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Quantitative Study of Growth of Some Dairy Psychrophiles

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To the Graduate Council:

I am submitting herewith a thesis written by Taher A. El-Farekh entitled "Quantitative Study of Growth of Some Dairy Psychrophiles." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

W. W. Overcast, Major Professor

We have read this thesis and recommend its acceptance:

J. T. Miles, B. J. Demott, M. R. Johnston

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

May 22, 1962

To the Graduate Council:

I am submitting herewith a thesis written by Taher A. El-Farekh entitled "Quantitative Study of Growth of Some Dairy Psychrophiles." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

W. W. Overcast
Major Professor

We have read this thesis
and recommend its acceptance:

J. T. Miles

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Melvin B. Johnston

Accepted for the Council:

Hilton A. Smith
Dean of the Graduate School

QUANTITATIVE STUDY OF GROWTH OF SOME DAIRY PSYCHROPHILES

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Taher A. El-Farekh

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T. A. F.

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CHAPTER I

INTRODUCTION

The psychrophilic bacteria have been of concern in the dairy field for the past half century. Their ability to grow at refrigeration temperatures in market milk and other dairy products is becoming more important because of the longer periods of holding before consumption. Growth and multiplication of these bacteria cause the undesirable changes in milk rather than their mere presence. The more rapidly they multiply, the quicker the changes in the milk take place; the longer the growth is delayed and the slower it is, the longer the milk may be retained in its fresh condition.

Although psychrophilic bacteria have been rather extensively studied, few studies (16, 30, 43, 56) have been conducted on the growth and growth characteristics of dairy psychrophiles in pure culture. Accurate quantitative interpretation of certain aspects of the growth phases is still lacking. This is due chiefly to the lack of comparable data on various organisms obtained under controlled reproducible environmental conditions.

The present investigation was undertaken to define more precisely the reproductive cycles of some of the common psychrophilic bacteria under controlled, comparable conditions. From these growth curves, certain generalized quantitative relationships are derived.

CHAPTER II

REVIEW OF LITERATURE

I. DAIRY PSYCHROPHILES

Terminology and Definitions

The first knowledge of the bacteria which grow at low temperature seems to be credited to Forster, 1887. Quoted by Ingraham (23), Forster stated:

> Our bacteria exhibit at certain temperatures a very special property which to my knowledge, at least with pure cultures, has not been previously observed. They grow almost as well in the ice box as at the usual room temperature and even when tubes of streaked nutrient gelatin are placed in a container packed with finely crushed ice in the ice box, that is, at 0° C.

A variety of names have been given to bacteria that can grow at low temperatures. These bacteria are called most commonly, psychrophiles, a term derived from the Greek words psychros, meaning cold, and philos, meaning loving, (cold-loving). The term cryophiles, of Greek origin also, has been used in the literature (25, 35).

Bacteria have been divided roughly into three classes, (19) depending upon their optimum temperature requirements for growth--namely, thermophiles, mesophiles, and psychrophiles. Thermophiles have an optimum temperature above 55° C., and do not grow at 40° C. Mesophiles thrive best between 37° C. and 40° C. They do not grow at 55° C. and are usually dormant at 20° C. Psychrophiles are organisms which grow best at 10° to 30° C. They do not multiply at 40° C.

In reviewing the literature of psychrophilic bacteria, one will immediately notice that the problem of these bacteria is comparatively simple if compared with the problem of their definition. Schultze and Olson (50) accepted the following definition: "The ability to multiply sufficiently rapidly to become a significant contributor to the microflora at a given low temperature." Kennedy and Weiser (26) defined psychrophiles as those organisms which have an optimum temperature range of 5° - 25° C. Ingraham (24) concluded that "probably the best definition of a psychrophile is an organism that grows reasonably well at 0° C. with generation time of less than 48 hours." Presenting different definitions, it seemed necessary to quote Zobell and Conn (60) stating that they have never encountered "true psychrophiles."

According to Standard Methods for the Examination of Dairy Products (2), psychrophiles are defined as those which are capable of relatively rapid growth at low temperatures, commonly within the range of 35° to 50° F. and are detected by incubating plates at 5° - 7° C. for 7 - 10 days.

Type and Ecology

The term psychrophile does not describe a particular taxonomic group of microorganisms, although most of them are Gram-negative, nonspore-forming rods (52).

Erdman (14) isolated 190 cultures of psychrophiles from milk and cream in Canada and obtained the following genera with decreasing frequency: Pseudomonas, Lactobacillus, Streptococcus (lactic acid streptococci), Aerobacter, Flavobacterium, and Escherichia, while there

was one culture of Alcaligenes viscosus (viscolactis).

Schultze and Olson (50) studied the dominating psychrophilic bacteria in commercially pasteurized samples of milk, cream, chocolate drinks and cottage cheese after storage at 4° C. Of the 586 cultures which were studied, 70.6% were species of the genus Pseudomonas, 7.9% Alcaligenes, 9.2% Achromobacter, and 0.7% Flavobacterium. Coliform bacteria were 10.8% and yeasts were .8%.

Writing about psychrophiles in general, Ingraham (23) stated that most psychrophiles appear to belong to the genus Pseudomonas and, to a much lesser extent, to the genera Achromobacter, Flavobacterium, Alcaligenes, and Micrococcus.

While most of the literature (14, 23, 25, 50, 58) reported the genus Pseudomonas as the leading psychrophiles found in commercially pasteurized and stored dairy products, Schultze and Olson (51) found that coliform bacteria are the dominant psychrophilic types in numerous samples of dairy products after storage at 4° C. for 1 week. Dahlberg (11) indicated that psychrophilic coliforms in pasteurized milk held at refrigeration temperatures are remarkably influenced by seasonal variations. After storage for 4 days at 45° - 50° F. and at 55° - 60° F. the coliform bacteria constituted about 5 per cent of the total count in October. During July and August the coliform count became 88 per cent of the total count after storage for 4 days at 45° - 50° F. and 50 per cent at 55° - 60° F.

Most available information (13, 14, 19, 24, 58) indicates that proper pasteurization will destroy the psychrophilic bacteria present in

raw milk. Watrous (58) showed that organisms capable of growth at 5° C. (psychrophiles) are destroyed in fluid milk products at a temperature of 62.8° C. for 30 minutes. Rogick and Burgwald (49) never found psychrophiles in 4.1 ml. of pasteurized milk taken from the vat method or from the high temperature-short time pasteurization system.

Kennedy and Weiser (26) accepted that mesophilic bacteria (with an optimum temperature range of 25° - 45° C.) are capable of adapting to growth at lower temperatures. The fact that most psychrophilic bacteria grow optimally at 21° - 32° C. made Lawton (30), Rogick (49) decide that such bacteria are facultative rather than obligate psychrophiles.

In a study made by Mikolajcik (33) on pure cultures of mesophilic-thermoduric, the results showed that only 8 out of 150 cultures studied showed psychrophilic tendencies. After a storage period of 7 days, 27 of the 150 showed psychrophilic tendencies. In this conversion, some organisms did develop the ability to grow at the lowered temperatures. However, growth at the lowered temperatures was not vigorous.

Source and Distribution

The present standing, as pointed out previously, emphasizes that psychrophilic bacteria are destroyed by pasteurization. Thus, their presence in pasteurized milk is due to post-pasteurization contamination or faulty pasteurization. The data of Thomas (55) presented in Table I illustrate the fact that psychrophilic organisms may be introduced at one or more points during post-pasteurization handling of the product.

Labots et al. (28) showed that air is of major importance in contaminating pasteurized milk while processing. The bacterial content

TABLE I

GEOMETRIC MEAN COLONY COUNTS/ML. AT 3° - 5° C.

Plant Series	Bulk Raw Milk	Off Holder	Off Cooler	From Filler	Bottled Milk
1	17,780,000	0	0	0	0
2	112,000	0	0	141	155
3	223,000	0	0	741	2,138
4	4,169,000	1	0	44	21,880
5	3,580,000	0	110,000	580,000	4,600,000

of the surrounding air during bottling varied for the several experiments from 20,000 to 60,000 per liter. The number of bacteria in the empty bottles were two to three times higher than the bacterial content of the corresponding volume of air. This large difference was explained tentatively as a result of sedimentation of bacteria in the bottles. Such findings might stimulate dairy industries to processing and filling milk under proper vacuum and under more sanitized conditions.

