INFLUENCE OF DIFFERENT PRESERVATION TECHNIQUES AND PACKAGING MATERIALS ON THE ACTIVITY OF STORED KEPI GRAINS

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Thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE

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August 2001

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

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ABSTRACT

Kepi is a refreshing, fermented dairy beverage that has been consumed for centuries and is traditionally made by incubating Kepi grains in milk. The Kepi grain is a complex starter culture consisting of a variety of lactic acid bacteria and yeasts. The successful marketing of the grains requires the effective preservation of the microbes present in the grains as well as an appropriate packaging that will retain the acidification activity of the preserved grains over an extended period of time. The aim of this study was to evaluate different preservation techniques and packaging materials in terms of their respective abilities to retain grain viability and activity over an extended storage period. Four different preservation techniques (freezing at -18°C, refrigeration at 4°C, air-drying and lyophilisation) and three packaging materials including a low density polyethylene film (LDPE), an oriented polyester film (OPET) and a metallised oriented polyester film (MOPET), were evaluated.

Activity tests were used to evaluate the impact of the preservation techniques in terms of the retainment of the acidification activity of the preserved grains, and the storage potential of the preserved and packaged grains. The activity tests included changes in pH, %TA, lactic acid production and lactose and volatile compound content over an 18 h fermentation period. In addition, the microbial viability of the packaged lyophilised grains after two months of storage, was also investigated. Frozen and refrigerated grains showed the best retainment of the acidification activity over a 10-month storage period. Air-drying and lyophilisation showed a good retention of the activity up to three months of storage, but the application of these techniques both resulted in a retarded initial acidification activity. After 10 months of storage, the air-dried and lyophilised grains showed only a low acidification activity. No volatile compounds could be detected during the course of the fermentation period, due to the relative short fermentation period of 18 h.

Overall, the best retainment of the fermentation activity was given by the LDPE and the OPET packaging films. However, the storage period had a considerable influence on the retention of the activity of the packaged lyophilised

grains. The viability study of the lyophilised packaged Kepi grains after two months of storage showed leuconostocs and lactobacilli to be the prevalent microbes in the grains. Low microbial counts were obtained from the lactococci-selecting medium for all three of the differently packaged Kepi grains, whereas no growth was observed on the media that selected for the propionibacteria and yeasts. The OPET packaging film provided the best preservation of the microbial composition.

It was, therefore, concluded that all four preservation techniques would be suitable for the preservation of Kepi grains and the subsequent storage at room temperature for three months. However, for storage periods of 10 months or longer the use of freezing and refrigeration are recommended as most suitable preservation techniques. All three of the packaging materials proved to be suitable for the packaging and storage of the lyophilised Kepi grains for periods of up to one month. However, for storage periods of two months or longer, the use of the OPET film for the packaging and retainment of the acidification activity of the lyophilised grains, can be recommended.

UITTREKSEL

Kepi is 'n eeu-oue verfrissende, gefermenteerde suiweldrankie wat tradisioneel vervaardig word deur Kepikorrels in melk te inkubeer. Hierdie Kepikorrels bestaan uit 'n komplekse samestelling van hoofsaaklik melksuurbakterieë en giste. Die effektiewe preservering en verpakking van die korrels is belangrike voorvereistes vir die suksesvolle bemarking daarvan. Dis belangrik dat die preservering en die verpakking van die korrels 'n positiewe bydrae sal lewer tot die behoud van die fermentasie-aktiwiteit van die mikrobes in die korrels oor 'n verlengde opbergingsperiode. Die doel van hierdie studie was om die opbergingspotensiaal van verskillend gepreserveerde en -verpakte Kepikorrels te evalueer in terme van die behoud van die lewensvatbaarheid en fermentasie-aktiwiteit van die samestellende mikrobes. Vier verskillende preserveringstegnieke (bevriesing by –18°C, verkoeling by 4°C, lugdroging en vriesdroging) en drie verskillende tipes verpakkingsmateriale, nl. 'n "low density polyethylene film" (LDPE), 'n "oriented polyester film" (OPET) en 'n "metallised oriented polyester film" (MOPET) was geëvalueer.

Aktiwiteitstoetsing was gebruik om die impak van die verskillende preserveringstegnieke en die verpakkingsmateriale op die behoud van die fermentasie-aktiwiteit van die Kepikorrels te ondersoek. Die verskillende aktiwitieitstoetse wat gedoen is, het die meting van die verandering in pH, %TA, melksuur- en laktosekonsentrasie oor 'n fermentasieperiode van 18 h ingesluit. Tesame met die aktiwitietstoetsing is die lewensvatbaarheid van die gevriesdroogde, verpakte Kepikorrels na twee maande van opberging ook ondersoek. Die bevrore en verkoelde Kepikorrels het die beste behoud van aktiwitiet na 'n 10-maande opbergingsperiode getoon. Die gelugdroogde en gevriesdroogde korrels het 'n goeie behoud van aktiwiteit getoon vir 'n opbergingstydperk van tot drie maande, maar beide die lugdroging- en vriesdrogingstegnieke het 'n aanvanklik vertraagde fermentasie-aktiwitieit getoon. Na 'n opbergingsperiode van 10 maande het beide die gelugdroogde en gevriesdroogde korrels egter 'n lae fermentasie-aktiwiteit getoon. As gevolg van 'n

relatiewe kort fermentasieperiode van 18 h kon geen vlugtige komponente in die Kepimonsters gevind word nie.

Die LDPE- en OPET-verpakkingsmateriale het die beste behoud van die fermentasie–aktiwiteit van die gevriesdroogde korrels getoon. Die opbergingsperiode het egter 'n aansienlike impak op die aktiwitietsbehoud van die korrels gehad. Die lewensvatbaarheidstudie het aangetoon dat *Leuconostoc*- en *Lactobacillus*-spesies die oorheersende mikrobes in die verpakte, gevriesdroogde Kepikorrels na 'n opbergingsperiode van twee maande was. Lae mikrobiese tellings vir al drie van die verpakkingsmateriale was gevind op die *Lactococcus*-selekterende medium, en geen mikrobegroei kon op die giste- en propionibakterieë-selekteringsmedium waargeneem word nie. Die beste behoud van die mikrobiese samestelling in die verpakte, gevriesdroogde Kepikorrels was gevind vir die OPET-verpakkingsmateriaal.

Die gevolgtrekking kan gemaak word dat al vier die preserveringstegnieke geskik is vir die preservering van die Kepikorrels en die daaropvolgende opberging van drie maande by kamertemperatuur. Vir opbergingsperiodes van 10 maande en langer word die gebruik van bevriesing en verkoeling aanbeveel as die mees geskikte preserveringstegnieke. Al drie verpakkingsmateriale kan gebruik word vir die verpakking en opberging van gevriesdroogde Kepikorrels vir 'n tydperk van een maand. Indien 'n opbergingsperiode van twee maande of langer verlang word, word die OPET-verpakkingsmateriaal aanbeveel vir die suksesvolle behoud van die fermentasie-aktiwiteit van die Kepikorrels.

dedicated to my parents

"The whole of science is nothing more than a refinement of everyday thinking."

- Albert Einstein -

CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

ACKNOWLEDGEMENTS

This research was done as part of the NRF program on fermented foods. I would like to express my sincere gratitude to the following people and institutions that formed an integral part of this research:

Professor T.J. Britz, my Study Leader and Chairman of the Department of Food Science, University of Stellenbosch, for his expert and continuous guidance, valuable criticism, patience and help throughout the course of my research and preparation of this thesis;

Dr. R.C. Witthuhn, Co-Study Leader and Lecturer at the Department of Food Science, University of Stellenbosch, for her ever-willing advice, patience and help during this study and preparation of this thesis;

The Nampak Group, Research and Development, South Africa, for supplying the packaging material for the preserved Kepi grains and especially Mr. F. Hannay and Mr. C. Linde for the help and advice provided with the packaging of the Kepi grains;

Mr. G.O. Sigge, Mrs. A.E. Maas and Mr. E. Brooks, for technical assistance during the execution of the experimental procedures, and Mrs. M.T. Reeves for her help with all the administrative work;

Ms. M. Cameron, Ms. T. Schoeman and Ms. A. Schoevers for their assistance with the experimental procedures;

Professor T.J. Britz and the National Research Foundation for a NRF grant holders bursary, Stellenbosch 2000 Merit Bursary and Harry Crossely for financial support throughout my post-graduate studies; and

My fellow post-graduate friends, for their help, untiring loyalty and support during my research.

CHAPTER 1

INTRODUCTION

Southern Africa has in recent years been characterised by a major demographic change partially due to the increased urbanisation of the Black African population (Bourne *et al.*, 1994; Mears, 1997) resulting in the formation of many informal low-income settlements (Myburgh, 1995). These communities are characterised by a limited purchasing power, which in some cases is less than R 5 per capita per day (Myburgh, 1996; Dr. A.S. Myburgh, 2000, Department of Agricultural Economics, University of Stellenbosch, personal communication). However, in spite of the urbanisation, the traditional food consumption profile of these low-income communities still resembles that of a traditional rural community.

Traditionally these communities enjoy soured milk products, mostly made from unpasteurised milk. It is, therefore, not surprising that these communities have a preference for unpasteurised (raw) milk that can easily be converted to fermented products like Maas/Amazi (Keller & Jordaan, 1990; Myburgh, 1996). However, unpasteurised milk is not readily obtainable on the South African consumer market due to new legislation that forbids the sale of unpasteurised milk unless it is to be used for further processing and, unfortunately, the production of traditional Maas is not regarded as 'further processing' (Anon., 1997; Vail, 1999). When pasteurised milk is allowed to sour naturally an unacceptable putrid taste and aroma develops. In addition to this, the high cost of commercially available similar products limits the consumption of these nutritious dairy products (Dr. A.S. Myburgh, 2000, Department of Agricultural Economics, University of Stellenbosch, personal communication).

A dietary study (BRISK study) was conducted by Bourne *et al.* in 1994 and they found that the milk intake of the urban Black African population was less than half of the recommended daily portion, presenting a particularly great challenge to the government, nutritionalists and the food industry (Bourne *et al.*, 1994; Myburgh, 1995; Shah, 1993). Another problem the low-income communities are faced with is the occurrence of lactose intolerance amongst almost 95% of them

(De Villiers, 1990). Fermented milks, such as Maas, are easily tolerated by lactose maldigesters, presumably due to the lower lactose content and the presence of ß-galactosidase derived from the bacterial starter culture used in the preparation of the product (Buttriss, 1997; Gurr, 1987).

The unavailability of raw milk for the production of their traditional soured milk products creates the demand in the low-income urban communities for a similar low cost fermented milk product that can easily be prepared with pasteurised milk, is safe and nutritious and has a reasonable shelf-life. In an attempt to enable these communities to produce a fermented dairy product, the use of lactic acid starters was considered (Mr. N. Robinson, 2001, Dairy Laboratory Elsenburg, personal communication), but it was found to be too expensive as a new culture is required for every product batch. In addition, the production and handling of the cultures require a highly technological approach, which is difficult or even impossible to maintain in low-income households (Mrs. E. Straus, 2000, Darleon (Pty.) Ltd., personal communication).

One fermented dairy beverage, Kepi (also known as kefir, kippi, khapov, knapon and hippe), can satisfy this demand. It is made with either pasteurised or unpasteurised milk and the use of an unique starter, the Kepi grains. Kepi has a taste comparable to that of traditional Maas, is nutritious, safe to consume, has an extended shelf-life and a wide range of proven health benefits (Marshall, 1993). This product has: a lower lactose content; the presence of ß-galactosidase that is beneficial to lactose-malabsorbing populations; the presence of partially hydrolysed proteins that leads to improved digestibility; the occurrence of higher levels of B vitamins due to yeast and acetic acid bacteria metabolism; antitumour activity; the inhibition of pathogenic bacteria by the low pH; and the presence of bacteriocins (Marshall, 1993; Özer & Özer, 2000; Van Wyk, 2000). Furthermore, Kepi production is not technologically complicated, nor does it require expensive facilities or a high energy input (Van Wyk, 2000).

The principle advantage provided by Kepi is that the product is made from reusable Kepi grains and thus, this will minimise production costs and simplify processing for the low-income communities. The major technological problem, however, is that Kepi grains are not easily available as their growth under normal conditions is extremely slow and can only develop from pre-existing grains (Saloff-

Coste, 1996). This slow Kepi grain biomass increase presents a problem in the marketing of the grains, as a large mass of grains needs to be available.

The Department of Food Science at the University of Stellenbosch has recently developed a technique of mass-culturing Kepi grains that may be used to produce large volumes of suitable grains. This mass-culturing technique, which can lead to biomass increases of up to 600% over a 10-day period (Schoevers, 1999), has been patented by the University of Stellenbosch (SA Patent 2000/2826). Hence, an adequate supply of Kepi grains is available for the marketing of the grains especially to the low-income communities and as this product has not previously been marketed in South Africa, this will present an excellent opportunity to launch it locally (Van Wyk, 2000). However, the marketing of the Kepi grains calls for the proper preservation and the subsequent packaging of the grains. It is not only important that the preserved grains retain their activity for an extended period, but an appropriate means of packaging to facilitate the easy dispersal of the grains is required.

The objectives of this study were, therefore, firstly to evaluate different preservation techniques based on the retention of a suitable level of grain activity over an extended storage period, and secondly, to find an appropriate packaging material to extend and preserve the storage potential of the grains.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Milk is a highly nutritious beverage, well supplied with carbohydrates, proteins, fats and vitamins (Marshall, 1993). It is a perishable product and preservation by fermentation has long been applied by various cultures, with evidence dating back to 2900 BC (Kurmann, 1984; Roginski, 1988). The lactic acid produced during the fermentation decreases the pH and increases the bacteriostatic characteristics of the beverage, preventing the growth of a wide range of bacteria (Bachmann, 1984; Garrote *et al.*, 2000). These changes contribute to a final fermented milk product with excellent physiological and rheological properties.

Kepi, also known as kefir, hippe, khapov, kephir, kiaphir, kiaphur, kefer, knapon, kippi and kaphir (Kon, 1972; Kwak *et al.,* 1996), has its origin in the south-eastern parts of the Russian Federation and the Caucasus, a mountain range bordered by the Caspian and Black Seas (Jacobs, 1951). For centuries Kepi remained unknown until travellers introduced it to Europe in the eighteenth and nineteenth centuries (Kurmann *et al.*, 1992). This beverage differs from other fermented milk products in that it is not the result of the metabolic activity of a single microbial species (Garrote *et al.*, 1997), but rather the mixed fermentation of lactic and acetic acid bacteria and yeasts (Rea *et al.*, 1996). The combined growth and metabolic activity of these starters result in a fermented product with distinct flavour characteristics.

Kepi is mildly alcoholic and has an effervescent character as a result of the production of carbon dioxide. It is this slight fizziness and low alcohol content that has earned Kepi its nickname - "the champagne of cultured dairy products" (Merin & Rosenthal, 1986). Kepi is further distinguished from other fermented milk products in that at the end of the fermentation process, the microbes can be collected as a solid matrix resembling a cauliflower floret (Rea *et al.*, 1996). These are called Kepi grains and are used repeatedly as starter culture in the production

of fresh Kepi. The grains contain a relatively stable and specific balance of microbes, which exist in a complex symbiotic relationship. The grains grow in the process of Kepi making only from pre-existing grains (Saloff-Coste, 1996). Kepi grains that are allowed to grow in milk, shed microbes from the grains into the milk where they continue to multiply, accompanied by physiochemical changes and the production of acid and flavour (Garrote *et al.*, 1998).

As with many fermented milk products, health benefits are also claimed for Kepi (Marshall, 1993). Fermented milks are low in fat, high in protein and high in nutritional value (Prentice & Neaves, 1986). During the fermentation of the milk the lactose is partially utilised and lactate is produced, which offers advantages to lactose-malabsorbing populations (Buttriss, 1997; Gurr, 1987; Keller & Jordaan, 1990; Roginski, 1988; Shah, 1993). The produced carbon dioxide in Kepi promotes the formation of a fine coagulum, which is more readily digestible than milk (Koroleva, 1988b). Kepi also has a high bacteriostatic activity against extraneous intestinal microbes, due to the presence of acetic acid bacteria and yeasts (Semenichina, 1984). Bacteriocins, produced by certain strains of the lactic acid bacteria, may also be present (Marshall, 1993). Bacteriocins antagonise related bacterial species and the presence of bacteriocin-producing bacteria in the gut may offer protection against pathogenic bacteria. Kepi has elevated levels of vitamins B_1, B_2 and folic acid as a result of the fermentation by yeast species (Alm, 1982; Libudzisz & Piatkiewicz, 1990; Marshall, 1993). The mild acidic taste and characteristic microbial composition of Kepi facilitates salivation, enzyme excretion in the stomach and pancreas and improves peristalsis (Koroleva, 1988b). Antitumour activity in mice has also been observed and is probably initiated by the water-insoluble polysaccharide, kefiran, in the Kepi grain (Marshall, 1993).

Fermented milks were originally developed as a means of preserving the nutrients and extending the shelf-life of the milk (Buttriss, 1997). The fermentation process also increases the wholesomeness and safety of the product (Kon, 1972), which is of considerable importance in developing countries, since it enables the production and storage of well-preserved products, without the need for refrigeration (Bachmann, 1984). In the warmer regions of the world where refrigeration is not always possible and the majority of people are lactose intolerant, the production of Kepi as a fermented milk product holds promising prospects.

B. KEPI MANUFACTURING TECHNOLOGY

History of Kepi manufacturing

The origin of the Kepi grains is still unknown (Koroleva, 1988b), but amongst the people of the northern slopes of the Caucasian Mountains there is a legend that Mohammed gave Kepi grains to the Orthodox people and taught them how to make Kepi (Grassick, 1999; Koroleva, 1988b). The "grains of the prophet" were guarded jealously and it was bequeathed from generation to generation as a source of family and tribal wealth (Grassick, 1999).

Traditionally Kepi was made in goats' or sheep's skin bags, by a continuous natural and uncontrolled fermentation (Duitschaever *et al.,* 1987; Duitschaever, 1989; Mann, 1985). Occasionally clay pots, wooden buckets or oak vats were also used instead of the leather bags (Grassick, 1999; Libudzisz & Piatkiewicz, 1990). Milk of various animals, principally from sheep, goats and cows, was used for the manufacturing of the Kepi beverage (Duitschaever, 1989; Fung, 1992; Jacobs, 1951). In the daytime the leather bags were subjected to sunlight and at night the sacks were brought into the house and hung near the door (Koroleva, 1988b). Everyone who entered or left the house was expected to push the sack with their feet in order to mix the liquid. The finished product was characterised by a high acidity and, depending upon the holding time, by a lower or higher carbon dioxide and alcohol content. The sacks were refilled with fresh milk and the process repeated. Over time this led to the formation of layers of microbes embedded in the protein and polysaccharide material inside the walls of the bags (Duitschaever *et al.*, 1987; Rea *et al.*, 1996).

The people of the northern Caucasus enjoyed this beverage for centuries without sharing the knowledge of Kepi making (Grassick, 1999). For centuries Kepi was unknown outside the Caucasus until news spread of its use for the treatment of tuberculosis and intestinal diseases. The first bottles of Kepi were offered for sale in Moscow in 1908, where it was mainly recommended for medicinal purposes (Grassick, 1999). Commercial manufacturing of Kepi began in the 1930's in the former USSR. The popularity of Kepi has since increased in countries like Poland, Hungary and the former Soviet Union. Currently Kepi is also being manufactured on a commercial scale in most European countries, as well as in Canada and parts of south-east Asia (Grassick, 1999; Kroger, 1993). Kepi is

also well-known in Greece, Israel and Brazil (Halle *et al.*, 1994) and is currently available in the United States and Japan, primarily as an ethnic drink (Saloff-Coste, 1996).

Preparation of the milk substrate

The milk, either full cream, skimmed milk or a mixture, used in the production of Kepi is subjected to microbiological, organoleptic and physical-chemical changes (Koroleva, 1988b; Liu & Moon, 1983). It is important that the milk for the making of Kepi has a total solid content of 8% (w/v) or higher (Marshall, 1993). Pasteurisation is the most important pre-treatment of the milk prior to inoculation. The pasteurisation, therefore, improves the keeping quality of the milk and inactivates pathogenic microbes and certain enzymes, including lipases (Merin & Rosenthal, 1986; Puhan, 1988) that are present in the milk. In addition, the heat treatment facilitates the loss of oxygen from the milk to make it a more suitable environment for the growth of the starter bacteria, as many lactic acid bacteria are micro-aerophilic and, therefore, grow and metabolise best under conditions of reduced oxygen tension (Robinson, 1995). The heat treatment of the milk used for the fermentation requires higher temperatures and longer holding times than for ordinary milk pasteurisation. This results in the denaturation of the whey proteins (Mann, 1979; Marshall, 1993; Puhan, 1988; Varnam & Sutherland, 1994) and contributes to the formation of a good coagulum, and an improved consistency and viscosity of the Kepi (Puhan, 1988).

Various pasteurisation temperature and time schedules have been recommended for the production of quality Kepi and heat treatments vary according to the particular dairy (Marshall, 1993). In studies conducted by Bondarev (1977) on the effects of heat treatment on the quality of Kepi, four different heat treatments of Kepi milk were compared. These treatments were: 85° - 87°C for 5 - 10 min; 92° - 95°C for 20 - 30 min; 110°C in an autoclave and a double pasteurisation involving a first treatment at 72° - 76°C in a plate pasteuriser, followed by a treatment at 85° - 87°C for 20 min in a tubular pasteuriser. Heating of Kepi milk at 92° - 95°C for 20 - 30 min was considered the optimal pasteurisation temperature-time combination. Berzhinskas *et al.* (1978) reported that the consistency of the Kepi made with the double-pasteurised milk was considerably improved, but was an uneconomical process. This treatment

increased the denaturation of the whey proteins, improved the dispersion of the casein micelles, reduced whey syneresis and increased the viscosity, elasticity and firmness of the curd. A level of whey protein denaturation of 80 to 85% or slightly more by the heat treatment is considered desirable (Puhan, 1988). This destruction of the proteins corresponds to a heat treatment of 95°C for 5 min or 80° - 85°C for 20 - 30 min. In order to inoculate the Kepi milk with a starter culture, the milk must first be cooled. The recommended chilled temperature ranges between 20° (Mann, 1979) and 25°C (Puhan, 1988; Saloff-Coste, 1996).

It is recommended that the milk used for the production of Kepi is homogenised (Mann, 1989; Mann, 1985; Marshall, 1993; Rašić, 1987; Varnam & Sutherland, 1994) as this decreases the size of the fat globules (Puhan, 1988). It further eliminates the creaming of the milk during incubation and it ensures a uniform distribution of the fat. Like pasteurisation, homogenisation also improves the consistency and viscosity of the product, while little or no whey separation occurs (Semenichina, 1984). Products made from homogenised milk appear whiter, have a more creamy taste, and are fuller and milder than those made from milk that was not homogenised. Homogenisation prior to pasteurisation breaks the bacterial clumps in the milk, which are then easily destroyed during the subsequent heating (Puhan, 1988).

Preparation of the starter culture

Most other fermented milk products can readily be made from the lactic acid bacteria present in the product. Kepi, however, can only be made from the Kepi grains and mother cultures prepared from the grains (Saloff-Coste, 1996). Kepi from which the grains have been removed is not recommended for use as a starter in order to obtain an acceptable product, since the original balance of the microbes has been disrupted (Garrote *et al.*, 1998; Kroger, 1993).

The Kepi culture, consisting of fresh Kepi grains, can be obtained from specialist starter companies as a suspension in sterile 0.9% (w/v) sodium chloride or as freeze-dried grains (Libudzsiz & Piatkiewicz, 1990; Marshall, 1993). The freeze-dried grains are standardised by the addition of yeast isolates, because the freezing and freeze-drying of the grains can result in the loss of more than 80% of the yeast content (Marshall, 1993). The mother culture contains approximately 80% lactococci, 5% lactobacilli and 5% yeasts (Marshall, 1993). Fresh grains are

added at a level of 10% (w/v) to cooled milk, although lower ratios (4 - 5%) of the Kepi grains can also be used (Kuo & Lin, 1999; Marshall, 1993). The grain cultivation is carried out at 20°C for 24 h, after which the grains are collected by sieving or filtering the fermented milk through cheesecloth. The collected grains are then washed with sterile water before being added to fresh milk. The requirements for the preparation of Kepi with a satisfactory composition include the fact that the milk must be renewed daily at the same time and that the ratio of the grains to milk should be kept in the range of 1:30 to 1:50 (Koroleva, 1988a; Özer & Özer, 2000). The milk used for the cultivation must be pasteurised at 95°C for 10 - 15 min and two to three agitations in the course of the cultivation are necessary (Koroleva, 1988a). In the case of lyophilised cultures, 1 g of the freeze-dried culture is added to 3 litres of heat-treated milk and incubated at 20°C for 20 h (Marshall, 1993). The reactivation of the lyophilised cultures requires at least three subculturings, with a higher incubation temperature and a larger inoculum than for grain and liquid starters (Kosikowski, 1982; Vedamuthu, 1982).

Production

Traditional methods

The existing production procedures for Kepi were developed by the industrialisation of traditional methods and the development of new starter cultures (Marshall, 1993). The traditional method of Kepi making is currently achieved by adding Kepi grains directly as a starter to milk that has been heat-treated, usually at 90°C for 30 min, and cooled to 20° - 25°C (Garrote *et al.*, 1998; Özer & Özer, 2000; Saloff-Coste, 1996). After inoculation the milk is gently mixed to facilitate proper distribution of the starter. The traditional manufacturing of Kepi involves two stages, namely a primary fermentation, where the Kepi grains are inoculated into the milk, followed by a ripening process (Marshall, 1993). Two different sets of temperature-time combinations are used in order to compensate for the different incubation requirements of the fermentation and the ripening stages. Incubation, the primary fermentation, is carried out at 19° - 23°C for 12 - 14 h, followed by a ripening period of 12 h at 8° - 10°C (Marshall, 1993; Roginski, 1988). Both these phases in the production of Kepi involve selected microbial activity, where lactic acid, alcohol and aroma are produced. After this fermentation of 24 h, the grains are removed by straining and the filtrate is refrigerated overnight before the

beverage is ready for consumption (Özer & Özer, 2000; Saloff-Coste, 1996). The quantity of the grains used for the fermentation vary between 0.55 - 10% (w/v) (Roginski, 1988; Saloff-Coste, 1996).

The traditional method of Kepi production can be altered for the industrialised production in that a grain-derived culture is used as a starter instead of the original grains. It involves a two-step fermentation, known as the Russian method, which is applied to stimulate the activity of the microbes and accelerate the changes in the milk (Özer & Özer, 2000; Saloff-Coste, 1996). In the first step, the Kepi culture is prepared by incubating the milk with 2 - 3% (w/v) grains to prepare a mother culture (Saloff-Coste, 1996). After fermentation (24 h at 20° - 25°C), the grains are removed and rinsed in cold water for re-use (Varnam & Sutherland; 1994). The resulting mother culture is added to fresh heat-treated milk (1 - 3% (v/v)), which is then fermented for another 12 - 18 h at 20° - 25°C (Halle *et al.*, 1994; Zourari & Anifantakis, 1988). The Kepi is kept for several hours in order to stabilise the coagulum before the beverage is ready for consumption (Varnam & Sutherland, 1994). In Fig. 1, the manufacturing of the above-mentioned starter culture (starter I) is given (Koroleva, 1988a). If a large quantity of milk is required, an intermediate or bulk starter (starter II, Fig. 1) is prepared from starter I (Bavina, 1971; Bavina & Rozhkova, 1973; Duitschaever, 1989). This method is also used in cases where the lack of equipment for separating Kepi grains from the starter impedes the recovery of the grains (Bavina, 1971). The milk is pasteurised at 95°C for 30 min, while it is continuously agitated. Inoculation of starter I (1 – 3% (v/v)) follows, and the milk is incubated for approximately 12 h. During the course of a slow cooling to 8°C over 12 - 24 h, the starter ripens (Fig. 1).

Kepi starters prepared from Kepi grains, as in the case with starter I and starter II, possess properties important from an industrial point of view, because it provides optimal conditions for the symbiotic growth of the microbes (Koroleva, 1988a). The starter is not affected by any seasonal fluctuations in the milk quality or by the presence of antibiotics or other inhibitors in the milk. Finally, the starter is also resistant to bacteriophages (Koroleva, 1982).

