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Microbiological and Sensory Effects of Milk Processed for Extended Shelf Life and the Development of Rapid Methods to Quantitate Spores and Lipase Activity

Michael R. Blake
Utah State University

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MICROBIOLOGICAL AND SENSORY EFFECTS OF PROCESSING MILK FOR EXTENDED SHELF LIFE AND THE DEVELOPMENT OF RAPID METHODS TO QUANTITATE SPORES AND LIPASE ACTIVITY

by

Michael R. Blake

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah
1996
ABSTRACT

Microbiological and Sensory Effects of Milk Processed for Extended Shelf Life and the Development of Rapid Methods to Quantitate Spores and Lipase Activity

by

Michael R. Blake, Doctor of Philosophy
Utah State University, 1996

Major Professor: Dr. Bart C. Weimer
Department of Nutrition and Food Sciences

The initial aim of this work was to evaluate processing conditions for extended shelf life (ESL) milk to have a shelf life at refrigeration temperature of 60 d. Milk was processed on a pilot-scale ultra-high-temperature processing plant and evaluated for microbial and sensory quality over 60 d at 7°C storage. Results of this study showed that lower process temperatures were preferable to minimize cooked flavors and that the minimum safe processing temperature was 134°C for 4 s as determined by the destruction of bacterial spores in the processed milk.

Consumer preference panel results indicated that consumers preferred milk processed at 134°C for 4 s (those recommended in this study for ESL processing) to commercial UHT milk although there was a slight preference for pasteurized milk. The critical sensory characteristic of the processed milk was a cooked flavor, which decreased with lower processing temperature and shorter storage time; however, a significant increase in flavors that could be associated with lipolytic activity was also noted.

This study highlighted deficiencies in existing methods for determining heat-stable bacterial products in thermal-processed foods. No rapid, sensitive assay for detection of
heat-stable spores or lipases in milk exists. If such assays were available, it would allow processors to determine lipase activity and bacterial spore counts before processing and direct raw milk with low spore counts and low lipolytic activity into long-shelf-life products. To this end, assays to rapidly quantitate spores and lipolytic activity in milk were developed.

The lipase assay relies on the hydrolysis of p-nitrophenyl caprylate liberating a yellow color that is detected using reflectance colorimetry. The assay is sensitive to 5 mUnits/ml and is linearly correlated to spectrophotometry ($r^2 = 0.93$) and release of titratable free fatty acids ($r^2 = 0.92$ to 0.97).

An immunocapture, enzyme-linked immunooassay coupled with a fluorescent detection system was developed for and resulted in a prototype spore assay using *Bacillus stearothermophilus* spores. This organism was selected because it is extremely heat resistant, is commonly found in milk, and is associated with spoilage of milk and milk products. The assay was able to quantitate spores down to $10^3$ cfu/ml in milk and other products in about 1.5 h. Other detection limits could be set if needed.
ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Bart Weimer—my friend and mentor—for his continuous support and guidance. I would like to thank the members of my committee for their ideas and advice. I would also like to thank Dairy Research and Development Corporation and the Western Center for Dairy Research and Technology for their financial assistance.

Above all, I would like to thank my wife, Sue, for her love and patience.

Michael Blake
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LIST OF SYMBOLS, NOTATION, DEFINITIONS

Abbreviation key:

ABC = avidin-biotin complex
ANOVA = analysis of variance
Anti-BsS = anti-Bacillus stearothermophilus spore IgG
DMSO = dimethylsulfoxide
ELISA = enzyme linked immunosorbent assay
ESL = extended shelf life
FFA = free fatty acid
HRP = horseradish peroxidase
HTST = high-temperature short-time pasteurized
IMB = immunomagnetic beads
OPA = o-phthalaldehyde
OPD = o-phenylenediamine
PCA = plate count agar
p-NP = p-nitrophenyl
pNP-C = p-nitrophenyl caprylate
UHT = ultra-high temperature processed
USU = Utah State University
CHAPTER I
INTRODUCTION

Milk is commonly processed by high-temperature short-time pasteurized (HTST) or ultra-high temperature processed (UHT) processing temperatures (Table 1). HTST milk is a nonsterile product and has a shelf life of about 14 d at 7°C and is usually spoiled by growth of psychrotrophic bacteria. UHT milk is considered sterile, may have a shelf life anywhere from 6 mo to 2 yr, and is spoiled by sensory or texture changes in part due to heat-stable enzymes during the long shelf life at room temperature or to the growth of extremely heat-tolerant bacterial spores.

Two common methods for UHT processing of milk are indirect heating of milk and direct steam injection heating of milk. Indirect UHT involves passing the milk past thin plates or through concentric tubes with superheated steam on the other side and allowing the milk to heat up via heat exchange across the plates. Cooked flavors resulting from burn-on of milk to heat exchange plates were a major concern (4) and initiated the development of a direct steam injection process.

Direct steam injection produces a shorter heating and cooling time since the milk is heated by superheated stream that is injected directly into the milk. This minimizes cooked flavors due to burn-on and shortens the total time that the milk is at high temperatures. Excess water from steam is removed from milk by flash evaporation immediately.

**TABLE 1. Common milk processing conditions.**

<table>
<thead>
<tr>
<th>Process</th>
<th>Heating Temperature (°C)</th>
<th>Heating Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTST</td>
<td>72</td>
<td>15</td>
</tr>
<tr>
<td>UHT</td>
<td>140</td>
<td>1 to 4</td>
</tr>
</tbody>
</table>
after heating. Although direct steam injection results in fewer cooked flavor defects, this
defect is still noticeable. Hence it would be of benefit to process milk at lower than UHT
temperatures, reducing cooked flavors, but maintaining a significantly longer shelf life than
HTST milk.

Production of milk using temperatures lower than those typically used for UHT have
been proposed to allow transport of refrigerated milk to remote regions. Studies to
determine the effects of temperature on milk processing are necessary before ESL milk can
be considered safe for consumption and be marketable. To demonstrate acceptability and
safety, it is desirable that ESL processed milk have acceptable sensory qualities and be
microbiologically safe for 60 d at ≤ 7°C storage.

In this study, milk was processed at different temperatures and holding times to
determine an acceptable set of process conditions dependent on microbial survival and
sensory deterioration during storage. Statistical analyses were used to determine the
correlations among sensory characteristics, microbial growth, and enzymatic deterioration
of the final product. From these data, a set of conditions was chosen for ESL processing
that had a minimum shelf life of 60 d with minimum safety risks. Further studies then
evaluated the consumer acceptability of this ESL milk.
CHAPTER II
LITERATURE REVIEW

FLAVOR ATTRIBUTES OF HEAT-PROCESSED MILK

Fresh milk should have a pleasing, slightly sweet flavor, little aroma, and a pleasant mouthfeel and aftertaste. UHT milk processing induces flavors most commonly characterized as “cooked” originating from volatile sulfides of heat-denatured milk proteins with reducing sugars via Maillard browning reactions. These sulfides and cooked flavors usually persist during aseptic storage for several months (60). Cooked and burned flavors have been reduced in UHT milk by using direct steam injection to heat milk since the increased rate of heating and cooling of the product before and after the holding period reduces the total heat treatment time (4). Additionally, burn-on associated with plate exchangers used with indirect UHT systems is also reduced (4). Disadvantages of direct steam injection may include reduced enzyme inactivation, allowing greater residual microbial proteolytic and lipolytic activity in milk during storage which are associated with the accumulation of off-flavors (31).

Light-induced flavors of heated milk commonly described as oxidized flavor have been associated with degradation of serum protein fractions, and photooxidation of lipids. This defect is easily minimized by reducing exposure to fluorescent lights during storage (60).

Microbially induced flavors in UHT products are almost exclusively due to either heat-stable enzymes associated with heat-labile raw-milk microbiota (especially pseudomonads) or growth of heat-stable Bacillus spores during storage (4, 40). Heat-stable lipases and proteases survive UHT treatments causing a slow, but cumulative increase in breakdown products associated with bitter, fruity, rancid, and unclean flavors in fluid dairy products (60, 39, 40).
RAW MILK MICROBIOTA

Many types of bacteria are normally found in raw and processed milk, which are dominated by psychrotrophic bacteria (43) (Table 2). Psychrotrophic organisms are defined as, "microorganisms that can grow at 7°C or less, irrespective of their optimal growth temperature" (9: 157). Several methods for inhibiting, or killing, bacteria in milk at the farm have been used and are commonly used in some countries, but are restricted by law in the US (18, 28, 42, 49, 58) (Table 3). Excluding post-processing contamination, several factors will dictate the microbiota of the final product:

1. The total number and type of organism in the raw milk. A higher number of cells in the raw milk will lead to more surviving organisms after thermal processing (4).

2. The organisms' ability to survive processing (thermal death kinetics). Heat is the most common choice for processing milk; however, bacteria have a wide range of tolerances to temperature; for example, some psychrophiles are not able to grow at room temperatures while some thermophiles survive and grow on the edges of boiling thermal pools. Some organisms—most notably Bacillaceae—have adapted to survive extreme temperatures by producing dormant spores which are the most heat-tolerant forms of microorganism known (38, 50, 68).

3. The organisms' ability to grow in the final product. To grow in processed milk the organism must be able to meet its nutritional requirements from milk. Nutritional requirements select for organisms able to utilize lactose. However, many organisms that are important in dairy products are unable to ferment lactose but use hydrolytic products
TABLE 2. Bacteria commonly found in milk (16).

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacteriaceae</td>
<td><em>Streptococcus</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactococcus</em></td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus</em></td>
</tr>
<tr>
<td></td>
<td><em>Microbacterium</em></td>
</tr>
<tr>
<td></td>
<td><em>Propionibacterium</em></td>
</tr>
<tr>
<td>Micrococcaceae</td>
<td><em>Micrococcus</em></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td><em>Escherichia</em></td>
</tr>
<tr>
<td></td>
<td><em>Aerobacter</em></td>
</tr>
<tr>
<td></td>
<td><em>Proteus</em></td>
</tr>
<tr>
<td></td>
<td><em>Salmonella</em></td>
</tr>
<tr>
<td></td>
<td><em>Citrobacter</em></td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella</em></td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td><em>Pseudomonas</em></td>
</tr>
<tr>
<td>Bacillaceae</td>
<td><em>Bacillus</em></td>
</tr>
<tr>
<td></td>
<td><em>Clostridium</em></td>
</tr>
<tr>
<td>Achromobacteriaceae</td>
<td><em>Alcaligenes</em></td>
</tr>
<tr>
<td></td>
<td><em>Achromobacter</em></td>
</tr>
<tr>
<td></td>
<td><em>Flavobacterium</em></td>
</tr>
<tr>
<td>Bacteriaceae</td>
<td><em>Brevibacterium</em></td>
</tr>
<tr>
<td>Corynebacteriaceae</td>
<td><em>Corynebacteria</em></td>
</tr>
<tr>
<td>Parvobacteriaceae</td>
<td><em>Brucella</em></td>
</tr>
</tbody>
</table>
TABLE 3. Treatments developed to reduce the initial microbiota in milk.

<table>
<thead>
<tr>
<th>Method</th>
<th>Treatment</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermization</td>
<td>Heat treatment 64 to 68°C for 10 to 15 s (at the farm)</td>
<td>Used in Netherlands at the farm.</td>
</tr>
<tr>
<td>Lactic Acid Bacteria*</td>
<td>Addition of spp. of <em>Lactobacillus, Pediococcus</em></td>
<td>Production of peroxide activates lactoperoxidase system.</td>
</tr>
<tr>
<td>Preservatives*</td>
<td>CO₂</td>
<td>Inexpensive, and effective especially against <em>Pseudomonas</em>.</td>
</tr>
<tr>
<td></td>
<td>Nisin</td>
<td>Inhibits cell growth, and delays spore germination, expensive.</td>
</tr>
<tr>
<td></td>
<td>Other chemical preservatives</td>
<td>Effective but illegal in the US.</td>
</tr>
</tbody>
</table>

*in the US milk packaging must list additive.

...of proteins or fats as their source of energy and carbon (16). Storage temperature of the processed product will further select for organisms best able to grow. Psychrotrophic organisms surviving processing may grow at refrigeration temperatures (4°C).

Psychrotrophic organisms capable of surviving pasteurization are usually members of the families Bacillaceae, Pseudomonaceae, and Achromobacteriaceae and are thought to be responsible for reduced shelf life in milk products. Therefore, the Mosley test has been used to predict milk shelf life based on the total plate count of raw milk (45). However, Bishop and White (1) noted that the predicted shelf life found in the Mosley test does not correlate to the actual shelf life. These observations suggest that factors other than total microbial load are involved in milk spoilage. Additional factors that may play a role in reducing the shelf life that are not accounted for in the Mosley test include heat-stable microbial metabolites such as spores and enzymes.
Processed milk may not contain vegetative organisms but may still have a limited shelf life due to viable spores of *Bacillus* species or heat-stable enzymes produced by many types of Gram negative bacteria during growth in the raw milk, especially from *Pseudomonas* species (Table 4; 34). The Queensland Food Research Institute—Australia characterized 205 lipolytic strains of various bacteria isolated from 36 raw milk samples (52) and found *P. fluorescens* and *P. fragi* (both of which are psychrotrophs) accounted for 63.9% and 31.2% of the bacteria isolated from raw milk. Thus, *Pseudomonas* species dominated the microbiota although species of *Aeromonas, Enterobacteriaceae, Bacillus,* and *Proteus* were also found.
TABLE 4. Characteristics of microbiota isolated from raw milk with a total proteolytic bacteria plate count $<10^5$ cfu/ml (34).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Psychrotrophic (cfu/ml)</th>
<th>Non-psychrotrophic (cfu/ml)</th>
<th>UHT tolerant products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter</em> /</td>
<td>165 (65%*)</td>
<td>44 (17%*)</td>
<td></td>
</tr>
<tr>
<td><em>Moraxella</em></td>
<td>1</td>
<td>3</td>
<td>Protease</td>
</tr>
<tr>
<td><em>Aeromonas</em></td>
<td>15</td>
<td>6</td>
<td>Protease, Lipase</td>
</tr>
<tr>
<td><em>Alcaligenes</em></td>
<td>8</td>
<td>4</td>
<td>Protease, Lipase</td>
</tr>
<tr>
<td><em>Chromobacteria</em></td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter</em></td>
<td>30</td>
<td>8</td>
<td>Protease, Lipase</td>
</tr>
<tr>
<td><em>Flavobacteria</em></td>
<td>7</td>
<td>5</td>
<td>Protease, Lipase</td>
</tr>
<tr>
<td><em>Gemella</em></td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>93</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><strong>Gram positive</strong></td>
<td>19 (8%*)</td>
<td>24 (10%*)</td>
<td></td>
</tr>
<tr>
<td><em>Aerococcus</em></td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus</em></td>
<td>6</td>
<td>6</td>
<td>Spores</td>
</tr>
<tr>
<td><em>Micrococcus</em></td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* percent of the total bacterial population
Enzymes in Milk

Bacterial enzymes, especially lipases and proteases produced by *P. fluorescens* and *Pseudomonas fragi*, survive milk processing and may cause flavor changes in milk stored for long periods (13, 57, 58). UHT milk is commonly held for long periods at room temperature, which is near optimum temperature of activity for these enzymes (57). The cumulative effect of low concentrations of enzyme and long holding periods can have a large impact on the physical condition and flavor of the stored UHT milk (40). Therefore, effort must be made to minimize the presence of heat-stable enzymes in raw materials going into long-shelf-life products. Furthermore, any assays used to detect these enzymes must be sufficiently sensitive to detect low levels of enzymes that can impart off-flavors during the storage time. Extensive work to characterize and control these enzymes (13, 19, 25, 33, 34, 57, 58, 59) shows the need for low psychrotrophic counts and good refrigeration of milk before processing, especially since the most prevalent psychrotroph in raw milk is *Pseudomonas*.

