Assessment of Dairy Product Quality Utilizing Bacterial Enumeration and Metabolite Detection.

Jay Russell Bishop
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/4041

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of “sectioning” the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106
ASSESSMENT OF DAIRY PRODUCT QUALITY UTILIZING BACTERIAL ENUMERATION AND METABOLITE DETECTION

The Louisiana State University and Agricultural and Mechanical Col. Ph.D. 1985

Copyright 1985 by Bishop, Jay Russell All Rights Reserved
ASSESSMENT OF DAIRY PRODUCT QUALITY UTILIZING
BACTERIAL ENUMERATION AND METABOLITE DETECTION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

Dairy Science

by

Jay Russell Bishop
B.S., Clemson University, 1977
M.S., Clemson University, 1982
May 1985
ACKNOWLEDGEMENTS

There are so many people to thank for their time, assistance, consideration and patience that I will not attempt to name them all. There are a few people that it is imperative be mentioned. Dr. Charles White has been a constant source of support and guidance under whose leadership I've learned more in the past 2 1/2 years than the previous 27. This is also true of each member of my committee who have been easy to work with and were willing to lend a hand whenever called upon.

A great deal of appreciation is due my wife, Mary K., for her continued support and encouragement throughout this entire process. This dissertation is dedicated to her.
# TABLE OF CONTENTS

| ACKNOWLEDGEMENTS | .................................................. | ii |
| LIST OF TABLES | .................................................. | v |
| LIST OF FIGURES | .................................................. | vii |
| ABSTRACT | .................................................. | ix |

## CHAPTER

### I. INTRODUCTION ..................................... 1

### II. LITERATURE REVIEW ................................ 4
- Keeping Quality ........................................ 4
- Bacterial Enumeration ................................... 8
- Psychrotrophic Bacteria ............................... 12
- Proteolysis ............................................ 17
- Preliminary Incubation ................................ 23
- Moseley Test .......................................... 25
- Shelf-life Test ....................................... 27
- Endotoxin (lipopolysaccharide) Detection .......... 31
- Impedance Detection .................................. 37

### III. MATERIALS AND METHODS ........................... 42

| Phase One |
| Sample collection | ............................................. | 42 |
| Microbiological procedures | ........................................ | 43 |
| Impedance measurements | ........................................ | 43 |
| Shelf-life determination | ........................................ | 45 |
| Statistical procedures | ........................................ | 45 |

| Phase Two |
| Sample collection | ............................................. | 45 |
| Microbiological procedures | ........................................ | 46 |
| Biochemical analyses | ........................................ | 46 |
| Impedance measurements | ........................................ | 48 |
| Shelf-life determination | ........................................ | 48 |
| Statistical procedures | ........................................ | 48 |
TABLE OF CONTENTS (Continued)

Phase Three
Sample collection .................................. 49
Microbiological procedures .................. 49
Biochemical analyses ......................... 49
Impedance measurements ..................... 51
Shelf-life determination .................... 51
Statistical procedures ...................... 51

IV. RESULTS AND DISCUSSION ...................... 53

Phase One .................................. 53
Phase Two ......................................... 59
Phase Three ...................................... 72

V. CONCLUSIONS ................................. 84

REFERENCES ......................................... 92

VITA ............................................. 111
<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flow chart of experimental methods used in Phases Two and Three involving psychrotrophic inoculation; ( ) = cottage cheese</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Correlation coefficients obtained from commercial milk samples (Phase One) (N=100)</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td>Correlations of bacterial enumerations to potential shelf-life of whole milk at 7°C (N=67)</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>Correlations of bacterial enumerations to potential shelf-life of skim milk at 7°C (N=72)</td>
<td>61</td>
</tr>
<tr>
<td>5</td>
<td>Linear and multiple (linear + quadratic) correlations studied to potential shelf-life of pasteurized fluid milk at 7°C</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>Components of a predictive regression equation estimating the potential shelf-life of pasteurized fluid milk in days with $r^2=0.919$</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>Components of a predictive regression equation estimating the potential shelf-life of pasteurized fluid milk in days with $r^2=0.939$</td>
<td>71</td>
</tr>
<tr>
<td>8</td>
<td>Linear relationships of bacterial enumerations to potential shelf-life of cottage cheese at 7°C (N=65)</td>
<td>73</td>
</tr>
<tr>
<td>9</td>
<td>Linear and multiple correlations of all parameters studied to potential shelf-life of cottage cheese at 7°C (N=65)</td>
<td>75</td>
</tr>
<tr>
<td>10</td>
<td>Components of a predictive regression equation estimating the potential shelf-life of cottage cheese in days with $r^2=0.904$</td>
<td>82</td>
</tr>
<tr>
<td>11</td>
<td>Components of a predictive regression equation estimating the potential shelf-life of cottage cheese in days with $r^2=0.938$</td>
<td>83</td>
</tr>
</tbody>
</table>
## LIST OF TABLES (Continued)

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Shelf-life differences between inoculation levels of <em>Pseudomonas fluorescens</em> P27 in dairy products stored at 7°C</td>
</tr>
<tr>
<td>13</td>
<td>Linear and multiple correlations of all parameters to potential shelf-life of products studied, and time required for each</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of impedance detection at 21°C to potential shelf-life of commercial whole milk at 7°C</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of impedance detection at 18°C to potential shelf-life of commercial whole milk at 7°C</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the linear relationship of the Moseley keeping quality test to potential shelf-life of commercial whole milk at 7°C</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of log modified psychrotrophic count to potential shelf-life of fluid milk at 7°C</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of proteolysis (ug glycine-leucine) to potential shelf-life of fluid milk at 7°C</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of endotoxin level to potential shelf-life of fluid milk at 7°C</td>
</tr>
<tr>
<td>7</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of impedance detection to potential shelf-life of fluid milk at 7°C</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of gram-negative bacteria (log) in crystal violet TTC agar to potential shelf-life of cottage cheese at 7°C</td>
</tr>
<tr>
<td>9</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of endotoxin level to potential shelf-life of cottage cheese at 7°C</td>
</tr>
<tr>
<td>10</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Scattergram of the quadratic relationship of proteolysis (ug glycine-leucine) to potential shelf-life of cottage cheese at 7°C</td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td>80</td>
</tr>
</tbody>
</table>

Scattergram of the quadratic relationship of impedance detection to potential shelf-life of cottage cheese at 7°C
ABSTRACT

A study was conducted to investigate the use of bacterial numbers and metabolites as estimators of the potential shelf-life of perishable dairy products, i.e., whole milk, skim milk and cottage cheese. The study was conducted in three phases. In the first phase, a method of impedance detection was performed at two incubation temperatures (18°C and 21°C), and its ability to estimate shelf-life of 100 commercially purchased milk samples compared to standard plate count (SPC), psychrotrophic bacteria count (PBC), modified psychrotrophic bacteria count (mPBC) and Mosely keeping quality test (MSPC). MSPC appeared to possess an adequate relationship to shelf-life (R=-0.839). Impedance detection time (IDT) at 21°C and 18°C proved to have the most significant relationships to shelf-life (R=0.877 and 0.868, respectively).

In the second and third phases of the study, samples of skim and whole milk, and cottage cheese were inoculated with Pseudomonas fluorescens P27 at levels of 0, 10³ and 10⁵ per ml or g. Samples were stored at 7°C. The milks were analyzed every 5 d and cottage cheeses every 7 d until determined to be organoleptically unacceptable. Each sample was analyzed for IDT at 21°C, PBC, proteolysis and endotoxin concentration. Each sample was subjected to preliminary
incubation (PI) of none, 21°C 7 h, 21°C 14 h, 13°C 18 h or 18°C 18 h, then enumerated for SPC, mPBC and crystal violet TTC count (CVT). All bacterial enumerations were significantly related to potential shelf-life of pasteurized fluid milks and cottage cheese but were of little predictive value. Proteolysis was also significantly related to potential shelf-life of products studied but proved to be of more value for estimation of cottage cheese quality. Endotoxin concentration and IDT were significantly related to shelf-life and proved valuable for the formulation of predictive regression equations. Impedance detection resulted in the preferred prediction equation suitable for pasteurized fluid milks and cottage cheese.
CHAPTER I

INTRODUCTION

Milk is defined as the whole, fresh lacteal secretion obtained by the complete milking of one or more healthy cows, excluding that obtained within 15 days before and five days after calving, or such longer period as may be necessary to render the milk practically colostrum-free. The average gross composition of cow's milk is: water, 87.0%; fat, 3.9%; lactose, 4.9%; proteins, 3.5%; ash (minerals), 0.7%; and trace amounts of vitamins. Milk's water content has been shown to be ideal for the growth of microorganisms as is its protein, carbohydrate, mineral and vitamin content, all of which are important to many forms of bacteria (59).

In the days prior to adequate refrigeration, bacterial spoilage ran rampant, and economic losses were staggering (5). Today, farm bulk tanks, every-other-day pick-up at farms, five-day-a-week plant operation, discontinuing of home delivery, and the purchasing of milk only on shopping days have increased the age of milk before consumption (146).

These processing methods and a more critical consuming public have caused the food industries to re-evaluate their quality standards. Quality means different things to different people and standards are ever changing. It has been pointed out that concepts of milk quality depend on
many factors, among them the following: (a) educational background of the individual, (b) economic and social status of the individual, (c) scientific information available, and (d) the ordinances, laws, regulations, etc., under which the dairy industry operates (59). Today, dairy products are subjected to more rigid quality standards than just bacterial counts. The milk industry has been a leader in establishing programs to give the consumer a product that is pure, of good flavor, of attractive appearance and of desirable keeping quality. These programs emphasize rigorous laboratory examination of milk and dairy products to ensure that this quality is maintained (59).

It is a universally accepted axiom that production of such superior quality dairy products possessing extended shelf-life requires high quality milk. Milk and other ingredients used should be free of offending odors and flavors, abnormal chemical and physical properties, and undesirable microorganisms and their metabolites, especially those microorganisms and their resultant metabolites that occur from post-pasteurization contamination (131).

In determining the presence of post-pasteurization contaminants, two major problems are encountered. First of all, time is critical. Rapid results are needed to find and eliminate sources of contamination. Second, the number of contaminants in a freshly pasteurized sample may not be present in detectable levels. The problem of detecting post-pasteurization contamination at a very early stage is
serious. There may never be a perfect test, but any improvement would be helpful, especially if results can be obtained sooner than with the presently used methods (16).

The major goal of any test or assay used to assess the quality of a perishable dairy product must be to provide reliable and accurate results within a test period that would allow for effective corrective measures, preferably less than 48 hours. As a result of this need, the objective of the following research was to utilize bacterial enumeration following various preliminary incubations and detection of bacterial metabolites to assess the quality, and estimate the potential shelf-life, of whole and skim milk and cottage cheese.
CHAPTER II

LITERATURE REVIEW

Keeping Quality

The period between processing/packaging and the time which milk becomes unacceptable to consumers is referred to as the "shelf-life" or "consumable life" of milk. Although it reflects the "keeping quality" of milk, Baker (7) states that there is no adequate working definition of shelf-life. Consumers of dairy products must have some indication of the expected or potential shelf-life of the products they buy. Therefore, to allow consumers to assess the age of products at the time of purchase, a date is placed on the container that indicates either the date of packaging or the last date that the product may be sold or offered for sale. The consumer then expects that a product purchased on any date up to that last date is of acceptable quality. Also, if properly treated, it should remain acceptable beyond the last date of sale (75).

On a daily basis, the consumer uses taste and smell to judge how long milk, kept under refrigeration, maintains an acceptable flavor. This, in effect, is the consumer's own test of keeping quality (12); therefore, flavor and keeping quality are of paramount importance to maintaining consumption of fluid milk (8). The most common descriptions given to unacceptable milk flavors are staleness, putrid, and/or curdled, bitter and fruity (7).
When dairy products leave the plant and are displayed in the supermarket showcases and, finally, reach the home refrigerator, low temperature refrigeration is the exception rather than the rule (146). Temperatures below 7°C are one of the key factors to extending shelf-life (10). It has been generally accepted that an increase in storage temperature of 5°F will decrease the shelf-life by approximately one-half (91). Hankin, et al. (76) concluded that keeping quality at any storage temperature was unrelated to the manufacturer's code date (last day product is to be sold). There was a significant correlation between keeping quality at 10.0°C and the other two storage temperatures of 1.7 and 5.0°C, suggesting a practical test to measure keeping quality at the lower temperatures.

