METHODS FOR ESTIMATING THE PSYCHROPHILIC POPULATIONS AND KEEPING QUALITY OF MILK

By
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ABSTRACT OF THESIS

Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and
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DOCTOR OF PHILOSOPHY

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Approved ..D.H.Wallmann

PART I

Psychrophilic organisms are best defined, from the practical aspect, as those organisms which are capable of relatively rapid growth at refrigeration temperatures (4.5 C). The most commonly encountered genera are Pseudomonas, Achromobacter, Flavobacterium, and Alcaligenes. Members of the coliform group are encountered to a lesser degree.

These organisms are responsible for deterioration of refrigerated dairy products and since they are destroyed by both H.T.S.T. and L.T.L.T. pasteurization, their presence in freshly pasteurized milk is indicative of post-pasteurization contamination.

Many investigators have reported that the initial numbers of psychrophilic organisms in freshly pasteurized milk cannot be used as a "yardstick" to predict keeping quality of milk stored at refrigeration temperatures. That this is apparently true, is evidenced by the observations that certain species of organisms cause deteriorative changes in milk in a relatively short time while others ("inert" psychrophiles) reach extremely high population levels over long periods of time without causing noticeable flavor defects.

To date, the most satisfactory method for the enumeration of psychrophilic organisms is a plate count on tryptone-glucose-extract agar incubated at 4.5 C for 7 days. This procedure is

obviously too long for the routine enumeration of psychrophilic organisms. Again, at the present time a rapid test for the prediction of keeping quality of milk at refrigerated temperatures is lacking.

It was the aim of this thesis to: (1) develop a medium for the rapid enumeration of psychrophilic organisms and (2) develop a method that would find application in the routine prediction of keeping quality of milk stored at refrigeration temperatures.

A screening procedure using a solid medium was adopted to determine the most suitable peptone for the growth of representatives of the four psychrophilic genera (Pseudomonas fluorescens, Flavobacterium rhenanus, Alcaligenes viscosus and Achromobacter superficiale). Growth determinations in liquid media were carried out to determine the effects of varying concentrations of the peptone, nutritional additives, and hydrogen ion concentration.

Since 20 C is the approximate optimum temperature for growth of these organisms, it was selected as the incubation temperature. However, this temperature allows the growth of various mesophilic organisms, which are part of the normal flora of milk. Consequently, it was necessary to find an inhibitor which would prevent the growth of these mesophilic types, without interfering with the growth of psychrophilic organisms.

Various dyes and surface active agents were tested and Nacconol N.R.S.F. (an anionic wetting agent) in a concentration of 1:1000 was selected.

The Phytone-Nacconol medium was formulated as follows:

Phytone	20.0 gm
yeast extract	5.0 gm
KH_2PO_4	0.1 gm
K_2HPO_4	5.0 gm
Nacconol N.R.S.F.	1.0 gm
agar	15.0 gm
distilled water	1,000 ml
pH	7.5

(incubate plates at 20 C for 48 hours)

This medium was tested against the procedure in Standard Methods for the Examination of Dairy Products, (10th Edition). A statistical analysis demonstrated that there was virtually little difference between the two techniques.

PART II

A keeping quality test is also presented whereby a 10 ml sample of pasteurized milk is aseptically pipetted in a tube containing 1 ml of sterile Nacconol-tetrazolium test solution. The mixture is then incubated at 20 C for 12, 24, 36 and 48 hours.

The appearance of a positive tube (rose red color) after 12 hours indicates milk will retain its acceptability under refrigeration for approximately 4.2 days; a positive tube after 24 hours indicates acceptability for 8.8 days; a positive tube after 36 hours or after 48 hours is indicative that the milk in question will remain acceptable for 12.6 and 15.6 days respectively.

The formula for Nacconol-tetrazolium test is as follows:

2, 3, 5 triphenyl tetrazolium chloride	0.1 gm
Nacconol N.R.S.F.	1.0 gm
K_2HPO_4	5.0 gm
KH_2PO_4	0.1 gm
distilled water to make	100 ml

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To My Wife

VITA

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PART I

A MEDIUM FOR THE RAPID ENUMERATION OF PSYCHROPHILIC ORGANISMS IN MILK

INTRODUCTION

To the early settlers, the family cow served as the milk supply. Industrial development, and increasing population necessitated the establishment of commercial milk supplies for the city dweller. From the original one or two cow operation, there emerged the larger specialized dairy operation. The dairy farmer was now producer, processor and distributor. Distribution at this time over established milk routes consisted of dispensing from earthen ware or copper transportation crocks via a pitcher to the consumer. This method presented a serious sanitation problem since it proved to be an excellent means of disease transmission.

Through the joint cooperation of sanitarians, milk producers, processors and equipment manufacturers a system was developed whereby milk could be handled safely from farm to consumer. In recent years emphasis has been directed toward a more efficient processing distribution and quality control.

Recent technological advances in processing and distribution of milk products have materially reduced costs. Paralleling this more efficient utilization of man power, there has been a lengthening in holding time of milk products prior to consumption. The advent of every other day deliveries at the consumer level, holding afternoon deliveries overnight for pasteurization the following day, the six day dairy operation, necessitating holding milk an additional day, and bulk handling of milk from the producer to the dairy have all contributed in the lengthening of time that milk follows

from "cow to consumer".

Add these factors to the recent trends in the market milk industry of distribution over a wide area from a centralized processing plant and extensive interstate traffic in fresh pasteurized milk and the problem of keeping quality or storage life becomes of prime importance.

Refrigeration has been used to prolong the shelf life of milk and other dairy products. In effect this serves to retard bacterial growth. However, certain microorganisms which may be present in dairy products as post-pasteurization contaminants are able to multiply with relative ease at refrigeration temperatures.

In recent years, attention has been focused on these psychrophilic microorganisms since they are responsible for deteriorative changes in milk and milk products stored at low temperatures. It is generally agreed that these organisms are eliminated by both H.T.S.T. and L.T.L.T. pasteurization and that their presence in pasteurized milk is a more critical index of post-pasteurization contamination than the presence of coliform organisms.

To date the selective culture of these organisms in freshly pasteurized milk requires an incubation temperature of 4.5 C for 7 days. This relatively long incubation temperature is impractical for the routine detection of these organisms as an indication of post-pasteurization contamination.

SCOPE OF INVESTIGATION

This study was undertaken in order to: (1) develop a plating medium for the enumeration of psychrophilic organisms in a shorter period of time than that currently used. A medium which yields visible colonies rapidly, could effectively be used to detect post-pasteurization contamination.

(2) Develop a method based on the growth of psychrophilic organisms to predict the keeping quality of pasteurized milk at refrigeration temperatures.

LITERATURE REVIEW

Definition of psychrophilic microorganisms

The general group of microorganisms which have received attention as the causative agents of food and dairy product spoilage at refrigeration temperatures are most commonly referred to as psychrophiles. Psychrophile or "cold loving" has the connotation, as Zobell (1946) points out, that the optimum conditions for growth are at "cold" temperatures. This does not hold true for most of the organisms which are generally considered as psychrophiles by the food and dairy industry. Temperatures from 5 C to 10 C serve as optimum for only a few of these. The majority, however, although capable of growth at refrigeration temperatures, appear to favor temperatures in the mesophilic range. Hence the term psychrophile, suggesting that optimum conditions for growth are at "cold" temperatures, may be a misnomer.

A controversy exists concerning optimum conditions for these microorganisms. As Dorn and Rahn (1939) reported, there exist four optimum temperatures for any organism on any medium; two are for metabolic activity and two are for growth. In each case, one optimum is for the rate of growth and the other for the amount of metabolic products or total crop yield formed on endpoint. If total crop is used as a criterion of growth, these bacteria are, as Greene and Jezeski (1954) found, truly psychrophilic. Or if minimum generation time is taken as a standard, these organisms

appear to favor temperatures within the range of mesophilic bacteria.

To further complicate the question of definition is the disagreement on the most favorable temperature for "obligate" as compared to "facultative" psychrophiles.

These arguments are, of course, of academic interest, but from the standpoint of practicality, a psychrophilic organism is one which is capable of relatively rapid growth at refrigeration temperatures.

Psychrophilic microorganisms found in dairy products

The psychrophilic group of organisms is comprised largely of gram negative non-spore-forming rods. Most frequently encountered are members of the genera Pseudomonas, Alkaligenes, Flavobacterium, and Achromobacter. Certain members of the coliform group are encountered to a lesser degree.

Van der Zant and Moore (1955) studied the growth characteristics and proteolytic activities of two psychrophilic cultures, Pseudomonas fluorescens and Pseudomonas putrifaciens. They recommended a plate incubation for three days at 25 C for the enumeration of bacteria growing in refrigerated milk and related products.

Lawton and Nelson (1954) isolated 50 psychrophilic organisms from commercially pasteurized milk using an incubation temperature of 3 C for seven days. Eight organisms were selected as being typical of the entire group. Seven of these were members of the genus Pseudomonas and one belonged to the genus Flavobacterium.

Studies on generation time at varying temperatures indicated that the temperature for optimum growth appeared to be about 21 to 32 C. Flavobacterium aquatile, however, had its optimum growth temperature at 10 C and was thus considered to be an obligate psychrophile.

Elliker (1954) and Lliker, Smith and Parker (1951) isolated organisms causing defects of cottage cheese at low temperatures. One was identified as Pseudomonas viscosa and caused a brown slime film on cottage cheese. Accompanying this visual defect was a "putrid" flavor and "rotten" odor. Another organism was identified as Pseudomonas fragi and caused a white gelatinous film, "fruity" odor and bitter taste. A third, Alcaligenes metalcaligenes, caused a white gelatinous film on cottage cheese but little change in odor or flavor. The source of these organisms was from soil and water.

Davis and Babel (1954) isolated organisms from dairy water supplies which were capable of producing a slimy defect on cottage cheese stored at low temperatures. In addition to the organisms cited before, they found members of the genera Achromobacter, Aerobacter and Proteus. Pasteurization temperatures destroyed these organisms within three minutes.

Greene and Jezeski (1951) and (1954) isolated organisms from deteriorated cottage cheese and creamery water supplies. They found the predominant causative organisms to be members of the genus Pseudomonas and Aerobacter aerogenes. Studies on the growth rates of these organisms at varying temperatures indicated that the lag phase at 0 C was 24 to 48 hours. As the temperature of incubation increased, the lag phase decreased while the growth rate was

accelerated.

Thomas and Chandra Sekar (1946) indicted members of the genera Achromobacter, Alcaligenes, Flavobacterium, Pseudomonas and various micrococci for spoilage of raw and pasteurized milk at refrigeration temperatures.

An investigation of "surface taint" of butter by Jezeski and Macy (1946) demonstrated that Pseudomonas was responsible for this defect in a majority of the cases. Alcaligenes and Flavobacterium, however, were found to be the causative agents in the remaining cases of "surface taint".

Sherman Cameron and White (1941) noted that the bacteria responsible for the spoilage of milk held just above the freezing point were gram negative, non spore-forming rods; primarily of the Pseudomonas group.

The low temperature spoilage of various cream samples were attributed to a Pseudomonas species by Anderson (1937). Long and Hammer (1941) described the isolation of Pseudomonas putrifaciens (formerly designated as Achromobacter putrifaciens) as the cause of a cheesy or putrid condition of salted butter. This organism was also isolated from raw milk, cream, soil and water.

Stark and Shieb (1936) studied the lipolytic and caseolytic bacteria isolated from butter. They reported that a relatively large percent were identified as Pseudomonas aeruginosa, Alcaligenes bookeri and Alcaligenes faecalis. Of these, only Alcaligenes bookeri failed to grow at 5 C, but did show evidence of growth at 10 C. A smaller percentage were identified as

Achromobacter aromafaciens and Achromobacter lipolyticum. Both grew at 5 C.

