MASS CULTIVATION AND ACTIVITY OF KEFIR GRAINS

ANINE SCHOEVERS

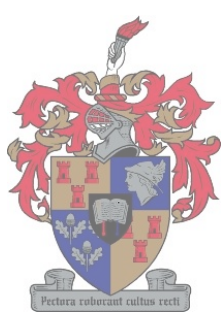
Thesis approved in fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE

In the Department of Food Science, Faculty of Agricultural Sciences
University of Stellenbosch

Study Leader: Professor T.J. Britz
Co-Study Leader: Dr. M. Manley

January 1999



DECLARATION

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

04 Feb. 2000

Anine Schoevers — Date

ABSTRACT

Consuming milk in the fermented form is an age-old tradition amongst the ethnic groups of Southern Africa and sour milk is usually made by leaving unpasteurised milk to ferment without the addition of any microbial starter culture. A recently introduced South African law, however, prevents the selling of unpasteurised milk. When pasteurised milk is used for the production of traditional sour milk (Amasi or Maas), the end-product has a putrid taste and aroma. The number of low-income communities in South Africa is increasing as a result of the high rate of unemployment as well as other factors and the inability to maintain a healthy diet often leads to malnutrition. An important need thus exists among these communities for a fermented milk product that is inexpensive, nutritious, palatable and easy to produce.

Kefir grains are the natural starter that is used during kefir production and consist of a complex variety of bacteria and yeasts trapped in kefiran, the water-insoluble polysaccharide that keeps the grain together. The nutritious fermented drink that is produced upon incubating kefir grains in milk is called kefir. This has a characteristic acid, mildly alcoholic and slightly yeasty taste. Kefir grains naturally grow in size during incubation in milk and the grain biomass increases between 60% and 100% over 10 days. This is too slow if the grains are to be produced commercially. Various factors that influence grain biomass increase were thus studied in order to develop a method for mass production of kefir grains. These factors included different incubation temperatures (18°, 22°, 25° and 30°C), enrichment of milk with different combinations of tryptose (2%), yeast extract (2%) and urea (0.5%), different volumes of replacement milk, agitation of the cultivation vessel in a shaking water bath and the use of active versus inactive kefir grains. Based on results obtained, final parameters resulting in optimum kefir grains biomass increase were the use of more than 1% active kefir grains as starter and cultivation of the grains at 25°C in milk containing added urea (0.5%) and yeast extract (2%), as well as agitating the cultivation vessel and replacing all the fermented milk daily. Kefir grain biomass increases up to 327% over 10 days were obtained using this method.

An important concern regarding the mass cultivation method was the influence on the activity of the grains. A quick, accurate and inexpensive method had to be developed to determine the grain activity based on the metabolic processes of the kefir grain micro-organisms. The metabolism of lactose to lactic acid is one of the most important metabolic conversions during the growth of lactic acid bacteria, consequently the measurement of the amount of lactose metabolised and lactic acid produced should give a good indication of the activity of these organisms.

A wide variety of analytical reference methods exist for the determination of lactose and lactic acid, but these are mostly time consuming, expensive and require a great deal of technical expertise. One of the main advantages of Fourier transform near infrared (FT-NIR) spectroscopy is that once the calibration has been derived, food constituents can rapidly and easily be determined with little or no sample preparation. The application of FT-NIR spectroscopy for the routine determination of lactose and lactic acid in kefir was thus studied. During calibration, the partial least squares algorithm was applied to the spectral data and acceptable calibration statistics (SEVC, r) were derived for lactose (0.3487 g.100 g^{-1}, 0.81), D+ lactic acid (0.1337 g.100 g^{-1}, 0.82) and L+ lactic acid (0.0797 g.100 g^{-1}, 0.93). The use of FT-NIR spectroscopy for the prediction of lactose and lactic acid in kefir can thus be successfully applied during more extensive activity testing of kefir grains.

UITTREKSEL

Gefermenteerde melk speel al vir dekades 'n belangrike rol in die tradisies en eetkulture van die etniese volke in Suidelike Afrika. Dikmelk word gewoonlik gemaak deur eenvoudig melk eenkant te laat staan om dik te word sonder die byvoeging van enige suurselkultuur. 'n Nuwe Suid-Afrikaanse wet is egter onlangs in werking gestel wat die verkoop van ongepasteuriseerde melk verhoed. Indien gepasteuriseerde melk vir die maak van tradisionele dikmelk (Amasi of Maas) gebruik word, lewer dit 'n eindproduk wat nie dieselfde kwaliteit en aroma het as dikmelk vanaf ongepasteuriseerde melk nie. As gevolg van die hoë werkloosheidsyfer en ander faktore in Suid-Afrika, is die aantal lae-inkomste gemeenskappe besig om toe te neem. Die onvermoë om 'n gebalanseerde dieet te handhaaf lei dan ook dikwels tot wanvoeding. In hierdie gemeenskappe bestaan daar dus 'n groot behoefte aan 'n gefermenteerde melkproduk wat goedkoop, voedsaam, smaaklik en maklik is om te maak.

Kefirkorrels is die natuurlike suursel wat tydens kefirproduksie gebruik word en bestaan uit 'n komplekse verskeidenheid van bakterieë en giste wat saamgebind word deur kefiraan, 'n polisakkaried wat onoplosbaar is in water. Die voedsame gefermenteerde drank wat geproduseer word tydens inkubasie van kefirkorrels in melk, word kefir genoem en het 'n karakteristieke suur, effens alkoholiese en ietwat gisserige smaak. Kefirkorrels vermeerder natuurlik tydens inkubasie in melk en korrel biomassa neem tussen 60% en 100% toe oor 'n tydperk van 10 dae. Hierdie toename is egter te stadig vir kommersiële produksie van die korrels. Verskeie faktore wat 'n invloed op biomassa toename het, is dus bestudeer om 'n metode te vind vir die massaproduksie van kefir korrels. Faktore wat bestudeer is, het verskillende inkubasie temperature (18°, 22°, 25° en 30°C), verryking van die melk met verskillende kombinasies van triptose (2%), gisekstrak (2%) en ureum (0.5%) en verskillende volumes vervangingsmelk ingesluit, asook skudding van die inkubasiehouers in 'n skud waterbad en die gebruik van aktiewe teenoor onaktiewe korrels. Die finale parameters wat gelei het tot optimum massaproduksie van kefirkorrels, was die gebruik van meer as 1% aktiewe korrels as suursel, inkubasie van die korrels by 25°C in melk waarby 0.5%

ureum en 2% gisekstrak bygevoeg is, skudding van die inkubasiehouers en daaglikse vervanging van al die gefermenteerde melk. 'n Toename van 327% in kefirkorrel biomassa is verkry na 10 dae met gebruik van hierdie metode.

'n Belangrike kwessie aangaande die massakwekingsmetode was die invloed op die aktiwiteit van die korrels. 'n Vinnige, akkurate en goedkoop metode was nodig om die aktiwiteit van die korrels te meet op grond van die metaboliese prosesse van die kefirkorrel mikroörganismes. Die metabolisme van laktose na melksuur is een van die belangrikste metaboliese omsettings tydens die groei van melksuurbakterieë en die bepaling van die hoeveelheid laktose verbruik en melksuur geproduseer behoort daarom 'n goeie aanduiding van die aktiwiteit van hierdie organismes te wees.

'n Verskeidenheid analitiese verwysingsmetodes bestaan vir die bepaling van laktose en melksuur, maar is meestal tydrowend, duur en vereis 'n hoë graad van tegniese vaardigheid. Een van die belangrikste voordele van Fourier transformasie naby infrarooi (FT-NIR) spektroskopie, na voltooiing van die kalibrasie, is die vinnige en eenvoudige bepaling van voedselbestanddele met minimale of geen monster voorbereiding. Die toepassing van FT-NIR spektroskopie tydens die roetine bepaling van laktose en melksuur in kefir is gevolglik bestudeer. PLS regressie is op die spektra toegepas tydens kalibrasie en aanvaarbare kalibrasie statistieke (SECV, r) is verkry vir laktose (0.3487 g.100 g^{-1}, 0.81), D+ melksuur (0.1337 g.100 g^{-1}) en L+ melksuur (0.0797 g.100 g^{-1}. 0.93). Die gebruik van FT-NIR spektroskopie kan dus suksesvol toegepas word vir die voorspelling van laktose en melksuur in kefir tydens meer volledige aktiwiteitstoetsing van kefirkorrels.

Without my work in natural science I should never have known human beings as they really are. In no other activity can one come so close to direct perception and clear thought, or realize so fully the errors of the senses, the mistakes of the intellect, the weaknesses and greatnesses of human character.

GOETHE

To my parents

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons and institutions for their invaluable contribution to the completion of this research:

Prof. Trevor Britz, Study Leader and Chairman of the Department of Food Science, University of Stellenbosch, for his unremitting advice, guidance and patience during this study and preparation of this thesis;

Dr. Marena Manley, Co-study Leader and Senior Lecturer at the Department of Food Science, for valuable advice and willing assistance during the course of my research and fulfilment of this thesis;

Mr. Gunnar Sigge, Mrs. Liezl Maas and Mr. Eben Brooks, for technical assistance during the execution of the experimental procedures;

Mrs. Marianne Reeves, for administrative duties performed and general advice given;

Mrs. Llewyn Jordaan and Mrs. Nicoleen Briers, for additional administrative support and interest showed in my research;

Mr. Dawie Basson and Ms. Corné van Schalkwyk, for experimental advice and encouragement throughout my research;

Mr. Rassie van Zyl, ARC-Infruitec/Nietvoorbij, Stellenbosch, for preparing the scanning electron micrographs;

Mr. Anton Jordaan, Photographical Services, University of Stellenbosch, for the photography of kefir grains;

The National Research Foundation for a Grant Holder Bursary, SAAFoST for the Brian Koeppen Memorial Scholarship, the University of Stellenbosch for the Harry Crossley Bursary and the Department of Food Science for financial support;

Ms. Anina van Zyl and Ms. Louise van Zyl, for guidance regarding FT-NIR spectroscopy and fellow post-graduate students, for continuous encouragement, interest and assistance with experimental work;

Ms. Melanie Steyn and Mrs. Liezl Maas, for editing parts of this manuscript; and

My family and close friends, for endless support and consideration throughout my studies.

CONTENTS

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

Chapter 1

INTRODUCTION

Man discovered the preservation effects of fermentation thousands of years ago by merely allowing a foodstuff (milk, meat, fish, vegetables, grain, etc.) to sour or decompose naturally (Marshall, 1987). Some fermentations were successful, while others were not. The most successful were likely to be those that involved micro-organisms that produced substantial amounts of acid, particularly lactic acid, and/or ethanol (Marshall, 1987). Many of the fermented milk products that are available today were derived from the results of the spontaneous lactic acid fermentation of milk caused by the naturally occurring lactobacilli and lactococci. Most microbes can utilise milk as a growth substrate (Marshall, 1993a; Steinkraus, 1996) due to the fact that milk is an excellent source of protein, calories in the form of fat and carbohydrates, vitamins and minerals (Gurr, 1987; Marshall, 1993b; Steinkraus, 1996). In the majority of developing countries it is rare that large quantities of cooled, fresh milk can be collected and distributed efficiently (Marshall, 1987). All-weather roads cannot always be relied on and great seasonal variations are common. All the above, as well as the influence of local traditions, have played an important role in the development of cultured milk products (Marshall, 1987; Buttriss, 1997).

A substantial part of the South African population is extremely poor due partially to the high rate of unemployment. An income of less than five rand per capita per day is common in townships where the density of people is very high (Myburgh, 1998). The lack of income, whether in cash or in kind, is the single most important determinant of nutritional status (Fincham *et al.*, 1993). Malnutrition therefore occurs frequently, with under nourishment and micro-nutrient deficiencies being the two most common forms. Nutritional risk in the black groups results in up to a third of children being underweight and stunted (Fincham *et al.*, 1993). Similarly, the inhabitants of the "homelands" are at nutritional risk. In contrast, a more acceptable nutritional state is found within

formal urban communities, such as those in Cape Town and Soweto (Fincham *et al.*, 1993). Thus, the choice of food products purchased are crucial due to the low income status, since the product has to meet several demands e.g. it has to be filling and nutritious, as well as conforming to cultural needs (Karaan & Myburgh, 1992; Myburgh 1998).

Fermented milk products are seen by the largest part of the South African population to be more palatable and can also serve as an economical source of a wide range of nutrients (Gurr, 1987). One of the main advantages of fermented milk products is that individuals who are lactose-intolerant are able to tolerate milk when consumed in the fermented form (Kilara & Shahani, 1975; Shahani & Chandan, 1979; Gurr, 1987, Marshall, 1993b). It has been estimated that as much as 95% of Africans are unable to digest lactose (De Villiers, 1990), therefore, the use of fermented milk products could prove to be a solution to this problem and it could also positively contribute to solving the malnutrition problem to some extent.

Due to the lack of cold storage and proper sanitation, the lower income segment store milk in the curdled form to prevent spoilage and putrefaction (Myburgh, 1998). These products are usually prepared from unpasteurised milk (Myburgh, 1998), but a recently introduced law prevents the selling of unpasteurised milk, except for further processing (Anon., 1997; Viall, 1999). Subsequently, pasteurised milk has to be used, but the problem is that putrefaction sets in before fermentation (due to the loss of natural lactic acid bacteria) in the absence of proper cold storage (Myburgh, 1998). Indigenous South Africans also claim that sour milk made from pasteurised milk has a putrid taste and aroma (Viall, 1999). In view of the very limited purchasing power in developing countries, it is obvious that certain dairy products should be as cheap as possible (Bachmann, 1984). An important technological advantage is that fermented milk can be produced in small dairies with relatively simple equipment, which could be manufactured in developing countries. Selling the product in bulk is possible without any hygiene risks and, if it is well suited to local conditions, could even be sold without need for refrigeration. Fermented milk could thus be produced and sold cheaply, especially if it can be sold without additional retail packaging (Bachmann, 1984). Amasi and yoghurt are generally liked and

accepted, but the price of these products limits their consumption by the lower socio-economic groups (Myburgh, 1998). Household production of yoghurt and amasi are further hampered by the fact that a new starter culture must be added to every new batch of milk and that the culture cannot be re-used. Knowledge of proper handling of such starter cultures is also very rarely found within impoverished communities. In the absence of artificial cold storage, there is a need for an inexpensive milk product that is easy to prepare, is nutritious and has an extended shelf-life.

Kefir is an ancient fermented milk beverage that is consumed widely in Eastern Europe, as well as in other parts in the world (Kwak *et al.*, 1996; Steinkraus, 1996; Garrote *et al.*, 1997). This product is produced when milk is fermented with kefir grains that have a complex composition, including yeasts, lactococci, leuconostoc, lactobacilli and sometimes even acetic acid bacteria (Angulo *et al.*, 1993; Rea *et al.*, 1996). Kefir has an characteristic taste which can be ascribed to the presence of yeasts, which are also responsible for the prickly sensation caused by the production of CO_2 (Libudzisz & Piatkiewicz, 1990; Steinkraus, 1996).

Kefir is highly nutritional and has high biological and dietic values as a result of it being a fermented milk product (Libudzisz & Piatkiewicz, 1990). The lactic acid bacteria, as well as certain enzymes present in the product, are capable of degrading the lactose, enabling lactose intolerant people to consume kefir with confidence (Marshall, 1993a and 1993b). Kefir is easy to prepare and has an extended shelf-life due to the preservative action of the lactic acid and alcohol (Steinkraus, 1996). Kefir is produced by simply incubating kefir grains in milk for 24 h and separating the grains from the fermented milk, after which the grains can either be stored or re-used directly by simply adding them to fresh milk. The energy requirements for kefir production are minimal: fermentation is carried out at room temperature (18° - 25°C) (Steinkraus, 1996), which could easily be accomplished in the hot South African climate. Kefir can thus be prepared without the use of expensive incubators or laboratory facilities (Keller & Jordaan, 1990). The grains slowly increase during every batch of kefir that is produced and could easily be handled by someone without technical knowledge. The same batch of grains can be used repeatedly and no expensive equipment is required to make

kefir. These benefits and the fact that two end-products – grains and kefir – are obtained, makes this a very economical process. A technical problem is that the natural grain biomass increase is too slow to obtain large amounts of grains quickly for wide scale distribution. Thus, enhancing the rate of grain biomass increase would make it possible to mass-produce kefir grains and to distribute grains amongst impoverished communities. This would enable them to manufacture an affordable but nutritious and filling product using pasteurised milk.

The objectives of this study were to determine the influence of environmental factors on the biomass increase of kefir grains during mass cultivation and to determine the activity of kefir grains by studying the metabolic behaviour of the specific microbial groups present in the grains.

References

Angulo, L., Lopez, E. & Lema, C. (1993). Microflora present in kefir grains of the Galician region (North-West of Spain). *Journal of Dairy Research,* **60**, 263-267.

Anonymous (1997). *Foodstuffs, Cosmetics and Disinfectant Act and Regulations. Act no. 54 of 1972, G.N.R. 1555/1997.* Johannesburg, South Africa: Lex Patria Publishers.

Bachmann, M.R. (1984). Fermented milk in the dairy development of the third world countries. *International Dairy Federation Bulletin*, **179**, 143-146.

Buttriss, J. (1997). Nutritional properties of fermented milk products. *International Journal of Dairy Technology*, **50**(1), 21-27.

De Villiers, F.P.R. (1990). The prevalence of lactose maldigestion in the rural Tswana. *South African Journal of Food Science and Nutrition*, **2**(4), 81-84.

Fincham, R., Harrison, D., Khosa, M. & Le Roux, I. (1993). *Nutrition and health in South Africa: The state of nutrition and the development of nutrition policy.* University of Cape Town, Rondebosch, South Africa: Southern Africa Labour and Development Research Unit.

Garrote, G.L., Abraham, A.G. & De Antoni, G.L. (1997). Preservation of kefir grains, a comparative study. *Lebensmittel-Wissenschaft und Technologie*, **30**, 77-84.

Gurr, M.I. (1987). Nutritional aspects of fermented milk products. *FEMS Microbiology Reviews*, **46**, 337-342.

Karaan, A.S.M. & Myburgh, A.S. (1992). Food distribution systems in the urban informal markets: The case of red meat marketing in the Western Cape townships and informal settlements. *Agrekon*, **31**(4), 289-293.

Keller, J.J. & Jordaan, I. (1990). Fermented milks for the South African market. *South African Journal of Dairy Science*, **22**(2), 47-49.

Kilara, A. & Shahani, K.M. (1975). Lactase activity of cultured and acidified dairy products. *Journal of Dairy Science*, **59**(12), 2031-2035.

Kwak, H.S., Park, S.K. & Kim, D.S. (1996). Biostabilization of kefir with a nonlactose-fermenting yeast. *Journal of Dairy Science*, **79**, 937-942.

Libudzisz, Z. & Piatkiewicz, A. (1990). Kefir production in Poland. *Dairy Industries International,* **55**(7), 31-33.

Marshall, V.M. (1987). Fermented milks and their future trends. I. Microbiological aspects. *Journal of Dairy Research*, **54**, 559–574.

Marshall, V.M. (1993a). Starter cultures for milk fermentation and their characteristics. *Journal of the Society of Dairy Technology*, **46**(2), 49-56.

Marshall, V.M. (1993b). Kefir. In: *Encyclopaedia of Food Science and Technology* (edited by Y. H. Hui), Volume 3. Pp. 1804-1808. Chichester, UK: John Wiley & Sons.

Myburgh, (1998). Food consumer behaviour of the low income communities in urban settlements. *Address at SAAFoST meeting held at ARC-Infruitec/Nietvoorbij*, 24 February 1998, Stellenbosch, South Africa.

Rea, M.C., Lennartson, T., Dillon, P., Drinan, F.D., Reville, W.J., Heapes, M. & Cogan, T.M. (1996). Irish kefir-like grains: their structure, microbial composition and fermentation kinetics. *Journal of Applied Bacteriology,* **81**, 83-94.

Shahani, K.M. & Chandan, R.C. (1979). Nutritional and healthful aspects of cultured and culture-containing dairy foods. *Journal of Dairy Science*, **62**, 1685-1694.

Steinkraus, K.H. (1996). Acid-fermented milk and milk/cereal foods. In: *Handbook of Indigenous Fermented Foods,* 2nd ed. Pp. 274-276 and 305-308. New York: Marcel Dekker, Inc.

Viall, J. (1999). New law "will milk small dairies dry". *Cape Argus, 18 October 1999*, p. 9.

Chapter 2

LITERATURE REVIEW

A. BACKGROUND

Kefir is a fermented milk drink that has been consumed for thousands of years. It originated in the village of Karatschajeff in the former Soviet Union (Duitschaever, 1989) and is still manufactured there under a variety of names (kephir, kiaphur, kefer, knapon, kepi, and kippi) (Kwak *et al.,* 1996).

Since time immemorial people who lived in the mountains learnt to make an invigorating drink from cow or goats' milk with the use of a special starter called "kefir grains" (Koroleva, 1988b). Traditionally, the Caucasians prepared kefir by fermenting milk either in animal hide bags, oak barrels or clay pots (Roginski, 1988). During the day, the milk sack was exposed to sunlight and at night it was taken into the house and hung near the door. Everyone who came in or went out had to push the sack with their foot in order to mix the liquid (Koroleva, 1988b). Fresh milk was added each time as the fermented milk was removed and over time this led to the build-up of layers of micro-organisms embedded in protein and polysaccharide material. After a few weeks of fermentation, this led to the formation of cauliflower-like grains (Duitschaever *et al.,* 1987). The fermented kefir drink was consumed while the kefir grains remained in the leather bag, resulting in a continuous fermentation process.

At the end of the nineteenth century Russian doctors reported that kefir had beneficial effects in the treatment of intestinal and stomach diseases. This nutritional and therapeutic information about kefir resulted in its becoming popular in Europe, as well as in the United States and Japan (Marshall & Cole, 1985; Koroleva, 1988b; Libudzisz & Piatkiewicz, 1990; Steinkraus, 1996; Kwak *et al.,* 1996). Kefir is still the most popular cultured product in Russia and forms about 65% of the total fermented products sold to the public (Steinkraus, 1996).

Kefir is obtained by lactic acid fermentation followed by alcoholic fermentation. Lactic acid bacteria form the lactic acid, while lactose and non-lactose fermenting yeasts produce alcohol and carbon dioxide, (Liu & Moon, 1983)

leading to the typical kefir flavour and taste. Other minor components that are formed during kefir production are traces of acetaldehyde, diacetyl and acetone (Vedamuthu, 1982). It is important to distinguish between the fermented kefir product and the kefir grains. Kefir is the fluid milk product that is obtained after separating the fermented milk mixture from the grains, while the kefir grains are the insoluble, white or yellowish masses that are held back from the milk after separation.

Kefir can be produced from any type of milk (cow, goat, sheep, camel and buffalo) and has a characteristic acid, mildly alcoholic, prickly and slightly yeasty taste (Duitschaever, 1989; Marshall, 1993; Steinkraus, 1996; Rea *et al.*, 1996). Kefir is self-carbonating due to the production of CO_2 by the yeasts (Kosikowski, 1977; Mann, 1989; Duitschaever, 1989; Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Steinkraus, 1996) and it is therefore fitting that kefir is known as "the champagne of cultured dairy products" (Merin & Rosenthal, 1986). The alcohol content can vary between 0.5 and 2.0% (v/v) and the fat content depends on the type of milk used as substrate (Duitschaever, 1989; Marshall, 1993). The acidity of the product depends on the method that is used for kefir production. Modern kefir, prepared in large volumes using lyophilised starters or using separate fermentation steps, has a 1% (v/v) titratable acidity and a pH of about 4.0 or lower which makes it more acidic than the traditional kefir. Traditional kefir, in contrast, is prepared with kefir grains and is "milder", with a pH of 4.4 or higher (Marshall, 1993). Kefir thus differs from other fermented dairy products in that, at the end of the fermentation process, the organisms can be recovered as a solid matrix resembling a cauliflower floret.

Kefir can be consumed with meals or as a snack before bedtime. It is served in a glass and can be either drunk or eaten with a spoon. Variations include sweetening with sugar (the same as yoghurt) or combining it with fruits or biscuits (Steinkraus, 1996). In addition to kefir made from full cream milk, there are also low-fat and skim milk kefirs, all usually containing not less than 11% non-fat solids. There is also kefir with a 6% fat content, but sometimes a higher fat content is preferred. Fruit kefir, kefir from buttermilk, and freeze-dried kefir are other products available on the European markets (Kurmann *et al.*, 1992).

B. KEFIR GRAINS

General characteristics

Kefir grains are moist, gelatinous, whitish, or yellowish, irregular cauliflower-like granules ranging from 0.5 to 3.5 cm in diameter (Marshall & Cole, 1985). Sheet-like structures and scroll-like forms can usually be distinguished from the cauliflower-like grains (Marshall *et al.,* 1984). All these structures are elastic and quite tough. The opposite surfaces of the sheet-like structures are different in nature, one side is normally smooth and flat while the other side is rough and convoluted (Marshall *et al.,* 1984). Kefir grains probably evolved through the curling of the flat sheet-like structures with subsequent folding and re-folding into a globular structure.

The grains grow in size as the associated micro-organisms multiply and insoluble carbohydrate compounds accumulate (Marshall, 1984). Active kefir grains float in milk (Koroleva, 1988b). Toba *et al.* (1990) reported that propagable kefir grains become non-propagable ones, even after a 3 - 4 day transfer to fresh milk. Non-propagable grains can still ferment milk and make kefir. The grains start out as very small and increase in size during the milk fermentation. Kefir grains can multiply in whole milk, skim milk, or even neutralised whey. La Rivière *et al.* (1967) reported that 50 g wet weight kefir grains can double their mass in 7 - 10 days if they are transferred to 500 ml fresh milk six times a week. Growth is nonetheless considerably retarded if the grains are rinsed with water after each separation (Steinkraus, 1996) and the reason for this probably lies in the marked decline of the main groups of grain micro-organisms (Koroleva, 1988a). As a result of a decrease in grain activity due to washing, a longer fermentation period will be necessary, and the kefir will have an inferior taste and consistency. It is still possible to re-establish the normal microbiological composition if the grains are subsequently incubated for 3 - 5 days. Koroleva (1988a) found that yeasts were the only microbial group that did not decline after grain washing.

It is important to remember that kefir grains grow only from pre-existing grains during kefir manufacture (Steinkraus, 1996). Despite the fact that intensive research had been done on the subject, no one has yet succeeded in producing

kefir grains from pure or the mixed microbial cultures that are normally present in the grains (Koroleva, 1975; Hirota & Kikuchi, 1976; Liu & Moon, 1983; Libudzisz & Piatkiewicz, 1990). The reason for this probably lies in the fact that very little, if anything, is known about the mechanism of grain formation.

A wide variety of micro-organisms can be found in and on the surface of kefir grains, depending on the circumstances under which the grains are propagated, their origin, storage conditions and handling patterns (Duitschaever, 1989; Pintado *et al.,* 1996). These micro-organisms live together in specific symbiosis, but the overall organisation of the micro-organisms in the grain is not completely known (Kosikowski, 1977; Toba *et al.,* 1987; Duitschaever, 1989; Libudzisz & Piatkiewicz, 1990; Angulo *et al.,* 1993; Rea *et al.,* 1996).

The micro-organisms in the grains are embedded in a water-insoluble polysaccharide matrix referred to as "kefiran" (La Rivière *et al.,* 1967). This holds the micro-organisms together and makes the grains extremely tough and resilient (Toba *et al.,* 1987; Duitschaever, 1989; Libudzisz & Piatkiewicz, 1990; Yokoi *et al.,* 1990).

The dry mass (10 - 16% (m/m)) of a fresh grain consists of 30% (m/m) protein and 25 - 50% (m/m) carbohydrates. Microbial cells account for the major part of the grain, together with autolysis products, curd proteins and carbohydrates like kefiran (Libudzisz & Piatkiewicz, 1990). The chemical composition of kefir grains is 890 - 900 $g.kg^{-1}$ water, 2 $g.kg^{-1}$ lipid, 30 $g.kg^{-1}$ protein, 60 $g.kg^{-1}$ sugar and 7 $g.kg^{-1}$ ash (Zourari & Anifantakis, 1988).

Microbial composition

The microbial composition is probably one of the areas of kefir processing that has been researched most extensively, however, the identification of many of the species remains unclear. This is further hampered by the differences that exist between grains from different regions. The composition of the grains is also subject to the conditions of propagation, handling and storage. Any modification to establish conditions for the cultivation of kefir grains will clearly lead to changes in the microbial composition (Koroleva, 1988a). It is thus essential to standardise the conditions under which kefir grains are cultivated in order to be able to draw comparable conclusions in relation to the microbial composition of different grain

samples. Asepsis is not maintained during kefir manufacture and therefore the presence of species of the genera *Leuconostoc*, *Streptococcus* and *Acetobacter*, as well as coliforms, could be attributed to different techniques employed during processing and storage (Marshall, 1984). If different grain samples are cultivated under the same conditions, their microbial composition becomes rather similar (Koroleva, 1988a), confirming the fact that kefir grains and their altering environment are capable of self-regulating its population.

The microbiological composition of general high quality kefir is shown in Table 1 (Koroleva, 1988b). Rea *et al.* (1996) found that lactococci were the dominant micro-organisms in kefir drink, followed by leuconostocs and yeasts. Mesophilic lactobacilli grew more slowly, while no thermophilic lactobacilli were detected. As a result of the presence of "*Streptococcus lactis*" and "*Streptococcus cremoris*", thermophilic lactic acid streptococci are able to propagate actively at kefir fermentation temperatures, which are lower than those normally optimal for these species (Semenichina, 1984).

In kefir grains from Poland, about 65 - 80% of the microbiological count consists of lactobacilli (homo- and heterofermentative, meso- or thermophilic), while 20% are streptococci (souring and aroma forming). Different species of lactose and non-lactose fermenting yeasts form about 5% of the microbiological count (Libudzisz & Piatkiewicz, 1990). The surface of kefir grains kept in milk for extended periods can sometimes be covered with a white mould *Geotrichum candidum* which impairs the taste and quality of the kefir (Koroleva, 1988a; Roginski, 1988). Contaminating yeasts of the *Mycoderma* type can also occasionally appear in kefir starters, again leading to an impairment of taste (Koroleva, 1988a).

When milk is inoculated with kefir grains, some micro-organisms are dispersed into the milk phase where they continue to grow with the production of acid, flavour and physico-chemical changes (Garrote *et al.*, 1997 and 1998). Various researchers have studied the concentrations of the different microbial groups present in the kefir grains and/or in the kefir drink at the end of fermentation and these findings are shown in Tables 2 and 3.

A kefir starter can be prepared by growing kefir grains in milk. According to Koroleva (1988a), such a starter consists of several functionally different groups of micro-organisms:

Table 1. Microbiological composition of high quality kefir (Koroleva, 1988b).

Type of micro-organism	Number per ml
	$1 \times 10^7 - 1 \times 10^8$
Thermophilic lactobacilli	
Homofermentative mesophilic lactic acid streptococci	1×10^9
Heterofermentative lactic acid streptococci	$1 \times 10^7 - 1 \times 10^8$
Yeasts	$1 \times 10^4 - 1 \times 10^5$
Acetic acid bacteria	$1 \times 10^4 - 1 \times 10^5$

Table 2. Concentration of micro-organisms in kefir grains at the end of the fermentation.

Lactic acid streptococci	Lactobacilli	Leuconostocs	Yeasts	Reference
$1 \times 10^8 - 1 \times 10^9.g^{-1}$	$1 \times 10^8 - 1 \times 10^9.g^{-1}$	-	$1 \times 10^8.g^{-1}$	Libudzisz & Piatkiewicz, 1990
$1 \times 10^7.g^{-1}$	$1 \times 10^9.g^{-1}$	-	$1 \times 10^8.g^{-1}$	Garrote *et al.*, 1997
-	$1 \times 10^9.g^{-1}$	-	$1 \times 10^8.g^{-1}$	Marshall, 1993
$<1 \times 10^6.g^{-1}$	$3 \times 10^9.g^{-1}$	$2.5 \times 10^7.g^{-1}$	$4 \times 10^8.g^{-1}$	Kandler & Kunath, 1983

Table 3. Concentration of micro-organisms in kefir drink at the end of the fermentation.