Commercially produced milk stored at 0°C averaged 31 d and 6 d at 7°C, with summer milks possessing twice the shelf-life of winter milks, as reported by Finley, et al. (52). Grosskopf and Harper (66) found that aseptically packaged and stored milk at 4°C had a shelf-life of 28 d. Hankin, et al. (76) discovered that keeping quality of milk samples collected in original containers from fillers and stored at 1.7, 5.6 and 10.0°C remained organoleptically acceptable, on the average, 17.5, 12.1 and 6.9 d, respectively. Harmon, et al. (79) found that the shelf-life of commercial samples of milk was 21 to 63 d when stored at 0.5°C, 7 to 17 d at 6°C, 3 to 7 d at 12°C, and only one day at 20°C. They found keeping quality to be directly related
to rate of development of bacterial populations. They added that the maximum number of microorganisms ultimately attained at spoilage was the same regardless of storage temperature. Janzen, et al. (91) noted a shelf-life of 9 d at 7°C. Langlois, et al. (107) indicated an average shelf-life of skim milk at 4.5°C was 13.3 d. Randolph, et al. (157) found that the average keeping quality of market milk purchased at retail outlets was 7.7 d at 7°C, and 6.2 d at 10°C. The samples were from 0 to 14 d old when purchased. The majority of the samples possessed "psychrophilic" (now known as "psychrotrophic") type of defects at the time the flavor quality was judged unacceptable. Sherman, et al. (178) reported that pasteurized milk had a shelf-life of 8 to 12 w at 0°C and spoilage marked by extensive proteolysis, was of bacterial origin, predominantly gram-negative pseudomonads. Shipe, et al. (179) discovered that the average keeping quality of commercial milk at 7.0°C was 13 d.

Shelf-life is materially affected by days held raw (92), pasteurization temperature, storage temperature and season as determined by taste panel and bacteriological tests (52). Gillis and Custer (63) found that repasteurization did not significantly extend the shelf-life beyond that of the same milk that was not repasteurized. Mistry and Kosikowski (135) reported that sorbate levels of 0.15 and 0.20% in milk at 7°C effectively prolonged quality
and reduced the rate of psychrotrophic bacterial growth, 19-20 d vs. 12-14 d. However, the maximum sorbate concentration allowed in foods is 0.01%.

There exist opinions that the consumer may exercise the most efficient indicator of keeping quality - flavor (7, 8, 72, 73). They report that the flavor score of freshly pasteurized milk, although subjective, is much better than standard microbiological and chemical tests as a predictor of keeping quality. Several researchers (13, 24, 65, 72, 205) conclude that the keeping quality of milk is generally unrelated to standard microbiological and chemical tests.

Differences of opinion are evident. Patel and Blankenagel (148) state that there is no doubt that in commercially pasteurized and packaged milk, post-pasteurization contamination is by far the most common cause of flavor defects of microbial origin. However, even in the absence of contaminants, off-flavors may be encountered if the raw milk contained large populations of psychrotrophs. Genera of bacteria most frequently involved in keeping quality problems in pasteurized milk include Pseudomonas, Achromobacter, Chromobacterium, Alcaligenes, Proteus, Escherichia and Enterobacter. The gelatinous curd and flavor defects of cottage cheese produced by Pseudomonas viscosa, Pseudomonas fragi (old nomenclature) and Alcaligenes metalcaligenes are well known. In milk as well as cottage cheese, Pseudomonas viscosa produces bitter, rancid and unclean flavors while that produced by
*Pseudomonas fragi* is a fairly common fruity defect (46). Elliker (47) found the major source of post-pasteurization contamination by bacteria to be the filler.

The appearance of off-flavors in raw milk, market milk, laboratory pasteurized milk and milk inoculated with *Pseudomonas fluorescens* coincided with psychrotrophic counts of $10^7$/ml. It is apparent that deterioration of pasteurized milk during storage is of microbiological origin, but may not be due to the growth of bacteria per se (7). Matoba, et al. (123) reported that the occurrence of a bitter flavor in milk appears to be related to the presence of a heat-stable protease. Hydrolysis of casein and lactalbumin yields bitter peptides. Therefore, keeping quality depends both on numbers of bacteria and their biochemical activities (118). This fact is illustrated by the research of Patel and Blankenagel (148) where milk with counts $>10^6$/ml before heating frequently developed objectionable flavors after pasteurization and subsequent storage. The most common defect was a bitter flavor which developed in spite of small numbers of organisms in the pasteurized products and in the absence of post-pasteurization contaminations.

**Bacterial Enumeration**

Microbiological tests done on milk have two main purposes: (1) to determine whether sanitary practices are adequate, and (2) to gain information that will aid in predicting keeping quality (118). The standard plate count
(121) is currently an acceptable method for determining the bacterial count of raw and pasteurized milk (199). The method was originally described in the first edition of Standard Methods, but has undergone several modifications since then (163). The basic underlying assumption of the standard plate count - as well as other plate count procedures - is that a single viable cell, when placed in an appropriate temperature, will multiply to the point where a visible colony is produced (187). However, in some cases colonies arise from cells which were originally aggregated in pairs, small chains, or small clusters and cannot be differentiated from colonies which arose from single cells (95, 121). Therefore, the basic assumption of plate count procedures is to some degree invalid (168).

Even though the standard plate count has historically been the main cultural procedure used to determine viable bacterial populations in dairy products (121), literature suggests that the scientific community is not entirely satisfied with this method of microbial enumeration. Aside from being inaccurate, the standard plate count has been criticized as being time consuming and expensive (12, 61, 62, 95, 151), not indicative of raw milk quality (12, 40, 81, 82, 98, 99, 108, 159, 166, 212, 213), and slow as a means of generating bacterial population estimates (62, 64). Regardless of the drawbacks or problems associated with the standard plate count, it is officially considered as a suitable method for measuring bacterial populations in most
types of dairy products (121). It is not a suitable method for detection of post-pasteurization contamination or indication of keeping quality as it does not differentiate between those organisms that survived the heat treatment and those that gained entry into the product after pasteurization (16).

The coliform count (121) is also used as an indication of contamination of dairy products. If coliform bacteria are present in pasteurized products, one can be sure post-pasteurization contamination has occurred. However, the absence of coliforms is no guarantee the product is free of contaminants. This method is limited due to the fact that only a relatively small group of all possible contaminants are coliforms (16).

Although all contaminants are important, the main concern of the dairy processor is the group of organisms that can grow at refrigeration temperatures - the psychrotrophs. These will be discussed in the next section. Zall, et al. (220) found significant correlation \((r=0.878)\) between standard plate count and psychrotrophic bacteria count values.

Determination of the pyruvate content of milk is a means of measuring bacteriological quality. One advantage of this approach is that the analysis can be automated to test 80-120 samples/h. The analysis involves the breakdown of pyruvate catalyzed by lactic dehydrogenase in the presence of reduced nicotinamide-adenine dinucleotide (NADH),
and it is the concentration of this cofactor which is measured spectrophotometrically and related to pyruvate concentration. This method relies essentially on two factors. First, that pyruvate production by all bacteria in milk is in some way equal. This is not true because some organisms do not produce pyruvate while others produce varying amounts depending on their physiological state. Second, the method uses the colony count procedure as its reference point (145). Ledford, et al. (110) reported a correlation between pyruvate and standard plate count of 0.72, while Marshall and Harman (118) found no association between standard plate count and pyruvate concentration.

Adenosine-5'-triphosphate (ATP) detection has been greatly simplified by the discovery of the luciferin-luciferase bioluminescent reaction which contains two key properties that render it applicable to the quantitative measure of ATP. First, the amount of light produced during the reaction is directly proportional to the concentration of luciferin and ATP in the reaction mixture (127). Secondly, ATP is the primary high energy nucleotide that participates in the bioluminescent reaction (168).

The luciferin-luciferase assay has previously been used to measure viable organisms in many diverse environments including water (114), soils (44, 94), rumen fluids (48, 49, 57, 217) and foods (21, 177). Several dairy applications of the luciferin-luciferase assay have been reported, including estimation of somatic cell and bacterial contents of raw
milk (18). Correlation of the ATP content of 48 tank truck samples and standard plate count was 0.93 (18). Correlation between the ATP content of 209 farm bulk tank samples and standard plate count was also 0.93 (168). Measurement of milk sample ATP content was considered to be an acceptable method of detecting high bacterial count milk (18,168).

Another measure of bacterial content was suggested by O'Toole (145) who stated that cell numbers are no more than a crude measure of cell mass. The dry mass of bacterial cells in a sample is probably the best measure of the bacterial content, if its determination could be achieved.

**Psychrotrophic Bacteria**

When considering the shelf-life of refrigerated milk and milk products, the concern is almost exclusively with those microorganisms which grow rapidly at storage temperatures. These bacteria were referred to as "psychrophiles", a term which means "cold-loving". This is a misnomer as they are not "cold loving" but rather "cold enduring"; while they are able to grow below 10°C, they grow much better at ambient or higher temperatures. A more appropriate name is "psychrotrophs" - "cold enduring" (97). These are defined in essentially four major ways based on (1) optimum growth temperature, (2) ability to grow at low temperatures, (3) method of enumeration and (4) criteria which are independent of the temperature (216).
Psychrotrophic microflora consist of microorganisms that can grow relatively rapidly at commercial refrigeration temperatures. This does not imply that the optimum temperature of growth of this group of organisms lies in the temperature range encountered in normal commercial refrigeration of foods. Optimum growth temperature of many psychrotrophs is in the mesophilic range (20-45°C). The term psychrotrophic was coined to accommodate the heterogeneous group that did not fall within the strict physiological definition of the psychrophilic group. Currently, the term psychrophilic is reserved for microorganisms that optimally grow at temperatures below 10°C (obligate psychrophiles) (200).

Psychrotrophs are mostly gram-negative, non-spore forming rods; they are usually aerobic; nearly all of them produce heat resistant metabolites; most of them are fairly resistant to penicillin and some other compounds. This group includes bacteria belonging to the genera Pseudomonas, Achromobacter, Flavobacterium and Alcaligenes, which are gram negative (216). Among the gram-positive bacteria, enterococci, micrococci and certain species of thermoduric Bacillus and Clostridium are important (204). Additionally, certain molds and yeasts are capable of rapid growth in refrigerated foods (200). Among psychrotrophs producing heat-resistant proteases, pseudomonads are the most common contaminants of milk (149).
Psychrotrophs are generally found in water and soil and are introduced into milk through these sources and become established on milk contact surfaces, equipment, flooring and drains in the processing plant. They may be introduced via wash water (200). Milk produced under sanitary conditions usually contains less than 10% of the total microbial flora as psychrotrophs, but milk produced under unsanitary conditions can contain more than 75% psychrotrophs (93).

While there have been a few reports of psychrotrophs surviving laboratory pasteurization in very small numbers, there is abundant evidence that in commercial operations this rarely happens. Consequently, there is almost a 100% certainty that their presence in a pasteurized product represents recontamination beyond the pasteurizer. In rare instances this recontamination may occur in the pasteurizer through leaking gaskets, hairline or pinhole cracks in plates, etc (97). With conventionally pasteurized milk samples held at 4.4 and 7.2°C, psychrotrophs increased from 1/ml to $10^8$/ml after 10 d at 7.2°C (131). El-Farekh (45) calculated the generation time of *Pseudomonas fluorescens* to be 7.2 h at 7.2°C.

The standard enumeration method for psychrotrophic bacteria involves pour-plating a sample in tryptone yeast extract agar (TGE), and incubation for 10 d at 7°C (121). A more rapid technique was developed by Oliveria and Parmalee (143), who reported that enumeration of psychrotrophic bacteria at 21°C for 25 h produced counts in very good
agreement with those obtained by standard psychrotrophic count. Correlation coefficients in raw milk were 0.992 and in pasteurized milk, 0.996. More specific detection and enumeration of gram-negative bacteria is accomplished by the use of inhibitors. Crystal violet and neotetrazolium chloride inhibits growth of gram-positive bacteria without causing inhibition of gram-negative bacteria (39, 184). Oehlrich and McKellar (141) evaluated an 18°C/45 h plate count technique for the enumeration of psychrotrophs with correlation values to the 7°C, 10 d method of 0.866 in raw milk and 0.936 in pasteurized milk.

Many psychrotrophs produce lipases which can degrade milk fat and cause flavor problems in dairy products (56). Most thermal processes may leave heat-stable lipases almost intact (3). The major producer of heat-stable lipases in milk appears to be *Pseudomonas* (*P.*) *fluorescens* (56). Frieden (60) stated the lipase from *P. fluorescens* is activated after a short heat treatment. Also, enzymes and enzymatic activities are connected with association to other molecules, e.g. proteins, and this activation is probably from dissociation of an enzyme-inhibitor-complex as a result of the heat treatment. The lipase was thermostable enough to be active in foods which were subjected to high-temperature-short-time (HTST) sterilizing processes. As a consequence, lipases can cause quality changes in foods and reduce their shelf-life (3, 165).
During psychrotrophic growth in milk, certain gross changes occur which are: (1) a decrease in the total protein content, (2) changes in the relative amounts of the protein fractions and (3) the appearance of two different atypical fractions (146). Psychrotrophic microorganisms may have an indirect as well as a direct effect on the quality of dairy products. Indirectly, psychrotrophs produce off-flavors and odors during growth in stored refrigerated raw milk which may carry over into the finished product even though the organisms fail to survive pasteurization. Directly, organisms surviving pasteurization or resulting from post-pasteurization contamination may multiply in sufficient numbers during manufacture and storage of dairy products so as to reduce the shelf-life, the quality and the quantity of the finished product (131). These bacteria cause bitter, fruity, rancid or yeasty flavors (33). Psychrotrophic levels in excess of $10^7$/ml are usually required before sensory changes are detected in milk (111, 131, 156).

