THE DIVERSITY AND TECHNOLOGICAL PROPERTIES OF YEASTS FROM INDIGENOUS TRADITIONAL SOUTH AFRICAN FERMENTED MILKS

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This thesis is dedicated to

my loving husband Deon and our son, Michael-Paul

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ABBREVIATIONS

Lb.	*Lactobacillus*
Lc.	*Lactococcus*
Str.	*Streptococcus*
Sacch.	*Saccharomyces*
L.	*Leuconostoc*
Kluyv.	*Kluyveromyces*
C.	*Candida*
T.	*Torulaspora*
D.	*Debaryomyces*
Tor.	*Torulopsis*
Br.	*Brettanomyces*
Zygosacch.	*Zygosaccharomyces*
Y.	*Yarrowia*
Rh.	*Rhodotorula*
Tr.	*Trichosporon*
Dek.	*Dekkera*
Gal.	*Galactomyces*
P.	*Pichia*
spp.	species
subsp.	subspecies
LAB	lactic acid bacteria
IDF	International Dairy Federation
cfu	colony forming units
h	hour (s)
min	minute (s)
YM	Yeast-Extract Malt-Extract Agar
NFM	reconstituted non-fat milk
m/v	mass per volume
v/v	volume per volume
cfu/ml	colony forming units per millilitre
cfu/g	colony forming units per gram
ml	millilitre
YNB	Yeast Nitrogen Base
a_w	water activity
ARC	Agricultural Research Council
CSIR	Council for Scientific and Industrial Research

PREFACE

Some aspects of the work conducted for this thesis have been published or presented as posters or papers elsewhere:

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Mostert, J.F., Beukes, E., Loretan, T., Viljoen, B.C. and Bester, B.H. 1998. Diversity and technological properties of microorganisms isolated from indigenous traditional South African fermented milks. 25th International Dairy Congress. Aarhus, Denmark, September 1998.

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CHAPTER 1

INTRODUCTION

Traditional fermented milks have a long history and are known and made all over the world, wherever milk animals are kept. Fermented milk products, especially in village environments, are usually made with empirical cultures whereby the inoculum from a previous product is used as the starter culture. The microorganisms utilized are mainly mesophilic or thermophilic strains of lactic acid bacteria (LAB) and yeasts which contribute to the specific sensory properties of the product. Yeasts, as many other microbes, are widely dispersed in the environment and are natural contaminants in milk and milk products. Due to their high tolerance towards low pH and temperatures and their ability to ferment carbon sources, they often occur as spoilage organisms in milk products. They also play a major role in certain dairy products, as part of the typical microbial population. In kefir the yeasts contribute to the development of a characteristic aroma and taste originating from mainly ethanol and carbon dioxide production during the fermentation of the milk. One of the major contributions of yeasts is the metabolism of lactic acid with a consequent increase in pH, thus, supporting the growth interactively of less acid tolerant microorganisms. Furthermore, yeasts are also able to produce proteolytic and lipolytic enzymes which are important in the formation of flavour compounds.

Traditional fermented milks are manufactured daily in many households and represent an important part of the diet of rural communities in South Africa. In many developing countries, village-art methods and age-old techniques are to a certain extent still used for food processing. These products are, however, steadily declining due to socio economic changes that are taking place. This means that some of the traditional techniques will eventually be lost, together with the associated fermentative microorganisms. These natural and wild organisms including yeasts, represent a unique genetic resource for food technology and biotechnology for the future. However, no scientific information exists on the diversity, properties and growth of yeasts in locally manufactured traditional fermented milks.

This study was undertaken to obtain knowledge about the ecobiology of yeasts in indigenous, traditional South African fermented milks and include the following:

(1) To isolate, enumerate and identify yeasts from traditional fermented milks, including kefir;

(2) To characterize technologically important properties, both physiological and biochemical;

(3) To study the fermentation characteristics of the yeasts and the interactions between lactic acid bacteria and the dominant yeasts associated with indigenous, traditional fermented milk.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Traditional fermented fresh milk products are all those with a substantial historic record. According to the Oxford dictionary, 'indigenous' means native or belonging naturally, and 'traditional' means custom handed down from generation to generation based on usage or experience. They have been deliberately made for many centuries as a means of preserving milk for later consumption and preventing spoilage (wild fermentation). In some countries of Europe, the Mediterranean Basin area, southwest Asia, and Africa, fermented milks are of great importance. They form the staple of numerous diets and their popularity is increasing. Yogurt and kefir are the best known samples.

Only products containing live microorganisms can be regarded as traditional fermented milk products. There are two main types: (1) products prepared with defined culture; and (2) products with a non-defined or empirical culture. In other words, the inoculum is obtained from a previous production and its microbial identity is unknown (Kurmann et al., 1992; Kroger et al., 1992).

Fermented milks, using raw milk from cows', sheep, goats, camels, or horses of the nomads roaming the areas have a history of thousands of years. The conversion of milk into fermented milk products has several important advantages. It is not only a means of preserving food, it also provides improved taste and better digestibility, especially where lactose intolerance is found among many individuals. It also allows for production of a variety of foods, and, in some instances, the process of fermentation and subsequent whey removal reduce the bulk of the starting material. The different types of traditional fermented milk products that have been developed in the course of history are defined by

the geographical culture and region from which they originated (Kurmann et al., 1992). They have come to be enjoyed everywhere in the world, because of their refreshing acid taste. Most of the fermented milk products include lactic acid fermentation incorporating lactic acid bacteria (LAB), but some also incorporate additional alcohol fermentation due to the presence of yeasts. The addition of lactic fungi prevents bacterial and mould contamination and improves storage properties. These kinds of products were the earliest examples of fermented milk products. Fortunately, the dominant bacteria present like lactococci and lactobacilli were fast growing organisms, which generally suppressed the spoilage and pathogenic organisms very effectively. Knowledge about the general occurrence and growth of yeasts in dairy products remains incomplete. More comprehensive ecological surveys are needed to determine the diversity of yeast presence in dairy products, and to establish the occurrence of any specific yeast-product associations, as well as yeast/lactic acid bacteria interactions (Kosikowski and Mistry, 1997). The biochemical mechanisms by which yeasts affect the sensory quality of dairy products are poorly understood. Research is required to determine the biochemical and physiological properties of the main yeasts species found in dairy products. The deliberate use of selected yeast species in the maturation of cheese or in the production of other fermented dairy products requires serious exploitation, and could give rise to new starter culture technology for the dairy industry. The advantages of fermented food products include the following:

(1) They can synthesize important ingredients (e.g. essential amino-acids, vitamins which enrich the human diet;
(2) An ability to produce flavour components that favour consumption of these foods in traditional and new markets;
(3) The ability to break down anti-nutritional factors;
(4) The inability to synthesize toxins and other undesirable secondary products;
(5) Store and supply energy;
(6) Contain proteins with higher nutritional value;

(7) In these products lactic acid bacteria convert the lactose, to which almost 90% of the South African population is intolerant or partly intolerant to, into the more digestible lactate (Kosikowski and Mistry, 1997).

The safety and shelf-life of fermented products may also be improved through the development of organisms that produce alcohols, antibiotics, or other substances that can inhibit the growth of undesirable organisms. Fermentations carried out in traditional vessels with unusual surface characteristics such as charred wood, semi-porous clay or gourds are difficult to replicate.

Isolation and characterization of predominant organisms are essential. According to a report from the advisory panel of an Ad Hoc Panel of the Board on Science and Technology for International Development, 'information should be collected on all traditional fermented foods (Kroger et al., 1992). A thorough microbiological, nutritional, and technical investigation should be carried out on each of the processes. The various microorganisms involved in each fermentation should be isolated, characterized, studied, and preserved. The biotechnological worth of each organism should be determined. Isolation should not be confined to the dominant organism because other microbes found in lower numbers might have an important function in the process. The role of each organism should be identified'.

2.2 HISTORICAL ACCOUNT OF FERMENTED MILKS

Milk has been with the human being for many centuries. Fermented milks originated in the Middle East before the Phoenician era. In an IDF Bulletin, Kurmann (1984) compiled a summary of the oldest mentions of fermented milks recorded by various authors: a cultured cream in the year 1300 B.C. in Mesopotamia (The Bible, Genesis 18.8); Koumiss in Russia dated back to 2000 B.C.; Laben occurs in an Arabian text book in the year 633 A.D.; yogurt in Turkey in the 8th century A.D.; buttermilk in India in the years 800-300 B.C.; Dahi in India in the years 800-300 B.C.; Airan in Central Asia in the years 1253-

1405 A.D.; and Tarho in Hungary in the 14th century. According to El-Gendy (1983) the traditional Egyptian fermented milks, Laban Rayeb and Laban Khad, and their instruments of manufacture were in use in Egypt in that period, perhaps as early as 7000 B.C. Asia also contributed to the early development and spread of sour and fermented milks by the Tartars, Huns and Mongols in their invasions of Russia and other European areas (Kosikowski and Mistry, 1997). Early representations of milking include that in a frieze at Ur (ca. 2900 B.C.), and on the sarcophagus of Kawit from Deir el-Bahari (Eleventh Dynasty). The regular milking of the animals must have quickly followed the use of butter, sour milk and cheese, for by accident alone these milk products must have occurred again and again.

Soured milk or curds have surely been consumed by many people of the earliest Neolithic times, but little remains as direct proof of this. Fermented milks were consumed in Mesopotamia and Palestine, and possibly in Egypt. Nevertheless, the Greeks and Romans also used soured milk, and three distinct kinds are mentioned (Brothwell and Brothwell, 1969). Around 8000 B.C., traditional homemade products, using empirical cultures, e.g. soured milk, yogurt, koumiss and kefir featured until the Middle ages. From there on until 1900, the development of specific products continued using empirical cultures. In the early 1900's, products were prepared using defined cultures, which consequently resulted in the development of industrial production. Consumption of fermented milk products evolved throughout the world from 1930 with the emphasis mainly towards human health and use of specific formulated cultures (Kurmann et al., 1992). Currently, consumption of fermented milk products in South Africa is valued at 208.1 million Rand or 3.4 million liters for 1995/1996 (Hermann, 1997).

2.3 TYPES OF FERMENTED AND CULTURED MILK FOODS

2.3.1 Classification of fermented milks

Many distinct forms of fermented milks exist (Fig. 2.1). Each fits a special circumstance of development catering to specific tastes or usage.

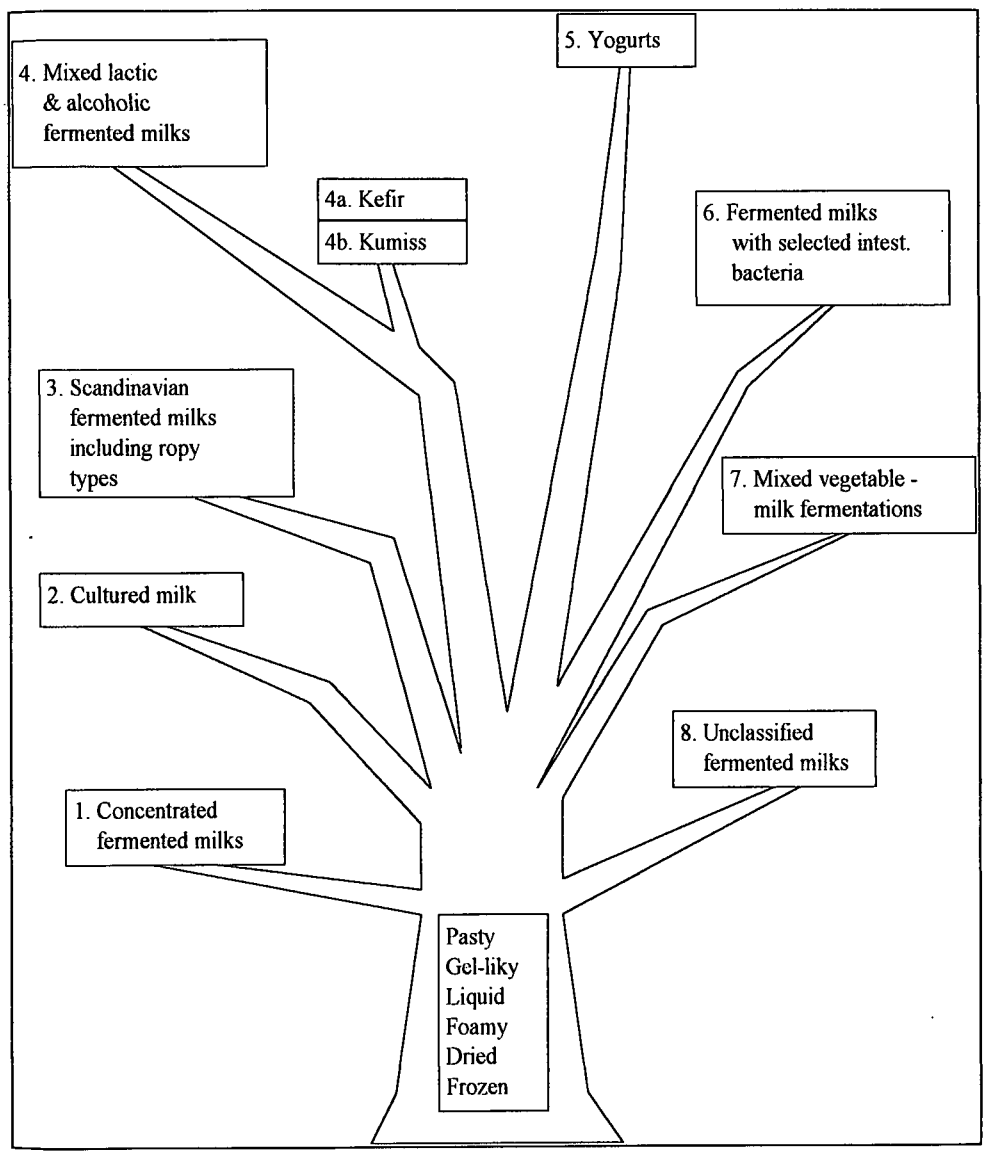

Fig. 2.1 Tree of fermented milk types.

(Roginski, 1988)

The following classification of fermented milks, according to organisms used for their manufacture, has been compiled by Kurmann (1984):

1. Fermented milks

1.1 Thermophilic bacteria, incubation temperature 30/35-40/45°C

1.1.1 Lactic acid fermentation, without producing appreciable amounts of gas and alcohol.

- Yogurt and similar traditional fermented milks: Yogurt (Bulgaria, Turkey etc.), Dahi (Indian). Diluted yogurt: eyran (Turkey), doogh (Iran), jub-jub (Lebanon). Concentrated yogurt: labneh, lebneh (Lebanon and other Arab countries), tan, than (Armenia), tulum, torba and kurut (Turkey), leben zeer (Egypt), laben raid (Saudi Arabia), zabady, zabady (Egypt and Sudan), roba, rob (Sudan and Iraq), matzoon, madzoon (Armenia), tiaourti (Greece), skyr (Iceland), tarho (Hungary)

1.1.2 Acid fermentation, without producing appreciable amounts of gas and alcohol, using mainly human intestinal bacteria:

- Mono-cultures: acidophilus milk, 'bifidus' milk, yakult.
- Mixed cultures of different formulae: BAT type (*Bifidobacterium* spp. *Lb. acidophilus, Str. salivarius* subsp. *thermophilus*); BAP type (*Bifidobacterium* spp., *L. acidophilus, Pediococcus* spp.) and other formulations.
- Types of fermented milk other than those mentioned previously.

1.2 Mesophilic bacteria, incubation temperature 10/15-20/30°C.

1.2.1 Lactic acid fermentation with slimy consistency:

- Nordic fermented milks (Scandinavian); karnmjolk, filmjolk and långfil (Sweden), viili (Finland), tettemilk and kjernemelk (Norway), tykmaelk (Denmark) and similar products.

1.2.2 Lactic acid fermentation using butter cultures:

- Fermented milks prepared with butter cultures.
- Artificial buttermilks: 'cultured buttermilk' (which is a type of cultured milk produced in North America) and similar products.

1.2.3 Concentrated fermented milks:

- Commercial products, e.g. ymer and lactofil (Scandinavia).
- Traditional homemade milks, e.g. Kellermilch and Legermilch in German-speaking areas of Europe.

1.2.4 Mixed lactic acid and ethanol fermentation:

- Koumiss (North Central Asia), leben, laban (Lebanon, Iraq and Egypt), and other similar products.
- Kefir (Caucasus) made of kefir grains,
- Artificial preparations, e.g. kefir made without grains.

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 claimed to be a specific carrier of specific microorganisms and/or enzymes:

- Nordic ropy milks.

1.4 Various unclassified fermented milks

2. Buttermilks

2.1 Conventional cultured buttermilks, a by-product of cultured butter manufacture.

2.2 Cultured buttermilk obtained by fermentation of a sweet buttermilk.

2.3 Yogurt buttermilk obtained by churning yogurt into butter and a liquid by-product.

3. Cultured creams

3.1 Cultured creams made with butter culture.

3.2 Cultured creams made with other bacteria

Kurmann et al. (1992) also compiled an encyclopaedia of fermented milk products of the world with all the relevant information regarding method of manufacture, organisms involved, etc. in the process of manufacture. According to Kosikowski and Mistry (1997), Russia produces the largest quantity of fermented and cultured milk products worldwide. Kefir is the most popular fermented milk beverage, while sour milk is now produced only in small volumes. Acidophilus milk with and without added bifidobacteria, is gaining popularity in Russia, Finland and Japan. Consumption of fermented milks in South Africa, other than yogurt and buttermilk, amounts to 2 kg per capita during 1966-1986 (IDF, 1990).

2.3.2 Traditional fermented milk of Southern Africa

2.3.2.1 Method of manufacture

From the Hottentots and Bushmen (Burchell, 1953), to the Zulu (Krige, 1965) Xhosa, Pondo, Thonga and Venda (Turner, 1909), to name a few, all had cattle and therefore milk. They all prepared sour milk and drank the whey. Butter prepared from the cream on top of the fresh milk, was smeared on their heads.

These people have used various containers and utensils for generation after generation and unintentionally preserved the unknown starter cultures. The Xhosa used small woven baskets initially and later milk-pails made out of wood (Soga, 1932). Perhaps the most used utensil was the calabash. 'Amasi' or sour milk was also prepared in stone jars, but according to Fox (1939), it does not impart the same flavour.

Flat leather pouches made out of cattle hide or bird skin was often used as described by Fehr (1968). The Nguni tribe also used milk sacks made out of ox-hides or other skin of animals or calabash vessels (Bohme, 1976). The Zulu used a gourd, whereby the Basuto

(Richards, 1932) and Bushmen (Burchell, 1953) used skin bags made of bird skin. According to Fox (1939), the Sotho used clay pots that impart a better flavour. 'Maas', 'amasi' or 'inkomasi' (all referring to traditional sour milk) were traditionally produced in clay pots and calabashes, but are now commercialized (Keller and Jordaan, 1990).

Fresh warm milk from the cow is normally poured directly into a calabash. The latter is then loosely stopped with the core of a maze cob and placed aside within the hut or during the winter-season outside in the midday sun. Sessile bacteria attached on the inner surface of the container are presumed to be responsible for the fermentation of the milk. Mixed fermentation of hetero- and homo-fermentative lactobacilli, streptococci, leuconostocs and yeasts have been reported to be dominant (Keller and Jordaan, 1990). After two to five days the curd has wholely separated from the whey. The whey, which is then allowed to run off through a small hole, hitherto securely plugged, situated in the bottom of the calabash, and the calabash is filled with fresh milk. Fermentation again sets within 2 to 3 hours when the 'amasi' is 'ripe', and froth is expelled from the mouth. The sour milk is now ready to be consumed after the whey has once been withdrawn.

Once a week or sometimes a fortnight, the milk vessel is washed, but not too often. Hot water and rough stones are poured into the vessel and roughly shaken a few times to remove the stale 'amasi' (Richards, 1932). It takes quite some time for the vessel to become 'seasoned', but once in order, it can be used for years (Fox, 1939). Sometimes some of the 'amasi' is left in the vessel to hasten the fermentation process, or to impart a special flavour.

The same method, with a few minor differences, is employed by other tribes: for example, the Basuto instead of using a gourd or calabash, have a skin bag in which they prepare their sour milk (Richards, 1932). However, milk bags made from hide and skin, can never be cleaned so perfectly to remove all taint of former sour milk. In a few hours, coagulation takes place and the milk is ready to use (Richards, 1932; Burchell, 1953). Small baskets that are skillfully woven by the woman from a fine kind of reed grass in such a way that it

is completely watertight, after it has previously been rubbed with grease, were also used. The milk is left to curdle and get sour, which are accomplished in a very short time in these baskets which have been repeatedly served to sour the milk, and which are, therefore, already acid (Fehr, 1968).

Over a wide range of cultures in the native tribes, there is a special significance attached to the use of sour milk or 'amasi'. Only members of the household or those connected by marriage may consume the 'amasi', and strangers may not share the dish. This is also applicable to the wife, who may only after their first year of marriage, consume from her husband's sack with permission (Turner, 1909; Soga, 1932). In the bushveld where milk is plentiful, grown men also consumed 'amasi', but in the middle and highveld, one seldom finds any but children taking 'amasi' (Sansom, 1974).

Milk is almost always consumed sour, though occasionally the woman and umfaas (small children) drink it fresh from the cow (Richards, 1932). In rural Zimbabwe, 'amasi' has been made for generations and the process been known to be a fairly effective method for converting milk into a more stable food product incorporated for processing small quantities of milk (Mutukumira, 1995).

2.3.3 Other traditional fermented milks

There are numerous types of traditional fermented milks known in the world. A comprehensive table of traditional fermented milk products throughout the world (Obermann, 1985), summaries all the different kinds of fermented milk products, country of origin, milk type and conditions, and the microflora present in each. Only a few are mentioned. A very similar product to the South African traditional sour milk, is made is Southern Ethiopia. 'Ititu' or concentrated fermented milk means sour milk because of the sour taste of the product. The milk is fermented in a vessel or 'gorfa' after it has been smoked with wood. When the small volume of milk (100-300 ml) has coagulated, the whey is removed and fresh milk is added to ferment (24-48h). This process is repeated

several times for up to 2 months until it is ready for consumption. It has a shelf life of about 2 months. During the fermentation period, the gourd is examined for mould growth, and if present, removed from the surface of the milk. To prevent further contamination, the gourd is again smoked with wood and the lid treated with the leaves of a special plant (Kassaye, 1991). The Masai of Northern Tanzania also store their milk in smoked gourds to give it a smoky flavour (Isono et al., 1994).

At the eastern sides of Mount Kenya, the Meru community applied very much the same tradition from generation to generation. The yogurt-like product they prepare is called 'Iria ri Matii'. The fermenting vessel or gourd is a mature fruit prepared the same way as the calabash. The inside of the vessel is carefully scratched with charcoal, and milk is fermented and discarded several times to impart the special product and flavour. When a batch is completed after incubation of three to four days, a fresh batch must be prepared immediately, or the gourd must be thoroughly cleaned and dried and the same process of preparation must be followed (Kimonye and Robinson, 1991).

'Nono' is another typical traditional fermented milk in Nigeria made with fresh cows' milk, although goats' milk has also been investigated as an alternative medium. Production takes place at domestic level in calabashes or any other suitable container, and are sold on farms, open-air markets and by hawkers (Atanda and Ikenebomeh, 1988; Bankole and Okagbue 1992; Olasupo, 1992).

In central Morocco, fresh cows' milk is used for the production of 'raib', their traditional fermented sour milk. Fermentation takes place at 15-30°C for one to three days (Hamama and Bayi, 1991). Very much the same applies in Lower Egypt where 'Laban rayeb' is manufactured in earthenware pots from cows' milk. It is produced on domestic level and sold at markets (El-Gendy, 1983; Khalafalla et al., 1988). Kurmann (1984) summaries the various microorganisms in relation to one another and their surroundings used in traditional fermented milks. It is interesting to note that various additives are used to start the fermentation process, e.g. other fermented milk products, such as butter and fresh

cheeses, bacteria from the gastro-intestinal tract, certain plants, germinating seeds and dew from certain plants. In Sudan, a few seeds of black cumin and a bulb of onion are added to fresh milk to start the fermentation process of one of their traditional milks made in leather bags, called "gariss" (Abdelgadir et al., 1998).

Other Asiatic countries add yeasts from beer, grated bread, etc. Peculiarities include the addition of horse bones, silver coins, fresh sheep-urine, ants, etc. Fermented milk has been consumed in Finland from ancient times. Milk left to sour naturally, is called 'fiili' or 'filli'. In West Sumatra, Indonesia, an ethnic group develops their traditional fermented milk called 'dadih' from buffalo milk by pouring it into fresh bamboo tubes and caps it with banana leaves. Microorganisms derived from the milk, banana leaves and bamboo tube ferment the milk within 2 to 3 days at room temperatures (Hosono et al., 1989).

Kefir is a fermented milk made from kefir grains. Kefir grains seem to be as old as humanity and some of these grains are known in South Africa as 'joghurt-plantjie' (yogurt plant. The kefir grains are added to milk in a suitable container, left at room temperature and fermented for 24 to 48 hours. The final product has a smooth texture, acid taste and is refreshing due to carbon dioxide formation during fermentation of the yeasts and heterofermentative organisms (Keller and Jordaan, 1990).

2.4 CHARACTERISTICS AND IMPORTANT PROPERTIES OF FERMENTED MILKS

2.4.1 Microbial composition

2.4.1.1 Lactic acid bacteria

A characteristic property common to all fermented milks are the presence of lactic acid. Kefir and Koumiss are additionally transformed by lactose-fermenting yeasts, which resulted in the production of ethyl-alcohol and carbon dioxide. Physical and flavour

differences are apparent too (Kosikowski and Mistry, 1997). Propagation of natural microflora is initiated with a small quantity of the previously coagulated milk to seed the fresh portion of milk. Milk is also acidified with a small piece of lamb or calf's stomach or with a portion of dried sour milk. In Bulgaria 'podkvasa', a kind of natural leaven is produced by shepherds from sheep milk, containing excellent naturally occurring souring strains used as starters for souring milk.

