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STUDIES OF SOME FACTORS AFFECTING THE GROWTH OF HEAT-RESISTANT PSYCHROTROPHS IN MILK

BY
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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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Dedicated to my father, the late Mr. Sing Kai Chan

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDIX 1

APPENDIX 2

APPENDIX 3

APPENDIX 4

Abstract

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Major Professor: R.R. Pereira.

The average psychrotrophic bacterial population of most commercially pasteurized, homogenized milk was found to be initially low; however, psychrotrophic bacterial count(PBC) could increase rapidly even at a temperature of 4.4°C. Milk samples stored at 4.4°C had a shelf-life twice as long as those samples stored at 7°C. All samples which received laboratory re-pasteurization or heat treatment at 80°C for 12 minutes at zero time(day of purchase) remained organoleptically acceptable after 21 days of storage at both 4.4°C and 7°C. The psychrotrophic thermoduric count and psychrotrophic sporeformer count obtained from these samples which received laboratory re-pasteurization or heat treatment at each of the experimental intervals averaged one log cycle higher than those samples receiving the same treatment at zero time. Furthermore, the study indicated that if the SPC or PBC was allowed to reach 10^7/ml in pre-heat-treated milk, a stimulatory effect on the growth of heat-resistant microflora was likely to occur. Such an effect was found unlikely to occur if the SPC or PBC was higher than 10^9/ml.

Supplementation of skim milk with 10% supernatant obtained from the skim milk in which proteolytic Ps. fluorescens PS3a had been growing was found to promote the growth of heat-resistant microflora at 7°C. Further study on supplementing hydrolyzed milk

proteins in skim milk showed a similar effect, suggesting that such an effect may be due to the breakdown of milk proteins by the proteolytic enzymes of Ps. fluorescens PS3a, thus increasing the availability of simple nutrients such as amino acids which could readily be utilized by heat-resistant microflora.

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1. Introduction

Current practices of alternate day collection of farm bulk tank milk may result in a substantial increase in undesirable psychrotrophic bacterial counts, but also in the accumulation of heat-resistant extracellular enzymes, and end product metabolites produced by the high microbial populations. Commercial pasteurization or ultra-high temperature (UHT) sterilization of raw milk are known to destroy vegetative cells, but the pre-formed heat-resistant enzymes, and bacterial end product metabolites can cause the deterioration of milk during subsequent storage (Adam et al., 1975; Gebre-Egziabher et al., 1980a; and Patel and Blankenagel, 1972).

Heat-resistant bacteria in milk have been reported to survive pasteurization and UHT treatment (Martin, 1981; Collins, 1981). Martin (1981) reported that mesophilic, thermoduric, and sporeforming bacteria had a marked effect on shortening the shelf-life of UHT-sterilized milk stored at 35°C. Psychrotrophic thermoduric and sporeforming bacteria have been reported to have a significant effect on the methylene blue keeping quality test for pasteurized milk stored at 1°C and 4°C (Coghill and Juffs, 1979). Overcast and Atmaram (1974) reported that excessive growth of Pseudomonas spp. in raw milk had a stimulatory effect on the growth of Bacillus cereus during subsequent storage at 5°C and 10°C.

Because there is some evidence in the literature to indicate that the growth of heat-resistant psychrotrophs could be stimulated by the previous growth of Pseudomonas sp. in raw milk, the

purpose of this study was to further investigate some factors which could affect the growth of heat-resistant microflora in milk.

2. Literature Review

2.1 Psychrotrophic Bacteria

The term "psychrophile" was first used by Schmidt-Nielson in 1902 to describe those organisms which were able to grow at 0°C. However, it has been demonstrated that those organisms which where capable of growing at low temperatures could grow more rapidly at elevated temperatures (Witter, 1961). Since the term "psychrophilic" (cold loving) implies optimum growth at low temperatures, it has been questioned whether this term should be used. In 1969, the International Dairy Federation suggested that the term "psychrotrophic" should be used; the term indicates organisms able to grow at a relatively rapid rate at or below 7°C irrespective of their optimum growth temperature.

2.2 Sources of Psychrotrophic Bacteria

The original sources of psychrotrophic bacteria are from soil, dust, and water. They are the predominant organisms in soil, and in the water from streams and rivers, the reason being their ability to grow at both moderate and low temperatures (Keogh and Dick, 1978). It has also been reported that water supplies, plants, animal feed, and bedding material are main sources of psychrotrophic bacteria which probably originated from soil (Mikolajcik, 1979).

Water supplies are important sources of psychrotrophic bacteria in the dairy industry (Witter, 1961). When such water comes in contact with dairy products and equipment it may cause serious psychrotrophic contamination of dairy products and result in

their rapid spoilage under refrigeration temperatures. Rakshy et al. (1971) reported that the dairy plant water supply for butter making contained coliforms, Pseudomonas sp., Achromobacter sp., Micrococcus sp., Bacillus sp., Enterobacter aerogenes, and Escherichia coli. Their results also revealed that stagnant water or water inoculated with Pseudomonas sp. resulted in a significant increase in free fatty acid contents of butter. Wash water was also reported as an important source of psychrotrophic bacteria in the cottage cheese industry (Tuckey, 1959), causing shortened shelf-life of the product. Morse et al. (1968b) reported that the psychrotrophic bacteria counts (PBC) of farm water supplies collected from three different Canadian cities ranged from < 10 to 270,000/ml, while the standard plate count (SPC) of the same water supplies ranged from < 10 to 930,000/ml.

Thomas and Thomas (1977) reported that poorly cleansed milk pipelines have been found to be one of the main sources of psychrotrophs in bulk collected milk. The PBC from these pipelines were reported as >100,000/ft^2. They also suggested the following guidelines for PBC of milking machines and pipelines: counts of <10,000/ft^2-very satisfactory; 10,000-50,000/ft^2 - satisfactory; 50,000-250,000/ft^2 - acceptable; and >250,000/ft^2 -unacceptable. Thomas and Thomas (1976) reported a high incidence of Gram-negative rods, especially in poorly cleaned bulk milk tanks. Their results indicated little difference in PBC between the microflora of hand cleaned and automatically cleaned tanks. The PBC from these bulk milk tanks ranged from <10,000 to >1,000,000/ft^2. Their results suggested the following guidelines for PBC of

cleaned farm bulk tanks: counts of <10,000/ft² -satisfactory; 10,000-100,000/ft² - fairly satisfactory; and >100,000/ft² - unsatisfactory.

2.3 Bacterial Population Levels in Milk

Thomas and Thomas (1973) reported that over 95% of the bulk raw milk samples had PBC of >10,000/ml; however, variability in the PBC was observed by different researchers. Morse et al. (1968 a) reported that the Standard Plate Count (SPC) of bulk tank milk samples from three major Canadian cities ranged from 3,000 to 100,000/ml. Patel and Blankenagel (1972) found that the SPC of raw milk samples ranged from <100 to 100,000,000/ml, and that milk with a SPC of >1,000,000/ml before heating frequently developed objectionable flavors after pasteurization and subsequent storage. Watrous et al. (1971 a) reported that the SPC of freshly collected raw milk samples ranged from <100 to >100,000 per ml, and that freshly pasteurized milk from the same milk fell in the range of 1 to 1000/ml. Jones and Langlois (1977) analysed the bacterial contents of different retail fluid milk products and their results indicated that 15.6% of whole milk samples, and 15.2% of low fat milk samples, 30.1% of skim milk samples, and 39.7% of chocolate milk samples had SPC of >20,000/ml. Watrous et al. (1971 b) reported that 96% of commercially pasteurized milk had SPC of <10,000/ml, and the SPC of laboratory pasteurized milk did not increase as a function of age and temperature. Watrous et al. (1971 b) further reported that with conventionally pasteurized milk samples held at 4.4° or 7.2°C, the PBC increased from <1/ml to >10^8/ml after 10 days

storage. These findings suggested that post-pasteurization contamination was an important source of psychrotrophs in heat-treated milk. However, it has been reported that psychrotrophic thermoduric and sporeforming organisms may survive pasteurization, grow during refrigeration storage and result in reduced shelf-life of the milk (Richter, 1981; Choudhery and Mikolajcik, 1971; Coghill and Juffs, 1979; and Watrous et al., 1977).

2.4 Types of Psychrotrophs

Three main types of psychrotrophic organisms are usually found in milk: Gram-negative heat-sensitive; Gram-positive thermoduric; and Gram-positive sporeforming psychrotrophs. The latter two groups of organisms may survive heat treatments such as high temperature short time (HTST) pasteurization and ultra high temperature sterilization (UHT), and cause spoilage in milk and milk products during refrigerated storage (Coghill and Juffs, 1979; Choudhery and Mikoljcik, 1971; Washam et al., 1977; Grosskopf and Harper, 1974; Franklin, 1970). Gram-negative heat-sensitive psychrotrophs are not usually found in properly heat-treated milk; Parker et al. (1953) concluded that the presence of such organisms is conclusive evidence of post-pasteurization contamination. Thomas and Druce (1969) have tabulated the psychrotrophic microflora of refrigerated raw milk as determined by different researchers, and found that the genus Pseudomonas accounted for approximately 50% of the total bacterial population in raw milk (Table 1).

2.4.1 Gram-negative Heat-Sensitive Rods

Table 1: Psychrotrophic Microflora of Refrigerated Raw Milk (Thomas and Druce, 1969)

Studies	No. of Cultures	Per Cent Cultures Classified as								
		Pseudomonas	Achromobacter	Alcaligenes	Flavobacterium	Arthrobacter	Aeromonas	Coliaerogenes	Micrococcus	Unclassified
A	79++	64.5	11.4	10.1	6.9	-	-	5.1	-	-
B	185++	33.5	-	1.6	17.3	40.0	-	3.8	3.2	0.6
C	235	36.6	58.0+	0.8	0.4	2.1	-	2.1	-	-
D	181++	56.9	7.2	1.1	-	6.1	-	20.4	3.9	4.4
E	142	47.1	8.6	-	2.8	-	6.3	33.1	2.1	-
F*+	275	61.0	6.0		10.0	-	2.5	3.6	11.0	5.9*

* Including Arthrobacter and Yeast
\+ Including non-fluorescent pseudomonads
*+ Cultures from dairy sources
++ Cultures from farm bulk tank milk

The bacterial genera most commonly found to be associated with heat-sensitive psychrotrophs in milk are Pseudomonas, Acinetobacter, Achromobacter, Aeromonas, Flavobacterium, Xanthomonas, and Alcaligenes (Witter, 1961; and Mikolajcik, 1979). The general consensus is that Pseudomonas is the most commonly encountered, and this is true not only in dairy products, but also in meat, fish, poultry, and eggs (Witter, 1961). The genus Pseudomonas predominates in liquid dairy products and it has been reported that they may account for 20.4-69% of the total bacterial population in raw milk (White et al., 1978), 62.1-70.1% in pasteurized milk (Thomas, 1970), and 46.3-62.8% in pasteurized cream (White et al., 1978).

2.4.2 Gram-positive Thermoduric and Sporeforming Microflora

Psychrotrophic thermoduric, non-sporeforming organisms are commonly found in milk. The most commonly encountered thermoduric bacteria belong to the genera Corynebacterium, Arthrobacter, Microbacterium, Streptococcus, Staphylococcus, Lactobacillus, and Streptomyces (Collins, 1981; Mikolajcik, 1979; and Washam et al., 1977). Martin (1981) reported that the micrococci comprised approximately 38% of the total thermoduric cultures isolated. Streptococci and Corynebacteria were the next most common, with 13 to 16% of the thermoduric isolates falling in these two groups.

Psychrotrophic sporeforming microflora are also commonly encountered in raw and heat-treated milk. Grosskopf and Harper (1974) isolated psychrotrophic strains of Bacillus from pasteurized and sterilized milk, aseptically packaged, and from raw

milk. These organisms were identified as B. cereus, B. circulans, B. coagulans, B. lentus, B. licheniformis, B. macerans, B. magaterium, B. pantothenticus, B. subtilis, and B. pumilus. Martin(1974) reported that Bacillus spp. accounted for almost 95% of the sporeforming bacteria in U.S. market milk. Of these, 43% belonged to the species B. licheniformis, and 37% to B. cereus. Among these, B. cereus strains were considered to be the predominant psychrotrophs. Shehata and Collins(1971) found that 25 to 35% of raw milk samples contained psychrotrophic Bacillus species. Coghill and Juffs(1979) found that 37% of pasteurized milk and cream samples processed in Queensland, Australia contained psychrotrophic sporeformers, and 65% of the isolates from these milk products belonged to B. cereus. Mikolajcik and Simon(1978) heated raw milk samples at 80°C for 12 min. and reported that approximately 88% of the samples contained 10 or more sporeformers per ml which were capable of outgrowth at 32°C and 21°C. Only 13% of the heated samples had psychrotrophic spore counts of 10 or more per ml(Table 2).

2.5 The Role and the Germination of Psychrotrophic Spores in Dairy Products

Heat-resistant psychrotrophs are often encountered in raw, and heat-treated milk and dairy products. Because of their ability to survive pasteurization or UHT-sterilization treatments, and to germinate and grow at refrigeration temperatures, they can affect the shelf-life of dairy products significantly(Coghill and Juffs, 1979). These bacteria can cause bitter, fruity, rancid, yeasty, and sweet curdling defects in milk(Collins, 1981).

Table 2

Distribution of Initial Mesophilic, Intermediate, and Psychrotrophic Spore Counts of Heated Milk[a]

Range of Counts	Mesophilic	Intermediate	Psychrotrophic
CFU/ml		% of Samples	
<1	1	2	39
≥1-<2	3	3	19
≥2-<10	9	7	29
≥10-<100	60	68	11
≥100-<10^3	24	20	2
≥10^3	3	0	0

[a] Data are for 109 samples

Mikolajcik and Simon, 1978.

Although the population level of psychrotrophic spores is greatly dependent on the time and temperature of heat-activation (Shehata and Collins, 1972). Typically, spores at 7°C had a lag phase of 8 to 14 days with the generation times of 22 to 26 hr. in the rapid growth phase (Chung and Cannon, 1971). Cannon (1974) reported that maximum spore germination with incubation at 7°C was obtained by heating the spore suspensions in milk at 74°C for 20 min.

Overcast and Adams (1966) investigated the influence of excessive psychrotrophic growth in raw milk on the growth of psychrophiles in post-pasteurized milk. Their results indicated that excessive growth had a slight stimulatory effect on the ability of some organisms to initiate growth as well as affecting the rate of growth for one to two days. Overcast and Atmaram (1974) reported that excessive growth of Ps. fragi or Ps. fluorescens in raw skim milk before processing had a stimulatory effect on the growth of two of three psychrotrophic B. cereus isolates with combination of heating at 80°C for 15 seconds.

2.6 Methods of Enumeration

2.6.1 Psychrotrophic Bacterial Count (PBC)

The official method for the enumeration of psychrotrophic bacteria is given by Standard Methods for the Examination of Dairy Products (A.P.H.A., 1978). The PBC determines the organisms in food samples capable of forming visible colonies on Standard Plate Count (SPC) medium incubated at 7±0.2°C for 10 days. The present recommended method for the enumeration of psychrotrophic

bacteria has the disadvantage of a protracted incubation period before results can be obtained. Alternate rapid methods have been reported by several researchers who have based their methodologies on the fact that most psychrotrophs in raw and heat-treated milk are predominantly Gram-negative bacteria, and that the measurement of the Gram-negative bacteria would in turn give an indication of the level of psychrotrophs (Witter, 1961). It was therefore suggested that for quality control purposes the test samples could use higher incubation temperatures for shorter times, and/or utilize Gram-positive bacterial inhibitors within the growth medium. A summary of various alternate rapid methods has been outlined by White (1982) and is presented in Table 3.

2.6.2 Psychrotrophic Thermoduric Count (PTC)

In the dairy industry, the term thermoduric bacteria applies to that group of bacteria which can survive pasteurization at 72°C for 16 seconds. Under laboratory conditions, the method for enumerating thermoduric organisms is given by the Standard Method (A.P.H.A., 1978), which is, laboratory pasteurization of the sample at 62.8°C for 30 minutes and then performing the PBC (A.P.H.A., 1978). Those organisms capable of forming visible colonies on SPC medium incubated at 7°±0.2°C for 10 days are considered to be psychrotrophic thermoduric organisms.

2.6.3 Psychrotrophic Spore Count (PSC)

The most common method used to determine PSC in a milk sample is to heat the sample at 80°C for 12 minutes, cool the sample in ice water, and then perform a PBC. Those organisms which are

Table 3

Rapid Methods for Estimating
Psychrotrophic Bacterial Count

1. Moseley Keeping Quality Test (Standard Test)	1. Sample incubation 5-7 days at 7C followed by SPC and flavor check.
2. Pre-incubated CVT Count	2. Plate pre-incubated (21C-16 hr.) resazurin-milk mixture, incubate ambient-48 hr. Develop rating system.
3. Hull or similar Test- Preliminary Incubation (PI) of sample at different temperatures- Hull Test	3. Measures degree of proteolysis.
4. Naeconol- TTC Test	4. Color change in hours correlated with approximate shelf-life.
5. PI-Gram Negative Agar Count	5. PI (13C-18hr.) followed by plating on SPC agar plus 10 IU penicillin G/ml agar.
6. PI-Proteolytic Count	6. PI (13C-18hr.) followed by plating on Spc agar+ 10%MSNF and incubation at 32C-48hr.
7. Metabolite indicators, e.g. pyruvate.	7. Measures change in pyruvate (20C-24hr.). Recommend fresh products.
8. Modified psychrotrophic count	8. Incubation of pour plates at 21 C-25hr.
9. PI-Instruments to detect bacterial numbers	9. PI (13C-18hr.) followed by estimation of microbial load.

White, 1982.

capable of outgrowth at 7°±0.2°C in 10 days on SPC medium are considered to be psychrotrophic sporeformers (Mikolajcik and Simon, 1978). An alternate method is to heat the milk samples at 80°C for 10 minutes (Coghill and Juffs, 1979). Cannon (1974) reported that maximum spore germination can be obtained by heating samples to 74°C for 20 minutes. However, Shehata and Collins (1972) found that heating at 87.8°C for 20 seconds was more effective in activating spores than heating at 76.7°C for 20 seconds.

2.7 Effects of Psychrotrophic Microflora on the Quality of Dairy Products

Psychrotrophic organisms can directly and indirectly affect the quality of dairy products. Directly, the organisms can survive pasteurization or other heat treatments, or re-enter the products as a result of post-pasteurization contamination. They may multiply in sufficient numbers during manufacture and/or refrigerated storage of the products and cause reduction of shelf-life, quality, and quantity of the finished product. Indirectly, psychrotrophic organisms can cause off-flavors and odors in raw milk which carry over to the finished product. Furthermore, psychrotrophic organisms are able to produce heat-stable extracellular enzymes which can result in the breakdown of milk protein and fat (Cousin, 1982).

2.7.1 Flavor Defects

Flavor defects caused by psychrotrophs in dairy products are often described as "bitter", "rancid", "stale", "fermented",

"putrid", "acid", "malty", "ropy", "sweet", "slimy", "fruity" "yeasty", and "unclean" (Mikolajcik, 1979; Shipe et al., 1978; Thomas and Druce, 1969, and Punch et al., 1965). Those flavor defects of particular signficance in psychrotrophic contaminated milk are rancid and bitter flavors, caused by lipase and protease action, respectively.

2.7.2 Pasteurized Milk

The principle off-flavors and defects in pasteurized milk associated with the growth of psychrotrophs are bitter, rancid, and sweet curdling (Overcast and Atmaram, 1974; Downey, 1980; and McKellar, 1981). Westhoff (1982) reported that as the PBC reached levels of 10^8/ml in pasteurized whole milk, 85% of the milk samples were judged unacceptable by the taste panelists (Table 4). The off-flavors described as bitter and rancid are usually caused by the heat-resistant extracellular proteases and lipases respectively, as produced by psychrotrophs. On the other hand, the defect known as sweet curdling is caused by the growth of a psychrotrophic strain of Bacillus cereus (Overcast and Atmaram, 1974). Choudhery and Mikolajcik (1971) reported that the sweet curdling effect was due to a rennin-like enzyme produced by B. cereus. This specific defect was commonly found in UHT-processed milk (Law et al., 1977). Patel and Blankenagel (1972) reported the flavor characteristics of some pasteurized milk samples with various SPC before and after heating and storage (Table 5), and their results indicated that milk exhibiting a SPC of $>10^6$/ml prior to heat treatment, frequently developed flavor defects after pasteurization and subsequent storage at 7°C for

Table 4

Comparison of Microbial Counts
and
Panelists' Responses

Range of Microbial Counts/ml		Good[1] to Fair	Fair[2] to Poor	Acceptable[3]	Not Acceptable
10^0-10^2	TPC[4]	100[6]	0	100	0
	PPC[5]	100	0	100	0
10^2-10^4	TPC	88	12	100	0
	PPC	87	13	100	0
10^4-10^6	TPC	68	32	100	0
	PPC	64	36	100	0
10^6-10^8	TPC	29	39	68	32
	PPC	30	38	68	32
10^8	TPC	0	24	24	76
	PPC	0	15	15	85

1,2 Good to fair equals to flavor scores of 3.0 to 2.0, Fair to poor equals flavor scores of 1.99to 1.0

3 Panelists indicated milk was acceptable

4 Total plate count

5 Psychrophilic plate count

6 Expressed as percent

Westhoff, 1982.

Table 5

Flavor Characteristics of Some Pasteurized Milk Samples with Various SPC Before and After Heating and Storage

Sample no.	Raw SPC/ml	Pasteurized		
		Weeks at 7C	SPC/ml	Flavor
1	16,000,000	1	400	Slightly Bitter
		2	27,000	Very Bitter
2*	6,000	1	300	Good
		2	100	Good
2**	27,000,000	1	100	Very Bitter
		2	300	Very Bitter
3	11,000,000	1	200	Good
		2	100	Good
4	5,000,000	1	1,500	Good
		2	30,000	Unclean

* Pasteurized immediately

** Held for 4 days at 7 C before pasteurization

Patel and Blankenagel, 1972.

one to two weeks.

2.7.3 Cheese

The principle off-flavors in cheese associated with the growth of psychrotrophs are bitter, rancid, unclean, and soapy (Law et al., 1976; Keogh and Dick, 1978; Mikoljcik, 1979). These off-flavors are mainly caused by the extracellular enzymes produced by psychrotrophs. Law et al., (1976) reported that Cheddar cheese which was manufactured from pasteurized milk and originally inoculated with 10^7 lipolytic Gram-negative psychrotrophs per ml became rancid after 4 months even though the organisms were destroyed by pasteurization. Yates and Elliot (1977) added a proteolytic psychrotroph to milk resulting in a loss of casein and hence an increased protein level in whey, which resulted in a 2.75 to 3.5% reduction in protein available for cheese making. Hicks et al. (1982) reported that the yield of direct-acid cheese manufactured from milk inoculated with psychrotrophs decreased as the psychrotrophic inoculation level increased. Furthermore, the cheese which was manufactured from the inoculated milk showed increased levels of free fatty acids, non-protein nitrogen, and whey protein, indicating the actions of certain enzymes.

2.7.4 Butter

The off-flavors defined as fruity and rancid are commonly found in butter contaminated with psychrotrophs (Keogh and Dick, 1978). A psychrotrophic strain of Pseudomonas fragi was found to cause these off-flavors. Rakshy et al. (1971) suggested that psychrotrophs in the water supply were found to be responsi-

ble for the deterioration of butter during subsequent storage.

2.7.5 Cultured Milk Products

Wang and Frank (1981) reported that psychrotrophic species of Pseudomonas, Enterobacter, Acinetobacter, Escherichia, and Actinobacillus were capable of reducing diacetyl, resulting in the loss of flavor in buttermilk. Sadovski et al. (1980) found that 72% of the psychrotrophs which were isolated from yogurt originated from the starter cultures, Streptococcus lactis, S. cremoris, and Leuconostoc cremoris. Organoleptic deterioration was detected after 17 days storage at 7°C.

Juffs and Babel (1975) reported that hydrogen peroxide produced by the lactic bacteria in cultured milk products inhibited the growth of psychrotrophs. Martin and Gilliland (1980) found a significant amount of inhibition of a psychrotrophic culture in autoclaved milk stored at 5.5°C when the milk contained at least 2.5×10^7 lactobacilli per ml.

2.8 Psychrotrophic Bacterial Enzymes

Although most of the Gram-negative heat-sensitive psychrotrophic bacteria are not present in properly heat-treated milk, they are however, capable of producing a wide variety of heat-resistant extracellular enzymes which primarily limit the keeping quality of dairy products. These enzymes include proteases, lipases, and phospholipases C; and because these enzymes are produced extracellularly, they can directly act on the milk proteins, fat globules, and phospholipids, resulting in the development of off-flavors (Law, 1979).

2.8.1 Protease-Producing Psychrotrophs

Law(1979) reported that protease producing psychrotrophic Gram-negative bacteria were often isolated from milk, with the Pseudomonas being the most frequently found genus, and Ps. fluorescens was the most common species. It has been reported that the heat-resistant protease produced by a strain of Ps. fluorescens was capable of gelling UHT-sterilized milk after 10 to 14 days storage at 20°C(Law et al., 1977). A rennin-like enzyme produced by a psychrotrophic strain of B. cereus, which was capable of developing a sweet curdling defect in milk was also reported(Choudhery and Mikolajcik, 1971; Overcast and Atmaram, 1974). Juven et al.(1979) reported that a psychrotrophic strain of Serratia liquefaciens produced a significant amount of proteolytic activity which led to a defect categorized as unclean flavor in refrigerated milk samples. A yeast species of Rhodotorula was also reported to have sufficient proteolytic activity to cause a fruity flavor in processed milk and cream(Patel et al., 1972).

The proteases produced by psychrotrophic bacteria have been reported to be extremely heat-resistant(Gebre-Egziabher et al., 1980b). These enzymes are not inactivated in milk by conventional pasteurization or UHT sterilization at 120°C to 149°C. A summary of the D values(time required to reduce enzyme activity by 90%) from different psychrotrophic bacteria isolated by different researchers is outlined in Table 6.

The stability of psychrotrophic proteases is particularly enhanced in milk(Mayerhofer et al., 1973). Heat resistance of the

Table 6

D Values of Proteases Produced
By Psychrotrophic Bacteria

Organism	Heat-Treatment (°C)	D Value (Min.)	Reference
Pseudomonas fluorescens P26	62.8	900	Mayerhofer et al. 1973.
	71.4	480	
	121.0	9	
Ps. fluorescens MC60	150	0.5	Barach et al., 1976a.
Pseudomonas sp. isolate # 22	121.0	10	Gebre-Egziabher et al., 1980b.
Pseudomonas sp. 21B	72.0	250	Downey, 1980.

enzyme in milk at high temperatures is dependent on the presence of Ca^{2+} (Barach et al., 1976). Barach and Adam, (1977) reported that the protease of Pseudomonas was similar to the classical heat-resistant protease of Bacillus thermoproteolyticus in its metal ion requirements, lack of cysteine, and degree of hydrophobicity. It is suggested that these properties allow the enzyme proteins to be flexible when denatured by extremes of temperature, then to refold quickly and accurately with the formation of Ca^{2-} bridges when the temperature is lowered again. Adam et al. (1976), DeBeukelar et al., (1977), and Gebre-Egziabher et al. (1980a) reported that the proteolytic activity of psychrotrophic bacteria did not affect whey protein appreciably. Gebre-Egziabher et al. (1980a) inoculated different cultures of psychrotrophs into raw milk, and found that the loss of k-, beta-, and alpha-casein ranged from 61%-91%, 62-89%, and 19%-76% respectively, after 9 days storage at 7°C. Five of these six isolates also showed significant degradation of beta- lactglobulin. DeBeukelar et al. (1977) reported that beta- casein was generally degraded more than alpha- casein while k- casein was not affected significantly by the proteolytic enzymes of psychrotrophs. However, Adam et al. (1976) and Gebre-Egziabher et al. (1980a) reported that k- and beta- casein were the most susceptible to the proteolytic psychrotrophs. Law et al. (1977) reported that milk samples in which Ps. fluorescens had grown and were subsequently UHT-sterilized and stored at 20°C, gelled after 10 to 14 days for those samples which had contained 5×10^7 colony forming units (CFU) per ml before sterilization gelled after 10 to

14 days. Starch electrophoresis of these samples showed that there was a considerable loss of alpha- and beta- casein; and k- casein was no longer detectable, suggesting complete breakdown to para-k-casein complexes.

Choudhery and Mikolajcik (1971) reported that sweet curd formation in skim milk was associated with the exponential growth phase of B. cereus and not with spore germination or sporulation. The characteristic proteolytic activity of B. cereus was evident by the disappearance of k- casein and appearance of para-k-casein followed by the release of sialic acid at the time of coagulation of skim milk.

It has been reported that UHT-sterilized milk coagulated in the presence of heat-resistant proteases, and k- casein degradation was detected by the release of sialic acid into the milk during storage (Bengtsson et al., 1973). Adam et al. (1976) suggested that degradation of k- casein might result in coagulation of fluid milk. Although k- casein comprises only a small percentage of the total protein and total casein in milk, it is important in stabilizing the casein micelle.

The degree of proteolysis was dependent on the composition of the psychrotrophic flora of milk since this factor was shown to influence the extent of proteolysis (Law, 1979). The liberation of peptides which can yield off-flavors such as bitterness has accompanied the digestion of milk proteins by proteases (Juven et al., 1979).

2.8.2 Lipase-Producing Psychrotrophs

The highest incidence of lipolytic activity among the psy-

chrotrophic bacteria of milk have been found in Pseudomonas fluorescens, Ps. fragi, (Law et al., 1976), Achromobacter lipolyticum (Khan et al., 1967), and Alcaligenes viscolactis (Driessen and Stadhouders, 1971). As with the proteases, the most active lipolytic psychrotroph was shown to belong to the genus Pseudomonas (Andersson et al., 1979).

Lipases produced by psychrotrophs are highly heat stable and result in the development of off-flavors in dairy products during subsequent storage (Downey, 1980; and Andersson et al., 1981). These enzymes are remarkably heat-resistant and are capable of surviving normal pasteurization and UHT-sterilization (Downey, 1980). A comparison of the heat stability of lipases produced by psychrotrophs is summarized in Table 7.

The mechanisms of thermal stability of the lipases are still not known, but there are at least two fundamentally different factors which, alone or in combination, can be offered as an explanation to heat resistance. Firstly, the primary structure of the enzyme molecule is important. Liu et al. (1973) suggested that a high content of hydrophobic amino acids in the enzyme molecule provided a compact structure which would not be denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds played an important role in stabilizing the molecule and thus could explain the high resistance to heat inactivation. Secondly, specific components in milk and in lipases, such as polysaccharides, and divalent cations, could stabilize the molecule.

The off-flavor defects arising from lipolytic psychrotrophic

Table 7

Comparison of the Percentage Remaining Enzyme Activity of Microbial Lipases at Various Times and Temperatures

Organisms	Heat Treatment (°C)	Lenght of Treatment (Min.)	Activity Remaining (%)	Reference
Ps. fluorescens				
22b	100	0.5	75	Fitz-Gerald,et al.,
30b	100	0.5	84	1982.
42	100	0.5	76	
9e	100	0.5	95	
49	100	0.5	85	
27a	100	0.5	83	
8b	100	0.5	90	
29a	100	0.5	100	
14a	100	0.5	100	
15c	100	0.5	84	
8	100	0.5	82	
7a	100	0.5	79	
37	100	0.5	80	
51	100	0.5	80	
Serratia marcescens 2	100	0.5	82	
Aeromonas 72	100	0.5	96	
Achromobacter lipolyticum	99	40	0	Khan et al.,1967.
Ps. fluorescens 22F	150	4.8	10	Driessen and Stadhouders, 1974.
Ps. fluorescens	140	2.0	10	Andersson et al., 1979.
Alcaligenes viscolactis 23al	84	0.17	7	Driessen and Stadhouders, 1971.

degradation of milk fats are seldom found in pasteurized milk, but rather in cream, cheese, and butter (Deeth and Fitz-Gerald, 1976). Law et al. (1976) reported that the lipase of Ps. fluorescens caused lipolytic rancidity in cheese, and their results showed that when butyric acid and medium chain (C6-C10) "soapy" fatty acids are released from milk fat, strong off-flavors can develop. These rancid flavors are also found in butter and cream. Andersson et al. (1981) found that the milk sample containing lipase showed a rapid increase in the acid degree values, and significant flavor changes appeared when the acid degree values exceeded 20 (Figure 1).

2.8.3 Phospholipase C-Producing Psychrotrophs

Psychrotrophic bacteria which produce extracellular phospholipase C include the genera of Pseudomonas, Seratia Acinetobacter, Alcaligenes, Aerobacter, Flavobacterium, Enterobacter, Citrobacter, and Bacillus (Fox et al., 1976). Approximately 40% of the total psychrotrophic bacterial populations produce this enzyme (Fox et al., 1976). The most active phospholipolytic psychrotrophs belong to the genera Pseudomonas and Bacillus (Law, 1979).

