AN INVESTIGATION INTO THE OCCURRENCE, GROWTH PROPERTIES AND CHARACTERISTICS OF PSYCHROTROPHIC COLIFORM ORGANISMS IN REFRIGERATED PASTEURISED BOVINE MILK IN THE WESTERN CAPE

LLEWELLYN GLENN FISHER

AN INVESTIGATION INTO THE OCCURRENCE, GROWTH PROPERTIES AND CHARACTERISTICS OF PSYCHROTROPHIC COLIFORM ORGANISMS IN REFRIGERATED PASTEURISED BOVINE MILK IN THE WESTERN CAPE.

BY

LLEWELLYN GLENN FISHER

Submitted for Complete Fulfilment of the requirements of the subject Food Project 4 (FPRJ 40) and Partial Fulfilment of the requirements for the Degree B.Tech (Food Technology) in the Department of Horticulture and Food Technology, Peninsula Technikon.

PROJECT LEADERS:

Mr. K. Woolward

Ms. J. van Wyk

DATE SUBMITTED:
NOVEMBER 1999

CONTENTS

ACKNOWLEDGEMENTS

The assistance of the following persons, and organisations made this research possible.

I appreciate all your efforts that have made this undertaking possible.

My wife Sheryl and my children for their support and inspiration and to whom I dedicate my work.

The management: Industrial partner.

Ms. M. Lottering: Laboratory Analyst.

Ms. N. Badrodien: Laboratory Analyst.

Ms. M. Brown: Laboratory Analyst.

The Peninsula Technikon.

Mr. L Dolley: H.O.D. Food Technology (Peninsula Technikon)

Mr. K. Woolward: Lecturer and Project Supervisor (Peninsula Technikon)

Ms. J. van Wyk: Senior Lecturer and Project Supervisor (Peninsula Technikon)

Ms. J Felix: Laboratory Technician (Peninsula Technikon)

Ms. G. Fransman: Administrative Assistant, (Peninsula Technikon)

Mr. M. Cloete: Student: In Service Trainee, (Peninsula Technikon)

CHAPTER 1

LITERATURE REVIEW

The Dairy industry, one of the larger food industries in South Africa processes probably the most perishable and possibly the most regulated foodstuff, namely milk.

The unique combination of vitamins, proteins, carbohydrates, lipids, moisture and near neutral pH, offers a suitable environment for the proliferation of microbes. Milk is therefore highly susceptible to microbiological activity resulting in the irreversible spoilage of this food (Frazier & Westhoff, 1988).

The coliform group of organisms comprises all aerobic and anaerobic, gram-negative, non-spore-forming rods that are able to ferment lactose with the production of acid and gas at 32°C within 48 hours (Richardson, 1985).

The primary purpose of the coliform detection test is to measure the quality of the practices used to minimise bacterial contamination of processed dairy products (Richardson, 1985).

IDF Standard 132A: (1991) defines psychrotrophic organisms as organisms forming countable colonies when incubated aerobically at 6.5°C for 10 days under the conditions specified in IDF standard 101A.

Shelf-life tests conducted in the fresh milk laboratory of a processing plant, revealed significant growth of coliforms in samples stored at 5°C. Lüch, (1985) reported that other contaminating psychrotrophs together with the coliforms reduce the shelf-life of the milk when the storage temperature thereof is above 10 °C.

Ledford & Senyk (1983) reported that, coliform counts exceeding 100 cfu/ml would be accompanied by unacceptable flavour defects. Frazier & Westhoff (1988) reported that coliforms produce "unclean" and "barny" flavours in milk.

Many retailers specify milk bacteriological standards based on "end of shelf-life determinations. Some retail outlets lodge claims with processors in terms of coliform counts and reject supplies that are then returned to the processors for credit.

Coliforms have been reported to grow at temperatures as low as -2°C and as high as 50°C. In foods the growth of coliforms is very slow at 5°C although authors have reported the growth of coliforms in foods at 3-6°C (Jay, 1996).

Coliforms do not usually survive pasteurisation and are frequently used as an indicator of inadequate processing or post pasteurisation contamination (Walker, 1988).

The key Regulation pertaining to milk is regulation number R258 as promulgated in terms of the Foodstuffs Cosmetics and Disinfectants Act (Act 54 of 1972). The main aim of the regulation is to protect the consumer from exposure to foodborne pathogens. In terms of the Act, health inspectors are appointed by the Municipal

Authorities to enforce these regulations. Samples are therefore taken at processing plants and retail outlets and tested in accordance with and for compliance with these Regulations.

CHAPTER 2

MOTIVATION FOR AND DESIGN OF THE STUDY

2.1 MOTIVATION FOR THE STUDY

Section 4 (5) of Regulation 258 of the Foodstuffs Cosmetics and Disinfectants Act (Act 54 of 1972) reads: "No person shall sell any pasteurised milk, which on application of the test described in paragraph 4 (4) of Annexure A, exceeds the most probable number (MPN) of 10 coliform bacteria per 1.0 ml". A preliminary study conducted on milk samples from retail outlets however, revealed that numerous milk samples exceeded the maximum permissible coliform count. (See Table 1 & Figure 1).

Tabulated in Table 1 are coliform detection test results of samples taken from the retail market. The identities of the processing plants and retail outlets from which these samples originate will not be revealed to protect the interests of the parties concerned.

The results in Table 1 clearly indicate that the above-mentioned regulation was contravened in 33 of the 80 samples tested. This amounts to 41.25%. It could therefore be argued that a substantial proportion of retailed pasteurised milk does not conform to legal requirements. The possible public health implications and the premature quality deterioration as a result of microbial spoilage should be a source of major concern amongst pasteurised milk processing operators.

As a consumer, one is deeply concerned about the high coliform counts found in samples tested from the retail market. The results of the preliminary study initiated the

research to investigate the growth of coliform organisms at refrigerated temperatures. Proving that these coliforms possess psychrotrophic/psychrophillic properties may hold grave consequences for both the processors and the monitoring authorities.

2.2 OBJECTIVES

It is therefore of great interest to the Dairy industry in general and myself in particular, to establish:

2.2.1 Whether coliforms actually grow in milk under refrigerated storage conditions.

2.2.2 Whether coliforms are still reliable spoilage indicator organisms for the dairy industry.

2.2.3 Whether the current coliform detection methods as prescribed in the Foodstuffs Cosmetics and Disinfectants Act are reliable for the control of post pasteurisation contamination for both the processors and the Health authorities.

2.2.4 The implications of non-detected coliform organisms in pasteurised milk for the industry and the product quality.

2.3 ASSUMPTIONS

For the purpose of this study, a sample with a coliform count exceeding 100 cfu/ml will be adjudged as spoilt. This was based on the findings of Ledford & Senyk, (1983). These authors reported that coliform counts of that order would be accompanied by unacceptable flavour defects. These flavour defects are attributable to the action of *Pseudomonas* species. These authors also reported close synergistic relationships

between coliforms and *Pseudomonas* species. Frazier & Westhoff (1988) reported that coliforms produce "unclean" and "barny" flavours in milk.

