MICROBIAL DIVERSITY OF NATURALLY FERMENTED MILK PRODUCED BY SMALLHOLDER MILK PRODUCERS IN

SOUTH AFRICA

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May 31, 2005

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By

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PHILOSOPHIAE DOCTOR

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Promoter: Prof. B.C. Viljoen

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"There is no question of being qualified in ourselves; we cannot claim anything as our own. The qualification we have comes from God"

2 Cor 3: 5

Dedicated to my children Hiwot Ameha and Brook Ameha and my wife Nigist Getahun whose love has been the source of inspiration and the strong motive for my patience and persistence

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DECLARATION

I, the undersigned, declare that the dissertation submitted hereby by me for the Ph.D. degree at the University of Free State is my own independent work and has not been previously submitted by me at another university or faculty. I furthermore cede copyright of the dissertation in favor of the University of the Free State.

_____	_____	_____
Name	Signature	Date

LIST OF ABBREVIATIONS, SYMBOLS AND CHEMICAL FORMULAE

α	Alpha
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
B.	*Bacillus*
BAP	*bifidus-acidophillus-pediococcus*
BAT	*bifidus-acidophillus-thermophilus*
B.C.	Before Christ
B-cells	Lymphocytes maturing in bone-marrow
β	Beta
C.	*Candida*
$C_4 – C_{20}$	Carbon four to carbon twenty
$C_{16}H_{17}N_2O_4SNa$	Benzylpenicillin sodium salt (Penicillin G)
$C_{22}H_{24}N_2O_9.2H_2O$	Oxytetracycline dihydrate
^oC	Degree Celsius
ca	circa
CB	Coliform bacteria
CFU	Colony forming units
Cl.	*Clavispora*
cm	Centimeter
CO_2	Carbon dioxide
CoA	Coenzyme A
Contd.	Continued
Control + OT	Oxytetracycline-containing non-yeast-inoculated control
Control + P	Penicillin-containing non-yeast-inoculated control
Cr.	*Cryptococcus*
D-	Dextrorotatory
$D_1 – D_2$	Day 1 to Day 5

D.	*Dekkera*
Deb.	*Debaryomyces*
DNA	Deoxyribonucleic acid
e.g.	Example
et al.	et alii (and others)
etc.	et cetera
EU	European Union
FAO	Food and Agriculture Organization
$FADH_2$	Reduced flavine adenine dinucleotide
Fig(s)	Figure(s)
FW	Formula weight
g	Gram
γ	Gamma
GLM	General linear model
H.	*Hansenula*
H_2O	Water
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
h/hrs	Hour/Hours
% I	Percentage increase
IDF	International Dairy Federation
i.e.	Id est (Latin phrase for “That is to say”)
IgA	Immunoglobulin A
IU	International unit
κ	Kappa
K.	*Kluyveromyces*
Kg	Kilogram
L	Liter
L-	Levorotatory
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*

Leu.	*Leuconostoc*
Log	Logarithm (base ten)
M17	Growth medium for lactic streptococci
ml	Milliliter
µ	Microgram
mm	Millimeter
mol % G+C	Mole percentage guanine-cytosine
MRL	Maximum residual limit
MRS	De Man, Rogosa and Sharpe growth medium for lactic acid bacteria
MYC	Mixed Yeast Culture
NaCl	Sodium chloride
NAD	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NFM	Naturally fermented milk
Oxy (OT)	Oxytetracycline
p	Probability
P.	*Pichia*
PCA	Plate count agar
Ped.	*Pediococcus*
Pen	Penicillin
pH	Negative logarithm of hydrogen concentration
r	Pearson's correlation coefficient
R.	*Rhodotorula*
% R	Percentage reduction
RBCA	Rose Bengal Chloramphenicol Agar
RP	Relative percentage
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S.	*Sacharomyces*

SD	Standard deviation
SE	Standard error
S.N.	Serial Number
Sp.	Species (singular)
Spo.	*Sporobolomyces*
Spp.	Species (plural)
St.	*Streptococcus*
T.	*Torulaspora*
TAM	Total aerobic mesophiles
TAMC	Total aerobic mesophilic count
TCA	Tri-carboxylic acid
T-cells	Lymphocytes maturing in thymus
UFS	University of Free State
U-NFM	Un-inoculated Naturally Fermented Milk
USA	United States of America
VRBA	Violet red bile agar
viz.	Videlicet (Latin word for 'Namely')
$\overline{X}$	Sample mean
Y.	*Yarrowia*
YM	Y-extract matose-extract glucose agar
Z.	*Zygosaccharomyces*

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CHAPTER I

Introduction and Literature Review

1. Introduction

Most foods are rich in easily accessible nutrients and as a result they are ideal substrates for growth of many different types of microorganisms. When microbes grow on such foods, they either produce undesirable changes or improve the taste, smell, texture and the preservation properties of the food. Thus, without even understanding the scientific basis, man has been using some of these microorganisms for thousands of years to produce cultured foods with improved keeping qualities and with characteristic flavors and textures that are different from the original food.

Foodstuffs that have generally gone through fermentation processes by specific microorganisms and/or their enzymes are known as fermented foods. Currently they contribute to about one-third of the diet worldwide (Campbell-Platt 1994). Some of these have been already scientifically studied for many decades and knowledge about them is keeping pace with development in technology (Ko, 1982). Others, particularly those from Asia and Africa, seem to have received little or no attention by scientists. Amongst these latter categories of fermented foods, some are still being manufactured according to traditional methods on a small village industrial scale or just at home for family consumption. These indigenous fermented foods are believed to have more attractive properties than the original agricultural raw materials from which they have been made. In addition to their external attractive properties, their nutritional values and keeping qualities are thought to be better than the raw materials. Because in most cases they do not require fuel for their processing, they are of particular advantage in most African countries where wood is a valuable and fairly

scarce resource (Hesseltine, 1979). If their manufacturing procedures are carefully studied and properly followed, such foods are generally believed to be safe for consumption. In addition, traditional methods of manufacturing fermented foods are not complicated, and expensive equipment is not required. Thus, fermentation of indigenous foods is considered as an inexpensive and effective means of food production that could be utilized in alleviating world food security problems (Pederson, 1979).

The most commonly used fermented foods in the African continent are cereal-based fermented products and fermented milk. Fermented cereals are important primarily in developing countries where the lack of resources limits the use of techniques such as vitamin enrichment of foods, and the use of energy and capital-intensive processes for food preservation.

Fermented milks and their products are also very important not only for their nutritional values, but also for their therapeutic values, for retarding the growth of spoilage and pathogenic microorganisms; for alleviating lactose intolerance and for being a source of income.

Although many of the indigenous fermented milks in Africa are named differently in different countries and localities within a country, they involve more or less similar processes except for minor differences seen in the technologies used in their production. Milk containers for instance may be made in different ways from different materials like animal skin, gourd, clay, wood etc. Calabashes made from gourd and/or clay pots are widely used in most parts of Ethiopia, Kenya, Sudan, and Egypt (Abdelgadir *et al.*, 2001; O'Mahony and Peters, 1987; Kimonye and Robinson, 1991; El-Gendy, 1983). In some African countries, milk vessels are smoked with smoldering woods of certain plant specimens primarily to improve the flavor and the keeping quality of the fermented milk (Kassaye *et al.*, 1991; Kimonye and

Robinson, 1991). Addition of fresh plant parts to the fermenting milk is also a common experience in some countries.

In South Africa, milk used to be fermented in milk-sacks, calabashes, clay pots, stone jars and baskets (Bohme, 1976; Bryant, 1967; Fehr, 1968; Fox, 1939; and Quinn, 1959). Some of these containers are still in use in the rural areas. However, with urbanization and modernity, these earlier technologies are to a large extent being abandoned and replaced with new ones (Coetzee *et al*., 1996). By abandoning such technologies it is possible to lose not only a wealth of indigenous knowledge but also the relevant microorganisms, which were probably best adapted to the conditions provided by those technologies. Thus, in order to promote the quality of the indigenous fermented milk and at the same time preserve and exploit the associated microorganisms, it is of paramount importance to study the scientific basis of these indigenous technologies.

Furthermore, since the fermentation processes involved in producing the indigenous fermented milks are spontaneous (Mutukumira, 1995, Jespersen, 2003), the products are often variable in quality (Sanni, 1993; Zulu *et al.* 1997). One way of controlling the variability and thereby achieving reproducibility is by using starter cultures. In addition, identifying and providing a practical means of using appropriate starter cultures is advantageous due to the competitive role of microorganisms and their metabolites in preventing the growth and metabolism of unwanted microorganisms. It is now generally understood that a strong starter reduces fermentation times, minimizes dry matter losses, avoids contamination with pathogenic and toxigenic bacteria and molds, and also minimizes the risk of incidental microflora causing off-flavors. Despite of such awareness, however, according to Holzapfel (1997) there has been no LAB starter culture commercially available to date for small-scale processing of traditional African foods. The development of such starter

cultures based on the indigenous product is, therefore, very essential to ensure the safe use of the fermented milks by the rural community. Achievement of this goal may be realized only through a thorough understanding of the composition and contribution of the microflora in the indigenous product. Currently, however, there is only limited information on the microflora of South African indigenous fermented milks.

The major groups of microorganisms involved in the production of fermented milks are lactic acid bacteria (LAB) and yeasts. The group name Lactic acid bacteria, refers to a large group of beneficial bacteria that have similar properties and the ability to produce lactic acid as an end product of the fermentation process. These bacteria are widespread in nature in both fermented and non-fermented foods and are also found in the human digestive system. Traditionally, they have been used as starter cultures for the production of fermented foods including fermented milk. The long history of human exposure to these bacteria through the consumption of fermented foods has led to the reasonable conclusion that they should generally be regarded as safe (Adams, 1999). As a result, LAB have been subjected to extensive studies. Currently, they are widely used as commercial starter cultures for large-scale production of a variety of fermented milks. In these products, LAB have two major functions, namely the achievement of certain beneficial physico-chemical changes in the food ingredients, e.g., acidification, curdling and production of flavor compounds, and inhibition of the outgrowth of microbial pathogens and spoilage microorganisms. These functions of LAB are mainly accomplished by the production of several metabolites. Of these, lactic acid is the major organic acid and is produced by all members of LAB. This organic acid lowers the pH and renders anti-microbial property to the food. It also functions as a permeabilizer of the gram-negative bacterial outer membrane and may act as a potentiator of the effects of other anti-microbial substances (Alakomi *et al*., 2000). The lowered pH, however, favors the growth of yeasts and molds

in the final product. Thus, the presence of significant populations of yeasts is not uncommon in naturally fermented milks.

There are a number of reports that indicate yeasts being frequently isolated from fermented milks. Their presence, however, has been mainly regarded as a nuisance and as a result their positive role in the final product has been given very little attention. However, recent works have shown that the yeasts interact with LAB and contribute to the characteristics of the final product (Cheirsilp *et al.*, 2003; Gadaga *et al*..2001; Narvhus *et al*., 2003; Roostita and Fleet,1996).

Thus, in order to improve the quality of the indigenous fermented milk prior understanding of the composition of the microflora and their interaction during the fermentation process is of vital importance.

The present study has, therefore, the following four major objectives.

- To isolate, identify and characterize the yeast microflora in the indigenous fermented milk
- To investigate how the yeast microflora influence the growth of the associated LAB in naturally fermented milks.
- To study the contribution of the indigenous African technologies in the fermentation of milk
- To investigate the effect of residual antibiotics on the growth of the LAB and yeast flora in naturally fermented milks

2. Literature Review

2.1. Fermentation

In 1857, a French scientist, Louis Pasteur, observed and demonstrated that living cells such as microorganisms can produce alcohol by fermentation of sugars. His work led to an understanding of the fundamental role of microorganisms in fermentation processes. This knowledge greatly assisted the development of the food industry in the world.

Fermentation is biochemically defined as the catabolism of glucose (or other sugars) in which the terminal hydrogen acceptor is an organic molecule. In lactic acid bacteria, excess hydrogens are “dumped” on to pyruvic acid, which is the breakdown product of glucose. This produces lactic acid. Our over-exercised muscles also do the same thing when the supply of oxygen is limited. In all fermentations, a hydrogen carrier (NAD) is freed up to assist further with glycolysis. Yeasts too perform fermentation, but with different terminal hydrogen acceptors (acetaldehyde) and products (CO_2 and ethanol).

Fermentation causes changes in food quality indices including texture, flavor, appearance, nutrition and safety. The benefits of fermentation may include improvement in palatability and acceptability by developing improved flavours and textures; preservation through formation of acidulants, alcohol, and antibacterial compounds; enrichment of nutritive content by microbial synthesis of essential nutrients and improving digestibility of protein and carbohydrates; removal of antinutrients, natural toxicants and mycotoxins; and decreased cooking times (Haard, 1999). Thus, food fermentation is an inexpensive method of modification of raw agricultural foodstuffs into more nutritious, palatable and safe products.

For convenience, food fermentations may be broadly divided into alcoholic fermentations carried out by yeasts, acid fermentations carried out by

bacteria, mixed alcoholic/acid fermentations, and fungal (mold) fermentations (Reed and Nagodawithana, 1991).

2.2. Lactic acid fermentation

Lactic acid fermentation is a form of anaerobic respiration that has a glucose-consuming catabolic pathway and is used by both bacteria and animals to produce ATP in the absence of oxygen. Lactic acid fermentation breaks down a glucose molecule into two molecules of pyruvate and combines them with hydrogen ions to form lactic acid. The energy released is stored in two ATP molecules and several NADH molecules. However, since there is no oxygen available to run the electron transfer chain, the energy of NADH cannot be transferred to ATP. Hence, lactic acid fermentation is by far inferior to cellular respiration as a way of generating energy.

In foods and beverages, lactic acid fermentation is performed by lactic acid bacteria, which are responsible for the sour taste.

2.3. Fermented foods

Fermented foods are foodstuffs derived from animal or plant tissues and subjected to the action of microorganisms and/or enzymes to give desirable biochemical changes. They contribute to about one-third of the diet worldwide (Campbell-Platt 1994).

The microorganisms involved in the production generally belong to three major groups of organisms namely, lactic acid bacteria, acetic acid-producing bacteria and certain alcohol-producing species of yeasts and molds. These microorganisms mostly convert the raw materials to products that have acceptable qualities. In the common fermented products such as sauerkraut and yogurt, for example, lactic acid is produced by lactic acid bacteria and is used to prevent the growth of undesirable microorganisms in

the non-sterile, raw materials making the products palatable, safe and shelf-stable (Ray and Daeschel, 1992).

Fermented foods may be produced through natural fermentations or through controlled fermentations using starter cultures. In a natural fermentation, the conditions are uncontrolled allowing the desirable microorganisms to grow and produce metabolic by-products, which result in the unique characteristics of the product. When the yield is unstable and where the desired microorganisms might not grow, or where pathogenic microorganisms might also grow, a controlled fermentation is used. In a controlled fermentation the fermentative microorganisms are isolated and characterized, then maintained for use as starter cultures. The latter are then added to the raw materials in large numbers and incubated under optimal conditions to produce the required fermented foods. In general, modern, large-scale production of fermented foods is dependent almost entirely on the use of such defined strain starters.

There is a wide variety of fermented foods in the world. Some are produced commercially at a large scale (Ko, 1982), whereas others are still being manufactured with undefined mixtures of microorganisms. Because they are so diverse, attempts have been made to classify them in different ways by different authorities (Yokotsuka 1982; Campbell-Platt 1987; Odunfa, 1988; Kuboye 1985; Steinkraus 1983, 1995, 1996, 1997). Of these, the scheme proposed by Steinkraus (1983, 1995, 1996, 1997) seems to be exhaustive. But since it is beyond the scope of this manuscript, the above references may be consulted for details.

2.3.1. African indigenous fermented foods

According to Steinkraus (1996), indigenous fermented foods constitute a group of foods that are produced in homes, villages, and small cottage industries at prices within the means of the majority of the consumers in the developing world. They are often manufactured according to traditional

methods on a small village industrial scale or just at home for family consumption. Although such traditional fermented foods are produced with undefined starters, they are still believed to have more attractive properties than the original agricultural raw materials from which they have been made (Odunfa and Oyewole, 1998). In addition to their beneficial effects often mentioned for fermented foods, such as improvement of flavor and texture, prolonged shelf-life, other effects include reduced loss of raw materials, reduced cooking time, improvement of protein quality and carbohydrate digestibility, improved bio-availability of micronutrients and removal of toxic and anti-nutritional factors such as cyanogenic glycosides (Addo *et al.* 1996; Onilude *et al.* 1999; Padmaja, 1995; Steinkraus, 1995, Svanberg and Lorri, 1997). Moreover, traditional methods of manufacturing fermented foods are not complicated, and expensive equipment is not required. Therefore, fermentation of indigenous foods is considered as an inexpensive and effective means of food production that could be utilized in alleviating food security problems in developing countries (Pederson, 1979).

African indigenous fermented foods are predominantly prepared from starch-rich plant materials and/or milk (Jespersen, 2003). The former includes mainly cereals and root crops, e.g., cassava, enset (Steinkraus, 1996).

2.3.1.1. Cereals

Cereal grains are dry fruits (caryopse) produced by the monocot plant family named Graminae. Although their protein content is generally poor, they are particularly rich in carbohydrates (Alais and Linden, 1991). Together with oil seeds and legumes, however, they supply the majority of the dietary protein, calories, vitamins, and minerals to the bulk of the populations in developing nations (Chaven and Kadam 1989).

Some examples of the most widely used cereals to produce fermented foods in Africa include maize, sorghum, barley, wheat, acha (*Digitaria esculenta.)* tef (*Eragrostis tef)*, and millet (*Eleusine* and *Pennisetum* species) (BOSTID,1992; 1996; Odunfa and Adeyele, 1987; Gashe *et al*., 1982, Gobbetti *et al*., 1995; Mensah *et al*., 1988). Of these, maize alone is used to produce over 20 different African fermented foods (Odunfa and Oyewole, 1998).

The nutritive and sensory values of these cereal grains and their products are, for the most part, inferior to animal food products. For this reason, some methods have been developed to improve their nutritive values. These include traditional genetic selection, genetic engineering, amino acid and other nutrient fortification, complementation with other proteins (notably legumes), milling, heating, germination and fermentation. Of these, fermentation is thought to be the least expensive method that can be readily used by underdeveloped countries (Haard, 1999).

2.3.1.2. Milk

Milk is a highly nutritious secretion of the mammary glands of mammals. Used for its original purpose of nourishing newly born animals, it is a near to perfect food designed for transfer from the producer to user in the most convenient and hygienic fashion imaginable. Historically man has used the milk of almost every domesticated animal for food.

2.3.1.2.1. Composition and physico-chemical properties of milk

About 87% of fresh milk is water, in which the other constituents are distributed in various forms based on the type and size of particle they form (Johnson, 1974). Fresh milk, therefore, will typically show four different kinds of solutions: emulsions, colloidal, molecular and ionic solutions. Its freezing point is determined by the amount of solids-not-fat component. Normally unadulterated milk will have a freezing point ranging from -0.53^0C to -0.55^0C (Jenness *et al.,* 1974).

Fresh cow's milk has a pH of between 6.5 and 6.7 at 25^0C. Values higher than 6.7 denote mastitic milk and values below pH 6.5 denote the presence of colostrum or bacterial deterioration. Because milk is a buffer solution, considerable acid development may occur before the pH changes. A pH, lower than 6.5, therefore, indicates that considerable acid development has taken place. This happens normally due to bacterial activity.

Measuring milk acidity is an important test used to determine milk quality and to monitor processes such as cheese making and yogurt-making. In both cases the titratable acidity is expressed in terms of percentage lactic acid. Fresh milk drawn from the udder of cow contains only traces of lactic acid and it normally shows an initial acidity of 0.14 to 0.16% when titrated using sodium hydroxide to a phenolphthalein end-point. After fermentation, however, the principal acid produced is lactic acid. Thus measurement of a significant amount of lactic acid is indicative of the microbial quality of milk.

The composition of cow's milk may vary considerably depending on the individual animal, stage of lactation, its breed, age and health status. Herd management practices and environmental conditions also influence milk composition. The range of composition of cow's milk is shown in Table 1.

The major components of milk are:

a) Water

Water is the main constituent of milk. On the average about 87% of whole milk is composed of water. In most cases milk processing is designed to remove water from milk or reduce the moisture content of the product.

b) Proteins

Milk proteins can be separated into two major fractions, the caseins and the whey, or serum, proteins.

When acid is added to milk, some of its protein precipitates in the form of a curd-like clot. This fraction of the milk, or curd, is known as casein. The

other protein remains dissolved in the liquid known as whey or milk serum (Whitney *et al.*, 1976). Casein consists of a mixture of proteins containing phosphate groups. These proteins can be separated by electrophoresis into α, β, and γ fractions with α fraction contributing about 66% of the total. α-casein is also consisting of a mixture of proteins including α-caseins, which are coagulated by calcium ions and k-casein, which is not calcium sensitive and which stabilizes casein micelle.

Casein is dispersed in milk in the form of micelles, which range in size from 40nm to 300nm. These micelles are stabilized by the K-casein and give the characteristic color and appearance to milk. Caseins are hydrophobic but K-casein contains a hydrophilic portion known as the glycomacropeptide and it is this that stabilizes the micelles.

Casein is easily separated from milk, either by acid precipitation or by adding rennin. In cheese-making most of the casein is recovered with the milk fat. Casein can also be recovered from skim milk as a separate product.

When the pH of milk changes, the acidic or basic groups of the proteins are neutralized. At the pH at which the positive charge on a protein equals exactly the negative charge, the net total charge of the protein is zero. This pH is called the iso-electric point of the protein (pH 4.6 for casein). If an acid is added to milk, or if acid-producing bacteria are allowed to grow in milk, the pH declines. As the pH declines, the charge on casein declines and consequently it precipitates. Hence milk curdles as it sours, or the casein precipitates more completely at low pH.

The whey proteins remaining in solution after the casein has been removed include albumins and globulins, and a number of enzymes including several phosphatases, lipases, and peroxidases. They also contain a proteose/peptone fraction and an antibody fraction consisting of immunoglobulins.

c) Lipids

The major constituents of lipids in milk are the triglycerides. In addition to these, milk consists of other lipids like diglycerides, monoglycerides, phospholipids, sterols, free fatty acids and traces of cerebrosides, squalene (a precursor of cholesterol), waxes and the fat-soluble vitamins.

The fatty acids of milk triglycerides are unusual. Over 9% of the fatty acids consist of the short-chain fatty acids, butyric, caproic, caprylic and capric acids (C_4, C_6, C_8 and C_{10} respectively). About 40% consist of the saturated C_{16} palmitic and C_{18} stearic acids. Oleic acid alone amounts to 30% while the polyunsaturates linoleic and linolenic acids make up 3% of the total. The remainder comprises mainly of lauric (C_{12}) and myristic acids (C_{14}) with smaller amounts of unusual odd-numbered or branched fatty acids.

The fats in milk, like in any other food, are subject to two types of deterioration that affect the flavor of milk products, namely hydrolytic rancidity and oxidative rancidity. Hydrolytic rancidity occurs when fatty acids are broken off from the glycerol molecule by lipase enzymes produced by milk associated bacteria. The resulting free fatty acids are volatile and contribute significantly to the flavor of the product. Oxidative rancidity occurs when fatty acids are oxidized. In milk products it causes tallowy flavors. For example, oxidative rancidity of dry butterfat causes off-flavors in recombined milk.

d) Lactose

Lactose is the major carbohydrate fraction in milk. It is made up of two sugars, glucose and galactose. The average lactose content of cow's milk varies between 4.4 and 5.2% (Nickerson, 1974), though milk from individual cows may vary more.

Lactose is less soluble in water than sucrose and is also less sweet. It can be broken down to glucose and galactose by bacteria that have the enzyme β-galactosidase. The glucose and galactose can then be fermented to lactic acid. This occurs when milk goes sour. Under controlled conditions they

can also be fermented to other acids to give a desired flavor, as in propionic acid fermentation in Swiss-cheese manufacture.

Some people are unable to metabolize lactose and suffer from an allergy as a result. This phenomenon is called lactose intolerance. Pre-treatment of milk with lactase enzyme breaks down the lactose and helps overcome this difficulty.

e) Salts

Milk salts are mainly chlorides, phosphates and citrates of sodium, calcium and magnesium. Although salts comprise less than 1 % of the milk, they influence its rate of coagulation and other functional properties. Some salts are present in true solution while the physical state of other salts is not fully understood. Calcium, magnesium, phosphorous and citrate are distributed between the soluble and colloidal phases. Their equilibria are altered by heating, cooling and by a change in pH.

In addition to the major salts, milk also contains trace elements. Some elements come to the milk from feeds, but milking utensils and equipment are important sources of such elements as copper, iron, nickel and zinc.

f) Vitamins

Milk contains the fat-soluble vitamins A, D, E and K in association with the fat fraction and water-soluble vitamins B complex and C in association with the water phase. Vitamins are unstable and processing can therefore reduce the effective vitamin content of milk. Particularly, the vitamin C is unstable and is readily destroyed by heat, by the catalytic action of traces of copper in the milk and by exposure to bright sunlight. As a result milk is not regarded as a reliable or important source of vitamin C in human diet.

2.3.1.2.2. Fermented milks

According to the IDF (1988), ‘fermented milk is a milk product prepared from milk, skimmed or not, with specific cultures; the microflora is kept alive until sale to the consumer and may not contain any pathogenic germ.’ In

short, fermented milks are whole or skim milk treated in several ways and curdled to beverage or custard-like consistency by lactic-acid-producing microorganisms.

The exact origin of the manufacture of fermented milks is difficult to establish. However, it is reasonable to speculate that it probably originated soon after man started milking cows, which is at least 10,000 years back (Narvhus, 2003). According to El Gendy (1983), there are evidences that indicate that the instruments of manufacture for the Egyptian fermented milks Laben rayeb and laben khad were in use around 7000 B.C. Written records also go to the dawn of civilization. For example, mention about fermented milks is made in the Holy Bible (see Genesis 18:8) and the Vedas (the sacred book of Hinduism). There are also ample evidences that show that the early nomadic herders, especially in Asia and South and Eastern Europe, Scandinavia, Africa, and South America, used many forms of fermented milks (Abdelgadir *et al.,* 1998*;* Chomakov *et al.,* 1973; Koroleva and Kondratenko, 1978; Tamime and Robinson, 1978; Rašić and Kurmann, 1978; El-Gendy, 1983). Such milks, in addition to forming the vital diet of the human population, were used for various other purposes including for curing disorders of the intestines, stomach and liver; stimulating the appetite; preservation of meat against spoilage during the summer; and serving as cosmetics (Rašić and Kurmann, 1978). In the early part of the 20th century, Metchnikoff's theory of 'longevity' that states about the beneficial health effect of yoghurt, significantly influenced the spread of the product to many countries of Europe and promoted extensive research in fermented milks in general and in yoghurt in particular (Rašić and Kurmann, 1978). Today, the diversity of fermented milks produced throughout the world totals approximately about 400 generic names, but in actual essence the list may only include very few varieties. Taking into account the microbial species that dominate the flora in the product including their principal metabolites, Kurman (1984) proposed a scheme of

classification for these fermented milks, which was later slightly modified by Roginski (1988) as outlined in Table 2.

In general, fermented milks may be classified in different ways but a system based on the type of starter microorganisms used such as shown above appears satisfactory.

2.3.1.2.2.1. Chemical composition of fermented milks

The chemical composition of fermented milks depends on the initial composition of the raw milk from which they are made and the specific metabolism of the microorganisms growing in the milk (Oberman, 1985). The main components are still protein, fats, carbohydrates, minerals, and vitamins, and are basically similar to the raw milk. Thus, the gross chemical composition alone indicates the potential nutritional value of fermented milks.

However, as can be seen from Table 1, the amounts of some of these components in fermented milks vary from those of the raw milk. In addition to these changes, a significant increase in the amounts of free amino acids, peptides, free fatty acids, folic acid, folinic acid, choline and decrease in the amounts of vitamin B_6 and B_{12} has been reported (Oberman, 1985; Rašić and Kurmann, 1978). Flavoring compounds such as diacetyl, acetaldehyde, acetoin etc., which are non-existent in raw milk, are present in fermented milks. Such a difference, obviously, results from the metabolic activities of microorganisms.

2.3.1.2.2.2. Artificial (New) fermented milks

The health aspects of fermented milks including yoghurt became apparent in the late 1800s and the early part of the 1900s; as a result of the views of Metchnikoff linking longevity of the Caucasians with high consumption of fermented milks, and the observations by Tisser regarding the beneficial role of certain microorganisms (*Bifidobacterium* spp.) that colonize the intestinal tract of newly born infants. This aspect generated great scientific interest among scientists and it wasn't until the 1930's that *Lactobacillus*

acidophilus has been identified to colonize and proliferate in humans rather than *Lactobacillus delbreuckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. As a consequence, research in this field has been enormous, and the knowledge acquired over the years in terms of human health benefits of fermented milks has helped to increase the consumption of these products in many countries. Gradually, in the 1950s, these products were even successfully used for the treatment of certain diseases.

Initially, such probiotic-containing fermented milks were manufactured using a single strain of *Bifidobacterium* sp. However, sensory assessment by trained panelists later revealed that such products were having certain drawbacks, i.e. their pH values were often at borderline (between 4.8 and 5.4), they were showing excessive whey separation, their coagulum was extremely weak, and in some cases, their odor and taste were unpleasant. In contrast, fermented milks manufactured using commercial blends of LAB and probiotic microorganisms were found to be superior to those produced using only single strains of bifidobacteria.

Over the past few decades, numerous probiotic fermented milk products have subsequently appeared in many markets in the world. Examples of some probiotic genera of microorganisms used in the production of fermented probiotic fermented milks in addition to *Bifidobacterium* include *Lactobacillus, Pediococcus, Enterococcus,* and to a lesser degree *Saccharomyces.*

Because many of these probiotic microorganisms grow slowly in milk, for rapid acid development, production of desirable flavor characteristics and stability of the product, the majority of the commercially available probiotic fermented milks still rely on the use of “conventional or traditional” starter cultures during the manufacture of these products. The probiotic microorganisms are, however, primarily used as therapeutic adjunct.

In fact, a variety of dairy products may serve as vehicles of probiotic microflora. Fermented milks are, however, the most convenient and frequently used vehicles for the implantation of the probiotic microflora in humans.

2.3.1.2.2.3. African fermented milks

In Africa, milk is produced in most agricultural production systems. Mostly, it is either consumed or sold fresh. When there is a surplus, however, it is left to ferment for some time until it becomes sour and is consumed later on, or processed into other products like butter, ghee and cheese. Sour milk is the most common product in many rural areas. There are three major reasons why milk is allowed to sour in Africa, namely;

a) To convert it into a more stable product that can be stored for a relatively longer period (20 days) compared to the shorter time needed (less than one day) for fresh milk. Fermented milks and their products are more stable than fresh milk because they are more acidic and/or contain less moisture. Numerous strains of bacteria are capable of converting lactose to lactic acid. The acid developed lowers the pH and retards the growth of lipolytic and proteolytic bacteria and therefore protects the fat and protein in the milk.

b) To improve the flavor of the milk

c) To facilitate butter extraction and cheese making

Casein, the predominant protein in milk, is soluble at a neutral pH, but insoluble in acid. Thus when milk sours, casein precipitates and thickens the product. This makes it easier to separate the casein proteins in the form of cheese from the whey proteins. In addition, in smallholder butter making, the acid developed assists in the extraction of fat during churning.

Although poorly studied, a variety of fermented milks are known to occur in Africa. Table 3 shows the list of some examples of fermented milks as

named by the local languages of their respective countries. Many of these varieties of fermented milks are very much alike but may slightly differ from one another due to differences in fermentation conditions, containers (vessels), milk composition and flavoring agents. In most cases the origin of the raw material is the cow. But in some countries like Somalia and Sudan, milk from sheep, goat and camel may be used. Some variations are also noted in the technologies employed in different countries. The daily residual fresh milk from domestic consumption is, generally, poured into a container covered with a lid. The kinds of the containers (vessels) used may differ from country to country or even between localities within a given country. No starters are used in most cases and acidification develops after a few days, either from the natural flora of milk when it is not boiled, or from the bacteria growing on the sides of the vessels. Milk is left to settle in a quiet place, often in a covered container sheltered from dust for usually 24–48 h. Coagulation time varies a lot depending on room temperature, which for instance may vary from 10°C in the highlands of Ethiopia to 40°C in Sudan.

In Kenya, fresh milk is usually boiled before natural fermentation. Some of these products (e.g. *mursik* and *mariwa*) are sometimes colored and flavored with charcoal powder from a particular tree called Senetwet. In both Kenya, and Ethiopia, the containers used for fermenting milk (e.g. *iri imata*, *irgo*, *mariwa*, *mazia*, *maivu*) are previously smoked to avoid mould growth. In Ethiopa, additionally, Irgo is flavored with fresh leaves of rue (*Ruta chalepensis* var *tenuifolia*) (FAO Animal Production and Health Paper, 1990).

The Roab (Sudan) and Rouaba (Chad) are sour buttermilks, which are by-products of the butter making from sour milk. These products have an acid taste with a yeast-fermented aroma. During production, raw or boiled milk is left to mature for 24 hours at room temperature in a clean closed container. The fermentation process is often initiated with either the normal microflora of the milk or by addition of 2-3% previously fermented milk. More or less, similar buttermilks are also produced in Egypt, Ethiopia, Somalia and Niger

and named as *laban khad/laban hamid*, *arrera*, *garoor* and *non mai*, respectively (FAO Animal Production and Health Paper, 1990; Morcos, 1977).

In some African countries, fermented milks are produced in the form of partly drained sour milks or concentrated milks. These include, *chambiko* (Malawi), *Ititu* (Ethiopia), *mabobo* (Madagascar), *madila* (Botswana), *mafi* (Lesotho), *mashoronga* (Zimbabwe), *mame* (Tanzania), *umlaza/mutivil* (Zimbabwe), *Laban zeer* (Egypt) (FAO, 1990; Demerdash, 1960). These concentrated fermented milks are sour milks obtained by spontaneous acidification of raw milk and are subsequently partly drained. The products are white to greenish like whey. Their texture is usually curdy or granular, but some may be semi-fluid when the curd is shaken. Again, the preparations of some of these concentrated fermented milks may involve addition of certain plant materials or their products into the fermented milk and/or smoking of the fermentation vessels (FAO, 1990; Kassaye, 1991; Isono *et al*., 1994). In Zimbabwe, for example, coagulation of milk is made possible by using vegetable enzymes. In other countries, inorganic substances like salt may be added to give the fermented milk a good keeping quality (e.g. *laban zeer* in Egypt).

In South Africa, although there is very limited literature available, the well-known traditional fermented milks, such as '*amasi*' and '*maas*' have been already commercialized (Keller and Jordaan, 1990). The early technologies and traditions of fermenting milk are, however, well documented by Fox (1939). According to Loretan *et al*. (2003), although rarely used, kefir grains are also used by a few South African households to ferment milk.

2.3.1.2.3. Microorganisms associated with milk fermentation

Since milk is a highly nutritious food, it provides a favorable environment for the growth of microorganisms. Yeasts, moulds and a broad spectrum of

bacteria can grow in milk, particularly at temperatures above 16°C. These microbes gain access into milk via the cow, air, feedstuffs, milk handling equipment and the personnel milking the cow. Once they get into the milk their numbers increase rapidly.

The initial bacterial count of milk may range from less than 1000 cfu/ml to 10^6cfu/ml. High counts (more than 10^5cfu/ml) are evidence of poor production hygiene.

In all developing countries, due to the rather simple conditions under which milking takes place, it would be expected that milk produced in the households would have very high initial bacterial contamination. However, due to the minimal use of equipment and the practice of milking directly into the milk storage vessels, milk produced under traditional systems tends to have lower initial bacterial counts than milk produced under mechanized milking in temperate countries (IDF, 1968). But according to FAO (1990), detailed information is required on the bacteriological quality of milk under traditional milking practices before making any kind of generalization on the potential shelf life of traditional fermented milks under un-cooled storage as in the tropical environment.

2.3.1.2.3.1. Bacteria

A variety of bacterial species are known to grow in milk. Table 4 indicates some of the most commonly encountered bacterial species and their significance in milk.Some of these bacteria are beneficial while others are harmful. The latter are either spoilage or pathogenic bacteria. The former includes predominantly the lactic acid bacteria (LAB). These bacteria (LAB) are the major groups of bacteria that are responsible for souring milk by changing lactose into lactic acid.

2.3.1.2.3.1.1. Lactic acid bacteria

The lactic acid bacteria, frequently termed as the “Lactics”, are more appropriately defined as gram-positive, non-sporulating, catalase negative, devoid of cytochromes, nonaerobic but aerotolerant, fastidious, acid-tolerant and strictly fermentative bacteria that produce lactic acid as a major or sole product from sugar fermentation (Axelsson, 1993). Since the group is formed only based on some morphological, metabolic and physiological similarities, the afore-mentioned definition may not hold true for all members of lactic acid bacteria. However, it is still useful for practical purposes and in reality many of the descriptions of genera or species center on this definition (Axelsson, 1993).

i) General characteristics

Lactic acid bacteria are primarily characterized by their ability to produce different optical isomers of lactic acid from the fermentation of glucose. Some produce the L (+) isomer while others produce the D(-) and /or DL (a mixture of L and D) isomers.

They are broadly defined as Gram-positive, non-sporulating, aerobic to facultatively anaerobic cocci or rod shaped bacteria which are oxidase, catalase, benzidine, and gelatinase negative, lacking cytochromes and incapable of utilizing lactates and of reducing nitrates to nitrites (Kandler, 1983; Andersson, 1988; Stackebrandt and Teuber, 1988, Madigan *et al*., 1997). However, except for the first two characteristics (i.e. Gram positive and non-sporulating) LAB may show certain variations with respect to the other characteristics. For example, some LAB may show catalase activity that is mediated by a non-heme pseudocatalase (Whittenbury, 1964; Kono and Fridovich, 1983). Due to this anomaly, Ingram (1975) suggested that the lack of cytochromes might be a more reliable characteristic in preliminary diagnosing than the commonly used catalase test. Nevertheless, there are still a number of reports showing that a true catalase and even cytochromes may be formed by some LAB, in some

cases resulting in respiration with a functional electron transport system (Whittenbury, 1964, 1978; Bryan-Jones and Whittenbury, 1969; Ritchey and Seeley, 1976; Wolf *et al*., 1991). Regardless of all these differences, all lactic acid bacteria grow anaerobically. But, unlike many anaerobes, most LAB are aerotolerant (Madigan *et al*., 1997).

These organisms are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities. Most species have multiple requirements for amino acids and vitamins. Because of this, lactic acid bacteria are generally abundant only in habitats where these requirements can be sufficiently provided. Habitats such as the animal oral cavities and intestines, plant leaves, cereals, vegetables, meat, dairy products and decaying plant or animal matter (e.g. rotting vegetables, fecal matter, compost, etc.) are thus rich sources of LAB (Axelsson, 1993; Hammes and Vogel, 1995; Stiles and Holzapfel, 1997).

Many of the lactic acid bacteria are beneficial because of their contributions to flavor, aroma, and increased shelf life of fermented products (Nes *et al*., 1996). Various members, for instance, are used commercially as starter cultures in the manufacture of food products, including dairy products (Salama *et al*.**,** 1995), fermented vegetables (Leisner *et al*., 1996), fermented doughs (Vogel *et al*., 1994), alcoholic beverages (Patarata *et al*., 1994; Pattison *et al*., 1998), probiotics in animal feeds (Castellanos *et al*., 1996), and meat products (Vogel *et al*., 1993). Because they have a long history of safe use, they are also thought to have probiotic properties in human beings (Salminen *et al*., 1998). Lactic acid bacteria have also been used for lactic acid fermentation of cereals used as infant-weaning foods (Lorri and Svanberg, 1994; Motarjemi *et al*., 1996; Olsen *et al*., 1995; Rombouts *et al*., 1995). However, there are also a number of nuisance members causing spoilage in food products (Jørgensen *et al*., 2000). For example *Lactobacillus curvatus* and *L. sake* have been reported as the major spoilage agents associated with vacuum packaged raw beef (Yang and Ray, 1994). Some LAB are even known to be pathogenic to man

(Aguirre and Collins, 1993; Gasser, 1994) and other animals (Collins *et al*., 1987), and still others have been implicated in diseases (Kandler and Weiss, 1986).

ii) Classification of Lactic Acid Bacteria

The early definition of lactic acid bacteria included the coliform bacteria (Stiles and Holzapfel, 1997). The definition was based on the ability of the bacteria to ferment and coagulate milk. But later on, when the genus *Lactobacillus* was described as consisting of gram-positive bacteria by Beijerinck in 1901, the coliforms were separated from the group "Lactic Acid Bacteria" (Axelsson, 1993). Orla-Jenssen in 1919 then divided the LAB into seven genera, namely *Betabacterium, Thermobacterium, Streptococcus, Betacoccus, Microbacterium,* and *Tetracoccus* based on some morphological and physiological characteristics i.e. shape, catalase reaction, nitrite reduction and end product of fermentation. He considered the LAB as a natural group consisting of bacteria that are gram positive, non-motile, non-spore-forming, rod- and coccus-shaped organisms that ferment carbohydrates and higher alcohols to form mainly lactic acid. Later, this classical approach of bacterial taxonomy was further expanded to include cell wall composition, cellular fatty acids, isoprenoid quinines and other characteristics. With the advent of molecular techniques, studies of characteristics like mol% G+C content of DNA, electrophoretic properties of gene products, DNA-DNA hybridization, and structure and sequence of rRNA revealed the need for reclassification of LAB and subsequently the earlier genera were revised (Schleifer, 1987). Thus, phylogenetically, LAB were redefined as bacteria that belong to a group of Gram-positive, low G+C containing, non-motile, non-spore forming, aerotolerant organisms that ferment hexoses to lactic acid. However, in view of the heterogeneity of LAB with respect to many other cultural characteristics, both the definition and the classification are still far from being precise (Vendamme *et al*., 1996).