Anderson and Hardenbergh (1932) described an organism resembling Achromobacter as being responsible for lipolytic spoilage of cream. It imparted a characteristic bitter flavor to the cream. The taste was a sensation similar to that of a sore throat. In later work by Anderson (1937), this gram negative, non-spore-forming rod was named Bacterium lipidis; but was later renamed Achromobacter lipidis N. sp. by Allison, Anderson and Cole (1938).

Rice (1936) isolated Alcaligenes viscosus as the causative organism of ropy cream. More recently Gainor and Wegmer (1954) isolated a psychrophilic bacterium resembling Alcaligenes viscosus from a ropy sample of pasteurized milk. This organism produced ropiness in test milk samples incubated at 5 C and 25 C but not at 36 C.

Boyd (1953) described Aerobacter aerogenes as one of the most common causes of flavor deterioration in milk stored at 4.5 C. This same organism was cited by Claydon (1943) as causing a medicinal flavor in market milk. This flavor became very intense after six days of refrigerated storage.

Stark and Shieb (1936) identified 11 of 188 gram negative caseolytic rods from butter as members of the Escherichia-Aerobacter group. The growth of these organisms at 5 C varied from none to moderate.

Hammer and Yale (1932) isolated 25 organisms from 17 samples of off-flavored butter. These were identified as members of the

Escherichia-Aerobacter group.

Olsen, Willoughby, Thomas and Morris (1953) and Nelson and Baker (1954) showed that coliform bacteria vary from sample to sample in their ability to grow at refrigeration temperatures. Some milk samples demonstrated a marked increase in coliform count during storage at 5 C while others gave little if any increase.

Primarily the gram negative non-spore-forming rods have appeared to be responsible in most cases of psychrophilic spoilage of dairy products. However, there have been occasional reports of organisms other than these that grow at refrigeration temperatures.

Sherman and Stark (1931) reported that Streptococcus glycerinaceus and Strep. liquefaciens isolated from milk and Strep. faecalis from swiss cheese were capable of growth at 10 C. Strep. glycerinaceus grew at 5 C while Strep. lactis did not. They also reported that Lactobacillus, although capable of growth at 10 C did not grow at 5 C.

Rogick and Burgwald (1950) stated that the psychrophilic flora in market milk was comprised of gram negative non-spore-forming rods and cocci. On the other hand, Thomas et al (1949) studying milk bacteria which grow at refrigeration temperatures found the presence of micrococci to be comparatively rare.

Thomas and Chandra Sekhar (1946) noted the occasional occurrance of various micrococci in a study of psychrophilic bacteria in raw and pasteurized milk.

Again, the most frequently encountered psychrophilic organisms belonged to the genera Pseudomonas, Achromobacter, Flavobacterium

and Alcaligenes. The coliform group appeared in somewhat fewer cases as the cause of dairy product spoilage at refrigeration temperatures.

The primary sources of these organisms are from deteriorated dairy products, raw milk, soil and water.

Shutt (1929), Provan (1941), Corley and Hammer (1942) and Wagenaar (1952) indicated that routine water analysis fails to detect psychrophilic organisms. These organisms are frequently found in coliform free water supplies. While coliform determinations on water supplies may prove the water to be safe from a public health standpoint, no information relative to the psychrophilic population of that water would result. Furthermore a standard plate count on water supplies may lead to erroneous conclusions as many psychrophiles do not grow at 35 C.

Erdman and Thornton (1951) reported that not one of 722 psychrophiles isolated grew at 35 C. Anderson and Hardenbergh (1932) showed that the Achromobacter and Alcaligenes groups did not grow at 35 C. Hiscox (1936) found 37 C incubation prevented the growth of certain Pseudomonas groups, while Greene and Jezeski (1954) observed that even 30 C was unfavorable for the growth of some Pseudomonas species. Jezeski and Macy (1946) stated that plate counts on creamery water supplies were higher at 20 C incubation than at 37 C.

Thus, the "total plate count", in many instances does not give a complete picture of the total viable organisms present.

Plate counts incubated at 22 C to supplement routine water

analysis was suggested by Provan (1941). Doetch and Scott (1951) indicated that the determination of proteolytic and lipolytic organisms should be performed in parallel with routine water examinations.

Elliker et al (1951), Doetch and Scott (1951), Olsen et al (1953) and Olsen, Parker and Mueller (1955) reported that proper sanitation and chlorination of suspect water supplies (2-3 ppm) were effective in minimizing the access of psychrophilic organisms in dairy products.

The influence of these organisms on the flavor of commercially pasteurized milk can generally be detected in 4 to 5 days. It is not uncommon, however, to find milk that is relatively free from flavor defects in 15 to 20 days storage.

Flavor deterioration by these organisms is caused by the utilization of the various milk constituents and the accumulation of metabolic by-products in the fluid milk as growth proceeds. The most commonly found flavors are putrid, rancid, cheesy, fermented, fruity and sour.

In many cases, there is a change in the physical state of the milk as a result of the growth of psychrophilic organisms. Usually this occurs after flavor spoilage becomes apparent. Occasionally a thickening of the milk occurs, a condition often associated with ropy milk. Curdling and proteolytic changes may also be detected. It is not uncommon to find green or yellow discoloration at the surface of the milk resulting from psychrophilic spoilage.

These or similar defects caused by psychrophilic organisms are also found in many other dairy products. Flavor defects in butter, cheeses, and other concentrated milk products are frequently caused by members of this group.

Effect of pasteurization

There has been some differences of opinion concerning the effect of pasteurization on psychrophiles. However, a review of the more recent literature reveals conclusively that psychrophilic organisms in milk are destroyed by proper pasteurization. The relatively few organisms, which might remain after pasteurization, would probably not be a factor in flavor deterioration over an extended period of refrigerated storage.

Roadhouse and Henderson (1941) state that certain psychrophilic organisms could withstand pasteurization at 143 F for 30 minutes. Kenedy and Weiser (1950), using laboratory pasteurization methods on pure cultures of psychrophilic organisms, reported that 8 of 15 showed a 90 per cent reduction in numbers occurred during pasteurization. Five exhibited a 50-90 per cent reduction in numbers, while 2 organisms exhibited definite thermoduric characteristics.

However, Erdman and Thornton (1951) observed that only 4 of 722 psychrophilic cultures survived laboratory pasteurization.

Jezeski and Macy (1946) noted that only 6 out of 41 cultures of psychrophilic bacteria could be recovered after pasteurization at 145 F for 30 minutes. Abel el Malek and Gibson (1952) isolated a heat tolerant psychrophile in 17 out of 28 samples of laboratory

pasteurized milk. Alcaligenes tolerans N. sp. was the name given to this organism which withstood 63 C for 30 minutes.

Kaufmann and Andrews (1954), using 2 species of Pseudomonas conducted thermal death time studies and concluded that the organisms were killed by L.T.L.T. and H.T.S.T. pasteurization. The margin of safety was considerably less with the latter when skim milk was used as the heating medium.

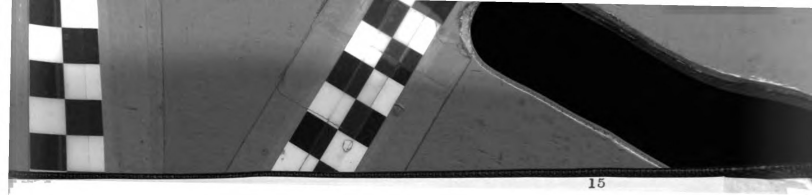
Sherman Cameron and White (1941) reported that good quality raw milk would keep for four weeks before spoilage was obvious; while good quality pasteurized milk would keep as long as eight weeks. Recontamination of the pasteurized milk with minimal quantities of raw milk significantly reduced the keeping quality. Presumably, this decrease in keeping quality was due to the presence of psychrophilic organisms in the raw milk. Having gained access to the pasteurized product, these organisms proliferated during refrigerated storage and thereby materially reduced its shelf life. They concluded that pasteurization effectively eliminated psychrophilic organisms.

Watrous, Doan and Josephson (1952) supplemented laboratory pasteurization studies with commercial L.T.S.T. pasteurization investigations. They reported that psychrophile counts of 75 samples immediately following laboratory pasteurization, and counts after 10 and 20 days of storage at 5 C, were less than one per milliliter. Psychrophilic bacteria were not detected in any sample from the holding vat when plated immediately or even after 5 C storage for 15 days.

Similar results were reported by Rogick and Burgwald (1952). A comparison of the effects of commercial L.T.L.T. and H.T.S.T. pasteurization on the elimination of psychrophiles showed that both methods resulted in the destruction of these organisms. Initially psychrophilic organisms could not be detected in sample quantities as high as 4.1 ml. After seven days storage at 4 to 7 C, these samples did contain psychrophilic organisms.

Olsen, Willoughby, Thomas and Morris (1953) reported that samples removed aseptically from H.T.S.T. pasteurizer did not contain any psychrophilic bacteria. The psychrophile counts did not reach a level of greater than 3 per ml. in seven days of storage at 4.5 C. Bottles of the finished product, although having relatively low psychrophilic counts initially, demonstrated a marked increase in numbers in five to seven days. This suggested the possibility that other heat tolerant mesophilic organism might become adapted to, and multiply at, refrigeration temperatures; or that all the psychrophilic bacteria were not eliminated by pasteurization.

Watrous, Doan and Josephson (1952) reported that there was little evidence to suggest that thermoduric organisms were readily adapted to psychrophilic conditions. These organisms did not show any evidence of growth in milk stored at 5 C. Studies on refrigerated storage of milk and cream by these investigators led them to the conclusion that organisms surviving pasteurization would not grow at refrigeration temperatures; and that the presence of psychrophilic organisms in pasteurized milk was the



result of post-pasteurization contamination.

Coliform vs. psychrophiles as indicators of post-pasteurization contamination.

During the course of the early work on the coliform group, many investigators, Gage and Stoughton (1906), Ayers and Johnson (1951), Minkin and Gurgwald (1935) and Long, Hedrick and Hammer (1944) concluded that certain strains of the coliform group exhibited varying degrees of heat tolerance. Many were not destroyed at pasteurization temperatures.

More recent investigations by Olsen, Macy and Halvorson (1952) demonstrated that the incubation temperature at which the test organism was grown, markedly influenced its thermal death time at various temperatures. They reported that Escherichia coli, after growth at 20 C, was destroyed by both H.T.S.T. and L.T.L.T. pasteurization. But after growth at 30 C to 37 C, its heat tolerance was increased to such a degree that the probability of its complete destruction, particularly by H.T.S.T. pasteurization, would be doubtful.

These findings, along with those of Craige (1946), were instrumental in explaining the variation in survival studies of many of the earlier reports.

Stark and Patterson (1936), Chilson, Yale, and Eglington (1936), Delay (1947), Frayer (1955) and others confirmed the earlier work that the test for coliform organisms is of merit as an aid for the detection of post-pasteurization contamination.

Buchbinder and Alff (1947) reported that there could not be

any practical significance attached to the so-called resistant coliforms in the routine performance of the coliform test of pasteurized milk. Dahlberg, Adams and Held (1953) reported the absence of thermoduric coliform bacteria in raw milk.

In 1937, Yale stated, with reference to the coliform test of pasteurized milk:

> "Bacteria forming red colonies upon these agar media, (violet-red-bile-agar or desoxycholate agar) regardless of whether they ferment lactose with gas formation, are significant if destroyed by proper pasteurization, since their presence in freshly pasteurized milk then indicates recontamination."

If this statement were modified in lieu of current knowledge to read, - any organism which is killed by proper pasteurization is significant, for their presence in freshly pasteurized milk would indicate contamination after pasteurization - then there is just cause to consider the presence of psychrophilic bacteria in freshly pasteurized milk as an indicator of post-pasteurization contamination.

