Growth and Proteolytic Activity of Selected Psychrotrophic Bacteria in Whole Milk and Whole Milk Retentate

Ravinder Reddy
GROWTH AND PROTEOLYTIC ACTIVITY OF SELECTED PSYCHROTROPHIC BACTERIA IN WHOLE MILK AND WHOLE MILK RETENTATE

BY

RAVINDER REDDY

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science Major in Dairy Science South Dakota State University 1987
GROWTH AND PROTEOLYTIC ACTIVITY OF SELECTED PSYCHROTROPHIC BACTERIA IN WHOLE MILK AND WHOLE MILK RETENTATE.

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

George S. Torrey / Date
Thesis Advisor

John G. Parsons / Date
Head, Dairy Science Department
ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. George S. Torrey, major professor and director of this research, for his guidance, advice and encouragement during the completion of this program.

I also wish to thank Dr. John G. Parsons for the opportunity to participate in this graduate program and for the research assistantship. A special thanks to Dr. Kenneth R. Spurgeon and to Dr. Robert J. Baer for their assistance during my studies. I would also like to thank Dr. Carl A. Westby, Dr. Vikram A. Mistry and Dr. Wayne Kennabach for serving in my examination committee. Thanks also to Dr. W. L. Tucker and Mr. Paul Evenson for their assistance in statistical analysis.

I wish to acknowledge and appreciate the cooperation of all the production faculty and staff members in the dairy science department.

To all my friends on 'third floor', I am indebted for their very enjoyable companionship.

Finally, I wish to express affectionate gratitude to my parents, for their love and sacrifice.

Ravinder
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>ULTRAFILTRATION</td>
<td>5</td>
</tr>
<tr>
<td>Theory</td>
<td>5</td>
</tr>
<tr>
<td>Ultrafiltration in the dairy industry</td>
<td>8</td>
</tr>
<tr>
<td>Ultrafiltration of whole milk</td>
<td>9</td>
</tr>
<tr>
<td>Chemical composition of whole milk retentates</td>
<td>12</td>
</tr>
<tr>
<td>Studies of Refrigerated Retentates</td>
<td>15</td>
</tr>
<tr>
<td>PSYCHROTROPHIC BACTERIA</td>
<td>18</td>
</tr>
<tr>
<td>Refrigeration and Psychrotrophs</td>
<td>20</td>
</tr>
<tr>
<td>Factors affecting incidence of psychrotrophic bacteria</td>
<td>23</td>
</tr>
<tr>
<td>Product contact surfaces</td>
<td>23</td>
</tr>
<tr>
<td>Storage temperature and time</td>
<td>26</td>
</tr>
<tr>
<td>Significance of psychrotrophs in milk</td>
<td>30</td>
</tr>
<tr>
<td>Detection of proteolysis</td>
<td>32</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>35</td>
</tr>
<tr>
<td>ARTICLE: Growth and Proteolytic Activity of Selected</td>
<td>45</td>
</tr>
<tr>
<td>Psychrotrophic Bacteria in Whole Milk and</td>
<td></td>
</tr>
<tr>
<td>Whole Milk Retentate</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>46</td>
</tr>
<tr>
<td>Introduction</td>
<td>46</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>48</td>
</tr>
<tr>
<td>Results and Discussions</td>
<td>53</td>
</tr>
<tr>
<td>References</td>
<td>69</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>72</td>
</tr>
<tr>
<td>APPENDIX: Supplemental information</td>
<td>75</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ARTICLE</strong></td>
<td></td>
</tr>
<tr>
<td>1 Composition of whole milk and retentates used for bacterial growth and proteolysis studies</td>
<td>59</td>
</tr>
<tr>
<td><strong>APPENDIX</strong></td>
<td></td>
</tr>
<tr>
<td>1 Colony forming units present in Tryptic Soy Broth cultures incubated for 18-24 at 25°C when absorbance at 420 nm was .3</td>
<td>76</td>
</tr>
<tr>
<td>2 Growth of psychrotrophic bacteria in whole milk and retentate incubated at 70°C for 5.5 d.</td>
<td>77</td>
</tr>
<tr>
<td>3 Tyrosine values in whole milk and retentates inoculated with proteolytic psychrotrophic bacteria and incubated at 70°C</td>
<td>78</td>
</tr>
<tr>
<td>4 TNBS bound free amino groups in whole milk and retentates inoculated with proteolytic psychrotrophic bacteria and incubated at 70°C</td>
<td>79</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Literature Review</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Representation of processes of Osmosis and Ultrafiltration</td>
</tr>
<tr>
<td><strong>Article</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Growth curve of isolate 1P</td>
</tr>
<tr>
<td>2</td>
<td>Growth curve of isolate 2P</td>
</tr>
<tr>
<td>3</td>
<td>Growth curve of isolate 11P</td>
</tr>
<tr>
<td>4</td>
<td>Growth curve of isolate 12P</td>
</tr>
<tr>
<td>5</td>
<td>Growth curve of isolate 17</td>
</tr>
<tr>
<td>6</td>
<td>Proteolytic activity of isolate 1P</td>
</tr>
<tr>
<td>7</td>
<td>Proteolytic activity of isolate 2P</td>
</tr>
<tr>
<td>8</td>
<td>Proteolytic activity of isolate 11P</td>
</tr>
<tr>
<td>9</td>
<td>Proteolytic activity of isolate 12P</td>
</tr>
</tbody>
</table>
INTRODUCTION

In the last twenty years a number of membrane separation processes have evolved from laboratory scale, through pilot plant investigation, to full scale production units. One such process, ultrafiltration, has been found particularly useful in the dairy industry. Ultrafiltration uses porous polymeric membranes to separate molecules, principally on the basis of their molecular weight. In milk, fat, protein and associated substances are retained on the membrane. Permeate, which passes through the membrane contains mainly water, lactose and other low molecular weight substances. There is interest in ultrafiltration of milk because of its potential importance in saving costs to both farmer and processor. The advantages of using ultrafiltration include incorporation of whey proteins in cheese, increased production capacity, reduced rennet requirements in cheesemaking, reduced energy requirements and overall low operating costs (52,53,67,68,93). Dairy farmers gain in reduced cooling and hauling costs, and can feed the permeate to cows (68,93). The technical and economic feasibility of ultrafiltration on the farm as well as at the plant has been successfully demonstrated (26,67,68,86,92,93), and ultrafiltration of milk has
already proven to be a desirable pretreatment step in the commercial manufacture of various dairy products (10,27,40).

The basic applications for ultrafiltration of whole milk are total concentration of milk, preconcentration of cheese milk and standardization of protein. The major objective of using ultrafiltration in the dairy industry is to remove bulk water and reduce volume, subsequently reducing operating costs. Reduction in volume allows the farmer to save on refrigeration and transportation costs. There is a great possibility that adaption of ultrafiltration in the dairy industry may induce changes in milk collection frequencies from the farm and operating schedules at the plant which may lead to extended refrigerated storage of retentates.

Extended refrigerated storage of milk and milk products is selective for the growth of psychrotrophic bacteria (5). Growth of psychrotrophic bacteria in these products is of major concern because most of these species produce extra-cellular enzymes, such as proteases and lipases, many of which are heat stable and even survive ultra-high temperature treatment. Thus, the living bacteria and/or their enzymes may cause spoilage in milk or heat sterilized dairy products (1,5,13,14,20,35,42,-85,90,91). The introduction of ultrafiltration may
potentially compound the problem of psychrotrophs since ultrafiltration directly or indirectly facilitates long holding periods.

Study of psychrotrophic bacterial growth in retentates is important because retentates as a microbial medium is entirely different from whole milk in chemical composition. Ultrafiltration of milk affects the relative as well as the absolute concentrations of milk constituents, because the membrane is permeable to low molecular weight materials. Much of the lactose and some water soluble minerals and vitamins pass through the membrane and therefore decrease in concentration or are only slightly concentrated in ultrafiltered milk (27,53,67,92). The membrane is completely impermeable to fat, protein, vitamin B₁₂ and folic acid in milk, so that these components are concentrated in inverse proportion to the volume decreased (30). Details of changes in mineral, vitamin and trace elements composition of milk during ultrafiltration are described by several authors (7,8,9,24,25,29,30).