Lactic acid streptococci	Lactobacilli	Leuconostocs	Yeasts	Acetic acid bacteria	Reference
$1 \times 10^9 - 1 \times 10^{10}.g^{-1}$	$1 \times 10^9 - 1 \times 10^{10}.g^{-1}$	-	$1 \times 10^6 - 1 \times 10^7.g^{-1}$	-	Garrote *et al.*, 1997
$1 \times 10^9.g^{-1}$	$1 \times 10^8.g^{-1}$	-	$1 \times 10^6.g^{-1}$	-	Marshall, 1993
$1 \times 10^9.g^{-1}$	$1 \times 10^5.g^{-1}$	$1 \times 10^8.g^{-1}$	$1.5 \times 10^6.g^{-1}$	$1 \times 10^5.g^{-1}$	Rea *et al.*, 1996
$1 \times 10^9.g^{-1}$	$1 \times 10^8.g^{-1}$	$1 \times 10^8.g^{-1}$	$1 \times 10^7.g^{-1}$	-	Kandler & Kunath, 1983

Mesophilic homofermentative lactic acid streptococci like "*Streptococcus lactis* subsp. *cremoris*" form the largest and most active part of the starter. These cultures ensure rapid acid production during the early stages of fermentation, but are inhibited at high acid levels (Koroleva, 1988a). Homofermentative lactic acid bacteria produce lactic acid as the major or sole end-product (70 - 90%) of glucose metabolism (IDF, 1992; Jay, 1996). All members of the genera *Streptococcus* (*Str.*) and *Lactococcus* (*Lacto.*) are homofermentative, along with some of the lactobacilli (Jay, 1996).

Several **Lactobacillus** species (*Lb.*) are also present in kefir grains. Lactobacilli are capable of growing at pH values lower than that required by *Lactococcus lactis* (pH 4.5). These organisms can therefore allow fermentation to continue and cause the pH to drop to 4.0 or lower (Jay, 1996). Koroleva (1982) found that the concentration of mesophilic lactobacilli did not exceed 10^2-10^3 ml^{-1} and concluded that they were not important in the quality of the product. Kandler & Kunath (1983), however, reported levels of $10^6.ml^{-1}$ mesophilic lactobacilli in milk freshly inoculated with kefir grains and $10^8.ml^{-1}$ at the end of fermentation. The species most frequently isolated are *Lb. brevis, Lb. casei* subsp. *rhamnosus, Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* (Koroleva, 1988a).

The formation of the specific aroma and taste of kefir are attributed to the presence of **mesophilic heterofermentative lactic acid streptococci** like *Leuconostoc mesenteroides* subsp. *dextranicum* (Koroleva, 1988a). Members of the genus *Leuconostoc* (*Leuc.*) as well as some lactobacilli are heterofermentative (Jay, 1996). Heterofermentative lactic acid bacteria (LAB) produce other compounds such as acetic acid, CO_2 and ethanol in addition to at least 50% lactic acid (IDF, 1992; Jay, 1996). Gas production is observed if growth of these organisms is excessive. Except for *Leuc. lactis*, leuconostocs normally grow poorly in milk, but their growth is stimulated by the addition of yeast extract, amino acids, Mn^{2+} or Mg^{2+} (Bellengier *et al.,* 1997). Associative growth with acid producers, such as streptococci, also leads to more favourable conditions for the growth of leuconostocs (Boquien *et al.,* 1988).

Various **yeasts** can be found in kefir grains. The majority in kefir grains are not able to ferment lactose (Saloff-Coste, 1996), but play an important role in promoting symbiosis among the micro-organisms, CO_2 production and the

development of the characteristic taste and aroma of kefir. Again excessive growth could lead to high gas levels and can cause packaging problems (Koroleva, 1988a). Glaeser & Hangst (1987) found that the characteristic yeasty flavour of kefir appears to be dependent on the yeast species rather than the total number of yeasts present in the kefir. An example of maintaining symbiosis is the ability of "*Torulopsis holmii*" (which appears in kefir grains) to preferentially utilise galactose, even in the presence of glucose (Ueda *et al.,* 1982). Further examples of yeasts that are important in kefir are *Kluyveromyces marxianus* subsp. *marxianus, Torulaspora delbrueckii, Saccharomyces cerevisiae* and *Candida kefir.*

The **acetic acid bacteria** (AAB), *Acetobacter aceti* and *A. rasens*, also play an important role in maintaining symbiosis amongst kefir grain micro-organisms. Members of the genus *Acetobacter* are able to produce acetic acid from ethanol (Jay, 1996). Acidification activity is increased when streptococci are cultivated together with acetic acid bacteria. AAB can also improve the consistency of kefir by increasing its viscosity (Koroleva, 1988a). These organisms are considered contaminants by some workers (Angulo *et al.,* 1993), however, they produce vitamins of the B group (Koroleva, 1988b; Zourari & Anifantakis, 1988), contribute to protein proteolysis and the accumulation of free amino acids and other products of protein hydrolysis. AAB may therefore stimulate other organisms in the grain (Koroleva, 1988b; Rea *et al.,* 1996). The presence of AAB in what is essentially an anaerobic fermentation, is unusual, given their highly aerobic nature. The cessation of growth of the AAB after ca. 20 h of incubation is probably a result of oxygen depletion (Rea *et al.,* 1996). The growth of AAB could be improved by stirring during fermentation (Häfliger, 1990). No AAB could be found in kefir grains studied by Pintado *et al.* (1996).

The quantitative composition of kefir grains and the resultant kefir are relatively stable throughout the year if a stable production cycle is used. The number of flavour-producing bacteria, yeasts and thermophilic lactobacilli stays constant, while the number of mesophilic lactic acid streptococci is slightly higher in spring and summer. In spring, the product viscosity sometimes decreases - this is in conjunction with a decrease in the number of AAB. This confirms the assumption that this group of micro-organisms plays an important role in product viscosity (Koroleva *et al.*, 1978).

It appears that the heterofermentative lactobacilli and the non-lactose fermenting yeasts are also very important in kefir grains. The presence of streptococci, lactococci and acetic acid bacteria are essential for flavour development during kefir manufacture, but are thought to play no part in grain formation (Marshall, 1993).

Kefiran

The micro-organisms in kefir grains are embedded in slimy materials of which kefiran, a polysaccharide described by La Rivière *et al.* (1967), makes up the highest percentage. Kooiman (1968) and Mukai et al. (1988) described the chemical structure of kefiran, and they reported that it contains equal amounts of glucose and galactose, as well as a repeating unit of 6-0-substituted galactose. Marshall (1984) postulated that organisms producing polymers of carbohydrate, such as kefiran, are necessary for the integrity of kefir grains.

La Rivière *et al.* (1967) reported that kefiran is a capsular material produced by "*Lactobacillus brevis*". Kandler & Kunath (1983) found *Lactobacillus kefir* to be the main heterofermentative lactobacillus in kefir grains, but it was probably not responsible for the kefiran production. In 1978, Rosi & Rossi suggested that the polysaccharides of kefir grains are produced by homofermentative lactobacilli, which they labelled as "atypical *Streptobacterium*". It thus remained unclear for many years which organism was responsible for the kefiran until 1987, when Toba *et al.* (1987) isolated polysaccharide-producing homofermentative lactobacilli from kefir grains. These workers found that the characteristics of the polysaccharides of the grains were similar to those of strains of an encapsulated homofermentative *Lactobacillus* species. These strains differed from that of the *Lb. kefir* strains described by Kandler & Kunath (1983). Based on these findings, Fujisawa *et al.* (1988) proposed a new species, *Lactobacillus kefiranofaciens*, as the main producer of kefiran. To further support this fact, Mukai *et al.* (1992) found kefiran to be the main accessory polymer in the cell wall of *Lb. kefiranofaciens*.

Various other attempts had been made to isolate polysaccharide-producing bacteria from kefir grains. In 1967, La Rivière *et al.* reported that "*Lb. brevis*"

produced kefiran, but the capsule-forming activity of this organism was lost with the first transfer to growth medium. Kunath & Kandler (1983) therefore concluded that "*Lb. brevis*" was not the main kefiran producer. Toba *et al.* (1986) and Fujisawa *et al.* (1988) isolated a capsule forming homofermentative bacterium, *Lb. kefiranofaciens,* from kefir grains with a newly developed medium containing wine. Yokoi *et al.* (1990) also reported on the isolation of a polysaccharide-producing organism (*Lactobacillus* sp. KPB-167B) which grew actively in a newly developed whey-medium. These bacteria produced the polysaccharide in yields of four to five times higher than found with *Lb. kefiranofaciens*, generating the possibility of applying these isolates in the industrial production of this polysaccharide. It was concluded that the polysaccharide was very likely kefiran, but the isolate differed slightly from *Lb. kefiranofaciens* (Yokoi *et al.*, 1991) and produced kefiran in higher concentrations in milk whey than *Lb. kefiranofaciens*. Although *Lb. kefiranofaciens* is able to grow and produce kefiran only in milk-whey-medium, it was found that *Lactobacillus* sp. KPB-167B could grow and produce kefiran in MRSL medium. These findings indicated that *Lactobacillus* sp. KPB-167B would be suitable for the mass production of kefiran (Yokoi *et al.,* 1991). Kefiran also possesses possible therapeutic properties for use in the treatment of tumorous diseases. Shiomi *et al.* (1982) and Murofushi *et al.* (1983) reported antitumour activity when kefiran was administered orally to mice.

Rheological studies have shown that kefiran has a very low viscosity in solution and is unable to form rigid gels in the absence of ethanol (Mukai *et al.,* 1990 and Mukai *et al.*,1991). However, Pintado *et al.* (1996) found that kefiran can form weak gels in conditions of low water activity and stated that this finding could have potential practical applications.

Other lactic acid bacteria ("*Leuconostoc mesenteroides*" and "*Streptococcus cremoris*") are also capable of producing extracellular polysaccharides (Brooker, 1976a; Brooker, 1976b; 1977) and a mucogenic strain of "*Streptococcus thermophilus*" has been shown to produce filaments of extracellular polysaccharide during fermentation of milk (Kalab *et al.*, 1983). In contrast to the carbohydrate produced by these organisms, the *Lactobacillus* strains of kefir do not appear to produce radial thickenings or filamentous

extensions of carbohydrate (Marshall *et al.*, 1984). These workers described the presence of electron-dense material as probable accumulation of an insoluble polymer caused by extracellular synthesis.

Cultivation

Kefir starters with any combination of micro-organisms can be brought about by changing the environmental factors under which kefir grains are cultivated e.g. milk renewal at regular intervals, cultivation temperature, grains to milk ratio, starter mixing, grain washing etc. The growth of homofermentative lactic acid bacteria, such as streptococci and lactobacilli, is accelerated when grain cultivation is performed at elevated temperatures (Koroleva, 1988a). Homofermentative and heterofermentative lactic acid streptococci are inhibited as a result of the pH being lowered to values inhibitory to their growth. Micro-organisms which are more acid resistant are therefore better suited to higher grain cultivation temperatures (Koroleva, 1988a).

Agitation two to three times during grain cultivation leads to a 10-fold increase in the amount of homofermentative lactic acid streptococci (Koroleva, 1988a), but this does not influence the concentration of heterofermentative LAB, thermophilic lactobacilli or AAB. Additional stirring does not affect the concentration of volatile fatty acids produced; it prohibits the growth of moulds on the grain-starter surface and assists in the even dispersion of microbial metabolites in the milk (Koroleva, 1988a).

Bondarev (1981) showed that the addition of calcium salts to the kefir milk leads to substantially increased bacterial counts compared to milk without extra calcium. This could have practical implications for the mass production of kefir grains. White & Kidney (1981) showed that *Saccharomyces cerevisiae* and *Lb. brevis* aggregate and co-sediment during beer brewing. This may have relevance to the kefir grain construction, particularly as these authors have shown that Ca^{2+} ions promote aggregation.

Modern production of grains is based on continuous cultivation in milk, resulting in an increase in biomass of 5 to 7% (m/m) per day (Libudzisz & Piatkiewicz, 1990). After fermentation, the grains are separated and the

recovered grains are directly introduced into fresh milk and washed once a week with cooled, sterile water. These workers mentioned that it is important to use stainless steel or glass vessels for cultivation of the grains. The kefir grain culture is produced in the form of fresh kefir grains suspended in a sterile solution of 0.9% NaCl or as a lyophilised culture made from kefir grains (Libudzisz & Piatkiewicz, 1990).

Storage

It is important to evaluate certain parameters in determining the best method of preserving kefir grains during storage. When the grains are taken out of storage to make kefir, it must be possible to produce a product of specified standard and quality. One of the main parameters to consider in evaluation of the preservation methods is the maintenance of all the microbial strains in their relative proportions in the grain. Equally important is the reproducibility of the desired characteristics of the kefir, such as rheological properties, acidity and CO_2 content after storage of the grains (Garrote *et al.*, 1997).

Kefir grains can be stored in clean, cold water at 4°C after the grains have been strained and washed and these grains will stay active for 8 to 10 days (Kosikowski, 1977). The grains can also be dried at room temperature for 36 to 48 h, placed in a paper envelope or in an aluminium pouch and stored in a cool, dry place for 12 to 18 months without losing activity (Kosikowski, 1977). Koroleva (1982) reported that kefir grains could retain their activity and ability to restore micro-organisms at unregulated temperatures if the grains are kept in sterile whey for a maximum of five days. The grains can also be stored in a 0.9% NaCl solution at 4°C for 8 to 10 days (Libudzisz & Piatkiewicz, 1990). Kefir grains do not stay viable if they are dehydrated with heat (Steinkraus, 1996). The grains may survive freeze-drying, but the best method to maintain viable kefir grains is to transfer them periodically to milk and keep them at refrigerator temperatures (4° to 7°C) (Steinkraus, 1996).

Garrote *et al.* (1997) studied the preservation of kefir grains under three different storage conditions. Two batches of grains were suspended in milk and frozen at -20°C and -80°C, while the third batch of grains was centrifuged, dried

with tissue paper and kept at 4°C in a petri dish sealed with Parafilm. After storage for 120 days and subsequent incubation in milk, four aspects were examined: weight increase of the stored grains, the microbial composition of the grains and the properties and microbial composition of kefir made from the stored grains.

Grains stored at -80°C and -20°C increased their weight at a rate comparable to that found with non-stored grains, but grains stored at 4°C did not increase in weight (Garrote *et al.* 1997). The viable yeast count of the kefir grains decreased with all three methods tested, although the decrease at -80°C was lower in respect to the control than the other two methods. The concentration of viable lactobacilli in the grains also decreased during storage at -20°C and -80°C, but increased during storage at 4°C. This was probably due to growth of cells during the first days of storage, or because of the breaking of the bacterial chains. The concentration of lactococci in the grains was not affected by the storage conditions tested.

Kefir obtained from the frozen grains (-80°C and -20°C) showed the same microbial composition, rheological behaviour, acidity, and carbon dioxide content as kefir obtained from non-stored grains (Garrote *et al.* 1997). Kefir made with grains stored at 4°C was unpleasant and did not have the acidity and viscosity of standard kefir. The final concentrations of yeasts, lactobacilli and lactococci in kefir fermented with grains stored at 4°C were lower than those in kefir obtained with frozen or non-stored grains (Garrote *et al.*, 1997). The concentration of lactococci in kefir obtained with frozen grains was higher than in kefir obtained with non-stored grains. This was attributed to the breaking of cell chains or to a diminution of the retention of these micro-organisms in the grain matrix after freezing and thawing. It was thus concluded that storage at -80°C and -20°C are good methods for storing kefir grains, and that the latter temperature is an especially good way of preserving kefir grains for household manufacture of kefir (Garrote *et al.*, 1997).

Brialy *et al.* (1995) studied the preservation of the intrinsic inhibitory power of kefir against *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Candida albicans* and *Saccharomyces cerevisiae*, after lyophilisation of kefir

grains. Fresh kefir actively inhibited the three bacterial strains, but did not have any effect on the growth of the yeasts (*S. cerevisiae* and *C. albicans*). Ribitol, sodium glutamate and glycerol were tested as cryoprotectors, and milk and water were tested as lyophilisation and regeneration media. The lyophilisation of kefir grains, without adding cryoprotective substances, led to the total loss of intrinsic inhibitory power, regardless of whether milk or water was used as lyophilisation or regeneration media. Lyophilisation of grains in water led to a loss of the intrinsic inhibitory power, no matter which cryoprotector was added or which medium was used for regeneration. Sodium glutamate was found unsuitable for the cryoprotection of kefir grains. Glycerol had no cryoprotective effect when the lyophilisation and regeneration substrate was water, but the intrinsic inhibitory power of the grains was preserved when milk was used as both the lyophilisation and regeneration medium. The regeneration substrate appeared to be less important in the case of glycerol being the cryoprotector, even if the use of water instead of milk slightly decreased the intrinsic inhibitory power. Satisfactory microbiological results were obtained when glycerol was used as both a lyophilisation and regeneration medium. It was thus found that glycerol was the best cryoprotector because of its high efficiency and low cost.

Lyophilised kefir cultures are becoming increasingly popular due to the ease of use in large production plants (Libudzisz & Piatkiewicz, 1990). These lyophilised cultures are standardised to 10% of the overall microbiological composition by adding yeasts isolated from the kefir grains (Libudzisz & Piatkiewicz, 1990). This supplementation is necessary because more than 80% of the yeasts can be lost during freezing and freeze-drying of kefir grains, resulting in cultures that consist almost entirely of streptococci (Marshall & Cole, 1985; Kramkowska *et al.,* 1986; Duitschaever *et al.,* 1988; Marshall, 1993). The main group of micro-organisms in frozen or freeze-dried cultures from Germany was streptococci, with very few yeasts or lactobacilli (Mann, 1983). Kefir made from a fast single-use freeze-dried kefir culture differed little in flavour, properties and biochemical characteristics from kefir made by traditional methods (Kramkowska *et al.*, 1982).

C. KEFIR PRODUCTION

Several methods exist for the production of kefir with the same properties as found in traditional kefir (Fig. 1), but without the disadvantages of the traditional production process (Saloff-Coste, 1996). Traditional kefir is made by inoculating heat-treated milk (pasteurised at 85° - 87°C for 5 - 10 min or 90° - 95°C for 2 - 3 min, homogenised and cooled to 20° - 25°C) with up to 5% (m/v) kefir grains (Koroleva, 1988b). After a period of fermentation lasting 24 h, the grains are removed and the kefir drink is ready for consumption (Kroger, 1993). The grains can then be used to inoculate fresh milk or can be stored for later use. Only small amounts of kefir can be produced by using the traditional method and the grains themselves are not always well understood or controlled (Saloff-Coste, 1996).

The "Russian" method entails two fermentation steps and is used to produce kefir in larger amounts (Hallé *et al.*, 1994). In the first fermentation step, 2 - 3% (m/v) kefir grains are added to milk and incubated, after which the grains are removed. The fermented milk without grains (mother culture or starter I) is then added in amounts of 1 - 3% (v/v) to fresh milk and then incubated for 12 to 18 h (Hallé *et al.*, 1994). Kefir produced in this way has the typical taste and aroma of traditional kefir (Koroleva, 1988a). It is, however, important to bear in mind that this mother culture cannot be used for subsequent inoculations to make an acceptable product, because the original balance of micro-organisms has been disrupted (Kroger, 1993; Marshall & Cole, 1985).

In factories where there is a lack of equipment for separating kefir grains from the mother culture, a different method is used (Koroleva, 1988a). In this case pasteurised milk is inoculated with the mother culture to produce a bulk starter (starter II). Amounts of 3 to 5% (v/v) of starter II is then used to make kefir (Koroleva, 1988a).

More recently manufacturers who want to avoid the difficulties associated with the before-mentioned methods have used lyophilised kefir cultures for kefir production. These concentrated starters are made from kefir grains and the mother culture is obtained by adding the contents of the package (one gram) to about three litres of milk (Libudzisz & Piatkiewicz, 1990). Another original method was developed by Merin & Rosenthal (1986), who prepared kefir by suspending kefir grains in a gauze bag and placing the bag in milk to prevent the escape of the grains into the

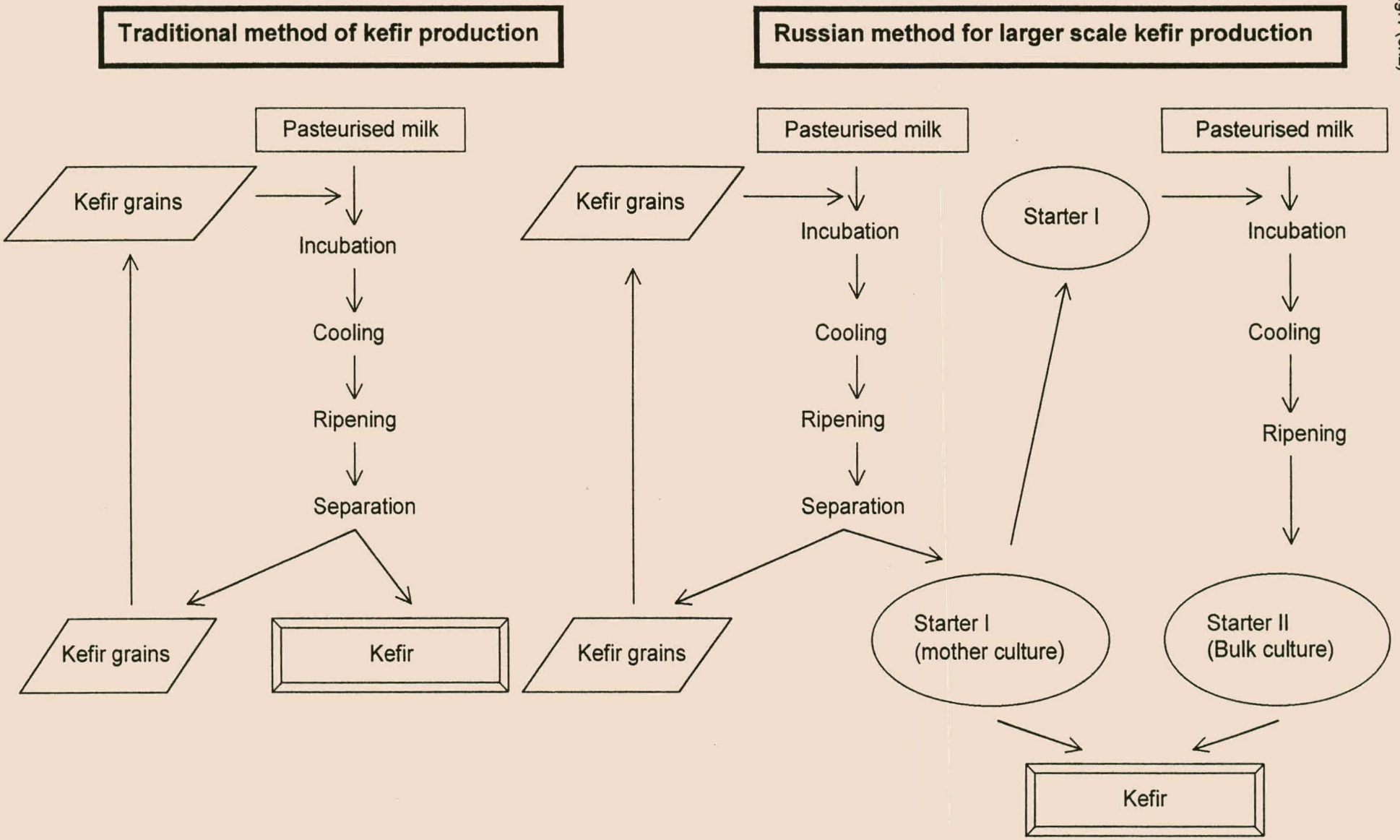


Figure 1. Different methods for the production of kefir (adapted from Koroleva, 1988a & Saloff-Coste, 1996).

milk. They found that the milk fermentation with the grains contained in the bag proceeded at the same rate and yielded the same quality product as milk fermented with the grains suspended freely in the liquid. The use of this type of method in the industry could facilitate the recovery of kefir grains from fermented milk during large-scale production of kefir.

D. FACTORS INFLUENCING KEFIR QUALITY DURING MANUFACTURE

Heat treatment of milk

Bondarev (1977) studied the effects of four different heat treatments of the milk used for kefir manufacture. The four different treatments were: 85° - 87°C for 5 - 10 min; 92° - 95°C for 20 - 30 min; 110°C in an autoclave; and "double" pasteurisation, involving treatment at 72° - 76°C in a plate pasteuriser, followed by treatment at 85° - 87°C for 20 min in a tubular pasteuriser. The milk was bulk inoculated at 20°C with 4% (m/v) starter, and the products tested for flavour, viscosity and whey separation. The results showed that greater denaturation of the whey proteins took place with increasing severity of the heat treatment. This resulted in a better coagulum and an improved kefir consistency. Heating kefir milk at 92° - 95°C for 20 - 30 min was considered optimal. Double pasteurisation also had favourable effects on the flavour and consistency of the kefir, but it was considered uneconomical.

Berzhinskas *et al.* (1978) studied the influence of multiple-stage pasteurisation of milk in kefir manufacture. This involved heating the milk to 87°C, cooling to 77°C, holding for 30 min and re-heating to 87°C. The kefir obtained by this method was compared to kefir made of milk pasteurised at 87°C for 30 min. The double pasteurisation treatment increased denaturation of the whey proteins, improved the dispersion of casein particles, and increased the firmness, elasticity and viscosity of the curd. Syneresis was reduced and the consistency of the kefir was improved considerably.

Starter concentration

Different reports in the literature show a wide range of grain to milk ratios for kefir manufacture. Twenty to fifty $g.l^{-1}$ appears to be the recommended amount of grains to be used (Bottazzi & Bianchi, 1980; Marshall & Cole, 1985; Merin & Rosenthal, 1986; Mann, 1989; Kroger, 1993). In contrast, Koroleva (1988a) used 20 - 100 $g.l^{-1}$ kefir grains and Marshall et al. (1984) and Neve (1992) used 50 - 100 $g.l^{-1}$. Rea *et al.* (1996) used one gram of grains per litre of milk as starter, while 200 g kefir grains are recommended by Chr. Hansen's Laboratory, Denmark, to ferment one litre of milk (Marshall & Cole, 1985)

The use of different amounts of kefir grains or starters obtained from kefir grains (1 - 5% m/v or v/v) do not cause significant quantitative of qualitative changes in the microbial composition of the finished product (Koroleva *et al.*, 1978). Coagulation is, however, prolonged if smaller amounts of grains or starter are used (1 - 2% m/v or v/v). Koroleva (1988b) recommended the use kefir grains as starter for kefir production and, at the same time, decreasing the amount of inoculum. Kefir made with 1 - 2% (m/v) kefir grains resulted in the maximum quantity of homofermentative mesophilic lactic acid streptococci and heterofermentative lactic acid streptococci (Babina & Rozhkova, 1973). These workers ascribed this to the more favourable conditions created by a smaller inoculum. The concentrations of other micro-organisms in kefir, especially thermophilic lactobacilli and AAB do not depend on the size of the inoculum (Babina & Rozhkova, 1973).

Garrote *et al.* (1998) studied the influence of different grain to milk ratios on the kefir characteristics. Kefirs obtained from incubating milk for 48 h with different grain to milk ratios showed large differences in final pH, lactococci concentration, apparent viscosity and CO_2 content. A high viscosity kefir was produced when milk was fermented with 10 - 50 $g.l^{-1}$ kefir grains, but milk inoculated with 100 $g.l^{-1}$ had a lower viscosity. Acidification of milk inoculated with 10 g kefir grains per litre was slower than with 100 $g.l^{-1}$. With a 100 $g.l^{-1}$ inoculum, a pH of 4.5 was reached after 5 h incubation, while a 10 $g.l^{-1}$ inoculum required 48 h incubation to reach the same pH. It appears that the concentrations of different micro-organisms are not dependent on the inoculum size in the 1 to 20 $g.l^{-1}$ range. In contrast, the final concentration of lactococci in fermented milks using an inoculum of 100 $g.l^{-1}$ is at least two log cycles lower (Garrote *et al.*,

1998). This could probably be explained by the sensitivity of the lactococci to low pH values. It was concluded that a ratio of 10 $g.l^{-1}$ should therefore be chosen when a viscous and not very acid product is desired. A ratio of 100 $g.l^{-1}$ will result in an acid beverage with low viscosity and a more effervescent taste (Garrote *et al.*, 1998).

Fermentation temperature

Korovkina *et al.* (1978) studied the influence of different incubation temperatures on the consistency and biochemical properties of kefir. Whole milk inoculated with 5% (m/v) kefir grains was incubated at temperatures ranging from 19° - 28°C. Carbon dioxide production, viscosity and acidity all increased with increasing temperatures. Incubation at 25°C was recommended for maximum production of ethanol and volatile fatty acids, and incubation at this temperature also resulted in kefir with a good specific flavour and consistency.

At higher temperatures (25° - 27°C), acid production proceeded quickly and the required acidity was reached in six to eight hours (Koroleva, 1988b). Heterofermentative lactic acid streptococci and yeasts could not develop in such a short time at these temperatures and their absence or lower numbers generally resulted in kefir with atypical flavours. It was found that the fermentation time was prolonged to 11 - 12 h when incubation temperatures of 20° - 22°C were used. It was also reported that if the coagulum was immediately cooled to 8° - 10°C, the heterofermentative lactic acid streptococci and yeasts once more could not develop, again resulting in atypical kefir (Koroleva, 1988b). Slow cooling of the coagulum over a 10 - 12 h period allowed these micro-organisms to grow and the characteristic taste and aroma of kefir developed.

Piechocka *et al.* (1977) found that kefir incubated at 22°C for 18 h and stored at 4°C for seven days had a better consistency, flavour and aroma than kefir incubated at 18°C for 18 h and also stored at 4°C for seven days. The coagulum that formed at 22°C was of a firmer consistency and was more resistant to whey separation.

Fermentation time

Large variations in the optimum fermentation time required for quality kefir are reported in the literature, and range from 8 - 24 h (Puhan, 1988). Koroleva (1988b) recommended that a total fermentation period of 24 h be used for quality kefir manufacture. It was recommended that this included fermentation at 20° to 22°C for 10 to 12 h , followed by ripening with slow cooling for the next 10 - 12 h until a temperature of 8° - 10°C was reached. The ripening stage of the kefir is very important for the development of kefir flavour due to the increase in the amount of diacetyl, acetaldehyde, ethanol, amylalcohol and n-propanol during this stage (Görner *et al.,* 1972).

Milk type

A variety of factors, such as the milk source, the fat content of the milk used, the composition of the grains and the conditions of production, can cause the composition and flavour of kefir to vary significantly (Zourari & Anifantakis, 1988). The type of milk (cow, goat, buffalo etc.) used for the fermentation plays a role in quality of the end-product due to differences in the content of the total solids, fat and lactose (Marshall, 1982). Kefir suited to local taste was developed in Egypt using skimmed buffalo milk. This product contained 0.85 - 1.09% (v/v) lactic acid, 0 to 0.17% (v/v) alcohol and 0.383 - 0.633% (v/v) acetaldehyde (Ismail *et al.*, 1983).

The fat content of kefir is dependant on the milk source (cow, goat, ewe or mare) as well as the milk fat content (whole fat, low fat or non-fat) (Saloff-Coste, 1996). Piechocka *et al.* (1977) found that the organoleptic score of kefir rose significantly with an increase in the fat content of milk from 0.06% to 2.0% (m/v). This can probably be accounted for by the fact that a lower fat content may cause the body and mouthfeel of the final product to be inferior (Brewer, 1995). The addition of 1 - 4% (m/v) non-fat milk solids (skim milk powder) can easily rectify this problem.

Chojnowski *et al.* (1978) found that kefir manufactured from homogenised mixtures of skim milk concentrated by ultrafiltration; whey protein concentrate prepared by ultrafiltration of whey; or cream, had better structure and organoleptic properties than traditionally made kefir and did not lead to whey separation. Chojnowski *et al.* (1981) studied the manufacture of kefir from various mixtures of

skimmed milk and whey. Cultured cream was added after the milk-whey mixtures had been concentrated by reverse osmosis. The best organoleptic and physico-chemical results were obtained when kefir was made from milk concentrated in a ratio of 1:2 and containing 20 - 30% (m/v) added whey proteins.

Kefir viscosity (consistency)

Semenichina (1984) found that lactic acid streptococci ("*Streptococcus cremoris*" and "*Streptococcus thermophilus*") form long cell chains and that this plays an important role during gel formation. An increased coagulum viscosity is caused by growth of these organisms in the milk. Lactic acid streptococci therefore affect the rheological properties of the coagulum by influencing the ability of the coagulum to restore its structure after agitation (Semenichina, 1984). Acetic acid bacteria can also improve the consistency of kefir (Koroleva, 1988b).

Korovkina *et al.* (1976) found that the viscosity of kefir depended on the pH of the fermented milk at the time of stirring. Kefir of typical consistency and stable body was produced, without any whey separation, when agitation was performed at pH 4.4 – 4.5. Kefir viscosity was also dependent on the method of cooling and ripening with the highest viscosity being obtained when slow cooling without agitation was used. Kefir with a better consistency can also be achieved by using higher milk pasteurisation temperatures, milk homogenisation, increasing the total solids content and adding stabilisers such as alginates and carraginates (Semenichina, 1984).

