THE THERMAL INACTIVATION OF PSYCHROPHILIC BACTERIA
IN MILK

by

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ABSTRACT

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To investigate the occurrence of thermo-
duric psychrophilic bacteria in milk after pasteur-
ization by the High Temperature, Short Time (HTST)
method, duplicate samples were removed in screw cap
test-tubes after the holding section from four
commercial HTST units. Standard Plate Counts (SPC)
incubated 2 days at 32°C. and psychrophilic plate
counts incubated 10 - 15 days at 4 - 5°C. were made
on one duplicate. The other duplicate was kept
un-opened at 4 - 5°C. for 10 days at which time
SPC and psychrophilic plate counts were made on it.
SPC and psychrophilic plate counts were also made on
the raw milk.

When no significant pattern of psychro-
philic survivors was found in pasteurized milk held
for 10 days, a limited laboratory experiment was
initiated. Duplicate one millilitre amounts of
milk containing high concentrations of three psy-
chrophilic strains of _Pseudomonas fluorescens_ were
given an HTST heat treatment in sealed glass con-
tainers made from glass tubing of 3 mm. bore and
1 mm. wall thickness. An innovation was the use of
a copper-constantan thermocouple sealed into some
tubes of milk and attached to a recording potentiometer

to show both the time and temperature of the entire
heating and cooling process.

Psychrophilic plate counts were made immed-
iately after pasteurization on one glass tube dupli-
cate at 4 - 5°C. for 10 - 15 days. The other glass
tube duplicate was held un-opened at 4 - 5°C. for
10 days and then used for a second psychrophilic
plate count.

No survivors were found immediately after
pasteurization among the three strains of Ps. fluor-
escens. One strain showed survivors at a low level
after the 10 days storage.

It is possible that some thermoduric strains
of psychrophilic bacteria do exist. However, when
the data of the first experiment is considered also,
it seems unlikely that a thermoduric psychrophile
will occur in normal raw milk supplies in sufficient
numbers to survive HTST pasteurization. Hence, it is
concluded that psychrophilic bacteria in milk are
more likely the result of post-pasteurization
contamination.

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INTRODUCTION

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Psychrophilic bacteria are a significant obstacle to keeping milk fresh under refrigeration. It has been assumed, generally, that these organisms gain entrance to the milk after pasteurization. If it can be shown that these bacteria also survive pasteurization, then psychrophilic bacteria deserve greater attention in assessing the quality of raw milk. Using four psychrophilic strains of <u>Pseudomonas</u> <u>fluorescens</u> and the Modified Small Plate Culture Method for samples exposed to High Temperature Short Time (HTST) type pasteurization as outlined in the 10th Edition of Standard Methods (1), Macaulay et al (56) found highly significant numbers of surviving test organisms in 5 ml. of milk held for ten days at refrigerated storage temperatures.

Prompted by Macaulay's findings, the first experiment was undertaken to detect and enumerate the presence in milk of viable psychrophilic bacteria immediately after an HTST pasteurization. The four commercial HTST pasteurizers used plate type heat exchangers to heat milk to a minimum of 161.5°F. and approved holding tubes to maintain this temperature for 16 - 20 seconds.

The second experiment grew out of the failure to detect any significant numbers of bacteria capable of growing at 4°C. in milk collected from the end of the holding tube of the four HTST pasteurizer units. Because the experiments of Macaulay had found survivors of Pseudomonas species in milk given a heat treatment in the range 162.5 - 163.9°F. (72.7 - 73.3°C.) for 16 - 20 seconds estimated time of exposure, it was decided to repeat his experiment in part using slightly different methods and materials for exposing the bacteria to the same time/temperature treatment.

The major innovation of this experiment is the adaptation of a semi-micro sealed glass tube, total immersion technique to an HTST treatment, and the control of the time and temperature of heating and cooling with the use of a thermocouple and recording potentiometer.

Because the numbers of psychrophiles surviving in pasteurized milk, if any, are few, a 10 day incubation period at 4°C. was decided on to give any survivors a chance to grow. It was considered that if the lag period of survivors was any greater, then for all practical purposes, they were inactive and not a keeping quality problem. To show the extent

of deviation in viable bacterial counts obtained

from freshly pasteurized milk as compared to counts

from the same milk held at 4°C. for 10 days, duplicate

samples were used. One was to be counted as soon as

possible, the other in ten days' time, with Standard

Plate counts (SPC) and Psychrophilic Counts being run

each time. This same pattern was followed in both

experiments.

LITERATURE REVIEW

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Various definitions have been advanced for the term "psychrophile" or "cold-loving" as applied to bacteria. Fabian (29) classifies cryophilic, mesophilic, and thermophilic bacteria according to their minimum, optimum, and maximum growth temperatures. Cryophilic bacteria are defined as having a minimum of 32°F., an optimum of 59°F. and a maximum of 86°F. Mesophiles are defined as having a minimum of 59°F., an optimum of 98.6°F. and a maximum of 113°F. Thermophiles have a minimum of 113°F., an optimum of 131°F and a maximum of 158°F.

Kennedy and Weiser (50) define psychrophiles as having an "optimum temperature range" of 5 - 25°C. They cite an optimum of 25 - 45°C. for mesophiles.

Olsen et al (65) cites 35 - 45°F. as the psychrophile range. Thomas (92) defines them as able to grow at less than 5°C. and unable to survive 63°C. for 30 minutes.

Oginsky and Umbreit (62) classify psychrophile's optimum temperature as 12 - 18°C. with an approximate minimum of 0°C. They place mesophile's optimum range as 25 - 37°C. or 37 - 50°C. with an approximate minimum of 15°C. Thermophiles have an

optimum of 55 - 60°C., a minimum of 40°C., and a maximum of 75°C. Ingraham and Stokes (46) define psychrophilic bacteria as those that grow well at 0°C. within two weeks' time.

Prouty (74) uses the adjective "facultative" to describe bacteria of mesophile optimum growth temperature which are capable of significant growth under psychrophilic conditions. Roth (82) has demonstrated with the genus <u>Arthrobacter</u> a continuous progression from psychrophile to mesophile of seven species' response to growth temperatures. Regardless of their definition, Elliker (23) says that these psychrophiles are the Dairy Industry's biggest problem.

LOW TEMPERATURE KEEPING QUALITY

It is generally recognized that keeping quality should be consistent. If a housewife uses a half pint of whipping cream over two weeks, she expects the next to keep as long. When all the dairy plants in a given area have the same keeping quality in their products, none of them is likely to suffer by comparison. Thus there is no incentive for improvement, even if the keeping quality of all of them is mediocre. As pointed out by Olsen (65), Boyd (8), Ford (30), Speck (87), and Elliker (23),

the trend to increased marketing areas, rising labour costs, the expense of automation and the investment in large scale equipment coupled with the handling of raw milk in bulk quantities and the transport of pasteurized milk over long distances all makes improved quality imperative.

In 1959, Ford (30) stated that four days was a common length of time between processing and consumption. With wider areas being served and larger size containers such as three quart cartons, the length of time between processing and consumption is increasing. Inventory control can be improved and the waste of scrapping older packages decreased where management has confidence in their product's keeping quality.

HIGH TEMPERATURE - SHORT TIME PASTEURIZATION

The heat treatment of milk known as Pasteurization was first used about 1890. From its first introduction, the equivalence of exposures using higher temperatures for shorter times to lower temperatures for longer times was recognized. Because the lethal length of time at higher temperatures became a matter of seconds and because of a tendency to disperse fat globules so that no cream layer formed, a lower temperature for a longer, more easily

controlled, time was chosen.

Since it was the most heat resistant patho-
gen likely in milk, the destruction of M. tuberculosis
was adopted as the standard by which equipment and
heat treatments were judged. The time and temperature
exposure first agreed to was 143°F. for 30 minutes.
This contained a ten minute safety margin over the
time required to kill M. tuberculosis and did not aff-
ect cream line formation. Much later, when the imp-
ortance of cream line formation had declined, Enright
(24) showed that Rickettsia burneti, the cause of Q
fever, would survive the Holder method (143°F. for
30 minutes). Raising the Holder method treatment to
145°F. for 30 minutes resulted in complete destruction
of R. burneti. The same author (25) found increased
temperature and time necessary where ice cream mix,
chocolate milk and cream were processed.

Holmquist (45) relates that the first li-
censed commercial High Temperature - Short Time unit
had two electrodes immersed in the milk for heating.
With fixed electrical input, control was by flow rate
from a pump with an automatic stop. The use of hot
water heating devices worked as well when it was
realized that "........the degree of heat and the
holding time rather than the method of applying the

heat are the important factors" (45, p. 14).

Weber (102) states that the initial time-
temperature treatment agreed upon for HTST was 160°F.
for 15 seconds. The introduction of the flow diver-
sion valve, a safety device to prevent sub-heated
milk from moving beyond the heating and holding sect-
ions of the apparatus, necessitated increasing the
length of holding time one second to 16 seconds.
This added second gave time for the valve to operate.
Kreuger (52) states that the temperature was raised
one degree (1°F.) as an added precaution. In check-
ing the original exposure, Workman (103) tested 17
strains of human and bovine tubercle bacilli, 74
strains of Brucella abortus, 218 strains of human and
186 strains of Bovine streptococci at 160°F. plus or
minus 5°F. for 15 seconds in a laboratory pasteuriza-
tion set up. In no case were any survivors found
among all these micro-organisms.