For the purpose of the study, it is assumed that with the passage of time, the coliform counts will increase when stored at temperatures between 4 - 9°C over the normal storage period of 7 days. This could have a significant effect on the coliform count of pasteurised milk when it eventually reaches the consumer (Asperger & Brandl, 1978).

For the execution of coliform detection tests it was assumed that the method for the detection of coliform organisms in milk as prescribed in Regulation 258 of the Foodstuffs Cosmetics and Disinfectants Act (Act number 54 of 1972) is the most appropriate method for the following reasons:

- The majority of dairy processing laboratories as well as the monitoring authorities uses this method.
- Since this method is prescribed by legislation one would assume that the method was carefully researched.
- The results of coliform tests conducted along these guidelines would be regarded as correct since these results are normally tabled in evidence during court proceedings regarding matters of transgressions of the above-mentioned act.

It is further assumed that coliform count data of milk from the single processing plant in the Western Cape represents the situation at the other similar plants. The processing plant targeted having the majority share of the retail market in the Western Cape, one would assume that the sanitation practices would be well established and carefully

managed. The coliform count data of milk from this processing plant is therefore significantly considered to be a representative sample.

2.4 HYPOTHESES

It is hypothesised that immediately after pasteurisation coliform organisms are present in milk but are undetected when milk is tested by the prescribed method in the above-mentioned regulations. These undetected coliforms, with the passage of time during refrigerated storage (5 ± 1°C) recover and propagate in pasteurised milk under these conditions of storage and contribute to the premature spoilage of pasteurised milk.

It is therefore further hypothesised that a significant proportion of pasteurised milk for sale at retail outlets does not comply with Section 4 (4) of Regulation 258 of the Foodstuffs Cosmetics and Disinfectants Act (Act number 54 of 1972) due to the growth of these coliform organisms.

2.5 DELIMITATIONS

The delimitations with respect to sample populations, laboratory equipment availability and techniques for the study were as follows:

2.5.1 The samples that were required for investigating the psychrotrophic growth properties were limited to one processing plant. The possible outcome of the study could have major implications in this market. Pasteurised milk quality being a major competitive advantage in this niche market, no other dairies were approached.

2.5.2 The sample population for the psychrotrophic growth curve construction was restricted to 40 samples and excluded milk from the retail trade. Milk from the retail outlets might have been exposed to temperatures higher than 5°C, which could cause premature deterioration of the milk.

2.5.3 Since the study involved just one laboratory, inter-laboratory experimental error was disregarded in terms of the statistical manipulation of data.

2.5.4 Experimental errors associated with the microbiological plate count methods were disregarded. Analysis was done in duplicate and the arithmetic average of the two counts was the count representing the data for that specific sample. The counts stipulated in the data were regarded as presenting 100% accuracy for practical purposes.

2.5.5 To establish the growth characteristics of coliforms in pasteurised, homogenised milk, 40 milk samples of packaged milk were stored at 5 ± 1 °C. Coliform detection tests were then conducted in duplicate and the arithmetic average of the duplicates recorded against that particular sample data.

2.5.6 Time interval ranges were 0 hours (i.e. immediately after sampling), 6 hours, 12 hours, 24 hours and then at 24-hour intervals for seven days after filling. The data collected was used to construct the growth curve of the coliform organisms in the natural medium, milk, for a period of 7 days. Serial dilutions of samples were done to accurately establish

the actual coliform organism counts. Coliform counts exceeding 300 organisms/ml were regarded as totally spoilt.

2.6 METHODS

The method that will be used to enumerate coliforms will be according to the pour plate method prescribed by the Regulation No. 258 (Anonymous, 1985a). The incubating temperature will be adjusted to 30 ± 1°C in accordance with the International Dairy Federation Standard; 73 A (Anonymous, 1985b).

Milk samples were stored at 5 ± 1°C for a period of 7 days (168 hours). The bacterial quality was evaluated in terms of coliform counts over this period in order to construe growth curves for this group of organisms.

Serial dilutions were done to ensure plates with coliform counts of < 300 colonies per plate.

The study involved the microbiological evaluation of 40 milk samples from a single pasteurisation plant.

Coliform organisms detected on the violet red bile agar plates were isolated using streak plate techniques and second purification was done on nutrient agar.

Pure cultures of Gram negative rod isolates will be identified using API 20E identification system method

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This section addresses the methods and materials used in the execution of the project to propagate the coliform organisms and to ensure proper storage and handling of samples

3.2 Sampling, sample handling and storage.

3.2.1 Sampling from retail outlets: (For the preliminary study).

3.2.1.1 Packaged milk from retail outlets were purchased and placed in a Styrofoam cooler box containing sufficient ice packs to maintain a temperature less than 5°C.

3.2.1.2 On arrival at the laboratory these samples were placed into a refrigerator at 5°C immediately.

3.2.1.3 Coliform tests were then executed within an hour of arrival.

3.2.2 Sampling from the production line.

3.2.2.1 The required amount of samples was collected from the line and placed in a Styrofoam cooler box containing ice packs.

3.2.2.2 On arrival at the laboratory these samples were immediately placed in a refrigerator at 5°C.

3.2.2.3 Coliform tests were executed within an hour of arrival.

3.3 Equipment and reagent requirements.

3.3.1 Equipment.

3.3.1.1 A cooler box manufactured from a suitable insulator material such as Styrofoam.

3.3.1.2 Ice packs were frozen prior to use and put in the cooler box for maintaining a temperature less than 5°C during the transportation of samples.

3.3.1.3 Incubator with the following minimum features:

- Fitted with digital temperature controls.
- The thermometer of the temperature control should be calibrated and traceable to a national calibration standard.
- Fitted with forced air circulation mechanism for proper thermal distribution.

3.3.1.4 Autoclave fitted with calibrated controls.

3.3.1.5 Waterbath capable of maintaining the water temperature within 1°C of the set temperature.

3.3.1.6 Refrigerator capable of maintaining the air temperature between 4°C and 6°C. The thermometer of the temperature control should be calibrated and traceable to a national calibration standard.

3.3.1.7 Calibrated digitally controlled pipetting equipment.

3.3.1.8 Glassware including Schott bottles, MacCartney bottles and general laboratory glassware.

3.3.1.9 Electronic top loading balance, calibrated, two decimal readout and fitted with a shield.

3.3.1.10 Colony counter with adequate light source.

3.3.1.11 General laboratory equipment as required, such as spatulas, beakers, pipettes, pasteur pipettes and microbiological loops.

3.3.2 Reagents and growth media.

3.3.2.1 Ringers tablets, Merck catalogue number HC 015525.001.

3.3.2.2 Violet red bile agar (Biolab). Merck catalogue number code C 23.

3.3.2.3 Glass distilled water of the highest purity.

3.3.2.4 Nutrient agar (Biolab). Merck catalogue number code C 1.

3.4 Preparation of reagents and growth media.

3.4.1 Preparation of single strength ringers solution:

- Two Ringer's tablets were dissolved in 1000 ml of glass distilled water.
- 9.0 ml aliquots of the solution were then dispensed into 28 ml wide neck MacCartney bottles.
- The bottles were then sterilised at 121°C/15 minutes in an autoclave.

