STUDIES ON THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF THE LACTOPEROXIDASE SYSTEM IN BOVINE MILK

BY

ABEDNEGO MFANUFIKILE DLAMINI

B.SC.(AGRIC - ED) UNIVERSITY OF SWAZILAND

SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE

OF THE UNIVERSITY OF GLASGOW

SCOTLAND

DEPARTMENT OF NUTRITION AND MICROBIOLOGY

SCOTTISH AGRICULTURAL COLLEGE

AUCHINCRUIVE

AYR

KA6 5HW

SCOTLAND, THE UNITED KINGDOM

SEPTEMBER, 1990

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DEDICATION

DEDICATED TO MY WIFE,

LANDILE

AND CHILDREN,

BUNYE AND NOMVUZO

FOR THEIR LOVE AND PERSEVERANCE

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to his supervisor, Dr J. Bruce, for his methodical directions, support, helpful comments and personal association. Also to Mr J, Weir, for helping with the biochemistry component of the project.

Profound gratitudes are extended to Ms E. Drysdale, Messrs S. Crawford and A. Gillespie for their personal participation in training the author on microbiology bench skills. The co - operation from Mrs M. Andrews and Mr P. Simpson is highly appreciated. Thanks are due to Mrs M. Patterson and Mr T.D Potter for their expertise on the analysis of lactoperoxidase and thiocyanate.

The Author is very thankful to the Computer Dept. staff, Messrs D. Arnot, G. Wallace, and A. McEwan, for helping with the Statistical Analysis and the printing of the of the thesis.

The author is much indebted to Dr R.C. Noble (Head of the Department) for granting permission for the study to conducted in the department, and to Dr D. West his kind guidance counselling and co - ordination of the Msc. program. Extended thanks are due to Dr H. Duncan of the Department of Agricultural Ghemistry, Glasgow University, for his generous counselling and encouragement.

Grateful acknowledgement is made to the Government of the Kingdom of Swaziland, and the British council, who sponsored the project.

Appreciation is extended to the colleagues of the author, Stephen Dunne, Aziz Taib, and Salome Mutayobe, for their support and encouragement.

Special gratitudes are due to the author's parents, brothers, sisters, family, relatives and friends, for their material and spiritual support and encouragement.

Finally to his wife and children, the author wishes to express his love.

PREFACE

THE ENVY OF THE ARK[1]

Is there a horse in England now
That would not rather be a cow?
Or a bleating sheep who, given the choice
Refuse a cow's more noble voice ?
Pigs would renounce their dirt and greed
To be a wholesome bovine breed.
Cows never whimper, whine or bark;
They were the envy of the ark:
They stand and chew and think and sigh-
To see the ambitious world rush by.
But even cows, yes even they,
Hope to improve themselves one day.
The common cow of black and white
Longs to run into, overnight,
The cream of cows, the cow supreme
The cow of every milkers dream,
The placid, boteous and , I vow,
The more than human Jersey cow.

[1]Stocker, E. The Jersey cattle society of the United Kingdom

ABSTRACT

STUDIES ON THE LACTOPEROXIDASE SYSTEM IN BOVINE MILK

Milk can be a vehicle of spoilage, food poisoning and pathogenic bacteria. While sanitation and refrigeration of the milk may reduce the proliferation of bacteria, in developing countries, due to economic or other reasons, refrigeration may not be possible. The Lactoperoxidase System (LPS) is currently an approved method for preserving milk.

In this study the effect of temperature on the activity of the LPS against specific bacteria found in milk was investigated. Fresh cultures of *Escherichia coli, Listeria monocytogenes, Salmonella enteritidis*, and *Yersinia enterocolitica*, diluted to give 10^4 cfu /ml were inoculated into samples of raw , and pasteurised / sterilised (p/s) milk mixture. The LPS was activated by adding 14 mg/l NaSCN and 30 mg/l $2Na_2CO_3.3H_2O_2$. Treatments were done at 7^oC, 15^oC, 22^oC, 25^oC, and 30^oC. Counts were done using a spiral plater on yeastrel milk agar and on appropriate selective agars.

The LPS was found to be bactericidal against *E.coli, S.enteritidis* and *Y.enterocolitica*, but was bacteriostatic against *L.monocytogenes*. *Yersinia* was the most susceptible to the bactericidal activity of the LPS, while, *Salmonella* was the least susceptible. In *E.coli* and *Yersinia* it was possible to observe D-values at all the levels of treatment, while in *S.enteritidis* treatments, counts were reduced by at least a log cycle in treatments done at 7^oC only. It was felt that this

apparent resistance of *S.enteritidis* to the LPS activity merits further investigation.

Temperature affected the persistency of the antibacterial activity of the LPS as well as the death rates of the bacteria. The persistency of the system was inversely related to temperature , whereas , the death rates of the bacteria were positively correlated to temperature. The recovery of bacterial cells from inhibition by the LPS was more rapid at higher temperatures. The differences between counts from selective and non selective agars in p/s milks, indicated that the LPS may result in injury of some bacterial cells, which may be responsible for the rapid recovery of the bacteria. However, it was suggested that this area requires further elucidation.

The antibacterial activity of the LPS was also affected by the milk media. At higher temperatures, the activity of the system persisted for a longer time in p/s than in raw milk samples. This could be attributed to the influence of background microflora present in the raw milk, or to the differences in chemical environments of raw and p/s milks e.g. there is less catalase in p/s than in raw milk.

In view of the improved activity of the LPS in p/s milks, it was suggested that a combination of thermization and activation of the LPS is worth consideration and that this possibility needs further study.

CONTENTS

1 CHAPTER ONE

1.1 INTRODUCTION.

The importance of the antimicrobial activity of the lactoperoxidase system can not be overemphasised. This system has been activated to control dental plaque (Alley *et al*, 1984; Carlson *et al*, 1983; Hoogendoorn, 1976; Tenovuo, 1978; Tenovuo *et al*, 1985). The system has also been found to be essential in the control of mastitis organisms in the udder (Marshall *et al*, 1986). There is evidence that the system works *in vivo* to control enterogenic pathogens (Reiter *et al*, 1981). It can also be activated in milk replacers to promote calf growth (Mullan *et al*, 1980; Reiter *et al*, 1981). Several experiments have demonstrated that the system can be employed as a temporary milk preservative (Bjorck, 1978; Chakraborty *et al*, 1986; Harnalv & Kandasamy, 1982; Kamau & Kroger, 1984; Korhonen, 1980; Martinez *et al*,1988; Thaker & Dave , 1986; Zajac *et al*, 1983).

A considerable amount of research has been done on the the lactoperoxidase system. Most of these studies were concerned with:

- the mode of action of the system and
- the identification of the microorganisms inhibited by the system.

Comparatively little has been published about the factors affecting the activity of the lactoperoxidase system (Tenovuo & Knuuttila, 1976; Thomas & Aune, 1978). In addition, although there is evidence that the effectiveness of the system when used as a milk preservative is greatly influenced by the milk storage

temperature (Reiter & Hanulv, 1982 ;Thaker & Dave, 1986), little has been reported on the effect of temperature on the survival / death rate of specific organisms exposed to the the system.

The purpose of the present study was to investigate the effect of temperature on the antibacterial activity of the lactoperoxidase system (LPS) on specific organisms. Such information will be beneficial particularly because the system is now approved by IDF (1988) for use as a temporary milk preservative. IDF specified that the LPS system should be used: in countries where there is not enough power for cooling the milk; under emergency cases such as when there has been electricity failures ; and when there is very little milk produced to justify use of electrical power.

1.2 MILK PRESERVATION

1.2.1 General

Milk preservation is concerned with the use of physical or chemical methods which kill or retard the growth of those microorganisms which are undesirable in the milk. These are the spoilage and pathogenic organisms (Singleton and Sainsburry, 1978). The latter group of organisms is of greatest concern because of the health hazards they pose, while the former group is of great concern from an economic point of view, because the spoilage they cause results in a reduced shelf life of the milk or its products, or in an unacceptable milk product. Examples of such spoilages include: the development of off-flavours such as fruity, rancid, bitter & unclean milk; discoloration, slime formation and coagulation. If the undesirable microbes are not

allowed to proliferate, the good quality of the milk is retained. Several practices can be employed to inhibit undesirable microbial growth.

1.2.2 Sanitation.

This is concerned with all the precautions taken to prevent microbial contamination of the milk during milking, storage and transportation. Even if the initial level of bacterial contamination can be kept at very low levels, storage of the milk at ambient temperature allows these organisms to multiply and reach an unacceptable level, particularly in warmer countries. Therefore it is imperative that sanitation needs to be supplemented with other preservative measures where refrigeration is not available.

1.2.3 Fermentation

This is the most primitive method of preventing the multiplication of undesirable bacteria in the milk. It was practised by the natives of Southern Africa, the Bantus. Here, milk sours very rapidly after milking due to the high ambient temperatures, and the presence of microorganisms in the milk which may have originated from the cow, the hands of the milker or the milking utensils. Two types of fermentation can be produced. Firstly is the undesirable fermentation which is brought about by the non-lactic-acid bacteria. The product of this fermentation is stale or insipid when consumed. Secondly is the lactic acid bacteria fermentation, which gives a more desirable product.

The traditional container for fermenting milk is the calabash. If milk is left undisturbed in this container, coagulation occurs as a result of the lactic acid bacteria activity. The coagulum is formed on the top while the whey settles on the bottom. The whey is emitted out though a special vent on the base of the calabash. This results in a concentrated, good tasting product, which has a longer shelf life, remaining in the calabash. Unfortunately the product of fermentation can not undergo further processing such as heat treatment, hence there is no guarantee that pathogenic bacteria are killed.

1.2.4 Cooling

This is the conventional method for controlling milk quality at farm level. Better refrigeration methods for raw milk storage at the farm, and at the processing plant have been developed. This has resulted in a change on the type of organisms that predominate in raw milk. Instead of the lactic acid bacteria that dominate in unrefrigerated milk, non acid producing and cold tolerant bacteria, or psychrotrophic bacteria predominate.

Although some strains of these bacteria can survive pasteurisation, thus causing deterioration of the pasteurised product (Cousin, 1982 ; Johnston & Bruce, 1982), most of these organisms are killed by pasteurisation. However, even this group which does not survive pasteurisation can still cause deterioration of the pasteurised product by action of heat resistant lipases and proteases which they produce on prolonged storage of raw milk. These enzymes degrade milk fat to produce

rancid defects, and milk proteins to produce a bitter product, consequently even in the refrigerator milk can not be stored for prolonged periods without incurring some defects, unless other methods of preservation are employed.

1.2.5 Bacterial Inhibition by Carbon Dioxide.

Psychrotrophic bacteria are strictly aerobic organisms. It has been established that if carbon dioxide is sparged into the milk the amount of oxygen will diminish. This can result in growth inhibition of the bacteria (King & Mabbit, 1982). However this method has received very little acceptance from dairy farmers.

1.2.6 On the Farm Heat Treatment.

Use of this method as a means for controlling psychrotrophic organisms has been suggested by Zall and Chen (1981). Raw milk at the farm is exposed to heat treatment prior to refrigerated storage. However this method may still be not feasible for use in countries where there is scarcity of heating power.

1.2.7 Use of Chemical Preservatives.

Chemical preservatives are used in milk under emergency cases, where milk quantities are too small for application of expensive cooling systems, and where there is not enough energy to operate these systems. Previously, the only chemical preservative approved by FAO was hydrogen peroxide (FAO 1957). This chemical provided temporary preservation because a number of organisms could survive exposure to the chemical. About 0.3% to 0.8% of the chemical could be used to extend the keeping quality of the milk for about 5 hours. This system is inactivated by addition of high catalase or heavy metals. It is more

effective at increased temperatures than at low temperatures, against lactic acid bacteria than against coliforms, and against anaerobic than aerobic bacteria. The hydrogen peroxide system has not gained much popularity in milk preservation because of the following limitations (Korhonen, 1980):

- it results in changes on biochemical activity and chemical composition of the milk which adversely affect its processing properties;
- residues of hydrogen peroxide on milk poses health hazards to consumers. The residual effect is due to the high application rate of the chemical required for preservation, about 300 to 800 ppm.

As a result of these disadvantages of the hydrogen peroxide system, attention has been focused in recent years on the indigenous occurring antimicrobial systems in milk. The objective had been to find out if these could be exploited in the preservation of raw milk.

The natural occurring antimicrobial systems can be divided into two broad groups.

A. Those systems with a specific form of inhibition:
   - -immunoglobulins
   - -cellular immunity
   - -complement mediated bactericidal activity.

B. Those systems with a non specific form of inhibition:
   - -colonisation of gastro-intestinal tract
   - -lysozomes
   - -lactoferrins

-properdin

-conglutinins

-vitamin-protein binder for B_{12} & folate

-the lactoperoxidase/ thiocyanate/ hydrogen peroxide system.

A detailed review of these natural occurring antimicrobial systems was done by Reiter (1978). In the present study, attention is focused on the antimicrobial effect of the lactoperoxidase - thiocyanate - hydrogen peroxide system (LPS) in bovine milk. This is important because the LPS is now recommended by IDF to be used as a milk preservative where refrigeration of milk is not feasible. It is hoped that the findings from this study will contribute to the accumulating knowledge about the antimicrobial activity of the LPS. Results from this study will contribute to a better understanding of the activity of the LPS under different temperature levels.

1.3 REVIEW OF THE LITERATURE

1.3.1 The Lactoperoxidase System

During the past two decades, basic research had been concentrating on how the lactoperoxidase - thiocyanate - hydrogen peroxide system can be used as a milk preservative. This system is in short known as "the lactoperoxidase system" (LPS). There are three components of the system, namely: lactoperoxidase(LPO), the catalyst; thiocyanate(SCN-),the ion donor; hydrogen peroxide (H_2O_2), the oxygen donor.

The LPS does not directly interfere with the growth of microorganisms but LPO, which is a naturally occurring enzyme in bovine milk, catalyses the oxidation of SCN- by H_2O_2 to produce antimicrobial substances, mainly hypothiocyanate ions (OSCN-).

1.3.2 Historical Background

Although the natural antibacterial potential of raw milk had been known ever since the late 19th century (Hesse, 1894), the importance of this antimicrobial potential in improving the keeping quality of raw milk, had not been given much attention until the past few decades (Korhonen,1973; 1977; 1980; Reiter, 1976; 1978; 1979).

The first person to suggest the involvement of a peroxidase in the antibacterial activity of milk was Hansen in 1924. However what this writer postulated was never confirmed until 1958, when Wright and Tramer (1958) found that the peroxidase was lactoperoxidase. Auclair (1959) confirmed the involvement of lactoperoxidase in the antimicrobial activity of milk. Three years later, Jago and Morison (1962), discovered that another component, hydrogen peroxide, was required for the system. A year later, Reiter et al (1963) established that lactoperoxidase was just catalysing the oxidation of hydrogen peroxide with another compound, which was identified as thiocyanate.

It is now well established that lactoperoxidase, thiocyanate and hydrogen peroxide are the three components of the lactoperoxidase system. It is also known that in bovine milk lactoperoxidase is the most abundant of the three components, thiocyanate is only present in trace amounts, while hydrogen

peroxide is not present in measurable amounts. This means that the LPS in bovine milk can be activated by the addition of minute quantities of SCN-, and an equimolar amount of hydrogen peroxide (about 12ppm and 8ppm respectively).

1.3.3 Components of the LPS.

1.3.3.1 Lactoperoxidase (LPO).

This is a peroxidase that is found in secretions of the exocrine gland such as saliva and milk (Wever *et al*, 1982). A peroxidase is defined by Webb *et al* (1974), as a compound which catalyses the transfer of oxygen from peroxides, especially hydrogen peroxide, to other substances. Reiter and Hanulv (1984), concurred with this definition and further stated that the peroxidases catalyse reduction of H_2O_2 by a variety of electron donors. In the case of the LPS, the electron donor is SCN-.

The presence of a peroxidase in milk was first detected by Anorld in 1881. It is now known that the main peroxidase of bovine milk is lactoperoxidase. It represents about 1% of the total whey proteins. LPO concentration in bovine milk may range from nil to about 50mg/litre, with an average of about 30mg/litre (Korhonen, 1980; Fox, 1982). The enzyme has been isolated and purified from bovine milk by several methods (Gothefers & Markland, 1975; Kiemerer & Kuhlmanu, 1972; Groves, 1971). This has made it possible for the chemical composition of this enzyme to be understood.

Fox (1982), described LPO as consisting of a glycoprotein to which a haemin is attached, hence the enzyme is called a haem protein. The iron content had been estimated by Webb *et al*,

(1974) to be 0.07%. The enzymatic activity is about 22 000 IU in milk, and 29 200 in colostrum. Sievers (1981) identified the presence of a prosthetic group in this enzyme as protohaeme and estimated the carbohydrate content to be 8% to 10%. This author also suggested that the enzyme consisted of a single polypeptide chain.

Sievers et al (1983) stated that the haemoprotein had a molecular weight of 78 500; that it contains a single molecule of protophyrin IX and a single polypeptide chain; and that the haem moeity is protohaem. The proximal ligand was suggested to be histidine, which in nature, was established as an imidazole. This is the *n*- ligand supplied by the protein side chain. The sixth ligand was established as the *O*-ligand carboxylate ion. Although at low temperatures imidazole ion derived from a distal histidine is most likely to be the sixth ligand. However the authors could not rule out the possibility of an amine, or thioether or the *s*-ligand, to be the proximal ligand. While the sixth axial ligand was confirmed as a carboxylate group. When modification of a carboxylate group located at the active site of the enzyme is done, the enzyme is inactivated.

LPO can exist in a solid state, or in a liquid state. Tenovuo and Kurkijarviv (1981) compared the enzymatic properties of these two forms of LPO. The immobilised LPO was found to be more stable than soluble LPO during storage at $4^{o}C$ and during heat inactivation at $60^{o}C$. When inactivated at $25^{o}C$ to $50^{o}C$, the inactivation energy was lower for immobilised LPO. However no difference was found in optimum pH of the two forms of LPO,

although solid LPO was found to be more resistant to external factors .

Although the optimum pH for LPO is 5, the enzyme can tolerate lower pH levels *in vitro*, as low as pH 3 (Wright & Tramer, 1958; Reiter, 1984). Bovine milk LPO is more heat stable than other whey enzymes (Figure 1). Lactoperoxidase retains its normal activity in normal pasteurisation ($63^{o}C$) for 30 minutes or($72^{o}C$) for 15 seconds. It seems there is not agreement among scholars about the minimum temperature for inactivation of the enzyme. Korhonen (1980) suggested $80^{o}C$ for 2.5 seconds, while Berg (1988) suggested $86^{o}C$ for 20 seconds.

Since raw bovine milk contains about 30mg LPO per litre of milk, and only 1 to 2 mg/L LPO is required to activate the LPS (Reiter, 1979; Bjorck, 1982; Korhonen, 1980), it is apparent that the enzyme is not a limiting factor for the activation of the LPS.

1.3.3.2 Thiocyanate (SCN-)

Thiocyanate is a goitrogenic chemical substance that is widely distributed in animal secretions. It can be found in saliva, urine, plasma, gastric juices, tears and milk (Reiter, 1984). It has an acute toxic effect if ingested in very high concentrations. Anderson and Chen (1940) reported that the LD_{50} of orally administered sodium thiocyanate was 764mg/kg. SCN- is also known to be a normal electrolyte in mammals blood (IDF,1988).

SOURCE: Jenness and Patton, (1959)

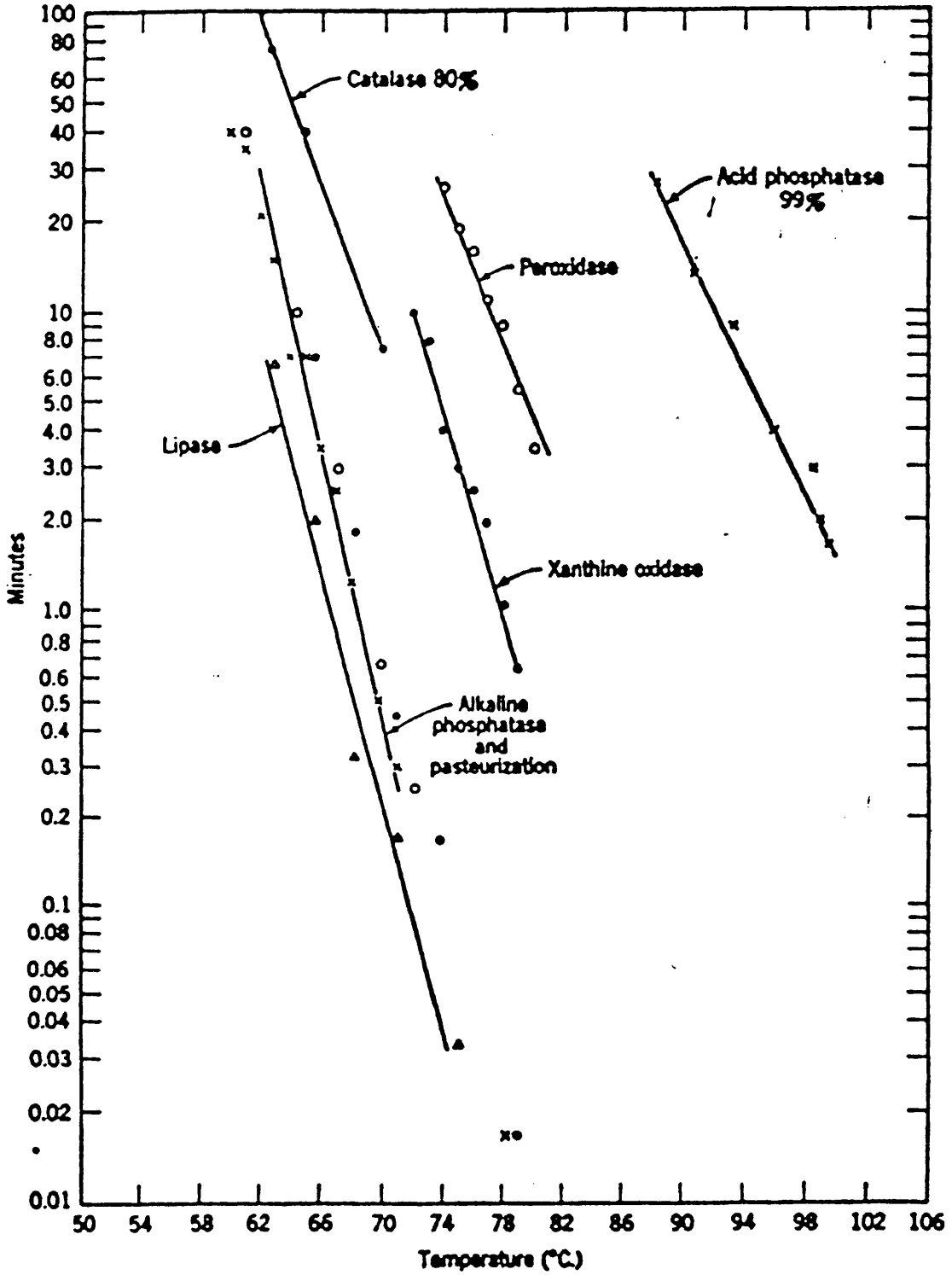


Fig. 1. Time-temperature relationships for inactivation of milk enzymes.

The levels of SCN- in bovine milk varies. IDF (I988) reported that normally the range is from 2-7 ppm, which is not in agreement with Lawrance, (1970). The latter reported a higher range of 10-15 ppm. The SCN- that is in milk is derived from the blood where it is normally 10 times higher than in the milk.

In ruminants the SCN- comes from three main sources: metabolism of S- containing amino acids; detoxification of glucosinolates; and detoxification of cyanogenic glucosides (Korhonen, 1982; Reiter, 1984; Virtanen, 1961; Wood, 1975). The last two sources constitute the dietary source of SCN-.

1.3.3.3 Glucosinolates.

These are chemical compounds found in vegetables belonging to the genus *Brassica*. All brassicas contain glucosinolates in their leaves and seeds. There are over 100 types of glucosinolates known, differing in the structure of the R-group. Their basic structure is shown below

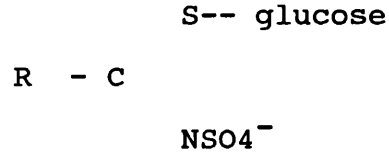


Table 1 shows the R- groups of the major glucosinolates found in rape seed (Bunting 1986).

TABLE 1

Trivial Name	**Semi-systemic Name**	**Structural Formula for R- group**
<u>The aliphatic glucosinolates:</u>		
Gluconapin	3-Butenyl-GSL	$CH_2 = CH - (CH)_2-$
Glucobrassicanapin	4-Pentenyl-GLS	$CH_2 = CH - (CH_2)_3-$
Progoitrin	2-Hydroxy-3--butenyl-GSL	$CH_2 = CH - CH - CH_2-$ OH
gluconapoleiferin	2-Hydroxy-4--pentenyl-GLS	$CH2 = CH - CH2 - CH - CH_2$ OH
<u>The aromatic glucosinolates:</u>		
Glucobrassicin	3-indolylmethyl-GSL	
Neo-glucobrassicin	N-methoxy-3-indolylmethyl-GSL	
	4-hydroxy-3-indolylmethyl-GSL	

Glucobrassicin breaks down completely to form thiocyanate ions. While the initial breakdown of progoitrin results in oxazolidinethione. Gluconapin and glucobrassicanapin breakdown results in stable isothiocyanates. Figure 2 show the steps in the breakdown of glucosinolates to goitrogenic products. The first step is always enzymatical removal of glucose, then the breakdown route becomes variable (Bunting, 1986).

1.3.3.4 Cyanogenic Glucosides

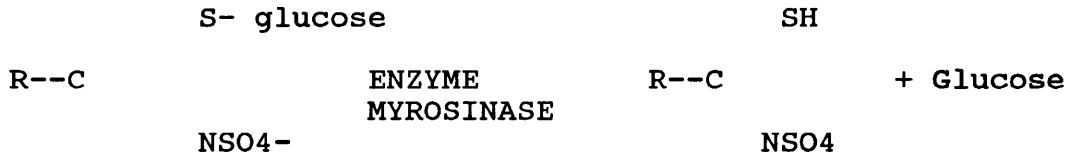
These chemical compounds can be found in a wide variety of crops: cereals - maize,millet, sugar cane; pulses - beans and peas; sweat potatoes and cassava; kernels of several fruits. On hydrolysis the glucosides from these plants release cyanide which on reaction with thiosulphate is detoxified and is converted to SCN-. This reaction is catalysed by rhodanase, an enzyme found in the liver, kidney or thyroid. An example of a cyanogenic glucoside is linamarin which can be found in cassava. The production of SCN from linamarin is shown in Figure 3.

Cyanide is converted to SCN- in the liver in the presence of methionine catalysed by rhodanase. In the initial step linamarin breakdown, the enzyme *B* glucosidase comes from the cassava.

To say that SCN- is goitrogenic means that if consumed in excessive amounts, about 200 - 400 ppm, it can cause goitre or can depress the hyperactivity of the thyroid gland in non ruminants (Reiter, 1985). The isothiocyanate and the oxazolidinethiones block the step of iodine addition to tyrosine during thyroid hormone synthesis, While thiocyanate prevents the thyroid hormone from taking up iodine. There is no evidence that

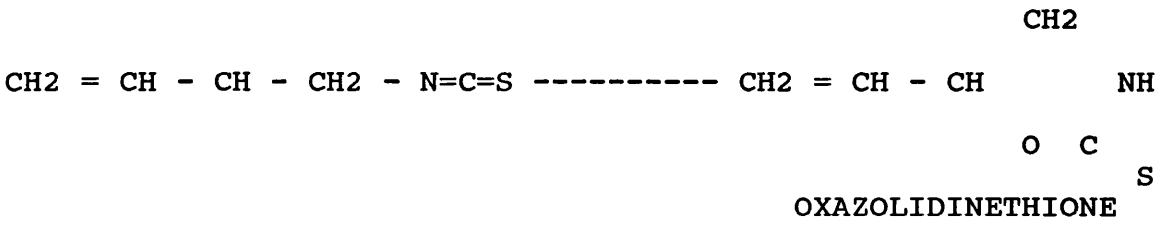
Figure 2 Breakdown route of glucosinolates to goitrogenic products.

1 Enzymic removal of glucose

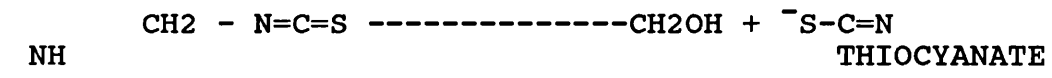


2.Rearrangement

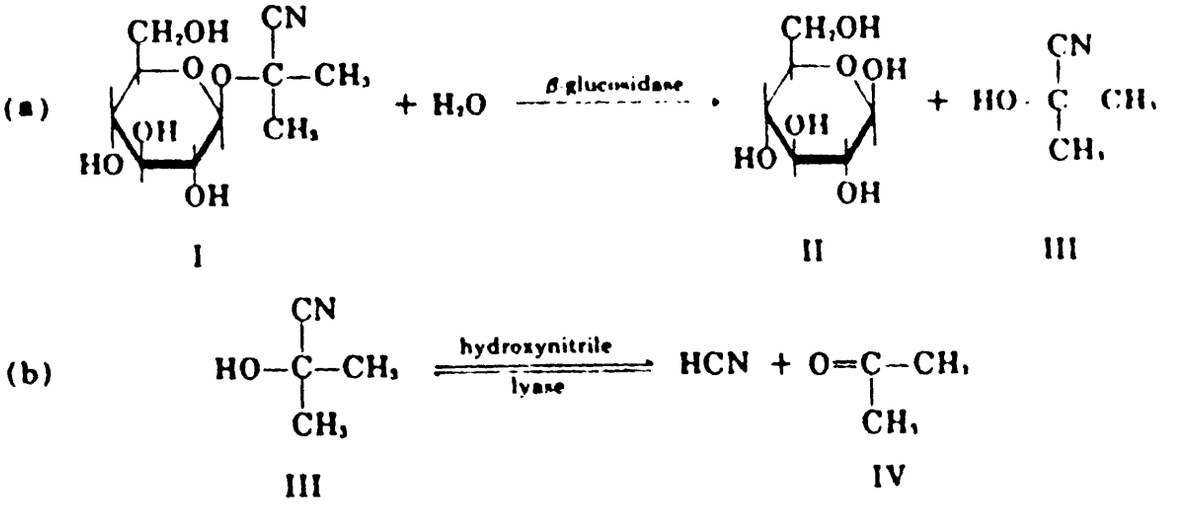
The isothiocyanate from Gluconapin and glucobrassicin are stable, but those from progoitrin cycles and form oxazolidinethione.



Isothiocyanates from Glucobrassicin breaks down to form free thiocyanate



SOURCE: Conn (1973)



The decomposition of linamarin by plant enzymes. In reaction (a), linamarin [2-(β-D-glucopyranosyloxy)isobutyronitrile] (I) is hydrolyzed by the β-glucosidase linamarase to form β-D-glucopyranose (II) and 2-hydroxyisobutyronitrile or acetone cyanohydrin (III). In reaction (b), the dissociation of the cyanohydrin to HCN and acetone (IV) is catalyzed by a hydroxynitrile lyase.

the SCN- found in milk can be responsible for thyroid problems. In an experiment where the level of milk SCN- was increased by about 8 ppm, Etten and Wolff (1973) found that there was a slight increase on the level of serum SCN-. However this increase was proportionate to an increase on SCN- excreted in the urine (Reiter, 1985).