In comparing plate counts of milk pasteur-
ized by the Holder method (143°F. for 30 minutes) and
by the HTST method, occasional high counts were found
in the HTST pasteurized milk. Parfitt (69), Prucha
and Parfitt (75), Fabian (29), and Kreuger (52)(53)
found that these were thermoduric organisms that orig-
inated on the farm and that they could be controlled

by improved sanitation, particularly in the care of
rubber milking machine parts.

Hileman (42) (43) in 1941 found a differ-
ence in kinds of microflora surviving when comparing
Holder and HTST methods. Increased numbers of alkali
producers found by litmus milk reactions were noted
in plant HTST milk. From commercial experience over
an eighteen month period, he dismissed them as being
of no importance as judged by complaints about undes-
irable flavours and odours. It is well to bear in
mind that delivery schedules were then more frequent.
KEEPING QUALITY RELATED TO TIME AND TEMPERATURE OF
STORAGE, BACTERIAL GROWTH AND FLAVOUR DEVELOPMENT

Numerous studies have been made in the past
of keeping quality or the length of time before bact-
erial growth causes off-flavours, odours, and other
changes in milk that has been pasteurized. Olsen
(65) lists flavours as "unclean, putrid, fruity,
unclean sour flavour". Further defects are thicken-
ing, ropiness, stringiness, and a slight green or
yellow colouration. Erdman and Thornton (26)(27)
reported bitter flavours in milk held 15 days at 4.5°C.
Kastli and Binz (48) found oxidized flavours in
samples stored at 2 - 3°C. after 2 - 3 days' storage.
Atherton et al (5) found off-flavour development

related to a decrease in the protein stability and that "stale" flavours were the most usual. Boyd (9) reported no oxidized flavours among the off-flavours due to growth of psychrophiles in milk.

Sherman (84), in studies using 0°C. storage, kept good quality pasteurized milk for periods of eight to twelve weeks. He further found that good quality raw milk, capable of keeping up to four weeks, could decrease the keeping quality of the pasteurized milk when injected in minute quantities into the latter. He found no spore formers growing, and blamed nearly all growth on Pseudomonas species.

In a more recent study of retail pasteurized milk in Lexington, Kentucky, Randolph et al (78) found that the average time for milk to keep a satisfactory flavour was 7.7 days. Further, it was found that after 10 days, 58% of samples were off-flavour, and after 14 days, 74% were off-flavour.

Boyd et al (9) found that keeping quality shown by flavour scores extended beyond the time that it took the psychrophile count to reach 50,000/ml. He found lowering the temperature of storage from 40°F. to 33°F. increased keeping quality time 11 to 14 days. Keeping quality at 33°F. was approximately the same using a 50,000/ml. standard and a flavour

score of 37. Broitman, Mallmann and Trout (10) found off-flavours in pasteurized milk with counts ranging from 8×10^6 to 1×10^8 per millilitre.

Other studies, involving the keeping quality of commercially pasteurized milk, are those of Day and Doan (20), Nelson and Baker (61), Rogick and Burgwald (81), and Burgwald and Josephson (11). They all indicate the association of off-flavour development and the growth of psychrophilic bacteria.

TYPES OF BACTERIA CAUSING STORAGE PROBLEMS

Elliker (23) lists the following genera as being the most frequently encountered in keeping quality problems of pasteurized milk and cream: Pseudomonas, Achromobacter, Chromobacterium, Alcaligenes, Proteus, Escherichia, and Aerobacter. The genus found most frequently to be the cause of both high counts and flavour defects is Pseudomonas. Punch et al (76) found that 24 pure cultures of Pseudomonas gave off-flavours in HTST pasteurized milk with counts ranging from 5.2 - 200 million. Using gas chromatography, Mickelson et al (59) found the flavour produced by a Pseudomonas fluorescens to consist of four different components.

From commercially pasteurized milks, Pseudomonas spp. have been isolated by the following:

Lawton and Nelson (54) who found 7 strains; Hempler
(41) after storage at 7°C. found Pseudomonas along
with Achromobacter and Alcaligenes; Baumann and Rein-
bold (6) found Pseudomonas in six out of ten samples
classified. Galesloot (34) states that they are the
principal cause of milk spoilage under refrigeration;
while Parker et al (71) state that the genus is the
most important to the whole dairy industry.

From raw milks, numerous investigators have
isolated psychrophiles of the Pseudomonas genus.
Thomas, Hobson, and Bird (93) found that of 102 psy-
chrophile isolates, 99 were gram negative rods and of
these, 35 out of the original 102 were Ps. fluorescens.
Andrey and Frazier (3) found that Pseudomonas spp.
were the second most frequent isolate. Morris (60)
found that Pseudomonas growth materially contributed
to the failure of the methylene blue test by certain
producers, and traced it to the farm water supply.
In an extensive study of farm bulk tanks, Marth and
Frazier (57) tested various genera isolated from raw
bulk tanks for growth ability at 38°F. when re-
inoculated into raw milk. Pseudomonas spp. grew more
rapidly than Achromobacter, Alcaligenes, and Aerobac-
ter toward the end of the four day test period. Also,
a difference in the ability of some cultures to grow

better in raw than in either pasteurized or sterilized milk was observed. Erdman and Thornton (27) identified 190 cultures out of 722 psychrophile isolates from raw milk and cream. Of these, Pseudomonas spp. accounted for 16.7% and also for over half of the Gram negative rods of which 62% exhibited fluorescence.

Defects in flavour and other qualities of both cottage cheese and butter have been caused by psychrophilic organisms. Large proportions of these have been Pseudomonas spp. traced to the water supply. With water and butter samples from Wisconsin and Minnesota creameries, Jezeski and Macy (47) found that twenty-eight out of forty-one cultures isolated were of the genus Pseudomonas and that a majority of these produced a green fluorescence in culture media. Druce and Thomas (21) studied farm butter made using un-chlorinated and contaminated water. Pseudomonas putrefasciens and fluorescent Pseudomonas spp. were found among the large number of psychrophilic bacteria counted. In his investigation of an intermittent surface taint of butter, Schutt (83) found that Pseudomonas fluorescens was the most important organism. Corley and Hammer (16) traced the spoilage of butter to high counts of Ps. fluorescens and Ps. putrefasciens in creamery water supplies.

Parker et al (70) and Davis and Babel (19) investigated a slimy curd defect of cottage cheese. Parker isolated a <u>Pseudomonas viscosa</u> that caused a yellowish or brownish slime and extensive proteolysis. The same author found that <u>Pseudomonas fragi</u> would cause a fruity odour. Davis and Babel found several <u>Pseudomonas</u> spp. that would cause a slimy curd defect. Bonner and Harmon (7) found <u>Pseudomonas desmolyticum</u>, <u>Ps. fragi</u>, <u>Ps. fluorescens</u>, and <u>Ps. tralucida</u> among samples of spoiled cottage cheese. In their survey of cottage cheese quality, Martin, Foltz, and Rutz (58) found that 44% of all samples examined had <u>Pseudomonas</u> species present. Like Parker above, they found that <u>Pseudomonas viscosa</u> gave a yellowish brown slime along with fruity, rancid, bitter, and flat flavour defects.

CHARACTERISTICS OF <u>PSEUDOMONAS</u> SPECIES AND OTHER PSYCHROPHILES

<u>Temperature of Growth</u>

Different Pseudomonas species have been found having a very wide range of growth temperatures. Sulzbacher (91) found isolates from frozen meat, apparently <u>Pseudomonas</u> spp., that grew on agar slants at -6°C. (21°F.). They produced a yellowish green pigment and alkaline reduction in milk.

Straka and Stokes (89) found isolates from Antarcti-
ca that grew on Trypticase Soy Agar at -7°C. Their
maximum growth temperature was 35°C. for 28 out of 31
strains. Only two grew at 37°C.

Olsen and Jezeski (63) grew a psychrophilic
strain of Pseudomonas fluorescens in both aerated and
stationary cultures at 4, 10, 15, 20, 25 and 32°C.
They found that aeration produced more rapid growth
at low temperatures. In their study of farm bulk
tank flora, Marth and Frazier (57) isolated six
species that grew well at 38°F. Their Pseudomonas
species' growth rates in milk at bulk tank tempera-
tures were such that initial counts in the range of
50,000 - 115,000 could reach 200,000/ml. in 24 hours.
An initial count of 30,000 - 70,000/ml. would reach
200,000/ml. after 48 hours under the same growth
conditions.

Castell and McDermott (12) investigated
the growth characteristics of organisms found in un-
chlorinated tap water from a 220 foot deep drilled
well. They found many psychrophilic types on plates
incubated at 3°C. that were active multipliers in the
water. After one week of growth in the well water,
100% resembled Pseudomonas fluorescens. Of 60 species
chosen for study the most dominant and important were

gram negative, alkaline forming, oxidase positive, proteolytic and lipolytic organisms with a relatively low temperature range.