E. END-PRODUCTS OF THE METABOLISM OF KEFIR MICRO-ORGANISMS

Ready-to-serve kefir contains lactic acid, mainly the L(+) form, in the amount of 0.8 - 0.9% (m/v), as well as formic, succinic and propionic acids, CO_2 (0.08 to 0.2% (v/v)), ethyl alcohol (0.035 - 2.0% (v/v)), different aldehydes (propionic, acetic) and trace amounts of isoamyl alcohol and acetone (Libudzisz & Piatkiewicz, 1990). Diacetyl is considered the main aroma forming substance in kefir, and is produced by the citrate utilising streptococcus "*Streptococcus lactis*

subsp. *diacetylactis*" and *Leuconostoc* sp. in amounts of approximately 1 $mg.l^{-1}$. Peptones make up 7% (m/m) of the nitrogen in kefir and amino acids 2% (m/m) of the nitrogen. Kefir contains more vitamin B_1, B_2 and folic acid than the milk it is made of (Libudzisz & Piatkiewicz, 1990).

Lactic acid

Lactic acid bacteria present in kefir grains, have complex and varying nutritional requirements. The main pathways of carbohydrate metabolism of the lactic acid bacteria are depicted in Fig. 2. The most important transformation during kefir manufacture is the conversion of lactose to lactic acid (Vedamuthu, 1982). Lactic acid is responsible for the sharp refreshing taste of kefir and although non-volatile, it serves as an excellent background for the more distinctive flavours and aromas characteristic of kefir. Lactose is the major milk sugar, consisting of units of D-galactose and D-glucose held together by a 1-4-β-linkage. A maximum of 1.5% acidity as lactic acid can be attained during the lactic acid fermentation and thus only about 30% (m/m) of the lactose content of milk is used, so there is an excess of substrate available after the fermentation is complete (Vedamuthu, 1982). "*Streptococcus lactis* subsp. *diacetylactis*" grows well in milk, can ferment lactose and accumulate up to 0.4 - 0.65% (v/v) lactic acid in 24 h at 30 °C (Vedamuthu, 1982).

L- and D-lactate are the major products formed during kefir fermentation and are produced by lactococci and leuconostocs, respectively (Rea *et al.,* 1996). Both isomers of lactate are produced during kefir fermentation, but the final level of L-lactate is ten times higher than that of the D-isomer. This correlated well with the increased numbers of lactococci found compared to leuconostocs. It is, however, important to remember that lactococci produce twice as much lactic acid as leuconostocs from the same amount of sugar.

According to the literature, the final concentration of total lactate and D-lactate in kefir can vary considerably (Rea *et al.,* 1996). The levels of D-lactate found by Rea *et al.* (1996) were much lower than those reported by other workers. A study by Thompson *et al.* (1990) on Irish kefir, showed a D-lactate concentration four times higher than the L isomer and a final pH of about 3.2, while the kefir studied by Rea *et al.* (1996) had a final pH of approximately 4.4.

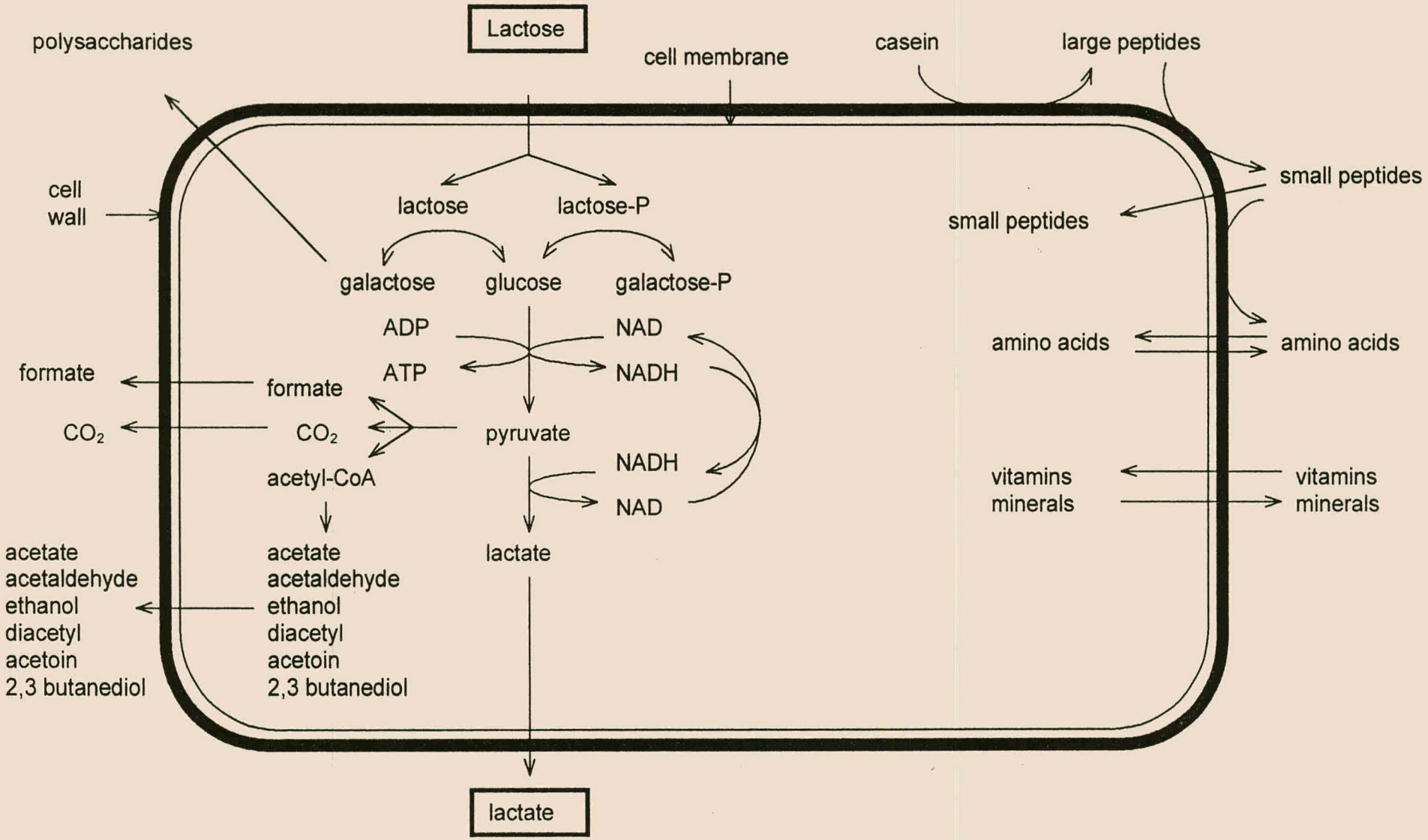


Figure 2. Main metabolic pathways of the lactic acid bacteria (from Saloff-Coste, 1996)

Yeast species present in kefir grains, such as *Candida kefir* and *Kluyveromyces marxianus* subsp. *marxianus,* are also able to metabolise lactose to ethanol and galactose (Moulin *et al.*, 1977; Saloff-Coste, 1996), while other yeast species such as *Saccharomyces cerevisiae* and *S. unisporus* are unable to utilise lactose as an energy source.

Diacetyl, acetate and acetoin

Although the predominant metabolite is lactic acid, it is clear that the minor metabolites are vital for product quality and identity (Marshall, 1984). These minor metabolites are often volatile and will therefore contribute to the flavour and aroma of kefir. Citrate metabolism is the second most important transformation of a milk component into an essential flavour component. Milk contains an average of 0.2% (m/v) citrate, but this value exhibits great seasonal fluctuation (Vedamuthu, 1982). The end-products of the metabolism of citrate are extremely important in determining the flavour and aroma of kefir. Vedamuthu (1982) and Garvie (1984) stated that "*Streptococcus lactis* subsp. *diacetylactis*" and "*Leuconostoc cremoris*" are the most important flavour bacteria in kefir.

"*Streptococcus diacetylactis*" is the only strain of "*Streptococcus lactis*" that is able to use citrate as an energy source and metabolise it to acetoin, diacetyl and CO_2 (Garvie, 1984). It is, however, possible for other streptococci to metabolise citrate rapidly if another fermentable energy source is also present (Cogan, 1981). Leuconostocs are less active than "*Streptococcus lactis* subsp. *diacetylactis*" and ferment milk citrate only when there is sufficient acid development and therefore requires associative growth with acid producing bacteria (Garvie, 1984). Leuconostocs are considered to be major acetate producers, rather than lactobacilli (Rea *et al.,* 1996).

Acetate is also considered to be a major product of the citrate metabolism of lactic acid bacteria with one mole of acetate being produced from one mole of citrate (Rea *et al.,* 1996). This compound can also be produced during sugar metabolism by heterofermentative lactic acid bacteria as well as from the oxidation of ethanol by the acetic acid bacteria. Häfliger (1990) stated that acetic acid bacteria at levels of more than 10^7 - 10^8 ml^{-1} were necessary for a measurable and sensorially detectable metabolic activity and that that level was unlikely to occur in

kefir preparations. It is therefore improbable that acetic acid bacteria oxidise ethanol to produce acetate because of the low levels of these bacteria in kefir grains ($<10^4$ ml^{-1}) (Rea *et al.*, 1996).

Diacetyl imparts the characteristic "buttery" aroma and flavour to fermented products. Citrate is converted to diacetyl by various flavour and aroma forming bacteria present in kefir and the grains, e.g. *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis* (Marshall, 1987). It is, however, important to remember that diacetyl and acetoin are produced only in acidic conditions (pH <5.0) (Cogan *et al.*, 1981). Formation of diacetyl during yeast fermentation is also well known (Suomalainen & Jannes, 1946). Leuconostocs produce little or no acetoin during co-metabolism of sugar and citrate (Cogan, 1981). *Saccharomyces cerevisiae* is also able to produce acetoin (Marshall, 1984).

Acetaldehyde

Acetaldehyde is an important flavour compound and is primarily derived from lactose, although other mechanisms for acetaldehyde production do exist (Vedamuthu, 1982). In some cultured products, acetaldehyde is undesirable because it imparts a "green" or "yogurty" flavour. Strains of "*Streptococcus lactis*" and a majority of "*Streptococcus lactis* subsp. *diacetylactis*" strains produce relatively high amounts of acetaldehyde (Vedamuthu, 1982). "*Leuconostoc cremoris*" possesses high concentrations of alcohol dehydrogenase and can thus convert acetaldehyde into ethanol at 5°C (Marshall, 1984). *Leuconostoc citrovorum* can also convert part of the acetaldehyde to form ethanol (Lees & Jago, 1976). Yeasts are another group of micro-organisms able to convert acetaldehyde to ethanol (Marshall, 1984).

Alcohol

Alcohol is an important component characteristic of kefir and is the end-product of sugar metabolism by leuconostocs and yeasts (Rea *et al.*, 1996). However, co-metabolism of lactose and citrate by leuconostocs results in no ethanol production. This suggests that mainly the yeasts produce ethanol in the kefir fermentation, but ethanol continues to be produced when growth of both

leuconostocs and yeasts has ceased (Rea *et al.*, 1996). During storage the ethanol concentration can increase from 20 mg.100 g^{-1} on day one to 200 mg.100 g^{-1} after 11 days (Alm, 1982a). Kefir with a higher concentration of ethanol generally has a more yeasty flavour (Marshall, 1984).

Carbon dioxide

Carbon dioxide is essential for flavour development of kefir. The gas is trapped in the thickened milk and provides a fizz or effervescence. Carbon dioxide is derived from lactose by heterofermentative LAB. Fermentation of citrate by aroma forming bacteria also yields considerable amounts of carbon dioxide in milk. A top-quality kefir should foam and fizz when agitated and form a head of carbon dioxide gas (Vedamuthu, 1982). Libudzisz & Piatkiewicz (1990) reported that Polish kefir contains 0.08 - 0.2% CO_2 (v/v).

Volatile fatty acids

Formic, acetic and propionic acids are the most important volatile fatty acids found in cultured dairy products. It had been reported that "*Str. lactis* subsp. *diacetylactis*" strains produce more volatile fatty acids from casein hydrolysate than other lactic streptococci (Vedamuthu, 1982). Although storage did not influence the concentration of acetic acid in kefir, the amount of ethanol increased from about 20 mg.100 g^{-1} kefir on day one to 200 mg.100 g^{-1} kefir on day 11 (Alm, 1982b).

F. ACTIVITY TESTING

Due to the variety of storage methods for kefir grains described in the literature, it would be natural to conclude that the activity of the grains would depend on the storage conditions and time. After storage, the success of kefir fermentation will depend on the activity of the grains at the moment of inoculation, not their activity during storage. The supplier of kefir grains should therefore be able to guarantee activity of the grains at the moment of selling, and likewise the buyer should be able to use a rapid, reliable test for checking their activity before use. The

supplier's estimate of the grain activity would, however, be meaningless if the grains were exposed to incorrect storage conditions or temperature abuse after manufacture and packaging.

The activity of a starter can be defined as the ability to produce acid under specified conditions (Kriel, 1978), as well as enzymatic reactions that lead to the production of aromatic components and/or thickening agents (Spinnler & Corrieu, 1989). A variety of factors can have an influence on the activity of the starter, including the physiological state of the culture(s), growth conditions, harvesting, packaging and storage conditions (Accolas & Auclair, 1970).

Kefir grains consist of a complex composition of various micro-organisms, including lactic acid bacteria, yeasts and acetic acid bacteria (Koroleva, 1988b). The conversion of lactose and citric acid to lactic acid and other metabolites is very important for growth of lactic acid bacteria (Chandan, 1982) and therefore the measurement of the lactate produced could be informative about the state of these organisms. It is also important to be able to determine the lactose concentration in fermented milk products owing to the high percentage of people who are lactose-intolerant or suffer from low levels of the enzyme lactase (Shah, 1993). The measurement of the conversion of lactose to lactic acid can thus be useful in determining the activity of milk starters and estimating the hydrolysis of lactose in milk.

Determination of lactose

Several analytical methods for the determination of lactose exist and include polarimetry (AOAC, 1995; Aurand *et al.*, 1987), spectrophotometry (Nickerson *et al.*, 1976; Narinesingh *et al.*, 1992), fluorimetry (Guilbault *et al.*, 1969; Chen *et al.*, 1992), gas and high-pressure liquid chromatography (Euber & Brunner, 1979; Harvey, 1988; Indyk *et al.*, 1996) and enzymatic methods (Beutler, 1984; Kleyn 1985; Galbán *et al.*, 1993; Mustranta & Östman, 1997).

The amount of lactose in milk can also be determined colorimetrically by using methylamine. Lactose reacts with methylamine in a hot alkaline solution to form a red complex with a maximum absorbance at 540 nm without interference from glucose or galactose (Fearon, 1942; Nickerson *et al.*, 1976; Katsu *et al.*, 1994). A microbial cell-based biosensor for determining lactose, glucose or

sucrose has recently been described by Švitel *et al.* (1998). This method is based on a Clark-type oxygen electrode covered with a membrane containing the microbial cells. Different microbial species are used according to the compound being measured. The determination of glucose together with another analogue saccharide, such as lactose, can be performed by using a biosensor that combines a specific enzyme with an electrochemical device (Katsu *et al.*, 1994). This concept is based upon three methods: the simultaneous use of two different enzyme electrodes (Pfeiffer *et al.*, 1980; Zhang & Rechnitz, 1993); the use of a glucose electrode and a suitable enzyme reactor (Masoom & Towshend, 1985); and the use of multiple enzyme membranes that rely on the differences in reaction time and diffusion rates between sugars (Mizutani & Asai, 1990; Watanabe *et al.*, 1991).

Determination of lactic acid

The measurement of pH during fermentation as a means of measuring acid production as a form of starter activity is widely used in the dairy and fermentation industries (Olsen, 1978; Heap & Lawrence, 1981; Okigbo *et al.*, 1985; Spinnler & Corrieu, 1989). Another measurement of the lactic acid produced is done by titration of a fermentation sample with sodium hydroxide (Accolas & Auclair, 1970; Horral & Elliker, 1947). A variety of techniques such as enzymatic assays (Leenheer & Jans, 1986), chemical methods (Barkar & Summerson, 1941; Burini, 1993), gas chromatography (Carlsson, 1973) and high-pressure liquid chromatography (Bouzas *et al.*, 1991; Fernandez-Garcia & McGregor, 1994) can also be used to measure lactic acid. The use of enzyme electrodes (Blaedel & Engstrom, 1980; Scheller *et al.*, 1987; Mascini *et al.*, 1984; Shu *et al.*, 1995) and biosensors (Luong *et al.*, 1989; Dremel *et al.*, 1990; Mulchandani *et al.*, 1995) based on whole cells and enzymes to measure lactate, have also been reported.

Near infrared spectroscopy

The official reference analytical methods for the determination of moisture, fat, protein and lactose are too time-consuming to be applied industrially for control purposes during the manufacture of dairy products (Frankhuizen, 1992). An

important need thus exists for the development of equipment or methods that can rapidly determine the above-mentioned quality components (Roberts, 1977; Weaver, 1978; Weaver, 1984). The use of near infrared reflectance (NIR) spectroscopy can potentially provide these benefits and is rapidly gaining popularity in the dairy industry (Frankhuizen, 1992; Giangiacomo & Nzabonimpa, 1994). Near-infrared reflectance spectroscopy can be used to determine fat, protein, lactose and solids in milk (Robert *et al.*, 1987), analyse dried products such as milk powder for fat, moisture and protein (Goulden, 1957; De Vilder & Bossuyt, 1983; Baer *et al.*, 1983) and examine other dairy products such as cheeses (Giangiacomo *et al.*, 1979), butter and casein (Goulden, 1957). The application of near infrared spectroscopy to fermented milks seems to be very limited despite the wide range of these products available (Rodriguez-Otero *et al.*, 1997a). Fat, protein and total solids in fermented milks were successfully determined by Rodriguez-Otero & Hermida (1996) by using near-infrared reflectance spectroscopy, but subsequent analysis using near-infrared transflectance spectroscopy yielded better results (Rodriguez-Otero *et al.*, 1997b). Activity testing of kefir grains will be greatly simplified if the metabolites to be tested could be quantified by means of near infrared spectroscopy. Considering that kefir is a fermented milk product, it seems reasonable to conclude that a substantial amount of work still needs to be done regarding the application of NIR on this product.

G. CONCLUDING REMARKS

The practice of kefir making is centuries old, but notwithstanding, some properties relating to this process still baffle man. Conclusive proof of this fact can for example be found in the difficulty in determining the interactions between the different micro-organisms leading to grain formation and the production of different metabolites. The nutritional benefits ascribed to kefir and kefir grains are another area that research still needs to be done on. Nobody is absolutely certain how

kefir grains are formed and substantial amounts of research will have to be completed if kefir grains are to be sold for commercial purposes.

Kefir is by no means an "old" product in our country and the opportunities to utilise this valuable and nutritious product seem endless. Being a combination first and third world country, poverty and malnutrition frequently occur in South Africa. An extreme climate further impedes the number and types of foodstuffs available. Enter kefir: a nutritious, palatable product that is inexpensive and easy to prepare, as well as being suited to the local climate and traditions. Only a small amount of kefir grains is necessary to start making one's own kefir. The grains are hardy, not easily contaminated if treated properly, can be used over and over again and are easy to store. All this makes kefir manufacture a very economical, viable process. Kefir grains can justly be called "white gold" !

REFERENCES

Accolas, J.P. & Auclair, J. (1970). Determination of the acid-producing activity of concentrated frozen suspensions of lactic acid bacteria. *Le Lait*, **50**, 609-626.

Alm, L. (1982a). Effect of fermentation on volatile acids and ethanol in Swedish dairy products. *Journal of Dairy Science*, **65**, 186-190.

Alm, L. (1982b). Effects of fermentation on curd size and digestibility of milk proteins in vitro of Swedish fermented milk products. *Journal of Dairy Science*, **65**, 509-514.

Angulo, L., Lopez, E. & Lema, C. (1993). Microflora present in kefir grains of the Galician region (North-West of Spain). *Journal of Dairy Research,* **60**, 263-267.

AOAC (1995). Official Method 896.01. Determination of lactose in milk: Polarimetric method. Final Action. In: *Official Methods of Analysis of the Association of Official Analytical Chemists*. Chapter 33, p. 17. Virginia: The Association of Official Agricultural Chemists.

Aurand, L.W., Woods, A.E. & Wells, M.R. (1987). Milk and milk products. In: *Food Composition and Analysis*. Pp. 543-595. New York: Van Nostrand Reinhold.

Babina, N.A. & Rozhkova, I.V. (1973). Quantitative compositions of kefir grains and kefir microflora at different times of the year. *Molochnaya Promyshlennost'*, **2**, 15-17.

Baer, R.J., Frank, J.F., Loewenstein, M. & Birth, G.S. (1983). Compositional analysis of whey powders using near infrared diffuse reflectance spectroscopy. *Journal of Food Science*, **48**, 959-961,989.

Barkar, S.B. & Summerson, W.H.J. (1941). The colorimetric determination of lactic acid in biological materials. *Journal of Biological Chemistry*, **138**, 535-554.

Bellengier, P., Richard, J. & Foucaud, C. (1997). Nutritional requirements of *Leuconostoc mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum* for growth in milk. *Journal of Dairy Research*, **64**, 95-103.

Berzhinskas, G., Urbene, S. & Puidokas, I. (1978). Influence of multiple-stage pasteurization of milk on the consistency of kefir. *XX International Dairy Congress*, **E**, 840-841.

Beutler, H.O. (1984). Metabolites. 1. Carbohydrates: Poly-, oligo- and disaccharides. In: *Methods of Enzymatic Analysis* (edited by H.U. Bergmeyer, J. Bergmeyer & M. Grassl), Volume VI, 3rd ed. Pp. 104-112. Weinheim: Verlag Chemie.

Blaedel, W.J. & Engstrom, R.C. (1980). Reagentless enzyme electrodes for ethanol, lactate, and malate. *Analytical Chemistry*, **52**, 1691-1697.

Bondarev, V.Y. (1977). Effect of heat-treatment on the quality of acid coagulum (kefir). *Molochnaya Promyshlennost'*, **5**, 29-30.

Bondarev, V.Y. (1981). Effect of changing calcium ions concentration on the development of kefir microflora. *Molochnaya Promyshlennost'*, **3**, 26-27.

Boquien, C., Corrieu, G. & Desmazeaud, M.J. (1988). Effect of fermentation conditions on the growth of *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ 1091 in pure and mixed cultures. *Applied and Environmental Microbiology*, **54**, 2527-2531.

Bottazzi, V. & Bianchi, F. (1980). A note on the scanning electron microscopy of micro-organisms associated with the kefir granule. *Journal of Applied Bacteriology*, **48**, 265-268.

Bouzas, J., Kantt, C.A., Bodyfelt, F. & Torres, J.A. (1991). Simultaneous determination of sugars and organic acids in Cheddar cheese by high-performance liquid chromatography. *Journal of Food Science*, **56**(1), 276-278.

Brewer, M.S. (1995). Kefir. *National Food Safety Database.* http://www.foodsafety.org/il/il105.htm, March, 1998.

Brialy, C., Rivalland, P., Coiffard, L. & De Roeck Holtzhauer, Y. (1995). Microbiological study of lyophilised dairy kefir. *Folia Microbiologica*, **40**(2), 198-200.

Brooker, B.E. (1976a). Surface coat transformation and capsule formation by *Leuconostoc mesenteroides* NCDO 523 in the presence of sucrose. *Archives of Microbiology*, **111**, 99-104.

Brooker, B.E. (1976b). Cytochemical observations on the extracellular carbohydrate produced by *Streptococcus cremoris*. *Journal of Dairy Research*, **43**, 283-290.

Brooker, B.E. (1977). Ultrastructure surface changes associated with dextran synthesis by *Leuconostoc mesenteroides*. *Journal of Bacteriology*, **131**, 288-292.

Burini, G. (1993). Indirect fluorimetric determination of lactic acid in wine. *Journal of AOAC International*, **76**, 1017-1021.

Carlsson, J. (1973). A simplified gas chromatography procedure for the identification of bacterial metabolic products. *Applied Microbiology*, **25**, 287-289.

Chandan, R.C. (1982). Other fermented dairy products. In: *Prescott and Dunn's Industrial Microbiology* (edited by G. Reed), 4th ed. Pp. 113-184. Westport, Connecticut, USA: AVI Publishing Co., Inc.

Chen, F., Liu, Y., Lu, J., Hwang, K.J. & Lee, V.H.L. (1992). A sensitive fluorometric assay for reducing sugars. *Life Sciences*, **50**(9), 651-659.

Chojnowski, W., Poznanski, S., Smietana, Z. & Bednarski, W. (1978). Production of fermented products from milk and whey using ultrafiltration. *XX International Dairy Congress*, **E**, 946.

Chojnowski, W., Poznanski, W., Bednarski, W. & Poliwko, I. (1981). Production of cultured beverages from milk and whey concentrated by ultrafiltration. *Roczniki Instytutu Przemyslu Mleczarskiego*, **21**(1), 25-33.

Cogan T.M. (1981). Constitutive nature of the enzymes of citrate metabolism in *Streptococcus lactis* subsp. *diacetylactis*. *Journal of Dairy Research*, **48**, 489-495.

De Vilder, J. & Bossuyt, R. (1983). Practical experiences with an InfraAlyzer 400 in determining the water, protein and fat content of milk powder. *Milchwissenschaft*, **38**(2), 65-69.

Dremel, B.A.A., Wansheng, Y. & Schmid, R.D. (1990). On-line determination of lactic acid during kefir fermentation based on a fibre-optic lactic acid biosensor and flow-injection analysis. *Analytica Chimica Acta*, **234**, 107-112.

Duitschaever, C.L., Kemp, N. & Emmons, D. (1987). Pure culture formation and procedure for the production of kefir. *Milchwissenshaft*, **42**(2), 80-82.

Duitschaever, C. L., Kemp, N. & Smith, A.K. (1988). Microscopic studies of the microflora of kefir grains and of kefir made by different methods. *Milchwissenschaft*, **43**(8), 479-481.

Duitschaever, C.L. (1989). What is kefir and how can it be made ? *Modern Dairy*, **68**(4), 18-19.

Euber, J.R. & Brunner, J.R. (1979). Determination of lactose in milk products by high-performance liquid chromatography. *Journal of Dairy Science*, **62**, 685-690.

Fearon, W.R. (1942). *Analyst*, **67**, 130 *(as cited by Nickerson et al. 1976).*

Fernandez-Garcia, E. & McGregor, J.U. (1994). Determination of organic acids during the fermentation and cold storage of yoghurt. *Journal of Dairy Science*, **77**, 2934-2939.

Frankhuizen, R. (1992). NIR analysis of dairy products. In: *Handbook of Near-Infrared Analysis* (edited by D.A. Burns & E.W. Ciurczak). Pp. 609-641. New York: Marcel Dekker, Inc.

Fujisawa, T., Adachi, S., Toba, T., Arihara, K. & Mitsuoka, T. (1988). *Lactobacillus kefiranofaciens* sp. nov. isolated from kefir grains. *International Journal of Systematic Bacteriology,* **38**(1), 12-14.

Galbán, J., de Marcos, S. & Castillo, J.R. (1993). Fluorometric-enzymatic lactate determination based on enzyme cytochrome b_2 fluorescence. *Analytical Chemistry*, **65**, 3076-3080.

Garrote, G.L., Abraham, A.G. & De Antoni, G.L. (1997). Preservation of kefir grains, a comparative study. *Lebensmittel-Wissenschaft und Technologie*, **30**, 77-84.

Garrote, L. G., Abraham, A.G. & De Antoni, G. (1998). Characteristics of kefir prepared with different grain:milk ratios. *Journal of Dairy Research,* **65**, 149-154.

Garvie, E.I. (1984). Taxonomy and identification of bacteria important in cheese and fermented dairy products. In: *Advances in the Microbiology and BioChemistry of Cheese and Fermented Milk* (edited by E.L. Davies & B.A. Law). Pp. 35-66. London: Elsevier Applied Science Publishers.

Giangiacomo, R., Torregioni, D., Frank, J.F., Loewenstein, H. & Birth, G.S. (1979). Analysis of Blue cheese using infrared reflectance spectroscopy. *Journal of Dairy Science*, **62**(suppl. 1), 39.

Giangiacomo, R. & Nzabonimpa, R. (1994). Approach to near infrared spectroscopy. *International Dairy Federation Bulletin*, **298**, 37-42.

Glaeser, H. & Hangst, E. (1987). Differentiation of yeast and micro-organisms producing acetic acid in kefir, and their effect on sensory properties. *Deutsche Molkerei Zeitung*, **108**(5), 108-111.

Görner, F., Palo, V. & Segin, M. (1972). Changes in the content of volatile compounds during kefir ripening. *Nahrung*, **16**(7), 777-785.

Goulden, J.D.S. (1957). Diffuse reflectance spectra of dairy products in the near infra-red region. *Journal of Dairy Research*, **24**, 242-251.

Guilbault, G.G., Sadar, S. & Peres, K. (1969). Fluorometric determination of carbohydrates. *Analytical Biochemistry*, **31**, 91-101.

Häfliger, M.J. (1990). Die Bedeutung der Essigsäurebakterien im Kefir. PhD Thesis, Eidgenössischen Technischen Hochschule, Zurich.

Hallé, C., Leroi, F., Dousset, X. & Pidoux, M. (1994). Les kéfirs: des associations bactéries lactiques-levures. In: *Bactéries lactiques: Aspects fondamentaux et technoligiques*, H. de Roissart, F.M. Luquet (eds.), Volume 2. Pp. 169-182. France: Lorica.

Harvey, J. (1988). A high performance liquid chromatography method for lactose determination in milk. *The Australian Journal of Dairy Technology*, **43**(1), 19-20.

Heap, H. A. & Lawrence, R.C. (1981). Recent modifications to the New Zealand activity test for Cheddar cheese starters. *New Zealand Journal of Dairy Science and Technology*, **16**, 91-94.

Hirota, T. & Kikuchi, T. (1976). Studies on kefir grains 1. Isolation and classification of micro-organisms from kefir grains and their characteristics. *Reports of research of Snow Brand Milk Products Co. Laboratory*, **74**, 63-82.

Horral, B.E. & Elliker, P.R. (1947). An activity test for Cheddar and cottage cheese starters. *Journal of Dairy Science*, **30**, 523-524.

IDF (1992). New technologies for fermented milks. *International Dairy Federation Bulletin*, **277**, 1-40.

Indyk, H.E., Edwards, M.J. & Woollard, D.C. (1996). High performance liquid chromatographic analysis of lactose-hydrolysed milk. *Food Chemistry*, **57**(4), 575-580.

Ismail, A.A. , El-Nockrashy, S.A. & Khorshid, M.A. (1983). A beverage from separated buffalo milk fermented with kefir grains. *Journal of the Society of Dairy Technology*, **36**(4), 117-118.

Jay, M.J. (1996). Fermentation and fermented dairy products. In: *Modern Food Microbiology*, 5th ed. Pp. 131-148. New York: Chapman & Hall.

Kalab, M., Allan-Wojtas, P & Phipps-Todd, B.E. (1983). Development of microstructure in set-style nonfat yoghurt. A review. *Microstructure,* **2**, 51-66.

Kandler, O. & Kunath, P. (1983). *Lactobacillus kefir* sp. nov., a component of the microflora of kefir. *Systematic Applied Microbiology*, **4**, 286-294.

Katsu, T., Zhang, X. & Rechnitz, G.A. (1994). Simultaneous determination of lactose and glucose in milk using two working enzyme electrodes. *Talanta*, **41**(6), 843-848.

Kleyn, D.H. (1985). Symposium: Role and significance of enzymes in dairy processing. Determination of lactose by an enzymatic method. *Journal of Dairy Science*, **68**, 2791-2798.

Kooiman, P. (1968). The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. *Carbohydrate Research*, **7**, 200-211.

Koroleva, N.S. (1975). *Technical Microbiology of Whole Milk Products*, Moscow, USSR: Pishchevaya Promyshlennost'.

Koroleva, N.S., Rozhkova, I.V. & Babina, N.A. (1978). Basic factors affecting the microflora and quality of kefir. *XX International Dairy Congress*, **E**, 843.

Koroleva, N.S. (1982). Special products (kefir, koumyss, etc.). *XXI International Dairy Congress*, 146-152.

Koroleva, N.S. (1988a). Starters for fermented milks. Chapter II. *International Dairy Federation Bulletin,* **227**, 35-40.

Koroleva, N.S. (1988b). Technology of kefir and kumys. Chapter VII. *International Dairy Federation Bulletin,* **227**, 96-100.

Korovkina, L.N., Patkul, G.M. & Maslov, A.M. (1976). Effect of pH, cooling method and chemical composition of the viscosity of kefir. *Izvestiya Vysshikh Uchebnykh Zavedenii, Pishchevaya Tekhnologiya*, **5**, 30-32.