In all countries where traditional lactic acid fermentation of milk exists at present, the original microflora act as a mixed, naturally stabilized population of undefined or only partially defined composition. As reported in many investigations, the original fermented milk samples showed the presence of various lactic acid bacteria, yeasts and moulds with *Str. thermophilus* and *Lb. bulgaricus* predominating. Other groups include micrococci, other lactic cocci, *Leuconostoc* spp., *Lc. lactis* and *Lc. cremoris*. The vast amount of different fermented milks produced with similar bacterial species, yet which differ very much from each other, confirm the large spectrum of metabolic activity and specificity of the strains used, even though they belong to the same species (Obermann, 1985). Traditional fermented milks contain, on the whole, mesophilic bacteria in regions with a cold and temperate climate and, on the whole, thermophilic bacteria in regions with hot and temperate or subtropical or tropical climate (Kurmann, 1984).

Naturally fermented milk is a result of spontaneous fermentation of milk set at temperatures around 25°C. The lactic acid bacterial cultures fall into two general categories, mesophilic cultures which grow optimally at >30°C and thermophilic cultures which grow best at 30°C (Schaack and Marth, 1988). Lactic acid bacteria produce large amounts of lactic acid from lactose. The resulting decrease in pH renders the medium in which they have grown unsuitable for the growth of most other microorganisms. The lactic acid bacteria (LAB) present in the final product have been reported as being dominated by lactobacilli, with the following species isolated in a study done on Zimbabwean traditional fermented milk: *Lb. helviticus, Lb. plantarum, Lb. delbrueckii* subsp. *lactis, Lb. casei* subsp. *casei* and *Lb. casei* subsp. *pseudoplantarum* (Feresu and

Muzondo, 1990). *Lactococcus, Lactobacillus* and *Leuconostoc* were the predominant LAB found by Mutukumira (1996) in traditional fermented milk produced in Zimbabwe. In Sudan, *Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis* were identified amongst the bacteria in 'gariss' (Abdelgadir et al., 1998). The lactic acid imparts a fresh flavour to fermented milks, assists in curd coagulation and texture formation, and the low pH helps to suppress the growth of pathogens and spoilage organisms. LAB also produce traces of flavourful aroma compounds and their proteolytic and, to a lesser degree lipolytic activity, aid the maturation of ripened dairy products (Sharpe, 1979).

2.4.1.2 Yeasts in traditional fermented milk

Some yeasts are considered food contaminants and in many cases they are responsible for product spoilage. Their presence, however, is common in natural cultures and they are able to establish positive relationships with many types of bacteria. Kefir grains represent the most important and interesting example of co-operation between yeasts and bacteria. A fundamental role is played by yeasts in this symbiosis (Dellaglio, 1988). Almost all the yeasts in fermented milk products grow in interaction with mainly lactic acid bacteria and other microorganisms.

Fermentations involving yeasts and lactic acid bacteria, and which are carried out in vessels will rapidly become anaerobic, acidic and saturated with carbon dioxide and alcohol. This kind of conditions will certainly be inhibitory to many spoilage microorganisms, including filamentous fungi and bacteria associated with various forms of food poisoning (Wood and Hodge, 1985). Yeasts in dairy products may interact with other microorganisms in three different ways: (i) they may inhibit or eliminate microorganisms which are undesired because they cause quality defects or possess potential pathogenic characters; (ii) they may inhibit the starter culture, or (iii) they may contribute positively to the fermentation or maturation process by supporting the function of the starter culture (Jakobsen and Narvhus, 1996).

According to the literature there are at least seven fermented milks that owe their production to both yeasts and lactic acid bacteria. In Table 2.1 an outline is given of traditional fermented milk products containing yeasts.Yeasts play undoubtedly an important role in these natural fermentations. Some properties of yeasts are very important in dairy fermentations and include the following: (1) fermentation or assimilation of lactose; (2) production of extracellular proteolytic enzymes; (3) production of extracellular lipolytic enzymes; (4) assimilation of lactic acid; (5) assimilation of citric acid; (6) growth at low temperatures and (7) tolerance of elevated salt concentrations (Fleet, 1990). There is, however, no scientific information available regarding the species diversity, biochemical and physiological properties and significance of yeasts in indigenous, traditional South African fermented milks.

The presence of various yeast and mould spp. in naturally soured milk products were reported by researchers investigating African fermented milk products (El–Sadek et al., 1972; Abou-Donia, 1984; Hosono et al., 1989; Abdelgadir et al., 1998; Kimonye and Robinson, 1991; Isono et al., 1994). Yeasts are normally present in lower numbers than the lactic acid bacteria. It is also clear that the presence of yeasts is influenced by the type of containers and processing methods used. Components of the smoke generated from the charcoal treatment of Iria ri Matii, a traditional yogurt-like drink from certain parts of Kenya, suppressed naturally occurring milk associated yeasts and moulds, thereby preventing a heavily contamination of yeasts in the product (Kimonye and Robinson, 1991). Isono et al. (1994) on the other hand, examined ten similar fermented milk products from different areas and found yeast counts between $1.00x10^6$ and $1.00x10^8$ cfu/g. Only *Saccharomyces* and *Candida* spp. could be identified. According to Abou-Donia (1984) various studies on Zabady, the national type of yogurt manufactured in Egypt, showed that yeasts and moulds are normally present in this product with numbers ranging from $5.00x10^4$ to $7.80x10^8$ cfu/ml. El–Sadek et al. (1972), reportedly found that the majority of species belonged to the genus *Candida* with only a few belonging to *Torulopsis*. The presence of yeasts in Zabady might well be due to contamination. The

presence of yeasts in various other traditional fermented milks have also been recorded (Hosono et al., 1989; Kassaye et al., 1991; Bankole and Okagbue, 1992).

2.4.1.3 Flavour development and flavour compounds

One of the important factors determining the specific identity of fermented milk products is the presence of specific flavour compounds. In sour milk and other products such as buttermilk and sour cream, diacetyl and acetoin are of particular importance. *Lc. diacetylactis* (*Lactococcus lactis* biovar *diacetylactis)* or *Leuconostoc* strains produce them from lactose, or by some bacteria from citrate. Diacetyl is a di-ketone with high aroma potential, especially in butter cultures, but also in other fermented dairy products even when present in very small amounts. It is also responsible for the characteristic 'buttery' nut-meat aroma in milks (Obermann, 1985). Another very important flavouring compound produced by lactic acid bacteria in fermented milks is acetaldehyde, the principal component in natural yogurt, but undesirable in excess in buttermilk (Lees and Jago, 1978a, b). A variety of other neutral and or acidic compounds may also contribute to the flavour and aroma of fermented milks. At low concentrations acetaldehyde is pleasing and suggests the aroma of butter cultures, whereas at higher concentrations it is pungent and rather objectionable (Hammer and Babel, 1943). An 'off-flavour' in non-yogurt cultured milk products could be ascribed to undesirable high concentrations of acetaldehyde in cultured buttermilk and sour cream. Several authors (Lindsay et al., 1965; Sandine et al., 1972), however, reported that flavour of such products is affected by diacetyl and acetaldehyde.

Acetoin, properly purified, has no odour, and at concentrations met in the dairy industry, probably has no effect on the taste of products. Acetoin is normally present at higher concentrations than diacetyl, which is an important aromatic compound in cultured dairy products. The compound is however, unstable as it degrades easily to acetoin (Seitz et al., 1963; Speckman and Collins, 1968; Hugenholtz, 1993).

Table 2.1

Fermented milks with yeast/lactic acid bacteria interaction

Traditional name	Country	Milk types, conditions	Microflora
Kefir	Caucasian mountains Russia	Goat, sheep, cow Kefir grains added, fermentation in skin bag/wooden barrels	*Str. lactis, Leuconostoc* spp., *Sacch. kefir*
Koumiss	Southern Russia, Asiatic steppes	Mare, camel or asses', fermentation in skin bags	*Lb. bulgaricus, Lb. acidophilus, Torula* yeast, *Sacch. lactis,* micrococci, Spore-forming bacilli
Taette/ Taet-mjölk	Northern European countries, Scandinavia	Cow inoculated with butterwort	Mixed lactic-acid (mesophilic strains), *Sacch. major taette*
Mazun (Matsun, Matzoni)	Armenia, Caucasus	Cow or buffalo	*Str. thermophilus,* Rod shaped bacteria, lactose fermenting yeast
Leben, Labneh	Tigris-Euphrate Valley, Lebanon, Egypt	Sheep, goat, cow, buffalo or a mixture. Addition of dried leben which has been cooked and cooled	*Str. lactis, S. thermophilus, Lb. bulgaricus* and lactose fermenting yeasts
Kuban	USSR (Bogdanoff, 1934)	Milk	*Str. hollandicus, Lb. bulgaricus, Mycoderma, Torula lactis* and other undefined yeasts
Dahi	India, Persia	Cow or buffalo Milk is boiled, cooled and inoculated with previous dahi	*Str. lactis, S. thermophilus, Lb. bulgaricus, L. plantarum,* lactose and non-lactose fermenting yeasts

(Obermann, 1985; Wood and Hodge, 1985)

Although yeasts play an important role in certain fermented milk products, e.g. kefir and koumiss, little is known about the nature of their flavour components. The use of yeasts for the promotion of flavour in cultured milk, similar to yogurt, has been suggested by Kuwabara (1970), after Margalith (1981). By introducing *Kloeckera africana* into

fermented milk, little ethanol, but appreciable amounts of aromatic substances were formed. Apparently, preliminary fermentation with yeasts improved the flavour and consistency of these products. However, no description of the aromatic substances is given. Equal or higher flavour scores were received when *Geotrichum candidum*, isolated from raw milk, was inoculated into pasteurized milk prior to cheesemaking (Irvine et al., 1954). Chen et al. (1998), reported on the use of yeast cultures as flavouring agents in yogurt-type products, with yeasts such as *Candida*, *Hansenula* and *Saccharomyces*.

Ethanol is produced in small amounts by *Leuconostoc* spp., which possess active alcohol dehydrogenase and is also the metabolic product of lactose fermenting yeasts present in kefir, koumiss and other similar products. Important acids are: formic, acetic, propionic, caproic, caprylic, capric, butyric and iso-valeric acids, provided by lactic acid fermentation and enzymatic transformation, and enzymatic transformation of amino acids. Carbon dioxide is responsible for carbonation of certain products. All of the above compounds are necessary to bring out the total flavour of fermented milk products. Even the minor metabolic products, being in trace concentrations, may have an important role in balancing the desirable flavour in milk (Sharpe, 1979; Stanley, 1980; Obermann, 1985). The majority of flavouring compounds are produced from lactose but some derived from the metabolism of other milk constituents.

2.4.2 Health properties and claims

Controversy exists over the special health-giving properties of fermented milk foods. Extremists claim a longer life expectancy for the consumers where these foods are staples. They point to the high percentage of centenarians in regions where fermented milks are consumed as, for example, Khrushchev, the late head of the former Soviet Union, once claimed that three times as many people lived to be over 100 years old in his country as in the United States. Others see nothing more in fermented milks than good basic foods (Roginski, 1993; Kosikowski and Mistry, 1997).

Known scientists of early ages, such as Hippocrates, Avicenna, Galen and others, considered milk not only a food product but a medicine as well. They prescribed sour milk for curing disorders of the stomach, intestines and other troubles (Obermann, 1985). There are good reasons to believe that milk could be a very effective means of preventing arteriosclerosis in particular. Sour milks were used also as cosmetics and preservants of food against spoilage. In the early part of the 20th century Metchnikoff (1845-1916) claimed that owing to lactic acid and other products present in sour milks fermented by lactic acid bacteria, the growth and toxicity of anaerobic, spore-forming bacteria in the large intestine are inhibited. Lactic acid is biologically active and capable of suppressing harmful microorganisms especially putrefactive ones and therefore has a favourable effect on human vital activities (Obermann, 1985). In many developing countries, diarrhoea is one of the major precipitating factors of child morbidity and mortality. Lactic acid fermentation, a traditional household-level technique, reportedly is effective in reducing or eliminating the growth of diarrhoea-causing pathogens. Possible antagonistic effects of lactic acid-producing bacteria on pathogens have been proposed (Gibbs, 1987). In a comprehensive article by Hitchins and McDonough (1989), many claims concerning prophylactic and therapeutic effects of fermented bovine milk consumption are mentioned, like increasing the digestibility of the milk proteins, anti-tumor effects, reducing serum cholesterol and lowering blood pressure. Mann and Spoerry (1974) and Richardson (1978) also supported these claims.

The low pH prevents growth and can even kill pathogens, and hence helps prevent food poisoning. Acidity may not always be enough to be bacteriocidal, therefore fermentations do not substitute improper food hygiene and food handling (Hitchins and McDonough, 1989).

2.5 CURRENT DEVELOPMENTS OF YEASTS IN FERMENTED MILKS

A limited number of in-depth-studies have been undertaken to identify the yeasts in traditional fermented milks throughout the world. Specific studies have, however, been

done on commercially available products such as 'Labaneh', a semi-solid dairy product made from set yogurt by removal of part of its whey (Yamani and Abu-Jaber, 1994). The other references to yeasts in fermented milks formed part of the overall microbiological survey done on these products. Most of the time the yeasts were considered as contaminants and unfavourable (Obermann, 1985; Hamama and Bayi, 1991; Kassaye et al., 1991; Kimonye and Robinson, 1991). From the study on the fermented milk of the Masai (Isono et al., 1994), yeasts were not considered part of the essential microorganism population in the product.

In a study on fermented milk in Indonesia on 'dadih', it was found that the yeast *Endomyces lactis* plays an important role in the fermentation of 'dadih', as this strain can utilize ethanol as a sole carbon source (Hosono et al., 1989). Yeasts as probiotics, receive a lot of attention today. The narrow and traditional definition of probiotics is that they serve the purpose of regulating the microbial colonization in the digestive tract (Gedek, 1991, after Jakobsen and Narvhus, 1996). However, in relation to current and future fermented dairy products, it seems more appropriate to define probiotic starter cultures as those that give fermented products an extra nutritional–physiological value. This may include a range of metabolites, partly degraded product constituents, various inhibitors, stimulants, enzymes and co-enzymes leading to an increase in nutritional value and anti-oxidant properties (Lambelet et al., 1992, Jakobsen and Narvhus, 1996). Interactions between yeasts and bacteria, include binding of pathogenic bacteria to the surface of the yeast and have been reported between cultures like *Sacch. cerevisiae* and enteric pathogens, e.g., enteropathogenic *Escherichia coli, Shigella* and *Salmonella.* The surface of the yeast cell is also reported to bind enterotoxins produced by enterobacteria through a mannose-specific reaction. Yeasts also produce metabolites, e.g. short-chain fatty acids and other specific compounds, with known toxic effects against undesired microorganisms in the intestinal tract. It appears that *Sacch. cerevisiae* can survive passage through the intestinal tract, with live cells detectable in the small intestine (Gedek, 1991; Jakobsen and Narvhus, 1996).

Based on the literature on reports concerning the microflora of original fermented milks, it is evident that the original microflora consist mainly of different types of lactobacilli and streptococci and of minor proportions of yeasts and milk associated moulds, being in associated growth and showing mutual symbiotic relationships (Obermann, 1985).

CHAPTER 3

YEASTS IN SOUTH AFRICAN HOUSEHOLD KEFIR

Abstract

Yeasts were isolated, identified and enumerated from seven kefir milks, fermented by different indigenous kefir grains collected throughout the country. All the samples revealed relatively high yeast populations, with counts exceeding $1.00x10^8$ cfu/ml. *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* were the dominating yeast species isolated. Other species encountered were *Saccharomyces unisporus, Saccharomyces rouxii, Torulaspora delbrueckii* and *Debaryomyces hansenii.* The yeast flora of traditional South African kefir grains is quite varied, consisting of non-lactose and lactose fermenting species, with the latter group dominating.

Keywords: Yeasts, kefir grains, fermented milks, *Saccharomyces cerevisiae, Kluyveromyces marxianus*

3.1 INTRODUCTION

3.1.1 Characteristics of the kefir beverage

Kefir is a fermented milk drink commonly consumed in the Commonwealth of Independent States (formerly USSR), Poland, Czechoslovakia, Hungary and the countries of Scandinavia. This fermented milk product originated in the Caucasian Mountains of Russia, which lie between the Black Sea and the Caspian Sea and was prepared in leather bags or oak vats. Nobody knows where and how these kefir grains originated, but according to legend, the kefir grains were given to the orthodox people by Mohammed, who also told them how it was to be used. Mohammed strictly forbade the secret of kefir preparation to be given away to other people or to pass the kefir grains to anybody for they would lose their magic strength. This legend explains why the method for kefir production has been kept a secret for such a long time (Koroleva, 1988). It is not a curdled product and is produced by adding kefir grains to milk. The fermentation is initiated by this white to yellow grains, resembling cooked rice or small cauliflower heads. The grains are insoluble in water, gelatinous, or irregular size, distributed on the inner surface of the vat or bag. Original kefir grains cannot be precisely reconstructed; they are recovered from sour milk and used repeatedly. When added to milk they swell and turn white, forming a slimy, jelly-like product (Obermann, 1985; Kosikowski and Mistry, 1997). Kefir is a self-carbonated beverage that can be made with any kind of milk, like cow, goat, sheep, camel, buffalo and according to Abraham and De Antoni (1999) even soya milk. The kefir beverage has a prickly, sharp acidic taste and yeasty flavour with a small percentage of alcohol (up to 2%). When agitated, kefir foams and fizzes (Wickerham, 1951; Obermann, 1985; Duitschaever, 1989).

3.1.2 Microbial population

Most fermented dairy products are produced making use of bacteria as a starter culture rather than yeasts. Kefir is an exception and is the product of a mixed fermentation with yeasts and bacteria (Bottazzi, 1983; Tamime et al., 1999). The grains contain lactobacilli, streptococci, micrococci and also yeasts in a specific symbiotic relationship to the bacteria. The kefir granules are held together by a polysaccharide, called kefiran.

The yeast flora of kefir vary with its source and production, but mixtures of lactose- and non-lactose fermenting species, identified as *Kluyv. marxianus, C. kefir, C. pseudotropicalis, Sacch. cerevisiae, Sacch. exiguus, P. fermentans* and *T. holmii* have been reported (Iwasawa et al., 1982; Engel et al., 1986; Marshall, 1986; Lin et al., 1999). Two other yeast species and bacteria have been identified: *Sacch. kefir* and *Torula kefir*; certain lactobacilli: *Lb. caucasicus* and *Lb. casei*; and cocci: *Leuconostoc* spp. As spoiling microorganisms were found: micrococci, spore-forming bacilli and coliforms (Obermann, 1985). Unfortunately, an authentic strain of *Lb. caucasicus* no longer exists and the epithet '*caucasicus* ' is not recognized. A reinvestigation of this commonly isolated strain has led to a renaming and it is now known as *Lb. kefir* (Roginski, 1993). A comprehensive summary (Table 3.1) of the yeasts associated with kefir from 1882, has been compiled by Häfliger (1990). Traditional kefir, according to Obermann (1985) contains 70% lactobacilli, 20% streptococci and 5% yeasts.

3.1.3 Kefir production

Kefir is prepared by adding kefir grains to cooled, boiled milk and incubating at 23-25°C overnight. The major end products of the fermentation are lactic acid (*ca.* 0.8%), ethanol and carbon dioxide (1%), traces of acetaldehyde, diacetyl and acetoin, plus other minor components influencing the flavour of kefir. For recovery, the kefir grains are washed with clean, cold water and stored in water at 4°C for up to ten days. They may also be stored in a dried state and still show activity for 12-18 months (Obermann, 1985). This makes for an unusual fermented product, both from a microbiological point of view and from a technical processing viewpoint. During fermentation, some members of the microbial population of the grains become planktonic and will be recovered from the milk. The types and the quantity of organisms present in kefir milk, depend on subsequent processing. For example, household kefirs examined in Germany showed a yeast population between $1.00x10^4$ and $1.00x10^6$/ml in the product immediately after removal of grains. In commercial kefirs, however, yeasts may be absent, or at a population of only $1.00x10^3$/ml (Roginski, 1993).

Table 3.1

Yeasts isolated from kefir

Organisms	Description by Kreger-van Rij (1984)
Sacch. cerevisiae	*Sacch. cerevisiae*
Sacch. kefir	*Kluyv. marxianus* var. *marxianus*
Sacch. kefir	*Kluyv. marxianus* var. *marxianus*
Torula kefir	*Kluyv. marxianus* var. *marxianus*
Sacch. fragilis	*Kluyv. marxianus* var. *marxianus*
Sacch. cerevisiae	*Sacch. cerevisiae*
Sacch. delbrueckii	*T. delbrueckii*
T. holmii	*C. holmii*
Sacch. lactis	*Kluyv. marxianus* var *lactis*
C. tenuis	*C. tenuis*
Sacch. calbergensis	*Sacch. cerevisiae*
C. pseudotropicalis	*C. kefir*
C. pseudotropicalis	*C. kefir*
Saccharomyces spp.	
Kluyv. lactis	*Kluyv. marxianus* var *lactis*
C. valida	*C. valida*
Br. anomalus	*Br. anomalus*
Sacch. unisporus	*Sacch. unisporus*
Sacch. delbrueckii	*T. delbrueckii*
Sacch. cerevisiae	*Sacch. cerevisiae*
Sacch. florentinus	*Zygosacch. florentinus*
C. kefir	*C. kefir*

Häfliger (1990)

Under South African conditions, a total different yeast population may be found in comparison to the native countries of kefir. Such differences might e.g. be due to adaptions to ecological factors (yeast-product associations), as suggested by Fleet (1990).

Two methods are currently used in Europe for the production of kefir: Firstly, those which have been developed by industrialization of traditional methods and secondly those that arise from new starter development. One of the problems encountered by the large-scale processing, is gas production. Kefir has a yeasty flavour with an alcohol and carbon dioxide content, which in itself create certain problems when manufactured for the retail market. Suitable containers therefore have to be available. Glass, crown-capped vessels are traditional, and these are returned to the dairies in eastern Europe. But, in the disposable culture of western European countries, alternatives had to be found. The foil-capped polystyrene container tends to blow and consumers perceive this as a defect. Attempts limiting the numbers of yeasts, thereby limiting gas production, were launched with the expectation that consumers would be happier. Consumers of typical kefir, however, abstained from consuming these products carrying the label 'kefir' in Europe, as it did not have the expected yeast flavour and effervescent liveliness. A number of closures have since been patented which allow for gas to escape, thus preventing bulging of the lids, yet retain the gaseous nature. The design of the aperture also protects entry of contaminating bacteria and dust. In South Africa, however, kefir is unknown to the public. Apart from the few households where kefir has been manufactured for years as a 'yogurt-plant' in the kitchen, many people are not aware of the existence of such a product.

3.1.4 Nutritional and health benefits

As with many fermented milks, health benefits are also claimed for kefir. Milk is a nutritious medium in its own right, well supplied with lactose, protein, fat and vitamins. During fermentation, some of the lactose is utilized and lactate is produced, offering advantages to lactose-malabsorbing populations. In kefir, more L(+)-lactate is produced than the D(+)-isomer. L(+)-lactate can be utilized in the human gastrointestinal tract (Marshall, 1993). Protein is hydrolized during the fermentation process and 7% of the

nitrogen is available as small peptides and 2% as free amino acids, which improves digestibility. Moreover, yeasts isolated from traditional cultured dairy products, reportedly manifested high activity against tuberculosis and coliform bacteria (Roginski, 1993). Kefir also has high nutritional, biological and dietetic value and is widely recommended for healthy people, as well as patients with gastro-intestinal and metabolic diseases, hypertension, ischaemic heart disease and allergy (Koroleva, 1988).

Kefir is not manufactured commercially or commercially available in South Africa. It has however, been established that kefir grains are used in a few households to ferment milk. The microbiological composition, and especially the diversity of yeasts in these products, are unknown. The purpose of this study was therefore to enumerate, isolate and characterize yeast strains present in kefir, manufactured with indigenous grains, and to compare with results reported elsewhere.

3.2 MATERIALS AND METHODS

3.2.1 Kefir production

Seven different kefir grains were obtained from households in different regions in the South Africa. The morphology of the seven kefir grains is shown in Fig. 3.1. They were maintained in the laboratory by preparing kefir every third day. The kefir grains were added to cold heat-treated milk (autoclaved at 121°C for 10 min), incubated at 25°C for 18 h and then cooled to 7°C. The kefir grains were recovered from the milk by means of a household sieve, washed in quarter-strength, sterile Ringer's solution (Merck) and transferred to fresh heat-treated milk.

3.2.2 Enumeration

Kefir milk with grains were sampled aseptically (100 ml) in sterile polythene sampling bags (Whirlpack, Nasco) and homogenized in a Colworth 400 Stomacher (Stomacher 400 Lab-blender, Seward Medical UAC House, London) for 2 min and serially diluted in sterile, quarter-strength Ringer's solution. In Table 3.2 the methods for the enumeration and isolation of the microorganisms in kefir, are summarized.