Psychrophiles able to grow in the absence of oxygen are reported by Upadhyay and Stokes (97) Under both aerobic and anaerobic conditions, decreasing incubation temperatures lengthened lag, exponential, stationary, and death phases. Elimination of oxygen increased the lag period, allowed the cells to survive much longer at low temperatures (but accelerated their death at $20^{\circ}C$. and higher), and finally, reduced the extent of growth. Maximal cell populations were obtained aerobically at $5^{\circ}C$., and anaerobically at $25^{\circ}C$. No species study was made. These findings contrast to the cold-loving, strictly aerobic organisms isolated by Straka and Stokes (89) from Antarctica.

Nutritional Requirements of Pseudomonas and other
Psychrophiles

The well water of Castell and McDermott (12) on chemical analysis showed these ions present: NH_4^+, Cl^-, Ca^{++}, and Mg^+. Trace amounts of carbon were detected when 100 ml. of the well water was evaporated to dryness. Carbohydrate fermenters, gram positive cocci, and spore forming bacilli were almost

entirely absent. The organisms were able to use
salts of organic acids.

In discussion of nutritional requirements
of psychrophiles causing spoilage in cottage cheese,
Harmon (38) says that Pseudomonas and Alcaligenes
genera are not fastidious. However, Straka and Stokes
(88) showed that with lower temperatures, Pseudomonas
fluorescens required a more complex media, Trypticase
Soy (contains trypticase, a pancreatic digest of cas-
ein, and phytone, a papaic digest of soya-bean meal).
A contrasting simple glucose-salts media supported no
growth. These authors suggest that the more complex
media overcomes "injury" caused by the low tempera-
ture. That heating above their normal temperature
range also injures is suggested by the findings of
Heather and Vanderzant (40) who showed that Ps.
fluorescens held at 55°C. showed most survivors of
longer heating times when grown on complex media.
These same authors (39),(40) report a greater lag
phase of heated cultures of Ps. fluorescens, Ps.
fragi, and Ps. putrefasciens.

Olsen and Jezeski (63) compared glucose,
monosodium citrate, and casamino acid as primary
carbon sources for a psychrophilic strain of Pseudo-
monas fluorescens. The most rapid growth at 4°C.

was with the casamino acids. The growth stimulation
of the casamino acids was due to two possible situa-
tions. They say that either the casamino acids pro-
vided substrate material as precursors needed in cell
synthesis or build-up, or they provided substrates
suited to catabolic or breaking down action by the
cell at low growth temperature.

Because high psychrophile counts have been
noted in the pasteurized product from plants whose
raw supply had a high count, investigation as to any
influence of growth prior to pasteurization on subse-
quent growth was made. Overcast and Adams (67)
found that excessive psychrophile growth in raw milk
stimulated Brevibacterium lipolyticum and a psychro-
phile isolate, inhibited Pseudomonas fragi after 1,
2, and 4 days growth, and had no stimulation for Ps.
fluorescens when these were grown in laboratory past-
eurized milk. Their failure to prove the subsequent
stimulation hypothesis is supported by earlier work
of Sinclair and Stokes (85). Using Ps. fluorescens
in a chemically defined liquid media containing 1%
glucose and 0.1% $(NH_4)_2SO_4$, they found that the
organism grew at 30°C. until it used up both comp-
ounds. If the energy and nitrogen sources were
added back to the filtrate of the growth media, the

Ps. fluorescens reinoculated would reach maximal
levels as in the original culture. After several re-
petitions, mineral deficiencies resulted. When re-
placed, growth occurred again. They concluded that
there was no evidence that auto-inhibitory substances
were made or that physical crowding limits growth.
In a similar experiment with Escherichia coli, organic
acids accumulated and inhibited growth.

Enzyme Activity of Pseudomonads and Psychrophiles

Lipolytic and proteolytic activity were
reported in the isolates of Castell and McDermott
(12). Overcast and Skean (68) found isolates of
Pseudomonas, Achromobacter, Alcaligenes, and Flavo-
bacterium genera all capable of hydrolyzing butter-
fat at 4°C. Thomas, Hobson, and Bird (93) found that
most of their gram negative isolates hydrolyzed tri-
butyrin but that only one-third hydrolyzed tri-olein.
Druce and Thomas (21) found that farm butter with a
high yeast and mould count and large numbers of psy-
chrophile and mesophile bacteria became rancid in two
or three days. Green and Jezeski (37) found two
isolates classified as Pseudomonas, one of which was
distinctively lipolytic, while the other was proteo-
lytic. Parker et al (70) observed extensive proteo-
lysis by a Pseudomonas viscosa.

In making detailed proteolytic studies of
their 102 psychrophile isolates of which 99 were gram
negative rods, Thomas, Hobson, and Bird (93) found
that a "high proportion" liquefied gelatin, a "major-
ity" showed slow proteolysis in litmus milk at 22°C.,
and slightly more than half showed caseolytic activity
on 30% milk agar. Vanderzant and Moore (100) suggest
that proteolytic activity of psychrophiles may vary
with temperature.

Further studies on the influence of tempera-
ture on the enzyme activities of psychrophiles were
carried on by Upadhyay and Stokes (98), (99). They
found that a formic hydrogenlyase, and a hydrogenase
with its enzyme forming system were different in
temperatures of optimum activity and inactivation.
The psychrophile enzyme source was a rod shaped, gram
negative bacterium that grew well in the range of 0 -
35°C. When the psychrophile hydrogenase (99) was
held at 60°C., there was 50% destruction of it after
2 hours as compared to only 25% destruction of a
mesophilic hydrogenase under the same conditions.

Heat and Pasteurization Studies of Psychrophiles

Heat studies on psychrophilic bacteria have
sought either to find the thermal death time of an
organism, or to determine its survival of pasteurization.

Pasteurization in the dairy industry may legally des-
scribe two time/temperature relationships. The first
is called Low Temperature, Long Time (or LTLT) past-
eurization; the second, High Temperature, Short Time
(or HTST) pasteurization. The Low Temperature, Long
Time pasteurization, also known as the Holder method,
raises every particle of the milk to a temperature of
62 - 63.5°C. for 30 - 35 minutes. The High Tempera-
ture, Short Time treatment is at a minimum of 71.6°C.
for 16 seconds. Under laboratory conditions and with
full size commercial pasteurizing units, the LTLT
method is usually a batch process while HTST is con-
tinuous.

Because temperature and time are easily con-
trolled, the LTLT treatment has been used the most to
study the thermoduric properties of psychrophilic
bacteria in the laboratory. When comparing different
studies, the initial number of organisms with their
previous growth and temperature history, and the heat-
ing, cooling and enumeration should be known. With
small numbers and long plate incubation times at low
temperatures, otherwise inconsequential contamination
grows in importance.

Influence of Initial Numbers on Survivors

Using glass tubes as a container in which

various concentrations of <u>Escherichia coli</u> were
heated, Craige (17) found survivors to a treatment
of $62 \pm 0.1^{\circ}C$. for 30 minutes. Various strains sur-
vived at concentrations ranging from $\log_{10}4.2$ -
$\log_{10}12$ organisms per millilitre. The enumeration
technique involved pouring the inoculated pasteurized
milk from a glass heating tube into lactose broth
fermentation tubes. Both Prouty (73) and Stumbo
(90) point out the relation borne by numbers of
bacteria to the time-temperature treatment needed
for their destruction. When number of survivors is
plotted logarithmically and time is plotted linearly
on semi-logarithmic paper, the numbers surviving for
successive units of time follows a straight line.
This gives a rate of destruction curve.

The use of thermal death time curves has
been made by Kaufman and Andrews (49), and Chaudhary,
Tuckey and Winter (13) to show that psychrophiles
are unlikely to survive pasteurization. Kaufman and
Andrews used two psychrophile organisms, one of them
a <u>Pseudomonas viscosa</u> - 3, at three different temper-
atures below LTLT temperature in order to determine
their thermal death curves. (Of 66 isolates from
milk and water, Kaufman and Andrews found none that
survived LTLT pasteurization in the laboratory).

Olsen, Macy, and Halvorsen (64) state that plotting "z" curves in the above manner explained differences in survivors of the two pasteurization heat treatments. However, in a recent discussion of "Ultra-High" temperature and time heat treatments for the pasteurization of milk, Robinson (79) cautions that extrapolation of thermal death time data gained at lower temperatures is not justified.

Many investigators have used an LTLT treatment under laboratory conditions to determine thermoduric or heat resistant properties of psychrophilic bacteria: (7), (47), (27), (50), (94), (95), (5), (70), (49), (67) . When the risk of extrapolation to the higher temperature used for HTST is borne in mind, the information from laboratory studies in regard to Pseudomonas species in particular and psychrophiles in general is vague. Jezeski and Macy (47) found that 6 out of 41 psychrophile isolates survived 65.5°C. for 30 minutes. Twenty-eight of the forty-one were Pseudomonas species. Nowhere do they state that any of the surviving six were Pseudomonas spp. Erdman and Thornton's (27) psychrophile survivors of 142°F. (61°C.) were gram negative non-fluorescing cocci. Kennedy and Weiser (50) had two of twelve pure cultures that

showed only 5% and 24% reduction of their numbers at 145°F. for 30 minutes. These had been isolated at 10°C.