The activation of the LPS is unlikely to cause goitre problems. This is particularly true because very little amount of SCN- (about 14mg of NaSCN per litre of milk) is recommended by IDF(1988). Yet a reduction of iodide uptake may occur when the dose is about 200 - 400 ppm. This level is equivalent to an intake of 10 - 20 litres per day of milk containing about 20 ppm SCN- (Reiter,1985; Van Etten and Wolff,1973).

1.3.3.5 Hydrogen Peroxide (H_2O_2)

This compound is formed from the oxidation of a reduced substance by dioxygen (O_2). This reaction occurs when an electron is transferred from the reduced substance to O_2, then superoxide (O_2-) is produced . O_2- undergoes dismutation to produce H_2O_2 (Thomas, 1985). The reaction can be shown in the following equation.

1. Electron transfer to dioxygen

   1 e- + O_2 -------- O_2- (super oxide)

2. Dismutation

   $2O_2$- + $2H_2$ --------- H_2O_2 + O_2

If two electrons are transferred from the reduced substance, hydrogen peroxide is formed directly, there is no dismutation.

2e + $2H^+$ + O_2 ---------- H_2O_2

It is possible for somatic cells to produce hydrogen peroxide. The interior of these cells is rich in reducing substances , and the extracellular environment contain O_2 and catalysts. The latter facilitate the transfer of electrons to O_2 so that H_2O_2 may be formed.

Some microorganisms classified as facultative anaerobes can produce large amounts of hydrogen peroxide and release it to the extracellular medium (Thomas, 1985). Organisms of the genera Streptococcus and Lactobacillus, the so called lactic acid bacteria, are found in this category. Thomas (1985) also gave the list of the various catalysts involved in the synthesis of hydrogen peroxide. These are:

Flavoprotein oxidases such as glucose oxidase, amino acid oxidases, metalloflavoprotein, xanthine oxidase, NADH, NADPH, FMN, FAD.

Copper containing oxidases such as non - hem iron proteins, non - hem proteins chelating Fe+++ or Fe++.

Haemoprotein peroxidases such horse radish peroxidase, catalase, myeloperoxidase and lactoperoxidase.

In milk the possible sources of hydrogen peroxide are polymorphonuclear leucoyctes, metabolic activity of lactic acid bacteria and somatic cells (Reiter, 1984). Although Reiter (1976) detected a very low concentration (2-4mg/ml) of hydrogen peroxide from aseptically canulated raw milk, the common trend is that in raw milk measurable amounts of the compound are not found. This is so because there is a continual reduction of hydrogen peroxide

by enzymes found in the milk, such as catalase and lactoperoxidase (IDF, 1988).

It is apparent that the amount of hydrogen peroxide in milk is a limiting factor for the lactoperoxidase system. In order to activate the lactoperoxidase system an equimolar amount of hydrogen peroxide to SCN- should be added in the milk. IDF (1988) recommended that 30mg of sodium percarbonate should be added per litre of milk, as a source of hydrogen peroxide.

For the activation of the lactoperoxidase system in milk all the three components of the system must be present in non limiting amounts. In bovine milk lactoperoxidase is always sufficient because only 1 - 2mg per litre is required. The two limiting factors are the quantities of hydrogen peroxide and thiocyanate. Increasing the concentration of SCN- to about 10 to 15 ppm followed by an equivalent increase on concentration of hydrogen peroxide on molar bases, is sufficient to activate the LPS (Korhonen, 1982).

1.3.4 Biochemical Activity of the Lactoperoxidase System

In the LPS, lactoperoxidase catalyses the oxidation of thiocyanate by hydrogen peroxide. The nature of the antibacterial product of the oxidation is not yet fully understood.

Reiter *et. al* (1964), suggested that thiocyanogen $(SCN)_2$ was the inhibitory compound produced as a short lived intermediary product of oxidation of SCN- by H_2O_2 . Later in 1966, Oram and Reiter suggested that sulphur dicyanide $S(CN)_2$, could produce the inhibitor for inactivation of hexokinase when the LPS is

activated. However they still felt that $S(CN)_2$ was not the inhibitor produced by the LPS.

When the bacterial cells and the LPS were separated by a semi-permeable membrane no inhibition could be observed. This resulted in Steel and Morrison (1969), suggesting that a complex binding between the cells and the system was required for inhibition. However, Hoogendoorn *et al* (1977), suggested that the lack of inhibition in the above experiment was due to an extremely low stability of the inhibitor compound.

Hogg and Jago (1970), found that even when lactoperoxidase was removed from the inhibitory mixture, the inhibitory properties were not completely lost, but were reduced. They also found that solutions could be stored for several days without loss of their inhibitory activities. Basing their conclusion on the amount of hydrogen peroxide consumed per mole of SCN-, these authors suggested that the inhibitor could either be cyanosulphurous acid (HO_2SCN) or cyanosulphuric acid (HO_3SCN).

Four years later, Hoogendoorn (1974) found that the inhibitor could not only inhibit hexokinase but also glycolytic enzymes containing essential thiol groups. With this knowledge, Hoogendoorn *et al* (1977) concluded that the inhibitor was a hypothiocyanous acid. They based their conclusion on the observation that SCN- can be estimated with a standard technique even in mixtures of $LPO/SCN/H_2O_2$. The capacity of such mixtures to oxidise thiol groups can be measured by the increase of SCN- after reduction by glutathione. This analytical technique made it possible for the inhibitor to be identified as hypothiocyanate

(OSCN-).This compound is more stable than the short lived products , HO_2SCN and HO_3SCN. This is in agreement with Hogg and Jago (1970) who found that the inhibitor formed by the LPS could be stored for several days. Hoogendoorn and his colleagues reaffirmed their findings by using exogenously prepared OSCN on *Streptococcus mutans*. The results of inhibition from this treatment were comparable to the results obtained when the LPS is activated.

Thomas and Aune (1977), concurred with Hoogendoorn and his colleagues on that OSCN- was the inhibitor produced by the LPS. They also found that besides OSCN- the oxidation of SCN- may yield $(SCN)_2$ which on hydrolysis produces hypothiocyanous acid. Basing their evidence on an observation that when *Escherichia coli* was exposed to preformed OSCN- or directly to the LPS similar responses were observed, Thomas and Aune concluded that it was not necessary to propose formation of short lived intermediates of the oxidation, to account for the antimicrobial action.

In 1980, Bjorck and Claesson suggested that the antimicrobial effect of the LPS did not come from the inhibitor OSCN- alone. They based their conclusion on some evidence that the bactericidal effect of the LPS was more stable than the non-enzymaticaly prepared OSCN- ions and the latter was inhibitory only to *Escherichia coli*. However it should be noted that Hoogendoorn et al (1977) used the non-enzymaticaly prepared OSCN- ions against *Streptococcus mutans*. Other reasons which made Bjorck and Claesson (1980) conclude that OSCN- was not

the only inhibitor produced by the LPS were:

- non enzymaticaly prepared OSCN- at concentration equal to enzymatic oxidation of SCN- did not give rise to a reduction in viable counts as the LPS;

- that the bactericidal effect of the LPS against *Escherichia coli* had a short duration (about 60 mins) despite the fact that the concentration of OSCN was still high in the medium (about 60 %).

As a result of the above observations the authors suggested that a more unstable agent causing irreversible inhibition was involved. This could possible be the higher oxyacids of thiocyanate, cyanosulfurous acids and cyanosulfuric acid . This is in agreement with the findings of Hogg and Jago, (1970), who suggested that the inhibitor could be cyanosulfurous acid since it is more stable than the latter.

When working to establish conditions under which OSCN- and HOSCN become bactericidal, Tenovuo (1984) found that at neutral pH HOSCN ions were the major products of the LPS that penetrated the cell wall of *Bacillus aureus* because these ions are uncharged.

From the preceding discussion it can be deduced that the inhibitor formed by the LPS might either be the major products of the system, OSCN, or the less stable intermediary products, HO_2SCN or HO_3SCN or both. The possibility of a complementary effect was suggested by Reiter(1984), who stated that pologràphic and kinetic studies suggested that the oxyacids may be formed

when hydrogen peroxide is present at higher than equimolar concentrations with SCN-. These higher oxyacids are better oxidising agents than OSCN-, and have a bactericidal effect against *E. coli* while the stable OSCN- is bacteriostatic. The products of LP--SCN--- oxidation are shown on following equations proposed by Thomas (1985).

$$H_2O_2 + 2SCN^- + 2H^+ \text{ ---------- } 2H_2O + (SCN)_2$$

$$(SCN)_2 + H_2O \text{ ---------- } HOSCN + H^+ + SCN^-$$

$$HOSCN \text{ ========== } H^+ + OSCN^-$$

The above equations show that the oxidation of SCN- can result in the formation of thiocyanogen, hypothiocyanous acid and hypothiocyanate ions. The production of the higher oxyacids is not shown. This is because these are the intermediates in the decomposition of thiocyanogen and hypothiocyanous acid. This can be shown in the following equations of Hogg and Jago (1970).

$$HOSCN + H_2O_2 \text{ ---------- } HO_2SCN + H_2O$$

$$HO_2SCN + H_2O \text{ ---------- } H_2SO_3 + HOCN$$

OR

$$HO_2SCN + H_2O_2 \text{ ---------- } HO_3SCN + H_2O$$

$$HO_3SCN + H_2O_2 \text{ ---------- } HOCN + H_2SO_4$$

As it can be seen from the above equations, the less stable oxyacids may be formed when there is an excess of hydrogen peroxide to oxidise the SCN-. However the short lived compounds may be less desirable than the more stable hypothiocyanate ions. This is true because the former have a broad reactivity, and can

be less effective as antimicrobial agents than the more stable compounds which react with a single class of essential biological materials (Thomas, 1985).

The factors that can affect the stability of OSCN- were discussed by Hoogendoorn *et al* (1977):

pH.

The decomposition of OSCN-is strongly dependent on the pH. As the pH of the solution is increased the percentage of OSCN- left after storage is increased with increase of storage time;

Light.

OSCN- ions were found to be sensitive to light, hence storage should be done on darkness or in diffused light;

Metal ions.

addition of ions such as Fe, Ni, Cu, Mn and compounds such as glycerol and ammonium sulphate decreased the stability of OSCN-.

Lactoperoxidase.

OSCN- stability was reduced when LPO was removed.

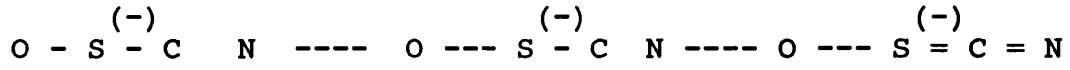
Concentration.

more concentrated solutions of OSCN- broke down more rapidly than less concentrated ones, even after the OSCN- had fallen to a concentration which is normally stable.

Heat.

OSCN- is remarkably stable to heat. 40 percentage OSCN- was recovered after heating at 100c for 30 minutes. although there is conflicting evidence to this (Hogg & Jago 1970).

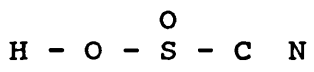
The stability of OSCN- is attributed to mesomeric effects. There are different resonance forms possible for the ionic forms (Hoogendoorn *et al* 1977):



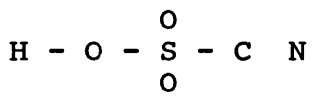
stable resonance forms for ionic forms

On the contrary, the higher oxyacids are devoid of the different resonance forms for ionic forms hence their instability (Hoogendoorn *et al* 1977; Hogg & Jago 1970).

cyanosulfurous acid



cyanosulfuric acid



1.3.5 The Antibacterial Action

1.3.5.1 Introduction

The key factor to the antibacterial action of the LPS is oxidation of sulfhydryl group of the bacterial enzymes (Reiter 1984; Aune and Thomas, 1978). The bacterial enzyme D- lactate dehydrogenase is also inhibited (Law & Johnson, 1981).

Marshall (1978) found that the primary effect of the LPS on *E. coli* was structural damage of the bacterial cytoplasmic membrane. This is followed by an immediate leakage of potassium ions, amino acids, and polypeptides. Then an inhibition of glucose uptake, purines, pyrimidines and amino acids follows. Synthesis of proteins, DNA and RNA are also inhibited. Damages on the structure of cytoplasmic membrane had also been reported on *Streptococci* (Marshall & Reiter 1980).

Before discussing the inhibition of various bacteria by the LPS , grouping of milk bacteria will be considered.

1.3.5.2 Classification of Milk Bacteria.

A simplified classification system of milk bacteria was originally founded by Bergy , and was later modified by Berg (1988). Although not all the bacteria found in milk are included in this classification, the major groups of bacteria that are important to the dairy industry are included (Table 2).

Most of the bacteria that are useful for milk processing are found on parts 14 & 16 , although even in other groups some bacteria that contribute to the processing of some dairy products can be found. e.g. *Propionbacterium*, which is useful in cheese production is found under part 17.

Classified under part 7, are the putrefactive bacteria and some gram-negative bacteria which are pathogenic to both man and ruminants, such as the *Brucella* species, while the pathogenic bacteria of milk are in general classified under part 8, and the spore forming bacteria are found on part 15. Animal and human

TABLE 2 Bacteria important for dairying.

Bergey's Manual	Family	Genus
Part 7. Gram-negative aerobic rods and cocci	I Pseudomonadaceae	*Pseudomonas*
	uncertain affilation	*Alcaligenes* *(Achromobacter)* *Acetobacter* *Brucella*
Part 8. Gram-negative facultatively anaerobic rods	I Enterobacteriaceae	*Escherichia* *Salmonella* *Klebsiella* *Enterobacter*
	II Vibrionaceae	*Aeromonas*
	uncertain affilation	*Flavobacterium* *Chromobacterium*
Part 14. Gram-positive cocci	I Micrococcaceae	*Micrococcus* *Staphylococcus*
	II Streptococcaceae	*Streptococcus* *Leuconostoc*
Part 15. Endospore-forming rods and cocci (gram-positive)	I Bacillaceae	*Bacillus* *Clostridium*
Part 16. Gram-positive non-sporeforming rod-shaped bacteria	I Lactobacillaceae	*Lactobacillus*
Part 17. Actinomycetes and related organisms (gram-positive)	Coryneform bacteria	*Corynebacterium* (human and animal parasites and pathogens) *Brevibacterium* *Microbacterium*
	I Propionibacteriaceae	*Propionibacterium*
	Order I I Actinomycetaceae II Mycobacteriaceae	*Actinomyces* 1. *Mycobacterium*

SOURCE: Berg, (1988)

pathogens of milk are found on part 17, for example the *Mycobacterium* pathogen for tuberculosis is found in this part.

From the above classification it can be seen that milk can contain:

the beneficial bacteria, which are those organisms that are required for milk processing, such as the lactic acid bacteria;

the spoilage bacteria, which are the organisms that causes deterioration of milk quality, such as *Escherichia coli* and *Pseudomonads* species;

the harmful bacteria, which include all the organisms that cause diseases to man and other animals. e.g. *Mycobacterium, Salmonella, Listeria and Yersinia*.

Another grouping of bacteria is a classification based on the sensitivity of the bacteria to temperature. (Berg, 1988 ; Barnwat, 1989). In this approach the bacteria are grouped as:

Psychrophiles- these are the bacteria which can grow under cold temperatures. They consist of two groups, the psychrophilic and the psychrotrophic bacteria. The former are the cold loving bacteria that grows within a temperature range of -10 to 20^{o}C, with an optimum temperature of 4 to 6^{o}C, while the latter are bacteria coming from various genera , but can tolerate low temperatures than 10^{o}C, although their optimum temperature might be higher.

Mesophiles- these bacteria have an optimum temperature of 25-35^{o}C

although their temperature for growth may range from 10°C to 45°C most of the beneficial bacteria bacteria to the dairy industry are found in this category.

Thermophiles and thermodurics- these are the bacteria that can survive at high temperatures. They consist of the thermophilic and the thermoduric bacteria. The former grows within the range of 30 to 60°C , with an optimum temperature of about 50°C. The latter refers to those bacteria that would survive normal pasteurisation. These organisms may have a lower optimum temperature , but producing heat resistant spores. However vegetative thermotolerants are also common.

1.3.5.3 Microorganisms Inhibited by the Lactoperoxidase System

The LPS has some antimicrobial properties against a wide range of micro-organisms. Belding *et. al* (1970), demonstrated that the lactoperoxidase - iodide - hydrogen peroxide system was able to kill an RNA (polio) and a DNA (vaccina) viruses. It is note worthy that these viruses are more resistant than many other viruses to the effect of drying, heating and disinfectants.

The LPS had also been found to have some mycoplasmacidal activity (Pruitt & Reiter,1985), and some fungicidal properties (Harmon & Klibanoff, 1973; Pruitt & Reiter, 1985).

However, what is of greatest concern for the purposes of this study is the antibacterial activity of the LPS.

1.3.5.4 Antibacterial Activity of the LPS.

1.3.5.4.1 Introduction

The LPS is bacteriostatic against most gram positive bacteria. This means that metabolic activities, hence growth of these bacteria are inhibited. Pruitt & Reiter (1985), stated that when these bacterial cells are exposed to the system, there is rapid inhibition of metabolic activities. This is followed by leakage of amino acids and inhibition of: potassium and carbohydrate transport and utilisation ; oxygen uptake ; amino acid and purine transport; production and excretion of extracellular products. The inhibition effect can be blocked by the presence of reducing compounds, and this may induce the recovery of the bacteria from inhibition by the LPS complex.

The activity of the LPS is bactericidal against most gram negative bacteria. This means that these organisms are killed when exposed to the system. This lethal effect is attributed to a more extensive damage to the inner membrane in these bacteria than in the gram positive organisms (Pruitt & Reiter, 1985).

The antibacterial effect of the LPS on various bacteria has been reported by Korhonen (1980). This is shown in Table 3.

1.3.5.4.2 Effect of the LPS on *Escherichia coli*.

E.coli is a species of chemoorganotrophic gram negative bacteria. They are facultative anaerobic rods of the family Enterobacteriaceae commonly found in the alimentary canal of warm blooded animals. The presence of the bacteria in milk may indicate faecal contamination. Some strains of *E. coli* can cause

3 Antibacterial activity of the LP/SCN^-/H_2O_2 system in milk or in a synthetic medium against different bacteria

terial species	Nature of effect	Reference
cultures		
ococcus cremoris	Bacteriostatic	Wright and Tramer (1958) Jago and Morrison (1962) Reiter, Pickering and Oram (1964)
nophilic lactobacilli	Bacteriostatic	Portmann, Gaté and Auclair (1962)
organisms		
ococcus agalactiae	Bacteriostatic	Reiter, Pickering and Oram (1964) Mickelson (1966)
ococcus uberis	Bacteriostatic	Reiter, Pickering and Oram (1964)
ylococcus aureus	Bactericidal	Reiter and Bramley (1975)
richia coli	Bactericidal	Reiter and Bramley (1975)
nic and enteropathogenic isms		
onella typhosa and *S. paphi*	Bactericidal	Hansen (1924)
onella typhimurium	Bactericidal	Reiter *et al.* (1976)
richia coli	Bactericidal	Reiter *et al.* (1976)
lomonas aeruginosa	Bactericidal	Reiter *et al.* (1976)
ococcus pyogenes	Bactericidal	Mickelson (1966)
oilage organisms		
lomonas fluorescens	Bactericidal	Björck *et al.* (1975)
-negative strains	Bactericidal	Björck *et al.* (1975)
milk flora	Bactericidal/ Bacteriostatic	Björck (1978)

SOURCE: Korhonen, (1980)

environmental mastitis. This occurs when toxins produced by these strains cause damage to the epithilial lining of the teat cistern. Enteropathogenic strain produces KA99 antigens which enable them to adhere on the intestinal lining of host animals. Here they produce toxins which causes dysentery, particularly in calves. (Blowey, 1988).

Data on the effect of the LPS on *E. coli* are ubiquitous. Marshall (1978), reported the bactericidal effect of LPS on several strains of *E. coli* that are known to be pathogenic to calves, lambs and piglets. The bactericidal action was enhanced at acidic pH. One of the first targets of the LPS in these bacteria is the inner membrane. This is followed by an immediate loss of potassium ions, and smaller molecule amino acids. The organism becomes unable to carry out energy transport processes, DNA, RNA and protein synthesis are inhibited and lysis becomes apparent within 2 - 3 hours of treatment.

There are many factors that can affect the activity of the LPS (Reiter *et al* 1976): the concentration of SCN- ; density of the inoculum; the strain of the *E. coli*. In the above study a concentration of 0.015mM SCN- was sufficient to cause a bactericidal effect when the inoculum was less than 10^7 cfu, which lasted for about 4 hours. When the inoculum was increased to greater than 10^7 no bactericidal effect was observed, until the concentration of the SCN- was increased to 0.225mM.

Other factors that can affect the susceptibility of the *E. coli* to the LPS include treatments that can alter the structure and permeability of the bacterial cell envelope. e.g. exposure

to: EDTA, lysozymes, and osmotic shock (Thomas & Aune 1978). The presence of sulfhydryl groups in the medium may inhibit the antimicrobial effect of the system (Klebanoff, 1967; Klebanoff, 1968; Mickelson, 1968). The failure of the inhibition in the presence of the -SH groups is attributed to the reason that hypothiocyanate reacts with bacterial -SH groups, therefore presence of these in the medium cause a competition between bacterial and medium -SH for hypothiocyanate ions.

Bjoerck & Claesson (1979) found that the bactericidal effect of the LPS lasted for a short period even though the concentration of the OSCN- was still high in the medium. (about 60% of initial concentration). A possible reason for this lack of inhibition suggested by Law & John (1980) is that some bacteria may fail to generate proton motive force across their membranes, which may prevent the inhibition of nutrient uptake in the LPS treated cells. There can also be a failure of D- lactate dependent uptake, which can be attributed to the rapid and irreversible inhibition of D- lactate dehydrogenase, which links this energy source to the proton translocating electron transport chain in the membrane.

In summary it can be stated that the literature shows that the LPS is bactericidal against strains of *E. coli*. This includes a wide range of serotype strains of human, bovine, and porcine origin. Included in this range are multiple antibiotic resistant strains (Reiter 1976). However there is not much information about the influence of temperature on the activity of the LPS against *E. coli*.

1.3.5.4.3 Effect of the LPS on Streptococcus Bacteria

Streptococcus is a genus of the family Micrococcaceae. These are gram positive chemooraganotrophic organisms. They can be either facultative or obligate anaerobic bacteria (Singleton & Sainsbury, 1978). Species of this genus are widely distributed in nature, and all are catalase negative. The common examples are: *S.agalactiae* - one of the main causal agents of bovine mastitis; *S.lactis* and *S.cremoris* - important agents in dairy products; *S.mutans* - responsible for dental plaque; *S.pneumonia* - a main causal agent for pneumonia; *S.salivaris* and *S.sanguis* - are responsible for production of dextrans and levans; *S.feacalis* which can be found in the intestines of both man and farm animals; lysogenic strains of *S.pyogens* - produces erythrogenic toxins.

As it can be deduced from the above discussion, many *Streptococcus* bacteria have undesirable effects, namely: acid production; plaque production; production of hydrogen peroxide, which can be toxic to mammalian cells; the production of mastitic milk. It would seem that it was against these or some of these effects that the antibacterial effect of the LPS against the *Streptococcus* bacteria was investigated by many researchers.

The LPS causes detoxification of H_2O_2 produced by oral streptococcus (Carlson *et. al*,1984).This occurs when the system causes blockage of glycolysis in the bacteria resulting in a reduction on acid production, oxygen production, hence H_2O_2 excretion. The target of the LPS inhibitor is 3-Phosphate dehydrogenase. The activity of hexokinase is also inhibited.

However some acid producing organisms have a high capacity to recover from inhibition. This is attributed to the low activity of NAD(P)H - OSCN oxyreductase in these bacteria. In other species like *S. sanguis* there is more acid inhibition even at lower level oxygen uptake. This is so because NADH oxidase and NADH - OSCN oxyreductase have higher affinity for NADH than lactate dehydrogenase (Figure 4).

Tenovuo and Knuttila (1977) observed that inhibition of *S. mutans* was achieved only when the cells were in contact with LPO. This suggest that in order for inhibition to occur, the enzyme must be adsorbed very rapidly into the bacterial cells.

The LPS can also inhibit the growth of mastitis organisms, thus reducing the contamination of milk and dairy products. The example of the organisms that can be inhibited are: *S. uberis*, *S. agalactiae* and *S. dysgalactiae*.

S.agalactiae is not completely inhibited by the LPS (Brown & Mickelson 1977). This is due to the presence of cysteine, high pH, high catalase, and high somatic cell counts. All these factors increase as the dry-off period progresses. *S.dysgalactiae* is more sensitive to the LPS than the other two species. But when the concentrations of cystine, cystein & glutathione are increased in the medium, the antibacterial effect is neutralised. *S.dysgalactiae* recovers quicker than the other two species. Such a difference in recovery can be attributed to the differences in capabilities of the organisms to restore cell sulfhydryl groups that were oxidised by the LPS (Mickelson & Brown, 1984).

SOURCE: Carlsson et al, (1983)

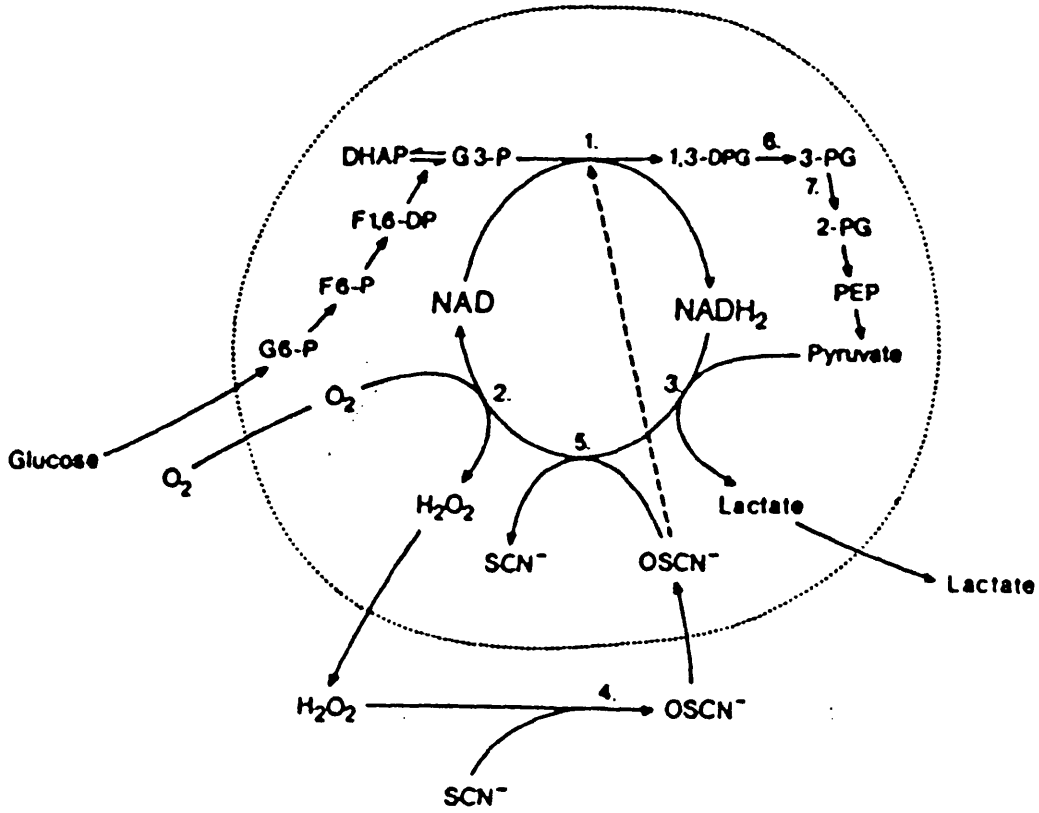


FIG. 4 Suggested scheme for regulation of glycolysis in *S. sanguis* ATCC 10556 in the presence of thiocyanate and lactoperoxidase. 1, glyceraldehyde 3-phosphate dehydrogenase; 2, NADH oxidase; 3, lactate dehydrogenase; 4, lactoperoxidase; 5, NADH-OSCN oxidoreductase; 6, phosphoglycerate kinase; 7, phosphoglyceromutase.

Marshall *et al* (1984), found that the LPS has a role in the defence of the mammary gland against *S. uberis*. However during the dry period, the activity of the LPS is reduced. This, is due to an increase of: cystine; dithiothreiol and sulphur containing proteins. All these tend to protect the bacteria against damage by the LPS.

The above discussion indicates that the LPS is bacteriostatic against most streptococcus bacteria. However there is evidence that the system can be bactericidal against members of this genus. Alley *et. al* (1984), reported a bactericidal effect of the system against *S. mutans*. These researchers observed that there was an increase on cell death when the concentration of OSCN was increased. Total inactivation of the bacterial cells was obtained with 2.5 mM SCN-, after 30 minutes. This was an irreversible bactericidal effect.

1.3.5.4.4 The Antibacterial Effect of the LPS Against Salmonellas

Salmonella is a genus of gram negative chemoorganotrophic asporogenous bacteria of the family Enterobacteriaceae (Singleton & Sainbury, 1978). Pathogenic strains of *Salmonella* causes the disease salmonellosis. Contamination of milk with *Salmonella* may come from man, water and infected animals (Berg 1988).

Data on the effect of the LPS on salmonellas are scarce. Wray & Mclaren (1987), tested the effect of the system against 24 strains of the species *S.typhimurium, S.dublin, S.bredeny, S.anatum* . The system was found to be bactericidal against all the strains tested. The magnitude of salmonella numbers decline was remarkable high in an acidified milk medium and in rough and

semi rough strains.

1.3.5.4.5 Effect of the LPS Against Listeria

The activity of the lactoperoxidase system so far has been tested against *Listeria monocytogenes*. These are rod shaped anaerobic bacteria, that are gram positive and non spore forming. Distribution of these bacteria is ubiquitous. They can can be found in the soil, vegetation, water and foods of animal origin.

L.monocytogenes can be pathogenic to both man and farm animals. In man known listeriosis problems include: septicemia ; meningitis and abortion. Death associated with food born listeriosis are not uncommon. There is evidence that milk (pasteurised) can also serve as a vehicle for pathogenic listeria resulting in death (Anonymous, 1988; Fleming *et al*, 1985; James *et. al*, 1985).

As regards the effect of the LPS on listeria species, there are conflicting reports. Earnshaw and Banks (1989) reported a non lethal effect of the system, but only a bacteriostatic effect. Later a bactericidal effect was reported by Denis and Ramet (1989). The possible reasons for these conflicting results could be the differences in time durations for these two experiments. While Earnshaw and Banks conducted their experiment for a shorter period, Denis and Ramet conducted their experiment for a longer period (1 to 30 days). During this period they observed that at the early stages the system was only growth inhibitive, and it was only after some days that it started to inactivate the cells completely . Probable Earnshaw and Banks did not carry out their

experiment long enough to observe a bactericidal effect. However the period of storage that enabled a complete inactivation of the listeria cells in the above experiment may not be practicable for storage of raw milk.