3.4.2 Preparation of violet red bile agar.

- 37 g of violet red bile agar was weighed into 1000 ml Schott bottles.
- 1000 ml of glass distilled water is added.
- The agar was allowed to dissolve completely by gentle mixing while being brought to boil in a waterbath.
- When the agar had dissolved the bottles were placed in a waterbath at 50°C.
- Kept at this temperature for 30 minutes, the agar would be ready for use.

3.4.3 Preparation of glass distilled water.

- Using a waterstill, water was brought to boil and the water vapour allowed to condense in a condenser.
- The condensate was collected in an aspirator jar.

3.4.4 Preparation of nutrient agar.

- 31 g of nutrient agar was weighed into 1000 ml Schott bottles.
- 1000 ml of glass distilled water was added.
- The agar was allowed to dissolve completely by gentle mixing while being brought to boil in a waterbath.
- When the agar had dissolved the bottles were sterilised in an autoclave at 121°C for 15 minutes.
- After sterilisation the bottles were placed in a waterbath at 50°C.
- Kept at this temperature for 30 minutes, the agar would be ready for use.
- Approximately 25 ml of agar was then pored aseptically into the petri dishes.
- The agar was allowed to solidify.
- The inverted dishes were placed at an angle onto the lids to allow the surface to dry.
- The surface of the plates would after drying be ready to be streaked.

3.4.5 Preparation of nutrient agar streak plates for isolation of organisms.

- After selecting colonies from coliform test plates (violet red bile agar plates) colonies were aseptically transferred using a sterile microbiological loop, to nutrient agar plates.
- These streaked and inverted nutrient agar plates were then aerobically incubated at 30°C for 48 hours.
- The isolates were then sub-cultured onto nutrient agar plates to ensure restoration of metabolic reactions that could have been affected by the selective media (violet red bile agar).
- These isolates were Gram stained. Only Gram negative rods were used for identification using the API 20E identification system for Enterobacteriaceae or API 20NE systems.

3.4.6 Determining growth properties of isolates at 4 ± 1°C and 30°C.

- Streaked nutrient agar plates of the most prominent isolate in terms of percentage population were incubated at both 4 ± 1°C and 30°C for a period of 96 hours.
- Colonies were described and recorded.

3.5 Milk sampling, preparation of samples and decimal sample dilutions.

3.5.1 Milk sampling.

3.5.1.1 Production line samples were taken from the lines and placed into Styrofoam cooler boxes, transported to the laboratory and placed in the 4 ± 1°C refrigerator immediately.

3.5.1.2 The primary packaging material serves as the sample container to prevent any contamination that may arise from the use of additional sample containers.

3.5.2 Preparation of samples and decimal sample dilutions.

3.5.2.1 No more than 10 samples were removed from the fridge during a testing session to prevent extended exposure to ambient temperature that could influence the growth of these organisms. Immediately after the required aliquots of the samples were dispensed for testing, these samples were put back in the refrigerator.

3.5.2.2 1 ml aliquots of the samples were dispensed into MacCartney bottles containing 9 ml of Ringers solution. 1ml aliquot from this solution would therefor represent 10^{-1} dilution of the original sample.

3.5.2.3 Repeating this process would result in decimal dilutions of the desired level.

3.5.2.4 To facilitate proper mixing, these containers were shaken by inverting the sample while moving it along a 45° plane with the horizontal over a distance of approximately 30 cm. Inverting the tube 15 times as stated would render the sample properly mixed.

3.6 Execution of pour plate test procedure.

3.6.1 Using a calibrated pipette, 1 ml aliquots were aseptically transferred to an appropriately marked petri dish.

3.6.2 Approximately 15 ml of violet red bile agar was aseptically poured into the petri dish.

3.6.3 The contents of the petri dish was mixed to distribute the sample over the petri dish area by swirling and rotating the petri dish until the contents appear uniform. This was done to ensure uniform distribution of the organisms in the sample to facilitate easy and accurate counting of the colonies.

3.6.4 The agar was allowed to cool and solidify.

3.6.5 After solidification of the agar, a 10 ml violet red bile agar overlay was poured onto the solidified agar in the plate. The agar was allowed to cool and solidify.

3.6.6 The petri dishes were inverted and put in an incubator set at 30°C for 24 hours.

3.6.7 After incubation the petri dishes were removed and the colonies that were typical in terms of the morphological description of coliforms were counted.

3.6.8 Petri dishes required for further tests were stored in a refrigerator at 4 ± 1°C.

3.7 Incubation / refrigeration temperature validation.

3.7.1 Thermometers standardised according to recognised techniques were used to ensure correct refrigeration and incubation temperature conditions.

3.8 Colony count method.

3.8.1 Inverted petri dishes were put in position on the Suntex colony counter.

3.8.2 Colonies appearing bright red in colour with a diameter greater than 1 mm were adjudged presumptive coliforms.

3.8.3 Plates with less than 30 and more than 300 colonies were discarded.

3.8.4 Colonies resembling the typical colony morphology were counted and recorded.

3.9 Isolation of organisms for identification.

3.9.1 Presumptive coliform colonies were picked off the violet red bile agar plates and transferred to nutrient agar streak plates.

3.9.2 Nutrient agar streak plates were incubated at 30°C for 48 hours.

3.9.3 Isolates were sub-cultured onto nutrient agar plates and incubated at 30°C for 48 hours.

3.9.4 Colonies were identified using API 20E identification system for Enterobacteriaceae and API 20NE.

3.10 API 20E identification system method.

3.10.1 Using a pre-flamed, cooled microbiological loop, a well isolated colony from the nutrient agar plate was removed and transferred to a tube containing either sterile 0.85% NaCl medium or sterile saline or sterile distilled water.

3.10.2 The contents of the tube/bottle were carefully mixed to achieve a homogeneous suspension of the bacterium.

3.10.3 With the aid of a sterile Pasteur pipette, cupules of the tests marked CIT, VP and Gel were filled with the bacterial suspension. The remainder of the tests was only filled up to the tube level.

3.10.4 Anaerobic conditions in the tests marked ADH, LDC, ODC, URE and H_2S were created. This was done by overlaying the cupule with mineral oil using a sterile Pasteur pipette.

3.10.5 Water was added to the tray housing to prevent dehydration of the test strips.

3.10.6 The incubation box was closed and incubated at 35-37°C for 18 – 24 hours. Further incubation for an additional 24 hours is advisable especially when less than 2 colony forming units are used in the inoculum.

3.10.7 After incubation the reaction results were read.

3.10.8 After the addition of the test reagents 10 minutes were allowed for the reagents to react prior to reading the test result.

3.10.9 Results were recorded on the API 20E results sheet by referring to the colour reaction chart.

3.10.10 The results were scored as follows; negative = 0 and positive = value of each individual test as indicated on the result sheet.

3.10.11 The numerical profile of the results were then established by calculating the sum of three consecutive test result scores. These were then recorded in the space provided for the numerical profile.