*Lipase*. Milk is a good medium for microbial lipase production (12) and lipolytic activity (35). Most known lipases are stimulated by adsorption to water/fat interphases, causing conformational changes in the enzyme, and exposing the active site (63). In milk, the fat globule membrane stabilizes the interface protecting against lipases, but is weakened during homogenization when serum proteins and caseins are adsorbed. Lipase activity is greatest immediately after homogenization (plateaus), but can be reactivated by a second or third homogenization (10). Lipase activity is also stimulated by calcium and magnesium ions and low levels of NaCl contributing to activation in milk (10, 15).

Flavor defects in milk associated with lipase activity are due to release of free fatty acids (FFAs) from the milk fat. Free fatty acids with chain lengths of C₄ to C₈ are associated with rancid flavors; C₁₀ to C₁₂ are associated with unclean, soapy flavors; and
C₁₄ to C₁₈ contribute little to flavor changes (58). Released FFAs are also susceptible to chemical oxidation to aldehydes and ketones that lead to cardboardy, oxidized, or metallic flavors (58). Detection thresholds for short chain FFAs are lower than those for longer chain FFAs, which is why rancid flavor predominates in milk spoiled by lipase activity (10).

Various methods to assay residual activity lipases in milk have been developed. However, most are too slow or lack the robustness and ease required for widespread factory use. Results from an assay should be obtained within a few hours if they are to be used to direct high-quality milk during processing. Deeth et al. (11) developed an extraction-titration method that measures the total FFAs released and correlated the amount of titratable FFAs to milk quality. This method is time consuming and does not lend itself to routine testing or automation. To improve the speed and ease of lipase detection, a fluorimetric method was applied to milk by Stead (56). This assay measures release of fluorescent 4-methylumbelliferone from an emulsion of oleate conjugated to non-fluorescent 4-methylumbelliferyl oleate. Stead noted this method was deficient when measuring opaque solutions, limiting its use in milk. de Monpezat (37) noted that 4-methylumbelliferyl oleate is unstable, limiting its usefulness in industry. The fluorescent substrate, umbelliferone oleate, offered as a stable alternative is not commercially available.

A colorimetric assay developed by McKellar (35) measures the release of β-naphthyl from β-naphthol caprylate as the colorless ester is hydrolyzed and a purple color develops. This procedure requires a quenching step with centrifugation and a secondary dye making it cumbersome for routine testing and offering little advantage over the extraction method of Deeth and associates.

Diffusion plate assays using tributyrin agar and butterfat agar are currently used in industry (51). These methods are simple to perform and interpret but lack sensitivity required to detect lipase activity in fresh milk and require long incubation periods (24 h to
48 h) to detect lipolysis. Even though this assay is used by industry, it does not meet industry needs for UHT products. To develop a rapid assay that is suited to milk, Richardson et al. (46) used reflectance colorimetry and tributyrin to monitor reduction of turbidity or change in pH. This method is able to measure pancreatic lipase activity rapidly, but the detection limit is too high for general use in thermally processed products.

Proteases. Many extracellular proteases from different bacteria are active after UHT processing and will therefore be of concern in long-shelf-life milk products. These proteases contribute to bitter flavors due to short peptides from enzymatic breakdown of milk proteins (19, 29) and age gelation due to their activity on α- and β-caseins (19, 24). However, age gelation does not occur before 60 to 80 d in milk stored at 7°C and is therefore of little concern in pasteurized or ESL milk.

*Pseudomonas* MC60, a raw milk isolate that is killed during pasteurization, produces a protease that is 4000 times more heat resistant than spores of *B. stearothermophilus*. This illustrates the heat tolerance of some microbial enzymes found in milk, even though the organisms that produce these enzymes are often killed by pasteurization (13). The most prolific protease producers found in milk are members of the genus *Pseudomonas*, in particular *P. fluorescens*. Although the optimum growth temperature of *Pseudomonas* is 30 to 40°C, the optimum conditions for protease production are at refrigeration temperatures and neutral pH, which describe conditions found in raw milk (12). To quantitate the levels of these proteases released into milk, several assays have been developed.

The most widely used assay involves labeling the amino terminal groups released from the action of proteolytic activity over a known period with o-phthalaldehyde (OPA) and β-mercaptoethanol (6). The OPA absorbs strongly at 340 nm and can be related to a standard curve for quantification of proteolysis. More recently, a method utilizing the degradation of Luciferase (the protein responsible for catalyzing the ATP/Luciferin light reaction) has been developed and is commercially available (47). An enzyme linked
immunosorbent assay (ELISA) specific to a protease from *P. fluorescens* has been developed but does not correlate with protease activity in milk during storage (8).

**Spores in Milk**

*Prevalence.* Some *Bacillus* species found in raw milk can survive, grow, and form spores that will survive heat processing conditions used with milk. The spore’s resistance to thermal destruction is primarily due to the low water content of the core, but also because of the heavy wall that protects the core, which is comprised of the cortex and spore coat. Psychrotrophic, sporeforming, spoilage organisms were first reported in milk by Grosskopf and Harper in 1969 (20), and since then many researchers have investigated spoilage of refrigerated milk products by sporeforming bacteria (14, 20, 28, 44, 64, 65). The primary source of spores in milk is contamination from silage. When a cow eats silage, spores enter the digestive tract, and although no growth occurs, they are found in the dung ten times more concentrated (55). Small traces of dung on the teats of the cows, which occurs even in modern dairies, lead to contamination of raw milk (55).

Burton (4) found spore counts in milk around the world to be variable depending on the climate and region (Table 5). *Bacillus* species, especially *Bacillus circulans*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus megaterium*, and *B. stearothermophilus*, dominate species found in milk (23, 36, 44, 48, 61, 65), some of which will grow at 10°C (54). *Bacillus stearothermophilus* is thermotrophic and, therefore, of less importance in refrigerated milk since it will not grow at refrigeration temperatures. However, *B. stearothermophilus* can grow in shelf-stable milk products exported to tropical regions without refrigeration (Personal comm., J. Manners, Bonlac Foods, 1995).

Spores from *B. stearothermophilus* are often used as indicators for heating systems because of their high heat tolerance. Recently a yet unidentified *Bacillus* species that is similar to *Bacillus badius* has been reported to survive UHT-processing in milk, flavored
<table>
<thead>
<tr>
<th>Country</th>
<th>Samples</th>
<th>Spore Count (cfu/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td></td>
</tr>
<tr>
<td>Barbados</td>
<td>5</td>
<td>&lt;1</td>
<td>&gt;22,000</td>
<td></td>
</tr>
<tr>
<td>Chile</td>
<td>5</td>
<td>28</td>
<td>1,500</td>
<td></td>
</tr>
<tr>
<td>Kenya</td>
<td>4</td>
<td>3</td>
<td>1,060</td>
<td></td>
</tr>
<tr>
<td>Iran</td>
<td>5</td>
<td>4</td>
<td>580</td>
<td></td>
</tr>
<tr>
<td>India</td>
<td>5</td>
<td>1</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>Lebanon</td>
<td>3</td>
<td>2</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Madagascar</td>
<td>5</td>
<td>&lt;1</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Pakistan</td>
<td>5</td>
<td>&lt;1</td>
<td>&gt;22,000</td>
<td></td>
</tr>
<tr>
<td>Senegal</td>
<td>2</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>England</td>
<td>0</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ohio, USA</td>
<td></td>
<td></td>
<td>&gt;25</td>
<td></td>
</tr>
</tbody>
</table>
milk, cream, and milk powder in many dairies across Europe (22). The organism is a mesophilic sporeformer with a $D_{147} = 5$ sec, which describes a single log reduction under severe UHT-processing conditions. Presence of this organism may not be easily detectable by the consumer since it can grow to $10^5$ cfu/ml without changing milk composition or sensory properties. However, this organism has led to the closing of a UHT plant and a dairy in Italy due to a new milk ordinance (EC-Directive 85/397) that sets a maximum colony count in UHT milk of 100 cfu/ml after 15 d at 30°C. There have been 52 confirmed cases of this organism in UHT milk reported across Europe, including Germany, France, Italy, Benelux, and Spain, and two cases outside of Europe (22).

**Bacterial Spore Detection.** No rapid assay currently exists that allows milk to be quickly assayed for the presence of less than $10^4$ cfu/ml of a specific spore before milk processing. If such a test existed, it would allow selection of high quality (i.e. low spore count) milk for use with long storage products. Current methods of detection involve heating the milk sample at 80°C for 10 to 15 min to destroy viable cells, then incubating the sample on nutrient agar at an appropriate temperature for 24 to 48 h before counting colonies. This method does not differentiate between spore species, which is of great importance to the milk industry since not all types of spores will survive process conditions and grow in milk. Additionally, this method relies on the assumption that all spores survive the heat shock and that all spores are immediately culturable. Additionally, multiple incubation temperatures are required since multiple species are present. To address the need for a rapid spore assay, Chang and Foegeding (5) developed a immunoassay for spores from *Bacillus*. The antibody used in this assay cross-reacted with all *Bacillus* spore types tested to varying degrees, although no other genera were tested, resulting in an assay with a sensitivity of $>10^6$ cfu/ml.

The optimum spore assay would have the following characteristics:
1. **Speed.** Result must be obtained in less than a few hours so that milk can be directed to process type. Longer times are expensive due to refrigeration costs of holding milk, and will allow growth of psychrotrophic organisms in milk, further spoiling the raw product. ELISA assays typically take less than an hour.

2. **Specificity.** Immunosorbent assays are typically species specific. Care must be taken to select antibody preparations that are specific to only the species of interest.

3. **Ease and robustness.** If the assay is to be performed in industrial settings, it should be simple and of use in many sample backgrounds.

4. **Low cost.** Cost of the assay and detection equipment is also an important factor although ELISA are already widely accepted in industry indicating that they can be acceptably priced.

The use of immunosorbent assays to quantify specific antigens is widespread in the biological and medical sciences. Recent developments have focused on increasing ELISA sensitivity and reducing detection time (26). These developments include:

1. **Labels.** The most popular label is still the enzyme label due to the amplification of signal through the turnover of the substrate. Focus of new label design is on luminescent and fluorescent labels due to increased signal detection.

2. **Enzymes.** New enzymes are being selected based on turnover and thermal stability, although suitable substrates are not yet available for their use.

3. **Signal amplification.** The avidin-biotin complex (ABC) system is commercially available and widely used. A recent development of this is the multilayered enzyme complex enhancement system (66). This
system allows a further 10-fold amplification of signal on top of the ABC system. Briefly it is a second (or third, etc.) ABC amplification of antibodies directed at the primary reporter enzyme. Detection limits of 0.6 to 1 zmol of alkaline phosphatase (500 molecules), corresponding to 17 fmol/L, were reported in a sandwich ELISA of proinsulin (26).

Cascade type reactions have also been developed. For example, the alkaline phosphatase dephosphorylation of NADP to NAD can be coupled to a two enzyme cascade using alcohol dehydroginase catalyzing NAD→NADH and then reoxidized to NAD by diaphorase. In this last reaction 4-iodonitrotetrazolium is converted to a colored formazan dye. This reaction has been applied to several detection systems allowing an alkaline phosphatase detection limit of 11 zeptomoles (7).

4. Immunomagnetic concentration of antigen. Immunomagnetic particles have been used to concentrate *Escherichia coli* O157 (17, 30, 41, 67), *Salmonella* (2, 27, 32, 53), *Listeria monocytogenes* (53), and *Vibrio parahaemolyticus* (62) from food samples. Most of these methods use plate count methods to quantitate the number of bound cells, which, although allowing for significant time reductions over traditional assays, still require 24-h incubation. Krusell and Skovgaard (27) and Gundersen et al. (21) used immunoassays for the detection of immunomagnetic-captured cells, although they still used an incubation step to amplify cell numbers. Most authors report an increase of 100-fold in their sensitivity using immunomagnetic capture of target organisms.
5. Sensors. Several biosensor systems have been developed (3), the most common employing electrochemical transducers. These include potentiometric, amphometric, capacitance, conductance, and piezoelectric devices. Thermal, paramagnetic, and optical devices have also been developed. Most of these devices are still in development stages and often require specialized equipment which limits their widespread use.

Although cost, availability, and the need for specialized staff may rule out some of these advances in immunodetection, some could easily be used to modify existing methods, for example, the use of better labels, faster reporter enzymes, or more sensitive detectors.

Good quality raw milk is important for the production of any processed milk but is critical for production of milk that will be stored for long periods such as ESL and UHT milk. If poor quality raw milk is used, heat-stable enzymes such as lipases and proteases may cause sensory defects in processed milk, or worse, heat-stable pathogens, such as *B. cereus* spores, will be present and survive to cause food poisoning outbreaks.

REFERENCES


CHAPTER III
SENSORY AND MICROBIAL QUALITY OF MILK
PROCESSED FOR EXTENDED SHELF LIFE
BY DIRECT STEAM INJECTION

FOREWORD

Sub-UHT conditions have been proposed for the production of ESL milk by the Australian dairy industry. Studies to determine the effects of temperature on milk processing are necessary before ESL milk can be considered to be safe for consumption and marketable. This chapter describes the effect of heat-processing milk at temperatures below those typically used for UHT processing of milk on residual milk microbiota, residual enzyme activity, and shelf life defined by sensory characteristics over 60 d. Questionnaires for sensory panels used in this study are included in Appendix B.

A concern with low processing temperatures is the survival of heat-tolerant microorganisms, the most heat-stable of which are the sporeforming genera Bacillus and Clostridium. To improve the efficiency of thermal processing of milk, we initially investigated the use of inline ultra-sonication to hydrate the spore core and lower its thermal resistance. Compression waves caused by sonication cause temporary openings in cell walls of microorganisms and allow water to be forced into a cell. The synergistic effect of ultra-sonication and thermal processing when combined has been described, but the efficiency of ultra-sonication drops as the temperature approaches boiling due to cavitation. However, these experiments were carried out at atmospheric pressure. We proposed that sonication of milk could be done at temperatures greater than the atmospheric boiling point if the pressure were sufficiently high. Direct steam-injection UHT processing heats milk at

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1 Coauthored by M. R. Blake, D. J. McMahon, P. Savello, and B. C. Weimer. Reprinted with permission from Journal of Food Protection. Copyright held by the International
140 to 150 psi, which allows superheating of milk and may allow sonication up to temperatures required to kill spores. To investigate this, a flow-through sonication cell was inserted into the holding tube of a pilot-scale direct steam injection UHT plant so that the holding time remained 4 s. *Bacillus stearothermophilus* spores were added to milk to be processed; however, problems with the sonicator caused by overheating stopped trials before useful results could be obtained. These experiments have not yet been repeated.

**ABSTRACT**

Heat treatments of milk between 100 and 145°C produce a new type of product with a shelf life of 15 to 30 d at 7°C, which is termed ESL milk. Little information is available on the safety, sensory qualities of this product. Extended shelf life milk is being processed commercially to expand the distribution area of fluid milk products. After arrival, this product still has the shelf life of a pasteurized product. Milk was processed by direct steam injection at temperatures between 100 and 140°C for 4 and 12 s. Holding time did not significantly affect the sensory quality of the milk. A trained taste panel found cooked flavor and other off-flavors varied significantly with increasing processing temperature and storage time. There were no significant differences noted in cooked or off-flavors between 132 and 140°C. Psychrotrophic *Bacillus* species were isolated from milk processed at and below 132°C, but no organisms were isolated from temperatures at or above 134°C. Consumer preference panels indicated consumers preferred milk processed at 134°C for 4 s to UHT-processed milk although there was a slight preference for HTST milk compared to milk processed at 134°C for 4 s. Higher temperatures had a less destructive effect on lipase activity, and storage time did not significantly affect lipase activity.