Korovkina, L.N., Patkul, G.M. & Maslov, A.M. (1978). Effect of milk fermentation temperature on biochemical properties and consistency of kefir. *XX International Dairy Congress*, **E**, 841-84.

Kosikowski, F.V. (1977). *Cheese and Fermented Milk Foods*, 2nd ed. Ann Arbor, Michigan, USA: Edwards Brothers, Inc.

Kramkowska, A., Kornacki, K., Bauman, B. & Fesnak, D. (1982). Method of production of a single-use kefir culture. *XXI International Dairy Congress*, 304-305.

Kramkowska, A., Fesnak, D., Kornacki, K. & Bauman, K. (1986). Production, characterization and use of expendable kefir starter culture in the kefir production process. *Acta Biotechnologica*, **361**, 73-75.

Kriel, J.B. (1978). Die bepaling van suurselaktiwiteit. *South African Journal of Dairy Technology*, **10**(4), 183-186.

Kroger, M. (1993). Kefir. *Cultured Dairy Products Journal*, **28**, 26-28.

Kunath, P. & Kandler, O. (1983). *Lactobacillus kefir* sp. nov., a component of the microflora of kefir. *Systematic Applied Microbiology*, **4**, 286-294.

Kurmann, J.A., Rasic, J.L.J. & Kroger, M. (1992). *Encyclopedia of Fermented Fresh Milk Products: an International Inventory of Fermented Milk, Cream, Buttermilk, Whey, and Related Products.* New York: Van Nostrand Reinhold.

Kwak, H.S., Park, S.K. & Kim, D.S. (1996). Biostabilization of kefir with a nonlactose-fermenting yeast. *Journal of Dairy Science*, **79**, 937-942.

La Rivière, J.W.M., Kooiman, P. & Schmidt, K. (1967). Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis. Archives of Microbiology*, **59**, 269-278.

Leenheer, K. & Jans, J.A. (1986). Enzymatic determination of the lactic acid & lactates content of dried milk. Chapter 5, IDF Standard 69A: 1984. *International Dairy Federation Bulletin*, **207**, 122-132.

Lees, G.J. & Jago, G.R. (1976). Acetaldehyde: an intermediate in the formation of ethanol from glucose by lactic acid bacteria. *Journal of Dairy Research*, **43**, 63-73.

Libudzisz, Z. & Piatkiewicz, A. (1990). Kefir production in Poland. *Dairy Industries International,* **55**(7), 31-33.

Liu, J.A.P. & Moon, N.J. (1983). Kefir – a "new" fermented milk product. *Cultured Dairy Products Journal*, **18**(3), 11-12.

Luong, J.H.T., Mulchandani, A. & Groom, C.A. (1989). The development of an amperometric microbial biosensor using *Acetobacter pasteurianus* for lactic acid. *Journal of Biotechnology*, **10**, 241-252.

Mann, E.J. (1983). Kefir & koumiss. *Dairy Industries International,* **48**(4), 9-10.

Mann, E.J. (1989). Kefir and koumiss. *Dairy Industries International*, **54**(9), 9-10.

Marshall, V.M. (1982). Flavour compounds in fermented milks. *Perfumer & Flavorist*, **7**, 27-34.

Marshall, V.M.E. (1984). Flavour development in fermented milks. In: *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk* (edited by F.L. Davies & B.A. Law). Pp. 153-186. London: Elsevier Applied Science Publishers.

Marshall, V.M. (1987). Fermented milks and their future trends. I. Microbiological aspects. *Journal of Dairy Research*, **54**, 559-574.

Marshall, V.M. (1993). Kefir. In: *Encyclopaedia of Food Science and Technology* (edited by Y. H. Hui), Volume 3. Pp. 1804-1808. Chichester, UK: John Wiley & Sons.

Marshall, V.M. & Cole, W. M. (1985). Methods for making kefir and fermented milks based on kefir. *Journal of Dairy Research*, **52**, 451-456.

Marshall, V.M., Cole, W.M. & Brooker, B.E. (1984). Observations on the structure of kefir grains and the distribution of the microflora. *Journal of Applied Bacteriology*, **57**, 491-49.

Mascini, M., Moscone, d. & Palleschi, G. (1984). A lactate electrode with lactate oxidase immobilized on nylon net for blood serum samples in flow systems. *Analytica Chimica Acta*, **157**, 45-51.

Masoom, M. & Towshend, A. (1985). Simultaneous determination of sucrose and glucose in mixtures by flow injection analysis with immobilized enzymes. *Analytica Chimica Acta.*, **171**, 185-194.

Merin, U. & Rosenthal, I. (1986). Production of kefir from UHT milk. *Milchwissenschaft*, **41**(7), 395-396.

Mizutani, F. & Asai, M. (1990). Simultaneous determination of glucose and sucrose by a glucose-sensing enzyme electrode combined with an invertase-attached cell. *Analytica Chimica Acta*, **236**, 245-250.

Moulin, G., Arthaud, J.F., Ratomahenina, R. & Galzy, P. (1977). Notes on kefir production. *Alimentaires et Agricoles,* **94**(5), 495-497.

Mukai, T., Toba, T., Itoh, T. & Adachi, S. (1988). Structural microheterogeneity of kefiran from kefir grains. *Japanese Journal of Zootechnology, Science and Biology*, **59**, 167 *(as cited by Yokoi et al., 1990).*

Mukai, T., Toba, T., Itoh, T., Nimura, T. & Adachi, S. (1990). Carboxymethyl kefiran: preparation and viscometric properties. *Journal of Food Science*, **55**, 1483-1484.

Mukai, T., Watanabe, N., Toba, T., Itoh, T. & Adachi, S. (1991). Gel-forming characteristics and rheological properties of kefiran. *Journal of Food Science*, **56**, 1017-1018, 1026.

Mukai, R., Onose, Y., Toba, T. & Itoh, T. (1992). Presence of glycerol teichoic acid in the cell wall of *Lactobacillus kefiranofaciens. Letters in Applied Bacteriology*, **15**, 29-31.

Mulchandani, A., Bassi, A.S. & Nguyen, A. (1995). Tetrathiafulvalene-mediated biosensor for L-lactate in diary products. *Journal of Food Science*, **60**(1), 74-78.

Murofushi, M., Shiomi, M. & Aibara, K. (1983). Effect of orally administered polysaccharide from kefir grain in delayed-type hypersensitivity and tumor growth in mice. *Japanese Journal of Medical Science and Biology*, **36**, 49.

Mustranta, A. & Östman, C. (1997). Enzymatic determination of lactose and galactose in foods: NMKL collaborative methods performance study. *Journal of AOAC International*, **80**(3), 584-590.

Narinesingh, D., Stoute, V.A., Davis, G., Persad, D. & Ngo, T.T. (1992). Improved spectrophotometric determination of lactose in milk using flow-injection analysis. *Analytica Chimica Acta.*, **258**, 141-149.

Neve, H. (1992). Analysis of kefir grain starter cultures by scanning electron microscopy. *Milchwissenschaft*, **47**, 275-278.

Nickerson, T.A., Vujicic, I.F. & Lin, A.Y. (1976). Colorimetric estimation of lactose and its hydrolytic products. *Journal of Dairy Science*, **59**, 386-390.

Okigbo, L.M., Oberg, C.J. & Richardson, G.H. (1985). Lactic culture activity testing using pH and impedance instrumentation. *Journal of Dairy Science*, **68**, 2521-2526.

Olsen, R.L. (1978). An improved activity test for lactic cultures and culture concentration, MS Thesis, University Logan, Utah State.

Pfeiffer, F., Scheller, M., Jänchem, M., Bertermann, K. & Weise, H. (1980). *Analytical Letters*, **13**, 1179.

Piechocka, M., Holmen, T.B. & Abrahamsen, R.K. (1977). Heat treatment, homogenization and incubation of milk for production of kefir, of various fat levels. *Nordeuropaeisk Mejeri Tidsskrift*, **43**(10), 334-343.

Pintado, M.E., Lopes Da Silva, J.A., Fernandes P.B., Malcata, F.X. & Hogg, T.A. (1996). Microbiological and rheological studies on Portuguese kefir grains. *International Journal of Food Science and Technology,* **31**, 15-26.

Puhan, Z. (1988). Results of the questionnaire 1785B "Fermented Milks". Chapter XII. *International Dairy Federation Bulletin*, **227**, 139, 160-161.

Rea, M.C., Lennartson, T., Dillon, P., Drinan, F.D., Reville, W.J., Heapes, M. & Cogan, T.M. (1996). Irish kefir-like grains: their structure, microbial composition and fermentation kinetics. *Journal of Applied Bacteriology,* **81**, 83-94.

Robert, P., Bertrand, D. & Devaux, M.F. (1987). Multivariate analysis applied to near infrared spectra of milk. *Analytical Chemistry*, **59**(17), 2187-2191.

Roberts, G.M. (1977). Infrared analysis of milk powders and casein products, Technical paper Pacific Scientific no. 4007 *(as cited by Frankhuizen, 1992).*

Rodriguez-Otero, J.L. & Hermida, M. (1996). Analysis of fermented milk products by near-infrared reflectance spectroscopy. *Journal of AOAC International*, **79**(3), 817-821.

Rodriguez-Otero, J.L., Hermida, M. & Centeno, J. (1997a). Analysis of dairy products by near-infrared spectroscopy: A review. *Journal of Agricultural and Food Chemistry*, **45**(8), 2815-2819.

Rodriguez-Otero, J.L., Centeno, J.A. & Hermida, M. (1997b). Application of near infrared transflectance spectroscopy to the analysis of fermented milks. *Milchwissenschaft*, **52**(4), 196-200.

Roginski, H. (1988). Fermented milks. *The Australian Journal of Dairy Technology*, **43**, 37-46.

Rosi, J. & Rossi, J. (1978). I microrganismi del kefir: I fermenti lattici. *Scienza e Tecnica Lattiero-Casearia*, **29**, 291-305 *(as cited by Garrote et al., 1997).*

Saloff-Coste, C.J. (1996). Kefir. *Danone World Newsletter* no. 11. http://www.danonenewsletter.fr/eng/news_11/sum.html, June, 1999.

Scheller, F.W., Pfeiffer, D., Schubert, D., Renneberg, R. & Kirsten, D. (1987). Application of enzyme-based amperometric biosensors to the analysis of real samples. In: *Biosensors: Fundamentals and Applications* (edited by A.P.F. Turner, I. Karube & G. Wilson). Pp. 315-346. Oxford: Oxford University Press.

Semenichina, Mrs. (1984). Other fermented milks. *International Dairy Federation Bulletin*, **179**, 120-122.

Shah, N. (1993). Effectiveness of dairy products in alleviation of lactose intolerance. *Food Australia*, **45**(6), 265-271.

Shiomi, M., Sasaki, K., Murofushi, M. & Aibara, K. (1982). Antitumor activity in mice of orally administered polysaccharide from kefir grain. *Japanese Journal of Medical Science and Biology*, **35**, 75.

Shu, H., Mattiasson, B., Persson, B., Nagy, G., Gorton, L., Sahni, S., Geng, L., Boguslavsky, L. & Skotheim, T. (1995). A reagentless amperometric electrode based on carbon paste, chemically modified with D-lactate dehydrogenase, NAD+, and mediator containing polymer for D-lactic acid analysis. I. Construction, composition, and characterization. *Biotechnology and Bioengineering*, **46**, 270-279.

Soumalainen, H. & Jannes, L. (1946). Fermentative formation of diacetyl. *Nature*, **157**, 336-337.

Spinnler, H.E. & Corrieu, G. (1989). Automatic method to quantify starter activity based on pH measurement. *Journal of Dairy Research*, **56**, 755 - 764.

Steinkraus, K.H. (1996). Acid-fermented milk and milk/cereal foods. In: *Handbook of Indigenous Fermented Foods,* 2nd ed. Pp. 275 and 305-308. New York: Marcel Dekker, Inc.

Švitel, J., Curilla, O. & Tkác, J. (1998). Microbial cell-based biosensor for sensing glucose, sucrose or lactose. *Applied Biochemistry*, **27**, 153-158.

Thompson, J.K., Johnston, D.E., Murphy, R.J. & Collins, M.A. (1990). Characteristics of a milk fermentation from rural Northern Ireland which resembles kefir. *Irish Journal of Food Science and Technology*, **14**, 35-49.

Toba, T. Abe, S., Arihara, K. & Adachi, S. (1986). A medium for isolation of capsular bacteria from kefir grains. *Agricultural and Biological Chemistry*, **50**, 2673-2674.

Toba, T., Arihara, K. & Adachi, S. (1987). Comparative study of polysaccharides from kefir grains, an encapsulated homofermentative Lactobacillus species and Lactobacillus kefir. *Milchwissenschaft,* **42**(9), 565-568.

Toba, T., Arihara, K. & Adachi, S. (1990). Distribution of micro-organisms with particular reference to encapsulated bacteria in kefir grains. *International Journal of Food Microbiology,* **10**, 219-224.

Ueda, M., Isasawa, S., Miyata, N & Ahiko, K. (1982). Hexosephosphorylating activities of *Torulopsis holmii* KY5 isolated from kefir grain. *Agricultural and Biological Chemistry* **46**, 2637-2643.

Vedamuthu, B.R. (1982). Fermented milks. In: *Fermented Foods in Economic Microbiology* (edited by A.H. Rose), Volume 7. Pp. 199-226. New York: Academic Press.

Watanabe, E., Takagi, M., Takei, S., Hoshi, M. & Shu-gui, C. (1991). Development of biosensors for the simultanenous determination of sucrose and glucose, lactose and glucose, and starch and glucose. *Biotechnology and Bioengineering*, **38**, 99-103.

Weaver, R.W.V. (1978). Infrared reflectance analysis applied to the dairy industry. In: *Proceedings of the 8th Technicon International NIRA Congress*. London, United Kingdom.

Weaver, R.W.V. (1984). Near infrared reflectance analysis applied to dairy products. In: *Proceedings of a seminar, University of Reading.* Pp. 91-102. London: Royal Society on Chemistry.

White, F.H. & Kidney, E. (1981). Yeast-bacterium interactions in the brewing industry. In: *Mixed Culture Fermentations* (edited by M.E. Bushell & J.H. Slater). Pp. 121-133. London: Academic Press.

Yokoi, H., Watanabe, T., Fuji, Y., Toba, R. & Adachi, S. (1990). Isolation and characterization of polysaccharide-producing bacteria from kefir grains. *Journal of Dairy Science*, **73**, 1684-1689.

Yokoi, H., Watanabe, T., Fuji, Y., Mukai, T., Toba, T. & Adachi, S. (1991). Some taxonomical characteristics of encapsulated Lactobacillus sp KPB-167B isolated from kefir grains and characterization of its extracellular polysaccharide. *International Journal of Food Microbiology*, **13**, 257-264.

Zhang, X. & Rechnitz, G.A. (1993). *Electroanalysis (as cited by Katsu et al., 1994).*

Zourari, A. & Anifantakis, E.M. (1988). Le kefir caracteres physico-chimiques, microbiologiques et nutritionnels. Technologie de production. Une revue. *Le Lait*, **68**, 373-392.

Chapter 3

INFLUENCE OF VARYING CULTURING CONDITIONS ON THE RATE OF KEFIR GRAIN BIOMASS INCREASE

Summary

This study was undertaken to determine the optimum conditions for kefir grain biomass increase. Kefir grain biomass normally increases between 60% and 100% over 10 days incubation in milk, but these increases are too slow for supplying kefir grains on industrial scale. Different incubation temperatures (18°, 22°, 25° and 30°C), milk enrichment with combinations of tryptose (2% m/v), yeast extract (2% m/v) or urea (0.5% m/v), different milk volumes (initial and replacement volumes) and different starter sizes, as well as agitation of the cultivation vessel in a shaking water bath (rpm = 130) and the use of active versus inactive grains, were studied to determine the influence on grain biomass increase.

Cultivation at 22°C resulted in the highest biomass increases, but was found unsuitable for the production for quality kefir and was subsequently replaced by 25°C as optimum incubation temperature. Initial grain starter sizes of more than 1% (m/v) was found to be necessary for grain biomass increase. Daily replacement of all the fermented milk with fresh milk resulted in biomass increases at least nine times more than when replacing only a portion of the fermented milk. It was concluded that the addition of yeast extract or tryptose to milk substantially improved the grain biomass increase. The addition of urea did not result in large biomass increases, but urea in combination with yeast extract led to biomass increases at least seven times more than found with the conventional method. Low fat milk resulted in the highest biomass increases during non-agitated cultivation, but no substantial difference in biomass increase was found when either low fat or full cream milk were used during agitated cultivation. Agitation of the cultivation vessel led to grain biomass increases at least six times more than conventional cultivation without agitation. The use of inactive kefir grains as initial inoculum resulted in a lag period of 20 days before

the grain biomass started to increase substantially. No lag phase was observed with the use of active grains as initial inoculum.

Parameters leading to optimum mass cultivation of kefir grains were the use of more than 1% active kefir grains as starter and cultivation at 25°C in milk containing added urea (0.5%) and yeast extract (2%), as well as agitating the cultivation vessel and replacing all the fermented milk daily. Maximum biomass increases of up to 327% over a 10-day period were observed with this method, compared to the 60% to 100% increases over the same period with the conventional grain cultivation method.

Introduction

Kefir grains are white to yellowish, moist and gelatinous granules with an irregular cauliflower-like appearance and are used as the natural starter during kefir manufacture. The size of the grain structure can be anything from 0.5 to 3.5 cm in diameter (Marshall & Cole, 1985). Kefir grains consist of a variety of micro-organisms entrapped in a water-insoluble carbohydrate (kefiran) which makes them extremely tough and resilient (La Rivière *et al.*, 1967). The grains are initially very small and increase in size during fermentation of milk, but can only grow from pre-existing grains (Steinkraus, 1996). Whole milk, skim milk or neutralised whey can be used as growth medium to grow the kefir grains in (Steinkraus, 1996).

Modern kefir grain production is based on continuous cultivation in milk, resulting in biomass increases of 5 - 7% per day (Libudzisz & Piatkiewicz, 1990). As soon as fermentation is completed the grains are removed from the milk by sieving and directly introducing them into fresh milk. La Rivière *et al.* (1967) reported that 500 g of wet kefir grains can double their weight in 7 - 10 days if they are transferred to 500 ml fresh milk six times a week. Growth is, however, greatly retarded if the grains are rinsed with water after each sieving (Steinkraus, 1996). The above biomass increases are, however, too small if kefir grains are to be produced commercially, and a new method will have to be developed to maximise grain biomass increase.

In spite of the fact that intensive research has been done to produce kefir grains from pure or mixed cultures normally present in the grains, no successful results have been reported (Koroleva, 1975; Hirota & Kikuchi, 1976; Liu & Moon, 1983; Libudzisz & Piatkiewicz, 1990). This failure can probably be ascribed to the fact that very little, if anything is known about the mechanism of grain formation. It is very likely that a combination of different factors have an influence on the biomass increase of the kefir grains, including the renewal of milk at regular intervals, cultivation temperature, grain washing and the presence of essential nutrients in the correct concentration in the growth medium.

The aim of this study was to investigate the influence of varying culturing conditions to obtain optimal conditions for kefir grain biomass increase.

Materials and methods

Milk pasteurisation

Fresh pasteurised milk purchased at local supermarkets was given a further heat treatment in a temperature-controlled water bath at 83° - 85°C for 20 min and the milk cooled to 3°C. Previous studies conducted by Human (1998) on commercially pasteurised milk showed that pasteurisation was not always performed satisfactorily, resulting in milk of an inferior microbiological quality (Human, 1998). It was, therefore, decided to re-pasteurise the milk to ensure reliable microbiological quality. Heat treatment of milk is also beneficial in that it makes the milk a more nutritious medium for the starter organisms due to the release of amino acids and other growth factors, reduction of the redox potential and elimination of inhibitory substances. The formation of hydrolytic rancidity is also prevented through the inactivation of lipases (Kessler, 1981). A re-pasteurisation treatment also increases the denaturation of whey proteins and the dispersion of casein particles. The firmness, elasticity and viscosity of the curd are improved, leading to improved kefir consistency. Syneresis is also reduced (Bondarev, 1977; Berzhinskas *et al.*, 1978).

Grain activation

Frozen kefir grains (-18°C) were allowed to defrost at room temperature, added to fresh pasteurised milk at 25°C and incubated at 25°C for 24 h. The grains were then retrieved using a sterilised stainless steel sieve (1.25% Milton solution for 30 min, then rinsed with sterile distilled water) and placed directly into fresh pasteurised milk at 25°C for 24 h. This procedure was repeated for three subsequent days before the grains were used.

Preparation of kefir

One litre of pasteurised milk (either low fat [2% fat m/v] or full cream [4% fat m/v]) was inoculated at 25°C with a specified mass of activated kefir grains and incubated at 25°C for 18 h. The grains were removed from the kefir with a sterilised sieve and placed in a sterile petri-dish for storage at 4°C until the next batch of kefir was made (within the next 24 h). The fermented kefir was incubated at 22°C for a further six hours and cooled to 4°C before evaluation. This procedure was repeated continuously before the follow-up studies were done. A summary of the experimental designs used in this study is given in Table 1.

Experimental Study I - *Biomass increase of kefir grains during incubation at different temperatures in full cream milk.*

Four 1 l sterile containers, each with 800 ml pasteurised full cream milk (4% fat m/v), were inoculated with 2 g unrinsed kefir grains (Table 1). Each container, with the milk-grain mixtures, was then incubated at a different temperature (18°, 22°, 25° and 30°C) for 24 h. The grains were removed after the incubation period using a sterilised sieve and dried lightly with towelling paper to remove residual milk. The grains were weighed in a sterile petri-dish, introduced into 800 ml fresh pasteurised full cream milk and again incubated at the specific temperatures. After every 24 h fermentation period the pH was monitored and the titratable acidity (TA) measured using the method of Dixon (1973). This procedure was repeated for eight days.

Table 1. Experimental designs used for Studies I - VIII.

Study	Description	Method	Temperature	Inoculum	Milk volume	Duration (days)	Milk type*
I	Biomass increase at different temperatures (full cream milk)	Sieving and re-inoculating into fresh milk every 24 h. (Mass, pH and TA)	18°, 22°, 25° and 30°C	2 g	800 ml, renewed daily with 800 ml fresh milk	8	F (4%)
II	Biomass increase at different temperatures (low fat milk)	Sieving and re-inoculating into fresh milk every 24 h. (Mass, pH and TA)	18°, 22°, 25° and 30°C	2 g	200 ml, renewed daily with 200 ml fresh milk	4	L (2%)
III	Biomass increase at different temperatures (low fat milk)	Sieving and re-inoculating into fresh milk every 24 h. (Mass, pH and TA)	18°, 22°, 25° and 30°C	2 g	200 ml, renewed daily with 200 ml fresh milk	10	L (2%)

Table 1. continue /...

Study	Description	Method	Temperature	Inoculum	Milk volume	Duration (days)	Milk type*
IV (i - iii)	Biomass increase in different milk types (non-agitation) (i - iii)	Sieving and re-inoculating into fresh milk daily (Mass every 24 h (i - iii))	25°C	4 g	i) 200 ml, renew daily with 200 ml fresh milk	i) day 0 - 28	i) a = L b = F c = FFP d = FY
					ii) 400 ml, renew daily with 400 ml fresh milk	ii) day 29 - 40	ii) a = LY b = FY c = FFP d = FYT
					iii) 400 ml, renew daily with 400 ml fresh milk	iii) day 41 - 62 Total = 62	iii) a = LY b = LT c = LYT d = FYT

Table 1. continue /...

Study	Description	Method	Temperature	Inoculum	Milk volume	Duration (days)	Milk type*
V (i - iii)	Cultivation with agitation in different milk types (i - iii)	Re-inoculating into fresh milk daily. (pH and TA every 24 h for whole period (43 days). Mass on days 0, 16, 23, 33 and 43)	25°C	40 g	i) 400 ml, renew daily with 100 ml fresh milk	i) day 0 - 16	i) a = L e = F b = LT f = FT c = LY g = FY d = LYT h = FYT
					ii) 400 ml, renew daily with 100 ml fresh milk	ii) day 16 - 23	ii) low fat
					iii) 400 ml, renew daily with 400 ml fresh milk	iii) day 23 - 43 Total = 43	iii) same as (i)
VI	Cultivation with agitation in different milk types (Repeat of V)	Re-inoculating into fresh milk daily. (pH and TA every 24 h. Mass on days 0, 10, 20 and 30)	25°C	40 g	Start with 400 ml, renew daily with 400 ml fresh milk	30	L LT LY LYT F FT FY FYT

Table 1. continue /...

Study	Description	Method	Temperature	Inoculum	Milk volume	Duration (days)	Milk type*
VII	Cultivation with agitation in different milk types	Sieving and re-inoculating into fresh milk daily (Mass on day 10, 20 and 40).	25°C	40 g (frozen grains)	Start with 400 ml, renew daily with 400 ml fresh milk	40	L(wa) F(wa) L F LY FY LU FU LUY FUY
VIII	Cultivation with agitation in different milk types	Sieving and re-inoculating into fresh milk daily (Mass on day 10 and 20)	25°C	40 g (active grains)	Start with 400 ml, renew daily with 400 ml fresh milk	20	L(wa) F(wa) L F LY FY LUY FUY

* L(wa) = Low fat milk without agitation
L = Low fat milk (2% fat)
LY = Low fat milk + 2% yeast extract
LT = Low fat milk + 2% tryptose
LU = Low fat milk + 0.5% urea
LYT = Low fat milk + 2% yeast extract + 2% tryptose
LUY = Low fat milk + 0.5% urea + 2% yeast extract

F(wa)= Full cream milk without agitation
F = Full cream milk (4% fat)
FY = Full cream milk + 2% yeast extract
FT = Full cream milk + 2% tryptose
FU = Full cream milk + 0.5% urea
FYT = Full cream milk + 2% yeast extract + 2% tryptose
FFP = Full cream milk + 2% full cream milk powder
FUY = Full cream milk + 0.5% urea + 2% yeast extract

Experimental Study II - *Biomass increase of kefir grains during incubation at different temperatures in low fat milk.*

This study was a repetition of Study I, except that 200 ml volumes of low fat milk (2% fat m/v), were used (Table 1). The containers were incubated at the same temperature range (18°, 22°, 25° and 30°C) for 24 h. After every 24-h period the grains were again separated, weighed, the pH monitored and TA measured. This study was performed over a period of four days.

Experimental Study III - *Biomass increase of kefir grains during incubation at different temperatures in low fat milk.*

This study was a repetition of Study II (Table 1), but performed over a 10-day period. The grains from Study II were separated and activated by placing in 200 ml fresh pasteurised, low fat milk at 25°C for 24 h. This was repeated three times before the grains were re-used as inoculum.

Experimental Study IV - *Biomass increase of kefir grains grown in different milk types (non-agitation).*

The grains from Study III were separated and activated at 25°C for 24 h. This was repeated twice before the grains were re-used (Table 1).

IV(i) In this study 4 g of kefir grains were inoculated into each of four sterile containers, with 200 ml of one of the following pasteurised milk types respectively: (a) low fat milk (L); (b) full cream milk (F); (c) full cream milk containing 2% full cream milk powder (FFP); and (d) full cream milk containing 2% yeast extract (FY).

Milk types c (FFP) and d (FY) were prepared by slowly blending the milk with either milk powder or yeast extract (Waring Blendor) for 30 sec. The four containers with the milk-grain mixtures were incubated at 25°C for 24 h, after which the grains were separated, weighed and re-inoculated into fresh milk of each type, followed by incubation at 25°C. This was done for 28 days (day 0 to 28).

IV(ii) Based on the data obtained during Study IV(i), the following experimental changes were made (from day 29 to 40) and the following pasteurised milk

types used: (a) low fat milk containing 2% yeast extract (LY); (b) full cream milk containing 2% yeast extract (FY); (c) full cream milk containing 2% full cream milk powder (FFP); and (d) full cream milk containing 2% yeast extract and 2% tryptose (FYT).

The grains used in Study IV(i) in milk type a (L) were now inoculated into a larger volume (400 ml) of the new milk type a (LY), previous milk type b (F) grains were now used in 400 ml of the new milk type b (FY) etc. Incubation proceeded at 25°C for 24 h periods with daily weighing of the grains as previously described.

IV(iii) Once again, based on the data obtained during Study IV(ii), changes were implemented and from day 41 to 62, 400 ml of the following pasteurised milk types were used: (a) low fat milk containing 2% yeast extract (LY); (b) low fat milk containing 2% tryptose (LT); (c) low fat milk containing 2% yeast extract and 2% tryptose (LYT); and (d) full cream milk containing 2% yeast extract and 2% tryptose (FYT).

The grains incubated in milk type a (LY) and milk type d (FYT) in study IV(ii) were incubated in the same milk types for this study. The grains that were previously incubated in the milk types b (FY) and c (FFP) were removed on day 40, mixed and incubated for 24 h in low fat milk at 25°C. On day 41 these grains were placed in the new milk types b (LT) and c (LYT) and incubated at the same temperature.

Experimental Study V - Cultivation of kefir grains in different milk types (with agitation).

Kefir grains from the previous study were separated and activated at 25°C for three days by replacing the milk every 24 h. Eight 1 l containers were prepared (Table 1), each with 400 ml of either one of the following milk types: (a) low fat milk (L); (b) low fat milk with 2% tryptose (LT); (c) low fat milk with 2% yeast extract (LY); (d) low fat milk with 2% tryptose and 2% yeast extract (LYT); (e) full cream milk (F); (f) full cream milk with 2% tryptose (FT); (g) full cream milk with 2% yeast extract (FY); and (h) full cream milk with 2% tryptose and 2% yeast extract (FYT).

Each container was inoculated with 40 g kefir grains and placed in a shaking water bath (rpm = 130) at 25°C for 24 h, over a 43-day period with grain mass determinations performed on days 0, 10, 16, 23, 33 and 43. For the first sixteen days (day 0 to 16), 100 ml fermented milk of each milk type was removed after every 24-h incubation period, replaced with 100 ml fresh milk of each type and the TA and pH of the fermented milk measured.

During the next seven days (day 16 to 23), all eight containers were replenished daily with 100 ml fresh, pasteurised low fat milk instead of the different milk types that were used during the first 16 days. Removal of the 100 ml samples was still performed daily, as well as the determination of the pH and TA.

During the next 20 days (day 23 to 43) all the fermented milk was removed from each container every 24 h and replaced with 400 ml of the different milk types as used in the first section of this study (Table 1). The pH and TA were measured daily.

Experimental Study VI - *Cultivation of kefir grains in different milk types (with agitation).*

This study was a partial repetition of Study V (Table 1). Eight 40 g batches of grains from Study V were placed in eight 1 l containers (400 ml milk) with the same milk types as used in Study V. The containers were incubated in a 25°C shaking water bath (rpm = 130) for 24 h periods with daily measurement of the pH and TA. All the fermented milk was removed after each 24-h period and replaced with 400 ml fresh, pasteurised milk of each type. The grain mass was determined on days 0, 10, 20 and 30.

Experimental Study VII - *Cultivation of frozen kefir grains in different milk types (with agitation).*

This study was similar to studies V and VI in that the influence of enrichment of the growth medium on kefir grain biomass increase was investigated, but in this case frozen instead of activated grains were used (Table 1). In this study, urea was used to replace tryptose as a nutrient source due to the unavailability of the latter nutrient. Forty grams of frozen grains (without any activation) from the previous study were defrosted and placed directly in 400 ml of either one of the following milk types (Table 1): (a) low fat milk without agitation

(L(wa)); (b) low fat milk (L); (c) low fat milk with 2% yeast extract (LY); (d) low fat milk with 0.5% urea (LU); (e) low fat milk with 0.5% urea and 2% yeast extract (LUY); (f) full cream milk without agitation (F(wa)); (g) full cream milk (F); (h) full cream milk with 2% yeast extract (FY); (i) full cream milk with 0.5% urea (FU); and (j) full cream milk with 0.5% urea and 2% yeast extract (FUY). The containers that were not agitated, were placed in a 25°C incubator and the rest in a shaking water bath (rpm = 130) at the same temperature. Cultivation was for 24-h periods and the milk was then replaced with 400 ml fresh milk of each type. The grain biomass was measured on days 0, 10, 20 and 40.

Experimental Study VIII - *Cultivation of active kefir grains in different milk types (with agitation).*

This study was basically a repetition of Study VII, but active kefir grains were used instead of frozen grains. The influence of growth medium enrichment and agitation was again investigated. At the completion of Study VII, the grains were taken immediately, divided into 40 g batches and placed in 400 ml of either one of the following milk types: (a) low fat milk without agitation (L(wa)); (b) low fat milk (L); (c) low fat milk with 2% yeast extract (LY); (d) low fat milk with 0.5% urea and 2% yeast extract (LUY); (e) full cream milk without agitation (F(wa)); (f) full cream milk (F); (g) full cream milk with 2% yeast extract (FY); and (h) full cream milk with 0.5% urea and 2% yeast extract (FUY). Milk enriched with only urea was omitted due to the weaker grain mass increase found in the previous study. Daily replacement of all the fermented milk with 400 ml fresh milk of each type was carried out in 24-h intervals and grain weighing on days 10 and 20.