3.10.12 With reference to the Analytical profile index the 7 – digit numerical index code obtained from the test results was used to obtain the identity of the organism.

3.10.13 The results were tabulated in Tables 5 and 7.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 RESULTS:

Tabulated in Table 1 below are the coliform colony count results obtained from analyses of forty samples from various milk processors sampled at retail outlets. (A1 to K10)

Table 1. Coliform counts of milk sampled at retail outlets.

Sample identity	Best Before days left	No. of coliforms /ml	Sample identity	Best Before days left	No. of coliforms /ml
A1	4	0	G4	1	0
A2	6	0	G5	7	0
A3	5	67	G6	5	0
A4	5	0	G7	5	0
A5	4	23	H1	5	1
A6	4	>300	H2	4	0
A7	4	0	H3	3	1
A8	4	0	H4	5	0
B1	1	0	H5	6	0
B2	0	224	H6	2	>300
B3	5	0	H7	4	7
B4	4	>300	H8	5	0
B5	2	0	I1	6	0
B6	5	0	I2	7	0
C1	4	0	I3	1	>300
C2	5	19	I4	2	>300
C3	5	0	I5	5	184
C4	6	32	I6	5	92
C5	-1 *	16	I7	7	2
C6	-3 *	10	I8	3	2
C7	7	4	I9	4	0
D1	2	0	I10	5	2
D2	2	0	J1	2	>300
D3	2	>300	J2	3	>300
D4	1	>300	J3	3	1
D5	7	0	J4	2	>300
D6	4	244	J5	6	30
E1	3	0	J6	6	1
E2	6	0	J7	3	>300
E3	4	0	J8	3	84
E4	5	88	K1	4	0
E5	6	21	K2	3	124
E6	3	0	K3	1	3
E7	5	0	K4	5	0
E8	5	>300	K5	6	>300
E9	4	66	K6	5	176
F1	2	>300	K7	2	0
G1	3	0	K8	3	15
G2	1	0	K9	4	21
G3	1	0	K10	4	73

* Negative best before values indicate that the milk has aged beyond its shelf life.

The data from Table 1 was used to construct a pie chart (Fig. 1) to graphically depict the magnitude of samples that:

- Exceed the maximum permissible number of coliforms per ml of pasteurised milk according to Regulation No. 258 Food stuffs Cosmetics and Disinfectants Act (Act 54 of 1972 as amended)
- Exceed 100 coliforms per ml, that is regarded as spoilt as reported by Asperger & Brandl (1978).

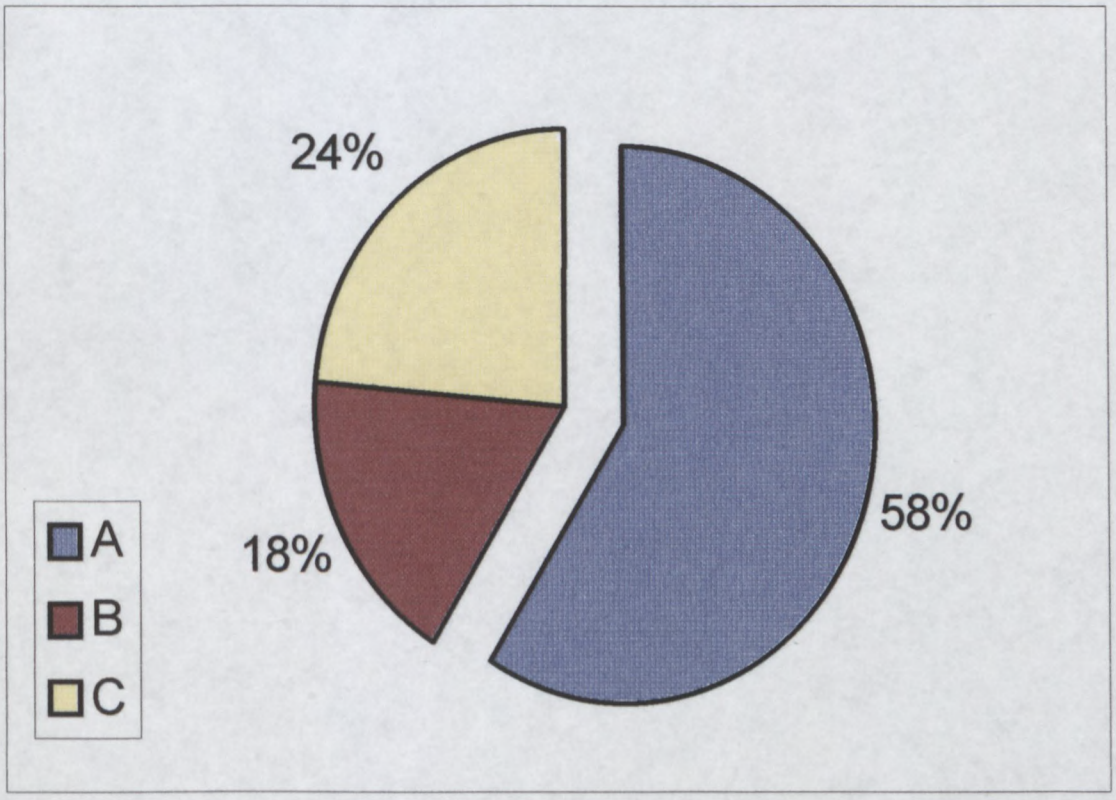

Figure. 1: Pie chart representing the data in Table 1 as per the groupings below.

A = Coliform counts within the maximum permissible limit (10/ml)

B = Coliform counts > 10 but less than 101 / ml

C = Coliform counts > 100 / ml

Tabulated in Table 2, below, are the coliform colony counts of milk sampled from a single processing line.

Table 2: The growth of coliforms in milk samples stored at 5 ± 1°C.