Association of Milk, Food and Environmental Sanitarians, Inc. See Appendix A for copyright clearance.
INTRODUCTION

Pasteurized milk typically has a 14-d shelf life at refrigeration temperatures with minimal flavor changes due to spoilage. Ultra-high temperature (UHT) processing (6, 26) allows milk to be stored at room temperature for 1 to 2 yr without microbial spoilage. However, increased temperature treatments used in UHT processing cause cooked or caramelized flavors due to Maillard browning reactions (22), which are objectionable to many US consumers but not to consumers in many other countries. UHT milk processed using direct steam injection results in less-cooked flavors compared to indirect heating but does not eliminate this defect (5). To further reduce cooked flavor, extend the shelf life beyond 14 d, and expand the transport capabilities of milk without spoilage, milk is being commercially processed around the world at temperatures above pasteurization, but below UHT processing, and has been termed ESL milk.

Heat treatments between 100 and 145°C produce ESL products with a shelf life of 15 to 30 d at 7°C, but little information is available on the safety, sensory qualities, or absolute shelf life of such milk. Franklin et al. (17), Griffiths et al. (18), and Guirguis et al. (20) demonstrated bacterial spores withstand these temperatures and are activated with heat treatments used in ESL processing. Conversely, Campylobacter, Yersinia enterocoliticia, E. coli O157:H7 (11), and heat shocked L. monocytogenes (4) are destroyed by pasteurization.

Heat-stable enzymes are associated with the accumulation of degradation products in long-shelf-life products leading to spoilage (27). Lipase isolated from P. fluorescens strains is thermostable to heat treatments used in pasteurization (14, 15, 16, 21), associated with milk proteins and the fat globule membrane (8), and contributes to the release of FFAs during storage (7, 13). Microbial survival, residual enzyme activity, and flavor must be considered in ESL dairy products. This chapter describes the effect of sub-UHT treatments
on milk microbiota, residual enzyme, and sensory characteristics activity over 60 d of storage at 7°C.

MATERIALS AND METHODS

Processing Conditions

All processing was done using an Alpha-Laval (Lund, Sweden), direct heating pilot-scale Sterilab® with Grade A raw milk, except where otherwise noted. Preheat temperature was 78°C for all processing trials (Table 6). Processing began by sterilizing the Sterilab® unit and discarding approximately 200 ml of processed milk before sample collection. Milk was packaged in 120-ml sterile plastic containers with approximately 10 ml of headspace and into 20-ml screw cap test tubes in a HEPA filtered, positive pressure laminar flow cabinet (Alpha-Laval Stericab®, Lund, Sweden). Previous to sample collection, the laminar flow cabinet was washed with 70% ethanol and allowed to dry. Before sample collection, sterile latex gloves were washed in 70% isopropyl alcohol followed by a foam sanitizer containing 62% ethyl alcohol.

Samples were stored in a 7°C incubator and tested for microbiological growth at 15-d intervals for 60 d. Commercially produced UHT whole milk control samples that had been processed by an indirect plate exchange process and packaged in 1-L brick pack containers were purchased from Gassner Foods Inc. (Logan, UT). High temperature, short time (HTST) pasteurization control samples, obtained from the Gary H. Richardson dairy processing laboratory at Utah State University (USU), were processed at 74°C for 16 s and packaged in half-gallon, gable top containers.

Sensory Evaluation

Test Conditions. Sensory evaluation was done in a sensory laboratory with individual booths under red lighting to mask slight differences in the color of the samples. Milk samples were presented at 20 ± 1°C to maximize sensitivity of panelists (1). Panelists were
TABLE 6. Processing conditions and sensory evaluation techniques used in this study.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Holding Time (s)</th>
<th>Process Temperature (°C)</th>
<th>Initial aerobic bacterial count (CFU/ml)</th>
<th>Sensory Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total plate count</td>
<td>Coloform count</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>120, 130, 140</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>4, 12</td>
<td>100, 110, 120, 130, 140</td>
<td>5.0 x 10^3</td>
<td>1.8 x 10^2</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>120, 122,* 124, 126,* 128, 130,* 132, 134,* 136, 138,* 140</td>
<td>7.4 x 10^3</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140</td>
<td>4.4 x 10^8</td>
<td>3.2 x 10^2</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>134</td>
<td>4.1 x 10^4</td>
<td>&lt;30</td>
</tr>
</tbody>
</table>

*These conditions were not tested in sensory evaluations of this trial.

nd = not determined
instructed not to swallow samples, and to rinse with water between samples. All samples were coded with random three-digit numbers and positioned on a serving tray using a balanced block design.

*Trained Sensory Panels.* Sixteen panelists, students and faculty of the Department of Nutrition and Food Sciences, USU, were selected on the basis of low identification threshold of cooked, sweet, rancid, fruity, and bitter flavors. Panelists were dismissed if they were not able to detect:

1. Cooked flavor in HTST milk boiled for 10 min,
2. Rancidity in HTST milk containing 75 ppm butyric acid,
3. Fruitiness in HTST milk 50 ppm powdered strawberry essence,

Selected panelists were trained to identify and record flavor characteristics with a mark on quantitative descriptive analysis line scales (examples of questionnaires are included in Appendix B). The distance from the beginning of the line scale to the panelist’s mark was measured (mm) and recorded as the descriptive score and later used for statistical analysis. A measure of 20 mm represented no defect and 100 mm represented extreme flavor defect.

Duplicate samples from milk processed at 120, 130, and 140°C for 4 and 12 s were stored at 7°C, and five sets of taste panels were conducted at 15-d intervals over 60 d to determine flavor differences associated with process holding time. Judges were asked to indicate intensity of cooked flavor, sweetness, rancidity, fruity, bitter, and other off-flavors on a quantitative descriptive analysis line scale from none to extreme. These panels were repeated using samples from milk processed in trial 3 (Table 6) to determine flavor differences associated with process temperature.

*Consumer Taste Panels.* Consumer taste panels consisted of 100 to 130 untrained panelists from USU students and faculty and Logan residents with a minimum age of 16
years. Judges were presented with five randomly coded processed milk samples, a UHT reference sample, and a HTST reference sample. Each judge was asked to compare the flavor of each sample on a line scale to the two references. This procedure was employed for five different milk samples using the same two blocked reference samples of UHT and HTST milk. The distance from the beginning of the line scale to the panelist’s mark was measured (mm) and recorded as the descriptive score and later used for statistical analysis.

Consumer preference evaluations involved comparisons of fresh commercial HTST milk to milk processed at 134°C for 4 s and stored for 15, 30, and 60 d at 7°C. Panels were conducted as paired preference tests using untrained judges. Judges were asked which they preferred and why they preferred that sample. At 60 d a hedonic scale was also used to rate acceptability with UHT milk included as a reference.

**Statistical Analysis**

Sensory results were analyzed by the analysis of variance (ANOVA) method using the JMP-SAS (SAS Institute, Inc., Cary, NC) computer software. Statistical significance at $\alpha = 0.05$ was used except where otherwise noted. Least squares difference tests were used to determine significant difference between means.

**Microbiological Assays**

*Total Plate Count.* A total count was done using the method of Zmarlicki et al. (32) as described by Standard Methods for the Examination of Dairy Products (28). Raw milk (200 µl) was mixed with 50 µl of Medium A (Wescor, Inc., Logan, UT; nutrient broth containing triphenyl tetrazolium chloride as a growth indicator) in sterile microtiter wells. Duplicate plates were sealed with sterile microtiter plate tape and incubated at 30°C for 24 h and monitored at 30-min intervals for development of red color (a*) in an automated reflectance colorimeter (Omnispec® 4000 bioactivity monitor; Wescor, Inc., Logan, UT). All positive samples were retested using a standard total plate count (28).
Total Psychrotrophic Count. Automated psychrotrophic counts were done according to the method of Zmarlicki et al. (32) as described by Standard Methods for the Examination of Dairy Products (28). Raw milk (200 µl) was mixed with 50 µl of Medium A (Wescor, Inc., Logan, UT) in sterile microtiter wells. Duplicate sealed plates were preincubated for 48 h at 7°C before transferring them to 30°C for 24 h to be monitored at 30 min intervals for development of red color (a*) in an automated reflectance colorimeter (Omnispec® 4000 bioactivity monitor; Wescor, Inc., Logan, UT). All positive samples were retested using a standard psychrotrophic total plate count (28).

Total Coliform Count. Coliforms were counted using the method of Zmarlicki et al. (32) and Yuan (31) as described by Standard Methods for the Examination of Dairy Products (28). One hundred microliters of Media C (Wescor, Inc., Logan, UT) was mixed with 100 µl of milk in sterile microtiter wells. Duplicate sealed plates were incubated at 30°C for 24 h and monitored at 30-min intervals for development of yellow color (b*) in an automated reflectance colorimeter (Omnispec® 4000 bioactivity monitor; Wescor, Inc., Logan, UT). All positive samples were tested on violet red bile agar using a standard coliform plate count (28).

Spore Count. Spores were counted according to the method described in Standard Methods for the Examination of Dairy Products (28). Samples were heated to 80°C for 15 min then grown aerobically on total plate count agar. Mesophilic counts were incubated at 30°C for 24 to 48 h; thermotrophic at 55°C for 24 h; and psychrotrophic at 7°C for 7 to 10 d.

Identification of Organisms. All post-processing isolates were single colony isolates from total plate count and spore count tests as previously described. Each isolate was grown in nutrient broth overnight, Gram stained for cell morphology, and identified using the Biolog MicroStation®, Version 3.0 (Biolog, Inc., Hayward, CA). Purified cultures were four-zone streaked onto BUGM® agar plus 1% Glucose (Biolog, Inc., Hayward,
CA, #6001) and grown at 30°C (except where strains' growth required different incubation temperatures) for 8 to 16 h. Colonies from the fourth zone were diluted into 0.85% saline to an OD$_{590}$ = 0.2. Cell suspensions were dispensed into a Gram-positive identification plate (Biolog, Inc., Hayward, CA) and incubated at 30°C for 4 to 24 h. Results were recorded as positive, negative, or borderline and entered into the Biolog MicroStation® database for identification.

**Heat-Stable Enzymes**

**Lipase Assay.** Lipase activity was done using chromogenic substrates of $p$-nitrophenyl ($p$-NP) esters in a method similar to that described by Blake and Weimer (2, 3) and Dias and Weimer (12). Briefly, 80 µl of 50 mM sodium phosphate buffer, pH 7.2 and 20 µl of 5.0 mM $p$-nitrophenyl caprylate ($p$NP-C) dissolved in dimethyl sulphoxide were mixed with 100 µl of milk in sterile 96-well microtiter plates. Duplicate assays were sealed with sterile microtiter plate tape and monitored for development of yellow color ($b^*$) every 5 min for 10 h at 37°C in an automated reflectance colorimeter (Omnispec® 4000 bioactivity monitor; Wescor, Inc., Logan, UT).

**Protease Assay.** Proteolytic activity was measured using the method of Church et al. (9). The method involves labeling the amino terminal groups released from the action of proteolytic activity over a known time period with OPA. The OPA adduct absorbs strongly at 340 nm and can be related to a standard curve for quantification of proteolysis.

**RESULTS AND DISCUSSION**

**Microbiology**

Milk with a high initial total plate count (4.4 x 10$^8$ cfu/ml) was processed to simulate the processing of poor quality milk (Table 6). Bacterial counts decreased significantly as processing temperatures increased (Table 7). No organisms survived 134 to 140°C process temperatures (Table 8). Coliforms were not isolated immediately after processing at 100°C
Table 7. Average microbiological counts on processed milk from trial 2 comparing process temperatures.

<table>
<thead>
<tr>
<th>Storage time (7°C)</th>
<th>Coliform Count (CFU/ml)</th>
<th>Psychrotrophic Count (CFU/ml)</th>
<th>Total Plate Count (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Process temp (°C)</td>
<td>Process temperature (°C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 110 120</td>
<td>100 110 120 130 140</td>
<td>100 110 120 130 140</td>
</tr>
<tr>
<td>Raw count</td>
<td>1.8 x 10²</td>
<td>1.7 x 10³</td>
<td>5.0 x 10³</td>
</tr>
<tr>
<td>0 d</td>
<td>&lt;10</td>
<td>10 35 &lt;10 &lt;10 &lt;10</td>
<td>&lt;10 &lt;10 &lt;10 &lt;10 &lt;10</td>
</tr>
<tr>
<td>15 d</td>
<td>10⁷</td>
<td>10⁶ 10⁶ 20 &lt;10 &lt;10</td>
<td>10⁷ 10⁶ 10³ &lt;10 &lt;10</td>
</tr>
<tr>
<td>30 d</td>
<td>10⁴</td>
<td>10⁷ 10⁵ 10³ &lt;10 &lt;10</td>
<td>10⁶ 10⁵ 10² &lt;10 &lt;10</td>
</tr>
</tbody>
</table>
TABLE 8. Number of samples (120 ml containers) found to contain psychrotrophic, mesophilic, and thermotrophic spores from milk processed for 4 s using direct steam injection from trial 3.

<table>
<thead>
<tr>
<th>Process temperature (°C)</th>
<th>7°C incubation (psychrotrophic)</th>
<th>30°C incubation (mesophilic)</th>
<th>55°C incubation (thermotrophic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>3 of 8</td>
<td>3 of 8</td>
<td>4 of 8</td>
</tr>
<tr>
<td>124</td>
<td>1 of 8</td>
<td>None recovered</td>
<td>3 of 8</td>
</tr>
<tr>
<td>128</td>
<td>None recovered</td>
<td>None recovered</td>
<td>1 of 8</td>
</tr>
<tr>
<td>132</td>
<td>None recovered</td>
<td>2 of 8</td>
<td>None recovered</td>
</tr>
<tr>
<td>134 to 140</td>
<td>None recovered</td>
<td>None recovered</td>
<td>None recovered</td>
</tr>
</tbody>
</table>

for 4 s, but they survived processing and grew to high numbers by 15 d. High total and psychrotrophic counts were obtained from milk processed at 100 and 110°C for 4 s by 15 d (Table 7). Isolates from milk processed at or below 110°C were not identified. Detection of coliforms in 100°C for 4-s heat treatment may be due to contamination during sample collection. However, we suggest that this is not the case since one would expect contamination at other temperatures as well which was not observed.