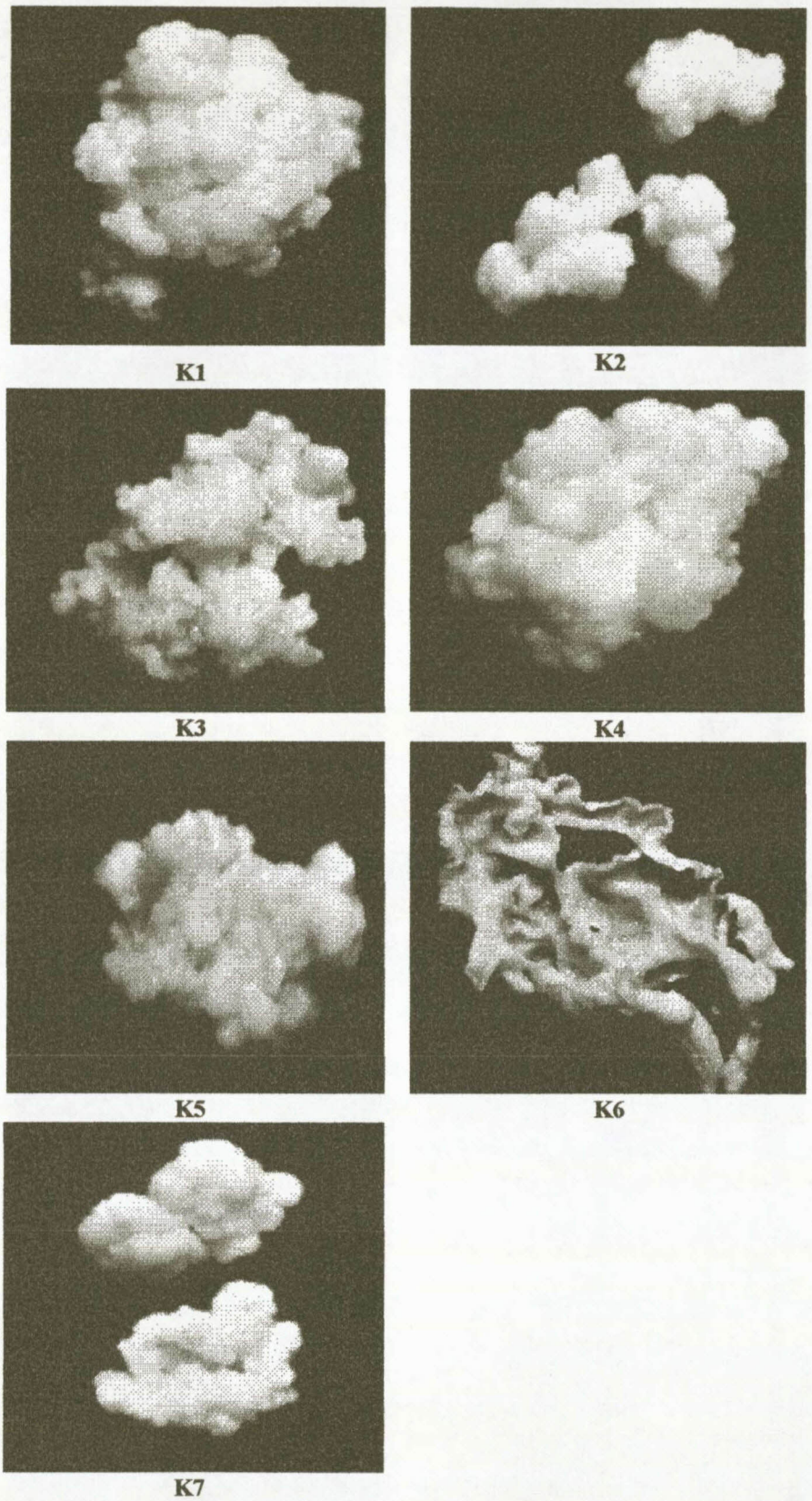

Fig. 3.1 The morphological characteristics of the seven kefir grains (x 2·5).

Table 3.2

Methodology applied for the microbiological analyses of kefir

Microorganisms	Media	Incubation time	Temperature	Atmosphere	Reference
Yeasts	YM	72 h	25°C	Aerobic	Wickerham (1951)
Thermophilic lactobacilli plus streptococci	MRS agar (Oxoid CM 361)	4 h	42°C	Aerobic	De Man et al. (1960)
Lactococci	M17 (Oxoid CM 785)	48 h	30°C	Anaerobic	Terzaghi and Sandine (1975)
Lactobacilli plus leuconostocs	Rogosa agar (Merck)	48 h	35°C	Anaerobic	Rogosa et al. (1951)
Acetic acid bacteria	MYP medium (2.5% D-mannitol, 0.5% yeast extract (Oxoid), 0.3% Peptone (Oxoid), and 2.5% agar)	5 d	28°C	Aerobic	Gosselé et al. (1983)

Viable plate counts were prepared by the spread-plate method, using Yeast-Extract Malt-Extract agar (YM) for the enumeration of yeasts (Wickerham, 1951). All plates were incubated at 25°C for 72 h.

Viable plate counts, according to the pour-plate method, for the lactic acid bacteria (LAB) and especially the thermophilic lactobacilli plus streptococci, were determined on MRS agar (Oxoid; CM 361) and incubated aerobically at 25°C for 72 h (De Man et al., 1960). The lactococci were determined on M17 agar (Oxoid; CM 785) and incubated anaerobically by means of a CO_2/H_2 gas-generating kit (Oxoid; BR 038 B) placed in an anaerobic jar at 30°C for 48 h (Terzaghi and Sandine, 1975), whereas the lactobacilli plus leuconostocs, were determined on Rogosa agar (Rogosa et al., 1951). The plates were also incubated anaerobically at 35°C for 48 h. For the determination of the acetic acid bacteria, MYP medium was used (Gosselé et al., 1983) and the plates incubated aerobically for 5 d at 28°C.

3.2.3 Isolation and identification

The predominant yeast isolates from the highest dilutions ($1.00x10^5$) on the YM plates were isolated and pure cultures were obtained by three successive streakings onto YM agar plates. Stock cultures were maintained on YM slants and kept at 4°C, until they were identified. The pH, physiological and biochemical characterization of the yeasts were performed as described by Kreger-van Rij (1984). The identifications were done by the keys proposed by Van der Walt and Yarrow (1984) and Barnett et al., (1990). Each isolate was inoculated into six fermentation media, 33 carbon source assimilation media and vitamin free medium (Van der Walt and Yarrow, 1984). Additional tests performed, included growth at 37°C, in 50% (m/v) D-Glucose medium, urea hydrolysis, splitting of arbutin and 0.01 and 0.1 % cycloheximide (Van der Walt and Hopsu-Havu, 1976). Assimilation of nitrogen compounds, as performed by means of the auxanographic method of Lodder and Kreger-van Rij (1952), was also included.

Ascospore formation was examined on McClary's Acetate agar, Potato Glucose agar, Gorodkowa agar, Corn Meal agar (Oxoid, CM103), and Malt Extract agar (Biolab, C10)

(Kreger-van Rij, 1984). The inoculated media were incubated at 18°C for 4 weeks and examined at 4 d intervals. Cell morphology and mode of reproduction were examined on Malt Extract agar (Biolab, C10) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium was examined on Corn Meal agar, according to the Dalmau plate technique (Wickerham, 1951).

3.3 RESULTS AND DISCUSSION

3.3.1. Enumeration

In Fig. 3.2 the overall microbiological composition of the kefir samples is outlined. Yeasts were found in all seven kefir samples. According to Mann (1989) yeast counts of $1.00x10^7$-$1.00x10^8$ cfu/ml were reported for kefir in Czechoslovakia, while lower counts ($1.00x10^2$-$1.00x10^4$ cfu/ml) were found by Marshall et al. (1984) in subcultured fermented kefir milk without grains. The counts also declined after the third subculture and no yeasts could be recovered after the fifth subculture. This is a strong indication of the important role of the kefir grain matrix in supporting the microbial composition of kefir. Yeast counts ranging between $6.40x10^6$ and $2.10x10^5$ cfu/ml were found by Kuo and Lin (1999), while in a study by Wyder (1998), yeast counts varied between $1.00x10^6$ and $1.00x10^9$ cfu/ml, respectively. In the present study, all the kefir samples had relatively high yeast counts that varied between $2.20x10^8$ and $3.00x10^8$ cfu/ml.

From Fig. 3.2, it is evident that the majority of the microbes were yeasts, lactic acid and acetic acid bacteria. The pH varied between 3.98 and 4.10, but no definite relationship was found between the yeast count and pH.

The LAB, namely the lactobacilli plus leuconostocs, thermophilic lactobacilli plus streptococci and lactococci, were also present in relatively high numbers. The dominating LAB were lactobacilli plus leuconostocs, however, in sample K3, the thermophilic lactobacilli plus streptococci and lactococci ($1.23x10^9$ and $1.20x10^9$ cfu/ml, respectively) were found in higher numbers.

Fig. 3.2 Microbiological composition of seven kefir milk samples.

The highest microbial counts of streptococci and lactobacilli, from a study by Kuo and Lin (1999), were $3.60x10^{11}$ and $8.30x10^{9}$ cfu/ml, respectively. The acetic acid bacteria were present in lower numbers than the yeasts and LAB, and varied between $1.17x10^{3}$ (K2) and $1.15x10^{5}$ cfu/ml for kefir K6.

3.3.2. Isolation and identification

Table 3.3 represents an outline of all the different yeast species isolated from the kefir samples. Four different genera were identified from 14 isolates, with 3 different species belonging to *Saccharomyces,* two different species to *Kluyveromyces* and one each to *Debaryomyces* and *Torulaspora.* The lactose fermenting yeast *Kluyv. marxianus* (5 isolates from 14 samples) was the most prevalent species followed by *Sacch. cerevisiae* (3) and *Kluyv. lactis* (2). The other four isolates were identified as *T. delbrueckii, D. hansenii, Sacch. rouxii* and *Sacch. unisporus,* a non-lactose fermenting yeast.

Table 3.3

Different yeast species isolated from kefir samples

Species	Total number	Kefir samples						
		K1	K2	K3	K4	K5	K6	K7
Kluyveromyces marxianus	5	2	-	1	1	1	-	-
Saccharomyces cerevisiae	3	-	1	-	-	-	-	2
Kluyveromyces lactis	2	-	-	-	-	1	1	-
Saccharomyces unisporus	1	-	-	-	1	-	-	-
Saccharomyces rouxii	1	-	1	-	-	-	-	-
Torulaspora delbrueckii	1	-	-	-	-	1	-	-
Debaryomyces hansenii	1	-	1	-	-	-	-	-

K2 and K5 were the only samples from which three different species were isolated. All the isolates were good candidates that one may expect in fermented milks. The studies of Angulo et al. (1993) showed that *T. delbrueckii* and *Sacch. cerevisiae* were the most frequently isolated species from kefir.

In Table 3.4 the species diversity is summarized. The dominating yeast species of the kefir samples were *Kluyv. marxianus* (36%), *Sacch. cerevisiae* (22%) and *Kluyv. lactis* (14%). The rest of the yeast species, namely *Sacch. unisporus*, *T. delbrueckii*, *D. hansenii* and *Sacch. rouxii* were each represented by 7%.

Apart from *Kluyv. lactis* and *Kluyv. marxianus*, all the yeasts isolated from the kefir samples were unable to ferment lactose, which made them dependent on lactic acid bacteria capable to hydrolyze this disaccharide. It is interesting to note that lactose-positive species appeared with lactose-negative yeasts in the samples, e.g. *Kluyv. lactis* (lactose-positive) and *T. delbrueckii* (lactose-negative).

Table 3.4

Species diversity of 14 yeasts from seven household kefir samples

Species	% Isolates
Kluyveromyces marxianus	36
Saccharomyces cerevisiae	22
Kluyveromyces lactis	14
Saccharomyces unisporus	7
Torulaspora delbrueckii	7
Debaryomyces hansenii	7
Saccharomyces rouxii	7

In a report on the kefir production in Poland, the non-lactose fermenting yeasts were found in the deeper layer of kefir grains while lactose fermenting yeasts were in the peripheral or outer layers of the kefir grain (Libudzisz and Piatkiewicz, 1990, Wyder, 1998). Koroleva (1988) in Russia also confirmed this observation.

According to Dellaglio (1988) and Koroleva (1988), *C. kefir*, the imperfect state of *Kluyv. marxianus,* is also frequently associated with kefir. In a very similar study performed on milk products, 66% of the isolates from kefir were identified as *Kluyv. marxianus*, and the other species belonged to *P. fermentans* (19%), *Sacch. cerevisiae* (9%) and *Sacch. dairensis* (5%) (Rohm et al., 1992).

3.4 CONCLUSIONS

All the yeasts isolated from kefir, were also found by other authors in traditional kefir (Iwasawa et al., 1982; Engel et al., 1986; Marshall, 1986; Koroleva, 1988; Roginski, 1988; Libudzisz and Piatkiewicz, 1990; Kroger, 1993; Roginski, 1993). The dominating yeast species were *Kluyv. marxianus, Sacch. unisporus* and *Sacch. cerevisiae.* The high numbers of yeasts and LAB in kefir, indicate that both may be important and part of the overall microflora. The symbiotic relationship observed between the yeasts and LAB is a common phenomenon, where the lactose is converted to lactic acid for use by the non-lactose fermenting yeasts. The rather similar yeast composition in all seven samples, shows the unique ability of the kefir grain for self-regulating its microflora (Koroleva, 1988). Wyder (1998) found varying yeast flora compositions and counts in kefir and kefir grains, thus revealing completely different ecological systems in these two products.

It can be concluded that the yeast flora of traditional South African kefir grains varied and consists of non-lactose and lactose fermenting species, with the latter group, dominating.

CHAPTER 4

DIVERSITY OF YEASTS IN TRADITIONAL FERMENTED MILKS

Abstract

Yeasts were enumerated, isolated and identified from different traditional fermented milks manufactured in households by small-scale dairy farmers in rural areas. A total of 50 different yeast strains were isolated from 14 sour milk samples. The yeast counts in the sour milk samples varied between $1.00x10^3$ and $1.00x10^6$ cfu/ml. Predominant species found in the fermented milks were *Torulaspora delbrueckii* (40%), *Debaryomyces hansenii* (22%) and *Kluyveromyces marxianus* (18%). Other species encountered were *Saccharomyces cerevisiae* (6%) and *Yarrowia lipolytica* (4%). *Dekkera anomala, Pichia membranaefaciens, Rhodotorula glutinis, Trichosporon beigelii* and *Galactomyces geotrichum* represented only a small percentage of the overall population. The lactic acid bacteria were present at counts varying between $1.00x10^5$ and $1.00x10^9$ cfu/ml.

Lactate assimilation proved to be a common feature for the yeast isolates. Furthermore, several species were capable of assimilating and fermenting lactose, and proteolytic breakdown of casein. A limited number of yeasts showed lipolytic activity. Most isolates were able to grow in the presence of 10% (m/v) NaCl whereas *Debaryomyces hansenii, Kluyveromyces marxianus* and *Yarrowia lipolytica* grew in the presence of 20% (w/v) NaCl. Three of the isolates showed antimicrobial activity against *Staphylococcus aureus* and were identified as *Kluyveromyces marxianus* (2 isolates) and *Debaryomyces hansenii.* Eighteen isolates produced tryptamine and tyramine when tested for amine production while not one strain produced histamine.

Key Words: Traditional fermented milks, yeasts, lactic acid bacteria, biochemical and physiological properties

4.1 INTRODUCTION

The nutritional and biotherapeutic properties of traditional fermented milks have stimulated research into various aspects of microbial metabolism, microbial composition and fermentation processes of these products (Gibbs, 1987; Hamama and Bayi, 1991; Kimonye et el., 1991; Jakobsen, 1994; Jakobsen and Narvhus, 1996). The preparation of traditional fermented milks in rural households implies spontaneous fermentation of milk by naturally occurring microbes (Fox, 1939; Kurmann, 1984; Hosono et al., 1989; Kimonye and Robinson, 1991). The "starter cultures" are introduced by the re-use of fermentation vessels and tools thereby ensuring a certain repeatability and stability in the fermentation process. It is, however, obvious that microorganisms, other than lactic acid bacteria (LAB), are also present which may contribute positively or negatively to the quality of these products. These organisms convert part of the lactose to lactic acid, which has a preservative effect on milk (Kosikowski and Mistry, 1997, Schaack and Marth, 1988). The low pH of cultured milk inhibits the growth of putrefactive bacteria and other undesirable organisms. Carbon dioxide, acetic acid, diacetyl, acetaldehyde and several other substances are formed during the conversion process, and these impart to the products their characteristic fresh taste and aroma.

Yeasts undoubtedly play an important role in these natural fermentations (Jakobsen, 1994). There is, however, no scientific information available regarding the species diversity, biochemical and physiological properties, and their significance in indigenous, traditional South African fermented milks. Yeasts in dairy products, apart from their negative role of causing spoilage, which is effectively controlled by proper implementation of hygienic and sanitation practices, have various desired characteristics (Sharpe, 1979; Jakobsen and Narvhus, 1996). The yeasts play a significant role as part of microbial interactions in cheese and fermented milk products like kefir (Leroi and Pidoux, 1993a, b; Robinson and Tamime, 1990) and contribute positively to the quality of these products. The yeasts also contribute to the development of a characteristic aroma and taste originating from mainly ethanol and carbon dioxide production. Furthermore, yeasts are also able to produce proteolytic and lipolytic enzymes which are important in the formation of flavour compounds (Sharpe, 1979). At the same time, the

yeasts contribute in the metabolism of lactic acid with a consequent increase in pH, thus, supporting the growth interactively of less acid tolerant microorganisms (Sharpe, 1979; Jakobsen and Narvhus, 1996).

This study was undertaken to establish the presence of yeasts in fermented milks and to identify the relevant species. Technologically important metabolic properties were also characterized.

4.2 MATERIALS AND METHODS

4.2.1 Sampling methods and selection of isolates

Fourteen traditional sour milk samples, made from raw milk in rural areas were collected. Due to the scarcity of these products, samples were collected with the help of missionaries in the Northern Province and Mpumalanga over a period of one year. Seven samples were collected from clay pots and seven from calabashes.

After collection, the sour milk samples were kept on ice and microbiologically analyzed within 48 h. Yeast enumerations were performed on each of the fermented samples. Serial dilutions were prepared as required in quarter strength Ringer's solution (Oxoid). Aliquots (0.1 ml) of the dilutions were spread inoculated on plates containing Yeast-Exteract Malt-Extract agar (YM) (Wickerham, 1951). Yeast colonies from the highest dilutions were isolated and subcultured on YM plates and incubated for 72 h at 25°C for control of purity by colony morphology and microscopy. The pure cultures were kept on YM slants at 4°C during the period of investigation.

Lactic acid bacterial (LAB) counts were performed on different selective media to establish the microbial composition of the various samples. MRS agar was used for the isolation of thermophilic lactobacilli and streptococci (Oxoid, CM361) (De Man et al., 1960), M17 agar for the isolation of lactococci (Oxoid, CM785) (Terzaghi and Sandine, 1975) and Rogosa agar for the isolation of lactobacilli and leuconostocs (Merck)

(Rogosa et al., 1951). The pour plates were incubated at various temperatures, (Chapter 3; Table 3.2) after which the colonies were counted.

4.2.2 Identification of the yeast isolates

The identification of the yeasts were performed by the keys proposed by Van der Walt and Yarrow (1984) and Barnett et al. (1990). Each isolate was inoculated into six fermentation media, 33 carbon source assimilation media and vitamin free medium (Van der Walt and Yarrow, 1984). Additional tests performed included growth at 37°C, in 50% D-Glucose medium, urea hydrolysis, splitting of arbutin and 0.01 and 0.1 % cycloheximide (Van der Walt and Hopsu-Havu, 1976). Assimilation of nitrogen compounds, as performed by means of the auxanographic method of Lodder and Kreger-van Rij (1952), was also included.

Ascospore formation was examined on McClary's acetate agar, Potato Glucose agar, Gorodkowa agar, Corn Meal agar (Oxoid, CM103) and Malt Extract agar (Biolab, C10) (Kreger-van Rij, 1984). The inoculated media were incubated at 18°C for 4 weeks and examined at 4 d intervals. Cell morphology, type of budding and mode of reproduction were examined on Malt Extract agar (Difco) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium were examined on Corn Meal agar according to the Dalmau plate technique (Wickerham, 1951).

4.2.3 Physiological and biochemical tests

Physiological and biochemical tests performed on the yeasts obtained from the fermented milk samples comprised yeast growth at pH values of 3, 3.5 and 4.0 on YM agar plates (pH adjusted with 36% (v/v) HCl), the utilization of lactose as a carbon source, lactose fermentation, utilization of lactate (Van der Walt and Yarrow, 1984) as well as proteolytic and lipolytic activity (Harrigan and McCance, 1966).

Proteolytic activity was determined on opaque milk agar, which consists of Nutrient agar (Biolab, C1) with the addition of 10% (m/v) reconstituted skim milk, sterilized by autoclaving for 20 min at 115°C.

Thin spread plates were inoculated with a loopful of fresh 24 h cultures of the different yeasts tested. Three isolates were inoculated per plate and incubated at 25°C for 2-3 d. The development of clear zones was monitored daily for 3 d. The hydrolysis of casein was confirmed when the plates were flooded with a 1% (v/v) HCl solution and the formerly clear zones precipitated. Lipolytic activity was determined on butterfat agar. Yeastrel agar (0.3% yeast extract, 0.5% peptone, 1.2% agar, pH 7.8) was mixed with 5% (v/v) sterile butterfat and thin, dry spread plates were inoculated with fresh yeast cultures and incubated for 2-3 d at 25°C. The plates were then flooded with 10 ml of a saturated copper sulphate solution for 10–15 min. The excess reagent was poured off and the plates gently washed in running water for one h to remove the excess copper sulphate. Lipolysis was indicated when the colony growth was surrounded by a blue-green coloured zone, due to insoluble copper salts of fatty acids set free on lipolysis (Harrigan and McCance, 1966).

Osmotolerance was determined in 10, 15 and 20 % (m/v) sodium chloride in YM agar. The test cultures were inoculated on spread plates and incubated for 2-3 d at 25°C. The ability of the yeasts to produce amines from amino acids was determined by streaking the cultures on a modified Niven medium (Niven et al., 1981) to which either histidine (2.7% (m/v)), tyrosine (0.1%) or tryptophan (1.1%) were added as substrate for decarboxylation. The plates were incubated at 25°C for 72 h.

A preliminary study on the anti-microbial properties of the yeasts against 11 food pathogens (Table 4.1) was also undertaken (Tagg and McGeven, 1971). Screening for the inhibitory activities of the yeast isolates was determined on YM agar by the agar well-diffusion method. YM agar was overlaid by 5 ml soft Nutrient agar (Biolab, C87) seeded by an overnight culture of each indicator organism. Overnight cultures (YM broth) of the test strains were added to wells bored into the indicator lawn, as well as the YM agar. The plates were then incubated at 25°C for 18 h.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Enumeration of yeasts and lactic acid bacteria

Table 4.2 illustrates the yeast and lactic acid bacteria counts of the fermented milk samples from clay pots and calabashes. The yeast counts from clay pots varied between $7.00x10^3$ cfu/ml in clay pot sample 1 (CL1) and $1.54x10^6$ cfu/ml in CL2.

Table 4.1

Test organisms used to determine the antimicrobial properties of yeast spp.

Organisms	
Staphylococcus aureus	*Salmonella* group D
SABS TCC Sta 56	SABS TCC Sal 34
Escherichia coli	*Serratia marcescens*
SABS TCC Esc 27	SABS TCC Ser 12
Klebsiella pneumonia	*Bacillus cereus*
SABS TCC Kle 2	SABS TCC Bac 10
Pseudomonas aeruginosa	*Listeria monocytogenes*
SABS TCC Pse 16	SABS TCC Lis 12
Proteus vulgaris	*Streptococcus faecalis*
SABS TCC Pre 8	SABS TCC Str 18
Proteus mirabilis	
SABS TCC Pre1	

Yeast counts of samples from the calabashes ranged between $5.20x10^5$ and $2.17x10^7$ cfu/ml. The lactic acid bacteria counts were, however, higher than the yeast counts. Thermophilic lactobacilli and streptococci counts on the MRS agar varied between $6.50x10^6$ and $2.40x10^9$ cfu/ml.

The lactococci varied between $1.41x10^6$ and $1.87x10^9$ cfu/ml (M17 agar) and the lactobacilli plus leuconostocs between $3.80x10^5$ and $5.10x10^8$ cfu/ml (Rogosa agar). Lower leuconostoc counts were thus encountered than lactobacilli plus streptococci and lactococci.

Table 4.2

Microbial counts of the fermented milk samples from clay pots and calabashes

Sample number	Counts (cfu/ml)			
	Yeasts	LAB		
		MRS	M17	Rogosa
CL1	$7.00x10^3$	$1.17x10^9$	$1.26x10^9$	$1.28x10^8$
CL2	$1.54x10^6$	$6.50x10^8$	$5.20x10^8$	$1.18x10^8$
CL3	$9.20x10^5$	$2.03x10^9$	$1.86x10^9$	$5.00x10^8$
CL4	$1.21x10^5$	$1.74x10^9$	$1.87x10^9$	$5.10x10^8$
CL5	$8.60x10^5$	$1.56x10^9$	$1.23x10^9$	$1.91x10^8$
CL6	$1.41x10^6$	$1.74x10^9$	$1.59x10^9$	$2.02x10^8$
CL7	$8.00x10^5$	$6.10x10^7$	$7.80x10^7$	$8.10x10^7$
CA1	$5.20x10^5$	$2.44x10^8$	$4.10x10^8$	$2.15x10^8$
CA2	$4.50x10^6$	$6.10x10^8$	$4.60x10^8$	$4.50x10^8$
CA3	$2.10x10^7$	$2.33x10^8$	$1.53x10^7$	$2.18x10^6$
CA4	$2.24x10^6$	$1.08x10^7$	$1.41x10^6$	$1.21x10^6$
CA5	$1.31x10^7$	$4.70x10^8$	$1.91x10^8$	$1.33x10^7$
CA6	$3.00x10^6$	$6.50x10^6$	$9.40x10^6$	$9.90x10^5$
CA7	$5.95x10^5$	$2.40x10^9$	$1.02x10^9$	$3.80x10^5$

CL = Clay Pot
CA = Calabash

4.3.2 Identification of yeast isolates

Fourteen sour milk samples manufactured by traditional methods were obtained from households in rural areas throughout the country. Fifty colonies representative of each sour milk sample (Table 4.3) were isolated and identified to species level. *T. delbrueckii* (40%), *D. hansenii* (22%) and *Kluyv. marxianus* (18%) represented the highest percentage of the overall yeast population. Other species encountered were *Sacch.*

cerevisiae (6%), *Y. lipolytica* (4%), *Dek. anomala* (2%), *P. membranaefaciens* (2%), *Rh. glutinis* (2%), *Tr. beigelii* (2%) and *Gal. geotrichum* (2%).

Table 4.3

Representation of the yeast species from 14 fermented milks

Species	n	% Isolates
Torulaspora delbrueckii	20	40
Debaryomyces hansenii	11	22
Kluyveromyces marxianus	9	18
Saccharomyces cerevisiae	3	6
Yarrowia lipolytica	2	4
Dekkera anomala	1	2
Pichia membranaefaciens	1	2
Rhodotorula glutinis	1	2
Trichosporon beigelii	1	2
Galactomyces geotrichum	1	2
Total	**50**	**100**

It is interesting to note that *Dek. anomala,* which favours growth in a high carbon dioxide concentration (Barnett et al. 1990), was isolated from a sample that appeared 'very fizzy' at the time of sampling. *Y. lipolytica,* a typical lipolytic yeast was isolated from another sample, which smelled rancid and 'old', because of the possible breakdown of fat (Tan and Gill, 1985; Keller and Jordaan, 1990).

