THE SURVIVAL OF MICROBIAL PATHOGENS IN DAIRY PRODUCTS

by

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“The wise also will hear and increase in learning, and the person of understanding will acquire skill and attain to sound counsel.”

Proverbs 1: 5

Dedicated to my wonderful family; my parents, my brother Sphee and most importantly my husband and son, Tsosane and Mohlomi Shabe

DECLARATION

“I ‘Mamajoro E. Lefoka declare that the dissertation hereby submitted by me for the Magister Scientiae degree at the University of the Orange Free State is my own independent work and has not previously been submitted by me at another university/faculty. I further more cede copyright of the dissertation in favor of the University of the Free State.”

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LIST OF ABBREVIATIONS

°C	Degree Celsius
ANOVA	Analysis of variance
Cfuml-1	Colony forming units per milliliter
CO_2	Carbon dioxide
d	Day
g/l	Grams per liter
h/hrs	Hours
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IDF	International Dairy Federation
LAB	Lactic acid bacteria
NADH	Nicotinamide adenine dinucleotide
pH	Negative logarithm of hydrogen concentration
spp	Species
TSB	Tryptic soy broth

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Over the last few years, food poisoning and food safety have become very topical subjects, eliciting a great deal of public concern to many people all over the world. This is a result of emerging foodborne pathogens that continue to cause outbreaks of food borne diseases in different countries. A wide variety of diseases can be caused by eating food contaminated with pathogenic microorganisms or their products; by no means all these diseases can be classed as food poisoning.

Foodborne disease outbreaks have heightened the awareness of foodborne pathogens as a public health problem in South Africa and around the world. Fig. 1 shows reports of food poisoning cases in South Africa in the past five years (2001-2005). A total of 1886 cases and 51 deaths were reported to the National Department of Health (NDoH). There was a peak in 2003 where 764 cases were reported. It should be noted, however, that the number of cases reported does not reflect the actual cases that occur in the country. Most cases of food poisoning are presented with mild symptoms, and therefore are less likely to be reported, as people are less likely to seek medical attention. Furthermore, when people do seek medical attention, health workers are less likely to report these less severe conditions. In the US reports have shown that foodborne illness account for approximately 76 million illnesses, 325 000 hospitalization, and 5000 deaths each year (Mead *et al*., 1999).

Virulence of a foodborne pathogen is determined by its ability to attach to the host, to invade host tissues, to produce toxins and to overcome the host's defence mechanisms. The ability of a foodborne pathogen to survive exposure to the acidic conditions encountered in the stomach is a key determinant of an organism's infectious dose (Gorden and Small, 1993), thus, acid plays an important role in bacterial enteric infection. Foodborne pathogens must survive in

the stomach (pH 3) for up to 2 hrs before passage to the intestinal tract, where colonization can occur (Giannella *et al*., 1972).

A broad spectrum of microbial pathogens can contaminate human food and water supplies and cause illness after they or their toxins are consumed. These include a variety of enteric bacteria, aerobes and anaerobes, viral pathogens and yeasts (Tauxe, 2002). During past decades, microorganisms such as *Staphylococcus aureus, Salmonella* spp., *Escherichia coli* 0157:H7, *Shigella* spp., *Listeria monocytogenes* and *Yersinia enterocolitica*, were reported as the most common foodborne pathogens that are present in many foods and able to survive in milk and fermented milk products (Alm, 1983; Ahmed *et al*., 1986; Ryser and Marth, 1988; Schaak and Marth, 1988; Pazakova *et al*.,1997; Canganella *et al*., 1998; Dineen *et al*., 1998; Gulmez and Guven, 2003; Tekinşen and Özdemir, 2006). In this review we discussed the survival of some of these pathogens associated with fermented milk products.

According to the definition proposed by the International Dairy Federation 'fermented milks' are products prepared from milk, skimmed milk or not, concentrated or not, with specific cultures; the microflora is kept alive until sale to the customers and may not contain any pathogenic microorganisms (IDF, 1988). Kosikowski (1966) added that the metabolic substances derived from fermentation such as diacetyl, CO_2, ethanol, acetoin, acetylaldehyde etc. must be present in fermented milks. This term “fermented milks” which has been commonly accepted in the dairy world implies a liquid or semi-liquid consistency of the product and therefore, in general understanding does not apply to cheeses. A multitude of fermented milks is nowadays commercially produced world-wide.

1.2 HISTORY AND BACKGROUND

The consumption of fermented milk products such as yoghurt dates back many centuries, although there is no precise record of the date when they were first made. In these ancient times the milk would ferment spontaneously by natural microflora. Fermentation did not take place in controlled systems or sterilized conditions, as a result contamination with yeasts and some pathogenic bacteria would normally occur. This caused fermented milks to become major vehicles of transmission for many foodborne pathogens. Foodborne diseases caused by these pathogens were problematic and must have continually preoccupied early humans.

1.2.1 Fermented milks

Fermentation of milk is a very ancient practice of man which has been passed down from generation to generation. Its aim was to obtain products with characteristic flavour, aroma and consistency and at the same time, could be stored unspoiled for a longer time than untreated raw milk. The actual origin of fermented milks is unknown but there is no doubt that their consumption dates back to prehistoric times (Helferich and Westhoff, 1980). Kosikowski (1966) believed that fermented milks originated in the Near-East, perhaps before Phoenician era, and spread through central and Eastern Europe. Moreover, it was added that the earliest example of fermented milk was warm, raw milk from cows, sheep, goats, camels or horses of the nomads roaming the area. This was also mentioned by Helferich and Westhoff (1980) who stated that ancient Eastern tribes who were nomadic shepherds preserved their milk from cows, sheep, goats, buffalos and camels in clay pots in warm temperatures (43 °C) and this combination set up ideal conditions that would inevitably cause the milk to ferment and produce an uncontrolled crude type of yoghurt.

Mention of cultured dairy products is found in some of the earliest writings of civilized man, eg. the Bible and the sacred books of Hinduism. There is also ample archaeological evidence to show that fermentation of milk has been known for millennia. Drawings and impressions on walls and rocks in caves show that early nomadic herders in Africa used many forms of fermented milk. According to Abdelgadir (1998) there is concrete evidence of milk fermentation by people of the ancient Kingdom of Meroe in Sudan. Meroe (690BC-AD 323) was quite advanced and thus left a wealth of articles and wall and rock drawings which were dominated by the cow. A milking scene has been left in an impressive drawing, showing a man carrying a milking pail presenting it to a queen-like figure of a woman seated in front of her hut.

A Sumerian relief in limestone found at Tell Ubaid in the Middle East which dates from about 2900-2460 BC depicts some detail of the manufacture of cultured butter (Kurmann, 1984). Another example is a sculptured Kumys amphora from the 4th century BC found at Certomlyk near the Dnieper River, not far from the Crimean Penninsula (Kurmann, 1984).

Even though composition and microbiology of fermented milks were not understood, their beneficial effects over fresh milk were recognised. Since ancient times, in Europe, Asia and Africa, sour milk was known as being more stable and advantageous than fresh milk. It preserved the high quality nutrients present in milks in a relatively stable form (Oberman, 1998). The fermented milk could be stored at warm temperatures and be safely consumed for several days (Helferich and Westhoff, 1980). Known scientists of early ages, such as Hippocrates, Avicenna, Galen and others, considered milk not only a food product but a medicine as well. They prescribed sour milks for curing disorders of the stomach, intestines and other illnesses (Oberman, 1998).

Metchnikoff's theory of longevity considerably influenced the spread of fermented milk products to many countries. The consumption of fermented milks has

increased considerably since 1964 in many countries (Rasic and Kurmann, 1978). The great popularity of fermented milks is attributed to their appealing taste as well as to their extended shelf life during which the survival of pathogenic microflora is reduced, particularly at low pH levels. Even though yoghurt has been around for many years, it is only recently that it has become a popular fermented milk product in Europe, Asia and Africa (Kosikowski, 1966). This increase in popularity and consumption is due to its beneficial influence on human health (Hattingh and Viljoen, 2001), its nutritional value and the use of sugar, fruits and flavours in its manufacture (Kroger, 1976).

1.2.2 Foodborne diseases

Food spoilage and food poisoning caused by microorganisms were problems that must have continually preoccupied early humans. One can imagine nomadic populations of hunters and gatherers who slaughtered wild animals and needed to preserve tons of meat. Foodborne disease surveillance began in the US in the early 1900s in response to morbidity caused by milk-transmitted typhoid fever and infantile diarrhoea (Cliver, 1990). In the first decades of the 1900s, some of the principle infections that were recognized as foodborne included typhoid fever, tuberculosis, brucellosis and septic throat, a zoonotic streptococcal infection (Tauxe, 2002).

In 1906, an aerobic, spore-forming bacillus was recognized as a cause of food poisoning (Hartman, 1997). In 1939, J. Schleifstein and M. B. Coleman described gastroenteritis caused by a bacterium that, in 1965, was named *Yersinia enterocolitica* by R. Sakazaki (Hartman, 1997). *Clostridium perfringens*, the causative agent of human gas gangrene infections, was first implicated as a cause of foodborne illness by E. Klein in 1885 (Hartman, 1997). The first confirmed report of listeric infection in humans appeared in 1929 (Cliver, 1990). The bacterium was isolated from three patients with infectious mononucleosis-like disease.

Many of these foodborne diseases that historically caused significant mortality and morbidity were largely eradicated in the industrialized world as a result of sanitation and pasteurization, disease control efforts in animals and other measures (Tauxe, 2002). Although many foodborne infections are controlled, the burden of emerging foodborne pathogens remains substantial.

1.3 EXAMPLES OF FERMENTED MILKS

A wide diversity of fermented milk products has been produced in various parts of Africa (Table 1) as a method of milk preservation. The majority of fermented milks are made from cow's milk, but sheep, goat, buffalo, camel and horse milk are also used in large quantities. Fermented milk was originally obtained by inoculating fresh milk with the remainder of the previous batch. This traditional method of preparing fermented milk is still used in some less sophisticated societies. Modern techniques of milk fermentation, on the other hand, use starter cultures with known characteristics. The advantage of modern techniques over the traditional methods is the production of consistent products that are less likely to spoil and are relatively safe.

1.3.1 African traditional fermented milks

Milk is a major component of the traditional diet in many regions in Africa. Most of the milk produced is consumed in the home and is rarely sold. In many African countries, refrigeration facilities are limited and milk stored at ambient temperatures is usually fermented rapidly by the natural flora.

Fermented milk produced in Ethiopia was described by Kurman *et al.* (1992) and it is known as *Ergo*. Milk is allowed to ferment naturally and is accumulated over a period until the desired acidity has been achieved. The product is viscous and usually supplements the main food. *Ititu* is a traditionally fermented milk consumed by pastoralists of Southern Ethiopia (Kurman *et al.,*1992). During its

production, the fermenting vessel (gorfa) is smoked with *Acacia nilotica* wood before the milk is added. According to some investigators, *ititu* contains the essential amino acids (Kassaye *et al.,* 1991). The pastoralist community in Somali produce *suusac* which is traditionally prepared by spontaneous fermentation of unheated camel's milk in smoke-treated gourds. The fermentation is carried out at ambient temperature (26-29 °C) for 1-2 days. Evidently, the fermented milk is characterized by low-viscosity, a distinct smoky flavour and an astringent taste (Berlin and Forsell, 1990; Kurman *et al*.,1992).

In the lower Egypt, farmers put fresh milk in earthenware pots and leave it undisturbed in a warm place until the cream rises and lower partially skimmed milk coagulates. The cream layer is removed and whipped by hand to butter while the remaining sour milk, often called 'Laban Rayeb' is either consumed as it is or is converted to a soft acid cheese - 'Karish cheese' (El-Gendy, 1983). Amongst the major fermented milk products of Sudan is *Rob* for its considerable economic and dietary importance to the people (Abdelgadir *et al*., 1998). It is mainly produced from surplus milk of the rainy season by nomadic tribes. During this season the housewife turns as much milk (about 80 %) into *rob* each evening. Abdelgadir *et al*. (1998) reported that the aim of souring milk into *rob* is not to obtain fermented milk for consumption, but to facilitate the extraction of butter from it. Hence, *rob* is the by-product of butter production and not the other way round as it is commonly held in the urban thought. Most of the *rob* is made from cow's milk but milk from sheep and goats can also be used. Another example of traditionally fermented milk is *Nono*, produced and consumed mainly by the 'Fulani', a nomadic cattle rearing tribe, in Nigeria (Atanda and Ikenebomeh**,** 1991). It is domestically prepared by naturally fermenting cow milk (or occasionally goat's milk). The cream that collects at the top of the container is Fulani butter, which is a by-product, and the remaining milk in the container is *nono*.

In South Africa, traditional fermented milks, *Maas* and *Inkomasi*, were described by Keller and Jordan (1990). The two products are traditionally produced in clay pots and calabash which are used repeatedly. Bacteria present on the inner surface of the container were presumed to be responsible for the fermentation of the milk. Feresu and Muzondo (1990) have mentioned the presence of a similar fermented milk, such as *amasi* in Zimbabwe. Amasi is produced by leaving fresh raw bovine milk to ferment naturally at ambient temperature in earthenware pots or any other suitable containers (Feresu and Muzondo 1990; Mutukumira *et al*., 1995). The microorganisms inherent in the milk, the container and the surrounding air are assumed to ferment the milk within 1-3 days depending on the ambient temperature.

1.4 CHEMICAL COMPOSITION AND DIETARY VALUE OF FERMENTED MILKS

The chemical composition of fermented milk products depends on the milk used and on the microorganisms and their specific action in metabolizing the milk components (Oberman, 1998). The typical composition of fermented milks (Table 2) was described by Fluckiger (1982) as cited by Oberman (1998).

Fermented milks have been acclaimed by some researchers for being more nutritious than fresh milk. The total free amino acid content in freshly drawn milk is fairly low and increase considerably during the manufacture of yoghurt (Rasic and Kurmann, 1978). In their study, Tamime and Deeth (1980) found the proline content to be 45 times higher in yoghurt than it was in fresh milk. The content of histidine, arginine, alanine, valine, methionine and isoleucine was about 4-9 times higher than that of original milk. Fermented milks are a good source of the B vitamins. Higher levels of folic acid, niacin, biotin, pantothenic acid, vitamin B_6 and vitamin B_{12} are found in certain cultured milk products than in fresh milk (Shahani and Chadan, 1979). However, a loss of vitamin B_{12} occurs during the manufacture of fermented milks such a biogurt, acidophilus milk, sour milk, kefir

and sour cream as a result of heat treatment of the milks and the consumption by lactic acid bacteria (Rasic and Kurmann, 1978). There are also claims that the digestibility of the milk proteins is improved by fermentation (Rasic and Kurmann, 1978).

Among the most important factors determining the specific identity of fermented milks are the flavour and aroma compounds. These products consist of acetylaldehyde, diacetyl, acetoin and, in some cases, alcohol. In cultured buttermilk and cultured cream the compound of particular importance is diacetyl produced by *Lactococcus lactis* ssp. *lactis* var. *diacetylactis* or *Leuconostoc* strains (Oberman *et al.,* 1998). At very low concentrations (3-5 mg/kg), diacetyl is responsible for the characteristic 'buttery' nut-meat aroma in milks. Furthermore, in yoghurt even small quantities of diacetyl contribute to the pleasant and delicate flavour and aroma, thus enhancing the principal aroma compound (Rasic and Kurmann, 1978). Another flavouring compound produced by lactic acid bacteria during fermentation is acetylaldehyde. It is a very important aroma compound in yoghurt and related fermented milks, and in order to produce a very good, typical and fresh aroma of yoghurt 10-15 mg/L is required. On the other hand, acetylaldehyde is undesirable in excess in buttermilk as it is responsible for the flavour defect described as 'green' or 'yoghurt-like'. Ethanol is also an important metabolic product of lactose-fermenting yeasts present in kefir, koumiss and other similar products. Assisted by the presence of formic, acetic, propionic acids etc. it is necessary to bring out the total flavour of the fermented products.

1.5 MICROBIAL COMPOSITION OF FERMENTED MILKS

The earliest information concerning the microbiological composition of fermented milk products was given at the end of the 19th century. The presence of a diverse range of microorganisms was reported in the early investigations. Metchnikoff (1907) (as cited by Oberman, 1998) pointed out the presence of *Bacillus bulgaricus*, cocci and yeasts in yoghurt. Grigoroff isolated rod-shaped bacteria

called *Bacillus* A from Bulgarican milk in 1905. The presence of lactobacilli, such as *Lactobacillus longus, Bacillus lebensis*, *Bacillus* exhibiting and not exhibiting granules, *Streptobacillus*, *Yoghurt bacillus*, was described by several other investigators.

With all the developments that have taken place over the years in food microbiology and the present level of knowledge, it is evident that the microflora of fermented milks consists of different strains of lactic acid bacteria belonging to *Lactobacillus, Lactococcus, Leuconostoc* and *Bifidobacterium* species and of minor proportions of yeasts and milk moulds in associated growth.

1.5.1 Lactic acid bacteria

As already mentioned previously, fermented milks are highly nutritious foods that supply most of the essential amino acids, carbohydrates and many other required nutrients such as fat, minerals and vitamins. Hence, they provide a favourable environment for the growth and propagation of a diversity of microorganisms.

Requirements for the growth of lactic acid bacteria include sugar such as lactose and a wide range of amino acids, vitamins and other growth factors (Oberman *et al*., 1998). Thus, fermented milk is a satisfactory medium for the growth of these organisms. In many investigations, LAB have been the most predominant of the microflora of several fermented milks. In South African traditional fermented milks the dominant LAB were found to be representatives of the genera *Leuconostoc, Lactococcus* and *Lactobacillus* (Beukes *et al*., 2001). The predominant species identified by most researchers include *Lactococcus lactis* subsp. *lactis*, *Lact. lactis* subsp. *lactis* biovar *diacetylactis, Lactobacillus paracasei* subsp. *paracasei*, *Lb. plantarum*, *Lb. acidophilus*, *Leuconostoc mesenteroids* subsp. *mesenteroides*, *Enterococcus faecum* and *Ent. faecalis*. These have been isolated from *Amasi* in Zimbabwe (Mutukumira, 1995; Feresu and Muzondo, 1990) and *Maasai* traditional fermented milk in Kenya (Mathara *et al.,* 2004). The

most common microbial species in yoghurt and *rob*, the fermented milk of Sudan, were found to be *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Abdelgadir *et al*., 1998; Rasic and Kurmann, 1978).

The primary role of lactic acid bacteria in all milk fermentations is the conversion of lactose to lactic acid which improves shelf life of fermented milks and lowers pH, thus, inhibiting growth of spoilage and pathogenic microorganisms (Kingamkono *et al.,* 1998; Garrote *et al*., 2000; Vandenberg, 1993). However, in the dairy industry, some LAB strains having probiotic activity may be used to supplement the original microflora for the production of new fermented milk products. According to Rasic and Kurmann (1978), they are classified as the non-essential microflora. These include *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Bifidobacterium longum* to mention a few. They are used in the production of yoghurt, acidophilus milk, A-B yoghurt, bifidus milk etc. which are well known for their health-promoting properties. LAB also play a major role in the development of flavour and aroma through the production of flavouring compounds such as diacetyl, acetoin and acetyaldehyde (Oberman *et al.*, 1998).

1.5.1.1 Microbial interactions of LAB in fermented milks

Yoghurt fermentation is an interesting example of an interaction between two LAB microorganisms in which there is mutual growth stimulation. The interaction between *L. bulgaricus* and *Streptococcus thermophilus* is mutualistic and growth and acid production is greater in a mixed culture as compared with monocultures (Rasic and Kurmann, 1978). Faster growth of streptococci at the beginning of fermentation brings about accumulation of moderate amounts of lactic and acetic acids, acetaldehyde, diacetyl and formic acid (Oberman *et al.*, 1998). The availability of formate and changes in the oxidation-reduction potential in the medium stimulate the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus*, which produces amino acids from the proteolysis of milk proteins. Hence, the stimulation of *Streptococcus thermophilus* growth occurs (Rasic and Kurmann,

1978; Tamime and Robinson, 1985). A desirable ratio of these organisms of 1:1 to 2:1 is maintained in the pasteurized milk used during yoghurt manufacture (Davis, 1971).

The metabolic activity of yoghurt bacteria during fermentation results in a considerable increase in cell numbers. At the end of fermentation the viable count of *L. bulgaricus* and *Streptococcus thermophilus* should be 10^6 cfuml^{-1} of fresh yoghurt (Rasic and Kurmann, 1978). However, at subsequent refrigerated cold storage of yoghurt there is a gradual decrease in the total number of viable culture bacteria, particularly *Streptococcus thermophilus*. The elimination of *Streptococcus thermophilus* is due to the low pH. *L. bulgaricus*, on the other hand, is acid tolerant and thus persists in the refrigerated yoghurt for weeks (Rasic and Kurmann, 1978).

1.5.2 Yeasts

Dairy products offer a special ecological niche that selects for the occurrence and activity of specific yeasts (Deak and Beuchat, 1996). Yeasts' growth in milk products is attributed to their ability to utilise milk constituents such as proteins, fat, lactose and citrate (Fleet, 1990).

Yeasts play an important role in dairy products: (i) in fermented milk products such as kefir, in which yeasts and LAB have a mutualistic interaction, yeasts enhance the growth of the LAB (Gadaga *et al*., 2001a). (ii) some yeast are known for their inhibitory role against undesirable microorganisms (Mathara *et al*., 2004), thus, contributing to the quality and safety of the fermented milk products. In an investigation done by Mathara *et al.* (2004), the Enterobacteriaceae were not detected simultaneously with yeasts during the fermentation of Maasai milk. (iii) yeasts also affect the quality of fermented milk by improving flavour through the production of flavour and aroma compounds (Jakobsen and Narvhus, 1996). *Saccharomyces cerevisiae* is well known for its function of converting

carbohydrates into alcohols and other aroma compounds such as esters, organic acids and carbonyl compounds (Torner *et al*., 1992). Also, the production of acetaldehyde was enhanced when *Candida kefyr* was in co-culture with LAB during the production of Zimbabwean naturally fermented milk (Gadaga *et al*., 2001b). (iv) some researchers, although few, showed that yeasts (*S. cerevisiae* in particular) may influence the nutritional value of fermented products. In an investigation where *S. cerevisiae* and *L. plantarum* were used as starter cultures to ferment various cereals in the production of weaning foods, an increase in the content of riboflavin, thiamine, niacin and ascorbic acid was noticed during fermentation (Sanni *et al*., 1999). The total contents of polyphenols, tannins and phytate were reduced by the fermentation, resulting in a better bioavailability of micronutrients.

Yeasts are apparently indigenous to labaneh, as its microbial flora forms an interesting microbial ecosystem. The high acid content of labaneh, which is enough to inhibit bacterial growth, and the limited access of air to the labaneh in the containers during refrigerated storage encourage a special habitat that is most suitable for the growth of yeasts. *S. cerevisiae* was found to be the predominant yeast species in Labaneh (Yamani and Abu-Jaber, 1994) and its predominance was explained by its ability to ferment glucose and galactose and to assimilate glucose, galactose and lactic acid, which occur in appreciable numbers in yoghurt whey.

1.5.2.1 Yeast- LAB interactions

Although fermented milk products are regarded as predominantly lactic acid bacterial fermentations, the frequent co-occurrence of yeasts and LAB has led to the suggestion that interactions may occur that can influence product characteristics and quality. These microbial interactions have been suggested in fermented products such as blue cheese, kefir, koumiss and *suusac* (Loretan *et al*., 2003; Lore *et al*., 2005)**.** Certainly, the presence of the yeasts is crucial for the

desirable properties of carbon dioxide and ethanol production in east European and Asian products such as kefir, koumiss and airag (Narvhus *et al*., 2003).

Symbiotic interactions have been reported between yeasts and LAB during the fermentation of some milk products. Gadaga *et al.* (2001a) investigated the growth and interaction of yeasts and LAB from Zimbabwean naturally fermented milk. The results suggested that *C. kefyr* stimulated the growth of *Lb. paracasei* subsp. p*aracasei* by providing essential metabolites such as pyruvate, amino acids and vitamins. On the other hand, the yeast utilized certain bacterial metabolites as carbon sources. Furthermore, in another study done by the same authors, when *L. lactis* subsp. *lactis* biovar *diacetylis* and *C. kefyr* grew mutually in co-culture the production of acetylaldehyde by the LAB was enhanced and the viability of LAB during storage was prolonged (Gadaga *et al*., 2001b).

Interaction between *Lb. hilgardii* and *S. florentinus* isolated from sugary kefir grains has also been reported, where yeasts stimulated the LAB through the production of carbon dioxide, pyruvate, propionate and succinate (Leroi and Pidoux., 1993a). In addition, some LAB release galactose into the medium, which may be used by galactose-assimilating but lactose-negative yeasts (Marshall, 1987).

Interactions of yeasts with LAB in some milk fermentations may result in inhibition or elimination of undesirable microorganisms. During the isolation of dominant microorganisms of *kule naoto*, the Maasai traditional fermented milk of Kenya, no Enterobacteriaceae were detected in any of the samples where yeasts were present (Mathara *et al*., 2004). Thus, a possible interaction between yeasts and bacterial flora in the fermentation of Maasai milk was observed. It is believed that a symbiotic relationship may occur when LAB produce organic acids such as lactic acid which lower the pH. The lower pH, being favourable for growth of many yeast species, causes the yeasts to become competitive in the immediate medium (Viljoen, 2001). Due to the low pH, the inhibitory metabolites produced,

and the strong competitive effects of yeast and LAB populations many spoilage and pathogenic microorganisms are inhibited. As a result, the shelf life of the fermented milks is extended.

1.6 ANTIMICROBIAL ACTIVITIES OF LACTIC ACID BACTERIAL CULTURES WITHIN FERMENTED MILKS

The antimicrobial effect of LAB has been appreciated by man for more than 10 000 years and has enabled him to extend the shelf life of many foods through fermentation processes. LAB exert strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens. This is done through the production of various metabolites such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bacteriocidal proteins during lactic acid fermentations (Sorels and Speck, 1970; Gilliland and Speck, 1977a; Pitt *et al*., 2000; Nasib *et al*., 2006; Price and Lee, 1970; Dahl *et al*., 1989; Vandebergh, 1993; Tagg *et al.*, 1976).

The direct antimicrobial effects of organic acids including lactic, acetic and propionic acids, which are end products of LAB fermentation, have been studied (Kingamkono *et al*., 1998; Ogawa *et al*., 2001b; Rubin and Vaughan, 1982; Garrote *et al*., 1999). The antagonistic actions of acids are believed to be; (i) interference with the maintenance of cell membrane potential, (ii) inhibition of active transport, (iii) reduction of intracellular pH, and (iv) inhibition of various metabolites functions (De Vuyst and Vandamme, 1994a). They have a broad mode of action and inhibit both Gram-negative and Gram-positive bacteria as well as yeasts and moulds.

Apart from their ability to produce organic acids, LAB produce hydrogen peroxide (H_2O_2) through the oxidation of reduced Nicotinamide adenine dinucleotide (NADH). H_2O_2 can have a strong oxidizing effect on membrane lipids and cellular proteins (Price and Lee, 1970; Gilliland and Speck, 1977a; Dahl *et al*., 1989;

Vandenbergh, 1993). Attaie *et al.* (1987) confirmed that *S. aureus* was inhibited by H_2O_2 produced by the starter cultures during production of acidophilus yoghurt. In addition to acids and H_2O_2, LAB starter cultures can produce a range of other antimicrobial metabolites such as ethanol and carbon dioxide from the heterofermentative pathway and diacetyl which is generated from excess pyruvate coming from citrate (Caplice and Fitzgerald, 1999).