Previous studies (1,6,73,87) of refrigerated retentates with natural mixed microbial flora indicate that retentates support good psychrotrophic growth, but that bacterial multiplication is greater in whole milk than in retentates after 2-3 days of incubation. Tayfour et al.
(73) observed that growth and proteolytic activity of \textit{Pseudomonas fluorescens} P28 was less in skim milk retentates concentrated five fold than in skim milk, however, skim milk concentrated 2 or 3 times showed better growth and proteolytic activity than did skim milk.

Characterization of growth of specific bacteria in whole milk retentate and data comparing changes in composition of whole milk and whole milk retentate caused by growth of specific bacteria is needed. The present study was undertaken to compare the growth and proteolytic activity of selected psychrotrophic bacteria in retentates to that in whole milk.
LITERATURE REVIEW

ULTRAFILTRATION

Ultrafiltration, (UF) provides a novel way of concentrating high molecular weight solutes present in solution, without the application of heat or the use of extreme chemical or physical conditions. Ultrafiltration makes use of porous polymeric membranes under a pressure gradient to separate molecules on the basis of molecular weight. Unlike ordinary filtration, the feed stream flows across the membrane surface and not perpendicular to the surface. It is a pressure-driven process (10-100 psig) for separating and concentrating suspended solids, colloids, and high molecular weight materials in solution. A selective, semi-permeable membrane retains high molecular weight solutes. The material passing through is called permeate; fluid stream and components retained by UF membranes are called retentate.

THEORY

Ultrafiltration is fundamentally similar to ordinary filtration except that the membrane pores are roughly 1,000
times larger than those of an ordinary filter. The theory of UF can be illustrated by a simple model in which a membrane is interposed between two liquids, one pure water and the other water with substances dissolved in it; for example milk. Initially, the membrane is easily permeable to water and completely impermeable to the high molecular weight solute. The magnitude and direction of flow through membranes is governed by the following four factors: osmotic pressure, applied pressure, permeability of the membrane, and membrane thickness. According to laws of thermodynamics, every solvent has a tendency to equalize its concentration through out the volume (tendency to attain equilibrium) and during this process osmotic pressure is developed. The magnitude of osmotic pressure developed is equal to the difference in the concentration of water on each side of the membrane. If the membranes were to be left intact pure water should flow towards the solution so as to dilute the milk. But, in the ultrafiltration of milk the opposite effect is desired i.e., water should flow out of the milk. So, it is necessary to oppose osmotic pressure with applied counter pressure. If the applied pressure exceeds osmotic pressure water will flow out of the milk, leaving behind concentrated milk solutes. The theory of ultrafiltration is summarized in the following Figure.
Average pore diameters in UF membranes range from less than one to about 10 nm. Constituents of the fluid with diameters greater than the membrane pore diameters are rejected during ultrafiltration. Because of their greater diameters, suspended particles (about 1000 nm), bacteria (5-10 um) and viruses (about 20 nm) are rejected. Water with a molecular weight of 18 and having an effective molecular diameter of about .2 nm can easily pass through UF membranes. Sugars and other low molecular weight, water-soluble substances also pass through membrane pores. Proteins, fat and other large molecules are excluded since they often have molecular weights of more than 100,000 and effective molecular diameters of several nanometers (31).
ULTRAFILTRATION IN THE DAIRY INDUSTRY

The first synthetic UF membrane was probably one made by the German chemist and biologist Moritz Traube in 1870 (31). By the 1920's polymeric UF membranes of various pore sizes had been developed, but for the next 40 years the process was rarely employed outside the laboratory chiefly because of membrane fouling. The most important application of UF in the food industry is for concentration of proteins from dilute solutions. Physical and chemical separation conditions are relatively mild and little denaturation of proteins takes place making UF attractive to the dairy industry (27).

Traditionally, cheese manufacturers have regarded whey as a disposal problem because of its high biological oxygen demand. Modern economic considerations led processors to seek a system to solve this disposal problem and recover whey proteins, and this search ultimately brought about the introduction of membrane filtration systems to the dairy industry. Since the introduction of UF for whey processing, other uses for UF have been recommended or evaluated by the dairy industry and include UF of whole and skim milk for the manufacture of cheese, or various cultured dairy products, protein enrichment of fluid products, enzyme recovery from lactose syrup
degradation and the concentration of raw milk on the farm.

ULTRAFILTRATION OF WHOLE MILK

Application of UF for concentrating milk was developed in 1969 by French researchers (52). This development became known as the "MMV concept" from the initials of the inventors' last names. Milk volumes were reduced by 50% or more during UF and the resulting product was called "precheese", a liquid product obtained on the retentate side of the membrane and having a composition very close or identical to that of high moisture cheese (53). Milk retentates have been used to make cheeses with high to medium moisture contents (10, 27, 40, 93), yogurt (10, 27) and cream (65).

Major areas of UF research include standardization of procedures for manufacture of products from UF whole milk retentates, studying the economics of UF, understanding the effects of process variables such as temperature, feed velocity, rejection coefficients and pressure during ultrafiltration of whole milk, and microbiological aspects of retentates.

Thompson and DeMan (86) studied the effects of product temperature, operating pressures, feed flow rate, and retentate concentration on permeate flux during UF of
whole milk and whey. Glover et al. (27) demonstrated the technical feasibility of twofold concentration of whole milk by UF in the laboratory. Chapman et al. (10) ultrafiltered whole milk to manufacture hard, medium and soft cheeses, and yogurt.

Yan et al. (92) used tubular UF membranes to concentrate whole milk up to 21.5% total solids. The behavior of whole milk in the UF process and effects of various parameters on UF of whole milk were studied. Concentration of milk fat lowered permeate flux but did not cause sufficient membrane fouling to exclude its applicability to whole milk ultrafiltration. However, UF of whole milk was observed to be limited by concentration and gel polarization, but could be done using high flow rates, relatively low pressures, and relatively high temperatures.

Garoutte and Amundson (26) demonstrated that UF of whole milk using hollow fiber membranes could be used to obtain a five fold concentration by volume. Flux was dependent on the pressure differential across the membranes and the flux declined slowly with increasing concentration. Reduction in flux was rapid after the solids concentration reached 25.0%. Hollow fibers used in this experiment are promising for ultrafiltering whole milk because of their high membrane surface area to volume ratio. Thompson and
DeMan (86) used hollow fibers to concentrate whole milk and whey to more than three fold. The effects of process variables on UF of the two materials were the same.

Another area of growing interest for application of UF in the dairy industry is on-farm milk concentration, which was again pioneered in France by Maubois's team in collaboration with Alfa-Laval (40). Milk on French farms was thermised (heated to 72°C for 15 s) before ultrafiltration. Ultrafiltration of milk has led to a new speciality cheese industry in France (40). The technology and microbiology of UF on French farms has been described (6,53).

Slack et al. (67) investigated UF of fresh raw milk in a controlled laboratory situation and in a farm environment in which feed to the unit was taken from automatic milking lines. This research demonstrated that it was technically possible to ultrafilter whole milk both at the farm and the plant, and also demonstrated that milk fat did not cause severe membrane fouling. The presence of milk fat did not cause an extreme decline in permeate flux, and the resulting permeates were clear and contained negligible protein and no fat. Storage studies indicated negligible rancidity in retentate stored up to 4 days without pasteurization. In another study, Slack et al. (68) reported that the farm ultrafiltration of raw milk was
economically favorable for dairy farms with 500 or more cows.

The first United States commercial-scale on-farm UF study was initiated at Adam Van Excel's 900 cow dairy farm near Lodi, California in late 1984 (2). The study was co-sponsored by the California Milk Advisory Board and Dairy Research Inc. In this experiment, every aspect of on-farm UF including technical applicability, economic feasibility, and cleanability of UF membranes was studied.

CHEMICAL COMPOSITION OF WHOLE MILK RETENTATES

Retention of whole milk components during ultrafiltration is dependent on many factors including membrane pore size, pressure differential, temperature of processing, fluid velocity, the concentration factor and concentration polarization (26,27,30,67,83,92). The percentage rejection of a particular feed component is defined by following formula:

\[
\% \text{ Rejection} = 1 - \frac{\text{Concentration in permeate}}{\text{Concentration in feed}}
\]

Rejection values range from 0-100% (83). A component with a percent rejection of zero will have the same concentration in the permeate as in the feed, whereas a substance with a percent rejection of 100% will have
zero concentration in the permeate (27).

Typically proteins and fats in milk have high rejection values. Protein rejection values may be as high as 99%, and range from 94 to 99%. Rejection percentages depend on membrane characteristics (27). Generally, protein rejection values of above 99% and fat rejection values of 100% are obtained during UF of whole milk (27,30,31,53). Lactose passes through membranes at approximately the same rate as water because of its low molecular weight. In trials by Glover et al. (27) only 10% of the lactose and 80% of the total nitrogen were retained.