4.3.3 Physiological and biochemical properties

The technologically important physiological and biochemical properties of the yeasts isolated from fermented milks are shown in Table 4.4. All the species showed growth between pH 3 and pH 4 (data not shown).

T. delbrueckii and *D. hansenii* usually lack the ability to ferment lactose (Barnett et al., 1990). In this study 7 strains from *T. delbrueckii* and 8 strains of *D. hansenii* were found to ferment lactose. Lactate assimilation was experienced with the majority of species. More than 50% of *T. delbrueckii* strains showed growth in the presence of 10 and 20%

Table 4.4
Some technologically important physiological and biochemical properties of yeasts isolated from fermented milks

Yeast species	n	Number of species with positive reactions							
		Lactose assimilation	Lactose fermentation	Lactate assimilation	Proteolytic activity	Lipolytic activity	NaCl tolerance		
							10%	15%	20%
Torulaspora delbrueckii	20	7	7	15	6	1	13	8	12
Debaryomyces hansenii	11	8	8	10	6	1	2	2	3
Kluyveromyces marxianus	9	9	9	9	7	0	2	2	3
Saccharomyces cerevisiae	3	2	2	3	2	2	3	3	3
Yarrowia lipolytica	2	0	0	1	2	2	2	2	2
Dekkera anomala	1	1	1	1	0	0	0	0	0
Pichia membranaefaciens	1	0	0	1	1	1	0	0	0
Rhodotorula glutinis	1	0	0	1	1	0	0	0	0
Trichosporon beigelii	1	1	0	1	0	1	1	1	0
Galactomyces geotrichum	1	0	0	1	0	1	1	1	0
Total	**50**	**28**	**27**	**43**	**25**	**9**	**24**	**19**	**23**

NaCl. *Sacch. cerevisiae* strains all grew in the presence of high salt concentrations as revealed by various authors (Kaminarides and Laskos, 1992; Tzanetakis et al., 1996). Roostita and Fleet (1996a), however, indicated that *Sacch. cerevisiae* grew weak at 10% NaCl and died off in 15% NaCl. *D. hansenii* is considered to be a salt tolerant yeast (Besançon et al., 1992; Kaminarides and Laskos, 1992; Jakobsen and Narvhus, 1996; Tzanetakis et al., 1996; Sørensen and Jakobsen, 1997), but in this study, the majority of strains of *D. hansenii* were unable to grow at higher NaCl concentrations. Three of the yeast isolates, however, were xerophilic, capable of growth at 20% NaCl, but not at 10 or 15% NaCl. These isolates were identified as *D. hansenii* (2 strains) and *Kluyv. marxianus*. According to Barnett et al. (1990), *Kluyv. marxianus* utilizes lactate but could either be lactose positive and/or negative. In this study, however, all the strains were found to utilize lactose. All nine species were lactate positive. *Y. lipolytica* failed to utilize or ferment lactose but utilized lactate which are in correspondence with results obtained by Barnett et al. (1990) and McKay (1992).

Twenty nine yeast strains belonging to 10 yeast species were screened for the qualitative production of tyramine, tryptamine and histidine on a differential agar medium (Table 4.5). A high number of the yeast species (61.2%) tested were capable of producing tyramine and tryptamine, whereas none of the strains. Amines play an role in causing spoilage of fermented foods by producing off flavours and putrid odours as well as their pronounced physiological effects on humans and animals (Bester and Mostert, 1993).

The fifty yeast isolates were screened for anti-microbial activity against 11 relevant food pathogens (Table 4.6). Only three isolates, two species of *Kluyv. marxianus* (isolates number 17 and 31) and one of *D. hansenii* (no 33) showed anti-microbial activities and only against *Staphylococcus aureus*. These results are only preliminary and further investigations are necessary in this regard.

Table 4.5

The production of tyramine, tryptamine and histamine by different yeast species

Species	n	Number of positive strains		
		Tyramine	Tryptamine	Histamine
Debaryomyces hansenii	8	7	7	0
Torulaspora delbrueckii	7	3	2	0
Kluyveromyces marxianus	5	3	4	0
Saccharomyces cerevisiae	2	NG	NG	NG
Yarrowia lipolytica	2	2	2	0
Dekkera anomala	1	1	1	0
Pichia membranaefaciens	1	NG	NG	NG
Rhodotorula glutinis	1	1	1	0
Trichosporon beigelii	1	NG	NG	NG
Galactomyces geotrichum	1	1	1	0
Total	**29**	**18**	**18**	**0**

NG = no growth

Table 4.6
Anti-microbial activities of the fifty yeast isolates against 11 relevant food pathogens

No	Yeast species	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	*Proteus mirabilis*	*Salmonella* group D	*Serratia marcescens*	*Bacillus cereus*	*Listeria monocytogenes*	*Streptococcus faecalis*
1	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
2	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
3	*Yarrowia lipolytica*	-	-	-	-	-	-	-	-	-	-	-
4	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
5	*Galactomyces geotrichum*	-	-	-	-	-	-	-	-	-	-	-
6	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
7	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
8	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
9	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
10	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
11	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
12	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
13	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
14	*Rhodotorula glutinis*	-	-	-	-	-	-	-	-	-	-	-
15	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
16	*Dekkera anomala*	-	-	-	-	-	-	-	-	-	-	-
17	*Kluyveromyces marxianus*	+	-	-	-	-	-	-	-	-	-	-
18	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
19	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
20	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
21	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
22	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
23	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
24	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
25	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
26	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
27	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-

Table 4.6
Anti-microbial activities of the fifty yeast isolates against 11 relevant food pathogens (continued)

No	Yeast specie	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	*Proteus mirabilis*	*Salmonella* group D	*Serratia marcescens*	*Bacillus cereus*	*Listeria monocytogenes*	*Streptococcus faecalis*
28	*Yarrowia lipolytica*	-	-	-	-	-	-	-	-	-	-	-
29	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
30	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
31	*Kluyveromyces marxianus*	+	-	-	-	-	-	-	-	-	-	-
32	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
33	*Debaryomyces hansenii*	+	-	-	-	-	-	-	-	-	-	-
34	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
35	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
36	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
37	*Pichia membranaefaciens*	-	-	-	-	-	-	-	-	-	-	-
38	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
39	*Kluyveromyces marxianus*	-	-	-	-	-	-	-	-	-	-	-
40	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
41	*Trichosporon beigelii*	-	-	-	-	-	-	-	-	-	-	-
42	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
43	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
44	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
45	*Saccharomyces cerevisiae*	-	-	-	-	-	-	-	-	-	-	-
46	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
47	*Debaryomyces hansenii*	-	-	-	-	-	-	-	-	-	-	-
48	*Torulaspora delbrueckii*	-	-	-	-	-	-	-	-	-	-	-
49	*Saccharomyces cerevisiae*	-	-	-	-	-	-	-	-	-	-	-
50	*Saccharomyces cerevisiae*	-	-	-	-	-	-	-	-	-	-	-

4.4 CONCLUSIONS

Yeast counts ranging between $1.00x10^3$ and $1.00x10^7$ cfu/ml were found among fourteen indigenous traditional fermented milk samples from calabashes and clay pots. These results are slightly higher than counts reported by other authors (Fahmi et al., 1966; Kimonye and Robinson, 1991; Mutukumira, 1995), although, Kassaye et al. (1991) and Hamama and Bayi (1991) and Bankole and Okagbue (1992), found yeast counts in the same order. Ten different yeast species were isolated and identified as *T. delbrueckii, D. hansenii* and *Kluyv. marxianus.* Other species were *Sacch. cerevisiae, Y. lipolytica, Dek. anomala, P. membranaefaciens, Rh. glutinis, Tr. beigelii* and *Gal. geotrichum.* These yeasts correspond with yeast species usually found in dairy products (Marshall, 1986; Fleet, 1990; Rohm et al., 1992; Viljoen and Greyling, 1995; Welthagen and Viljoen, 1998). The lactic acid bacterial counts were higher than the yeasts', with counts varying between $1.00x10^5$ and $1.00x10^9$ cfu/ml, as was found by other authors (Hosono et al., 1989, Hamama and Bayi, 1991; Kassaye et al., 1991; Kimonye and Robinson, 1991).

CHAPTER 5

INTERACTIONS AND FERMENTATION CHARACTERISTICS OF YEASTS ASSOCIATED WITH INDIGENOUS FERMENTED MILK

Abstract

Fermentative and growth characteristics of three predominant yeasts, *Kluyveromyces marxianus, Debaryomyces hansenii* and *Torulaspora delbrueckii*, originally isolated from naturally fermented milks in South Africa were determined at different pH values (4, 4.5, 5 and 6) and temperatures (7, 15, 25 and 35°C), as well as in different lactose (3, 4.5, 5.5 and 10% (m/v)) and lactate (0.6, 0.85, 1.25 and 1.5% (m/v)) concentrations.

Growth studies at pH values 4.5 to 6.0, generally showed an increase in viable yeast cell numbers of all three species. At pH 4.0, however, only *Torulaspora delbrueckii* exhibited positive growth. Although the optimum growth temperature of the yeasts were 25°C, all three species grew at temperatures between 7 and 35°C. With the exception of *Torulaspora delbrueckii,* which failed to utilize lactose, all the species showed marked increases in cell numbers in the presence of varying lactose and lactate concentrations at 25°C. The properties of these yeasts suggested a metabolically active role during, as well as at the end of lactic acid fermentation of milk.

Differences in the growth patterns of the individual yeast species and a commercial fermented milk culture (CHN-22) (at different yeast:bacteria ratios) in non-fat milk, were observed during incubation for 48h.

Good and sensory acceptable fermented milk products were manufactured during this study, although no specific relationship was found between specific combinations of yeast:lactic acid bacteria and flavour development, or the presence of flavour compounds produced. The desirable products were mostly achieved when incubation took place at

22°C. Acetone and acetaldehyde were detected in all the combinations, while acetoin was detected in two combinations and diacetyl in six combinations.

Appreciable amounts of CO_2 and ethanol were produced at 22°C and 32°C by the yeasts *Kluyveromyces marxianus* and *Debaryomyces hansenii*, whereas *Torulaspora delbrueckii* lacked the ability to produce ethanol at any of the temperatures. The best CO_2 production was experienced at 32°C with *Kluyveromyces marxianus*.

Key Words: *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Torulaspora delbrueckii*, fermentation characteristics, interactions, yeasts, lactic acid bacteria, indigenous fermented milks

5.1 INTRODUCTION

Yeasts are significant in foodstuffs, because they can cause spoilage or lead to useful fermentations (Rossi, 1994). Depending on their properties and concentrations, yeasts may have positive (reduction in acidity, aroma development) or negative (unpleasant taste or appearance, gas production, yeasty flavour and other off-flavours, discoloration and changes of texture) effects (Lee and Lim, 1988a, b; Eliskase-Lechner and Ginzinger, 1995; Jakobsen and Narvhus, 1996; Leclercq-Perlat et al., 1999). The role of yeasts as spoilage organisms in dairy products is linked to their nutritional requirements, certain enzymatic activities and the ability to grow at low temperatures, low pH-values, low water activities (a_w) and high salt concentrations (Fleet and Mian, 1987; Seiler, 1991; Rohm et al., 1992; Jakobsen, 1994). Summarizing several studies, the following trends in the occurrence of yeasts in milk and dairy products were revealed: *Cryptococcus* spp. and in particular *C. diffluens* and *C. famata* (the imperfect state of *D. hansenii)* are common contaminants of some pasteurized milks, *Rhodotorula glutinis* and *Rhodutorula rubra,* in particular, are associated with products containing milk fats; representatives of yeast genera associated with cheese and yogurts include *D. hansenii, Kluyv. marxianus* var. *lactis, Kluyv. marxianus* var. *marxianus* (the perfect state of *C. kefyr), Kluyv. bulgaricus, Tor. sphaerica, Yarrowia* and *Candida* species and *Sacch. cerevisiae* (Besançon et al., 1992; Jakobsen, 1994; Rossi, 1994; Roostita and Fleet, 1996b).

In the specific case of dairy products, yeasts can degrade casein and its primary derivates (amino acid decarboxilation and deamination), as well as fat, and they can ferment lactose or sucrose when fruit is present in the product. Proteolytic and lipolytic yeasts are reasonably frequent in cheese where they normally have a positive contribution to flavour and structure development during the ripening stage. Yeasts can also grow in fermented milk and traditional products, both representing a selective environment (Jakobsen, 1994; Rossi, 1994). In yogurt, yeasts like *C. famata, C. krusei, C. lusitaniae, Kluyv. marxianus* and *Sacch. cerevisiae* can cause spoilage (Suriyarachchi and Fleet, 1981; Green and Ibe, 1987; McKay, 1992; Jakobsen, 1994), but lately, yeast cultures are used as flavouring agents in yogurt-type products, mainly contributing to the formation of ethanol and the development of a characteristic flavour (Chen et al., 1998).

Some essential attributes for use of yeasts as a starter culture, is their ability to grow and compete with other naturally occurring yeasts, as well as their compatibility with lactic acid bacteria (LAB) and possible stimulating action when co-inoculated (Guerzoni et al., 1996). Eliskase-Lechner and Ginzinger (1995) recommended that *D. hansenii,* selected for its acid-reducing and aromatic properties, be added to starters for soft smear cheeses. Martin et al. (1999) reported on fruity odours and flavours generated in cheese curd by co-culturing, among others, *Kluyv. lactis, D. hansenii, Y. lipolytica* and five bacteria. *Y. lipolytica* produced more volatile compounds, thereby inducing cheesy flavours, than *D. hansenii* and *Kluyv. lactis* (Martin et al. 1999). This characteristic of *Y. lipolytica* was also noted by Wyder (1998). Several yeasts could assist the starter cultures in cheese by proteolytic and lipoltic activities and possibly the formation of amines, thereby directly taking part in the maturation, including the formation of aromatic compounds (Joosten, 1988; Jakobsen, 1994; Vivier et al., 1994; Chen et al., 1998). Other desired technological characteristics of yeasts functioning as spontaneous or controlled starter cultures for cheese, include fermentation of lactose, assimilation of lactate and positive interactions with the primary starter cultures (Deiana et al., 1984; Jakobsen, 1994). Osmotolerance, as investigated in basic studies with *D. hansenii* and other osmotolerant yeasts, are important factors in controlling the activities in cheese (Jakobsen, 1994).

The characteristics of a fermented milk beverage are not only dependent on the type of microorganism present in the milk (Suzzi et al., 1996). Aromatic compounds, carbon dioxide (CO_2) production and ethanol contents also play a substantial role in the outcome of the final product. Ethanol is important due to its capacity to interact with other molecules to form esters, which in turn, contribute significantly to the organoleptic characteristics of fermented products, especially cheese (Suzzi et al., 1996). The appearance of ester molecules is principally due to yeast activity (Molimard and Spinnler, 1996). The formation of flavour and aromatic compounds in fermented milk products consists of very complex mechanisms and various factors play a role in the development of a desirable aroma and flavour. Such factors include starter culture composition, temperature, pH of medium and various metabolites or substances, i.e. acids, added to the medium. The proper choice of a starter culture is important for cheese, and especially for low fat cheese, which flavour is frequently not sufficiently pleasing (Guerzoni et al., 1996). The meganisms of

aroma/flavour –forming compounds of lactic acid starter organisms is well documented (Irvine et al., 1954; Mizuno and Jezeski, 1959; Keenan et al., 1966; Pack et al., 1968; Speckman and Collins, 1968; Turaç, et al., 1969; Hamdan et al., 1971; Collins, 1972; Walsh and Cogan, 1973; Lees and Jago, 1978a, b). A study by Subramanian and Shankar (1985) on the commensalistic interaction between LAB and yeasts in the preparation of acidophilus-yeast milk, emphasized on the stimulating effect of the yeast on the LAB present in the milk.

In this study, only the basic aromatic compounds (acetaldehyde, diacetyl, acetoin and acetone) were examined for their presence or absence and whether they increased or decreased during the time of incubation. In addition, CO_2 and ethanol production were monitored, as they represent an important characteristic of fermented milks. The growth kinetics of the yeasts in association with LAB under simulated fermentation conditions, and the interaction between the different microbial populations, were also determined.

5.2 MATERIALS AND METHODS

I. Growth and technological characteristics of yeasts in simulated environmental fermented milk conditions

5.2.1 Cell preparation and culture media

Three representative yeasts, isolated and identified previously and proved to be predominant during the fermentation of milks, namely *Kluyv. marxianus, D. hansenii* and *T. delbrueckii*, were used. The yeasts were streaked out on plates containing Malt Extract agar (MEA, Biolab C10) to get a thick overlay of active growing cells. Plates were incubated at 25°C for 72 h to obtain cells in the stationary phase.

5.2.2 Growth of yeasts at variable environmental conditions

Growth studies of the yeasts at different pH values and different temperatures were conducted in 10% (m/v) reconstituted non-fat milk (NFM). Volumes of 300 ml NFM were

sterilized by autoclaving at 121°C for 10 min in 500 ml Schott bottles and immediately cooled to 30°C to prevent discoloration and denaturation of the casein. A loopful of each of the three representative (5.2.1) yeasts was inoculated aseptically in the cooled milk, after it was adjusted to the different conditions as described under sections 5.2.2.1 and 5.2.2.2. Samples (1 ml) of the milk were taken consecutively at 6 h intervals and analyzed for viable yeast counts. Yeasts were enumerated by pour plate inoculation of diluted samples, prepared as required, in 1% Ringer's solution and plated on MEA. The plates were incubated at 25°C for 72 h and all visible colonies counted on the highest dilutions.

5.2.2.1 Growth of yeasts at different pH values

The sterilized milk (5.2.2) was adjusted with 35% (m/v) HCl to obtain pH values of 4.0, 4.5, 5.0 and 6.0, respectively. The inoculated milks were incubated in a waterbath at 25°C for 60 h on every sampling occasion, the pH was measured with a Radiometer/Copenhagen PHM 83 Autocal pH meter. The temperatures of the waterbaths were monitored throughout the survey.

5.2.2.2 Growth of yeasts at different temperatures

The sterilized milk inoculated with the relevant yeasts (5.2.1), was incubated for 60h in waterbaths, without shaking, at 7, 15, 25 and 35°C, respectively.

5.2.2.3 Growth of yeasts at different concentrations of lactose and lactate

Yeast Nitrogen Base (YNB, Difco, 0392-15-9) (6.7% m/v in distilled water) suspensions with final lactose concentrations of 3, 4.5, 5.5 and 10% (m/v) (Associated Chemical Enterprises), and lactate concentrations of 0.6, 0.85, 1.25 and 1.5% (m/v) (Sigma, 96%) were filter sterilized (0.45µm, Sartorius) and used as growth medium. The yeast cells on the MEA plates (~4g) were scraped from the plates and suspended in 20 ml sterile 1% Ringer's solution. Tubes containing the relevant carbohydrate suspensions, were inoculated with 2-3 drops of a 2% freshly prepared cell-suspension of the different yeasts. A control without added yeasts was included for both variables. The test tubes were incubated in a waterbath

at 25°C for 192 h. Absorbency was measured in an Eppendorf PCP 6121 photometer at a wavelength of 568nm, at 6 h intervals.

II. The interaction between yeasts and lactic acid bacteria in milk

5.2.3 Microorganisms, culture media and cell preparation

A commercial mesophilic heterofermentative lactic starter culture was selected for the interaction studies with the different yeasts. CHN-22 (CHR Hansen) is a mixed culture comprising of *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *cremoris, Leuconostoc pseudomesenteroides* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis.* Three representative yeast species, previously isolated from indigenous fermented milks and identified as *Kluyv. marxianus, D. hansenii* and *T. delbrueckii,* were cultivated in 200 ml sterile YNB in Erlenmeyer flasks (500 ml). The cultures were incubated in a shaking waterbath at 25°C for 48 h. Viable yeast cells were harvested by centrifugation at 10 000 rpm, using a Beckman J2-21 centrifuge (Beckman Instruments, Ireland). A 2% (m/v) yeast solution for each species was prepared in sterile 1% Ringer's solution. Similar procedures were followed for the cultivation of LAB, using MRS broth (Biolab, C87). The freeze-dried starter culture was inoculated into MRS broth and incubated at 25°C without shaking, for 48 h.

5.2.4 Interaction studies of mixed cultures

Different ratios of yeast and LAB were inoculated into 600 ml sterile 10% (m/v) NFM. Cell suspensions (1%) of each yeast strain and CHN-22 were added to the milk in 1*l* Schott-bottles resulting in final yeast:LAB ratios of 1:1, 3:7 and 1:9 respectively. The inoculated milk samples were incubated at 7, 22 and 32°C for 48 h, without shaking.

Serial dilutions, as required, were prepared in Ringer's solution and pour-plated consecutively on a 12 h basis. During each sampling occasion, milk samples were collected for the determination of lactose and lactate (20 ml), ethanol (1 ml) and aroma (20 ml) analyses. The samples were immediately frozen and stored at –21°C, until chemical

analyses were conducted. The sensory analyses of the milks were limited to odour and appearance only. After the incubation period, the smell ("nose") and appearance of the samples were described. The pH was measured throughout the incubation period at every sampling occasion.

5.2.5 Chemical analyses

For the production of CO_2, 300 ml NFM in 500 ml Erlenmeyer flasks were inoculated (5.2.4) and a rubber stopper with a tube, was inserted into the flask (Fig. 5.1). The tube was then inserted into an inverted graduated glass cylinder filled with water and the level of the water recorded. The flasks were incubated in waterbaths set at 7, 22 and 32°C, respectively. The amount of CO_2 produced, was regularly measured over 48 h and the cylinder, when necessary, refilled with water to the initial level.

The analyses of the lactose (Smit and Nel, 1987) and lactate content (Pryce, 1969) were performed by the accredited analytical laboratory of the ARC-Animal Nutrition and Animal Products Institute, Irene. Production of alcohol, aroma profiles (diacetyl, acetoin, acetone and acetaldehyde) were determined by a contracted laboratory at the CSIR in Pretoria with a Genesis Equilibrium Head-space Sampler. The different profiles were characterized with a Varian 3800 Gas Chromatograph (GC).

5.2.6 Microbial analyses

Reconstituted NFM (600 ml) was sterilized in 1λ Schott bottles at 121°C for 10 min and immediately cooled to 30°C. For the enumeration of the microorganisms, pour plates of Chloramphenicol agar (Biolab, C98) was used for the enumeration of the yeasts and MRS Agar for the enumeration of the LAB. All plates were incubated at 25°C for 72 h. Dilutions

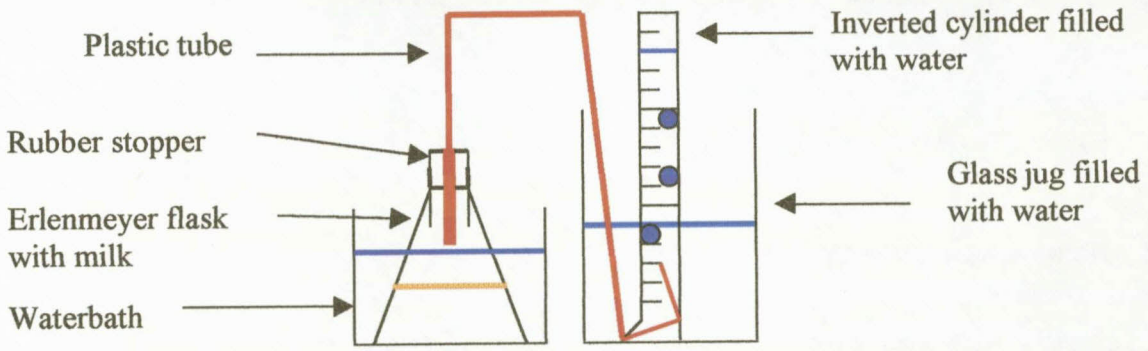

Fig. 5.1 A schematic presentation of the apparatus used for the determination of CO_2 production.

were prepared in sterile 1% Ringer's solution and colonies on the highest dilutions were counted.

5.3 RESULTS AND DISCUSSIONS

I. Growth and technological characteristics of yeasts in simulated environmental fermented milk conditions

5.3.1 Growth at different pH values

The growth patterns of the yeast species in NFM at different pH values, are presented in Fig. 5.2. At pH 4, the cell numbers of *T. delbrueckii* remained constant with log units ranging from 6.42 (0 h) to 6.35 after 54 h. After 60 h the yeast numbers increased to 7.33. *Kluyv. marxianus* and *D. hansenii,* on the other hand, showed a substantial decrease in viable cell numbers ranging from 6.82 and 3.99 (0 h) to 1.60 and 1.30 (60 h) log units, respectively. Based on these results, it is evident that *T. delbrueckii* prefers a lower pH value to grow.

If the growth patterns of the three species were compared at higher pH values (4.5, 5.0, 5.5 and 6.0), slight differences in the yeast counts were observed. However, at pH 5.5 cell numbers of *Kluyv. marxianus* and *T. delbrueckii* increased during incubation by 1.29 and 1.25 log units, respectively. At pH 6.0, only *Kluyv. marxianus* showed an increase in numbers (1.29 log units) after 60 h at 25°C. It is also evident that, although not substantially, *Kluyv. marxianus* was able to grow in milk between pH 4.5 and 6.0. The growth of the yeasts at low pH values suggested that the species might play a metabolically active role during, as well as at the end (especially *T. delbrueckii*) of lactic acid fermentation of milk. In a study conducted by Sørenson and Jakobsen (1997), changes in pH had little effect on growth of *D. hansenii*.

