AN ABSTRACT OF THE THESIS OF

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Title: Identification and Characterization of Some

Psychrotrophic Heat Resistant/Sporeforming Bacteria in the

Grade A Raw Milk Supply of Oregon

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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 proper-
ties. 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% demon-
strated 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

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_____

Head of Department of Foods and Nutrition

_____

Dean of Graduate School

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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 spore-forming 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 psychrotro-phic 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.

REVIEW OF LITERATURE

Identification and Incidence of Thermoduric Psychrotrophs

The trend toward prolonged refrigerated storage of raw milk
before processing, application of higher pasteurization temper-
atures, and extended refrigeration before consumption has marked-
ly 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 invest-
igated. 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, milk-
ing 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. subtilus, 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 identifi-
cation of the genus Bacillus included the absence of adequate
differential tests for species identification and lack of coop-
eration 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 psy-
chrotrophic 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.

ORGANISM	REFERENCE	ORGANISM	REFERENCE
B. brevis	1,2,5,7	B. lentus	3,7
B. cereus	2,3,4,5,6,7,8	B. licheniformis	1,3,5,7
B. cereus var.		B. macerans	1,2,3,4,5
mycoides	7	B. megaterium	1,2,3,5
B. circulans	1,2,3,5,6,7	B. polymyxa	1,2,4,5,6,7
B. coagulans	1,2,3,5,6,7	B. pumilus	1,3,6,7
B. firmus	2,7	B. subtilus	1,3,4,6,7
B. laterosporus	1,6,7		

REFERENCES: 1. Shehata and Collins (1971); 2. Chung and Cannon
(1971); 3. Grosskopf and Harper (1974); 4. Washam
et al. (1976); 5. Johnson and Bruce (1982);
6. Sharma et al. (1984); 7. Phillips and Griffiths
(1986).

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 pasteur-

ization (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 Examin-

ation 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 occured 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. subtilus, 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 char- acteristics found in between psychrophilic Pseudomonas and mesophilic bacteria.

Mikolajcik and Koka (1968) investigated the influence of heat treatment of milk upon germination, outgrowth, and sub- sequent vegetative cell growth of Bacillus cereus spores. B. cereus spores were inoculated at a rate of 10^4/mL with and with- out 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 ger-
minated 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 germin-
ation. 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 incu-
bation 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 affect-
ing 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 meosphilic 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 spore-
formers 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 psy-
chrotrophic; 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.

Organism	D value (min)	Thermal Resistance at 95 C Z value (C)	Ref.
B. licheniformis A-1	17.76	6.4	1
B. licheniformis A-5	20.50	6.8	1
B. licheniformis ATCC 10716	12.10	7.8	1
B. cereus 1	10.16	9.6	1
B. cereus 7	14.40	7.1	1
B. pumilus	4.03	7.5	1
B. cereus var. mycoides	10.90	7.6	1
B. coagulans ATCC 7050	6.90	9.1	1
B. laterosporus	5.95	7.0	1
B. circulans	4.75	11.5	1
B. megaterium 9	6.50	8.4	1
B. sphearicus	7.60	9.1	1
B. cereus	1.80	9.4	2
B. pumilus	1.40	9.7	2
B. laterosporus	2.10	10.1	2

References: 1. Mikolajcik (1970)
            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. B̲. macerans, B̲. polymyxa, B̲. laterosporus,
B̲. subtilus, B̲. lentus, B̲. cereus, and B̲. 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 B̲. circulans; fruity was the primary flavor
defect, along with the unclean defect, for both B̲. coagulans
and B̲. laterosporus. Bacillus cereus and B̲. 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 susceptability 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 (Donnely and
Busta, 1981).

Tinuoye and Harmon (1975) inoculated thermoduric psychro-
trophs (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 thermohilic 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 modifi-
cations 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 occuring 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 intra-venously. 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 detec-tion 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 casse-
roles.

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 (21×10^7
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 precipation (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 subtilus has also been implicated in outbreaks of food poisoning in the literature. Bacillus subtilus, 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. subtilus 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 proced-ures 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 iso-

lation and subsequent genus and species identification. Cellular

and colony morphology, physiology, and reaction to selected bio-

chemical 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. subtilus #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.

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

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 30×10^{4} 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 4. Plate counts on raw and heat treated milk
samples from commercial processing plants.

Milk	Plate Procedure	Count CFU/mL	No.of Samples	% of TOTAL
Raw	SPC[a]	<5,000	27	46
		5,000-20,000	24	41
		20,000-50,000	6	10
		>50,000	2	3
Lab Pasteurized	SPC[b]	<100	32	54
		100-500	19	32
		500-1,000	1	2
		>1,000	7	12
	PSC[c]	<100	36	61
		100-500	7	12
		500-1,000	3	5
		1,000-10,000	4	7
		>10,000	9	15
	PPSC[d]	<100	45	76
		100-500	4	7
		500-1,000	2	3
		1,000-10,000	2	4
		>10,000	6	10

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.