Bacteriocins from generally regarded as safe (GRAS) LAB have arisen a great deal of attention as a novel approach to control pathogens in foodstuffs. Their use as natural food preservatives has been widely studied (Benkerroum *et al*., 2002; Attaie *et al*., 1987; Farais *et al*., 1994; Davies *et al*., 1997). These proteinaceous inhibitors target the cell membrane and depolarize it, and also inhibit synthesis of the cell wall (Ross *et al*., 2002). Since Gram-negative bacteria have a protective barrier provided by the lipopolysaccharide (LPS) layer of the outer membrane, bacteriocins are generally most inhibitory against Gram-positive bacteria (Savadogo *et al*., 2004a). Common targets in context of fermentation include *Bacillus* and *Enterococcus* spp., *Staphylococcus aureus, Listeria monocytogenes* and *Clostridium* spp.

Besides the production of inhibitory compounds, high numbers of lactic acid bacteria (10^6 $cfuml^{-1}$) compete with the pathogens for nutrients during the fermentation process (Pitt *et al*., 2000). The combined influence of large numbers of competing LAB and the resulting decrease in pH produce an unfavourable environment for many pathogens such as *Listeria monocytogenes* (Pitt *et al*., 2000). Adhesion of pathogenic bacteria to mucosal surfaces is considered to be the first step of intestinal infections (Tuomola *et al*., 1999). Some probiotic bacteria with beneficial health effects have been found to adhere to the intestinal mucosa. Therefore, adhesive probiotics could inhibit mucosal adherence and invasion by pathogens (Tuomola *et al*., 1999).

Each antimicrobial compound produced during fermentation provides an additional hurdle for pathogens and spoilage bacteria to overcome before they

can survive and/or proliferate in the food from the time of manufacture to the time of consumption. Since any microorganisms may produce a number of inhibitory substances, its antimicrobial potential is defined by the collective action of its metabolic products on undesirable bacteria.

Addition of lactic starter cultures for the production of yoghurt and other milk products has been shown to provide a measurable defence against pathogens. However, a number of pathogens tolerate the acidic conditions created by these starter cultures (Foster *et al*., 1990; Leyer *et al.,* 1995; Cormac *et al*., 1996). Therefore, fermented products manufactured with starter cultures should not be assumed to be free of pathogens.

1.7 ENVIRONMENTAL FACTORS OF YOGHURT AND OTHER FERMENTED MILKS THAT FAVOUR THE GROWTH AND SURVIVAL OF PATHOGENS.

The growth of most foodborne pathogens is controlled by refrigeration, pasteurization, addition of lactic acid bacteria and/or addition of selected antimicrobial agents. However, there are environmental factors that allow the growth of some spoilage and pathogenic organisms in yoghurt, the most important being nutrients, pH, and temperature.

During lactic acid fermentation, yoghurt bacteria (*L. bulgaricus* and *Streptococcus thermophilus*) metabolise lactose to produce lactic acid (Tamime and Robinson, 1985). Furthermore, probiotic bacteria were also found to produce both lactic acid and acetic acid (Samona *et al*., 1996). The presence of these organic acids results in a low pH. Hence, the final pH of commercial yoghurt ranges between 3.7 and 4.3 (Hamann *et al*., 1983). Many reports have shown that this low pH has an advantage of inhibiting growth of undesirable organisms (Rubin *et al*., 1982; Giraffa *et al*., 1994). However, many pathogens adapt and survive this condition. Leyer and Johnson (1992) demonstrated that acid adaptation of *E. coli*

O157:H7 increased its survival in acid foods. *L. monocytogenes* is a lactic-acid producing bacterium that can resist several stresses including low pH (Cole *et al*., 1990). Cormac (1996) confirmed this finding by demonstrating the enhanced survival of acid-adapted strains in foods containing lactic acid such as yoghurt. *Salmonella spp* and *Staphylococcus aureus* are also known to adapt to acidic environments and this promotes their survival in dairy products (Leyer *et al*., 1995; Pazakova *et al*., 1997). The mechanism of acid tolerance has not been fully elucidated yet.

Fermented milks, such as yoghurt and Amasi, are usually kept at refrigerated cold storage at temperatures ranging between 0 - 10 °C (Rasic and Kurmann, 1978). Even though these temperatures are inhibitory to growth of many undesirable microorganisms, some investigators showed that pathogens are able to grow at this range. Massa *et al*., (1997) studied the survival of *E. coli* O157:H7 in yoghurt during preparation and storage at 4 °C and found that the organism lost its viability rather slowly during refrigeration (Table 3).

Yersinia enterocolitica survived in yoghurt for 3-5 days during storage at 4 °C (Binnet, 1983). Moreover, Ahmed *et al*. (1986) showed that *Y. enterocolitica* could survive in yoghurt for 5 days at 5 °C when the organism was inoculated into the milk after the addition of starter cultures. Donnelly and Briggs (1986) demonstrated that *L. monocytogenes* survives well at refrigerated temperatures. This pathogen was found to grow at temperatures as low as 4 °C (Ryser and Marth, 1988). Interesting to note are the results of Dalu and Feresu (1996) who investigated the survival of *L. monocytogenes* in traditionally fermented unpasteurized and pasteurized milk, and industrially fermented milk marketed in Zimbabwe. They found that more cells of *L. monocytogenes* survived in all three fermented milk products when they were stored at 5 °C than at 20 °C.

Fermented milks are nutritious foods supplying most of the essential amino acids, carbohydrates and many other required nutrients such as fat and vitamins (Rasic and Kurmann, 1978). Usually, their nutritional value is determined by the nutritive

value of milk from which it is made. Lactose and sucrose are present in yoghurt as major carbohydrates where the lactose concentration is approximately 4 % and sucrose concentration for fruit and flavoured yoghurt may vary between 5 -10 % (Davis, 1974). Davis (1971) indicated that glucose and fructose may occur in yoghurts through the use of invert sugar by some manufacturers and that small amounts of galactose may arise from the bacterial metabolism of milk lactose.

The presence of these carbohydrates, thus, encourages the growth of pathogens in yoghurt. Carbohydrates are essential for growth of *L. monocytogenes*, with glucose serving as carbon and energy source (Cliver, 1990). The introduction of fruit and sugar into yoghurts amplifies the risk of contamination by pathogens. In the case of staphylococcal poisoning which occurred in France the high sugar content favoured the development of *Staphylococcus aureus* and toxin formation while inhibiting the lactic acid bacteria (Mocquot and Hurel, 1970).

1.8 HEALTH BENEFITS OF FERMENTED MILKS

Traditionally, fermented milks have been accepted for their flavour, taste and texture (Rasic and Kurmann, 1978). In recent years, however, a substantial expansion of the market for yoghurt and other fermented milks is caused by people's belief in the nutritional and healthy value of these products. The value of yoghurt and similar fermented milks in human health is not only based on the high nutritional value related to the great variety of components in milk, but also on the beneficial effects of probiotic and other yoghurt bacteria present (Wood, 1998). The consumption of probiotic products, such as yoghurt and acidophilus milk, is beneficial in alleviating lactose intolerance, inhibiting microbial pathogens, and enhancing digestibility of nutrients and the immune system.

1.8.1 Alleviation of lactose intolerance

Many humans lack the enzyme β-galactosidase in their intestine and thus are unable to digest lactose efficiently (Kim and Gilliland, 1983). Lactic acid bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* that are used as starter cultures and probiotic bacteria simultaneously produce this enzyme which hydrolyses lactose, thus resulting in enhanced tolerance for dairy products (Kim and Gilliland, 1983). Furthermore, during the manufacture of yoghurt, lactose content is decreased by 20-30 % or sometimes more (Rasic and Kurmann 1978). The reduced content of lactose in yoghurt is an important factor for better tolerance in fermented milks than ordinary milk by lactose intolerant people.

1.8.2 Inhibition of microbial pathogens

Probiotic bacteria such as bifidobacteria and lactobacilli exhibit antimicrobial properties. These have been demonstrated against foodborne pathogens (Gilliland and Speck, 1977a). Mechanisms responsible for the inhibition of pathogens include competition for nutrients, adhesion of sites, production of inhibitory metabolites such as organic acids and hydrogen peroxide and the stimulation of the immune system (O' Sullivan *et al*., 1992). *Lactobacillus acidophilus* binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria (Bernet *et al*., 1994).

1.8.3 Anticarcinogenic activity

People of Finland consume large amounts of yoghurt and it has been suggested that they harbour numerous intestinal lactobacilli that have anticarcinogenic properties (O' Sullivan *et al*., 1992). Suppression of tumour cells can also be mediated indirectly through an activation of the immune system whereby whole cells as well as cell-wall fragments of LAB can activate the macrophages in the host. A review indicating that some LAB and fermented milk play a significant role in suppressing carcinogenesis has been published by Adachi (1992).

1.8.4 Enhancement of the immune system

Lactic acid bacteria activate macrophages and lymphocytes and enhance Ig A (Fooks *et al*., 1999) in order to protect the host against infection by enteric pathogens and tumour development. Simone *et al*. (1989) also found that *Lb. acidophilus*, *Lb. delbrueckii* ssp. *bulgaricus* and bifidobacteria influence the regulation of γ-interferon production by human peripheral blood lymphocytes in-vitro. γ-Interferon exhibits antiviral and anti-proliferative effects and can activate killer cells.

1.8.5 Enhancement of digestibility and utilization of nutrients

The lactic acid produced by LAB has demonstrated a number of physiological and biological advantages including improvement of digestibility of milk proteins by precipitating them in their curd particles, acceleration in the release of stomach contents, improvement of calcium, phosphorus and iron utilization, and provoking the secretion of gastric juice (Rasic and Kurmann, 1978).

1.8.6 Decrease of cholesterol level in blood

There is an increasing awareness that the risk of heart attacks and serum cholesterol correlates. The beneficial influence of fermented milk on serum cholesterol has been acclaimed by Mann and Spoerry (1974) who stated that after the consumption of a large quantity of fermented milk by Maasai men, the level of serum cholesterol decreased.

1.9. BACTERIAL FOODBORNE PATHOGENS AND THEIR SURVIVAL IN FERMENTED MILKS

Foodborne pathogens prevailed for a number of decades and some continue to emerge. Many of these organisms have been studied including enteric bacteria, aerobes and anaerobes, viral pathogens and yeasts. A broad spectrum of microbial pathogens can contaminate human food and water supplies, and cause

illness after they or their toxins have been consumed. Many of them have been known to cause outbreaks in many countries.

Contamination by these pathogens in fermented milks, in particular yoghurt, may occur through air, but it is mainly due to contaminated packaging material, ingredients such as fruit concentrates, honey, and chocolate, stabilizing agents such as thickeners and poor hygiene of the processing lines. The most important and dominant pathogens, their characteristics and diseases they cause and their survivals in fermented milks are discussed.

Two types of food poisoning have been described. These include infections which involve food poisoning caused by the ingestion of live microorganisms when the organisms grow in the gastrointestinal tract to produce the disease. They also include intoxications in which the organism grows in the food and release a toxin from the cells. Most of the foodborne pathogens are categorized according to the type of food poisoning they cause.

1.9.1 Bacteria causing infections

1.9.1.1 *Escherichia coli*

This organism is considered to be part of the normal microflora of the intestinal tract of humans and other mammals. The species is Gram-negative, a non-spore-forming rod which is motile. Organisms of this species are generally lactose fermentors, but sometimes the lactose fermentation is delayed (Cliver, 1990). Most strains of *E. coli* are harmless, however some are pathogenic and cause diarrhoeal diseases (Meng *et al*., 2001).

There are principally four different groups of *E. coli* which are pathogenic and have been implicated in foodborne disease outbreaks. These are categorized based on virulence properties, mechanisms of pathogenicity and clinical

symptoms. These categories include the enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), and the enterohaemorrhagic (EHEC) groups. The latter is the most important in terms of severity of foodborne illnesses.

EHEC were first recognized as human pathogens in 1982 when *E. coli* O157:H7 was associated with two outbreaks of hemorrhagic colitis (Riley *et al*., 1983). Since then more foodborne disease outbreaks due to *E. coli* O157:H7 have been reported. In the US it is estimated that each year *E. coli* O157:H7 causes approximately 73 000 illnesses and 61 deaths (Mead *et al*., 1999). The principal foods linked to transmission of the organism were shown to be ground beef and raw milk (Griffin and Tauxe, 1991). However, recent reports of outbreaks showed increased variation in vehicles of transmission such as, apple cider (Besser *et al*., 1993), mayonnaise (Weagant *et al*., 1994), even yoghurt which is generally regarded as safe (Morgan *et al*., 1993).

Human infection with *E. coli* O157:H7 can result in non-bloody diarrhoea and haemorrhagic colitis in which the stools contain red blood. It is also the leading cause of haemolytic uremic syndrome which causes renal failure in children. The pathogenicity of this organism seems to be connected with the ability to produce attaching and effacing adherence to the large bowel and the production of verotoxin or Shiga-like toxin (Griffin and Tauxe, 1991).

1.9.1.2 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, non-spore forming rod which is facultatively anaerobic. *L. monocytogenes* is psychrotrophic and although it grows best at 30-37 °C, the organism thrives at refrigerated temperatures i.e. at temperatures as low as 4 °C (Cliver, 1990). As a result, this pathogen is an important food-borne hazard because of its ability to replicate slowly at

refrigerated temperatures. The pH range for growth was thought to be 5.6 to 9.6, although recent investigations indicate that the organism can initiate growth in laboratory media at pH values as low as 4.4. *L. monocytogenes* grows optimally at water activity (a_w) ≥0.97. For most strains, the minimum a_w for growth is 0.93, but some strains may grow at a_w values as low as 0.90. Furthermore, the bacterium may survive for long periods at values as low as 0.83 (Swaminathan, 2001).

L. monocytogenes is widespread in the environment and has been isolated from a number of environments including decaying vegetation, soil, animal feed, sewage and water. It is also found in various foods, both raw and processed. These foods include pasteurized milk and milk products, meat and meat products, fermented raw-meat sausages as well as raw vegetables (White *et al*., 2002).

The pathogenesis of *L. monocytogenes* centres on its ability to survive and multiply in the phagocytic host cells (Forsythe, 2000) and to the production of a haemolysin called β-listeriolysin (Eley, 1992). Listeriosis has emerged as one of the major foodborne diseases during the last decade. However, it is not a new disease, the first reported human case occurred in a soldier of the First World War who suffered from meningitis (Rocourt and Cossart, 1997). Between 1930 and 1950, a few human listeriosis cases were reported. However, there are now hundreds of human cases reported every year (White *et al.*, 2002).

Listeriosis is the general name given to a variety of illnesses caused by the consumption of *L. monocytogenes* contaminated foods. Listeriosis is normally present in humans as septicemia or meningitis. Pregnant women are particularly susceptible to the onset of this illness and infection may result in spontaneous abortion or stillbirth of the fetus. It is most common in newborns, the elderly and immunocompromised hosts (Donnelly, 1990).

1.9.1.3 *Salmonella* spp.

Salmonella is a genus of the Enterobacteriaceae family. They are Gram-negative, facultative anaerobic, non-sporeforming rods. *Salmonell*a, with the exception of *Salmonella tyhpi*, ferment glucose with the production of acid and gas (Cliver, 1990). However, they are unable to metabolize sucrose and lactose. They actively grow within a wide temperature range with the optimum growth at 38 °C and the minimum growth at 5 °C. The pH influences the growth and survival of *Salmonella*, the range in which this pathogen survives is about pH 4-9 (Cliver, 1990).

At present *Salmonella* spp. is the most common reported cause of food poisoning in the UK and the USA, being also important in Japan and other parts of the world. The most common serotype in the UK was *Salmonella* Typhimurium until in 1998 when it was overtaken by *Salmonella* Enteriditis which has been on the increase for a number of years (Eley, 1992). A wide range of contaminated foods are associated with *Salmonella* food poisoning including raw meats, poultry, and milk and dairy products (Forsythe, 2000). Contamination is through poor temperature control and handling practices, or cross-contamination of processed foods from raw ingredients.

The primary reservoir is the intestinal tract of humans and animals. This pathogen is excreted in the faeces and can remain viable in the faecal material for several years. The principal source of infection is ingestion of contaminated food.

The disease caused by *Salmonella* is generally called salmonellosis. Characteristic symptoms of this syndrome include diarrhoea, nausea, abdominal pain, mild fever and chills, occasional vomiting and headache. *Salmonella* Typhi and *Salmonella* Paratyphi produce typhoid and typhoid-like fever in humans. Typhoid fever is fatal. The organism multiplies in the submucosal tissue of the

ileal epithelium and then spreads throughout the body via macrophages. Various internal organs such as the spleen and liver become infected. The pathogenicity of *Salmonella* is thought to be due to its enterotoxin and a cytocin (Cliver, 1990).

1.9.1.4 *Shigella* spp.

These are Gram-negative, facultative, non-spore-forming bacilli that ferment many carbohydrates (but usually not lactose) with the production of acid (Cliver, 1990). The genus, consists of four species (*S. dysenteriae, S. flexneri, S. boydii and S. sonnei*), is a member of the family Enterobacteriaceae and is closely related to *E. coli*. The optimal temperature for growth is 37 °C and the pH for growth is about 4.0- 4.5, but can only survive for 30 min at pH 3.5 (Cliver, 1990).

Shigella is the major cause of gastrointestinal illness throughout the world. According to surveys by the Centres for Disease Control in the US, *Shigella* ranks third among bacterial foodborne pathogens in the number of illness cases (Mead *et al*., 1999). The incidence of infection with this pathogen is estimated at 448 000 cases per year, with 20 % of these cases being due to foodborne transmission of the pathogen (Mead *et al*., 1999). Shigellae have no known nonhuman reservoir and are usually transmitted from person to person through poor personal hygiene, although contaminated food and water have been associated with outbreaks of shigellosis (Smith, 1987).

Shigella dysenteriae causes classic bacillary dysentery, which is the most severe form of shigellosis (Lampel and Maurelli, 2001). *Shigella sonnei* causes the mildest infection, while *Shigella flexneri* and *Shigella boydii* infections can be either mild or severe. Symptoms of shigellosis vary from an asymptomatic infection to mild diarrhoea to fulminating dysentery. In severe cases symptoms include bloody stools with mucus and pus, dehydration, fever, chills, toxemia, and vomiting (Cliver, 1990).

1.9.2 Bacteria causing intoxications

1.9.2.1 *Staphylococcus aureus*

These are Gram-positive, facultatively anaerobic, non-sporeforming cocci. They were described in 1897 (Forsythe, 2000). This pathogen produces a wide range of pathogenicity and virulence factors like staphylokinase, hyaluronidases, coagulases and haemolysins (Forsythe, 2000). Staphylococcal food poisoning is caused by the ingestion of food containing pre-formed toxins, named enterotoxins secreted by this pathogen.

Staphylococcus aureus exists in air, dust, sewage, water, milk and food. Although this pathogen is transmitted to food from a human source, equipment and environmental surfaces can also be sources of contamination. Foods that are frequently associated with staphylococcal food poisoning include meat and meat products, bakery products and milk and dairy products. The type of food poisoning caused by *Staphylococcus aureus* is characterized by nausea, vomiting, and abdominal cramps, often with diarrhoea but without fever. The onset of the symptoms is rapid, often appearing 1-6 h after ingestion of the contaminated food (Clive, 1990).

Staphylococcus aureus is involved in essentially all staphylococcal foodborne disease outbreaks. A recent survey revealed that *Staphylococcus aureus* was involved in 15 % of recorded foodborne illnesses caused by dairy products in eight developed countries (De Buyser *et al*., 2001). According to the same report, *Staphylococcus aureus* was responsible for more than 85 % of the dairy-borne diseases in France. Yoghurt was regarded as hygienically safe against pathogens due to high acidity and milk pasteurization which were thought to be effective barriers against contamination by *Staphylococcus aureus* (Benkerroum *et al*., 2002). However, it is now being established that *Staphylococcus aureus*

can actually occur and survive in yoghurt during processing and post-contamination (Pazakova *et al*., 1997).

Pazakova *et al.* (1997) also found that during fermentation, the concentration of *Staphylococcus aureus* cells remained unchanged but was reduced during the cold storage due to the presence of inhibitory substances produced by the yoghurt starter culture. The size of inoculum affects the survival of *Staphylococcus aureus* in yoghurt. For example, milk contaminated with 10^3 $cfuml^{-1}$ showed an absence of *Staphylococcus aureus* after 48 h storage period. Moreover, no *Staphylococcus aureus* species was observed during the fermentation and storage of yoghurt made from milk inoculated with 10^2 $cfuml^{-1}$ of *Staphylococcus aureus*.

Benkerroum *et al*., (2002) studied the behaviour of *Staphylococcus aureus* in yoghurt fermented with a bacteriocin-producing thermophilic starter and found that this pathogen survived yoghurt processing and 10 days of storage at refrigeration temperature. A significant increase in numbers of *Staphylococcus aureus* was even noted in the first 2 h of fermentation. The same behaviour was reported by Pazakova (1997).

1.10 SURVIVAL OF BACTERIAL FOODBORNE PATHOGENS IN FERMENTED MILK

For *E. coli* O157:H7 to be transmitted through food, it must be able to survive the processing and the storage of the food. It must also be able to survive environmental conditions of the food. *E. coli* O157:H7, when inoculated at high levels, survived in mayonnaise (pH 3.6-3.9) for 5-7 weeks at 5 °C (Zhao and Doyle*,* 1994) and survived in apple cider (3.6-4.0) for 10 to 31 days at 8 °C (Zhao *et al.,* 1993). The isolation of *E. coli* O157:H7 from a wide spectrum of high acid foods demonstrates the potential of this pathogen to survive harsh conditions,

including low pH, low a_w and refrigerated storage. As most cultured dairy products, including yoghurt, rely on these conditions combined with pasteurization for microbial preservation and safety, cultured milk products may be at risk for transmitting *Escherichia coli* O157:H7 infection (Guraya *et al*., 1998).

Growth and survival of *E. coli* O157:H7 in fermented milks has been investigated by many researchers over the years. In a study done by Gulmez and Guven (2003) the strains survived for up to 21 days during cold storage (5-7 °C) of kefir. In commercial dairy products inoculated with 10^3 cfuml^{-1}, *E. coli* O157:H7 was recovered for up to 12 days in yoghurt (pH 4.0), 28 days in sour cream (pH 4.3), and at levels >10^2 cfuml^{-1} at 35 days in buttermilk (pH 4.1) (Dineen *et al*., 1998). The survival of *E. coli* O157:H7 cells for up to several weeks in fermented dairy products, specifically cheese, sour cream, yoghurt, kefir, and buttermilk, illustrates the potential health risks associated with post-processing contamination of even low levels of this organism in various dairy foods (Dineen *et al*., 1998).

Persistence of *E. coli* O157:H7 in acidic foods such as fermented milks shows the ability of this pathogen to tolerate and adapt to acidic environments. Acid tolerance of *E. coli* O157:H7 is a general characteristic shared by many enteric bacteria such as *E. coli* and *Shigella* spp. and its acid adaptation can enhance the survival of this organism in acidic dairy foods during fermentation (Gahan *et al*., 1996). Hsin-Yi and Chou (2001) stated that acid adaptation improved the survival of *E. coli* O157:H7 in Yakult and low-fat yoghurt stored at 7 °C.

L. monocytogenes is a lactic-acid producing bacterium that can resist several environmental stresses, including low pH, temperature and osmolarity (Cole *et al*., 1990). *L. monocytogenes* grows well at refrigeration temperatures and minimal nutrients. Behaviour of this bacterium during fermentation and subsequent storage of various fermented milk products has been studied.

Survival of this pathogen in yoghurt depends on the size and starter culture inocula, the final pH reached, the temperature and duration of the fermentation, and the strain (Schaak and Marth, 1988). The pathogen survives between 9-15 h during the fermentation and then can survive from1-12 days during refrigerated storage of yoghurt as shown by the results of Schaak and Marth (1988) (Table 2). Furthermore, Schaack and Marth (1988) believed that it was because of the casein in yoghurt which exerts a protective effect that *L. monocytogenes* was able to survive in yoghurt. In contrast to Schaak and Marth (1988) results, Choi *et al.* (1988) observations showed that *L. monocytogenes* survived longer (from 13-27 days) in plain and flavoured yoghurt stored at the same temperature. During the souring of Ergo, traditional Ethiopian fermented milk, a substantial number of *L. monocytogenes* strains still survived even though the pH had markedly decreased to as low as 3.9 (Ashenafi, 1994). Also, Gohil *et al*. (1996) found that the pathogen survived for 7 days in unsalted labneh (pH 4.5) stored at 4 °C and for 5 days when stored at 10 °C. In cultured buttermilk held at 4 °C *L. monocytogenes* survived from 18 days to 26 days (Choi *et al*., 1988).

Reports have shown that *Salmonella* Typhimurium induce adaptive responses to acids, salts, and temperature, and these adaptive responses may enhance survival in harsh environments (Ingraham, 1987). Leyer and Johnson (1992) showed that it has the ability to adapt to acidic conditions and this adaptation enhanced their resistance to organic acids, thus increasing their survival in fermented dairy products. This further showed that acid-adapted cells were more resistant to the lactoperoxidase system (Leyer and Johnson 1993).

In previous studies studying the survival of *Salmonella* spp. during the preparation and curing of cheese, it was demonstrated that when milk became contaminated with *Salmonella* spp. after pasteurization the pathogen could survive the cheese-making process and persist for several months in cheese (Geopfert *et al*., 1968). Lactic acid produced in yoghurt is shown to be responsible for the death of the most prevalent milk pathogens, including

Salmonella Typhimurium (Rubin and Vaughan, 1979). However, Rubin *et al*., (1982) demonstrated that the elimination of this pathogen in acid-milk was reduced by casein, which exerted a protective effect towards the salmonellae. From these findings, it is clear that the ability of pathogenic microorganisms to survive prolonged periods of time in dairy products containing high amounts of casein might be due to the protective action of casein.

In a study done by Hal-Haddad (2003), *S. infantis* was able to survive during storage at 4 °C in both traditional and 'bio-yoghurt' for up to 5 days when the yoghurt was at pH < 4.5, but up to 10 days when the pH was ≥ 4.5. These results suggested that the pH of yoghurt is crucial in restricting the survival of a pathogen such as *S. infantis*. Another serovar of *Salmonella*, Typhimurium, was shown to survive for up to 9 days in refrigerated cultured skim milks (Park and Marth, 1972).

1.11 YEASTS AS EMERGING FOODBORNE PATHOGENS IN DAIRY PRODUCTS

Dairy products offer a special ecological niche that selects for the occurrence and activity of specific yeasts (Deak and Beuchat, 1996). The occurrence of yeasts in dairy products is significant because they can cause spoilage, effect desirable biochemical changes and they may adversely affect public health (Fleet and Mian, 1987).

Yeasts play an important role in dairy products: (i) the processing of certain fermented products and in the ripening of certain cheeses; (ii) the spoilage of milk and dairy products; and (iii) the usage of yeasts to ferment whey, a major by-product of cheese making (Marth, 1987).

In recent times, the spoilage of yoghurts by yeasts has emerged as a significant problem (Suriyarachchi and Fleet, 1981). Yoghurts become contaminated with yeasts through contaminated ingredients such as fruits, nuts, and honey which are added to the fermented yoghurt base just before packaging (Davis, 1975) and the development of yeasts on the surfaces of production equipment (Fleet, 1992).

The presence of yeasts as the primary contaminants of yoghurt is encouraged by the high acidity, sugar content, low storage temperature and the types of preservatives used (Green and Ibe, 1987). Furthermore the growth of yeasts in yoghurt has been attributed to the ability of the yeasts to ferment lactose or, in fruit- flavoured yoghurt, to ferment added sucrose or to invert sugar used as sweetner (Suriyarachchi and Fleet, 1981). Growth of some yeasts can be inhibited by the addition of sorbic acid as a preservative to yoghurt, but many strains capable of growth in the presence of high sorbate have been isolated from yoghurts (Suriyarachchi and Fleet, 1981). The properties of yeasts that exhibit growth in yoghurt are indicated in Table 5.

A total of 73 yeast strains were isolated from yoghurt and identified as belonging to eight genera of *Torulopsis, Kluyveromyces, Saccharomyces, Candida, Rhodotorula, Pichia, Debaryomyces and Sporobolomyces* (Suriyarachchi and Fleet, 1981). Of these yeasts, *Torulopsis candida, Kluyveromyces fragilis, Saccharomyces cerevisiae, Kluyveromyces lactis* and *Rhodotorula rubra* were found to be the most frequently isolated species. Green and Ibe (1987) confirmed this in their study with the addition of other yeasts such as *Candida rugosa* and *Candida lusitaniae*. Most of these yeasts are spoilage organisms, however, some of these species are of medical importance since they are found to be opportunistic fungal pathogens in humans.