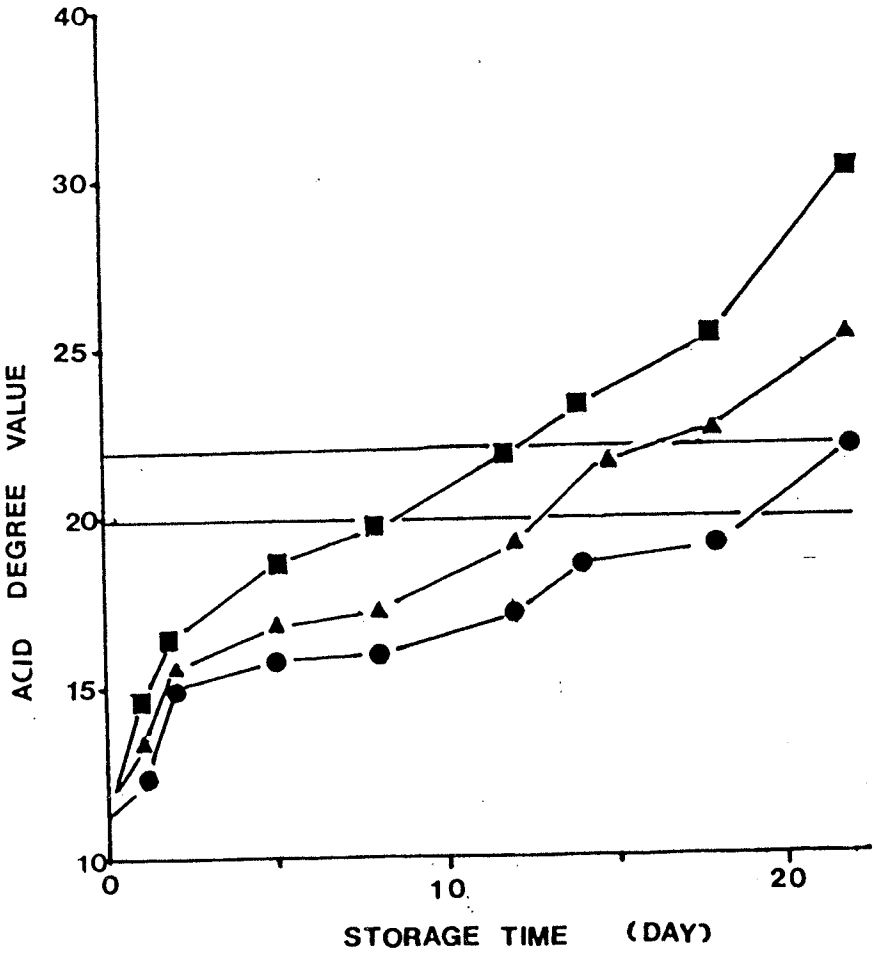
The heat resistance of phospholipase C is not as great as for psychrotrophic proteases or lipases. Doi and Nojima (1971) reported that a Phospholipase C from a strain of Ps. fluorescens lost activity completely at 80°C for 5 minutes.

Phospholipids, especially lecithin (Phosphotidyl choline), are oriented at the interface between serum and fat globules in milk where they serve as part of a protective barrier and emulsifier.

Figure 1: Acid Degree Value (ADV) of Sterilized milk During Storage at 8°C Containing Inactivated Lipase and Two Levels of Lipase (.1 and .3 unit Lipase/ml), Andersson et al., 1981.

● Inactivated Lipase
▲ .1 Unit Lipase/ml
■ .3 Unit Lipase/ml

The area between the two horizontal lines shows the ADV range where significant flavor changes were detected.



Phospholipase C hydrolyses phosphorylcholine from the diglyceride moiety of lecithin. It can also hydrolyze about 90% of the lipid phosphorous of both low- and high-density lipoprotein fractions of milk (O'Mathony and Shipe, 1972). This enzyme can cause various types of flavor defects described as bitter, sour, and fruity in raw and pasteurized milk. It has been reported that at least 75% of the bacteria which cause bitter flavor in milk produce phospholipase C (Fox et al., 1976).

Furthermore, it has been reported that phospholipase C enhances the activities of both native milk lipase and microbial lipase (Chrisope and Marshall, 1976). It is believed that phospholipase C causes the breakdown of fat globule membrane and this results in releasing and exposing of diglycerides which lipases can attack readily (Law, 1979).

2.8.4 Inactivation of Psychrotrophic Bacterial Enzymes

The ability of heat-resistant psychrotrophic enzymes to survive high temperatures has been well documented (Egziabher et al., 1980b; Law et al., 1977; Patel et al., 1972; Andersson et al., 1979; and Driessen and Stadhouders, 1971). Barach et al. (1976a, 1978) reported that extracellular protease from Pseudomonas fluorescens MC 60 could be inactivated up to 90% by heating at 55°C for 60 minutes. Marshall and Marstiller (1981) reported that the protease from Ps. fluorescens M5 was completely inactivated at 40°C for 60 minutes. However, the enzyme retained 66% of its activity after heating at 50°C for 30 minutes. Fitz-Gerald et al. (1982) reported that 85% of the lipases from psychrotrophs isolated from raw milk showed less stability to heating at 55°C

for 1 hour as compared to heating at 100°C for 30 seconds. Barach et al. (1978) suggested that low temperature inactivation of proteases appeared to be a two-step process. The first step involved a conformational change of the enzyme structure, followed by formation of an enzyme-casein complex. This resulted in little reversible loss of enzyme activity. Barach et al. (1978) also suggested that using low-temperature inactivation of psychrotrophic bacterial enzymes would increase the shelf-life of UHT-sterilized milk.

2.9 Prevention of Spoilage in Milk and Dairy Products Due to Psychrotrophic Bacteria

Thorough cleaning and sanitizing procedures for the bulk milk tanks, milking pipelines and equipment are important in reducing the level of contaminants in milk (Dommett et al., 1980 and Thomas and Thomas, 1977). Keogh and Dick (1978) suggested that strict attention to plant hygiene, the storage of milk for the shortest possible time and lowest possible temperatures, and filtration and chlorination of water supplies at factory and farm can reduce the level of psychrotrophic contamination in milk and dairy products.

Juffs and Babel (1975) reported that the addition of lactic cultures to milk was found to be effective in restricting psychrotrophic growth. The inhibition of psychrotrophs due to lactic culture was decreased by the addition of catalase, thus suggesting that hydrogen peroxide was the inhibitor. Martin and Gilliland (1980) reported that a significant inhibitory effect on psychrotrophs was observed when at least 2.5×10^7 lactobacilli per

ml were added to milk and the inhibitory effect was more pronounced at increasing oxygen concentrations.

Bjorck (1978) found that activation of the antibacterial lactoperoxidase system in milk, such that increasing the thiocyanate concentration to 0.25mM and adding an equimolar amount of hydrogen peroxide, would result in a substantial reduction of bacterial flora in milk. This treatment has been reported to have no effect on the physico-chemical properties of milk.

Scope of Investigation

This study was conducted to investigate the interrelationships among three main groups of psychrotrophic microflora in commercially pasteurized, homogenized milk: these groups are the heat-sensitive, thermoduric, and sporeforming organisms. The role of heat-sensitive organisms on the growth of thermoduric and sporeforming organisms was also investigated. Specifically, the following areas were studied:

a) To investigate the shelf-life and psychrotrophic bacterial levels of commercially pasteurized, homogenized milk.

b) To determine the growth characteristics of heat-sensitive and heat-resistant psychrotrophic microflora in milk when grown together at 7°C.

c) To determine the subsequent growth characteristics of thermoduric and sporeforming organisms in skim milk previously grown with *Pseudomonas fluorescens* PS3a at 7°C.

d) To determine the effects of supplementing skim milk with supernatant of skim milk previously grown with *Ps. fluorescens* PS3a, on the growth of milk microflora at 7°C.

e) To investigate the effects of supplementing skim milk with commercially available milk fractions on the growth of psychrotrophic thermoduric and sporeforming milk microflora at 7°C.

3. Materials and Methods

The bacterial cultures used throughout this study were:

1. Pseudomonas fluorescens P26
2. Pseudomonas fluorescens PS3a
3. Microflora including heat-sensitive, thermoduric, and spore-forming organisms isolated from commercially pasteurized homogenized milk.

Ps. fluorescens P26 was obtained from Dr. R.T. Marshall, Department of Food Science and Nutrition, University of Missouri, Columbia, Missouri: 65201, U.S.A..

Ps. fluorescens PS3a was isolated from a commercially pasteurized homogenized milk sample.

3.1 Microbiological Analysis

3.1.1 Standard Plate Count (SPC)

Enumeration of aerobic mesophilic organisms was performed according to Standard Methods for the Examination of Dairy Products (A.P.H.A., 1978). Appropriate serial dilutions were made with phosphate buffer according to A.P.H.A. (1978). Standard plate count medium (SPC, Difco) was prepared according to manufacturer's specification. All dilutions were plated in duplicate and resulting counts were expresed as the mean. Incubation was carried out at 32±0.2°C for 48 hours. All media used in this investigation were routinely sterilized at 121°C for 15 minutes, unless otherwise specified. Counts ranging between 30 and 300 colonies were used for analysis. In some instances, where very low counts were obtained, it became necessary to use the data from the plates

exhibiting less than 30 colonies.

3.1.2 Psychrotrophic Bacterial Count (PBC)

The procedure for enumerating aerobic psychrotrophic bacteria was carried out according to A.P.H.A. (1978). Culture plates were incubated at 7±0.2°C in a refrigerated incubator (Puffer Hubbard) for 10 days before plate counts were performed (A.P.H.A., 1978).

3.1.3 Mesophilic Thermoduric Count (MTC)

Commercial milk samples were laboratory pasteurized at 62.8±0.2°C for 30 minutes in a constant temperature water bath. Those organisms which survived this heat treatment. and which were capable of growing on SPC medium incubated at 32°C for 48 hours, were designated as mesophilic thermoduric organisms. Laboratory pasteurization was performed according to A.P.H.A. (1978). Exactly 10 ml samples of milk were aseptically transferred to 20 X 125 mm sterile capped test tubes and subsequently placed into a water bath set at 62.8±0.2°C. A pilot tube containing a thermometer with 10 ml of milk was used to determine the "come-up" time. When the temperature in the pilot tube reached 62.8°C, the required holding period was timed. At the end of the holding period, the test tubes were rapidly cooled in an ice-water bath before bacteriological analyses were performed.

3.1.4 Psychrotrophic Thermoduric Count (PTC)

The procedure of enumerating aerobic psychrotrophic thermoduric organisms was performed according to A.P.H.A., (1978). The plates were incubated at 7±0.2°C for 10 days.

3.1.5 Mesophilic Spore-forming Bacteria Count (MSC)

The procedure for the enumeration of mesophilic aerobic sporeforming organisms in milk was carried out according to the method employed by Mikolajcik and Simon (1978). Exactly 10 ml samples of milk were aseptically transferred into 20 X 125 mm sterile capped test tubes, and heat-treated at 80±0.2°C for 12 minutes in a water bath. A pilot tube equipped with a thermometer conatining 10 ml milk was used to monitor come up time. At the end of the holding period, the contents of test tubes were rapidly cooled in an ice-water bath before bacteriological analysis were performed. Those organisms which survived this heat treatment, and which were capable of forming visible colonies on SPC medium at 32°C after 48 hours incubation, were considered to be mesophilic sporeforming organisms.

3.1.6 Psychrotrophic Sporeformer Count (PSC)

The procedure for the enumeration of psychrotrophic aerobic sporeforming organisms was performed according to the method employed by Mikolajcik and Simon (1978). Culture plates were incubated at 7±0.2°C for 10 days.

3.2 Chemical Analyses

3.2.1 Estimation of Total Protein

The estimation of total protein was carried out according to the Folin-Ciocalteu Procedure (Lowry et al., 1951). Absorbance was measured at 500 nm using a Hitachi Perkin-Elmer UV-Vis spectrophotometer.

3.2.2 Determination of Tyrosine Value

The tyrosine value of milk was determined by the method of Hull (1947). Five ml of 0.72 N Trichloroacetic acid was then pipetted into the milk sample and mixed in a vortex apparatus for 1 min. The mixture was allowed to stand for 10 minutes and then filtered (Whatman #42 filter paper). Five ml of filtrate was then transferred into a 50 ml Erlenmeyer flask to which 10ml of sodium carbonate reagent was added. The latter was prepared by dissolving 75 grams of anhydrous sodium carbonate and 10 grams of sodium metaphosphate in deionized water to a final volume of 500 ml. The mixture was then vortexed for 30 seconds followed by the addition of 3 ml of 1:2 diluted phenol reagent prepared according to Folin-Ciocalteu procedure (Lowry et al., 1951). The entire mixture was then mixed for 30 seconds and allowed to stand for 5 minutes in order to allow maximum color development. Absorbance was measured at 650 nm using a Hitachi Perkin-Elmer spectrophotometer.

3.2.3 Determination of Titratable Acidity

The titratable acidity of skim milk was determined by titration using a 9 ml sample as outlined by Atherton and Newlander (1981). Sodium hydroxide (0.1 N), was used as the titrant. Samples were titrated to the phenolphthalein end-point.

3.2.4 pH Determination

The pH of milk samples was monitored using a Radiometer pH meter Model 51. The pH meter was routinely standardized before use with Fisher certified Gram-Pac buffer salts, pH 6.86±0.01 at

25°C.

3.3 Preparation and Maintenance of Bacterial Cultures

3.3.1 Reviving Cultures

Cultures of Ps. fluorescens P26 and Ps. fluorescens PS3a were routinely maintained on nutrient agar slants (Difco) stored at 7±0.2°C. A loopful of culture from these slants was then aseptically transferred into 100 ml sterile nutrient broth (Difco) and incubated at 21°C for 24 hours.

3.3.2 Standardization of Initial Inoculum

Inocula obtained in section 3.3.1. resulted in approximately 9.0×10^7 Ps. fluorescens PS3a/ml and 4.0×10^8 Ps. fluorescens P26/ml. Standardization of these inocula was performed by diluting 1.0 ml of the active culture with 99 ml of phosphate buffer. A 0.1 ml aliquot was then added to 50 ml of 10% sterile skim milk (Difco).[1] Inocula of approximately 8.4×10^3/ml of Ps. fluorescens P26 and 1.8×10^3/ml of Ps. fluorescens PS3a were subsequently obtained.

3.3.3 Maintenance of Cultures

Cultures of Ps. fluorescens P26 and Ps. fluorescens PS3a were maintained on nutrient agar slants grown at 21±0.2°C for 24 hours. and stored at 7±0.2°C. To ensure viability, the cultures were subcultured to fresh nutrient slants weekly. The organisms were also subcultured onto casein agar (SPC medium + 1% T.S. skim

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[1]Volumes of aliquot and skim milk were altered according to conditions of the experiment.

milk) and incubated at 21°C for 48 hours. This procedure was used to monitor proteolytic activity and culture purity. Microscopic examination of resulting colonies was routinely performed.

3.3.4 Preparation of Groups of Microflora from Commercially Pasteurized, Homogenized Milk

A specific brand of milk was purchased from a retail store. The milk sample was inmediately stored in crushed ice in a styrofoam cooler after purchase.

3.3.4.1 Mixed Population

Cultures obtained directly from the milk were designated as mixed cultures.

3.3.4.2 Thermoduric Organisms

The cultures obtained after laboratory pasteurization were used as thermoduric organisms.

3.3.4.3 Spore-forming Organisms

The cultures obtained after heat treatment at 80°C for 12 min. were used as sporeforming organisms.

3.4 Experimental Methods

All analysis were performed in duplicate. Results are expressed as an average of two separate trial runs.

3.4.1 Survey of Psychrotrophic Bacterial Contents of Six Commercially Pasteurized, Homogenized Milk Samples

Duplicate samples of six different brands of one-liter milk cartons were purchased at different local retail stores and were

immediately stored in crushed ice in a styrofoam cooler. One set of samples (six) was stored at 4.4°C, and the other was stored at 7±0.2°C. Both sets of milk samples were enumerated for PBC, PTC, and PSC at time intervals of 0, 7, 14, and 21 days as previously described (0 time was designated as day of purchase). Organoleptic qualities of these milk samples were also assessed at the respective intervals.

In order to determine the growth characteristics of psychrotrophic thermoduric and sporeforming organisms, twenty-eight 10 ml portions of each commercial milk sample were aseptically transferred to sterile capped test tubes, and these were divided equally into two groups. The first group consisting of 14 tubes, was laboratory re-pasteurized to determine PTC and the second group was heat-treated to determine PSC as previously described. Duplicate tube samples from each group were plated out to determine the initial PTC and PSC. The remaining tube samples from each group were divided equally into 2 sets, one set was stored at 4.4°C, and the other was stored at 7°C. Duplicate tube samples from each group held at 4.4°C and 7°C were used to determine the PTC, PSC, and organoleptic qualities at time intervals of 7, 14, and 21 days.

3.4.2 Growth Patterns of Heat-Sensitive and Heat-Resistant Bacterial Flora from Commercially Pasteurized, Homogenized Milk, when Grown together at 7°C

In order to determine the growth patterns of psychrotrophic heat-sensitive and thermoduric milk microflora at 7°C, and their ability to grow at 32°C, duplicate milk samples were purchased

and handled as outlined in section 3.3.4. Thirty-10 ml portions of milk samples were aseptically transferred into sterile capped test tubes, and were divided equally into 3 groups. Tube samples from the first group were used to determine the initial SPC and PBC, and the remaining tubes were stored at 7°C, plate counts were performed on duplicate samples at 5, 10, 15, and 20 day intervals. Tube samples from the second group were laboratory re-pasteurized as described previously to determine the initial PTC and MTC, the remaining tubes were stored at 7°C. The tube samples were laboratory re-pasteurized at time intervals of 5, 10, 15, and 20 days, followed by performing PTC and MTC. All tube samples from the third group were laboratory re-pasteurized at zero time, and PTC and MTC were performed on duplicate tube samples at 0, 5, 10, 15, and 20 days. All samples were stored at 7°C.

3.4.3 Growth Patterns of Ps. fluorescens P26 and Heat-Resistant Bacterial Flora from Commercially Pasteurized Homogenized Milk, when grown together at 7°C

Milk samples were purchased and handled as in section 3.3.4. Ten ml portions of milk were aseptically transferred into thirty sterile capped test tubes, and were subjected to laboratory re-pasteurization. This procedure was used to destroy all heat-sensitive organisms present in milk samples. After pasteurization, tube samples were divided equally into 3 groups, designated as A,B and C.

Both group A and B were inoculated with Ps. fluorescens P26 to a level ca. 8.4 X 10^3/ml, and all samples were stored at 7°C.

Samples from group A were plated out to determine the SPC and PBC at time intervals of 0,.5, 10, 15, and 20 days.

In order to determine the growth patterns of psychrotrophic thermoduric organisms at 32°C and at 7°C, duplicate tube samples of group B were laboratory re-pasteurized to determine the MTC and PTC at time intervals of 0, 5, 10, 15, and 20 days.

Group C was used as the uninoculated control group. In order to ensure that the thermoduric organisms in this group were exposed to the same environmental conditions as in group B, duplicate tube samples were laboratory re-pasteurized to determine the MTC and PTC at time intervals of 0. 5, 10, 15, and 20 days.

3.4.4 Isolation of Pseudomonas fluorescens PS3a from a Commercially Pasteurized, Homogenized Milk

Milk samples were purchased and handled as outlined in section 3.3.4. Appropriate serial dilutions were performed using phosphate buffer. Culture plates poured with SPC medium were incubated at 7°C for 10 days. Individual colonies were picked off from the plates and were then inoculated into 10 ml nutrient broth and incubated at 21°C for 24 hours. The broth cultures were streaked onto SPC medium augmented with 10% skim milk, and incubated at 21°C for 48 hours. Culture plates were treated with 1% HCl in order to distinguish false positive proteolytics (A.P.H.A., 1978). Isolates showing proteolytic activity were selected for further examination. Proteolytic isolates were inoculated into 50 ml sterile 10% skim milk, and incubated at 7°C. (The initial inoculum size of these bacterial cultures was

first standardized as in section 3.3.2). Milk samples were organoleptically assessed daily, and those cultures which exhibited the greatest bitterness in the shortest period of time were selected for further identification. Identification procedures for these organisms were carried out according to Bergey's Manual Determinative Bacteriology (Buchanan and Gibbons, 1975), and Cowen and Steel (1970).

3.4.5 Effect of Pseudomonas fluorescens PS3a on the Physiochemical properties of 10% Skim Milk at 7°C

A 500 ml volume of 10% skim milk (sterile) was inoculated with Ps. fluorescens PS3a to a level of ca. 2.63 X 10^3/ml in duplicate one-litre sterile capped Erlenmeyer flasks. Revival and standardization of inocula were performed as outlined in sections 3.3.1 and 3.3.2. Standard plate count, tyrosine value, pH, and titratable acidity of the inoculated skim milk were performed as outlined in section 3.1.1, 3.2.2, 3.2.4, and 3.2.3 respectively, at time intervals of 0, 5, 10, 15, and 20 days. Organoleptic qualities of the milk were also assessed.

3.4.6 Effect of End-Product Metabolites of Pseudomonas fluorescens PS3a on the Subsequent Growth of Heat-Resistant Microflora in Skim Milk at 7°C

A culture of Ps. fluorescens PS3a was prepared by the procedure as outlined in section 3.3.1, and standardized as outlined in section 3.3.2. Twenty-four 125 ml Erlenmeyer flasks capped with foam stoppers wrapped in aluminum foil were used, each containing 50 ml of 10% sterile skim milk. Ps. fluorescens PS3a

were inoculated into each flask to a level of ca 2.63×10^3, the latter being kept at 7°C. Standard plate count (SPC), tyrosine value (TV), titratable acidity (TA), and pH were determined on 4 flasks, at time intervals of 0, 1, 2, 3, 4, and 5 days. At each time interval four flasks were removed and heated at 121°C for 10 minutes, cooled in an ice water bath and then stored at 7°C until the five incubation periods were completed. After completing the above procedure at all six time intervals, all twenty four flasks were divided equally into two groups.

Flasks from group A, previously grown with Ps. fluorescens PS3a for 0, 1, 2, 3, 4, and 5 days at 7°C, were inoculated with exactly 1 ml of thermoduric culture as obtained by the procedure outlined in section 3.3.4.2. All flasks were stored at 7°C, and MTC, and PTC were performed at time intervals of 0, 5, 10, 25, and 20 days.

Flasks from group B, previously grown with Ps. fluorescens PS3a for 0, 1, 2, 3, 4, and 5 days at 7°C, were inoculated with exactly 1 ml of Spore-former culture as obtained by the procedure outlined in section 3.3.4.3. All flasks were stored at 7°C; and MSC and PSC were performed at time intervals of 0, 5, 10, and 20 days.

3.4.7 The Effects of Supplementing Skim Milk with Supernatant of Skim Milk Previously Grown with Pseudomonas fluorescens PS3a on the Growth of Milk Microflora in Skim Milk at 7°C

One liter of reconstituted skim milk (sterilized) was inoculated with Ps. fluorescens PS3a to a level of ca 1.84×10^3/ml and incubated at 21°C for 5 days. A standard plate count was per-

formed to determine the final bacterial concentration as outlined in section 3.1.1. The culture broth was then centrifuged using sterile plastic centrifuge containers, at 2600 X g for 30 minutes at 5°C in a Sorval Superspeed RC-2B centrifuge. The supernatant was carefully decanted and filtered through a sterile Whatman #42 filter paper. The clear supernatant was then filtered through a 0.45 um sterile membrane filter (Millipore, Bedford, Mass.) to remove any particles (including bacterial cells) larger than 0.45 um. The sterile supernatant was kept in a sterile plastic container at 7°C (Yates and Elliot, 1977). Estimation of total protein, pH and titratable acidity were then performed.

In order to determine the effects of supplementing 10% skim milk with the supernatant of Ps. fluorescens PS3a on the growth of milk microflora, eighteen sterile capped 125 ml Erlenmeyer flasks were used and divided into three groups. The first group, serving as control, contained 50 ml of skim milk. The second group contained 47.5 ml of skim milk into which 2.5 ml of supernatant was added (5% V/V). The third group contained 45 ml of skim milk into which 5 ml of supernatant was added (10% V/V). One ml of mixed culture obtained from commercially pasteurized, homogenized milk was aseptically transferred into duplicate control, 5%, and 10% supernatant-supplemented flasks, and were stored at 7°C. Standard Plate Count and PBC were performed on all flasks, and everyday thereafter for 5 days.

Exactly one ml of thermoduric culture obtained as outlined in section 3.3.4.2 was aseptically transferred into duplicate control, 5%, and 10% supernatant-supplemented flasks, and were

stored at 7°C. Mesophilic Thermoduric Count and PTC were performed on all flasks at time intervals of 0, 2, 4, 6, 8, 10, and 15 days.

Exactly one ml of sporeforming culture obtained as outlined in section 3.3.4.3 was aseptically transferred into duplicate control, 5%, and 10% supernatant-supplemented flasks, and were stored at 7°C. MSC and PSC were performed on all flasks at time intervals of 0, 2, 4, 6, 8, 10, and 15 days.

3.4.8 The Effects of Supplementing Skim Milk with Commercially Available Milk Fractions on the Growth of Thermoduric and Sporeforming Milk Microflora at 7°C

Three commercially available milk fractions were used in this study: tryptone (hydrolyzed milk casein), peptonized milk (hydrolyzed skim milk), and whey protein. All fractions (10% W/V) were prepared in deionized water, sterilized, and cooled to room temperature before use. Bacterial cultures were obtained by the procedure outlined in section 3.3.4.

3.4.8.1 Effect of Tryptone

Twelve sterile Erlenmeyer flasks were divided into 3 groups. The first group, serving as control contained 50 ml of skim milk. The second group contained 49.5 ml of skim milk into which was supplemented with 0.5 ml of 10% tryptone (1% V/V). The third group contained 47.5 ml of skim milk into which was supplemented with 2.5 ml of 10% tryptone (5% V/V). All flasks were stored at 7°C between plating intervals of 0, 2, 4, 6, 8, 10, and 15 days after the inoculation of bacterial cultures.

One ml of thermoduric culture obtained as outlined in section 3.3.4.2 was aseptically inoculated into duplicate control, 1%, and 5% tryptone-supplemented flasks. Mesophilic Thermoduric Count and PTC were performed.

One ml of sporeforming culture obtained as outlined in section 3.3.4.3 was aseptically inoculated into duplicate control, 1%, and 5% tryptone-supplemented flasks. Mesophilic Sporeformer Count and PSC were performed at each of the plating intervals.

3.4.8.2 Effect of Peptonized Milk

The procedure used was exactly the same as outlined in section 3.4.8.1 except that flasks were supplemented with peptonized milk.

3.4.8.3 Effect of Whey Protein

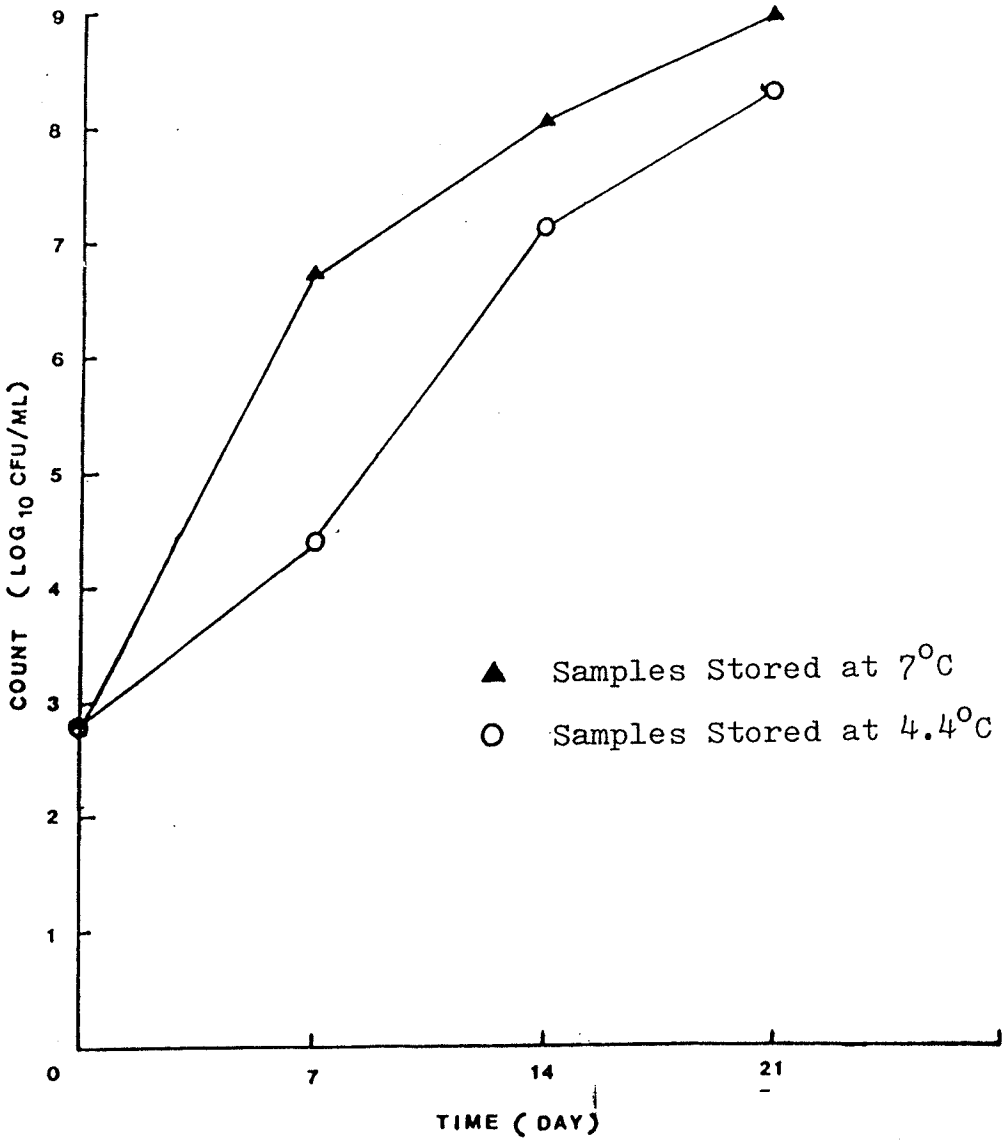
The procedure used was exactly the same as outlined in section 3.4.8.1 except that flasks were supplemented with whey protein.

4. Results and Discussion

4.1 Survey of Psychrotrophic Bacterial Population of Six Commercially Pasteurized, Homogenized Milk Samples

This study was carried out to determine the psychrotrophic bacterial population as well as the keeping quality of milk samples obtained from retail outlets and stored at 4.4°C and 7°C. The results of the effects of storage temperature and time on PBC are presented in Figure 2. The results presented are expressed as the means of six different samples. The initial PBC of six different commercially pasteurized, homogenized milk samples averaged less than 8×10^2/ml. After 7 days of storage, the average PBC increased more than 3 fold at 7°C and only by one fold at 4.4°C. Thereafter, up to 21 days, the PBC increased gradually to a maximum of ca. 9.55×10^8/ml at 7°C, and ca. 2.24×10^8/ml when the samples were stored at 4.4°C. As would be expected, the PBC increased as a function of time and higher storage temperature. The results indicated that the mean PBC of the samples stored at 4.4°C averaged ca. one log cycle less than those stored at 7°C. These results also indicated that although the initial PBC was low, psychrotrophic organisms could grow rapidly even at the low temperature of 4.4°C. The study also found that many of these commercially pasteurized milk samples may have been contaminated to some degree with psychrotrophs after pasteurization. It is this aspect of poor processing procedures which may result in extensive psychrotrophic bacterial growth in a short period of time under refrigeration, thus severely affecting the keeping quality of the milk.

Figure 2: The Effect of Storage Temperatures and Time on Psychrotrophic Bacterial Count



It is generally accepted that most psychrotrophic bacteria in milk, except for some heat-resistant psychrotrophs, are destroyed by conventional HTST pasteurization at 72°C for 16 seconds, or laboratory pasteurization at 62.8°C for 30 minutes (White et al., 1978; Patel and Blankenagel, 1972). Thus, the presence of psychrotrophs in commercially pasteurized milk is a result of post-pasteurization contamination.

The results from this study also showed that the initial psychrotrophic bacterial counts on commercially pasteurized milk samples are of little or no practical value for predicting the keeping quality of milk. Samples held at 4.4°C or 7°C, which were examined over a period of time, presented a more accurate assessment of keeping quality of pasteurized milk, which reflected the processing and packaging conditions. White et al. (1978) reported that PBC, in contrast to SPC, more accurately indicated whether or not the milk was processed under sanitary conditions.

The levels of psychrotrophic thermoduric and sporeforming organisms were also examined in these six milk samples. Their levels were determined by two different methods. In one case, the milk samples were laboratory re-pasteurized to determine the PTC or heat-treated at 80°C for 12 minutes to determine PSC at zero time. Samples were then separated into two portions and incubated at 4.4°C and 7°C for a period up to 21 days. The other method of determining the PTC and PSC involved incubating the purchased milk samples at 4.4°C and 7°C for 7, 14, and 21 days and determining the PTC and PSC at those intervals.

The results of the effect of storage temperature and time on

PTC are presented in Figure 3. The initial PTC of milk samples which were laboratory re-pasteurized at each of the experimental intervals was ca. 3 X 10^1/ml. The PTC of those samples stored at 4.4°C and 7°C increased logarithmically to the maximum of ca. 1.1 X 10^5/ml and 8.9 X 10^4/ml respectively, after 21 days of storage. The results showed a more rapid increase in the mean PTC at 7°C as compared to that at 4.4°C. The PTC of those milk samples receiving laboratory re-pasteurization at zero time, increased gradually to ca. 3.7 X 10^3/ml at 4.4°C and 3.3 X 10^4/ml at 7°C after 21 days of storage. The data indicated that the PTC of milk samples which were laboratory re-pasteurized at each of the experimental intervals, was higher than those received the same treatment at zero time.