Cottage cheese manufactured from milk in which psychrotrophs had outgrown to levels >$10^7$/ml and then pasteurized at 62.8°C for 30 min was found to have a firmer curd and to require less manufacturing time than control milk. However, the cottage cheese was organoleptically unacceptable (85). Mohamed and Bassette (137) attributed cottage cheese vat failure to milk heavily contaminated with psychrotrophs whether the cheese was manufactured by the direct-acid-set method or by the conventional starter culture procedure.
The formed curd disintegrated and shattered during the manufacturing procedure. It was found that average cottage cheese yields decrease (6, 83). Principal psychrotrophic defects in cottage cheese are slime formation, surface discoloration, off-odors and off-flavors (120). Stone and Naff (188) found increases in soluble nitrogen and bitter flavor to be associated with increases in psychrotrophic bacteria in cottage cheese. Contaminated water has been shown to be a source of cottage cheese spoilage organisms since the curd is washed and held at low temperatures (139).

Psychrotrophic coliforms' survival in a cultured product was found to be limited, and it was concluded that they have little effect on shelf-life (170).

**Proteolysis**

The first indication of naturally occurring proteolytic enzymes in milk was published as early as 1879 (161). Storrs and Hull (189) also contributed substantially to the belief that the protease enzyme is a natural constituent of milk. Harper (80) stated that raw milk usually (but not always) contains a small and variable amount of protease enzyme, and that proteolysis is slight. The most frequently represented protease-producing psychrotrophic gram-negative bacterial genus is *Pseudomonas*, with *P. fluorescens* as the most common species (109).

Shahani (176) reported that at least 19 enzymes have been found in normal cow's milk. Enzymes occurring naturally
in milk could be classified as follows: (a) enzymes acting as a hydrolytic group, (b) enzymes having a physiological role, (c) enzymes associated with the microsomal particles of milk and (d) enzymes having an unknown role. Proteinases and peptidases constitute the primary enzyme forms in bacteria responsible for proteolysis of milk proteins (206).

Most of the work on identification of native milk proteinases have been with the trypsin-like enzymes (27, 43, 106, 196). These enzymes belong to the group of serine proteinases and predominantly hydrolyze b-casein (67). Results indicate that the trypsin-like enzyme system consists of two serine proteinases and, in addition, a chymotrypsin-like enzyme. Gamma-caseins and related phosphoproteins are fragments of b-casein. However, gamma-casein formation during storage of milk would be expected to be as slow as the enzyme reaction would be limited by association of both substrate (b-casein) and enzyme with casein micelles.

An entirely different situation exists during cold-storage of bulk milk at 2 to 6°C. Large quantities of b-casein dissociate from micelles into milk serum primarily because of its hydrophobicity and to changes in the salt equilibrium (160). Behavior has been similar for proteinases associated with micelles. Proteolytic activity brings about a modification of the electrophoretic pattern of dairy products (87).
Pennington, et al. (150) made the observation that when milk was held at 0°C, the following phenomena were noted: (a) proteolysis of casein was primarily of bacterial origin, (b) proteolysis of lactalbumin was due primarily to native enzymes of the milk and (c) both enzyme systems combined gave rise to more rapid proteolytic changes than either system alone.

Warner and Polis (203) stated that proteolysis that occurred in casein solutions was attributed to the presence of an enzyme on the basis of the following evidence: (a) the activity was affected by heat, (b) the activity had a definite optimum pH, (c) the activity could be concentrated and (d) the proteolysis proceeded in sterile solutions. Peterson and Gunderson (152), in studying certain characteristics of proteolytic enzymes from P. fluorescens, reported that extracellular, proteolytic enzyme elaboration was inversely proportional to the temperature, at least from 0 to 30°C. It would appear temperature plays an important role in the effect of proteolytic activity on dairy quality.

Due to heat resistance, Sandvik (172) concluded that the interpretation of the food spoilage potentialities of proteolytic bacteria should include the possible residual effect of the enzyme after pasteurization or sterilization. Extracellular enzymes, produced by microbes in refrigerated foods prior to heat treatment, may not be completely inactivated by the heating and may be active in the stored product (126). Protease enzymes survived 70°C for 30 min; however,
when heating was at 40°C for 30 min, proteolytic activity decreased significantly (30). Several researchers have shown protease inactivation at 63°C for 15 h, 71°C for 8 h and 121°C for 9 min (1,119,126). Adams (1) compared inactivation of MC 60 protease at 149°C to inactivation of bacterial spores to establish the ultra-high-temperature (UHT) sterilization parameters. The protease was more than 4,000 times more resistant that Bacillus stearothermophilus spores. The resistance of psychrotrophic proteases suggests that their destruction by heat is impractical.

The destruction of heat-resistant bacterial proteases at sub-sterilization temperature may be feasible. Maximum low temperature inactivation occurred at 55°C. For low temperature inactivation to be beneficial, it is essential that inactivation occur at all protease concentrations. The extent of protease inactivation appeared to be independent of protease concentration and, therefore, could occur at the low protease levels which might be in raw milk (9). The practicality of this procedure in a dairy plant is questionable.

Optimum temperature and pH ranges for protease activity are 5 to 50°C and pH 6 to 8 (1, 29, 119). Therefore, it is evident that environmental factors cause variability of protease activity. Half (46%) of the variability may be explained by milk pH (26%), by psychrotrophic bacteria (9%), and by the stage of lactation (11%). The second half (54%)}
of this variability in activity could be due only to variations of the native milk proteinase system (88).

The degree of measurable proteolysis is directly correlated with the incidence of the naturally occurring "bitter" flavor (36, 84, 105, 207, 209, 210, 211). Janzen, et al. (93) reported a significant relationship between protease activity and flavor score of whole and skim milk \((r=-0.936\) and \(-0.917\), respectively) at \(7^\circ\text{C}\). Also concluded in the study was that, because the shelf-life of skim milk was significantly less than that of whole milk, and protease activity was significantly higher in skim milk as compared to whole milk, the increased protease activity in the skim may be partially responsible for its decreased shelf-life.

The successful use of UHT treatment of milk can be hindered by heat stable enzymes in milk (138). White and Marshall (208), and Thomas and Mills (195) showed a reduction in shelf-life of Cheddar and cottage cheese due to the addition of a heat-stable protease enzyme.

Speck and Adams (186) introduced the following methods for controlling heat-stable proteases in milk: (1) prevent contamination by psychrotrophs; (2) prevent growth and metabolism by psychrotrophs by lower temperature and decreased aeration, addition of antibiotics and other inhibitors, and addition of starter organisms; (3) UHT inactivation; and (4) inactivation at sub-pasteurization temperatures.
There exists many methods for the detection of proteolysis. The trichloroacetic acid (TCA) method of Hull (86) is the most widely used to date, but not necessarily the best. Samples, et al. (171) and McKellar (128) found the trinitrobenzene sulfonic acid (TNBS) to be more suitable for detecting proteolysis than the Hull procedure. A sensitive assay for protease activity based on the reaction of primary amino groups of trichloroacetic acid-soluble peptides and amino acids with fluorescamine was found to be suitable for determining protease activity in general (28). Halambeck, et al. (69) stated that because the Hull procedure is specific to the release of aromatic amino acids, it lacks sufficient sensitivity to detect small differences in the release of other amino acids and peptides. More sensitive methods are based on amine reaction with TNBS or fluorescamine. Church, et al. (31) introduced a spectro-photometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. Because all hydrolytic products are assayed, the method is more accurate than the Hull procedure which depends upon properties of aromatic residues. Furthermore, the o-phthaldialdehyde assay is more rapid and convenient than methods using ninhydrin, TNBS or fluorescamine. Hutkins and Marshall's (89) immunological detection procedure of protease activity did not show promise or utility. The same was the case with the dialysis method of Mistry and Kosikowski (136).
Preliminary Incubation

Preliminary incubation (PI) is based upon the theory that as the holding temperature of milk is lowered, a point is reached where the udder flora no longer multiplies while many of the psychrotrophic contaminants grow actively. Preliminary incubation encourages the growth of bacterial contaminants, and aids in detecting milks where good cooling masks insanitary production and handling practices. Experimental evidence has indicated that the ratio of bacteria detected before and after PI is closely related to the cleanliness of milking equipment, especially milking machines (96).

The PI count is simply a standard plate count (SPC) following incubation of a raw milk sample for 18 h at 12.8°C (121). The test is designed to detect sanitation problems (169). Chalmers (26) in Scotland had suspected that efficient cooling in farm bulk tanks might cover up insanitary practices. To check this, he held samples at 15°C for 21 h, then ran a second set of plate counts at 37°C. He concluded that 24% of the 103 samples gave initial counts under 10,000/ml which did not reflect the probable true hygienic conditions of milk production. Other Scottish workers (181), following Chalmers' (26) lead, repeated the analysis on similar samples after holding for 24 h at 12.8-15.6°C. Because certain samples with initial counts under 10,000/ml showed a hundred-fold increase following PI, they concluded that there would seem to be little correlation between
production methods and the relative increase in count. Meany (129) applied PI at 15.6°C for 24 h to a series of samples from selected farms in the Chicago area. Differences in the degree of increase were related to the conditions of production; when milking equipment was thoroughly cleaned, much smaller increases in counts were noted. Davis and Killmeier (41) reported excellent results using 12.8°C for 18 h.

Several workers (81, 96, 99, 162) have reported that the PI count is a better indicator of raw milk microbiological quality than the SPC. A large magnitude of change in the total bacterial count following PI indicates psychrotrophic contamination (99, 158, 212). Using the PI count, 100,000-200,000/ml is suggested as a desirable standard. This would serve to emphasize thorough cleaning along with efficient cooling in the production of quality milk (11, 96, 121, 169, 197).

Additionally, the PI count is considered to provide a good indication of potential keeping quality of milk and potential shelf-life of dairy foods processed from raw milk (11, 212, 213, 214, 215). Phillips, et al. (154) introduced a test involving preincubation of samples at 21°C for 25 h in the presence of a mixture of nisin:penicillin:crystal violet to prevent growth of gram-positive organisms to identify post-pasteurization contamination of milk and single cream. This test (P-INC test) could successfully
predict the level of contamination after storage at 6°C for 7 d in 85% of milk samples.

Ledford (112, 113) examined the practice of PI prior to coliform enumeration in violet red bile agar. He found that PI 14 d at 6.7°C indicated coliforms in 75% of the samples. Unsatisfactory flavor scores were observed in 91% of the samples positive for coliform. Shorter PI periods resulted in much lesser percentages of related flavor scores. Preliminary incubation at 25°C for 20 h milk samples resulted in no significant increases in tyrosine values, as an indication of proteolysis, compared to unincubated samples (104).

Johns (96) stated that it is not contended that PI will detect every type of unsanitary condition or practice. Nevertheless, it is believed that PI furnishes more information regarding the care taken in production and handling than can be obtained from analyzing a freshly taken sample of milk, no matter which of the several current testing methods is employed.

Moseley Test

Over 25 years ago, the W.K. Moseley Laboratory at Indianapolis began conducting a second SPC on processed samples after holding them at 7.2°C for 5 d (97). The basis of the Moseley Keeping Quality Test is that practically all of the bacteria that grow well in milk in 5-7 d at 7°C are destroyed by pasteurization. By conducting this test, two
important types of information can be obtained. First, if the bacterial numbers increase appreciably during storage, the evidence is strong that post-pasteurization contamination has occurred. Second, if the plate count on the fresh sample is high, thermoduric bacteria are probably in the raw product which is a situation that occurs less frequently (46).

Application of the 5 d at 7.2°C keeping quality test followed by careful study of contamination sources has greatly improved shelf-life of pasteurized fluid milk and has represented a real economic advantage to plants adopting the program (46). Several researchers (51, 107, 157, 183) have reported a highly significant correlation between the keeping quality of pasteurized fluid milk and the Moseley test, with the highest correlation value being -0.70. But, each have pointed out the fact that, even though Moseley test results are correlated to keeping quality, the time necessary for obtaining test results is prohibitive to adequate quality control. By the end of 7-9 d, the product is already in the home of the consumer. Thus, it is no longer under the control of the producer where corrective measures could be taken.
Shelf-life tests

If there would be such a thing as an ideal test for determining post-pasteurization contamination, and, therefore, determining potential shelf-life, it would have the following characteristics: (1) the ideal test would be accurate, i.e. it would indicate exactly the number of organisms which enter the product after pasteurization. It would differentiate between thermoduric bacteria that survived the heat treatment and post-pasteurization contaminants, regardless of how few there may be. (2) The ideal test would provide results within the shortest time possible. There is little value in a method that tells us the quality of a container of milk sold two weeks ago. A little accuracy could be sacrificed to get results the next morning after processing. (3) Such a test would be simple to do and it would be economical (16).