Candida lusitaniae was found to be strongly resistant to amphotericin B and was causally associated with septicemia and death in a patient after peritonitis

(Guinet *et al*., 1983). *C. rugosa* is associated with infections of skin and nails (Cook, 1958). *K. fragilis* was found to be the cause of pulmonary infection in an immuno- suppressed cardiac transplant patient by Lutwick *et al*., (1980). *S cerevisiae* was reported as a nosocomial cause of septicemia in a hyper-alimented burned man (Eschete and West, 1980).

1.12 CONCLUSION

The survival of foodborne pathogens for up to several days or weeks in fermented dairy products, specifically cheese and yoghurt, illustrates the potential health risks associated with post-processing contamination of these pathogens in various dairy products. For this reason, dairy product manufacturers should design comprehensive programs to ensure that these pathogens are absent from post-pasteurization portions of their operations. Features of a fermentation program designed to prevent the presence of bacterial pathogens in the finished product include starter culture management, pH control during and after manufacture, salt concentration monitoring, and maintenance of a controlled sanitary curing environment (Dineen *et al*., 1998).

There is a need for the investigation of the survival period of these foodborne pathogens in yoghurt before the finished product reaches the retail market, in order to heighten the awareness of post-processing contamination in the dairy industry.

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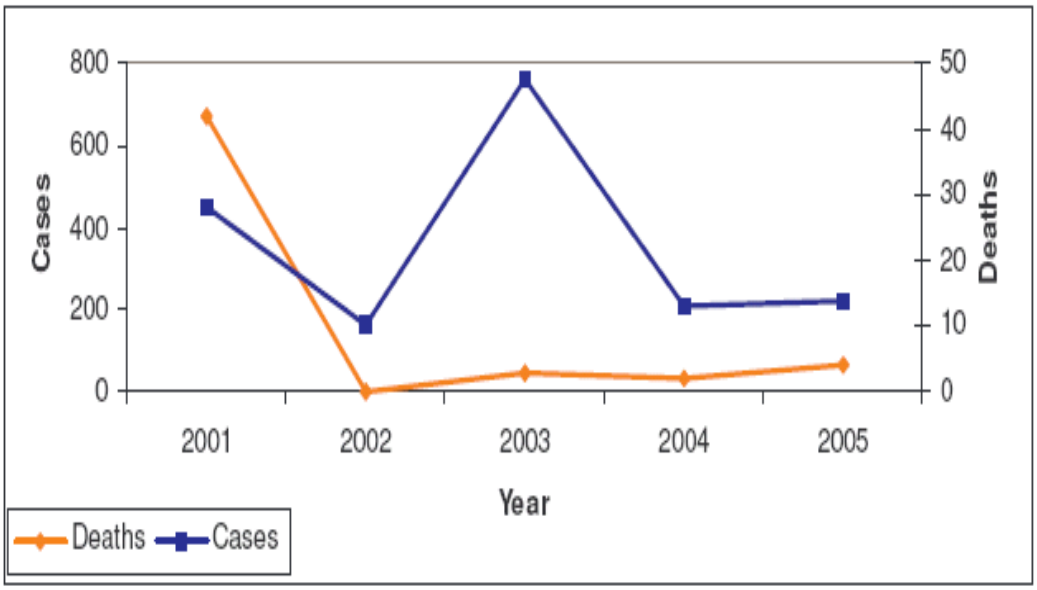
Figure 1: Reported cases of food poisoning, South Africa, 2001-2005



Source: Epidemiology and Surveillance Directorate, NDoH

Table 1. Some fermented milk products in Africa

Local name	**Country**	**Reference**
Susa	Kenya	Kurmann *et al*., (1992)
Maas, Inkomasi,Amasi)	South Africa	Keller and Jordan (1990)
Nono	Nigeria	Atanda and Ikenebomeh (1991)
Laben rayeb	Egypt	Kurmann *et al*., 1992
Ergo and Ititu	Ethiopia	Kurmann *et al*., 1992
Rob	Sudan	Abdelgadir *et al*., 1998
Amasi	Zimbabwe	Mutukumira, 1995
Suusaac	Somalia	FAO (1990)

Table 2. The typical composition of fermented milks

Content	Percentage
Dry matter	ca. 14-18%
Protein	4-6%
Fat	0.1-10%
Lactose	2-3%
Lactic acid	0.6-1.3%
Carbohydrates	5-25%
pH	3.8-4.6%
Alcohol content: in kefir	0.5-2%
In koumyss	2-3%

Natural aromatic compounds, complex and specific for the particular milk product cited by Oberman (1998)

Table 3. Survival of *Escherichia coli* O157:H7 during preparation and storage of traditional (TY) and bifido (BY) yoghurt after low level inoculation.

Time after inoculation	**Temperature (°C)**	**pH TY***	**TY+**	**pH BY***	**BY+**
0 h	42	6.6	3.52	6.6	3.49
5 h	4	5.1	3.04	5.2	3.46
12 h	4	4.9	3.36	5.0	3.43
24 h	4	4.7	3.30	4.8	2.94
2 d	4	4.7	2.93	4.8	2.91
3 d	4	4.5	2.86	4.6	2.89
4 d	4	4.5	2.83	4.6	2.86
5 d	4	4.5	2.79	4.6	2.80
6 d	4	4.5	2.74	4.6	2.76
7 d	4	4.5	2.72	4.6	2.73

* Mean of three values.

+ *Escherichia coli* **O**157:**H**7 (log_{10} $cfuml^{-1}$) recovered; mean of three values.

(Massa and Altieri, 1997)

Table 4. Survival of *L. monocytogenes* in yoghurt mix fermented at 45 °C and subsequently stored at 4 °C.

Strain	**Trial No.**	**Initial no. of *Listeria*/ml**	**Initial pH at refrigeration**	**Final pH**	**Survival (days)**
V7	1	TNTC	4.78	3.94	12
	2	3.14×10^4	4.85	4.09	7
	3	6.63×10^4	4.68	4.08	9
SA	1	TNTC	4.87	3.99	5
	2	2.93×10^3	4.88	4.09	4
	3	6.54×10^4	4.68	4.11	12
OH	1	TNTC	4.89	3.93	12
	2	2.35×10^3	4.86	4.08	7
	3	3.28×10^4	4.62	4.09	9
CA	1	TNTC	4.79	3.88	5
	2	1.65×10^3	4.83	4.13	1
	3	2.06×10^4	4.59	4.11	5

(Schaak and Marth, 1988)

Table 5. Properties of yeasts that exhibit growth in yoghurt.

Yeast species	Property						
	Fermentation of:		Casein hydrolysis	Utilization of lactic acid	Growth at		Growth in
	Sucrose	Lactose			5°C	10°C	Sorbate 100µg/ml
T. candida	+	-	+	+	+	+	+
K. fragilis	+	+	+	+	W	+	+
S. cerevisiae	+	-	-	+	W	+	+
K. lactis	+	+	-	+	W	+	+
T. versitilis	+	+	W	+	W	+	+
C. krusei	-	-	-	+	-	+	+
P. toletana	-	-	-	+	-	+	-

W, weak response

(Suriyarachchi and Fleet, 1981)

CHAPTER 2

SURVIVAL OF FOODBORNE PATHOGENS IN COMMERCIAL YOGHURT DURING STORAGE AT 4 °C

Abstract

The survival of *Escherichia coli* O157:H7, non pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp.* and *Shigella spp* in plain and fruit yoghurt during cold storage was investigated. Fresh yoghurt was inoculated with approximately 10^4 cfu ml^{-1} of each pathogen separately and stored at 4 °C. The inoculated yoghurt was sampled consecutively each day for the enumeration of the presumptive pathogens, LAB, yeasts, and determination of pH. Viable cells of the non-pathogenic *E. coli* and *E. coli* O157:H7 were detected for up to 11 days in plain yoghurt and 14 days in fruit yoghurt. The *S. aureus* cells were most sensitive within the yoghurt environment being detectable in yoghurt for only 24 hrs in both types of yoghurt.. *Shigella spp* survived for 3 days in fruit yoghurt and for 4 days in plain yoghurt. *L. monocytogenes* survived for no longer than 6 days in both types of yoghurt. *Salmonella* survived for no longer than 7 days in both types of yoghurt. LAB numbers remained stable at an average of 8 log cfu ml^{-1} in both types of yoghurt throughout cold storage. Yeasts counts also remained stable at counts below 10 cfu ml^{-1}. A slight reduction in pH was also noticed during storage of the inoculated yoghurt with an average decrease from 4.3-4.1 and 4.2-4.1 in fruit yoghurt.

2.1. INTRODUCTION

Yoghurt is a fermented product obtained by lactic acid fermentation of milk by the action of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococccus thermophilus.* It is usually manufactured with heat-treated milk of standardised composition after inoculation and incubation at 45 °C (Rasic and Kurmann, 1978). It is a unique and interesting dairy product that has gained much popularity all over the world. It is interesting to note the trends in its consumption over the past decades, while some cultured dairy products have shown a steady decline in consumption, yoghurt, on the other hand, has shown a vast increase.

Apart from the traditional yoghurt starter cultures, probiotic cultures representative of *Str. thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* are also added in co-culture with the starter cultures for the production of bio-yoghurts. These products are widely accepted in the market for their beneficial health effects (O'Sullivian *et al*., 1992).

In yoghurts and other fermented milks there are an accumulation of metabolites derived from LAB capable of inhibiting the growth of undesirable microorganisms (Oberman *et al.*, 1998). For example, lactic acid, which is the main product, lowers the pH of the yoghurt, and thus inhibiting the growth of pathogens such as *Staphylococcus aureus* and *Salmonella* spp. (Attaie *et al*., 1987; Pazakova *et al*., 1997; Nassib *et al*., 2006). Inhibition by lactic acid bacteria has also been attributed to their ability to produce specific antimicrobial peptides called bacteriocins (Benkerroum *et al*., 2002). Some lactic acid bacteria also inhibit the growth of Clostridi*a*, Staphylococci and some psychrotrophs by the production of hydrogen peroxide which has strong oxidizing effects on the bacterial cells (Wheater *et al*., 1952; Price and Lee, 1970; Gilliland and Speck, 1977a). Apart from the production of inhibitory compounds, high numbers of lactic acid bacteria ($>10^6$ cfuml^{-1}) compete with the pathogens for nutrients during the fermentation process (Pitt *et al*., 2000).

Although LAB have shown to provide measurable defence against food-borne pathogens, several investigators have demonstrated that pathogens are able to survive during fermentation and storage of yoghurt. Massa and Alteiri (1997) studied the survival of *E. coli* O157:H7 in yoghurt during preparation and storage at 4 °C and found that the organism survived preparation and for 7 days during storage of the yoghurt. *Yersinia enterocolitica* also survived yoghurt manufacture and more than 1 week during storage at 5 °C (Ahmed *et al*., 1986). From the data presented by Schaak and Marth (1988), *Listeria monocytogenes* survived between 9-15 h during fermentation and then for 7-12 days during refrigerated storage within yoghurt.

The survival of these food-borne pathogens in yoghurt and yoghurt-like products is supported by their ability to adapt to the environmental factors. During lactic acid fermentation, yoghurt bacteria metabolise lactose to produce lactic acid and hence a low pH (Tamime and Robinson, 1985). The final pH of commercial bio-yoghurt ranges between 3.7 and 4.3 (Hamann *et al*., 1983). Many pathogens are capable to adapt and survive this environmental stresses. Leyer and Johnson (1992) demonstrated that acid adaptation of *E. coli* O157:H7 increased its survival rate in acid foods. *L. monocytogenes* can resist several stresses including low pH (Cole *et al*., 1990). Cormac (1996) confirmed this finding by demonstrating the enhanced survival of acid-adapted strains in foods containing lactic acid such as yoghurt. *Salmonella* spp. and *Staphylococcus aureus* are also known to adapt to acidic environments and this promotes their survival in dairy products (Leyer *et al*., 1992; Pazakova *et al*., 1997). The mechanism of acid tolerance has not yet been fully elucidated.

Yoghurt is a nutritious food supplying most of the essential amino acids, carbohydrates and many other required nutrients such as fat and vitamins (Rasic and Kurmann, 1978). Usually, the nutritional value of yoghurt is determined by the nutritive value of milk from which it is made. Lactose and sucrose are

present in yoghurt as major carbohydrates; lactose concentration is approximately 4 % and sucrose concentration for fruit and flavoured yoghurt may vary between 5 -10 % (Davis, 1974). Davis (1971) indicated that glucose and fructose may occur in yoghurts through the use of invert sugar by some manufacturers and also from the fruit, and that small amounts of galactose may arise from the bacterial metabolism of milk lactose.

The presence of these carbohydrates, thus, encourages the growth of pathogens in yoghurt. Carbohydrates are essential for growth of *L. monocytogenes*, with glucose serving as carbon and energy source (Cliver, 1990). The introduction of fruit and sugar into bio-yoghurts after fermentation increases the risk of contamination by microorganisms including pathogens. In the case of staphylococcal poisoning from yoghurt which occurred in France (Mocquot and Hurel, 1970), the high sugar content favoured the development of *Staphylococcus aureus* and toxin formation while inhibiting the lactic acid bacteria.

In several major outbreaks of food-borne diseases, dairy products have been implicated as vehicles of transmission. Considerable effort has been made to control these pathogens in dairy products. However, yoghurt has received limited attention due to the fact of pasteurization and the product's high acidity which were thought to be effective barriers to the growth of many pathogens. Consequently, the possibility of survival and persistence of these pathogens in yoghurts and bio-yoghurts could be of great concern.

For that reason, the aims of this study were to determine the survival of these presumptive pathogens, the pH of the yoghurt, and the behaviour of LAB and yeasts during storage of plain and flavoured yoghurt at 4 °C. In addition, the microbial kinetics of these foodborne pathogens during storage of yoghurt at 4 °C were also determined.

2.2. MATERIALS AND METHODS

2.2.1 Cultures

Microbial strains were isolated from infected patients and were supplied by the Medical Faculty of the University of Free State. These comprise strains of *Staphylococcus aureus, Shigella spp., Listeria monocytogenes, Salmonella spp.,* the non-pathogenic *E. coli* and that of serotype O157:H7. All were grown on selective media. Each strain was maintained on nutrient agar (Biolab, Germany) slants at 4 °C with a monthly transfer. Long term preservation was done in 15 and 80 % glycerol in Cryotube™ vials and were stored at -20 °C.

2.2.2 Test product

Freshly made yoghurt samples were obtained from a dairy plant in Bloemfontein, Free State. Plain and strawberry-flavored types of yoghurt packed in 175 ml containers were chosen for the investigation. Samples were transported to the laboratory on ice directly after processing and stored at 4 °C. Microbiological analysis was done after 24 h of cold storage at 4 °C.

2.2.3 Inoculation of microorganisms

During packaging of the product, contamination can occur (post-processing contamination). Therefore, before the fresh yoghurt was used in the investigation it was tested for the presence of any contaminating bacteria. In particular, it was tested for the presence of all the pathogens that were to be used in this study. This was done by plating out 0.1 ml of each sample on selective media (Table 1) and incubated at 37 °C for 24 hrs.

A pre-inoculum was prepared by inoculating one loopfull of cells from each pure culture in 10 ml Tryptic Soy Broth (TSB). This was incubated for 6-8 h at 37 °C. The pre-inoculum (0.1 ml) was then inoculated into 1 L Erlenmeyer flasks containing 200 ml of TSB and incubated at 37 °C for a further 8 h. A cell concentration of 10^4 cfu ml^{-1} of each microorganism was inoculated into the bio-yoghurt, the lid of the yoghurt containers was re-sealed and stored at 4 °C.

2.2.4 Enumeration of food-borne pathogens and lactic acid bacteria.

During each sampling day, 1 ml of the yoghurt was aseptically drawn from the test yoghurt and dispensed in 9 ml peptone water. For each test sample, serial dilutions (10^{-1},10^{-2}, 10^{-3}) were carried out for microbial assays in 9 ml peptone water and plated out in duplicate using the spread plate technique onto selective agar (Table 1). 0.1ml of undiluted bio-yoghurt sample was also plated out on the selective agar. The plates were then incubated aerobically at 37 °C for 24 h. After incubation, typical colonies (Table 1) of the presumptive pathogens were counted and the results interpreted as cfu ml^{-1}. Microbiological analysis of the foodborne pathogens were done every day until no pathogenic cells was detected on the plates. The lactic acid bacteria were enumerated using De Man Rogosa agar (MRS) (Biolab) and the plates incubated aerobically at 30°C for 48 h. Yeasts were enumerated on Rose Bengal Chloramphenicol Agar (RBCA) and incubated aerobically at 30 ^{o}C for 5 days.

2.2.5 Determination of pH

The pH of each sample was determined using a Cybersan pH meter, model 500.

2.2.6 Kinetics of microbial survival

The survival curve of each food-borne pathogen was determined at cold refrigeration. Linear regression analysis of each curve was carried out to obtain

the slope or specific death rate (μ') using Microsoft Excel software. The death equation was used to calculate the length of survival of all pathogens if initial concentration was 100 cfu.ml^{-1}.

Death equation:

$$\mathbf{X_t = X_o . e^{-kd.t}}$$

Where: X_t is the final concentration of viable cells

$\mathbf{X_o}$ is the initial concentration of viable cells

kd is the specific death rate

t is time

2.2.7 Statistical Analysis

The results were statistically evaluated using STATISTICA 2000 software. Pearson correlation test and one way ANOVA were used for analysis.

2.3. RESULTS AND DISCUSSION

2.3.1 Survival of microorganisms

None of the presumptive food-borne pathogens being tested were found in any of the un-inoculated yoghurt control samples. Behavior of all the pathogens, lactic acid bacteria (LAB), yeasts and the pH values during storage of plain and fruit yoghurt at 4 °C are shown in Fig. 1 and Fig. 2. The slopes of the presumptive foodborne pathogens during their survival in plain and fruit yoghurt were constructed (Figs. 3 and 4) and the specific death rates were determined. These are shown in Tables 2 and 3.

Salmonella spp.

A rapid decrease in cell numbers from an initial count of 4.24 - 2.16 log cfu ml^{-1} in plain yoghurt (Fig. 1) and of 4.73 - 2.23 log cfu ml^{-1} in fruit yoghurt (Fig. 2) was noticed during the first 3 days of storage at 4 °C. In the 3 days cells died off at a rate of 0.068 h^{-1} in plain yoghurt and at a rate of 0.0776 h^{-1} in fruit yoghurt. There was a steady decline in the counts of *Salmonella* spp. from 3-8 d in plain yoghurt and from 3-7 d in fruit yoghurt. During this period, cells died at a rate of 0.0298 h^{-1} and 0.0281 h^{-1} in plain and fruit yoghurt respectively. Cells could no longer be detected after 9 d in plain yoghurt and at 8 d in fruit yoghurt. Results of the survival of *Salmonella* spp. were similar in both plain and flavored yoghurt. Statistical analysis also showed that there was no significant difference (p>0.0001) in the survival of *Salmonella* spp. in plain and flavored yoghurt.

Research on the survival of *Salmonella* spp. has also been done by other researchers and similar results in the duration of survival have been found. *Salmonella* serovar Typhimurium showed a progressive decline in viability during cold storage (4 °C) of yoghurt in a study carried out by Nassib *et al.* (2006), and was undetectable after 7 days. Hal-Haddad (2003) found that *Salmonella infantis*

was able to survive at 4 °C during storage of both traditional and 'bio-yoghurt' for up to 5 days when the yoghurt pH was < 4.5, but up to 10 days when the pH was ≥ 4.5.

The pH decreased from 4.31-4.2 in plain yoghurt and from 4.2-4.11 in fruit yoghurt. The decline in pH was rapid during the first 3 days of yoghurt storage. The cells seemed to have been sensitive to the lowering temperature, causing them to die off at a much faster rate. After 3 days the rate at which the pH declined decreased and the death rate of the cells also decreased. At this point *Salmonella* had adapted to the acidic environment of yoghurt. This characteristic of *Salmonella* spp. of acid-adaptation has been observed by other studies (Leyer and Johnson, 1992; Shen *et al*., 2007).

Staphylococcus aureus

The strain of *S. aureus* used in this study was the most sensitive to the yoghurt environment compared to all the other pathogens tested as it survived for the shortest period in both plain and fruit yoghurt. Counts of *S. aureus* decreased from 3.97 to 1 log cfu ml^{-1} within 24 hrs. The cells decreased rapidly at a death rate of 0.274 h^{-1}. This meant that at a specific time the numbers were decreasing by 27.4 % of the total number of cells at that point. In fruit yoghurt a rapid decline was also seen from 4.18 to 1 log cfu ml^{-1} within 24 hrs of yoghurt storage. No cells could be detected in the yoghurt after 24 hrs in both plain and fruit yoghurt. There was no significant difference (p>0.0001) in the length of survival of *S. aureus* in plain and fruit yoghurt.

In a study done by Minor and Marth (1972), staphylococci were completely inactivated within 24 hrs in yoghurts inoculated with 100-300 cells g^{-1}. No microorganisms were detected after 2-4 days of storage when yoghurt received the large inoculums (10^5 cfu/ ml) of *S. aureus*. In some yoghurts, 99 % or more of

the microorganisms were inactivated within 24 hrs of storage. In another study a 1-2 log reduction was observed during storage of yoghurt at 4 °C (Pazakova, 1997). No *S. aureus* was detected in yoghurt produced from milk contaminated with 10^3 cfu ml^{-1} after 48 hrs of cold storage. Moreover, no *S. aureus* was observed during the fermentation and storage of yoghurt made from milk inoculated with 10^2 cfu/ ml cells of the microorganism.

Results on the pH level of yoghurt inoculated with *S. aureus* showed a decline from 4.18-4.14 in plain yoghurt and 4.2-4.11 in fruit yoghurt. Amongst the pathogens, *S. aureus* was the most sensitive pathogen to low pH (pH< 4.2) as it died within 24 h of storage of the inoculated yoghurt. Several investigators support this finding. Attaie *et al*. (1987) observed that when the pH of acidified yoghurt reaches a pH of 4.8 and lower, the low pH became inhibitory to *S. aureus* and reduced the growth rate. Moreover, Minor and Marth (1970) observed inhibition of *S. aureus* in pasteurized acidified milk over an 8 h period. They showed that lowering the pH of the medium to 4.8 and 4.0 caused a reduction in growth of about 0.2 and 3.0 log cycles, respectively.

Listeria monocytogenes

This bacterium was detected for 6 days in both types of yoghurt. The cells decreased by approximately 3 log units from 3.95-1 log in plain yoghurt and 4-1 log units in fruit yoghurt. The cells died at a rate of 0.0437 h^{-1} and 0.0415 h^{-1} in plain and flavored yoghurts respectively. The type of yoghurt had no apparent effect ($p>0.0001$) on the survival of *L. monocytogenes* as the death rate in both yoghurts during storage at 4 °C was similar.

Choi *et al*. (1988) found that *L. monocytogenes* could survive from 20-27 days during storage of yoghurt with a pH of 4.0 at 4 °C. Although the initial counts of the pathogen were similar, the survival in their study was longer than we found in our study. *L. monocytogenes* survived between 1-12 days in a study done by

Schaak and Marth (1988). This was still longer compared to our results. Siragusa and Johnson (1988) observed that low pH (4.1), high acidity, and 1.5 % lactic acid of yoghurt were apparently able to kill off low (10^1-10^2 cfuml^{-1}) and high numbers (10^6-10^7 cfuml^{-1}) of *L. monocytogenes* cells at 3 and 9 days respectively. The difference in the length of survival observed with our results and others could be due to different strains, yoghurts, pH levels, and the types and numbers of the starter cultures used.

The pH decreased from 4.31-4.2 in plain yoghurt and 4.19-4.12 in fruit yoghurt. The results clearly showed a correlation between the decline in pH levels and the corresponding decrease in cell counts of *L. monocytogenes.*

Shigella spp.

The strains of *Shigella* spp. used could no longer be detected after 4 days in plain yoghurt and after 3 days in fruit yoghurt. The counts decreased from an initial 4.08 log to 1 log unit in plain yoghurt and from 3.85 to 1 log unit in fruit yoghurt within 4 and 3 days respectively. The rate at which the cells died off was similar in plain (0.0819 h^{-1}) and fruit (0.0840 h^{-1}) yoghurts. There was also no significant difference (p>0.0001) in the survival of *Shigella* spp. in plain and fruit yoghurt. The pH declined from 4.31-4.24 in plain yoghurt and 4.2-4.17 in fruit yoghurt. As the pH level decreased, counts of *Shigella* spp. also decreased rapidly.

Non-pathogenic *Escherichia coli* and *E. coli* O157:H7

The non-pathogenic strain of *E. coli* tested is considered as the normal microbiota of the gastro-intestinal tract. It was used in this study to compare its survival rate to the pathogenic strain of *E. coli* O157:H7. The two *E. coli* strains survived much longer in both types of yoghurt compared to other pathogens. Cell counts of *E. coli* decreased from 4.69-1 log unit and *E. coli* O157:H7 decreased

from 4.33-1 log unit within 11 days in plain yoghurt. In fruit yoghurt, cell numbers of *E. coli* and *E. coli* O157:H7 decreased from 4.36-1 log and 4.58-1 log units respectively within 14 days of yoghurt storage. The statistical analysis also showed that there were no significant differences ($p>0.001$) in the survival of *E. coli* O157:H7 and non-pathogenic *E. coli* in either plain or fruit yoghurt.

In accordance with the results obtained in our study, Bachrouri *et al*. (2006) observed that *E. coli* O157:H7 survived in yoghurt for 20 days and could not be found at day 21 when stored at 4 °C. Furthermore, Bachrouri *et al*. (2006) could not find viable cells of non-pathogenic *E. coli* after 21 days of storage at 2-4 °C. Again, a longer time of survival compared to our results was observed. A possible reason for this is the difference in the inoculum levels of the two strains of *E. coli*. After inoculation of *E. coli* O157:H7 in yoghurt at approximately 3 log units, Dineen *et al*. (1998) recovered counts < 10 $cfug^{-1}$ after 12 days at 4 °C. Massa *et al.* (1997) on the other hand, found that cells of *E. coli* O157:H7 lost its viability much slower during storage, which is similar to our results. The population of this bacteria decreased by about 1-2 log cycles at 4 °C storage at the end of 7 days. This strain, however survived for a longer period in a study done by Guraya *et al.* (1998), when cells were undetectable (<10 cfu ml^{-1}) after storage for 35 days Canganella *et al.* (1998) investigated the survival of the non-pathogenic *E. coli* in fruit yoghurts and found that at 4 °C *E. coli* strains exhibited a higher tolerance to the yoghurt environment. Cells were still detectable in the samples after 21 days of storage.

Our results showed a similar trend in the rate at which the cells die-off in *E. coli* and *E. coli* O157:H7 during the first 7-8 days in plain yoghurt and 8-10 days in fruit yoghurt. The cells died off gradually at a rate of 0.0142 h^{-1} and 0.0139 h^{-1} in plain yoghurt and 0.012 h^{-1} and 0.0089 h^{-1} in fruit yoghurt respectively. After this period, cells of both types died off at a much faster rate. In plain yoghurt *E. coli* died off at 0.0633 h^{-1} and O157:H7 at 0.0578 h^{-1} whereas in fruit yoghurt *E. coli* died off at 0.0309 h^{-1} and *E. coli* O157:H7 at 0.0463 h^{-1}.