Lubert et al. (32) found that Pseudomonas fluorescens, an important psychrophile, that was present in waters of the province, occurred almost universally in such products as raw milk and cream and was frequently involved with production of rancidity in products. Morrison and Hammer (36) considered Pseudomonas fragi, another important psychrophile, as being widely distributed in waters and dirts, and emphasized the importance of farms as a source of the organisms, and did not consider dairy plant equipments as the main source of this organism. Erdman and Thornton (13) stated that the major source of the psychrophilic bacteria in general, is non-sterile utensils and their presence in freshly pasteurized milk indicates inefficient plant sanitation. Claydon et al. (8) found that the addition of certain amounts of water to milk created more suitable conditions for initial development to some bacterial species than was provided in undiluted milk. This finding might put more emphasis on the partially cleaned utensils as a great source of psychrophilic contamination.

Although hypochlorites are considered effective against psychrophilic bacteria and used widely in sanitizing water and equipments in

the dairy plants. Kristoffersen (27) reported a strain of Pseudomonas fluorescens which proved to be relatively resistant to sanitizers of all types.

Growth and Defects

The development of defects in dairy products due to the growth of psychrophiles was first investigated by Conn (9), 1903, who stated that "milk preserved at 50° F. or lower will keep sweet for a long time, but it becomes filled with bacteria of a more unwholesome type than those that grow at higher temperatures." Numerous defects may be attributed to their activity. Some of the more common flavor and aroma defects were described by Erdman (14) as stale and oxidized, sour, and bitter predominating. An unusual bitter flavor was found to be caused by certain strains of the genus Pseudomonas (Pseudomonas fluorescens) in milk held at 4.5° C. for 15 days. Hussong et al. (22) studied the significance of Pseudomonas fragi in dairy products. They indicated the ability of this organism to cause ropiness, May-apple odor and later the rancidity of milk. Both the Pseudomonas fluorescens and Pseudomonas fragi are noted frequently in the literature (18, 36, 43) for their strong lipolytic activity and cause of rancidity.

Overcast and Skean (43) studied twenty-five pure psychrophilic cultures which belonged to the genera, Pseudomonas, Achromobacter, Alcaligenes, and one yeast. They observed the lipolytic ability of all the cultures within fifteen days at 5° C., 10 days at 10° C., and 2 days at 21° C.

In addition to the ability to attack milk constituents and produce undesirable flavors and odors, some psychrophiles are capable of growth on the surface of products like butter and cause discoloration (21). However, certain species in milk produce no detectable changes, but may multiply rapidly during refrigerated storage and may raise the bacterial count above that acceptable as a legal maximum (16).

Nicholas and Anderson (41), studying the keeping quality of pasteurized milk with a bacterial content ranging from 3,000 to 92,000 per ml., found that such milk, if kept in the home refrigerator at 40° F., would retain high quality from 10 days to two weeks. The same milk was removed daily, shaken and permitted to stand at room temperature for one hour before being returned to storage.

Boyd et al. (4) reported that the keeping quality of commercially pasteurized and homogenized milk stored at 40° F. will be retained 13 to 18 days based on a 50,000 per ml. bacterial standard. When stored at 33° F., the average keeping quality of the milk was extended an additional 11 to 14 days. Flavor deterioration was correlated with the growth of psychrophilic bacteria at both storage temperatures. Burgwald and Josephson (6) found that initial bacterial counts (standard agar plate) of the milk did not always indicate its potential keeping quality. One lot of pasteurized milk samples which contained bacterial count of 61,000 per ml. remained sweet for 25 days at temperatures below 40° F. and for 21 days at a temperature about 40° F. on the half pints and for 13 to 15 days in the quarts. Another lot of samples held below 40° F. and having an initial bacterial count of 7,150 per ml. remained sweet for only 16

days. The half pints and quarts held above 40° F. remained sweet for only 12 days.

Ford (15) stated that 73% of the samples of freshly bottled milk which contained psychrophiles had plate counts greater than 100,000 per ml. after storage for 5 days at 45° F.; 39% of the samples in which no psychrophiles were detected in 1 ml. quantities had counts greater than 100,000 per ml. after storage for 5 days at 45° F. After storage for 7 days at 45° F., flavor scores in the range of 35 to 36.5 were given to 33% of the samples having psychrophiles in 1 ml. quantities when fresh, and to 42% of the samples have no psychrophiles in 1 ml. when fresh.

Watrous (58) and Rogick (49) found psychrophiles in all commercially pasteurized milk of their study by the end of one week of storage at refrigeration temperatures.

Burgwald (7) considered psychrophilic bacteria as being responsible for the acid produced in milk during storage at 40° F. This consideration agrees with the data of Watrous (58) by which the extent of growth of acid producing bacteria (coliforms) is shown.

Dahlberg (12) observed a slight decrease in standard plate count of milk stored one day at 45° - 50° F., while coliform bacteria did not decrease and showed a slight increase in the 2-day-old sample. After 4 days of storage the positive samples ranged from 7 to 10 of the 18 samples. The flavor score for milk held for 7 days was "good" with a numerical average of 38.0. The pasteurized cooled milk held for 6 hours at room temperature showed slightly higher bacterial counts and more positive

coliform tests than shown by the plant samples. In another study by Dahlberg (10) it was found that bacterial counts of milk held at 35° - 40° F. were slightly lower at 4 days than the counts on the same milk when fresh. At 0 day, the average Standard Plate Count was 12,000 as compared to 9,000 at 4 days. Milk of the same initial Standard Plate Count, which was held at 45° - 50° F., showed a Standard Plate Count of 210,000 at 4 days of storage.

Burgwald and Josephson (7) stated that milk of good quality can be expected to retain excellent bacteriological and flavor qualities for at least 4 days during the summer months and 6 to 7 days during winter months if refrigerator temperatures are maintained near 40° F. It was also shown that mesophilic populations are small when compared with psychrophilic populations, and the amount of acid produced by mesophiles is also smaller.

Studying raw milk, Babel (3) found that the bacterial flora of milk held at 4.4° C. for 2 to 4 days are largely of psychrophilic bacteria which resulted in changes of milk acidity. He also pointed out that the rate of coagulation of milk by rennet varies considerably with extended holding at low temperature and shows a direct relation to the type of bacteria present. It was concluded that rennet coagulation time increases with an increase in holding time for 2 to 3 days, and then decreases. The decrease was thought to be associated with measurable changes in pH. Morris (35) observed a pH change in samples of morning milk which failed to pass the methylene blue test after being held overnight in a refrigerator at 4° C.

Olson et al. (42) made an extensive study on the keeping quality of pasteurized milk. They presented data in which it was pointed out that commercially pasteurized milk frequently keep for 7 to 10 days of storage, and the rapidity with which deterioration takes place will depend largely upon the initial number of organisms present. The rate of growth and the capability to cause organoleptic defects are influenced largely by the initial number of these organisms. The different capability to cause organoleptic changes is particularly evident from the data shown, where one plant had a milk sample with extensive growth occurring but with no concurrent detectable flavor deterioration. This serves as an excellent example of relatively inert bacterial activity.

Greene and Jezeski (16) studied the biochemical activities of three psychrophilic cultures of Aerobacter aerogenes, and two pseudomonads, and found a definite relationship between observed deteriorative changes and the numbers of viable cells present, as well as, the stage of growth of the cultures. Proteolysis occurred when the cell count was between 1.1×10^8 to 1.5×10^9 cells per ml. Lipolytic activity became measurable when the cell count ranged from 1.3×10^8 to 1.3×10^9 per ml. The titratable acidity showed its initial rise when the population reached 8.4×10^7 to $7.5 \times 10.^8$ per ml.

Van der Zant and Moore (56), studying the influence of storage temperatures on growth of four pseudomonad cultures, found an increase in soluble nitrogen, tyrosine, and tryptophan within 24 hours of storage at 25° and 21° C. when the cell count was about 10^7 cells per ml. At 10° and 5° C., however, proteolysis was negligible during the first 5 days

but became detectable when the population reached 10^6 to 10^7 cells per ml.

Using lard, Alford (1) observed that Pseudomonas fluorescens cells grown at 31° C. produced very little lipase during a given incubation period, whereas appreciable quantities were produced at 5° and 20° C. Nashif and Nelson (38) stated that the maximum production of extracellular lipase by Pseudomonas fragi occurred in 3 days at 15° C. or below, while little or no detectable lipase was produced at 30° C., although the bacterial counts were over a billion per milliliter. They (37) also noted that lipase produced by the same organism is more stable when allowed to stand at 3° to 5° C. On the other hand, Peterson and Gunderson (47) reported that an increase in temperature from 0° C. to 20° C. increased proteolysis.