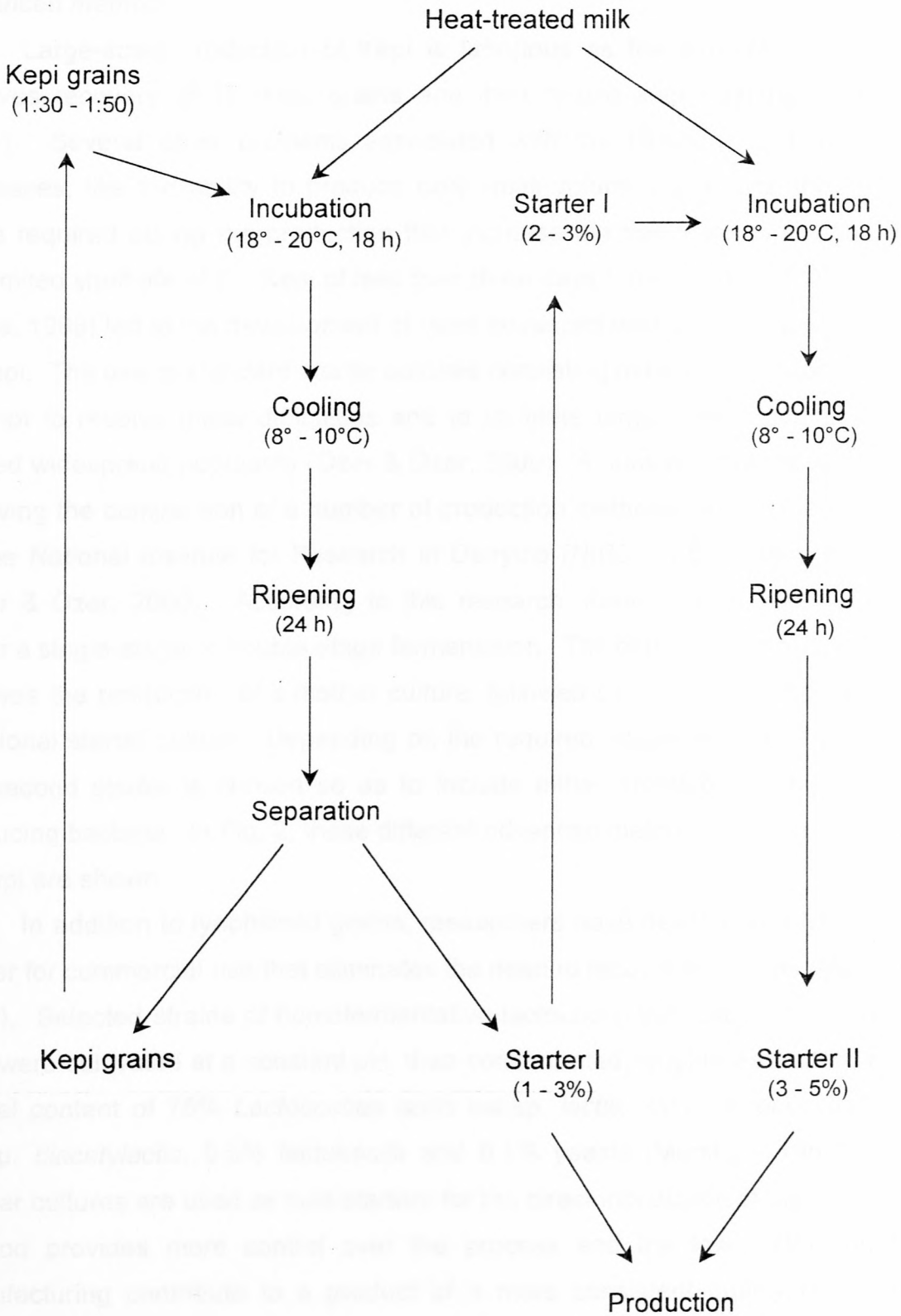

Figure 1. Preparation of Kepi starters for the use in larger scale production (Koroleva, 1988a).

Advanced methods

Large-scale production of Kepi is laborious as the fermentation of Kepi involves recovery of the Kepi grains and their re-use after washing (Marshall, 1987). Several other problems associated with the traditional manufacturing processes, like the ability to produce only small volumes at a time, the several steps required during the production that increase the risk of contamination and the limited shelf-life of the Kepi of less than three days (Özer & Özer, 2000; Saloff-Coste, 1996) led to the development of more advanced methods for the production of Kepi. The use of standard starter cultures consisting mainly of lactococci, as an attempt to resolve these difficulties and to facilitate large-scale production, has gained widespread popularity (Özer & Özer, 2000). A number of intensive studies involving the comparison of a number of production methods has been conducted by the National Institute for Research in Dairying (NIRD) in the United Kingdom (Özer & Özer, 2000). According to this research, Kepi production can involve either a single-stage or double-stage fermentation. The double-stage fermentation involves the production of a mother culture, followed by the fermentation with an additional starter culture. Depending on the required characteristics of the Kepi, the second starter is chosen so as to include either aroma-producing or acid-producing bacteria. In Fig. 2, these different advanced methods for the production of Kepi are shown.

In addition to lyophilised grains, researchers have developed a lyophilised starter for commercial use that eliminates the need to recover the grains (Marshall, 1987). Selected strains of homofermentative lactococci, lactobacilli and *Candida kefir* were cultivated at a constant pH, then concentrated, lyophilised and mixed to a final content of 75% *Lactococcus lactis* subsp. *lactis,* 24% *Lactococcus lactis* subsp. *diacetylactis*, 0.5% lactobacilli and 0.1% yeasts (Marshall, 1987). The mother cultures are used as bulk starters for the direct inoculation of the milk. The method provides more control over the process and the fewer steps in the manufacturing contribute to a product of a more consistent quality (Zourari & Anifantakis, 1988). Using this starter, the final Kepi beverage was judged to have a better aroma and flavour, due to higher levels of diacetyl and a less prominent yeasty flavour. However, the lower numbers of yeasts resulted in a product with less ethanol and less carbon dioxide (Marshall, 1987).

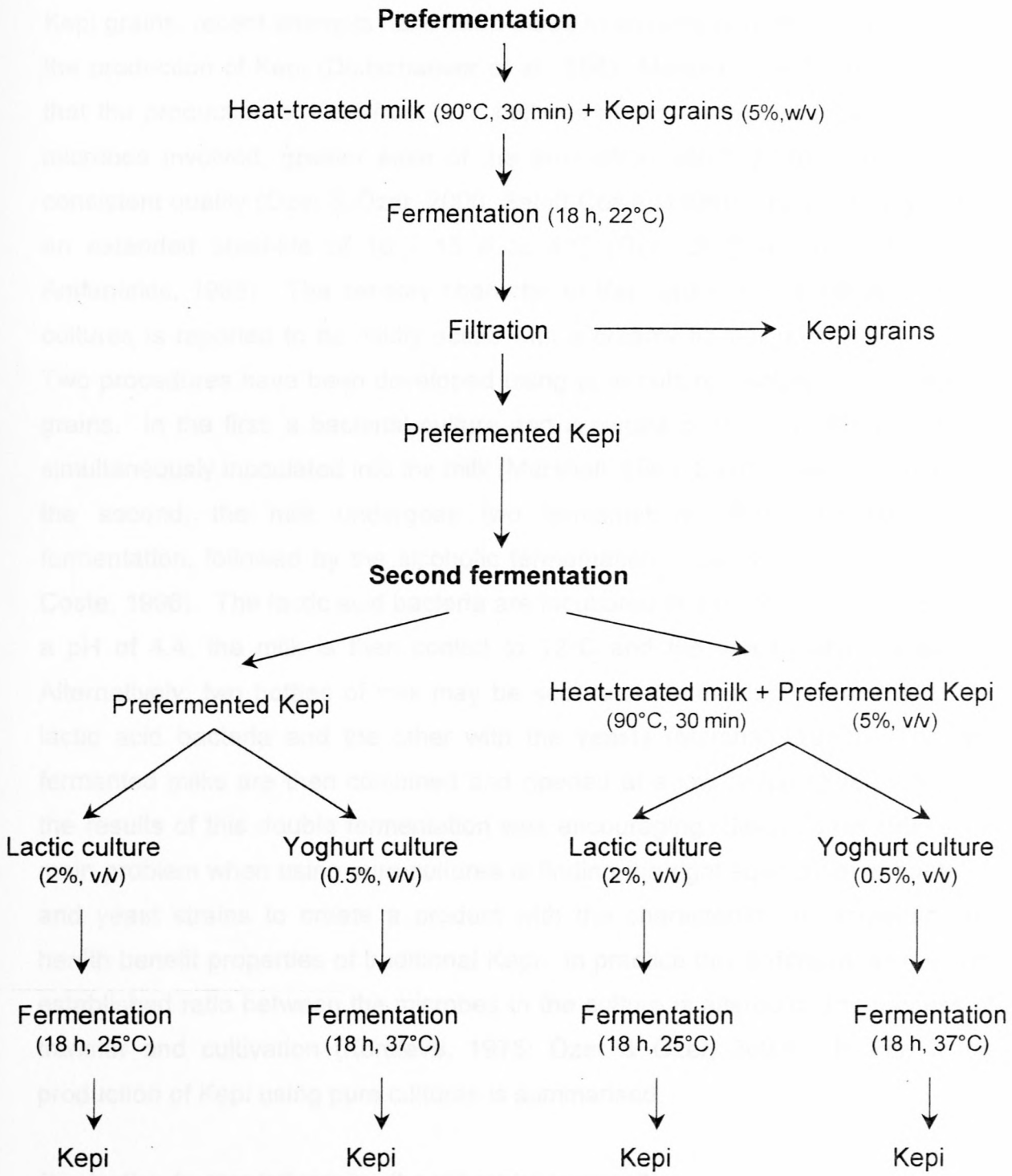

Figure 2. Advanced Kepi production methods (Özer & Özer, 2000).

Pure cultures

Due to the extremely variable character of the microbial composition of the Kepi grains, recent attempts have been made to develop pure defined cultures for the production of Kepi (Duitschaever *et al.*, 1987; Marshall, 1993). It is believed that the production of Kepi from pure cultures allows for the better control of the microbes involved, greater ease of the production and a product with a more consistent quality (Özer & Özer, 2000; Saloff-Coste, 1996). The product also has an extended shelf-life of 10 - 15 d at 4°C (Özer & Özer, 2000; Zourari & Anifantakis, 1988). The sensory character of Kepi produced by the use of pure cultures is reported to be mildly acidic with a creamy flavour (Muir *et al.*, 1999). Two procedures have been developed using pure cultures isolated from the Kepi grains. In the first, a bacterial culture and a culture containing Kepi yeasts are simultaneously inoculated into the milk (Marshall, 1993; Saloff-Coste, 1996) and in the second, the milk undergoes two fermentations, firstly the lactic acid fermentation, followed by the alcoholic fermentation (Özer & Özer, 2000; Saloff-Coste, 1996). The lactic acid bacteria are incubated at 24° - 27°C for 18 - 20 h to a pH of 4.4, the milk is then cooled to 12°C and the yeast culture is added. Alternatively, two bottles of milk may be separately fermented, the one with the lactic acid bacteria and the other with the yeasts (Marshall, 1993). The two fermented milks are then combined and ripened at a low temperature. Although the results of this double fermentation was encouraging (Saloff-Coste 1996), the main problem when using pure cultures is finding the right equilibrium of bacterial and yeast strains to create a product with the characteristic organoleptic and health benefit properties of traditional Kepi. In practice this is difficult, as the pre-established ratio between the microbes in the culture is altered by the process of transfer and cultivation (Koroleva, 1975; Özer & Özer, 2000). In Fig. 3 the production of Kepi using pure cultures is summarised.

Production factors influencing the microbial composition

The proportions of microbes in Kepi grains and starters are influenced by numerous factors during the production of the beverage (Rašić, 1987). The optimal inoculation ratio is in the range of 1:30 - 1:50 (culture:milk) (Koroleva, 1988a; Özer & Özer, 2000). High inoculation ratios of Kepi grains (1:10 - 1:20) shorten the fermentation process, but the growth rates of the yeasts and the

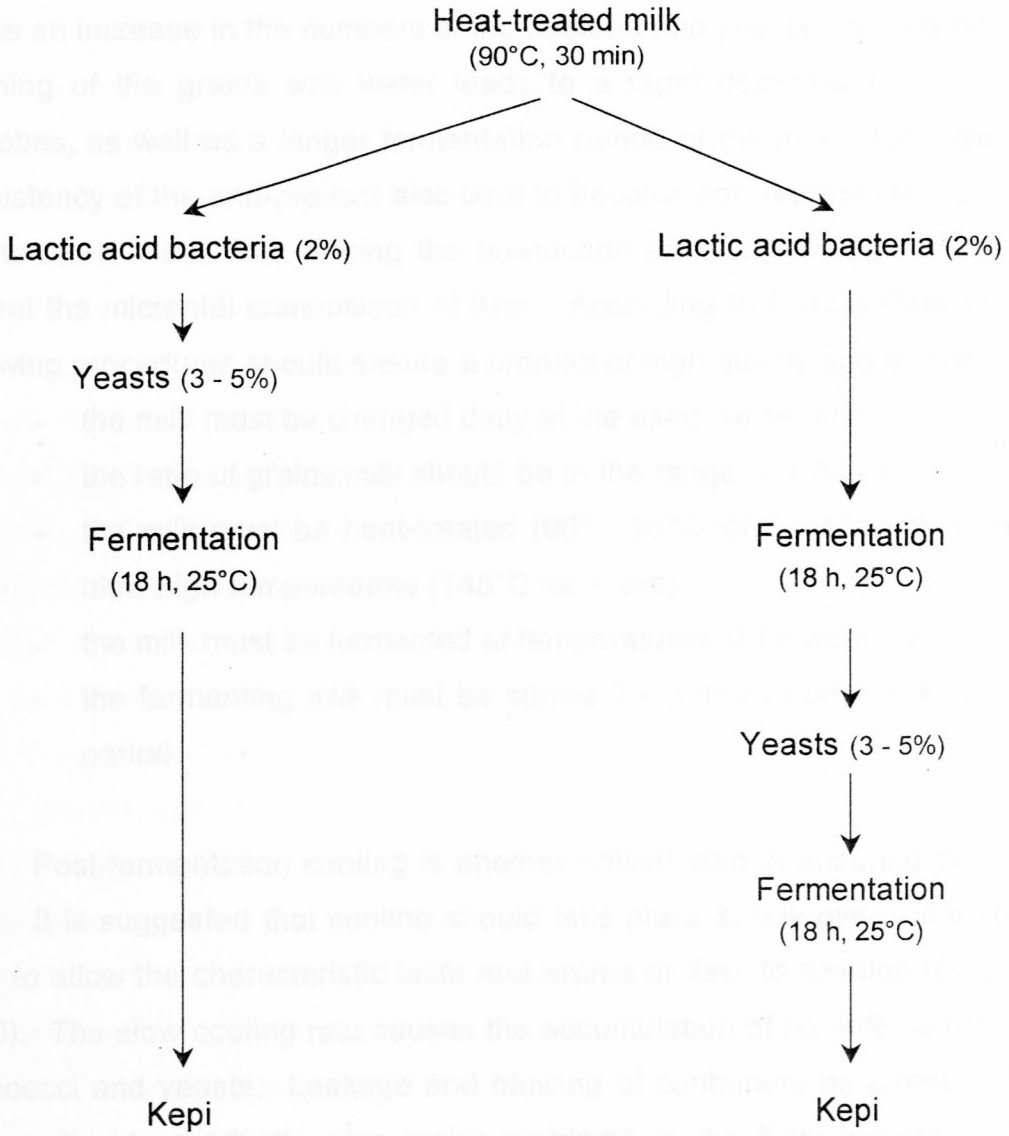

Figure 3. Kepi production using pure cultures (Özer & Özer, 2000).

homo- and heterofermentative lactococci decrease compared with the growth at lower inoculation ratios (e.g. 1:50). Agitation also affects the balance of the microbes in the Kepi grains. Frequent agitation during the fermentation may cause an increase in the numbers of the bacteria and yeasts. In contrast, frequent washing of the grains with water leads to a rapid decrease in the number of microbes, as well as a longer fermentation period of the milk. The taste and the consistency of the end-product also tend to become non-representative of Kepi. It is, therefore, clear that altering the production and fermentation conditions can control the microbial composition of Kepi. According to Özer & Özer (2000), the following procedures should ensure a product of high quality and standardisation:

- the milk must be changed daily at the exact same time;
- the ratio of grains:milk should be in the range of 1:30 - 1:50;
- the milk must be heat-treated (90° - 95°C for 5 - 15 min) or treated at ultra-high temperatures (145°C for 4 sec);
- the milk must be fermented at temperatures of between 18° - 20°C; and
- the fermenting milk must be stirred 2 - 3 times during the fermentation period.

Post-fermentation cooling is another critical step in ensuring the quality of Kepi. It is suggested that cooling should take place slowly over a period of 10 – 12 h to allow the characteristic taste and aroma of Kepi to develop (Özer & Özer, 2000). The slow cooling rate causes the accumulation of homofermentative lactic lactococci and yeasts. Leakage and blowing of containers as a result of excess carbon dioxide production are major problems in the Kepi industry. To avoid these, it is recommended that non-lactose-fermenting yeasts should be used, in which case sucrose supplementation is recommended (Kwak *et al.*, 1996; Özer & Özer, 2000)

Properties of the final product

The composition, flavour, viscosity and effervescence of Kepi varies significantly, based on the source and fat content of the milk used, the composition of the grains or starters, the size of the inoculum, and the technological conditions of the production (Duitschaever *et al.*, 1988; Saloff-Coste, 1996; Steinkraus, 1996). The final Kepi beverage is characterised by a compact and uniform curd,

with a creamy consistency and a discernible effervescence (Marshall, 1993; Oberman & Libudzisz, 1998). The final product should have a refreshing acidity, with a mildly alcoholic, buttery and yeasty aroma (Liu & Moon, 1983; Marshall, 1993). The flavour compounds include: lactic (0.8 - 0.9% (w/w)), formic, succinic, acetic and propionic acids; ethyl alcohol (0.5 - 2%); and different aldehydes, including acetaldehyde and trace amounts of iso-amyl alcohol, acetoin and acetone (Guzel-Seydim *et al.*, 2000; Marshall, 1993; Oberman & Libudzisz, 1998; Steinkraus, 1996). Diacetyl, the main aroma-forming substance, is present in amounts of 1 $mg.l^{-1}$ (Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Oberman & Libudzisz, 1998). The effervescent character is due to the presence of carbon dioxide (0.08 - 0.2%) (Marshall, 1993; Oberman & Libudzisz, 1998), mainly as a result of the yeast metabolism (Marshall, 1993). About 7% of the nitrogen present in Kepi is in the form of peptones and 2% is in the form of amino acids, as a result of proteolysis (Libudzisz & Piatkiewicz, 1990; Oberman & Libudzisz, 1998). Kepi also has reduced lactose content and an increased level of ß-galactosidase as a result of the fermentation (Saloff-Coste, 1996).

The pH of the final Kepi usually ranges between 4.2 and 4.6 (Marshall, 1982; Saloff-Coste, 1996), but may have a pH below 3 (Steinkraus, 1996). The acidity of Kepi can differ, based on whether the Kepi is prepared in large volumes using traditional or industrial methods or by the use of lyophilised starters and separate fermentation steps. When using the industrial method for production of Kepi, the product may be expected to have a titratable acidity of 1% with a pH below 4.0, whereas the traditionally prepared Kepi is milder in taste and has a pH of above 4.4. In addition, the alcohol and carbon dioxide levels in Kepi produced by modern manufacturing methods are comparatively low, but can easily be increased by changing the composition of the starter, temperature, duration of the fermentation and the package sealing method (Koroleva, 1982; Koroleva, 1988b; Steinkraus, 1996).

Several attempts have been made to nutritionally enrich Kepi. Kepi that had been enriched with 0.6 - 0.7% (w/v) sodium caseinate was found to have an increased protein content and viscosity, and a reduced whey syneresis (Mann, 1979; Mann, 1983). Kepi made from milk, fortified with calcium ions, had an increased bacterial count, a higher titratable acidity and a greatly reduced fermentation period. In addition, the enriched Kepi had a more pronounced,

specific, sharper flavour than the control Kepi (Mann, 1983). Procedures exist whereby sucrose is added to the milk to encourage the yeast fermentation (Marshall, 1993). This is necessary for starters that contain non-lactose-fermenting yeasts, such as *Saccharomyces cerevisiae* and *Pichia fermentans* (Kwak *et al.*, 1996; Lin *et al.*, 1999). Attempts have also been made to enrich Kepi milk with the B-vitamins (Mann, 1979). The ability of propionic acid bacteria to synthesise vitamin B_{12} has for several years been used in research for the enrichment of fermented milk beverages (Cerná & Hrabová, 1977). *Propionibacterium shermanii,* the most widely applied species for the vitamin B_{12} enrichment of Kepi utilises lactose and has no adverse effect on the organoleptic properties of Kepi (Cerná & Hrabová, 1977). Evidence of a sixty-fold enrichment of vitamin B_{12} in Kepi was obtained with this method. Successful vitamin B_{12} enrichment of Kepi has also been obtained by using a culture of *Propionibacterium pettersonni* in combination with Kepi grains (Mann, 1979).

A high quality Kepi beverage has a microbial composition of 10^7 - 10^8 colony forming units (cfu) thermophilic lactobacilli, 10^9 homofermentative mesophilic lactic acid lactococci, 10^4 - 10^5 yeasts and 10^4 - 10^5 acetic acid bacteria per ml of the beverage (Koroleva, 1988b; Rea *et al.*, 1996). The presence of these microbes, especially the thermophilic lactobacilli, yeasts and acetic acid bacteria, actively inhibit the growth of coliforms and pathogenic bacteria like *Shigella*, *Salmonella* and *Escherichia coli* in the beverage (Garrote *et al.*, 2000; Koroleva, 1988a; Koroleva, 1988b; Libudzisz & Piatkiewicz, 1990; Saloff-Coste, 1996). In addition to this, the Kepi beverage is inhibitory against microbes such as *Staphylococcus aureus* (Brialy *et al.*, 1995).

C. THE KEPI GRAIN

Properties

The Kepi grains are the focal point of Kepi technology (Roginski, 1988). The mass of the Kepi grain is made up of microbial cells grouped in a highly organised manner, products of their autolysis, and a curd consisting of milk proteins and carbohydrates (Oberman & Libudzisz, 1998; Özer & Özer, 2000). Kepi grains are characterised by an irregular form and an uneven surface (Oberman & Libudzisz, 1998) and have a structure similar to tiny cauliflower

florets, which vary in size from 0.3 to 3.5 cm in diameter (Garrote *et al.*, 1997). The grains are moist, gelatinous (Roginski, 1988; Vedamuthu, 1982), slimy, extremely resilient (Steinkraus, 1996), and have a white, slightly yellow or cream colour (Koroleva, 1988a; Liu & Moon, 1983; Roginski, 1988; Vedamuthu, 1982). They are insoluble in water and common solvents, but when added to milk they swell, turn white (Liu & Moon, 1983; Vedamuthu, 1982), and initiate dual lactic acid and yeast fermentation (Mann, 1979; Özer & Özer, 2000). Active Kepi grains float on the milk surface (Koroleva, 1988a; Roginski, 1988). The grains start out as very small granules that gradually grow in size. A mass of 500 g wet weight Kepi grains can double in weight in 7 to 10 days, if the grains are daily transferred to 500 ml fresh milk (La Rivière *et al.*, 1967). The Kepi grains consist of 89 - 90% water, 6% carbohydrates, 3% protein, 0.7% ash and 0.2% lipids (Garrote *et al.*, 1997; Mann, 1985).

The microbes present in the Kepi grains include lactic acid bacteria (lactobacilli, lactococci, leuconostocs and *Streptococcus thermophilus*) at a concentration of 10^8 - 10^9 cells.g^{-1}, lactose fermenting and non-lactose fermenting yeasts at approximately 10^8 cells.g^{-1}, and acetic acid bacteria at 10^8 cells.g^{-1} (Kurmann *et al.*, 1992; Liu & Moon, 1983; Oberman & Libudzisz, 1998; Özer & Özer, 2000; Saloff-Coste, 1996; Tamime *et al.*, 1999). Mycelial fungal species such as *Geotrichum candidum* has also been identified as part of the Kepi microbes (Garrote *et al.*, 1997; Pintado *et al.*, 1996; Tamime *et al.*, 1999). In Table 1 the microbes that have most frequently been isolated from Kepi grains are listed. Generally, lactobacilli (homo- and heterofermentative, meso- or thermophilic) constitute about 65 - 80% of the microbial content (Oberman & Libudzisz, 1998). The remaining 20% consist of acid and aroma forming lactococci and different lactose fermenting and non-lactose fermenting yeasts (about 5%) (Oberman & Libudzisz, 1998).

The microbes in the grains are embedded in a matrix of fibrillar material composed largely of a resilient polysaccharide matrix called kefiran (Angulo *et al.*, 1993; Duitschaever, 1989; Saloff-Coste, 1996). This insoluble polysaccharide is composed of equal amounts of glucose and galactose and is a result of the microbial metabolism (Angulo *et al.*, 1993; Marshall, 1993; Özer & Özer, 2000). This slimy, but extremely resilient embedding material is of great survival value for the microbes (Liu & Moon, 1983). Kefiran is produced by several

Table 1. The compositional microbes of the Kepi grain.

Lactic Acid Bacteria	References
Lactobacillus	
Lactobacillus kefir *	Oberman & Libudzisz, 1998
L. casei ssp. *alactosus*	Oberman & Libudzisz, 1998
L. casei ssp. *rhamnosus*	Oberman & Libudzisz, 1998
L. casei ssp. *tolerans*	Angulo *et al.*, 1993
L. casei ssp. *pseudoplantarum*	Angulo *et al.*, 1993
L. delbreukii ssp. *bulgaricus*	Marshall, 1993; Keller & Jordaan, 1990
L. delbreukii ssp. *lactis*	Oberman & Libudzisz, 1998
L. acidophilis	Marshall, 1984; Oberman & Libudzisz, 1998
L. helviticus ssp. *jugurti*	Kwak *et al.*, 1996
L. kefiranofaciens	Marshall, 1993; Saloff-Coste, 1996
L. plantarum	Kwak *et al.*, 1996
L. paracasei ssp. *paracasei*	Saloff-Coste, 1996
L. gasseri	Angulo *et al.*, 1993; Garrote *et al.*, 1997
L. cellobiosus	Marshall, 1993; Oberman & Libudzisz, 1998
L. lactis	Marshall, 1993
L. viridescens	Angulo *et al.*, 1993
L. fermentum	Angulo *et al.*, 1993; Garrote *et al.*, 1997
L. kefirgranum	Takizawa *et al.*, 1994
L. parakefir	Takizawa *et al.*, 1994
Lactococcus/Streptococcus	
Lactococcus lactis ssp. *lactis*	Oberman & Libudzisz, 1998
L. lactis ssp. *lactis var. diacetylactis*	Oberman & Libudzisz, 1998
L. lactis ssp. *cremoris*	Oberman & Libudzisz, 1998
Streptococcus salivarius ssp. *thermophilis*	Angulo *et al.*, 1993; Kwak *et al.*, 1996
S. filant	Kwak *et al.*, 1996; Oberman & Libudzisz, 1998
S. durans	Oberman & Libudzisz, 1998
Leuconostoc mesenteroides ssp. *mesenteroides*	Saloff-Coste, 1996
L. mesenteroides ssp. *dextranicum*	Oberman & Libudzisz, 1998
L. mesenteroides cremoris	Oberman & Libudzisz, 1998
L. lactis	Saloff-Coste, 1996
L. kefir	Kwak *et al.*, 1996
Enterococcus durans	Angulo *et al.*, 1993

* formerly known as *Lactobacillus brevis*

Table 1. (continued)

Yeasts	References
Kluyveromyces lactis	Oberman & Libudzisz, 1998
K. marxianus ssp. *bulgaricus*	Oberman & Libudzisz, 1998
K. marxianus ssp. *marxianus*	Oberman & Libudzisz, 1998
K. fracilis	Kwak *et al.*, 1996
Saccharomyces floretinus	Oberman & Libudzisz, 1998
S. globosus	Oberman & Libudzisz, 1998
S. unisporis	Oberman & Libudzisz, 1998
S. carlsbergensis	Oberman & Libudzisz, 1998
S. cerevisiae	Saloff-Coste, 1996; Steinkraus, 1996
S. lipolytic	Garrote *et al.*, 1997
Candida pseudotropicalis	Oberman & Libudzisz, 1998
C. kefir	Oberman & Libudzisz, 1998
C. friedrichii	Angulo *et al.*, 1993
C. holmii	Angulo *et al.*, 1993
C. valida	Mann, 1979
Torulaspora delbreukii	Oberman & Libudzisz, 1998
T. holmii	Brialy *et al.*, 1995; Steinkraus, 1996
Pichia fermentans	Angulo *et al.*, 1993; Lin *et al.*, 1999
Bretlanomyces anomalus	Mann, 1979
Acetic Acid Bacteria	
Acetobacter aceti	Marshall, 1993; Tamime *et al.*, 1999
*A. pasteurianus***	Marshall, 1993; Tamime *et al.*, 1999
Moulds (mycelial fungi)	
Geotrichium candidum	Pintado *et al.*, 1996; Tamime *et al.*, 1999

** formerly known as *Acetobacter ransens*

homofermentative lactobacilli, including *Lactobacillus kefiranofaciens* and *Lactobacillus kefir,* which form an integral part of the grain (Fujisawa *et al.*, 1988; La Rivière *et al.*, 1967; Marshall *et al.*, 1984b; Rea *et al.*, 1996; Saloff-Coste, 1996; Toba *et al.*, 1987; Yokoi *et al.*, 1990). In the absence of the kefiran producing microbes, Kepi grains are not propagated, although non-propagable grains still retain their Kepi-producing capacity (Rea *et al.*, 1996; Saloff-Coste, 1996; Toba *et al.*, 1990).