Again, a decline in pH levels was detected during storage of yoghurt at 4 °C. The pH levels of the yoghurt decreased from 4.2-4.08 on average in fruit yoghurt and from 4.31-4.6 in plain yoghurt. The results indicated that the two strains of *E. coli* were the most resistant to acidic conditions within yoghurt. Despite a rapid decline in pH levels, both microorganisms continued to show a much more gradual decline in cell numbers compared to the other pathogens. Acid tolerance of the *E. coli* strains is a characteristic that has been shown to enhance their survival in acid food. Leyer *et al*. (1995) showed that *E. coli* O157:H7 has an acid-adaptive response, and the expression of this system enhances survival in the presence of lactic acid and in acidified food products such as fermented sausage and apple cider. Hsin-Yi and Chou (2001) stated that acid adaptation improves the survival of *E. coli* O157:H7 in Yakult and low-fat yoghurt stored at 4 °C. Similarly, the microorganisms became more sensitive to pH levels <4.12 in plain yoghurt and <4.2 in fruit yoghurt.

2.3.2 Behavior of Yeasts and Lactic acid bacteria (LAB)

Literature has shown that yoghurts are selective environments that encourage the growth of yeasts due to their low pH (Suriyarachchi and Fleet, 1981). The introduction of fruit and sugar into yoghurts has enhanced the risk of spoilage by yeasts by providing additional sources of contamination and fermentable substrates. However, yeasts rarely grow in refrigerated dairy products due to the competitive growth of the rapidly growing psychrotrophic bacteria (Cousin, 1982). Our results corresponded partially with the absence of yeast growth at low temperatures. During storage of yoghurt, the yeast counts remained stable below 10 cfu ml^{-1} (Figs. 1 and 2). Yeasts counts ranged from 2-3 cfu ml^{-1}. These levels were not high enough to cause yoghurt spoilage. When produced by good manufacturing practice, yoghurts should contain no greater than 1 yeast cell per g, and if stored under refrigeration conditions, it should not undergo spoilage by yeasts (Davis, 1975). This was evident in our results as the yoghurts did not spoil.

The counts of LAB remained high as expected being the starter culture at an average of 8 log units in both plain and fruit yoghurt (Figs. 1 and 2). The counts remained stable during the storage of yoghurt. Bachrouri *et al*. (2006) found similar counts of LAB during storage. It is well known that lactic acid bacteria are inhibitory to the growth and survival of pathogenic and spoilage microorganisms. Lactic acid bacteria utilize the lactose present in the yoghurt and by the lactic acid fermentation produces organic acids, mainly lactic acid. These organic acids cause a decline in pH and thereby inhibiting the growth of pathogens (Gilliland and Speck, 1977a). This characteristic of lactic acid bacteria has been evident in our study. The high levels of lactic acid bacteria resulted in a decline in pH which resulted in the inhibition of pathogens.

2.3.3 Kinetics of microbial death

Death rate (*k')* and specific death rate (*μ')* are both reflections of an exponential decreasing population. However, there is a difference since specific death rate is the fraction of the original number of cells by which the population decreases per unit time. It measures the rate of death when it is maximal and is it is the slope of a straight line, but does not reflect the behavior of the population over a period of time. In contrast, k' is an average value for the population death rate over a finite period of time.

The linear regression equations and the regression coefficients were obtained (Figs. 3 and 4) and the slopes of the equations were determined as the specific death rates. The specific death rate of each pathogen is shown in Tables 2 and Table 3. It should be noted that the non-pathogenic, the pathogenic *E. coli* and *Salmonella* spp. have two specific death rates. For the first 7-8 days in plain yoghurt and 8-10 days in fruit yoghurt, non-pathogenic *E. coli* and *E. coli* O157:H7 had low specific death rates, meaning the die-off was low but the death rate increased thereafter. On the other hand, the death rate of *Salmonella* was high at first and then decreased. The pH played a major role in this change in the

specific death rate of these pathogens. The decrease in pH and the cold temperature were not optimum for growth of the cells, therefore, the cells became stressed, and as a result causing a rapid death of cells for the two *E. coli* strains. In comparison with other pathogens, *S. aureus* had the highest death rates in plain and flavored yoghurt.

Generally, the infective dose of these enteric bacteria ranges between 10-10^7 cells. In our study we used high inoculums of the microorganisms 10^4 cfu/ ml cells in order to construct death curves and also because these numbers are high enough to cause food poisoning. On the other hand, cells present as low as 100 cfu/ ml are known to cause disease. From the death curves we were able to calculate the time of survival of each pathogen in yoghurt stored at 4 ˚C. Results are shown in Figs. 5 and 6. It should be noted that, in our study and in studies done by many other researchers cited in this study, the time of survival is defined by the time the viable cells are ≥10 cfu/ ml. On the different selective agar plates, microorganisms are detectable only when there are ≥10 cells/ ml, but when there were <10 cfu/ ml they could not been detected with certainty or seen on plates.

Based on the data, 100 cells of *Shigella* spp. survived for 2 days in both plain and fruit yoghurt. *L. monocytogenes* remained viable for 3 days and *Salmonella* also for 2 days in both types of yoghurt. When using the specific death rate determined for 100 cells of non-pathogenic *E. coli* within the first days of storage, it remained viable for almost 8 days in plain and fruit yoghurt, but if we used the specific death rate after the cells were stressed, they remained viable for only 2 days in plain yoghurt and 4 days in fruit yoghurt. *E. coli* O157:H7 cells (100 cells) survived for 8 days in plain yoghurt and 12 days in fruit yoghurt.

At dairy plants where yoghurt is produced, contamination levels as high as 10^4-10^7 cfu/ ml of pathogenic cells are highly unlikely because of good hygiene practices and HACCP. However, contamination levels of 10-100 cells are possible. When contamination of these numbers occurs, yoghurt is normally

stored at low temperature for 3 days to allow death of the pathogens before distribution to the market. Based on our results, we clearly indicated that sufficient pathogenic cells survived to cause food-borne diseases. Based on the death kinetics calculations (Tables 4 and 5), *L. monocytogenes*, *Shigella* spp. *Salmonella* spp. and *S. aureus* would survive for less than 3 days in plain and flavored yoghurts, but the pathogenic *E. coli* survived for 8 days and non-pathogenic *E. coli* for 8 and 11days in plain and fruit yoghurt if their initial concentration of cells was only 100 cfu ml^{-1}.

2.4. CONCLUSION

The study has shown that foodborne pathogens of the Enterobacteriaceae family can survive for long periods during cold storage of yoghurt at 4 °C. When inoculated with high (10^4 cfu ml^{-1}) or low (10^2 cfu ml^{-1}) concentrations in the yoghurt, *E. coli* O157:H7 remained viable for longer periods compared to the other pathogens. This provides clear evidence that this bacterium is highly tolerant to acidic foods such as yoghurt and may pose serious concerns. *S. aureus* was the most sensitive pathogen to low pH levels within the yoghurt. It could not survive periods longer than 24 hrs in yoghurt. *L. monocytogenes*, *Shigella* spp. and *Salmonella* spp. can survive for 3-8 days in yoghurt.

When inoculated at low levels of 100 cells in yoghurt, most pathogens in this study excluding *E. coli* O157:H7, survived for 1-3 days in plain and fruit yoghurt. *E. coli* O157:H7 survived for >1 week in the yoghurt. These results are of high public health significance, since its survival is long enough to present a hazard for the consumer especially as the infectious dose is extremely low.

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Table 1. Selective broth and agar used for the enumeration of the presumptive pathogens, and the description of colonies of each pathogen.

Microorganism	Selective broth	Selective agar	Description of colonies
E. coli	Tryptic soy broth (Merk, Germany)	*VRB-Mug agar	Pink to dark red colonies, fluorescence under UV
E. coli O157:H7	Tryptic soy broth	*VRB-Mug agar	Pink to dark red colonies
L. monocytogenes	Tryptic soy broth	Rapid' L. Mono agar (Bio-rad, France)	Green- blue colonies
Salmonella spp.	Tryptic soy broth	*XLD agar	Transparent, sometimes black-centered
Shigella spp.	Tryptic soy broth	*MacConkey agar	Colourless, translucent colonies
S. aureus	Tryptic soy broth	*Baird-Parker agar	Black, glossy and convex with a white margin surrounded by a clear zone

Note: * media from (Biolab, Germany)

Table 2. The specific death rates of the presumptive food-borne pathogens in Plain yoghurt

Plain Yoghurt			
Pathogen	**Period (days)**	**Specific death rate (d^{-1})**	**Specific death rate (h^{-1})**
L. monocytogenes	0d - 6d	1.0479	0.0437
Shigella spp.	0d - 4d	1.9661	0.0819
Salmonella spp	0d - 3d	1.6326	0.0680
Salmonella spp	3d - 8d	0.7140	0.0298
E. coli	0d - 7d	0.3403	0.0142
E. coli	8d - 11d	1.5190	0.0633
E. coli O157:H7	0d - 8d	0.3345	0.0139
E. coli O157:H7	9d - 11d	1.3863	0.0578
S. aureus	0d - 1d	6.5803	0.2742

Table 3. The specific death rates of the presumptive food-borne pathogens in Fruit yoghurt.

Fruit Yoghurt			
Pathogen	**Period (days)**	**Specific death rate (d^{-1})**	**Specific death rate (h^{-1})**
L. monocytogenes	0d - 6d	0.9963	0.0415
Shigella spp.	0d - 3d	2.0160	0.0840
Salmonella spp	0d - 3d	1.8630	0.0776
Salmonella spp	4d - 7d	0.6754	0.0281
E. coli	0d - 10d	0.3039	0.0127
E. coli	11d - 14d	0.7419	0.0309
E. coli O157:H7	0d - 8d	0.2146	0.0089
E. coli O157:H7	9d - 14d	1.1101	0.0463
S. aureus	0d - 1d	7.0276	0.2928

Table 4. Calculations showing the time (days) that the pathogens would survive in plain yoghurt stored at 4°C if the initial inoculums were 100cfuml^{-1}

E.coli; t = - (ln 9 – ln 100) 0.3403 = **7.1 days**	*E.coli*; t = - (ln 9 – ln 100) 1.5190 = **1.6 days**
E. coli O157:H7; t = - (ln 9 – ln 100) 0.3345 = **7.2 days**	*E. coli O157:H7*; t = - (ln 9 – ln 100) 1.3863 = **1.7 days**
Salmonella spp.; t = - (ln 9 – ln 100) 1.6326 = **1.5 days**	*Salmonella* spp.; t = - (ln 9 – ln 100) 0.7140 = **3.4 days**
Shigella spp; t = - (ln 9 – ln 100) 1.9661 = **1.22 days**	*L. monocytogenes;* t = - (ln 9 – ln 100) 1.0479 = **2.3 days**
S. aureus t = - (ln 9 – ln 100) 6.5803 = **0.37 days (8.9h)**	

Table 5. Calculations showing the time (days) that the pathogens would survive in fruit yoghurt stored at 4°C if the initial inoculums were 100cfuml^{-1}

E.coli; t = - (ln 9 – ln 100) 0.3039 = **7.9 days**	*E.coli*; t = - (ln 9 – ln 100) 0.7419 = **3.3 days**
E. coli O157:H7; t = - (ln 9 – ln 100) 0.2146 = **11.2 days**	*E. coli O157:H7*; t = - (ln 9 – ln 100) 1.1101 = **2.2 days**
Salmonella spp.; t = - (ln 9 – ln 100) 1.8630 = **1.3 days**	*Salmonella* spp.; t = - (ln 9 – ln 100) 0.6754 = **3.6 days**
Shigella spp; t = - (ln 9 – ln 100) 2.016 = **1.19 days**	*L. monocytogenes;* t = - (ln 9 – ln 100) 0.9963 = **2.4 days**
S. aureus t = - (ln 9 – ln 100) 7.0276 = **0.34 days (8.6h)**	

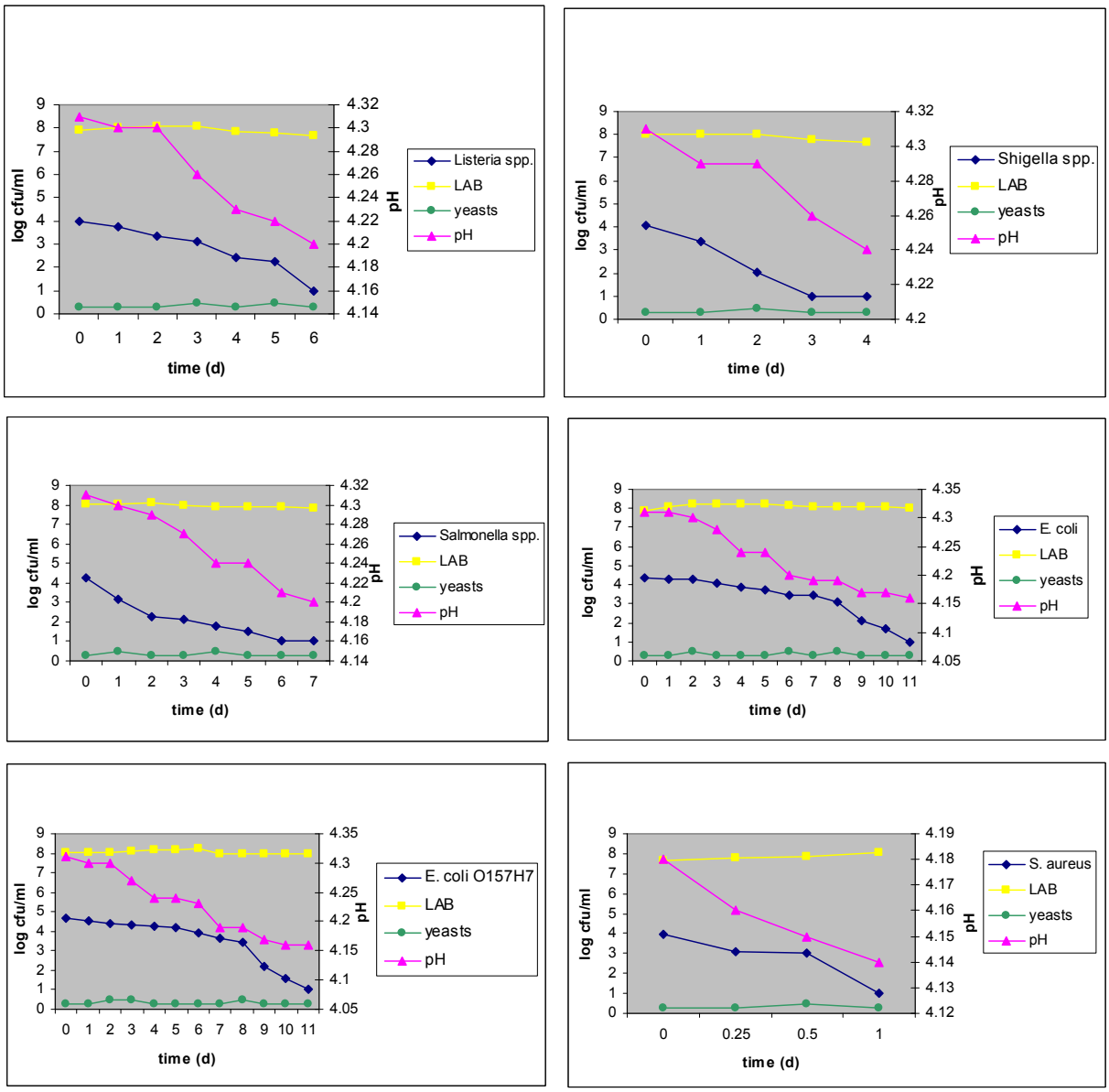

Fig. 1. Survival curves of presumptive food-borne pathogens, behaviour of LAB and yeasts and change in pH in plain yoghurt during cold storage at 4 °C

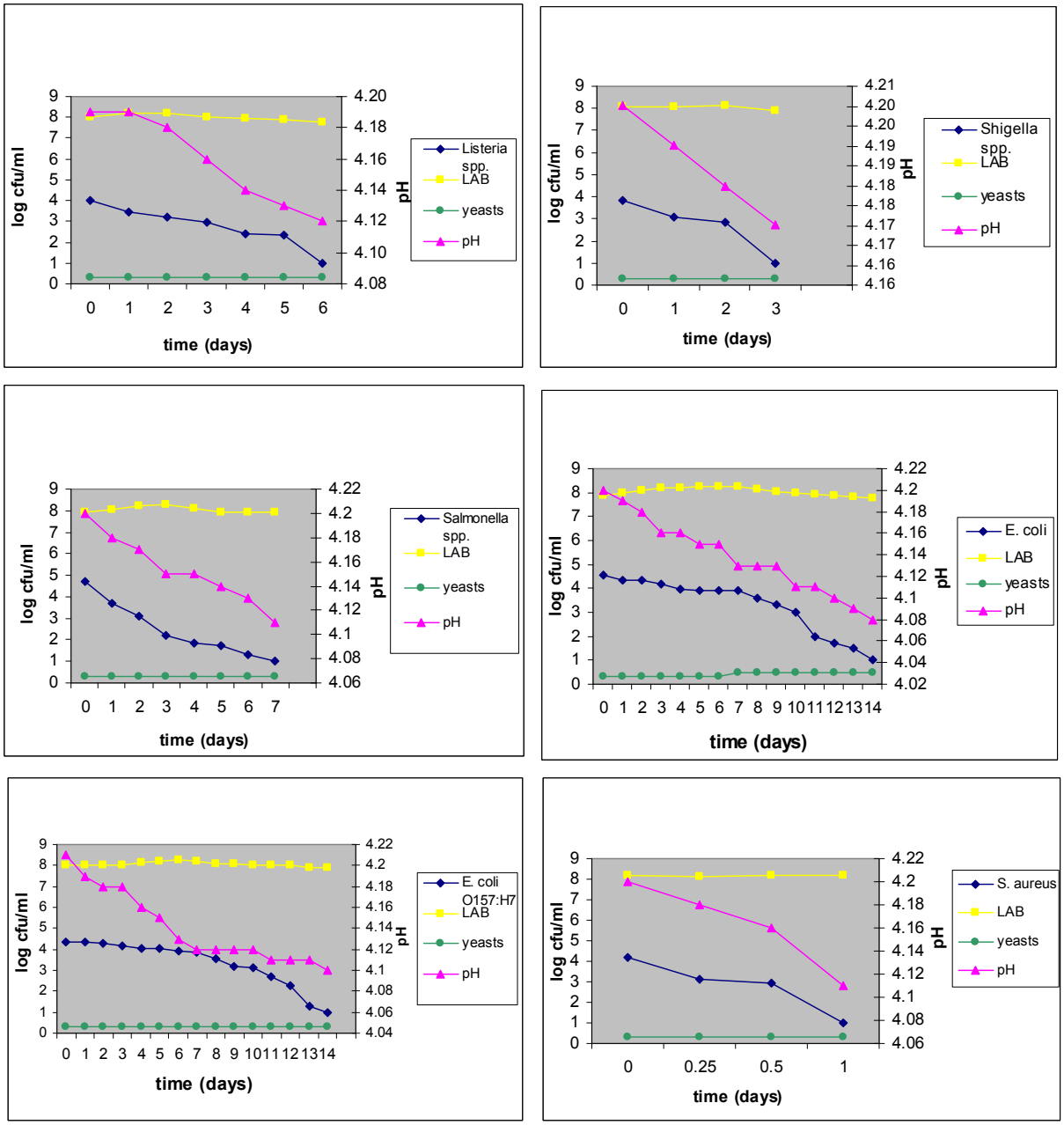

Fig. 2. Survival curves of presumptive food-borne pathogens, behaviour of LAB and yeasts and change in pH in fruit yoghurt during cold storage at 4 °C

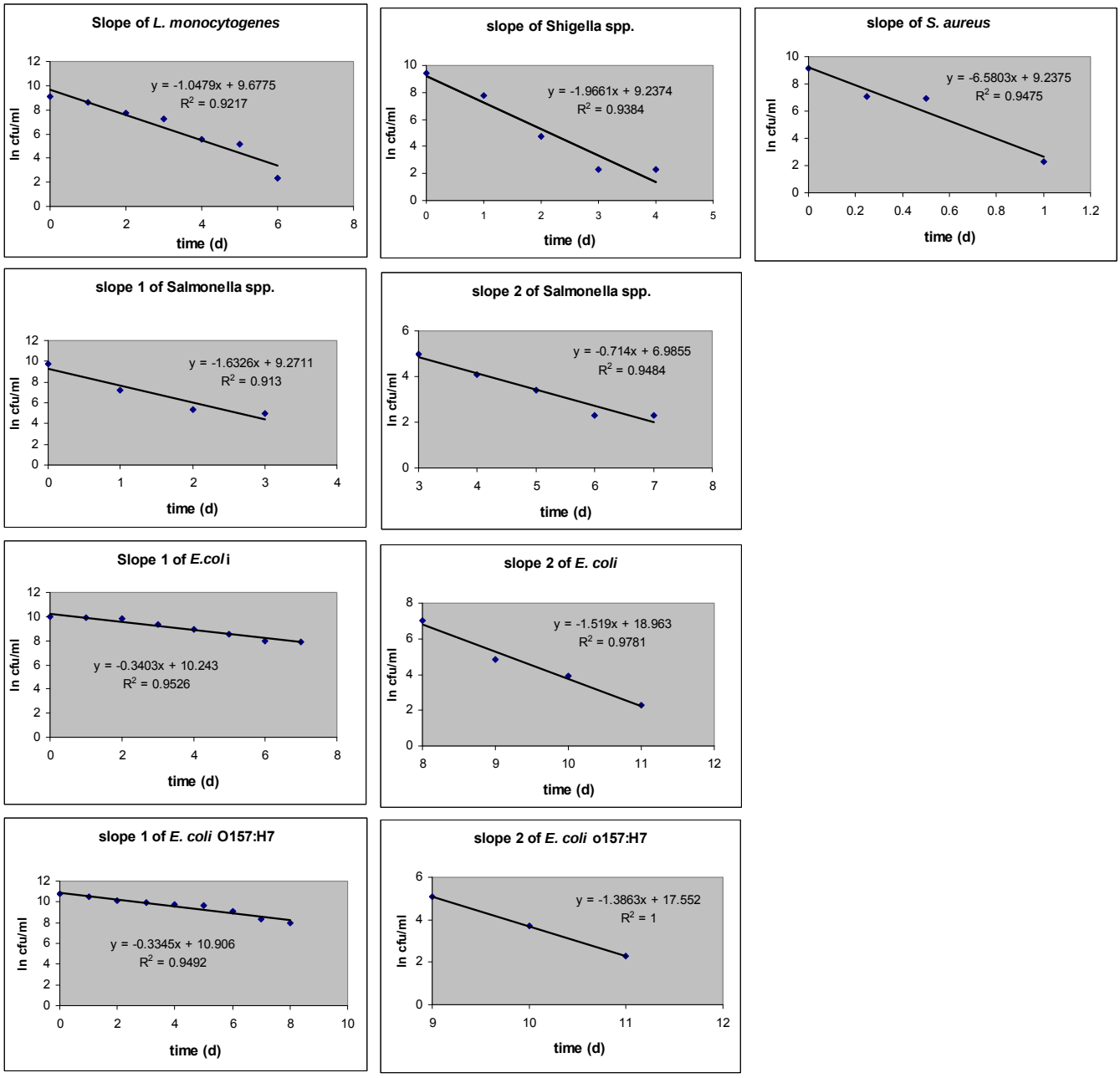

Fig. 3. Slopes of presumptive food-borne pathogens during storage of plain yoghurt at 4 °C

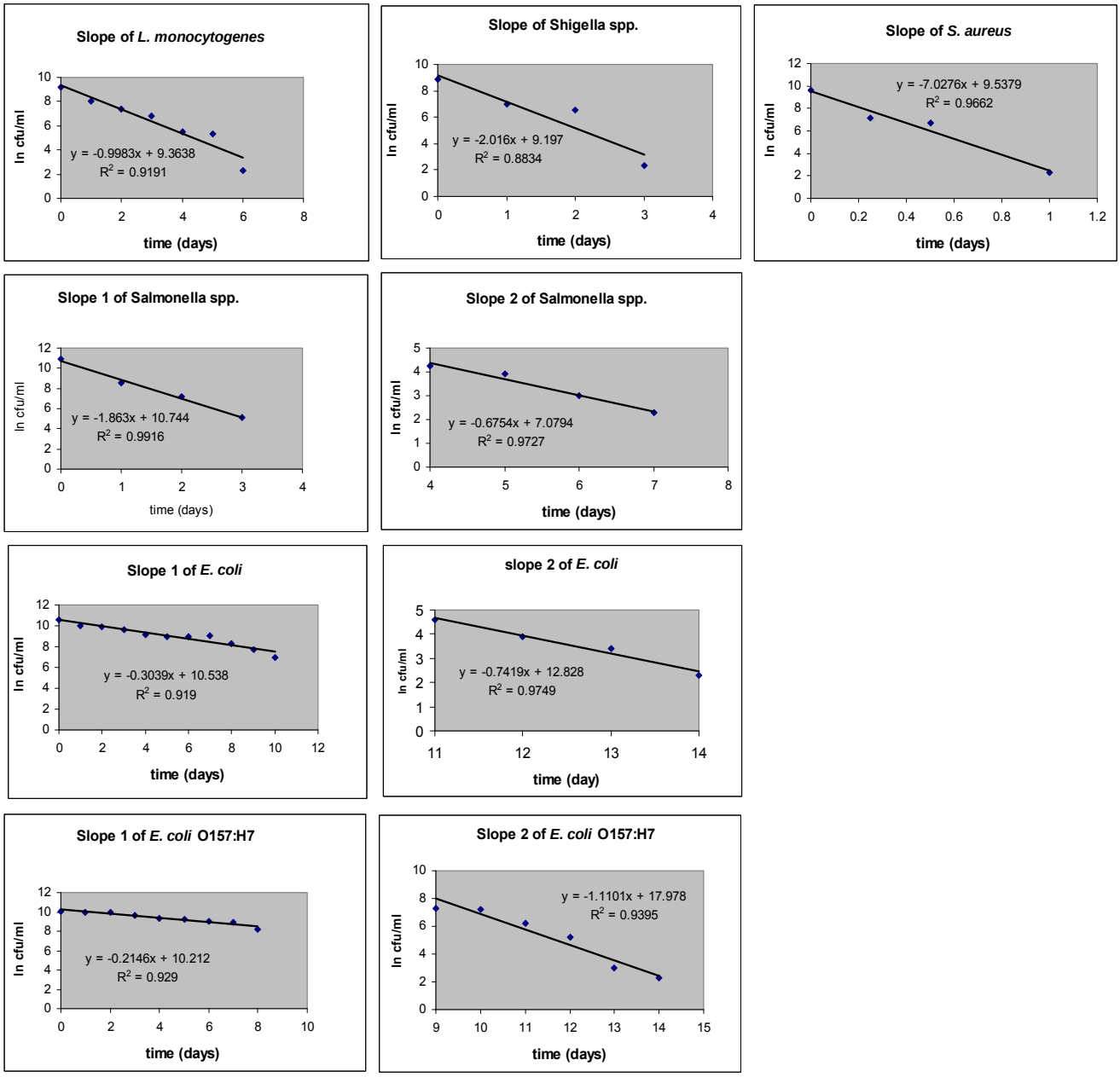

Fig. 4. Slopes of presumptive food-borne pathogens during storage of fruit yoghurt at 4 °C

Death equation: $\mathbf{X_t = X_o . e^{-kd.t}}$

Where; X_t is the final concentration of viable cells

$\mathbf{X_o}$ is the initial concentration of viable cells

kd is the specific death rate

t is time

Therefore; $\mathbf{t = \frac{- (\ln X_t - \ln X_o)}{k_d}}$

CHAPTER 3

THE EFFECT OF TEMPERATURE ABUSE ON THE SURVIVAL OF FOODBORNE PATHOGENS IN YOGHURT

Abstract

The survival of *Escherichia coli* O157:H7, non pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp. and *Shigella* spp. in fruit yoghurt during temperature abuse at 25 °C, 12 °C and 37 °C was investigated. At higher temperatures pathogens are inhibited at a much faster rate. Yoghurt inoculated with foodborne pathogens individually was stored at 25 °C untill pathogens could no longer be detected. There was a rapid die off of pathogenic microorganisms. *L. monocytogenes*, *Salmonella* spp. and *Shigella* spp. survived for 2 days in the yoghurt. Both *E. coli* O157:H7 and non pathogenic *E. coli* survived for 3 days. *S. aureus* was the most sensitive of all the foodborne pathogens, it survived for only 24 hrs in the yoghurt. A rapid decline in pH was noticed throughout the storage period. Lactic acid bacteria increased to levels as high as 9 log units and the yeasts cell counts increased to levels as high as 4 log units. Two sets of yoghurt samples inoculated with the same pathogens were stored at 12 °C and 37 °C for 4 and 6 hrs respectively, and thereafter stored at refrigerated temperature of 4 °C. During the cold storage, yoghurts abused at higher temperatures exhibited that pathogens died off faster with the death rates ranging between 0.0718-0.7413 h^{-1} at 12 °C and 0.2223-0.9473 h^{-1} at 37 °C. When placed back at 4 ^{o}C, the die off was lower with death rates ranging between 0.0188-0.997 h^{-1} in yoghurt initially stored at 12 °C and 0.0152-0.0979 h^{-1} in yoghurt initially stored at 37 °C. In yoghurt abused at 12 °C the levels of yeasts initially remained low (<10 cfu ml^{-1}), but quickly increased to levels as high as 2 log units at the end. A similar behavior was observed for yoghurt samples abused at 37 °C, but in contrast yeast levels increased more rapidly from the onset. LAB numbers increased in both sets of yoghurt when stored at 4 ^{o}C.