1.3.5.4.6 The Effect of the LPS on Other Species of Bacteria.

The lactoperoxidase system inhibited the growth of *Bacillus cereus*. A complete inhibition of these bacteria growth was achieved with 10 mM H_2O_2, and was associated with extracellular release of collagenase activity (Tenovuo *et al*, 1985). The antibacterial effect was improved when cells were grown aerobically, and when cells were treated during their growing phase rather than their resting phase.

Beumer *et al*, (1985) found that the LPS was bactericidal against *Campylobacter jejuni*. It is not clear whether the bacteria were inhibited by the LPS or by the enzyme LPO, because in the above experiment the organisms were inactivated in raw milk which was not supplemented by any source of SCN or H_2O_2

There is also evidence from the literature that the LPS has some antimicrobial effect against spoilage bacteria of the genus *Pseudomonas*. The bactericidal effect of the system had been confirmed on: *Pseudomonas fluorescens*, (Bjorck *et. al*, 1975); *Pseudomonas auruginosa* (Reiter *et. al* 1976). Bjorck (1978), demonstrated the antimicrobial effect of the system against a wide range of psychrotrophic bacteria. This indicates that the system can enhance yield of milk products such as cheese (Reiter & Harnulv, 1982).

1.3.5.5 Use of the LPS as a Milk Preservative

The Lactoperoxidase system had been used to preserve raw milk, pasteurised milk, and powdered milk.

Banks and Board (1985), found that *Enterococcus, Pseudomonas*, and Enterobacteriaceae bacteria could not grow in milk infant formula where the LPS was activated. The inhibition was about 48 hours when storage was done at 30°C. However yeast growth was not inhibited.

When the LPS was activated in refrigerated raw milk, prior to and post pasteurisation, the shelf life of the pasteurised milk was significantly improved (Martinez *et al* 1988).

Several studies have been carried out as regards the use of the LPS as a milk preservative. Milk preservation using the LPS is required where cold storage is not feasible or where cold storage must be extended for more than 48 hours (Bjorck, 1979; Zajaca, *et. al*, 1983). Temperature inversely affect the antibacterial activity of the LPS. In raw milk the effect of the system is mainly bacteriostatic. At 30°C, 25°C, 20°C, 15°C, 10°C, 7°C, and 4°C, the preservative effect of the LPS lasted for 7 to 8 hours, 11 to 12 hours, 15 to 16 hours, 24 to 26 hours, 48 hours 72 hours and 104 hours respectively. (Reiter & Harnulv, 1982 ; Bjorck *et. al*). Better results from the system are obtained when: the initial bacterial count in the milk is lower than 10^5 ; the ambient temperature is below 20°C; the amounts of SCN and H_2O_2 are not less than 10:10 ppm.

CHAPTER TWO

2.1 AIM AND OBJECTIVES

The main aim of this study was to investigate how changes in the milk storage temperature can affect the activity of the lactoperoxidase system on specific food poisoning bacteria. The experiment was carried out in bovine milk. Specifically the objectives of the study were:

1. To determine the effect of the milk storage temperature on the activity of the LPS on: *Salmonella enteritidis, Listeria monocytogenes, Yersinia enterocolitica & Escherichia coli.*

2. To determine the death rate of the above bacteria in bovine milk that has been activated with the LPS and stored at different temperatures.

3. To compare and contrast the antibacterial effect of the LPS on these bacteria when activated in raw milk, and in pasteurised/sterilised (P/S) milk mixture.

2.2 MATERIALS AND METHODS

2.2.1 The Preliminary Study

In a study preliminary to the main research project, the level of thiocyanate in fresh milk and in milk samples activated with the LPS was determined. The purpose of this exercise was two fold : to determine if the amount of NaSCN recommended by IDF (1988) is adequate to activate the LPS ; and to determine if the amount of NaSCN used does not result in an excessive

accumulation of SCN residues in treated samples.

The level of lactoperoxidase was measured in fresh raw milk samples, in raw milk samples stored at -18°C for 20 days, in pasteurised milk samples and in milk samples prepared by mixing pasteurised and sterilised milk (section 2.2.2.1.2). The purpose of this exercise was to determine the stability of lactoperoxidase to heat treatment and to storage at -18°C.

2.2.1 Reagents and equipment

The following reagents were used for the analysis of SCN.

(i) 20% (w/v) trichloroacetic acid (TCA).

(ii) Ferric nitrate reagent, which was prepared by dissolving 16 g Fe $(NO_3)_3$ in 50 ml 2 M HNO_3 and then diluted with distilled water to 100 ml.

The following reagents were used for the analysis of lactoperoxidase.

(i) Buffer which was prepared by dissolving 13.608 g of sodium acetate trihydrate in 800 ml distilled water, and then adjusting the pH to 6 by addition of acetic acid.

(ii) ABTS reagent which was prepared by dissolving 0.05487 g of 2, 2 Azino - di (3 ethylbenzthiazoline sulphonic acid) in 1 litre of the above buffer.

(iii) 10 mM hydrogen peroxide

2.2.1.3 Chemical analysis

The concentration of SCN was determined in milk samples using the method recommended by IDF (1988) with the following alterations:

8 ml of milk was mixed with 4 ml of TCA

3 ml of filtrate was mixed with 3 ml of the ferric nitrate reagent.

3 ml of the ferric nitrate reagent was mixed with 3 ml distilled water to make the blank solution.

The procedure for the analysis of lactoperoxidase was as follows:

10 ml of milk was diluted to 50 ml with distilled water.
20 l of sample was added to 3 l buffered reagent and after 1 minute 100 l was added and absorbance measured at 412 nm.

2.2.2 The Main Experiment

2.2.2.1 Preparation of Milk Samples

2.2.2.1.1 Sources of milk samples

Fresh milk was obtained from the college dairy farm immediately after the afternoon milking. The milk was aseptically collected from the bulk tank in sterile 1 litre volume bottles. Within a few minutes after collection the milk was apportioned into 29.7 ml volumes of Raw (unprocessed) milk and pasteurised/sterilised milk mixture. These small volume samples were stored in a deep freezer at $-18^{o}C$.

2.2.2.1.2 Milk processing

The pasteurised /sterilised milk mixture was made by mixing pasteurised milk with steam sterilised milk in the ratio of 1:2. Pasteurised milk was prepared in the laboratory by heating in a water bath 50 ml volumes of fresh milk, contained in 100 ml screw capped polystyrene containers, at 63^{o}C for 30 minutes. Sterilised milk was prepared by heating in a steamer 300 ml volumes of fresh milk, contained in 500 ml volume Duran bottles at 100^{o}C for 40 minutes. The reason for using pasteurised/ sterilised milk mixture (p/s) was to obtain a milk medium which has a very low total bacterial count yet with sufficient lactoperoxidase for activating the LPS system.

2.2.2.2 Preparations of Bacterial Cultures

The four species of bacteria that were used in this experiment were: *Salmonella enteritidis, Listeria monocytogenes, Yersinia enterocolitica* and *Escherichia coli*. All the four bacteria were obtained from the department of nutrition and microbiology in the college. Prior to use, the cultures were subcultured, in duplicate and one of subcultured slopes kept in the refrigerator as a replacement culture in case the inuse slope became contaminated. All cultures were subcultured on Yeastrel milk agar slopes contained in screw capped universal bottles. In order to facilitate standardised inoculation of the bacteria into milk samples, the cultures were grown in nutrient broth and incubated as explained under individual experiments in the next chapter.

2.2.2.3 Chemicals

All the chemicals used in the experiments were of analytical grade. Sodium thiocyanate was purchased from BDH (UK), and this chemical served as a source of thiocyanate. Sodium percarbonate was obtained from Interox (Cheshire, England). This chemical was used as a source of hydrogen peroxide. The specifications of these chemicals are given in Appendix 1

2.2.2.4 General Design of the Experiments

29.7 ml volumes of raw milk and p/s milk mixture were prepared in 100 ml screw capped sterile polystyrene containers. To all samples, 0.3 ml of an overnight (18hrs) bacteria culture that had been diluted at 10^{-2} in 1/4 strength ringer solution was added. This amount and dilution of the culture was enough to give approximately 10^4 colony forming units of bacteria per ml.

In control samples only the bacterial culture was added, while in the treated samples the LPS was activated by addition of sodium percarbonate and sodium thiocyanate. These chemicals were added at a rate of 30 and 14 mg per litre of milk respectively. This was in accordance with IDF (1988) recommendations.

However to minimise errors that could be incurred when weighing the very small amount of the chemicals for these 30 ml samples, the chemicals were prepared and added to the samples in solution forms. The chemical solutions were prepared as follows:

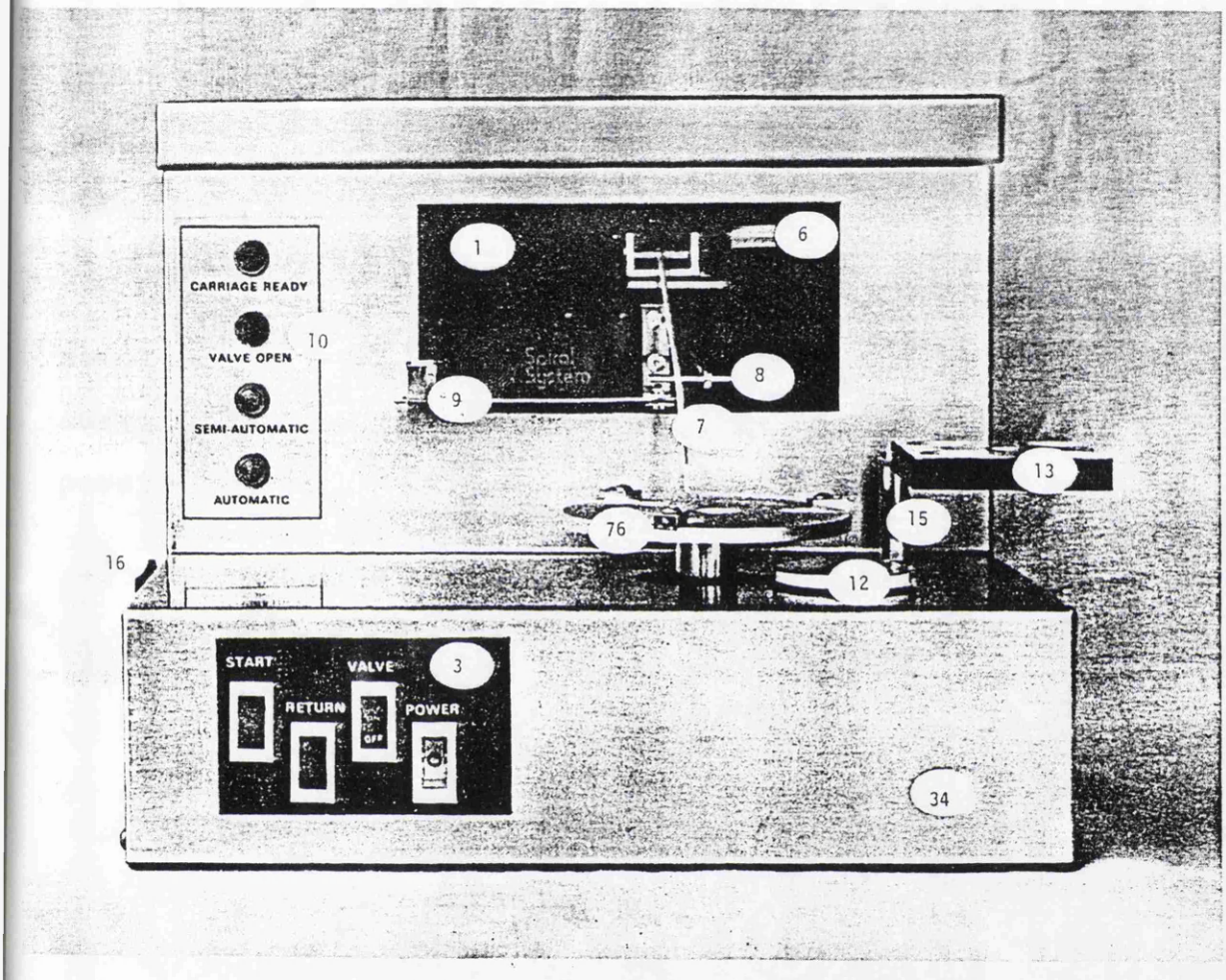
A solution of NaSCN that could give the rate of 14 mg / L when added into the milk + bacteria sample was prepared by : dissolving 28 mg of NaSCN in 100 ml sterile

distilled water; and adding 1.5 ml of this solution into the sample (30 ml).

A solution that could give 30 mg Sodium percarbonate per litre of milk when added into the treated samples was prepared by: dissolving 60 mg of sodium percarbonate in 100 ml sterile distilled water ; and adding 1.5 ml of this solution into the samples.

In order to activate the LPS in treated samples the above chemicals were added in the manner described in the order recommended by IDF (1988). NaSCN was added first and was thoroughly mixed with the milk sample, then sodium percarbonate was added and again a thorough mixing was done.

The samples were diluted in 1/4 strength Ringer solutions, and plated using the spiral plater (Don Whitley Scientific Ltd.) and following the procedure outlined by Hannah Research Institute (1984). Counts were done on dry plates of Yeastrel milk agar (YMA) and on appropriate selective agar plates as outlined under individual experiments. The reason for using YMA was to determine the effect of the antimicrobial activity of the LPS on the total bacterial counts (TBC) counts and compare this with the effect of the system on the specific bacteria. Counts were measured starting from time zero hours, i.e. time period just prior to activation of the LPS, and thereafter at intervals specified under individual experiments. Plates were incubated as outlined under individual experiment, and a colony counter was used for counting the colonies.



SPIRAL PLATER - MODEL D

1. Shutter
3. Switch panel
6. Sight glass
7. Stylus
8. Stylus lift arm
9. Shutter-anchored sample cup holder
10. Indicator lights
12. Turntable height adjuster
13. Sample cup holder
16. Carriage positioning knob
34. Case
76. 10-cm turntable

The activity of the LPS against all the four species of bacteria was tested at the following storage temperatures : $7^{o}C$, $15^{o}C$, $22^{o}C$, $25^{o}C$, $30^{o}C$.

2.2.2.5 Statistical Analysis

The Minitab statistical computer package, from the Scottish Agricultural College, **Auchincruive**, was used for analysis of the results. Correlation coefficient values and regression equations were established, where it was appropriate, using this package.

Calculation of bacterial death rates from LPS activated samples were determined graphically by plotting $\log_{10}$ cfu /ml surviving cell population versus storage time. D - values where possible were estimated from the regression equations.

CHAPTER THREE

3.1 EXPERIMENT I: THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF THE LPS ON *ESCHERICHIA COLI*

3.1.1 Introduction

Escherichia coli is one of the most abundant bacteria of the intestinal tract of warm blooded animals. Traditionally the presence of this organism in raw milk is viewed as an indication of faecal contamination. In pasteurised dairy products the presence of detectable *E. coli* is viewed as an indication of underpasteurisation of product or post pasteurisation contamination.

The presence of the organism in milk and dairy products is also viewed as as a serious health hazard for these main reasons: Firstly, the presence of the organism may indicate that faecal contamination has occurred therefore other enteropathogenic bacteria, e.g. *Salmonella* may be present. Secondly, some strains of *E.coli* are pathogenic and these have been found in milk and dairy products (Mehlman & Romers, 1982).

There is also evidence that some of these enteropathogenic strains can grow at refrigeration temperatures (Olsvik & Kkapperud, 1982 ; Witter, 1961). It is this psychrotrophic ability of these bacteria which make them a health hazard in raw milk that has been refrigerated for a prolonged period.

The antibacterial activity of the LPS on *E.coli* is well documented (Reiter *et al*, 1976 ; Marshall, 1978 ; Bjorck & Claesson, 1980). At present it is well accepted that the LPS is lethal to *E. coli*, although recovery of the bacterial cells is

still not fully understood. Currently the influence of temperature on the antibacterial activity of the LPS on *E. coli* has not been well investigated.

The main objective of this experiment was to determine the effect of storage temperature on the bactericidal effect of the LPS on *E.coli* in milk.

3.1.2 Materials and Methods

3.1.2.1 Bacterial Cultures

The antibacterial activity of the LPS was tested on *E.coli* Nutrient broth cultures were grown by incubation at 37^{o}C for 18 hours.

3.1.2.2 Plating medium

Counts were done in yeastrel milk agar and in violet red bile agar (VRBA) plates. The VRBA plates were prepared using the so - called basal and overlay method (Hartman *et al*, 1975).

3.1.2.3 Milk samples and sampling of counts

5 samples of raw milk and a mixture of pasteurised and sterilised milk (p/s) were prepared and activated with the LPS as previously explained (section 2.2.2.1). An equal number of control samples were also prepared. Bacterial counts were done at 0 hours, and thereafter sampled at different intervals in samples stored at different temperatures as shown in table 4.

TABLE 4

TABLE SHOWING SAMPLING PERIODS OF *E.COLI COUNTS* FROM SAMPLES STORED AT DIFFERENT TEMPERATURES

Incubation temperature (^{o}C)	Sampling periods (hours)
7	0 20 or 25 46 or 51 72.5 or 75.5 92 or 100 144.5
15	0 3 or 4 6 or 8 9 or 10.5 11 26 or 27 30 47
22	0 3 6 9 11 or 13 14 or 15 16 or 17 18
25	0 2 or 3 4 or 6 8 or 9 10 12
30	0 2 or 3 4.5 or 6 6.5 9 or 9.5 12

Key to Figures 5 - 9

1.RWVATR = counts in raw milk + LPS on Violet red bile agar

2.RWYATR = '' '' '' '' on Yeastrel milk agar

3.PSVATR = '' '' p/s '' '' Violet red bile agar

4.PSYATR = '' '' '' '' '' Yeastrel milk agar

5.RWVACON = counts in raw milk control VRBA

6.RWYACON = '' '' '' ''' '' YMA

7. PSVACON = '' '' P/S '' VRBA

8. PSYACON = '' '' '' '' YMA

E.COLI COUNTS AT 7 C
Treated & control samples

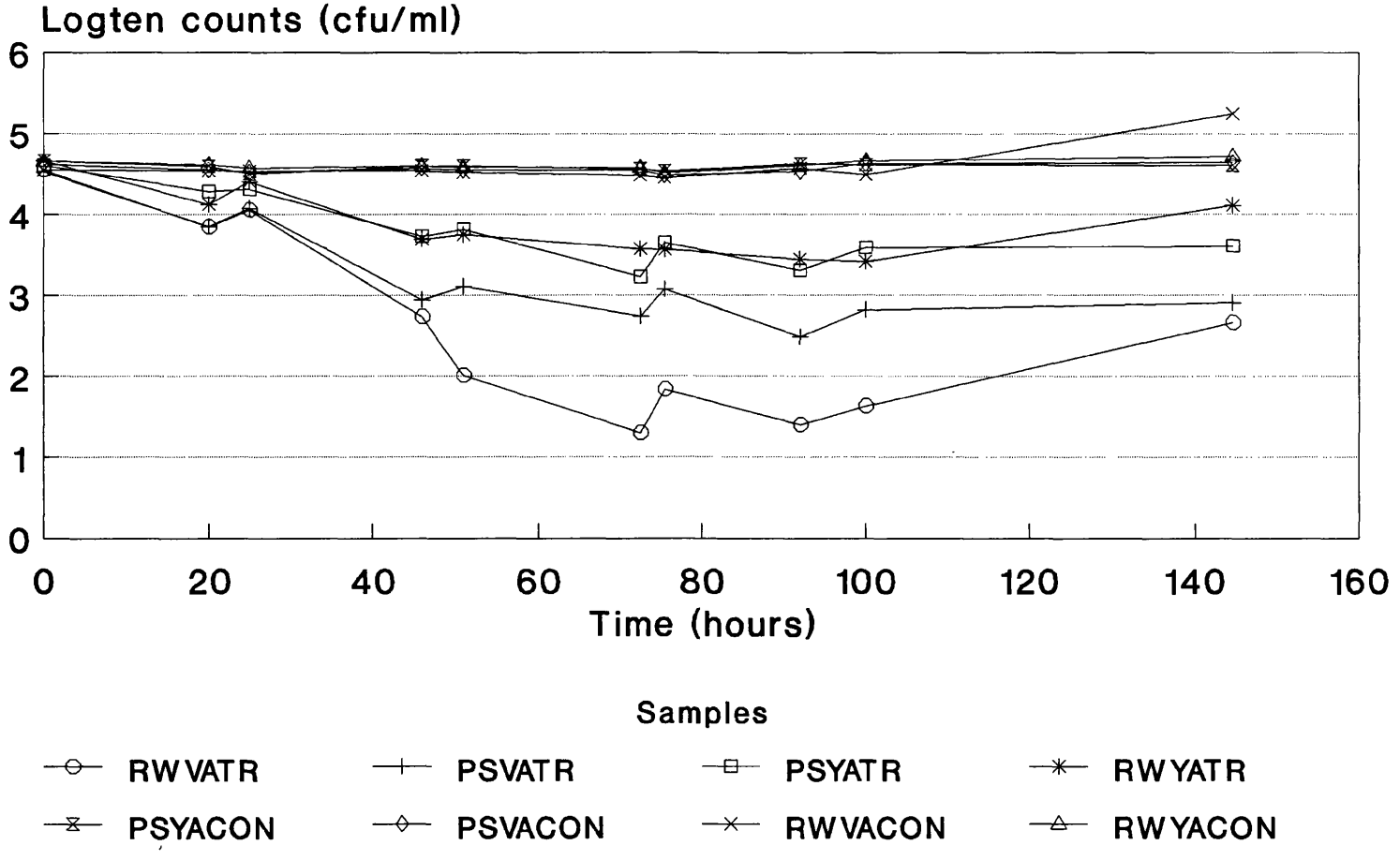


figure 5

E.COLI COUNTS AT 15 C
(Treated and control samples)

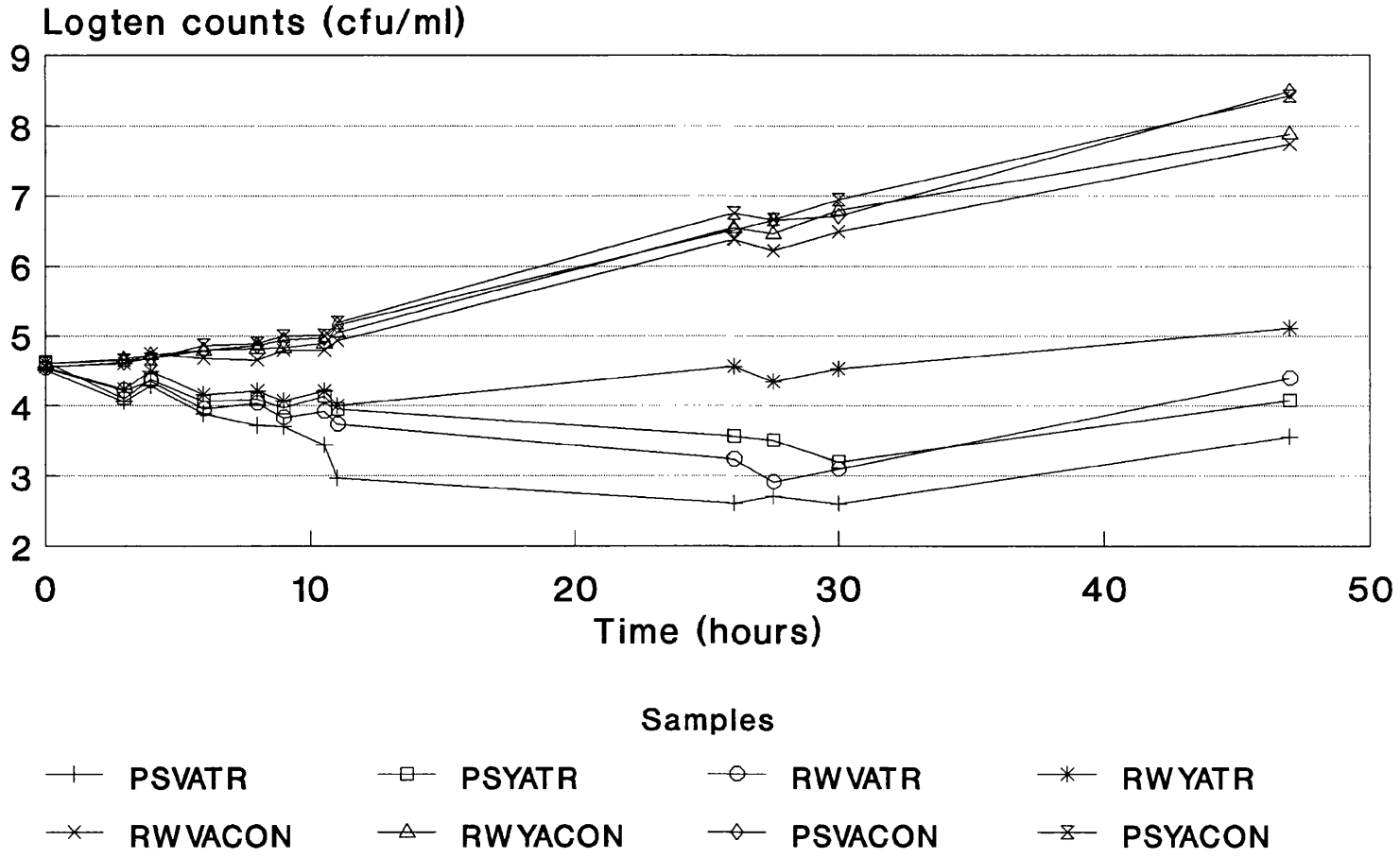


figure 6

E.COLI COUNTS AT 22 C
(Treated and control samples)

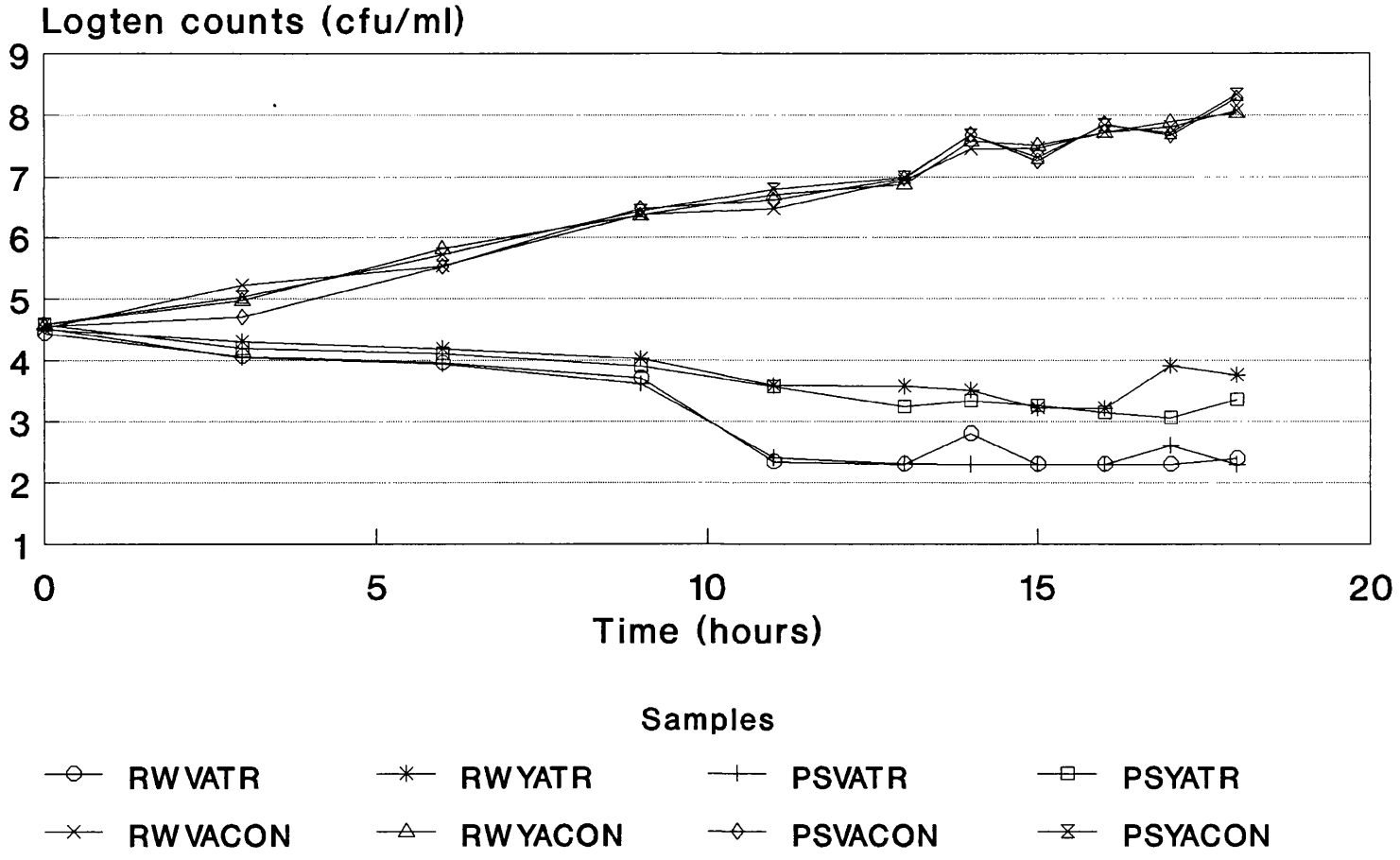


figure 7

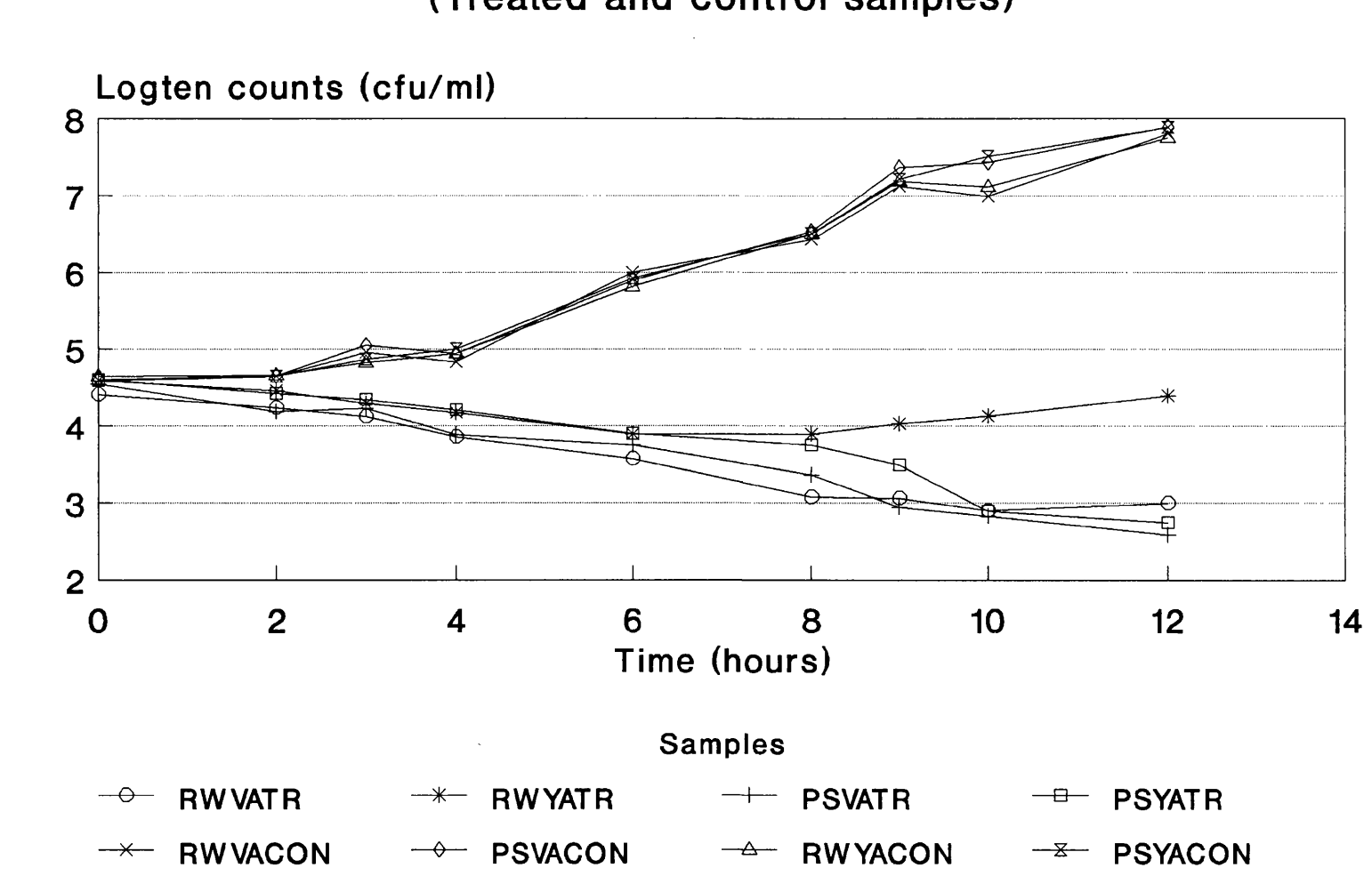

E.COLI COUNTS AT 25 C
(Treated and control samples)
Logten counts (cfu/ml)
8
7
6
5
4
3
2
0
2
4
6
8
10
12
14
Time (hours)
Samples
RWVATR
RWYATR
PSVATR
PSYATR
RWVACON
PSVACON
RWYACON
PSYACON

figure 8

E.COLI COUNTS AT 30 C
(Treated and control samples)