The retention of low molecular weight substances including water-soluble vitamins, calcium, magnesium, phosphates, and trace minerals is dependent on the proportion of substances bound to macromolecules (27,30,92). Vitamin B₁₂ and folic acid are retained during UF and concentrated in inverse proportion to the volume decrease because they are protein bound (27). Tomita et al. (84) and Green et al. (30) determined the rejection coefficients of individual vitamins during UF of whole milk.

The retention of non-protein nitrogenous fractions has also been determined (27). Proteose-peptone components are concentrated to a slight extent indicating that they are retained by membranes. The low molecular weight
nitrogenous compounds including urea, amino acids, and ammonia are not concentrated (27). Green et al. (27) reported that the concentration factors for calcium, magnesium, zinc, iron, and copper are dependent on the proportion bound to protein. Fukuwatari (24) measured retention of elements and found that membrane permeability to Fe, Zn, and Mn was low compared to Cu. Most minerals were concentrated to a lesser extent in milk containing added citrate or acid indicating that the minerals were partly solubilized from micelles by these treatments. Brule and Fauquant (7) found conditions increasing mineral binding to milk proteins resulted in smaller losses of minerals from retentates.

Green and Potter (29) determined retention of various milk components using membranes of different composition, membranes with different molecular weight cut-off points, and milks pretreated differently. Samples were collected at different stages of processing and at concentration factors of 1.5 and 2.0. They observed that the component retentions were not affected by different membrane composition or different molecular weight cut-off and also retention did not differ for pasteurized and homogenized milks.

As UF is increasingly applied in dairy processing, there is a growing need for compositional data to answer
many questions about heat stability of retentates, nutritional quality of dairy products manufactured from retentates, and standardization of the process. Perhaps the most important question concerns the effect of compositional changes from milk to retentate on microbial growth of spoilage bacteria and desirable organisms.

STUDIES OF REFRIGERATED RETENTATES

Although from the bacteriological point of view, cold storage of raw milk has been the subject of a great deal of research, very few publications (6, 25, 73, 87) mention the refrigeration of raw retentates. There are four reasons why there is a need for more research in this area: (1) Growth and activities of microorganisms may not be the same in milk and retentate since they are two different media, (2) Retentate is a concentrated medium which may either stimulate growth of some organisms or contain inhibitory concentrations of milk components which may inhibit microbial growth, (3) Microbial growth during ultrafiltration may cause spoilage during subsequent storage, (4) Ultrafiltration will increase holding time at the farm or in the plant and potentially increase the problem of psychrotrophs.

Veillet-Poncet et al. (87) studied growth patterns
of mixed microflora of aerobic mesophiles, psychrotrophs, caseolytic psychrotrophs; and coliforms in naturally contaminated retentates at 4, 7 and 12°C. Bacterial multiplication was more pronounced in milk than in the retentate stored at 7°C and 12°C, while the reverse was observed at 4°C. Psychrotrophs dominated the microflora of retentate and milk samples at all storage temperatures irrespective of the initial proportions. Some inhibition of growth of all types of microorganisms was observed during the first 24h of incubation at 7°C. After one day at 4°C and two days at 7°C, the entire microbial flora was psychrotrophic, and about half was caseolytic. This indicates that retentates like milk support good psychrotrophic growth when stored at refrigerated temperatures.

Garcia-Ortiz et al. (25) studied the physico-chemical aspects of cold storage of retentates. Skim milk retentates were stored at 4, 7, and 12°C for 10 d. and observed for changes in acidity, pH and casein degradation. Initially, there was not much difference in acidity and pH of retentate and skim milk but thereafter acidity of skim milk increased rapidly compared to retentates. The increase in non-casein nitrogen during storage was markedly less in retentate than in skim milk.
Benard et al. (6) compared microbial growth in raw milk, raw retentate and thermized retentate stored at 2, 4, and 6°C stored for 7 d. The types of organisms detected were aerobic mesophiles, psychrotrophs and coliforms. Storage temperatures and storage times had a profound effect on natural flora in all the three products. Lag phases for growth ranged from 24h at 6°C, to 72h at 2°C. Bacterial growth ranged from 0.2 log per d at 2°C to 1 log per d at 6°C. A mixture of thermized retentates collected over a period of 4 d contained approximately 5,000 aerobic mesophiles per ml vs 25,000 per ml in raw milk; counts of psychrotrophs were 3,000 to 4,000 per ml in the thermized retentates and 12,000 per ml in raw milk.

Tayfour et al. (73) inoculated Pseudomonas fluorescens P28 into different concentrations of skim milk retentates which were stored at 4, 7, and 10°C. Growth in all retentates was similar until the end of log phase. During stationary phase, cell populations were less in retentate concentrated five times than in retentates concentrated only two or three times, or in skim milk. Proteolytic activity determined by gel electrophoresis was detected when bacterial cell counts reached 10⁸ CFU per ml. Proteolysis was less in retentates concentrated four or five times compared to retentates concentrated two and three times and to skim milk.
Similar results were also seen in storage studies of reverse osmosis concentrates (15,17). In these publications the authors postulate that the reduced growth may be due to either concentration of some inhibitory substances during ultrafiltration or depletion of some essential micro-nutrients which might have been lost in the permeate.

PSYCHROTROPHIC BACTERIA

The terminology "psychrophiles and psychrotrophs" has often been a major controversy. Ingraham and Stokes (36) defined psychrophiles as microbes which are able to multiply at 0°C. Literal meaning of the word psychrophile is "cold loving" which suggests a preference for growth at lower temperatures. Food and Dairy industries recognized an important difference between the terms "cold loving" and "cold thriving". Association of spoilage in refrigerated foods with the literal meaning of the psychrophile is not appropriate (85). In 1960, Eddy (18) coined the term "psychrotrophs" for those microorganisms which are capable of growth at refrigeration temperatures but do not meet the classical temperature classification requirements of psychrophiles. In the dairy industry, psychrotrophs are defined as organisms which are able to multiply at 7°C or below irrespective of their optimum temperatures (13).
The predominance of cool temperatures in the natural environment has a great influence on the dominant microflora of a specific environment. As emphasized by Morita (56) much of our environment is cold, and a natural habitat for low temperature organisms predominates. Stokes and Redmonds (72) have consolidated this concept of a cold environment by demonstrating the presence of psychrophiles in streams, rivers and lakes and determined the percentages of psychrophiles in various soils and foods. They stated that large numbers of psychrophilic bacteria were present in dairy products such as milk, ice cream, cream, butter, and cheese, and also in sea foods, meats, and chicken. Widespread distribution of psychrophiles in nature has created innumerable sources for dairy product contamination. These sources include soil, vegetation, water, and air.

The microbial flora of raw milk can vary greatly in numbers and types depending on how milk is contaminated. Milk production conditions and basic animal husbandry methods are by far the most important factors in this regard. The proximity of soil and vegetation contributes substantially towards psychrotrophic contamination of milk. Most commonly found species of psychrotrophs in milk have been isolated from soil (78). Grass, hay, barley and oats may contain up to 100 million psychrotrophs per gram (74).
Farm and processing plant water supplies have been shown to contain many lipolytic and proteolytic microorganisms such as *Pseudomonas*, *Achromabacter*, *Alcaligenes*, and *Flavobacterium* (74).

Besides feed and water, environmental conditions at the farm have a role in the microbiological quality of milk. Air-born contamination, poor milking conditions, udder health, dust, improper ventilation of milking facilitates, and infected personnel contribute to unacceptable quality of milk. Regarding the control of psychrotrophs and other spoilage organisms at their origin (e.g. soil, vegetation, water and air), contamination from these sources can be minimized but not completely eliminated. Sterile milk cannot be collected from the cow, however, understanding the subsequent handling of milk will help to understand how milk is contaminated and will aid in improving milk quality.

**REFRIGERATION AND PSYCHROTROPHS**

The history of modern dairying may be divided into two chapters, pre-refrigeration and refrigeration eras. The refrigeration era began with the advent of efficient refrigeration systems used at every stage of milk handling. Refrigeration is an invaluable method of food preservation...
because it does not affect the wholesomeness of the product. The lower temperature resulting from refrigeration slows metabolic rates in cells and decreases reproduction rates.