The grains appear to arise from the curling of flat sheet-like structures, with a subsequent folding and refolding into a globular structure (Marshall, 1993). Scanning electron microscopy photographs of the Kepi grains have shown a more particulate and grainy material on the exterior of the grain and a more fibrillar material on the interior (Rea *et al.*, 1996). The grainy material is due to the presence of particles of clotted milk (Marshall, 1982; Rašić, 1987). The structure is more even at the edges of the grain, making it easier for the microbes to be transferred into the surrounding milk (Mann, 1985).

Marshall *et al.* (1984a) showed a distinction between sections of the grain and provided evidence of the asymmetric arrangement of the microbes in the Kepi grain. The non-lactose fermenting yeasts are found in the deep layers of the Kepi grains, while lactose fermenting yeasts are present mainly in the peripheral layers (Bottazzi & Bianchi, 1980; Koroleva, 1988a; Lin *et al.*, 1999; Mann, 1989; Oberman & Libudzisz, 1998). The surface microbes of the Kepi grains consist of mesophilic lactococci, mesophilic and thermophilic lactobacilli, and acetic acid bacteria (Bottazzi & Bianchi, 1980; Garrote *et al.*, 1997; Koroleva, 1988a). Yeasts and short lactobacilli are predominantly found on the convoluted sides of the sheet-like structures of the matrix and short lactobacilli are almost exclusively in the smooth side with long curved lactobacilli in the zone between these two populations (Duitschaever *et al.*, 1988; Neve, 1992; Rea *et al.*, 1996; Toba *et al.*, 1990). In addition, it was found that kefiran producing lactobacilli are embedded in the centre of the sheet-like structures, with the non-kefiran producing lactobacilli and yeasts each predominating on separate sides of the sheets (Marshall, 1984; Marshall *et al.*, 1984a; Toba *et al.*, 1990; Varnam & Sutherland, 1994). The kefiran producing microbe, *Lactobacillus kefiranofaciens,* is found in the centre of the grains where its growth is favoured by the anaerobic conditions and the presence of ethanol (Varnam & Sutherland, 1994). *Lactobacillus kefir*, the most

common lactobacilli in Kepi, is present only in small numbers at the surface of the grain (Marshall, 1984; Varnam & Sutherland, 1994). Despite the fact that large numbers of lactococci are present in Kepi, their presence in the grains are limited (Neve, 1992; Rea *et al.*, 1996). This may indicate that they are only lightly bound to the Kepi grain and are easily washed off. This confirms that Kepi grains should be cultured without excessive washings due to the surface distribution of the microbes (Liu & Moon, 1983). The small number of lactococci inside the Kepi grain can also be contributed to the very low pH in this part of the grain (Rea *et al.*, 1996).

The Kepi grain, with its complex microbial composition, functions as a single organism (Oberman & Libudzisz, 1998; Rašić, 1987). Very little is known about the mechanisms of grain formation and, therefore, Kepi grains can not be precisely reconstructed (Marshall, 1984; Özer & Özer, 2000; Steinkraus, 1996). In spite of many efforts, there has been no success in producing new grains by separately growing the mixed cultures of the Kepi microbes and then recombining them (Marshall, 1993; Oberman & Libudzisz, 1998; Rea *et al.*, 1996).

Metabolic activity of the compositional microbes

Fermented milks share the common taste of sourness or tartness, but the different milk products have unique flavour and aroma characteristics that distinguish them from each other (Marshall, 1987). The volatiles acetaldehyde and diacetyl are, respectively, the major aroma compounds of buttermilk and yoghurt, whilst Kepi contains both and is only slightly carbonated. These flavour components are the end-products of the bacterial metabolism (Marshall, 1987). They are usually the consequence of the reduction of pyruvate, thereby regenerating pyridine nucleotides for the lactose catabolic pathways (Marshall, 1987). During fermentation, the lactose in the milk is utilised by homo- and heterofermentative lactic acid bacteria, via the glycolytic (Embden-Meyerhof pathway) and D-tagatose-6-pathways, and by the Leloir and phosphoketolase pathways (Litopoulou-Tzanetaki & Tzanetakis, 2000). In addition, lactose is also utilised by the lactose-fermenting yeasts present in the grains. Citrate is also utilised, particularly by the aroma-producing microbes, which include the *Leuconostoc* species (Guzel-Seydim *et al.*, 2000; Litopoulou-Tzanetaki & Tzanetakis, 2000). The most important volatile components formed in Kepi

include acetaldehyde, propionaldehyde, acetone, ethanol, 2-butanone, n-propyl alcohol, diacetyl and amyl alcohol (Litopoulou-Tzanetaki & Tzanetakis, 2000). Some vitamins are synthesised by both the lactic acid bacteria and the yeasts (Kneifel & Mayer, 1991; Litopoulou-Tzanetaki & Tzanetakis, 2000). The levels of some vitamins increase during the fermentation process, while others are utilised by the microbes, e.g. ortoric acid is lost during the fermentation, while the B vitamins and the riboflavenoid group vitamins accumulate in Kepi (Alm, 1982; Kneifel & Mayer, 1991; Litopoulou-Tzanetaki & Tzanetakis, 2000). *Propionibacterium shermanii* produces propionic acid (Kosikowski, 1982) and is the most widely applied strain for the vitamin B_{12} enrichment of Kepi (Cerná & Hrabová, 1977). This implies that the presence of these microbes, as with the acetic acid bacteria, can stimulate the growth of other microbes present in the Kepi by supplying certain growth factors to the microbes (Semenichina, 1984). The last step during the maturation is the proteolytic activities exhibited by the yeasts and the acetic acid bacteria, resulting in the development of a yeasty aroma (Litopoulou-Tzanetaki & Tzanetakis, 2000). In Fig. 4 the major homo- and heterolactate fermentation pathways of the lactic acid bacteria is illustrated and in Fig. 5 the diacetyl and acetoin production pathways are shown.

Lactic acid is the main metabolite of the fermentation (Rea *et al.*, 1996). The Kepi microbes are dominated by the mesophilic, homofermentative lactic acid lactococci, which include strains of the species *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *diacetylactis* and *Lactococcus lactis* subsp. *cremoris.* This group is the largest and most active microbial part of the Kepi culture. The L- and D-lactic acid isomers are produced by lactococci and leuconostocs, respectively. The high concentration of the lactococci explains the 10-fold higher amount of the L(+)-lactic acid isomer in comparison to the D(-)-isomer at the end of the fermentation (Dellaglio, 1988; Koroleva, 1988a; Litopoulou-Tzanetaki & Tzanetakis, 2000; Rea *et al.*, 1996). Lactococci provide rapid acid development during the first hours of fermentation, but the strains are inhibited by high acidity levels (Dellaglio, 1988; Koroleva, 1988a; Rea *et al.*, 1996), indicating a microbiological shift during the fermentation process (Neve, 1992). In addition to the souring function, these microbes also contribute to aroma formation in the Kepi beverage (Oberman & Libudzisz, 1998). *Lactococcus lactis* subsp. *diacetylactis* has the ability to utilise citric acid and to convert it to diacetyl, carbon dioxide and

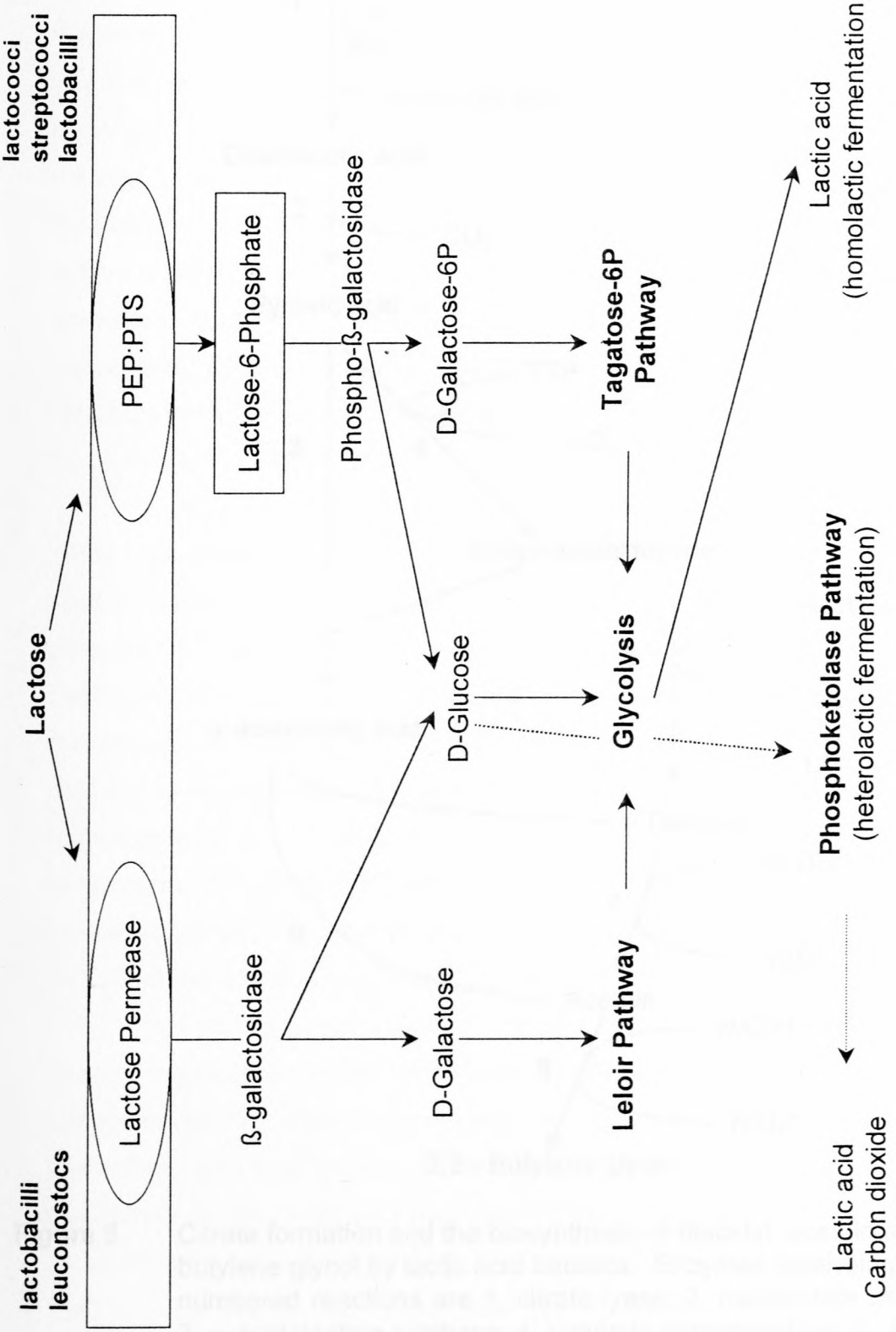

Figure 4. Major pathways of lactose utilisation by lactic acid bacteria (Arihara & Luchansky, 1994; Kandler, 1983).

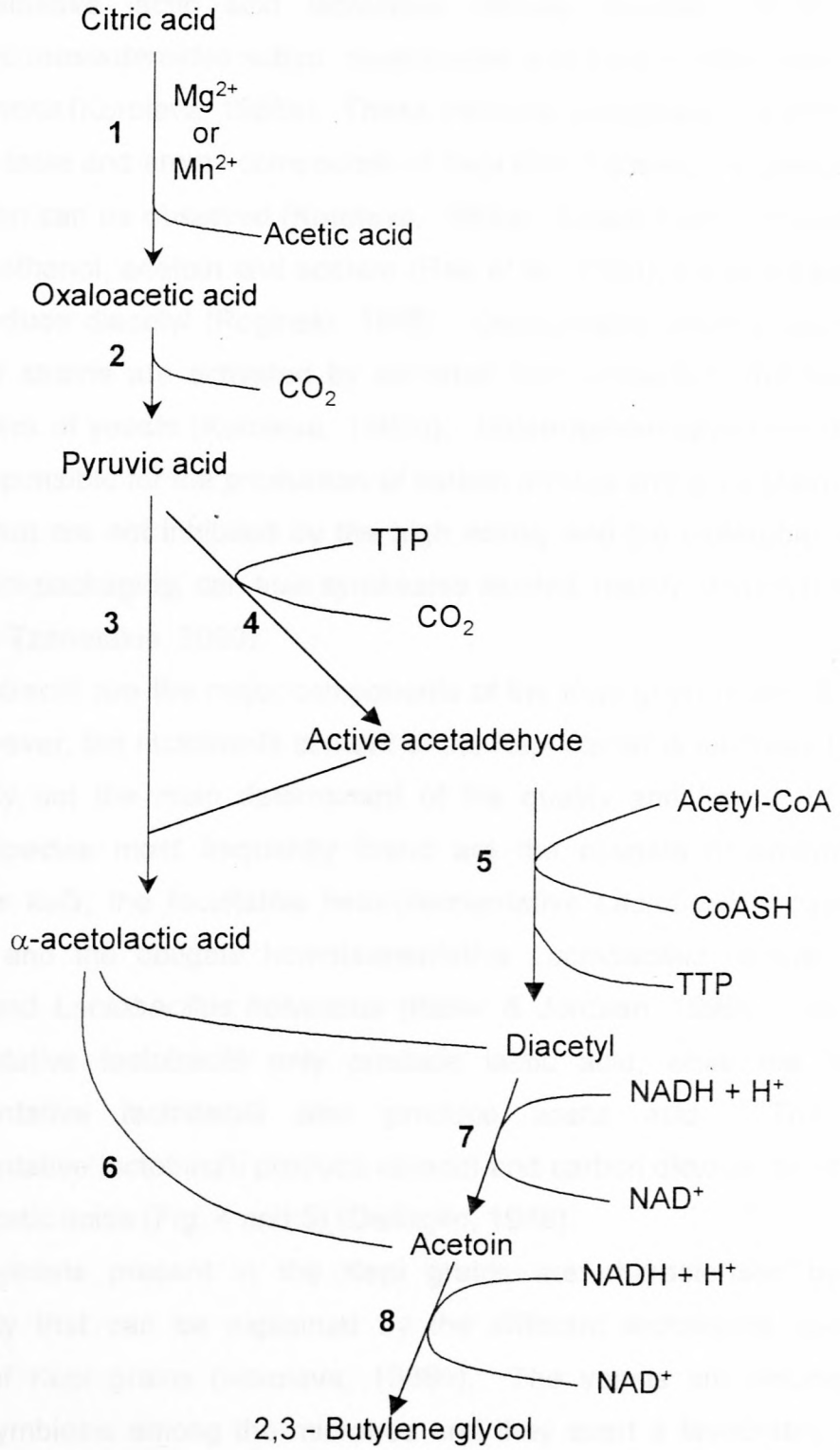

Figure 5. Citrate formation and the biosynthesis of diacetyl, acetoin and butylene glycol by lactic acid bacteria. Enzymes catalysing the numbered reactions are 1, citrate lyase; 2, oxalacetate decarboxylase; 3, α-acetolactate synthase; 4, pyruvate decarboxylase; 5, diacetyl synthase; 6, α-acetolactate decarboxylase; 7, acetyl reductase; 8, acetoin reductase (Human, 1998).

2,3-butylene glycol (Dellaglio, 1988; Kosikowski, 1982). The mesophilic, heterofermentative lactic acid lactococci include members of the species *Leuconostoc mesenteroides* subsp. *dextranicum* and *Leuconostoc mesenteroides* subsp. *cremoris* (Koroleva, 1988a). These microbes participate in the formation of the specific taste and aroma compounds of Kepi and in cases of excessive growth, gas formation can be observed (Koroleva, 1988a). *Leuconostoc* species produce lactic acid, ethanol, acetoin and acetate (Rea *et al.*, 1996), utilise citrate at a low pH and produce diacetyl (Roginski, 1988). *Leuconostoc mesenteroides* subsp. *dextranicum* strains are activated by elevated temperatures in the presence of large numbers of yeasts (Koroleva, 1988a). Heterofermentative lactic lactococci are also responsible for the production of carbon dioxide and in addition, the lactic lactococci that are not inhibited by the high acidity and the anaerobic conditions resulting from packaging, can also synthesise alcohol, mainly ethanol (Litopoulou-Tzanetaki & Tzanetakis, 2000).

Lactobacilli are the major components of the Kepi grain (Keller & Jordaan, 1990). However, the lactobacilli content of the Kepi starter is relatively low and is subsequently not the main determinant of the quality and flavour of the final product. Species most frequently found are the obligate heterofermentative *Lactobacillus kefir,* the facultative heterofermentative *Lactobacillus casei* subsp. *rhamnosus* and the obligate homofermentative *Lactobacillus delbreukii* subsp. *bulgaricus* and *Lactobacillus helveticus* (Keller & Jordaan, 1990). The obligate homofermentative lactobacilli only produce lactic acid, while the facultative heterofermentative lactobacilli also produce acetic acid. The obligate heterofermentative lactobacilli produce ethanol and carbon dioxide, apart from the lactic and acetic acids (Fig. 4 and 5) (Dellaglio, 1988).

The yeasts present in the Kepi grains are characterised by a wide heterogeneity that can be explained by the different techniques used in the cultivation of Kepi grains (Koroleva, 1988a). The yeasts are responsible for promoting symbiosis among the microbes and they exert a favourable effect on the activity of the lactic acid bacteria, providing them with growth stimulants and metabolising some of the produced lactic acid (Keller & Jordaan, 1990; Koroleva, 1982). The yeasts are responsible for the formation of carbon dioxide, the production of low concentrations of alcohol, and the development of the characteristic tastes and aromas in the Kepi beverage (Koroleva, 1988a). Excess

growth of the yeasts can lead to high levels of gas being formed in the product and subsequent packaging problems (Koroleva, 1988a). Some components of the Kepi microbial composition, particularly lactose-fermenting yeasts like *Torulaspora spp.*, have bacteriostatic and bactericidal effects on coliforms (Litopoulou-Tzanetaki & Tzanetakis, 2000). However, the total antibiotic activity of the Kepi microbes is more effective than that of its individual components (Litopoulou-Tzanetaki & Tzanetakis, 2000; Oberman & Libudzisz, 1998).

The acetic acid bacteria are considered to play an important role in maintaining symbiosis between the Kepi grain microbes (Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990). The production of vitamin B_{12} by these microbes stimulates the growth of other microbes in the grains (Rea *et al.*, 1996) and the presence of these acetic acid bacteria results in an improved consistency of the Kepi by increasing the viscosity (Bachmann, 1984; Libudzisz & Piatkiewicz, 1990; Oberman & Libudzisz, 1998). Their presence, in what is essentially an anaerobic fermentation, is unusual given their highly aerobic nature. The cessation of the growth of acetic acid bacteria after approximately 20 h is probably a result of oxygen depletion (Rea *et al.*, 1996) and it has been shown that stirring of the milk during the fermentation process improves the growth of these bacteria (Rea *et al.*, 1996).

In an investigation into the chemical changes that occur during fermentation and the ripening of Kepi (Gawel & Gromadka, 1978), it was found that the main chemical changes in the Kepi occur during the first day of the fermentation. In this period, there is a considerable increase in the volatile activity, simple nitrogen compounds, acetoin and carbon dioxide. The ethanol content is low and the concentrations of orotic acid and citric acid is lowered. This is because the orotic acid is used for microbial growth and results in the synthesis of nucleotides, while the citric acid is converted to diacetyl and acetoin. The content of acetaldehyde and diacetyl, the compounds responsible for the yoghurty and buttermilk aroma in Kepi, show a large variation between different Kepi samples (Driessen & Puhan, 1988).

D. PRESERVATION OF KEPI GRAINS AND CULTURES

Many aspects regarding the cultivation techniques and the environment must be considered if Kepi grains are to be cultivated to obtain sufficient grains of a standard and desirable quality. The most important consideration is the sustainability of the activity of the microbial grain population when they are not actively used in the fermentation process.

Kepi grains are usually preserved by the application of freezing and freeze-drying techniques. Storage at 4°C, wet or dried, has been proposed as an alternative method of preserving Kepi grains (Garrote *et al.*, 1997). In addition, air-drying of the grains in a warm oven has also been applied by some researchers (Vedamuthu, 1982). Additional preservation techniques may include osmotic dehydration (Barbosa-Cánovas & Vega-Mercado, 1996) and fluidised-bed drying (Barbosa-Cánovas & Vega-Mercado, 1996). Although these techniques have not been commercially applied to Kepi grains, fluidised-bed drying has successfully been applied for the preservation of yeasts (Mr. G. Reed, 1999, Anchor Yeast, personal communication).

Preservation techniques

Cold storage

The Kepi grains must be removed from the fermented product, since a prolonged fermentation may lead to excessive acid production and result in gradual damage to the viable organisms (Kroger, 1993). Refrigeration of the grains at 4°C inhibits acid production, but the organisms tend to lose their activity after some time (Oberman & Libudzisz, 1998). It has been reported, however, that grains can remain active for several months if washed with cold water and stored in sterile water or a solution of 0.9% (w/v) sodium chloride at a temperature of 4°C (Marshall, 1993; Oberman & Libudzisz, 1998; Tamime, 1981). Garrote *et al.* (1997) showed that Kepi grains that are stored at 4°C do not show a weight increase when re-inoculated into the milk and the product obtained after re-inoculation does not have the acidity or viscosity of the standard product. Steinkraus (1996) suggested that the most effective way of maintaining viable Kepi grains is by transferring them periodically into milk that is at a constant temperature of between 4° and 7°C. This subculturing allows for a good quality

product, but the Kepi grains are susceptible to contamination (Garrote *et al.*, 1997) and an increased risk of mutations and modifications of the microbes exist (Mocquot & Hurel, 1970).

Air-drying

A long term storage method which allows the grains to remain active for well over a year, is to wash the grains with clean, cold water, and to either dry the grains with cheese cloth or paper for two days at room temperature (Kosikowski, 1982) or to dry them in a warm oven (Vedamuthu, 1982). The grains are then stored in aluminium foil or in a paper envelope in a cool, dry place (Kroger, 1993; Vedamuthu, 1982). The grains that have been dried in this manner have a brownish colour and have a lag-phase of approximately three days upon re-introduction into milk (Marshall, 1982; Marshall, 1984; Pintado *et al.*, 1996). This lag-phase is consistent with the commonly accepted belief that microbes in a dried form take a considerable time to re-adapt their metabolic system to the production of biomass (Pintado *et al.*, 1996). In addition, this crude method of preservation can lead to contamination of the dried grains, and it is possible that the proportions of the symbiotic microbes may be altered (Tamime, 1981).

Freezing

The use of freezing as a preservation method for starter cultures began during the mid-1950's (Fennema *et al.*, 1973). Initial attempts involved two main approaches (Fennema *et al.*, 1973). The first approach was to freeze a ripened culture, followed by incubation in milk to produce approximately 0.85% titratable acid before freezing. The second approach involved the freezing of an unripened culture, incubated briefly or not at all, before freezing. Several studies claimed success when both ripened and unripened cultures were used directly to produce bulk starters (Fennema *et al.*, 1973). However, it has been shown that lactic acid cultures, when frozen as described, tend to lose their activity (Fennema *et al.*, 1973). Even though these procedures were not entirely successful for preparing conventional lactic acid starter cultures, it has been claimed that Kepi grains can effectively be preserved by freezing and storage at -18°C (Fennema *et al.*, 1973). Grains held at -18°C for nine months can easily be re-activated to undergo a normal alcohol and lactic acid fermentation (Fennema *et al.*, 1973). A fast rate of

freezing is recommended as this limits the damage caused by ice-crystal formation (Stanley, 1998). Lower temperatures of storage improves the shelf-life of the cultures and temperatures of -70° to -80°C are commonly used (Stanley, 1998).

In a more recent study, the effect of freezing on the grains was investigated (Garrote *et al.*, 1997). Kepi grains stored at -20° and -80°C were found to maintain their microbial composition and activity needed to ferment milk for up to 7 - 8 months. It was also found that the grains increased their weight by successive subculturings at a rate comparable to that found with unstored grains. The Kepi prepared with the frozen and stored grains showed the same microbial populations, viscosity, level of acidity and carbon dioxide content as was normally obtained with unstored grains. A good product can be obtained with grains stored at -20° and -80°C and this method avoids tedious subculturing and subsequent risk of contamination. This implies that storage of Kepi grains at household freezer temperatures is a good method to preserve the necessary metabolic activity (Garrote *et al.*, 1997).

Freeze-drying

Kepi grains cannot be dehydrated with heat and survive, but they may survive freeze-drying preservation techniques (Steinkraus, 1996). This makes freeze-drying the single most important dehydrating preservation method for Kepi grains.

Freeze-drying requires very low pressures or high vacuum in order to produce a satisfactory drying rate (Liapis & Bruttini, 1995). Since the water in the grains exist in a combinational state or solution, the product must be cooled below 0°C to keep the water in a solid phase. Most freeze-drying is done at -10°C or lower and at absolute pressures of about 2 mm Hg or less (Liapis & Bruttini, 1995).

Cryoprotectants have also been used to prevent or reduce culture inactivation during the freezing or freeze-drying processes. Cryoprotectants used in the production of starter cultures include carbohydrates such as lactose and sucrose, monosodium glutamate and glycerol (Stanley, 1998). However, in a study conducted by Brialy *et al.* (1995) monosodium glutamate was found to be unsuitable for the use as a cryoprotector for Kepi, in contrast with earlier conclusions by Pereda-Arladin (1990). It was also shown that glycerol and ribitol are suitable cryoprotective substances, and serve as a means of preservation of

the intrinsic inhibitory effect of dairy Kepi (Brialy *et al.*, 1995). Glycerol can be used in two different ways, either as an additive substance in milk or as a lyophilisation substrate and cryoprotective substance (Brialy *et al.*, 1995).

Freeze-drying, with the aid of cryoprotectants, may be an effective preservation technique for the long-term storage of Kepi. Such freeze-dried grains retain their activity for up to 12 - 18 months (Oberman & Libudzisz, 1998). The preserved grains are in the form of a powder or small crystals, and after re-activation, the grains start to form in the growth medium (Tamime, 1981). The re-activation requires several subculturings, with an incubation temperature and an inoculum a little higher than for undried cultures (Mocquot & Hurel, 1970).

Lyophilised Kepi cultures have also been made available and can replace the Kepi grain starter as prepared by the traditional procedure (Duitschaever, 1989). These cultures consist almost entirely of lactococci and difficulties are frequently encountered with the survival of the yeast population. More than 80% of the yeasts can be lost during freezing and freeze-drying of the Kepi grains (Marshall, 1993). Due to this loss of the yeast cells, freeze-dried grains are stabilised by the addition of yeast isolates.