3.1 INTRODUCTION

Safety and quality of food are topics of much concern in the food industry and to consumers all around the world. This is a result of emerging foodborne pathogens that continue to cause outbreaks of foodborne diseases in different countries. These outbreaks have heightened the awareness of these pathogens as a major public health hazard.

During the past decades, microorganisms such as *Staphylococcus aureus, Salmonella* spp.*, Escherichia coli* O157:H7*, Shigella* spp. and *Listeria monocytogenes* etc., were reported as the most common foodborne pathogens that are present in many foods and are able to survive in milk and fermented milk products (Alm, 1983; Ahmed *et al*., 1986; Ryser and Marth, 1988; Canganella *et al*., 1998). In recent times, the dairy industry has been concerned with the presence of these pathogens in dairy products, with common sources of contamination as hands and clothing of food processors, and dirty surfaces of equipment. Amongst dairy products yoghurt has always been considered as safe due to the fact that milk pasteurization and the product's high acidity were thought to be effective barriers to the growth of many pathogens.

Yoghurt is a coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* from milk and milk products (Rasic and Kurmann, 1978). Probiotic species such as *Bifidiobacterium bifidum* and *Lactobacillus acidophilus* are also added to yoghurt for their beneficial health effects. Yoghurt is a unique and interesting dairy product which is a perfect alternative to junk and snack foods. In most countries its consumption and popularity have increased considerably since 1964, owing much to its beneficial effects on human health and its nutritional value (Kroger, 1976; Rasic and Kurmann, 1978).

Most commonly yoghurt is stored at 4 °C after its production. Many investigators have shown that some foodborne pathogens are able to survive in this yoghurt stored at low temperature (Ryser and Marth, 1988; Massa *et al*., 1997; Ogwaro *et al*., 2002; Bachrouri *et al*., 2006). Many of the local communities in Africa lack refrigeration and thus store yoghurt at ambient temperatures (25-37 °C). Also some local people do not know the correct storage temperatures of yoghurt and other dairy products. In everyday life in South Africa yoghurt is stored incorrectly by consumers. Typical examples include the following:

- In one instance a person goes shopping and buys yoghurt but instead of immediate storage the yoghurt is kept in the car for 6 hrs at very high summer temperatures (37 °C) while the person continues to do other things.
- In another instance, because electricity cut-offs have become common in South Africa and certain households experience this and the refrigerator is switched off for 4 hrs. During this time the temperature in the yoghurt increases (12 °C).

Little scientific research has been done on the effect of different storage temperatures on the survival of foodborne pathogens in South African yoghurt. Therefore, the aim of this study was to apply the instances mentioned above to determine the survival of foodborne pathogens during temperature abuse and during storage at ambient temperatures (25 °C) where there are no refrigerators.

3.2 MATERIALS AND METHODS

3.2.1 Cultures

Microbial strains were isolated and identified from infected patients and supplied by the Medical Faculty of the University of Free State. These were representatives of the species *Staphylococcus aureus, Shigella* spp*., Listeria*

monocytogenes, Salmonella spp*.,* the non-pathogenic *E. coli* and that of serotype O157:H7. All were grown on selective media (Table 1). Each strain was maintained on nutrient agar (Biolab, Germany) slants at 4 °C with a monthly transfer. Long term preservation was done in 15 and 80 % glycerol in Cryotube™ vials and were stored at -20 °C.

3.2.2 Test product

Freshly made yoghurt samples were obtained from a dairy plant in Bloemfontein, Free State. Plain and strawberry-flavored types of yoghurt packed in 175 ml containers were chosen for the investigation for comparison. Samples were transported to the laboratory on ice within 1 h and stored in the refrigerator at 4 °C. Microbiological analysis was done after 24 h of cold storage at 4 °C in the laboratory.

3.2.3 Inoculation of microorganisms

During packaging of the product, contamination can occur (post-processing contamination). Therefore, before the fresh yoghurt was used in the investigation it was tested for the presence of any contaminating bacteria. In particular, it was tested for the presence of the pathogens that were to be used in this study. This was done by plating out 0.1 ml of each sample on selective media (Table 1) and incubated at 37 °C for 24 hrs.

A inoculum was prepared by inoculating one colony from each pure pathogenic culture in 10 ml Tryptic Soy Broth (TSB). This was incubated for 6-8 h at 37 °C. 0.1 ml of the inoculum was inoculated into 200 ml of the TSB and incubated at 37 °C for a further 6-8 h. A concentration of approximately 10^4 cfu ml^{-1} of each pathogen was inoculated into the yoghurt, the lid was then re-sealed and inoculated samples were stored at varying temperatures.

3.2.4 Sampling and Enumeration of food-borne pathogens, yeasts and lactic acid bacteria.

In this study there were 3 sets of yoghurt. The first set comprised of 6 yoghurt samples inoculated with the test pathogens and stored at 25 °C for the duration of the experiment to simulate yoghurts kept by people with no refrigerator facilities. The second set consisted of 6 yoghurt samples inoculated with the test pathogens and abused at 12 °C for 4 hrs (to simulate power cuts) before storing at 4 °C until the pathogens could not be detected in the yoghurt. The third set comprised of 6 yoghurt samples inoculated with the test pathogens and abused at 37 °C for 6 hrs (simulating consumer kept yoghurts in car) before storing at 4 °C until no pathogens was detected.

Each day 1 ml was aseptically drawn from each test yoghurt and dispensed in 9 ml peptone water. For each test sample, serial dilutions (10^{-1},10^{-2}, 10^{-3}) were carried out for microbial assays in 9 ml peptone water and plated out in duplicate using the spread plate technique onto selective agar (Table 1). 0.1ml of undiluted bio-yoghurt sample was also plated out on the selective agar. The plates were incubated aerobically at 37 °C for 24 h. After incubation, typical colonies (Table 1) of the presumptive pathogens were counted and the results interpreted as cfu ml^{-1}. Microbiological analysis of the foodborne pathogens were done each day until pathogenic counts were no longer detected on the plates. The lactic acid bacteria were enumerated using De Man Rogosa agar (MRS) (Biolab) and the plates incubated aerobically at 30 °C for 48 h. Yeasts were enumerated on Rose Bengal Chloramphenicol Agar (RBCA) and incubated aerobically at 30 °C for 5 days.

3.2.5 Determination of pH

On each sampling occasion, the pH of each sample was determined by a Cybersan pH meter, model 500.

3.2.6 Statistical Analysis

The results were statistically evaluated using STATISTICA 2000 software. Pearson correlation test and one way ANOVA were used for analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 Survival of microorganisms in yoghurt stored at 25 °C

Survival of foodborne pathogens tested is shown in Fig.1. Slopes of pathogens were constructed (Fig. 2) and the specific death rates determined (Table 2). In general, the length of survival ranged between 1-3 days. A rapid decrease in counts over a short period of time was noticed. *L. monocytogenes* survived for 2 days with counts decreasing rapidly (0.0709 h^{-1}) from 3.477-2 log units. The pathogen could no longer be detected in yoghurt after 3 days. *Shigella* spp. decreased from 4.716-1 log units within 2 days. The cells died-off at a rate of 0.1783 h^{-1}. *Salmonella* spp. survived for 2 days decreasing from an initial log count of 4.8 to 1 at a rate of 0.1847 h^{-1}. Within 3 days, non pathogenic *E. coli* and *E. coli O157:H7* cell counts decreased from 4.505-1 log and 5.02-1 log unit respectively. The cells of non pathogenic *E. coli* and *E. coli O157:H7* died off at a rate of 0.1138 h^{-1} and 0.1176 h^{-1} respectively. The highest death rate compared to the other pathogens was exhibited by *S. aureus* of 0.2208 h^{-1} decreasing from 3.3-1.0 log unit within 24 hrs.

An increase in LAB levels was noticed in all the yoghurt samples. On average, LAB counts increased by approximately 1 log unit from 8-9 log cfu ml^{-1}. Yeasts also grew well in the yoghurt samples reaching levels as high as 4 log units in yoghurt containing *Shigella* spp. and 5 log units in yoghurt containing non pathogenic *E. coli*. At these levels the yoghurt showed signs of spoilage and instead of the semi-liquid state it became liquid. Viljoen *et al.* (2003) observed maximum yeasts counts of 6.95 log units in fruit yoghurts stored at high temperatures of 15-25 °C. A rapid decline in pH over a short period of time was also observed. The pH decreased from an average of 4.1-3.96. However, the pH decreased to as low as 3.78 in yoghurt containing non pathogenic *E. coli* and *E. coli O157:H7*.

A correlation between yeasts and LAB was evident in the study (Fig. 1). Yeast numbers increased simultaneously with an increase in LAB numbers which may be attributed to a symbiotic interaction between the organisms. This stimulating interaction has been reported in fermented milks by some researchers. The results by Gadaga *et al*. (2001) suggested that yeasts provided essential metabolites such as pyruvate, amino acids and vitamins. On the other hand, the yeasts utilized certain bacterial metabolites as carbon sources.

It has also been reported that interactions between yeasts and LAB in some milk fermentations may result in inhibition or elimination of pathogenic microorganisms (Mathara *et al*., 2004). LAB produce organic acids such as lactic acid which lower the pH. The lower pH, being favourable for growth of many yeast species, causes the yeasts to become competitive in the immediate medium (Viljoen, 2001). Due to the low pH, the inhibitory metabolites produced, and the strong competitive effects of yeasts and LAB populations, many spoilage and pathogenic microorganisms are inhibited.

When comparing the results of yoghurt stored at cold storage at 4 °C to those of yoghurt stored at room temperature at 25 °C we found that the pathogens were inhibited more rapidly at 25 °C. At 4 °C the time of survival of pathogens ranged between 1-14 days while at 25 °C it ranged between 1-3 days. Initial counts of each pathogen differed at the different temperatures, therefore, we used statistical analysis and the specific death rates to further compared their survival rates. Statistical analysis showed that the decrease in the viable cells of the pathogens at 25 °C was significantly ($p<0.0001$) when compared to that at 4 °C. Death rates of pathogens were also much higher at 25 °C than at 4 ^{o}C. LAB counts increased to levels as high as 9 log units at 25 °C while they remained stable at 8 log units at 4 °C. Little or no growth of yeasts at 4 °C was observed but at 25 °C the yeasts grew to levels as high as 4 log units.

Studies have shown that pathogens die off faster at room temperatures (± 25 °C) than at 4 °C storage. Dalu and Feresu (1996) observed that most cells of *L. monocytogenes* survived when fermented milks products were stored at 4 °C compared to those at 20 °C. When fermented milk inoculated with *E. coli* O157:H7 was stored at 4 °C and 25 °C, a slight reduction of 0.5-1.0 log unit in viable cells occurred after 5 days of storage at 4 °C. However, at 25 °C the pathogen failed to maintain its contamination level and was not recovered after 5 days of storage (Ogwaro *et al*., 2002).

The comparison of yoghurts stored at different temperatures showed that yoghurt stored at cold refrigeration temperatures (4 °C) may remain at good quality without any spoilage, however, the yoghurt may not be safe for consumption as foodborne pathogens were able to survive for longer periods. On the other hand, yoghurts stored at room temperature (25 °C) may be safe for consumption as pathogens die off at a much faster rate surviving for a shorter period of time. However, the yoghurt may be of poor quality as a result of spoilage due to high levels of contaminating yeasts.

3.3.2 Survival of pathogens in yoghurt stored at 4 °C after temperature abuse at 12 °C

Yoghurt inoculated with foodborne pathogens was stored at 12 °C for 4 hrs and then placed back at 4 ^{o}C until no pathogens could be recovered. The results are shown in Fig. 3. All of the pathogens except *S. aureus* decreased by <0.5 log units during the 4 hr abuse of yoghurt at 12 °C. *S. aureus* decreased by 1.3 log units. The slopes of the survival rate of the pathogens were constructed (Fig.4) and the specific death rates determined (Table 3). The death rates were high, showing that the die off of the pathogens was faster. *S. aureus* had the highest death rate of 0.7413 h^{-1} and could not be recovered after the 4 hrs of abuse at 12 °C whereas *L. monocytogenes* had the lowest death rate of 0.0718 h^{-1}. At 4 °C the counts of pathogens decreased by 1-2 log units over a longer period

(Table 3). Much lower death rates were observed with *Salmonella* spp. having the lowest (0.0188 h^{-1}). *L. monocytogenes* survived for 8 d, *Shigella* spp. for 3 d, *Salmonella* spp. for 11 d, *E. coli* for 11d, *E. coli O157:H7* for 8 d and *S. aureus* for 4 hrs.

There was a rapid decrease in pH levels during the experiment and much more enhanced during abuse at 12 °C. This suggested that at high temperatures the pH decreases faster and this has an effect of inhibiting growth of pathogens at a much faster rate than at low temperatures. Gohil *et al*. (1996) observed a similar behavior with *L. monocytogenes* when Labneh (a traditional yoghurt from the United Arab Emirates) was stored at 4 °C and 10 °C. Some cells survived for 7days at 4 °C and for 5 days at 10 °C. They suggested that one of the reasons for the poor survival of *L. monocytogenes* at higher temperatures may have been an increasing level of lactic acid generated by the starter culture. Tetteh and Beuchat (2003) also showed that *Shigella flexneri* is able to survive longer at 4 °C than at 12 °C regardless of pH (3.5-7.3). The behavior of *E. coli* O157:H7 inoculated in 10 % rehydrated non-fat dry milk was determined at 4 and 12 °C (Guraya *et al*., 1998). Better survival at 4 ^{o}C than at 12 ^{o}C was observed in the study.

During storage at 12 °C the yeast levels remained stable below 1 log cfu ml^{-1} except in yoghurt samples inoculated with *S. aureus* where they increased to 2 log cfu ml^{-1} after 4 hrs. Studies have shown that at higher temperatures yeasts showed enhanced numbers (Viljoen, 2001; Suriyarachchi and Fleet, 1981; Salji *et al*., 1987) but it was not the case in our study. However, Viljoen *et al*. (2003) showed that at 10 °C no yeast growth was observed initially in yoghurt samples. Our results corresponded with these findings. If the yoghurt samples were monitored for a longer period (more than 7 days), the number of yeasts in yoghurt at 12 °C might have changed.

An increase in the levels of LAB was observed when the yoghurts were placed back at refrigerating temperatures. The LAB numbers proliferate to as high as 9 log units during storage at 4 ^{o}C. The high levels of LAB both at 12 ^{o}C and 4 ^{o}C contributed to the inhibition of the pathogens.

3.3.3 Survival of pathogens in yoghurt stored at 4 °C after temperature abuse at 37 °C

The survival of pathogens during storage at 37 °C and at 4 °C is shown in Fig 5. At 37 °C the pathogens died off quickly. The decrease in counts of pathogens ranged between 0.5-1.8 log units during the 6 hrs. The slopes of the pathogens were constructed (Fig.6) and the specific death rates determined (Table 4). The death rates of the pathogens were much higher at this temperature ranging from 0.2223-0.9273 h^{-1}. *S. aureus* had the highest specific death rate and could no be recovered in the yoghurt after 4 hrs. After 6 hrs of abuse at 37 °C the same yoghurt samples were stored at 4 °C (Fig 5). At this temperature, the decrease in pathogen counts ranged between 1-2.2 log units. The die off of pathogens was slower compared to that at 37 ^{o}C, the death rate ranged between 0.0152-0.0979 h^{-1}. This clearly indicated that the survival of pathogens is affected more intensively at 37 °C compared to at 4 °C. *L. monocytogenes* survived for 2 d, *Shigella* spp. for 3 d, *Salmonella* spp. for 9 d, *E. coli* for 9 d, *E. coli O157:H7* for 10 d and *S. aureus* for 4 hrs.

The pH decreased rapidly at both temperatures. The low pH was more effective in inhibiting growth of pathogens at 37 °C than at 4 °C. From the onset of the experiment growth of yeasts was observed. The yeasts grew as high as 2 log cfu ml^{-1} within most yoghurt samples and were highest in yoghurt samples inoculated with *E. coli* O157:H7. There was a rapid increase in yeast counts during abuse at 37 °C and these were more pronounced in yoghurt samples inoculated with *Salmonella* spp. and *E. coli*. At 37 °C the increase in yeast counts remained steady. The LAB in the yoghurt samples increased slightly and remained stable

throughout the storage period. The counts were higher at 8-9 log cfu ml^{-1} compared to the control only kept at refrigeration temperatures.

Behavior of microorganisms in sections 3.3.2 and 3.3.3 was compared. Statistical analysis showed that there was a great significant difference ($p<0.001$) in the survival of pathogens in the different instances. The death rates of the pathogens were much higher at 37 °C than at 12 °C. This meant that the die off of pathogens was more affected at 37 °C than at 12 °C. The survival of pathogens at 4 °C in both instances was similar and the difference in the death rates varied. In general pathogens died off quicker in yoghurt that was abused at 37 °C compared to those at 12 °C. *S. aureus* was the most sensitive pathogen in both instances being completely inhibited within 4 hrs after abuse. Low counts (<1 log cfu ml^{-1}) were observed at 12 °C whereas at 37 °C an increase in yeast counts was observed from the onset. Similar behavior of LAB and pH was observed in both instances.

3.4 CONCLUSION

Foodborne pathogens survived in yoghurt at various temperatures. The time period of their survival was affected by pH, LAB and the storage temperatures. Pathogens survived for a shorter period at high temperatures (25 °C) than at low temperatures (4 °C). The increased levels of LAB and the rapid decline in pH as a result of production of organic acids played a major role in the inhibition of foodborne pathogens. When yoghurt was initially abused at higher temperatures (12 °C and 37 °C) the pathogens die off faster, having higher death rates than when they were later stored at 4 °C. At low temperatures (4 °C) the pathogens died off slowly and the death rates decreased. This showed that the antagonistic activity of LAB and low pH on the survival of pathogens was much more enhanced at high temperatures.

At high temperatures (25 °C and 37 °C) yeast counts grew to levels as high as 4 log cfu ml^{-1}. At these levels, the yeasts contributed to the spoilage of the yoghurts. At low temperatures the number of yeasts remained below 10 cfu/ ml but can increase in numbers after prolonged periods of time to levels not high enough (2 log cfu ml^{-1}) to cause severe spoilage. Storage of yoghurt at ambient temperatures (25 °C), thus, remained safe of pathogens but a poor quality product was observed since yeasts and other organisms caused spoilage.

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Table 1. Selective broth and agar used for the enumeration of the presumptive pathogens, and the description of colonies of each pathogen.

Microorganism	Selective broth	Selective agar	Description of colonies
E. coli	Tryptic soy broth (Merk, Germany)	*VRB-Mug agar	Pink to dark red colonies, fluorescence under UV
E. coli O157:H7	Tryptic soy broth	*VRB-Mug agar	Pink to dark red colonies
L. monocytogenes	Tryptic soy broth	Rapid' L. Mono agar (Bio-rad, France)	Green- blue colonies
Salmonella spp.	Tryptic soy broth	*XLD agar	Transparent, sometimes black-centered
Shigella spp.	Tryptic soy broth	*MacConkey agar	Colourless, translucent colonies
S. aureus	Tryptic soy broth	*Baird-Parker agar	Black, glossy and convex with a white margin surrounded by a clear zone

Note: * media from (Biolab, Germany)

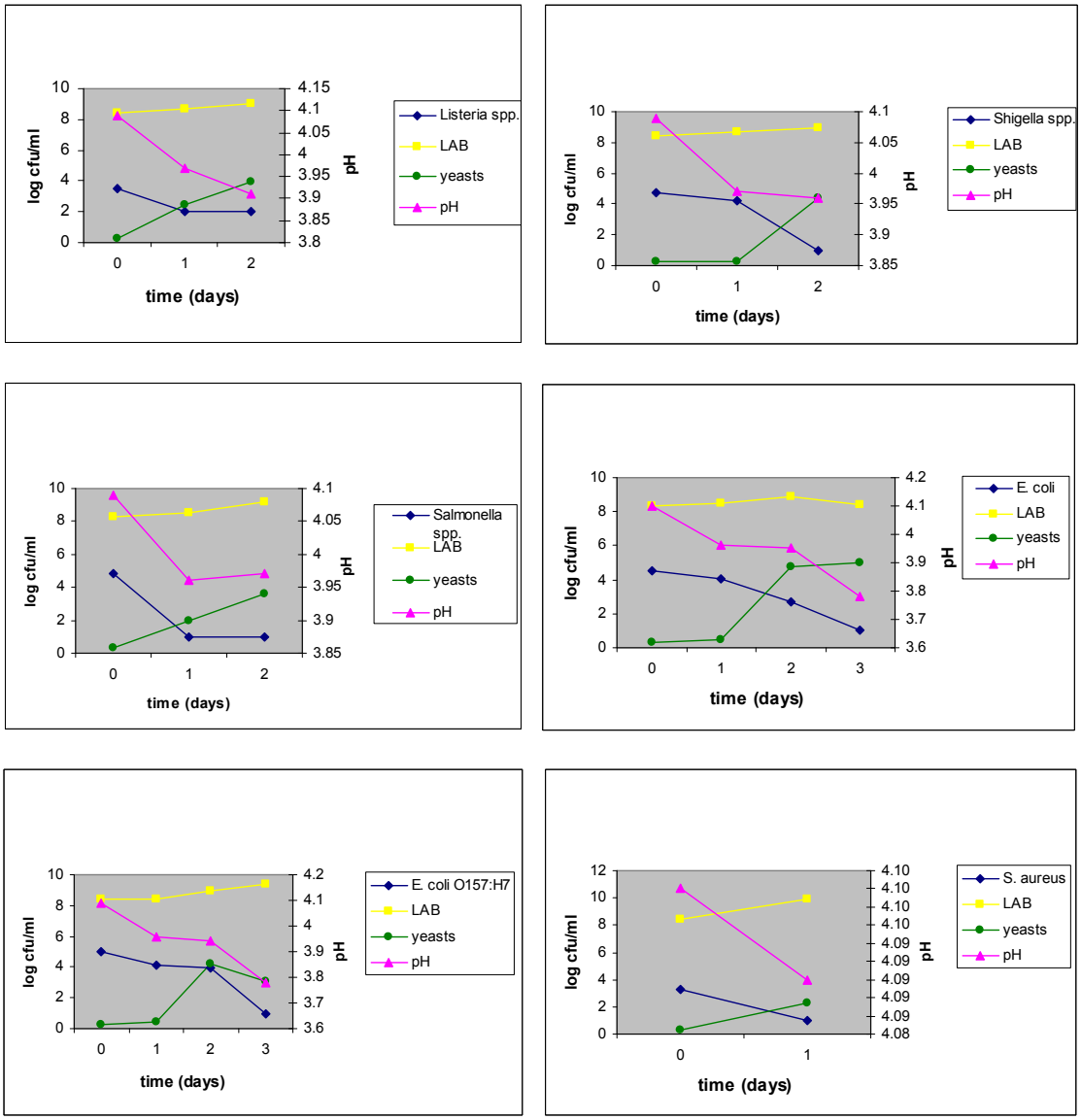

Fig. 1 Survival of foodborne pathogens during storage of yoghurt at 25 °C

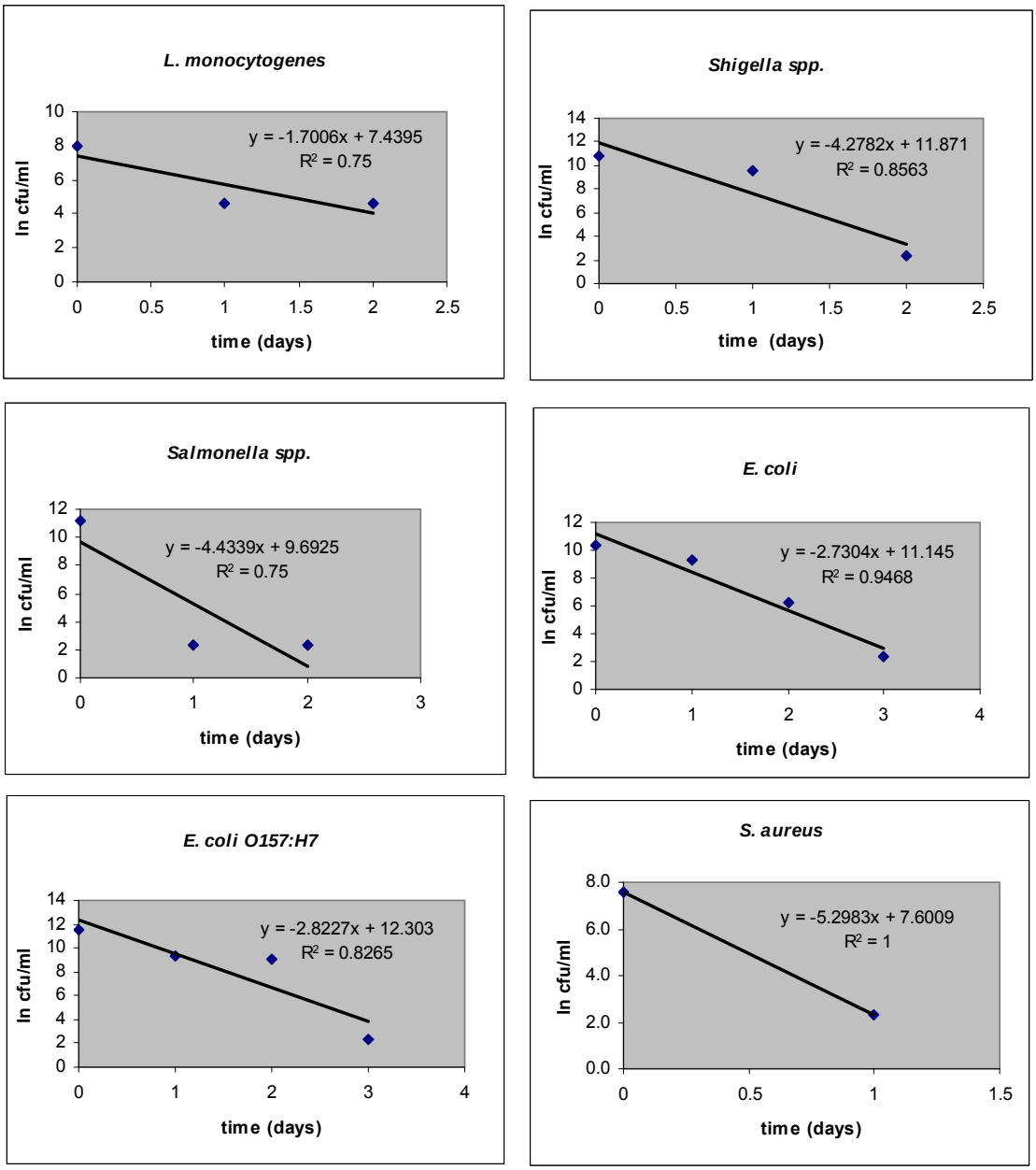

Fig. 2 Slopes of presumptive food-borne pathogens during storage at 25 °C

Table 2. The specific death rates of the presumptive food-borne pathogens in yoghurt at 25°C.