Currently the group “LAB” generally consists of about 12 genera of food interest, *viz*. *Aerococcus* (*A.*), *Carnobacterium* (*C.*), *Enterococcus* (*E.*), *Lactococcus* (*Lc.*), *Lactobacillus* (*Lb.*), *Leuconostoc* (*Leuc.*), *Oenococcus* (*O.*), *Pediococcus* (*P.*), *Streptococcus* (*S.*), *Tetragenococcus* (*T.*), *Weissella* (*W.*) and *Vagococcus* (*V.*) (Bottazzi, 1988; Stiles and Holzapfel, 1997; Axelsson, 1998). Most of these genera have been defined on the basis of cell morphology, DNA base composition, and type of fermentative metabolism. Some genera like *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* consist of members with fairly similar DNA compositions showing little variation from strain to strain while the heterogeneous *Lactobacillus* comprises members with widely diverse DNA compositions (Madigan *et al*., 1997).

Of the twelve genera listed above, five of them are of particular importance in fermented milks. These genera and some of their respective representative species are shown in Table 5.

iii) Metabolism in LAB

A relatively simple sugar fermentation pathway that, by definition, results in the formation of lactic acid characterizes lactic acid bacteria. However, they are also well recognized by their capacity to convert a great number of substrates in industrial food fermentations. The end products of these conversions provide crucial protection against spoilage, contribute to the production of the desired flavors, and add to the texture of the final products. Despite the vast amount of organic molecules that collectively can be metabolized by lactic acid bacteria, most individual species or strains have the capacity to use only a limited number of substrates as sole energy source (London, 1990). Most commonly they obtain their energy from sugars and related fermentable compounds. However, despite their ability to extract energy from a variety of fermentable compounds, lactic acid bacteria usually have very limited biosynthetic ability. Consequently, they

have a complex nutritional requirement including the need for amino acids, vitamins, purines, and pyrimidines (Madigan *et al*., 1997; Axelsson, 1993).

Often, the lactic acid bacteria are grouped as homofermenters and heterofermenters based on the end product of hexose fermentation. The homofermenters possess the enzyme aldolase, one of the key enzymes in glycolysis and ferment hexoses more directly to lactic acid than the heterofermenters. The heterofermenters on the other hand, since they lack the enzyme aldolase, cannot split fructose bisphosphate to triose phosphate. Instead, they convert the hexose to pentose (i.e. they oxidize glucose 6-phosphate to 6-phosphogluconate and then decarboxylate it to pentose phosphate), which is in turn broken down to triose phosphate and acetylphosphate using the enzyme phosphoketolase. The triose phosphate is ultimately changed to lactic acid with a net gain of 1 mol of ATP, while the acetylphosphate is converted to ethanol by accepting electrons from NADH generated in the process. Thus, the heterofermenters produce only one mole of ATP unlike the homofermenters that produce 2 moles of ATP per mol of glucose consumed. Because of this metabolic difference, the heterofermenters often produce half as much cell mass as the homofermenters. In addition, the heterofermenters produce a number of products besides lactic acid, including carbon dioxide, acetic acid, and ethanol (Wood and Holzapfel, 1995). Often, when they convert hexoses to pentoses, they also produce aldehyde and diacetyl, which are highly desirable aromatic and flavor enhancing compounds in dairy products (Jay, 1986; Sharpe and Fryer, 1965; Frosbisher *et al*. 1974).

In general, the simplest and most direct way for lactic acid bacteria to generate metabolic energy is substrate level phosphorylation. The two substrate level phosphorylation reactions, involving phosphoglycerate kinase and pyruvate kinase, which operate in the glycolytic pathway, are well known and found in all lactic acid bacteria. Moreover, the acetate kinase pathway, generating ATP and acetate from acetyl-phosphate, is also widely distributed (Poolman, 1993; Konings *et al*., 1995).

Apart from this, many lactic acid bacteria are capable of co-metabolizing citrate in the presence of another carbon source, such as glucose or lactose (Kennes *et al*., 1991; Starrenburg and Hugenholtz, 1991; Salou *et al*., 1994; Garcia-Quintáns *et al*., 1998; Palles *et al*., 1998; Goupry *et al*., 2000). Citrate metabolism plays an important role in many food fermentations involving lactic acid bacteria, since it occurs in many natural substrates, such as milk, vegetables, and fruits. Its degradation results in the formation of metabolic end products such as acetate, diacetyl, acetaldehyde, and acetoin, which have very distinct aroma properties and significantly influence the quality of fermented foods (Hugenholtz, 1993).

iv) Importance of LAB

Lactic acid bacteria are industrially important in the development of starter cultures that can be used in the manufacture of different dairy products (e.g. yoghurt, acidophilus milk, cottage cheeses, soft cheeses and hard cheeses) (Jay, 1986) and in the processing of meats, alcoholic beverages and fermented vegetables (Jay, 1986; Kandler and Weiss, 1986). Milk alone, for instance, can be fermented to more than 1000 products using different starter cultures (Mäyrä-Mäkinen and Bigret, 1993).

Currently, consumers are constantly demanding reliable supplies of high quality foods. To meet these demands, biotechnologists are routinely producing high-performing, reliable strains of specially selected lactic acid bacteria that can be used as starter cultures for making different fermented-milk, -meat, -fish, -vegetable and -cereal products. In many developed countries, computer-controlled fermentations are commonly used to produce the lactic acid bacteria with the most desirable and consistent properties. This ensures that every batch of fermented product is just as good as the last one because not only has there been optimized fermentation conditions but also because standard starter cultures have been in use.

Not all fermentations by lactic acid bacteria are, however, useful in food production and processing. Some microbial fermentation processes cause food spoilage by producing off flavors (Kandler & Weiss, 1986; Kandler *et al*., 1983; Cai *et al*., 1998) or food poisoning by producing harmful toxins. Indeed control of spoilage of foods in many societies has been achieved by deliberate fermentation of the foods by addition of particular microorganisms such as lactic acid bacteria to prevent spoilage of the food by others.

Lactic acid bacteria have this major potential for use in biopreservation because they are safe to consume and during storage they naturally dominate the microflora of many foods. Their growth lowers both the carbohydrate content of the foods that they ferment and the pH due to lactic acid production. It is this acidification process which is one of the most desirable side effects of their growth. The pH may decline to as low as 4.0, low enough to inhibit the growth of most spoilage microorganisms, thus allowing these foods prolonged shelf life (Cooke *et al*., 1987). The fermentation (and growth of the bacteria) is, however, self-limiting due to the sensitivity of lactic acid bacteria to such acidic pH values. Even in raw meats and fish that are chill stored under vacuum or in an environment with elevated carbon dioxide concentration, for instance, the lactic acid bacteria become the dominant population and preserve the meat with a ‘hidden' fermentation. The same applies to processed meats provided that the lactic acid bacteria survive the heat treatment or they are inoculated onto the product after heat treatment (Stiles, 1996). Furthermore, some LAB produce nisin, a peptide metabolite that is known to have antimicrobial properties against many spoilage and pathogenic bacteria (Ray and Daeschel, 1992).

The antimicrobial effects of fermentation are not confined to spoilage organisms alone and can also affect pathogens that might be present in the food. Thus, traditional food fermentations can take potentially hazardous raw materials, such as raw meat and milk, and transform them into products

with both improved keeping qualities and a reduced risk of causing illness. The lactic acid bacteria promote health through one or more of the following ways:

i. Production of organic acids mainly lactic acid and small amounts of acetic and formic acids. These organic acids cause a decline in pH and thereby growth inhibition of food spoiling or poisoning bacteria, killing of certain pathogens, detoxification by degradation of noxious compounds of plant origin (usually in combination with plant-derived enzymatic activities) (Caplice and Fitzgerald, 1999; Sinha, 1986; Ray and Daeschel, 1992).

ii. Production of antimicrobial compounds such as bacteriocins, H_2O_2, reuterin (Juven *et al*., 1992; Weber, 1994) diacetyl and CO_2 (Weber, 1994).

It should be remembered that the antimicrobial effects of fermentation are not confined to spoilage organisms alone and can also affect pathogens that might be present in the food. Thus, traditional food fermentations can take potentially hazardous raw materials, such as raw meat, cereal-based foods and milk, and transform them into products with both improved keeping qualities and a reduced risk of causing illness. However, such an activity in fermentation is largely directed at bacterial pathogens. Studies using food borne viruses or their surrogates have suggested that food-borne viral hazard are largely unaffected by the pH and acidity levels occurring in fermented foods (Hermann and Cliver, 1973 ; Dethmers, 1975; Kantor and Potter, 1975; Nout *et al*., 1989 and Wood & Adams, 1992) and there is limited information on the prevalence and survival of parasites in fermented foods (Warnekulasuriya *et al*., 1998; Perez *et al*., 2001).

iii. Probiotic effects as live organisms in food.

Some of the probiotic lactic acid bacteria have great potential in the preparation of functional foods that can contribute to health promotion.

Their potential health benefits include protection against enteric infections including diarrhoeagenic bacteria (Mensah *et al*., 1991; Svanberg *et al*., 1992), use as an oral adjuvant, immunopotentiator in malnutrition (Saavedra, 2001), and prevention of chemically induced tumors (Liu *et al*., 2002). Some of these were evidenced by the observation that *Lactobacillus casei* could prevent enteric infections and stimulate secretory IgA in malnourished animals and that yogurt could inhibit the growth of intestinal carcinoma through increased activity of IgA, T cells, and macrophages (Perdigon *et al*., 1995). Their protective role may also be the result of their ability to compete with potentially harmful microorganisms (Kandler and Weiss, 1986; Daeschel, 1989; Fernandez *et al*., 1987).

Additional health benefits of lactic acid bacteria also include alleviation of problems of lactose intolerance in humans through enhanced lactose digestion (Gilliland, 1991) by assisting to increase the peristaltic movement of parts of the digestive system (Yokokura, 1977), prevention and/or treatment of rotavirus and antibiotic induced diarrhoea (Saavedra, 2001), reduction in serum cholesterol, and reduction in hypertension. Some researchers have also reported that lactic acid bacteria and their cell wall components show anticancer activity (Bogdanov *et al*., 1975; Ebina *et al*., 1995), antimutagenic activity (Bodana and Rao, 1990), anti-infectious effect (Perdigon *et al*., 1986; Perdigon *et al*., 1987; Popova *et al*., 1993) and the ability to induce cytokine production (Pereyra *et al*., 1991; Davidkova *et al*., 1992; Guencheva *et al*., 1992; Popova *et al*., 1993; De Simone *et al*., 1993). The immuno-stimulatory effect of yogurt has been proposed and investigated by using animal models (Gill *et al*., 2000; Shu *et al*., 2000; Meydani and Ha, 2000) and humans (Arunachalam *et al*., 2000). Recent work by Kitazawa *et al*. (2003) has also demonstrated that DNA from *L. bulgaricus* NIAI B6 induces a significant proliferation of peyer's patch and splenic B cells.

In milk, brined vegetables, many cereal products and meats with added carbohydrates, the growth of lactic acid bacteria produces also a new food product. The acidity caused by LAB also changes the texture of the foods due to precipitation of some proteins, and the biochemical conversions involved in growth enhance the flavor and thereby improving the palatability of the food. Flavor compounds particularly are widely used in the food industry. These products can be obtained either by chemical synthesis and therefore are called synthetic or by fermentation thus called natural flavors. Currently there is a tendency towards the use of natural products by the consumer, therefore there is an increasing industrial interest in flavor compounds produced by LAB. One of these products is diacetyl, an important component in the flavor of butter, although other flavour compounds like acetoin, acetaldehyde and 2,3-butanediol play a role in the flavor, they are present in trace amounts. The mixture of all these compounds produced during the lactic acid fermentation in suitable proportions is what gives butter its characteristic flavor (Escamilla-Hurtado *et al*., 1996).

LAB not only improve the palatability but also the digestibility and the nutritional value of certain foods by increasing nutrient availability, modifying or reducing anti-nutritional food components like tannins, glucosides, polyphenols, phytates, cellulose, hemicellulose, glucuronic and polygalacturonic acids (Kalantzopoulos, 1997; Steinkraus, 1994) and by adding more protein, amino acids, and vitamins to the food product (Hesseltine, 1979). For example, fermented grain foods generally have a protein content of 8-20%, as opposed to 1-2% protein in an unfermented grain dish. These fermented foods also have enhanced values of thiamine, riboflavin, niacin, isoleucine/leucine, histidine, arginine, valine, tryptophan, lysine, tyrosine, cystine, and methionine (Hesseltine, 1979). Similarly, literatures show that fermented milks consist of more vitamins (Kneifel *et al*., 1992), peptides

and free amino acids such as valine, histidine, serine and proline than raw milk (Tamime and Deeth, 1980; Vaitheeswaran and Bhat, 1988).

2.3.1.2.3.2. Yeasts

Based on the vegetative growth characteristics and on the nature of the spores, if formed, all organisms belonging to the group fungi are divided into four classes: Phycomycetes (Zygomycetes), Ascomycetes, Basidiomycetes, and Deuteromycetes (Fungi Imperfectii). Most yeasts in nature belong to the subdivision Ascomycetes (sporogenous) and Fungi Imperfectii (asporogenous). A few belong to the Basidiomycetes. Under certain cultural conditions, Phycomycetes could also show the yeast morphology. However, since their normal existence is in the filamentous form, they are not regarded as yeasts.

Yeasts are generally unicellular fungi that reproduce sexually and/or asexually by budding or fission. These organisms are widely distributed in nature being present on plant surfaces, in the air, the soil and in the intestinal tract of animals. Like bacteria, yeasts can have beneficial and non-beneficial effects in foods.

Although there is a large diversity of yeasts and yeast-like fungi, (approximately 700 species according to Wyder, 2001), only a few are commonly associated with the production of fermented foods. They are all either ascomycetous yeasts or members of the genus *Candida*. Of these, the most beneficial yeasts in terms of desirable food fermentation are from the Family *Saccharomycetaceae*, especially the Genus *Saccharomyces* (Walker, 1988).

Their importance in the food industry is mainly in their ability to produce enzymes that favor desirable chemical reactions such as the leavening of bread and the production of alcohol and invert sugar. In addition, some yeasts are chromogenic and produce a variety of pigments, including green,

yellow and black. Others are capable of synthesizing essential B group vitamins and thus contribute to the improvement of the nutritional value of foods. Apart from these beneficial attributes, many yeasts can be a nuisance and may cause spoilage in foods. Table 6 shows some examples of undesired yeasts in foods.

Although the yeasts have been reported to occur in large numbers in cheese (Addis *et al*. 2001), their populations in fermented milks are generally low (ca < 10^4 CFU/ml) compared with bacteria. According to Roostita and Fleet (1996), possible explanations for this could be the competitive utilization of substrates by bacteria and the presence of antagonistic metabolites produced by bacteria.

Since only limited information is available on the interaction between LAB and yeasts in fermented milk, a detailed study is needed in this respect before any kind of conclusion can be made.

i) Characteristics of yeasts related to the food environment

Most yeasts require a supply of sugar and an abundance of oxygen for growth. Although, some yeasts can ferment sugars to alcohol and carbon dioxide in the absence of air, they require oxygen for growth. Yeasts are active in a very broad temperature range - from 0 to 50^0C, with an optimum temperature range of 20^0C to 30^0C.

Even though the optimum pH for most microorganisms is near the neutral point (pH 7.0), yeasts are usually acid tolerant and are therefore associated with the spoilage of acidic foods. They can generally grow in a pH range of 4 to 4.5 (Mountney and Gould, 1988).

They are fairly tolerant of high concentrations of sugar and grow well in solutions containing 40% sugar. At higher concentrations, only a certain group of yeasts, the osmophilic ones can survive. There are only a few

yeasts that can tolerate sugar concentrations of 65-70% and they grow very slowly under such conditions (Board, 1983). Some yeasts, for example members of *Debaryomyces,* can tolerate high salt concentrations up to 25%. A particular species that can tolerate high salt concentrations and low water activity is *Zygosaccharomyces rouxii.* This species is often associated with fermentations in which salting is an integral part of the process.

In terms of water requirements, yeasts are intermediate between bacteria and moulds. Bacteria have the highest demands for water, while moulds have the least need. Normal yeasts require a minimum water activity of 0.85 or a relative humidity of 88%.

ii) Metabolism in yeasts

Although all yeasts are known to derive their chemical energy (ATP) from the breakdown of organic compounds, there is metabolic diversity in the types of the substrates used and in how these organisms generate and consume energy from these substrates.

Most yeasts employ sugars as their main carbon and energy source. But there are particular yeasts, which can utilize non-conventional carbon sources. Crude gas oil or preferentially purified n-paraffin fractions with chain lengths of C_{10} – C_{20} may, for instance, be used as substrates by many yeast species (Champgnat *et al.,* 1963). *Candida lipolytica*, *C. tropicalis*, and some species of *Rhodotorula* and *Trichosporon* are examples of such hydrocarbon-assimilating yeasts. Amongst the Ascomycetes, such an ability is recorded in *Debaryomyces*, *Endomyces*, *Metschnikowia*, and *Pichia* (Reed and Nadgodawithana, 1991).

The major source for energy production in the yeast is, however, glucose and glycolysis is the general pathway for conversion of this substrate to pyruvate, whereby production of energy in the form of ATP is coupled to the

generation of intermediates and reducing power in form of NADH for biosynthetic pathways.

Metabolically, yeasts are predominantly facultative aerobes, capable of growing either in the absence of air (fermentative) or in its presence (oxidative). Thus, in the presence of aeration, yeasts have the ability to shift its energy-yielding metabolism from fermentative to oxidative. In the presence of oxygen and absence of repression, pyruvate enters the mitochondrial matrix where it is oxidatively decarboxylated to acetyl CoA by the pyruvate dehydrogenase multi enzyme complex. This reaction links glycolysis to the citric acid cycle, in which the acetyl CoA is completely oxidized to give two molecules of CO_2 and reductive equivalents in the form of NADH and $FADH_2$. However, the citric acid cycle is an amphibolic pathway since it combines both catabolic and anabolic functions. The latter results, for example, from the production of intermediates for the synthesis of amino acids and nucleotides. Replenishment of compounds necessary to drive the citric acid cycle, such as oxaloacetate and α-ketoglutarate, are done through the fixation of CO_2 to pyruvate by the actions of the enzymes pyruvate carboxylase (ATP-dependent) and phosphoenolpyruvate carboxykinase and the glyoxalate cycle (a shortcut across the citric acid cycle), which is important when yeasts are grown on two-carbon sources, such as acetate or ethanol.

Under anaerobic conditions, yeasts are known to perform predominantly the alcoholic fermentation, producing two moles each of ethanol and CO_2 from each mole of glucose. Because this pathway is so inefficient, yeast multiplication under such conditions is minimal.

Yeasts, like LAB, utilize also the hexose phosphate pathway (the pentose phosphate cycle) as an alternative mode of glucose oxidation. This pathway provides the cell with pentose sugars and cytosolic NADPH, necessary for

biosynthetic reactions, such as the production of fatty acids, amino acids and sugar alcohols.

With regard to nitrogen metabolism, most yeasts are capable of assimilating a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell, such as amino acids (and consequently peptides and proteins), polyamines, nucleic acids and vitamins. By and large, ammonium ions, either supplied as nutrient or derived from the catabolism of other nitrogenous compounds, can be directly assimilated into a couple of amino acids, notably glutamate and glutamine, which can then serve as donors of the amino group in other amino acids

Many yeasts have also different kinds of proteases that have different specificity for proteins and polypeptides. The ones used in catabolism tend to be non-specific and attack many different peptide bonds between amino acids. The small peptides produced can then be transported into the cell where they are further degraded into amino acids. Several of the amino acids are structurally so similar to important intermediates in the TCA cycle and other major metabolic pathways that it has become simpler for cells to convert them into "central metabolites". In most cases this involves removal of the amino group (deamination), for example, glutamate, aspartate and alanine can be deaminated to produce 2-oxoglutarate, oxaloacetate, and pyruvate, respectively, which are important central metabolites for many biosynthetic processes. Some yeast species have also the capability to degrade lipids by lipase activity.

iii) Contribution of yeasts in dairy products

Yeasts in cheese are considered as insignificant at the earlier stages of cheese production, but are an important component of the microflora of many, if not all, cheese varieties in later stages (Welthagen and Viljoen, 1999), being present as natural contaminants in the curd during maturation.

a) Enhancement of microbial growth

Some yeasts are capable of enhancing the growth of other organisms when grown in co-cultures (Jakobsen and Narvhus, 1996; Rossi *et al*., 1997).

Enhancement of the growth of *Y. lipolytica* and *K. marxianus* by numerous (but not all) strains of *D. hansenii* and that of *Y. lipolytica* and *K. marxianus* by *Sac. cerevisiae* has been observed on "spot-on-lawn" plates (Addis *et al.* 2001). According to these authors, both yeast-yeast and yeast-bacteria interactions are strain specific. In their studies they observed that two of the strains of *D. hansenii* enhanced the growth of isolates of *S. xylosus* while four of the other strains enhanced the growth of *E. faecium* and *E. faecalis*. The report further showed that only one strain of *Y. lipolytica* enhanced the growth of *S. xylosus* whereas the growth of *Enterococcus* spp. was enhanced by strains of *Y. lipolytica*, *K. marxianus* and *Sac. cerevisiae*

b) Inhibition of growth (antagonistic activity)

In dairy products, some yeasts have been known also to inhibit both the desirable (e.g. starter microorganisms) and the undesirable microorganisms (Jakobsen and Narvhus, 1996; Rossi *et al*., 1997). The reports of Addis *et al*. (2001) also indicate that some strains of *Y. lipolytica* show weak inhibition against *L. monocytogenes* and *B. cereus* (Addis *et al*., 2001).

c) Improvement of flavor and texture

Yeasts play a key role in improving the flavor and texture of dairy products. In some cheese types, for instance, they make a positive contribution to the development of flavour and texture during the stage of maturation. Their main contribution to the maturation process is their ability to utilize lactic acid, which in turn may lead to an increase in pH, favouring bacterial growth and initiating the second stage of cheese ripening (Rhom *et al*., 1992).

According to Dumont and Adda (1979), Fox *et al*., (1995), McSweeney and Sousa (2000) and Smit *et al*., (2002) flavor compounds are produced from three major milk constituents: lactose, lipids and proteins. The main mechanisms by which yeast growth influences the flavor and texture and thereby the final quality of cheese are, thus, fermentation of lactose, utilization of lactic acid, lipolytic and proteolytic activities. Over-ripening during maturation could, however lead to spoilage. Despite this drawback, the use of yeasts in the dairy industry could determine potential advantages including production of flavour components and acceleration of ripening by means of its lipolytic and proteolytic properties, fermentation of lactose, assimilation of lactate and positive interactions with the primary starter cultures.

d) Spoilage

Yeasts may also act as spoilage organisms causing typical defects such as fruity, bitter or yeasty off-flavours, loss of texture quality, discoloration changes, excessive gas formation and increased acidity (Walker, 1988; Fleet, 1990).

As indicated above, some yeasts may have a negative role by causing over-ripening of the product during maturation. Over-ripening is in most cases interpreted as a sign of spoilage. It is now well established that continued lactose fermentation could lead to increased acidity, gassiness and fruity flavours, while continued hydrolysis of protein and fat could contribute to bitter and rancid flavours as well as a softening of product texture.

e) Improvement of the nutritional value of the products

Yeasts are excellent sources of lysine, riboflavin, niacin, and thiamin, and other amino acids and vitamins. The release of these substances by autolysis or other mechanisms greatly enhances the nutritional properties of the fermented food product.

2.3.1.2.4. Interaction between microflora

In a complex microbial ecology there exists a complex array of interactive associations between microorganisms (Boddy and Wimpenny, 1992). These interactions may be classified on the basis of effects, as direct or indirect interactions (Viljoen, 2001). Indirect interaction refers to competition, commensalism, mutualism, ammensalism or neutralism (Linton and Drozd, 1982), and direct interaction to predation and parasitism (Fredrickson, 1977; Bull and Slater, 1982). In naturally fermented milks, neither predation nor parasitism is known to occur at least between the major groups of microorganisms (LAB, yeast and molds) that contribute to the quality of the product. Thus, in this manuscript, emphasis is given much to the indirect type of interaction occurring especially between and within LAB and yeasts.

2.3.1.2.4.1. Bacterial interaction (Interaction between LAB)

LAB interact with one another in antagonistic or beneficial ways. The beneficial interaction may be done in the form of commensalism or mutualistic interdependence. A good example of the later form of interaction is seen between *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salvarius* subsp. *thermophilus* in yoghurt. These two organisms live and grow in the afore-mentioned product symbiotically and produce the characterstic flavor and texture. The interaction is evidenced by the fact that the rate of acid production is always greater when the two species are grown together rather than when grown separately as monocultures (Dellaglio, 1988). Similarly, a mutualistic co-existence was also observed in a mixed culture of *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis* (Dahiya and Speck, 1963). In commensalism, however, the growth of only certain strains of bacteria is stimulated as a result of the production of metabolites by co-existing microorganisms. Such a stimulation can occur when there is a release of amino acids in the medium as a result of the proteolytic activity of the partner microorganism.

Ample evidences are also available for the antagonistic interactions occurring between LAB. Mesophilic lactococci and leuconostocs are known to produce a wide variety of inhibitory substances which can act against spoilage microorganisms as well as against other strains in mixed-strain starters (Hugenholtz, 1986). By and large this kind of interaction was mostly shown between bacteriocin-producing LAB and the susceptible LAB of their own close relatives. According to Lbudzisz, as cited by Oberman and Libudzisz (1998) the ability to induce an antagonistic effect in relation to other mesophilic LAB is frequently observed among *Lactococcus lactis* ssp. *lactis* var. *diacetylactis* strains. About 50% of these strains were found to inhibit the growth of other lactococci and leuconostoc strains. The production of antagonistic substances is not, however, limited to only closely related species. The recently identified proteinaceous active substance produced by *Lactobacillus paracasei* subsp. *paracasei* strain M3 has been shown to display a strong activity against several *L. delbrueckii* species, *Helicobacter pylori* NCIPD 230 and *Bacillus subtilis* ATCC 6633 (Atanassova *et al*., 2003). Similarly, nisin has been reported to have a wide spectrum activity against a number of spoilage and pathogenic bacteria.

2.3.1.2.4.2. Yeast-Lactic Acid Bacteria Interaction

Many types of yeasts are known for their symbiotic associations with LAB and influence the characteristics of many kinds of fermented foods including bread leavens, some fermented milk (e.g. kefir, koumiss etc.), ginger ‘beer’, kvass, and some un-hopped ales ((Boraam *et al*., 1993, Wood and Hodge, 1985). These associations are in most cases highly stable to the extent that they are able to resist contamination from other organisms.

In co-culture model systems, in which microorganisms grow in separate compartments but metabolites can diffuse from one compartment to the other, it has been shown that *Lactobacillus sanfranciscensis*, a key sourdough lactic acid bacterium, did not grow in the absence of either

isoleucine or valine. In co-culture, however, with *S. cerevisiae* or *S. exiguus* the dominant yeasts in sourdough, the strain reached a final cell number similar to that obtained when all the amino acids were present in the medium (Gobbetti *et al*., 1994). The stimulation of bacterial growth was related to the excretion of valine and isoleucine by the yeasts either during growth or as a consequence of accelerated autolysis, resulting from the high level of those amino acids found in the bacterial compartment of the co-culture.

Studies on the interactions between lactic acid bacteria and yeasts in grape must also have shown a stimulation of the bacterial growth by vitamins, amino acids and peptides released by yeasts as a consequence of an accelerated autolysis caused by bacterial enzymes (Lonvaud-Funel *et al*., 1988). Moreover, some authors have even identified a growth stimulant factor for *L. sanfranciscensis* in a small penta-peptide contained in freshly prepared yeast extractives (Berg *et al*., 1981).

In the preparation of beverages from cereal grains, if the conditions are acidic and anaerobic, the yeasts act on maltose to produce alcohol and CO_2. The carbon dioxide produced by yeasts maintains an anaerobic condition, which is conducive for the growth of LAB in the sealed container. Whereas the acidic conditions of the beer which encourage the growth of yeasts are produced by the fermentative action of lactic acid bacteria, primarily *Lactobacillus, Leuconstoc, Streptococcus,* and *Pediococcus* (Hesseltine, 1979).

Among the fermented milks, there are a number of traditional products that are known to involve yeast-LAB interaction. Table 7 shows the list of some of these fermented milks with the major species of LAB and yeasts needed for their production.

In naturally fermented milk, Gadaga *et al.* (2001) reported enhanced production of acetaldehyde, ethanol and CO_2 when *Candida kefyr 23* was grown in co-culture with *Lactococcus lactis* subsp. *lactis* biovar. d*iacetylactis* C1 and *Lactococcus lactis* subsp. *lactis* Lc261.

Antagonistic interactions are also known to exist between lactic acid bacteria and yeasts. According to Atanassova *et al*. (2003) the substance produced by the starter culture of the Bulgarian yellow cheese, *Lactobacillus paracasei* subsp. *paracasei* strain M3, has also a fungistatic activity against *Candida albicans*, *C. pseudointermedia* NBIMCC 1532, *C. blankii* NBIMCC 85 and *Saccharomyces cerevisiae* NBIMCC 1812.

2.3.1.2.4.3. Interaction between yeasts (Yeast- yeast interaction)

A typical example of yeast-yeast interaction is shown in the Swedish yeast process described by Jarl (1969) where starch is shown to be converted to yeast protein by growing *C. utilis* in symbiosis with the amylolytic yeast *Endomycopsis fibuligera* (Lindner) Dekker. *Candida humicola* increases the yield of L-serine (Yamada *et al*. , 1971) while *Hansenula anomala* accumulates L- tryptophan (Terui and Niizu, 1969).

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Table 1: Composition of cow's milk before and after fermentation

Main constituent	Whole milk, Raw[1] (%)	Fermented milk[2] (%)
Water	85.5 – 89.5	v
Total solids	10.5 – 14.5	~14-18
Fat	2.5 – 6.0	0.1-10
Proteins	2.9 – 5.0	4-6
Lactose	3.6 – 5.5	2-3
Lactic acid	0.14 – 0.16	0.6 – 1.3
Minerals	0.6 – 0.9	~ 0.6 - 0.9

[1] Source: O'Mahony (1988) [2] Source: Oberman (1985)

v = variable depending on the extent of whey removal

Table 2: Proposed scheme of classification of fermented milks by Roginski (1988) with slight modifications shown in shaded cells.

Type	Example	Country of origin
1. Fermented milks		
1.1. Thermophilic bacteria, incubation temperature 30/35-40/45ºC		
1.1.1. Lactic acid fermentation, without producing appreciable amounts of gas and alcohol	Yoghurt, diluted yoghurt, eyran, doogh, dehydrated yoghurt, kashk, kaskg, jub-jub, concentrated yoghurt, labneh, lebneh, tan, than, torba, kurut, laben raid, zabaday, zabady, roba, rob, matzoon, madzoon, dahi, tiaourti, tarho, tulum	Armenia, Bulgaria, Egypt, Greece, Hungary, India, Iran, Iraq, Lebanon, Saudi Arabia, Sudan, Turkey,
1.1.2. Acid fermentation, without producing appreciable amounts of gas and alcohol, using mainly human intestinal bacteria.		
1.1.2.1. Single strain	Acidophilus milk, bifidobacteria milk (formerly bifidus milk), yakult	Germany, Japan, USA*
1.1.2.2. Mixed strain	BAT type (*Bifidobacterium* sp., *Lactobacillus acidophilus, Streptococcus thermophilus*), BAP type (*Bifidobacterium* sp., *L. acidophilus, Pedicoccus* sp.)	Germany, Japan, USA*
1.2. Mesophilic bacteria, incubation temperature 10/15-20/30ºC.		
1.2.1. Lactic acid fermentation with simultaneous production of slime.	Scandinavian fermented milks (Karnmjolk, filmjolk, viili, tettemelk, kjernemelk, tykmaelk	Sweden, Finland, Norway, Denmark
1.2.2. Lactic acid fermentation using butter cultures.	Fermented milks prepared with butter cultures, artificial buttermilks (cultured buttermilk and similar products).	Czechoslovakia, Germany, Netherlands, USA, USSR*

Contd.

1.2.3. Concentrated fermented milks.		
1.2.3.1. Traditional home-made milks	Kellermilch, Lagermilch	German-speaking areas of Europe
1.2.3.2. Commercial products	Skyr, ymer,l actofil	Scandinavia
1.2.4. Mixed lactic acid and ethanol fermentation.	Kumys (Koumiss), leben, laban, Kefir made with and without grains	North Central Asia, Egypt, Iraq, Lebanon, Caucasus
1.3. Mixed material plant-milk fermentations.		
1.3.1. Products where plant material is a substrate for fermentation.	Kishk	Egypt
1.3.2. Products where plant material is a carrier of specific microorganisms and/or enzymes.	Nordic ropy milks	German-speaking areas of Northern Europe
1.4. Various unclassified fermented milks.	Acidophilus yeast milk*	USSR*
2. Buttermilks		
2.1. Conventional buttermilk	By-product of cultured butter manufacture.	Finland, Switzerland, USA, USSR*
2.2. Cultured buttermilk obtained by fermentation of a 'normal' buttermilk.	Buttermilk, Commercial buttermilk, Cultured skim milk*	Czechoslovakia, Germany, Netherlands, USA, USSR*
2.3. Other buttermilks	Yoghurt buttermilk, Lassi	Bulgaria, India*
3. Cultured creams		
3.1. Cultured creams made with butter culture.	Bulgaricus cultured cream*	USA*
3.2. Cultured creams made with other bacteria.	Acidophilus cream (cultured), Czechoslovakia buttermilk*	USA, Bulgaria*

* Source for information in shaded cells: Kurmann *et al.* (1992), Oberman and Libudzisz (1998)

Table 3: Some examples of African fermented milks

Local name of the fermented milk	Country	Reference
Iria ri matii	Kenya	Kimonye and Robinson (1991)
Susa	Kenya	Kurmann (1992)
Maas, Inkomasi, Amasi,	SouthAfrica	Keller and Jordan (1990)
Nono	Nigeria	Atanda and Ikenebomeh (1991), Bankole and Okagbue (1992), Olasupo and Azeez (2001)
Suusaac	Somalia	FAO (1990)
Garoor	Somalia	FAO (1990)
Laben rayeb	Egypt	Kurmann *et al.* (1992)
Irgo	Ethiopia	O'Mahony and Peters (1987a & b); Kurmann *et al.* (1992).
Arrera	Ethiopia	FAO (1990)
Ititu	Ethiopia	Bekele and Kassaye (1987); Kassaye *et a*l. (1991); Kurmann *et al*. (1992)
Amasi	Zimbabwe	Mutukumira *et al.* (1995)
Rob	Sudan	Abdelgadir *et al.* (1998)
Kadam	Mali	FAO (1990)

Contd.

Nyaamme	Ghana	FAO (1990)
Ikuvugota	Zaire	FAO (1990)
Lait caille	Mauritania	FAO (1990)
Pindidaam	Cameroon	FAO (1990)

Table 4: Bacterial species commonly associated with milk.

Family/Genus/Species	Role in the milk	Source of microorganism
Pseudomonas spp.	Spoilage	Milking environment and milk handling equipment
Citrobacter spp.	Spoilage	Milking environment
Hafnia spp.	Spoilage	Milking environment
Enterobacteriaceae	Pathogenic and spoilage	Infected udder, personnel, Milk handling equipment and the milking environment
Staphylococcus aureus	Pathogenic	Infected udder
Streptococcus agalactiae	Pathogenic	Infected udder
St. thermophilus	Acid fermentation	Milk handling equipment and the milking environment
Lc. lactis ssp. *lactis*	Acid fermentation	Milk handling equipment and the milking environment
Lactococcus lactis ssp.*lactis* variety *diacetylactic*	Flavor production	Milk handling equipment and the milking environment
Lc. lactis ssp. *cremoris*	Acid fermentation	Milk handling equipment and the milking environment
Leuconostoc lactis	Acid fermentation	Milk handling equipment and the milking environment
Bacillus cereus	Spoilage	Milking environment

Contd.

B. subtilis	Spoilage	Milking environment
B. stearothermophils	spoilage	Milking environment
B. coagulans	Spoilage	Milking environment
B. megaterium	Spoilage	Milking environment
Clostridium spp.	Spoilage	Milking environment
Lb. lactis	Acid production	Milk handling equipment and the milking environment
Lb. delbrueckii ssp. *bulgaricus*	Acid production	Milk handling equipment and the milking environment
Lb. acidophilus	Acid production	Milk handling equipment and the milking environment
Propionibacterium spp.	Acid production	Milk handling equipment and the milking environment
Mycobacterium tuberculosis	Pathogenic	Infected cow

Source: Rašić and Kurmann (1978), Oberman (1985), Wood and Hodge (1985), Ray and Daeschel (1992), Mäyrä-Mäkinen and Bigret (1993), Ray (1996), Oberman and Libudzisz (1998), Gran *et al*. (2003).

Table 5: Common genera of lactic acid bacteria found in fermented milk

Genus	Species	Examples of fermented milks
Lactobacillus	*Lb. delbrueckii, Lb. delbrueckii* spp. *lactis, Lb. delbrueckii* spp. *bulgaricus, Lb. helveticus, Lb. acidophilus, Lb. fermentum, Lb. brevis, Lb. kefir*	Bulgarian butter milk, Yoghurt
Lactococcus	*Lc*. *lactis* ssp. *lactis, Lc*. *lactis* ssp. *lactis* variety *diacetylactis, Lc*. *lactis* ssp. *cremoris*	Buttermilks, cultured cearm, filmjolk, lattif, langfil, viili, taetmjolk, ymer, kjaddermilk
Leuconostoc	*Leu. mesenteroides, Leu. mesenteroides* ssp. *cremoris*, *Leu. mesenteroides* ssp. *dextranicum, Leu. lactis*	Buttermilks, filmjolk, lattif, langfil, viili, taetmjolk, ymer, kjaddermilk
Streptococcus	*St. thermophilus*	Yoghurt
Pediococcus	*Ped. pentosaceus, Ped. acidilactici*	Biokys (BAP type probiotic milk)

Source: Lee and Wong (1993), Mäyrä-Mäkinen and Bigret (1993), Oberman and Libudzisz (1998).

Table 6: Some examples of nuisance yeasts in foods.

Yeast	Type of food/beverage	Type of problem they cause	Reference
Some strains of *S. cerevisiae*	Wine and other beverages	Spoilage (off flavor turbidity, high amounts of sulphite and H_2S production)	Campbell, 1996; Reed & Nagodawithana, 1991; Rauhut, 1993
Z. bailii	Soft drinks	Spolage	Thomas and Davenport, 1985
Yarrowia lipolytica	Yoghurt, cheese	Spoilage (off flavor)	Westall and Filtenborg, 1998
S. diastaticus	Beverages	Spolage (off flavor and turbidity	Reed & Nagodawithana, 1991
R. glutinis	Sauerkraut	Undesirable pink color formation	Mountney and Gould, 1988
Dekkera intermedia	Beer	Spoilage	Reed & Nagodawithana, 1991
Candida spp.	Bread	Spoilage	Reed & Nagodawithana, 1991
C. pseudotropicalis	Yoghurt	Spoilage (gas and alcohol production)	Rašić & Kurmann, 1978
C. krusei	Yoghurt	Spoilage (oxidize lactic acid to CO_2 and H_2O)	Rašić & Kurmann, 1978
Torulopsis spp.	Bread	Spoilage	Reed & Nagodawithana, 1991
Kloeckera spp.	Wine	spoilage	Reed & Nagodawithana, 1991

Contd.