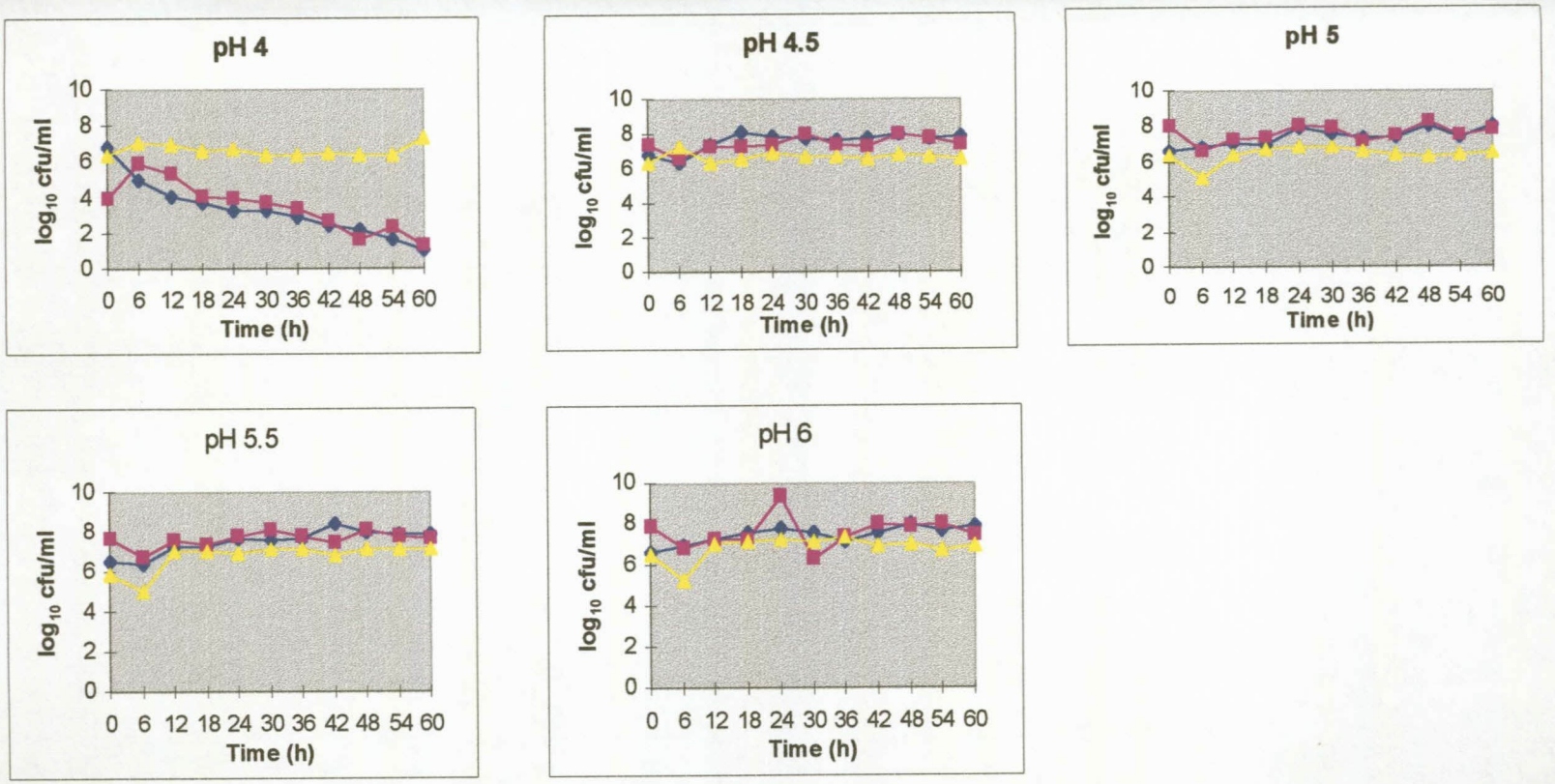

Fig. 5.2 Growth patterns of *Kluyveromyces marxianus* (◆), *Debaryomyces hansenii* (■) and *Torulaspora delbrueckii* (▲) in non-fat milk at different pH values incubated at 25°C.

5.3.2 Growth at different temperatures

In Fig. 5.3 the different growth patterns of the three yeast species at 7, 15, 25 and 35°C, are presented. All species grew in milk at 7°C, but with decreased rates and cell yields, as compared to growth at 25°C. At 7°C, only *T. delbrueckii* showed a substantial increase in growth of log 1.1 units. *Kluyv. marxianus* and *D. hansenii* showed insignificant growth with increasing log values of 0.4 and 0.2, respectively.

The maximum cell population of the yeasts obtained when grown in milk at 15°C, was *ca.* $1.00x10^7$ cfu/ml. *D. hansenii* exhibited good growth in milk at 15°C, dominating the other yeasts in reaching log counts as high as 8.03 units. High cell densities by *T. delbrueckii* were obtained only after 60 h of growth, while *Kluyv. marxianus* gradually increased in cell density reaching its maximum of log 7.45 units after 54 h. Similar growth patterns were observed at 25°C and 35°C, although higher final cell densities were obtained at 25°C. Maximum values of growth for *D. hansenii, Kluyv. marxianus* and *T. delbrueckii* at 25°C of 8.21, 7.69 and 8.90 log units, respectively, were obtained.

At 35°C, *T. delbrueckii* exhibited the highest cell density reaching a maximum of log 7.92 after 48 h. *Kluyv. marxianus* and *D. hansenii* both reached maximum cell numbers exceeding $1.00x10^7$ cfu/ml. Despite the faster growth of yeasts at higher temperatures, they started to die off after reaching maximum populations much earlier, in comparison to yeasts grown at lower temperatures, when growth was sustained over longer periods (Roostita and Fleet, 1996a, b). Roostita and Fleet (1996a, b) found higher maximum populations at lower temperatures. At higher temperatures the maximum specific growth rate of *D. hansenii* increased with increasing pH (Sørenson and Jakobsen, 1997).

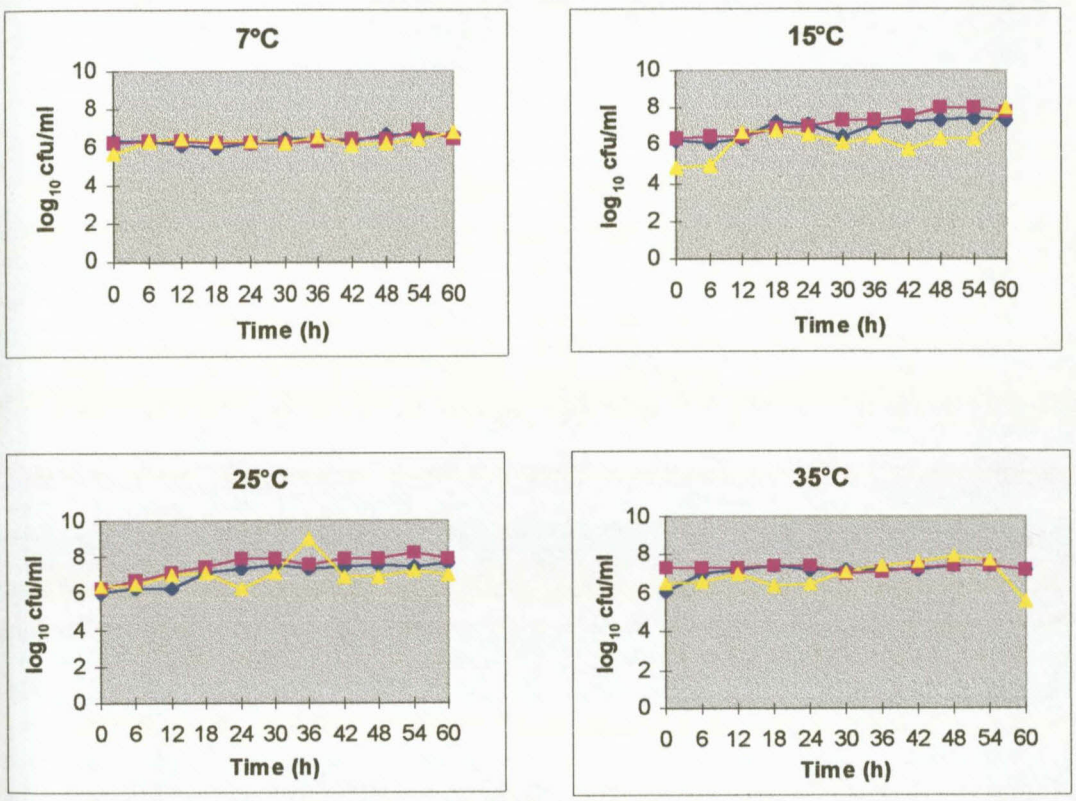

Fig. 5.3 Growth patterns of *Kluyveromyces marxianus* (◆), *Debaryomyces hansenii* (■) and *Torulaspora delbrueckii* (▲) in non-fat milk at different temperatures.

5.3.3 Growth of yeasts at different lactose concentrations

Each yeast species was examined for cell growth in YNB media, respectively containing 3, 4.5, 5.5 and 10% lactose, at 25°C. These growth studies are represented in Fig. 5.4. *Kluyv. marxianus* is characterized as a typical dairy associated yeast, capable of fermenting lactose

(Fleet, 1990), whilst *D. hansenii* exhibit variable results regarding the utilization of lactose (Kurtzman and Fell, 1998). *T. delbrueckii* lacks the ability to utilize lactose (Barnett et al., 1990).

The substantial growth of *Kluyv. marxianus* and *D. hansenii* in all the lactose concentrations correlated with its ability to utilize lactose. Significant changes in cell density for *Kluyv. marxianus* and *D. hansenii* were detected with increasing lactose concentration. Similar growth rates were observed when *Kluyv. marxianus* and *D. hansenii* were grown in the different lactose concentrations at 25°C, but increasing lactose contents correlated with increased cell densities. Lactose was not utilized by *T. delbrueckii* and no growth was detected. In a study by Wyder (1998) on smear ripened cheese, *D. hansenii* A and B, and *Kluyv. marxianus,* assimilated lactose.

5.3.4 Growth of yeasts at different lactate concentrations

Lactic acid or lactate is the principal flavour compound of cultured milk products. It is an odourless, non-volatile acid that creates the typical acidulous sensation of fermented dairy products (Margalith, 1981). Despite reduced growth rates and total cell numbers in comparison to the growth of *Kluyv. marxianus* and *D. hansenii* in lactose, all the yeast species selected were able to utilize lactate as a carbon source (Fig. 5.5). Their rates and cell densities decreased correspondingly as the concentration of lactate was decreased from 1.5 to 0.6%. *D. hansenii* exhibited good growth in all the lactate concentrations, yielding the highest cell numbers at all occasions. Similar growth patterns, in comparison to *D. hansenii,* when grown at different lactate concentrations were observed for *Kluyv. marxianus.*

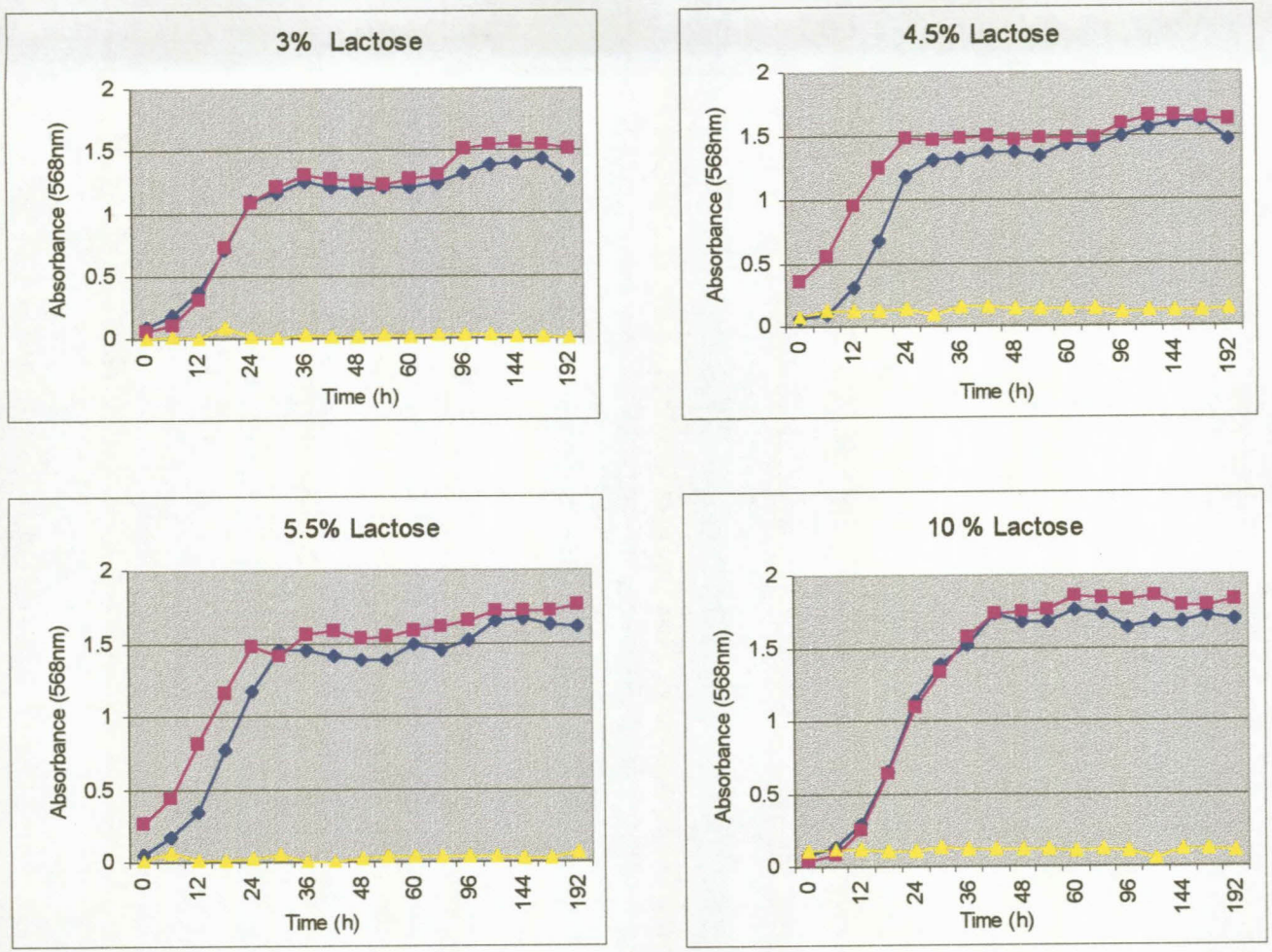

Fig. 5.4 Growth of *Kluyveromyces marxianus* (◆), *Debaryomyces hansenii* (■) and *Torulaspora delbrueckii* (▲) in YNB with 3, 4.5, 5.5 and 10% lactose at 25°C.

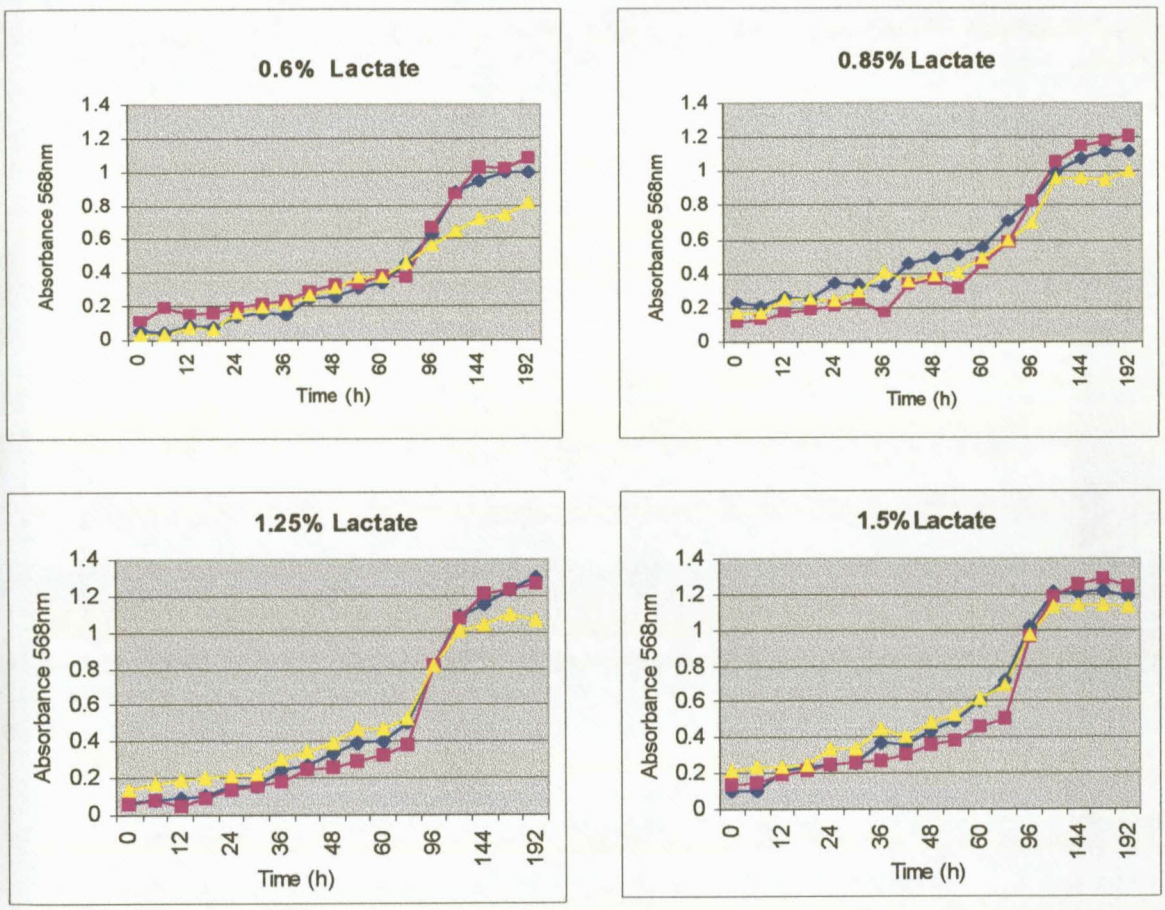

Fig. 5.5 Growth of *Kluyveromyces marxianus* (◆), *Debaryomyces hansenii* (■) and *Torulaspora delbrueckii* (▲) in YNB with 0.6, 0.85, 1.25 and 1.5% lactate at 25°C.

The weakest growth was observed with *T. delbrueckii*, yielding the lowest cell numbers after 192 h of growth at 25°C. Besançon (1992) found that most of the isolates of *D. hansenii* isolated from Roquefort cheese were able to metabolize lactose and lactic acid. One strain of *D. hansenii* was, however, able to metabolize lactose, but not lactic acid.

II The interactions between yeasts and lactic acid bacteria in milk

5.3.5 Microbial interaction

Lactic acid bacteria and yeasts were grown in sterilized NFM milk at different temperatures and ratios in order to determine the beneficial or detrimental effects of individual yeast species on the final product. When equal ratios of the individual yeast species and LAB were grown in milk at 7°C, reduced growth rates and cell densities were observed (Fig. 5.6). In correspondence with the low growth rate, minimal amounts of lactose were utilized, which resulted in reduced lactic acid production and consequently, also minimal variance in pH. Despite equal ratios of inoculation, LAB predominated during growth. An increase in the viable cell counts of the LAB was observed when the cell density of the yeast species increased (Figs. 5.6, 5.7, 5.8). *Kluyv. marxianus* yielded the highest cell densities compared to *D. hansenii* and *T. delbrueckii,* when grown at 7°C. Similar growth patterns of the yeast and LAB species were obtained with the 3:7 and 1:9 ratios of individual yeast species: LAB grown at 7°C. The 1:1 ratio, however, resulted in higher LAB counts that may be an indication of increased stimulating influences of yeasts on the LAB. Stimulating effects incurred by yeasts on LAB were also reported by Subramanian and Shankar (1985) during the preparation of acidophilus milk, and by Yamauchi et al. (1975) during the growth of LAB and yeasts in cheese prepared from skim milk. Higher survival of LAB cultures and better texture of cheese prepared with yeasts and LAB were observed by Lee and Lim (1988a).

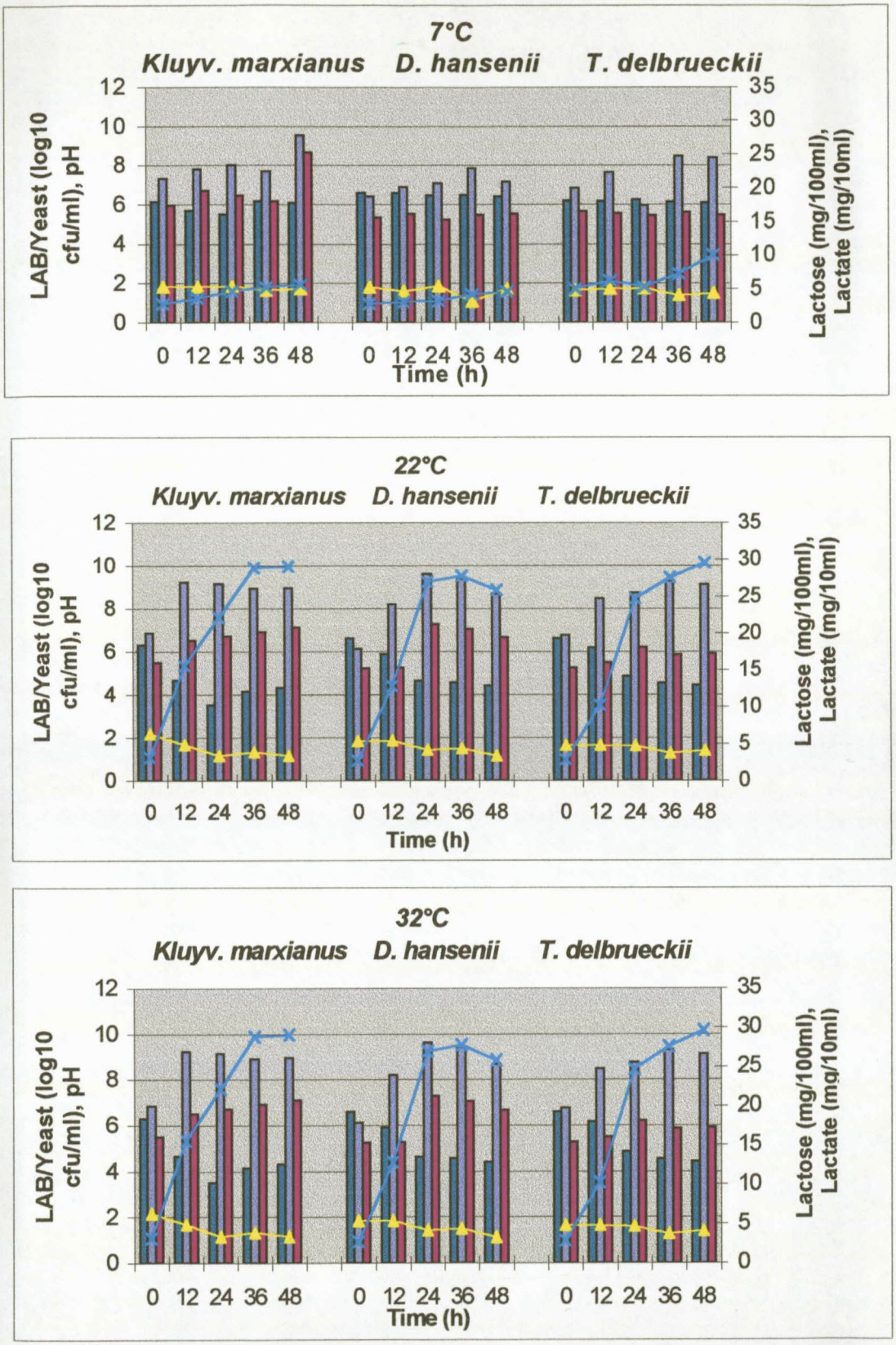

Fig. 5.6 Growth of yeasts (■) and lactic acid bacteria (LAB) (■) in non-fat milk at 7, 22 and 32°C as well as changes in pH (■), utilization of lactose (■) and production of lactate (■) during incubation for 48 h. Culture composition: Commercial fermented milk culture with either *Kluyveromyces marxianus, Debaryomyces hansenii* or *Torulaspora delbrueckii* in 1:1 ratio.

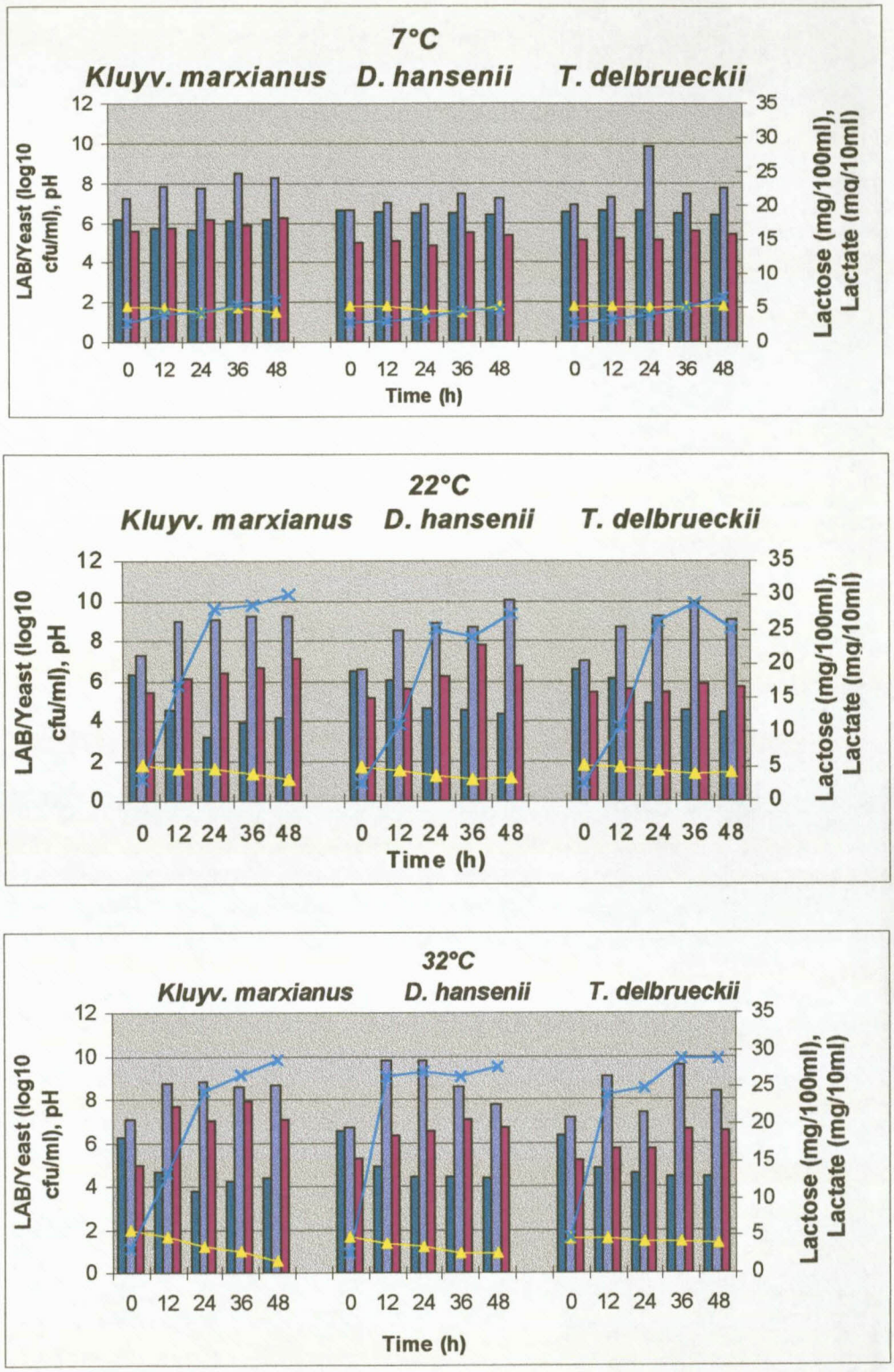

Fig. 5.7 Growth of yeasts (■) and lactic acid bacteria (LAB) (■) in non-fat milk at 7, 22 and 32°C as well as changes in pH (■), utilization of lactose (■) and production of lactate (■) during incubation for 48 h. Culture composition: Commercial fermented milk culture with either *Kluyveromyces marxianus*, *Debaryomyces hansenii* or *Torulaspora delbrueckii* in 7:3 ratio.