The effects of storage temperature and time on PSC are presented in Figure 4. The initial mean PSC of milk samples which were heat-treated at each of the experimental intervals was ca. 1 X 10^1/ml. The PSC of those samples held at 4.4°C and 7°C increased to ca. 1.7 X 10^4/ml and 4.68 X 10^4/ml respectively after 21 days storage. The PSC of the samples held at 4.4°C was ca. 1, 1/2, and 1/2 log cycles less than those samples held at 7°C at time intervals of 7, 14, and 21 days respectively.

The initial PSC of milk samples which received heat treatment at zero time was ca. 1 X 10^1/ml; and the counts increased gradually to ca. 1.1 X 10^3/ml when held at 4.4°C and ca. 2.3 X 10^3/ml when stored at 7°C after 21 days of storage. The PSC of those samples held at 4.4°C was ca. 1/2 log cycle less than those held at 7°C at time intervals from 7 to 21 days.

Figure 3: The Effect of Storage Temperatures and Time on Psychrotrophic Thermoduric Count

I. Milk samples laboratory re-pasteurized at the experimental intervals

▲ Samples stored at 7°C

■ Samples stored at 4.4°C

II. Milk samples laboratory re-pasteurized at zero time

○ Samples stored at 7°C

⬢ Samples stored at 4.4°C

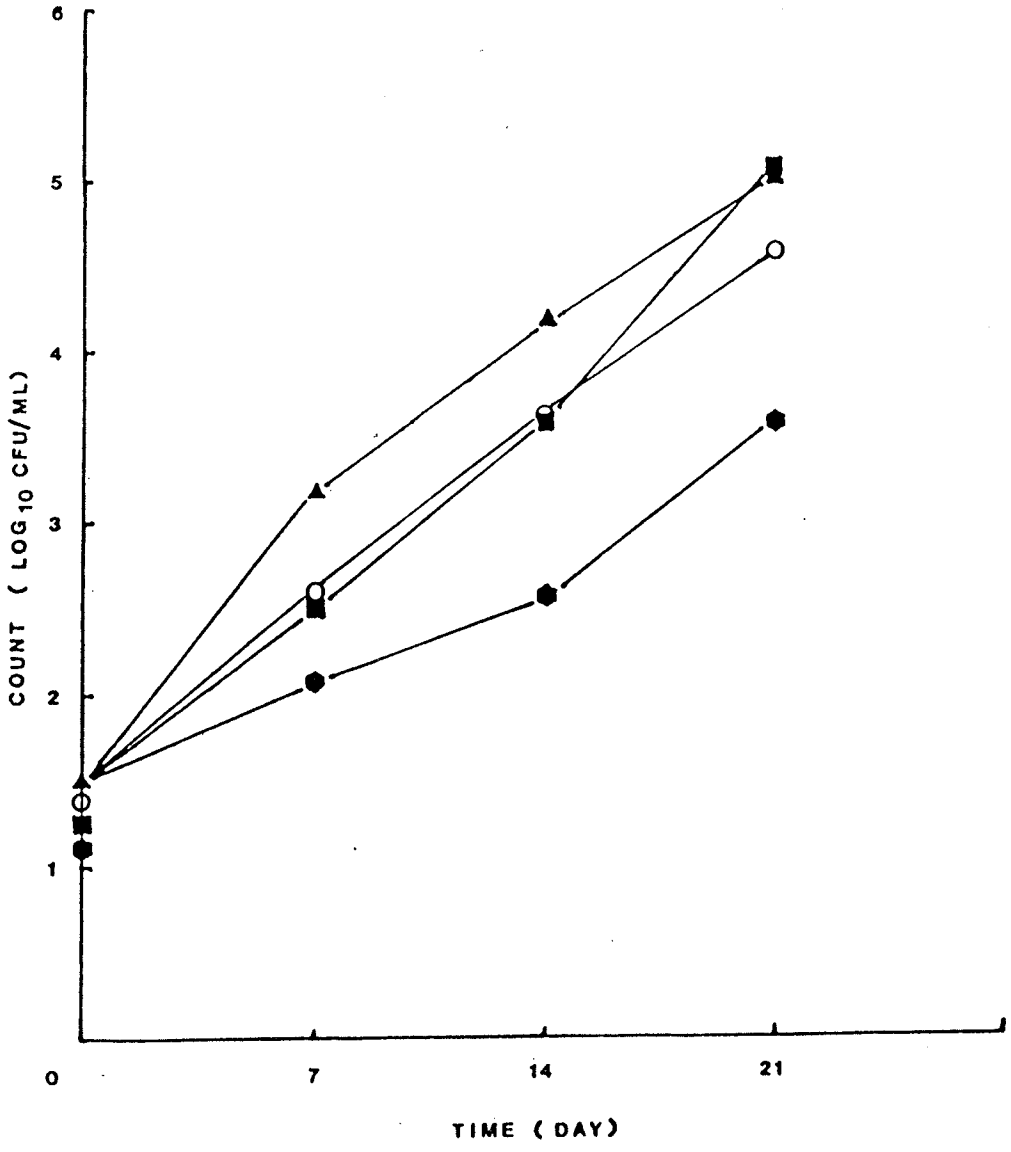


Figure 4: The Effect of Storage Temperatures and Time on Psychrotrophic Sporeformer Count

I. Milk samples heat-treated at the experimental intervals

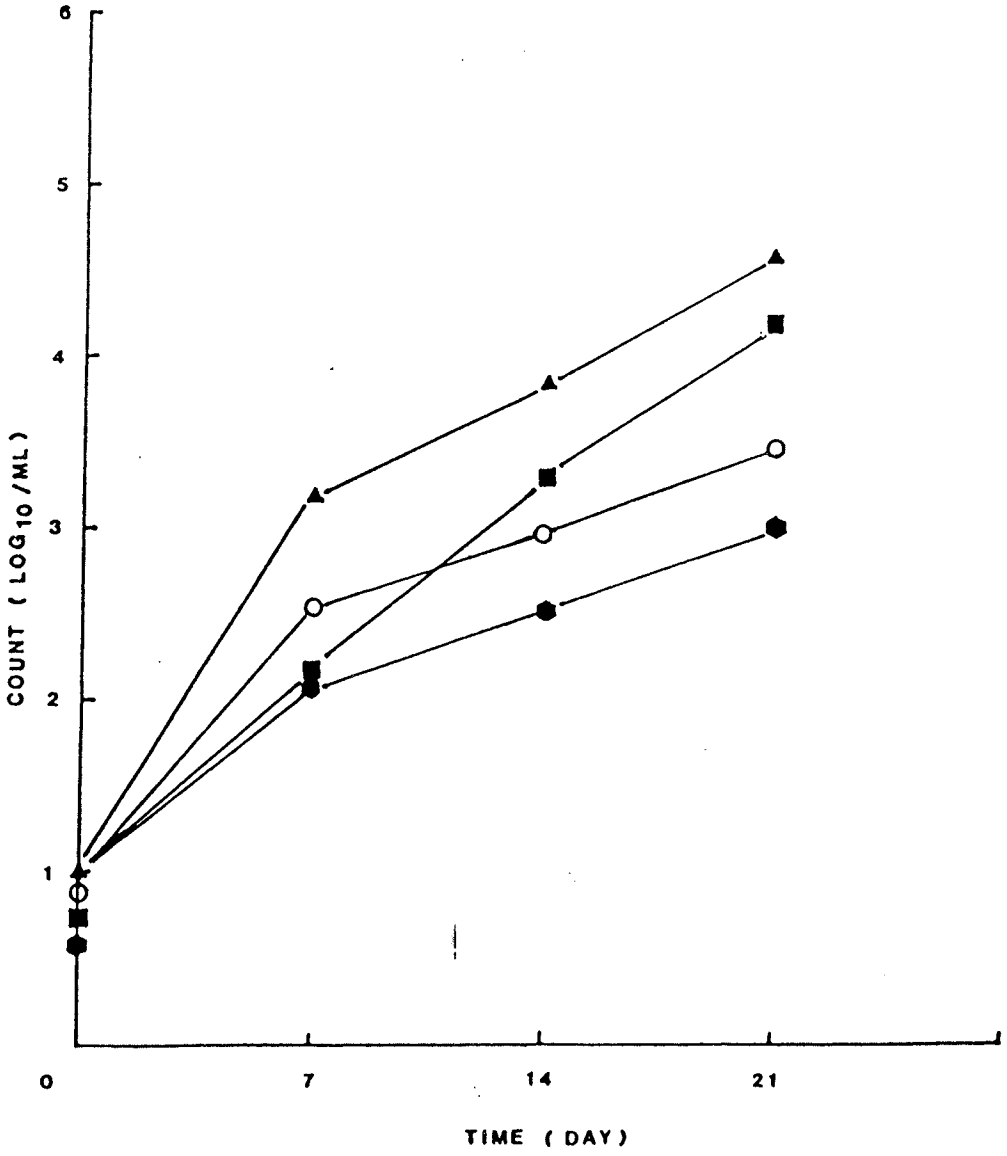
▲ Samples stored at 7°C

■ Samples stored at 4.4°C

II. Milk samples heat-treated at zero time

○ Samples stored at 7°C

⬢ Samples stored at 4.4°C



These results were similar to those observed for PTC, thus indicating that the PSC of the milk samples which were heat-treated at each of the experimental intervals, was higher than those receiving the same treatment at zero time by ca. 1 log cycle for those samples stored at 7°C, and 4.4°C between time intervals from 7 to 21 days.

As would be expected, both PTC and PSC increased as a function of time and higher storage temperature. The results also showed that the PTC and PSC from the milk samples held at 4.4°C were significantly less than those stored at 7°C. The data also revealed that the PTC and PSC of milk samples, which were laboratory re-pasteurized or heat-treated at each of the experimental intervals were higher than those samples that received the same treatment at zero time. It is possible that the growth of heat-sensitive psychrotrophs could have a slight stimulatory effect on the ability of heat-resistant psychrotrophs to initiate growth. Most of the heat-sensitive psychrotrophs were eliminated in samples which were heat-treated or laboratory re-pasteurized at zero time; therefore, the level of heat-sensitive psychrotrophs in those samples was much lower when compared with those samples which received the heat treatment at each of the time intervals. Law(1979) reported that psychrotrophic bacteria are strongly proteolytic and lipolytic; and most of these psychrotrophs belong to the genus Pseudomonas which is a well documented heat-sensitive psychrotroph. The proteolytic and lipolytic activities of these psychrotrophs on milk proteins and lipids resulted in increased peptide, amino acid, and short-chained fatty acid concentrations

in milk. These simpler nutrients could be utilized by the heat-resistant psychrotrophs, and result in an increased growth rate of these organisms. These stimulatory effects were further examined in subsequent sections of this thesis.

Besides the psychrotrophic bacterial population determinations, the organoleptic qualities of the milk samples were also assessed at each of the experimental intervals and the results are presented in Table 8. The organoleptic qualities of all milk samples were judged acceptable on the day of purchase(zero time). After 7 days of storage, 50% of the milk samples held at 7°C had developed some flavor defects, and the PBC of these milk samples was $>10^7$/ml. Detectable flavor changes were also found in two milk samples held at 4.4°C for the same period of time. After an additional 7 day storage period, 80% of the milk samples held at 7°C were spoiled and/or coagulated, and the PBC of these samples was $>10^8$/ml. Fifty percent of the milk samples held at 4.4°C were found to have flavor defects, and the PBC of these samples was $>10^7$/ml. After 21 days of storage, all milk samples held at both temperatures except for one sample were spoiled. These results indicated that milk samples with PBC greater than 10^7/ml develop undesirable flavors, and greater than 10^8/ml result in coagulation. Patel and Blankenagel(1972) reported that milk exhibiting SPC greater than 10^6/ml prior to heat-treatment, frequently developed offensive flavor after pasteurization and subsequent storage. Westhoff(1982) found that milk samples exhibiting PBC of greater than 10^8/ml were found unacceptable to taste panelists. Our results reaffirmed the findings of other researchers that the

Table 8: Organoleptic Qualities[1] of Milk Stored at 7° and 4.4°C

Sample Code	Temperature (°C)	Time (Day) 0	7	14	21
1	4.4	OA	OA	OA	Coagulated
	7.0		OA	Coagulated	-
2	4.4	OA	Unclean	Coagulated	-
	7.0		Coagulated	-	-
3	4.4	OA	Cheesy Odor	Coagulated	-
	7.0		Coagulated	-	-
4	4.4	OA	OA	CA	Coagulated
	7.0		OA	OA	Unclean
5	4.4	OA	OA	Coagulated	-
	7.0		Bitter	Coagulated	-
6	4.4	OA	OA	OA	OA
	7.0		OA	OA	Fruity

[1] Milk samples which were subjected to re-pasteurization or heat-treatment at zero time and stored at 4.4 and 7C were remained organoleptically acceptable after 21 days storage.

OA Organoleptically Acceptable

storage of milk at 4.4°C or lower could extend the shelf-life by a factor twice as long as those milk samples held at 7°C for one week. Watrous et al. (1971 b) indicated that pasteurized milk should be held at 4.4°C or below to achieve maximum shelf-life. The results of this study indicated that milk samples which were subjected to either heat-treatment at 80°C for 12 minutes or laboratory re-pasteurization at 62.8°C for 30 minutes at zero time remained organoleptically acceptable after 21 days storage at 4.4°C and 7°C. Both PTC and PSC of these milk samples were less than 10^5/ml after 21 days storage at both temperatures. The data indicated that the level of heat-resistant psychrotrophs were not sufficiently high to cause any undesirable flavor changes. Watrous et al. (1971 a) reported that in freshly laboratory-pasteurized milk, no recoverable psychrotrophs were found in the milk. The results indicated that 89.1% of milk samples still had counts <1/ml after 10 days of storage at 7.2°C. Patel and Blankenagel (1972) found that no thermoduric psychrotrophic growth was found in the pasteurized milk during the first week of storage at 7°C. Watrous et al. (1971 a) pointed out that these heat-resistant psychrotrophs required a long period of time for recovery after heat-treatment. Maxcy (1967) suggested that heat-resistant psychrotrophs were of minor significance in the spoilage of pasteurized milk as compared to the Gram-negative heat-sensitive rods of post-pasteurization contamination. The results obtained from this study are in general agreement with these researchers, and suggest that heat-sensitive psychrotrophs which re-enter pasteurized milk as post-pasteurization contaminants are mainly respon-

sible for reducing the shelf-life of processed milk.

4.2 Growth Patterns of Heat-Sensitive and Heat-Resistant Psychrotrophs Isolated from Commercially Pasteurized, Homogenized Milk, When Grown at 7°C on Commercially Pasteurized, Homogenized Milk

This study was carried out to determine the growth patterns of heat-sensitive, heat-resistant and a combination of the two latter groups of microorganisms when grown in milk at 7°C. The results are presented in Figures 5 and 6. In Figure 5, the initial heat-sensitive psychrotrophic count (HSPC) which was obtained by subtracting the PTC of the control samples (in which only thermoduric psychrotrophs were growing) from the PBC, was found to be 6.6×10^2/ml. Heat-sensitive psychrotrophs multiplied exponentially without any evidence of a lag phase, and reached counts of ca. 2.2×10^8/ml after 15 days. Counts gradually increased to a maximum of 2.5×10^8/ml after twenty days of incubation. The initial PTC obtained from the samples containing both heat-sensitive and heat-resistant psychrotrophs, was ca. 3.4×10^2/ml. After approximately ten days of lag phase, the thermoduric psychrotrophic populatiom increased to a maximum of 3.2×10^5/ml at the end of a 20 days of storage. The inital PTC of the control samples was ca. 3.4×10^2/ml, and counts increased slightly to a maximum of 1.6×10^3/ml after 20 days. These results indicated that the PTC obtained from the samples containing both heat-sensitive and heat-resistant psychrotrophs increased more rapidly than those samples containing only heat-resistant psychrotrophs during storage at 7°C.

Figure 5: Growth Patterns of Heat Sensitive and Thermoduric Psychrotrophs in Commercially Pasteurized, Homogenized Milk Stored at 7°C

\+ Psychrotrophic Bacterial Count

* Heat Sensitive Psychrotrophic Count

△ Psychrotrophic Thermoduric Count obtained from samples containing both heat sensitive and thermoduric psychrotrophs

□ PTC obtained from samples containing only thermoduric psychrotrophs (Control)

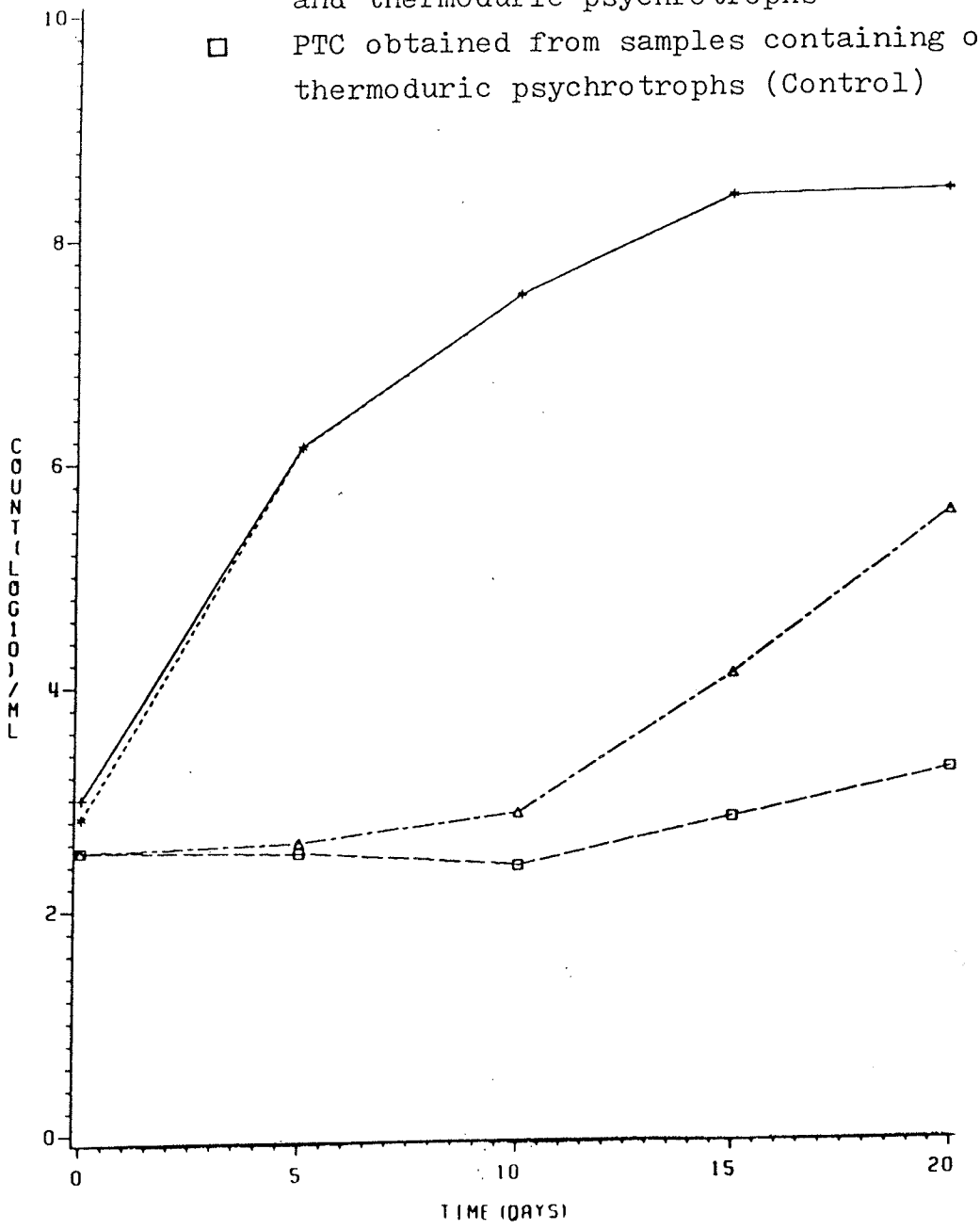


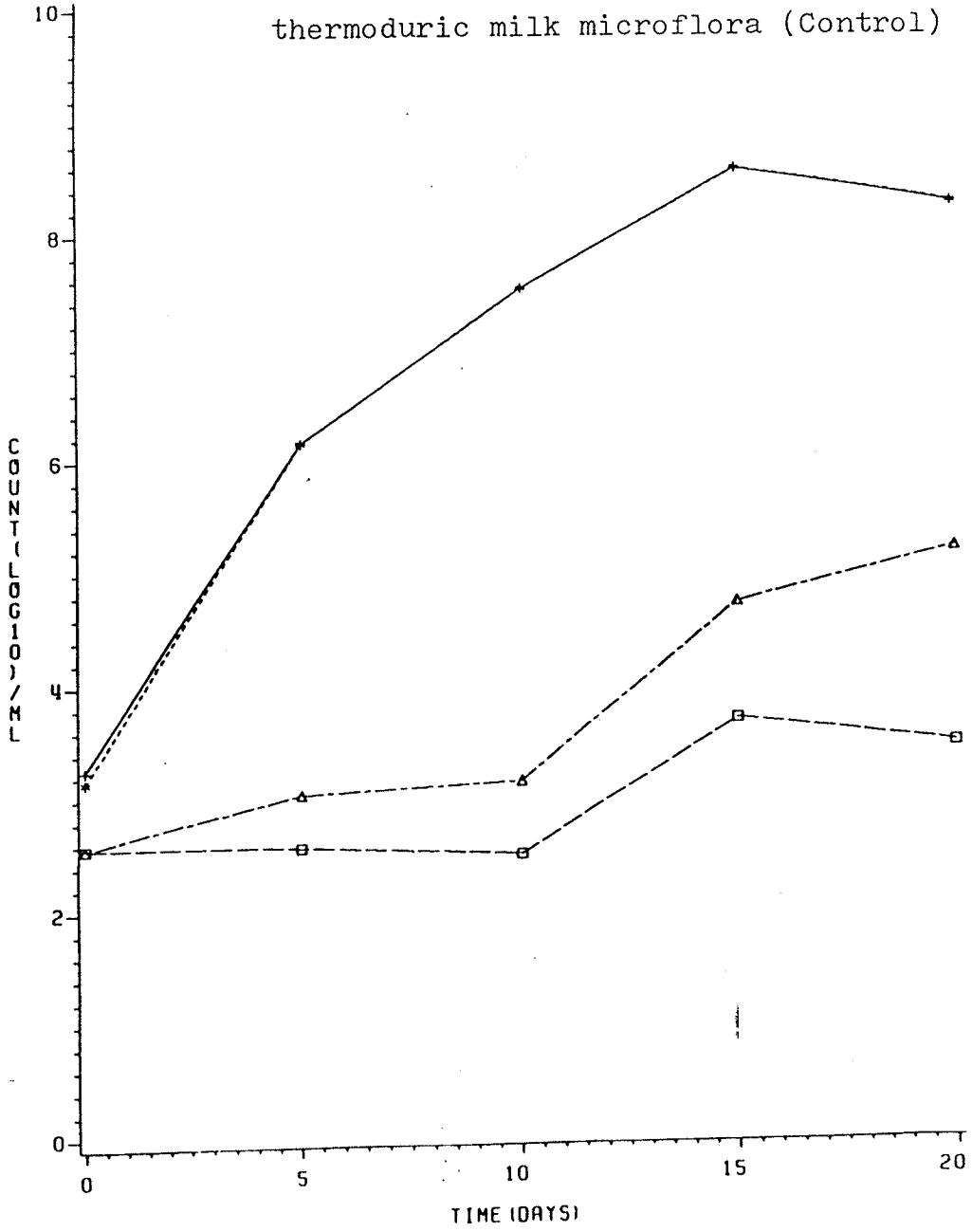
Figure 6: The Ability of Heat Sensitive and Thermoduric Milk Microflora to Grow at 32°C

+ Standard Plate Count

* Heat Sensitive Mesophilic Count

△ Mesophilic Thermoduric Count obtained from samples containing both heat sensitive and thermoduric milk microflora

□ MTC obtained from samples containing only thermoduric milk microflora (Control)



The growth pattern of heat-sensitive and thermoduric milk microflora at 32°C is illustrated in Figure 6. The initial heat-sensitive mesophilic count (HSMC) which was obtained by subtracting the MTC of the control samples from the SPC, was ca. 1.4×10^3/ml. The HSMC increased substantially to a maximum of ca. 3.2×10^8/ml after 15 days without any evidence of a lag phase. Thereafter, counts decreased slightly to 1.5×10^8/ml at the end of 20 days of incubation. The initial MTC which was obtained from the samples containing both heat-sensitive and thermoduric microflora was ca. 3.7×10^2/ml. After a lag phase of 10 days, the MTC increased to a maximum of ca. 1.3×10^5/ml after 20 days of incubation. The MTC of the control samples was initially 3.7×10^2/ml, and these counts increased only slightly to 4.4×10^3/ml after 15 days, and remaining essentially unchanged at the end of twenty days. These results were similar to those observed at psychrotrophic conditions. The MTC obtained from the samples containing both heat-sensitive and thermoduric milk microflora increased more rapidly than those samples containing only heat-resistant milk microflora during storage at 7°C.

The results from this study showed that heat-sensitive psychrotrophs are not only capable of growing rapidly at low temperature but also at 32°C. Heat-sensitive psychrotrophs grew much faster than heat-resistant psychrotrophs under similar storage conditions. Thermoduric psychrotrophs, when grown together with heat-sensitive psychrotrophs, increased in numbers more rapidly than those thermoduric psychrotrophs growing alone under the same storage conditions. Therefore, the results suggest that heat-sen-

sitive psychrotrophic bacteria may have a stimulatory effect on initiating the growth of thermoduric psychrotrophs. Similar results were also observed under mesophilic conditions. Overcast and Atmaram(1974) reported that excessive growth of Ps. fragi or Ps. fluorescens in raw skim milk had a stimulatory effect on the growth of two of three psychrotrophic Bacillus cereus isolates when combined with heat activation. Their results indicated that the stimulatory effect was most pronounced when heat activation at 80°C for 15 seconds was used. Our results indicate that if the HSPC or HSMC is allowed to exceed 10^7/ml, a stimulatory effect on the growth of heat-resistant psychrotrophs is likely to occur.

4.3 Growth Patterns of Ps. fluorescens P26 and Heat-Resistant Bacterial Flora from Commercially Pasteurized,Homogenized Milk, when Grown Together at 7°C

In section 4.2, the results have indicated that heat-sensitive milk microflora may be able to stimulate the growth of heat-resistant organisms in milk when grown at 7°C. The group of heat-sensitive organisms used in that particular study was obtained from a commercially pasteurized milk, and no effort was made to identify that particular group of organisms. However, in this study, Ps. fluorescens P26 obtained from Dr. R.T. Marshall, the University of Missouri, U.S.A., was used to determine whether this psychrotroph could possibly stimulate the growth of heat-resistant milk microflora when grown at 7°C.

Ps. fluorescens P26 is well characterised as a heat-sensitive psychrotroph with a D value of 2.6 minutes at 62.8°C (Mayerhofer et al.,1973). This organism was found to be strongly proteolytic,

and its protease which was found to be extremely heat-resistant, required 9 minutes at 121°C for complete inactivation. The determination of the growth patterns of thermoduric organisms in this study was carried out by 2 different methods. In the first method, the milk samples designated as the control samples, which contained only thermoduric milk microflora during storage at 7°C, were used to determine the levels of MTC and PTC. In the second method, the milk samples designated as the experimental samples, which contained both Ps. fluorescens P26 and thermoduric microflora during storage, were used to determine the levels of MTC and PTC.

Figures 7 and 8 illustrate the growth patterns of Ps. fluorescens P26 and thermoduric microorganisms in milk at 7°C and their ability to grow at 32°C respectively. The HSPC was obtained by subtracting the PTC of the control samples from the PBC. The growth patterns of the PBC, HSPC, PTC of the control samples, and the PTC of the experimental samples are presented in Figure 7. The initial HSPC was ca. 8.7×10^3/ml, and the counts increased substantially to ca. 2×10^7/ml after only 5 days of incubation. Thereafter, the HSPC increased gradually to a maximum of ca. 2×10^9/ml after 20 days of storage. The initial PTC of the experimental samples was 3.2×10^2/ml, and the counts increased gradually up to a maximum of 5×10^4/ml at 15 days. Thereafter, the PTC decreased slightly to 2.4×10^4/ml after 20 days of incubation. The PTC of the control samples did not increase throughout the entire 20 day incubation period.

The HSMC was obtained by subtracting the MTC of the control

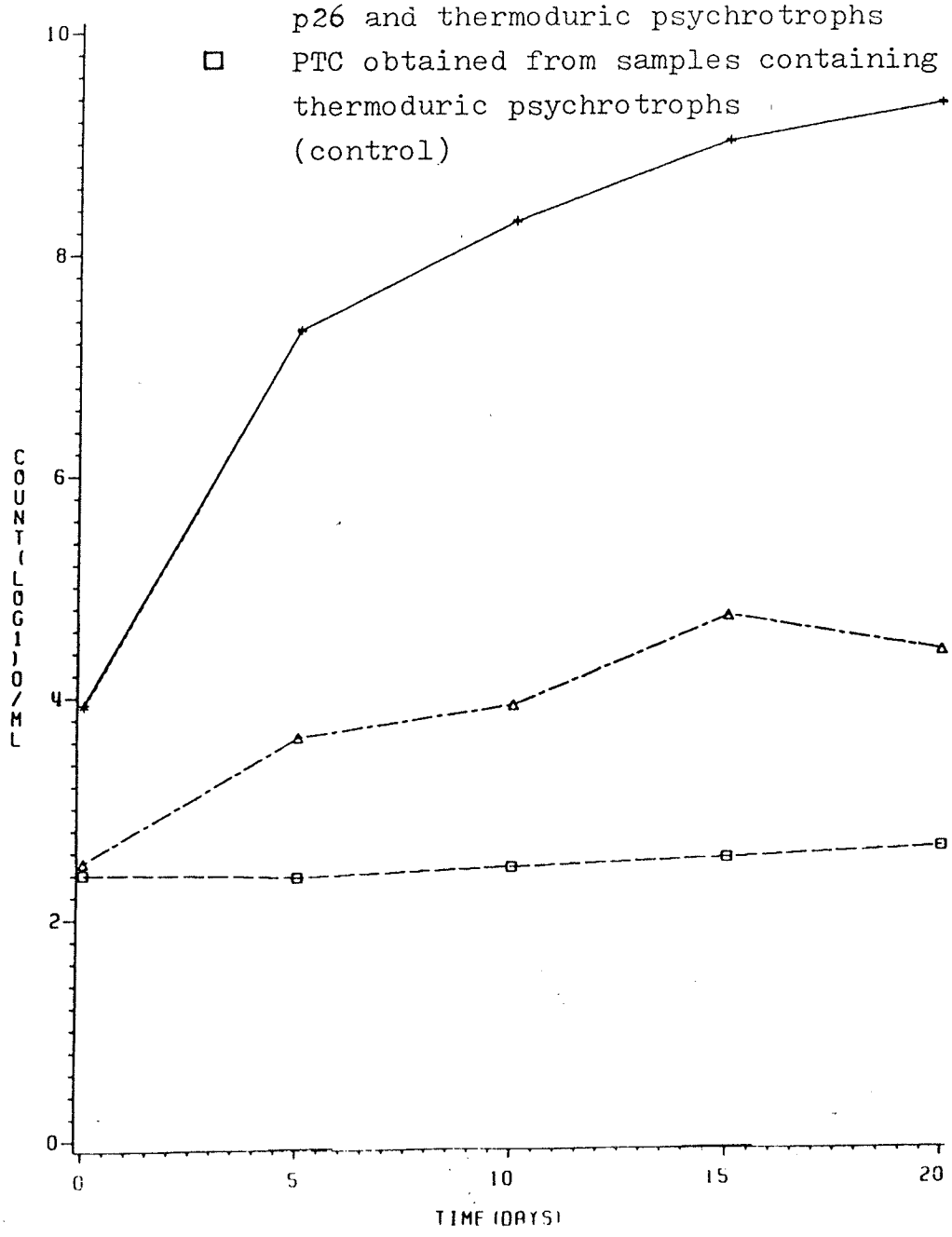
Figure 7: Growth Pattern of Ps. fluorescens p26 and Thermoduric Milk Microflora, When Grown Together in Milk at 7°C

+ Psychrotrophic Bacterial Count

* Heat Sensitive Psychrotrophic Count

Δ Psychrotrophic Thermoduric Count obtained from samples containing both Ps. fluorescens p26 and thermoduric psychrotrophs

□ PTC obtained from samples containing only thermoduric psychrotrophs (control)



samples from the SPC. The ability of Ps. fluorescens P26 and thermoduric microorganisms to grow at 32°C is presented in Figure 8. The initial HSMC was ca. 8.9×10^3/ml, and the population increased exponentially to 2.5×10^7/ml without any evidence of a lag phase after 5 days of incubation. Thereafter, the HSMC increased gradually to a maximum of 1.8×10^9/ml after 20 days. Thereafter, the MTC decreased approximately one log cycle to 8.5×10^3/ml after 20 days. After a ten day lag phase, the MTC of the control samples increased up to 2.2×10^4/ml after 20 days from an initial level of 3.6×10^2/ml.

