Heat resistant sporeforming psychrotrophic bacteria were isolated from raw milk samples from 59 Grade A farms in Oregon. Forty-nine of the 59 (83%) raw milk samples in this survey contained sporeforming psychrotrophic bacteria; isolates from twenty-four (40%) of the samples exhibited proteolytic properties. Populations of sporeforming psychrotrophic bacteria ranged from <10 to >10,000 CFU/mL for all samples. One hundred-two isolates were identified as *Bacillus* species. Twelve different *Bacillus* species were identified with *B. licheniformis* being the most predominate (18% of the samples) and *B. laterosporus* the least frequently isolated species, (2%). Fifty-eight percent of the bacilli isolates produced a bitter off-flavor and putrid odor, while 42% produced a fruity and/or rancid off-flavor when inoculated into sterile whole milk. Based on biochemical activity tests, 83% of the thermoduric isolates hydrolysed casein while 56% were proteolytic (in litmus milk), 57% demonstrated lipolytic activity and 31% produced acid in litmus milk.
Forty-eight isolates that tested positive for proteolysis were evaluated quantitatively for activity, which ranged from 0.93 to 1.93 units (expressed as mM of alanine). Isolates of *Bacillus cereus* var. *mycoides* demonstrated significantly higher (p>0.05) proteolytic activity than other *Bacillus* species isolated.
Identification and Characterization of Some Psychrotrophic Heat Resistant/Sporeforming Bacteria in the Grade A Raw Milk Supply of Oregon

by

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Typed by researcher for Ralph R. Meer
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Special thanks to Sam Beattie, Ph.D. student Department of Food Science, for taking time to answer my many questions and providing instructions for the final preparation of this thesis.

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INDENTIFICATION AND CHARACTERIZATION OF SOME PSYCHROTROPHIC
HEAT RESISTANT/SPOREFORMING BACTERIA IN THE
GRADE A RAW MILK SUPPLY OF OREGON

INTRODUCTION

The objectives of this research were to identify and characterize the psychrotrophic, heat resistant, sporeforming bacteria isolated from selected samples of Grade A raw milk from Oregon producers. This microbiological analysis of raw milk may serve as an important index for estimation of potential shelf life of fluid milk and milk products. Although there has been considerable previous effort directed at isolating and classifying these particular microorganisms in various studies, detailed knowledge concerning the extent and nature of proteolytic sporeforming bacteria in the Oregon milk supply has been limited.

A quality survey conducted by Bodyfelt (1986) on the Grade A raw milk supply of Oregon documented the occurrence of psychrotrophic sporeforming bacteria to be a significant concern. This research project is an extension of the prior survey.

Psychrotrophic bacteria have been recognised as a recurring problem in the refrigerated storage of fluid milk and perishable dairy products for a long time. Prior emphasis has been on post-pasteurization contaminants that are psychrotrophic, such as Pseudomonas, Flavobacterium, and Alcaligenes (Cousins, 1982). These gram-negative, non-sporeforming, heat sensitive organisms can be found on equipment and in water supplies. Although these organisms are heat sensitive, many of their pro-
teolytic and lipolytic enzymes can withstand moderate to severe heat treatments and result in product spoilage (Collins, 1979). Today, with increased success in controlling post-pasteurization contamination with non-heat-resistant psychrotrophs, attention has turned to sporeforming bacteria which have developed psychrotrophic growth characteristics. The predominant microorganisms which comprise this category are *Bacillus* species. These microorganisms have made their way into the raw milk supply as contaminants of water, from teats of cows (Mc Kinnon and Pettipher, 1983), from soil and milkstone deposits in bulk tanks and pipeline gaskets (Cannon, 1972), and/or as post-pasteurization contaminants (Phillips and Griffiths, 1986). In the spore state, these types of organisms can easily survive pasteurization temperatures with subsequent germination and outgrowth of vegetative cells. These organisms have been shown to produce degradative enzymes (e.g. lipases, phospholipases, and proteases) similar to those of non-sporeforming psychrotrophs, which result in flavor and quality defects in dairy products (Cousins, 1982).

The combination of both thermoduric and psychrotrophic properties within the same microorganism represents marked potential for causing spoilage in perishable milk products. The use of higher pasteurization temperatures and extended refrigerated storage of raw and pasteurized milk and cream products exacerbates substantially the significance of this group of microorganisms.
Identification and Incidence of Thermoduric Psychrotrophs

The trend toward prolonged refrigerated storage of raw milk before processing, application of higher pasteurization temperatures, and extended refrigeration before consumption has markedly increased the significance and importance of a group of microflora described as "thermoduric psychrotrophs". The presence of thermoduric or sporeforming bacteria in raw and processed milk has been well documented and extensively investigated. Reviews of the early studies on thermoduric bacteria (Hitemann, 1940; Thomas et al., 1950; Jayne-Williams, 1960; and Franklin, 1960) reported on the incidence, significance, influence of heat treatment, and effect of the organisms on fluid milk quality. These first reports emphasized the ubiquitous nature of sporeforming organisms, principally Bacillus, and their unlimited points of entry (animals, feed, bedding, pasture, milking equipment, transport vehicles, processing equipment) during the production and processing of milk and milk products. The sporeforming organisms discussed in these reviews were primarily mesophilic in nature, although thermophilic sporeformers were also mentioned which required incubation temperatures in the range of 55-65 C for growth and enumeration. A variety of Bacillus species had been isolated from both raw and pasteurized milk at this time with B. subtilis, B. licheniformis, and B. cereus most often encountered, with B. cereus var. mycoides, B.
circulans, B. coagulans, and B. megaterium isolated less often (Thomas et al., 1950). Problems encountered in early identification of the genus Bacillus included the absence of adequate differential tests for species identification and lack of cooperation between taxonomists investigating these organisms (Hilemann, 1940). Also of note are the number of countries that have identified and reported Bacillus in their milk supply, including the United States, Britain, Australia, India, France, Italy, Holland, and Japan. The defects associated with these organisms were recorded as early as 1903 and the linkage of B. cereus and B. cereus var. mycoides with sweet curdling and bitty cream was reported in 1938 (Jayne-Williams, 1960). It was also shown that the development of bitty cream could be inhibited if the product was stored at 5 C, which indicated the lack of psychrotrophic properties in these isolates. These early reviews provided no indication of bacteria which simultaneously possessed both thermoduric and psychrotrophic properties.

The word "psychrotrophic" bacteria was defined at the 1976 International Dairy Federation meeting as microorganisms that can grow at 7 C or less, irrespective of their optimal growth temperature (Collins, 1981). Evidence seems to indicate that the thermoduric psychrotrophs may be variants of mesophilic organisms that have adapted to growth at lower temperatures. Grosskopf and Harper (1974) developed this hypothesis based on the observation that isolated Bacillus species lost their ability to grow at refrigeration temperatures when stored at 21 C and on
the broad spectrum of psychrotrophic *Bacillus* that has been isolated.

The presence of psychrotrophic, sporeforming bacteria in raw and pasteurized milk was first reported by Grosskopf and Harper (1969). This study attributed the loss of quality of pasteurized milk stored at 4°C for four weeks to the outgrowth of *Bacillus coagulans*. Subsequently, three other psychrotrophic bacilli, *B. brevis*, *B. cereus*, and *B. licheniformis* were isolated. Since this first report, many researchers have isolated thermoduric psychrotrophs from milk (Table 1).

**Table 1.** Psychrotrophic *Bacillus* species commonly isolated from raw and pasteurized milk.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>REFERENCE</th>
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<tbody>
<tr>
<td><em>B. brevis</em></td>
<td>1,2,5,7</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>2,3,4,5,6,7,8</td>
</tr>
<tr>
<td><em>B. cereus var.</em></td>
<td>7</td>
</tr>
<tr>
<td>mycoides</td>
<td></td>
</tr>
<tr>
<td><em>B. circulans</em></td>
<td>1,2,3,5,6,7</td>
</tr>
<tr>
<td><em>B. coagulans</em></td>
<td>1,2,3,5,6,7</td>
</tr>
<tr>
<td><em>B. firmus</em></td>
<td>2,7</td>
</tr>
<tr>
<td><em>B. laterosporus</em></td>
<td>1,6,7</td>
</tr>
<tr>
<td><em>B. lentus</em></td>
<td>3,7</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>1,3,5,7</td>
</tr>
<tr>
<td><em>B. macerans</em></td>
<td>1,2,3,4,5</td>
</tr>
<tr>
<td><em>B. megaterium</em></td>
<td>1,2,3,5</td>
</tr>
<tr>
<td><em>B. polymyx</em></td>
<td>1,2,4,5,6,7</td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>1,3,6,7</td>
</tr>
<tr>
<td><em>B. subtilus</em></td>
<td>1,3,4,6,7</td>
</tr>
</tbody>
</table>


Although the majority of sporeforming organisms isolated in these studies were identified as *Bacillus*, this does not signify the absence of anaerobic sporeformers. Bhadsville et al. (1972) reported isolating four *Clostridium* species from 48 raw milk
samples and Martin (1974) reported 5% of the sporeformers in milk to be of the genus *Clostridium*. Other thermoduric species which do not form spores have also been isolated from raw and pasteurized milk. Washam et al. (1977) isolated non-sporeforming heat resistant bacteria belonging to the genera *Arthrobacter*, *Microbacterium*, *Streptococcus*, and *Corynebacterium* from pasteurized milk. Stadhouders (1975) indicated the presence of thermoduric microorganisms belonging to the genera *Alcaligenes*, *Streptococcus*, *Microbacterium*, and *Micrococcus*. Johnson and Bruce (1982) also isolated thermoduric species of *Microbacterium* and *Micrococcus*. Although the non-sporeforming microorganisms are capable of withstanding the moderate heat treatment of pasteurization (63 °C for 30 min), they typically cannot survive the more extreme temperatures used to isolate the sporeforming group of organisms (80 °C for 12 min), (Standard Methods for the Examination of Dairy Products, APHA 1985).

