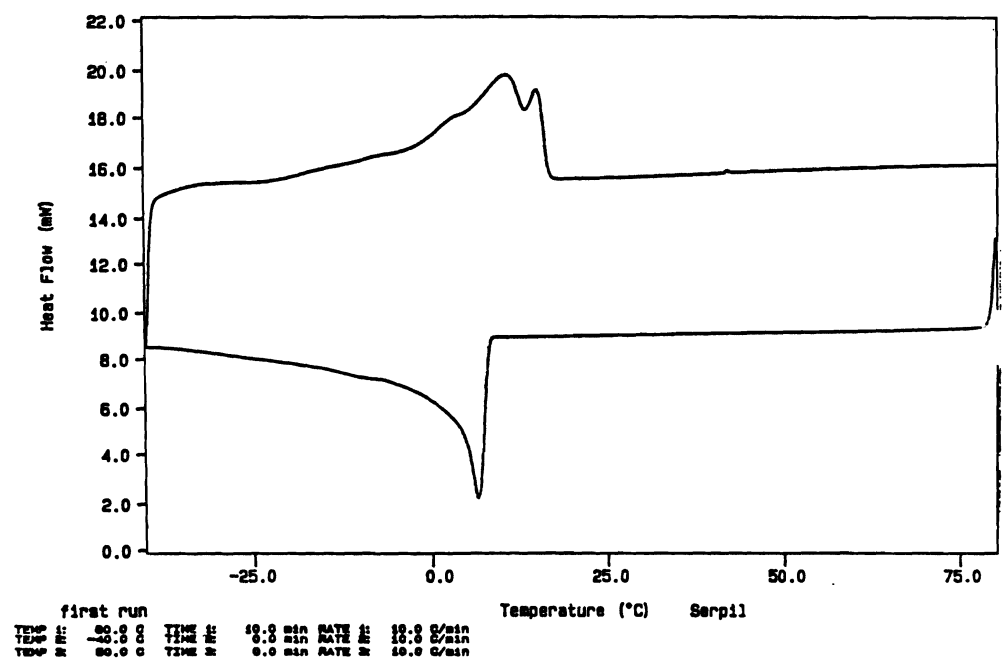


Figure 6. DSC freezing and melting curves of middle melting fraction (20-30°C).

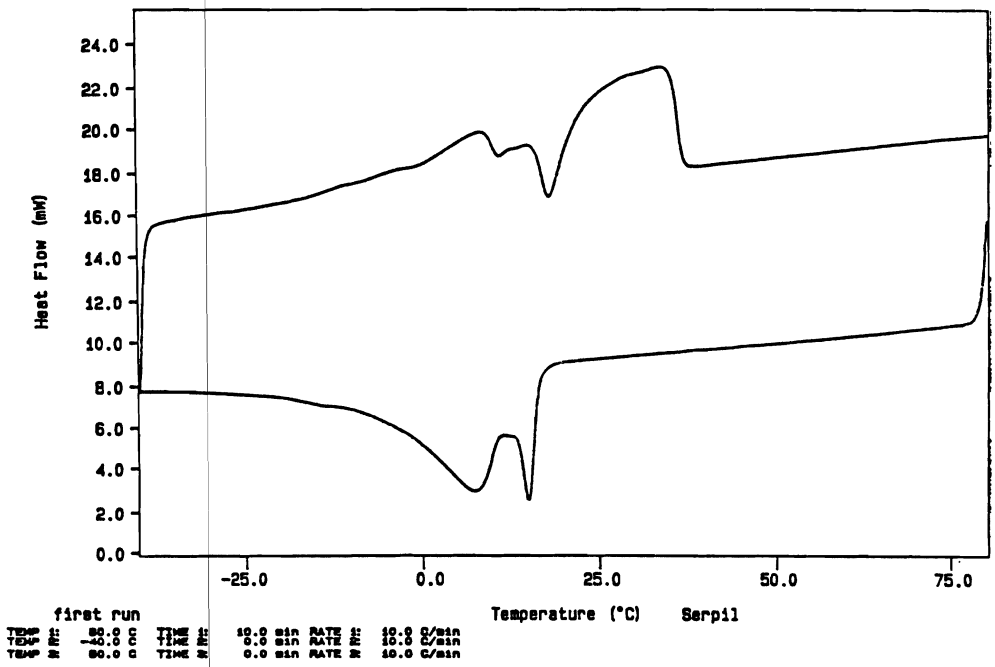


Figure 7. DSC freezing and melting curves of cocoa butter.

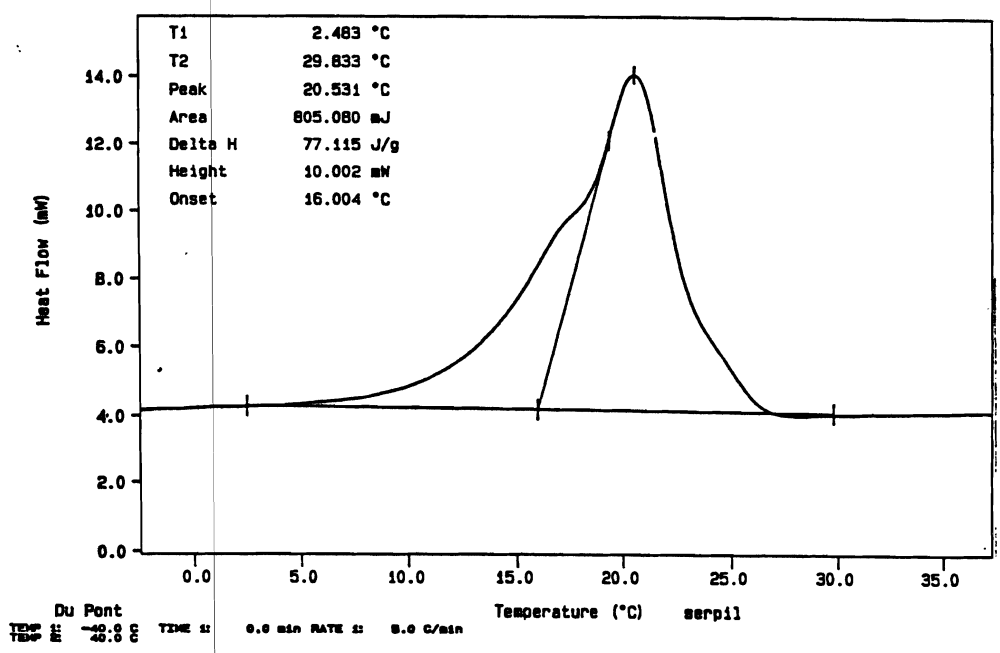
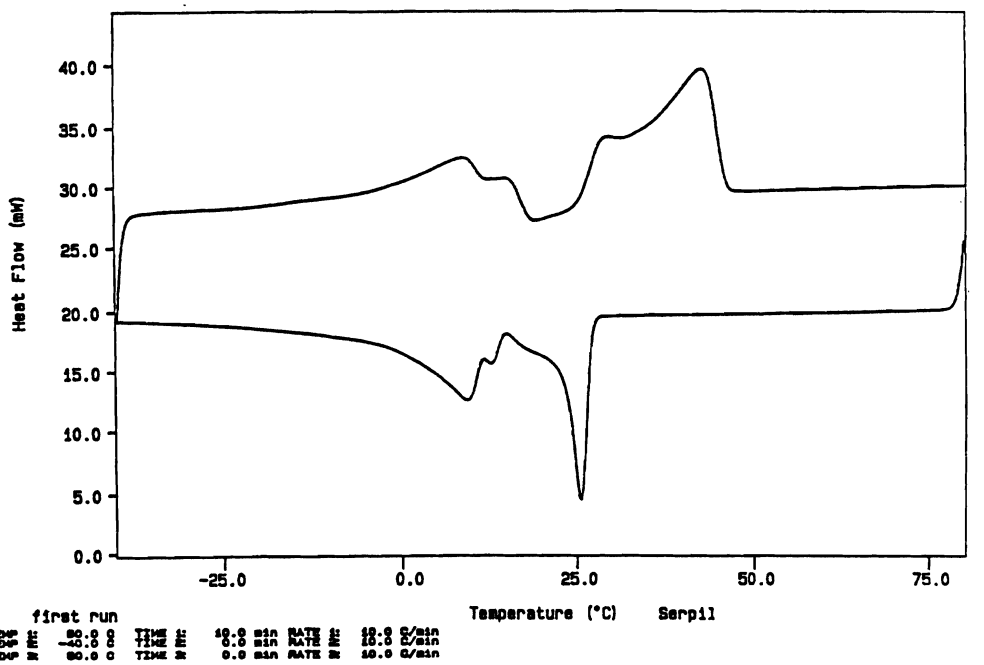


Figure 8. DSC freezing and melting curves of high melting fraction (30°C).



DSC Freezing and Melting Profiles for Butterfat

Freezing and melting profiles for anhydrous butterfat, each of the three butterfat fractions, and cocoa butter have been determined using the DSC. These curves show interesting differences. Figure 4 shows the DSC heating and cooling curves for anhydrous milkfat. In the melting curve (lower curve on figure), two distinct peaks can be seen at about 10°C and 18°C. The liquid fraction obtained from crystallization of butterfat at 20°C (t_m < 20 fraction) contains just the lower peak at about 10°C (Figure 5). The higher melting component of the butterfat has been separated by this fractionation process. The middle fraction (between 20° and 30°C) contains both peaks (Figure 6), while the high-melting fraction (crystals from 30°C separation) shows a preponderance of the higher melting component. These results serve to verify the differences in physical properties between butterfat fractions. Cocoa butter, on the other hand, shows a much simpler DSC profile due to its relative simplicity in triglyceride structure. Figure 7 shows a melting profile for cocoa butter. This can be compared to the melting profiles for butterfat (upper curve in Figure 4). Future work in this area will involve studying the freezing and melting curves for each of the mixtures of butterfat fractions and cocoa butter.

Future work:

The efforts on characterizing these blends using DSC and NMR is continuing. Evaluation of various butterfat fraction-cocoa butter mixtures will be extended to include a variety of butterfat fractions produced by melt crystallization as well as by supercritical extraction/ fractionation.

In the upcoming year, the X-ray diffraction will be performed on each of these blends to determine the crystal structure changes that occur. These results should give some insight into the physical mechanisms for the softening effect.

Objective 2:

The main objective of this project is to enhance the utilization of butterfat by studying the use of appropriate butterfat fractions in milk

chocolate. Not only would there be an economic advantage to replacing cocoa butter with a butterfat fraction, but there may be textural and/or flavor advantages as well. In this portion of the project, various chocolates are made with incorporation of butterfat fractions and evaluated for physical and organoleptic characteristics.

The research, thus far, has focused primarily on sample preparation and developing methods to analyze various chocolate characteristics that could be affected by altering the fat composition of the product. These characteristics include tempering profile, viscosity, and hardness or "snap".

Methods:

Preparation of Research Chocolate

In an initial trial to incorporate butterfat into chocolate, some research chocolate was prepared in the pilot plant of a local chocolate company. Ten pounds of a control containing 35% fat were prepared in a typical chocolate processing fashion. Ten pounds of a test chocolate were prepared in which the total fat content was still 35% but the milk fat accounted for 32% w/w of the fat phase, as opposed to 22% w/w in the control. This was accomplished by adding an appropriate portion of a high-melting butterfat fraction. These chocolates were acceptable, but a method of producing research chocolate in the UW-Madison Food Science pilot plant would be preferred. Because the UW pilot plant lacks chocolate refining equipment, arrangements were made with another chocolate manufacturing company to have a base chocolate prepared.

Six hundred pounds of a base milk chocolate were prepared for this work. This base was 26.5% fat, which is somewhat lower than the fat content of an average milk chocolate. The total fat content was readjusted to the desired level in the pilot plant at the UW. Several pounds were melted and mixed to uniformity in the Hilliard's Automatic Tempering Melter. Pure cocoa butter can be added to produce a control chocolate. A mixture of cocoa butter and butterfat fraction can be added to produce a test chocolate. This method allows the flexibility to produce chocolates with a range of total fat contents, as well as a range of ratios of butterfat to cocoa butter.

Tempering the Chocolate

Chocolate is first tempered by hand in a double boiler to determine the proper tempering conditions. Then, in order to monitor the tempering process, chocolate is tempered in a jacketed beaker in a semi-automatic system. The Master Servodyne drive system is set at 75 rpm to mix the chocolate mass. As fat crystals form during tempering, the viscosity of the chocolate increases. The viscosity increase is proportional to an increase in the torque that is required to turn the stirring shaft. The shaft torque output is sent from the Servodyne system to a data acquisition system so that it can be collected on an Apple IIe computer. A Masterflex peristaltic pump circulates water at 45°C through the jacketed beaker. A type-T thermocouple placed in the chocolate monitors the temperature change which is also recorded by the Apple IIe. When the chocolate has been stirring at 43°C for 30 minutes the pump is shut-off and a second pump circulates water at 24°-26°C through the beaker. When the chocolate reaches 26°C the second pump is shut off so that the chocolate can be mixed at its minimum temperature for several minutes to promote fat crystal nucleation. The first pump is then switched on again and the chocolate is rewarmed to 30°C to melt the unstable crystals that have formed. When the mass reaches 30°C, pump #1 is turned off so that the chocolate can mix at this temperature for several minutes to allow the remaining stable crystals to grow. The chocolate is then molded and cooled in a desiccator in a walk-in cooler at 8°C.

Chocolates that differ in formulation require different temperature profiles for tempering and, thus, it is difficult to compare their respective degrees of temper. Because the torque is proportional to viscosity, which increases with increasing fat crystal formulation, two chocolates are tempered to the same degree if they have the same torque profile.

Therefore, comparing the torque profiles of different chocolates allows one to compare their degrees of temper.

Development of Method to Evaluate Chocolate Hardness

A method using the Instron Universal Testing Machine (model 1130) is being developed to objectively evaluate chocolate hardness. A small (1"x 1"x 1/2") sample of tempered chocolate is placed on a flat, stainless steel platform. This is sheared apart by a wedge-shaped plunger attached to the Instron crosshead. The pounds of force required to break the sample are registered as a peak maximum on the chart recorder.

Results:

Two chocolates were manufactured for qualitative comparison. One chocolate incorporated the high-melting butterfat fraction while the second was the control (no butterfat fraction). Preliminary evaluation suggested that the experimental chocolate had a more distinct buttery flavor than the control, and that it also had a distinctly different mouthfeel. That is, the experimental chocolate, made with a high-melting fraction of butter, retained some sensation in the mouth after the cocoa butter had melted. This sensation was different from the waxy feel of a compound coating made with high-melting fats. It has been proposed that the butterfat may be dissolving in the mouth rather than melting, giving the chocolate a different feel. This idea needs to be investigated further.

The base chocolate method of sample preparation has proven to be successful for manufacturing and comparing chocolates. Two test chocolates have been prepared, each with its corresponding control chocolate. Test chocolate I had 36.6% fat with milkfat constituting 38.55% of the fat phase. The middle-melting fraction of butterfat (obtained by crystal separation between 30° and 25°C) replaces 28.8% of the cocoa butter found in control chocolate I. Test chocolate II has 31.9% fat with milkfat constituting 32.1% of the fat phase. The high-melting fraction of butterfat (solid fraction at 30°C) replaces 18.8% of the cocoa butter in control chocolate II. Both test chocolates and their controls have been successfully hand-tempered.

Sensory Evaluations of Chocolates

Preliminary sensory evaluations of these chocolates have been performed using the graduate students in my research group. The general consensus is that these chocolates are of reasonably good quality. Test chocolate I is slightly softer than the control but has only a slight flavor difference. Test chocolate II (high melting fraction), again, has a distinct buttery flavor and produces an aftertaste in the mouth associated with the higher melting temperature.

Instron Method Results

The chocolate evaluation tests under development have not been used for these experimental chocolates. At this point, the Instron method demonstrates variations within individual samples that are too high. Therefore, it has not been used to evaluate these test chocolates. However, the Instron has been able to distinguish hardness differences between tempered and untempered chocolate. A distinct peak is obtained when a sample of tempered chocolate is sheared by the instrument. There are no distinct peaks produced when softer, untempered chocolate is sheared.

Tempering Profiles

The temperature/torque history curves, for chocolates of the same formulation, illustrate the effect of a change in tempering temperature profile on the final temper of the product. That is, different tempering profiles result in slightly different product textural characteristics depending on the fat crystal formation. Further work on this analysis is underway to determine the relationship between the temperature/torque history profiles and the optimal temper of various chocolates.

Future Work:

The investigations on chocolates of various formulations will continue. Additional butterfat fractions will be made and incorpo-

rated into the base chocolate for evaluation of sensory qualities and textural properties. Changes in the necessary tempering conditions for each chocolate will be monitored by tempering in a semi-automatic system and producing temperature/torque history curves.

It is desirable to know the actual values for apparent viscosity as these help determine flow properties under processing conditions. The Casson viscosity method for chocolate will be used to determine plastic viscosity and yield value for each chocolate.

Future Instron work will involve modifying the method to improve reproducibility of the results. The modified method will be used to quantify the effect of the butterfat fractions and their addition levels on the hardness of the test chocolates. More complete sensory studies will be done to help relate subjective evaluation of the samples to the hardness values obtained with the Instron.

Objective 3:

Incorporation of these butterfat fractions into confectionery products is an important goal of this research. In the past year, an undergraduate student was involved in setting up the equipment necessary to manufacture caramels with the addition of these butterfat fractions. These studies have, thus far, not resulted in any data since the student was mainly concerned with the equipment setup and the development of the experimental procedures. In the next year, additional undergraduate students will be enlisted to carry this research further.

Project Title:

Modification of Milk Fat Composition by Production of Null Mutants for Acetyl-CoA Carboxylase in Transgenic Animals

Personnel:

Robert Bremel, professor, Dept. of Dairy Science (co-PI); and Ki-Han Kim, professor, Dept. of Biochemistry, Purdue University, Lafayette, Indiana (co-PI)

Funding:

National Dairy Promotion and Research Board

Funding Code:

89-1

Dates:

July 1, 1989 - June 30, 1992

Objectives:

1. To produce anti-sense genes for acetyl-CoA carboxylase, the controlling enzyme for fat synthesis in the mammary gland, in gene constructs driven by mammary-specific promoter and enhancer sequences (Research carried out at Purdue University).

2. To produce transgenic mice expressing anti-sense acetyl-CoA carboxylase gene in their mammary gland and to analyze the composition of the milk produced by these animals (Research conducted at the University of Wisconsin-Madison).

Summary of Findings:

Genetic Engineering of RNA

RNA molecules with catalytic cleavage action against sequence specific RNA targets (ribozymes) have been produced by using recombinant DNA plasmids that place the intended transcript under the transcriptional control of a specific promoter. In the present studies, the plasmids were designed and constructed so that generated ribozymes can specifically cleave rat acetyl-CoA carboxylase (ACC) mRNA.

Oligonucleotides employed in the construction of the recombinant DNA plasmids containing anti-ACC ribozyme sequences were synthesized in the Laboratory for Macromolecular Structure at Purdue University using an Applied Biosystems 380A DNA synthesizer. The hammerhead ribozyme structure is encoded in the central 22 bases of the insert and is flanked by bases 136-145 and 147-157 of the ACC ORF in the case of ribozyme #2, and by bases 147-158 and 160-167 in the case of ribozyme #3.

At the moment, both pBiRb6;7 and pGEM3Z/Rb7(7) active ribozymes are active in vitro against ACC mRNA synthetic transcripts, cleaving the substrates at the predicted sites. The control transcripts generated from pGEM3Z/Rb7(7), using SP6 RNA polymerase, are inactive against the same ACC mRNA transcripts.

Ribozymes have been placed in the SmaI site of a-lactalbumin cassette for testing in cell culture systems and in transgenic animals.

Significance to the Dairy Industry:

Changing Milk through Genetic Engineering

Substantial changes in the composition of milk have been possible through advances in genetic engineering. Although presently there are only a few examples, transgenic animals have been produced which demonstrate that it is possible to place genes for virtually any protein of interest under the control of the genetic controlling elements of a milk protein and to then have this protein expressed in the mammary gland. For example, using this technique, it is possible to express sheep ß-lactoglobulin, and several other non-milk proteins, in the milk of mice. The genes for these new proteins behave as Mendelian dominants, and animals produce the proteins only in their milk during lactation.

Altering the Fat Composition of Milk

Recently, using similar techniques, it has been shown to be possible to produce so called 'null mutants', animals in which a gene has been selectively inactivated by inserting a gene containing the sequence complementary to the sequence of the mRNA coding for the gene to be nullified. The objective of this project is to produce anti-sense genes for a key enzyme in the synthesis of milkfat which are driven by mammary specific promoters. With these genetic constructions it should be possible to dramatically alter the fat composition of milk.

Seminars:

Governor's Biotechnology Task Force, Oconomowoc, Wisconsin, March 1990

Fat and Cholesterol Reduced Foods, IBC USA, New Orleans, Louisiana, March 1990

First Bulgarian-American Biotechnology Seminar, Borovitz, Bulgaria, May 1990

Application of Biotechnology in Food Systems, American Society of Clinical Nutrition, Washington D.C., May 1990.

North East American Dairy Science Association-Animal Science Meeting, July, 1990

Publications and Theses:

Bremel, R.D., G. Bleck and H.C. Yom. (1989). Altering Milk Composition using Molecular Genetics. *J.Dairy Sci* 72:2826-2833

Bleck, G.T., Robert D. Bremel. (1990). Isolation and cloning of a gene encoding bovine a-lactalbumin. *J. Dairy Sci. Suppl* 1. p242

Project Title:

Expression of Bovine Alpha S(1) Casein in Milk and Tissues of Transgenic Mice

Personnel:

Robert Bremel, professor, Dept. of Dairy Science (co-PI); and Neal First, professor, Dept. of Meat and Animal Sciences (co-PI)

Funding:

Wisconsin Milk Marketing Board

Funding Code:

IV-64

Dates:

Sept. 1, 1987 - Aug. 31, 1990 (extension requested of WMMB)

Objectives:

1. Alpha S(1) casein transgenic production and expression;

2. Produce a general purpose test system with which we can determine the utility of various promoter and/or other genetic constructs prior to their use with milk proteins; and

3. Study the expression of kappa-casein under the control of a known mammary specific promoter.

Summary of Findings:

Our work, in the original phases of this project, involved the engineering of alpha S(1) casein cDNA in such a way as to make it possible to produce transgenic animals with specific genetic constructs (Objective 1). Second, because we recognized that the promoter systems we planned to use in our initial experiments were not optimal, and certainly were not the type which would be applicable to use in dairy cattle, we set out to produce a general-purpose promoter construct. This construct could be used as a genetic test system to determine the utility of various promoter and/or other genetic constructs prior to their use with milk proteins (Objective 2). And, lastly, because we had kappa casein cDNA available to us, and because kappa casein is so important to the Wisconsin cheese industry, we chose to study the expression of kappa-casein under the control of a known mammary specific promoter (Objective 3). The actual accomplishments of the project have been:

- Produced clones of alpha-S1 casein cDNA driven by MMTV promoter (MMTV-alpha-S1 casein(cDNA))
- Transfected and expressed genes in human mammary cell line MCF7
- Developed Polymerase Chain Reaction (PCR) techniques to rapidly screen potential transgenic animals
- Produced MMTV-alpha-S1 casein (cDNA) transgenic mice
- Obtained a genomic clone from beta casein for further engineering studies
- Isolated a genomic clone of bovine alpha-lactalbumin containing extensive 5' and 3' flanking regions
- Developed a PCR screening system to permit screening of animals using oligonucleotide primers for the signal peptide and 770 nucleotides (nt) upstream in the 5' flanking region of the gene
- Currently are sequencing the 5' flank ing region of the gene to provide information needed for further genetic constructions

Significance to the Dairy Industry:

Use of Genetic Engineering to Alter Milk Components

The basic premise on which the initial pro-

posal was made was that, despite interest in modifying the composition and qualities of milk, change has been slow, difficult, and ultimately limited by the inherent genetic correlation between fat and protein in milk. There is good reason to believe that the only way in which the genetic correlation between these components can be broken is through genetic engineering. Advances in molecular biology, particularly relevant to this area of research, have been occurring at a rapid rate primarily because of advances in genetic medicine. It is the long-term goal of this laboratory to use this technology as a means of altering the components in milk to provide new product/marketing opportunities for the dairy industry.

Recent advances in molecular biology have made altering milk through genetic engineering a reality, a variety of proteins have already been expressed in mammary secretions. Work, to date, has concentrated on using laboratory animals. There is actually very little work done with bovine genes primarily because the emphasis of most of the work has been on producing pharmaceuticals in milk. This laboratory is one of only perhaps 3 or 4 laboratories in the world dedicated to working with cattle and modifying the properties of milk for the dairy industry's advantage. Although it is clear that it will be possible to produce pharmaceuticals by this means, it is also clear that production of these materials will have little or no impact on the dairy industry, other than providing an end-product with a sufficiently high enough economic value to provide an incentive for the requisite technological development to take place.