The data obtained from this study showed that Ps. fluorescens P26 was capable of growing rapidly at 7°C as well as at 32°C without any evidence of a lag phase. Ps. fluorescens P26 counts obtained at 32°C were similar to those obtained at 7°C, suggesting that Ps. fluorescens P26 was able to grow at 32°C at a similar rate as at 7°C. The results from Figure 7 showed that the PTC of the experimental samples was at least one log cycle higher than those of the control samples between the time intervals of 5 to 15 days. Similar results were also obtained in Figure 8. Comparing the results of Figure 7 to 8, there is no difference between the HSPC and HSMC, however the MTC of the control samples was one log cycle and 1.5 log cycles higher than the PTC of the control samples at time intervals of 15 and 20 days respectively. Therefore, the results suggest that most of the thermoduric organisms were growing better under a mesophilic temperature, but not in a psychrotrophic environment. Boyd et al. (1954) reported that only 17.9% of thermoduric isolates from milk grew at 5°C.

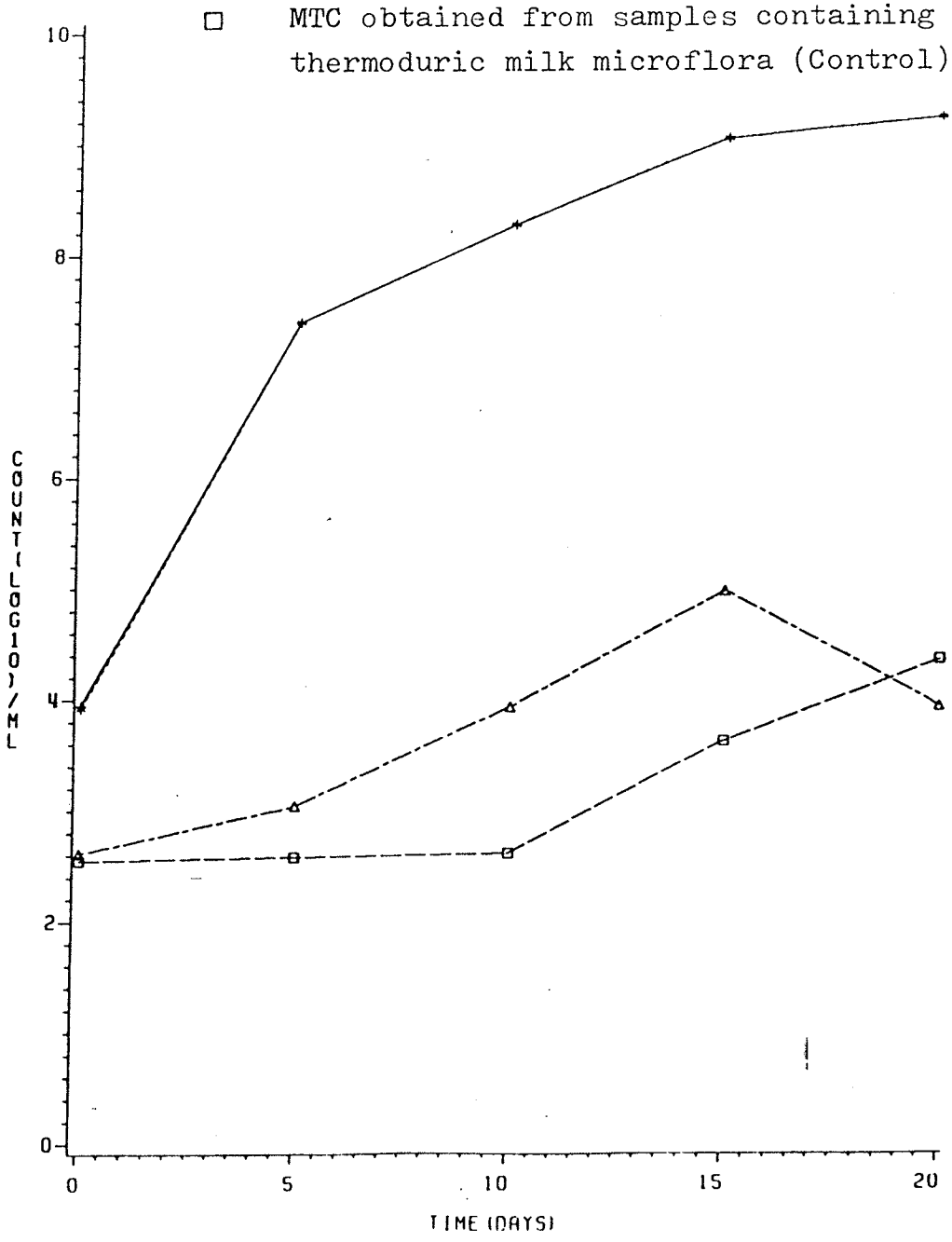
Figure 8: The Ability of Ps. fluorescens p26 and Thermoduric Milk Microflora to Grow at 32°C

+ Standard Plate Count

* Heat Sensitive Mesophilic Count

△ Mesophilic Thermoduric Count obtained from samples containing both Ps. fluorescens p26 and thermoduric milk microflora

□ MTC obtained from samples containing only thermoduric milk microflora (Control)



Both MTC and PTC obtained from the experimental samples showed increased cell conentrations after 15 days of incubation at 7°C when the thermoduric organisms were growing together with Ps. fluorescens P26. The present study also showed that both MTC and PTC of the experimental samples were higher than those of the control samples up to the time interval of 15 days. Therefore, there is evidence here to suggest that Ps. fluorescens P26 exhibits a stimulatory effect on the growth of heat-resistant milk microflora at 7°C. However, both MTC and PTC of the experimental samples decreased after 20 days, and the corresponding Ps. fluorescens P26 counts in the experimental samples reached ca. 10^9/ml. This may be due to the fact that at a such high Ps. fluorescens P26 concentration, the organisms produced toxic end-products which could inhibit the growth of heat-resistant organisms. Therefore the results of this study suggest that if Ps. fluorescens P26 is allowed to increase to ca. 10^7-10^8/ml, a stimulatory effect on the growth of heat-resistant milk microflora may occur. However, if Ps. fluorescens P26 count is higher that 10^9/ml, this stimulatory effect is unlikely to occur. The results of this study support the findings of Overcast and Adams (1966). They reported that excessive pre-pasteurization Ps. fragi growth was not stimulatory for post-pasteurization growth of psychrotrophic organisms, in fact, their work indicated that the excessive growth was found to be inhibitory to post-pasteurization psychrotrophic growth.

4.4 Isolation of a Proteolytic Psychrotroph

The isolation procedure was listed in section 3.4.4. Five

different psychrotrophic isolates were obtained from commercially pasteurized,homogenized milk. Four of these isolates showed no proteolytic activities on SPC agar fortified with 10% skim milk when incubated at 21°C for 48 hours. The remaining isolate showed proteolytic activity and was subsequently inoculated at a level of ca. 3 X 10^3/ml into 50 ml of 10% skim milk and incubated at 7°C. After 10 days of incubation, bitterness was detected organoleptically in the skim milk. The isolate was then identified according to the procedure listed in Bergey's Manual (Buchanan and Gibbons, (1975), and Cowan and Steel (1970). The results are presented in Table 9. The isolate was identified as Pseudomonas fluorescens and was labelled with a code number, PS3a.

4.5 The Effects of Ps. fluorescens PS3a on the Physico-Chemical Properties of 10% Skim Milk at 7°C

Pseudomonas fluorescens PS3a was inoculated at a level of ca. 2.63 X 10^3/ml in 10% sterile skim milk and stored at 7°C. Microbiological and chemical analyses were performed at time intervals of 0,5,10, 15 and 20 days. The results are presented in Figure 9.

After 5 days of incubation, the Ps. fluorescens PS3a count increased substantially to ca. .6 X 10^7/ml, without any evidence of a lag phase. Thereafter, counts increased gradually up to 1.6 X 10^9/ml at the end of the twenty day incubation period.

The initial pH, titratable acidity, and tyrosine value of skim milk were 6.65, 0.175% (expressed as %lactic acid), and 50.63 ug/ml respectively. Both the pH and titratable acidity did not increase significantly during the incubation period of 20 days,

Table 9

Morphological and Biochemical Characteristics of the Proteolytic Isolate

Tests	Results
Microscopic Analyses	Non-sporeforming rods, motile, Gram's negative.
Pigment	Diffusible fluorescent pigment on Pseudomonas F agar
Growth on SPC agar at 4 C	+ve*
Growth on SPC agar at 41 C	-ve**
Growth on MacConkey's agar	+ve
Catalase	+ve
Oxidase	+ve
Hugh-Leifson O/F broth	Oxidative
Acid from Glucose	+ve
Acid from Xylose	+ve
Citrate Utilization	+ve
Hydrolysis of Casein	+ve
Utilization of Nitrogen	-ve
Urea Utilization	-ve
Indole	-ve
Arginine Decarboxylase	+ve

* Positive
** Negative

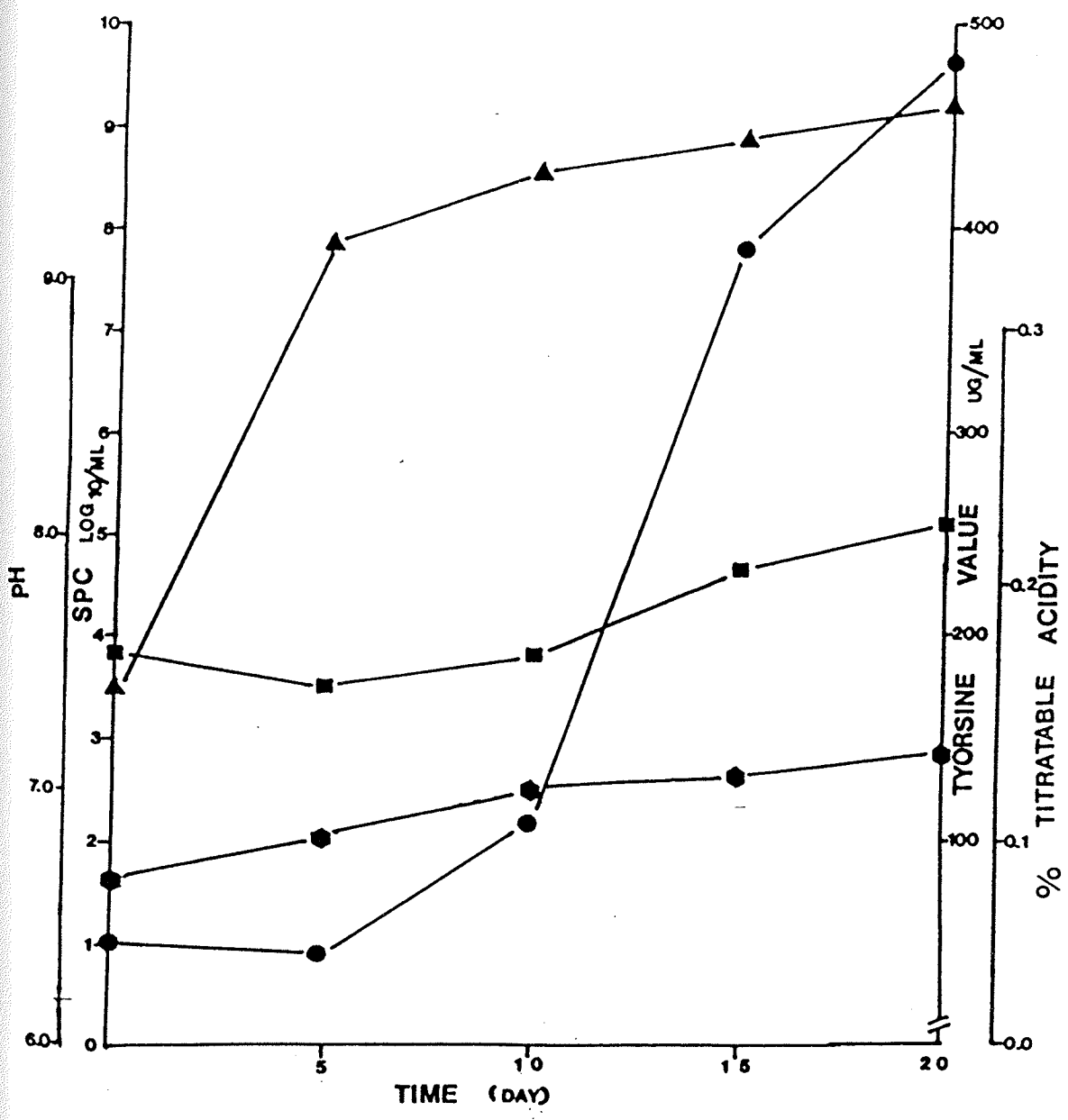
Figure 9: The Effect of Ps. fluorescens ps3a on the Physio-Chemical Properties of 10% Skimmilk at 7°C

▲ SPC at 32°C

⬢ pH

● Tyrosine Value

■ Titratable Acidity



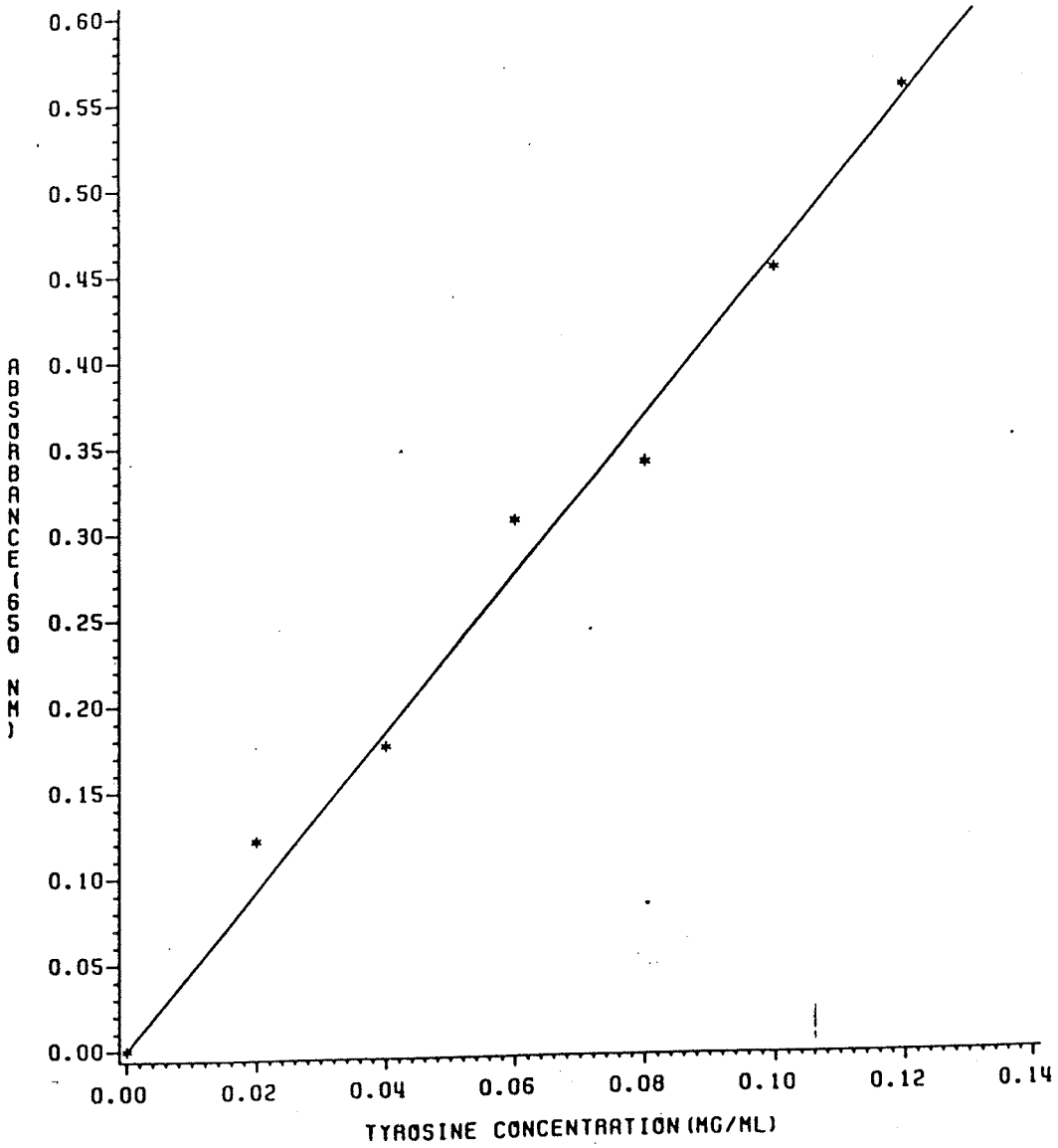
and these values reached 7.14 and 0.223% respectively at the end of the incubation. The tyrosine value varied slightly after 5 days, but increased substantially to ca. 480 ug/ml at the end of 20 days. Bitter flavor was not detected organoleptically even though the level of Ps. fluorescens PS3a reached ca. 10^7/ml after 5 days, but the corresponding tyrosine value was only 47.5 ug/ml. However, bitter flavor was detected after 10 days, and the corresponding bacterial count and tyrosine value were 3 X 10^8/ml and 117 ug/ml respectively. Mayerhofer et al.(1973) reported that the increase of free tyrosine and tryptophan which was caused by the protease action of Ps. fluorescens P26, was directly related to the presence of bitterness in skim milk.

The results indicate that Ps. fluorescens PS3a is capable of growing rapidly under refrigeration temperature, and it increased to approximately 4 log cycles after only 5 days of storage at 7°C. Pseudomonads are generally regarded as heat-sensitive psychrotrophs (Mikolajcik, 1979); and Parker et al.(1953) concluded that the presence of heat-sensitive organisms in processed milk was evidence of post-pasteurization contamination. Watrous et al.(1971 b) reported that laboratory pasteurization destroyed and/or inactivated coliforms and psychrotrophs which were usually responsible for spoilage of commercially processed milk. The results obtained from this study therefore suggest that precautions should be taken to minimize post-processing contamination of milk with heat-sensitive psychrotrophs. This study also showed that as the bacterial population was increasing, more free amino acids and peptides were released due to the increase in proteo-

lytic activities of these organisms, resulting in an increase in pH. Juff (1976) reported that the proteolytic activity of Ps. fluorescens and Ps. aeruginosa resulted in peptonization and alkaline reactions in milk. The degree of proteolysis reported here is measured in terms of ug/ml of free tyrosine by using Hull's method, and the standard curve is presented in Figure 10.

The results of this experiment also suggest that both pH and titratable acidity determinations are not good parameters to relate to the bacterial population. There is also no good correlation between the tyrosine value and the bacterial counts after 5 days of storage. Juffs (1973 a) reported that no significant relationship was found between tyrosine value and psychrotrophic or proteolytic psychrotrophic count. However, after 10 days, a positive relationship between tyrosine value and bacterial count was found. Therefore, the results suggest that the use of tyrosine values as a measure of initial psychrotrophic bacterial count was not sensitive enough to estimate counts in freshly pasteurized milk or when PBC was less than 10^7/ml in milk. Juffs (1973 b, 1975) found that bacterial counts (total bacteria, psychrotrophs, or proteolytic psychrotrophs) generally exceeded 10^6/ml before a definite increase in tyrosine value could be detected or any deterioration in organoleptic quality was observed. Therefore, this study suggests that the measure of tyrosine value is only useful as a general index of the bacteriological and organoleptic qualities of milk which has been subjected to prolonged cold storage.

Figure 10: Standard Curve for Tyrosine Value Determination by Measurement of the Absorbance at 650 nm.



4.6 The Growth Characteristics of Heat-Resistant Milk Microflora in 10% Skim Milk Previously Grown with Pseudomonas fluorescens PS3a at 7°C

This phase of the study involved examining the effects of skim milk in which Ps. fluorescens PS3a had previously grown for one to five days, on the growth of heat-resistant milk microflora at 7°C. The Ps. fluorescens PS3a counts, pH, titratable acidity, and tyrosine value of skim milk were analyzed at 0,1,2,3,4, and 5 days before the skim milk was autoclaved at 121°C for 10 minutes to remove the Ps. fluorescens PS3a. The results are presented in Table 10. After the first day of incubation, the cell concentration increased to ca. 2×10^4/ml from an initial count of 2.63×10^3/ml. Thereafter, the counts increased by 4.25 log cycles, reaching a maximum of 7.2×10^7/ml after 5 days at 7°C.

The initial tyrosine value was found to be 50.63 ug/ml. This high initial value was possibly due to heat denaturation of milk proteins during sterilization of the skim milk. The tyrosine value decreased gradually to a low of 39.38 ug/ml on the fourth day. However, the tyrosine value then increased slightly to 46.25 ug/ml after 5 days of incubation. The results indicated that as Ps. fluorescens PS3a started multiplying, the organisms utilized the simplest available nutrients in the medium, such as free amino acids and small peptides. Stanier et al. (1976) reported that simple carbon sources such as glucose are metabolized more rapidly and support more rapid bacterial growth than the complex carbon sources such as acetate. After these simple nutrients were used up, Ps. fluorescens PS3a started to break down more complex

Table 10: The Effects of Skimmilk in Which Ps. fluorescens ps3a Had Previously Grown for One to Five Days at 7°C

Time (Day)	SPC (Log_{10}/ml)	Tyrosine Value (ug/ml)	pH	Titratable Acidity (% Lactic Acid)
0	3.24	50.63	6.65	0.175
1	4.32	52.88	6.70	0.180
2	5.87	48.00	6.70	0.180
3	7.28	47.25	6.70	0.170
4	7.67	39.38	6.78	0.165
5	7.86	46.25	6.82	0.160

milk proteins. This is evident from the increase in tyrosine value after 5 days of incubation. However, the break down of milk proteins appeared to be relatively small. Because both the pH and the titratable acidity remained relatively constant throughout the 5 day incubation period.

The results of the growth of thermoduric and sporeforming milk microflora at 7°C, in skim milk in which Ps. fluorescens PS3a had previously grown for 0,1,2,3,4, and 5 days at 7°C, are presented in Figures 11,12,13, and 14.

The growth patterns of mesophilic and psychrotrophic thermoduric microflora are illustrated in Figures 11 and 12, respectively. The results from these two figures showed relatively similar growth patterns. In general, as the incubation period of Ps. fluorescens PS3a increased prior to autoclaving the skim milk the faster was the subsequent growth of thermoduric organisms when grown in the same milk at 7°C. The maximum increases of mesophilic and psychrotrophic thermodurics were 1.53 and 1.72 log cycles respectively in skim milk in which Ps. fluorescens PS3a had previously grown for 5 days at 7°C prior to sterilization at 121°C for 10 minutes, compared to only 0.81 and 0.97 log cycles in the control samples after 20 days. Even though the increases of the MTC and PTC are small compared to the control samples, analysis of variance showed a significant difference in MTC and PTC among treatments at $P \leq 0.005$. Comparison of the difference between means indicated a significant difference in MTC and PTC between the samples in which Ps. fluorescens PS3a had previously grown for 5 days at 7°C, and the control sample at $P \leq 0.05$, and no

Figure 11: The Subsequent Growth Characteristics of Mesophilic Thermoduric Organisms in 10% Skimmilk Previously Grown with Ps. fluorescens ps3a at 7°C

+ Ps. fluorescens ps3a had grown for 0 day (Control)

* Ps. fluorescens ps3a had grown for 1 day

□ Ps. fluorescens ps3a had grown for 2 days

◇ Ps. fluorescens ps3a had grown for 3 days

△ Ps. fluorescens ps3a had grown for 4 days

X Ps. fluorescens ps3a had grown for 5 days

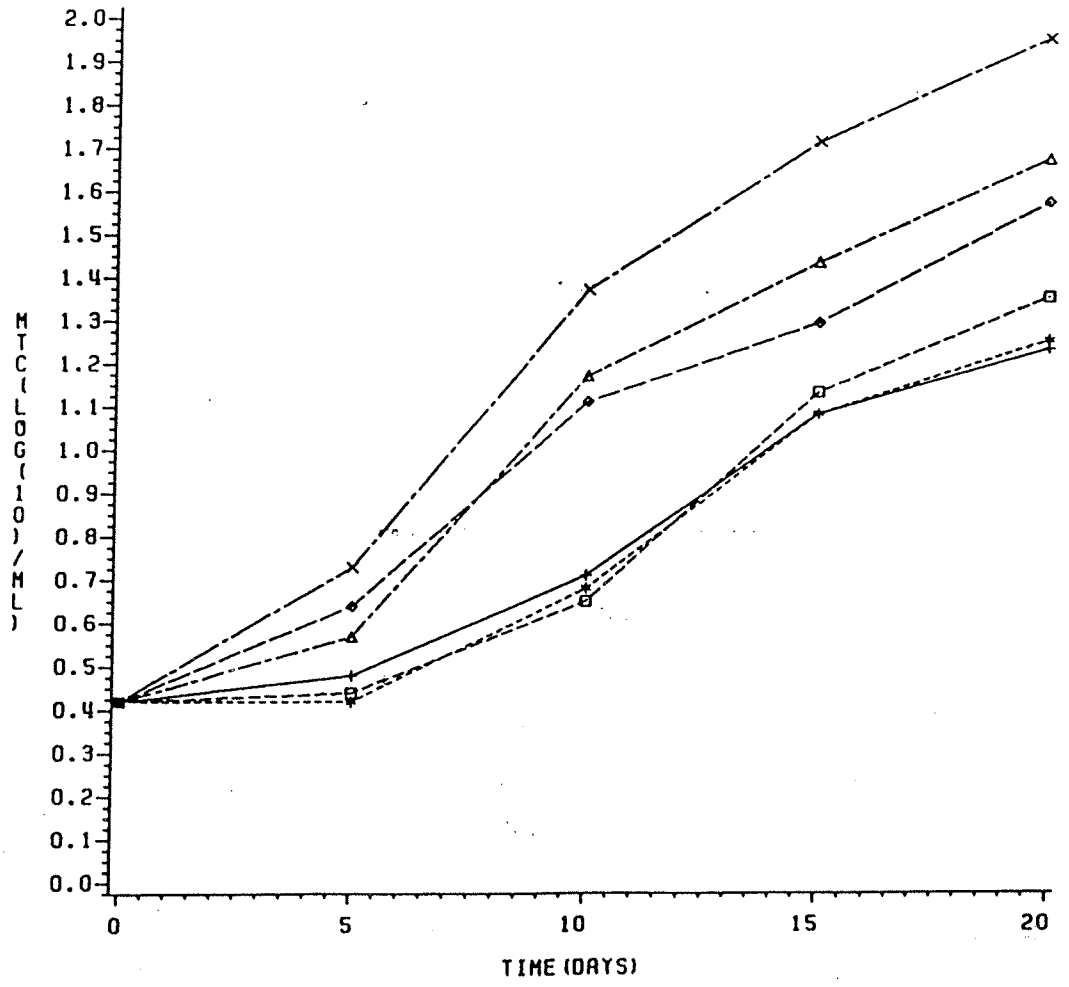
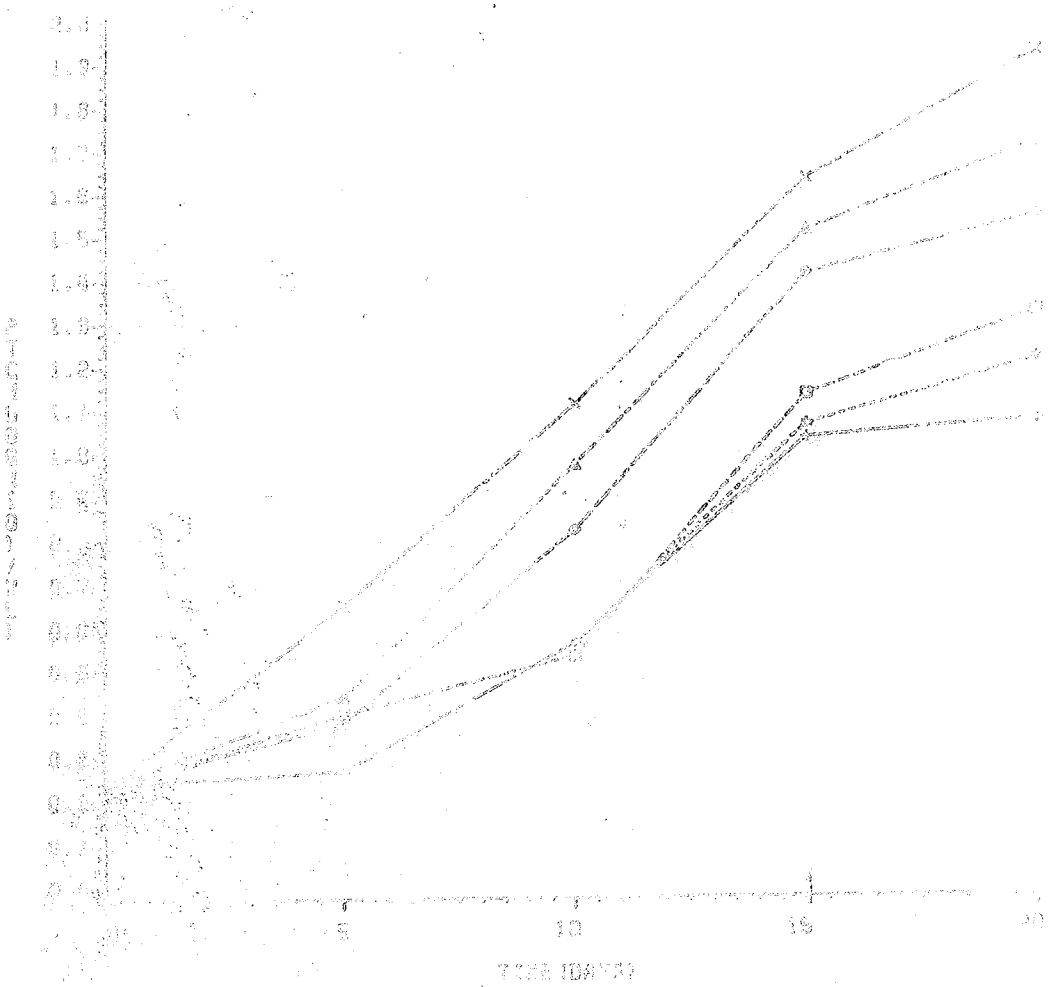


Figure 12: The Subsequent Growth Characteristics of Psychrotrophic Thermoduric Organisms in 10% Skimmilk Previously Grown with Ps. fluorescens ps3a at 7°C

- + Ps. fluorescens ps3a had grown for 0 day (Control)
- * Ps. fluorescens ps3a had grown for 1 day
- □ Ps. fluorescens ps3a had grown for 2 da
- ◇ Ps. fluorescens ps3a had grown for 3 da
- △ Ps. fluorescens ps3a had grown for 4 da
- X Ps. fluorescens ps3a had grown for 5 da



difference between the others (Appendix 2 Tables 1 and 2). Both MTC and PTC obtained from Figures 11 and 12 respectively were similar, suggesting that psychrotrophic thermoduric milk microflora were also capable of growth at 32°C.

Figures 13 and 14 illustrate the growth curves of mesophilic and psychrotrophic sporeformers, respectively, grown in the skim milk in which Ps. fluorescens PS3a had previously grown for 0,1,2,3,4, and 5 days at 7°C. These results are very similar to those reported for the thermoduric microflora. In general, the longer Ps. fluorescens PS3a growth occurred prior to sterilization, the faster was the subsequent growth of sporeforming microorganisms. The increases in the MSC and PSC however, were small, both the MSC and PSC from the samples in which Ps. fluorescens PS3a had previously grown for 5 days at 7°C prior to sterilization showed the largest increase of ca. 1.70 and 1.65 log cycles respectively, after 20 days of incubation, compared to only 0.8 log cycles in both MSC and PSC of the control samples. An analysis of variance showed a significant difference in both MSC and PSC among treatments at $P \leq 0.005$. Comparison of the differences between treatment means indicated that there was a significant difference in both MSC and PSC between the control sample and the samples in which Ps. fluorescens had previously grown for 5 days at 7°C at $P \leq 0.05$. No difference between the others were indicated (Appendix 2 Tables 3 and 4).