A wide range of values has been reported for the incidence of psychrotrophic, sporeforming bacteria found in both raw and pasteurized milk and milk products. Bodyfelt (1980) suggested that 20-25% of shelf life problems associated with pasteurized milk may be linked to heat resistant psychrotrophs. A recent survey conducted by Bodyfelt (1986) which analysed Oregon Grade A raw milk for flavor quality and shelf life potential found heat resistant sporeforming psychrotrophs at a level of ≥100 CFU/mL in 25% of 555 samples. Martin et al. (1961) found sporeforming bacteria in all but two samples out of a collection of approx-
imately 300 raw milk samples. Of 350 representative isolates, 94% were from the genus *Bacillus* and 6% from the genus *Clostridium*. Grosskopf and Harper (1969) isolated *Bacillus coagulans* that grew in pasteurized milk stored at 2°C for 13-17 days. They found the generation time of *B. coagulans* under these conditions to be 24 to 30 hr. They isolated psychrotrophic sporeformers from about 25% of milk supplies tested. Chung and Harper (1971) found that 83% of raw milk samples collected from 18 producers contained spores of psychrotrophic bacteria, ranging from 2 to 900 spores/mL. Typical spores at 7°C had a lag phase of 8 to 14 days with a generation time of 22-26 hr in the rapid growth phase. Contrary to the long generation times observed by the previously listed researchers, Langeveld (1973) reported the generation time of psychrotrophic *Bacillus* isolates in milk to be 12 hr at 5°C and Shehata et al. (1971) showed the generation time of psychrotrophic *Bacillus* to be 5 hr at 7.2°C. Mikolajcik and Simon (1978) investigated the microbiological quality of 109 Ohio raw milk samples; microorganisms were isolated after a heat treatment of 80°C for 12 min and subsequent incubation at 1, 2, and 4 weeks. At time 0, the psychrotrophic spore count ranged from less than 1/mL in 39% of the samples to a maximum of 140/mL. After incubation for 14 days, 34% of the samples had counts >1 million/mL, and after 28 days 71% of the samples had counts >1 million/mL. Coghill and Juffs (1979) found sporeforming, psychrotrophic bacteria in 31% of 167 pasteurized milk and cream samples in Queensland. They also indicated that
for *B. cereus* (and probably for other *Bacillus* species), spore germination generally occurred after treatment of milk and noted that germination was optimal at temperatures of 65-75°C.

**Growth of Sporeforming Psychrotrophs in Milk**

Shehata et al. (1971) measured the growth rates, doubling times, and temperature characteristics of 12 cultures (9 species) of *Bacillus* isolated from milk and demonstrated growth at refrigeration temperatures. The cultures were divided into two groups. Group A, which consisted of *B. subtilis*, *B. circulans* RH3 and *B. coagulans* TS3 and TS4, grew at 0°C and showed growth characteristics similar to those of psychrophilic strains of *Pseudomonas*. Group B, which consisted of eight cultures (*B. licheniformis*, *B. circulans* F7, *B. coagulans* F8, *B. pumilus*, *B. laterosporus*, *B. brevis*, *B. megaterium*, and *B. cereus*) had minimal growth temperatures of 5 to 7°C and other growth characteristics found in between psychrophilic *Pseudomonas* and mesophilic bacteria.

Mikolajcik and Koka (1968) investigated the influence of heat treatment of milk upon germination, outgrowth, and subsequent vegetative cell growth of *Bacillus cereus* spores. *B. cereus* spores were inoculated at a rate of 10^4/mL with and without heat shock (80°C for 12 min) into 4 types of milk; raw, pasteurized (62.8°C for 30 min), high heat (100°C for 10 min) and autoclaved (121°C for 10 min). After a 2 hr incubation at 35°C, 27.3, 89.7, 65.2, and 15.6% of the non-heat shocked spores and
67.8, 99.5, 94.9, and 83.5% of the heat shocked spores had germinated in the raw, pasteurized, high heat, and autoclaved milk, respectively. These results indicated that heating of spores and milk in which they are germinated resulted in increased germination. These researchers also inoculated cells that were in their exponential growth phase into various milk systems. Although the growth of vegetative cells in raw milk was one-half that of the heated milks for the first 30 min, continued incubation resulted in no differences in generation time or number of generations. The authors concluded that heat treatment of the spores from this group of microflora stimulated germination and subsequent outgrowth, while heat treatment of the milk affected the initial rate of active cell multiplication. Donovan (1959) demonstrated that while milk was not a good sporulation medium for *Bacillus* species, thin stationary films of milk on surfaces presented ideal conditions for sporulation. It was also shown that the dilution of milk increased the percentage of sporulating organisms. This condition is typical of that found after use of cleaning in place (CIP) systems on raw milk storage tanks which are not allowed to drain or dry sufficiently. Factors affecting germination and growth of spores in milk reported by Phillips and Griffiths (1986) include: occurrence of either fast or slow germinating species or strain, seasonal variations, and the exact point of contamination of the milk with spore source. Additional factors contributing to the rate of germination indicated by Davies (1977) included: somatic cell count, interaction between
milk components, and severity of heat treatment.

It has been reported by a number of authors (Labots and Hup, 1964b; Wilkinson and Davies, 1973; Stewart, 1975) that the rate at which spores germinate depends on their source. While spores isolated from soil and feces tend to be fast germinators, those isolated from raw milk and milking equipment appear to be comparatively slow germinators. It has been suggested by Davies (1975) that large numbers of B. cereus spores in pasteurized milk can be contaminants from within the dairy processing plant instead of the dairy farm itself. It was suggested that B. cereus spores isolated from raw milk were comparatively slower germinators than spores isolated from pasteurized milk, thus indicative that B. cereus isolates from pasteurized milk were more likely derived from contamination at the dairy plant. This theory was also supported by research conducted by Phillips and Griffiths (1986). These researchers reported that the average shelf life of those milks from which Bacillus organisms were associated with post-pasteurization contamination was approximately 2.5 days less than milk which was relatively free from such contaminants. The percent of isolates identified as Bacillus species in the milks was similar, 80.4% in the post-heat treatment contaminated and 74.3% in the non-contaminated samples, after 14 days storage at 6 C.

Johnson and Bruce (1982) reported thermoduric psychrotrophs in 27.2% of raw milk samples collected from 1040 farms in western Scotland. Eighty-five percent of the microorganisms isolated
were identified as belonging to the genus *Bacillus*, while 9% were assigned to the Coryneform group. The sampling period in this study was from March to July. Seasonal variation in the incidence of *Bacillus* organisms was noted. During the period March to April, when most of the cows were kept indoors, the level of contamination was 27.3% compared to 16.7% from April to July when cows were kept outside during the day and night. This trend was also seen by Ridgway (1954) who investigated the keeping quality of 3,753 bottles of commercially sterilized milk incubated at 2 different temperatures, 37-38°C and 23-24°C. In both cases the highest incidence of unsatisfactory bottles occurred during the winter months (Nov.-April). Defects also developed in milk more rapidly during these months. It is believed the cause was increased contamination of the teats by bedding. Contrary to the two reports, Phillips and Griffiths (1986) found that although the total mesophilic count varied little during the year from both creamery silo and bulk farm tank milk samples, thermoduric sporeformers were isolated in greater numbers during the winter while psychrotrophic sporeformers were higher during the summer months. In agreement with these results, Mc Kinnon and Pettipher (1983) demonstrated that the thermoduric spore counts in farm bulk tanks, collection tankers, silos, and pasteurized milks were lower in the summer than in the winter. These researchers also demonstrated that pasture reflected a higher proportion of psychrotrophic, sporeforming organisms than bedding and concomitantly, milk produced from cows
on pasture had a higher proportion of psychrotrophic sporeformers than cows housed indoors on bedding.

Shehata and Collins (1972) measured the heat resistance of psychrotrophic spores, isolated from pasteurized milk, in sterilized skim milk. Corresponding D values in minutes at 90°C for each isolate were *B. circulans*, 4.4; *B. brevis*, 4.8; *B. pumilus*, 5.1; *B. megaterium*, 5.2; *B. coagulans*, 5.5; *B. cereus*, 5.8; *B. licheniformis*, 6.2; and *B. laterosporus*, 6.4. Spores from three of the organisms, *B. cereus*, *B. pumilus*, and *B. laterosporus* were also tested for their thermal resistance at 95°C. These values, along with ones from *Bacillus* sp. tested by Mikolajcik (1970) are listed in Table 2. The organisms tested by Mikolajcik (1970) were ones previously isolated in the department by Martin et al. (1962) from milk, except for *B. licheniformis* ATCC 10716, *B. coagulans* ATCC 2050, and *B. megaterium* 9. Skim milk was also used for the measurement of thermal resistance in the later study. Shehata and Collins (1972) concluded from these data that psychrotrophic spores were less heat resistant than mesophilic spores. However, Mikolajcik (1970) made no reference in his research as to whether his spores were mesophilic or psychrotrophic; and, as stated previously, all but 3 were isolated from raw milk. Laine (1970) suggested that the temperature at which the spore is formed is an important determinant of heat resistance (cited in Law, 1979). Research done by Law et al. (1979) with *B. sphaericus* isolates showed that spores produced at lower temperatures have lower thermal resistance than those
produced at higher temperatures.

Table 2. Thermal resistance of selected *Bacillus* species at 95°C.

<table>
<thead>
<tr>
<th>Organism</th>
<th>D value (min)</th>
<th>Z value (°C)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. licheniformis</em> A-1</td>
<td>17.76</td>
<td>6.4</td>
<td>1</td>
</tr>
<tr>
<td><em>B. licheniformis</em> A-5</td>
<td>20.50</td>
<td>6.8</td>
<td>1</td>
</tr>
<tr>
<td><em>B. licheniformis</em> ATCC 10716</td>
<td>12.10</td>
<td>7.8</td>
<td>1</td>
</tr>
<tr>
<td><em>B. cereus</em> 1</td>
<td>10.16</td>
<td>9.6</td>
<td>1</td>
</tr>
<tr>
<td><em>B. cereus</em> 7</td>
<td>14.40</td>
<td>7.1</td>
<td>1</td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>4.03</td>
<td>7.5</td>
<td>1</td>
</tr>
<tr>
<td><em>B. cereus</em> var. mycoides</td>
<td>10.90</td>
<td>7.6</td>
<td>1</td>
</tr>
<tr>
<td><em>B. coagulans</em> ATCC 7050</td>
<td>6.90</td>
<td>9.1</td>
<td>1</td>
</tr>
<tr>
<td><em>B. laterosporus</em></td>
<td>5.95</td>
<td>7.0</td>
<td>1</td>
</tr>
<tr>
<td><em>B. circulans</em></td>
<td>4.75</td>
<td>11.5</td>
<td>1</td>
</tr>
<tr>
<td><em>B. megaterium</em> 9</td>
<td>6.50</td>
<td>8.4</td>
<td>1</td>
</tr>
<tr>
<td><em>B. sphæricus</em></td>
<td>7.60</td>
<td>9.1</td>
<td>1</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>1.80</td>
<td>9.4</td>
<td>2</td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>1.40</td>
<td>9.7</td>
<td>2</td>
</tr>
<tr>
<td><em>B. laterosporus</em></td>
<td>2.10</td>
<td>10.1</td>
<td>2</td>
</tr>
</tbody>
</table>