Pathogen	Period (days)	Specific death rate (d^{-1})	Specific death rate (h^{-1})
Listeria	0-2	1.7006	0.0709
Shigella	0-2	4.2782	0.1783
Salmonella	0-2	4.4339	0.1847
E. coli	0-3	2.7304	0.1138
O157:H7	0-3	2.8227	0.1176
S. aureus	0-1	5.2983	0.2208

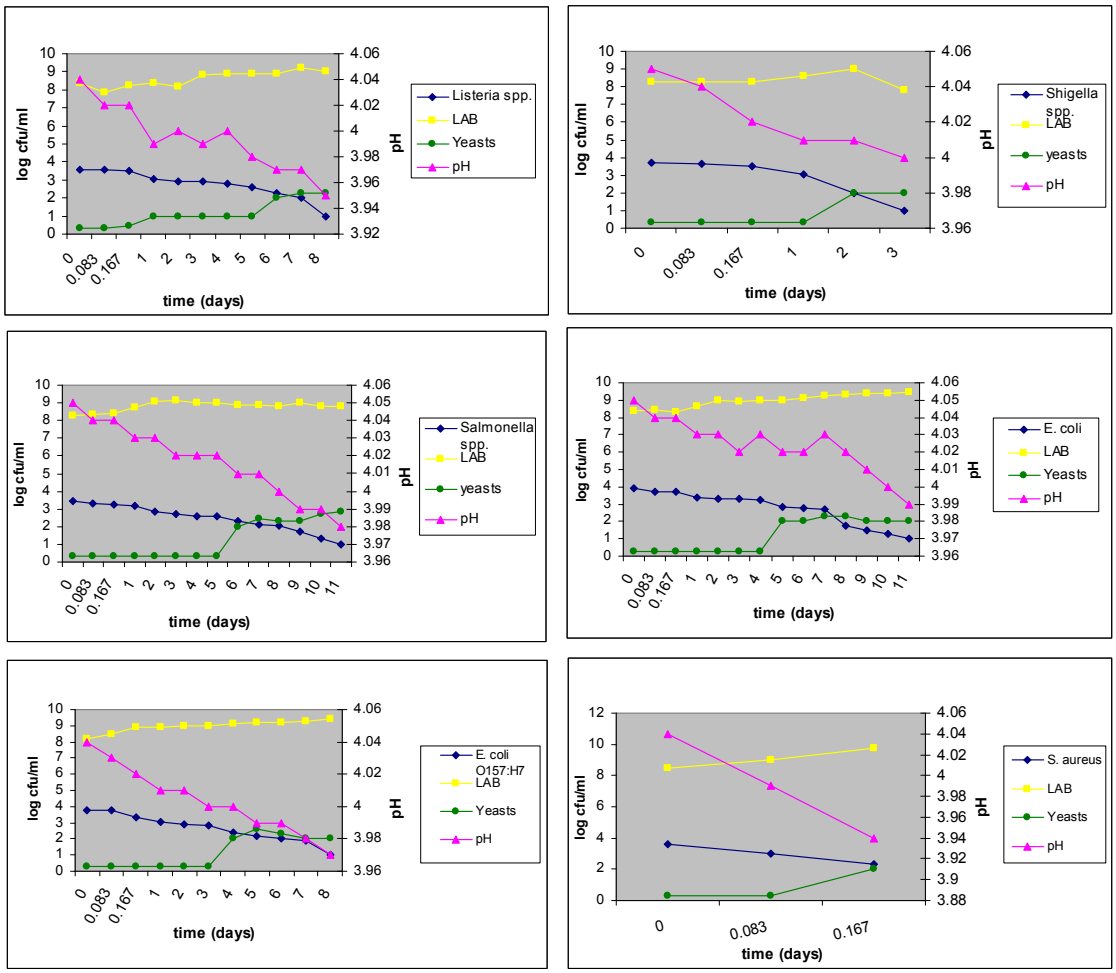

Fig. 3 Survival of foodborne pathogens during abuse of yoghurt at 12°C and then stored at 4°C

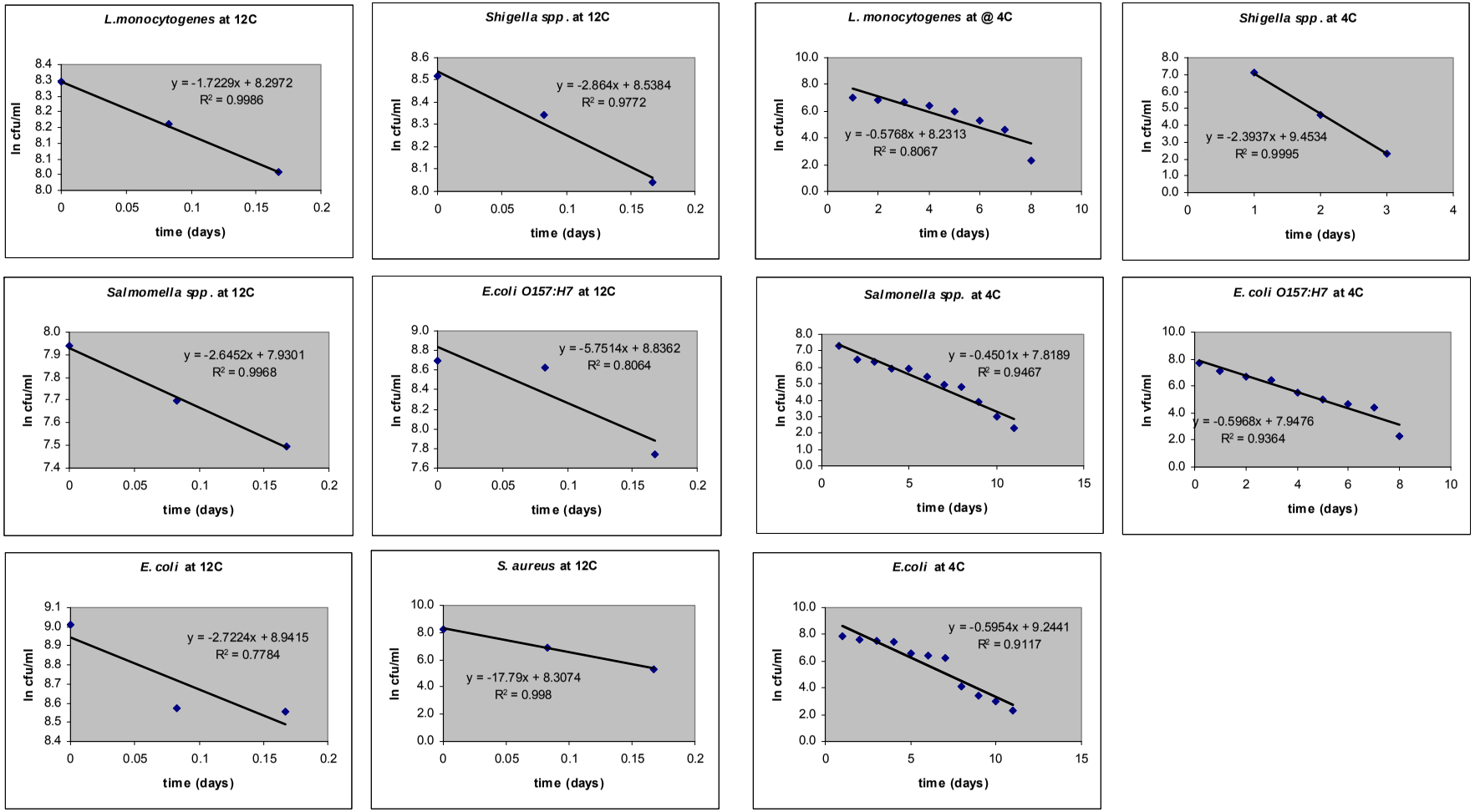

Fig 4 Slopes of foodborne pathogens during yoghurt storage at 12°C and then at 4°C

Table 3. The specific death rates of the presumptive food-borne pathogens in yoghurt at 12°C and 4°C

Pathogens	**death rate at 12C (d^{-1})**	**death rate at 12C (h^{-1})**	**death rate at 4C (d^{-1})**	**death rate at 4C (h^{-1})**
Listeria spp.	1.7229	0.0718	0.5768	0.0240
Shigella spp.	2.864	0.1193	2.3937	0.0997
Salmonella spp.	2.6452	0.1102	0.4501	0.0188
E. coli	2.7224	0.1134	0.5954	0.0248
E. coli O157:H7	5.7514	0.2396	0.5968	0.0249
S. aureus	17.79	0.7413		

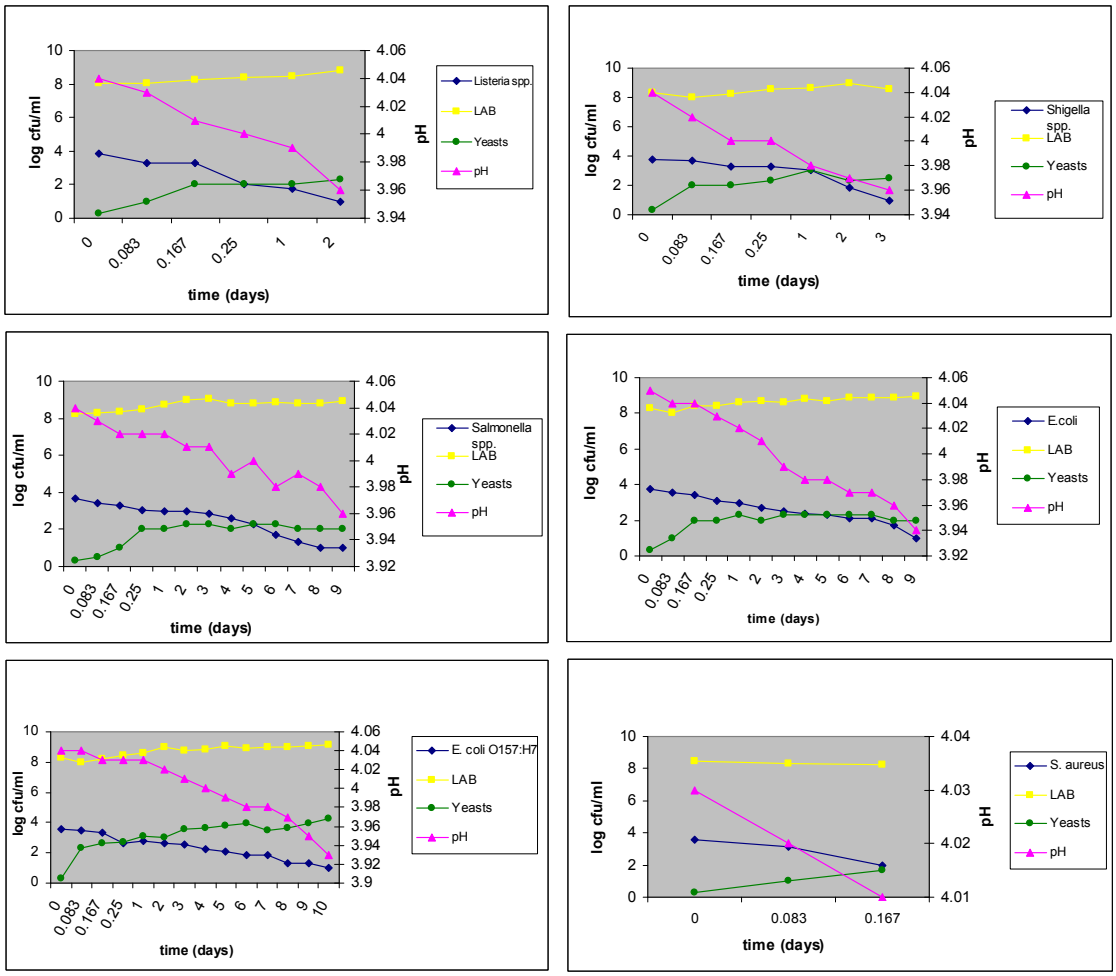

Fig. 5. Survival of foodborne pathogens during abuse of yoghurt at 37°C and then stored at 4°C

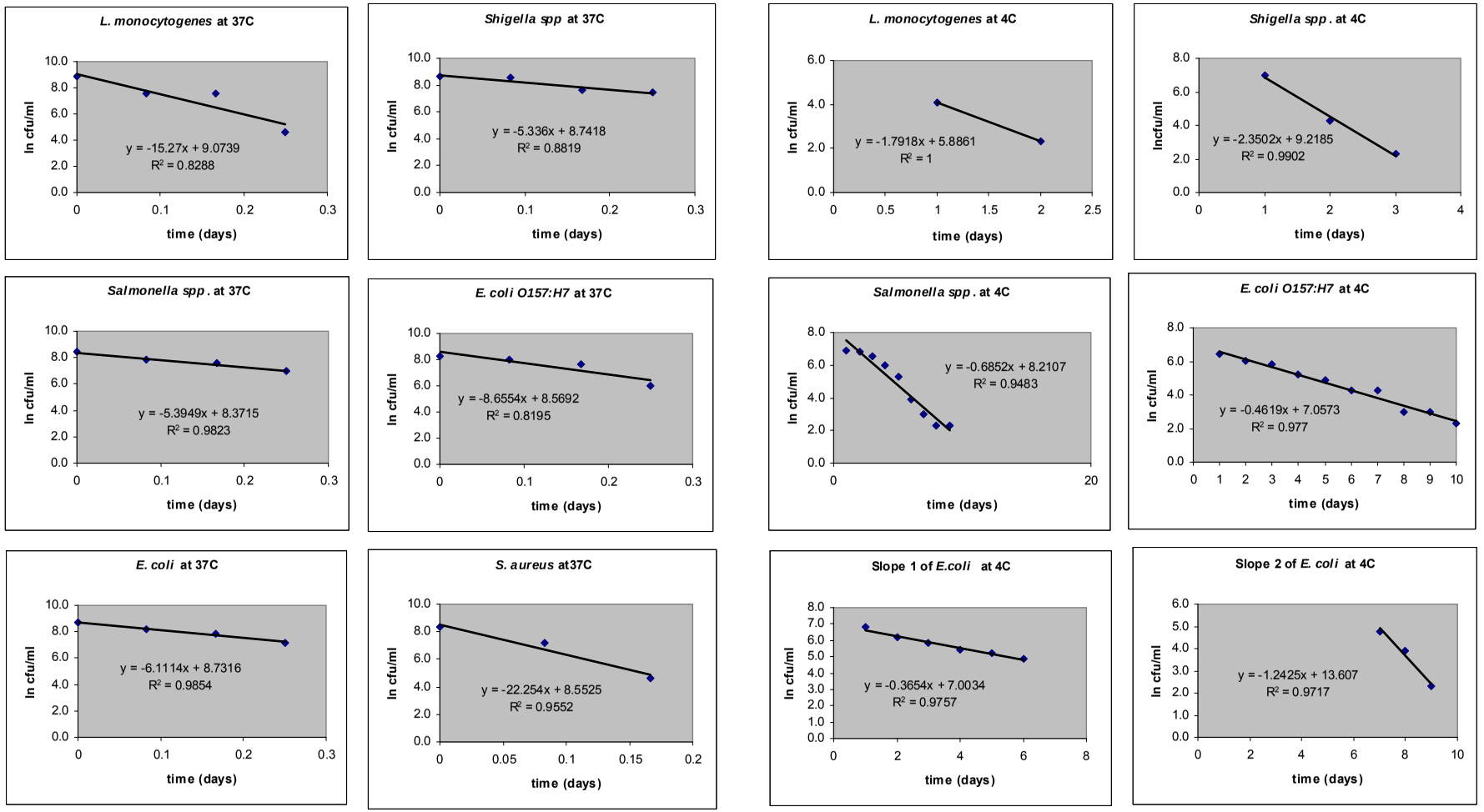

Fig 5 Slopes of foodborne pathogens during yoghurt storage at 37°C and then at 4°C

Table 4. The specific death rates of the presumptive food-borne pathogens in yoghurt at 37°C and 4°C

Pathogens	**death rate at 37C (d^{-1})**	**death rate at 37C (h^{-1})**	**death rate at 4C (d^{-1})**	**death rate at 4C (h^{-1})**
Listeria spp.	15.27	0.6363	1.7918	0.0747
Shigella spp.	5.336	0.2223	2.3502	0.0979
Salmonella spp.	5.3949	0.2248	0.6852	0.0286
E. coli	6.1114	0.2546	0.3654	0.0152
E. coli			1.2425	0.0518
E. coli O157:H7	8.6554	0.3606	0.4619	0.0192
S. aureus	22.254	0.9273		

CHAPTER 4

THE SURVIVAL OF PRESUMPTIVE FOODBORNE PATHOGENS DURING THE PRODUCTION OF SETHEMI

Abstract

The survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., and *Shigella* spp. during natural fermentation of fresh raw milk into Sethemi at 25 ˚C and 37 ˚C was investigated. The behavior of Lactic acid bacteria (LAB), yeasts and the change in lactose, galactose, glucose and lactic acid were also investigated. All the foodborne pathogens survived the fermentation process. At 25 ˚C counts of the *Salmonella* spp. increased from 3.477 to 5.4 log cfu ml^{-1} during the first 24hrs of fermentation. At the end of fermentation they decreased to 3.0 log $cfuml^{-1}$. Counts of *Shigella* spp. increased from 2.8 to 3.7 log cfu ml^{-1} and these decreased to 1 log $cfuml^{-1}$ at the end of fermentation. Counts of *S. aureus* increased from 4.6 to 7.1 log $cfuml^{-1}$ and decreased to 5.8 log $cfuml^{-1}$ at the end of fermentation. *E. coli* O157:H7 counts increased from 4.5 to 5.0 log cfu ml^{-1} and decreased to 4.2 log cfu ml^{-1}. Counts of *L. monocytogenes* remained constant throughout the fermentation process. The behavior of these pathogens at 37 ˚C was similar. Counts of *Salmonella* spp. increased from 2.9 to 3.7 log cfu ml^{-1} during the first 24 hrs of fermentation. After 18 hrs counts remained constant at 1 log cfu ml^{-1} until the end of fermentation. Counts of *Shigella* spp. increased from 2.7 to 3.9 log $cfuml^{-1}$ and remained constant at 1 log cfu ml^{-1} until the end of fermentation. Counts of *S. aureus* increased from 4.5 to 7.5 log cfu ml^{-1} and decreased to 6.1 log cfu ml^{-1}. *E. coli* O157:H7 counts increased from 3.6 to 7.3 log cfu ml^{-1} but decreased to 2.0 log cfu ml^{-1} at the end of fermentation. Counts of *L. monocytogenes* remained constant throughout the fermentation process. The yeast counts remained almost constant thoughout the fermentation process at both temperatures except in milk inoculated with *L. monocytogenes*; at 37C the counts increased from 2.8 to 5.9 log cfu ml^{-1} but decreased to 2.2 log cfu ml^{-1} at the end of fermentation, at 25 ˚C the counts increased from 2.85 to 5 log cfu ml^{-1} and decreased to 1.9 log cfu ml^{-1} at the end of fermentation. LAB also increased during milk fermentation reaching levels as high as 8-9 log cfu ml^{-1} at both temperatures. The lactose produced by LAB decreased with time at both

temperatures, there was a slight increase in galactose in all containers at both temperatures, and lactic acid increased rapidly. The low pH of the milk was inhibitory to most pathogens; inhibition was more effective at the temperature of 37 ˚C. At 25 ˚C *L. monocytogenes* could not be inhibited and persisted in the milk.

4.1. INTRODUCTION

Milk is a major component of the traditional diet in many regions of Africa. Most of the milk produced in communities is consumed at home and is rarely sold (Ogwaro *et al*., 2002). Lack of refrigeration facilities in households and high temperatures during summer days has resulted in the conversion of any surplus liquid milk to stable products such as yoghurt or sour milk in households in Africa. These stable products generally have a longer shelf life than their original state and their ultimate spoilage is different in character.

The process of conversion of the milk to these stable products is natural fermentation. Naturally fermented milk (NFM) is produced by spontaneous fermentation of unpasteurized milk by naturally occurring microbes (Narvhus and Gadaga, 2003). In South Africa this naturally fermented milk is called Sethemi. It is commonly produced by Sotho people living in and around the Free State Province (Kebede *et al*., 2005). Sethemi is produced by allowing fresh raw milk to sour in a container (clay pot, plastic or nickel) at room temperature, 25-30 ˚C, for 3-4 days depending on the coagulation of milk. It is clear that under these conditions limited hygiene is practiced in preparation of this product. Milk can be contaminated from sources such as diseased animals, the milk handler or contaminated equipment.

It is believed that most fermented foods owe their origin to the fact that processes used in their production are inhibitory to many microorganisms (Adams and Mitchell, 2002). The antimicrobial effects of fermentation are not confined to spoilage organisms alone; they can also affect pathogens that might be present (Pitt *et al*., 2000; Mufandaedza *et al*., 2006; Nassib *et a*l., 2006). Thus, traditional fermentations can take potentially hazardous raw milk and transform it into a product with both improved keeping qualities and a reduced risk of causing illness.

Although fermented milk products have been considered intrinsically safe due to their low pH and high acidity, several investigators have demonstrated that microorganisms such as *Listeria monocytogenes* and *Escherichia coli* O157:H7 can survive in fermented milks over several days and weeks (Hal-Haddad, 2003; Gulmez and Guven, 2003; Ogwaro *et al*., 2002; Ashenafi, 1994; Mufandaedza *et al*., 2006). These organisms are some of the most important bacterial foodborne pathogens that can lead to foodborne diseases through consumption of contaminated milk and fermented milks (Mead *et al*., 1999). Therefore, the survival of these pathogens for up to several weeks illustrates the potential health risks associated with pre-and-post processing contamination of fermented milks.

The behavior of some pathogens of concern to food safety has been extensively investigated in acidified dairy products such as yoghurt; however, a limited number of studies have been carried out on the survival of these foodborne pathogens in Sethemi, a naturally fermented milk of South Africa (Beukes *et al*., 2001; Narvhus and Gadaga, 2003).

Therefore, the objectives of the present study were to investigate the behavior of five foodborne pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Shigella* spp, *Salmonella* spp, *Staphylococcus aureus*) during the fermentation period of Sethemi under two different temperatures of coagulation, 25 ˚C and 37 ˚C; to investigate the behavior of naturally occurring Lactic Acid Bacteria (LAB) and Yeasts during the fermentation period of Sethemi; and also to monitor change in pH, lactic acid produced during fermentation , and sugar content in milk.

4.2. MATERIALS AND METHODS

4.2.1 Cultures and their maintenance

Microbial strains that were supplied by the Medical Faculty of the University of Free State for the investigation of Survival of foodborne pathogens in plain and fruit yoghurt were used as test microorganisms in this investigation. These were; *Staphylococus aureus*, *Shigella spp*, *Listeria monocytogenes*, *Salmonella spp*, and *E. coli O157:H7*. Each strain was maintained on nutrient agar (Biolab, Germany) slants at 4 ˚C with a monthly transfer. Long term preservation was done in 15 and 80% glycerol in Cryotube™ vials and were stored at -20 ˚C.

4.2.2 Inoculation of microorganisms and Milk fermentation

A dairy farm in Bloemfontein was selected where 7L of fresh raw milk were collected in a sterile plastic bottle. The milk was transported to the laboratory where it was kept in refrigeration at 4 ˚C until analysis. Small plastic containers were rinsed with clean sterile water and 500 ml of the milk was dispensed into each container. Prior to inoculation of foodborne pathogens in the milk, the counts of microorganisms in the milk were determined. These included Lactic acid bacteria (LAB), yeasts and pathogens.

The test cultures mentioned earlier were inoculated into the milk. An inoculum was prepared by inoculating one colony from each pure culture in 10 ml tryptic soy broth (TSB). This was then incubated for 24hrs at 37 ˚C. Only 0.1 ml of the inoculum was inoculated into 500 ml of milk to make an initial concentration of 10^4cfu ml^{-1}. A control was also done in which no fooborne pathogens were inoculated. Containers were then covered with aluminum foil as it could not remove easily and to prevent contamination by dust and insects. The traditional method of Sethemi production without employing starter cultures was carried out

(Kebede, 2005). Some of the containers were kept at ambient temperatures (25 ˚C) in the laboratory and others at incubation temperatures of 37 ˚C for 2 days.

Samples were collected consecutively at 6hr intervals for 24 hrs during fermentation and then at 48hrs, at the end of fermentation. Milk was aseptically collected in 20 ml McCartney bottles starting immediately after inoculation. Milk was thoroughly mixed and for each sample 1 ml was aseptically withdrawn, dispensing into 9 ml sterile peptone water (Merck, Darmstadt, Germany) and serially diluted for plate counting. The remaining milk samples were stored at -20 ˚C for chemical (HPLC) analysis to determine carbohydrate and lactic acid content.

4.2.3 Enumeration of microorganisms in Sethemi

Each foodborne pathogen was enumerated on selective agar. Table 1 shows selective agar that were used for the enumeration of the pathogens and the description of their colonies. The plates were incubated aerobically at 37 ˚C for 24 hrs. Lactic acid bacteria were enumerated by spread plating on De Man Rogosa (MRS) agar (Biolab). The plates were incubated aerobically at 30 ˚C for 48 hrs. Yeasts were enumerated by spread plating on Rose Bengal Chloramphenicol Agar (RBCA) (Biolab). The plates were incubated aerobically at 25 ˚C for 72 hrs.

4.2.4 Determination of pH

The pH of each sample for both raw and fermented milk was determined by a Cyberscan pH meter, model 500. The pH was monitored every time samples were collected for enumeration procedures.

4.2.5 Carbohydrate and Lactic acid Determination

The sugar and lactic acid content were measured by means of a waters HPLC system.

4.3. RESULTS AND DISCUSSIONS

4.3.1 Survival of pathogenic bacteria

Survival of the foodborne pathogens is depicted in Figs. 1 and 2. The presence of these pathogens in raw unpasteurized milk as a result of contamination prior to inoculation was tested. The milk was free of *L. monocytogenes*, *Salmonella spp*., *Shigella spp*., *Ecoli O157:H7* but not of *S. aureus*. The counts of this pathogen found in the milk prior to inoculation and during fermentation are shown in controls in Figs. 1 and 2.

Salmonella spp.

Figure 1 shows that counts of *Salmonella* spp. increased from an initial value of 3.5 log cfu ml^{-1}at inoculation to maximum value of 5.4 log cfu ml^{-1} after 24 hrs of fermentation at 25 ˚C. At the end of fermentation counts had decreased to 3.0 log cfu ml^{-1}. At 37 ˚C (Fig. 2) counts increased from 3.0 log cfu ml^{-1} to 3.7 log cfu ml^{-1} within the first 6hrs of fermentation. The counts also decreased to 1 log cfu ml^{-1} at the end of fermentation. At 25 ˚C there was gradual growth over a period of 24 hrs until the maximum counts were reached. On the other hand, growth at 37 ˚C was rapid with maximum counts being reached in a short period of 6 hrs. This suggested that the growth rate of *Salmonella* spp. was higher at 37 ˚C. *Salmonella* spp. actively grows within a wide temperature range with the optimum

growth at 37 ˚C (Cliver, 1990) which would explain the higher growth rate at 37 ˚C.

According to Fig. 1 the counts of *Salmonella* spp. decreased by only 0.5 log units, from 3.5 to 3.0 log cfu ml^{-1}, at the end of fermentation at 25 ˚C. The decrease in Fig. 2 was of 1.9 log units at the end of fermentation at 37 ˚C. At the same temperature the decrease in pH was rapid and continued to decrease even further at the end of fermentation whereas at 25 ˚C the decrease in pH became steady. We found out that at 25 ˚C pH became inhibitory to growth of the pathogen at value 4.29 and at 37 ˚C it became inhibitory at value 5.95. Thus, the *Salmonella* spp. was inhibited more effectively by low pH at 37 ˚C than at 25 ˚C.