Possible Gene Insertion Techniques

We have reviewed various strategies for using molecular genetics to alter milk, especially using transgenic animals, and considered various candidate mammary or milk proteins, or enzymes, which might be targeted. At present, the primary impediment to implementation in dairy cattle is economic. However, due to the advances in 'gene therapy' in human medicine, we can now reasonably expect alternatives to germ-line insertion of genes. Also, we are aware of work being done to make it possible to use ballistic techniques (literally firing little bullets of DNA), similar to those used to inject genes into plant cells, to insert genetic material into sperm. It seems that this is clearly a possible alternative which would make the insertion of genetic material through normal artificial insemination a reality.

Future Plans

Given the rate at which the capabilities in these areas have been progressing, it is a reasonable assumption that this technology will be available to us in the near future. Therefore, we must address the question of what genetic characteristics we can engineer that will provide new opportunities for the dairy industry in the future. As we have pointed out elsewhere, progress in these areas will require a detailed knowledge of the genes that will be used and of the useful constructions that can be used in various experiments. It is our goal to provide this knowledge towards applying these techniques in cattle.

Seminars:

- Governor's Biotechnology Task Force, Oconomowoc, WI, March 1990
- Fat and Cholesterol Reduced Foods, IBC USA, New Orleans, LA,March 1990
- First Bulgarian-American Biotechnology Seminar, Borovitz, Bulgaria, May 1990
- Application of Biotechnology in Food Systems, American Society of Clinical Nutrition, Washington D.C., May 1990.
- North East American Dairy Science Association-Animal Science Meeting, July 1990

Publications and Theses:

Bremel, R.D., G. Bleck & H.C. Yom. (1989). Altering Milk Composition using Molecular Genetics *J.Dairy Sci.* 72:2826-2833

Bleck, G.T., Robert D. Bremel. (1990). Isolation and cloning of a gene encoding bovine alpha-lactalbumin *J. Dairy Sci. Suppl. 1* . p242

Project Title:

Conversion of Whey Components to Commercially Valuable Products

Personnel:

D. C. Cameron, assistant professor, Dept. of Chemical Engineering; E.N. Lightfoot, professor, Dept. of Chemical Engineering; Timothy Cooper and James Flatt, graduate students, Dept. of Chemical Engineering

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-53

Dates:

July 1, 1988 - June 30, 1990

Objectives:

1. Develop a database of products that can be derived from lactose and whey minerals.

2. Identify products for which whey components are the preferred substrates.

3. Perform an economic evaluation to identify the most promising products for further development.

4. Develop technologically feasible laboratory-scale processes for the production of selected products.

5. Make recommendations for further development and commercialization of the laboratory processes.

Summary of Findings:

Polysaccharide Production from Whey

We have successfully completed our program to isolate proprietary microorganisms capable of producing novel, commercially-useful polysaccharides from whey. Three strains from over 3,000 lactose-utilizing organisms were identified as producing significant quantities of exopolysaccharides. Soil samples from Wisconsin farm fields, regularly spread with whey, were screened for microorganisms able to produce useful polysaccharide gums from lactose. An organism, identified as *Rhizobium* sp. ATCC 55046, was isolated from a soil sample from a farm near Mineral Point, Wisconsin. In a synthetic whey medium containing 45 g/l lactose, the organism gave a maximum growth rate of 0.6 hr^{-1}, and produced 16 g/l polysaccharide with a yield on lactose of 0.4 g/g and a productivity of 0.3 g/l hr. Polysaccharide production is primarily growth associated. Studies with various carbohydrates showed that polysaccharide production is greater on disaccharides than monosaccharides, with the best production on lactose. Hence, the polysaccharide potentially can be produced most economically on whey or whey permeate.

Polysaccharide Composition

The composition of the polysaccharide as determined by HPLC, proton NMR, and enzymatic assays was mannose, 47-50 mol%; galactose, 27-30 mol% and galacturonic acid, 18-23 mol%. This composition makes the polysaccharide an anionic galactomannan. No organic modifying groups were detected. The weight average molecular weight, estimated by GPC with dextran standards and referenced to xanthan gum, was 2.5×10^6 daltons. The polysaccharide has shear thinning behavior and can be modeled by the power law equation. The polysaccharide efficiently builds viscosity at low concentrations (ca. 0.1%), but less efficiently at high concentrations. The viscosity is stable between pH 2 and 11, and the polysaccharide has good thermostability in the absence of salts. The polysaccharide shows elastic flow behavior.

Potential Applications of Polysaccharides

The combination of these properties suggest that potential applications would include food and non-food products requiring a moderate degree of thickening, wet-end additives and

coating agents for paper products, binders for building materials and ceramics, and detergents. The galactomannan seems to be particularly well-suited as a resoiling inhibitor for detergent formulations since the requirements for superior resoiling inhibition agents include anionic character, good alkaline and temperature stability, and relatively low thickening efficiency. This polysaccharide is biodegradable; hence, it is environmentally advantageous versus existing resoiling inhibitor additives.

We are currently investigating conversion of lactose to another valuable polysaccharide, however, we are unable to publicly disclose this research until a patent application, which is in progress, has been filed.

Significance to the Dairy Industry:

Successful completion of this project will result in the development of small-scale processes for the conversion of lactose in whey permeate to several commercially useful polysaccharides. Successful completion of a future scale-up program could then lead to the construction of one or more small plants for fermentation of whey permeate to polysaccharides. Initially, the total permeate utilized would be small, however, as the market penetration of these polysaccharides increases, the amount of permeate utilized could become significant.

More generally, the findings from this research program have provided the economic framework for identification of products which could feasibly be produced from lactose in whey. We hope that this work can play a role in the development of a whey-based chemicals industry.

Presentations:

1. Flatt, J. H., R. S. Hardin, J. M. Gonzalez, D. C. Cameron (1990), "Polysaccharide Production from Lactose by a Newly Isolated Soil Bacterium," Poster presented at the NATO Advanced Research Workshop on New Biosynthetic Biodegradable Polymers of Industrial Interest from Microorganisms, Sitges, Spain on May 25, 1990.

2. Cameron, D. C. (1990), "Microbial Production of Polysaccharides from Lactose," Presented at the Midwest Biotechnology Symposium, St. Paul, MN on May 23, 1990.

3. Cooper, T.A., J. H. Flatt, E.N. Lightfoot and D.C. Cameron (1989), "Production of Exopolysaccharides from Lactose by Wildtype *Zoogloea ramigera* and a Capsule Minus Mutant Strain Isolated by Buoyant Density Centrifugation," Presented at the ACS 1989 Fall Meeting, Sept. 9, 1989., Paper 104.

4. Brummel, S.E. and K. Lee (1989), "Use of Soluble Hydrocolloids for Fat Replacement in Process Cheese Spreads," IFT Annual meeting, Chicago IL.

5. Flatt, J. H., T. A. Cooper, D. C. Cameron, E. N. Lightfoot (1989) "Enhancement of Polysaccharide Production from *Z. ramigera* Fermentation of Whey," Presented at the CDR Cheese Research and Technology Conference, March 30, 1989, Madison, WI.

6. Brummel, S.E. and K. Lee, 1989. ibid. Presented at CDR's annual conference in Madison WI on March 30.

7. Flatt, J. H., T. A. Cooper, D. C. Cameron, E. N. Lightfoot, (1988) "Utilization of Dairy Waste: Microbial Production of Galactose-Containing Polysaccharides from Lactose in Whey," Presented at the AIChE 1988 Annual Meeting, Dec. 2, 1988, Paper 105c, Washington, D.C.

Publications and Theses:

Hardin, R. S., J. H. Flatt, D. C. Cameron, Polysaccharide Producing Organism and Novel Galactomannan Polysaccharide, U.S. Patent Application, Filed May 21, 1990.

Other manuscripts are presently in preparation, and will be submitted following filing of relevant patent applications.

Project Title:

Recovery of Lactoferrin and Immunoglobulin G from Cheese Whey by Affinity Microfiltration

Personnel:

J.P. Chen, assistant scientist, Center for Dairy Research (PI); C. H. Wang, graduate student, Dept. of Food Science

Funding:

Wisconsin Milk Marketing Board: Basic Research

Funding Code:

89-29, 1C

Dates:

July 1, 1989 to June 30, 1990

Objectives:

1) To characterize the general properties of heparin sepharose, protein G sepharose, and protein G bearing streptococcal cells in terms of binding capacity and binding constant for lactoferrin (Lf) and immunoglobulin G (IgG).

2) To use the above resins (cell) in a new method combining affinity chromatography and microfiltration to isolate Lf and IgG in a highly purified and active form from cheese whey.

Summary of Findings:

Uses for Lactoferrin and Immunoglobulin G

Lf and IgG are two of the minor components of cheese whey with proven antimicrobial activities. A proposed use of these proteins is in calf feed supplements as antidiarrhea agents. Lf is also used in infant formula supplement because of high levels of this protein in human milk, and it may also play a role in leukemia treatment. In this study Lf was purified by heparin sepharose and IgG was purified by protein G sepharose and protein G bearing streptococcal cells (heat-killed, formalin fixed Group C *Streptococcus* sp.) from Cheddar cheese whey.

Binding Experiments

Binding experiments were first carried out for each adsorbent to characterize its binding capacity and its binding constant for the target protein. These parameters are important in terms of efficiently utilizing the ligands and employing the microfiltration technique. The binding parameters were found by carrying out batch adsorption experiments with pure Lf and IgG standard solutions and fitting the data with a Langmuir isotherm equation:

$$Q = \frac{Q_m}{K_d + P} \qquad (1)$$

where Q is the amount of bound protein per ml of resin (cell), Q_m is the maximum binding capacity of the resin (cell), K_d is the apparent binding constant (dissociation constant) and P is the free protein concentration in solution at equilibrium. A typical result is shown in Figure 1 for a binding study between IgG and protein G sepharose resin. A curve representing the best-fit to the experimental data was calculated by non-linear regression according to the form in equation (1) to give values of Q_m and K_d. This procedure was repeated for each adsorbent and the calculated binding parameters are summarized in Table 1. The bindings between adsorbents and adsorbates are all fairly strong for all three systems, judging from their low K_d values (10^{-6} to 10^{-7}). This also indicates the feasibility of using these adsorbents in affinity microfiltration as the binding strength of the adsorbent for the adsorbate is strong enough to prevent its leakage during the filtration process.

Heparin Sepharose Binding Capacity

Heparin sepharose exhibited very large binding capacity for Lf (over 120 mg of Lf per ml of resin), making it an efficient and economic choice for isolating this protein. Protein

Figure 1. Binding study between IgG and Protein G Sepharose.

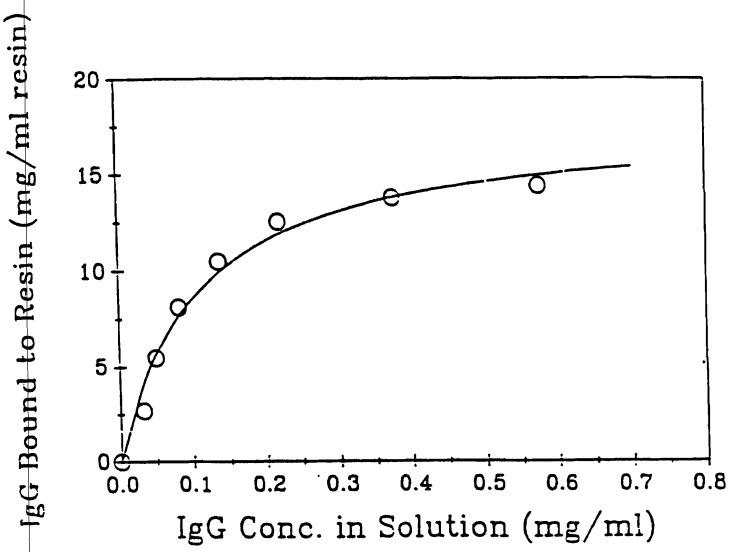


Table 1. Binding parameters of Heparin Sepharose, Protein G Sepharose and Protein G bearing Streptococcal cells.

Parameter	Heparin Sepharose	Protein G Sepharose	Protein G Cells
Q_m [a]	124 mg Lf/ml resin	17.8 mg IgG/ml resin	148 µg IgG/ml cell [c]
K_d [b]	5.4×10^{-6} M	3.5×10^{-7} M	1.1×10^{-7} M

[a]maximum binding capacity
[b]apparent binding constant (dissociation constant)
[c]10% cell suspension

Figure 2. Procedures for purifying Lf and IgG by affinity microfiltration.

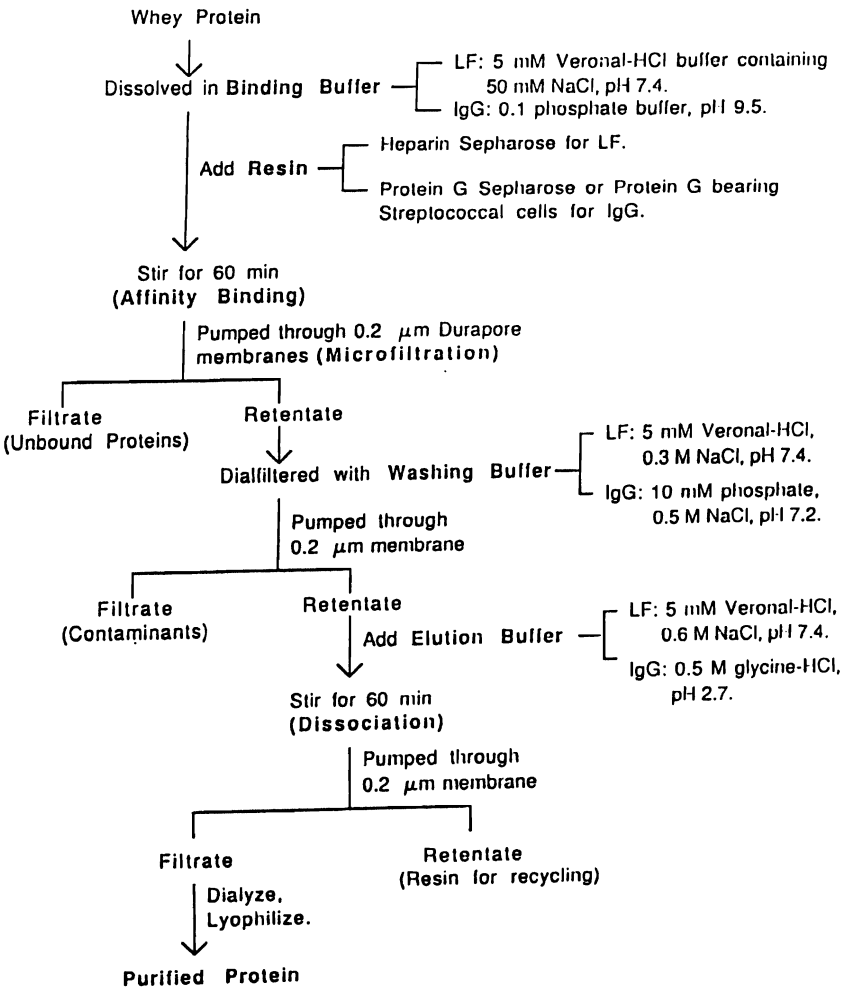


Table 2. Purity, biological activity and yield of Lf and IgG.

Protein	Purity[c] (%)	Biological Activity(%)	Yield (per liter of cheese whey)
Lactoferrin	>95	92[d]	80 mg
IgG (PS)[a]	90	86[e]	40 mg[f]
IgG (PC)[b]	68	N.D.	N.D.

[a]Purified by Protein G Sepharose.
[b]Purified by Protein G bearing Streptococcal Cells.
[c]Determined by SDS-PAGE.
[d]Determined by iron-binding capacity.
[e]Determined by immunochemical analysis.
[f]From 5 ml of resin.

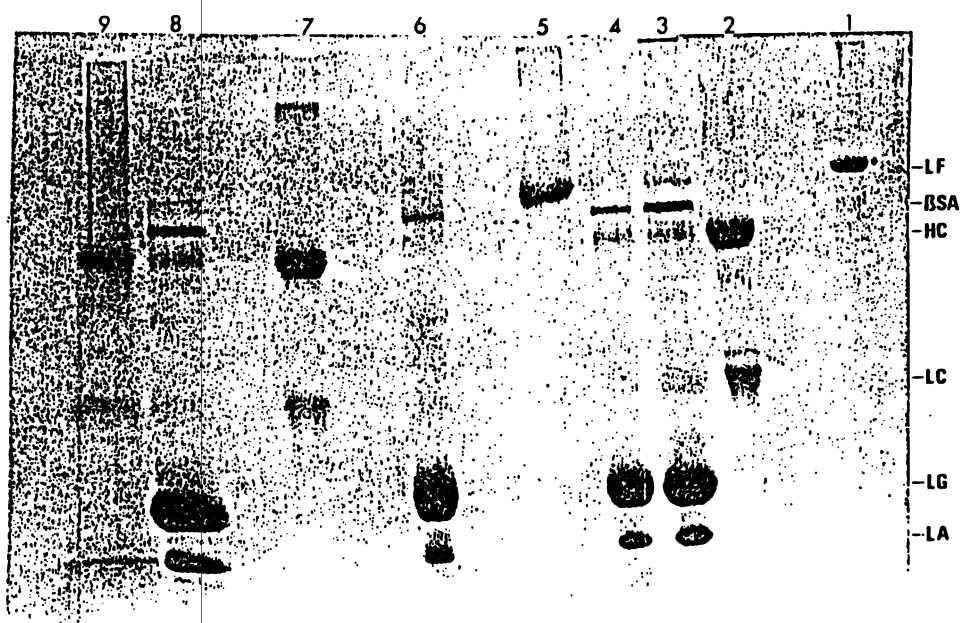
G bearing cells showed two orders of magnitude less binding capacity than protein G sepharose, which may partially be due to the limited outer surface area of a cell compared to the large surface area of a porous sepharose gel particle. Nonetheless, compared to affinity resin, cells can be easily mass produced in house and they may have higher rigidity and less problems with fouling and ligand stability.

Purification of Lf and IgG

After characterizing the binding properties, we then used these adsorbents to purify Lf and IgG by affinity microfiltration following the procedures outlined in Figure 2. Whey proteins dissolved in the binding buffer were first contacted with the adsorbent, the solution was then pumped through 0.2 μm Durapore low-protein-binding microfiltration membranes in cross-flow filtration configuration to separate unbound contaminating proteins from the target protein, followed by diafiltering the retentate with a washing buffer to further eliminate any residual contaminants. The target protein was subsequently desorbed from the adsorbent with a suitable elution buffer and collected in the filtrate during a second microfiltration step. The final purified product can be deemed sterilized by microfiltration during the process.

The purity, biological activity, and yield of purified Lf and IgG are shown in Table 2 and Figure 3. From SDS-PAGE analysis of protein purity, the isolated Lf in lane 5 showed a single band with the same molecular weight as the standard Lf in lane 1. The purity will be >95% considering the detection limit of the Coomassie Blue staining method employed.

Figure 3. SDS-PAGE of purified proteins.



1: Standard lactoferrin; 2: Standard immunoglobulin G; 3: Cheese whey; 4: Heparin unbound whey proteins; 5: Purified lactoferrin by Heparin Sepharose; 6: Protein G Sepharose unbound whey proteins. 7: Purified immunoglobulin G by Protein G Sepharose; 8: Protein G cell unbound whey proteins; 9: Purified immunoglobulin G by protein G cell. LF=lactoferrin; BSA=bovine serum albumin; HC and LC=heavy and light chain of immunoglobulin G, respectively; LG=β-lactoglobulin; LA=α-lactalbumin.

Compared to the original cheese whey in lane 3, whey proteins unbound to heparin sepharose in lane 4 did not have the band corresponding to Lf, indicating that all lactoferrin in cheese whey was absorbed by heparin sepharose and this resin can efficiently and selectively remove Lf from cheese whey. For protein G sepharose-purified IgG, the two bands in lane 7 were with the same molecular weights as those of standard IgG in Lane 2, which represent the heavy and light chain of IgG. Purity of this protein isolate was estimated to be 90% by densitometer readings. On the other hand, IgG purified by protein G bearing cells in lane 9 showed a major contamination band with the same molecular weight as a-lactalbumin to give a purity of 70%.

Purification Results Differences

The difference in purification results with different protein G-containing adsorbents is due to the fact that the microbial cell contains native protein G in its cell wall, while protein G sepharose used in the experiments contains recombinant protein G as the ligand, which had the albumin-binding region of native protein G deleted genetically to avoid the unspecific binding. The biological activities of purified proteins were estimated by measuring iron-binding capacity of Lf and performing radial immunodiffusion analysis for IgG, respectively. Both protein isolates showed reasonably high activities at around 90%, meaning that 95% of the Lf or IgG is active in the final product. The high iron-binding capacity can confer purified Lf an antimicrobial activity by chelating the essential iron for growth of certain bacteria and an effective iron-carrying capability for iron-fortification of foods or infant formula.

Significance to the Dairy Industry:

Results from this study clearly indicate that affinity microfiltration is a specific and gentle technique and is feasible for isolating Lf and IgG from cheese whey to give products with high purity and biological activity. Since the composition of cheese whey remains the same after the isolation steps, this process seems to be profitable by producing valuable antimicrobial whey proteins without disturbing the same whey for possible downstream conventional whey processing, if selling prices of purified Lf or IgG can offset the additional processing cost. An advantage of this process is that it can be easily scaled up and is possible to be operated on a continuous mode in industry.

Presentations:

Wang, C.H. and J.P. Chen, "Isolation of Lactoferrin and Immunoglobulin G from Cheese Whey by Affinity Microfiltration," paper presented at 51th IFT annual meeting, Anaheim, California, June, 1990.

Publications:

Chen, J.P. and C.H. Wang, Purification of Lactoferrin and Immunoglobulin G from Cheese Whey by Affinity Cross-Flow Filtration, *Journal of Food Science* (submitted for publication).

Project Title:

Freeze Concentration of Fluid Dairy Products

Personnel:

Richard W. Hartel, assistant professor, Dept. of Food Science; Leon Espinel, graduate student; Myong-Soo Chung, graduate student; Yuping Shi, visiting Chinese scholar.

Funding:

Wisconsin Milk Marketing Board

Funding Code:

IV-56

Project Dates:

July 1, 1988 - June 30, 1991

Objectives:

1. The optimum nucleation and growth conditions of ice crystals in dairy products must be found in order to optimize the freeze concentration process.

2. An important distinction must be made between two mechanisms of nucleation of ice.

3. Once the nucleation and growth kinetics have been established for dairy products, a computer simulation can then be implemented to find reactor configurations that result in large ice crystals with a narrow size distribution to facilitate the washing step.

4. Another important aspect for developing a reliable continuous freeze concentration system for dairy products is that of long-term stability of nucleation and growth.

Nucleation Experiments:

Objectives:

Nucleation of ice crystals is critical to the development of freeze concentration technologies. Providing the seed stock from which to grow large ice crystals suitable for separation is crucial to the efficient operation of the process. In addition, understanding the mechanisms of nucleation that may occur during the suspension growth of ice crystals to large size is also important. Defining the conditions under which nucleation becomes important during the growth process will allow operation of the suspension type crystallizer at optimal growth conditions. For example, lower temperatures will cause the ice crystals to grow more rapidly. However, nucleation will occur under these conditions resulting in an unacceptable product crystal size distribution. Outlining the conditions under which nucleation is suppressed will allow the most rapid growth conditions that yield a suitable size distribution for separation. In order to study these phenomena, several experimental techniques were utilized to observe the basic mechanisms of nucleation as well as the practical sense of the conditions under which nucleation will occur in a suspension crystallizer.

Experimental:

Photomicroscopic Cell Experiments

Two sets of experiments were conducted in this project. The first involved the use of a photomicroscopic cell suitable for cold temperature work. In these experiments, a seed crystal generated by freezing a droplet of water on the end of a microsyringe was slid across a glass cover slip in the photomicroscopic cell under controlled temperature conditions. This gentle sliding contact was found to cause the formation of secondary nuclei under certain conditions of type of product and temperature in the solution. The formation and subsequent growth of these nuclei were observed by photomicroscopy.

Batch Crystallizer Experiments

The second set of experiments involved the use of a batch crystallizer for observation of the onset of secondary nucleation. It was found that most dairy products, especially at higher concentrations, were sufficiently

translucent that the photomicroscopic technique was not suitable for observation of the onset of nucleation by a contact mechanism. Therefore, a batch procedure was implemented that enabled the detection of the critical temperature below which secondary nuclei were formed due to collisions of a seed crystal with the stirring impeller and container walls.

The experimental procedure involved preparation of suitable dairy products at various concentrations including whey permeate, whey powder and skim milk . In addition, a solution made up of whey extract and whey protein concentrate was used. These solutions were maintained in the batch crystallizer in a constant temperature water bath for sufficient time that equilibrium was attained and no nucleation by any mechanism was observed. That is, the solutions were held at temperatures high enough that no spontaneous nucleation would occur. Agitation at a constant stirring rate using a marine propeller was initiated. A single seed crystal, similar in nature to those used in the photomicroscopic cell experiments described previously, was dropped into the stirred solution at a specific temperature and bulk subcooling (freezing temperature minus bulk temperature). After a period of several minutes and for an hour thereafter, samples were removed from the crystallizer and placed under the microscope stage to determine if nucleation had occurred.