Most of the above studies used some sort of tube immersed in a water bath which experience has shown is well suited to LTLT studies. The necessity to study milk and its flora at higher temperatures has resulted in a variety of other means and equipment. To study creaming and phosphatase tests for pasteurization with HTST in the early 1940's, Holland and Dahlberg (44) used a thin walled copper container, 12 by 20 by 1 centimeter, that they immersed in water. Franklin (32) has developed a similar device quite recently that heats the milk between surfaces only 5/32 inch apart.

The use of liquid media into which large numbers of organisms could be injected and then retrieved at timed intervals from a constant temperature state are described by Gilcreas (35), Kaufman and Andrews (49), Chaudhary et al (13) and Foster (31) This method has been found suited to thermal death time studies (49), (13) where automatic devices to remove bacteria and media from the test chamber were devised (35). Enright (25) devised a more sophisticated apparatus where liquid media or milk was placed

in a 3 millimeter clearance between two concentric
cylinders. The inner cylinder rotated inside the
sealed outer cylinder giving constant and continuous
agitation. A thermocouple and continuous recorder
gave time and temperature curves of the heat treat-
ment when the apparatus was heated by immersion in a
water bath.

The use of commercial units to test for the
survival of psychrophiles in HTST and LTLT treatments
was made in "in plant" studies by Rogick and Burgwald
(80), (81). In neither treatment could they find
any bacteria in 4.1 millilitres of milk immediately
after pasteurization with plates incubated at 4 - 7°C.
for twelve days. However, after 7 days' storage in
a refrigerator at 4 - 7°C., all their samples showed
some psychrophile count (on plates again incubated at
4 - 7°C.). However, it should be noted that the counts
made after 7 days' storage in a refrigerator, were
made on the same samples used for the counts made
immediately after pasteurization.

With initial numbers not reported, Olsen
et al (65) found no survivors when sealed glass tubes
of milk were totally immersed in a water bath for LTLT
pasteurization. The psychrophile counts were all zero.
These same authors withdrew milk aseptically from

between the flow diversion valve and the regeneration section of an HTST unit operated at 162°F. for 16 seconds holding time, stored it for seven days under refrigeration, and found psychrophile counts of three and zero.

In more recent trials comparing a High Temperature, Short Time treatment of 172°F. and a 16 second holding time to milk given an Ultra-High heat treatment, Glazier (36) found higher increases in counts with the HTST milk. Although the holding time used was 0.6 second and therefore not a directly comparable treatment to HTST, Evans, Lachman and Litsky (28) using 10°F. steps from 160 - 260°F. found significant psychrophile growth in milk processed in the 160 - 200°F. range and stored at 40°F.

Enumeration of Psychrophiles

Olsen et al (66) point out that bacteria that survive pasteurization show little growth at temperatures of 45°F. (7.2°C.) or lower. Consequently, psychrophilic counts on freshly pasteurized milk free of recontamination are nearly always negative if incubated at 40 - 45°F. for 7 - 10 days. This is supported by Thomas et al (94). Olsen et al (66) also point out the close relationship of plate counts made at 25, 32, and 35°C. and that 25°C. for 3 days will

include nearly all types of bacteria in the milk, including psychrophiles.

One method for detecting the presence of psychrophilic bacteria in pasteurized milk is the Moseley count (4) most recently favoured by Elliker (22). It involves storage at 45°F. (7.2°C.) for 5 days and then plating the milk from its retail container with plate incubation at either 25 or 32°C.

Attempts to selectively speed up the detection of psychrophiles have been made. Leesment (55) used preincubation at higher than usual temperatures, immediately after plating, before placing the plates in the more usual lower temperature surroundings. A surface plate technique of Punch and Olsen (77) reduced the time for colonies to appear by 2 - 3 days at 6°C. Day and Doan (20) and Csenge and Doan (18) developed the use of neotetrazolium under vacuum conditions as a dye reduction test capable of indicating psychrophile spoilage in advance of flavour defects. Collins (14) has used Violet Red Bile Agar as recommended by Elliker, to grow subsurface colonies in 48 hours at 25°C. of Pseudomonas fragi, Ps. viscosa, and Alcaligenes metalcaligenes. In a preliminary report, Freeman, Nanavati and Glen (33) have attempted to inhibit growth of gram positive organisms in order to

allow enumeration at a higher temperature of gram
negative bacteria. They tested a number of chemicals
with some measure of success.

Chemical Control of Psychrophiles and Pseudomonas

Castell and McDermot (12) reported that
3% salt (NaCl) and 1.5 parts per million (ppm) of
chlorine would control the growth of the organisms
found by them in well water. Collins (15) found
that pH had a distinct bearing on the effectiveness
of chlorine added to alkaline waters containing
Pseudomonas species. Post and Krishnamurty (72)
found considerable lysis of Pseudomonas fluorescens
in the presence of sodium hexametaphosphate which
is a common chelating or water softening agent for
calcium and magnesium ions in hard water. Elliker
(23) recommends wide-spread use and high (200 ppm)
concentrations of chlorine to counteract the intro-
duction of psychrophiles into dairy products under
plant conditions.

EXPERIMENTAL METHOD

EXPERIMENTAL METHOD

Experiment 1 - HTST Plant Pasteurization and Psychrophiles

The purpose of these experiments was to detect the presence of any psychrophilic bacteria in milk immediately after heating in a plate type heat exchanger to a minimum of 161.5°F. and holding for 16 - 20 seconds in an approved holding tube.

Each raw storage tank was taken as the basic unit or lot of milk. Duplicate raw samples were taken from the end of the holding tube for each tank of milk at the beginning, middle, and end of the time required to process it. The samples were labelled and placed in wire racks in ice water. On return to the laboratory, SPC and psychrophile counts were made on one-half of each duplicate sample while each of the other halves were held at 4°C. for 10 days. At the end of the 10 days holding, SPC and psychrophile counts were repeated.

Sampling Technique

The sampling cock used at the end of the holding tube (as shown in Fig. 2) was a valve manufactured by the Aluminum Pressure Vessel (APV) firm of equipment manufacturers for the purpose of timing the holding period of their HTST units. The valve

threads into a normally plugged opening with a rubber
O-ring gasket to seal it. The valve is equipped with
a curved side arm that opens upward above the valve
seat (in the opposite direction from the downward
pointing delivery tube). This side arm allowed the
delivery tube below the valve seat proper to be sani-
tized before each sample was taken, as shown in Fig. 2.

Prior to being taken to the dairy, the valve
was sterilized in the autoclave at 248°F. for 20 min-
utes plus well wrapped in several layers of brown
wrapping paper. At the dairy, the cock was fastened
by the pasteurizing room employees to the end of the
holding tube before the HTST unit was sanitized for
the day's operation. Then the dairy's usual hypo-
chlorite sanitizer was run through the cock for five
to ten minutes. Before each sample was taken, the
cock was sprayed with an iodine sanitizer all over
its outside surface, and through the delivery tube
and the side arm. Milk was allowed to run from the
cock for about thirty seconds in order to rinse any
sanitizer from the delivery tube before taking each
split sample. The samples were collected in screw
cap test-tubes which had been previously sterilized
in the autoclave. Their capacity was approximately
25 millilitres and they were filled with about

20 millilitres of milk. Care was taken to see that the ice water level was higher than the level of the milk in the tubes.

Plating Procedure

Plate counts were run on both Plate Count Agar (Difco) and on Trypticase Soy Agar (B.B.L.) for the last three plant trials. Prior to this, counts were made on Plate Count Agar only. Each plate, on each media was made in duplicate. Poured plates were incubated for the SPC at 32°C. for 48 hours, and for 10 - 15 days for the Psychrophile Count at 4°C. Otherwise, the procedures followed were as outlined in Standard Methods (2).

Experiment 2 - HTST Laboratory Pasteurization
of Psychrophiles

Test Organisms

Three Pseudomonas fluorescens strains as used by Macaulay et al (56) were used in this experiment:

Pseudomonas fluorescens

Strain - Spiers   Catalogue No. 3756
Strain - 85 Groombridge Catalogue No. 9428
Strain - 25/8 ATCC 13525 Catalogue No. 10038

These three cultures were obtained through the courtesy of the National Collection of Type Cultures, Central Public Health Laboratory, Colindale,

London, NW 9. Obtained in lyophilized form, they
were started in Trypticase Soy Broth, and then main-
tained on Trypticase Soy Agar Slants at 4 - 5°C. They
were tested for growth at 37°C. and found to conform
to Macaulay's information about them, that they were
true psychrophiles unable to grow at 37°C.