Refrigeration with its many benefits caused many changes in the dairy industry. Most visible of these was introduction of the farm bulk tank. Improved cooling and bulk storage of milk made alternate day collection possible. Some effects of refrigeration were longer holding periods, long distance transportation, five-day work schedules in processing plants, and extended shelf life of pasteurized milk and dairy products. Coincident with the improvements brought on by refrigeration was a change in the spoilage flora of milk. Before the advent of refrigeration, raw milk had high bacterial counts composed primarily of mesophilic bacteria, particularly lactic acid bacteria, and milk was often spoiled by souring or curdling (85). Once widespread application of refrigeration in the dairy industry became common and much improved sanitary practices were introduced, the problems caused by these lactic acid bacteria in raw milk largely disappeared. The lower holding temperatures of milk for longer times on the farm, in transportation, in the plant, and by the consumer however, provided ample opportunities for psychrotrophic bacteria to grow and deteriorate product quality. The
absence of large numbers of lactic acid bacteria may have played a role in the evolution of problems caused by psychrotrophs since some lactic acid bacteria are able to use oxygen and produce hydrogen peroxide, which suppresses growth of psychrotrophs (13,20). Extended holding periods for farm milk and five day working schedules at processing plants are essential facets of modern dairying, which have compounded the problem of psychrotrophs and milk quality.

Morita (56) has reviewed studies in which raw milk stored at 100°C developed a microflora that was mostly lactic streptococci; whereas raw milk stored at 0°C developed a microflora dominated by gram negative psychrotrophs. Hence, we can conclude that continuous refrigeration of milk creates an environment that favors growth of psychrotrophic bacteria. Since it is well known that soil, vegetation, water and air are the natural sources of contamination of milk by psychrotrophs, it might seem that the efficiency of modern dairying practices would make the impact of these natural factors minimal and all but eliminate the problem. This has not happened. The presence and survival of microbes particularly psychrotrophs in milk is dependent on the sanitary quality of product contact surfaces, storage temperatures, and time.
FACTORS AFFECTING PRESENCE AND SURVIVAL OF PSYCHROTROPHIC BACTERIA IN MILK

(A) PRODUCT CONTACT SURFACES

The numbers of bacteria present in aseptically drawn milk will commonly be below 1000 CFU/ml. These bacteria are derived from the teat conduits. Other sources of infection, as already mentioned are ambient air, the cow and the milker. A significant source of contamination after the milk is drawn is product contact surfaces, such as milking equipment, pipelines, and bulk tank surfaces. Proper cleaning and sanitizing of equipment is vital for production of high quality milk (74,75,76,77,78,79,80). Milk produced under sanitary conditions usually contains less than 10% of the total microbial flora as psychrotrophs, but milk produced under unsanitary conditions may contain more than 75% of the microbial flora as psychrotrophs (78). Raw milk collected from farms which practiced good hygiene methods had less than 1000 CFU of psychrotrophs per ml(13).

The main environmental factor that affects growth and survival of psychrotrophs on product contact surfaces is the temperature (3-10°C) at which surfaces of the equipment, particularly that of tanks, are maintained.
Smooth stainless steel surfaces have small microscopic crevices which serve as niches for accumulation of the milk residues and microbes. Likely sources of bacterial flora in milk contact surfaces are initial rinsing water, contaminated brushes used to apply detergent and sanitizer, air in the dairy which can harbor milk residues, accessories such as agitators, dipsticks, thermometers, valves in pipes and tanks, milking machine parts and outlet and sampling ports (74,79). In summary, milk may become contaminated with large numbers of undesirable types of bacteria during milking or from poorly cleaned milking facilities and pipelines (74,79,80).

Thomas and Thomas (79,80) observed that bulk collected milk produced and handled under strictly hygienic conditions had a low incidence of gram-negative rods. However, large numbers of gram-negative rods were found in milk from farms with poorly cleaned dairy equipment. The lowest proportion of these bacteria was found in efficiently sanitized milking machines whereas they formed an appreciable part (39%) of the microflora of rinses containing small numbers of bacteria from farm bulk milk tanks. The psychrotrophic bacterial population of farm milk tanks is generally much less than that of pipeline milking plants (74), whereas the proportion of psychrotrophs of the total bacterial count in tanks is
often much more than in the case of milking plants. Earlier, Thomas and Thomas (82) had observed that poorly cleaned pipeline milking plants had 44% of the total flora as psychrotrophs, compared to 82% of the total flora in low count rinses of the surfaces of the farm milk tanks and 92% of the flora in high count rinses of equipment.

Methods of cleaning and sanitizing can also play a role in the survival of psychrotrophs on surfaces. In British studies when steam was used as the sanitizer, only 10% of the survivors were gram-negative rods, whereas when quaternary ammonium compounds were used as sanitizers predominantly gram-negative rods survived (13, 82). Manual or automatic cleaning does not make any differential impact on psychrotrophs (74). Also, cleaning temperatures did not have any significant effect on the psychrotrophic flora (4). In the British study of Thomas and Thomas, (82) it was observed that milking equipment was the major source of bacterial contamination of raw milk with the next major source being bulk tank surfaces. These results agree with the results of Ogawa (60) who concluded that the psychrotrophic bacterial count (PBC) of raw milk obtained by hand milking (10-100 CFU/ml) was less than the PBC in raw milk obtained by machine milking (1000 CFU/ml). Similarly, samples of milk collected in buckets contained fewer psychrotrophs than samples from pipeline milk (13).
The importance of cleanliness or sanitation of product contact surfaces lies in the fact that unsanitary surfaces may contribute actively multiplying psychrotrophs to products. Milk produced under sanitary conditions usually does not support a rapid increase in bacterial numbers when held at 4°C or less. However, milk produced under unsanitary conditions often displays a rapid increase in growth of psychrotrophic organisms. This rapid increase is not the result of initial numbers of psychrotrophs but rather the presence of actively multiplying psychrotrophs (13,74).

(A) STORAGE TEMPERATURE AND TIME

The holding temperature and time greatly influence the types and numbers of organisms present in milk since optimum growth temperatures vary with bacterial species. In order to improve the keeping quality, raw milk is cooled. Standard plate counts of raw, manufacturing grade milk are significantly higher in can milk than in bulk tank milk (16). This was because bulk tank milk rarely exceeded 4.4°C, whereas can milk exceeded 10°C at the time of collection at the farm. Milk stored in milk tanks was cooled to 4.4°C, but at this temperature growth was not entirely excluded. Usually at 4.4°C there is little growth
for at least 3-5 days. Sometimes, it is possible for undesirably high numbers to develop at 4°C, depending upon the types and numbers of initial contaminants. Efficient and rapid cooling immediately after milking is important. A few hours of storage at higher temperatures (70°C or higher) leads to a considerable reduction of the lag phase, and as a result bacterial numbers increase more rapidly when the temperature is reduced to 4°C. Microbial spoilage and keeping quality of milk are basically dependent on the length of the lag phase. As the storage temperature increases, the length of the lag phase decreases, and the keeping quality decreases. Therefore, not only are the bacterial numbers important but also the physiological condition of these microorganisms (71).

The importance of storage temperature as well as the holding time of the milk has been stated by LaGrange (45). Manufacturing grade milk received into a plant with bacterial counts in the low millions, requires little time before the counts increase dramatically. This is especially true for milk received at 70°C or more where the generation time is 8h or less, compared to 12h at 50°C and 16h at 20°C. Species of the genus *Pseudomonas*, the dominant genus in cold stored milk illustrate the dramatic effect of varying temperatures on generation times. Green and Jezeski (28) studied generation times of different strains
of *Pseudomonas* spp. at 0-20°C, 4-60°C, 10°C and 20°C. Bacterial generation times at these temperatures were 27-29 h, 12-14 h, 5-6 h and 1.2-1.7 h, respectively. From this it is clear that slight changes in temperature below 10°C are critical for milk quality. Freshly drawn milk should therefore be promptly cooled to 5°C or below and also held at that temperature until processed. Generation times of psychrotrophs at different temperatures have been reviewed in detail by Cousin (13).