Freeze-dried starters should be kept at temperatures of lower than 5°C (Oberman & Libudzisz, 1998). Despite the capability of freeze-drying to provide a high quality dehydrated product, it remains an expensive and time-consuming form of dehydration.

Osmotic dehydration

As far as known, no reference is available in the literature where osmotic dehydration is used for the preservation of Kepi grains, but it could possibly be applied as a means of a pre-processing step prior to drying. In addition to the removal of part of the excess water in the grains, osmotic dehydration simultaneously can, depending on the composition of the osmotic solution, supply the compositional microbes with nutrients.

Osmotic dehydration involves the concentration of a food product by means of the immersion of the product in a hypertonic solution of carbohydrates, salt, sorbitol or glycerol (Barbosa-Cánovas & Vega-Mercado, 1996) and is the process where molecules in a solution move through a semi-permeable membrane to another solution that has a lesser concentration of the particular molecules.

Compared to air-drying or freeze-drying, osmotic dehydration is faster, because the removal of water occurs without a phase change. This process has received considerable attention over the last few years, because of potential industrial applications. Osmotic dehydration is usually applied in conjunction with the hurdle technology that involves the use of additional preservation techniques (Barbosa-Cánovas & Vega-Mercado, 1996).

Osmotic dehydration and hurdle technology have successfully been applied in the processing of fruit, vegetables, fish and meat and in the production of intermediate moisture foods (Barbosa-Cánovas & Vega-Mercado, 1996). Osmotic dehydration can be used as a pre-processing step prior to a regular drying process. In other instances, the preservation utilises hurdle technology by reducing the water activity, using certain osmotic agents, and adding small amounts of antimicrobial agents or changing the pH (Barbosa-Cánovas & Vega-Mercado, 1996).

Fluidised-bed drying

The use of fluid-bed drying for granular materials is well established and large numbers of fluid-bed dryers are operational throughout the food and chemical industries (Hovmand, 1995). Fluidised-bed drying involves the drying of particles by passing a gas upward through a layer of particles supported by a grid. The advantage of the fluidised and vibrio-fluidised systems is that they are isothermal, due to the intensive mixing of the solid state, which prevents local overheating of the particles (Adamiec *et al.*, 1995). However, disadvantages of this drying method are the risk of mechanical damage to the cells and the formation of agglomerates and deposits on the walls, which disturb the uniform bed fluidisation and cause local overheating and thermal inactivation of the product. The drying of vegetative and spore cultures by this method is not recommended, because the abrasion can damage the microbes or mechanically disrupt the biopolymer chains (Adamiec *et al.*, 1995).

To be suitable for fluidised-bed drying, food materials must have an average particle size of between 0.01 and 20 mm, a narrow particle size distribution, a regular particle shape and have an easy disintegration of lumps of particles upon fluidisation (Sokhansanj & Jayas, 1995). It is important that the particles should not be sticky at the processing temperatures (Barbosa-Cánovas &

Vega-Mercado, 1996). Although Kepi grains do not meet up all the suitability requirements for fluidised-bed operations, a possible application of this drying method on the Kepi grains could be very effective, provided that the air temperature do not exceed 75°C (Mocquot & Hurel, 1970).

E. PACKAGING OF KEPI AND KEPI GRAINS

The packaging of fermented milk products and the cultures used in the production of these products must preserve the quality and allow for the increase of the storage life between the production and the consumption (Odet, 1988). Kepi and Kepi starters are yeast containing fermented milk products in which the yeasts produce carbon dioxide during storage. This is of particular importance when suitable packaging methods and materials are considered. The presence of a large population of living lactic bacterial cells is considered a fundamental criteria of fermented milks and their cultures. Therefore, the materials used for the packaging must be compatible with the special physical, chemical and bacteriological properties of the fermented milks. The International Dairy Federation Technical Guide (1982) recommends that the following criteria be taken into consideration when selecting packaging for milk and milk products:

- migration
- odour and taste
- mechanical strength
- material stability
- permeability to gasses and water vapour
- permeability to flavour
- light transmission
- disposal of used packages
- recycling of packaging materials.

The materials used for packaging of fermented milks are normally: glass; polyethylene; complex cardboard-polyethylene; polypropylene; polystyrene and coextrusion plastic complexes. The packaging materials that will be used for the preservation of dried grains should be barriers against air and moisture. Suggested materials are foil pouches or metal cans (Mr. G. Reed, 1999, Anchor

Yeast, personal communication; Mr. A.N. Starke, 1999, Nampak, personal communication). In addition, methods used for the packaging of dried yeasts have also been suggested for the dried Kepi grains (Mr. G. Reed, 1999, Anchor Yeast, personal communication). These include the packaging of the dried yeasts under either vacuum or flushed with nitrogen gas in polyethylene terephthalate (PET) foil pouches, which are impermeable to both moisture and oxygen. It has also been suggested that the dried packaged grains be stored at -20°C to ensure a longer shelf-life. This was based on experience gained by the preservation of yeast grains packaged under vacuum and stored in a cool and dry place, which led to a shelf-life of more than three years (Mr. G. Reed, 1999, Anchor Yeast, personal communication).

The suggested packaging for the frozen and cold stored Kepi grains are plastic tubs similar to that used in yoghurt packaging (Mr. G. Reed, 1999, Anchor Yeast, communication). The grains are then either stored under vacuum or at atmospheric pressure in these containers. Alternatively, carton boxes can also be used. It is suggested that the frozen culture must be stored at -60°C to ensure a shelf-life of at least one year (Mr. J. Strydom, 1999, Miles Seravac, personal communication).

F. REGULATORY ISSUES

The only two known standards relating to the composition of Kepi (kefir) were issued in California, USA in 1991 as part of the Food and Agriculture Code, and by the International Dairy Federation as part of the IDF 163 documentation of 1992. The descriptions of Kepi, however, are clearly not true Kepi (kefir) in the traditional sense and are referred to as "Kepi milk". The making of true Kepi involves yeast cells, a dual lactic and alcoholic fermentation, and the presence of yeast cells in the final product. This fact is contradicted in item 38871 of the 1991 Food and Agricultural Code. The sections dealing with Kepi (not including fruit Kepi) are cited from Kroger (1993), and appear as follows:

Article 36. Kepi (Kefir) Milk - A Cultured Milk

38871. Market milk or market milk combined with non-fat milk from market milk, with or without added milk solids, flavouring, or seasoning, which is

certified raw milk or has been pasteurised and afterwards fermented by *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus brevis* (formerly known as *Lactobacillus caucasicus*) may be sold as Kepi, low-fat Kepi, or non-fat Kepi, or such names as may be characteristic for the product and approved by the director. Such product may contain harmless edible stabiliser not to be exceeded six-tenths of one percent. It shall contain no more than 10 coliform bacteria per gram and shall be free of moulds, yeasts, and other fungi, and other objectionable bacteria, which may impair the quality of the product.

38872. Kepi shall contain not less than 3.5% milk fat. Low fat Kepi shall contain not less than 1.9%, or more than 2.1% milk fat. Non-fat Kepi shall contain a maximum of twenty-five hundredths of one percent milk fat. Kepi made from goat milk shall contain not less than 2.8% of milk fat.

38873. When offered for sale, Kepi, low-fat Kepi, and non-fat Kepi shall be labelled, on a principal panel of the container, with the name and address of the manufacturer or distributor, and a statement whether it is made from pasteurised milk or certified raw milk. If the name and address of the distributor are used, the factory license number of the manufacturer shall also appear on the carton or container. Kepi shall be labelled "Kepi a Cultured Milk". Low-fat Kepi shall be labelled "Low-fat Kepi a Cultured Milk". Non-fat Kepi shall be labelled "Non-fat Kepi a Cultured Milk".

38874. Kepi, low-fat Kepi, or non-fat Kepi, in liquid form, and with or without fruit added, shall be made from market milk and may be labelled as "Certified Raw", "Certified Pasteurised", or "Grade A".

In the General Standard of Identity for Fermented Milks as found in the IDF Standard 163 of 1992, the definition of fermented milks is given, followed by definitions and raw materials, cultures, ingredients and additives, followed by a classification system consisting of four categories for fermented milks, under which Kepi is classed in the lactic acid and alcoholic fermentation category. The section dealing with the quality requirements for some typical fermented milks devotes the following two paragraphs to Kepi (IDF Standard 163, 1992):

Culture: Starter prepared from Kepi grains, whose microbial population is constituted by yeasts both lactose fermenting (*Kluyveromyces marxianus*)

and non-lactose fermenting (*Saccharomyces unisporus, Saccharomyces cerevisiae* and *Saccharomyces exiguus*), *Lactobacillus kefir*, species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter* growing in a strong, specific relationship.

Composition: Acidity not lower than 0.60% per weight expressed as lactic acid. Minimum counts of specific microbes at the time of sale: lactic acid bacteria 10^7 cfu.g^{-1} and yeast 10^4 cfu.g^{-1}.

G. DISCUSSION

Even though Kepi has been known for many centuries, research still needs to be done on the Kepi grains and the Kepi technology for the successful marketing of this fermented milk product. Kepi as a fermented milk product with an enjoyable taste, high nutritional value and a convenient and easy method of preparation and storage, and has a positive marketing potential. A successfully preserved and stable culture that can easily be propagated in fresh milk will further expand the boundaries of this beverage. Not only will this offer endless possibilities to the disadvantaged populations of the third world countries, but it will also prompt Kepi to take its rightful place as an excellent health beverage all over the world.

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CHAPTER 3

EVALUATION OF DIFFERENT PRESERVATION TECHNIQUES ON THE STORAGE POTENTIAL OF KEPI GRAINS

Abstract

Kepi is a refreshing, fermented dairy beverage that has been consumed for centuries and is traditionally made by incubating Kepi grains in milk. The successful marketing of the grains requires the effective preservation of the microbes present in the grains. The aim of this study was to evaluate four different preservation techniques of Kepi grains and to investigate the influence of different storage periods on the activity of the preserved grains. Four different preservation techniques including freezing (-18°C); refrigeration (4°C); air-drying; and lyophilisation, were evaluated. Activity tests were used to evaluate the impact of the preservation techniques in terms of the retainment of the acidification activity of the preserved grains, and the storage potential of the preserved grains. The activity tests included the change in pH, %TA, lactic acid production and lactose content over an 18 h fermentation period. Frozen and refrigerated grains showed the best retainment of the acidification activity over the 10-month storage period. Air-drying and lyophilisation showed a good retention of the activity up to three months of storage, but the application of these techniques both resulted in a slow initial acidification activity. After 10 months of storage, the air-dried and lyophilised grains showed a low acidification activity. No volatile compounds could be detected during the course of the fermentation period, due to the relative short fermentation period of 18 h. It can, therefore, be concluded that all four of the preservation techniques are suitable for the preservation of Kepi grains and the subsequent storage for three months. However, for storage periods of 10 months or longer the use of freezing and refrigeration are recommended as most suitable preservation techniques.

Introduction

Kepi is an acidic, mildly alcoholic fermented dairy beverage produced by the fermentation of milk with a grain-like starter culture known as Kepi grains (Koroleva, 1988; Merin & Rosenthal, 1986). These grains usually contain a relatively stable and specific balance of microbes which exist in a complex symbiotic relationship (Obermann & Libudzisz, 1998; Saloff-Coste, 1996). The microbes are embedded in a matrix of fibrillar material composed largely of a resilient polysaccharide, called kefiran (Angulo *et al.*, 1993; Duitschaever, 1989; Saloff-Coste, 1996).

The different groups of microbes present in the grains are active at different stages of the fermentation (Koroleva, 1982). Each of the microbial groups has different physiological properties and may show great variation in quantitative ratios due to possible changes that can occur in the cultivation conditions of the Kepi grains. The lactococci, including *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetilactis* provide rapid acid development during the first hours of the fermentation (Koroleva, 1982; Litopoulou-Tzanetaki & Tzanetakis, 2000). As the acidity of the milk increases it provides favourable conditions for the excessive growth of the lactobacilli, causing the lactococci to rapidly die off (Dellaglio, 1988; Koroleva, 1988; Rea *et al.*, 1996). The yeasts, acetic acid bacteria and the aroma-producing microbes, mainly leuconostocs, have a much slower growth rate than the lactic acid producers, resulting in the slow production of the aroma compounds and the gradual increase in the concentration of these substances in the later stages of the fermentation (Koroleva, 1982).

In the past the preservation of the complex microbial populations present in the Kepi grains was achieved by various preservation methods that include freezing (Garrote *et al.*, 1997), lyophilisation (Oberman & Libudzisz, 1998), air-drying (Kroger, 1993; Vedamuthu, 1982) and refrigeration (Marshall, 1993). Research has shown that grains preserved by air-drying and lyophilisation retain their activity for up to 12 - 18 months (Kosikowski, 1982; Oberman & Libudzisz, 1998). Frozen grains stored at -20°C were found to maintain the microbial activity for up to 7 – 8 months, whereas grains stored at refrigerated temperatures showed a decreased activity after about 10 days (Oberman & Libudzisz, 1998). The use of

the various preservation techniques to preserve the fermentation activity of the Kepi grains over an extended storage period is an important consideration in the viable application of grains in the production of the Kepi beverage.

The aim of this study was to compare four different techniques to preserve Kepi grains. The activity of the preserved grains was evaluated at different time intervals using four activity measurements, including changes in substrate pH, the percentage titratable acidity (%TA), the determination of the utilised lactose and produced lactic acid, and the presence of volatile compounds in the Kepi beverage.

Materials and methods

Kepi grain activation

Kepi grains were obtained from the Department of Food Science, University of Stellenbosch, Stellenbosch, South Africa. Fresh, pasteurised, full cream milk purchased at local supermarkets was given a further heat treatment in a temperature-controlled waterbath at 83° - 85°C for 20 min and then cooled to 4°C. The purpose of this double pasteurisation was to obtain milk of a reliable microbiological quality, as previous studies on commercially pasteurised milk showed that pasteurisation was not always performed satisfactorily, resulting in milk of an inferior microbiological quality (Human, 1998). In addition, it is also known that the heat treatment of milk is beneficial in that it renders a more nutritious medium for starter organisms (Robinson, 1995). Eighteen grams of grains were aseptically added to 500 ml of the double pasteurised milk and incubated at 25°C. After 24 h of incubation the grains were recovered from the milk mixture by straining through a sterilised stainless steel kitchen sieve (1.25% Milton solution for 30 min, then rinsed with sterile distilled water). The recovered grains were then added to double pasteurised milk at 25°C for 24 h. This procedure was repeated seven times prior to the application of the four different preservation techniques.

Preservation techniques

Fresh activated Kepi grains were divided into samples (triplicate units) of 18 g each and used for the application of the following four preservation techniques:

I frozen storage at –18°C;

II refrigeration at 4°C;

III air-drying at room temperature for three weeks in a desiccator; and

IV freeze-drying and storage at 4°C.

The frozen and refrigerated grain samples were placed in 100 ml glass containers and stored in moisture tight jars. The air-dried grain samples were allowed to dry in desiccators and stored in petri dishes in moisture-tight jars in the presence of silica gel. After lyophilisation (Heto CT 60e Freeze Dryer, Denmark), the grain samples were aseptically transferred to 100 ml glass containers and also stored in moisture-tight jars. All Kepi grains were stored in the dark.

Activity tests and weight loss determination

Activity tests were carried out on the Kepi grains directly after the application of the four different preservation techniques (M_0), and after 1 month (M_1), 3 months (M_3) and 10 months (M_{10}) of storage. All activity tests were done in triplicate. At each time interval, before the activation of the grains, the mass of the preserved grains was determined and the weight loss over the specific storage period calculated.

The activation of the air-dried, lyophilised and refrigerated grains were obtained by the direct transfer of the grains to 500 ml double pasteurised, full cream milk at 25°C, followed by the incubation for 24 h. The frozen grains were first allowed to defrost at room temperature before the inoculation into the milk. All the preserved grains were recovered by sieving with a sterilised stainless steel kitchen sieve and then transferred to 1 ℓ of double pasteurised, full cream milk. The activity tests, including the determination of the pH, %TA, lactose utilisation, lactate production and the volatiles present in the Kepi beverage were carried out on the produced Kepi after 0, 10 and 18 h of incubation at 25°C.

The pH of the Kepi samples was measured with a Knick pH meter (pHB-4, Merck). The %TA of the Kepi samples was determined by the titration of a 10 ml

Kepi sample with standardised 0.1 M NaOH to the phenolphthalein endpoint (Dixon, 1973). This determination was done in duplicate for each sample.

The lactose content was measured colorimetrically with the use of methylamine as an indicator (Katsu *et al.*, 1994). The determination of the lactose content in the Kepi was based on the reaction of the lactose with methylamine in a hot alkaline solution and the subsequent formation of a red complex with a maximum absorbance at 540 nm. A Spectronic 20, Genesys spectrophotometer (Spectronic Instruments, USA) was used in the determination of the absorbance.

The lactic acid was measured enzymatically with the D-/L+ Lactic Acid Test Combination Kit (AEC Amersham, Germany). In addition, the lactose and lactic acid content of the Kepi samples were also predicted from absorbance spectra recorded using the FT-NIR Spectrum Identicheck™ spectrophotometer (Perkin-Elmer, Germany). The samples were scanned from 10 000 – 4 000 cm^{-1} with wavelength increments of 4 cm^{-1}, which produced a total of 1 501 points per spectrum. The spectral data was collected at a resolution of 32 cm^{-1} using a 0.2 nm path length quartz cuvette (Schoevers, 1999).

The volatile compounds present in the Kepi were determined by measuring 9.75 ml of each sample into a 20 ml glass vial and adding n-buthanol as internal standard to a final concentration of 200 $mg.l^{-1}$. Each vial was sealed with a butyl rubber stopper and aluminium cap and then incubated for 30 min in a waterbath at 60°C. A 250 µl sample was withdrawn using a gas-tight syringe and injected into the gas chromatograph (Fisons Instruments, Milan, Italy) (Mutukumira, 1996). Quantitative determination of the compounds was done by integration of the peak areas, using an internal standard calibration (Mutukumira, 1996). Identification of the unknown compounds was achieved by comparing the retention times to those of the analytical grade standard compounds. An aqueous stock solution was prepared as standard and contained 200 $mg.l^{-1}$ of acetaldehyde (Merck), acetone (Riedel-de Haën), ethanol (Saarchem), diacetyl (Aldrich), 2-butanone (Merck) and n-buthanol (internal standard) (Merck) (Ulberth, 1991). A Fison GC 8000 gas chromatograph equipped with a flame ionisation detector was used with helium as carrier gas at 1.2 ml min^{-1}. The temperature profile was 35°C at 2 min, increased by 5°C.min^{-1} to 220°C with a holding time of 10 min. The temperatures of the injector and the detector were 150° and 200°C, respectively. A 30 m fused silica

capillary column with a bonded methyl 5% phenyl silicone phase (0.25 µm film) (Qyadrex Corporation, USA) (Ulberth, 1991) was used.

Results and discussion

Characteristics of the preserved grains

The 'frozen' (-18°C) and 'refrigerated' (4°C) grains retained the typical appearance, colour and resilient nature of the fresh grains (Fig. 1B and C). However, after 10 months of storage the 'refrigerated' grains had a clearly detectable acidic, alcoholic odour. This can be ascribed to the possible acid and alcoholic fermentation by the microbes present in the moist grains. Traces of mycelial fungal growth were also detected on the grains after the 10-month storage period. The 'lyophilised' grains retained the appearance and colour of the fresh grains, but were found to be extremely brittle (Fig. 1A). The 'air-dried' grains developed a brownish colour and a foul odour, together with a tough structure due to the moisture loss (Fig. 1D). Mycelial fungal growth on the 'air-dried' grains was observed after approximately two weeks of the drying process.

Weight loss

The weight loss over the 10-month period for the 'frozen' and 'refrigerated' samples was fairly small, with a total weight loss of 4.81% (0.865 g) and 9.16% (1.648 g), respectively (Fig. 2). Air-drying of the grains resulted in a 5-fold (23.42%) decrease in sample weight, from 18.000 to 4.215 g after the preservation treatment (Fig. 2). However, only a slight decrease in the weight of the 'air-dried' grains was observed over the total storage period, which was ascribed to additional moisture loss during the storage period in the desiccators. The mass of the 'air-dried' grains after 1, 3 and 10 months of storage were 16.56% (2.981 g), 16.22% (2.919 g) and 14.66% (2.639 g) of the original sample weight. In the case of the 'lyophilised' grains an initial weight loss of 81.12% (14.599 g) was observed. However, as opposed to the 'air-dried' grains, the 'lyophilised' grains had a constant weight throughout the rest of the storage period (~ 3.450 g) (Fig. 2). Apart from the 'air-dried' grains the weight of the preserved grains was found to be stable in the containers throughout the storage period.

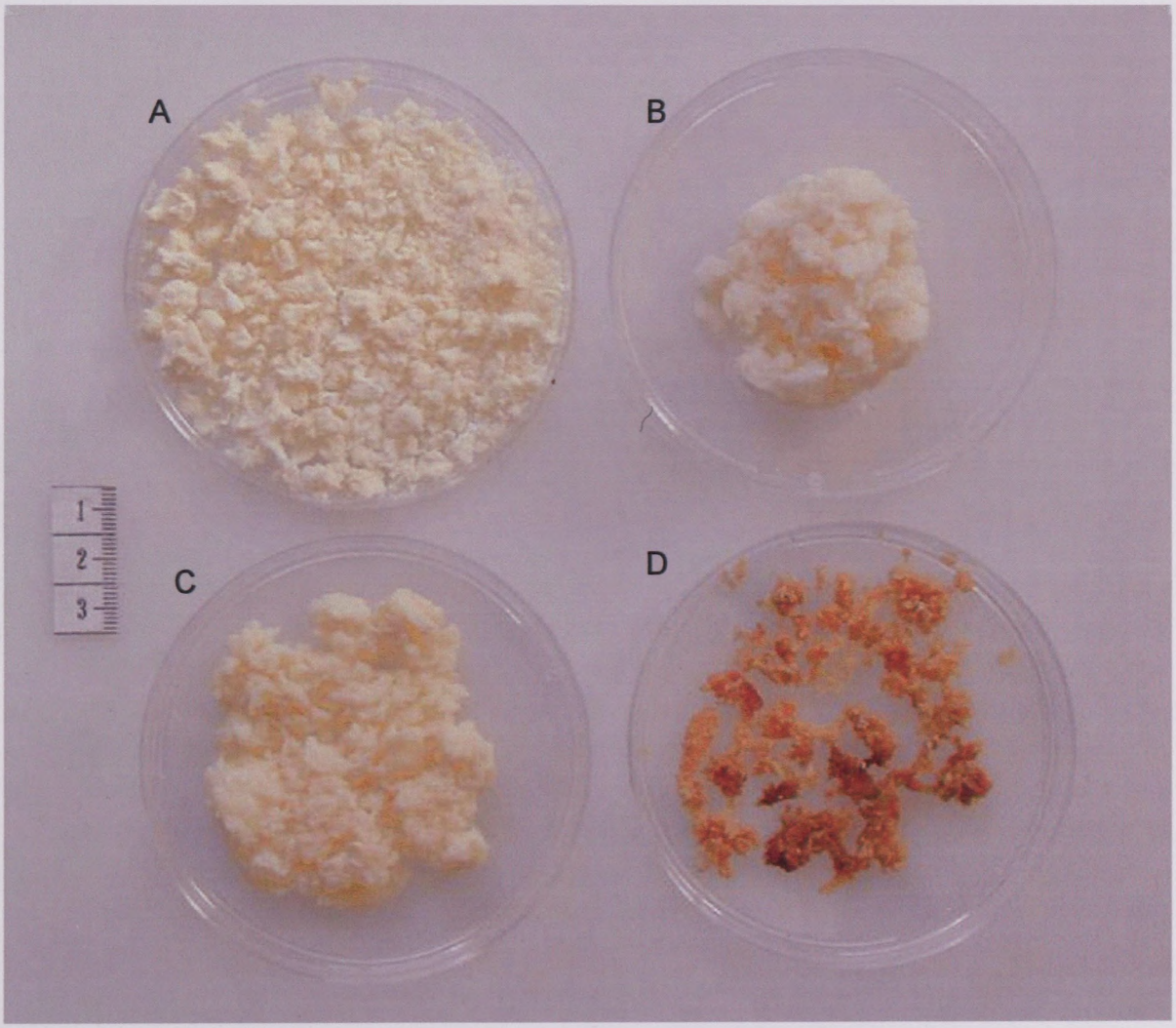

Figure 1. Examples of Kepi grains preserved by the four different preservation technique: lyophilisation (A); freezing (B); refrigeration (C); and air-drying (D).

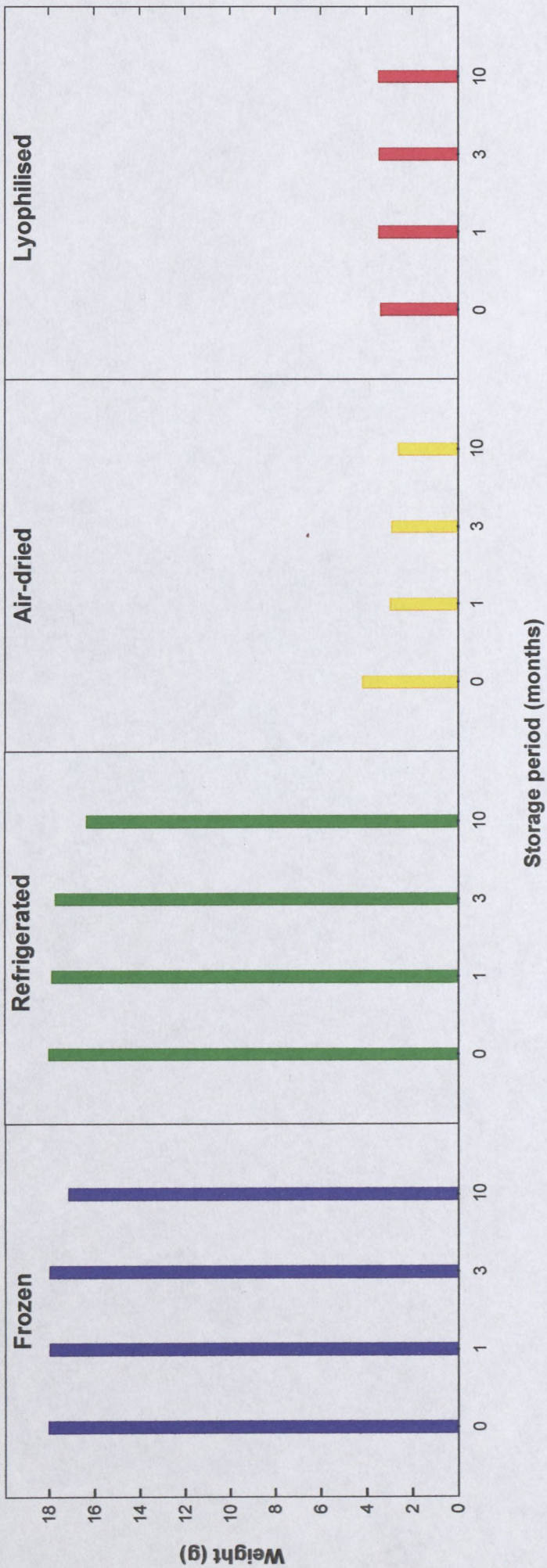

Figure 2. Weight loss profiles of the 18 g Kepi grain samples preserved by the four different preservation techniques over the 10-month storage period. Storage period 0 = after the application of the preservation techniques (M_0); 1 = 1 month of storage (M_1); 3 = 3 months of storage (M_3); 10 = 10 months of storage (M_{10}).