This group of organisms appears to be a much more logical indicator of post-pasteurization contamination, since it is possible to have a product contaminated after pasteurization by psychrophiles without coliform organisms. Under certain conditions, dairy water supplies, acceptable from a public health standpoint, may harbor psychrophilic organisms which could conceivably gain access to pasteurized products. This was demonstrated by Shutt (1929), Provan (1941), Coreley and Hammer (1942) and

Wagenaar (1952). Here there would not be any evidence of post-pasteurization contamination using the presence of the coliform group as an indicator. Whereas, this contamination might readily be detected if the presence of psychrophiles were observed.

In the current literature, Nelson and Baker (1954) stated that the coliform test should be retained as a quick index of contamination but negative results should be interpreted conservatively, since the possibility exists that this test will not detect some important types of contamination.

Watrous, Doan and Josephson (1952) reported that:

> "It seems obvious that determination of psychrophilic bacteria is a more critical index of post pasteurization contamination of milk than the coliform determination."

Many current reports have hinted at the possibility of using psychrophiles as an indicator of post-pasteurization contamination. These opinions have been conservative owing to the length of time required to enumerate psychrophilic organisms at present. Again, there is the general reluctance to discard a test as well established as the coliform test for post-pasteurization contamination.

Methods for estimating psychrophilic organisms

Recently, Atherton et al (1954) described some attempts at possible methods to ascertain psychrophilic deterioration prior to the development of off-flavors.

The resazurin reduction time of refrigerated bottled milk proved to be so long at both 20 C and 37 C incubation, that it gave

only slight information concerning psychrophilic activity. A test for the detection of phosphatase produced during the growth of these organisms proved to be of little value. As acid production is not a normal characteristic of psychrophiles in milk at low temperatures, changes in pH were too slight to be of practical significance.

The Storrs test, a measure of protein stability, appeared to be the most promising as a rapid method for the detection of psychrophilic activity. A 10 point drop in stability value below the normal for fresh milk was usually followed by off-flavor development. This test, however, would be of limited application inasmuch as this drop in stability occurred too close to the time of spoilage.

The most frequently used method for the determination of psychrophilic populations in milk has been the agar plate method.

Pennington (1908) incubated agar plates prepared from raw milk for 4 to 6 weeks at 0 C. Thomas and Chandra Sekhar (1946) reported that higher counts were obtained at the end of 21 days than at 7 to 14 days incubation at 3 C to 5 C. Kenedy and Weiser (1950) incubated plates at 10 C and suggested that psychrophiles appeared within 3 days, while adaptive mesophiles required 3 to 5 days before colonies were visible. Burgwald and Josephson (1947) and Dahlberg et al (1953) employed a 10 day incubation period at 8 to 10 C. Watrous et al (1952) used a 10 day incubation time at 5 C. Boyd et al (1954) recommended that psychrophiles be enumerated by incubation of plates at 5 C for at

least 10 days but preferably 20 days. Standard Methods for the Analysis of Dairy Products, (APHA 1953) states that psychrophiles be enumerated by incubation of plates at 5 C for 7 days. Nelson and Baker (1954) suggested an incubation temperature of 25 C for 3 days or 21 C for 4 days on the basis that these times and temperatures detected samples giving high counts on plates incubated 10 days at 5 C in all cases.

This last recommendation should be tempered with a word of caution. Although this method is the most rapid to date, it is subject to some degree of interpretation. Psychrophilic counts (incubation of plates at 4.5 C for 7 days) on freshly pasteurized milk are generally very low. Higher counts are obtained by incubation at 20 C and 25 C since these temperatures would permit the growth of mesophilic types as well as psychrophiles. During the course of refrigerated storage, the 4.5 C counts increase. This increase in psychrophilic organisms is reflected by the increase in counts at 21 C and 25 C but only partially reflected in the 35 C because only a few psychrophilic types are capable of growth at this temperature. Usually after 5 to 6 days storage, the psychrophilic population greatly overshadows the non-psychrophilic population, hence the close agreement among the 4.5 C, 21 C and 25 C counts.

A. SELECTION OF A PEPTONE

EXPERIMENTAL

The problem of developing a selective plating medium must be considered along four lines of attack. Primarily, the medium should permit the growth of a given organism so as to obtain readily a visible colony in the shortest time possible. Secondly, it should allow the maximum recovery of viable organisms. Third, it should allow these organisms to exhibit a very short lag phase. Finally the inhibitory agent used must be capable of eliminating, as nearly as possible, all organisms exclusive of the desired group without exhibiting adverse effects upon the desired group.

Representatives of four psychrophilic genera commonly isolated from milk namely, Achromobacter superficiale, Alcaligenes viscosus, Pseudomonas flourescens and Flavobacterium rhenanus were used as test organisms.

Colony size determination, total counts, and inhibition studies were carried out on solid media while the relative effects of various nutritional additives were determined in liquid media.

There is a large number of peptones on the market that vary in their nutritive values. They have not been tested in media for growing psychrophiles. In an attempt to determine the most suitable peptone for the growth of psychrophilic organisms, a screening procedure was undertaken. A peptone, incorporated into a solid medium, which allowed the given organisms to exhibit maximum colony size in the shortest time and also permit the

maximum percentage recovery of the viable organisms would be the most desirable. The following peptones were used in this study: peptonized milk, trypticase, lactalysate, proteose peptone, milk protein hydrolysate, polypeptone, myosate, tryptose, thiotone, and phytone. To eliminate variables, the various media were made with two per cent of each peptone serving as a sole source of nutrition. Each medium was buffered with monobasic and dibasic potasium salts to a pH of 6.8 so that the total amount of both salts approximated 0.5 per cent.

A 24 hour culture was transferred for each day for 4 days in brain heart infusion broth and diluted to approximately 50 to 100 organisms per ml. This dilution was then plated out in triplicate on the various media containing the peptones described above. The plates were incubated at 20 C and counted at 24, 36 and 48 hours.

At each counting, the diameter of a representative number of colonies was measured. Since great variation occurred in the diameter of subsurface colonies owing to such factors as 0_2 tension and thickness of the medium, only the diameters of surface colonies were measured.

Percentage recovery of viable organisms on each peptone was determined by comparison to a 48 hour plate count on tryptone-glucose-extract agar incubated at 20 C.

This procedure was carried out five times on each peptone medium and with each of the four representative organisms. The results of these five trials were then averaged as shown in Tables 1 to 4 inclusive.

TABLE 1

Average colony diameter and percentage recovery of Alcaligenes viscosus at 24, 36, and 48 hours at 20 C using various peptones incorporated into a solid medium

Peptone	Average colony diameter in mm at			Percentage recovery based on 48 hr plate count of TGE agar at 20 C		
	24 hrs	36 hrs	48 hrs	24 hrs	36 hrs	48 hrs
Trypticase	0	0	0.1	0	0	21
Peptonized Milk	0	<0.1	<0.1	0	3	3
Proteose Peptone	0	0.4	0.9	0	41	86
Milk Protein Hydrolysate	0	0.6	0.9	0	7	38
Lactalysate	0	0.3	0.9	0	14	79
Polypeptone	0	0.7	1.1	0	41	69
Tryptose	0.4	0.9	1.6	8	79	103
Thiotone	0.4	1.0	1.4	7	28	69
Myosate	0	0.6	1.4	0	86	121
Phytone	0.4	1.1	1.9	14	90	121

TABLE 2

Average colony diameter and percentage recovery of Flavobacterium rhenanus at 24, 36, and 48 hours at 20 C using various peptones incorporated into a solid medium

Peptone	Average colony diameter in mm at			Percentage recovery based on 48 hr plate count of TGE agar at 20 C		
	24 hrs	36 hrs	48 hrs	24 hrs	36 hrs	48 hrs
Trypticase	<0.1	0.1	0.1	0	2	11
Peptonized Milk	0	0.4	0.9	0	56	105
Proteose Peptone	0	0.5	1.0	4	65	95
Milk Protein Hydrolysate	<0.1	0.4	1.3	3	74	104
Lactalysate	0.1	1.3	2.1	42	105	119
Polypeptone	0.1	1.6	2.2	16	86	95
Tryptose	0.1	1.8	2.2	10	79	93
Thiotone	<0.1	1.3	2.3	21	88	91
Myosate	0.5	1.6	2.3	46	90	96
Phytone	1.1	2.9	3.4	86	111	112

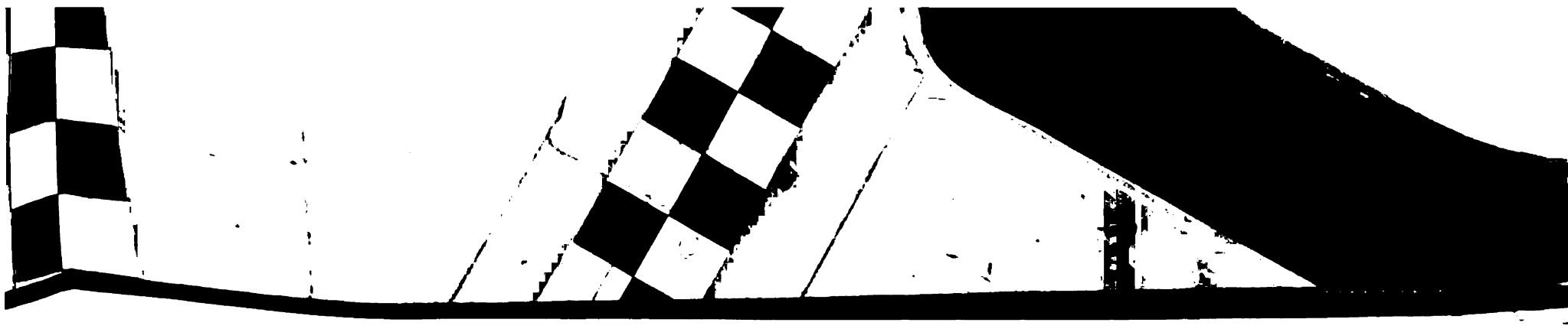


TABLE 3

Average colony diameter and percentage recovery of Achromobacter superficiale at 24, 36, and 48 hours at 20 C using various peptones incorporated into a solid medium

Peptone	Average colony diameter in mm at			Percentage recovery based on 48 hr plate count of TGE agar at 20 C		
	24 hrs	36 hrs	48 hrs	24 hrs	36 hrs	48 hrs
Trypticase	0	0.6	1.4	0	10	81
Peptonized Milk	0	0	1.5	0	0	133
Proteose Peptone	0	0.4	1.1	0	12	90
Milk Protein Hydrolysate	0.3	0.6	0.9	5	5	38
Lactalysate	0	0.8	1.6	0	105	112
Polypeptone	0.3	0.5	1.2	5	57	95
Tryptose	0.3	0.6	1.5	10	81	114
Thiotone	0	0.5	1.1	0	43	62
Myosate	0.5	0.9	1.6	10	48	86
Phytone	0.5	0.9	2.0	10	100	114

TABLE 4

Average colony diameter and percentage recovery of Pseudomonas fluorescens at 36 and 48 hours at 20 C using various peptones incorporated into a solid medium

Peptone	Average colony diameter in mm at		Percentage recovery based on 48 hr plate count of TGE agar at 20 C	
	36 hrs	48 hrs	36 hrs	48 hrs
Trypticase	0	<0.1	0	13
Peptonized Milk	1.0	2.8	50	65
Proteose Peptone	0.5	0.9	51	89
Milk Protein Hydrolysate	1.0	1.4	64	91
Lactalysate	1.4	1.6	73	108
Polypeptone	1.4	1.9	65	112
Tryptose	1.0	1.3	47	96
Thiotone	1.4	1.9	61	105
Myosate	1.1	1.6	82	99
Phytone	1.8	2.3	99	127

RESULTS

Examination of Table 1 demonstrates that the average colony diameter of Alcaligenes viscosus is not significantly different through the 36 hour measurement on the media containing tryptose, thiotone, and phytone. At 48 hours, however, the average colony diameter on the medium containing phytone is 0.3 mm greater than that on the tryptose and 0.5 mm greater than that on the thiotone.