In similar studies by other researchers a similar trend in the behavior of *Salmonella* spp. was observed (Nassib *et al*., 2006; Mufandaedza *et al*., 2006). Farber and Peterkin (1991) noted that *S. enteriditis* can grow substantially in fermented milks because they have the ability to adapt and proliferate between pH 2 and 4. In a study carried out by Shen *et al*., (2007) the viable population of *Salmonella* Typhimurium, that were not acid adapted, increased from 6 log cfu ml^{-1} to 9 log cfu ml^{-1} during the initial 24 hrs of fermentation at 37 ˚C. As the duration of fermentation was further extended the organism's counts were reduced by LAB and high acidity to 4.5 log cfu ml^{-1}.

Shigella spp.

Similar behavior in the survival of *Salmonella* spp. was observed with the *Shigella* spp. Figure 1 shows that initial concentration of *Shigella* spp was 2.8 log cfu ml^{-1} and grew as high as 3.7 log cfu ml^{-1} during 12 hrs of fermentation at 25 ˚C. The counts decreased to 1.0 log cfu ml^{-1} at the end of fermentation, this was a decrease of 1.8 log units from the beginning of fermentation to the end. At 37˚C counts increased from 2.7 to 3.9 log cfu ml^{-1}. After 12 hrs counts decreased to 1 log cfu ml^{-1} and remained the same until the fermentation was completed. Growth

was gradual at 25 ˚C and rapid at 37 ˚C. Again the growth rate and the death rate were also higher at 37 ˚C than at 25 ˚C. The pH was inhibitory to the growth of the microorganism at 5.1 at 25 ˚C and at 5.74 at 37 ˚C. As a result, pH was more inhibitory to *Shigella* spp. at 37 ˚C. The results showed that low pH inhibited the growth of this organism and the inhibition was more pronounced at a temperature of 37 °C. Due to lack of information and work done on the survival of *Shigella* spp. in indigenous fermented milks, our results were not comparable with any relevant studies on the subject.

Staphylococcus aureus

As was already shown, *S. aureus* was present in the uninoculated milk prior to inoculation of foodborne pathogens. Several studies support these findings. Gran *et al*., (2003a) studied the presence of selected pathogens in 21 samples of naturally fermented milk in Zimbabwe and found out that 20 samples contained *S. aureus* and 15 of these contained over log 5 cfu g^{-1}. The organism was also isolated from 15 samples of traditional fermented milk produced in a plastic container, claypot and a calabash gourd from individual households in South Africa and Namibia (Beukes *et al*., 2001).

Counts of *S.aureus* increased from 5.4 to 6.6 log cfu ml^{-1} within 24 hrs of fermentation in the uninoculated raw milk (Fig.1 control). After 48 hrs the counts decreased to 6.4 log cfu ml^{-1} at which the final pH was 4.29. From the beginning of fermentation to the end *S. aureus* increased by 1 log unit. Thus, pH was not effective in inhibiting the growth of this pathogen at 25 ˚C. The behavior of this pathogen at 37 ˚C was different; initial counts of 5.1 log cfu ml^{-1} increased to 7.2 log cfu ml^{-1} after 18 hrs but decreased thereafter to 1 log cfu ml^{-1} at the end of fermentation, at pH 3.67 (Fig. 2 control). According to these findings pH is only inhibitory to *S. aureus* at levels below 4 and at 37 ˚C. In milk inoculated with *S. aureus* the initial counts were expected to increase because of the already

present counts in the raw milk (Fig. 1). However, the initial counts were 4.60 log cfu ml^{-1} which increased to 7.1 log cfu ml^{-1} within 24 hrs of fermentation. After 24 hrs growth was inhibited and counts were decreased to 5.81 log cfu ml^{-1}. The trend was similar to that in uninoculated milk; pH was only inhibitory between 24 and 48 hrs of fermentation, but in general the growth and survival of *S. aureus* during the production of Sethemi was not inhibited by LAB at 25 ˚C. At 37 ˚C growth increased by 3 log units from initial concentration of 4.5 to 7.5 log cfu ml^{-1}, during the first 18 hrs. This decreased to 6.1 log cfu ml^{-1} after 48 hrs of Sethemi fermentation at pH 3.73. The results indicate that generally LAB was not inhibitory to *S. aureus* during fermentation of milk inoculated with the microorganism at both temperatures as counts were still higher at the end of fermentation than they were at the beginning of fermentation. The survival of this organism pre-and-post fermentation poses a serious health threat to the consumers of Sethemi.

Information on the survival of *S. aureus* in raw milk during fermentation is limited. However studies on the survival of this pathogen in other dairy products are found. *S. aureus* was enumerated during the manufacture and ripening of cheese and there was substantial multiplication of *S. aureus* cells during processing at 30 ˚C (Erkmen, 1995). From milk to curd processing the numbers increased by 2.3 log units. The milk used in that study was, however, pasteurized. Benkerroum *et al*., (2002) studied the behavior of S. aureus during yoghurt processing; a significant increase in numbers of *Staphylococci* was noted in the first 2 hrs of fermentation. Extrinsic and intrinsic growth parameters during fermentation were encouraging to the growth of this organism. In most cases pH had no effect in inhibiting growth of *S. aureus*. Research has shown that the decrease in pH has a high inhibitory effect on this microorganism at low temperatures 4 ˚C (Attaie *et al*., 1987; Pazakova *et al*., 1997). Therefore, temperatures of 25 ˚C and 37 ˚C are not a limiting factor for growth of *S. aureus* during milk fermentation. From the nutritional standpoint milk also provides an adequate culture medium for growth. The high initial concentrations of *S. aureus*, resulting from inoculation of *S.*

aureus into milk that already contained a high concentration of cells, certainly accounted for the persistence of the pathogen in Sethemi. It is well documented that the quantity of the initial inoculum has an effect on the inhibition of pathogens in foods (Benkerroum *et al*., 2000; Laukova *et al*., 1999; Stiles, 1996).

***Escherichia coli* O157:H7**

The survival of *E. coli* O157:H7 during the production of Sethemi is shown in Figs. 1 and 2. At the beginning of fermentation at 25 ˚C counts of the pathogen were 4.5 log cfu ml^{-1}. They increased to 6.7 log cfu ml^{-1} after 18 hrs of fermentation but decreased to 4.2 log cfu ml^{-1} at the end of fermentation. The pH decreased from 6.63 to 4.21. From counts of 3.6 log cfu ml^{-1} E. coli O157:H7 cells increased up to 7.3 log cfu ml^{-1} at 37 ˚C (Fig. 2). When pH reached a value of 5.2 the cells decreased with the decrease in pH until they reached 2.0 log cfu ml^{-1} at the end of fermentation. At this point pH had decreased to 3.66. The results showed that *E. coli* O157:H7 survived well in Sethemi. Feresu and Nyathi (1990) have previously shown that some strains of pathogenic *E. coli* could survive in both traditionally fermented milk and in pasteurized milk and multiply to high numbers. Similar trends in survival of this pathogen were observed by Ogwaro *et al*., (2002) during fermentation of full cream pasteurized milk. At 25 ˚C, E. coli O157:H7 grew to cell densities of 10^8 cfu ml^{-1} during the initial 24 hr incubation stage with pH of the milk declining from an initial 6.8 to 5.1. At 37 ˚C the pathogen grew from 10^5 cfu ml^{-1} to 10^{10} cfu ml^{-1} during the initial 12 hrs of incubation and subsequently maintained the population size. The pH declined from 6.8 to 4.9. This microorganism grows easily in the early stages of fermentation because pH and other antimicrobial substances produced by LAB are limited (Benkerroum *et al*., 2002). Also *E. coli* O157:H7 is well known for its acid-tolerance characteristic (Park *et al*., 1999; Hsin-Yi and Chou, 2001; Leyer *et al*, 1995) and its acid adaptation can enhance the survival of this organism in acidic dairy foods during fermentation (Gahan *et al*., 1996).

Listeria monocytogenes

L. monocytogenes exhibited a different behavior of survival compared to other pathogens. Figure 1 shows the survival of this pathogen during fermentation at 25 ˚C. The counts of the microorganism were almost constant throughout the fermentation process. They range between 3.5, 4.1, and 3.5 log cfu ml^{-1}. The pH declined from 6.6 to 4.32 and did not have any effect in inhibiting L. monocytogenes cells. Dalu and Feresu (1996) determined the behavior of *L. monocytogenes* during fermentation of amasi at ambient temperature. Similar results to ours were found; they found out that although the microorganism grew only marginally during fermentation, $>10^2$ cells ml^{-1} could still be detected at the end of fermentation. The results we obtained suggested that growth factors were limited to encourage the growth of this pathogen. The highest counts of LAB were recorded in milk inoculated with *L. monocytogenes*, even at the end of fermentation. LAB competed with the pathogen during the fermentation process (Pitt *et al*., 2000). LAB outnumbered the *L. monocytogenes* by 4 to 5 log units from 12 hrs of fermentation. *L. monocytogenes* grows best at 30-37 ˚C; therefore it must be the temperature was also not favorable for growth. The combined influence of large numbers of competing LAB and the temperature may have produced an unfavorable environment for growth of the pathogen.

During 18 hrs of fermentation at 37 ˚C, counts on *L. monocytogenes* increased from 3.6 log cfu ml^{-1} to 4.83 log cfu ml^{-1} (Fig. 2). After the pH declined from an initial value of 6.6 to 4.45 the counts declined to 1 log cfu ml^{-1} at the end of fermentation, the pH at this point was 3.97. At this temperature the low pH was effectively inhibitory to the microorganism as only 10 cells ml^{-1} were detected at the end of fermentation. It is evident that three factors played a major role in inhibiting the pathogen; these were the low pH, the high LAB numbers and the temperature.

4.3.2. Changes in pH

In general a similar trend in pH was seen in all containers; pH decreased with fermentation time. The pH decreased from values of 6.72 and 6.53 in raw milk to pH values of 4.25 and 3.82 in the final Sethemi product at 25 ˚C. Towards the end of fermentation pH became stable in all the containers kept at 25 ˚C. At 37 ˚C pH decreased from initial values of 6.64 and 6.57 to 3.97 and 3.62 after 48 hrs. In comparison to milk kept at 25 ˚C a rapid decrease in the pH was noticed and pH continued to increase even further towards the end of fermentation. The pH was lower at 37 ˚C (3.71) than at 25 ˚C (4.30) at the end of fermentation.

4.3.3 Behavior of LAB

The lactic acid bacteria increased in numbers at the beginning (within 18 hrs) of fermentation reaching to maximum numbers of 9.1 and 9.2 log cfu ml^{-1} in milk inoculated with *L. monocytogenes* during fermentation at 25 °C and 37 °C simultaneously. In milk inoculated with other pathogens (Figs. 1 and 2) the maximum levels ranged between 8.5 log cfu ml^{-1} in *E. coli* O157:H7-inoculated milk and 8.7 log cfu ml^{-1} in *S. aureus*- inoculated milk at 25 ˚C. At 37 ˚C the counts ranged between 8.0 log cfu ml^{-1} in *E. coli* O157:H7-inoculated milk and 8.4 log cfu ml^{-1} in *Shigella*-inoculated milk. Towards the end of fermentation the levels of LAB started decreasing in containers kept at both temperatures although the levels were still very high. The counts compared favorably with findings of similar studies on fermented milks by other workers. According to Beukes *et al*., 2001 numbers of LAB found in South African traditional fermented milk were recorded with mean values of 8.0 log cfu ml^{-1}. In a study carried out by Mufandaedza *et al*., 2006 the lactic acid bacteria in spontaneously fermented raw milk grew to maximum population of about 8.9 log cfu ml^{-1} over 48 hrs. Results by Kebede, 2005 showed that the counts of LAB increased from initial levels of 4.94 and 5.53 log cfu ml^{-1} to maximum levels of 9.27 and 9.21 log cfu ml^{-1} during the fermentation period of Sethemi. Increased growth of LAB may have been due

to the abundant availability of nutrients in the fermenting milk. There is a mutualistic interaction between LAB and yeasts during fermentation in which yeasts stimulate growth of LAB by providing growth factors like amino acids, vitamins and other compounds, thus, consequently leading to elevated acid production (Leroi and Pidoux, 1993a; Loretan, 1999; Viljoen, 2001). This, therefore, explained the decrease in pH during fermentation. The lactic acid bacteria present in milk utilized the lactose available (Figs. 5 and 6) further converting it into lactic acid, one of the major organic acids produced by the LAB.

4.3.4 Behavior of Yeasts

The behavior of yeast in Sethemi is shown in Figs. 1 and 2. In most of the containers kept at both temperatures a stable linear growth of yeasts was noticed. There was an increase in numbers to some extent; however, the increase was not significant. In containers with milk inoculated with *Salmonella* spp., *Shigella* spp., and *E. coli* O157:H7 stored at 25 °C numbers of yeasts increased by 1 log unit. Counts in *S. aureus*-inoculated milk grew from initial values of 1.477 log cfu ml^{-1} to 2.88 log cfu ml^{-1} at 25 °C. At 37 °C counts of yeasts grew by 1 log unit in all containers except for the container with milk inoculated with *L. monocytogenes.* In milk inoculated with *L. monocytogenes* at both temperatures levels of yeasts were much higher in comparison with those in other containers. The numbers increased from initial values of 2.85 log cfu ml^{-1} to maximum values of 5.00 log cfu ml^{-1} at 25 °C and from 2.83 log cfu ml^{-1} to 5.93 log cfu ml^{-1} at 37 °C. A similar trend was noticed in the growth of yeasts in the uninoculated milk and the milk inoculated with pathogens (Figs. 3 and 4). Towards the end of fermentation at 25 °C counts of yeasts were slightly higher in milk inoculated with *S. aureus, Shigella* spp., and *E. coli* O157:H7. The same trend was noticed at 37 °C in milk inoculated with *E. coli* O157:H7.

Many species of yeasts are able to grow in milk and fermented milk products. Yeasts' growth in milk products is attributed to their ability to utilize milk

constituents such as proteins, fat, lactose, galactose, glucose, citrare and the low pH (Suriyarachchi and Fleet, 1981; Fleet, 1990). The source of yeasts in raw milk and naturally fermented milk has been assumed to be chance contamination from the animals, the milk handler or the contaminated equipment. Other researchers have reported varying counts of yeasts. In a study on South African naturally fermented milk, it was found that the yeasts counts had a mean value of 4.1 log cfu ml^{-1} (Loretan., 1999). In the naturally fermented raw milk of Zimbabwe the wild yeasts grew to about 7.9 log cfu ml^{-1} (Mufandaedza *et al*., 2006). These values are much higher compared to those we found. The dominant and faster growing bacteria (LAB and pathogens) which are evident in our study (Figs. 1 and 2) and other studies (Beukes *et al.*, 2001) were responsible for restricting the growth of yeasts through competition for available constituents in the milk.

Higher numbers of yeasts were noticed in milk contaminated with *L. monocytogenes*. The milk which was used to inoculate *L. monocytogenes* was not the same milk used for all the other pathogens, reason being the experiment on this pathogen was carried out on a different day but under similar conditions. It is possible that the raw milk was from a different cow and different milking equipment might have been used. Thus, the microbial composition of the milk was different from the previous milk used; perhaps the lactose-utilizing yeasts and galactose-assimilating strains were more dominant in the milk being able to adapt quickly to the conditions and increasing in numbers (Fleet, 1990; Kebede *et al*., 2005).

4.3.5 Changes in sugar and lactic acid concentrations

Changes in the levels of major sugars and lactic acid present during fermentation of raw milk in different containers at different temperatures are depicted in Figs. 5 and 6. The results indicated a linear and steady decrease in lactose concentrations over time during milk fermentation at 25 ˚C. In the uninoculated milk lactose decreased from initial concentrations of 36.1g/L to 23.5 g/L on the

last day of fermentation. In containers inoculated with inoculated milk lactose decreased from an average value of 34 g/L to an average value of 27 g/L after 48hrs of fermentation at 25 ˚C. At 37 ˚C decrease in levels of lactose concentrations is higher compared to that at 25 ˚C. For instance, in the uninoculated milk the lactose concentration decreased from 31.55 g/L to 13.17 g/L after 48hrs of fermentation. In milk inoculated with *E.coli* O157:H7 lactose levels decreased from 36.3g/L to 15.6 g/L after 48hrs of fermentation. After a certain time (6, 12, 18 hrs) in different containers levels of galactose began to increase rapidly from being undetected in the milk to levels as high as 11.8 and 15 g/L after fermentation of milk inoculated with E.coli O157:H7 at 25 ˚C and 37 ˚C simultaneously. At 25 °C lactic acid could not be detected in the milk during the first 6-18hrs, however after the 18hrs the lactic acid content began to increase rapidly reaching high levels ranging between 7.4-13.6 g/L. The increase in the lactic acid content was higher at 37 °C where levels ranged between 7.4-17.86 g/L. This would explain the increased rate on inhibition of pahtgens at this temperature. In all the containers at both temperatures glucose could not be detected in the milk throughout the fermentation process. A similar trend in the changes in lactose, galactose, and glucose concentrations during milk fermentation were found by other researchers (Alm, 1982; Kebede, 2005; Borregaard, E. and Arneborg, N., 1998). Alm (1982) found out that the lactose content decreased from about 4.8g/100g in unfermented milk to about 2.4g/100g in yoghurt. There was also a gradual increase in the galactose content from traces in milk to about 1g/100g. Only traces of free glucose were found in all the products.

During lactic fermentation of milk LAB mainly metabolize lactose to produce lactic acid, glucose and galactose, thus, resulting in a decreased concentration of lactose as was evident in our results. While glucose is further metabolized to lactic acid, galactose is expelled through a lactose-galactose antiport mechanism and accumulates in the media (Hutkins and Morris, 1987; Marshall and Tamime, 1997). This would explain why no traces of glucose were detected in our

experiment; with the high numbers of LAB present in the milk during fermentation (Figs. 1 and 2), all the available glucose was metabolized to produce lactic acid at high concentrations (Figs. 5 and 6). Furthermore, glucose is used as the main carbon and energy source by many foodborne pathogens (Cliver, 1990), therefore, it may have also been used up by the pathogens in the milk. The galactose that was released into the media may be used by galactose-assimilating, but lactose-negative yeasts (Fleet, 1990). The increase in lactose content in the control (Fig. 6a) and during survival of *S. aureus* (Fig. 6c) within the first 6 hrs of fermentation could not be explained, however, a possible reason may have been experimental error.

4.4. CONCLUSION

The lactose content in milk decreased during fermentation because it was metabolized by the lactic acid bacteria for growth, consequently increasing the counts of LAB during the production of Sethemi. Counts of LAB were as high as 8-9 log cfu ml^{-1} at both temperatures of fermentation. Metabolism of lactose produced galactose and lactic acid which increased with time during fermentation at both temperatures. Glucose was, however, not detected in the milk. The increase of lactic acid also resulted with decrease in pH with time. A marginal growth of yeasts was observed during fermentation, the increase and decrease was not significant. However, in milk inoculated with *L. monocytogenes* the yeasts by approximately 3 log units and decreased by 3 log units at both temperatures. The behavior of LAB and yeasts in raw uninoculated milk and milk inoculated with foodborne pathogens were similar. Counts of LAB and values of pH were almost the same; however, counts of yeasts in most containers were comparable to those in milk inoculated.

All the foodborne pathogens tested in this study were able to survive during fermentation of raw milk at 25 ˚C and 37˚ C in the production of Sethemi, South African fermented milk. In most containers, during the first 24 hrs of fermentation,

viable populations of these pathogens increased by numbers ranging between 1 and 4 log units. Growth of these pathogens was encouraged by the abundant supply of growth nutrients in the milk. Again towards the end of fermentation the growth of the foodborne pathogens was inhibited by a high lactic acid content which resulted in a low pH. Inhibition of the pathogens by LAB was more effective at 37 ˚C where counts of viable populations decreased to levels as low as 1 log cfuml^{-1} and 2 log cfu ml^{-1}. When milk was fermented at 25 ˚C high counts of 3.0 log cfu ml^{-1} of *Salmonella* spp., 5.8 log cfu ml^{-1} of *S. aureus*, 4.2 log cfu ml^{-1} of *E. coli* O157:H7 and 3.5 log cfu ml^{-1} of *L. monocytogenes* could still be detected at the end of fermentation. At this temperature *L. monocytogenes* was not affected by the fermentation process as the numbers remained constant throughout. The high numbers of these pathogens at the end of fermentation poses a great health threat to local people of South Africa who still rely on the natural and uncontrolled fermentation for preservation milk.

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Table 1 Selective broth and agar used for the enumeration of the presumptive pathogens, and the description of colonies of each pathogen.

Microorganism	**Selective broth**	**Selective agar**	**Description of colonies**
E. coli	Tryptic soy broth (Merk, Germany)	*VRB-Mug agar	Pink to dark red colonies, fluorescence under UV
E. coli O157:H7	Tryptic soy broth	*VRB-Mug agar	Pink to dark red colonies
L. monocytogenes	Tryptic soy broth	Rapid' L. Mono agar (Bio-rad, France)	Green- blue colonies
Salmonella spp.	Tryptic soy broth	*XLD agar	Transparent, sometimes black-centered
Shigella spp.	Tryptic soy broth	*MacConkey agar	Colourless, translucent colonies
S. aureus	Tryptic soy broth	*Baird-Parker agar	Black, glossy and convex with a white margin surrounded by a clear zone

Note: * media from (Biolab, Germany)

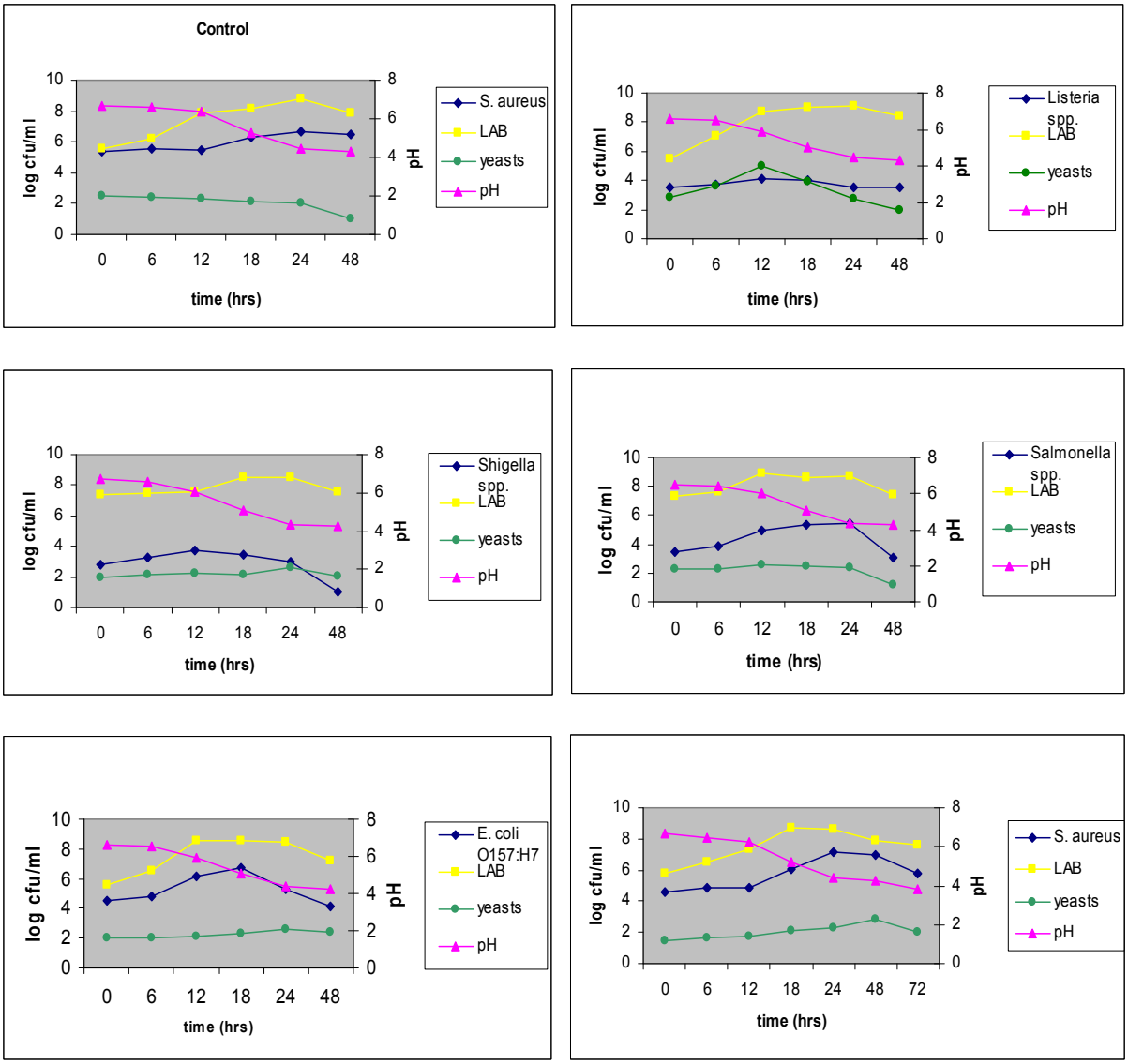

Fig.1 Survival of foodborne pathogens in raw milk during fermentation at 25˚C. The control is the uninoculated raw milk.

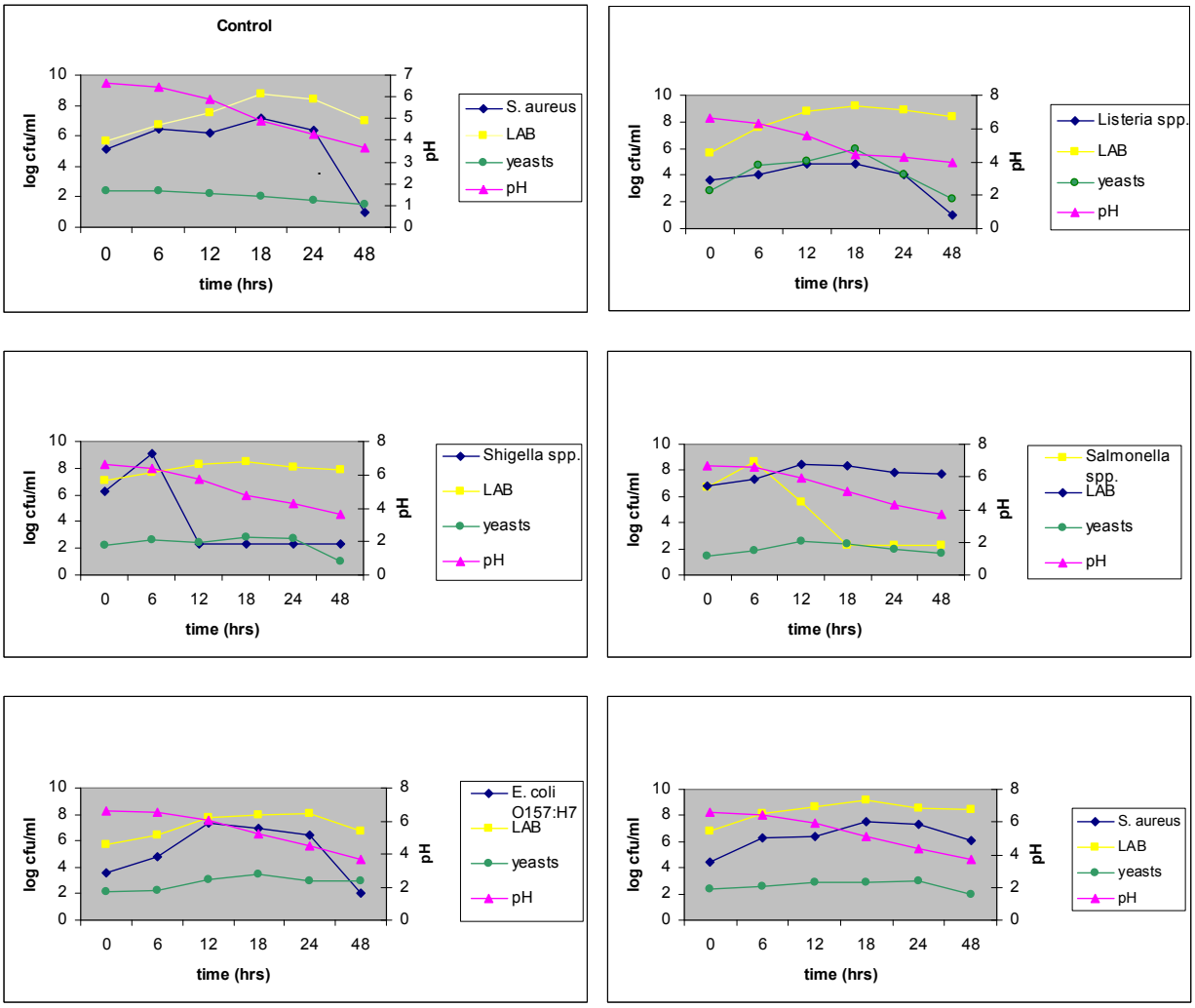

Fig.2 Survival of foodborne pathogens in raw milk during fermentation at 37˚C. The control is the uninoculated raw milk.