Activity tests

The results of the activity tests (pH, %TA, lactose and lactic acid content and the presence of volatile compounds) for each preservation technique and storage period resulted in a large dataset. The separate pH, %TA, lactose and lactic acid profiles for the different preservation techniques (Fig. 3 – Fig. 6) have been included as an Appendix at the end of this chapter.

pH and %TA profiles

The pH of the samples is a measurement of the concentration and activity of the hydronium ion (H_3O^+) and gives an indication of the effective acidity of the Kepi (Newlander & Atherton, 1964). The titratable acidity of the samples represents the hypothetical 'end-point' which is the amount of alkali necessary to shift the protein and other buffer systems to that pH value at which the indicator changes colour (Johnson, 1974). The titration value of the samples is given as the percentage titratable acidity (%TA) expressed in terms of percentage lactic acid (James, 1995). The %TA is thus a measurement of the change in acidity that occurs in the Kepi samples, but it does not always give a true indication of the effective acidity as obtained by the pH. Although some general relationship exists between the acidity and the pH of a sample, it is not possible to convert the %TA directly into pH and each measurement must, therefore, be used and interpreted independently (Newlander & Atherton, 1964).

The pH of the fresh milk used in this study was found to be 6.53 and served as the 0 h control (Fig. 7). The pH of the milk fermented with the active Kepi grains decreased from pH 6.53 to 4.40 (control for 10 h) during the first 10 h and from pH 4.40 to 4.13 (control for 18 h) during the next 8 h of the fermentation (Fig. 7).

Similar pH results for the different storage periods (M_0 to M_{10}) were obtained for the 'frozen' grain samples. At period M_0 the decrease in the pH for the frozen samples after 10 h of fermentation was from pH 6.53 to 4.93, indicating that freezing has a marked influence on the initial acidification activity of the Kepi grains. However, the corresponding pH values of the frozen samples that were stored for periods of up to 10 months were noticeably lower than that of M_0 (4.93), with a pH value of 4.59 observed for both M_1 and M_3 and a still lower value of 4.44 for M_{10}. This is probably due to changes in the starter composition of the

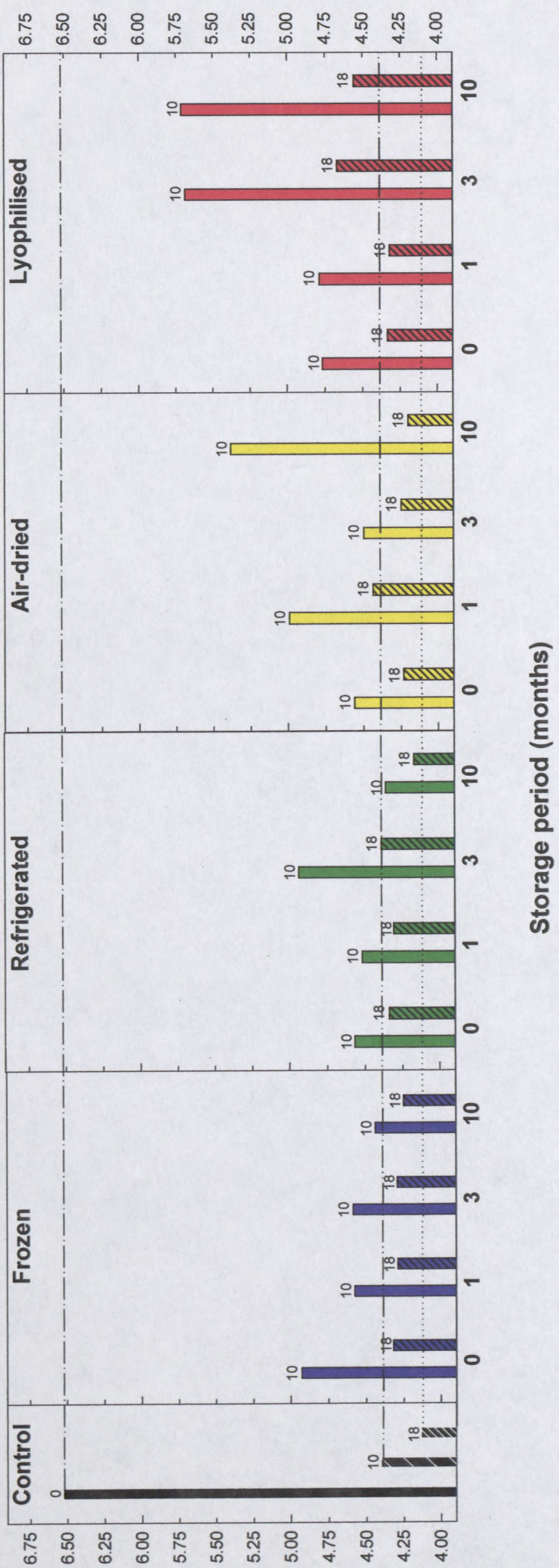

Figure 7. The average pH values (of triplicate samples) of the Kepi prepared from the preserved grains for each of the time intervals: t = 10 h and 18 h and storage periods: directly after the application of the preservation techniques (M_0); after 1 month of storage (M_1); 3 months of storage (M_3); and 10 months of storage (M_{10}). The pH values for t = 0 h (control = 0 h) is that of fresh milk and the average pH values for the active grains (controls = 10 h, 18 h) served as controls.

preserved (frozen) grains during the storage period, causing the stabilisation of the starter microbes or a possible higher activity of the acid-producing bacteria. The storage period had very little effect on the final pH as the pH of all the frozen samples after 18 h of fermentation were very similar (~pH 4.30). Freezing may, therefore, be used as an effective technique for the preservation of Kepi grains. This is in agreement with the results obtained by Garrote *et al.* (1997).

'Refrigeration' of the Kepi grains showed a good retainment of the initial acidification activity as a decrease in the pH from 6.53 to 4.57 was noted during the first 10 h of fermentation at M_0. The decrease in the pH over the first 10 h of fermentation at M_1 was similar to the decrease in the pH observed at M_0 (4.57). However, at M_3 and M_{10} the decrease in the pH after 10 h of fermentation differed markedly, with pH values of 4.94 and 4.36, respectively. These varying pH values for the refrigerated grains at M_3 and M_{10} are possibly due to changes in the microbial activity of the preserved grains over the 10-month storage period. The pH of the samples after 18 h of fermentation was very similar for M_0 (4.34), M_1 (4.31) and M_3 (4.39) and was slightly lower for M_{10} (4.17). From the data it is clear that a low final pH throughout the 10-month storage period was maintained for the refrigerated grains, which is in accordance with results of Pintado *et al.* (1996).

The 'air-dried' grains showed a pH decrease of 6.53 to 4.56 for the first 10 h of fermentation and from pH 4.56 to 4.24 after the next 8 h of fermentation at M_0. This indicates a good retainment of the initial acidification activity. At M_1 a decrease in the activity of the grains was observed as the pH of the samples after 10 h of fermentation was 5.0 and only 4.44 after 18 h of fermentation. At M_3, however, the pH had a sharp decrease to 4.50 after 10 h of fermentation and to pH 4.25 after 18 h of fermentation, indicating a possible increase in the microbial activity. At M_{10} the microbial activity of the grains decreased as a small decrease in pH (6.53 to 5.39) over the first 10 h of fermentation was observed. However, the microbial activity appeared to recover over the 18 h fermentation time, as a final pH of 4.20 was reached suggesting that a change in the population had occurred, but the acid producers were able to recover over the longer incubation time.

The 'lyophilised' grains showed the lowest retainment of the activity of the Kepi grains. After 10 h of fermentation the lyophilised grains led to a decrease in the pH from 6.53 to 4.77 and from pH 4.77 to 4.34 after 18 h of fermentation at M_0.

The pH values for M_1 were similar to those obtained for M_0. At M_3 and M_{10}, a decrease in the activity of the lyophilised grains was observed, with respective pH values of 5.70 and 5.72 after 10 h and 4.67 and 4.56 after 18 h of fermentation, respectively. The decrease in the pH over the first 10 h of the fermentation for the lyophilised grains was significantly slower than for the frozen and refrigerated grains. The slow initial rate of acid production by the dried (lyophilised and air-dried) grains could be ascribed as a consequence of a prolonged lag-phase of the microbes induced by the complete dehydration of the grains (Tamime, 1981). The dehydration treatment may have caused injuries to the microbial cells present in the Kepi grain and time was thus required for the reparation of reversible cellular injuries prior to growth and lactic acid production (To & Etzel, 1997). This lag time was expected in view of the fact that microbes in a dried state take a considerable time to re-adapt their metabolism to biomass production (Pintado *et al.*, 1996). Reports in the literature have indicated that reactivation of the dried Kepi grains requires at least three subculturings (Kosikowski, 1982). The total decrease in the pH after 18 h of fermentation is in the same order for all the preserved samples, indicating a rapid acid production by the remaining lactic acid bacteria, particularly in the dehydrated samples. The retention of the acidification activity in the dried grains (air-dried and lyophilised) is consistent with the findings by Kosikowski (1982) and Oberman & Libudzisz (1998) who reported that dried grains can remain active for a period of 12 - 18 months.

The %TA of fresh milk (control = 0 h) was found to be 0.22% and after 10 h of fermentation (control for 10 h) with active Kepi grains the %TA reached 0.76% and then increased to 0.88% after 18 h (control for 18 h) of fermentation (Fig. 8). The %TA values of all the preserved Kepi grain samples were high for M_0, M_1 and M_3 (with the exception of the lyophilised grains), but had much lower values after 10 months (M_{10}) of storage (Fig. 8).

The %TA profiles of the milk after 10 h of fermentation with the 'frozen' Kepi grains were similar for M_0, M_1, and M_3 (~0.77%). The corresponding value for M_{10}, however, was lower at 0.69%. The same trends were observed for the 18 h fermentation periods, with values of 0.91%, 0.94% and 0.87% for M_0, M_1 and M_3, respectively, and a much lower %TA (0.45%) for the M_{10}. The low %TA value observed at M_{10} after 18 h of fermentation was even lower than the %TA value

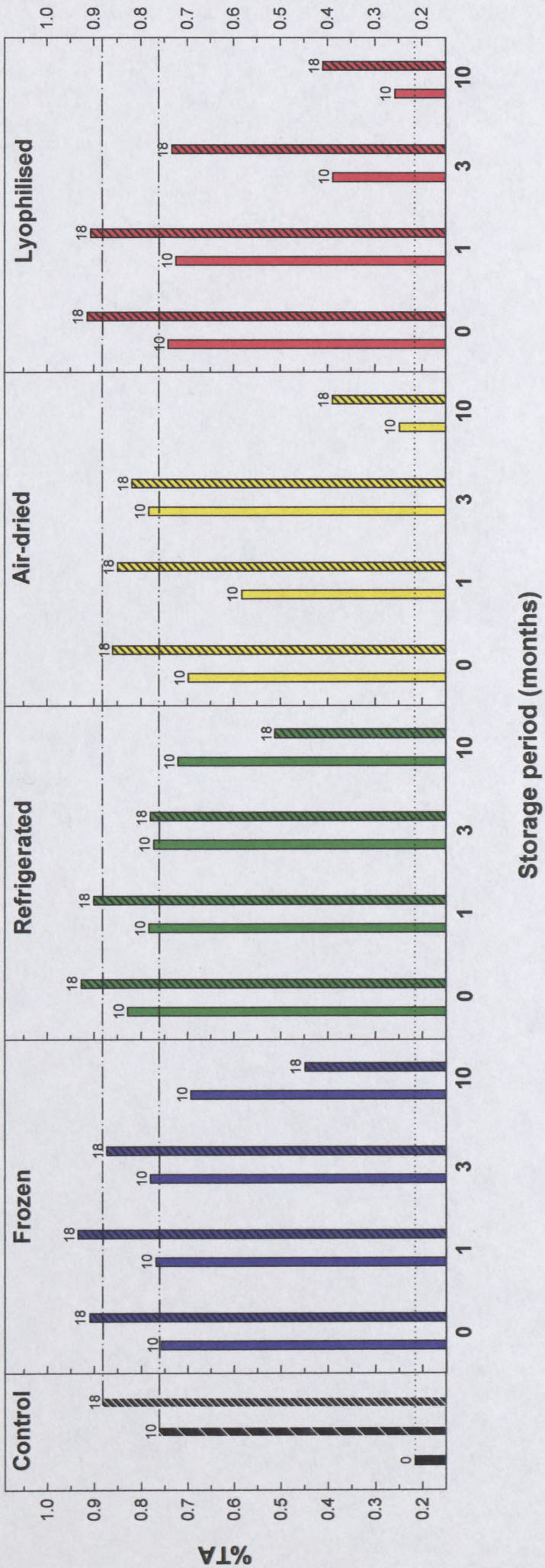

Figure 8. The average %TA values (of triplicate samples) of the Kepi prepared from the preserved grains for each of the time intervals: t = 10 h and 18 h and storage periods: directly after the application of the preservation techniques (M_0); after 1 month of storage (M_1); 3 months of storage (M_3); and 10 months of storage (M_{10}). The %TA values for t = 0 h (control = 0 h) is that of fresh milk and the average %TA values for the active grains (controls = 10 h, 18 h) served as controls.

obtained after the 10 h of fermentation (0.69%). No explanation can be offered for this tendency.

Similar %TA values were recorded for the 'refrigerated' samples after 10 h of fermentation throughout the 10-month storage period (~ 0.78%). A gradual decrease in the %TA values of the samples after 18 h of fermentation was noted with values of 0.93%, 0.90% and 0.78% for M_0, M_1 and M_3, respectively. However, the %TA for the 18 h fermentation period at M_{10}, as was found for the frozen samples, had a low value (0.51%). The low %TA values at M_{10} were observed for both the frozen and refrigerated grains despite a low pH and a high lactic acid content (data discussed later) of the samples. This confirms the observation made by Harper (1976) that no direct relationship exists between the total lactic acid and the titratable acidity in cultured products.

The %TA values obtained for the 'air-dried' grains at M_0 were comparable to the values of the active grains with values of 0.70% and 0.86% for the 10 h and 18 h fermentation periods. The %TA value for the 10 h fermentation period at M_1 showed a decrease (0.58%), but recovered to 0.78% after 3 months of storage. The values for the 18 h fermentation period for both M_1 and M_3 remained stable (0.86%). At M_{10}, however, a very sharp decrease in the %TA values were observed with values of 0.25% and 0.39% after 10 and 18 h of fermentation, respectively. These low %TA values after 10 months of storage were probably due to a prolonged lag phase of the Kepi grain microbes and the consequent decreased activity of the starter microbes present in the dried grains. In addition, the low %TA values can also possibly be ascribed to a lower number of viable bacterial cells present in the dried grains at the end of the 10-month storage period.

Similar %TA values, comparable to the active grains (control), were recorded for the 'lyophilised' grains for the 10 h (~ 0.74%) and 18 h (~ 0.92%) fermentation periods at both M_0 and M_1. However, a noticeable decrease in the %TA values was observed at M_3 (10 h = 0.39%, 18 h = 0.73%) and M_{10} (10 h = 0.26%, 18 h = 0.41%). As in the case of the air-dried grains, these low acidity values probably relate to a longer starter lag phase, a lower activity of the microbial population in the lyophilised Kepi grains and a decreased concentration of lactic acid (data discussed later) in the fermented samples. This loss in acidification activity by the Kepi grains could also be due to damage and even cell

death caused by the extreme dehydration and the extensive storage period of the grains.

Lactose utilisation and lactate production

The lactose and lactic acid content values for fresh milk (control = 0 h), as obtained by the NIR absorbance spectra, were 4.812 and 0.156 g.100 g^{-1} Kepi beverage, respectively (Fig. 9 and 10). In this study it was found that the lactose content decreased to 4.19 g.100 g^{-1} during the first 10 h of the fermentation (control = 10 h) and further decreased to 3.34 g.100 g^{-1} during the last 8 h of the fermentation period (control = 18 h), whereas the lactic acid content increased from 0.16 to 0.50 g.100 g^{-1} (control = 10 h) and from 0.50 to 0.60 g.100 g^{-1} (control = 18 h) for the corresponding fermentation periods.

The lactose content values obtained for the Kepi samples (Fig. 9) at the different stages of fermentation showed an increasingly lower lactose utilisation trend from M_0 to M_{10} for all the preservation techniques. The Kepi samples showed a small decrease in their lactic acid content over the 10-month storage period (Fig. 10) indicating the gradual loss in activity of the lactose utilising and lactic acid producing microbes over the 10-month storage period. In most cases the Fourier Transform Near Infrared (FT-NIR)-derived data showed the expected tendencies of decreased lactose and increased lactic acid content during the fermentation. However, it was found that the values for the lactose and lactic acid content were not really representative of the actual values and were thus not taken into further consideration.

As a result of the above lactose and lactic acid discrepancies, the lactose and lactic acid values of the Kepi samples for M_{10} were compared using both the NIR absorbance spectra and colorimetric and enzymatic techniques (Fig. 11). A summary of the data is also given in Table 1, where it can be seen that the lactose and lactic acid values for the same samples varied markedly between the different techniques.

The lactose content of the Kepi samples for M_{10}, obtained with the use of the colorimetric determination method, is shown in Fig. 11B. The average lactose content of triplicate samples of the unfermented milk as determined by this method was 5.00 g.100 g^{-1} Kepi. The data given in Fig. 11B clearly indicates that, with lactose utilisation taken as the measure of activity, no significant difference

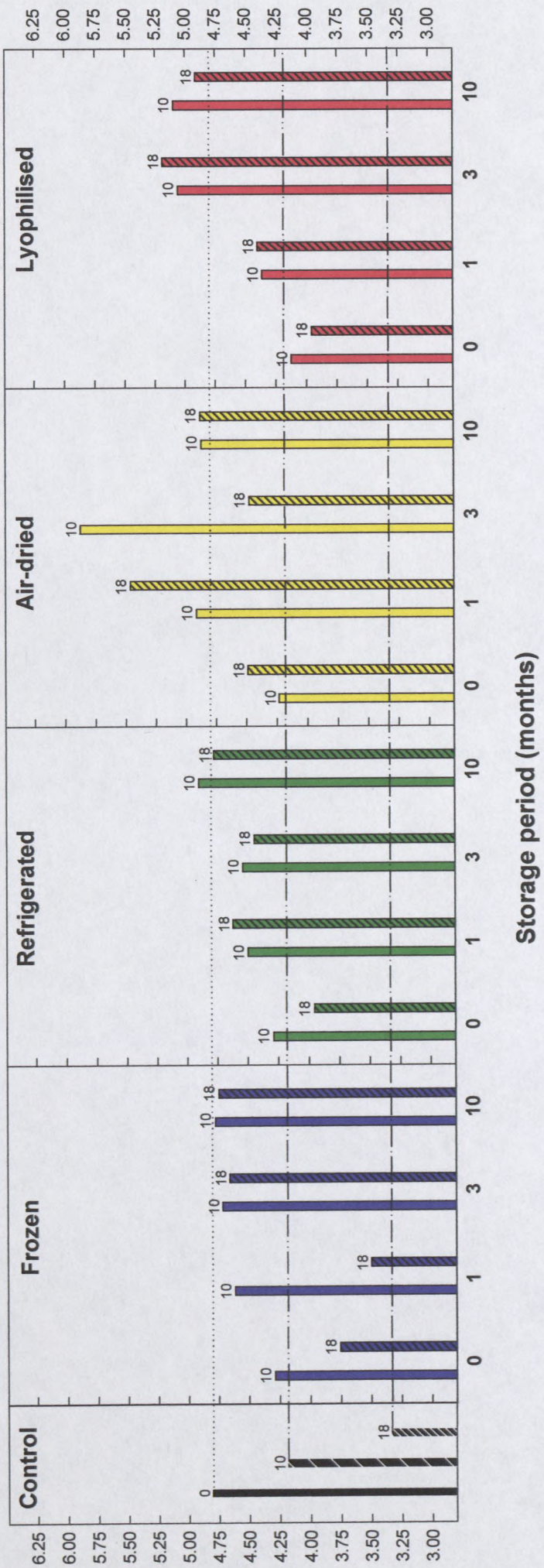

Figure 9. The average lactose content (g.100 g^{-1}) (of triplicate samples) of the Kepi prepared from the preserved grains for each of the time intervals: t = 10 h and 18 h and storage periods: directly after the application of the preservation techniques (M_0); after 1 month of storage (M_1); 3 months of storage (M_3); and 10 months of storage (M_{10}). The lactose content values for t = 0 h (control -= 0 h) is that of fresh milk and the average lactose content for the active grains (controls = 10 h, 18 h)) served as controls.

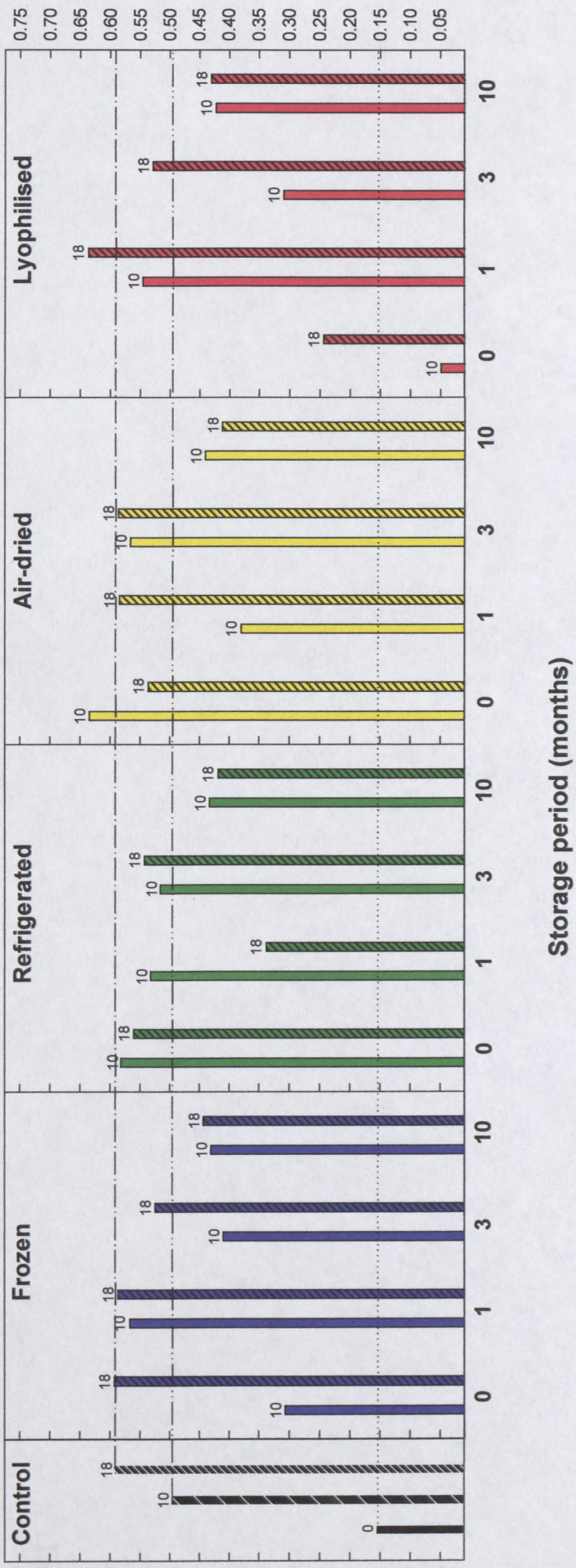

Figure 10. The average lactic acid content (g.100 g^{-1}) (of triplicate samples) of the Kepi prepared from the preserved grains for each of the time intervals: t = 10 h and 18 h and storage periods: directly after the application of the preservation techniques (M_0); after 1 month of storage (M_1); 3 months of storage (M_3); and 10 months of storage (M_{10}). The lactic acid content values for t = 0 h (control = 0 h) is that of fresh milk and the average lactic acid content for the active grains (controls = 10 h, 18 h) served as controls.

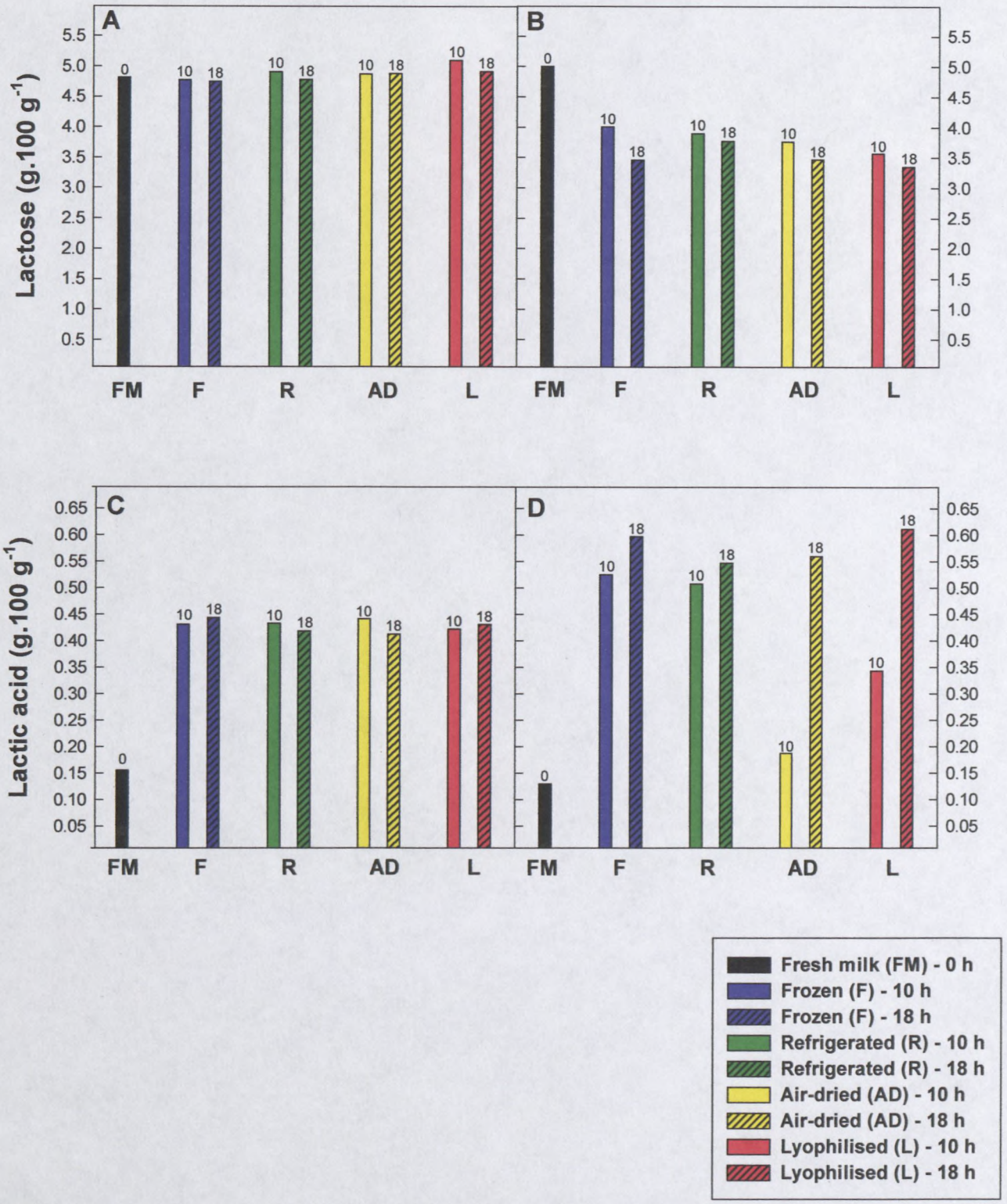

Figure 11. The average lactose and lactic acid content (g.100 g^{-1}) (of triplicate samples) of Kepi prepared with the four different preserved grains for each of the time intervals (t = 10 h and t = 18 h) after 10 months of storage as obtained by the NIR absorbance spectra (A and C) and the colorimetric (B) and enzymatic techniques (D). The average lactose and lactic acid values for t = 0 h is that of fresh milk.

Table 1. Comparison of lactose and lactic acid content of Kepi derived from the colorimetric and enzymatic methods and the FT-NIR spectra.

	Colorimetric/Enzymatic methods			NIR Spectroscopy	
	T0	**T10**	**T18**	**T10**	**T18**
Lactose content (g.100 g^{-1})[a]					
Frozen	4.81	4.00	3.46	4.77	4.75
Refrigerated	4.81	3.90	3.77	4.91	4.78
Air-Dried	4.81	3.77	3.47	4.88	4.88
Lyophilised	4.81	3.57	3.35	5.10	4.92
Lactic Acid content (g.100 g^{-1})[b]					
Frozen	0.16	0.53	0.60	0.43	0.44
Refrigerated	0.16	0.51	0.55	0.43	0.42
Air-Dried	0.16	0.19	0.56	0.44	0.41
Lyophilised	0.16	0.34	0.61	0.42	0.43

[a] colorimetric method

[b] enzymatic method

between the final values of the different preservation techniques after 10 months of storage was observed. The total lactose content of the milk (5.00 g.100 g^{-1}) was reduced to 3.77 g.100 g^{-1} by the 'refrigerated' grain samples, 3.47 g.100 g^{-1} with the 'air-dried' samples, 3.46 g.100 g^{-1} using the 'frozen' grain samples and 3.35 g. 100 g^{-1} by the 'lyophilised' samples (Fig. 11B).