No.	0 hours	6 hours	12 hours	18 hours	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
1	30	18	6	33	113	>300	>300	>300	>300	>300
2	25	30	26	59	31	>300	>300	>300	>300	>300
3	21	22	9	44	17	>300	>300	>300	>300	>300
4	28	29	13	25	18	>300	>300	>300	>300	>300
5	31	45	30	43	22	>300	>300	>300	>300	>300
6	27	17	16	43	10	>300	>300	>300	>300	>300
7	22	18	17	22	9	>300	>300	>300	>300	>300
8	27	22	0	29	9	212	>300	>300	>300	>300
9	28	24	0	43	25	218	>300	>300	>300	>300
10	38	17	0	47	44	>300	>300	>300	>300	>300
11	0	0	0	0	0	12	113	>300	>300	>300
12	0	0	0	0	4	17	112	>300	>300	>300
13	0	0	0	0	0	57	107	>300	>300	>300
14	0	0	0	0	0	17	78	>300	>300	>300
15	0	0	0	0	0	48	160	>300	>300	>300
16	0	0	0	0	0	24	70	>300	>300	>300
17	0	0	0	0	0	26	80	>300	>300	>300
18	0	0	0	0	0	33	73	>300	>300	>300
19	0	0	0	0	0	41	170	>300	>300	>300
20	0	0	0	0	0	28	66	>300	>300	>300
21	4	9	15	36	156	216	>300	>300	>300	>300
22	7	14	16	41	124	223	>300	>300	>300	>300
23	9	13	19	51	160	198	>300	>300	>300	>300
24	4	15	21	35	111	156	>300	>300	>300	>300
25	0	0	0	24	127	176	>300	>300	>300	>300
26	3	14	17	34	138	205	>300	>300	>300	>300
27	12	15	22	36	77	169	>300	>300	>300	>300
28	9	19	24	43	99	144	>300	>300	>300	>300
29	6	13	20	41	84	153	>300	>300	>300	>300
30	7	20	28	51	93	175	>300	>300	>300	>300
31	0	0	0	29	47	76	123	>300	>300	>300
32	0	0	0	11	43	85	154	>300	>300	>300
33	0	0	0	5	41	111	162	>300	>300	>300
34	0	0	0	11	63	119	121	>300	>300	>300
35	0	3	6	17	77	102	133	>300	>300	>300
36	0	2	7	22	55	98	178	>300	>300	>300
37	0	0	0	16	41	124	163	>300	>300	>300
38	0	4	4	35	53	97	155	>300	>300	>300
39	0	0	0	18	68	104	171	>300	>300	>300
40	0	0	0	13	56	78	136	>300	>300	>300
Ave.	**8.5.**	**9.6**	**7.9**	**23.9**	**50.4**	**101.7**	**126.3**	**>300**	**>300**	**>300**

No.- indicates the different samples tested.
Results: These are the coliform colony counts done in duplicate at the indicated time intervals between 0 and 144 hours. All samples were stored at 5 ± 1°C.

The arithmetic average of the results in Table 1 was used to construct the graph (Fig. 2). Figure 2 graphically represents the growth curve of coliform organisms at 5 ± 1°C in milk.

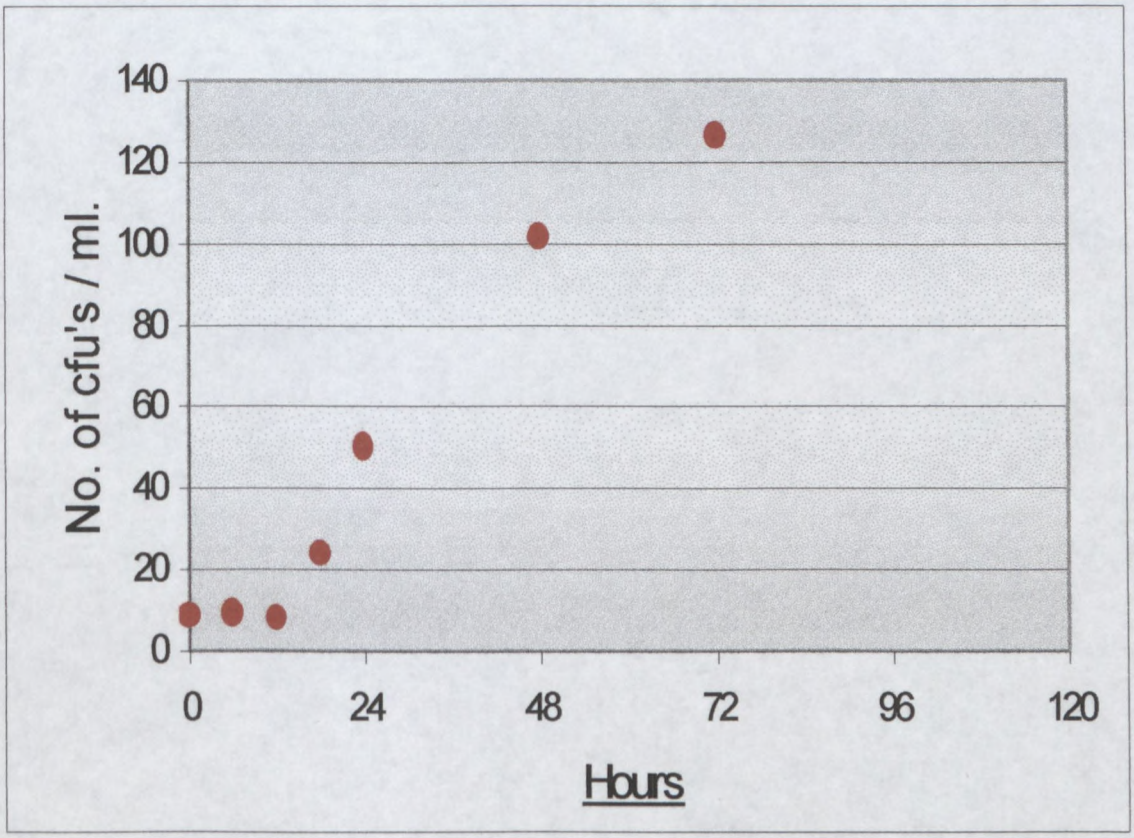

Figure 2: Coliform growth curve at 5 ± 1°C.

Following the determination of the growth properties at 5 ± 1°C the next step was to:

a. Repeat the growth of these coliforms using different samples of unknown microbiological quality for the purpose of validating previous data.

b. To isolate and identify these organisms.

Table 3 below, represents the data collected of the validation tests. Due to the refrigerator operating at 4 ± 1°C the growth of these organisms was retarded. This resulted in a shift of the growth curve in relation to time.

Table 3: Confirmation of coliform counts of milk samples from a single processing line that were stored at 4 ± 1°C for validation purposes.

Sample identity	0 hours	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours
A	0	0	0	0	0	0	69	1270
B	0	0	0	0	0	3	2	29
C	0	0	0	0	0	5	85	1080
D	0	0	0	0	0	0	16	65
E	0	0	0	0	0	0	32	1180
Average (A-E)	0	0	0	0	0	2	41	725

Control tests

Positive control	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
Negative control	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

The data collected during the execution of the validation set of samples was used for the construction of figure 3 (Fig. 3).