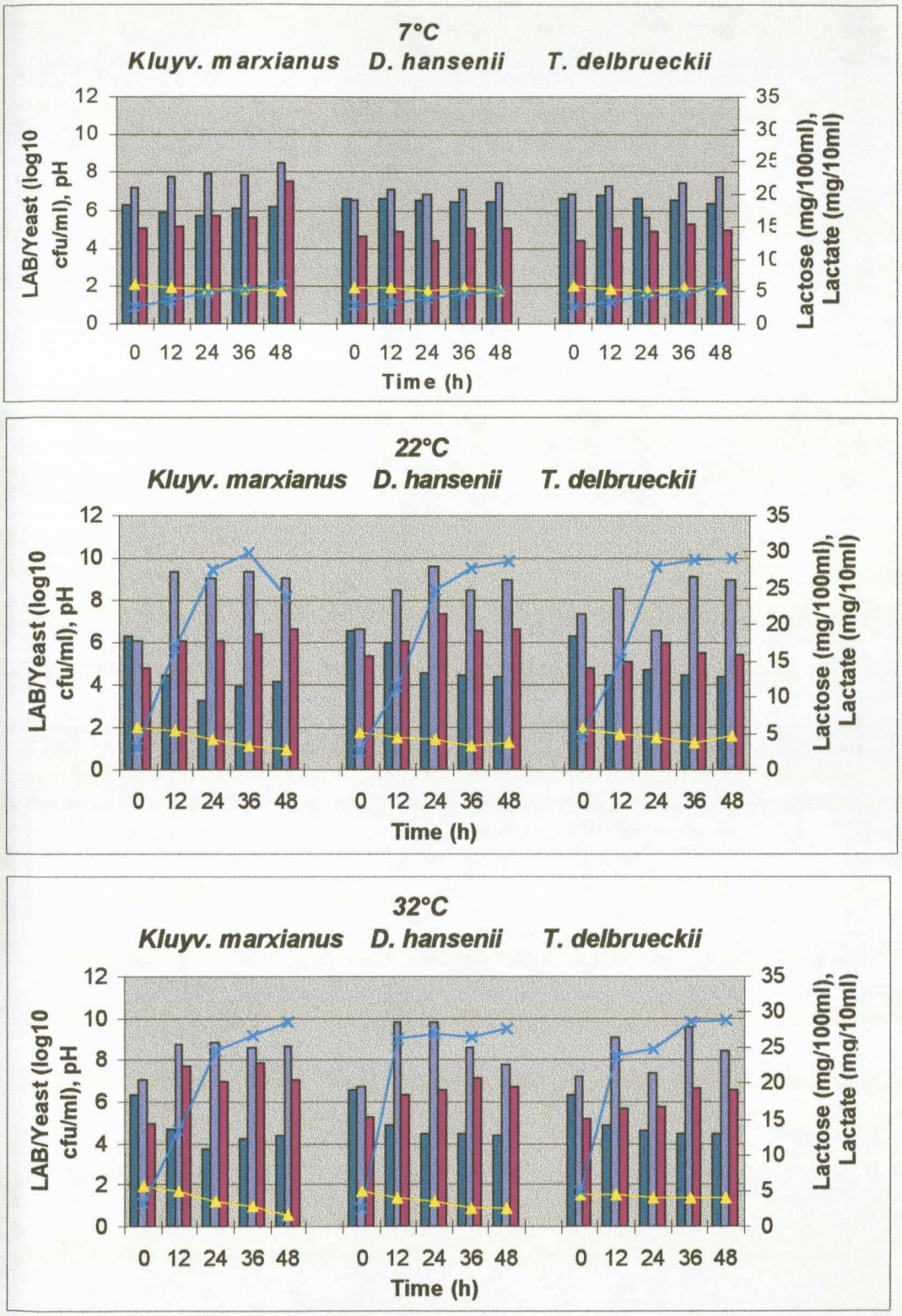

Fig. 5.8 Growth of yeasts (■) and lactic acid bacteria (LAB) (■) in non-fat milk at 7, 22 and 32°C as well as changes in pH (■), utilization of lactose (■) and production of lactate (■) during incubation for 48 h. Culture composition: Commercial fermented milk culture with either *Kluyveromyces marxianus*, *Debaryomyces hansenii* or *Torulaspora delbrueckii* in 9:1 ratio.

Lee and Lim (1988b) and Macedo (1995) also indicated that yeasts present in the cheese, might contribute directly and indirectly to cheese ripening, based on a higher survival of LAB organisms due to the decreasing acidity of the cheese, and by increasing the ripening ratio by means of a synergistic action between yeasts and the lactic starter bacteria. Leroi and Pidoux (1993a) found a better survival of LAB cultures when inoculated in mixed cultures with yeasts, after studying the synergism between the microorganisms associated with sugary kefir grains. The low temperature (7°C) also had a substantial effect on the growth rate and cell densities of the LAB and yeasts in this study, which resulted in decreased values compared to growth at 22 and 32°C. Deiana et al. (1984), found that *D. hansenii* added to the LAB starters in the production of cheese, utilized the lactic and acetic acids in the center of the cheese.

The number of viable cells of the LAB in the milk grown at 32°C, was higher with all the different yeast:LAB ratios in comparison to results obtained when incubating at 22°C. *Kluyv. marxianus* and *T. delbrueckii*, both attained higher cell densities when grown at 32°C compared to growth at 22°C, whereas no significant differences in the growth of *D. hansenii* at these temperatures were observed. The increased cell numbers obtained at the higher temperatures corresponded with the increased utilization of lactose, and higher amounts of lactic acid produced. The increased lactic acid production consequently resulted in the decline in pH (Figs. 5.6, 5.7 and 5.8 at 22 and 32°C). An increase in viable cell numbers of yeasts and LAB at higher temperatures, correlated with results obtained by Subramanian and Shankar (1985) during the production of acidophilus-yeast milk cultured at 37°C. The increase in lactic acid production was attributed to the increased numbers of LAB and the yeast populations. Production of various organic acids by dairy associated yeasts has been reported (Suriyarachchi and Fleet, 1981; Subramanian and Shankar, 1985; Fleet and Mian, 1987). In general, higher amounts of lactic acid were observed in the presence of *Kluyv. marxianus*. This may be an indication of enhanced acid production of the species compared to the other yeasts, or stimulation of the LAB by the species, resulting in higher organic acid production.

The number of viable LAB cells was generally higher when grown in association with *Kluyv. marxianus*. Guerzoni et al. (1996) observed stimulation of LAB by *Y. lipolytica*

when applied as a starter culture with LAB in cheese. *Y. lipolytica* possesses some of the essential attributes for use as a starter culture for cheese production, like the ability to grow and compete with other naturally occurring yeasts and the compatibility with LAB and possible stimulating action when co-inoculated. Macedo et al. (1995) also indicated that the yeasts present in cheese contribute mainly to the ripening process by the utilization of lactic acid. This leads to an increase in the pH and consequently encourages growth of the bacteria sensitive to acidic environments, and therefore initiating the second stage of maturation. *D. hansenii* (lactose-utilizing yeast) was the predominant yeast in both the curd and 35 d old ripened cheese (Macedo et al., 1995).

5.3.6 Sensory analyses and production of volatile aromatic components

The sensory analyses performed in this study, were limited to odour and appearance only. No established methods were used for the sensory analyses, but the samples were smelled and the appearance visually monitored. The fermented milk samples were evaluated after 48 h and the observations are outlined in Table 5.1. Only six of the 27 yeast:LAB combinations showed good characteristic sensory properties associated with traditional fermented milks (Table 5.2). These fermented milks were characterized generally as having a fresh, pleasant acid aroma, slightly fizzy with a relatively thin body. The best aroma developments were obtained at an incubation temperature of 22°C. However, an exception occurred at 32°C with yeast:LAB combination at a 1:9 ratio, which comprised of *Kluyv. marxianus*. It is interesting to note that all three yeast species (*Kluyv. marxianus, D. hansenii* and *T. delbrueckii*), in combination with the LAB, produced fermented milk products with characteristic sensory (odour) properties associated with fermented milk.

The production of acetaldehyde and acetone with 1:1, 3:7 and 1:9 yeast:LAB ratios, are illustrated in Figs. 5.9, 5.10 and 5.11. With every yeast:LAB combination, higher amounts of acetone compared to acetaldehyde was produced.

Table 5.1

Flavour and appearance of milk samples inoculated with different yeast:lactic acid bacteria ratios and incubated at three different temperatures after 48 h

Yeast spp.	Temp	Yeast:LAB	Remarks
Kluyveromyces marxianus	7°C	1:1	Lacks acid (Flat)
		3:7	Lacks acid (Flat)
		1:9	Lacks acid (Flat)
	22°C	1:1	Lacks acid (Flat)
		3:7	Lacks freshness, slightly putrid
		1:9	Refreshing piquant acid smell, very foamy. Slightly yeasty. Whey separation
	32°C	1:1	Unclean, not characteristic. Whey separation
		3:7	Less pleasant smell, slightly sweaty and unclean. Whey separation
		1:9	Refreshing acid smell, very characteristic. Whey separation
Torulaspora delbrueckii	7°C	1:1	Musty smell. Whey separation and very thick
		3:7	Musty, unclean. Whey separation
		1:9	Musty, unclean. Not coagulated
	22°C	1:1	Very pleasant fresh sharp smell. Uniformly thick and homogenate
		3:7	Very pleasant fresh sharp smell. Uniformly thick and homogenate
		1:9	Putrid smell. Whey separation
	32°C	1:1	Putrid smell. Whey separation
		3:7	Putrid smell. Whey separation
		1:9	Putrid smell
Debaryomyces hansenii	7°C	1:1	Cooked smell. Not coagulated.
		3:7	Cooked smell. Not coagulated
		1:9	Cooked smell. Not coagulated
	22°C	1:1	Very pleasant fresh sour smell
		3:7	Very pleasant smell
		1:9	Slightly putrid and unclean
	32°C	1:1	Very musty
		3:7	Unclean
		1:9	Unclean

LAB = Culture CHN-22 (*Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *cremoris, Leuconostoc pseudomesenteroides* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis)*

Due to its volatile nature, no continuous production of acetaldehyde or acetone was observed with any of the combinations. A general decrease in the amount of acetaldehyde and acetone was experienced with each combination. According to Pack et al. (1968), a greater decrease in flavour is experienced at 30°C, whereas a rapid accumulation or increase in flavour compounds during growth of aroma bacteria in mixed-strain lactic

starter cultures at 5°C and 21°C is achieved. However, in this study, the different temperatures had little or no effect on the production of acetaldehyde and acetone.

Table 5.2

Combinations of yeasts and lactic acid bacteria resulting in a desirable sensory profile for fermented milk

Temperature °C	Combination Yeast:LAB	Yeast species
22	1:1	*Torulaspora delbrueckii*
22	3:7	*Torulaspora delbrueckii*
22	1:9	*Kluyveromyces marxianus*
32	1:1	*Kluyveromyces marxianus*
22	1:1	*Debaryomyces hansenii*
22	3:7	*Debaryomyces hansenii*

The production of diacetyl and/or acetoin was observed in six combinations (Table 5.3). These specific yeast:LAB combinations differed from the yeast:LAB combinations responsible for the production of the typical sensory properties associated with fermented milk (Table 5.2). According to Collins (1972), yeasts produce diacetyl by a different mechanism compared to most other bacteria.

Despite the inability of some strains of *Sacch. cerevisiae* and bacteria to produce diacetyl, they produce appreciable amounts of acetoin (Chuang and Collins, 1968). Acetoin was produced in only two samples and at much higher concentrations compared to diacetyl. Collins (1972) and Walsh and Cogan (1973) also indicated on the higher production of acetoin, compared to diacetyl. No diacetyl was found in Laban, a popular fermented milk in Lebanon, although acetaldehyde and acetoin were found (Baroudi and Collins, 1976). Their results also showed that *Streptococcus thermophilus* produced acetaldehyde, but its production was mostly attributed to *Kluyv. fragilis*. There were also traces of ethanol in the absence of *Sacch. cerevisiae*. In this study, however, *Kluyv. marxianus* produced no diacetyl or acetoin.

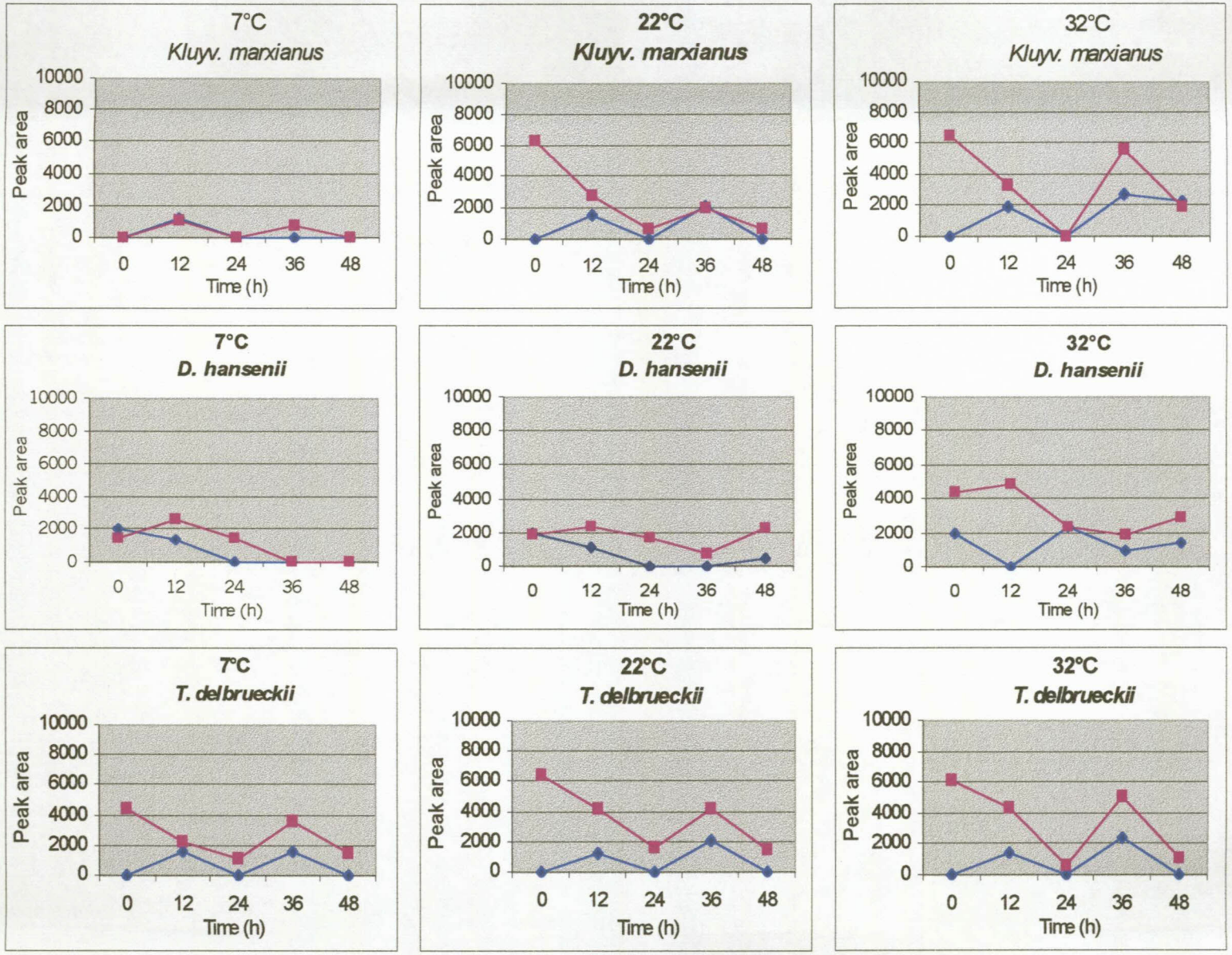

Fig. 5.9 Acetaldehyde (■) and acetone (■) production by *Kluyveromyces marxianus*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* in non-fat milks with 1:1 yeasts:LAB ratio at different temperatures.

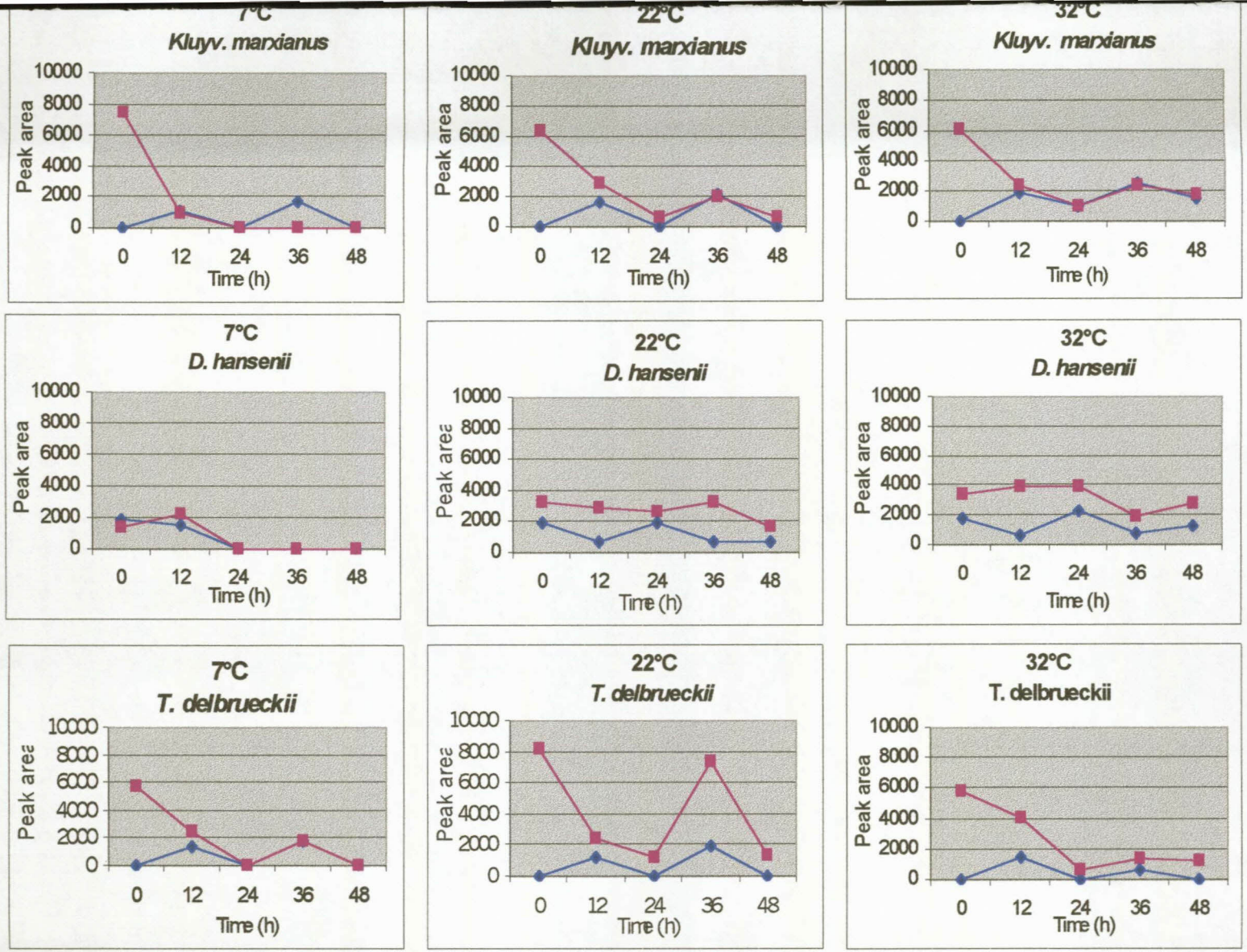

Fig. 5.10 Acetaldehyde (■) and acetone (■) production by *Kluyveromyces marxianus*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* in non-fat milk with 3:7 yeast:LAB ratio at three different temperatures.

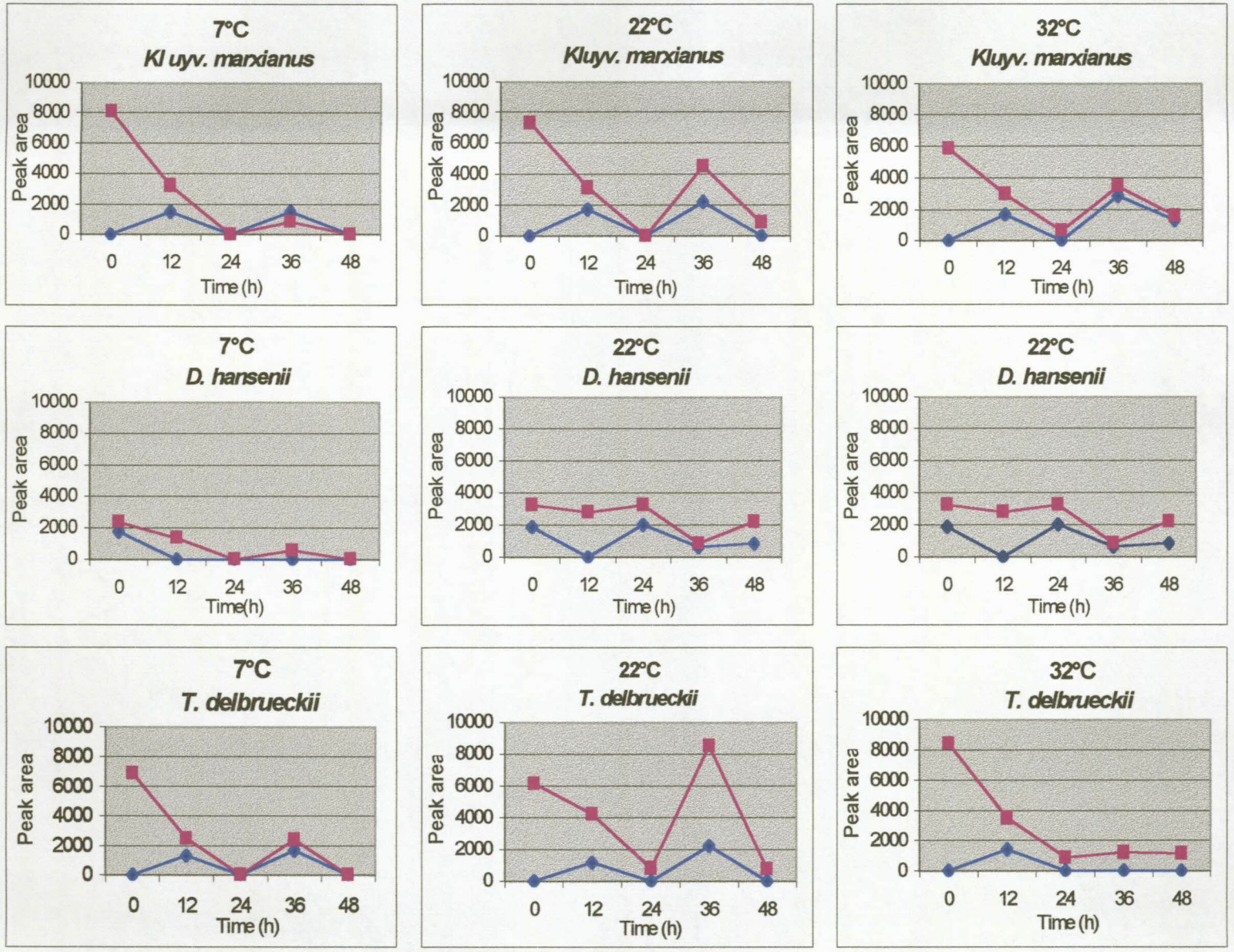

Fig. 5.11 Acetaldehyde (■) and acetone (■) production by *Kluyveromyces marxianus, Debaryomyces hansenii* and *Torulaspora delbrueckii* in non-fat milk with 1:9 yeast:LAB ratio at different temperatures.