The 'frozen' and the 'refrigerated' grain samples, using the colorimetric technique as measurement parameter showed the highest production of lactic acid after 10 h of the fermentation, with values of 0.53 and 0.51 g.100 g^{-1} (Fig. 11D). The 'air-dried' and 'lyophilised' grains had a much slower initial lactic acid production rate for this time period. The 'air-dried' grains showed a lactic acid production of only 0.06 g.100 g^{-1}, whereas the 'lyophilised' grains produced 0.21 g.100 g^{-1} lactic acid over the first 10 h of the fermentation period. However, after 18 h of fermentation the lactic acid content of the Kepi samples were more alike, indicating a relative similar activity for all of the preserved grain samples at the end of the fermentation period. The highest lactic acid production for this time period was recorded for the 'lyophilised' grains (0.61 g.100 g^{-1}), followed by the 'frozen' (0.60 g.100 g^{-1}), 'air-dried' (0.56 g.100 g^{-1}) and 'refrigerated' grains (0.55 g.100 g^{-1}).

Volatile compounds

Attempts to determine the volatile compounds present in the Kepi were made with the use of gas chromatography, but no positive results were obtained. The reason for this is probably due to the low content of the volatile compounds in the Kepi beverage brought about by the relative short fermentation period of 18 h. The production of Kepi usually requires an activation period followed by a 24 h fermentation period (18 h at 25°C followed by 6 h at 23°C) of which the last 6 h of fermentation mainly acts as a ripening period. As the activity tests were preformed only during the first 18 h of the fermentation period the optimal ripening and the consequent formation of the various volatile compounds in the Kepi beverage were, therefore, not attained. In addition, the absence of volatile compounds in the Kepi beverage can possibly also be ascribed to the loss of part of the yeast population, the main aroma-producing microbes in the Kepi grain, as a result of the preservation of the grains (Marshall, 1993). However, this is an aspect that will have to be determined in future follow-up studies.

Conclusions

The use of Kepi grains as a starter culture for the production of Kepi has widespread possibilities in South Africa, but a reliable and economic grain preservation technique are required to facilitate the successful marketing of this unique fermented beverage. The aim of this study was thus to evaluate four different preservation techniques in terms of pH, %TA, lactic acid production, lactose utilisation and the production of volatile compounds over a period of 10 months.

Based on the data obtained in this investigation it was concluded that, with the retention of the acidification activity in the preserved Kepi grain taken as the main activity measure, all four of the preservation techniques proved to be suitable for the preservation of the grains. 'Freezing' and 'refrigerated' storage showed a good retainment of the acidification activity throughout the 10-month storage period. The storage period had no significant influence on the activity of the 'frozen' and 'refrigerated' grains and both these preservation techniques resulted in a low final pH (~ 4.3) throughout the 10-month storage period. The 'air-drying' and 'lyophilisation' techniques also proved to be successful in retaining the acidification activity of the grains, however, an increased lag phase and a lower initial rate of pH decrease were observed for both techniques with the prolonged storage period. In addition, the results from the activity testing clearly showed that no direct relationship exists between both the pH and the total lactic acid content and the %TA values of the preserved grain samples. It is possible that the %TA values are influenced by the various buffer systems present in the fermented milk product.

The use of FT-NIR spectroscopy for a rapid and less expensive prediction of the lactose and lactic acid content in Kepi has great potential as an alternative to existing analytical methods, provided that an acceptable and reliable calibration dataset is used. However, in this study the use of the colorimetric and enzymatic techniques as analytical methods proved to be more accurate and reliable. It is hence suggested that further research still needs to be done to ensure the future success of the application of the Near Infrared technology in the determination of the metabolites in fermented milk products.

To be able to successfully market Kepi grains the preserved grains will also require an appropriate packaging material to facilitate the distribution of the grains. It is also important that the packaging will lead to the simultaneous retainment of the activity of the mass-cultured preserved grains over an extended period of time.

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APPENDIX

TO CHAPTER 3

To simplify the discussion of the results, the data illustrated in Fig. 3 – 6 have been included in this Appendix.

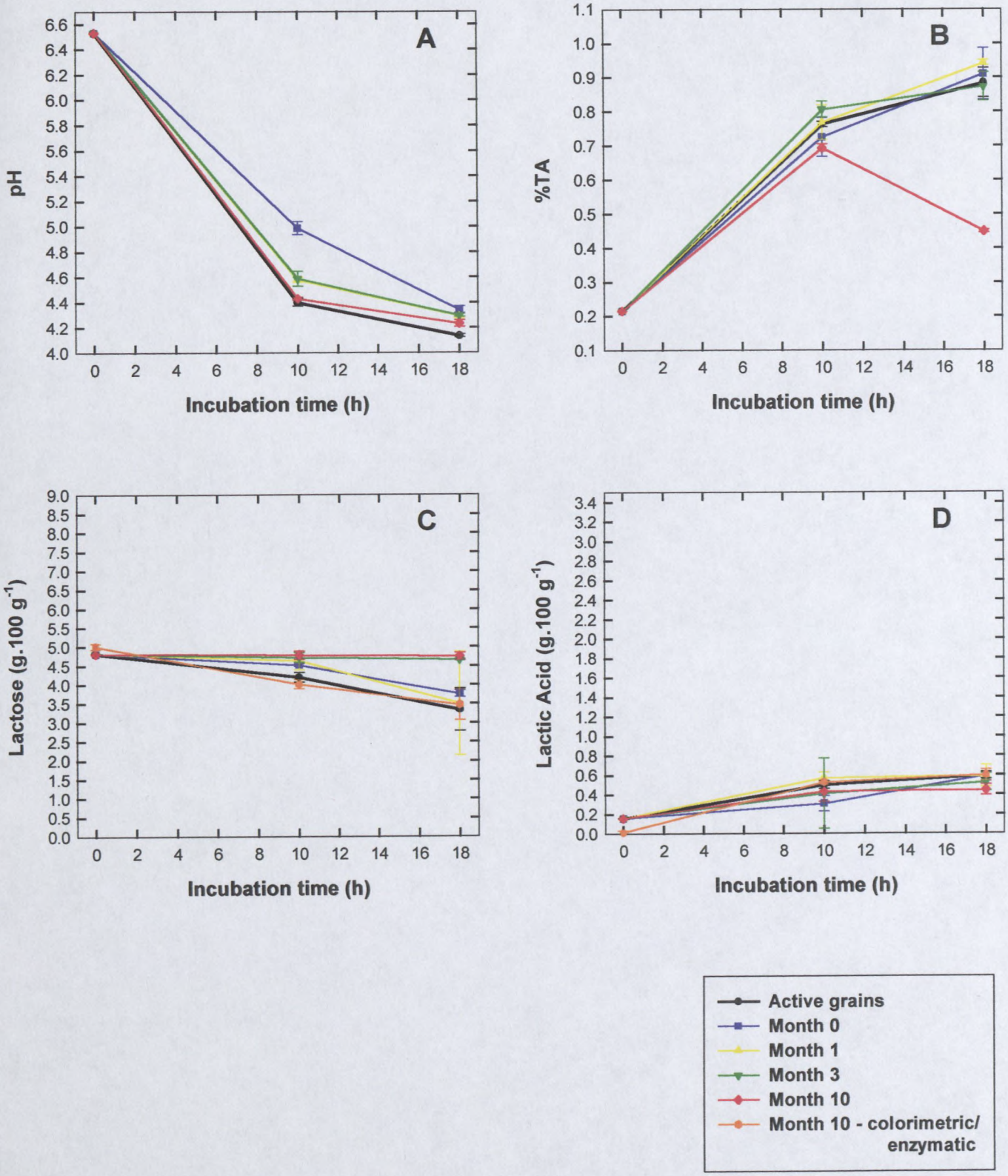

Figure 3. Separate pH (A), %TA (B), lactose (C) and lactic acid (D) content profiles of the frozen grain samples for the different storage periods M_0, M_1, M_3 and M_{10} (studies were done in triplicate). The standard deviation was used as the error-bar length.

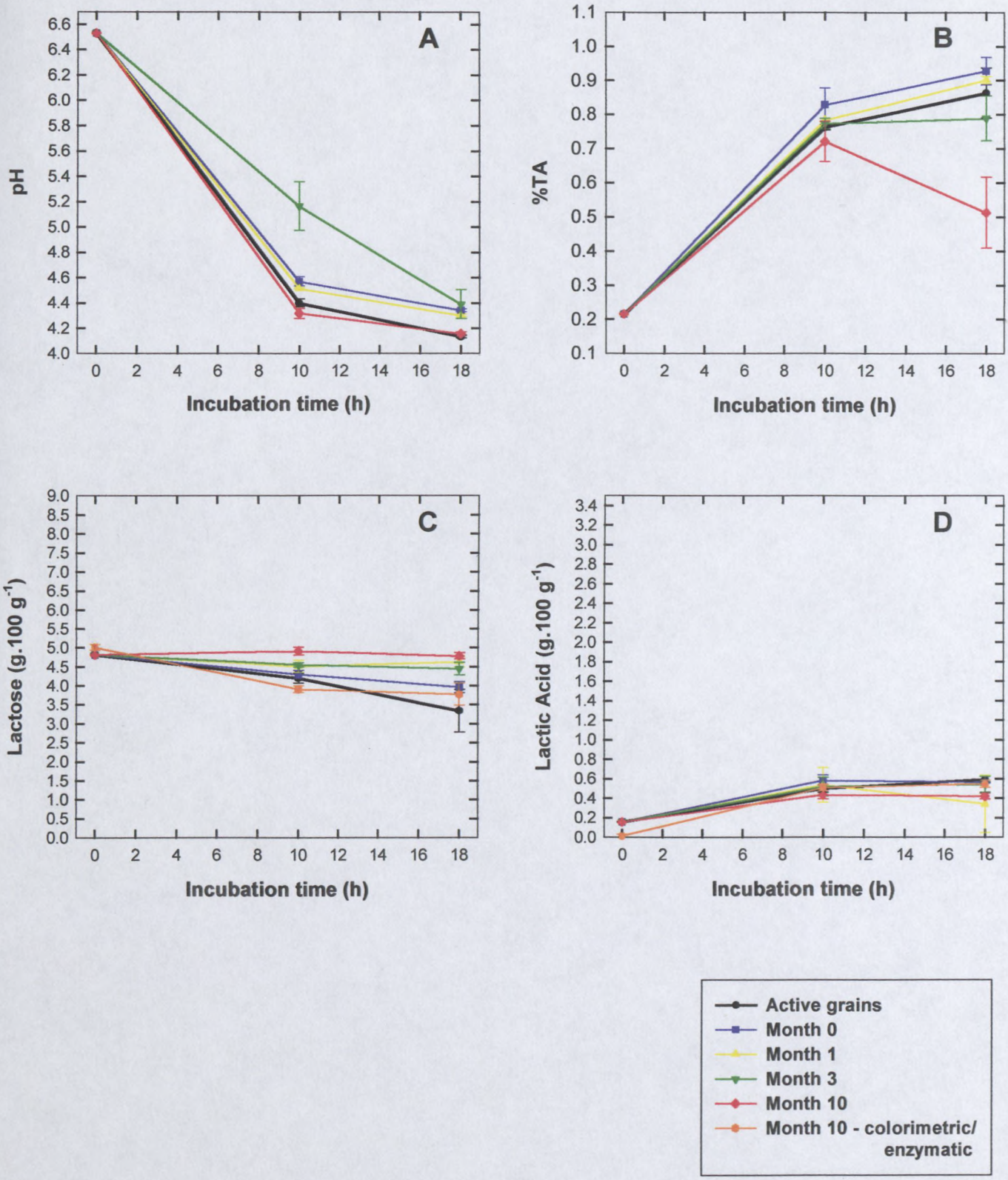

Figure 4. Separate pH (A), %TA (B), lactose (C) and lactic acid (D) content profiles of the refrigerated grain samples for the different storage periods M_0, M_1, M_3 and M_{10} (studies were done in triplicate). The standard deviation was used as the error-bar length.

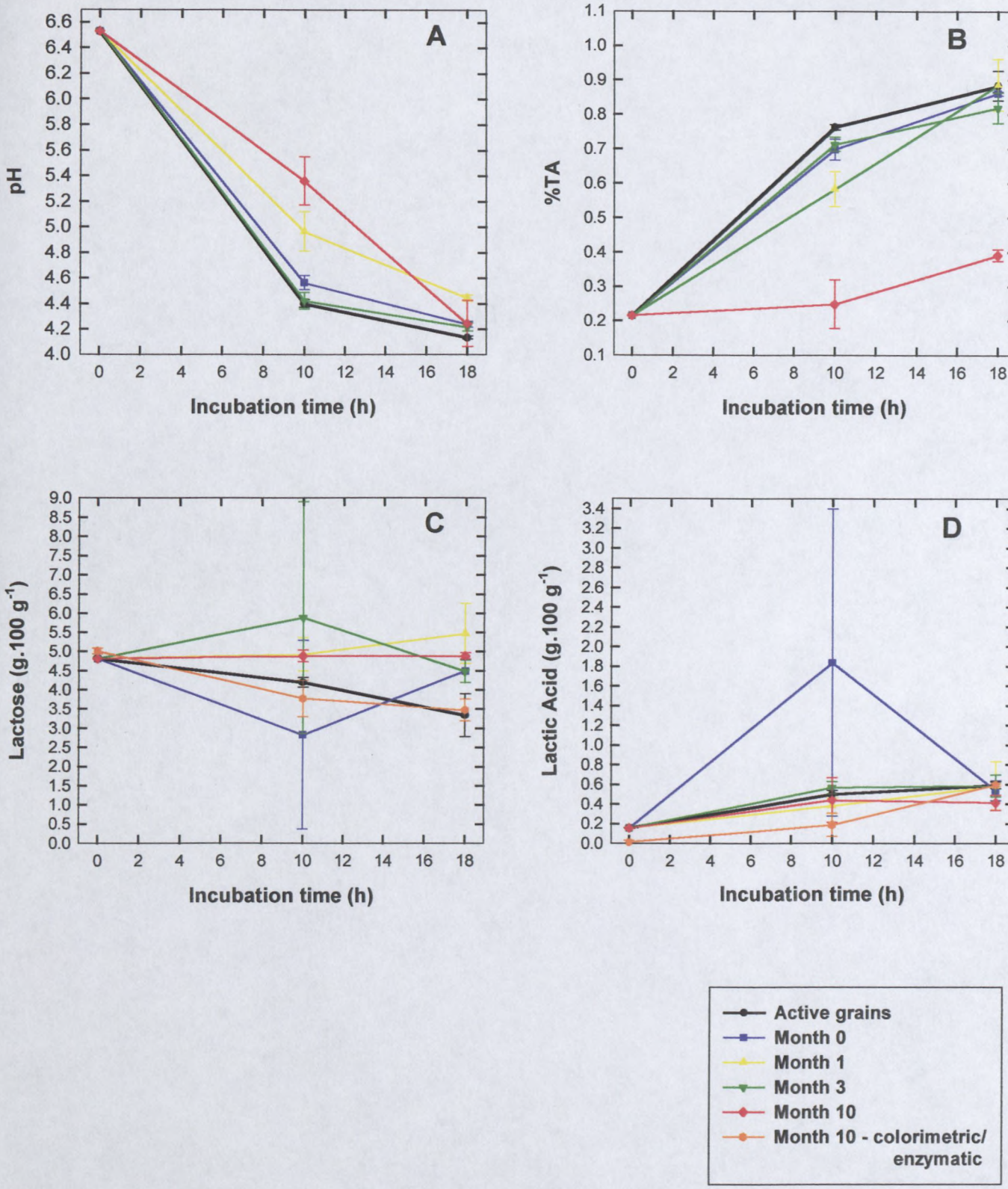

Figure 5. Separate pH (A), %TA (B), lactose (C) and lactic acid (D) content profiles of the air-dried grain samples for the different storage periods M_0, M_1, M_3 and M_{10} (studies were done in triplicate). The standard deviation was used as the error-bar length.

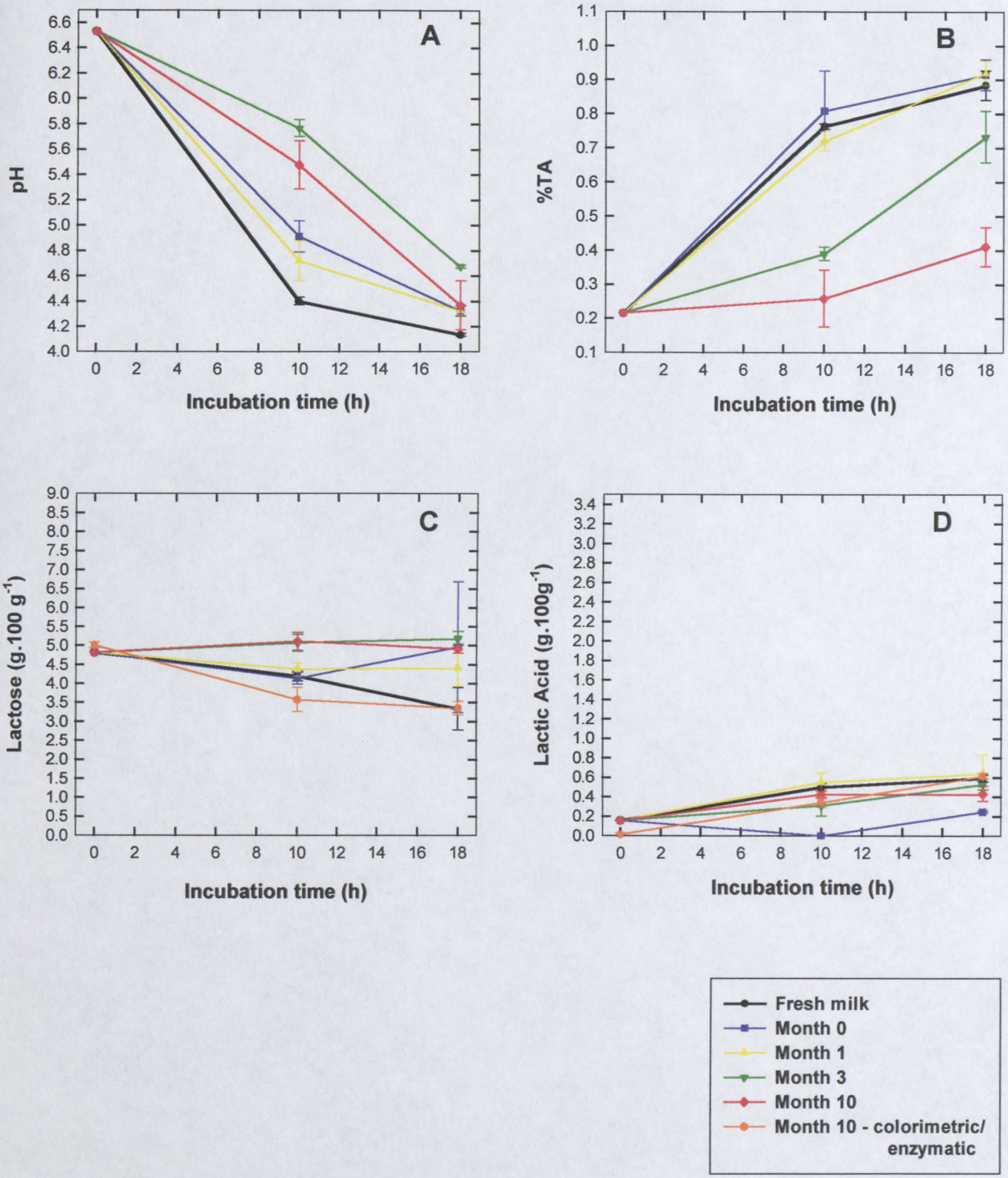

Figure 6. Separate pH (A), %TA (B), lactose (C) and lactic acid (D) content profiles of the lyophilised grain samples for the different storage periods M_0, M_1, M_3 and M_{10} (studies were done in triplicate). The standard deviation was used as the error-bar length.

CHAPTER 4

INFLUENCE OF DIFFERENT PACKAGING MATERIALS ON THE MICROBIAL ACTIVITY OF LYOPHILISED KEPI GRAINS

Abstract

The Kepi grain is a complex starter culture consisting of a variety of lactic acid bacteria and yeasts. These grains can effectively be preserved by lyophilisation and the successful distribution of these lyophilised grains will require appropriate packaging that will preserve the dried state and the acidification activity of the grains. The aim of this study was to evaluate three different packaging materials in terms of their ability to retain the viability and activity of the grains over an extended storage period. Three different packaging materials including a low density polyethylene film (LDPE), an oriented polyester film (OPET) and a metallised oriented polyester film (MOPET), were evaluated. Activity tests, including the monitoring of the pH, %TA, lactose and lactic acid content over an 18 h fermentation period, were used to evaluate the acidification activity of the lyophilised grains. Overall, the best retainment of the fermentation activity was found for the LDPE and the OPET packaging films. The storage period had a considerable influence on the retention of the activity of the packaged lyophilised grains. The viability study of the lyophilised packaged Kepi grains after two months of storage revealed leuconostocs and lactobacilli to be the predominating microbes of the grains. Low microbial counts were obtained from the lactococci-selecting medium for all three of the differently packaged Kepi grains, whereas no growth was observed on the media that selected for the propionibacteria and yeasts. The OPET packaging film provided the best preservation of the microbial composition.

Introduction

The unique and complex character of the Kepi grain contributes to the very distinguished taste and aroma of the Kepi beverage. These grains are normally moist, gelatinous granules with a white to yellowish colour (Vedamuthu, 1982) and have a cauliflower-like appearance varying in size from 0.5 - 3.5 cm (Marshall & Cole, 1985). The microbes present in the grains are firmly held together in the water-insoluble carbohydrate kefiran complex, which contributes to the extremely tough and resilient nature of the grains (La Rivière *et al.*, 1967; Marshall, 1993).

The preservation of Kepi grains can be achieved by various techniques, including freezing, lyophilisation, air-drying and refrigerated storage (Garrote *et al.*, 1997; Libudzisz & Piatkiewicz, 1990; Vedamuthu, 1982). In the previous study (Chapter 3 of this thesis) these four preservation techniques were compared and it was found that the frozen and refrigerated grains showed the best retainment of the Kepi microbial activity after a storage period of 10 months. In a recent marketing study conducted by Van Wyk (2000), the Kepi target market was identified as the low-income urban African communities. These communities often do not have cooling and freezing facilities at their disposal and it is, therefore, important to market and supply the Kepi grains in the least perishable state. Dried grains can either be obtained by air-drying or by lyophilisation. Due to an unacceptable odour, colour change and mould (mycelial fungus) growth on the air-dried grains, air-drying as a preservation method was considered unsuitable. It was, therefore, decided that grains in the lyophilised state would be the most suitable method to distribute and market the grains. The successful marketing and distribution of the lyophilised Kepi grains will of course require an appropriate packaging material that will positively contribute to the retention of the acidification activity of the mass-cultured preserved grains over an extended period of time.

The aim of this study was to determine the impact of three different packaging materials on the retainment of the microbial activity and the resulting acidification activity of lyophilised Kepi grains. In addition, the survival of the different groups of microbes present in the lyophilised packaged Kepi grains after two months of storage, was also investigated.

Materials and methods

Preservation and packaging of mass cultured Kepi grains

Frozen mass cultured Kepi grains, obtained from the Department of Food Science, University of Stellenbosch, Stellenbosch, South Africa, were lyophilised (Heto CT 60e Freeze Dryer, Denmark). The lyophilised Kepi grains were weighed into units of 4 g, representing the 18 g of fresh Kepi grains required to prepare 1 ℓ of Kepi beverage.

The grains were sealed in three different packaging materials including low-density polyethylene (LDPE), oriented polyester film (OPET) and metallised oriented polyester film (MOPET). These packaging materials were kindly supplied by the Nampak Group R&D, South Africa (Mr. F. Hannay, 2000, Nampak Group R&D, personal communication). The dimensions of the pouches were 10 cm x 8 cm and were heat-sealed under atmospheric conditions. The preserved grains comprised 50% of the sealed pouches. The packaged lyophilised grains were then stored in desiccators at room temperature. In Fig. 1, samples of the three different packaging materials are shown and the permeability properties of these packaging films are listed in Table 1.

Activity testing

Activity tests were performed on the Kepi grains directly after lyophilisation (M_0), and after 1 month (M_1), 2 months (M_2) and 3 months (M_3) of storage in the three different packaging materials. All activity tests were done in triplicate. At each time interval the lyophilised grains were activated by incubating the grains in 500 ml double pasteurised (80°C for 20 min) full cream milk for 36 h at 25°C. The grains were recovered by sieving through a sterile stainless steel kitchen sieve and then transferred to 1 ℓ of double pasteurised full cream milk. Activity tests were performed after 0, 10 and 18 h of incubation at 25°C and included the determination of the pH, the percentage titratable acidity (%TA), the lactose utilisation and lactate production.

The pH of the Kepi samples was measured with a Knick pH meter (pHB-4, Merck). The %TA of the samples was determined using the method of Dixon (1973). This determination was performed in duplicate for each sample.

Figure 1. Examples of the three different packaging materials: low density polyethylene (LDPE); oriented polyester film (OPET); and metallised oriented polyester film (MOPET) that were used for the packaging of the lyophilised Kepi grains.

Table 1. Moisture vapour transmission rates and oxygen permeability values of the three different packaging materials tested (Mr. F. Hannay, 2000, Nampak Group R&D, personal communication).

Film[a]	MVTR[b] ($g.m^{-2}$)	O_2-permeability[c] ($cm^3.m^{-2}$)	Costs (R/kg)
90 micron LDPE milk film (milk sachet)	5	~2000	15.06
12 micron OPET / 50 micron LDPE laminate (spirit sachet)	9	110	38.64
12 micron MOPET / 70 micron LDPE laminate (wine laminate)	0.8	1	48.90

[a] LDPE = low density polyethylene
OPET = oriented polyester film
MOPET = metallised polyester film

[b] Moisture vapour transmission rate measured at standard conditions of 38°C at 90% relative humidity and expressed as $g.m^{-2}$ per day.

[c] O_2-permeability measured at 23°C, 50% relative humidity and expressed as $cm^3.m^{-2}$ per day at 1 atm.

The lactose content of each Kepi sample was measured colorimetrically (Katsu, *et al.*, 1994) with the maximum absorbance being measured at 540 nm with the use of a Spectronic 20, Genesys spectrophotometer (Spectronic Instruments, USA). The lactic acid content of each Kepi sample was measured enzymatically with the use of the D-/L+ Lactic Acid Test Combination Kit (AEC Amersham, Germany).

Microbial content

The number of viable microbes present in the lyophilised grains after two months of storage at 25°C in the different packaging materials was determined on the various selective media given in Table 2. The selective media included the De Mann, Regosa and Sharpe Agar (MRS), Potassium Carboxymethyl Cellulose Agar (KCA), Acetobacter Perodoyans Medium (APM), Yeast Extract Lactose Medium (YELN), Pal Propiobac Medium (Pal-P) and Yeast Extract Chloramphenicol Agar (YEC). Ten grams of activated Kepi grains from each of the three different packaging materials were homogenised in 90 ml (1:10) sterile saline solution (0.85% (w/v) NaCl) using a Stomacher (BagMixer, Interscience, France) and used in the preparation of a dilution series (10^{-1} – 10^{-6}). A 1 ml volume from each of the diluted samples was plated on the different media. The media that selected for the lactobacilli, lactococci, leuconostocs and propionibacteria were incubated anaerobically using the Anaerocult A system (Merck) for 5 to 10 d at 30°C. The plates with the media that selected for the yeasts and the acetic acid bacteria were aerobically incubated for 3 to 5 d at 25°C. The microbial counts obtained were expressed as colony forming units per gram (cfu.g^{-1}) of Kepi grains.