Four species of psychrotrophic, spore-forming organisms were identified from milk processed between 120 and 132°C and stored at 7°C. Most organisms were isolated after 30 d of incubation. Isolates included *Bacillus insolitus, Bacillus cereus/thuringiensis, B. coagulans,* and *B. licheniformis. Bacillus licheniformis* was isolated from milk containing > $10^8$ cfu/ml that was processed at 120 and 122°C and stored for 30 d. There was no bacterial growth during the 60-d storage period associated with any of the other processing conditions used in this study.
Presence of *B. insolitus* (a psychrophilic spore-former) in the raw milk reflects the location of Logan, which is located at 1368 m with an average mean-daily winter temperature of -2.2°C (Climate Center, USU, Logan). Optimum growth temperature for *B. insolitus* is between 0 and 10°C (29); however, *B. insolitus* is not a prime candidate for growth in ESL milk because it has no β-galactosidase activity and has limited ability to metabolize other carbon sources (29). *Bacillus cereus/thuringiensis* and *B. coagulans* were also isolated from samples processed ≤128°C. *Bacillus cereus* spores are widespread and commonly found in milk and cream products (10). It is considered a food pathogen and 11 to 89% of the strains grow at 10°C but not at 5°C (29), suggesting this organism may be a pathogen in ESL products. *Bacillus coagulans* spores are scarce in soil, but they are common in silage and have been reported in raw milk (24, 25) and processed milk stored at 4°C for 28 d (19). Growth is inhibited at 10°C (29), but we isolated colonies from psychrotrophic plates incubated at 7°C. Samples containing *B. coagulans* had psychrotrophic plate counts between 50 and 150 cfu/ml, indicating limited growth during storage for 60 d at 7°C.

*Bacillus licheniformis* is normally a common mesophilic soil organism and typically does not grow at ≤10°C, but produces heat-resistant spores (29). Although this organism is commonly found in dairy products (30), one would expect limited growth in refrigerated products (29).

**Enzymatic Spoilage**

**Lipolytic Activity.** Process temperature significantly influenced (*P < 0.0001*) residual lipase activity in the processed milk (Figure 1). Our data support the findings of Driessen (13), Fitz-Gerald et al. (15), and Kumura et al. (21), who demonstrated increased processing temperatures are less destructive to thermostable lipase of *Pseudomonas*. The level of lipase in all trials was observed to be near the detection limit of the assay (2, 3), which lead to the variation in observed lipase activity.
Figure 1. Least squared mean of residual lipase activity of milk processed for 4 s by direct steam injection at various temperatures. Error bars represent the standard error of the mean.
Storage time did not have a significant effect on lipase activity ($P = 0.62$), which remained constant at $2.2 \Delta b^*/h$ through day 60. Choi and Jeon (7) found release of free fatty acids to continue during storage of UHT milk containing residual lipase. These suggest that once lipase is present it will remain active during long storage periods at refrigeration temperatures, suggesting that raw milk containing heat-stable lipase may not be suitable for processes that are intended for ESL or UHT milk processing.

Proteolytic Activity. No significant protease activity was noted in any of the process temperatures.

Trained Sensory Evaluation

ANOVA tables describing the trained panel sensory data are tabulated in Appendix C. Examples of questionnaires used in these panels are listed in Appendix B.

Temperature Effect. Trained taste panelists perceived cooked flavor to be the dominant flavor change associated with the processing conditions (Figure 2). Cooked flavor increased from $120$ to $128^\circ C$ then remained constant to $140^\circ C$. Other off-flavors were more noticeable at processing temperatures below $128^\circ C$ than at processing temperatures above $132^\circ C$. There was no significant interaction between cooked flavor and sweetness (see table of correlations, Appendix C), which is known to occur in Maillard browning reactions (22). Rancid flavor was significantly less in processed milk samples than the HTST sample; however, rancid, fruity, and bitter flavors, which are also commonly associated with microbial and enzyme spoilage, did not change significantly between process temperatures.

Storage Effect. The most pronounced effects of storage time on sensory qualities in milk (Figure 3) were decreased cooked flavor ($P < 0.0001$) and increases in other off-flavors ($P < 0.0001$). Initially, other off-flavors may have been masked by intense
Figure 2. Least squared mean of the flavor intensity of milk processed at different temperatures by direct steam injection and stored at 7°C over 60 d. Samples were judged by a trained taste panel at 15-d intervals using quantitative descriptive analysis with a value of 20 mm being associated with no defect and 100 mm being associated with extreme flavor defect. Error bars represent the standard error of the mean. An example questionnaire is listed in Appendix B; ANOVA and correlation tables are listed in Appendix C.
cooked flavors. The increase in ratings for other off-flavors after cooked flavors stabilized suggested that other chemical changes occurred. Some panelists described these other off-flavors as “unclean” and “stale,” possibly due to rancidity from the slow accumulation of thermotolerant lipases produced by psychrotrophic microorganisms in raw milk before processing. These flavors have been attributed to free fatty acids with chain lengths of C_{10} to C_{12} (23). Rancid, fruity, and bitter flavors often attributed to enzyme activity did not change significantly. Although formal taste panels of this milk were conducted up to 60 d, the milk was held at 7°C and tasted every month by a few panelists who judged the milk as acceptable at 240 d.

**Consumer Sensory Evaluation**

Results from the consumer discrimination panel determined milk processed at 120 to 140°C for 4 s by direct steam injection (Table 6) had a flavor between that of HTST and UHT milk (Figure 4). Analysis of data from panelists who had described processed milk as more like HTST or UHT milk revealed that milk processed at 120 and 130°C tasted significantly more like HTST milk than did milk processed at 140°C. Milk processed at 120°C was still significantly different from HTST milk. A trained taste panel perceived no significant difference in the flavor of milk processed at 120 to 140°C for 4 or 12 s, and stored for 60 d, indicating that either holding time could be used without changing the flavor. Milk processed below 120°C was unacceptable for use in taste panels after 15 d because of high psychrotrophic and total plate counts (Table 7).

The sensory and microbiological data were used to select a set of conditions recommended for ESL processing. Trained panelists thought milk processed at 134°C for 4 s had flavor characteristics more like HTST processed milk when evaluated over 60 d. We conducted a forced choice consumer preference panel to determine whether consumers preferred milk processed at 134°C for 4 s to HTST milk and whether their preference changed after storage at 7°C. Panelists preferred HTST milk to ESL milk (Figure 5). The
Figure 4. Least squared mean of the sensory score of different process temperatures compared to HTST and commercial UHT milk. Samples were judged 24 h after processing by an untrained consumer discrimination panel. HTST-processing conditions were 74 °C for 16 s. Error bars represent the standard error of the mean. Small letter superscripts on the process temperature indicate significant differences in flavor between treatments. Example questionnaire is listed in Appendix B; ANOVA and Fisher pairwise comparison tables are listed in Appendix C.
Figure 5. Least square mean of consumer hedonic rating (1 associated with extreme dislike and 9 associated with extreme like), and consumer preference (ranked with 1 associated with least preferred and 3 most preferred) comparing HTST (74°C for 16 s), ESL (134°C for 4 s, direct steam injection), and commercial UHT (indirect plate-exchange heating) processed milk. Small letters indicate significant differences in consumer acceptance or preference between treatments. Example questionnaire is listed in Appendix B; ANOVA and Fisher pairwise comparison tables are listed in Appendix C.
panelists listed several reasons for disliking ESL milk although no single reason predominated.

A hedonic scale and a ranking test were used to determine the consumer acceptability of ESL milk at 60 d. Consumers significantly preferred HTST milk to ESL milk, but preferred both HTST and ESL to UHT milk (Figure 5). Consumer ranking indicated HTST was equivalent to ESL in consumer acceptance, and HTST and ESL samples were preferred to UHT milk. These data indicate that there were small but perceptible differences between HTST and ESL processed milk; however, consumers prefer either HTST or ESL milk to UHT milk.

This study suggests that ESL milk may be useful in the market place to offer consumers a fluid product that tastes like HTST pasteurized milk, but can be transported under refrigeration to distant locations that do not have a readily available supply of fluid milk. The extended shelf life allows the product to be in transit for many days and still have a typical HTST pasteurization product shelf life after it arrives on location. However, care must be taken during processing to assure that high quality milk is used to reduce the risk of disease, as ample opportunity is present for psychrotrophic pathogens to grow during transport and shelf life. Heat treatments below 134°C for 4 s produced acceptable flavored milk, but the possibility for survival and growth of pathogens exists.

REFERENCES


CHAPTER IV
A SEMIAUTOMATED REFLECTANCE COLORIMETRIC METHOD
FOR THE DETERMINATION OF LIPASE ACTIVITY IN MILK

FOREWORD

To quantitate the activity of lipolysis in milk samples, we initially tried the method of McKellar and Cholette (13), which measures the release of 8-napthol from 8-napthyl caprylate. The method is a stopped reaction requiring a secondary dye, but it is cumbersome to use with many samples. We modified the procedure to allow it to be run in microtiter plates in a reflectance colorimeter but found it unsatisfactory since the chromogen sedimented, leading to an inaccurate estimation of enzyme activity.

The following chapter describes a colorimetric assay based on the release of p-nitrophenol from the colorless pNP-C that we developed to measure the lipase activity in milk.

ABSTRACT

Measurement of heat-stable lipase activity in dairy products relies on methods that are slow or that cannot be used in turbid solutions, which limit their industrial value. A need exists for a rapid, simple, informative assay to detect lipase activity in dairy products. In this work we observed that hydrolysis of p-nitrophenyl esters, monitored by reflectance colorimetry, was linearly correlated to spectrophotometry ($R^2 = 0.93$) and release of titratable FFAs ($R^2 = 0.92$ to .97) indicating that chromogenic substrates were useful in determining lipase activity. However, FFAs, at the concentrations reported in milk, inhibited p-nitrophenyl caprylate hydrolysis leading to an underestimation of lipase activity in milk with previous lipolysis. Milk fat also significantly reduced hydrolysis of the

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1 Coauthored by M. R. Blake, R. Koka, and B. C. Weimer. The copyright clearance and
chromogenic substrates tested but could be accounted for in activity calculations, and a correction equation was developed. To demonstrate the use of this assay, lipase activity in UHT skim milk inoculated with *Pseudomonas fluorescens* AFT36 was followed using reflectance colorimetry and tributyrin agar. Lipase activity increased with cell numbers during 106 h of incubation. Extracellular lipase activity was detected after 10 h of incubation using reflectance colorimetry while tributyrin agar required 28 h. Reflectance colorimetry and chromogenic substrates allowed a rapid, sensitive, and meaningful detection of esterase and lipase activity in milk.

**INTRODUCTION**

The widespread practice of storing cooled bulk milk before collection and again before processing has heightened the importance of psychrotrophic bacteria as a factor in the quality of dairy products. Raw milk is commonly held for 24 to 48 h at 5 to 7°C before processing, which favors growth of psychrotrophic bacteria (11, 12). Raw milk psychrotrophic microflora are typically dominated by *Pseudomonas* species (with *P. fluorescens* predominating), which account for approximately 50 to 75% of the Gram-negative organisms (17). Even though pseudomonads are killed during pasteurization, extracellular lipase and protease produced during growth are thermostable and remain active during storage of finished products (18). Residual lipase activity that led to flavor changes has been found in HTST and UHT milk (11, 17). The long shelf life of UHT-processed dairy products allows slow, but cumulative changes such as off-flavors (18) and age gelation (11). Additionally, the inability of some milk to form a stable foam is attributed to lipolytic activity in milk used to make Cappuccino-style coffee (4).

Various methods have been developed to determine residual activity of heat-stable protease and lipase in fluid and powdered milk; however, those assays tend to be unsuited
for widespread factory use. Deeth et al. (3) developed a method of extraction and titration that measures the release of total FFAs. The amount of titratable FFA is related to milk quality, but this method does not lend itself to routine testing or automation. An assay developed by McKellar and Cholette (13) measures the release of β-naphtol from β-naphtol caprylate. That procedure requires a quenching step with centrifugation and a secondary dye, making the assay slow and difficult for routine use. A sensitive fluorimetric method exists that rapidly determines psychrotrophic lipase activity by measuring the release of 4-methylumbelliferone from an emulsion of oleate conjugated to nonfluorescent 4-methylumbelliferyl oleate (8). Stead (16) noted that the method was deficient in opaque solutions, limiting its use in milk.

Use of spectrophotometry with chromogenic substrates to detect enzyme activity rapidly in clear solutions is well documented (1, 7, 10); however, the food industry often requires enzyme assays to be done in turbid solutions for which spectrophotometry is inadequate. Hydrolysis of colorless p-NP esters releases the corresponding FFA and the yellow chromogen p-nitrophenol, which can be detected using a variety of methods, including spectrophotometry and reflectance colorimetry. Richardson et al. (15) and Yuan (20) exploited reflectance colorimetry to monitor microbial growth in food. Richardson et al. (15) used reflectance colorimetry and tributyrin to measure added lipase activity in milk by monitoring the reduction in turbidity and the change in pH. That method was useful for detection of pancreatic lipase activity, but the detection limit was too high for use with products containing low concentrations of lipase. Dias and Weimer (5) used chromogenic substrates and reflectance colorimetry in milk containing cell free extracts of *Lactobacillus helveticus* CNRZ32. Those investigations highlight the use of reflectance colorimetry in dairy products and suggest these techniques may be useful for detection of lipase activity in dairy products.
In this study, we used reflectance colorimetry to monitor hydrolysis of chromogenic substrates with added commercial lipase and lipase produced by *P. fluorescens* during growth in milk. The effects of milk fat and FFAs on hydrolysis of the substrates were also reported.

**MATERIALS AND METHODS**

**Culture Growth**

*P. fluorescens* AFT36 was grown in 10 ml of nutrient broth (Difco, Detroit, MI) for 16 h at 30°C with aeration in a shaking incubator (200 rpm). The entire culture volume was centrifuged (4100 x g at 4°C for 15 min), washed twice with 10 ml of sterile 0.85% saline, and resuspended in sterile 0.85% saline to A$_{590}$ = 0.2. This preparation was then used to inoculate (0.1% vol/vol) 500 ml of UHT skim milk (Gossner Foods, Inc., Logan, UT), which was incubated at 15°C with agitation (200 rpm) for 106 h. Samples were collected during culture incubation and split for cell count and lipase activity. Sampling was stopped after 50 h of growth in the stationary phase. Cells were counted using the pour plate technique with plate count agar (Difco) and aerobic incubation at 30°C for 24 h.

Estimation of lipase activity produced during growth in UHT skim milk was done using 1.5 ml volumes that were centrifuged (16,000 x g at 4°C for 15 min) to pellet cells, and the supernatant was filter-sterilized with a 0.45-µm syringe filter (model 21053-25; Corning Glass Works, Corning, NY). The filtrate was collected and split for lipase assays using reflectance colorimetry and tributyrin agar.

**Lipase Activity Estimation**

*Chromogenic Substrates.* Stock solutions of pNP-fatty acid esters (Sigma Chemical Co. St. Louis, MO) were dissolved to 5 mM in dimethyl sulfoxide (DMSO) and stored at -20°C. Substrates were thawed, equilibrated to room temperature (25°C), and added to
assays immediately prior to incubation. Esters of p-NP included: butyrate, caprylate, laurate, myristate, palmitate, and stearate.

**Spectrophotometric Assay.** To 50 mM sodium phosphate buffer (pH 7.2) was added 0 to 100 mU lipase/ml; *P. fluorescens* lipase (Fluka-No. 62312; EC 3.1.1.3; Fluka, Ronkonkoma, NY) was used. The pNP-caprylate (pNP-C) was added to a final concentration of 0.5 mM in triplicate assays with a spectrophotometer (DU®-65; Beckman Instruments, Fullerton, CA) in a total volume of 1.0 ml. Release of p-nitrophenol was detected spectrophotometrically (410 nm) every 2 min for 4 h at 37°C using 1.5 ml, UV-rated, disposable cuvettes (Baxter Diagnostic, Inc., Deerfield, IL).

**Color Saturation Determination.** Curves for color saturation were done in 50 mM sodium phosphate buffer (pH 7.2) and UHT skim milk using an automated reflectance colorimeter (Omnispec® 4000 bioactivity monitor; Wescor, Inc., Logan, UT) with p-nitrophenol. The absolute color of solutions containing 0 to 5 mM p-nitrophenol were measured in triplicate at 37°C using the b* color value (blue to yellow) after mixing.