2. Shehata and Collins (1972)

Defects Associated With Thermoduric Psychrotrophs in Milk and Milk Products

The effect of thermoduric psychrotrophs in fluid milk and milk products is similar to that of other spoilage bacteria. Off flavors, e.g. bitter, putrid, unclean, stale, rancid, fruity, yeasty, and sour, have been associated with these microorganisms (Washam et al., 1977; Mikolacik and Simmon, 1978; Collins, 1981; Cousins, 1982; Coghill, 1982). Washam et al. (1977) inoculated isolated *Bacillus* sp. into sterile milk at a 0.5% level and
incubated at 7.2 C. *B. macerans*, *B. polymyxa*, *B. laterosporus*, *B. subtilus*, *B. lentus*, *B. cereus*, and *B. sphericus* were associated with the following defect combinations: fruity and sour; sour, yeasty and gassy; sweet curdling, bitter and unclean; sweet curdling and bitter; sour; sweet curdling and bitter; sour and unclean; respectively. Shehata et al. (1971) associated a fruity off-flavor, often accompanied by a rancid note, to milk contaminated with *B. circulans*; fruity was the primary flavor defect, along with the unclean defect, for both *B. coagulans* and *B. laterosporus*. *Bacillus cereus* and *B. cereus* var. *mycoides* have been associated with additional defects such as sweet curdling in fluid milk and bitty cream which are caused by the production of proteolytic and lipolytic enzymes, respectively (Overcast and Atmarm, 1974; Cox, 1975; Mikolajcik, 1978; Bodyfelt, 1980; and Cousins, 1982).

The proteases produced by thermoduric psychrotrophs have been shown to attack principally casein and to a lesser extent whey proteins (Bengtsson et al. 1973), which lead to gelation of UHT milk, sweet curdling of milk, bitter and unclean off-flavors in cheese, decreases in cheese yield, and such textural and body defects as wheying off in cultured dairy products (Cousins, 1982; Dairy Research Review, 1986). Collins (1981) reported (based on a California survey) that in the spring season about 25% of carton bottom surfaces of homogenized milk (stored at 7.2 C for 12 days) will have large microbial colonies (buttons or pellicles) approximately .3 to .6 cm in diameter. Furthermore,
if the cartons are stored for 19-20 days, about 90% will contain buttons. Often, the presence of these "buttons" goes unnoticed by the consumer of the affected product. Phillips et al. (1981) measured the growth and associated enzymatic activity of spoilage bacteria in pasteurized cream and found that *Bacillus* species which were isolated were mainly proteolytic. None were solely lipolytic, but a number of the isolates demonstrated both proteolytic and lipolytic activity.

Lipolytic activity, or the production of lipases by thermoduric psychrotrophs, has been demonstrated to produce both rancid and fruity off-flavors. The fruity note arises from the esterification of free fatty acids (Cousins, 1982). Thermoduric psychrotrophs have been shown to produce phospholipases, particularly phospholipase-C or lecithinase (Fox et al., 1976). It is believed that these phospholipases have a degradative action on the fat globule membrane, which results in increased susceptibility to the action of lipases (Fox et al., 1976). It has also been suggested that the degradation of the fat globule membrane by lecithinase results in the aggregation of fat globules leading to the "bitty cream" defect which is so frequently observed in cream products (Cox, 1975; Coghill, 1979; Mikolajcik, 1978; Cousins, 1982).

*Bacillus* species isolated by Johnson and Bruce (1981) demonstrated a variety of biochemical capabilities which would be of significance in milk spoilage: 84% hydrolysed casein, 73% were proteolytic in litmus milk, 77% were lecithinase positive,
57% hydrolysed cream, and 8% fermented lactose. Anaerobic spore-formers, particularly *Clostridium tyrobutyrium*, which produce gas, can produce defects in cheese known as "blowing", "late blowing" or "late gas" since the defect usually does not show up until 2 to 3 weeks following manufacturing. Butyric acid, hydrogen sulfide, and putrefactive end products produced by clostridia can also produce flavor and odor defects (Donnelly and Busta, 1981).

Tinuoye and Harmon (1975) inoculated thermoduric psychrotrophs (200 to 1000 organisms/mL) into sterile whole and skim milk. Organoleptic defects were detected in samples with populations of 3 or 4 million/mL. This level of growth was reached within 6 days at 7.2 C. Punch et al. (1965) showed that psychrotrophic microbial populations of 5 to 20 million/mL in samples of pasteurized milk held at 6-20 C were associated with physical and flavor defects. However, Tinuoye and Harmon (1975) noted that the temperature for organisms to produce degradative enzymes, i.e. proteases, lipases, and carbohydrases, is usually lower than the optimum temperature for cell division. Thus it is possible for milk that is held at refrigeration temperatures to develop off-flavors caused by microbially produced enzymes, even though the microbial population remains below that normally associated with microbial defects.
Proteolytic Activity of Sporeforming Bacteria

A study conducted by Sharma et al. (1974) examined the presence of proteolytic sporeforming psychrotrophic bacteria in 51 raw milk samples. Twenty of the samples had proteolytic psychrotrophic spore counts in the range of 1-5 CFU/mL, 3 samples had more than 5 CFU/mL, and 28 samples were negative. However, after subsequent preincubation (7 C for 7 days), 14 samples had proteolytic psychrotrophic spore counts in the range of 1-5 CFU/mL, 14 had 6-10 CFU/mL, 11 had 11-15 CFU/mL, and 1 sample showed a count of more than 15 CFU/mL. The proteolytic activity ranged from 20 to 480 units/mL. One unit of enzyme activity was defined as the amount of enzyme required to release TCA soluble fragments that produced a blue color equivalent to 1 ug of tyrosine/hr at 37 C. The method used to harvest the protease enzyme and measure activity was that of Keay and Wildi (1970) with modifications. Twelve of the isolates (24%) demonstrated protease activity that ranged from 51-100 units/mL and 10 isolates (20%) had enzyme activity greater than 300 units/mL.

Chopra et al. (1984) measured the proteolytic activity of 171 strains of thermophilic bacterial cultures isolated from various fluid milk samples and milk products. The criteria used to distinguish organisms that were thermophilic was their ability to grow at 55 C (facultative) and 65 C (obligate) when plated on tryptone dextrose yeast extract agar (TDYA) and incubated for 48 hr. These were recorded as thermophilic bacteria count (TBC). A proteolytic thermophilic count (PTC) was made on TDYA with a 10%
addition of sterilized skim milk. Market raw milk contained the highest viable count (plated on TDYA for 48 hr at 37 C), although the TBC and PTC were greater in pasteurized products than in raw milk samples. The method of Kaey and Wildi (1970) with modifications was used to harvest the enzyme and measure proteolytic activity. Fifty of the 171 isolates demonstrated a proteolytic activity greater than 100 units/mL at 55 C. Only 10 isolates had a proteolytic activity >100 units/mL at 65 C. All of the 50 isolates demonstrating a proteolytic activity >100 units/mL at 55 C were identified as belonging to the genus *Bacillus*. Twenty-nine were *B. stearothermophilus*, 12 were *B. coagulans*, 5 were *B. circulans*, and 4 were *B. licheniformis*. The heat resistance of the isolated protease enzymes was also tested. It was found that enzymes produced by 5 *B. stearothermophilus* organisms and 1 *B. licheniformis* retained 100% of their activity at temperatures up to 95 C for 30 minutes.

**Pathogenesis and Health**

Although the main concern with presence of *Bacillus* species in milk and milk products is the extension of shelf life, public health issues also need to be considered. *Bacillus cereus* has been shown to cause two different forms of gastro-enteritis as well as being capable of causing mastitis, systemic infection, and gangrene (Johnson, 1984). The two foodborne illnesses are caused by 2 distinct toxins; one which is responsible for emetic outbreaks characterized by nausea and vomiting within 0.5 to 6 hr
after ingestion of contaminated food and the other which is associated with the onset of watery diarrhea and abdominal cramps and pain occurring 6 to 15 hr after consumption of contaminated food. In this latter syndrome, nausea may occur but vomiting rarely happens.

A summary of the current knowledge of *B. cereus* toxins was provided by Gilbert and Kramer (1984). The diarrheal toxin, a true enterotoxin, is capable of causing fluid accumulation in ligated rabbit ileal loops, altering vascular permeability of rabbit and guinea pig skin, and killing mice when injected intravenously. Oral administration of the enterotoxin to rhesus monkeys causes diarrhea and in high concentrations is capable of causing necrosis in skin and intestinal mucosa. Laboratory detection of the enterotoxin includes ligated rabbit ileal loop, vascular permeability assay, tissue culture assay, immuno-gel diffusion and aggregate haemagglutination. The enterotoxin is synthesized and released during the late exponential phase of growth, at a temperature optimum of 32-37 C and a pH range of 4-11. This emphasizes the importance of proper temperature control of dairy products in addition to other sanitation measures for maximizing shelf life. The emetic toxin requires mesophilic temperatures for its production (25-30 C) and is stable in the pH range of 2-11. The production of the emetic toxin occurs during the stationary growth phase. The chemical mechanism of the emetic toxin is not known and laboratory detection is conducted by monkey feeding trials.
Emetic outbreaks seem to be almost entirely associated with rice, although a study done by Johnson et al. (1984) found that rice did not appear to select for survival of emetic strains over diarrheal strains. Outbreaks of emetic illness have also been reported in other starchy food such as macaroni and cheese and vanilla slices (cream puffs). Unconfirmed cases due to feta cheese and skim milk powder may have involved *B. cereus* (Johnson, 1984). Foods involved in diarrheal type outbreaks are varied and range from vegetables and salads to meat dishes and casseroles.

Cases of *B. cereus* food poisoning in milk-based products have been reported in eastern Europe. An outbreak of *B. cereus* food poisoning was reported which resulted from the ingestion of ice cream (mainly by school children) which was manufactured under faulty conditions (Bulyga et al., 1973). Another case involved children who drank milk heavily contaminated (21 X 10 cells/mL) with *B. cereus*. Approximately 81% of the children who drank the milk became ill within 8-11 hr and the symptoms disappeared within 5-10 hr without drug treatment (Vlad and Vlad, 1972).