The results obtained from this portion of the study indicated that if the number of Ps. fluorescens PS3a is allowed to increase to a level of greater than 10^7/ml in skim milk prior to sterili-

Figure 13: The Subsequent Growth Characteristics of Mesophilic Sporeformers in 10% Skimmilk Previously Grown with Ps. fluorescens ps3a at 7°C

+ Ps. fluorescens ps3a had grown for 0 day (Control)

* Ps. fluorescens ps3a had grown for 1 day

□ Ps. fluorescens ps3a had grown for 2 days

◇ Ps. fluorescens ps3a had grown for 3 days

△ Ps. fluorescens ps3a had grown for 4 days

X Ps. fluorescens ps3a had grown for 5 days

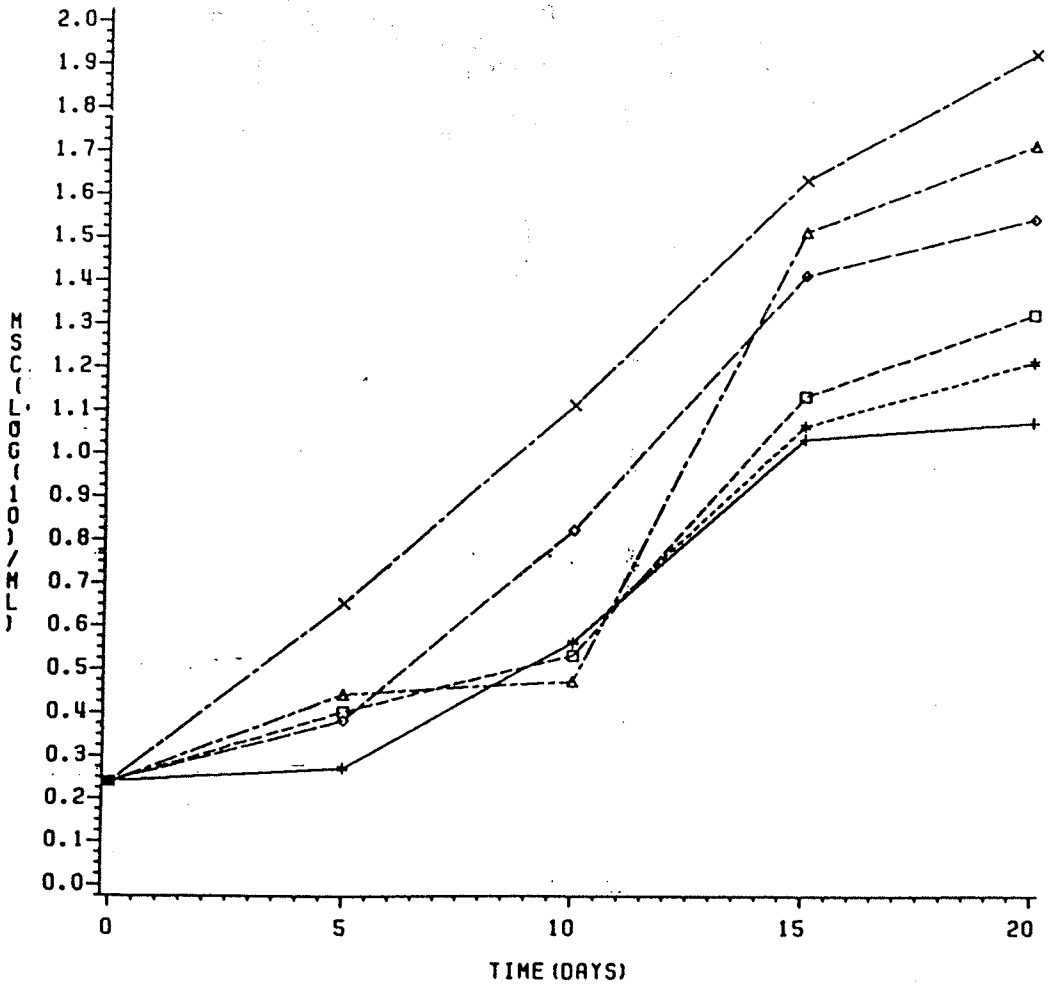


Figure 14: The Subsequent Growth Characteristics of Psychrotrophic Sporeformers in 10% skimmilk previously Grown with Ps. fluorescens ps3a at 7°C

+ Ps. fluorescens ps3a had grown for 0 day (Control)

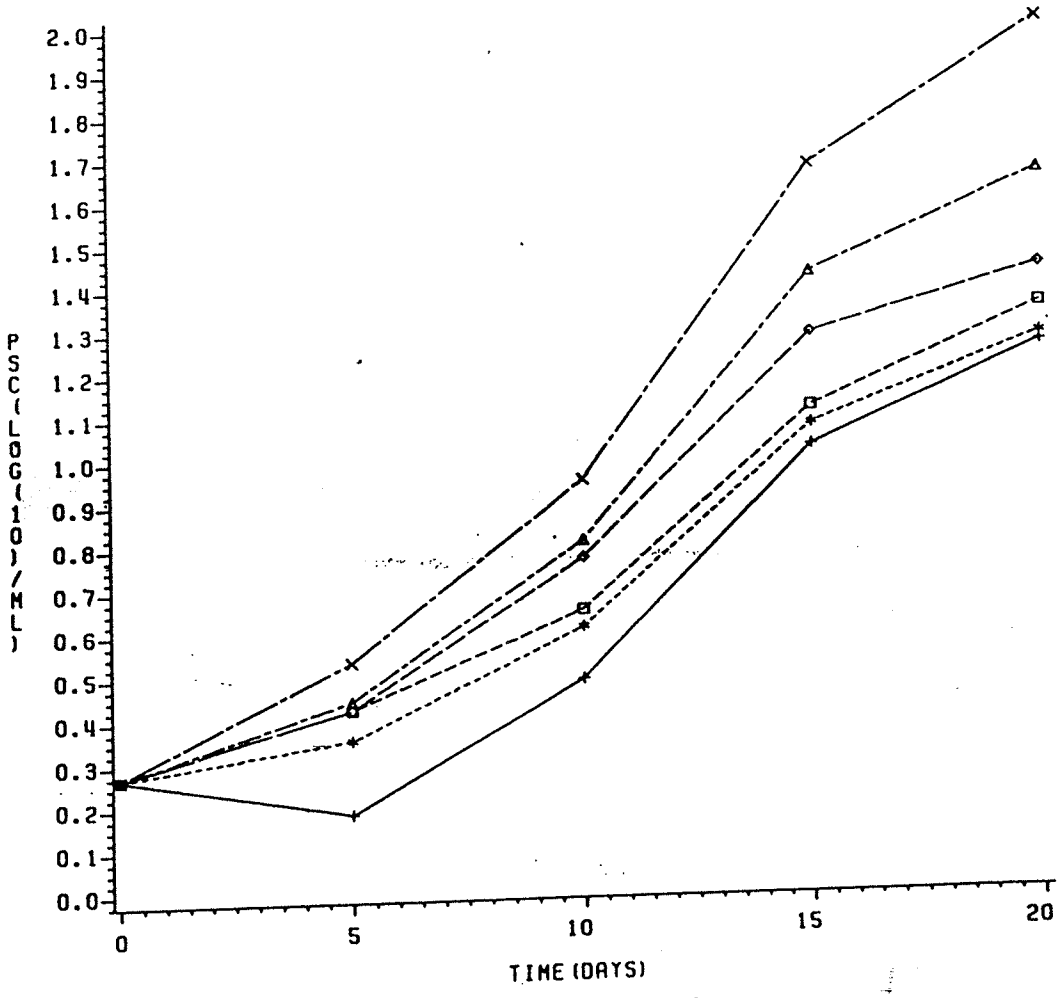
* Ps. fluorescens ps3a had grown for 1 day

□ Ps. fluorescens ps3a had grown for 2 days

◇ Ps. fluorescens ps3a had grown for 3 days

△ Ps. fluorescens ps3a had grown for 4 days

X Ps. fluorescens ps3a had grown for 5 days



zation, there is a definite stimulatory effect on the growth of heat-resistant milk microflora in the same milk stored at 7°C after sterilization. However, such stimulatory effects are not likely to occur in those skim milk samples containing less than 10^7/ml of Ps. fluorescens PS3a prior to sterilization. Mikolajcik and Simon (1978) found it unlikely that gram-negative counts of less than 25,000/ml would have enough stimulatory substances for outgrowth of spores. In our study, if one allows the gram-negative counts to increase to greater than 10^7/ml, the gram-negative organisms such as Pseudomonas fluorescens can produce enough stimulatory substances to cause the growth of heat-resistant microflora in skim milk at 7°C. The results obtained from this study also showed that most psychrotrophic thermoduric and sporeforming milk microflora are capable of growth at 32°C. This may be particularly important in UHT-sterilized milk. It has been reported that heat-resistant psychrotrophs are capable of surviving HTST pasteurization or UHT-treatment (Coghill and Juffs, 1979). Therefore, if the initial bacterial count of raw milk is high before UHT-treatment is administered, then the surviving heat-resistant microflora may grow more rapidly by utilizing the stimulatory substances which were produced by previous bacterial growth prior to the heat treatment. This would then result in a reduction of the shelf-life of UHT-treated milk.

4.7 The Effects of Supplementing Skim Milk with Supernatant of Skim Milk Previously Grown with Ps. fluorescens PS3a on the Growth of Milk Microflora at 7°C

Approximately 1.84 X 10^3/ml of Ps. fluorescens PS3a were ino-

culated into one liter of 10% sterile skim milk in a three liter Erlenmeyer flask and incubated at 21°C for 5 days; the final cell concentration was ca. 7 X 10^7/ml. The initial pH and titratable acidity of skim milk was found to be 6.65 and 0.174%, respectively. After 5 days of incubation, the skim milk was completely coagulated. The preparation of supernatant was carried out using the procedure listed in section 3.4.7. The physico-chemical properties of the supernatant are presented in Table 11. The total soluble protein of the supernatant was measured by the Folin-Ciocalteu procedure, and the standard curve is presented in Figure 15. Extensive changes in the milk proteins were evident after coagulation had occurred and there was an increase in pH as well as titratable acidity. Figures 16-21 illustrate the effect of supplementation of skim milk with supernatant of coagulated skim milk on the growth of milk microflora. All data were subjected to the analysis of variance and mean comparison tests, and the results are presented in Appendix 3 Tables 1 through 6.

Figures 16 and 17 illustrate the effects of supplementing skim milk with supernatant on the SPC and PBC respectively. Both figures indicate that there was little or no apparent difference between the control and the 5% supernatant-supplemented flasks with respect to the growth of both SPC and PBC. However, in 10% supernatant-supplemented flasks, both SPC and PBC were slightly higher than the control flasks(in which no supernatant supplementation was used) during the storage period from one to five days. Comparing the results in Figure 16 to Figure 17, there was no difference between the SPC and PBC until the fourth day. The PBC

Table 11: Characteristics of Supernatant Obtained From Skimmilk in Which *Pseudomonas fluorescens* ps3a Had Been Grown for 5 Days at 21 C

Ps. fluorescens ps3a Counts/ml in Skimmilk		Supernatant		
Initial	Final	pH	Titratable Acidity (% Lactic Acid)	Soluble Protein (mg/ml)
1.84×10^3	3×10^7	7.15	0.21	7.9

Figure 15: Standard Curve for Protein Determination By Measurement of the Absorbance at 500 nm.

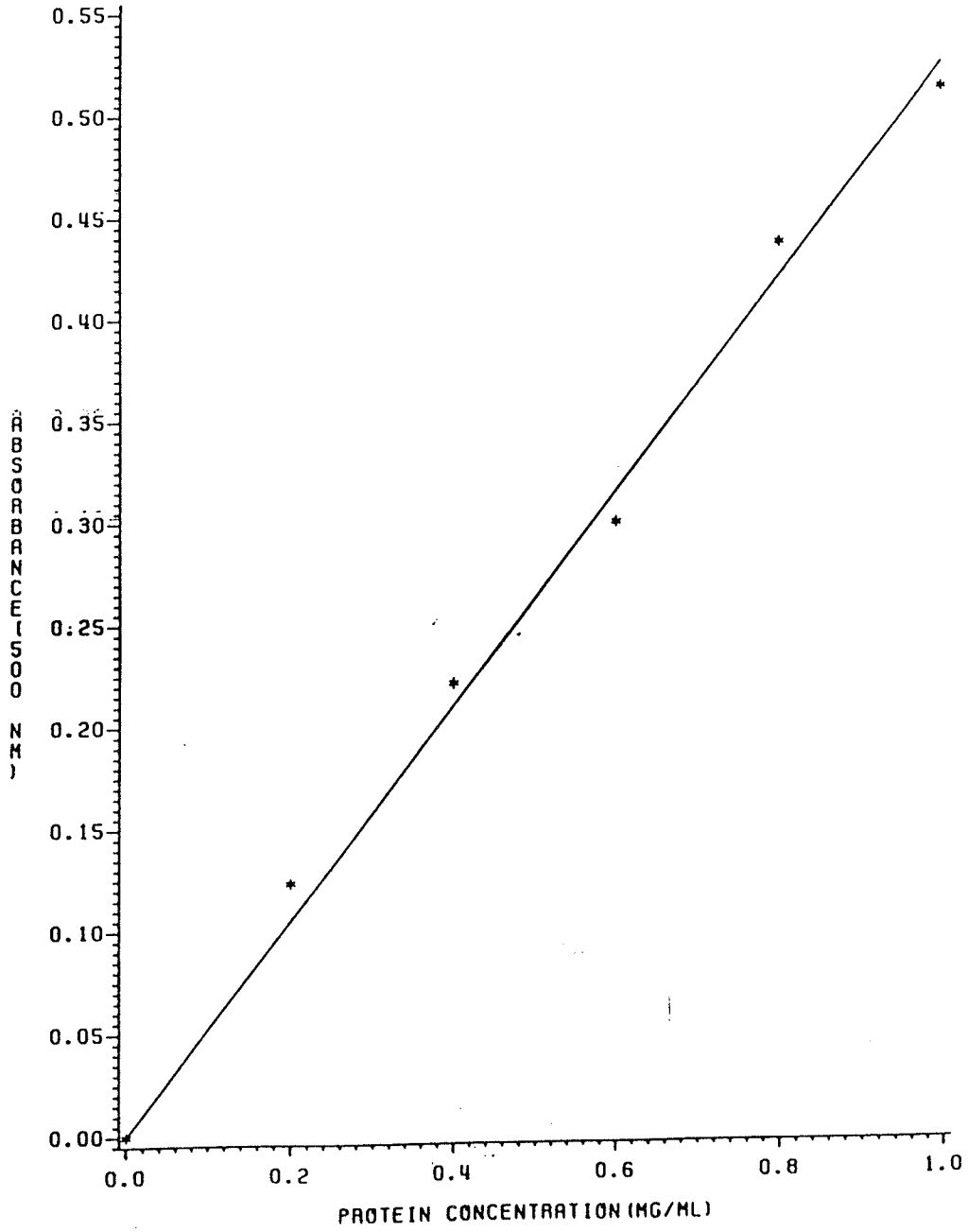


Figure 16: The Effect of Supernatant Obtained from the Skimmilk in Which Ps. fluorescens ps3a Had Grown, on the Growth of Mesophilic Milk Microflora Obtained from Commercially Pasteurized, Homogenized milk, in 10% Skimmilk Stored at 7°C

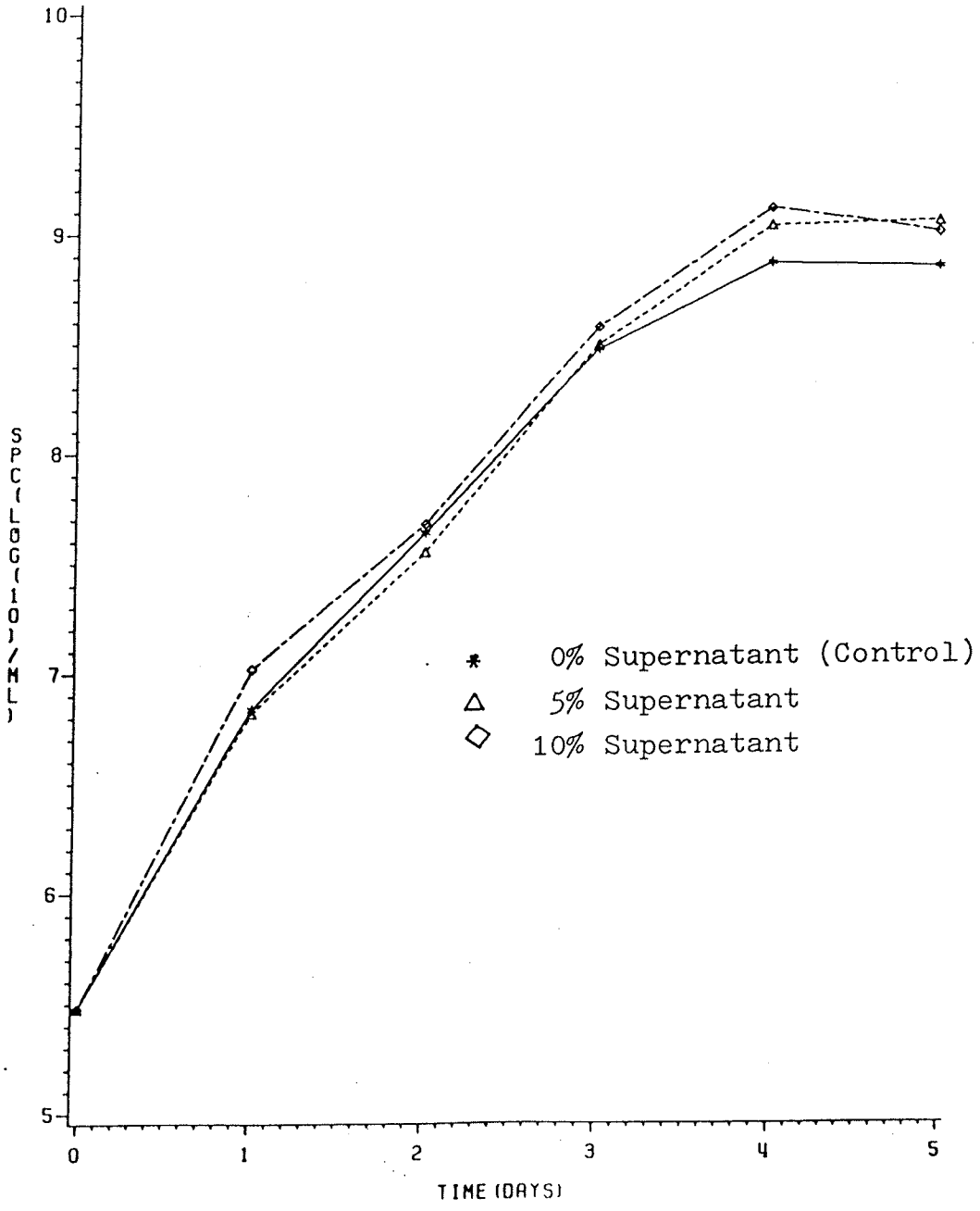
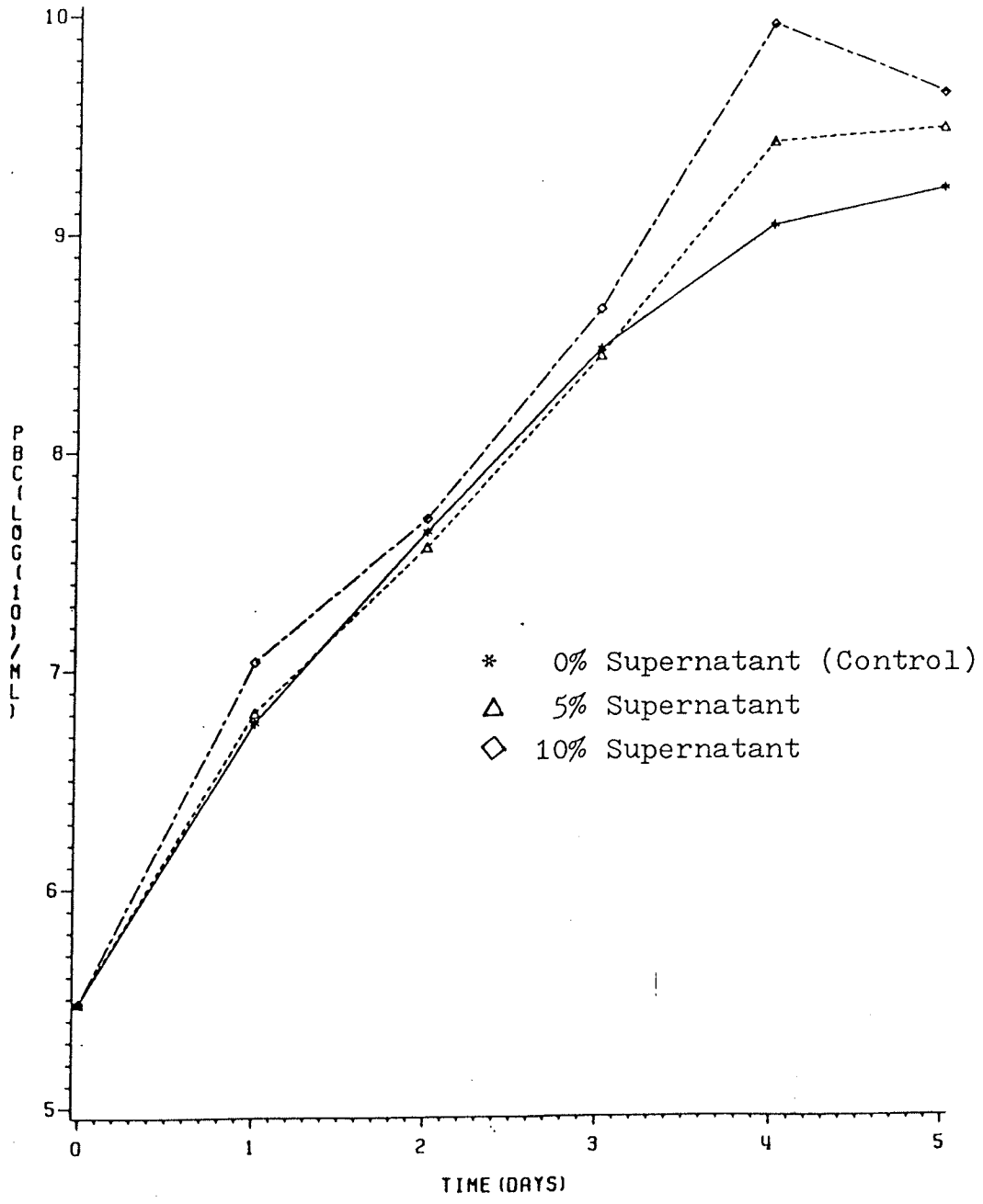


Figure 17: The Effect of Supernatant Obtained from the Skimmilk in Which Ps. fluorescens ps3a Had Grown, on the Growth of Psychrotrophic Milk Microflora Obtained from Commercially Pasteurized, Homogenized Milk, in 10% Skimmilk Stored at 7°C



of the control, 5% and 10% supernatant-supplemented flasks were ca. 0.25, 0.5 and 1 log cycles, respectively, higher than SPC. Analysis of variance showed that there was a significant difference in the SPC and PBC between the control, as well as 5% and 10% supernatant-supplemented flasks at $P \leq 0.05$. A comparison between treatment means indicated that there was a significant difference in SPC and PBC between the control and 10% supernatant-supplemented flasks at $P \leq 0.05$ (Appendix 3, Tables 1 and 2).

The effects of supplementing skim milk with supernatant on the MTC and PTC are illustrated in Figures 18 and 19, respectively. Figure 19 shows that psychrotrophic thermoduric organisms in both the control and 5% supernatant-supplemented flasks remained in the lag phase throughout the first eight days of incubation, the counts reached a maximum of 3×10^4/ml and 1×10^5/ml respectively after 15 days of incubation. However, in 10% supernatant-supplemented flasks, after an initial 6 day lag phase, the level of psychrotrophic thermoduric organisms incubated at 7°C increased significantly to ca. 10^7/ml after 15 days. The ability of psychrotrophic thermoduric organisms to grow at the mesophilic temperature of 32°C is shown in Figure 18. The results are quite similar to that observed for PTC. The MTC of the control and 5% supernatant-supplemented flasks remained constant after 10 days, however, the counts increased to ca. 1×10^4 CFU/ml and 1×10^5 CFU/ml repectively after 15 days. After 6 days of inactivity, the MTC obtained from 10% supernatant-supplemented flasks increased substantially after 15 days of incubation to ca. 10^7/ml. Analysis of variance indicated that

Figure 18: The Effect of Supernatant Obtained from the Skimmilk in Which Ps. fluorescens ps3a Had Grown, on the Growth of Mesophilic Thermoduric Organisms Obtained from Commercially Pasteurized, Homogenized Milk, in 10% Skimmilk Stored at 7°C

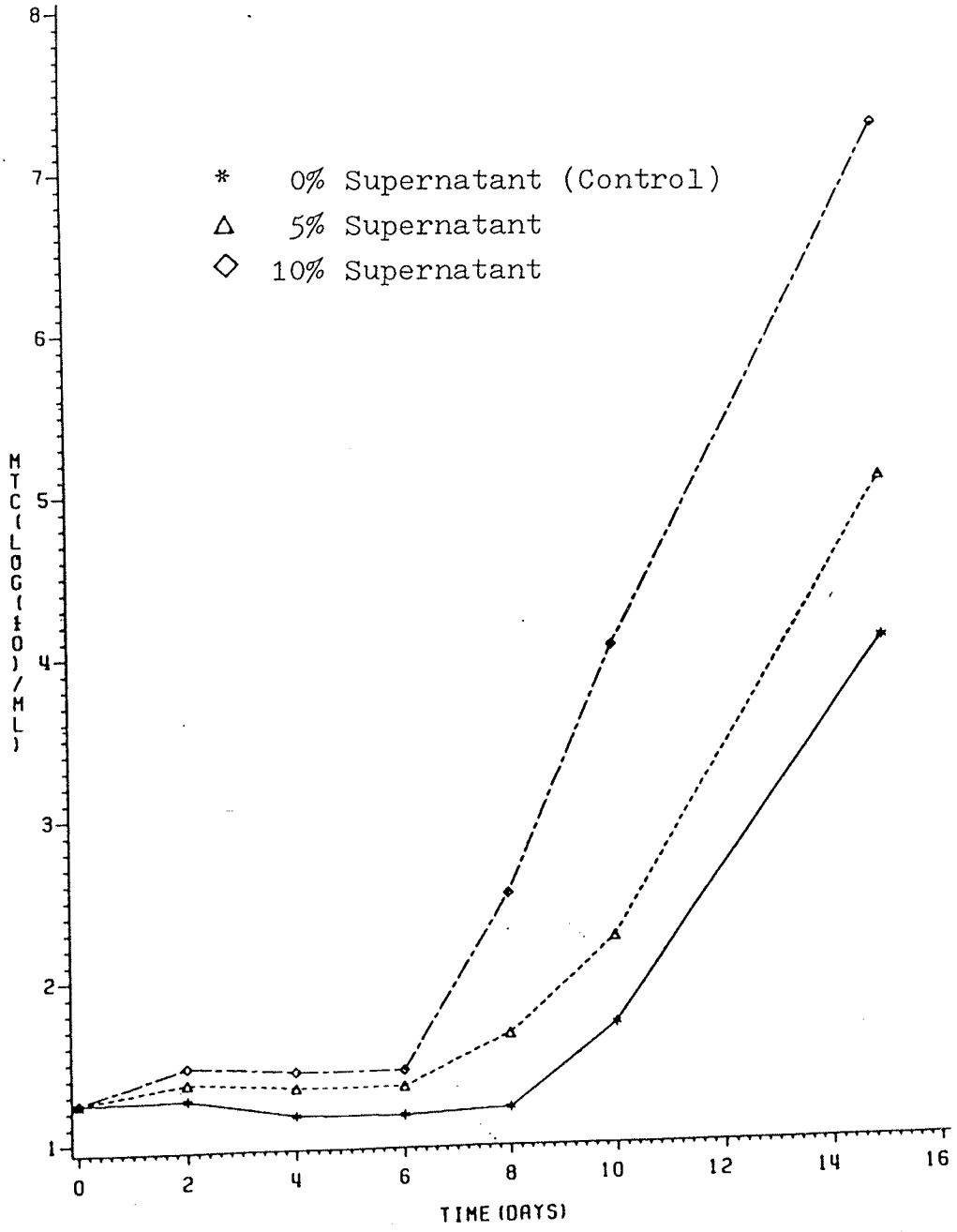
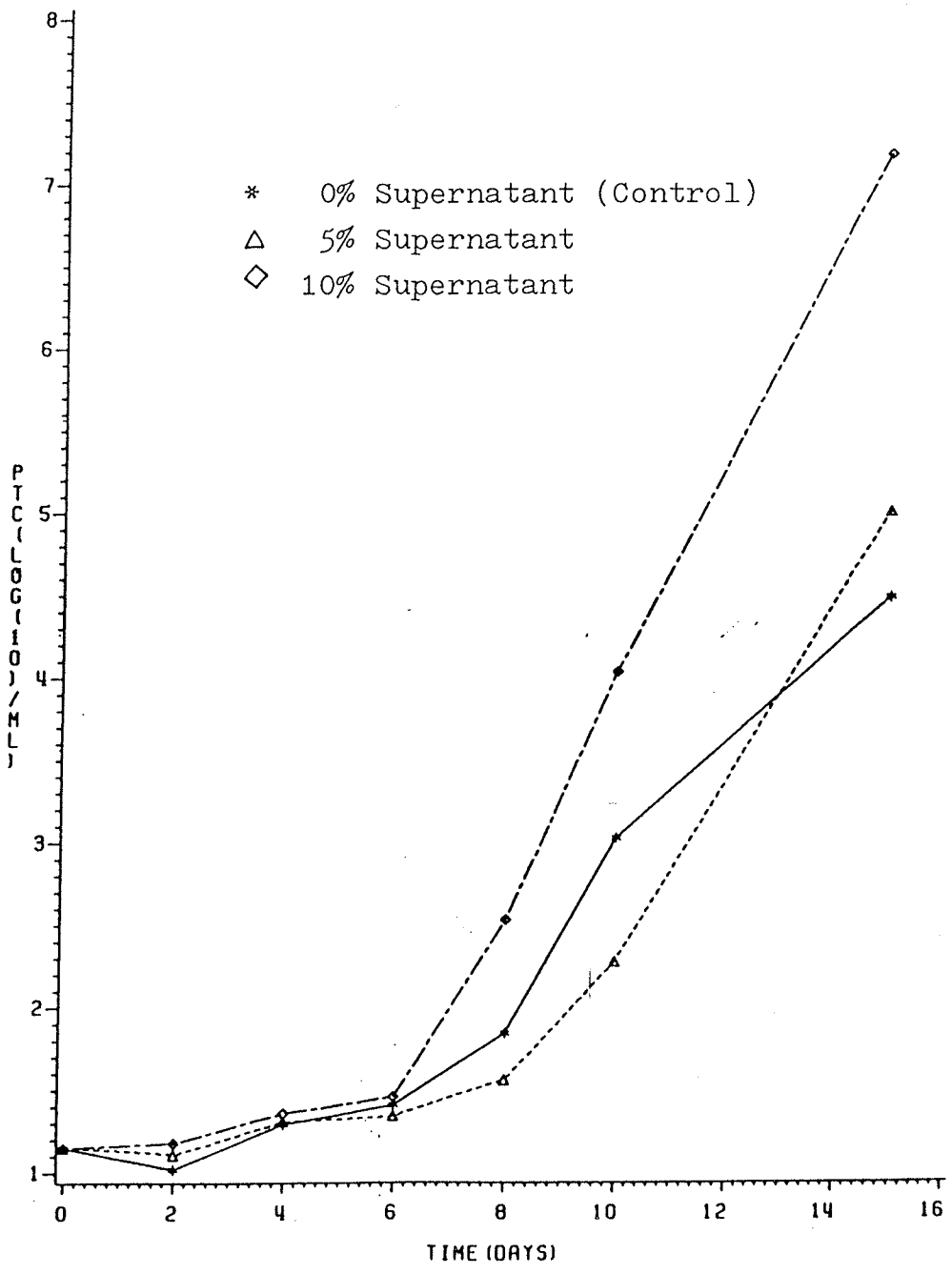


Figure 19: The Effect of Supernatant Obtained from the Skimmilk in Which Ps. fluorescens ps3a Had Grown, on the Growth of Psychrotrophic Thermoduric Organisms Obtained from Commercially Pasteurized, Homogenized Milk, in 10% Skimmilk Stored at 7°C



there was no significant difference in PTC among the treatments, however a significant difference among treatments in MTC was found at $P \leq 0.05$. Comparison of differences between treatment means indicated that there was a significant difference in MTC between the control and 10% supernatant-supplemented flasks, and no difference was found in PTC at $P \leq 0.05$ (Appendix 3, Tables 3 and 4).