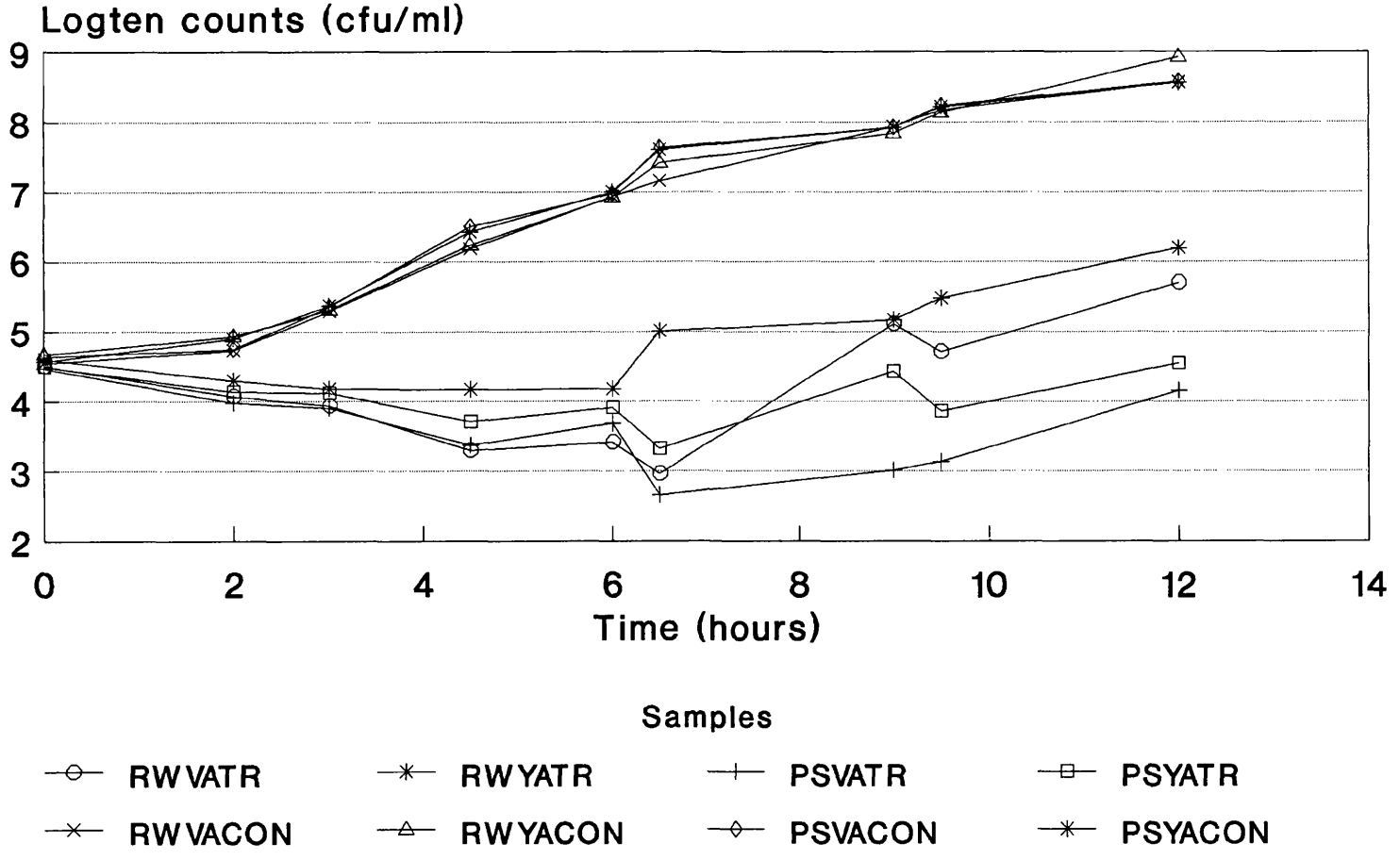


figure 9

3.1.3 Results

The results presented in this section show the effect of the bactericidal effect of the LPS activity on *E.coli* as the storage temperature was increased.

3.1.3.1 The activity of the LPS on *E.coli* at 7°C.

3.1.3.1.1 Controls

In all the control samples the *E.coli* populations showed a decline during the experiment as indicated by the negative correlation coefficient values (Table 8). The decline in numbers was very slight and tended to take place during the first 20 hours. After 100 hours there was an indication that numbers were increasing.

3.1.3.1.2 LPS activated samples

In contrast with the control samples , LPS activated samples showed a marked decline in *E.coli* numbers from 10^4 to $< 2 \times 10^1$ in the raw milk - VRBA counts over a period of 70 hours. Meanwhile in p/s samples counts after 70 hours had fallen to 10^2 (Figure 5). During the period 70 hours to 100 hours the bacterial counts remained constant at 10^2 or less and then started to show an increasing trend after 100 hours. Counts recovered on YMA in both p/s and raw milk showed a decline in bacterial populations. However the rate and extent of the population decline was not as marked as was observed on VRBA where only *E.coli* was being counted.

The bactericidal effect of the LPS on *E.coli* at this temperature is confirmed by the high negative r - values. These values are slightly higher in raw milk samples (-0.966 & -0.936),

than in p/s samples (-0.906 & -0.936).

The D-values were lower in VRBA counts than in YMA counts. The lowest value was observed in raw milk VRBA counts (26.01 hours) while the highest value was observed on raw milk YMA counts (65.55 hours).

3.1.3.2 The activity of the LPS on *E.coli* at 15^{o}C

3.1.3.2.1 Controls

E.coli grew slightly better in p/s milk than in raw milk at 15^{o}C. In the control samples (Figure 6). The growth of this bacterium was slow during the first ten hours of incubation, but After 27 hours, counts had increased to 10^{7}.

3.1.3.2.2 LPS treated samples

All LPS treated samples showed a decline in bacterial numbers compared with the corresponding control samples. The VRBA counts show that the E.coli populations declined during the first 27 - 30 hours of incubation. On the other hand raw milk YMA counts were already increasing after 27 hours of incubation, indicating a rapid recovery of TBC from the antimicrobial activity of the LPS.

The death rate of bacterial populations was slightly quicker in p/s milk samples than in raw milk samples. This is indicated by the lower D-values for p/s milk samples (12,41) contrasted with (17.2) of raw milk VRBA counts. The lowest correlation coefficient value was observed in raw milk YMA counts (-0.792). However in all the treated samples the correlation coefficient were negative values, indicating that bacterial populations were decreasing as the incubation time was increasing.

3.1.3.3 The activity of the LPS on *E.coli* at 22°C

3.1.3.3.1 Controls

In all control samples *E.coli* increased from 10^4 to 10^7 within 17 hours. r values were also high figures in control samples (0.981 to 0.994).

3.1.3.3.2 LPS treated samples

Results of *E.coli* counts in p/s and raw milk samples activated with the LPS and stored at 22°C, are presented in Figure 7.

The results show that *E.coli* populations in VRBA counts decreased from 10^4 to $<10^2$ after 15 hours. While in the YMA plates TBC counts decreased from 10^4 to 10^3 after 15 hours.

There were no significant differences between raw and p/s milk samples D-values. However the D-values were slightly higher in YMA counts than in VRBA counts, indicating a slower death rate on TBC than *E.coli* cells (Table 8).

3.1.3.4 The activity of the LPS on *E.coli* at 25°C

Results showing the behaviour of *E.coli* in the presence and absence of the LPS in milk stored at 25°C are presented in Figure 8.

3.1.3.4.1 Controls

In control samples the bacterial populations grew from 10^4 to a maximum of 10^7 cfu/ml after 12 hours of incubation. As it can be seen from the results, all the control samples had an initial lag phase of approximately 2 hours.

3.1.3.4.2 LPS activated samples

In samples activated with the LPS, *E.coli* populations decreased steadily from 10^4 to 10^2 after 12 hours. It was observed that YMA counts started to increase after 8.0 hours, hence a higher D-value was observed from these counts. The results indicate that the bacterial cell death rate was quicker in VRBA counts than in YMA counts from p/s and raw milk samples. This can be seen in the lower D-values from these counts. Figure 8 shows that counts were already increasing in raw milk VRBA after 12 hours , yet in p/s samples counts were still at a low level. This suggest that the activity of the LPS persisted longer in p/s samples than in raw milk samples at 25°C.

3.1.3.5 The activity of the LPS on *E.coli* at 30°C.

3.1.3.5.1 Controls

The results presented in Figure 9 show that in all control samples, *E.coli* populations were increasing during the entire treatment period. Increases from an initial level of 10^4 to 10^9 after 12 hours were observed.

3.1.3.5.2 LPS activated samples

Figure 9 also shows that *E.coli* cells were dying in all the samples activated with the LPS. This bactericidal effect lasted for at least 6 hours. Lower D-values were observed in VRBA counts than on YMA counts. In raw milk YMA counts, no D-value could be observed because the death phase of the bacteria were shorter, and counts had already increased to > 10^4 after 6 hours. In p/s samples the bactericidal effect of the system lasted slightly longer. It was only after 9 hours that the counts from these samples started to show a slight increase. Recovery was quicker

in raw milk samples because after 12 hours counts had reached 10^6 meanwhile in p/s samples counts had only reached 10^4 at the same period.

It was also observed that in p/s treated samples the population of *E.coli* declined from an initial level of 10^4 to a minimum level of 10^2. Meanwhile in raw milk treated samples a less marked decrease was observed, from 10^4 to 10^3 after 6 hours. This behaviour again indicates that the bactericidal effect of the LPS persisted for a longer time in p/s than in raw milk samples.

3.1.4 Discussion

When comparing the untreated with the treated samples, the populations of *E.coli* decreased significantly in treated samples at all temperatures. The kill of *E.coli* by the LPS is a positive proof that the system worked under conditions of this experiment.

Temperature affected the persistency of the bactericidal effect of the LPS, as well as the death rate of *E.coli*. At high temperatures (22-30^{o}C) the bactericidal effect lasted for a shorter period than was the case at lower temperatures (7 & 15^{o}C). The death rate was faster at higher temperatures than at lower ones. This is indicated by D-values calculated from samples for the various incubation temperatures employed in the study (Table 8)

It is not fully understood why *E.coli* recovers from inhibition by the LPS. Bjorck (1979), discounted the possibility that the bacteria may develop some resistance to the system. The

results from this experiment indicate that temperature has a great influence on the recovery of the bacteria cells from the activity of the LPS. At lower temperatures recovery was severely retarded, while as the temperature increased recovery was enhanced. This might be attributed to the fact that at the lower temperatures, the *E.coli* cells were not growing optimally, and consequently the bactericidal activity of the LPS is more noticeable than it would be if the cell are rapidly multiplying. cThe presence of foreign microflora in the milk may have an effect on the duration of the activity of the LPS on *E.coli*. This is indicated by the behaviour of counts from YMA. The decline in numbers recovered on YMA is not as marked as was observed on VRBA where only *E.coli* was being counted. Presumably the higher levels of bacteria surviving exposure to the LPS can be accounted for by the initial microflora of the raw and p/s milk growing during the experimental period. It was also observed that D-values from TBC were significantly higher than were in *E.coli* counts particularly at higher temperatures, where the D-values could not be observed during the experimental periods. Such a behaviour of YMA counts can be attributed to the non selective nature of the agar medium, and the fact that raw milk is contaminated with foreign bacteria. Therefore it can be deduced that the presence of other bacteria in the milk samples, rather than *E.coli* tends to reduce the persistency of the LPS activity.

Another possible reason for YMA counts to behave differently from the VRBA counts in the LPS treated samples, could be attributed to a tendency of the selective agar to inhibit colony formation from injured bacterial cells (Mcdonald *et al*, 1983).

The results showed that the bactericidal effect of the LPS persisted for a longer time in p/s than in raw milk samples. A possible reason for this behaviour could be the differences in chemical environments of p/s and raw milk samples. Probably catalase and the SH groups were responsible. These points will be fully discussed in the next chapter.

3.2 EXPERIMENT II: THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF THE LPS ON *LISTERIA MONOCYTOGENES*

3.2.1 Introduction

Listeria monocytogenes is at present considered as a cause of various, often severe disorders in man such as: meningo-encephalitis , septicaemia during perinatal period, pneumonia, endocarditis, urethritis, and abortion (Gray & Killinger, 1966).

Various food stuffs have been incriminated as agents of listeriosis infection: Coleslaw (Schlech *et al*,1983) ; pasteurised whole and semi - skimmed milk (Fleming *et al*, 1985) ; Mexican style white cheese (James *et al*, 1985). In the listeriosis problems where dairy products were incriminated, fatalities have been reported, particularly with members of the most vulnerable groups such as: pregnant women, young children, the elderly, persons who had an underlying illness or were taking immunosuppressants.

Contamination of milk with listeria could represent a significant health hazard. This is particularly true because of the ability of the pathogen to thrive and multiply under refrigeration temperatures. In milk, Bearns and Girard (1958)

reported that the organism is capable of growth at 4 to 6°C. There is evidence that *L. monocytogenes* is more prone to cause food poisoning problem in foods stored at lower temperatures. (Gray & Kilinger, 1966; Palumbo, 1986). Some reports even suggest that these organisms may survive pasteurisation (Flemming *et al*,1985). However conflicting reports on this point exist (Bearns & Girard, 1959 ; Bradshaw *et al*, 1985 ; Domelly & Briggs, 1986) and the current view held by IDF is that normal pasteurisation should kill the bacteria.

More recently, a few research studies have been done to investigate the antibacterial activity of the LPS on *L. monocytogenes*. Conflicting results have been obtained. e.g. Earnshaw & Banks (1989), reported that the system was bacteriostatic, while Denis & Ramet (1989), reported that the activity of the system was bactericidal. However in both reports the activity of the system was tested in UHT milk.

The main objective of the present research was to investigate the influence of temperature on the activity of the LPS on *L. monocytogenes* in raw and in p/s milk media.

3.2.2 Materials and Methods.

3.2.2.1 Bacteria Cultures

The antibacterial activity of the LPS was tested on *L. monocytogenes* serotype 1. Cultures were prepared as outlined in section 2.2.2.2 above. Broth cultures were grown in nutrient broth by incubation at 37°C for 18 hours.

3.2.2.2 Plating media

Counts were done on Yeastrel milk agar and on Oxford agar plates.

3.2.2.3 Milk samples and sampling of counts

5 samples of raw milk and 5 samples of p/s milk were prepared as outlined in section 2.2.2.1 above , and were activated with the LPS. An equal number of control samples were also prepared.

Counts were measured at time 0 and at varying intervals in samples stored at different temperatures as shown in Table 5.

3.2.3 Results

The results presented in this section show that the antibacterial activity of the LPS on *L. monocytogenes* was mainly bacteriostatic.

3.2.3.1 The activity of the LPS on *L.monocytogenes* at 7°C

3.2.3.1.1 Controls

Figure 10 shows the behaviour of L. monocytogenes in the control samples stored at 7°C. The results show that there was an initial lag phase of 50 hours after which cells started to increase from 10^4 to 10^6 after 200 hours. As can be seen from the graphs, in p/s samples listeria cells tended to grow quicker than in raw milk. The correlation coefficients are all positive figures. The lowest value was observed in raw milk samples Oxford agar counts (0.609). The correlation coefficient values indicate that the listeria cells were growing as the incubation time was increased at 7°C.

TABLE 5

TABLE SHOWING SAMPLING PERIODS OF *LISTERIA MONOCYTOGENES* COUNTS IN SAMPLES STORED AT DIFFERENT TEMPERATURES

Incubation Temperature (°C)	Sampling periods (hours)						
7	0	45	117 or 118	141 or 147	167	189	212 280
15	0	4	8	11	24	30	45
22	0	4 or 5	8	11	18 or 22	24 or 26	30
25	0	2 or 2.5	4 or 5.5	6 or 8	11		
30	0	2	4 or 5	6	8 or 8.5	11	

Key to symbols of Figures 10 - 14

1.PSOATR = COUNTS FROM PS MILK + LPS ON OXFORD AGAR

2.PSYATR = COUNTS '' '' '' '' '' YMA

3.RWOATR = '' '' RAW '' '' '' OXFORD AGAR

4.RWYATR = '' '' '' '' '' '' YMA

5.RWOACON = '' '' RAW MILK CONTROLS OXFORD AGAR

6.RWYACON = '' '' '' '' '' YMA

7.PSOACON = '' '' PS MILK CONTROLS OXFORD AGAR

8.PSYACON = '' '' '' '' '' YMA

L.MONOCYTOGENES COUNTS AT 7 C
(Treated and control samples)

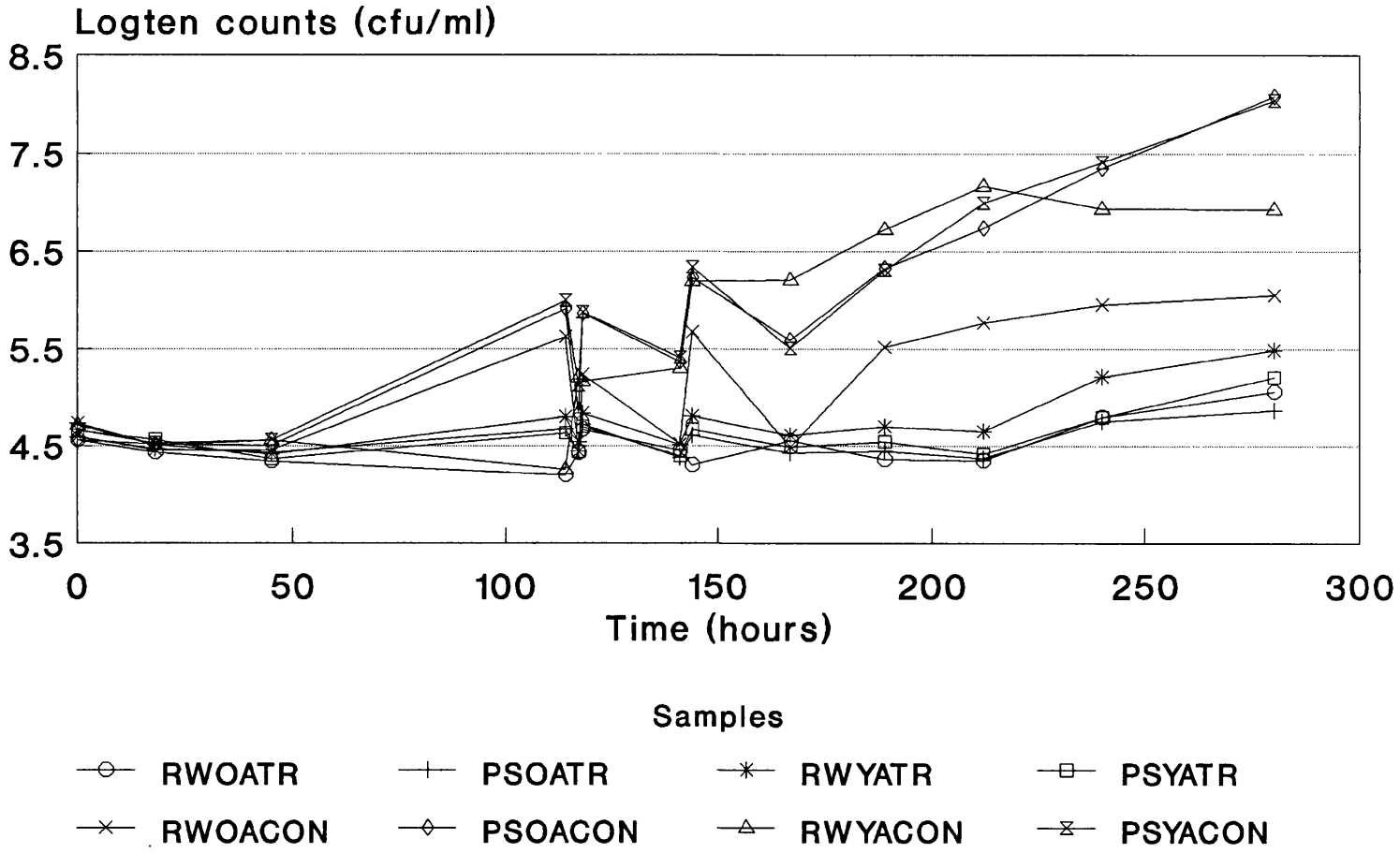


figure 10

L.MONOCYTOGENES COUNTS AT 15 C
(Treated and control samples)

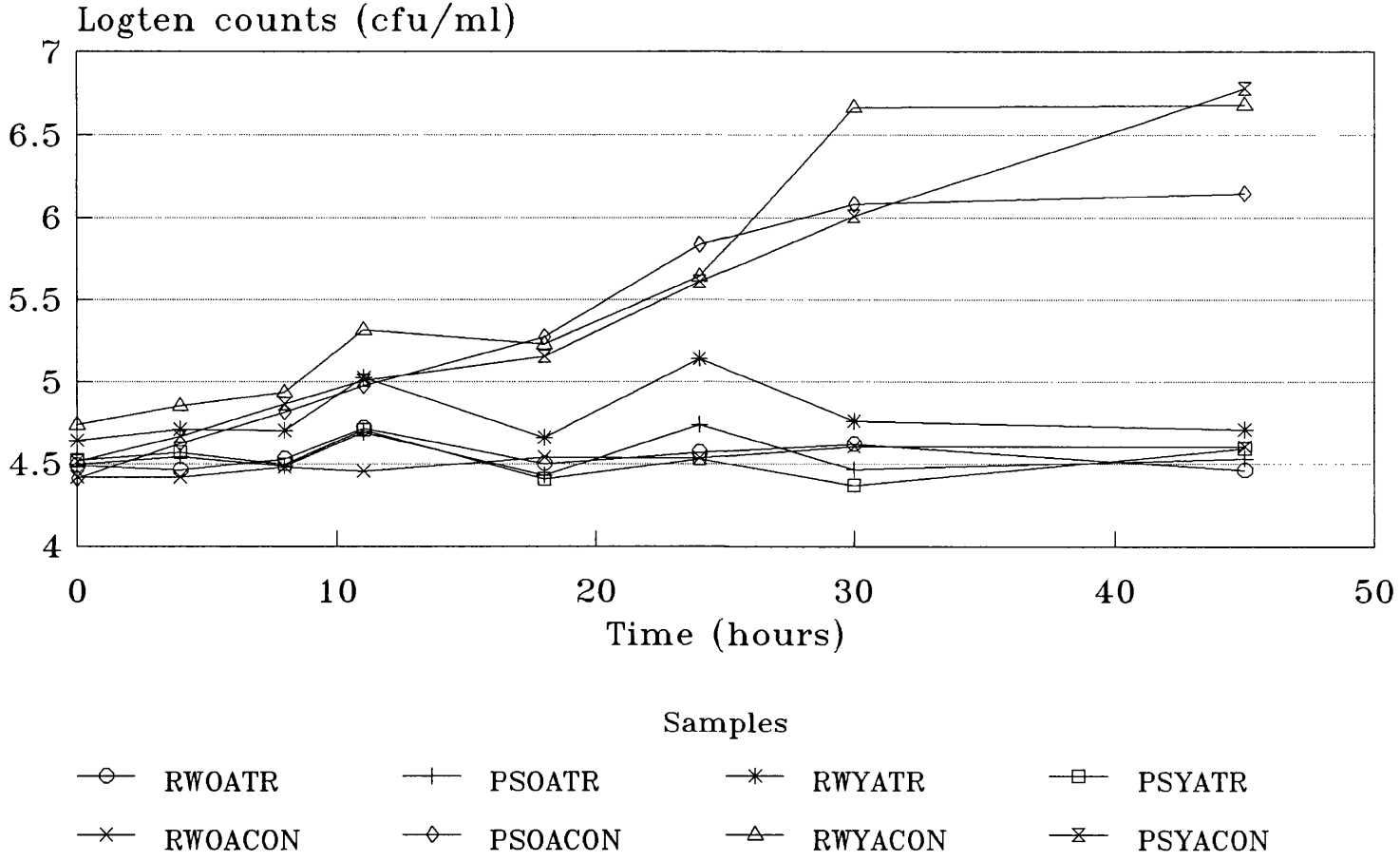


figure 11

L.MONOCYTOGENES COUNTS AT 22 C
(Treated & control samples)

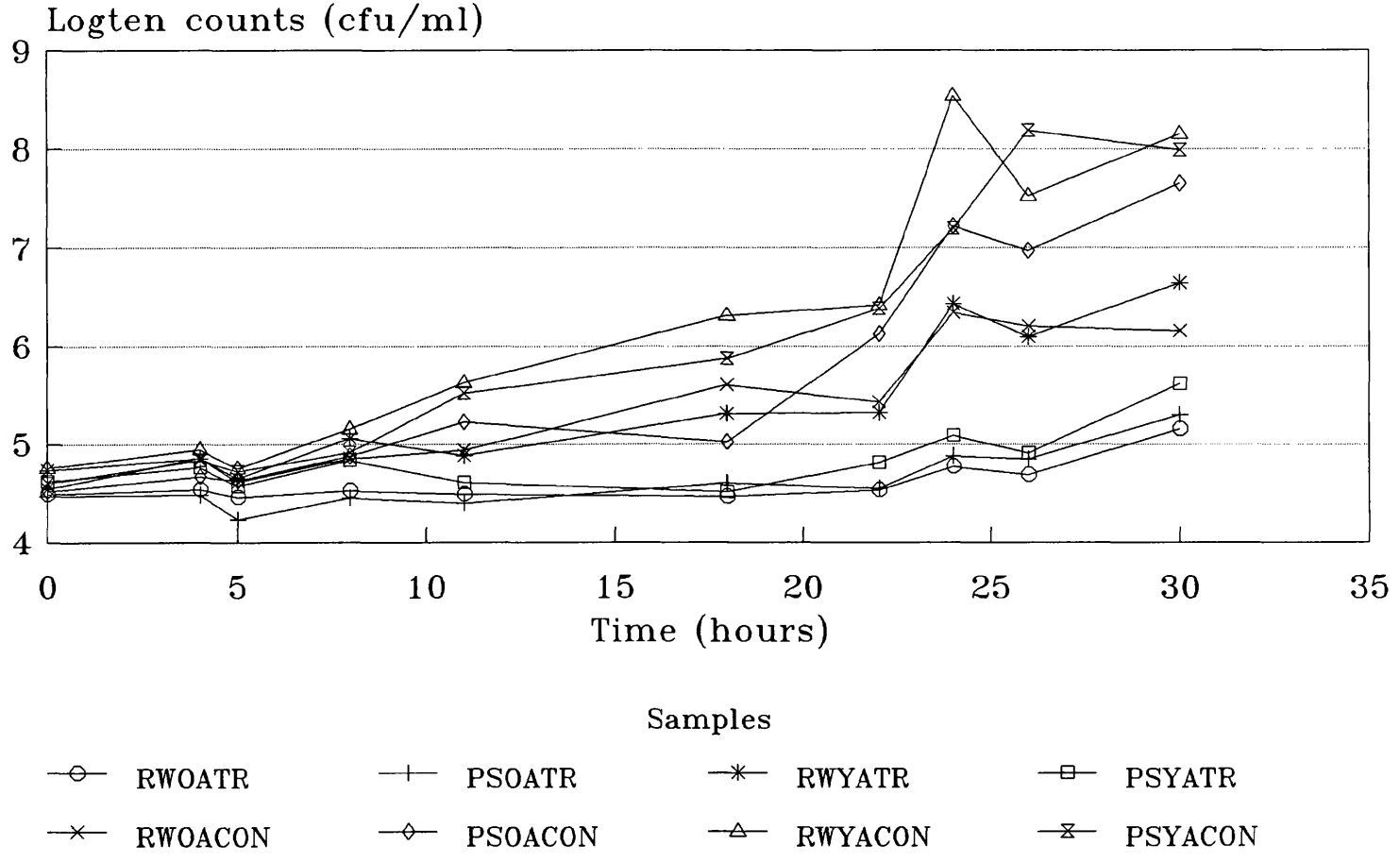


figure 12

L.MONOCYTOGENES COUNTS AT 25 C
(Treated and control samples)