Results and discussion

Experimental Study I - *Biomass increase of kefir grains during incubation at different temperatures in full cream milk.*

In this study the biomass increase of kefir grains in full cream milk, at four different incubation temperatures, was monitored (Fig. 1 - 3). In all four cases the

Fig1-3(ch3)

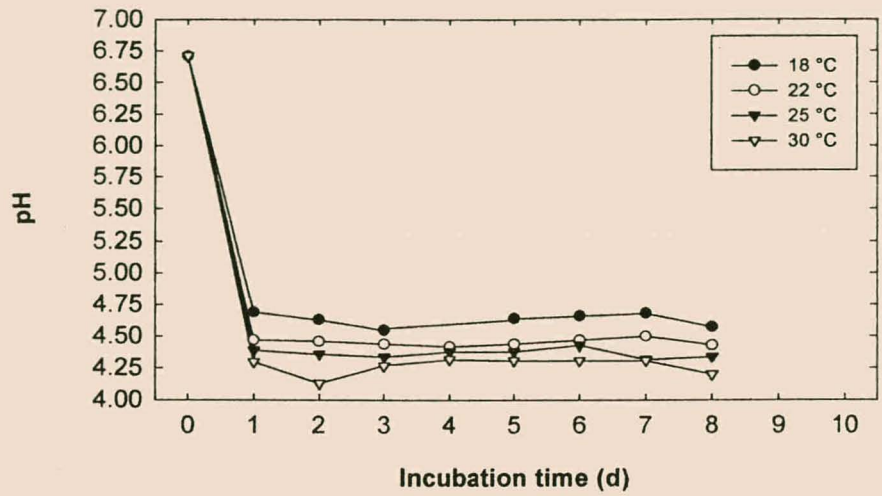


Figure 1. Influence of different incubation temperatures on the pH of full cream kefir (Study I).

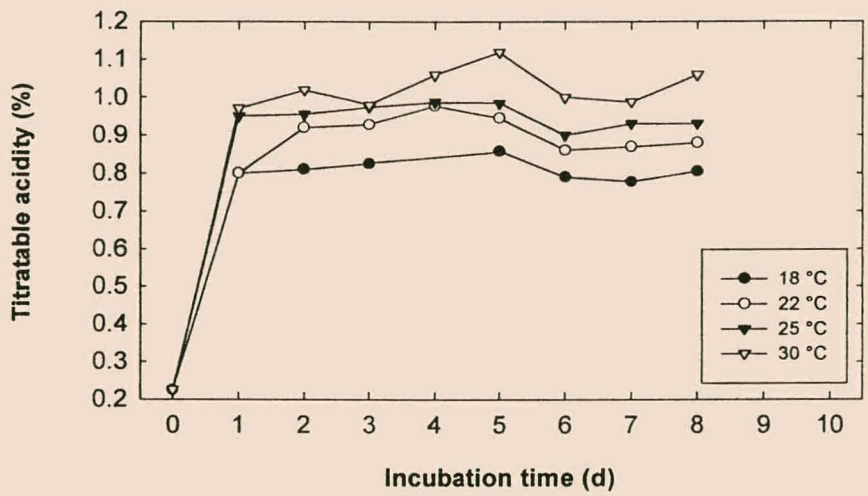


Figure 2. Influence of different incubation temperatures on the development of titratable acidity in full cream kefir (Study I).

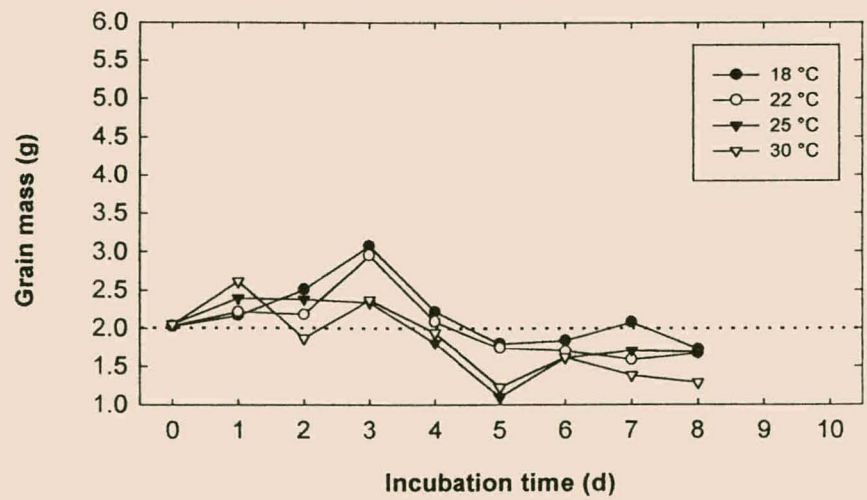


Figure 3. Influence of different incubation temperatures on the biomass increase of kefir grains incubated in full cream milk (Study I). The dotted line indicates the original inoculum size.

pH dropped sharply from 6.7 to below 4.8 during the first 24 h and stabilised for the duration of the study. During the eight-day incubation period the milk incubated at 30°C reached the lowest pH (4.3), with higher pH values measured for the milk incubated at lower temperatures. It was consequently concluded that a higher amount of lactic acid was produced at higher incubation temperatures. The stabilisation of the pH after the first fast drop could probably be attributed to the daily depletion of the same amount of fermentable substrate (i.e. equal volumes of milk).

In all four cases the most dramatic change in TA also occurred during the first 24 h of incubation (Fig. 2). The milk incubated at the higher temperatures led to higher TA values than milk incubated at lower temperatures, indicating that more lactic acid is produced as the incubation temperature is increased. The measured TA is generally concluded to correspond to the lactic acid produced (James, 1995), therefore, it was assumed that almost the same amount of lactic acid was produced every 24 h. After the first major increase, the TA values stabilised and did not show any major changes for the duration of the incubation period.

At the end of the study (day 8), the grain biomass in all four cases was found to be lower than the original inoculum (Fig. 3), but no definite pattern in biomass change was discernible. A possible explanation for this could be that some of the grains were so small that they were able to pass through the sieve-mesh and as a result, were lost. The volume of milk (800 ml) could also have been too large to create a favourable environment for grain propagation. It is, however, interesting to note that although the grains varied in weight they still produced lactic acid at a relatively constant rate for each incubation temperature.

Experimental Study II - Biomass increase of kefir grains during incubation at different temperatures in low fat milk.

Due to the variations in kefir grain biomass found in the previous study, it was decided to decrease the milk volume to 200 ml and change the milk type from full cream to low fat. In this study, almost the same pH pattern was observed (Fig. 4) as in Study I, with a sharp pH decrease within the first 24 h and stabilisation after that. However, the incubation at 18°C resulted in a more gradual decrease

Fig4-6(ch3)

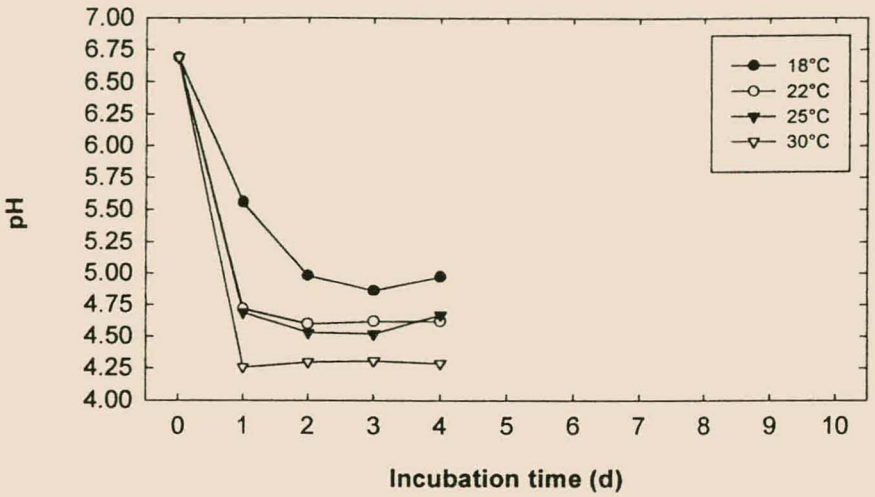


Figure 4. Influence of different incubation temperatures on the pH of low fat kefir (Study II).

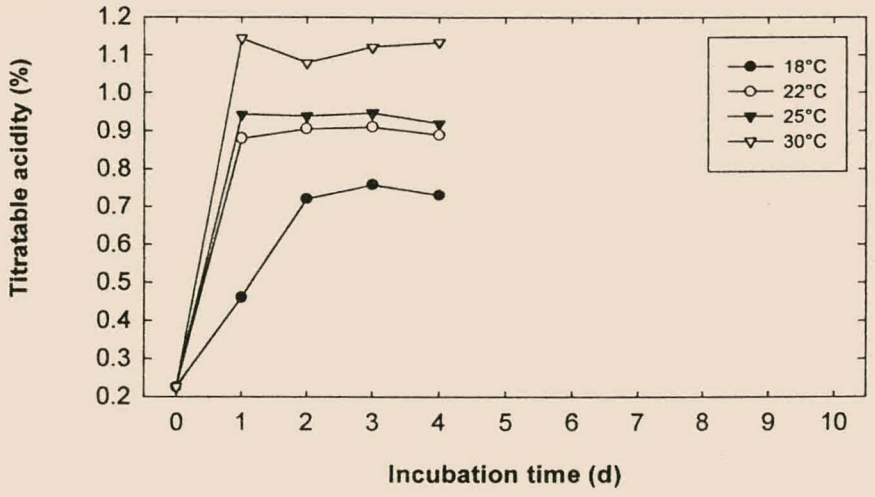


Figure 5. Influence of different incubation temperatures on the development of titratable acidity in low fat kefir (Study II).

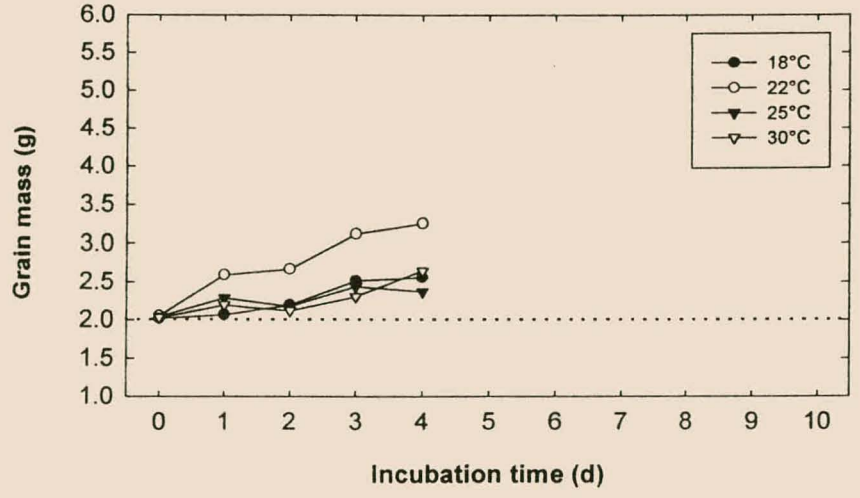


Figure 6. Influence of different incubation temperatures on biomass increase of kefir grains incubated in low fat milk (Study II). The dotted line indicates the original inoculum size.

in pH. Incubation at 30°C resulted in the attainment of the lowest pH (4.3), while incubation at the lower temperatures resulted in pH values ranging from 4.5 - 4.9. Incubation at 22° and 25°C showed very similar pH profiles.

Incubation at 22°, 25° and 30°C again resulted in a rapid increase in TA during the first 24 h, and stabilised for the duration of the study (Fig. 5). The TA, during incubation at 18°C, only stabilised after 48 h, again leading to the conclusion that incubation at 18°C is less favourable for acid production. The highest TA value (1.14%) was obtained by incubation at 30°C and corresponded well with the data from Study I, where the maximum TA at 30°C was 1.12%. Incubation at lower temperatures again resulted in lower TA values, the lowest being for incubation at 18°C (0.73% after four days). In this study, in comparison to Study I, smaller volumes of milk were used (200 ml instead of 800 ml), but almost the same TA was measured as in Study 1. Thus, it was assumed that only a certain amount of lactic acid can be produced per period if the same grain mass is used to ferment different volumes of milk. The amount of lactic acid produced is therefore dependent on the amount of lactic acid producing bacteria present in the grains.

In this study, compared to Study I, a definite increase in grain biomass was found. Incubation at 22°C led to the largest increase in grain biomass (59% over the four day period) compared to the rest (16 - 31% increase over the same period) (Fig. 6). It was thus concluded that incubation at 22°C could be the optimum incubation temperature in terms of kefir grain biomass increase. A possible explanation for grain mass increase observed only during the second study, could be that the grains were not active enough during the first study, but gradually became more active with succeeding transfers to fresh milk. The decrease in milk volume from 800 ml to 200 ml could also have created a more favourable environment for the micro-organisms responsible for grain propagation. In addition, low fat milk appears to be more favourable for grain propagation than full cream milk. It was, therefore, decided to extend this study over a longer period of time in order to confirm the above assumptions.

at different temperatures in low fat milk

This study was similar to the previous two studies, but was performed over a longer period. The same pH trends were observed (Fig. 7) - a sharp pH drop from pH 6.7 to below pH 4.9 within the first 24 - 48 h, again with the exception of the grains incubated at 18°C, followed by pH stabilisation for the remainder of the study. It was found that higher incubation temperatures resulted in lower pH values.

Incubation at all the temperatures (18° - 30°C) again resulted in TA values that increased sharply and then stabilised (Fig. 8). However, values were slightly lower than those for Study II. Incubation at 22° and 25°C did not differ significantly in terms of TA, but incubation at 18°C yielded TA values significantly lower than the rest.

Incubation at 22°C again showed the highest grain biomass increase (130% over the 10-day period) (Fig. 9). This was followed by incubation at 25°C (113% over 10 days). Grains incubated at 18° and 30°C showed the lowest increases in biomass during this study. Although it was found that incubation at 22°C resulted in the highest biomass increase, it was decided to change the incubation temperature to 25°C for the Studies IV to VIII. This was based on the fact that kefir produced at 25°C had a better taste and aroma than kefir produced at 22°C (Calefato, 1998).

Experimental Study IV - Biomass increase of kefir grains grown in different milk types (non-agitation)

In this study the influence of enrichment of the growth medium (milk) on grain biomass increase, was investigated (Fig. 10). For days 0 to 28 there was a definite grain biomass increase for all four milk types. However, grains cultivated in milk types a (L) and b (F) showed lower growth increases (928% and 671%, respectively, over the 28 day period) than type c (FFP)(1056%) or type d (FY)(1099%). The addition of yeast extract and milk powder consequently resulted in a greater increase of the kefir grain biomass. The data also clearly showed that low fat milk was more advantageous to grain cultivation than full

Fig7-9(ch3)

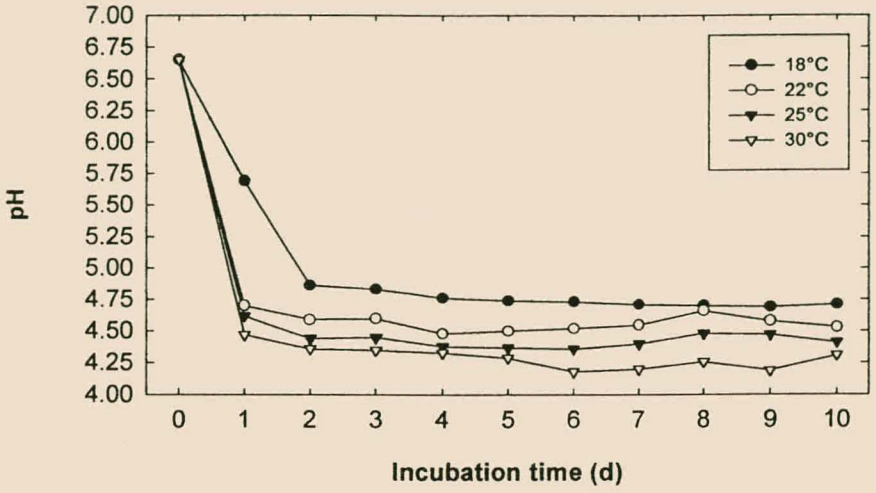


Figure 7. Influence of different incubation temperatures on pH of low fat kefir (Study III).

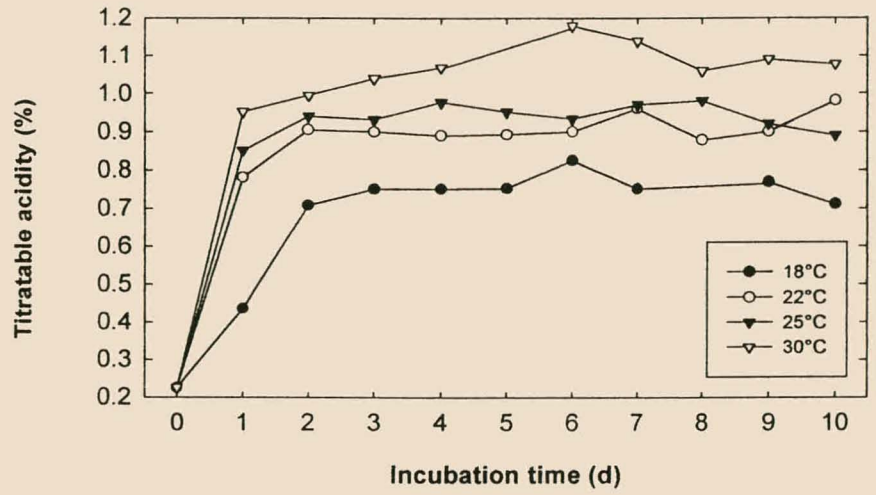


Figure 8. Influence of different incubation temperatures on the development of titratable acidity in low fat kefir (Study III).

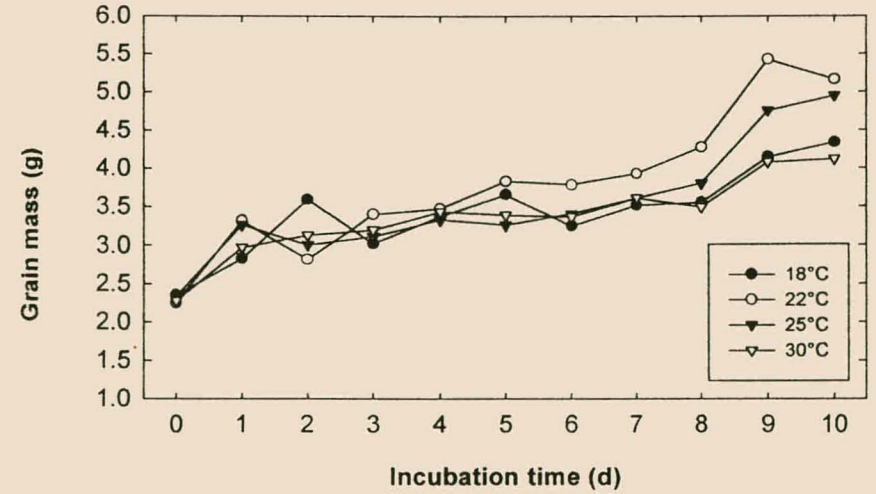


Figure 9. Influence of different incubation temperatures on biomass increase of kefir grains incubated in low fat milk (Study III).

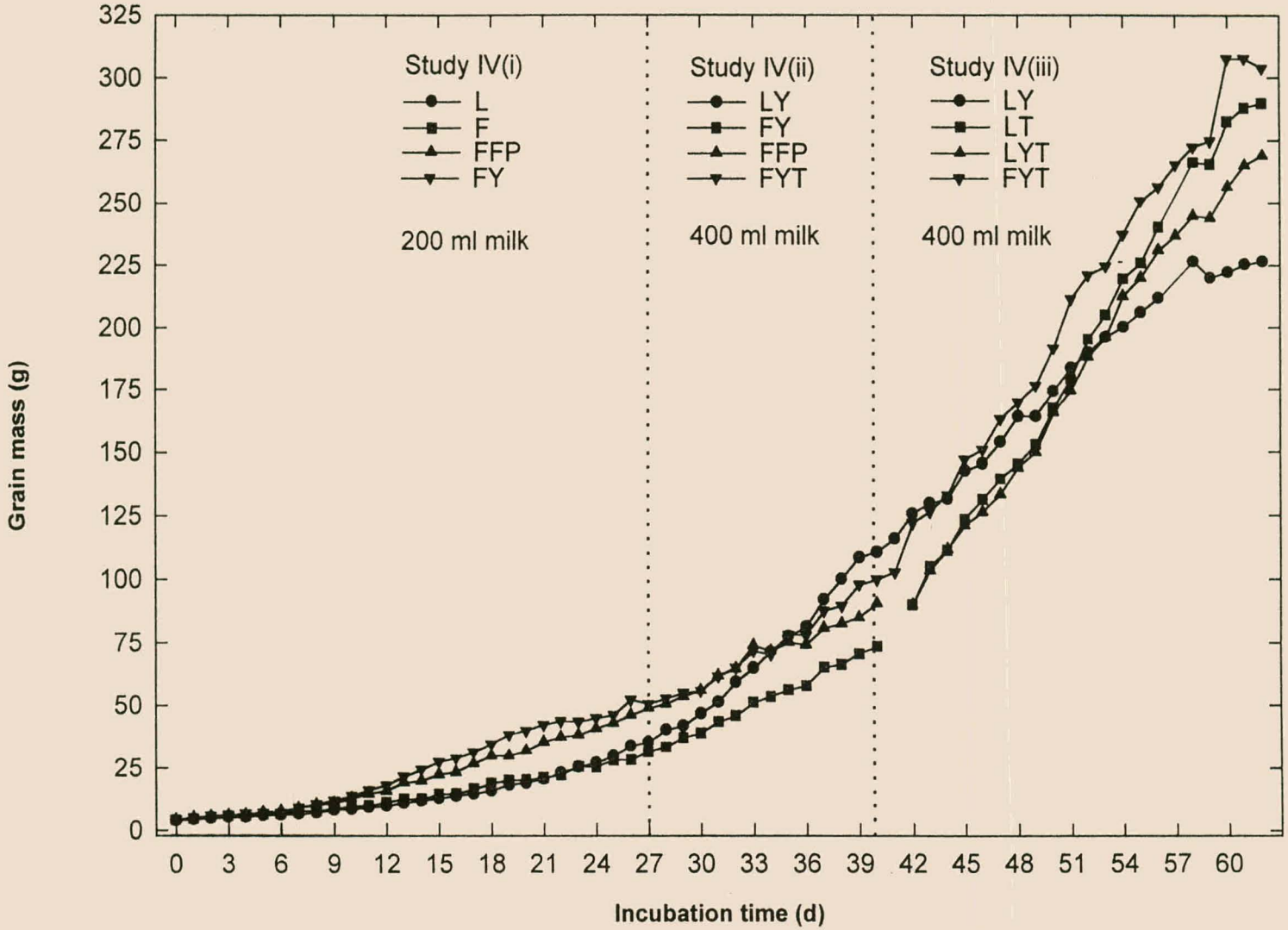


Figure 10. The influence of different milk types on kefir grain biomass increase during non-agitated grain cultivation. (Study IV). The dotted lines indicate changes in the experimental design.

L = low fat milk (2%); LY = low fat milk + 2% yeast extract; LT = low fat milk + 2% tryptose; LYT = low fat milk + 2% yeast extract + 2% tryptose; F = full cream milk (4%); FY = full cream milk + 2% yeast extract; FT = full cream milk + 2% tryptose; FYT = full cream milk + 2% yeast extract + 2% tryptose; FFP = full cream milk + 2% full cream milk powder.

cream milk, confirming the conclusion reached in Study II.

During the next period from day 29 to 40 it was decided to determine if bigger milk volumes and different milk enrichments had any influence on grain biomass increase. Thus, milk types a, b and d were further enriched. The biomass increase in milk type b (FY) showed a similar increase (98% during this second period) compared to the corresponding milk type during the preceding 11-day period in Study IV(i) (101%; day 17 to 28). The addition of yeast extract, plus an increased milk volume, did not greatly enhance the biomass increase of the grains. Milk type d (FYT) showed an increase of 82% from day 29 to 40, compared to the 69% increase during the previous 11 days. The addition of 2% tryptose to previously enriched milk did not lead to a substantial increase in grain biomass. Cultivation of grains in milk type c (FFP) resulted in an increase of 70% in biomass during this period, which was nearly the same (69%) as during the previous 11-day period, suggesting that the addition of full cream milk powder did not really enhance grain increase. Milk type a (LY) showed the highest increase in grain biomass for this period (165%), but the increase was found to be lower than for the period from day 17 to 28 (173%). It was, thus, found that the addition of yeast extract did not lead to a substantial increase in grain biomass. Milk types b (FY), c (FFP) and d (FYT) were composed of full cream milk and showed lower biomass increases than that found for milk type a (LY). It could thus be possible that the higher fat content of full cream milk might have had an inhibitory effect on grain propagation.

From days 41 to 62, milk types b and c were changed to low fat milk with altered nutrient additions. Subsequently, for this period the highest increase in biomass was found for grains cultivated in milk type b (LT) (222% over the 20-day period). Grain increase calculations were based on the difference in mass between the start and end masses of each period. Cultivation of grains in milk type c (LYT) showed an increase of 199% for this period, and therefore, it was concluded that the addition of yeast extract to milk already containing tryptose, did not substantially increase the grain biomass. Further cultivation of grains in milk type a (LY) resulted in a mass increase of only 81% over the 20-day period (compared to the 165% increase for the previous 11 day period). Continued cultivation of grains in milk type d (FYT) resulted in a further increase of 149% for this period, compared to the 82% increase for the previous 11-day period.

Biomass comparison of milk types a (LY) and d (FYT) showed that grains in type d (FYT) had a higher mass increase compared to type a (LY), although both types had almost the same grain mass on day 42. This could probably be due to the addition of tryptose to milk already containing yeast extract. Another possible explanation could be that the combination of both nutrients (tryptose and yeast extract) caused a higher formation of extracellular polymers (ECP) and subsequent weight increase, than milk with only yeast extract. At the end of the 62-day period it was difficult to conclude exactly which milk type resulted in the highest grain biomass increase. This was due to the influence of other parameters, such as different grain masses at the start of each period, as well as different milk volumes used in these periods. However, at the conclusion of this study (Fig. 10), the milk types containing tryptose (LT, LYT and FYT) had the highest grain biomass

It is of interest to note that the formation of ECP was found in milk containing yeast extract after about 30 days of cultivation. These grains were slimy and formed a solid mass. This was also found for grains cultivated in milk containing tryptose, but to a lesser degree. This phenomenon was the most pronounced with milk containing both nutrients. Grains cultivated in milk without nutrients did not produce ECP that was visibly detectable.

Experimental Study V - Cultivation of kefir grains with agitation and different milk types.

In this study the influence of growth medium enrichment, a larger inoculum size and agitation on kefir grain biomass increase was investigated (Fig. 11 - 13 and Table 2). Tryptose and/or yeast extract was used for the enrichment and agitation was carried out in a shaking water bath (rpm = 130). The pH for all eight milk types dropped considerably during the first 24-h period and stabilised in the 3.3 - 3.6 range after the fourth day of incubation at 25°C (Fig. 11). The stabilised pH for milk type a (L) (pH 3.3 - 3.4) was significantly lower than the stabilised pH (pH 4.4 – 4.6) obtained during the fermentation of the same milk type without agitation at the same temperature (Studies II and III; Fig. 4 and 7). This was probably as a result of the larger inoculum concentration (10% = 40 g

Fig 11-12(ch3)

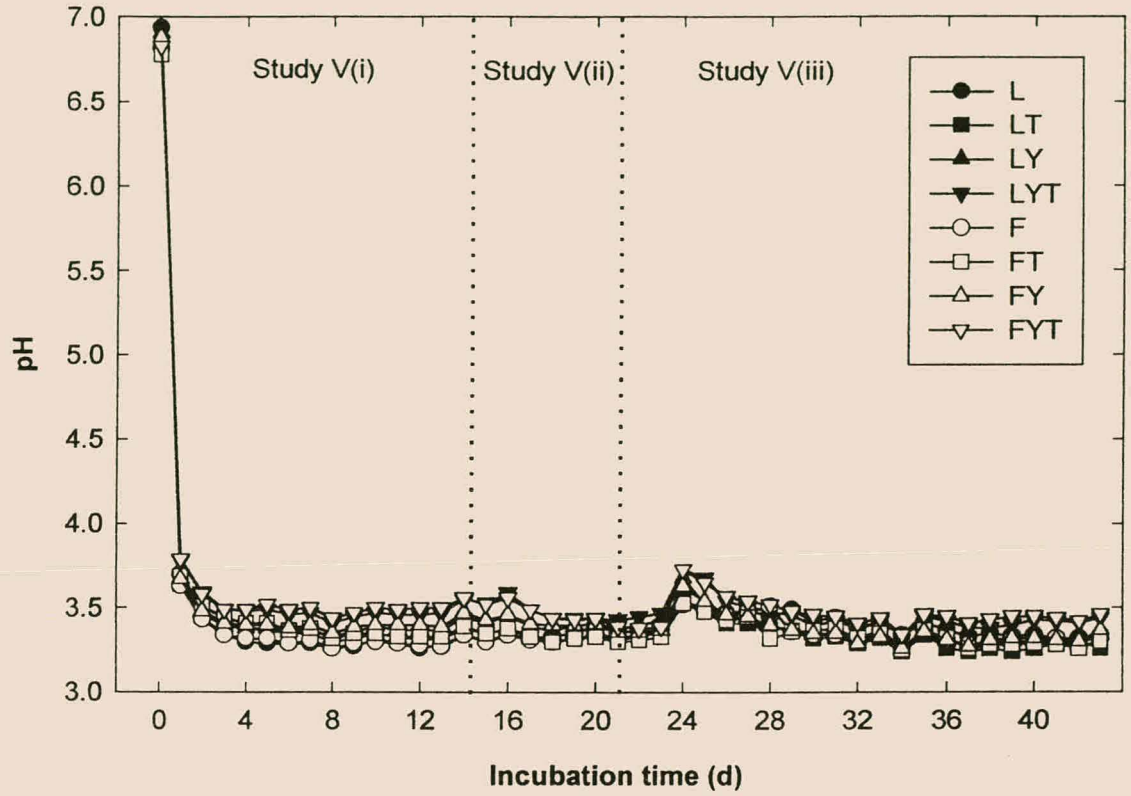


Figure 11. Influence of different milk types on pH during agitated kefir grain cultivation (Study V). The dotted lines indicate changes in the experimental design.

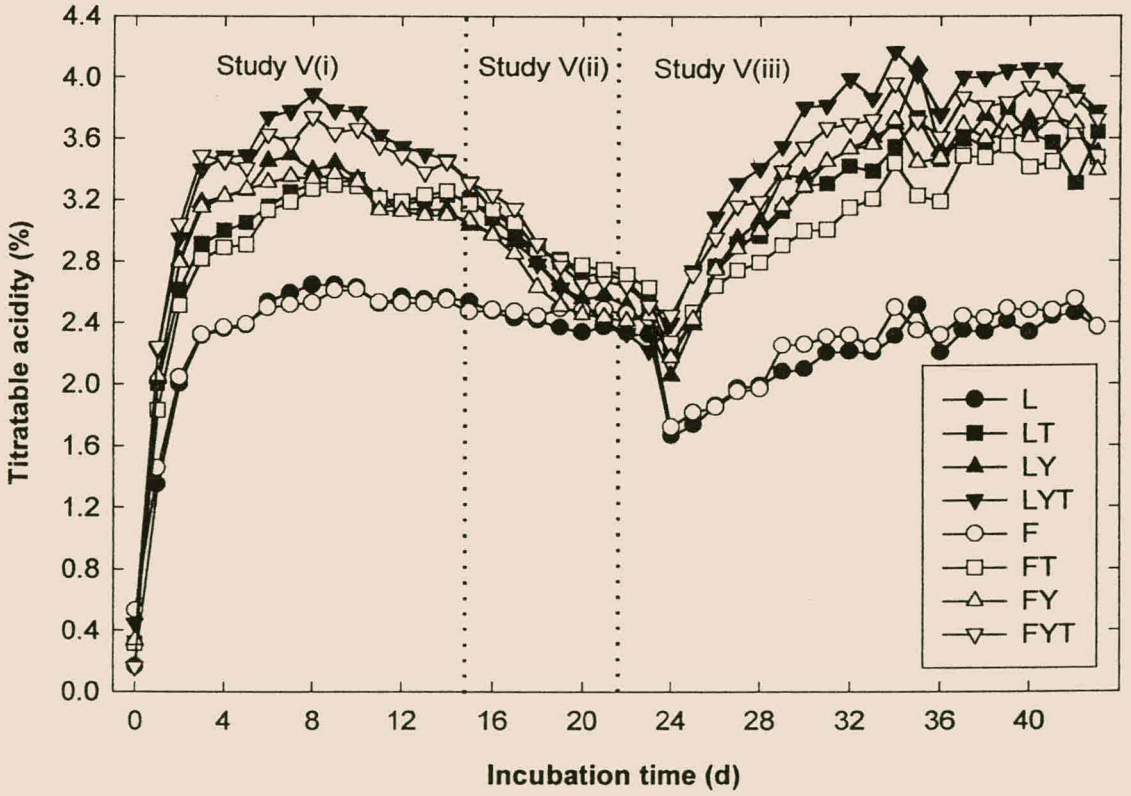


Figure 12. Influence of different milk types on titratable acidity during agitated kefir grain cultivation (Study V). The dotted lines indicate changes in the experimental design.