The effects of supplementing skim milk with supernatant on MSC and PSC are illustrated in Figures 20 and 21. The results obtained from both figures are very similar. There was a significant difference in the PSC among the control, 5% and 10% supernatant-supplemented flasks for the incubation intervals from 4 to 6 days. During this period, PSC in the 5% and 10% supernatant-supplemented flasks were ca. 0.87 log cycle higher than that of the control, however, there was little or no difference in counts after 8 days of incubation. An analysis of variance indicated a significant difference among treatments at $P \leq 0.01$. Comparison between treatment means indicated that there was a significant difference between the control and 10% supernatant-supplemented flasks; no difference between the others at $P \leq 0.05$ was found (Appendix 3, Table 5). Figure 20 shows the ability of psychrotrophic sporeformers to grow at 32°C. The results are very similar to those for PSC. Mesophilic sporeformers in the controls and 5% supernatant-supplemented flasks remained in a two day lag phase, thereafter MSC increased progressively to ca. 5×10^8/ml. After a two day lag phase the MSC obtained from the 10% supernatant-supplemented flask increased rapidly to ca. 7×10^8/ml after

Figure 20: The Effect of Supernatant Obtained from the Skimmilk in which Ps. fluorescens ps3a Had Grown, on the Growth of Mesophilic Sporeformers Obtained from Commercially Pasteurized, Homogenized Milk, in 10% Skimmilk Stored at 7°C

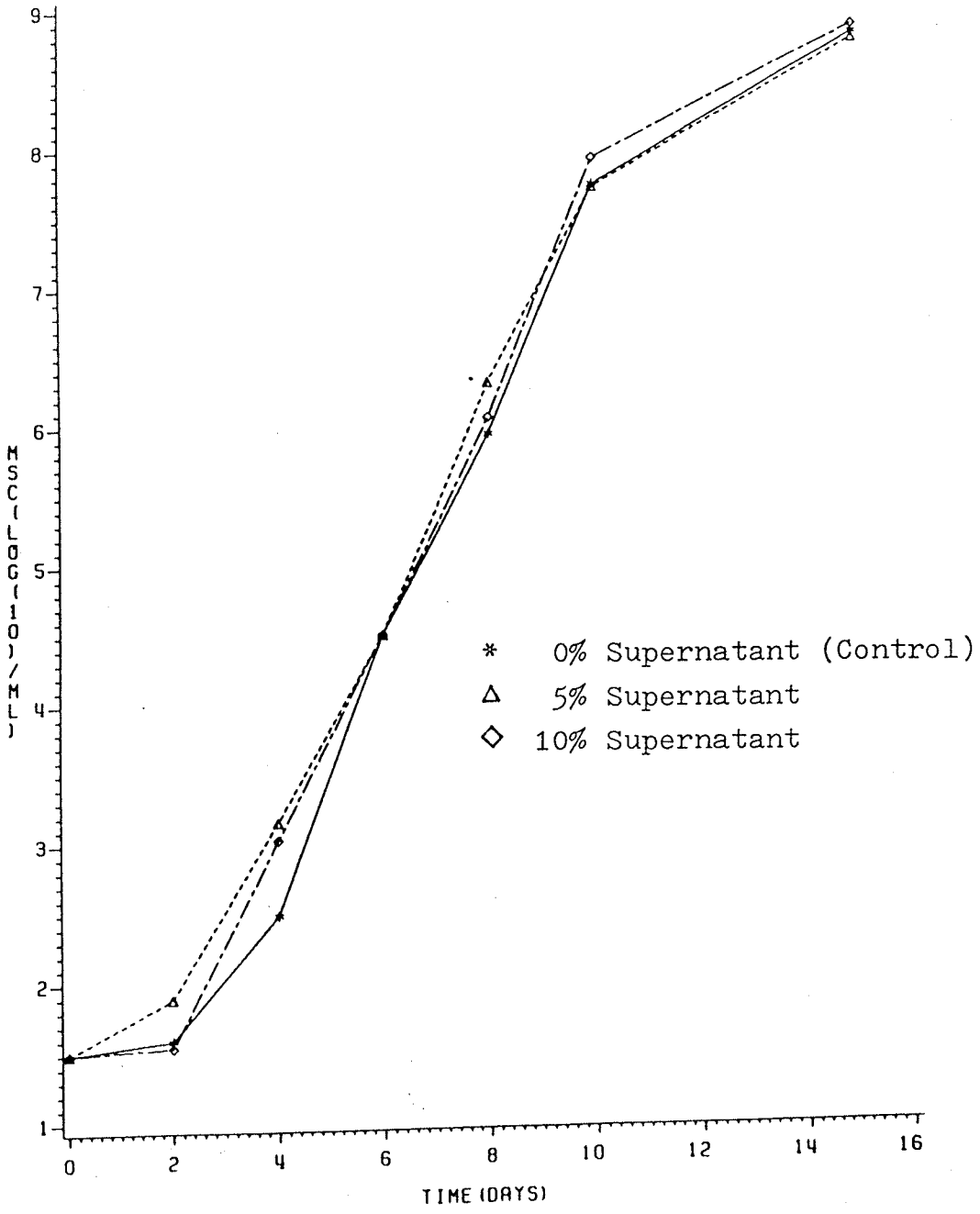
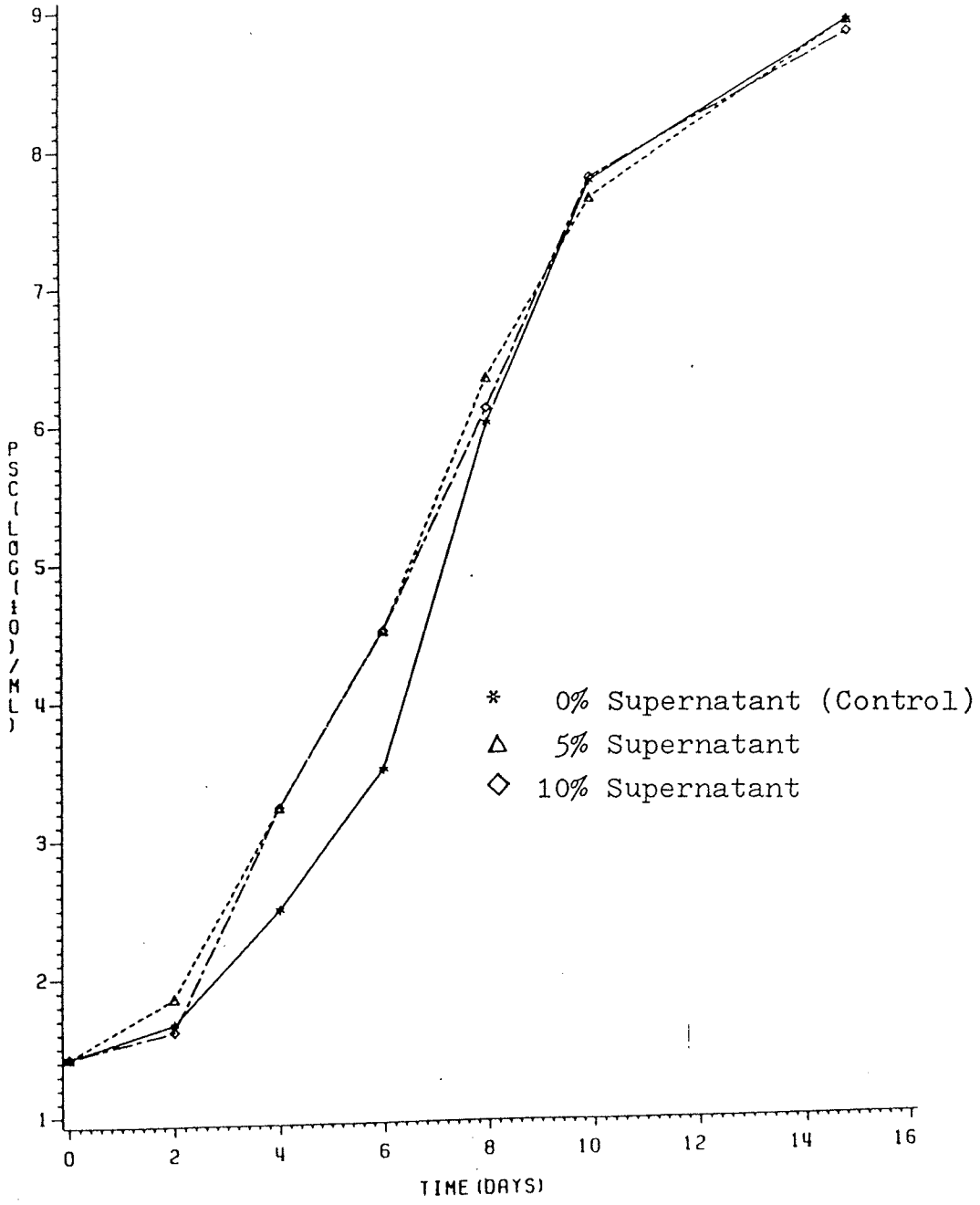


Figure 21: The Effect of Supernatant Obtained from the Skimmilk in which Ps. fluorescens ps3a Had Grown, on the Growth of Psychrotrophic Sporeformers Obtained from Commercially Pasteurized, Homogenized Milk, in 10% Skimmilk Stored at 7°C



15 days of incubation. The control, 5% and 10% supernatant-supplemented flasks were significantly different for MSC at the fourth day. The MSC for both 5% and 10% supernatant-supplemented were ca. 0.87 log cycle higher than the control. Counts for 5% and 10% supernatant-supplemented flasks were not significantly different over the entire incubation period. Statistically, the analysis of variance and comparison between treatment means shows no significant difference in MSC between the controls, 5% and 10% supernatant-supplemented flasks at $P \leq 0.05$ (Appendix 3, Table 6).

The results obtained from this study indicated that supplementation of skim milk with 10% supernatant obtained from the growth skim milk medium in which Ps. fluorescens PS3a had been grown, produced a stimulatory effect on initiating the growth of psychrotrophic milk microflora at 7°C. The results also indicated that microflora in flasks which contained 10% supernatant generally had a shorter lag phase than those in the control and 5% supernatant-supplemented flasks. Yates and Elliott (1977) reported that supernatant containing a proteolytic psychrotroph showed a marked increase of whey protein in milk after storage for 6 days at 5°C. Janzen et al. (1982) reported that proteases obtained from the milk supernatant had a significant ability to produce flavor defects in skim and whole milk stored at 4.5°C and 7°C. Their results also indicated that skim milk had a shorter shelf-life than whole milk stored under the same conditions. Our study therefore suggests that proteolytic enzymes in the supernatant of Ps. fluorescens PS3a caused a breakdown of the complex milk pro-

teins which could be utilized more readily by the milk microflora. Consequently, the lag phases of the milk microflora were significantly shortened. Shortening of lag phases were found in all 10% supernatant-supplemented flasks. Although our statistical analyses on the results of PTC and MSC were not significant, the addition of 10% supernatant to skim milk results in shortening the lag phases of the organisms. The results also indicated that most psychrotrophic milk flora are capable of growing at 32°C and that their growth curves at 32°C are similar to those at 7°C. As mentioned previously, this is particularly important in UHT-treated milk, both proteases and sporeformers are known to survive UHT-treatment (Adams et al., 1975; Martin, 1981); and since the storage of UHT-milk is usually at room temperature, the proteases which survive from UHT-treatment can act on the milk proteins and cause the release of simple molecules. Sporeforming organisms can therefore utilize these simple nutrients and start multiplying, and cause the reduction of shelf-life of UHT-milk.

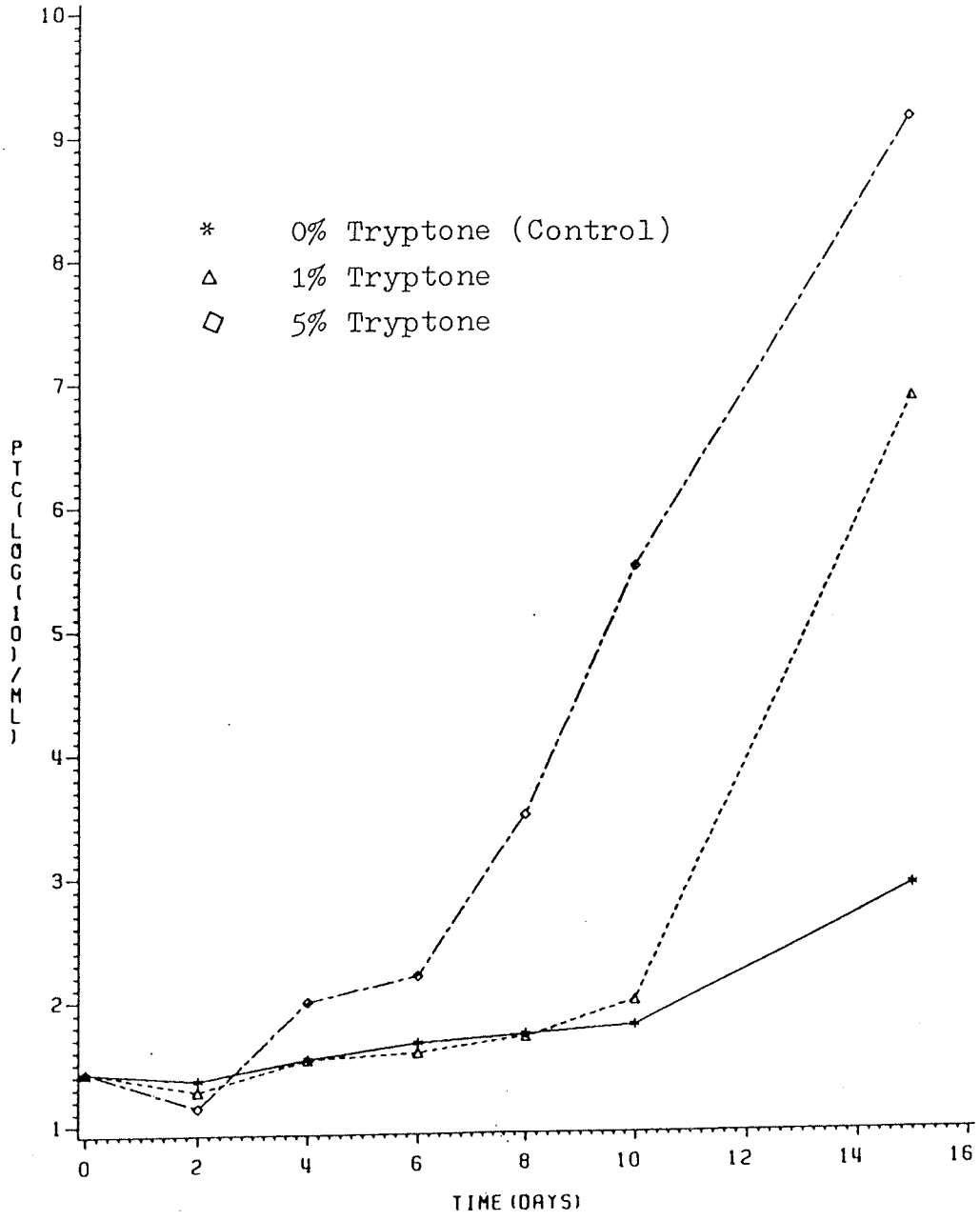
4.8 The Effects of Supplementing Skim Milk with Commercially Available Milk Fractions on the Growth of Thermoduric and Sporeforming Milk Microflora at 7°C

4.8.1 Effects of Tryptone

Figures 22-25 illustrate the effects of supplementing skim milk with 1% and 5% tryptone on the growth of heat-resistant microflora at 7°C.

The growth curves of psychrotrophic thermoduric organisms at

Figure 22: The Effect of Supplementing Skimmilk with Tryptone on Psychrotrophic Thermoduric Count



7°C are shown in Figure 22. The initial PTC of the control (no tryptone added), 1% and 5%-tryptone supplemented flasks was ca. 2.69 X 10^1/ml. PTC of the control flasks showed little or no significant increase after 10 days of incubation, thereafter, count increased slowly to ca. 5 X 10^2/ml at 15 days. The PTC of the 1% tryptone supplemented flask showed a significant increase to ca. 6.8 X 10^6/ml after 15 days preceded by a 10 day lag phase. The PTC of the 5% tryptone supplemented flask showed a substantial increase to ca. 1.3 X 10^9/ml after 15 days with an initial six day lag phase.

Figure 23 reveals the ability of psychrotrophic thermoduric organisms to grow at 32°C. The initial MTC of the control, 1% and 5% tryptone supplemented flasks was ca. 3.98 X 10^1/ml. The MTC of the control flask showed little or no significant increase during the entire 15 day incubation period. After an eight day lag phase, the MTC of the 1% tryptone supplemented flask showed a significant increase to a maximum of ca. 3.2 X 10^6/ml at 15 days. The MTC of the 5% tryptone supplemented flask showed a substantial increase to a maximum of ca. 5 X 10^8/ml at 15 days after an initial eight day lag phase. An analysis of variance indicated that there was a significant difference in both MTC and PTC among the treatments (control, 1% and 5% tryptone supplemented) at $P \leq 0.05$ (Appendix 4 Tables 1A and 1B). Comparison between means also indicated that there was a significant difference in both MTC and PTC between the control and 5% tryptone supplemented flasks at $P \leq 0.05$, and no difference between the others (control and 1%; 1% and 5% tryptone supplemented flasks).

Figure 23: The Effect of Supplementing Skimmilk with Tryptone on Mesophilic Thermoduric Count

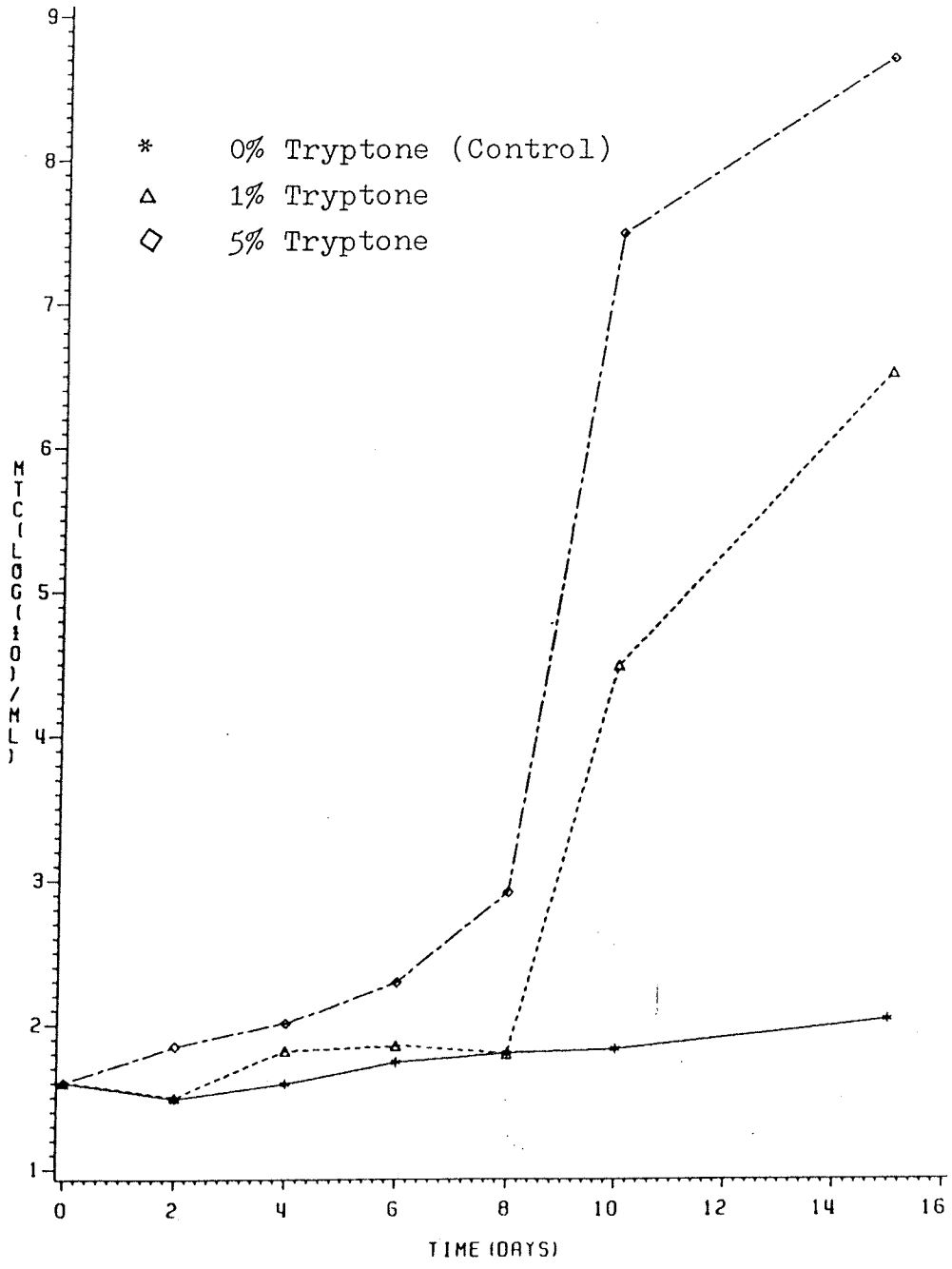


Figure 24 shows the growth curves of psychrotrophic sporeforming organisms at 7°C. The initial PSC of the control, 1% and 5% tryptone supplemented flasks was ca. 1.86 X 10^1/ml. After 15 days of incubation at 7°C, the PSC of the control, 1% and 5% tryptone supplemented flasks increased slightly to ca. 2.95 X 10^2/ml, 2.14 X 10^2/ml and 2.75 X 10^2/ml, respectively. Increases of counts in all 3 treatments were very small over the entire 15 day incubation period; however, an analysis of variance showed that there was a significant difference among treatments at $P \leq 0.01$. A comparison of the treatment means indicated that there was a significant difference in PSC between the control and 5% tryptone supplemented flasks, but no difference between the others at $P \leq 0.05$ (Appendix 4 Table 1D).

The ability of psychrotrophic sporeforming organisms to grow at 32°C is illustrated in Figure 25. The initial MSC of the control, 1% and 5% tryptone supplemented flasks was ca. 3.47 X 10^1/ml. After 15 days of incubation at 7°C, the MSC of all three treatments increased ca. only one log cycle over the entire 15 day incubation period; however, an analysis of variance indicated that there was a significant difference in MSC among treatments at $P \leq 0.01$. A comparison of treatment means showed that there was a significant difference in MSC between the control and 5% tryptone supplemented flasks, but no difference between the others at $P \leq 0.05$ (Appendix 4 Table 1C).

Results from this study indicated that 5% tryptone-supplemented skim milk definitely showed a significant stimulatory effect on the growth of thermoduric psychrotrophs and a similar

Figure 24: The Effect of Supplementing Skimmilk with Tryptone on Psychrotrophic Sporeformer Count

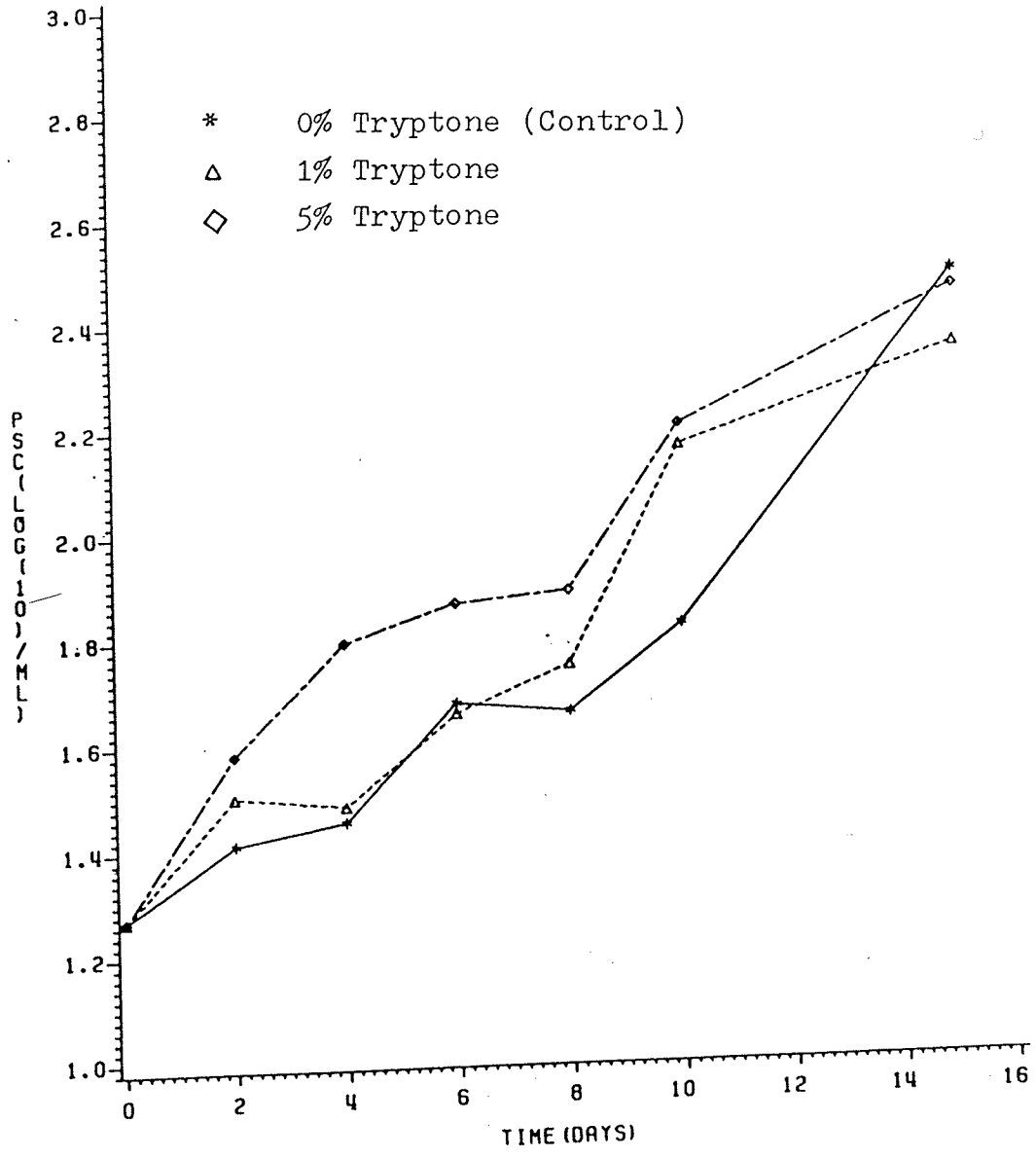
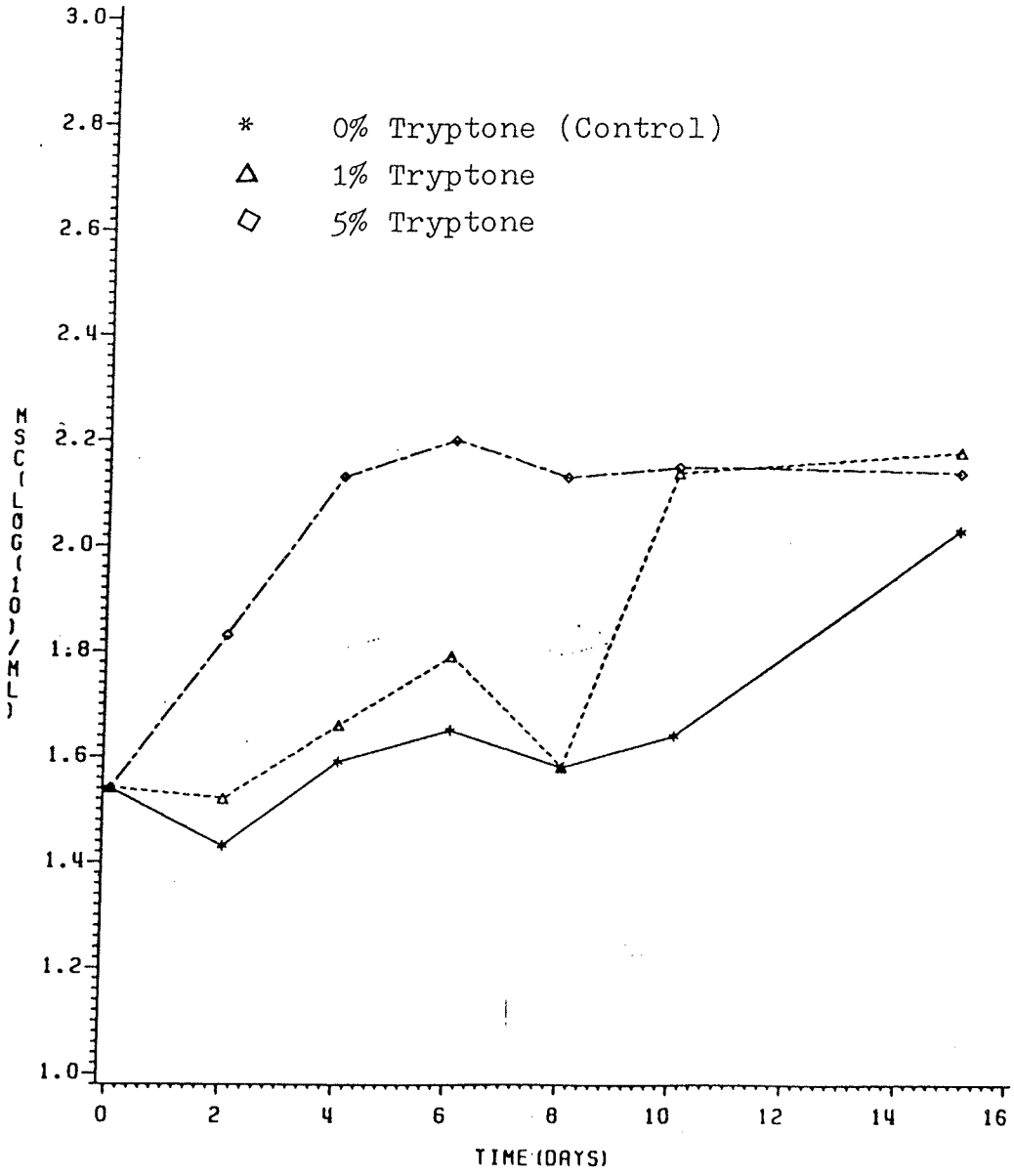


Figure 25: The Effect of Supplementing Skimmilk with Tryptone on Mesophilic Sporeformer Count



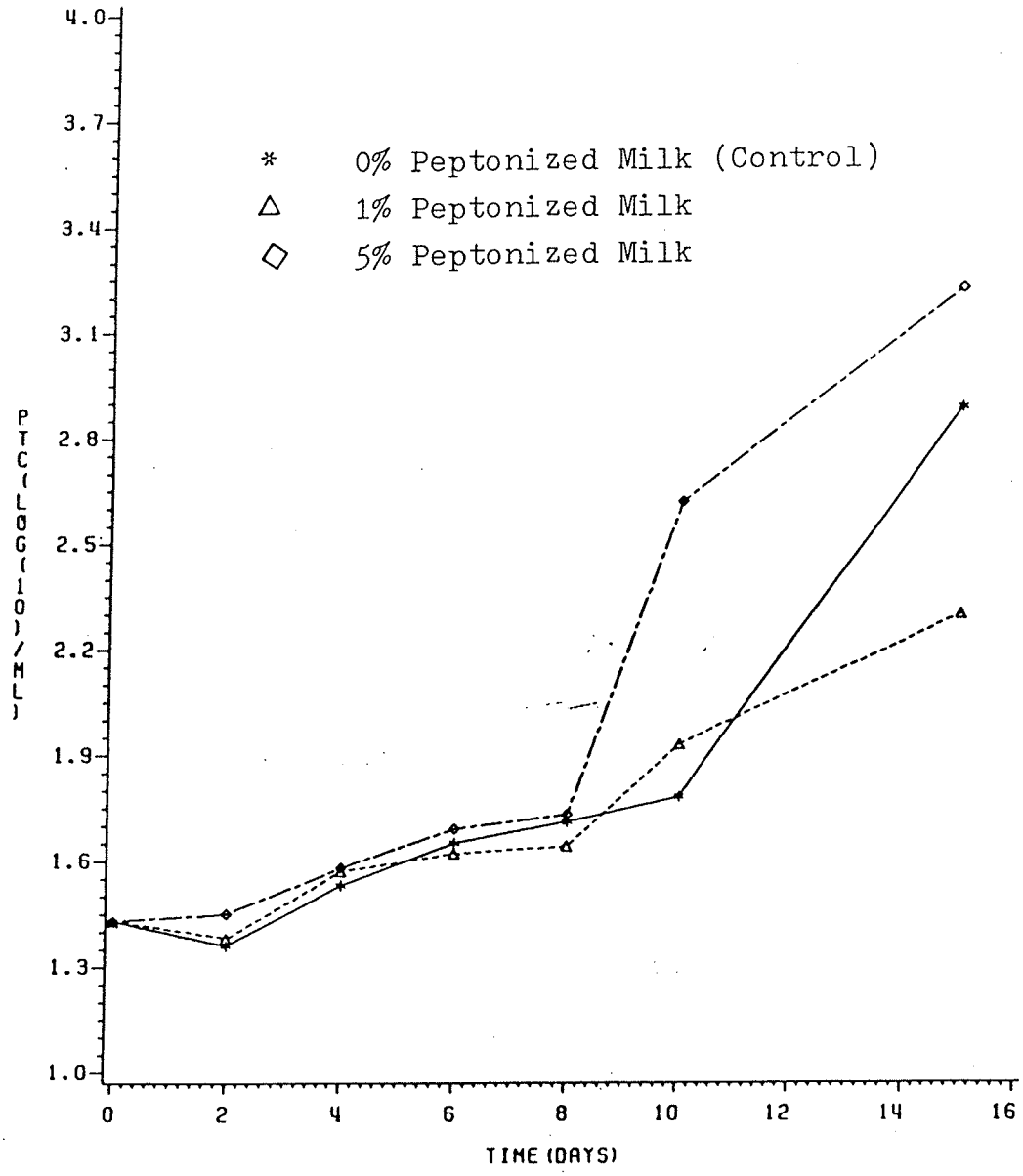
but lesser effect on sporeforming psychrotrophs at both 7°C and 32°C. According to the Difco Manual(1974) tryptone is prepared from hydrolyzed milk casein, and is a good source of essential amino acids and peptides. Yates and Elliott(1977) reported a significant increase of whey protein in milk which was inoculated with proteolytic psychrotrophs, and resulted in a significant loss of casein. Proteolytic activity of thermoduric and sporeforming psychrotrophs has been reported by a number of researchers(Overcast and Atmaram,1974; Washam et al.,1977; and Choudhery and Mikolajcik,1971). Overcast and Atmaram(1974) reported that the proteolytic activity of B. cereus was found to be similar to that of rennin which causes the formation of sweet curdling defect in milk. Choudhery and Mikolajcik(1971) reported that B. cereus caused the breakdown of casein in skim milk. Their results indicated that casein was the main source of protein for thermoduric and sporeforming psychrotrophs. Our results therefore suggest that supplementation of 10% skim milk with 5%(v/v) tryptone can stimulate the outgrowth of thermoduric and sporeforming psychrotrophs in skim milk stored at 7°C.