**Reflectance Colorimetric Assay in Phosphate Buffer.** To 50 mM sodium phosphate buffer (pH 7.2) were added 0 to 100 mU of *P. fluorescens* lipase/ml. The p-NP-esters were added to a final concentration of 0.5 mM in duplicate in a 96-well microtiter plate to a total volume of 200 µl. Release of p-nitrophenol was monitored in an automated reflectance colorimeter every 2 min at 37°C for 4 h using b* color units.

**Chromogenic Substrate Concentration Optimization.** To 50 mM sodium phosphate buffer (pH 7.2) were added 1.6 mU of *P. fluorescens* lipase/ml and pNP-C to a final concentration of 0 to 5.0 mM. Assays were mixed in sterile 96-well microtiter plates, to a total volume of 200 µl, and monitored in duplicate for yellow color development (b*) every 10 min for 10 h at 37°C in an automated reflectance colorimeter.

**Reflectance Colorimetric Assay in Milk.** To 100 µl of HTST whole milk containing 0 to 25 mU of *P. fluorescens* lipase/ml were added 80 µl of 50 mM sodium phosphate
buffer (pH 7.2) and 20 µl of 5.0 mM pNP-ester in DMSO (Table 9). Duplicate assays were mixed in sterile 96-well microtiter plates, sealed with sterile microtiter plate tape, and monitored for color development (b*) every 10 min for up to 10 h at 37°C in the automated reflectance colorimeter. If no activity was observed in the first 4 h, then activity was monitored to 10 h.

TABLE 9. Activity and correlation of hydrolysis of butyrate, caprylate, laurate, myristate, palmitate, and stearate esters of p-nitrophenol with titration of FFA. Calculations used means from triplicates.

<table>
<thead>
<tr>
<th>pNP-ester</th>
<th>Ratio of pNP-ester</th>
<th>Correlation$^2$</th>
<th>Ratio of release$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrate (4:0)</td>
<td>4.6</td>
<td>0.954</td>
<td>0.5444</td>
</tr>
<tr>
<td>Caprylate (8:0)</td>
<td>29.4</td>
<td>0.919</td>
<td>1.0073</td>
</tr>
<tr>
<td>Laurate (12:0)</td>
<td>16.7</td>
<td>0.968</td>
<td>0.6441</td>
</tr>
<tr>
<td>Myristate (14:0)</td>
<td>5.2</td>
<td>0.955</td>
<td>0.9639</td>
</tr>
<tr>
<td>Palmitate (16:0)</td>
<td>2.7</td>
<td>0.954</td>
<td>0.7077</td>
</tr>
<tr>
<td>Stearate (18:0)</td>
<td>6.3</td>
<td>0.931</td>
<td>0.3851</td>
</tr>
</tbody>
</table>

$^1$Ratio of p-nitrophenyl-ester (p-NP) concentration added (0.5 mM) to fatty acid in full cream milk (3.6%), if the triacylglycerides were fully hydrolyzed.

$^2$Correlation of reflectance colorimetry to titrated FFA for measuring lipolysis.

$^3$Ratio of p-nitrophenol released during hydrolysis to the titrated FFA liberated in milk.

Reflectance Colorimetric Lipase Activity Calculation. Calculations for lipase activity were similar to those used by Dias and Weimer (5) for aminopeptidase activity (Equation 1). Lipase activity was calculated by subtracting the b* color value of a control containing 0.5 mM pNP-C in the appropriate sample background. These adjusted b* color values were then used to calculate the initial enzyme rate by integration of running means (14).
\[
\text{Adjusted } b^* = \frac{b^*_{[\text{assay}]} - b^*_{[\text{substrate control}]} \times (K_1 - K_2)}{(b^*_{[p-\text{NA standard}]} - b^*_{[\text{water standard}])}} \tag{1}
\]

where \(K_1\) = Mean of >50 1 mM \(p\)-nitroanaline (\(p\)-NA) readings for three colorimeters (20.74), and \(K_2\) = Mean of >50 water readings for three colorimeters (-1.457).

**Tributyrin Agar Diffusion Assay.** Lipase activity in UHT skim milk was determined as described by Fox and Stepaniak (6). The tributyrin agar contained 0.25% tributyrin (Sigma Chemical Co.) emulsified by sonication for 6 min in 1.5% agar (Difco). Each plate contained 20 ml of agar with four 4-mm diameter wells to which 30 \(\mu\)l of sterile supernatant from the inoculated UHT skim milk were added. Lipase activities were determined during aerobic incubation at 30°C for 24 h by measuring the diameter (mm) of the zones of clearing after subtracting the well diameter.

**FFA Titration**

The titration method of Deeth et al. (3) was used to measure the release of FFAs in milk. Briefly, triplicate 10-ml samples of whole milk were incubated with 0 to 3.2 mU of \(P.\) fluorescens lipase/ml for 1 h at 37°C after which 5-ml samples were mixed with 10 ml of extraction mixture (40:10:1, isopropanol: petroleum ether: 4 \(N\) \(H_2\)SO\(_4\)), 6 ml of petroleum ether, and 4 ml of water; the mixture was shaken vigorously for 15 s. The layers were allowed to separate for 10 min at room temperature, and 5 ml of the upper layer was then titrated with 0.02\(N\) methanolic KOH using 50 \(\mu\)l of methanolic phenolphthalein as an indicator. The FFA content was determined using the formula

\[(\text{TN} / \text{PV}) \times 10^3,\]

where \(T\) is the net titration volume, \(N\) is the normality of the methanolic KOH, \(P\) is the proportion of the upper layer titrated (i.e., volume of aliquot withdrawn divided by the total volume of upper layer), and \(V\) is the volume (in milliliters) of the original milk.
Inhibition of Lipase Activity by FFAs

L-Isomers of laurate, myristate, palmitate, stearate, and oleate (Sigma Chemical Co.) were dissolved in DMSO to 5 mM. Each fatty acid was added to 50 mM sodium phosphate buffer (pH 7.2) to a final concentration of 0 to 5 mM, containing 0.625 mU (final concentration) *P. fluorescens* lipase/ml, and pNP-C at a final concentration of 0.5 mM. Microtiter plates were sealed and monitored for yellow color development every 10 min for 10 h at 37°C in the automated reflectance colorimetry.

Influence of Milk Fat on Lipase Activity

Grade A pasteurized skim milk was autoclaved (121°C for 15 min) to destroy milk lipase and minimize residual bacterial lipase activity; some milk caramelization was observed. UHT cream was added to sterile skim milk at 0 to 4.0 % milk fat and homogenized. *P. fluorescens* lipase was added to 50 mM phosphate buffer (pH 7.2) at 0 to 158 mU/ml. Each assay contained 100 µl of autoclaved milk containing UHT cream, 80 µl of phosphate buffer containing lipase, and 20 µl of 5 mM pNP-C. Plates were sealed and monitored for color development (b*) every 10 min for 10 h at 37°C in an automated reflectance colorimeter.

Statistical Analysis

Student’s t test used in this study is described in Moore and McCabe (14). ANOVA was done using the JMP-SAS (SAS Institute, Inc., Cary, NC) computer software. Statistical significance was set at *P* ≤ 0.05 on least squares means.

RESULTS AND DISCUSSION

Correlation of Reflectance Colorimetry and Spectrophotometry

The b* color change with addition of *p*-nitrophenol to milk and buffer increased linearly to 0.5 mM in milk and to 0.1 mM in phosphate buffer (Figure 6). Measured b*
Figure 6. Saturation of color in milk (□) and sodium phosphate buffer (○) with addition of p-nitrophenol (p-NP) using reflectance colorimetry (b* color values). Data points represent the means of three replicates. Inset figure describes the change in b* color up to 5 mM p-NP.
color values did not begin at the origin because microtiter plates and liquid backgrounds used in the assay contained a small, but detectable, yellow color. This observation does not influence the usefulness of the assay because the change in $b^*$ color value, rather than the absolute color value, was used to calculate differences in enzyme activity. Final substrate concentrations used in further experiments were 0.5 mM, unless otherwise noted, to avoid nonlinear color detection during chromogenic substrate hydrolysis, allowing increased incubation times to detect lower concentrations of lipase. Alternatively, the substrate concentration could be increased and the incubation time shortened, but this format may hinder detection of low levels of lipase that are important in long-shelf-life products.

Substrate at 0.5 mM was selected to demonstrate the utility of reflectance colorimetry in determining lipase activity in complex, turbid mixtures. The concentration of $p$NP-C used in this study may not be suitable in other food products or experiments with controlled interface surface area. This leads to a need for determination of a new optimum substrate concentration for each product type being tested. Although this assay could be used to analyze kinetics of lipases or esterases, it would be necessary to measure water-fat interface surface areas since microbial lipases are known to have non-Michaelis-Menton kinetics and require an interface for activation for lipid substrates (9). We were not able to measure surface tension and, therefore, did not attempt to estimate kinetic parameters of the lipase we were using in this study. Additionally, water-fat interface surface area would not be controlled in industrial product testing, and kinetic measurements in foods containing multiple lipases would be of little value to industry.

Assays containing added commercial lipase and $p$NP-esters were monitored with spectrophotometry and reflectance colorimetry to determine whether these two detection methods measured the same enzymic reaction. Color liberated by hydrolysis of $p$NP-C in phosphate buffer monitored with reflectance colorimetry was linearly correlated to the enzyme activity monitored by spectrophotometry ($r^2 = 0.92$), indicating reflectance
colorimetry and spectrophotometry were measuring release of $p$NP due to lipase activity in phosphate buffer. However, spectrophotometric detection of the enzyme activity was less reproducible due to slight increased turbidity when the substrate was added to the buffer. The increase in turbidity was accounted for in the spectrophotometric assay by subtracting a control of the substrate alone since the turbidity increase occurred when the substrate was added to the phosphate buffer.

Differences in $b^*$ color value in milk and buffer with addition of $p$-nitrophenol (Figure 6) indicate that a control well containing a known concentration of $p$-nitrophenol in the product being tested is required to account for differences in reflected light. Additionally, the buffering capacity of the assay buffer must be controlled because $p$-nitrophenol is a common pH indicator (Figure 7), and fluctuations in the pH decrease accuracy and reproducibility. If these controls are not employed, color detection could be independent of enzyme activity, thereby misrepresenting lipase concentration.

After it was determined that reflectance colorimetry could linearly detect $p$-nitrophenol in milk up to 0.5 mM, standard curves were constructed with varying substrate concentrations to determine the optimal substrate concentration (Figure 8). Final concentrations of 0.5 mM $p$NP-C were acceptable for the determination of lipase activity. These data indicate lipase activity can be determined at substrate concentrations that are practical for use in dairy products. The $V_{max}$ and $K_m$ concentrations were not determined since Michaelis-Menten kinetics may be misleading in this assay format and misrepresent the data (9, 19).

**Lipase Detection Using Chromogenic Substrates and FFA Titration**

Hydrolysis of each chromogenic substrate after addition of commercial *P. fluorescens* lipase to whole milk was linearly correlated ($R^2 = 0.92$ to 0.98) to titratable FFAs assay (Table 9), indicating that use of chromogenic substrates in milk is an acceptable alternative
Figure 7. Influence of pH on the b* color (blue (-60) to yellow (+60)) value of p-nitrophenol in sodium phosphate buffer. Data points represent the means of duplicates; error bars represent the standard deviation of duplicates.
Figure 8. Effect of pNP-caprylate (pNP-C) concentration in sodium phosphate buffer on the activity of *P. fluorescens* lipase measured by colorimetry. Data points represent the mean of duplicates. Inset figure describes the effect of added lipase on detected activity in sodium phosphate buffer at 0.5 mM pNP-C. Activity is expressed as the rate of change of color from blue to yellow (Δb*/h).
to using FFA titration to determine lipase activity. When color development from the hydrolysis of the chromogenic substrates in milk was compared with liberation of titratable FFAs, pNP-C had a high correlation ($R^2 = 0.92$) and had the greatest ratio of color units liberated per unit of titrated FFAs (Table 9).

In 10 h at 37°C, the minimum detectable concentration of commercial *P. fluorescens* lipase added to milk, monitored using pNP-C as the substrate, was 5 mU/ml (determined by Student’s t test). We concluded from these data that reflectance colorimetry was an appropriate method to monitor *P. fluorescens* lipase activity in dairy products and that pNP-C was a suitable substrate.

*p*-Nitrophenyl-propionate and pNP-butyrate were unstable in the reaction conditions used in this study (data not shown), and a detectable color change occurred without added enzyme; however, a high correlation with FFA titration was obtained after the control values were subtracted in the calculation of enzyme activity. These observations indicate that pNP-propionate and pNP-butyrate can be used to detect lipolytic activity but are prone to error. After further use of these substrates, we observed that the stock solutions, which were dissolved in DMSO, solubilized the polystyrene microtiter plates, which caused autohydrolysis of short chain pNP-esters. A control containing all assay materials was used to ensure substrate autohydrolysis of pNP-C—possibly due to DMSO—was not mistaken for lipase activity. These controls demonstrated no detectable change in buffer over a 10-h assay at the final DMSO concentrations (10% v/v) used in these reactions (data not shown).

The CV for the reflectance colorimetric determination of commercial lipase was 14.2% in buffer and 6.2% in milk, which is similar to the findings of Dias and Weimer (5) using reflectance colorimetry and chromogenic amino acid substrates for aminopeptidase activity.
Influence of FFAs on Lipase Detection

The activity of *P. fluorescens* lipase decreased with addition of FFAs to assay mix (Figure 9). The extent of the inhibition varied with each fatty acid and did not correlate to the FFA chain length or abundance in milk fat. The extent of inhibition and activity underestimation depended on the concentration of each FFA at the time of testing. Choi and Jeon (2) found fatty acid concentrations in UHT-treated milk increased during storage due to residual lipase activity; concentrations began at 3.8 to 33.4 ppm. The rate of fatty acid release was faster with higher storage temperatures and with longer chain length fatty acids, increasing to 5.4 to 42.1 ppm after 12 wk of storage at 35°C (2). The extent of lipase inhibition with the five most abundant fatty acids in milk fat was estimated by regression using the initial and stored FFA concentrations observed by Choi and Jeon (2) (Table 10). From these data we suggest that lipolysis in milk previous to testing will lead to inhibition of *P. fluorescens* lipase and, consequently, an underestimation of residual lipase activity in finished dairy products.

Influence of Milk Fat on the Hydrolysis of *pNP-C* in Milk

Addition of up to 4% milk fat to HTST skim milk significantly (*P < 0.0001*) decreased all levels of added lipase activity as determined by ANOVA (Figure 10). This could have been due to competitive inhibition or differences in substrate availability. Observed lipase activities using added commercial lipase in the presence of increasing milk fat concentrations could be expressed by Equation [2], derived by ANOVA, which relates lipase concentration to lipase activity in the presence of milk fat, making it possible to assay milk without removing fat or associated lipase with *pNP-C* and reflectance colorimetry.
Figure 9. Effect of FFA concentration on hydrolysis of $p$NP-C in sodium phosphate buffer. Data points represent the mean of duplicates.
Figure 10. Effect of milk fat and added lipase on observed activity of total lipase in heat-treated milk measured by reflectance colorimetry. Bars represent the means of duplicates.
TABLE 10. Influence of FFAs added individually at concentrations found in fresh milk and milk that had been stored for 12 wk (2) on the activity of added lipase in skim milk. Lipolysis was measured by hydrolysis of pNP-caprylate using reflectance colorimetry. Calculations used means from duplicates.