Product	Date Received	Standard Plate Count[a] (per mL)	Sporeforming Psychrotrophs[b] (per mL)	Proteolytic Sporeforming[c] Psychrotrophs (per mL)	Spoilage Time (days)	Defect[e]
Whole	2/15	120^d	$<10^d$	$<10^d$	-	-
2%	2/15	190^d	20^d	$<10^d$	-	-
Whole	3/2	60^d	$<10^d$	10^d	20	bitter,unclean
2%	3/2	80^d	30^d	20^d	20	sl. bitter
Whole	3/10	70^d	3200	3600	25	sl. bitter
2%	3/10	490	60^d	20^d	25	bitter
Whole	3/15	490	30^d	10^d	21	sweet curdled bitter
2%	3/15	790	20^d	10^d	21	bitter,unclean

[a] Standard Methods for the Examination of Dairy Products APNA (1985) p. 133
[b] 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)
[c] 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)
[d] Estimated Standard Plate Count (ESPC) Standard Methods for the Examination of Dairy Products APNA (1985) p. 143
[e] 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 incuba-
tion 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 field-
man, 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 equip-
ment 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 <u>Bacillus</u> (102 total strains) were isolated. <u>Bacillus licheniformis</u>, found in 18% of the samples, was the most common species isolated. The pattern of <u>Bacillus</u> species isolated from raw milk samples was similar to <u>Bacillus</u> 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 <u>Bacillus firmus</u> (43.6%), <u>B. megaterium</u> (23%), <u>B. brevis</u> (15.7%), and the remainder as <u>B. coagulans</u>, <u>B. polymyxa</u>, <u>B. macerans</u>, <u>B. circulans</u>, and <u>B. cereus</u>. Johnson and Bruce (1982) identified <u>Bacillus</u> sp. isolated from lab pasteurized raw milk as <u>B. cereus</u> (65.7%), <u>B. licheniformis</u> (19.9%), <u>B</u> <u>coagulans</u> (10.1%) and the balance as <u>B. megaterium</u>, <u>B, circulans</u>, <u>B. polymyxa</u>, <u>B. brevis</u>, and <u>B. macerans</u>.

Contrary to most other reported studies of sporeforming psychrotrophs, no <u>Bacillus cereus</u> 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 <u>B. cereus</u>, <u>B circulans</u>, and <u>B. cereus</u> var. <u>mycoides</u> were present in greatest numbers in the months of April to September, while <u>B. licheniformis</u>, <u>B. pumilus</u>, and <u>B. subtilus</u> were generally higher during November to February. However, the seasonal variation of sporeforming psychrotrophic <u>Bacillus</u> 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

Table 6. Identifcation of psychrotrophic, sporeforming isolates from raw and commercially processed samples.

ORGANISM	NUMBER OF SAMPLES CONTAINING ORGANISMS		% OF TOTAL SAMPLES
	raw	pasteurized	
Bacillus brevis	5	0	9
B. cereus mycoides	4	0	6
B. circulans	5	2	11
B. coagulans	1	1	3
B. firmus	2	1	5
B. latersporus	1	0	2
B. licheniformis	11	1	18
B. macerans	3	0	5
B. megaterium	6	1	11
B. polymyxa	5	1	9
B. pumilus	9	0	13
B. sphaericus	5	0	8
Total Samples	59	8	

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

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 character-

istics 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 characteris-

tics indicated, the appropriate morphological and microbiological

characteristics (with exception of having lower growth tempera-

tures) 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 con-
tributing 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 pro-
duction, 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 spore-
formers 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 <u>Streptococcus lactis</u> var. <u>maltigenes</u>.
These two organisms closely fit the description in Bergey's Manual
for <u>S</u>. <u>lactis</u>, 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 sur-
viving the 80 C for 10 min heat treatment. However, Bergey's
Manual indicates a heat resistance of <u>Streptococcus lactis</u> of
60 C for 30 min. These two microbial isolates were identified
as belonging to the variety <u>maltigenes</u>, 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 avail-
able within the research laboratory, so alternately milk
samples were obtained from a local dairy processer 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. <u>Bacillus</u> 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.

Day 1	Raw	Past.	Organism	Day 3 Cont.	Raw	Past.	Organism
		Milk Sample No.				Milk Sample No.	
	1		B. sphaericus		3		B. circulans
	2		B. polymyxa		4		B. pumilus
	3		-			5 (2%)	B. coagulans
	4		B. sphaericus				B. brevis
			B. circulans			6 (whole)	B. circulans
	5		B. circulans				B. firmus
	6		-				
		7 (2%)	B. pumilus	Day 4	1		-
		8 (whole)	-		2		B. cereus var. mycoides
					3		B. polymyxa
Day 2	1		-		4		B. pumilus
	2		B. brevis		5		-
	3		-		6		B. polymyxa
	4		-		7		B. laterosporus
		5 (whole)	B. licheniformis				B. firmus
		6 (2%)	B. megaterium			8 (whole)	-
						9 (2%)	B. polymyxa
Day 3	1		B. licheniformis				
			B. polymyxa				
			B. firmus				
	2		B. licheniformis				
			B. coagulans				
			B. circulans				

pasteurization of the raw milk samples, psychrotrophic spore-
formers were detected in 5 (16.7%) of the samples. Psychrotro-
phic 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 sporeform-
ing 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 pasteuriza-
tion with subsequent growth at 7.2 C from pasteurized milk
products but was unable to isolate any psychrotrophic sporefomers
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 pasteur-
ized milk. Phillips and Griffiths (1986) demonstrated that the
average shelf life of milk from which Bacillus organisms were
associated with post-pasteurization contamination was approx-
imately 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) contamin-
ation 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 past-
eurized 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)

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

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

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).