Observing the growth of Pseudomonas species in butter, Hiscox (21) observed no pigment production at 22° C. (optimum growth temperature for the studied species), while at 1° - 3° C. growth was slow but abundant pigmentation resulted.

Pereira and Morgan (45) showed that the production of esters responsible for the fruity aroma in milk cultures is markedly affected by the previous nutrition and/or incubation temperature of Pseudomonas fragi. In another report they (46) detected fruity aroma in all cultures in which alanine, or glutamic acid was the carbon source or in which threonine was the nitrogen source.

Alford (1), Lubert (32), and Peterson (47) observed that alkaline pH stimulates extracellular enzyme production. Lubert (32) pointed out that the optimum pH for lipase enzyme activity is between 8 and 9. While

Parker (44) observed that pH 5 effectively retarded Pseudomonas fragi and shaking of the growth medium twice daily was noticed by Nashif and Nelson (38) to lower both cell population and lipase production.

II. GROWTH

Concerning the growth phenomenon of bacteria, Henrici's (20) quotation of D'Arcy Thompson is fitting.

> It is perfectly true that all changes in form inasmuch as they necessarily involve changes of actual or relative magnitude, may in a sense be properly looked upon as phenomena of growth; and it is also true, since the movement of matter must always involve an element of time, that in all cases the rate of growth is a phenomenon to be considered.

Smith et al. (53) stated that "bacterial growth consists of a succession of phases characterized by variations in the rate of change of the population." This statement, in addition to factors affecting the growth phases, will be the definition applied to the following review.

The Growth Curve

When a suitable nutrient medium is inoculated with viable bacteria and the rate of growth of the culture followed, a characteristic growth curve may be obtained. The growth of a population as a function of time usually is plotted on a semi-logarithmic scale; the logarithm of the number of bacteria per milliliter being plotted against time. Most of the growth curves (19, 34, 53, 54) which were reviewed were hypothetical. They represented in a general way the growth behavior of bacteria subsequent to their being transferred from an unfavorable environment to one which is favorable for their growth.

Figure 1 (5, 29, 53) represents a typical growth curve in which the cycle has been divided into four phases. Monod (34) believed that the life cycle of bacteria is more complicated than this, thus he subdivided the curve into six phases, Figure 2.

It should be emphasized that a curve representing the growth behavior of a given species of bacteria under one set of environmental conditions will never be identical in form to a curve representing the behavior of the same species under any other set of conditions. Inoculating Mycobacterium tuberculosis, strain BCG, into broth tubes under certain conditions, Volk (57) obtained a linear growth curve. When these controlled conditions were modified, a typical logarithmic growth curve was obtained.

Factors Affecting Growth

From the standpoint of dairy bacteriology, the chief interest lies on factors influencing the length of the lag phase and the rate of growth of bacteria in the rapid growth phase. Therefore, only the lag phase and the rapid growth phase will be discussed further.

Greene (17) found that the incubation temperature of the mother culture had a definite effect on the response of the inoculums to their subsequent incubation temperatures. There was a better response at 20° C. when inoculums were taken from 5° and 20° C. than when taken from 37° C. He also observed that exposure of pasteurized milk to a low temperature (2° C.) followed by exposure to a higher temperature (8° C.) resulted, in every case, in a greater count than did exposure to a higher temper-

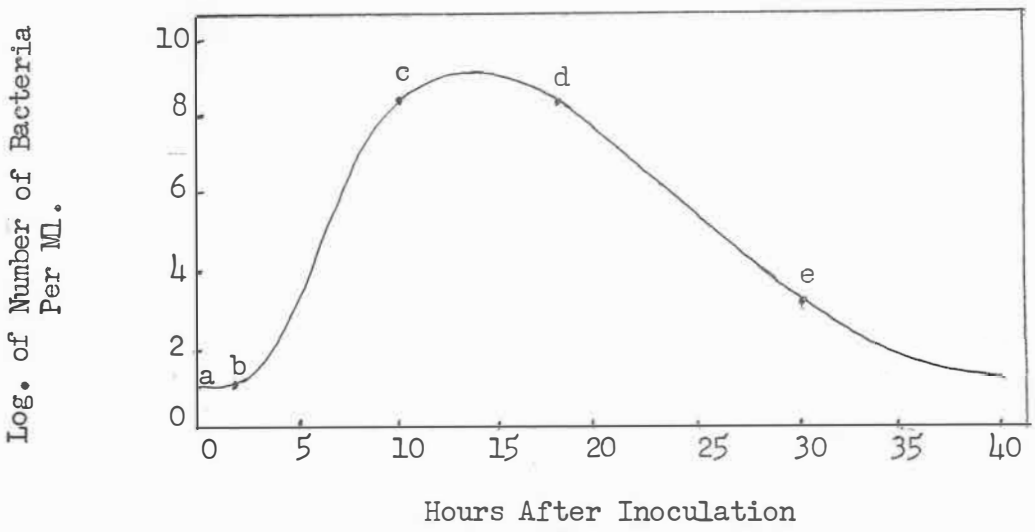

Figure 1. Typical growth curve (53). The four phases of growth are presented:

a to b, an initial period of slow or of no growth

b to c, a period of regular growth

c to d, a period when the numbers remain more or less stationary

d to e, a period when the numbers of living bacteria are diminishing.

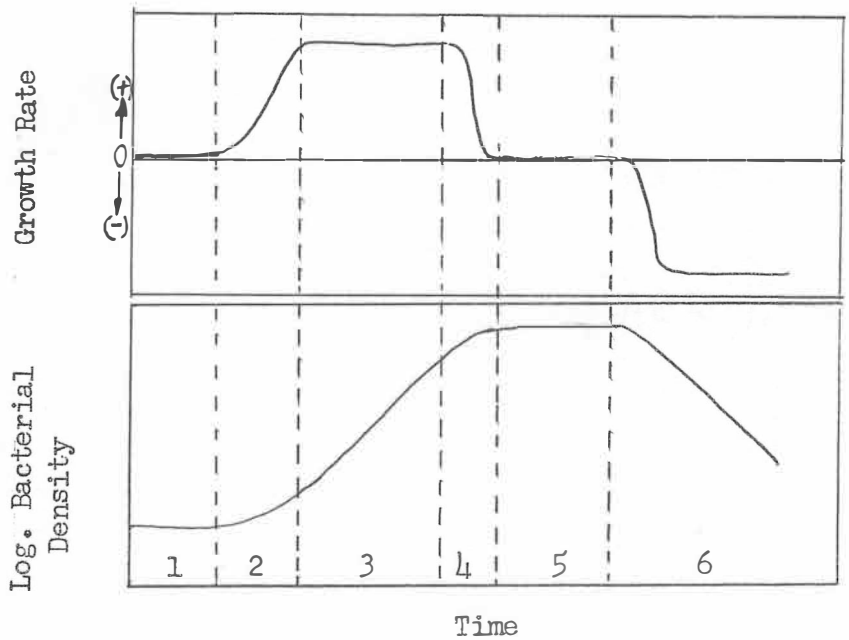

Figure 2. Phases of Growth (34).

1. Growth phase, growth rate null
2. Acceleration phase, growth rate increases
3. Exponential phase, growth rate constant
4. Retardation phase, growth rate decreases
5. Stationary phase, growth rate null
6. Phase of decline, growth rate negative

ature followed by exposure to a lower one.

Stumbo (54) recognized that the size of the inoculum affects both the length of the logarithmic phase and the rate of growth. Stumbo (54) stated that the greater the number of organisms present, the shorter will be the lag phase of growth and the more rapidly will bacterial multiplication occur in the rapid growth phase. Lawton (30) showed that if the milk supply is contaminated with as little as a 20 cells of physchrophilic bacteria per ml. they tend to increase, even at 5° C., to about 10 million after only 3 - 4 days.

The age of bacteria has been considered in explaining the lag phase by several workers (5, 20, 54). The older the organisms, the longer their lag phase will be. Comparing organisms entering food with dust with the same numbers of organisms entering with small amounts of food which have remained for several hours on improperly cleaned equipment, Stumbo (54) found that organisms entering with the dust will ordinarily be relatively old and will show considerable growth lag. Organisms in the food left on the equipment will, on the other hand, very likely be quite young and after entering the fresh food may demonstrate very little or no growth lag.