<table>
<thead>
<tr>
<th>FFA</th>
<th>Observed lipase inhibition at FFA concentrations found in fresh milk</th>
<th>Observed lipase inhibition at FFA concentrations found in milk after 12 wk at 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Laurate</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Myristate</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>Palmitate</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>Stearate</td>
<td>34</td>
<td>39</td>
</tr>
<tr>
<td>Oleate</td>
<td>37</td>
<td>38</td>
</tr>
</tbody>
</table>

1 FFAs were added at concentrations determined by Choi and Jeon (2). FFAs were added individually, and the percent change in lipase activity measured.

\[ \text{[Lipase Equivalents]} = -92.42 + 40.94 \times (\% \text{fat}) + 16.99 \times (\text{lipase activity}) \]  \[2\]

Lipase equivalents are calculated in milliunits per milliliter; fat concentration in percent fat; and lipase activity in the change in \(b^*\) per hour.

**Lipase Activity Determination in UHT Skim Milk During Growth of *P. fluorescens* AFT36**

The activity lipase produced during growth of *P. fluorescens* AFT 36 in UHT skim milk, monitored using reflectance colorimetry and tributyrin agar, followed the growth curve during 106 h of incubation (Figure 11). When reflectance colorimetry was used, lipase activity was first observed at 10 h of culture incubation which corresponded to 9.6 x 10^4 cfu/ml. However, tributyrin agar required 28 h of culture incubation (4.5 x 10^6 cfu/ml) to detect lipase activity. This demonstrated that reflectance colorimetry could detect
Figure 11. Plate count (○) and lipase activity measured by reflectance colorimetry (◇) and zone diameter on tributyrin agar (□) of *P. fluorescens* AFT36 grown in skim milk. Data represent the means of duplicates.
lipase activity in dairy products contaminated with *Pseudomonas* lipase earlier than traditional agar assays would detect.

**CONCLUSIONS**

We used reflectance colorimetry to detect lipase activity in buffer and milk and compared the observed activity to a titratable FFA method and a tributyrin agar zone assay. The reflectance colorimetric method was more rapid than either of these methods and was more simple to perform than the titratable FFA method. Different fatty esters of the chromogenic substrate were evaluated, and 0.5 mM *pNP-C* was chosen because of its sensitivity and stability. We found FFAs and milk fat in the sample significantly inhibited substrate hydrolysis and must be accounted for when quantitating lipolytic activity in food products. Using reflectance colorimetry, we detected lipase activity after 10 h of growth (9.6 x 10^4 cfu/ml) of *P. fluorescens* AFT 36 in skim milk, suggesting that this assay could be of use to detect lipase production by microorganisms during growth in dairy products.

**REFERENCES**


CHAPTER V
AN IMMUNOCAPTURE SANDWICH-ELISA FOR QUANTIFICATION OF BACTERIAL SPORES¹

FOREWORD

We evaluated several methods for detecting spores in milk before adopting the immunocapture sandwich-ELISA described in the following chapter. These other methods are described briefly.

β-D-Glucosidase and α-D-Galactosidase Assays

β-D-Glucosidase and α-D-galactosidase are present and active on the surface of B. stearothermophilus spores and are slightly more heat resistant than the spore. This characteristic may make them useful indicators of heat destruction of B. stearothermophilus spores (23). To test their utility as indicators of spore presence, we used p-nitrophenyl-α-D-glucoside and p-nitrophenyl-β-D-galactoside to quantitate α-D-glucosidase and β-D-galactosidase activity, respectively. The relationship of enzyme activity to spore concentration in solution is shown in Figure 12. The minimum detectable number of B. stearothermophilus spores using this method was 10⁶ cfu/ml, which is too insensitive to be used in foods, even if a concentration step such as centrifugation or immunocapture were used in conjunction with the assay. Additionally, Bacillus cereus and B. subtilis spores were tested for β-D-glucosidase and α-D-galactosidase activity, but neither showed any color change at 10⁷ cfu/ml. This suggests that these spore coat enzymes may not be good as reporter enzymes since they are not found on the spore surface across the genera. To improve sensitivity, and to allow detection of different species of spore, we adopted an immunolabeling approach.

¹ Coauthored by M. R. Blake and B. C. Weimer.
Figure 12. Quantification of *B. stearothermophilus* spores in milk using p-nitrophenyl-β-D-glucoside and p-nitrophenyl-α-D-galactoside to quantify β-D-glucosidase and α-D-galactosidase activity respectively found in the spore coat of *B. stearothermophilus*. 
In our second approach to spore detection, we hypothesized that detection of single spores may be possible by immunolabeling after filtration (Figure 13). If so, it should be possible to filter spores from a solution and detect their presence by a precipitating ELISA. To test this hypothesis we filtered *B. stearothermophilus* spores from water using a 0.22 µm pore size filter and bound to the spores, biotinylated antibodies specific to *B. stearothermophilus* (anti-BsS). To amplify the signal, an ABC-horseradish peroxidase complex (Pierce, Rockford, IL) was attached to the bound antibodies, and a precipitating substrate (ImmunoPure® metal enhanced DAB, Pierce, Rockford, IL) was used to label spores. We could not distinguish the presence of spores over background noise in 10 h of incubation. To improve the signal-to-noise ratio, more effective blocking of the filter paper was required to reduce nonspecific binding of antibodies to the filter membrane. Several blocking agents were used to minimize background noise, including 2% BSA (Sigma), skim milk powder in Blotto® (Pierce, Rockford, IL), and casein in Blotto® (Pierce, Rockford, IL) blotting solution. None of these blocking agents reduced signal-to-noise ratio great enough to observe activity in 10 h. This may have been due to loss of enzyme activity or to insufficient activity in 10 h to enable the spores to be seen.

Other methods used to increase signal such as selecting a more active reporter enzyme complex, or by further amplification of the enzyme complex would theoretically not improve sensitivity since the ratio of specifically to nonspecifically bound enzyme would remain the same, and, therefore, the signal-to-noise ratio would remain unchanged. Since we could not improve the effectiveness of blocking any further using the best known
blocking agents, we concluded that the sensitivity of an ELISA was insufficient to detect a single spore bound to filter paper, and we modified the assay format to include spore concentration through immunocapture.

**Antibody Excess ELISA of Spores Bound to Glass Beads**

Immuno capture is the capture of an antigen—the spore—by an antibody that is bound to a solid matrix. Immobilization of antibodies to hydroxyl groups on silica surfaces with heterobifunctional crosslinkers has been reported by Bhatia et al. (1). We used this method to bind anti-BsS to 6 mm diameter glass beads with N-γ-maleimidobutyryloxy succinimide ester. The beads were added to milk containing *B. stearothermophilus* spores and incubated for 2 h to allow spore capture. Plate counts of the spores in the milk were done at regular intervals to determine if spores were being captured from solution and bound to the beads. No significant differences were observed in spore numbers, indicating that less than 10% of the spores were being bound from solution.

To estimate the number of spores bound to the beads, we chose to use an antibody excess ELISA (Figure 14) based on its ease of use. To quantitate the number of spores bound, we measured the number of unbound antibodies and tried to correlate that to the number of spores bound. A horseradish peroxidase (HRP) labeled anti-IgG was used to label unbound anti-BsS. No significant reduction in the number of unbound anti-BsS was observed when beads were added to buffer containing spores over adding beads to buffer not containing spores. This may be due to only a few spores being bound and, therefore, the number of unbound antibodies did not significantly change, or no spores were bound,
or the bound spores were competitive displaced by the anti-IgG used to label unbound anti-BsS. Reasons for lack of spore capture by the anti-BsS include insufficient avidity of the antibody to the spore, allowing the spore to disassociate after being captured due to mechanical disruption, or an inability of the antibody to bind to the antigen on the spore surface. This may be due to steric hindrance between the spore and the bead, which could theoretically be overcome with a sufficiently long spacer, or due to the inability of the antibody to orientate itself to the antigen because of a lack of flexibility in the spacer.

Realizing that the percent spore capture from the solution was lower than expected, we switched from the antibody excess assay format to a sandwich assay format to improve sensitivity. Also to improve spore capture, we began using small immunomagnetic beads to allow greater dispersal of beads throughout the sample which would permit a greater bead-surface area to be used. These factors increase the probability of the antibody and the antigen coming in contact with each other, thereby increasing the likelihood of spore capture. The immunomagnetic beads we chose to use have a diameter of 2.8 µm. Therefore, $4 \times 10^6$ immunomagnetic beads (IMB)—which corresponds to about 5 µl of beads—have the equivalent surface area to one 6 mm bead.

**Immunocapture of B. stearothermophilus Spores**

Initially, strepavidin-coated super paramagnetic beads (Dynal, Lake Success, NY) were used to bind biotinylated-anti-BsS. The antibodies were biotinylated using NHS-LC-biotin, a long chain derivative of biotin that minimizes steric hindrance between the biotinylated antibody and strepavidin. Approximately three biotin molecules were attached to the surface of each IgG, which was determined by the HABA® assay. To ensure that biotinylation did not
interfere with spore binding, two ELISAs were done using biotinylated and non-biotinylated antibodies at equal concentrations on spores electrostatically bound to a microtiter plate. No difference was observed between the two antibody preparations, indicating that biotinylation did not inhibit antibody binding to the spore. This does not necessarily mean that the avidity of the antibodies has not been altered; it may just be a function of antibody titer.

Using IMB prepared using biotinylated antibodies, we could detect no captured spores after addition of IMB for 1 h to a solution of $10^6$ cfu/ml of *B. stearothermophilus* spores (Figure 15). To further eliminate possible effects of biotinylation on the avidity of the biotinylated anti-BsS, we bound anti-BsS to tosyl activated paramagnetic beads, allowing covalent coupling of the antibodies via a surface amine group on the antibody surface. This coupling results in tight binding of antibodies to the surface of the bead. No spores were captured using this method of coupling, suggesting that factors such as steric hindrance between the spore and the bead inhibit tight binding and are responsible for the lack of spore capture. If this were the case, expected observations would include antibody/antigen binding when both are in solution, but no binding when the antibody is bound to a matrix, which matches our observations.

We hypothesized that steric interference between the surface of the bead and the spore may interfere with the antibody/antigen interaction since the spore is approximately 0.5 µm in diameter, and the bead, 2.8 µm. To overcome this interference, we bound anti-IgG to the bead to act as a protein spacer (Figure 16). We bound biotinylated anti-mouse-IgG to strepavidin-coated beads, and non-biotinylated anti-mouse-IgG to tosyl activated beads and then captured anti-BsS. The
resulting IMB were added to a solution of 10⁶ cfu/ml of *B. stearothermophilus* spores for 1 h to bind spores. No spores were captured by the IMB, suggesting that either antibody avidity was too weak to hold the spore during washing or that the avidity was great enough, but the antibody was not able to orientate itself with the antigenic site. Incorrect orientation may be due to either insufficient spacer length and therefore not overcoming steric interference, or to lack of flexibility in the spacer leading to partial bonding, or to strained bonding between the antibody and its antigen. Hermanson et al. (10) noted that with rigid support materials a spacer molecule may provide greater flexibility, allowing the immobilized antibody to move into position to establish the correct binding orientation with its antigen. We concluded that although we may have minimized steric hindrance between the bead and the spore, the spacer immunoglobulin we had chosen did not allow enough flexibility, impeding correct orientation of the antibody to its antigenic site on the spore surface. Others have used hydrocarbons to increase the degrees of freedom of antibody movement (10); however, hydrocarbon linkers may increase hydrophobic interactions with the polystyrene bead.

To provide a sufficiently long spacer that allows the antibody to orientate itself to surface antigens on the spore, we constructed a flexible, hydrophilic, 60 nm-long poly-threonine spacer (MW(vis) 12,100; Figure 17). Poly-threonine (Sigma Chemical, St. Louis, MO) was chosen because of its solubility in water, allowing easier binding chemistries. All other poly-amino acids required an organic solvent, which is unacceptable since we were trying to bind them to polystyrene beads, which are destroyed by organic solvents.
We bound the poly-threonine spacer to the tosyl-activated groups on the bead via the terminal amine. We then attached the anti-BsS, via the carbohydrate moiety, to the carboxyl end of the poly-threonine spacer. We assumed the poly-threonine spacer forms no secondary or tertiary structures, which would allow approximately 20 nm of space between the Fc portion of the antibody and the matrix and should allow the flexibility required for correct orientation of the antibody to the spore.

This configuration successfully allowed capture of *B. stearothermophilus* spores from solution and was used to develop an immunoassay for quantifying spores. The following chapter describes the immunocapture, sandwich ELISA we developed using this antibody immobilization method to quantify *B. stearothermophilus* spores in milk and other food and environmental samples.

**ABSTRACT**

There are no methods currently for rapid and sensitive detection of microbial spores that could be used to direct foods containing high spore loads away from sensitive products. Existing methods require either an overnight incubation, or cannot detect spores below $10^5$ cfu/ml and are not specific to particular species. This work describes a method to specifically detect $<10^4$ cfu/ml of bacterial spores within 2 h. Polyclonal antibodies to *B. stearothermophilus* spores were attached to 2.8-μm diameter magnetic-polystyrene beads using a poly-threonine spacer via the antibody carbohydrate moiety. A biotin-avidin amplified sandwich ELISA coupled to a fluorescent substrate was used to quantitate captured spores. The concentration of *B. stearothermophilus* spores in samples was linearly correlated to fluorescent activity ($R^2 = 0.99$) with a lower detection limit of $8 \times 10^3$ cfu/ml and an upper detection limit of $8 \times 10^5$ cfu/ml. The detection limits can be changed by varying the immunomagnetic bead concentration. Several food and environmental samples were tested to illustrate the assays' versatility.
INTRODUCTION

Bacterial spores are the most heat-stable form of microorganisms and are, therefore, of great concern in food products that receive extensive heat treatments to prolong shelf life. Spores are ubiquitous in the environment, which is a source of pre- and post-contamination for foods. Burton (3) found the spore counts in milk from around the world varied between zero and >22,000 cfu/ml depending on the climate of the region. *B. stearothermophilus* spores are one of the most heat-resistant bacterial spores found in high numbers in soil and water, which contaminate foods. Contaminating *B. stearothermophilus* spores survive extreme heating to germinate and grow at elevated product storage temperatures—as often occurs in foods transported in equatorial regions of the world.

While *B. stearothermophilus* is not commonly a problem, other bacilli often lead to food-borne illness or spoilage in a variety of food stuffs. *B. cereus, B. licheniformis, B. subtilis,* and *Bacillus pumilus* have all been implicated in outbreaks of food-borne illness and are commonly isolated from raw and pasteurized milk (7). *B. cereus* is also responsible for a sweet curdling defect in milk (18). Recently, *B. badius* has been reported to survive extreme temperature processing in milk ($D_{121} = 5$ sec) (9). *B. badius* is a mesophilic organism and grows readily at room temperature, making it a likely candidate for spoiling temperature-processed foods. Confirmed cases (fifty-two) of *B. badius* in UHT milk have been reported across Europe including Germany, France, Italy, Benelux, and Spain, and two cases outside of Europe (9). A contributing factor to this problem is the lack of a rapid assay to determine spore counts of specific species in milk before processing (11). Such an assay could be used to monitor critical control points in a hazard analysis critical control point (HACCP) plan and would allow raw materials with high spore loads to be used in products that will not pose a food safety risk to consumers.