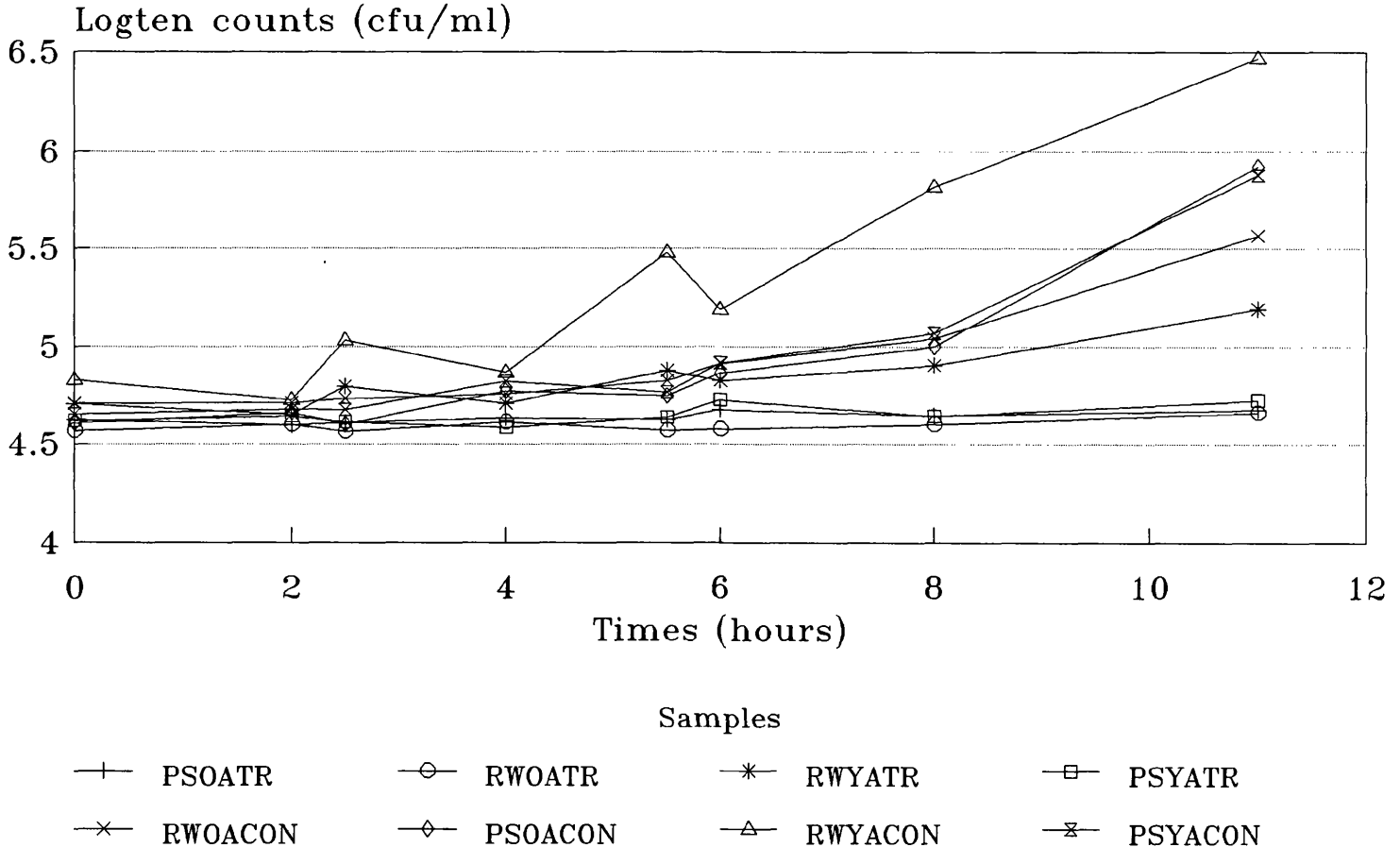


figure 13

L.MONOCYTOGENES COUNTS AT 30 C
(Treated and control samples)

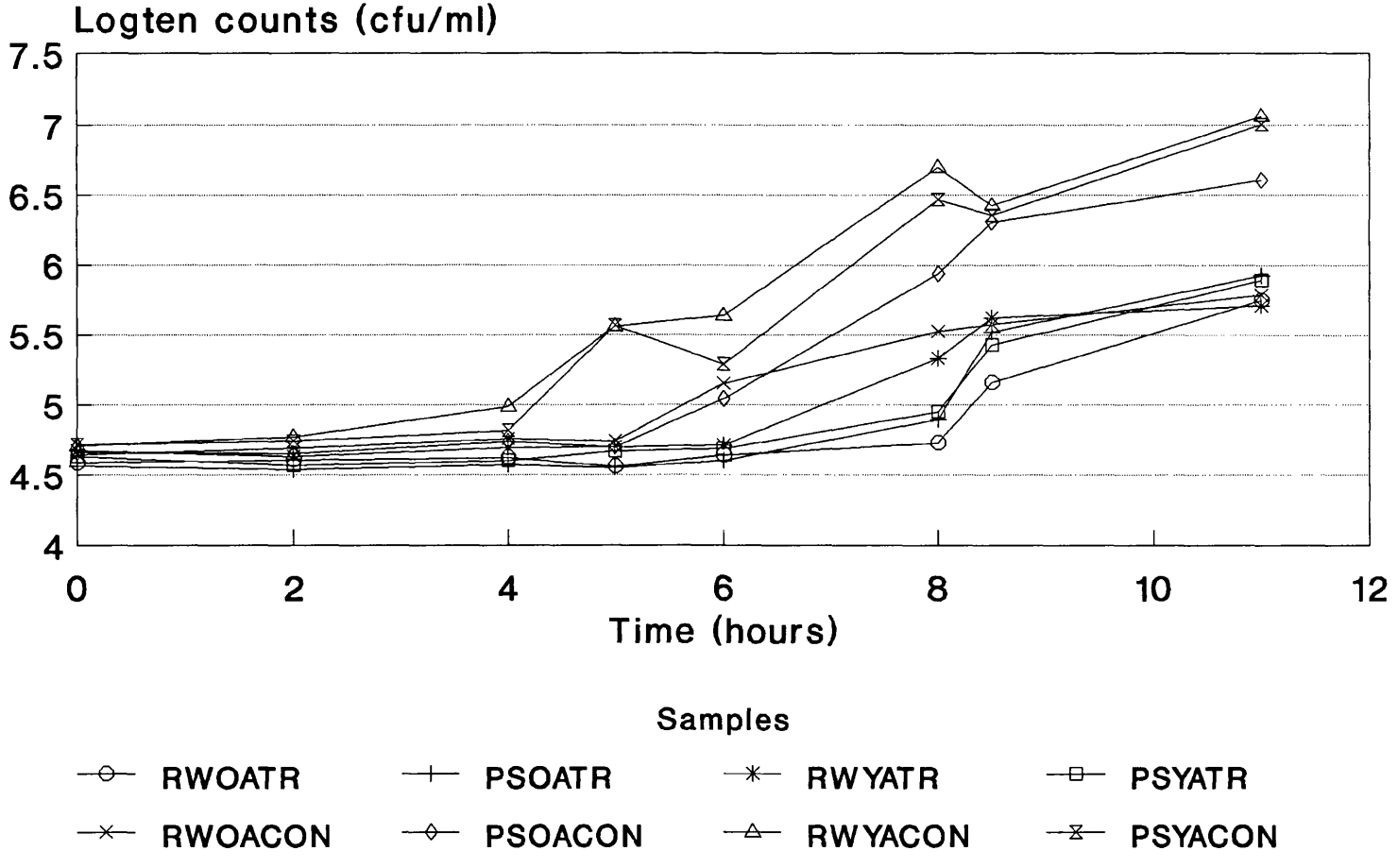


figure 14

3.2.3.1.2 LPS treated samples

Figure 10 also shows the inhibition of *L.monocytogenes* by the activity of the LPS on samples stored at 7°C. As it can be seen from the results, at 7°C a slight reduction in numbers was observed in the treated samples for the first 40 hours, and thereafter numbers tended to increase slightly and stabilised for up to 200 hours. After 200 hours of incubation, there was again a tendency for numbers to increase.In all cases LPS treated samples have lower numbers of bacteria counts compared with their corresponding controls. This is indicated by the lower r values in all the treated samples.

3.2.3.2 The activity of the LPS on L.monocytogenes at 15°C

3.2.3.2.1 Controls

The control results presented In Figure 11 show that *Listeria* cells were growing steadily during the treatment period. Increases were rapid in cells growing in p/s samples where counts rose from 10^4 to 10^6 after 45 hours. YMA counts from raw milk showed similar dynamics to p/s counts, however there was very little increase *L.monocytogenes* populations on Oxford agar plates from raw milk. The slight increase was from 1 x 10^4 to 4 x 10^4 after 45 hours.

3.2.3.2.2 LPS treated samples

Figure 11 also shows the the behaviour of *L.monocytogenes* in LPS activated samples stored at 15°C. These results show that the LPS inhibited the growth of the *Listeria* for at least 40 hours. the low r values (table 8) confirm this observation. All the values are closer to 0 rather than to 1.

3.2.3.3 The activity of the LPS on *L.monocytogenes* at 22°C

3.2.3.3.1 Controls

The results showing the behaviour of Listeria cells in samples stored at 22°C are presented in Figure 12 . In these results the Listeria cells increased markedly in p/s than in raw milk samples. In p/s samples counts from Oxford agar plates had increased from 10^4 to 10^8 after 30 hours, meanwhile in the raw milk samples counts had reached 10^6 after 30 hours. The magnitude of increase was the same in TBC from both p/s and raw milk samples.

3.2.3.3.2 LPS treated samples

The behaviour of *L.monocytogenes* in the presence of the LPS in samples stored at 22°C is shown in the results presented in Figure 12 . From the results it can be seen that LPS inhibited the growth of *Listeria* for approximately 22 hours. This is indicated by the very low correlation coefficient values from p/s samples and raw milk Oxford agar count. However on raw milk YMA counts the results show that the inhibition effect lasted for a shorter time. This is confirmed by the slightly higher correlation coefficient value (0.251) .

3.2.3.4 The activity of the LPS on *L.monocytogenes* at 25°C

3.2.3.4.1 Controls

In control samples *Listeria* cells increased steadily at 25°C. The results show that in both p/s and raw milk samples counts had increased by at least one log cycle at 11 hours. TBC increase was more marked in raw milk samples (Figure 13). In all the control samples high positive r values were observed.

3.2.3.4.2 LPS treated samples

Results showing the antibacterial activity of the LPS on *L.monocytogenes* at 25°C are presented in Figure 13 . As it can be seen from these results, the LPS inhibited the growth of *Listeria* for at least 11 hours. This is confirmed by the very low correlation coefficient values in p/s and in raw milk Oxford agar counts (0.042 to 0.111). However it was noted that the correlation coefficient value of raw milk YMA counts was slightly higher (0.447). From these results it can be deduced that the inhibitory effect of the LPS at 25°C persisted for a short time in raw milk TBC.

3.2.3.5 The activity of the LPS on L.monocytogenes at 30°C

3.2.3.5.1 Controls

In control samples, TBC increased from 10^4 to 10^7 after 11 hours. In these counts an initial lag phase of 3 hours was observed. This lag phase was slightly longer in *Listeria* counts (5 hours). It was also noted that *Listeria* growth was slower in raw milk samples than in p/s . In raw milk counts slightly increased from 10^4 to 10^5 after 11 hours, meanwhile in the p/s samples counts had exceeded 10^6 at 11 hours (Figure 14).

3.2.3.5.2 LPS treated samples

On samples stored at 30°C, the LPS inhibited the growth of *Listeria* for approximately 6 hours. This is more noticeable on the Oxford agar counts (Figure 14). The correlation coefficient values from these counts are very close to 0.0 (-0.014 & 0.056). However counts from the YMA plates shows slightly higher correlation coefficient values. This indicates that the antibacterial activity of the LPS against TBC lasted for short

time at this temperature of incubation.

3.2.4 Discussion

The results of this experiment show that the antibacterial effect of the LPS was mainly bacteriostatic to *L. monocytogenes*. The persistency of this effect was inversely related to temperature i.e. it decreased as the incubation temperature was increased. It appears as if the LPS extended the lag phase on the listeria growth curve because at the end of the inhibitory phase, the bacteria started to grow rapidly.

However the results from this experiment do not corroborate earlier work by Denis & Ramet (1989), who reported that the LPS caused growth inhibition, and eventually complete lysis of the bacteria cells. Accurate comparisons can not be made between this previously reported work and the work reported here because of the differences in the experimental procedures used in these two studies. The present study was conducted in raw milk and in p/s milk, which have different environmental conditions from UHT milk that was used by Denis and Ramet as a medium. The reasons for the failure of the system to induce lysis on the bacterial cells, particularly at lower temperatures where longer storage was possible, could not be established in this experiment. However Earnshaw and Banks (1989) suggested that *L. monocytogenes* may produce specific agents that effectively neutralises the bactericidal effect of the LPS such as : an enzyme that catalysis oxidation of $NADH_2$ by OSCN- ; an increase in cell sulphydryl groups ; phosphoenolpyruvate dependent phosphotransference system sugar transport mechanisms. Although

our results do not permit a decision as to which of the LPS antagonistic agents is liberated by *L.monocytogenes* in LPS activated milk, they do support the view that *L.monocytogenes* is not as susceptible to the LPS as some other bacteria.

3.3 EXPERIMENT III: THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF THE LPS ON *SALMONELLA ENTERITIDIS*.

3.3.1 Introduction

The presence of *Salmonella* in foods has become a matter of public concern in recent years. Recently, in Britain one government official made a vague statement about the presence of *Salmonella* in eggs. This was met by massive consumer boycott of eggs, consequently the officer had to sacrifice her job. Elsewhere in the world *Salmonella* is also a major problem e.g. in 1985, 7 500 cases of salmonellosis were reported in Canada (Mclean, 1987). In Illinois (USA), 1 600 people contracted salmonellosis (Lecos, 1986).

Milk and other dairy products have been incriminated as vehicles of human salmonellosis. (Bryan, 1983 ; Lecos,1986 ; Tacket *et al*, 1985). Consumption of raw milk had been strongly associated with human infection with *Salmonella*. Evidence for this association has been presented by West *et al* (1988), who studied the correlation of infection in inhabitants from known *Salmonella* positive dairy farms.

Contamination of bulk milk supplies may arise from a number of sources e. g. shedding of *Salmonella* from mammary glands of infected cows (Giles and Kay, 1987 ; Ogilve, 1986 ; Woods *et al*,

1984). Environmental contamination via dung and unwashed udders is also possible.

Even though pasteurisation can effectively kill this bacterium, the presence of *Salmonella* in milk still remains a matter of public health concern. This is true because some reports have been made of salmonellosis outbreak involving pasteurised dairy products , suggesting faulty pasteurisation or post pasteurisation contamination (D'Aoust, 1985 ; Lecos, 1986 ; Rowe *et al*, 1987). The tendency of some farm families to consume raw milk makes this hazard worse.

There is very little information about the antibacterial activity of the LPS on *Salmonella* in milk. Wray and Mclaren (1987) investigated the antibacterial activity of LPS on salmonellas using UHT milk as a medium. However no similar work has been done on raw or pasteurised milk.

The objective of this experiment was to determine the effect of temperature on the antibacterial activity of the LPS on *Salmonella enteritidis* in raw and p/s milk.

3.3.2 Methods and Materials: Bacteria cultures

The antibacterial activity of the LPS was tested on *Salmonella enteritidis* phage type 4. Broth cultures were grown by incubation at 37^{o}C for 18 hours.

Bacterial counts

Counts were done on YMA and on XLD agar (XLDA) plates. Plates were incubated at 37^{o}C for 24 hours.

Milk samples and sampling of counts

6 samples of raw milk and of p/s milk were inoculated with the *Salmonella* culture and activated with the LPS. An equal number of control samples were also prepared. Counts were measured at 0 hours of incubation, and at varying intervals in samples stored at different temperatures as shown in Table 6.

3.3.3 Results

When the activity of the LPS was tested on *S. enteritidis*, the results show that temperature affected the persistency of the LPS inhibitory effect as well as the death or survival rate of the bacterial cells.

3.3.3.1 The antibacterial effect of the LPS on *S.enteritidis* at $7^{o}C$

3.3.3.1.1 Controls

In control samples there was an initial lag phase of approximately 123 hours. After this phase, counts started to increase (Figure 15). Highest rises were observed in raw milk samples. Here counts grew from 10^4 to 10^6 after 200 hours. Meanwhile in p/s samples counts just rose from 10^4 to 10^5 after 200 hours. The positive correlation coefficient values indicate that in control samples counts were already growing rather than dying.

3.3.3.1.2 LPS treated samples

In samples stored at $7^{o}C$, the LPS was bactericidal to *S. enteritidis*. The results presented in Figure 15 show that the bacterial populations declined from 7×10^4 to 2×10^3 after 200 hours in XLDA counts. In raw milk bacterial cells were dying

TABLE 6

TABLE SHOWING SAMPLING PERIODS OF *S.ENTERITIDIS* COUNTS IN SAMPLES STORED AT DIFFERENT TEMPERATURES

Temperature (^{o}C)	Sampling periods (hours)					
7	0	18	24	48	73	
	96 or 97	118	141	or 145	166	
	214					
15	0	2.5	6	10	17	
	21.5	25	30 or 32	49		
22	0	1 or 2	3 or 4	5 or 7		
	8 or 9	10 or 11				
25	0	1 or 1.5	2.5	3	4	
	5					
30	0	1 or 1.5	2.5	3	4	
	5					

Key to symbols in Figures 15 - 19

1.PSXATR = COUNTS FROM P/S MILK + LPS ON XLD AGAR

2.PSYATR = '' '' '' '' '' ON YMA

3.RWXATR = '' '' RAW MILK '' '' ON XLD AGAR

4.RWYATR = '' '' '' '' '' '' ON YMA

5.PSXACON = '' '' P/S MILK CONTROLS ON XLD AGAR

6.PSYACON = '' '' '' '' '' '' YMA

7.RWXACON = '' '' RAW '' '' '' XLD AGAR

8.RWYACON = '' '' '' '' '' '' YMA

S.ENTERITIDIS COUNTS AT 7 C
(Treated and control samples)

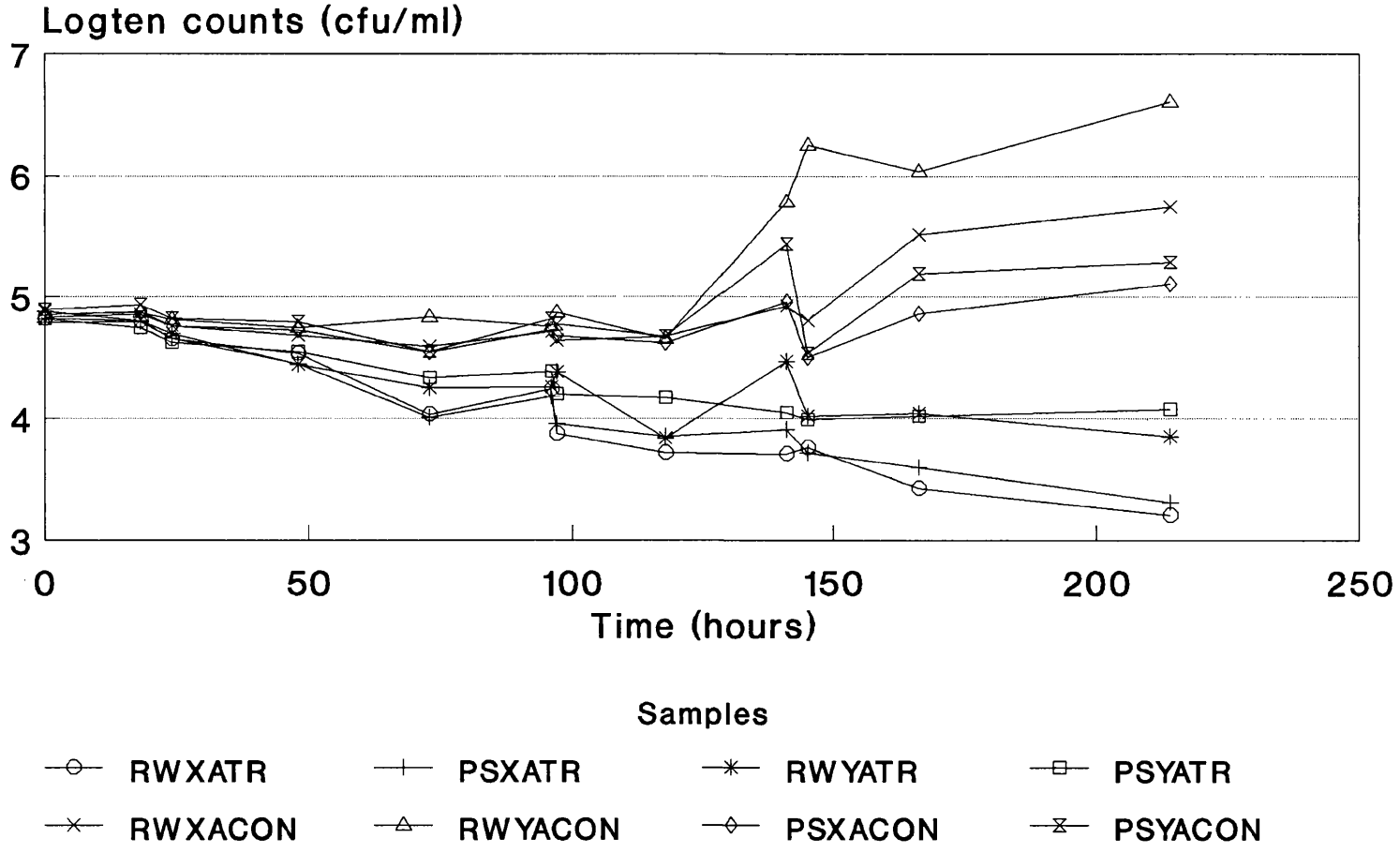


figure 15

S.ENTERITIDIS COUNTS AT 15 C
(Treated and control samples)

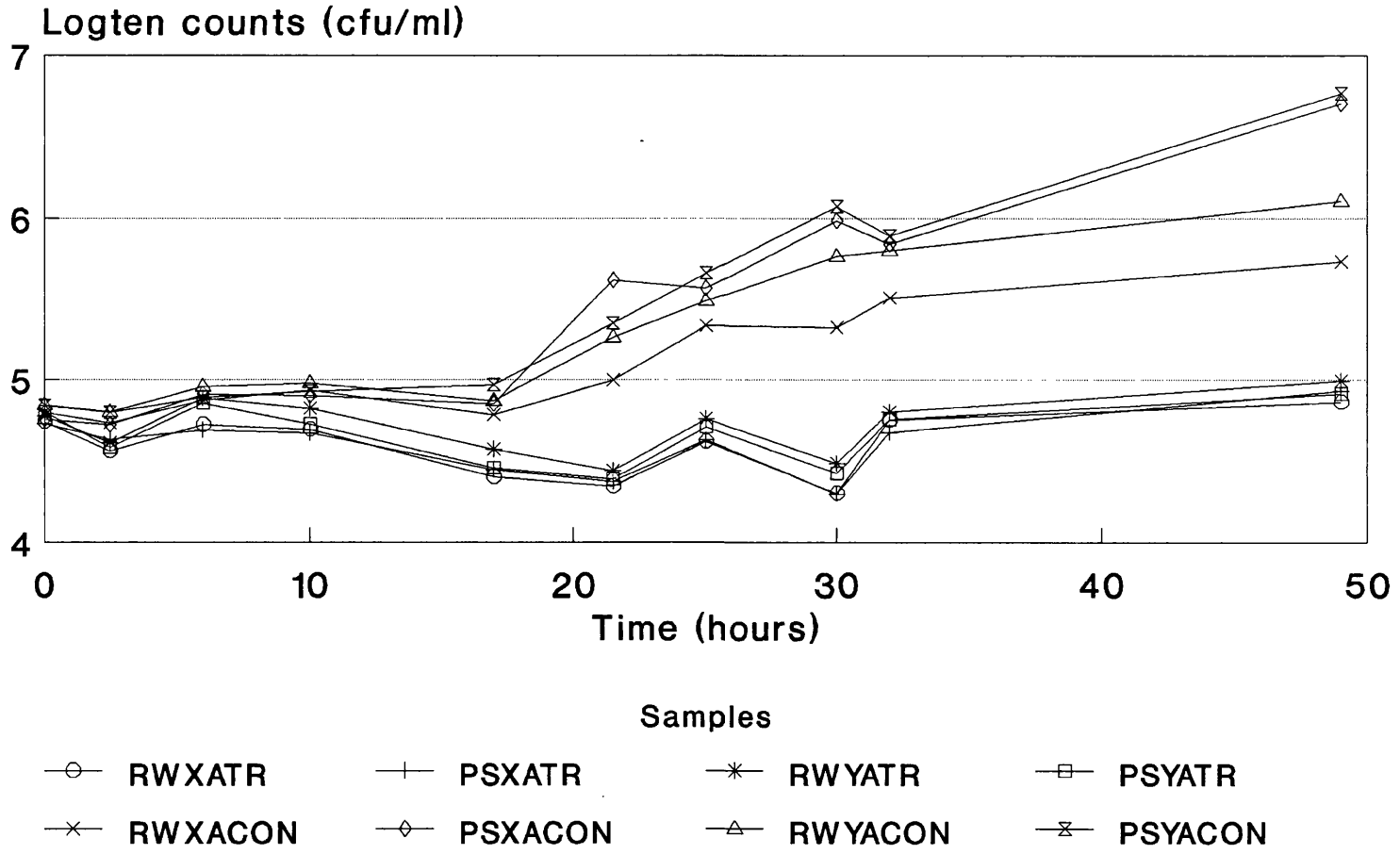


figure 16

S.ENTERITIDIS COUNTS AT 22 C
(Treated and control samples)

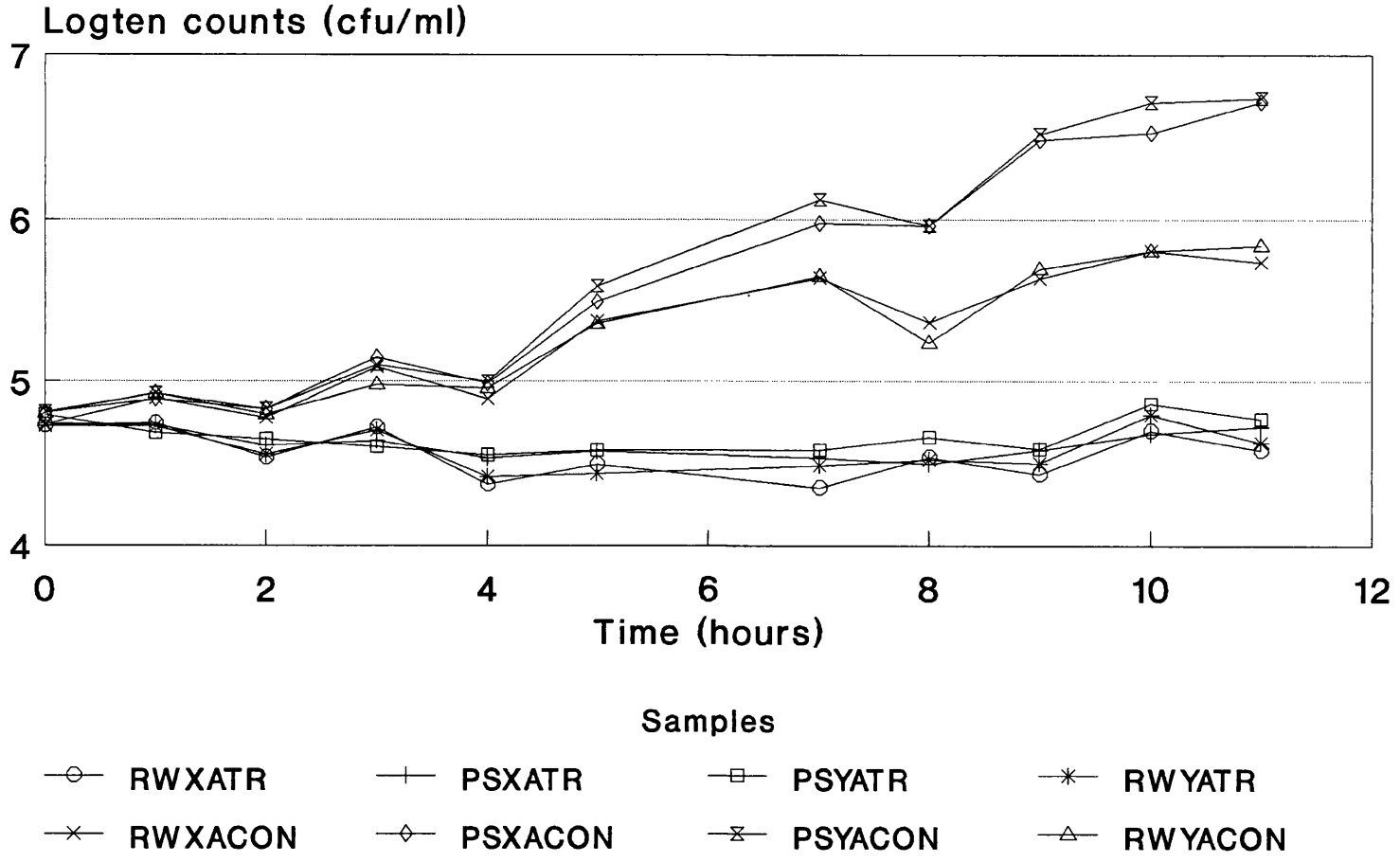


figure 17

S.ENTERITIDIS COUNTS AT 25 C
(Treated and control counts)

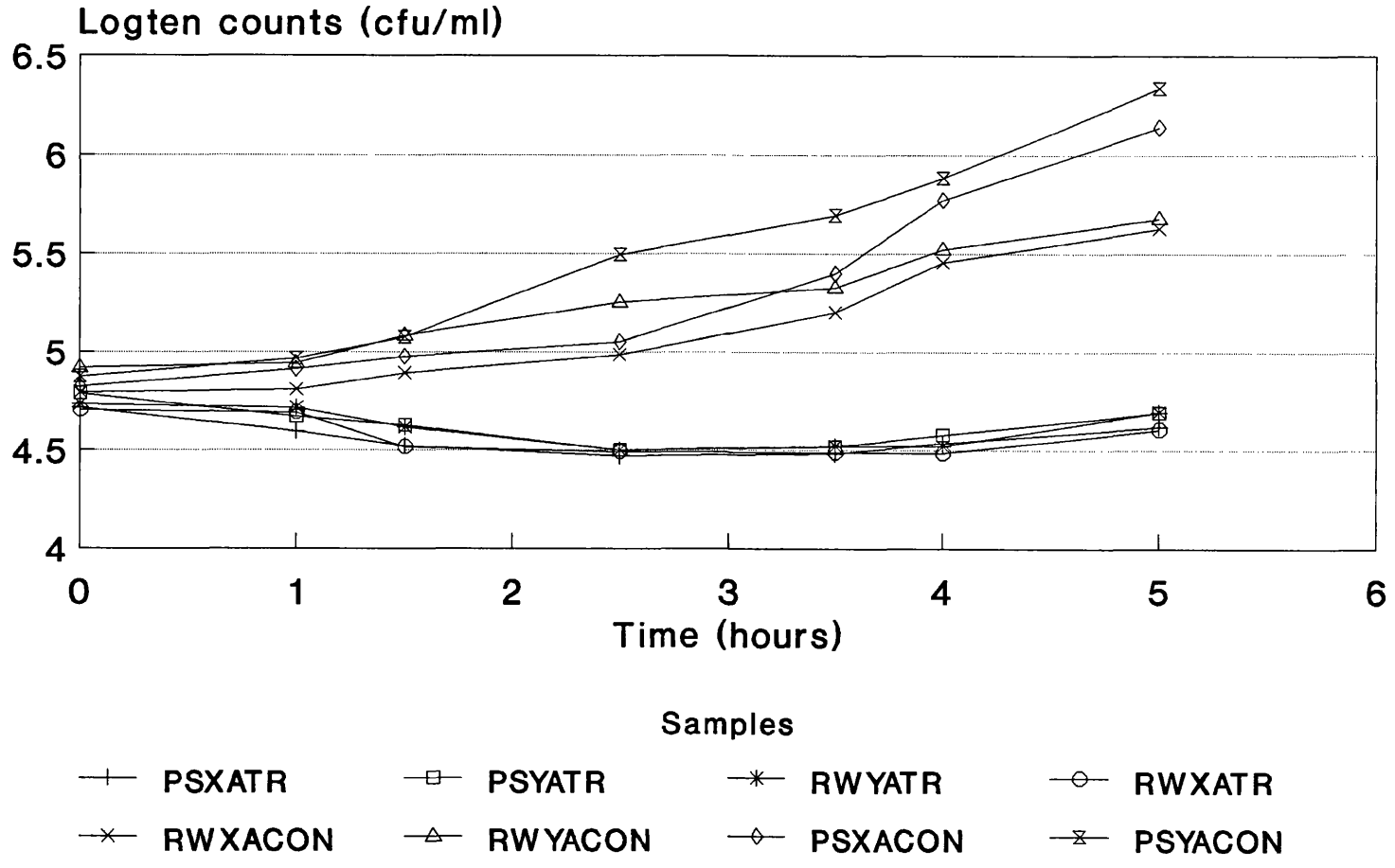


figure 18

S.ENTERITIDIS COUNTS AT 30 C
(Treated and control samples)