The lag phase is more distinct with sporulating microorganisms (5) when a suspension of bacterial spores are placed in a suitable culture medium microscopic observation will show that growth does not apparently begin immediately. There will be no increase in numbers until the spores have germinated and begun to multiply.

It should be emphasized that the lag phase does not mean a complete

dormancy of bacterial cell. During the early hours of growth (lag phase) the cells increase considerably in size at a time when little or no cell division is occurring (53). Lichstein (31) pointed out that during the lag phase increases in cell mass are detectable almost immediately, whereas cell division may be delayed substantially. Thus, during the initial growth phase optical density measurements of culture turbidity are preferred in measuring growth to viable counts. It should be noted, however, that turbidity is affected by changes in the refractive index of the cells, as well as by changes in the shape of the cells, and that dead cells contribute to the total turbidity. Therefore, it is clear that methodology plays an important role in determining the early period of the growth cycle namely the lag phase.

The more suitable the environment the shorter will be the lag period or phase of adjustment and the more rapidly the growth. Of the many environmental factors operative Lichstein (31) discussed temperature, pH, and oxidation-reduction potential. These have been demonstrated to affect remarkably the nutritional requirements of microorganisms as well as the initiation of growth. These factors and others were pointed out previously under dairy psychrophiles growth and defects. A further explanation of the mechanics of pH and oxidation-reduction potential presented by Lichstein (31) is:

1. pH: pH may exert some of its effect by controlling the permeability of the bacterial cell to some substance essential for growth initiation.

2. Oxidation-reduction: It is recognized that a reduced potential must be developed before such micro-organisms can commence growth.

The rate of growth of aerobic bacteria in a stationary culture can be a function of the rate of which oxygen diffuses into the medium has been demonstrated by several groups (38, 57). In such cases forced aeration or mechanical agitation of the culture will improve both the initiation and the subsequent rate of growth. It should be emphasized that relatively minor changes in the environment may affect markedly the ability of microorganisms to initiate growth and the rate of this growth.

Watrous (58) isolated 35 colonies from plates incubated at 5° C. for 10 days. These plates represented samples of chocolate milk, cream and skim milk which had been held at 5° C. for 10 days before plating. He incubated these cultures at 35°, 25°, and 5° C. Growth was observed in 24 cultures at 25° and 5° C., but not at 35° C.; two cultures failed to grow on plates incubated at 5°, 25°, or 35° C., and four grew at 35°, 25° and 5° C. These findings show the inadequacy of using growth range as a definition of psychrophiles.

Greene and Jezeski (16) observed that the temperature coefficients (Q10) for both growth and biochemical activity were considerably higher near the minimum temperatures studied than they were at the higher levels. In certain cases these values indicated that cooling from 5° to 0° C. increased the keeping time of milk by approximately the same number of days as did cooling from 30° to 5° C. This study, in addition to several other studies (4, 9, 13, 19, 30, 33), emphasized clearly that milk should not be stored at temperatures higher than 5° C.

Stumbo (54) stated that food packing may well be considered as representing a battle between man and microbe. Therefore, application of

knowledge concerning the growth behavior of bacteria should constitute the initial step in the packer's effort to "out wit" the microbe. Bacterial decomposition of food or food components is brought about by the growth of bacteria in the food. An understanding of the factors which influence bacterial growth is for this reason essential for successful food plant control.

CHAPTER III

EXPERIMENTAL METHODS

The psychrophilic bacteria used in this study were Pseudomonas fluorescens (13525), Pseudomonas fragi (4973) obtained from the American Type Culture Collection, Washington, D. C., and Brevibacterium lipolyticum obtained from the Culture Collection of the Dairy Microbiology Laboratory at the University of Tennessee. Two trials using Pseudomonas fluorescens are designated A and B and one trial each with Pseudomonas fragi and Brevibacterium lipolyticum are designated C and D, respectively. Culture E represents the flora of milk that results from post pasteurization contamination. The trial with culture E was skim milk taken directly from the bottle filler and treated as a pure culture study.

All milk used in this study except for culture E was commercially pasteurized skim milk which was placed in one liter quantities, in sterile two-liter Erlenmeyer flasks. The flasks were closed with parchment covered rubber stoppers in such a manner as to aid in the prevention of external contamination during sampling. Three flasks of milk were used in each trial and steamed for 25 minutes at approximately 100° C. to assure a minimum of vegetative cells in the milk.

Stock cultures of the organisms were maintained at 4° C. in screw-cap tubes on Milk-Protein Hydrolysate (M-PH) agar slants. Prior to use each culture was transferred twice to M-PH agar slant and incubated at 25° C. for 24 hours. The third transfer was made on a large agar surface formed by placing 30 to 35 ml. of agar in a 6 oz. screw-cap

bottle and allowing the agar to solidify on the side of the bottle. The growth from the third transfer was washed from the surface with 5 ml. of dilution water as prepared by Naylor and Smith (39). This cell suspension was used to prepare the inoculum. Inocula were standardized by using a Spectronic-20 Colorimeter manufactured by Bausch and Lomb. One ml. of the cell suspension was diluted in 69 ml. of water. This dilution gave a transmission of 83% at 600 mu wave length, and repeatedly had a bacterial count of 500 million organisms per ml. Each inoculum was prepared on this basis except culture A which had a 77% transmission in order to obtain a larger initial cell population.

The prepared milk was inoculated with 10^{-4} ml. quantities (5×10^4 cells) of the diluted cells. The flasks of milk were stored at $4 \pm 1^{\circ}$ C. immediately after inoculation and maintained at this temperature throughout the study period. One ml. samples were taken at each test period and plated at the appropriate dilutions on M-PH agar and incubated at 25° C. for three days as recommended by Nelson and Baker (40). The samples were taken aseptically and the flasks at the time of sampling were maintained in iced water to prevent temperature fluctuation of milk. Several dilutions were plated at each sampling period, but only 2 plates were chosen per flask for counting, thus having a total of 6 countable plates for each culture at each test time. Wilson (59) reports that such a sampling procedure gives statistically reliable data.

CHAPTER IV

RESULTS AND DISCUSSION

The quantitative study of the growth of four pure cultures of psychrophilic bacteria and the mixed flora of commercially processed skim milk are presented in the form of growth curves. These growth curves were drawn by visual inspection from the logarithmic averages of 6 counts. The data from which these curves were derived are presented in the appendix. The following generalized quantitative relationships are derived from these growth curves.

Generation Time

The generation time of the logarithmic growth phase for each curve was calculated using the following formula:

$$g = \frac{T \log 2}{\log b - \log a}$$

Where g is the generation time in hours, a is the number of viable cells at the start of the phase and b the number after the time T. The points a and b are the limits of the straight line in the exponential growth phase where the growth rate is constant.

The generation times of the Pseudomonas fluorescens cultures, A and B Figures 3 and 4 were the same, 7.22 hours. This indicates that the generation time was not affected by the difference in size of the initial inoculum. (The initial inoculum for culture A averaged 1916 cells