Effect of storage temperature on milk quality was studied by Finley et al (21). They stated that 81% of the total milk samples held at 0°C remained acceptable for over three weeks, but only 15% of the milk samples held at 7.2°C were acceptable for more than one week. Similar results were reported by Hankin et al (33). Milk stored at 1.7°C was judged good for 17.5 days and was spoiled primarily by proteolytic organisms, but milk stored at 5.6°C was spoiled by proteolytic and acid producing microorganisms within 12.1 days. Only 4% of the milk was acceptable after one week at 10°C because acid producers and coliforms caused flavor defects (34). Holding milk at 7°C was also compared to milk held at 4.4°C. Results indicated a more rapid increase of psychrotrophs (75). Rapid cooling to below 4.4°C after production is a deterrent for growth during subsequent refrigeration.
Duration of low temperature storage of milk before processing is limited depending upon the initial contamination with actively multiplying proteolytic and lipolytic strains of psychrotrophs, and the composition of the microflora. Milk produced under hygienic conditions could be safely held at 4.4°C for 72 h before processing. Milk with heavy initial contamination held at 7°C or above showed a relatively rapid build up of psychrotrophs and developed an unclean or rancid flavor within 48 h of milking (75,76,77). Thomas et al. (74) reported that 3 h after milking psychrotrophic counts ranged from 0-13,000 CFU/ml and after 72 h of holding at 3-5°C, the numbers ranged from 10-29,000,000 CFU/ml.

Introduction of farm bulk tank milk perhaps made alternate day milk collection possible and stimulated growth of psychrotrophs in milk. Alternate day collection of milk resulted in a higher psychrotrophic bacterial count (PBC) than in milk collected daily and the difference in PBC was deemed insignificant when the refrigeration was good (74,77). Psychrotrophic bacterial counts for raw milk stored at 5°C for 1,2 or 3 days were 400,000, 2.1 million and 11 million CFU/ml, respectively (50). Psychrotrophic bacterial counts of 50 CFU/ml of milk have been reported immediately after milking, whereas PBCs of 1,700-49,000 CFU/ml in one-day old milk and 4,300-71,000 CFU/ml in two
day-old milk were observed (50).

A comprehensive assessment of the influence of alternate day collection of milk on bacteriological quality would be very important in the future, since there is a possibility that pick-up operations could change from alternate day to once or twice a week. This possibility is foreseen as the introduction of on-farm UF seems imminent. A farmer with an UF unit on the farm will have less to store in the bulk tank every day, and a hauler will have less to carry, thereby increasing the bulk storage capacity. This results in extended refrigerated storage and so the bacteriological quality and the effects of several associated factors such as milking hygiene, and efficiency of cleaning of dairy equipment becomes very pertinent.

SIGNIFICANCE OF PSYCHROTROPHS IN MILK

Psychrotrophs are now considered a very significant spoilage problem in the dairy industry. While food storage at refrigeration temperatures obviously prolongs shelf life, these conditions select for psychrotrophic bacteria. Many psychrotrophs are potent producers of extra-cellular, heat stable lipases and proteases capable of causing extensive spoilage, due to breakdown of fat and protein
Most psychrotrophs in raw milk are heat-sensitive, gram-negative rods, but some heat-sensitive, gram-positive species have also been isolated (13). The latter organisms cause spoilage of stored heat treated milk and milk products by their growth, but involvement of specific enzymes has not been reported. Biochemical changes in refrigerated milk depend upon types and numbers of bacteria, duration of storage, and efficiency of refrigeration (13,63). Off-flavors resulting from bacterial growth have been detected organoleptically in pasteurized milk stored for periods less than 5 days at 1 to 4.4°C (13). Contrary to this, Ogawa (60) observed that organoleptic changes were seldom detectable in milk stored at 5-7°C after 7 days, when the populations reached $10^7$ to $10^8$ per ml.

Changes in milk flavor caused by proteolytic activity of bacteria occur in the following order of increasing severity: lacks freshness, staleness, rancid, fruity and bitter flavor (32,61,90,91). The most common defects in milk initiated by proteolysis were found to be fruity and rancid flavors (14,32,66). Also, putrid, potato, cheesy, bitter, unclean, soapy and fishy flavors have been associated with proteolysis and/or lipolysis by psychrotrophs. Psychrotrophs are also capable of producing acid, gas and pigmentation in many dairy products (5,54).
Age gelation in ultra-high temperature treated (135 to 150°C for few seconds) milk is a serious problem. Possible causes include physico-chemical changes, indigenous milk proteases or extra-cellular proteases from psychrotrophs (46, 47, 48). The shelf life of UHT milk may be extended by inactivation of psychrotrophic proteases by low temperature treatment (88, 89).

The quality and yield of Cottage and Cheddar cheeses were significantly reduced when the psychrotrophic bacterial count exceeded one million per g. (3, 55); and many vats of product fail to coagulate properly. The rennet coagulation time of milk is reported to be slightly decreased by the presence of large numbers of psychrotrophs (14). Growth of Psychrotrophs of 10^8 CFU/ml resulted in greater losses of nitrogen in whey although the curd was firmer and less fragile (14). Recent studies (35, 64) have determined that extensive bacterial growth in raw milk can result in reduced cheese yields. Rancidity, bitterness and other flavor defects traceable to psychrotrophs of raw milk appear in ice cream, butter and yogurt.

DETECTION OF PROTEOLYSIS

Psychrotrophs cause spoilage by biochemically altering the constituents of milk. Milk proteins act as
substrates for psychrotrophic proteases. There is information available on the characteristics of these enzymes, conditions for enzyme activity, and types of organisms contributing enzymes important to the quality of milk and dairy products. Recent reviews by Cousin (13), Law (47), Fox (23) and Fairbairn and Law (20) describe aspects of proteolytic enzymes related to quality in the dairy industry.

Proteolysis in milk can be detected by two different methods: 1). Detection of changes in concentration and presence of various milk proteins, 2) quantification of end products of proteolysis. The following discussion is restricted to the later method. It is generally known that proteolytic enzymes degrade milk proteins releasing peptides and amino acids. Quantification of these end products in cold-stored raw milk should be an index of the bacterial activity in that milk, and a guide to both history in production and storage and to the future storage potential of the milk. If proteolysis measurements are to be effectively utilized as an index for milk quality then, quantification of amino acid and peptide end products should be sensitive and reliable.

Many procedures have been used to detect proteolysis in milk and dairy products, such as determination of casein-nitrogen, non-casein-nitrogen, non-protein-nitrogen,
formal-nitrogen, pyruvic acid and ammonia (13). Studies have shown that the rate of formation of end products of proteolysis differ for various psychrotrophic bacteria (22,58,61).

Traditionally, the method most widely used to detect proteolysis in milk is that of Hulls, developed in 1947 (37). Hull's test relies on release of tyrosine and tryptophan containing peptides from milk protein that react with the Folin-Ciocalteau reagent. Juffs (37) used the Lowry modification (49) of Folin's procedure to estimate trichloroacetic acid-soluble amino acids and peptides. Proteolysis was expressed in terms of color equivalent to that of a tyrosine standard and the degree of proteolysis was represented as tyrosine values (TVs). Use of TVs for determining proteolysis as a routine test for milk quality has limited application since bacterial counts greater than one million per ml of milk are necessary before changes in the values are detected (38). Juffs further found out that natural variation among the TVs in raw milk samples complicated the use of the test. Juffs (37) studied TVs as a method for detecting proteolysis in milk, but found no relation between psychrotrophs or proteolytic psychrotrophs or total bacterial counts and the TVs.
The need for a simple, sensitive, chemical procedure for detecting deterioration of cold stored milk and milk products has made the determination of proteolysis an important area of research. The reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS) has been used to measure proteolysis in milk (54). Koops et al. (41) developed a method for determining nitrogen in milk that used a colorimetric determination of ammonia after sample digestion. Snoeran and Both (69), and Snoeran et al. (70) used Koop's (41) method to determine nitrogen fractions as ammonia. Church et al. (12) developed a spectrophotometric assay using O-pthalaldehyde for determination of proteolysis in milk. Kwan et al. (43) compared different methods and concluded that the fluorescamine (11) method was the most reliable and sensitive.

REFERENCES


Growth and Proteolytic activity of Selected Psychrotrophic Bacteria in Whole Milk and Whole Milk Retentate

R. Reddy\(^2\) and G. S. Torrey

Dairy Science Department
South Dakota State University
Brookings, SD 57007-0647

KEY WORDS: ultrafiltration, retentates, psychrotrophic bacteria, growth, proteolysis, tyrosine values, free amino groups.