Examples of attempts to develop less time-consuming tests: (1) use preincubation to build up relatively large populations that are easier to enumerate; (2) use certain selective media that permit growth of contaminants but inhibit growth of other bacteria; (3) test for certain end-products of microbial metabolism or changes in the substrate; (4) apply the sample on the surface of agar media to accelerate growth of aerobes; (5) conduct flavor tests; and (6) use of combination of 2 or more of these (16, 213).

The entire concept of shelf-life is based on the well-documented supposition that the primary cause of
shelf-life deterioration is that of gram-negative psychrotrophic bacteria. Most of these bacteria are members of the genus *Pseudomonas*. Consequently, most of the tests which have been designed to predict or estimate the shelf-life of fluid dairy products are geared to measure these pseudomonads (213).

The chief objection to Moseley's storage quality test (121) has been that a week or more elapses before the results are available. Various workers have suggested different ways of shortening the waiting period. Gyllenberg (68) used ammonium lactate agar and ammonium lactate crystal violet agar, and incubated plates 2 to 4 d at 28°C, for determining the incidence of organisms responsible for the spoilage of refrigerated milk. Freeman, et al. (59) investigated the inhibitory action of various chemicals on gram-positive organisms. They reported that 0.5% sodium desoxycholate (SDC) was the most effective of all those tested.

Olson (144) recommended the CVT test, in which 2 ppm of crystal violet plus 50 ppm of 2,3,5-triphenyltetrazolium chloride (TTC) are incorporated into the plate count agar to repress gram-positive bacteria and also make gram-negative colonies more distinctive. Sing, et al. (181) reported poor correlation between CVT counts and SPC values after 7 d storage at 7.2°C, with only 15% of 90 samples agreeing. Lightbody (117) used 10 IU penicillin plus TTC to suppress gram-positive organisms and found a highly significant
correlation between count and samples after holding 24 h at 20°C, 4 d at 5°C, or 4 d at 10°C with plates incubated 3 d at 30-32°C. Blankenagel and Humbert (15) described a surface disc method using crystal violet as an inhibitor.

Taking advantage of the fact that gram-negative contaminants are catalase positive, strongly aerobic, and not repressed by surface-active agents, Maxcy (124) developed the following procedure: (1) plates are poured with nutrient agar containing 0.5% alkyl aryl sulfonate; (2) plates are dried 48 h at 32°C, then 0.5 ml milk is carefully "spotted" onto the surface in separate droplets; (3) plates are allowed to stand undisturbed for 30 min while the milk is absorbed before being incubated in an upright position for 16-20 h at 32°C, plus 1 h at 7°C with covers removed; and (4) plates are then flooded with 5% solution of hydrogen peroxide. Catalase-positive colonies decompose the hydrogen peroxide and are recognized by the gas bubbles released; only these colonies are counted. This count is related to post-pasteurization contamination. Hankin and Dillman (70) used the fact that pseudomonads are strongly oxidase-(+) to correlate the oxidase test with keeping quality. A gram-negative psychrotrophic bacteria count has been highly correlated (74, 214, 215) and not correlated at all (130) to keeping quality. Hankin and others (71, 76, 77) found an inverse correlation between flavor score and SPC and the oxidase count.
Janzen (90) suggested that shelf-life of pasteurized milk is influenced by somatic cell concentration in raw milk. High somatic cell concentrations are indicative of abnormalities that frequently involve increased microbial flora.

Boyko and Blankenagel (20) utilized the findings of Freeman, et al. (59) that 0.5% SDC was the best inhibitor of gram-positive organisms. They found that all 45 test organisms which grew in milk containing SDC were killed by laboratory pasteurization and, therefore, assumed that organisms present in processed milk which grew in the presence of SDC were contaminants. Catchick and Gibon (25) sought a way to shorten the SDC test to 16 h so that results would be available the next day. They modified the SDC test by adding resazurin. The advantages of this resazurin reduction test are: (1) use of a large (9 ml) sample, (2) use of a PI to build up small numbers, (3) detection of only gram-negative organisms, and (4) providing results the next morning (16). The methylene blue test after pre-incubation at 18°C for 18 h was a good index of keeping quality, as determined by Jooste and Groeneveld (100). Custer and Knight (38) found the Early Detection (SDC + resazurin) test to be as accurate as the Moseley test in measuring the shelf-life and the results can be obtained 6 d sooner. Waes and Bossuyt (201) reported that the benzalkon-crystal violet-ATP method was useful for predicting keeping quality.
Marshall and Harmon (118) found pyruvate concentration to be of limited value as a quality test for milk due to great variability. Senyk, et al. (175) and Shipe, et al. (179) obtained correlation values of flavor score to pyruvate content of -0.81 and -0.78, respectively. Asher and Sargent (4) revealed the best fitted regression equation ($r^2=0.615$) with data on changes of acidity, pH, acid degree value and free fatty acids as related to keeping quality. Shipe and Hsu (180) measured fluorescent compounds in milk to provide an index of milk quality.

For any of the aforementioned shelf-life tests to be effective, there must exist heterogeneity of samples. Maxcy and Wallen (125) stated that a single package provides low probability for predicting behavior of the entire production lot. The extreme differences in spoilage rates of individual units with sample sets indicated sensory evaluation of multiple samples to be the most logical, simple criterion for evaluating shelf-life.

The aim of shelf-life tests should be to detect and eliminate all sources of recontamination. When this has been accomplished, shelf-life should no longer be a problem (97).

Endotoxin (lipopolysaccharide) Detection

Levin and Bang (115) originally described the ability of amoebocyte lysate from *Limulus polyphemus*, the horseshoe crab, to form a gel in the presence of minute amounts of
endotoxin. Since that time, the Limulus assay has been used as a method of detecting endotoxin in patients with gram-negative septicemia (23, 116, 164), for the study of experimental endotoxemia and shock in animals (37), and as a method of detecting pyrogen in parental pharmaceuticals (34). This method has been shown to be the most sensitive method available for the detection of endotoxin (35, 101).

According to the reported mechanism of the Limulus Amoebocyte Lysate (LAL) assay, endotoxin activates a proenzyme-enzyme cascading reaction resulting in activation of coagulase (142, 192). Coagulase hydrolyzes coagulen, a clottable heat-stable protein (191, 193), at arginine-glycine and arginine-threonine linkages, resulting in the removal of the peptide-C chain to form a gel (coagulin) (198).

With this particular assay, there included the following "grading of lysate gelation": 4+ - firm gel with considerable opacity; 3+ - soft gel with moderate to considerate opacity; 2+ - weak gel with slight to moderate opacity and adhesion of starch-like floccules to sides of the tube when slanted; 1+ - very weak gel with slight opacity and with some starch-like floccules adhering to sides of the tube; negative - no visible increase in viscosity or opacity. As little as 100 pg/ml of certain endotoxins may be reliably detected (100).

DiLuzio and Friedman (42) suggested the Limulus assay might be used for detection of bacterial endotoxin in
drinking water and other surface waters. Jorgensen, et al. (102) reported that the Limulus assay procedure was easily adapted to the test of water samples for endotoxin. Measured endotoxin concentrations varied from 0.78 ng/ml to 1,250 ng/ml. The Limulus assay is currently permitted by the Food and Drug Administration to determine possible endotoxin contamination of ingredients used to prepare parental and biological products in the pharmaceutical industry. The great advantage of the assay is its rapidity— a total test time of less than 2 h.

The Limulus endotoxin assay was compared to the SPC and coliform count for assessment of the bacteriological quality of reclaimed waste water by Jorgensen, et al. (103). LAL assays were technically simpler to perform and provided results much sooner than conventional culture methods. However, the endotoxin values did not correlate extremely well with determinations of viable bacterial numbers. This lack of correlation may have been due to alterations in the normal ratio of viable gram-negative cells to endotoxin caused by water reclamation procedures. Seiter and Jay (174) determined endotoxins in ground beef by the LAL assay.

Clark (32) evaluated various methods to enumerate psychrotrophic bacteria in fluid milk and concluded that the LAL test could be used as a simple shelf-life projection test. Mikolajcik and Brucker (132) studied the LAL assay's potential for detection of psychrotrophic spoilage organisms.
in food systems. They stated that the LAL assay is a rapid and highly sensitive method to detect endotoxins in biological systems. Endotoxin activity is associated with the Lipid A segment of lipopolysaccharides constituting the outer cell membrane of gram-negative bacteria (GNB). Both viable and non-viable GNB are detected which makes this test particularly useful in tracing the history of the milk supply. The LAL assay is finding increasing use as a rapid indirect indicator of gram-negative spoilage organisms in refrigerated meat and dairy products. The LAL assay will detect $10^3 - 10^4$ GNB/ml of milk. Mikolajcik and Brucker (134) also found that for each log increment in GNB population, the lipopolysaccharide (LPS) titers also increased one log cycle with a correlation coefficient of 0.88. Average LPS values increased from 0.86 ng/ml to 4,350 ng/ml as log increments of GNB/ml increased from 1.0 to 8.0 with correlation coefficient of 0.95. Currently used GNB enumeration procedures can detect viable GNB in the product resulting from post-pasteurization contamination but not GNB which were present prior to pasteurization. Pasteurization did not affect the LPS titer as it is heat-stable. When the mean GNB counts of commercial pasteurized milk were plotted for each LPS value, a correlation coefficient of 0.99 was obtained. For 186 individual trials, a correlation coefficient of 0.98 was obtained.

Mikolajcik (133) revealed that the LAL assay will detect within one hour as few as 100 GNB/ml of milk. The
accuracy of the test improves where counts exceed 10,000 GNB/ml of milk. The outstanding feature of the procedure is its ability to detect the quality of the raw milk supply even after pasteurization. Thus, it furnishes information about the number of GNB which were present in the raw milk supply and the possibilities that proteases and lipases might be present in the milk which would affect the keeping quality of the finished product.

Sudi (190) discovered that, because of the heat stability of GN-LPS, the evidence of bacterial growth in raw milk cannot be destroyed by an UHT treatment, as detected by the LAL assay. In fact, there was an apparent 3.6-fold increase of GN-LPS concentration following UHT treatment. It is proposed that the main advantage of the Limulus assay lies in the assessment of the bacteriological quality of heat treated dairy products, like UHT milk, where cultural methods are negative.

Evans, et al. (50) modified the Limulus lysate assay so that the reaction of lysate and endotoxin formed a turbid suspension instead of a firm clot. Absorbancy at 360 nm is measured. The firm-clot method was found to be a less sensitive and reproducible technique for the detection of endotoxin than was the spectrophotometric modification of the Limulus lysate assay. Tsuji and Steindler (198) increased the LAL sensitivity to endotoxin approximately 20 to 30-fold when lyophilized LAL is reconstituted with depyrogenated natural seawater. Magnesium was identified as
the component present in natural seawater and commercial sea
salts which is responsible for the increased sensitivity.
The greatest endotoxin sensitivity for the LAL reagent was
achieved with solutions of magnesium ranging from 50 to
60mM. Magnesium may increase the LAL sensitivity by: (1)
acting at the initial endotoxin recognition phase by
modifying the size of endotoxin for easier recognition by
LAL, (2) accelerating the rate of proenzyme-enzyme cascading
reaction, (3) contributing to the formation of a firmer gel
or increased turbidity by strongly bridging gel micelles, or
all three.

Bodyfelt, et al. (17) reported the LAL test included
the following limitations: expensive reagents, lack of
sufficient sensitivity to micro-quantities of endotoxins,
and complexity of the procedure. This lack of sensitivity
was alleviated by Bishop, et al. (14) by removing the
problem of varied background turbidity by "blanking" each
sample with an unincubated duplicate. They also detected
significant linear relationships between LPS content of milk
and psychotrophic bacteria count \( r = 0.715 \), flavor score
\( r = -0.918 \) and days of storage at 4.5°C \( r = 0.939 \).

Sudi (190) pointed out that the Limulus test may easily
develop into an entirely new double-purpose method of food
analysis. The test may not only prove to be suitable for
the detection of manipulations of raw materials, but it may
also provide means for demonstrating that good manufacturing
practices have been followed as closely as possible.
Mikolajcik (133) feels the Limulus test, because of its sensitivity and accuracy and because it yields results quickly, will be of tremendous value to the dairy industry as a rapid screening test for raw milk and as a guide for predicting the shelf-life of pasteurized and UHT milks.