Brettanomyces spp.	Wine	spoilage	Reed & Nagodawithana, 1991
H. annomala	Bread	spoilage	Seiler, 1980
Pichia burtonii	Bread	spoilage	Seiler, 1980
S. bailii	Myonnaise and salad dressings	spoilage	Reed & Nagodawithana, 1991
Cr. neformans	Fruits and vegetables	Disease (Cryptococcosis)	Green and Bulmer (1979); Pal and Mehrotra (1985)

Table 7: Fermented milks produced by both LAB and yeasts

Name of fermented milk	Country of origin	Source of milk	Important LAB	Important yeast species/ groups
Kefir	Caucasus	Sheep, cow, goat	*Lb. brevis, Lb. kefir, Lb. casei, Lb. cellobiosus, Lb. delbrueckii, Lb. helveticus, Lc. Lactis, St. thermophilus*	*K. marxianus* ssp. *marxianus, K. lactis, S. florentinus, S. globosus C. kefyr, T. delbrueckii*
Koumiss	Asiatic steppes	Mare, camel or ass	*Lb. delbrueckii ssp. bulgaricus, Lb. acidophilus*	*K. marxianus* ssp. *marxianus, C. kefyr*
Continental acidophilus	United states of America	Cow	*Lb. acidophilus*, *Lb. caucasicus*	Lactose fermenting yeast
Taette (Taet-mjolk)	Scandinavian countries	Cow	*St. lactis* resembling var. *holandicus*	*Saccharomyces* sp.
Mazun (Matsun, Matzoni)	Caucasus, Armenia	Sheep, goat, buffalo	*St. thermophilus*	Lactose fermenting yeasts
Leben (Labneh)	Lebanon and some Arab countries	Goat or sheep	*St. thermophilus, St. lactis, Lb. bulgaricus*	Lactose fermenting yeasts
Brano milk	Bulgaria	Sheep or cow	*Lb. delbrueckii ssp. bulgaricus, St. thermophilus*	Lactose fermenting yeasts
Dahi	India, Persia	Cow, buffalo	*St. thermophilus, St. lactis, Lb. bulgaricus, Lb. helveticus, Lb. plantarum*	Lactose fermenting yeasts
Maconi	Caucasus	-	*Lb. delbrueckii ssp. bulgaricus, Lb. kefir*	*S. lactis*

Source: Wood and Hodge (1985), Oberman (1985), Oberman and Libudzisz (1998), Wyder (2001).

CHAPTER II

The diversity of yeasts in Sethemi (a South African naturally fermented milk) and the associated changes in microbial load during fermentation

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Abstract

A total of 116 yeast strains were isolated from raw milk, the production of fermented milk and the final product of Sethemi (a naturally fermented milk produced in South Africa) collected in the Free State area. A high diversity of yeasts was observed, representing 12 different genera and 22 distinct species. Despite the high diversity during the initial stages of fermentation, only seven species proved to be dominant at all stages being present at high numbers in the final product. The predominant isolates were identified as *Candida(C) albicans*, *Cryptococcus(Cr.) curvatus*, *Debaryomyces(Deb.) hansenii*, *Kluyveromyces(K) marxianus*, *Clavispora(Cl.) lusitaniae*, *Saccharomyces cerevisiae(S.)* and *Yarrowia(Y.) lipolytica*.

The growth of the associated bacterial flora was also studied. The results showed that Sethemi was predominated by aerobic mesophilic bacteria (AMC), lactobacilli-leuconostocs (LAB on MRS) and lactococci (LAB on M17), which increased from 5.56 – 9.27, 4.94 – 9.21, and 5.3 – 9.20 $\log_{10}$ CFU/ml, respectively, during the five days fermentation period. Coliform bacteria and yeasts, on the other hand, were found at lower numbers as compared to mesophiles and LAB and increased from 3.56 and 3.81 to 6.83 and 6.12 $\log_{10}$ CFU/ml, respectively.

1. Introduction

Yeasts occur widely in dairy products (Suriyarachchi and Fleet, 1981; Fleet and Mian, 1987; Fleet, 1990; Roostita and Fleet, 1996; Narvhus and Gadaga, 2003). Their functions may vary depending on their nature, the accompanying microflora and the type of substrate provided by the raw material and/or product. Lactic acid bacteria (LAB) are known to be the predominant microorganisms responsible for most of the desirable characteristics in fermented milk. However, a number of reports also indicate that the yeasts are also involved in some ways to contribute either positively or negatively to the product characteristics by bringing about certain biochemical changes (Fleet and Mian, 1987; Fleet, 1990; Jacobsen and Narvhus, 1996; Gadaga et al., 2001; Narvhus and Gadaga, 2003). Their contribution in the fermentation of milk and milk products may be either by supporting the LAB (Jakobsen and Narvhus, 1996) and/or inhibiting undesired microorganisms that may cause quality deterioration (Deiana et al., 1984), or adding value to the final product by means of desirable biochemical changes like the production of aromatic compounds, proteolytic and lipolytic activities (Szumski and Cone, 1962; Machota et al., 1987; Fernandez et al., 1988; Fleet, 1990; Besançon et al., 1992).

In some cheeses, for instance, yeasts contribute positively to the development of desirable flavors and texture during maturation, while in others they cause spoilage by producing off flavors, bitter and buttery taste (Brocklehurst and Lund, 1985; Fleet, 1990; Rohm et al., 1992; and Tudor and Board, 1993).

Despite the availability of reports highlighting their positive roles, there is still a general tendency of considering yeasts as less important or a nuisance in the production of fermented milks. As a result, little attention has been given to identifying and characterizing the yeast microflora especially in naturally fermented milks (Fleet, 1990). However, because some studies have indicated that the quality of naturally fermented milks is poor and variable, recently there is a growing interest to improve this product through the development of starter

cultures (Gadaga et al., 1999; Holzapfel, 1997 & 2002; Narvhus and Gadaga, 2003). Achievement of this goal depends, however, upon a thorough understanding of the microflora of the naturally fermented milks. Limited research work has been done to identify and characterize the microflora of some of the South African traditional fermented milks (Keller and Jordaan, 1990; Beukes et al., 2001; Loretan et al., 2003). Sethemi is a naturally - fermented milk commonly produced by the Sotho people living in and around the Free State Province of South Africa. The fermented milk is traditionally produced by allowing approximately 4L of raw milk to ferment in a clay pot (5 L pots) at room temperature for a period of 3-4 days, depending on the coagulation of the milk determined by the daily temperature. The clay pot is prepared by cooking maize meal porridge in it prior to the addition of raw milk. Between milk fermentations, the pot is only rinsed with cold water. To our knowledge there is no documented literature on the microflora of Sethemi. Thus, the present study was undertaken to investigate the extent to which yeasts and bacteria occur in this traditionally fermented milk product and identify the important yeast species with the ultimate objective of characterizing and evaluating their potential use in future small-scale starter culture development.

2. Materials and methods

2.1. Sample collection

Twenty four samples of raw milk and 15 samples of Sethemi were collected from different sources around Bloemfontein, including households, small scale Sethemi producers, and selected dairy farmers supplying raw milk to the Sethemi producers. All the milk farmers practiced hand-milking with limited numbers of cows (3-8) and some also produced Sethemi on a small scale for their own household use. In each case, 4 L of hand-milked raw milk or 4 L of fermented milks were purchased and transported to the laboratory in cooler boxes covered with ice. The samples were stored at refrigerator temperatures (7^{o}C) until required for analysis. All microbial analysis were performed within 1 hr after collecting the

samples. Additional samples of Sethemi were obtained by fermenting the raw milk in clay pots purchased from the local market and prepared for milk fermentation by the Sethemi producers. In all milk fermentations carried out in the laboratory, the traditional approach was followed as prescribed by the local Sethemi producers.

2.2. Milk fermentation

Raw milk obtained from homesteads and the dairy farmers (5 randomly selected) were spontaneously fermented in the laboratory using the traditional fermentation method. The clay pots (5L capacity) used in the fermentations had been conditioned for the test fermentation by carrying out three trial runs.

The raw milk (4L) was poured into a clean, dry clay pot. After closing the pot loosely with a piece of cloth, the milk was allowed to ferment at ambient temperature (25-30^{O}C) for six days. At 24h intervals, aliquots of the fermenting milk (20ml) were aseptically withdrawn and transferred into sterile McCartney tubes. The milk was thoroughly mixed using a vortex mixer (Vortex V1 Plus, Boeco, Germany) and a portion (1ml) serially diluted using peptone water (Merck, Darmstadt, Germany) for microbiological analysis. pH was also determined on each sampling during the fermentation. The remaining portion was stored frozen (-20^{o}C) for determination of carbohydrate contents. The experiment was repeated 5 times making use of five different milk samples.

2.3. Microbial counts in naturally fermented Sethemi

Lactic acid bacteria (LAB) were enumerated by spread plating onto MRS agar (Merck, Darmstadt, Germany) and M17 agar (Merck). The plates were incubated at 30^{o}C for 48 hrs. Yeasts were enumerated by spread plating on Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Bassingstoke, UK). The plates were incubated at 25^{o}C for 72 hrs.

Total aerobic mesophilic bacteria and coliform bacteria were enumerated by spread plating on Plate Count Agar (PCA) (Oxoid) and Violet Red Bile Agar

(VRBA) (Oxoid), respectively. The PCA plates were incubated at 30^{o}C for 48 hrs, while the VRBA plates were incubated at 37^{o}C for 24 hrs. In all cases, a portion of the appropriately diluted fermented milk samples (0.1ml) was used for spreading on to the surfaces of the respective duplicate plates. All plates containing between 25 and 150 colony forming units (CFU) on the highest dilution (or the highest number if below 25) were counted and the mean values determined from duplicate plates.

2.4. Isolation of Yeasts

At every sampling interval during the fermentation, colonies with visually different morphological characteristics were picked from the RBCA plates and purified by streaking on Yeast Extract-Malt Extract-Glucose Agar (YM) (Merck). The pure cultures were then stored at 4^{o}C on YM agar (Merck) slants until characterization.

2.5. Characterization and Identification of yeasts

All the yeast isolates were characterized according to the profiles of van der Walt and Yarrow (1984). Production of pigments, vegetative reproduction (budding, fission) and formation of pseudohyphae or true hyphae were examined. Conventional methods of tube culture were used to determine the biochemical activities of the isolates (van der Walt and Yarrow, 1984). These included fermentation of sugars and assimilation of carbon compounds, assimilation of nitrogen compounds, growth on vitamin free media, resistance to 0.01% and 0.1% cycloheximide, urea hydrolysis, growth in 10% NaCl, growth in 50% glucose medium, growth at 37^{o}C and splitting of arbutin. The results were compared with a computer based database for yeast identification (Barnett et al., 1990). Further confirmation of the identity of the species was performed through sequence analysis of the D1/D2 domain using primer pairs NL-1(5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG (Kurtzman and Robert, 1998). Sequencing reactions were performed with the ABI Prism^{TM} Big Dye terminator^{TM} V3.1 cycle

sequencing ready reaction kit and data collected on an ABI Prism 377 DNA sequencer (Applied biosystems). Data was analyzed using sequencing analysis V3.3 and sequences assembled using Auto-assembler V1.4.0.

2.6. Determination of pH

The pH of both raw and fermented milk was determined at 24ºC using a digital pH meter (Cyberscan 500, Eutech Instruments, Germany) fitted with an FC 200 electrode (CE, Singapore). The pH meter was calibrated using commercial buffers (Merck) of pH 4 and 7.

2.7. Determination of sugars

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Bouzas et al., 1991). A Biorad-aminex 87H column with a 0.01N H_2SO_4 at 0.6 ml/min. eluent was used (Bouzas et al., 1991). The concentrations of each sugar were then calculated with reference to the peak area of a 1.0%, (w/v) standard solution of each sugar (Sigma-Aldrich, USA).

2.8. Statistical analysis

The results were statistically evaluated using NCSS 2000 statistical soft ware (Hlbtze, 1998). Pearson correlation test, GLM and one-way ANOVA were used for analysis. Comparisons between the mean values of the different microbial groups as well as between the mean values of the different fermentation times were made using the Tukey-Kramer Multiple Comparison Test.

3. Results

3.1. Microbial counts in naturally fermented milk

At the end of the seven days fermentation period, aerobic mesophilic bacteria and lactic acid bacteria were the predominant microorganisms in the naturally

fermented milk prepared in the laboratory (Fig. 1). The aerobic mesophilic counts (AM), presumptive lactobacilli-leuconostocs (LAB on MRS) and presumptive lactococci (LAB on M17), increased from initial levels of 5.56, 4.94 and 5.3 to maximum levels of 9.27, 9.21 and 9.20 $\log_{10}$ CFU/ml, respectively, during the fermentation period. The mean counts of LAB and aerobic mesophiles were significantly ($p < 0.01$) higher than those of coliform bacteria and yeasts. The coliform bacteria and yeasts increased from an initial count of 3.56 and 3.81 to 6.83 and 6.12 $\log_{10}$ CFU/ml, respectively, during the fermentation period (Fig. 1).

The different groups of microorganisms reached maximum population numbers at different times during fermentation. The highest mean counts for coliform bacteria were obtained after 2 days, whereas the highest counts for LAB and aerobic mesophiles were obtained after 3 days. The highest yeast counts were recorded after 4 days (Fig. 1).

The Tukey-Kramer Multiple Comparison Test showed that for coliform bacteria, Day 2 was significantly different from all other days, suggesting that the second day of fermentation is the most likely time at which these bacteria attain maximal growth in naturally fermented milk under the specified experimental conditions. After Day 2, there was a gradual decrease in the coliform counts. This change coincided with a decline in the pH of the fermenting product (Fig. 1). The aerobic mesophilic bacteria and LAB (both on MRS and M17) growth patterns were similar throughout the fermentation period, with almost similar counts recorded (Fig. 1). After three days of fermentation, the counts remained stable. Similarly, but at lower numbers, yeasts reached optimum numbers after three days and remained stable for the remainder of the fermentation period.

3.2. Changes in pH

The pH of the milk decreased gradually with fermentation time, declining from an initial value of 6.76 to 4.16 after 7 days (Fig. 1). Analysis of variance showed that the mean values varied significantly ($p<0.01$) with time (data not shown). With the

exception of the declining numbers of coliform bacteria, none of the other microbial populations tested exhibited a decrease in numbers, although aerobic mesophilic and lactic acid bacterial numbers reached the stationary phase at lower pH values.

3.3. Changes in sugar concentrations

The effect of microbial growth on milk sugar levels is depicted in Fig. 2. The concentration of lactose decreased from 46g/L in the raw milk to 29g/L at the end of the fermentation period. At the same time, the concentration of galactose increased from 0.13 g/L to reach a maximum of 1.52 g/L on Day 5. Glucose was not detected in the fermenting milk until Day 5 where the concentration was recorded as 0.35g/L.

3.4. Isolation and identification of yeasts

A total of 116 representative yeasts were isolated from raw milk, the processing of fermenting milk and naturally fermented milk samples as obtained from households. The strains isolated during processing were obtained at the different time intervals during fermentation as indicated in Fig. 1. Critical biochemical and physiological characteristics supporting the growth of the dominant isolates are shown in Table 1**.** The total of 116 yeast isolates were representatives of 12 genera and 22 species. The diversity of the yeasts characterized, obtained from raw and fermented milk is shown (Table 2). As indicated, yeast isolates from raw milk comprised of eight distinct species, the processing and final product consisted of 21 different species, whereas isolates only from the final product included 9 different species. Yeast diversity was the highest at the initial stages of Sethemi processing representing 18 different yeast species. The high yeast diversity rapidly disappeared during the later stages of processing resulting in a final group of seven dominant species (Table 2). The dominant species, including *C. albicans, Cl. lusitaniae, Cr. curvatus, Deb. hansenii, K. marxianus, S. cerevisiae* and *Y. lipolytica* were regularly present during processing, in the final product and in Sethemi households samples (Table 2). Of the dominant species, only *S.*

cerevisiae and *Y. lipolytica* were not recovered from the raw milk. The dominant yeast species numbers of the different yeast types increased with time (results not shown). The final composition of laboratory fermented milk samples and samples from households showed very similar yeast diversity results, with the exception of *R. glutinis* and *R. mucilaginosa* which were not found in laboratory results.

Debaryomyces hansenii, Kluyveromyces marxianus, Saccharomyces cerevisiae, Candida albicans, Yarrowia lipolytica, Cryptococcus curvatus, and *Clavispora lusitaniae* in that order were the most frequently isolated species representing more than 90% of the total yeast population in the raw and fermented milk.

3.5. Characterization of yeasts isolated from raw and fermented milk

The ability of the yeast isolates to ferment and assimilate sugars and/ or some organic acids present in raw and fermented milk was studied. Accordingly, 62.5%, 41.67%, and 8.33 % of the total species were found to be positive for glucose, galactose and lactose fermentation, respectively. Assimilation tests showed that 100%, 95.83%, 29.17%, 58.33% and 70.83% of the species were positive for glucose, galactose, lactose, lactate, and citrate assimilation, respectively (Data not shown). The results clearly indicated that all species isolated from raw and naturally fermented milks had the ability to utilize one or more of the milk constituents or fermented product. The accumulation of galactose and glucose during fermentation should have further encouraged yeast proliferation.

Despite the high frequency of utilization of milk constituents, however, the pattern of yeast development over time (5 days) showed that only nine of the 22 yeast species appeared in at least three of the five fermentation days (60% of the time) (Table 2). The majority of the yeasts appeared only on the first day of milk fermentation.

4. Discussion

The predominant microorganisms in naturally fermented milk proved to be aerobic

mesophilic bacteria and lactic acid bacteria (LAB), followed by coliform bacteria and yeasts. The mean counts obtained for most of the microbial groups are similar to those reported for other African traditional fermented milks (Mutukumira, 1995; Loretan *et al*., 1999; Abdelgadir *et al.*, 2001; Beukes *et al*., 2001; Narvhus and Gadaga, 2003). Coliform bacteria were inhibited but not totally eliminated. The decrease in coliform counts is attributed to the decreasing pH as confirmed by the negative correlation between the two parameters after two days of fermentation (Data not shown). However, when all values of both parameters were considered in the statistical test, there appeared to be no significant correlation between the two because of the fact that the coliform bacteria were growing well among with the LAB during the first two days of fermentation (Table 3, Fig. 1). The fact that coliforms were still detected after five days of fermentation raises serious questions about the safety of the product. A number of researchers have reported that the survival and growth of pathogenic organisms may be inhibited by lactic acid fermentation (Holzapfel *et al*., 1995; Adams and Nicolaides, 1997; Holzapfel, 2002) whereas others have shown that some coliform bacteria may fail to survive lactic acid fermentation (Feresu and Nyati, 1990; Simango, 1995; Gran *et al*., 2002 & 2003). The high numbers of coliforms in the Sethemi, however, reinforces the need for fast acid producing starter cultures which can retard the proliferation of the coliforms before they reach high numbers. The high coliform numbers may be detrimental to the product by causing off-flavours, shortening the shelf-life and adding to the quality risk factor.

Raw and pasteurized milks are frequently contaminated with yeasts (Engel, 1986; Fleet and Mian, 1987) since the chemical composition of milk will support their growth. However, the populations reported from raw milk are usually low ($<10^3$ cfu/ml) as compared to populations reported for bacteria (Fleet, 1990; Deak, 1991). Our results regarding the numbers of yeasts in raw milk are similar to those reported elsewhere (Roostita and Fleet, 1996). It was observed that the faster growing psychrotropic bacteria are responsible for restricting the growth of yeasts through competition for available constituents in the milk (Cousin, 1982). Higher

numbers of yeasts were, however, observed during the fermentation of the milk (Fig. 1) indicating that yeasts grow better under restricted environments (Viljoen, 2001) when intrinsic factors like a lower pH play a substantial role. Viljoen (2001) also indicated on the mutualistic effect that exists between lactic acid bacteria and yeasts whereby the lactic acid bacteria encourage the growth of yeasts.

A high diversity of yeast strains was detected at the initial stages of the fermentation (Table. 2) comprising of 18 different species at day one. Only eight of these strains originated from the raw milk while the majority probably originated from the clay pots or the immediate environment. Only *S. cerevisiae* and *Y. lipolytica* which were not isolated from the raw milk developed as predominant species in the final product. Despite the high diversity on day one of fermentation, most of these primary strains, however quickly disappeared probably due to selective pressures exerted by the biotic factors present (Viljoen, 2001). Those that possessed the proper attributes to counteract the ecological determinants developed into yeast communities and eventually predominated in the final product (Table 2). The lactose utilizing yeasts and strains with lipolytic and proteolytic activity usually adapt quickly to the specific environmental conditions and are likely to increase in numbers compared to their negative counterparts (Eliskases-Lechner and Ginzinger, 1995). This phenomenon has been reported in cheese studies revealing that the composition of the yeast microflora appears to be more heterogeneous in the young cheese than in the older ones (Eliskases-Lechner and Ginzinger, 1995).

Many different types of yeasts can grow in fermented milk using the end products of lactose breakdown, namely glucose and galactose. Growth and predominance, however, are also dependent on the ability of the strains to assimilate organic acids, and on their proteolytic and lipolytic activities in the milk (Fleet and Mian, 1987). In this study, of the 22 different characterized yeast species, only *K. marxianus* was capable of fermenting lactose, which is the major carbohydrate fraction of milk. The remainder of the yeast strains probably depended on end products of the metabolism of lactose, proteins or fats for obtaining energy. Of the

remaining predominant species, *C. albicans, Cl. lusitaniae, Deb. hansenii, Cr. curvatus, Y. lipolytica* and *S. cerevisiae* can utilize other available sugars during fermentation like glucose and galactose, whereas *Y. lipolytica* also has exceptional lipolytic and proteolytic activity.

K. marxianus and *Deb. hansenii* have been shown to have varying abilities to metabolize milk protein and fat (Fleet, 1990). *S. cerevisiae* does not have the ability to utilize milk lactose, protein or fat (Fleet and Mian, 1987; Fleet, 1990), but is commonly isolated from milk (Gadaga *et al*., 2000; Abdelgadir *et al*., 2001; Jespersen, 2003). It seems, therefore, that *S. cerevisiae* depends on other microorganisms in the milk for its survival and growth. *S. cerevisiae* ferments and assimilates glucose and galactose. The concentration of both glucose and galactose was observed to increase at varying levels during the fermentation period (Fig. 2). Previous studies showed that during the alcoholic fermentation of Koumiss by strains of *S. cerevisiae,* some of the LAB only metabolized the glucose moiety from lactose and excrete the galactose moiety (Montanari *et al*., 1996). This phenomenon was further demonstrated in other fermented products (Marshall, 1987; Marshall and Tamime, 1997; Gadaga *et al*., 2001).

While the predominant yeast strains *Deb. hansenii, K. marxianus, S. cerevisiae,* and *Y. lipolytica* have been isolated from dairy products on many occasions (Fleet, 1990; Yamani and Abu-Jaber, 1994; Viljoen and Greyling, 1995; Loretan et al., 1998, 2003; Wyder, 1998; Roostita and Fleet, 1999; Sserunjogi, 1999; Gadaga et al., 2000; Corbo *et al*. 2001; Abdelgadir et al., 2001; Jespersen, 2003), to our knowledge, the occurrence of *C. albicans* at high levels has not been reported from fermented milks so far. Yeast mastitis has been known to occur in 2-3% of all cases of mastitis in dairy cows (Stanojevic and Krnjajic, 2003), and the main yeasts associated with it are *C. albicans* and *Cr. neoformans*. It is possible, therefore, that the high prevalence of *C. albicans* could be attributed to milk samples derived from cows with mastitic udders.

Although some yeasts like *Candida albicans* and *Cryptococcus neoformans* have long been known to be pathogenic to human beings, they are not believed to be transmitted through foods (Fleet, 1990). However, according to Kockova-Kratochvilova (1990), *C. albicans* and the related species such as *C. tropicalis*, *C. rugusa*, and *C. parapsilosis* occur in humans as commensals or saprophytes only as long as the host is not providing them suitable conditions. Reports also showed an increase in the incidence of an invasive disease caused by *Candida lusitaniae* (the anamorphic stage of *Clavispora lusitaniae*) (Hadfield *et al.*, 1987; Merz, 1984). Consequently, all the above species, being opportunistic pathogens, can turn out to cause serious disease under certain conditions of the host. In addition to the pathogenic *Candida,* reports also indicated some species of *Pichia* and *Cryptococcus* encountered as rare and emerging causative agents of opportunistic diseases that develop in immunocompromised patients or cases with other predisposing factors (Araissie *et al*., 1989; Chakrabarti *et al*., 2001; Ikeda *et al.,* 2002). *Cr. curvatus* and *Cr. laurentii* have been isolated from AIDS or cancer patients suffering from myeloradiculitis and fungemia (Dromer, *et al*., 1995; Johnson *et al*., 1998; Kunova and Krcmery, 1999). Therefore, since these yeasts are potentially opportunistic pathogens their high percentage of occurrence in fermented milks, especially that of *Cr. curvatus*, *C. albicans* and *C. parapsilopsis*, may pose a serious health threat in view of the very high prevalence of immuno-compromised people amongst the African population.

5. Conclusion

The microflora of naturally fermented milk is predominated by aerobic mesophilic bacteria and lactic acid bacteria. However, considerable growth of yeasts and coliform bacteria can also be observed during the fermentation process. Neither the growth of lactic acid bacteria nor the lower pH was correlated to the sufficient inhibition of coliform bacteria and therefore pH alone may not be a dependable means of controlling the growth of coliform bacteria.

Several species of yeast exist in *Sethemi* including many potential pathogens. Therefore, the development of starter cultures and making them commercially available to control these undesirable microorganisms would be of major importance in the improvement of the quality as well as the safety of *Sethemi*.

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Table 1: Characteristic fermentation and assimilation properties of the dominant yeast species associated with naturally fermented milk (Sethemi).

SPECIES	FERMENTATION OF SUGARS			ASSIMILATION				
	Glucose	Galactose	Lactose	Glucose	Galactose	Lactose	Lactate	Citrate
C. albicans	+	+	-	+	+	-	-	+
Cl. lusitaniae	+	+	-	+	+	-	+	-
Cr. curvatus	-	-	-	+	+	+	+	+
Deb. hansenii	-	-	-	+	+	+	+	+
K. marxianus	+	+	+	+	+	+	+	-
S. cerevisiae	+	+	-	+	+	-	-	-
Y. lipolytica	-	-	-	+	+	-	+	+

Table 2: Yeast diversity within raw milk, during the processing of Sethemi, and Sethemi samples obtained from households. (Presence of yeasts indicated as positive and the relative percentage of incidence in brackets; raw milk is representative of 24 samples; fermentation represents 5 repetitions; Sethemi households are representative of 15 samples).

YEAST SPECIES	RAW MILK	FERMENTATION					SETHEMI - HOUSEHOLDS
		Day 1	Day 2	Day 3	Day 4	Day 5	
C. albicans	+	+	+	-	+	+	+
C. ernobi	+	-	-	-	-	-	-
C. parapsilosis	+	+	-	+	+	-	-
C. rugosa	+	+	-	-	-	-	-
C. sake	-	+	-	-	-	-	-
C. tenuis	-	+	-	-	-	-	-
C. tropicalis	-	-	-	-	-	+	-
Cl. lusitaniae	+	+	-	+	-	+	+
Cr. curvatus	+	+	+	+	-	+	+
Cr. laurentii	-	+	-	-	-	-	-
D. anomala	-	-	+	-	-	-	-
Deb. hansenii	+	+	+	+	+	+	+
K. marxianus	+	+	+	+	+	+	+
P. anomala	-	+	-	-	-	-	-
P. pini	-	+	-	-	-	-	-
P. scolyti	-	+	-	-	-	-	-
R. glutinis	-	-	+	-	-	-	+
R. mucilaginosa	-	+	-	-	-	-	+
S. cerevisiae	-	+	+	+	+	+	+
Spo. roseus	-	+	+	-	-	-	-
T. delbrueckii	-	+	+	-	-	-	-
Y. lipolytica	-	+	-	+	+	+	+

Table 3. Correlation matrix showing relations between variables (Pearson correlation test)

		CB	LAB (MRS)	LAB (M17)	YEASTS	TAMC	pH
CB	r	1.000000	0.245647	0.107967	-0.058760	0.099637	-0.073444
	P	0.000000	0.003440	0.204177	0.490435	0.241493	0.388478
	N	140	140	140	140	140	140
LABMR	r	0.245647	1.000000	0.772665	0.320876	0.789850	-0.630455
	P	0.003440	0.000000	0.000000	0.000111	0.000000	0.000000
	N	140	140	140	140	140	140
LABM1	r	0.107967	0.772665	1.000000	0.212777	0.685919	-0.582104
	P	0.204177	0.000000	0.000000	0.011602	0.000000	0.000000
	N	140	140	140	140	140	140
YEAST	r	-0.058760	0.320876	0.212777	1.000000	0.227218	-0.449030
	P	0.490435	0.000111	0.011602	0.000000	0.006939	0.000000
	N	140	140	140	140	140	140
TAMC	r	0.099637	0.789850	0.685919	0.227218	1.000000	-0.474873
	P	0.241493	0.000000	0.000000	0.006939	0.000000	0.000000
	N	140	140	140	140	140	140
pH	r	-0.073444	-0.630455	-0.582104	-0.449030	-0.474873	1.000000
	P	0.388478	0.000000	0.000000	0.000000	0.000000	0.000000
	N	140	140	140	140	140	140

Cronbach's Alpha = 0.704081 Standardized Cronbach's Alpha = 0.341608
CB = Coliform bacteria, LABMRS = Lactic acid bacteria count on MRS, LABM17 = Lactic acid bacteria count on M17, TAMC = Total aerobic mesophilic count
r = Pearson's correlation coefficient, P = Probability, N = Number of samples

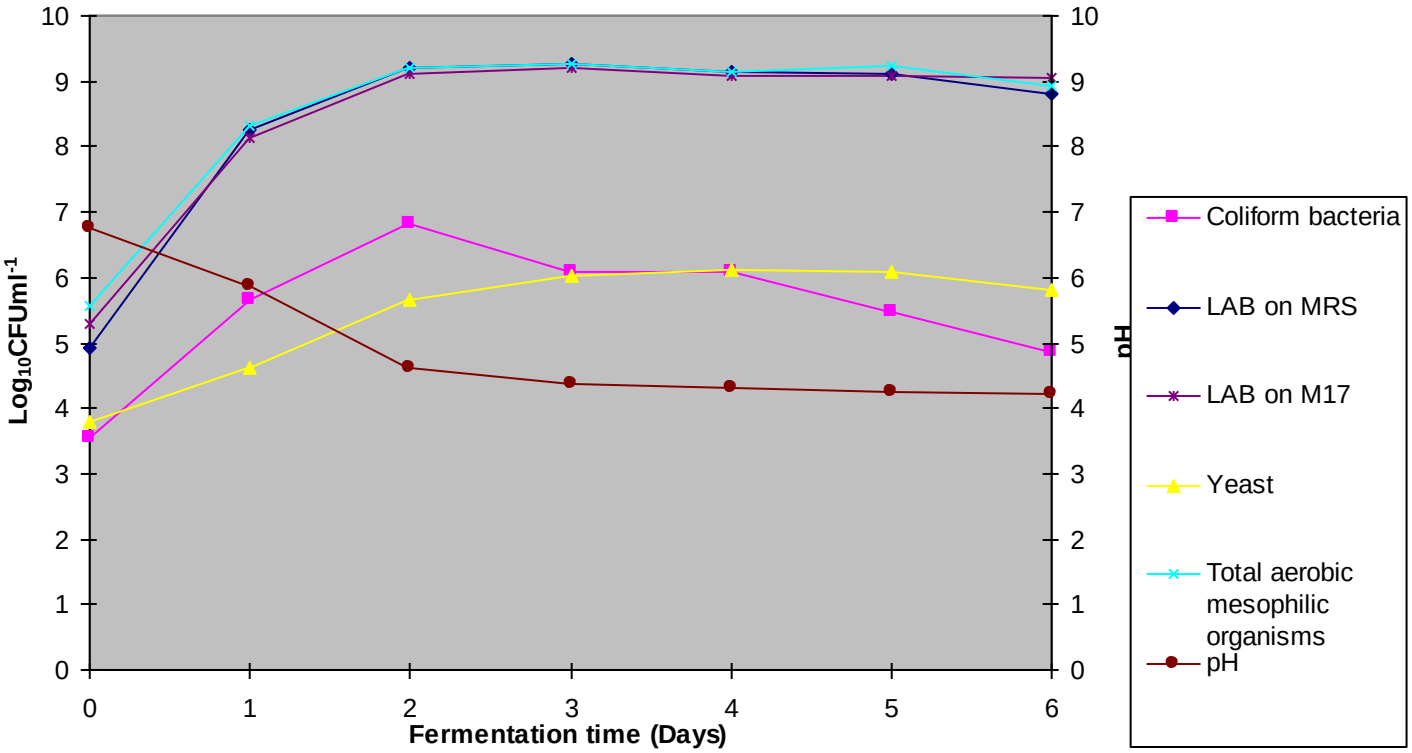

Fig .1: Growth of microorganisms and changes in pH during the production of the indigenous fermented milk Sethemi.

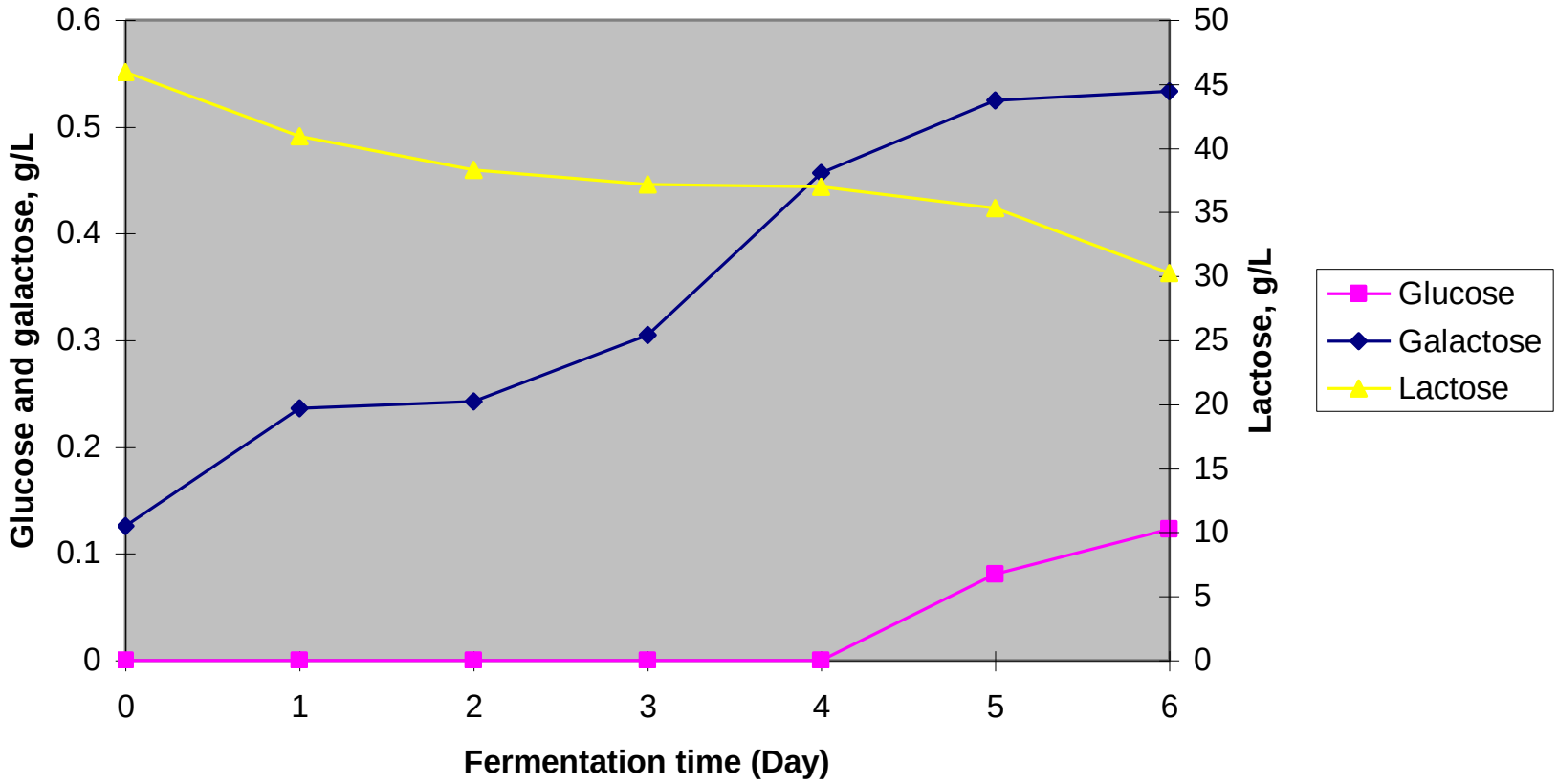

Fig. 2: Sugar levels during spontaneous fermentation of milk

CHAPTER III

Differences in the development of microbial loads during the production of Sethemi (naturally fermented milk) using different containers

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Abstract

Sethemi (A South African naturally fermented milk) was produced in the laboratory using four different containers, namely clay pot, gourd, nickel jar and plastic bowl, by applying the traditional method of fermentation. The development of bacteria and yeasts in all fermented milk samples in the different containers was followed. The results showed that naturally fermented milk produced from all containers were predominated by aerobic mesophilic bacteria (TAM), lactobacilli-leuconostocs (LAB on MRS) and lactococci (LAB on M17). No significant differences were found in the number of microbial loads between containers.

The different containers had, however, differences with respect to the time at which the microbial groups attained maximum numbers, the types of dominant yeast species, and the diversity of yeast species. Obtaining maximum growth of lactobacilli-leuconostocs, lactococci, and total aerobic mesophilic bacteria in milk fermented in the nickel container needed less time compared to fermentations in the other containers.

In milk fermented in the clay pot and gourd the most dominant yeast species were *Debaryomyces hansenii* and *Saccharomyces cerevisiae*, respectively, while *Cr. curvatus* predominated in both the nickel and plastic containers. The highest yeast diversity was obtained from milk samples fermented in the clay pot represented by

15 species. Of the 23 species isolated in total from all the containers and raw milk, only *Deb. hansenii*, *Cr. humicola* and *K. marxianus* were isolated from both raw and naturally fermented milks of all containers.

1. Introduction

Fermentation is one of the oldest food processing technologies in the world. It has been in use for millennia in the production of a number of food products including the present day most coveted dairy products. The art of this early technology has been passed down for generations from parent to child and now belongs to a body of knowledge known as "indigenous knowledge". According to Walshe *et al.* (1991), most of this knowledge has not been documented and is in danger of being lost as technologies evolve and families move away from traditional food preservation practices.

Amongst the few that have been documented, are the technologies used in the production of some varieties of bread, alcohol and dairy products mainly from Europe and partly from Asia (Hesseltine, 1979; Oberman, 1985; Steinkraus 1983, 1996, 1997; Yokotsuka, 1985). Examples of dairy fermented products include yogurts in the Western world, Russian kefir, Middle-East yogurts, liban (Iraq), Indian dahi, Egyptian laban rayab, laban zeer, Malaysian tairu (soybean milk), lactic acid fermented cheeses in the Western world, Chinese sufu or furu, and lactic acid fermented yogurt/wheat mixtures (Hesseltine, 1979; Steinkraus, 1996; 1997).

In Africa, although there are a number of traditionally fermented foods (Odunfa and Oyewole, 1998) including naturally fermented milks that are still being used by several rural and urban communities (Abdelgadir *et al.,* 1998; El-Gendy, 1983; FAO, 1990; Gran *et al*., 2002; Mutukumira, 1995), only limited information is available on the quality and diversity of these products as well as on the indigenous technologies used to produce them. Since milk is highly perishable and bulky to transport over long distances, in most African rural communities the bulk of it is consumed immediately either as fresh or in sour form (Ganguly *et al.*, 1999; Ryoba and Kurwijila, 1995; Mshangama and Ali, 1995). Only a fraction of the milk production enters the commercial sector in many of these countries (Walshe *et al*., 1991; Ganguly *et al.*, 1999). According to these authors, in southern and eastern Africa, except in Kenya and Zimbabwe, the milk that is consumed in the traditional way

within the farming community constitutes 80-90% of the total milk produced. Thus, converting surplus milk immediately into the less perishable product i.e., naturally fermented milk, ghee, butter and in few instances cottage cheese, at the house-hold level is a common practice and plays a significant role in the diet of their respective communities (Chikoto, 1999; Kerven, 1987a; 1987b; Leeuw *et al*., 1984; Majubwa, 1987). The technologies used in the preparation of these dairy products may involve a number of processes as indicated in Table 1.

In all of these processes, containers play a key role in the production of the particular required dairy product. In Africa, most of the containers used in the preparation as well as storage of the dairy products are the same containers used in the production of fermented milks. These vessels are generally simple and mostly made from locally available cheap materials such as woven grass, wood fiber, calabash, hollowed wood or animal skin bags (FAO, 1990). Calabashes made from gourd, clay pots and wood are widely used in most parts of Ethiopia, Kenya, Sudan and Egypt (Bonfoh *et* al., 2003; Abdelgadir *et al.*, 2001; O'Mahony and Peters, 1987; Kimonye and Robinson, 1991; El-Gendy, 1983). Literature also shows that in the past, in many parts of South Africa, milk used to be fermented in milk-sacks, calabashes, clay pots, stone jars and baskets (Fox, 1939; Quinn, 1959; Bryant, 1967; Fehr, 1968; Bohme, 1976). Flask-shaped calabashes which were mainly made of gourd but named differently in different localities were mostly used as milk containers by the *Xhosa, Thembu, Bomvana, Mpondo, Mpondomise, Mfengu,* and *Bhaca* people in South Africa. Though some of these may still be in use in the rural areas, many specimens are currently available as museum collections (Bohme, 1976). *Amasi*, one of the most widely consumed traditional fermented milks by the Bantu people used to be prepared by pouring raw milk straight into calabashes made of gourd or into stone jars. According to Fox (1939), the flavor of *Amasi* produced using stone jar*,* was different from that produced using calabash (gourd)*.* The Sotho people also used clay pots which are believed to impart better flavor than calabashes (Fox, 1939). Although these earlier traditional containers are currently being replaced by new commercially produced and readily available ones, such as plastics (Coetzee *et al.*,

1996) and nickel jars and/or pots (personal communication with local people), some like calabashes (gourd) and clay pots are still in use in the rural areas (Beukes *et al.*, 2001). In rural South Africa, as in the tropics, the average milk producer produces milk in small quantities (FAO, 1990; Ganguly *et al.*, 1999). Therefore, as long as the low milk production potential continues to prevail, milk processing revolving around natural fermentation using the afore-mentioned simple technologies will also continue to be exercised at the household level (FAO, 1990). These small-scale traditional fermentation technologies at the household level offer considerable potential for stimulating development in the food industry of the poor communities in light of their low cost, scalability, minimal energy and infrastructural requirements and the wide acceptance of fermented products by the local people if used properly with adequate understanding of their scientific basis (Nout, 2004; Rolle and Satin, 2002).