\(^1\) Published with approval of the Director of the South Dakota Agriculture Experiment Station as publication No-\(-\)-of the Journal Series.
\(^2\) Department of Dairy Science, Mississippi State University, Mississippi State, MS 39762.
ABSTRACT

Raw whole milk (12.0 ± .5% total solids) was ultrafiltered at 50-54°C to obtain 25.0 ± .5% total solids in whole milk retentate. Whole milk and whole milk retentates were dispensed into 500 ml flasks fitted with screw caps and heated (65°C/35min) in a water bath. Samples were cooled to 70°C and inoculated to contain about $10^3$ CFU/ml of pure cultures of psychrotrophic bacteria. For each psychrotroph, proteolytic activity as estimated by tyrosine values and free amino groups, and growth were compared in whole milk and whole milk retentate. Growth and proteolytic activity were similar in milk and retentates during the logarithmic phase of bacterial growth; but during the stationary phase bacterial numbers and proteolytic activity were less in retentates than in milk.

INTRODUCTION

A major application of ultrafiltration (UF) to cheese making is on-farm or in-plant concentration of milk. The objectives of UF are to remove water and reduce milk
volume, thereby reducing operating costs related to refrigeration and transportation. Adaption of UF of whole milk in the dairy industry should reduce frequency of milk collection from the farm and change processing schedules at the plant. These changes will cause extended storage of retentates. Technical and economic feasibility studies that evaluated advantages and disadvantages of on-farm or in-plant UF of whole milk have been reported (8,20,21,26).

Extended storage of refrigerated milk and milk products favors growth of psychrotrophic bacteria. Most psychrotrophic bacteria in milk and milk products produce extra-cellular, heat-stable enzymes which may survive ultra-high temperature treatment (3,16). Thus, these lipolytic and proteolytic enzymes may cause spoilage in milk and in heat sterilized dairy products (3,16). Introduction of UF may compound the spoilage problem, since UF of whole milk may result in doubling the period of refrigerated storage.

Refrigerated storage of raw milk has been the subject of a great deal of research (3). Psychrotrophic growth in retentates is important because whole milk and retentate are different microbiological media as UF alters both the relative and absolute concentrations of milk constituents (6,9,10,24). Studies (2,25) have indicated that bacterial growth of mixed microflora was less in
retentates than in milk during refrigerated storage. Tayfour et al. (23) observed growth and proteolytic activity of *Pseudomonas fluorescens* P28 was less in skim milk retentates concentrated five times than in skim milk, however, growth and proteolytic activity in skim milk retentates concentrated two or three times was higher than in skim milk.

Characterization of growth of specific bacteria in whole milk retentate and data comparing changes in the composition of whole milk and whole milk retentate caused by growth of specific bacteria is needed. The present study was undertaken to investigate growth and proteolytic activity of psychrotrophic bacteria in whole milk and whole milk retentate.

**MATERIALS AND METHODS**

**Ultrafiltration**

Raw whole milk was obtained within 48 h after milking from the dairy farm at South Dakota State University and heated with continuous agitation to 50-54°C to increase the flux during UF. The heated milk was immediately ultrafiltered at the same temperature in an Abcor model 1/1 sanitary pilot plant containing a spiral
wound ultrafiltration module (Abcor Inc., Wilmington, MA). The unit contained cellulose acetate membranes having a total surface area of 4 m². Inlet pressure on the membrane system during operation was maintained at 2.8 Kg/cm², and outlet pressure was 1.4 Kg/cm². Previous experimentation showed that whole milk concentrated 3 times (initial feed volume to final feed volume) yielded retentates with approximately 25% total solids (TS). When the concentration of milk approached 3 times, retentate samples were taken in sterile bottles from the concentrate return flow every two min, and sample bottles containing retentate were maintained in ice to suppress growth of natural contaminants. Total solids of milk and retentate were determined by the Mojonnier method (1). Retentate samples with 25.0 ± .5% TS were chosen for growth and proteolysis studies with psychrotrophic bacteria. When collected samples were outside the desired range of 25.0 ± .5% TS, two samples within the range of 25.0 ± 1.5% TS were mixed to obtain a sample having 25% TS.

Samples

Two hundred milliliters of whole milk or whole milk retentate were dispensed into separate sterile 500 ml Erlenmeyer flasks fitted with screw caps. Samples of whole
milk and retentate were heated at 65°C for 35 min in a water bath and immediately cooled to 70°C. The heat treatment was done to reduce natural contamination in samples before inoculation with pure cultures. Bacteria surviving the heat treatment of whole milk and retentate were determined not to increase in numbers during incubation for 5.5 d at 70°C. Samples of heated whole milk and retentate were tested for fat (1), total protein (15), and ash content (15).

Cultures and Inoculation

Isolation, characterization and identification of proteolytic, psychrotrophic bacteria used in this study are described by Roberts (18). Isolates 1P and 2P were identified as different strains of Pseudomonas fluorescens; isolates 11P, 12P and 17P were identified as different strains of Pseudomonas spp. Inocula were prepared by growing each culture statically in Tryptic Soy Broth (TSB) at 25°C for 24 h. One tenth of a milliliter of culture was transferred to 10 ml of fresh TSB and incubated at 25°C for 18 h. The concentration of cells of each culture was measured by diluting the culture with sterile TSB to an absorbance of .3 at a wavelength of 420 nm using a Bausch and Lomb Spectronic 20 spectrophotometer (Bausch and Lomb,
Rochester, NY.). Cell numbers for each isolate were determined previously in this laboratory (Table 1A). The cultures were then diluted to attain a bacterial concentration of 1,000 CFU/ml in 200 ml milk or retentate.

Growth Studies

Pure cultures of isolates 1P, 2P, 11P, 12P or 17P were inoculated into flasks containing heat-treated retentate or whole milk and flasks were incubated statically at 70°C for 5.5 d. Growth experiments in both whole milk and retentate were in duplicate. Samples were taken from flasks twice daily to determine growth by plating an appropriate dilution of whole milk or retentate in Standard Methods Agar (15). Plates were counted after incubation at 25°C for 48 ± 3h.

Proteolysis

Pure cultures of bacterial isolates were inoculated into heated whole milk and retentate and these were incubated statically at 70°C for 15 d. Samples were taken at 0, 5, 10, and 15 d for chemical analyses and bacterial growth (15). Tyrosine values were determined by the method described by Juffs (13,14), as modified by Senyk et al
(19). In the case of retentate samples, a large amount of precipitate was observed after alkaline copper tartrate was added, hence the assay mixture was refiltered (Whatman # 1) to remove the precipitate and prevent erroneous results in colorimetry. A second method (16), measuring 2,4,6-trinitrobenzene sulfonic acid (TNBS)-bound free amino groups was used to estimate proteolysis resulting from growth of bacterial isolates. During measurement of TNBS-bound free amino groups, trichloroacetic acid filtrates of some 10 d and all 15 d samples contained precipitates, which were removed by centrifugation of filtrates at 1,958 X g for 5 min in a Sorvall SH-MT rotor (Dupont Co., Newtown, Conn.).

Statistical Analysis

Growth of isolates in whole milk and retentate was compared by dividing growth curves into two linear parts representing logarithmic and stationary phases. A comparison for each of these phases of growth in whole milk and retentate was made using multiple regression analysis (22). Analysis of Covariance was used to measure treatment effects by removing, by regression, certain portions of experimental error caused by initial differences in microbial populations of the samples and sampling error.
Analysis of variance (22) was used to compare proteolysis as estimated by tyrosine values and TNBS-bound free amino groups in whole milk and whole milk retentate.

RESULTS AND DISCUSSION

The chemical composition of whole milk and retentate used for experiments is presented in Table 1. Total solids and ash contents of retentates were twice that of milk, fat and protein concentrations were about three times greater than milk. Ultrafiltration of whole milk to greater than 25.0% TS has been reported to cause operating problems such as membrane fouling and reduction in flux (8). Reduction in flux approaches 20% at 25.0% TS and increases rapidly thereafter (8). Therefore 25.0% TS concentration was chosen for the study of growth and proteolytic activity of psychrotrophic bacteria. Whole milk and retentate were heat treated (650°C/35 min) to reduce natural contamination, which might have interfered with studies of pure cultures.

Growth Studies

Results of growth experiments for five isolates incubated at 70°C for 5.5 d are summarized in Figures 1-5. As indicated, growth of each isolate in whole milk was not
different from that in retentate during the logarithmic phase. However, during the stationary growth phase, the bacterial concentration in whole milk was significantly different ($P < .05$) from that in retentates. Figures 1-5 indicate that growth in retentates reached lower cell populations during stationary phase than did the same isolates during growth in whole milk. Growth and the time required to reach the end of log phase were different for each isolate as indicated by slopes and intercepts.