After 48 hours, only 69 per cent of the total count on tryptone-glucose-extract agar occurred on the medium containing thiotone, while both the tryptose and phytone containing media yielded counts after 48 hours of over 100 per cent.

In Table 2 there was little difference between the effect of the peptones thiotone and myosate on colony size and percentage recovery of Flavobacterium rhenanus. The percentage recovery on the phytone medium, although over 100 per cent after 48 hours, is not significantly different from the other two peptones in question. However, a significant margin of difference may be noted with regards to colony diameters. The average colony diameter on the phytone medium was 0.6 mm, 1.3 mm and 1.1 mm greater at 24, 36 and 48 hours respectively, than the average diameter of those colonies appearing on the myosate medium.

In Table 3, the medium containing phytone yielded larger colonies of Achromobacter superficiale than did the myosate medium. The results indicate a difference of 0.4 mm in colony size as well as a 28 per cent difference in percentage recovery. The number of colonies of Achromobacter superficiale on lactalysate after 48 hours

was similar to that on the phytone medium. However, growth on lactalysate medium was not visible in 24 hours, while on the phytone medium there was a 10 per cent colony recovery in 24 hours. This may be due to either an extended stationary phase by the organism the lactalysate medium or a more rapid logaritimic phase on the phytone medium. In any event, the phytone medium was the most suitable peptone for the growth of Achromobacter superficiale.

Pseudomonas fluorescens in Table 4 had a slightly larger colony on the medium containing peptonized milk, than on the medium containing phytone. The percentage recovery of colonies on the former is less than half of the latter. The phytone medium also gave a slight advantage for the growth of Pseudomonas fluorescens over the medium containing polypeptone.

DISCUSSION

In a general way there appears to be a similarity in the nutritional requirements of the four test organisms, as all appear to demonstrate larger colonies and higher percentage recovery on the phytone medium. Interesting to note is the observation that the peptones tryptose, myosate and phytone foster larger colonies of Alcaligenes viscosus, Flavobacterium rhenanus and Achromobacter superficiale, than do the other peptones. Again this serves to demonstrate a closer similarity in growth requirements of these three organisms. More striking perhaps is the marked similarity of the sequence of poor to best peptones for the growth of Alcaligenes viscosus and Flavobacterium rhenanus.

The effects of phytone might be due to the presence of certain

nutritional elements which promoted a more rapid growth. Possibly these nutrients were lacking or in lesser amounts in the other peptones, and thus account for the less luxuriant growth on the other peptones. The presence of toxic products in the other peptones could conceivably account for the differences in growth of the test organisms on the peptones tested.

B. EFFECT OF NUTRITIONAL ADDITIVES

EXPERIMENTAL

Having selected phytone as the base medium peptone, the next step was to ascertain the optimum concentration of the peptone, the effect of additional metabolites and the effect of hydrogen ion concentration.

The method of Darby and Mallmann (1939) of minimal inoculum was most suitable for these determinations. The effect of addition or **omission** of nutrients in a medium for the growth of an organism is most markedly demonstrated during its early phases of growth. Generally accepted is the idea that the lag phase is the most critical stage of growth. Huntington and Winslow (1937) showed that during this stage, the organisms exhibit all the characteristics of physiological youth. Generally, these young cells are more susceptible to adverse conditions. Thus, it is considered of prime importance to know if an organism can adjust to its new environment rapidly, undergo division, and survive.

Accordingly, a diagnostic medium should not be evaluated in terms of the final crop of organisms, but rather on the basis of the behavior of these organisms during the lag and early logarithmic stages. Consequently, the following studies have been conducted utilizing minimum number of organisms which were stored at 4.5 C for 24 hours prior to use.

The interpretation of results which follows are based on the

behavior of the organisms during the lag and early logarithmic stages.

Representatives of the four psychrophilic genera, namely Pseudomonus fluorescens, Alcaligenes viscosus, Flavobacterium rhenanus, and Achromobacter superficiale were used as the test organisms.

The procedure for determining the growth rates of these organisms was as follows: a 24 hour culture of each organism was transferred each day for 3 days in brain heart infusion broth.

Incubation was at 20 C. Following the 24 hour incubation of the third transfer, each culture was placed in a refrigerator at 4.5 C for 24 hours. It was felt that this 24 hour refrigeration period would more closely approximate the actual conditions of psychrophilic organisms in refrigerated milk. After 24 hour refrigerated storage, each culture was diluted so that between 20 and 100 organisms per ml were present. These were seeded into flasks containing 100 ml of the respective media. Initial plate counts were made immediately after the original inoculation and every three hours thereafter up to and through the ninth hour. Another count was then made after 24 hours of incubation.

These flasks were incubated at 20 C and subjected to a mechanical shaking for two minutes prior to sampling. Plating was made with tryptose-glucose-extract agar and incubated for 72 hours at 20 C. Due to the relatively slight thermal resistance of these organisms, the plating agar was always cooled to 48 C prior to plating.

This procedure was repeated four times for each additive in the medium. Any great degree of variation in one or more of the trials in this series was deemed adequate grounds for repetition of the entire series. The results of the four trials were then averaged and the averages listed in Tables 5 to 24 inclusive.

RESULTS

It may be noted in Table 5 that there was virtually no effect on the early stages of growth of Alcaligenes viscosus with increasing concentrations of phytone. This serves to illustrate that although a stimulatory effect was absent, the peptone was not toxic in concentration up to 3 per cent.

Tables 6 and 7 clearly illustrates that lactose 0.5 per cent, dextrose 0.5 per cent and sodium chloride 0.5 per cent and 0.25 per cent did little to accelerate growth in the early stages of growth of Alcaligenes viscosus. The same was true of beef and yeast extract as seen in Table 8.

Table 9 demonstrates that Alcaligenes viscosus was capable of growth over a wide pH range. However, this organism had an optimum pH between 7.3 to 7.5. In these pH ranges, Alcaligenes viscosus had appeared in slightly greater numbers after 9 hours of growth than in the corresponding period at pH 6.5 to 6.8; and after 24 hours the number of cells in this pH range was approximately three times the number of organisms growing in the range of pH 6.5 to 6.8.

The effect of varying concentrations of phytone on the lag phase of Flavobacterium rhenanus is shown in Table 10. Again there was apparently no stimulatory effect as the peptone concentration

TABLE 5

Influence of phytone concentration on the early stages of growth of Alcaligenes viscosus

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 1.5 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.5 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 3.0 K_2HPO_4 0.275 KH_2PO_4 0.275
0	27	22	33	28
3	75	84	89	81
6	456	400	456	430
9	2,530	2,870	3,000	3,360

TABLE 6

Influence of lactose and dextrose on the early stages of growth of Alcaligenes viscosus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Lactose 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Dextrose 0.5
0	22	22	26
3	84	95	100
6	400	553	513
9	2,870	3,660	3,430

TABLE 7

Influence of sodium chloride on the early stages of growth of Alcaligenes viscosus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.25
0	22	33	28
3	84	96	95
6	400	407	540
9	2,870	2,930	3,200

TABLE 8

Influence of beef and yeast extract on the early stages of growth of Alcaligenes viscosus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Beef ex. 0.50	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Yeast ex. 0.50
0	22	39	35
3	84	95	100
6	400	573	613
9	2,870	4,333	2,950
24	9,200,000	12,000,000	7,800,000

TABLE 9

Influence of pH on the early stages of growth of Alcaligenes viscosus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.0
0	38	35	34
3	114	100	120
6	577	613	667
9	2,662	2,950	4,333
24	6,870,000	7,800,000	12,200,000

TABLE 9 (continued)

Influence of pH on the early stages of growth of Alcaligenes viscosus

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.3	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 8.0
0	29	40	29	28
3	141	136	111	88
6	696	637	640	420
9	5,700	5,067	2,900	3,000
24	21,400,000	20,000,000	11,300,000	11,000,000

TABLE 10

Influence of peptone concentration on the early stages of growth of Flavobacterium rhenanus

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 1.5 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.5 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 3.0 K_2HPO_4 0.275 KH_2PO_2 0.275
0	73	72	74	64
3	183	177	150	156
6	983	827	765	760
9	4,830	5,600	4,600	4,600

TABLE 11

Influence of lactose and dextrose on the early stages of growth of Flavobacterium rheinmus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Lactose 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Dextrose 0.5
0	72	64	58
3	177	143	135
6	827	810	1,027
9	5,600	7,500	7,000

TABLE 12

Influence of sodium chloride on the early stages of growth of Flavobacterium rhenanus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.25
0	72	65	51
3	177	120	120
6	827	703	703
9	5,600	6,000	6,000

TABLE 13

Influence of beef and yeast extract on the early stages of growth of Flavobacterium rhenanus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Beef ext. 0.50	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Yeast ext. 0.50
0	72	73	73
3	177	154	167
6	827	649	683
9	5,600	4,200	5,833
24	25,000,000	45,000,000	69,000,000

TABLE 14

Influence of pH on the early stages of growth of Flavobacterium rhenanus

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.0	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.2
0	53	73	47	56
3	115	167	116	127
6	513	683	533	773
9	5,200	5,833	5,233	7,433
24	62,000,000	69,000,000	67,000,000	70,000,000

TABLE 14 (continued)

Influence of pH on the early stages of growth of Flavobacterium rhenanus

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 8.0
0	66	75	75
3	176	201	209
6	893	963	853
9	11,134	7,233	7,400
24	81,000,000	69,000,000	64,000,000

TABLE 15

Influence of peptone concentration on the early phases of growth of Achromobacter superficiale

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 1.5 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.5 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 3.0 K_2HPO_4 0.275 KH_2PO_4 0.275
0	32	31	30	32
3	76	86	79	85
6	580	573	570	493
9	2,300	3,970	2,500	2,530

TABLE 16

Influence of lactose and dextrose on the early stages of growth of Achromobacter superficiale

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Lactose 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Dextrose 0.5
0	31	29	27
3	86	86	95
6	573	647	593
9	3,970	3,433	3,733

TABLE 17

Influence of sodium chloride on the early stages of growth of Achromobacter superficiale

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.25
0	31	29	28
3	86	99	101
6	573	470	543
9	3,970	3,100	3,700

TABLE 18

Influence of beef and yeast extract on the early stages of growth of Achromobacter superficiale

Hours	Organisms per ml.		
	Medium in percent concentration		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Beef ex. 0.50	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Yeast ex. 0.50
0	31	28	24
3	86	89	85
6	573	607	700
9	3,970	4,700	3,500
24	13,000,000	12,000,000	12,000,000

TABLE 19

Influence of pH on the early stages of growth of Achromobacter superficiale

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.0	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.2
0	37	24	33	36
3	154	84	120	120
6	777	700	643	693
9	3,867	3,500	4,667	5,733
24	11,700,000	12,000,000	9,800,000	18,000,000

TABLE 19 (continued)

Influence of pH on the early stages of growth of Achromobacter superficiale

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 8.0
0	35	29	23
3	150	112	82
6	673	567	400
9	7,334	3,367	2,300
24	22,400,000	12,000,000	8,500,000

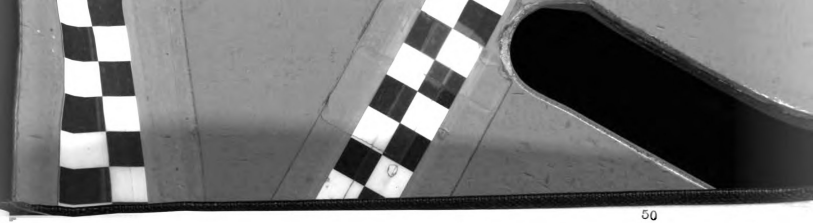


TABLE 20

Influence of peptone concentration on the early stages of growth of Pseudomonas fluorescens

Hours	Organisms per ml.							
	Medium in percent composition							
	Phytone	1.5	Phytone	2.0	Phytone	2.5	Phytone	3.0
	K_2HPO_4	0.275	K_2HPO_4	0.275	K_2HPO_4	0.275	K_2HPO_4	0.275
	KH_2PO_4	0.275	KH_2PO_4	0.275	KH_2PO_4	0.275	KH_2PO_4	0.275
0		21		20		15		25
3		39		39		38		45
6		80		140		140		127
9		133		450		433		370