For use with the glass tube heat treatment,
these cultures were grown in Trypticase Soy Broth for
24 hours at 25°C. and then stored in the refrigerator.
Transfers were made from these broth cultures with a
.01 ml. loop into 250 ml. of Trypticase Soy Broth in
500 ml. capacity flasks. The flask contained a
Teflon coated stirring magnet that was sterilized
along with the media in the flask in the autoclave for
15 minutes at 15 psi. These flasks were stoppered
with cotton plugs. Incubation was for 18 - 24 hours
at 25°C. in a circulating, temperature controlled
water bath. The magnetic stirrer was operated at a
speed fast enough to form a slight vortex in the con-
tents of the flask without the formation of foam.

To separate the organisms from the Trypti-
case Soy Broth, 40 ml. portions were pipetted into
sterile plastic centrifuge tubes having a capacity
of 50 ml. and centrifuged on a Clinical model centri-
fuge at 1560 g. The broth was decanted,and the cells

resuspended in 0.85% saline solution to the amount
of 20 ml., and centrifuged down. The saline was then
decanted. After a second saline washing using 20 ml.,
the cells were placed in two of the centrifuge tubes
and spun down. The second saline washing was decanted,
and a third amount of saline added to the amount of
3 - 5 ml. to give as high a concentration of cells as
possible. This last step was performed using sterile
Pasteur pipettes. This concentrate was placed in a
clear glass test-tube, 17 mm. in diameter and a percent
transmittance reading taken in a Lumetron Colorimeter
at 620 mu. Then the suspension was examined in a
Petroff-Hauser counting chamber using crystal violet
(saturated alcoholic solution - 10 ml., placed in 100
ml. of distilled water) as recommended in the direc-
tions of the maker of the counting chamber (51)

Milk was obtained with different levels of
test organisms as follows: using the counting chamber
estimate of the number of organisms in the salt sus-
pension, serial dilutions were made using 9 ml.
quantities of homogenized milk which had been meas-
ured with a pipette into rubber stoppered test-tubes
and sterilized for 5 minutes at 248°F. One millilitre
of suspension was placed in the first tube of milk,
mixed by inverting twenty-five times, and then one

millilitre of this was placed in the second 9 ml. of milk. This series of 1/10th dilutions continued so that the range of numbers of organisms in milk was from one-tenth the number in the original salt suspension to a concentration in the last tube of approximately 1000/ml. This series of suspensions in milk supplied the material to be sealed in glass tubes for the heat treatment. Psychrophile counts were made on those tubes used for pasteurizing material.

Sealed Glass Tube Pasteurization

The glass tubing used for heating and storing the milk was a standard type of soft flint glass with the following specifications:

Outside diameter	5 mm.	Softens	- 700°C.
Inside diameter	3 mm.	Anneal	- 520°C.
Wall thickness	1 mm.		

Coefficient of Expansion:
92 x 10^{-7}/1°C. from 0 - 300°C.

This glass tubing came in 90 cm. lengths. These were cut to 23 cm. lengths. By filling a 900 mm. length of the tubing with water at room temperature, and measuring the amount delivered into a 10 ml. graduate, it was calculated that by filling the tube to a length of 14.1 cm., the amount delivered would be approximately 1 millilitre. This checked using the mathematical formula for the volume of a cylinder;

$$V = \pi r^2 D$$

where V = volume of cylinder
       r = radius of cylinder
       D = length of cylinder

Substituting: V = 1,000 cubic millimeters
               r = 1.5 millimeters
               D = unknown
               π = 3.14159

$$1,000 = 3.14159 \times 1.5 \times 1.5 \times D$$
$$D = 141.4 \text{ mm.}$$
$$= 14.1 \text{ cm.}$$

The remainder of the length of the tube
interior was used up partly by sealing and partly for
an air cushion of 4 - 5 cm. that allowed for expansion
and contraction of the milk during heating and cooling.
Prior to being placed in pipette holders for steriliz-
ing in the autoclave, one end of each tube was closed
by holding and rotating it in an ordinary gas (Bunsen)
flame until a solid glass plug formed. The milk sus-
pensions were placed in the tubes with a sterile Past-
eur pipette and filled from the sealed end to a distance
of 14 cm. The milk was then sealed in the tube using
the gas flame. When the hottest possible flame was
used, the tube could be sealed without any heat being
conducted to the milk. The operator could hold the
tube at or beyond the level of the milk during the
sealing without his bare fingers feeling any heat.

To hold the tubes in the hot water bath and
then in the cooling ice bath, a plated wire test-tube

holder was adapted so that it would hold the tubes in a horizontal position. This was important in order to insure that all five tubes being heated came in contact with the heating water and the cooling ice water at the same instant. The test-tube rack was modified by using four zig-zag wires fastened to the middle platform. The tubes of milk were held in place by the spring of the wire and spaced approximately 1/8 inch apart. Two of the wires were used at either end of each set of tubes. With this arrangement, the five tubes could be immersed at a time. One of these tubes was a probe tube containing a thermocouple and also filled with milk. Thus all five tubes were close enough together that they would be heated and cooled the same amount.

The tubes were identified by the number of their position in the rack and so labelled during their 10 day storage.

Temperatures of cooling baths, heating, and incubation were measured using a mercury bulb thermometer. The measurement of the temperature of the milk in the glass tubes as well as the duration of the High Temperature, Short Time pasteurization exposure was accomplished with the use of a thermocouple and recording potentiometer. The copper-constantan

37

junction (the thermocouple) was welded on an impulse
spot welder and so positioned in the middle of the
3 mm. diameter bore of the glass tubing that the wires
leading to it did not touch the side for at least 1 cm.
Centering the thermocouple was accomplished by bending
the wires. This position could be checked by rotating
the tube held up to the light. If it was off center,
an eccentric wobble could be observed. The lead wires
were sealed into the tubes at 2 cm. from the end in
such a way that the sealing gas flame did not impinge
directly on the wires at the point of sealing. The
glass tube with the wires inside lying parallel to each
other was heated until the enamel insulation evaporated
(the glass was slightly incandescent) and then pinched
twice very quickly with fine nose pliers (see Fig. 3,
II and III). The probe tubes were sterilized in the
same manner as the plain glass tubes in the autoclave.

The fine copper and constantan wires used
to make the thermocouple gave a rapid response to
temperature change because the speed of response of
a thermocouple is inversely proportional to its mass.
The conduction of heat to the couple along the length
of the wires was minimized by their small size. To
check the amount of heat conduction and the speed of
response, preliminary observations using a bare couple

in a water bath were made. These showed that when the couple was moved even 1/8 to 1/16 inch out of the water, with the leads still in the water, that its temperature dropped immediately. Hence, the fact that the leads to the couple were in contact with the side of the tube had no bearing on the reading given by the probe. In other words, the probe accurately reflected the temperature and changes in that temperature which pertained to the medium in which the couple was situated. For the above reasons, it is believed that the thermocouple accurately measured the temperature of the milk at any given instant bearing in mind the 2-second full scale speed of the recorder used.

The length of time involved in the heating and cooling of the samples of milk in the glass tubes was estimated from the moving chart during the heating process. Because heating occurred in one water bath, and cooling took place in an ice water bath beside it, the length of time that the milk was exposed in the heating bath was only controllable to the nearest two seconds. After the experiment, the exact length of time could be measured from the chart with the use of dividers and a ruler.

To aid in the measurement of the 16 seconds that was aimed for, an "L" shaped piece of wire was

fastened to the recording pen so that it extended a
distance equivalent to 16 seconds in the direction
that the chart paper was moving. Thus, when the place
where the recording line had crossed the minimum temp-
erature line on the chart was reached by the wire, the
milk had been held at or above that temperature for
sixteen seconds and was a signal to lift the tubes out
of the hot water bath and plunge them into the ice
water for cooling.

Enumeration

Counts on the material in the tubes after
pasteurization were made according to Standard Methods
(2) with the following modifications:

In order to remove the milk from the sealed
glass tubes, it was necessary to break them open.
This was done in the region of the air cushion left
between the milk and the end of the tube sealed after
the milk was placed in it. First, the glass was
nicked or scratched using a three sided file. Then
the nick and the outside of the glass were wiped with
tissue soaked in methyl alcohol, the end of the tube
and the nick quickly flamed, and the glass broken at
the nick with a flamed pair of needle nose pliers.
A twist of the wrist gave a clean break each time.
The milk was then tapped into a sterilized dilution

blank giving a 1:100 dilution of the contents of the tube. It was sometimes necessary to swing the blank and the tube in an arc to completely drain the tube. To facilitate the removal of the milk in any future modification, bending the tubes into a flattened "U" shape with an air cushion in each arm would allow the tube to be opened at both ends allowing the milk to drain more easily out of the narrow bore.