Nucleation was detected when there was a sufficient number of ice crystals present in the crystallizer caused by the contact nucleation of the seed crystal. The temperature and subcooling at which nucleation was first observed was recorded as the critical subcooling required for the onset of secondary nucleation. These temperatures were much higher than the temperatures required for onset of spontaneous nucleation by some heterogeneous mechanism.

Results:

Crystal Development

When a seed crystal was slid across a glass cover slip in sugar solutions at the appropriate subcoolings, a crop of secondary nuclei could be observed along the path of contact. These nuclei were generated by the sliding collision of the seed crystal and the surface and were likely due to the disruption of a semi-ordered layer at the surface of the seed crystal. This adsorption layer during crystal growth is thought to be where the water molecules are rearranging from the liquid water phase to become incorporated into the crystal lattice structure. Any contact at this point will cause small clusters of water molecules to be passed into the bulk solution where, under the appropriate conditions, they will survive and grow to appear as secondary nuclei. Similar results have been observed for a variety of crystal systems including sucrose and lactose in aqueous solutions.

Temperature and Concentration Effect on Nucleation

Table 1 shows the critical temperatures necessary for secondary nucleation in lactose and sucrose solutions. Several repeats were performed on each solution to determine the critical subcooling temperature and the standard deviation of this temperature. Some differences were observed between lactose solutions of varying concentration, however, these changes were not large. In Figure 1, the raw data from Table 1 are converted into critical subcoolings for each solution. It appears that there is some effect from lactose concentration up to a point and that sucrose gives an effect similar to lactose.

A similar table can be developed for the critical subcooling for secondary nucleation in the batch crystallizer for lactose solutions, as shown in Figure 1. Here, it appears that there is an effect of lactose concentration on the critical subcooling for nucleation although the standard deviations of the measurements are quite high compared to the changes between lactose concentrations. Figure 1 compares how this critical subcooling changes with lactose concentration for both the photomicroscopic cell and the batch crystallizer.

Figure 1. The Effects of Lactose Concentration on the Critical Subcooling Temperature.

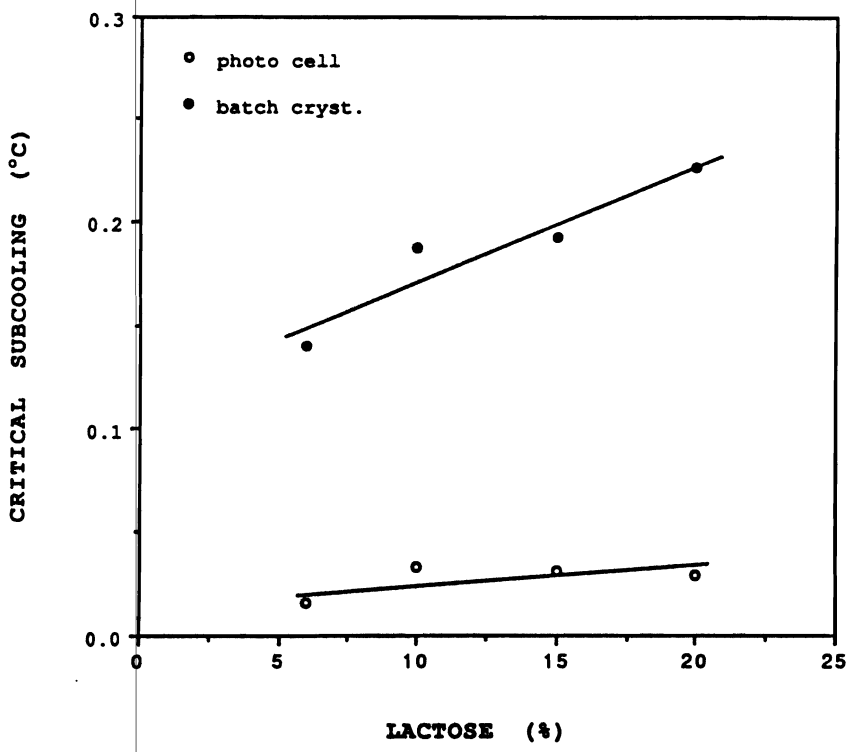


Figure 2. Effects of Whey Protein in Aqueous Dairy Products on the Critical Subcooling Temperature for Contact. Nucleation

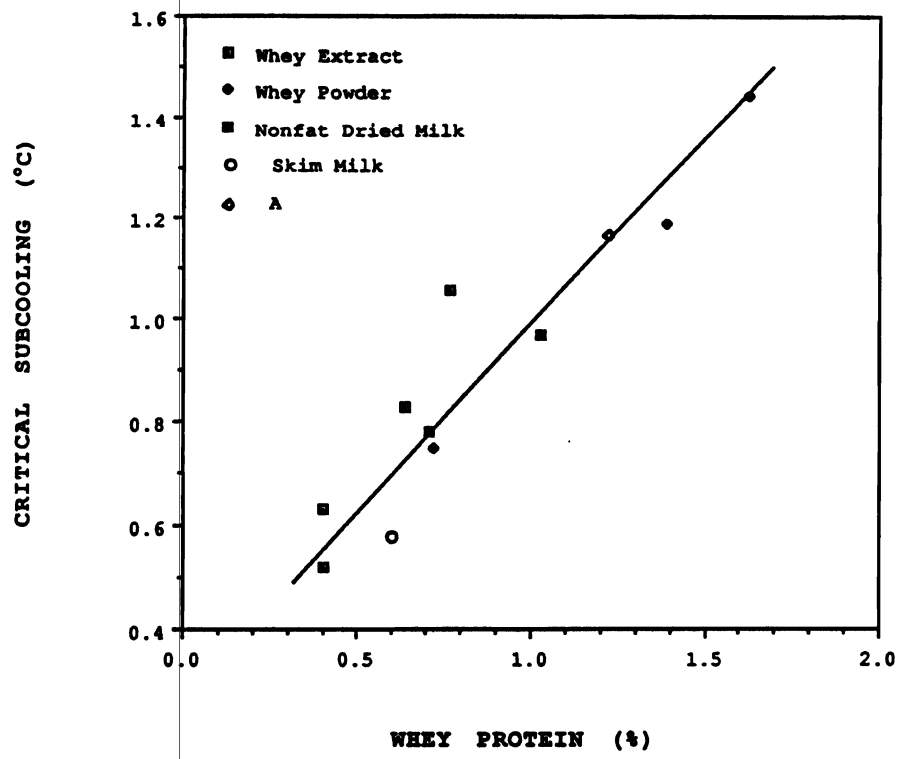


Table 1. Occurrence of contact nucleation in aqueous sugar solutions as determined using the photomicroscopic technique.

ΔT (°C)	6% lactose	10% lactose	15% lactose	20% lactose	6% sucrose
-0.025	N	-	-	-	-
-0.006	N	-	-	-	-
0.003	N	-	-	-	-
0.005	N	N	N	-	-
0.008	N	N	-	N	-
0.010	N	-	N	-	-
0.011	N	-	-	-	-
0.013	NNNYNNNN	-	N	N	N
0.014	NY--NY-N	N	-	N	N
0.015	Y-NYN-NN	-	-	-	N
0.017	---N--Y	-	-	N	NYYNN
0.018	--N-N---	-	-	-	N---N
0.019	---N---	-	-	-	N--N-
0.020	--N-Y-N-	N	N	-	Y--Y-
0.021	Y-Y---N-	-	N	N	---N
0.022	-----Y-	-	N	-	---N
0.023	-	N	YNN	N	---Y
0.024	-	N	--N	NNNNY	-
0.025	-	-	---	Y-N--	-
0.027	-	N	-NN	---N-	-
0.028	-	NYNN	-NN	-NNN-	-
0.029	-	N---	-N-	-N-Y-	Y
0.030	Y	N-N-	-Y-	-Y---	-
0.032	-	Y-NN	--N	--N--	-
0.034	-	--N-	--N	--N--	-
0.035	-	--YN	---	-----	-
0.037	-	---N	--N	--N--	-
0.038	-	---Y	---	Y-N--	-
0.040	-	-	--N	--Y--	-
0.041	Y	-	Y-N	-	Y
0.043	Y	-	-YY	-	-
0.049	Y	-	Y	-	Y

N - No nucleation
Y - Nucleation

Table 2. Critical subcooling temperatures, ΔT_c, for contact nucleation of ice in fluid dairy products as determined using the batch crystallization technique. (Whey Extract: 5.8% whey protein, 83.2% lactose, 11% other on dry basis. Whey Powder: 11.8% whey protein, 76.3% lactose, 11.9% other on dry basis. Non-fat dry milk: 34.1% protein, 52.6% lactose, 13.3% other on a dry basis.)

Sample	Freezing Point (°C)	Number of Replicates	ΔT_c (°C)	SD[1]
Whey Extract				
6.8%	-0.59	10	0.632	0.032
11.0%	-0.97	5	0.829	0.040
13.8%	-1.16	5	1.057	0.034
Whey Powder				
6.1%	-0.59	10	0.751	0.034
11.8%	-1.05	5	1.185	0.042
13.8%	-1.32	5	1.446	0.025
Nonfat Dry Milk				
6.9%	-0.36	5	0.519	0.029
12.4%	-0.65	5	0.783	0.016
17.8%	-0.92	5	0.969	0.035
Skim Milk				
9.8%	-0.56	5	0.583	0.019
A[2]				
9.1%	-0.68	4	1.170	0.030

[1] Standard deviation of ΔT_c.
[2] Solution A contains 100 ml of 6.8% whey extract and 2.5 g of whey protein concentrate (34.5% protein).

Variation in Methods of Measurement of Nucleation

A large difference can be observed in the value of the critical subcooling for secondary nucleation, depending on the method of measurement. These differences can be attributed to the differences in the type of contact encountered, the energy of the contact and the method of observation of the contact nuclei. The gentle sliding contact has a lower contact energy than in the batch crystallizer, however, the contact is a direct event and the observation is immediate through the microscope objective. In the batch crystallizer, there may be a higher contact energy, however, the chances of a contact event are much reduced as compared to the photomicroscopic cell technique. In addition, many more nuclei are probably required for observation of a nucleation event in the batch crystallizer as compared to the photomicroscopic cell. These results serve to demonstrate the condition specific nature of these critical subcooling nucleation experiments. Nevertheless, useful information regarding the occurrence of secondary nucleation can be obtained from these techniques.

Critical Subcooling and Total Solids Content in Fluid Dairy Products

Table 2 shows the critical subcoolings obtained for several fluid dairy products at various concentrations. The critical subcooling was seen to increase with increasing total solids content for each product classification. However, significant differences were observed between products. Solutions made from nonfat dried milk (NFDM) powders resulted in the lowest critical subcooling while whey extract solutions had the highest subcooling. Skim milk from the UW Dairy Plant had a critical subcooling value slightly below the equivalent NFDM solution probably due to the difference in the protein structure. The constituents of these fluid dairy products were identified and correlated with the critical subcooling using a multiple regression analysis. It was found from the statistical results that the critical subcooling was significantly affected by the whey protein content of the dairy product, with lactose having no significant effect. Total protein and total solids had an effect as related to the whey protein content. Figure 2 shows the effects of whey protein content in each of the dairy products on the critical subcooling temperature. A direct relationship was observed where the critical subcooling increased directly as the whey protein content increased regardless of the product classification.

Summary:

These results demonstrate the nature of the secondary nucleation event of ice crystals in fluid dairy products. It has been observed that the magnitude of the critical subcooling temperature above which secondary nucleation is negligible depends on the type of contacting system used to observe the nucleation event. This indicates that, at this point, each freeze concentration system will require an individual study to determine the critical subcooling point for any given dairy product. Further work will be required to fully understand the mechanism of secondary nucleation so this event can be predicted from first principles. It has also been observed that the magnitude of the critical subcooling depends on the type of fluid dairy product and its concentration. That is, this boundary will be continually changing during the freeze concentration process as the product becomes more and more concentrated. The results indicate that the whey protein content plays the dominant role in determining the magnitude of the critical subcooling with an increase in whey protein content resulting in a direct increase in the critical subcooling temperature for secondary nucleation. Further work will be necessary to detail the exact mechanism of this interaction.

Determination of this critical subcooling for contact nucleation is important for the operation of suspension crystallizers. Operation of the crystallizer below this critical subcooling point will result in the formation of new nuclei. These nuclei will cause a decrease in the mean crystal size and produce a wider size distribution that will be more difficult to separate.

Growth Experiments

Objectives:

The three-stage freeze concentration process involving nucleation, growth, and separation is limited by the rate at which large crystals can be grown from the nucleated seeds. The size and distribution of these product crystals also determine, to a large extent, the efficiency of the separation device. For the most efficient separation, large ice crystals of uniform shape (ideally spherical) with a narrow size distribution are required. Optimization of this growth process for fluid dairy products plays an important role in future development of freeze concentration systems for dairy products and is the main focus of this research.

Previous work in the past year has developed the technique for controlling the rate of ice crystal growth in a suspension crystallizer by controlling the heat balance conditions existing in the crystallizer. That is, the rate of heat generation caused by the latent heat of formation during the phase change must be balanced by the rate of heat transfer removal from the crystallizer through the walls of the vessel. When these heat terms are balanced, it has been shown that large, spherical ice crystals with a narrow size distribution can result. The objectives in the past year have been to extend this experimental technique to the study of the concentration of skim milk in an effort to determine the conditions for optimal ice crystal growth.

Experimental:

Growth of seed crystals in skim milk has been studied in a controlled batch crystallizer. In brief, skim milk is brought to a constant temperature below its freezing point and equilibrated at a given subcooling in a constant temperature water bath. Seeds, nucleated separately from a 10% lactose solution in a colder water bath, are added to the skim milk at some initial time. The growth of these ice nuclei into large product-sized crystals is followed by photomicroscopy.

Samples are taken from the crystallizer periodically and analyzed for size distribution using image analysis of the resulting photomicrographs. The change in average size of the distributions are used as a measure of the rate of growth of these ice crystals. Conditions within the crystallizer are maintained such that a heat balance exists throughout the experiment.

In order to maintain heat balance conditions, several parameters were adjusted as the crystallization progressed. These were the refrigerant temperature, the total surface of ice crystals, and the stirring rate. The refrigerant temperature was continually lowered so as to maintain a relatively constant rate of heat transfer removal from the system. As the suspension builds up, that rate of heat generation increases. This can be counterbalanced by reducing the refrigerant temperature as well as by periodic removal of ice crystals from the suspension. In addition, as the concentration of the remaining liquid increases, the heat and mass transfer coefficients continually decline unless the agitation rate is increased accordingly. Therefore, each of these three parameters was varied in order to maintain approximately constant heat balance conditions within the crystallizer and thereby maximize the rate of ice crystal growth throughout the duration of the experiment.

Results:

Ice Crystal Growth Under Near Balance Conditions

Table 3 shows a summary of the experimental conditions and results for ice crystal growth under heat balance conditions for skim milk in batch crystallization. Ice crystal products with average crystal sizes ranging from 0.5 to 2.5 mm can be grown in a period of several hours depending on the initial growth conditions. Final product concentrations ranging from 13% to 29% total solids were obtained under these conditions.

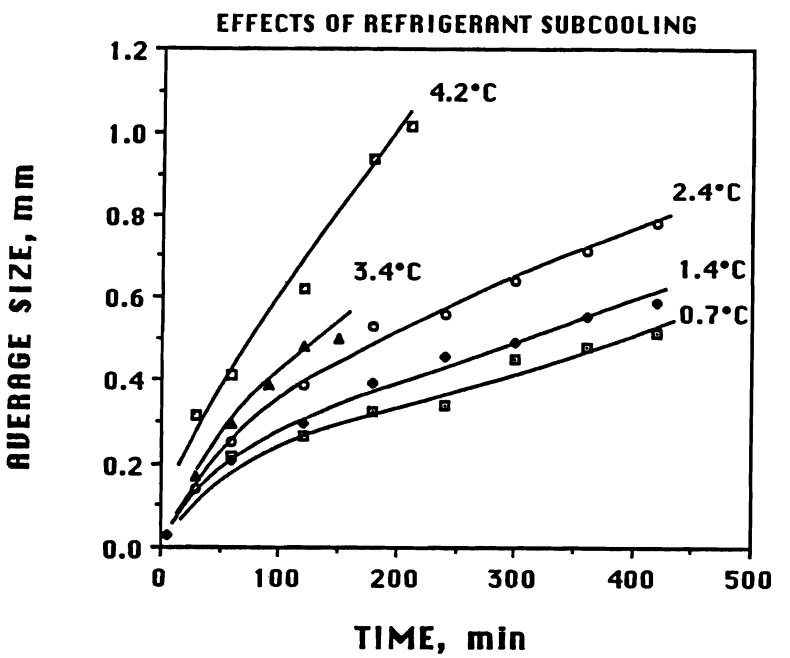
Typical curves for average size vs. time are shown in Figure 3. These curves demonstrate the efficiency of growing ice crystals under these conditions. For comparison, typical batch crystallization experiments result in size vs. time curves that start out with high slopes

Table 3. Experimental Conditions and Results for Ice Crystal Growth Under Heat Equilibrium Conditions in Skim Milk in Batch Crystallization.

Run	Seeds (ml)	$(T_b - T_c)$ (°C)	Holding Time (h)	C_f (%)	L_f (mm)	Nucleation
RM6	20	0.7	7.0	13.3	0.52	No
RM2	20	1.4	7.0	16.9	0.59	Few
RM11	10	1.5	6.5	17.9	0.64	Yes
RM12	5	1.5	7.0	21.2	2.57	No
RM13	10	1.5	6.5	17.3	1.32	No
RM3	20	2.4	6.0	19.4	0.42	Yes
RM4	20	2.4	6.0	22.6	0.71	Few
RM10	10	2.5	6.5	21.6	0.84	Few
RM14	1	2.5	5.5	21.9	1.34	No
RM5	20	3.4	6.5	26.6	0.57	Yes
RM9	10	3.4	2.5	20.3	0.50	Few
RM7	20	1-4.2	4.0	27.4	1.35	No
RM8	7	4.3	4.5	29.1	0.53	Yes

* Seeds formed by spontaneous nucleation in 10% lactose and added as slurry.
* $(T_b - T_c)$ - bulk solution temperature minus refrigerant temperature
* C_f - final concentration; L_f - final average crystal size

Figure 3. Growth of Ice Crystals in Skim Milk at Various Refrigerant Subcoolings under Heat Balance Conditions. Refrigerant subcooling is bulk solution temperature minus the refrigerant temperature.



but then the increases in average size with time start to decrease as the equilibrium conditions are approached. Eventually, the ice crystals will stop growing as a thermal equilibrium is attained. By maintaining constant heat balance conditions, the growth rate of ice crystals can be maintained for long periods of time resulting in large crystal sizes in relatively short periods of time.

Considerations for Development of Technology

Although these results are quite promising, several difficulties must be overcome prior to the development of this technology. These include:

1) control of the process and experimental conditions,

2) elimination of product foaming,

3) development of a continuous process based on these principles.

These experiments were controlled manually based on the experience of the operator. Currently, alternative techniques are being developed and evaluated for control of these processes. Future commercialization of this technology will depend on this control development. Another problem associated with the agitation rates necessary to maintain optimal rates of heat and mass transfer arises due to foaming of the skim milk product. This was evidenced by a multitude of fine bubbles incorporated in the milk product during the concentration. These air bubbles may have had some impact on any nucleation events that took place in the suspension crystallizer. Current work involves operation at lower stirrer speeds along with the use of antifoaming agents in the milk. Future work will necessitate the development of a freeze concentration technology that minimizes foaming of the product due to high agitation rates. The current experiments operate in a semi-batch mode, meaning that some product is withdrawn during the course of the experiment, but no new material is fed in.

Commercialization of this technology will likely require that the process be made into a fully continuous one such that dilute product is continually fed in at one end and the concentrate is continually removed at the other. Conversion of this heat balance technique to continuous operation will require the development of crystallizers that enable a continuous change in conditions to be implemented. That is, the refrigerant temperature must change throughout the course of the crystallization.

Summary:

The current process for growing large ice crystals based on maintaining a high level of heat balance in a suspension crystallizer shows great promise for future developments in freeze concentration technology. Further work is required for development of this unique technology and for outlining the ice crystal growth kinetics for a variety of fluid dairy products under a variety of conditions. A significant amount of effort will be required to develop this technique into a commercial technology. Nevertheless, the potential gains from an economic freeze concentration process warrant continued efforts in this area.

Significance of Findings to the Dairy Industry:

Freeze concentration is a relatively new technique for concentrating fluid foods. It has several advantages, particularly for heat-sensitive foods, over the traditional techniques of evaporation and reverse osmosis due to the low temperature operation. At the present time, however, the economics of freeze concentration do not compare favorably with evaporation or reverse osmosis. Further research on the fundamentals of ice nucleation and growth in fluid dairy products is necessary to allow the full advantages of freeze concentration to be determined. The results of this study show that control of the heat balance in an ice crystallizer can result in rapid growth of ice crystals to product size for efficient separation. These results may allow the development of more economically attractive means of concentrating fluid dairy products using freeze concentration.

Publications:

1. Shi, Y.,B. Liang and R.W. Hartel. 1990. Crystallization of Ice From Aqueous Solutions in Suspension Crystallizers, ACS Symposium Series (in press).

Presentations:

1. Shi, Y.,B. Liang and R.W. Hartel. 1989. "Ice Crystal Morphology During Growth From Aqueous Solutions in a Suspension Reactor," presented at American Institute of Chemical Engineers National Meeting, San Francisco, CA, November 1989.

2. Shi, Y., B. Liang and R.W. Hartel. 1989. "Crystallization of Ice From Aqueous Solutions in Suspension Crystallizers," presented at PACIFICHEM meeting (joint ACS), Honolulu, HA, December 1989.

3. Shi, Y,B. Liang and R.W. Hartel. 1990. Nucleation and Growth of Crystallization symposium to be held in September 1990, Garmisch, West Germany.

Theses:

1. Chung, Myong-Soo. 1990. Formation of Contact Secondary Nuclei From Crystals in Sugar Solutions and Fluid Dairy Products, MS thesis, UW-Madison.

Project Title:

New Dairy Foods with Added Fiber and Added Calcium

This project involves three principal investigators with the common objective of developing a nutritionally significant new dairy food. This is a final report and is comprised of a report from each principal investigator. The editors.

Personnel:

Owen Fennema, professor, Dept. of Food Science (co-PI), Janet Greger, professor, Dept. of Nutritional Sciences (co-PI); Ken Lee, associate professor, Dept. of Food Science (co-PI); Susan M. Kaup, (PhD) research associate, Dept. of Nutritional Sciences; Ni Luh Puspitasari, graduate student, Dept. of Food Science; Joey Ann Hendrick, graduate student, Dept. of Food Science, Olafur Reykdal, graduate student, Dept. of Food Science.

Funding:

National Dairy Promotion and Research Board

Funding Code:

88-4

Dates:

July 1, 1988 - June 30, 1990

Objectives:

This project represents a collaborative venture. The common objective is to create new dairy foods and dairy ingredients using fortification techniques. Specific objectives are:

1. Study milkfat and fiber interactions to provide insight on the use of fiber in dairy products and the effect of fiber on milkfat utilization. (Fennema)

   - Although the addition of some hydrocolloids may allow claims for dietary fiber enrichment, it is not our intent to provide significant quantities of fiber in dairy foods. The intent is to develop new dairy foods using these ingredients. Any additional dietary fiber introduced into the diet as a result of this work may be considered a positive side effect.

2. Develop calcium-fortified dairy products with high consumer appeal and high biological value. (Greger)

   - Methods to raise calcium levels in existing dairy products with no adverse affects on texture or flavor will be explored.

   - The bioavailability of calcium in fortified dairy products will be measured.

3. Develop new functional properties for dairy ingredients and new dairy products based on chemical interactions between hydrocolloids (dietary fibers) and dairy components. (Lee)

   - Create new dairy foods using high moisture and hydrocolloid addition. The ability of hydrocolloids to hold water opens the possibility of the development of new dairy foods which have significantly fewer calories with acceptable sensory properties.

Project Title:

New Dairy Foods with Added Fiber and Added Calcium

Report from Dr. Owen Fennema

Personnel:

Owen Fennema, professor, Dept. of Food Science (co-PI), Joey Ann Hendrick, graduate student, Dept. of Food Science

Objectives:

1. Study milkfat and fiber interactions to provide insight on the use of fiber in dairy products and the effect of fiber on milkfat utilization. (Fennema)

- Although the addition of some hydrocolloids may allow claims for dietary fiber enrichment, it is not our intent to investigate the nutritional benefits of fiber in dairy foods. The intent is to develop new dairy foods using these ingredients. Any additional dietary fiber introduced into dairy products as a result of this work may be considered a positive side effect.

Refinement of original objective:

-The purpose of this research was to assess the effects of various types of fiber, both soluble and insoluble, on lipase-catalyzed hydrolysis of triacylglycerols in an in vitro system simulating conditions prevailing in the human duodenum. Few studies of this kind have been attempted and the procedures, when properly validated, will be extremely useful as a rapid, economical means for determining the effects of various fibers on the rates and extents of lipolysis, especially hydrolysis of milkfat, and perhaps on other matters of interest, such as the relationship between fiber consumption and blood composition (cholesterol, lipids).