TABLE 21

Influence of lactose and dextrose on the early stages of growth of Pseudomonas fluorescens

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Lactose 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Dextrose 0.5
0	21	16	19
3	39	35	32
6	140	77	90
9	450	160	280

TABLE 22

Influence of sodium chloride on the early stages of growth of Pseudomonas fluorescens

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.5	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 NaCl 0.25
0	21	13	17
3	39	31	36
6	140	83	96
9	450	200	400

TABLE 23

Influence of beef and yeast extract on the early stages of growth of Pseudomonas fluorescens

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Beef ex. 0.50	Phytone 2.0 K_2HPO_4 0.275 KH_2PO_4 0.275 Yeast ex. 0.50
0	20	32	29
3	39	64	76
6	140	293	333
9	450	993	1,253

TABLE 24

Influence of pH on the early stages of growth of Pseudomonas fluorescens

Hours	Organisms per ml.			
	Medium in percent composition			
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 6.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.0	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.2
0	84	29	66	75
3	123	76	116	143
6	227	333	333	587
9	430	1,253	867	1,220
24	100,000	151,000	133,000	700,000

TABLE 24 (continued)

Influence of pH on the early stages of growth of Pseudomonas fluorescens

Hours	Organisms per ml.		
	Medium in percent composition		
	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.5	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 7.8	Phytone 2.0 Yeast ex. 0.5 K_2HPO_4 KH_2PO_4 pH 8.0
0	64	62	
3	152	101	
6	550	427	
9	1,300	912	
24	1,450,000	820,000	

was increased from 1.5 per cent to 3.0 per cent. Toxic effects of the peptone, however, are lacking.

Lactose, dextrose and sodium chloride exhibited little if any influence on the early phase of growth of Flavobacterium rhenanus, (Tables 11 and 12).

While beef extract and yeast extract offered little by way of accelerating growth during the early stages of growth, yeast extract did show a slight effect on the cell crop of Flavobacterium rhenanus after 24 hours incubation. In Table 13, the number of organisms in the medium containing yeast extract was greater than twice the numbers in the control medium

Table 14 demonstrates that a similar situation existed with Flavobacterium rhenanus as with Alcaligenes viscosus. Namely that the organism was capable of growth over a relatively wide range of pH and that the optimum range for growth was within the range of pH 7.3 to 7.5.

The same pattern of growth demonstrated by both Alcaligenes viscosus and Flavobacterium rhenanus also occurred with Achromobacter superficiale.

An increase in peptone concentration, although not exhibiting any stimulatory effects on the early phase of growth of Achromobacter superficiale, did not demonstrate any marked toxicity up to a 3 per cent concentration (Table 15). Addition of lactose, dextrose, sodium chloride, beef extract and yeast extract to the buffered phytone medium (Tables 16 through 18) was virtually without effect on accelerating the early logarithmic period of growth.

Table 19 reveals that the optimum pH for growth was at pH 7.3 to 7.5, although Achromobacter superficiale is capable of growth over a relatively wide range. Even at the optimum pH, the count at 24 hours was only twice the count at pH 6.5 and about three times the count at pH 8.0.

In Table 20 using Pseudomonas fluorescens, an increase in concentration of phytone from 1.5 per cent to 2 per cent almost doubled the count after six hours of incubation and tripled the number of cells after nine hours of growth. Although differences among these counts are slight, these differences remained constant for each replicate trial.

The addition of lactose, dextrose and sodium chloride to the buffered phytone base medium in Tables 21 and 22 did little to enhance the growth of Pseudomonas fluorescens during the early stages of growth.

The addition of beef extract to the base medium was responsible for increasing the count to twice that of the control medium as seen in Table 23. Here also, a stimulatory effect of yeast extract was noted. The count after nine hours incubation in the medium with yeast extract was almost three times greater than the control medium.

The optimum pH for the growth of Pseudomonas fluorescens in the phytone medium (Table 24) was at pH 7.5. During the first nine hours of growth, the counts at pH 7.2, 7.5 and 7.8 were not significantly different. However, after 24 hours of growth, the counts obtained on the medium buffered to a pH of 7.5, showed

almost twice the numbers obtained at pH 7.8 and 7.2 and 15 times greater numbers than that at pH 6.5.

DISCUSSION

The minimal inoculation studies performed in liquid media were originally designed by Darby and Mallmann (1939) to detect changes in the lag phase of growth of the coliform organisms. Psychrophilic organisms, however, when grown at 20 C exhibit an extremely slight, if any, lag phase, (Greene and Jezeski 1951), Van der Zant and Moore, 1955). Consequently, the minimal inoculum technique was used in this study to detect changes in the early logarithmic period of growth.

Studies on the comparison of peptones for the growth of Escherichia coli by Darby and Mallmann (1939) indicated the possibility of toxic effects as a result of increased peptone concentration. Accordingly, experiments were carried out to determine the toxicity effect, if any, of phytone on the growth of the psychrophilic test organisms. There was no evidence of toxicity of this peptone on any of the test organisms. A two per cent concentration of phytone was believed to be optimum for the growth of these organisms.

Studies by Bowers and Hucker (1934) showed an increase in number and size of colonies by the addition of a fermentable carbohydrate as well as yeast extract to standard media for routine milk control work.

These observations prompted the experimental work concerned with the effect of lactose, dextrose, beef and yeast extract on the

early phase of growth of the psychrophilic test organisms.

In this study, the carbohydrates were virtually without effect on the early stages of growth of the test organisms. Yeast extract was found to be slightly stimulatory to the growth of Pseudomonas fluorescens.

The addition of sodium chloride has been reported by Dunham (1939) to enhance the recovery of coliform organisms from eosin-methylene blue medium. Mooney and Winslow (1935) also demonstrated a stimulatory effect of sodium chloride on the growth of Salmonella pullorum. Darby and Mallmann (1939) noted a marked increase of growth in both the early and late stages of growth of coliform organisms with the addition of sodium chloride to a tryptose base medium. Sherman and Holm (1922) reported that the addition of salt to a one per cent peptone medium enabled Alcaligenes faecalis to maintain a rapid growth rate over a wide pH range.

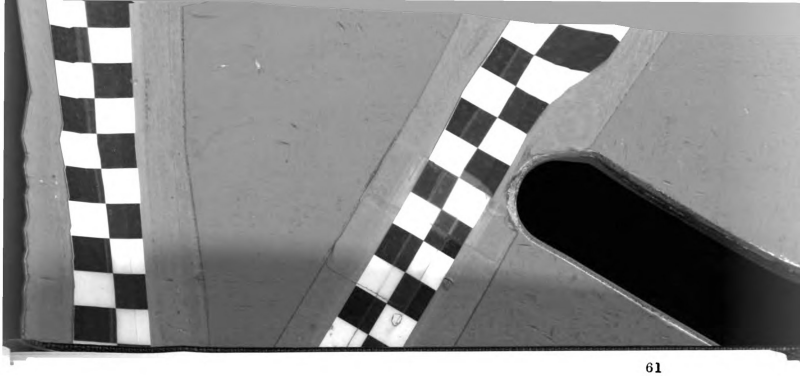
For these reasons, the influence of sodium chloride on the early stages of growth of the psychrophilic test organisms was investigated. It was found that sodium chloride did not accelerate the growth of any of the test organisms.

The investigation of the effects of pH on the growth of these organisms showed quite conclusively that the optimum pH for all these organisms was in the vicinity of 7.5.

In following the general nutritional trends of these four organisms, either they are not exceptionally fastidious in their nutritional requirements, or that the peptone, phytone, is an almost complete nutritional source for these organisms. In any

event, the observation may be made that nutritional additives did little to encourage a more rapid rate during the early stage of growth. Once again this trend serves to point out the close nutritional relationship among these organisms. In all cases, a pH of 7.5 was found to be optimum.

On the basis of these findings, a medium containing 2.0 per cent phytone, 0.5 per cent yeast extract and buffered to pH 7.5 with 0.5 per cent, K_2HPO_4 and 0.01 per cent, KH_2PO_4 was prepared. Agar (1.5 per cent) was added to this medium and it was then employed as a base for the further experimentation on a selective media.



C. SELECTION OF AN INHIBITOR

EXPERIMENTAL

Since this base medium as such, would permit the growth of various mesophilic as well as psychrophilic organisms, it was necessary to find an inhibitor which would be relatively non-toxic for psychrophiles yet prevent the growth of mesophilic organisms.

Because the gram negative flora of milk is predominately psychrophilic, inhibitors for gram positive organisms were tested as selective agents. The dyes, methyl violet, ethyl violet, brilliant green, crystal violet, and the surface active agents sodium lauryl sulfate and Nacconol N.R.S.F.* (an alkyl aryl sulfonate) were incorporated in varying concentration into the solid base medium described before.

Preliminary studies indicated that the dyes were not suitable. In general, the concentrations of the various dyes necessary for the inhibition of the gram positive test organisms were also inhibitory to the psychrophilic organisms.

It was also found that sodium lauryl sulfate in concentrations greater than 1:10,000 crystalized and became ineffective at 20 C. Nacconol N.R.S.F. was unaffected. This compound was found to be equally as inhibitory as lauryl sulfate against gram positives in studies by Mallmann and Darby (1941). Accordingly tests are reported on this compound.

To test the inhibitory action of Nacconol N.R.S.F. against

* Product of Allied Chemical and Dye Corporation

a representative gram positive flora of milk, representative organisms were seeded into the base medium containing concentrations of Nacconol N.R.S.F. of 1:100,000, 1:50,000, 1:10,000, 1:5,000 and 1:1000.

The following organisms, representative of the gram positive flora of milk, were used: Streptococcus lactis, Streptococcus liquefaciens, Streptococcus faecalis, Micrococcus sp. 102, Bacillus subtilis, Leuconostoc mesenteroides, Leuconostoc citroverum, Lactobacillus casei and Lactobacillus acidophilis.

A 1 ml dilution, containing 50 to 100 organisms of a 24 hour culture which had been transfered for three days in brain heart infusion broth was used as the inoculum. Three separate trials, using triplicate plates were made with the base medium containing various concentrations of the dyes and surface active agents.

The plates were incubated at 20 C and observed after 48 and 72 hours, the counts were averaged and recorded in terms of percentage recovery as compared to the counts obtained on the base medium alone.

This procedure was repeated using the four representative psychrophilic organisms Alcaligenes viscosus, Flavobacterium rhenanus, Achromobacter superficiale and Pseudomonas fluorescens.

RESULTS AND DISCUSSION

The results are shown in Table 25. Nacconol N.R.S.F. at a concentration of 1:10,000 in the base medium completely inhibited the gram positive test organism for 72 hours. On the other hand Alcaligenes viscosus, Achromobacter superficiale, Flavobacterium

TABLE 25

Effect of Varying Concentrations of Nacconol N.R.S.F. incorporated into a solid base medium on the percentage recovery of gram positive test organisms

Conc. of Nacconol in base medium	Per cent recovery of viable organisms based on 72 hour plate count								
	S. actis	S. faecalis	S. liquefaciens	B. subtilis	Micro-coccus sp.102	L. mesenteroides	L. citrovorum	L. casei	L. acidophilus
0	100	100	100	100	100	100	100	ng*	ng*
1:100T	100	100	100	100	100	100	100		
1:50T	92	94	92	45	43	94	93		
1:10T	0	0	0	0	0	0	0		
1:5T	0	0	0	0	0	0	0		
1:1T	0	0	0	0	0	0	0		

* no growth

rhenanus and Pseudomonas fluorescens were uneffected at concentrations as great as 1:1,000.

The investigation thus far suggested that the base medium containing 1:10,000 Nacconol N.R.S.F. might be used as a selective medium for psychrophilic organisms in milk.