4.8.2 Effects of Peptonized Milk

Figures 26-29 illustrate the effects of supplementing skim milk with 1% and 5% peptonized milk on the growth of heat-resistant microflora at 7°C.

The growth curves of psychrotrophic thermoduric organisms at 7°C are shown in Figure 26. The initial PTC of the control(no peptonized milk added), 1% and 5% peptonized milk supplemented flasks was ca. 2.69 X 10^1/ml. The PTC of the control flask did

Figure 26: The Effect of Supplementing Skimmilk with Peptonized Milk on Psychrotrophic Thermoduric Count



not show any significant increase after a ten day lag phase, and the count reached a maximum of ca. 7.76 X 10^2/ml after 15 days of incubation at 7°C. The PTC of 1% peptonized milk supplemented flasks increased very little to a maximum of 2.0 X 10^2/ml after 15 days. The PTC of 5% peptonized milk supplemented flasks showed the greatest increase following an eight day lag phase to ca. 1.70 X 10^3/ml after 15 days. An analysis of variance and comparison of the difference between means indicated that there was no significant difference in PTC among treatments at $P \leq 0.05$ (Appendix 4 Table 2B).

The ability of psychrotrophic thermoduric organisms to grow at 32°C is also shown in Figure 27. The initial MTC of the control, 1% and 5% peptonized milk supplemented flasks was ca. 3.98 X 10^1/ml. Both the MTC of the control and the 1% peptonized milk supplemented flasks did not show any significant increase oven the entire incubation period of 15 days. However, the MTC of 5% peptonized milk supplemented flasks showed a significant increase after an eight day lag phase with the count reaching a maximum of ca. 2.14 X 10^3/ml after 15 days. An analysis of variance showed a significant difference in MTC among treatments at $P \leq 0.05$. Comparison of treatment means indicated that there was a significant difference in MTC between the control and 5% peptonized milk supplemented flasks, but no difference between the others at $P \leq 0.05$ (Appendix 4 Table 2A).

Figure 28 illustrates the growth curves of psychrotrophic sporeforming organisms at 7°C. The initial PSC of the control, 1% and 5% peptonized milk supplemented flasks was ca. 1.86 X 10^1/ml.

Figure 27: The Effect of Supplementing Skimmilk with Peptonized Milk on Mesophilic Thermoduric Count

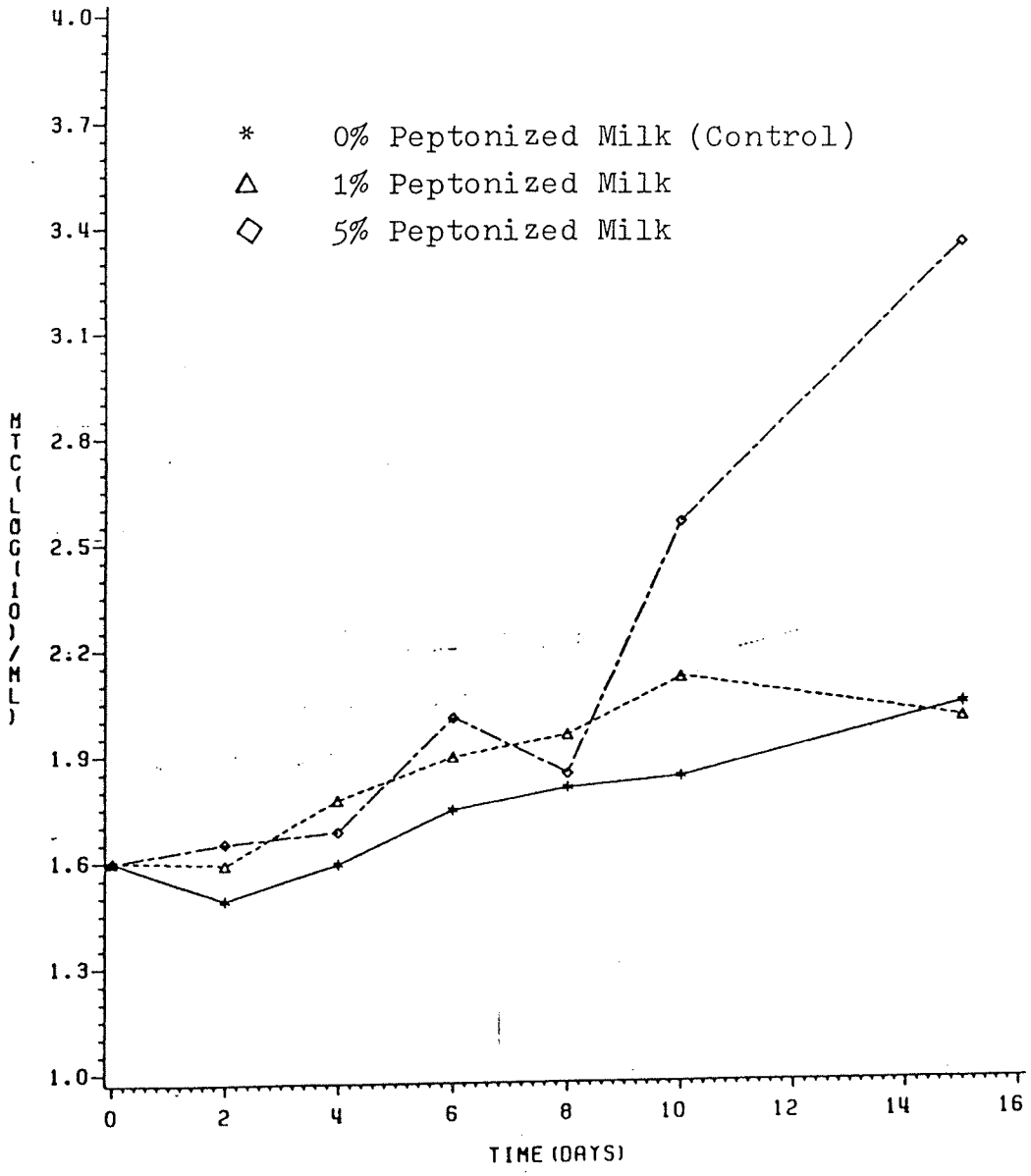
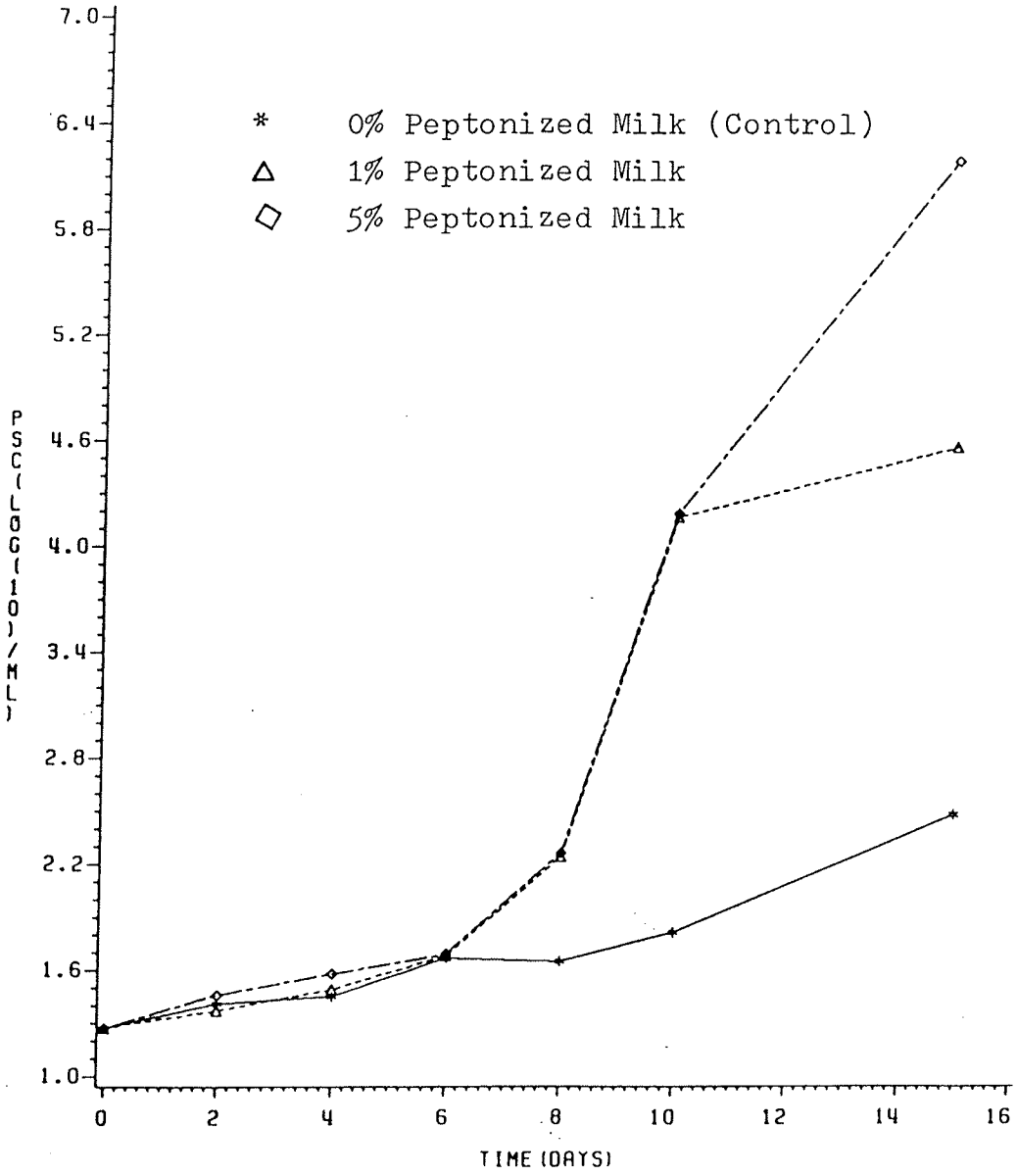


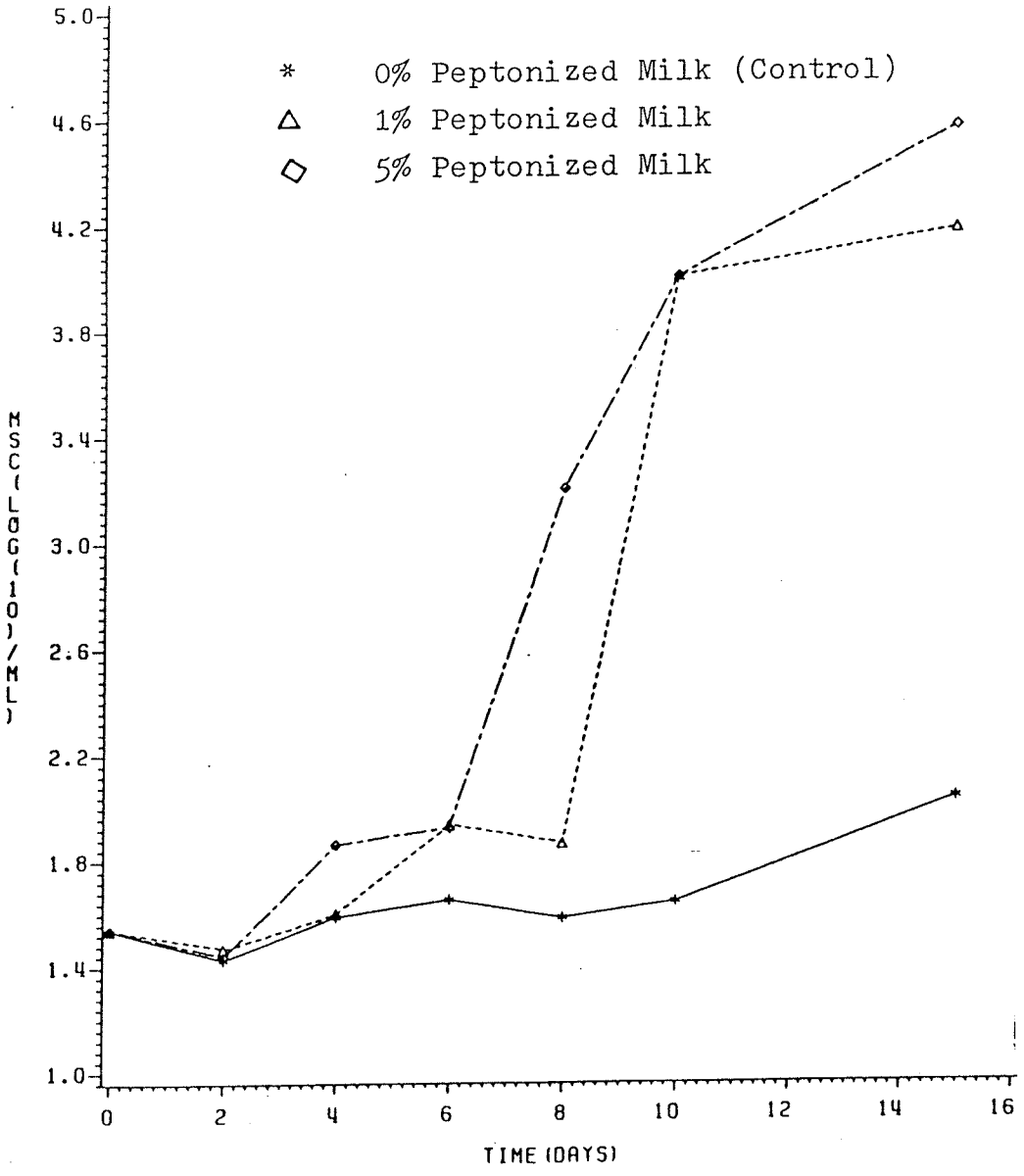
Figure 28: The Effect of Supplementing Skimmilk with Peptonized Milk on Psychrotrophic Spore-former Count



The PSC of the control flask increased slowly to a maximum of ca. 2.95 X 10^2/ml at the end of the 15 day incubation period. PSC of the 1% peptonized milk supplemented flask showed a significant increase after an eight day lag phase, with the count increasing to a maximum of ca. 3.55 X 10^4/ml after 15 days. The PSC of the 5% peptonized milk supplemented flask also increased significantly after an eight day lag, and the count reached a maximum of ca. 1.51 X 10^6/ml at the end of the 15 day incubation period. An analysis of variance showed a significant difference in PSC among treatments at $P \leq 0.05$. A comparison of treatment means indicated that there was a significant difference PSC between the control and 5% peptonized milk supplemented flasks, but no difference between the others at $P \leq 0.05$ (Appendix 4 Table 2D).

Figure 29 shows the ability of psychrotrophic sporeforming organisms to grow at 32°C. The initial MSC of the control, 1% and 5% peptonized milk supplemented was ca. 3.47 X 10^1/ml. The MSC of the control increased only 0.43 log cycle during the entire 15 day incubation period to a maximum of ca. 1.07 X 10^2/ml at the end of 15 days. However, both the MSC of 1% and 5% peptonized milk supplemented flasks showed a significant growth after a definite lag phase. The MSC of the 1% peptonized milk supplemented flask increased to a maximum of 1.51 X 10^4/ml at 15 days after an eight day lag phase while the MSC of the 5% peptonized milk supplemented flask increased to a maximum of ca. 3.72 X 10^4/ml at 15 days after a six day lag phase. An analysis of variance indicated that there was no significant difference in MSC among treatments at $P \leq 0.05$. A comparison of treatment means also shows a signifi-

Figure 29: The Effect of Supplementing Skimmilk with Peptonized Milk on Mesophilic Sporeformer Count



cant difference in MSC between the control and 5% peptonized milk supplemented flasks, and no difference between the others at $P \leq 0.05$ (Appendix 4 Table 2C).

The results from this study indicated that the supplementation of skim milk with 5% peptonized milk exerted a stimulatory effect on the outgrowth of thermoduric and sporeforming psychrotrophs at 7°C. The results also showed that the stimulatory effect on sporeforming psychrotrophs is much greater than on thermoduric psychrotrophs. According to the Difco Manual (1974), peptonized milk, which is hydrolyzed skim milk, contains the degradation products of proteins, albumins, and globulins of milk, and its nitrogen is more readily available for bacterial assimilation than the native milk protein. Law (1979) reported that addition of proteolytic Pseudomonas sp. during cheese making resulted in a marked increase of acid production by the starter cultures. Such an effect indicated that the proteolytic activity of Pseudomonas caused the breakdown of milk protein and released small peptides and amino acids which the starter cultures could utilize immediately. Therefore, if psychrotrophs are allowed to break down milk proteins before pasteurization or UHT-treatment, the hydrolyzed milk proteins can be readily utilized by the surviving heat-resistant organisms after heat-treated.

4.8.3 Effects of Whey Protein

The effects of supplementing 10% skim milk with 1% and 5% whey protein on the growth of heat-resistant milk microflora at 7°C are illustrated in Figures 30-33.

The growth curves of psychrotrophic thermoduric organisms are

shown in Figure 30. The initial PTC of the control, 1% and 5% whey flasks was ca. 2.69 X 10^1/ml. After a ten day lag phase, the PTC of the control sample reached a maximum of ca. 7.76 X 10^2/ml at the end of the 15 day incubation period. After an eight day lag phase, the PTC of 1% and 5% whey samples increased to a maximum of ca. 3.02 X 10^3/ml and 2.82 X 10^4/ml, respectively, after 15 days. An analysis of variance showed that there is a significant difference in PTC among these 3 treatments at $P \leq 0.05$. A comparison of treatment means indicated that there is a difference in PTC between the control and 5% whey samples, and no difference between the others (the control and 1%; 1% and 5% whey samples) at $P \leq 0.05$ (Appendix 4 Table 3B).

The ability of thermoduric organisms to grow at 32°C is also illustrated (Figure 31). The initial MTC of the control, 1%, and 5% whey samples was ca. 3.98 X 10^1/ml. The MTC of all 3 samples did not show a significant increase during the entire 15 day incubation period. However, the MTC of the 5% whey samples at 7°C were at least 1/2 log cycle higher than those of the control sample throughout the entire 15 day period. An analysis of variance showed a significant difference in MTC among the three treatments at $P \leq 0.01$. A comparison of treatment means indicated a difference in MTC between the control and 5% whey samples, and no difference between the others at $P \leq 0.05$ (Appendix 4 Table 3A).

Figure 32 illustrates the growth curves of psychrotrophic sporeforming organisms at 7°C. The initial PSC of the control, 1% and 5% whey flasks was found to be ca. 1.86 X 10^1/ml. The PSC of both control and 1% whey supplemented samples increased slightly

Figure 30: The Effect of Supplementing Skimmilk with Whey Proteins on Psychrotrophic Thermoduric Count

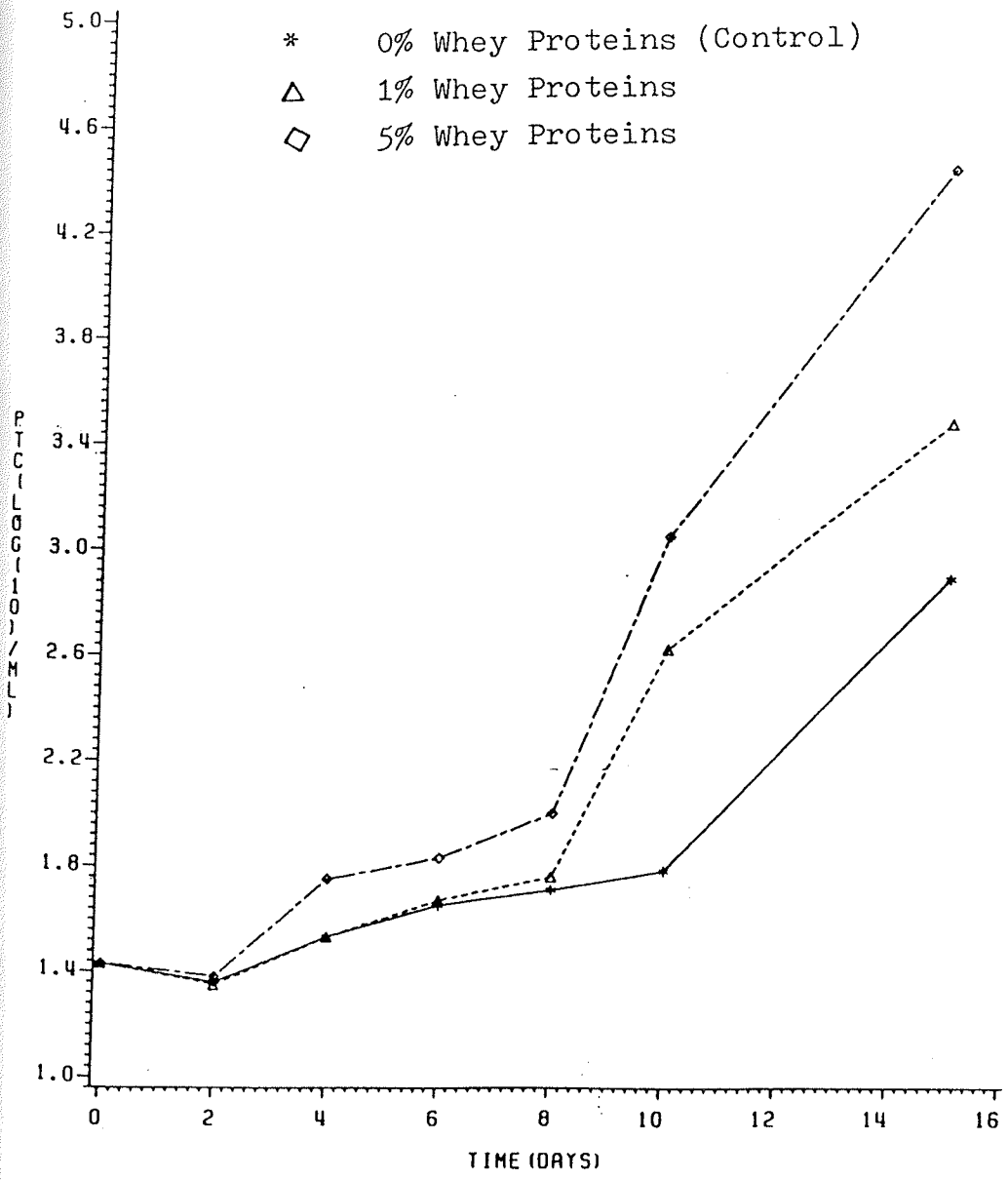
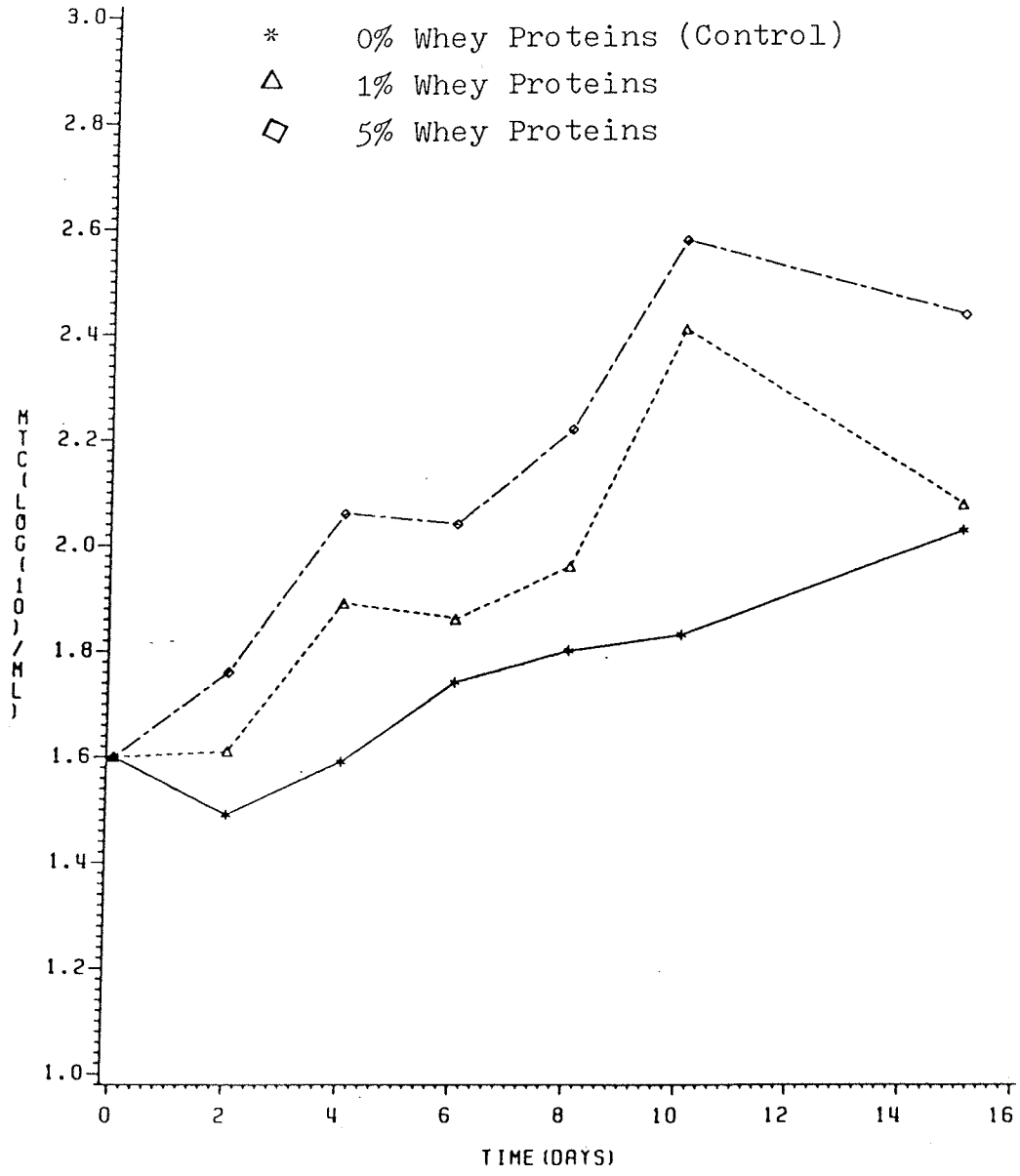


Figure 31: The Effect of Supplementing Skimmilk with Whey Proteins on Mesophilic Thermoduric Count



to the maximum of ca. 2.95 X 10^2/ml and 3.1 X 10^2/ml, respectively, after 15 days storage at 7°C. After an eight day lag phase, however, the PSC of the 5% whey supplemented sample increased significantly to ca. 2.82 X 10^4/ml after 15 days. An analysis of variance indicated a difference in PSC among these 3 treatments at $P \leq 0.05$. A comparison of treatment means also showed a significant difference in PSC between the control and 5% whey supplemented flasks, and no difference between the others (the control and 1% ; 1% and 5% whey samples) at $P \leq 0.05$ (Appendix 4 Table 3D).

The ability of sporeforming organisms to grow at 32°C is illustrated in Figure 33. The initial MSC of the control, 1% and 5% whey supplemented flasks was ca. 3.47 X 10^1/ml. After 15 days of incubation, the MSC of both control and 1% whey samples did not show significant increase; counts reached a maximum of ca. 1.07 X 10^2/ml and 1.78 X 10^2/ml, respectively. However, the MSC of the 5% whey supplemented sample increased gradually to a maximum of ca. 8.13 X 10^3/ml after the same period of time. A significant difference in MSC among these 3 treatments was indicated at $P \leq 0.01$. A comparison of the treatment means revealed that there was a difference in MSC between the control and 5% whey-supplemented samples, and no difference between the others at $P \leq 0.05$ (Appendix 4 Table 3C).

The data indicated that supplementation of 10% skim milk with 5% whey protein provided a slight stimulatory effect on the growth of thermoduric and sporeforming psychrotrophs at 7°C. However, very little effect was evident on MTC, and on those sam-

Figure 32: The Effect of Supplementing Skimmilk with Whey Proteins on Psychrotrophic Spore-former Count

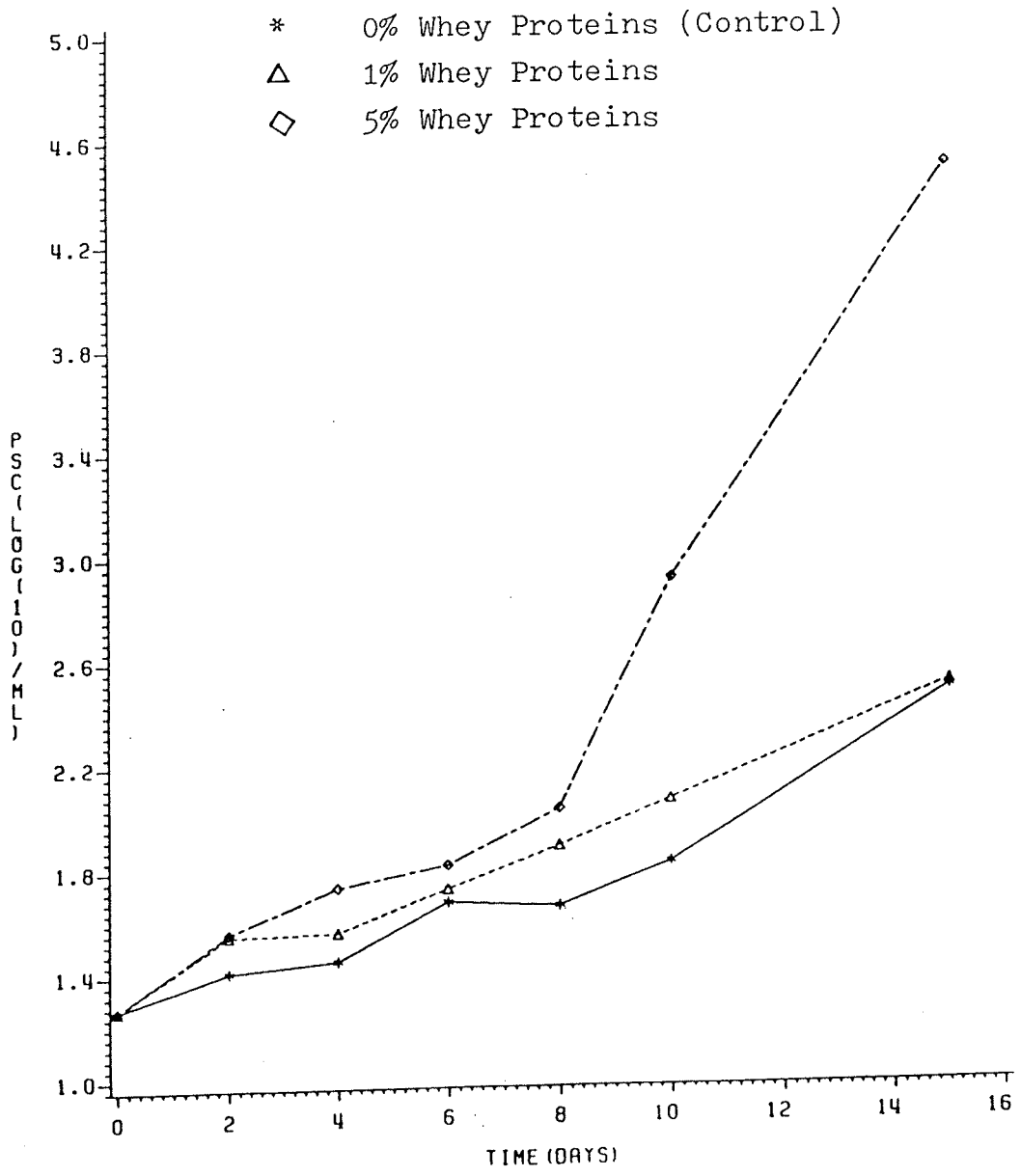
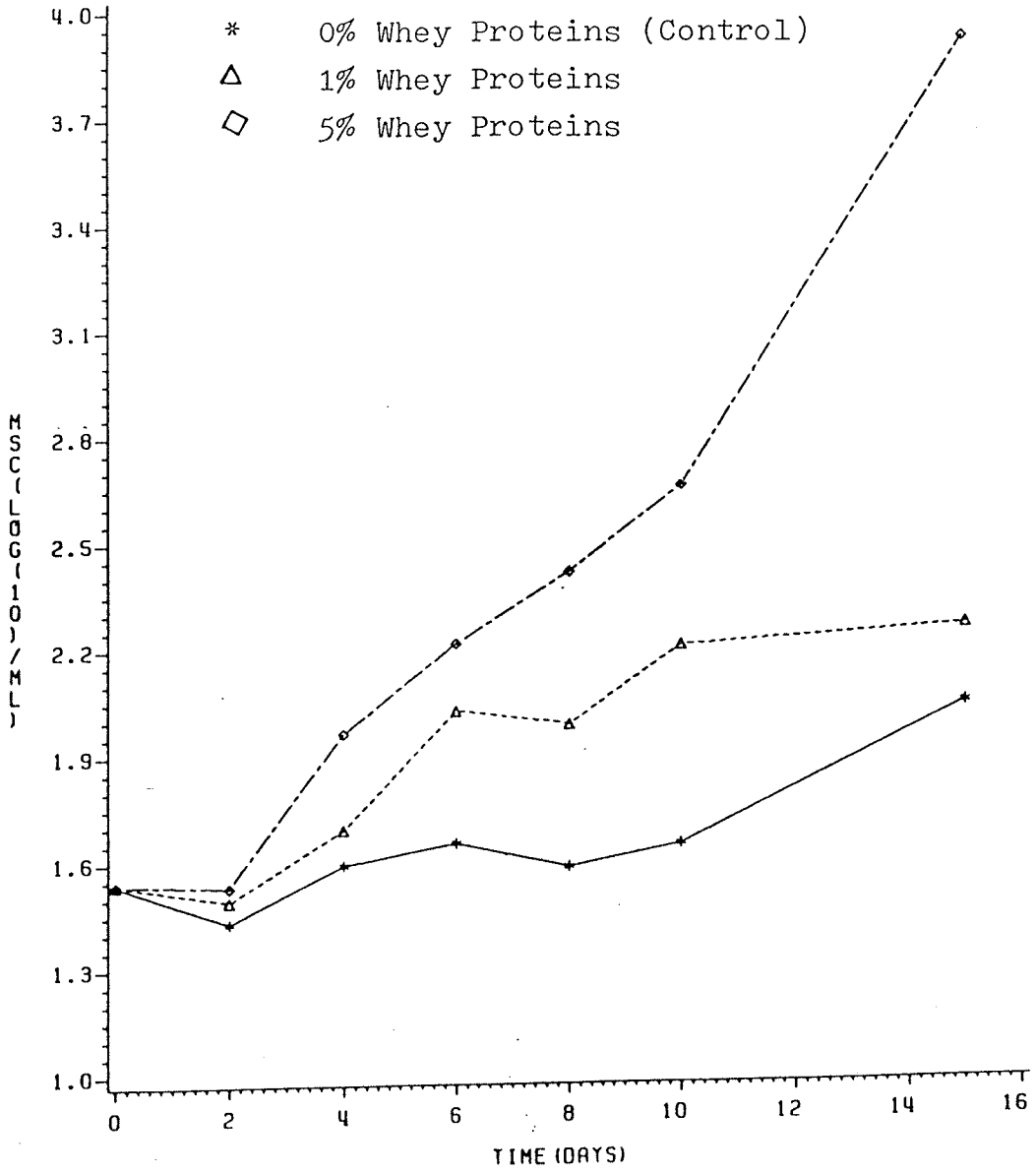


Figure 33: The Effect of Supplementing Skimmilk with Whey Proteins on Mesophilic Sporeformer Count



ples supplemented with 1% whey protein. This stimulatory effect was not found to be as great as on those samples supplemented with tryptone or peptonized milk. Whey protein, which makes up about 20% of total protein in milk and consists mainly of alpha and beta lactoglobulin, has been well recognized as being unaffected by the proteolytic activity of psychrotrophic organisms in milk (Law, 1979). However, our results indicated that whey protein shows some stimulatory effect on the growth of heat-resistant organisms at 7°C. This is probably due to the fact that the whey protein used in this particular study was subjected to sterilization at 121°C for 15 minutes; causing some heat denaturation. The denatured whey protein could then be used by the heat-resistant organisms. It was reported by deWit (1981) that whey protein would be denatured at 70°C, with an unfolding of the residual protein structure at 130°C. Although there is no literature information available to show the stimulatory effect of whey protein on the growth of heat-resistant milk microflora, this particular study suggests that denatured whey protein can slightly stimulate the growth of thermoduric or sporeforming organisms in skim milk at 7°C.