Executive Summary

The first studies were done using tributyrin (contains short-chain fatty acids) as a substrate. This fat was selected since the fatty acids released upon hydrolysis are water soluble and can be directly and continuously monitored with an automatic titrimeter. These experiments were undertaken because they permitted a quick assessment of whether fibers of the types tested had an inhibitory effect on fat hydrolysis. Of the ten fibers tested, red and white wheat bran, oat bran, and sugarbeet fiber caused the rate of lipase-catalyzed hydrolysis of fat (tributyrin) to decrease substantially.

Tests with fats containing medium- to long-chain fatty acids (all commercial fats including milkfat) have not yet been conducted because of problems with methodology. Since the fatty acids released during hydrolysis are insoluble, they cannot be directly and continuously titrated. Existing tests that are supposedly suitable for this situation have proven to be unsuitable. Considerable effort has, therefore, been expended in developing a new test, and designing and fabricating suitable reaction vessels. The methodology is almost perfected to the point where studies of milkfat can begin.

Effects of Fiber on Tributyrin Hydrolysis

Development of Methods:

The development of suitable methodologies was required since few of the needed procedures and conditions had been established previously. The composition of the reaction mixture was determined after a thorough review of the literature dealing with the physiology of digestion. Thus, sample pH, temperature, ionic strength, types of electrolytes, types, and concentrations of bile salts, and the concentrations of lipase, co-lipase, fat, and fiber all were selected to conform with conditions that are normally encountered in the human duodenum.

For the first studies, an automatic titrimeter was chosen to monitor the release of butyric acid from tributyrin. This method was validated based on recovery studies done by adding known amounts of butyric acid to test samples with and without fiber.

A stirring speed of 150 rpm was chosen since the rate of tributyrin hydrolysis in the absence of fiber was unaffected by changes in stirring

speed over the range of 50 to 150 rpm. This speed ensured sample homogeneity and was found to provide the most severe test of inhibition of lipolysis by fibers.

A 30-minute reaction time was chosen to assure that substrate availability was not a rate limiting factor and because linear plots could be obtained over this time period. In control samples (no fiber) about one-third of the lipid was hydrolyzed during this period.

Several levels (4%, 8%, and 12% wt/vol on a wet basis) of lipids were tested and a concentration of 4% was arbitrarily selected. This represented about 80% lipid on a dry basis when 1% fiber was present, and a 1:4 ratio of fiber to lipid. This is within the range encountered in human diets.

Several methods were tested for plotting the data in an attempt to obtain linearity. A plot of the logarithm of substrate remaining versus time produced the best linearity (corresponds to first-order reaction kinetics), and was therefore selected.

Results:

The following fibers were tested at a 1% (wt/vol wet basis; fiber-fat ratio of 1:4 on a dry basis) concentration: red and white wheat bran, oat bran, sugarbeet fiber, psyllium seed, pectin LM 12 CG, carrageenan, carboxymethylcellulose, gum arabic, and pectin slow set. Four of these fibers exhibited a significant ability to inhibit the rate of tributyrin hydrolysis. Listed in decreasing order of inhibition they were: red wheat bran, white wheat bran, oat bran, and sugarbeet fiber (Figure 1).

Effects of Fiber on Triolein Hydrolysis

Development of Methods:

The analytical procedure used for monitoring release of butyric acid could not be used to measure release of oleic acid from triolein because oleic acid is insoluble in the test medium. Thus, an extraction procedure involving organic solvents was selected from the published literature. A stirring device was designed and constructed for these triolein studies. This device enabled six samples in test tubes to be stirred simultaneously at constant speed and constant temperature, and to be started or stopped independently, if desired. The stirring paddles were also custom made to accommodate samples with high viscosity. After testing stirring speeds ranging from 50-150 rpm, a speed of 150 rpm was selected for the reasons stated above.

Many attempts were made to obtain linear plots of reaction progress versus time but this was never achieved. At least two circumstances contributed to this lack of success: the lipid was present as an emulsion rather than in solution and the analytical method was less accurate and precise than that used for tributyrin. Because of this lack of plot linearity, the effects of fibers were compared at fixed reaction times.

Results:

The following fibers were tested at a 1% (wt/vol wet basis; fiber-fat ratio of 1:3 on a dry basis) concentration: oat bran, soy fibers (Fibrin [TM] 2000 and 1450 NI), red and white wheat bran, and psyllium seed. Two of these fibers exhibited a significant ability to inhibit the rate of hydrolysis of triolein. Listed in decreasing order of inhibition they were: Oat bran and soy fiber (1450 NI).

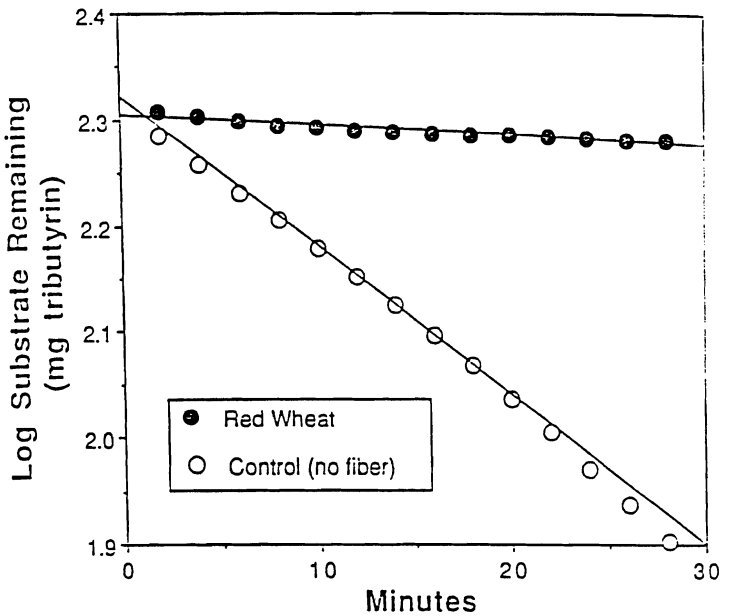
The results obtained from these studies indicate that certain fibers have inhibitory effects on the rate of lipase-catalyzed hydrolysis of both tributyrin and triolein. The mode of inhibition remains largely unknown.

Improvement of the Analytical Procedure for Determining Free Oleic Acid in the Presence of Calcium.

Calcium was present in the original reaction mixture and this caused the freed oleic acid to become insoluble. The analytical procedure obtained from the literature was found to recover only about 80% of known amounts of oleic acid added to the samples used in this study. Considerable effort has been expended to alter the procedure and thereby achieve greater recoveries. The results of our efforts are shown in Table 1, and the improved

Figure 1. Lipase-catalyzed hydrolysis of tributyrin in the presence of: A) red wheat bran, B) white wheat bran, C) oat bran, D) sugarbeet fiber. Each symbol represents a mean of three observations. Tributyrin 4 % (wt/vol wet basis), red wheat bran 1 % (wt/vol wet basis; fiber to fat ratio of 1:4.1 on dry basis). Samples reacted for 30 minutes at 37°C; data first two minutes omitted.

1A.



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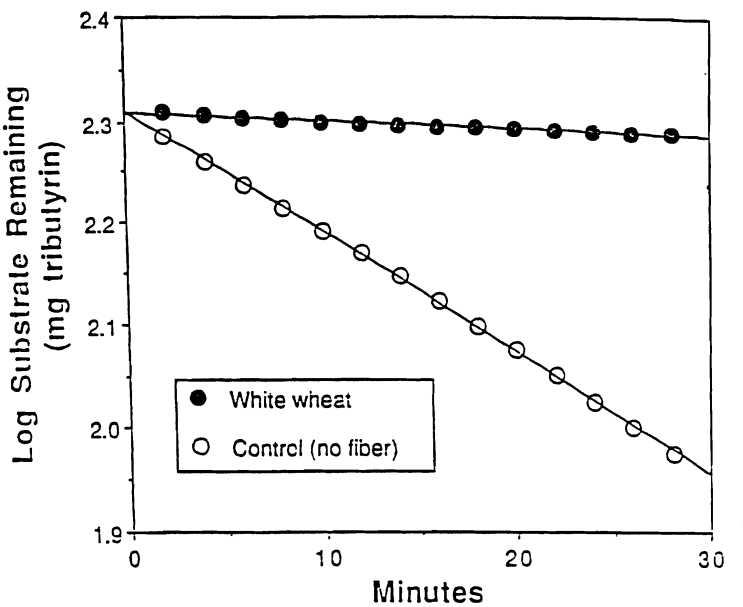

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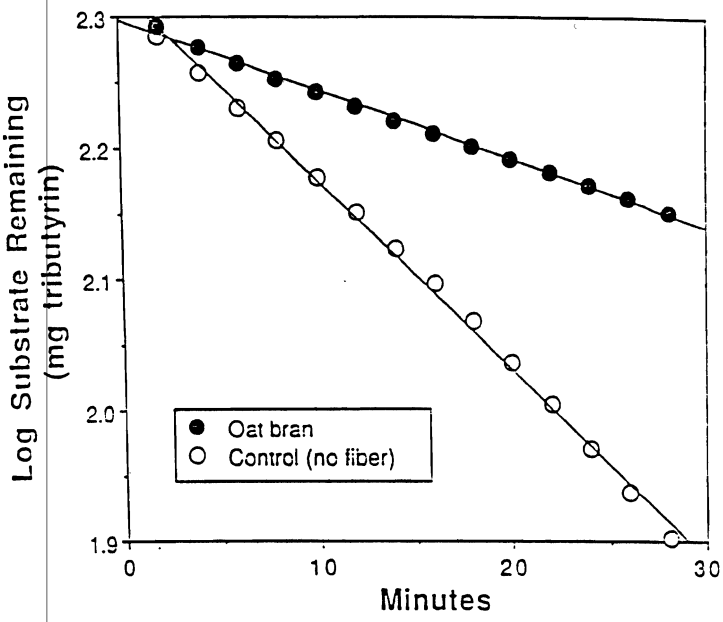
Log Substrate Remaining
(mg tributyrin)
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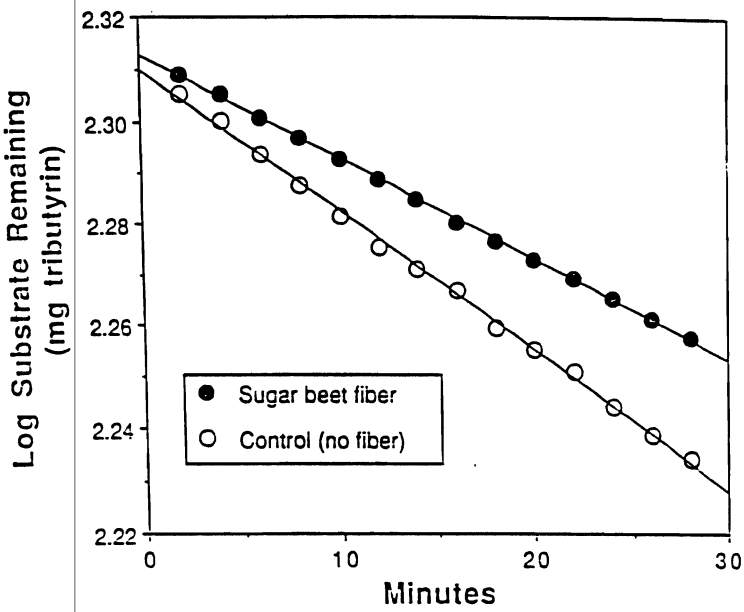
2.2
2.1
2.0
1.9
0
10
20
30
Minutes
Oat bran
Control (no fiber)

1D.


Log Substrate Remaining
(mg tributyrin)
2.32
2.30
2.28
2.26
2.24
2.22
0
10
20
30
Minutes
Sugar beet fiber
Control (no fiber)

procedure will be used in all future studies involving long-chain fatty acids.

Implications of Research:

Dietary lipids represent the major source of calories in the diet. Furthermore, their consumption has a proven relationship to other measures of human health such as levels of triglycerides and cholesterol in the blood. Based on in vivo studies, it is known that consumption of fiber can lower blood levels of cholesterol and can increase the concentration of lipids in the feces (lessen bioavailability of fat). The development of in vitro procedures to accurately monitor the effects of various fiber types and levels on rates and extent of lipolysis could be important for several reasons:

1) This test will enable interactions between milkfat and fiber to be easily and accurately studied. Of special interest is whether the presence of fiber effects the hydrolysis of milk fat in different ways than that observed for plant triacylglycerols.

2) The in vitro test might serve as a simple, accurate, and inexpensive means of predicting results in vivo, thus enabling the vitro test to be used to easily select a concentration and type of dietary fiber that will achieve a desired objective in vivo (e.g., lower blood cholesterol).

3) The mechanisms by which various fibers influence rates and extents of lipolysis can be most easily determined by means of in vitro tests where experimental variables are subject to adequate control.

4) Added understanding of fiber-lipid interactions obtained from studies in vitro may allow lipids and fibers to be manipulated (interaction of lipid and fiber prior to consumption) so that lipid bioavailability can be reduced well below what is now possible.

Publications from project:

Hendrick, Joey Ann. 1989. Effect of various fibers on lipase-catalyzed hydrolysis of lipids in vitro. M.S. thesis, University of Wisconsin-Madison.

Table 1. Recovery of Oleic Acid[a]

Fiber type	Fiber Concentration (wt/vol)	% Recovery	X	S
Pectin (type LM 12CG)	2%	94.4 96.0 95.1	95.2	0.8
Psyllium	1%	95.8 92.7 92.0	93.5	2.0
Oat Bran	1%	90.4 90.1 84.7	88.4	3.2
Control (2-10-90)[b]	0%	99.9 97.2 98.3	98.5	1.3
Control (2-17-90)[b]	0%	102.2 101.2 102.3	101.9	0.6

a = The amount of oleic acid added was equivalent to that produced by 50% hydrolysis of 4% (wt/vol) triolein or 0.200 g per sample. b =Mean and standard deviation for combined control trials were 100.2 and 2.1 respectively.

Project Title:

New Dairy Foods with Added Fiber and Added Calcium

Report from Dr. Janet Greger

Personnel:

Janet L. Greger, professor, Dept. of Nutritional Sciences and Susan M. Kaup, postdoctoral associate, Dept. of Nutritional Sciences.

Objectives:

2. Develop calcium-fortified dairy products with high consumer appeal and high biological usefulness. (Greger)

- Methods to raise calcium levels in existing dairy products with no adverse affects on texture or flavor will be explored.
- The bioavailability of these calcium-fortified dairy products will be measured.

Executive Summary

The nutritional properties of cottage cheese fortified with calcium lactate or with a calcium lactate guar complex were reevaluated in a rat model. Apparent absorption of calcium from the cottage cheese was very efficient (>90%). However, overall calcium retention and to a lesser extent, bone calcium content, increased with each incremental increase in dietary calcium. Despite the sensitivity of the model to variation in calcium intakes, no effect of guar gum on calcium utilization was noted. This indicates that the guar gum can be used to mask bitterness induced by the calcium fortification without any deleterious effects on calcium bioavailability.

Incrementally increasing the amount of calcium added to the cottage cheeses increased phosphorus retention, did not antagonize the utilization of magnesium and zinc, and only slightly decreased iron utilization in animals. Overall, this work demonstrates that cottage cheese could be fortified with calcium to produce a product in which calcium is highly bioavailable with little negative effects on the utilization of other minerals. These types of data are needed when nutritional claims are made for a food, especially a new or modified one.

Materials and Methods:

Cottage cheese was manufactured in the university dairy plant to contain 37.5% dressing and 62.5% curd. In prior work, we determined that cottage cheese fortified with food grade calcium lactate (Ballord-Schlesinger, Carle Place, NY) and a hydrocolloid solution of low viscosity guar gum (Guar TICOLV, TIC Gum, Inc., Belcamp, MD) had more acceptable flavor and sensory properties than cottage cheese fortified with other calcium salts (calcium chloride, calcium phosphate, calcium carbonate, and calcium citrate). Moreover, the guar gum succeeded in masking bitterness of added calcium better than other hydrocolloids (gum arabic, carrageenan, xanthan gum, locust bean gum). Cottage cheeses (four with 0.28% guar gum and graded levels of calcium and one without gum or calcium additives) were freeze-dried so that they could be incorporated into animal diets.

Forty-eight weanling male Sprague Dawley rats (Harlan Sprague Dawley, Madison, WI) (n=6/treatment) rats were fed for 28 days, one of eight diets that contained 20% protein as supplied by dried cottage cheese with graded levels of calcium. Graded levels of calcium were provided by either a calcium lactate-guar gum complex added to the cream of the cottage cheese during processing (Diets Guar 2.1, Guar 2.8, Guar 3.4, and Guar 4.1) or by calcium lactate added directly to the cottage cheese-based diet (Diets-Control 2 .2, Control 2.9, Control 3.6 and Control 4.4). The exact concentration (mg/g) of calcium in each diet as determined by analysis is indicated by the name of the diet, ie., Diet Guar 2.1 contained 2.1 mg Ca/g diet. The cottage cheese-based diets were determined by analyses to provide 2.85 mg P/g diet, 0.59 mg Mg/g diet, 35ug Zn/g diet, 37 ug Fe/g diet, 7.2 mg Na/g diet, 9.4 mg Cl/g diet, and 4.4mg K/g diet.

Results and discussion:

At the beginning of this study, rats weighed 48 +/- 2 g (Mean +/- SEM); at the end rats

weighed 222 +/- 3 g. There were no significant differences in growth or food intake among rats fed the eight diets in this study. Average feed intake of rats was 12.2 +/- 0.1 g/day; the average digestibility of the diets was 93 +/- 0.1%.

Calcium Bioavailability

In general, the rats absorbed calcium from the cottage cheese very efficiently (>90%), but the level of calcium in the cottage cheese significantly ($p<0.001$) affected apparent absorption of calcium (Figure 1a). Despite significantly ($p<0.001$) increased urinary losses (Figure 1b), calcium retention increased significantly ($p<0.001$) with each incremental increase in the level of calcium in the cottage cheese (Figure 1c).

The presence of guar gum in the cottage cheese had no effect on calcium absorption, urinary excretion, or on overall calcium retention.

The growth of the tibias and calcium concentration and content of tibias were all significantly ($p<0.001$) affected by the level of calcium in the cottage cheese but were not statistically affected by the presence of guar gum (Figure 2a-c). All of these data are consistent. Pearson correlation coefficients of calcium retention as calculated by balance techniques with the tibia calcium content ($r=0.938$, $p<0.0001$), with tibia weight ($r=0.903$, $p<0.0001$), and with tibia calcium concentration($r=0.677$, $p<0.0001$) were extremely strong. This suggests that in this model, in which adequate (as determined by overall body growth) but less than recommended levels of calcium are fed, tibia weight and tibia calcium content are especially good indicators of overall calcium utilization and bioavailability.

Phosphorus Utilization

Previously we and others have observed that kidney calcification occurred in rats fed moderate (4-5 mg P/g diet) levels of phosphorus with casein. Thus, we reduced the level of dietary phosphorus in this study to 2.80 mg P/g diet so that kidney calcification did not become a confounding factor. Accordingly, kidney calcium concentrations were unaffected by dietary treatments. They averaged 64.4+/- 0.8 mg Ca/g wet weight. Moreover, creatinine clearance of all rats were within a normal range.

Table 1. Phosphorus utilization of rats fed cottage cheese supplemented with guar gum and calcium.

Dietary treatments	Apparent absorption, of P	Urine P	Apparent retention of P	Tibia P
	(% intake)			(mg/g)
Guar 2.1	93 ± 1[bcd]	35 ± 1[ab]	58 ± 1[e]	53 ± 1[c]
Guar 2.8	95 ± 1[b]	25 ± 1[c]	70 ± 1[d]	58 ± 1[b]
Guar 3.4	93 ± 1[bcd]	17 ± 1[d]	76 ± 0[c]	61 ± 1[b]
Guar 4.1	91 ± 1[de]	9 ± 1[e]	83 ± 1[b]	61 ± 1[b]
Control 2.2	93 ± 1[cd]	38 ± 3[b]	54 ± 3[e]	50 ± 1[c]
Control 2.9	94 ± 1[bc]	25 ± 1[c]	69 ± 1[d]	59 ± 1[b]
Control 3.6	92 ± 1[d]	15 ± 1[d]	77 ± 1[c]	59 ± 1[b]
Control 4.4	90 ± 1[e]	11 ± 1[e]	79 ± 1[bc]	61 ± 1[b]

[a]Mean ± SEM (n=6).

[b-e]Means in a column without a common superscript letter are significantly ($p<0.05$) different.

Table 2. Magnesium utilization by rats fed cottage cheese supplemented with guar gum and calcium.

Dietary treatments	Apparent absorption of Mg	Urine Mg	Apparent retention of Mg	Tibia Mg
	(% intake)			(mg/g)
Guar 2.1	74 ± 3[a,c]	39 ± 1	35 ± 3[d]	1.95 ± 0.03[d]
Guar 2.8	83 ± 1[b]	42 ± 2	42 ± 2[bcd]	2.16 ± 0.04[bc]
Guar 3.4	82 ± 2[b]	44 ± 1	38 ± 2[cd]	2.28 ± 0.04[b]
Guar 4.1	84 ± 1[b]	40 ± 1	44 ± 2[bc]	2.22 ± 0.06[bc]
Control 2.2	76 ± 2[c]	35 ± 3	42 ± 3[bcd]	1.83 ± 0.05[e]
Control 2.9	83 ± 1[b]	36 ± 4	47 ± 3[b]	2.12 ± 0.05[c]
Control 3.6	85 ± 1[b]	38 ± 3	48 ± 3[b]	2.13 ± 0.03[c]
Control 4.4	83 ± 1[b]	40 ± 1	44 ± 1[bc]	2.15 ± 0.06[c]

[a]Mean ± SEM (n=6)

[b-e]Means in a column without a common superscript letter are significantly ($p<0.05$) different.

Table 3. Zinc and iron utilization of rats fed cottage cheese supplemented with guar gum and calcium.

Dietary treatments	Apparent absorption of Zn	Tibia Zn	Plasma Zn	Apparent absorption of Fe	Tibia Fe
	(% of intake)	(μg/g)	(μg/100 ml)	(% of intake)	(μg/g)
Guar 2.1	15 ± 10[a]	137 ± 4[bc]	180 ± 5[b]	78 ± 2[de]	67 ± 3[b]
Guar 2.8	31 ± 4	143 ± 4[b]	165 ± 7[bcd]	83 ± 4[bcd]	60 ± 2[bcd]
Guar 3.4	22 ± 2	142 ± 4[b]	156 ± 6[cd]	76 ± 3[e]	56 ± 3[cd]
Guar 4.1	22 ± 4	127 ± 2[c]	153 ± 9[d]	82 ± 4[cde]	56 ± 2[cd]
Control 2.2	25 ± 7	129 ± 4[c]	174 ± 9[bc]	88 ± 2[bc]	62 ± 3[bc]
Control 2.9	34 ± 5	143 ± 2[b]	171 ± 3[bcd]	89 ± 2[b]	59 ± 3[cd]
Control 3.6	25 ± 5	137 ± 4[bc]	154 ± 7[d]	88 ± 2[bc]	55 ± 4[cd]
Control 4.4	20 ± 7	137 ± 4[bc]	162 ± 6[bcd]	79 ± 3[de]	53 ± 1[d]

[a]Mean ± SEM (n=6).

[b-e]Means in a column without a common superscript letter are significantly ($p<0.05$) different.

Urinary excretion of phosphorus was reduced and phosphorus retention as determined by balance was increased with each incremental increase in calcium intake (Table 1). Tibia phosphorus concentrations were reduced among rats fed the lowest levels of calcium (<2.9 mg Ca/g diet). None of these variables were significantly affected by the ingestions of guar gum in the cottage cheese.

Magnesium Utilization

Sometimes calcium fortification of foods results in reduced utilization of magnesium and trace elements. We wanted to ascertain if this would be a problem with these cottage cheeses.

Rats fed the lowest levels of calcium (Guar 2.1, Control 2.2) absorbed magnesium less efficiently than rats fed the other six diets (Table 2). Overall retention of magnesium as determined by balance technique was not consistently affected by calcium intake, but rats fed the lowest level of calcium (Guar 2.1 and Control 2.2) retained lower concentrations of magnesium in bone than the other rats. The dietary treatments had no effects on kidney or plasma magnesium concentration. These data suggest that the lowest level of calcium fed was inadequate for optimal magnesium utilization but the highest levels fed were not so great to be antagonistic to magnesium utilization, as is sometimes noted.

Zinc and Iron Utilization

Overall, increasing the amount of calcium or guar gum in the cottage cheese did not consistently affect zinc utilization (Table 3). However, tibia iron concentrations decreased as calcium ingestion increased so that rats fed Diets Guar 3.4 and Guar 4.1 had lower tibia iron concentrations than rats fed Guar 2.1. Similarly rats fed Diet Control 4.4 had lower tibia iron concentrations than rats fed Diet Control 2.2. Apparent absorption of iron was lower among rats fed the control cottage cheese when fed the highest level of calcium (Diet Control 4.4 vs. Diets Control 2.2, Control 2.9, Control 3.6). The presence of guar gum appeared to mask the effect of calcium level on iron absorption.