Fig13(ch3)

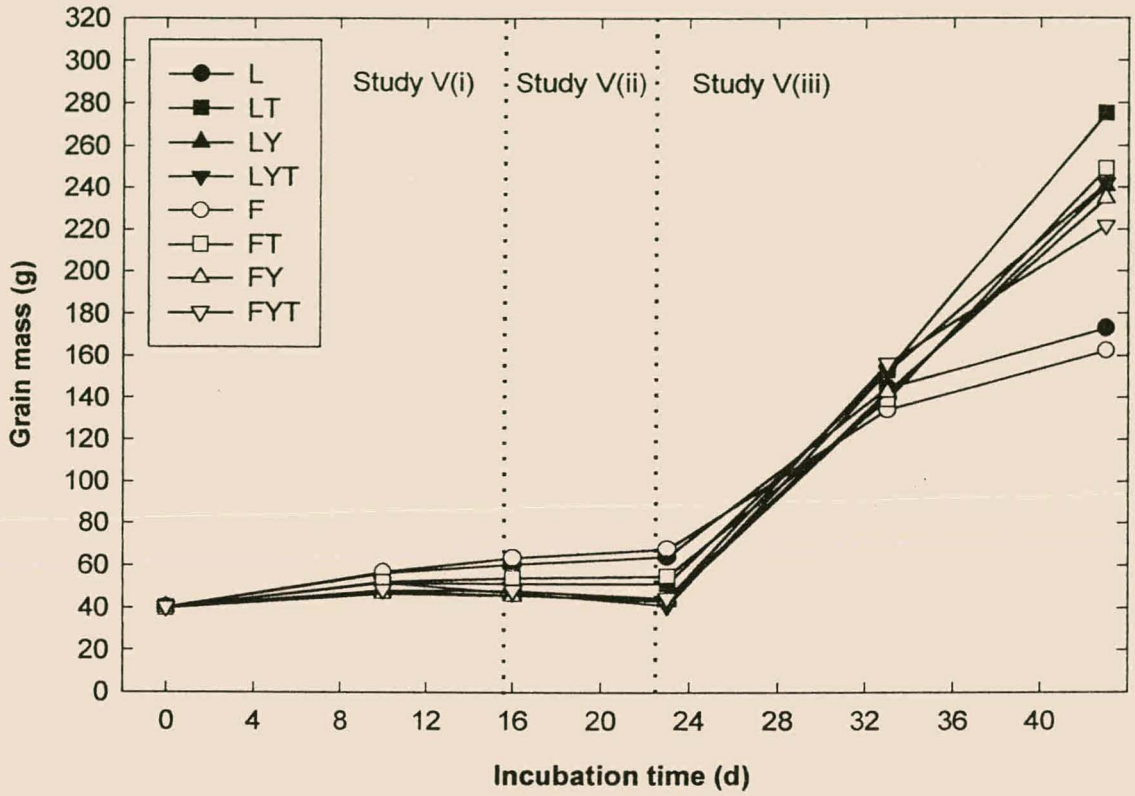


Figure 13. Influence of different milk types on grain mass increase during agitated kefir grain cultivation on grain biomass increase (Study V). The dotted lines indicate changes in the experimental design.

Table 2. Change in kefir grain biomass during agitated cultivation in different milk types as found in Study V (- = decrease).

Milk type*	Mass (g) (day 0)	Mass (g) (day 10)	% Change (0-10)	Mass (g) (day 16)	% Change (10-16)	Mass (g) (day 23)	% Change (16-23)	Mass (g) (day 33)	% Change (23-33)	Mass (g) (day 43)	% Change (33-43)	% Change (23–43)	% Change (0-43)
L	40.1	56.4	40.6	60.4	7.2	64.3	6.3	144.7	125.1	173.0	19.6	169.2	331.4
LT	40.3	51.7	28.5	51.3	-0.7	51.5	0.4	153.1	197.1	275.2	79.8	434.1	582.9
LY	40.0	47.6	19.0	46.0	-3.4	43.1	-6.3	141.4	227.9	240.7	70.2	458.0	501.8
LYT	40.1	51.9	29.4	47.1	-9.4	41.0	-12.8	152.7	272.1	242.0	58.5	489.9	503.5
F	40.2	56.9	41.4	63.6	11.7	68.0	6.9	134.4	97.6	162.4	20.8	138.9	304.0
FT	40.0	52.4	31.0	54.3	3.5	55.0	1.3	139.3	153.4	249.1	78.8	353.2	522.8
FY	40.0	46.6	16.3	45.6	-2.1	44.3	-2.9	142.8	222.6	234.8	64.4	430.4	487.0
FYT	40.2	48.5	20.7	48.2	-0.5	44.6	-7.6	156.4	251.1	221.7	41.7	397.6	451.5

* L = Low fat milk (2% fat)
LY = Low fat milk + 2% yeast extract
LT = Low fat milk + 2% tryptose
LYT = Low fat milk + 2% yeast extract + 2% tryptose
F = Full cream milk (4% fat)
FY = Full cream milk + 2% yeast extract
FT = Full cream milk + 2% tryptose
FYT = Full cream milk + 2% yeast extract + 2% tryptose

instead of 1% = 2 g as used in Studies II and III). Agitation of the cultivation vessels also probably improved nutrient exchange between the kefir grains and the growth medium, leading to more active grains and a lower pH. During the period of day 16 to 23, only 100 ml low fat milk was added each day and the pH remained stable at 3.3 - 3.4 for all eight milk types.

To determine the influence of different milk replacement volumes on grain biomass increase, all the fermented milk was removed daily and replaced with 400 ml fresh milk of each type from day 23 onwards. A slight increase in pH was observed for the first two days due to the smaller acid to milk volume ratio. A period of about six days was necessary before the pH again stabilised at 3.3 - 3.4.

The TA again showed a fast increase initially and then a slower increase from day 4 to 8, for all eight milk types (Fig. 12). The TA decreased slowly thereafter for milk with both nutrients [types d (LYT) and h (FYT)] and milk with yeast extract [types c (LY) and g (FY)] (Fig. 12). The TA of the milk without any nutrients [types a (L) and e (F)] as well as those with tryptose [types b (LT) and f (FT)] decreased gradually from day 8 to day 16. It is interesting to note that the corresponding milk types [a (L) and e (F), b (LT) and f (FT), c (LY) and g (FY), d (LYT) and h (FYT)] showed essentially similar TA values. It was thus found in this study that there was no clear difference between low fat milk and full cream milk in terms of the TA production. The highest TA value (3.7%) was found when grains were cultivated in milk containing both nutrients (LYT and FYT). Grain cultivation in milk without any nutrients (L and F) showed the lowest TA value (2.6%). It was thus concluded that acid production was stimulated by the addition of nutrients like tryptose and yeast extract. Agitation of low fat milk without added nutrients also resulted in higher TA values than those found for low fat milk without agitation (maximum TA = 2.6% in this study compared to approximately 1% in Studies II and III, Fig. 5 and 8).

During the period from day 16 to 23 (100 ml low fat milk added each day instead of 100 ml of each milk type) the TA decreased further for all eight milk types to values ranging from 2.2 - 2.6%. This subsequent decline in the amount of acid produced can probably be attributed to the reduced availability of fresh milk combined with the increase in grain biomass. Daily removal of all the fermented milk and replacement with 400 ml fresh milk every day (day 23 to 43) led to an increase in TA that reached maximum values around day 34. It was

again found that the highest amount of acid was produced when grains were cultivated in milk containing both nutrients (4.0 - 4.2%). More variation in TA was observed during this period (day 23 to 43) than during the first period (day 0 to 16). The same trends in terms of TA were again found for comparable milk types a (L) and e (F), as well as c (LY) and g (FY), but this was less prominent with the remaining milk types b (LT), d (LYT), f (FT) and h (FYT). The TA appears to have stabilised at a value slightly less than the maximum value and for the duration of the study no significant decreases were found for the majority of the milk types. The data again showed that the organisms in milks, without nutrient addition (L and F), produced the lowest TA.

During the first 10 days of this study (Fig. 13), the highest biomass increase was observed for grains cultivated in milk without any nutrients (L and F), and the lowest increase for milk with yeast extract (LY and FY) (Fig. 13 and Table 2). During the next period (day 10 to 23, replacing with 100 ml fresh low fat milk daily), increases in biomass were observed only for milk types a (L), e (F) and f (FT), the rest all showed a decrease in biomass. Daily replacement of all the fermented milk with 400 ml fresh milk resulted in a dramatic increase in mass for all the milk types (day 23 to 43). During the period from day 23 to 33, the highest mass increases were observed when grains were cultivated in milk containing tryptose and yeast extract (LYT and FYT). This was followed by milk containing yeast extract (LY and FY), milk with tryptose (LT and FT) and milk without nutrients (L and F). The same patterns in biomass increase could be seen for the low fat and full cream milk types containing similar nutrient combinations (e.g. LYT and FYT), with the grains in the low fat milk types having slightly higher biomass increases than grains in the full cream milk types.

During the last 10 days of this study (day 33 to 43), further increases in biomass were observed, the lowest being for milk without any nutrients (Table 2). These increases were, however, less than for the previous 10-day period (day 23 to 33). On day 33, the mass of grains in the different milk types were in the same range (140 - 156 g), but these showed dramatic changes for the remainder of the study. At the end of the study, grains cultivated in milk without any nutrients were significantly less than the rest. At the end of the 43-day period, the highest increases in grain biomass were found when grains were cultivated in milk containing tryptose (Table 2). Cultivation in milk without any nutrients resulted in

the lowest increase. In this study it was thus found that cultivation in low fat milk gave higher yields than cultivation in full cream milk.

There appears to be a limiting factor that determines the amount of grains formed per volume of milk replaced - during the period of day 0 to 23, only 100 ml replacement milk was added each day and as a result the grain increase ceased or was very low. From day 23 to 43, all the milk was replaced daily and the data showed that the grains increased during the whole period, although more slowly during the later stages (probably reaching a maximum limit). This can be seen as a classical example of "Liebig's law of the minimum" which states that "the total yield or biomass increase of any organism will be determined by the nutrient present in the lowest (minimum) concentration in relation to the requirements of that organism" (Atlas & Bartha, 1993).

It was noticed that, although the grain mass increased, the pH stayed constant at approximately 3.3 - 3.4 (Fig. 11 and 13). It was also clear that there was no direct relationship between pH and TA (Fig. 11 and 12). No apparent conclusion could thus be drawn in regard to the relationship between TA and biomass increase of the kefir grains (Fig. 12 and 13).

Grains cultivated in milk containing yeast extract and/or tryptose again formed ECP during the experimental period. This occurrence was the same as that found in Study IV.

It was concluded from data obtained in this study that the volume of the replacement milk played an important role in grain biomass increase, and that the volume should be increased as the grain biomass increases. The data also clearly showed that agitation of the growth medium enhanced grain mass increase considerably in comparison to non-agitation, and that the addition of tryptose to the growth medium led to the highest increases in grain biomass.

Experimental Study VI - Cultivation of kefir grains with agitation and different milk types

This study was a repetition of the previous study in order to verify the results of Study V. The influence of enrichment of the growth medium, replacement of all the milk and agitation on the rate of kefir grain biomass changes was once more investigated (Fig. 14 – 16, Table 3). A significant pH drop was again observed during the first day with stabilisation at 3.4 - 3.5 after the

fourth day (Fig. 14) and then the pH remained virtually unchanged for the duration of the study.

In the case of the TA (Fig. 15) it was found, as for Study V, that the corresponding milk types followed the same patterns, confirming that there is no difference between full cream and low fat milk in terms of amount of TA produced (Fig. 15). Once again, the milk types without nutrition addition resulted in the lowest TA values.

When the biomass increases, during the last 20 days of Study V, were compared to the increases measured during the first 20 days of this study (Fig. 16), it was found that these biomass increases were lower than those found during Study V (Tables 2 and 3). It was also found in this study that grain cultivation in milk containing yeast extract resulted in the highest biomass increases (Table 3). Cultivation of grains in milk without added nutrients again resulted in the lowest increase in biomass, confirming the results found in Study V. In this study, however, cultivation in full cream milk gave moderately higher biomass yields than cultivation in low fat milk. This is the opposite to that found in Study V and no explanation for the differences could be given. Grains cultivated in milk containing yeast extract and/or tryptose again formed ECP. This was similar to Studies IV and V.

Experimental Study VII - *Cultivation of frozen kefir grains with agitation and different milk types.*

This study was performed to determine the influence of using frozen kefir grains instead of activated grains during grain cultivation. Cultivation was carried out with agitation, full volume replacement and enrichment of the growth medium. Urea was used as nutrient source instead of tryptose. The data from this study showed, probably due to the fact that frozen kefir grains were used, that a prolonged lag period (20 days) was present before the grain biomass started to increase. It is, therefore, important that the grains must be as active as possible

Fig14-15(ch3)

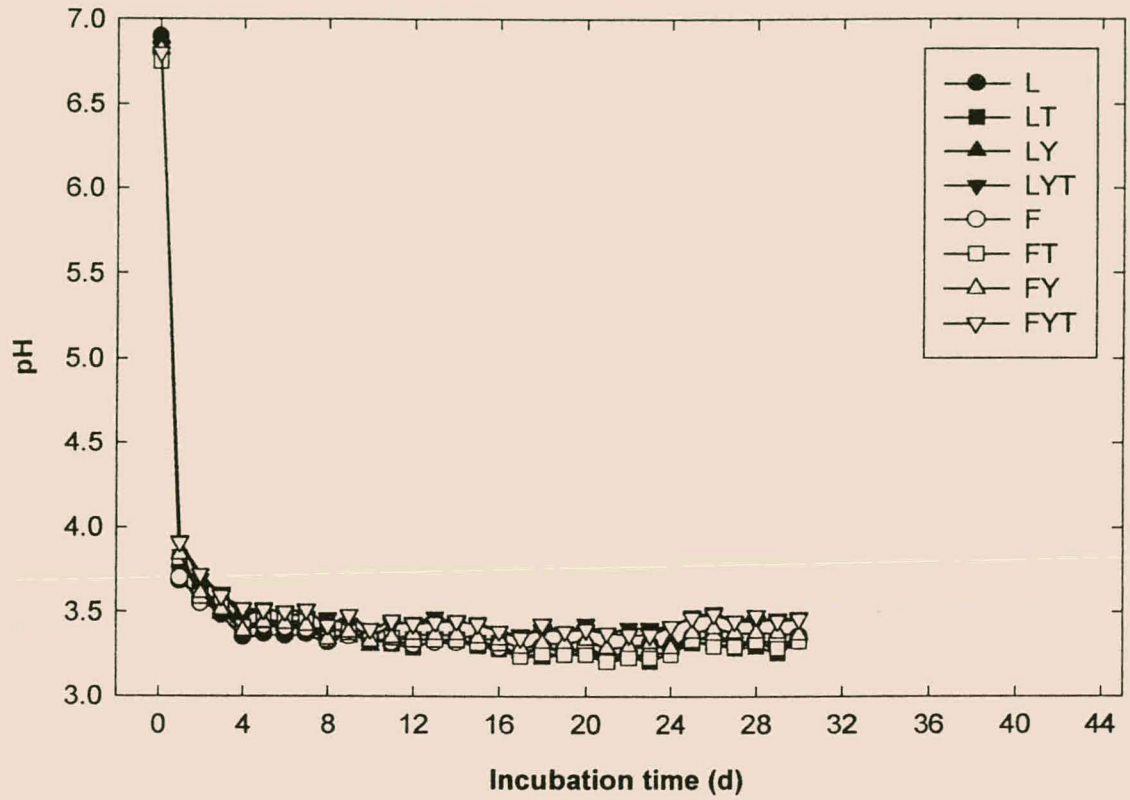


Figure 14. Influence of different milk types on pH during agitated kefir grain cultivation (Study VI).

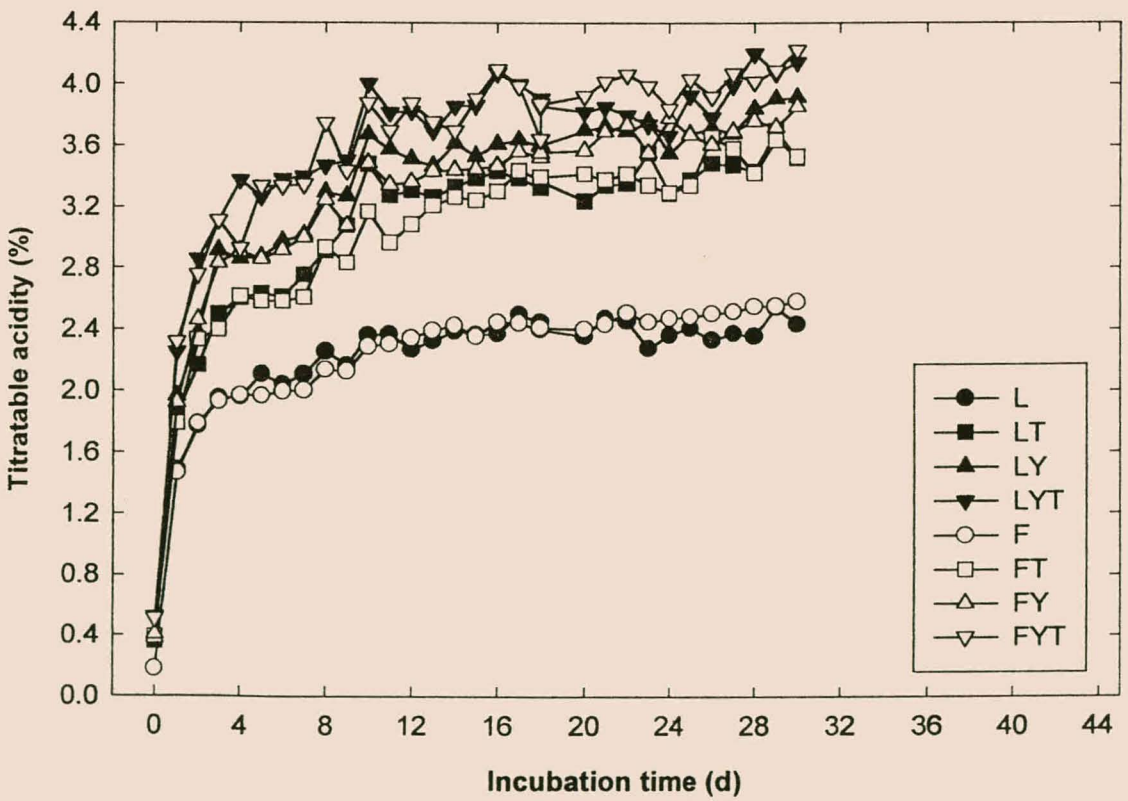


Figure 15. Influence of different milk types on titratable acidity during kefir grain cultivation (Study VI).

Fig16(ch3)

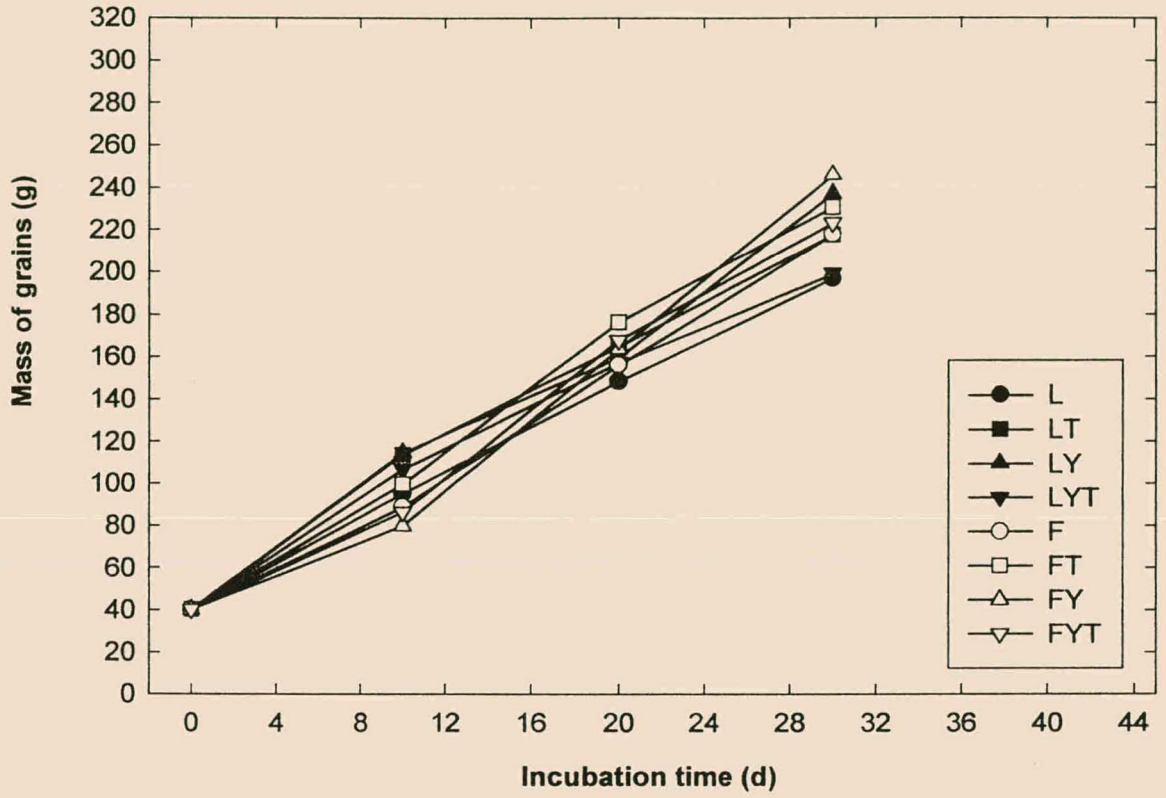


Figure 16. Influence of different milk types on grain mass increase during agitated kefir grain cultivation (Study VI).

Table 3. Kefir grain biomass increase during agitated cultivation in different milk types as found in Study VI.

Milk type*	Mass (g) (day 0)	Mass (g) (day 10)	% Change (day 0-10)	Mass (g) (day 20)	% Change (day 10-20)	% Change (day 0 - 20)	Mass (g) (day 30)	% Change (day 20-30)	% Change (day 0–30)
L	40.3	95.1	135.8	148.4	56.1	267.9	196.8	32.6	388.3
LT	40.2	113.7	182.8	164.8	44.9	309.8	217.6	32.0	441.3
LY	40.3	114.7	184.7	159.8	39.3	296.6	237.0	48.3	488.1
LYT	40.2	106.8	165.7	157.4	47.4	291.5	199.2	26.6	395.5
F	40.3	88.8	120.3	156.2	75.9	287.5	217.7	39.4	440.2
FT	40.2	99.6	147.5	176.6	77.3	338.9	230.8	30.7	474.1
FY	40.3	79.8	98.2	163.8	105.3	306.9	246.1	50.2	510.7
FYT	40.3	86.2	113.8	167.9	94.8	316.5	223.8	32.9	455.3

* L = Low fat milk (2% fat)
LY = Low fat milk + 2% yeast extract
LT = Low fat milk + 2% tryptose
LYT = Low fat milk + 2% yeast extract + 2% tryptose
F = Full cream milk (4% fat)
FY = Full cream milk + 2% yeast extract
FT = Full cream milk + 2% tryptose
FYT = Full cream milk + 2% yeast extract + 2% tryptose

before mass cultivation is started so as to optimise biomass increase.

During this study it was observed that the containers which were not agitated (L(wa) and F(wa)) showed lower increases in grain mass than any of the other milk types (Fig. 17 and Table 4). It was thus obvious that agitation of the cultivation vessel led to increased grain growth.

The data also showed that the fat content of the milk did not influence the grain mass increase, for corresponding milk types showed almost the same biomass values. During the study, it was found that milk containing both nutrients (LUY and FUY) showed the highest increase in grain biomass, while milk containing urea (LU and FU) showed less increases than milk with yeast extract (LY and FY) (Fig. 17 and Table 4). It was, therefore, concluded that when added separately, yeast extract was more beneficial for grain growth than urea.

Experimental Study VIII - Cultivation of active kefir grains with agitation and different milk types.

This study was essentially a repetition of Study VII, but was performed with active kefir grains instead of frozen grains. Based on the results of Study VII, it was decided to exclude the milk types with 0.5% added urea (LU and FU) as these milk types had led to lower increases in grain biomass.

During the whole 20-day cultivation period of this study, the containers which were not agitated (L(wa) and F(wa)) again showed the lowest biomass increases, even lower than for the milks without any added nutrients (L and F). These results confirm that agitation of the cultivation vessel definitely enhances grain biomass increase (Fig. 18 and Table 5). However, in this study a definite pattern could not be distinguished in regard to the influence of the fat content of the milk on biomass increase. At the end of the cultivation period it was found that the low fat milk, in most cases, enhanced grain biomass increase only slightly more than that found for the full cream milk (Table 5). In comparison, data from Study IV, where no agitation was used, showed that low fat milk enhanced grain mass increase much more than full cream milk. It was concluded that in the last three studies (V - VIII), where agitation was used, the influence of the milk fat content on grain biomass increase became less prominent. Agitation could

Fig17-18(ch3)

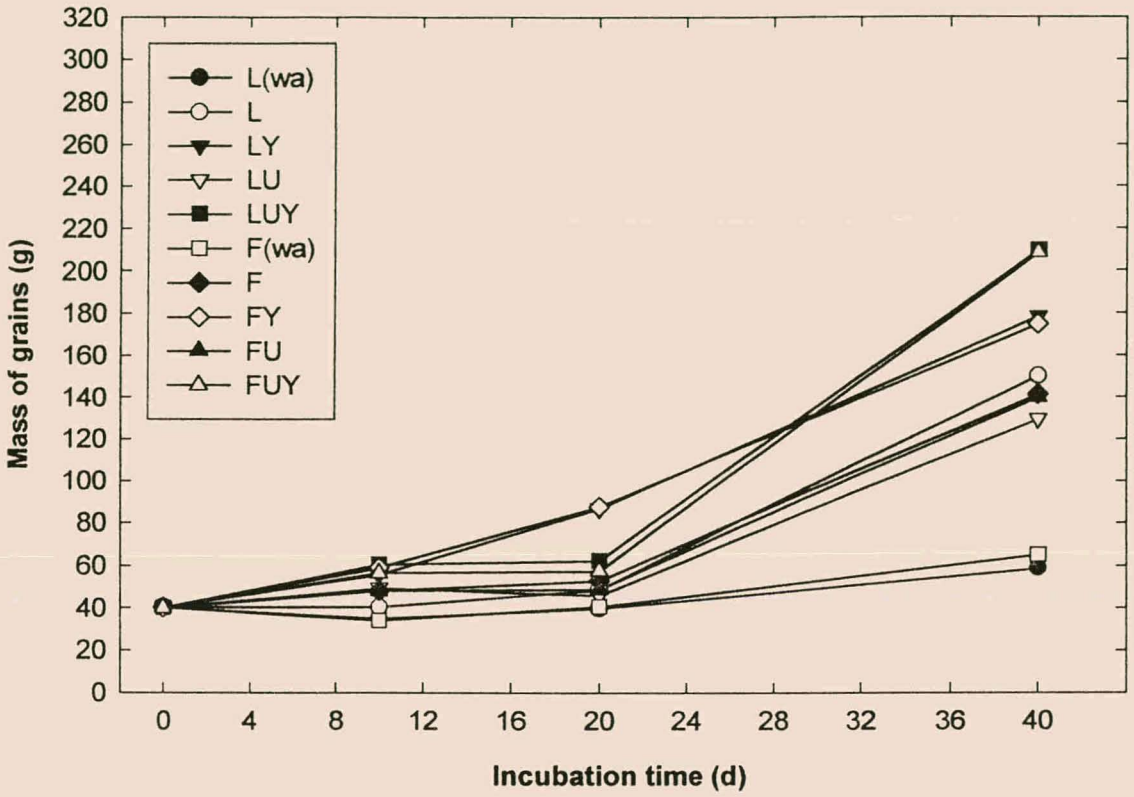


Figure 17. Influence of different milk types on biomass increase of frozen kefir grains during agitated cultivation (Study VII).

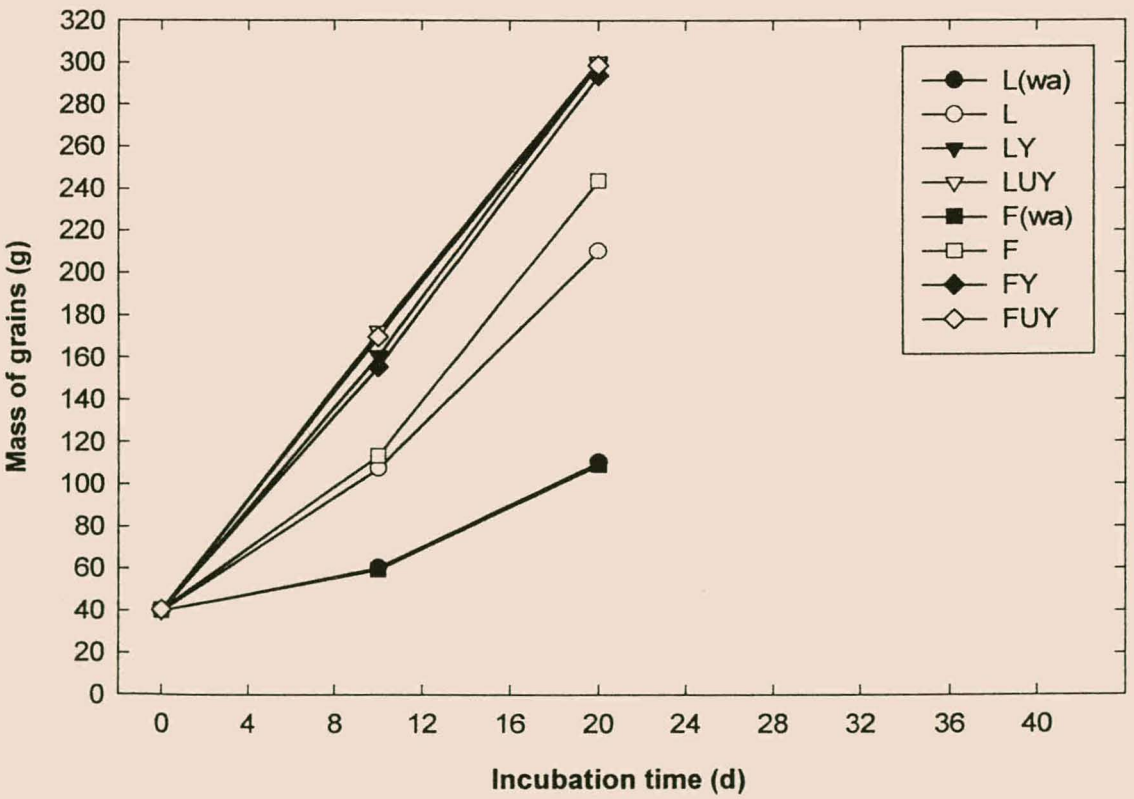


Figure 18. Influence of different milk types on biomass increase of active kefir grains during agitated cultivation (Study VIII).

Table 4. Change in kefir grain biomass during agitated cultivation in different milk types as found in Study VII (- = decrease).