**Impedance Detection**

Several rapid automated methods have been proposed to overcome the disadvantages inherent in plate counts. These include: ATP photometry (219), fluorescence microscopy (153), radiometric measurements (155) and impedance detections (22). The impedance method has been considered to be the most promising of the instrumental methods (218).

Impedance is the resistance to the flow of an alternating electrical current through a conducting material, and has been shown to be a complex entity composed of a resistive (or conductive) component and a reactive component (capacitance). The impedance technique relies on the fact that metabolizing microorganisms alter the chemical composition of the growth medium and that these chemical changes cause a change in the impedance of the medium (2, 147). Total impedance change represents the vector sum of changes in the resistance and capacitance components of the growth medium. Impedance changes are detectable when the concentration of microorganisms exceeds a threshold level of \(10^6\) to \(10^7\) cells per milliliter. The time required for the initial inoculum to reach the threshold level is designated
as the detection time and is a function of both the initial concentration and the specific growth kinetics of the organism in the given medium. By comparing the detection time obtained to the results of a standard calibration curve, an estimate of the initial concentration of microorganisms can be made. The impedance detection time (IDT) is defined as the accelerating change in impedance associated with microbial growth and metabolism. The computer uses a complex algorithm that determines the onset of acceleration in the impedance curve. The calculation of the IDT from impedance curves is a critical part of the impedimetric method. In order to obtain consistent interpretations of IDT by the computer algorithm, it is important to generate impedance curves with smooth baselines and sharp accelerations. It is important to realize that the basis of the impedimetric estimation is metabolic change, while that of a colony count estimation is biomass production. The consequences of this difference must be carefully considered in the preparation of samples for impedimetric estimation of the number of microorganisms (53).

Impedance measurements are particularly useful for rapidly screening various foods to determine whether they meet the desired microbiological criterion. Rowly, et al. (167) found the impedance method to be promising as a rapid screening technique to determine if cooked meats have less than 1 coliform per gram. Hardy, et al. (78) used the impedance method to rapidly assess whether a sample of
frozen vegetable contains greater or less than $10^5$ organisms per gram. Firstenberg-Eden and Klein (54) obtained a correlation coefficient of 0.90 between the impedimetric test and violet red bile agar (VRBA) plate counts for coliform using a total of 91 meat samples.

O'Connor (140) used an impedance method for the determination of bacteriological quality of raw milk, and found that an IDT of 8.5 h differentiated samples with less than or greater than $10^5$ bacteria per milliliter. Gnan and Luedecke (64) stated that impedance detection offers a definite time advantage, approximately 7 h vs. 48 h for the SPC, when examining raw milk samples. A classification system of selecting a cut-off detection time of approximately 7 h is helpful in screening out samples likely to have a SPC exceeding $10^5$/ml.

Waes and Bossuyt (202) described a simple impedimetric method to detect, within 2 h, complete failure of a starter due to bacteriophages in the manufacture of Cheddar cheese. This method is based on the observation that about $10^5$ disturbing bacteriophages per milliliter, which cause complete failure of the starter, inhibit the normal impedance of lactic starter bacteria.

Firstenberg-Eden and Tricarico (55) revealed that a rapid impedimetric determination for total, mesophilic and psychrotrophic counts in raw milk showed correlations between IDT and SPC of -0.96, -0.95 and -0.96 respectively. Mesophiles were most often seen as the predominant
population. The impedimetric method allowed for these samples containing above $10^5$ cfu/ml to be screened out within 4 h. Psychrotrophic levels of $10^5$/ml and above were screened within 21 h, while total concentration of samples containing above $10^5$ cfu/ml were screened within 16 h.

Cady, et al. (22) stated that present keeping quality tests, which try to predict spoilage based on presence of psychrotrophic organisms, have two severe limitations. First, spoilage is not always directly related to the number of organisms present (156). Second, it appears that psychrotrophs are only part of the milk spoilage problem. Poor flavor and keeping quality can also be attributed to the presence of microbial enzymes and metabolic products (146, 148) from organisms present before pasteurization. Thus, present methods, although offering some useful information, are too slow and often too inaccurate to meet the needs of milk producers and processors. An optimal microbiological test would provide counting and keeping quality estimates within a period of time allowing for effective corrective measures. Cady, et al. (22) also found that detection time correlates better with the shelf-life than do the SPC and psychrotrophic count. These early data show promise as a 9-14 h impedance-based keeping quality prediction. Bossuyt and Waes (19) determined that impedance measurements are useful to trace post-pasteurization contamination in pasteurized milk. Martins (122) evaluated a rapid impedimetric method for determining the keeping
quality of milk and concluded that positive correlation coefficients indicate that short shelf-lives tend to produce early impedance response detection times and samples with long shelf-lives tend to produce late detection times.
CHAPTER III

MATERIALS AND METHODS

The following research was conducted in three phases. The initial phase involved determining the value of impedance detection as a predictor of potential shelf-life of commercial whole milks. The second phase studied various parameters as predictors of potential shelf-life of pasteurized whole and skim milk samples inoculated with psychrotrophs. The third phase studied these same parameters as predictors of potential shelf-life of artificially contaminated (psychrotrophs) cottage cheese. The word "potential" is a necessary term in any shelf-life study as the experiment progresses under ideal conditions, which would exclude activities such as temperature abuse, unsanitary handling, etc., as could possibly occur in the home.

Phase One
Sample Collection

A total of 100 samples of pasteurized whole milk was obtained from retail outlets and from four local dairies. Samples were stored at 7°C for 0-7 d then analyzed as day zero to facilitate the widest possible range of shelf-life values of the milks studied. This day "zero" corresponds to the actual time a particular container was opened, and evaluation began, after storage.
Microbiological Procedures

Standard plate count (SPC) and psychrotrophic bacteria count (PBC) were determined using the procedures recommended by the American Public Health Association (121) by plating in Tryptone Glucose Yeast Extract (TGE) agar and incubating for 48 h at 32°C and 10 d at 7°C, respectively. The modified psychrotrophic bacteria count (mPBC) (143) was determined by plating in TGE agar and incubating at 21°C for 25 h. Diluent used was the phosphate buffer of Standard Methods (121). This enumeration was conducted after preliminary incubation (PI) at 18°C for 18 h. The Moseley keeping quality test consisted of incubation of a sample at 7°C for 5-7 d, then plating for SPC (121) and evaluating for flavor.

Impedance Measurements

Impedance measurements were carried out on the Bactometer M120SC Microbial Monitoring System (Bactomatic, Princeton, NJ). Modified Plate Count Agar (MPCA) containing (in g/L): yeast extract, 20; tryptone, 20; dextrose, 4; and agar, 10, was used for the impedance analysis. Modules used in the instrument were filled by pipetting 0.5 ml of MPCA into each module well. This medium produced the highest quality impedance detection curves with a single acceleration (55). Growth on the surface of the agar shortened the generation time of the milk flora. The modules were gently agitated to allow for even distribution
of the agar. They were then placed in individual bags which were sealed. The modules were placed at 4°C until needed.

After being adequately mixed, 5.0 ml of each milk sample were added to 5.0 ml of sterile Plate Count Broth (PCB), mixed, and pre-incubated at 18°C for 18 h. At the completion of the PI, each sample was again mixed and 0.5 ml was inoculated into two wells of each of two modules. One module was incubated at 18°C during impedance measurement and the other at 21°C. These temperatures were used because 18°C is the temperature at which mesophiles and psychrotrophs have the most similar generation times (55). By using this temperature, one group of organisms will be unable to outgrow the other during PI and impedance detection. The 21°C temperature was used as an attempt to obtain an early detection time due to accelerated growth, and to provide a temperature more suitable to the growth of gram-negative psychrotrophs. After the entry of the modules into the Bactometer Processing Unit, continuous automatic data collection was carried out by the instrument. During the test, the impedance detection times (IDTs) of the samples were automatically determined by the instrument. The IDTs for the duplicate wells were averaged. This average IDT was compared to the shelf-life of the sample tested.

All media and media constituents used were from Difco Laboratories (Detroit, MI).
Shelf-life Determination

The shelf-life of each milk sample was determined by daily sensory evaluation as performed by two trained and two semi-trained panelists, i.e., when an objectionable flavor was detected, the previous day was considered to be the shelf-life of the product. This objectionable flavor was a recognition of an off-flavor which would correspond to a "5" or lower on the ADSA score card for milk. This would approximately correspond to the point at which the product should no longer be offered for sale. Thus, an off-flavor noted on day 15 would have a reported shelf-life value of 14 d. When there was any doubt concerning the presence of an objectionable flavor, that particular sample was tasted again the next day for confirmation. The day the off-flavor was confirmed constituted the day a sample was determined unacceptable.

Statistical Procedures


Phase Two

Sample Collection

Whole and skim milk, bottled in paperboard 1.9 L cartons, were obtained the day of processing from two local manufacturers. This milk was purchased in sufficient quantities to allow for separate duplicate cartons being
used for each analysis period. For each milk, three groups were initially inoculated with a pure culture of *Pseudomonas fluorescens* P27 at levels of: 0/ml, 1,000/ml, and 100,000/ml. Milks were stored at 7°C for up to 20 d, with samples obtained for microbiological and biochemical analyses every five days.

**Microbiological Procedures**

Bacterial enumerations conducted were: SPC (32°C 48 h), PBC (7°C 10 d) (121), mPBC (21°C 25 h) (143), and gram-negative count on crystal violet TTC agar (TGE agar + 1 ppm crystal violet + 50 ppm TTC at 21°C for 72 h - CVT) (185). Diluent used was the phosphate buffer of *Standard Methods* (121). Samples were subjected to PI prior to bacterial enumerations, except for PBC. Preliminary incubations were: none, 21°C 7 h, 21°C 14 h, 13°C 18 h, and 18°C 18 h.

**Biochemical Analyses**

Proteolysis of each sample was determined by the o-phthaldialdehyde (OPA) method of Church, et al. (31). The o-phthaldialdehyde reagent solution was made daily by combining the following reagents and diluting to a final volume of 50 ml with distilled water: 25 ml of 100mM sodium tetraborate; 2.5 ml of 20% (wt/wt) sodium dodecyl sulfate; 40 mg of OPA (dissolved in 1 ml of methanol); and 100 ml of B-mercaptoethanol. To 5.0 ml of a milk sample were added 1 ml water and 10 ml 0.75 N trichloroacetic acid (TCA) while
vortexing. After 10 min, the solution was filtered (Whatman #2 filter paper), and from this TCA filtrate, a 100 ml sample was added to 3.0 ml OPA reagent. The solution was mixed briefly by inversion and incubated for 2 min at ambient temperature, and the absorbance at 340nm was measured. A standard curve was prepared by adding a series of volumes of a 1.0mM Leu-Gly solution to 3.0 ml OPA reagent.

Endotoxin (lipopolysaccharide) levels were evaluated by the modified spectrophotometric Limulus Amoebocyte Lysate (LAL) assay (Worthington Biochemicals, Millipore, Freehold, NJ). Milk samples were initially clarified by adding 2 ml of 1% sodium citrate solution to 18 ml milk, then centrifuging at 8,500 x g for 20 min. The supernatant was poured off and the pellet resuspended in 20 ml sterile pyrogen-free Mg-saline solution. A 50 mM Mg solution has been found to increase the sensitivity of the assay (198). To each cuvette was added 0.3 ml of the resuspension solution and 3.0 ml LAL reagent. Absorbance was determined at 360 nm after incubation at 37°C for exactly 1 h. A standard curve was prepared using E. coli endotoxin of known concentrations. To remove variation of background turbidities of the milk samples, each incubated test sample was "blanked" with an identical unincubated test sample.
Impedance Measurements

Impedance measurements were carried out on the Bactometer M120SC Microbial Monitoring System (Bactomatic, Princeton, NJ). Modified plate count agar (MPCA) and a modified CVT agar (MPCA + 1 ppm crystal violet + 50 ppm TTC - MCVT) were used for the impedance analysis. Modules used in the instrument were filled by pipetting 0.5 ml of MPCA and MCVT into separate wells of a module (8 wells of each module with each agar). After being adequately mixed, 5.0 ml of each milk sample were added to 5.0 ml of sterile PCB, mixed, and PI at 18°C for 18 h. At the completion of the PI, each sample was again mixed and 0.5 ml was inoculated into duplicate wells of each agar. Impedance detection was conducted at 21°C. Average IDT values for each agar were compared to the shelf-life of the sample tested.

All media and media constituents used were from Difco Laboratories (Detroit, MI).

Shelf-life Determination

The shelf-life of each milk was determined by daily sensory evaluation.