TABLES AND FIGURES

Table 1

MEAN STANDARD PLATE COUNTS OF COMMERCIAL MILK

Plant	Tank	Raw Plate Count	Pasteurized Plate Counts	
			Immediate	After Storage*
1.	1	4.5×10^7	2.9×10^5	1.9×10^5
	2	5.3×10^7	4.0×10^5	2.6×10^5
	3	7.6×10^6	1.3×10^5	7.0×10^4
2.	1	1.2×10^5	3.7×10^2	4.2×10^1
	2	1.5×10^5	6.5×10^2	2.1×10^2
	3	3.1×10^4	4.9×10^2	1.7×10^2
1.	1	1.9×10^7	1.0×10^5	6.5×10^4
	2	3.6×10^7	2.1×10^5	1.5×10^5
	3	6.1×10^6	1.6×10^5	1.2×10^5
3.	1	2.0×10^7	5.9×10^4	8.7×10^4
	2	1.2×10^7	5.6×10^4	9.2×10^4
	3	1.5×10^6	5.2×10^4	5.2×10^4
4.**	1	7.4×10^5	7.4×10^4	5.1×10^4
	2	8.4×10^5	5.1×10^4	4.3×10^4
4.**	1	2.2×10^6	9.1×10^4	5.1×10^4
	2	3.9×10^6	9.9×10^4	5.4×10^4
4.**	1	2.9×10^6	8.7×10^4	7.0×10^4
	2	2.5×10^6	1.3×10^5	1.0×10^5

* 10 days at 4 - 5°C. (39.2 - 41°F.)
** Milk from Plant No. 3 processed in 320 lb. lots at
   Dept. of Food Science, University of Manitoba

Table 2

MEAN PSYCHROPHILIC COUNTS OF COMMERCIAL MILK

Plant	Tank	Raw Plate Count	Pasteurized Plate Counts	
			Immediate	After Storage*
1.	1	7.6×10^6	1.3×10^1	3.3×10^0
	2	3.8×10^7	9.1×10^0	8.3×10^0
	3	2.1×10^6	7.5×10^0	4.1×10^0
2.	1	3.0×10^5	7.5×10^0	0.0
	2	2.3×10^5	1.6×10^1	0.0
	3	5.0×10^3	0.0	1.6×10^0
1.	1	8.8×10^5	4.1×10^0	2.5×10^0
	2	1.0×10^6	0.0	3.1×10^0
	3	1.5×10^5	1.0×10^1	1.6×10^0
3.	1	2.3×10^7	1.2×10^0	1.4×10^4
	2	1.5×10^7	6.6×10^{-1}	6.4×10^3
	3	1.9×10^6	1.4×10^0	3.7×10^3
4.**	1	9.0×10^5	1.1×10^0	0.0
	2	1.3×10^6	5.0×10^{-1}	0.0
4.**	1	2.9×10^6	1.2×10^0	0.0
	2	1.1×10^6	1.4×10^0	0.0
4.**	1	1.6×10^6	8.8×10^{-1}	0.0
	2	2.9×10^6	1.8×10^0	0.0

* 10 days at 4 - 5°C. (39.2 - 41°F.)

** Milk from Plant No. 3 processed in 320 lb. lots at
Dept. of Food Science, University of Manitoba

Table 3

PLATE COUNTS OF PSYCHROPHILIC STRAINS OF
Pseudomonas fluorescens

Strain	Trial	Before Pasteurization Counting Chamber Estimate	Plate Count	After Pasteurization Immediate Plate Count	After Storage** Plate Count
3756	1	1.98×10^9	-	Negative	200
		1.98×10^{11}	-	"	100
		1.98×10^{10}	-	"	100
	2	1.98×10^7	-	"	200
		1.98×10^5	-(L.A.)	"	1.87×10^{5}*
9428	1	2.33×10^8	1.5×10^7	"	0
		2.33×10^6	2.9×10^5	"	0
	2	2.33×10^4	-	"	0
		2.33×10^9	3.3×10^8	"	0
		2.33×10^5	2.9×10^4	"	0
10038	1	2.60×10^9	3.7×10^8	"	500
		2.60×10^7	9.1×10^6	"	900*
		2.60×10^7	9.1×10^6	"	4200
	2	2.60×10^8	8.4×10^7	"	950
		2.60×10^4	9.6×10^3	"	3800*
		2.60×10^4	9.6×10^3	"	2500
3756	1	3.29×10^8	6.0×10^8	"	0
		3.29×10^2	3.0×10^2	"	0
		3.29×10^5	6.3×10^5	"	0

** 10 days at $4 - 5^{\circ}$C. ($39.2 - 41.0^{\circ}$F.)
 * Tube with thermocouple leads
(L.A.) = leak past thermocouple leads

Table 3 - Continued

Strain	Trial	Before Pasteurization Counting Chamber Estimate	Plate Count	After Pasteurization Immediate Plate Count	After Storage ** Plate Count
3756	2	3.29×10^6	6.3×10^6	Negative	0
		3.29×10^3	6.0×10^3	"	0
		3.29×10^3	6.0×10^3	"	0
9428	1	1.27×10^9	7.8×10^8	"	150
		1.27×10^9	7.8×10^8	"	0 *
		1.27×10^6	6.3×10^5	"	0
	2	1.27×10^7	1.0×10^7	"	0
		1.27×10^3	8.0×10^2	"	0
		1.27×10^3	8.0×10^2	"	0 *

** 10 days at 4 - 5°C. (39.2 - 41.0°F.)
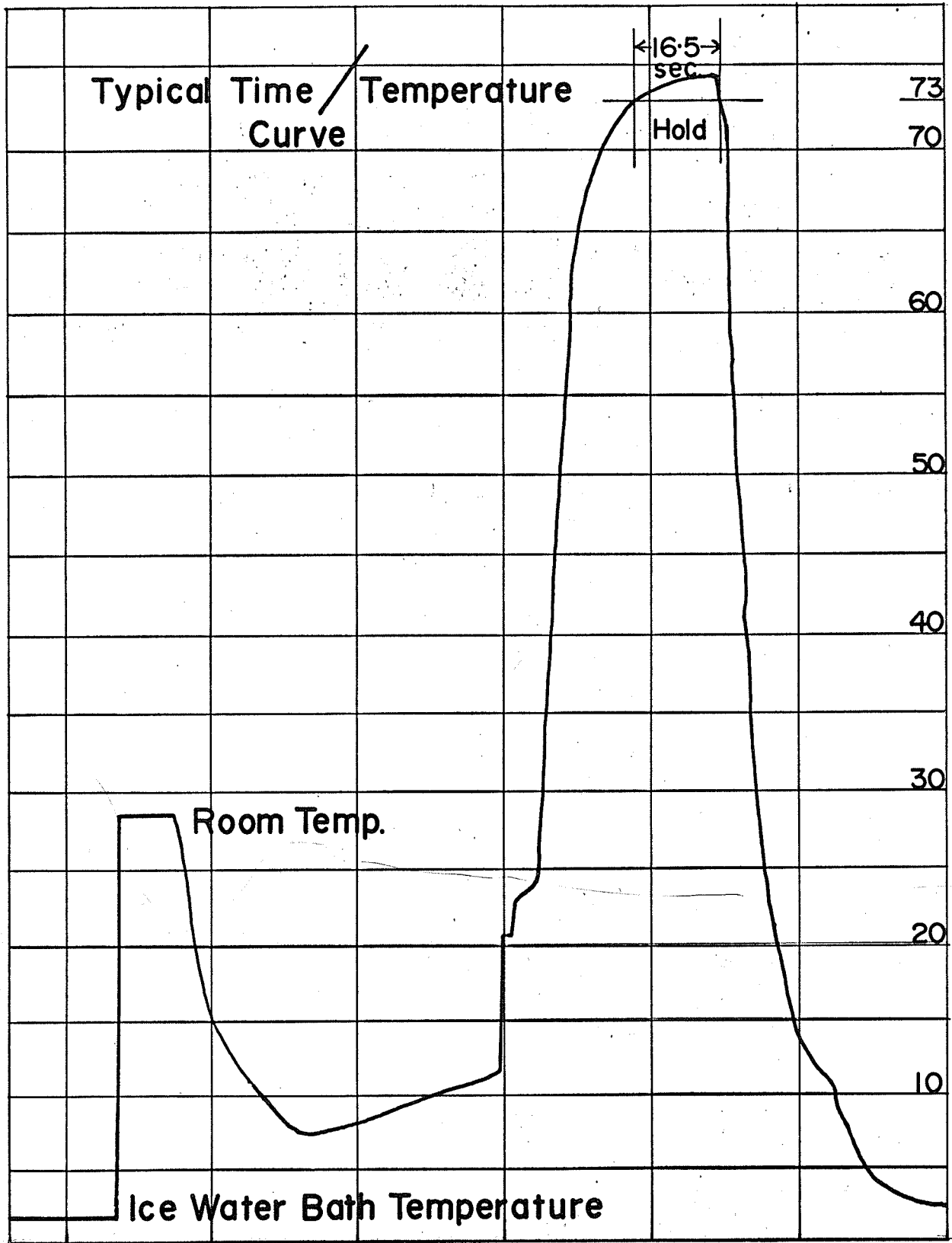 * Tube with thermocouple leads

Fig. I   HTST Pasteurization

Typical Time / Temperature Curve

16·5 sec.

Hold

Room Temp.