At present, the contribution of traditional technologies to the characteristics of the indigenous fermented milk, relative to the metabolism of the microorganisms present, has not been assessed (Narvhus, 2003). Consequently, as part of the strategy that must be followed to promote the traditional technology used in the production of Sethemi, a study was initiated to investigate the influence of containers on the microbiology of naturally fermented milks. The information obtained would allow primarily the rural development agents to emphasize on the promotion of the container(s) that may best suit the needs of the low-income rural population. The main objective of this study was, therefore, to investigate the contribution of containers (milk vessels) on the development of the dominant milk microflora associated with the production of Sethemi (A South African naturally fermented milk).

2. Materials and methods

2.1. Milk sample collection

Samples of raw milk were collected from selected dairy farmers supplying raw milk to the Sethemi producers in the vicinity of Bloemfontein, South Africa. All the milk farmers practiced hand-milking of a limited numbers of cows (3-8) and some also

produced Sethemi on a small scale for their own household use. In each sampling case, 4 x 4 L of hand-milked raw milk were purchased and transported in sterile 5 L Erlenmeyer flasks to the laboratory in cooler boxes covered with ice. The samples were immediately divided amongst the four different containers and kept closed at ambient temperature throughout the fermentation time. In all milk fermentations carried out in the laboratory, the traditional approach was followed as prescribed by the local Sethemi producers.

2.2. Milk containers (vessels)

Four different types of containers, namely clay pot, gourd (container made from the dried fruit wall of the plant *Lagenaria peucantha* by removing the internal contents), nickel container (the type that is commonly used by the local dairy farms) and plastic bowl were obtained from the surrounding local markets or purchased from small scale household Sethemi producers. The gourd (calabash), nickel container and the plastic bowl had been in active household use for an undetermined period of time before applied in the study. The clay pot, which was obtained as a new container from the market, had been "acclimatized" by the Sethemi producers, running three batches of milk fermentation prior to conducting the actual experiment. With the exception of the nickel container (10 L) all containers had a volume capacity of 5 L. These containers were selected as fermentation vessels in this study since a preliminary survey indicated that these vessels are the most frequently used containers by the local people living around Bloemfontein.

2.3. Preparation and sampling of fermented milks

Five repetitions of milk fermentations in each container were carried out using the traditional method of Sethemi production without employing starter cultures.

On each occasion, 4 L of the raw whole milk samples were poured into the four different containers after properly rinsing the containers with sterile water. These were kept closed and undisturbed in the laboratory at ambient temperature (25-

30^{O}C) for a period of seven days. Samples were collected consecutively at 24hr intervals during processing starting immediately after the milk was poured into the containers. Aliquots of the fermenting milk (20ml) were aseptically withdrawn and transferred into sterile McCartney tubes. The milk was thoroughly mixed using a vortex mixer (Vortex V1 Plus, Boeco, Germany) and a portion (1ml) serially diluted using peptone water (Merck, Darmstadt, Germany) for microbiological analysis. Microbial analyses were performed in triplicate. pH was also determined on each sampling during the fermentation. The remaining portion was stored frozen (-20^{o}C) for determination of carbohydrate contents.

2.4. Enumeration of microbial loads in Sethemi

Lactic acid bacteria (LAB) were enumerated by spread plating onto MRS agar (Merck, Darmstadt, Germany) and M17 agar (Merck) respectively. The plates were incubated at 30^{o}C for 48 hrs. Yeasts were enumerated by spread plating on Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Bassingstoke, UK). The plates were incubated at 25^{o}C for 72 hrs.

Total aerobic mesophilic bacteria and coliform bacteria were enumerated by spread plating on Plate Count Agar (PCA) (Oxoid) and Violet Red Bile Agar (VRBA) (Oxoid), respectively. The PCA plates were incubated at 30^{o}C for 48 hrs, while the VRBA plates were incubated at 37^{o}C for 24 hrs. In all cases, a portion of the appropriately diluted fermented milk samples (0.1ml) was used for spreading on to the surfaces of the respective duplicate plates. All plates containing between 25 and 150 colony forming units (CFU) on the highest dilution (or the highest number if below 25) were counted and the mean values determined from duplicate plates.

2.5. Isolation of yeasts associated with Sethemi

During each sampling occasion, including all four containers, at least five representative colonies from the yeast enumeration medium, Rose Bengal Chloramphenicol Agar (RBCA), were selected based on their colony morphologies

and streaked on Yeast Extract-Malt Extract-Glucose Agar Medium (YM) as primary cultures. These cultures were sub-cultured and purified at least 3 times on YM plates before designating them as isolates and proceeding with identification. All the resulting isolates were subsequently maintained on YM at 4^{o}C and periodically transferred to freshly prepared slants.

2.6. Characterization and Identification of yeasts

All isolates were characterized according to van der Walt and Yarrow (1984). Production of pigments, vegetative reproduction (budding, fission) and formation of pseudohyphae or true hyphae were examined. The conventional method of tube culture was used to analyze the biochemical activities of the isolates (van der Walt and Yarrow, 1984). The distinguishing tests included biochemical and physiological tests such as fermentation (6 compounds), assimilation of different carbon compounds (30 compounds), assimilation of various nitrogen compounds (5 compounds), growth on vitamin free media, resistance to 0.01% and 0.1% cycloheximide, urea hydrolysis, growth in 10% NaCl, growth in 50% glucose medium, growth at 37^{o}C, and splitting of arbutin. The results were compared with a computer database using the yeast identification software (Barnett *et al*., 1990).

2.7. Determination of pH

The pH of both raw and fermented milk was determined at 24ºC using a digital pH meter (Cyberscan 500, Eutech Instruments, Germany) fitted with an FC 200 electrode (CE, Singapore). The pH meter was calibrated using commercial buffers (Merck) of pH 4 and 7. The pH of the fermented milk in different containers was monitored at regular intervals as indicated during enumeration studies.

2.8. Carbohydrate determination

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Bouzas et al., 1991). A Biorad-aminex 87H column with a 0.01N H_2SO_4 at 0.6 ml/min. eluent was used (Bouzas et al., 1991). The

concentrations of each sugar were then calculated with reference to the peak area of a 1.0%, (w/v) standard solution of each sugar (Sigma-Aldrich, USA).

2.9. Statistical analysis

The results were statistically evaluated using Analysis ToolPak provided by Microsoft Excel 2000 and NCSS 2000 statistical soft ware (Hlbtze, 1998). Pearson correlation test was used to determine relationships between pH and microbial growth as well as between the numbers of the different microbial groups. A single factor ANOVA was used to statistically evaluate differences between the highest mean microbial counts of the different containers. GLM ANOVA was also used to determine the presence of significant differences between containers in their combined mean microbial counts that have been obtained from the mean microbial counts of all fermentation days. Comparisons between the mean values of fermentation days were also determined using the Tukey-Kramer multiple comparison tests.

3. Results and Discussion

3.1. Enumeration of bacteria and yeasts from Sethemi produced in different containers

The development of the microbial numbers including the lactobacilli-leuconostocs, lactococci, coliform bacteria, total aerobic mesophilic bacteria and yeasts were determined over a six day fermentation period during the production of Sethemi, using four different containers. The microbial enumeration results associated with each container are shown in Figs. 1 a - e. The optimum mean number of live cells counted for each microbial group in the four respective containers as obtained at the specific day are indicated in Table 2, whereas the average cell numbers (combined mean) obtained for each microbial group per fermentation in the different containers are indicated in Table 3. As shown in Table 2, comparisons between microbial numbers associated with the different containers exhibited no significant differences (F-critical > F-ratios, $p > 0.05$). However, when the

combined mean microbial counts obtained from the mean counts of all fermentation days were compared significant differences were observed between containers with respect to the number of lactobacilli-leuconostocs (LAB on MRS) (F-critical < F-ratio, $p < 0.05$), lactococci (LAB counts on M17) (F-critical < F-ratio, $p < 0.05$), and yeasts (F-critical < F-ratio, $p < 0.05$) (Table 3). No significant differences between containers with respect to the combined mean values of total aerobic mesophilic counts (TAMC) and coliform bacteria were obtained (Table 3).

Due to the lack of information, our results were not comparable with any relevant studies based on microbial numbers associated with different containers. To our knowledge, the only available literature that attempted to study microbial numbers in fermented milks using different containers was the work done by Beukes *et al*., (2001). These authors collected a limited number of indigenous fermented milk samples produced in a clay pot, calabash and plastic container from individual rural households and enumerated different groups of bacteria. The data, however, were only shown for the clay pot and gourd samples. According to these authors, the highest coliform counts were obtained from clay pot samples whereas the mean counts of total aerobic mesophilic bacteria, LAB on MRS and LAB on M17 in the clay pot were higher than those in gourd. In the current study, although gourd and clay pot microbial samples were significantly different ($p < 0.05$) from nickel and plastic based on the combined mean counts of LAB on MRS, LAB on M17 and yeast, no significant difference ($p > 0.05$) in microbial numbers was observed between clay pot and gourd milk samples in either the maximum mean counts or the combined mean counts of coliform bacteria, total aerobic mesophilic bacteria, yeasts, LAB on MRS and LAB on M17 (Table 2 and 3).

A variation in the time elapsed before reaching the optimum microbial numbers was detected, however between samples obtained from the nickel container and the remaining three containers. As indicated in Table 2, the time elapsed before maximum microbial numbers is attained by each microbial group of organisms is reduced by one day in samples from the nickel container. Consequently, the results generally suggested that the nature of the container may affect the time for

reaching optimum numbers, but not the maximum number of microbes during Sethemi production. The results, therefore, proved that at ambient temperature, a more or less similar product of Sethemi with respect to the microbial loads can be produced with any of the four types of containers.

As expected, LAB and total aerobic mesohphilic bacteria were the predominant microorganisms present in Sethemi samples associated with all containers. The mean counts for lactobacilli-leuconostocs (LAB on MRS), lactococci (LAB on M17) and total aerobic mesophilic bacteria (TAMC) in all containers (Table 2) were higher than the mean counts reported for the South African traditional fermented milk (Beukes *et al.*, 2001) and the Maasai (Kenyan) traditional fermented milk (Mathara *et al.*, 2004). The differences observed in the mean microbial counts could be attributed to differences in microbial composition (species difference), milk composition, fermentation conditions, the methodologies employed and the time of sampling. Despite the possible existence of such variations, the microbial loads reported in this study corresponded with other reports from East Africa (Isono *et al*., 1994; Mathara, 1999; Miyamoto *et al.*, 1986), Ethiopia and Zimbabwe (Beyene, 1994; Gran *et al*., 2002; Mutukumira, 1995). Since the occurrence of coliform bacteria in fermented milks is often associated with potential health hazard (Gran *et al*., 2003), the presence of high levels of coliform bacteria in Sethemi present in all containers strongly supports the need for undertaking adequate sanitary measures. Yeast numbers obtained agreed with results reported by Isono et al. (1994) and Mathara et al. (2004) who indicated numbers ranging from 6.0 to 8.0 $\log_{10}$ cfu/ml and 4.3 to 7.4 $\log_{10}$ CFU ml/ml in Tanzanian and Kenyan traditional fermented milks, respectively.

3.2. Effect of containers on the pH profile of Sethemi

The pH of the fermented milk showed generally a similar decreasing trend with fermentation time in all containers (Fig. 1 a-e). Values decreased from 6.77 in the raw milk to values ranging from 4.10 ± 0.13 to 4.32 ± 0.06 in the final Sethemi produced in different containers (Table 4). A single factor ANOVA showed that

there was a significant difference ($p < 0.01$) between fermented milk in the different containers. The pH levels of Sethemi produced in gourd and nickel were found to be significantly lower than that produced in the clay pot and plastic bowl (Table 4). This difference is mainly attributed to the rapid decline in pH values of milk in the gourd and nickel containers after day 1. As depicted in Fig 1 (a-e), the pH of the fermented milk in the gourd and nickel was much lower compared to the fermented milk in the clay pot and plastic bowl on this day. This difference was also supported by the Tukey-Kramer multiple comparison test which showed that the pH of the milk in the clay pot and plastic bowl were different from samples in the gourd and nickel based on the combined mean values of pH. In addition, GLM ANOVA showed that the pH values showed significant variations as a result of interaction between the specific container and fermentation time ($p <0.01$). Accordingly, the milk fermentation time elapsed in each container had a significant effect on the pH of Sethemi.

In general, the lowest pH values of Sethemi in all four containers were obtained after 4 days of fermentation and the means ranged between 4.10 ± 0.13 to 4.32 ± 0.06, which were higher than that reported for the Zimbabwean traditional fermented milk of 3.88 ± 0.22 (Gran *et al*. 2003) but within the range reported for Kenyan traditional fermented milk (Mathara *et al*., 2004).

3.3. Changes in sugar levels of Sethemi during fermentation

Changes in the levels of the major sugars present in the raw milk and during fermentation in the different containers were depicted in Fig. 2 (a-d). As indicated, the lactose concentration during milk fermentation showed a linear decrease with time in all containers mainly attributed to the growth of the lactic acid bacteria. The lactose decreased from an initial concentration of 44.68g/L in the raw milk to 29.0, 34.6, 24.2, and 33.2 g/L after six days of fermentation in the clay pot, gourd, nickel and plastic bowl, respectively. At the same time, the galactose concentration increased from 0.13g/L in the raw milk to values of 1.52, 0.26, 0.26 and 0.55g/L after fermentation in the clay pot, gourd, nickel and plastic containers, respectively.

These differences in available carbohydrates suggest that the composition of the lactic acid bacterial flora may also vary between containers. Furthermore, based on its high galactose content the milk fermented in the clay pot may have a relatively large number of galactose excreting LAB compared to the milk in the other containers since literature established that some LAB have the ability to excrete galactose into the media during the metabolism of lactose (Marshall, 1987; Marshall and Tamime, 1997). In all containers, except in the fermented milk produced in the gourd, galactose accumulation continued to increase whereas the contents in the gourd followed a rapid decline after day 2 (Fig. 2 b). Glucose was not detected in the raw milk but appeared in the fermented milk of clay pot starting from day 5 and reached a concentration of 0.35g/L at day 6 (Fig. 2 a). Similarly, low levels of glucose (0.14g/L) were detected in the plastic bowl at the end of the fermentation time (Day 6) (Fig. 2 d). In contrast, no glucose was detected in milk samples fermented in the gourd and nickel containers throughout the fermentation period (Fig. 2 b and c). The decline in galactose during fermentation as well as the complete absence of glucose in the fermented milk in the gourd cannot be explained. The galactose and glucose can be utilized by yeasts (Fleet and Mian, 1987; Fleet, 1990) and bacteria, but similar microbial counts were observed in all milk samples.

3.4. Yeast diversity in Sethemi produced using different containers

A total of 112 yeast strains were isolated from raw milk and Sethemi produced in the laboratory using four different containers. Of these, 29, 35, 14 and 16 isolates were obtained from fermented milk produced in the clay pot, gourd, nickel and plastic bowl, respectively, whereas the remaining 18 isolates were obtained from raw milk. The isolates belonged to 12 genera and 23 different species. The diversity and distribution of the representative yeast species of the milk fermented in the four different types of containers and raw milk are shown in Table 5. As indicated, the isolates from raw milk and the fermented milk of the clay pot, gourd, nickel, and plastic bowl comprised of 8, 15, 10, 4 and 5 different species, respectively. Of the eight distinct species present in the raw milk, only some were

recovered from the fermented milk produced in the clay pot (6), gourd (6), nickel (3) and plastic bowl (4) (Table 5). The low percentage of yeasts originating from the milk clearly indicated that most of the yeast species (65% of the total identified yeast species) might have been introduced as contaminants originating from the containers or immediate environment. The low recovery of raw milk associated yeasts might also indicate that containers had different influences on the survival of different yeast species. These may be attributed to either the physico-chemical changes brought about by the specific nature of the container or the increased competition resulting from the additionally introduced contaminant yeast species originating from the containers.

An evaluation of the proportion of yeasts identified from each group as an indication of yeast diversity in the raw milk (8/18), fermented milk in the clay pot (15/29), gourd (10/35), nickel (4/14) and plastic bowl (5/16) showed that yeast diversity was higher in the fermented milk of the clay pot compared to fermented milk in the other containers and the raw milk. The lowest diversity of yeast species was obtained from samples fermented in gourd and nickel containers.

The distribution of the yeast species amongst the containers revealed that only 13.04% of the yeast species were identified from fermented milk samples of all containers whereas 60.87% of the identified species were obtained from only a single container. Yeasts species present in the fermented milk of all four of the containers, comprised *Cr. curvatus*, *Deb. hansenii*, and *K. marxianus*. In contrast, *C. ernobi* and *Cr. albidus* were isolated only from the raw milk. All of these results corroborated the fact that the conditions provided by specific containers might have a selective role in favoring the growth of specific microorganisms as previously suggested by Beukes et al. (2001).

Different yeast species proved to be predominant in fermented milks produced in the four different containers. Fermented milk in the clay pot was represented by highest percentage of *Deb. hansenii* (20.7%) followed by *K. marxianus* (13.8%). In gourd, the most dominant yeast species were representatives of *S. cerevisiae*

(22.9%), *K. marxianus* (17.1%) and *C. albicans* (17.1%), and *Cl. lusitaniae* (11.4%) in that order. In milk fermented in the nickel and plastic bowl, the most predominant species comprised of *Cr. curvatus*, *Deb. hansenii* and *K. marxianus*. In general, except in fermented milk of the gourd where *Cr. curvatus* and *Deb. hansenii* were found at lower proportions, in all other containers the most frequently occurring representative species were *Cr. curvatus*, *K. marxianus*, and *Deb. hansenii*.

4. Conclusion

The use of different containers, namely clay pot, gourd (calabash), nickel jar and plastic bowl, for the production of Sethemi has no significant effect on the microbial loads of the product. It is, therefore, possible to produce a more or less similar fermented milk (Sethemi) with respect to the number of microbes actively playing role during fermentation. Differences in yeast diversity, however, were detected between the milk samples fermented in the different containers. These differences in the type of species present may in turn contribute to differences in taste, smell, texture etc. of the final product. Similarly, the specific type of lactic acid bacteria associated with a specific container may further contribute to differences in the final product and needs to be investigated.

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Table 1: Different technologies applied in the preparation of indigenous fermented dairy products.

S.N.	Technology	Country	References
1.	Making suitable containers mostly from gourd, woven grass, animal skin, clay and hollowed wood	Ethiopia	FAO (1990)
2.	Seasoning the containers before use	Kenya, Ethiopia	FAO, 1990; Ganguly *et al.*, 1999
3.	Cleaning the milk vessels using plant materials and pebbles		FAO, 1990; Gonfa *et al*., 2001
4.	Disinfecting the vessels using wood smoke	Ethiopia , Kenya	Kurwijila, 1989; Kassaye et al., 1991; Kimonye and Robinson, 1991; FAO, 1990
5.	Back slopping (using a portion of the previous batch of fermented milk as starters)	Egypt	El-Gendi, 1983; FAO, 1990
6.	Curdling by lactic acid fermentation without employing defined starter cultures and prior heat treatment	Zimbabwe	FAO, 1990; Mutukumira *et al*., 1995
7.	Flavouring fermented milk using smoke or extracts of plant materials	Kenya, Ethiopia	Kurwijila, 1989; Kassaye *et al.*, 1991
8.	Milk coagulation using special enzymes from plant materials	Tanzania	FAO, 1990; Ganguly *et al.*, 1999
9.	Whey removal using specially made holes in the milk vessels or using siphons made from plants with hollow stalks	Ethiopia, South Africa	FAO, 1990; Kassaye *et al.*, 1991
10.	Concentrating the total milk solids by repeated removal of whey and addition of fresh milk	Egypt, Ethiopia	El-Gendy, FAO, 1990; Kassaye *et al.*, 1991
11.	Extracting the fat globules by shaking the curdled or coagulated milk to produce fresh butter		Ganguly *et al.*, 1999
12.	Cottage cheese made by heating the butter milk	Ethiopia	Ganguly *et al.*, 1999
13.	Heating the fresh butter and adding flavouring spices as well as colorants to produce ghee, a product with long shelf-life	Ethiopia	FAO, 1990
14.	Addition of flour made from cereals to settle the impurities during ghee-making		FAO, 1990

Table 2: Comparison between the highest mean values of microbial counts in fermented milks produced in four different containers using a single factor ANOVA.

Organism	**Highest mean values of microbial counts for each container (Log_{10}CFU/ml)**								F-ratio	P-value	F-critical
	Clay	T	Gourd	T	Nickel	T	Plastic	T			
Coliform bacteria	6.92	2	6.54	2	6.91	2	6.86	2	0.20	0.89	3.24
LAB on MRS	9.20	3	9.21	3	9.29	2	9.38	3	1.26	0.32	3.24
LAB on M17	9.14	3	9.15	3	9.19	2	9.33	3	0.71	0.56	3.24
Yeast	5.93	5	6.26	5	6.20	4	6.34	4	0.83	0.50	3.24
TAMC	9.20	3	9.24	3	9.24	2	9.37	3	0.82	0.50	3.24

T = Time (Days), LAB = lactic acid bacteria, TAMC = Total aerobic mesophilic count

Table 3: Comparison between combined mean values of microbial counts of fermented milks produced in different containers using GLM ANOVA

Organism	**Combined mean values of microbial counts (Log_{10}CFU/ml) for each container**				**F ratio**	**Probability level**	**F-critical**
	Clay	Gourd	Nickel	Plastic			
Coliform bacteria	6.16	5.86	6.37	6.10	0.78	0.51	3.69
LAB on MRS	8.94	8.90	9.06	9.08	5.60	0.001	3.69
LAB on M17	8.88	8.85	9.02	9.05	4.59	0.005	3.69
Yeast	5.54	5.86	5.83	5.99	3.92	0.011	3.69
TAMC	8.93	8.94	9.10	9.13	2.06	0.109	3.69

LAB = lactic acid bacteria, TMAC = Total aerobic mesophilic count

Table 4: Comparison between the lowest pH values of Sethemi produced in different containers

Container	Lowest pH (Mean ± SD)	Time (Day)	F-ratio	Probability level	F-critical
Clay pot	4.26 ± 0.07	6	5.23	0. 01	3.24
Gourd	4.10 ± 0.13	6			
Nickel	4.16 ± 0.10	6			
Plastic bottle	4.32 ± 0.06	4			

Table 5: Yeast diversity within raw milk and *Sethemi* in four different containers (Presence of yeasts indicated as positive; fermentation represents 5 repetitions in each container).

YEAST SPECIES	RAW MILK	FERMENTED MILK			
		Clay pot	Gourd	Nickel	Plastic
C albicans	+	+	+	-	+
C. ernobi	+	-	-	-	-
C. parapsilosis	+	+	+	-	-
C. rugosa	-	+	-	-	-
C sake	-	+	-	-	-
C. tenuis	-	-	-	-	+
C. tropicalis	-	-	+	-	-
Cl. lusitaniae	+	+	+	-	-
Cr. albidus	+	-	-	-	-
Cr. curvatus	+	+	+	+	+
Cr. laurentii	-	-	-	-	+
D. anomala	-	-	+	-	-
Deb. hansenii	+	+	+	+	+
K. marxianus	+	+	+	+	+
P. anomala	-	+	-	-	-
P. pini	-	+	-	-	-
P. scolyti	-	+	-	-	-
R. glutinis	-	+	-	-	-
R. mucilaginosa	-	+	-	-	-
S. cerevisiae	-	-	+	-	-
Sp. roseus	-	+	-	-	-
T. delbrueckii	-	-	+	-	-
Y. lipolytica	-	+	-	+	-

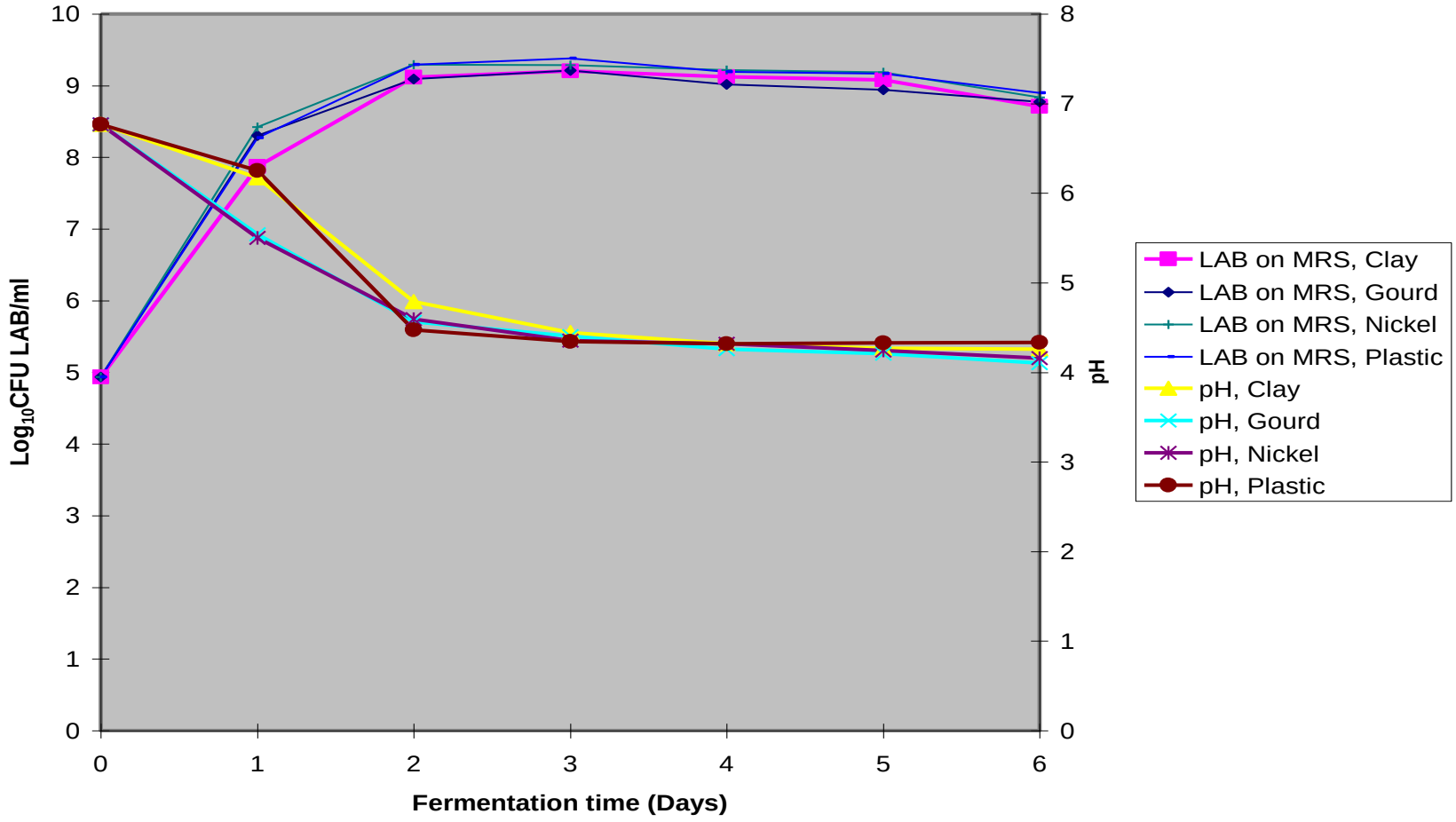

Fig. 1 a: LAB counts (LAB on MRS) and pH profile in *Sethemi* produced at 25ºC using four different containers

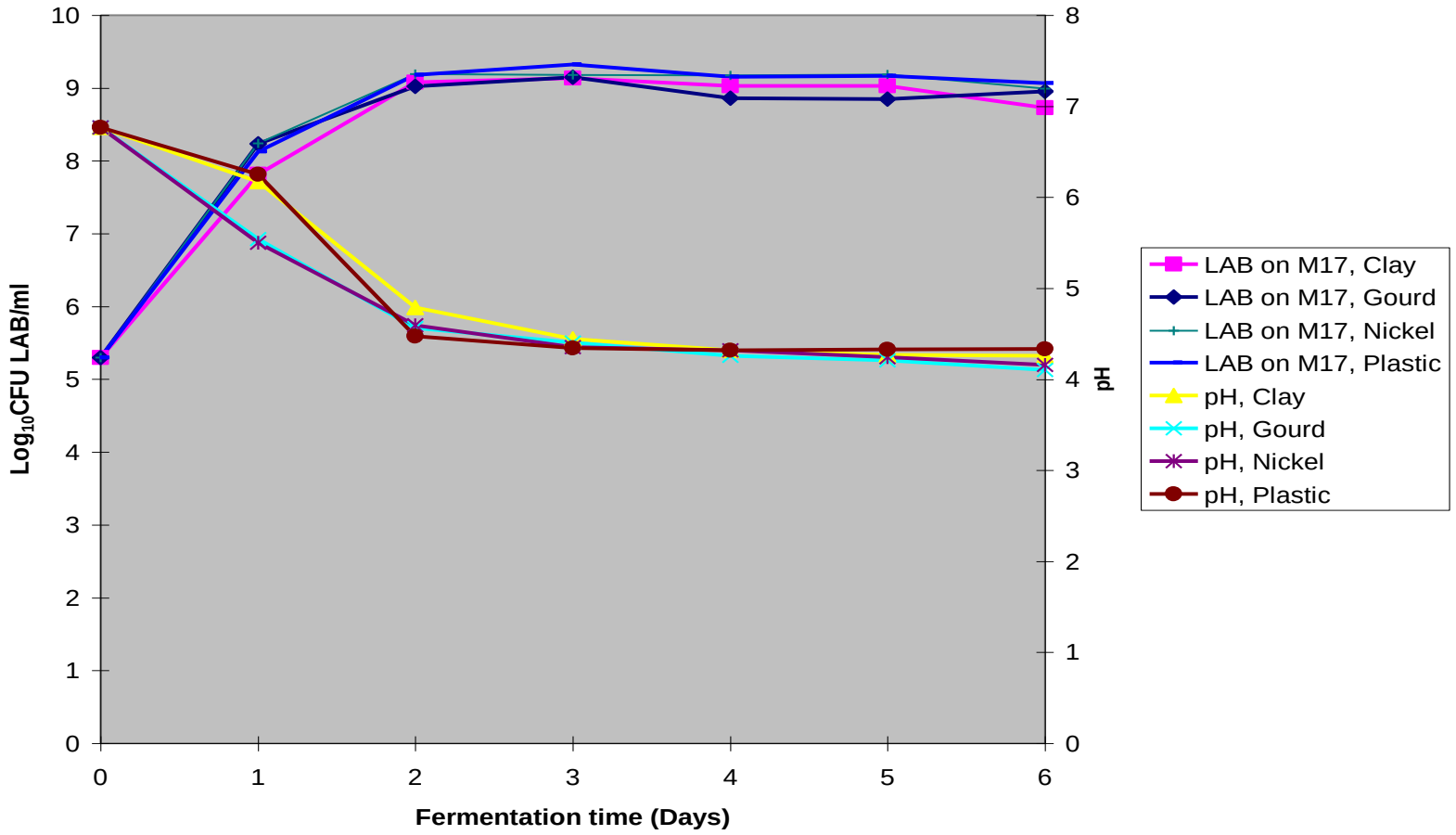

Fig. 1b: LAB counts (LAB on M17) and pH profile in sethemi produced at 25oC using four different containers

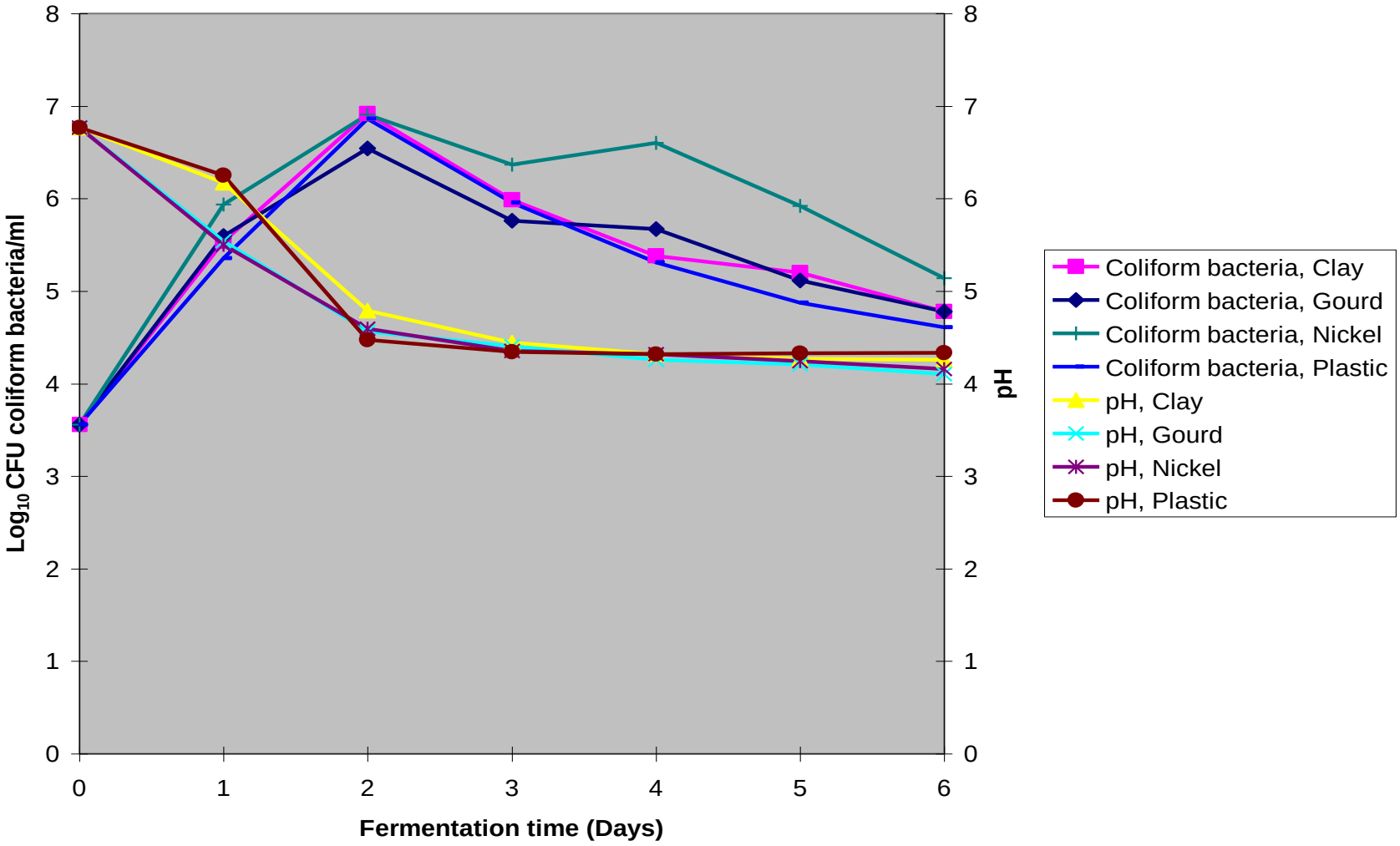

Fig. 1c: Coliform bacteria counts and pH profile in Sethemi produced at 25oC using four different containers

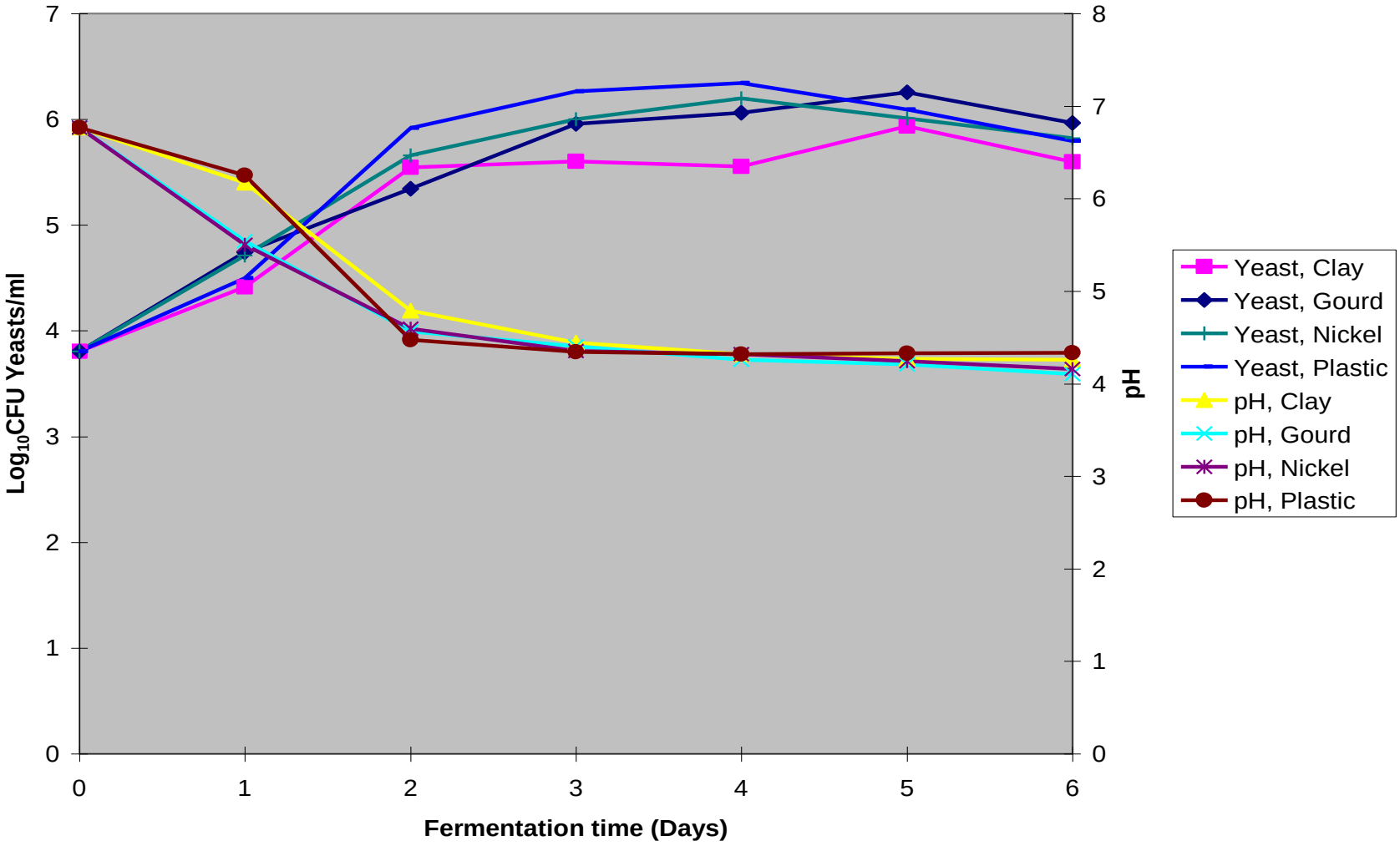

Fig. 1d: Yeast counts and pH profile in Sethemi produced at 25oC using four different containers