Isolation of *B. cereus* from food involves plating on differential and selective media. The most commonly used media formulations include; mannitol-egg yolk-polymyxin (MYP) agar, KG agar, blood agars, polymyxin-pyruvate-egg yolk-manitol-bromothymol blue agar (PEMBA), and a similar formula substituting bromocresol purple for bromothymol blue (PEMPA). Several of
these formulas used polymyxin as an inhibitory agent for competitive organisms and are designed to use the mannitol negative, lecithin-hydrolyzing nature of B. cereus for differentiation. Typical B. cereus colonies on the PEMBA plates are peacock blue with a surrounding zone of precipitation, while colonies on the plates containing bromocresol purple are mauve in color with the same zone of precipitation (Szabo et al., 1984). In a study done by Harmon et al. (1984), researchers concluded that MYP was slightly superior to trypticase-soy-polymyxin blood agar and PEMBA because the B. cereus colonies on this medium were more easily differentiated from those of other species. Szabo et al. (1984) whose research group was responsible for modifying the PEMBA plates to the PEMPA concept, indicated the latter medium was more advantageous based on decreased incubation time to 18-22 hr with PEMPA compared to 24-48 hr with PEMBA and an increased ease of recognizing presumptive colonies.

Bacillus subtilis has also been implicated in outbreaks of food poisoning in the literature. Bacillus subtilis, an organism responsible for causing ropy bread, has been linked as the causative agent of a specific food poisoning in individuals eating bread that was contaminated heavily with this organism (Riemann, 1969). Other foods that have been linked with B. subtilis food poisoning include fish, pickled fish, and turkey. Symptoms associated with this organism in order of severity include; diarrhea, abdominal cramps, nausea, prostration, and vomiting (Riemann, 1969).
MATERIAL AND METHODS

Sample Collection

Raw milk samples were collected from producers located primarily in the Willamette Valley of western Oregon. Selection of processing sites to obtain samples was based on the expressed cooperation of plant personnel through prior contact by the Oregon State University Extension Specialist in Dairy Processing. A representative of the processing plants exercised discretion as to which samples would be obtained for the study. Samples were taken by state licensed milk samplers (haulers) and transported under refrigeration (4.2 C) to respective processing sites. Processed milk samples (pasteurized and homogenized) were obtained directly from the dairy processor. The samples were transported in ice chests to a microbiology laboratory at Oregon State University. Milk samples were stored at 1 C prior to commencing analysis, which was conducted within 24 hr of receiving samples. A total of fifty-nine raw milk and eight processed milk samples were collected throughout the time period from November 1986 to March 1987.

Plate Counts of Raw Milk

Standard plate counts and counts following laboratory pasteurization were made on all raw samples based on methods in Standard Methods for the Examination of Dairy Products (APHA, 1985). A modification of the Standard Methods was employed to
conduct the Psychrotrophic Sporeforming Count (PSC) and the Proteolytic Psychrotrophic Sporeforming Count (PPSC), *Standard Methods for the Examination of Dairy Products* (APHA, 1985).

Approximately 15 mL of raw milk were transferred aseptically to sterile 15 mm screw cap test tubes, heat-treated for 10 min at 80°C (excluding the heating "come-up" time), cooled instantly in an ice water bath and then incubated at 7.2°C for 10 days.

The heat treatment was carried out in an 80°C water bath equipped with a shaker (New Brunswick Scientific Co.). The temperature was monitored using a control tube which contained the same amount of sample and a thermometer. After incubation, standard plate counts were conducted and incubated for 48-72 hr at 21°C, the colonies observed were recorded as the PSC. All plating procedures were conducted with standard methods agar (DIFCO).

The heat treated and incubated (10 days at 7.2°C) samples were also used to plate the PPSC using the Casein Agar Method, *Standard Methods for the Examination of Dairy Products* (APHA, 1985 p 191-193). Plates were incubated at 21°C and counted after 48-72 hr. Colonies surrounded by a white zone or halo of casein precipitate were considered proteolytic and were recorded as the PPSC.

*Microbiological Evaluation of Processed Milk*

The processed milk samples were treated the same as the raw samples in conducting plate counts (i.e. SPC, PSC, and PPSC), except that the lab pasteurization count was excluded. The bulk
of the sample was stored at 7.2 C and monitored by sensory analysis (flavor and odor) up to and beyond (when appropriate) the pull date. Sensory monitoring of the samples was conducted by the investigator, who has had training and experience in dairy products sensory evaluation.

Identification of Isolated Microorganisms

Selected colonies from both the PSC and PPSC plates (based on statistical selection techniques recommended by Harrigan and McCance, 1966) were streaked on standard methods agar for isolation and subsequent genus and species identification. Cellular and colony morphology, physiology, and reaction to selected biochemical tests (Table 3) were used to identify proteolytic and nonproteolytic sporeforming psychrotrophic organisms, according to the diagnostic key from Skerman (1967) and Gordon et al. (1973) and using standard taxonomic procedures and descriptions from Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1985). Reference strains used were obtained from the American Type Culture Collection, Rockville, MD. and included: *Bacillus cereus* #14579, *B. circulans* #4513, *B. subtilis* #6051, and *B. megaterium* #4513.

Selected heat resistant psychrotrophic isolates were tested for their ability to survive 10 min at 80 C heat treatment. A 0.1 mL cell or spore suspension was used to inoculate 15 mL of sterile whole milk and then subjected to the heat treatment. Standard plate counts were conducted immediately and after an
incubation period of 10 days at 7.2 °C to measure survival and
growth at refrigeration temperatures. The ability of the same
isolates to survive two subsequent heat treatments: (1) simulated
HTST pasteurization (16 sec at 78.6 °C) followed by (2) reheating
at 80 °C for 10 min was also tested. These temperatures/time
conditions represented a simulation of the pasteurization or
treatment incurred by the processed milk.

Table 3. A list of physiological characteristics and biochemical
tests used to identify thermoduric psychrotrophs.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Test Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>gram reaction</td>
<td>growth at pH 5.7</td>
</tr>
<tr>
<td>presence of spores</td>
<td>growth at pH 9.7 *</td>
</tr>
<tr>
<td>cell and colony morphology</td>
<td>growth at 45 &amp; 65 °C</td>
</tr>
<tr>
<td>anaerobic growth</td>
<td>hydrolysis of starch</td>
</tr>
<tr>
<td>catalase production</td>
<td>hydrolysis of arginine *</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>decomposition of casein</td>
</tr>
<tr>
<td>final pH of V-P broth</td>
<td>decomposition of tyrosine</td>
</tr>
<tr>
<td>growth in 0, 5, 7, and 10 % NaCl</td>
<td>reaction in litmus milk</td>
</tr>
<tr>
<td>acetyl methylcarbinol production</td>
<td>production of indole</td>
</tr>
<tr>
<td>acid production from:</td>
<td>reduction nitrate to nitrite</td>
</tr>
<tr>
<td>arabinose</td>
<td>deamination of phenylalanine</td>
</tr>
<tr>
<td>fructose *</td>
<td>utilization of propionate</td>
</tr>
<tr>
<td>glucose</td>
<td>utilization of citrate</td>
</tr>
<tr>
<td>glycerol *</td>
<td>lipolytic activity</td>
</tr>
<tr>
<td>mannitol</td>
<td>growth in milk + 10% methyl blue *</td>
</tr>
<tr>
<td>sorbitol *</td>
<td>growth in broth + 40% bile *</td>
</tr>
<tr>
<td>trehalose</td>
<td>liquefaction of gelatin *</td>
</tr>
<tr>
<td>xylose</td>
<td></td>
</tr>
</tbody>
</table>

References:
"Manual of Methods for General Bacteriology" ASM 1981
"The Genus Bacillus" Agricultural Handbook # 427 USDA 1971
* additional tests for cocci
Determination of Effects on Fluid Milk

Isolated colonies from both proteolytic and nonproteolytic plates grown in nutrient broth (DIFCO) for 48 hr at 30 C were used to inoculate at a level of 0.5% (v/v), 125 mL of sterile commercial whole milk (heat treated for 30 min at 63 C to germinate spores and then autoclaved for 15 min at 12 p.s.i. (83 Kpa). The number of cells contained in the inoculum was not determined.

Determination of Proteolytic Activity

Selected isolates that tested positive for proteolysis from the proteolytic sporeforming psychrotrophic plates were screened for proteolytic activity. Isolates that were stored on standard methods agar slants at 7.2 C were used to inoculate 5 mL of nutrient broth and then incubated for 48 hr at 30 C to produce actively growing cells. Five mL of broth with cells were then added aseptically to 20 mL of nutrient broth, which was then incubated at 30 C for 48 hr in a shaking water bath. Following incubation the broth (containing cells) was centrifuged, the cells washed, recentrifuged, and resuspended in dilute 0.1% peptone water (DIFCO). Cell numbers were standardized by prior optical density readings on a Bausch Lomb Spectronic 20 at 440 nm and plate counts made of the diluted cell suspension. Approximately 30 X 10⁴ CFU/mL were used to inoculate 50 mL of 11% (w/v) sterile nonfat dry milk. The inoculated samples were then stored for 14 days at 7.2 C. After incubation, 5 mL portions
were used to determine proteolytic activity by the method of Church et al. (1983), (see appendix figure pg. 60). Statistical analysis system (SAS) was used to compare the mean proteolytic activity measurement. The Duncan procedure (SAS, 1979) was used to execute the least significant difference test (p>0.05).
RESULTS AND DISCUSSION

Plate Count Results on Raw and Commercially Pasteurized Milk Samples

Standard plate counts (SPC) for the 59 raw milk samples are summarized in Table 4. These counts ranged from <100 to 96,000 CFU/mL; 86% of the samples had counts <20,000 CFU/mL. All the raw samples met the USPHS/FDA requirements for Grade A raw milk, i.e. <100,000 CFU/mL. The SPC results for the raw milk samples following laboratory pasteurization (63 C for 30 min) and for the 8 commercially processed milk samples are summarized in Tables 4 and 5, respectively. All of these bacterial counts met the requirements for Grade A pasteurized milk (i.e. <20,000 CFU/mL).