D. PRELIMINARY TEST OF THE PHYTONE-NACCONOL MEDIUM

EXPERIMENTAL

It was now necessary to determine if this concentration (1:10,000) of Nacconol N.R.S.F. would be adequate in preventing the growth of the gram positive flora of milk under practical conditions. Nacconol N.R.S.F. in final concentrations of 1:10,000, 1:5,000 and 1:1000 was added to the base medium. These media were then tested on 29 milk samples collected from the Lansing area.

One ml quantities were plated out on three media and incubated at 20 C for three days. Colonies were picked at random and streaked on tryptone-glucose-extract agar slants and incubated at room temperature for 48 hours.

The cultures were then gram stained and transfered onto another tryptone-glucose-extract slant and incubated at 4.5 C for seven days in order to determine if the organisms isolated from the Phytone-Nacconol medium were psychrophilic.

These studies indicated that the media containing Nacconol N.R.S.F. in a concentration of 1:10,000 and 1:5,000 were not sufficiently selective. The relatively large number of gram positive isolates from these two media justified discarding these in favor of the medium containing a concentration of Nacconol N.R.S.F. of 1:1,000. This latter medium demonstrated marked selectivity as demonstrated in Table 26.

TABLE 26

Isolation of organisms from solid base medium with 1:1,000 concentration of Nacconol N. R. S. F.

	Number	Per Cent
Organisms isolated	204	
Gram negative rods	186	91
Other organisms*	18	9
Organisms showing growth at 4.5 C in 7 days	164	84

* Gram variable cocci

Gram positive cocci

Gram negative cocci

RESULTS AND DISCUSSION

Although a 1:10,000 concentration of Nacconol N.R.S.F. was shown to be effective against a gram positive flora in pure culture studies, it did not exhibit sufficient inhibitory properties against the gram positive flora in milk. Presumably this is due to the "tying up" of Nacconol N.R.S.F. with various constituents of milk or the presence of gram positive organisms in the milk which are resistant to the action of Nacconol N.R.S.F.

In Table 26, it may be seen that of the 204 isolates from the base medium with a 1:1000 concentration of Nacconol N.R.S.F., 91 per cent were identified as gram negative rods while 84 per cent were capable of growth at 4.5 C.

From the practical viewpoint, these results were considered to be adequate for a selective medium. Thus, the base medium containing Nacconol N.R.S.F. in a final concentration of 1:1000 was then employed in a field test.

E. PHYTONE-NACCONOL AGAR VS. TRYPTONE-GLUCOSE-EXTRACT AGAR

EXPERIMENTAL

In order to test the effectiveness of the medium as compared to the method described in Standard Methods for the Examination of Dairy Products for the enumeration of psychrophilic bacteria, the following investigation was carried out.

Forty-five fresh pasteurized milk samples were obtained from dairies in the Lansing area. Replicate samples were plated according to Standard Methods on tryptone-glucose-extract agar and on Phytone-Nacconol agar of the following composition:

Phytone	20.0 gms
Yeast Extract	5.0 gms
K_2HPO_4	5.0 gms
KH_2PO_4	0.1 gms
Nacconol N.R.S.F.	1.0 gms
Agar	15.0 gms.
Distilled water	1000 ml

Replicate tryptone-glucose-extract agar plates were incubated at 35 C for 2 days, 25 C for 3 days, 20 C for 4 days and 4.5 C for 7 days. Phytone-Nacconol agar plates were incubated at 20 C for 2 days.

RESULTS

A representative sample of the data is shown in Table 27. While this table does not include all the results obtained, those not included demonstrated the same trend.

A count on tryptone-glucose-extract agar at 25 C for 3 days and 20 C for 4 days has been recently recommended for the estimation of

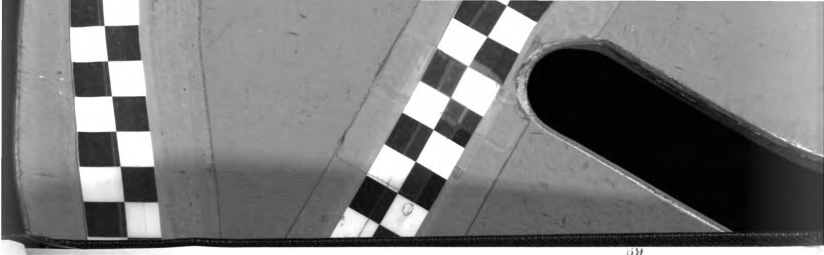


TABLE 27

Comparison of counts obtained on tryptose glucose extract agar incubated at 35 C for 2 days, 25 C for 3 days, 20 C for 4 days and 4.5 C for 7 days

Sample #	35 C	25 C	20 C	4.5 C
1	2100	3800	3500	520
2	4500	11200	2000	4
3	15000	20500	7500	6
4	2800	3200	1700	1
5	20300	7200	1350	4
6	1300	3100	2500	210
7	13000	21100	14400	2
8	80	70	60	1
9	300	260	160	3
10	1800	1570	710	0
11	17100	20400	12000	12300
12	15100	16700	11000	2600
13	19800	22500	17200	6400
14	7300	7700	9100	4
15	5800	8900	6300	10
16	220	58	31	2
17	1700	1700	370	17
18	3400	6200	3800	1
19	6400	10400	6200	1
20	4900	8100	6300	90
21	2100	3200	1600	0

TABLE 28

Statistical analysis of psychrophile counts on tryptose glucose extract agar and Phytone-Nacconol agar

Sample #	P-N agar	TGE agar	Difference	Sample #	P-N agar	TGE agar	Difference
1	3	0	3	24	0	0	0
2	42	40	2	25	70	30	40
3	50	40	10	26	24	15	9
4	21	6	15	27	0	0	0
5	1000	1200	-200	28	2	520	-518
6	60	53	7	29	3	4	-1
7	67	61	6	30	27	6	21
8	17	2	15	31	13	1	12
9	5	3	2	32	4	4	0
10	1500	1200	-300	33	120	210	-90
11	38	9	29	34	13	2	11
12	5600	6200	-600	35	0	1	-1
13	27	43	-16	36	5	3	-2
14	23	7	16	37	3	0	3
15	210	580	-370	38	30	4	26
16	210	21	189	39	31	10	21
17	570	620	-50	40	9	2	7
18	26	130	-104	41	18	17	1
19	200	320	-120	42	0	1	-1
20	17	3	14	43	15	1	14
21	80	10	70	44	82	90	-8
22	7	0	7	45	6	0	6
23	40	40	0				

$\bar{d} = -1825$

$\Sigma d^2 = 985,755$

$$t = \frac{\bar{d} - \mu_d}{\sqrt{\frac{s_d^2}{n}}} = \frac{-40.556 - 0}{\sqrt{\frac{39,224.861}{45}}}$$

$$t = -1.373$$

of psychrophilic organisms. These incubation times and temperatures yield reliable information on stored milk with high psychrophilic counts. However, it is questionable if the same reliability exists for the enumeration of psychrophilic organisms in freshly pasteurized milk. For this reason, the 25 C count for 3 days and the 20 C count for 4 days have been included in this study.

It may be seen that counts at 25 C for 3 days and 20 C for 4 days on tryptone-glucose-extract agar on freshly pasteurized milk are for the most part a reflection of the 35 C count. As such, these counts offer little information concerning the psychrophilic population.

Table 28 represents a statistical analysis of the counts obtained at 20 C for 2 days on Phytone-Nacconol agar medium as compared to the counts obtained by incubation for 7 days on tryptone-glucose-extract agar at 4.5 C.

DISCUSSION

In the preparation of this table, the t test as described by Stearman (1955) was used to test the differences in the two methods. The formula for the t test is as follows:

$$t = \frac{\bar{d} - nd}{\sqrt{\frac{s^2 d}{n}}}$$

where $\bar{d} = \frac{\text{sum of the differences}}{\text{number of samples}}$

$$s^2 d = \frac{\text{sum of } (\text{differences} - \bar{d})^2}{\text{number of sample} - 1}$$

n = number of samples

The hypothesis on this test is that there is no difference be-

ween the two treatments; with this hypothesis $\mu_d=0$. Alternatives to this hypothesis are that the mean is greater than zero, in which case the counts obtained on the Phytone-Nacconol medium will be higher; or that the mean is less than zero, whereupon the counts on tryptone-glucose-agar will be higher. Using a 5 per cent level of significance and since there are 45 differences in the sample, there will be 45 -1 = 44 degrees of freedom. From the t table, t with 5 per cent level of significance and 44 degrees of freedom is 2.016. Thus the critical region of the test will be values of t which are less than -2.016 or greater than +2.016.

The value of t as calculated by the equation is -1.373. This does not fall into the critical region of the test; therefore the mean plate counts of the two different methods are not significantly different, statistically.

F. THE USE OF TETRAZOLIUM SALTS AS A COLORING AGENT FOR PSYCHROPHILES ON PHYTONE-NACCONOL AGAR

EXPERIMENTAL

During the course of this investigation, extreme care was exercised in counting colonies, particularly on those plates where 1 ml quantities of milk were used. The opacity of the medium in these cases definitely impeded counting. In an attempt to alleviate this situation, various dyes were screened as coloring agents for psychrophilic organisms. A reduction indicator, 2, 3, 5 triphenyl tetrazolium chloride (TTC) was selected. When .001 gm was incorporated into 100 ml of tryptone-glucose-extract agar and used to enumerate psychrophiles at 4.5 C for 7 days, it was found that almost without exception all psychrophiles reduced triphenyl tetrazolium chloride from colorless to red.

This concentration of triphenyl tetrazolium chloride was incorporated into the Phytone-Nacconol medium. Forty-five milk samples were used to determine psychrophilic numbers with this medium as compared to the Phytone-Nacconol medium without triphenyl tetrazolium chloride.

RESULTS AND DISCUSSION

It was observed that there was little if any significant differences between the two media. Although no attempt was made to measure the size of the colonies on the two media, it appeared that the colonies on the medium containing the tetrazolium salts were larger. Presumably this was an optical illusion resulting

from the vivid red colonies on a white background.

There could be no dispute that the addition of the tetrazolium salts to Phytone-Nacconol agar definitely facilitated the counting of colonies.

SUMMARY

1. Of the peptones tested, phytone was found to be the most suitable peptone for the growth of Alcaligenes viscosus, Flavobacterium rhenanus, Achromobacter superficiale and Pseudomonas fluorescens.
2. A 2 per cent concentration of phytone was found to be optimum, while concentrations up to 3 per cent did not demonstrate any marked toxicity for the growth of the test organisms.
3. The addition of 0.5 per cent yeast extract to the medium was slightly stimulatory for the growth of Pseudomonas fluorescens.
4. Marked differences were not apparent during the early stages of growth of these organisms grown on media adjusted from pH 6.5 to 8.0. A pH of 7.5, however, was found to yield a decidedly higher colony count at 24 hours with all the organisms tested.
5. Nacconol N.R.S.F. incorporated into the solid base medium in a final concentration of 1:1000 was found to be inhibitory to the gram positive flora of milk. There was no apparent effect on the representative psychrophilic organisms.
6. To facilitate counting, 2, 3, 5 triphenyl tetrazolium chloride was added to the medium as a coloring agent. Almost without exception, psychrophilic organisms reduced TTC. A comparison of media with and without TTC showed little, if any, difference in the total numbers of colonies obtained.
7. Phytone-Nacconol agar is suggested as a rapid selective method for the enumeration of psychrophilic organisms in freshly pasteurized milk.

8. 2, 3, 5 triphenyl tetrazolium chloride, (1 cc of a sterile 1:100 solution per 100 ml of media) may be added to Phytone-Nacconol agar just prior to pouring plates. Psychrophiles will reduce TTC. The red colored colonies thus formed facilitates counting.

9. When tested, plate counts on this medium were similar statistically to those obtained by incubation at 4.5 C for 7 days on tryptose-glucose-extract agar.