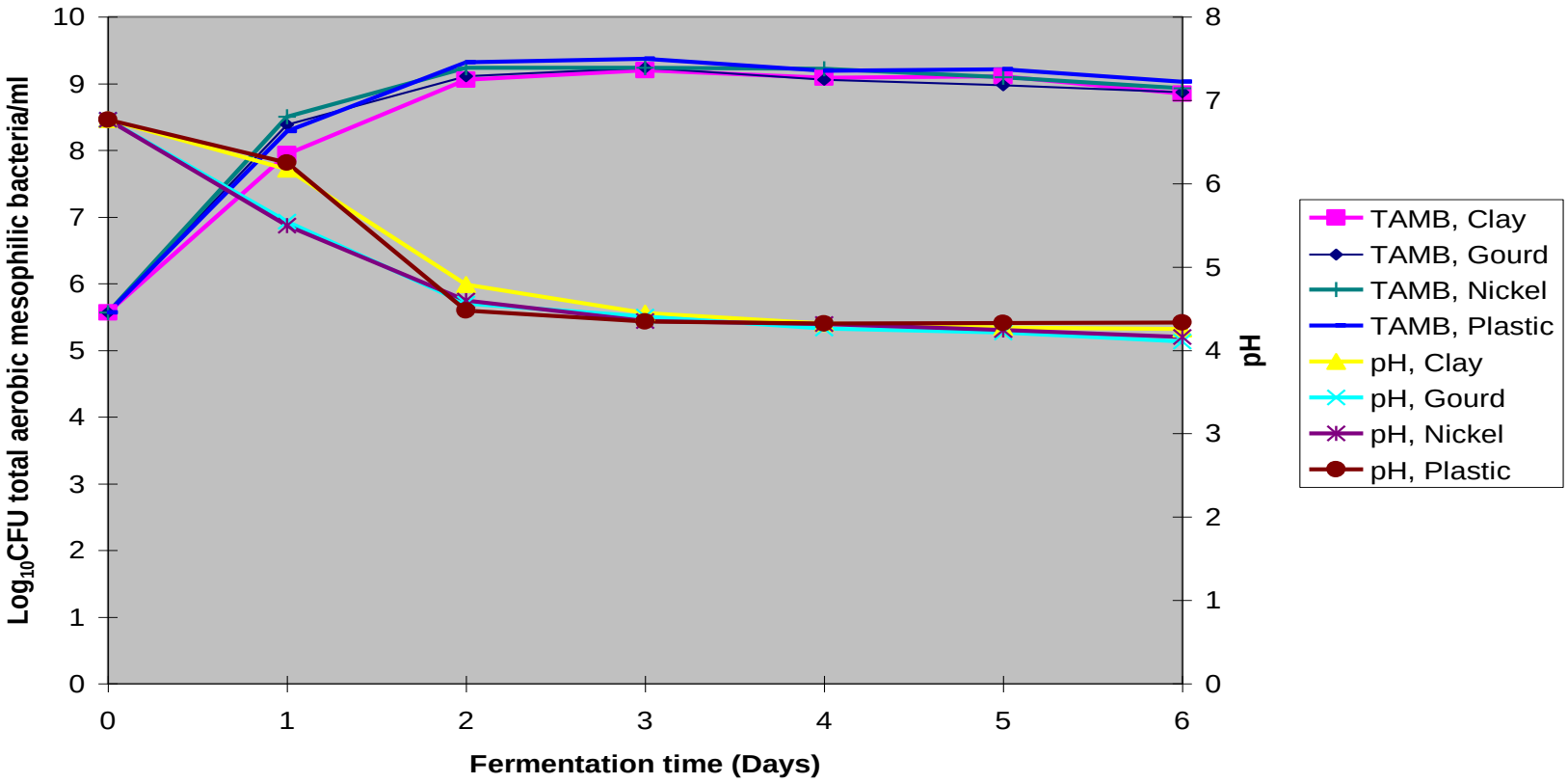

Fig. 1e: Total aerobic mesophilic bacteria counts and pH profile in Sethemi produced at 25oC using four different containers

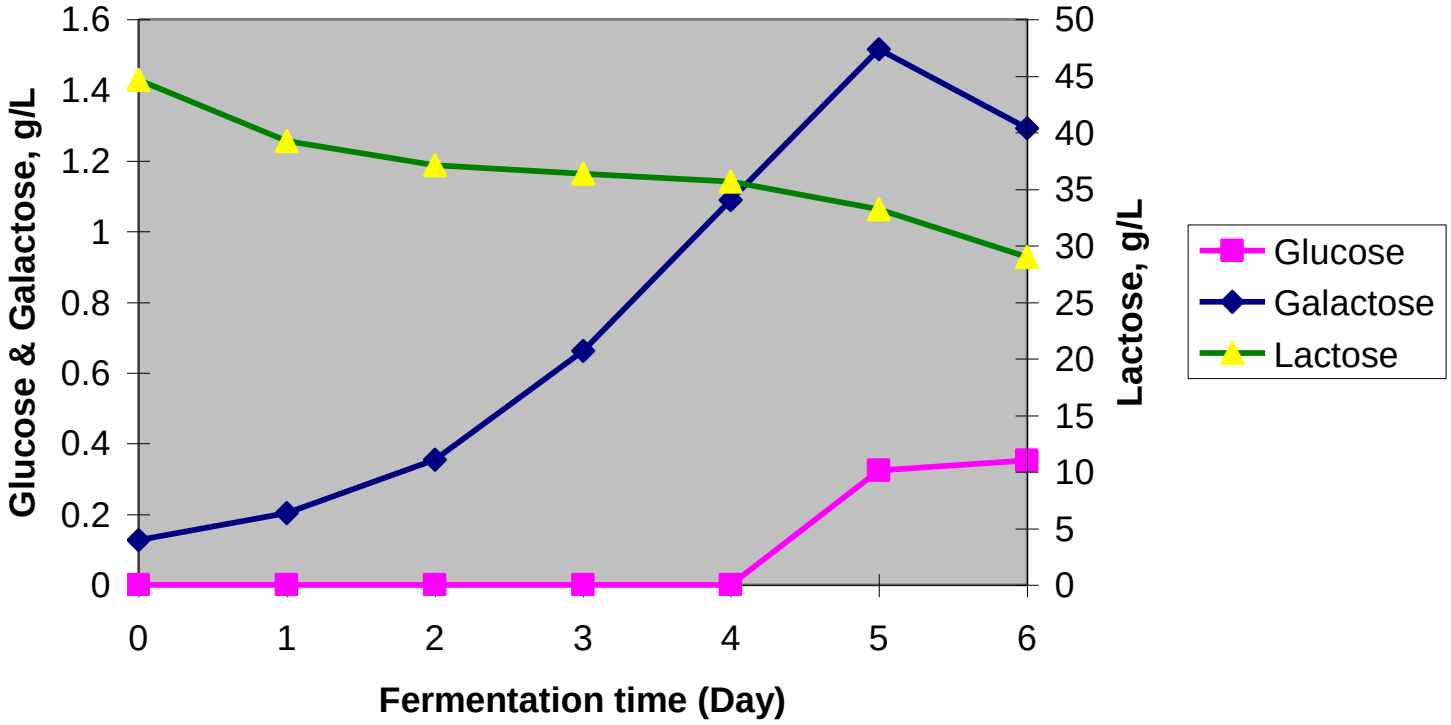

Fig. 2a: Levels of sugars during milk fermentation in clay pot

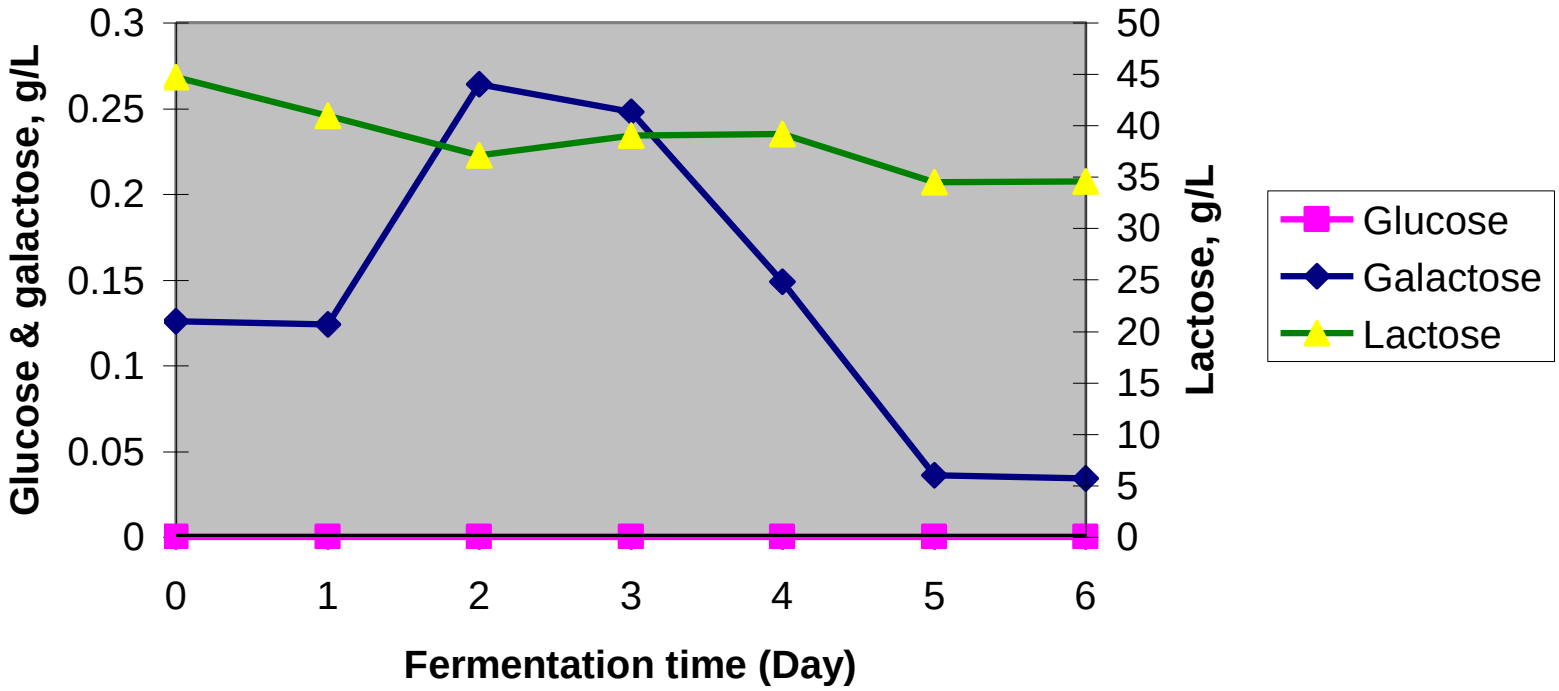

Fig. 2b: Sugar levels during milk fermentation in gourd

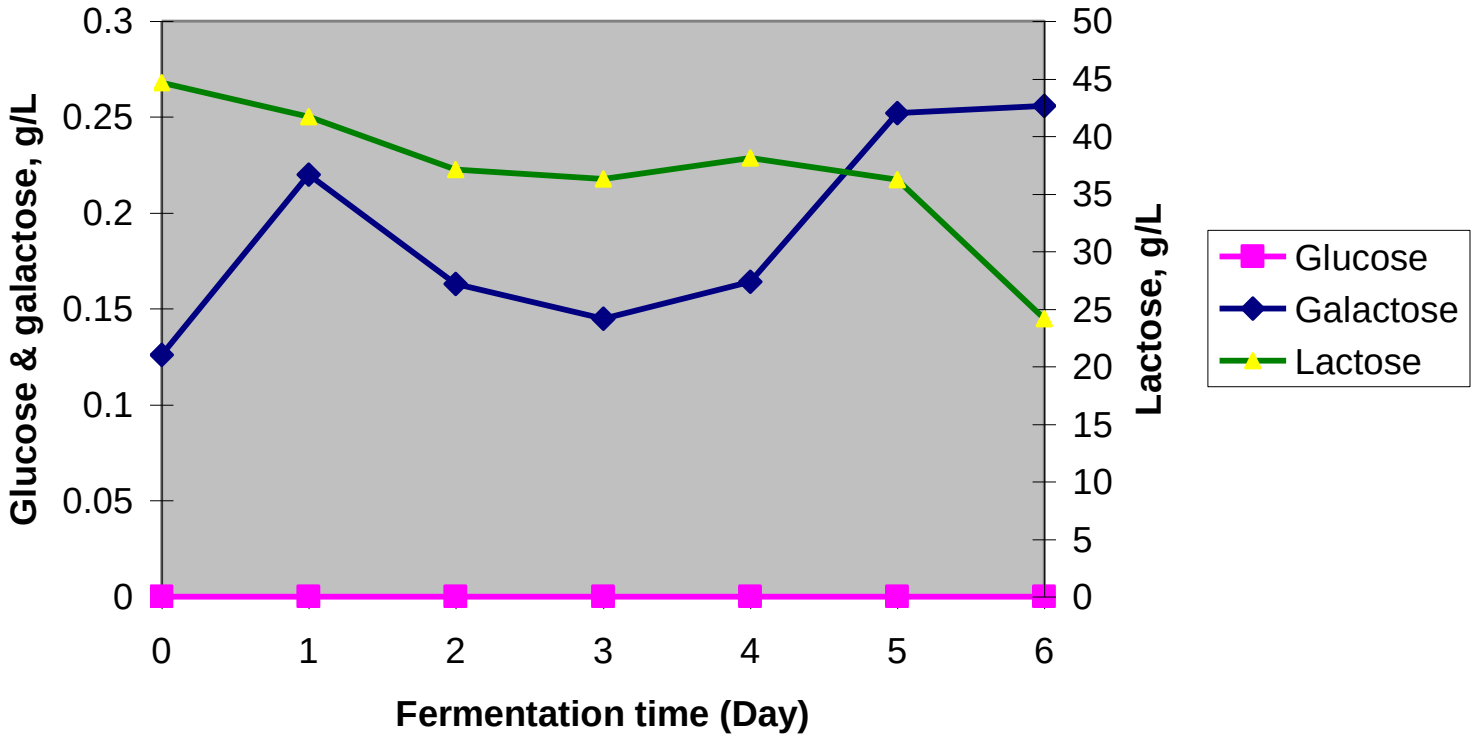

Fig. 2c: Sugar levels during milk fermentation in nickel container

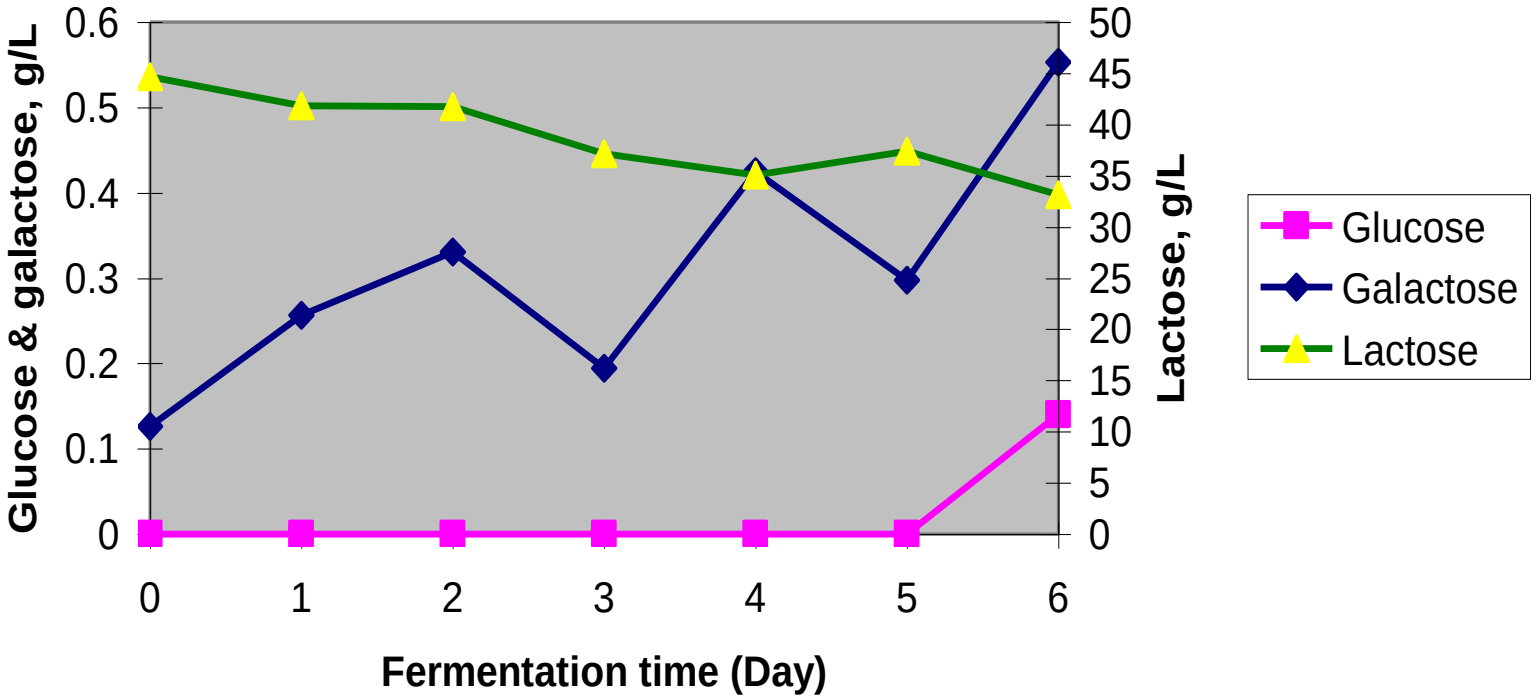

Fig. 2d: Sugar levels during milk fermentation in plastic bowl

CHAPTER IV

The effect of incubation temperature on the survival and growth of yeasts in *Sethemi*, a naturally fermented milk

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Abstract

The growth of yeasts in raw milk incubated at 7, 15, 25 and 37^{o}C, was monitored over a period of 40 days. The different temperatures were selected to represent the average ambient temperatures around Bloemfontein during winter, spring, summer and in the human body, respectively. *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Candida albicans* and *Debaryomyces hansenii* previously isolated from *Sethemi*, a naturally fermented South African milk product, were inoculated individually and in mixture into the raw milk and growth patterns were monitored. The growth of lactic acid bacteria in the inoculated raw milk and un-inoculated raw milk was also monitored. It was observed that the yeasts grew in the raw milk at all temperatures. The lactic acid bacteria also grew to high numbers (9 $\log_{10}$ CFU ml^{-1}) both in the un-inoculated and the inoculated milk samples. Addition of the different yeast strains seemed to stimulate the growth of the LAB and hence the rapid reduction of pH, except in the case of milk inoculated with *C. albicans*. This yeast seemed to have an inhibitory effect on the LAB. Of the four yeast species studied, *S. cerevisiae* showed the most substantial stimulatory effect on the numbers of

LAB. In addition, final yeast counts in samples inoculated with *C. albicans* and incubated at 37^{o}C were significantly ($p<0.05$) lower than in the other samples, suggesting that *C. albicans* did not grow well in the milk at this temperature. Overall, it was observed that 25^{o}C was the ideal temperature for the spontaneous fermentation of the milk to give Sethemi with a clean, viscous coagulum.

Key words: yeasts, naturally fermented milk, Sethemi, lactic acid bacteria

1. Introduction

Yeasts are widely distributed in nature and as a result they have a number of opportunities for entering into both commercially and traditionally produced fermented milks during preparation. This is clearly evidenced by the fact that they have been frequently encountered and isolated from a variety of fermented milks. Counts ranging from 10^3–10^7 CFU ml^{-1} have been reported by several researchers (van Uden and Carmo Sousa, 1957; Fleet and Mian, 1987; Suriyarachchi and Fleet, 1981; Green and Ibe, 1986; Viljoen *et al*., 2003). Depending on the type of the fermented milk produced, their contribution may either be positive or negative. In yoghurt, their occurrence is mainly a consequence of contamination and hence they are the major cause of spoilage (Fleet, 1990), whereas in the commercial production of Kefir and Koumiss they are deliberately introduced into milk to bring about the desired aroma and flavor of the final product (Chandan *et* al., 1969; Wyder, 1998). In naturally fermented milks they occur as members of the indigenous microflora (Gadaga *et al.*, 2000; 2001; Abdelgadir *et al*., 2001). Yeast counts of up to 6.1 $\log_{10}$ CFU ml^{-1} were reported in Sethemi, a South African naturally fermented milk product.

Sethemi is a fermented milk product with pH ranging from 4.1 to 4.3 and is produced by allowing raw milk to spontaneously ferment in gourds or clay pots. The characteristics of Sethemi are influenced by the type of the predominant fermenting microorganisms including lactic acid bacteria and yeasts. There is a lot of manual handling during the traditional preparation of Sethemi and the hygienic conditions are often poor. This will result in high levels of contamination with all sorts of microorganisms including yeasts. Thus, a non-agitated Sethemi, in both winter and summer is very likely to support the growth of yeasts.

In a previous study, seven yeast species, namely*, Debaryomyces hansenii*, *Candida albicans*, *Cl. lusitaniae*, *Cryptococcus curvatus*, *Kluyveromyces*

marxianus, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica* were found to be the common yeast species associated with Sethemi (Chapter 2). Of these, *Deb. hansenii*, *C. albicans*, *K. marxianus* and *S. cerevisiae* were the most predominant and were selected for further study. Previous studies have shown that some of these species metabolise different constituents of milk. For example, *D. hansenii* assimilates lactate, while *K. marxianus* is capable of fermenting lactose and breaks down milk protein and fat (Fleet, 1990; Ferreira and Viljoen, 2003). *S. cerevisiae,* on the other hand, does not have the ability to utilize milk lactose, protein or fat (Fleet and Mian, 1987; Fleet, 1990), but it is commonly associated with fermented milks (Gadaga *et al*., 2000; Abdelgadir *et al*., 2001; Jespersen, 2003). *Candida albicans* is an opportunistic pathogen that can cause superficial, localized, and/or systemic infection (Ryan, 1990; Bodey, 1993). The extent to which this potentially dangerous yeast can grow in naturally fermented milks under different environmental conditions must be thoroughly studied before any kind of conclusion can be made concerning the safety of Sethemi.

The objective of the current study was, therefore, to investigate the growth behavior of these selected yeasts under different temperatures and in association with lactic acid bacteria in order to understand the mechanisms that can be used to improve the quality of naturally fermented milks.

2. Materials and methods

2.1. Materials

The natural fermented milk trials were done at the University of the Free State (UFS), Department of Microbial, Biochemical and Food Biotechnology. Raw milk collected in sterile 5L bottles from a dairy farm near Bloemfontein, South Africa, was transported to the laboratory in a cooler box and kept in the refrigerator for less than 1 h before analysis.

Actively growing pure cultures of *D. hansenii, C. albicans, K. marxianus* and *S. cerevisiae* strains previously isolated and identified from Sethemi were used. These were kept at the UFS on Yeast extract-Malt extract (YM) agar slants at 5^{o}C. The cultures were purified by plating 3 times on YM agar and incubating them for 72 hrs at 25^{o}C.

2.2. Preparation of yeast inoculum

A pure colony of each of the four yeast strains was picked and transferred into YM broth (50ml) in 200ml Erlenmeyer flasks. The cultures were incubated at 25^{o}C for 24h with shaking. A portion of this culture (1ml) was further transferred to another flask containing YM broth (50ml) and incubated at 25^{o}C for 48h. The microbial load in each flask was estimated by making appropriate serial dilutions of portions of the broth culture and spread plating on YM agar. Volumes of the broth culture were calculated for each strain for direct inoculation into raw milk to give approximately 3 log CFU ml^{-1}. A mixed culture was obtained by mixing appropriate volumes (0.25ml) of the yeast strains to obtain a final volume (1ml) that would give approximately 3 log CFU ml^{-1} of yeast in the inoculated milk.

2.3. Fermentation

A calculated volume of each yeast strain was inoculated into raw milk (200 ml). For each strain, four different sets of inoculated milk were prepared. After mixing thoroughly, the inoculated milk was incubated at 7, 15, 25 and 37^{o}C, representing the mean temperatures for winter, spring/autumn, summer seasons in the Bloemfontein area, and that of the human body, respectively. The treatments were coded 1-6 as indicated below:

1. *K. marxianus*
2. *S. cerevisiae*

3. *C. albicans*
4. *D. hansenii*
5. Mixed yeast culture
6. Control

A portion (1ml) of the fermenting milk was withdrawn from each flask every day for day 1 to day 6, for microbiological and chemical analysis. From Day 7 to Day 20, the sampling interval was 2 days. Thereafter, samples were taken after every 4 days up to Day 40. At each sampling stage, yeast and LAB counts as well as pH were determined. The experiment was repeated three times.

2.4. Microbiological analysis

The yeast counts were determined by spread plating appropriate dilutions (0.1ml) of the fermenting milk onto Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Basingstoke, UK), and incubating at 25^{o}C for 72 hrs. LAB counts were determined by spread plating a similar volume onto MRS agar (Merck, Darmstadt, Germany), and incubating at 30^{o}C for 48h.

2.5. Determination of pH

pH was determined using a Hanna Instruments, HI 9321 pH meter (HANNA Instruments), calibrated using standard buffers at pH 4 and 7.

2.6. Statistical analysis

The differences in counts between the different treatments were compared using one way Analysis of Variance using the NCSS 2000 statistical software (Hlbtze, 1998). Pearson correlation test and the t-test were also determined using the same package.

3. Results and discussion

The results are presented as the mean of 3 replicate trials. Significant difference refers to $p<0.05$.

Sethemi was prepared using the traditional method by incubating raw milk in containers until a thick coagulum was formed. Because of the environment under which it is prepared, Sethemi is often contaminated with various types of yeasts and lactic acid bacteria. This study simulated typical fermentation conditions of the different seasons in Bloemfontein. Raw milk was also inoculated with single strains of four different yeast species or a mixed culture of the yeasts and allowed to ferment spontaneously.

At temperatures as low as 7 and 15^{o}C, the LAB showed a prolonged lag phase compared to the growth response at 25 and 37^{o}C, both with and without added yeast cultures (Fig. 1). In the un-inoculated naturally fermented milk (U-NFM), the highest LAB counts at 7^{o}C were about 9.8 $\log_{10}$ CFU ml^{-1}. This number was obtained after 12 days of fermentation (Fig. 1a). In the presence of *K. marxianus*, *S. cerevisiae*, *D. hansenii* and the mixed yeast culture (MYC), the maximum LAB counts were much higher than the U-NFM. This probably indicates some level of stimulation of the LAB by the yeasts. However, at the same temperature, the LAB counts in the presence of *C. albicans* were lower than in the U-NFM, suggesting possible inhibition due to the added yeast. In the presence of *K. marxianus*, the LAB counts were observed to decline especially after 20 days of fermentation. The final LAB counts in this case were significantly lower than all the other treatments.

A similar trend was observed at 15^{o}C (Fig. 1b). However, the lag phase was significantly reduced to less than 24h. The maximum LAB counts in the inoculated milk were much higher than the control (U-NFM). This difference was quite obvious after Day 5 at 15^{o}C. The LAB counts in all the yeast

inoculated milks also declined, and the lowest counts were recorded in the presence of *K. marxianus*.

At both 25ºC and 37ºC, growth of the LAB was rapid, with the highest counts (11.8 $\log_{10}$ CFU ml^{-1}) in the former case reached after 48h, while in the later (11 $\log_{10}$ CFU ml^{-1}), after 12h. The highest LAB counts at the two temperatures were therefore not significantly ($p>0.05$) different. What was obviously different was the time taken to achieve this number. The shorter time has implications on the quality of the fermented milk. Although a thick coagulum was obtained at 37ºC in all cultures, it only remained smooth for a very short period of time. After about 6h, there was evident gas production, disruption of the coagulum and separation of serum. At 25ºC, the highest LAB counts were obtained in the presence of *K. marxianus* and *S. cerevisiae*. However, after the fourth day, the LAB counts started to decline in all cases, especially in the presence of *K. marxianus*. For some reason, the LAB counts in the presence of the MYC did not decline in numbers as much as in the other cultures, up to day 10.

Yeast growth in the spontaneously fermented milk was also influenced by the incubation temperatures (Fig. 2a-d). The highest total yeast counts were recorded at 25ºC (Fig. 2c). Although growth was rapid at 37ºC, the yeast counts gradually declined to less than 5 $\log_{10}$ CFU ml^{-1} after Day 10. By this time, the milk incubated at 37ºC had already shown excessive gas production, separation of serum and development of off-odors.

It was interesting to note that at 25ºC, the yeast counts in the inoculated milk displayed similar trends as the total yeast count in the U-NFM. However, *K. marxianus* seemed to reach higher numbers (8 $\log_{10}$ CFU ml^{-1}) than the other strains. This was expected as *K. marxianus* is known to be lactose fermenting and can breakdown milk proteins and fat. *D. hansenii* had the longest lag phase but eventually had maximum populations of about 8 $\log_{10}$ CFU ml^{-1}. For *D. hansenii*, MYC, and U-NFM, the yeast counts

were similar at all temperatures. *S. cerevisiae* also grew well in the milk. However, it is important to note that these trends were actually reflecting the growth of total yeast. The inoculated milk had a head-start compared to the U-NFM. At all temperatures, the yeast counts in the milk inoculated with *C. albicans* were not significantly different from the counts in U-NFM, probably suggesting that *C. albicans* does not grow well in the milk. However, in general, when yeast was inoculated into the milk, the time to reach maximum populations for all cultures was significantly reduced.

All the yeasts seemed to survive in the milk on prolonged fermentation (Fig. 2a-d), indicating their ability to withstand the conditions in the fermenting milk.

Fig. 3 (a-d) shows the changes in pH in the fermenting milk at the different temperatures and the different yeast cultures. The pH of the fermented milk decreased significantly faster at the higher incubation temperatures. At 37^{o}C the pH dropped sharply from an average of 6.69 to about 4.3 in about 12h, while at 25^{o}C the pH was reduced to about 4.5 during the same period. At 15^{o}C the reduction in pH was less pronounced and took longer than at the higher temperatures. The lowest pH values were obtained in the milk inoculated with *D. hansenii* and incubated at 37^{o}C. This also corresponds to the lowest LAB counts recorded. In general, there were significant differences ($p<0.05$) in the final pH values of all the cultures at the different temperatures. The main effect on the final pH was therefore determined by the incubation temperature and not the type of yeast culture. This means that pH was mainly a function of LAB growth.

It is clear from the above that 25^{o}C seemed to be the ideal temperature for preparing Sethemi. It was possible to produce a smooth coagulum with high numbers of LAB in a reasonably short time. At this temperature, the yeast flora is also shown to grow to significantly higher levels, reaching maximum numbers about 2 days later than LAB. The gap between these fermentation

times, i.e. between the time of maximum LAB counts and that of the yeast counts, matches with the time at which a thick coagulum without any visually detectable gas production and alcoholic smell was formed. The implication of this result is that, during summer when the temperature in Bloemfontein and the surroundings approaches 25^{o}C, an organoleptically acceptable *Sethemi* could be produced but should be consumed within 2 to 4 days of natural fermentation. This will also depend on the hygienic practices during preparation. At 37^{o}C, on the other hand, the pH continued to drop until about the third day of incubation resulting in good growth of yeasts as well as in a sharp decline in the population of LAB. Since the yeasts are known to tolerate acidic conditions better than the bacteria, it appeared that they took advantage of the acidic condition and attained high levels of growth which was comparable to that observed at 25^{o}C incubation (Fig. 2c, d). Thus, in view of the sharp decline of LAB and the high growth of yeasts, depending on the dominant yeast species occurring in the milk, this particular fermented milk is very likely to be spoiled within 12 hrs after the coagulum formation. If consumed before this time, however, it might be safer than milk fermented at either 15^{o}C or 7^{o}C because its lower pH would be more inhibitory to the growth of pathogenic bacteria. Thus, during the hottest seasons, it would be advisable not to keep Sethemi on the shelf longer than one day. Longer incubation times required at 15^{o}C or 7^{o}C would result in the yeasts and psychrotrophic bacteria growing to high numbers and thereby spoiling the milk.

3.1. Potential yeast-LAB interaction

Interaction between the yeast and LAB was reflected by either stimulating growth of LAB or by retarding the rate of decline and thereby ensuring the survival of LAB for a relatively longer period of time in the fermented milk. The later type of interaction has been reported in cheese production where the yeasts were shown to increase the survival of LAB and thereby enhance the proteolytic process for cheese maturation (Devoyod, 1990;

Fleet, 1990; Lee and Lim, 1988; Yamauchi *et al*., 1975). La Rivière (1969) also reported that appreciable growth of some lactic acid bacteria occurred only in the presence of a yeast.

The existence of a symbiotic relationship would probably explain the growth and survival of all the yeasts in this study, since only *K. marxianus* is able to utilize lactose. As for the other yeasts, they are able to utilise either glucose or galactose or both (Drathen, 1987; Koroleva, 1984; 1991; Pintado *et al.,* 1996; Rosi, 1991). Therefore the LAB can assist the yeast flora by splitting the lactose into glucose and galactose and supplying the carbon sources or their metabolic end products. Furthermore, lactic acid bacteria possess a complex proteolytic system composed of proteinases, which initially cleave the milk protein to peptides (Kunji *et al*., 1996) and peptidases which cleave the peptides to small peptides and amino acids (Law and Haandrikman, 1997). These mechanisms would, therefore, make the environment inhabitable for yeasts like *S. cerevisiae* which are incapable of either utilizing end products of glucose metabolism or producing proteolytic enzymes. The yeasts in return may provide the LAB with growth factors. Studies have shown that the production of growth factors by yeasts promotes the growth and development of the bacterial flora (Valde´s-Stauber, 1997).

Similar stimulation of LAB by yeast has been reported in studies on ruminant diets, where *S. cerevisiae* is believed to assist in the digestion of the animal and thereby stimulating the growth of rumen bacteria. The yeast is thought to supply the bacteria with important metabolites (Martin & Nisbet, 1992; Dawson & Girard, 1997). However, *S. cerevisiae* is known to be lactose negative, non-lipolytic and non-proteolytic. It is therefore not clear how it was able to stimulate the LAB. It is however possible that the growth stimulation of lactic acid bacteria in this work could have resulted from both live cells of *S. cerevisiae* as well as from metabolites of autolysed cells of *S. cerevisiae*. Extensive research work showing the growth

promoting properties of yeast extracts originated from *S. cerevisiae* (Bibal et al. 1989; Milton et al. 1991; Jensen and Hamer, 1993; Ibrahim and Bezkororainy, 1994; Olmos-Dichara et al. 1997) and *K. marxianus* (Baralle and Borzani, 1988; Amrane and Prigent, 1993; Borzani *et al*., 1993) are available. This autolyzate has been found to contain important molecules like enzymes and B group vitamins among other substances (Vasallo *et al.*,1992; Chen *et al*., 1992; Schwarz and Hang, 1994; Hensing *et al*., 1994) that support lactic acid bacterial growth.

The observed decline of the LAB population after a short period of enhancement in the presence of *K. marxianus*-inoculated fermented milk (Fig. 1) is not unexpected. This can be explained by the fact that *K. marxianus* produced excessive gas and higher levels of alcohol compared to the other yeast species. Green and Ibe, (1986) previously reported that high levels of alcohol in fermented milk retard the growth of LAB.

Despite the presence of reports indicating that the growth of *C. albicans* is inhibited by compounds produced by LAB (Shahani, 1976) and lactoferrin (Andersson *et al.* 2000, Soukka *et al.*, 1992; Xu *et al.,* 1999), results obtained showed substantial growth of this species at 25^{o}C in the presence of a large number of LAB in the laboratory-produced Sethemi. Despite being an opportunistic human pathogen, with expected optimum growth at 37^{o}C, enhanced growth was detected at 25^{o}C whereas only minimal growth was observed at 37^{o}C. According to literature, the species is susceptible to medium-chain free fatty acids and their corresponding monoglycerides (Kabara et al., 1972; Bergsson *et al*., 2001). According to Bergsson *et al*. (2001), capric acid, a 10-carbon saturated fatty acid, and Lauric acid, a 12-carbon saturated fatty acid, were effective in killing *C. albicans*. Thus, there is speculation that the above-mentioned fatty acids and monoglycerides could have been produced in sufficient quantities at 37^{o}C. Further studies are, however, required in this regard to ascertain the real cause of the discrepancy.