Significance to the Dairy Industry:

With this animal model, we demonstrated that the bioavailability of calcium was high from our calcium-fortified cottage cheese whether or not guar gum was added. The amount of guar gum added was insufficient to decrease mineral utilization.

This model would be useful in substantiating health claims concerning calcium to the FDA. Current discussions indicate that such substantiation will be necessary in the future if health claims are made.

A typical 4 oz. (113g) serving of the cottage cheese used to make Diet Guar 4.1 would provide 249 mg calcium; an 8 oz . glass of milk provides 291 mg calcium. Thus these cottage cheeses with guar gum have sensory acceptance and can provide highly bioavailable calcium in quantities approximating those in milk.

Seminars and Publications:

Greger, J.L., S.M. Kaup, C.F. Powers, K. Lee. Bioavailability of calcium from calcium fortified cottage cheese. *FASEB J* 1990;4:1474.

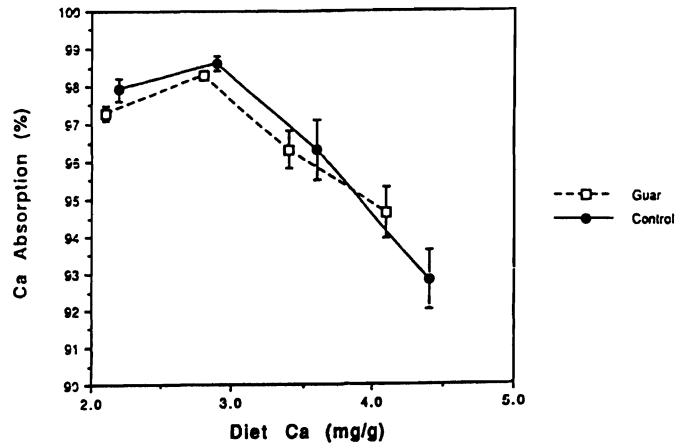
Kaup, S.M., J.L. Greger, K. Lee. Evaluation of the nutritional properties of cottage cheese fortified with calcium and guar gum in an animal model (Submitted for review).

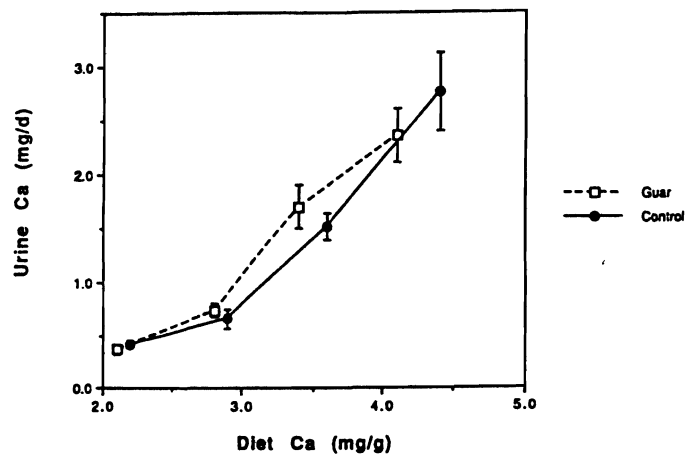
Puspitasari, N.L., K. Lee, J.L. Greger. Calcium fortification of cottage cheese with hydrocolloid control of bitter flavor defects (Submitted for review).

On Dairy-Related Topics but Not Funded by this Project

Lewis, N.M., M.S. Marcus, A.R. Behling, J.L. Greger. Calcium supplements and milk effects on acid-base balance and on retention of calcium, magnesium and phosphorus. *Am J Clin Nutr* 1989;49:527-533.

Figure 1. Apparent absorption of calcium expressed as a percentage of intake (a), urinary excretion of calcium expressed as mg Ca excreted per day (b), and apparent retention of calcium expressed as mg Ca retained per day (c) of rats fed various cottage cheeses with different levels of calcium and guar gum.





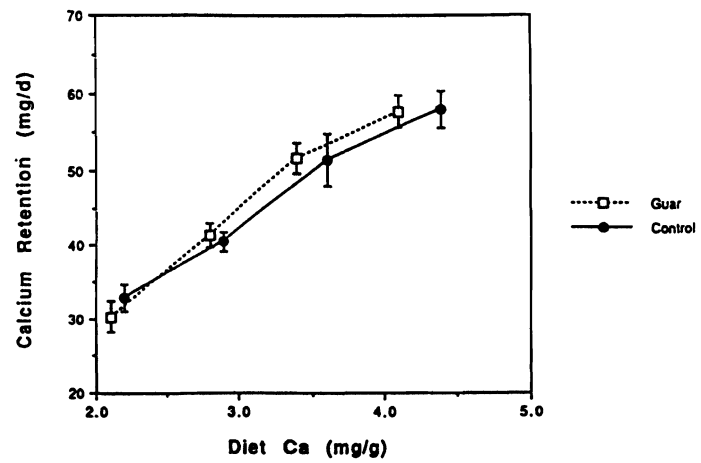
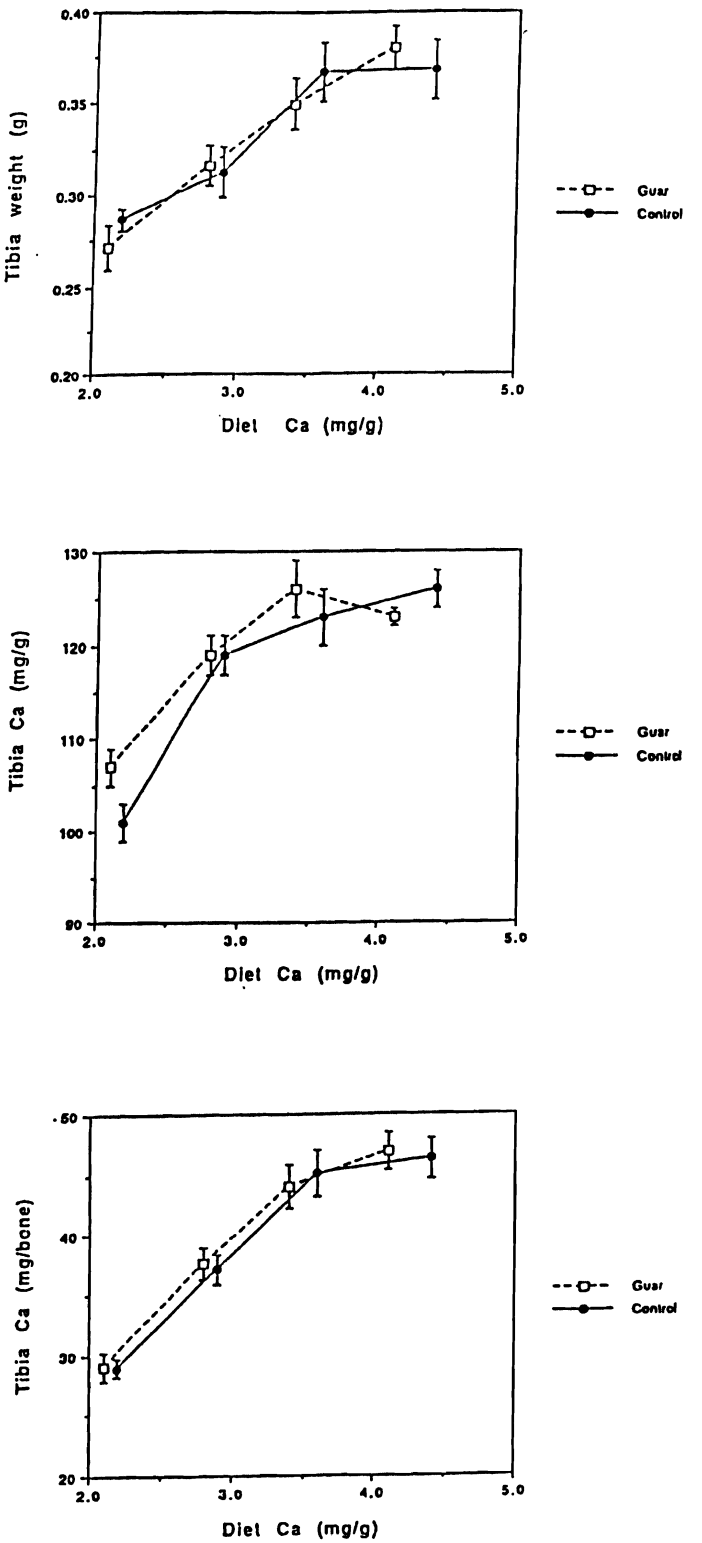


Figure 2. Tibia weights expressed as grams (a), tibia calcium concentrations expressed as mg Ca/g wet bone weight (b), and tibia calcium contents expressed as mg Ca/bone (c) of rats fed cottage cheeses with different levels of calcium and guar gum.



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Greger, J.L., M.S. Marcus, N.M. Lewis, A.R. Behling. Chloride, sodium and potassium utilization by subjects fed milk and calcium supplements. *Nutr Res* 1990;9:1307-1311.

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Project Title:

New Dairy Foods with Added Fiber and Added Calcium

Report from Dr. Ken Lee

Personnel:

Ken Lee, associate professor, Dept. of Food Science (co-PI), Ni Luh Puspitasari and Olafer Reykdal, graduate students, Dept. of Food Science

Objectives:

2. Develop new functional properties for dairy ingredients and new dairy products based on chemical interactions between hydrocolloids (dietary fibers) and dairy components. (Lee)

- Create new dairy foods using high moisture and hydrocolloid addition. The ability of hydrocolloids to hold water opens the possibility of development of new dairy foods which have significantly less calories with acceptable sensory properties.

The work of Dr. Lee goes beyond the initial objective of formulating and organoleptically testing a dairy product with added hydrocolloid and calcium. In an associated project, Reykdal and Lee identify and correlate chemical measurement of calcium bioavailability with the rat bioassay measurements obtained by Greger and Kaup. The editors.

Summary of Findings:

A high calcium cottage cheese containing soluble dietary fiber was developed and tested in the CDR/Food Science laboratories. The cheese wasn't bitter, bitterness being a common defect in calcium-fortified foods. The calcium was highly bioavailable as shown in a bioassay done in the Dept. of Nutritional Sciences laboratories. Several chemical measures of calcium availability were evaluated as predictors of bioavailability of calcium from dairy products. One measure, ionic dialysable calcium without predigestion, stood out as highly correlated (0.99) with animal results.

Masking Bitter-Tasting Ca Salts Added To Cottage Cheese

Several hydrocolloids (soluble dietary fibers) were tested for their ability to mask bitter tasting Ca salts added to cottage cheese. Cottage cheese dressing was made containing hydrocolloids and was fortified with common calcium sources, including calcium chloride, calcium phosphate, calcium lactate, calcium carbonate, or calcium citrate. Creamed cottage cheese was made from these dressings and tested for bitterness at equal calcium levels. Calcium lactate proved to be the least bitter salt and was studied further. Solutions of gum arabic (20%), low viscosity guar gum (2.5%), locust bean gum (2.5%), carrageenan (1.5%), and xanthan gum (.2%) constituted 10% to 30% of cottage cheese dressing. In preliminary tests with a single judge, cottage cheese containing 7.5 mg/g guar gum and locust bean gum had no bitter taste at 117 mg added Ca/100 g and 73 mg added Ca/100 g, respectively. Cottage cheese containing 7.5 mg/g mixture (1:1) of guar gum and locust bean gum had no bitter taste at 73 mg added Ca/100 g. Sensory analysis (n=35) data indicated that bitterness of cheese with added calcium and guar was not different from a negative control (without added calcium and guar) and was significantly less bitter than a positive control (with added calcium and without added guar). The added guar caused a decrease in overall preference attributable to a foreign taste in the guar extract used.

Bioavailability of Calcium from Cottage Cheese

A calcium profile (dialyzable, ionic dialyzable, soluble, and ionic soluble calcium) was used to predict biological availability of calcium from the cottage cheese containing added calcium lactate and soluble fiber. The bioavailability was also determined by rat bioassay in a co-investigation under the direction of Prof. J.L. Greger. Cottage cheese was fortified at 4 levels of calcium lactate, with or without guar gum to control bitterness. Chemical tests were done on both simulated gastric digests and on non-digested aqueous extracts. Two different methods were tried: 1) dialyzable and ionic

dialyzable calcium; and 2) soluble and ionic soluble calcium. Calcium added to cottage cheese significantly increased the percent calcium measured by these tests. Chemical measures of calcium in rat diets containing the same cheese, with and without guar, correlated highly to bioassay results. Ionic dialyzable, ionic soluble, and soluble calcium correlated significantly with accepted measures of bioavailability, particularly with rat tibia calcium and tibia weight. Ionic dialyzable calcium in non-digested diets best predicted bioavailability, with a correlation coefficient of 0.99. This correlation was not improved by multiple regression analysis. Simulated digestions were not better predictors than simpler non-digestion procedures. These chemical measures accurately predicted bioavailability from a calcium-fortified food.

Accomplishments:

Several levels and types of calcium salts were added to cottage cheese cream dressing, and several soluble fiber sources were used to sequester the bitter flavor introduced at these high levels of calcium addition. Since dairy products are generally believed to be excellent sources of calcium, and cottage cheese is relatively low in calcium, it is a responsible measure to add calcium to it in a bioavailable form.

All cottage cheese in these experiments was made in the UW-Madison Dairy Plant and consisted of 37.5% dressing and 62.5% curd. The dressing contained 14.0% fat and 24.5% total solids; therefore, the fat content was 5.25%. Six food grade calcium salts were added directly to dressing to determine which salt had the least off-flavor. Cottage cheese contains 60 mg Ca/100 g and salts were added at 28, 117, and 205 mg calcium/100 g. Different concentrations of hydrocolloid solutions were used as shown in Table 1. Hydrocolloid solutions consisted of 10, 20, and 30% (wt/wt) of cottage cheese dressing.

Sensory Test Results

Cottage cheeses were evaluated in preliminary tests by an experienced dairy judge. The most acceptable cheese from the preliminary tests was selected for a detailed descriptive sensory evaluation using a trained panel at the Sensory Analysis Laboratory, UW-Madison. Cottage

Table 1. Hydrocolloid Concentrations in the Final Product.

Hydrocolloid	Aqueous concentration (mg/g)	Final concentration (mg/g) in cottage cheese at different level of dressing replacements		
		10%	20%	30%
Gum arabic	200	7.50	15.0	22.5
Guar gum (GG)	25	0.938	1.87	2.81
Carrageenan	15	0.562	1.12	1.69
Xanthan gum	2	0.075	0.15	0.22
Locust bean gum (LBG)	25	0.938	1.87	2.81
GG + LBG (1:1)	25	0.938	1.87	2.81

cheeses to which hydrocolloid-calcium mixtures were added were compared to cheese with an equivalent amount of calcium without hydrocolloid and to cheese with no additives. Panelists were asked to indicate attribute intensity using a continuous scale with the lower limit defined as 1 and the upper limit as 7.

Cottage cheese with added calcium phosphate was the same as the control at 28 mg Ca/100 g but was sour, slightly chalky, and slightly bitter at 117 mg Ca/100 g. Cottage cheese with added calcium lactate was equal to the control at 28 mg Ca/100 g and was slightly sour and chalky at 117 mg Ca/100 g. Other cheeses with calcium chloride, calcium carbonate, and calcium citrate had unacceptable tastes even at 28 mg Ca/100 g. Calcium lactate was used in subsequent experiments because of its least bitter taste. Cottage cheese dressing contained 14.0% fat. Lipid could influence solubility of calcium in the dressing; this is one possible reason why different salts gave different degrees of bitterness here.

Cheeses containing 2.5, 5.0, and 7.5 mg guar/g did not differ from the control at 117 mg Ca/100 g. Cheeses with 5.0 and 7.5 mg/g locust bean gum and a 1:1 mixture of guar and locust bean gum were undistinguished from the control at 73 mg Ca/100 g. Dressings with gum arabic, xanthan gum, and carrageenan produced cottage cheeses with unacceptable flavors at 28 mg Ca/100 g, worse than cheese with no hydrocolloids at the same calcium level. Cheese with gum arabic did not have an acceptable taste at the highest concentration used (60 mg/g). The viscosity of the solution was low (200 cp) and insufficient to mask bitterness. A higher concentration of gum arabic resulted in a pronounced caramel-like flavor. Cheese made with carrageenan had a dry and crumbly dressing, possibly due to interaction with milk protein. Cottage cheese with guar and locust bean gum gave the best results.

Table 2. Descriptive Sensory Evaluation (n=35) of Cottage Cheese with and without Added Calcium Lactate and Added Guar Gum (0.28%).

Sensory Attribute	Negative Control No added Guar No added Ca	Positive Control (223 mg Ca/ 100g Cheese)	With Both Ca and Guar (223 mg Ca/ 100g Cheese)	Graphical Descriptor Scale Extremes 1	7
Bitterness	3.15±1.05a	4.56 ±1.23b	3.51 ±1.25a	Absent	Very Pronounced
Foreign taste	2.41 ± .63a	3.85 ±1.41b	4.79 ±1.21c	Absent	Very pronounced
Saltiness	3.51 ±1.10a	4.19 ±1.24b	3.56 ±1.24ab	Not Salty	Very Salty
Dairy culture flavor	3.57 ±1.07a	3.32 ±.18a	3.19 ±1.30a	Very Weak	Very Strong
Richness	4.20 ±.96a	3.89 ±1.07b	3.03 ± .94b	Lacks Richness	Very Rich
Tartness	3.48 ±1.11a	3.89 ±1.35a	3.39 ±1.30a	Not Tart	Very Tart
Overall Preference	4.67 ±1.05a	2.67 ± .93b	2.69 ±1.00b	Dislike Extremely	Like Extremely

a,b,c Mean scores with same sensory attribute (row) with same letter are not different at $p < 0.05$.

Table 3. A Calcium Profile of Digested Cottage Cheese Diets Prepared for a Rat Bioassay on Calcium Bioavailability Values are means ± S.D., n=3. Means in the same column with the same letter are not different (p<0.05).

	Calcium Profile			
	Method One		Method Two	
Calcium Content mg/100g	Ionic Dialyzable % (ID)	Dialyzable % (D)	Ionic Soluble % (IS)	Soluble % (S)
Guar Gum Diets				
11.3	5.78±0.88^{e}	10.4±1.29^{ab}	10.8±0.5^{de}	63.7±3.9^{abc}
14.6	6.35±0.91^{de}	10.3±0.83^{ab}	12.5±2.0^{cd}	68.2±2.3^{abc}
18.0	7.02±0.68^{cd}	10.7±0.82^{ab}	13.0±0.9^{cd}	65.9±4.3^{abc}
20.7	7.68±0.98^{bc}	10.2±0.50^{b}	16.5±1.5^{ab}	70.0±4.2^{ab}
Control Diets				
11.1	6.30±0.54^{de}	11.0±0.80^{ab}	8.9±1.5^{e}	60.6±6.7^{c}
15.0	6.92±0.63^{cde}	11.6±1.26^{a}	11.6±2.3^{de}	61.9±6.3^{bc}
18.3	8.38±0.46^{ab}	11.3±0.16^{ab}	14.6±0.3^{bc}	65.5±6.5^{abc}
21.3	9.09±0.24^{a}	11.2±0.17^{ab}	17.4±2.3^{a}	72.4±6.4^{a}

Table 4. Correlation (Pearson's first-order coefficients (r) of means, n=8) *between Chemical Measures in Diets and Rat Bioassay Measurements*

Coefficients in **boldface** represent a better correlation than control with bioassay.

	Bioassay Measures		
Chemical Measures	mg Ca/g tibia	mg Ca/tibia	mg tibia
Calcium Content (control)	0.899 **	0.979 **	0.972 **
Calcium in Non-Digested Diets			
% Ionic Dialyzable (ID)	**0.913** **	**0.990** **	**0.984** **
%Dialyzable (D)	0.820 *	0.897 **	0.894 **
% Ionic Soluble (IS)	0.853 **	0.954 **	0.959 **
% Soluble (S)	0.899 **	0.971 **	0.964 **
Calcium in Digested Diets			
% Ionic Dialyzable (ID)	0.715 *	0.836 **	0.861 ^{**a}
% Dialyzable (D)	0.096 ^{NS}	0.065 ^{NS}	0.058 ^{NS}
% Ionic Soluble (IS)	0.830 *	0.912 **	0.907 **
% Soluble (S)	0.714 *	0.756 *	0.734 *

* $p<0.05$
** $p<0.01$
^{NS} Not significant
^{a} Correlation underestimated due to a significant ($p<0.05$) fiber effect.

Critical Concentration and Flavor Masking

Viscosity of hydrocolloid and dressing mixtures were measured to explore the relationship of critical concentration (c*) and flavor masking. Critical concentration is the point at which the viscosity of gum solutions increase noticeably due to the onset of coil entanglement and overlap. Guar, locust bean, and their mixture had higher viscosities than other hydrocolloids. However, when mixed with dressing the viscosity was lower than the dressing itself (490 cp), likely a dilution effect. A concentration of 2.5% was higher than the c* for guar and locust bean gums (Figure 1). As shown, there is no evidence of exceeding the c* of guar gum in the cheese dressing. More hydrocolloid was added to increase viscosity of the mixture to c*. When raised above 3%, the flavor of guar gum became pronounced and produced dry dressing. The ability of hydrocolloids to mask calcium bitterness may be due to the combined effects of fat in the dressing and interference at receptor sites.

Differences in Saltiness

A preliminary sensory evaluation showed significant differences in saltiness between the control and the cottage cheese with added hydrocolloid and calcium. Therefore 0.35% sodium chloride was added to control this difference. A subsequent descriptive sensory analysis with 35 panelists is shown in Table 2. No significant differences between the control and the cottage cheese with added calcium and guar gum in bitterness, saltiness, and dairy culture flavor were found (Table 2, Figure 2).

There were differences in foreign taste between all samples. Although guar gum did suppress the bitter taste of calcium, the overall preference of the cheese with added calcium and guar gum was lower than control. Use of purified or flavor neutral guar may yield a more acceptable product. Added guar gum suppressed richness of the cheese but had no effect on dairy culture flavor and tartness. The low score in richness may be caused by the difference in fat content of each treatment. Cottage cheeses with 30% dressing replaced by guar gum, locust bean gum, and guar gum and locust bean gum mixture (1:1) solutions had no bitter tastes. Calcium analysis showed these cheeses contained 223 ± 3.0 mg Ca/100 g, 197 ± 3.8 mg Ca/100 g, and 210 ± 3.4 mg Ca/100 g, respectively. Control cottage cheese contained 92 ± 1.1 mg Ca/100 g. Our results indicated that guar gum and locust bean gum could be used to mask the bitter taste of added calcium, producing cottage cheese with up to 20% of the RDA per 100 g serving.

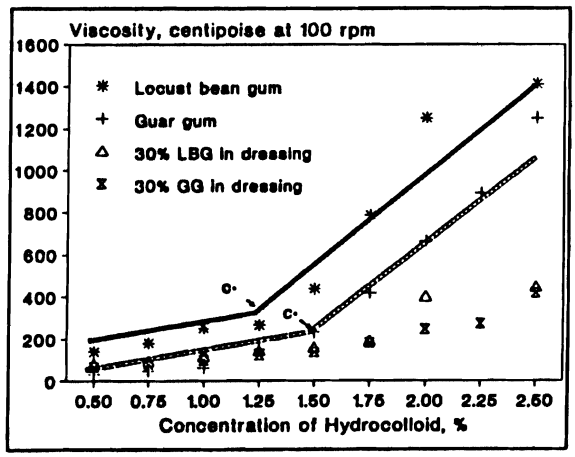
Calculating Bioavailability - In vitro vs. Bioassays

In vitro tests which estimate calcium bioavailability from dairy products are needed. In vitro methods are rapid and inexpensive compared to bioassays. It is possible to control experimental conditions tightly and avoid the variability of bioassays. In vitro procedures can provide insight into the mechanisms of bioavailability change in foods during processing and storage. However, in vitro tests for calcium bioavailability must be validated by correlation with in vivo data. The purpose here was to evaluate soluble, dialyzable and ionic calcium in calcium-fortified cottage cheese and rat diets prepared with the same cottage cheese. Results were correlated with in vivo data to find predictors of calcium bioavailability.

Calcium Profile Measurements

The calcium profile is a general term which includes dialyzable (D), ionic dialyzable (ID), soluble (S), and ionic soluble (IS) calcium. D and S-Ca represent both ionic and bound calcium. The calcium profile was measured in both cottage cheese and rat diets prepared with cottage cheese as diagramed in Figure 3. Cottage cheese without guar gum or calcium additions along with cottage cheese with 0.28 % guar gum fortified at four levels with calcium lactate (0, 50, 100, and 130 mg calcium added/100g cheese) were prepared at the UW-Madison Dairy plant (Puspitasari et al., 1990). Rat diets were prepared at the UW-Madison Department of Nutritional Sciences with freeze-dried cottage cheese (Kaup et al., 1990).