Results and discussion

Activity tests

To simplify the discussion of the results, the pH, %TA, lactose and lactic acid profiles for the different packaging materials (Fig. 2 - 4) have been included as an Appendix at the end of the chapter.

Table 2. The selective media used to determine the microbial content if the Kepi grains.

Isolation medium[a]	Selected microbes
MRS-agar (Biolab) with 3% (w/v) ethanol (Merck) and 0.5% (w/v) filter sterilised cycloheximide (Merck).	Lactobacilli (MRS-medium)
KCA-medium (Nickels & Leesment, 1964) ($g.l^{-1}$): tryptone (Biolab) 20.0; yeast extract (Saarchem) 5.0; gelatine (Merck) 2.5; glucose (Merck) 5.0; lactose (Merck) 5.0; sodium chloride (Saarchem) 4.0; tri sodium citrate.$2H_2O$ (Saarchem) 2.0; calcium lactate.$5H_2O$ (Saarchem) 8.0; agar (Biolab) 15.0; calcium citrate (Saarchem) 10.0 and carboxymethyl cellulose (Merck) (1.5% w/v) 100 ml. 10 ml filter sterilised TTC (Oxoid) was added.	Lactococci (KCA+TTC-medium)
KCA-medium with 30 µg filter sterilised vancomycin (Fluka) (Benkerroum *et al.*, 1993).	Leuconostocs (KCA+V-medium)
APM-medium (Biolab) ($g.l^{-1}$): malt extract 15.0; yeast extract (Saarchem) 5.0 and agar (Biolab) 15.0. 60 ml filter sterilised ethanol (Merck) (50% v/v) was added.	Acetic acid bacteria (APM-medium)
YELN-medium ($g.l^{-1}$): yeast extract (Biolab) 5.0; sodium lactate (Saarchem) (60% v/v) 20.0; agar (Biolab) 15.0 and Tween 80 (Merck) 1.0 ml. 10 ml filter sterilised naladixic acid (0.02%) was added (Riedel *et al.*, 1994).	Propionibacteria (YELN-medium)
Pal Propiobac-medium (Thierry & Madec, 1995).	Propionibacteria (Pal-P-medium)
YEC Medium (Biolab) (Rea *et al.*, 1996)	Yeasts (YEC-medium)

[a] The following abbreviations were used: MRS = De Mann Regosa and Sharp Agar, KCA = Potassium Carboxymethyl Cellulose Agar, TTC = Triphenyltetrazolium Chloride, APM = Acetobacter Peroxydans Medium, YELN = Yeast Extract Lactate Naladixic Medium, YEC = Yeast Extract Chloramphenicol Agar.

pH profiles

An increase in the pH values, as shown in Fig. 5, from month one (M_1) to month three (M_3) were observed for all three of the differently preserved lyophilised grains, indicating a gradual loss in the acidification activity over the 3-month storage period. The control sample (freshly lyophilised grains) gave a decrease in the pH of the fresh milk from pH 6.53 to pH 4.50 after 10 h of the fermentation and a further decrease from pH 4.50 to pH 4.28 during the next 8 h of the fermentation period. The pH values of the Kepi samples prepared with the lyophilised grains packed in the three different packaging materials after one month of storage (M_1) showed little variation between the different packaging materials. After 10 h of fermentation at M_1 the pH values of the packaged grains (LDPE = pH 4.88; OPET = pH 5.08; MOPET = pH 5.03) were higher than the corresponding value for the control sample (pH 4.50). However, after 18 h of fermentation similar pH values (~ 4.20) were observed for the control as for the stored grains. This indicates an excellent retainment of the activity of the packaged grains after one month of storage. The slow initial drop in the pH could probably have been due to a prolonged lag phase of the microbes in the lyophilised Kepi grains.

After two months of storage (M_2) similar pH values were obtained from the grains stored in the LDPE (10 h = 5.50; 18 h = 4.46) and OPET (10 h = 5.54; 18 h = 4.49) films. The lyophilised grains that had been stored in the MOPET film for two months, however, showed higher pH values (10 h = 6.23; 18 h = 5.27) compared to the corresponding pH values of the LDPE and the OPET films.

Higher pH values for all the packaged grain samples were observed after three months of storage (M_3), indicating a decrease in the acidification activity of the packaged Kepi grains over the extended storage period. The pH values of the MOPET stored grains, however, remained stable and did not show a decrease from M_2 to M_3. The pH values obtained for M_3 were 6.24, 6.34 and 6.08 after 10 h of fermentation and 5.73, 6.03 and 5.40 after 18 h of fermentation for the LDPE, OPET and MOPET films, respectively.

%TA profiles

A sharp decrease in the %TA values was observed from M_1 to M_3 for all three the packaging materials, indicating a decrease in the acid producing ability of

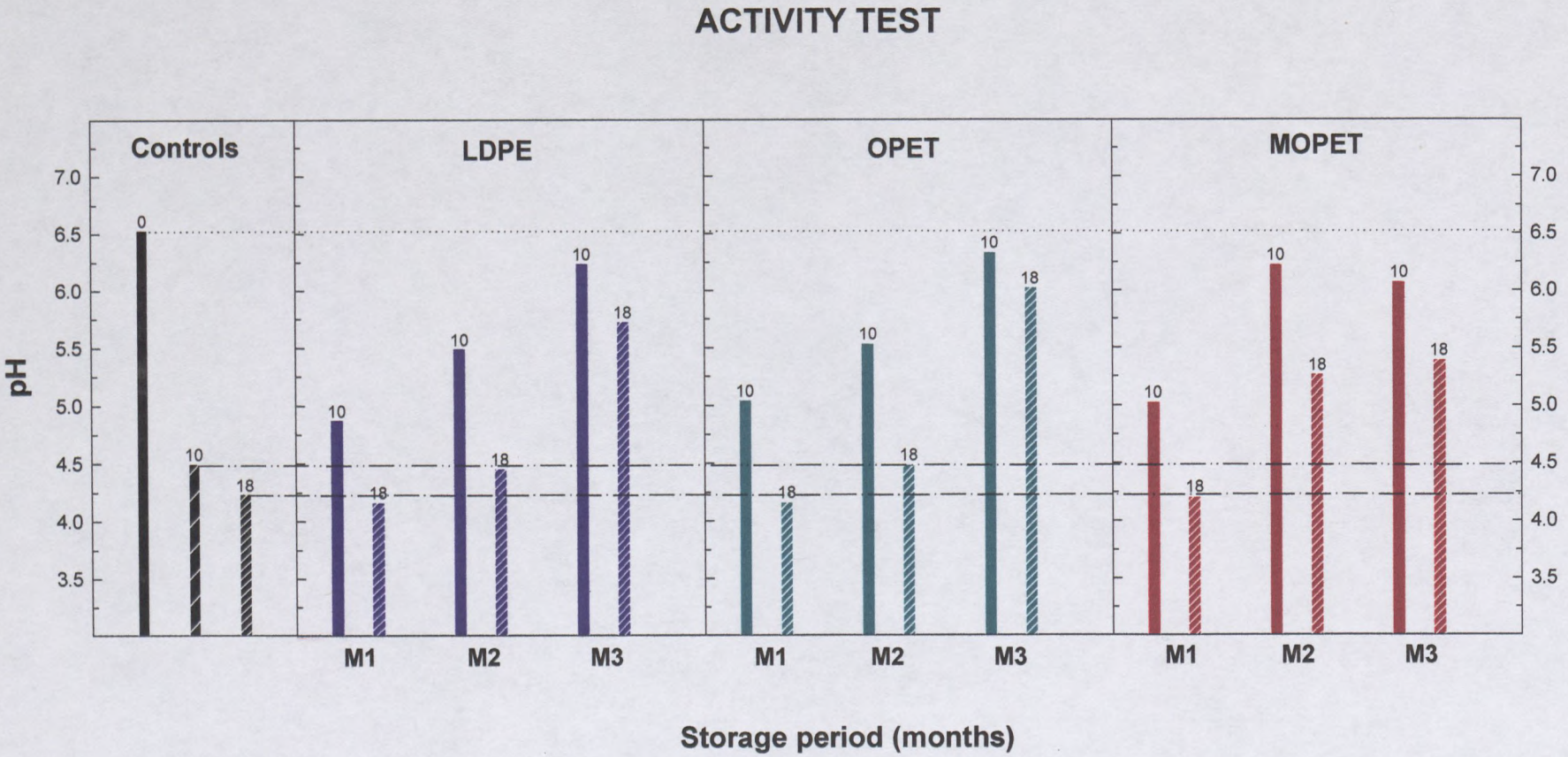

Figure 5. The average pH values (of triplicate samples) of the Kepi beverage prepared from the packaged lyophilised grains for each of the time intervals (10 h and 18 h) and the storage periods of 1, 2 and 3 months (M_1, M_2 and M_3). The pH value for t = 0 h (control = 0 h) is that of fresh milk and the average pH values for active Kepi grains served as controls (controls = 10 h, 18 h). (LDPE = low density polyethylene; OPET = oriented polyester; MOPET = metallised oriented polyester).

the packaged Kepi grains (Fig. 6). The %TA values for the control sample showed an increase from 0.22 to 0.71% over the first 10 h of the fermentation and an increase from 0.71 to 0.81% during the last 8 h of the 18 h fermentation period.

At M_1, %TA values that were similar to that of the control sample were observed for all three of the packaging materials tested. These results are in accordance with the relatively low pH values that were recorded for the grains after one month of storage (Fig. 5). Increasingly lower %TA values were recorded after two (M_2) and the three months (M_3) of storage.

The lowest %TA values at M_2 were observed for the MOPET stored grains (10 h = 0.25%; 18 h = 0.41%). In contrast the LDPE (10 h = 0.35%; 18 h = 0.52%) and OPET (10 h = 0.39%; 18 h 0.50%) packaging materials showed a slightly better retainment of the acidification activity.

At M_3 very low %TA values, compared to that of the control sample, were obtained for the LDPE (10 h = 0.23%; 18 h = 0.34%) and OPET (10 h = 0.23%; 18 h = 0.27%) packaged grains. The acidification activity of the MOPET stored grains, however, remained stable at M_3 as %TA values similar to that of M_2 were recorded. These low % TA values can be attributed to the relatively high storage temperature of 25°C.

Lactose utilisation

The lactose content of the Kepi beverage prepared from the packaged lyophilised grains showed progressively higher residual values with prolonged storage, indicating a gradual loss in the lactose utilisation activity of the stored grains (Fig. 7). A sharp decrease in the lactose content of the control sample from 5.05 to 3.35 g.100 g^{-1} was observed during the course of the 18 h fermentation. The LDPE stored grains showed a relative stable acidification activity fairly similar to that of the control sample at M_1 and M_2 (10 h = ~ 4.00 g.100 g^{-1}; 18 h = ~ 3.40 g.100 g^{-1}). At M_3, however, the lactose content of the prepared Kepi samples was higher (10 h = 5.58 g.100 g^{-1}; 18 h = 5.08 g.100 g^{-1}) indicating a lower acidification activity. The OPET and MOPET stored grains showed lower lactose utilisation results over the 3-month storage period compared to the LDPE film. Little variation between the lactose content values of M_1 and M_2 was observed for the OPET stored grains with lactose content values of ~ 4.50 and ~ 4.10 g.100 g^{-1} for the 10 h and the 18 h fermentation periods, respectively. After three months of

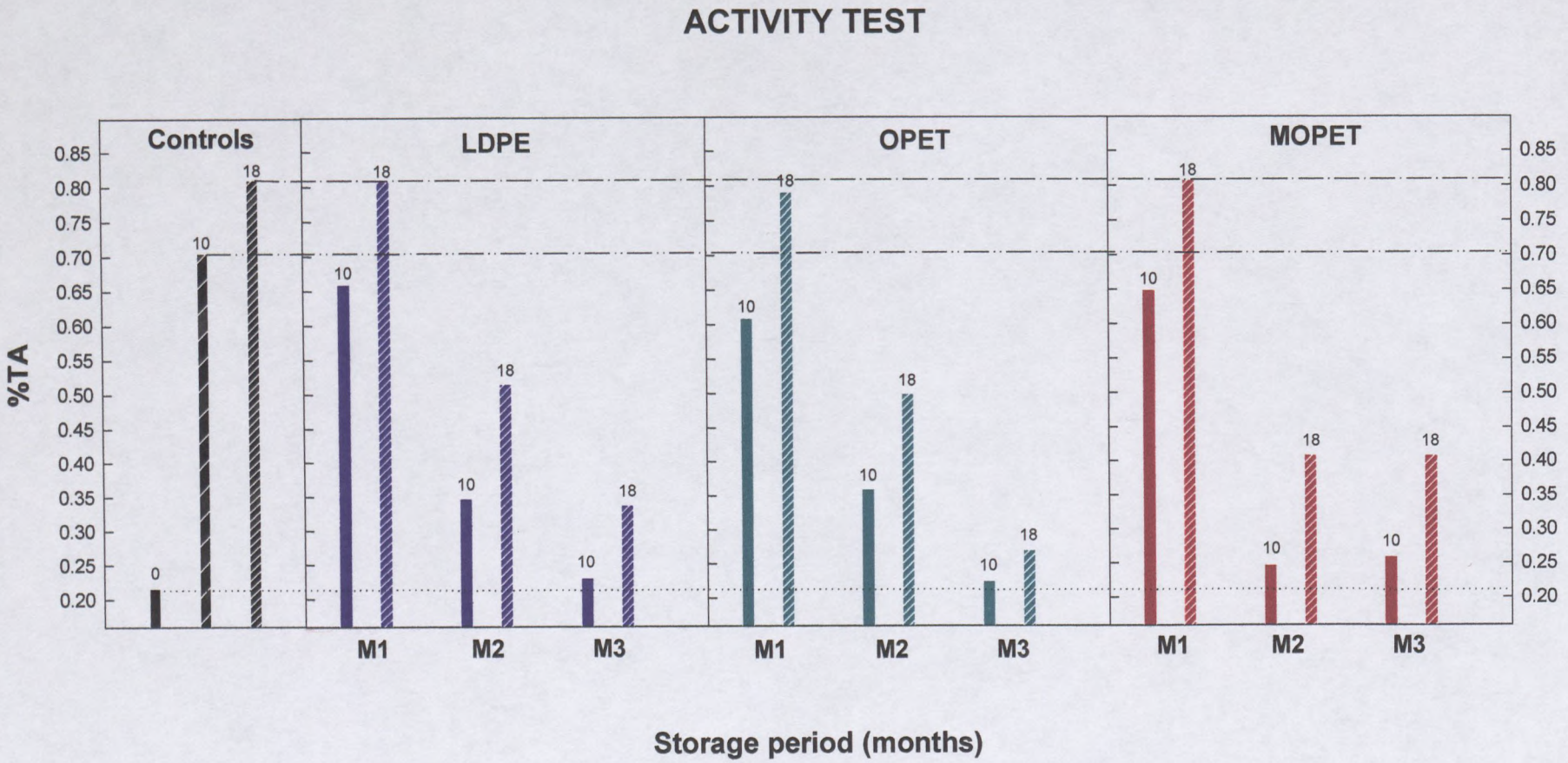

Figure 6. The average %TA values (of triplicate samples) of the Kepi beverage prepared from the packaged lyophilised grains for each of the time intervals (10 h and 18 h) and the storage periods of 1, 2 and 3 months (M_1, M_2 and M_3). The %TA value for t = 0 h (control = 0 h) is that of fresh milk and the average %TA values for active Kepi grains served as controls (controls = 10 h, 18 h). (LDPE = low density polyethylene; OPET = oriented polyester; MOPET = metallised oriented polyester).

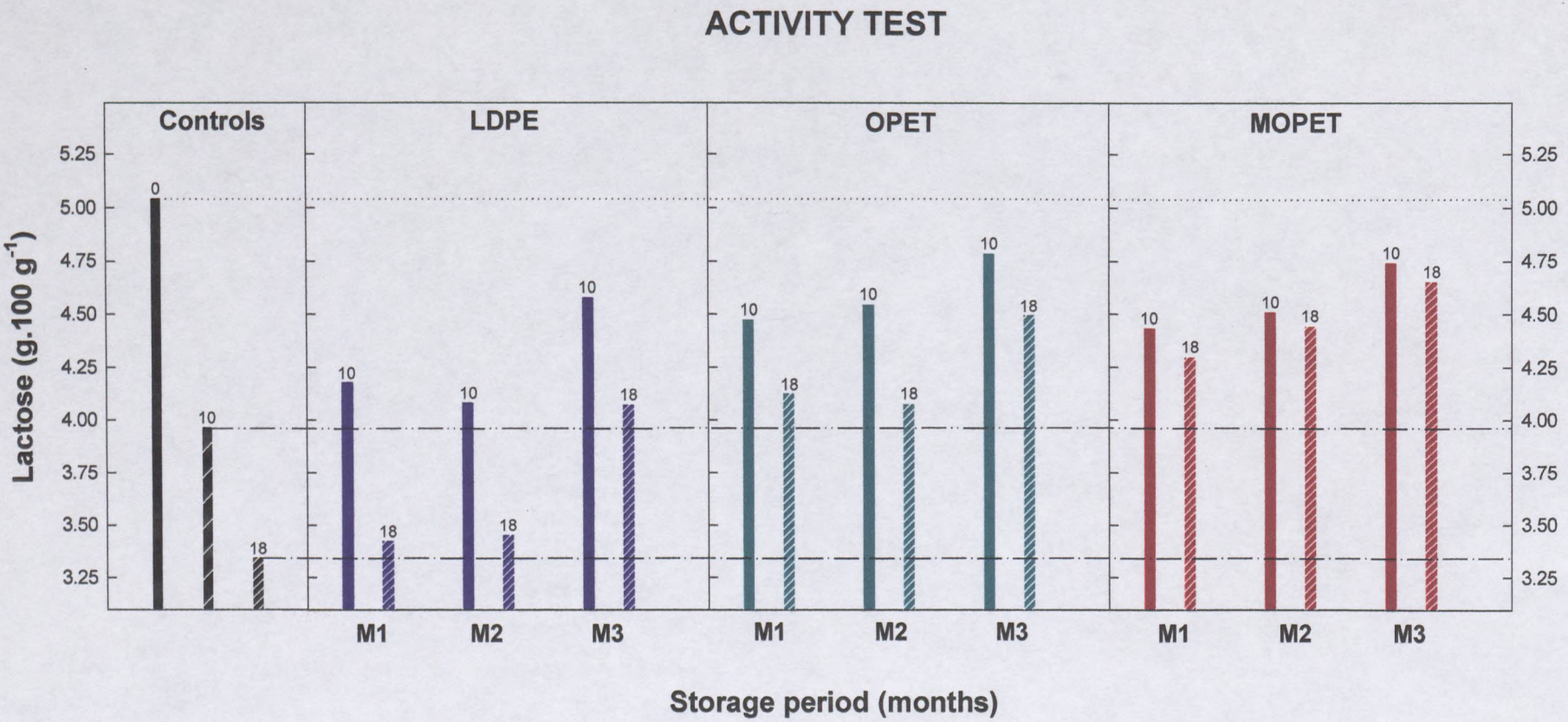

Figure 7. The average lactose content (g.100 g^{-1}) (of triplicate samples) of the Kepi beverage prepared from the packaged lyophilised grains for each of the time intervals (10 h and 18 h) and the storage periods of 1, 2 and 3 months (M_1, M_2 and M_3). The lactose content of the Kepi for t = 0 h (control = 0 h) is that of fresh milk and the average lactose content values for active Kepi grains served as controls (controls = 10 h, 18 h). (LDPE = low density polyethylene; OPET = oriented polyester; MOPET = metallised oriented polyester).

storage in the OPET film, high lactose content values (10 h = 4.79 g.100 g^{-1}; 18 h = 4.50 g.100 g^{-1}) were recorded. The lactose utilisation by the MOPET stored grains showed a similar trend to that of the grains that had been stored in the OPET bags, as comparable lactose content values were recorded at M_1 and M_2 (10 h = ~ 4.45 g.100 g^{-1}; 18 h = ~ 4.35 g.100 g^{-1}) followed by a higher lactose content at M_3 (10 h = 4.75 g.100 g^{-1}; 18 h = 4.66 g.100 g^{-1}).

Lactate production

The lactic acid content of the samples was found to increase with the prolonged fermentation (to 18 h) but progressively decreased over the 3-month storage period (Fig. 8). The lactic acid content of the control sample showed an increase from 0.01 to 0.40 g.100 g^{-1} for the first 10 h of the fermentation and a further increase to 0.57 g.100 g^{-1} over the last 8 h of the fermentation.

The lyophilised grains stored in the LDPE film bags had a gradual decrease in lactic acid production over the first two months of the storage period, followed by a sharp decrease in the lactic acid production after three months of storage. The lactic acid content of the Kepi samples at the end of the fermentation period was 0.48 g.100 g^{-1} and 0.45 g.100 g^{-1} for M_1 and M_2, respectively, whereas the lactic acid content of the 18 h sample at M_3 was only 0.14 g.100 g^{-1}.

The Kepi grains stored in the OPET film bags also showed the gradual decrease in the lactic acid content over the 3-month storage period with total lactic acid content values of 0.52 g.100 g^{-1}, 0.41 g.100 g^{-1} and 0.29 g.100 g^{-1} at 18 h for M_1, M_2 and M_3, respectively.

Likewise, a decreasing trend in the total lactic acid content was observed for the MOPET stored grains over the 3-month storage period. After 18 h of the fermentation the lactic acid content values of 0.50 g.100 g^{-1}, 0.40 g.100 g^{-1} and 0.22 g.100 g^{-1} were recorded at 18 h for M_1, M_2 and M_3, respectively.

In all cases a slow initial rate of lactic acid production was observed for the packaged grains, indicating a possible extended lag phase of microbial growth induced by the lyophilisation treatment and the storage period and storage conditions. In addition, an increasing lag phase for each of the differently packaged lyophilised Kepi grain samples was observed for the 3-month storage period. It is possible that this dehydration treatment in combination with the extended storage period and storage conditions may have caused injury to the microbial cells present in the Kepi grains or even led to cell death. The data

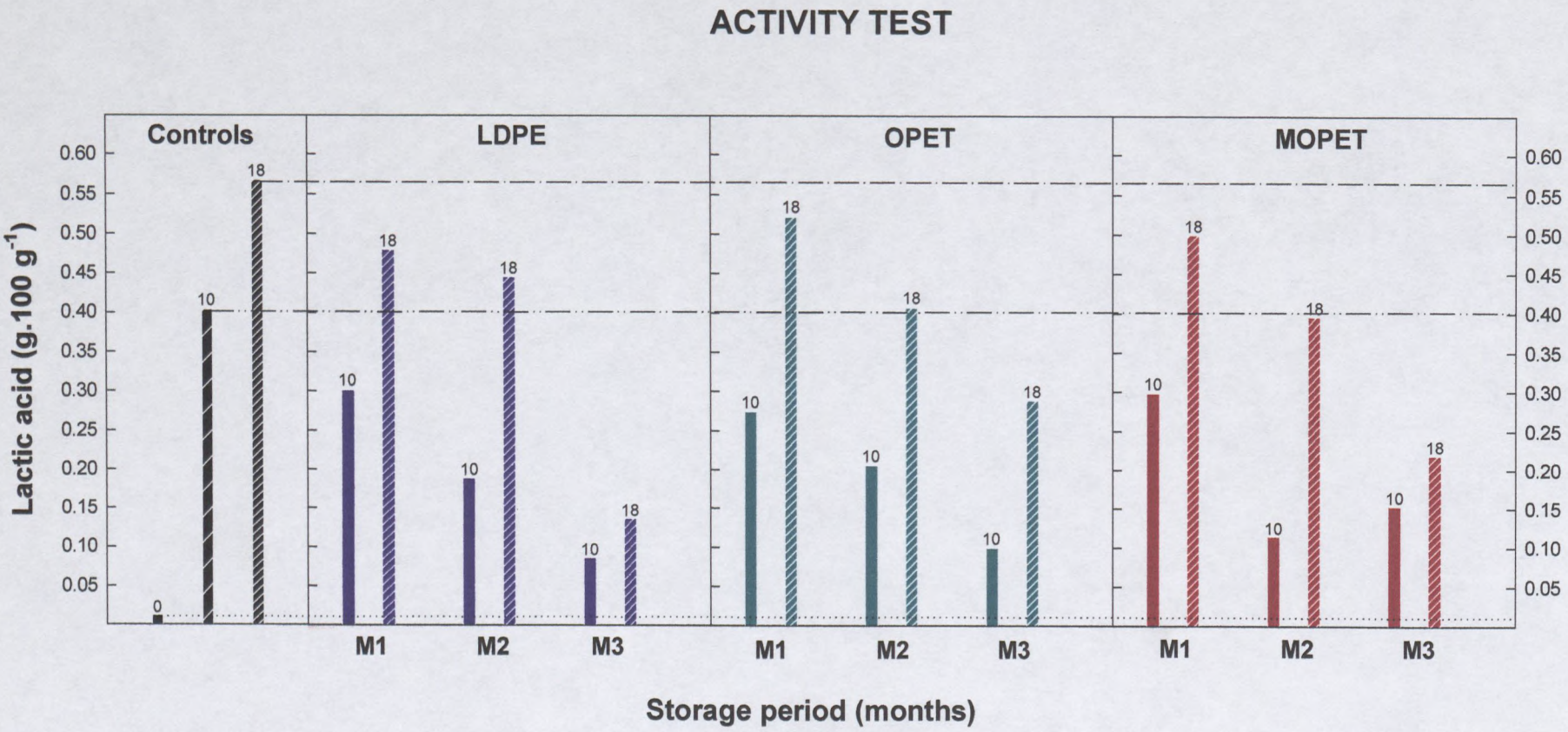

Figure 8. The average lactic acid content (g.100 g^{-1}) (of triplicate samples) of the Kepi beverage prepared from the packaged lyophilised grains for each of the time intervals (10 h and 18 h) and the storage periods of 1, 2 and 3 months (M_1, M_2 and M_3). The lactic acid content of the Kepi for t = 0 h (control = 0 h) is that of fresh milk and the average lactic acid content values for active Kepi grains served as controls (controls = 10 h, 18 h). (LDPE = low density polyethylene; OPET = oriented polyester; MOPET = metallised oriented polyester).

obtained clearly showed that time may have been required to repair any "reversible cellular injury" prior to growth and lactic acid production (To & Etzel, 1997).

Microbial content of packaged, lyophilised Kepi grains

In this study a similar trend in the enumeration values for the differently packaged lyophilised grains was observed, with the OPET film persistently giving a higher microbial count than the LDPE and the MOPET films (Table 3, Fig. 9). After an activation period of three days the highest microbial counts (average of triplicates) obtained for the LDPE stored grains were on the APM (2.6×10^6 cfu.g^{-1}) and KCA+V-media (1.7×10^6 cfu.g^{-1}). A lower count of 7.3×10^5 cfu.g^{-1} was obtained with the MRS-medium and the lowest microbial count for the Kepi grains packaged in the LDPE films was found on the KCA+TTC-medium (1.1×10^4 cfu.g^{-1}). No growth was found on either the YELN, Pal-P or YEC-media.

The highest enumeration values (average of triplicates) for the OPET stored grains were obtained from the KCA+V-medium (6.5×10^6 cfu.g^{-1}), followed by the APM (3.0×10^6 cfu.g^{-1}) and the MRS-media (1.4×10^6 cfu.g^{-1}). As with the LDPE stored grains, the lowest average microbial counts were found on the KCA+TTC-medium (4.3×10^4 cfu.g^{-1}). Again, no growth was observed on the YELN, Pal-P and YEC-media.

A similar pattern was observed for the grains that had been packaged in the MOPET films. A high average microbial count was obtained on the KCA+V (3.2×10^6 cfu.g^{-1}) and APM-media (1.8×10^6 cfu.g^{-1}). The average microbial counts obtained from the MRS-medium was lower at 5.6×10^5 cfu.g^{-1}, while the lowest average count for the MOPET stored grains was obtained from the KCA+TTC-medium (8.1×10^3 cfu.g^{-1}). Again, no growth was found on the YELN, Pal-P and YEC-media.