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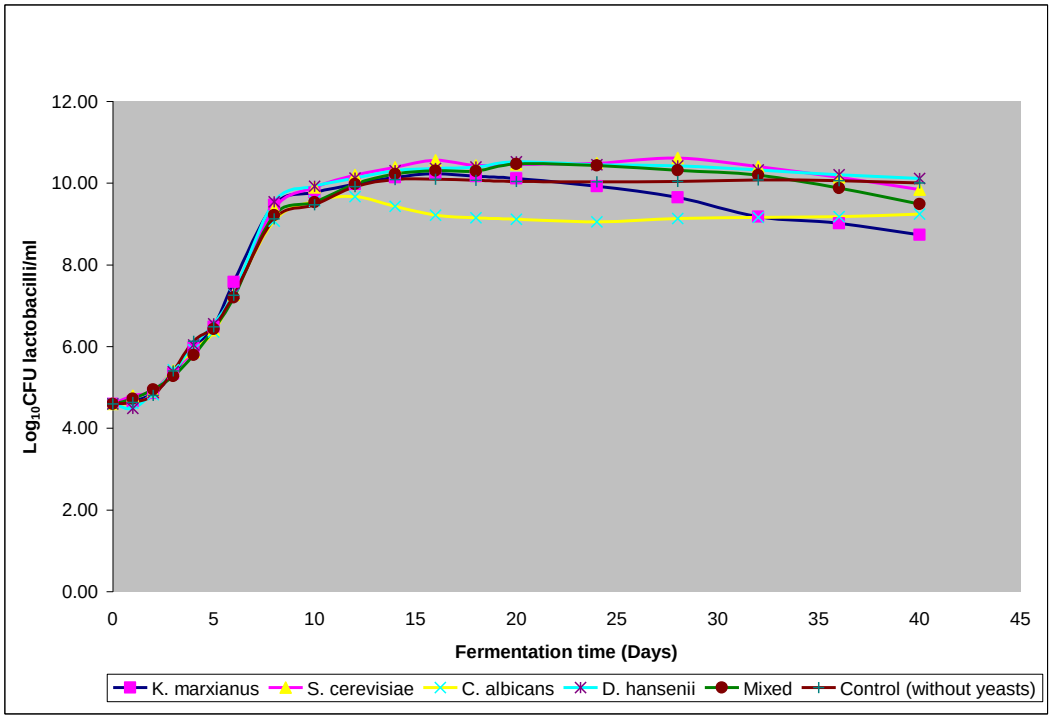
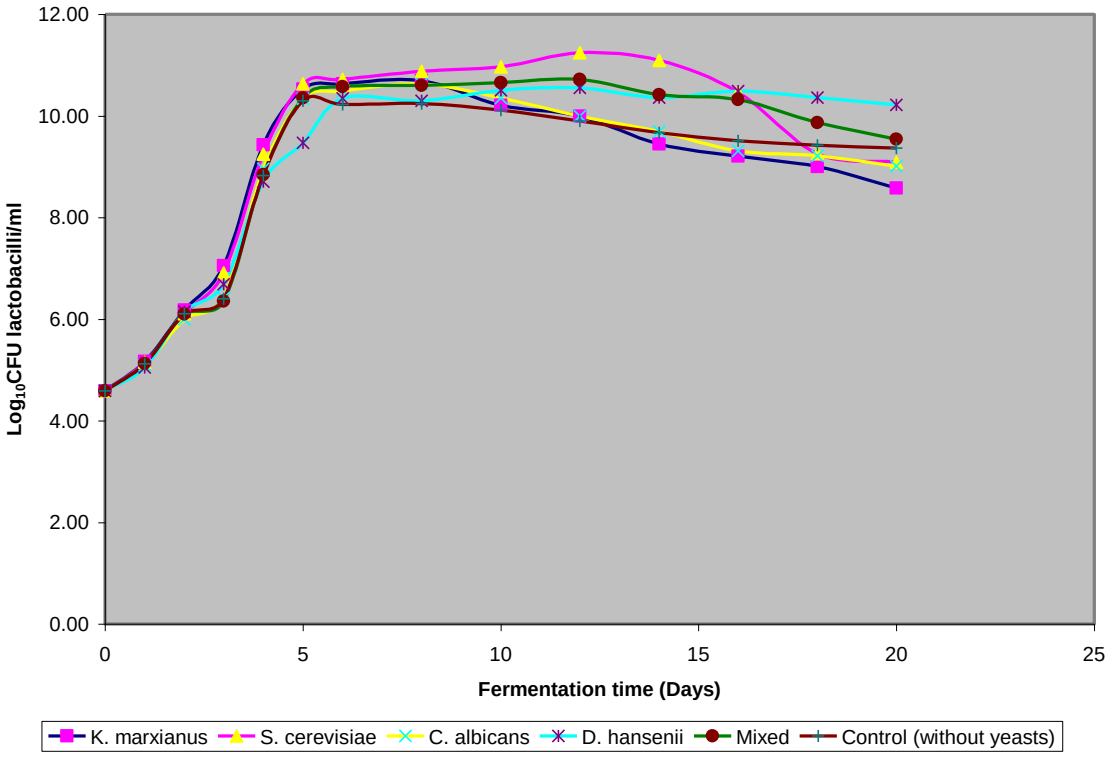


Fig. 1(a): Changes in numbers of lactobacilli-leuconostocs in yeast-inoculated and control NFM incubated at 7^{o}C



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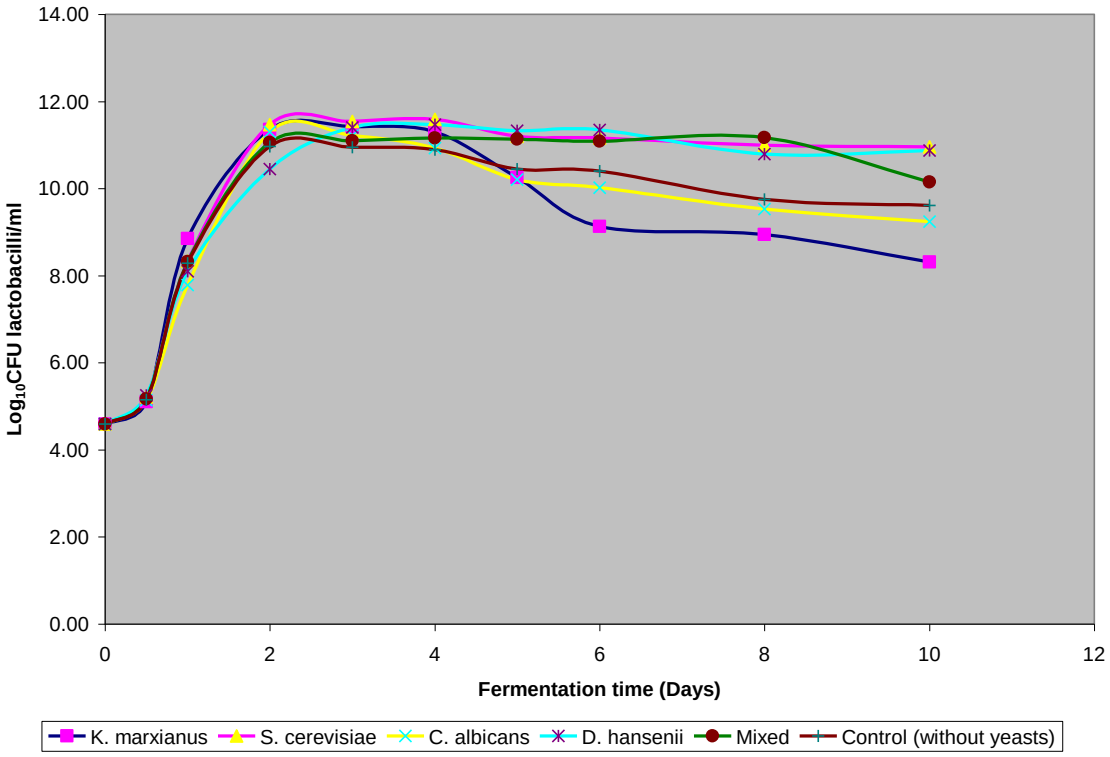

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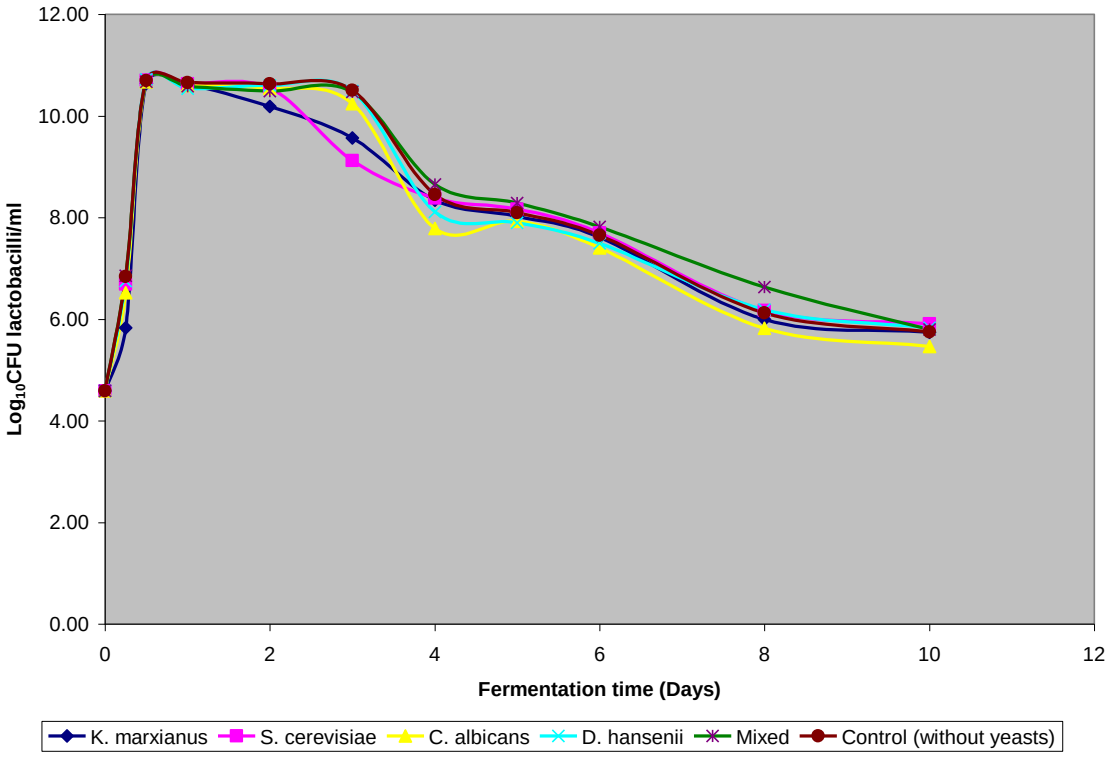

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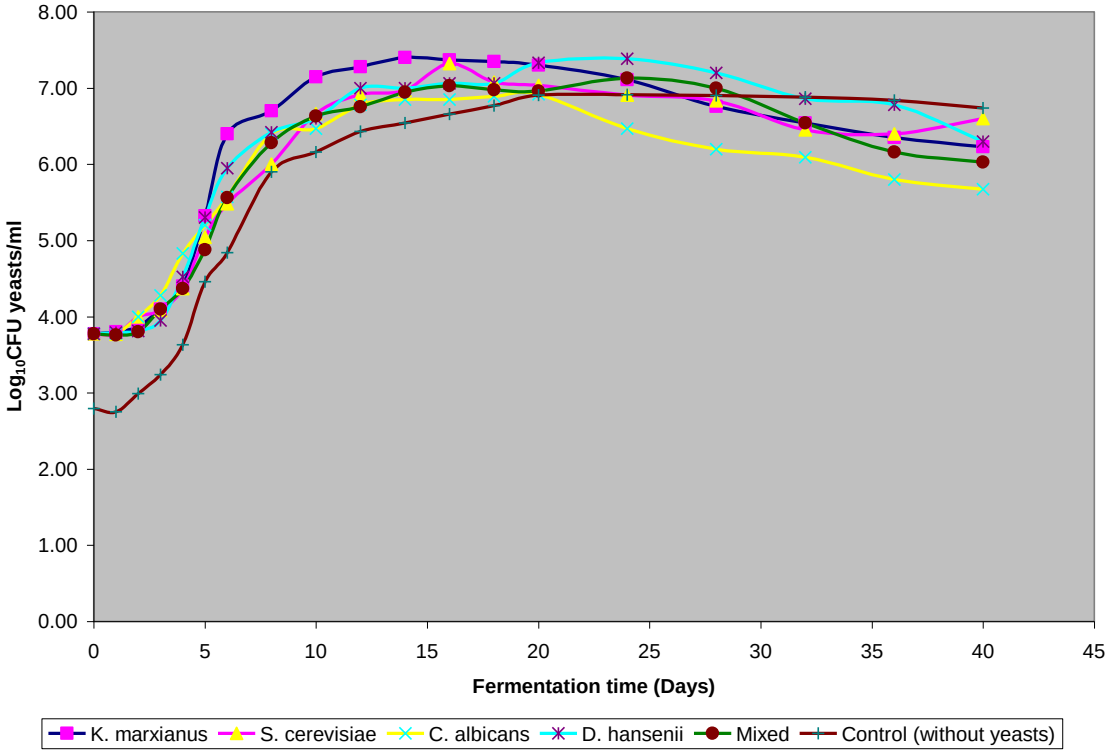
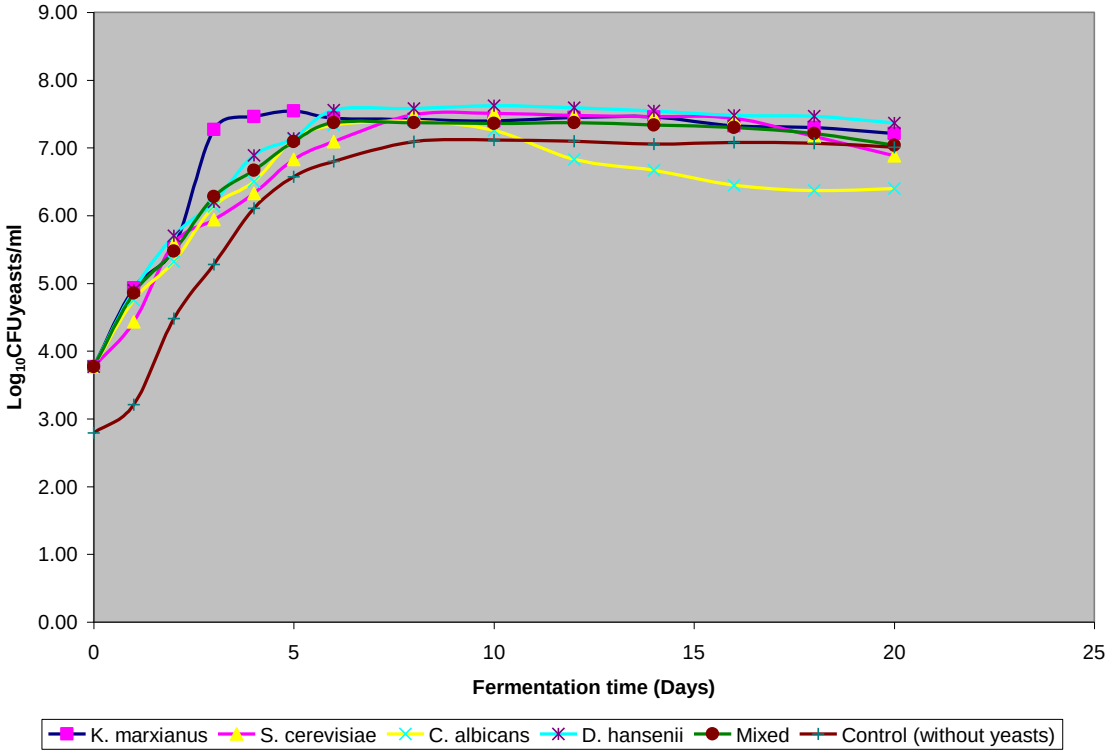


Fig. 2(a) Changes in total yeast counts in yeast-inoculated and control NFM incubated at 7ºC



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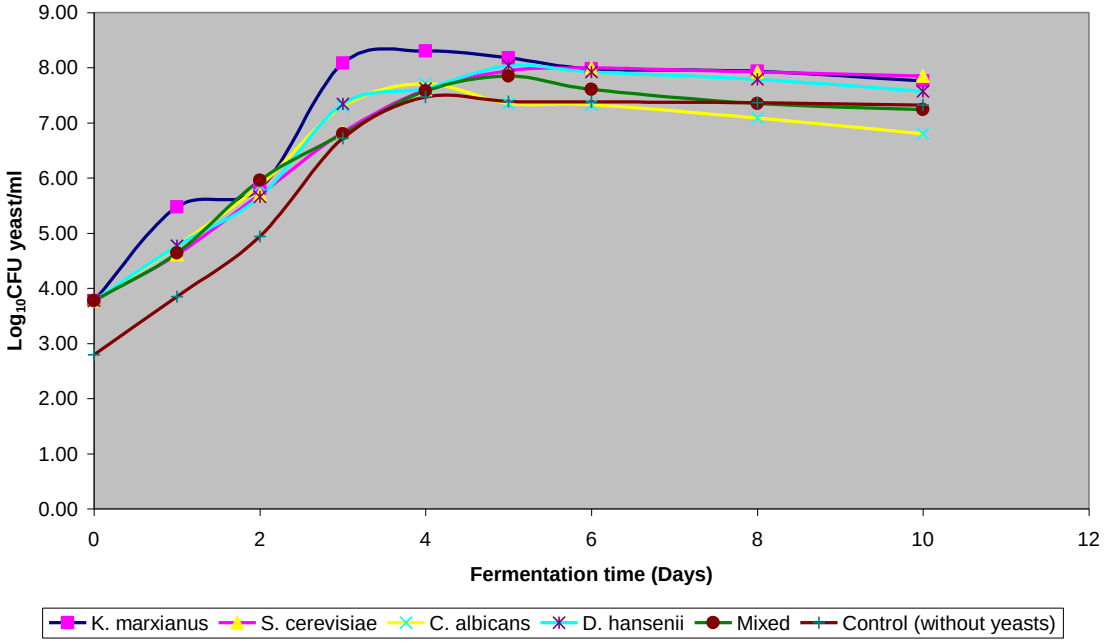
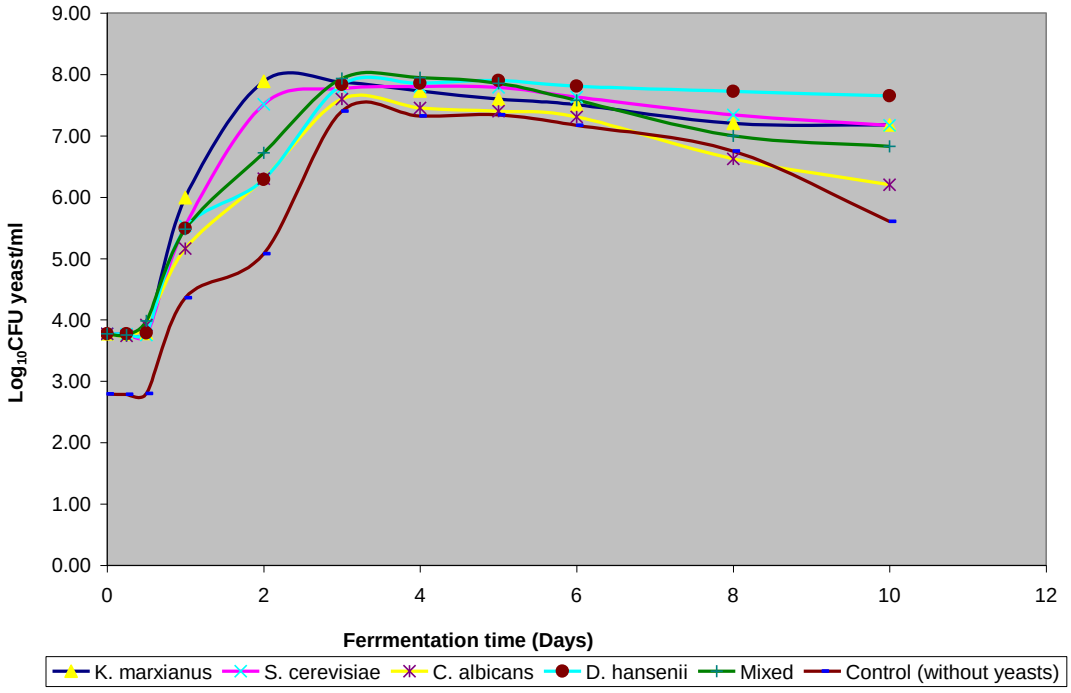


Fig. 2(c) Changes in total yeast counts in yeast-inoculated and control NFM incubated at 25^{o}C



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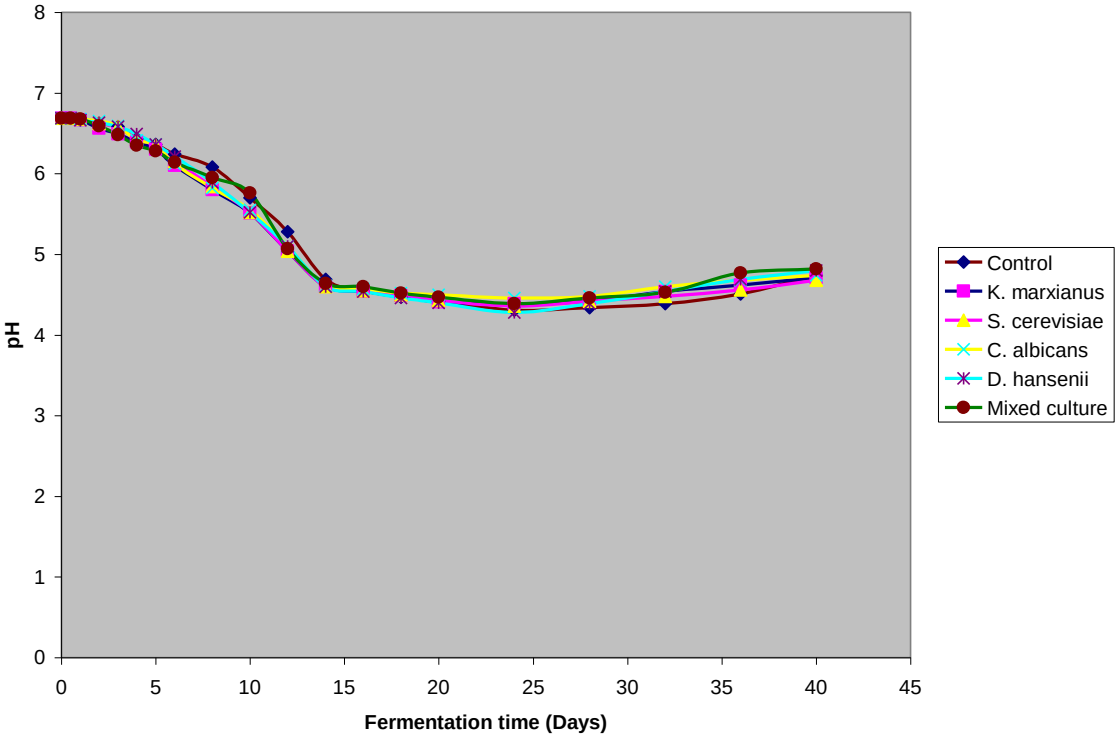

Fig. 3(a) Comparison between the pH profiles of 7°C incubated non-inoculated and yeast-inoculated NFMs

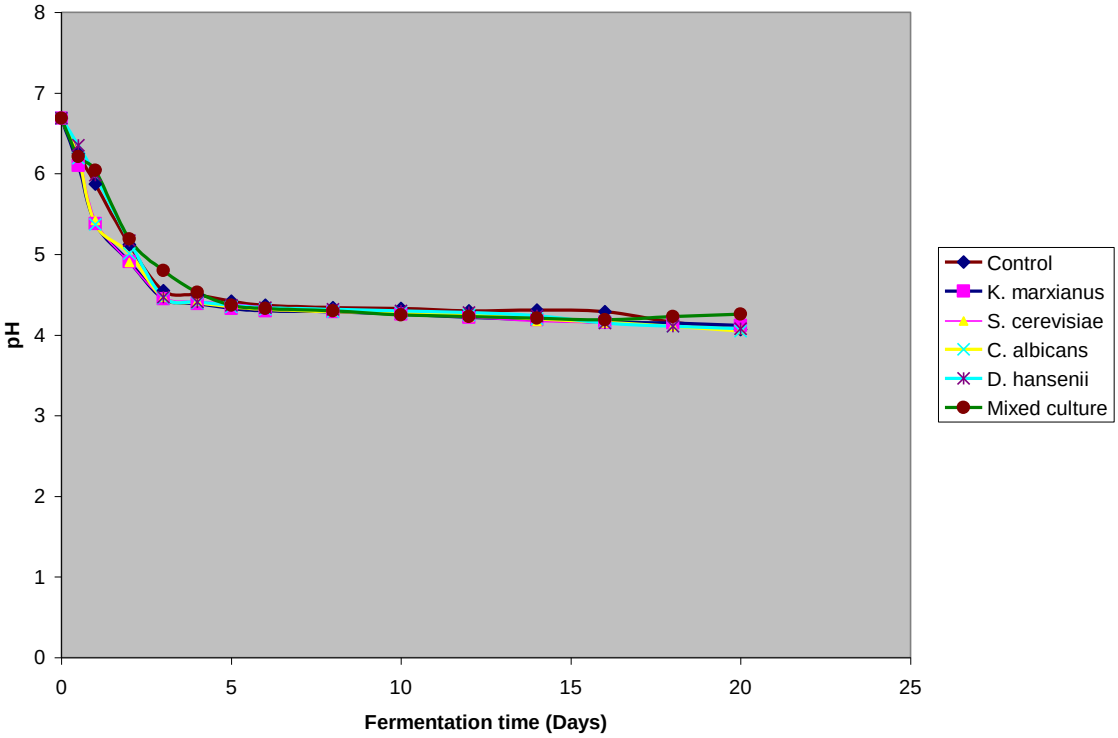

Fig. 3(b): Comparison between the pH profiles of 15^{o}C incubated non-inoculated and yeast-inoculated NFMs

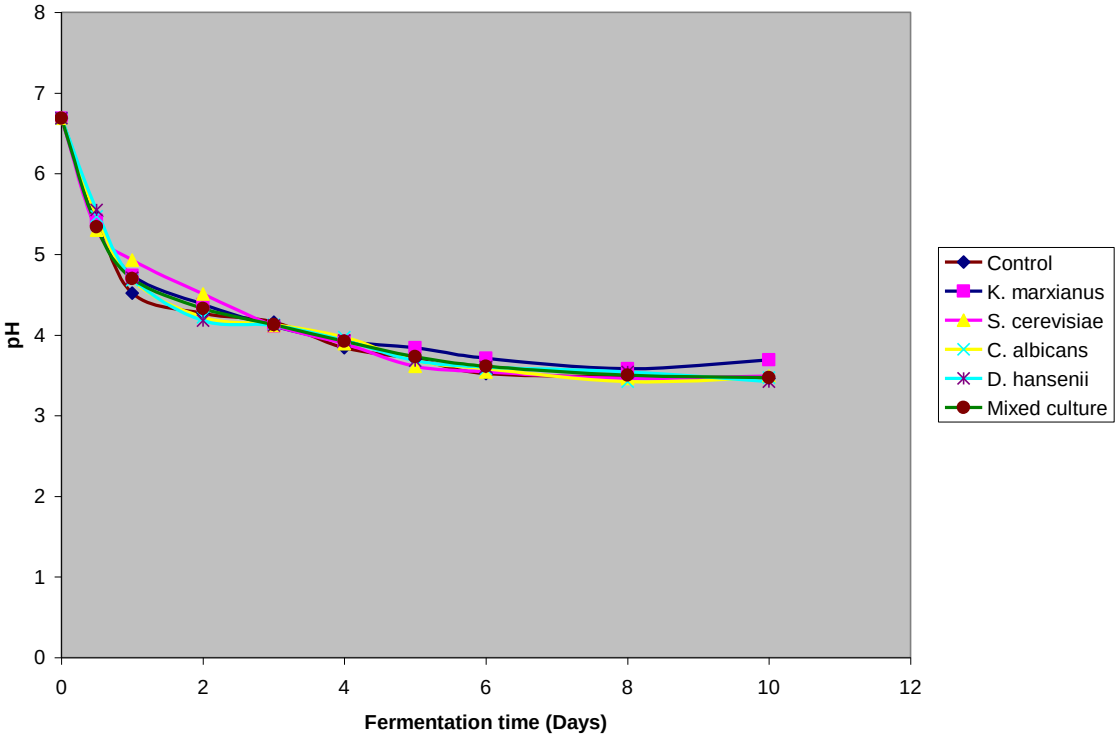

Fig. 3(c): Comparison between the pH profiles of 25^{o}C incubated non-inoculated and yeast-inoculated NFMs

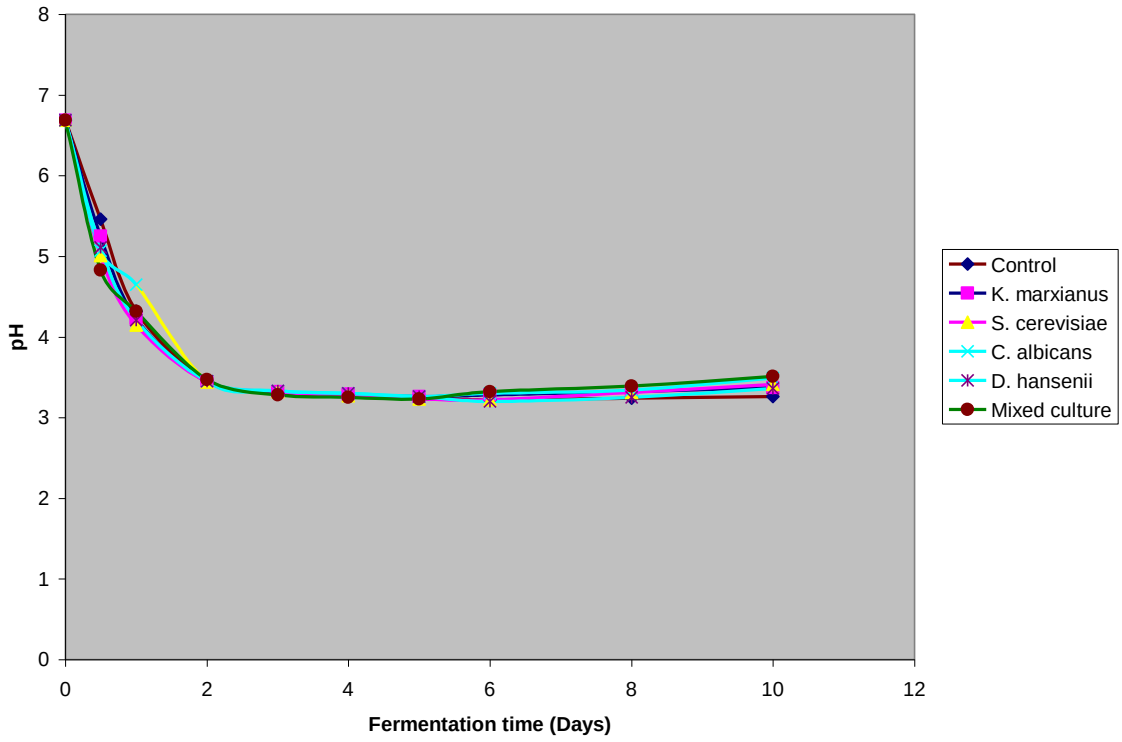

Fig. 3(d): Comparison between the pH profiles of 37^{o}C incubated non-inoculated and yeast-inoculated NFMs

CHAPTER V

The effect of residual antibiotic on the growth of LAB and yeasts in Sethemi (South African Naturally Fermented Milk)

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Abstract

The effect of residual levels of oxytetracycline and penicillin G on the growth of LAB and yeasts in raw and yeast-inoculated naturally fermented milks was studied. The results showed that in raw fermented milk, oxytetracycline and penicillin G significantly inhibited the growth of both lactobacilli-leuconostocs and lactococci at minimal concentrations allowed. In both antibiotic treatments, lactococci were more inhibited. Penicillin G was more inhibitory than oxytetracycline on the growth of LAB.

In oxytetracycline-treated fermented milks, the addition of *S. cerevisiae* and *D. hansenii* significantly reduced the inhibition while the addition of *C. albicans* and *K. marxianus* contributed to the inhibition. In contrast, in penicillin-treated milk, all four yeast species, namely *S. cerevisiae, D. hansenii, C. albicans* and *K. marxianus* added to the inhibition of the growth of LAB.

Yeast counts were stimulated by the addition of antibiotic treatments. Oxytetracycline-treatment resulted in a higher increase in yeast counts. The

highest increase numbers was usually associated with the highest concentration of antibiotics added.

1. Introduction

One of the major concerns of both large and small-scale dairy farms is ensuring high production of good quality milk products. Good quality milk and milk products, as measured by consumers and defined by researchers, are wholesome, of good appearance, good predictable taste and flavor, maintaining original nutritional qualities, safe from harmful microorganisms and substances, and having a long shelf-life (Loewenstein and Speck, 1992).

The quality and quantity of milk production are adversely affected by several factors of which disease is a major contributing factor at the dairy farms (Aiumlamai, 1999). The most common disease associated with dairy cows is mastitis (Gilmour, 1999), an inflammation of the udder caused by a variety of microorganisms, mostly bacteria, which gain access to the interior of the mammary glands through the teat canal. Currently, the disease is being recognized as a major animal health problem causing a serious wastage and undesirable milk quality in the tropics (Aiumlamai, 1999; Thirapatsakun, 1999). Both sub-clinical and clinical mastitis can cause a significant reduction in the amount of milk production in addition to affecting the quality of milk (Beck *et al.* 1992; Owens *et al.* 1997). At a chronic stage, the infection in a single quarter of the udder alone may result up to 25% loss in milk production apart from being a source of infection and causing a recurring health problem in the herd (Thirapatsakun, 1999). As a rule, cows with chronic mastitis case must be culled to control the spread of the disease (Browning *et al.*, 1994; Seguya *et al.*, 1998). However, small holder farmers are not willing to cull their cows (Aiumlamai, 1999) instead they tend to treat them with antibiotics repeatedly for as long as the disease continues to reappear. Often, milk produced from such cows as well as

from clinical and sub-clinical mastitic cows that are under treatment has been reported to contain levels of residual antibiotics. According to Jones (1999a) some of the suspected reasons for drug residues in milk are extended usage or excessive dosage of approved drugs, poor records of treatment, milker or producer mistakes of accidental transfer into bulk tanks, failure to observe recommended label withdrawal time, lack of advice on withdrawal period, prolonged drug clearance, treated-animal identification problems, multiple dosing, products not used according to label directions, withholding milk from treated quarters only, contaminated milking equipment, early calving or short dry periods, purchase of treated cows and use of dry cow therapy to lactating cows.

The residues belong to one or more of six major groups of antibiotics that are commonly used in veterinary medicine like aminoglycosides, penicillins and cephalosporins, macrolides, quinolones and fluroquinolones, sulphonamides, and tetracyclines (Mullan, 2001). Each group consists of a wide variety of antibiotics which are often known by different trade marks. Although a variety of these drugs may be in use, in South Africa, as in many developing countries, penicillin G and oxytetracycline are among the most frequently used ones by small holders and their use is highly unregulated (Hart and Kariuki, 1998; verbal communication with a local veterinarian). They are easily available in the marketplace and therefore small holders have easy access to purchase them from drug stores over the counter. Over-the-counter antibiotics are meant to be used in accordance with the label instructions under the supervision of veterinarians. However, since the supervision of a veterinarian incurs additional expense, administration of the drugs is often done by small holders without the supervision of the veterinarian. For this reason, the risk of antibiotic misuse and the resulting residue is expected to be very high in the rural areas.

Oxytetracycline belongs to a group of antibiotics called tetracycline and is routinely used in an extra-label manner for intrauterine therapy (Dinsmore

et al., 1996). It is a broad-spectrum antibacterial which is active against a wide range of Gram positive and Gram negative bacteria interfering with the production of proteins that the bacteria need to multiply and divide (Chopra *et al*., 1981).

Penicillin, on the other hand, is one of a group of bactericidal antibiotics, inhibiting the growth of some gram-positive bacteria and some spirochetes. There are many different types of penicillins, including synthetic ones, and their effectiveness varies for different organisms. Although most are still used clinically, their value has been diminished by the widespread development of resistance among target microorganisms and also by some people's allergic reaction to penicillin.

The penicillin's bactericidal effect is attributed to the inhibition of bacterial cell wall synthesis (Donowitz and Mandell, 1988). Penicillin G must penetrate the cell wall to attach to specific proteins on the inner surface of the bacterial cell membrane. In actively growing cells, the binding of penicillin within the cell wall leads to interference with production of cell wall peptidoglycans and subsequent lysis of the cell in a hypo- or iso-osmotic environment (Wright and Wilkowski, 1983; Papich, 1987; Donowitz and Mandell, 1988).

Although the rate of excretion and the concentrations of penicillin G and oxytetracycline found in milk may vary depending on the route of administration, dosage and the type of solvent used, both are believed to be distributed and excreted into milk in quantities sufficient to cause problems in health and production (Korsby *et al.,* 1962; Canon *et al.,* 1972; Anifantakis, 1982; Prescott and Baggot, 1993; Jones, 1999a; Katla *et al.,* 2001). In general, the amount of antibiotic excreted into milk may vary from eight to 80%, usually averaging about 50% (Mullan, 2001). This amount is high enough to evoke concerns over the use of antibiotics. Consequently, a number of researchers reported the potential danger of the irresponsible

use of these and other antibiotics in agriculture for promoting growth of animals as well as for preventing infection rather than for curing (French *et al*., 1987; Harvey and Mason, 1998; Khachatourians, 1998; Witte, 1998). According to these authors, the greatest concern of antibiotic misuse is the possibility that resistance developed by some microorganisms might be passed onto human pathogens through plasmids and make treatment of diseases difficult in the future. Notably, the risk becomes apparent when the antimicrobial used in animals is also used in humans or displays cross resistance with an antimicrobial used in human medicine (McKellar, 1998).

Apart from causing development of drug resistance in microbes, antibiotic residues may have other potential health risks such as allergic reactions in susceptible individuals, possible carcinogenicity upon prolonged exposure and direct toxic effects (Erkine, 1978; Varnam and Sutherland, 1994; Jones, 1999b; Bagley, 2004). In addition, residual antibiotics represent the most important group of exogenous inhibitory factors in milk and therefore interfere with the manufacture of several dairy products (Anifantakis, 1982; Varnam and Sutherland, 1994; Jones, 1999b; Tamime and Robinson, 1999). Often, concentrations above the MRL (Maximum Residue Limit) inhibit bacteria used in the fermentation process employed by the dairy industry (Tamime and Robinson, 1999). Furthermore, antibiotics are also known to decrease the acid and flavor production associated with butter manufacture, reduce the curdling of milk and cause improper ripening of cheeses (Jones, 1999a).

However, unlike with bacteria, antibacterial antibiotics have very little adverse effect on fungi [Poultry Industry Association of New Zealand (Inc.), 2002]. In fact, in human beings, administration of antibiotics often results in fungal infections of the mouth, digestive tract and vagina as a result of the destruction of the competing bacterial flora which help to prevent overgrowth of other organisms (Life Research Universal, 2001-2004). But it is not exactly known to what extent such residues affect the growth of

yeasts in naturally fermented milks. In a previous work four dominant yeast species isolated from Sethemi, i.e., *K. marxianus, S. cerevisiae, C. albicans,* and *D. hansenii* have been selected for their potential significance in health and production of fermented milks (Chapter II and Chapter IV). The impact of residual antibiotics on the growth of these yeasts as well as on the lactic acid bacteria in Sethemi has not been assessed. Thus, the objective of the present study is to investigate the effect of different levels of oxytetracycline and penicillin G on the survival of the yeasts and lactic acid bacteria in Sethemi.

2. Materials and Methods

2.1. Raw milk

Raw milk was collected from different sources around Bloemfontein, including households, small scale Sethemi producers, and selected dairy farmers supplying raw milk to the Sethemi producers. All the milk farmers practiced hand-milking of a limited numbers of cows (3-8). On three occasions, 7.2 L of hand-milked raw milk was purchased and transported to the laboratory in cooler boxes covered with ice. The samples were stored at refrigerator temperatures (7^{o}C) until required for analysis. All microbial analyses were performed within 1 hr after collecting the samples.

2.2. Antibiotics

Two commonly used antibiotics for the treatment of dairy cattle (Jones, 1999a), i.e., oxytetracycline [oxytetracycline dihydrate ($C_{22}H_{24}N_2O_9.2H_2O$), FW = 496.5, O.5750, SIGMA Chemical Co., USA] and penicillin G (Benzylpenicillin sodium salt, $C_{16}H_{17}N_2O_4SNa$, FW = 356.4, 1730 units penicillin G base per mg, P-3032, SIGMA Chemical Co., USA) were applied in this study to evaluate their effect on the survival of lactic acid bacteria and yeasts in naturally fermented milks. Three levels of each antibiotic were prepared by dissolving the antibiotics in sterile distilled water. The MRL

(Maximum Residual Limit) recommended for the respective antibiotics was the minimum residual level used in this study. The MRL values for oxytetracycline and penicillin following the recommendations of the European Union (EU) are 100 μg and 4 μg, respectively (Commission Regulation, EU, 1990). Levels 100, 500, 2500 μg and 4, 20, 100 μg of oxytetracycline and penicillin, respectively, were selected to simulate the range of levels of residual antibiotic occurrence in raw milk.

2.3. Yeast cultures

Yeast species, previously isolated from laboratory-produced Sethemi and determined as dominant species were used as test organisms. These included *Debaryomyces hansenii, Candida albicans, Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. The cultures were maintained on YM slants (Yeast Extract-Malt Extract-Glucose Agar Medium consisting of: Glucose, 10g; Yeast extract, 3g; Peptone, 5g; Malt extract, 3g; Agar, 20g per L distilled water) in screw-capped McCartney bottles at 5^{o}C and transferred once in a month to freshly prepared YM to ensure viability. All cultures were re-plated three times on YM agar and incubated for 72 hrs at 25^{o}C in order to activate the yeasts. Pure activated colonies were used as live yeast cells for ensuing inoculum preparation. All experiments were done in triplicate.

2.4. Inoculum preparation

A pure colony of each of the four yeast strains was picked and transferred into YM broth (50ml) in 200ml Erlenmeyer flasks. The cultures were incubated at 25^{o}C for 24h with shaking. A portion of this culture (1ml) was further transferred to another flask containing YM broth (200ml) and incubated at 25^{o}C for 72h. The microbial load in each flask was estimated by making appropriate serial dilution of portions of the broth culture and spread plating on YM agar. Volumes of the broth culture were calculated for

each strain for direct inoculation into raw milk to give approximately 5 X 10^5 CFU/ml yeast cells.

2.5. Preparation of fermented milk

A calculated volume of each yeast strain was inoculated into raw milk (200 ml). For each strain, different sets of inoculated milk were prepared. After mixing thoroughly, the inoculated milk was spiked with different residual levels of antibiotics as indicated in Table 1. Control fermentations were performed without the addition of yeasts and antibiotics, without antibiotics but added yeasts and without yeasts but added antibiotics to cover the full spectrum. In total 48 fermentations were performed in triplicate.

A portion (1ml) of the fermenting milk was withdrawn from each flask every day for a period of ten days for microbiological and chemical analysis. At each sampling stage, the yeast and LAB counts and the pH were determined.

2.6. Microbiological analysis of milk samples

The procedures used for the microbial analysis of milk samples follow the methods outlined in “Laboratory Methods in Food and Dairy Microbiology” by Harrigan and McCance (1976). Serial dilutions were prepared by diluting the milk samples in 9 ml volumes of 0.1% Bacteriological Peptone (Oxoid L37, Basingstoke, England). Appropriate dilutions were spread plated on to duplicate plates of M17 (Oxoid CM 785) and MRS agar plates and incubated at 30 °C for 48 h. For enumerating yeasts RBCA (Oxoid) was used and incubated for 72 hrs at 25^{o}C. Counts were recorded as logarithmic values of colony-forming units per ml of the milk sample (Log_{10}CFU ml^{-1}).

2.7. pH Determination

The pH of raw milk, non-yeast-inoculated-antibiotic-free/treated fermented milks, and yeast-inoculated-antibiotic-free/treated fermented milks were determined at regular intervals using a calibrated digital pH meter (Cyberscan 500, EUTECH Instruments) with an FC 200 electrode, CE Sigapore.

2.8. Statistical analysis

Comparisons between the mean values of microbial counts of the controls as well as between the mean values of the controls and the yeast-inoculated-antibiotic-treated fermented milks were made and statistically evaluated using the paired T-test.

3. Results and Discussion

3.1. The effect of residual levels of antibiotics on the growth of lactobacilli-leuconostocs and lactococci in non-yeast-inoculated naturally fermented raw milk

The development of LAB in naturally fermented raw milk was significantly ($p<0.05$) reduced with the addition of oxytetracycline and penicillin G at all concentrations (Figs.1b-d, 2b-d, 3a-c, 4b-d). Increased concentrations of oxytetracycline resulted in enhanced inhibition, although no significant differences ($p>0.05$) in LAB numbers were obtained when milk was fermented with the addition of 500 or 2500 µg oxytetracycline (Table 1). Maximum LAB counts were obtained after two days of fermentation under normal circumstances whereas longer times (up to 4 days) were needed with increased concentrations (Table 1). Although both lactobacilli-leuconostocs (LAB on MRS) and lactococci (LAB on M17) significantly

decreased at higher concentrations of oxytetracycline, the effect was more prominent on lactococci resulting in a reduction of 3.4 log units in bacterial cells at the highest concentration (Table 1).

Similar trends in lactic acid bacterial reductions were observed with the addition of penicillin G (Figs. 3a-c and 4b-d). Again lactococci were more susceptible to the presence of the antibiotic compared to the lactobacilli-leuconostocs (Table 2). Inhibition of LAB was most prominent during the initial stages (day 1 to 3) of fermentation with the highest inhibition occurring on day 1 resulting in extended lag phases (compare Fig. 4a with Figs. 4b-d). This is not unexpected considering the sensitivity of penicillin G to low pH values. According to Grunwald and Petz (2003), most of the penicillin is degraded and converted to penillic acid when milk is coagulated by lactic acid having a pH of 4.0. Furthermore, the maximum LAB loads was again prolonged from 2 days to 4 days (Table 2) resulting in a visible delay of coagulum formation in all cases. The result is also in agreement with the observations of Grunwald and Petz (2003) in yoghurt. According to these authors, coagulation of milk was negatively influenced by increasing concentrations of residual benzylpenicillin.