Statistical Procedures

Statistical handling involved calculation of simple linear and multiple correlation coefficients, and regression analyses using SAS (173). Stepwise regression was used to develop prediction models from combinations of the measured variables.
Phase Three
Sample Collection

Dry curd from culture-set cottage cheese and cream dressing were obtained the day of processing from a local manufacturer. The dressing was inoculated with *Pseudomonas fluorescens* P27 at levels to yield a bacterial content of 0/g, 1,000/g, and 100,000/g after combining the curd and dressing at a 58:42 (curd:dressing) ratio. The cottage cheese was then packaged in 454 g quantities in sufficient numbers to allow for each analysis period. Cottage cheese containers were stored at 7°C for up to 35 d, with samples obtained for microbiological and biochemical analyses every seven days.

Microbiological Procedures

Bacterial enumerations conducted were: SPC (32°C 48 h), PBC (7°C 10 d) (121), mPBC (21°C 25 h) (143), and CVT (21°C 72 h) (185). Diluent used was 2% sodium citrate (121), with samples blended in a sterile stainless steel Waring blender. Samples were subjected to PI prior to bacterial enumerations, except for PBC. Preliminary incubations were: none, 21°C 7 h, 21°C 14 h, 13°C 18 h, and 18°C 18 h.

Biochemical Analyses

Proteolysis of each sample was determined by the OPA method of Church, et al. (31). The OPA reagent was prepared daily by combining the following reagents and diluting to a
final volume of 50 ml with distilled water: 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% sodium dodecyl sulfate; 40 mg of OPA (dissolved in ml methanol); and 100 ml of B-mercaptoethanol. To 5.0 g of a blended cottage cheese sample were added 1 ml water and 10 ml 0.75 N TCA while vortexing. After 10 min, the solution was filtered (Whatman #2 filter paper), and from this TCA filtrate, a 100 ml sample was added to 3.0 ml OPA reagent. The solution was mixed briefly by inversion and incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured. A standard curve was prepared by adding a series of volumes of a 1.0 mM Leu-Gly solution to 3.0 ml OPA reagent.

Endotoxin (lipopolysaccharide) levels were evaluated by the gelation Limulus Amoebocyte Lysate (LAL) assay (Difco Laboratories, Detroit, MI). Five grams of cottage cheese were blended with 5 ml pyrogen free Mg-saline. Next, 0.1 ml of the blended sample was added to the Pyrotest tube and swirled gently. Dilutions of $10^{-1}$, $10^{-2}$, and $10^{-3}$ of each sample were also tested. Tubes were allowed to incubate at 37°C for exactly 1 h. At the end of the incubation period, each tube was observed for gelation. Each test kit was initially tested for its sensitivity with known concentrations of \textit{E. coli} endotoxin, and the endotoxin content was determined by multiplying the level of sensitivity times the dilution factor.
Impedance Measurements

Impedance measurements were carried out on the Bactometer M120SC Microbial Monitoring System (Bactomatic, Princeton, NJ). Modified plate count agar (MPCA) and modified crystal violet TTC (MCVT) agar were used for impedance analysis. Modules used in the instrument were filled by pipetting 0.5 ml of MPCA and MCVT into separate wells of a module (8 wells of each module with each agar). For each cottage cheese container, half of the cottage cheese was aseptically removed and replaced with sterile PCB. The sample solution was then adequately mixed and PI at 21°C 18 h. At the completion of PI, each sample was again mixed and 0.5 ml was inoculated into duplicate wells of each agar. Impedance detection was conducted at 21°C. Average IDT values for each agar were compared to the shelf-life of the tested sample.

All media and media constituents used were from Difco Laboratories (Detroit, MI).

Shelf-life Determination

The shelf-life of each cottage cheese was determined by daily sensory evaluation.

Statistical Procedures

Statistical handling involved calculation of simple linear and multiple correlation coefficients, and regression analyses using SAS (173). Stepwise regression was used to develop prediction models from combinations of the measured variables.
Table 1. Flow chart of experimental methods used in Phases Two and Three involving psychrotrophic inoculation; ( ) = cottage cheese.

Milk/Cottage cheese

Inoculate with *P. fluorescens* P27

0/ml (g) 10^3/ml (g) 10^5/ml (g)

Storage at 7°C

0 d 5 d 10 d 15 d 20 d
(0) (7) (14) (21) (28)

Experimental sample

Proteolysis Endotoxin PI PBC Sensory analysis

None 21°C 7 h 21°C 14 h 13°C 18 h 18°C 18 h

(SP C mPBC CVT) (SP C mPBC CVT) (SP C mPBC CVT) (SP C mPBC CVT)
CHAPTER IV

RESULTS AND DISCUSSION

Phase One

Correlation coefficients of all shelf-life relationships of this study are shown in Table 2. Illustrated here is the fact that bacterial numbers obtained in the conventional ways are of limited value in predicting shelf-life. This would further prove the fact that microbial activity, and not necessarily microbial counts, are more closely related to product spoilage, as has been shown previously (93, 122). These correlation values are somewhat higher than have previously been presented in publications. This is probably due to the wider range of shelf-life values obtained in this study, which resulted in more accurately calculated coefficients.

Displayed in Figure 1 is the high multiple correlation coefficient obtained by assessing the quadratic relationship between IDT's at 21°C and shelf-life (r=0.877). This coefficient was virtually equal to that of the linear relationship (r=0.868). These coefficients were slightly higher than those of IDT's at 18°C related to shelf-life. Correlation coefficients for the quadratic model (Fig. 2) and linear only relationship were 0.868 and 0.851, respectively.

The above coefficients were significantly (p<0.05)
Table 2. Correlation coefficients obtained from commercial milk samples (Phase One) (N=100).

<table>
<thead>
<tr>
<th>Relationship to shelf-life</th>
<th>Correlation Coefficients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linear</td>
<td>Multiple</td>
</tr>
<tr>
<td>IDT(^a) 21(^\circ)C</td>
<td>0.868</td>
<td>0.877</td>
</tr>
<tr>
<td>IDT 18(^\circ)C</td>
<td>0.851</td>
<td>0.868</td>
</tr>
<tr>
<td>MSPC(^b)</td>
<td>-0.770</td>
<td>-0.773</td>
</tr>
<tr>
<td>SPC(^c)</td>
<td>-0.524</td>
<td>-0.560</td>
</tr>
<tr>
<td>PBC(^d)</td>
<td>-0.583</td>
<td>-0.642</td>
</tr>
<tr>
<td>mPBC(^e)</td>
<td>-0.629</td>
<td>-0.668</td>
</tr>
</tbody>
</table>

\(^a\)IDT=Impedance detection time.
\(^b\)MSPC=Moseley plate count.
\(^c\)SPC=Standard plate count (32\(^\circ\)C, 48 h).
\(^d\)PBC=Psychrotrophic bacteria count (7\(^\circ\)C, 10 d).
\(^e\)mPBC=Modified psychrotrophic count (18\(^\circ\)C, 18 h followed by 21\(^\circ\)C, 25 h).
Figure 1. Scattergram of the quadratic relationship of impedance detection at 21°C to potential shelf-life of commercial whole milk at 7°C.
Figure 2. Scattergram of the quadratic relationship of impedance detection at 18°C to potential shelf-life of commercial whole milk at 7°C.
greater than those obtained from the relationship of the Moseley test to shelf-life. The linear (Fig. 3) and quadratic model correlation coefficients from the Moseley test were $-0.770$ and $-0.773$, respectively, which are relatively high. This the method used with the most frequency since its introduction in the 1950's. The correlation coefficient obtained in this study was higher than that obtained by Smith, et al. (182). Also, their (182) predicted values of shelf-life from the Moseley test were quite different from the values of this study. For example, a Moseley count of $1 \times 10^6$ predicted a shelf-life of 17 d in their study as opposed to only 8 d in this study.

The Moseley test can be used to "categorize" the milk samples into potentially "poor", "good" or "marginal" (Fig. 3). A major drawback of this test has always been the time required for acquisition of data. By doing the SPC after storage at 7°C for 5-7 d, one does not have results for 7-9 d.

When comparing resultant shelf-life correlations from impedance detection at 18°C to 21°C, there was no significant difference. However, since incubation at 21°C resulted in quicker IDT's it should be the temperature of choice.

By using the impedance method the results were available in 25-38 h after processing (18 h PI + 7-20 h impedance detection). Therefore, this method alleviated the problem encountered with the Moseley test of 7-9 d until results are obtained. In addition, this procedure required much less
Figure 3. Scattergram of the linear relationship of the Moseley keeping quality test to potential shelf-life of commercial whole milk at 7°C.
work than the Moseley test (or any other microbiological plating method), and its correlation coefficients were higher.

It was also possible to "categorize" milks as to their potential shelf-life by using IDT's. This capability was due to the high correlation of IDT to actual shelf-life as determined organoleptically. From Fig. 1 it would appear that if the IDT <6.1 h, the potential shelf-life of the milk would be <9 d, and if the IDT >12.4 h, the potential shelf-life would be >9 d. There existed a range of IDT's between 6.1 and 12.4 h where the shelf-life values were near 9 d. These samples could not be classified as having <9 d or >9 d of shelf-life by this method, but the precision of the impedance method exceeded any other method studied.

Phase Two

Correlations to potential shelf-life of whole milk at 7°C (Table 3) indicated PI 21°C 14 h, with mPBC, was the superior bacterial enumeration technique (r=-0.827). All correlations presented were significant (p<0.01), but since mPBC had the highest correlation value and required the least amount of time (25 h + 14 h PI = 39 h), it was preferred. This was also true for skim milk (Table 4), with mPBC after PI 21°C 14 h having a correlation to potential shelf-life of -0.743, and for combined data (whole + skim milk) of -0.782. There was a slight increase in correlation values when the quadratic relationship of bacterial
Table 3. Correlations of bacterial enumerations to potential shelf-life of whole milk at 7°C (N=67).

<table>
<thead>
<tr>
<th>PI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PBC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SPC&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CVT&lt;sup&gt;d&lt;/sup&gt;</th>
<th>mPBC&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-0.703</td>
<td>-0.805</td>
<td>-0.760</td>
<td>-0.757</td>
<td></td>
</tr>
<tr>
<td>21°C 7h</td>
<td>-0.810</td>
<td>-0.758</td>
<td>-0.760</td>
<td>-0.760</td>
<td></td>
</tr>
<tr>
<td>21°C 14h</td>
<td>-0.814</td>
<td>-0.778</td>
<td>-0.827</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13°C 18h</td>
<td>-0.807</td>
<td>-0.740</td>
<td>-0.754</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18°C 18h</td>
<td>-0.810</td>
<td>-0.760</td>
<td>-0.801</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>PI=Preliminary incubation.

<sup>b</sup>PBC=Psychrotrophic bacteria count (7°C, 10 d).

<sup>c</sup>SPC=Standard plate count (32°C, 48 h).

<sup>d</sup>CVT=Crystal violet TTC count (21°C, 72 h).

<sup>e</sup>mPBC=Modified psychrotrophic bacteria count (21°C, 25 h).
Table 4. Correlations of bacterial enumerations to potential shelf-life of skim milk at 7°C (N=72).

<table>
<thead>
<tr>
<th>PI</th>
<th>Bacterial enumerations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBC\textsuperscript{b}</td>
</tr>
<tr>
<td>None</td>
<td>-0.609</td>
</tr>
<tr>
<td>21°C 7h</td>
<td>-0.707</td>
</tr>
<tr>
<td>21°C 14h</td>
<td>-0.729</td>
</tr>
<tr>
<td>13°C 18h</td>
<td>-0.723</td>
</tr>
<tr>
<td>18°C 18h</td>
<td>-0.729</td>
</tr>
</tbody>
</table>

\textsuperscript{a}PI=Preliminary incubation.
\textsuperscript{b}PBC=Psychrotrophic bacteria count (7°C, 10 d).
\textsuperscript{c}SPC=Standard plate count (32°C, 48 h).
\textsuperscript{d}CVT=Crystal violet TTC count (21°C, 72 h).
\textsuperscript{e}mPBC=Modified psychrotrophic bacteria count (21°C, 25 h).
enumerations with potential shelf-life was added. For the preferred mPBC, with 21°C 14 h PI, correlation values were -0.860, -0.755 and -0.814 for whole, skim and combined, respectively (Table 5). This quadratic effect is better illustrated in Fig. 4. Bacterial enumerations were of limited predictive value due to relatively large shelf-life deviations about the calibration curve associated with relatively low correlation coefficients (r=-0.827). However, with only limited laboratory facilities where plating is the only means of quality determination, the selected mPBC method may offer valuable information not previously available.