Table 5.3

The production of diacetyl and acetoin in non-fat milk with different yeast:lactic acid bacteria ratios incubated at different temperatures (time when detected indicated in brackets)

Combination Yeast:LAB		Incubation temperature	Peak area			
			Diacetyl		Acetoin	
T. delbrueckii	1:1	7°C	2511	(36 h)	0	
T. delbrueckii	1:1	32°C	943	(36 h)	1587	(12 h)
D. hansenii	3:7	32°C	540	(12 h)	0	
T. delbrueckii	3:7	7°C	24310	(48 h)	0	
T. delbrueckii	3:7	22°C	1491	(36 h)	0	
D. hansenii	1:9	32°C	1543	(12 h)	4767	(12 h)

According to Macedo et al. (1995) yeasts are able to synthesize lipolytic and proteolytic enzymes which may also contribute to the development of aroma and flavour during ripening. The results obtained by Deàk and Beuchat (1996) and Roostita and Fleet (1996b) indicated that the fermentation or assimilation of lactose by lactose fermenting yeasts, influenced the aroma of cheese by the formation of ethanol, limiting acidification due to the utilization of lactic acid, affecting the texture of cheese and the formation of CO_2.

5.3.7 Production of ethanol

From the three yeast:LAB combinations (1:1, 3:7 and 1:9) in Fig. 5.12, only *Kluyv. marxianus* produced substantial amounts of ethanol, especially when incorporated in 1:1 and 1:9 ratios at 32°C. After 48 h of incubation at 32°C, peak areas of 4 051 100 (1:1 ratio) and 3 141 500 (1:9 ratio) were recorded. *D. hansenii* produced lesser amounts of ethanol at

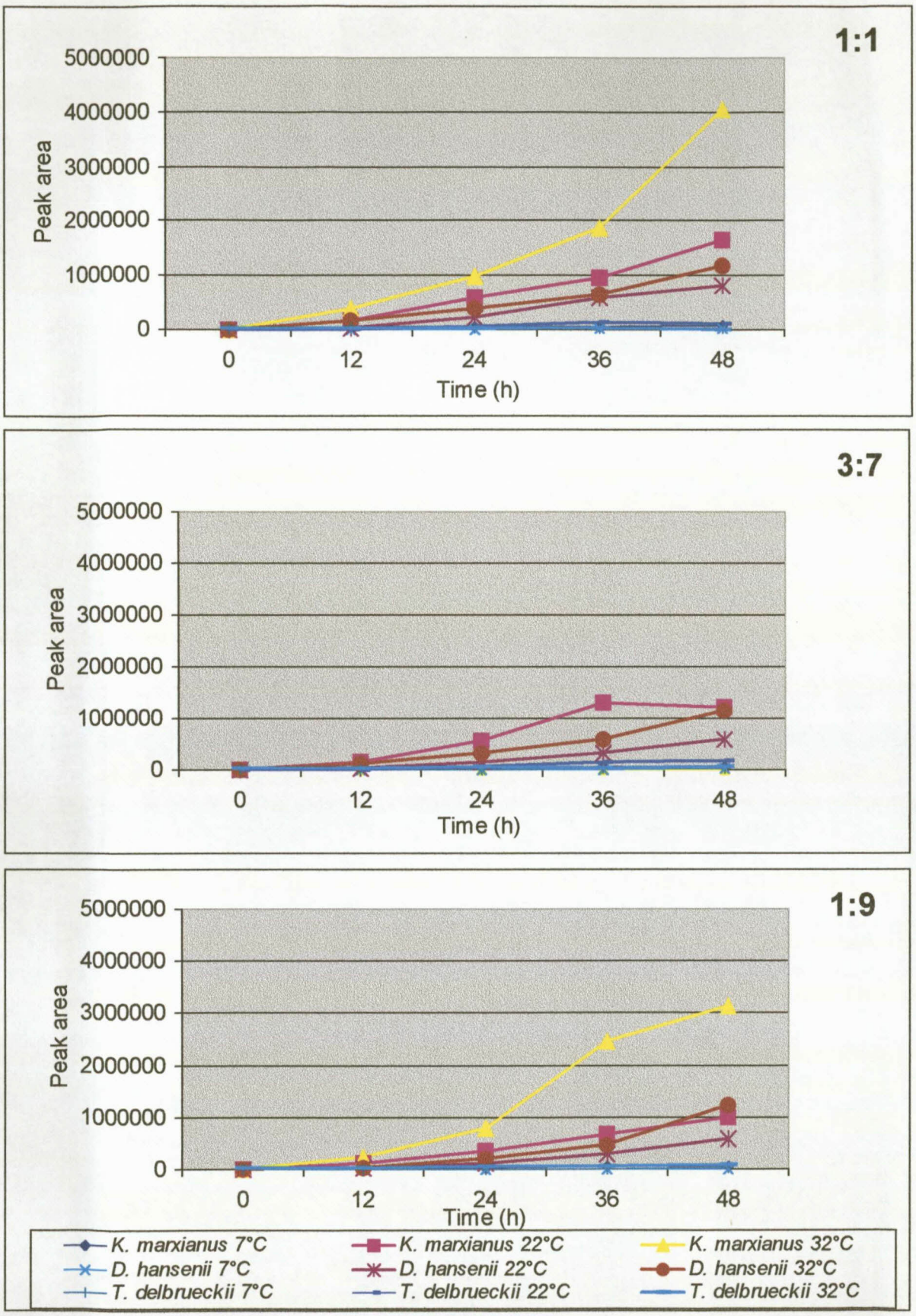

Fig. 5.12 Ethanol production by *Kluyveromyces marxianus*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* in non-fat milk with different yeast:LAB ratios at three different temperatures.

22°C and 32°C compared to *Kluyv. marxianus,* whereas *T. delbrueckii* showed no significant production of ethanol at any of the temperatures.

Low amounts of ethanol production were generally observed with all the yeasts incubated at 7°C. *Kluyv. marxianus* var. *lactis* reportedly produced relatively high amounts of ethanol in the head space of inoculated milk after different fermentation times at 30°C. Suzzi et al. (1996) found that *Sacch. cerevisiae* and *Kluyv. marxianus* var. *marxianus* and var. *lactis,* amongst others, were able to produce aromatic compounds, including ethanol.

5.3.8 Production of carbon dioxide

The total volumes of CO_2 produced by the three yeast:LAB combinations after 48 h of incubation at 7, 22 and 32°C, are outlined in Fig. 5.13. (Only the yeast:LAB combinations, which produced CO_2 at the different temperatures, are shown). At 7°C, only *T. delbrueckii* produced small volumes of CO_2 (18–32 ml) in all three combinations. With 1:9 ratio *D. hansenii* and *T. delbrueckii* produced less than 250 ml of CO_2 after the 48 h growth period. The highest production of CO_2, however, was experienced at 32°C with *Kluyv. marxianus* at 1:9 (878 ml), 3:7 (846 ml) and 1:1 (662 ml) ratios, respectively. *D. hansenii* produced approximately 250 ml, and only very small amounts with the 1:1 ratio. *T. delbrueckii* produced the same amount of CO_2 with 1:9 ratio as *D. hansenii* (234 ml) at 3:7 ratio, but less than 1 ml with the 1:1 ratio. Apart from providing a refreshing ("prickling") taste sensation in fermented milk beverages, the production of CO_2 opens the cheese structure of blue cheese and provides space for the development of *Penicillium roqueforti* (Van den Tempel and Jakobsen, 1996, after Fox and Law, 1991).

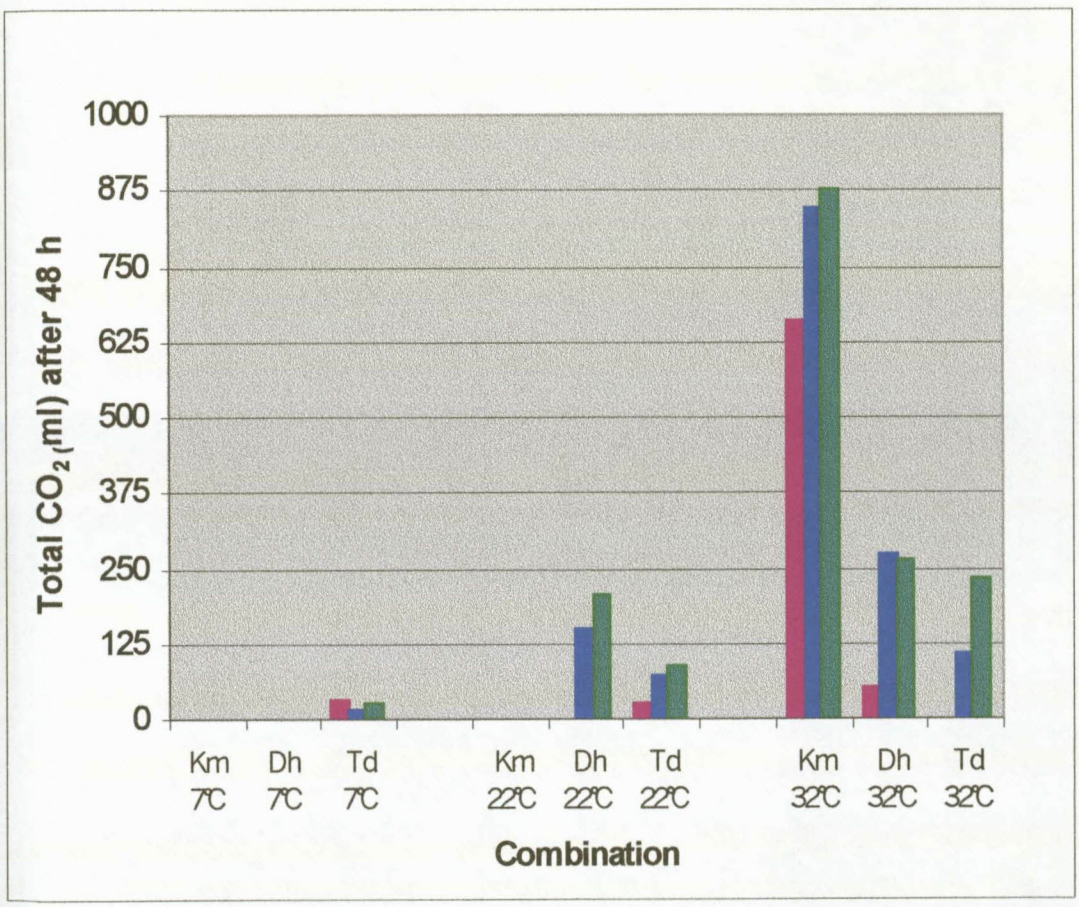

Fig. 5.13 Carbon dioxide production by *Kluyveromyces marxianus* (Km), *Debaryomyces hansenii* (Dh) and *Torulaspora delbrueckii* (Td) after 48 h in non-fat milk with yeast:LAB ratios of 1:1 ( ), 3:7 ( ) and 1:9 ( ) at three different temperatures.

5.4 CONCLUSIONS

In order to determine the growth patterns and the characteristics of the three dominant yeast species associated with indigenous fermented milk products, i.e. *Kluyv. marxianus, D. hansenii* and *T. delbrueckii* (4.3.2), growth studies at different environmental conditions were undertaken. The yeast strains were cultured individually and in different combinations with a commercial fermented milk culture (CHN-22), used in yogurt production. Growth of the yeasts was determined at four different pH values and four temperatures, in conjunction with growth at four different concentrations of lactose and lactate.

The growth of the selected yeast species at low pH values suggested that these yeasts may play a metabolically active role during, as well as at the end of lactic fermentation of milk. Both *Kluyv. marxianus* and *D. hansenii* were able to progress in milk at pH values of 4.5 and 6.0, whereas no growth was experienced at pH<4.5. The survival of *T. delbrueckii* at pH 4.0 is noteworthy and indicated a possible metabolic role in fermented milks at low pH values.

T. delbrueckii showed a substantial increase in growth at 7°C, whereas *Kluyv. marxianus* and *D. hansenii* only survived at 7°C. Maximum cell densities for all three yeasts were experienced at 25°C. Despite faster growth of yeasts at higher temperatures (35°C), they started to die off earlier.

The growth of *Kluyv. marxianus* and *D. hansenii* is directly proportional to lactose concentrations between 3 and 10%. Leclercq-Perlat et al. (1999) also reported on the correlation between lactose concentrations and *D. hansenii* concentration in the inner part of surface-ripened soft cheese. *T. delbrueckii* showed little or no growth in the presence of lactose. However, *T. delbrueckii* was able to utilize lactate and consequently may grow in a commensialistic interaction with *Kluyv. marxianus* and *D. hansenii,* as they are able to break down lactose to lactate. The growth rates and cell densities of all three yeasts decreased correspondingly, as the concentration of lactate was decreased from 1.5 to 0.6%. The utilization of lactate at high concentrations may play a role in spoilage of fermented products with a high lactate content, like yogurts.

To determine the interactions between the amount of yeasts and lactic acid bacteria (LAB) in fermented milk, three different ratios of yeast:LAB were used. These different combinations were combined with changes in the temperature of incubation, while changes in pH, lactose and lactate utilization were monitored. In all, 27 different combinations, involving the mentioned parameters, were determined over a period of 48 h. Reduced growth rates and cell densities were observed when the individual yeast species and LAB in 1:1, 3:7 and 1:9 ratios, were grown in the milk at 7°C. The 1:1 yeast:LAB ratio, in general, resulted in higher LAB populations, that may be an indication of increased stimulating influence of yeasts on the LAB. The number of viable cells of the LAB in the milk grown at 32°C, were higher with all the different ratios than that obtained at 22°C. No differences were observed in the growth of *D. hansenii* at 22 and 32°C, while *Kluyv. marxianus* and *T. delbrueckii* both increased their cell numbers at 32°C. The higher amount of lactic acid production in the presence of *Kluyv. marxianus* might be an indication of the enhanced acid production of the species compared to the other two yeasts, or enhanced stimulation of the LAB by the species. The number of viable LAB cells was also higher when grown in association with *Kluyv. marxianus.*

Good and sensory acceptable products were manufactured during this study, although no specific relationship was found between specific combinations of yeast:LAB and flavour development or -levels produced. The desirable products were predominantly achieved when incubated at 22°C. Production of flavour compounds like acetone and acetaldehyde was observed in all the combinations, whereas diacetyl and acetoin production were detected in only a few combinations.

Appreciable amounts of CO_2 and ethanol were produced at 22 and 32°C by all the yeasts, whereas *T. delbrueckii* lacked the ability to produce ethanol. The highest CO_2 production was experienced at 32°C, with the inclusion of *Kluyv. marxianus.* The high production of CO_2 can be desirable in products like kefir, where a fresh taste is required and in other products i.e. blue-veined cheese where the CO_2 tends to open the cheese structure for the mould to develop. In contrast, it may cause undesirable effects in yogurt related products.

Potential and important advantages from a deliberate use of yeasts for fermentation and maturation of dairy products are evident. The advantages mentioned comprise beneficial microbial interactions between different desirable organisms, including LAB starter cultures and the production of aroma component. More controlled studies are, however, necessary to determine the specific influence of yeasts and LAB on the sensory characteristics of fermented milks. Although the three yeast spp. used in the interaction studies were dominant, other yeasts e.g. *Y. lipolytica* and *Gal. geotrichum* should also be studied in order to determine their role on the characteristics of fermented milk products, or for use as starter cultures in other products.

CHAPTER 6

GENERAL RESULTS AND DISCUSSIONS

Yeasts were found in all the kefir samples obtained from households around the country with relatively high yeast counts that varied between $2.20x10^8$ and $3.00x10^8$ cfu/ml. Four different genera were identified from 14 isolates. All the yeasts isolated from kefir were good candidates that one would expect to find in traditional kefir. The lactose fermenting yeast *Kluyv. marxianus* (5 isolates) was the most prevalent species, followed by *Sacch. cerevisiae* (3) and *Kluyv. lactis* (2). The other isolates were identified as *T. delbrueckii, Debaryomyces hansenii, Sacch. rouxii* and *Sacch. unisporus.* It was found that the yeast flora of traditional South African kefir grains is quite varied consisting of non-lactose and lactose fermenting species, with the latter group, dominating.

Relatively high yeast numbers were also found among the 14 indigenous traditional fermented milk samples from calabashes and clay pots, ranging from 10^3 to 10^7 cfu/ml. From the fifty yeast colonies, ten different yeast species were isolated, consisting of predominant isolates of *T. delbrueckii, D. hansenii* and *Kluyv. marxianus.* Other species encountered were *Sacch. cerevisiae, Y. lipolytica, Dek. anomala, P. membranaefaciens, Rh. glutinis, Tr. beigelii* and *Gal. geotrichum.* These yeasts correspond with yeast species usually encountered in dairy products and those found by various authors. The lactic acid bacteria dominated with counts between $1.00x10^5$ and $1.00x10^9$ cfu/ml which were substantially higher than the yeast counts. There was notably no differences in the yeast and lactic acid bacteria counts between samples from clay pots and calabashes.

The majority of the yeast species assimilated lactate, while some of the strains of *T. delbrueckii* and *D. hansenii* (conventionally considered as lactose-negative) fermented lactose. *Sacch. cerevisiae* and more than half of *T. delbrueckii* strains showed growth in NaCl concentrations of 10 and 20%, although the opposite was encountered by Roostita and Fleet (1996a). *Debaryomyces* which is considered to be salt tolerant, did not perform

well in high NaCl concentrations. Most of the yeast species were able to produce tyramine and tryptamine but non of the strains tested were able to decarboxylate histidine. Although these findings are preliminary, it is important to consider that amines are important because of their role in causing spoilage of fermented foods by producing off flavours and putrid odours, as well as physiological effects on humans and animals. When tested for anti-microbial activity against 11 relevant food pathogens, three isolates identified as *Kluyv. marxianus* (2 species) and *D. hansenii* (1) showed positive results against *Staphylococcus aureus*.

The growth of the selected yeast species at low pH values (pH 4.5 – 6.0) suggested that these yeasts may play a metabolically active role during, as well as at the end of lactic fermentation of milk. *T. delbrueckii* survived at pH 4.0 and showed a substantial increase in growth at 7°C. These findings indicated on a possible metabolic role in fermented milks at low pH values and temperatures. *Kluyv. marxianus* and *D. hansenii* survived at 7°C, with maximum cell densities experienced at 25°C. When grown in different concentrations of lactose, *Kluyv. marxianus* and *D. hansenii* were found to be directly proportional to lactose concentrations between 3 and 10%, while *T. delbrueckii* showed little or no growth in the presence of lactose. The growth rates and cell densities of all three yeasts decreased correspondingly as the concentration of lactate was decreased from 1.5 to 0.6%. The utilization of lactate at high concentrations may play a role in spoilage of fermented products with a high lactate content.

Reduced growth rates and cell densities were observed when the individual yeast species and LAB in 1:1, 3:7 and 1:9 ratios were grown in milk at 7°C. The 1:1 yeast:lactic acid bacteria ratio, generally resulted in higher LAB populations, that may be an indication of increased stimulation influence of yeasts on the LAB. While *Kluyv. marxianus* and *T. delbrueckii* both increased their cell populations at 32°C, no significant difference in the growth of *D. hansenii* at 22 and 32°C were experienced with all three ratios used. Although no specific relationship was found between specific combinations of yeast:LAB and flavour development or flavour levels produced, sensory acceptable products were manufactured during this study. The desirable products were predominantly made at 22°C, while

production of flavour compounds like acetone, acetaldehyde, diacetyl and acetoin were also detected in some products.

The highest production of CO_2 was experienced with *Kluyv. marxianus* at 32°C, which can be desirable in products like kefir where a fresh acid taste is required and in products like blue-veined cheeses, where CO_2 are responsible for the open structure, necessary for the development of the mould.

More controlled studies are, however, necessary to determine the specific influence of yeasts and LAB on the sensory characteristics of fermented milks. According to an Advisory Panel (1992) for Application of Biotechnology to Traditional Fermented Foods, it was recommended that the biotechnological worth of each organism be determined and the isolation of microorganisms from fermented products not be confined to the dominant organisms, because other microbes found in lower numbers might also have important functions. Thus, although the three yeasts spp. used in the interaction studies were dominant, the other yeasts e.g. *Y. lipolytica* and *Gal. geotrichum* should also be studied further to determine their role on the characteristics of fermented milk and other products.

CHAPTER 7

SUMMARY

Yeasts are widely dispersed in nature and considered as natural inhabitants of the dairy environment. They are also natural contaminants in milk and milk products causing either spoilage or leading to fermentation with beneficial effects. In kefir, yeasts belong to the typical microflora and are responsible for the prickly and refreshing taste due to carbon dioxide production. In a number of other fermented dairy products, yeasts play an important role in metabolic interactions, thereby ensuring a product with typical sensoric properties.

In this study, yeasts from kefir milks fermented by seven indigenous kefir grains were isolated and characterised in order to determine the diversity of the yeast flora in South African kefir. These kefir grains were inoculated into full cream milk and incubated at 25°C for 18 h. The fermented kefir milks revealed yeast counts between $2.00x10^8$ and $3.00x10^8$ cfu/ml. The representative yeast strains were dominated by *Kluyveromyces marxianus, Kluyveromyces lactis* and *Saccharomyces cerevisiae.* Other species encountered were *Saccharomyces unisporus, Saccharomyces rouxii, Torulaspora delbrueckii* and *Debaryomyces hansenii.*

Furthermore, a total of 50 yeasts were isolated and identified from 14 different traditional fermented milks manufactured in households by small-scale dairy farmers in rural areas. Growth patterns and the characteristics of *Kluyveromyces marxianus, Debaryomyces hansenii* and *Torulaspora delbrueckii,* the three dominant yeast species encountered in the fermented milk samples, were determined. A commercial lactic acid bacteria (LAB) culture was used in various combinations with the yeasts in the growth and fermentation studies.

Growth at different pH values suggested that these yeasts (especially *T. delbrueckii)* play a metabolically active role during, as well as right to the end of the lactic acid fermentation of

milk. *T. delbrueckii* also showed significant growth at 15 °C, but was not able to ferment lactose, as was the case with *Kluyv. marxianus* and *D. hansenii*. The growth of these two yeasts was also directly proportional to the concentration of lactose. All three yeasts were able to grow in the presence of 0.6, 0.85, 1.25 and 1.5 % lactate. Although the best aroma profiles were experienced at 22 °C, no specific yeast or combination was responsible for the production of a desirable aroma. The highest alcohol and CO_2 production resulted from a yeast:LAB combination with *Kluyv. marxianus*.

These results could not highlight a specific combination of yeasts and LAB of all the variables tested to include in a fermented milk product to enhance or improve its characteristics. Some combinations produced desirable aromas while other combinations produced better alcohol or CO_2 yields. However, it indicated that yeasts play a role in the improvement of the characteristic fresh pungent flavour encountered with traditional fermented milks.

Key Words: Yeasts, traditional fermented milks, indigenous kefir, lactic acid bacteria, lactic fermentation, metabolic interactions

OPSOMMING

Giste kom wyd verspreid voor in die natuur en word algemeen in suiwel ekosisteme aangetref. Giste is gevolglik natuurlike kontaminante in melk en melkprodukte wat bederf kan veroorsaak of tot voordelige fermentasie kan lei. In kefir is giste deel van die tipiese mikroflora wat verantwoordelik is vir die kenmerkende prikkelende en verfrissende smaak, a.g.v. die produksie van koolstofdioksied. In verskeie ander gefermenteerde melkprodukte speel giste 'n belangrike rol in die metaboliese interaksies waardeur verseker word dat gewenste sensoriese eienskappe ontwikkel.

In hierdie studie is giste uit kefir melk, wat deur sewe verskillende kefir korrels gefermenteer is, geïsoleer en gekarakteriseer om die spesie diversiteit te bepaal. Die kefir korrels is in volroom melk geïnokuleer en vir 18 h by 25°C geïnkubeer. Gistellings van tussen $2.00x10^8$ en $3.00x10^8$ kolonie vormende eenhede is bepaal. *Kluyveromyces marxianus, Saccharomyces cerevisiae* en *Kluyveromyces lactis* was die mees dominante spesies, terwyl *Saccharomyces unisporus, Saccharomyces rouxii, Torulaspora delbrueckii* en *Debaryomyces hansenii* in laer getalle voorgekom het.

Vyftig verteenwoordigende giste uit 14 verskillende tradisioneel-gefermenteerde suurmelk monsters, wat in huishoudings in landelike gebiede vervaardig is, is voorts geïsoleer en identifiseer. Groeistudies is onder verskillende omgewingstoestande uitgevoer om die groeipatrone en eienskappe van die drie dominante giste nl. *Kluyveromyces marxianus, Debaryomyces hansenii* en *Torulaspora delbrueckii* te bepaal. 'n Kommersiële melksuurbakterie-kultuur (MSB) is in verskillende kombinasies met die giste gebruik in die groei- en fermentasie studies.

Groei by verskillende pH waardes dui daarop dat dié giste, (veral *T. delbrueckii*), 'n metabolies-aktiewe rol speel gedurende en tot aan die einde van die melksuurfermentasie van die melk. *T. delbrueckii* het ook die beste groei getoon by 15°C maar was, inteenstelling met *Kluyv. marxianus* en *D. hansenii,* nie in staat om laktose te fermenteer

nie. Die groei van die twee giste was direk eweredig met die konsentrasie van laktose. Al drie giste kon in die teenwoordigheid van 0.6, 0.85, 1.25 en 1.5 % laktaat groei. Alhoewel die beste geur profiele by 22°C bepaal is, was geen spesifieke gis of kombinasie verantwoordelik vir die gewenste geur nie. Die vinnigste alkohol en koolstofdioksied produksie is met 'n gis:MSB kombinasie met *Kluyv. marxianus* verkry.

Hierdie resultate kon nie 'n spesifieke gis of kombinasie van giste en MSB uitwys wat al die gewenste eienskappe in 'n gefermenteerde melkproduk verbeter nie. Sommige kombinasies het gewenste geur en smaak geproduseer, terwyl ander weer meer alkohol of CO_2 gelewer het. Hierdie resultate dui egter daarop dat giste 'n belangrike rol speel in die verbetering van die vars prikkelende smaak, kenmerkend van tradisioneel-gefermenteerde melkprodukte.

Sleutelwoorde: Giste, tradisioneel-gefermenteerde melk, inheemse kefir, melksuurbakterieë, melksuurfermentasie, metaboliese interaksies

CHAPTER 8

REFERENCES

Abdelgadir, W.S., Ahmed, T.K., Dirar, H.A., 1998. The traditional fermented milk products of the Sudan, a Review. Int. J. Food Microbiol. 44, 1-13.