Milk type*	Mass (g) (day 0)	Mass (g) (day 10)	% Change (day 0 – 10)	Mass (g) (day 20)	% Change (day 10 – 20)	Mass (g) (day 40)	% Change (day 20 – 40)	% Change (day 0 – 40)
L(wa)	40.1	34.9	-13.0	39.8	14.0	58.8	47.7	46.6
L	40.5	40.4	-0.0	48.6	20.3	150.1	208.9	270.6
LY	40.4	55.7	37.8	86.8	55.9	178.4	105.5	341.6
LU	40.1	49.3	23.1	45.9	-6.9	129.5	182.1	222.9
LUY	40.1	60.5	50.9	62.5	3.3	209.6	235.4	422.7
F(wa)	40.2	34.1	-15.2	40.6	19.2	65.2	60.6	62.2
F	40.4	48.0	18.8	53.0	10.4	141.3	166.6	249.8
FY	40.2	59.1	46.8	87.7	48.5	174.9	99.4	335.1
FU	40.1	48.0	19.6	49.0	2.1	139.9	185.5	248.9
FUY	40.1	56.5	41.0	57.6	1.9	208.4	261.8	419.7

* L = Low fat milk (2% fat)
L(wa) = Low fat milk without agitation
LY = Low fat milk + 2% yeast extract
LU = Low fat milk + 0.5% urea
LUY = Low fat milk + 0.5% urea + 2% yeast extract
F = Full cream milk (4% fat)
F(wa) = Full cream milk without agitation
FY = Full cream milk + 2% yeast extract
FU = Full cream milk + 0.5% urea
FUY = Full cream milk + 0.5% urea + 2% yeast extract

Table 5. Change in kefir grain biomass during agitated cultivation in different milk types as found in Study VIII.

Milk type*	Mass (g) (day 0)	Mass (g) (day 10)	% Change (day 0 – 10)	Mass (g) (day 20)	% Change (day 10 – 20)	% Change (day 0 – 20)
L(wa)	40.1	60.4	50.6	110.5	83.0	175.6
L	40.0	107.4	168.5	210.4	95.9	426.0
LY	40.1	160.7	300.8	299.0	86.1	645.6
LUY	40.4	172.3	326.5	300.4	74.4	643.6
F(wa)	40.0	59.6	49.0	109.1	83.1	172.8
F	40.6	113.4	179.3	243.8	115.0	500.5
FY	40.2	155.6	287.1	293.8	88.8	630.8
FUY	40.5	169.7	319.0	298.8	76.1	637.8

* L = Low fat milk (2% fat)
L(wa) = Low fat milk without agitation
LY = Low fat milk + 2% yeast extract
LUY = Low fat milk + 0.5% urea + 2% yeast extract
F = Full cream milk (4% fat)
F(wa) = Full cream milk without agitation
FY = Full cream milk + 2% yeast extract
FUY = Full cream milk + 0.5% urea + 2% yeast extract

probably have led to increased availability of nutrients to the grain micro-organisms.

At the end of the study (day 20), the highest biomass increases were found for agitated milk containing both nutrients (LUY and FUY) and milk with yeast extract (LY and FY) (Table 5). The biomass increases for these milk types were very similar, confirming the conclusion reached in Study VII that the addition of urea does not strongly enhance grain biomass increase. In this study, no clear lag phase in biomass increase was observed probably due to the fact that active kefir grains were used during cultivation instead of frozen grains. In order to achieve maximum kefir grain biomass increase in the shortest time, it is thus important to use active grains, together with growth medium enrichment and agitation.

Kefir grain morphology

After completion of all the research, photographs and scanning electron micrographs (SEM) were taken of the surface and interior of typical mass cultivated kefir grains to illustrate morphological variations of the grains (Fig. 19 to 24). The characteristic cauliflower-like shape of kefir grains can clearly be seen in Fig. 19 to 21. The flat structure depicted in Fig. 19 visually shows the difference between the smooth and convoluted sides of a grain, while the grain pictured in Fig. 21 could be a rolled-up and condensed version of the structure depicted in Fig. 19. The surface of the grain in Fig. 22 also shows a cauliflower-like appearance or what could be small bubbles of matter clumped together. Predominantly rod-shaped lactobacilli were found in the interior of the mass cultivated grains (Fig. 23 and 24). In Fig. 24, strings of ECP can clearly be seen between the kefir micro-organisms. The formation of ECP was observed in Studies IV to VIII where yeast extract and/or tryptose was used as additional nutrient sources.

Conclusions and recommendations

One of the pre-requisites for the successful implementation of kefir production on industrial scale, is the regular supply of active kefir grains *en masse*. The aim of

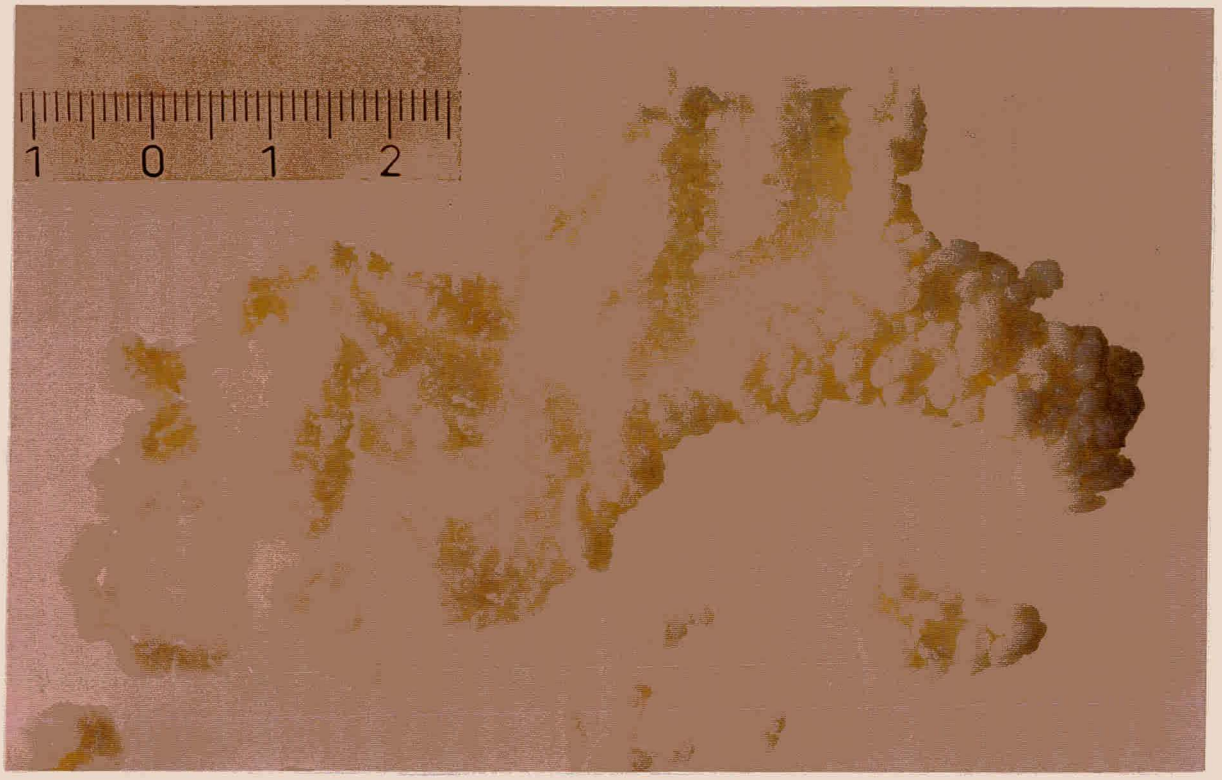


Figure 19. A typical mass cultivated kefir grain.

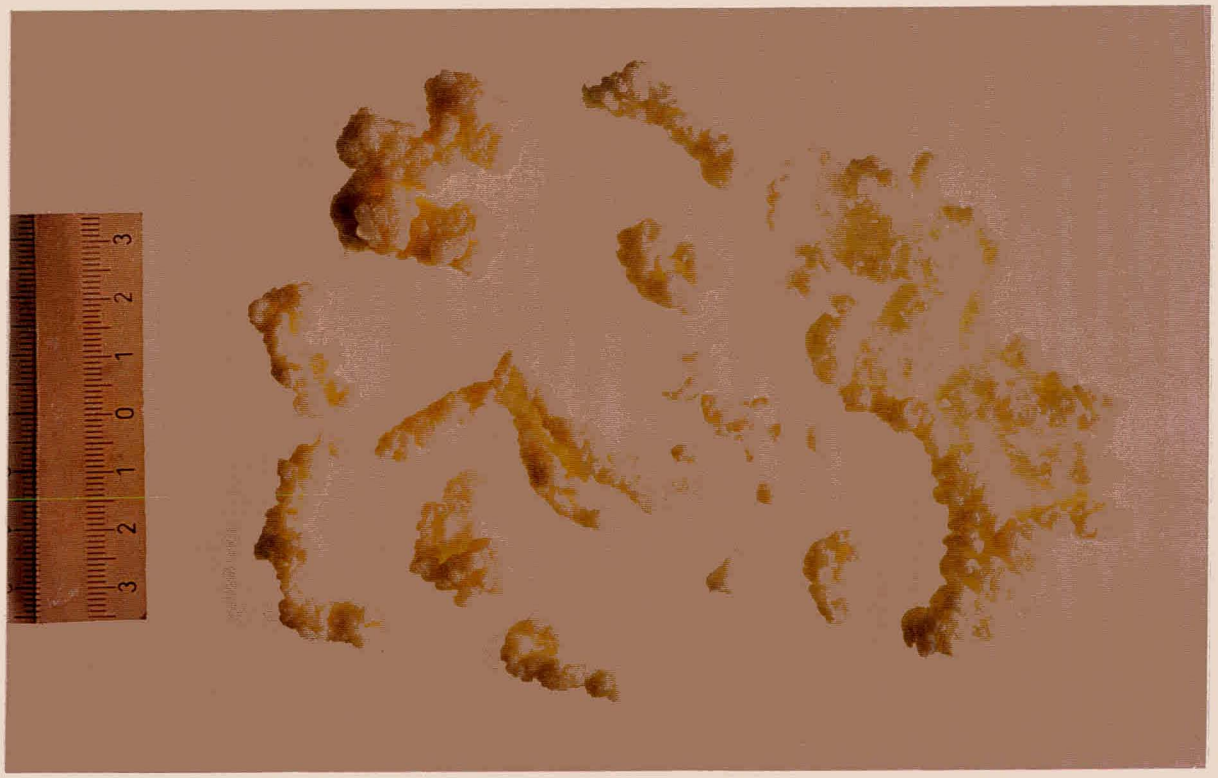


Figure 20. A variety of kefir grains formed during mass cultivation.

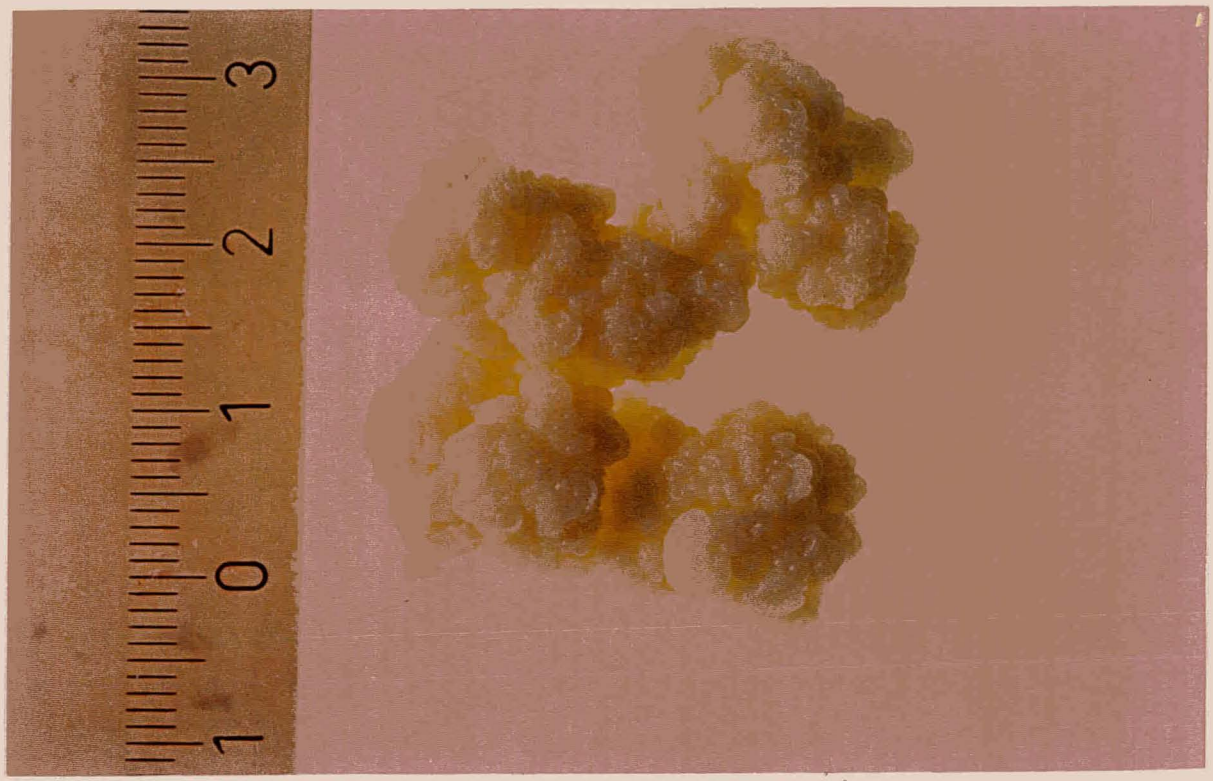


Figure 21. A mass cultivated kefir grain.

Figure 22. A scanning electron micrograph (40 X) of the surface of a mass cultivated kefir grain.

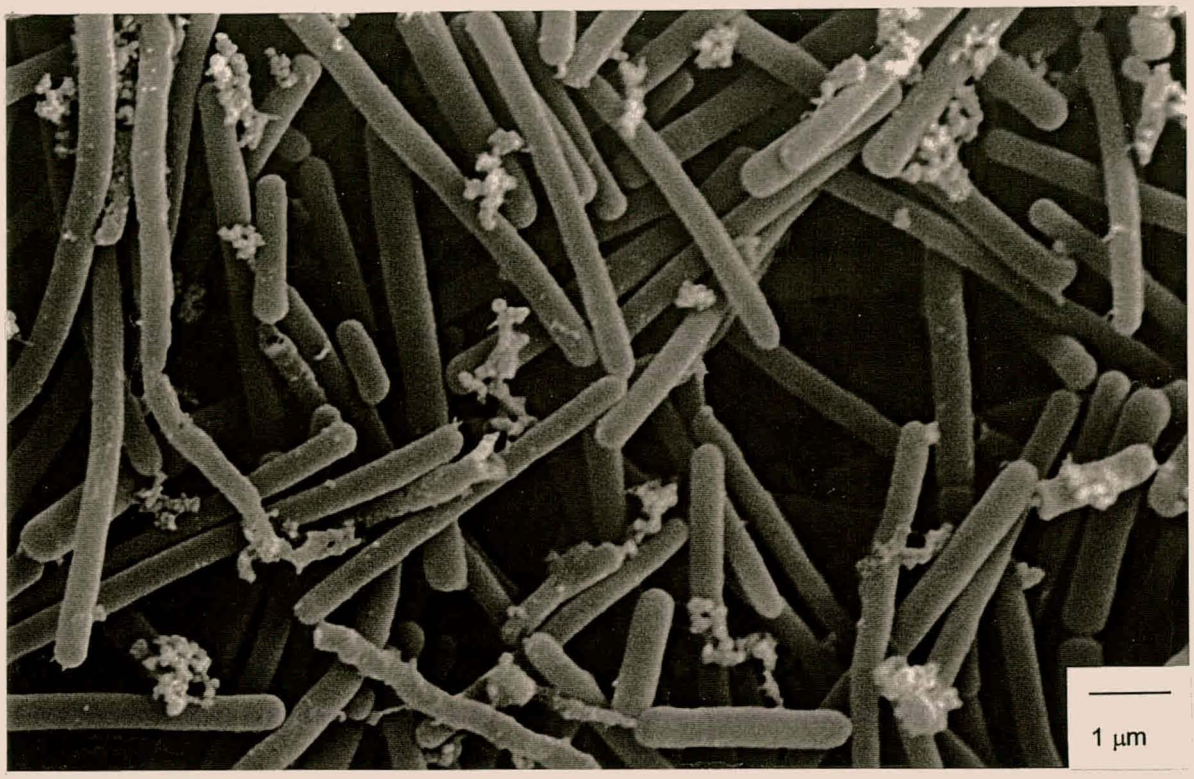


Figure 23. A scanning electron micrograph (6 500 X) of the interior of a mass cultivated kefir grain.

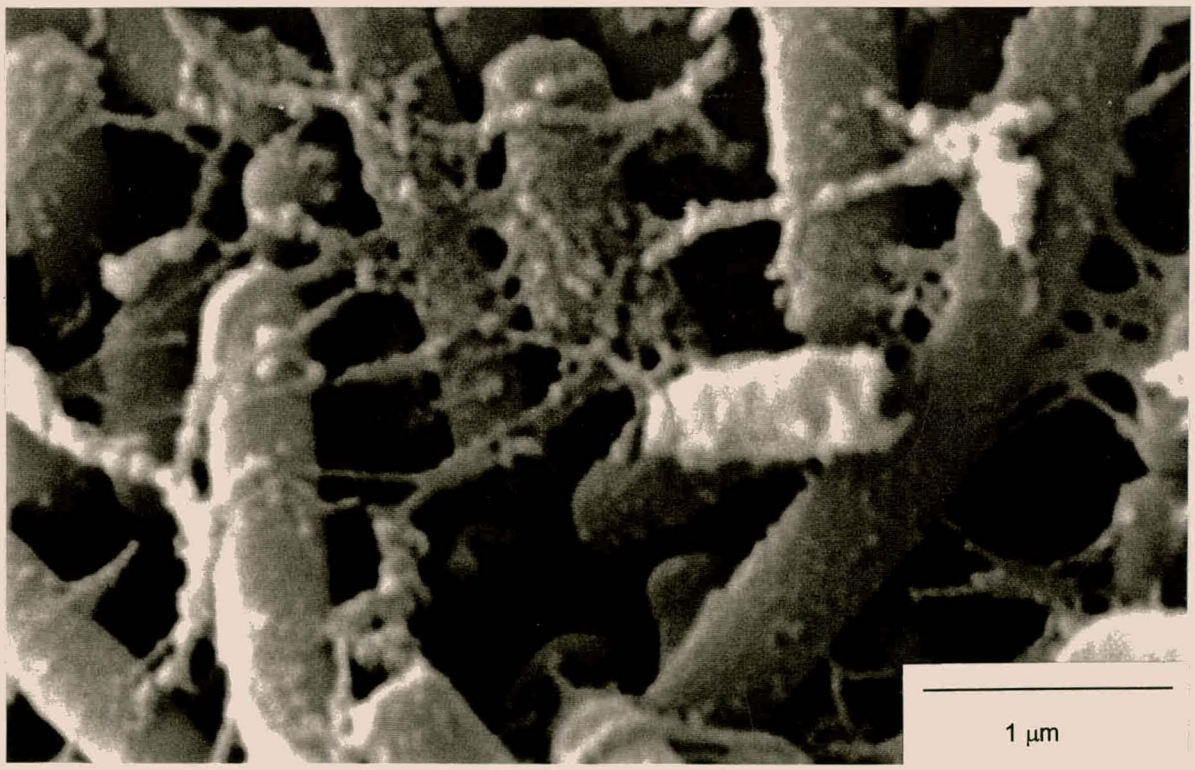


Figure 24. A scanning electron micrograph (22 000X) of the interior of a mass cultivated kefir grain.

this study was thus to investigate different culturing conditions to optimise kefir grain biomass increase. The results from the different experimental studies clearly showed that during cultivation of grains in different milk types, no relationship existed between the pH and the TA values. Any relationship between grain mass increase and changes in TA was unclear and the acid production may have been influenced by other lactose-utilising, but non acid-producing, yeasts and lactic acid bacteria.

Initial grain starter sizes of less than 1% (m/v) resulted in a decrease of kefir biomass. This was ascribed to the ratio of milk volume to grain starter mass being too large and as a result creating unfavourable conditions for grain mass increase. More favourable conditions were created when the grain starter size was at least 1% (m/v), resulting in a positive biomass increase.

The optimum temperature for grain biomass increase was found to be 22°C, but as previously reported and found in this study, the quality of kefir produced at 22°C was inferior to kefir produced at 25°C. Grain cultivation at 25°C led to a biomass increase of 113% over 10 days compared to the 130% increase at 22°C. It was thus concluded that 25°C was the better temperature for maximum grain biomass increase and the production of quality kefir.

The influence of milk fat content on biomass increase could not be clearly defined, but the fat content appeared to play a more important role during non-agitated cultivation than agitated cultivation. In the studies where the cultivation vessels were not agitated, it was found that the use of low fat milk as growth medium resulted in higher biomass increases than cultivation in full cream milk. It appeared as if the milk fat inhibited nutrient exchange. In contrast, during agitation of the cultivation vessels there was no major difference in biomass increase when grains were cultivated in either full cream or low fat milk.

The use of inactive kefir grains as initial inoculum resulted in a lag period of 20 days before the grain biomass started to increase. No lag period was observed when active grains were used as initial inoculum and it was thus concluded that the use of active grains during mass cultivation was essential to prevent this lag period. In the case of frozen grains or grains that had been stored for a substantial period, it is important to activate the grains before using them for mass cultivation.

For low fat milk it was found that addition of either tryptose or yeast extract greatly enhanced grain biomass increase. Addition of both these nutrients to the milk also led to substantial increases, but was lower than when each nutrient was added separately. In the latter stages of the study, tryptose had to be replaced with urea due to unavailability of the former nutrient. The addition of urea alone did not enhance biomass increase, however, urea combined with yeast extract resulted in the best biomass increases of the whole study.

It was found in all the studies where agitation was used that large amounts of ECP were produced. ECP production was very notable in cases where yeast extract and/or tryptose were used as additional nutrient sources.

Based on the data obtained in the investigation, it is thus recommended that for maximum kefir grain biomass increase, more than 1% active grains must be used as starter and the grains cultivated in either full cream or low fat milk for at least 20 days with agitation at 25°C and daily replacement of all the fermented milk.

References

Atlas, R.M. & Bartha, R. (1993). Effects of abiotic factors and environmental extremes on microorganisms. In: *Microbial Ecology: Fundamentals and Applications*, 3rd ed. Pp. 212-245. New York: The Benjamin/Cummings Publishing Company, Inc.

Berzhinskas, G., Urbene, S. & Puidokas, I. (1978). Influence of multiple-stage pasteurization of milk on the consistency of kefir. *XX International Dairy Congress*, **E**, 840-841.

Bondarev, V.Y. (1977). Effect of heat-treatment on the quality of acid coagulum (kefir). *Molochnaya Promyshlennost'*, **5**, 29-30.

Calefato, J. (1998). Department of Food Science, University of Stellenbosch, Personal communication.

Dixon, A. (1973). Die bepaling van die suurheid van melk, room , suursels en wei. Opleidingsreeks nr. 4, *South African Journal of Dairy Technology*, **5**(1), 27-30.

Hirota, T. & Kikuchi, T. (1976). Studies on kefir grains. 1. Isolation and classification of micro-organisms from kefir grains and their characteristics. *Reports of research of Snow Brand Milk Products Co. Laboratory*, **74**, 63-82.

Human, M.E. (1998). Optimisation of maas process parameters to achieve traditional flavour, aroma and texture. M.Sc Thesis. Stellenbosch: University of Stellenbosch, South Africa.

James, C.S. (1995). General food studies. In: *Analytical Chemistry of Foods* . Pp. 168-169. Great Britain: Chapmann & Hall.

Kessler, H.G. (1981). Food Engineering and Dairy Technology. Verlag A. Kessler, Freising, FRG *(As cited by Roginski, 1988).*

Koroleva, N.S. (1975). *Technical Microbiology of Whole Milk Products*, Moscow, USSR: Pishchevaya Promyshlennost'.

La Rivière, J.W.M., Kooiman, P. & Schmidt, K. (1967). Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis. Archiv für Mikrobiologie*, **59**, 269-278.

Libudzisz, Z. & Piatkiewicz, A. (1990). Kefir production in Poland. *Dairy Industries International,* **55**(7), 31-33.

Liu, J.A.P. & Moon, N.J. (1983). Kefir – a "new" fermented milk product. *Cultured Dairy Products Journal*, **18**(3), 11-12.

Marshall, V.M. & Cole, W. M. (1985). Methods for making kefir and fermented milks based on kefir. *Journal of Dairy Research*, **52**, 451-456.

Steinkraus, K.H. (1996). Acid-fermented milk and milk/cereal foods. In: *Handbook of Indigenous Fermented Foods,* 2nd ed. Pp. 275, 305-308. New York: Marcel Dekker, Inc.

Chapter 4

THE USE OF FOURIER TRANSFORM NEAR INFRARED (FT-NIR) SPECTROSCOPY IN THE DETERMINATION OF THE LACTOSE AND LACTIC ACID CONTENT IN KEFIR AS INDICATION OF KEFIR GRAIN ACTIVITY

Summary

Kefir is a refreshing, fermented drink produced when kefir grains are incubated in milk. The success of the fermentation depends on the metabolic activity of the kefir grains. The activity of the grains can be determined by monitoring the metabolic processes during fermentation, for example the conversion of lactose to lactic acid. Measurement of the amount of lactose and lactic acid in a kefir sample would therefore give an indication of the activity of the grains. Most of the time, official analytical methods for these constituents are expensive, time consuming and require technical expertise. It was thus necessary to develop a rapid and less expensive method for the routine analysis of metabolic end-products in kefir samples. The application of Fourier transform near infrared (FT-NIR) spectroscopy for the prediction of lactose and lactic acid content in kefir samples was studied. With the use of the partial least squares algorithm, acceptable calibration statistics (SECV, r) were obtained for determining lactose (0.3487 g.100 g^{-1}, 0.81), D+ lactic acid (0.1337 g.100 g^{-1}, 0.82) and L+ lactic acid (0.0797 g.100 g^{-1}, 0.93). It was concluded that FT-NIR spectroscopy can be used successfully for the rapid and less expensive prediction of the lactose and lactic acid content in kefir and can be applied for the reliable rapid determination of the activity of kefir grains.

Introduction

Kefir is a refreshing fermented milk drink that has been consumed for centuries and is traditionally made by incubating kefir grains in milk. The unique microbial composition of the grains imparts a very distinguished taste to kefir, which can be described as acid, mildly alcoholic, prickly and slightly yeasty (Duitschaever, 1989; Marshall, 1993; Steinkraus, 1996; Rea *et al.*, 1996). Lactic acid fermentation takes place and leads to the formation of lactic acid by the lactic acid bacteria. This is followed by the production of alcohol and carbon dioxide by the lactose and non-lactose fermenting yeasts during alcoholic fermentation. Traces of acetaldehyde, diacetyl and acetone are other minor components that contribute to the characteristic kefir flavour (Vedamuthu, 1982).

Measuring the metabolic end-products produced is normally one of the best ways of determining the activity of micro-organisms. One of the most important metabolic processes for the growth of lactic acid bacteria is the conversion of lactose and citric acid to lactic acid and other metabolites (Chandan, 1982). A reliable indication of the activity of these organisms could thus be obtained by measuring the concentration of lactose and lactic acid present in a kefir sample.

A variety of analytical methods exist for the determination of lactose and lactic acid, but these are either very expensive, complicated or too tedious to be used as a routine measurement of kefir grain activity. A need thus exists for a method that can determine the conversion of lactose to lactic acid quickly and accurately.

The application of near infrared (NIR) spectroscopy in the determination of food constituents is rapid, requires little or no sample preparation and is easy to use by technicians with little training once the calibration has been derived (Rodriguez-Otero *et al.*, 1997a; Wetzel, 1998). Opaque samples, like kefir, can be analysed by measuring the light passing through the sample or reflected by its surface (Giangiacomo & Nzabonimpa, 1994). In order to perform a NIR spectroscopy analysis, an empirical, linear equation has to be developed in which the concentration of the analyte is related to optical measurements. These measurements are generally expressed as absorbance or, in the instance of reflectance measurements, as log 1/R, where R is reflectance (Wetzel, 1998). The method involves a set of samples for which known values have been obtained

by a reference method. As soon as spectral data have been collected for these samples, mathematical treatment of the optical data is performed and a final calibration is obtained (Wetzel, 1998). This calibration model can then be used to predict unknown sample values.

NIR spectroscopy has been used in the dairy industry since 1957, when the first spectra of casein, fat, lactose and powdered milk was obtained (Goulden, 1957). The widest application of NIR to dairy products are found in the analysis of milk powder due to its uniform particle size and shape, combined with its consistent formulation (Frankhuizen, 1992; Rodriguez-Otero *et al.*, 1997a). Other applications include the analysis of milk, cream, butter, whey powder and various cheese types (Frank & Birth, 1982; Baer *et al.*, 1983a; Baer *et al.*, 1983b; Frankhuizen & Van der Veen, 1985; Robert *et al.*, 1987; Wehling & Pierce, 1988; Kamishikiryo-Yamashita *et al.*, 1994; Diaz-Carrillo *et al.*, 1993; Molt & Kohn, 1993; Rodriguez-Otero *et al.*, 1995; Rodriguez-Otero *et al.*, 1997b). Moisture, fat, protein, casein, total solids and lactose are measured most frequently by NIR analysis of dairy products (Rodriguez-Otero *et al.*, 1997b). Despite the wide variety of fermented milk products available, very little work has been done regarding the application of NIR spectroscopy in the analysis of these products. Rodriguez-Otero & Hermida (1996) and Rodriguez-Otero *et al.* (1997a) performed the determination of fat, protein and total solids of different fermented milk products in Spain. No reference could be found regarding the application of Fourier transform near infrared (FT-NIR) spectroscopy analysis to the analysis of fermented dairy products.

The aim of this study was to determine the amount of lactose, D+ lactic acid and L+ lactic acid in kefir samples by means of FT-NIR spectroscopy. In future, the measurement of the conversion of lactose to lactic acid could be incorporated into activity testing of kefir grains.

Materials and methods

Samples

Different kefir samples were obtained at various stages of the kefir production process and analysed for lactose, D+ lactic acid and L+ lactic acid. Full cream milk (4% fat m/v), low fat milk (2% fat m/v) and non-fat milk (reconstituted

from milk powder) were used for kefir production in order to obtain a good variation between the samples. A total of 111 samples were analysed for lactose, while 94 samples were analysed for D+ lactic acid and 91 for the L+ isomer.

Reference Analysis

The lactose content was measured colorimetrically by using methylamine (Katsu *et al.*, 1994). The lactose reacts with methylamine in a hot alkaline solution and forms a red complex with a maximum absorbance at 540 nm. The lactic acid was measured enzymatically with the D+/L+ Lactic acid Test Combination from Boehringer Mannheim (cat. no. 1112821). All determinations were performed in duplicate.

FT-NIR spectral measurements

Absorbance spectra were recorded using the FT-NIR Perkin-Elmer Spectrum IdentiCheck™ spectrophotometer. Samples were scanned from 10 000 to 4000 cm^{-1} with wavelength increments of 4 cm^{-1} which produced a total of 1501 points per spectrum. The spectral data was collected at a resolution of 32 cm^{-1} using a 0.2 mm path length quartz cuvette.

Pre-processing

The partial least squares (PLS1) algorithm (Martens & Jensen, 1983; Marten & Naes, 1988) was applied to the spectral data and cross validation was used to determine the optimum number of PLS factors to use for each calibration. Individual QUANT+ methods were built and calibrated for each constituent tested using the Spectrum QUANT+™, Version 4.10, software programme. For lactose, multiplicative scatter correction (MSC) was carried out on second derivative spectra with a gap of nine points. Second derivative spectra (width = 49 and gap = 13 points) were used for D+ lactic acid, while the data for L+ lactic acid were pre-processed by applying standard normal variate (SNV) transformation, without de-trending, to second derivative spectra (width = 49 points).

The aim of a full-spectrum method, like PLS, is to decrease the amount of data, without discarding any useful information, in order to reduce the risk of overfitting (Osborne *et al.*, 1993). Linear combinations of the original spectral data are used to construct a small number of factors, after which regression on these

factor scores are used to determine the prediction equation (Osborne *et al.*, 1993). During construction of factors with PLS, the variation within the reference data is measured by comparing it to the spectral data, and thus, only spectral data that relates to variation in the reference data will be take into consideration (Osborne *et al.*, 1993; Anon., 1997). It is possible to account for the majority of spectral variance, due to the fact that property values are usually accompanied by noise, and spectral attributes from one property overlap others (Anon., 1997).

No separate validation set is required when performing full cross validation (Osborne *et al.*, 1993). One sample is taken from the calibration set, while the remaining samples are used to perform a calibration. This calibration is then used to make a prediction for the separate sample. The whole process is repeated until each sample has been treated this way. One set of samples thus provides both the calibration and the validation set (Osborne *et al.*, 1993). Due to the fact that one test sample is left out in each repeated calibration, this method can be very effective in recognising certain types of outliers. Standard outlier techniques are not always as proficient in identifying these outliers (Naes & Ellekjaer, 1993).