Bacterial populations of all strains growing in retentates reached more than $10^7$ CFU/ml at the end of 5.5 d of incubation, indicating that retentates support psychrotrophic growth. Similar results were obtained by Veillet-Poncet et al (25). They observed that during refrigeration of retentates, psychrotrophic bacteria comprised 95-100% of the total microflora. They also found retentates stored for 72h at $4^0$C, 48h at $7^0$C or 24h at $12^0$C, contained $6 \times 10^5$, $3 \times 10^5$ and $6.5 \times 10^5$ CFU per ml respectively, and were comprised 100%, 100% and 95% of psychrotrophs. As in this study, Veillet-Poncet et al. (25) reported that at $7^0$C multiplication of natural mixed microflora was more pronounced in milk than in retentate. Similarly, Benard et al.(2) observed that pasteurized retentates stored at $6^0$C contained more than $10^7$ CFU/ml of psychrotrophic bacteria at the end of five days.
incubation. Tayfour et al. (23) studied growth and proteolytic activity of *Pseudomonas fluorescens* P28 in skim milk retentates of various concentrations at 70°C and found that growth was highest in retentates concentrated two or three times and lowest in retentate concentrated five times. Proteolysis was less in highly concentrated milks. Haggerty and Potter (11) and Hicky et al. (12) reported that growth of mesophiles unlike psychrotrophs is not affected in their growth by ultrafiltration.

Concentration by ultrafiltration causes changes in composition of milk and these changes may have some adverse affect on microbial growth. Storage studies (4,5) of reverse osmosis concentrates illustrate that psychrotrophic growth in concentrates was less compared to that in milk during stationary phase, agreeing with our results, even though the compositional changes caused by reverse osmosis and ultrafiltration are different. This may suggest the concentration process itself is a potential factor affecting microbial growth.

Proteolysis Studies

Proteolytic activity of psychrotrophic bacteria was determined by measuring end products of proteolysis: tyrosine and free amino groups. Figures 6-9 show TVS in
whole milk and retentates that resulted when strains of psychrotrophic bacteria were grown in whole milk and retentate samples. Initial TVs of whole milk fall within the normal range for whole milk (13,14). Initial TVs of the retentates used in the studies of isolate 11P and 12P are significantly higher (P < .05) than whole milk. Initial free amino groups in whole milk and concentrates are not different and fall within the normal range for heat treated milk and concentrates (10). However, free amino groups determined for heat-treated, whole milk are higher than those observed by McKeller (16). In the first five days of incubation there was a decrease in TVs and free amino groups indicating no proteolysis. The decrease in TVs and free amino groups was probably due to microbial uptake of these during growth. Methods in this experiment measure end products as indicators of proteolysis as opposed to the detection of structural changes of proteins observed in electrophoretic methods. During the later part of incubation both TVs and free amino groups increase very rapidly from day 5 to day 15. Proteolytic activity of each isolate in whole milk was significantly higher (P < .05 at day 10 and P < .01) than that of the same isolate grown in retentate. Previous research (23) also showed that the proteolysis was lower in highly concentrated retentates compared with less concentrated retentates or milk. As
indicated in Figures 6-9, both TVs and free amino groups appear not to increase until growth has entered stationary phase.

In milk products, proteolysis can be detected once the microbial population reaches about $10^7$ CFU/ml (13,14), and off-flavors associated with proteolysis will appear at approximately the same population (3). Figure 6 indicates the proteolytic activity of *Pseudomonas fluorescens* isolate 1P in whole milk and retentate and illustrates the increase in TVs and free amino groups after the bacterial populations reaches $10^8$ CFU/ml.

Several explanations were offered (4,5,7,23,25,27) for the reduced growth and proteolytic activity of psychrotrophic bacteria in retentate, such as (a) different chemical composition of retentates, (b) loss of nutritionally essential micronutrients in permeate, (c) depletion of some nutrients during growth, (d) some kind of inhibitory affect of concentration, and (e) all of these in combination. Reduced proteolytic activity of psychrotrophs in retentates may be due to higher concentration of amino acids in the retentates which will meet nutritional requirements of the bacterial populations (25). Concentration of a nutrient may be inhibitory to synthesis of extra-cellular protease (25). Concentration of phosphates during ultrafiltration may be implicated, as it
has been reported that extra-cellular production of protease in skim milk is inhibited by addition of polyphosphates (17). Garcia-Oritz et al. (7) stated that increase in protein concentration causes a decrease in proteolysis.

Results of this research and other reports (4, 5, 7, 23, 25, 27) indicate that retentate has potentially an inhibitory effect on psychrotrophic growth and proteolytic activity. With the numerous advantages of ultrafiltration in dairy industry, reduced activity of psychrotrophs will be an additional incentive and it can also be a way to control problems caused by psychrotrophs. However, better understanding of the mechanisms and factors affecting psychrotrophic growth in retentates is needed.
Table 1. Composition\(^1\) of whole milk and retentates used for bacterial growth and proteolysis studies.

<table>
<thead>
<tr>
<th></th>
<th>Fat</th>
<th>Protein</th>
<th>Ash</th>
<th>Lactose(^2)</th>
<th>Total solids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>3.20(.13)(^3)</td>
<td>3.10(.26)</td>
<td>0.70(.01)</td>
<td>4.80</td>
<td>12.2(.35)</td>
</tr>
<tr>
<td>Retentate</td>
<td>10.19(.05)</td>
<td>9.19(.1)</td>
<td>1.40(.01)</td>
<td>3.80</td>
<td>25.0(.08)</td>
</tr>
</tbody>
</table>

\(^1\) Means of eight observations  
\(^2\) By difference.  
\(^3\) Standard deviation.
\[ y = 7.3 + 0.01x \]

\[ y = 6.9 + 0.01x \]

\[ y = 3.7 + 0.06x \]
Figure 1. Growth of Isolate 1P in whole milk (■) and whole milk retentate (▲) incubated at 70°C. Each point represents the mean of data from three experiments, each done with duplicate growth flasks. Colony forming units in whole milk were significantly higher (P<.05) than in retentate after 80 h of incubation.
Figure 2. Growth of Isolate 2P in whole milk (□) and whole milk retentate (▲) incubated at 70°C. Each point represents the mean of data from three experiments, each done with duplicate growth flasks. Colony forming units in whole milk were significantly higher (P<.05) than in retentate after 50 h of incubation.
Figure 3. Growth of Isolate 11P in whole milk (□) and whole milk retentate (▲) incubated at 70°C. Each point represents the mean of data from three experiments, each done with duplicate growth flasks. Colony forming units in whole milk were significantly higher (P<.05) than retentate after 75 h of incubation.
Figure 4. Growth of Isolate 12P in whole milk (□) and whole milk retentate (▲) incubated at 70°C. Each point represents the mean of data from three experiments, each done with duplicate growth flasks. Colony forming units in whole milk were significantly higher (P<.05) than in retentate after 80 h incubation.
\[ \hat{y} = 7.4 + 0.01x \]

\[ \hat{y} = 7.1 + 0.01x \]

\[ \hat{y} = 3.4 + 0.06x \]
Figure 5. Growth of Isolate 17P in whole milk (□) and whole milk retentate (▲) incubated at 70°C. Each point represents the mean of data from three experiments, each done with duplicate growth flasks. Colony forming units in whole milk were significantly higher (P<.05) than in retentate after 50 h of incubation.
\[ \hat{y} = 5.8 + 0.01x \]
\[ \hat{y} = 4.4 + 0.01x \]
\[ \hat{y} = 3.0 + 0.05x \]
Figure 6. Growth and proteolytic activity of isolate 1P in whole milk (open) and whole milk retentate (closed) incubated at 70°C. Each point represents the mean of two experiments, each done with duplicate flasks. Tyrosine values (△,▲), free amino groups (○,●) and bacterial growth (□,■). Tyrosine values in whole milk are significantly higher (P<.05 on d 10 and P<.01 on d 15) than whole milk retentate. Values for free amino groups analyses in whole milk are significantly higher (P<.05 on d 10 and 15) than in whole milk retentate.
Figure 7. Growth and proteolytic activity of isolate 2P in whole milk (open) and whole milk retentate (closed) incubated at 7°C. Each point on the plot is an average of two replications taken from duplicate flasks. Tyrosine values (Δ, ▲), Free amino groups (○, ●), and Bacterial growth (□, ■). Tyrosin values in whole milk are significantly higher (P<.05 on d 10, P<.01 on d 15) than whole milk retentate. Free amino groups in whole milk are significantly higher (P<.05 on d 10, 15) than in whole milk retentate.
Figure 8. Growth and proteolytic activity of isolate llP in whole milk (open) and whole milk retentate (closed) incubated at 70°C. Each point on the plot is an average of two replications taken from duplicate flasks. Tyrosine values (△,▲), Free amino groups (○,●), and Bacterial growth (□, ■). Tyrosin values in whole milk are significantly higher (P<.05 on d 10, P<.01 on d 15) than whole milk retentate. Free amino groups in whole milk are significantly higher (P<.05 on d 10, 15) than in whole milk retentate.
Figure 9. Growth and proteolytic activity of isolate 12P in whole milk (open) and whole milk retentate (closed) incubated at 70°C. Each point on the plot is an average of two replications taken from duplicate flasks. Tyrosine values (△, ▲), Free amino groups (○, ●), and Bacterial growth (□, ■). Tyrosine values in whole milk are significantly higher (P<.05 on d 10, P<.01 on d 15) than whole milk retentate. Free amino groups in whole milk are significantly higher (P<.05 on d 10, 15) than in whole milk retentate.
REFERENCES