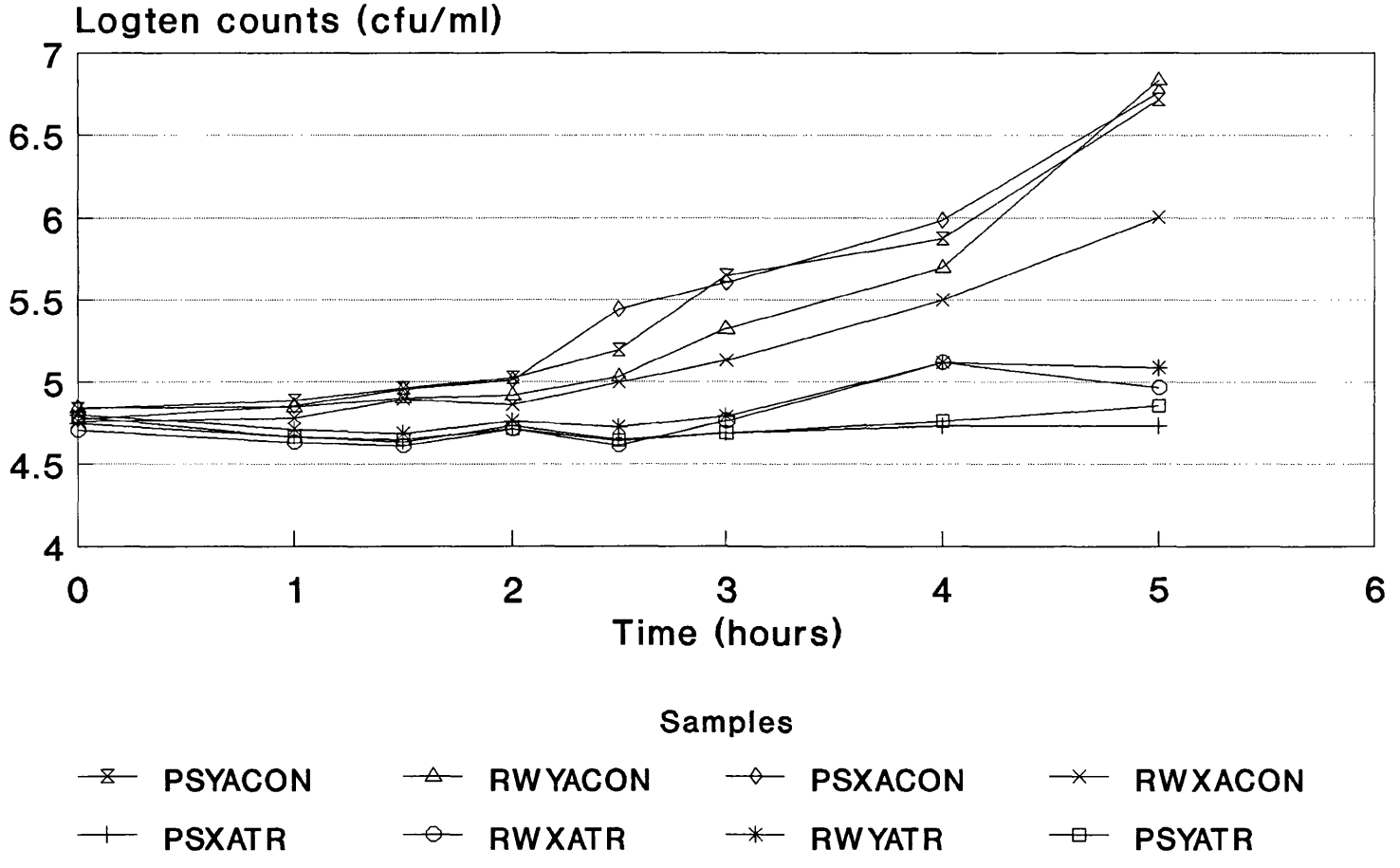


figure 19

quicker than in p/s samples. This is indicated by the lower D - value (123.92 hours) in the former compared with (145.8 hours) in the latter. The D - values from YMA counts are significantly higher than those from XLDA (Table 8). In this case, this may be attributed to the presence of indigenous milk flora.

However in all the samples , the correlation coefficient values were high (-0.832 to -0.971). This is an indication that as the incubation time was increasing the bacterial cells were decreasing.

3.3.3.2 The activity of the LPS on *S. enteritidis* at 15^{o}C

3.3.3.2.1 Controls

The results presented in Figure 16 show the behaviour of *S.enteritidis* in the absence of the LPS. In these control samples, *Salmonella* populations increased from 10^4 to 10^6 after 48 hours. The dynamics of the increase were similar in raw milk and in p/s milk samples. In all control samples, there were initial lag phases of approximately 18 hours.

3.3.3.2.2 LPS treated samples

In Figure 16 the results showing the behaviour of *S.enteritidis* in the presence of the LPS in samples stored at 15^{o}C are presented. The results show that in the treated samples, the bacterial populations decreased slightly from 6 x 10^4 to 2 x 10^4 after 30 hours. The dynamics of decrease was similar in all the treated samples. In all the treated samples, the bacterial populations were significantly lower than in the corresponding controls.

3.3.3.3 The antibacterial activity of the LPS on *S.enteritidis* at 22°C

3.3.3.3.1 Controls

Figure 17 shows that in the control samples a lag phase was observed during the first 4 or 5 hours. After the lag phase counts in p/s samples grew from 10^4 to 10^6 after 11 hours, while in raw milk samples the counts reached 10^5 after 11 hours.

3.3.3.3.2 LPS treated samples

In LPS treated samples, the populations of salmonella cells decreased slightly for the first 7 hours of incubation (Figure 17). The correlation coefficient values are higher in p/s samples than in raw milk samples (Table). This may suggest that the bacterial cells died better in p/s samples than in raw milk samples at this temperature of storage. However it was observed that in all the treated samples counts remained stable at 10^4 even after 11 hours.

3.3.3.4 The activity of the LPS on *S.enteritidis* at 25°C

3.3.3.4.1 Controls

In control samples (Figure 18.) there was a lag phase of 1 hour after which counts increased from 10^4 to 10^5 or 10^6 after 5 hours was observed. Even in these samples correlation coefficient values were higher in p/s samples than in raw milk samples.

3.3.3.4.2 LPS treated samples

The results presented in Figure 18 show that the *Salmonella* counts declined very slowly in samples that were treated with the LPS. The correlation coefficient values are slightly higher in p/s samples than in raw milk samples, although the difference is not significant.

Even though the counts from LPS treated samples ceased to decrease after 3 hours, they remained stable at 10^4 cfu /ml for up to 5 hours. Meanwhile in the control samples counts had already reached 10^6 after 5 hours.

3.3.3.5 The activity of the LPS on *S.enteritidis* at 30°C.

3.3.3.5.1 Controls

In the control samples counts had increased from 10^4 to 10^6 after 5 hours.

3.3.3.5.2 LPS treated samples

At 30°C the decrease in the bacterial populations was infinitesimally small and short-lived. Figure 19 shows that in all the treated samples counts declined slowly for the first 1.5 hours and then remained constant at 10^4 . In raw milk samples counts started to increase slightly after 3 hours, while in p/s samples the counts remained constant for up to 5 hours. However in all the LPS treated samples, counts were significantly lower than in the controls for up to 5 hours of incubation.

3.3.4 Discussion

The results show that the activity of the LPS on *S.enteritidis* was bactericidal. This bactericidal effect was affected by temperature and the type of milk sample used. Estimation of counts was also affected by type of plating agar used.

At lower temperatures the activity of the LPS persisted for a longer time, and the death rate of the bacterial cells was also slower. It was possible to observe D-values only at 7°C and it was noted that these D-values were also high, indicating a slow

death rate. At 7°C it was possible to calculate D-values because bacterial numbers continued to declined over a relatively long period (>200 hours). At higher incubation temperatures accurate calculation of D-values was impaired due to the recovery and growth of cells after a relatively short period of declining numbers.

At higher temperatures the bactericidal effect of LPS lasted for a short time, and this was associated with an increase in the growth rate of the bacterial cells in the control samples. It can be suggested that at the higher temperatures the recovery from growth inhibition of surviving cells is quicker than the bactericidal strength of the LPS against *S.enteritidis*.

The activity of the LPS was also affected by the type of milk medium used as samples. At lower temperatures, (7 & 15 °C), the death rate of bacterial cells was quicker in raw milk than in p/s samples. But at higher temperatures, the inverse was true. This might be attributed to the presence of background microflora in the milk samples. In raw milk samples the initial bacterial counts were higher than in p/s samples. This means that there were more foreign bacteria rather than the *Salmonella* in the former than in the latter. At lower temperatures there is less interference from these bacteria on the LPS activity because of their restricted growth. While at higher temperatures these foreign bacteria are growing freely, hence their interference on the LPS activity becomes obvious. This suggestion is also supported by the fact that YMA counts from raw milk were higher than the counts from the other samples, particularly at higher

temperatures.

As it was mentioned previously in the case of *E.coli* it is possible that catalase is playing a part in counteracting the activity of the LPS against the *Salmonella* in raw milk.

It is not clearly understood why the results of this experiment are slightly different from previously reported results (Wray & Mclaren, 1987), who observed that *Salmonella* died quicker in LPS treated samples. However, it can be noted that the work of Wray and Mclaren was conducted under different conditions. In the present experiment, a different species of *Salmonella* was used, test samples were prepared from raw milk and p/s milk rather than UHT milk, and the pH of the test samples was not altered. Probably it can be suggested that *S.enteritidis* is more resistant to the bactericidal effect of the LPS than the other species tested by the above authors.

3.4.0 EXPERIMENT IV: THE EFFECT OF TEMPERATURE ON THE ANTIBACTERIAL ACTIVITY OF THE LPS ON *YERSINIA ENTEROCOLITICA*

3.4.1 Introduction

The genus *Yersinia* belongs to the family Enterobacteriaceae. *Yersinia enterocolitica* is a facultative anaerobic, gram negative, short rod shaped bacterium (Cowan, 1974). At present this bacterium is recognised as a cause of gastroenteritis, mesenteric lymphademitis, and pseudoappendicitis in small children, while in adults it causes acute abdominal disorders, diarrhoea, arthritis and erythema nodosum which is the most striking symptom in the older age group (Winbald,

1973 ; Palumbo, 1986). Foods of animal origin are the major sources of human Yersinosis infection.

Although *Y.enterocolitica* can grow readily in the presence of other meat microflora in beef and pork (Hanna *et al*,1977), Stern *et al* (1980) found that when inoculated into raw milk these bacteria were poor competitors. However , evidence of cases of *Y.enterocolitica* that had been isolated from raw and from pasteurised milk do exist (Hughes, 1979 ; Moustafa *et al*, 1983). These bacteria have also been isolated from other dairy products such as ice cream, cream, cheese, and chocolate milk drinks (Lee, 1977). Most recently it has been reported that these organisms can survive in yoghurt (Ahmed *et al*, 1986).

In several countries milk and other dairy products have been incriminated as active vehicles of Yersinosis. In Canada, raw milk was responsible for an infection of 58 school children with *Y.enterocolitica* (Bryan, 1982). In New York (USA), chocolate milk drink was implicated as an epidemiological vehicle in Yersiniosis outbreaks (Black *et al*, 1978). Kruumbiegel (1970), reported that pasteurised milk could be implicated as a vehicle of *Y.enterocolitica* infection that resulted in several cases of appendectomies. Even reconstituted powdered milk was incriminated as a potential carrier of *Y.enterocolitica* (Shangani *et al*, 1983).

The fact that *Y.enterocolitica* had been isolated from pasteurised dairy products means that this organism is a potential public health hazard. This is made worse by the fact

that the bacteria can grow well even under normal refrigeration temperatures i.e. 1 to 7°C (Hanna *et al*, 1977). Also this organism can survive alkaline and slight acid condition, a point which raises some uncertainties about the effectiveness of some of the dairy equipment cleaning systems as means of controlling these bacteria (Aulisio *et al*, 1980 ; Stern *et al*, 1980).

The antibacterial activity of the LPS has been reported on many food poisoning and spoilage bacteria such as : *Salmonella* (Wray & Mclaren, 1987) ; *L.monocytogenes* (Denis & Ramet, 1989 ; Earnshaw & Banks, 1989) ; *C.jejuni* (Beumer *et al*, 1985 ; Borch *et al*, 1989). However nothing has been reported about the effect of the antibacterial activity of the LPS on *Y.enterocolitica*.

The objective of this experiment was two fold : to determine the sensitivity of *Y.enterocolitica* to the LPS ; and to determine the effect of temperature on the antibacterial activity of the LPS on *Y.enterocolitica*.

3.4.2 Materials and Methods

3.4.2.1 Bacterial Cultures

The antibacterial activity of the LPS was tested on *Yersinia enterocolitica* serotype 0:5,27 a milk isolate. Broth cultures were grown by incubation at 25°C for 18 hours.

3.4.2.2 Bacterial counts

Counts were done on YMA and on Bacto Yersinia selective agar (BYSA) from (DIFCO laboratories Detroit Michigan USA). Plates were incubated at 25°C for 48 hours.

3.4.2.3 Milk samples and sampling of counts

Five samples of raw and of p/s milk were prepared and activated with the LPS. An equal number of control samples were also prepared.

Measurement of counts was done at 0 hours of incubation, and there after at different intervals on samples stored at different temperatures, as shown in Table 7.

3.4.3 Results

The antibacterial activity of the LPS on *Y.enterocolitica* was tested in raw and p/s milk samples stored at different temperatures. The results shows that the activity of the LPS was bactericidal to *Y.enterocolitica* in all the samples stored at different temperatures. The results also shows that temperature affected the persistency of the bactericidal effect of the system, as well as the death rate of the *Yersinia* cells.

3.4.3.1 The antibacterial activity of the LPS on *Y.enterocolitica* at $7^{o}C$

3.4.3.1.1 Controls

In the control samples, there was moderate increase of *Yersinia* populations during the incubation period. Figure 20 shows that in the raw milk samples BYSA counts increased from 10^4 to 10^6 after 95 hours, and the YMA counts had increased to 10^8 after the same period of incubation. In p/s samples there was a slight increase of BYSA the counts from 10^4 to 10^7 after 95 hours, while TBC increased to 10^8.

It can be pointed out that in the counts from raw milk control samples an initial lag phase of approximately 40 hours

TABLE 7

TABLE SHOWING SAMPLING PERIODS *Y.ENTEROCOLITICA* COUNTS FROM SAMPLES STORED AT DIFFERENT TEMPERATURES

Incubation temperature (^{o}C)	sampling Periods (hours)				
7	0	24	42.5 or 50.5	77	99
15	0	5	10	16	22 or 23
	29	41	48		
22	0	3.5	6.5	9	12.5
	16	19	24		
25	0	1.5 or 2	4 or 5	6 or 7.5	
	9	11			
30	0	1.5 or 2	4 or 5	6 or 7.5	
	9	11			

Key to symbols on Figures 20 - 24

1.PSBATR = COUNTS FROM P/S + LPS MILK ON BYSA

2.PSYATR = '' '' '' '' '' '' YMA

3.RWBATR = '' '' RAW MILK + LPS ON BYSA

4.RWYATR = '' '' '' '' '' '' YMA

5.PSBACON = '' '' P/S MILK CONTROLS ON BYSA

6.PSYACON = '' '' '' '' '' '' YMA

7.RWBACON = '' '' RAW MILK '' '' BYSA

8.RWYACON = '' '' '' '' '' '' YMA

Y.ENTEROCOLITICA COUNTS AT 7 C
(Treated and control samples)

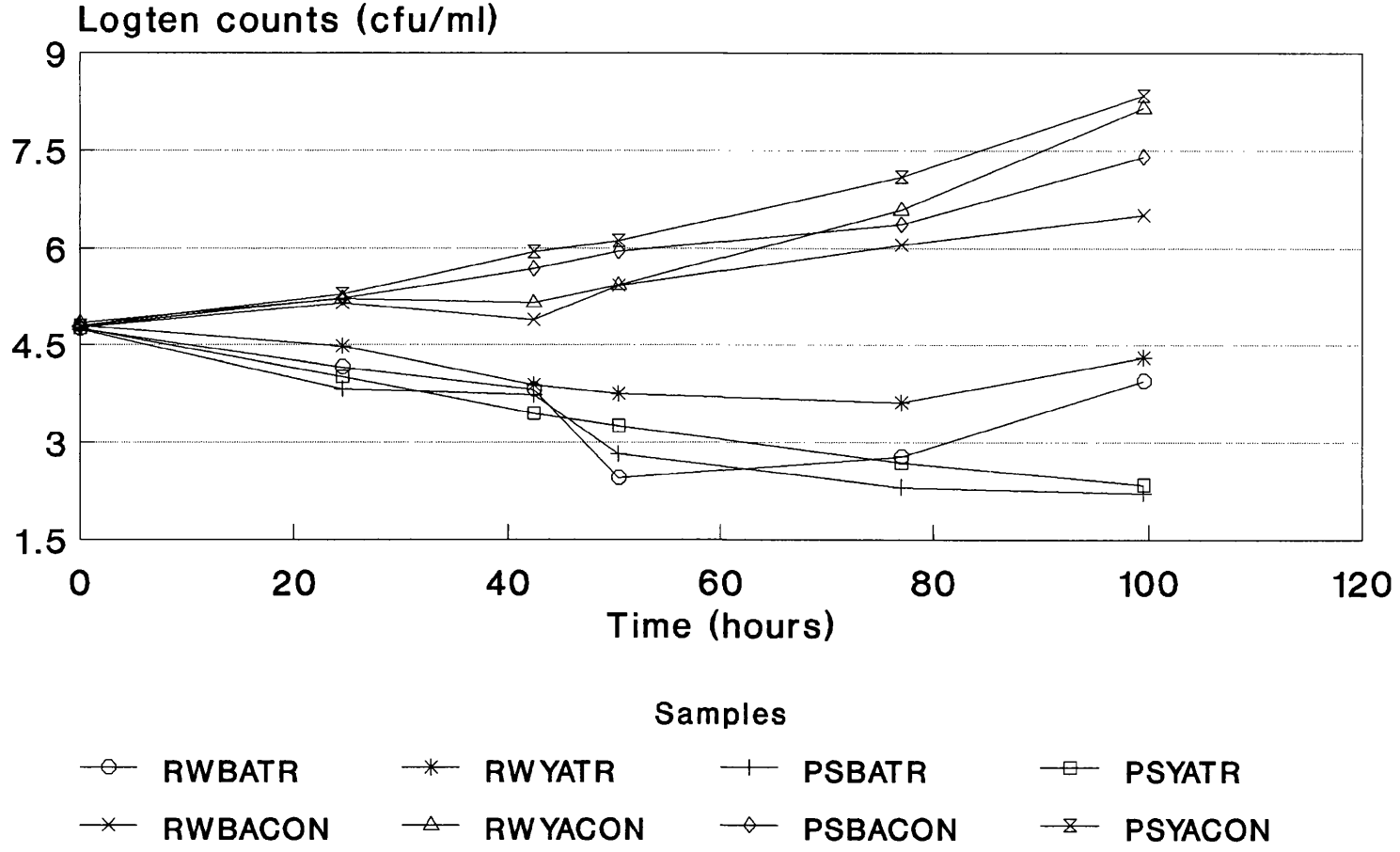


figure 20

Y.ENTEROCOLITICA COUNTS AT 15 C
(Treated and control samples)

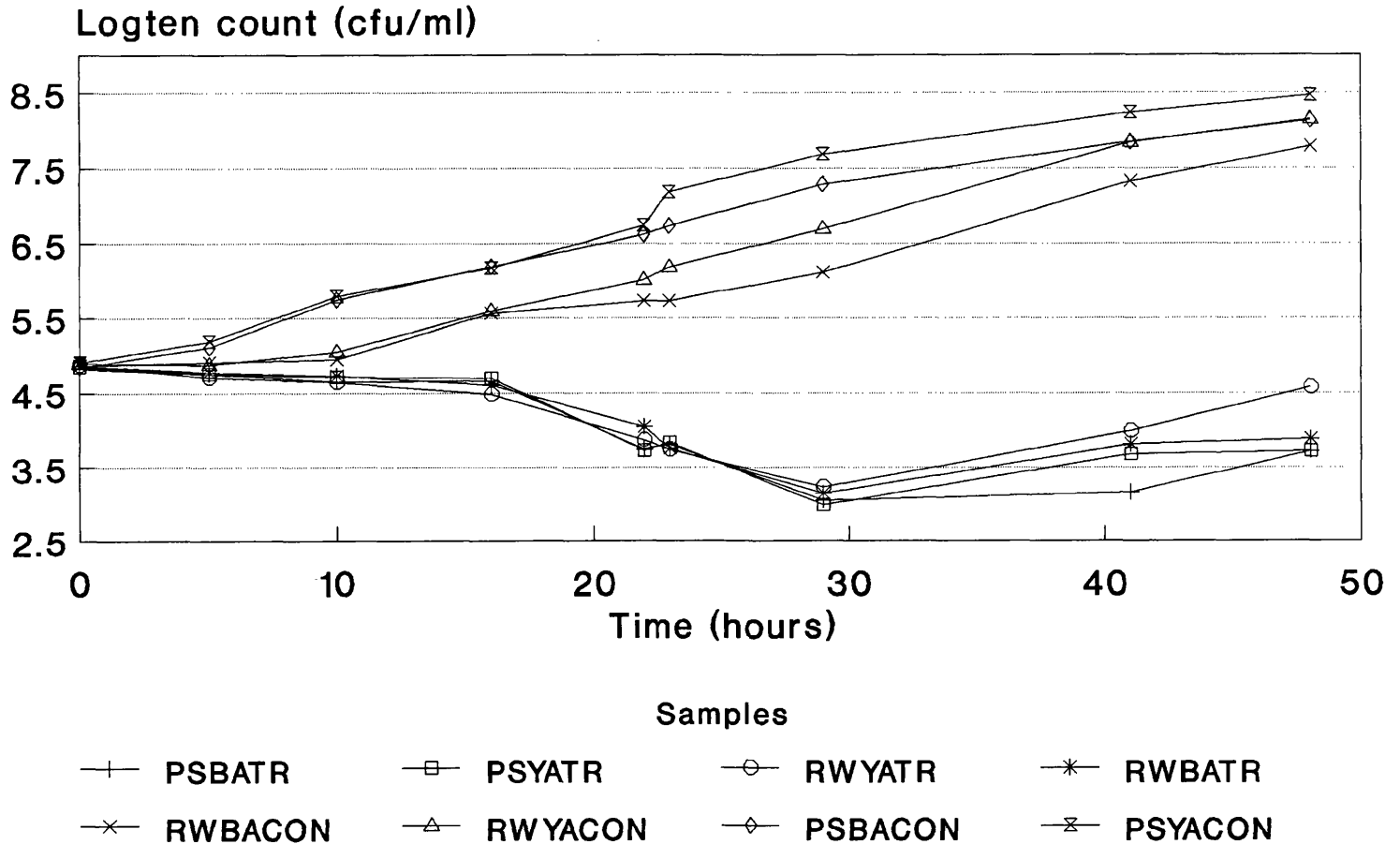


figure 21

Y.ENTEROCOLITICA COUNTS AT 22 C
(Treated and control samples)

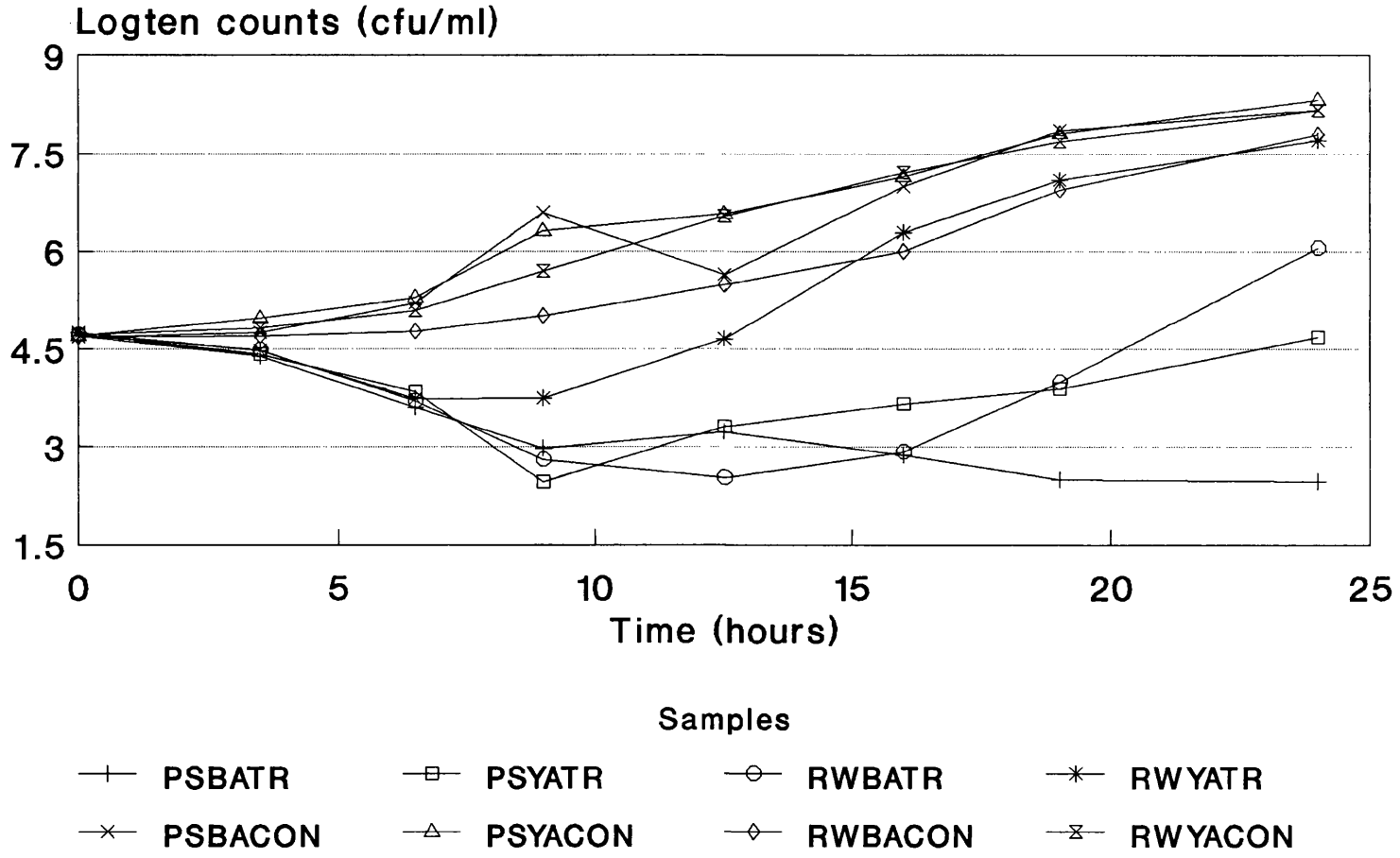


figure 22

Y.ENTEROCOLITICA COUNTS AT 25 C
(Treated and control samples)

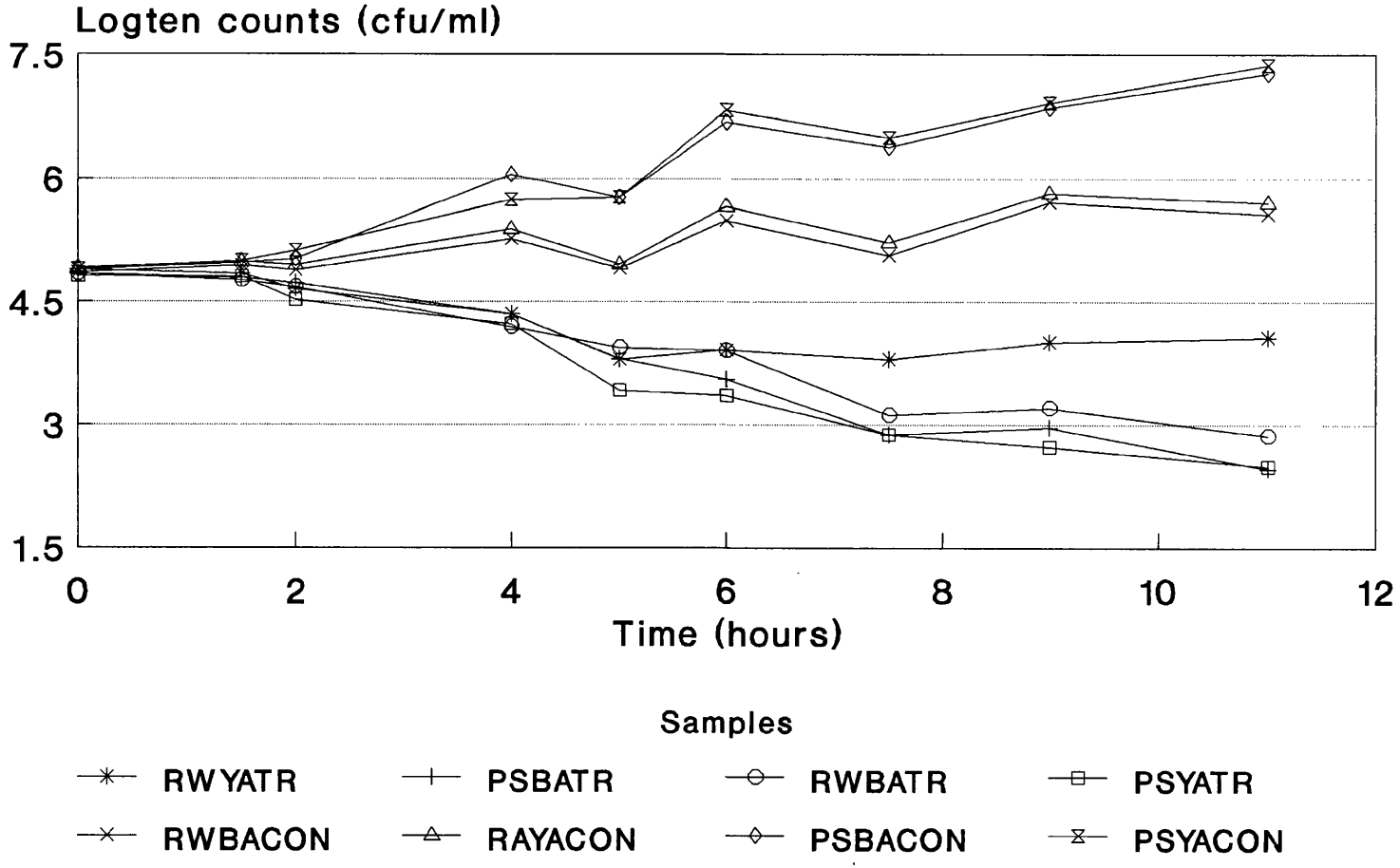


figure 23

Y.ENTEROCOLITICA COUNTS AT 30 C
(Treated and control samples)

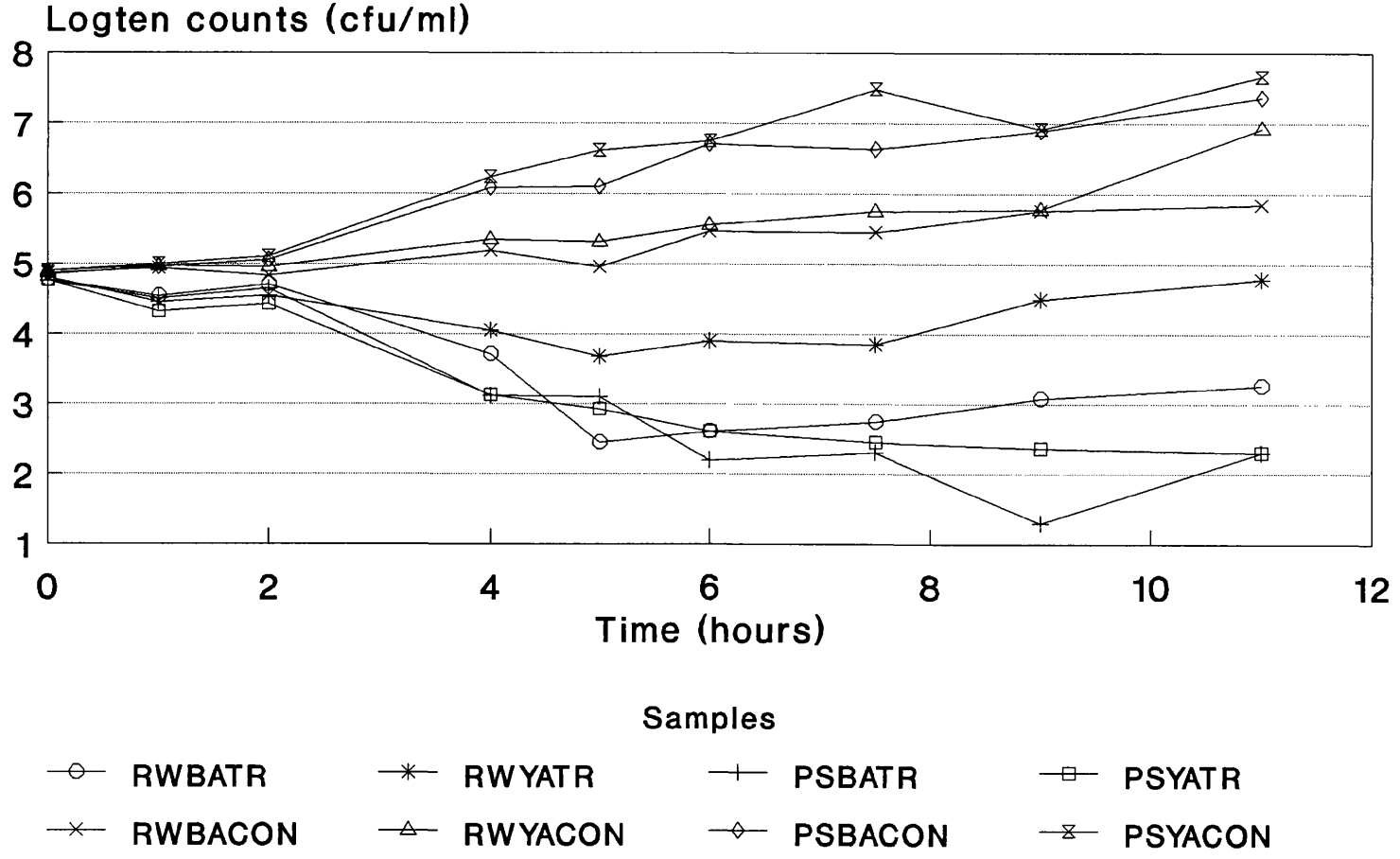


figure 24

was observed. Probably this lag phase is responsible for the slightly lower correlation coefficient values from these samples than the values from p/s milk samples.