MSC is a pre-processing parameter that compensates for light scattering variations that are wavelength-dependent and which occurs during reflectance spectroscopy (Anon., 1997). During application of MSC, the scatter of each standard is determined in relation to the mean standard. The standard is then normalised so that each standard has the same scatter as the mean standard, after which each standard is treated in the same way during a prediction (Anon., 1997). When applying MSC, each spectrum is rotated so that it fits as closely as possible to the mean spectrum (Geladi *et al.*, 1985; Ilari *et al.*, 1988; Isaksson & Naes, 1988). The use of MSC results in simpler calibrations that are more easily interpreted than log 1/R data (Isaksson & Naes, 1988; Osborne, 1988).

The multiplicative interferences of scatter and particle size can be removed by applying SNV transformation to near-infrared, diffuse reflectance spectra (Anon., 1997). De-trending can be used in conjunction with SNV, and accounts for the variation in baseline shift and curvilinearity that could be encountered in the above mentioned spectra (Anon., 1997).

Smoothing is particularly useful when the data are very noisy and when it is important to preserve the shape of the spectral bands (Buco, 1994). When using smoothing, the signal to noise ratio is improved by applying a Savitzky-Golay

function to the data (Anon., 1997). A predicted value from a polynomial equation replaces the log 1/R value at each spectral point. This equation fits an interval with the use of the least squares method (Buco, 1994).

It is possible to separate overlapping bands and remove baseline shifts with the use of the second derivative of a spectrum (Buco, 1994). Using derivatives is particularly useful where broad peaks are observed instead of well defined, sharp peaks (Wetzel, 1998). Derivative spectra usually have sharper features than the original spectra and can therefore be used to reduce the effects of overlapping bands (Anon., 1997). If three points along the spectrum are considered, a baseline is drawn between the outer two points and the centre point is corrected to this baseline. The difference of the centre point from the baseline is then plotted against the wavelength (Wetzel, 1998). This type of correction removes baseline offset and linear slope. Due to the fact that second derivative spectra have sharp minima where there are maxima in the original spectra, this parameter of pre-processing can be used to identify band positions in complex regions (Anon., 1997). The effects of particle size can also be substantially reduced by the use of derivatives (Osborne *et al.*, 1993), leading to a simplified calibration procedure that is more robust to particle size variations.

The accuracy of a full cross validation is expressed as the root mean square error of cross validation (RMSECV) (equation 1).

$$\text{RMSECV} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}} \qquad \ldots 1$$

Where:

n = the number of samples in the calibration set

y_i = the measured constituent value for the i^{th} sample, determined by a reference method

$\hat{y}_i$ = the predicted value for the i^{th} sample when it was dropped from the regression

The standard error of cross validation (SECV) measures the random errors and precision of the NIR method after adjusting for the lack of accuracy (bias) (Buco, 1994) (equation 2).

$$SECV = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i - bias)^2}{n-1}} \quad ...2$$

Where: n = the number of samples in the calibration set

y_i = the measured constituent value for the i^{th} sample, determined by a reference method

$\hat{y}_i$ = the predicted value for the i^{th} sample when it was dropped from the regression

$$bias = \frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)}{n} \quad ...3$$

The bias (equation 3) is interpreted as the average difference between $\hat{y}_i$ and y_i in the prediction set (Naes & Isaksson, 1991) and represents the systematic error (or lack of accuracy) in the NIR calibration equation (Buco, 1994). If the insignificant effect of n-1 to determine SECV instead of n is ignored, the relationship between SECV and RMSECV can simply be defined as (Naes & Isaksson, 1991):

$$RMSECV^2 = SECV^2 + bias^2$$

The goodness of fit of the line between actual and predicted sample values can be measured by the correlation coefficient (r) (equation 4). This parameter explains the extent to which y-values vary from the fitted straight-line relationship (Osborne *et al.*, 1993). If r = 1 or r = -1, then the points fall exactly on the line, but if r = 0, then there is no relationship between the actual and predicted samples (Buco, 1994).

$$r = \sqrt{1 - \frac{\sum_{i=1}^{n_s}(\hat{y}_i - y_i)^2}{\sum_{i=1}^{n_s}(y_i - \bar{y}_i)^2}} \quad \ldots 4$$

Where:

n_s = the number of samples in the calibration set

y_i = the measured constituent value for the i^{th} sample, determined by a reference method

$\hat{y}_i$ = the predicted value for the i^{th} sample obtained from the calibration equation

$\bar{y}_i$ = the mean of y_i

The proportion of the total variance in the y-values explained by the fitted line is thus given by the coefficient of determination (r^2), and this value lies between 0 and 1 (Osborne *et al.*, 1993).

The accuracy with which the duplicate samples were analysed in the same laboratory can be measured by standard error of laboratory of the reference method (SEL) (equation 5).

$$SEL = \sqrt{\frac{\sum(y_1 - y_2)^2}{2n}} \quad \ldots 5$$

Where:

y_1 = the first analysis

y_2 = the second analysis

n = the total number of duplicate samples (not total number of analyses)

Results and discussion

A characteristic FT-NIR spectrum of a kefir sample is illustrated in Fig.1. The FT-NIR statistics, presented in Table 1, demonstrated acceptable calibrations

Fig1-2(ch4)

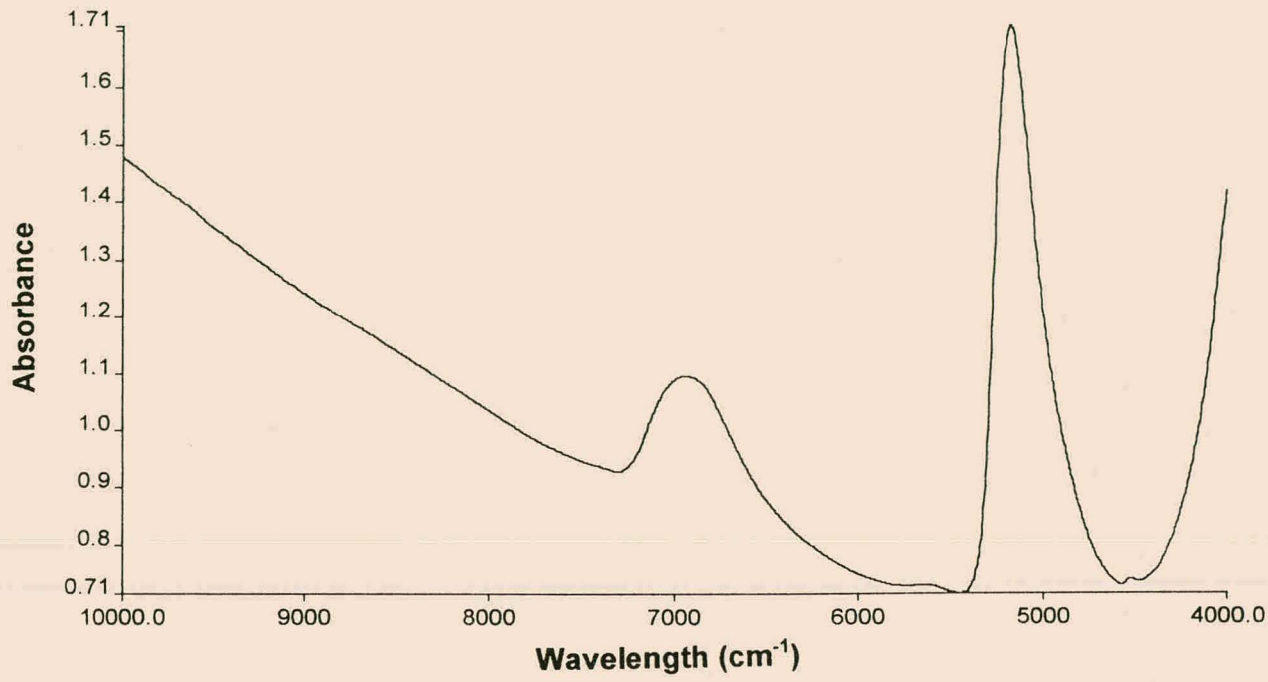


Figure 1. A typical FT-NIR spectrum of a kefir sample.

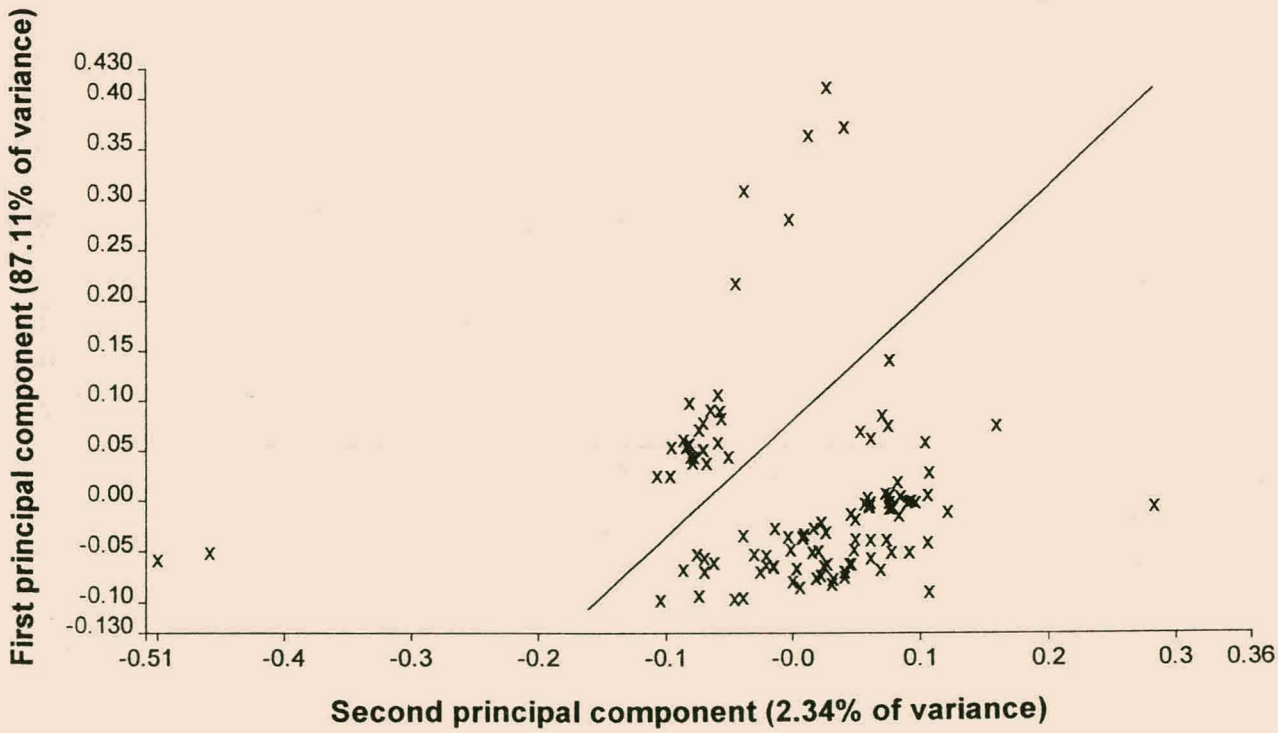


Figure 2. Score plot of the first versus second principal components for second derivative FT-NIR lactose spectra.

Table 1. FT-NIR spectroscopy results for the determination of lactose, D+ lactic acid and L+ lactic acid content in kefir.

	Lactose	D+ lactic acid	L+ lactic acid
Range ($g.100\ g^{-1}$)	2.8847 – 5.2797	0 – 1.1011	0 – 0.6000
n	111	94	91
PLS factors	5	7	7
Mean ($g.100\ g^{-1}$)	4.0700	0.1638	0.2891
SECV ($g.100\ g^{-1}$)	0.3487	0.1337	0.0797
RMSECV ($g.100\ g^{-1}$)	0.5522	0.2055	0.1328
bias	-0.0062	0.02704	0.0017
SD	0.5755	0.2196	0.1963
SEL ($g.100\ g^{-1}$)	0.1307	0.0393	0.0294
r	0.81	0.82	0.93
F-value	40.06	26.07	73.88

PLS factors = number of factors used in the calibration
SECV = standard error of cross validation corrected for bias
RMSECV = standard error of cross validation
bias = average residuals
SD = standard deviation
SEL = standard error of laboratory
r = correlation coefficient

obtained for all three constituents tested, with the respective SECV and r values for lactose being 0.3487 g.100 g^{-1} and 0.81, 0.1337 g.100 g^{-1} and 0.82 for D+ lactic acid, as well as 0.0797 g.100 g^{-1} and 0.93 for L+ lactic acid. Comparison of the SECV with the standard deviation of the individual reference methods showed that the former values were not substantially higher than the latter, indicating acceptability of the calibrations. The significant difference between the property variance accounted for by the calibration model and the residual property variance is measured by the F-value, and is satisfactory for L+ lactic acid (73.88), reasonable for lactose (40.06), but could be improved for D+ lactic acid (26.07). Performing a principal component analysis (PCA) (Fig. 2) on the data indicated that transformation of the data set resulted in a certain degree of clustering of the spectra according to milk type. This was probably due to the fact that the non-fat milk samples were reconstituted from milk powder, while fresh milk was used for the full cream and low fat milk samples. The relationship between reference sample values and FT-NIR predicted sample values for lactose and both isomers of lactic acid is illustrated in Fig. 3 to 5, and indicates that the FT-NIR calibration model is reasonably accurate in predicting sample values.

Conclusions and recommendations

The suitability of applying FT-NIR spectroscopy to the determination of lactose and lactic acid content in kefir was determined in this study and found to be favourable for incorporation into activity testing of kefir grains. Using FT-NIR spectroscopy instead of official analytical methods for the determination of lactose and lactic acid in kefir will be considerably less expensive and time-consuming, and can be performed by anyone with basic laboratory skills.

References

Anonymous (1997). Perkin Elmer, Spectrum QUANT+ User's Reference. United Kingdom: The Perkin-Elmer Corporation.

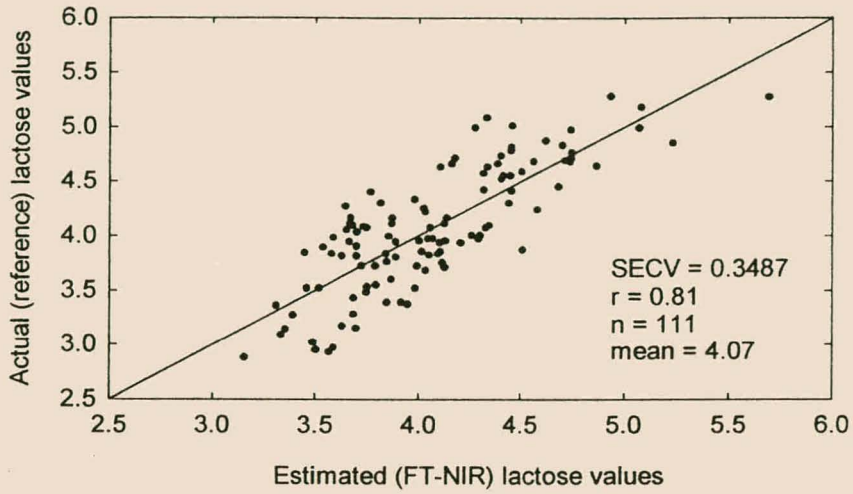


Figure 3. Scatterplot of lactose in kefir predicted by FT-NIR spectroscopy versus actual values measured by the colorimetric method.

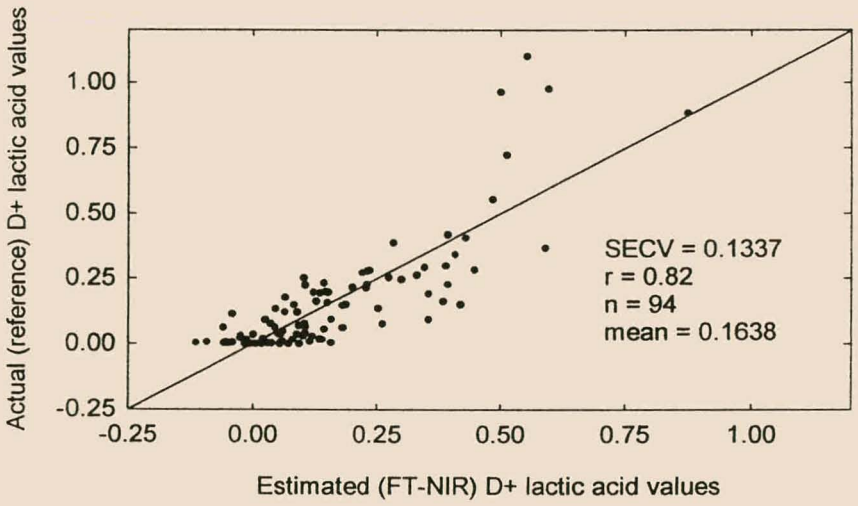


Figure 4. Scatterplot of D+ lactic acid in kefir predicted by FT-NIR spectroscopy versus actual values measured by the enzymatic method.

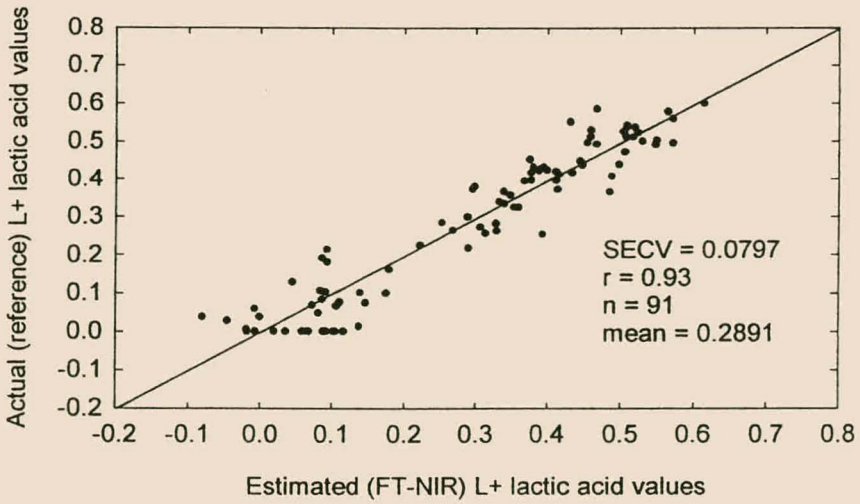


Figure 5. Scatterplot of L+ lactic acid in kefir predicted by FT-NIR spectroscopy versus actual values measured by the enzymatic method.

Baer, R.J., Frank, J.F., Loewenstein, M. (1983a). Compositional analysis of nonfat dry milk using near infrared diffuse reflectance spectroscopy. *Journal of AOAC International*, **66**, 858-863.

Baer, R.J., Frank, J.F., Loewenstein, M. & Birth, G.S. (1983b). Compositional analysis of whey powders using near infrared diffuse reflectance spectroscopy. *Journal of Food Science*, **48**, 959-961.

Buco, S.M. (1994). Pre-conference course on Multivariate Statistics in Diffuse Reflectance Spectroscopy. *7th International Diffuse Reflectance Spectroscopy Conference*. 13-14 August 1994. Chambersburg, Pennsylvania, USA.

Chandan, R.C. (1982). Other fermented dairy products. In: *Prescott and Dunn's Industrial Microbiology* (edited by G. Reed), 4th ed. Pp. 113-184. Westport, Connecticut, USA: AVI Publishing Co., Inc.

Diaz-Carrillo, E., Muñoz-Serrano, A., Alonso-Moraga, A. & Serradilla-Manrique, J.M. (1993). Near infrared calibration for goats milk components: protein, total casein, α-casein, β-casein, κ-casein, fat and lactose. *Journal of Near Infrared Spectroscopy*, **1**, 141-146.

Duitschaever, C.L. (1989). What is kefir and how can it be made ? *Modern Dairy*, **68**(4), 18-19.

Frank, J.F. & Birth, G.S. (1982). Applications of near infrared reflectance spectroscopy to cheese analysis. *Journal of Dairy Science*, **65**, 1110-1116.

Frankhuizen, R. (1992). NIR analysis of dairy products. In: *Handbook of Near-Infrared Analysis* (edited by D.A. Burns & E.W. Ciurczak). Pp. 609-641. New York: Marcel Dekker, Inc.

Frankhuizen, R. & Van der Veen, N.G. (1985). Determination of major and minor constituents in milk powders and cheese by near infrared reflectance spectroscopy. *Netherland Milk and Dairy Journal*, **39**, 191-207.

Geladi, P., MacDougal, D. & Martens, H. (1985). Linearization and scatter correction for near-infrared reflectance spectra of meat. *Applied Spectroscopy*, **39**, 491-500.

Giangiacomo, R. & Nzabonimpa, R. (1994). Approach to near infrared spectroscopy. *International Dairy Federation Bulletin*, **298**, 37-42.

Goulden, J.D.S. (1957). Diffuse reflectance spectra of dairy products in the near infra-red region. *Journal of Dairy Research*, **24**, 242-251.

Ilari, J.L., Martens, H. & Isaksson, T. (1988). Determination of particle size in powders by scatter correction in diffuse near-infrared reflectance. *Applied Spectroscopy*, **42**, 722-728.

Isaksson, T. & Naes, T. (1988). The effect of multiplicative scatter correction (MSC) and linearity improvement in NIR spectroscopy. *Applied Spectroscopy*, **42**, 1273-1284.

Kamishikiryo-Yamashita, H., Oritani, Y., Takamura, H. & Mabota, T. (1994). Protein content in milk by near infrared spectroscopy. *Journal of Food Science*, **59**, 313-315.

Katsu, T., Zhang, X. & Rechnitz, G.A. (1994). Simultaneous determination of lactose and glucose in milk using two working enzyme electrodes. *Talanta*, **41**(6), 843-848.

Marshall, V.M. (1993). Kefir. In: *Encyclopaedia of Food Science and Technology* (edited by Y. H. Hui), Volume 3. Pp. 1804-1808. Chichester, UK: John Wiley & Sons.

Martens, H. & Jensen, S.Å. (1983). Two-stage partial least squares regression on latent factors: a new NIR calibration method. In: *Proceedings of the Seventh World Cereal and Bread Congress, Progress in Cereal Chemistry and Technology* (edited by Holas, J. & Kratochvil, J.), Part A. Pp. 607-647. Amsterdam: Elsevier.

Martens, H. & Naes, T. (1988). Multivariate calibration by data compression. In: *Near Infrared Technology in the Agricultural and Food Industries*, (edited by Williams, P.C. & Norris, K.). Pp. 57-87. St. Paul: American Association of Cereal Chemists Inc.

Molt, K. & Kohn, S. (1993). Near-infrared spectroscopy. Chemometric method for quality control and process analysis of processed cheese products. *Deutsche Milchwissenschaft.*, **44**, 1102-1107.

Naes, T. & Isaksson, T. (1991). SEP or RMSEP, which is best ? *NIR News*, **2**(4), 16.

Naes, T. & Ellekjaer, M.R. (1993). Cross-validation and leverage-correction revisited. *NIR News*, **4**(1), 8.

Osborne, B.G. (1988). Comparitive study of methods of linearization and scatter correction in near infrared reflectance spectroscopy. *Analyst*, **113**, 263-267.

Osborne, B.G., Fearn, T. & Hindle, P.H. (1993). *Practical NIR Spectroscopy with Applications in Food and Beverage Analysis*, 2nd ed. Essex, England: Longman Scientific & Technical.

Rea, M.C., Lennartson, T., Dillon, P., Drinan, F.D., Reville, W.J., Heapes, M. & Cogan, T.M. (1996). Irish kefir-like grains: their structure, microbial composition and fermentation kinetics. *Journal of Applied Bacteriology,* **81**, 83-94.

Robert, P., Bertrand, D., Devaux, M.F. & Grappin, R. (1987). Multivariate analysis applied to near infrared spectra of milk. *Analytical Chemistry*, **59**, 2187-2191.

Rodriguez-Otero, J.L., Hermida, M. & Cepeda, A. (1995). Determination of fat, protein and total solids in cheese by near infrared reflectance spectroscopy. *Journal of AOAC International*, **78**, 802-806.

Rodriguez-Otero, J.L. & Hermida, M. (1996). Analysis of fermented milk products by near infrared reflectance spectroscopy. *Journal of AOAC International*, **79**, 817-821.

Rodriguez-Otero, J.L., Centeno, J.A. & Hermida, M. (1997a). Application of near infrared transflectance spectroscopy to the analysis of fermented milks. *Milchwissenschaft*, **52**(4), 196-200.

Rodriguez-Otero, J.L., Hermida, M. & Centeno, J. (1997b). Analysis of dairy products by near-infrared spectroscopy: A review. *Journal of Agricultural and Food Chemistry*, **45**(8), 2815-2819.

Steinkraus, K.H. (1996). Acid-fermented milk and milk/cereal foods. In: *Handbook of Indigenous Fermented Foods,* 2nd ed. Pp. 275 and 305-308. New York: Marcel Dekker, Inc.

Vedamuthu, B.R. (1982). Fermented milks. In: *Fermented Foods in Economic Microbiology* (edited by A.H. Rose), Volume 7. Pp. 199-226. New York: Academic Press.

Wehling, R.L. & Pierce, M.M. (1988). Determination of moisture in Cheddar cheese by near infrared reflectance spectroscopy. *Journal of AOAC International*, **71**, 571-574.

Wetzel, D.L.B. (1998). Analytical Near Infrared Spectroscopy. In: *Instrumental Methods in Food and Beverage Analysis* (edited by D.L.B. Wetzel and G. Charalambous). Pp. 141-194. Amsterdam: Elsevier.

Chapter 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Background

During the past years, the South African population has become increasingly urbanised and this subsequently led to the establishment of more informal urbanised communities (Myburgh, 1998). Cooling and/or storage facilities are severely limited in these environments and food products are usually bought and consumed in the same day. The purchasing power of a considerable part of the South African population is extremely weak and the decision whether to buy a product or not, even the most basic items, is crucial. Daily income can be as little as five rand per capita per day and the inability to maintain a healthy diet is frequently the cause of malnutrition (Fincham *et al.*, 1993; Myburgh, 1998). Consumers are very aware of what they are buying and food products should comply with strict criteria, which includes giving a full feeling in the stomach, being nutritious and palatable as well as providing energy (Karaan & Myburgh, 1992; Myburgh, 1998).

Fermented milk has for centuries played an important cultural and nutritional role in the daily life of indigenous tribes of Southern Africa. Raw milk was easily available due to the presence of large cattle herds and milk was often left to thicken on its own to make traditional sour milk. Living in informal urban settlements prevented ownership of large cattle herds and unpasteurised milk became harder to obtain. A new South African law that was introduced recently prevents the selling of unpasteurised milk (Anon., 1997; Viall, 1999), but the use of pasteurised milk to make traditional sour milk results in a product with a putrid taste and aroma. A need thus exists for an inexpensive fermented milk drink that can be made from pasteurised milk, does not require cooling and is easy to prepare.

Introducing kefir to the low-income consumer market could be the solution to this problem as kefir is a fermented product that is very easy to make and requires no more facilities than what is normally found in a low-income family's kitchen. The grains that are used as starter culture can be re-used over and over again and can even be shared amongst families as the grain mass increases during fermentation. Fermentation takes place at room temperature and pasteurised milk is used during production. The cost of making kefir would only be the price of the milk purchased and the initial acquisition of the kefir grains.

Mass production of kefir grains

Kefir grain biomass increases only 5% to 7% per day under normal incubation conditions, which creates a problem for the distribution of kefir grains on industrial scale. It thus became necessary to develop a method that would improve biomass increase to such an extent that a supplier of kefir grains will be able to provide grains at an acceptable rate to customers.

Factors that could have an influence on kefir grain biomass increase were studied. The grains were incubated in milk and the influence of different incubation temperatures (18°, 22°, 25° and 30°C), different milk types (full cream and low fat milk) and different starter sizes on grain biomass increase were evaluated during non-agitated cultivation. Cultivation at 22°C and in low fat milk resulted in the highest increase in grain biomass, but kefir produced at this temperature was inferior in quality. Cultivation was proceeded at 25°C in low fat milk and resulted in good quality kefir and acceptable biomass increases. Initial grain starter sizes of more than 1% (m/v) were necessary for grain biomass increase.

The next step was to study the influence of milk enrichment, agitation of the cultivation vessel, different milk replacement volumes and the use of active versus inactive grains on grain biomass increase. Tryptose, yeast extract and urea were tested as additional nutrient sources and agitation was carried out at 25°C in a temperature-controlled shaking water bath. Agitation of the cultivation vessel showed a definite enhancement of grain biomass increase as did increasing the

volume of replacement milk during cultivation. The fat content of the milk did not have the same impact on biomass increase during agitated cultivation as it did during non-agitated cultivation. The addition of yeast extract or tryptose to improved the grain biomass increase milk considerably. The addition of urea did not result in large biomass increases, but urea in combination with yeast extract led to the best biomass increases of the whole investigation. The use of active kefir grains as initial starter was necessary to reduce the lag period in biomass increase that was found with the use of inactive grains. A maximum of 327% increase in biomass was obtained with this method compared to the 60% to 100% increase under normal conditions. Extracellular polymers (ECP) were formed during cultivation in milk enriched with yeast extract and/or tryptose.

Activity testing of kefir grains

One of the most critical areas during kefir manufacture is the activity of the kefir grains used. Grains that are not fully active will result in kefir of an inferior quality, taste and aroma. Suppliers selling kefir grains must also be able to guarantee the activity of the grains at the moment of sale. A mass cultivation method was developed, but the effect of enhanced biomass increase on metabolic activity was still unclear.

Ways of examining the activity of micro-organisms almost invariably include monitoring their metabolic processes, of which the conversion of lactose to lactic acid is probably the most important in the case of kefir grain micro-organisms. Activity of kefir grains could thus be determined by measuring the lactose and lactic acid content in kefir samples. Due to the fact that official reference analytical methods for lactose and lactic acid are time consuming, expensive or require high levels of technical expertise, it was decided to use Fourier transform near infrared (FT-NIR) spectroscopy for the determinations. Calibrations were derived for lactose, D+ lactic acid and L+ lactic acid using Perkin Elmer IdentiCheck™ and QUANT+™ software programmes. Acceptable calibrations were obtained for all three constituents and it was concluded that FT-NIR spectroscopy was a rapid and reliable method to be incorporated into activity testing of kefir grains.

Recommendations

A certain amount of refining of the mass cultivation method still needs to be performed. The precise incubation temperature for optimum kefir grain biomass increase will have to be clarified. Yeast extract was one of the added nutrients that resulted in the best biomass increases, but contains of a variety of unknown ingredients and is too expensive to use during mass cultivation of grains on industrial scale. It will, therefore, be necessary to examine each ingredient separately or in combination to find the ingredient(s) in yeast extract that leads to the enhancement of grain biomass increase. This ingredient(s) can then be used more cost effectively for mass cultivation.

The sensorial quality of kefir produced with the use of mass cultivated grains needs to be examined carefully to determine whether mass cultivated grains impart undesirable characteristics to kefir or not. The extracellular polymers (ECP) produced during mass cultivation of the grains still needs to be identified and its role during grain propagation determined. Possible industrial applications of the ECP in other industries can also be included in further work.

The best storage method for kefir grains still needs to be clarified, as well as the effect storage has on the activity of the grains. The determination of lactose and lactic acid with FT-NIR spectroscopy can be successfully applied during activity testing of this nature.

Although excellent opportunities exist for both kefir and kefir grains, virtually no marketing has been done to promote these products amongst different cultural groups in South Africa. This must still be followed up.

Performing PCA analysis on the spectral data during FT-NIR spectroscopy showed that grouping of the data occurred for different milk types. If kefir made from different milk types is to be analysed regularly for lactose and lactic acid, it might be more advantageous to perform separate calibrations for each milk type instead of performing one calibration for all the different milk types.

References

Anonymous (1997). *Foodstuffs, Cosmetics and Disinfectant Act and Regulations. Act no. 54 of 1972, G.N.R. 1555/1997.* Johannesburg, South Africa: Lex Patria Publishers.

Fincham, R., Harrison, D., Khosa, M. & Le Roux, I. (1993). *Nutrition and health in South Africa: The state of nutrition and the development of nutrition policy.* University of Cape Town, Rondebosch, South Africa: Southern Africa Labour and Development Research Unit.

Karaan, A.S.M. & Myburgh, A.S. (1992). Food distribution systems in the urban informal markets: The case of red meat marketing in the Western Cape townships and informal settlements. *Agrekon*, **31**(4), 289-293.

Myburgh, (1998). Food consumer behaviour of the low income communities in urban settlements. *Address at SAAFoST meeting held at ARC-Infruitec/Nietvoorbij*, 24 February 1998, Stellenbosch, South Africa.

Viall, J. (1999). New law "will milk small dairies dry". *Cape Argus, 18 October 1999*, p. 9.