Protease activity was also significantly (p<0.05) related to potential shelf-life with linear correlations of -0.508 for whole milk and -0.525 for skim milk. There was a significant (p<0.05) increase in correlation values from linear to the quadratic effect, as quadratic relationships were -0.722 for whole milk and -0.671 for skim milk, but these values decreased with combined data (Table 5). The scattergram in Figure 5 of this quadratic effect reveals that apparently at low and medium levels of proteolysis, potential shelf-life was quite varied, and not until relatively high levels of proteolysis were detected was the potential shelf-life consistently short. Therefore, protease activity is also of limited predictive value, even though it is certainly a cause for product quality degradation but not a viable initial indicator.
Table 5. Linear and multiple (linear + quadratic) correlations studied to potential shelf-life of pasteurized fluid milk at 7°C.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Whole (N=67)</th>
<th>Skim (N=72)</th>
<th>Combined (N=139)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.703</td>
<td>-0.802</td>
<td>-0.609</td>
</tr>
<tr>
<td>SPC&lt;sup&gt;b&lt;/sup&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>-0.814</td>
<td>-0.821</td>
<td>-0.729</td>
</tr>
<tr>
<td>CVT&lt;sup&gt;c&lt;/sup&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>-0.778</td>
<td>-0.779</td>
<td>-0.690</td>
</tr>
<tr>
<td>mPBC&lt;sup&gt;d&lt;/sup&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>-0.827</td>
<td>-0.860</td>
<td>-0.743</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>-0.508</td>
<td>-0.722</td>
<td>-0.525</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>-0.897</td>
<td>-0.898</td>
<td>-0.914</td>
</tr>
<tr>
<td>IDT-MPCA&lt;sup&gt;e&lt;/sup&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>0.869</td>
<td>0.908</td>
<td>0.879</td>
</tr>
<tr>
<td>IDT-MCVT&lt;sup&gt;f&lt;/sup&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>0.900</td>
<td>0.923</td>
<td>0.912</td>
</tr>
</tbody>
</table>

<sup>a</sup>PBC=Psychrotrophic bacteria count.
<sup>b</sup>SPC=Standard plate count.
<sup>c</sup>CVT=Crystal violet TTC count.
<sup>d</sup>mPBC=Modified psychrotrophic bacteria count.
<sup>e</sup>IDT-MPCA=Impedance detection time in modified plate count agar.
<sup>f</sup>IDT-MCVT=Impedance detection time in modified crystal violet TTC agar.
<sup>g</sup>After corresponding preliminary incubation.
Figure 4. Scattergram of the quadratic relationship of log modified psychrotrophic bacteria count to potential shelf-life of fluid milk at 7°C.
Figure 5. Scattergram of the quadratic relationship of proteolysis (ug glycine-leucine) to potential shelf-life of fluid milk at 7°C.
Endotoxin concentrations resulted in significantly (p<0.01) high correlation values to potential shelf-life, with -0.897 for whole milk, -0.914 for skim milk and -0.896 for combined milks' linear relationship, and -0.898 for whole, -0.926 for skim, and -0.913 for combined milks' quadratic effect (Table 5). These correlation values agree with earlier work (14). Endotoxin concentrations appeared to be useful for the prediction of potential shelf-life of:

Shelf-life = 12.02 - 0.19(E) + 0.0008(E)^2,

for combined whole and skim milk data with shelf-life in days and endotoxin concentration in ng/ml, and an associated r^2=0.805 (Fig. 6).

Impedance detection times were highly related (p<0.01) to potential shelf-life as illustrated by linear correlation values of 0.869, 0.879 and 0.874 in MPCA, and 0.900, 0.912 and 0.906 in MCVT agar for whole, skim and combined milks, respectively (Table 5). There existed a slight increase in correlation values when the quadratic effect was added (0.915 in MPCA and 0.930 in MCVT agar). The MCVT agar had higher correlation values than the MPCA with no increase in detection times, as both averaged 6.6 h for this study. This superiority of MCVT agar was not totally unexpected as it was obviously supporting the growth of only gram-negative bacteria which have been proven to cause the majority of problems with keeping quality.

The quadratic relationship between IDT and potential shelf-life of fluid milks at 7°C, as illustrated in Figure
Figure 6. Scattergram of the quadratic relationship of endotoxin level to potential shelf-life of fluid milk at 7°C.
7, could be used to easily "categorize" milk samples as to their potential quality. An IDT <6 h indicated a potential shelf-life of <9 d, and an IDT >9 h indicated a potential shelf-life of >11 d. This agrees well, especially the 6 h IDT, with work completed in Phase One. IDT was also very useful as a predictor of potential shelf-life of pasteurized fluid milk with an equation of:

\[
\text{Shelf-life} = 0.560 + 1.400(\text{IDT}) - 0.032(\text{IDT})^2,
\]

with shelf-life in days and IDT in hours, and an associated \( r^2 = 0.852 \).

Utilizing all parameters studied (Table 5), regression equations were formulated to estimate the potential shelf-life of pasteurized fluid milks. When only IDT and endotoxin were included in the equation (Table 6), the coefficient of determination was 0.919, whereas when a total of seven components were included (Table 7), the coefficient of determination was 0.939. This increase was not significant which illustrated the fact that impedance detection and endotoxin concentration were parameters most important to potential shelf-life, with impedance detection showing greater value by the higher \( F \) value in Table 6.

When comparing the predictive values of endotoxin vs. impedance detection, the endotoxin method has several limiting factors. As evident in Figure 6, at low levels of endotoxin, days shelf-life were quite varied, with a range of 8 to 18 d. Also, its predictive equation had an associated shelf-life deviation of 3 d as compared to 2 d.
Figure 7. Scattergram of the quadratic relationship of impedance detection to potential shelf-life of fluid milk at 7°C.
Table 6. Components of a predictive regression equation estimating the potential shelf-life of pasteurized fluid milk in days with $r^2=0.919$.

<table>
<thead>
<tr>
<th>Component</th>
<th>Estimate</th>
<th>F-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prob F&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>5.9021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDTC</td>
<td>0.5300</td>
<td>134.91</td>
<td>0.0001</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>-0.0049</td>
<td>87.86</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup>, 65 df.

<sup>b</sup> Level of significance.

<sup>c</sup> IDT = Impedance detection time.
Table 7. Components of a predictive regression equation estimating the potential shelf-life of pasteurized fluid milk in days with $r^2=0.939$.

<table>
<thead>
<tr>
<th>Component</th>
<th>Estimate</th>
<th>F-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prob F&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-2.6339</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2857</td>
<td>11.53</td>
<td>0.0012</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>-0.0200</td>
<td>13.11</td>
<td>0.0006</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>0.2323</td>
<td>8.51</td>
<td>0.0050</td>
</tr>
<tr>
<td>mPBC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-3.3664</td>
<td>10.34</td>
<td>0.0021</td>
</tr>
<tr>
<td>(Proteolysis)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-0.0008</td>
<td>13.35</td>
<td>0.0005</td>
</tr>
<tr>
<td>Endotoxin * Proteolysis</td>
<td>0.0001</td>
<td>6.68</td>
<td>0.0122</td>
</tr>
<tr>
<td>Proteolysis * mPBC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0145</td>
<td>8.17</td>
<td>0.0058</td>
</tr>
</tbody>
</table>

<sup>a</sup>7, 60 df.

<sup>b</sup>Level of significance.

<sup>c</sup>IDT=Impedance detection time.

<sup>d</sup>mPBC=Modified psychrotrophic bacteria count.
with impedance detection. One factor in favor of endotoxin detection was the performance time required of just 2 h, although laboratory analysis was quite extensive. It was apparent from the higher multiple correlation and regression coefficients obtained with IDT vs. endotoxin as related to potential shelf-life of pasteurized fluid milks that impedance detection revealed the most useful information concerning potential shelf-life estimation by a predictive equation.

The seven-component regression equation (Table 7) did prove important in that it gave insight into factors affecting potential shelf-life, and to what extent each did. Therefore, an effort could be made in the future to manipulate, and possibly eliminate, these factors in order to produce a fluid dairy product with the longest possible potential shelf-life.

Phase Three

Linear relationships to potential shelf-life of cottage cheese at 7°C (Table 8) indicated that bacterial enumerations were of no practical value for estimation of shelf-life. All presented correlations were significant (p<0.05), but those values were too low, with too much associated variation, to be reliable initial indicators of cottage cheese quality. The gram-negative bacteria count on CVT agar had the highest multiple correlation value to potential shelf-life (-0.606) when the quadratic effect was included (Fig. 8 and Table 9).
Table 8. Linear relationships of bacterial enumerations to potential shelf-life of cottage cheese at 7°C (N=65).

<table>
<thead>
<tr>
<th>PI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bacterial enumerations</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SPC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CVT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>mPBC&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>-0.515</td>
<td>-0.527</td>
<td>-0.592</td>
<td>-0.495</td>
</tr>
<tr>
<td>21°C 7h</td>
<td>-0.558</td>
<td>-0.586</td>
<td>-0.495</td>
<td></td>
</tr>
<tr>
<td>21°C 14h</td>
<td>-0.538</td>
<td>-0.578</td>
<td>-0.531</td>
<td></td>
</tr>
<tr>
<td>13°C 18h</td>
<td>-0.588</td>
<td>-0.580</td>
<td>-0.574</td>
<td></td>
</tr>
<tr>
<td>18°C 18h</td>
<td>-0.579</td>
<td>-0.523</td>
<td>-0.514</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>PI=Preliminary incubation.
<sup>b</sup>PBC=Psychrotrophic bacteria count.
<sup>c</sup>SPC=Standard plate count.
<sup>d</sup>CVT=Crystal violet TTC count.
<sup>e</sup>mPBC=Modified psychrotrophic bacteria count.
Figure 8. Scattergram of the quadratic relationship of gram-negative bacteria (log) in crystal violet TTC agar to potential shelf-life of cottage cheese at 7°C.
Table 9. Linear and multiple correlations of all parameters studied to potential shelf-life of cottage cheese at 7°C (N=65).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Linear</th>
<th>Multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC\textsuperscript{a}</td>
<td>-0.515</td>
<td>-0.532</td>
</tr>
<tr>
<td>mPBC\textsuperscript{bg}</td>
<td>-0.574</td>
<td>-0.586</td>
</tr>
<tr>
<td>SPC\textsuperscript{c}</td>
<td>-0.588</td>
<td>-0.590</td>
</tr>
<tr>
<td>CVT\textsuperscript{dg}</td>
<td>-0.592</td>
<td>-0.606</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>-0.728</td>
<td>-0.811</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>-0.860</td>
<td>-0.868</td>
</tr>
<tr>
<td>IDT-MCVT\textsuperscript{eg}</td>
<td>0.845</td>
<td>0.874</td>
</tr>
<tr>
<td>IDT-MPCA\textsuperscript{fg}</td>
<td>0.860</td>
<td>0.897</td>
</tr>
</tbody>
</table>

\textsuperscript{a} PBC=Psychrotrophic bacteria count.
\textsuperscript{b} mPBC=Modified psychrotrophic bacteria count.
\textsuperscript{c} SPC=Standard plate count.
\textsuperscript{d} CVT=Crystal violet TTC count.
\textsuperscript{e} IDT-MCVT=Impedance detection time in modified CVT agar.
\textsuperscript{f} IDT-MPCA=Impedance detection time in modified plate count agar.
\textsuperscript{g} After corresponding preliminary incubation.
Endotoxin (lipopolysaccharide) concentration was also significantly \( (p<0.01) \) related to potential shelf-life as evidenced by the linear and multiple (quadratic added) correlation coefficients of \(-0.728\) and \(-0.811\), respectively. The gelation LAL method was used in favor of the spectrophotometric LAL method due to problems of clarifying the cottage cheese sample in order to obtain a relatively particulate-free resuspension solution as is needed for the spectrophotometric assay. By using the gelation method, values lacked specificity due to their being dependant upon the dilution series. This may partially explain these correlation values being lower than previously obtained in milk \((14)\). Due to this effect, endotoxin concentration could not be used to predict potential shelf-life of cottage cheese, but could possibly be used to "categorize" cottage cheese samples as to their potential shelf-life. Samples with \(<\ 100 \ ng/g\) indicated a potential shelf-life of \(>12 \ d\), whereas \(>800 \ ng/g\) indicated a potential shelf-life of \(<7 \ d\), as illustrated in Figure 9.

Proteolysis was found to be significantly \( (p<0.01) \) related to potential shelf-life of cottage cheese. The linear correlation coefficient of \(-0.860\) was the highest obtained in Phase Three, with a slight increase to \(-0.868\) when the quadratic effect was added (Table 9). It became apparent from Figure 10 that, although the multiple correlation value was relatively high, proteolysis was of limited value in the prediction of potential shelf-life of
Figure 9. Scattergram of the quadratic relationship of endotoxin level to potential shelf-life of cottage cheese at 7°C.
Figure 10. Scattergram of the quadratic relationship of proteolysis (ug glycine-leucine) to potential shelf-life of cottage cheese at 7°C.
cottage cheese because of the variability associated with the scattergram. Sample "categorization" was also not practical.