3.4.3.1.2 LPS treated samples

The results presented in Figure 20 show that the LPS was bactericidal to *Y.enterocolitica* in samples stored at 7°C. In the treated raw milk samples, *Yersinia* counts declined from 10^4 to a minimum of 10^2 after 50 hours, but after 90 hours counts had increased to 10^3. Meanwhile in p/s treated samples, *Yersinia* populations decreased from an initial level of 10^4 to 1 x 10^2 after 99.5 hours.

From the results it would seem that the bactericidal effect of the LPS at 7°C persisted longer in p/s samples than in raw milk samples. However the D-values from BYSA counts were not significantly different on p/s and raw milk samples. (31.4 and 35.97 hours). The D-value of raw milk YMA counts was slightly higher. It may be suggested that the death rate of TBC in raw milk was slow.

In all the treated samples stored at 7°C it was observed that the correlation coefficient values were higher than -0.900. This indicated strong linearity of declining cell counts with increase of incubation time.

3.4.3.2 The activity of the LPS on *Y.enterocolitica* in samples stored at 15°C

3.4.3.2.1 Controls

In the control samples, it was observed that on raw milk counts, there was an initial lag phase of 10 to 12 hours after

which counts started to rise from 10^4 to 10^6 after 29 hours. Yet in p/s control samples such a lag phase was not noticeable. Instead, counts were increasing steadily from these samples, starting from 10^4 at time 0 hours to 10^8 after 40 hours. All the correlation coefficient values from the control samples were higher than 0.900

3.4.3.2.2 LPS treated samples

Figure 21 shows the behaviours of *Y.enterocolitica* in raw and p/s milk samples that were activated with the LPS and stored at 15°C. These results show an unusual feature in that the rate of bacterial population decrease was slower during the first 12 to 18 hours of incubation, after which counts started to decrease sharply from 10^4 to 10^2 after 29 hours of incubation.

The results also show that after 41 hours, the counts from the treated samples had started to rise. There is no significant difference in the dynamics of *Yersinia* decrease between raw and p/s milk samples. In all the treated samples the correlation coefficient values were higher than -0.900, indicating a strong linearity of decreasing cells with increase of incubation time. There were not significant differences observed amongst D-values of raw and p/s milk samples, and of YMA and BYSA counts. However the D- values from YMA counts were slightly higher than those from BYSA.

3.4.3.3 The activity of the LPS on *Y.enterocolitica* in samples stored at 22°C

3.4.3.3.1 Controls

The behaviour of *Y.enterocolitica* in control samples stored

at 22°C is shown in Figure 22 . As it can be seen from these results, in all milk samples there was an initial lag phase of approximately 5 hours. After this phase counts rose from 10^4 to 10^7 after 24 hours in the raw milk samples. Meanwhile in the p/s samples counts rose from 10^4 to 10^8 after 24 hours.

3.4.3.3.2 LPS treated samples

The results presented in Figure 22 show the behaviour of *Y.enterocolitica* in the presence of the LPS at 22°C. As it can be seen from the results , the antibacterial activity of the LPS on *Yersinia* persisted for up to 24 hours of incubation in p/s samples. On the contrary, in raw milk samples the persistency of the system was shorter. After 12 hours, *Yersinia* counts from these samples started to increase. However there were no significant differences observed on D- values of raw and p/s milk samples, although these values were slightly higher in raw milk than in p/s samples.

There was a remarkable difference in the recovery of surviving *Yersinia* cells from growth inhibition by the LPS in raw milk and in p/s milk samples. In the former counts had increased to 10^6 after 24 hours, meanwhile in the latter samples, counts were still trailing at 10^2 after 24 hours.

It was also observed that on raw milk TBC the antibacterial activity persisted for a short time. These counts started to increase after 8 hours of incubation.

3.4.3.4 The activity of the LPS on *Y.enterocolitica* in samples stored at 25°C

3.4.3.4.1 Controls

Figure 23 presents the results from control samples stored at 25°C. It is noteworthy that growth was relatively poor in raw milk controls compared with p/s controls. The raw control sample counts increased after an initial lag phase of 3 to 4 hours from 10^4 to 10^5 after 11 hours, meanwhile in p/s samples the increase of bacterial populations were more noticeable, and the counts had increased from the initial level of 10^4 to 10^7 in 11 hours. The correlation coefficient values were slightly higher in these samples (0.945 & 0.957) than the values from raw milk samples (0.769 & 0.812). All the above observations indicate that the growth of *Y.enterocolitica* was better in p/s samples than on raw milk samples.

3.4.3.4.2 LPS treated samples.

Results showing the behaviour of *Y.enterocolitica* in samples activated with the LPS stored at 25°C are presented in Figure 23. The results show that in raw milk YMA counts there was a sharp decline of bacterial counts from 10^4 to 10^3 after 5 hours. This was followed by a period of 5 hours during which numbers remained approximately constant. This contrast with the results obtained in p/s milk on YMA and the BYSA counts from p/s and raw milk samples which all demonstrated a steady decline throughout the incubation period of 11.5 hours.

There is no significant difference among the D-values from these samples. However it was observed that the D value from raw

milk YMA counts was slightly higher and the correlation coefficient from these counts was slightly lower (-0.808).

3.4.3.5 The activity of the LPS on *Y.enterocolitica* at 30°C

3.4.3.5.1 Controls

The results of the control samples (Figure 24) show that the growth of *Yersinia* was better in p/s than in raw milk samples. In the latter, after an initial lag phase of two hours, cells increased slightly from 10^4 to 10^6 after 11 hours, meanwhile in p/s samples counts had increased from 10^4 to 10^7 after 11 hours.

3.4.3.5.2 LPS treated samples

Results showing the behaviour of *Y.enterocolitica* in samples activated with the LPS and stored at 30°C are presented in Figure 24. The results show that the bactericidal effect of the LPS persisted longer (11 hours) in p/s samples than in raw milk samples (5 to 6 hours). In p/s samples *Yersinia* counts decreased slowly during the first 1.5 or 2 hours, then gradually decreased from 10^4 to 2 x 10 after 8 hours. On the contrary, in raw milk samples, the *Yersinia* counts decreased sharply after the initial slow decline, from 10^4 to 10^2 after 5 hours. This was followed by a short lag phase for 2 hours , after which counts started to show an increase .

The highest D-value was observed in raw milk YMA counts (6.67hours). In overall the D-values were lower in p/s samples (2.56 and 3.61 hours), than in raw milk samples (2.87 and 6.67 hours). From these results it can be deduced that the antibacterial activity of the LPS on *Y.enterocolitica*, at 30°C,

persisted longer in p/s samples than raw milk samples. And also the death rate of the bacteria was quicker in the former than in the latter.

3.4.4.0 Discussion

Y.enterocolitica is a peculiar food born pathogen in that it grows even under a storage temperature as low as 1 to 7^{o}C (Hanna *et al* 1977). The presence of this bacteria in raw milk may be considered a public health hazard because there have been several cases of Yersiniosis outbreaks where pasteurised dairy products were incriminated.

Previous reports have investigated possibilities of exploiting the LPS in improving the hygienic quality of dairy foods by controlling food pathogens such as *Salmonellas, L.monocytogenes*, and *C.jejuni*. Most of these bacteria were found to be susceptible to the antibacterial activity of the LPS, however no similar work has been done on the bacteria *Yersinia enterocolitica*. In the present experiment the antimicrobial activity of the LPS and the influence of temperature on the activity of this system against *Y.enterocolitica* were investigated.

The results from this study have shown that the antibacterial activity of the LPS was bactericidal to *Y.enterocolitica*. The storage temperature of treated samples affected the persistency of the bactericidal effect as well as the death rate of the bacterial cells.

The antibacterial activity of the LPS complex persisted longer at lower temperatures than at higher temperatures, 77 to 99 hours and 6 to 11 hours in samples stored at 7°C and 30°C respectively. The bacterial death rates were higher in samples stored at higher temperatures than those stored at lower temperatures, however the recovery of surviving cells from the antibacterial activity of the LPS was quicker in samples stored at higher temperatures than those stored at lower ones. A quicker recovery was observed in raw milk samples than in p/s milk samples.

The results also shows that the antibacterial activity of the LPS varied between raw and p/s milk samples. In p/s milk samples the bactericidal effect of the system persisted for a longer time than in raw milk samples at all the incubation temperatures. It was also observed that the recovery of surviving cells was more rapid in raw milk than in p/s samples.

The reasons for the better performance of the LPS activity in p/s samples than in raw milk samples are still obscure. One possible reason could be the effect of the initial background microflora. It would seem that presence of this background microflora in the raw milk samples interfered with the bactericidal effect of the LPS, possible by competing with the *Yersinia* for the LPS bactericidal complex (OSCN-). This suggestion is supported by the observation that in the non selective YMA counts from raw milk samples, the antibacterial activity of the LPS was even shorter, and recovery of surviving cells from inhibition, was more rapid than in the counts from

selective agar samples where only *Yersinia* cells were counted.

Another possible reason for the poor performance of the LPS in raw milk samples could be attributed to the differences in the chemical environments of the raw and p/s milk samples. Due to the high heat treatment involved in the preparation of p/s milk samples, it would be expected that these samples may have lower concentration of enzymes such as catalase than the raw milk samples. This means that there will be less competition for the H_2O_2 between the catalase and SCN- in the p/s than in the raw milk samples , hence more antibacterial ions (OSCN-) will be available in the former than in the latter.

However the reasons why *Yersinia* recover from inhibition by the LPS activity, are still not well understood. But the results from this experiment have demonstrated that the antibacterial activity can be exploited to control the problems caused by *Y.enterocolitica* in milk, particularly in pasteurised milk.

CHAPTER IV: DISCUSSION AND CONCLUSIONS

4.1 Introduction

The antimicrobial activity of the LPS had been investigated on many milk spoilage bacteria such as *Pseudomonads* and *E.coli* (Bjorck *et al*, 1975). Zajac *et al* (1983), investigated the possibility of using the LPS to extend the keeping quality of pasteurised milk. Meanwhile the activity and mechanism of the LPS against known food pathogens such as *Salmonella* (Wray & Mclaren, 1987), *Listeria* (Earnshaw & Banks, 1989), and *Campylobacter* (Borch *et al*, 1989) have been investigated using UHT milk as substrate. There are some reports which indicate that the system can improve the hygienic quality of cheeses (Denis & Ranet, 1989 ; Reiter & Harnulv, 1982).

Previous studies have reported that factors such as pH, concentration of SCN- and initial bacterial count, can affect the activity of the LPS (Reiter *et al*, 1975). Zajac *et al* (1983) observed that temperature affected the influence of the the LPS on the keeping quality of raw and pasteurised milk. However the effect of temperature on the activity of the LPS on specific food poisoning bacteria had not been investigated.

The main purpose of this study was to investigate the effect of temperature on the antibacterial activity of the LPS on *E.coli, Y.enterocolitica, S.enteriditis* and *L.monocytogenes*. In this study it was noted that temperature affected the activity of the LPS on individual species of bacteria differently, and also affected the behaviour of the bacteria in different milk medium activated with the LPS.

4.1.1 The influence of temperature on the activity of the LPS.

In order to investigate the influence of temperature on the antibacterial activity of the LPS, experiments were carried out at 7°C, 15°C, 22°C, 25°C, and 30°C. The results show that temperature affected the persistency of the LPS antibacterial activity, the death rate of bacteria, as well as the recovery of surviving bacteria from inhibition by the LPS.

At lower temperatures, the antibacterial activity of the LPS persisted longer, but as incubation temperatures were increased, the persistency of the antibacterial activity decreased. This observation paralleled previous reports, which stated that the duration of the antibacterial activity of the LPS is inversely related to temperature (Korhonen, 1982 ; Martinez *et al*, 1988).

However the results from this study do not concur with earlier reports by Bjorck *et al* (1975), who stated that bacterial cells were already multiplying after 24 hours in samples activated with the LPS and stored at 5°C, while in the present experiments bacterial inhibition by the LPS persisted for over 90 hours in samples stored at 7°C. It is difficult to make accurate comparisons between the above experiments, because of the different conditions under which the experiments were conducted. Bjorck and his colleagues prepared their sample media from whey, and used glucose oxidase as a source of H_2O_2, while in the present experiment the activity of the LPS was investigated in raw and p/s milk samples, and sodium percarbonate was used as a source of H_2O_2.

The prolonged activity of the LPS observed in samples stored at lower temperatures in this study, can be attributed to the greater stability of the active oxidative product (OSCN-) at lower temperatures than at higher temperatures (Bjorck *et al*, 1975), or it can be attributed to the slower recovery of surviving bacterial cells at lower than at higher temperatures (Bjorck, 1977).

Temperature also affected the death rate of the bacterial species that were susceptible to the bactericidal effect of the LPS. The death rates increased with increases in storage temperatures. This observation is illustrated by the lower D-values observed from samples stored at higher temperatures (Table 8) . This behaviour caused by the LPS can be attributed to the effect of temperature on the enzymatic activity of lactoperoxidase (LP). The optimum temperature for the activity of LP is approximately 20^{o}C (Morrison & Huttquist, 1963), hence at temperatures around 20^{o}C the peroxidation of SCN- and H_2O_2 to yield antibacterial ions is enhanced , and this in turn induces a quicker kill of the bacteria by the LPS.

The influence of temperature was also observed in the recovery of bacterial cells from the antibacterial activity of the LPS. In samples stored at lower temperatures, the recovery of surviving bacteria was slower, but as the storage temperatures were increased, the recovery of the bacteria became quicker. This could possibly be attributed to the influence of temperature on the growth of bacteria. Since at lower temperatures bacterial growth of most bacteria is inhibited or reduced, while at higher

TABLE 8

SAMPLES AND TEMPERATURE	REGRESSION EQUATION	D-VALUES (HRS)	CORRELATION COEFFICIENT LPS TREAT.	CONTR.
I E.COLI.				
7°C				
P/SVRBA	Y = 4.40 - 0.0221X	39.55	- 0.906	- 0.402
RWVRBA	Y = 4.55 - 0.0385X	26.01	- 0.966	- 0.206
P/SYMA	Y = 4.58 - 0.0153X	63.78	- 0.949	- 0.226
RWYMA	Y = 4.55 - 0.0136X	65.55	- 0.936	- 0.470
15°C				
P/SVRBA	Y = 4.27 - 0.O609X	12.41	- 0.933	0.983
RWVRBA	Y = 4.40 - 0.0483X	17.24	- 0.952	0.971
P/SYMA	Y = 4.44 - 0.0386X	20.80	- 0.925	0.980
RWYMA	Y = 4.51 - 0.0434X	23.04	- 0.792	0.980
22°C				
P/SVRBA	Y = 4.50 - 0.141X	6.93	- 0.950	0.981
RWVRBA	Y = 4.46 - 0.134X	7.61	- 0.926	0.988
P/SYMA	Y = 4.53 - 0.0832X	11.4	- 0.926	0.981
RWYMA	Y = 4.46 - 0.0617X	15.57	- 0.873	0.988
25°C				
P/SVRBA	Y = 4.62 - 0.169X	5.91	- 0.985	0.971
RWVRBA	Y = 4.49 - 0.158X	6.29	- 0.979	0.971
P/SYMA	Y = 4.75 - 0.157X	6.35	- 0.972	0.976
RWYMA	Y = 4.62 - 0.117X	8.58	- 0.980	0.967

TABLE 8 (cont)

30°C				
P/SVRBA	Y = 4.48 - 0.230X	4.40	- 0.911	0.961
RWVRBA	Y = 4.51 - 0.227X	4.43	- 0.965	0.951
P/SYMA	Y = 4.49 - 0.155X	6.42	- 0.925	0.975
RWYMA	Y = 4.60 - 0.147X	6.80	- 0.958	0.968
II *Y.ENTEROCOLITICA*				
7°C				
P/SBYSA	Y = 4.74 - 0.0315X	31.44	- 0.941	0.973
RWBYSA	Y = 4.71 - 0.0269X	35.97	- 0.903	0.866
P/SYMA	Y = 4.73 - 0.0294X	33.44	- 0.936	0.962
RWYMA	Y = 4.83 - 0.0211X	47.19	- 0.958	0.904
15°C				
P/SBYSA	Y = 5.02 - 0.0567X	21.02	- 0.922	0.994
RWBYSA	Y = 5.06 - 0.0587X	20.52	- 0.916	0.955
P/SYMA	Y = 5.05 - 0.055X	21.50	- 0.906	0.907
RWYMA	Y = 5.00 - 0.0542X	20.75	- 0.956	0.907
22°C				
P/SBYSA	Y = 4.79 - 0.189X	5.83	- 0.941	0.865
RWBYSA	Y = 4.86 - 0.202X	5.65	- 0.913	0.793
P/SYMA	Y = 4.91 - 0.226X	5.21	- 0.906	0.907
RWYMA	Y = 4.76 - 0.125X	8.15	- 0.896	0.904
25°C				
P/SBYSA	Y = 4.98 - 0.212X	4.72	- 0.972	0.957
RWBYSA	Y = 4.91 - 0.171X	5.84	- 0.979	0.769
P/SYMA	Y = 4.95 - 0.253X	3.68	- 0.972	0.945
RWYMA	Y = 4.96 - 0.179X	5.56	- 0.933	0.812

30°C

P/SBYSA	Y = 4.99 - 0.428X	2.34	- 0.953	0.962
RWBYSA	Y = 5.01 - 0.394X	2.54	- 0.920	0.838
P/SYMA	Y = 4.86 - 0.382X	2.62	- 0.990	0.839
RWYMA	Y = 4.79 - 0.174X	5.57	- 0.872	0.915

III *S.ENTERITIDIS*

7°C

P/SXLDA	Y = 4.71 - 0.00718X	145.8	- 0.964
RWXLDA	Y = 4.83 - 0.00807X	123.9	- 0.971
P/SYMA	Y = 4.80 - 0.00557X	180.9	- 0.960
RWYMA	Y = 4.82 - 0.00534X	187.3	- 0.812

KEY TO SYMBOLS USED

P/S VRBA = *E. coli* counts in p/s milk on Violet red bile agar

RWVRBA = " " " " raw " " "

P/SYMA = " " " " P/S " " Yeastrel milk agar

RWYMA = " " " " raw " " "

P/SBYSA = *Yersinia* counts in p/s milk on yersina selective agar

RWBYSA = " " raw " " "

P/SYMA = " " P/S " Yeastrel milk agar

RWYMA = " " RAW " " "

P/SXLDA = *Salmonella* counts in p/s milk on XLD agar

RWXLDA = " " RAW " " "

P/SYMA = '' '' p/s @ on Yeastrel milk agar

RWYMA = '' '' RAW '' '' ''

D-VALUE is defined as the time (hours) taken to reduce the number of bacteria in the treated samples by one log cycle at specific temperatures (Zall, 1990 ; Denis and Ramet, 1989)

tenperatures it is accelerated, it can be inferred that the regrowth of surviving bacteria is also intensified at higher tenperatures as soon as the antibacterial activity of the LPS is depleted.

The quick recovery of bacterial cells from the antibacterial activity at higher temperatures confirms that the system has a short life span in samples stored at higher temperatures. Pruitt & Reiter (1985), suggested that this could be attributed to the poor ability of the electron donor (SCN-) to facilitate synthesis of OSCN- at temperatures above 20^{o}C. It would therefore appear that the antibacterial effect at the higher temperatures is brought about mainly by the short-lived higher oxyacids (HO_2SCN and HO_3SCN), where else complementary interaction between OSCN- and the higher oxyacids may possibly be responsible for the prolonged antibacterial activity of the LPS at lower temperatures.

4.1.2 The influence of temperature on the antibacterial activity of the LPS on different species of bacteria

The sensitivity of the different species of bacteria to the antibacterial activity of the LPS was variable, and was affected by temperature.

It was observed that the LPS was mainly bacteriostatic to *L.monocytogenes*. This means that the system only caused growth inhibition of the bacteria and no noticeable decline in numbers were observed.

This observation agrees with earlier reports (Korhonen, 1982; Oram and Reiter, 1966), which stated that in most gram positive bacteria the LPS temporarily inhibits growth , after which the bacteria recover and grow. In this connection it is noteworthy that Denis and Ramet (1989) observed a bactericidal effect by the LPS on *L.moncytogenes*. The differences in conditions under which these experiment were conducted makes it difficult for an accurate comparison to be made.

In the present study, temperature affected the persistency of the bacteriostatic effect of the LPS on *L.monocytogenes* . At lower temperatures (7 & 15^{o}C) the bacteriostatic activity of the LPS persisted longer, while at higher temperatures (22 - 30^{o}C) regrowth of the inhibited bacteria occurred very rapidly. Pruitt and Reiter (1985), stated that some gram positive bacteria recover spontaneously from inhibition by the LPS, when they are exposed to Lactoperoxidase systems generating lower concentration of OSCN-. This may raise some concern about whether the amount of sodium thiocyanate recommended by IDF to activate the LPS is high enough to produce sufficient OSCN- concentration, particularly for milk samples stored at higher temperatures.

The results of this study have showed that the activity of the LPS is bactericidal to *E.coli, Y.enterocolitica,* and *S. enteritidis*. This was in agreement with the earliest reports (Hesse, 1894 ; Hansen, 1924) and the most resent reports (Bjorck, 1982 ; Korhonen, 1982) on the bactericidal activity of the LPS on gram negative bacteria.

There was variation in the susceptibility of the three species of bacteria to the bactericidal activity of the LPS. *Y.enterocolitica* was the most susceptible , while *S. enteritidis* showed the most resistance to the bactericidal activity of the LPS. This is illustrated by the observation that in *Yersinia*, the antibacterial activity of the LPS persisted for relatively longer period and the death rates were relatively rapid (Table 8), while in *Salmonella*, the persistency of the LPS was markedly shorter, particularly at higher temperatures and death rates were slower. To substantiate the above points, the D-values were comparatively lower in *Y.enterocolitica*, than they were in the other bacteria. While in *S.enteritidis*, regrowth of surviving cells was so spontaneous such that it was not possible to observe D-values from samples stored at all the other incubation temperatures except 7^{o}C. Even in those samples stored at 7^{o}C, the *S.enteritidis* D-values were comparatively higher than the values for other bacteria and the counts were not decreased by more than 2 log cycles.

The fact that *S.enteritidis* is showing a greater resistance to the the bactericidal activity of the LPS than the other two closely related genera of bacteria is of particular interest. In this connection it is noteworthy that Wray & Mclaren (1987), working with several strains of *S.typhimurium* and *S.dublin* found these strains to be more susceptible to the bactericidal activity of the LPS. This observation may indicate that *S.enteritidis* is particularly resistant to the antibacterial action of the LPS, and it can be considered that this aspect of the work merits

further investigation.

Although *Y.enterocolitica* proved to be the most susceptible bacterium to the bactericidal activity of the LPS, it was observed that regrowth of surviving bacterial cell at 7°C, was quicker in *Yersinia* than in *E.coli*, particularly in raw milk samples. However this behaviour of *Yersinia* can be attributed to the ability of the bacterium to grow at lower temperatures such as 7°C. This may mean that as soon as the antibacterial activity of the LPS was depleted the *Yersinia* cells started to grow while the *E.coli* cells could not grow because of inhibition by the low temperature.

The results also showed that the counts from non selective agars were higher than counts enumerated on selective agars in the treated samples. Previous works (Hurst, 1980 and Mcdonald *et al*, 1983) showed that when stressed bacterial cells are enumerated on selective and non selective media, only uninjured cells are enumerated on the selective medium, whereas the non selective medium recover both injured and uninjured cells. If this case is true in the results of the LPS activated samples, it may be implied that the system results in injury of some of the bacteria, rather than killing them and this tendency may have a great influence on the recovery of the bacteria from the so - called bactericidal activity of the system. However, this work merits further elucidation.

4.1.3 The effect of temperature on the antibacterial activity of the LPS in different milk mediums

Temperature affected the behaviour of bacteria differently in raw and in p/s samples activated with the LPS. At higher temperatures the antibacterial activity of the LPS persisted longer in p/s than in raw milk samples, however in samples stored at lower temperatures, the difference in persistency of the LPS in raw and p/s samples was not noticeable.

This longer persistency of the activity of the LPS at higher temperatures in p/s milk is noticeable despite the fact that the bacteria tended to show better growth in p/s milk compared with raw milk in the untreated controls. This observation indicates that the activity of the LPS was better in p/s than in raw milk samples.

The reasons for the bacteria to behave differently in raw and p/s samples activated with the LPS are not clearly understood. However, the influence of the type and load of the background microflora in the raw milk samples can be considered. There is a possibility that some of the bacteria initially present in the raw milk might be resistant to the antibacterial activity of the LPS. At higher temperatures these resistant strains could multiply rapidly such that their presence interferes with the antibacterial activity of the LPS on the susceptible strains.

A mechanism by which resistant strains of bacteria interfere with the activity of the LPS has been suggested by Pruitt and

Reiter (1985). These writers proposed that when LPS treated samples are contaminated with LPS resistant strains of bacteria, the resistant strains are able to reverse the inhibition of susceptible strains. This is possible because the resistant strains utilise an $NADH_2$ - oxidising enzyme to catalyse the oxidation of $NADH_2$ by OSCN- , consequently the OSCN- generated by the LPS is kept below bacterial inhibitory levels. However the results from this study show that this influence of the background microflora could be possible affecting counts from samples stored at higher temperatures, because it was at this temperatures that significant difference on TBC counts from raw and p/s milks were observed.

The differences in the behaviour of the LPS activity in raw and p/s milk samples may also be attributed to the differences in the chemical environmental conditions of these two milks. Since p/s milk samples were prepared by high heat treatment, it can be expected that these samples should contain very little enzymes such as catalase which may interfere with the antibacterial activity of the LPS (Thomas and Aune 1978). Even the low heat treatment (63 - 65^{o}C) used to prepare pasteurised milk will destroy catalase (Griffiths, 1986).

4.2 A note on the stability of lactoperoxidase and SCN- in milk samples

In a study preliminary to the main research project, the stability of lactoperoxidase was investigated in milk samples stored at -18^{o}C for about 20 days, and in pasteurised and p/s milk samples. It was observed that frozen storage of milk for 20

days did not change the activity of lactoperoxidase. However in pasteurised milk the concentration of lactoperoxidase was slightly reduced compared with fresh milk and was even lower in p/s milk samples (Appendix). However the concentration in the p/s samples, was still greater than the minimum level of lactoperoxidase required for the activation of the lactoperoxidase system (Korhonen 1980 ; IDF, 1988).

When the concentration of SCN- was investigated, fresh milk samples were found to contain approximately 3.5 mg/l SCN-. This level of SCN- was supplemented by addition of 14 mg/l NASCN, as recommended by IDF (1988). It was found that after the LPS had gone to completion at the end of the experimental period, a residual level of approximately 9.5 mg/l SCN- remained. This indicated that SCN was not a limiting factor for the LPS. This residual level of SCN- is unlikely to cause health hazards (Reiter, 1984).

4.3 CONCLUSION AND RECOMMENDATIONS

In conclusion the following findings from this research project can be summarised.