The sporeforming psychrotrophic counts and the proteolytic sporeforming psychrotrophic counts for raw milk are also included in Table 4. These bacterial counts reflect both survival and subsequent cell multiplication during 10 days at 7.2 C. Bacterial counts obtained immediately following the 80 C heat treatment would have included any viable cells or spores able to survive the heat treatment and subsequently recover on the plating media. Standard Methods (APHA, 1985) recommends plating of samples immediately following heat treatment (80 C for 12 min) followed by incubation of plates at 7.2 C for 10 days. However, in this study the milk samples themselves were incubated at 7.2 C.
<table>
<thead>
<tr>
<th>Milk</th>
<th>Plate Procedure</th>
<th>Count CFU/mL</th>
<th>No. of Samples</th>
<th>% of TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>SPC</td>
<td>&lt;5,000</td>
<td>27</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,000-20,000</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20,000-50,000</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;50,000</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Lab</td>
<td>SPCb</td>
<td>&lt;100</td>
<td>32</td>
<td>54</td>
</tr>
<tr>
<td>Pasteurized</td>
<td></td>
<td>100-500</td>
<td>19</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500-1,000</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;1,000</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>PSCc</td>
<td></td>
<td>&lt;100</td>
<td>36</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100-500</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500-1,000</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,000-10,000</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10,000</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>d</td>
<td>PPSC</td>
<td>&lt;100</td>
<td>45</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100-500</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500-1,000</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,000-10,000</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10,000</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

a and b  Standard Methods for the Examination of Dairy Products APHA p 133 and 189, respectively.

c  Psychrotrophic sporeforming count, sample heat treated at 80 °C for 10 min and incubated at 7.2 °C for 10 days, followed by plating on standard methods agar for 48-72 hr at 21 °C.

d  Proteolytic psychrotrophic sporeforming count, same treatment as PSC but plated on casein-agar for 48-72 hr at 21 °C.
Table 5. Plate counts on commercially pasteurized milk products after refrigeration for 24 hr, spoilage time at 7.2 C and defect characteristic.

<table>
<thead>
<tr>
<th>Product</th>
<th>Date Received</th>
<th>Standard Plate Count</th>
<th>Sporeforming Psychrotrophs</th>
<th>Proteolytic Sporeforming</th>
<th>Spoilage Time (days)</th>
<th>Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>2/15</td>
<td>120&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2%</td>
<td>2/15</td>
<td>190&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Whole</td>
<td>3/2</td>
<td>60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20</td>
<td>bitter, unclean</td>
</tr>
<tr>
<td>2%</td>
<td>3/2</td>
<td>80&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20</td>
<td>slight bitter</td>
</tr>
<tr>
<td>Whole</td>
<td>3/10</td>
<td>70&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3200</td>
<td>3600</td>
<td>25</td>
<td>slight bitter</td>
</tr>
<tr>
<td>2%</td>
<td>3/10</td>
<td>490</td>
<td>60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25</td>
<td>bitter</td>
</tr>
<tr>
<td>Whole</td>
<td>3/15</td>
<td>490</td>
<td>30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21</td>
<td>sweet curdled bitter, unclean</td>
</tr>
<tr>
<td>2%</td>
<td>3/15</td>
<td>790</td>
<td>20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21</td>
<td>bitter, unclean</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard Methods for the Examination of Dairy Products APNA (1985) p. 133
<sup>b</sup> Sample heat treated at 80 C for 10 min and incubated for 10 days at 7.2 C, followed by plating on Standard Methods Agar (48-72 hr at 21 C)
<sup>c</sup> Sample heat treated at 80 C for 10 min and incubated for 10 days at 7.2 C, followed by plating on Casein Agar (48 hr at 21 C)
<sup>e</sup> Monitored by researcher trained in Dairy Products Evaluation
for 10 days following the heat treatment and then standard plate counts were conducted on the incubated samples. Permitting the spores to germinate and the subsequent outgrowth of vegetative cells within the milk sample served to simulate conditions as they occur in commercial products more than the pattern of germination and cell growth on culture media. Counts at day 0 would not have taken into account injured cells which required an extended lag phase prior to growth in a supportive medium. Otherwise the result would be lower plate counts which would not reflect a true microbial or spore count. The use of the 80 C heat treatment, followed by the incubation period (10 days at 7.2 C), is selective in that typical survivors will: (1) represent sporeforming bacteria and (2) be psychrotrophic in nature. The longer lag phase and generation times associated with psychrotrophic bacteria are the basis for the 10 day incubation period.

Sporeforming psychrotrophs were found in 49 of 59 samples (83%). Counts ranged from <10 to >10,000 CFU/mL. Mikolajcik and Simmon (1978) reported the sporeforming psychrotrophic counts of 51 raw milk samples, following incubation of 7 days at 7 C, to range from <1 to >10,000 CFU/mL. These researchers found that psychrotrophic counts higher than 10 CFU/mL were found in 58% of the samples after incubation at 7 C for 7 days. Sharma et al. (1984) reported the psychrotrophic spore count of 51 raw milk samples to range from <1 to >100 CFU/mL after incubation of the heat treated samples at 7 C for 7 days. Sporeforming psychro-
trophs were found in 92% of their set of samples. The spore-
forming psychrotrophic counts on the commercially processed 
samples are listed in Table 5. These counts ranged from <10 to 
3200 CFU/mL. Sporeforming psychrotrophs were found in 6 out of 
8 (75%) of the pasteurized samples.

The proteolytic psychrotrophic sporeforming counts (PPSC) 
from the raw and commercially processed milk samples are listed 
in Table 4 and 5, respectively. The PPSC's on the raw milk, 
following incubation for 10 days at 7.2 C, ranged from <10 to 
>10,000 CFU/mL. Standard Methods for the Examination of Dairy 
Products (APHA, 1985) indicates that plates that contain a 
proteolytic count in excess of 80 CFU/mL are not generally con-
sidered readable. It was our experience in this study that 
plates containing >100 CFU/mL were difficult to read, and hence 
estimated counts had to be employed. Proteolytic sporeforming 
psychrotrophs were found in 73% of all raw milk samples tested. 
Sharma et al. (1984) reported proteolytic sporeforming psychro-
trophs counts on 51 raw milk samples ranging from <1-15 CFU/mL, 
after incubation of samples for 7 days at 7 C, and reported a 78% 
incidence rate of proteolytic sporeformers in their samples. 
The proteolytic sporeforming psychrotrophic counts on the 
commercially processed samples (Table 5), after incubation for 10 
days at 7.2 C, ranged from <10 to 3600 CFU/mL; 6 out of 8 (75%) 
of the samples tested contained this form of microorganism.

Several producer farms that were identified by a raw milk 
quality survey (Bodyfelt, 1986) as having elevated psychrotrophic
sporeforming counts (PSC) on more than one sampling date were visited. This visit was made by the Oregon State University Extension Specialist in Dairy Processing, the local dairy fieldman, and this investigator. The purpose of this visit was to identify conditions responsible for the high PSC's. In each case, after conversation with the producer, factors were identified that could have been responsible for the high PSC's. These causative factors typically involved build-up of milkstone within the bulk tank in areas not accessible by the cleaning in place system and failure to adequately clean outlet valves. Another problem area associated with raw milk bulk tanks is the presence of (diluted) stationary films of milk. Donovan (1959) showed that this condition was favorable for sporulation of Bacillus species. Milk itself is not a good sporulation medium. It is believed that increased sporulation in diluted milk occurs because of increased oxygen content and decreased concentrations of nutrients (Donovan, 1959). This emphasizes the importance of immediate rinsing, cleaning, and sanitizing of equipment surfaces on the farm and the need to prevent equipment from standing after rinsing without draining off the rinse water.

**Identification of Microorganisms**

The identification and percentage of occurrence of the sporeforming psychrotrophic organisms isolated from raw and commercially processed milk samples analyzed in this study are
listed in Table 6. Twelve different species of *Bacillus* (102 total strains) were isolated. *Bacillus licheniformis*, found in 18% of the samples, was the most common species isolated. The pattern of *Bacillus* species isolated from raw milk samples was similar to *Bacillus* categories identified by other workers as listed previously (Table 1). Psychrotrophic sporeforming bacteria isolated from raw milk by Chung and Cannon (1971) were identified as *Bacillus firmus* (43.6%), *B. megaterium* (23%), *B. brevis* (15.7%), and the remainder as *B. coagulans*, *B. polymyxa*, *B. macerans*, *B. circulans*, and *B. cereus*. Johnson and Bruce (1982) identified *Bacillus* sp. isolated from lab pasteurized raw milk as *B. cereus* (65.7%), *B. licheniformis* (19.9%), *B. coagulans* (10.1%) and the balance as *B. megaterium*, *B. circulans*, *B. polymyxa*, *B. brevis*, and *B. macerans*.

Contrary to most other reported studies of sporeforming psychrotrophs, no *Bacillus cereus* were identified from the milk samples examined in this study. A possible explanation may be seasonal variation. In this study raw milk samples were examined from November to March. Phillips and Griffiths (1986) reported that *B. cereus*, *B circulans*, and *B. cereus* var. *mycoides* were present in greatest numbers in the months of April to September, while *B. licheniformis*, *B. pumilus*, and *B. subtilis* were generally higher during November to February. However, the seasonal variation of sporeforming psychrotrophic *Bacillus* due to differences in types of feed and methods of housing the cows (i.e. pasture as opposed to barn housing and harvested feed)
would be of a lesser concern in the Willamette Valley of Oregon, since dairy farms typically do not pasture cows irrespective of the time of year. Chance contamination with sporeformers from dust during the dry summer (late spring to early fall season) and mud during the wet winter (mid-fall to mid-spring season) are equally as great. Another possible explanation is that the

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NUMBER OF SAMPLES CONTAINING ORGANISMS</th>
<th>% OF TOTAL SAMPLES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>raw</td>
<td>pasteurized</td>
</tr>
<tr>
<td>Bacillus brevis</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>B. cereus mycoides</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>B. circulans</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B. firmus</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>B. laterosporus</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>B. macerans</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>B. polymyxa</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>B. sphaericus</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total Samples</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>
incubation temperature (7.2 C) used was too low for outgrowth of this particular species within our study. Although other previously mentioned investigators have isolated *B. cereus* species capable of growth at 7 C, Bergey's Manual (9th ed.) indicates that the minimum growth temperature for *B. cereus* is 10 C for 11 to 86% of the strains tested, with no growth at 5 C.