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 trypto-
phan 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 signif-
icant 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 acitivity of <u>Bacillus</u> species isolated
from milk samples expressed as mM of alanine. 1

ORGANISM	NO.OF ISOLATES	MEAN + SD 2 (mM of alanine)
<u>Bacillus</u> <u>brevis</u>	4	1.24 $\pm$ 0.044 [c]
<u>B</u>. <u>cerus</u> <u>mycoides</u>	9	1.93 $\pm$ 0.065 [a]
<u>B</u>. <u>circulans</u>	2	1.32 $\pm$ 0.042 [b c]
<u>B</u>. <u>coagulans</u>	2	1.10 $\pm$ 0.035 [d]
<u>B</u>. <u>firmus</u>	3	1.08 $\pm$ 0.081 [d]
<u>B</u>. <u>licheniformis</u>	5	1.33 $\pm$ 0.073 [b]
<u>B</u>. <u>megaterium</u>	3	1.32 $\pm$ 0.051 [b c]
<u>B</u>. <u>polymyxa</u>	8	1.28 $\pm$ 0.066 [b c]
<u>B</u>. <u>pumilus</u>	9	0.93 $\pm$ 0.057 [e]
<u>B</u>. <u>sphaericus</u>	3	1.25 $\pm$ 0.053 [b c]

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 actvity 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 3.0×10^5 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-pthaldialdehyde and B-mercaptoethanol, form an adduct with alpha-amimo 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 tempera-ure 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 <u>Bacillus</u> species with a corresponding proteolytic activity. This information allows the identification of certain <u>Bacillus</u> 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 Steptococcus. 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, respective-
ly. 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 pro-
eolytic 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, process-
ing 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 B. cereus and possibly other Bacillus species in relation to their ability to cause foodborne illness from milk and dairy products.

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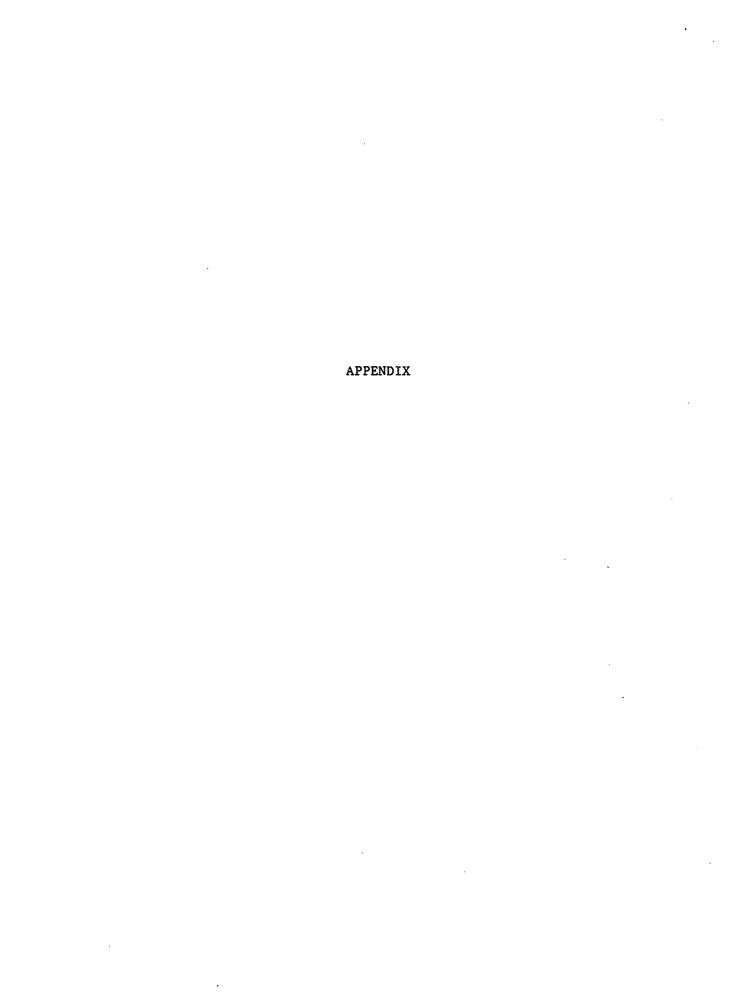in foods. J. Food Protection 47:856-860.

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APPENDIX

Appendix Figure.   Standard curve used to determine alanine liberated by proteolysis of milk proteins by Bacillus species [a]

a Church et al. (1983)