Comparison between the effects of oxytetracycline and penicillin G on the microbial numbers of lactobacilli-leuconostocs and lactococci showed that penicillin was responsible for enhanced inhibition during the first three days of fermentation (compare Fig.1b with Fig. 3c and Fig. 2b with Fig. 4d). However, the effect of penicillin G on the maximum LAB numbers was less compared to that of oxytetracycline. At equal concentrations (100 μg/kg milk), the latter antibiotic was responsible for a 0.90 and 1.50 log unit reduction (Table 1) whereas penicillin G caused a 0.70 and 0.80 log unit reduction in numbers of lactobacilli-leuconostocs and lactococci, respectively, in raw fermented milk (Table 2).

3.2. The effect of residual levels of antibiotics on the growth of lactic acid bacteria in yeast-inoculated fermented milk

LAB counts on MRS and M17 were generally significantly higher in yeast-inoculated antibiotic-free naturally fermented milks than in yeast-inoculated-oxytetracycline-treated fermented milks (Table 1, Figs. 1a-d and 2a-d). Despite higher numbers of LAB in naturally fermented raw milk inoculated with the different yeasts, only the addition of *Debaryomyces hansenii* and *Saccharomyces cerevisiae* resulted in higher LAB numbers when oxytetracycline was added at all concentrations (Figs. 1b-d and 2b-d). Both yeast species were responsible for supporting the survival of LAB reducing the inhibiting effect of oxytetracycline. This supporting role of the yeasts may prevent antibiotic-induced diarrhea (Elmer *et al.*, 1996; Schellenberg *et al*., 1994) as indicated in vitro by Bernet *et al*. (1994) whereby *S. cerevisiae* was shown to play a role by encouraging the growth of LAB. Whether a similar role can be played by *D. hansenii* is not known and attention should be given to this organism for detailed studies in this regard.

In contrast, the results also indicated that the addition of *Kluyveromyces marxianus* and *Candida albicans* to the fermented milk resulted in enhanced reductions of LAB in the presence of oxytetracycline. The time to reach maximum LAB counts, however were not affected as a result of the yeasts but rather to the presence of the antibiotic since similar time periods were observed in the milk without yeasts to reach maximum numbers. Lactococci were again reduced at higher levels than lactobacilli-leuconostocs.

In the presence of penicillin, LAB numbers were lower when raw milk was inoculated with yeasts and allowed to naturally ferment compared to fermented milks without yeasts (Figs. 3a-c and 4b-d). This observation suggested that the ability of *S. cerevisiae* and *D. hansenii* to reduce the inhibition of LAB numbers is dependent on the type of antibiotic used.

Furthermore, unlike the supporting role in oxytetracycline-spiked milks, these two species aggravated the inhibition by probably competing with the highly stressed LAB for available nutrients in penicillin-treated milks.

The addition of residual quantities of penicillin G to yeast inoculated fermented milk showed similar patterns to the yeast-inoculated-oxytetracycline-spiked fermented milks with respect to the time of reaching maximum LAB numbers and the significant reduction of LAB counts. The only difference was in *K. marxianus*-inoculated fermented milk where the growth of LAB with the addition of 20 μg of penicillin to milk showed a longer lag phase taking four days (Table 2) to attain maximum numbers. Thus, the addition of penicillin G proved to reduce the LAB numbers in yeast inoculated fermented milks more compared to the addition of oxytetracycline. Again, lactococci were more sensitive to penicillin G treatment in the presence of yeasts (Table 2).

3.3. The effect of residual levels of antibiotics on the growth of yeasts in the non-yeast-inoculated fermented raw milk and yeast-inoculated fermented milk

Comparison between antibiotic-free naturally fermented raw milk and antibiotic-treated fermented milk showed that residual levels of antibiotics were responsible for significant increases ($p<0.05$) in the number of yeast cells (Compare Fig. 5a with Figs. 5b-d and 6a-c). This clearly indicated that yeast growth is favored by the presence of antibiotics which on the other hand contributed to lower LAB numbers. In other words the yeasts had a competitive advantage under the environmental conditions and therefore grew to higher levels in the milk. Significant differences in yeast numbers were generally obtained at the highest concentration of antibiotics added with the exception of *S. cerevisiae*-inoculated milk with added penicillin G. The most substantial increase in maximum numbers was also observed in

C. albicans-inoculated fermented milks with the addition of either oxytetracycline or penicillin G (Table 3).

In all yeast inoculated fermented milks treated with penicillin G, no significant differences in yeast numbers were obtained at penicillin concentrations of 4 and 20 μg /kg milk. However, the yeast numbers obtained at 100 μg of penicillin/kg milk were significantly higher than those obtained at 4 or 20 μg /kg milk except for *S. cerevisiae* inoculated milk. Again in *S. cerevisiae* inoculated fermented milk treated with oxytetracycline, differences in yeast numbers between the different antibiotic concentrations added were insignificant, but all were significantly higher than the yeast numbers obtained in naturally fermented milks (the controls) (Table 3).

Important to notice, is that the yeast numbers significantly out-competed the LAB numbers when grown in oxytetracycline treated fermented milks. In penicillin G treated fermented milks, all yeast inoculated milks, except *S. cerevisiae*-inoculated milk, showed higher yeast numbers than LAB numbers at the highest concentration. Previous studies also showed that treatment with antibiotics commonly leads to an overgrowth of yeasts, especially *Candida albicans* and may cause serious diseases (Elmer *et al.*, 1996). In this study the results indicate that the presence of antibiotics in fermented milk may contribute to a product which may be classified as unhealthy for human consumption. Therefore the need to take appropriate measures to prevent such fermented milks from being consumed by the rural populations becomes very important.

4. Conclusion

Residual oxytetracycline and penicillin G as small as their MRL values can slow the coagulation of naturally fermented milks by inhibiting the growth of LAB and affecting the time required to reach the desired pH. By reducing

the competing LAB, they also allow excessive growth of spoilage and pathogenic yeasts. Thus, the presence of antimicrobial residues in milk, even at concentrations equal to MRL values, can have undesirable consequences both from the health as well as from the manufacturing point of view as they interfere with the production of safe and quality fermented milks with the shortest time possible.

S. cerevisiae and *D. hansenii* behave differently in oxytetracycline- and penicillin G-treated fermented milks. It is possible that these two yeast species could have direct effect on oxytetracycline but not on penicillin G. Thus, further studies are suggested to understand the mechanisms by which they reduce inhibition of growth of LAB in oxytetracycline-treated fermented milks.

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Table 1. Maximum LAB counts in yeast-inoculated naturally fermented milk spiked with different concentrations of oxytetracycline

YEASTS	OXYTETRACYCLINE							
	0 μg/kg milk		100 μg/kg milk		500 μg/kg milk		2500 μg/kg milk	
	LAB on MRS	LAB on M17	LAB on MRS	LAB on M17	LAB on MRS	LAB on M17	LAB on MRS	LAB on M17
K. marxianus	10.6 (2)	11.0 (2)	9.1(3)	9.0 (3)	8.0 (3)	7.4 (3)	7.5 (4)	7.2 (4)
S. cerevisiae	10.7 (4)	11.2 (4)	9.8(3)	9.5 (3)	8.7 (3)	8.0 (3)	8.1 (4)	7.8 (4)
C. albicans	10.4 (2)	10.7 (2)	9.3 (3)	9.2 (3)	8.3 (3)	7.6 (3)	7.8 (4)	7.3 (4)
D. hansenii	10.7 (4)	11.2 (4)	9.9 (3)	9.6 (3)	8.7 (3)	8.2 (3)	8.3 (4)	7.8 (4)
Control	10.3 (2)	10.8 (2)	9.4 (3)	9.3 (3)	8.4 (3)	7.7 (3)	7.7 (4)	7.4 (4)

- LAB = Lactic acid bacteria, Control = raw milk with oxytetracycline as indicated
- Number in parenthesis indicates day of reaching maximum number
- Results shown in $\log_{10}$ CFU/ml are the means of three repetitions
- Control = raw milk with added antibiotics as indicated

Table 2: Maximum LAB counts in yeast-inoculated naturally fermented milk spiked with different concentrations of penicillin G.

YEASTS	PENICILLIN G							
	0 μg/kg milk		4 μg/kg milk		20 μg/kg milk		100 μg/kg milk	
	LAB on MRS	LAB on M17	LAB on MRS	LAB on M17	LAB on MRS	LAB on M17	LAB on MRS	LAB on M17
K. marxianus	10.6 (2)	10.2 (2)	9.4 (3)	9.1 (3)	9.2 (4)	8.8 (4)	9.0(4)	8.3 (4)
S. cerevisiae	10.7 (4)	11.2 (4)	9.9 (3)	9.5 (3)	9.6 (3)	9.0 (3)	9.5(4)	8.6 (4)
C. albicans	10.6 (2)	10.2 (2)	9.7 (3)	9.3 (3)	9.4 (3)	8.9 (3)	9.3(4)	8.5 (4)
D. hansenii	10.9 (4)	10.6 (4)	9.8 (3)	9.3 (3)	9.5 (3)	8.9 (3)	9.4(4)	8.5 (4)
Control	10.3 (2)	9.9 (2)	9.9 (3)	9.6 (3)	9.7 (3)	9.3 (3)	9.6(4)	9.1 (4)

- LAB = Lactic acid bacteria, Control = raw milk with oxytetracycline as indicated
- Number in parenthesis indicates day of reaching maximum number
- Results shown in $\log_{10}$ CFU/ml are the means of three repetitions
- Control = raw milk with added antibiotics as indicated

Table 3. Maximum yeast numbers in yeast-inoculated naturally fermented milk spiked with different concentrations of oxytetracycline and penicillin G.

YEASTS	OXYTETRACYCLINE				PENICILLIN G			
	0 μg/kg	**100 μg/kg**	**500 μg/kg**	**2500 μg/kg**	**0 μg/kg**	**4 μg kg**	**20 μg kg**	**100 μg/kg**
	YC*	YC*	YC*	YC*	YC*	YC*	YC*	YC*
K. marxianus	8.9 (5)	9.2 (5)	9.6 (5)	9.9 (5)	7.9 (5)	8.2 (6)	8.3 (5)	8.9 (4)
S. cerevisiae	8.3 (6)	8.9 (6)	8.9(6)	8.9 (7)	7.3 (4)	8.1 (5)	8.2 (6)	8.2 (5)
C. albicans	7.2 (6)	8.5 (6)	8.9 (5)	8.9(5)	7.2 (4)	8.2 (6)	8.2 (7)	8.7 (6)
D. hansenii	8.3 (6)	8.6 (6)	8.9 (5)	9.2 (5)	7.4 (4)	8.2 (6)	8.2 (7)	8.8 (6)
Control	7.2 (5)	8.4 (6)	8.6 (6)	8.9 (6)	6.9 (5)	7.4 (4)	7.6 (6)	7.6 (4)

- YC = Yeast count
- Number in parenthesis indicates day of reaching maximum number
- Results shown in $\log_{10}$ CFU/ml are the means of three repetitions
- Control = raw milk with added antibiotics as indicated

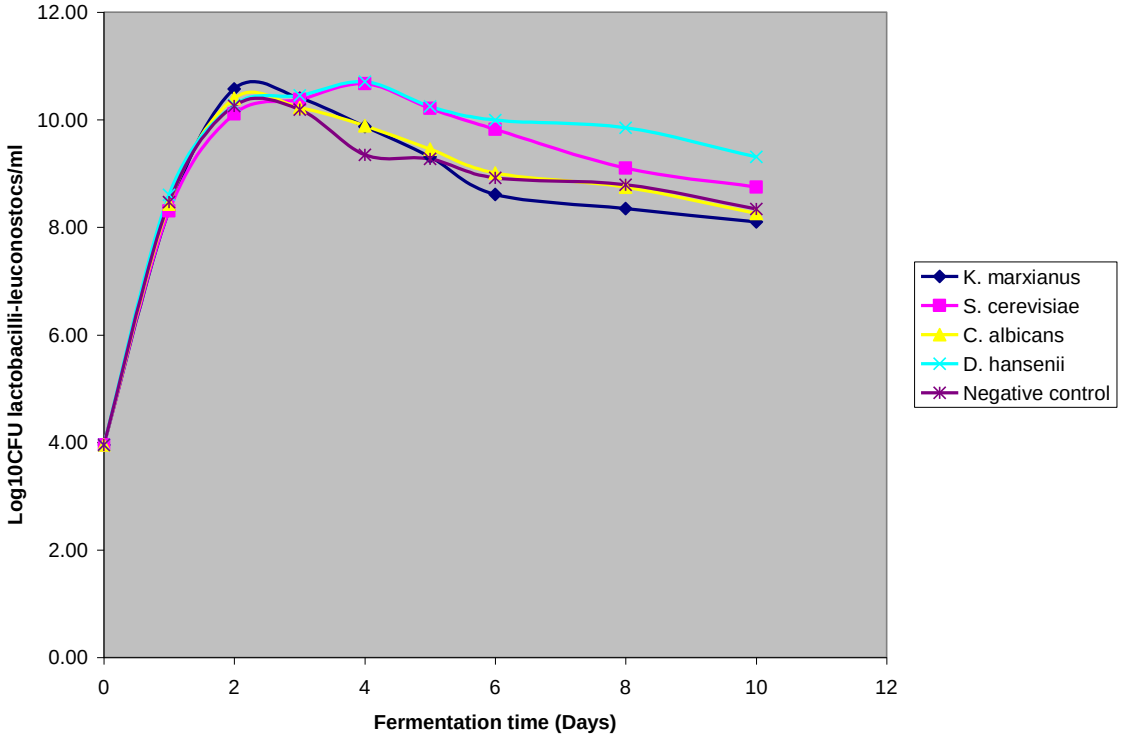

Fig. 1a: Changes in lactobacilli-leuconostocs numbers during the fermentation of yeast-inoculated-antibiotic-free raw milk at ambient temperature

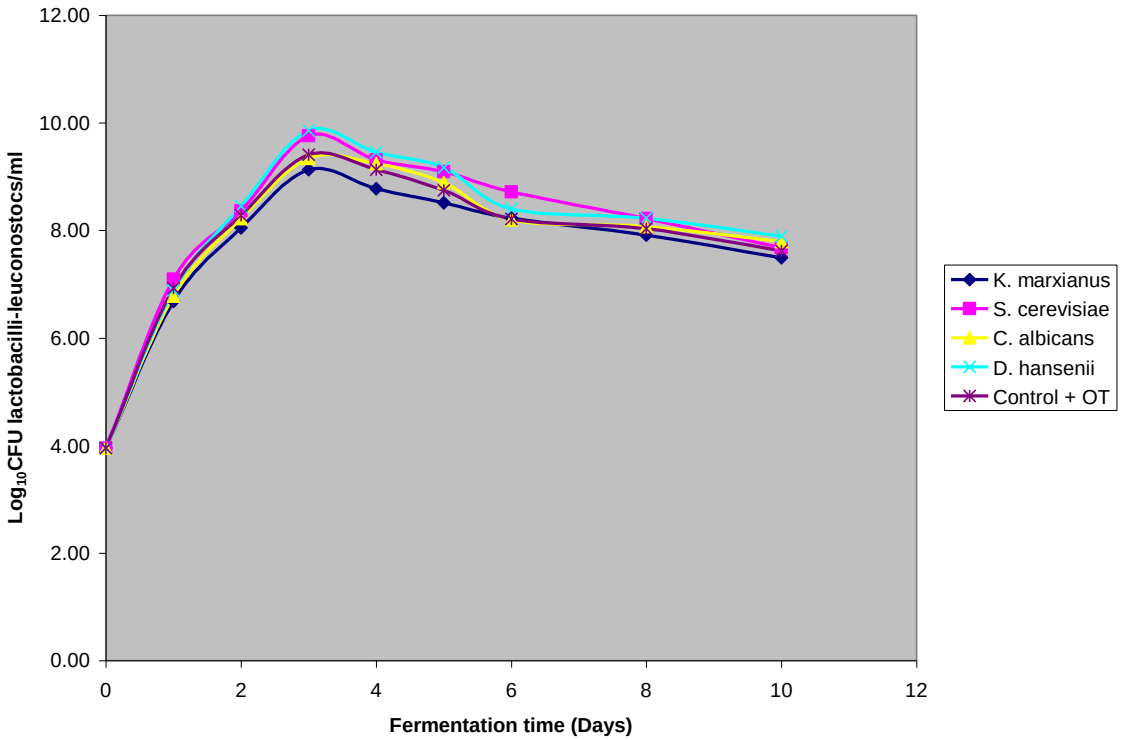

Fig. 1b: Changes in lactobacilli-leuconostocs numbers during the fermentation of yeast-inoculated milk containing 100 µg/kg of oxytetracycline at ambient temperature

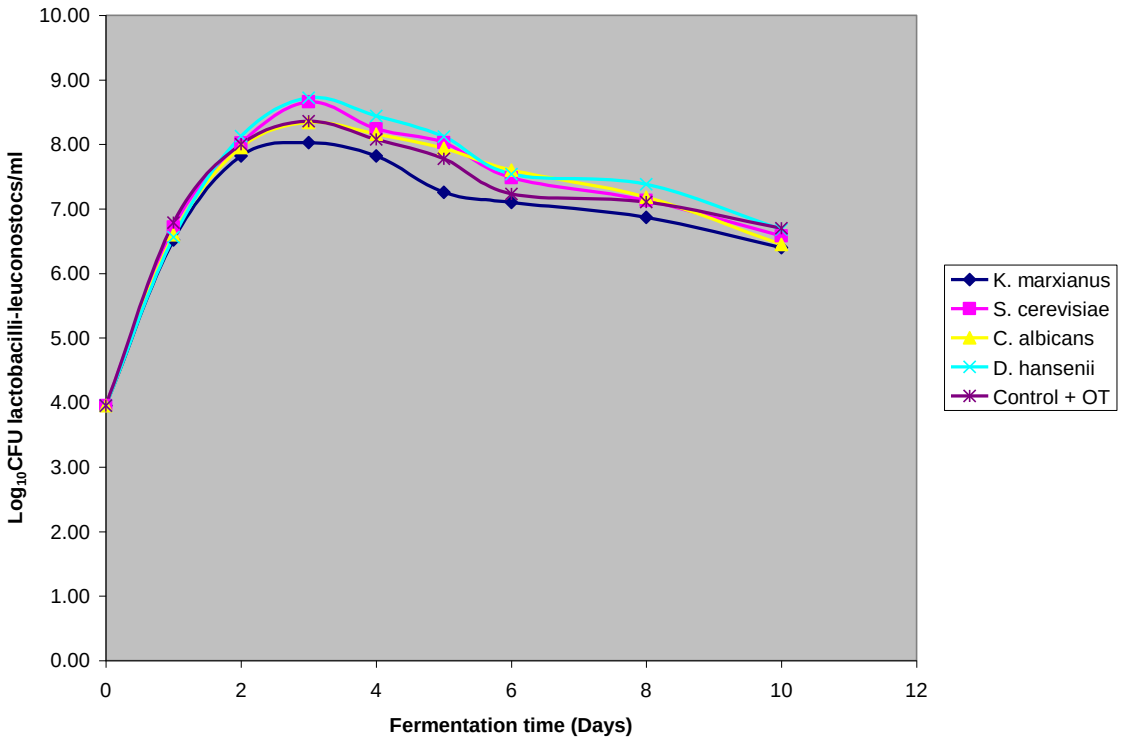

Fig. 1c: Changes in lactobacilli-leuconostocs numbers during the fermentation of yeast-inoculated milk containing 500 µg/kg of oxytetracycline at ambient temperature

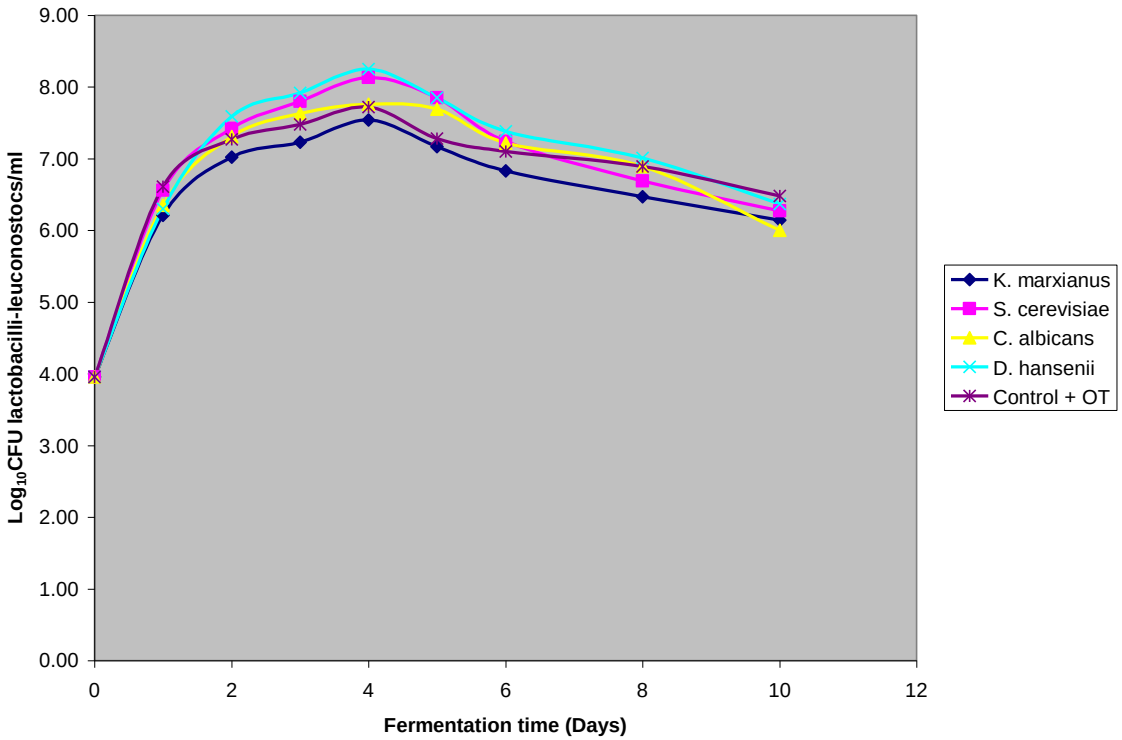

Fig. 1d: Changes in lactobacilli-leuconostocs numbers during the fermentation of yeast-inoculated milk containing 2500 µg/kg of oxytetracycline at ambient temperature.

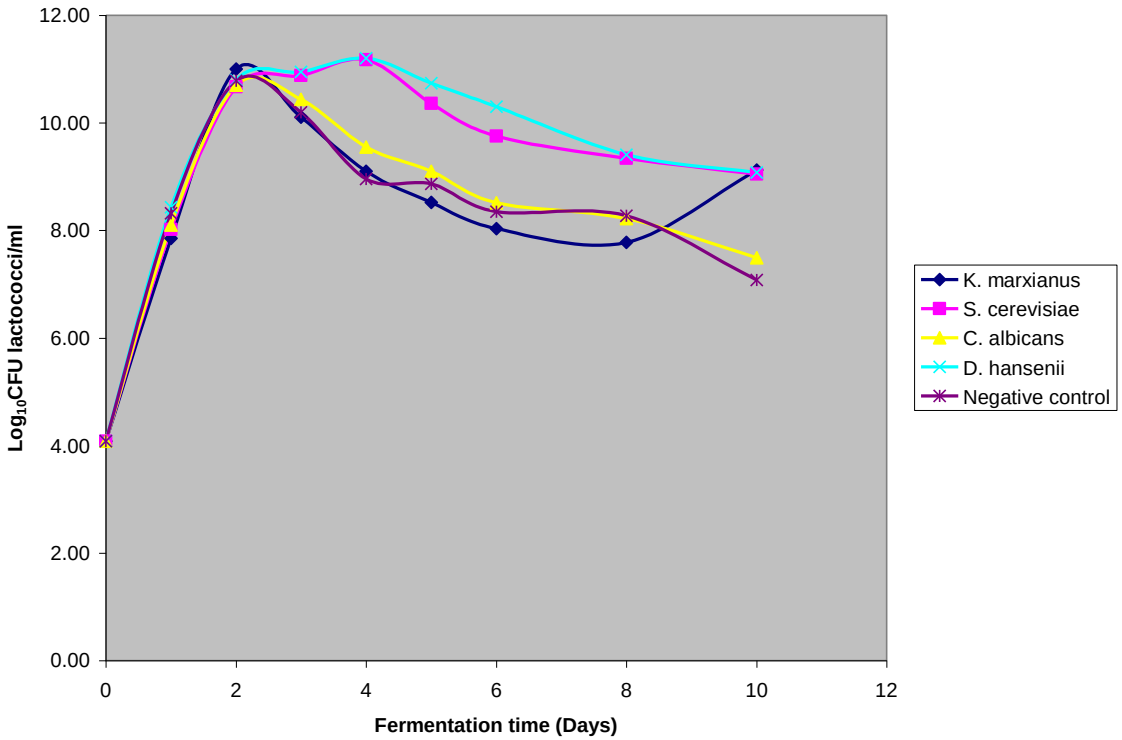

Fig. 2a: Changes in lactococci numbers during the fermentation of yeast-inoculated-antibiotic-free raw milk at ambient temperature.

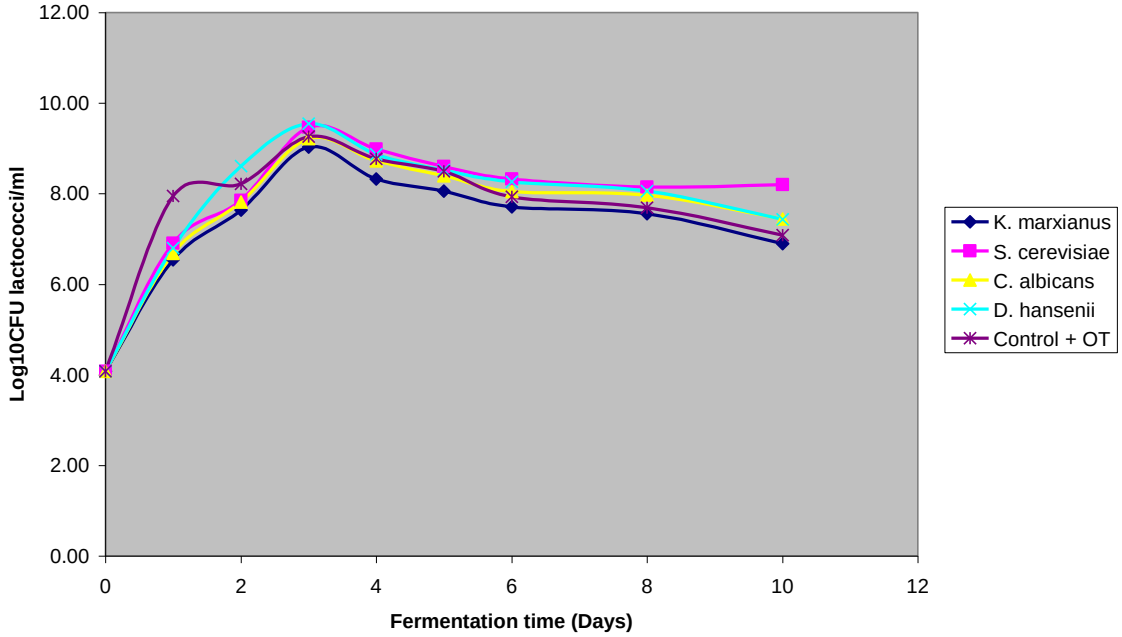

Fig. 2b: Changes in lactococci numbers during the fermentation of yeast-inoculated milk containing 100 µg/kg of oxytetracycline at ambient temperature.

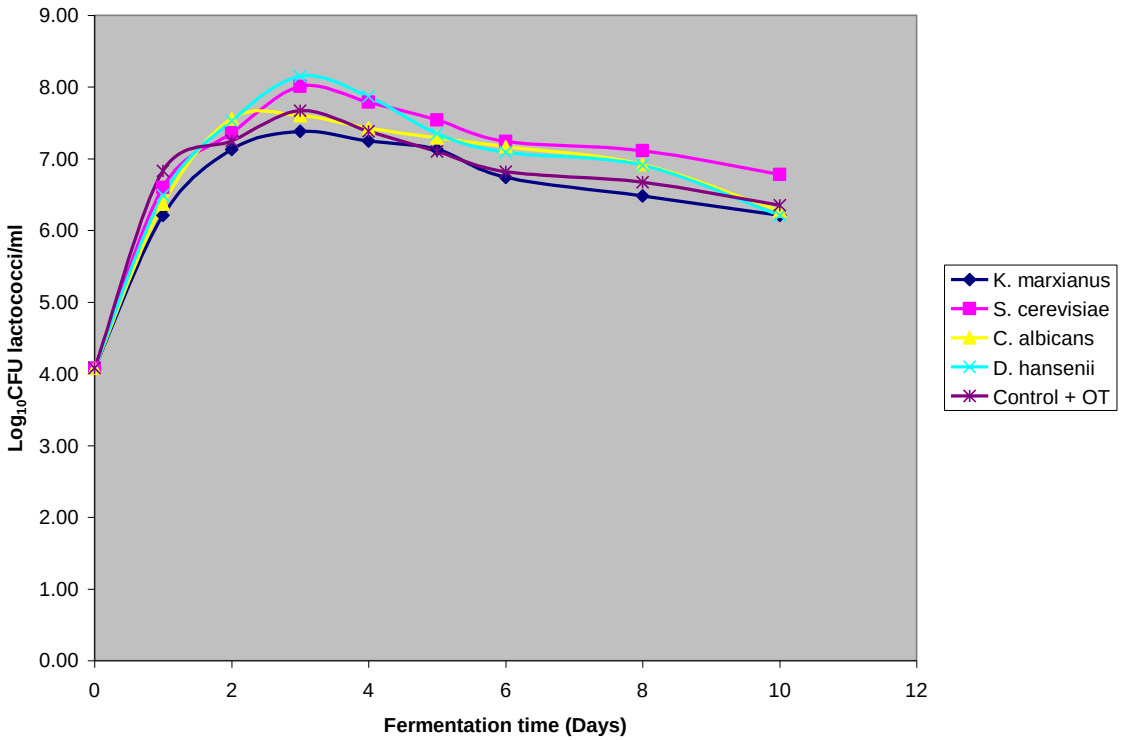

Fig. 2c: Changes in lactococci numbers during the fermentation of yeast-inoculated milk containing 500 µg/kg of oxytetracycline at ambient temperature.

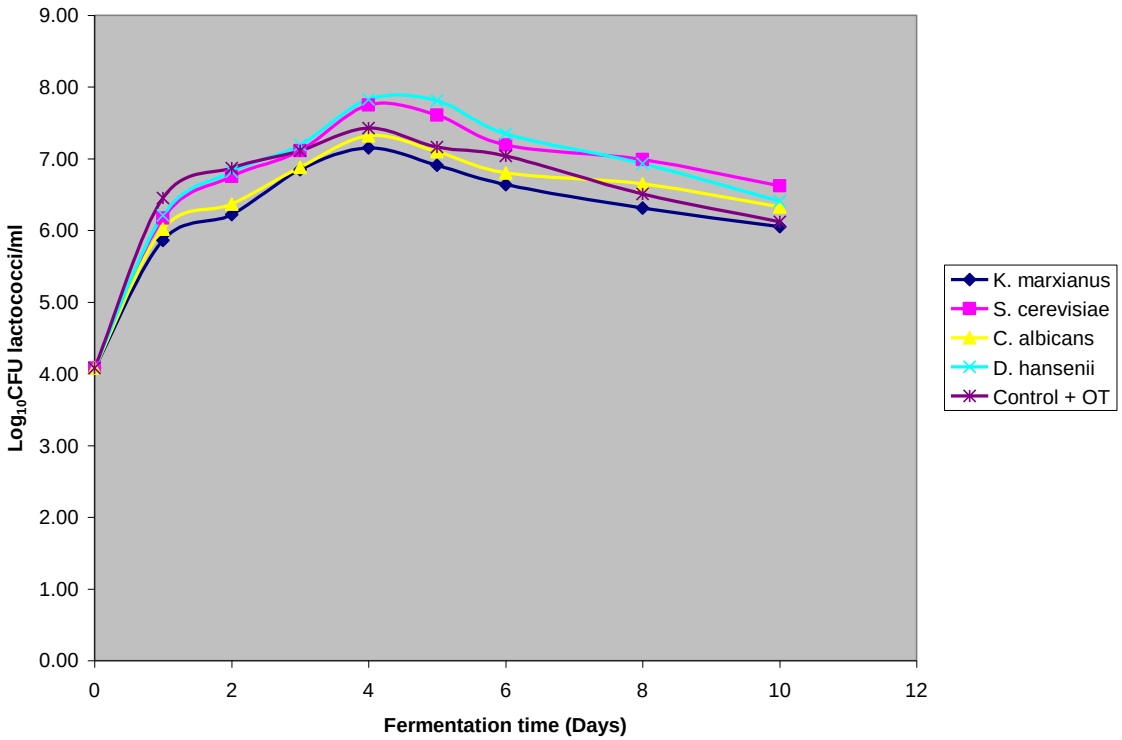

Fig. 2d: Changes in lactococci numbers during the fermentation of yeast-inoculated milk containing 2500 µg/kg of oxytetracycline at ambient temperature.

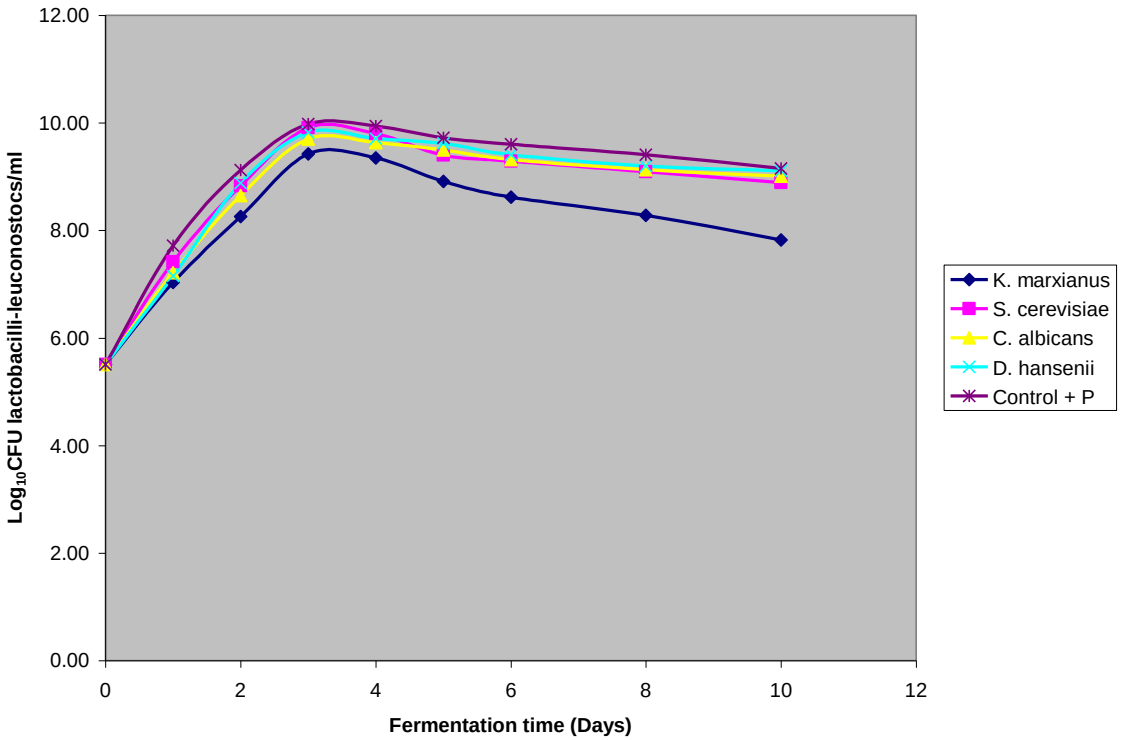

Fig. 3a: Changes in lactobacilli-leuconostocs counts with time during the fermentation of yeast-inoculated and 4 micrograms/kg of penicillin-treated milk at ambient temperature

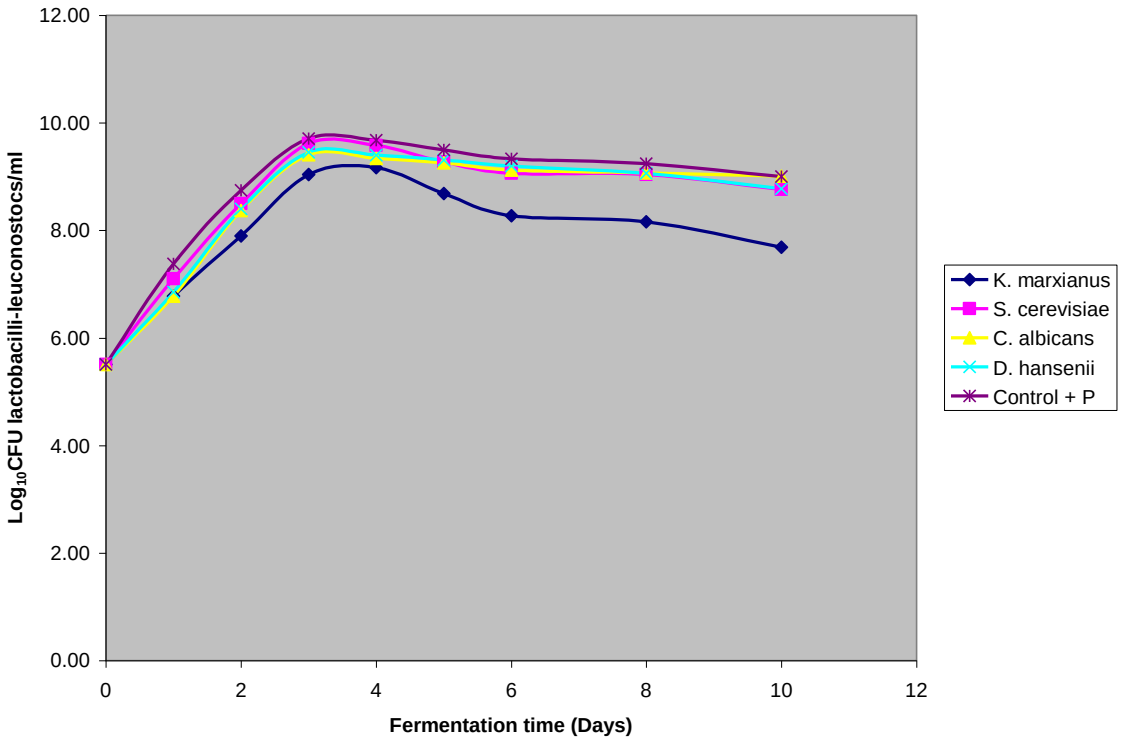

Fig. 3b: Changes in lactobacilli-leuconostocs counts with time during the fermentation of yeast-inoculated and 20 micrograms/kg of penicillin-treated milk at ambient temperature

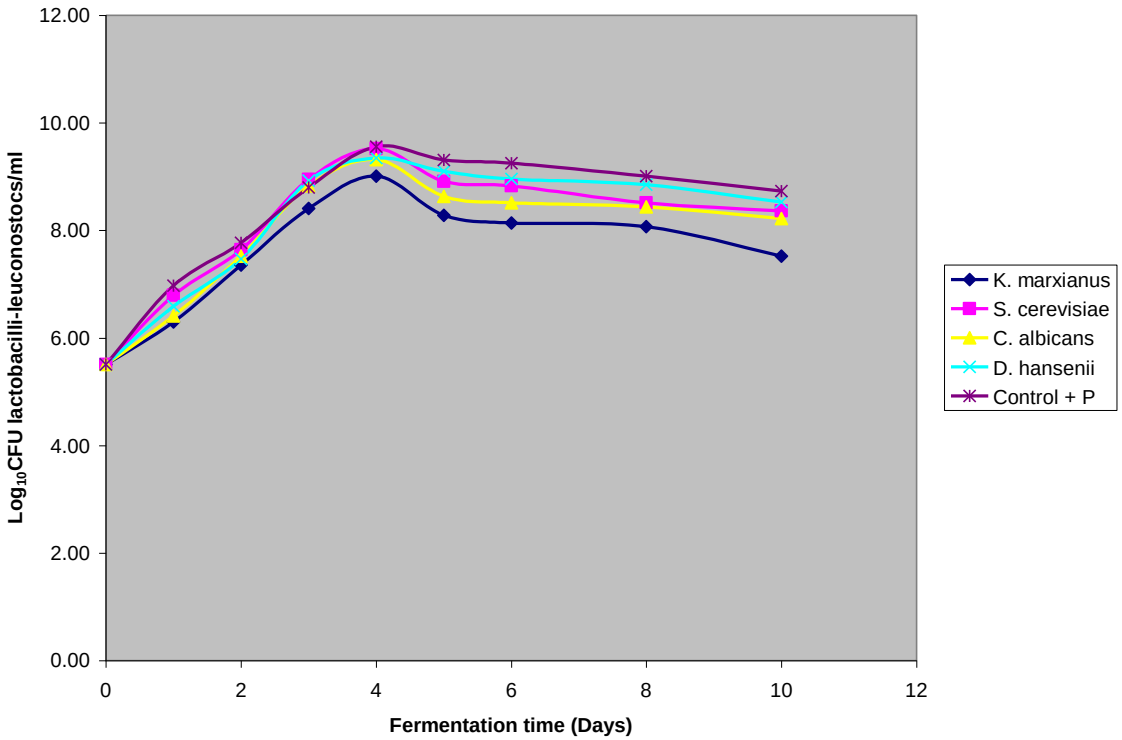

Fig. 3c: Changes in lactobacilli-leuconostocs counts with time during the fermentation of yeast-inoculated and 100 micrograms/kg of penicillin-treated milk at ambient temperature