Ice Water Bath Temperature

°C.
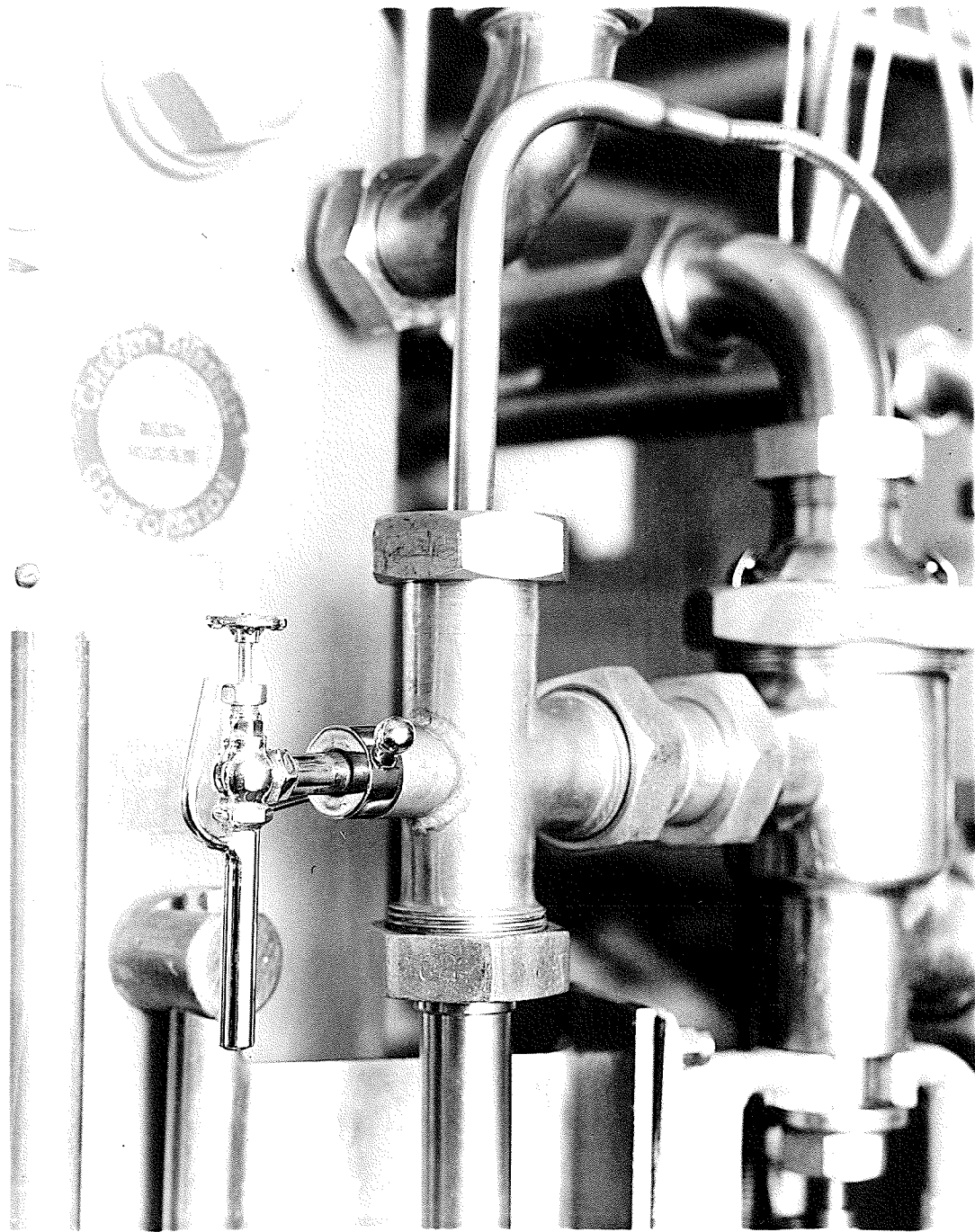
73
70
60
50
40
30
20
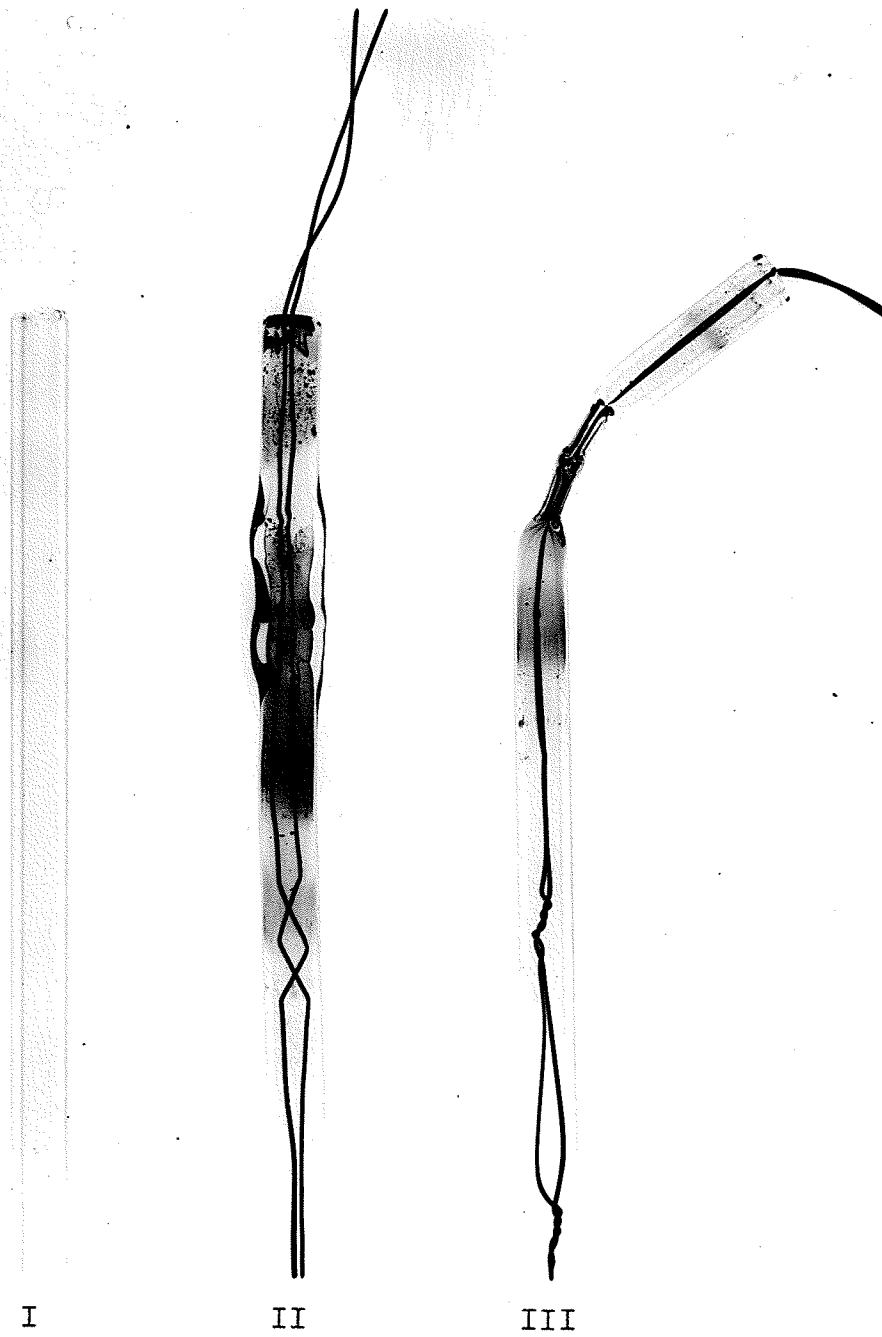10

Figure 2

APV SIDE-ARM SAMPLING VALVE - on HTST pasteurizer

Figure 3

I   II   III

GLASS TUBES FOR LABORATORY PASTEURIZATION   x 1.5
I - flint tube, 5 mm. O.D., 3 mm. I.D.
II & III - thermocouple leads in temperature
sensing tube

RESULTS AND DISCUSSION

Results and Discussion

The psychrophilic bacteria that limit the
keeping quality of pasteurized milk have generally
been assumed to be the result of post-pasteurization
contamination. Macaulay et al (56) gave four psychro-
philic strains of Pseudomonas fluorescens an HTST
pasteurizing treatment in 5 ml. of milk and found
survivors in highly significant numbers after storage
for 10 days at 3 - 5°C. If psychrophilic strains are
thermoduric with regard to present HTST pasteurizing
treatments, then serious reassessment of raw milk's
bacterial quality is needed. To show whether Macaulay's
work was of any practical commercial significance, the
first experimental series was proposed.

The original hypothesis was that pasteur-
ized milk taken aseptically from the end of the hold-
ing tube of an HTST pasteurizer contains micro-
organisms capable of growth in refrigerated milk.
Because the numbers of these organisms would be very
small initially, a period of growth at refrigerated
temperatures in the milk was recognized as necessary.

For this hypothesis to be valid, demonstra-
ble changes would have to occur during the refrigerated
period. The following standards were proposed for
acceptance: 1. Standard Plate Count (SPC) should

show an increase between samples counted immediately
and those held for 10 days at 4 - 5°C. 2. Psychro-
philic Plate Counts should be able to show both the
presence and numbers of the bacteria that grow at
4 - 5°C.