The *Bacillus* species listed in Table 6 were matched by their morphological, microbiological and biochemical characteristics with descriptions from "The Genus Bacillus" U.S. Dept. of Agriculture Handbook No. 427 and "Bergey's Manual of Determinative Bacteriology" (9th ed.). Although every one of our *Bacillus* isolates did not match identically with the specific characteristics indicated, the appropriate morphological and microbiological characteristics (with exception of having lower growth temperatures) were consistent for each species. According to Bergey's Manual, all *Bacillus* species observed in this study, with the exception of *B. brevis*, *B. coagulans* *B. laterosporus*, and *B. licheniformis* are capable of growth at 10 C. In this study all *Bacillus* isolates were capable of growth at 7.2 C, including the species previously listed. Bergey's Manual also states that only two of the isolates identified in this study are capable of growth at 5 C, *B. polymyxa* and *B. megaterium*. Shehata and Collins (1971) identified *B. subtilus*, *B. circulans*, and *B. coagulans* strains that are capable of growth within two weeks at 0 C. They also isolated strains of *B. brevis*, *B. cereus*, *B. laterosporus*, *B. circulans*, *B. macerans*, *B. megaterium*, *B.
licheniformis, B. polymyxa, B. pumilus, and B. subtilus that exhibited minimum growth temperatures of 5-7 C. The difference in minimum growth temperatures observed in these isolates stresses the importance of monitoring and studying factors contributing to the adaptability of mesophilic Bacillus species, as changes in the production, storage and processing methods influence the ecology of microflora in milk.

The biochemical characteristics which were most variable within each group of species included hydrolysis of starch, decomposition of casein and tyrosine, acetylmethyl carbinol production, and production of acid from various carbohydrates. However, no pattern of variation between species or within each species was identified in comparing the characteristics of the isolates with reference descriptions.

The isolation procedures used in this study were primarily for aerobic sporeforming bacteria, therefore anaerobic sporeformers that belong to the genus Clostridium were not identified. Procedures for isolation of Clostridium species would have included incubation of milk samples and plates under anaerobic conditions. Bhadsville et al. (1972) identified 4 catalase negative, gram-positive, motile, sporeforming, rod shaped bacteria from 48 raw milk samples as belonging to the genus Clostridium. Two of the isolates were identified as C. hastiforme, which were able to grow at temperatures above 4 C, and 2 as C. carnis, which were able to grow slowly at 0 C. However, according to Bergy's Manual (9th ed.), these two
Clostridium species demonstrate little or no growth below 25 C. The only Clostridium species listed in Bergey's Manual as being able to grow at refrigeration temperatures is Clostridium putrefaciens, which has been isolated from pork. Martin (1974) reported 5% of the sporeformers in milk from his study to be of the genus Clostridium. These two reports seem to indicate that Clostridia capable of growth at refrigeration temperatures are less common in milk than are psychrotrophic Bacillus, although psychrotrophic variants of mesophilic Clostridium could be a concern.

Along with the Bacillus isolates, 18 thermoduric cocci, identified as Micrococcus luteus, M. varians, Streptococcus faecalis, and S. lactis var. maltigenes were isolated. These organisms have been isolated from raw and pasteurized milk samples previously (Stadhouders, 1975; Washam et al., 1977). These reported incidences of other bacterial genera were obtained from high heat-treated (80 C for 10 min) samples, followed by incubation at 7.2 C for 10 days. Since these organisms do not produce spores, inadequate heat-treatment or contamination of the samples or plates was suspected. This group of cocci, along with selected bacilli, were retested by the investigator for their ability to survive simulated HTST pasteurization (78.6 C for 16 sec), high heat-treatment (80 C for 10 min) and subsequent growth at refrigeration temperatures (7.2 C). All the Bacillus species retested were able to survive both heat treatments and grew at 7.2 C. All of the cocci group were able to survive the
pasteurization temperature and grew at 7.2 C; however, only two of the isolates survived the high heat treatment. These two cocci were identified as Streptococcus lactis var. maltigenes. These two organisms closely fit the description in Bergey's Manual for S. lactis, except that morphologically the cells existed singularly as well as groups of two and small chains and they lacked the ability to grow in 40% bile. Results from this study indicated that these two isolates of cocci were capable of surviving the 80 C for 10 min heat treatment. However, Bergey's Manual indicates a heat resistance of Streptococcus lactis of 60 C for 30 min. These two microbial isolates were identified as belonging to the variety maltigenes, based on their ability to produce a distinct malty off-flavor when they were inoculated into sterile whole milk (Morgan, 1976). The ability to produce a malty off flavor was tested twice, (with positive results), early in the study and approximately 3 months later, after storage of the two cultures of this organism on standard plate count agar slants at 7.2 C. These two organisms were isolated from a common grouping of raw samples, but from separate raw milk samples within the group.

An attempt was made to make a connection between the sporeforming psychrotrophic bacteria isolated from raw milk samples and those isolated from the corresponding pasteurized milk products. The resources required to make this comparison included an ample supply of raw milk, adequate collection facilities, cold storage facilities, an operating pasteurizer
that complied with quality standards for product processing and subsequent cleaning and sanitizing, container filling machines and aseptic product containers. These resources were not available within the research laboratory, so alternately milk samples were obtained from a local dairy processor that had expressed a willingness to cooperate in such an overview study. Milk samples were obtained on four separate days and consisted of raw samples in addition to processed milk samples, assumed to have originated (at least in part) from the same source as the raw milk samples. However, it was not possible to obtain a written record of the exact identity of the raw samples (by producer number) that comprised the pasteurized milk source of samples. The *Bacillus* species isolated from both the raw and processed milk samples are listed in Table 7. On days 1 and 2 of this study, there were no similar species isolated from the raw and processed samples. On day 3 *B. coagulans* was isolated from the 2% lowfat sample as well as the raw sample no. 2. *B. circulans* which was found in raw milk samples no. 2 and no. 3, and *B. firmus* which was found in raw sample no. 1, were both isolated from the whole milk sample. On day 4, *B. polymyxa* was isolated from the 2% lowfat sample as well as raw milk samples no. 3 and no. 6.

A similar attempt at such a comparison of raw milk supply and processed milk was undertaken by Coghill and Juffs (1979). These researchers examined 30 raw milk samples and found 7 (23%) to contain psychrotrophic sporeformers. Following laboratory
Table 7. Bacillus species isolated from raw milk and pasteurized samples after heat-treatment (80 C for 10 min.) and subsequent incubation (10 days at 7.2 C) obtained from one dairy.

<table>
<thead>
<tr>
<th>Milk Sample No.</th>
<th>Organism</th>
<th>Milk Sample No.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 Raw</td>
<td></td>
<td>Day 3 Cont. Raw</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>B. sphaericus</td>
<td>3</td>
<td>B. circulans</td>
</tr>
<tr>
<td>2</td>
<td>B. polymyxa</td>
<td>4</td>
<td>B. pumilus</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>5 (2%)</td>
<td>B. coagulans</td>
</tr>
<tr>
<td>4</td>
<td>B. sphaericus</td>
<td>6 (whole)</td>
<td>B. circulans</td>
</tr>
<tr>
<td>5</td>
<td>B. circulans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>B. firmus</td>
</tr>
<tr>
<td>7 (2%)</td>
<td>B. pumilus</td>
<td>Day 4</td>
<td></td>
</tr>
<tr>
<td>8 (whole)</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td>2</td>
<td>B. cereus var. mycoides</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>4</td>
<td>B. polymyxa</td>
</tr>
<tr>
<td>2</td>
<td>B. brevis</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>6</td>
<td>B. polymyxa</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>7</td>
<td>B. laterosporus</td>
</tr>
<tr>
<td>5 (whole)</td>
<td>B. licheniformis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (2%)</td>
<td>B. megaterium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td>8 (whole)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>B. licheniformis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>B. licheniformis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B. polmyxa</td>
<td>9 (2%)</td>
<td>B. polymyxa</td>
</tr>
<tr>
<td>4</td>
<td>B. firmus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>B. coagulans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>B. circulans</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
pasteurization of the raw milk samples, psychrotrophic sporeformers were detected in 5 (16.7%) of the samples. Psychrotrophic sporeformers were detected in only two samples when raw milk and the same milk sources were laboratory pasteurized. In addition to the problem of effective monitoring of raw milk samples to determine their presence in the final product, the probability of post-pasteurization contamination with sporeforming psychrotrophs cannot be overlooked. Davies (1975) indicated that large numbers of *B. cereus* spores in pasteurized milk were derived from the processing plant and not the farm. Coghill (1982) isolated *Bacillus* species capable of surviving pasteurization with subsequent growth at 7.2 C from pasteurized milk products but was unable to isolate any psychrotrophic sporeformers from the raw milk. These results indicated that those organisms found in pasteurized milk products were present as a result of post-pasteurization contamination.

Establishing a connection between spores found in raw milk with those in the processed sample could ultimately determine the potential shelf life of the milk or dairy product. Davies (1975) indicated that *B. cereus* spores isolated from raw milk have slower germination times than those isolated from pasteurized milk. Phillips and Griffiths (1986) demonstrated that the average shelf life of milk from which *Bacillus* organisms were associated with post-pasteurization contamination was approximately 2.5 days less than milk which was free from such contamination. This indicates that the presence of spores as a
result of post-pasteurization contamination could result in a product with a shorter shelf life even more so than spores that originated from the raw milk. Processing conditions associated with post-pasteurization contamination may include; (1) improper cleaning and sanitizing of processing equipment, (2) contamination of water supplies and (3) overall problems with sanitary conditions, especially airborne contamination.

**Effects and activity of psychrotrophic sporeformers in milk**

The various flavor defects judged to be associated with the thermoduric isolates from both raw and commercially pasteurized milk samples in this study are summerized in Table 8. The flavor defects produced in milk by the *Bacillus* organisms parallel those found by other authors (Washam et al., 1977; Mikolajcik and Simmon, 1978; Collins, 1982; Coghill, 1982). Fifty-eight percent of the isolates were judged to produce a bitter taste and/or putrid odor, which is indicative of the presence of proteolytic enzymes produced by these organisms. The remainder of the bacilli tended to produce fruity, yeasty or rancid off-flavors, which is indicative of the production of lipolytic enzymes. The production of proteolytic and lipolytic enzymes can vary with strain (Gordon et al., 1973). Shehata et al. (1971) isolated *B. circulans*, *B. laterosporus*, and *B. coagulans* that produced fruity flavors in sterile milk at 7.2 C, followed by the production of rancidity by *B. coagulans* and unclean flavors by the other two organisms. In this study *B.*
circulans and B. coagulans were observed to produce both fruity and rancid off-flavors. However, our B. laterosporus isolate produced bitter and unclean off-flavors. Washam et al. (1977) also isolated B. laterosporus, which produced bitter and unclean off-flavors and "sweet curdle" (coagulation). In this study isolated species of B. cereus var. mycoides produced a bitter taste and putrid odor, but sweet curdling (gelation) was not observed. Additionally, no B. cereus or B. cereus var. mycoides were isolated from the whole milk sample from day 4 (Table 5) which had exhibited coagulation (sweet curdle).