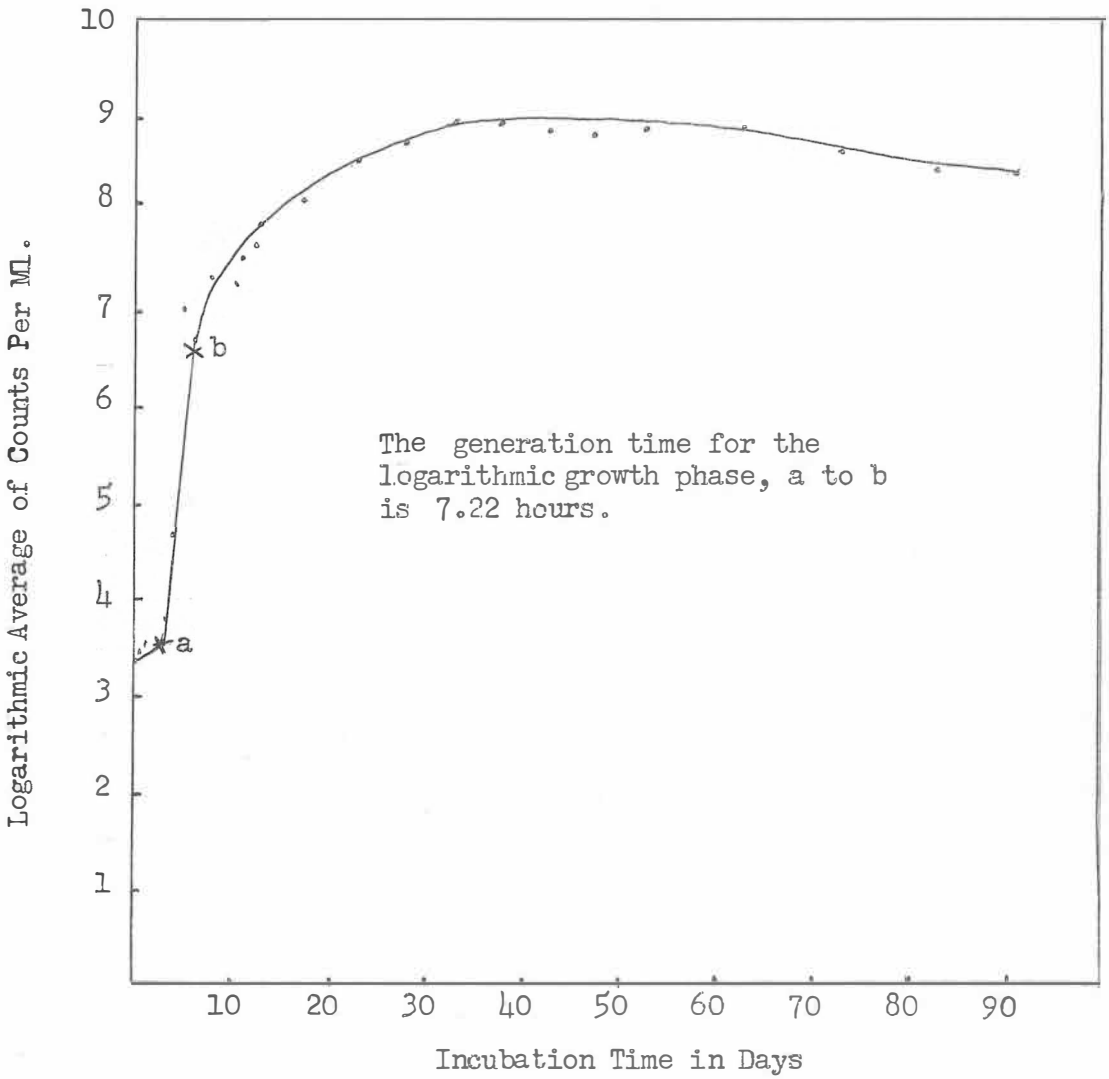

Figure 3. Growth curve of a pure culture of Pseudomonas fluorescens (A), 13525, grown in skim milk at 4° C.

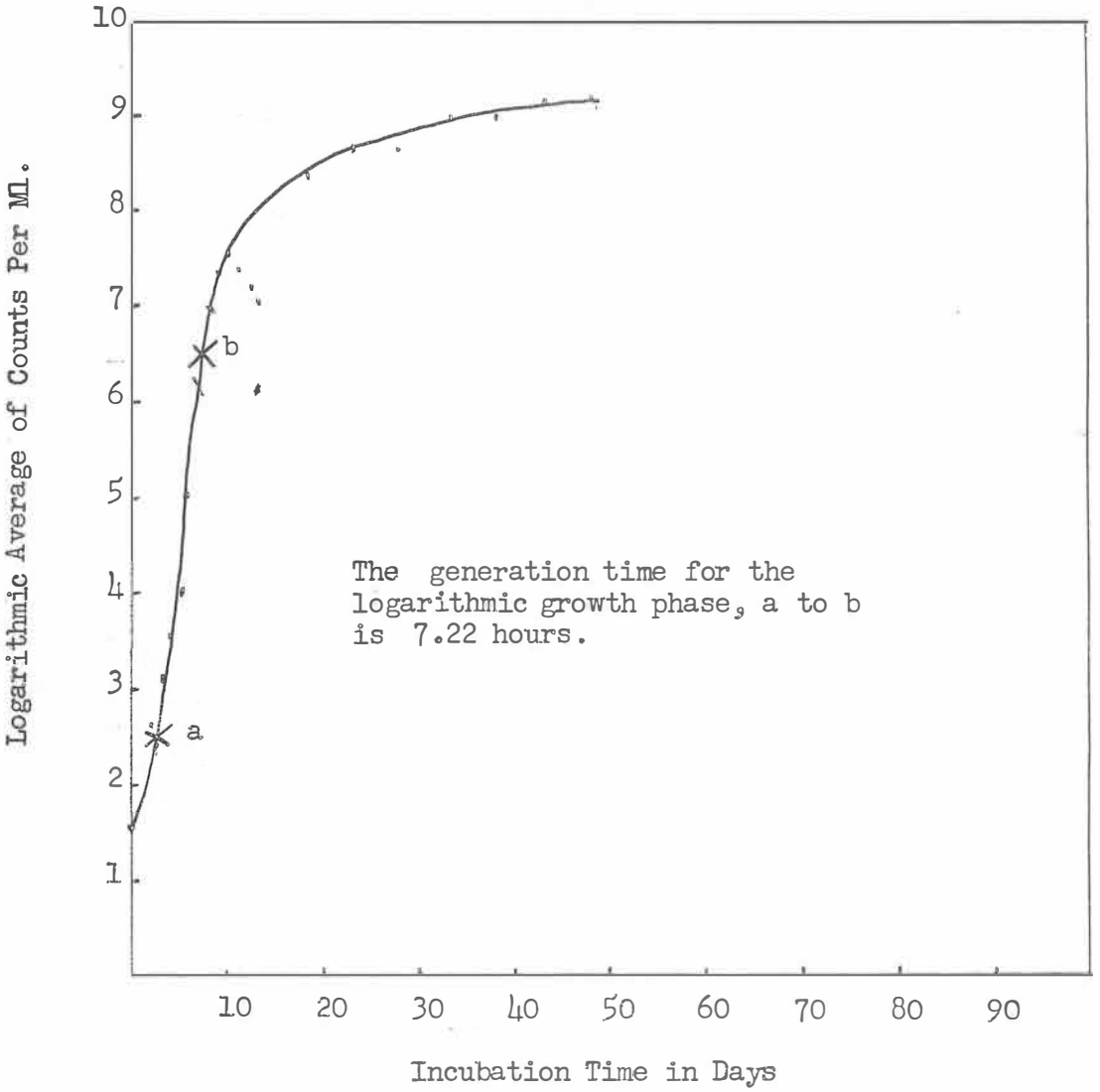

Figure 4. Growth curve of a pure culture of Pseudomonas fluorescens (B), 13525, grown in skim milk at 4° C.

per ml. compared to an average of 26 cells per ml. in culture B). Van der Zant and Moore (56) obtained a generation time of 432 minutes (7.20 hours) for a tentatively identified culture of Pseudomonas fluorescens grown in skim milk at 5° C. with initial inoculum of about 3500 cells per ml.

The generation time for Pseudomonas fragi, Figure 5, was 5.55 hour; Brevibacterium lipolyticum, Figure 6, 11.24 hours; and for culture E, Figure 7, 8.08 hours. Knowing the generation time of a pure culture and the initial inoculum, one could determine the approximate bacterial count of the culture after a given period of time, and finally the extent of changes in the growth media that may be anticipated.

Length of Lag Phase

It is evident from the standpoint of dairy bacteriology, that the chief interest lies on factors influencing the length of the lag phase. Some of these factors are: the size of the inoculum (30), the age of bacteria (20), and the most important factor is temperature of storage (17). These factors and others were reviewed previously.

The lag phases in this study were the portions of the curves between the initial inoculum point and the point a, the beginning of the exponential phase. The length of the lag phase was 3 days for cultures A and B, Figures 3 and 4. This indicates that the difference in the two inocula was not large enough to cause any difference in the lag phase as pointed out previously (30) or the size of the inocula may not have any effect on the lag phase as shown in Figures 1 and 2.