The enumeration values for the differently packaged lyophilised Kepi grains as obtained with the different selective media showed fairly similar trends (Fig. 9, Table 3). The microbial counts for the lyophilised grains stored in the OPET films (2.7×10^6 cfu.g^{-1}) were higher than found for either the MOPET (1.4×10^6 cfu.g^{-1}) or the LDPE (1.25×10^6 cfu.g^{-1}) films for every group of Kepi microbes selected. It can thus be assumed that the unique moisture vapour transmission rates and the oxygen permeabilities of the three different packaging materials did have an

Table 3. The average (of triplicate samples) microbial counts ($cfu.g^{-1}$) obtained from lyophilised packaged Kepi grains after two months of storage.

Medium	Active grains	Packaging materials		
		LDPE	OPET	MOPET
APM	1.8×10^8	2.6×10^6	3.0×10^6	1.8×10^6
KCA+V	3.9×10^4	1.7×10^6	6.5×10^6	3.2×10^6
MRS	1.4×10^8	7.3×10^5	1.4×10^6	5.6×10^5
KCA+TTC	1.9×10^8	1.1×10^4	4.3×10^4	8.1×10^3
YELN	1.9×10^8	0	0	0
Pal-P	1.3×10^4	0	0	0
YEC	1.0×10^3	0	0	0

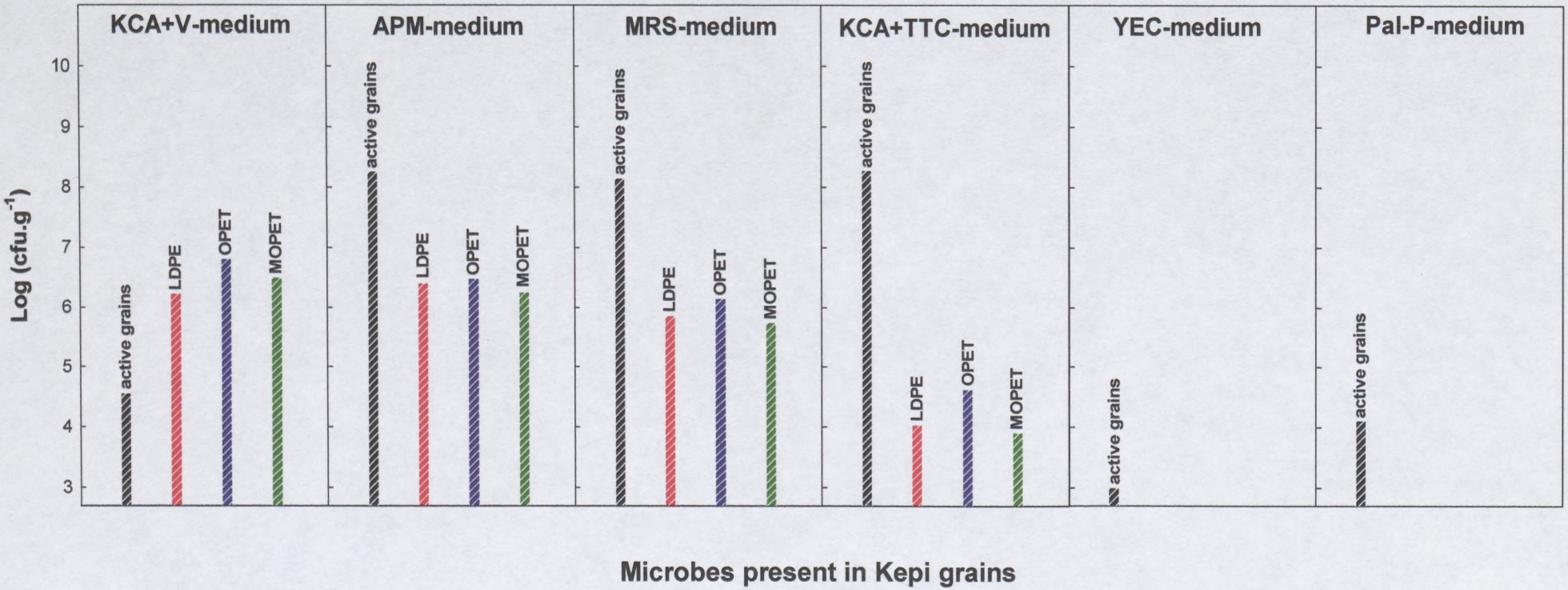

Figure 9. Enumeration values (cfu.g^{-1}) of the microbes present in the lyophilised and differently packaged Kepi grains that were stored for two months (control = active Kepi grains). (Packaging materials: LDPE = low density polyethylene; OPET = oriented polyester; MOPET = metallised oriented polyester).

influence on the number of viable microbes present in the packaged lyophilised Kepi grains after two months of storage. Furthermore, the much lower enumeration values that were recorded for the packaged lyophilised Kepi grains compared to the corresponding values obtained from active Kepi grains (control) (Fig. 9, Table 3) indicated the adverse effect of the storage period on the viability of the Kepi grain microbes.

Low enumeration values were obtained from the KCA+TTC-medium that is generally recommended for the selection of the lactococci. A possible explanation can be that the lactococci, which are situated on the surface layers (Varnam & Sutherland, 1994) of the Kepi grain, could have been washed off into the milk during the 3-day activation period. In addition, as a result of the known rapid growth of the lactococci a longer activation period should allow for the recovery of the lactococci population.

The absence of viable yeasts in the lyophilised grains as was shown by the absence of growth on the YEC-medium was expected, as research has shown that more than 80% of the yeasts present in Kepi grains can be lost on lyophilisation of the grains (Marshall, 1993). However, it is possible that the yeasts may only have been present in very low concentrations at this early stage of Kepi production or that they might not have been metabolically active. Furthermore, Bottazzi *et al.* (1980) reported that yeasts normally dominate in the centre of the Kepi grains, justifying their low presence. Once again, it is possible that the negative yeasts count after the lyophilisation and packaging is only temporary and that a longer period of Kepi grain activation and subsequent Kepi production as well as a more rigorous making of the Kepi grains would lead to an increase in the enumeration values, but this will still have to be confirmed in future studies.

No propionibacteria were found to be present in the lyophilised Kepi grains even though positive counts were found on the Pal-P-medium. This observation was expected as the presence of propionibacteria in the Kepi grain has not been reported in the past. However, the presence of propionibacteria in Kepi has been detected with the use of a rapid polymerase chain reaction (PCR) method (Ms. T. Schoeman, 2001, Department of Food Science, Stellenbosch, personal communication), but unfortunately only high concentrations ($> 1 \times 10^6$ cfu.g^{-1}) of propionibacteria can be detected using this method. It is possible that propionibacteria in the Kepi are only present in small numbers or are bound by the

kefiran in the kepi grain and can, therefore, not easily be isolated. In addition, since it is known that propionibacteria are slow-growing microbes (Perez-Chaia *et al.*, 1994) it is also possible that inadequate time is allowed for the development of these microbes during the production of Kepi.

In this study, the selective media used to isolate lactic acid bacteria (MRS, KCA+TTC and KCA+V) were found not to be very specific as lactobacilli species were found (Ms. T. Schoeman, 2001, Department of Food Science, Stellenbosch, personal communication) on all three of the selective media. In addition, lactobacilli were also isolated from the Pal-P and APM-media. It is, therefore, important not to derive conclusions about the microbial content of a fermentation environment entirely on media selectivity alone.

Conclusions

In Chapter 3 of this thesis the use of lyophilisation was found to provide an acceptable preservation technique for mass-cultured Kepi grains. These mass-cultured grains have promising marketing potential to provide a dried but stable starter culture. However, with an appropriate packaging as a prerequisite for the successful marketing of the lyophilised Kepi grains it was, therefore, necessary to evaluate the applicability of different packaging films in terms of their respective abilities to preserve the acidification activity of the lyophilised Kepi grains.

Based on the data obtained from this study it was concluded that there was a difference between the three different packaging materials in terms of their ability to preserve the acidification activity of the lyophilised Kepi grains. The packaging and storage of the grains had a considerable impact on the viability and survival of the Kepi grain microbes, causing a substantial decrease in the lactic acid bacteria counts and loss of the yeast population. It was also found that similar acidification activities were recorded for the lyophilised grains packaged in all three the different packaging materials after one month of storage. However, after two and three months of storage the best retainment of activity was found with the LDPE and OPET packaging films. The storage period of three months had a considerable negative influence on the activity of the lyophilised grains, resulting in decreased activity and prolonged lag phases of the Kepi grain microbes.

The survival of the microbes present in the lyophilised grains after two months of storage were clearly impacted by the three different packaging materials, with the highest retainment of the microbial composition found in the OPET packaging film. It was, therefore, concluded that all three of the packaging materials are suitable for the packaging and storage of the lyophilised Kepi grains, but only for periods of up to one month. However, if storage periods of two months or longer are necessary, the use of the OPET film for the packaging and preservation of the acidification activity and the retainment of an acceptable microbial level of the lyophilised grains can be recommended.

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APPENDIX

TO CHAPTER 4

To simplify the discussion of the results, the data illustrated in Fig. 2 - 4 have been included in this Appendix.

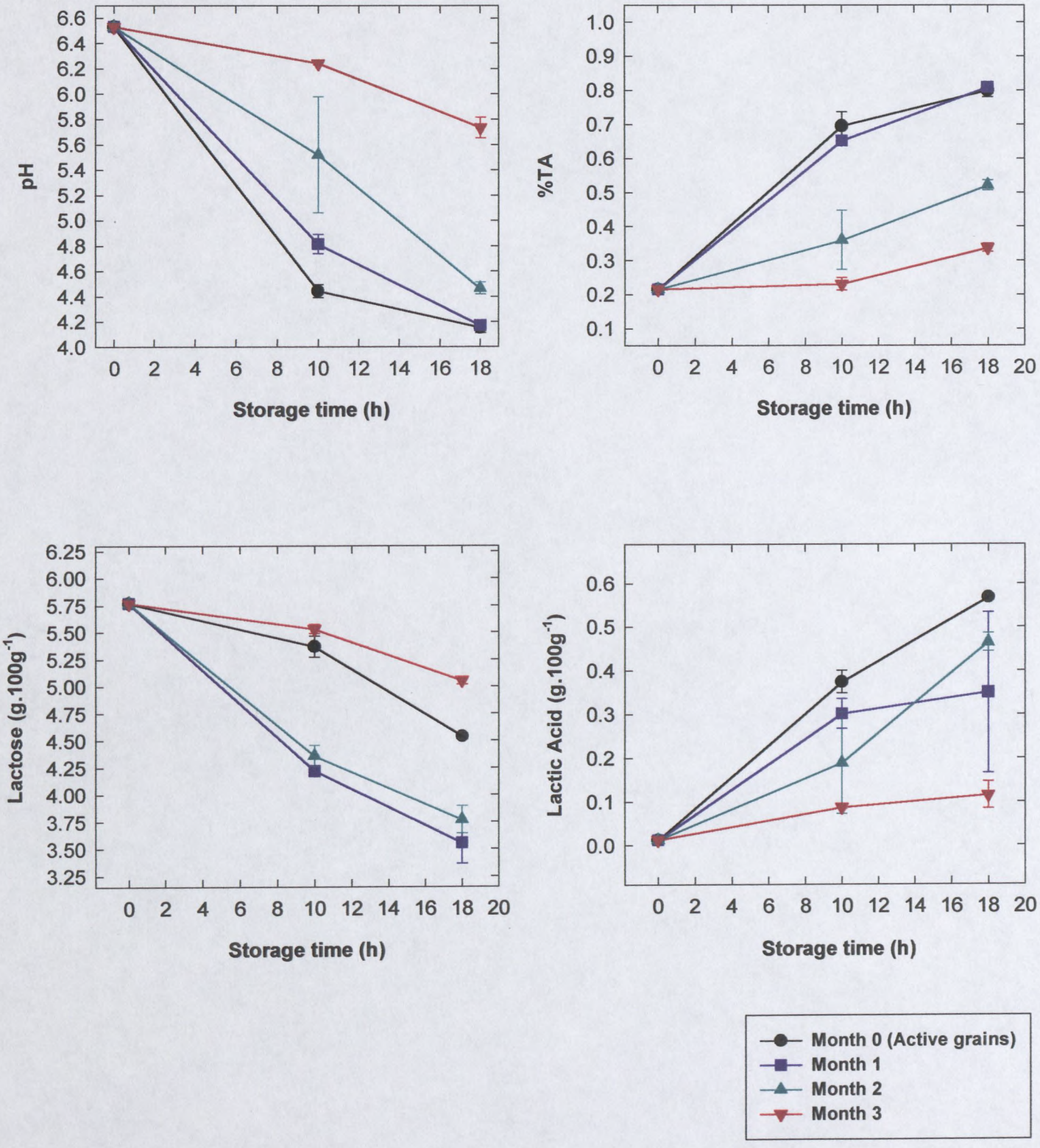

Figure 2. The pH, %TA lactose and lactic acid content profiles of the lyophilised Kepi grains that were packaged in the low density polyethylene (LDPE) film bags for the different storage periods M_0, M_1, M_2 and M_3 (studies were done in triplicate). The standard deviation was used as the error-bar length.

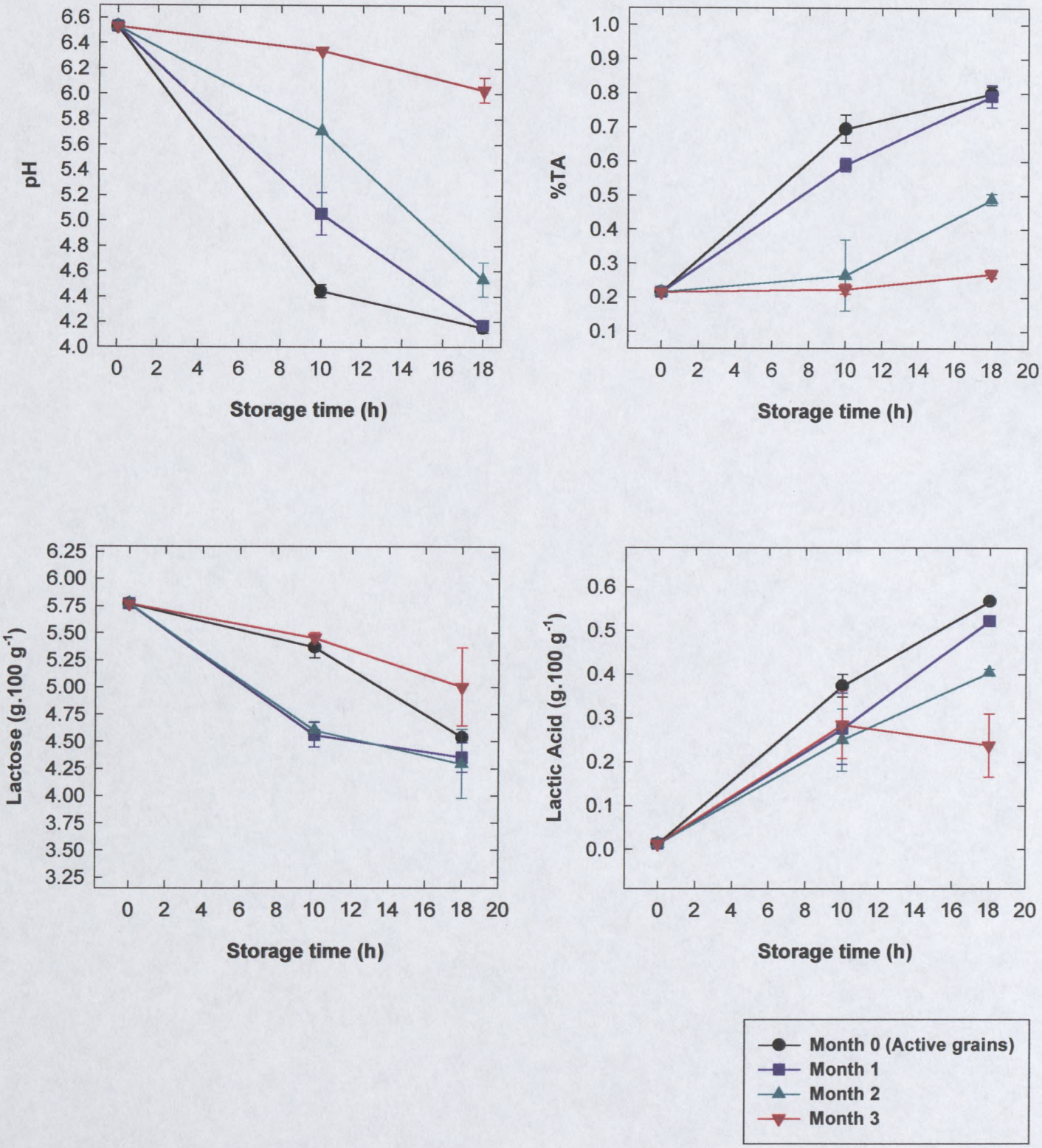

Figure 3. The pH, %TA lactose and lactic acid content profiles of the lyophilised Kepi grains that were packaged in the oriented polyester (OPET) film bags for the different storage periods M_0, M_1, M_2 and M_3 (studies were done in triplicate). The standard deviation was used as the error-bar length.

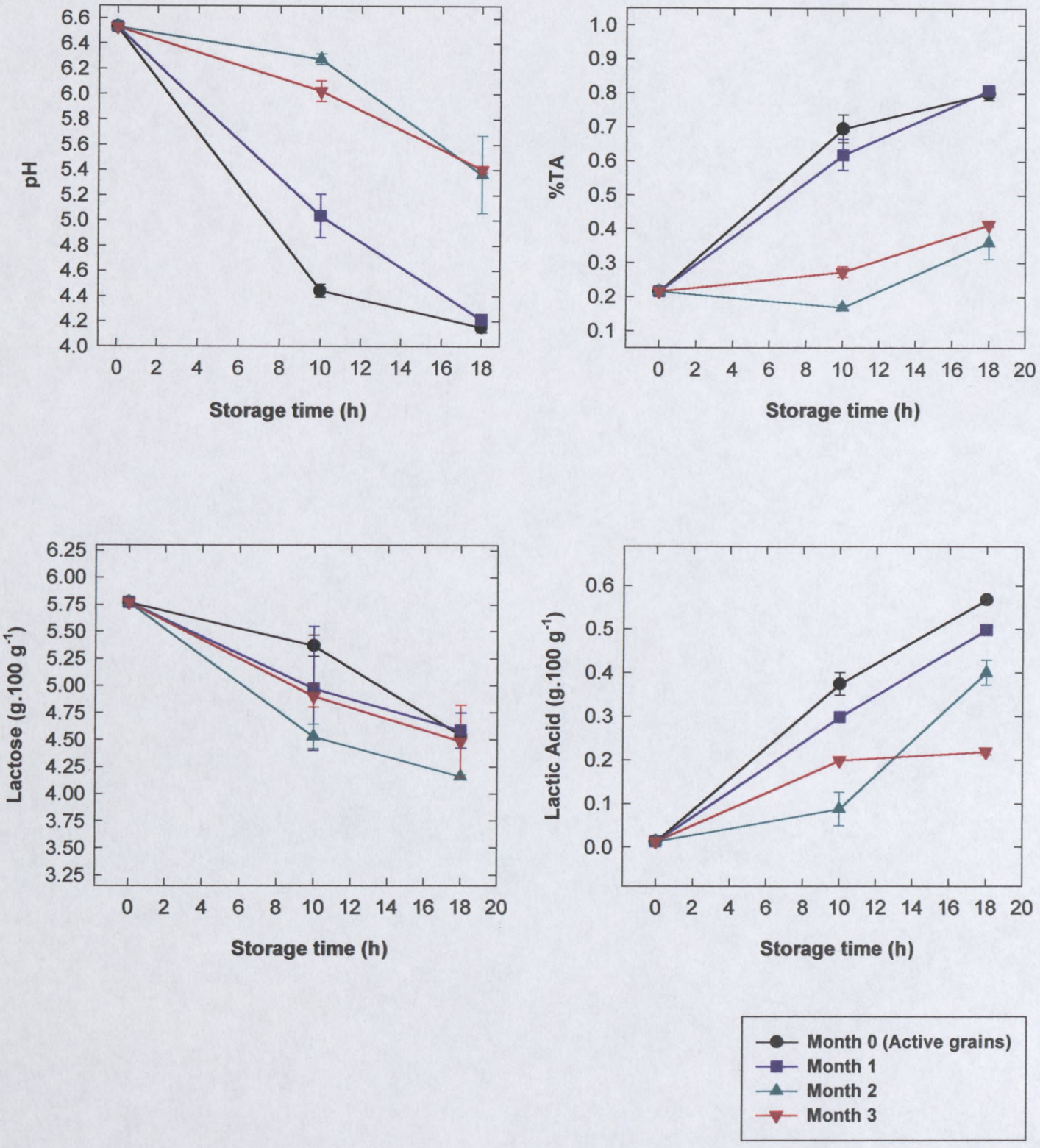

Figure 4. The pH, %TA lactose and lactic acid content profiles of the lyophilised Kepi grains that were packaged in the metallised oriented polyester (MOPET) film bags for the different storage periods M_0, M_1, M_2 and M_3 (studies were done in triplicate). The standard deviation was used as the error-bar length.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Background

The rapid urbanisation of the rural Black African population of Southern Africa during the past few years has resulted in the establishment of large impoverished urban communities (Myburgh, 1995). In spite of the urbanisation and partly due to the high population density, the food consumption profile of these communities still resembles that of a traditional rural community (Myburgh, 1995). Maas, a fermented milk product prepared from unpasteurised milk, is an essential part of their daily diet. This product provides all the nutrients found in fresh milk and is easily tolerated by lactose maldigesting individuals, who comprise up to 95% of these communities (De Villiers, 1990). Unfortunately, several factors such as legislation that forbids the selling of unpasteurised milk without a licence and the consequently growing unavailability of this milk prevents the low-income Africans from making Maas (Anon., 1997). Furthermore, as pasteurisation of milk destroys the lactic acid bacteria responsible for the natural souring of the milk, traditional Maas cannot be prepared from pasteurised milk. In addition, a limited buying power prevents these communities from purchasing the more costly commercially available Maas. As Maas is the main dairy product consumed by these communities, this situation results in incidences of malnutrition (Bourne *et al.*, 1994).

Thus, a demand for a low cost nutritious fermented milk product that can easily be prepared with pasteurised milk, is safe to consume and has a taste similar to traditional Maas, exists in the low-income urban African communities. A fermented dairy beverage with the ability to satisfy this demand is Kepi. Kepi is made with the use of an unique starter, the Kepi grains, which are re-usable and will, therefore, minimise the production costs and simplify processing. The Department of Food Science at the University of Stellenbosch has recently developed a technique for the mass-culturing of the Kepi grains that renders

biomass increases of up to 600% over a 10-day period (SA Patent 2000/2826). Hence, an adequate supply of Kepi grains is available for the marketing of the grains.

Preservation of Kepi grains

The successful marketing and distribution of the Kepi beverage and Kepi grains necessitates the application of a reliable and economic preservation technique. The appropriate preservation technique(s) will need to preserve the fermentation activity of the Kepi grains over an extended storage period of up to 6 months. In this study four different preservation techniques were evaluated in terms of the retention of the fermentation activity of the Kepi grains over a period of 10 months.

It was found that the four preservation techniques (freezing at -18°C, refrigeration at 4°C, air-drying and lyophilisation both stored at 4°C) all showed a good retention of the acidification activity throughout the 10-month storage period. It was also found that the storage period had no adverse effects on the activity of the frozen and refrigerated Kepi grains. However, with prolonged storage, an increased lag phase and a lower initial rate of pH decrease were observed for the lyophilised and air-dried grains. Despite this slow initial acidification activity observed for the lyophilised and the air-dried grains, the total decrease in the pH after 18 h of fermentation was in the same order for all the preserved samples. It was, therefore, concluded that all four of the preservation techniques would be suitable for the preservation of Kepi grains and the subsequent storage for three months or possibly longer. However, for storage periods of 10 months or longer, the use of freezing and refrigeration are recommended as the most suitable preservation techniques.

One negative aspect was found. The presence of volatile compounds could not be detected in the Kepi samples. This absence of the volatile compounds is probably due to an inadequate fermentation period (18 h), subsequently preventing the optimal ripening and production of the volatile compounds in the Kepi beverage. In addition, part of the yeast population, the main aroma-producing microbes in the Kepi grains, was lost or minimised during the preservation of the grains, resulting in a lower content or even absence of volatile compounds in the Kepi.

Packaging of lyophilised Kepi grains

In a recent marketing study conducted by Van Wyk (2000), the target market of Kepi was identified as the low-income urban African communities. As these communities often do not have the proper cooling and freezing facilities at their disposal, it is essential to market the Kepi grains in its least perishable state, which is the lyophilised grains. Due to an unacceptable odour, colour change and mould (mycelial fungus) growth that developed during the drying period on the air-dried grains this method was considered unsuitable for marketing. It was hence decided to market and distribute the grains in the lyophilised state. However, an appropriate packaging, which will positively contribute to the retention of the activity of the mass-cultured lyophilised Kepi grains over an extended period, is needed to facilitate the successful marketing of the grains. Three different packaging materials (low density polyethylene - LDPE, oriented polyester - OPET and metallised polyester - MOPET) kindly supplied by the Nampak Group R&D, South Africa, were thus evaluated in terms of the above-mentioned criteria.

Similar acidification activities were recorded for the lyophilised grains packaged in the three different packaging materials after one month of storage. However, after two and three months of storage at room temperature under atmospheric conditions the best retainment of activity was found with the LDPE and OPET packaging films.

The survival of the microbes present in the lyophilised grains after two months of storage was influenced by the three different packaging materials, with the OPET packaging film giving the best retainment of the microbial counts. The storage period had a marked influence on the activity of the lyophilised grains, resulting in a gradually decreasing activity and prolonged lag phase over the 3-month period. In addition, the storage period and lyophilisation treatment had a considerable effect on the viability and survival of the Kepi grain microbes, causing a substantial decrease in the lactic acid bacteria counts and loss of the yeast population.

In conclusion, for storage periods longer than one month the use of the OPET film for the packaging and preservation of the acidification activity and the microbial content of the lyophilised grains is recommended. For the production of

an organoleptically acceptable and good quality Kepi beverage from these packaged, lyophilised Kepi grains, optimisation of the production processes will probably have to be implemented.

Recommendations and future research

In Chapter 3 of this thesis the use of lyophilisation was found to provide an acceptable preservation technique for mass-cultured Kepi grains that has promising marketing potential to provide a dried but stable starter culture. With packaging as a prerequisite for the successful marketing of the lyophilised Kepi grains the appropriate packaging for the grains were evaluated in Chapter 4 of this thesis.

The improved retainment of the fermentation activity in the preserved and packaged Kepi grains can be achieved by the application of additional treatments to the grains prior to preservation, followed by improved storage conditions. It is suggested that the Kepi grains be activated prior to the preservation treatment as it was found that active grains showed a much higher retention of activity than grains that were not activated (data not shown). In addition, the storage of the preserved grains under vacuum will probably contribute to a higher retention of the activity of the grains due to the elimination of atmosphere-related influences. The use of cryoprotective substances e.g. glycerol and ribitol might also lead to the satisfactory lyophilisation and/or freezing of the Kepi grains (Brialy *et al.,* 1995). In a study conducted by Fonseca *et al.* (2000), it was found that the use of a cryoprotective agent resulted in a lower rate of loss in the acidification activity of lactic acid bacteria by effectively reducing their sensitivity to freezing during frozen and lyophilised storage.

In this study (Chapter 3), Fourier Transform Near Infrared (FT-NIR) spectroscopy was also used for the rapid and easy prediction of the lactose and lactic acid content in the Kepi samples. However, the predicted lactose and lactic acid content values were not representative of the actual values when compared to data obtained with the use of a colorimetric and an enzymatic method. It, therefore, was not been feasible to use any of the FT-NIR-derived data. One of the main difficulties encountered referencing the FT-NIR spectroscopy data in the prediction analysis included the finding of a suitable sampling method for the

opaque Kepi samples and the compilation of an acceptable and reliable calibration dataset. It is thus recommended that these aspects be addressed in future studies.

Finally, the microbial interaction in mixed starters is very complex and is still not completely understood. Kepi grains contain up to five different microbial species consisting of a mixture of eukariotic (yeast) and prokaryotic (bacterial) cells. Therefore, a closer look into the interspecifc relationships in the Kepi grain will provide information that can be useful in optimising the preservation conditions of Kepi grains.

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