The activity of the LPS was found to be bactericidal to *Y.enterocolitica, E.coli,* and *S.enteritidis* , in the order of susceptibility.

The activity of the LPS was found to be mainly bacteriostatic to *L.monocytogenes*.

The persistency of the antibacterial activity of the LPS was inversely related to temperature.

The death rate of the three susceptible species of bacteria was affected by the storage temperature. The death rate was positively correlated with incubation temperature.

Temperature affected the regrowth of surviving bacterial cells from the antibacterial action of the system. As temperature was increased , so was the recovery rate of surviving cells.

Temperature affected the response of the bacteria to the antibacterial activity of the LPS differently in different milk samples. A better response of the bacteria to the antibacterial activity was observed in p/s samples compared with raw milk samples at higher temperatures. While samples stored at lower temperatures there were no significant differences.

Based on the observations made from this study it can be recommended that further research should be carried out to investigate the reasons why susceptible species of bacteria recover from the antibacterial activity of the LPS. Possibilities of prolonging the life span of the antibacterial action by reactivating the system prior to regrowth of bacteria in samples stored at different temperatures, needs to be investigated.

The apparent resistance of *S.enteritidis* to the activity of the LPS merits further study.

The results have shown that in addition to improving the keeping quality of raw milk, the antibacterial activity of the LPS can be used to improve the hygienic quality of pasteurised

milk by killing pathogenic bacteria such as *Y.enterocolitica*.

In view of the improved LPS activity observed in p/s milk, it may be worth considering a combination of thermization and LPS to extend the keeping quality of raw milk and this possibility merits further study.

In final conclusion it can be pointed out that although the LPS is an acceptable method of preserving milk, this method should not substitute for good hygienic and sanitary practices in the dairy industry but rather supplement these measures where cold storage of milk is not feasible. This is particularly true because the results from this study have shown that the antibacterial activity of the system results in temporary inhibition of the bacteria in milk samples, but not in a permanent eradication.

REFERENCES

ADAMSON, M. & CARLSSON, J. (1982). Lactoperoxidase and thiocyanate protect bacteria from hydrogen peroxide. Infect. Immun., **35**:20 -24.

ADAMSON, M. & PRUITT, K.M. (1978). Incorporation of SCN into hexokinase by the lactoperoxidase system. Fed. Proc., **37**: 1579.

ADAMSON, M. & PRUITT, K.M. (1981). Lactoperoxidase catalysed inactavation of Hexokinase. Bioch. Biophys. Acta, **658**: 238 - 247.

AHMED, A.H.; MOUSTAFA, K.M. AND EL-BASSONY, T.A. (1986). Growth and survival of Yersinia enterocolitica in yogurt. J. Food Prot., **49**: 983 - 985.

ALLEY, T.; ADAMSON, M. AND ARNOLD, R.R. (1984). Bactericidal effect of the lactoperoxidase system on Streptococcus mutans. J. Dent. Res., **63**: 243.

ANDERSON, & CHEN, (1940). Absorption and toxicity of sodium and potasium thiocyanate. J. Am. Pharm. Assoc., **29**: 152.

ANONYMOUS, (1985). Listeriosis transimitted by contaminated jalisco - brand cheese. Calif. morbid., **46**: nov. 22.

ARNOLD C. (1881). Arch. Pharm., **xvi** no1:41

AUCLAIR, (1954). J. Dairy Res., **21**: 323

AULISIO, C.C.G.; MEHLMAN, I.J. AND SANDERS, A.C. (1980). Alkali method for rapid recovery of Yersinia enterocolitica and Yersinia pseudotubercolosis from foods. Appl. Environ. Microbiol., **39**: 135 - 140.

AUNE, T.M. & THOMAS, E.L. (1977). Accumulation of hypothiocyanate ions during peroxidase - catalysed oxidation thiocyanate ions. Eur. J. Biochem., **80:** 209 - 214.

BANKS, J.G. & BOARD, R.G. (1985). Preservation by the lactoperoxidase system (LP-S) of a contaminated infant milk formula. Let. Appl. Microbiol., **1:** 81 - 85.

BARNWART, G.T. (1989). Basic food microbiology. 2nd ed. New York, Van Nostrand Reinhold.

BEARNS, R.E. AND GIRARD, K.F. (1958). The effect of pasteurisation on Listeria monocytogenes. Can. J. Microbiol., **4:** 55 - 61.

BELDING, M.E.; KLEBANOFF, S.J. & RAY, G.C. (1970). Sience, **167:** 195.

BERG, J.C.T. van den. (1988). Dairy technology in the tropics and subtropics. PUDOE, Wageningen, the Netherlands.

BEUMER, R.R.; NOOMAN, A; MARIJS, J.A. AND KAMPELMACHER, E.H. (1984). Antibacterial action of the lactoperoxidase system on Campylobacter jejuni in cows milk. Neth. Milk Dairy J., **39:** 107 - 114.

BJORCK, L. (1978). Antibacterial effect of the lactoperoxidase system on psychrotrophic bacteria in milk. J. Dairy Res., **45:** 109

BJORCK, L (1979). Enzymatic stabilisation of milk - utilization of milk peroxidase for preservation of raw milk. Proc. IDF Annual sessions, Montreux.

BJORCK, L. & CLAESSON, O. (1980). Correlation between concentration of hypothiocyanate and antibacterial effect of the lactopreoxidase system against Escherichia coli. J. Dairy Sci., **63** : 919 - 922.

BJORCK, L. CLAESSON, O. AND SCHULTHES, W. (1979). The lactoperoxidase / thiocyanate / hydrogen peroxide system as a temporary milk preservative in developing countries. Milchwissenschaft, **34**: 726 - 729.

BLACK, R.E.; JACKSON. R.T.; TSAI, T.; MEDVESKY, M.; SHAYEGANI, M. FREELY, J.C.; MCLEOD, K.E. AND WAKELER, A.M. (1978). Epidemic Yersinia enterocolitica infection due to contamination of chocolate milk. N. Engl. J. Med., **298**: 70.

BLOWEY, R.W. (1988). A veterinary book of dairy farmers. Page Bros. Norwich.

BORCH, E.; WALLENTINI, C.; ROSEN, M. AND BORCK, L. (1989). Antibacterial effect of lactoperoxidase / thiocyanate / hydrogen peroxide system against strains of Campyalobacter isolated from poultry. J. Food Prot., **52**: 638 - 641.

BRADSHAW, J.G.; PEELER, J.T.; CORWIN, J.J.; HUNT, J.M.; TIENERY, J.T.; LARKIN, E.P. AND TWEDT, R.M. (1985). Thermal resistance of Listeria monocytogenes. J. Food Prot., **48**: 743 - 745.

BRADSHAW, J.G.; PEELER, J.T.; CORWIN, J.J. BARNET, J.E. AND TWEDT, R.M. (1987). Thermal resistance of diseases associeted Salmonella tyhpimurium. J. Food Prot., **50**: 95 - 96.

BROWN, R.W. & MICKELSON, M.N. (1979). Lactoperoxidase thiocyanate and free cystein in bovine mammary secretions in early dry period and at start of lactation and their effect on Streptococcus agalactiae growth. Am. J. Vet. Res., **40**: 250 - 255.

BRYAN, F.L. (1983). Epidemiology of milk borne diseases. J. Food. Prot., **46** : 637 - 649.

BUNTING, E.S. (1986) Oilseed rape in perspetive. in Oil seed rape. (ed) Scarisbrick D.H and Daniels R.W. Collins prof. tech. books, London.

CARLSSON, J.; EDLUND, M.B.K. AND HANSTROM, L. (1984). Bactericidal and cytotoxic effect of hypothiocyanate - hydrogen peroxide mixtures. Infect. Immun., **44**: 581 - 586.

CARLSSON, J.; IWAMI, Y. & YAMADA,T. (1983). Hydrogen peroxide excretion by of oral *Streptococci* and effect of the lactoperoxidase - Thiocyanate - hydrogen peroxide system. Infect. & Immun., **40**: 70 - 80.

CHAKRABORTY, B.K.; CHAUNDRY, S.S.; ALEX, K.A.; JACOB, G. AND SON, G.J. (1986). Application fo the lactoperoxidase system for preserving buffalow milk produced in Indian villages. Milchwissenschaft, **41**: 16 - 18.

CONN, E.E.(1973). Cyanogenic Glucosides. in Toxicants occurring naturally in foods, 2nd ed. National academy of sciences, Washington D.C.

COUSIN, M.A. (1982). Presence and activity of pschrotrophic microoganssms in milk and dairy products. J. Food Prot., **45**: 172 - 207.

COWAN, S.T. (1974). Family 1. Enterobacteriaceae pp 290 - 293. In RE Buchanan and N.E Gibbons (ed) Bergey's manual of determintive bacteriology. 8th ed. Williams , Wilkins & Baltimore.

D'AOUST, J.Y. (1985). Infection dose of Salmonella typhimurium in chedder cheese. Am. J. Epidemiol., **122**: 717 - 721.

DENIS, F. AND RAMET, J.P. (1989). antibacterial activity of the lactoperoxidase on Listeria monocytogenes in trypticase soy broth, UHT milk and French cheeses. J. Food Prot., **52**: 702 - 711

DOMELLY, C.W. & BRIGGS, E.H. (1986) . Psychrotrophic growth and inactivation of listeria moncytogenes as a function of milk composition. J. Food Prot., **49**: 994 - 998.

EARNSHAW, R.G & BANKS, J.G. (1989). A note on the inhibition of Listeria monctogenes in by activation of the Lactoperoxidase system. Lett. Appl. Microbiol., **8**: 203 - 205.

FAO, (1957). Report on the meeting of experts on the use of hydrogen peroxide and other preservatives in milk. Doc.57/11/8655.

FLEMING, D.W.; COCHI, S.L.; MCDONALD, K.A.; BRONDUM, J.J.; HAYES, P.S.; PLIKAYTIS, B.D.; HOLMES, M.B.; AUNDURIER, A.; BROOM, C.V. AND REINGOLG, A.L. (1985). pasteurised milk as a vehicle of infection in outbreak of listeriosis. N. Engl. J. Med., **312**: 404 - 407.

FOX, P.F. (1982). In Developement in dairy chemistry. (ed) Fox, P.F. **vol. 1.**, Appl. Sci. Publishers, London.

GILES, N. & KING, S.C. (1987). Excretion of S. typhimurium from cow's udder. Vet. Rec., **118**: 23.

GRAY, M.L. & KILLINGER, A.H. (1966). Listeria monocytogenes and listeric infection. Bacteriol. Rev., **30**: 309 - 383.

GRIFFITHS, M.W.(186). J. Food Prot., **49**: 696.

GROVES, M.L. (1971). Milk proteins. In Chemistry and molecular biology vol 2, (ed) MCKENZIE, H.A. Academic Press, New York.

HANNA, M.O.; STEWART, J.C.; ZINC, D.L.; CARPENTER, Z.L. AND VANDERZANT, C. (1977). Development of Yersinia enterocolitica on raw and cooked beef and pork at different temperatures. J. food Sci., **42**: 1180 - 1184.

HANNAH RESAERCH INSTITUTE (1984). Rapid detection of post pasteurisation contamination. Bulletin **no.10**. University of Glasgow.

HANSEN, F.W. (1924). The bactericidal property of milk. Br. J. Exp. Path., **5**: 271.

HARNULV, B.G. & KANDASAMY, C. (1982). Increasing the keeping quality of milk activation of its lactoperoxidase system. Results from Sri Lanka. Milchwissenschaft, **37**: 454 - 457.

HARTMAN, P.A.; HARTMAN, P.S. AND LANZ, W.W. (1975). Violet red bile 2 agar for stressed coliforms. Appl. Microbiol., **29**: 537 - 539.

HESSE, W. (1894). Ueber die beziehungen zwischen kuhmilch und cholerabacillen. Z. Hyg. Infektkr., **17**: 238.

HOGG, D.M. & JAGO, G.R. (1970). The antibacterial action of lactoperoxidase. The nature of the bacterial inhibitor. Biochem J., **117**: 779 - 790.

HOOGENDOORN, H . (1976) . Inhibitor active aginst streptococus bacteria. Carier Res,. **10**: 151.

HOOGENDOORN, H.; PIERSSENS, J.P.; SHOLTY, W. & STODARD, L.A. (1977). Hypothiocyanite ions , the inhibitor formed by the system lactoperoxidase - thiocyanate - hydrogen peroxide. Caries Res., **11**: 77 - 84.

HUGHES, D. (1979). Isolation of Yersinia enterocolitica from milk and dairy farms in Australia. J. Appl. Bacteriol., **46**: 125 - 130.

HURST, A. (1980). Injury and its effect on survival and recovery pp 205 - 214. In International commission on microbiological specification for foods. (ed) Microbial ecology of foods. Academic Press Inc. New York.

IDF, (1988). Temporary preservation of raw milk by activation of lactoperoxidase system.Bulletin of the International Dairy Federation no. 234. Report F - Doc 96. Oslo July, 1983.

JAGO, G.R. & MORRISON, M. (1962). Antistreptococcal activity of lactoperoxidase. Proc. Soc. Exp. Biol. Med., **111**: 585.

JAMES, S.M.; FANNIN, S.L.; AGREE, B.A.; HALL, B.; PARKER, E.; VOGT, J.; RUN, G.; WILLIAMS, J.; LIEB, L.; SALMINEN, C,; PRENDERGAST, T.; WERNER, S.B. AND CHIN, J. (1985). Listeriosis outbreak assciated with Mexican style cheese. Calif. Morbid. Mortal Weekly Rep., **34**: 357 - 359.

JENNESS, R. & PATTON, S (1959). Principles of dairy chemistry. John Willey & sons, Inc.

JOHNSTON, D.W. & BRUCE, J. (1982). Incidence of thermoduric psychrothrophs in milk produced in the West of Scotland. J. Appl. Bacteriol., **52**: 333.

KING, J.S. & MABBIT, L.A. (1982). Preservation of raw milk by the addition of carbon dioxide. J. Dairy Res., **43**: 439.

KLEBANOFF, S.J. (1967). Iodination of bacteria, a bactericidal mechanism. J. Exp. Med., **126**: 1063 - 1078.

KLEBANOFF, S.J. (1968). Myeloperoxidase - halide - hydrogen peroxide antibacterial system. J. Bacteriol., **95**: 2131 - 2138

KORHONEN, H. (1977). Antimicrobial factors in bovine colostrum. J. Sci. Agric. Soc. Finland, **49**: 434

KORHONEN, H. (1980) . A new method for preserving raw milk, the lactoperoxidase system. World animal review, **35**: 23

KRUMBIEGEL, E.R. & WISNIESWIKI, H.J. (1970). Q fever in the Milwaukee area II consumption of infected milk by human volunteers. Arch Environ. Health, **21** : 63 - 65.

LARA, R.; MENDOZA, A.; CRUZ, J.D. AND GARCIA, H.S. (1987). Effect of the lactoperoxidase system on yield and characteristics of fresh cheese. Milchwissenchaft, **42** : 773 - 775.

LAW, B.A. (1979). Review of the progress of dairy science. Ezymes of psychrotrophic bacteria and their effect on milk products. J. Dairy Res., **46**: 573.

LAW, B.A. AND JOHN, P. (1981). Effect of the lactoperoxidase bactericidal system on formation of electro chemical proton gradient on E.coli. Fems. Microbiol. Lett., **10** : 67 - 70.

LAWRENCE, A.J. (1970). The thiocyanate content of milk. xvii. Intl. Dairy Congr. Sydney, **1E**: 99.

LECOS, C. (1986). Of microbes & milk probing Americas worst Salmonella outbreak. FDA Consumer, **20** 18 - 21.

LEE, W.H. (1977). An assement of Yersinia eneterocolitica and its presence in foods. J. food Prot., **40**: 486 - 489.

MARSHALL, V.M. (1978) In vitro and in vivo studies on the effect of the lactoperoxiase - thiocyanate - hydrogen peroxide system on Escherichia coli. PhD Thesis, Reading University.

MARSHALL, V.M. & REITER, B. (1976). The effect of the lactoperoxidase - thiocyanate - hydrogen peroxide on metabolism of Escherichia coli. Proc. Soc. Gen. Microb., **3**: 109 (abst)

MARSHALL, V.M.; COLE, W.M. AND BRAMLEY, A.J. (1986). Influence of the the lactoperoxidase system on susceptibility of the udder to Streptococcus uberis. J. Dairy Res., **53** : 507 - 514.

MARTINEZ, C.E.; MENDOZA, P.G.; ALACRON, F.J. AND GARSHIA, H.S. (1988). Reactivation of the lactoperoxidase system during raw milk storage and its effect on the characteristics of pasteurised milk. J. food Prot., **51** : 558 - 561.

MCLEAN, D.M. (1987). Enviromental aspect of Salmonella in humans. National Research Council of Canada. Ottawa.

MCDONALD, L.C.; HACKNEY, C.R. AND RAY, B. (1983). Enhanced recovery of Escherichia coli by compounds that degrade hydrogen peroxide or block its formation. Appl. Environ. Microbiol., **45**: 360 - 365.

MEDINA, M.; GAYA, P. AND NUMEZ, M. (19890). The lactoperoxidase system in ewe's milk: levels of lactoperoxidase and thiocyanate. Lett. Appl. Microbiol., **8**: 147 - 149.

MEHLMAN, J.J. & ROMERO, A. (1982). Enteropathogenic Escherichia coli method for recovery from foods. Food technol., **36**: 73.

MICKELSON, M.N. (1966). Effect of lactoperoxidase and thiocyanate on the growth of *Streptococcus agalactiae* in a chemically defined culture medium. J. Gen. Microbiol., **43**: 31.

MORRIS, P.W.; KELLY, A.M. AND LAGAS, W.G. (1979). Inactivation of bovine lactoperoxidase. Experientia, **35**: 589.

MRISSON, M. AND HUTTQUIST, D.E. (1963). J. Biol. Chem., **283**: 2847.

MOUSTAFA, M.K.; AHMED, A.H. AND MARTH, E.H. (1983). Occurrence of Yersinia enterocolitica in raw and pasteurised milk. J. Food Prot., **46**: 276 - 278.

MULLAN, W.M.A.; WATERHOUSE, A. & WADE, V.N. (1980). The production and storage of lactoperoxidase containing feeding material for calf rearing experiment. Dairy Ind. Intl., **45** (12): 15 - 18.

OGILVE, T.H. (1986). The persitant isolation of Salmonella typhimurium from the mammary gland of dairy cows. Can. Vet. J., **27**: 329 - 331.

OLSVIK, O. & KAPPERUD, G. (1982) Enterotoxin production in milk at 22 and 4^{o}C by Escherichia coli and Yersinia enterocolitica. Appl. Environ. Microbiol., **43**: 997 - 1000.

ORAM, J.D. & REITER, B. (1966). The inhibition of Streptococci by lactoperoxidase - thiocyanate - hydrogen peroxide, the oxidation of thiocyanate and the nature of the inhibitory compound. Biochem. J., **100** : 382 - 388.

PALUMBO, S.A. (1986). Is refrigeration enough to restrain foodborne pathogens. J. food Prot., **49** : 1003 - 1009.

PAUL, K.G.; OHLSSON, P.I. AND HENRIKSSON, A. (1980). The isolation and some liganding properties of lactoperoxidase. Febs. Lett., **110** (2): 200 - 204.

POLIS, B.D. & SMUKLER, H.W. (1953). J. Biol. Chem., **210**: 475

PRUITT, K.M.; ADAMSON, M. & ANORLD, R. (1979). Lactoperoxidase binding to Streptococci. Infect. Immun., **25**: 304 - 309.

PRUITT, K.M. & REITER, B. (1985). Biochemistry of peroxidase systems and antimcrobial effects. in The lactoperoxidase system chemistry and biological significance. (ed) Pruitt, K.M. and Tenovuo, J. pp 144 - 178. New York, Marcel Dekker.

REITER, B. (1976). Bacterial inhibitors in milk and other biological secretions with special reference to the complement - antibody, tranferrin - lactoferrin and lactoperoxidase - thiocyanate - hydrogen peroxide sysytem Symposium Series Soc. Appl. Bact., **5**: 31.

REITER, B. (1978). Antimcrobial systems in milk. J. Dairy. Res., **45**: 131.

REITER, B. (1979). The lactoperoxidase thiocyanate hydrogen peroxide antibacterium system. Excerpta Medica., **90**: 285 - 293.

REITER, B. (1982). in Symposium on mastitis control. ed Bassalk - chabial ska. L. Jablomma, Polland. p 351.

REITER, B. (1984). The biological significance and exploitation of some of the immune systems in milk - a review. Microbiolgie - Ailments - Nutrition, **2**: 1 - 20

REITER, B. (1985). The lactoperoxidase system in bovine milk. in The lactoperoxidase system chemistry and biological significance. (ed) Pruitt, K.M. and Tenovuo, J. pp 123 - 143. New York, Marcel Dekker.

REITER, B.; PICKERING, A.; ORAM, J.D. AND POPE, G.S. (1963). Peroxidase - Thiocyanate inhibition of Streptococci in raw milk. J. Gen. Microbiol., **33**: xii

REITER, B.; PICKERING, A. AND ORAM, J.D. (1964). An inhibitory system - lactoperoxidase - thiocyanate - peroxide in raw milk. In Microbial inhibitors (ed) MOHN, N. Uppsala, Sweeden, Almquist and Wicksell.

REITER, B.; MARSHALL, V.M.E.; BJORCK, L. AND ROSEN, C.G (1976). The non specific bactericidal activity of the lactoperoxidase - thiocyanate - hydrogen peroxide system of milk against E.coli and some gram negative pathogens. Infect. Immun., **13**: 800.

REITER, B.; MARSHALL, V.M. AND PHILIPS, M.S. (1980). The antibiotic activity of the lactoperoxidase thiocyanate hydrogen peroxide system in the calf abomasum. Res. Vert. Sci., **28** : 116 - 112

REITER, B.; FULFORD, R.J.; MARSHALL, V.M.; YURROW, N. AND DUCKER, M.J. (1981). An evaluation of growth promoting effect of the lactoperoxidase system in new born calves. Anim. Prod., **32**: 297 - 306.

REITER, B. & HARNULV, B.G. (1982). The preservation of refrigerated and uncooled milk by its natural lactoperoxidase system. Dairy Indust. Intl., **47** (5): 13 - 19.

REITER, B. AND HARNULV, G. (1984). Lactoperoxidase antibacterial system : natural occurrence, biologicl functions and practical aplications. J. Food Prot., **47**: 724 - 732.

ROWE ,B.; HUTCHSON, D.N.; GILBERT, R.J.; HALES, B.H.; BEGG, N.J.; DAWKINS, H.C.; JACOB, N.; RAE, F.A. AND JEPSON, M. (1987). Salmonella ealing. infection associated with consumption of infant dried milk. Lancet, **II** : 900 - 903.

SCHLECH, W.F.; RAVIGUE, P.M.; BORTOLUSSI, R.A.; ALLEN, A.C.; HALDANE, E.V.; WORT, A.J.; HIGHTOWER, A.W.; JOHNSON, S.E.; KING, S.H.; NICHOLLS, E.S. AND BROOME, C.V. (198). Epidemic listeriosis - evidence for transmittion by food. N. Engl. J. Med., **308**: 203 - 206.

SHAYAGANI, M.; MORSE, I.; De FORGE, I.; ROOL, T.; PARSON, L.M. AND MOUSPIN, P.S. (1983). Microbiology of major food outbreaks of gastroenteritis caused by Yersinia enterocolitica serogroup 0 : 8. J. Clin. Microbiol., **17**: 35.

SIEVERS, G (1979). The prosthetic group of milk lactoperoxidase is protoheme ix. Biochimica et Biophysica acta. , **579**: 181 - 190

SIEVERS, G. (1981). Structure of milk lactoperoxidase. Febs. letters, **127**: 253 - 256

SIEVERS, G.; GADSBY, P.M.; PETERSON, J. AND THOMSON, A.J. (1983). Assignment of the axial ligands of the haem in milk lactoperoxidase using magnatic circular Dichroism Spectroscopy. Biochimica et Biophysica Acta, **742**: 659 - 668.

SINGLETON, P. & SAINSBURY, D. (1978). Dictionary of microbiology. John Wiley & sons, Bath.

STEELE, W.F. & MORRISON, M. (1969). Antistreptococcal activity of lactoperoxidase. J. Bacteriol., **97**: 635 - 639.

STERN. N.J.; PIERSON, M.D. AND KOTULA, A.W. (1980). Growth and competitive nature of Yersinia enterocolitica in whole milk. J. Food Sci., **45**: 972 - 974.

TENOVUO, J. (1985) in The lactoperoxidase system: chemistry and biological significance. (ed) Pruiit, K.M and Tenovuo, J. Marcel Dekker, New York. pp 102 - 122.

TENOVUO, J. & KNUUTTILA, M.L.E. (1977). Antibacterial activity of salivary peroxidases on cariogenic strains of Streptococcus mutans. J. Dent. Res., **56**: 1608 - 1613.

TENOVUO, J. & KURKIJARVI, K. (1981). Comparison of soluble and soilid state lactoperoxidase. Caries Res., **15** (2): 197.

TENOVUO, J.; MAKIEN, K. AND SIEVERS, G. (1985). Antibaterial activity of lactoperoxidase and myeloperoxidase against Bacillus cereus. Antimicrob. Agents Chemother., **27**: 96 - 101.

THAKAR, R.P & DAVE, J.M. (1986). Application of the activated lactoperoxidase thiocyanate and hydrogen peroxide system in enhancing the keeping quality of raw buffalow milk at high temperatures. Milchwissenschaft, **41**: 20 - 22.

THOMAS, E.L. & AUNE, T.M. (1978). Lactoperoxidase, peroxidase, thiocyanate antimicrobial system: correlation of sulfhydryl oxidation with antimicrobial action. Infect. Immun., **20**: 456.

THOMAS, E.L. (1985). Bacterial hydrogen peroxide production. in The lactoperoxidase system chemistry and biological significance. (ed) Pruitt, K.M. and Tenovuo, J. pp 179 - 202. New York, Marcel Dekker.

VAN ETTEN, C.H. AND WOLFF, I.A. (1973). Natural sulphur compounds. in Toxicants occurring naturally in foods. 2nd ed. pp 210 - 234. National academy of Sciences, Washington. D.C.

VIRTANEN, A.I. (1961). Ueber die chemieder Brassica faktoren ihre wirkung auf die funkton der schilldruse und ihr uebergehen in die milch. Experientia, **17**: 241

WATERHOUSE, A, & MULLAN, W.M.A. (1980). Re - inclusion of an active lactoperoxidase system in a milk substitute diet for calves. Anim. Prod., **30**: 458 (abst).

WEBB, B.H.; ARNOLD, H.J. & ALFORD, J.A. (1974). Fundamentals of dairy chemistry. AVI publishing company, Connecticut.

WEST, A.M.; MARTIN, S.W.; MCEWAN, S.A., TAMBLYU, S.E. AND CLARK, R.C. (1988). Factors associated with presence of Salmonella spp in the farm families and bulk milk supply of dairy farms South Western Ontario. Can. J. Publ. Health, **79**: 119 - 123

WEVER, R.; KAST, W.M.; KASINOEDIN, J.H. AND BOELENS, R. (1982). The peroxidation of thiocyanate catalysed by myeloperoxidase and lactoperoxidase. Biochimica et Biophysica acta, **709**: 212 - 219.

WINBALD, S. (1973). The clinical panorama of Yersinia enterocolitica. Contrib. Microbiol. Immunol., **2** : 129 - 132.

WITTER, L.D. (1961). Psychrophilic bacteria - review. J. dairy Sci., **44**: 983 - 1015.

WOOD, J.L. (1975). Biochemistry. in Chemistry and biochemistry of thiocyanic acid and its derivatives. Newman A.H.(ed). pp 145 - 221. Academic press, London.

WOODS, D.S.; COLLINS-THOMSON, D.L.; IRVINE, D.M. AND MYHR, A.N. (1984). Sources of persistence of Salmonella muester in naturally contaminated milk. J. Food Prot., **47** : 20 - 27.

WRAY, C. & MCLAREN, I. (1987). A note on the effect of the lactoperoxidase system on Salmonella in vitro and in vivo. J. Appl. Bacteriol., **62**: 115 - 118.

WRIGHT, R.C & TRAEMER, J.J. (1958). Factors influencing the activity of cheese starters. The role of milk peroxidase. J. Dairy Res., **25**:104.

ZAJACK, M.; GLADYS, J.; SKARZINSKA, M.; HARNULV, G. AND BJOCK, L. (1983). Changes in bacteriological quality of raw milk stabilised by activation of the lactoperoxidase system and stored at different temperatures. J. Food Prot., **46** :1065 - 1068.

ZALL, R.R. (1990) . Control and destruction of microorganisms. in Dairy microbiology The microbiology of milk 1. 2nd ed, (ed), Robison, R.K. Elsevier Science publishers ltd., Essex. pp. 115 - 161

ZALL, R.R. & CHEN, J.H. (1981). Heating and storing milk in dairy farms before pasteurisation in milk plants. J. Dairy Sci., **64**: 1540.

ZALL, R.R.; CHEN, J.H. AND DVURECK, D.J. (1983). Milchwissenschaft, **38**: 203 - 208.

APPENDIX RESULTS OF A PRELIMINARY STUDY

ROW	SAMPNO	LPRAW	LPPAST	LPP/S	SCNSAM	SCNRW	SCNTRM
1	1	14.68	10.09	4.43	1	2.50	9.8
2	2	14.94	8.60	5.18	2	2.50	8.7
3	3	15.97	9.98	4.21	3	2.50	9.8
4	4	16.82	10.46	4.21	4	2.50	8.7
5	5	13.47	8.60	5.99	5	4.50	8.7
6	6	15.16	7.03	4.14	6	2.50	9.8
7	7	20.02	11.32	3.73	7	5.00	9.9
8	8	12.63	7.88	4.40	8	5.76	9.9
9	9	18.69	8.32	5.17	9	6.40	8.2
10	10	12.41	9.81	6.40	10	3.50	10.0
11	11	18.34	10.23	4.50	11	3.50	8.4
12	12	16.25	9.27	4.98	12	5.40	8.5
13	13	11.74	7.30	6.08	13	3.50	9.5
14	14	12.71	9.62	4.50	14	3.00	8.5
15	15	15.01	9.01	7.11	15	4.20	9.0
16	16	17.46	10.25	4.74	16	5.70	10.4
17	17	15.29	10.05	4.70	17	5.70	9.4
18	18	16.70	8.00	4.26	18	4.00	10.0
19	19	16.82	9.21	3.93	19	3.50	8.8
20	21	15.04	8.41	4.10	20	2.50	9.0
21	22	12.28	8.89	4.80			
22	23	15.89	9.71	3.60			
23	24	15.89	9.05	5.07			
24	25	15.97	9.70	5.34			
25	26	12.83	8.42	6.18			

KEY. SAMPNO= SAMPLE NUMBERS

LPRAW = LACTOPEROXIDASE LEVEL IN RAW MILK (mg/l)

LPPAST = LACTOPEROXIDASE LEVEL IN PASTEURISED MILK (mg/l)

LPP/S = LACTOPEROXIDASE LEVEL ON P/S MILK (mg/l)

SCNSAMP= SAMPLE NUMBER

SCNRW = LEVEL OF SCN IN UNTREATED SAMPLES (mg/l)

SCNTR = LEVELS OF SCN IN LPSTREATED SAMPLES (mg/l)