The lag phases for culture C, Figure 5, was 1 day; culture D,

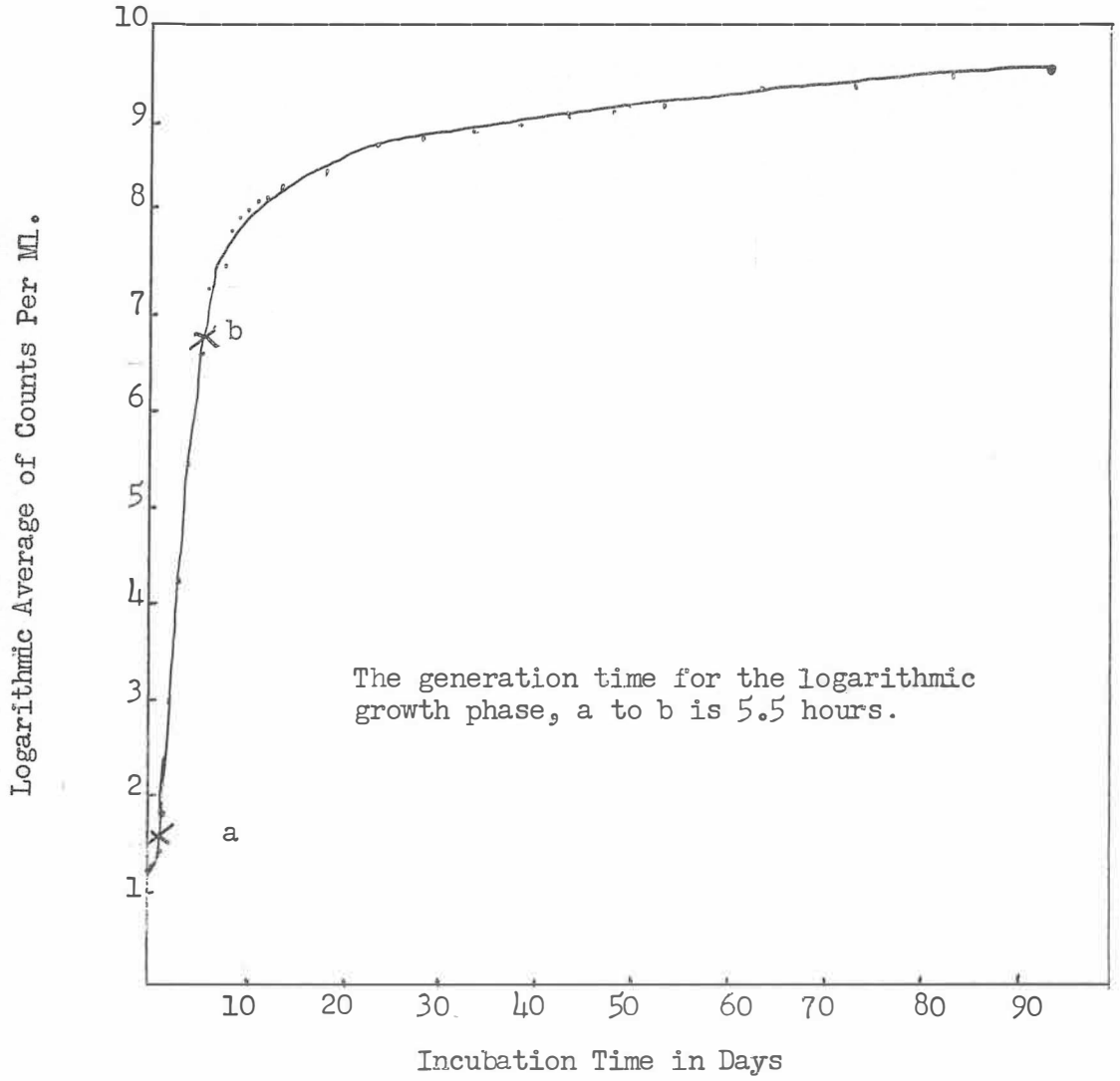

Figure 5. Growth curve of a pure culture of Pseudomonas fragi (C),(4973), grown in skim milk at 4° C.

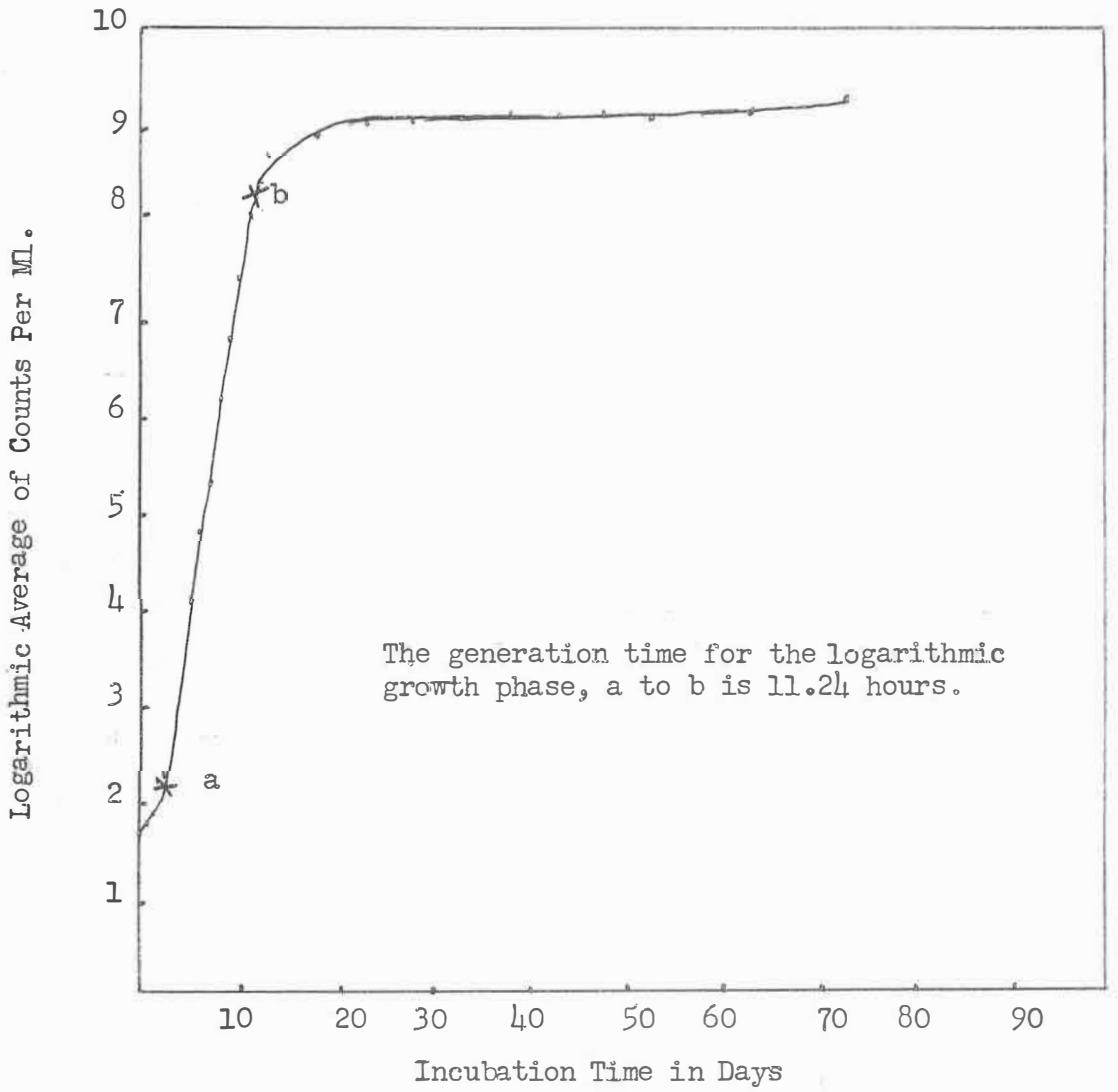

Figure 6. Growth curve of a pure culture of Brevibacterium lipolyticum (D), grown in skim milk at 4° C.