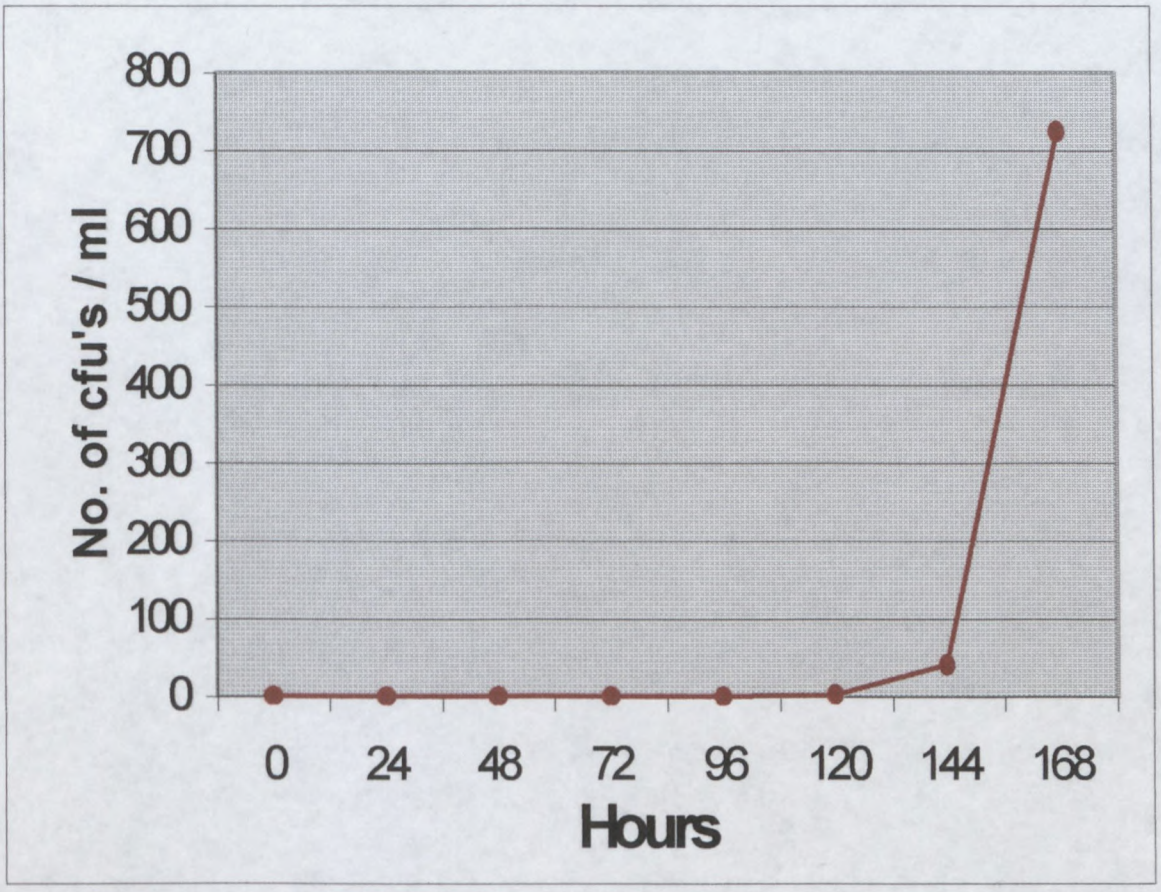

Figure 3: Coliform growth curve at 4 ± 1°C from data in Table 3. The graph depicts the average coliform count of the five samples tested.

The test plates were then examined for distinctively different colony morphologies for isolation purposes and subsequent identification.

Three distinctly different colonies were picked off the violet red bile agar plates and streaked for isolation of organisms. The respective colony morphologies were recorded. The percentage population of the different colony morphologies on the violet red bile agar plates was also determined. This data is reflected in Table 4.

Table 4: Percentage isolate population in milk based on colony morphology.

Colony morphology	Percentage population in milk	Isolate
X Typical bright red colony measuring ca. 1 mm in diameter	80%	Isolate A Isolate B Isolate E Isolate F Isolate G Isolate H
Y Typical bright red colony measuring ca. 1 mm in diameter with a light red haze of ca. 1 mm thick surrounding the colony	10%	Isolate I
Z Typical bright red colony measuring ca. 1.5 - 2.0 mm in diameter with a light red haze of ca. 1 mm thick surrounding the colony	10%	Isolate C Isolate D

Colonies X, Y & Z were propagated on nutrient agar plates and the colonies were subjected to the API 20E identification system for Enterobacteriaceae. Colonies X and Z were not pure. Therefore further isolation was required. Organisms (A to I) were isolated, purified and identified.

Table 5 represents the reactions of these colonies (X,Y & Z) from these plates when subjected to the API 20E identification system for Enterobacteriaceae.

Table 5: API 20E identification results of isolates after 48 hours.

Colony Y	Colony X	Colony Z
Profile index number: 5100112	Profile index number: 1004173	Profile index number: 0007173
Hafnia alvei 1	*Enterobacter agglomerans*	*Erwinia nigrifluens*
➢ 92.4% confidence id. ➢ Good discrimination	➢ 92.2% confidence id. ➢ Good discrimination	➢ 87.9% confidence ➢ Good discrimination

(Refer to Appendix for detailed reaction results)

Colony X (cfu X) was streaked onto nutrient agar plates and aerobically incubated at both 4 ± 1°C and 30 ± 1°C. Plates were evaluated for visible colony development.

Growth was detected on the plates incubated at 30 ± 1°C within 24 hours. In contrast, at 4 ± 1°C, visible growth was only detected after 72 hours. This clearly indicates that the growth of cfu. X is not optimum at lower temperatures i.e. it does not display typical psychrotrophic properties. (See table 6)

Table 6: Growth properties of colony X (cfu. X) at 4 ± 1°C and 30 ± 1°C.

D = Detected and ND = Not Detected

Incubation period intervals	4 ± 1°C incubation	30 ± 1°C incubation
24 hours	ND	D
48 hours	ND	D
72 hours	D	D
96 hours	D	D

When reading the API test strips, it was noted that some reactions had changed from positive to negative and vice-versa between 24 hr and 48 hr incubation periods. It was

therefore clear that colonies X and Z were not pure cultures and further sub-culturing was required. The original colonies from the violet red bile agar plates were streaked onto nutrient agar plates. The isolates as indicated in Table 4 were then identified by application of the API system for Enterobacteriaceae.

The results of these tests are tabulated below.

Table 7: Identification of isolates A-I

Isolate	API profile index	Positive identification	Identification status
A	0005042	*Acinetobacter*	Acceptable identification. (87.1%)
B	2005042	*Pseudomonas flourescence / putida*	Good identification (97.8%)
C	0000000	None	Set up API (NE)
D	0003000	*Sphingomonas paucimobilis* *Pseudomonas species* *Flavimonas oryzihibitans* *Acinetobacter species*	Poor identification 38% 20% 13.7% 12.8%
E	0001000	None	Set up API (NE)
F	2205042	*Cryseomonas luteola*	Good Identification 96.2%
G	0205042	*Flavimonas oryzihabitans* *Acinetobacter species* *Cryseomonas luteola*	Good identification 54.5% 38.0% 7.3%
H	0001000	None	Set up API (NE)
I	4305112	*Hafnia alvei 1*	Excellent identification 99.9%

Isolate origin: Refer to Table 4

It is evident from Table 7 that none of these isolates are coliforms. *Hafnia alvei 1* is a member of Enterobacteriaceae, but not a coliform. The organisms that produced a poor identification profile will produce a better result when the API 20NE is used.

Further observations:

After the violet red bile agar plates were counted they were stored in the refrigerator for possible later use. However, growth of colony forming unit that mimics the colony morphology of coliforms on Violet Red Bile Agar was observed. This cfu was isolated. Both the isolates (C, E & H) that produced no positive identification on application of the API 20E test strips and the unknown cfu that mimics the colony morphology on violet red bile agar plates are currently in the process of being positively identified for possible further investigation.