Table 8. Flavor and odor defects associated with isolates from raw and pasteurized milk samples inoculated into steriled whole milk and incubated at 7.2 C

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>DEFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus brevis</td>
<td>bitter, putrid, unclean</td>
</tr>
<tr>
<td>B. cereus mycoides</td>
<td>bitter, putrid</td>
</tr>
<tr>
<td>B. circulans</td>
<td>fruity, rancid, yeasty</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>fruity, rancid, yeasty</td>
</tr>
<tr>
<td>B. firmus</td>
<td>fruity, yeasty</td>
</tr>
<tr>
<td>B. laterosporus</td>
<td>bitter, unclean</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>bitter, putrid</td>
</tr>
<tr>
<td>B. macerans</td>
<td>bitter, putrid</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>bitter, putrid</td>
</tr>
<tr>
<td>B. polymyxa</td>
<td>fruity, rancid, yeasty</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>bitter, unclean</td>
</tr>
<tr>
<td>B. sphaericus</td>
<td>sour, rancid, unclean</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>rancid</td>
</tr>
<tr>
<td>M. varians</td>
<td>bitter, putrid</td>
</tr>
<tr>
<td>Stretococcus faecalis</td>
<td>bitter, putrid</td>
</tr>
<tr>
<td>S. lactis maltigenes</td>
<td>malty</td>
</tr>
</tbody>
</table>

Table 5. Organisms associated with sour, rancid, unclean flavors and gelation.
Several thermoduric cocci were also isolated from milk samples in this study and are listed in Table 7. *Micrococcus luteus* produced a rancid off-flavor in sterilized whole milk incubated at 7.2°C, while the *M. varians* and *Streptococcus faecalis* isolates produced a bitter taste and a putrid odor. As should be expected, *S. lactis* var. *maltigenes* produced a malty off-flavor. Specific strains of *S. lactis* have been known to produce a malty off-flavor in certain dairy products by metabolizing leucine to 3-methyl butanal (Buchanon and Gibbons, 1974; Morgan, 1976).

Examination of the biochemical test results of the thermoduric isolates in this study showed that 83% of them hydrolysed casein, 56% were proteolytic in litmus milk, 57% demonstrated lipolytic activity, and 31% produced acid in litmus milk (fermented lactose). These results are reasonably similar to those of Johnson and Bruce (1982), who examined the biochemical capabilities of 277 psychrotrophic *Bacillus* species isolated from raw milk in West Scotland and found that 84% hydrolysed casein, 73% were proteolytic in litmus milk, 77% were lecithinase positive, 57% hydrolysed cream and 8% fermented lactose. The metabolic activities of the bacilli demonstrate marked potential for spoilage of dairy products.

Of the 102 *Bacillus* isolates in this study, 48 tested positive for proteolysis on casein agar plates. This group of isolates was further analyzed for proteolytic activity by the method described by Church et al. (1983). The Church method for
assessing proteolysis was chosen because it is considered a simple and rapid test and requires the addition of only one reagent solution for color development. This assay method for proteolysis is also regarded as being more sensitive since o-pthaldialdehyde and B-mercaptoethanol form adducts having similar high absorptivities with all but 2 alpha-amino groups (a weak reaction with cysteine and none with proline). In comparison, the method proposed by Hull (1947) depends on the reaction of the Folin-Ciocateau reagent with tyrosine and tryptophan for measuring the extent of proteolysis. Furthermore, this method requires the addition of both sodium carbonate-tetraphosphate and phenol reagent for color development.

The relative proteolytic activities of Bacillus species isolated from milk samples (expressed as mM of alanine) are summarized in Table 9. Statistical analysis system (SAS) was used to compare the means for each group of species. The Duncan procedure (SAS, 1979 ed.) was used to execute the least significant difference test (p>0.05). There were five groups which were significantly different at the p>0.05 level. Bacillus cereus var. mycoides (group A) demonstrated significantly higher proteolytic activity while B. pumilus (group E) demonstrated significantly less proteolysis than the other Bacillus isolates. Four of the Bacillus species were placed in both groups B and C.

Sharma et al. (1984) determined that the proteolytic activity of 50 psychrotrophic Bacillus isolates ranged from 20 to 480 units/mL, wherein one unit of enzyme activity was
defined as the amount of enzyme required to release sufficient
TCA-soluble fragments to produce a blue color equivalent to 1 ug
of tyrosine/hr at 37 C. Their proteolytic assay was a modifica-
tion of the method used by Keay and Wildi (1970), which was based
on the frequently employed method of Hull (1947). Chopra et al.

Table 9. Proteolytic activity of Bacillus species isolated
from milk samples expressed as mM of alanine. 1

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NO. OF ISOLATES</th>
<th>MEAN ± SD (mM of alanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus brevis</td>
<td>4</td>
<td>1.24 ± 0.044</td>
</tr>
<tr>
<td>B. cerus mycoides</td>
<td>9</td>
<td>1.93 ± 0.065</td>
</tr>
<tr>
<td>B. circulans</td>
<td>2</td>
<td>1.32 ± 0.042</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>2</td>
<td>1.10 ± 0.035</td>
</tr>
<tr>
<td>B. firmus</td>
<td>3</td>
<td>1.08 ± 0.081</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>5</td>
<td>1.33 ± 0.073</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>3</td>
<td>1.32 ± 0.051</td>
</tr>
<tr>
<td>B. polymyxa</td>
<td>8</td>
<td>1.28 ± 0.066</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>9</td>
<td>0.93 ± 0.057</td>
</tr>
<tr>
<td>B. sphaericus</td>
<td>3</td>
<td>1.25 ± 0.053</td>
</tr>
</tbody>
</table>

1 Church et al. (1983)
2 Means with the same letter are not significantly
different from each other at the p>0.05 level using the
least significance difference test, (SAS, 1979).

(1984) also used a modification of the Keay and Wildi (1970)
method to measure the proteolytic activity of thermophilic
bacterial isolates from a variety of milk and milk products. The proteolytic activity of 171 isolates ranged from 20-500 units per mL. All 50 isolates that exhibited >100 "enzyme unit"/mL were identified as belonging to the genus *Bacillus*. It is difficult to directly compare the proteolytic activity results from other investigators to those obtained in this study. This is due to the difference in assay methods used to measure proteolytic activity and the units of activity expressed. The other investigators did not associate a specific bacterial isolate (genus or species) with its corresponding proteolytic activity level, but instead merely indicated a range of proteolytic activity associated with a grouping of organisms.

The results from this study demonstrated that under the given growth conditions, *Bacillus* species seemed to exhibit different levels of activity. It was not determined whether the variation in activity was due to: (1) a faster rate of growth of some isolates under the assay conditions, which could have resulted in increased concentration of protease enzyme (i.e. more cells produce more enzyme) or whether (2) the amount of enzyme production varied between species, irrespective of cell numbers. Making a distinction between the amount or rate of enzyme production and its proportionality to cell number under given conditions for a given species could be a focus for future study.

The assay conditions employed in this study involved the inoculation of washed cells (at a rate of $3.0 \times 10^5$ organisms per mL) into sterile nonfat dry milk followed by incubation at 7.2 C
for 14 days. The inoculum stage was chosen to obtain cells in the exponential growth phase where enzyme production and cell growth would be optimum. The temperature, 7.2°C (45°F), was used to simulate typical storage conditions of milk and milk products. The time of incubation was selected based on previous trials at 5, 7, and 10 days which resulted in activity values too low to accurately measure for many isolates.

Use of the Church method to measure proteolytic activity in this study proved sufficient, but was not without limitations. As mentioned previously the reagents in this method, o-pthalialdehyde and B-mercaptoethanol, form an adduct with alpha-amino groups of 20 different amino acids. Hence, this method measures the end products of protein degradation, small peptides and amino acids. By the time a fluid milk or cream product reaches this stage of protein deterioration, the product usually has quite objectionable sensory characteristics. Most of the inoculated milks had developed strong off-flavors and odors (primarily bitter and putrid, respectively) before proteolytic activity could be detected using the Church method of measuring proteolysis. By measuring some intermediate products between the initial protein and subsequent free amino acids, (i.e. peptones and large peptides) a more rapid and appropriate assessment of proteolytic activity could be performed. The development of assays and methods to isolate and quantitate protease (and quite possible lipolytic and phospholipolytic) enzymes could also expedite assessment of proteolytic activity of
potential spoilage organisms in milk and milk products.

Additional research to measure quantitatively the enzymatic (proteolytic and lipolytic) activity associated with all heat resistant psychrotrophic organisms as well as individual microbial species is needed. Understanding the conditions under which this activity occurs could prove quite useful in planning alternative pasteurization or ultra-high pasteurization procedures to help maximize the shelf life potential of perishable milk products.

Additionally, development of rapid and sensitive methods for measuring the presence or absence of the "critical" enzymes in raw and pasteurized milk could be used as an indicator or prediction of potential shelf life. Since the optimum temperature for microorganisms to produce enzymes (lipases, proteases, and carbohydrases) is usually lower than the optimum temperature for cell growth (Tinuoye and Harmon, 1975), there is sufficient opportunity for milk held for several days at refrigeration temperature to develop off-flavors induced by microbial enzymes, even though the bacterial population remains below that normally associated with microbial defects. With the increasing tendency to store milk and milk products for longer and longer periods, all psychrotrophic bacteria can only increase in importance.