The standard method for quantifying spores in milk (20) involves heat-shocking the sample for 10-15 min, followed by a total plate count. This assay requires 24 to 48 h and
multiple incubation temperatures, which is time-consuming and yields historical information industrially. The food industry is increasingly requiring microbiological assays to yield predictive information for use in HACCP analysis and risk assessment. An ELISA capable of detecting $10^6 \text{cfu/ml}$ of *B. cereus* spores in foods demonstrated the possibility of using an immunoassay; however, the assay was not practical for industrial use because of an unacceptable detection limit and antibody cross-reactivity (4).

Techniques to increase sensitivity of immunosorbent assays have focused on more efficient reporter labels, such as faster catalyzing reporter-enzymes; signal amplification, such as avidin- or strepavidin-biotin enzyme complexes; and better detectors, such as luminescence and fluorescence (12, 19). Immunomagnetic antigen capture has been used extensively to separate and identify *Escherichia coli* and *Salmonella* from foods. However, these methods involve either a preincubation or a subsequent incubation step—usually 18 to 24 h—to increase the cell numbers for detection (2, 5, 13, 14, 15, 16, 17, 21, 24). Use of immunomagnetic capture to supplement plate counting has greatly shortened *E. coli* and *Salmonella* testing, but long incubation times limit this method in a proactive or predictive way.

The object of this work was to develop a rapid assay for detection of *Bacillus* spores. To do this we used immunomagnetic capture to concentrate *B. stearothermophilus* spores from foods, a biotin-strepavidin complex to amplify the signal, and fluorescent detection.

**MATERIALS AND METHODS**

**Bacterial Spores**

Commercial preparations of spores of *B. stearothermophilus* ATCC 10149, *B. cereus* ATCC 11778, and *B. subtilis* 6633 (Fisher Scientific, Pittsburgh, PA) were used for antibody production. Viable spore numbers and germination estimates were obtained by plating on PCA overnight at 65°C, 30°C, and 30°C, respectively. All other spores (Table
11) were prepared by spread-plating a single colony isolate on PCA and incubating the covered plate at 30°C for approximately 2 wk. Spores were swabbed from the surface of the dried agar and washed repeatedly in distilled water to remove water-soluble components. Spores were pelleted and separated from cell debris by centrifugation (1,500 x g for 20 min) (6). Presence of spores was confirmed by heating to 80°C for 15 min then plating on PCA (20).

**Polyclonal Antibodies Production**

Polyclonal anti-*B. stearothermophilus* spore antibodies (anti-BsS) were made at the Utah State Biotechnology Center (Logan, UT). BALB/c mice were injected in the intraperitoneal cavity with 1x10⁷ cfu/ml *B. stearothermophilus* spores in sterile physiological saline (0.5 ml) three times at 3-wk intervals (8). Total serum IgG’s were purified using a protein A/G column (Pierce Chemical, Rockford, IL). Antibodies were desalted and concentrated to 1 mg/ml in 0.1 M NaPO₄, pH 7.0 in a 30 kD Centricon® (Amicon, Beverly, MA) at 4,500 x g at 4°C.

**Antibody Specificity**

Antibody specificity was tested by measuring the cross reactivity against *Bacillus* spores listed in Table 11 using a standard ELISA. A suspension of each spore type (10⁶ cfu/ml), suspended in 50 mM NaCO₃ (pH 9.5), was nonspecifically bound to wells of a microtiter plate for 12 h at 4°C. Wells containing spores were blocked with BSA (2% in PBS) for 4 h at 25°C, and washed four times with PBS containing 0.1% Tween 20 (PBST). Anti-BsS (1:10,000 serum dilution in PBS) was added to wells, slowly agitated for 2 h at 25°C, and washed four times with PBST. HRP-labeled anti-whole mouse IgG (Sigma) was added to label anti-BsS for 2 h, then washed four times with PBST. OPD color development was measured using a b* color scale (blue to yellow) at 37°C for 1 h in
### TABLE 11. Incubation temperature and source of *Bacillus* species used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Incubation temperature (°C)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>65</td>
<td>ATCC 10149&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>30</td>
<td>ATCC 11778&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>30</td>
<td>ATCC 6633&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. circulans</em></td>
<td>30</td>
<td>ATCC 4513&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. coagulans</em></td>
<td>30</td>
<td>ATCC 7050&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>30</td>
<td>Raw milk isolate&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. mascerans</em></td>
<td>30</td>
<td>Raw milk isolate&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>30</td>
<td>ATCC 842&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. pumulis</em></td>
<td>30</td>
<td>Raw milk isolate&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Fisher scientific.  
<sup>2</sup>Purchased from ATCC.  
<sup>3</sup>Kindly donated by Floyd Bodyfelt, Oregon State University.

an automated reflectance colorimeter (Omnispec® 4000 bioactivity monitor; Wescor, Inc., Logan, UT).

**Antibody Biotinylation**

Antibodies purified from total serum were biotinylated with NHS-LC-Biotin® (Pierce Chemical, Rockford, IL). Efficiency of surface biotinylation was determined using the HABA® assay (Pierce), but was modified to remove the β-mecaptoethanol step so that antibodies were not denatured. This modified procedure gave the number of surface biotin moieties per antibody (Sigma Technical Support).
Antibody/Bead Conjugation

Antibody Oxidation. Sodium meta-periodate (5 mg) was used to oxidize carbohydrate moieties on the antibodies (10). Sodium meta-periodate was removed after oxidation by washing five times with 0.1M NaPO₄, pH 7.0 in a 30 kD Centricon® (4,500 x g, 4°C) and immediately crosslinking to magnetic beads as described below.

Crosslinking Antibodies to Beads. Poly-threonine (MW(vis) 12,100; Sigma Chemical, St. Louis, MO) was covalently coupled to 2.8-µm, tosyl-activated polystyrene Dynabeads® (Dyna!, Lake Success, NY) in 50 mM borate buffer (pH 9.5) via the terminal amine as described by the bead product instructions. Four washes (three times for 10 min, and once for 30 min) with TBS buffer (pH 7.5) were used to block remaining tosyl-active sites. Adenine dihydrazine (ADH; 0.5 M in 0.1 M MES, pH 4.75; Sigma) was linked to the carboxy terminal of the bound poly-threonine using an ethylene diamine carbodiimide mediated reaction (10). Oxidized antibodies were mixed with the ADH activated beads at room temperature for 12 h to allow crosslinking between the oxidized carbohydrate moiety of the IgG and the ADH terminal of the poly-threonine linker (10). After crosslinking, the immunomagnetic beads (IMB) were stored rotating (= 50 rpm) in PBST with 0.02% sodium azide at 4°C until used.

Immunocapture

IMB (3 x 10⁷) were added to 1 ml of sample containing spores and allowed to rotate (= 50 rpm) for 30 min at 25°C. The IMB were removed from the sample with a magnetic particle concentrator (Dynal MPC-E-1®) and washed four times with PBST. PBST was used as a wash buffer to stop IMB clumping and to block spore adhesion to tube walls (24). After each wash, IMB were transferred to a new microfuge tube. The presence of bound spores on IMB was confirmed by plate counts and examination with a phase contrast microscope.
Determination of Optimum IMB Binding Temperature

IMB were added to UHT skim milk containing $5 \times 10^4$ B. stearothermophilus spores and incubated between 4°C to 55°C while rotating (= 50 rpm) for 30 min. The IMB were washed four times with PBST, plated on PCA, and incubated overnight at 65°C. B. stearothermophilus colonies were counted to quantitate bound spores.

Fluorescent Detection of Captured Spores

Spores bound to IMB were labeled with a secondary biotinylated anti-BsS. The IMB were then washed with PBST and resuspended in an ABC-alkaline phosphatase complex solution (Vector Laboratories, Inc., Burlingame, CA) for 30 min. The IMB were washed three times with PBST and resuspended in 100 µl of 0.2 M Tris buffer containing 0.1% BSA (pH 8.5) to remove unbound enzyme complex. A 40-µl suspension of the IMB was added to 3 ml of Fluorophos® substrate (Advanced Instruments, Norwood, MA) and fluorescence monitored for 2 min at 38°C in an Flurophos® FLM200 fluorometer (Advanced Instruments, Norwood, MA).

Product Testing

Fluid products were tested with no modification. Powdered products were dissolved to 1 g/ml. IMB ($3 \times 10^6$) were added to 1 ml of each product and mixed gently for 30 min at 25°C. Bound spores were quantitated using fluorescent detection.

RESULTS AND DISCUSSION

Detection of bacterial spores in food and environmental samples is currently unacceptable for use in a factory HACCP plan because it yields historical data that have limited value. The standard method of killing viable cells and plating surviving spores is too slow, taking at least an overnight incubation at multiple incubation temperatures to allow colonies to be counted. Additionally, it does not readily differentiate between spore
types. The ELISA method of Chang and Foegeding (4) is rapid, but not sensitive enough for routine testing (the lower detection limit is $10^6$ spores/ml), and also does not differentiate between spore species.

The anti-BsS were directionally oriented via the carbohydrate moiety to beads using a poly-threonine spacer. Several other methods of binding antibodies were tried before adopting this procedure but were not acceptable because the IMB-bound anti-BsS did not capture spores from solution (Table 12). In all cases, we confirmed the presence of bound antibodies on the surface of the IMB by ELISA using HRP-labeled anti-IgG. Only antibodies bound via the poly-threonine spacer allowed capture of *B. stearothermophilus* spores.

The IMB specifically captured *B. stearothermophilus* spores from PBST containing equal numbers of *B. stearothermophilus* and *B. subtilis* spores (Figure 18A). About 99% of nonspecifically bound organisms were removed during each wash, leaving only *B. stearothermophilus* spores captured after four washes (Figure 18B). Captured *B. stearothermophilus* spores were specifically labeled with biotinylated-anti-BsS, which minimized potential background created by nonspecifically bound organisms and were used to bind the avidin-biotin-alkaline phosphatase complex.

Using this immunocapture-sandwich ELISA, we quantified spores in UHT skim milk down to $8 \times 10^3$ cfu/ml in 2 h with no pre-enrichment steps and no sample preparation. Increasing the number of beads in the assay increased the fluorescent activity, suggesting that this could be used to increase the assay sensitivity further (Figure 19) (5, 22).

We also demonstrated the immunocapture of spores in a variety of complex backgrounds such as fluid milk, powdered milk, baby formula, spices, soil, and sand to show the versatility of the assay in the food industry (Figure 20). The slope of the generated curves was similar for all samples tested, indicating that sample background did not influence antigen binding. Therefore, approximate spore loads can be obtained without
TABLE 12. Method of anti-Bs IgG attachment to IMB and its influence on the number of spores captured from PBST containing 10^7/10^8 cfu/ml *B. stearothermophilus* spores.

<table>
<thead>
<tr>
<th>Method of Ab attachment to magnetic beads</th>
<th>Antibody modification</th>
<th>Antibody orientation</th>
<th>Number of spores bound(^1) from PBST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin-Strepavidin</td>
<td>NHS-LC-Biotinylation(^2) (3.4 biotins / Ab)</td>
<td>non-directional</td>
<td>0/0</td>
</tr>
<tr>
<td>Ab amine groups bound to Tosyl activated -OH groups on bead surface</td>
<td>none</td>
<td>non-directional</td>
<td>0/0</td>
</tr>
<tr>
<td>Anti-Fc IgG spacer</td>
<td>none</td>
<td>directional</td>
<td>0/0</td>
</tr>
<tr>
<td>Poly-threonine-ADH crosslinker</td>
<td>Carbohydrate oxidation</td>
<td>directional</td>
<td>160/3600</td>
</tr>
</tbody>
</table>

\(^1\)Determine by plate counts after four washes with PBST.
\(^2\)Biotinylation did not inhibit binding as determined by ELISA.

calibrating the assay to each product. Foods containing fat, such as raw whole milk, required careful handling when removing the beads from the sample since the beads can easily be removed with the supernatant and lost. Separation of IMB from fatty products required 5 min rather than the 2 min used for nonfat samples. Soil samples containing a high percentage of iron fines interfered with bead recovery, although other soil types tested did not. These data support the use of this assay to test for *B. stearothermophilus* spores in food and environmental samples.

Since the assay has been designed to be used with raw ingredients that may vary in temperature, we tested the ability of the IMB to capture *B. stearothermophilus* spore from milk at refrigeration temperature to the germination temperature of *B. stearothermophilus* spores (4 to 50°C). The number of spores captured from UHT skim milk containing 5 x 10^4 *B. stearothermophilus* spores did not vary significantly between 4 and 50°C. This suggests that the assay could be performed on products within this range with no difference in assay sensitivity, which helps avoid sample preparation time.
Figure 18. These figures demonstrate the selective capture of *B. stearothermophilus* spores in the presence of *B. subtilus* spores. The bar graph shows the concentration of spores in the milk and PBST samples before adding IMB and compares the number of spores bound to the IMB after four washes. The line graph illustrates the removal of nonspecifically bound spores during the four washes.
Figure 19. Fluorescent detection of captured *B. stearothermophilus* spores in skim milk by a biotin-avidin amplified sandwich ELISA using $3 \times 10^6$ and $1.4 \times 10^7$ IMB. Data points represent the mean of two replications. Error bars represent standard error of the means.
Figure 20. Fluorescent detection of captured *B. stearothermophilus* spores various food and environmental samples using $3 \times 10^6$ IMB. Data points represent the mean of two replications. Error bars represent standard error of the means.
This spore assay is about a 1000 times more sensitive than the only other rapid spore assay (4) and is about 10 times faster than any spore assay with equivalent sensitivity (20). This assay will enable users to rapidly test raw ingredients for high spore numbers and will yield predictive information.

CONCLUSIONS

The described immunomagnetic-capture sandwich-ELISA is specific to spores of *B. stearothermophilus*, which allows spores to be quantitated in food and environmental samples. The assay is sensitive down to $8 \times 10^3$ cfu/ml of spores in UHT skim milk and can be completed in about 2 h. Sensitivity can be adjusted by adding varying amounts of IMB. Soil samples containing a high percentage of fines interfered with bead recovery, and care must be taken with fatty samples to avoid bead loss. Sample temperatures between 4 and 50°C did not affect spore capture by the IMB. This assay can be used to detect high spore counts in raw ingredients and assist processors to predict poor quality raw materials.

REFERENCES


CHAPTER VI
SUMMARY

We processed milk by direct steam injection at temperatures between 100 and 140°C for 4 and 12 s and stored the milk for 60 d at 7°C to quantitate the sensory and microbiological qualities under these conditions. We found that lower process temperatures had less-cooked flavors and less lipolytic activity during storage. Additionally, we found that spores of psychrotrophic bacteria in the raw milk survived and grew after processing below 132°C but that milk processed at 134°C was not spoiled by these organisms. Based on these observations, processing conditions of 134°C for 4 s were recommended for ESL milk to avoid survival of food-borne pathogenic sporeformers and to minimize cooked and off-flavors due to lipolysis.

These data reflected a need for rapid and sensitive assays for heat-stable enzymes and bacterial spores since existing assays were slow, cumbersome, and have inadequate sensitivities. To this end, a lipase assay was developed that could detect down to 5 mUnits/ml of lipolytic activity in milk, which is more sensitive and more rapid than currently used assays, and required no sample preparation. The utility of the assay was demonstrated by measuring lipolytic activity in milks containing added lipase and 0 to 4% fat and by monitoring the activity of lipase released during growth of P. fluorescens in skim milk.