SUMMARY AND CONCLUSIONS

The purpose of this study was to compare growth and proteolytic activity of selected psychrotrophic bacteria in ultrafiltered milk with growth and proteolytic activity of the same isolate in whole milk.

Ultrafiltration of whole milk was carried out in a spiral wound pilot plant at 50-54°C to attain 25.0% total solids in retentate. Retentates and whole milk were heat treated (65°C/35 min.), inoculated with one of five selected strains of Pseudomonas, and incubated at 70°C. For each psychrotroph, proteolytic activity as estimated by tyrosine values and free amino groups, and growth was compared in whole milk and whole milk retentate. Results obtained during these studies indicate that there was no significant difference between growth in whole milk and ultrafiltered milk during initial stage of incubation. However, after 2-4 days of incubation (onset of stationary phase was different for each isolate) growth in ultrafiltered milk lagged behind growth in whole milk. This study also showed that there is reduction in activity of proteases elaborated by the gram-negative psychrotrophs in retentates compared to whole milk.
From this study it is apparent that whole milk retentates have inhibitory an affect on *Pseudomonas* spp, however, further investigations are needed to determine the reasons for such action of retentates. Also, it is important to understand factors affecting the possible inhibitory action and reasons for inhibition only during stationary phase of bacterial growth and not in logarithimic phase. Psychrotrophic bacterial populations of retentates in the present study and in previous studies reached $10^7$ CFU/ml or more, indicating that retentates are as good microbiological media as milk in the initial stages of of incubation. But, this similarity ends with the onset of stationary phase, which appears premature in retentates compared to that of milk. It may be assumed that a difference in composition between milk and retentate is the major influencing factor on the growth of psychrotrophs. Apart from differences in composition, whole milk and whole milk retentate have a different history of heat treatments; this is because milk is ultrafiltered at 540°C for at least 20 to 25 min to obtain 25% TS in the retentate. The affect of ultrafiltration and heat treatment before inoculation might have caused heat injury to constituents of retentate which ultimately affects the bacterial growth. Information is required to understand affects of heat-treatments (of ultrafiltration process and of thermization) on the
composition of retentates and on bacterial growth in retentates. Studies are also needed to prove that the inhibition of growth and proteolytic activity of *Pseudomonas* spp. in retentates is true for all psychrotrophic bacteria. From the present study it is difficult to come to a conclusion about keeping quality of retentates and its usefulness in prevention of problems caused by psychrotrophic bacteria. In this study the differences observed in bacterial numbers in whole milk and retentates are small and usually the bacterial concentrations of retentates observed in this study would be enough to cause spoilage. Therefore, the difference observed may not be of practical significance in solving the problems caused by psychrotrophic bacteria.

Information about growth, proteolytic and lipolytic activity of psychrotrophic bacteria in retentates would be valuable for the introduction of ultrafiltration into the dairy industry, and possibly will help to prevent problems caused by psychrotrophs in refrigerated milk and milk products in general.
APPENDIX

Data in appendix supports information presented in the article section of this thesis. Table 1 presents the data relating absorbance of pure cultures and cell concentrations (See "Cultures and Inoculation" of Materials and Methods). Table 2 is used to construct figures 1 to 5, and tables 3 and 4 are used to construct figures 6 to 9.
Table 1. Colony forming units present in Tryptic Soy Broth cultures incubated for 18-24 at 25°C when absorbance at 420 nm was .3

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>CFU/ml on SMA&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P</td>
<td>$1.54 \times 10^8$</td>
</tr>
<tr>
<td>2P</td>
<td>$6.42 \times 10^7$</td>
</tr>
<tr>
<td>11P</td>
<td>$7.12 \times 10^7$</td>
</tr>
<tr>
<td>12P</td>
<td>$1.08 \times 10^8$</td>
</tr>
<tr>
<td>17P</td>
<td>$9.61 \times 10^7$</td>
</tr>
</tbody>
</table>

<sup>1</sup>Standard Methods Agar
Table 2. Growth\(^1\) of psychrotrophic bacteria in whole milk (W) and retentate (R) incubated at 70\(^\circ\)C for 5.5 d.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Time of incubation, h</th>
<th>Log CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>1P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3.40</td>
<td>4.91</td>
</tr>
<tr>
<td>R</td>
<td>3.41</td>
<td>4.93</td>
</tr>
<tr>
<td>2P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3.01</td>
<td>4.34</td>
</tr>
<tr>
<td>R</td>
<td>3.00</td>
<td>4.42</td>
</tr>
<tr>
<td>11P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3.37</td>
<td>5.33</td>
</tr>
<tr>
<td>R</td>
<td>3.37</td>
<td>5.31</td>
</tr>
<tr>
<td>12P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3.45</td>
<td>5.17</td>
</tr>
<tr>
<td>R</td>
<td>3.44</td>
<td>5.17</td>
</tr>
<tr>
<td>17P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3.09</td>
<td>4.50</td>
</tr>
<tr>
<td>R</td>
<td>3.00</td>
<td>4.09</td>
</tr>
</tbody>
</table>

\(1\) Means of three replicates
Table 3. Tyrosine values\(^1\) in whole milk(W) and retentates(R) inoculated with proteolytic psychrotrophic bacteria and incubated at \(70^\circ\text{C}\).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Time of incubation, days.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>mg/ml</td>
</tr>
<tr>
<td>1p</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.81(.13)(^4)</td>
</tr>
<tr>
<td>R</td>
<td>1.11(.13)</td>
</tr>
<tr>
<td>2p</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.78(.15)</td>
</tr>
<tr>
<td>R</td>
<td>1.09(.15)</td>
</tr>
<tr>
<td>11p</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>1.03(.09)(^2)</td>
</tr>
<tr>
<td>R</td>
<td>1.52(.09)</td>
</tr>
<tr>
<td>12p</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>1.02(.13)(^2)</td>
</tr>
<tr>
<td>R</td>
<td>1.52(.13)</td>
</tr>
</tbody>
</table>

1 Means of two replicates.
2 Tyrosine values in whole milk and retentate are significantly different, \(P=.05\)
3 Tyrosine values in whole milk and retentate are significantly different, \(P=.01\).
4 Standard deviation.
Table 4. TNBS bound free amino groups<sup>1</sup> in whole milk(W) and retentates(R) inoculated with proteolytic psychrotrophic bacteria and incubated at 70°C.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Time of incubation, days.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>umoles/ml</td>
</tr>
<tr>
<td>1p W 1.11(.21)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.55(.21)</td>
</tr>
<tr>
<td>R 1.21(.21)</td>
<td>0.63(.23)</td>
</tr>
<tr>
<td>2P W 1.05(.24)</td>
<td>0.54(.29)</td>
</tr>
<tr>
<td>R 1.18(.26)</td>
<td>0.58(.26)</td>
</tr>
<tr>
<td>11P W 1.04(.12)</td>
<td>0.94(.14)</td>
</tr>
<tr>
<td>R 1.06(.12)</td>
<td>0.85(.14)</td>
</tr>
<tr>
<td>12P W 1.03(.04)</td>
<td>0.85(.14)</td>
</tr>
<tr>
<td>R 1.11(.04)</td>
<td>0.85(.04)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means of two replicates.
<sup>2</sup>TNBS bound free amino groups in whole milk and retentate are significantly different, P<.01
<sup>3</sup>Standard deviation.