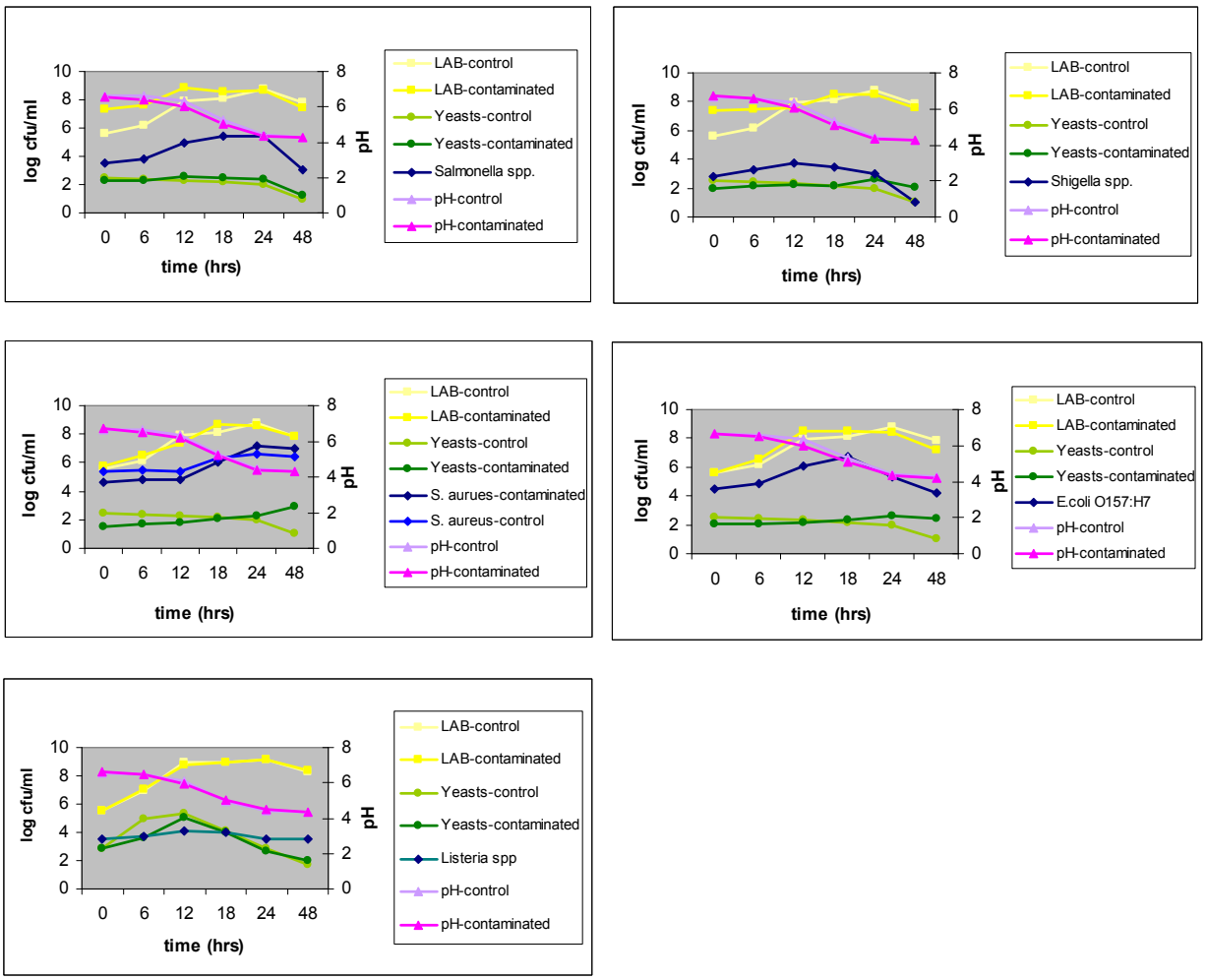

Fig. 3 Uninoculated raw milk vs raw milk inoculated with foodborne pathogens during fermentation at 25˚C.

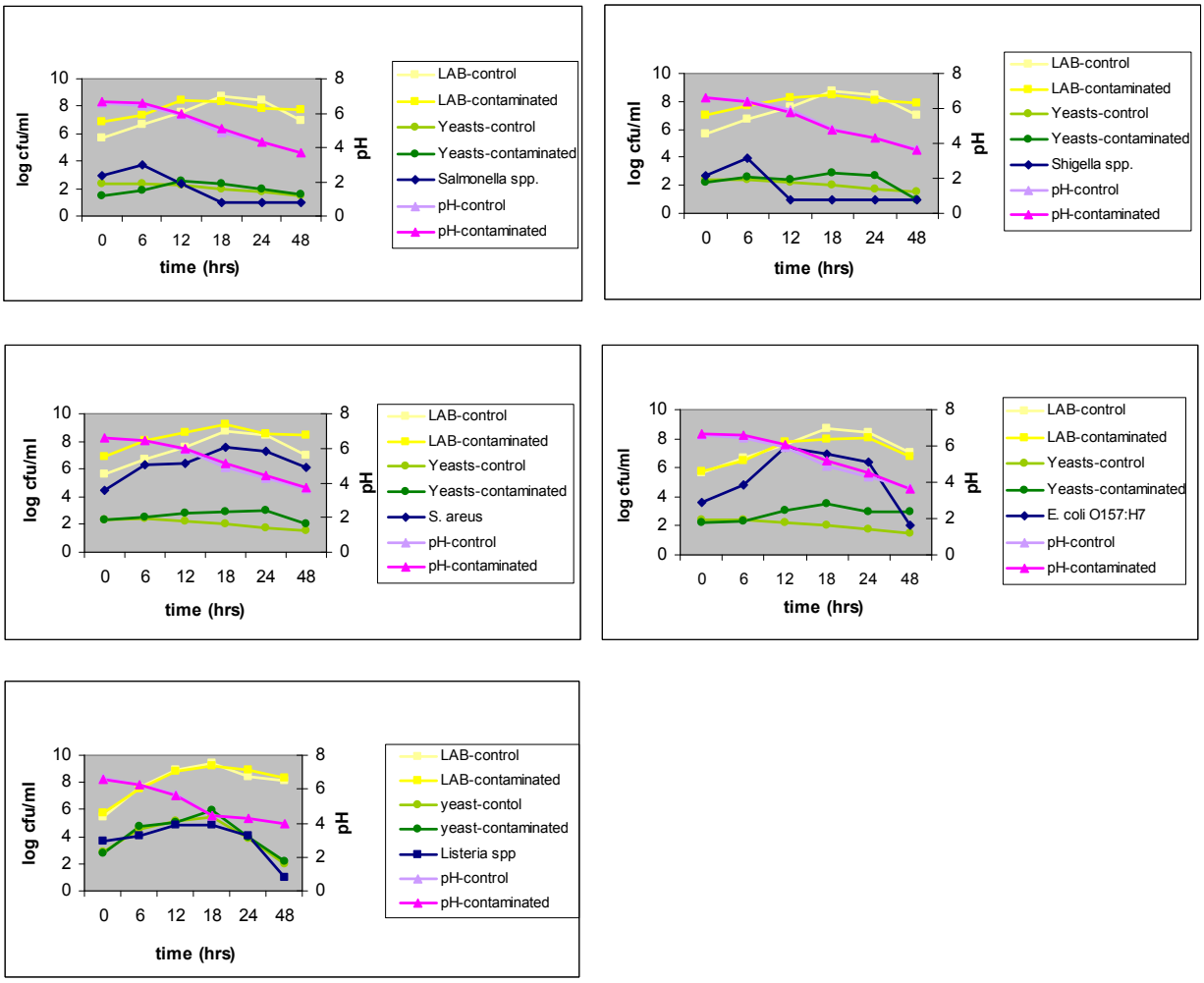

Fig. 4 Uninoculated raw milk vs raw milk inoculated with foodborne pathogens during fermentation at 37˚C.

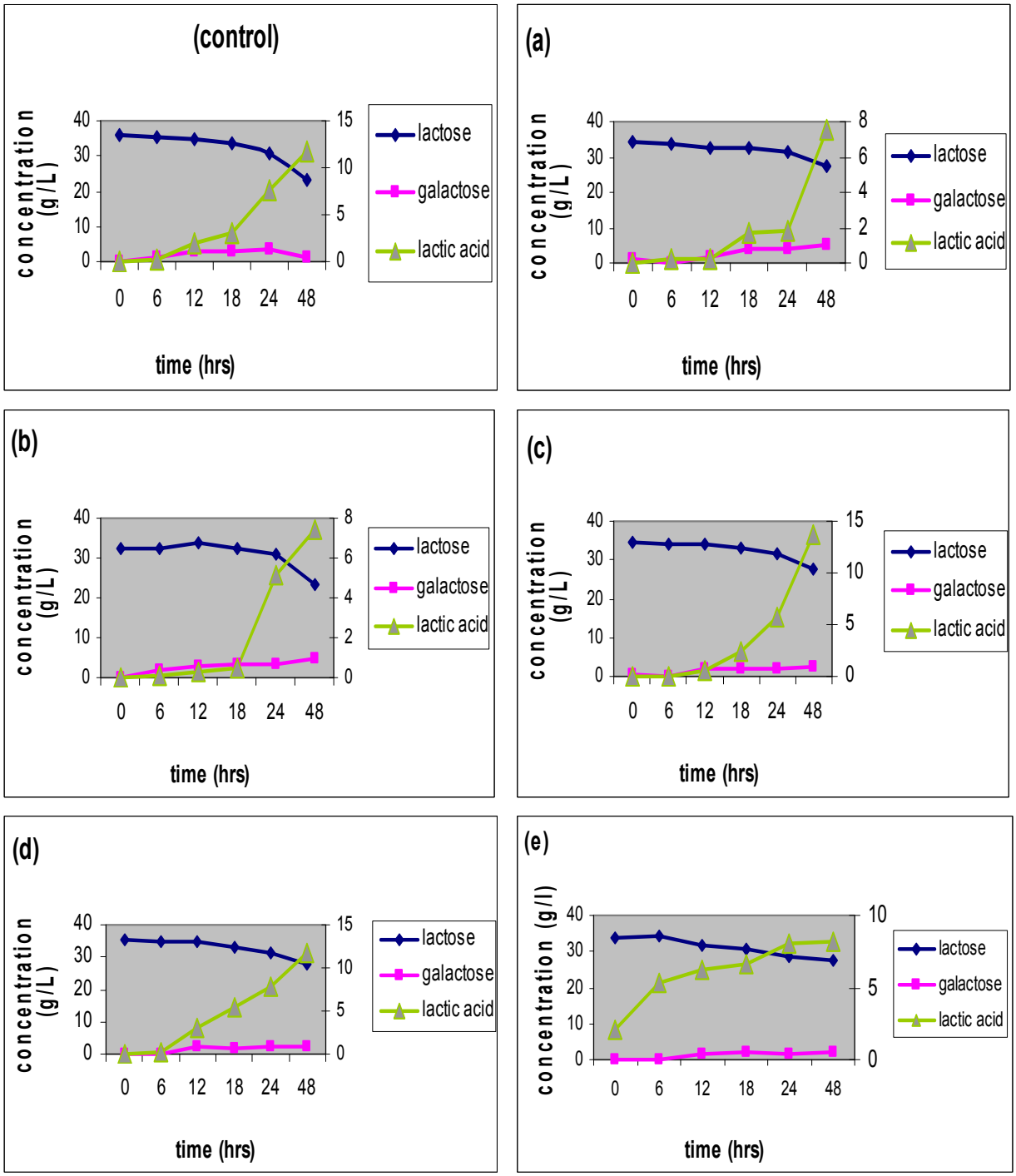

Fig. 5 Concentrations of main sugars and lactic acid in raw milk inoculated with (a) *Shigella* spp. (b) *Salmonella* spp. (c) *E. Coli* O157:H7 (d) *S. aureus* and (e) *L. monocytogenes* and in the control during fermentation at 25˚C.

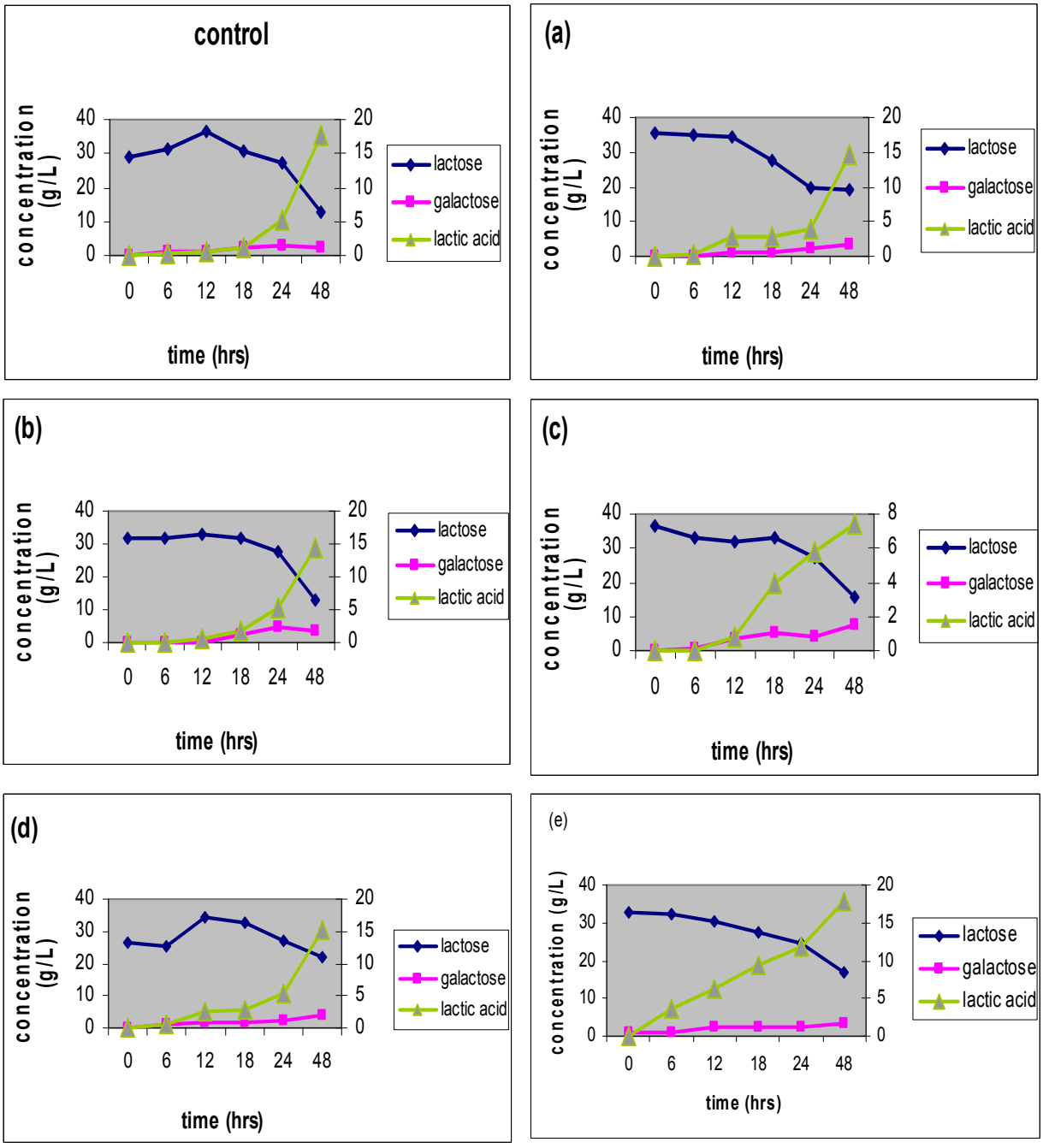

Fig. 6 Concentrations of main sugars and lactic acid in raw milk inoculated with (a) *Shigella* spp. (b) *Salmonella* spp. (c) *E. Coli* O157:H7 (d) *S. aureus* and (e) *L. monocytogenes* and in the control during fermentation at 37˚C.

CHAPTER 5

GENERAL DISCUSSIONS AND CONCLUSIONS

During the past decades, microorganisms such as *Staphylococcus aureus, Salmonella* spp.*, Escherichia coli* O157:H7*, Shigella* spp. and *Listeria monocytogenes* etc., were reported as the most common foodborne pathogens that are present in many foods and are able to survive in milk and fermented milk products (Alm, 1983; Ahmed *et al*., 1986; Ryser and Marth, 1988; Canganella *et al*., 1998). This is because milk and other fermented milk products such as yoghurt and Sethemi are nutritious food supplying most of the the essential amino acids, carbohydrates and many other required nutrients such as fat and vitamins (Rasic and Kurmann, 1978). The presence of these carbohydrates, thus, encourages the growth of pathogens in yoghurt and Sethemi.

The survival of these foodborne pathogens was determined during storage of some of the fermented milk products, yoghurt and Sethemi, at different temperatures. These are the most common milk products in Free State, South Africa.

5.1 SURVIVAL OF FOODBORNE PATHOGENS IN YOGHURT

5.1.1 Survival of pathogens in yoghurt stored at 4 ˚C and 25 ˚C

Survival of *S. aureus*, *Salmonella spp.*, *E. coli O157:H7*, non pathogenic *E. coli*, *Shigella spp*. and *L. monocytogenes* was determined in yoghurt stored at cold chain refrigeration (4 ˚C). Results showed that these pathogens were able to survive for some time in both plain and fruit yoghurt. The length of survival ranged between 1-11 days in plain yoghurt and 1-14 days in fruit yoghurt when initial counts were ± 4 log cfu ml^{-1}. The specific death rates of the pathogens were also low at this temperature. *S. aureus* was the most sensitive pathogen as it could not be recovered after only 24 hrs of yoghurt storage*. E. coli O157:H7* and the non pathogenic *E. coli* survived the longest at 4 ˚C and exhibited their characteristic of acid tolerance and adaptability (Hsin-Yi and Chou, 2001).

There was a decrease in pH with time during the storage of yoghurt and there seemed to have been a correlation between pH and counts of pathogens. The

pathogen counts decreased with the decrease in pH. The LAB were high at 8 log cfu ml^{-1} levels and these remained fairly stable throughout the study. LAB and low pH played a major role in the inhibition of the pathogens. During lactic acid fermentations, LAB utilise lactose (in the yoghurt) producing organic acids, mainly lactic acid. This acids cause the pH of the yoghurt to decline, thus, resulting in the inhibition of pathogens. The yeasts counts were low (<10 cfu ml^{-1}) in the yoghurt and remained low throughout storage. Yeasts rarely grow in refrigerated dairy products due to the competitive growth of the rapidly growing psychrotrophic bacteria (Cousin, 1982) and this was evident in this experiment. Literature has also shown that when produced by good manufacturing practice, yoghurt should contain no greater than 1 yeast cell/g and if stored under refrigeration, it should not undergo spoilage. This proved to be the case in this experiment.

In the dairy industry yoghurt is normally stored at 4 ˚C for 3 days to allow complete inhibition of contaminating pathogens by the yoghurt environment and the cold temperatures before it can be distributed to the market place. The death kinetics we did in the study showed that all the other pathogens except *E. coli O157:H7* will not be recovered after the 3 days if the common contamination levels of 100 cfu ml^{-1} are present in the yoghurt. The two *E. coli* strains will, however, survive for 8 days in the yoghurt. Thus, if yoghurt samples in the dairy industry are contaminated by *E. coli O157:H7* and the yoghurt samples are distributed to the market after only 3 days as suggested, then this would cause a great public health hazard as consumption of this yoghurts would possibly cause foodborne diseases.

Many rural households lack refrigeration and in most cases fermented milk products are stored at ambient temperatures (25 ˚C). This led us to investigate the survival of the same foodborne pathogens during storage of yoghurt at 25 ˚C. The survival of these pathogens at this temperature was decreased as the pathogens died off at a much faster rate. Their specific death rates were much higher than they were when the yoghurt was stored at 4 ˚C. The LAB increased

by 1 log cfu ml^{-1} to reach levels of 9 log cfu ml^{-1}. This increase in LAB resulted in a rapid decrease in pH, thus resulting in a much higher death rate of pathogens. This showed that the antagonistic activity of LAB and low pH against pathogens is enhanced at higher temperatures.

At the same temperature the levels of yeasts increased to levels as high as 4 log cfu ml^{-1}. A symbiotic interaction between LAB and yeasts has been reported (Gadaga *et al*., 2001). An increase in LAB results in an increase in yeasts counts. It has also been reported that this interaction of yeasts with LAB in some milk fermentations may result in inhibition or elimination of pathogenic microorganisms (Mathara *et al*., 2004). The yeasts in the yoghurt resulted in yoghurt being spoilt as the yoghurt containers swelled and the yoghurt became liquid instead of the normal semi-liquid state.

The overall results showed that at 4 ˚C we have yoghurt that is of good quality (unspoilt) but not safe for consumption whereas at 25 ˚C we have yoghurt that is of poor quality (spoilt) but safe for consumption.

5.1.2 Effect of temperature abuse at 12° C and 37 °C on the survival of pathogens in yoghurt

The survival of foodborne pathogens was monitored during 4 hr storage at 12 °C and subsequent storage at 4 °C. At 12 °C the pathogens died off much quicker than when they were stored at 4 °C. The same behaviour was noticed when the yoghurt was stored at 37 °C for 6 hrs and subsequently at 4 °C. The specific death rates of the pathogens were higher at 12 °C and 37 °C. The death rates, however, were much higher at a higher temperature (37 °C). *S. aureus* was still the most sensitive pathogen compared to the others. In both instances it survived for only 4 hrs having the highest death rate at 37 °C. When the yoghurts were stored at 4 °C their death rates decreased and the survival of the pathogens was lengthened. As was shown in the previous experiments pathogens are inhibited at a faster rate at high temperatures than at low temperatures.

There was a rapid decrease in pH in both instances but it was more enhanced in the initial 4-6 hrs when yoghurt was stored at higher temperatures. The pH continued to decrease rapidly even after storage at 4 °C although not at the same rate. There was a slight increase in the levels of LAB reaching counts of 9 log cfu ml^{-1}. As noted from the previous experiments the combination of high LAB counts, low pH and storage temperatures played a major role in the inhibition of pathogens. Yeast counts remained below 10 cfu ml^{-1} in yoghurt that was initially stored at 12 °C, in yoghurt that was initially stored at 37 °C the yeasts grew from the onset of the experiment and reached levels as high as 4 log cfu ml^{-1} in yoghurt contaminated with *E. coli O157:H7*. This shows that at high temperatures (25 °C and 37 °C) the yeasts grow to levels high levels that even cause spoilage. At low temperatures little or no growth of yeasts is observed.

5.2 SURVIVAL OF FOODBORNE PATHOGENS IN SETHEMI

The raw milk was tested for prior contamination of the test pathogens. *S. aureus* was detected in the milk showing that this pathogen is a common contaminant of raw milk. Contamination might have occurred when the cows were milked and the sources of contamination might have been either the udder of the cows, the hands of the milk handlers or the milking equipment.

All the pathogens tested in this study were able to survive milk fermentation at 25 °C and 37 °C during the production of Sethemi. Within the first 12-18 hrs of fermentation pathogens in most containers, increased by numbers ranging between 1-4 log units. Growth of these pathogens was encouraged by the abundant supply of growth nutrients, particularly carbohydrates in the milk. After 18 hrs the pathogens counts began to decrease. At this period the lactic acid content had increased to very high levels which resulted in a very rapid drop in pH, thus inhibiting the growth of pathogens. Inhibition of pathogens was greater at 37 °C where counts decreased to levels as low as 1 log cfu ml^{-1} than at 25 °C. Towards the end of fermentation at 25 °C counts of pathogens as high as 5.8 log

cfu ml^{-1} could still be detected. This poses a great health hazard to local people who still rely in the traditional way of milk preservation. Fermentation did not have any effect on the survival of *L. monocytogenes*. The yeasts counts were fairly stable throughout fermentation at both temperatures except in milk inoculated with *L. monocytogenes*. Growth of LAB to levels as high as 8-9 log cfu ml^{-1} was observed during fermentation. The LAB played a major role in the inhibition of pathogens by producing lactic acid.

5.3 REFERENCES

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CHAPTER 6

SUMMARY

The history and the background of dairy products, specifically fermented milk products and foodborne diseases were reviewed. The constructed literature review also gave details of the microbial and nutritional compostion of fermented milks and examples of African traditional fermented milks. Health benefits of these dairy products were also included. Furthermore, the most common foodborne pathogens and their survival in dairy products were discussed.

The survival of *Escherichia coli* O157:H7, non pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp.* and *Shigella spp* in plain and fruit yoghurt during cold storage at 4 °C was investigated. The survival of these microbial pathogens ranged between 1-14 days in both types of yoghurt, with *Staphylococcus aures* being the most sensitive pathogen to the yoghurt enviroment. Lactic acid bacteria and yeasts counts remained fairly stable at an average of 8 log cfuml^{-1} and below 10cfuml^{-1} respectively. A reduction in pH was noticed during yoghurt storage. The high numbers of LAB and low pH played a major role in the inhibition of the food borne pathogens over time in both plain and fruit yoghurt.

The survival of the same microbial pathogens in fruit yoghurt during temperature abuse at 25°C was investigated. There was a rapid die-off of the food borne pathogens at this temperature. Their survival in the yoghurt ranged between 1-3 days. There was a rapid decrease in pH and an increase in the LAB and these together with the high temperature played a major role in the higher death rate of the foodborne pathogens in the fruit yoghurt. Yeasts grew to high levels that caused the yoghurt to spoil.

When comparing yoghurt stored at 4 °C and 25°C a conclusion was made: yoghurt stored at 4 °C will be of good quality but will not be regarded as safe as pathogens survival for a long period, and yoghurt stored at 25°C will be regarded as safe but will not be of good quality as high yeasts counts will result in spoilage.

The microbial pathogens were also inoculated into yoghurt and their survival at 4 °C after temperature abuse at 12 °C and 37 °C for 4-6 hours was studied. The pathogenic microorganisms died-off at a higher death rate during temperature abuse at high temperatures than at a low temperature at 4 °C. At 4 °C the death rate decreased and the survival in yoghurt ranged between 1-11 days. A rapid decrease in pH and a slight increase in LAB were observed during and after temperature abuse. The yeast increased during the storage of yoghurt. This study showed that inhibition of foodborne pathogens is high at higher temperatures.

The survival of the microbial pathogens in milk during the production of Sethemi at 25 °C and 37 °C was studied. The pathogens increased, growing to high counts during the first 24hrs of fermentation. After 24hrs growth of some pathogens was inhibited, however, all the pathogens could still be detected even at the end of fermentation at both temperatures. Yeasts grew to some extend and the lactic acid bacteria grew to high levels during the fermentation of milk at both temperatures. The lactose produced by LAB decreased with time and there was a slight increase in galactose. A rapid increase in lactic acid was noted after a few hours of fermentation, this caused a decline in pH which resulted in the inhibition of pathogens. The inhibition was most effective at 37 °C.

(Key words: foodborne, inhibition, microbial, pathogens, dairy products, yoghurt, sethemi, survival)