PART II

A TEST FOR DETERMINING THE KEEPING QUALITY OF MILK

LITERATURE REVIEW

The keeping quality of refrigerated pasteurized milk is largely determined by the number and activity of the psychrophilic population of the milk. In general, the number of psychrophiles in freshly bottled milk is not considered a reliable index of the shelf life of the product, (Burgwald and Josephson 1947), Dahlberg et al (1953), Olsen et al (1953), Atherton et al (1954).

Wide variations occur in both the initial number of psychrophiles and the number at the time of spoilage. The organisms vary in their action on the various milk constituents as well as in the amount of by-products contributing to flavor defects. Occasionally, so-called "inert" psychrophiles reach high population levels without noticeable flavor defects. The psychrophilic population of the milk prior to pasteurization reduces the shelf life of the pasteurized product. Weber (1956) reported that milk which had a high psychrophilic count before pasteurization would not keep as long as milk with relatively low counts prior to pasteurization. Added to these factors are inherent differences in growth rates at refrigeration temperatures of the various psychrophiles.

Day and Doan (1956) studied chemical tests for measuring flavor defects of milk. Changes in protein stability, acidity, and variations in nitrogen distribution of the non-casein fractions were too slight and inconsistent. Significant changes occurred too near the time of pronounced flavor defects to be of practical value.

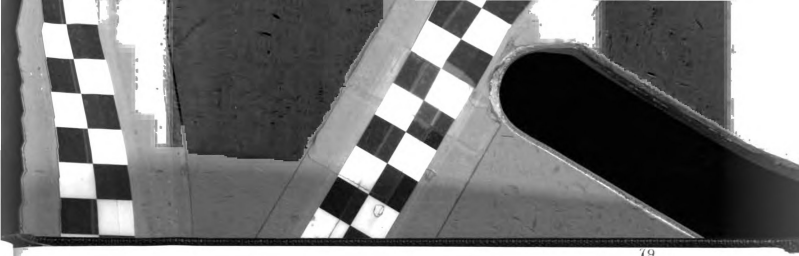
The methylene blue test has been used for many years as an

indicator of bacterial activity and as such, gives a misleading indication of keeping quality. In some early studies, Hiscox et al (1932) deviated from the usual standard of acid production as a criterion of keeping quality and used instead taste determination. They demonstrated a marked correlation between the reduction of methylene blue at 15.5 C and the keeping quality of milk at the same temperature. Almost without exception, as soon as the reduction was complete, a flavor defect could be detected in a portion of milk reserved for tasting. This modification of the methylene blue test differed from others in the temperature of incubation. Thus, the test measured only the biochemical activity of organisms capable of relatively rapid growth at low temperature. The close agreement between reduction time and keeping quality provided a clue to the present investigation.

A test which would indicate an extremely small population, i.e.- less than one organism per ml - as well as a relatively high population, and give an estimate of the activity of psychrophilic organisms, might be used in predicting the keeping quality of milk. A test of such delicacy could be accomplished best by an enrichment technique using the natural substratum, the milk under test.

EXPERIMENTAL

Earlier studies were conducted using 2, 3, 5 triphenyl tetrazolium chloride (TTC) as a coloring agent to facilitate counting of colonies where opacity of the medium was a problem. Without exception, psychrophiles growing on tryptose-glucose-extract agar, reduced TTC. A series of tests with pure cultures of psychrophiles



comparing resazurin and TTC indicated that the latter was more sensitive and thus would be more suitable. On the basis of these studies, TTC in a concentration of 1-10,000 was selected as the reduction indicator for the demonstration of bacterial activity.

To shorten the test time, the activity of the organisms had to be accelerated. As the minimum generation time of these organisms is at approximately 20 C, this temperature was selected as the incubation temperature for the test.

An incubation temperature of 20 C is not selective for psychrophiles and consequently the interfering orgamisms had to be inhibited.

In the process of developing a selective plating medium for the enumeration of psychrophiles, various surface active agents were tried. Nacconol N.R.S.F.* was shown by Mallmann and Darby (1941) to have similar inhibitory properties as sodium lauryl sulfate. Sodium lauryl sulfate, when tested, was found unsuitable for this plating medium because at the inhibitory concentration, it had a tendency to crystalize at 20 C. Consequently, Nacconol N.R.S.F. in a concentration of 1-1000 was selected. At this concentration the surface active agent inhibited the gram positive organisms without exhibiting any adverse effect on the gram negatives. Because the gram negative populations of milk is predominantly psychrophiles (Boyd, 1953), those not psychrophilic were minor and would likely not interfere seriously with the test.

A buffer system was found necessary because some psychrophiles,

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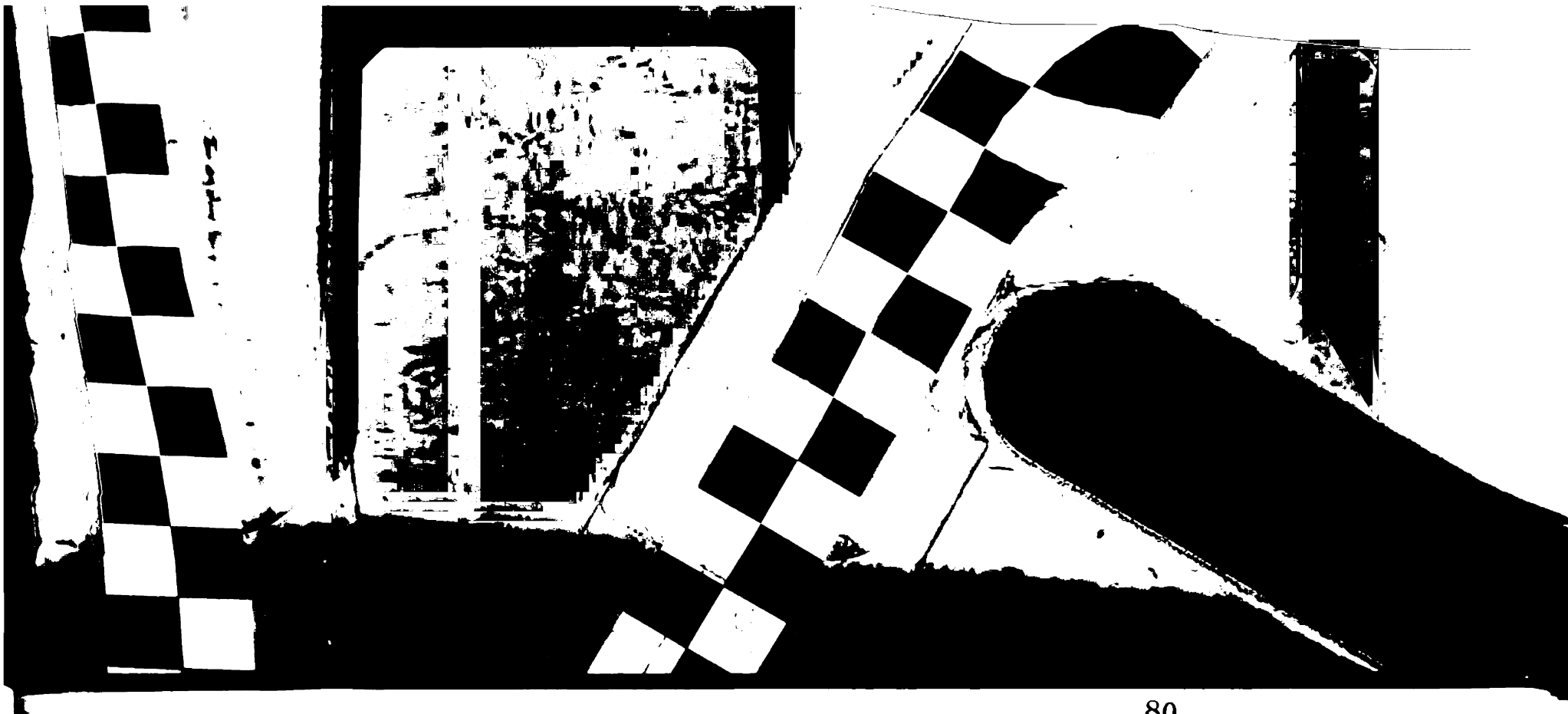


being acid producers, lowered the pH of the milk and consequently interferred with the reduction of TTC. The addition of 0.5 per cent K_2HPO_4 and 0.001 per cent KH_2PO_4 would maintain the pH of the milk from 7 to 7.5 depending upon the milk in question.

The indicator solution was prepared as follows:

2, 3, 5 Triphenyl tetrazolium chloride	0.1 gm.
Nacconol N.R.S.F.	1.0 gm.
K_2HPO_4	5 gm.
KH_2PO_4	0.1 gm.
Distilled water to make	100.0 ml.

Solution placed in dark bottle and autoclaved at 121 C for 15 minutes

Sterile solution stored at room temperature

In the performance of the test, 1 ml quantities of the Nacconol - TTC solution were pipetted into sterile tubes to which were added 10 ml samples of the milk in question. The tubes were shaken and incubated at 20 C and examined after 12, 24, 36 and 48 hours. The presence of a pale pink to rose red color was reported as positive at the particular time of reading. Occasionally a pink button was formed at the bottom of the tube. This was considered negative in the absence of a pale pink color throughout the tube of milk.

Twenty-three lots of fresh pasteurized milk were obtained from 12 dairies in the Lansing area. Three quarts of each lot were pooled in a large sterile vessel to eliminate possible variations in different quart bottles. This milk was then dispensed into 10 sterile half pint bottles which were chilled prior to filling. The bottles were capped aseptically and immediately stored at 4.5 C.

Duplicate Nacconol - TTC tests were made on all samples

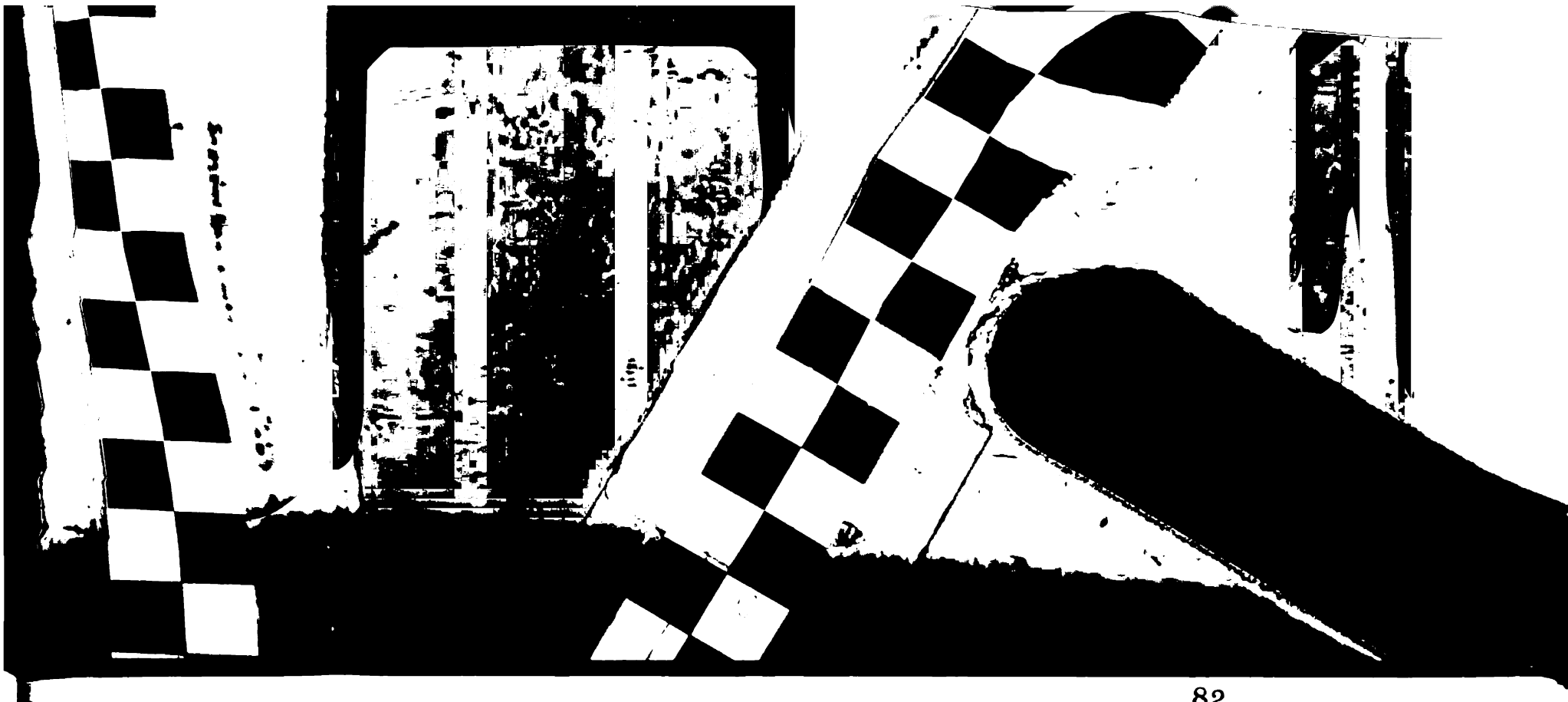
immediately following bottling. Replicate platings were made on tryptose-glucose-extract milk agar. Sets of plates were incubated at 35 C for 2 days, 25 C for 3 days, 20 C for 4 days and 4.5 C for 7 days.