4.2 DISCUSSION:

4.2.1 Definitions to consider:

4.2.1.1 Coliform bacteria:

> Bacteria that, in the presence of bile salts or other equivalent selective agents, can grow and produce acid and gas from lactose when incubated at 35 or 37°C (Harrigan & McCance, 1986).

4.2.1.2 Coli-aerogenes bacteria:

Bacteria that, in the presence of bile salts or other equivalent selective agents, can grow and produce acid and gas from lactose when incubated at 30°C (Harrigan & McCance, 1986).

4.2.1.3 Total Enterobacteriaceae:

Bacteria that, in the presence of bile salts, will grow and produce acid and gas from glucose as determined by use of Violet Red Bile Glucose agar (Harrigan & McCance, 1986).

4.2.2 Documented properties of the Enterobacteriaceae isolates identified.

4.2.2.1 Properties of *Hafnia alvei 1:*

4.2.2.1.1 General information:

This organism is a Gram negative rod group under the genus *Hafnia*. *Hafnia alvei* was formally grouped under the genus *Enterobacter* as *Enterobacter hafnia* named after the old name of Copenhagen. *Hafnia alvei* is the sole specie of this genus (Krieg & Holt, 1984).

4.2.2.1.2 General properties:

Hafnia alvei have the following properties that are of interest to this investigation: The members of this genus conform to the general definition of the family Enterbacteriaceae. Gram negative rods measuring 1.0µm in diameter and 2.0-5.0µm in length. They are motile by peritrichous flagella at 30°C. Members of the genus *Hafnia* are able to grow at 35°C but many

of their biochemical and physiological activities at this temperature are irregular. The maximum temperature for growth is usually 40 - 42°C. No growth occurs at 5°C.

Lactose in not fermented, but plasmid-mediated lactose positive strains may occur. Members occur in faeces of man and other animals including birds. They are also found in soil, sewage, water and dairy products (Krieg & Holt, 1984).

Hafnia alvei is also an opportunistic pathogen for humans in blood, urine, or wound infections in patients with underlying illness or predisposing factors (Holt et al, 1994).

4.2.2.1.3 Their significance in terms of this investigation:

The findings of this investigation clearly indicate the delayed growth properties of *Hafnia alvei* at 4 ± 1°C. This statement is supported by data in Table 7 as well as deductions from Tables 3 and 6. This is contrary to the documented property of this genus regarding no growth at 5°C.

Their presence in milk could also indicate possible faecal contamination, since they are found in the human and animal faeces. As stated above these organisms are opportunistic pathogens and could delay the healing process of an internal wound, e.g. ulcers in the intestinal tract.

Since their numbers are low (Table 4), their milk spoilage action could be limited in comparison with the other isolates. They are members of the family Enterobacteriaceae and are also good spoilage indicator organisms. Harrigan & McCance, (1986) support this statement.

4.2.3 Properties of other isolates other than Enterobacteriaceae:

4.2.3.1 General information:

Isolates in question are the following:

Acinetobacter species; *Pseudomonas flourescence; Pseudomonas putida; Sphingomonas paucimobilis; Pseudomonas* species; *Flavimonas oryzihibitans* and *Cryseomonas luteola.*

4.2.3.2 General properties and grouping:

The genera *Acinetobacter*, *Cryseomonas*, *Flavimonas*, *Pseudomonas* and *Sphingomonas* (*Sphingobacterium*) are members of Group 4 Gram-negative Aerobic/ Microaerophilic rods and cocci, sub-group 4a (Aerobic) according to Holt & Krieg, (1994).

According to Holt & Krieg (1994), this group includes rods and cocci that can grow under air atmosphere (21% oxygen). Some genera are microaerophillic under nitrogen fixing conditions but, when supplied with a source of fixed nitrogen, they grow as aerobes

4.2.3.3 Their significance in terms of this investigation:

It is an established fact that members of this group contribute to the spoilage of milk at refrigerated temperatures (Ledford & Senyk, 1983). *Pseudomonas* is responsible for flavour defects in milk.

Some genera are also commensals of warm-blooded animals and humans in which they may occasionally prove pathogenic according to Holt & Krieg, (1994).

4.2.4 Evaluation of findings in terms of the objectives of this investigation.

4.2.4.1 **Objective 1:**

Do coliform organisms actually grow in milk under refrigerated storage conditions?

No coliform organisms were found.

One Enterobacteriaceae viz. *Hafnia alvei 1* was detected. The results clearly showed that low storage temperatures retards the growth of this organism.

Seven non-Enterobacteriaceae (non-coliforms) were isolated. These organisms originally yielded false positive results for the recognised coliform test. Even though they are not coliforms, they are spoilage organisms.

An important implication of the false positive result on violet red bile agar is the fact that mixed colonies appeared as single colonies, yielding an erroneous result in terms of numbers. Therefore the prescribed test seems suspect.

4.2.4.2 **Objective 2:**

Are coliforms still reliable indicator organisms for the industry?

The detection of coliform organisms in pasteurised milk at the processing plants still indicate the possible contamination of other spoilage and possibly pathogenic organisms. Of concern however, as found in this investigation is that coliforms were not detected immediately after processing and filling but that other organisms that mimic typical coliform colony morphology on Violet Red Bile Agar, develop during refrigerated storage. Therefore the reliability and validity of the test result is questionable in the light of the findings of this investigation.

Walker (1988) reports that some coliforms are psychrotrophic and are also responsible for spoilage of dairy products. The findings of this investigation do not support this statement. It would however be of interest to establish whether the coliform organisms that were found by Walker (1988) are present in milk in the Western Cape area. Confirming the findings of Walker (1998) would pose a problem to dairy processors.

To compound the problem of detecting coliforms, Richardson (1985) reports that the total viable coliform count of milk is questionable due to the possibility of stressed organisms as a result of sub-lethal injury. The use of selective media used for the enumeration of these organisms can be inhibitory. Table 1 (Samples 1-10 & 21-30) does indicate the detection of coliforms immediately after processing. This investigation however questions the identity of these cfu's due to the false positive results obtained.

The findings of my investigation have established the delayed growth of non-coliform Enterobacteriaceae members. With reference to Tables 2 and 3 it is clear that after a storage period of 168 hours the number of these bacteria increased significantly. The question raised by this finding is: Does milk support the resuscitation of sub-lethally stressed organisms and could this action be extended to similarly stressed pathogens that may survive pasteurisation heat processing conditions. Harrigan & McCance (1986) report that the presence of any of the members of the bacterial groups defined in the section on definitions above, in heat treated foods is indicative of one or more of the following:

(a) "The initial concentration of the bacteria was so high that the heat treatment was inadequate to reduce the concentration to an in-detectably low level."

(b) "Post heating conditions allowed multiplication of survivors until their numbers reached detectable levels."

(c) "Contamination occurred subsequent to the heat treatment."