Figure 1. Viscosity of Hydrocolloid Solutions and Cottage Cheese Dressing Mixtures as a Function of Hydrocolloid Concentration (n=3). C is the critical concentration of hydrocolloid solutions.*



% S Ca = ((mg Ca/ g supernatant)(g supernatant)(100%))/Total mg Ca

% IS Ca = ((mg ionic Ca/ g supernatant)(g supernatant)(100%))/ Total mg Ca

Control diets contained calcium lactate at 4 levels and cottage cheese without additions. Two chemical methods which each gave two distinct measurements were tested: 1) Dialysis for D and ID-Ca; and 2) Solubility for S and IS-Ca. Details of these methods are in submitted manuscripts (Reykdal and Lee, 1990ab). A calcium profile was measured in digested and non-digested samples. Digestion consisted of a simulated gastrointestinal digestion. Calcium profiles of diets were compared to rat bioassay data from the same diets. S and IS-Ca were calculated as shown in the equations above.

Dialyzable (D), ionic dialyzable (ID), soluble (S), and ionic soluble (IS) calcium in digested and non-digested cottage cheese, and in rat diets containing these cheeses were measured (Table 3). Table 3 is given as a typical sample of the data that was obtained.

Comparison of the calcium profile in cottage cheese alone, and rat diets containing cottage cheese indicates the effect of diet preparation. The calcium profile in non-digested diets was lower ($p<0.01$) compared to non-digested cheese. However, diet preparation increased ($p<0.01$) the calcium profile in digested diets compared to digested cheese.

Calcium profile data was correlated against rat bioassay data and indicated chemical predictors of calcium bioavailability. This study has the marked advantage of measurements on the same diets used in a rat bioassay. Data compared to bioassay measurements include D, ID, S, and IS-Ca (both as an absolute concentration and as a % of total) and calcium content of samples. Analysis of covariance showed that guar gum had no influence on correlations except for one case, so data were pooled.

Highly significant correlations between calcium profile in diets (except % D-Ca) and calcium concentration in rat tibia (mg/g bone), calcium in tibia (mg/bone), and tibia weight were found (Table 4). As expected, total calcium content of rat diets correlated well with bioassay measurements. Calcium profile data correlating **better** than total calcium content (control) are in **boldface** in Table 4. These measures, with coefficients larger than the control, are desirable predictors of calcium bioavailability.

Bioassay Measurements

In general, ID and IS-Ca were better correlated to bioassay measurements than D or S-Ca. ID-Ca, in non-digested diets, was consistently better correlated than the control and was the best predictor of calcium bioavailability (r=0.99). This is an unusually high correlation for chemical versus bioassay data. These same trends were found when the calcium profile of cottage cheese, rather than rat diets containing cottage cheese, were correlated. Correlations were generally improved when logarithmic or negative reciprocal transformations were used for the calcium profile and control. Products, sums, or ratios of calcium profiles did not improve correlations.

Correlations did not improve by digestion of samples. Dialysis simulated gastrointestinal digestion more closely than solubility measures. However, D-Ca in digestion did not correlate better than S-Ca with in vivo data. These results show that simulation of gastroi-ntestinal conditions may not yield correct results.

Both chemical measures and bioassay measurements increased with calcium content, thus a calcium profile can predict calcium bioavailability from cottage cheese. Correlation analysis show that ID and IS-Ca were the best predictors of calcium bioavailability.

Recommended In Vitro Calcium Availability Tests

Since correlations were not improved by digestion, a simpler approach to screening of calcium availability should be used. Two in vitro tests for calcium availability are recommended: Estimation of calcium profile (especially ionic calcium) in 1) non-digested samples; or 2) pepsin digests after adjustment of pH to simulate duodenal conditions (pH 5) where most calcium is absorbed. By this approach, formation of calcium-soaps is avoided and rapid, accurate results are obtained. The high calcium bioavailability of dairy products appears to be maintained when calcium is added. The addition of a soluble fiber to the cheese had no effect on the availability of calcium.

Figure 2. Sensory Profile of Cottage Cheese with or without Added Calcium Lactate (Positive and Negative Controls) Versus the Same Cheese to which Calcium Lactate and Guar Gum were Added.

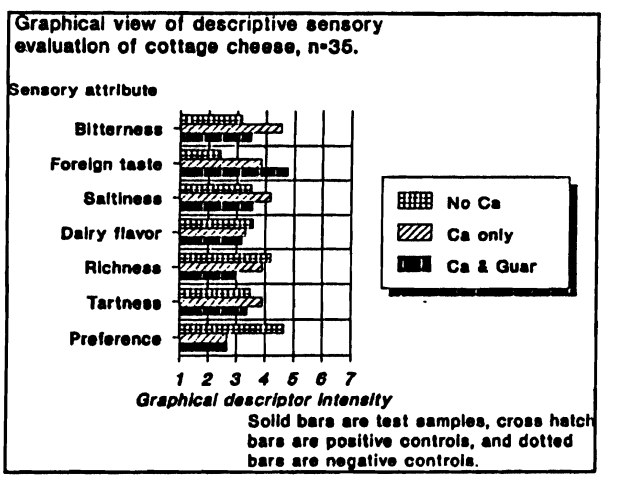
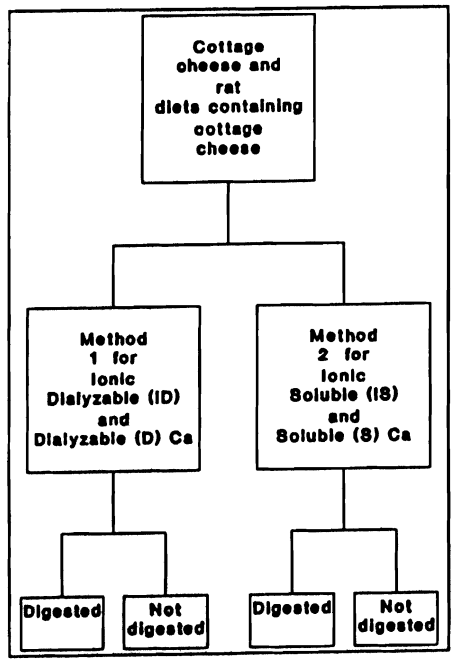


Figure 3. General Experimental Design for Evaluation of a Calcium Profile in Dairy Foods.



Publications:

Puspitasari, N.L., J.L. Greger, and K. Lee. 1990. Calcium fortification of cottage cheese with hydrocolloid control of bitter flavor defects. *J. Dairy Sci.* [accepted].

Kaup, S.M., J.L. Greger, and K. Lee. 1990. Evaluation of the nutritional properties of cottage cheese fortified with calcium and guar gum in an animal model. *J. Food Sci.* [submitted].

Greger, J.L., S.M. Kaup, C.F. Powers, and K. Lee. 1990. Bioavailability of calcium from calcium-fortified cottage cheese. *FASEB J.* 4(3):1474.

Reykdal, O. and K. Lee. 1990. Soluble, dialyzable and ionic calcium in calcium fortified cottage cheese with correlation to bioassay. *J. Food Sci.* [submitted].

Reykdal, O. and K. Lee. 1990. Soluble, dialyzable and ionic calcium in raw and processed skim milk, whole milk and spinach. *J. Food Sci.* [submitted].

Puspitasari, Ni Luh. M.S. Thesis, 1990. Suppression of bitter flavors in calcium fortified cottage cheese with hydrocolloids.

Reykdal, Olafur. M.S. Thesis, 1990. Soluble, dialyzable and ionic calcium in milk, spinach and calcium fortified cottage cheese with correlation to bioassay.

Presentations:

Puspitasari, N.L. and K. Lee. March 29, 1989. "Hydrocolloids as a means for calcium fortification of cottage cheese." Proceedings of the Cheese Research and Technology Conference, p. 109. Center for Dairy Research, University of Wisconsin-Madison.

Puspitasari, N.L. and K. Lee, July 1989. "The use of hydrocolloids as a vehicle for calcium supplementation of cottage cheese." IFT 50th annual meeting, Chicago. Abstr.

Lee, Ken, J.L. Greger and N.L. Puspitasari. 1990. "Minimizing Bitter Flavor Defects in Cottage Cheese Fortified with Calcium. 23rd International Dairy Congress Abstracts."

Project Title:

Demand Analysis of Dairy Products Using Time-Series and Cross-Sectional Data

Personnel:

Brian W. Gould, associate scientist, Center for Dairy Research and Dept. of Agricultural Economics; Federico Perali, graduate student, Dept. of Agricultural Economics

Funding:

Wisconsin Milk Marketing Board: Basic Research

Funding Code:

89-29, 2A

Dates:

July 1, 1989 - June 30, 1990

Objectives:

Analyze the demand for a variety of dairy products, while incorporating demographic characteristics into demand systems. Use methodologies developed employing both cross-sectional and aggregate time-series data for fluid milk and hard manufactured products.

Summary:

Demand for Whole and Lowfat Milk Analyzed

Gould, Cox, and Perali (1990) estimated a time-series model of the demand for a variety of fluid milk products. A demand systems approach was used in which the demand for whole and lowfat milk was analyzed. The authors examined the trend of reduced per capita whole milk consumption and increased low-fat and skim milk consumption. The demographic variables included in the analysis were: percent of the population under the age of 5 years, percent of the population between the ages of 5 and 13, percent of the population over the age of 65, the median number of years of schooling completed, and the percent of the population that is nonwhite.

Demand for Butter Analyzed

Using a similar method, the demand for butter was analyzed in a second paper (Gould, Cox and Perali, 1990). Quarterly U.S. disappearance data for the years 1962-1987 were used in the analysis. Five food fats and oils commodities were delineated in the demand system: butter, vegetable shortenings, cooking and salad oils, margarine, and lard.

The unique aspects of this research were that we included a variable representing government butter donations so as to isolate the impacts of these donations on commercial disappearance of food fats and oils. The authors also analyzed the impacts of changes in the demographic profile of the U.S. population on the intake and type of dietary fat obtained directly from food fats and oils. Gould is currently extending this research to analyze the factors affecting the U.S. demand for cheese.

Cheese Consumption Analyzed

The above analyses were used to develop several methodologies that will be used in cross-sectional demand analysis when household level data become available. A third area of research initiated by Gould uses cross-sectional data to examine the at-home consumption of cheese, but unlike the previous work, a single equation approach is used. The methodology that has traditionally been used to account for censoring in commodity demand analysis is the Tobit model. The underlying assumption associated with this model is that the same stochastic process affects both the decision whether or not to purchase a commodity, and the level of purchase. This assumption, which implies that zero expenditure values represent corner solutions, does not allow for the situation where the household does not report household expenditures, even though consumption does occur. This is an important consideration when expenditure

data is used in applied consumption analysis, in that a zero expenditure value need not imply that consumption of a durable or semi-durable good is zero.

Based on a comparison on recent cheese expenditure and household consumption data, there is evidence that for many households the usual one-week survey period associated with cross-sectional household level surveys may be shorter than the frequency of purchase of this commodity, given the relatively long shelf-life for many varieties. In the 1972 and 1987 Consumer Expenditure Surveys, 48% and 42% of the households, respectively, reported that they had purchased cheese over a one-week survey period. Over a similar length of time, data from the 1977 USDA Household Food Consumption Survey indicates that over 81% of the households had consumed cheese at home. This large difference in percentages supports the hypothesis that in the case of the consumption of cheese, the frequency of purchase for many households may be greater than one week.

Infrequency of Purchase Model Use

An infrequency of purchase model was used to examine the factors affecting the at-home consumption of cheese in the U.S. (Gould, 1990). Unlike the previous models that have examined at-home cheese demand, this model allows us to separately identify those factors affecting the probability of observing a non-zero expenditure level and those affecting the level of expenditure, given a decision to purchase the commodity. This is important both from the methodological reasons discussed above concerning the validity of attributing a zero expenditure value to zero consumption and in terms of making available a continuing series of annual expenditure data for applied consumption analysis.

The principle source of U.S. household expenditure data for small, frequently purchased items such as food and beverages, housekeeping supplies and services, and personal care products and services, is the Diary Component of the Bureau of Labor Statistic's (BLS) Continuing Consumer Expenditure Survey (CEX). This information has been collected on an annual basis since 1979, with quarterly data available for each year. The 1987 Diary Survey is used in this analysis. For this survey, each participating household was asked to keep two one-week diaries of expenditures over consecutive weeks. Because each week's diary was treated as statistically independent of the other week's diary during the data collection process, we continued to use this assumption in the present application. The original data set contained observations on 13,098 households. Because of their unique characteristics, households where the reference person was a college student or resided in transient housing or rooming houses were omitted from the study. We also excluded those individuals who did not completely report household income. With these restrictions, our final sample size was 10,023 households.

The econometric models presented in Gould (1990) lead to a rejection in the null hypothesis that the Tobit specification adequately models the demand for cheese, vis-a-vis, the infrequency of purchase model. This results lends support to the conclusions of previous analyses that recommend a multi-stage analysis of the consumption process. In spite of substantial differences between the estimated coefficients obtained under the traditional Tobit and the infrequency of purchase, the estimated income and demographic elasticities are quite similar. Under the infrequency of purchase model, however, we obtain additional insights into the purchase vs. consumption decision that is not available under the Tobit specification.

Significance to the Dairy Industry:

The dairy industry is facing a dramatic change in the types of commodities being demanded by consumers. In order to understand the reasons for such changes in demand and possible future directions, it is essential that household level consumption data be analyzed. The above research projects have developed several methodologies that will allow for future investigations into the demand for dairy products. Over the next few years, several household level data sets will be

released where the methodologies developed above will be used to examine the demand for a wide variety of dairy products.

Publications:

Gould B.W., T.L. Cox, and F. Perali. The Demand for Fluid Milk Products in the U.S.: A Demand Systems Approach, forthcoming, *Western Journal of Agricultural Economics*, July 1990.

Gould, B.W., T.L. Cox, and F. Perali. Determinants of the Demand for Food Fats and Oils: The Role of Demographic Variables and Government Donations forthcoming, *American Journal of Agricultural Economics*, November 1990.

Gould, Brian W. At Home Consumption of Cheese: An Analysis Using an Infrequency of Purchase Model, submitted for publication, *American Journal of Agricultural Economics*, June 1990.

Project Title:

Development of an Economic Engineering Microcomputer Model to Analyze New Dairy Processes and Products

Personnel:

Brian W. Gould, associate scientist, Center for Dairy Research and Dept. of Agricultural Economics; Michael Thomsen, graduate student, Dept. of Agricultural Economics

Funding:

Wisconsin Milk Marketing Board: Basic Research

Funding Code:

89-29, 2B

Dates:

July 1, 1989 to June 30, 1990

Objectives:

Develop a microcomputer model to evaluate the economic feasibility of alternative dairy processes and products.

Summary of Findings:

Computer Program to Evaluate Plant Data

Research was initiated to develop computer models that allow for an economic analysis of new processes and products. ECOANAL, a computer program written in PASCAL code, is the first model that has been developed as a result of this research. ECOANAL allows the user to input plant-specific data such as cheese yields, hours of labor, capacity utilization of plant, milk price, cheese price, etc. From this data, single period net return values are calculated. To be as "user friendly" as possible, the program was designed using a spreadsheet-type of format to input and modify data. The user can also recall previously entered data; update or modify any input requirement, process change, price change etc.; and analyze the economic impacts of such changes. The model has been developed for use in cheese plants.

New Program Features Being Added

Over the next year, the computer model will be extended in several areas. A user's manual will be developed, and pretesting of the model will be conducted using data from several cheese plants.

Extensions to the model will include incorporating a milk standardization routine to automatically calculate cheese yields based on milk characteristics and the type of cheese being produced. Currently, the model requires the user to specify cheese yields in advance. In addition, the model will allow the user to determine the most economical method to standardize milk, given raw milk, cream, and non-fat dry milk prices.

Another area of extension to the model will be the incorporation of financial variables, such as cash flow and internal rates of return. This addition will allow for a dynamic analysis of a dairy plant over several years.

Significance to the Dairy Industry:

Often, new dairy products or processes are developed with little attention paid to their economics. The microcomputer model developed over the past year was designed for the plant manager considering a change in production, be it changing products involved or processes used.

Applying the basic model developed over the past year, we will explore its use in a variety of types of dairy plants. Eventually, we will have developed a library of economic analysis software useful in analyzing a variety of dairy products.

Project Title:

Economic Benefits of Increasing the Quality of Milk Used for Cheese in Wisconsin

Personnel:

Edward V. Jesse, professor, Dept. of Agricultural Economics (co-PI); Matthew T. Holt, assistant professor, Dept. of Agricultural Economics (co-PI); Hugo DeGroote, research assistant, Dept. of Agricultural Economics;

Cooperator:

Wisconsin Dairy Herd Improvement Cooperative

Funding:

Wisconsin Milk Marketing Board

Funding Code:

88-50

Dates:

July 1, 1988 - June 30, 1990 (Extension to September 30, 1990 requested)

Objectives:

1. Identify factors associated with variation in the casein content of milk used for cheese.

2. Measure the economic benefits of changes in farm and dairy plant practices associated with high casein content.

Summary of Findings:

The first 15 months of the project were devoted to collection of individual cow and aggregate herd test data for 45 northwest Wisconsin dairy herds, averaging 45 cows per herd. Information on feeding and management practices was also obtained monthly from September 1988 to October 1989. Test data included standard Dairy Herd Improvement Cooperative (DHIC) composition and quality measures, as well as casein levels.

Cow and herd test data were merged with information on feeding and management practices and with individual cow descriptive data (e.g. age, breed, stage of lactation) in central DHIC files.

Most of the data analysis to date has involved producing a comprehensive set of descriptive statistics. Several regression equations have been estimated defining the physical relationship between casein and milk quality as measured by somatic cell count (SCC).

For the bulk tank (herd) data, the estimated equations account for seasonal variations (i.e., month of sample), fat content, standard plate count, protein levels, the amount of solids nonfat, lactose level, and the SCC. The preliminary results are somewhat mixed, as they indicate that SCC does not have a strong statistical relationship with casein levels. However, only linear model specifications have been estimated to date; we have not yet adequately examined the potential interactions between SCC and the other explanatory variables mentioned above. It is comforting, however, that the data do indicate a strong positive relationship between plate count and casein, and protein and casein.

Significance to the Dairy Industry:

Three quarters of Wisconsin milk is used for cheesemaking. Higher cheese yields through adoption of farm and plant practices consistent with high casein recovery will positively impact Wisconsin dairy farmers and the cheese industry.

Publications:

None

Project Title:

Optimization of Cheese Manufacture Production Planning

Personnel:

John P. Norback, associate professor, Dept. of Food (PI); Hong K. Chung, graduate student, Dept. of Food Science

Funding:

Cheese Research Institute

Funding Code:

3-89

Dates:

July 1, 1988 - June 30, 1990

Objectives

1. Develop a framework for a production planning model;

2. Design the model optimizing the allocation of production resources to the multiple products with multi-processing stages.

Summary of Findings:

Need for a Production Planning Framework for the Dairy Processor

Production planning is a demanding assignment for a dairy processor. The processor must continually decide on the allocation of resources to products, timing of resource and product release, and product mix to obtain goals such as enhancing productivity and profitability. The decision-making is more complicated when the supplies of common resources are limited or multi-staged processing and intermediate byproducts are used. Without an integrated planning framework, a production manager may have to depend on his intuition or experience to make decisions. Decisions stemming from the lack of integrated information or poor planning will negatively impact the profitability. For example, excessive inventory ties up capital and increases wastes, while insufficient production reduces customer satisfaction and potential profits. By handling the information from an overall perspective, the manager can effectively allocate the resources, manage overall costs and have a sound basis for detail level decisions.

Because of distinct characteristics of industries and products, it is crucial to analyze the characteristics of a specific industry and examine their implications for production planning. The characteristics of the dairy industry associated with production planning are summarized in Exhibit 1.

This research looked at the parameters that must be considered when developing a computer program that can assess the effects of various parameters on the profitability of a Cheddar and process cheese production plant. The computations associated with the parameters were integrated into a model that optimizes the profitability of this type of plant. For reprints of the computations and model development contact CDR (608-262-2217) or the principal investigator, Dr. John Norback, at the UW-Madison (608-263-4949).

Exhibit 1. The Characteristics of the Dairy Industry Associated with the Production Planning

1. The dairy industry usually demands a short lead time due to the perishability of raw milk, fluid milk products and other products (cream, ice cream, cottage cheese and cultured products).

2. Dairy processing plants use small number of ingredients, while various options of flavors, sizes and packages lead to the product differentiation. Intermediate products are often used for preservation, while some byproducts are sources of revenue or the input resources for the end products. These aspects make it inefficient to follow the practices which the discrete manufacturing industry uses to establish the bill of material (BOM). Rather, an integrated BOM systematically containing the products and ingredients would be more desirable.

3. Many resources require precise measurement of units into several decimal places for more accurate production and inventory costs.

4. The unit of an ingredient form often varies with the stages of purchasing, processing, or storage. To effectively control the production and inventory, the conversion relations between several forms of units must be organized and defined.

5. The changes in availability, quality, and prices of ingredients or consumers' food consumption trend often make it necessary to change product formula (recipe) or use substitutes. These changes should be timely and efficiently manipulated in order to assess the impact on profitability trigger when narrow profit margin of dairy products are considered.

6. Product yield may vary with the changes in quality of ingredients or the use of substitute.

In addition, dairy processors should consider the seasonality of milk supplies, dairy product production and consumption, and uses of batch processes in production planning. The production planning framework addresses these aspects.

Chapter 3

Worldwide Information and Technology Exchange Program

An Overview of WITEP

Since January 1988, the Worldwide Information and Technology Exchange Program has been an integral part of the scope and goals of CDR. The fundamental purpose of WITEP is *communication.* Building the vital structure of communication networks between dairy scientists, dairy research centers, and between the researchers and industry is the purpose underlying WITEP activities. Through dialogue, scientists collaborate and exchange ideas to spur basic research efforts which can then lead to new product development.

WITEP is comprised of four areas of activity: the scientist exchange program, the seminar series, the annual research conference, and the information center. Each of these help to transfer dairy research information to the researchers and industry personnel who use it to further research efforts and to develop new uses for milk and its components.

In the following sections, the past year's progress in these four areas is summarized. If you have ideas or questions about WITEP, please contact Sarah Quinones, WITEP Coordinator, at 608/262-2217.

Scientist Exchange Program

Through the exchange of scientists, CDR promotes dialogue between dairy research organizations. WITEP provides funds for UW dairy researchers to invite scientists to come to the UW and cooperatively pursue dairy research projects and similarily will provide for UW scientists to make research visits at other dairy research programs. The visiting scientists strengthen the research program by bringing their research experience and training. In addition to the research contribution the visiting scientist makes, a communication link to another research organization is made. Ultimately, these improved communications will lead to more joint research projects and less replication of research.

The following lists the scientists that have spent research visits at CDR in the past year.

Scientists visiting CDR

S.K. Lee

Researcher, Dept. of Fermentation,
DooSan Research Institute
1-3 Kusan Dong Eunpyung Ku
Seoul, Korea 122

Sponsoring professor:
Elmer H. Marth, professor, Dept. of Food Science

Period of stay:
March 1, 1989 - February 28, 1990

Project title:
Examination of heat resistance of *Borrelia burgdorferi*. Use of *Micrococcus* sp. to improve flavor of lowfat Cheddar cheese.

Martinius A. van Boekel

Senior researcher and lecturer,
Dept. of Food Science
Wageningen Agricultural University
P.O. Box 8129
6700 EV Wageningen, The Netherlands

Sponsoring Professor:
Robert C. Lindsay, professor, Dept. of Food Science

Period of stay:
April 1 - October 1, 1989

Project title:
Flavor development in Cheddar cheese from protein degradation products: the influence of heating milk on the formation of volatile sulfur compounds during aging of cheese.

Hyong Joo Lee

Associate professor, Dept. of Food Science and Technology
College of Agriculture
Seoul National University
Suwon, 440-744 Korea

Sponsoring Professor:
Norman F. Olson, professor, Dept. of Food Science

Period of stay:
June 28 - August 21, 1989

Project title:
Characterization of extracellular proteinases of *Lactococcus lactis ssp. lactic* ML8.

Moustafa El-Shenawy

Researcher
National Research Center
Food and Dairy Research Laboratories
Dokki-Cairo, Egypt

Sponsoring professor:
Elmer H. Marth, professor, Dept. of Food Science

Period of stay:
July 1 - October 31, 1989.

Project title:
Antilisterial effect of the lactoperoxidase system of milk.

Morsi El Soda

Professor, Agricultural Industries,
University of Alexandria,
Alexandria, Egypt
Visit duration: June 1, 1989 - September 31, 1989

Sponsoring professor:
Norman Olson, professor, Dept. of Food Science

Project title:
Acceleration of ripening of low-fat Cheddar cheese using the cell-free extract of non-starter bacteria.

Geraldine Farrell

Lecturer
St. Angela's College of Education
Lough Gill
Sligo, Ireland

Sponsoring professor:
Elmer H. Marth, professor, Dept. of Food Science

Period of stay:
September 1, 1989 - August 30, 1990

Project title:
The growth and survival of *Borrelia burgdorferi* in milk.

José Coca

Professor, Dept. of Chemical Engineering
University of Oveido,
33071 Oveido, Spain

Sponsoring professor:
C.G.Hill, professor, Dept. of Chemical Engineering

Period of stay:
October 15, 1989-November 15, 1989
July 1 - July 30, 1990

Project Title:
Electrodialysis of whey and fractionation of whey proteins.