A spore assay capable of quantitating spores of B. stearothermophilus down to 8 x 10³ cfu/ml was developed using immunocapture with a fluorescent detection system. The assay was specific to B. stearothermophilus spores. The assay was applied to fluid, powder, and solid products including milk, powdered milk, baby formula, pepper, clay, and sandy soil. Samples containing a high concentration of iron fines, such as some soil types, were unacceptable since the fines interfered with the magnetic removal of IMB. Total time for the assay is 2 h.
These assays will allow the milk producer to direct milk containing high lipase or spore concentrations into products other than long-shelf-life products and will help minimize the risk of product spoilage. Furthermore, the spore assay can be used to quickly assess the quality of environmental sources of bacterial contamination for the presence of spores. For processors of ESL milk, these assays will allow better quality control, which will lead to better flavored, longer life ESL milk and will minimize the risk of food poisoning due to milk contaminated with a pathogenic sporeformer.
APPENDICES
Appendix A:
Copyright Clearances and Coauthor Release Letters
January 15, 1996

Michael R. Blake
Dept. Nutrition and Food Sciences
Utah State University
Logan, UT 84322-8700

Dear Michael:

I am writing in response to your request for permission to use an article from *Journal of Food Protection*.

Permission to use this article is granted provided the printed material will include the following: Reprinted with permission from *Journal of Food Protection*. Copyright held by the International Association of Milk, Food and Environmental Sanitarians, Inc. These materials are to be used only for the purpose outlined in your January 12 fax.

If you have any questions, please contact me or Carol Mouchka at (515) 276-3344 or by Fax (515) 276-8655.

Sincerely,

Michelle L. Sproul
Publication Assistant
Leslie B. Hansen  
J. Dairy Science Editor  
Department of Animal Science  
University of Minnesota  
130 Haecker Hall  
St. Paul MN 55108

Dear Dr. Hansen,

I would like to include the following manuscript as a chapter in my dissertation:

"A semiautomated reflectance colorimetric method for the determination of lipase activity in milk" MS# 5263

I am requesting permission to include this paper with your approval.

Please inform me of any fees or wording that you would like to accompany the chapter. I am in the final stages of preparation of my dissertation, so if possible could you fax a copy of the copyright clearance.

Thank you in advance for your assistance.

Mick Blake  
Dept. Nutrition and Food Sciences  
Utah State University  
Logan UT 84322-8700  
Phone: (801) 797 2123  
Fax: (801) 797 2379

Permission granted for this use only  
Editor, Journal of Dairy Science
Dear Rathna,

I would like to include the following paper in my dissertation with your permission:


Thank you,

Michael Blake

Permission Granted
Appendix B:
Example Questionnaires
EXAMPLE QUESTIONNAIRES

Panel Questionnaire 1 (pg. 103)
Questionnaire designed as a modified scalar difference test so that judges can compare each processing temperature to both UHT and HTST milk at the same time. This format allowed the judge to give a magnitude of estimation for comparative analysis. The scale extended beyond references to minimize central tendency. Samples were presented in a balanced block design.

Panel Questionnaire 2 (pg. 104)
Questionnaire designed as a Quantitative Descriptive analysis, with five specific sensory characteristics being tested for and one general off-flavor characteristic to account for unexplained characteristics. The scale extended from 0 mm to 120 mm to minimize central tendency. The identical form was used for all trained panels. Samples were presented in a balanced block design. All treatments and taste panels were designed as duplicate, balanced block designs for statistical analysis.

Panel Questionnaire 3.1 (pg. 105)
Questionnaire was conducted as a consumer preference questionnaire to determine whether consumers preferred the ESL processed milk or HTST pasteurized milk. Questions were aimed at determining if the panelist liked cooked flavors in milk.

Panel Questionnaire 3.2 (pg. 106)
At 60 days a hedonic scale was added to questionnaire 3.1 to also determine acceptability at the end of the proposed shelf life.
You have been presented with five milk samples and two reference samples (R1 & R2). For each sample place a slash anywhere on its line scale to represent how it compares with the two reference samples.

Sample __

____________________________________
R1 R2

Sample __

____________________________________
R1 R2

Sample __

____________________________________
R1 R2

Sample __

____________________________________
R1 R2

Sample __

____________________________________
R1 R2
You have been presented with six milk samples. Answer all questions for each milk sample by putting a slash on the line scale with the sample number over it to represent the strength of the characteristic.

**Cooked flavor**

| none | slight | moderate | strong | extreme |

**Sweetness**

| none | slight | moderate | strong | extreme |

**Rancidity**

| none | slight | moderate | strong | extreme |

**Fruity**

| none | slight | moderate | strong | extreme |

**Bitter**

| none | slight | moderate | strong | extreme |

**Other off-flavor**

| none | slight | moderate | strong | extreme |

Thank you, Mick
Please read the following carefully, answering all questions.

Do you like the flavor of milk?  
Y  N

Do you like the flavor of powdered milk?  
Y  N  Never Tried

Do you like the flavor of canned milk?  
Y  N  Never Tried

Do you like the flavor of Long Life Milk (UHT Milk)?  
Y  N  Never Tried

Taste both of the samples and check the sample you prefer

I prefer:

___ 250  ___ 763

Why did you prefer this sample? (Please check one or more)

- It tasted less cooked
- It tasted sweeter
- It tasted creamier
- It tasted fresher
- It tasted better (please explain)

thank you for your time
You have been given three milk samples.

First rate them from best flavor to worst flavor.

Sample____  Best Flavor
Sample____  Next Best
Sample____  Worst Flavor

Then, using the following scale rate the samples from 1 to 9.

<table>
<thead>
<tr>
<th>Rating</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Like Extremely</td>
<td>9</td>
</tr>
<tr>
<td>Like Very Much</td>
<td>8</td>
</tr>
<tr>
<td>Like Moderately</td>
<td>7</td>
</tr>
<tr>
<td>Like Slightly</td>
<td>6</td>
</tr>
<tr>
<td>Neither Like nor Dislike</td>
<td>5</td>
</tr>
<tr>
<td>Dislike Slightly</td>
<td>4</td>
</tr>
<tr>
<td>Dislike Moderately</td>
<td>3</td>
</tr>
<tr>
<td>Dislike Very Much</td>
<td>2</td>
</tr>
<tr>
<td>Dislike Extremely</td>
<td>1</td>
</tr>
</tbody>
</table>

Sample  Score
_______  _______
_______  _______
_______  _______
Appendix C:
Tables of Statistical Analysis
STATISTICAL ANALYSIS OF DATA FROM TRAINED PANELS

Correlation between flavor scores by trained panelists on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

<table>
<thead>
<tr>
<th></th>
<th>Cooked</th>
<th>Sweet</th>
<th>Rancid</th>
<th>Fruity</th>
<th>Bitter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweetness</td>
<td>0.380</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rancid</td>
<td>-0.010</td>
<td>-0.014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruity</td>
<td>0.083</td>
<td>0.366</td>
<td>0.268</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bitter</td>
<td>-0.048</td>
<td>-0.018</td>
<td>0.233</td>
<td>0.150</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>-0.107</td>
<td>-0.092</td>
<td>0.154</td>
<td>0.022</td>
<td>0.104</td>
</tr>
</tbody>
</table>

Split plot ANOVA for cooked flavor scored by a trained panel on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>F_0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (temp)</td>
<td>6</td>
<td>8423.5</td>
<td>8834.8</td>
<td>1472.5</td>
<td>*8.37</td>
<td>4.28</td>
</tr>
<tr>
<td>Replication</td>
<td>1</td>
<td>4.6</td>
<td>9.8</td>
<td>9.8</td>
<td>0.06</td>
<td>5.99</td>
</tr>
<tr>
<td>Error A</td>
<td>6</td>
<td>1113.4</td>
<td>1055.7</td>
<td>175.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storage time (ST)</td>
<td>4</td>
<td>15452.6</td>
<td>15452.6</td>
<td>3863.2</td>
<td>10.27</td>
<td>2.71</td>
</tr>
<tr>
<td>ST x Temp</td>
<td>24</td>
<td>7635.1</td>
<td>7635.1</td>
<td>318.1</td>
<td>0.85</td>
<td>1.91</td>
</tr>
<tr>
<td>Error B</td>
<td>28</td>
<td>5118.5</td>
<td>5118.5</td>
<td>376.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Judge</td>
<td>938</td>
<td>264293.5</td>
<td>264293.5</td>
<td>281.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1007</td>
<td>302041.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Bolded cells indicates significant difference between treatments at \( \alpha = 0.05 \). Fisher pairwise comparison tables for significant cells are in the following tables.
Fisher pairwise comparison of temperature treatments for cooked flavors. Ranges encompassing zero are not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>75</th>
<th>120</th>
<th>124</th>
<th>128</th>
<th>132</th>
<th>136</th>
</tr>
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<tbody>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td><strong>-9.09</strong> to <strong>-1.17</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td><strong>-11.45</strong> to <strong>-3.53</strong></td>
<td><strong>-6.32</strong> to <strong>1.60</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>132</td>
<td><strong>-12.67</strong> to <strong>-4.75</strong></td>
<td><strong>-7.54</strong> to <strong>0.38</strong></td>
<td><strong>-5.18</strong> to <strong>2.74</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td><strong>-12.97</strong> to <strong>-5.05</strong></td>
<td><strong>-7.84</strong> to <strong>0.08</strong></td>
<td><strong>-5.48</strong> to <strong>2.44</strong></td>
<td><strong>-4.27</strong> to <strong>3.65</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td><strong>-11.69</strong> to <strong>-3.77</strong></td>
<td><strong>-6.56</strong> to <strong>1.36</strong></td>
<td><strong>-4.20</strong> to <strong>3.72</strong></td>
<td><strong>-2.98</strong> to <strong>4.94</strong></td>
<td><strong>-2.68</strong> to <strong>5.24</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Bolded cells indicates significant difference between treatments at $\alpha = 0.05$*
Fisher pairwise comparison of storage time treatments for cooked flavors. Ranges encompassing zero are not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>6.14 to 12.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>8.38 to 15.21</td>
<td>-0.90 to 5.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>6.19 to 12.81</td>
<td>-3.08 to 3.18</td>
<td>-5.54 to 0.95</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>4.79 to 11.74</td>
<td>-4.49 to 2.13</td>
<td>-6.94 to -0.12</td>
<td>-4.54 to 2.08</td>
</tr>
</tbody>
</table>

*Bolded cells indicates significant difference between treatments at $\alpha = 0.05$

Split plot ANOVA for rancid flavor scored by a trained panel on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>F_{0.05}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (temp)</td>
<td>6</td>
<td>2478.4</td>
<td>2583.8</td>
<td>430.6</td>
<td>*6.46</td>
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<tr>
<td>Replication</td>
<td>1</td>
<td>8.2</td>
<td>10.1</td>
<td>10.1</td>
<td>0.15</td>
<td>5.99</td>
</tr>
<tr>
<td>Error A</td>
<td>6</td>
<td>349.1</td>
<td>400.5</td>
<td>66.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storage time (ST)</td>
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<td>1055.6</td>
<td>1055.6</td>
<td>263.9</td>
<td>1.49</td>
<td>2.71</td>
</tr>
<tr>
<td>ST x Temp</td>
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<td>1588.6</td>
<td>1588.6</td>
<td>66.2</td>
<td>0.37</td>
<td>1.91</td>
</tr>
<tr>
<td>Error B</td>
<td>28</td>
<td>2314.4</td>
<td>2314.4</td>
<td>177.4</td>
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<tr>
<td>Judge</td>
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<td>114288.4</td>
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<td>121.8</td>
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<td>122082.6</td>
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</table>

*Bolded cells indicates significant difference between treatments at $\alpha = 0.05$
Fisher pairwise comparison of temperature treatments for rancid flavors. Ranges encompassing zero are not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>75</th>
<th>120</th>
<th>124</th>
<th>128</th>
<th>132</th>
<th>136</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>*1.69 to 6.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>2.64 to 7.69</td>
<td>-1.58 to 3.48</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>128</td>
<td>1.90 to 6.96</td>
<td>-2.31 to 2.74</td>
<td>-3.26 to 1.79</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>1.20 to 6.26</td>
<td>-3.01 to 2.04</td>
<td>-3.96 to 1.09</td>
<td>-3.23 to 1.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>1.26 to 6.31</td>
<td>-2.96 to 2.10</td>
<td>-3.91 to 1.15</td>
<td>-3.17 to 1.88</td>
<td>-2.47 to 2.58</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>1.92 to 6.98</td>
<td>-2.29 to 2.76</td>
<td>-3.24 to 1.81</td>
<td>-2.51 to 2.55</td>
<td>-1.81 to 3.25</td>
<td>-1.86 to 3.19</td>
</tr>
</tbody>
</table>

*Bolded cells indicates significant difference between treatments at $\alpha = 0.05$*
Split plot ANOVA for sweet flavor scored by a trained panel on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

<table>
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<tr>
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<td>1668.4</td>
<td>1715.4</td>
<td>285.9</td>
<td>2.83</td>
<td>4.28</td>
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Split plot ANOVA for fruity flavor scored by a trained panel on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

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Split plot ANOVA for bitter flavor scored by a trained panel on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

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Split plot ANOVA for other off-flavors scored by a trained panel on milk processed between 120 and 140°C and sampled at 15 d intervals over 60 d.

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*Indicates significant difference between treatments at α = 0.05
ANOVA of HTST, ESL and UHT milk ranked in order of preference by consumer panel.

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<tr>
<th>SOURCE</th>
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*Indicates significant difference between treatments at α = 0.05

Fisher pairwise comparison of treatments. Ranges encompassing zero are not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>ESL</th>
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<tr>
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</tr>
<tr>
<td>UHT</td>
<td>-0.9453 to -0.5445</td>
<td>-1.1392 to -0.7384</td>
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</table>
ANOVA of hedonic scoring of HTST, ESL and UHT milk by consumer panel.

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
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*Indicates significant difference between treatments at $\alpha = 0.05$

Fisher pairwise comparison of treatments. Ranges encompassing zero are not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>ESL</th>
<th>HTST</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTST</td>
<td>-1.327  to -0.143</td>
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</tr>
<tr>
<td>UHT</td>
<td>1.081  to 2.266</td>
<td>1.816 to 3.000</td>
</tr>
</tbody>
</table>
CURRICULUM VITAE

Michael R. Blake
(February 1996)

CAREER OBJECTIVE:

To obtain a position as an applied research scientist in food microbiology that requires technical expertise, leadership, and communication skills. Special areas of interest: food microbiology, biosensors, and application of biotechnology in the food industry.

EDUCATION:

BSc in Microbiology and Biochemistry, LaTrobe University, Victoria, Australia. 1987
PhD in Food Science, Utah State University, Utah, USA. Research emphasis in dairy microbiology and biosensors with industrial application emphasis. March-1996

EXPERIENCE

POSTDOCTORAL ASSOCIATE, Utah State University, Department of Nutrition and Food Sciences, Logan, Utah

Duties: Development of rapid assays for the food industry used for specific quantification of bacteria, bacteriophage, and bacterial enzymes in foods and other complex systems.

LABORATORY TECHNICIAN, Melbourne University, Department of Biochemistry, Parkville, Victoria

Duties: Assisted in studies of the interaction between cheese starter bacteria and their bacteriophage and the related phenotypic and genotypic changes.
LABORATORY TECHNICIAN/SUPERVISOR, Bonlac Foods Ltd., Cororooke, Victoria.


Duties: Laboratory supervisor and technician in a factory QC laboratory. Responsibilities included maintaining cheese starter cultures, and product quality control.

SCIENTIFIC PUBLICATIONS


SCIENTIFIC PRESENTATIONS