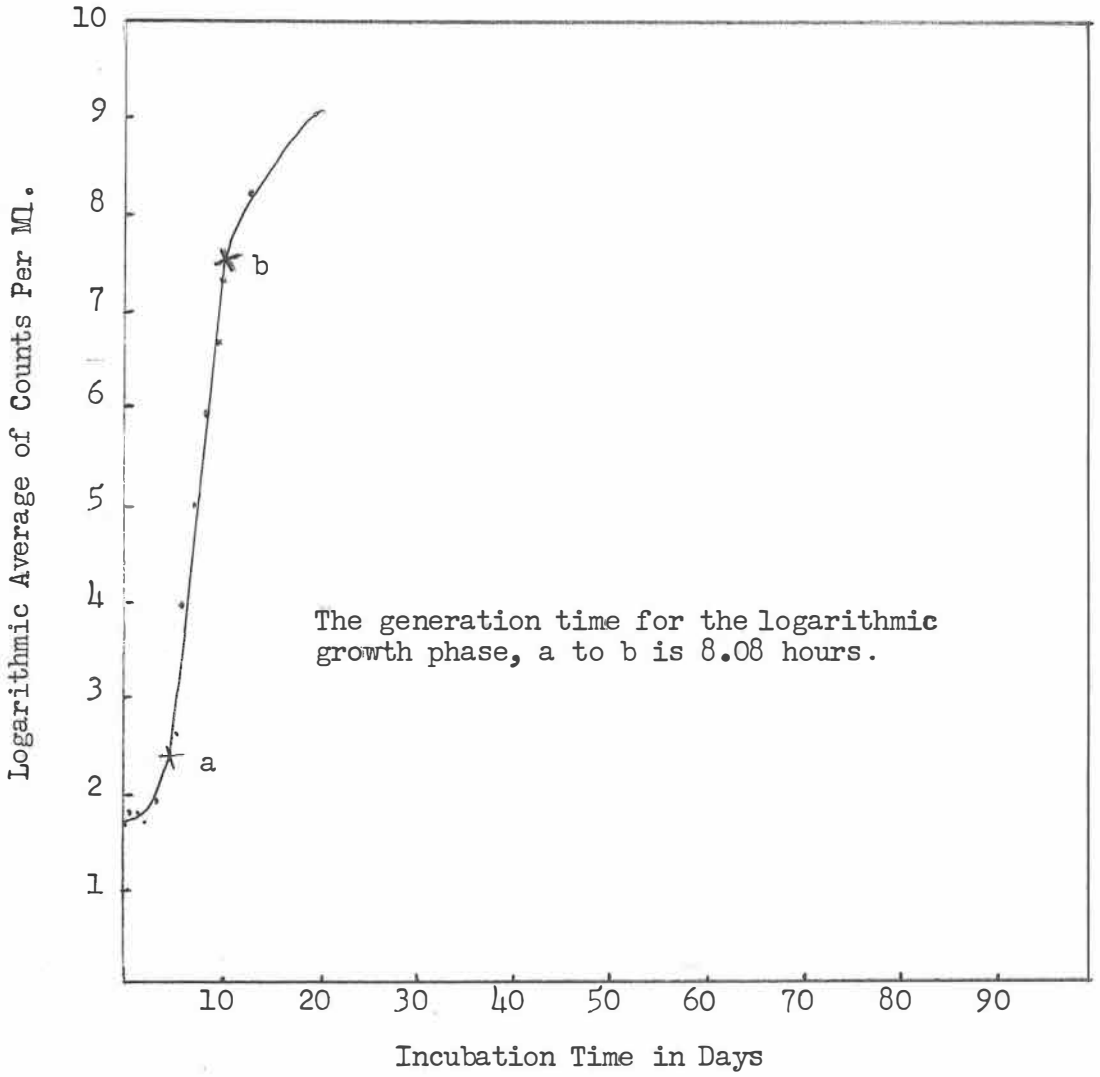

Figure 7. Growth curve of the mixed flora of commercially processed skim milk (culture E) grown at 4° C.

Figure 6, 2 days; and culture E, Figure 7, 4.2 days. The data presented in this study and other studies (16, 30, 43, 56) indicate that psychrophilic bacteria grow very rapidly at cold storage temperatures and may possess very short lag phases.

The lag phase in the pure cultures ranged from 1 day for culture C to 3 days for cultures A and B. The pre-inoculation treatment was the same for all pure cultures. Any explanation for this difference may be the genetic differences which enable certain psychrophilic bacteria to adjust and commence growth in a shorter time than others. Culture E, a mixed flora, had the longest lag phase of all cultures studied 4.2 days. This might be explained by more than one reason: firstly, in the pure cultures study, the inocula were taken from activated culture as previously described, thus younger cells were obtained. The age of bacteria has been considered in explaining the lag phase by several workers (5, 20, 54). Stumbo (54) stated that organisms entering with the air will ordinarily be relatively old and will show considerable growth lag. Labots et al. (28) showed that air is of major importance in contaminating pasteurized milk while processing.

Secondly, pasteurized milk is contaminated with mesophilic bacteria which apparently take considerable time in adjusting to growth at lower temperatures (26). In a study made by Mikolajcik (33) on pure cultures of mesophilic-thermoduric organisms, the results showed that only 8 out of 150 cultures studied showed psychrophilic tendencies. After a storage period of 7 days, 27 of the 150 showed psychrophilic tendencies.

Counts After 7 Days of Storage

The storage of market milk for 7 days is not uncommon practice in the dairy industry. This storage time is also significant because commercially pasteurized milk keeps in good quality for an average of 7 days (42). However, pure cultures of psychrophilic bacteria inoculated in pasteurized milk were found (43) to cause changes in milk flavor at 4 to 8 days of storage.

The average bacterial counts after 7 days of storage for culture A was 1.6×10^7 cells per ml., and 2.3×10^6 cells per ml. for culture B. At 10 days of storage both cultures obtained the same bacterial count, but a sudden unexplainable drop in bacterial count of culture B was observed at the 11th., 12th. and 13th. day of storage. The counts of culture B were increased and by the 18th. day it reached the same level of growth as culture A.

The average bacterial counts after 7 days of storage was 3.0×10^7 cells per ml. for culture C; 1.9×10^5 cells per ml. for culture D; and 1.7×10^5 cells per ml. for culture E. Comparing the pure cultures B, C, and D which had about the same initial inoculum size of less than 50 cells per ml., we find that culture C obtained the highest bacterial count followed by culture B and the least by culture D.

The incubation times of the cultures studied ranged from 20 days for culture E to 93 days for culture C. Culture A was the only one to reach the phase of decline, Figure 3, while the other cultures were still in the retardation phases, Figures 4, 5 and 7, and in the stationary phase for culture D, Figure 6.

Greene and Jezeski (16), in their study on some psychrophiles found that proteolysis occurred when the cell count was between 1.1×10^8 to 1.5 to 10^9 cells per ml. Lipolytic activity became measurable when the cell count ranged from 1.3×10^8 to 1.3×10^9 per ml. Comparing the counts of all cultures used in this study with the data of Greene and Jezeski (16) we find that none of the cultures grew to a large enough population at 7 days of storage to cause any detectable change by their standards. However, Overcast and Skean (43) detected rancidity at 4 days of storage in pasteurized milk inoculated with pure cultures of the Pseudomonas genus.

Undoubtedly differences exist in different species or different strains within the species in their ability to bring about noticeable changes in the milk. A count of 3.3×10^6 after 4 days at 40° F. in the study by Overcast and Skean (43) produced a detectable rancid flavor and a decided increase in the free fatty acid value. This count is considerably lower than the count reported by Greene and Jezeski (16) as necessary to produce detectable lipolysis.

Quantitative studies on the growth of psychrophilic bacteria common in milk furnish the dairy industry with experimental data on which to base their decisions for the handling of milk. It is evident from this study with generation times as short as 5.5 hours and lag phases as short as one day extreme precautions must be exercised in order to prevent contamination with psychrophilic bacteria. Since psychrophiles do not survive current pasteurization temperatures, emphasis on post pasteurization contamination is necessary in order to process milk with a maximum

shelf life. The trend toward ultra high-temperature pasteurization can aid in attaining a maximum shelf life provided the packaging of the product can be accomplished under a system of essentially asceptic conditions.

CHAPTER V

SUMMARY AND CONCLUSIONS

Samples of pasteurized skim milk were steamed for 25 minutes at approximately 100° C. and inoculated with pure cultures of Pseudomonas fluorescens, A and B, at different levels, Pseudomonas fragi, Brevibacterium lipolyticum. The fifth culture was the normal flora of commercially pasteurized skim milk treated as a pure culture. All samples were stored at 4° C. Bacterial growth was determined using agar plates incubated at 25° C. for 3 days.

The generation time was calculated for all cultures which ranged from 5.55 hours for Pseudomonas fragi to 11.24 hours for Brevibacterium lipolyticum. The generation time was found the same (7.22 hours) for Pseudomonas fluorescens cultures A and B regardless of differences in inoculum size for the two cultures.