W. James Harper

Professor Emeritus, Ohio State University
3563 South Old 3-C Road
Galena, OH

Sponsoring professor:
Norman F. Olson, professor, Dept. of Food Science

Period of stay:
October 15 - November 14, 1989
June 1 - June 30, 1989

Project title:
Fractionation of milk proteins and proteolysis of cheese; participant in CDR's scientist mentor program.

CDR-affiliated scientists visiting other dairy research programs

Richard Hartel,
Assistant Professor, Dept. of Food Science
Mark E. Johnson,
Senior Scientist, CDR
and Robert Lindsay
Professor, Dept. of Food Science

Visit to a dairy research program
STELA
Laval University
Quebec City, Canada

Dates of visit:
January 8-10, 1990

Sarah H. Quinones
WITEP coordinator, CDR

Visits to dairy research programs
Research centers in Germany, France, Denmark, and The Netherlands

Dates of visits:
March 1-21, 1990

CDR-affiliated scientists attending conferences or training/ courses

Jyh-Ping Chen
Assistant Scientist, CDR

Training -- Name of course and location:
Massachusetts Institute of Technology special summer program:
"Biotechnology: Principles and Processes," August 14 - August 18, 1989.
Boston, Massachusetts

Susan M. Kaup
Postdoctoral Research Associate working with Dr. Janet Greger,
Dept. of Nutritional Sciences

Conference -- name and location:
Federation of American Societies for Experimental Biology,
74th Annual Meeting
April 1-5, 1990; Washington D.C.

Denise M. Ney
Assistant Professor, Dept. of Nutritional Sciences

Conference -- name and location:
81st American Oil Chemists Society (AOCS) Annual Meeting,
April 22-25, 1990. Baltimore, Maryland.

Visiting scientist reports

S.K. Lee

Sponsoring professor:
Elmer H. Marth, professor, Dept. of Food Science

Visiting scientist's address:
1-3 Kusan Dong Eunpyung Ku
Seoul, Korea 122

Period of stay:
March, 1989 - February, 1990

Project title:
Examination of heat resistance of *Borrelia burgdorferi*. Use of *Micrococcus* sp. to improve the flavor of lowfat Cheddar cheese.

Project's objectives/conclusions:

The first objective was to determine heat resistance of *Borrelia burgdorferi*. This objective was accomplished and results are summarized in the following paragraph.

Borrelia burgdorferi strain EBNI was cultivated in BSK-II medium at 34°C, then cultures at different physiological states were heat-treated at temperatures in the range of 50 to 70°C. Numbers of survivors were estimated by the Most Probable Number technique. Log MPN was plotted against treatment time, and resulting survivor curves were linear. Estimated D-values for cultures incubated at 34°C. for 7 d before heat-treatment were 5.5, 4.3, 2.7, .47, and .14 min at 50, 55, 60, 65, and 70°C, respectively. Spirochetes in the lag phase had greater resistance to heat than those in the stationary phase, with the latter being more resistant to heat than spirochetes in the same phase of growth but refrigerated at 4°C for 3 d. D-values for *B. burgdorferi* are generally less at 50°C and greater at 70°C than those reported for other non-sporeforming pathogens. When log10 MPN was plotted against the treatment temperature, two linear segments for each thermal death curve were obtained. Our data show the spirochete had higher z- values than most non-sporeforming pathogens. The pH of the medium, in the range of 5.0 to 7.6, did not affect resistance of *B. burgdorferi* to heat.

The second objective was to evaluate a proteolytic and peptidolytic strain of *Micrococcus* sp. for possible use to improve the flavor of lowfat Cheddar cheese. This objective also was accomplished and results are summarized in the following paragraph.

Micrococcus sp. LL3 was chosen for this work because earlier research indicated this bacterium had intracellular protease and aminopeptidase. Cheese was made from milk with 1.9% milkfat. Either cheese milk or cheese curd was treated with live cells, freeze-dried cells, or a cell-free extract of *Micrococcus* sp. LL3. Six-month-old cheese made with a

micrococcus preparation:
(A) Contained 14 to 28% more TCA-soluble nitrogen than did the control cheese, (B) contained 11 to 29% more PTA-soluble nitrogen than did control cheese, (C) contained ca. 10^3 *Micrococcus* sp. LL3/g when live cells of the bacterium were added so fresh cheese contained ca. 10^7/g, (D) exhibited more hydrolysis of casein at and after 4 months of age than did control cheese, (E) had a higher score for typical Cheddar flavor than did control cheese.

How did these efforts contribute to CDR's program?

The work done by Mr. Lee addressed two concerns of the CDR research program. His research on *Borrelia burgdorferi* relates to the safety of milk and milk products. Evidence is beginning to accumulate that *B. burgdorferi* can cause infection, at least in experimental animals, when ingested orally. Further more, dairy cows can be infected by the spirochete. Thus a question arises about the safety of milk and milk products if they happen to be contaminated with this pathogen. The second phase of Mr. Lee's work dealt with a possible way of enhancing the flavor of lowfat Cheddar cheese, which is clearly important in the research efforts of CDR.

Publications produced as a result of research:

1. Lee, S.K., A.E. Yousef, and E.H. Marth. 1990. Thermal inactivation of *Borrelia burgdorferi*, the cause of Lyme's disease. *J. Food Prot.* 53:296-299.

2. Lee, S.K., M.E. Johnson, and E.H. Marth. 1990. Characteristics of lowfat Cheddar cheese made by addition of *Micrococcus* species LL3.

Martinius A. van Boekel

Sponsoring Professor:
Robert C. Lindsay, professor, Dept. of Food Science

Visiting scientist's address:
Dept. of Food Science
Wageningen Agricultural University
P.O. Box 8129
6700 EV Wageningen, The Netherlands

Period of stay:
April 1, 1989 - October 1, 1989

Project title:
Flavor development in Cheddar cheese from protein degradation products: the influence of heating milk on the formation of volatile sulfur compounds during aging of cheese.

Project objectives/conclusions

1. To investigate the rate of production of volatile sulphur compounds in aging Cheddar cheese prepared from milk receiving various heat treatments.

Milk was subjected to 4 different treatments:
a. none (raw milk)
b. pasteurized (12 s 73° C)
c. thermalized (1 min 55° C)
d. high-pasteurized (1 min 82° C)

In a period of 5 months the cheeses were analyzed for volatile sulphur. There were not many differences as far as volatile sulphur contents were concerned, except for the high-pasteurized milk which had only very limited production of methanethiol. The other cheeses showed gradual development of carbonylsulfide, (H_2S, COS), methanethiol (CH_3SH), and dimethylsulfide (CH_3-S-CH_3).

2. To evaluate the Cheddar flavor intensity in the cheeses made from milk with different heat treatments.

Some flavor differences were noted by a taste panel, in that the thermalized milk heat treatment gave a somewhat better flavor. The raw-milk cheese initially also developed a good flavor, but after 5 months an unclean flavor developed. It was also very clear that the cheese made from high-pasteurized milk had no cheese flavor at all.

3. To investigate the role of sulfur-containing amino acids in Cheddar flavor.

It was clearly demonstrated that low molecular weight, highly-volatile sulphur compounds (H_2S, COS, CH_3SH, and H_3-S-CH_3) did not constitute

Cheddar flavor, nor did they do this in combination with dairy flavor compounds such as diacetyl, acetaldehyde, and acetoin. There were some indications, however, that higher molecular weight sulphur compounds may be involved in cheese flavor (Cheddar).

How did these efforts contribute to CDR's research program?

In general, more information about cheese ripening and flavor development has been obtained, with an emphasis on the role of sulfur containing amino acids.

Also, a better headspace analysis method has been developed than was previously available. This method is quantitative and could also be useful for volatiles other than sulfur compounds. Although the effect of heat treatment of milk on flavor development in the resulting cheeses is unclear, the results certainly justify further research in this area.

Publications produced as a result of research:

1. T. Bhowmik, B. Riesterer, M.A.J.S. van Boekel and E.H. Marth. Characteristics of low-fat Cheddar cheese made with added *Micrococcus* or *Pediococcus* species. Submitted to *Milchwissenschaft*.

2. M.A.J.S. van Boekel and R.C. Lindsay. Formation of volatile sulphur in cheese and model systems and their relation with Cheddar cheese flavor. (Submitted to *J. of Dairy Science, 1990.*)

3. M.A.J.S. van Boekel and R.C. Lindsay. Effect of heat treatment of cheesemilk on flavor development in Cheddar cheese. (Submitted to *J. of Dairy Science, 1990.*)

4. M.A.J.S. van Boekel and R.C. Lindsay. Distribution of volatiles in vapor over fat and aqueous phases. (Submitted to *J. of Dairy Science, 1990.*)

Note from the WITEP coordinator:
This scientist exchange led to another scientist exchange which was suggested by Dr. van Boekel. A graduate of Dr. van Boekel's will be spending four months doing research on "Inactivation of intracellular enzymes during spray-drying of starter cultures" with Dr. Mark Etzel of the UW-Madison Dept. of Food Science in the spring/summer of 1991.

Hyong Joo Lee

Sponsoring Professor:
Norman F. Olson, professor, Dept. of Food Science

Visiting scientist's address:
Dept. of Food Science and Technology
College of Agriculture
Seoul National University
Suwon, 440-744 Korea

Period of stay:
June 28 - August 21, 1989

Project title:
Characterization of extracellular proteinases of *Lactococcus lactis ssp. lactic* ML8.

Project's objectives/conclusions:
The *Lactococcus lactis ssp. lactis* contains plasmids of 1.0, 1.5, 4.5, and 34 Mdal, respectively. The 34 Mdal plasmid encodes the extracellular proteinase which is directly related to the bitter peptide formation.

The objective of the project is to characterize the extracellular proteinases. DEAE Sephadex chromatography showed that the proteolytic enzymes consist of two major fractions, i.e., F-I and F-I. F-II is the fraction that contains the extracellular proteinase and this fraction has at least two different enzymes.

Further research on such properties of the enzyme as the molecular weight, optimum conditions for activity, and reaction kinetics would be desirable.

How did these efforts contribute to CDR's research program?

Information obtained from this work may contribute to the development of better starter strains which retain a good level of acid production but produce less bitter peptides.

Publications produced as a result of research:

None at this time.

Moustafa El-Shenawy

Sponsoring professor:
Elmer H. Marth, professor, Dept. of Food Science

Visiting scientist's address:
National Research Center
Food and Dairy Research Laboratories
Dokki-Cairo, Egypt

Period of stay:
July 1 - October 31, 1989

Project title:
Antilisterial effect of the lactoperoxidase system of milk.

Project objectives/conclusions:

Listeria monocytogenes is a pathogen that has been involved in food-related outbreaks of listeriosis in 1981, 1983, 1985 and 1987. All but one of these outbreaks resulted from consumption of contaminated milk or dairy products. Listeriosis is a disease that affects infants, pregnant women and adults. The mortality rate of listeriosis in these outbreaks was about 30%. The lactoperoxidase system (LPS) is one of the natural antimicrobial mechanisms present in milk. The system is composed of three components: (1) lactoperoxidase which is found naturally in milk in concentrations greater than needed for the system to operate, (2) thiocyanate which occurs in milk in various concentrations depending on the feeding regime of the animal, and (3) hydrogen peroxide which must be added to activate the system. The detailed mode of action of the system is not known yet, but it is likely that its effect against the bacteria results mainly from an oxidation of SH-groups that occur in various essential proteins of the bacterial cell wall which interferes with metabolic processes of the bacteria.

According to the literature, the LPS is active against most foodborne pathogens. However, there was no information about its effect against *L. monocytogenes*. A study was done to determine the effect of the system against different strains of the pathogen (strain V7, a milk isolate; strain California, isolated from Mexican-style cheese implicated in a 1985 outbreak of listeriosis in California; and strain Scott A, a clinical isolate).

The test culture was inoculated at three different levels into raw milk, a culture medium, or a buffer solution, and incubated at 35 or 4°C. Results indicate that presence of the activated LPS in the culture medium, buffer, or milk resulted in some inactivation of the pathogen. Generally, this antilisterial effect, in part, depended on the initial level of contamination; the pathogen was completely inactivated within a few hours at 35°C if present in small numbers (30-50 CFU/ml). When populations of ca. 10^4 or 10^7 were present, a bactericidal effect occurred with a decrease in population of about one log cycle. At 4°C the same behavior was observed, but the reduction in population was about one-half log cycle.

The susceptibility of the bacterium was greatest when grown and treated in the culture medium, followed by milk and buffer. Strain California was more susceptible to the LPS than were Scott A and V7. We can conclude that under some circumstances activation of the LPS in milk is useful for control of *L. monocytogenes.*

How did these efforts contribute to CDR's research program?

A major concern of the Center for Dairy Research is the safety of milk and milk products. Raw milk may become contaminated with *L. monocytogenes,* and during the transportation and storage of milk, the population of Listeria may increase.

Activation of the LPS which occurs naturally in milk may inactivate or control growth of *L. monocytogenes* in milk for several hours. This can minimize the risk associated with this pathogen. Thus the LPS becomes another viable means for control of *L. monocytogenes* provided conditions under which raw milk is handled and stored warrant application of this technology.

Publications resulting from research:

El-Shenawy, M.A., H.S. Garcia, and E.H. Marth, 1990. Inhibition and inactivation of Listeria monocytogenes by the lactoperoxidase system in raw milk, buffer or a semi-synthetic medium. *Milchwissenschaft* (in press)

Morsi El Soda

Sponsoring professor:
Norman Olson, professor, Dept. of Food Science

Visiting scientist's address:
Dept. of Agricultural Industries
Faculty of Agriculture
Alexandria University
Alexandria, Egypt

Period of stay:
June 1, 1989 - September 30, 1989.

Project title:
Detection, characterization, and evaluation of accelerated ripening of cheese using the peptide hydrolase system of several non-starter bacteria.

Project objectives/conclusions:

1. To compare the peptide hydrolase system of several non-starter bacteria and to evaluate the potential of their crude cell-free extract for the acceleration of cheese ripening.

It was possible to determine the levels and numbers of aminopeptidases (AP) and dipeptidyl peptidases (DPP) in several non-starter bacteria (NSB) during the four-month research visit. Partial purification of the APs and DPPs was accomplished using gel filtration chromatography. Some of the properties of the different enzymes were then studied.

The results revealed the presence of AP of different specificities in *Lactobacillus casei, Propionibacterium shermanii, Brevibacterium linens, Pediococcus* sp., and *Leuconostoc mesenteroides.* The DPP activity was detected in *L. casei, P. shermanii,* and *Pediococcus sp.* See Chapter 2 *(Research Reports)* of this annual report, for more detailed information on the results of this study.

The optimum temperature for the different enzymatic preparations varied from 30-50°C while the optimum pH was close to neutrality. Most of the AP were inhibited by O-phenanthroline and P-hydroxymercuribenzoate while phenylmethylsuphonyl fluoride had a strong inhibitory effect on DPP.

An attempt was also made to reduce the curing time of a reduced-fat cheese using either freeze-shocked cells or the lyophilized extracts of NSB. The research showed that non-starter lactic acid bacteria possess enzymes that might be of interest for the acceleration of cheese ripening. It was also possible to conclude that freeze-shocked cells are more effective than the lyophilized extracts in accelerating the ripening process.

In addition to the previously mentioned work, attempts were also made for the entrapment of calcium and iron in different types of liposomes as a part of Lauren Jackson's work (graduate student of Dr. Ken Lee, Dept. of Food Science) on the fortification of cheese with minerals.

How did this research contribute to CDR's research program?

One of the objectives of CDR's research program is obtaining a better understanding of the cheese ripening process in order to provide the dairy industry with the appropriate cultures and enzymes.

Publications resulting from this research:

M. El Soda, A. Macedo, and N. Olson. Aminopeptidase and dipeptidyl aminopeptidase activities of several cheese-related microorganisms. (In preparation to be submitted to *Milchwissenschaft*)

C. Chen, M. El Soda, B. Riesterer, and N. Olson. Acceleration of reduced-fat cheese ripening using lyophilized extracts or freeze-shocked cells of some cheese-related microorganisms. (In preparation to be submitted to *Milchwissenschaft*).

The following were sent as brief communications to the International Dairy Congress, Montreal, 1990:

1. Detection of the peptide hydrolase system of several non-starter bacteria.

2. Partial purification and characterization of aminopeptidase and dipeptidyl peptidase activities from several non-starter bacteria.

3. Acceleration of reduced-fat Cheddar cheese ripening using lyophilized crude cell free extract of several non-starter bacteria.

José Coca

Sponsoring professor:
C.G.Hill, professor, Dept. of Chemical Engineering

Visiting scientist's address:
Department of Chemical Engineering
University of Oveido,
33071 Oveido, Spain

Period of stay:
October 15, 1989-November 15, 1989
July 1, 1990 - July 31, 1990

Project Title:
Electrodialysis of whey and fractionation of whey proteins.

Project objectives/conclusions:

The aim of this project is to use electrodialysis to study the demineralization of whey and whey permeates, and the fractionation of major whey proteins. At present, the focus is on the following topics:

1. Demineralization of both whey and whey permeate, obtained by ultrafiltration.
2. Demineralization of whey as a preparatory step to protein fractionation.
3. Effect of electrical inversion and hydraulic inversion on cleaning of the membrane surface.
4. Fractionation of whey proteins by ionic chromatography.
5. Influence of operating conditions on the mass transfer parameters of electrodialysis and chromatography for the scale-up of lactose and protein recovery.

The stay at CDR served the purpose of permitting Dr. Coca to conduct a thorough literature search in the area. Preliminary experiments were conducted using the ionics electrodialysis unit available. The project is being continued at the University of Oviedo using a Stantech unit. The electrodialysis of synthetic solutions of ultrafiltration whey permeates has been almost completed.

Contributions to CDR's Research Program:

A seminar summarizing the results of this research was presented to the staff of the CDR and the Dept. of Chemical Engineering on July 24, 1990. The research will contribute to both the collaborative research efforts between the UW and the University of Oviedo and the further development of the dairy research program of the Dept. of Chemical Engineering at the University of Oviedo, located in the province of Asturias, the dairyland of Spain.

Presentations/publications resulting from this research:
CDR seminar: "Membrane Separation Research at the Dept. of Chemical Engineering, University of Oviedo," Oviedo, Spain. July 24, 1990

W. James Harper

Sponsoring professor:
Norman F. Olson, professor, Dept. of Food Science

Visiting scientist's address:
Professor Emeritus, Ohio State University
3563 South Old 3-C Road
Galena, Ohio

Period of stay:
October 15 - November 14, 1989
June 1 - June 30, 1990

Project title:
Fractionation of milk proteins and proteolysis of cheese; participant in CDR's scientist mentor program.

Project's objectives:

1. To consult with Dr. J. P. Chen of CDR and Drs. C. H. Hill and J. Coca of the Dept. of Chemical Engineering in regard to their research projects relating to the fractionation and recovery of whey proteins.
2. To consult with Drs. N. F. Olson and M. Johnson; and Brian Riesterer, two Ph.D. candidates, and technical staff on research projects relating to cheese ripening.
3. Carry out collaborative research projects on whey fractionation with Drs. Coca and Chen.
4. Provide input to WITEP in respect to program development and the development of international collaborative research networks.

Activities during visit:

Two seminars were given for CDR, the Depts. of Food Science and Chemical Engineering staff and graduate students to provide fundamental background on whey proteins and protein functionality. Individual sessions were held with Drs. Coca, Chen, and Srinivasan to discuss their research programs; input was provided on these programs during both visits to CDR. Assistance was given to Dr. Coca in developing his research program and a preliminary plan of continuing collaboration developed. Overall needs for future protein research were developed for evaluation of possible implementation.

Met with staff and graduate students in respect to ongoing programs in cheese research and participated in research discussions and cheese evaluations. Presented two seminars to staff and graduate students in relation to cheese ripening.

Reviewed research programs in this area and suggested possible directions for future research to be reviewed and evaluated for possible implementation. Initiated a preliminary research project to develop a means of controlling undesirable microflora in cheese slurries, so that the

slurry approach might be developed into a tool to predict ripening in cheese in order to reduce research time requirements. The potential of this approach will be evaluated for possible future involvement.

Developed criteria for the establishment of an international collaborative research network in the areas of milkfat, protein, and cheese ripening. The priority of developing such networks were: (a) milkfat, (b) cheese ripening, and (c) proteins. Prepared a proposal for a research workshop for scientists in milkfat research, as a first step in the development of a research scientists network in this field. An initial evaluation has been made of developing collaborative research on butter flavor in baked goods between the UW-Madison(CDR) and the New Zealand Dairy Research Institute.

Note from the WITEP coordinator:
A scientist from the New Zealand Dairy Research Institute is spending two weeks in October, 1990 at the UW-Madison Food Science Dept. to develop a a proposal for a collaborative research project with Dr. Robert Lindsay on the use of butterflavors in baked goods.

A series of 6 databases were developed to assist in program management in CDR and WITEP. These data bases included: (a) Visiting scientists, (b) CDR research projects and project management, (c) Staff training and exchanges, (d) Seminars, (e) Directory of dairy research scientists and (f) Senior scientists. These data bases are developed (in *Foxbase* for the Macintosh), but still require the inputting of records in some of the databases.

How did these efforts contribute to CDR's research program:

1. The effectiveness of the program on whey proteins has been improved through providing CDR research with a better understanding of the fundamentals of whey proteins and suggestions for research project improvement.

2. Input into the cheese research program has provided insights into possible new directions to better meet the future objectives of CDR in respect to the area of cheese ripening.

3. Improved the operation of CDR and WITEP by providing better tools for program and project management.

Publications resulting from this visit:

One review article has been written in respect to the "Functionality of Whey Proteins" for an IDF monograph, and one review article on "Acceleration of cheese ripening with slurries" is in preparation.

Seminar Series

The CDR seminar series is designed to bring together scientists to discuss developments in dairy foods research. Researchers from CDR/UW and from industry make suggestions regarding speakers that they would like in the series. In addition to promoting collaborative research, the seminars are mechanism for transferring research to the dairy industry.

Dr. Timothy M. Cogan
National Dairy Research Center
Moorepark, Fermoy, Ireland.
"Citrate metabolism and diacetyl production by lactic acid bacteria."
July 13, 1989.

Dr. Hyong Joo Lee
Associate Professor, Dept. of Food Science and Technology, Seoul National University, Suwon, Korea.
"Bitter peptide formation with Prt- mutants of *Lactococcus lactis* ML_8 selected by curing of 34 Mdal plasmid."
August 17, 1989.

Dr. M.A. van Boekel
Senior lecturer, Dept. of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands.
"Milk protein content measurement using infrared spectrophotometry."
August 29, 1989.

Dr. M.A. van Boekel
Senior lecturer, Dept. of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands.
"Relation between volatile sulphur compounds and Cheddar cheese flavor."
September 25, 1989.

Dr. Morsi El-Soda
Professor, Dept. of Agricultural Industries, Alexandria University, Alexandria, Egypt.
"Pediococci - the cheese starters for the 21st century?"
September 25, 1989.

Dr. Souzan El-Kest
PhD graduate student, Dept. of Food Science, UW-Madison.
"Death and injury of frozen *Listeria monocytogenes* as affected by glycerol and milk components."
October 17, 1989.

Dr. Moustafa El-Shenawy
Researcher, Dairy and Food Microbiology Laboratory
National Research Center, Cairo, Egypt.
"Control of *Listeria monocytogenes* with heat, gamma irradiation, and the lactoperoxidase-thiocyanate system."
October 17, 1989.

Mr. Robert Masella
Asst. Director, STELA Dairy Sciences Research Center, Laval University, Quebec City, Quebec, Canada.
"Working with the Dairy Industry - a University Research Center Perspective."
October 24, 1989.

Dr. W. James Harper
Professor emeritus, Dept. of Food Science and Nutrition, Ohio State University
"Functional properties of beta-lactoglobulin."
October 26, 1989

Dr. W. James Harper
Professor emeritus, Dept. of Food Science and Nutrition, Ohio State University
"Functional properties of alpha-lactoglobulin."
October 30, 1989

Dr. L.K. Creamer
Head, Protein Chemistry Section
New Zealand Dairy Research Institute, Palmerston North, New Zealand.
"Casein micelle structure and chemistry of proteins in cheese."
December 12, 1989.

Dr. Ed Jesse
Chairperson, Dept. of Ag Economics, University of Wisconsin-Madison.
"Reconstituted Milk - Opportunities & Constraints."
December 13, 1989.

Dr. J.P. Chen
Assistant scientist, Center for Dairy Research, University of Wisconsin-Madison.
"Affinity Purification of Food Proteins - the Broad Picture."
January 18, 1990.

Dr. Arun Kilara
Dept. of Food Science, Pennsylvania State University, University Park, Pennsylvania.
"Microemulsions and reverse micelles and their applications in the dairy and food industries."
January 29, 1990.

Dr. Rafael Jimenez-Flores
Research Associate, Dept. of Food Science and Technology, University of California - Davis.
"Cholesterol Removal and Other Technological Advances at the California Dairy Foods Research Center."
March 13, 1990.