This research effort was the first study to examine in detail the incidence, identification, and characterization of sporeforming psychrotrophic bacteria as they relate to the fluid milk industry in Oregon. This study was also the first to
associate specific *Bacillus* species with a corresponding proteolytic activity. This information allows the identification of certain *Bacillus* species that may have a greater role in milk or dairy product spoilage.
SUMMARY

Heat resistant sporeforming psychrotrophic bacteria isolated from Grade A raw milk and commercially pasteurized milk were identified and characterized. The method used to isolate these organisms involved heat treatment of the milk samples (80°C for 10 min) followed by subsequent incubation at 7.2°C for 10 days. Organisms able to survive the heat treatment followed by growth at refrigeration temperatures met the criteria for heat resistant/sporeforming psychrotrophic bacteria. These bacteria were isolated from 59 raw milk and 8 commercially processed samples which consisted of four 2% and four whole milk samples obtained from processing plants in western Oregon. Standard plate counts for most of the raw and all the pasteurized samples met standards for Grade A raw and pasteurized milk with counts of <80,000 and <20,000, respectively. Sporeforming psychrotrophs were found in 49 of 59 (83%) of the raw milk samples. Sporeforming psychrotrophic counts ranged from <10 to >10,000 CFU/mL after incubation as a heat treated milk sample for 10 days at 7.2°C. Sporeforming psychrotrophs were found in 6 out of 8 (75%) of the commercially processed milks, in which the counts after incubation in the milk ranged from <10 to 3200 CFU/mL. Proteolytic, sporeforming psychrotrophs were found in 73% of the raw and 75% of the processed milk samples. Based on the casein agar method, proteolytic sporeforming psychrotrophic counts on the raw and processed samples (after incubation in the milk samples for 10 days at 7.2°C) ranged from <10 to >10,000.
CFU/mL and <10 to 3600 CFU/mL, respectively.

Of the 120 microbial isolates, 102 were gram-positive or gram-variable rods belonging to the genus *Bacillus*. Eighteen gram-positive cocci were identified as belonging to the genus *Micrococcus* and *Streptococcus*. Twelve different *Bacillus* species were identified with *B. licheniformis* the most predominant, (18% of the samples) and *B. laterosporus* the most infrequent species isolated, (2% of the samples). Fifty-eight percent of the *Bacillus* isolates produced a bitter taste and a putrid odor; 42% produced a fruity and/or rancid off-flavor when inoculated into sterile whole milk and incubated at 7.2 C, which indicates the production of proteolytic and lipolytic enzymes, respectively. A review of the biochemical activities of the thermoduric isolates showed that 83% hydrolysed casein, 56% were proteolytic in litmus milk, 57% exhibited lipolytic activity and 31% produced acid in litmus milk (fermented lactose). Forty-eight isolates screened for proteolysis, (i.e. isolates testing positive for proteolysis on the casein agar plates) were further evaluated to quantitate their proteolytic activity. As a group the proteolytic activity of the *Bacillus* isolates ranged from 0.93 to 1.93 units, expressed as mM of alanine. *B. cereus* var. *mycoides* demonstrated significantly higher proteolytic activity while *B. pumilus* demonstrated significantly less proteolysis (p>0.05) than the other *Bacillus* isolates.

This study helped reconfirm that a considerable portion of the Grade A raw milk supplies in western Oregon does contain heat
resistant sporeforming psychrotrophic bacteria, especially *Bacillus* species. The rate of incidence of these organisms in raw milk and the particular *Bacillus* species isolated in this study of Grade A raw milk supply were similar to the pattern of thermoduric psychrotrophs observed in other studies. The ability of these organisms to produce proteolytic and lipolytic enzymes was evident by the off-flavors produced when they were inoculated into sterile whole milk. All of the microbial isolates were capable of growth at 7.2 C. Variations in biochemical reactions observed by the isolates identified in this study with standard descriptions (e.g. Bergey's Manual) could represent metabolic adaptation by these microorganisms to cope with growth at low temperatures, assuming these isolates are variants of mesophilic species. Measurement of the proteolytic activity of these organisms demonstrated that under the given conditions, some species of *Bacillus* showed greater proteolytic activity than others; this is indicative that the given species is as important or more so than cell population. With modern trends of milk production and marketing that include the increasing use of refrigeration and prolonged periods between production, processing and eventual consumption, the thermoduric psychrotrophs are certain to become a more challenging quality assurance problem for the dairy industry of the United States.

Although no *Bacillus cereus* species were isolated from the raw and processed milk samples in this study this species remains a principal concern because of its ability to produce toxins and
their association with foodborne illness. Further research is needed to address the significance of \textit{B. cereus} and possibly other \textit{Bacillus} species in relation to their ability to cause foodborne illness from milk and dairy products.
BIBLIOGRAPHY


Coghill, D. and Juffs, H.S. 1979

Collins, E.B. 1981

Cousin, M.A. 1982

Cox, E.A. 1975

Credit, C., Hedeman, R., Heywood, R., and Westhoff, D. 1972
Identification of bacteria isolated from pasteurized milk following refrigeration storage. J. Milk Food Tech. 35:708-709.

Davies, F.L. 1975

Donnelly, L.S. and Busta, F.F. 1981

Donovan, D.O. 1959
The occurance of Bacillus cereus in milk and on dairy equipment. J. Applied Bacteriology 22:131-137.

Fox, C.W., Chrisope, G.L., and Marchall, R.T. 1976

Gilbert, R.J. and Kramer J.M. 1984


Role of psychrotrophic sporeformers in long life milk. J. Dairy Sci. 52:89.

Comparison of selective plating media for enumeration of Bacillus cereus in foods.

Harrigan, W.F., and McCance, M.E. 1966
Laboratory Methods in Microbiology.

Hileman, J.L. 1940
Thermoduric bacteria in pasteurized milk. A review of literature.

Hull, M.E. 1947
Studies of milk proteins. II. Colorimetric determination of the partial hydrolysis of the proteins in milk.
J. Dairy Sci. 30:881-884.

Jayne-Williams, D.J. and Franklin, J.G. 1960
Bacillus spores in milk. Part I and II

Johnson, D.W. and Bruce J. 1982

Johnson, K.M., Nelson, C.L., and Busta, F.F. 1984
Influence of heating and cooling rates on Bacillus cereus spore survival and growth in a broth medium and in rice.

Johnson, K.M. 1984
Bacillus Cereus foodborne illness: An up-date.

Keay, L. and Wildì, B.S. 1970
Proteases of the genus Bacillus. I Neutral proteases.
Biotechnology Bioengineering 12:179-212.

Labots, H. and Hup, G. 1946b
Bacillus cereus in raw milk II. The occurrence of slow and fast germinating Bacillus cereus in milk and their significance in the enumeration of Bacillus cereus spores.
Laine, J.J. 1970
Studies on psychrophilic bacilli of food origin.
Annales Academiae Scientiarum Fennicae, A. IV Biologica

Langeveld, L.P.M., Cuperus, F. and Stadhouders, J. 1973
Bacteriological aspects of the keeping quality at 5 C of
reinfected and non-reinfected pasteurized milk.

Law, B.A., Cousins, C.M., Sharpe, M.E., and Davies, F.L. 1979
Psychrotrphys and their effects on milk and dairy products.
Society for Applied Bacteriology Symposium Series No. 13
London Academic Press p 137-152.

American Society of Microbiology Washington, D.C.

McKinnon, C.H. and Pettipher G.L. 1983
A survey of sources of heat-resistant bacteria in milk with
particular reference to psychrotrophic sporeforming bacteria.

Sporeforming microorganisms in selected milk supplies.

Martin, J.H., Stahly, D.P., and Harper, W.J. 1961
The incidence and nature of the sporeforming microorganisms

Martin, J.H. 1974
Significance of bacterial spores in milk.
J. of Milk Food Tech. 37:94.

Martin, J.H. 1981
Symposium: Heat resistant microorganisms in dairy food systems.
J. Dairy Sci. 64:149-156.

Mikolajcik, E.M. and Koka, M. 1968
Bacillus in milk I. Spore germination and growth.

Mikolajcik, E.M. 1970
Thermodestruction of Bacillus spores in milk.
J. of Milk Food Tech. 33:61-63.

Mikolajcik, E.M. 1978
Psychrotrophic sporeformers: A possible keeping quality problem
in market milk. Am. Dairy Review 40:34A.
Mikolajcik, E.M. and Simon, N.T. 1978
Heat resistant psychrotrophic bacteria in raw milk and their growth at 7 C. J. Food Protection 41:93-95.

Overcast, W.W. and Atmaram, K. 1974
The role of Bacillus cereus in sweet curdling of fluid milk. J. Milk Food Tech. 37:233-236.


Phillips, J.D. and Griffiths, M.W. 1986
Factors contributing to the seasonal variation of Bacillus sp. in pasteurized products. J. Applied Bacteriology 61:275-285.

Punch, J.D., Olson, J.C., and Thomas, E.L. 1965

Ridgway, J.D. 1954

Riemann, H. 1969

Duncan Procedure p 191-194
SAS Institute Inc., Cary, N.C.

Sharma, J.K., Malik, R.K., and Mathur, D.K., 1984


Shehata, T.E. and Collins E.B. 1971
Isolation and identification of psychrophilic species of Bacillus from milk. Applied Microbiology 21:466.

Shehata, T.E. and Collins E.B. 1972

Skerman, V.B.D. 1967
A Guide to the Identification of the Genera of Bacteria. Williams and Wilkins, Baltimore, M.D.
Smith, N.R., Gordon, R.E., and Clark, F.E. 1952
Aerobic Sporeforming Bacteria. Agricultural Handbook No. 16
USDA, Washington, D.C..

Stadhouders, J. 1975.
Microbes in milk and dairy products: An ecological approach.

Steel, R.G.D. and Torrie, J.H. 1960
Principles and Procedures of Statistics. p 113-114

Stewart, D.B. 1975
Factors influencing the incidence of Bacillus cereus

Twenty-four hour isolation and confirmation of Bacillus cereus
in foods. J. Food Protection 47:856-860.

1950 Thermoduric organisms in milk. Part I. A Review of the
37:27-64.

Tinuoye, O.L. and Harmon, L.G. 1975
Growth of thermoduric psychrotrophic bacteria in refrigerated

Vlad, A. and Vlad, A. 1973
Food poisoning due to Bacillus cereus.
Microbiologia, Parazibologia, Epidemiologia, No.6:531-536

Washam, C.J., Olson, H.C., and Vedamuthu, E.R. 1977
Heat resistant psychotrophic bacteria isolated from

Wilkinson, G. and Davies, F.L. 1973
Germination of spores of Bacillus cereus in milk and milk
J. Applied Bacteriology 36:485-496.
Appendix Figure. Standard curve used to determine alanine liberated by proteolysis of milk proteins by Bacillus species.

Absorbance at 340 nm

0 1 2 3 4 5 6 7 8 9 10 11 12

mM alanine

Church et al. (1983)