The first experiment used four different
HTST units and duplicate samples were removed from
the ends of their holding tubes. One duplicate was
given SPC and psychrophilic plate count soon after
pasteurization and the other held at 4 - 5°C. for 10
days before SPC and psychrophilic plate counts were
made again. Macaulay also held his sample 10 days but
at 3 - 5°C. Because there was no way of choosing
between bacteria from the milk's natural flora which
had merely lain dormant at 4 - 5°C. for 10 days and
those that had actively grown in it during this time,
incubation of the psychrophilic plates was also at
4 - 5°C. This is in contrast to Macaulay who could
incubate his plates at 20°C. since he dealt with a
single psychrophilic strain. Results of this present
study took longer to be evident.

In the second experiment, the glass tubes
were used first, to eliminate any chance of any part-
icle of milk, as well as its pasteurizing container,
not receiving the full HTST heat treatment; and second,

to eliminate all chance of post-pasteurization contamination during the 10 day storage period. In contrast, Macaulay used stainless steel tubes closed with rubber stoppers for heating and cooling containers. For the 10 days storage, the milk had to be transferred to glass test-tubes.

In Table 1, the results from 18 tank lots of commercial milk are summarized and compared using the Standard Plate Count. The raw, post=pasteurized, and after storage pasteurized counts show: the effect of pasteurization in the change between raw and pasteurized; and the effect of the 10 days storage in the change between pasteurized and the after storage pasteurized counts. In Table 2, these same 18 tank lots are similarly summarized and compared using a psychrophilic plate count.

The key counts are those made after 10 days storage. These samples were not opened after being taken from the end of the holding tube. Rogick and Burgwald (81), (80) examined samples immediately after HTST pasteurization and found no psychrophiles. On re-examination after 7 days at $4 - 7^{o}C.$, these same samples all showed evidence of growth. This was attributed to psychrophiles. By using a duplicate un-opened tube, possible contamination was stopped.

Tables 1 and 2 show no general pattern of growth during the 10 day storage in either SPC or psychrophilic counts. They also show rather surprisingly high counts in both raw and pasteurized milk. These may be due to the warmer months in which this work was done. Large numbers of psychrophilic bacteria are to be noted in the raw milk from Table 2 in company with similar high counts by SPC for the same milk in Table 1.

Plant No. 3 shows increases. The chief cause was a refrigeration failure in the cold storage room where plates and samples were stored. A blown fuse stopped the compressors and allowed samples and plates to reach 15°C. (60°F.) before discovery and correction. These results show the importance of continuous low storage temperatures to prevent growth of bacteria able to grow at temperatures just above those that allow psychrophiles to grow. Thus even brief removal of milk containers from cold storage to a warm environment has its effect and allows growth.

In none of the three tank lots processed at Plant No. 3 did the psychrophilic count reach levels likely to cause objectionable flavor as indicated by Boyd et al (9).

When milk from Plant No. 3 was later brought

to the University Food Science Department for process-
ing at 72.2°C. (162°F.), the legal minimum, there were
no significant increases. Instead, the mean counts
declined.

Discussing similar work, Olsen (65) points
out that processing plants with good records have less
psychrophile trouble. Plant No. 2 is an example.
Here, well organized sanitizing, operating, and clean
up applied by trained workers is checked by in-plant
and external laboratories. Their low raw counts re-
sult from continuous conscientious effort by field
staff on the farm coupled with bonuses for low raw
milk counts.

How much influence has the raw SPC on sub-
sequent keeping quality? It has been suggested that
high count raw milk is more suited to psychrophilic
growth after pasteurization. Overcast and Adams (67)
investigated this theory and found no stimulation
for Ps. fluorescens. They did find stimulation for a
Brevibacterium lipolyticum and one other psychrophile
isolate. An inhibitory effect on Ps. fragi was shown
after 1, 2, and 4 days. Their conclusion, the same
as Erdman and Thornton's (26), (27), was that psychro-
philes in milk are a sanitation problem.

The results for experiment 2 are shown in Table 3. The immediate post pasteurization counts are all zero. The counts for the majority made 10 days after pasteurization are below the limit of 30 per plate using a 1:100 dilution. The first trial used Ps. fluorescens strain 3756 and the four tubes had mean counts of 2, 1, 1, and 2 respectively. When a trial using this organism was repeated, the after storage counts were uniformly zero.

The results using Ps. fluorescens strain 10038 show survivors after storage. This shows that some organisms were able to survive the heat treatment. Macaulay (56) consistently found survivors immediately after pasteurization with all three organisms. Mac- aulay's pre-pasteurization counts ranged from approx- imately 200,000 to more than 1,000,000. This is the chief difference between the results of the two labor- atory investigations. This explains why Macaulay's counts after 10 days storage were so much higher than those in this study. The recording chart for the first trial of 10038 shows an exposure of 16 sec. at 73°C. or above, with maximum temperature reached 73.5°C., and a bath temperature of 74°C. During the second trial of 10038, the maximum temperature was 1°C. lower. No effect is evident from this.

Comparison of HTST time - temperature treatment in Exp. 2 with a commercial HTST shows that the glass tube method took about 26 seconds to reach $73^\circ C$. ($162^\circ F$.). Franklin (32) indicates 40 seconds for this in a HTST unit. Cooling was abrupt from $73^\circ C$. as shown in Figure 1. Thus this method gave considerably less heat treatment than normal HTST units at temperatures below that of the $73^\circ C$. - 16 second treatment.

The sealed glass tube method's chief advantage was its absolute guarantee of freedom from contamination during the 10 day incubation at $4 - 5^\circ C$. A further advantage was the exact record of heat treatment from the thermocouple tube. The semi-micro nature of the tubes gave rapid temperature change with simple and inexpensive apparatus.

Disadvantages encountered with the method were: awkward removal of milk from the tubes to dilution blanks, the anaerobic conditions in the sealed tube, and the problem of giving identical exposures to separate lots. The first could be remedied by having longer Pasteur pipettes. Mechanical devices might overcome the third since this depends, in part, on the eye-hand co-ordination of the operator in the transfer from heating to cooling bath. That the

sealed tube is much different from conditions at the
bottom of a full milk bottle or carton is doubtful.
Sinclair and Stokes (86) found higher yields or org-
anisms from liquid media at lower temperatures due to
the greater amount of oxygen dissolved. Most modern
HTST units have vacuum sections for flavor removal
that also remove air and oxygen. Because of autoclav-
ing and mixing by inverting rather than shaking, little
air was likely to be incorporated in the milk prior
to pasteurizing in the glass tubes. Thus, there is
little to indicate any difference between the oxygen
tension in the sealed glass tubes, the screw cap
tubes used in Exp. 1, and the ordinary milk container.

Upadhyay and Stokes (97) state that as in-
cubation temperature is lowered, lag phase of growth
as well as exponential, stationary, and death phases
are lengthened. If this is the reason for no growth
in either experiment during the 10 day period at 4 -
5°C., then the keeping quality problem is one of
maintaining uniformly low temperatures. This suggests
that taking samples in the same way as Experiments 1
and 2, incubating at 15 - 20°C. for 2 - 3 days would
speed up the lag phase. Incubating psychrophilic
plates at 4 - 5°C. for 15 - 20 days would follow.
This was not done in this study because Macaulay's

results indicated survivors without it. If, when starting with natural populations, survivors from such a procedure are found, then, either these are facultative psychrophiles, or the higher incubation temperature has allowed recovery of cold temperature growth ability.

Possibly the heat shock of the HTST treatment in Experiment 2 caused damage that only a few isolated cells were able to overcome. Perhaps the damage was such that the medium used in plating did not meet the more demanding nutritional needs of the heat shocked bacteria. Of course, it could also be that the milk itself no longer met these increased nutritional needs and the bacteria died. If so, then they didn't survive pasteurization.

A possible survival hypothesis is that a few bacteria repair their heat damaged enzyme systems and other disorganization in the cell. As with all chemical and cellular processes, low temperatures would slow this repair. Higher temperatures within their growth range might allow a recovery of low temperature growth ability through such repair being done faster.

From the results of these experiments, Macaulay's contention that psychrophiles are

thermoduric is entirely possible. However, its sig-
nificance relative to practical HTST pasteurization
is open to question because the occurrence of large
enough numbers of any one particular thermoduric
organism in raw milk is unlikely. Thus, isolated
cases of psychrophiles surviving commercial HTST
pasteurization are possible, but the more likely cause
of psychrophiles in pasteurized milk is post-
pasteurization contamination.

CONCLUSIONS

Conclusions

In Experiment 1, natural populations of psychrophilic bacteria were found in commercial raw milk using psychrophilic plate counts. In milk taken from the ends of HTST holding tubes, these same bacteria failed to be detected in any significant amount, even after 10 days incubation in milk at 4 - 5°C. as indicated by ability to grow on solid media at this same temperature.

In Experiment 2, pasteurization of selected strains of psychrophilic Ps. fluorescens in high concentrations by an HTST treatment in 3 mm. bore glass tubes failed to show any pattern of survival generally but with a specific organism a low survival rate was observed on a solid medium at 4 - 5°C.

From these two experiments, it is concluded that psychrophilic growth in milk after pasteurization is not likely to be caused by survivors of pasteurization but by post-pasteurization contamination.

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