Dr. Earl Hammond
Professor, Dept. of Food Technology, Iowa State University.
"Swiss Cheese Flavor - A Review of Studies Done at Iowa State University in the Past Fifteen Years."
April 6, 1990.

Dr. Jim Harper
Professor emeritus, Dept. of Food Science and Nutrition, Ohio State University.
"Accelerated cheese ripening using slurries."
June 5, 1990.

Dr. Jim Harper
Professor emeritus, Dept. of Food Science and Nutrition, Ohio State University.
"Effect of minor compounds on whey protein functionality."
June 7, 1990.

Research Conferences

CDR hosts an annual research conference each year which focuses on a dairy research topic. The research theme alternates yearly between cheese research and basic dairy research topics such as lactose or milkfat. CDR also served as a sponsor of the UW Dairy Manufacturer's Conference in 1990.

1990 Dairy Products Technical Conference

April 25-26, 1990
Chicago O'Hare Marriott Hotel
Chicago, Illinois

CDR's third annual conference was jointly sponsored by CDR and the American Dairy Products Institute (ADPI), Chicago, IL. This conference focused primarily on developments in whey processing technology and research, and also addressed other technical dairy product issues. (The program is shown below.) Attendance of the conference was excellent with about 290 people registered. Proceedings for the conference were published and are available from CDR or ADPI. CDR distributed its annual research report and other informational handouts on CDR activities at the conference.

The 1990 Conference Program:

"The Dairy Industry: Present & Future," R.A. Freemore

"A Look at Future Food Labeling," G.M. Burditt

"Dairy Product Nutrition & Quality," R.G. Bursey

"Dairy Products & Consumer Image," E.W. Speckman

"Advances in Processing Technology/An Overview," M. Cheryan

"Manufacture and Use of Milk Protein Concentrate," Z. Puhan

"Manufacture and Use of High Protein Whey Products," D. Gleeson

"Collection, Handling, & Processing of Salt Drippings Using the Ultra-Osmosis Membrane," D.P. O'Shea

"Whey Proteins -- Present Status," C.V. Morr

"Whey Proteins -- Functionality," M. Mangino

"Simplesse -- A Unique Food Ingredient," N.S. Singer

"Permeate Utilization," A.K. Keller

"Dietary Lactose Reduces Salmonella Concentration & Colonization

in Poultry," J.R. DeLoach

"Utilization of Dairy Products in Animal Feeds," D.J. Schingoethe

"Nutrition and Dairy Products," Banquet speaker: David Kritchevsky, MD

"Whey Processing in the Future," Luncheon speaker: W. James Harper.

UW Dairy Manufacturer's Conference

May 23, 1990
The Mead Inn
Wisconsin Rapids, Wisconsin

The Center for Dairy Research was a sponsor of the UW Dairy Manufacturer's Conference. The conference was coordinated by Dr. Bill Wendorff of the Dept. of Food Science Extension, UW-Madison. WITEP assisted with funding of speakers, and developed the proceedings and an informational brochure, *Cheesemaker's Guide to Wisconsin Resources*, distributed at the conference. Program is shown below.

"Infections and Intoxications with Milk and Dairy Products in the UK," Richard J. Gilbert

"Update of NR214: Landspreading of Whey and Whey Permeates," Micheal Witt

"Eliminating Crystal Formation in Package Cheese," Dr. Norman Olson

"CDR -- Your Support Team for the Industry, " Sarah Quinones and Dr. Norman Olson

"Recovery Systems for Whey Components," David Hibbard

"Whey and Whey Permeate for Soil Improvement," Dr. Art Peterson

"Whey Components in Milk Replacers," Dr. Trevor Tomkins

"The Future of Whey Components in the Food Processing Industry," Dr. W. James Harper

Information Center

The Information Center within WITEP includes activities such as the development of the annual research report, conference proceedings, the *Dairy Pipeline* newsletter, other informational handouts, and various databases.

Conference proceedings
Proceedings were developed and published for the *Dairy Products Technical Conference*, April 25-26, 1990. A compilation of the abstracts (conference proceedings) for the *UW Dairy Manufacturer's Conference* May 23, 1990 were published and distributed at the conference.

Cheesemaker's Guide to Wisconsin Resources
This brochure was developed in particular for distribution at the *UW Dairy Manufacturer's Conference* held May 23, 1990 at Wisconsin Rapids, Wisconsin. This brochure lists the names of UW dairy extension personnel and other Wisconsinresources for cheesemakers.

UW Dairy Pipeline Newsletter
This newsletter is published 2-3 times per year and addresses technical issues of current concern to the Wisconsin dairy processing industry. It features articles on technology and research developments at the UW, a cheesemaking question-and-answer column, and a calendar of CDR events.

Annual Report
The CDR annual report consists of the following:

- a compilation of progress reports for all CDR-affiliated research projects underway at the UW,
- a summary of scientist exchanges and events/programs sponsored by WITEP,
- a publication list of all dairy -related research publications published in the past year,
- an overview of the structure and staff comprising CDR,
- and additional dairy foods research survey articles.

The annual report is distributed to:

- the other five dairy foods research centers and our funding agencies,
- the Wisconsin dairy industry (made available at our annual research conference),
- numerous international dairy foods research centers,
- and to a mailing list of individuals who have requested our annual report.

CDR Reprint Collection
A collection of reprints of all publications generated from research funded through CDR has been developed. This listing begins at 1976.

Chapter 4

Dairy Foods Research Abstracts

Note from the editors: This chapter contains the abstracts of several research publications thought to be of interest to the dairy industry. If you would like the complete reprint, write to:

Center for Dairy Research
WITEP, Rm. 241
1605 Linden Dr.
Madison, WI 53706

or fax: WITEP 608-262-1578

Title:

Role of *Micrococcus* and *Pediococcus* Species in Cheese Ripening: A Review

Tarun Bhowmik and Elmer H. Marth, Department of Food Science and The Food Research Institute, University of Wisconsin-Madison, *Journal of Dairy Science* 73:859-866

Abstract:

The secondary flora of cheese contain several kinds of microorganisms, including micrococci and pediococci. Micrococci constitute a major portion of the raw milk flora. Some thermoduric micrococci survive pasteurization and occur in cheese made from pasteurized milk. Micrococci or pediococci may be present naturally in cheese milk, or when added deliberately may improve and enhance flavor development in Cheddar cheese. The beneficial role of these bacteria has been related to their proteolytic, lipolytic, and esterolytic activities and to some metabolites they produce during the ripening process. The possible advantages and disadvantages of using them as agents for accelerated cheese ripening are described. (Key words: *Micrococcus, Pediococcus,* cheese ripening).

Title:

Lactobacilli - Their Enzymes and Role in Ripening and Spoilage

Noraini M. Khalid and Elmer H. Marth, Department of Food Science and Food Research Institute, University of Wisconsin-Madison, *Journal of Dairy Science* 73:2669-2684

Abstract:

Lactobacilli commonly occur in natural cheese because they are used as a starter culture (e.g., Swiss cheese) or enter milk, and thus cheese, as post-pasteurization contaminants (e.g., Cheddar cheese). Proteinases (cell wall-bound, intracellular, and extracellular) occur in lactobacilli; those from some species of *Lactobacillus* preferentially hydrolyze α_{s1}-casein, whereas those from others prefer ß-casein. Peptides released from casein by proteinases are subsequently hydrolyzed by peptidases inside cells of lactobacilli. The intracellular peptidases are a vital part of the mechanism by which lactobacilli make free amino acids that are precursors of some cheese flavor compounds. Aminopeptidase, dipeptidase, carboxypeptidase, and endopeptidase activities have been associated with lactobacilli, although largely intracellular, membrane-associated peptidases have been noted. Intracellular lipases and esterases also occur in lactobacilli, but activity of these enzymes has been designated as "weak." Despite this, they probably contribute to flavor development in some varieties of cheese. Certain lactobacilli can cause defects such as formation of white crystals of calcium lactate on the surface of cheese, or of biologically active amines, sometimes causing illness in consumers. (Key words: lactobacilli, enzymes, cheese)

Title:

Microbiological Safety of Cheese Made from Heat-Treated Milk.

Johnson, Eric A., J.H. Nelson and Mark Johnson. Food Research Institute and the Walter V. Price Cheese Research Institute, University of Wisconsin-Madison,
Journal of Food Protection, vol. 53, No. 5:441-452 (Part I of a three-part paper)

Abstract:

Research on pasteurization of milk for cheesemaking was begun in the late 1800's. Early equipment was crude and control devices non-existent. Consequently, early pasteurization processes were not well verified. Commercial application was slow, except in New Zealand, where almost the entire cheese industry converted to pasteurization in the 1920's. In the United States, debate on the merits of pasteurization continued for years. Demand for cheese during World War II and foodborne disease outbreaks caused by cheese stimulated promulgation of government standards which included the options of milk pasteurization or 60 d holding at a minimum temperature of 2°C (35°F). The cheese industry has continued to improve technology, including that which is safety related. United States production of cheese has continued to expand, from just over 1 billion pounds in 1948 to 5.4 billion pounds in 1987. Thirty-eight percent of the 1987 total comprised varieties wherein heat-treated milk is frequently utilized.

Executive Summary:

The heat-treatment of raw milk can exert a significant role in producing microbiologically safe cheese. Recent, thorough research has affirmed that milk heat-treatment at 65.0-65.6°C (149-150°F) for 16-18 s will destroy virtually all pathogenic microorganisms which are major threats to the safety of cheese.

An extensive review of epidemiological literature identified only six illness outbreaks transmitted via U.S.-produced cheese during 40 years, 1948-1988. During these four decades, the United States cheese industry produced over 100 billion pounds of natural cheese (not including cottage and related varieties). The most frequent causative factor in U.S. and Canadian cheese-related outbreaks was post-pasteurization contamination. Faulty pasteurization equipment or procedures were implicated in one outbreak each in the U.S. and Canada. Use of raw milk was a factor in one outbreak in each country. Inadequate time-temperature combinations used for milk heat treatment were not implicated.

The epidemiology of cheese-related outbreaks in the U.S., Canada, and Europe demonstrated that soft surface-ripened cheeses, e.g., Camembert and Brie, are at significantly greater risk to transmit pathogens than other cheeses. No outbreaks were linked to hard Italian varieties, e.g., Parmesan, Romano, and Provolone. Varieties such as Cheddar and Swiss were infrequently involved.

Pathogens were prioritized as high, medium, or low risk in cheese. Three organisms, *Salmonella, Listeria monocytogenes,* and enteropathogenic *Escherichia coli,* were judged to be high risk threats to the cheese industry. *Staphylococcus aureus* was listed as low risk because growth and toxin production is readily suppressed by modern lactic culture technology and acidity (pH) control in cheese.

Recently published research (*98,99,126*) comprehensively defined the effect of raw milk heat-treatment on pathogen survival. Multi-strain or species mixtures of pathogens were inoculated into raw milk at levels of 10^5/ml. Inoculated milk was heat-treated in a commercial HTST pasteurizer - mean holding time 17.6 s, minimum 16.2 s. All strains of *Yersinia enterocolitica, Campylobacter* sp., *E. Coli* 0157:H7, and all but one *Salmonella* species were destroyed at 65°C (149°F). *Salmonella senftenberg* was inactivated at 69°C (156.2°F). *S. senftenberg* is rarely isolated from cheese. *L. monocytogenes* in naturally contaminated milk at levels of 10^4 organisms per ml was inactivated at 66°C (150.8°F); laboratory-cultured inoculum at levels of 10^5 organisms per ml required 69.0°C (156.2°F).

A large cheese factory which heat-treats milk for cheesemaking at 64.4°C (148-149°F) for 16 s with concurrent concentration at 16-17% total solids provided data on aerobic plate count (APC) and coliform for several hundred production days. The average APC of 1.4 million/ml in raw milk was reduced 1.71 logs to an average of 28,000/ml in milk entering the vat. Coliform counts, which averaged 121,000/ml in raw milk were almost all <10/ml in heat-treated milk, with a maximum of 50/ml. All temperatures studied were above 60°C (140°F), the minimum hot food holding temperature specified in FDA's good manufacturing practice regulations.

A multiplicity of practices other than pasteurization or heat-treatment contribute significantly to the microbiological safety of cheese. Some, such as milk quality management, lactic culture management, pH control, salt addition, and controlled curing conditions are established technologies. Others represent potential opportunities, such as natural inhibitory substances in milk, and antibacterial substances, e.g., nisin and lysozyme. It is imperative that the relationships of established and potential safety technologies be better defined to enable the articulation of proven safety systems geared to the characteristics and safety risks of various cheese varieties. Neither pasteurization nor any other single technology can assure safe cheese.

The National Cheese Institute should encourage and support research on cheese safety. Three NCI actions are recommended:

1) Establish a guideline that the minimum heat-treatment of milk for cheesemaking be 64.4°C (148°F) for 16 s or equivalent with adequate process control.

2) Evaluate current safety technology and practice for cheese manufacture. Define, prioritize, and support research with primary emphasis on the combined effect of heat-treatment and other current cheese technologies.

3) Evaluate technologies not currently utilized in cheese manufacture for safety potential.

Title:

***Listeria monocytogenes* and Listeriosis Related to Milk, Milk Products and Dairy Ingredients: A Review**

F.E. El-Gazzar and E.H. Marth, Department of Food Science and Food Research Institute, University of Wisconsin -Madison, *Milchwissenschaft* (in press)

I. *Listeria monocytogenes*, Listeriosis, and Responses of the Pathogen to Environmental Conditions

Abstract:

Recent outbreaks of listeriosis associated with consumption of milk products contaminated with the psychrotrophic *Listeria monocytogenes* have led to renewed scientific interest in this bacterium. The pathogen can cause abortion in pregnant women and meningitis and encephalitis in neonates and susceptible adults. *L. monocytogenes* sometimes occurs in leukocytes in milk of mastitic animals and thus gains some protection so that under certain circumstances it can survive the recommended high-temperature, short-term pasteurization process. The pathogen tolerates NaCl and grows at low temperatures and at both acidic and alkaline pH values.

II. *Listeria monocytogenes* and Dairy Technology

Abstract:

Listeria monocytogenes is of major concern to the dairy industry since the pathogen has been isolated from various dairy products in the marketplace. The pathogen behaves differently in different kinds of cheese; it survived for more than one year in Cheddar, 140 days in Colby, 90 days in Feta, and grew in Camembert cheese. Behavior of the pathogen in cheese depends mainly on the strain of *L. monocytogenes*, and on different conditions of cheese manufacture, ripening, and storage. Also, composition and pH of ingredients affects the fate of the pathogen in dairy ingredients. Use of appropriate hygienic procedures during milk processing should reduce the likelihood of listeriosis outbreaks associated with dairy foods.

Title:

Reducing the Sodium Content of Foods: A Review

K. Anjan Reddy and Elmer H. Marth; Dept. of Food Science and The Food Research Institute, University of Wisconsin-Madison; *Journal of Food Protection* (in press).

Abstract:

Salt (sodium chloride), a substance essential for life processes, is the second most used food additive. It is added to foods as a flavoring or flavor enhancing agent, a preservative, or an ingredient responsible for desired functional properties in certain products. Excessive dietary sodium is believed to contribute to hypertension and cardiovascular disease, which afflicts ca. 60 million Americans. During the last decade the food industry has responded to the dietary needs of Americans concerned with sodium consumption by providing processed foods without added salt or with reduced amounts of sodium. Other foods are available in which some or all of the salt has been replaced by a salt substitute. If properly used, common salt substitutes, including potassium chloride, certain herbs, spices, organic acids, autolyzed yeast products, and hydrolyzed vegetable protein, can result in products that are consumer-acceptable. Commercially available foods with less than the normal amount of salt include natural cheeses, pasteurized process cheeses, cottage cheese, butter, buttermilk, ice cream, cured meat products, fresh sausages, cereal products, vegetables, salad dressing, smoked fish, fish sauces, soy sauce, and miso.

Title:

The Impact of Lactic Acid Bacteria on Cheese Flavor

Norman F. Olson, Center for Dairy Research and Dept. of Food Science, University of Wisconsin-Madison, FEMS Microbiology Reviews 87 (1990) 131-148

Summary:

Cheese flavor is a manifestation of complex interactions of volitile and non-volitile flavor-active compounds plus tactual perception. Numerous agents, including lactic acid bacteria, produce the flavor sensations. The effect of lactic acid bacteria is more dominant in cheese varieties with limited growth of secondary flora. This review describes the indirect and direct impacts of lactic acid bacteria in cheese with emphasis on carbohydrate fermentation, changes in oxidation-reduction potential, interactions with non-starter bacteria, autolysis, proteolytic and peptidolytic activities, transport of metabolites, and flavor production.

Chapter 5

Dairy-Related Research Publications

Note from the editors: This chapter lists most, although not all, of the dairy-related papers published at the University of Wisconsin-Madison during the past year (1989). Some publications are listed in more than one department due to joint appointments of some faculty.

Department of Agricultural Economics

Barrows, R. (Reviewed) Extension Science: Information Systems in Agricultural Development, by Niels Roling. *American Journal of Agricultural Economics* (August 1989): 833-34.

Barrows, Richard L. and D. Henneberry. Capitalization of Exclusive Agricultural Zoning into Farmland Prices. *Land Economics*, 66: 3 (Aug. 1990) 249-254

Bentley, Susan E. and William Saupe. Involuntary Farm Exit in Wisconsin, Longer Term Adjustments Were Less Negative. *Choices* Third Quarter (1989): 30.

Bentley, Susan E., William A. Saupe, et al. Involuntary Exits From Farming: Evidence From Four Studies. *Agricultural Economic Report* 625. Economic Research Service, USDA (November 1989): 12 pages.

Bock, C. Allen and Phillip E. Harris. *1989 Income Tax Workbook*. Champaign-Urbana, Illinois: University of Illinois (1989): 651 pages.

Buse, Reuben C., A.C. Johnson, and M. Johnson. Econometrics: Basic and Applied. Singapore: *Maxwell-MacMillan International Edition* (1989).

Campbell, Gerald R., Pricing Relative to Costs. *Direct Marketing Newsletter 9*. Platteville, Wisconsin: University of Wisconsin- Extension (January 1989): 1-3.

Campbell, Gerald R., Grain Outlook. In E. Jesse (editor), *Status of Wisconsin Farming*, 1989. Madison, Wisconsin: University of Wisconsin, Department of Agricultural Economics and Cooperative Extension Service (January 1989): 33-38.

Chavas, J.-P. Information Issues in the Coordination of Agricultural and Resource Policies. In R. E. Just and N. Bockstael, (eds.), *Commodity and Resource Policies in Agricultural Systems*. New York, New York: Springer-Verlage (1989).

Chavas, J.-P. and M.T. Holt. Acreage Decisions Under Risk: The Case of Corn and Soybeans. *American Journal of Agricultural Economics* 71 (1989).

Cox, T.L. Advertising and Demand Stimulation: Current Research. In C.S. Thraen and D.E. Hahn (eds.), *Advertising, Promotion and Consumer Use of Dairy Products: Insights from Economic Research*. Ames, Iowa: The Ohio State University Press (January 1989): 169-8 3.

Glauber, J., P. Helmberger and M. Miranda. Four Approaches to Commodity Market Stabilization: A Comparative Analysis. *American Journal of Agricultural Economics* 71: 326-337 (1989).

Gould, Brian W., William E. Saupe and Richard M. Klemme. Conservation Tillage: The Role of Farm and Operator Characteristics and the Perception of Soil Erosion. *Land Economics* 65:2 (May 1989): 167-182.

Gould, Brian W. and William E. Saupe. Off-Farm Labor Market Entry and Exit. *American Journal of Agricultural Economics* 72:4 (November 1989): 960-969.

Harris, Phillip E. Income Tax Consequences of Debt Reduction Under the Agricultural Credit Act of 1987. *Agricultural Law Update* 7:1 (October 1989): 4-7.

Harris, Phillip E. and W.A. Tinsley. Tax Management: Taking Taxes into Account. *Yearbook in Agriculture.* USDA (1989): 156-159.

Jesse, E.V. Imports: Dairy Products Limited, but Casein Unrestricted. *Hoard's Dairymen* (October 1988): 1 page.

Jesse, E.V. Guess the Government in 1989-90. *Proceedings, Wisconsin Forage Council 1989 Symposium* (January 1989): 3 pages.

Jesse, E.V. (editor) Status of Wisconsin Farming, 1989. Madison, Wisconsin: University of Wisconsin, *Department of Agricultural Economics and Cooperative Extension* (February 1989): 57 pages.

Jesse, E.V. America's Fat Surplus Keeps Growing. *Hoard's Dairyman* (April 25, 1989): 1 page.

Jesse, E.V. Solving the Butterfat Problem. *Hoard's Dairyman* (May 10, 1989): 1 page.

Jesse, E.V. On the Economics of Milkfat. Nutrition and Other Factors Affecting the Composition of Animal Products. *Proceedings, 1989 Nutrition Institute.* Oak Brook, Illinois: National Feed Ingredients Association (June 6, 1989): 9 pages.

Jesse, E.V. World Butterfat Situation and Outlook. Milkfat -Trends and Utilization. *Proceedings, 1988 Dairy Research Conference.* Madison, Wisconsin: University of Wisconsin, Center for Dairy Research (July 1989): 11 pages.

Jesse, E.V. Issues in Multiple Component Pricing of Milk. Milkfat — Trends and Utilization. *Proceedings, 1988 Dairy Research Conference.* Madison, Wisconsin: University of Wisconsin, Center for Dairy Research (July 1989): 7 pages.

Jesse, E.V. Trade and Pricing Challenges in the 1990's: Legislating, Regulating, Marketing, and Pricing Dairy Products. *Proceedings, 44th Annual Midwest Milk Marketing Conference* (July 1989): 11 pages.

Jesse, Edward V. and Lydia Zepeda. Socio-Economic Implications of rBST Use. *Discussion Paper Bovine Somatotropin (rBST),* Madison, Wisconsin: University of Wisconsin, College of Agricultural and Life Sciences (August 29, 1989).

Klemme, Richard. Components of Sustainable Agricultural Systems. *Proceedings, Conference on Sustainable Agriculture for Today and Tomorrow: Wisconsin Research for Wisconsin Farmers,* Eau Claire, Wisconsin, March 22, 1988; Appleton, Wisconsin, March 23, 1988.

Klemme, Richard. Cost Cutting Survival: How to Cut Costs and Remain Competitive Beyond the Next Century. *Dairy Herd Management,* Miller Publishing Company April (1988).

Luening, R.A. and B.L. Jones. Characteristics of U.S. Farm Managers Chapter 3 in *1989 Yearbook of Agriculture: Farm Management* (USDA): 12-14.

Marion, Bruce W., Robert L. Wills, and L.J. Butler. *The Social and Economic Impact of Biotechnology on Wisconsin Agriculture.* Madison, Wisconsin: University of Wisconsin, College of Agriculture and Life Sciences (April 1988): 154 pages.

Marion, Bruce W. The Concentration-Price Relationship in Food Retailing. In L.W. Weiss (editor), *Concentration and Price.* Cambridge, Massachusetts: The MIT Press (1989): 183-193.

Marion, Bruce W. Food Retailing and Wholesaling in the United States: Organization, Trends and Competition. In Y. Nakano and T. Yamanaka (eds.), *An International Comparison of Wholesale and Retail Marketing,* Vol. 1 (1989): 193-214.

Marion, Bruce W. Is the Family Farmer Being Squeezed Out of Business by Monopolies? In Gary Comstock (editor), *Concentration and Price.* Cambridge, Massachusetts: The MIT Press (1989): 213-218.

Pulver, Glen C. Developing A Community Perspective on Rural Economic Development Policy. *Journal of the Community Development Society* 20:2 (Fall 1989): 1-14.

Pulver, Glen C. and Ayse C. Somersan. Building on Economic Development Outreach Programs in a Land-Grant University. *New Alliances For Rural Economic Development.* University Park, Pennsylvania: Pennsylvania State University, Northeast Regional Center for Rural Development (February 1989): 24-28.

Shaffer, Ron. *Community Economics: Economic Structure and Change in Smaller Communities.* Ames, Iowa: Iowa State University Press (May 1989).

Wills, R.L. and W.F. Mueller. Brand Pricing and Advertising. *Southern Economic Journal* 56 (October 1989): 383-395.

Zepeda, Lydia. An Ex Ante Adoption Model of Bovine Somatotropin by California Milk Producers. *American Journal of Agricultural Economics* 71 (December 1989).

Economic Issues Series 1987 Through 1989

No. 108 - January 1989 - Kada, Ryohei. The Future of U.S. -Japan Agricultural Trade Relations: A Japanese Perspective

No. 111 - May 1989 - Carter, Michael R. U.S. Farm Exports and Third World Agricultural Development

No.112 - November 1989 - Zepeda, Lydia. Issues and Policy Options Surrounding Bovine Somatotropin

Marketing And Policy Briefing Papers - 1987 Through 1989

No. 27 - March 1989 - Jesse, Edward V. and Gerald R. Campbell. A Futures Contract for Cheese - - Could It Work?

No. 28 - August 1989 - Cropp, Bob and Ed Jesse. Modernization and Nationalization of Federal Milk Marketing Orders.

No. 29 - September 1989 - Jesse, Edward V. 1989 Wisconsin Farm Policy Preference Survey.

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