Abou-Donia, S.A., 1984. Egyptian fresh fermented milk products. New Zealand J. Dairy Sci. Technol. 19, 7-18.

Abraham, A., De Antoni, G.L., 1999. Characterization of kefir grains grown in cow's milk and in soya milk. J. Dairy Res. 66, 327-333.

Advisory Panel, 1992. Research priorities in traditional fermented foods. In: Applications of Biotechnology to Traditional Fermented Foods. Report of an Ad Hoc Panel of the Board on Science and Technology for International Development, ISBN 0 309 046858. National Academy Press, Washington, D.C.

Angulo, L., Lopez, E., Lema, C., 1993. Microflora present in kefir of the Galician region (North-West of Spain). J. Dairy Res. 60, 263-267.

Atanda, O.O., Ikenebomeh, M.J., 1988. Changes in the acidity and lactic acid content of 'Nono', a Nigerian cultured milk product. Lett. Appl. Microbiol. 6, 137-138.

Bankole, M.O., Okagbue, R.N., 1992. Properties of 'Nono', a Nigerian fermented milk food. Ecol. Food. Nutr. 22, 145-149.

Barnett, J.A., Payne, R.W., Yarrow, D., 1990. Yeasts: Characteristics and Identification, 2nd edn. Cambridge University Press, Cambridge.

Baroudi, A.A.G., Collins, E.B., 1976. Microorganisms and characteristics of Laban. J. Dairy Sci. 59 (2), 200-202.

Besançon, X., Smet, C., Chabalier, C., Revile, M., Reverbel, J.P., Rotomahenina, R., Galzy, P., 1992. Study of surface yeast flora of Roquefort cheese. Int. J. Food Microbiol. 17, 9-18.

Bester, B.H., Mostert, J.F., 1993. Biogenic amines in food. S. Afr. J. Food Sci. Nutr. 5, 103-111.

Bogdanoff, V.M., 1934. An investigation of Kuban, a fermented milk. J. Dairy. Res. 5, 153-159.

Bohme, H.E., 1976. Some Nguni crafts. Ann. S. Afr. Mus. 70, 8-15.

Bottazzi, V., 1983. Other fermented dairy products. In: Reed, G. (Ed.), Biotechnology, Food and Feed Production with Microorganisms, Verlag, Weinheim, Vol. 5, pp. 315-363.

Brothwell, D., Brothwell, P., 1969. Food in antiquity - a survey of the diet of early peoples, In: Ancient Peoples and Places. Thames and Hudson, London, pp. 50-53.

Burchell, W.J., 1953. Travels in the interior of Southern African. The Batchworth Press, London, Vol. II, pp. 416-419.

Chen, H-L., Su, H-P., Lin, C-W., 1998. Characterization of yeast cultures for a flavouring agent in a yogurt-type product. J. Food Sci. 63(5), 897-900.

Chuang, L.F., Collins, E.B., 1968. Biosynthesis of diacetyl in bacteria and yeast. J. Bacteriol. 95, 2083-2089.

Collins, E.B., 1972. Biosynthesis of flavour by microorganisms. J. Dairy Sci. 55 (7), 1022-1028.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl. Bact. 23, 130-135.

Deàk, T., Beuchat, L.R., 1996. Handbook of food spoilage. CRC Press, New York

Deiana, P., Fatichenti, F., Farris, G.A., Mocquot, G., Lodi, R., Todesco, R., Cecchi, L., 1984. Metabolization of lactic and acetic acids in Pecorino Romano cheese made with a combined starter of lactic acid bacteria and yeast. Le Lait. 64, 380-394.

Dellaglio, F., 1988. Starters for fermented milks. IDF Bulletin No 227, Brussels, Belgium. pp. 7-17.

Duitschaever, C.L., 1989. What is kefir and how can it made? Mod. Dairy. 68, 18-19.

El-Gendy, S.M., 1983. Fermented foods of Egypt and the Middle-East. J. Food. Prot. 46, 358-367.

Eliskases-Lechner, F., Ginzinger, W., 1995. The yeast flora of surface-ripened cheeses. Milchwissenschaft. 50, 458-462.

El-Sadek, M.G., Naguib, Kh., Negm, A., 1972. Microbiological and chemical studies on zabady. Milchwissenschaft. 27 (9), 570-572.

Engel, G., Krusch, V., Teuber. M., 1986. Microbiological composition of kefir. I. Yeasts. Milchwissenschaft. 41, 418-421.

Fahmi, A.H., Abdel-Malek, Y., Hassouna, M.M., Fahmi, M., Samia, A.A., 1966. Microbiological studies on fermented milks. I. Laban Zabadi. J. Microbiol. U.A.R. 1 (1), 133-142.

Fehr, W., 1968. Ludwig Fehr's account of the tribel life and customs of the Xhosa in 1807 (from the original manuscript in german of The natives of the south coast of Africa). AA Balkema, Cape Town, pp. 22-27.

Feresu, S.B., Muzondo, M.I., 1990. Factors affecting the development of two fermented milk products in Zimbabwe. MIRCEN J. Appl. Micro. Biotech. 5, 349-355.

Fleet, G.H., 1990. Yeasts in dairy products. J. Appl. Bacteriol. 68, 199-211.

Fleet, G., Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. Int. J. Food Microbiol. 4, 145-155.

Fox, F.W., 1939. Some Bantu recipes from the Eastern Cape Province, In: Bantu Studies. S. Afr. Inst. Med. Res. Annu. Rep. Johannesburg, pp. 71-73.

Fox, P.F., Law, J., 1991. Enzymology of cheese ripening. Food Biotechnol. 5, 239-262.

Gedek, B.R., 1991. Reguliering der Darmflora über die Nahrung. Zbl. Hyg. 191, 277-301.

Gibbs, P.A., 1987. Novel uses for lactic acid fermentation in food preservation. J. Appl. Bacteriol. Symp. Suppl. 16, 515-85.

Gosselé, F., Swings, J., Kersters, K., De Ley, J., 1983. Numerical analysis of phenotypic features and protein gel electropherograms of *Gluconobacter* Asai 1935 emend. mut. char. Asai, Iizuka, and Komagata 1964. Int. J. Syst. Bacteriol. 33, 65-81.

Green, M.D., Ibe, S.N., 1987. Yeasts as primary contaminants in yogurts produced commercially in Lagos, Nigeria. J. Food Prot. 50(3), 193-198.

Guerzoni, M.E., Gobbetti, M., Lanciotti, R., Vannini, L. and Lòpez, C.C., 1996. *Yarrowia lipolytica* as potential ripening agent in milk products. IDF –ref. S.I. 9801. Yeast in the dairy industry: positive and negative aspects. Proceedings of the symposium organized by group F47, held in Copenhagen (Denmark), 23-33.

Häfliger, M.J., 1990. Die Bedeutung der Essigsäurebakterien im Kefir. Dissertation der Eidgenössischen Technischen Hochschule, Zürich.

Hamama, A., Bayi, M., 1991. Composition and microbiological profile of two Moroccan traditional dairy products. J. Soc. Dairy Technol. 44, 119-120.

Hamdan, I.Y., Kunsman, Jr., J.E., Deane, D.D. 1971. Acetaldehyde production by combined yogurt cultures. J. Dairy Sci. 54 (7), 1080-1082.

Hammer, B.W., Babel, F.J., 1943. Bacteriology of butter cultures. A Review. J. Dairy Sci, 26, 84.

Harrigan, W.F., McCance, M.E., 1966. Laboratory methods in microbiology. Academic Press, London and New York pp. 52, 63, 268

Hermann, M.N., 1997. The dairy industry in Southern Africa and especially South Africa. Food Ind. S. A. 50, 3, 5, 16, 20, 24.

Hitchins, A.D., McDonough, F.E., 1989. Prophylactic and therapeutic aspects of fermented milk. Am. J. Clin. Nutr. 49, 675-684.

Hosono, A., Wardjojo, R., Otani, H., 1989. Microbial flora in 'Dadih', a traditional fermented milk in Indonesia. Lebensm-Wiss. Technol. 22, 22-24.

Hugenholtz, J., 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Reviews. 12(1-3), 165-178.

IDF., 1990. Consumption statistics for milk and milk products 1988. IDF Bulletin No. 246, Brussels, Belgium, pp.21.

Irvine, O.R., Bullock, D.H., Sproule, W.H., 1954. Flavour development in pasteurized milk cheddar cheese. I. The effect of inoculating milk with *Geotrichum candidum*. J. Dairy Sci. 37 (6), 633-639.

Isono, Y., Shingu, I., Shimizu, S., 1994. Identification and characteristics of lactic acid bacteria isolated from Masai fermented milk in Northern Tanzania. Biosci. Biotechnol. Biochem. 58, 660-664.

Iwasawa, S., Ueda, M., Miyata, N., Hirota, T., Ahiko, K., 1982. Identification and fermentation character of kefir yeast. Agric. Biol. Chem. 46, 2631-2636.

Jakobsen, M., 1994. Yeasts. International Dairy Federation, F-Doc 245, Report of subject F47, 1-15.

Jakobsen, M., Narvhus, J., 1996. Yeasts and their possible beneficial and negative effects on the quality of dairy products. Int. Dairy. J. 6, 755-768.

Joosten H.M.L.J., 1988. The biogenic amine contents of Dutch cheese and their toxicological significance. Neth. Milk Dairy J. 42, 25-42.

Kaminarides, S.E., Laskos, N.S. 1992. Yeasts in factory brine of Feta cheese. Aust. J. Dairy Technol. 47, 68-71.

Kassaye, T., Simpson, B.K., Smith, J.P., O'Connor, C.B. 1991. Chemical and microbiological characteristics of Ititu. Milchwissenschaft. 46, 649-653.

Keenan, T.W., Lindsay, R.C., Morgan, M.E., Day, E.A., 1966. Acetaldehyde production by single strain lactic streptococci. J. Dairy Sci. 49, 10-14.

Keller, J.J., Jordaan, I., 1990. Fermented milks for the South African market. South African J. Dairy Sci. 22, 47-49.

Khalafalla, S.M., Fayed, E.O., Aly, A.A., El-Samragy, Y.A., 1988. Composition and microbiological properties of laban rayeb produced in Lower Egypt. Ecol. Food. Nutr. 22, 297-302.

Kimonye, J.M., Robinson, R.K., 1991. Iria ri Matii - a traditional fermented milk from Kenya. Dairy Ind. Int. 56, 34-35.

Koroleva, N.S. 1982. Special products (kefir, Koumyss, etc.) 21st Int, Dairy Congress Miscow, Vol. 2, 146-152.

Koroleva, N.S., 1988. Starters for fermented milks. Bulletin of the IDF No 227, Brussels, Belgium, pp. 35-40.

Kosikowski, F.V., Mistry, V.V., 1997. Cheese and fermented milk foods. Vol. 1. 3rd edn., F.V. Kosikowski, L.L.C.,Westport, Connecticut, 728 pp.

Kreger-van Rij, N.J.W., 1984. The Yeasts: a Taxonomic Study. 3rd edn., Elsevier Science, Amsterdam.

Krige, E.J., 1965. The social system of the Zulus. Shuter and Shooter, Pietermaritzburg, pp. 208, 397.

Kroger, M., 1993. Kefir. Cult. Dairy. Prod. J. 28, 26-29.

Kroger, M., Kurmann, J.A., Rasic, J.L., 1992. Fermented milks – Past, present, and future. In: Applications of Biotechnology to Traditional Fermented Foods. Report of an Ad Hoc Panel of the board on Science and Technology for International Development, ISBN 0 309 046858. National Academy Press, Washington, D.C.

Kuo, C-Y., Lin, C-W., 1999. Yaiwanese kefir grains: their growth, microbial and chemical composition of fermented milk. Aust. J. Dairy Technol. 54, 19-23.

Kurmann, J.A., 1984. Aspects of the production of fermented milks. IDF Bulletin No 179, Brussels, Belgium, pp.16-26.

Kurmann, J.A., Rašic, J.Lj., Kroger, M., 1992. Encyclopedia of fermented fresh milk products: An international inventory of fermented milk, cream, buttermilk, whey and related products. Van Nostrand Reinhold, New York, NY, 367 pp.

Kurtzman, C.P., Fell, J.W., 1998. The Yeasts, A taxonomic study. Fourth revised and enlarged edition. Elsevier Science B.V., The Netherlands.

Kuwabara, S., 1970. Fermented milk. Brit. Pat. 1, 197, 257.

Lambelet, P., Löliger, J., Saucy, F., Bracco, U., 1992. Antioxidant properties of coenzyme Q_{10} in food systems. J. Agric. Food. Chem. 40, 581-548.

Leclercq-Perlat, M-N., Oumer, A., Bergere, J-L., Spinnler, H-E., Corrieu, G., 1999. Growth of *Debaryomyces hansenii* on a bacterial surface-ripened soft cheese. J. Dairy Res. 66, 271-281.

Lee, J-T., Lim, J-W., 1988a. Studies on the ripening of cheese made with lactic acid bacteria and yeasts. I. Changes of physico-chemical composition. Kor. J. Anim. Sci. 30/31, 170-177.

Lee, J-T. , Lim, J-W., 1988b. Studies on the ripening of cheese made with lactic acid bacteria and yeasts. II. Proteolysis of cheese. Kor. J. Anim. Sci. 30(4), 258-263.

Lees, G.J., Jago, G.R., 1978a. Role of acetaldehyde in metabolism: A review 1. Enzymes catalyzing reactions involving acetaldehyde. J. Dairy Sci. 61, 1205.

Lees, G.J., Jago, G.R., 1978b. Role of acetaldehyde in metabolism: A review 2. The metabolism of acetaldehyde in cultured dairy products. J. Dairy Sci. 61, 1216.

Leroi, F., Pidoux, M., 1993a. Detection of interactions between yeasts and lactic acid bacteria isolated from sugary kefir grains. J. Appl. Bacteriol. 74, 48-53.

Leroi, F., Pidoux, M., 1993b. Characterization of interactions between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from sugary kefir grains. J. Appl. Bacteriol. 74, 54-60.

Libudzisz, Z., Piatkiewicz, A., 1990. Kefir production in Poland. Dairy Ind Int. 55, 31-33.

Lin, C-W., Chen, H-L, Liu, J-R., 1999. Identification and characterisation of lactic acid bacteria and yeasts isolated from kefir grains in Taiwan. Aust. J Dairy Technol.54, 14-18.

Lindsay, R.C., Day, E.D., Sandine, W.E., 1965. Green flavour defect in lactic starter cultures. J. Dairy Sci, 48, 863.

Lodder, J., Kreger-van Rij, N.J.W., 1952. The Yeasts, a Taxonomic Study. Elsevier North-Holland Publishing Co., Amsterdam.

Macedo, A.C., Malcata, F.X., Hogg, T.A., 1995. Microbiological profile in Serra ewe's cheese during ripening. J. Appl. Bacteriol. 79, 1-11.

Mann, E.J., 1989. Kefir and koumiss. Dairy Ind. Int. 54, 9-10.

Mann, G.V., Spoerry, A., 1974. Studies of a surfactant and cholesteremia in the Masai. Am. J. Cl. Nutr. 27, 464-469.

Margalith, P.Z., 1981. Flavor microbiology. Charles C Thomas Publisher, Illinois, p 32-118.

Marshall, V.M., 1986. The microflora and production of fermented milks. In: Adams, M.R. (Ed.), Prog. Ind. Microbiol. Elsevier, Amsterdam, Vol. 23, pp. 1-44.

Marshall, V.M., 1993. Fermented milks – Kefir. In: Macrae, R., Robinson, R.K. and Sadler, M.J. (Eds.), Encyclopaedia. of Food Science, Food Technology and Nutrition. Academic Press, London, Vol. 3, pp. 1804-1808.

Marshall, V.M., Cole, W.M., Brooker, B.E., 1984. Observations on the structure of kefir grains and the distribution of the microflora. J. Appl. Bacteriol. 57, 491-497.

Martin, N., Savonitto, S., Molimard, P., Berger, C., Brousse, M., Spinnler, H.E., 1999. Flavor generation in cheese curd by coculturing with selected yeasts, mold and bacteria. J. Dairy Sci. 82, 1072-1080.

McKay, A.M., 1992. Growth of fermentative and non-fermentative yeast in natural yoghurt, stored in polystyrene cartons. Int. J. Food Micrbiol. 15, 383-388.

Mizuno, W.G., Jezeski, J.J., 1959. Studies on starter metabolisms. IV. Effect of various substrates on the formation of acetoin by a mixed strain starter culture. J. Dairy Sci. 42, 251-263.

Molimard, P., Spinnler, H.E., 1996. Review: Compounds involved in the flavour of surface mold-ripened cheeses: origins and properties. J. Dairy Sci. 79, 169-184.

Mutukumira, A.N., 1995. Properties of "amasi" a natural fermented milk produced by smallholder milk producers in Zimbabwe. Milschwissenschaft 50, 201-204.

Mutukumira, A.N., 1996. Investigation of some prospects for the development of starter cultures for industrial production of traditional fermented milk in Zimbabwe. Doctor Scientiarum Thesis 1996:5, ISSN 0802-3220, Agricultural University of Norway, As, Norway.

Niven, C.F., Jeffrey, M.B., Corlett, D.A., 1981. Differential plating medium for the detection of histamine producing bacteria. Appl. Environ. Microbiol. 41, 321-322.

Obermann, H., 1985. Fermented milks, In: Wood, B.J.B. (Ed.), Microbiology of fermented foods. Elsevier Applied Science Publishers, London and New York, Vol. 1, pp. 167-195.

Olasupo, N.A., 1992. Nono, a Nigerian study. Dairy. Ind. Int. April, p37.

Pack, M.Y., Vedamuthu, E.R., Sandine, W.E., Eliker, P.R., Leesment, H., 1968. Effect of temperature on growth and diacetyl production by aroma bacteria in single-and mixed strain lactic cultures. J. Dairy Sci. 51, 339-344.

Pryce, J.D., 1969 A modification of the Barker-Summerson method for the determination of Lactic Acid, Anal. 94, 1151.

Richards, A.I., 1932. Hunger and work in a savage tribe - A functional study of nutrition among the southern bantu. George Routledge and Sons, LTD., London, pp.20 -26.

Richardson, T., 1978. The hypocholesteremic effect of milk – A review. J. Food. Prot. 41, 226-235.

Robinson, R.K., Tamime, A.Y., 1990. Microbiology of fermented milks. In: Robinson, R.K. (Ed.), The Microbiology of Milk Products, 2nd ed. Elsevier, London, pp. 291-343.

Roginski, H., 1988. Fermented milks. Aust. J. Dairy. Technol. 43, 37-45.

Roginski, H., 1993. Fermented foods – types of fermented milks. In: Macrae, R., Robinson, R.K. and Sadler, M.J. (Eds.), Encyclopaedia of Food Science, Food Technology and Nutrition. Academic Press, London, Vol. 3, pp. 1795-1813.

Rogosa, M., Mitchell, J.A. and Wiseman, W.E., 1951. A selective medium for the isolation of oral and fecal lactobacilli. J. Bacteriol. 62, 132-133.

Rohm, H., Eliskases-Lechner, E., Bräuer, M., 1992. Diversity of yeasts in selected dairy products. J. Appl. Bacteriol. 72, 370-376.

Roostita, R., Fleet, G.H., 1996a. Growth of yeasts in milk and associated changes to milk composition. Int. J. Food Microbiol. 000, 00.

Roostita, R., Fleet, G.H., 1996b. The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. Int. J. Food Microbiol. 28, 393-404.

Rossi, J., 1994. Yeasts. International Dairy Federation, F-Doc 245, Report of subject F47, 16-25.

Sandine, W.E., Daly, C., Elliker, P.R., Vedamuthu, E.R., 1972. Causes and control of culture-related flavour defects in cultured dairy products. J. Dairy Sci, 55, 1031.

Sansom, B., 1974. Traditional economic systems. In: W.A. Hammond-Tooke (Ed.), The Bantu-Speaking Peoples of Southern Africa, 2nd edn., Routlidge and Kegan Paul, London, pp.151-167.

Schaack, M.M., Marth, E.H., 1988. Interaction between lactic acid bacteria and some foodborne pathogens: A Review. Cult. Dairy Prod. J. 14-19.

Seiler, H., 1991. Some additional physiological characteristics for the identification of food-borne yeasts. Neth. Milk Dairy J. 45, 253-258.

Seitz, E.W., Sandine, W.E., Elliker, P.R., Day, E.A., 1963. Studies on diacetyl biosynthesis by *Streptococcus diacetilactis*. Can J. Microbiol. 9, 431.

Sharpe, E.M., 1979. Lactic acid bacteria in the dairy industry. J. Soc. Dairy. Technol. 32, 9.

Smit, L.E., Nel, M.M., 1987. Vergelyking tussen die standaardkoperreduksie, chloramien T-metodes en 'n gewysigde hoëdrukvloeistofchromatografiese metode vir die bepaling van laktose in melk- en melkprodukte. S.Afr. J. Dairy Sci, 19 (2), 62-64.

Soga, J.H., 1932. The Ama-Xhosa: Life and customs. Lovedale Press, Lovedale, pp. 355-399.

Sørensen, B.B., Jakobsen, M., 1997. The combined effects of temperature, pH and NaCl on growth of *Debaryomyces hansenii* analyzed by flow cytometry and predictive microbiology. Int. J. Food Microbiol. 34, 209-220.

Speckman, R.A., Collins, E.B., 1968. Diacetyl biosynthesis in *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol. 95 (1), 174-180.

Stanley, G., 1980. Variation in mixed single strain starters as used in the preparation of fermented dairy products. In: B.E. Kirsop (Ed.), The stability of industrial micro-organisms. Kew, Surrey, England, p. 20-31.

Subramanian, P., Shankar, P.A., 1985. Commensalistic interaction between *Lactobacillus acidophilus* and lactose-fermenting yeasts in the preparation of acidophilus-yeast milk. Cult. Dairy Prod. J. 20 (4), 17-26.

Suriyarachchi, V.R., Fleet, G.H., 1981. Occurrence and growth of yeasts in yogurts. Appl. Environ. Microbiol. 42 (3), 574-579.

Suzzi, G., Gardini, F., Fiore, C., Caruso, M., Lanorte, M.T., 1996. A survey of yeasts isolated from water-buffalo mozzarella cheese produced in Basilicata. IDF –ref. S.I. 9801. Yeast in the dairy industry: positive and negative aspects. Proceedings of the symposium organized by group F47, held in Copenhagen (Denmark), 44-49.

Tagg, J.R., McGeven, A.R., 1971. Assay system for bacteriocins. J. Appl. Microbiol. 21, 943.

Tamime, A.Y., Muir, D.D., Wszolek, M., 1999. Kefir, koumiss and kishk. Dairy Ind. Int. 64(5), 32-33.

Tann, K.H., Gill, C.O., 1985. Batch growth of *Saccharomycopsis lipolytica* on animal fats. Appl. Microbiol. Biotechnol. 21, 292-298.

Terzaghi, B.E., Sandine, W.E., 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29, 807-813.

Turà, M., Rašić J., Canić V., 1969. Influence of *Str. thermophilus* and *Lb. bulgaricus* culture on volatile acids content in the flavour components of yogurt. Milschwissenschaft. 24, 277-281.

Turner, G.A., 1909. The diet of the South African natives in their kraals. Governments printing and stationery office, Pretoria, pp. 96-195.

Tzanetakis, N., Hatzikamari, M., Litopoulou-Tzanetaki, E., 1996. Yeasts of the surface microflora of feta cheese. IDF –ref. S.I. 9801. Yeast in the dairy industry: positive and negative aspects. Proceedings of the symposium organized by group F47, held in Copenhagen (Denmark), 34-34.

Van den Tempel, T., Jakobsen, M., 1996. The technological characteristics of *Candida famata* isolated from Danablu. IDF –ref. S.I. 9801. Yeast in the dairy industry: positive and negative aspects. Proceedings of the symposium organized by group F47, held in Copenhagen (Denmark), 59-69.

Van der Walt, J.P., Hopsu-Havu, V.K., 1976. A colour reaction for the differentiation of ascomycetous and hemibasidiomycetous yeasts. Antonie Leeuwenhoek. 42, 157-163.

Van der Walt, J.P., Yarrow, D., 1984. Methods for the isolation, maintenance, classification and identification of yeasts. In: Kreger-van Rij, N.J.W. (Ed.), The Yeasts - a Taxonomic Study, 3rd edn., Elsevier, Amsterdam, pp. 45-104.

Viljoen, B.C., Greyling, T., 1995. Yeasts associated with Cheddar and Gouda making. Int. J. Food Microbiol. 28, 79-88.

Vivier, D., Rivemale, M., Reverbel, J.P., Ratomahenina, R., Galzy, P., 1994. Study of the growth of yeasts from feta cheese. Int. J. Food Microbiol. 22, 207-215.

Walsh, B., Cogan, T.M., 1973. Diacetyl, acetoin, and acetaldehyde production by mixed-species lactic starter cultures. Appl. Microbiol. 26 (5), 820-825.

Welthagen, J.J., Viljoen, B.C., 1998. Yeast profile in Gouda cheese during processing and ripening. Int. J. Food Microbiol.41, 185-194.

Wickerham, L.J., 1951. Taxonomy of yeasts. U.S. Dept. Agr. Tech. Bull. 1029.

Wood, J. B., Hodge, M.M., 1985. Yeast-lactic acid bacteria interactions and their contribution to fermented foodstuffs, In: Wood, B.J.B. (Ed.), Microbiology of Fermented Foods. Elsevier Applied Science Publishers, London and New York, Vol. 1, 263-293.

Wyder, M-T., 1998. Identification and characterization of the yeast flora in kefyr and smear ripened cheese – Contribution of selected yeasts to cheese ripening. Doctorate Thesis, Diss. ETH No. 12842, 152 pp.

Yamani, M.I., Abu-Jaber, M. M., 1994. Yeast flora of Labaneh produced by in-bag straining of cow milk set yogurt. J. Dairy. Sci. 77, 3558 - 3564.

Yamauchi, K., Kang, K.H., Kamingogawa, S., Komagata, K., Fatichenti, F., Bergere, J.L., Deiana, P., Farris, G.A., 1975. Effects of yeasts isolated from cheese on the growth of lactic acid bacteria in skim milk. Jpn. J. Zootechn. Sci. 46, 78-80.