To summarize the 3 parts of this study, the results suggest that supplementation of 10% skim milk at the rate of 5% tryptone, peptonized milk, or whey protein can stimulate the outgrowth of heat-resistant milk microflora at 7°C. Supplementing skim milk with 5% tryptone showed the greatest stimulatory effect on the growth of thermoduric organisms and very little effect on the growth of sporeformers, while supplementing skim milk with 5%

peptonized milk showed the greatest stimulatory effect on the growth of sporeformers, and very little effect on thermodurics. Supplementation with 5% whey protein showed the least stimulatory effect on the growth of both thermoduric and sporeforming organisms at 7°C. The significance of this study is to illustrate the fact that hydrolyzed milk proteins such as small peptides and/or amino acids are very important in stimulation of the growth of thermoduric and sporeforming organisms.

5. Summary

To summarize the overall investigation, most commercially pasteurized,homogenized milk is contaminated to some degree with psychrotrophs after processing. Since these psychrotrophs can multiply at a rapid rate, extensive psychrotrophic growth can occur in a short period of time, severely affecting the keeping quality of the milk during subsequent storage.

The extensive growth of psychrotrophic organisms in milk is responsible for the subsequent growth of thermoduric and sporeforming milk microflora. Such a stimulatory effect can occur when the previous PBC or SPC is higher that 10^7/ml. However, if the previous PBC or SPC is higher than 10^9/ml, a stimulatory effect is unlikely to occur. This may be due to the accumulation of toxic end product metabolites and/or exhaustion of nutrients by the previous excessive growth of psychrotrophic organisms.

The results of this study also indicated that supplementation of skim milk with 10% supernatant of skim milk in which Ps. fluorescens had previously grown to ca. 7 X 10^7/ml, resulted in the extensive growth of both psychrotrophic thermoduric and sporeforming milk microflora at 7°C. The results also showed that most of these psychrotrophic heat-resistant organisms grew well at 32°C. This is particularly important in UHT-sterilized milk. Proteases produced by Ps. fluorescens, as well as the heat-resistant milk microflora are known to survive both HTST pasteurization and UHT treatment and because the storage of UHT-sterilized milk is usually at room temperature, proteolytic activity can increase and produce enough stimulatory substances for the growth

of heat-resistant organisms during storage. Supplementation of skim milk with hydrolyzed casein or peptonized milk provided a stimulatory effect on the growth of heat-resistant milk microflora during storage at 7°C; however a lesser effect was found using a whey protein supplement.

The importance of this study was to demonstrate that the practice of alternate day or longer collection of farm bulk tank milk can promote the growth of psychrotrophs during storage and transportation. Even though the conventional HTST pasteurization or UHT-sterilization can destroy most of the heat-sensitive psychrotrophs, their end product metabolites including proteases and lipases are known to survive these treatments as described previously. The findings of this study indicate that milk samples which contain high SPC or PBC before pasteurization or autoclaving at 121°C for 10 minutes, can promote the outgrowth of heat-resistant organisms at 7°C.

6. Conclusion

This investigation was initiated to study the role of heat-sensitive psychrotrophs on the growth of heat-resistant milk microflora in milk stored at 7°C. The findings of this study include:

(1) Commercially pasteurized, homogenized milk is usually contaminated with psychrotrophic organisms to some degree, necessitating milk storage at temperatures below 4.4°C, since these psychrotrophs can grow very rapidly at temperatures above 4.4°C.

(2) The initial PBC of milk is of little or no practical use in predicting keeping quality. PBC should be performed over a period of time in order to present a more accurate picture of processing and packaging conditions, and keeping quality.

(3) The MTC and PTC of the milk samples which were laboratory re-pasteurized at zero time were higher than for those samples which received the same treatment at each of the experimental intervals.

(4) The initial tyrosine value, titratable acidity, and pH of milk are not correlated with psychrotrophic bacterial content. Therefore, they should not be used as rapid methods for estimation of the initial PBC or SPC of milk.

(5) If the SPC or PBC is allowed to reach 10^7/ml in pre-heat-treated milk, a stimulatory effect on the growth of heat-resistant milk microflora during subsequent storage of heat-treated milk at 7°C is likely to occur. However, such effects are unlikely to occur when the SPC or PBC is greater than 10^9/ml.

(6) Milk samples which had previously been grown with *Ps. fluorescens* PS3a for 5 days at 7°C were able to stimulate the growth of thermoduric and sporeforming milk microflora during subsequent storage of heat-treated milk at 7°C.

(7) Supplementation of 5% hydrolyzed casein (tryptone) or peptonized milk can stimulate the growth of thermoduric and sporeforming organisms in skim milk stored at 7°C.

(8) The recommendations of this study are:

(a) to develop strict hygienic methods of milk production including effective cleaning and sterilization of all farm and creamery equipment involved in production, thereby minimizing the chance of psychrotrophic contamination in processed milk,

(b) to store the processed or raw milk at temperatures below 4.4°C. High bacteriological quality of raw milk is essential for increasing the shelf-life of finished products.

(9) A prospect for further research and development would be to develop a rapid method for estimation of PBC, and to identify specific substances which are responsible for the stimulatory effects on the outgrowth of heat-resistant microflora. Future research could also focus on the removal of such substances from milk which has been contaminated with heat-sensitive psychrotrophs.

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APPENDIX 1

Table 1: The effect of storage temperatures and time on PBC (Means of Six Samples)

Count (Log10 CFU/ml)

Time (Days)	Temp. °C	Sample Code 1	2	3	4	5	6	Row Average
0	7.0	1.81	5.28	2.52	2.76	2.47	2.45	2.88
0	4.4	1.81	5.28	2.52	2.76	2.47	2.45	2.88
7	7.0	6.28	7.15	7.63	7.68	7.70	3.78	6.70
7	4.4	3.53	5.11	4.85	5.18	4.65	2.90	4.37
14	7.0	8.17	8.67	8.66	8.30	8.85	5.68	8.06
14	4.4	7.84	8.34	8.72	5.61	8.90	4.28	7.21
21	7.0	8.72	9.32	9.76	8.57	9.56	7.93	8.98
21	4.4	8.73	9.17	9.04	7.01	9.54	6.58	8.35

Table 2: The effect of storage temperatures and time on PTC (Means of Six Samples)

Treatment	Time (Days)	Temp. °C	Count (Log10 CFU/ml) Sample Code 1	2	3	4	5	6	Row Average
	0	-	1.30	1.30	1.81	1.60	1.40	1.48	1.48
	7	4.4	1.95	3.45	2.57	2.64	2.33	1.98	2.49
	7	7.0	2.19	4.02	4.02	3.00	3.11	3.04	3.23
A[1]									
	14	4.4	3.72	4.31	4.61	3.16	3.74	2.20	3.62
	14	7.0	3.02	5.03	4.53	4.10	4.51	4.04	4.21
	21	4.4	4.96	6.33	4.95	4.39	5.13	4.51	5.05
	21	7.0	4.56	5.44	5.59	4.72	5.08	4.29	4.95
	0	-	1.30	1.30	1.81	1.60	1.40	1.48	1.48
	7	4.4	1.54	2.19	2.64	2.11	2.08	1.98	2.09
	7	7.0	1.70	3.04	2.90	2.95	3.13	1.65	2.56
B[2]									
	14	4.4	2.18	3.10	3.32	2.41	2.84	2.26	2.69
	14	7.0	2.60	4.97	3.54	3.41	3.41	3.39	3.55
	21	4.4	2.54	4.39	4.48	3.20	3.50	3.32	3.57
	21	7.0	3.62	5.63	4.84	4.52	4.71	3.77	4.52

[1]Laboratory-pasteurized at Experimental Intervals
[2]Laboratory-pasteurized at Zero-Time

Table 3: The effect of storage temperatures and time on PSC (Means of Six Samples)

Treatment	Time (Days)	Temp. °C	Count (Log10 CFU/ml) Sample Code 1	2	3	4	5	6	Row Average
	0	-	1.00	1.18	1.00	1.00	1.00	1.00	1.03
	7	4.4	2.10	2.45	2.27	2.29	2.06	2.08	2.21
	7	7.0	2.08	3.27	4.82	3.45	3.20	2.30	3.19
A[1]									
	14	4.4	2.28	5.49	2.27	2.13	5.72	2.51	3.40
	14	7.0	2.61	5.08	4.53	3.51	4.71	2.28	3.79
	21	4.4	3.00	5.62	3.93	3.98	5.83	3.00	4.23
	21	7.0	4.22	5.69	6.04	4.16	4.88	3.00	4.67
	0	-	1.00	1.18	1.00	1.00	1.00	1.00	1.03
	7	4.4	1.70	2.45	2.08	2.16	2.06	2.13	2.10
	7	7.0	2.02	2.74	2.93	2.70	3.04	1.78	2.54
B[2]									
	14	4.4	2.31	2.48	2.53	2.60	2.76	2.26	2.49
	14	7.0	2.50	3.16	3.23	3.10	3.24	2.16	2.90
	21	4.4	2.62	3.45	3.19	3.28	3.18	2.52	3.04
	21	7.0	3.02	3.45	3.43	3.70	3.51	3.04	3.36

[1]Laboratory-pasteurized at Experimental Intervals
[2]Laboratory-pasteurized at Zero-Time

Table 4A: Growth patterns of heat-sensitive and heat-resistant Psychrotrophs isolated from commercially pasteurized,homogenized Milk, When Grown at 7°C

COUNT (LOG 10/ml)

Time (Days)	PBC	PHSC	PTC[1]	PTC[2]
0	3.00	2.83	2.53	2.53
5	6.14	6.13	2.60	2.50
10	7.48	7.48	2.85	2.38
15	8.35	8.35	4.07	2.79
20	8.40	8.40	5.51	3.21

[1]Counts were obtained from the samples in which heat-sensitive and thermoduric psychrotrophs were growing together at 7°C.

[2]Counts were obtained from the control samples in which only thermoduric psychrotrophs were growing at 7°C.

Table 4B: The ability of heat-sensitive and heat-resistant to grow at 32°C.

COUNT (LOG 10/ml)

Time (Days)	SPC	MHSC	MTC[1]	MTC[2]
0	3.26	3.16	2.57	2.57
5	6.14	6.14	3.03	2.56
10	7.48	7.48	3.12	2.48
15	8.50	8.50	4.67	3.64
20	8.18	8.18	5.12	3.40

[1]Counts were obtained from the samples in which heat-sensitive and thermoduric psychrotrophs were growing together at 7°C.

[2]Counts were obtained from the control samples in which only thermoduric psychrotrophs were growing at 7°C.

Table 5A: Growth patterns of Ps. fluorescens P26 and heat-resistant Bacterial Flora from commercially pasteurized, homogenized Milk, when grown together at 7°C

COUNT (LOG 10/ml)

Time (Days)	PBC	PHSC	PTC[1]	PTC[2]
0	3.94	3.92	2.51	2.40
5	7.31	7.31	3.63	2.37
10	8.28	8.28	3.91	2.45
15	8.98	8.98	4.70	2.53
20	9.32	9.32	4.38	2.62

[1]Counts were obtained from the samples in which Ps. fluorescens P26 and thermoduric psychrotrophs were growing together at 7°C.

[2]Counts were obtained from the control samples in which only thermoduric psychrotrophs were growing at 7°C.

Table 5B: The ability of heat-sensitive and heat-resistant to grow at 32°C.

COUNT (LOG 10/ml)

Time (Days)	SPC	MHSC	MTC[1]	MTC[2]
0	3.95	3.93	2.62	2.55
5	7.40	7.40	3.04	2.58
10	8.27	8.27	3.93	2.61
15	9.05	9.05	4.97	3.62
20	9.25	9.25	3.93	4.35

[1]Counts were obtained from the samples in which heat-sensitive and thermoduric psychrotrophs were growing together at 7°C.

[2]Counts were obtained from the control samples in which only thermoduric psychrotrophs were growing at 7°C.

Table 6: The effects of Ps. fluorescens on the physico-chemical properties of 10% skim milk at 7°C

Time (Days)	SPC (32°C)	TCA[1] Soluble TEV[2]	pH	Titratable Acidity (1% Lactic Acid)	Organoleptic Qualities
0	3.42	50.63	6.65	0.175	O.A.
5	7.86	46.25	6.82	0.160	O.A.
10	8.52	117.50	7.00	0.173	Bitter
15	8.90	389.38	7.05	0.206	Extremely Bitter
20	9.21	482.50	7.14	0.223	Extremely Bitter, Protein Ppt., Production of Greenish Pigment

[1] TCA- Trichloroactic Acid
[2] TEV- Tyrosine Equivalent Value

Table 7: Standard curve for tyrosine value determination

Tyrosine Concentration (mg/ml) [1]	Absorbance (650 nm)
0.00	0.000
0.02	0.121
0.04	0.175
0.06	0.305
0.08	0.388
0.10	0.450
0.12	0.555

[1]Standard Solution: 0.2 mg/ml

Table 8A: The subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. Fluorescens PS3a at 7°C (Mesophilic Thermoduric Count).

	MTC (Log10/ml)					
	Days that Ps. fluorescens was grown in Skim Milk prior to sterilization					
Time (Days)	0	1	2	3	4	5
0	0.42	0.42	0.42	0.42	0.42	0.42
5	0.48	0.42	0.44	0.64	0.57	0.73
10	0.71	0.68	0.65	1.11	1.17	1.37
15	1.08	1.08	1.13	1.29	1.43	1.71
20	1.23	1.25	1.35	1.57	1.67	1.95

Table 8B: The subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. Fluorescens PS3a at 7°C (Psychrotrophic Thermoduric Count).

	PTC (Log10/ml)					
	Days that Ps. fluorescens was grown in Skim Milk prior to sterilization					
Time (Days)	0	1	2	3	4	5
0	0.27	0.27	0.27	0.27	0.27	0.27
5	0.18	0.35	0.42	0.42	0.44	0.53
10	0.48	0.60	0.64	0.76	0.80	0.94
15	1.01	1.06	1.10	1.27	1.41	1.66
20	1.24	1.26	1.33	1.42	1.64	1.99

Table 8C: The subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. Fluorescens PS3a at 7°C (Mesophilic Sporeformer Count).

	MSC (Log10/ml)					
	Days that Ps. fluorescens was grown in Skim Milk prior to sterilization					
Time (Days)	0	1	2	3	4	5
0	0.24	0.24	0.24	0.24	0.24	0.24
5	0.27	0.27	0.40	0.38	0.44	0.65
10	0.56	0.56	0.53	0.82	0.97	1.11
15	1.03	1.06	1.13	1.41	1.51	1.63
20	1.07	1.21	1.32	1.54	1.71	1.92

Table 8D: The subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. Fluorescens PS3a at 7°C (psychrotrophic sporeformer Count).

	PSC (Log10/ml)					
	Days that Ps. fluorescens was grown in Skim Milk prior to sterilization					
Time (Days)	0	1	2	3	4	5
0	0.27	0.27	0.27	0.27	0.27	0.27
5	0.18	0.35	0.42	0.42	0.44	0.53
10	0.48	0.60	0.64	0.76	0.80	0.94
15	1.01	1.06	1.10	1.27	1.41	1.66
20	1.24	1.26	1.33	1.42	1.64	1.99

Table 9: Standard curve for protein determination

Protein Concentration (mg/ml) [1]	Absorbance (500 nm)
0.00	0.000
0.20	0.123
0.40	0.220
0.60	0.298
0.80	0.434
1.00	0.510

[1]Standard Solution: 1.0 mg bovine serum albumin/ml

Table 10A. The effect of supernatant obtained from the Skim Milk in which Ps. fluorescens PS3a had grown, on the growth of Milk Microflora obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (Standard Plate Count)

Time (Days)	SPC (Log10/ml) Percent Supernatant Added (v/v) 0	5	10
0	5.48	5.48	5.48
1	6.84	6.82	7.02
2	7.64	7.55	7.68
3	8.47	8.49	8.57
4	8.86	9.03	9.11
5	8.85	9.06	9.01

Table 10B. The effect of supernatant obtained from the Skim Milk in which Ps. fluorescens PS3a had grown, on the growth of Milk Microflora obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (psychrotrophic bacterial Count)

Time (Days)	PBC (Log10/ml) Percent Supernatant Added (v/v) 0	5	10
0	5.48	5.48	5.48
1	6.76	6.81	7.04
2	7.63	7.56	7.69
3	8.47	8.44	8.65
4	9.03	9.41	9.95
5	9.20	9.48	9.64

Table 10C. The effect of supernatant obtained from the Skim Milk in which Ps. fluorescens PS3a had grown, on the growth of Milk Microflora obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (Mesophilic Thermoduric Count)

Time (Days)	MTC (Log10/ml) Percent Supernatant Added (v/v) 0	5	10
0	1.25	1.25	1.25
2	1.26	1.36	1.46
4	1.15	1.32	1.42
6	1.14	1.32	1.42
8	1.17	1.63	2.49
10	1.68	2.21	4.00
15	4.01	5.00	7.18

Table 10D. The effect of supernatant obtained from the Skim Milk in which Ps. fluorescens PS3a had grown, on the growth of Milk Microflora obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (Psychrotrophic Thermoduric Count)

Time (Days)	PTC (Log10/ml) Percent Supernatant Added (v/v) 0	5	10
0	1.15	1.15	1.15
2	1.02	1.11	1.18
4	1.29	1.31	1.36
6	1.41	1.34	1.46
8	1.84	1.56	2.53
10	3.02	2.27	4.03
15	4.48	5.00	7.18

Table 10E. The effect of supernatant obtained from the Skim Milk in which Ps. fluorescens PS3a had grown, on the growth of Milk Microflora obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (Mesophilic Sporeformer Count)

Time (Days)	MSC (Log10/ml) Percent Supernatant Added (v/v) 0	5	10
0	1.50	1.50	1.50
2	1.59	1.89	1.54
4	2.48	3.15	3.02
6	4.48	4.48	4.48
8	5.91	6.28	6.03
10	7.69	7.67	7.88
15	8.76	8.72	8.82

Table 10F. The effect of supernatant obtained from the Skim Milk in which Ps. fluorescens PS3a had grown, on the growth of Milk Microflora obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (psychrotrophic sporeformer Count)

Time (Days)	PSC (Log10/ml) Percent Supernatant Added (v/v) 0	5	10
0	1.43	1.43	1.43
2	1.66	1.85	1.61
4	2.48	3.22	3.22
6	3.48	4.48	4.48
8	5.97	6.30	6.08
10	7.71	7.59	7.74
15	8.85	8.85	8.76

Table 11A. The effects of supplementing skim milk with tryptone, on the growth of heat-resistant milk microflora at 7°C

	Counts (Log10) /ml											
	MTC			PTC			MSC			PSC		
Time (Days)	Percent Tryptone Added (v/v)											
	0	1	5	0	1	5	0	1	5	0	1	5
0	1.60	1.60	1.60	1.43	1.43	1.43	1.54	1.54	1.54	1.27	1.27	1.27
2	1.49	1.50	1.85	1.36	1.27	1.14	1.43	1.52	1.83	1.41	1.50	1.58
4	1.59	1.82	2.01	1.53	1.52	1.99	1.59	1.66	2.13	1.45	1.48	1.79
6	1.74	1.85	2.29	1.65	1.57	2.19	1.65	1.79	2.20	1.67	1.65	1.86
8	1.80	1.79	2.91	1.71	1.69	3.48	1.58	1.58	2.13	1.65	1.74	1.88
10	1.83	4.48	7.48	1.78	1.98	5.48	1.64	2.14	2.15	1.81	2.15	2.19
15	2.03	6.51	8.70	2.89	6.83	9.10	2.03	2.18	2.14	2.47	2.33	2.44

Table 11B. The effects of supplementing skim milk with peptonized milk, on the growth of heat-resistant milk microflora at 7°C

	Counts (Log10) /ml											
	MTC			PTC			MSC			PSC		
Time (Days)	Percent Peptonized Milk Added (v/v)											
	0	1	5	0	1	5	0	1	5	0	1	5
0	1.60	1.60	1.60	1.43	1.43	1.43	1.54	1.54	1.54	1.27	1.27	1.27
2	1.49	1.59	1.65	1.36	1.38	1.45	1.43	1.47	1.44	1.41	1.37	1.46
4	1.59	1.77	1.68	1.53	1.57	1.58	1.59	1.60	1.86	1.45	1.49	1.58
6	1.74	1.89	2.00	1.65	1.62	1.69	1.65	1.93	1.92	1.67	1.68	1.69
8	1.80	1.95	1.84	1.71	1.64	1.73	1.58	1.86	3.20	1.65	2.24	2.26
10	1.83	2.11	2.55	1.78	1.93	2.62	1.64	4.00	4.00	1.81	4.16	4.18
15	2.03	1.99	3.33	2.89	2.30	3.23	2.03	4.18	4.57	2.47	4.55	6.18

Table 11C. The effects of supplementing skim milk with whey proteins, on the growth of heat-resistant milk microflora at 7°C

	Counts (Log10/ml)											
	MTC			PTC			MSC			PSC		
Time (Days)	Percent whey proteins Added (v/v)											
	0	1	5	0	1	5	0	1	5	0	1	5
0	1.60	1.60	1.60	1.43	1.43	1.43	1.54	1.54	1.54	1.27	1.27	1.27
2	1.49	1.61	1.76	1.36	1.35	1.38	1.43	1.49	1.53	1.41	1.55	1.56
4	1.59	1.89	2.06	1.53	1.53	1.75	1.59	1.69	1.96	1.45	1.56	1.73
6	1.74	1.86	2.04	1.65	1.67	1.83	1.65	2.02	2.21	1.67	1.72	1.81
8	1.80	1.96	2.22	1.71	1.76	2.00	1.58	1.98	2.41	1.65	1.88	2.02
10	1.83	2.41	2.58	1.78	2.62	3.05	1.64	2.20	2.65	1.81	2.05	2.90
15	2.03	2.08	2.44	2.89	3.48	4.45	2.03	2.25	3.91	2.47	2.49	4.48

APPENDIX 2

Table 1. Statistical analyses of the subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. fluorescens PS3a at 7°C for MTC

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	4	73.83		
Treatments	5	1.78	0.356	5.56*
Error	50	3.22	0.064	
Total	59	78.83		

* Significant at P≤0.005

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Days)

Control	1	2	3	4	5
0.78a	0.77ab	0.80ab	1.01ab	1.05ab	1.24b

Table 2. Statistical analyses of the subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. fluorescens PS3a at 7°C for PTC

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	4	67.22		
Treatments	5	1.26	0.252	5.14*
Error	50	2.46	0.049	
Total	59	70.95		

* Significant at P≤0.005

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Days)

Control	1	2	3	4	5
0.64a	0.71ab	0.75ab	0.83ab	0.91ab	1.08b

Table 3. Statistical analyses of the subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. fluorescens PS3a at 7°C for MSC

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	4	69.11		
Treatments	5	1.77	0.354	5.53*
Error	50	3.19	0.064	
Total	59	74.07		

* Significant at P≤0.005

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Days)

Control	1	2	3	4	5
0.63a	0.67ab	0.72ab	0.88ab	0.97ab	1.11b

Table 4. Statistical analyses of the subsequent growth characteristics of heat-resistant organisms in 10% skim milk previously grown with Ps. fluorescens PS3a at 7°C for PSC

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	4	74.87		
Treatments	5	2.07	0.414	5.75*
Error	50	3.62	0.072	
Total	59	80.56		

* Significant at P≤0.005

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Days)

Control	1	2	3	4	5
0.62a	0.70ab	0.82ab	0.93ab	0.99ab	1.18b

APPENDIX 3

Table 1. Statistical analyses of the effect of Supernatant Obtained from Skim Milk in which Ps. fluorescens PS3a had grown on the growth of milk microflora Obtained from a commercially pasteurized,homogenized Milk in 10% Skim Milk stored at 7°C(SPC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	5	2274.86		
Treatments	2	0.09	0.045	4.09*
Error	28	0.30	0.011	
Total	35	2275.25		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	5%	10%
7.69a	7.74ab	7.81b

Table 2. Statistical analyses of the effect of Supernatant Obtained from Skim Milk in which Ps. fluorescens PS3a had grown on the growth of milk microflora Obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (PBC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	5	2713.06		
Treatments	2	0.53	0.265	3.93*
Error	28	1.89	0.068	
Total	35	2715.48		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	5%	10%
6.65a	6.74ab	6.92b

Table 3. Statistical analyses of the effect of Supernatant Obtained from Skim Milk in which Ps. fluorescens PS3a had grown on the growth of milk microflora Obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (MTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	358.24		
Treatments	2	8.51	4.255	4.96*
Error	33	28.33	0.858	
Total	41	395.24		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	5%	10%
1.67a	2.01ab	2.75b

Table 4. Statistical analyses of the effect of Supernatant Obtained from Skim Milk in which Ps. fluorescens PS3a had grown on the growth of milk microflora Obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (PTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	393.20		
Treatments	2	2.96	1.480	2.44 n.s.
Error	33	20.02	0.607	
Total	41	416.18		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	5%	10%
2.03a	1.96a	2.56a

Table 5. Statistical analyses of the effect of Supernatant Obtained from Skim Milk in which Ps. fluorescens PS3a had grown on the growth of milk microflora Obtained from a commercially pasteurized, homogenized Milk in 10% Skim Milk stored at 7°C (MSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	1545.81		
Treatments	2	0.24	0.120	2.69 n.s.
Error	33	1.47	0.045	
Total	41	1547.51		

n.s. Not Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	5%	10%
4.63a	4.81a	4.75a

Table 6. Statistical analyses of the effect of Supernatant Obtained from Skim Milk in which Ps. fluorescens PS3a had grown on the growth of milk microflora Obtained from a commercially pasteurized,homogenized Milk in 10% Skim Milk stored at 7°C(PSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	1537.62		
Treatments	2	0.75	0.375	5.51*
Error	33	2.26	0.068	
Total	41	1540.63		

* Significant at P≤0.01

II. Comparison of the Differences Between Means(Tukey's Test)

Treatments(Level of supplement)

Control	5%	10%
4.51a	4.82b	4.76b

APPENDIX 4

Table 1A. Statistical analyses of the effect of supplementing skim milk with tryptone, on the growth of heat-resistant milk microflora at 7°C (MTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	527.81		
Treatments	2	31.13	15.57	3.95*
Error	33	129.95	3.941	
Total	41	688.89		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.73a	2.79ab	3.83b

Table 1B. Statistical analyses of the effect of supplementing skim milk with tryptone, on the growth of heat-resistant milk microflora at 7°C (PTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	493.50		
Treatments	2	23.17	11.59	3.80*
Error	33	100.49	3.05	
Total	41	617.16		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.76a	2.33ab	3.54b

Table 1C. Statistical analyses of the effect of supplementing skim milk with tryptone, on the growth of heat-resistant milk microflora at 7°C (MSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	139.99		
Treatments	2	1.04	0.52	6.50*
Error	33	2.64	0.080	
Total	41	143.67		

* Significant at $P \leq 0.05$

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.64a	1.77ab	2.02b

Table 1D. Statistical analyses of the effect of supplementing skim milk with tryptone, on the growth of heat-resistant milk microflora at 7°C (PSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	139.67		
Treatments	2	0.24	0.12	6.00*
Error	33	0.17	0.02	
Total	41	140.62		

* Significant at P≤0.01

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.68a	1.73ab	1.86b

Table 2A. Statistical analyses of the effect of supplementing skim milk with peptonized milk, on the growth of heat-resistant milk microflora at 7°C (MTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	156.97		
Treatments	2	0.99	0.50	3.30*
Error	33	5.01	0.15	
Total	41	162.97		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.73a	1.84ab	2.09b

Table 2B. Statistical analyses of the effect of supplementing skim milk with peptonized milk, on the growth of heat-resistant milk microflora at 7°C (PTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	155.15		
Treatments	2	0.53	0.27	3.01 n.s.
Error	33	2.90	0.09	
Total	41	158.58		

n.s. Not significant at $P \leq 0.05$

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.76a	1.70a	1.96a

Table 2C. Statistical analyses of the effect of supplementing skim milk with peptonized milk, on the growth of heat-resistant milk microflora at 7°C (MSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	259.00		
Treatments	2	7.62	3.81	4.38*
Error	33	28.59	0.87	
Total	41	295.21		

* Significant at $P \leq 0.05$

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.64a	2.37ab	2.65b

Table 2D. Statistical analyses of the effect of supplementing skim milk with peptonized milk, on the growth of heat-resistant milk microflora at 7°C (PSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	313.18		
Treatments	2	7.26	3.63	3.30*
Error	33	36.27	1.10	
Total	41	356.71		

* Significant at $P \leq 0.05$

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.68a	2.39ab	2.66b

Table 3A. Statistical analyses of the effect of supplementing skim milk with whey proteins, on the growth of heat-resistant milk microflora at 7°C (MTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	158.62		
Treatments	2	0.98	0.49	8.91*
Error	33	1.80	0.06	
Total	41	161.40		

* Significant at P≤0.01

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.73a	1.92ab	2.10b

Table 3B. Statistical analyses of the effect of supplementing skim milk with whey proteins, on the growth of heat-resistant milk microflora at 7°C (PTC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	214.41		
Treatments	2	1.81	0.91	4.33*
Error	33	6.89	0.21	
Total	41	223.11		

* Significant at $P \leq 0.05$

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.76a	1.98ab	2.27b

Table 3C. Statistical analyses of the effect of supplementing skim milk with whey proteins, on the growth of heat-resistant milk microflora at 7°C (MSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	171.84		
Treatments	2	3.31	1.66	5.73*
Error	33	9.53	0.29	
Total	41	184.68		

* Significant at P≤0.01

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.64a	1.88ab	2.32b

Table 3D. Statistical analyses of the effect of supplementing skim milk with whey proteins, on the growth of heat-resistant milk microflora at 7°C (PSC)

I. ANALYSIS OF VARIANCE

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Time	6	189.97		
Treatments	2	2.43	1.22	4.25*
Error	33	9.44	0.29	
Total	41	201.84		

* Significant at P≤0.05

II. Comparison of the Differences Between Means (Tukey's Test)

Treatments (Level of supplement)

Control	1%	5%
1.68a	1.86ab	2.25b