Preliminary testing had demonstrated that most of the milks in the Lansing area were of relatively good quality. Therefore, in order to approximate conditions of poor quality, samples from each source were held at refrigeration temperatures for varying periods to allow an increase of psychrophiles prior to testing.

Milk samples were tested organoleptically on alternate days. Questionable samples were tested again the following day. An undisturbed bottle, representative of each lot, was used for each sampling. Bottles were coded to avoid bias. Prior to tasting, a portion was removed aseptically for measuring the number of psychrophiles present.

RESULTS AND DISCUSSION

In general, the initial psychrophilic bacterial counts were not directly correlated with keeping qualities of the milk samples tested. These results are similar to those obtained by other investigators cited. There is, however, a correlation if broad ranges of psychrophiles are bracketed. Milks with initial psychrophilic counts of less than 10 per ml exhibited a greater refrigerated storage life than milks with counts in the range of 10 to 10,000 per ml. These in turn showed a longer refrigerated shelf life than those which had counts ranging from 10,000 to 100,000,000 per ml.



There is a relationship between the psychrophilic counts of the milk at the time of spoilage, with few exceptions, and the presence of off-flavors. The counts at the time of the appearance of off-flavors ranged from 8×10^6 to 1×10^8 per ml. However, (Table 4) initial psychrophile counts in milk approaching the above range did not necessarily have flavor defects. These high initial counts were a good indication that the samples refrigerated shelf life would be very short.

Samples 31, 34, 36 and 39 (Table 3) had initial counts in the millions, yet they remained acceptable for 11, 10, 9 and 8 days respectively. These exceptions serve to point out the importance of the various types of organisms involved.

These same samples demonstrate the importance of the difference in growth rates of various species. Assuming the generation time of a psychrophilic population to range from 4 to 14 hours, it would take 20 to 70 hours for a population of 5×10^6 (acceptable flavor) to change to a populations of 1.6×10^8 per ml (flavor already unacceptable). Within reasonable limits, the initial and final psychrophilic counts, presented in Table 4, appear to fit into the above groupings. However, an examination of samples 31, 34, 36 and 39 illustrate some obvious discrepancies, because between 8 and 11 days were needed for various initial populations of 2×10^6, 3×10^5, 6.9×10^6 and 2.7×10^6 to final populations of 3.3×10^8, 1.2×10^6, 1.8×10^7 and 3.3×10^8 respectively. These observations demonstrate the importance of generation time of a psychrophilic population.

The results presented in Table 1 to 4 inclusive show the relationship between the positive Nacconol - TTC test and the keeping quality of various samples of milk. When a positive test was obtained in 48 hours, (Table 1) the average keeping time of 5 samples was 15.6 days. The average keeping time of 14 samples showing a positive test in 36 hours (Table 2) was 12.6 days. Eleven samples which gave a positive test in 24 hours (Table 3) had an average keeping-time of 8.8 days, while the 9 samples which exhibited a positive test in 24 hours (Table 4) had a refrigerated shelf life of 4.2 days. The data in Table 4 appear to confirm the work of Day and Doan (1956) who demonstrated reduction of neotetrazolium 3.75 days in advance of flavor spoilage.

A direct relationship between reduction time and keeping quality is demonstrated in Figure 1 in which the average keeping time of the various milk samples was plotted against the period in which a positive test was obtained.

Of the 19 samples (Table 1 and 2) which have a predicted keeping quality of 12.6 days or more, only 4 failed to meet this prediction, but even these had a refrigerated shelf life of 9 days. On the other hand, of the 20 samples (Tables 3 and 4) judged to have poor keeping quality, 19 exhibited a refrigerated shelf life of less than 12 days.

On the basis of these results, milk samples which exhibited a positive test in 24 hours or less had poor keeping quality (a shelf life at 4.5 C of less than 12.6 days). A positive test in 12 hours would indicate very poor keeping quality. A negative test in 24

TABLE 1

Measurement of keeping quality by the Nacconol-TCC test of commercially bottled milk stored at 4.5 C

Samples showing positive test in 48 hours

Sample #	Total keeping time in days	Initial count		Count at spoilage 4.5 C	Off flavor
		37 C	4.5 C		
8	18	80	1	210,000,000	bitter
10	18	1,800	0	510,000,000	fermented
16	17	220	2	330,000,000	sour
17	10	1,700	17	420,000,000	fruity
18	15	3,400	1	690,000,000	sour
Average	15.60				

TABLE 2

Measurement of keeping quality by the Nacconol-TCC test of commercially bottled milk stored at 4.5 C

Samples showing positive test in 36 hours

Sample #	Total keeping time in days	Initial count		Count at spoilage 4.5 C	Off flavor
		37 C	4.5 C		
1	9	2,100	520	890,000,000	sour
2	13	4,500	4	80,000,000	bitter
3	19	15,000	6	12,000,000	fruity
4	18	2,800	1	7,000,000	fermented
5	13	20,000	4	260,000,000	bitter
7	13	13,000	2	180,000,000	fruity
9	9	300	3	270,000,000	rancid
12	10	15,100	11,000	460,000,000	rancid
15	12	5,800	10	100,000,000	bitter
19	13	6,400	1	250,000,000	bitter
20	9	4,900	90	210,000,000	bitter
21	15	2,100	0	220,000,000	fermented
23	10	1,000	1,000	210,000,000	bitter
26	13	25,000	8,000	12,000,000	fermented
Average	12.57				

TABLE 3

Measurement of keeping quality by the Nacconol-TCC test of commercially bottled milk stored at 4.5 C

Samples showing positive test in 24 hours

Sample #	Total keeping time in days	Initial count		count at spoilage 4.5 C	Off flavor
		37. C	4.5 C		
6	10	1,300	210	340,000,000	sour
11	9	17,100	12,000	1,000,000,000	bitter
13	10	19,800	6,400	1,100,000,000	bitter
25	7	23,000	510,000	80,000,000	bitter
29	12	1,800	47,000	210,000,000	fermented
31	11	2,400,000	20,000,000	330,000,000	sour
33	4	330,000	3,500,000	80,000,000	bitter
34	10	100,000	3,000,000	12,000,000	fermented
36	9		69,000,000	180,000,000	fruity
39	8	6,900,000	27,000,000	330,000,000	sour
40	7	100,000	100,000	7,000,000	fermented
Average	8.82				

TABLE 4

Measurement of keeping quality by the Nacconol-TTC test of commercially bottled milk stored at 4.5 C

Samples showing positive test in 12 hours

Sample #	Total keeping time in days	Initial count		count at spoilage	Off flavor
		37 C	4.5 C	4.5 C	
22	3	1,200,000	27,000,000	250,000,000	bitter
24	3	3,800,000	30,000,000	890,000,000	sour
30	3	540,000	24,800,000	1,000,000,000	bitter
35	2	14,000,000	190,000,000	340,000,000	sour
14	4	100,000	33,000,000	260,000,000	bitter
37	7	14,000,000	25,000,000	512,000,000	fermented
38	5	3,200,000	80,000,000	690,000,000	sour
41	6	164,000,000	190,000,000	210,000,000	fermented
43	5	140,000,000	106,000,000	210,000,000	bitter
Average	4.22				

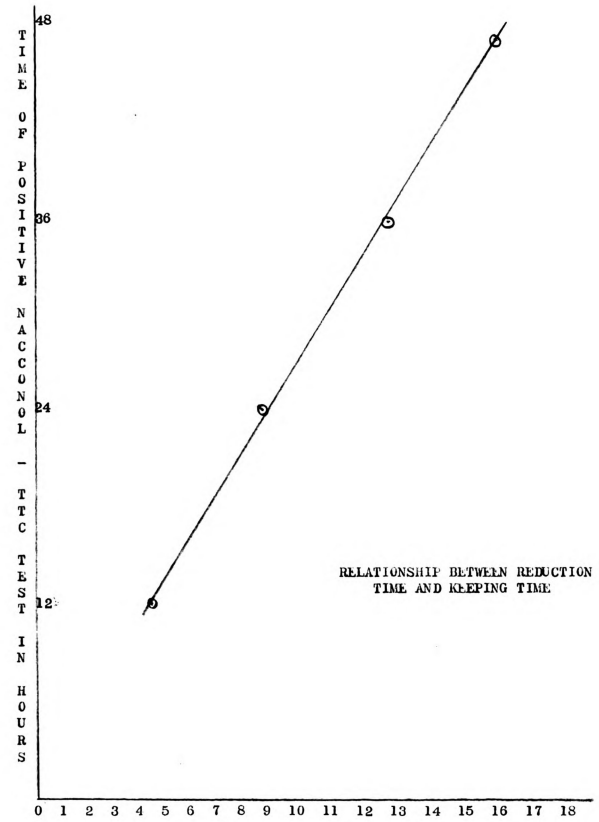

TIME OF POSITIVE NACCONOL - TTC TEST IN HOURS
48
36
24
12
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
KEEPING TIME IN DAYS
RELATIONSHIP BETWEEN REDUCTION TIME AND KEEPING TIME

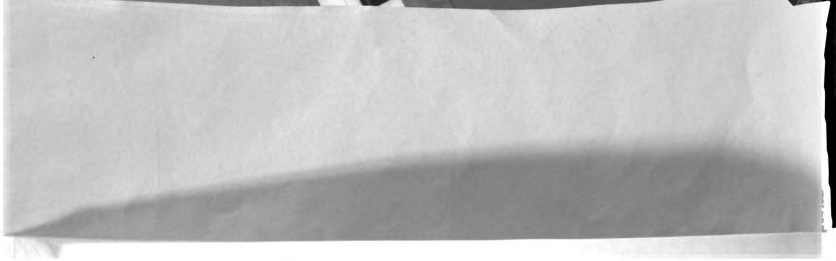
Figure 1

hours would indicate good keeping quality.

The test could be used in the dairy routinely to check storage life of raw and freshly pasteurized milk and to check sanitation of post pasteurization equipment and disposition of the product.

SUMMARY

A method is described whereby a 10 ml sample of milk, with the addition of 1 ml of a Nacconol - TTC solution, was incubated at 20 C and read at 12, 24, 36 and 48 hour intervals. The appearance of a positive tube (pale pink to rose red) is dependent upon psychrophilic bacterial activity). A positive test at 12 hours indicated a 4.5 C shelf life of approximately 4 days, a positive test at 24 hours, 9 days, a positive test at 36 hours, 12 days, and a positive test at 48 hours, 15 days.



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