Impedance detection was also significantly (p<0.01) related to potential shelf-life of cottage cheese as illustrated by linear correlation values of 0.845 in MCVT agar and 0.860 in MPCA. Adding the quadratic term did not greatly increase the degree of relationship (Table 9). The MPCA appeared to be superior to the MCVT agar. The quadratic relationship between IDT and potential shelf-life of cottage cheese (Fig. 11) could be used to "categorize" cottage cheese samples as to their potential quality. One sample which presents somewhat of a problem - at (16, 6) - was noted to have a defect due to an "unclean" taste thought to be associated with poor quality non-fat dry milk powder. With this sample included, and IDT <6 h indicated a potential shelf-life of <9 d, and an IDT >16 h indicated a potential shelf-life of >11 d. Without the sample in question, and IDT >13 h indicated a potential shelf-life of >11 d.

Impedance detection was also useful as a predictor of potential shelf-life of cottage cheese with an equation of:

\[ \text{Shelf-life} = -0.61 + 1.21(\text{IDT}) - 0.018(\text{IDT})^2, \]

with shelf-life in days and IDT in hours, and \( r^2 = 0.805 \).

Utilizing all parameters studied (Table 9), regression equations were formulated to estimate the potential shelf-life of cottage cheese. When only IDT and proteolysis
PRODUCT CODE: CCPC

SAMPLES: 65

SPECIFIED SHELF-LIFE: 9.0

MULT. CORR. = 0.90

SHELF-LIFE = -0.0176T^2 + 1.21T - 0.61

CUTOFF TIME 5.78

CAUTION TIME 12.52

Figure 11. Scattergram of the quadratic relationship of impedance detection to potential shelf-life of cottage cheese at 7°C.
were included in the equation (Table 10), the coefficient of
determination was 0.904, whereas when a total of
five-components were included (Table 11), the coefficient of
determination was 0.938. From the two-component equation it
appears IDT and proteolysis are of the most value in
predicting potential shelf-life of cottage cheese from the
almost equal F-values, but the five-component equation would
tend to emphasize the value of impedance detection as
evidenced by its higher F-value.
Table 10. Components of a predictive regression equation estimating the potential shelf-life of cottage cheese in days with $r^2=0.904$.

<table>
<thead>
<tr>
<th>Component</th>
<th>Estimate</th>
<th>F-value(^a)</th>
<th>Prob F(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>23.619</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDT(^c)</td>
<td>-0.436</td>
<td>106.68</td>
<td>0.0001</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>-0.079</td>
<td>107.81</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

\(^a\)2, 62 df.
\(^b\)Level of significance.
\(^c\)IDT=Impedance detection time.
Table 11. Components of a predictive regression equation estimating the potential shelf-life of cottage cheese in days with $r^2=0.938$.

<table>
<thead>
<tr>
<th>Component</th>
<th>Estimate</th>
<th>F-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prob&gt;F&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>51.989</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.568</td>
<td>28.81</td>
<td>0.0001</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>-0.002</td>
<td>10.93</td>
<td>0.0016</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>-0.330</td>
<td>19.03</td>
<td>0.0001</td>
</tr>
<tr>
<td>(IDT&lt;sup&gt;c&lt;/sup&gt;)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-0.006</td>
<td>4.35</td>
<td>0.0413</td>
</tr>
<tr>
<td>(Proteolysis)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.001</td>
<td>12.43</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

<sup>a</sup>5, 59 df.

<sup>b</sup>Level of significance.

<sup>c</sup>IDT=Impedance detection time.
CHAPTER V

CONCLUSIONS

Almost all psychrotrophically inoculated samples of whole and skim milk, and cottage cheese developed a bitter flavor as expected (126, 207). This was the initial defect noted which caused an end to the respective sample's shelf-life in most cases. Cottage cheese samples also developed a fruity flavor which was probably due to psychrotrophic growth. These resultant flavor defects allowed for the range of shelf-life values necessary for comparison and interpolation, as well as extrapolation, of parameters studied for the estimation of potential shelf-life of perishable dairy products.

Although the differences in shelf-life values as influenced by psychrotrophic inoculation levels was not the major emphasis of this study, it is appropriate to illustrate the effect this treatment did have on the dairy products analyzed (Table 12). Days shelf-life for each inoculation level, of each product, were significantly different (p 0.05). These data tend to prove the point that psychrotrophic contamination does cause degradation of dairy product quality.

As discussed earlier, bacterial enumeration was of limited value for the estimation of the potential shelf-life of dairy products. The psychrotrophic bacteria count produced correlation values of -0.754 for milk and -0.532
Table 12. Shelf-life differences between inoculation levels of *Pseudomonas fluorescens* P27 in dairy products stored at 7°C.

<table>
<thead>
<tr>
<th>Inoculation levels/ml</th>
<th>Days shelf-life</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Milk</td>
<td>Skim Milk</td>
<td>Cottage Cheese</td>
</tr>
<tr>
<td>0</td>
<td>15.1</td>
<td>14.2</td>
<td>16.0</td>
</tr>
<tr>
<td>1,000</td>
<td>10.6</td>
<td>11.5</td>
<td>10.5</td>
</tr>
<tr>
<td>100,000</td>
<td>7.8</td>
<td>10.0</td>
<td>8.7</td>
</tr>
</tbody>
</table>
for cottage cheese and required a total of 10 d to perform (Table 13). The time factor alone negates the value of the PBC even if the relationship to shelf-life had been greater. The standard plate count produced correlation values of -0.780 for milk and -0.590 for cottage cheese and required 62 h, which included 14 h PI. Again the time period is too long and the relationships too low. This was also true for the gram-negative count in CVT agar with values of -0.735 for milk and -0.606 for cottage cheese and required 86 h, which also included 14 h PI. This was somewhat of a surprise to have such low correlation coefficients as related to shelf-life. Gram-negative psychrotrophs are the usual cause of product quality degradation, especially in this case of specific psychrotrophic inoculation. Thus, one would expect a relatively high relationship between the CVT count and shelf-life. This may further support the supposition of product quality being affected by bacterial metabolism, and not necessarily bacterial numbers. The modified psychrotrophic bacteria count appeared to be of some value for shelf-life prediction of milk, but not necessarily cottage cheese, as evidenced by the correlation value of -0.814, and, just as important, the time required of 39 h, including 14 h PI. The mPBC had several advantages. It was simple to perform as the sample was plated as if for SPC but incubated at 21°C for 25 h. As this temperature is just slightly less than normal room temperature of 22°C, it is possible the enumeration could be
Table 13. Linear and multiple correlations of all parameters to potential shelf-life of products studied, and time required for each.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Milk</th>
<th>Cottage cheese</th>
<th>Time Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.650</td>
<td>-0.754</td>
<td>-0.515</td>
</tr>
<tr>
<td>mPBC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.782</td>
<td>-0.814</td>
<td>-0.574</td>
</tr>
<tr>
<td>SPC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.771</td>
<td>-0.780</td>
<td>-0.588</td>
</tr>
<tr>
<td>CVT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.729</td>
<td>-0.735</td>
<td>-0.592</td>
</tr>
<tr>
<td>Proteolysis</td>
<td>-0.474</td>
<td>-0.556</td>
<td>-0.860</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>-0.896</td>
<td>-0.913</td>
<td>-0.728</td>
</tr>
<tr>
<td>IDT-MPCA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.874</td>
<td>0.915</td>
<td>0.860</td>
</tr>
<tr>
<td>IDT-MCVT&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.906</td>
<td>0.930</td>
<td>0.845</td>
</tr>
</tbody>
</table>

<sup>a</sup>PBC=Psychrotrophic bacteria count.
<sup>b</sup>mPBC=Modified psychrotrophic bacteria count.
<sup>c</sup>SPC=Standard plate count.
<sup>d</sup>CVT=Crystal violet TTC count.
<sup>e</sup>IDT-MPCA=Impedance detection time in modified plate count agar.
<sup>f</sup>IDT-MCVT=Impedance detection time in modified CVT agar.
<sup>g</sup>After corresponding preliminary incubation.
conducted without the benefit of an incubator. One disadvantage was the difficulty of enumerating the pin-point colonies which formed within the 25 h period.

An interesting occurrence throughout the study was the "ceiling" bacteria enumeration of 8.51 (log CFU/ml) as an average for each enumeration of each product. This was the count related to end of shelf-life, which is higher than the previously reported (111, 131, 156) value of 10^7/ml. Even when samples were "plated" past the shelf-life period, counts did not increase significantly. This was evident in milk as well as cottage cheese, leading to a possible conclusion that the available nutrients for bacterial growth and metabolism are nearly equal in whole milk, skim milk and cottage cheese.

Proteolysis, as detected by the o-PTHALDIALDEHYDE method (31), was of limited value in milk, but of increased value in cottage cheese as evidenced by the correlation values of -0.556 and -0.868, respectively (Table 13). This discrepancy is difficult to explain except for the possibility of increased available protein in the cottage cheese, which would be in conflict with the "equal nutrient" theory proposed to explain the bacteria "ceiling" count being the same for milk and cottage cheese. This assay required 2 h for analysis which was a definite advantage. Another advantage of this particular proteolysis detection method over the Hull method (86) was that all hydrolytic products are assayed, not just aromatic residues. There-
fore, the method used in this study for proteolysis detection was rapid, simple, accurate, and of value for shelf-life prediction of cottage cheese.

Endotoxin (lipopolysaccharide) levels were determined by two methods of the LAL assay—spectrophotometric and gelation. It was possible to determine endotoxin levels in milk by spectrophotometric method after clarification of the sample, with a resultant correlation value of -0.913 (Table 13). This proved to be a very valuable assay for shelf-life estimation of milk. When endotoxin levels were determined in cottage cheese, clarification of the sample was not possible due to non-selective centrifugation because of the semi-solid nature of the product. This inability resulted in the application of the LAL gelation method, which was simpler to conduct but not nearly as accurate as the spectrophotometric method due to concentration being calculated on a dilution basis. This may have been the reason for the decreased correlation value of -0.811. Another "plus" for this assay was the time requirement of only 2 h. These relatively high correlation values were expected as endotoxin production would be indicative of gram-negative bacterial metabolism, which has been shown to be the probable cause of product quality degradation.

Impedance detection's value for shelf-life estimation was evidenced by the high correlation values on MPCA and MCVT agar of 0.915 and 0.930, and 0.897 and 0.874 for milk and cottage cheese, respectively (Table 13). Due to the
principle for impedance detection of bacterial produced chemical changes in media resulting in the impedance of an AC current, this method was able to be dependant upon bacterial numbers as well as bacterial metabolism, therefore resulting in a more realistic evaluation of product quality. As expected, MCVT agar produced higher correlation values than MPCA for milk due to the selected detection of gram-negative bacterial growth. This was not the case for cottage cheese. An explanation of this phenomenon may have been the greater variety of reasons for flavor defects of cottage cheese. Advantages for shelf-life estimation by impedance detection are numerous. The method was simple, automatic and accurate. The system allows for 128 to 512 samples to be tested simultaneously at two to eight different incubation temperatures. The impedance detection system also has the capacity to determine standard plate count, mesophilic plate count, psychrotrophic bacteria count, coliform count and the presence of yeasts and salmonella. In addition, presence of antibiotic residues and starter culture activity can be determined. Therefore, shelf-life determination is only one of the assets of impedance detection techniques.

In conclusion, bacterial enumeration was of limited value for estimation of potential shelf-life of perishable dairy products. Proteolysis was also of limited value for estimation of potential shelf-life of milk, although it was certainly a cause for product quality degradation. It did
prove to be of value for estimation of potential shelf-life of cottage cheese. Endotoxin concentration was of great value for prediction of potential shelf-life of perishable dairy products, probably due to its association with gram-negative bacterial metabolism. Impedance detection was of greatest value for prediction of potential shelf-life of perishable dairy products. As stated earlier, it was recommended that results be available within a time period which would allow for effective corrective measures, preferably less than 48 h. Ironically, the four methods studied which revealed data within this time frame were the top four methods as related to potential shelf-life - impedance detection (30 h, R=0.930), endotoxin (2 h, R=-0.913), proteolysis (2 h, R=-0.868), and mPBC (39 h, R=-0.814).
REFERENCES


VITA

Jay Russell (Rusty) Bishop, son of Mr. and Mrs. Robert G. Bishop, was born January 16, 1955 in Orangeburg, South Carolina. Upon completion of high school from Orangeburg-Wilkinson High in 1973, he enrolled at Clemson University in Microbiology. He received the B.S. degree in 1977.

In July, 1977 he was appointed Research Technician, and later Research Specialist, with the Dairy Science Department at Clemson. He began a M.S. program in Nutrition in January of 1978, which was completed in August of 1982 on a part-time basis while working full-time.

He began his Ph.D. program in Dairy Microbiology at L.S.U. under the supervision of Dr. Charles H. White in August of 1982 until the present. Major research emphasis has been in the area of estimation of dairy product quality.
EXAMINATION AND THESIS REPORT

Candidate: Jay Russell Bishop

Major Field: Dairy Science

Title of Thesis: Assessment of Dairy Product Quality Utilizing Bacterial Enumeration and Metabolite Detection

Approved:

[Signatures]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

January 21, 1985