In the pure cultures, bacterial count after 7 days of storage was the highest for Pseudomonas fragi, 3.0×10^7, and the lowest for Brevibacterium lipolyticum, 1.9×10^5 cells per ml. However, the mixed flora culture obtained a slightly lower count, 1.7×10^5 cells per ml.

The lag phases for the pure cultures studied ranged from 1 day for Pseudomonas fragi to 3 days for both cultures of Pseudomonas fluorescens. The mixed flora culture showed still a longer lag phase of 4.2 days.

Of all cultures studied, Pseudomonas fragi was found to be the most actively growing culture. It had the shortest generation time, the highest counts after 7 days of storage, and the shortest lag phase.

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APPENDIX

TABLE II

DUPLICATE COUNTS OF THREE CULTURES OF PSEUDOMONAS FLUORESCENS (A) GROWN IN MILK STORED AT 4° C.

Age of Culture In Days	Culture I		Culture II		Culture III	
	1	2	1	2	1	2
	Bacterial Counts x 10^4 per ml.					
0	0.195	-	0.205	-	0.175	-
½	0.290	-	0.300	-	0.250	-
1	0.310	-	0.320	-	0.380	-
2	0.320	-	0.290	-	0.350	-
3	0.710	0.810	0.650	0.600	0.640	0.690
4	6.2	6.5	5.8	6.3	3.2	3.6
5	1260	1220	1160	1030	800	848
6	830	950	141	150	107	101
7	1630	1560	1500	1610	1700	1570
8	1680	1730	1690	1720	1850	1870
9	1040	1130	1170	1090	1210	1150
10	1900	1790	1800	1930	2010	1880
11	3300	3600	3200	3600	3100	3500
12	5200	6900	5700	4500	4200	3700
13	7100	8100	7200	7300	9200	8300
18	14500	15100	9500	8900	15600	16100
23	34900	34000	33000	32000	37000	35900
28	50800	52500	55900	58000	49400	49100
33	78000	86000	112000	114000	88000	97000
38	89000	73000	88000	90000	95000	85000
43	78000	73000	53000	46000	87000	85000
48	86000	68000	71000	62000	63000	67000
53	66000	77000	87000	81000	79000	63000
63	76000	87000	83000	65000	88000	91000
73	40000	39000	40000	35000	66000	65000
83	23000	30000	27000	20000	45000	39000
92	22000	18000	33000	36000	44000	40000

TABLE III

DUPLICATE COUNTS OF THREE CULTURES OF PSEUDOMONAS FLUORESCENS (B) GROWN IN MILK STORED AT 4° C.

Age of Culture In Days	Culture I		Culture II		Culture III	
	1	2	1	2	1	2
	Bacterial Counts x 10^4 per ml.					
0	.0021	.0029	.0029	.0026	.0027	.0024
2	.0403	.0417	.0392	.0413	.0443	.0456
3	.11	.125	.113	.146	.130	.152
4	.355	.380	.403	.391	.411	.430
5	.9	1.01	1.13	1.07	1.45	1.57
6	7.2	6.5	12.8	10.06	15.0	13.6
7	40.6	38.4	220.0	205.0	454.0	436.0
8	1250	1270	1040	995	572	600
9	2100	2300	2160	1980	2070	1880
10	1910	1800	4600	5400	4800	4400
11	1820	1650	1920	1620	5000	4200
12	1250	1170	940	960	2320	2160
13	1130	1310	1260	1500	530	740
18	20900	22900	33400	32100	25100	23600
23	38000	40000	48000	45000	46000	47000
28	35000	32000	52000	44000	36000	42000
33	88000	97000	90000	81000	120000	113000
38	68000	59000	99000	95000	124000	135000
43	150000	140000	163000	144000	131000	132000
48	132000	145000	141000	150000	146000	160000

TABLE IV

DUPLICATE COUNTS OF THREE CULTURES OF PSEUDOMONAS FRAGI (C) GROWN IN MILK STORED AT 4° C.

Age of Culture In Days	Culture I		Culture II		Culture III	
	1	2	1	2	1	2
	Bacterial Counts x 10^4 per ml.					
0	0.0016	0.0019	0.0012	0.0015	0.0015	0.0016
½	0.0022	0.003	0.0028	0.0033	0.0029	0.0036
1	0.004	0.006	0.007	0.008	0.005	0.009
2	0.105	0.111	0.094	0.087	0.093	0.1
3	1.77	1.83	1.66	1.53	1.46	1.53
4	27	26.5	27.2	27.3	28	27.6
5	355	366	273	259	302	316
6	1850	1680	1320	1450	1900	1750
7	3150	3420	2550	2600	3200	3200
8	7000	7200	6100	5700	7400	6500
9	9700	8500	9600	8600	9600	8800
10	12100	11500	11200	10800	11900	11200
11	12900	14600	12500	11900	14300	14000
12	15200	15100	15000	13800	15200	15500
13	18500	18300	18000	10100	18200	19300
18	27300	29000	31000	33500	33400	34800
23	42000	47000	51000	55000	59000	68000
28	68000	57000	59000	66000	64000	53000
33	78000	89000	62000	68000	83000	82000
38	84000	94000	96000	99000	78000	93000
43	115000	119000	96000	105000	104000	113000
48	123000	121000	117000	129000	127000	131000
53	142000	136000	131000	139000	112000	125000
63	177000	174000	189000	203000	184000	201000
73	233000	231000	196000	206000	215000	221000
83	245000	232000	260000	271000	272000	283000
93	338000	352000	318000	298000	288000	297000

TABLE V

DUPLICATE COUNTS OF THREE CULTURES OF BREVIBACTERIUM LIPOLYTICUM (D) GROWN IN MILK STORED AT 4° C.

Age of Culture In Days	Culture I		Culture II		Culture III	
	1	2	1	2	1	2
	Bacterial Count x 10^4 per ml.					
0	0.0034	0.0044	0.0039	0.0038	0.0056	0.0051
½	0.0054	0.0062	0.0056	0.0052	0.0058	0.0063
1	0.0080	0.0076	0.0091	0.0086	0.0071	0.0068
2	0.0177	0.0163	0.175	0.0182	0.0150	0.0170
5	0.98	0.91	1.38	1.25	1.42	1.52
6	5.6	7.4	6.3	5.6	6.3	5.6
7	20.5	22.5	14.1	14.9	21.8	23.5
8	144	139	131	133	156	161
9	585	562	595	625	605	634
10	2490	2440	2410	2450	2730	2850
11	10300	10300	11000	10400	10600	9800
12	22600	22600	21500	20900	24600	23900
13	45200	52000	50400	50000	52200	54600
18	78000	82000	78000	87000	68000	83000
23	113000	106000	103000	108000	106000	94000
28	111000	106000	99000	113000	107000	106000
33	104000	115000	95000	99000	116000	111000
38	133000	114000	104000	104000	98000	96000
43	117000	118000	118000	130000	123000	126000
48	123000	136000	140000	134000	98000	106000
53	114000	111000	115000	118000	117000	120000
63	149000	142000	156000	148000	149000	140000
73	175000	173000	160000	152000	148000	155000

TABLE VI

DUPLICATE COUNTS OF THREE CULTURES (E) OF THE MIXED FLORA OF COMMERCIALLY PROCESSED SKIM MILK GROWN AT 4° C.

Age of Culture In Days	Culture I		Culture II		Culture III	
	1	2	1	2	1	2
	Bacterial Counts x 10^4 ml.					
0	.0051	.0054	.0044	.0034	.0111	.0098
½	.0063	.0068	.0041	.0044	.0085	.0096
1	.0062	.0065	.0048	.0037	.0073	.0101
2	.0055	.0056	.0036	-	.0065	-
4	.0109	.0104	.0060	.0062	.0090	.0097
5	.104	.0970	.0210	-	.0260	.0220
6	3.5	4.0	.32	.37	.40	-
7	23.5	25.4	26.2	25.1	2.0	2.3
8	105	-	99	-	85	-
9	1630	1590	210	320	300	200
10	5500	5100	17100	1750	1630	1640
12	11500	12000	5600	6500	8400	8400
13	13000	14800	15100	17000	11900	12700
14	49000	48000	28500	19800	19100	20400
20	133000	139000	127000	133000	102000	106000