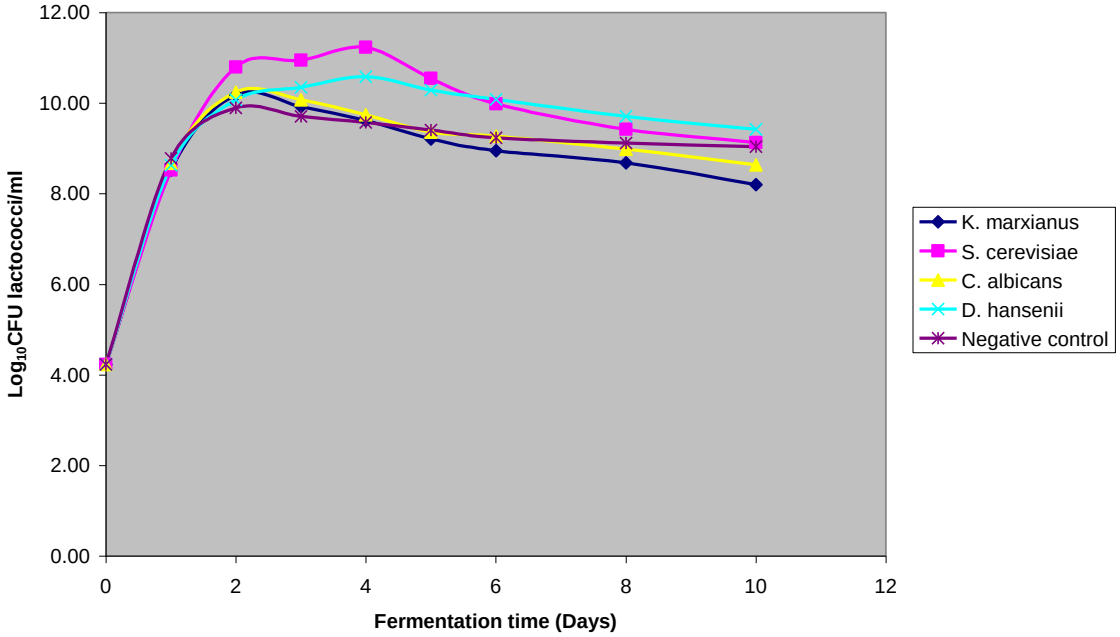

Fig. 4a: Changes in lactococci counts with time during the fermentation of yeast-inoculated-antibiotic-free milk at ambient temperature

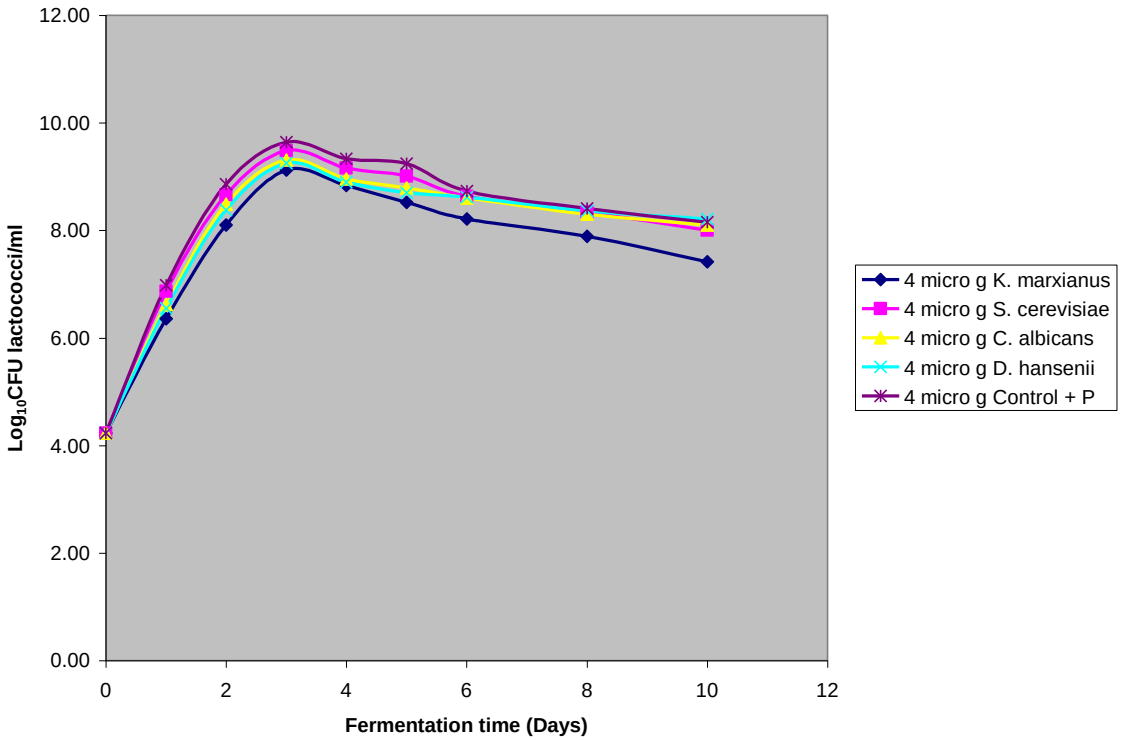

Fig. 4b: Changes in lactococci counts with time during the fermentation of yeast-inoculated and 4 micrograms/kg of penicillin-treated milk at ambient temperature

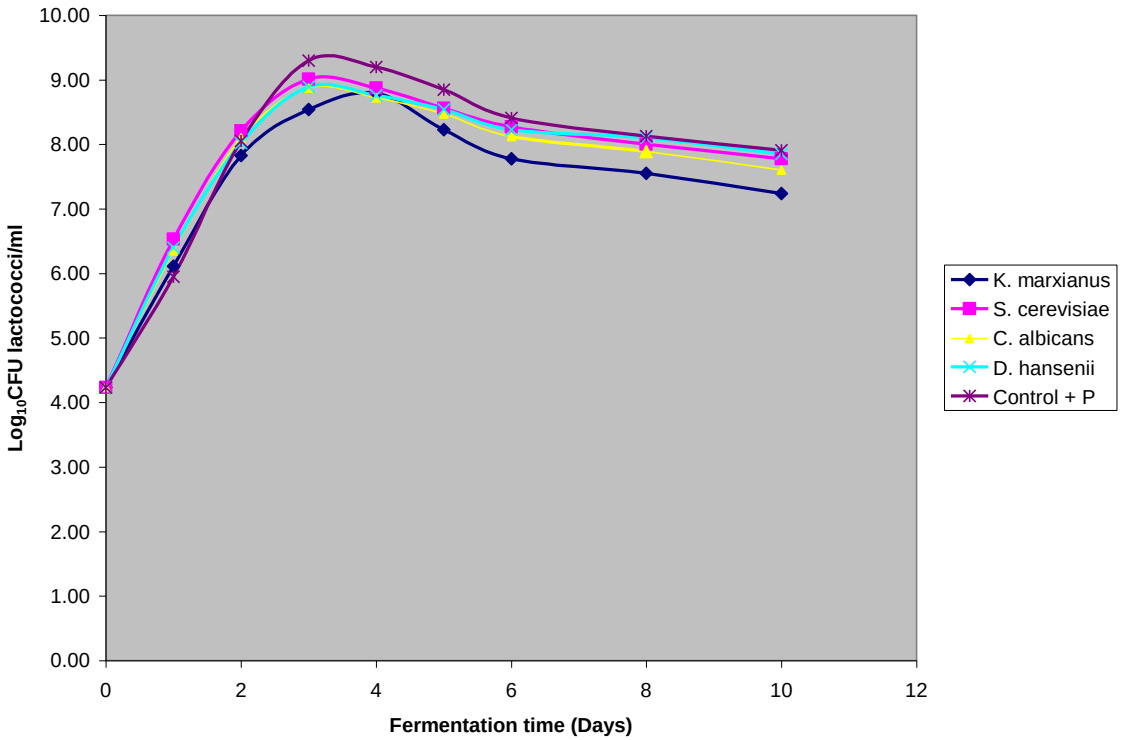

Fig. 4c: Changes in lactococci counts with time during the fermentation of yeast-inoculated and 20 micrograms/kg of penicillin-treated milk at ambient temperature

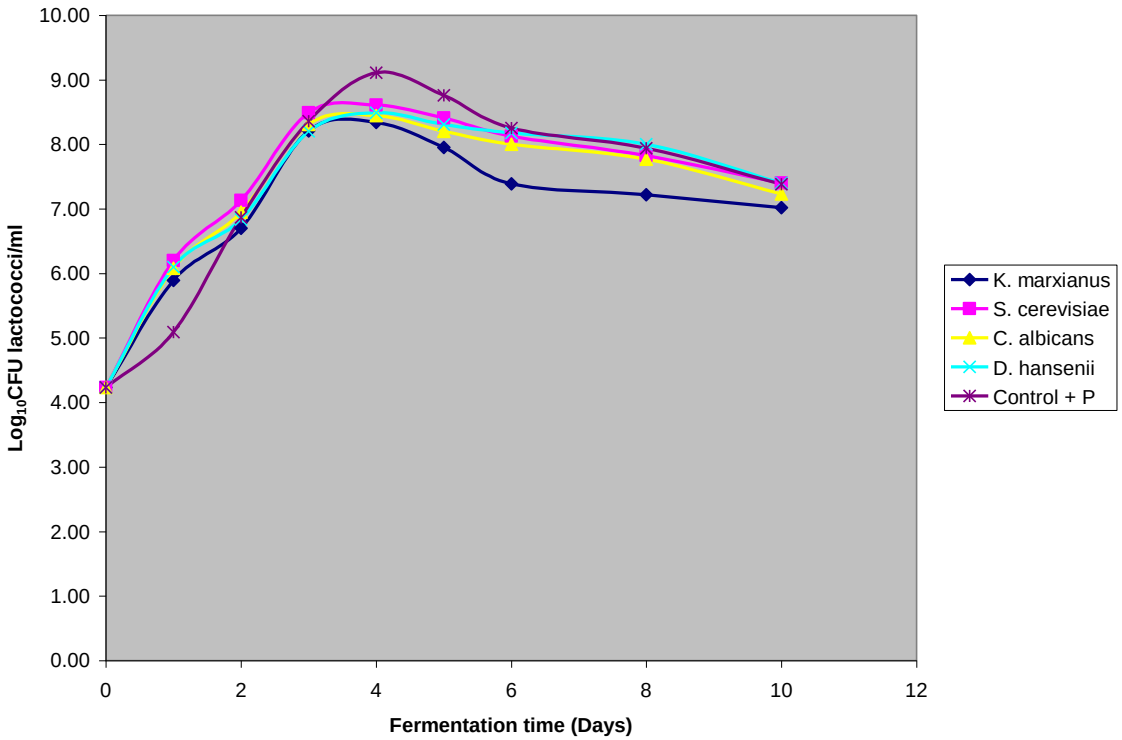

Fig. 4d: Changes in lactococci counts with time during the fermentation of yeast-inoculated and 100 micrograms/kg of penicillin-treated milk at ambient temperature

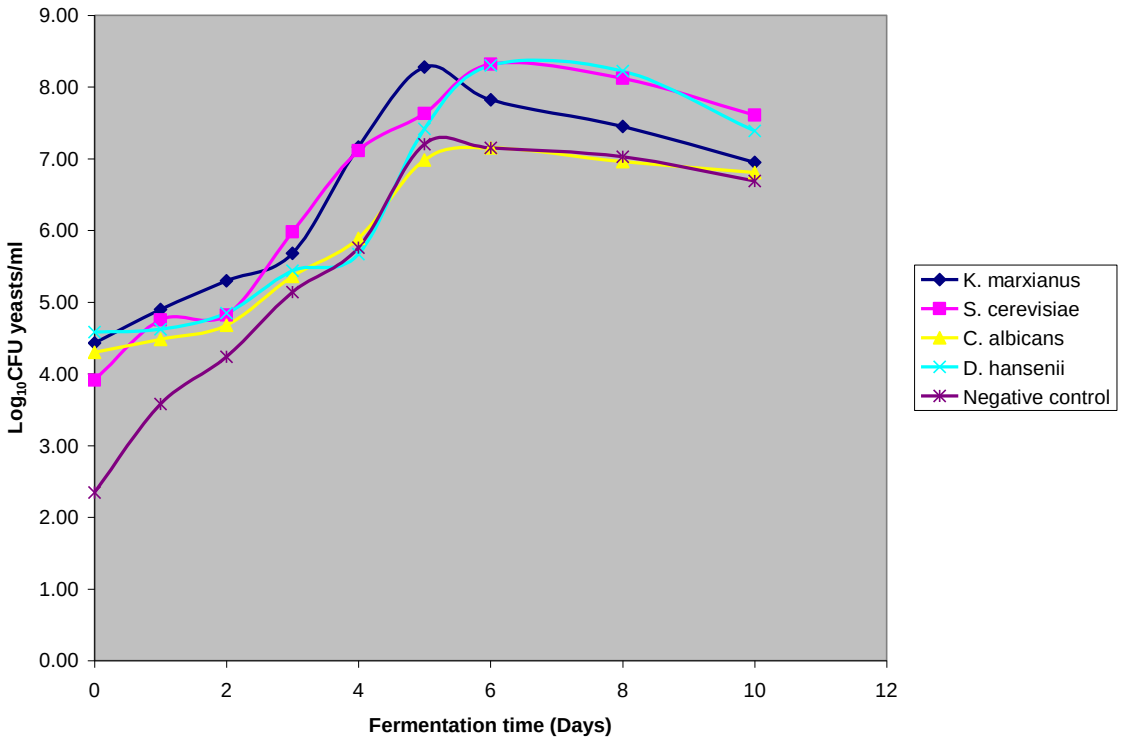

Fig. 5a: Changes in total yeasts during the fermentation of yeast-inoculated-antibiotic-free milk at ambient temperature

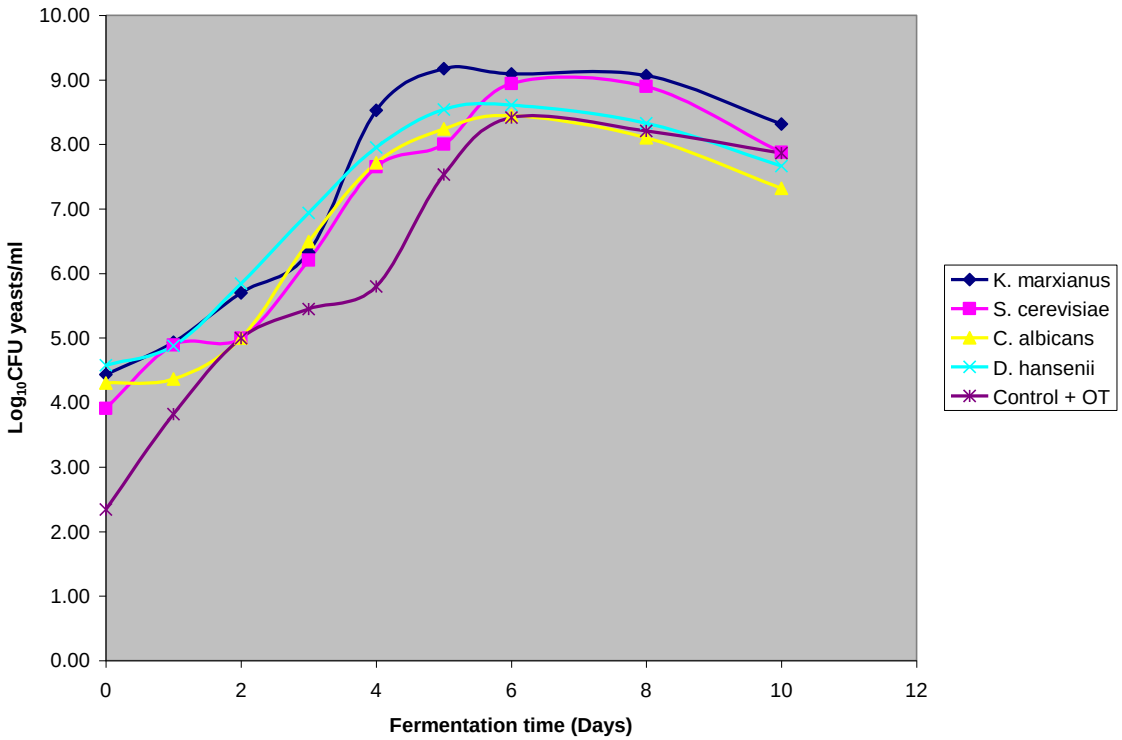

Fig. 5b: Changes in total yeast counts during the fermentation of yeast-inoculated and 100 micrograms/kg of oxytetracycline-treated milk at ambient temperature

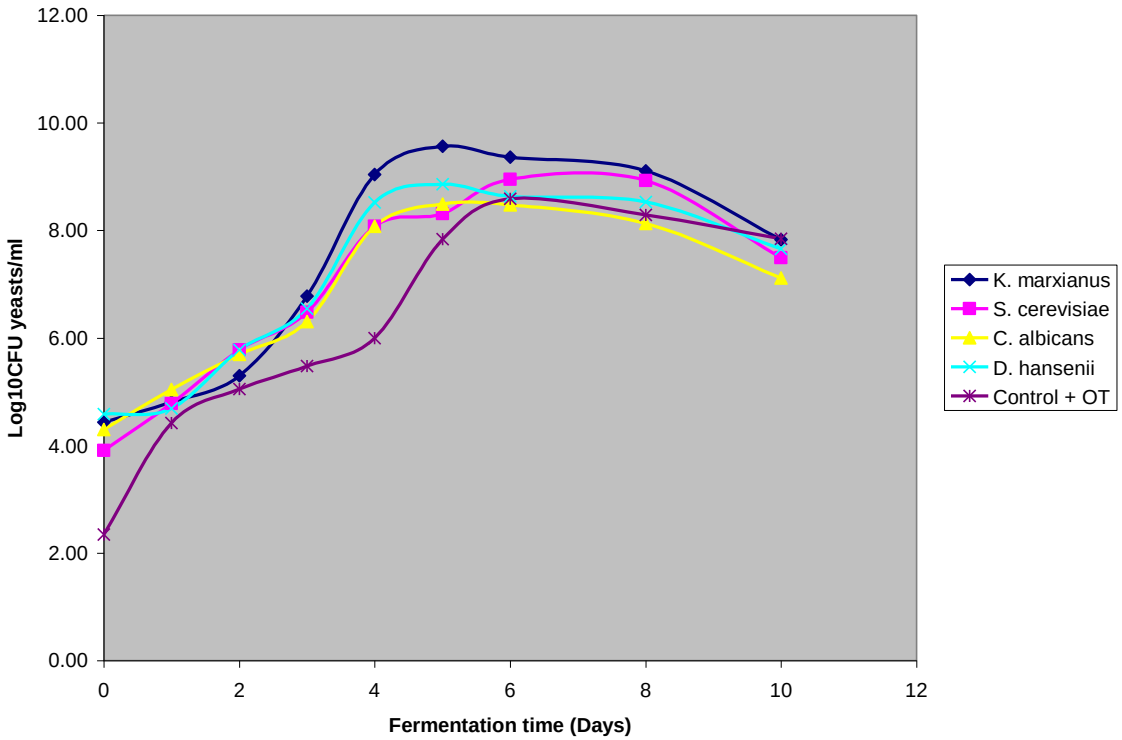

Fig. 5c: Changes in total yeast counts during the fermentation of yeast-inoculated and 500 micrograms/kg of oxytetracycline-treated milk at ambient temperature

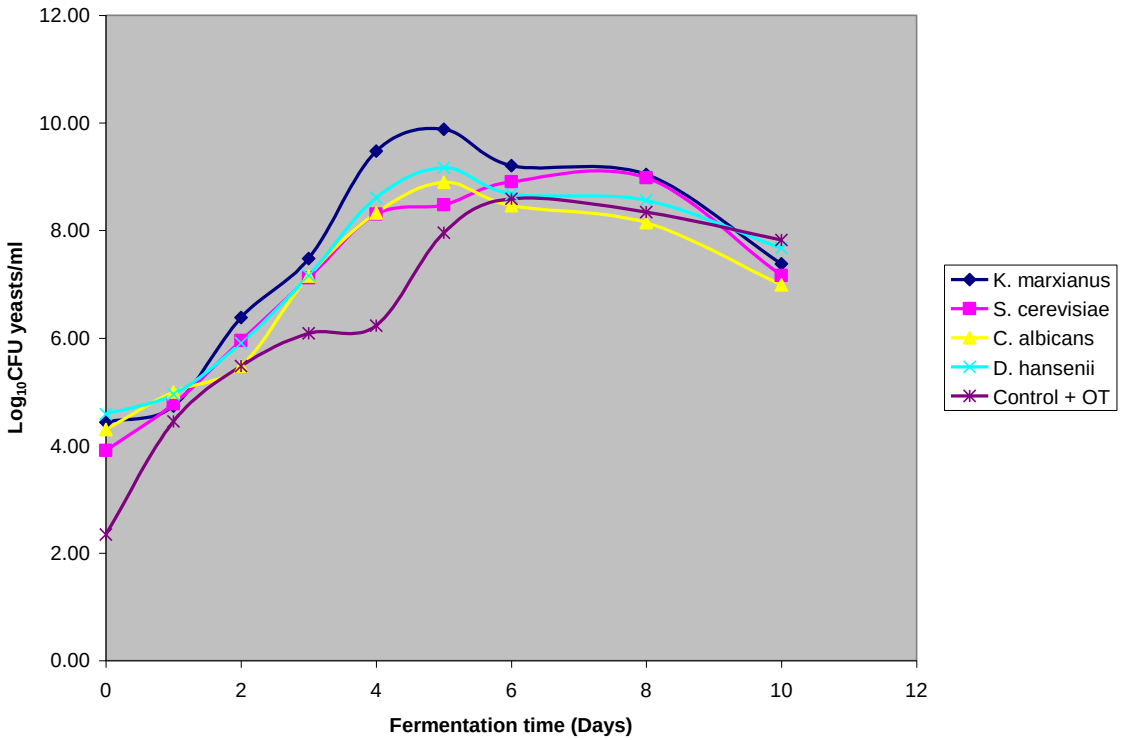

Fig. 5d: Changes in total yeast counts during the fermentation of yeast-inoculated and 2500 micrograms/kg oxytetracycline-treated milk at ambient temperature

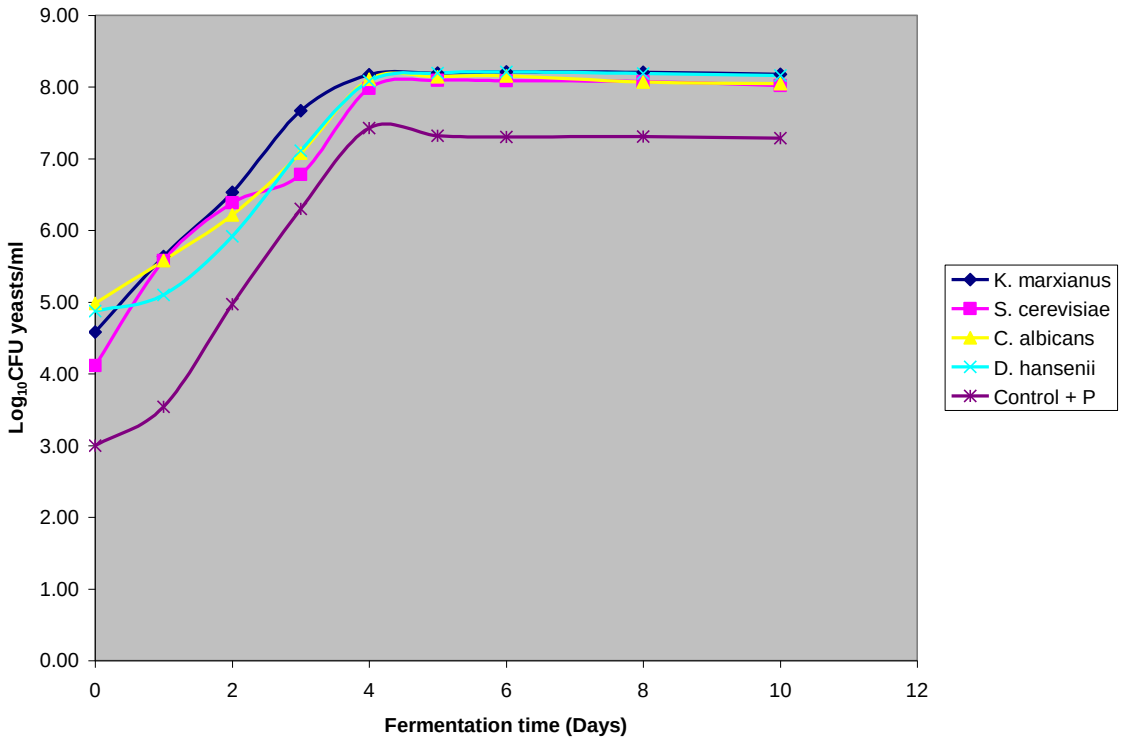

Fig. 6a: Changes in total yeast counts during the fermentation of yeast-inoculated and 4 micrograms/kg ofpenicillin-treated milk at ambient temperature

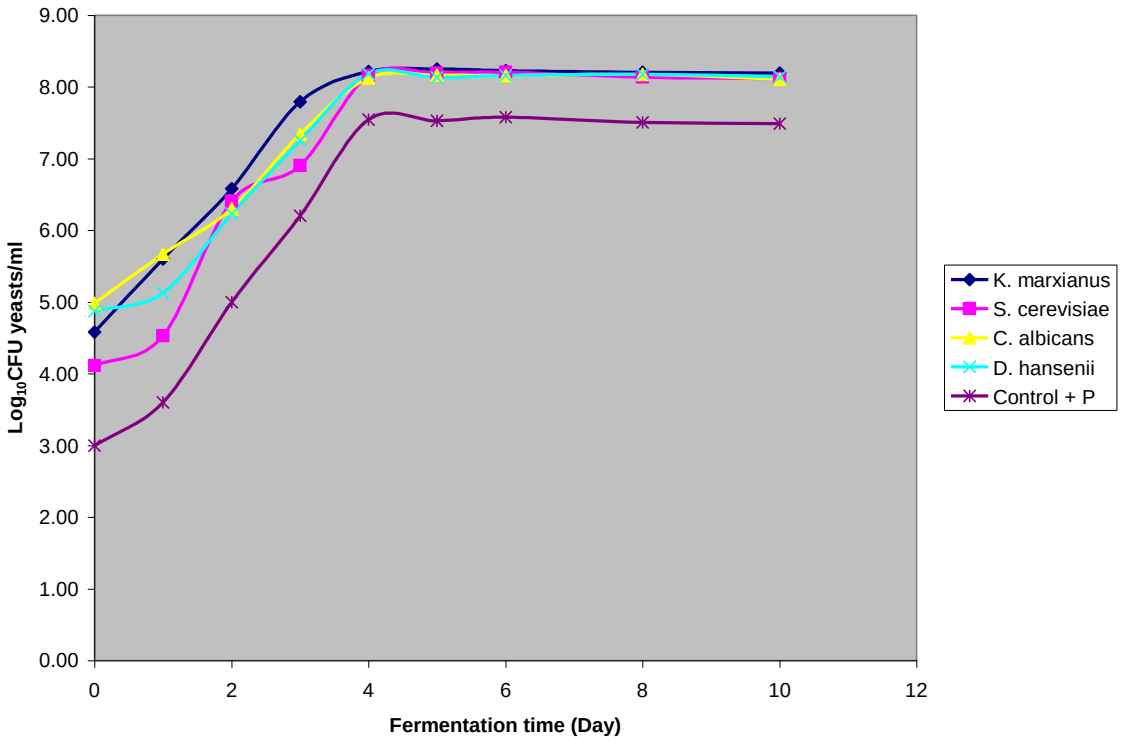

Fig. 6b: Changes in total yeast counts during the fermentation of yeast-inoculated and 20 micrograms/kg of penicillin-treated milk at ambient temperature

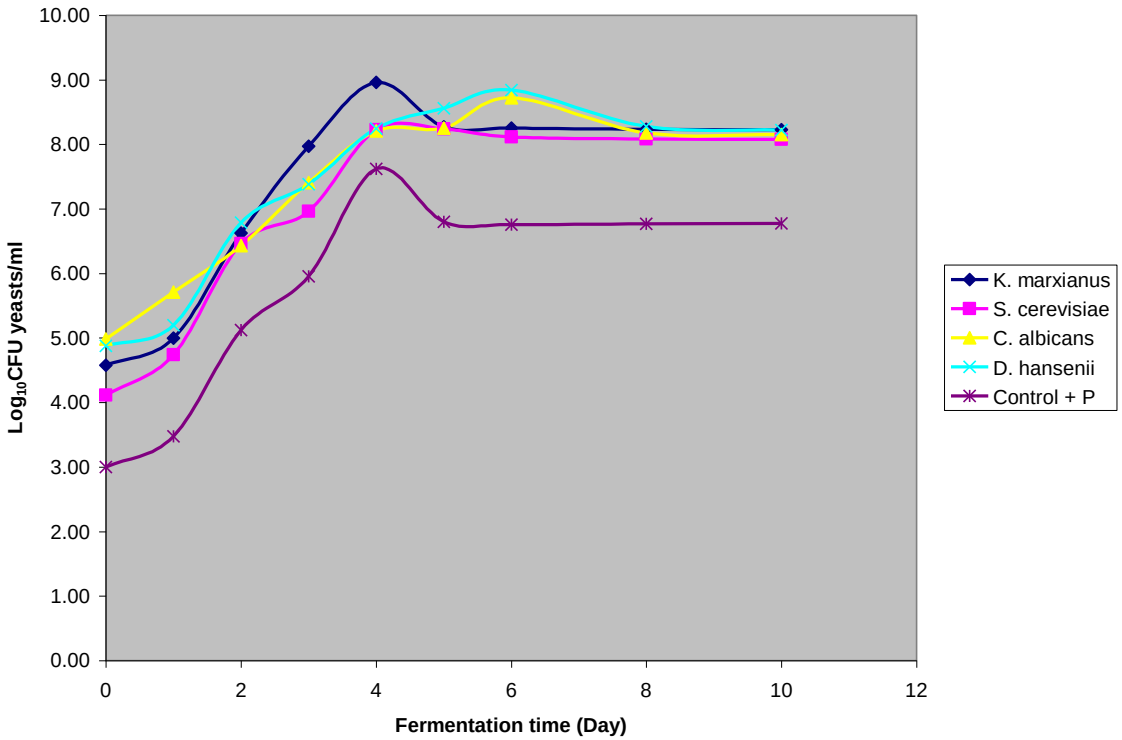

Fig. 6c: Changes in total yeast counts during the fermentation of yeast-inoculated and 100 micrograms/kg of penicillin-treated milk at ambient temperature

CHAPTER VI

General discussion and conclusions

Indigenous fermented milks have been produced and transmitted from generation to generation for several centuries and consequently they are currently regarded as part of a regional intangible cultural heritage (Nout, 2004; UNESCO, 2005). Cultural heritages provide the people with a sense of identity and continuity and thus deserve attention to be preserved and promoted (UNESCO, 2005). Apart from this, because indigenous fermented milks are well known and accepted by consumers, they provide an appropriate basis for development of a local food industry, which not only preserves the raw milk but also stimulates and supports agro-industrial development (Nout, 2004).

Homemade fermented milks and their products, especially in nomadic or village environments, are still occasionally made by spontaneous fermentation, though in most cases they may be made using an empirical culture obtained from a previous batch of production serving as an inoculum. In both the spontaneous fermentation and the deliberate fermentation of milk using an empirical culture, however, the microbial identity remains unknown (The Advisory Panel of National Academy of Sciences, 2004) and as a result the quality of the products is often variable. Production of a non-standardized variable product would have undoubtedly drawbacks both from the economic and public health point of view by causing wastage of resources and resulting food borne diseases, respectively. Thus, at present, there is a growing interest to produce wholesome and safe naturally fermented milk that has still maintained the qualities that made it palatable and acceptable for centuries by many people in Africa.

Most of the indigenous fermented milks known in Africa are naturally fermented milks (Kassaye *et al.*, 1991; Beyene, 1994; Kurwijila *et al.*, 1995; Mutukumira, 1995; Beukes *et al*., 2001; Gonfa *et al*., 2001). The literature shows that some studies have already been made to identify and characterize the microflora for few of these naturally fermented milks (Kimonye and Robinson, 1991; Kurwijilla et al., 1995; Mutukumira, 1995; Gadaga et al., 1999; Abdelgadir, 2001). A number of them, however, have not been studied so far. Even in those few studied ones emphasis was mainly given to the identification and characterization of the LAB. In this work, attempts have been made to identify, characterize and enumerate the yeast microflora of Sethemi (a naturally fermented milk produced by the Sotho people living around Bloemfontein, South Africa) along with their influence on the growth of other microflora, particularly LAB. The contribution of containers in the development of these microbial groups in Sethemi has also been assessed. In addition, simulation studies have been made to assess the effect of residual antibiotics on the growth of the LAB and yeast flora of Sethemi.

The results showed that the predominant organisms in Sethemi were aerobic mesophilic bacteria and lactic acid bacteria (LAB) followed by coliform bacteria and yeasts. The mean counts obtained for most of the microbial groups favorably compare with those reported previously from Zimbabwe (Mutukumira, 1995), Sudan (Abdelgadir, 2001), South Africa (Loretan et al., 1999; Beukes et al., 2001) and recently by Narvhus & Gadaga (2003). Coliform bacteria were to some degrees inhibited but not totally eliminated.

A number of researchers have reported that the survival and growth of pathogenic organisms may be inhibited by lactic fermentation (Holzapfel *et al.*, 1995, Adams and Nicolaides, 1997; and Holzapfel, 2002). Additionally, others have shown that coliform bacteria may not be necessarily eliminated by lactic acid fermentation (Feresu and Nyati, 1990; Simango, 1995; Gran

et al., 2002; 2003). In this study, although the growth of coliform bacteria was generally inhibited after Day 2, the number of viable cells remaining until the sixth day of fermentation was still very high (4.8 $\log_{10}$CFU/ml). This may indicate that the coliform bacteria, though they are indicators of fecal contamination, may not necessarily be indicators of the safety of fermented milks.

Yeast counts in this study were generally significantly lower than the bacterial counts. The diversity of yeast composition was, on the other hand, higher in the first day of fermentation than in any other fermentation days. Of the 22 identified yeast species, only one species, i.e., *K. marxianus* was capable of fermenting lactose, which is the major carbohydrate fraction of milk. The remaining species were probably dependent on end products of the metabolism of lactose, proteins or fats for obtaining energy. Most of the identified yeast species were shown to have been derived from sources other than milk, but adapted to conditions of the ecological niche provided by milk.

Of the total yeast species identified in Sethemi, about 32% are known as potential human pathogens belonging to the genera *Candida* and *Cryptococcus.* In view of the presence of high number of immuno-compromised people in South Africa (The World Fact Book, 2001), the occurrence of *Candida albicans, C. tropicalis*, *C. rugosa*, *C. parapsilosis, C. lusitaniae, Cr. curvatus* and *Cr. laurentii* in Sethemi may pose a serious health threat (Merz, 1984; Hadfield *et al.*, 1987; Araissie et al., 1989; Kockova-Kratochvilova ,1990; Dromer, *et al*., 1995; Johnson *et al.*, 1998; Kunova and Krcmery, 1999; Chakrabarti *et al.*, 2001; Ikeda, 2002) amongst the disadvantaged groups.

Statistical evaluations of the performance of microbial loads in a clay pot, gourd, nickel can and plastic bowl revealed containers had no significant difference based on the maximum counts of LAB, coliform bacteria, total

aerobic mesophilic bacteria and yeasts. Marked differences, however, were observed based on the time period to reach these numbers. Reducing the fermentation time can be valuable since it can optimize equipment use (The advisory panel of National Academy of Sciences, 2004). Consequently, the nickel container appears to be more advantageous than the others and furthermore has a smooth surface for easy cleaning to maintain improved sanitary qualities.

The effect of temperature on the growth of LAB and yeasts in raw milk and yeast-inoculated naturally fermented milk was studied in Chapter 3. Results of the raw fermented milks incubated at different temperatures showed that thick and smooth coagulum-forming fermented milk that contains high numbers of live LAB and requires a reasonably short production time can be produced at 25^{o}C. This product would remain acceptable if consumed within 2 to 4 days after natural fermentation. At 37^{o} incubation, however, Sethemi is very likely to be spoiled within 12 hrs after the coagulum formation.

Raw milk inoculated with selected yeast species, namely *K. marxianus, S. cerevisiae, C. albicans and D. hansenii,* and incubated at different temperatures showed that all these yeasts are capable of growing in varying degrees at all incubation temperatures. In addition, the growth of the introduced yeasts was found to enhance the growth of LAB. Enhancement of growth was reflected by either stimulating growth at an earlier stage before the LAB attain maximum growth or by retarding the rate of decline ensuring the survival of LAB for longer periods. The survival and growth of *C. albicans,* being a pathogen, in naturally fermented milk needs special consideration.

The effect of residual antibiotics on LAB and yeast flora of Sethemi was investigated in Chapter 5. The study showed that the presence of residual levels of oxytetracycline and penicillin G in quantities as small as their

respective MRL values can result in a significant reduction in the number of LAB and encourage the growth of competing yeasts.

S. cerevisiae has been shown to stimulate the growth of LAB in antibiotic added naturally fermented milks. This may be a possible mechanism to prevent antibiotic-induced diarrhea assisting the gut to re-establish the protective microflora. Therefore, *S. cerevisiae* may be applied as a possible probiotic adjunct. Similar results were obtained with *D. hansenii.* The highest increase in yeast numbers in the presence of antibiotics was observed in *C. albicans*-inoculated fermented milks and since this species proved to be growing in fermented milk, the enhanced numbers may lead to serious infections during human consumption. Also of importance to note, yeast numbers out-competed LAB numbers at the highest concentrations of antibiotics added.

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CHAPTER VII

SUMMARY

The microbial ecology of Sethemi is complex and determines its quality and safety. Raw milk and Sethemi (a naturally fermented milk from South Africa) samples were collected in the vicinity of Bloemfontein, South Africa, to determine the development and diversity of all relevant microorganisms. Simulated milk fermentations were executed in the laboratory to study environmental influences on the microbial development.

A total of 116 yeasts were isolated and identified using conventional methods and sequence analysis of the D1/D2 domain. The isolates belonged to 12 different genera and 22 distinct species. Despite the high diversity during the initial stages of fermentation, only seven species proved to be dominant at all stages being present at high numbers in the final product. The predominant species were identified as *C. albicans, Cryptococcus curvatus, Debaryomyces hansenii, Kluyveromyces marxianus, Clavispora lusitaniae, Saccharomyces cerevisiae and Yarrowia lipolytica.* Aerobic mesophilic bacteria, lactobacilli-leuconostocs and lactococci being present at numbers in excess of 9 $\log_{10}$ CFU/ml, however, predominated during processing and in the final product.

Sethemi was also produced in the laboratory in four different containers, namely clay pot, gourd, nickel jar and plastic jar, using the traditional methods of milk fermentation. The development of bacteria and yeasts was

similar in all containers but differed in the time to reach maximum numbers. Optimum growth of LAB was obtained sooner in nickel containers. The highest yeast diversity was, however, obtained in the clay pot dominated by the yeasts *Deb. hansenii*, *Cr. humicola* and *K. marxianus*, which were commonly found in the raw and naturally fermented milks.

Considering their industrial or health importance, four dominant yeast species, namely *K. marxianus, S. cerevisiae, C. albicans*, and *Deb. hansenii* were selected for environmental studies. *S. cerevisiae* showed the highest stimulatory effect on the growth of LAB and promised further study whereas the good growth of *C. albicans*, an opportunistic yeast pathogen, was alarming. The best Sethemi was produced at 25^{o}C and must be consumed within two to four days. Fermentation at higher temperatures, however, resulted in early spoilage.

The effect of residual levels of oxytetracycline and penicillin G on the growth of LAB and yeasts in the presence or absence of the four selected yeast species in naturally fermented milks was studied. The results showed that oxytetracycline and penicillin G at the lowest values significantly inhibited the growth of lactobacilli-leuconostocs and lactococci. In both antibiotic treatments, lactococci were more inhibited than lactobacilli-leuconostocs. In oxytetracycline-treated fermented milks, the addition of *S. cerevisiae* and *D. hansenii* significantly reduced the inhibition while the addition of *C. albicans* and *K. marxianus* contributed to the inhibition. In contrast, in penicillin-treated milk, all four yeast species added to the inhibition of the growth of LAB. The yeast growth was generally favored by the presence of both antibiotics and even out-competed the LAB in some instances indicating that it might pose a serious health problem amongst the immunocompromised if it is a potentially pathogenic one.