The same authors suggest the use of Lactose Resuscitation Broth to resuscitate metabolically damaged Salmonellae. These authors also suggest that when dried foods with a very high solids content such as dried milk are analysed in terms of non-selective resuscitation, a 10% suspension in sterile distilled water should be incubated for 24 hours at 37°C. Prolonged storage of the milk samples actually may facilitate the resuscitation of the isolates established through this investigation. Whether this action could be extended to coliforms is a matter that requires further investigation.

4.2.4.3 **Objective 3:**

Is the current coliform detection method as prescribed in the Foodstuffs Cosmetics and Disinfectants Act reliable for the control of post pasteurisation contamination for both the processors and the Health authorities?

This investigation brought to light the possibility of false-positive coliform results on application of the prescribed method. Therefore the monitoring of milk at the end of its shelf life as implied by the said regulation is impossible by using this technique considering the findings of this investigation. The reliability of the prescribed method is questioned and therefore this is a matter that requires further investigation.

4.2.4.4 **Objective 4:**

What are the implications of non-detected coliform organisms in pasteurised milk on the industry and the product quality?

This investigation failed to detect and positively identify coliforms that grow at refrigerated temperatures during storage. The reliability of the prescribed method is questioned as a result of these results.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

In conclusion; this investigation has failed to detect the presence of psychrotrophic coliforms. It however has managed to open the doors to further future research along the following areas of interest:

5.1 The degree of selectivity and the possible significant differences of various types of media in respect to the detection of coliforms.

5.2 The mechanisms that provide non-coliforms to mimic typical coliform colony morphology on violet red bile agar plates.

5.3 To investigate the findings of Walker (1988) in terms of the greater Western Cape area regarding the presence of psychrotrophic coliforms in milk.

5.4 To investigate the extent to which bovine milk under refrigerated conditions aid in the resuscitation of sub-lethal heat-stressed organisms.

5.5 To investigate whether milk supports the resuscitation of sub-lethally stressed pathogens that may survive pasteurisation.

5.6 To establish the effect of the individual and combined isolates (i.e. *Acinetobacter species, Pseudomonas flourescence, Pseudomonas putida, Sphingomonas paucimobilis, Flavimonas oryzihabitans, Cryseomonas luteola, and Hafnia alvei*) in terms of milk spoilage.

REFERENCES

Anonymous, (1985a). Regulation No. 258 of The Foodstuffs Cosmetics and Disinfectants Act (Act 54 of 1972).

Anonymous, (1985b). Enumeration of coliforms - colony count technique and most probable number technique at 30°C. International Dairy Federation Standard; 73 A.

Anonymous, (1991). Milk: Estimation of numbers of Psychrotrophic micro-organisms; Rapid colony count technique, 25 hours at 21°C. International Dairy Federation Standard 132A.

Anonymous, (1992). API 20E Analytical Profile Index for Enterbacteriaceae.

Asperger, H. & Brandl, E. (1978). The coliform count as a microbiological criterion for refrigerated market milk. International-Dairy-Congress-[XX-Symposium] Food Science and Technology Abstracts (1978) 10 (10) pp. 1408.

Frazier, W. C. & Westhoff, D. C. (1988). Food Microbiology: Fourth Edition, USA, McGraw-Hill, Inc.

Harrigan, W. F & McCance, M. E. (1986). Laboratory methods in Food and Dairy Microbiology: Academic Press Inc. (London) LTD.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). Bergey's Manual of Determinative Bacteriology: Ninth Edition, USA, Williams & Wilkens.

Jay, J. M. (1996). Modern Food Microbiology: Fifth Edition, USA, International Thomson Publishing: pp. 390-405.

Krieg, N. R. and Holt, J. G. (1984). Bergey's Manual of Systematic Bacteriology: Volume 1, USA, Williams & Wilkens.

Ledford, R. A., Senyk, G. F., Shipe, W. F., Kotsides, E. & Wolff, E. T. (1983). Influence of growth of coliforms on flavour acceptability of commercially processed milk samples. Journal of Dairy Science, 66 (8): pp. 1611-1615.

Lüch, H. (1985). Are the quality specifications laid down in Regulation No. 258 of February 1985 achievable?. South African Journal of Dairy Technology, 17 (4): pp. 125-142.

Richardson, G. H. (1985). Standard Methods for the examination of dairy products: Fifteenth Edition, USA, American Public Health Association.

Walker, S. J. (1988). Major spoilage organisms in milk and dairy products. Journal of the Society of Dairy Technology, 41(4): pp. 91-92.

APPENDIX

Violet Red Bile Agar (Biolab): Merck catalogue number code C 23: Batch number: 1005169

Ringers tablets: Catalogue number HC 015525.001: Batch number: TP 321325-816; Expiry date 30-04-2003.

Nutrient Agar (Biolab): Merck catalogue number code C1: Batch number: 1008765.

API 20 E identification system for Enterobacteriaceae test kits: Lot number: 726449601: Expiry date: 15 July 2000.

Memmert waterbath: Model: WB 350 T

Hussmann display fridge: Model: GDC 41/2

Memmert incubator: Model: BE 500

Mettler balance: Model: PC 4400 Delta Range

Suntex colony counter: Model: 560

Eastern autoclave: Model: EA 630 Vertical Type

Addis 25 L cooler box.

Gilson variable volume Pipetman: Item ID: D-83-10914

Eppendorf Varispenser Plus Variable volume: 1 to 10 ml

Vortex Genie 2 Orbital vortex mixer.

Detailed reaction results of the API 20E tests for organisms listed in Table 5.

Test	Reaction result	Score	Index	Test	Reaction result	Score	Index	Test	Reaction result	Score	Index
ONPG	+	1		**ONPG**	+	1		**ONPG**	-	0	
ADH	-	0	5	**ADH**	-	0	1	**ADH**	-	0	0
LDC	+	4		**LDC**	-	0		**LDC**	-	0	
ODC	+	1		**ODC**	-	0		**ODC**	-	0	
CIT	-	0	1	**CIT**	-	0	0	**CIT**	-	0	0
H_2S	-	0		**H_2S**	-	0		**H_2S**	-	0	
URE	-	0		**URE**	-	0		**URE**	-	0	
TDA	-	0	0	**TDA**	-	0	0	**TDA**	-	0	0
IND	-	0		**IND**	-	0		**IND**	-	0	
VP	-	0		**VP**	-	0		**VP**	+	1	
GEL	-	0	0	**GEL**	-	0	4	**GEL**	+	2	7
GLU	-	0		**GLU**	+	4		**GLU**	+	4	
MAN	+	1		**MAN**	+	1		**MAN**	+	1	
INO	-	0	1	**INO**	-	0	1	**INO**	-	0	1
SOR	-	0		**SOR**	-	0		**SOR**	-	0	
RHA	+	1		**RHA**	+	1		**RHA**	+	1	
SAC	-	0	1	**SAC**	+	2	7	**SAC**	+	2	7
MEL	-	0		**MEL**	+	4		**MEL**	+	4	
AMY	-	0		**AMY**	+	1		**AMY**	+	1	
ARA	+	2	2	**ARA**	+	2	3	**ARA**	+	2	3
OX	-	0		**OX**	-	0		**OX**	-	0	

oooOOOooo